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Wim Soetaert and Erick J. Vandamme

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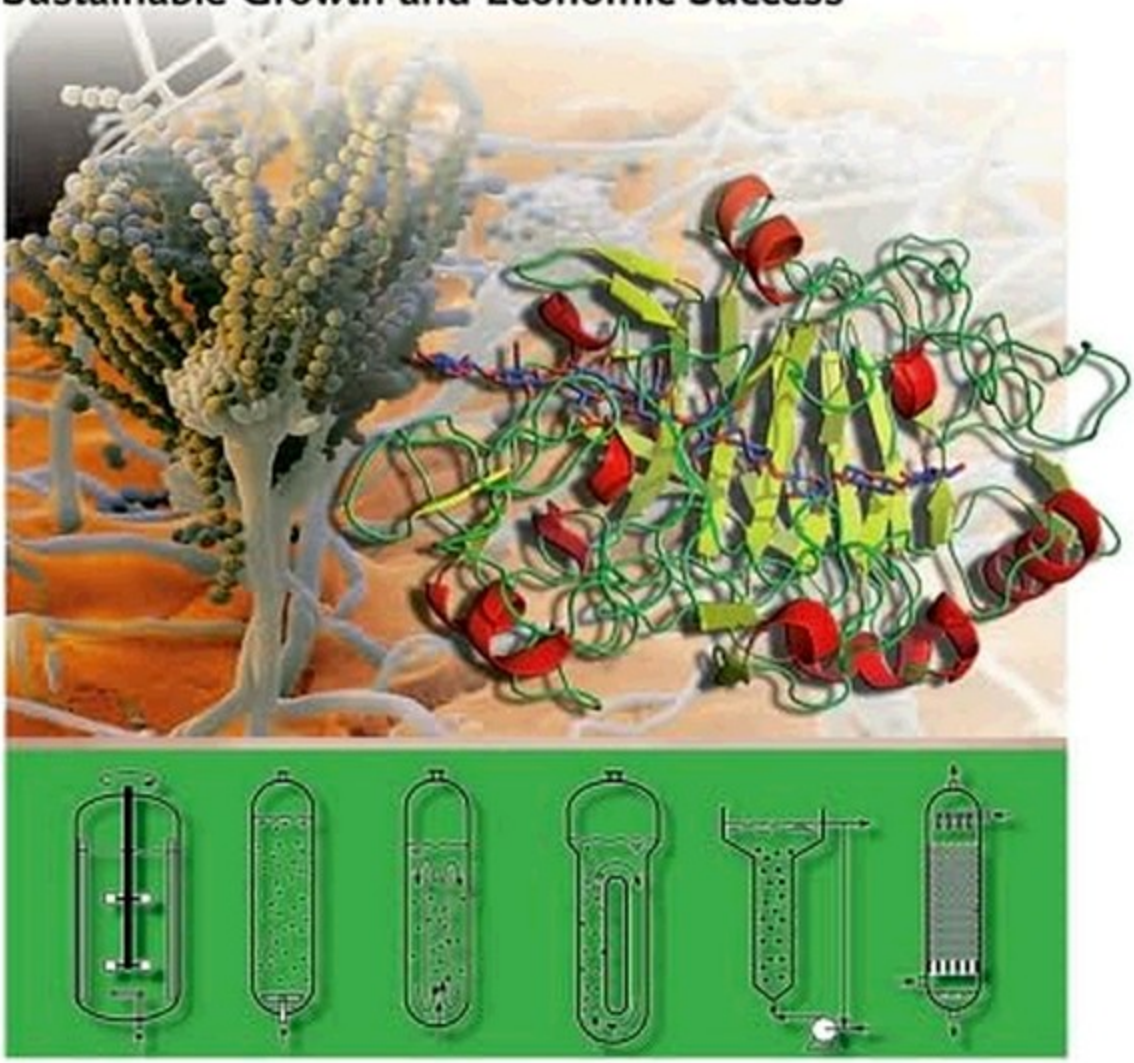
Industrial Biotechnology

Sustainable Growth and Economic Success



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Preface

Presently, a third wave of biotechnology – coined industrial or white biotechnology – is developing at full speed. It has positioned itself distinctly and firmly from red biotechnology, which is aimed at the medical field, and from green biotechnology, focusing on genetically modified crops and plants.

Industrial biotechnology exploits (micro)biological systems for the productions of fine and bulk chemicals, materials, fibers and energy. The underpinning technologies are based on biocatalysis and enzyme technology (the use of cells and enzymes to catalyse chemical reactions) and on fermentation technology (the directed and controlled mass production of microbial and higher cells, their enzymes and metabolites). Scientific breakthroughs in high throughput screening, molecular genetics, directed evolution, enzyme and cell engineering, metabolic engineering, novel culture techniques and integrated downstream processing have boosted the field dramatically over the past decade.

Industrial biotechnology has developed into a main contributor to sustainable technologies, leading towards green chemistry, reuse technology and the bio-based economy; indeed, not fossil, but renewable resources such as sugars and cellulosics, plant and animal derived oils, but also agro and food processing residues can now be transformed, using enzyme technology and/or fermentation processes, into a wide range of chemical compounds, such as fine and bulk chemicals, solvents, pharmaceuticals, bioplastics, vitamins, pigments, food additives, biopesticides, biofuels and many more.

It is obvious that the growing implementations of industrial biotechnology offer significant ecological advantages. Since renewable agricultural crops or their residues are the preferred starting materials, this technology consequently has an overall beneficial effect on greenhouse gas emissions and global climate change, and at the same time supports the agricultural sector, the basic provider of these raw materials.

Also industrial biotechnology now already often outcompetes conventional petrochemical technology in process performance benefits, such as high reaction rate, increased conversion efficiency and specificity, improved product purity, lower overall energy input and lower waste generation.

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These ecological and technological benefits will lead to a rapid penetration of industrial biotechnology in all production sectors of the chemical, agro-, food- and environment- related industries.

This will result in the formation of new production facilities, so called biorefineries, where the chemical industry and the agro-industry will have to integrate their knowledge and technology to the benefit of society and its planet.

This volume aims to cover the enabling technologies of industrial biotechnology as well as its application domains in a comprehensive manner. It starts with introductory chapters on the scope, impact and the history of industrial biotechnology. Basic and enabling technologies involved such as microbial genomics, bioinformatics, metabolic engineering and modelling, directed evolution of enzymes, fermentation technology and biocatalysis, systems biology, nanotechnology and downstream processing are covered in following chapters.

Subsequently, applications in the food and feed sector, the chemical and pharmaceutical industry, the paper and pulp sector, industrial enzyme production, bioplastics, biofuels are reviewed.

To conclude, environmental, economical and societal aspects of industrial biotechnology are discussed as well.

All mentioned scientific and technological aspects are treated by renowned experts in their field. Links between the technical, economical and ecological aspects are clearly expressed throughout the volume.

The editors are indebted to the John Wiley & Sons staff (Dr. Rainer Muenz, Zoe Mills and Dr. Martin Graf) for their invaluable help all along our editorial task. The secretarial help of Dr. Dominique Delmeire (Ghent University, Laboratory of industrial microbiology and biocatalysis) who kept the editors and authors abreast of the progress of the manuscript, was essential to keep all involved on track.

The editors hope that this volume will encourage chemists, physicists and biotechnologists from academia and from industry and agriculture to integrate their minds and efforts, so as to advance the impact of industrial biotechnology in the coming decades to the benefit of our society and to the health of our planet.

Ghent, January 2010

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The Scope and Impact of Industrial Biotechnology

Wim Soetaert and Erick J. Vandamme

Introduction

Industrial or white biotechnology has recently positioned itself firmly as distinct from red biotechnology, which is aimed at the medical sector, and green biotechnology, which focuses on genetically modified crops. Industrial biotechnology uses biological systems for the production of chemicals, materials, and energy. This technology is mainly based in biocatalysis (the use of enzymes to catalyze chemical reactions) and in fermentation technology (directed use of microorganisms), in combination with breakthroughs in molecular genetics, directed evolutions, and enzyme engineering and metabolic engineering of microorganisms and cells.

The term "white biotechnology," initially proposed by decision-making bodies of the European Union, is now gaining momentum worldwide; it covers the field of industrial biotechnology, with "white" also referring to the positive environmental aspects linked to the application of industrial biotechnology. This new biotechnology has developed into a main contributor to the so-called "green chemistry" area, in which renewable resources such as sugars or vegetable oils are transformed into a wide variety of chemical substances such as fine and bulk chemicals, pharmaceuticals, biocolorants, solvents, bioplastics, vitamins, food additives as well as biofuels such as bioethanol and biodiesel [1–5].

The implementation of industrial or "white" biotechnology offers significant ecological advantages. Renewable agricultural crops are the preferred starting materials, instead of dwindling fossil resources such as crude oil and natural gas. This technology consequently has a beneficial effect on greenhouse gas emissions and at the same time supports the agricultural sector, which is the provider of these raw materials. Moreover, industrial biotechnology frequently shows significant performance benefits compared with conventional chemical technology, such as a higher reaction rate, increased conversion efficiency, improved product purity, lowered energy consumption, and significant decrease in chemical waste generation. The combination of these factors has led to the recent strong penetration of industrial biotechnology in all sectors of the chemical industry, particularly in fine chemicals but equally so for bulk chemicals such as plastics

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and fuels. In 2003, the penetration of biotechnological production processes in the chemical industry was estimated at 5%, and will have increased to 10% by the year 2010 [6].

This development is now mainly driven by the laws of market economy in view of the higher efficiencies obtained by biotechnology production processes. In the near future, a number of societal and technological changes are expected to reinforce this trend even further, such as the depletion of crude oil reserves, the increased demand of a growing world population for raw materials and energy, the demand for sustainability and efficiency in chemical production systems and changes in agricultural policy [7, 8].

The strong development of industrial biotechnology is of immediate interest to the economically important chemical and agro-industries. From the collaboration of these two industries, entirely new chemical activities can be created in the form of biorefineries. Industrial biotechnology may also contribute significantly to the future of agriculture worldwide.

Furthermore, efforts to increase public awareness about industrial biotechnology are needed, with the added benefit that this is likely to improve the consumer's perception of biotechnology as a whole, in view of the clear link between industrial biotechnology and the sustainable development of our society [9, 10].

Sustainable or Green Chemistry

The chemical industry is a very important production sector, but at the same time a big user of fossil resources and a significant source of waste. This industrial sector produces a broad range of compounds that can roughly be divided into the following groups: fine chemicals, pharmaceutical products, bulk chemicals, plastics, and fuels.

Researchers, chemists, and chemical engineers face major challenges for developing sustainable chemical processes that respect the environment, improve our quality of life, and at the same time are competitive in the marketplace. This includes the development of new production processes, which reduce or eliminate the use of dangerous or hazardous substances, minimize energy consumption and waste generation, and start as much as possible from renewable raw materials. The ultimate goal is the development of a clean chemical technology, starting from renewable raw materials and energy, with minimal waste generation, and maximal productivity and competitiveness.

Sustainable chemistry is based on a range of different technologies, ranging from more efficient conventional chemical processes, the use of better catalysts, innovative separation methods such as membrane processes, recycling and re-use technology, and last, but not least, the use of industrial biotechnology. The latter technology is increasingly impacting the chemical sector, a reflection of the fact that biotechnology is naturally suitable for sustainable chemistry [6, 7, 11]. Whereas the use of renewable raw materials is rather difficult in conventional petrochemical processes, industrial biotechnology can handle these renewable raw materials with

amazing ease. Low waste generation and energy consumption, the use of non-hazardous, harmless, and renewable raw materials and high efficiency guarantee the sustainability of this technology. Industrial biotechnological processes are gradually penetrating the chemical industry, with very positive results with regard to sustainability as well as industrial competitiveness.

It is important to stress that industrial biotechnology is not the sole technology in this quest for sustainability. The most sustainable chemistry consists of an interplay between different technologies. In fact, it is common to obtain the best results from a suitable combination of conventional chemical technology and industrial biotechnology. New processes increasingly seem to consist of so-called combi-syntheses, consisting of a number of chemical and biotechnological steps. Also innovative separation technologies such as membrane technology and the use of supercritical solvents are being increasingly integrated and help to increase the eco-efficiency of this "green chemistry" [1, 6, 7, 11].

Industrial Biotechnology as a Multidisciplinary Technology

Multidisciplinary technology encompasses the integrated application of disciplines such as biochemistry, bio-informatics, molecular genetics, and process technology to develop useful processes and products, based on microbial, animal, or plant cells, their organelles or enzymes as biocatalysts. Microorganisms in particular have received a lot of attention as a biotechnological instrument and are used in so-called fermentation processes. Numerous useful bacteria, yeasts, and fungi are widely found in nature, but seldom find the optimum conditions for growth and product formation in their natural environment. In artificial (*in vitro*) conditions, the biotechnologist can intervene in the microbial cell environment (in a fermenter or bioreactor), as well as in their genetic material (DNA), to better control and direct the cell metabolism during these fermentation processes. Because of their extremely high synthetic versatility, ease of using renewable raw materials, high rate of microbial reactions, quick growth, and relatively easy to modify genetic material, many microorganisms are extremely efficient and in many cases indispensable workhorses in the various sectors of industrial biotechnology.

Industrial biotechnology has been practiced for a long time in a number of sectors, including healthcare, food industry, and fine chemistry. At present, this technology increasingly penetrates into areas such as bulk chemistry and energy supply, in a world where sustainable development is the key word.

The McKinsey study has indicated that the market share of industrial biotechnology will strongly increase in all areas by 2010, but particularly in fine chemicals production. The degree of penetration in 2010 is estimated to lie between 30% and 60% for fine chemicals and between 6% and 12% for polymers and bulk chemicals. Taken over the whole of the chemical industry, the penetration of biotechnology was estimated at 5% in 2003 and has increased to 10% in 2010, and will strongly increase even further afterwards. The speed of penetration will depend mainly on a number of factors such as the prices of crude oil and

agricultural raw materials, technological developments, and the political will to support and structure this new technology.

Use of Renewable Versus Fossil Resources

The use of renewable resources as raw material for technical (non-food) purposes is certainly not new [8, 11, 12]. People have used such materials from the first civilizations onwards. Humans have always employed plant- and animal-based raw materials to meet their basic needs, including natural fibers for clothing, wood for heating, animal fat for lighting, and natural dyes for textiles and artworks, etc.

The first industrial activities were also largely based on the use of renewable resources. This continued until the industrial revolution, when there was a fundamental change brought about in the nineteenth century by the emergence of carbochemistry (based on coal, aromatics and synthesis gas) and in the twentieth century by the development of petrochemistry. The use of renewable raw materials declined significantly, mainly as a consequence of the extremely low prices for petrochemical resources. During this period, the strongly developing chemical industry was systematically based on petrochemical resources. Nowadays, a large part of the chemical industry is based on petrochemical resources and our energy needs are also largely met by fossil fuels such as coal, petroleum, and natural gas. Currently, 95.8% of all organic chemical substances produced in Europe (including fuel) are based on fossil resources.

Nevertheless, a fair number of important industries are nowadays still based on renewable raw materials. Half of the fibers used in the textile industry are natural fibres (cotton, wool, flax, etc.), the oleo-chemical industry supplies our daily hygienic needs for soap and detergents based on vegetable oils, the building industry still uses a lot of wood and other natural fibers as construction material, etc. Moreover, petrochemistry does not offer a realistic alternative for the use of renewable raw materials in several important applications. For example, almost all antibiotics are still made by fermentation processes, starting from natural sugars and about half of our drugs are still isolated from living organisms.

The oil crisis between 1973 and 1979, when OPEC raised oil prices from US$2 to US$30 per barrel (1 barrel = 159l), gave rise to a renewed interest in renewable resources. As a result of this crisis, serious concern grew about our increasing dependency on fossil resources and the fact that these are not infinitely available. This concern was largely channeled politically into the energy question and resulted in many studies concerning the development of alternative energy sources. The results of these studies underlined that renewable raw materials were not (yet) competitive and the enthusiasm for renewable raw materials quickly disappeared when the oil price dropped again and the economy turned back to business as usual.

In the 1990s, the discussions around sustainable development and the greenhouse effect as well as the emergence of the green political parties provided new impulses. The problems related to food surpluses in the European Union were

also an important driving force. Because of the huge costs arising from these food surpluses, the EU strongly intervened in the European Common Agricultural Policy (CAP). For this purpose, the EU developed the "set-aside" land concept in 1992. According to this principle, subsidies were given to farmers for not planting anything on parts of their land, in order to limit overproduction. Then, within the European CAP, possibilities were created to use this land for non-food applications. Thus, farmers could earn additional revenue from this land.

With the increasing awareness and concern about industrial waste and its effects on the environment, the need arose for better biodegradable intermediates and final end-products. These biodegradable products can naturally degrade into components that are absorbed back into the natural cycle, in contrast to persistent products that do not (or only after an unacceptably long period) disappear from the environment or from the food chain. Biodegradability was the focal point of many products and these were frequently based on renewable resources, in view of their intrinsic biodegradability. Such applications are, for example, chemical substances that will almost certainly end up in the environment, such as lubricating oils for tree saws and agricultural machinery, detergents, etc. Green detergents such as alkylpolyglucosides have already achieved a significant market share and are made entirely from renewable resources (fatty acid alcohols and glucose).

The world's crude oil reserves will not last forever [13, 14]. With regard to fossil reserves, we are now faced with the paradoxical situation that, while crude oil (petroleum) is being consumed faster than ever, the "proven oil reserves" have remained at about the same level for 30 years as a consequence of new oil finds. Nevertheless, these "proven oil reserves" are located in increasingly difficult to reach places. Therefore, the cost for extracting the crude oil rises continuously, reflected in increasing oil prices. In sharp contrast to this, agricultural raw materials such as wheat and corn are becoming cheaper as a fundamental consequence of the rising agricultural yields. This trend will most likely continue for some time, also as a consequence of the realizations of the "green" biotechnology. This long-term trend may be perturbed by the transitory effects of market imbalances and politics but for a growing number of applications the economic balance is tipping towards the use of renewable resources, including in the segment of (inexpensive) bulk chemicals.

On a weight basis, renewable resources are about half as expensive as fossil resources (Table 1). Agricultural by-products such as straw are even 10 times less expensive than petroleum. It is also quite remarkable that the current world

Table 1 Average world market price of some fossil and renewable resources.

Fossil	Price (€/ton)	Renewable	Price (€/ton)
Petroleum	380	Corn/wheat	120
Coal	40	Straw	20
Ethylene	800	Sugar	400

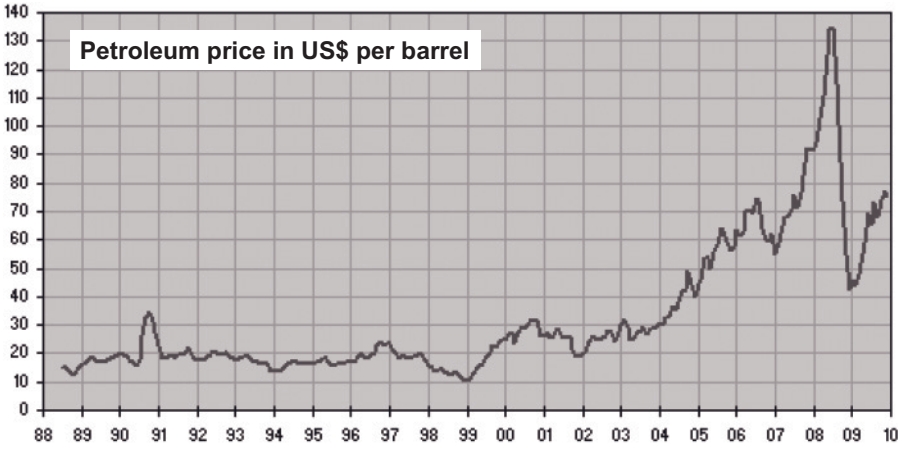

Figure 1 Crude oil price from 1988 to 2010. Copyright oilenergy.com.

market prices for petroleum and sugar are about the same, despite the fact that sugar is a very pure (99.8%) and refined product and petroleum is a non-refined crude raw material, consisting of a very complex mixture of hydrocarbons and other compounds. On an energy base, as renewable resources have about half the energy content of fossil resources, renewable and fossil resources are roughly equal in price.

At the end of 2009, the oil price was about 75$ per barrel, which is 380€ per ton, about 3 times as much as the price of agricultural commodities such as wheat or corn (around 120€/ton). It is increasingly becoming clear that we are faced with a long-term trend in increasing high petroleum prices instead of a transitional effect (Figure 1) and it is obvious that the use of renewable raw materials has significant growth potential.

Renewable Raw Materials for the Chemical Industry

Renewable raw materials are essentially based on the use of "biomass", the sum of all substances that the living world is made of. Its fundamental basis is plant growth and production, which is fueled by the photosynthesis process, and possibly via the intermediate step of animal production, resulting in a large variety of available biomass.

The total annual biomass production on our planet is estimated at 170 billion tons and consists of roughly 75% carbohydrates (sugars), 20% lignins, and 5% other substances such as oils and fats, proteins, terpenes, alkaloids, etc. [14]. Of this biomass production, 6 billion tons (3.5%) are presently being used for human needs, distributed as:

- 3.7 billion tons (62%) for human food use, possibly via animal breeding as an intermediate step;
- 2 billion tons of wood (33%) for energy use, paper and construction needs;
- 300 million tons (5%) to meet the human needs for technical (non-food) raw materials (clothing, detergents, chemicals, etc.).

The rest of the biomass production is used in natural ecosystems (feed for wild animals), is lost when biomass is obtained for humans (especially by burning), or is lost as a result of natural mineralization processes.

The renewable raw materials discussed here are almost all provided by agriculture and forestry. The animal breeding sector and fisheries also contribute (mainly animal fat), but are clearly less significant both in terms of volume and in view of the low conversion efficiencies of plant to animal (about 10–25%).

A range of different technologies can be used to industrially convert this available biomass into renewable raw materials or energy carriers. This industrial activity is often linked or connected to the food sector, in view of the fact that food ingredients and renewable raw materials for technical use can be made within the same factory from the same agricultural raw materials. For example, sugar or glucose are produced for human food use and are also the most important raw materials for industrial fermentation processes.

The following industrial sectors currently supply the most important renewable raw materials:

- The sugar and starch sector: this produces carbohydrates such as sugar, glucose, starch, and molasses from plant raw materials such as sugar beet, sugar cane, wheat, corn, potatoes, sweet cassava, rice, etc.
- Oil and fat-processing sector: this produces numerous oleo-chemical intermediates such as triglycerides, fatty acids, fatty alcohols, and glycerol from plant raw materials like rape seeds, soybeans, palm oil, coconuts, and animal fats.
- The wood-processing sector, particularly the cellulose and paper industry: this produces mainly cellulose, paper and lignins from wood.

These industries process plant raw materials in order to break them down into separate components such as sugar, starch, cellulose, glucose, proteins, oils, and lignins. They make use of two technological pillars:

- Fractionation technology: this is primarily based on physical and chemical separation methods to separate agricultural raw materials into their separate components.
- Enzymatic technology: this intervenes during the transformation of agricultural raw materials. In practice, mainly hydrolytic enzymes are used, for example amylases, that hydrolyze starch to glucose.

Although these technologies are clearly very different in nature, the interaction between them is particularly decisive for success. For example, the fractionation technology is strongly influenced by the use of hydrolytic enzymes.

The pure basic products obtained (sugar, starch, cellulose, oils) are then converted into a very broad range of products, employing physical, chemical, and biotechnological processes. For example, starch and cellulose are chemically modified to derivatives that find many uses in our daily lives. Sugars such as sucrose and glucose are chemically coupled to oleo-chemicals to obtain detergents and emulsifiers.

With respect to industrial biotechnological processes, fermentation technology needs to be specifically mentioned. This very important key technology makes use of microorganisms (bacteria, yeasts, micro-algae, and fungi) to convert basic raw materials such as sugars and oils into an almost unlimited range of products. By simple use of another production organism, the raw material (for example sugar) can be converted into totally different products, ranging from products with a chemical structure that is very close to the raw material (e.g., gluconic acid from glucose) to products that have virtually nothing in common with the starting material (for example, antibiotics, enzymes, etc.).

This whole chain of different process steps implies the use of very different technologies, often within the same factory or industry complex. These are increasingly referred to as "biorefineries," analogous to the petrochemical crude oil refineries.

For orientation, the estimated world production figures and indicative world market prices of a number of renewable and petrochemical raw materials are given in Table 2. The comparison clearly shows that their volumes and prices are quite comparable.

Table 2 Estimated world production figures and indicative world market price of a number of renewable and petrochemical raw materials.

World production (million tons/year)		World market price (€/ton)
Renewable raw material		
Cellulose	320	500
Sugar	140	250
Starch	55	250
Glucose	30	300
Bioethanol	38	400
Glutamic acid	1	1500
Petrochemicals		
Ethylene	85	500
Propylene	45	350
Benzene	23	400
Terephthalic acid	12	700
Isopropanol	2	700
Caprolactam	3	2000

Bioprocesses in Industrial Biotechnology

Fermentation Processes

Industrial biotechnology is used to produce a wide variety of bulk and fine chemicals such as alcohol, lactic acid, citric acid, vitamins, amino acids, solvents, antibiotics, biopolymers, biopesticides, industrial enzymes, biocolorants, biosurfactants, alkaloids, steroids, etc. Industrial fermentation is the main technology here, whereby microorganisms (bacteria, yeasts, and fungi) are cultivated that efficiently convert sugars into useful products. It is the only industrial production method for several of these products and some are produced in very significant quantities [3, 4, 12]. Table 3 compiles the production figures and prices for a number of these fermentation products. The range varies from inexpensive bulk products to very expensive fine chemicals.

Thanks to recombinant DNA technology, one can now specifically intervene into the genetic material of these microorganisms. On the one hand, the metabolism of microorganisms can be modified or even completely changed (so-called "metabolic engineering"). On the other hand, genes from higher organisms (plants and animals) or other microorganisms (yeast, bacteria, virus, algae) can be inserted into industrial microorganisms and brought to expression. Thus, new direct gene products can be made or new metabolic pathways can be created to produce chemical substances with high efficiency via industrial fermentation processes.

In practise, well-known, productive, and harmless production organisms are used that, equipped with the new genetic information, will produce the desired

Table 3 World production figures and prices for a number of fermentation products.

	World production (ton/year)	World market price (€/kg)
Bioethanol	50 000 000	0.40
L-Glutamic acid (MSG)	1 500 000	1.50
Citric acid	1 500 000	0.80
L-Lysine	800 000	1.5
Lactic acid	250 000	1.5
Vitamin C	80 000	8
Gluconic acid	50 000	1.50
Antibiotics (bulk products)	30 000	150
Antibiotics (specialities)	5 000	1 500
Xanthan	20 000	8
L-Hydroxyphenylalanine	10 000	10
Dextran	200	80
Vitamin B_{12}	3	25 000

chemical products in high yield and efficiency. A major advantage is that these genetically modified microorganisms do their work under controlled conditions in a fermenter or bioreactor, carefully contained and separated from the outside world. They cannot escape from the factory, thus avoiding ecological problems and concerns with regard to the release of genetically modified organisms into the environment.

Enzymatic Processes and Enzyme Technology

Enzymes are catalytically active proteins that have evolved and were perfected over billions of years of evolution. As very specific and efficient catalysts, they direct the chemistry of life without needing the extreme temperatures, high pressures, or corrosive conditions that are often required in chemical synthetic processes. Enzymes are the machinery of the living world and their amazing properties are increasingly used for industrial applications. This technical discipline is referred to as biocatalysis [15].

Enzymes have become very important in a wide range of industrial sectors to carry out biocatalytic reactions. Typically microbial enzymes are used, produced by the previously mentioned fermentation processes. New technologies for enzyme engineering such as directed evolution allow new enzymes to be tailor-made, whereas metagenomics vastly expands the range of natural enzymes to be exploited. These developments can greatly improve this technology or even expand it to totally new applications.

Conventional applications are the large-scale use of enzymes in the starch sector, not coincidentally the sector at the source of glucose, one of the most important renewable raw materials. A key enzyme is α-amylase, a very thermostable enzyme used to hydrolyze starch at a temperature of 105 °C. Such thermostable enzymes allow bioreactions to take place at high temperatures, considerably increasing the reaction rate. Glucose isomerase is another important enzyme in this sector. This enzyme converts glucose to fructose. It is used in immobilized form and maintains its catalytic activity up to 2 years when used industrially. The world production of glucose and fructose syrups with the help of this enzyme exceeds 15 million tons per year.

The detergent sector is another big application area for enzymes. Here, proteases and lipases are used to break down protein and fat stains on clothing.

The animal feed industry is another important market. For example, phytase from the fungus *Aspergillus niger* is employed to release phosphate from phytic acid in animal feed, reducing the need for additional phosphates, with considerable environmental benefits. Other enzymes are used to greatly improve food conversion, with equally positive ecological benefits.

Enzymes are increasingly penetrating the chemical industry to catalyze numerous reactions. The specificity of the enzymatic reaction is very important here. When compared with conventional chemical catalysts, the specificity is often very high. Besides a high degree of reaction specificity, chirality has also stimulated the application of biocatalysts in the chemical industry. The use of enzymes (used in

free or immobilized form) for very specific organochemical reactions is rapidly developing. These are mostly one-step reactions, carried out with high efficiency, specificity, and reaction rate. This scientific domain is often referred to as "biocatalysis" and the processes used are described as "bioconversions" or "biotransformations." These bioconversions are normally performed at normal temperatures and pressures, so that no dangerous intermediate products are needed and no dangerous waste products generated. Typically, the reactions take place in "green" solvents such as water, ethanol, or supercritical CO_2, though enzymes are also active in "conventional" chemical solvents such as methanol, acetone, chlorinated solvents, etc. It should also be mentioned here that the enzymes used in industrial biotechnology and biocatalysis are practically all derived from microorganisms via fermentation. Industrial enzymes represent a 2 billion dollar sector in industrial biotechnology [4, 16].

Economical and Ecological Advantages of Industrial Biotechnology

Introducing biotechnological process steps into chemical syntheses often results in significant ecological advantages such as considerably reduced waste generation, reduced energy requirement, decreased use of solvents, elimination of dangerous intermediate products, etc. However, these ecological advantages are typically not the reason for the technology switch. The technological process improvements and accompanying cost reduction are almost always the driving force for such a decision; the ecological advantages are a pleasant side-effect and, by themselves, are not sufficient to motivate decision-makers to introduce a new technology (with associated failure risk). The way industrial biotechnology combines both economic and ecological progress is quite typical: the increased efficiency and reduced production cost of such biotechnological processes nearly always results in a greatly decreased ecological impact and generally leads to improved competitiveness.

In a 2001 OECD report, 21 case studies were presented [17], each convincingly illustrating the economic and ecological advantages offered by industrial biotechnology. It should be pointed out that in most cases the processes described have been implemented in industrial practise and are competitive; in no way are they limited to theoretical studies or research projects.

Expected Changes in Society and Technology

Several changes in both society and technology are expected to happen in the coming years that may seriously modify the present order. Admittedly, these changes are likely to come about gradually, but may nevertheless give rise to some real "shock waves." If we are to overcome these changes, the technological basis of our society will have to change radically.

Changes in the Supply of Primary Raw Materials

Currently, most organic chemical substances are based on petrochemical resources and our energy needs are also largely met by fossil fuels such as coal, petroleum, and natural gas. This is mainly a consequence of the very low prices for petrochemical resources in the past. However, the world's crude oil reserves will not last forever. The increasing demand for crude oil from a growing world population is now faced with a stagnating production rate. Even though the known oil reserves have remained at about the same level for 30 years as a consequence of new oil findings, these oil reserves are increasingly located in difficult to reach places. Therefore, the costs for extracting the crude oil rise continuously, as reflected in increasing oil prices.

In sharp contrast to this, locally produced agricultural raw materials such as wheat and corn are becoming continually cheaper as a fundamental consequence of rising agricultural yields, gradually tipping the economic balance towards the use of renewable resources. This trend will most likely continue for some time.

Today, on a weight basis, fossil resources are about two to three times more expensive than comparable renewable resources such as corn. Agricultural by-products such as straw are even 10 times cheaper than petroleum. Renewable resources thus offer excellent potential as a raw material for both our chemical needs and our energy needs (biofuels), for reasons of cost, self-sufficiency, sustainable development, and the conservation of natural resources [5, 18, 19]. However, the technologies for the efficient conversion of these renewable resources into useful products are still a major limitation and industrial biotechnology is the key technology in that respect. Whereas conventional chemical processes have reached a high technological maturity and efficiency in using fossil resources, they typically have serious difficulties in using renewable raw materials. Industrial biotechnology processes, in contrast, can handle these renewable resources with amazing ease as microorganisms have no difficulty in converting raw materials, such as, for example, carbohydrates into a wide variety of useful products. Industrial biotechnology is thus an essential technology that needs to be developed for the transition from our present-day fossil-based society to a future sustainable bio-based society.

Increased Demand of a Growing Population for Raw Materials and Energy

Currently, around 80% of all available raw materials and energy are used by approximately 20% of the world population. Naturally, it can be expected that the other 80% of the world population will do everything possible to improve their living standards and thus require much more raw materials and energy than before. Also, one should not forget that the world population keeps growing at an alarming rate. The often cited and erroneously interpreted assertion of "reduced population growth" will only take effect in a few generations at the earliest, an unfortunate consequence of the peculiarities of population growth dynamics. For the time being, the world population continues to grow faster than ever. In

particular, the dynamics of China and India must be taken into consideration, in view of the fact that these population-rich countries are expected to improve their living standards in the short term.

All these effects will inevitably lead to a strong increase in the demand for raw materials and energy. A fair redistribution of the available raw materials is unlikely, so that in the end far more raw materials will be used globally. This will deplete the remaining fossil reserves and other raw materials even faster and bring renewable raw materials and energy to the forefront of attention.

Increased Demand for Efficiency in Chemical Production Systems

The laws of the market economy provide strong pressures to continuously improve the efficiency of all production systems. Wasteful production systems that produced large quantities of waste were still economic in the past, either because this waste could be dumped into the environment or the cost of clean-up was shifted to the society. Nowadays, "the polluter pays" principle means that such processes are doomed. Waste costs money, both to get rid of and because it represents a yield loss with all its associated extra costs. Consequently, a high degree of efficiency and performance is required of all chemical processes today. Biocatalytic methods are particularly efficient and specific, with less waste, raw material use, and energy consumption as a result. The penetration of industrial biotechnology into the chemical industry is almost always motivated by normal economic principles such as cost saving, increased efficiency, etc.

Furthermore, as it can be expected that the factors discussed previously will inevitably result in further price increases for raw materials and energy, the need for efficient chemical processes will grow even stronger. The further penetration of industrial biotechnology into the chemical industry and its synergistic cooperation with conventional chemical technology will no doubt be strongly stimulated.

Growing Need for Sustainability of the Production Systems

The world is faced with the major challenge of developing clean and sustainable production processes that respect the environment, improve our quality of life and at the same time are competitive in the marketplace. This includes the development of new production processes that reduce or eliminate the use of dangerous or hazardous substances, minimize energy consumption and waste generation, and ideally start from renewable raw materials. Industrial biotechnology is particularly well positioned to play a key role in this quest for sustainability. Bioprocesses generally produce less waste and the use of toxic and dangerous chemicals can sometimes be completely eliminated by using an enzyme. In general, industrial biotechnology typically leads to a significantly reduced environmental footprint of industrial manufacturing.

Since the Kyoto treaty, most industrialized nations have been obliged to respect a number of base criteria with respect to raw material use and energy policy. Many

countries have undertaken efforts within the framework of the Kyoto treaty. The negotiability of CO_2 emission rights is now a fact and the first penalties for exceeding the norms soon will become effective. This is expected to result in a fundamental change in perception on the use of raw materials and energy consumption. It is clear that renewable raw materials that are CO_2 neutral, such as biomass, will benefit from this development.

Changing Consumer Perceptions and Behavior

In most developed societies, there is a growing demand from consumers for information about the products they buy and this goes beyond quality and price. Nowadays, a growing segment of consumers also care more about the production systems by which their products are made (i.e., organic farming, animal welfare, etc.) and about what happens with them after their use (waste, degradability, etc.). Production systems that cause ecological damage or animal suffering, are based on inequitable trade or exploitation (such as child labor), etc. are being increasingly rejected by consumers, even if they happen far away and strictly speaking do not directly burden or harm them directly. Consumers are looking for goods and services that are obtained and used under socially and economically acceptable circumstances, and do not raise any ethical or emotional dilemmas.

The European public's refusal of genetically modified crops is of this nature. It is ironic that the consumers who reject genetically modified crops are often exactly the same consumers that use without grumbling and even with enthusiasm products made by means of fermentation processes, such as fermented milk products or Quorn, a mycoprotein from fungi. Consumer perception of chemical products in foods (preservatives, colorants, antioxidants) is also very negative and natural alternatives are demanded. One can therefore expect increasing replacement of these "chemical" products by products obtained by industrial biotechnology.

Industrial biotechnology has the potential to be perceived as working in concert with nature. People generally like the idea of using biomass instead of petroleum and biological processes instead of more conventional synthesis. So bio-processes are not only cost-competitive and offer ecological benefits, they also have a public acceptance edge over classical processes. Furthermore, industrial biotechnology and its processes are mostly performed in contained production systems and the used microorganisms are harmless or cannot survive in nature.

Changes in the Agricultural System

It is clear that the agricultural sector will need to adapt continuously to new needs and problems, under the pressure of consumers, governments as well as foreign forces that wish to reduce import restrictions [20]. Particularly in Europe, the expansion of the European Union with a large number of new member-states from Eastern Europe will put a lot of additional pressure on the system.

Some guidelines for the change can be clearly distinguished. What is needed is:

- agriculture that is less oriented to (mass) production but more directed towards high-quality agricultural products;
- agriculture with more respect for the environment;
- reduction or abolition of production-related subsidies for farmers;
- reduced import taxes for a number of agricultural products to allow developing countries to market their products in Europe;
- more diversity of agricultural systems, such as new agricultural crops and new production systems, such as organic agriculture;
- stimulation for the production of agricultural crops for non-food purposes.

The use of agricultural raw materials as a renewable raw material for the chemical industry and for fuel clearly meets the many expectations. Consequently, these developments are warmly welcomed, particularly by the agricultural community that has clearly understood the importance of industrial biotechnology.

Conclusions and Perspectives

Industrial biotechnology can synthesize a broad range of chemical substances, usually using useful microorganisms and their enzymes. The recent wave of new applications seems to indicate that only the tip of the iceberg has been touched. The microbiologist Jackson Foster predicted in 1964: "Never underestimate the power of the microbe" and has been proven right so far.

For now, some bottlenecks still remain. It is self-evident that replacing a "hydrocarbon economy" with a "carbohydrate economy" is not going to be easy or cheap! In addition to convincing the petrochemical sector, it remains important to know the total production costs and return of biomass production and use for the different applications, before investments can be made. The potential for valorization of waste biomass and by-products from other bio-processes may in many cases have a significant impact on economic feasibility.

This technology, already strongly developed in conventional domains of the food and healthcare industry, now also strongly penetrates the chemical industry with applications in fine and bulk chemistry, polymer synthesis, pharmaceutical industry, and the energy sector. As these processes and products are largely based on renewable raw materials and possess substantial ecological benefits, this provides them with a major advantage in the perspective of sustainable development.

Science, industry, and policy people alike should give more attention to this green chemistry and its bio-products. Successful innovation through a biotechnological product or process is never solely defined by technology and science but equally by other factors such as acceptance by the general public, the innovation climate, and support by the authorities through a consistent R&D policy.

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1
History of Industrial Biotechnology

Arnold L. Demain

1.1
Early History

The practise of industrial biotechnology has its roots deep in antiquity. Long before their "discovery," microorganisms were exploited to serve the needs and desires of humans, for example to preserve milk, fruits, and vegetables, and to enhance the quality of life by producing beverages, cheeses, bread, pickled foods, and vinegar. The use of yeasts dates back to ancient days. The oldest fermentation know-how – the conversion of sugar to alcohol by yeasts – was used to make beer in Sumeria and Babylonia as early as 7000 BC. By 4000 BC, the Egyptians had discovered that carbon dioxide generated by the action of brewer's yeast could leaven bread. Ancient peoples are also known to have made cheese with molds and bacteria.

Another ancient product of fermentation, wine, was made in Assyria in 3500 BC and reference to wine can be found in the Book of Genesis, where it is noted that Noah consumed a bit too much of the beverage. According to the Talmud, "a man without salt and vinegar is a lost man." In the field of human health, vinegar has a long history of use: the Assyrians used it to treat chronic middle ear diseases, Hippocrates treated patients with it in 400 BC, and, according to the New Testament, vinegar was offered to Jesus on the cross.

The use of microorganisms in food also has a long history. In 100 BC, Ancient Rome had over 250 bakeries which were making leavened bread. As a method of preservation, milk was fermented to lactic acid to make yogurt and also converted into kefyr and koumiss using *Kluyveromyces* species in Asia. The use of molds to saccharify rice in the koji process dates back at least to AD 700. By the fourteenth century AD, the distillation of alcoholic spirits from fermented grain, a practise thought to have originated in China or the Middle East, was common in many parts of the world. Vinegar manufacture began in Orleans, France, at the end of the fourteenth century and the surface technique used is known as the Orleans method.

In the seventeenth century, Antonie van Leeuwenhoek, a Dutch merchant with no university training but a keen amateur interest in the construction of microscopes, turned his simple lens to the examination of water, decaying matter,

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and scrapings from his teeth. There he reported on the presence of tiny "animalcules," moving organisms less than a thousandth the size of a grain of sand. Leeuwenhoek's lack of university connection might have caused his discoveries to go unrecognized had it not been for the Royal Society in England and its secretary, Henry Oldenburg, who corresponded with European science amateurs. From 1673 to 1723, Leeuwenhoek's observations as a microscopist were communicated to the Society in a series of letters.

Most scientists at the time thought that microbes arose spontaneously from non-living matter. What followed was a 100-year debate over spontaneous generation, aptly called the "War of the Infusions." Proponents had previously claimed that maggots were spontaneously created from decaying meat, but this theory was disputed by Italian physician, Francesco Redi. By this time, the theory of spontaneous generation, originally postulated by Aristotle, among others, had been discredited with respect to higher forms of life, so the proponents concentrated their arguments on bacteria. The theory did seem to explain how a clear broth became cloudy via growth of large numbers of "spontaneously generated microorganisms" as the broth aged. However, others believed that microorganisms only came from previously existing microbes and that their ubiquitous presence in air was the reason that they would develop in organic infusions after gaining access to these rich liquids.

In the early nineteenth century, three independent investigators – Charles Cagniard de la Tour of France, Theodor Schwann, and Friedrich Traugott Kützing of Germany – proposed that the products of fermentation, chiefly ethanol and carbon dioxide, were created by a microscopic form of life. This concept was bitterly opposed by the leading chemists of the period (such as Jöns Jakob Berzelius, Justus von Liebig, and Friedrich Wöhler), who believed fermentation to be strictly a chemical reaction; they maintained that the yeast in the fermentation broth was lifeless, decaying matter.

Organic chemistry was flourishing at the time, and these opponents of the theory of living microbial origins were initially quite successful in putting forth their views. Interest in the mechanisms of these fermentations resulted in the later investigations by Louis Pasteur (Figure 1.1), which not only advanced microbiology as a distinct discipline, but also led to the development of vaccines and concepts of hygiene which revolutionized the practise of medicine.

In 1850, Casimir Davaine detected rod-shaped objects in the blood of anthrax-infected sheep and was able to produce the disease in healthy sheep by inoculation of such blood. In the next 25 years, Pasteur of France and John Tyndall of Britain finally demolished the concept of spontaneous generation and proved that existing microbial life came from preexisting life.

The work of Pasteur originated in his work as a chemist on chirality. In the 1850s, Pasteur had detected two optical types of amyl alcohol, that is, D and L, but he was not able to separate the two. For this reason, he began to study living microbes carrying out fermentation which led to his conclusion, in 1857, that fermentation was a living process of yeast. In 1861, Pasteur proved the presence of microbes in the air, which discredited the theory of spontaneous generation of

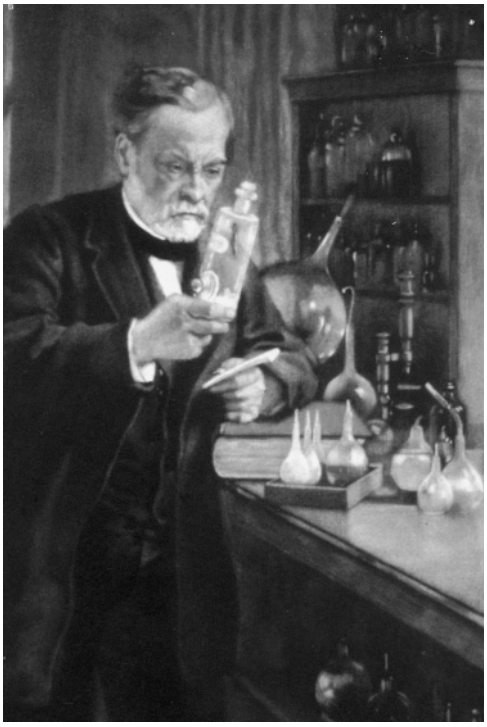

Figure 1.1 Louis Pasteur.

microbes, and it was at this point that fermentation microbiology was born. Nevertheless, it took almost two decades, until 1876, to disprove the chemical hypothesis of Berzelius, Liebig, and Wöhler (i.e., that fermentation was the result of contact with decaying matter).

In 1876, the great German microbiologist Robert Koch (Figure 1.2) proved that bacteria from anthrax infections were capable of causing the disease. His contributions involving the growth of microbes in pure culture led to the decline of the pleomorphism theory, which postulated that one form of bacteria developed into another. It was mainly Koch's work that led to the acceptance of the idea that specific diseases were caused by specific organisms, each of which had a specific form and function. In 1884, his students Gaffky and Loeffler were able to confirm the etiologic role of infectious bacteria in typhoid fever and diphtheria.

Pasteur was called on by the distillers of Lille to find out why the contents of their fermentation vats were turning sour. Using his microscope, he noted that the fermentation broth contained not only yeast cells, but also bacteria, and he already knew that these could produce lactic acid. This observation led to his suggestion that such souring could be prevented by a mild heat treatment, which later became known as "pasteurization."

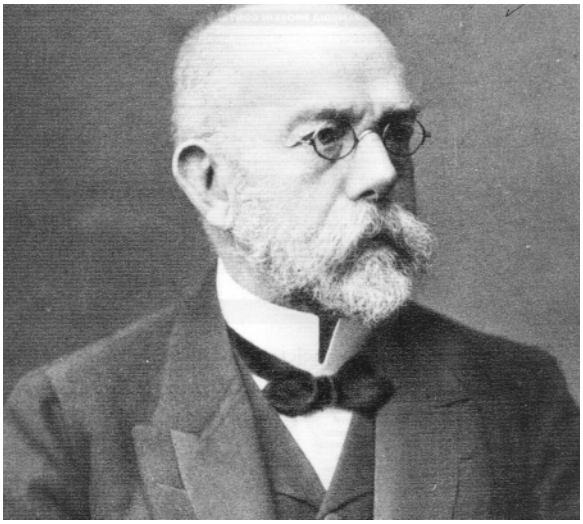

Figure 1.2 Robert Koch.

One of Pasteur's greatest contributions was to establish that each type of fermentation was mediated by a specific microorganism. Furthermore, in a study undertaken to determine why French beer was inferior to German beer, he demonstrated the existence of life forms that were strictly anaerobic. Interest in the mechanisms of these fermentations resulted in the later investigations by Pasteur which not only advanced microbiology as a distinct discipline, but also led to the development of vaccines and concepts of hygiene which revolutionized the practise of medicine.

With the establishment of the germ theory of disease by Pasteur and Koch, the latter half of the nineteenth century was characterized by the fight against disease and the attention of microbiologists was directed towards aspects of microbiology concerned with medicine and sanitation. This resulted in the discovery that the human body has its own defenses in the fight against pathogenic microbes. Pasteur and Koch, among others, found that upon invasion by a bacterium, proteins (i.e., antibodies) are formed in the bloodstream that can specifically neutralize the invading organism. Thus, the science of immunology was founded. By injecting either dead forms or attenuated forms of the disease-producing bacterium, Pasteur could render the individual immune to the disease. The production of these vaccines occupied much of the early research in microbiology.

During Pasteur's life, the application of antiseptics was introduced. It had been shown in 1846 by Ignaz Semmelweis that chlorine could control infection, and in 1865, Joseph Lister showed that the same could be done with carbolic acid. Later, Paul Ehrlich used synthetic dyes and established the concept of the "magic bullet." Towards the end of the nineteenth century, Ehrlich began testing many synthetic compounds. He achieved success in 1909, curing relapsing fever, syphilis, and

trypanosomiasis with an arsenical product called Salvarsan or Compound 606 (because it was his 606th attempt to produce an arsenical compound which killed the syphilis bacterium *in vivo* without harming the host). This was the first chemotherapeutic drug ever discovered and he coined the term "chemotherapy."

This use of drugs selectively toxic to the parasite but not harmful to the host opened an entirely new field for the curing of human diseases. In 1927, this work was continued by Gerhard Domagk in Germany [1] along with his collaborators Mietzsch and Klarer. They were working at the I.G. Farbenindustrie which was the result of a 1924 merger between Bayer and BASF. Their work resulted in the development of the red-colored molecule "prontosil rubrum." This compound was active in mice against streptococci but strangely was not active *in vitro*. Then in 1935, Trefouel and coworkers in France discovered that the red dye was broken down in the animal to the colorless and inhibitory sulfanilamide. This established the important concept that chemicals could kill or inhibit bacteria without toxicity to humans. Although the Nazi government refused to permit Domagk to accept the Nobel Prize in 1939, it was later accepted by him in 1947. Other synthetic chemotherapeutic drugs gained widespread use over the years, including isonicotinic acid hydrazide and para-aminosalicylic acid, both for tuberculosis.

Another discovery in the nineteenth century concerned the way microorganisms interact with one another. For thousands of years, moldy cheese, meat, and bread had been employed in folk medicine to heal wounds. Then in the 1870s, Tyndall, Pasteur, and William Roberts, a British physician, directly observed the antagonistic effects of one microorganism on another. Pasteur, with his characteristic foresight, suggested that the phenomenon might have some therapeutic potential. For the next 50 years, various microbial preparations were tried as medicines, but they were either too toxic or inactive in live animals. This led to the pivotal moment in microbiological history when, in 1927, Alexander Fleming discovered penicillin (see Section 1.2).

In 1877, Moritz Traube proposed that (i) protein-like materials catalyzed fermentation and other chemical reactions and (ii) they were not destroyed by doing such things. This was the beginning of the recognition of what we call enzymes today. He also proposed that fermentation was carried out via multistage reactions in which the transfer of oxygen occurred from one part of a sugar molecule to another, finally forming some oxidized compound (e.g., carbon dioxide) and a reduced compound (e.g., alcohol).

The field of biochemistry became established in 1897 when Eduard Buchner found that cell-free yeast extracts, lacking whole cells, could convert sucrose into ethanol. Thus, the views of Pasteur were modified and it became understood that fermentation could also be carried out in the absence of living cells.

During World War I, the need for glycerol, used to manufacture ammunition, resulted in the application of yeast to convert sugars into glycerol. This development led after the war to an exhaustive study by Neuberg of the mechanisms involved in these reactions and those converting sugars to ethanol. This was followed by the studies of Dutch scientists in Delft looking at oxidation/reduction reactions and the kinetics of enzyme-catalyzed reactions.

Figure 1.3 Chaim Weizmann.

Also during World War I, Chaim Weizmann (Figure 1.3) at the University of Manchester applied the butyric acid bacteria used for centuries for the retting of flax and hemp, for production of acetone and butanol. His use of *Clostridium* to produce acetone and butanol was the first non-food fermentation developed for large-scale production; with it came the problems of viral and microbial contamination that had to be solved. Although use of this fermentation faded because it could not compete with chemical methods of solvent production, it did provide a base of experience for the development of large-scale cultivation of fungi for production of citric acid. Soon after World War I, an aerobic process was devised in which *Aspergillus niger* was used. Not too many years later, the discoveries of penicillin and streptomycin and their commercial development heralded the start of the antibiotic era.

1.2 The Penicillin Story

The golden era of antibiotics began with the accidental discovery of penicillin by Alexander Fleming in 1929 in England [2]. He noted that some of his plates con-

Figure 1.4 Howard W. Florey.

taining *Staphylococcus aureus* were contaminated with a mold, *Penicillium notatum*, and was surprised to see that none of the bacterial colonies could grow in the vicinity of the mold. Fleming concluded that the mold was producing some kind of inhibitory agent. He also observed that filtrates of the mold lyzed the staphylococci and were non-toxic in animals. He named the agent penicillin. Since the activity was very unstable and Fleming could get no encouragement from his fellow scientists concerning the usefulness of such material, the project was abandoned.

The importance of Fleming's discovery was that it led to penicillin, the first successful chemotherapeutic agent produced by a microbe, thus initiating the golden age of the wonder drugs. However, the road to the development of penicillin as a successful drug was not an easy one. For a decade, it remained as a laboratory curiosity, and an unstable curiosity at that. Attempts to isolate penicillin were made in the 1930s by a number of British chemists, but the instability of the substance frustrated their efforts. When World War II arrived and many British soldiers were dying on the battlefield from bacterial infections after being wounded, a study of penicillin began in 1939 at the Sir William Dunn School of Pathology of the University of Oxford by Howard W. Florey (Figure 1.4), Ernst B. Chain (Figure 1.5), Norman Heatley (Figure 1.6), Edward Abraham, and their colleagues.

Figure 1.5 Ernst B. Chain.

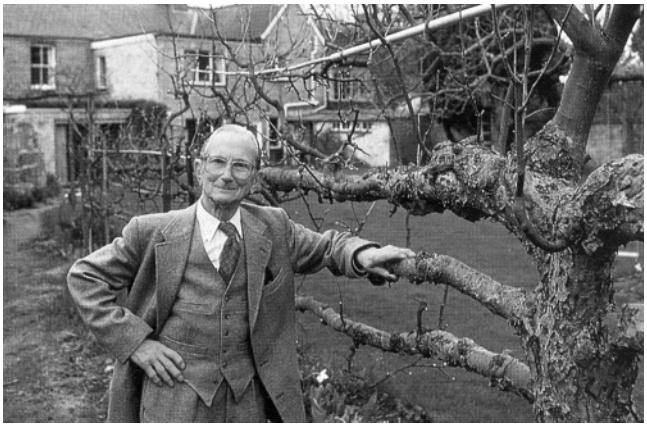

Figure 1.6 Norman Heatley.

This effort led to the successful preparation of a stable form of penicillin and the demonstration of its remarkable antibacterial activity and lack of toxicity in mice.

Unfortunately, production of penicillin by the strain of *P. notatum* in use was so slow that it took over a year to accumulate enough material for a clinical test

on humans [3]. When the clinical tests were found to be successful, however, large-scale production became essential, prompting Florey and his colleague to go to the United States in the summer of 1941 to seek assistance. There they convinced the Northern Regional Research Laboratory (NRRL) of the US Department of Agriculture (USDA) in Peoria, Illinois, and several American pharmaceutical companies (including Merck, Squibb, and Pfizer) to develop the commercial production of penicillin. Heatley remained for a period at the NRRL to work with Moyer and Coghill [4]. Thus began a momentous cooperative effort among university and industrial laboratories in the United States and academic institutions in the United Kingdom which lasted throughout the War.

The result was that thousands of lives were saved, on and off the battlefield. The discovery and development of the beta-lactam antibiotics was among the most powerful achievements of modern science and technology. Since Fleming's accidental discovery of the penicillin-producing mold, years of steady progress followed, and today, the beta-lactam group of compounds is one of the most successful examples of natural product application and chemotherapy.

In the 1940s, a period of intense development in microbial genetics began [5]. Although Fleming's original strain produced only traces of penicillin, "brute force" genetic manipulation made tremendous strides in production ability and led to a whole new technology known as "strain improvement." These early genetic studies concentrated heavily on the production of mutants and the study of their properties. The ease with which "permanent" characteristics of microorganisms could be changed by mutation and the simplicity of the mutation technique had tremendous appeal to microbiologists. A cooperative "strain-selection" program was established between the workers at the USDA in Peoria, the Carnegie Institution, Stanford University, and the University of Wisconsin.

Strain selection began with *Penicillium chrysogenum* NRRL 1951, the well-known isolate from a moldy cantaloupe obtained in a Peoria market. This strain was capable of producing 60 μg/ml. Cultivation of spontaneous sector mutants and single-spore isolations led to more productive cultures from NRRL 1951. One of these, NRRL 1951-1325, produced 150 μg/ml. It was next subjected to X-ray treatment by Demerec of the Carnegie Institution at Cold Spring Harbor, New York, and mutant X-1612 was obtained. This yielded 300 μg/ml. Workers at the University of Wisconsin then obtained ultraviolet-induced mutants of Demerec's strain. One of these, Q-176, which produced 550 μg/ml, became the ancestor of all of the strains used in industry. The "Wisconsin family" of superior strains became well known all over the world, some producing over 1800 μg/ml. The penicillin improvement effort was the start of a long engagement between genetics and industrial microbiology which ultimately demonstrated that mutation was the major factor involved in the hundred- to thousand-fold increases obtained in the production of microbial metabolites.

Originally, penicillin had been produced in surface culture, but it was found that titers were very low. Submerged culture soon became the method of choice. By the use of strain improvement and medium modifications, such as the use of corn steep liquor as additive, the yield of penicillin was increased by 100-fold in

Figure 1.7 Marvin Johnson.

just a few years. Much of the understanding of the physiology of *P. chrysogenum* in relation to penicillin production was achieved by Professor Marvin Johnson (Figure 1.7) and his students at the University of Wisconsin. Further clinical successes were demonstrated in both the United Kingdom and the United States, and finally, in 1943, penicillin was used to treat those wounded in battle.

By the 1950s, it was realized that *P. chrysogenum* could use additional acyl compounds as side-chain precursors (other than phenylacetic acid for penicillin G) and produce new penicillins; one of these, penicillin V (phenoxymethylpenicillin), achieved commercial success. Its commercial application resulted from its stability to acid which permitted oral administration, an advantage it held over the accepted article of commerce, penicillin G (benzylpenicillin). Penicillin G and penicillin V became the main penicillins of commerce. In commercial production, the usual medium had been a complex one, composed of glucose, corn steep liquor, side-chain precursor (phenylacetic acid for penicillin G or phenoxyacetic acid for penicillin V), and mineral salts. The earliest recognition that glucose had a negative effect on penicillin biosynthesis was made by Johnson and his students [6, 7]. Because they found glucose to be excellent for growth but poor for penicillin formation, while lactose showed the opposite pattern, they devised a medium containing both sugars in which growth occurred at the expense of glucose and when it

was exhausted, the mass of cells began to produce the antibiotic at the expense of lactose. Unlike glucose, lactose was utilized slowly and did not exert carbon catabolite repression on the process. Then, Davey and Johnson [8] found that intermittent or continuous feeding of the less expensive glucose could replace batch feeding of lactose. This represented the birth of the fed-batch fermentation which is commonplace in the fermentation industry today.

The biosynthesis of penicillin from its precursors, L-cysteine, L-valine, and phenylacetic acid, was actively studied during the 1950s, 1960s, and 1970s. Of great interest was the relationship between L-lysine and penicillin formation. In 1947, David Bonner had made the observation that 25% of the lysine auxotrophs that he had made from *P. chrysogenum* failed to make penicillin and he predicted that (i) there was some relationship between the antibiotic and the amino acid and (ii) there was a common precursor of the two compounds. That he was absolutely correct was established ten years later when Demain [9] found that lysine was a potent inhibitor of penicillin biosynthesis. That the inhibition could be reversed by L-α-aminoadipic acid led to the postulations [10] that (i) L-α-aminoadipic acid was involved in penicillin biosynthesis although it did not end up in the final penicillin molecule, (ii) penicillin was derived from α-ketoglutarate and acetyl-CoA via the fungal lysine biosynthetic pathway, and (iii) lysine inhibition of penicillin biosynthesis was due to feedback inhibition by lysine of its own biosynthetic pathway, thus limiting L-α-aminoadipic acid formation. Independently, Arnstein and colleagues [11] detected the tripeptide δ-(α-aminoadipyl)-cysteinylvaline (ACV) as an intracellular compound in *P. chrysogenum*. Results in several laboratories established L-α-aminoadipic acid as an important precursor of all penicillins. Soon, δ-(α-L-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) was established as the crucial intermediate of penicillin biosynthesis. The reaction sensitive to feedback inhibition by lysine was later proven to be the initial step of lysine biosynthesis in fungi, that is, the homocitrate synthase reaction [12, 13].

During the 1950s, the future of penicillins became doubtful as resistant strains of *Staphylococcus aureus* emerged in hospital populations. The staphylococcal population was building up resistance to penicillin via selection of penicillinase-producing strains, and new drugs were clearly needed to combat these resistant forms. The penicillins described up to this point were solvent-soluble, exhibiting a high degree of activity against Gram-positive organisms, but were much less active against Gram-negatives. Fortunately, two developments occurred which led to a rebirth of interest in the penicillins and related antibiotics. One was the 1959 discovery in Japan by Koichi Kato of the accumulation of the "penicillin nucleus" in *P. chrysogenum* broths to which no side-chain precursor had been added [14]. In the United Kingdom, Batchelor *et al.* [15] isolated the compound 6-aminopenicillanic acid (6-APA) which was the "penicillin nucleus" discovered by Kato. 6-APA was used to make "semi-synthetic" (i.e., chemical modification of a natural antibiotic) penicillins with the benefical properties of resistance to penicillinase and to acid, plus broad-spectrum antibacterial activity.

The second development was the discovery of a completely different type of penicillin, a hydrophilic type, showing equivalent activity against both classes of

microorganisms (i.e., penicillin N). This compound was discovered independently by two groups of workers. Brotzu in 1948 [16] published his work in an unknown Sardinian journal on the isolation of an antibiotic-producing culture of the fungus *Cephalosporium acremonium* (later reclassified as *Acremonium chrysogenum*) from sewage. Unable to purify the antibiotic material, he sent the culture to Florey at Oxford where, some ten years earlier, *P. notatum* had been at a similar stage in history. While the British scientists were studying the components of this antibiotic complex, workers at the Michigan Department of Health in the United States [17] announced that a species of *Tilachlidium* produced a new antibiotic which they called "synnematin." After the culture was reclassified as *Cephalosporium salmosynnematum* [18], it was shown that synnematin was a complex of two components, A and B. Almost nothing was ever published on the A component, and we have no knowledge today about its structural relationship to synnematin B. While the above work was going in Michigan, the British announced [19, 20] that Brotzu's strain produced two antibiotics, that is, "cephalosporin P," active only against Gram-positive organisms and "cephalosporin N," which was active against both Gram-positive and Gram-negative bacteria. "Cephalosporin P" was found to be of steroidal nature and not a β-lactam at all. "Cephalosporin N," on the other hand, was found by Abraham and coworkers [21] (Figure 1.8) to be a true penicillin possessing an α-D-aminoadipyl side-chain, and to be identical to synnematin B [22]. It was renamed "penicillin N." In comparison to penicillin G, it was only 1% as active against Gram-positive forms, but had equal to or somewhat greater activity against Gram-negative bacteria. The hydrophilic nature of penicillin N and its roughly equivalent activity against Gram-positive and Gram-negative bacteria were due to the carboxyl group in the side-chain.

Figure 1.8 Guy G.F. Newton and Edward P. Abraham.

The tremendous success attained in the battle against disease with penicillin G not only led to the Nobel Prize being awarded to Fleming, Florey, and Chain, but to a new field of antibiotic research, and a new antibiotic industry. Penicillin opened the way for the development of many other antibiotics, and it still remains the most active and one of the least toxic of these compounds. Today, about 100 antibiotics are used to combat infections in humans, animals, and plants.

1.3 The Coming of the Cephalosporins

A key breakthrough was the finding in Edward Abraham's laboratory at Oxford that a second antibiotic was produced by Brotzu's strain of *A. chrysogenum*. After his important contributions as part of the Florey penicillin team, Abraham had established an independent laboratory at Oxford. Abraham and Newton [23] found the new compound to be related to penicillin N in that it consisted of a β-lactam ring attached to a side-chain which was identical to that of penicillin N, that is, D-α-aminoadipic acid. It differed, however, from the penicillins in containing a six-membered dihydrothiazine ring in place of the five-membered thiazolidine ring of the penicillins. It was called cephalosporin C. Thus, the era of the cephalosporins was launched.

The nucleus of cephalosporin C was named 7-aminocephalosporanic acid (7-ACA). Cephalosporin C strongly absorbed ultraviolet light, was stable to acid and to penicillin β-lactamase, was non-toxic, and had *in vivo* activity in mice. Its mode of action was the same as that of the penicillins; that is, inhibition of bacterial cell wall formation. Although neither penicillin N nor cephalosporin C was ever commercialized, they led to important knowledge on the biosynthesis of these compounds and the development of many powerful semi-synthetic cephalosporins of great use in medicine.

The stability of cephalosporin C to penicillin β-lactamase was a very attractive property. The main disadvantage of the molecule was its weak activity; it had only 0.1% of the activity of penicillin G against sensitive staphylococci, although its activity against Gram-negative bacteria equaled that of penicillin G. However, by chemical removal of its D-α-aminoadipic acid side-chain and replacement with phenylacetic acid, a penicillinase-resistant semi-synthetic compound was obtained which was 100 times as active as cephalosporin C. Many other new cephalosporins with wide antibacterial spectra were developed in the ensuing years, for example, cephalothin, cephaloridine, and cephaloglycin, making the semi-synthetic cephalosporins the most important group of antibiotics at that time. The stability of the cephalosporins to penicillinase was evidently a function of its dihydrothiazine ring since (i) the D-α-aminoadipic acid side-chain did not render penicillin N immune to attack and (ii) removal of the acetoxy group from cephalosporin C did not decrease its stability to penicillinase. Cephalosporin C competitively inhibited the action of penicillinase from *Bacillus cereus* on penicillin G. Although it did not have a similar effect on the *S. aureus* enzyme, certain of its derivatives did. Another

advantage was that cephalosporins could be given to some patients who were allergic to penicillins.

From a biosynthetic sense, the relationship of penicillin N and cephalosporin C was of great interest. An important development that led to rapid progress in this area was the subcellular work done by Abraham and his Oxford colleagues in the 1970s [24]. They used protoplast lysates of *A. chrysogenum* to convert labeled valine into a penicillin. This led to the discovery at Massachusetts Institute of Technology in 1976 of the ring expansion reaction [25], catalyzed by the "expandase" (deacetoxycephalosporin C synthase; DAOCS) enzyme. For many years, it had been thought that penicillin N and cephalosporin C were products of different biosynthetic branches in *A. chrysogenum*. However, the discovery of the ring expansion enzyme showed that cephalosporins were produced from a penicillin. This was confirmed by Yoshida *et al.* in 1978 [26], who showed that the ring expansion enzyme converted penicillin N to deacetoxycephalosporin C. Extracts of mutants which fermentatively produced penicillin N but not cephalosporins failed to carry out this reaction, whereas early blocked mutants (negative for production of both penicillin N and cephalosporins) did expand the ring.

From the 1970s through the 1980s, knowledge accumulated concerning fermentation and biosynthesis of cephalosporin C. Of major importance was the (i) stimulation by DL-methionine via a regulatory mechanism unrelated to its ability to contribute the sulfur atom to the antibiotic [27], (ii) use of acetate as precursor to the acetoxy group [28], (iii) L-cysteine and L-valine [29] as precursors of the nucleus and (iv) L-α-aminoadipic acid as precursor of the D-α-aminoadipyl side-chain of cephalosporin C [30]. An important step forward was provided by Banko *et al.* [31] when they demonstrated that the cell-free activity of the enzyme from *A. chrysogenum* formed the important tripeptide precursor of all penicillins and cephalosporins, that is, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV). The enzyme ACV synthetase was proven to be a single multifunctional enzyme acting on L-α-aminoadipic acid, L-cysteine, and L-valine to produce LLD-ACV. Also of importance was the isolation by Hollander *et al.* [32] of pure isopenicillin N synthetase ("cyclase") which converted the LLD-ACV to isopenicillin N.

The long-held notion that β-lactams were produced only by fungi was shattered by a report from Merck & Co. that a streptomycete produced penicillin N [33]. This provocative finding was presented at the 1962 American Society for Microbiology Annual Meeting and was only published as an abstract. Although much doubt was cast on this report, two reports from Eli Lilly & Co. and Merck published some 9–10 years later [34, 35] reported that various species of *Streptomyces* and *Nocardia* produced cephalosporins modified at C7 (= cephamycins) and/or at the side-chain attached to C3. The discovery of cephamycin C led to much research on and development of prokaryotic cephalosporins since the presence of the methoxy group on the β-lactam ring made the molecule more active against Gram-negative and anaerobic pathogens and more resistant to Gram-negative β-lactamases. For the first time in the history of the β-lactams, molecules were available which showed a high degree of stability to these troublesome enzymes. Like fungal cephalosporin C, cephamycin C was never used clinically but was employed

for semi-synthesis of many medically useful compounds. A more potent semi-synthetic cephamycin, cefoxitin, was rapidly commercialized by Merck, to be followed later by cefmetazole, temocillin, cefotetan, and other semi-synthetic cephalosporins.

In the 1970s to the 1980s, the pathways to the penicillins and the cephalosporins including cephamycin C were worked out, especially after cell-free systems became available [24, 36]. Late in the 1970s came reports on the production of β-lactam antibiotics which were neither penicillins nor cephalosporins. The most important was clavulanic acid from streptomycetes, which possessed only weak antibiotic activity but was an excellent inhibitor of β-lactamase (see Section 1.8.1). It became a blockbuster compound by being co-formulated with broad-spectrum semi-synthetic penicillins that are susceptible to β-lactamase, for example, with amoxycillin, the combination is known as Augmentin.

Another important development in the history of the β-lactam antibiotics was the discovery at Merck of the the carbapenems. The first, called thienamycin, was discovered by Kahan *et al.* [37] with a screening protocol based on inhibition of peptidoglycan synthesis. The antibiotic was produced by *Streptomyces cattleya*, which also made cephamycin C. Carbapenems resembled the penicillins in having a β-lactam ring fused to a five-membered ring. They differed in that the five-membered ring was unsaturated and contained a carbon atom instead of the sulfur. Sulfur was, however, present in another location in all the carbapenems produced by streptomycetes. A large number of carbapenems were reported, but thienamycin was the most important. Indeed, it was the most potent, most broad-spectrum and non-toxic natural antibacterial agent ever found. It inhibited cell wall synthesis, as did the penicillins and cephalosporins, and was relatively resistant to microbial β-lactamases.

The development of commercial process technology for the penicillin nucleus (6-APA) and the cephalosporin nucleus (7-ACA) opened the way for chemical acylation with various side-chains yielding many improved semi-synthetic penicillins and cephalosporins with broader antibacterial activity and improved pharmacokinetic properties. Broad-spectrum penicillins and cephalosporins became the best-selling antibacterial agents in the pharmaceutical arena.

1.4 The Waksman Era

The advent of penicillin, which signaled the beginning of the antibiotic era, was closely followed in the 1940s by the discoveries of Selman A. Waksman (Figure 1.9), a soil microbiologist at Rutgers University. He and his students, especially H. Boyd Woodruff, Albert Schatz, and Hubert Lechevalier, succeeded in discovering many new antibiotics from the filamentous bacteria, the actinomycetes, such as actinomycin D, neomycin, and the best-known of these new "wonder drugs," streptomycin. These discoveries on the antibiotic-producing abilities of the actinomycetes occurred long before the developments, described above, of

Figure 1.9 Selman A. Waksman and H. Boyd Woodruff.

β-lactams produced by these filamentous bacteria. Waksman and Woodruff published in 1940 on the discovery of the actinomycins, which were chromooligopeptides [38]. One such compound, actinomycin D, was used for years to combat Wilms tumor in children and became a very important tool in the development of molecular biology as an inhibitor of RNA polymerase.

After its monumentous discovery in 1944 by Waksman, Schatz, and Bugie [39] as a product of *Streptomyces griseus*, streptomycin was used against tuberculosis caused by *Mycobacterium tuberculosis* and also against Gram-negative bacteria; bacterial meningitis was also treated with streptomycin. Its major impact on medicine was recognized by the award of the Nobel Prize to Waksman in 1952. As the first commercially successful antibiotic produced by an actinomycete, this aminoglycoside led the way to the recognition of these organisms as the most prolific producers of antibiotics. Streptomycin also provided a valuable tool for studying cell function. After a period of time during which it was thought to act by altering permeability, its interference with protein synthesis was recognized as its primary effect. Its interaction with ribosomes provided much information on their structure and function; it not only inhibited their action but also caused misreading of the genetic code and was required for the function of ribosomes in streptomycin-dependent mutants.

With Lechevalier, Waksman reported on the discovery of neomycin in 1948 [40] and candicidin in 1953 [41]. Neomycin, an aminoglycoside produced by *Streptomyces fradiae*, served as a topical antibacterial, and the polyene candicidin, made by *S. griseus*, found use as a topical antifungal antibiotic.

Cooperation on the development of industrial processes between Rutgers University, Princeton University, Columbia University, and Merck & Co., Inc. led to the birth of the field of biochemical engineering. With royalties on streptomycin turned over to Rutgers University by the manufacturer, Merck, Waksman was able to build the world-famed Institute of Microbiology.

The discoveries of the aminoglycosides at Rutgers ushered in the antibiotic era and resulted in the discovery of many more "wonder drugs" such as chloramphenicol in 1947 [42], the tetracyclines in 1948 [43], macrolides such as erythromycin in 1952 [44], glycopeptides such as vancomycin in 1956 [45], additional aminoglycosides such as gentamicin in 1963 [46], β-lactams such as cephamycins in 1970 [34, 35] and carbapenems in 1979 [37], ansamycins such as rifamycin in 1957 [47], and polyene macrolides such as nystatin in 1950 [48]. Approximately 15 000 microbial secondary metabolites have been discovered; of these, about 12 000 are antibiotics. Their unusual chemical structures included β-lactam rings, cyclic peptides containing "unnatural" and non-protein amino acids, unusual sugars and nucleosides, polyenes, and large macrolide rings. Although most were useless for humans, being either too toxic or inactive in higher organisms, others were life savers. The antibiotics were virtually the only drugs utilized for chemotherapy against pathogenic microorganisms and were crucial in the increase in average life expectancy in the United States from 47 years in 1900 to 74 for men and 80 for women in 2000.

For some reason, the actinomycetes were amazingly prolific in the number of antibiotics they could produce. Roughly 70% of all antibiotics were obtained from these filamentous prokaryotes, and 75% of those were in turn made by a single genus, *Streptomyces*. It is quite amazing that strains of *Streptomyces hygroscopicus* produced over 180 different secondary metabolites. About 10% of the antibiotics were made by unicellular bacteria and about 20% by fungi [49]. New bioactive products from microbes were discovered at an amazing pace: 200–300 per year in the late 1970s increasing to 500 per year by the 1990s. Accompanying the natural product antibiotics in the pharmaceutical arena were synthetic antimicrobials such as the quinolones and fluoroquinolones. Even these synthetics traced their discovery back to a natural product, that is, quinine. The first quinolone, nalidixic acid, was modeled after quinine. Commercialization of antibiotics, however, slowed down in the 1980s and only three, daptomycin, caspofungin acetate, and the synthetic oxazolidinone, were commercialized in the ensuing decades.

1.5 Strain Improvement

The experiences described above in which penicillin-producing cultures were mutagenized and screened for improved producers led the way to the extensive use of genetics to improve production ability [50]. From the 1950s on, genetic manipulation such as mutagenesis/screening was replaced by mutagenesis/selection/screening in which various selective means were used to decrease the number

of strains that had to be screened for improved production. Then, it was found that new derivatives, some better than the parent molecule, could be produced by mutants. This was first discovered by Kelner in 1949 [51] but the more active derivatives were not isolated and identified. However, the medically useful metabolites demethyltetracycline [52] and doxorubicin [53] were discovered later by mutation of the cultures producing tetracycline and daunomycin, respectively. In 1969, the technique of "mutational biosynthesis" (= mutasynthesis) was devised by University of Illinois Professors Kenneth Rinehart and David Gottlieb and student W.T. Shier [54]. In this process, a mutant blocked in secondary metabolism was fed analogs of the moiety whose biosynthesis was blocked. If successful, the mutant (called an "idiotroph") produced a new secondary metabolite. Mutational biosynthesis was used for the discovery of many new secondary metabolites. The most well known was the commercial antihelmintic agent doramectin, production of which employed a mutant of the avermectin producer, *Streptomyces avermitilis* [55] (see Section 1.8.5).

For strain improvement, genetic recombination was virtually ignored in industry before 1975, mainly due to the low frequency of recombination, as low as 10^{-6}. However, use of polyethyleneglycol-mediated protoplast fusion in actinomycetes by Okanishi *et al*. [56] changed the situation markedly. Okanishi's work on protoplast formation, fusion, and regeneration accelerated the use of genetic recombination. From then on, there was a heightened interest in the application of genetic recombination to the production of important microbial products. Frequencies of recombination increased to even greater than 10^{-1} in some cases. After 1985, many strain improvement programs routinely included (i) transposition mutagenesis, (ii) targeted deletions and duplications by genetic engineering, and (iii) genetic recombination by protoplast fusion and plasmid transformation. Much was known about genetics and regulation in the actinomycetes due to the elegant research on *Streptomyces coelicolor* by David Hopwood, Keith Chater, Mervyn Bibb, and their colleagues at the John Innes Institute in Norwich, England (sometimes referred to as the "temple of *Streptomyces* genetics") [57]. Their favorite organism made at least five secondary metabolites (an A-factor-like molecule, and the antibiotics actinorhodin, undecylprodigiosin, methylenomycin A, and "calcium-dependent antibiotic" or CDA).

These efforts and others by geneticists in academia and industry throughout the world in the 1970s and 1980s revealed that the genes encoding most antibiotic biosynthetic pathways were clustered into operons, thus facilitating transfer of entire pathways from one organism to another. Such clusters were found to also include regulatory and resistance genes. In 1985, "combinatorial biosynthesis" was born [58]. An international effort from the United Kingdom, Japan, and the United States resulted in the cloning of a pathway from one streptomycete producing the isochromanequinone antibiotic actinorhodin into strains producing granaticin, dihydrogranaticin, and mederomycin (which are also isochromanequinones). This resulted in the discovery of two new hybrid antibiotics, mederrhodin A and dihydrogranatirhodin. Combinational biosynthesis became a widely used technique used for discovery of new hybrid drugs [59] by recombinant DNA (rDNA) technol-

ogy. New antibiotics were also created by changing the order of the genes of an individual pathway in its native host.

Progress in strain development has recently involved extensive use of new genetic techniques such as (i) metabolic engineering, accomplishing quantification, and control of metabolic fluxes, and including inverse metabolic engineering and transcript expression analyses, such as association analysis and massive parallel signature sequencing; (ii) directed evolution (see Section 1.11); (iii) molecular breeding including DNA shuffling and whole genome shuffling; and (iv) combinatorial biosynthesis. These efforts are facilitating not only the isolation of improved strains but also the elucidation and identification of new genetic targets to be used in product discovery.

1.6
Semi-Synthetic Antibiotics to Combat Resistant Microbes

Although there was a feeling expressed by many in the late 1970s that the era of product discovery for bacterial disease was ending, the battle against resistant microbes continued and featured some surprising developments. These included: (i) semi-synthetic variations of old antibiotics (ketolides, clarithromycin, azithromycin, glycylglycines); (ii) older underutilized antibiotics (teicoplanin); (iii) new derivatives of previously undeveloped narrow-spectrum antibiotics (streptogrammins); and (iv) a few newly developed antimicrobial agents (caspofungin, daptomycin, and the synthetic epothilones).

Of great success was the development of semi-synthetic erythromycins [60]. These included clarithromycin, roxithromycin, azithromycin, and the ketolide telithromycin. Whereas the first two showed improved acid stability and bioavailability over erythromycin A, they showed no improvement against resistant strains. On the other hand, azithromycin and telithromycin acted against macrolide-resistant bacteria. All the above semi-synthetic erythromycins were effective agents for upper respiratory tract infections and could be administered parentally or orally. Telithromycin was bacteriostatic, active orally, and of great importance for community-acquired respiratory infections. Of particular interest was its low ability to select for resistance mutations as well as to induce cross-resistance. It also did not induce MLS_B resistance, a problem with other macrolides.

For more than 35 years, the glycopeptides vancomycin and teicoplanin were virtually the only natural antibiotics active against multidrug-resistant Gram-positive bacteria. Their use was cut down by the increase in multidrug resistance. To the rescue came a number of new semi-synthetic antibiotics, one called Synercid. Synercid was composed of a synergistic (by 100-fold) pair of narrow-spectrum streptogramins, that is, quinupristin and dalfopristin, both being semi-synthesized from natural compounds made by a single strain of *Streptomyces pristinaespiralis* [61]. The pair was constituted by a (Group A) polyunsaturated macrolactone containing an unusual oxazole ring and a dienylamide fragment and a (Group B) cyclic hexadepsipeptide possessing a 3-hydroxypicolinoyl exocyclic

fragment. Although the natural streptogramins were poorly water-soluble and could not be used intravenously, the Synercid components were both water-soluble. They inhibited protein synthesis, and were active against vancomycin-resistant *Enterococcus faecium* (VREF), methicillin-resistant *S. aureus* (MRSA), glycopeptide-resistant *S. aureus*, and β-lactam-resistant *Streptococcus pneumoniae*. Synergistic action of the streptogramins was due to the fact that the B component blocked binding of aminoacyl-tRNA complexes to the ribosome while the A component inhibited peptide bond formation and distorted the ribosome, promoting the binding of the B component. Synercid was approved by the US Food and Drug Administration (FDA) in 1999.

A semi-synthetic tetracycline, that is, a glycylcycline, was successfully developed for use against tetracycline-resistant bacteria [62]. The 9-*t*-butylglycylamido derivative of minocycline called tigecycline, was active against resistant Gram-positive, Gram-negative, and anaerobic bacteria possessing the ribosomal protection resistance mechanism or the active efflux mechanism.

1.7 The Primary Metabolites

The development of penicillin fermentation in the 1940s marked the true beginning of what might be called the golden age of industrial microbiology. The work of Louis Pasteur pointed to the importance of the activity of non-pathogenic microbes in wine and beer in producing alcohol. This realization resulted in a large number of microbial primary metabolites of commercial importance being produced by fermentation. Primary metabolism involves an interrelated series of enzyme-mediated catabolic, amphibolic, and anabolic reactions which provide biosynthetic intermediates and energy, and convert biosynthetic precursors into essential macromolecules such as DNA, RNA, proteins, lipids, and polysaccharides. It is finely balanced and intermediates are rarely accumulated. By deregulating primary metabolism, overproduction of many primary metabolites was achieved in the fermentation industry. Commercially, the most important primary metabolites were amino acids, vitamins, flavor nucleotides, organic acids, and alcohols.

1.7.1 Amino Acids

Production of amino acids amounted to 2.3 million tons in 2002. The most important acids made at least partly by biological methods were glutamate (1.6 million tons per year), lysine-HCl (700 000 tons), threonine (70 000 tons), phenylalanine (13 000 tons, including that by chemical synthesis), aspartic acid (10 000 tons made enzymatically), and tryptophan (3000 tons including that made enzymatically). High titers were achieved in fermentation (e.g., 170 g/l L-lysine-HCl).

Monosodium glutamate (MSG) is used as a potent flavor enhancer. The glutamic acid fermentation was discovered in Japan in 1957 by Kinoshita, Udaka, and

Shimono [63] of the Kyowa Hakko Kogyo Company. It is of interest that the founder of the company (Dr, Benzaburo Kato), worrying about the diet of the Japanese population and trying to reduce dependence on rice, had charged Kinoshita with the task of producing edible proteins by fermentation. Instead, the researchers came up with a fermentation process yielding an extracellular L-amino acid from non-proteinaceous material. This major discovery was crucial for the development of the amino acid fermentation industry. MSG was manufactured using various species of the genera *Corynebacterium* and *Brevibacterium*.

Normally, glutamic acid overproduction would not be expected to occur because of feedback regulation. Glutamate feedback controls include repression of PEP carboxylase, citrate synthase, and NADP-glutamate dehydrogenase; the last-named enzyme is also inhibited by glutamate. However, by decreasing the effectiveness of the barrier to outward passage, glutamate was pumped out of the cell, thus allowing its biosynthesis to proceed unabated. The excretion of glutamate freed the glutamate pathway from feedback control until excessive levels accumulated.

Glutamate excretion was intentionally effected by various manipulations, such as limitation of biotin in *Corynebacterium glutamicum*; all glutamate overproducers were natural biotin auxotrophs. Biotin is a cofactor of acetyl-CoA carboxylase which is essential for biosynthesis of fatty acids. The surprising report [64] that the addition of penicillin to cells grown in high biotin resulted in excretion of glutamic acid led Shiio and coworkers [65] to postulate (i) that growth of the glutamate-overproducing bacterium in the presence of non-limiting levels of biotin resulted in a cell membrane permeability barrier restricting the outward passage of intracellular amino acids out of the cell and (ii) that inhibition of cell wall biosynthesis by penicillin altered the permeability properties of the cell membrane and allowed glutamate to pass out of the cell. The commonality in the various manipulations that were found to bring about high-level production of L-glutamic acid, that is, (i) limitation of biotin, (ii) addition of penicillin, or (iii) fatty acid surfactants (e.g., tween 60) to exponentially growing cells, was recognized and the permeability mechanism was strongly supported [66]. Apparently, all of these manipulations result in a phospholipid-deficient cytoplasmic membrane, which favors active exit of glutamate from the cell. This view was further supported by the discoveries that oleate limitation of an oleate auxotroph [67] and glycerol limitation of a glycerol auxotroph [68] brought about glutamate excretion. Both oleate and glycerol were precursors of phospholipids. Glutamate-excreting cells were later found to have a major decrease in cell lipids, especially phospholipids [69]. It thus became clear that high level glutamate excretion required (i) growth inhibition in the presence of unlimited carbon and energy sources and (ii) a change in strain on the membrane caused by deficiency of biotin, oleate or glycerol, or addition of certain agents.

Despite the above evidence, the leaky plasma membrane hypothesis was discounted by certain investigators in favor of an efflux system specific for glutamate and regulated by the energy state of the cell. The action of biotin was attributed to effects on intermediary metabolism, correlating with the activity of fatty acid syn-

thetases. Additional opinions discounting the permeability hypothesis attributed glutamate overproduction to a decrease in the activity of α-ketoglutarate dehydrogenase caused by biotin limitation or by addition of penicillin or surfactants. In 2001, however, the permeability modification hypothesis was supported. The various manipulations leading to glutamate overproduction were shown to cause increased permeability of the mycolic acid layer of the cell wall [70]. The glutamate-overproducing bacteria are characterized by a special cell envelope containing mycolic acids which surrounds the entire cell as a structured layer and is thought to be involved in permeation of solutes. The mycolic acids esterified with arabinogalactan and the non-covalently bound mycolic acid derivatives formed a second lipid layer, the cytoplasmic membrane being the first. As stated by these authors, "The concepts of 'permeability of the cell wall' as originally used in the very first work on L-glutamate production more than forty years ago now takes on a new meaning." Nampoothiri *et al.* [71] provided evidence that overexpression or inactivity of genes involved in lipid synthesis changed glutamate efflux dramatically, altered the chemical and physical properties of the cytoplasmic membrane, and that this was necessary to achieve efflux of L-glutamate. They state "that altering the phosphoplipid content alone is sufficient to enable L-glutamate efflux." Burkovski and Kraemer [72] further stated that "There is no doubt that stimulation of glutamate excretion in *C. glutamicum* is directly or indirectly related to membrane and/or cell wall integrity."

Since the bulk of the cereals consumed in the world were deficient in L-lysine, this essential amino acid became an important industrial product. The lysine biosynthetic pathway is controlled very tightly in an organism like *Escherichia coli* which contains three aspartate kinases, each of which is regulated by a different end-product (lysine, threonine, and methionine). In addition, after each branch point, the initial enzymes were inhibited by their respective end-products. However, in lysine fermentation organisms (e.g., mutants of *C. glutamicum* and its relatives), there is only a single aspartate kinase, which is regulated via concerted feedback inhibition by threonine plus lysine. By genetic removal of homoserine dehydrogenase, a glutamate-producing wild-type *Corynebacterium* was converted into a lysine-overproducing mutant that could not grow unless methionine and threonine were added to the medium [73].

E. coli strains were constructed with plasmids bearing amino acid biosynthetic operons. Plasmid transformation was also accomplished in *Corynebacterium*, *Brevibacterium*, and *Serratia* so that rDNA technology could be used to improve these commercial amino acid-producing strains [74]. Especially useful was the concept of metabolic engineering, that is, the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or introduction of new ones with the use of rDNA technology [75, 76]. Its essence was the combination of analytical methods to quantify fluxes and the control of fluxes with molecular biological techniques to implement suggested genetic modification. Metabolic control analysis revealed that the overall flux through a metabolic pathway depended on several steps, not just a single rate-limiting reaction [77]. Metabolic flux studies of wild-type *C. glutamicum* and four improved lysine-

producing mutants showed that yield increased in the series from 1.2 to 24.9% relative to the glucose flux [78]. It was found that (i) the pentose phosphate pathway had increased, (ii) anaplerotic net flux increased almost twofold by concerted control of C3 carboxylation and C4 decarboxylation, (iii) the relative flux through isocitrate dehydrogenase decreased from 83 to 60%, and (iv) NADPH demand increased from 109 to 172%.

The value of genetic engineering can be appreciated in the following example on overproduction of threonine. By introduction of feedback-resistant threonine dehydratases and additional copies of genes encoding branched amino acid biosynthetic enzymes, various groups converted their lysine- or threonine-producing strains into L-isoleucine-producing strains. An engineered strain of *E. coli* (made by mutating to isoleucine auxotrophy, cloning in extra copies of the *thrABC* operon, inactivating the threonine-degrading gene *tdh*, mutating to resistance to high concentrations of L-threonine and L-homoserine) produced 80 g/l L-threonine in 1.5 days at a yield of 50% [79].

1.7.2 Nucleotides

Commercial interest in nucleotide fermentations developed due to the discovery of A. Kuninaka in Japan that certain purine ribonucleoside 5′-monophosphates, namely guanylic acid (GMP), inosinic acid (IMP), and xanthylic acid (XMP) were enhancers of flavor for foods, beverages, and seasonings [80, 81]. Interestingly, AMP had no such activity. The intensity of flavor enhancement by these compounds surpassed that of MSG by several orders of magnitude. Combination of a flavor nucleotide with MSG had a synergistic effect on flavor enhancement. Originally, the nucleotides were made by enzymatic hydrolysis of yeast RNA but this was an expensive process. Auxotrophic mutants of the glutamic acid producer, *C. glutamicum*, were then found to produce IMP and XMP [82, 83]. By further mutation of the IMP producer, a strain was obtained which could produce a mixture of the two most potent nucleotides, GMP and IMP [84]. Direct fermentation became the method of choice for the industry [85], although some companies produced the nucleosides (inosine and guanosine) by fermentation and then enzymatically (via phosphokinase catalysis) converted these to the 5′ nucleotides. Production of 5′-flavor nucleotides amounted to about 40 g/l.

1.7.3 Vitamins

Vitamins are made at a rate of 70 000 tons per year by synthesis and fermentation. Of these, riboflavin was produced by these two methods at an annual rate of 4000 tons. Most fungi produce enough riboflavin (vitamin B_2) to satisfy their growth requirements, but a few were natural overproducers of this vitamin. This tendency of uncontrolled synthesis of riboflavin was found primarily in two species of fungi, *Eremothecium ashbyi* and *Ashbya gossypii*. Overproduction in natural

strains of *E. ashbyi* was discovered in 1935 by A. Guilliermond and coworkers [86]. They noted the yellow color of the colonies of both cultures, *E. ashbyi* being the more intense. During the stationary phase of growth, the vacuoles became yellowish and, in some vacuoles, rosettes of needle-shaped crystals of riboflavin were observed. This led to the use of *E. ashbyi* for the industrial production of riboflavin in animal feed formulae [87].

L.J. Wickerham and coworkers at the Peoria NRRL/USDA lab received, in 1943, a culture of *A. gossypii* from W.J. Robbins, Director of the New York Botanical Garden and designated it as NRRL Y-1056. This culture produced pale yellow colonies, but in 1944, Wickerham and his colleagues noted the emergence of a variant with bright orange-yellow colonies [88]. *E. ashbyi* was replaced in industry by the more stable *A. gossypii* [89] which produced, after genetic manipulation, over 20 g/l of the vitamin.

An rDNA process was developed for riboflavin in *Corynebacterium ammoniagenes* by cloning and overexpressing the organism's own riboflavin biosynthesis genes and its own promoter sequences [90]. The resulting culture produced 15 g/l riboflavin in 3 days. Genetic engineering of a *Bacillus subtilis* strain already containing purine analog-resistance mutations led to production of 15 g/l riboflavin [91]. This strain of *B. subtilis* was produced by inserting multiple copies of the *rib* operon at two different sites in the chromosome, expressing these constitutively from strong phage promoters located at the 5′-end and in an internal site in the operon, making purine analog-resistance mutations to increase guanosine triphosphate (GTP; a precursor) production and a riboflavin analog (roseflavin)-resistance mutation in *ribC* that deregulated the entire pathway [92]. The limiting gene in this strain was found to be *ribA*, encoding both 3,4-dihydroxy-2-butanone 4-phosphate synthase (N-terminal half of RibA) and GTP cyclohydrolase (C-terminal domain) [93]. One additional copy of *ribA* increased riboflavin titer much beyond 15 g/l and also raised the yield from glucose by 25%. Due to these efforts, *B. subtilis* became important for the industrial production of riboflavin.

Bacterial formation of vitamin B_{12} by bacteria is a very old phenomenon. The anaerobic pathway is about 4 billion years old whereas the aerobic pathway evolved when our atmosphere became enriched with oxygen about 2 billion years ago [94]. In the late 1940s, a Merck scientist [95] discovered that *Streptomyces griseus* and *Pseudomonas denitrificans* could form vitamin B_{12} [96]. Other studies showed that the vitamin could also be produced by *Propionibacterium shermanii* as well as other bacteria. *P. denitrificans* and *P. shermanii* became the industrial producing organisms. Production of vitamin B_{12} reached levels of over 200 mg/l using *P. denitrificans*. Fermentation was used exclusively at a rate of 12 tons/year in the early 2000s. This may seem like a small number but no more is needed since vitamin B_{12} is such a potent molecule.

At one time, ascorbic acid (vitamin C) was produced by isolation from lemons. This was replaced by the seven-step Reichstein synthesis in 1933 [97], which included a biosynthetic step. After chemical conversion of D-glucose to D-sorbitol, *Gluconobacter oxydans* was used to convert the latter to L-sorbose. L-Sorbose was

then converted in several chemical steps to 2-keto-L-gulonic acid (2-KLGA). 2-KLGA was then chemically converted by acid or base to ascorbic acid. The yield from glucose to 2-KLGA was 50%. The Reichstein process was used for 70 years but newly developed fermentation processes which converted D-glucose, D-sorbitol, or L-sorbose to 2-KLGA became competitive [98].

Some of the microbiological vitamin C processes used single organisms such as species of *Gluconobacter*, *Acetobacter*, or *Pseudomonas*. Mixed culture processes included one developed in China as far back as 1969 and used widely there to convert D-sorbitol to 2-KLGA. In another process, D-glucose was converted to 2,5-diketo-D-gluconic acid by *Erwinia* or *Acetobacter* which was then transformed into 2-KLGA by *Corynebacterium* [99]. Another similar process involved mutants of *G. oxydans* and *Bacillus megaterium* [100]. A genetically engineered strain of *Erwinia herbicola* produced 120 g/l of 2-KLGA [101], whereas recombinant *G. oxydans* made 130 g/l [102]. Ascorbic acid is used in the pharmaceutical, food, beverage, and feed industries. Annual production is 110 000 tons by synthesis and fermentation.

1.7.4 Organic Acids

Organic acids have been an important product of biotechnology. Much information on the history of the organic acid fermentations can be found in the reviews of Miall [103], Mattey [104], Roehr [105], and Magnuson and Lasure [106]. The most important commercial organic acids are citric, acetic, and lactic acids. Fermentation processes are also available for production of succinic, gluconic, oxogluconic, pyruvic, itaconic, shikimic, malic, propionic, butyric, oxalic, kojic, fumaric, erythorbic, *trans*-epoxysuccinic, tartaric, itatartaric, and long-chain α, ω-dicarboxylic acids.

Citric acid production has historic significance since it was the first industrial fermentation to be developed. It had been exclusively produced via isolation from lemons. In 1916, citric acid production by black aspergilli was described by Charles Thom and J.N. Currie [107]. Currie joined Chas. Pfizer and Co. in Brooklyn, NY and developed a commercial production process in 1923. The 1927 patents of Fernbach *et al.* [108, 109] were the basis of citric acid production in England by John & E. Sturge, Ltd. The process was improved in the 1930s by Raistrick's group [110] and by Doelger and Prescott [111]. The crucial metal requirements of the *Aspergillus niger* culture were studied by a number of groups including Tomlinson and coworkers [112] and Adiga *et al.* [113].

During the early years, the acid was produced solely by surface culture in flasks for laboratory studies and in trays for commercial production. However, Amelung [114] and Kluyver and Perquin [115] found that submerged culture was better. Further development of the citric acid fermentation depended greatly on the work of Professor Marvin Johnson with colleagues David Perlman [116] and Ping Shu [117] at the University of Wisconsin during the same years that Johnson was contributing so greatly to the development of the penicillin fermentation. The use

of invert molasses (high-test molasses), treated to reduce its iron content, was pioneered by Miles Laboratories. Mutants producing higher concentrations were obtained by Miles Laboratories [118], James *et al.* [119] and Hannan *et al.* [120].

About 1.5 million tons of citric acid are produced by *A. niger* per year. The commercial process employs *A. niger* in media deficient in iron and manganese. A high level of citric acid production was also associated with an increased intracellular concentration of fructose 2,6-biphosphate, an activator of glycolysis. Other factors contributing to high citric acid production were the inhibition of isocitrate dehydrogenase by citric acid, and the low pH optimum (1.7–2.0). In approximately 4–5 days, the major portion (80%) of the sugar provided was converted to citric acid, titers reaching about 100 g/l. Alternative processes were developed for the production of citric acid by *Candida* yeasts, especially from hydrocarbons. Such yeasts were able to convert *n*-paraffins to citric and isocitric acids in extremely high yields (150–170% on a weight basis). Titers as high as 230 g/l were reached.

The acetic acid bacteria are Gram-negative, obligately aerobic bacteria composed of species of *Acetobacter*, *Gluconoacetobacter*, and *Frateuria*. Their activity as the agents of wine spoilage has been a problem since at least 10 000 BC. *Acetobacter suboxydans* was used to produce vinegar as far back as 4000 BC. Indeed, the Latin word "acetum" means sour wine or sharp wine. Production of vinegar, an aqueous solution of acetic acid, is best carried out with species of *Gluconacetobacter* and *Acetobacter* [121]. A solution of ethanol was converted to acetic acid in which 90–98% of the ethanol was attacked, yielding a solution of vinegar containing 12–17% acetic acid. Industrial production of acetic acid was carried out solely by the conversion of sugar until the latter part of the 1800s when the distillation of wood became competitive with the fermentation process. Petroleum then became a major source of synthetic acetic acid. The first chemical plant for acetic acid production was established in 1916. Acetic acid made by fermentation from corn was mainly used by the food industry. Production of acetic acid amounts to 7.5 million tons per year by synthesis and fermentation.

Today, acetic acid fermentation is a two-step process in which the yeast *Saccharomyces cerevisiae* converts glucose to ethanol and *Acetobacter aceti* produces acetic acid from the ethanol. Cloning of aldehyde dehydrogenase from *Acetobacter polyoxogenes* on a plasmid vector into *A. aceti* subsp. *xylinum* increased the rate of acetic acid production by over 100% (1.8 g/l per h to 4 g/l per h) and titer by 40% (68–97 g/l) [122].

Another group of organisms considered for acetic acid production has been the anaerobic, thermophilic anaerobes of the genus *Clostridium*. In 1940, Wieringa isolated *Clostridium aceticum* [123] which was subsequently lost. However in 1942, Fontaine *et al.* [124] isolated *Clostridium thermoaceticum* which converted sugar quantitatively to acetic acid via the Embden–Meyerhof pathway to pyruvate which was then converted to acetate. As a result, 1 mole of glucose was converted into 3 moles of acetate. Experience with this fermentation showed that 0.85 g of acetic acid could be produced from 1 g of glucose [125] and titers of 83–102 g/l were reached with improved mutants [126]. Production by *C. thermoaceticum* was reviewed by Cheryan *et al.* [127].

Lactic acid production has been used to retard food spoilage for centuries, but the way in which it does this was not known until the Pasteur discovery of 1857 that this favorable activity was caused by microorganisms. In 1878, Joseph Lister [128] isolated the first pure culture of any bacterium which he called *Bacterium lactis*, later to be renamed *Lactobacillus lactis* ssp. *lactis*. The lactic acid bacteria are Gram-positive anaerobes that produce and excrete lactic acid into the medium. They were among the first microbes to be used in the manufacture of foods. Today, lactic acid is used in the food industry as a preservative and as flavor enhancer and in the chemical and pharmaceutical industries. Important applications of L-lactic acid are in the manufacture of polylactide (see Section 1.7.6) and that of the environmentally benign solvent ethyl lactate. About 250 000 tons of lactic acid are produced annually.

In addition to the lactic acid bacteria, the fungus *Rhizopus* is also a producer of the acid. It is sometimes preferred because it does not require supplements such as yeast extract, corn steep liquor, or whey which make product recovery expensive. Also, *Rhizopus oryzae* synthesizes solely the L-(+) isomer of lactic acid, whereas most lactobacilli produce mixed isomers of the acid. Some recombinant lactobacilli can produce individual isomers but yields are low. A six-step strain improvement program resulted in a *R. oryzae* strain producing over 130 g/l of L-(+)-lactic acid and a yield from glucose of near 90% [129].

Although most lactobacilli produce mixed isomers, a strain of *L. lactis* has been isolated which makes 195 g/l of L-lactic acid from 200 g/l glucose [130]. A recombinant *E. coli* strain produced optically pure D-lactic acid from glucose with a yield almost at the theoretical maximum yield (i.e., two molecules from one molecule of glucose) [131]. The organism was engineered by eliminating genes of competing pathways encoding fumarate reductase, alcohol/aldehyde dehydrogenase, and pyruvate formate lyase, and by a mutation in the acetate kinase gene.

1.7.5
Alcohols

Ethanol production is probably the oldest fermentation process known. For 6000 years, since the Sumerian and Egyptian days, the conversion of sugar in fruits and grains to ethanol has been an important process. Up until the 1980s, it was mainly used for to make alcoholic beverages, but in recent years, it has become an important clean and neat feedstock and fuel, especially for automobiles. Ethanol also has applications as: (i) a solvent in the laboratory, in pharmaceuticals, and in cosmetics, (ii) a cosurfactant in oil–water emulsions, and (iii) a sterilizing agent and antiseptic.

It was J.L. Gay-Lussac who, in 1810, first reported ethanol and CO_2 as the principal products of sugar breakdown by yeast (see the fascinating review by F. Schlenk [132]). In 1837, the physicist Charles Cagniard-Latour described the features of yeast cells found in fermented beverages including their shape, reproduction, their need for both fermentable carbohydrate and a nitrogen source for multiplication, and differences between yeasts of wine production and those of

the beer-making process. At about the same time, Theodor Schwann, trained in medicine, found that yeasts were required for alcoholic fermentation. The work of Cagniard-Latour and Schwann was confirmed by Friedrich Traugott Kuetzing, a pharmacist and college teacher. However, the work of these pioneers was rejected by the chemists J.J. Berzelius, J. Liebig, and F. Woehler, who believed the process to be strictly chemical in nature. Interestingly, one of the severest critics of the chemists was Moritz Traube, who had been a student of Liebig. He proposed that fermentation was carried out by oxidizing and reducing cellular components which he called "ferments." It was Pasteur in 1860 who proved that the yeast cells were absolutely required to carry out the series of (bio)chemical reactions. Finally, Eduard Buchner in 1897, with the help of his brother Hans, was able to carry out cell-free synthesis of ethanol with extracts of yeast. For this, he won the Nobel Prize in chemistry in 1907.

Ethyl alcohol is produced via fermentation of sugars (or a polysaccharide that can be depolymerized to a fermentable sugar) by *S. cerevisiae* in the case of hexoses, and *Kluyveromyces fragilis* or *Candida* species with lactose or a pentose, respectively. *S. cerevisiae* and other yeasts were chosen and adapted for specific ethanol fermentations. They included baker's yeast (different strains for compressed and active dry yeast), wine yeasts (including special flocculent strains for the production of champagne and film-forming strains for the production of flor sherry), sake yeast, top and bottom fermenting brewing strains (varying in the degree of flocculation occurring during fermentation), and distiller's strains used for alcohol production from cereal starch. About 2 million tons of yeast are produced annually for the distilling, brewing, and baking industries each year. The production of beverage alcohol was restricted to the use of microorganisms (e.g., yeast) but that of industrial and fuel alcohol was usually carried out by chemical synthesis from petroleum; this eventually changed in favor of yeasts. Under optimum conditions, approximately 10–12% ethanol by volume was obtained in yeast fermenations within 5 days. Such a high concentration slowed down growth and the fermentation ceased. With special (saki) yeasts, the fermentation could be continued to alcohol concentrations of 20% by volume but these concentrations were attained only after months of fermentation.

In 1977, yeast production of beverage, fuel, and fuel alcohol was 20% less than by chemical synthesis. However, by 1984, yeasts provided 87% more ethanol than did chemical synthesis. The percentage of total alcohol made by yeasts continued to increase over the years. By 2006, 13.2 million tons of ethanol were produced from corn annually in the United States by fermentation compared with 0.65 million tons by synthesis. Due to the elimination of lead from gasoline, ethanol was substituted as a blend to raise gasoline's octane rating. Later, it was added to gasoline as an oxygenate to reduce CO_2 emissions by improving the overall oxidation and performance of gasoline. This was due to the phasing out of the use of methyl tert-butyl ether (MTBE) as oxygenate, as ruled by many state legislatures in the United States. Ethyl alcohol was produced in Brazil from cane sugar at a rate of over 4 billion gallons per year and was used either as a 25% blend or as a pure fuel.

Fuel ethanol produced from biomass is being considered as a means to provide relief from air pollution caused by use of gasoline without contributing to the greenhouse effect [133] and of eliminating the dependence of the United States on foreign sources of petroleum. The available feedstock in the United States could supply 20 billion gallons of fuel ethanol. New processes have been developed to convert biomass to ethanol and rDNA technology has been used to convert *E. coli* and its close relatives into efficient producers of ethanol (43% yield, v/v) [134]. Alcohol dehydrogenase II and pyruvate decarboxylase genes from *Zymomonas mobilis* were inserted in *E. coli* and became the dominant system for NAD regeneration. Ethanol represented over 95% of the fermentation products in the genetically engineered strain. Some genetically engineered *E. coli* strains made as much as 60 g/l of ethanol. By cloning and expressing the same two genes into *Klebsiella oxytoca*, the recombinant strain was able to convert crystalline cellulose to ethanol in high yield when fungal cellulase was added [135]. Maximum theoretical yield was 81–86% and titers as high as 47 g/l of ethanol were produced from 100 g/l cellulose.

Bacteria such as clostridia and *Zymomonas* have also been reexamined for their utility in ethanol production after years of neglect. *Clostridium thermocellum*, an anaerobic thermophile, converts waste cellulose (i.e., biomass) and crystalline cellulose directly to ethanol, without the need for fungal cellulase [136–139].

Butanol is another alcohol which could help solve the problem of overdependence on petroleum as a motor fuel [140, 141]. Cloning of its *ace* operon genes *adc* (encoding acetoacetate decarboxylase), *ctfA* and *ctfB* (two genes encoding coenzyme A transferase) on a plasmid containing the *adc* promoter into *Clostridium acetobutylicum* resulted in a 95% increase in production of acetone, a 37% increase in butanol, a 90% increase in ethanol, a 50% increase in solvent yield from glucose, and a 22-fold lower production of acids [142].

An *E. coli* culture has been developed that grows on glucose and produces 1,3-propanediol (PDO; trimethylene glycol; 3G) at 135 g/l, with a yield of 51% and a rate of 3.5 g/l per h [143]. This effort was jointly carried out by scientists from Genencor International and DuPont and was achieved by introducing eight new genes to convert dihydroxyacetone phosphate (DHAP) into PDO. These included yeast genes converting dihydroxyacetone to glycerol and *Klebsiella pneumoniae* genes converting glycerol to PDO. The researchers improved production in the recombinant by modifying 18 *E. coli* genes, including regulatory genes. PDO was the monomer used to chemically synthesize polyurethanes and the polyester fiber Sorono™ by DuPont (see Section 1.7.6). PDO is also used as a polyglycol-like lubricant and as a solvent.

Other alcohols that can be made by fermentation are glycerol, erythritol, mannitol, sorbitol, and xylitol.

1.7.6 Polymers

Thirty thousand tons of the polysaccharide xanthan are produced annually for use in the oil, pharmaceutical, cosmetic, paper, paint, and textile industries. Genetic

manipulation increased titers of xanthan by twofold and increased pyruvate content by 45% [144]. Cloning genes, which complemented xanthan-negative mutants, into wild-type *Xanthomonas campestris* increased xanthan production by 15% [145].

A solution to the polluting effects of chemically produced plastics was provided by a group of microbially produced biodegradable plastics, known as polyhydroxyalkanoates (PHAs). One PHA, polyhydroxybutyrate, became available from microorganisms in the 1980s [146]. PHAs accumulate intracellularly to levels of 30–80% of cell dry weight and under certain conditions, in *Alcaligenes eutrophus,* reach 96% of the cell material [147].

Another new bioplastic is polytrimethylene terephthalate (3GT polyester; 3G+), made by reacting terephthalic acid with PDO produced by fermentation [148]. 3G+ is a new, environmentally friendly polyester. Another environmentally friendly plastic is polylactide, made chemically from fermentation-derived L-(+)-lactic acid [149].

1.7.7
Specialty Sugars, Sugar Alcohols, L-Sugars, Oligosugars, Novel Extracellular Polysaccharides, Biopigments, Cosmetics Including Fragrants, and Microbial Enzymes for Chiral Synthesis and Other Applications

An important specialty sugar is L-ribulose [150] which can be made by bioconversion, that is, dehydrogenation of ribitol. This compound is then converted to L-ribose which is used for the synthesis of nucleoside analogs as antiviral agents. A useful homopolysaccharide of glucose is dextran, which is secreted by strains of *Leuconostoc, Streptococcus,* and *Lactobacillus*. Dextran can be produced by use of dextransucrase [151]. Cosmetics (personal care products) were used by ancient Egyptians, Greeks, Romans, Incas and Aztecs centuries ago and are extensively used today [152]. They include hyaluronic acid, chitosan, xanthan, ceramides, amino acids, ectoines, provitamins, dihydroxyacetone, *Clostridium botulinum* toxin, organic acids, cyclodextrins, biosurfactants, indigo, biopigments, fatty acids, and microbial enzymes. Important biopigments are riboflavin, β-carotene, astaxanthin, zeaxanthin, and monaascin. Biofragrants include a peach aroma 4-decalactone, a butter aroma *R*-δ-dodecanolide, and cheese aromas butyric acid and its ethyl ester [153]. Biocatalysis (see Sections 1.12 and 1.13) has been extensively used in the bulk and fine chemicals areas and also in the environmental sector [154,155].

1.8
The Shift from Antibiotics to Pharmacological Agents

During the 3 billion years in which bacteria have inhabited the earth, Nature has developed a unique chemistry in the form of hundreds of thousands of identified and isolated novel secondary metabolites. These natural products, with structures much more spatially complex than those of synthetic chemicals, have been an

overwhelming success in their usage by humans. Secondary metabolites of microorganisms have reduced pain and suffering, and revolutionized medicine. Natural products have been the most important anti-infective and anticancer agents. The inhibitors of HIV reverse transcriptase and protease used in combination against AIDS were all modeled after leads obtained from natural products at the National Cancer Institute of the United States. The anti-herpes agent acyclovir and the non-Hodgkin's lymphoma drug cytarabine derive from sponges. At the turn of the century, over half of the approved drugs available were either natural products or were related to them, and that did not even include biologicals such as vaccines and monoclonal antibodies.

Of all the traditional products made by fermentation, the most important to human health are the secondary metabolites (idiolites). These (i) are often produced in a developmental phase of batch culture (idiophase) subsequent to growth, (ii) have no function in growth, (iii) are produced by narrow taxonomic groups of organisms, (iv) have unusual and varied chemical structures, and (v) are often formed as mixtures of closely related members of a chemical family. In nature, their functions serve the survival of the strain, but when the producing microorganisms are grown in pure culture, the secondary metabolites have no such role. Thus, production ability in industry is easily lost by mutation ("strain degeneration"). In general, both the primary and the secondary metabolites of commercial interest have fairly low molecular weights, that is, less than 1500 Da.

Whereas primary metabolism is basically the same for all living systems, secondary metabolism is mainly carried out by plants and microorganisms and is usually strain-specific. The best-known secondary metabolites are the antibiotics, as discussed above.

In the latter half of the twentieth century, more attention was placed on the use of microbial secondary metabolites as pharmacological agents. No longer were microbial sources looked upon solely as potential solutions for microbial diseases. With great vision, Hamao Umezawa (Figure 1.10) began, in the 1960s, his pioneering efforts to broaden the scope of industrial microbiology to low molecular weight secondary metabolites which had activities other than, or in addition to, antibacterial, antifungal, and antitumor action. He and his colleagues at the Institute of Microbial Chemistry in Tokyo focused on enzyme inhibitors [156, 157] and over the years, discovered, isolated, purified, and studied the *in vitro* and *in vivo* activity of many of these novel compounds. Similar efforts were conducted at the Kitasato Institute in Tokyo led by Satoshi Omura [158]. This change in screening philosophy was followed by ingenious applications of molecular biology to detect activities of compounds from microbes and plants for non-antibiotic applications.

1.8.1 Enzyme Inhibitors

The most important group of enzyme inhibitors are the statins, used for cholesterol-lowering in humans. These extremely successful agents also have antifungal

Figure 1.10 Hamao Umezawa.

activities, especially against yeasts. Independently, Brown *et al.* [159] in the United Kingdom and Endo in Japan [160] discovered the first member of this group, compactin (ML-236B; mevastatin) as an antibiotic product of *Penicillium brevicompactum* and *Penicillium citrinum*. Endo *et al.* discovered compactin in broths as an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the regulatory and rate-limiting enzyme of cholesterol biosynthesis. Later, Endo [161] and Alberts *et al.* [162] (at Merck, USA) independently discovered the more active methylated form of compactin known as lovastatin (monacolin K; mevinolin) in broths of *Monascus ruber* and *Aspergillus terreus*, respectively.

Lovastatin (mevinolin) was produced by *Aspergillus terreus* and in its hydroxyacid form (mevinolinic acid), was a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase from liver. Lovastatin led the way to the development of pravastatin, Zocor (simvastatin), and Lipitor (atorvastatin). Pravastatin was produced by bioconversion, that is, hydroxylation of compactin using actinomycetes such as *Streptomyces carbophilus* [163] and *Actinomadura* sp. [164]. Interestingly, the enzymatic mechanism in the two actinomycetes was found to be quite different [165]. Zocor was prepared by semi-synthesis from lovastatin, whereas Lipitor was made by total synthesis, modeled after the structure of the other statins.

The statins were found to inhibit *de novo* production of cholesterol in the liver, the major source of blood cholesterol. High blood cholesterol leads to atheroscle-

rosis which is a causal factor in many types of coronary heart disease, a leading cause of human death. Statins were a success because they reduced total plasma cholesterol by 20–40%, whereas the previously used fibrates only lowered it by 10–15%. Statins were not only useful for reduction in the risk of cardiovascular disease, they also prevented stroke, reduced development of peripheral vascular disease, and had antithrombotic and anti-inflammatory activities. It is obvious why this group of compounds became the leading drugs of the pharmaceutical industry.

Anti-enzyme screens also led to the discovery of the pseudotetrasaccharide acarbose, a natural inhibitor of intestinal α-glucosidase and sucrase [166]. Acarbose was produced by an actinomycete of the genus *Actinoplanes*. It decreased hyperglycemia and triacylglycerol synthesis in adipose tissue, liver, and the intestinal wall of patients with diabetes, obesity and type IV hyperlipidemia. Additional valuable enzyme inhibitors included lipstatin, used to combat obesity and diabetes by inhibiting gastrointestinal absorption of fat. It was a pancreatic lipase inhibitor produced by *Streptomyces toxytricini*. The commercial product was called Orlistat (tetrahydrolipstatin) [167].

Also discovered at this time was a very important enzyme inhibitor for medicine called clavulanic acid [168] (see Section 1.3) and several for agriculture such as the polyoxins [169] and the phosphinothricins (see Section 1.8.5). Polyoxins are antifungal agents which inhibit cell wall formation via inhibition of chitin synthetase.

Inhibitors of metal uptake also became important. One, called desferal, was a siderophore produced by *Streptomyces pilosus* which was used in iron-overload disease (hemochromatosis) and for aluminum overload in kidney dialysis patients [170].

1.8.2 Immunosuppressants

Umezawa's concepts also led to development of the hugely important immunosuppressants, such as ciclosporin, tacrolimus, sirolimus, and mycophenolic acid, which revolutionized the field of organ transplantation. Ciclosporin (initially known as cyclosporine A) was originally discovered as a narrow-spectrum antifungal peptide produced by the mold *Tolypocladium niveum* (previously *Tolypocladium inflatum*) [171]. Discovery of its immunosuppressive activity led to its use in heart, liver, and kidney transplants and to the overwhelming success of the organ transplant field.

Although ciclosporin had been the only product on the market for many years, two other products from actinomycetes provided further opportunities. These were sirolimus (rapamycin) and tacrolimus (FK-506). Both were narrow-spectrum polyketide antifungal agents, which were 100-fold more potent than ciclosporin as immunosuppressants and less toxic.

Sirolimus was discovered by a small group of scientists at Ayerst Laboratories in Montreal, Canada, under the leadership of Claude Vezina [172]. In 1964, a

Canadian scientific expedition had traveled to Easter Island (Rapa Nui) in the South Pacific to gather samples of soils and plants. Fortunately, they shared the soil samples with the Ayerst team and in 1972, the latter isolated sirolimus (called rapamycin at that time) from *Streptomyces hygroscopicus*. This remarkable molecule exhibited potent activity against the pathogenic yeast *Candida albicans* and other yeasts. Due to the efforts of Suren Sehgal, it was later found to have both immunosuppressive and anticancer activities [173]. Rapamycin was patented in 1975, but because of corporate lack of interest, it was not commercialized as an agent to facilitate organ transplantation until 1999. Sirolimus did not exhibit the nephrotoxicity of ciclosporin and tacrolimus, and was synergistic with both compounds in immunosuppressive action. The drug found a new use in cardiology when it was used for impregnation of stents because sirolimus-impregnated stents were less prone to proliferation and restenosis, which usually occur after treatment of coronary artery disease.

Developments on the fermentative production of sirolimus and the production of new analogs of this multipotent molecule were aided by the discoveries in 1991–1993 by Paiva and coworkers [174–176] of its biosynthetic precursors (i.e., acetate, propionate, methionine, pipecolic acid, and shikimic acid).

Tacrolimus was discovered by the Fujisawa Pharmaceutical Co. (now Astellas) [177] but almost abandoned after initial animal studies showed dose-associated toxicity. However, Dr. Thomas Starzl of the University of Pittsburgh, realizing that the immunosupressant was 30- to 100- fold more active than ciclosporin, tried lower doses which were very effective and non-toxic, thus saving the drug and many patients after that, especially those that were not responding to ciclosporin. Since its introduction in 1993 in Japan and 1994 in the United States, tacrolimus has been used for transplants of liver, kidney, heart, pancreas, lung, intestines, and for prevention of graft-versus-host disease. A topical preparation was shown to be very active against atopic dermatitis, a widespread skin disease.

A very old broad-spectrum antibiotic compound, mycophenolic acid, has an amazing history. The unsung hero of the story is Bartolomeo Gosio, the Italian physician who discovered the compound in 1893 [178]. Gosio isolated a fungus from spoiled corn which he named *Penicillium glaucum*, and which was later reclassified as *P. brevicompactum*. He isolated crystals of the compound from culture filtrates in 1896 and found it to inhibit growth of *Bacillus anthracis*. This was the first time an antibiotic had been crystallized and the first time that a pure compound had ever been shown to have antibiotic activity. The work was forgotten but fortunately the compound was rediscovered by Alsberg and Black in 1913 [179] and given the name mycophenolic acid. They used a strain originally isolated from spoiled corn in Italy called *Penicillium stoloniferum*, a synonym of *P. brevicompactum*. The chemical structure was elucidated many years later by Raistrick and coworkers in England. Mycophenolic acid has antifungal, antiviral, antitumor, antipsoriasis, and immunosuppressive activities. It was never commercialized as an antibiotic because of its toxicity, but its 2-morpholinoethylester was approved as a new immunosuppressant for kidney transplantation in 1995 and for heart transplants in 1998. The ester is called mycophenolate mofetil (CellCept) and is a prodrug which is hydrolyzed to mycophenolic acid in the body.

1.8.3 Antitumor Agents

Natural products became the leading chemotherapeutic drugs against cancer. Cytarabine (Cytostar®) for non-Hodgkin's lymphoma was isolated from a sponge [180]. Most of the other important antitumor compounds were microbially produced secondary metabolites. These included actinomycin D, mitomycin, bleomycins, and the anthracyclines daunorubicin and doxorubicin. The bark of the Pacific Yew tree (*Taxus brevifolia*) yielded taxol (paclitaxel), the microtubule stabilizer discovered by Wall and Wani [181] with excellent activity against breast and ovarian cancer. Of particular interest is that it is also produced by endophytic fungi isolated from the same source [182]. Taxol was the first antitumor drug known to act by blocking depolymerization of microtubules. Another plant compound used for cancer and discovered by Wall and Wani is camptothecin [181].

For a number of years, scientists worked on the "magic bullet" approach which would utilize monoclonal antibodies specific for tumor cells to bring very toxic chemotherapeutic agents into intimate contact with tumor cells and thus provide a specific, and hopefully a safe, way to kill these cells. Problems included less than required specificity, immunological complications, and poor penetration of antibody–toxin conjugates into the cancer cells. However, the combination of monoclonal antibodies with chemotherapeutic agents had promise. In the 1990s, the microbially produced toxic enediyne antitumor drug calicheamicin was attached to a humanized monoclonal antibody and was approved for use against acute myeloid leukemia (AML) [183]. The monoclonal antibody was designed to direct it to the CD33 antigen which is a protein expressed by myeloid leukemic cells. The conjugate was called Mylotarg (or gemtuzumab ozogamicin). It became a marketed product in 2001.

1.8.4 Ergot Alkaloids

Broad screening led to the development of ergot alkaloids for various medical uses such as for uterocontraction, hypertension, serotonin-related disturbances, and migraine headaches, among others. These plant alkaloids had been traditionally produced by extraction from sclerotia from parasitic species of the fungal genus *Claviceps*. Later, these alkaloids became products of fungi in submerged commercial fermentations [184].

1.8.5 Agricultural Compounds

The new efforts in pharmacology also paid dividends in agriculture. New products included bioherbicides, antiparasitic agents, bioinsecticides, agricultural antifungal agents, plant growth stimulators, and animal estrogenic agents.

The agricultural use of synthetic chemicals as herbicides had worried many environmentalists as several widely used herbicides were reported to cause cancer

in long-term animal tests. To fill the void, antibiotics were considered for use as agricultural herbicides. One such herbicide, bialaphos (*N*-{4[hydroxyl[methyl] phosphinoyl] homoalanyl}alanylalanine), which was active against broad-leaved weeds, was developed in Japan in 1973 [185]. This *Streptomyces viridochromogenes* product had been discovered one year earlier by Zahner's group in Germany as a broad-spectrum antibiotic active against bacteria and *Botrytis cinerea* [186]. Its hydrolysis product, DL-homoalanin-4-yl (methyl) phosphinic acid (DL-phosphinothricin) is a glutamine synthetase inhibitor which is manufactured by Hoechst as glufosinate (Basta). Of great interest to environmentalists was that bialaphos was easily degraded in the environment, having a half-life only 2 h.

The gibberellins, plant growth stimulators, are produced commercially by *Gibberella fujikuroi*, the conidial state of the fungus *Fusarium moniliforme*, and are used as plant regulators and in the malting of barley in the brewing process [187]. They increase the yield of vegetables and accelerate the development of biennials.

One of the major economic diseases of poultry is coccidiosis caused by species of the parasitic protozoan *Eimeria*. For years, this disease was treated solely by synthetic chemicals and indeed only synthetic compounds were screened for coccidiostat activity. Although they were generally effective, resistance developed rapidly in the coccidia and new chemical modifications of the existing coccidiostats were pursued. Surprisingly, a parenterally toxic and narrow-spectrum antibiotic, monensin, was found to have extreme potency against coccidia [188]. The polyether antibiotics, especially monensin (produced by *Streptomyces cinnamonensis*), lasalocid (produced by *Streptomyces lasaliensis*), and salinomycin (produced by *Streptomyces albus*), have dominated the commercial coccidiostat field ever since.

An interesting sideline in the monensin story is the discovery of its further use as a growth promotant in ruminants. For years, synthetic chemicals had been screened for activity in cattle and sheep diets to eliminate wasteful methane production and increase volatile fatty acid formation (especially propionate) in the rumen, which would improve feed efficiency. Although the concept was sound, no useful products resulted. Experimentation with monensin showed that polyethers had this activity and these compounds are now widely used [189].

Another antiparasitic agent is avermectin which replaced the synthetic products previously used as antihelmintics/endectocides. Direct *in vivo* screening of fermentation broths against nematodes in mice led to the discovery of the potent activity of the avermectins against disease-causing helminths in animals. Avermectin's antihelmintic activity was an order of magnitude greater than that of previously developed synthetic compounds. The *Streptomyces avermitilis* culture, which was isolated by Yoko Takahashi and Satoshi Omura and coworkers at the Kitasato Institute in Japan (described in [190]), produced a family of secondary metabolites having both antihelmintic and insecticidal activities which were named "avermectins" and developed by Merck in the United States [191]. They are disaccharide derivatives of macrocyclic lactones with exceptional activity against parasites, that is, at least 10 times higher than that of any synthetic antihelmintic agent known. Despite their macrolide structure, avermectins lack antibiotic activity

against bacteria and fungi, do not inhibit protein synthesis nor are they ionophores; instead they interfere with neurotransmission in many invertebrates. The activity of avermectins against both nematode and arthropod parasites in sheep, cattle, dogs, horses, was 1000 times more active than that of the previously used synthetic compound, thiobenzole. A semi-synthetic derivative, ivermectin, was soon established as a commercial veterinary product.

A fortunate fallout from the work with avermectin was the finding that ivermectin had activity against the black fly vector of human onchocerciasis ("river blindness") [192]. It interferes with transmission of the filarial nematode, *Onchocerca volvulus*, to the human population. Since 40 million people were affected by this disease, the decision by Merck in the 1980s to supply ivermectin free of charge to the World Health Organization for use in humans in the tropics was met with great enthusiasm and hope for conquering this parasitic disease. Ivermectin is also effective in the Asian human disease strongyloidiasis.

A family of bioinsecticides called the spinosyns were discovered at Eli Lilly and commercialized by Dow AgroSciences [193]. These were non-toxic, non-antibiotic, environmentally friendly tetracyclic macrolides produced by *Saccharopolyspora spinosa* with activity against insects of the orders Coleoptera, Diptera, Hymenoptera, Isoptera, Lepidoptera, Siphonoptera, and Thysanoptera.

Another important bioinsecticide was a non-antibiotic large molecule, the protein crystal of *Bacillus thuringiensis*. The selective toxicity of this protein (delta-endotoxin; BT toxin) against insects of the order Lepidoptera has been exploited successfully in agriculture. The strong selectivity of its toxicity against these insects meant that it did not harm the environment and only little resistance has developed over the years. Other commercialized agricultural products include the bioinsecticide nikkomycin, the agricultural antifungal group known as polyoxins, and the animal estrogenic agent zearelanone.

1.9
The Biopharmaceutical Revolution

Major milestones in genetics include the findings of Gregor Mendel in the mid-nineteenth century on the inheritance of characters in peas. In 1944, a major discovery, that DNA was the genetic material, was made by Avery, McCloud, and McCarty at the Rockefeller Institute. Two years later, Lederberg and Tatum discovered sex in bacteria. The revolution in biotechnology was sparked by the 1953 discovery of the double-stranded structure of DNA by Watson and Crick (Figure 1.11). The use of microorganisms and their antibiotics as tools of basic research is mainly responsible for the remarkable advances in the fields of molecular biology and molecular genetics. The biotechnology revolution did not come immediately but required some 15 years for additional basic discoveries to be made by others in molecular biology/genetics. In 1956, Alexander Rich (Figure 1.12) and David Davies reported that two single-standed RNA molecules could spontaneously organize themselves to form a double helix. Although this was doubted by

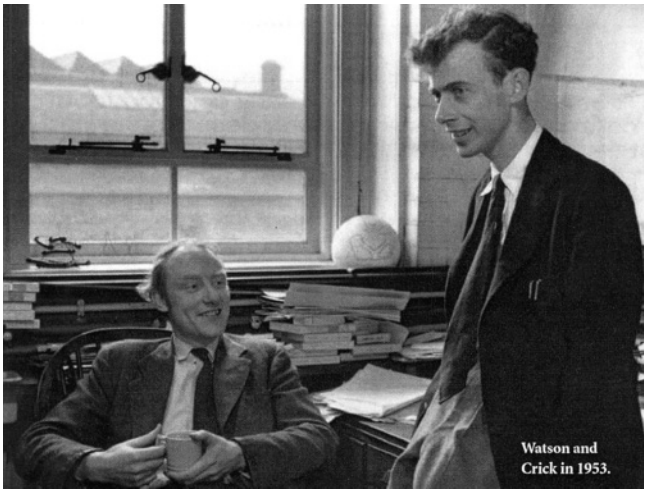

Figure 1.11 James D. Watson and Francis Crick.

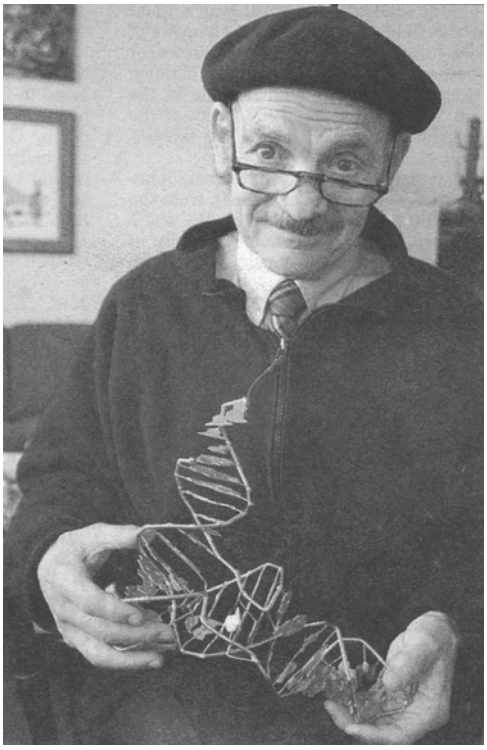

Figure 1.12 Alexander Rich.

many, the RNA double helix led about 40 years later to the discovery of micro RNAs and RNA interference. Beadle and Tatum received the Nobel Prize in 1958 for demonstrating the relationship between genes and enzymes. Three years later, Monod and Jacob discovered the regulation of gene expression and, in 1962, Smith and Arbor described restriction endonucleases.

The year 1966 was a very important one, during which the genetic code was deciphered by Nirenberg, Matthei, Leder, Khorana, and Ochoa. Shapiro and Beckwith isolated a gene in 1969 and Khorana chemically synthesized a gene in 1970. Until this point, genetic recombination was recognized to occur only between organisms of the same species or of closely related species. Even in the laboratory, protoplast fusion was restricted to genetically related species. All organisms had restriction endonucleases that recognized foreign DNA and destroyed it so that "illegitimate recombination" would not occur.

Then in 1972–1973, the development of recombinant DNA by Berg, Cohen, and Boyer at Stanford University and the University of California, San Francisco, triggered the birth of modern biotechnology [194]. These workers discovered how to use restriction enzymes to cut DNA molecules, how to use another enzyme, DNA ligase, to join DNA molecules from different organisms, and how to introduce the rDNA via a vehicle (e.g., plasmid, phage) into *E. coli*. They thus defied nature and carried out recombination across species barriers. This propelled biotechnology to new heights and led to the establishment of a new biopharmaceutical industry in the United States and around the world.

The revolutionary exploitation of basic biological discoveries, which began in 1971, did not take place in a vacuum but heavily depended upon the solid structure of the fermentation industry. At that time, a physician (Peter Farley), a biochemist (Ronald Cape, Figure 1.13), and a Nobel Laureate physicist (Donald Glaser), with several others, conceived of the commercialization of rDNA technology and established the Cetus Corporation in Berkeley, California, in 1971. Thus began one of the most exciting adventures in the history of industrial biotechnology. The vision of these Cetus founders led to the establishment of a major biotechnology industry, serving the needs of patients throughout the world and revolutionizing the practise of industrial microbiology.

The second biotechnology company was established in 1976, across the bay from Cetus in South San Francisco, by Herbert Boyer and Robert Swanson. In that same year, a human gene was expressed in bacteria and yeast DNA was replicated and expressed. By 1978, Genentech had developed human insulin and tissue plasminogen activator (tPA). Also in 1978, bacterial DNA was successfully inserted into yeast chromosomes and Biogen was founded in Cambridge, Massachusetts. In 1979, yeast protoplasts were transformed by a hybrid *E. coli*/yeast plasmid. Amgen was founded in southern California in 1980, the same year that a monumental ruling was made by the US Supreme Court stating that living organisms could be patented. This was based on the work of Ananda Chakrabarty.

In 1981, Genetics Institute, Chiron, and Genzyme were formed and the first recombinant diagnostic kit was approved by the FDA. In 1982, recombinant human insulin was ready for the marketplace as a Genentech/Eli Lilly endeavor.

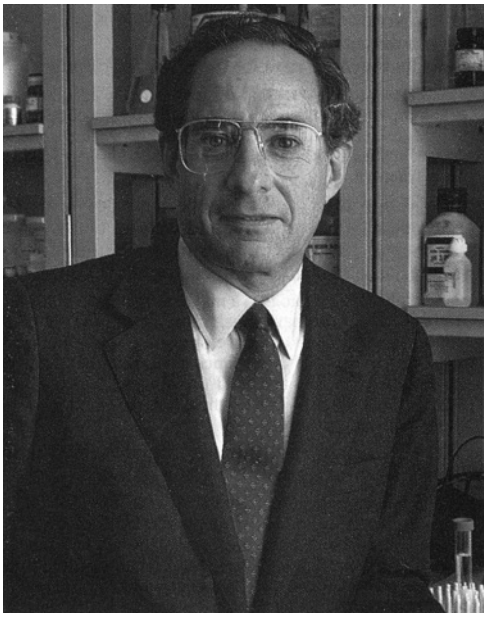

Figure 1.13 Ronald Cape.

Other products soon followed: human growth hormone in 1983; α-interferon, and recombinant hepatitis B vaccine in 1986; tPA in 1987; erythropoietin (EPO) in 1989; granulocyte colony-stimulating factor (G-CSF) in 1991; Factor VIII in 1992; and β-interferon in 1993.

Although Cetus is no longer in existence as an independent corporate entity, having been incorporated into Chiron Corporation in 1991, it should long be remembered as the founder of modern biotechnology and the developer of the polymerase chain reaction (PCR) in 1985, a technique of enormous importance today. Indeed, the PCR principal investigator, Cary Mullis, holds the only Nobel Prize (awarded in 1993) ever given to a scientist for their work in the biotechnology industry.

Since then, thousands of companies have been established, including Immunex, Centocor, MedImmune, etc. Many of them invested in modern biotechnology with no clear idea of the future but with the faith that genetics would lead to products that could not even be conceived of at the time; indeed this dream came true in a major way. This led to an explosion of investment activity in new companies, mainly dedicated to innovation via genetic approaches. Newer companies entered the scene in various niches such as microbiological engineering and downstream processing.

By 1988, there were about 440 biotechnology companies and 70 large pharmaceutical, chemical, and energy corporations in the United States devoting all or part of their resources to biotechnology. In 1993, the field served the following

areas: 41% therapeutics, 27% diagnostics, 15% supplies, 9% agricultural, and 8% chemical, environmental and services. The number of US companies increased to about 1500 by 2003. The number of US employees was 191 000 in 2002. R&D spending on biotechnology in the United States in 2000 amounted to US$21 billion and revenues were US$36 billion.

A significant number of biotechnology companies and departments of large companies were also established in Europe and Asia. The 2005 world market for rDNA products amounted to US$43 billion. Today, large pharmaceutical companies have major holdings in some of these companies and biopharmaceutical revenues have reached over US$60 billion.

The rDNA pharmaceutical market dealt with four principal areas: (i) blood products (thrombolytics, dismutases, septic shock drugs, clotting agents, erythropoietin); (ii) immunotherapy products (α-, β-, and γ-interferons, interleukins, colony-stimulating factors; (iii) infectious disease combattants (hepatitis B vaccine, AIDS vaccine); (iv) growth factors for mammalian cells (epidermal growth factor, insulin-like growth factors, fibroblast growth factors, transforming growth factors, platelet-derived growth factor, growth hormone releasing factor, lung surfactants, and tumor necrosis factor).

Many benefits to society have come from biotechnology [195]. (i) Diabetics no longer have to fear producing antibodies to animal insulin. (ii) Children deficient in growth hormone no longer suffer from dwarfism or fear the risk of contracting Creutzfeldt–Jakob syndrome. (iii) Children who have chronic granulomatous disease are able to have a normal life by taking γ-interferon therapy. (iv) Patients undergoing cancer chemotherapy or radiation therapy recover more quickly with fewer infections when they use granulocyte colony-stimulating factor (G-CSF).

The success of the biopharmaceutical revolution can be seen in the following data. Between 1997 and 2002, 40% of the drugs introduced into medical practise came from biotechnology companies. The five largest pharmaceutical companies in-licensed from six to ten products from biotechnology or specialty pharmaceutical companies yielding 28–80% of their revenue. The biotechnology industry had two drug/vaccine approvals in 1982, none in 1983/1984, one in 1985, and 32 in 2000! The number of patents granted to biotechnology companies rose from 1500 in 1985 to 9000 in 1999. The major products of the biopharmaceutical industry are discussed below.

1.9.1 Human Insulin (Novolin, Humulin)

Human insulin will always be remembered as the product that launched the biopharmaceutical industry. The first recombinant protein produced and then approved by FDA, human insulin was developed by Genentech in 1979 and produced industrially in 1982 by Eli Lilly. Previously, the process required extraction from the pancreas of dead cattle and pigs and the product was not identical to human insulin. Furthermore, the animal products contained impurities that

caused allergic reactions. These problems were all solved by recombinant human insulin.

1.9.2 Erythropoietin (Epogen, Procrit)

Erythropoietin (EPO) is a bone marrow factor for kidney disfunction and for chemotherapy patients used for treatment of chronic renal failure in patients using kidney dialysis. It acts to ameliorate certain anemias by stimulating production and differentiation of red blood cells. The product entered clinical trials in 1985 and was approved in 1989. It is also useful for anemia caused by azidothymidine (AZT) for AIDS and by chemotherapeutics for cancer. EPO is also given to patients who want to use their own banked blood instead of the blood of others.

1.9.3 Interferons

α-Interferon (Intron-A, Roferon) was cleared by the FDA for use against Kaposi's sarcoma, chronic myeloid leukemia, genital warts, and hairy cell leukemia. It became useful in antiviral therapy. By 1992, it had been approved for hepatitis B and C. β-Interferon ("Betaseron," recombinant interferon β-1a, Avonex) was approved by the FDA for multiple sclerosis in 1993 and γ-interferon in 1990 for treatment of chronic granulomatous disease.

1.9.4 Human Growth Hormone (Somatotropin, Somatropin; Humatrope, Nutropin, Protropin, Somatren, Serostim)

An early effort of the Genentech organization was the production of human growth hormone (hGH) which had immediate application in the treatment of abnormally small children. Up to 1984, hGH had been produced from pituitary glands of human cadavers. It was very expensive and, in some cases, the material was contaminated with Creutzfeldt–Jakob virus, resulting in death. Since then, recombinant hGH has replaced the pituitary material and this has eliminated the problem. Although hGH was originally approved only for treating dwarfism, it was later approved for 11 indications; much of it is sold for burns and as an anti-aging product.

1.9.5 Tissue Plasminogen Activator (Activase, Alteplase)

Tissue plasminogen activator (tPA) dissolves blood clots in human coronary arteries and is prescribed for rapid cessation of heart attacks (acute myocardial infarction), deep vein thrombosis, pulmonary embolism, and stroke. It was

introduced on the market in late 1987. Use of tPA was extended to stroke patients in 1996.

1.9.6 Interleukins

Interleukin 2 (IL-2, proleukin) showed activity against renal cell cancer and was approved in 1992. In early 1998, FDA approved recombinant IL-11 (Neumega) for treatment of cancer chemotheraphy-related thrombocytopenia (i.e., low platelet count), due to its ability to stimulate platelet formation.

1.9.7 Factor VIII

Patients with hemophelia traditionally received blood coagulant products derived from human plasma to correct their deficiency of the blood-clotting protein Factor VIII. Unfortunately, 60% of these patients became infected with HIV, hepatitis, or other diseases whose viruses contaminate such products. Clinical trials began in 1989 on recombinant Factor VIII for use in hemophelia. The FDA approved the product in 1993.

1.9.8 Colony-Stimulating Factors

Colony-stimulating factors are bone marrow factors for kidney disfunction and for chemotherapy patients. Granulocyte colony-stimulating factor (G-CSF; Neupogen, Filgrastim, Leukine) was approved in 1991 for chemotherapy-induced white blood cell deficiency (neutropenia). Granulocyte macrophage colony-stimulating factor (GM-CSF) was also approved in 1991 for stimulation of white cell growth in autologous bone marrow transplants.

1.9.9 Human DNase (Pulmozyme)

Human DNase was approved by the FDA in 1994 for cystic fibrosis (CF). It was the first new drug in 30 years for CF, a disease that affects 30 000 people in the United States. In clinical trials, DNase has also shown efficacy in chronic bronchitis, a disease that affects 2 million people in the United States.

1.9.10 Glucocerebrosidase (Cerezyme)

Glucocerebrosidase was approved in 1994 for the genetic disorder Gaucher's disease. Patients lacking this enzyme cannot prevent lipid accumulation in vital organs and bones.

1.9.11 Monoclonal Antibodies

Monoclonal antiobodies (mAbs) were discovered by Georges Kolter and Cesar Milstein in the United Kingdom in 1975 [196]. They fused a mouse skin cancer cell ("myeloma") with an antibody-producing white cell. The result was a hybrid cell ("hybridoma") which produced a pure specific antibody. The two immunologists were awarded the Nobel Prize in 1984. Previously, polyclonal antibodies (pAbs) had been used but they contained varying specificities and affinities and were very variable. They had been produced by the entire immune system of the animal, whereas mAbs were produced by single cells of the immune system.

For mAb production, mice were immunized with a single antigen, allowed to show an immune response, their spleens were removed, cells extracted, fused with cells of mouse lymphoma cell line (immortal cancerous). Then, the fused "hybridoma" cells were cloned and screened to isolate those hybridoma cells that excreted the specific desired antibody. Since the human body could react in an undesirable way to mouse sequences, the mAbs were "humanized" by genetic engineering techniques. The mice were genetically engineered so that human genes encoding human heavy chains and human kappa light chain replaced the relevant mouse genes which were eliminated. Monoclonals could also be made in human immune cells. They were used to bind or block the binding of a target protein as a "magic bullet" in which a drug or a radioisotope is brought to a designated target.

After 2000, mAbs became the fastest growing therapeutic protein class, reaching a market of US$6.8 billion in 2006. ReoPro was the first successful therapeutic mAb, being approved in 1994 for inhibition of platelet aggregation (blood clotting). It successfully prevented complications of angioplasty such as death, heart attack, and need for repeat angioplasty. It was followed by four more monoclonals in 1998: (i) infliximab (Remicade) inhibited tumor necrosis factor (TNF) and was approved for Crohn's disease and also for rheumatoid arthritis; (ii) basiliximab (Simulect) was used prophylactically against acute organ rejection in patients receiving renal transplantation, along with ciclosporin and corticosteroids; (iii) trastuzumab (Herceptin) targeted the epidermal HER2 growth factor receptor protein oncogene and was used for late-stage metastatic breast cancer in 25–30% of the women with this disease and whose tumors overexpressed HER2; (iv) palivizumab (Synagis, MEDI-493) for prevention of lower respiratory tract disease caused by the respiratory syncytial virus. This was the first mAb against an infectious disease and was used to stop respiratory syncytial virus (RSV) leading to serious lower respiratory tract disease in pediatric patients. Adalimumab (Humira) was approved in 2003 for rheumatoid arthritis. Another very important monoclonal is retuximab (Rituxan), used for non-Hodgkin's lymphoma.

1.9.12 Additional Biopharmaceuticals

Other important products include: (i) etanercept (Enbrel), approved in 1998 for rheumatoid arthritis via its binding and inhibition of TNF, a protein involved in

inflammation; and (ii) imatinib (Gleevec, Glivec), which is active against chronic myelogenous leukemia (CML). CML is a disease caused by genetic translocation between chromosomes 9 and 22, generating an abnormal protein, Brc-Ab1, which causes uncontrolled proliferation of white blood cells resulting in leukemia. Imatinib blocks the action of Brc-Ab1 and is also active against gastrointestinal stromal tumor (GIST).

1.10 Recombinant Hosts

High cell density fermentation of microorganisms reaches levels of 233 g dry cell weight/l for bacteria and 268 g dry cell weight/l for yeasts [197]. Mammalian polypeptides are produced in these microbes at levels up to 70% of cell protein and concentrations as high as 15 g/l.

1.10.1 *E. coli*

The first most popular bacterial system for production of recombinant proteins was *E. coli*. Early in the era of biopharmaceuticals, it was realized that the same milligram quantities of mammalian polypeptides that were being produced in a few liters of recombinant *E. coli* broth previously had to be extracted from the brain tissue of half a million sheep. The benefits of *E. coli* as a recombinant host, in addition to high cell densities and elevated product yields, included the following: (i) it was easy to quickly and precisely modify the genome; (ii) growth was rapid; (iii) culture conditions were simple; (iv) protease activity was easily reduced; (v) avoidance of incorporation of amino acid analogs was possible; (vi) promoter control was simple; (vii) plasmid copy number could be altered easily; (viii) alteration of metabolic carbon flow was not a problem; (ix) formation of intracellular disulfide bonds was easy; (x) accumulation of heterologous proteins amounted to as much as 50% of dry cell weight; (xi) survival was possible under a wide variety of environmental conditions; (xii) expensive medium ingredients were not required; and (xiii) performance was reproducible especially with computer control [195].

One problem with *E. coli* was the formation of the heterologous proteins in the form of inclusion bodies. In this form, the recombinant proteins were inactive, aggregated, and insoluble, usually possessing non-native intra- and intermolecular disulfide bonds and unusual free cysteines. To produce active protein, these bodies had to be removed from the cell by homogenization, washing, and centrifugation, solubilized by denaturants (guanidine HCl, urea, sodium dodecyl sulfate) which unfolded the protein, and treated with reducing agents which broke the disulfide bonds. Then, refolding was carried out by removal of denaturant and reducing agent. The renaturation processes used were (i) air oxidation, (ii) the glutathione reoxidation system, and (iii) the mixed disulfides of protein-*S*-sulfonate and protein-*S*-glutathione systems. Heterologous recombinant proteins were also made

in biologically active soluble form at high levels by fusing their genes to the *E. coli* thioredoxin gene. Many human proteins were produced at levels of 5–20% of total proteins as fusions in *E. coli* cytoplasm. Some fusions retained the thioredoxin properties of (i) being released by osmotic shock or freeze/thaw methods and (ii) high thermal stability.

Another useful method of reducing the formation of inclusion bodies containing heterologous proteins in *E. coli* is to lower the temperature of growth from 37 to 30 °C. Products made in *E. coli* include human insulin, human growth hormone, α β γ-interferons, and G-CSF [195].

Almost all polypeptides excreted by eukaryotes are glycosylated. Glycosylation is species-, tissue-, and cell-type specific. Unfortunately, *E. coli* does not glycosylate proteins. In some cases, a normally glycosylated protein is active without the carbohydrate moiety and can be made in bacteria. This was found to be the case with γ-interferon. In cases where glycosylation is necessary for stability or proper folding (e.g., erythropoeitin), proteins can often be provided by recombinant yeast, mold, insect, or mammalian cells.

1.10.2 Yeasts

Yeasts offer certain advantages over bacteria as a cloning host. (i) They can secrete heterologous proteins into the extracellular broth when proper signal sequences are attached to the structural genes. (ii) They carry out glycosylation of proteins. However, glycosylation by *S. cerevisiae* is often unacceptable for mammalian proteins because the *O*-linked oligosaccharides contain only mannose whereas higher eukaryotic proteins have sialylated *O*-linked chains. Furthermore, *S. cerevisiae* overglycosylates *N*-linked sites which led to reduction in both activity and receptor-binding, causing immunological problems.

The methylotrophic yeast *Pichia pastoris* was found to possess advantages over *S. cerevisiae* as a host for heterologous genes. (i) This yeast could be grown at extremely high cell densities in protein-free media. (ii) It had a higher level of protein productivity. (iii) It did not overglycosylate. (iv) Foreign genes were incorporated in multiple copies into the chromosome. (v) The levels of protein production by these yeasts were relatively high. For example, *P. pastoris* can produce extracellularly 4 g/l of intracellular IL-2, 4 g/l of human serum albumin, and 10 g/l of TNF. (vi) The expression cassette was stably integrated into the host genome at specific locations. (vii) *P. pastoris* was haploid and amenable to traditional mutagenesis.

1.10.3 Molds

When foreign genes are introduced via plasmids into filamentous fungi, they integrate stably into the chromosome as tandem repeats. As many as 100 copies of a gene are observed. Production of bovine chymosin by recombinant *A. niger*

var. *awamori* amounts to 1 g/l and that of human lactoferrin by *A. awamori* is 2 g/l of extracellular protein.

1.10.4 Insect Cells

Insect cells in culture are good hosts for recombinant protein production [198]. Recombinant insect cell cultures have yielded over 200 proteins encoded by genes from viruses, bacteria, fungi, plants, and animals. Expression vectors have been prepared from the baculovirus which attacked invertebrates but not vertebrates or plants, thus insuring safety. The most widely used baculovirus is the nuclear polyhedrosis virus (*Autographa californica*) which contains circular double-stranded DNA, is naturally pathogenic for lepidopteran cells, and can be grown easily *in vitro*. The virus contains a gene encoding the protein polyhedrin which is normally made at very high levels and is not necessary for virus replication. The gene to be cloned was placed under the strong control of the viral polyhedrin promoter and suitable levels of proteins were produced with many of the posttranslational modifications of higher eukaryotes, including phosphorylation, glycosylation, correct signal peptide cleavage, proteolytic processing, palmitylation, and myristylation.

The usual host is the fall armyworm (*Spodoptera frugiperda*) in suspension culture. Alternatively, a larval culture is used which is much cheaper than cell culture. Larval systems have produced 600 mg/l of recombinant protein.

1.10.5 Mammalian Cells

The use of mammalian cell culture, chiefly immortalized Chinese hamster ovary (CHO) cells, was mandated by the need for EPO and tPA production in the early 1980s [195]. The development of mammalian cell culture was facilitated by prior developments in microbial fermentation technology. Mammalian cell cultures were useful in that the proteins were made in a properly folded and glycosylated form, thus eliminating the need to renature them. The production of recombinant proteins by mammalian cells was also done in N50 murine myeloma cells, baby hamster kidney cells, green monkey kidney cells, and human embryonic kidney cells. Mammalian cell culture became the leading source of recombinant biopharmaceuticals and was used for production of human growth hormone, GM-CSF, G-CSF, EPO, and Pulmozyme, among others. CHO cell processes were developed that yielded 3–5 g/l of recombinant protein.

1.10.6 Transgenic Animals

Transgenic animals were developed as production systems for recombinant peptides. tPA was made in milk of transgenic goats at a level of 3 g/l. Cows produce 30l of milk per day, of which protein amounts to 35 g/l; thus the total protein

produced per day is 1 kg. Production titers are 2 g/l of antithrombin III and 4 g/l of human growth hormone in the milk of mice, 5 g/l of recombinant fibrinogen in sheep milk, 8 g/l of α-glucosidase in rabbit milk, 14 g/l of antithrombin III in goat milk, 35 g/l of α-1-antitrypsin in sheep milk, and 40 g/l of hemoglobin in pigs; all genes were human in origin.

In most cases, the protein is as active as the native protein. Transgenic goats produce a tPA with glycosylation different from that produced in cell culture and with a longer half-life than native tPA. One of the negative points in production of proteins by transgenic animals is the length of time needed to assess production levels. This takes 3.5 months in mice, 15 months in pigs, 28 months in sheep, and 32 months in cows.

1.10.7
Transgenic Plants

Transgenic plants could also be used to produce valuable products, including β-glucuronidase (GUS), avidin, laccase, and trypsin. Oilseed rape plants have been used to produce enkephalin and a neuropeptide. Recombinant proteins can be produced in transgenic plants at levels as high as 14% of total tobacco-soluble protein (in the case of phytase from *A. niger*) and 1% of canola seed weight (hirudin from *Hirudo medicinalis*). Potential advantages include satisfactory glycosylation, targeting, compartmentalization, and natural storage stability.

1.11
Enzymes

The term "enzyme" was first coined by Kuhne in 1877 meaning "in yeast." When the field of biochemistry was born in 1897 via Buchner's discovery that cell-free extracts of yeast could carry out the production of ethanol from sugar, Buchner referred to the glycolytic enzyme complex as "zymase," meaning "the enzyme of yeast itself." Enzymes became valuable in manufacturing because of their rapid and efficient action at low concentrations under mild pH values and temperatures, their high degree of substrate specificity (which reduced side-product formation), their low toxicity, and the ease of terminating their action by mild treatments. Some microbial strains produced very high concentrations of extracellular enzymes. Wild strains of *Bacillus licheniformis* produced 5 g/l of protease and commercial strains made 20 g/l. High-yielding strains of *Aspergillus* produced 20 g/l of glucoamylase.

Additional reasons for using microbial cells as sources of enzymes were as follows: (i) enzyme fermentations were quite economical on a large scale due to short fermentation cycles and inexpensive media; (ii) screening procedures were simple and thousands of cultures could be examined in a reasonably short time; and (iii) different species produced somewhat different enzymes catalyzing the same reaction, allowing one flexibility with respect to operating conditions in the

reactor. This versatility is illustrated by the fact that α-amylase from *Bacillus amyloliquefaciens*, a commercial enyzme used for years for hydrolysis of starch at a temperature as high as 90 °C, was forced to compete in 1972 with a similar enzyme from *B. licheniformis* which could operate at 110 °C The optimal temperatures for the *B. amyloliquefaciens* and the *B. licheniformis* α-amylases were 70 °C and 92 °C respectively.

In the 1980s and 1990s, microbial enzymes were increasingly used for applications which traditionally employed plant and animal enzymes. These shifts included the partial replacement of (i) amylases of malted barley and wheat in the beer, baking, and textile industries by amylases from *Bacillus* and *Aspergillus*; (ii) plant and animal proteases by *Aspergillus* protease for chill-proofing beer and tenderizing meat; (iii) pancreatic proteases by *Aspergillus* and *Bacillus* proteases for leather bating and in detergent preparations; and (iv) calf stomach rennet (chymosin) by *Mucor* rennins for cheese manufacture. Later, cloning of mammalian chymosin became of interest to cheese manufacturers and tests on cheese made with the recombinant enzyme showed commercial success. Recombinant chymosin was approved in the United States and its price was half that of natural calf chymosin. Important industrial enzymes included the following: (i) glucose isomerase for production of high-fructose corn sirup; (ii) penicillin acylase for production of semi-synthetic penicillins; (iii) peroxidase for manufacture of phenolic resins (which could replace synthetic phenol-formaldehydes); and (iv) nitrile hydrolase for hydration of acrylonitrile to acrylamide. Glucose isomerase was used in conjunction with α-amylase and glucoamylase to convert starch to mixtures of glucose and fructose known as "high fructose corn syrup." The development of glucose isomerase permitted the corn wet milling industry to capture 30% of the sweetener business from the sugar industry in the 1970s. In the United States alone, high fructose corn syrup is produced at 30 billion pounds per year.

The industrial enzyme market reached US$2 billion in 2000 divided into the following application areas: food, 45% (of which starch processing represents 11%); detergents, 34%; textiles, 11%; leather, 3%; pulp and paper, 1.2%. This does not include diagnostic and therapeutic enzymes. The world market for products of enzyme reactions were as follows: high fructose corn syrup, US$1 billion; aspartame, US$800 million; acrylamide, US$300 million; 6-aminopenicillanic acid (6-APA) and 7-aminodeacetoxycephalosporanic acid (7ADCA), US$200 million.

Certain microorganisms ("extremophiles") can grow in extreme environments such as 100 °C, 4 °C, 250 atm, pH 10, pH 2, or 5% NaCl. "Extremozymes," that is, enzymes from these diverse organisms, have industrial significance. A commercial example is Cellulase 103 from an alkaliphile. The enzyme broke down the microscopic fuzz of cellulose fibers which trapped dirt on the surface of cotton textiles. The enzyme was commercialized by Genencor International in 1997 for use in detergents to return the "newness" of cotton clothes even after many washings.

With the development of rDNA methodology it became possible to clone genes encoding microbial enzymes and express them at levels hundreds of times higher than those naturally produced. The industrial enzyme business adopted rDNA

methods eagerly to increase production levels and to produce enzymes from non-industrial microorganisms in industrial organisms, such as species of *Aspergillus* and *Trichoderma*, as well as *Kluyveromyces lactis*, *S. cerevisiae*, *Yarrowia lipolytica*, and *B. licheniformis*. Over 50% of the market is provided by recombinant processes. Sixty per cent of the calf rennin (chymosin) used for cheese making in the United States is supplied by recombinant *E. coli* and the two lipases used industrially (i.e., *Humicola* lipase produced in *Aspergillus* and *Pseudomonas* lipase) are both recombinant. Heat-stable amylase from *B. licheniformis* has been made in a gene-amplified strain of the same species. Plant phytase (produced in recombinant *A. niger*) is used as a feed for 50% of all pigs in Holland. A 1000-fold increase in phytase production was achieved in *A. niger* by use of recombinant technology. Scientists at Novo Nordisk have isolated a very desirable lipase for use in detergents from a species of *Humicola*. For production purposes, the gene was cloned into *A. oryzae* where it produced 1000-fold more enzyme [199]. It became a commercial product for laundry cleaning, for interesterification of lipids and for esterification of glucosides producing glycolipids which have applications as biodegradable non-ionic surfactants for detergents, skincare products, contact lenses, and as food emulsifiers.

Virtually all laundry detergents now contain genetically engineered enzymes and much cheese is made with genetically engineered microbes. Over 60% of the enzymes used in the detergent, food, and starch processing industries are recombinant products [200].

The properties of many enzymes have been altered by genetic means. "Brute force" mutagenesis and random screening of microorganisms over the years have led to changes in pH optimum, thermostability, feedback inhibition, carbon source inhibition, substrate specificity, V_{max}, K_m, and K_i. This information has been exploited by the more rational techniques of protein engineering. Single changes in amino acid sequences have yielded similar types of changes in a large variety of enzymes. For example, a protease from *Bacillus stearothermophilus* was increased in heat tolerance from 86 °C to 100 °C, that is, it was made resistant to boiling! The enzyme was developed by site-directed mutagenesis [201]. Only eight amino acids had to be modified. Temperature stability at 100 °C was increased 340-fold and activity at lower temperature was not decreased. All eight mutations were far from the enzyme's active site.

An excellent method for improving enzymes is directed evolution (also known as applied molecular evolution or directed molecular evolution [202]. DNA shuffling, one type of directed evolution, has achieved significant improvement of catalytic activity, modified specificity, and improved stability of enzymes. This method of pooling and recombining parts of similar genes from different species or strains has yielded remarkable improvements in enzymes in a very short period of time. The procedure actually mimicks nature in that mutation, selection, and recombination were used to evolve highly adapted proteins, but it was much faster than nature. Enzyme activity has been improved up to 32 000-fold (TEM-1 β-lactamase), substrate specificity by 1000-fold (β-galactosidase), protein folding by 48-fold (green fluorescent protein), antibody activity by over 400-fold, expression by 100-fold, arsenate resistance by 40-fold, atrazine degradation by 80-fold, etc. [203].

Proteins from directed evolution work first went on the market in 2000. These were green fluorescent protein of Clonetech and Novo Nordisk's LipoPrime® lipase. Directed evolution provided β-glucosidase activity to a β-galactosidase, converted a β-glucuronidase into a β-galactosidase, gave phospholipase activity to a lipase, and converted an indole-3-glycerol-phosphate synthase into a phosphoribosylanthranilate isomerase [204].

1.12 Bioconversions

The first example of the use of a biological process to compete with a chemical process in the petrochemical industry was the production of acrylamide, made at 200 000 tons per year as a flocculant, a component of synthetic fibers, a soil conditioner, and a recovery agent in the petroleum industry [205]. The chemical process involving copper salt catalysis of the hydration of acrylonitrile had problems associated with it. A bioconversion using *Pseudomonas chlororaphis* B23 or *Rhodococcus rhodochrous* J1, in which nitrile hydratase was induced by methacrylamide and catalyzed the hydration, competed with the chemical reaction. The conversion yield was over 99.99%, was carried out at 10 °C, and the cells were used many times. The titer was 656 g/l after 10 h. Today, bioconversions are used widely in chemical manufacture. They have also become essential to the fine chemical industry because of the demand for single-isomer intermediates.

1.13 Vaccines

Protein antigens for vaccines have been made by cloning and expressing genes coding for surface antigens of viruses, bacteria and parasites. The first subunit vaccine on the market was that of hepatitis B virus surface antigen which was produced in yeast. In 1994, the first recombinant live veterinary vaccine was approved by the USDA. The VectorVax FP-N vaccine produced by Syntro Corp. used a fowlpox virus vector whose two disease-causing genes were deleted to produce a vaccine against both fowlpox virus and Newcastle disease.

1.14 Systems Microbiology

"Systems microbiology" emerged as a term and a scientific field to describe an approach that considers genome-scale and cell-wide measurements in elucidating processes and mechanisms carried out by microbial cells [206]. An expanded view of the microbial cell became possible due to the impressive advances in (i) genomics and in other "omic" techniques (e.g., proteomics, metabolomics) and

(ii) high-throughput technologies for measuring different classes of key intracellular molecules. For discovery of novel active secondary metabolites of commercial importance, genomics have provided a huge group of new targets against which natural products are being screened. The human genome has 30 000–35 000 genes, less than 50% having a putative function. These genes have the potential to produce over 100 000 proteins. Estimates of the number of proteins acting as useful targets range from 600 to 10 000.

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2
Industrial Systems Biology

José Manuel Otero and Jens Nielsen

2.1
Introduction

The chemical industry is currently undergoing a dramatic change driven by demand for more sustainable processes for the production of fuels, chemicals, and materials, as a result of both financial and environmental constraints. The industry is therefore looking into the use of biotechnological processes where the feedstock is renewable plant materials, as a replacement for traditional chemical processes based on petroleum feedstocks. In biotechnological processes different microorganisms can be exploited, and in particular the large diversity of metabolic reactions represents a rich source for design of chemical conversion processes that leads to efficient production of desirable products. However, it is often found that microorganisms that produce a desirable product, either naturally or because they have been engineered through insertion of heterologous pathways, have low yields and productivities, and in order to establish an economically viable process it is necessary to improve the performance of the microorganism.

Here metabolic engineering is the enabling technology. Through metabolic engineering the metabolic landscape of the microorganism is engineered such that there is an efficient conversion of the raw material, typically glucose, to the product of interest. This process may involve insertion of new enzymes activities or deletion of existing enzyme activities, but often also deregulation of existing regulatory structures operating in the cell.

In order to rapidly identify the optimal metabolic engineering strategy the industry is to an increasing extent looking into the use of tools from systems biology. This involves both "x-ome" technologies such as transcriptome, proteome, metabolome, and fluxome analysis, and advanced mathematical modeling tools such as genome-scale metabolic modeling. Here we look into the history of these different techniques and review how they find application in industrial biotechnology, which will lead to what we here define as industrial systems biology.

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2.2 Industrial Biotechnology

The term "industrial biotechnology" first widely appeared in the literature in the early 1980s when genetic engineering, propelled by recombinant DNA technology, was searching for applications beyond health care and medical biotechnology [1, 2]. Today, industrial biotechnology represents a well-defined field with significant academic, government, and corporate representation. Formally, industrial biotechnology is the bioconversion, either through microbial fermentation or biocatalysis, of organic feedstocks extracted from biomass or their derivatives to chemicals, materials, and/or energy. Biomass is the result of photosynthetic carbon fixation by plants to form organic polymers that may be digested, enzymatically or chemically, to carbohydrate, protein, and lipid monomers. Industrial biotechnology, often referred to as "white biotechnology" in Europe [3], aims to provide cost-competitive, environmentally friendly, self-sufficient alternatives to existing or newly proposed petrochemical processes.

Processes that exploit industrial biotechnology have recently garnered increasing global attention with traditional petrochemical processing under scrutiny as a result of increasing raw material costs, environmental constraints, and decreasing self-sufficiency.

Industrial biotechnology has experienced unprecedented growth with bio-based production processes representing 5% of the total chemical production sales volume. By 2010, several studies have estimated that the total fraction will increase to 20%, representing US$310 billion of a projected total sales volume of US$1600 billion. Industrial biotechnology will continue to capture significant sales volume percentages in the arenas of basic chemicals and commodities (2–15%), specialty or added-value chemicals (2–20%), and polymers (1–15%). However, the greatest percentage gain is likely to occur in the fine chemical market (16–60%), where industrial biotechnology platforms enable complex chemistry that are presently produced via complex synthetic or combinatorial routes [4].

Furthermore, industrial biotechnology is enabling new products, particularly novel therapeutic agents such as polyketides and specialty chemicals not previously identified, such as the diverse polyunsaturated fatty acids and biopolymers produced by microalgae [5].

Industrial biotechnology is by no means a new field, with fermentation processes for antibiotics (penicillin production by *Penicillium chrysogenum*; annual market size exceeding US$1.5 billion), vitamins (L-ascorbic acid production by the Reichstein process and biocatalysis by *Gluconobacter oxydans*; annual market size exceeding US$600 million), organic acids (citric acid production by *Aspergillus* sp.; annual market size exceeding US$1.5 billion), and amino acids (L-glutamate and L-lysine production by *Corynebacterium glutamicum*; annual production exceeding 600 000 tons) well established [5].

In each of these examples, host organisms well suited for production of the target compound were isolated naturally. Furthermore, under controlled environments, random mutagenesis followed by screening, selection, and traditional

bioprocess development were used to enhance production yields, titers, productivities, and robustness. Despite the fact that this method provides little to no mechanistic understanding of which specific genetic perturbations lead to improved strains so that they could be further exploited, it has proven to be commercially successful as illustrated by the more than 1000-fold improvement in penicillin titer by *P. chrysogenum* [6].

The significant increases in fundamental research and development, and commercialization at industrial scales of biotechnological processes may be attributed to several key factors, which can be grouped into four broader factors that are important to consider in connection with development of a new bio-based process:

1) process economics
2) biotechnology process development
3) environmental impact
4) sustainability and self-sufficiency.

Each of these broad factors involve several identifiable and quantitative drivers fueling the application of industrial biotechnology to processes previously exclusive to the petrochemical industry or for the production of new chemicals. Figure 2.1 shows an overview of how these four factors needs to be considered and evaluated before development of any industrial biotechnology process begins.

Industrial biotechnology, as a means of developing petrochemical alternative processes that are cost-effective, commercially viable, sustainable, self-sufficient, and environmentally favorable, has recently received significant attention with crude petroleum prices between January 2007 and 2008, increasing 41.2% [7]. Furthermore, between 1997 and 2007, natural gas, a common feedstock for commodity and added-value chemicals production, witnessed a well-head price increase of 175.4% [8]. Figure 2.1 summarizes the four key factors that are often evaluated when considering substitution of a petrochemical process with a biotechnology process, or its implementation for production of a novel chemical. Process economics, as compared to petrochemical equivalents or other benchmarking processes, are critical in establishing commercial viability, with particular focus paid to long-term operating costs. Next, biotechnology development costs, resources, and development efforts are considered, with initial analysis focused on establishing pilot-plant scale proof-of-concept. This review will in particular highlight the impact metabolic engineering and systems biology have had on upstream process development, referring to a game-changing paradigm referred to as "industrial systems biology." The final two factors to be critically evaluated include sustainability and self-sufficiency, and environmental impact. Sustainability and self-sufficiency not only relate to process-specific considerations, such as feedstock availability, or the opportunities for further expansion through biorefinery integration, but also include focus on public perception and the socio-political landscape. There is no question that the recent infusion of effort and resources into biotechnology processes would not have been possible, even under the current economic climate, if the significant government policy initiatives, coupled to broad social

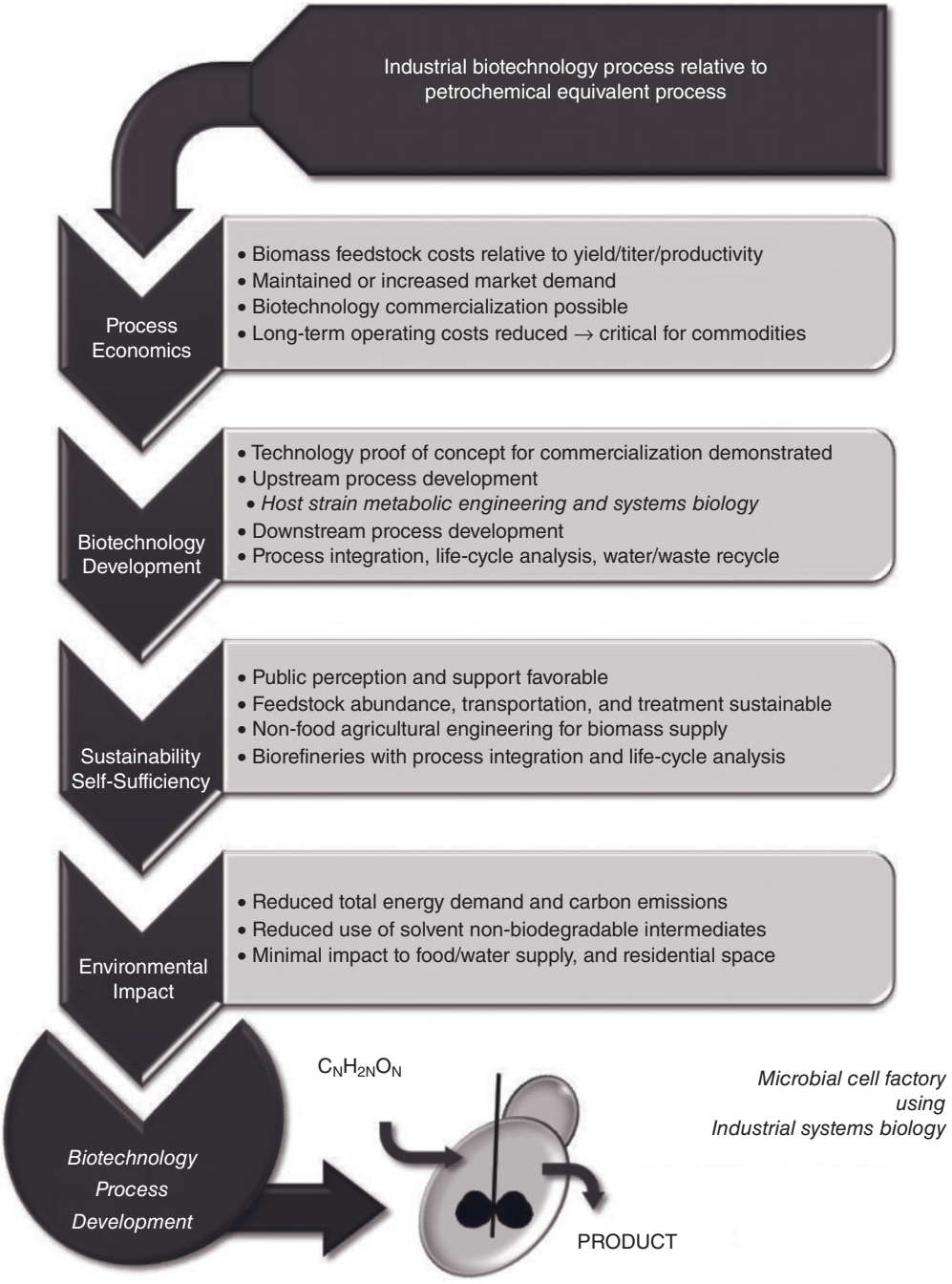


Figure 2.1 Four drivers of industrial biotechnology.

support, were not present. This is also noted when considering environmental impact, particularly in light of the recent scientific confirmation of global climate change resulting from the increase in greenhouse gas emission [9]. Careful consideration of these four general sectors, will ultimately determine whether proceeding with biotechnology process development is warranted or not. While not immediately obvious to most research and development scientists or engineers, it is critical to not divorce the impact these considerations may have on process development, particularly in designing strategies for construction of a microbial cell factory. It is such analysis that often defines the constraints, boundaries, targets, and viable metabolic engineering strategies, including which systems biology approaches should be exploited to experimentally demonstrate proof-of-concept.

This review aims to provide a historical perspective of industrial biotechnology process development, and in particular, focus on the rapid deployment of metabolic engineering and systems biology technologies that first emerged from academic research groups driven by the human health and medical biotechnology sectors. Specifically, mature, recently launched and in-development examples of products that have benefited from this novel *modus operandi*, that is systems biology, will be highlighted for motivation. Based on such examples, and *de novo* processes presently in proof-of-concept, we here define a new term, industrial systems biology, acknowledging that tools established in the rapidly growing field of systems biology, often applied to metabolic engineering, are prevalent in two forms. Enterprises are reshaping existing or forming new process development groups with industrial systems biology capabilities and expertise, or they are outsourcing process development to small, recently formed entities that specialize in industrial systems biology.

Figure 2.2 provides a more focused schematic overview of industrial systems biotechnology, and the significant level of data integration and analysis required to ultimately yield a viable commercial process. Industrial systems biology is a dynamic interaction between various disciplines and approaches. At the core is a platform technology based on a production host, for which a genome sequence is available, and subsequent annotations based on existing literature review, database query, comparative genomics, and experimental data, where available, are completed. The annotations may vary in types of functional genomics data assigned to specific fields; however, a standard skeleton syntax structure of defining a gene, the gene product (e.g., metabolic enzyme), the metabolites serving as reactants and products (including any cofactors and intermediates), and the resulting stoichiometry is often applied. This genome annotation may then have additional fields defined, such as experimentally determined transcriptome levels, intracellular or extracellular metabolite concentrations, isotope-based flux measurements, and protein translation levels. This framework, referred to as a genome-scale metabolic network reconstruction, may then be used for stoichiometric or kinetic modeling. Often, because kinetics parameters such as the forward and reverse reaction rates at physiologically relevant conditions have not been experimentally determined for a significant fraction of the network, flux balance analysis (FBA)

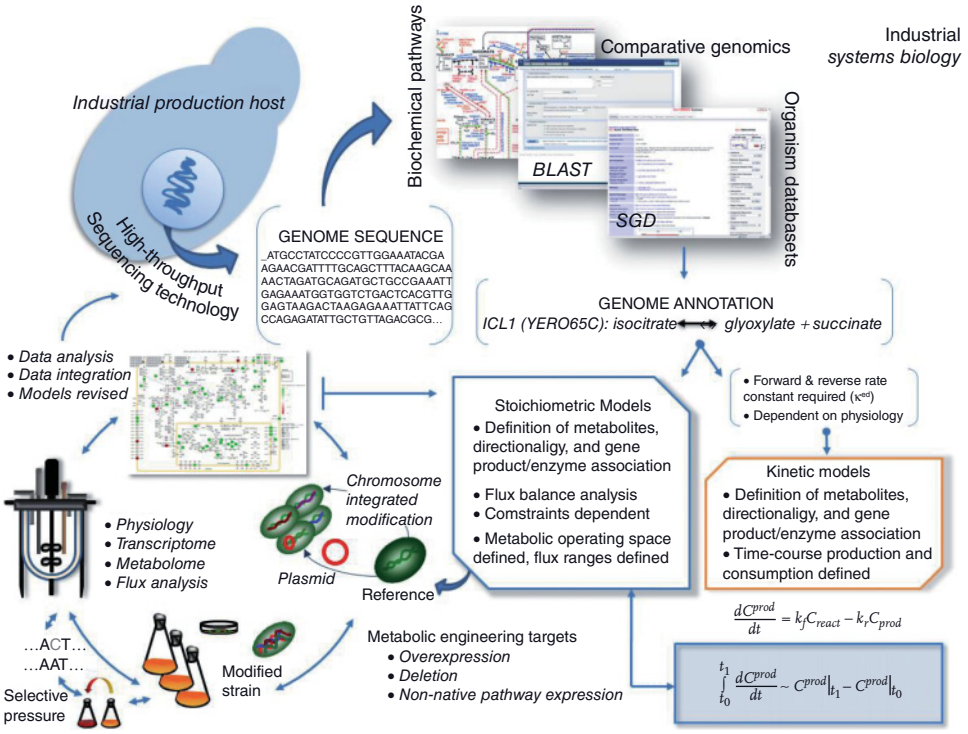


Figure 2.2 Industrial systems biology.

is used for predictive modeling as it only depends on the stoichiometry and network constraints (e.g., precise stoichiometric definition of biomass, ATP maintenance terms, glucose uptake rate).

Such modeling allows two essential functions for metabolic engineers to be fulfilled. First, it creates a structured archive of microbial metabolism that may be inspected, interrogated, and further optimized through visual displays. Second, it provides a framework for predictive simulations, under specified constraints, narrowing the metabolic engineering strategies considered, which are often based on classical biochemical pathway knowledge or de novo strategies that arose as a result of simulation results. Once a high probability of success (HPOS) metabolic engineering strategy has been identified, often requiring gene overepxression, deletion, or non-native pathway reconstruction, genetic engineering is performed on the production host, yielding a modified strain. The modified strain is initially characterized, and may undergo directed evolution or other non-targeted approaches to yield an improved phenotype. The resulting modified strain is then characterized under well-controlled fermentation conditions, where physiological parameters, such as maximum specific growth rate, substrate consumption rates, product yields and titers, by-product formation, and morphology are determined.

Furthermore, functional genomics characterization, often requiring transcriptome, proteome, metabolome, and fluxome measurements is completed. Bioinformatics, coupled with data integration, are then required for analysis of the resulting modified strain, and to identify opportunities for a second round of metabolic engineering.

Furthermore, the analysis should lead to a revised model with improved predictive power that may yield promising strategies for further phenotype improvement. While this approach has often been referred to as the metabolic engineering cycle, we here compliment the traditional cycle to include integrative approaches and data sets from systems biology. Together, when applied to industrial biotechnology products, this is referred to as industrial systems biology.

The sections that follow will provide a brief overview of (i) the market drivers for industrial biotechnology and (ii) industrial systems biology. Subsequent sections will further focus on exploring industrial systems biology from the perspective of modeling microbial metabolism, and in particular, providing case studies of diverse products that have benefitted tremendously from milestones in metabolic engineering and systems biology.

2.3
Market Drivers for Industrial Biotechnology

The four factors referred to in Figure 2.1 are highly interconnected, and there is significant debate as to the ranking of these factors in terms of priority and impact. Process economics are often estimated by quantitative modeling that includes major process costs, both operating and capital, and process value, dictated by the product's estimated market price, demand growth rate, market share, and any competitive advantages that may exist. For commercialization it is reasonable to assume that process economics must be favorable before any further effort can continue in considering biotechnology process development, sustainability, or environmental impact (see Figure 2.1).

Sustainability and self-sufficiency are perhaps less well-defined, noting that many of the issues considered, including raw material availability and the potential for process integration into a biorefinery, are likely to be discussed in the context of process economics. It is included in Figure 2.1 and discussed as a separate category simply because of the recent focus it has received in the background of significantly increasing petroleum and feedstock prices. Self-sufficiency and sustainability may actually trump process economics in cases where issues such as national security play a role (e.g., dependence on a foreign state for significant proportions of energy).

While it may seem unorthodox to discuss market drivers in the context of a chapter focused on the progress in metabolic engineering and systems biology that have elucidated microbial metabolism, enabling its exploitation, it is in fact among the features that distinguishes industrial biotechnology from many other disciplines. Market drivers, and a clear understanding of their impact, will

significantly guide which metabolic engineering strategy may be preferred, and consequently, drive research and development. For example, selection of the appropriate feedstock, such as glucose, xylose, or glycerol, or complex feedstocks such as lignocellulose, for an industrial biotechnology process will significantly drive which metabolic engineering strategy is to be selected, and consequently which systems biology tools are to be utilized. Certainly, feedstock selection is closely tied to the sectors highlighted in Figure 2.1.

A more concrete example is provided in a recent review of the impact that biofuels, and more specifically, bioethanol, has had on the field of industrial biotechnology. It provides context and a clear linkage between market drivers and subsequent milestones in industrial systems biology, and their impact on process development [10].

2.4 Industrial Systems Biology

Systems biology is the quantitative collection, analysis, and integration of whole genome scale data sets enabling biologically relevant and often predictive mathematical models to be constructed. With genome sequences becoming readily available for production organisms, process development has been a benefactor of the scientific achievements in systems biology, particularly in the areas of transcriptomics, proteomics, metabolomics, and fluxomics. Such developments today encompass a systems biology toolbox that may be further exploited for production of metabolic intermediates that often serve as desirable precursors in the petrochemical sector. Given the many definitions and extensive nomenclature that has evolved in systems biology, a glossary of x-omic terminology is given in Table 2.1, and it is these definitions that will be used here.

The examples to be discussed here will largely focus on upstream process development, with particular attention paid to the metabolic engineering strategy employed, and how functional genomics data and analysis provided clear advantages. The examples cited will draw examples from numerous fermentation organisms, with focus, however, on *Saccharomyces cerevisiae*.

It will be of little surprise that the largest industrial biotechnology product in the world, recently garnering unprecedented corporate, social, and government support, is bioethanol. In 2005, total world production of bioethanol was 46 billion liters, with the production volume and the total number of refineries built between 2005 and 2006 in the United States increasing by 2.6 billion liters and 14, respectively [11].

S. cerevisiae today is the preferred bioethanol production host, among other industrial biotechnology products, primarily as a result of proven industrial process robustness and exceptional physiological and x-omics characterization [12–15]. The *S. cerevisiae* genome sequence, consisting of 6604 total open reading frames (4437 verified; 1343 uncharacterized; 834 dubious) [16], was first made publicly available in 1996 largely through André Goffeau's coordination of the European

Table 2.1 X-omic glossary.

Systems biology	A multidisciplinary approach relying on the integration and interrogation of diverse data types that share a common scaffold based on genomics and functional genomics characterization of a biological system, with particular focus on development of predictive and quantitative mathematical models.
Metabolic engineering	A field encompassing both forward and inverse metabolic engineering. Metabolic engineering is the gene-targeted, rational, and quantitative approach to redirection of metabolic fluxes to improve the yield, titer, productivity, and/or robustness associated with a specific metabolite in a biological system.
Industrial systems biology	The application of numerical or experimental methods developed as a result of individual or combined x-ome analysis to bioprocess development. Bioprocess development encompasses strain or expression system improvements in terms of final product titer, yield, or productivity, or improvements in process robustness and efficiency.
Forward metabolic engineering	This is targeted metabolic engineering representing the linear progression from modeling to target gene identification to strain construction and characterization. Inherent to this strategy is specific and hypothesis-driven genetic manipulations that are based on predictive metabolic modelling, from simple biochemical pathway stoichiometric balancing to more sophisticated kinetic models.
Inverse metabolic engineering	A host strain constructed via random or directed mutagenesis and/or evolution is examined via systems biology tools to determine the genetic perturbation(s) that lead to the desired phenotype.
X-omics	A general term referring to collection and analysis of any global data set whereby any type of informational pathway with reference back to the cell's genome is investigated. X-ome analysis and data collection requires the whole cell genetic sequence, preferably annotated. May also be considered synonymous with functional genomics.
Genomics	The comprehensive study of the interactions and functional dynamics of whole sets of genes and their products, often species and strain specific.
Transcriptomics	The genome-wide study of mRNA expression levels in one or a population of biological cells for a given set of defined environmental conditions.
Metabolomics	The measurement of all metabolites to access the complete metabolic response of an organism to an environmental stimulus or genetic modification. Here, a metabolite is defined as being any substrate or product participating in a reaction catalyzed by any gene product.
Fluxomics	The study of the complete set of fluxes that are measured or calculated in a given metabolic reaction network. A metabolic flux is defined as a quantitative measurement of the rate of conversion of reactants to products, where rate may be defined as the mass or concentration per unit time of reactant consumption and product formation.
Proteomics	The large-scale analysis of the structure and function of proteins as well as of protein–protein interactions in a cell.
Metagenomics	The study of the genomes and associated x-omes in organisms recovered from the environment as opposed to laboratory cultures. Organisms recovered from the environment are often difficult to culture in controlled laboratory conditions, but may reveal interesting characteristics accessible through functional genomics.

yeast research community [17]. Soon thereafter, in 1997 and 1998, respectively, the first cDNA spotted microarray exploring metabolic gene regulation and the first commercial platform (Affymetrix) microarray data exploring mitotic cell regulation were reported [18, 19].

The genome sequence, coupled with extensive annotation based on fundamental biochemistry, peer-review literature, and available transcription data, enabled publication of the first genome-scale metabolic model for *S. cerevisiae* in 2003 [20]. The genome-scale metabolic model represents an integration of extensive amounts of data into an annotated, defined, and uniform format, permitting simulations of engineered genotypes to elicit desired phenotypes [20, 21].

Strain development has classically been dominated by random mutagenesis, largely by chemical mutagens and radiation, of a production host followed by screening and selection in controlled environments for a desired phenotype. Although this methodology has had tremendous success, it has largely been end-product driven with minimal mechanistic understanding. Today, with the exponential increase in genome sequences of existing and future production hosts, coupled with tools from bioinformatics that enable integration and interrogation of x-omic data sets, it is possible to identify high-probability targeted genetic strategies to increase yield, titer, productivity, and/or robustness [22–24]. It is also now possible to perform inverse metabolic engineering, where previously successful production systems may be x-omically characterized to elucidate key metabolic pathways and control points for future rounds of targeted metabolic engineering [25]. In both forward and inverse metabolic engineering, systems level models and simulations are accelerating bio-based process development, resulting in reduced time to commercialization with significantly less resource commitment.

Today, industrial biotechnologists are no longer considering singular products, but rather diverse portfolios of petrochemical commodity, added-value, high added-value, and specialty chemicals to be produced using biotechnology. The term "biorefinery" was first used in 1999, when it was suggested that lignocellulosic raw materials converted to numerous bio-commodities via integrated unit processes may offer competitive performance to existing petrochemical refineries [26].

If the biorefinery platform model is to evolve from academic conception to industrial reality it will require two essential driving forces. First, the economic and socio-political landscape must continue to support and warrant the significant financial investment, favorable legislative policy, and consumer-driven demand required. Second, the advances and tools developed within systems biology for metabolic engineering must be successfully applied in commercial environments. Several examples, such as bioethanol, have suggested that biorefineries are viable commercially; however, the diverse product streams that will be required continue to demand more sophisticated, native and non-native, multigene metabolic engineering approaches. These approaches may only be realized through advanced interrogation and integration of microbial metabolic space using systems biology tools.

2.5
Metabolic Models

2.5.1
Microbial Metabolism – A Historical Perspective

There have been extensive reviews about the application of random mutagenesis and directed evolution for novel development or enhancement of existing microbial cell factories for the production of a wide range of industrial biotechnology products [27–32]. What is often referred to as "classical strain development" is dependent on the capacity to induce and promote genetic diversity, under controlled laboratory conditions, in a desirable production host organism that can be selectively screened, isolated, cultured, and preserved based on phenotypic criteria. Genetic diversity may be induced using mutagenic chemical agents, radiation, ultraviolet light exposure, intercalating agents, or through genetic recombination [27]. While resulting modified strains may then be further physiologically characterized, the specific and targeted genetic alterations that lead to the improved phenotype are not known, preventing any mechanistic understanding from being applied to future rounds of strain improvement.

Microbial metabolism, the working arena of modern metabolic engineering, has been characterized and built up a scientific body of knowledge for nearly a century; however, if one were to establish a time-line, then perhaps 1932 serves as a suitable starting point, as it coincides with the publication in *Science* of Professor Albert Jan Kluyver's (1888–1956) article, "Microbial metabolism and its bearing on the cancer problem" [33]. Like recombinant DNA technology first pioneered in the early 1970s at Stanford University and the University of California at San Francisco [34], the first applications of microbial metabolism were related to human health and medicine. Also in 1932, published five months prior to Kluyver *et al*. and receiving significantly less attention at the time, another article offered one of the first examples of the role microbial metabolism would play in industrial biotechnology. This article, by Pulley and Greaves, entitled, "An application of the autocatalytic growth curve to microbial metabolism," appeared in the *Journal of Bacteriology* [35]. In a 22 page manuscript, with four references, the authors made two notable observations. The first is a simple, yet governing observation of microbial growth kinetics, neatly summarized by the authors in the opening paragraph:

> Growth does not take place at a constant rate in living organisms. In bacterial cultures it is initially exceptionally slow, then increasingly rapid, and finally exceptionally slow. This is most conspicuous if the initial inoculum into fresh media is very small. We have found that the rate of accumulation of microbial metabolic products likewise is not constant but begins slowly, increases rapidly, and again slows down [35].

The second observation is that a cross-disciplinary approach is possible in which kinetic differential reaction equations, first applied to monomolecular autocata-

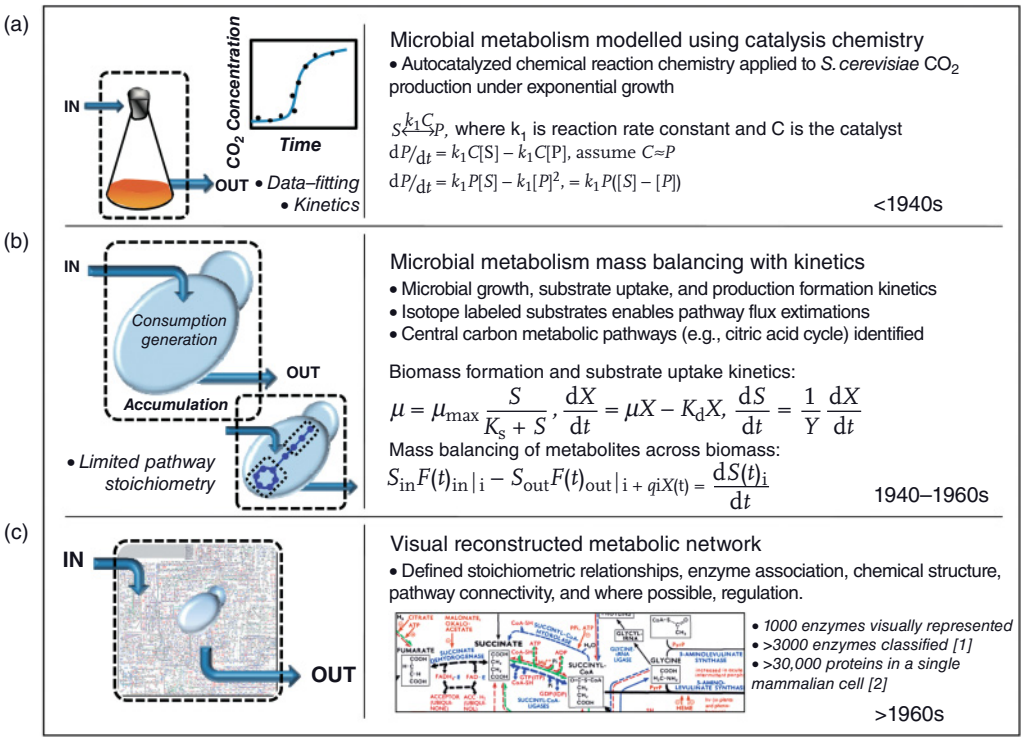


Figure 2.3 Microbial metabolism.

lyzed chemical reactions, and later to the growth of plants and animals [36], can be used to fit nitrate and carbon dioxide accumulation data in soil bacteria and *Saccharomyces cerevisiae*, respectively [35]. This is the first clear example of where mathematical models were used to fit existing microbial metabolic data, and consequently, yield a predictive relationship between the accumulation of metabolic products, (e.g., nitrates and carbon dioxide), and time, governed by a reaction rate constant (Figure 2.3). Microbial metabolism has been extensively investigated since the 1930s, when classical reaction rate expressions to describe autocatalyzed reaction chemistry were applied to carbon dioxide formation in *S. cerevisiae*. This approach required metabolite concentration profiles as functions of time, and the starting metabolite concentration to determine the reaction rate constant. This was a data-fitting approach with minimal focus on predictive power; however, semi-quantitatively explored the relationship between metabolite consumption and production rates, with relation to growth rate [35]. In the next 20 years, significant progress in elucidating glycolysis, the citric acid cycle, and fatty acid oxidation was made, and growth kinetics, the relationship between growth rate, substrate utilization, and product formation rate was further developed. This approach relied on classical mass balancing with emphasis on resolution of kinetic parameters. From

the 1960s onward, the approach of describing metabolism with mass balancing and kinetic parameters estimation vastly expanded to include the majority of metabolic space (Figure 2.3b). A significant milestone was the publication of *Biochemical Pathways* that provided the first visual representation of major components of metabolism. In the recently published 4th edition of *Biochemical Pathways* there are over 1000 enzyme-catalyzed reactions depicted with specific annotation, including stoichiometry, chemical structure, pathway connectivity, compartmentalization, and where possible, regulation [37]. The reconstructed microbial network, particularly provided in a singular, graphical representation, permitted the first generation of metabolic engineering strategies to be devised based on rational, hypothesis-driven, strategies.

Although Jacques Monod is often credited with the modern mathematical characterization of growth kinetics, commonly referred to as "Monod growth kinetics," which includes a kinetic relationship for biomass formation as a function of substrate concentration and affinity that evolved from his seminal work on enzyme kinetics using β-galactosidase in *Escherichia coli*, as summarized in a series of publications culminating with a 1953 publication in *Nature* [38],, it was Pulley *et al*., nearly 20 years earlier who evaluated the microbial metabolic relationships that would ultimately serve as the foundations for early industrial fermentations. During the same period numerous other scientists, including Meyerhof [38–45], Embden [46], Parnas [47], Warburg [48, 49], Cori [50–52], Harden [53–57], and Neuberg [58], were elucidating metabolic pathways, primarily involved in anaerobic fermentation, again with a particular focus on medical applications.

During the same time, Hans A. Krebs was working on the primary components of central carbon metabolism, such as amino acid metabolism (e.g., glutamic acid, proline), and the citric acid cycle, today often referred to as the Krebs cycle [59–70], highlighting the cell's capability for aerobic metabolism and metabolite oxidation. It is interesting to note that baker's yeast once again played a critical role in the pursuit of this line of research, when ^{14}C-labeled acetate was supplemented and it was observed that the dicarboxylic acids remained unlabeled, suggesting that different modes of fermentation and oxidation result from different types of carbon source supplied [69]. In many respects, one could argue that this was the beginning of metabolic flux measurements for pathway elucidation. (Note: Isotope labeling was used extensively in the 1940s to study the distribution of carbon atoms in the fatty acids and acetoacetate that were shown to appear in the citric acid cycle. It was shown, consequently, that dicarboxylic acids are intermediates in the complete oxidation of fatty acids [70].) In 1953, Krebs shared the Nobel Prize in Physiology with Fritz A. Lipmann (credited with the discovery of coenzyme-A) for their elucidation of intermediate metabolism. While the focus remained on human health, in his Nobel address in 1953, Krebs mentioned in reference to a debate regarding the oxidation of acetate and its relation to the citric acid cycle:

> It is true that these results may not be looked upon as conclusive because permeability barriers might prevent the mixing of substances arising as intermediates with those that are present in other compartments of the cell,

> and at present it is best to regard the terminal pathway of oxidation in yeast, and certain other microorganisms, e.g. *E. coli*, as an open problem, even though the reactions of the cycle occur in these materials [68].

By then, baker's yeast and *E. coli*, two of the leading industrial biotechnology hosts, were already being evaluated as model organisms, and one of the key distinguishing features of these organisms that to this day affects metabolic engineering strategies was noted: compartmentalization.

The foundations of modern microbial metabolism can continue to be traced through the literature, but the small subset of examples provided here intends only to provide perspective and context for the early industrial biotechnology processes. Prior to the development of recombinant DNA technology, there was a key development in the metabolism literature that to this day continues to impact metabolic engineering and systems biology approaches; however, is often not discussed in this context. In 1965 the first edition of *Biochemical Pathways* was published by the Boehringer Mannheim GmbH (Mannheim, Germany) company, created and led by Dr. Gerhard Michal. This was the first comprehensive, visual, graphic representation of metabolism, which was initially in wall chart form (Figure 2.3) but has recently been converted to book format [37]. (Note: As of this writing there have been four editions of the *Biochemical Pathways* wall chart, including the reference book, *Biochemical Pathways*, published in 2005, all continuing to be edited by Dr. Gerhard Michal. The wall chart continues to be made available by Roche Diagnostics GmbH (Mannheim, Germany), at https://www.roche-applied-science.com/techresources/publications_req.jsp.) This integration of biological data, which included stoichiometric relationships, chemical structure of all reactants, products, intermediates, and cofactors, assignment of enzymes to specific reactions, definition of compartments and transport, and where possible, regulatory data or interactions, was first compiled for central pathways such as glycolysis, the citric acid cycle, synthesis and degradation of fatty acids, amino acids, and nucleotides. Perhaps most importantly, it provided one of the first global, visual representations of the reconstructed metabolic network, where connectivity between metabolites, pathways, and compartments could be realized. A systematic visual representation of the metabolic network permitted intuitive, hypothesis-driven metabolic engineering approaches to be developed, based on driving carbon flux in given directions, or redirecting other metabolic fluxes in desirable directions. This metabolic map frequented the walls of all major academic and industrial research centers of industrial biotechnology, and provided metabolic engineers with a comprehensive overview of the landscape within which they were operating.

Of course, the critical piece of information missing was the association between genes, gene products (e.g., functional enzymes), and the metabolic pathways on which they act. Furthermore, the *Biochemical Pathways* wall chart, while it has evolved to include organism-specific detail, has largely been treated as a summary of all metabolic reactions in the many different organisms studied. Again, it would ultimately be comparative genomics, coupled with bioinformatics efforts to create organism-centered databases that would provide the required specificity.

2.5.2
Genome Sequencing and Functional Genomics

Several reviews have provided historical perspectives with respect to the formation of the multidisciplinary field of systems biology, and its impact on metabolic engineering, or more broadly industrial biotechnology, often focusing on milestone publications [71–75]. A supplementary approach more focused on dissecting which milestones were critical for commercialization of industrial biotechnology is to inspect the patent literature that in addition to cataloging the specific scientific or technological achievement also suggests their industrial importance. The sequence of inventions described below, by no means exhaustive, provides a temporal context of some of the milestones in the fields of industrial biotechnology and recombinant DNA technology that ultimately culminated in the first major added-value product produced via extensive forward metabolic engineering.

In 1948, the Cold Spring Harbor Laboratory (Cold Spring Harbor, New York, USA) was granted a US patent entitled, "Production of penicillin," which states, "It is an object of this invention to produce penicillin in extremely highly yields. Another object is to produce mutations of molds of the genus mycetes capable of yielding extremely large amounts of antibiotic substances" [76]. In 1982, Genentech, Inc. (South San Francisco, California, USA), was granted a patent entitled, "Method for microbial polypeptide expression," that cited somatostatin, an inhibitor of growth hormone, as an example polypeptide, and went on to state:

> Despite wide-ranging work in recent years in recombinant DNA research, few results susceptible to immediate and practical application have emerged. This has proven especially so in the case of failed attempts to express polypeptides and the like coded for by "synthetic DNA", whether constructed nucleotide by nucleotide in the conventional fashion or obtained by reverse transcription from isolated mRNA (complimentary or "cDNA"). In this application we describe what appears to represent the first expression of a functional polypeptide product from a synthetic gene, together with related developments which promise widespread application [77].

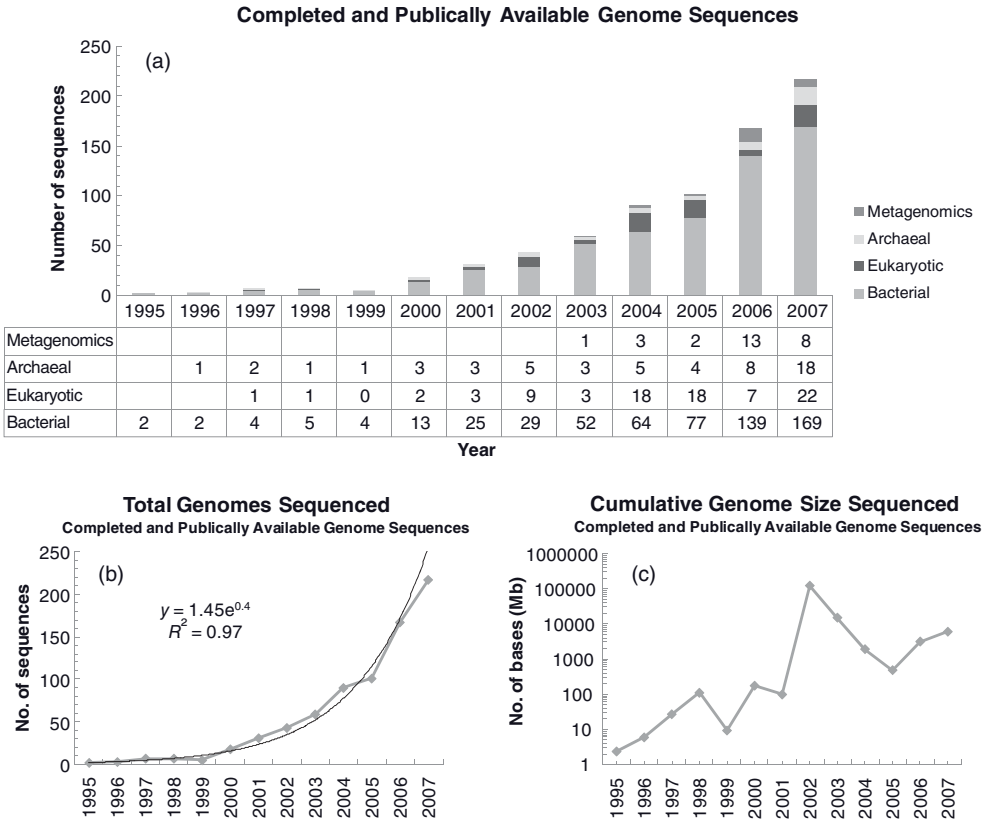
In 1985, the Purdue Research Foundation (West Lafayette, Indiana, USA) was granted a patent entitled, "Direct fermentation of D-xylose to ethanol by a xylose-fermenting yeast mutant," wherein they claimed, "a process for producing yeast mutants capable of utilizing D-xylose to ethanol in high yields is described," [77] the yeast mutants being *Candida* sp. XF 217 and *S. cerevisiae* SCXF 138. In 1997, the E.I. Du Pont de Nemours and Company (Wilmington, Delaware, USA) was granted a US patent entitled, "Bioconversion of a fermentable carbon source to 1,3-propanediol by a single microorganism [78]." This patent went on to comprehensively describe the metabolic pathways present in naturally producing microorganisms (e.g., *Citrobacter* sp., *Clostridium* sp., *Klebsiella* sp.), and which specific enzyme activities were both required for carbon flux redirection and redox balancing (e.g., NAD^+ regeneration). In one of the first major successes of modern

metabolic engineering, it went on to describe the specific expression vectors and cloning techniques used to construct a recombinant strain of *E. coli* capable of high-yielding production of 1,3-propanediol [78, 79]. (Note: There have been several US patents issued to E.I. Du Pont de Nemours and Company, and collaborating enterprises, such as Genencor International, in the development of 1,3-propanediol; however, the US patent issued in 1995 represents the first of that series of patents.)

This rather brief survey of the extensive biotechnology patent literature suggests that for nearly 40 years (1948–1985), development of industrial biotechnology processes was relegated to methods of mutant selection. However, within approximately 10 additional years (by 1997), gene-targeted approaches were in use to construct microbial cell factories capable of producing high added-value chemicals.

Those with industrial experience will recall that in the late 1980s and early 1990s, with recombinant DNA technology emerging from medical biotechnology, we witnessed expression of compounds previously produced via synthetic routes now being attempted in production organisms [28, 80–82]. These compounds included L-glutamic acid (1 000 000 tons annually), citric acid (1 000 000 tons annually), L-lysine (350 000 tons annually), lactic acid (250 000 tons annually), food-processing enzymes (100 000 tons annually), vitamin C (80 000 tons annually), gluconic acid (50 000 tons annually), antibiotics (35 000 tons annually), feed enzymes (20 000 tons annually), xanthan (10 000 tons annually), L-hydroxyphenylalanine (10 000 tons annually), vitamin F (1000 tons annually), and vitamin B_{12} (12 tons annually), to name a few [5, 83–85]. This was made possible by the introduction of genetic sequences encoding for enzymes that were likely to catalyze desired reactions, or the deletion of genes that would downregulate undesired reactions and pathways. These approaches were largely hypothesis driven, resource intensive, and low throughput, minimizing the probability of successfully identifying a genotype that would elicit a significantly improved phenotype. The real advantage of random mutagenesis, screening, and selection, was the relatively large experimental space that could be covered, even if mechanistic understanding was sacrificed. The other advantage was its track record – it worked. Fast-forward approximately 10 years, and what has changed?

Although techniques that permitted manipulation of recombinant DNA existed, the annotated genome sequences of industrially relevant production hosts were not available. Figure 2.4 highlights the exponential increase in published genome sequences that first started in 1995 and has continued to expand through 2008 with a total of 730 published genome sequences, and 905, 1763, and 91 bacterial, eukaryotic, and archaeal sequence projects on-going, respectively. Figure 2.4a shows a plot of the number of completed genome sequences, broken down according to organism classification (archael, eukaryotic, bacterial, and metagenomics). Although an increasing number of eukaryotic and archaeal genomes and metagenomes have been sequenced, the overwhelming majority of organisms sequenced continue to be bacterial. As suggested in Figure 2.4b, the number of genome sequences has doubled approximately every 1.7 years, although data from 2006, 2007, and 2008 (not shown) suggest that this genome sequencing rate is declining.



	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
Metagenomics									1	3	2	13	8
Archaeal		1	2	1	1	3	3	5	3	5	4	8	18
Eukaryotic			1	1	0	2	3	9	3	18	18	7	22
Bacterial	2	2	4	5	4	13	25	29	52	64	77	139	169

Total number of completed genome sequences are doubling approximately every 1.7 years.

Figure 2.4 Total completed and publicly available genome sequences. A summary of the characteristics of all completed and publicly available genome sequences on a per year basis between 1995 and 2007. The data are adapted from the Genomes OnLine Database [86].

While Figure 2.4b considers only completed genome sequences, Figure 2.4c presents the cumulative size of the genomes sequenced. Specifically, the size of each genome sequenced was summed across all the genomes sequenced in a given year (the coverage of each genome sequenced is not considered in this calculation). The cumulative genome size increased robustly until 1998; however, between 1999 and 2001, there was marked decrease, culminating with a large increase in 2002. Between 2002 and 2005 the cumulative size decreased significantly. Between 2005 and 2007 there has been an increase, although still below 2002 levels. Figure 2.4b,c suggest that while there has been an increase in the number of total completed genome sequences publicly available, the size of those genomes has not been increasing. This is consistent with the observation that smaller genomes, such as those represented by metagenomes and bacterial organisms have dominated most recent sequencing efforts.

Table 2.2 Characteristics of publicly available genome sequences published between 1995 and 1999.

Date	Domain[a)]	Organism	Relevance	Size (kb)	Number of ORFs	Publication
Jul-95	B	*Haemophilus influenzae*	Medical, human pathogen	1830	1657	*Science* 269, 496–512
Oct-95	B	*Mycoplasma genitalium*	Medical, human pathogen, animal pathogen	580	477	*Science* 270, 397–403
Jun-96	B	*Synechocystis* sp.	Biotechnological, environmental, ocean carbon cycle	3573	3172	*DNA Res.* 3, 109–136
Sep-96	A	*Methanocaldococcus jannaschii*	Biotechnological, energy production	1664	1729	*Science* 273, 1058–1073
Nov-96	B	*Mycoplasma pneumoniae*	Medical, human pathogen	816	689	*Nucleic Acids Res.* 24, 4420–4449
May-97[b)]	**E**	***Saccharomyces cerevisiae***	**Model organism**	**12069**	**5860**	***Nature* 387,5–105**
Aug-97	B	*Helicobacter pylori*	Medical, human pathogen	1667	1576	*Nature* 388, 539–547
Sep-97	**B**	***Escherichia coli***	**Medical**	**4639**	**4243**	***Science* 277, 1453–1474**
Nov-97	A	*Methanothermobacter thermoautotrophicus*	Biotechnological, energy production	1751	1873	*J. Bacteriol.* 179, 7135–7155
Nov-97	**B**	***Bacillus subtilis***	**Biotechnological**	**4214**	**4105**	***Nature* 390, 249–256**
Nov-97	A	*Archaeoglobus fulgidus*	Biotechnological	2178	2420	*Nature* 390, 364–370
Dec-97	B	*Borrelia burgdorferi*	Medical, human pathogen	910	851	*Nature* 390, 580–586
Mar-98	B	*Aquifex aeolicus*	Biotechnological	1551	1529	*Nature* 392, 353–358
Apr-98	A	*Pyrococcus horikoshii(shinkaj)*	Biotechnological	1738	1955	*DNA Res.* 5, 55–76
Jun-98	B	*Mycobacterium tuberculosis*	Medical, human pathogen, animal pathogen	4411	4402	*Nature* 393, 537–544
Jul-98	B	*Treponema pallidum pallidum*	Medical, human pathogen	1138	1036	*Science* 281, 375–388

Table 2.2 Continued.

Date	Domain[a)]	Organism	Relevance	Size (kb)	Number of ORFs	Publication
Oct-98	B	*Chlamydia trachomatis*	Medical, human pathogen, animal pathogen	1042	895	*Science* 282, 754–759
Nov-98	B	*Rickettsia prowazekii*	Medical, biothreat, human pathogen	1111	835	*Nature* 396, 133–140
Dec-98	E	*Caenorhabditis elegans*	Model organism	100272	23209	*Science* 282, 2012–2018
Jan-99	B	*Helicobacter pylori*	Medical, human pathogen	1643	1491	*Nature* 397, 176–180
Apr-99	B	*Chlamydophila pneumoniae*	Medical, human pathogen	1230	1052	*Nat. Genet.* 21, 385–389
Apr-99	A	*Aeropyrum pernix*	Biotechnological	1669	1700	*DNA Res.* 6, 83–101
May-99	B	*Thermotoga maritima*	Biotechnological, energy production, evolutionary	1860	1858	*Nature* 399, 323–329
Nov-99	B	*Deinococcus radiodurans*	Environmental, bioremediation	3060	2637	*Science* 286, 1571–1577

a) Bacterial (B), Eukaryotic (E), Archaeal (A).
b) The sequences highlighted in bold represent key organisms that have been used extensively for industrial biotechnology applications.

This genomic revolution was mainly driven by the medical research field, as illustrated in Table 2.2, which presents characteristics of those genomes sequenced between 1995 and 1999. It is particularly interesting to note that while industrial biotechnology products such as citric acid, penicillin, and amino acids were actively manufactured during this time, genome sequences of industrial production hosts were not available. The remaining organisms, as highlighted by their relevance, are applicable to medical biotechnology and human medicine [86]. It can be seen that of the 24 sequences made available, only three could be considered to have broad applicability to the industrial biotechnology sector: *Saccharomyces cerevisiae*, *Escherichia coli*, and *Bacillus subtilis*, while the rest were driven by the medical community. One can even argue that sequencing of these three genomes was also mainly motivated by their medical relevance, either as a eukaryote model organism, pathogen, or model pathogen.

If we move beyond 1999, as indicated in Table 2.3, many more industrially important cell factories have been genome sequenced, and with the substantial reduction in sequencing costs even genome sequencing has become a tool to

Table 2.3 Characteristics of publicly available genome sequences published in 2007.

Date	Domain[a)]	Organism	Relevance	Size (kb)	Number of ORFs	Publication
Jan-07	B	*Prochlorococcus marinus*	Carbon cycle, Environmental, MMI	1669	1921	*PLoS Genet.* 3, e231
Jan-07	B	*Prochlorococcus marinus*	Carbon cycle, Environmental, MMI	1704	1906	*PLoS Genet.* 3, e231
Jan-07	A	*Hyperthermus butylicus*	Biotechnological	1667	1602	*Archaea* 2, 127–35
Jan-07	B	*Prochlorococcus marinus*	Carbon cycle, Environmental, MMI	1864	2193	*PLoS Genet.* 3, e231
Jan-07	B	*Prochlorococcus marinus*	Environmental, MMI, Carbon cycle	2682	2997	*PLoS Genet.* 3, e231
Jan-07	E	*Aspergillus niger*	Biotechnological, Fermentation, Citric acid production	33900	14165	*Nat. Biotechnol.* 25, 221–231
Jan-07	B	*Rubrivivax (Methylibium) gelatinosus (petroleiphilum)*	Environmental, Bioremediation	4044	3819	*J. Bacteriol.* 189, 931–45
Jan-07	B	*Lactococcus lactis cremoris*	Food industry, Biotechnological	2529	2434	*J. Bacteriol.* 189, 3256–3270
Feb-07	E	*Ustilago maydis*	Agricultural, FGI, Plant pathogen	20500	6902	*Nature* 444, 97–101
Feb-07	B	*Streptococcus sanguinis*	Medical, Human pathogen	2388	2270	*J. Bacteriol.* 189, 3166–3175
Feb-07	B	*Actinobacillus pleuropneumoniae*	Animal pathogen, Medical, Swine pathogen	2274	2012	*J. Bacteriol.* 190, 1495–1496
Feb-07	E	*Pichia stipitis*	Biotechnological, Energy production, Ethanol production, Fermentation	15426	5816	*Nat. Biotechnol.* 25, 319–326

Table 2.3 Continued.

Date	Domain[a)]	Organism	Relevance	Size (kb)	Number of ORFs	Publication
Mar-07	B	*Acinetobacter baumannii*	Human pathogen, Medical	3976	3352	*Genes Dev.* 21, 601–614
Mar-07	A	*Uncultured methanogenic archaeon RC-I*	Biotechnological	3179	3085	*Science* 313, 370–372
Mar-07	B	*Prochlorococcus marinus*	Carbon cycle, Environmental, MMI	1641	1907	*PLoS Genet.* 3, e231
Mar-07	B	*Herminiimonas (Cenibacterium) arsenicoxydans*	Biotechnological, Detoxification, Environmental	3424	3325	*PLoS Genet.* 13, e53
Mar-07	B	*Saccharopolyspora erythraea*	Biotechnological, Antibiotic production, Erythromycin production	8212	7198	*Nat. Biotechnol.* 25, 447–453
Mar-07	B	*Francisella tularensis tularensis*	Animal pathogen, Medical	1898	1634	*PLoS ONE* 2, e947
Mar-07	B	*Geobacillus thermodenitrificans*	Environmental, Biotechnological	3607	3392	*PNAS* 104, 5602–5607
Apr-07	B	*Corynebacterium glutamicum*	Biotechnological, Glutamate production, Amino acids production, Food industry	3300	3052	*Microbiology* 153, 1042–1058
Apr-07	M	*Marine microbial communities*	Environmental, MMI	N/A	6123395	*PLoS Biol.* 5, e77
Apr-07	E	*Macaca mulatta*	N/A	2871189	34023	*Science* 316, 222–234
Apr-07	B	*Salinispora tropica*	Biotechnological, Cancer treatment, Medical	5183	4536	*PNAS* 104, 10376–10381
Apr-07	B	*Streptococcus suis*	Medical, Human pathogen, Swine pathogen, Animal pathogen	2095	2189	*PLoS ONE* 2, E315
Apr-07	B	*Streptococcus suis*	Medical, Human pathogen, Swine pathogen, Animal pathogen	2096	2186	*PLoS ONE* 2, E315

Table 2.3 Continued.

Date	Domain[a)]	Organism	Relevance	Size (kb)	Number of ORFs	Publication
Apr-07	A	*Metallosphaera sedula*	Bioremediation, Biotechnological	2 191	2 256	*Appl. Environ. Microbiol.* Epub
May-07	B	*Bradyrhizobium sp*	Agricultural	7 456	6 717	*Science* 316, 1307–1312
May-07	E	*Ostreococcus lucimarinus*	N/A	13 200	7 651	*PNAS* 104, 7705–7710
May-07	B	*Dichelobacter nodosus*	Medical, Animal pathogen	1 389	1 280	*Nat. Biotechnol.* 25, 569–575
May-07	B	*Orientia (Rickettsia) tsutsugamushi*	Medical, Animal pathogen	2 127	1 182	*PNAS* 104, 7981–7986
May-07	W	*Monodelphis domestica*	N/A	N/A	18 022	*Nature* 447, 167–177
May-07	B	*Bradyrhizobium sp*	Agricultural	8 264	7 394	*Science* 316, 1307–1312
May-07	B	*Staphylococcus aureus aureus*	Medical, Human pathogen, Animal pathogen, Cattle pathogen, Poultry pathogen	2 906	2 697	*PNAS* 104, 9451–9456
May-07	B	*Clavibacter (Corynebacterium) michiganensis michiganensis*	Agricultural, Plant pathogen	3 297	2 984	*J. Bacteriol.* 190, 2138–2149
May-07	B	*Vesicomyosocius okutanii*	Evolutionary	1 022	937	*Curr. Biol.* 17, 881–886
May-07	B	*Clostridium botulinum*	Medical, Human pathogen, Biothreat	3 886	3 574	*Genome Res.* 17, 1082–1092
Jun-07	M	*Simulated microbial communities*	N/A	262 860	352 230	*Nat. Methods* 4, 495–500
Jun-07	A	*Methanobrevibacter smithii*	Medical, HGMI	1 853	1 793	*PNAS* 104, 10643–10648
Jun-07	M	*Oral TM7 microbial communities*	Medical	3 450	4 078	*PNAS* 104, 11889–11894

Table 2.3 Continued.

Date	Domain[a)]	Organism	Relevance	Size (kb)	Number of ORFs	Publication
Jun-07	E	*Aedes aegypti*	Animal pathogen, Human pathogen, Medical	1 380 000	N/A	*Science* 316, 1718–1723
Jun-07	B	*Flavobacterium psychrophilum*	Medical, Fish pathogen	2 861	2 412	*Nat. Biotechnol.* 25, 763–769
Jun-07	B	*Bacteroides vulgatus*	Medical, Human pathogen, Animal pathogen, HGMI	5 163	4 065	*PLoS Biol.* 5, E156
Jun-07	B	*Parabacteroides distasonis*	Medical, Human pathogen, Animal pathogen, HGMI	4 811	3 850	*PLoS Biol.* 5, E156
Jun-07	B	*Staphylococcus aureus*	Animal pathogen, Cattle pathogen, Human pathogen, Medical, Poultry pathogen	2 906	2 747	*PNAS* 104, 9451–9456
Jul-07	B	*Staphylococcus aureus aureus*	Animal pathogen, Human pathogen, Medical	2 878	2 614	*J. Bacteriol.* 190, 300–310
Jul-07	B	*Janthinobacterium* sp.	Environmental, Medical	4 110	3 697	*PLoS Genet.* 3(8), e138
Jul-07	M	Soil microbial communities	N/A	N/A	N/A	*ISME J.* 1, 283–290
Jul-07	B	*Clostridium kluyveri*	Biotechnological	3 964	3 838	*PNAS* 105, 2128–2133
Jul-07	B	*Clostridium botulinum A*	Biothreat, Human pathogen, Medical	3 863	3 552	*PLoS ONE* 2, e1271
Jul-07	B	*Clostridium botulinum A*	Biothreat, Human pathogen, Medical	3 760	3 407	*PLoS ONE* 25, 1281–1289
Jul-07	B	*Sulfurovum* sp.	Environmental	2 562	2 438	*PNAS* 104, 12146–12150
Jul-07	B	*Nitratiruptor* sp.	Environmental	1 877	1 843	*PNAS* 104, 12146–12150
Jul-07	B	*Yersinia pseudotuberculosis*	Medical, Human pathogen	4 723	4 124	*PLoS Genet.* 3(8), e142

Table 2.3 Continued.

Date	Domain[a)]	Organism	Relevance	Size (kb)	Number of ORFs	Publication
Aug-07	B	*Bacillus amyloliquefaciens*	Biotechnological, Antibiotic production, Suppresses Plant pathogens, Agricultural	3 918	3 693	*Nat. Biotechnol.* 25, 1007–1014
Aug-07	E	*Vanderwaltozyma polyspora*	Evolutionary	14 661	5 367	*PNAS* 104, 8397–8402
Aug-07	E	*Nematostella vectensis*	Model organism	297 398	N/A	*Science* 317, 86–94
Sep-07	E	*Fusarium (Gibberella) graminearum (zeae)*	Agricultural, Plant pathogen, FGI	36 000	N/A	*Science* 317, 1400–1402
Sep-07	M	Marine Plankton communities	Environmental	7 200	N/A	*PLoS ONE* 2(9), e914
Sep-07	B	*Campylobacter jejuni jejuni*	Medical, Human pathogen	1 628	1 626	*J. Bacteriol.* Epub
Sep-07	B	*Prochlorococcus marinus*	Carbon cycle, Environmental	1 738	1 983	*PLoS Genet.* 3, e231
Sep-07	E	*Homo sapiens*	Medical	2 782 357	N/A	*PLoS Biol.* 5, e254
Sep-07	E	*Brugia malayi*	N/A	90 000	11 500	*Science* 317, 1756–1760
Sep-07	B	*Bacillus pumilus*	Biotechnological, Bioenergy, Medical	3 704	3 681	*PLoS ONE* 2, e928
Sep-07	E	*Vitis vinifera*	N/A	475 000	N/A	*Nature* 449, 463–467
Sep-07	B	*Arcobacter butzleri*	Human pathogen, Medical	2 341	2 259	*PLoS ONE* 2, e1358
Oct-07	E	*Giardia lamblia (intestinalis)*	N/A	11 192	13 100	*Science* 317, 1921–1926
Oct-07	E	*Chlamydomonas reinhardtii*	N/A	100 000	15 256	*Science* 318, 245–250
Oct-07	B	*Acaryochloris marina*	Biotechnological	6 503	6 254	*PNAS* 105, 2005–2010
Oct-07	E	*Babesia bovis*	N/A	8 200	3 671	*PLoS Pathog.* 3, e148

Table 2.3 Continued.

Date	Domain[a)]	Organism	Relevance	Size (kb)	Number of ORFs	Publication
Oct-07	B	*Sorangium cellulosum*	Biotechnological	13 033	9 384	*Nat. Biotechnol.* 25, 1281–1289
Nov-07	B	*Lactobacillus helveticus*	Biotechnological, Food industry	2 080	1 610	*J. Bacteriol.* 190, 727–735
Nov-07	E	*Hemiselmis andersenii*	Evolutionary	571	N/A	*PNAS* 104, 19908–19913
Nov-07	B	*Staphylococcus aureus aureus*	Human pathogen, Medical	2 872	2 657	*BMC Microbiol.* 7, 99
Nov-07	B	*Bartonella tribocorum*	Medical, Rat pathogen	2 619	2 074	*Nat. Genet.* 39, 1469–1476
Nov-07	M	Gut microbiome	Biotechnological, Energy production	N/A	N/A	*Nature* 450, 560–565
Nov-07	E	*Malassezia globosa*	Human pathogen, Medical	9 000	4 285	*PNAS* 104, 18730–18735
Nov-07	E	*Laccaria bicolor*	Agricultural	65 000	N/A	*Nature* in press
Dec-07	B	*Neisseria meningitidis*	Human pathogen, Medical	2 153	2 020	*Genomics* 91, 78–87
Dec-07	M	Gut microbiome	Medical	727 000	N/A	*DNA Res.* 14, 169–181
Dec-07	B	*Candidatus Sulcia muelleri*	Agricultural	245	227	*PNAS* 104, 19392–19397
Dec-07	E	*Vitis vinifera* L.	N/A	504 600	29 585	*PLoS ONE* 2, e1326
Dec-07	E	*Monosiga brevicollis*	N/A	38 648	N/A	*Nature* 51, 783–788
Dec-07	B	*Microcystis aeruginosa*	Animal pathogen, Environmental, Human pathogen, Medical	5 842	6 312	*DNA Res.* 14, 247–256

Table 2.3 Continued.

Date	Domain[a)]	Organism	Relevance	Size (kb)	Number of ORFs	Publication
Jan-08	B	*Clavibacter (Corynebacterium) michiganensis sepedonicus*	Agricultural, Plant pathogen	3258	3058	*J. Bacteriol.* 190, 2150–2160
Jan-08	B	*Actinobacillus pleuropneumoniae*	Medical, Animal pathogen, Swine pathogen	2242	2036	*PLoS ONE* 3, e1450
Jan-08	B	*Rickettsia rickettsii*	Human pathogen, Medical	1268	1384	*Infect. Immun.* 76, 542–545
Jan-08	B	*Chlamydia trachomatis*	Animal pathogen, Human pathogen, Medical	1038	874	*Genome Res.* 18, 161–171
Jan-08	B	*Chlamydia trachomatis*	Animal pathogen, Human pathogen, Medical	1038	874	*Genome Res.* 18, 161–171
Jan-08	B	*Xanthomonas campestris campestris*	Plant pathogen, Agricultural	5079	4471	*J. Biotechnol.* Epub
Jan-08	E	*Physcomitrella patens patens*	N/A	453929	N/A	*Science* 319, 64–69
Feb-08	B	*Escherichia coli*	Human pathogen, Medical	4686	4305	*J. Bacteriol.* Epub
Feb-08	B	*Candidatus Cloacamonas acidaminovorans*	Biotechnological, Wastewater treatment	2246	1820	*J. Bacteriol.* Epub
Feb-08	B	*Finegoldia magna*	N/A	1797	1631	*DNA Res.* Epub
Feb-08	B	*Leuconostoc citreum*	Acetate production, Biotechnological, Ethanol production, Food industry, Lactic acid production	1796	1702	*J. Bacteriol.* Epub
Feb-08	A	*Halobacterium salinarum*	Biotechnological	2000	N/A	*Genomics* in press
Feb-08	B	*Lysinibacillus sphaericus*	Insect pathogen, Agricultural	4639	4786	*J. Bacteriol.* Epub

a) Bacterial (B), Eukaryotic (E), Archaeal (A), Microbial (M).
MMI = Marine Microbial Initiative; FGI = Fungal Genome Initiative; GMI = Human Gut Microbiome Initiative

analyze cell factories with different phenotypes. Table 2.3 provides a summary of all publicly available genome sequences published between January 2007 and February 2008 [86]. During this period a total of 96 genomes were published with 34 classified as having relevance to biotechnology and/or the environment. Of particular interest is the number of organisms sequenced specifically driven by industrial biotechnology processes, where specific products such as citric acid, bioethanol, lactic acid, and amino acids (e.g., glutamate) are cited. Although a large fraction, approximately two-thirds, of genome sequences continue to be driven by medical biotechnology, the number of genome sequenced of relevance for industrial biotechnology is increasing. This is readily confirmed by review of the respective fiscal year 2008 operating budgets of the US National Institutes of Health, at US$29.5 billion, compared with the US Department of Energy's Division of Energy Efficiency and Renewable Energy, at US$1.7 billion [87, 88]. Nearly all of the genome sequences cited to have biotechnological relevance have been funded by the US Department of Energy, suggesting that functional genomics and systems biology are recognized as a key advantage in advancing industrial biotechnology. This was certainly not the case less than a decade ago according to Table 2.2. The presence of complete genome sequences has clearly allowed better targeting of genetic modifications, and information about the complete parts lists of a given cell factory is extremely valuable.

With genome sequences for several industrial model organisms in hand, it was the annotation of those sequences that bridged the gap between expanding knowledge-based databases (e.g., genome sequence collections) and the data-driven databases (e.g., application of the genome sequences for annotation, model development, and further understanding) [89]. The annotation of genome sequences has evolved into a well-defined discipline referred to as functional genomics, which focuses on developing numerous experimental and theoretical tools for determination of gene function [90]. Functional genomics, through linking gene products (e.g., enzymes) to gene functions (e.g., reaction stoichiometry) has permitted the development of genome-scale models for various data types, such as reconstructed metabolic network models.

2.6
Reconstructed Metabolic Network Models

2.6.1
Introduction

Even though genome sequencing has clearly facilitated the use of targeted genetic modifications for construction of cell factories with desirable phenotypes, the major step forward has been the introduction of metabolic engineering – the enabling science for cell factory design and construction. Metabolic engineering involves the identification of specific and targeted genetic modifications (gene deletions, overexpression, or modulation) followed by implementation of these

modifications via molecular biology tools that lead to redirection of fluxes to enhance production or robustness of a given product or organism, respectively [32, 91–95] (Figure 2.2). A key technology in the successful application of metabolic engineering is the availability of a well-annotated genome, including the quantitative tools that permit careful inspection and manipulation of the genome. Among those tools has been the recent development of genome-scale metabolic models (GSMMs). To develop a model of cellular metabolism that enables the prediction of concentration profiles as functions of time, the stoichiometry and kinetic reaction rates for each biochemical reaction in a cell at physiological conditions would be required. At present, this information is not available, either via estimation or experimental measurement. Through careful annotation based on existing biochemical knowledge, literature review, and experimentation; however, it is possible to associate known genes with known biochemical reactions and their corresponding stoichiometry. The result is a biochemical model describing the formation and depletion of each metabolite. By providing mass–balance boundary conditions, this makes possible constraint-based simulations of how the metabolic network operates under different conditions. In simpler terms, using basic stoichiometry these models can be used to predict the relationships between genes with function in the metabolic network operating in a cell. If cells are fed x grams of glucose it is possible through the use of linear programming and the biochemical model to predict the maximum y_i grams of formed product i.

It is also clear that GSMMs can be used to predict a theoretical landscape of genetic perturbations that can maximize product and biomass formation, even under different growth conditions (i.e., growth on alternative carbon sources). GSMMs have been developed for several model production organisms, and were a major step in not only allowing model-guided metabolic engineering, but also integration of different x-ome data in order to obtain detailed metabolic characterization. Table 2.4 provides a comprehensive summary of all the GSMMs completed to date. With a total of 34 networks reconstructed, there is a large diversity in organisms, ranging from the simplest bacterium (e.g., *Escherichia coli*), to higher order and complicated eukaryotes (e.g., *Homo sapiens*). However, what is equally critical to note is that while these reconstructions provide a scaffold for further annotation using functional genomics data and a systematic methodology for simulation of metabolism, there are significant opportunities for network expansion. Combined, all of the metabolic reconstructed networks have an average genome coverage of 14.6 ± 8.1% (n = 29). If *Saccharomyces cerevisiae*, the most well-characterized eukaryote, is isolated as an example, the most recent metabolic reconstructed network has genome coverage of 13.6%, while 4691 of the 6608 total ORFs (70.9%) have a verified function [A]. Although not all organisms share an equally high level of characterization, *S. cerevisiae*, GSMMs continue to illustrate the vast improvement in coverage that may still be realized. Furthermore, expanded coverage in future genome-scale reconstructions are likely to include less-characterized zones of metabolism, such as lipids, and pathways not traditionally associated with central carbon metabolism, such as protein synthesis, folding, and transport.

Table 2.4 Genome-scale metabolic reconstructed networks.

Genome sequenced	Reference	Genome dimensions		Metabolic network characteristics						Reference
		Size (kb)	Total ORFs	Total reactions (unique)[a)]	Total metabolites (unique)[b)]	Total genes (enzymes)[c)]	Percent genome covered[d)]	Compartments[e)]	Model ID[f)]	
Escherichia coli										
Escherichia coli K-12 MG1655	Blattner *et al.*, *Science*, 1997 [180]	4639	4243	(627)	(438)	660	15.6	Cytoplasm, extracellular	iJE660	Edwards and Palsson, *PNAS*, 2000 [199]
Escherichia coli K-12 MG1655	Blattner *et al.*, *Science*, 1997 [180]	4639	4243	(931)	(625)	904	21.3	Cytoplasm, extracellular	iJR904	Reed *et al.*, *Genome Biol.*, 2003 [200]
Escherichia coli K-12 MG1655	Blattner *et al.*, *Science*, 1997 [180]	4639	4243	2077 (1339)	1668 (1039)	1260	29.7	Cytoplasm, periplasm, extracellular	iAF1260	Feist *et al.*, *Mol. Syst. Biol.*, 2007 [201]
Saccharomyces cerevisiae										
Saccharomyces cerevisiae S288C	Goffeau *et al.*, *Nature*, 1996 [17]	12069	5860	1175 (842)	584	708	12.1	Cytoplasm, mitochondria, extracellular	iFF708	Förster *et al.*, *Genome Res.*, 2003 [202]

Table 2.4 Continued.

Genome sequenced	Reference	Genome dimensions		Metabolic network characteristics						Reference
		Size (kb)	Total ORFs	Total reactions (unique)[a)]	Total metabolites (unique)[b)]	Total genes (enzymes)[c)]	Percent genome covered[d)]	Compartments[e)]	Model ID[f)]	
Saccharomyces cerevisiae S288C	Goffeau *et al.*, *Nature*, 1996 [17]	12 069	5 860	1489 (1149)	646	750	12.8	Cytoplasm, mitochondria, peroxisome, nucleus, endoplasmic reticulum, Golgi apparatus, vacuole, extracellular	iND750	Duarte *et al.*, *Genome Res.*, 2004 [203]
Saccharomyces cerevisiae S288C	Goffeau *et al.*, *Nature*, 1996 [17]	12 069	5 860	1038	636	672	11.5	Cytoplasm, mitochondria, extracellular	iLL672	Blank *et al.*, *Genome Biol.*, 2005 [204]
Saccharomyces cerevisiae S288C	Goffeau *et al.*, *Nature*, 1996 [17]	12 069	5 860	1431	1013	795	13.6	Cytoplasm, mitochondria, extracellular	iIN795	Nookaew *et al.*, *BMC Syst. Biol.*, 2008 [205]
Saccharomyces cerevisiae S288C	Goffeau *et al.*, *Nature*, 1996 [17]	12 069	5 860	There is currently a community-wide effort to consolidate the *S. cerevisiae* models iFF708, iND750, and iLL672, into one common reconstructed network model. This common model is expected to be made available in 2008.						Jens Nielsen, personal communication, 2008.

Haemophilus influenzae										
Haemophilus influenzae	Fleischmann *et al.*, *Science*, 1995 [181]	1830	1657	461	451	400	24.1	Cytoplasm, extracellular	iCS400	Schilling and Palsson, *J. Theor. Biol.*, 2000 [206]
Helicobacter pylori										
Helicobacter pylori 26695	Tomb *et al.*, *Nature*, 1997 [182]	1667	1576	388	403	291	18.5	Cytoplasm, extracellular	iCS291	Schilling *et al.*, *J. Bacteriol.*, 2002 [207]
Helicobacter pylori 26695	Tomb *et al.*, *Nature*, 1997 [182]	1667	1576	476	485	341	21.6	Cytoplasm, extracellular	iIT341	Thiele *et al.*, *J. Bacteriol.*, 2005 [208]
Plasmodium falciparum										
Plasmodium falciparum 3D7	Gardner *et al.*, *Nature*, 2002 [183]	22900	5268	697	525	(816)	NA	Cytoplasm, extracellular	iIY816	Yeh *et al.*, *Genome Res.*, 2004 [209]
Mannheimia succiniproducens										
Mannheimia succiniciproducens MBEL55E	Hong *et al.*, *Nat. Biotechnol.*, 2004 [160]	2314	2380	373	352	329	0.1	Cytoplasm, extracellular	iSH329	Hong *et al.*, *Nat. Biotechnol.*, 2004 [160]
Mannheimia succiniciproducens MBEL55E	Hong *et al.*, *Nat. Biotechnol.*, 2004 [160]	2314	2380	686 (638)	519	425	17.9	Cytoplasm, extracellular	iTK425	Kim *et al.*, *Biotechnol. Bioeng.*, 2007 [163]

Table 2.4 Continued.

Genome sequenced	Reference	Genome dimensions		Metabolic network characteristics						Reference
		Size (kb)	Total ORFs	Total reactions (unique)[a]	Total metabolites (unique)[b]	Total genes (enzymes)[c]	Percent genome covered[d]	Compartments[e]	Model ID[f]	
Methanococcus jannaschii										
Methanococcus jannaschii DSM 2661	Bult *et al.*, *Science*, 1996 [184]	1664	1729	609	510	(436)	NA	Cytoplasm, extracellular	iST436	Tsoka *et al.*, *Archaea*, 2004 [210]
Streptomyces coelicolor										
Streptomyces coelicolor A3(2) M145	Bentley *et al.*, *Nature*, 2002 [185]	8667	7769	971 (700)	500	711	9.2	Cytoplasm, extracellular	iIB711	Borodina *et al.*, *Genome Res.*, 2005 [211]
Aspergillus niger										
Aspergillus niger CBS 513.88	Pel *et al.*, *Nat. Biotechnol.*, 2007 [186]	33900	14165	355	284	20	0.1	Cytoplasm, mitochondria, glyoxysome, extracellular	iHD20	David *et al.*, *Eur. J. Biochem.*, 2003 [212]
Aspergillus niger CBS 513.88 and *Aspergillus niger* ATCC 9029[g]	Pel *et al.*, *Nat. Biotechnol.*, 2007 [186]	33900	14165	2443	2349	(988)	NA	Cytoplasm, extracellular	iJS988	Sun *et al.*, *Genome Biol.*, 2007 [213]
Aspergillus niger CBS 513.88 and *Aspergillus niger* ATCC 1015[h]	Pel *et al.*, *Nat. Biotechnol.*, 2007 [186]	33900	14165	2240 (1190)	1045 (782)	871	6.1	Cytoplasm, mitochondria, extracellular	iMA871	Andersen *et al.*, *Mol. Syst. Biol.*, 2008 [214]

Aspergillus nidulans										
Aspergillus nidulans FGSC A4	Galagan *et al.*, *Nature*, 2005 [187]	31 000	9 500	1213 (794)	732 (551)	666	7.0	Cytoplasm, mitochondria, glyoxysome, extracellular	iHD666	David *et al.*, *Genome Biol.*, 2006 [215]
Aspergillus oryzae										
Aspergillus oryzae RIB40	Machida *et al.*, *Nature*, 2005 [188]	37 000	12 074	(1679)	1040	1184	9.8	Cytoplasm, mitochondria, extracellular	iWV1184	Vongsangnak *et al.*, *BMC Genomics*, 2008 [216]
Lactococcus lactis										
Lactococcus lactis IL1403	Bolotin *et al.*, *Genome Res.*, 2001 [189]	2 365	2 321	621	509 (422)	358	30.6	Cytoplasm, extracellular	iAO358	Oliveira *et al.*, *BMC Microbiol.*, 2005 [217]
Lactobacillus plantarum										
Lactobacillus plantarum WFCS1	Kleerebezem *et al.*, *PNAS*, 2003 [190]	3 308	3 009	704	670	210 (710)	23.6	Cytoplasm, extracellular	iBT710	Teusink *et al.*, *Appl. Environ. Microbiol.*, 2005 [218]
Bacillus subtilis										
Bacillus subtilis 168	Kunst *et al.*, *Nature*, 2007 [191]	4 214	4 105	1020	988	844	20.6	Cytoplasm, extracellular	iYO844	Oh *et al.*, *J. Biol. Chem.*, 2007 [219]
Bacillus subtilis 168	Kunst *et al.*, *Nature*, 2007 [191]	4 214	4 105	563	NA	534	13.0	Cytoplasm, extracellular	iAG534	Goelzer *et al.*, *BMS Syst. Biol.*, 2008 [220]

Table 2.4 Continued.

Genome sequenced	Reference	Genome dimensions		Metabolic network characteristics						Reference
		Size (kb)	Total ORFs	Total reactions (unique)[a]	Total metabolites (unique)[b]	Total genes (enzymes)[c]	Percent genome covered[d]	Compartments[e]	Model ID[f]	
Staphylococcus aureus										
Staphylococcus aureus N315 (MRSA)	Kuroda *et al.*, *Lancet*, 2001 [192]	2813	2588	640	571	619	23.9	Cytoplasm, extracellular	iSB619	Becker and Palsson, *BMC Bioinform.*, 2005 [221]
Corynebacterium glutamicum										
Corynebacterium glutamicum Nakagawa	Ikeda and Nakagawa, *Appl. Microbiol. Biotechnol.*, 2001 [193]	3309	2993	(446)	411	446	14.9	Cytoplasm, extracellular	iKK446	Kjeldsen *et al.*, *Biotechnol. Bioeng.*, 2008 [222]
Mycobacterium tuberculosis										
Mycobacterium tuberculosis H37Rv (lab strain)	Cole *et al.*, *Nature*, 1998 [194]	4411	4402	939	828	661	15.0	Cytoplasm, extracellular	iNJ661	Jamshidi and Palsson, *BMC Syst. Biol.*, 2007 [223]
Mycobacterium tuberculosis H37Rv (lab strain)	Cole *et al.*, *Nature*, 1998 [194]	4411	4402	849	739	726 (723)	16.5	Cytoplasm, extracellular	iDB726	Beste *et al.*, *Genome Biol.*, 2007 [224]

Methanosarcina barkeri										
Methanosarcina barkeri Fusaro	Maeder *et al.*, *J. Bacteriol.*, 2006 [195]	4837	3606	619	558	692	19.2	Cytoplasm, extracellular	iAF692	Feist *et al.*, *Mol. Syst. Biol.*, 2006 [225]
Rhizobium etli										
Rhizobium etli CFN42	Gonzalez *et al.*, *PNAS*, 2006 [196]	4381	4035	387	371	363	9.0	Cytoplasm, extracellular	iRO363	Resendis-Antonio *et al.*, *PLOS Comp. Biol.*, 2007 [226]
Homo sapiens										
Homo sapiens	Lander *et al.*, *Nature*, 2001 [197]	3200000	26966	3311	2712	1496	5.5	Cytoplasm, mitochondrion, Golgi apparatus, endoplasmic reticulum, lysosome, peroxisome, nucleus, extracellular	Homo sapiens Recon 1	Duarte *et al.*, *PNAS*, 2007 [227]
Homo sapiens **mitochondria**[i)]										
NA	NA	NA	NA	189 (153)	230	(298)	NA	Cytoplasm, mitochondron, extracellular	iTV298	Vo *et al.*, *J. Biol. Chem.*, 2004 [228]

Table 2.4 Continued.

Genome sequenced	Reference	Genome dimensions		Metabolic network characteristics						Reference
		Size (kb)	Total ORFs	Total reactions (unique)[a]	Total metabolites (unique)[b]	Total genes (enzymes)[c]	Percent genome covered[d]	Compartments[e]	Model ID[f]	
***Mus musculus* cardiomyocyte**										
Mus musculus C57BL/6J	Mouse Genome Sequencing Consortium *et al.*, *Nature*, 2002 [198]	2 500 000	24 174	1220	872	473	2.0	Extracellular space, cytosol, mitochondria	iKS473	Sheikh *et al.*, *Biotechnol. Prog.*, 2005 [229]

Genome-scale reconstructed metabolic networks report several different parameters characterizing the network. Absolute consistency among reconstructions is not feasible; however, where possible common parameters have been defined providing dimensions of the reconstructed networks.

a) Total reactions, as defined by the authors, includes intracellular, extracellular, and exchange reactions. Where available, unique reactions are defined as the total number of reactions absent of any isoenzyme-catalyzed reactions, where the reaction stoichiometry is identical.

b) Total metabolites, as defined by the authors, includes all reactants, products, cofactors, catalysts, and intermediates involved in any stoichiometric reaction. Unique metabolites are defined as those unique in chemical structure, since a fraction of metabolites with identical chemical structure may be found in multiple compartments.

c) In most reconstructed networks the number of genes included in the model, as defined by the open reading frames (ORFs) producing a gene product that catalyzes a defined stoichiometric reaction, are provided. However, several reconstructions only include the gene product (e.g., enzymes) with no indication of ORF association.

d) In most reconstructed networks the percentage of the sequenced genome annotated by the model is provided. Here, the percentage genome covered is calculated based upon the total number of ORFs from the originally seqeuenced organism, and the total genes included in the reconstruction. Values may differ slightly from the original publication of the reconstructed network if the sequenced genome of the organism has been updated.

e) In most reconstructed networks the compartmentalization used is provided. However, in several models no mention of compartments is provided, therefore, annotatio of this parameter is provided here based on inspection of the model.

f) The common nomenclature used for model identification is *i-First name-Last name-Number of ORFs represented*. In several cases there has been deviation from this nomenclature and the model name used in the original publication is provided for consistency.

g) Strain *Aspergillus niger* ATCC 9029 was genome sequenced, threefold coverage, by Integrated Genomics (Chicago, USA); however, this genome sequence is not presently, publicly available.

h) Strain *Aspergillus niger* ATCC 1015 was genome sequenced by the Department of Energy's Joint Genome Institute; however, this genome sequence is not presently listed as completed.

i) This reconstructed model was not based on a genome sequence, but rather included analysis of a proteome network constructed for human mitochondria.

GSMMs provide an appropriate scaffold for further expansion and data integration, because of their easily manipulated mathematical framework. While that framework has been described previously, it warrants a brief review in the following section.

2.6.2 Genome-Scale Reconstructed Network Process

Given the relatively large number of genome-scale reconstructions now available (Table 2.4), a robust methodology has been established for *de novo* model construction. There are numerous reviews describing the process of genome-scale network reconstruction, including the initial biochemical annotation performed, the mathematical framework employed for describing metabolism, the resulting system of linear differential equations, the assumptions and constraints required for simplification, and ultimately numerical solution methods [20, 96–98]. Although the history of quantitative flux balance analysis has early roots in various fields, particularly if one considers the previously discussed isotope-labeled substrate experiments performed in the 1950s, it is widely accepted that flux balance analysis first became widespread in the 1960s and 1970s. Early attempts focused on specific enzyme kinetics, such as the characterization of yeast pyruvate kinase *in vitro* for calculation of glycolytic flux under anaerobic cultivation conditions [99]. Furthermore, through the work of Michael Savageau and other groups in the development of systems analysis of biochemical processes, the broader framework for what today is commonly referred to as biochemical systems theory emerged during the 1960s through a series of seminal publications in the *Journal of Theoretical Biology* [100–102]. Along similar lines two independent research groups – Kacser and Burns (1973) [103] and Heinreich and Rappoport (1974) [104–107] – developed a mathematical framework for quantitative analysis of how flux control in metabolic pathways is distributed, a concept that today is referred to as metabolic control analysis. However, it should be noted that flux balance analysis, particularly with genome-scale resolution, has largely been developed since the late 1990s with significant computational tools and methodologies developed aimed at extracting more predictive power from the collection of models available [23, 108–111].

With nearly 30 genome-scale metabolic models constructed, and numerous others proposed, it is relevant to investigate the fundamental approach to flux balance analysis. The simplified mathematical framework presented here has been adapted from an excellent presentation of flux balance analysis [112]. To use the power of this methodology, let us define a hypothetical metabolic system composed of unique metabolites A, B, C, D, and E, and a two-compartment biochemical reaction space (compartments 1 and 2) (Figure 2.5). The reactions and stoichiometry are clearly defined, and included in the stoichiometry is annotation of the compartmentalization. For purposes of this example, R_1, R_2, R_5, R_6, and R_7 will be referred to as catalyzing reactions, while R_3, R_4, R_8, R_9 are considered exchange transport reactions. It is important to note that the nomenclature and approach may vary in model construction, but, for example, internal transport of

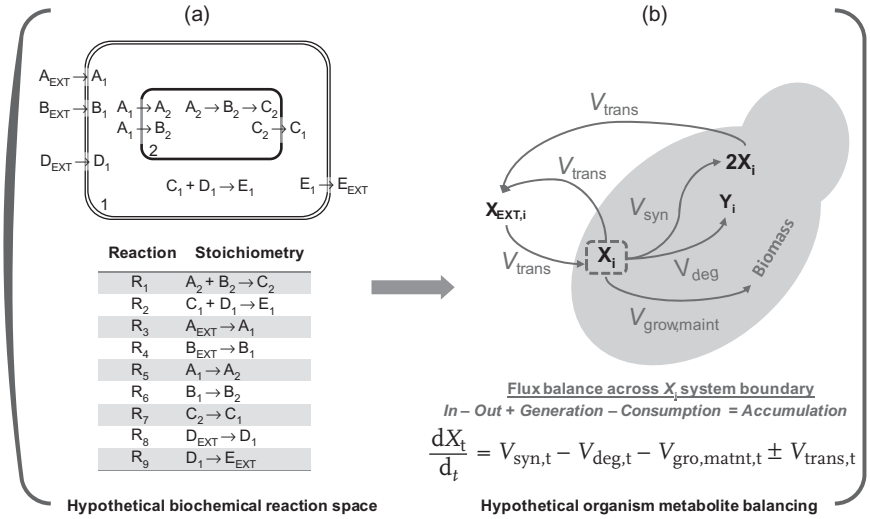


Figure 2.5 Biochemical reaction space as described in the text.

metabolite A_1 from compartment 1 to compartment 2 results in a new metabolite, A_2, being defined. Consequently this reaction is defined as catalysis, because R_5 may be modeled as the depletion of A_1 to form A_2 even though these metabolites are not in fact chemically unique.

Figure 2.5 depicts both a hypothetical biochemical reaction space (a), and a hypothetical organism metabolite balancing (b). In (a), a two-compartment bioreaction chemical space is suggested, with unique metabolites A, B, C, D, and E, considered in a network of exchange transport reactions, catalysis reactions, and internal transport reactions. Catalysis reactions in this context are defined as catabolic or anabolic reactions, and include internal transport reactions. This may be most reasoned by noting that while A_1 and A_2 are chemically identical metabolites (e.g., metabolite A is considered a single unique metabolite), for modeling purposes they are considered independent metabolites, and the transport of A from compartment 1 (A_1) to compartment 2 (A_2) is equivalent to the conversion of A_1 to A_2. Therefore, R_1, R_2, R_5, R_6, and R_7 are, in fact, considered metabolic reactions. Reactions R_3, R_4, R_8, and R_9 are then considered exchange transport reactions, where the external metabolite (A_{EXT}) enters the system. In (b), the principles applied to the theoretical biochemical reaction space are then framed in the context of cellular metabolism. A flux ($V_{n,i}$) balance across metabolite X_i is considered, and four categorical fluxes considered include: transport fluxes ($V_{trans,i}$), synthesis fluxes ($V_{syn,i}$), degradation fluxes ($V_{deg,i}$), and a flux representing a depletion of metabolite X_i to satisfy growth and maintenance requirements ($V_{gro,main,i}$). These fluxes may be summed to determine the accumulation of metabolite X_i within the system boundary considered with respect to time. However, for most flux balance analysis applications the time-scales of dynamic changes in metabolite pools are often

significantly faster than the time-scales associated with growth, therefore, a steady-state assumption is often applied ($dX_i/dt = 0$).

In a genome-scale network reconstruction each of these reactions would be further annotated by assigning function to a specific open reading frame (ORF), and subsequently a comprehensive list of all reactions, metabolites, and their assigned ORF are reconstructed, including identifying those reactions and metabolites that are unique (e.g., independent of compartmentalization, and representing novel chemical entities and their catabolic or anabolic reactions). The methodology then employed is derived from the classical principles of chemical engineering, where essentially a mass balance is performed across a defined system boundary. (Note: In modern educational terms, mass and energy balancing is most commonly associated with the field of chemical engineering; however, in a biological context flux balance analysis originates from earlier enzymatic characterization and biochemical pathway analysis, as discussed above. In historical terms it is worth noting that mass and energy balances extend from the laws of mass and energy conservation, which were originally formalized in the 1700s by the chemist John Dalton, and experimentally demonstrated by another chemist Mikhail Lomonosov [113].)

A mass balance approach for a given metabolite may be considered of the qualitative form:

$$\text{In} - \text{Out} + \text{Generation} - \text{Consumption} = \text{Accumulation} \tag{2.1}$$

From a biochemical reaction perspective the above mass balance may formalized mathematically into the expression:

$$\frac{dX_i}{dt} = V_{syn,i} - V_{deg,i} - V_{gro,maint,i} \pm V_{trans,i} \tag{2.2}$$

In Equation 2.2, the accumulation of metabolite X_i with respect to time, is defined as the rate of synthesis (V_{syn}), minus the rate of degradation (V_{deg}), minus the rate of consumption related to growth or maintenance of existing biomass ($V_{gro,maint}$), and then plus or minus the rate of transport (V_{trans}) across a defined biological boundary (e.g., membrane). Equation 2.2, presently in scalar format, may then be written in matrix format, yielding Equation 2.3.

$$\frac{dX}{dt} = \mathbf{S} \cdot V \pm V_{trans} \tag{2.3}$$

Equation 2.3 represents a mass balance across all metabolites in the biochemical reaction space considered, having concentration X, and then defining a vector of all the metabolic reactions, V, and a stoichiometric matrix, $\mathbf{S}$. Biological time-scales associated with changes in metabolite concentrations are often very rapid, and significantly faster than time-scales associated with growth (e.g., for *S. cerevisiae* the doubling time is about 2 h). It is therefore reasonable to assume that the concentrations of all the intracellular metabolites are in a steady state, yielding Equation 2.4:

$$0 = \mathbf{S} \cdot V \pm V_{trans} \tag{2.4}$$

Equation 2.4 may be further simplified by considering that the rate of transport of all metabolites, X, may be reduced to a constant value equivalent to the net transport of metabolites into or out of the bioreaction space. This simplification, converts V_{trans} to a constant term, $\boldsymbol{b}$, a vector representing the net exchange flux of metabolites. This constant value, $\boldsymbol{b}$, for each metabolite, in matrix format is expressed in Equation 2.5, noting the use of the identity matrix, **I**.

$$0 = \mathbf{S} \cdot \boldsymbol{V} + \boldsymbol{b} \cdot \mathbf{I} \tag{2.5}$$

Prior to further simplifications, it is appropriate to take Equation 2.5 and apply it to the system described in Figure 2.5. The resulting stoichiometric matrix, **S**, and the vector describing all of the metabolic reactions, $\boldsymbol{V}$, are presented below as Equation 2.6. Furthermore, the vector, $\boldsymbol{b}$, representing the net exchange fluxes for each metabolite is also represented in Equation 2.6 (for simplification purposes the identity matrix is not included since $\boldsymbol{b} \cdot \mathbf{I} = \boldsymbol{b}$). For clarity purposes, the top row of each matrix serves as a column header. In the case of the vector $\boldsymbol{b}$, while all metabolites are designated, only certain metabolites have a net transport, previously defined as R_3, R_4, R_8, and R_9. Those metabolites are designated with the corresponding transport reaction (R_n) as depicted in Figure 2.4.

$$\begin{array}{|c|ccccc|} \hline \boldsymbol{A_1} & 0 & 0 & -1 & 0 & 0 \\ \boldsymbol{B_1} & 0 & 0 & 0 & -1 & 0 \\ \boldsymbol{C_1} & 0 & -1 & 0 & 0 & 1 \\ \boldsymbol{D_1} & 0 & -1 & 0 & 0 & 0 \\ \boldsymbol{E_1} & 0 & 1 & 0 & 0 & 0 \\ \boldsymbol{A_2} & -1 & 0 & 1 & 0 & 0 \\ \boldsymbol{B_2} & -1 & 0 & 0 & 1 & 0 \\ \boldsymbol{C_2} & 1 & 0 & 0 & 0 & -1 \\ \hline \end{array} \bullet \begin{array}{|c|} \hline \boldsymbol{R_n} \\ \hline R_1 \\ R_2 \\ R_5 \\ R_6 \\ R_7 \\ \hline \end{array} + \begin{array}{|c|} \hline \boldsymbol{b_n} \\ \hline (R_3)A1 \\ (R_4)\ B_1 \\ C_1 \\ (R_8)\ D_1 \\ (R_9)\ E_1 \\ A_2 \\ B_2 \\ C_2 \\ \hline \end{array} \tag{2.6}$$

$$\boldsymbol{S} \quad \bullet \quad \boldsymbol{V} \quad + \quad \boldsymbol{b} \quad = \quad 0$$

Equation 2.5 may be further rearranged, particularly focusing on separation of those metabolites that have a net exchange flux. This is readily accomplished by definition of a new vector, $\boldsymbol{b}_t$, that will only include rows of metabolites that have a net exchange flux (e.g., not all metabolites are transported across the system boundary). Consequently, the columns of the identity matrix will be reduced to the same number of rows as vector $\boldsymbol{b}_t$, and be renamed $\mathbf{I}_t$. Furthermore, the stoichiometric matrix, **S**, may be subdivided to include the stoichiometry for reactions that are related to metabolic catabolism or anabolism resulting in the net accumulation or transport of a metabolite, defined as $\mathbf{S}_r$, and into reactions that constitute biomass formation and maintenance, defined as $\mathbf{S}_{b,m}$. Lastly, the modified stoichiometric matrices are multiplied by the corresponding flux vectors, $\boldsymbol{V}_{b,m}$ and $\boldsymbol{V}_r$. The final resulting equation is included below, as Equation 2.7.

$$0 = \mathbf{S}_r \cdot \boldsymbol{V}_r + \mathbf{S}_{b,m} \cdot \boldsymbol{V}_{b,m} + \boldsymbol{b}_t \cdot \mathbf{I}_t \tag{2.7}$$

Using more familiar matrix notation, Equation 2.7 may be reformulated to Equation 2.8:

$$\begin{aligned} \mathbf{S}' &= \langle \mathbf{S}_{\mathrm{r}} | \mathbf{S}_{\mathrm{b,m}} | \mathbf{I}_{\mathrm{t}} \rangle \\ V' &= \begin{matrix} \boldsymbol{V}_{\mathrm{r}} \\ \boldsymbol{V}_{\mathrm{b,m}} \\ \boldsymbol{b}_{\mathrm{r}} \end{matrix} \\ \mathbf{S}' \cdot V' &= 0 \end{aligned} \tag{2.8}$$

The above form (2.8) is what is most commonly used in the literature to represent the flux balance of a stoichiometrically defined bioreaction space, similar to what is provided in Figure 2.5. From this point forward, additional constraints that are often specific to the bioreaction space being considered and the organism are included. These considerations will include but not be limited to the metabolite and reaction compartmentalization, the reversibility of the reactions, the net biomass equation (e.g., summation of all metabolite precursors, redox cofactors, and energy cofactors in stoichiometric quantities), the theoretical minimum and maximum metabolite fluxes, the minimum and maximum growth rates, the amount of ATP (or equivalent energy currency) required for maintenance, and the amount of starting fluxes for input exchange fluxes (e.g., glucose uptake rate). Lastly, an objective function, to be maximized or minimized, must be defined and typically takes the form of Equation 2.9, where Z is an objective function equal to the summation of the product of a unit vector, $\boldsymbol{q}_{\mathrm{i}}$, and the metabolic fluxes, $\boldsymbol{V}_{\mathrm{i}}$, where $\boldsymbol{q}_{\mathrm{i}}$ is typically the growth rate flux or glucose uptake rate. Both of these fluxes serve as suitable maximization parameters for modeling *in vivo* microbial metabolism where under conditions of excess nutrients and limited substrate, the specific growth rate of microbes, μ, will approach $\mu_{\max}$. Note that included in Equation 2.9 are constraints on metabolites, $\boldsymbol{V}_{\mathrm{i}}$, which typically range from a minimum (a) to a maximum (b):

$$\begin{aligned} Z &= \sum \boldsymbol{q}_i \cdot \boldsymbol{V}_i \\ a &\le \boldsymbol{V}_i \le b \end{aligned} \tag{2.9}$$

The resulting system of linear equations, for a given objective function, may be solved using linear programming methods, for which several numerical solution packages are available. The result is a solution space that may be represented on a minimum of two or maximum of three dimensions, from which a specific phenotypic phase place is defined. Figure 2.6 highlights what is commonly referred to as the phenotypic phase plane (PhPP), where a two-dimensional or three-dimensional solution space is considered for a simulation where the maximization of an objective function is considered under specific constraints, such as the optimization of growth rate under a constant glucose uptake rate (q_{gluc}), and oxygen uptake rate (q_{O2}).

Figure 2.6 is a synopsis of the phenotypic phase planes that result from flux balance analysis, which has its origins in performing a steady-state mass balance of metabolites across a defined system boundary. As depicted in Figure 2.6a, the resulting reconstructed metabolite network may be mapped onto two or three dimensions. The y-axis represents an objective function, which can be either

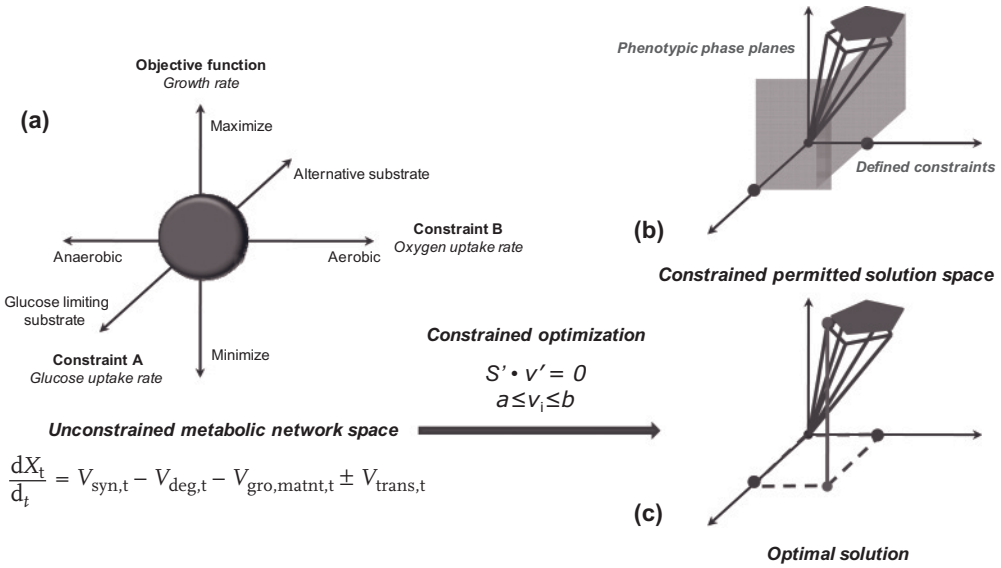


Figure 2.6 Flux balance analysis and phenotypic phase planes.

maximized or minimized, while the other two dimensions (*x*-axis, *z*-axis) represent flux constraints. Common constraints include the glucose and oxygen uptake rates, which will create a bound solution space where the objective function, often growth rate, can be maximized as shown in Figure 2.6b. The resulting phenotypic phase plane (Figure 2.6c), then yields an optimal solution that satisfies the optimization criteria.

The approach described here in flux balance analysis has been applied to numerous organisms as described in Table 2.4, and in particular, has served as a critical tool in metabolic engineering approaches, and more recently, systems biology. Systems biology is the quantitative characterization of genetic, transcription, protein, metabolic, signaling, and other informational pathway responses to a clearly defined perturbation of a biological system. The perturbation may be in terms of a genetic, chemical, or environmental stimulus. At the core of systems biology is the transformation of quantitative, typically large-scale data sets, into *in silico* models that provide both interpretation and prediction. GSMMs provide a framework of how x-ome data may be organized and overlaid on the metabolic network. As technologies have become more accessible for transcriptome (DNA oligonucleotide and cDNA microarrays), proteome (two-dimensional gel electrophoresis coupled to MS or direct MS analysis), fluxome (isotopically labeled substrates coupled to detection by GC-MS), and metabolome (numerous analytical methods including LC-MS and GC-MS) measurements, enormous data sets have been generated that require bioinformatics and quantitative models to be developed for data analysis, interpretation, and prediction. Industrial biotechnol-

ogy is beginning to exploit the benefits of these tools, realizing that metabolic engineering strategies for improved process development may first be screened *in silico*, producing a reduced list of specific genetic perturbations with a high probability of success that should be experimentally validated. The process is highly iterative, with strain construction and characterization providing new x-ome data that can be used to improve the models (i.e., experimental quality control of *in silico*) and metabolic engineering strategies (Figure 2.2).

2.7 Industrial Systems Biology Case Studies

There have been extensive reviews and literature describing industrial biotechnology, noting several prominent case studies [73, 75, 114–115]. As previously suggested, industrial systems biology is prevalent in two forms: existing companies are either building their own infrastructure through reshaping in-house competences or forming new process development groups with industrial systems biology capabilities and expertise, or they are out-sourcing process development to small, recently formed entities that specialize in industrial systems biology. Examples of such enterprises focused on providing industrial systems biology expertise to more traditional process development groups include METabolic EXplorer (France, founded in 1999), Genomatica (USA, founded in 2000), Fluxome Sciences (Denmark, founded in 2002), and Microbia Precision Engineering (USA, a subsidiary of Ironwood Pharmaceuticals, formerly Microbia). Although small, these companies have significant collaborations with many of the major chemical manufacturing, nutraceutical, pharmaceutical, and petrochemical companies.

Industrial systems biology, while in its infancy, has already had significant impact on tangible products produced using industrial biotechnology. Although several products may be presented as case studies, perhaps a more appropriate context to gage the impact of industrial systems biology is to consider three broader product classes: (i) mature and developed (e.g., bioethanol), (ii) recently launched and rapidly growing (e.g., 1,3-propanediol), and (iii) in-development (e.g., succinic acid). All three of these products given as examples have been significantly impacted by the application of systems biology for development of commercialized microbial cell factories.

2.7.1 A Mature and Developed Industrial Biotechnology Product: Bioethanol

As highlighted previously, the largest industrial biotechnology product in the world, both in terms of volumetric production and total sales, is bioethanol, which continues to endure phenomenal expansion fueled by unprecedented corporate, social, and political support [10]. In 2007, the total number of biorefineries producing bioethanol in the United States increased from 110 operating in 19 states to 139 spread across 21 states. In the same year, annual production capacity increased

by approximately 7.6 billion liters to a total United States annual capacity of 29.9 billion liters, representing the largest fraction of the 49.6 billion liters of total world production capacity. On December 19, 2007, the United States adopted the Energy Independence and Security Act (EISA) of 2007, which is an expansion of the earlier Renewable Fuels Standard (RFS) that was enacted as part of the Energy Policy Act of 2005. The most recently adopted legislation stipulates a mandatory time-course adoption of renewable biofuels between 2008 and 2022, where the mandatory total renewable fuels products must grow from 34.1 to 136.3 billion liters, respectively. Furthermore, the 136.3 billion liters of renewable fuels must be composed of 41.6% renewable biofuel and 58.3% advanced biofuels, and at least 44% or 60.6 billion liters of advanced biofuels must be derived from cellulosic biomass [116]. For the foreseeable future bioethanol will continue to expand as the most mature and developed biotechnology product, with unprecedented demand for more advanced metabolic engineering strategies and application of systems biology tools to enhance all segments of bioethanol process development.

The producers of bioethanol are using a variety of fermentation platforms; however, *S. cerevisiae* is among the more popular because of its robustness for large-scale (>300 000 l) fermentation processes. The first eukaryotic genome-scale metabolic model was reported in *S. cerevisiae* in 2003 [20]. This genome-scale network reconstruction, by using a relatively simple synthetic medium, could predict 88% of the growth phenotypes correctly. In a subsequent evaluation, the same metabolic network model was used to generate a phenotypic phase plane (PhPP) analysis that describes yeast's metabolic states at various levels of glucose and oxygen availability [117]. Examination of the *S. cerevisiae* PhPP has led to the identification of two lines of optimality: LO_{growth}, which represents optimal biomass production during aerobic, glucose-limited growth, and $LO_{ethanol}$, which corresponds to both maximal ethanol production and optimal growth during microaerobic conditions. The predictions of the *S. cerevisiae* PhPP were compared with independent experimental data, and the results showed strong agreement between the computed and measured specific growth rates, uptake rates, and secretion rates. Thus, metabolic network reconstructions and simulation can be used to systematically reconcile existing data available for *S. cerevisiae*, particularly now that yeast resources, databases, and tools for global analysis of genomic data have been expanded and made publicly available, such as the *Saccharomyces* Genome Database [16, 118–120].

The use of metabolic models for optimization of bioethanol production in *S. cerevisiae* has been demonstrated in two studies. A simple metabolic model was used to identify the deletion of NADPH-dependent glutamate dehydrogenase and overexpression of the NADH-dependent glutamate dehydrogenase, which resulted in increased ethanol production coupled with a 40% reduction in the production of the by-product glycerol [121]. In a second study the above mentioned genome-scale metabolic model was used to identify a new target for improving bioethanol production by insertion of an NADPH-forming glyceraldehyde dehydrogenase. This resulted in increased bioethanol with reduced glycerol formation [122]. In

both of these examples modification of the redox metabolism was performed, and this has in general proved to be effective for improving many bioprocesses as the redox cofactors NADH and NADPH participate in a large number of reactions.

With the experimental mechanics of collecting transcriptome data becoming commonplace, attention and focus are now being placed on data analysis methods and integration with other x-ome data sets. It has become apparent that standalone transcriptome data, unless used for environmental screening or quality control (i.e., confirming that an engineered genotype is producing the corresponding transcription profile), provide limited mechanistic biological insight for metabolic engineering approaches. Several studies have coupled transcriptome with metabolome and fluxome data [18, 123–125]. For example, elementary flux modes for three carbon substrates (glucose, ethanol, and galactose) were determined using catabolic reactions from a genome-scale metabolic model of *S. cerevisiae*, and then used for gene-deletion phenotype analysis. Control-effective fluxes were used to predict transcript ratios of metabolic genes for growth under each substrate, resulting in a high correlation between the theoretical and experimental expression levels of 38 genes when ethanol and glucose media were considered [126]. This example demonstrates that incorporating transcriptional functionality and regulation into metabolic networks for *in silico* predictions provides both more biologically representative models, and a means of bridging transcriptome and fluxome data.

In another example, the topology of the genome-scale metabolic model constructed for *S. cerevisiae* is examined by correlating transcriptional data with metabolism. Specifically, an algorithm was developed enabling the identification of metabolites around which the most significant transcriptional changes occur (referred to as reporter metabolites) [230]. Because of the highly connected and integrated nature of metabolism, genetic or environmental perturbations introduced at a given genetic locus affect specific metabolites and then propagate throughout the metabolic network. Using transcriptome experimental data, predictions of which metabolites are likely to be affected can be made, and serve as rational targets for additional inspection and metabolic engineering. This algorithm has been recently extended to include reporter reactions, whereby transcriptional data is correlated with the metabolic reactions of the reconstructed *S. cerevisiae* genome-scale metabolic network model to identify those reactions around which a genetic or environmental perturbation conferring transcriptional changes cluster [127].

There have been several examples where flux measurements and analysis has significantly contributed to bioethanol strain development, particularly with respect to engineering xylose- and pentose-consuming fermentations. As highlighted earlier, bioethanol conversion from cellulosic biomass sources will be critical to meeting renewable fuel standards, and xylose represents the most abundant pentose sugar in hemicellulose, hardwoods and crop residues, and the second most abundant monosaccharide after glucose [128]. Because *S. cerevisiae* fails to consume pentose sugars efficiently, compared to glucose, significant research has

been carried out in metabolically engineering such strains. For example, Grotkjær *et al*. compared the flux profile of two recombinant *S. cerevisiae* strains, TMB3001 and CPB.CR4, both expressing xylose reductase (XR) and xylitol dehydrogenase (XDH) from *P. stipitis*, and the native xylulokinase (XK), but CPB.CR4 included a *GDH1* deletion and *GDH2* being put under a *PGK* promoter [129]. Expression of XR, XDH, and XK leads to highly inefficient xylose utilization because of a cofactor imbalance, where excess NADH must be regenerated via xylitol production, resulting in reduced ethanol yield. Therefore, metabolic engineering of the ammonium assimilation through deletion of the NADPH-dependent glutamate dehydrogenase (*GDH1*) and overexpression of the NADH-dependent glutamate dehydrogenase (*GDH2*) resulted in a 16% higher ethanol yield because of a 44% xylitol reduction [129, 130]. Using a reverse metabolic engineering approach, metabolic flux analysis was used to characterize the intracellular fluxes for both strains based on experimental data of anaerobic continuous cultivations using a growth-limited feed of ^{13}C-labeled glucose, confirming that XR activity shifted from being mostly NADPH to partly NADH dependent in the CPB.CR4 strain.

Furthermore, the analysis revealed, unexpectedly, activation of the glyoxylate cycle in CPB.CR4, generating the question of whether glyxoylate cycle activation may be preferred for ethanol yield. It was only through flux measurements and analysis, based on a reduced reconstructed metabolic network that the distribution of carbon believed to have been altered via targeted genetics could be confirmed.

In a separate example, again addressing the issue of redox balance resulting from xylose fermentation, metabolic flux analysis was used to predict that activation of the phosphoketolase pathway (PKP), which leads to the net reoxidation of one NADH per xylose converted to ethanol, would be preferred [131]. The PKP converts xylose-5-P to acetyl-P and glyceraldehyde-3-P, enabling the maximum theoretical yield of 0.51 g ethanol/g xylose without affecting the NADPH/NADH consumption ratio of the XR reaction. A functional PKP was reconstructed in strain TMB3001c and the ethanol yield was increased by 25% as a result of minimization of xylitol formation; however, metabolic flux analysis predicted that only about 30% of the optimum flux required to eliminate xylitol and glycerol accumulation was present. Further overexpression of PKP, however, led to increased acetate and a reduced xylose consumption rate, prompting the investigators to overexpress the acetaldehyde dehydrogenase *ald6*. This reduced acetate formation, and produced a strain with 20% higher ethanol yield and a 40% higher xylose consumption rate compared with the reference strain [132].

Metabolic flux analysis serves two purposes: a priori determination of preferred metabolic engineering targets and experimental confirmation of carbon flux distributions – neither of which are possible based on visual inspection of biochemical pathways.

There are many examples of the integration of various x-ome technologies with genome-scale reconstructions of microbial metabolism to elucidate previously poorly understood phenotypes, or for *de novo* prediction of metabolic engineering strategies in the context of bioethanol process development [10, 133].

2.7.2
A Recently Launched and Rapidly Growing Industrial Biotechnology Product: 1,3-Propanediol

1,3-Propanediol (PDO), produced by DuPont's new technology platform, DuPont Bio-Based Materials, and called Bio-PDO™, is an example of a recently launched product, previously produced via petrochemical conversion and now made possible by industrial biotechnology. PDO is a critical intermediate in the production of terephthalic acid and PDO polymers, commonly used in the clothing, fiber, and carpet industries, and serves as an intermediate for DuPont's new polymer platform, Sorona®. DuPont has partnered with Tate & Lyle plc to produce Bio-PDO using a proprietary fermentation platform based on *E. coli* conversion of D-glucose to dihydroxyacetone phosphate (DHAP), then to glycerol, and finally to PDO.

The development of the microbial cell factory was completed in collaboration with Genencor International. (Note: Genecor International, founded in 1982, was a joint venture between Genentech and Corning Glassworks (Dow Corning) and is responsible for several notable milestones in recombinant DNA technology. In 2005, Genecor International was acquired by the Danish food ingredient company Danisco.) The manufacturing facility was completed in 2006 with production beginning in November 2006. Using corn as the principal feedstock, it will produce 45 million kg/year at full capacity [134].

A life-cycle assessment of the production of nylon-6 polymer versus the production of renewably sourced Sorona with Bio-PDO results in 30% less energy usage and 63% less greenhouse gas emissions (including bio-based content stored in the product) [135]. The Sorona family of polymers, composed of Bio-PDO and consequently capable of inducing an inflammatory response, has been verified to be non-cytotoxic and non-inflammatory [136]. The estimated demand for polymers composed of PDO is 500 000–1 000 000 tons per annum [137].

PDO is among the oldest fermentation products. It was first identified by August Freund in 1881 in a glycerol-fermenting mixed culture of *Clostridium pasteurianum* [138], and later quantitatively analyzed at the microbiology school of Delft [139] and continued at Ames, Iowa [140]. The original petrochemical processes operated by Shell Chemical Company (Division of the Shell Group, The Netherlands) and Degussa (Germany; the process later acquired by DuPont), the survey of PDO production by a large variety of bacteria, including *Citrobacter*, *Clostridium*, *Enterobacter*, *Klebsiella*, and *Lactobacillus* species, and the bioreaction engineering analysis initially performed to evaluate the feasibility of industrial biotechnology production of PDO have been extensively reviewed [137, 141–143]. In the native PDO-producing organisms briefly mentioned, PDO formation is the result of anaerobic fermentation of glycerol where excess reducing equivalents in the form of NADH are regenerated (NAD^+) via glycerol dehydratase (*dhaB1-B3*) activity followed by 1,3-propanediol oxidoreductase (*dhaT*) activity [137]. Because of the significant cost-benefit of utilizing glucose feedstocks, a metabolic engineering strategy was developed requiring the heterologous expression of pathways forcing carbon flux redirection from DHAP to 1,3-propanediol. The key genetic modifica-

tions implemented in an *E. coli* microbial scaffold exhibiting no accumulation of PDO and relatively little accumulation of glycerol (as compared to acetate or succinate), as detailed by Nakamura and colleagues, included:

- overexpression of the non-native (*S. cerevisiae*) glycerol-3-phosphate dehydrogenase (DAR1) and glycerol-3-phosphate phosphatase (GPP2), for glycerol accumulation
- overexpression of the non-native (*K. pneumonia*) glycerol dehydratase (*dhaB1, dhaB2, dhaB3*) and the vitamin B_{12} reactivating factors (*dhaBX, orfX*) for conversion of glycerol to 3-hydroxypropionaldehyde
- overexpression of the native oxidoreductase (*yghD*) to complete the conversion of 3-hydroxypropionaldehyde to PDO; however, using NADPH as opposed to NADH
- deletion of the native glycerol kinase (*glpK*), glycerol dehydrogenase (*gldA*), and triosephophate isomerase (*tpi*) (essential to ensure maximizal carbon flux from glucose to DHAP, with minimal reversion of carbon back to glyceraldehydes-3-phosphate and, consequently, the TCA cycle and respiration)
- downregulation of the native phosphotransferase system (PTS) and glyceraldehydes dehydrogenase (*gap*), the former replaced with an exclusively ATP-dependent phosphorylation (elimination of phosphenolpyruvate dependence) using the native galactose permease (*galP*) and glucokinase (*glk*), and the later augmented with a reconstituted native *tpi*. The reconstitution of *tpi* in the background of *gap* deletion provides a useful flux control point.

The integration of the above modifications, coupled with additional strategies that remain proprietary, resulted in an *E. coli* under fed-batch conditions capable of producing PDO with a final yield of 51% (w-PDO/w-glucose), titer of 135 g/l, and productivity of 3.5 g/l per h [137].

Bio-PDO is among the first success stories for metabolic engineering and industrial biotechnology in the added-value chemical industry. Yet, it should be realized that over 10 years of development and significant resources were invested to reach this milestone. Furthermore, based on the available literature and conference presentations, the process described here required an enormous development of recombinant DNA technology, enzyme characterization and profiling, and classical bioreaction pathway analysis that was unavailable during development. Consequently, many of the systems biology tools currently available were not employed during the metabolic engineering strategy design and implementation stage.

Today, metabolic modeling of microbial metabolism is being applied to the Bio-PDO to confirm the expected phenotype of intracellular flux distributions, and identify potential opportunities for second-generation metabolic engineering strategies. Specifically, metabolic flux analysis (MFA) has been developed to enable dynamic measurement of intracellular flux distributions using isotopically labeled [1-^{13}C]-glucose supplemented to fed-batch fermentations of *E. coli* K12

(strain overproducing Bio-PDO) in a ratio of 3:1 naturally enriched [U–^{13}C]-glucose. A detailed metabolic reconstructed network of *E. coli* metabolism has been completed, which includes 75 reactions and 74 metabolites, encompassing five substrates (glucose, citrate, O_2, NH_3, SO_4), five products (PDO, biomass, CO_2, acetate, and ATP), and 63 balanced intracellular metabolites [144].

Previously developed MFA have suffered from the limitation of assuming an isotopic steady state because of the relatively short time-scales of intracellular metabolite concentration pools. Antoniewicz *et al.* extended the scope of flux resolution from steady-state to dynamic environments through a modeling strategy that employed derivatives of isotopmer spectral analysis, and classical MFA based on the mass isotopomer distributions of amino acids determined by elementary metabolite unit (EMU) modeling [144, 145]. For the first time, the time-profile *in vivo* fluxes of the fed-batch industrial process for Bio-PDO production were resolved, consisting of 82 redundant measurements across 20 distinct time-points.

Intracellular flux distributions were found to change extensively over the course of the fed-batch profile, showing a decrease in the split ratio between glycolysis and the pentose phosphate pathway (PPP) of 70/30 at 20 h to 50/50 at 43 h, and a decrease in the flux from glyceraldehyde-3-phosphate to 3-phosphoglycerate of approximately 21% across the same time interval. The flux from DHAP to glycerol-3-phosphate and ultimately PDO increased by approximately 10% and remained relatively constant at 132. However, the efflux of PDO had a large variation increasing from 78 (18.6 h) to 138 (28.6 h) and then decreased to 130 (40.7 h) (all flux values were normalized to the glucose uptake rate of 100). During the same time interval the primary energy-producing pathway, the TCA flux, remained relatively constant at 46.

Metabolic modeling in the context of flux estimations and a reconstructed metabolic network provide verification that the genetic engineering believed to confer a desired phenotype does so. Furthermore, these data provide an *in vivo* opportunity to assess opportunities for further metabolic engineering, such as targeting the discrepancy between the efflux of PDO and the intracellular PDO formation flux. Yet another potential metabolic engineering target is that the metabolic model was incapable of accounting for all of the net ATP produced (176 excess ATP flux for P/O ratio of 3, and 123 excess ATP flux with a P/O ratio of 2) compared with the ATP-consuming reactions. Given the modifications made to the *E. coli* K12 PTS and the fact that the TCA cycle flux, the primary source of ATP under aerobic conditions, remained constant, there may be opportunities to redirect excess ATP to higher biomass formation, consequently increasing the productivity of PDO.

PDO production using industrial biotechnology will continue to accelerate, mature, and it is feasible that PDO will reach commodity chemical status as market demand and sustainable, cost-effective supply increase. Furthermore, it should be noted that microbial metabolic modeling, primarily in the form of MFA, is also being pursued in other production organisms, such as *Klebsiella pneumoniae* [146–150].

2.7.3
An In-Development Industrial Biotechnology Product: Succinic Acid

The global chemical manufacturing industry experienced a 15% increase to US$665.6 billion in total sales between 2004 and 2005, with European companies contributing 45.1% (US$300.5 billion) [151]. In that same period the primary raw material for chemical manufacturing, crude petroleum, rose in price by approximately 30% (US$31 per barrel in January 2004 to US$41 per barrel in January 2005), and if we consider the change in price between 2004 and 2006, the price increase is nearly 80% (US$56 per barrel in January 2005). The rapid increase in price was not only driven by surging demand, but instability in and threats to supply.

It is estimated that a US$10 increase in the price of a barrel of oil increases variable costs to the US chemical industry by approximately US$2.6 billion per year [152]. The chemical manufacturing industry is therefore actively searching for cost-effective, environmentally friendly, renewable, and sustainable raw material feedstocks that will not only enable production of key chemical building blocks, but can also serve as a platform for future products (Figure 2.1). Industrial biotechnology is a promising alternative with microbial organisms engineered to produce key products from biomass.

In 2004, based upon a critical analysis to identify the top building blocks that may be produced from biomass and subsequently converted to high-value bio-based chemicals, the US Department of Energy identified succinic acid as a top 10 building block [153]. In the same year, 160 million kg of succinic acid were synthesized from petrochemical conversion of maleic anhydride (10% of total worldwide maleic anhydride production). If bio-based succinic acid production becomes more commonplace, global market demand is estimated to increase to US$2 billion per annum with a total energy savings of 2 800 000 MWh/year. Succinic acid is used in a variety of products and serves as a critical starting material or intermediate in the production of useful chemicals for solvents and polymers.

Figure 2.7 illustrates the diverse chemical synthesis opportunities offered by using succinate and succinic anhydride as a building block chemical. Presently, succinic acid is primarily used in the specialty chemical sector amounting to a total annual demand of 15 000 tons, and is produced from the conversion of maleic anhydride. Approximately 10% of the total maleic anhydride produced (1.6 billion kg per annum) is dedicated towards succinate production. Maleic anhydride is widely used in housing, construction, and automotive manufacturing industries, which as a result of their recent strong demand, has caused the price to increase from US$0.95 per kg (2003) to US$1.21 per kg (2005). This price increase has resulted in increased petrochemical maleic anhydride production facilities, where the raw material, *n*-butane is utilized. Consequently, there has been significant price pressure to design a cost-effective succinic acid industrial biotechnology process in the face of increasing maleic anhydride supplies. It is estimated that the market for succinate is in excess of US$2 billion per

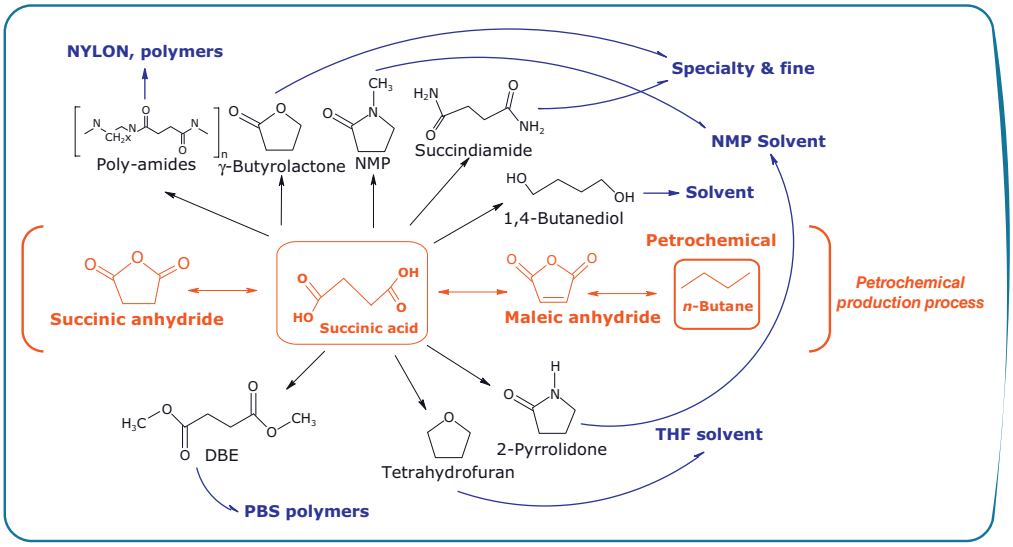


Figure 2.7 Succinic acid–a sustainable building block chemical.

annum, assuming a minimum productivity of 2.5 g/l per h may be reached via fermentation.

Succinic acid ($C_4H_6O_4$, MW 118.09 g/mol, pK_{a1} 4.21, pK_{a2} 5.72) is a polyprotic dicarboxylic acid that occurs in nature. It is soluble in water (100 mg/ml), yielding a clear, colorless solution. Succinate, the anion of succinic acid, is a citric acid cycle intermediate produced from the GTP-coupled oxidation of succinyl-CoA by succinyl-CoA synthetase, and in many cases, as a by-product of the isocitrate lyase–catalyzed conversion of isocitrate to glyoxylate. Succinic acid is then further oxidized to fumarate by succinate dehydrogenase, co-producing $FADH_2$. There are numerous biomass-based production platforms, all prokaryotic, including *Anaerobiospirillium succiniciproducens*, *Actinobacillus succinogenes*, *Succinivibrio dextrinosolvens*, *Corynebacterium glutanicum*, *Prevotella ruminocola*, a recently isolated bacterium from bovine rumen, *Mannheimia succiniciproducens*, and a metabolically engineered succinic acid–overproducing *E. coli*.

Several extensive reviews have discussed the succinic acid market in detail and have comprehensively presented the various metabolic engineering strategies coupled with application of systems biology that have been employed to date [154–157]. The two organisms that have been most significantly engineered from native isolations are *E. coli* and *M. succiniciproducens*. For illustrative purposes, only *M. succiniciproducens* will be highlighted here as it epitomizes the potential for industrial systems biology.

M. succiniciproducens MBEL55E is a capnophilic Gram-negative bacterium first isolated in 2002 from a bovine rumen in Korea that natively accumulates large amounts of succinic acid under glucose-supplemented anaerobic (100% CO_2) fermentation conditions (0.68 g succinic acid/g glucose) [158]. Shortly following

the isolation, classical batch and continuous fermentation of sodium hydroxide–treated wood hydrolysate was examined and resulted in a succinic acid productivity of 1.17 g/l per h (yield: 56%) and 3.19 g/l per h (yield: 55%), respectively [159]. These were certainly the highest productivities reported at the time, and were particularly promising given the lignocellulosic feedstock used (mixed substrate glucose and xylose, batch and continuous cultivations were also performed as controls, with similar productivities and yields resulting).

In the same year, the 2 314 078 base pair genome sequence of *M. succiniciproducens* MBEL55E was reported, co-currently with the genome-scale reconstructed metabolic network [160]. The genome-scale reconstructed metabolic network, consisting of 373 reactions (121 reversible and 252 irreversible) and 352 metabolites, predicted, using MFA, a theoretical production of 1.71 and 1.86 moles of succinic acid for every mole of glucose under CO_2 and CO_2–H_2 atmospheres, respectively [160]. As a consequence of the simulations, the authors note, "Based on these findings, we now design metabolic engineering strategies for the enhanced production of succinic acid; one such strategy will be increasing the PEP carboxylation flux while decreasing the fluxes to acetic, formic, and lactic acid" [160].

In 2006, the authors constructed a series of knockout mutants of *M. succiniciproducens* MBEL55E that included disruption of three CO_2-catalyzing reactions (PEP carboxykinase, PEP carboxylase, malic enzyme) and disruption of four genes responsible for by-product formation of lactate, formate, and acetate (*ldhA*, *pflB*, *pta*, and *ackA* genes) [161]. Their results confirmed that a mutant capable of virtually no lactate, fumarate, or acetate formation was feasible, and that PEP carboxykinase was most critical for anaerobic growth and maximizing succinic acid production [161].

The resulting metabolically engineered strain, *M. succiniciproducens* LPK7 under batch fermentation conditions produced 0.97 mol succinic acid per mol glucose, and under fed-batch fermentation conditions reached a maximum titer, productivity, and yield of 52.4 g/l, 1.8 g/l per h, and 1.16 mol succinic acid per mol glucose, respectively [161]. The theoretical carbon yield of succinate under excess reducing power and CO_2 carboxylation is 2 mol succinic acid per mol glucose ($\Delta G^{o\prime} = -317$ kJ/mol) [154].

In 2006 the complete proteome of *M. succinciproducens*, was reported [162]. This was one of the first examples of proteomics applied to industrial biotechnology process development. Using two-dimensional electrophoresis coupled with mass spectrometry, identification and characterization of 200 proteins distributed across whole cellular proteins (129), membrane proteins (48), and secreted proteins (30), was described. Characterization of cell growth and metabolite levels in conjunction with proteome measurements during the transition from exponential to stationary growth was carried out.

Two interesting conclusions could be drawn from such analysis that were not previously possible. First, a gene locus previously annotated as the succinate dehydrogenase subunit A (*sdhA*) is likely to be the fumarate reductase subunit A (*frdA*) based on comparative proteome analysis supported by physiological data. Second, two novel enzymes were identified as likely metabolic engineering targets for

future improvements in succinic acid production. PutA and OadA are enzymes responsible for acetate formation and conversion of oxaloacetate to pyruvate, respectively, and their deletion is likely to induce higher flux towards succinic acid through minimization of by-product formation [162]. This is a clear example of where proteome measurement and analysis not only provided novel information for future metabolic engineering strategies, but also served as a quality-control check for two critical assumptions: First, that genome annotation is error-free, and second, that mRNA expression directly correlates with protein expression and activity.

Most recently, in 2007, an updated genome-scale reconstructed network of *M. succiniciproducens* was presented that included 686 reactions and 519 metabolites based on re-annotation and validation experiments [163]. The refined reconstructed network, in conjunction with constraints-based flux analysis, was verified using comparative experimental data of the maximum specific growth rate and metabolic production formation rate for various MBEL55 mutants. In all simulation cases, the maximum specific growth rate was correctly predicted while the rate of succinic production, for a fixed glucose uptake rate, was in relatively good agreement (between 7.8 and 30.4%, depending on the genotype simulated *in vivo*). The model was further used to evaluate additional gene-deletion strategies likely to improve succinic acid production, and simulations were compared with strategies previously reported in genome-scale simulation of the *E. coli* reconstructed metabolic network [163, 164]. The comparative analysis of these genome-scale model simulation results suggested that the positive effect of various gene deletions on succinic acid production was more pronounced in *M. succiniciproducens* than in *E. coli*, and that the metabolic performance, defined as the absolute flux of succinic acid production, was higher in *M. succiniciproducens* because of the higher observed glucose consumption rate under anaerobic conditions [163].

In approximately 5 years (2002–2007), a previously unknown microbe, *M. succiniciproducens*, was transformed into a leading microbial cell factory candidate for succinic acid production as a result of the thorough application of systems biology tools: genome sequencing, genome-scale metabolic network reconstruction, fluxomics, proteomics, and subsequent model revision. It should be noted that similar approaches for *E. coli* and *A. succiniciproducens* have been reported; however, given the relative lack of a priori knowledge, short development time, and diversity of x-ome data collected and integrated, *M. succiniciproducens* remains a prominent example of successfully applied industrial systems biology.

2.8
Conclusion and Future Perspectives

Applying a mathematical framework to microbial metabolism, beginning in earnest as early as the 1930s, has provided a scaffold for large data sets, most recently associated with the emerging field of systems biology (transcriptomics, proteomics, fluxomics, metabolomics), to be integrated, interrogated, analyzed,

and ultimately, reformulated into predictive models referred to as genome-scale metabolic reconstructed networks. These networks, presently numbering 30 and growing, in conjunction with accessible and easily applied recombinant DNA technology, have offered metabolic engineers the ability to define genetic targets for redirection of carbon flux from renewable, sustainable, and cost-effective substrates to high added-value and commodity chemical production with clear and high probability of success. The construction of microbial cell factories to meet industrial biotechnology process development needs, previously relegated to classical methods of directed evolution, screening, selection, isolation, and propagation, are now being constructed faster and more efficiently through the use of systems biology toolboxes.

Here then, we define a new term, industrial systems biology, which includes the specific application of genome-scale technologies, both experimental and *in silico*, to industrial biotechnology process development. The impact of industrial systems biology is apparent over a broad cross-section of products, which may be classified as mature and developed (e.g., bioethanol), recently launched and rapidly growing (e.g., 1,3-propanediol), and in development (e.g., succinic acid).

It took the E.I. Du Pont de Nemours and Company more than 10 years of development and a large number of resources to ultimately and successfully commercialize industrial biotechnology production of 1,3-propanediol. The challenge for the field, and in particular metabolic engineers and industrial system biologists, is to develop similar cost-effective, scalable, sustainable, renewable, and robust processes with a fraction of the resources. So what are the largest areas for increased technology development likely to aid in reaching the aforementioned objective?

Although a large number of genome-scale metabolic network reconstructions are available, what is interesting to observe is the relatively poor coverage of microbial metabolism that these reconstructions offer. A close inspection of Table 2.4 reveals that all of the metabolic reconstructed networks combined have an average genome coverage of 14.6 ± 8.1% (n = 29). If *Saccharomyces cerevisiae*, the most well-characterized eukaryote, is isolated as an example, the most recent metabolic reconstructed network has a genome coverage of 13.6%, while 4691 of the 6608 total ORFs, 70.9%, have a verified function [16, 120]. From a more general perspective, the problem of metabolic gap closing is exacerbated by the relatively large number of orphan metabolic activities, where 30–40% of the known metabolic activities that are classified by the Enzyme Commission have no associated genomic sequences in any organism [111, 165, 166]. There is currently significant effort underway to extend pathway reconstructions to regions of metabolism that are poorly understood or that have been functionally neglected to a large degree [89, 111].

Industrial biotechnology has largely focused on the production of added-value and commodity chemicals; however, the largest expected growth sector is in the area of specialty and fine chemicals, where industrial biotechnology offers simpler routes for complex synthetic chemistry, or the possibility of *de novo* chemicals that may offer similar or enhanced application [4, 5]. Specialty and fine chemical

entities are typically present as metabolic intermediates in secondary and tertiary regions of metabolism, and are often poorly annotated and rarely included in genome-scale network reconstructions. A clear example is lipid metabolism in *S. cerevisiae*, where a recent update to the existing genome-scale metabolic reconstruction, iN795, included 118 previously unreported lipid reactions relative to iND750 (Table 2.4). Of those 118 lipid metabolism participating reactions, 28 were assigned to ergosterol esterification and lipid degradation – previously not represented (I. Nookaew and J. Nielsen, personal communication, 2008).

In addition to refined annotation and extension of metabolic models into uncharted metabolic pathways, the computational methods used for predictive simulations and model analysis are rapidly improving. For example, systematic evaluation of a diverse range of objective functions, including, maximization of biomass yield, maximization of ATP yield, minimization of the overall intracellular flux, maximization of ATP yield per flux unit, maximization of biomass yield per flux unit, minimization of glucose consumption rate, minimization of the number of reaction steps, minimization of the redox potential, minimization of ATP producing fluxes, maximization of ATP producing fluxes, and minimization of reaction steps, when compared with experimental *in vivo* ^{13}C-determined fluxes can provide insight to which optimization function best represents the metabolic network [167].

Yet another example employed a bi-level programming framework for identification of optimal gene deletions resulting in overproduction of a desired product by stoichiometrically including a drain towards biomass formation, thereby coupling production and biomass formation. This approach, called OptKnock, revealed non-intuitive metabolic engineering strategies for succinate, lactate, and 1,3-propanediol production, and in particular, provided strategies that lend themselves to improvement via directed evolution, where growth selection and adaptation are now directly linked to growth [164]. These, and other examples, are pushing the limits of high-value non-intuitive metabolic engineering strategies that may be deciphered from genome-scale reconstructed networks [168–178].

As with any mathematical framework that incorporates large collections of diverse biological data that are constantly being investigated, updated, re-annotated, re-analyzed, and debated, clear modeling objectives must be set forth. From an industrial biotechnology perspective, focused on identifying high-yield, robust, and easy to implement non-intuitive metabolic engineering strategies, microbial metabolic modeling must continue to expand upon constraint-based stoichiometric flux balance analysis that incorporates experimental verification, and subsequent model updating and expansion. Perhaps the emerging availability of kinetic parameters will enable fully dynamic metabolic reconstructions to be realized in the future, but for now, the full benefits of stoichiometric metabolic modeling have yet to be realized in constructing next generation microbial cell factories.

Industrial systems biology is a new approach to a challenge of epic proportions: how do we develop processes for production of chemicals, materials, and energy that are cost-effective, renewable, sustainable, scalable, and environmentally favorable?

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3
Fermentation Technology

Yusuf Chisti

3.1
Introduction

Fermentation processes use microorganisms to convert a substrate to some product. Conversions or biotransformations that are brought about *in vitro* through the action of cells of animals and plants are also thought of as fermentation processes. In view of the tremendous variety of possible substrates, microorganisms, and products, fermentation processes can be extremely diverse. Examples of the many different kinds of products produced by fermentation include the following: bread, cheese, wine, beer, coffee, medicinal and industrial enzymes, amino acids, antibiotics, soy sauce, compost, biopolymers, bioplastics, microbial oils, flavors, colorants, specialty chemicals, vaccines, therapeutic proteins, and numerous other products.

A fermentation is initiated by inoculating a substrate with the desired microorganism. The inoculated substrate is held under environmental conditions that favor its conversion to the desired product. The crude product may be used directly, or it may be processed further to isolate specific molecular entities from it.

3.2
Types of Fermentations

Most commercially useful fermentations may be classified as either solid-state or submerged cultures. In solid-state fermentations, the microorganisms grow on a moist solid with little or no "free" water, although capillary water may be present [1]. Examples of solid-substrate fermentations are seen in the making of cheese, bread, coffee, and compost [1]. Submerged fermentations [2, 3] may use a dissolved substrate (e.g., sugar solution) or a solid substrate suspended in a large amount of water to form a slurry. Submerged fermentations are used in producing penicillin, recombinant insulin, beer, and many other products.

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Solid-state and submerged fermentations may each be further subdivided into oxygen-requiring aerobic processes and anaerobic processes that must be conducted in the absence of oxygen. Examples of aerobic submerged fermentations include production of the antibiotic penicillin using the fungus *Penicillium chrysogenum*. Fermented meat products such as pepperoni and salami are produced by anaerobic solid-state fermentations. A submerged anaerobic fermentation occurs in yogurt making.

Fermentations may require only a single microbial species to effect the desired biochemical change, that is they may be monoseptic. For monoseptic fermentations the substrate must be sterilized to kill unwanted microorganisms prior to inoculation with the desired species. Monoseptic fermentations or monocultures are used to produce many pharmaceutical products such as insulin. Many food fermentations and biological waste treatment processes require the participation of several microbial species, or mixed cultures, acting simultaneously and/or sequentially.

3.3 Fermentation Process

Industrial fermentations may be carried out either batchwise, as fed-batch operations, or as continuous cultures (Figure 3.1) [2]. Batch and fed-batch operations are the most common; continuous fermentation is relatively rare. Treatment of wastewater by the activated sludge method is generally carried out as a continuous

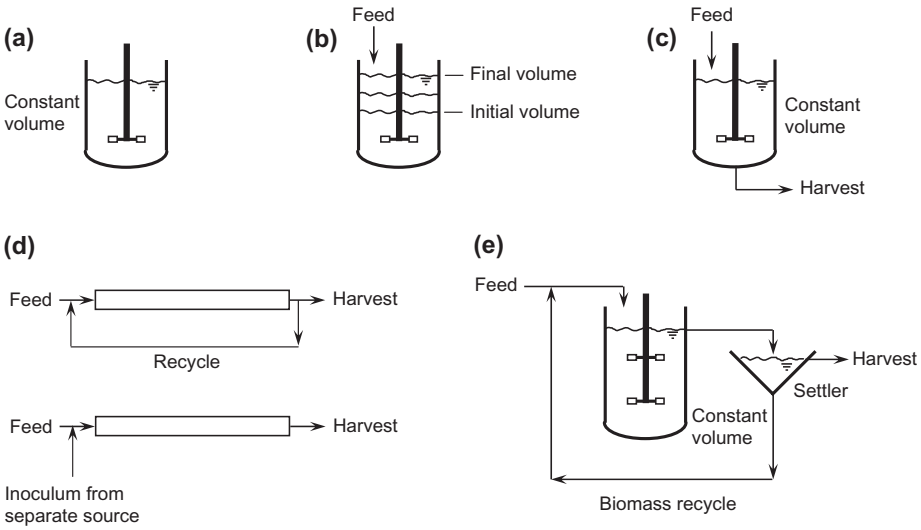

Figure 3.1 Fermentation methodologies. (a) Batch fermentation; (b) fed-batch culture; (c) continuous flow well mixed fermentation; (d) continuous plug flow fermentation with and without recycle; (e) well-mixed continuous fermentation with recycle of concentrated biomass.

fermentation. Brewing of beer and most antibiotic fermentations are conducted in batch or fed-batch modes of operation.

In batch processing (Figure 3.1a), a batch of culture medium in the fermenter is inoculated with a microbial culture, or the "starter culture." The fermentation proceeds for a certain duration (the "batch time" or "fermentation time") and the product is harvested. Batch fermentations typically extend over 4–5 days, but some traditional food fermentations may last for months. In fed-batch fermentations, sterile culture medium is added either continuously or periodically to the inoculated fermentation batch (Figure 3.1b). The volume of the fermenting broth increases with each addition of the medium. The batch is harvested after the batch time. The composition of the feeding medium may vary with time.

In continuous fermentations, sterile medium is fed continuously into a fermenter and the fermented product is continuously withdrawn, so the fermentation volume remains unchanged (Figure 3.1c). Typically, continuous fermentations are started as batch cultures and feeding begins after the microbial population has reached a certain concentration. In some continuous fermentations, a small part of harvested culture may be recycled, to continuously inoculate the sterile feed medium entering the fermentation (Figure 3.1d). Whether continuous inoculation is necessary depends on the type of mixing in the fermenter. "Plug flow" fermentation devices such as long tubes that do not allow back mixing, must be inoculated continuously. Elements of the fluid moving along in a plug flow device behave like tiny batch fermenters. Hence, true batch processes are relatively easily transformed into continuous operations in plug flow fermenters, especially if pH control and aeration are not required. Continuous cultures are particularly susceptible to microbial contamination, but in some cases the fermentation conditions may be selected (e.g., low pH, high alcohol, or salt content) to favor the desired microorganisms instead of any potential contaminants.

In a "well-mixed" continuous fermenter (Figure 3.1c), the feed rate of the medium should be such that the dilution rate, that is, the ratio of the volumetric feed rate to the constant culture volume, remains less than the maximum specific growth rate of the microorganism in the particular medium and at the particular fermentation conditions. If the dilution rate exceeds the maximum specific growth rate, the microorganism will be washed out of the fermenter. In some well-mixed continuous fermentations a part of the biomass in the harvest stream is concentrated and recycled to the fermentation vessel (Figure 3.1e). Biomass recycle enables the dilution rate to be increased to above the value that would result in washout in the absence of biomass recycle. A high dilution rate increases the throughput of the fermenter. Continuous well-mixed fermentation with biomass recycle is used commonly in wastewater treatment by the activated sludge method.

3.3.1 Inoculum Generation

Industrial fermentations are mostly batch operations. Typically, a pure starter culture (or seed), maintained under carefully controlled conditions, is used to

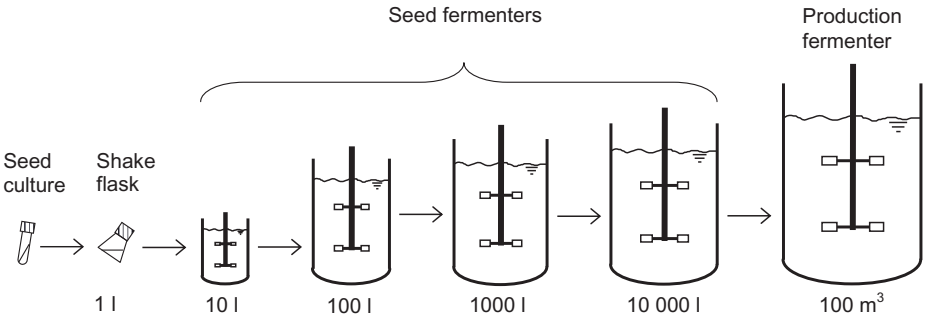

Figure 3.2 Generation of inoculum for a large production fermenter.

inoculate sterile Petri dishes or liquid medium in shake flasks. After sufficient growth, the preculture is used to inoculate the "seed" fermenter. Because industrial fermenters can be quite large (e.g., 150–250 m^3), the inoculum is built up through several successive stages, to 5–10% of the working volume of the production fermenter. This strategy ensures optimal use of the production fermenter by minimizing the batch time in this vessel. A train for producing inoculum for a 100 m^3 production fermenter is shown in Figure 3.2. An excessively long fermentation time (or batch time) reduces productivity (i.e., the amount of product formed per unit time per unit volume of fermenter), and increases costs. Sometimes inoculation spores, produced as seeds, are blown directly into a large fermenter with the ingoing air. In animal cell culture, the inoculum size is generally selected to give an initial cell count of $2–4 \times 10^5$ cells/ml.

3.3.2 Growth and Product Formation

Microbial growth in a newly inoculated batch fermenter typically follows the pattern shown in Figure 3.3. Initially, in the lag phase, the cell concentration does not increase very much. The length of the lag phase depends on the growth history of the inoculum, the composition of the medium, and the amount of culture used for inoculation [3]. An excessively long lag phase ties up the fermenter unproductively, therefore the length of the lag phase should be minimized. Short lag phases occur when: the concentration of the medium and the environmental conditions in the seed culture and production vessel are identical (hence less time is needed for adaptation); the dilution shock is small (i.e., a large amount of inoculum is used); and the cells in the inoculum are in the late exponential phase of growth [3]. The lag phase is essentially an adaptation period in a new environment. The lag phase is followed by exponential growth, during which the cell mass increases exponentially. Eventually, as nutrients are exhausted and inhibitory products of metabolism build up, the culture enters a stationary phase. Ultimately, starvation causes cell death and lysis, and hence the biomass concentration declines.

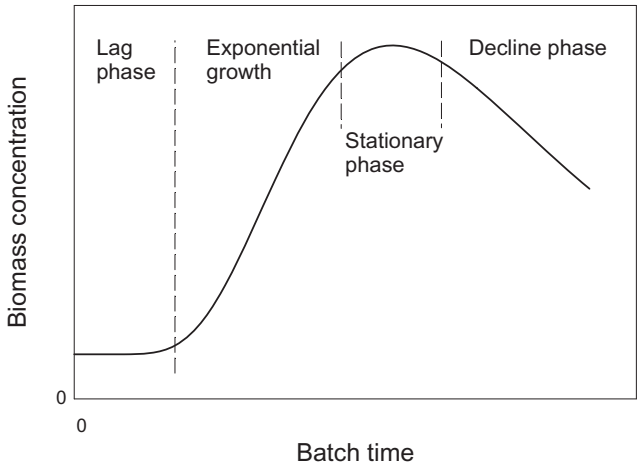

Figure 3.3 Typical growth profile of microorganisms in a submerged batch culture.

Table 3.1 Typical doubling times.

Cell type	t_d (min)
Bacteria	20–45
Yeasts	90
Molds	160
Protozoa	360
Hybridomas	630–1260
Plant cells	3600–6600

During exponential growth, the time required by the biomass to double (i.e., doubling time, t_d) depends on the specific growth rate (i.e., growth rate per unit cell mass, μ), as follows:

$$t_d = \frac{\ln 2}{\mu} \tag{3.1}$$

Typical doubling times for various kinds of microorganisms and cells are shown in Table 3.1 [3]. Bacteria generally grow faster than yeasts, and yeast multiply faster than molds. The maximum biomass concentration in submerged microbial batch fermentations is typically 40–50 kg/m^3 [2]. The maximum final cell concentration in batch suspension culture of animal cells tends to be about 4×10^6 cells/ml, or lower.

An excessively high substrate concentration can limit growth, for instance by lowering the water activity. Moreover, certain substrates inhibit product formation and, in yet other cases, a fermentation product may inhibit biomass growth. For

example, ethanol produced in the fermentation of sugar by yeast can be inhibitory cells. Multiple lag phases (or diauxic growth) are sometimes seen when two or more growth-supporting substrates are available [3]. As the preferentially utilized substrate is exhausted, the cells enter a lag phase while the biochemical machinery needed for metabolizing the second substrate is developed. Growth then resumes.

Microbial products may be classified into primary and secondary metabolites. Primary metabolites are products that are essential to survival of microbial cells. Synthesis of primary metabolites is usually directly related with the rate of biomass growth. Citric acid and amino acids are examples of primary metabolite. Secondary metabolites are products that are non-essential to survival but serve various useful functions. Examples of secondary metabolites include most antibiotics. Often secondary metabolites are produced after the growth has ceased. The conditions that favor growth may be quite different to conditions that favor the production of a secondary metabolite. Understanding the relationship between biomass growth and formation of the product, is essential to successful conduct of fermentations.

3.4 Fermentation Medium Design

A well-designed fermentation medium must be inexpensive, readily available, of a consistent quality, and must have all the chemical elements that are required to produce a given quantity of biomass and metabolites. Often, the media contain an excess of nutrients. Microorganisms and cells have the general formula of $CH_xN_yO_z$. Values of the variables x, y, and z for common types of cells are shown in Table 3.2 [3]. Therefore, to support growth and product formation, a fermentation medium must have sources of carbon, nitrogen, and oxygen. In most cases, oxygen is supplied by continuous aeration of the fermentation broth. Carbon is generally provided in the form of sugars or starch. Nitrogen may be provided in the form of inorganic salts or as organic compounds that contain nitrogen (e.g., protein hydrolysate). A medium may require micronutrients such as vitamins. Trace amounts of elements such as phosphorus, iron, zinc, and sulfur are required.

A fermentation medium may be "defined," that is, its chemical composition is fully known. Alternatively, fermentation media may be "complex", or without a precisely known composition. Media based on complex natural products such as molasses and yeast extract are often not fully defined in terms of the specific

Table 3.2 Values of the variables in $CH_xN_yO_z$.

Cell type	x	y	z
Yeasts and bacteria	1.65–1.85	0.12–0.25	0.26–0.56
Cultured plant cells	1.60–2.00	0.11–0.17	0.62–0.83

compounds they contain. Complex media are used in many industrial fermentations because they are generally less expensive than defined media. Defined media are used in many animal cell culture processes.

Media for obtaining optimal growth and product formation may be different. Production of certain metabolites requires the presence of specific precursor compounds in the medium.

3.5 Sterilization of Air and Fermentation Medium

In most industrial fermentations, the medium and any air that is used for supplying oxygen must be sterilized to prevent contamination with unwanted microorganisms. Air is invariably sterilized by filtering through hydrophobic membrane filters [3, 4]. Membrane filters that retain particles as small as 0.1 μm, or essentially all microorganisms, are available.

The culture medium may be sterilized together with the fermenter, or a separately sterilized batch of medium may be filled in a presterilized fermenter. Fermenter and media are commonly sterilized by heating to 121 °C and holding at this temperature for 20–30 min. Heat-sensitive media that do not contain any suspended solids are sterilized by filtration through absolute rated hydrophilic membrane filters. Media that are used to culture animal cells are commonly sterilized by membrane microfiltration. In some microbial fermentations, a part of the medium may be sterilized by heat and the heat-sensitive components are then sterilized separately by filtration. Water and salt solutions are inexpensively sterilized by microfiltration.

Sterilizing a large volume of medium as a batch in the fermenter is time consuming as heating and cooling processes are slow. To get around this, a continuous sterilization operation is often used (Figure 3.4) [3]. In continuous sterilization, the raw medium is preheated from room temperature to some higher

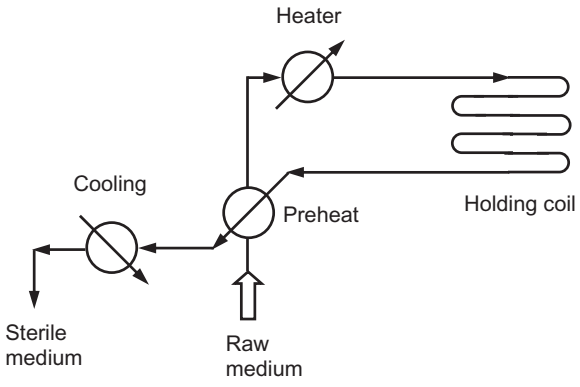

Figure 3.4 Continuous sterilization of fermentation medium.

temperature. The preheated medium then flows through a heater that raises the temperature further to around 150 °C. The hot medium then moves through a "holding coil" that ensures that the medium is held at 150 °C for a few seconds (e.g., 2–4 s). As the hot sterilized medium exits the holding coil, the heat in it is transferred through a metal wall to the incoming raw medium in the preheater (Figure 3.4). The hot medium is cooled further to fermentation temperature and flows directly into the fermenter that has been presterilized empty.

3.6 Environmental Factors

A fermentation is influenced by numerous factors, including temperature, pH, ionic strength, nature and composition of the medium, dissolved oxygen, dissolved carbon dioxide, and shear rates [5] in the fermenter. The method of operation (e.g., batch, fed-batch, continuous, precursor feeding) and mixing (cycling through varying environments) also influence fermentations [2]. Variations in these factors may affect: the rate of fermentation; the product spectrum and yield; the organoleptic properties of the product (appearance, taste, smell, and texture); the generation of toxins [6]; nutritional quality and other physico-chemical properties. Many industrial fermentations are carried out under highly controlled conditions of temperature, pH, dissolved oxygen, and other possible factors. In contrast, traditional food fermentations are often conducted with minimal control. The specific conditions required in a fermentation depend on the nature of the microorganism, the nature of the substrate and the characteristics of the desired product.

Oxygen requirements of a fermentation depend on the microbial species, the concentration of cells, and the type of substrate [2, 3]. Oxygen supply must at least equal the oxygen demand or the fermentation will be oxygen limited. Oxygen demand is especially difficult to meet in viscous fermentation broths and in broths containing a large concentration of the oxygen-consuming cells. As a general guide, oxygen supply capability of a fermenter depends on the aeration rate, the agitation intensity, and the properties of the culture broth. In large fermenters, oxygen transfer becomes difficult when demand exceeds 4–5 kg/m^3 per h [2].

Below some critical concentration of dissolved oxygen, the amount of oxygen limits microbial growth. The critical dissolved oxygen level depends on the microorganism, the culture temperature, and the substrate being oxidized. The higher the critical dissolved oxygen value, the greater the likelihood that oxygen transfer would become limiting. Under typically used culture conditions, fungi such as *P. chrysogenum* and *Aspergillus oryzae* have a critical dissolved oxygen value of about 3.2×10^{-4} kg/m^3 [2]. For baker's yeast and *Escherichia coli*, the critical dissolved oxygen values are 6.4×10^{-5} and 12.8×10^{-5} kg/m^3, respectively [2].

All fermentations generate heat. In submerged cultures, 3–15 kW/m^3 of the heat output typically comes from microbial activity [3]. In addition, mechanical agitation of the broth produces up to 15 kW/m^3 [3]. Consequently, a fermenter must be cooled to prevent temperature rise and damage to culture. Heat removal tends

to be difficult because, typically, the temperature of the cooling water is only a few degrees lower than that of the fermentation broth. Industrial fermentations are commonly limited by the heat transfer capability. The ability to remove heat depends on the surface area available for heat exchange, the temperature difference between the broth and the cooling water, the properties of the broth and the coolant, and the turbulence in these fluids. The geometry of the fermenter determines the heat exchange area that can be provided. Because metabolic heat generation depends on the oxygen consumption rate, heat removal in large vessels becomes difficult as oxygen consumption rate approaches 5 kg/m^3 per h [2].

Compared with submerged culture, biomass levels in solid state fermentations are lower at 10–30 kg/m^3 [1, 2]. Nevertheless, because there is little water, the heat generation per unit fermenting mass tends to be much greater in solid state fermentations than in submerged culture [1, 2]. Temperature can rise rapidly because there is little water to absorb the heat. Cumulative metabolic heat generation in koji fermentations for a variety of products has been noted at 419–2387 kJ/kg solids [1]. Koji fermentations are solid-state fermentations of steamed soybeans or grain. These fermentations are widely used in making Asiatic fermented products such as soy sauce and saké. Higher values, up to 13 398 kJ/kg, have been observed during composting. Peak heat generation rates in koji processes range over 71–159 kJ/kg per h, but the average rates are more moderate at 25–67 kJ/kg per h. Methods for temperature control of solid-state fermentations are discussed elsewhere [1].

3.7 Fermentation Kinetics

3.7.1 Batch Fermentation

During exponential growth in a well-mixed batch fermenter, the biomass concentration increases with time as follows:

$$\frac{dX}{dt} = \mu X \tag{3.2}$$

where X is the biomass concentration at time t and μ is the growth rate constant, or specific growth rate. The biomass concentration at any time can be calculated by integrating Equation 3.2; thus,

$$\int_{X_o}^{X} \frac{dX}{X} = \mu \int_{0}^{t} dt \tag{3.3}$$

or

$$\ln \frac{X}{X_o} = \mu t \tag{3.4}$$

where X_o is the biomass concentration at the beginning of exponential growth. The specific growth rate μ can be determined as the slope of a plot of the left-hand side of Equation 3.4 against time. For exponential growth, the time to double the biomass can be estimated by Equation 3.1. Batch cultures are relatively easy to carry out aseptically, but they cannot maintain a high growth rate for long because of depletion of nutrients and accumulation of waste products.

3.7.2 Continuous Culture

Continuous culture may be carried out in a well-mixed fermenter or a plug flow fermenter. Continuous cultures are fed and harvested continuously. The volume of the broth in the fermenter remains constant as the rates of feeding and harvest are identical. A constant culture environment is maintained. This allows for a high and constant rate of production over extended periods.

In a well-mixed continuous culture of volume V_L, fed at a constant volume flow rate Q with a feed that has a fixed composition, the biomass balance on the fermenter is as follows:

$$\underbrace{V_L \frac{dX}{dt}}_{\substack{\text{Change in total}\\ \text{amount of}\\ \text{biomass in}\\ \text{reactor}}} = \underbrace{QX_o}_{\substack{\text{Inflow}\\ \text{of biomass}}} - \underbrace{QX}_{\substack{\text{Outflow}\\ \text{of biomass}}} + \underbrace{V_L \mu X}_{\substack{\text{Increase in}\\ \text{biomass}\\ \text{by growth}}} \tag{3.5}$$

In Equation 3.5, X_o is the biomass concentration in feed, μ is the specific growth rate and X is the biomass concentration in the well-mixed fermenter. The feed does not normally contain any biomass, that is, X_o is zero, except at start-up. Equation 3.5 can be rearranged to the following:

$$\frac{dX}{dt} = \frac{Q}{V_L}(X_o - X) + \mu X \tag{3.6}$$

At steady state, the biomass concentration in the fermenter does not change, that is,

$$\frac{dX}{dt} = 0 \tag{3.7}$$

and Equation 3.6 becomes

$$\frac{Q}{V_L}(X_o - X) = -\mu X \tag{3.8}$$

The ratio of feed flow rate Q and the volume of the broth V_L in the fermenter is known as the dilution rate, D. When $X_o = 0$, the usual case, Equation 3.8 simplifies to:

$$D = \mu \tag{3.9}$$

Hence, in any well-mixed continuous culture operating at steady state with a finite biomass concentration in the fermenter, the dilution rate necessarily equals the specific growth rate. If the dilution rate exceeds the maximum specific growth rate for the microorganism, the culture will be washed out, that is, no biomass could be grown in steady-state operation.

In a plug flow fermenter, so long as the conditions are such that the specific growth rate remains constant, the biomass concentration X_e at the exit of the fermenter depends on dilution rate D, as follows:

$$X_e = X_o \exp\left(\frac{\mu}{D}\right) \tag{3.10}$$

where X_o is the biomass concentration at the inlet of the fermenter, μ is the specific growth rate and D is the dilution rate. Clearly, a plug flow fermenter needs to be inoculated continuously (i.e., $X_o > 0$), or no biomass will be produced.

3.8 Fermentation Equipment

3.8.1 Submerged Fermentation

Submerged fermentation equipment is commonly designed for monoseptic cultures and is capable of being sterilized by steam under pressure [3, 4]. The major types of submerged fermenters are the stirred tank fermenter, bubble column, airlift fermenter, fluidized-bed fermenter, and trickle-bed fermenter (Figure 3.5). These are explained below.

3.8.1.1 Stirred Tank Fermenter

Stirred tank fermenter [3] is one of the most commonly used types because of its flexibility. It consists of a cylindrical vessel with a working height-to-diameter ratio (aspect ratio) of 3–4 with a central shaft that supports 3–4 impellers placed about 1 impeller-diameter apart (Figure 3.5a). Various types of impellers that direct the flow axially (parallel to the shaft) or radially (outward from the shaft) may be used. Sometimes axial- and radial-flow impellers are used on the same shaft. The vessel is provided with four equally spaced vertical baffles that extend from near the walls into the vessel. Typically, the baffle width is 8–10% of the vessel diameter.

3.8.1.2 Bubble Column

This consists of a cylindrical vessel with a working aspect ratio of 4–6 (Figure 3.5b). It is sparged at the bottom and compressed gas provides agitation. Although simple, it is not widely used because of poor performance relative to other systems [2, 3]. It is not suited to very viscous broths or those containing large amounts of solids [2, 3].

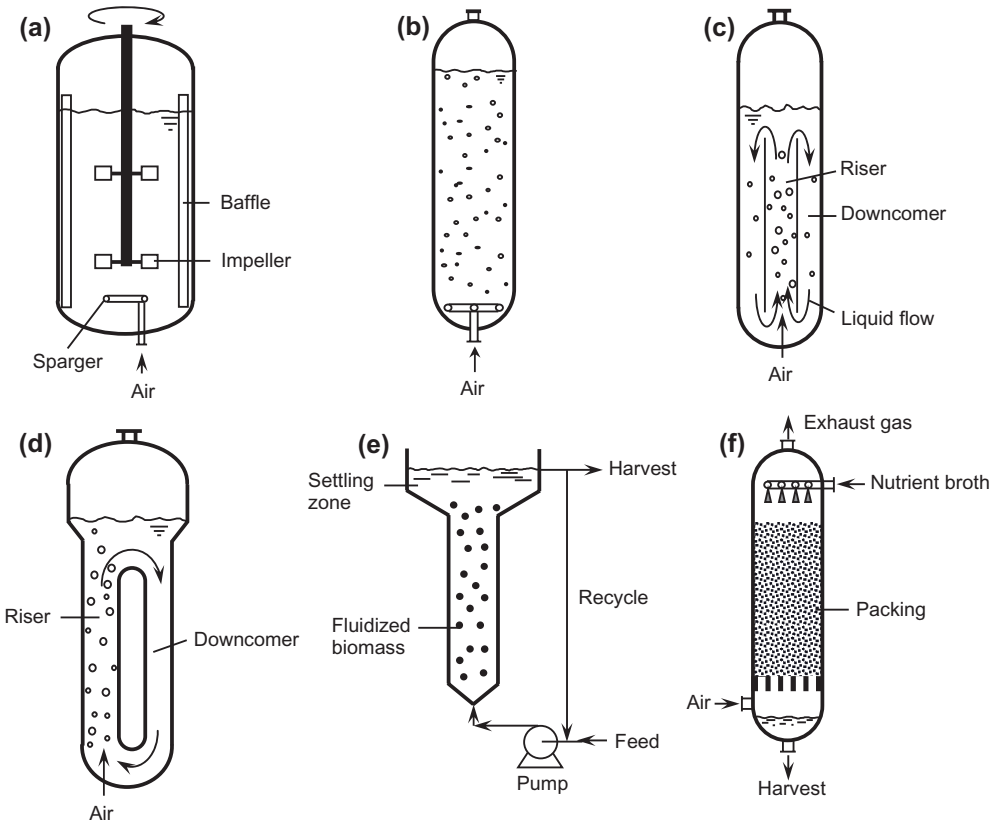

Figure 3.5 Fermenters for submerged culture. (a) Stirred tank fermenter; (b) bubble column; (c) internal-loop airlift fermenter; (d) external-loop airlift fermenter; (e) fluidized bed fermenter; (f) trickle-bed fermenter.

3.8.1.3 **Airlift Fermenters**

These come in internal-loop (Figure 3.5c) and external-loop (Figure 3.5d) designs [2, 3]. In the internal-loop configuration the aerated riser and the unaerated downcomer are contained in the same shell. In the external-loop configuration, the riser and the downcomer are separate tubes that are linked near the top and the bottom. Liquid circulates between the riser (upflow) and the downcomer (downflow). The working aspect ratio of airlift fermenters is six or greater. In general, these are very capable fermenters, except for handling the most viscous broths. Their ability to suspend solids, transfer oxygen and heat is good. The hydrodynamic shear is low. The external-loop design is relatively little used in industry.

3.8.1.4 **Fluidized Bed Fermenter**

These are similar to bubble columns with an expanded cross-section near the top (Figure 3.5e). Fresh or recirculated liquid is continuously pumped into the bottom

of the vessel at a velocity that is sufficient to fluidize the solids or maintain them in suspension. Fluidized beds need an external pump. The expanded top section slows the local velocity of the upward flow such that the solids are not washed out of the bioreactor.

3.8.1.5 **Trickle-Bed Fermenter**

These consist of a cylindrical vessel that is packed with support material (e.g., woodchips, rocks, plastic structures) (Figure 3.5f). The support has large open spaces, for the flow of liquid and gas. The microorganisms grow attached to the solid support. A liquid nutrient broth is sprayed onto the top and trickles down the bed. Air may flow up the bed, countercurrent to liquid flow. These fermenters are used in vinegar production, as well as other processes. They are suitable for liquid with low viscosity and few suspended solids [2].

All fermenters or bioreactors for monoseptic submerged culture have certain common features, as shown in Figure 3.6 [7]. The fermenter vessel is provided with side ports for pH, temperature, and dissolved oxygen sensors. Retractable sensors that can be replaced during operation are used commonly. Connections for acid and alkali (for pH control), antifoam agents, and inoculum are located above the broth level in the reactor vessel. The liquid level can be easily seen through a vertical sight glass located on the vessel's side (Figure 3.6). A second sight glass is located on the top of the vessel and an externally mounted light can be used to illuminate the inside of the bioreactor. The sight glass on top can be internally cleaned by a jet of steam condensate. The vessel may be placed on a load cell to obtain a precise indication of the amount of material it contains.

When mechanical agitation is used, either a top or bottom entering agitator may be employed. The bottom entry design is more common and it permits the use of a shorter agitator shaft, often eliminating the need for support bearings inside the vessel. The shaft of the agitator is provided with steam sterilizable single or double mechanical seals [7]. Seals prevent leakage from the fermentation vessel and ingress of potential contaminants from the environment. Double seals are preferred, but they require lubrication with cooled clean steam condensate, or other sterile fluid. Alternatively, when torque limitations allow, magnetically coupled agitators may be used thereby eliminating the mechanical seals [7].

An air (or other gas mixture) sparger supplies oxygen (and sometimes carbon dioxide or ammonia for pH control) to the culture. Aeration of fermentation broth generates foam. Typically, 20–30% of the fermenter volume must be left empty to accommodate the foam and allow for gas disengagement [7]. Foaming in bioreactors is controlled by a combination of chemical and mechanical methods. Chemical antifoaming agents are commonly mixed with the broth at initiation of fermentation. Further additions of antifoam agent are made from time to time, as needed. Typical antifoams are silicone oils, vegetable oils, and substances based on low molecular weight poly(propylene glycol) or poly(ethylene glycol). Emulsified antifoams are more effective because they disperse better in the fermenter.

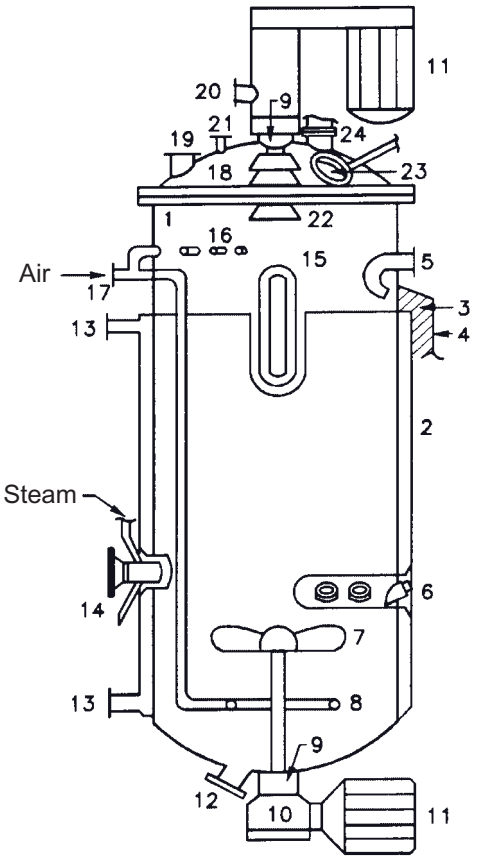

Figure 3.6 A typical submerged culture fermenter: (1) reactor vessel; (2) jacket; (3) insulation; (4) protective shroud; (5) inoculum connection; (6) ports for pH, temperature and dissolved oxygen sensors; (7) agitator; (8) gas sparger; (9) mechanical seals; (10) reducing gearbox; (11) motor; (12) harvest nozzle; (13) jacket connections; (14) sample valve with steam connection; (15) sight glass; (16) connections for acid, alkali, and antifoam agents; (17) air inlet; (18) removable top; (19) medium feed nozzle; (20) air exhaust nozzle (connects to condenser, not shown); (21) instrumentation ports for foam sensor, pressure gage, and other devices; (22) centrifugal foam breaker; (23) sight glass with light (not shown) and steam connection; (24) rupture disc nozzle.

Excessive use of antifoams may interfere with some downstream separations such as membrane filtrations. Hydrophobic silicone antifoams are particularly troublesome, as they foul membrane filters and chromatography media [3]. The use of antifoam chemicals is minimized by combining it with mechanical breakage of foam. A mechanical "foam breaker" may be installed in the headspace of the fermenter, as shown in Figure 3.6 [7]. The device in Figure 3.6 separates the foam – a dispersion of gas in liquid – into its components by centrifugal action, as explained in Figure 3.7. The operation of the foam breaker and the addition

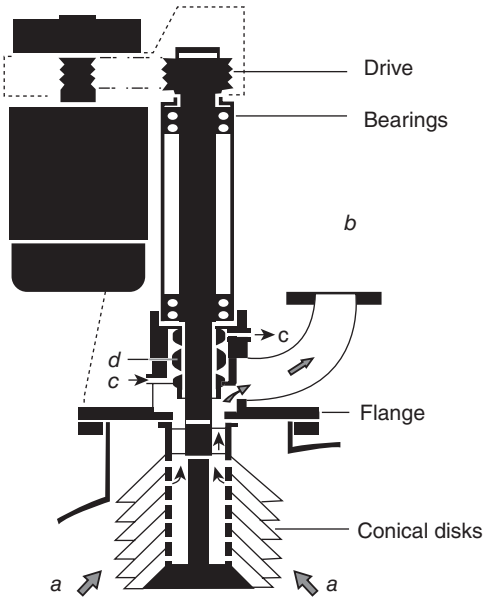

Figure 3.7 A mechanical foam breaker. The motor, drive, and shaft assembly are used to rotate the stack of conical discs at a high speed. The foam enters the spaces between the rotating discs at *a* and is separated into gas and liquid by the centrifugal force. The liquid spins into the bioreactor and liquid-free gas exhausts through the nozzle *b*. The mechanical seal *d* prevents leakage into and out of the sterile bioreactor. The seal is lubricated by sterile cooling water *c*.

of antifoam chemicals are controlled by signals from a foam sensor that extends into the bioreactor from the top. The shaft of the high speed mechanical foam breaker must also be sealed using double mechanical seals as explained for the agitator.

In most instances, the bioreactor is designed for a maximum allowable working pressure of 3.78–4.10 bar (absolute pressure) at a design temperature of 150–180 °C [7]. The vessel is designed to withstand full vacuum. In North America the design conforms to the American Society of Mechanical Engineers (ASME), Section VIII, Division 1, Boiler and Pressure Vessel Code. Other codes may be acceptable in other locations. The reactor can be sterilized in-place using saturated clean steam at a minimum pressure of 2.1 bar (absolute pressure). Overpressure protection is provided by a rupture disk located on top of the bioreactor. The rupture disk is piped to a contained drain. Usually a graphite burst disk is used because it does not crack or develop pinholes without failing completely. Other items located on the head plate of the vessel are nozzles for media or feed addition and for sensors (e.g., the foam electrode), and instruments (e.g., the pressure gage).

The vessel is designed to drain completely and a harvest nozzle is located at the lowest point on the reactor vessel (Figure 3.6). The reactor is either provided with a manhole, or the top is removable. Flat head plates are commonly used in smaller

vessels, but a domed construction of the head is less expensive for larger bioreactors (Figure 3.6) [7].

The bioreactor vessel should have few internals; the design should take into account the clean-in-place [7–9] and sterilization-in-place needs. There should be a minimum number of ports, nozzles, connections, and other attachments consistent with the current and anticipated future needs of the process. The bioreactor should be free of crevices and stagnant areas where pockets of liquids and solids may accumulate. Attention to design of such apparently minor items as the gasket grooves is important [7]. Easy-to-clean channels with rounded edges are preferred. As far as possible, welded joints should be used in preference to sanitary couplings. Steam connections should allow for complete displacement of all air pockets in the vessel and associated pipework, for sterilization. Even the exterior of a bioprocess plant should be cleanly designed with smooth contours, minimum bare threads, and so forth.

The reactor vessel is invariably jacketed. In the absence of special requirements, the jacket is designed to the same specifications as the vessel [7]. The jacket is covered with chloride-free fiberglass insulation which is fully enclosed in a protective shroud as shown in Figure 3.6. The jacket is provided with overpressure protection through a relief valve located on the jacket or its associated piping [7].

For the great majority of applications, austenitic stainless steels are the preferred material of construction for bioreactors [7]. The bioreactor vessel is usually made in Type 316L stainless steel, while the less expensive Type 304 (or 304L) is used for the jacket, the insulation shroud, and other non-product contacting surfaces. The L grades of stainless steel contain less than 0.03% carbon, which reduces chromium carbide formation during welding and lowers the potential for later intergranular corrosion at the welds [7]. The welds on internal parts should be ground flush with the internal surface and polished. Welds are difficult to notice in high-quality construction.

In addition to the materials of construction, the surface finish also requires attention. The finish on surfaces which come in contact with the product material and, to some extent, the finish on external surfaces affects the ability to clean, sanitize, and sterilize the bioreactor and the general processing area [7]. The surface finish has implications on stability and reactivity of the surface, and it may have process implications relating to microbial or animal cell adhesion to surfaces.

The mill finished surface of stainless steel sheet is unsatisfactory for use in bioreactors [7]. Minimally, the surface should receive a mechanical polish. Mechanical polish is achieved by abrasive action of a sandpaper type material on metal. The surface finish may be specified by grit number, for example, 240 grit polish, which refers to the quantity of particles per square inch of the abrasive pad [7]. The higher the grit number, the smoother the finish. More quantitative measures of surface finish rely on direct measurement of roughness in terms of "arithmetic mean roughness," Ra, or "root mean square roughness." Microscopic examination of even a highly smooth mechanically polished surface reveals a typical pattern of grooves and ridges that provide sites for microbial attachment. For example, a 320

grit polished surface will have an Ra of the order of 0.23–0.30 μm. Hence, for internal surfaces of bioreactors, an electropolished surface is preferable to mechanical polish alone [7].

Electropolishing is an electrolytic process which preferentially removes the sharp microscopic surface projections arising from mechanical polishing; the result is a much smoother finish [7]. Electropolishing significantly reduces the metal surface area and, hence, the product–metal contact area. The treatment imparts corrosion resistance to stainless steel by removing microscopic regions of high local stress; it creates a passivated steel surface, rich in protective chromium oxide [7]. To attain a suitable electropolished finish, the surface should be previously mechanically polished; however, there is little advantage to starting with a much better than 220 grit (Ra ≈0.4–0.5 μm) polished surface.

If mechanical polish alone must be used, it should be at least 240 grit, and the direction of polish should be controlled to produce a vertical grain for good drainage [7]. The surface should receive a nitric acid wash treatment as a minimum. The orientation of the grain does not seem to be of consequence if the surface is to be electropolished.

3.8.2 Solid-State Fermentation

Solid-state fermentation devices [1, 2] vary in technical sophistication from the very primitive banana leaf wrappings, bamboo baskets, and substrate heaps to the highly automated machines used mainly in Japan. Some "less sophisticated" fermentation systems – for example, fermentation of cocoa beans in heaps – are quite effective in large-scale processing. In contrast, some of the continuous, highly mechanized, soy sauce fermentation processes that have proven successful in Japan are not suited to less-developed locations in Asia. Some commonly used solid-state fermenters are shown in Figures 3.8–3.13. Solid-state fermenters are discussed below.

3.8.2.1 Tray Fermenter

These are simple and widely used in small- and medium-scale koji operations of Asia [1, 2]. Trays are made of wood, metal, or plastic, often with perforated or wire mesh bottom for improved aeration. Substrate is fermented in shallow (≤0.15 m deep) layers. Trays may be covered with cheese cloth to reduce contamination.

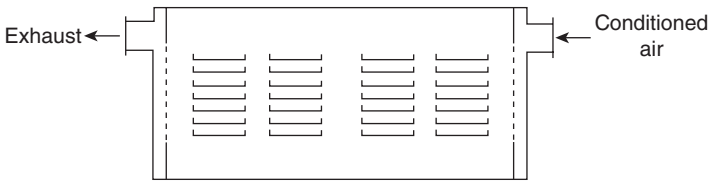

Figure 3.8 Tray fermenter.

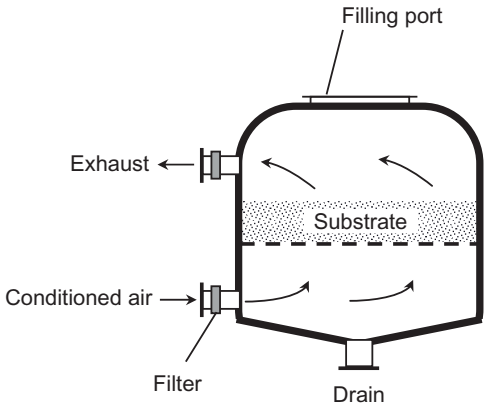

Figure 3.9 Static-bed fermenter.

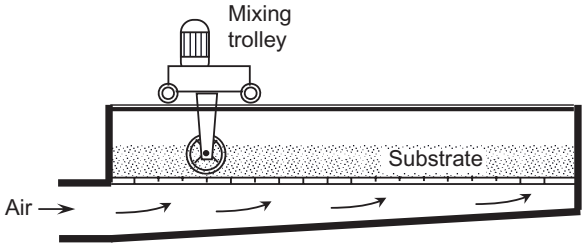

Figure 3.10 Tunnel fermenter.

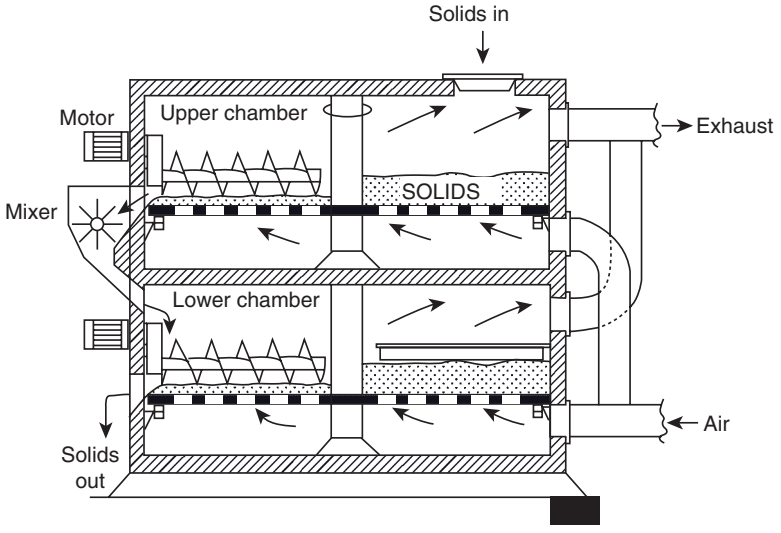

Figure 3.11 Rotary disk fermenter.

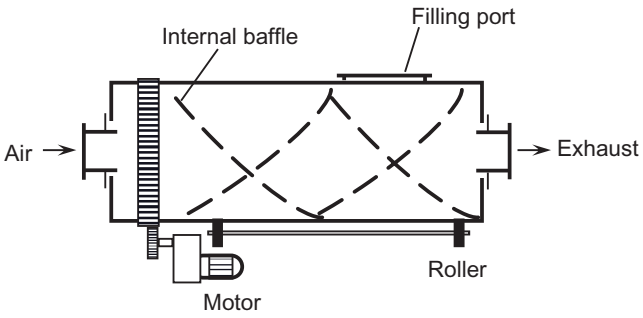

Figure 3.12 Rotary drum fermenter.

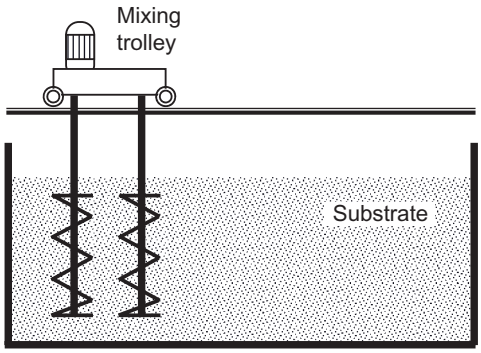

Figure 3.13 Agitated tank fermenter.

Processing is non-sterile. Single or stacked trays may be located in temperature and humidity controlled chambers (Figure 3.8), or simply in ventilated areas [1, 2]. Inoculation and occasional mixing are done manually. Sometimes, tray handling, filling, emptying, and washing may be automated. Despite some automation, tray fermenters are labor intensive, and require a large area. Tray fermenters have a limited scalability.

3.8.2.2 **Static Bed Fermenter**

This is an adaptation of the tray fermenter. It employs a single, larger and deeper, static bed of substrate located in an insulated chamber (Figure 3.9) [1, 2]. Oxygen is supplied by forced aeration through the bed of substrate.

3.8.2.3 **Tunnel Fermenter**

This is an adaptation of the static bed device. Typically, the bed of solids is quite long, but normally no deeper than 0.5 m (Figure 3.10) [1, 2]. Tunnel fermenters may be highly automated with mechanisms for mixing, inoculation, continuous feeding, and harvest of substrate [1, 2].

3.8.2.4 Rotary Disk Fermenter

The rotary disk fermenter [1, 2] consists of upper and lower chambers, each with a circular perforated disk to support the bed of substrate (Figure 3.11). A common central shaft rotates the disks. Inoculated substrate is introduced in the upper chamber, and slowly moved to the transfer screw. The upper screw transfers the partly fermented solids through a mixer, to the lower chamber where further fermentation occurs. Fermented substrate is harvested using the lower transfer screw. Both chambers are aerated with humidified, temperature-controlled air. Rotary disk fermenters are used in large-scale koji making in Japan.

3.8.2.5 Rotary Drum Fermenter

The cylindrical drum of the rotary drum fermenter [1, 2] is supported on rollers, and rotated (1–5 rpm) around the long axis (Figure 3.12). Rotation may be intermittent, and the speed may vary with the fermentation stage. Straight or curved baffles inside the drum aid in tumbling the substrate, hence, improving aeration and temperature control. Sometimes the drum may be inclined, causing the substrate to move from the higher inlet end to the lower outlet during rotation. Aeration is through coaxial inlet and exhaust nozzles.

3.8.2.6 Agitated Tank Fermenter

Either one or more helical screw agitators are mounted in cylindrical or rectangular tanks to agitate the fermenting substrate (Figure 3.13). Sometimes, the screws extend into tanks from mobile trolleys that ride horizontal rails located above the tanks. Another stirred tank configuration is the paddle fermenter. This is similar to the rotary drum device, except that the drum is stationary, and periodic mixing is provided by motor-driven paddles supported on a concentric shaft [1, 2].

3.8.2.7 Continuous Screw Fermenter

Sterilized, cooled, and inoculated substrate is fed at the inlet of the unaerated chamber (Figure 3.14). Fermenting solids are moved toward the harvest port by

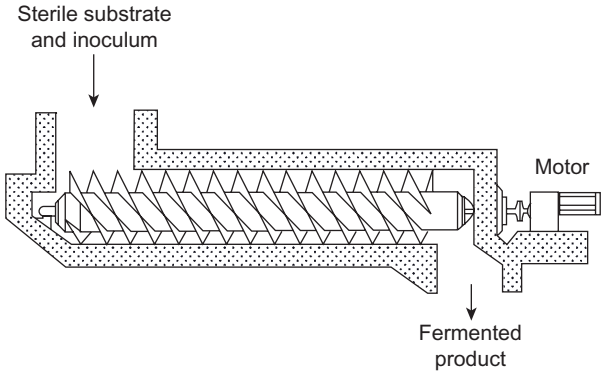

Figure 3.14 Continuous screw fermenter.

the screw (Figure 3.14). Rotational speed and length of the screw control fermentation time. Continuous screw fermenters are suitable for continuous anaerobic or microaerophilic fermentations [1, 2].

Unlike in submerged culture, pressure vessel construction is not the norm for solid-state fermenters. Large concrete or brick fermentation chambers, or koji rooms, may be lined with steel, typically Type 304 stainless steel. However, more corrosion-resistant construction in Type 304L and 316L stainless steels is also used.

3.9 Recovery of Fermentation Products

Crude broth produced by fermentation requires further downstream processing for recovering the desired product in a suitably pure state. Downstream processing typically consists of a series of steps that eventually provide the product at the desired level of purity. Downstream processing operations are discussed in some depth in Chapter 8. This discussion is limited to factors that must be considered in developing any economically viable product purification and concentration scheme based on a small selection of the many available processing operations [10]. Product recovery and purification often contribute 70–80% to the final cost of producing a product [10]; therefore, attention to design of an economic downstream process is important. Purifying a product to more than the level required in a given application is wasteful and expensive. Realistic purity specifications that are consistent with the intended use need to be established and adhered to in downstream processing.

To the extent possible, the requisite purification and concentration should be achieved with the fewest processing steps; generally, no more than 6–7 downstream processing steps are used in recovering products of industrial fermentations [10]. Use of a minimum number of processing steps minimizes the capital outlay and operational expenses. Furthermore, use of a large number of processing steps reduces overall yield of the process. This is because the overall yield of an n-step process with a step yield of x% is only $(x/100)^n$. For example, a processing train of only five steps, each with 90% step yield, would reduce the overall recovery to less than 60% [11]. Use of high resolution separations such as chromatography early in a recovery process minimizes loss of yield.

Attempts should be made to concentrate the product-containing part of the broth as early as possible during downstream processing [10]. Doing so reduces the size and cost of equipment that is required for subsequent processing stages. Fermentation broths can be quite viscous and, therefore, difficult to pump, mix and filter. Processing is greatly facilitated if the viscosity of the broth is reduced, for example, by digesting any unwanted polymers and removing any unnecessary suspended solids. Downstream process should be designed to maximize the speed of processing [10]. Rapid processing reduces the expense associated with time and minimizes loss of product that is associated with prolonged exposure to the often adverse processing conditions.

Recovery and separation processes make use of differences in physical and chemical properties of the components of a mixture, to separate them. For example, recovery of cells by filtration from a broth, makes use of the difference between the size of the cell and the fluid molecules, to achieve separation. Generally, best overall separation outcome will be achieved if the individual steps used in the downstream process make use of differences in as many physical–chemical properties of the mixture components as possible, to achieve separation [10]. As an example, when two chromatographic steps in series are to be used, gel filtration, which separates based on molecular size, and ion exchange chromatography, which separates based on difference in charge on the molecules, may be a superior combination compared with using two stages of ion exchange chromatography.

3.10 Concluding Remarks

This chapter provided an overview of fermentation technology as used in industrial processes. Fermentations can be extremely diverse depending on the substrate, microorganism, and product. Most industrial fermentation are operated batchwise or as fed-batch operations, but other modes of operation are also used. Successful conduct of a fermentation requires attention design and pretreatment of fermentation medium, generation of inoculum and the environment conditions that are necessary for growth and product formation. In monoseptic fermentations, attention to prevention of microbial contamination is important. Although stirred tank fermenters are commonly used for submerged fermentations, many different types of fermenters are available. A suitable fermenters needs to be selected with reference to the nature of a specific fermentation, the scale of operation, and the engineering and operational issues involved.

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4
Directed Evolution of Industrial Biocatalysts

Marlen Schmidt, Dominique Böttcher, and Uwe T. Bornscheuer

4.1
Introduction

Biocatalysts are extensively used in the industrial production of bulk chemicals and pharmaceuticals and over 300 processes have been implemented in industry [1–4]. Up to now, most applications of enzymes involve the use of hydrolases. However, this is expected to change in the near future, as biocatalysts from other enzyme classes (i.e., lyases and oxidoreductases) have been the subject of intensive research in the past decade and major breakthroughs have been already made.

Independent of the reaction system and enzyme investigated for a given biocatalytic process, very often the enzyme does not meet the requirements for a large-scale application and its properties have to be optimized. This usually includes the chemo-, regio-, and especially stereoselectivity of the biocatalyst, but also process-related aspects such as long-term stability at certain temperatures or pH-values and activity in the presence of large substrate concentrations need to be optimized to achieve highest productivity.

In addition to the classical strategies such as immobilization, additives, or process engineering, molecular biology techniques are now the most important methodologies used to tailor-design enzymes for given processes. Two different (but often complementary) strategies are used: rational protein design and directed (molecular) evolution. A general scheme of these approaches is given in Figure 4.1. This chapter focuses on the major achievements made in the past decade in the area of directed evolution, but rational protein design is also included. Examples covered include industrial processes as well as important contributions from academic research dealing with industrially relevant biocatalysts. As one can expect that not all biocatalyst improvements made in industry are published, it is anticipated that there are several further processes employing enzymes optimized by directed evolution approaches in existence.

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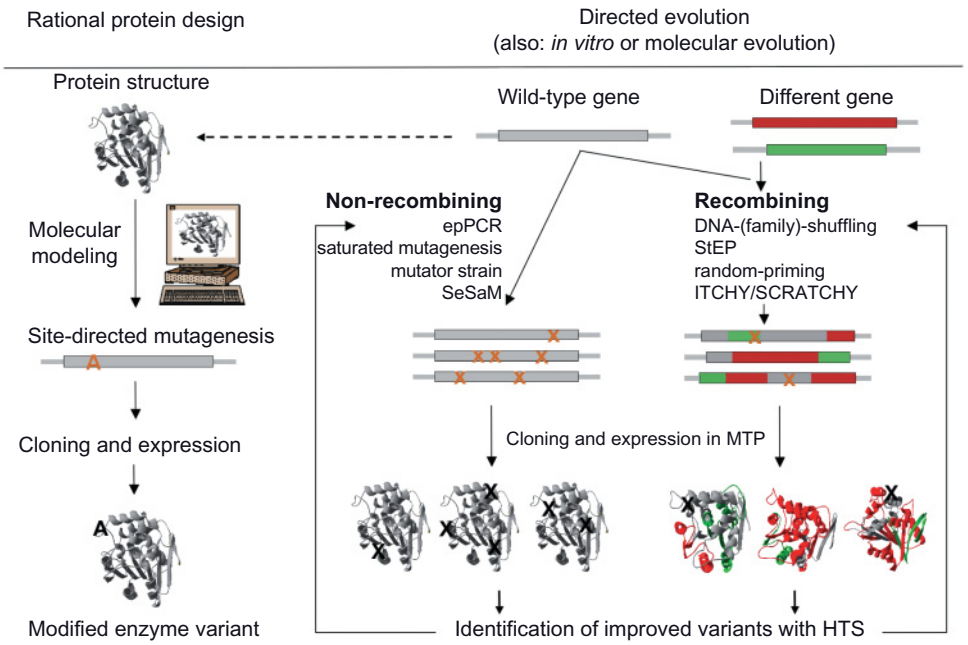

Figure 4.1 Schematic comparison of rational protein design and directed evolution. Rational design starts from a protein structure (or a homology model), from which key amino acid residues are identified. These are then introduced at the gene level and the resulting mutant is produced and verified for desired properties. Directed evolution starts from one or several (homologous) genes, which are subjected to a range of random mutagenesis methods. From the resulting libraries of mutants or chimeras, desired variants are – after production in a microtiter plate format – then identified by screening or selection. For abbreviations, see text.

4.2 Strategies for Protein Design

4.2.1 Rational Protein Design

Our growing understanding of how to engineer the properties of enzymes is steadily making rational protein design more efficient (Figure 4.1). Nevertheless, the need for the availability of the protein structure (or at least a sufficiently good homology model) and a thorough understanding of the catalytic mechanism are still the major obstacles for rational protein design. From the structure, certain amino acids (hot spots) that are thought to be involved in the desired properties can be identified by molecular modeling techniques. Site-directed mutagenesis (SDM) is then performed at these positions using, for instance, the QuikChange™ SDM method from Stratagene. Rational protein design has been especially suc-

cessful where properties such as altered activity or enantioselectivity have been addressed and numerous examples can be found in the literature [5].

4.2.2 Directed Evolution

Directed evolution (also named *in vitro* evolution or molecular evolution) is a technique of protein alteration and selection of the fittest individual. Within just a decade, this technology has emerged as an extremely powerful and widely used tool to improve biocatalysts. In general, a directed evolution strategy comprises random mutagenesis followed by a high-throughput screening (HTS) and/or selection step (Figure 4.1).

The process begins with the selection of a known enzyme – which must be available in recombinant form and having a suitable expression system at hand – and in the identification of the property that needs to be optimized. A large mutant library, as unbiased as possible, is prepared by random mutagenesis of the protein-encoding gene, according to the techniques detailed below. After cloning and expression, a huge collection of enzyme variants, typically in the range of 10^4–10^6 individuals, is generated. This library is finally subjected to the identification of mutants with the evolved desired property using screening or selection methods. The best hits might subsequently serve as improved starting points for additional rounds of mutagenesis to accumulate beneficial mutations. Often the information derived from mutant analysis can serve as a basis for rational protein design approaches.

In the following sections, the most important mutagenesis methods and suitable HTS systems are introduced, followed by examples of the successful application of directed evolution experiments to the production of improved biocatalysts.

4.2.2.1 Mutagenesis Methods

Two different strategies for the generation of mutant libraries can be followed: (i) asexual (non-recombining) evolution, in which a parent gene is subjected to random mutagenesis to yield variants with point mutations, and (ii) sexual (recombining) evolution, in which several parental genes are randomly fragmented and then recombined to yield a pool of chimeras.

In the last 15 years a plethora of methods have been developed and some will be briefly described in the following paragraphs. For a deeper and more detailed insight many reviews and books are available [6–10].

The most widely used asexual method is error-prone polymerase chain reaction (epPCR). Here, non-optimal reaction conditions are used to create a mutant library [11, 12]. For example, increasing the Mg^{2+} concentration, adding Mn^{2+} and usage of unbalanced dNTP concentrations, can increase the error rate of *Taq*, the commonly used polymerase from *Thermus aquaticus*, from 0.001% to 1%. However, a homogeneous mutational spectrum (unbiased library) cannot be created using *Taq* DNA polymerase with Mn^{2+} and unbalanced nucleotides as the "polymerase or error bias," will result in a tendency to exchange the desoxynucleotides A or T

in preference to G or C. Improved DNA polymerases that exhibit a more homogeneous mutational spectrum are now commercially available, for example Mutazyme™ I and II from Stratagene with a higher error rate, as well as *Taq*-Pol I614K [13] or *Pfu*-Pol (exo-) D473G [14]. A further bottleneck introduced by the ligation of the mutated epPCR product into a cloning or expression vector is partially solved by the "bringer" technique developed by Bichet *et al.* [15]. This method can be regarded as a variation of Stratagene's QuikChange™ method, because it is based on amplifying the whole plasmid under mutagenic conditions. A related technique is MEGAWHOP, in which the mutated gene serves as a megaprimer in a second PCR; restriction digestion and ligation is therefore no longer necessary [16, 17]. Recently, an epPCR was combined with rolling circle amplification (RCA) [18], which uses a polymerase from bacteriophage ϕ29 in an isothermal amplification [19].

Another major problem in epPCR is the so-called "codon bias," resulting from the degenerate genetic code, which means that a specific amino acid change will be much less common than others. For example, a single mutation in a valine codon results in only six alternative amino acid substitutions. Thus, the encoding of all 20 proteinogenic amino acids requires the exchange of all three (adjacent) codons, which is a rather unlikely event when an entire protein encoding gene (i.e., 1000 bp) is subjected to epPCR at an error rate of 1%. [8].

An easier alternative to create random mutations involves the use of mutator strains. Strains such as *Epicurian coli* XL1-Red are deficient in three of the primary DNA repair pathways (*mut*D (interferences in 3′5′ exonuclease activity), *mut*S (deficient mismatch repairing), and *mut*T (no hydrolysis of 8-oxodGTP)) and exhibit a mutation rate approx. 5000 times that of the wild-type host strain. On the one hand, with this approach the ligation step is avoided, but on the other hand, there is no control on the location of the mutation, which can affect the promotor present in the plasmid, its copy number, and other features of the vector. There are several references that describe successful creation of mutants using this technique [20–23].

When beginning from already selected variants or genes with sufficient homology, sexual recombination methods are preferred because they may combine the best features of both parental donors. The first example was developed by Stemmer *et al.* [24] and termed DNA or gene shuffling. It consists of a DNase-dependent degradation step and subsequent recombination of the fragments without primers (self-priming PCR) followed by a final PCR with primers. This method was further refined in the last decade and also named DNA family shuffling or molecular breeding. One alternative is the staggered extension process (StEP) developed by Zhao *et al.* [25]. This consists of the amplification of short fragments of the parental genes, so that in subsequent cycles, the resulting short fragments can anneal on any other of the parental genes. Thus, the bias introduced by DNase I digestion can be avoided. A further improvement on the classic shuffling approach was developed by Coco *et al.*, who devised an alternative DNA shuffling method called random chimeragenesis on transient templates (RACHITT), based on the ordering, trimming, and joining of randomly cleaved single-stranded parental gene

fragments annealed onto a transient full-length single-stranded template. This method exhibited higher recombination frequencies and 100% chimeric products [26]. Clearly, similarity of sequences between the parental genes is a necessary constraint to the application of recombination techniques. However, methodologies have been introduced to recombine several parental sequences without the need for high sequence homology, which is necessary to combine genes with high structure but little sequence homology, such as P450 monooxygenases, for example. The first homology-independent method was developed by Ostermeier *et al.* [27, 28]. ITCHY (incremental truncation for the creation of hybrid enzymes) is based on the fusion of fragments of different size of two genes, which were generated by an exonuclease III and S1-nuclease digestion. A major disadvantage is that there is only one recombination point. Once again this method was optimized and several spin-off-methods were developed, for instance thio-ITCHY [28, 29], Gene Reassembly™ method [30], non-homologous random recombination (NRR) [31, 32] and sequence homology-independent protein recombination (SHIPREC) [33].

A completely different strategy to evolve enzymes without introducing mutations, circular permutation (CP), was proposed by Lutz and colleagues [34]. They linked the native N- and C-termini of the gene encoding lipase B from *Candida antarctica* and subsequently linearized it by random digestion to yield variants bearing alternative N- and C-termini. Surprisingly, this not only led to active lipase, some variants also showed higher catalytic efficiency than the wild type (up to 11-fold against *p*-nitrophenol butyrate and 175-fold against 6,8-difluoro-4-methylumbelliferyl octanoate, while K_m values were nearly the same). For the most active variant (cp283) kinetic experiments demonstrated, that CP of this enzyme does not compromise the enantioselectivity in the resolution of some chiral secondary alcohols [35].

As outlined above, a wide range of techniques are available to randomly mutate genes. In order to predict and compare the quality of the mutant library, several software tools have been developed to facilitate the planning of directed evolution experiments. Often the ratio between transitions and transversions is used to evaluate a library, but using a mutagenesis assistant program (MAP) (available at http://map.iu-bremen.de), Wong *et al.* showed that this often fails as an estimation [36]. The authors proposed instead a protein structure indicator, an amino acid indicator (complemented by codon diversity coefficient), and a chemical diversity indicator as a better benchmark for library comparisons.

As recently reviewed [37, 38], various computational tools have also been reported to simulate the mutation processes and to rationalize published experimental results. PEDEL (program for estimating diversity in epPCR libraries) and LDP (library diversity program) are examples of computational models used to simulate random mutagenesis methods, especially epPCR [38]. Another useful program is GLUE (program for libraries comprising a random sampling of equally probable variants), which was developed to estimate how many variants a library contains and how large a library has to be in order to sample all variants [39]. GLUE, as well as PEDEL and DRIVeR are available at http://guinevere.otago.ac.nz/stats.

html [39, 40]. Moore and Maranas modeled the shuffling method and thus developed the eShuffle and eSCRATCHY programs [41–44] (http://maranas.che.psu.edu/software.html), as well as the SIRCH procedure to characterize the complete residue–residue coupling consistent with a given protein structure [45]. Wong *et al.* developed a guide (random mutagenesis strategy flowchart (RaMuS) that should help researchers – especially those who just enter the field of (directed) evolution – to pick a suitable random mutagenesis method adjusted to their needs and skills [10].

4.2.3 Focused Directed Evolution

The question whether rational design or directed evolution is the better strategy has often been discussed and no simple conclusion can be drawn. In fact, rational design is hampered by the complexity of proteins and limited by our knowledge of sequence–function relationships. Also, it often does not provide the desired result and yields sometimes very surprising variants, which can be far away from the needed property. For example, thermostability is difficult to predict rationally and therefore directed evolution appears to be the better choice [46].

Recently, combinations of directed evolution and rational design have been proposed, which have been named semi-rational design or rational evolution, although we prefer the term focused directed evolution. The successful application of iterative saturation mutagenesis (ISM) was described by Reetz and coworkers. This yields increased thermostability of a lipase (subcategory B-FIT) [47, 48], increased enantioselectivity, and expanded substrate acceptance, as exemplified for an epoxide hydrolase (subcategory CASTing) [47–49]. In the ISM approach the protein structure (or homology model) is first analyzed to identify all the amino acid positions that contribute to a certain property. Next, all sites are (separately) subjected to saturation mutagenesis followed by screening of the libraries for the property of interest. Subsequently, the best hit of each library is used as a template for further rounds of saturation mutagenesis and this strategy is then iteratively continued until the desired improved biocatalyst has been found (Figure 4.2).

CASTing (combinatorial active-site saturation test) as a subcategory of ISM follows this strategy to identify hot spots, especially for properties such as enantioselectivity or substrate specificity. Here, the area around the catalytically active site in a radius of ~10 Å is used to identify all the amino acid residues that interact with the substrate. For the systematic design and screening of focused mutant libraries around the whole binding pocket, a program (CASTER) was developed. This is freely available on the Internet (http://www.kofo.mpg.de/kofo/institut/arbeitsbereiche/reetz/deutsch/reetz_forschung1.html).

Researchers at Codexis used a similar approach, but unlike CASTing their strategy is 3D-structure or homology model independent and they achieved a substantial improvement in catalytic function. ProSAR is an extension of SAR (structure–activity relationship) for molecular protein optimization and therefore the relationship between the structure of the interacting molecule (enzyme) and

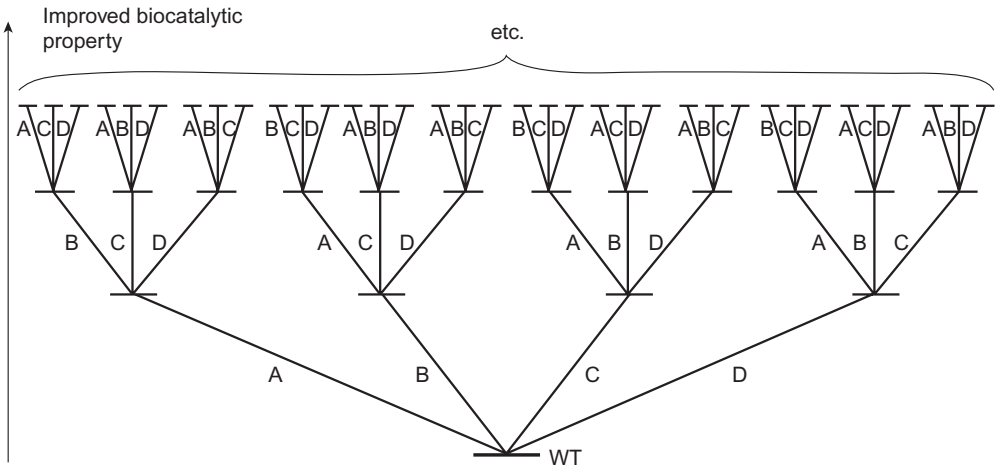

Figure 4.2 Principle of iterative saturation mutagenesis.

the measurable property of interest is analyzed and formulated in an equation [50]. Mutants of libraries from different mutagenesis methods were analyzed by activity and sequencing and then ranked as "beneficial," "potentially beneficial," "neutral," or "deleterious." Those in the last categories were discarded and the beneficial ones were kept and used as parental enzymes for the next round of mutations. Others mutations were retested and may also serve as parents. The improvement of a halohydrin dehalogenase, which is important for the production of a precursor of the cholesterol-lowering drug atorvastatin, is an excellent example for the usefulness of this method (see Section 4.4.3).

Semi-rational attempts with recombining mutagenesis methods are SISDC (sequence-independent site-directed chimeragenesis) [51] and SCOPE (structure-based combinatorial protein engineering) [52] where crossover positions are identified based on the three-dimensional structure of the proteins.

4.3 Assay Systems

The key to a successful directed evolution experiment is a rapid and highly reliable assay system. Because of the huge number of possible variants that are created in the random mutagenesis approach, desired hits are difficult to identify after individual expression of variants using classical analytical methods, such as gas chromatography or HPLC. Consequently, suitable high-throughput tools must be available to identify desired biocatalysts. The strategies can be subdivided into either selection or screening approaches, as outlined in detail below. Both approaches have their advantages and disadvantages and a decision on which method is more appropriate has to be made on a case-by-case basis. A broad

overview of recently described tools can be found in the book by Reymond [53] and in a number of reviews [54–58].

4.3.1 Selection

Biological selections are based on complementation of auxotrophy or resistance to cytotoxic agents like antibiotics. When applicable, selection is a very powerful tool for screening large libraries and the discovery of protein mutants. However, the usage of phenotypic selection is limited to the isolation of catalysts for reactions that are of direct biological relevance or can be indirectly linked to a selectable phenotype. Such selections can be carried out *in vitro* or *in vivo* and either in solid phase or microtiter plates. Mutagenesis techniques create libraries often ranging between 10^5 and 10^{10} individuals. In order to screen a significant amount of sequence space, inactive or uninteresting individuals can be discarded to reduce the size of the library.

4.3.1.1 Display Techniques

Phage display is one of the most common techniques for *in vitro* selection of the fittest mutants in a large library. It consists of the cloning of the gene of interest (in this case each individual in a library of mutants) in fusion with a gene encoding a coat protein of the virion. When the phage is assembled, the foreign protein is displayed on its surface. Therefore, a physical linkage between gene and expression product is achieved by means of a phage particle. The phages are then captured by affinity interaction of the displayed enzyme with an immobilized ligand. The nature of this binder depends on the enzyme, for example, the fusion tag can be a substrate, or a suicide-substrate coupled to biotin (which is subsequently captured on streptavidin beads), or an immobilized transition state analog. The selected phages are eluted, replicated, and amplified by simple infection [59].

Danielsen *et al*. illustrated the selection of Lipolase® (a lipase)-displaying phages with a biotinylated phosphorylating inhibitor that enabled the library to be enriched 180-fold in a single round [60].

Protein libraries can also be displayed on bacteria and yeast as well as on the surface of bacteriophages. Bacterial display has certain advantages over the much more widespread phage display. First, only one host is needed to propagate the library compared with two, the bacteriophage and bacterium, in phage display. Second, the selected variants can be directly amplified without further transfer of the genetic material to another host. Third, the risk of affinity artifacts because of avidity effects might be less pronounced. Bacterial display of an esterase from *Burkholderia gladioli* was achieved by Schultheiss *et al*. using an artificial gene composed of the esterase gene and the essential autotransporter domains in *E. coli*. The esterase activity was successfully directed to the outer membrane fraction as confirmed by different techniques [61].

Bacterial cell surface display coupled with flow cytometric screening has also been adapted to the screening of enzyme libraries [62]. In fact, this technology

currently represents the only general approach for the quantitative examination of enzyme catalytic activity at the single cell level and in very large populations of mutants. Furthermore, the display of enzymes on the bacterial surface provides free access of synthetic substrates to the enzyme.

The ability to form a physical link between a fluorescent product of a reaction and the cell that expresses the respective enzyme on its surface proved to be the key for the quantitative determination of catalytic activity at the single cell level. Several routes are now available to display enzymes on the microbial cell surface, most of which have been developed for *E. coli* [63].

One example is using the EstA, an outer membrane-anchored esterase from *Pseudomonas aeruginosa*. An inactive EstA variant was used as an anchoring motif for the *Escherichia coli* cell surface display of lipolytic enzymes. Flow cytometry analysis and measurement of lipase activity revealed that *Bacillus subtilis* lipase LipA, *Fusarium solani pisi* cutinase and *Serratia marcescens* lipase were all efficiently exported by the EstA autotransporter and also retained their lipolytic activities upon cell surface exposition [64]. Very recently, Kolmar and coworkers showed that *E. coli* bacteria that display esterases or lipases on their cell surface together with horseradish peroxidase (HRP) are capable of hydrolyzing carboxylic acid esters of biotin tyramide. The tyramide radicals generated by the coupled lipase–peroxidase reaction were short lived and therefore became covalently attached to reactive tyrosine residues that were located in close proximity on the surface of a bacterial cell that displayed lipase activity. Differences in cellular esterase activity were found to correlate well with the amount of biotin tyramide deposited on the cell surface. This selective biotin tyramide labeling of cells with lipase activity allowed their isolation by magnetic cell sorting [65].

4.3.1.2 *In Vivo* Selection

In vivo selection can be performed when the target activity is essential for viability and growth, for example, overcoming increasing concentrations of antibiotics or providing an essential nutrient [66].

The principle was shown by Reetz and Rüggeberg with an example in which survival was coupled to the hydrolysis of a certain enantiomer that releases a growth-inhibiting compound [67]. Thus, microorganisms expressing the lipase variant with the adequate enantioselectivity cannot cleave this compound and therefore survive, promoting an effective enrichment of the culture in the enzyme variant with the desired enantioselectivity.

Sometimes selection is performed as a complementation approach: only a mutated enzyme variant is produced (i.e., an essential metabolite) [68, 69].

Stemmer's group subjected four genes of cephalosporinases from *Enterobacter*, *Yersinia*, *Citrobacter*, and *Klebsiella* species to epPCR or DNA shuffling. Libraries from four generations (a total of 50 000 colonies) were assayed by selection on agar plates with increasing concentrations of moxalactam (β-lactam antibiotic). Only clones that were able to hydrolyze the β-lactam antibiotic could survive. The best variants from epPCR gave only a eight-fold increased activity, but the best chimeras from multiple gene shuffling showed 270–540-fold higher resistance to moxalactame [70].

For libraries expressed in microorganisms, HTS can be performed on colonies growing in a solid culture like an agar plate. Assays on agar-plated colonies typically enable the screening of $>10^4$ variants in a matter of days, but they are often limited in sensitivity: soluble products diffuse away from the colony and hence only very active variants are detected. Assays based on insoluble products have higher sensitivity, but their scope is rather limited.

Solid-phase screening relies on product solubilization following an enzymatic reaction that gives rise to a zone of clearance, a fluorescent product, a pH shift visualized by a pH indicator or a strongly absorbing (chromogenic) product like X-gal or α-naphthyl acetate and Fast Blue/Fast Red as an example for esterase activity detection [71, 72].

The assay may detect the enzyme product directly or may be coupled to a second enzyme whose product can in turn be easily monitored, as demonstrated by the successful coupling of cytochrome P450 with horseradish peroxidase [73]. If the assay is toxic or requires cell lysis, then a portion of the cells from a colony is transferred onto a filter membrane, where the assay is performed [74]. In recent years, digital imaging has been increasingly employed to achieve higher throughput and quantitative signal evaluation [73, 75, 76].

Lipolytic activity can still be screened in a high-throughput format on plates, with triolein and tributyrin-agar through halo formation. Alternatively, a high-throughput assay in solid phase was recently developed by Babiak and Reymond using esters of coumarin [77].

4.3.2 Screening

Many assays cannot be applied in a solid-phase format. Thus, individual clones must be grown and assayed in microtiter plates. These assays are significantly more time-consuming than solid-phase assays. However, by using robot automation and colony-picking technology the throughput can be substantially increased. A major advantage is that screening provides significantly more information than a selection approach as the activity can be directly and quantitatively measured, and even allowed to determine the kinetics. Furthermore, screening enables the direct determination of the enantioselectivity of an enzyme, which is very often the key property that needs to be improved for industrial biocatalysis.

4.3.2.1 Hydrolase Assays

Lipases and Esterases Hydrolytic activity of esters can be determined using a wide variety of substrates, preferentially using the "true" compounds of interest compared with surrogates (i.e., non-natural substrates designed to provide an intense, detectable signal when they are converted by the enzyme). Nevertheless, not all activity assays can be implemented in the high-throughput format required for the screening of the vast libraries created by the mutagenesis protocols used in directed

evolution. An example is a simple pH-stat assay using tributyrin or triolein emulsions as substrates. Colorimetric and fluorometric assays are undoubtedly the most widespread screens to determine hydrolytic activities. They involve the cleavage of an ester to yield a chromophore/fluorophore that is measured. The most commonly used chromophores/fluorophores are *p*-nitrophenol, fluorescein, resorufin, or coumarin. The major disadvantages of using these artificial substrates are that they differ from the true substrate and hence can lead to false positive hits, and that they are often not commercially available.

One assay in which acetates can be directly used as "true" substrate is based on a commercially available "acetic acid test" (R-Biopharm GmbH, Darmstadt, Germany). This couples the hydrolysis of acetates with an acetate-dependent enzymatic cascade leading to the stoichiometric formation of NADH [78]. If enantiomerically pure chiral (*R*)- and (*S*)-acetates are used in separate experiments but using the same enzyme variant, the method allows the determination of enantioselectivity. This assay could thus be successfully used to identify an esterase variant with high enantioselectivity in the synthesis of (*S*)-butyn-2-ol [79].

An important disadvantage of hydrolytic activity assays of lipases and esterases is the rather poor solubility of most substrate in aqueous media and the risk of strong autohydrolysis at extreme pH or elevated temperature using chromogenic or fluorogenic substrates.

To circumvent this problem, two strategies have been described in the literature. First, the esters of *p*-nitrophenol or coumarin were replaced by the corresponding acyloxymethylethers, or diacylglycerol analogs. This makes the substrate much more stable, as the ester susceptible to enzymatic cleavage is separated from the chromophore (or fluorophore), avoiding autohydrolysis since the alcohol moiety is now a worse leaving group compared with coumarin or the *p*-nitrophenoxide ion. Depending on its particular structure, the cleaved alcohol is then directly decarboxylated or first oxidized with periodate and then subjected to BSA-catalyzed β-elimination in order to release the chromophore/fluorophore [80–82]. This methodology is also applicable to the screening and characterization of enantioselective enzymes [83]. Disadvantages are the need for synthesis of the specifically designed substrates and that only end-point measurements are possible rather than quantification of enzyme kinetics. A variation of the method described above uses back-titration with epinephrine of the sodium periodate consumed in the oxidation of the diol generated by enzymatic cleavage [84, 85].

In 1997, Janes and Kazlauskas described the first HTS method for enantioselectivity based on the separate hydrolysis of the enantiomers of *p*-nitrophenyl esters of a chiral acid, named Quick E. In this method they determine the enantioselectivity E by measuring initial rates of hydrolysis of pure enantiomers of 4-nitrophenyl-2-phenylpropanoate and a reference compound (resorufin tetradecanoate). Advantages are the short measurement time, the need of much smaller amount of hydrolase, and the easy measurement of high enantioselectivities. There are also some disadvantages like requirement of pure enantiomers, need for a clear solution, and that the test can only be used for chromogenic substrates

[86]. The Quick E method has been applied successfully in order to test the substrate selectivity of a series of hydrolases [87–89].

In order to use a racemate as substrate, a separation technique is needed prior to quantification of each enantiomer. This separation can be according to chirality or, in the case of isotopically labeled substrates, according to molecular mass. HPLC and gas chromatography have been adapted to high-throughput in order to be used [90]. With this setup, about 700 measurements per day were carried out to screen a mutant library from *Pseudomonas aeruginosa* lipase for the enantioselective esterification of 2-phenylpropanol. Mass spectrometry (MS) is used with one isotopically labeled compound in an enantiomer pair in kinetic resolutions (a pseudo racemate) [91] or in the biotransformation of a meso-compound. Capillary electrophoresis using chiral selectors (e.g., cyclodextrins) as pseudo-stationary phase in the electrolyte has also been adapted to process as many as 96 samples in parallel, allowing the determination of 7000 samples of derivatized chiral amines per day [92].

4.3.2.2 Oxidoreductase Assays

Most assays for oxidoreductases follow NAD(P)H formation or depletion by measurement of absorption at 340 nm. Although the concentration of the cofactor is directly linked to the substrate turnover, care must be taken, as the data can be strongly influenced by background reactions taking place in crude cell lysates, often making the purification of the enzyme of interest necessary.

Dehydrogenases The activity of dehydrogenases is commonly measured by cascade reactions to couple NAD(P)H generation with formation of colored compounds. Examples are tetrazolium dyes like nitroblue tetrazolium (NBT). An NBT/phenazine methosulfate (PMS) assay was described for the detection of dehydrogenase activity [93]. The assay was shown to work in liquid and solid phase and most importantly could be applied to HTS [94]. Another assay uses the reduced form of the cofactor. In the so-called "alkali assay" the $NAD(P)^+$ is decomposed under strong alkaline conditions to form a highly fluorescent product [95].

Oxidative activity can be monitored by coupling enzymatic H_2O_2 production to an HRP reaction. The enzyme HRP, for example, reacts in the presence of H_2O_2 with compounds like ABTS and produces a soluble green product that can be detected spectrophotometrically at 405 nm [96]. As alternatives to ABTS, other chromogenic substrates such as *o*-dianisidine can be used [97].

In a recent example, directed evolution libraries from *Aspergillus niger* expressing glucose oxidase were screened for improved pH and thermostability. For this, Schwaneberg and coworkers have developed a medium-throughput 96-well microtiter plate assay, in which oxidation of glucose mediated by oxidized ferrocenemethanol shows a color change from blue to pale yellow that can be recorded at 625 nm [98].

ABTS and *o*-dianisidine can also be directly used for measurement of peroxidase activity. The application for HTS of enzyme libraries has been demonstrated in a number of examples [99–102]. Another assay for the determination of peroxidase

activity represents the guaiacol assay, which was used for screening of improved cytochrome *c* peroxidase [103]. The colorless guaiacol (2-methoxyphenol) is oxidized to the phenoxy radical in the presence of peroxidase. The radical is polymerized to the brown tetraguaiacol and can be detected at 470 nm.

Several assays for oxygenase activity, such as P450 heme monooxygenase, have been published. For example the *p*-nitrophenoxy analog assay (pNA) has been used in a range of directed evolution experiments [104–106]. Recently, Schwaneberg and coworkers published a product-based screening system using the colorimetric agent *p*-nitrothiophenolate (pNTP) for the directed evolution of epoxygenases as exemplified for the P450 BM3 monooxygenase. pNTP is a yellow chromophore that reacts with an epoxide through nucleophilic attack, resulting in ring opening and a colorless product [107].

4.3.2.3 **Hydroxynitrile Lyase Assays**

Hydroxynitrile lyases (HNL) catalyze the cleavage of cyanohydrins and usually the activity is determined by the cleavage of HCN from mandelonitrile catalyzed by the enzyme. The benzaldehyde produced can then be determined spectrophotometrically at 280 nm. Although this assay is applicable for high throughput, it is restricted to aromatic substrates. Another option to detect the activity is the conversion of benzaldehyde with an alcohol dehydrogenase and the determination of the increasing NADH concentration [108]. Alternatively, the released HCN could be detected as described by Selmar *et al.* [109], based on a reaction described by König [110], which can be regarded as an all-purpose screening assay without restriction to any specific substrate. In this assay the CN^- is first oxidized with chlorosuccinimide, then attached to pyridine, and after hydrolysis to glutaconic aldehydes occurs. Finally in the König reaction, with a primary amine and barbituric acid as a coupling reagent, a colored compound that can be detected at 580 nm, is formed. Recently, this method was further developed for activity measurements in microtiter plates, and is thus suitable for high-throughput systems [111]. The assay is theoretically useful to detect the activity and enantioselectivity of HNLs towards any cyanohydrin substrate.

Limitations might occur in the case of hydrophobic substrates due to poor water solubility. This problem can be overcome by the use of emulsifying agents such as gum arabic. As tested, the increased turbidity has no influence on the formation and spectrophotometric detection of the dye. Another main disadvantage is that until now the assay can only be used for end-point detection and not for kinetic measurements. For this purpose one still has to use the conversion of mandelonitrile as mentioned above.

The principle of another assay is based on the Feigl–Anger method [112], which results in a blue color when HCN gas comes into contact with filter paper impregnated with a mixture of copper(II)ethylacetoacetate and tetra base [113]. The blue-colored salt is an oxidation product of tetra base, being formed in the presence of hydrocyanic acid and copper(II)ethylacetoacetate. The screening procedure itself was implemented as a sandwich assembly, in which the membrane-blotted colonies, which are incubated to the substrate solution on the bottom, are separated

from the Feigl–Anger test paper on the top by a permeable nylon tissue. This assembly exclusively allowed the gaseous HCN to reach the detection paper and results in a distinct dark blue spot directly above an HNL-producing colony.

4.4 Examples

4.4.1 Esterases, Lipases, and Phospholipases

The majority of industrially used enzymes are hydrolases (65%) and thus many examples of evolved hydrolases can be found in literature. One of the first examples was an esterase variant from *Bacillus subtilis* (BsubpNBE) with activity 150 times higher in 15% dimethylformamide (DMF) than the wild-type, created by combining epPCR and shuffling. The enzyme is applicable for the deprotection of a precursor in the production of the antibiotic loracarbef in the presence of DMF as cosolvent [114].

The thermostability of the same enzyme was also increased by 14 °C in eight rounds of epPCR and recombination [115].

A similar esterase, BS2, which differs only by 11 amino acids from BsubpNBE, was evolved by rational design in our group and the enantioselectivity towards the tertiary alcohol 2-phenyl-3-butin-2-yl acetate could be increased sixfold to $E = 19$, and towards linalyl acetate inverted from (*R*)- to (*S*)- preference with $E = 6$ [116]. In a later study, this mutant (G105A) showed a good enantioselectivity towards 2-phenyl-3-butin-2-yl acetate ($E = 54$) in 20% v/v DMSO, and an *E*-value of >100 towards the trifluoromethyl analog [117] (Figure 4.3). Another mutant, E188D, gave similar high enantioselectivity towards both substrates as well as a series of other tertiary alcohol acetates [118].

Using a focused random approach we were also able to invert the enantioselectivity [119]. While screening with the acetate assay described above, a double mutant (E188W/M193C) with an (*S*)-preference and an *E*-value of ~70 towards 1,1,1-trifluoro-2-phenylbut-1-yn-3-ol was identified. Notably, the single mutants E188W or M193C, which could have also been obtained by random mutagenesis, show only $E = 16$ for the (*S*)-enantiomer or low (*R*)-selectivity, respectively, and only the combination, which was unlikely be obtained by "normal" random

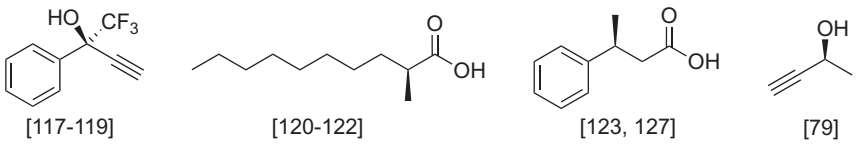

Figure 4.3 Selected examples of chiral compounds obtained using designed esterases or lipases.

mutagenesis, results in the substantial inversion of enantioselectivity. This synergistic result is thus an excellent argument for using focused directed evolution (Figure 4.3).

Shortly after the first evolved esterase from the Arnold group was reported, directed evolution of a lipase from *Pseudomonas aeruginosa* was reported by Reetz and coworkers. The initial enantioselectivity in the kinetic resolution of 2-methyldecanoic acid *p*-nitrophenyl ester (MDA) was $E = 1.1$ (in favor of the (*S*)-acid), and after four rounds of epPCR an $E = 11$ was obtained. Further mutants were created by combining mutations on the positions identified to be critical in the generation of the best variants for every round, which led to the identification of a more enantioselective variant ($E = 21$) [120]. For this same reaction, a DNA shuffling approach proved effective, yielding a variant that exhibited $E = 32$. Furthermore, a modified version of Stemmer's combinatorial multiple-cassette mutagenesis was applied to two of the obtained mutants and a mutagenic oligocassette, which allowed simultaneous randomization at previously determined "hot spots." This resulted in the most enantioselective variant (X, with six mutations), displaying a selectivity factor of $E = 51$. In addition, variants with good (*R*)-selectivity ($E = 30$) were also identified [121]. Interestingly, only one mutation is located next to the active site, whereas all other substitutions are remote. After a theoretical study, 10 new variants were prepared and the double mutant M8 (S53P/L162G), which was supposed to show high enantioselectivity, gave an *E*-value of 64 at a conversion of 20% [122] (Figure 4.3).

Attempts to resolve racemic mixtures of esters of secondary alcohols with mutants of an esterase from *Pseudomonas fluorescens* (PFE-I) have been reported as well. Our group used the mutator strain *E. coli* XL-1 Red, to develop an enantiopreference in the hydrolysis of methyl 3-phenylbutyrate [123]. The same enzyme was also evolved by epPCR and the acetate assay for its application in the enantioselective hydrolysis of the very important building block (*S*)-3-butyn-2-ol with enantiomeric excess (ee) >99% [79]. Here only a single round of epPCR was necessary to obtain a variant containing three point mutations that showed increased enantioselectivity without overhydrolysis. Subsequently, the effect of each mutation on the enantioselectivity, the reaction rate, and the solubility of the mutant were studied. From this work the final conclusion was that mutations close to the active side can also have a substantial (negative) effect on protein folding, which should receive more attention in protein engineering.

Candida antarctica lipase B (CAL-B) is one of the most used biocatalyst in organic chemistry [124]. Nevertheless, it was not possible to obtain more thermostable variants by rational mutagenesis. When the directed evolution approach was used, after two rounds of mutation by epPCR, variants were found that were 20-fold more stable at 70 °C than the wild type. Positions 221 and 281 were found to be critical to prevent irreversible inactivation and protein aggregation of these enhanced variants, which were also proved to be more active against *p*-nitrophenyl butyrate and 6,8-difluoro-4-methylumbelliferyl octanoate [125].

CAL-B was also engineered by shuffling its gene with those of lipases from *Hyphozyma* sp. CBS 648.91 and *Crytococcus tsukubaensis* ATCC 24555 in order to

create a lipase B variant with increased activity in the hydrolysis of diethyl 3-(3′,4′-dichlorophenyl)glutarate, that yields a chiral synthon for the preparation of an NK1/NK2 dual antagonist [126].

The enantioselectivity of *Burkholderia cepacia* KWI-56 lipase was evolved towards (*R*)-enantioselectivity in the hydrolysis of 3-phenylbutyric acid *p*-nitrophenyl ester using a novel technique for the construction and screening of a protein library by single-molecule DNA amplification by PCR followed by *in vitro* coupled transcription/translation system. The library was generated by saturating four positions (L17, F119, L167, and L266), and then diluting until only five molecules of DNA were present per well of a microtiter plate. These molecules were amplified using a single-molecule PCR product and expressed *in vitro*, since each gene fragment already carried a T7 promoter, a ribosome-binding site, and T7 terminator. The DNA corresponding to active wells showing the desired enantioselectivity was once again diluted to give one molecule per well, then re-amplified and re-checked. The best mutant exhibited a selectivity factor of $E = 38$ towards the (*R*)-enantiomer, whereas the wild type exhibited $E = 33$ for the opposite enantiomer [127] (Figure 4.3).

Phospholipase A1 activity – already present in many lipases as promiscuous activity – was substantially enhanced in the lipase from *Staphylococcus aureus* by sequential rounds of epPCR. After four rounds, two products were obtained, displaying a 5.9- and 6.9-fold increase in phospholipase/lipase activity ratio. A final round of DNA shuffling with these two products and wild-type lipase was performed to combine beneficial mutations and to eliminate neutral or deleterious mutations. This procedure yielded a variant containing six amino acid mutations displaying a 11.6-fold increase in absolute phospholipase activity and an 11.5-fold increase in phospholipase/lipase ratio compared with the starting point [128]. In a similar way, a single round of epPCR yielded a 17-fold increased phospholipase/lipase ratio of the thermoalkalophilic lipase from *Bacillus thermocatenulatus* [129].

The chain-length selectivity of lipases can be altered by site-directed mutagenesis, as shown by Joerger and Haas for the *Rhizopus oryzae* (formerly *Rhizopus delemar*) lipase [130], but also by directed evolution [60]. While trying to isolate new enzyme variants of the extracellular lipase from *Thermomyces lanuginosa* with enhanced activity in the presence of detergent, Danielsen *et al*. randomized nine amino acids in two regions flanking the flexible α-helical lid. A S83T mutation was found in six of the seven most active variants, which in the homologous *Rhizopus oryzae* lipase had been proven to alter the chain-length preference.

4.4.2 Nitrilase

A substantially improved nitrilase that can be used for the industrial production of (*R*)-4-cyano-3-hydroxybutyric acid (Figure 4.4), an important atorvastatin (Lipitor) intermediate, was created by evolutive desing using the gene site saturation mutagenesis (GSSM) method developed by Diversa Inc. [131]. In GSSM, each

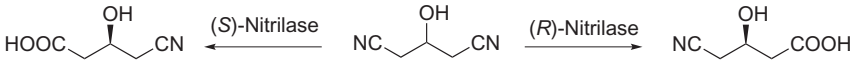

Figure 4.4 Desymmetrization of prochiral 3-hydroxyglutaronitrile using evolved nitrilases.

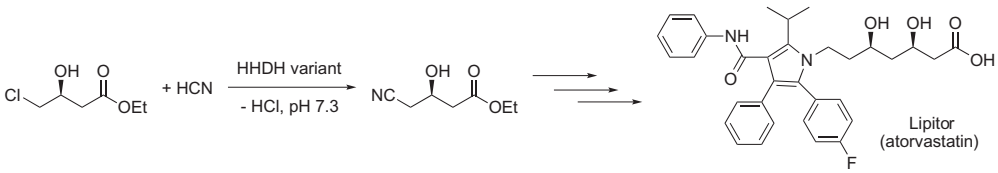

Figure 4.5 Synthesis of a key precursor of Lipitor using a halohydrin dehalogenase (HHDH). The best variant contained 35 mutations.

amino acid of a protein is replaced with each of the 19 remaining proteinogenic amino acids [132, 133]. This is accomplished at the genetic level with a primer set including either 32 or 64 codon variants for each amino acid residue of the wild-type enzyme. Thus, GSSM permits unbiased access to all codon variants. Nevertheless, this method may not be applicable to most academic laboratories because of its high cost and the lack of sophisticated HTS equipment. In this example, the wild-type nitrilase catalyzed the desymmetrization of 3-hydroxyglutaryl nitrile with a product ee of 95% at 100 mM substrate concentration. But using the industrially more relevant concentration of 3 M, an unsatisfying enantiomeric excess of 87.6% ee was observed. Therefore a library that accesses every single amino acid variant of the 330-amino-acid wild-type enzyme with a theoretical number 10 528 variants was created and screened with three times oversampling in an HTS based on mass spectrometric detection using a mixture of the natural and an isotopically N-labeled prochiral substrate. This led to the identification of a variant with a single mutation (A109H) showing excellent enantioselectivity and enabling a volumetric productivity of 619 g/l per day. This would have been very unlikely using epPCR or gene recombination, because a mutation of alanine to histidine requires the exchange of two bases in the codon triplet.

4.4.3 Halohydrin Dehalogenase

The catalytic function of a halohydrin dehalogenase (HHDH) from *Agrobacterium radiobacter* was improved by ProSAR-driven evolution, introduced by Fox *et al.*, to obtain ethyl (*R*)-4-cyano-3-hydroxybutyrate, which is again involved in the synthesis of atorvastatin (Lipitor) (Figure 4.5) [50]. The new enzyme had a 4000-fold volumetric productivity in the cyanation process compared with the wild type. This example also demonstrates the efficiency of combining theoretical consideration with laboratory methods for the improvement of biocatalyst function.

4.4.4
P450 Monooxygenase

A very useful enzyme for industry is the cytochrome P450 monooxygenase and several techniques involving the mutagenesis of P450 BM-3 (from *Bacillus megaterium*, a natural fusion protein of P450 monooxygenase and reductase peptides) have led to a variety of biocatalysts with features of industrial interest (drug development, biodegradation processes, and biocatalysis). For example, directed evolution was used by Wong *et al*. to increase the catalytic activity of cytochrome P450 BM-3 in organic cosolvents. The starting enzyme was a previously isolated mutant, P450 BM-3 F87A, with higher activity [105]. Mutants were obtained using epPCR followed by saturation mutagenesis and their catalytic activity was screened in a 96-well plate format with and without the organic cosolvents dimethylsulfoxide (DMSO) and tetrahydrofuran (THF). The mutant F87A5F5, isolated in the second round of random mutagenesis, displayed increased specific activity (5.5-fold in 10% DMSO; 10-fold in 2% THF). The mutant W5F5, where the alanine at position 87 was mutated back to a phenylalanine, gave a nearly six-fold higher specific activity in 25% DMSO, but only a 3.4-fold increase in 2% THF. The specific activities of the mutants were also determined in four other water-miscible cosolvents: acetone, acetonitrile, dimethylformamide (DMF), and ethanol. In all cases the activity was increased and comparable to the improvements observed for DMSO and THF.

4.4.5
Aldolases

A broad range of aldolases has been described in literature, which allow the C-C-bond formation between an aldehyde and a donor molecule (i.e., dihydroxyacetone phosphate (DHAP), pyruvate, phosphoenolpyruvate, etc.) creating thereby up to two stereogenic centers in one reaction step. This makes these biocatalysts very attractive for organic synthesis [134, 135]. However, narrow substrate ranges and enzyme inactivation under synthesis conditions represented major obstacles for the large-scale application of aldolases.

Again, these limitations could be overcome by directed evolution. A very elegant route for the aldolase-catalyzed synthesis of a key intermediate of Lipitor was initially developed by the Wong group [136] in which DERA (2-deoxy-D-ribose 5-phosphate aldolase) catalyzed the highly enantioselective tandom aldol reaction between chloroacetaldehyde and two equivalents of acetaldehyde (Figure 4.6).

However, the enzyme lacks sufficient stability in the presence of acetaldehyde and the reaction was improved in a collaboration with Diversa Inc. [138] and by researchers at DSM. They identified more stable variants in mutant libraries by screening for higher stability and improved productivity. By combination of the best variants, a 10-fold improvement under industrially relevant conditions was achieved [137]. The Wong group also described an *in vivo* selection assay for the directed evolution of an L-rhamnulose aldolase starting from an L-rhamnulose-1-phosphate aldolase (RhaD) [139]. This assay enabled the identification of the

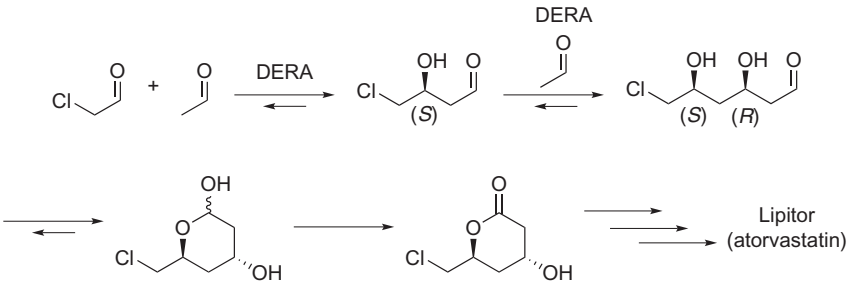

Figure 4.6 Synthesis of a key precursor of Lipitor using 2-deoxy-D-ribose 5-phosphate aldolase (DERA) in a tandem aldol reaction with chloroacetaldehyde and two equivalents of acetaldehyde as starting materials [137].

desired RhaD variant out of an epPCR-derived library, which in contrast to the wild type accepted dihydroxyacetone instead of the very expensive and unstable phosphorylated analog DHAP as donor. Also, a KDPG-(D-2-keto-3-deoxy-6-phosphogluconate) aldolase could be improved with respect to its catalytic efficiency, substrate specificity, and stereoselectivity by means of directed evolution [140]. A related enzyme utilizing galactose derivatives (KDPGal) was subjected to a combination of epPCR, DNA shuffling and site-directed mutagenesis, yielding a variant with 60-fold improved catalytic efficiency in the synthesis of a shikimate pathway product [141].

A broad range of further examples of aldolases improved by directed evolution can be found in a number of recent reviews [142–144].

4.5 Conclusions

Only a few years after directed evolution was first described for protein engineering, this technology has emerged as a very powerful tool for the design and alteration of the properties of enzymes. This method quickly found its application in a broad range of proteins, with the vast majority being of interest for biocatalysis. Consequently, a diverse set of molecular biology tools to create well-balanced mutant libraries as well as suitable HTS methods have been developed to make the application of directed evolution easier and more feasible. Within just a decade, directed evolution has emerged as a standard methodology in protein engineering and therefore can be used complementary or in combination with rational protein design to meet the demand for industrially applicable biocatalysts exhibiting the desired chemo-, regio-, and stereoselectivity, as well as withstanding process conditions (i.e., high substrate concentrations, solvents, temperatures, long-term stability). Furthermore, this methodology already found its way into various industrial applications as exemplified above for selected detailed examples and as summarized in Table 4.1.

Table 4.1 Selected examples of biocatalysts improved by directed evolution methods.

Enzyme (origin)	Target	Mutagenesis method	Assay	Improved property	Applications	References
P. fluorescens esterase	Enantioselectivty against 3-butyn-2-ol	epPCR	Acetate assay	Increased enantioselectivity	Production of (*S*)-3-butyn-2-ol with >99% eep (chiral building block)	[79]
P. fluorescens esterase	Enantioselectivity against methyl-3-bromo-2-methylpropionate	Saturation mutagenesis near the active site	pNPA assay, QuickE	5-fold higher enantioselectivity	Production of MBMP (important chiral synthon)	[89]
P. fluorescens esterase	Enhanced stability	epPCR and StEP	In MTP with heat treatment	Increase in thermostability and decrease in substrate inhibition	(*S*)-ketoprofen	[145]
P. aeruginosa lipase	Enantioselectivity	epPCR Saturation mutagenesis Cassette mutagenesis	In MTP with chiral *p* -nitrophenyl esters	E increase from 1 to 64	–	[120–122]
B. subtilis esterase	High activity in DMF towards pNB-esters of loracarbef	epPCR Saturation mutagenesis Shuffling	In MTP with *p*-nitrophenyl esters	Activity increased 150-fold in 15% DMF, increased temperature stability	Industrial biocatalysis	[114]
Coprinus cinereus heme peroxidase	Stability and activity under laundry conditions	epPCR Saturation mutagenesis Shuffling	Incubation in MTP at high pH with H_2O_2, measuring residual activity	100-fold oxidative stability 170-fold thermal stability	Laundry industry	[100]

Arthrobacter sp. Hydantoinase	Inverted enantioselectivity	epPCR Saturation mutagenesis	pH indicator	L-Selective and 5-fold more active	Industrial L-amino acid production	[146]
Subtilisin	Thermostability	epPCR Saturation mutagenesis	Synthetic peptide *p*-nitroanilide (s-AAPF-pNa)	Increase to 60 °C	Laundry industry	[147]
25 *Bacillus* sp. subtilisins (Savinase®)	Activity Thermostability Organic solvent-tolerance pH-profile	Family shuffling	Clearing halo in milk agar FRET (BODIPY-FL-casein)	Improved characteristics for all parameters	Laundry industry	[148, 149]
Aspergillus fumigatus phytase	Improved activity at lower pH	Rational design Site-directed mutagenesis	*p*-Nitrophenyl phosphate	Decreased pH optimum by 0.5–1.0 units	Animal feed industry	[150]
Trichoderma reesei xylanase	Thermotolerance Altered pH optimum	Rational design Site-directed mutagenesis	White halos in xylan-containing agar plates coupled to Remazol brilliant blue	4- to 5-fold increased thermostability at 60–65 °C	Pulp bleaching	[151]
Soybean β-amylase	Altered pH optimum	Rational design Site-directed mutagenesis		pH shift from 5.4 to 6.0–6.6	Food industry	[152, 153]
B. amyloliquefaciens α-amylase	Specific activity and activity at alkaline pH	Site-directed mutagenesis epPCR Shuffling	Modified Phadebas assay (Pharmacia)	Improved activity at pH 10	Laundry industry	[154]

Table 4.1 Continued.

Enzyme (origin)	Target	Mutagenesis method	Assay	Improved property	Applications	References
Thermotoga neapolitana xylose isomerase	Improved activity with glucose at lower temperature and pH	epPCR	MTP assay using resorcinol-ferric $(NH_4)_2SO_4$/HCl method	Lowered optimal temperature (60 °C) 12-fold higher activity	Industrial biocatalysis	[155]
P450 BM3	Hydroxylation of short-chain alkanes such as octane	epPCR StEP	Selection with an octane analog Screening NADH consumption	100-fold enhancement for octane Fastest alkane hydroxylase known to date	Biocatalysis and biodegradation	[156]
P450 BM3	Regio- and stereoselective hydroxylation of linear alkanes	StEP Site-directed mutagenesis epPCR Shuffling	Screening NADPH consumption with propane	Improved regio- and stereoselectivity in the synthesis of (*S*)- and (*R*)-octanol	Asymmetric synthesis	[157]
P450 BM3	Activity in organic solvents	epPCR and saturation mutagenesis	In MTP with pNCA and cosolvents	5.5-fold increase of activity in DMSO, 10-fold in THF	Oxidative biocatalysts	[105]
B. cepacia/P. pseudoalcaligenes biphenyl dioxygenase	Substrate specificity	Shuffling / StEP	Colorimetric agar plate assay using biphenyl or PCB	Enhanced activity towards polychlorinated biphenyls, benzene and toluene	Biodegradation of persistent pollutants	[158]

B. cepacia toluene monooxygenase	Improved conversion of naphthalene and degradation of chlorinated pollutants (TCE)	Shuffling	Colorimetric MTP assay diazo dye formation from tetraazotized *o*-dianisine	6.4-fold faster conversion / 2-fold increase in TCE degradation	Biodegradation of persistent pollutants	[159]
Rhodococcus sp. haloalkane dehalogenase	Improved degradation of chlorinated pollutants (TCP)	epPCR Shuffling	pH indicator	8-fold increase in k_{cat}/K_M for trichloropropane (TCP) dehalogenation	Degradation of toxics and persistent pollutants	[160]
A. tumefaciens *N*-carbamoylase	Thermo- and oxidative stability	Shuffling	pH indicator	Increased thermo- and oxidative stability	Industrial D-amino acid production	[161]
Pseudomonas sp. glutaryl acylase	Substrate specificity	Spiked oligo PCR Saturation mutagenesis	Growth assay (selection on adipyl-serine)	3- to 10-fold increase of adipyl-acylase activity	Production of semi-synthetic antibiotics	[162, 163]
P. putida benzoylformate decarboxylase	Substrate specificity	epPCR	pH indicator	Improved ee from 82 to 93% Novel carboligase activitiy on *o*-substituted benzaldehydes	Biosynthesis of chiral precursors	[164]
P. diminuta phosphotriesterase *A. radiobacter* phospho-triesterase	Substrate specificity	epPCR Shuffling Site-directed mutagenesis	Agar-plate assay using coumaphos-*o* -analog	Enhanced activity towards organophosphates	Bioremediation of wastewater	[165]

Table 4.1 Continued.

Enzyme (origin)	Target	Mutagenesis method	Assay	Improved property	Applications	References
Nitrilase	Enantioselectivity / volumetric productivity	GSSM	Mass spectrometric detection of isotopically different products	Higher activity and enantioselectivity in 3 M substrate solution	Synthesis of Lipitor intermediate	[131]
Monoamine oxidase from *Aspergillus niger* (MAO-N)	Activity and enantioselectivity	Mutator strain *E. coli* Xl1-Red	HRP-coupled assay	47-fold higher activity, 5.8-fold increased selectivity	Deracemization of of α - methylbenzylamine and cyclic secondary amines	[21, 22, 166, 167]
Halohydrin dehalogenase (HHDH) from *Agrobacterium radiobacter*	Volumetric productivity	ProSAR-driven evolution	Different activity tests	4000-fold improvement	Synthesis of Lipitor intermediate	[50]
Epoxide hydrolase from *Agrobacterium radiobacter*	Enantioselectivity	epPCR and shuffling	Agar plate assay and activity assay towards *p* - nitrophenyl glycidyl ether (pNPGE)	Up to 13-fold increased enantioselectivity against pNPGE, as well as towards epichlorohydrin and 1,2-epoxyhexane	Production of chiral precursor for pharma and fine chemicals	[168–171]

MTP, microtiter plate.

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5
The Industrial Production of Enzymes

Reeta Rani Singhania, Anil Kumar Patel, and Ashok Pandey

5.1
Introduction

Enzymes are protein molecules (except ribozymes which are RNA molecules) that catalyze biochemical reactions. In enzymatic reactions, the molecules at the beginning of the process, called substrates, are converted into different molecules, the products. The enzymes speed up the reaction by forming transition state complexes with the substrate which reduces the activation energy of the reaction. Enzymes are attractive for industrial purposes because they are efficient and selective in the chemistries they accelerate and they act in a similar way to the inorganic catalysts used in the chemical industry.

Enzyme technology is an interdisciplinary field, and enzymes are routinely used in many environmental friendly industrial sectors. Recent advancements in biotechnology, especially in the areas of genetics and protein engineering, have opened a new arena for the application of enzymes in many industrial processes. The industrial enzymes represent the heart of biotechnology processes. A series of major R&D initiatives have seen not only the development of a number of new products but also improvements in the process and performance of several existing processes. Almost all processes in a biological cell need enzymes to occur at significant rates. Since enzymes are selective for their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathways occur in that cell.

Enzymes have been used throughout human history in cheese manufacturing and in food manufacturing indirectly via yeasts and bacteria. Isolated enzymes were first used in detergents in 1914, although their protein nature was not proven until 1926, and their large-scale microbial production started in the 1960s. The industrial enzyme business is steadily growing due to improved production technologies, engineered enzyme properties, and new applications.

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5.2
Enzyme Production

Most of the enzymes are commercially produced by microorganisms through submerged fermentation though some are produced by solid-state fermentation. The major industrial enzymes are produced by GRAS (generally recognized as safe)-status microorganisms in large biological reactors called fermenters. However, some enzymes are still extracted from animal or plant tissues. Plant-derived commercial enzymes include the proteolytic enzymes papain, bromelain, and ficin and some other specialty enzymes, such as lipoxygenase from soybeans. Animal-derived enzymes include proteinases like pepsin and rennin. Usually the production organism and often also the individual enzyme have been genetically modified for maximum productivity and the desired enzymatic properties. The downstream processing steps for industrial enzymes depend on the degree of purity required, which in turn depends on the application. Large-volume industrial enzymes are not usually purified but sold as concentrated liquids or granulated dry products. Enzymes used in special applications, such as diagnostics or DNA technology, need to be highly purified.

The enzyme production process can be divided into phases as shown in Figure 5.1.

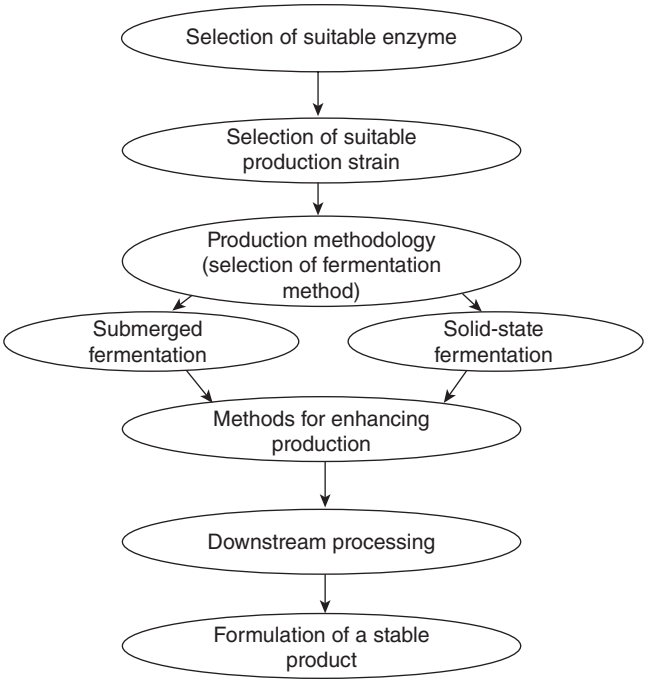

Figure 5.1 Steps involved in enzyme production.

5.2.1
Selection of a Suitable Enzyme

Criteria used in the selection of an industrial enzyme include specificity, reaction rate, pH and temperature optima and stability, effect of inhibitors, and affinity to substrates. Specific enzymes are required with specific properties based on particular applications. For example, enzymes used in the paper industry should not contain cellulose-degrading activity as a side-activity because this would damage the cellulose fibers. The enzymes used in the animal feed industry must be thermotolerant to survive in the hot extrusion process used in animal feed manufacturing but at the same time they must have maximal activity at the body temperature of the animal. Enzymes used in industrial applications must usually be tolerant against various heavy metals and have no need for cofactors.

5.2.2
Selection of a Suitable Production Strain

Several aspects have to be considered in the selection of suitable source for enzyme production. Microorganisms are the preferred source for industrial enzymes, rather than plants or animals, because of their fast multiplication rate and ease of culture. During the past few decades, the use of filamentous fungi for the production of primary and secondary metabolites has increased rapidly. In general, the extracellular enzyme producers are preferred to intracellular producers because the recovery and purification processes are much simpler; intracellular enzymes have to be purified from thousands of different cell proteins and other components. Second, the production host should have GRAS-status, which means that it is "generally recognized as safe." This is especially important when the enzyme produced by the organism is used in food processes. Third, the organism should be able to produce large amounts of the desired enzyme in a reasonable time-frame.

New developments in screening have made it possible to start with the optimal parameters of the process, establish the specifications of the desired biocatalyst (enzyme), and shift through the natural biodiversity for the ideal biocatalyst [1]. In addition, the available biocatalysts can be pushed to the extremes of operating conditions and the physical limitations of the proteins by directed evolution or gene shuffling [2–4]. The development of efficient methods to create and handle diversity creates new challenges in screening methodologies and is causing a trend towards robotic handling, new equipment (e.g., parallel capillary electrophoresis, thermistor arrays), new fluorophores and chromophores, and new approaches to screening for stereoselectivity [5]. In addition, numerous new sources of the enzymes such as the extremophiles [6–10], non-culturable organisms (www.diversa.com), and completely sequenced genomes (www.ncbi.com) have become available.

The use of the wild type of organisms has several advantages as they often produce mixture of enzymes required to degrade a complex substrate. But some

wild-type organisms are not easy to scale-up, and some may pose risks for safety. In most cases, the genetically modified organism possess a greater range of enzymes with varying properties, such as improved activities or specificity, safe to handle and reduced content of foreign proteins. The industrial strains typically produce over 50 g/l extracellular enzyme proteins. Most of the industrial enzymes are produced by a relatively few microbial hosts, such as *Aspergillus* and *Trichoderma* fungi, *Streptomyces* fungi imperfecti, and *Bacillus* bacteria. Yeasts are not good producers of extracellular enzymes and are rarely used for this purpose.

5.2.3 Production Methodology

Once the organisms has been selected, the production process has to be developed. Submerged fermentation has been extensively used for the industrial production of enzymes to date, but solid-state fermentation is rapidly gaining interest worldwide for the production of primary and secondary metabolites [11–13]. The use of agro-industrial residues offers potential advantages in solid-state fermentation processes [14–17]. The optimization of a fermentation process includes choice of media composition, cultivation type, and process conditions irrespective of the type of bioprocess and considerable effort and time needs to be expended to accomplish these tasks. Before starting with any bioprocess, several aspects have to be looked at: Is the organism in question safe? Are extra precautions needed? What kind of nutrients does the organism need and what are their optimal/economic concentrations? How should the nutrients be sterilized? What kind of reactor is needed (mass transfer, aeration, cooling, foam control, sampling)? What variables need to be measured and how should the process be controlled? What method of cultivation is best for this organism (batch, fed-batch, or continuous cultivation)? What are the optimal growth conditions, the specific growth and product formation rate, the yield and volumetric productivity? How can cell concentration be maximized in the reactor? How should the cell be degraded if the product is intracellular and how should the product be recovered, purified, and preserved? Choice of the fermentation process is also critical and depends exclusively on the final product and its ultimate application.

5.2.3.1 Submerged Fermentation

Most of the industrial production of the enzyme are carried out by submerged fermentation, generally employing genetically modified strains. Bioreactors for submerged fermentation are well developed and offer online control over several parameters and there is no problem for mass transfer and heat removal. The large-volume industrial enzymes are produced in 50–500 m^3 fermenters. The medium in submerged fermentation is liquid which remains in contact with the microorganism. A supply of oxygen is essential in submerged fermentation.

There are four main ways of growing microorganisms in submerged fermenters: batch culture, fed-batch culture, perfusion batch culture, and continuous culture. In batch culture the microorganisms are inoculated in fixed volume of

medium. In the case of fed-batch culture, the concentrated components of the nutrient are gradually added to the batch culture. In perfusion batch culture, the addition of the culture and withdrawal of an equal volume of used cell-free medium is performed. In continuous culture, fresh medium is added into the batch system during the exponential phase of microbial growth with a corresponding withdrawal of the medium containing the product. Continuous cultivation gives near-balanced growth, with little fluctuation in nutrients, metabolites, cell numbers, or biomass.

Extracellular enzymes are often recovered after cell removal (by vacuum drum filtration, separators or microfiltration) by ultrafiltration. If needed, purification is carried out by ion exchange or gel filtration. The final product is either a concentrated liquid with necessary preservatives such as salts or polyols or alternatively granulated to a non-dusty dry product. It should be remembered that enzymes are proteins, and as such can cause and have caused in the past allergic reactions. Therefore protective measures are necessary in their production and application.

5.2.3.2 **Solid-State Fermentation**

The high cost of enzyme production by submerged fermentation makes it uneconomical to use many enzymes in several industrial processes. To reduce the cost of production, solid-state fermentation is an attractive alternative. This is a low-cost fermentation process, particularly suitable for the development of bioprocesses using agro-residues. Solid-state fermentation appears to possess several biotechnological advantages, such as higher fermentation productivity, higher end-concentration of products, higher product stability, lower catabolic repression, cultivation of microorganisms specialized for water-insoluble substrates or mixed cultivation of various fungi, and last but not least, lower demand on sterility due to low water activity [18]. Although at present mostly operated on a laboratory scale. solid-state fermentation is becoming popular as a large number of industrial enzyme production involves fungal strains which are better suited for solid-state fermentation as it more resembles their natural environment. Submerged fermentation does not replicate the natural habitat of wild-type microorganisms.

Scale-up, purification of end-products, and biomass estimation are the major challenges that have led the researchers to search for solutions. Scale-up in solid-state fermentation has long been a limiting factor, but recently with the advent of biochemical engineering a number of bioreactors have been designed that overcome the problems of scale-up and, to an extent, also the on-line monitoring of several parameters, as well as heat and mass transfer.

Although product recovery and purification processes are more expensive in solid-state fermentation, employing natural supports, their utilization supposes a reduction in production costs and usually much higher activities are obtained [19]. Hence, an economic evaluation of the overall process should be done before determining its feasibility for a specific purpose. This system is especially suitable for the production of high-value products such as enzymes. There are particular applications where concentrated end-products with high titers are required rather than purified products. For example, bioconversion of biomass requires

concentrated crude cellulase, and in the leather industry crude proteases are enough to remove the hair from the leather.

Separation of biomass is a big challenge in solid-state fermentation, and this is essential for kinetic studies. Certain indirect methods are available, such as glucosamine estimation, ergosterol estimation, protein (kjeldahl) estimation, DNA estimation, dry weight changes, and CO_2 evolution, but all of these have their own weaknesses. Recently, digital image processing has been developed as a tool for measuring biomass in solid-state fermentation. The images are acquired by stereomicroscope and a digital camera and processed using KS400 software [20]. In recent times, the estimation of oxygen intake and carbon dioxide evolution rate have been considered to be most accurate for the determination of growth of the microorganism [21–22].

Tengerdy [23] advocated that solid-state fermentation was particularly suitable for lignocellulosic enzyme production for various agro-biotechnological applications. To illustrate this, cellulase production was compared in submerged fermentation and solid-state fermentation [23, 24]. In the submerged fermenter, the cellulase yields were generally about 10 g/l, and the average fermentation cost in a stirred tank bioreactor was about US$200/m^3. Thus, the production cost of crude fermentation by the submerged fermentation was about US$20/kg. In the solid-state fermenter, the average production level was about 10 mg/g substrate and the average fermentation cost only about US$25/mt. Thus, the unit cost of cellulase produced by solid-state fermentation was about US$0.2/kg [16, 23].

Much published information is available on the production of enzymes of industrial importance, such as proteases, cellulases, ligninases, xylanase, pectinase, amylase, glucoamylase, inulinases, phytases, tannase, phenolic acid esterase, microbial rennet, aryl-alcohol oxidase, oligosaccharide oxidase, tannin acyl hydrolase, α-L-arabinofuranosidase, in submerged fermentation or solid-state fermentation [25–34].

5.2.4
Downstream Processing

The cost for the purification and conditioning of enzymes for final use – that is the downstream processing of enzymes – accounts for more than 50% of the total enzyme production cost. The cost of downstream processing depends on the degree of purity required, hence on the end use of the enzyme. The downstream processing cost for a therapeutic enzyme will clearly be higher than that for a technical enzyme because of the purity required and in order to reduce the production cost downstream processing must be improved. The preliminary steps involved in enzyme purification are shown in Figure 5.2.

After the clarification step the solution containing intracellular and extracellular enzymes is further purified by ultrafiltration. For this particular step the molecular weight of the protein to be purified needs to be known so that impurities such as ions and other proteins of lower molecular weight can be removed. Even after ultrafiltration, the concentrated enzyme solution will still contain other proteins, nucleic acids, and polysaccharides. The removal of these impurities depends

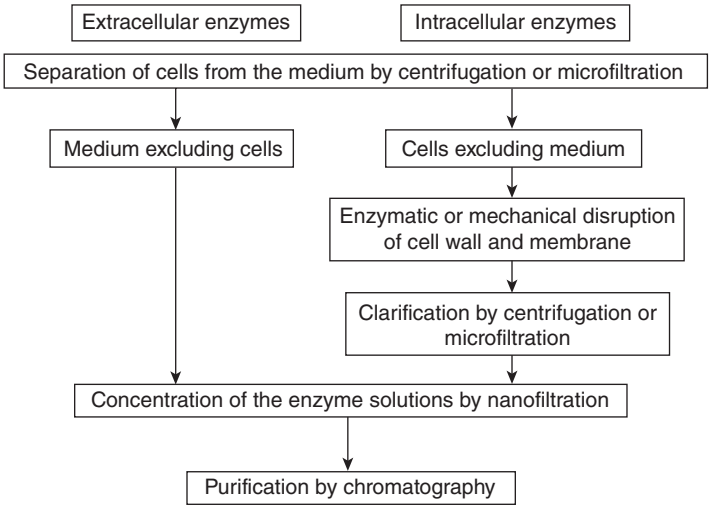

Figure 5.2 Preliminary steps involved in downstream processing of enzymes.

strictly on the use of the enzyme and also depends on whether the impurities inhibit the enzyme action as a catalyst in a particular process. For recombinant enzyme, removal of nucleic acid is necessary to minimize the risk of unwanted gene transfer. Enzyme concentration favors chromatographic purification also as less adsorbent is required to adsorb the protein when it is in a concentrated form. Enzymes are generally purified by chromatography. Different enzyme properties, such as molecular size, surface charge, and hydrophobicity of enzyme surface, are used for their separation with different chromatographic adsorbents.

Purification of an enzyme from a concentrated clarified homogenate previously used to involve at least five separation steps including chromatography, and 10% of the enzyme could be lost during each purification step, leading to low enzyme recovery. Hence to reduce the cost of enzyme, increase in the enzyme recovery and thereby reduction in the number of purification steps was very important. In recent years process engineering of chromatography has improved considerably, including the development of continuous chromatographic process such as simulated moving bed and continuous separation, which have been recently introduced in the downstream processing of proteins [35]. Thus it is possible to purify the enzymes now with a high recovery rate and limited number of steps, which helps in reducing the cost of the enzyme.

5.3 Enzyme Improvement

Although highly attractive for biological and chemical synthesis, enzymes may not possess the desired properties when operated on an industrial scale. For example,

they may lose their stability or tolerance to the changes in the operating parameters, their high activity in non-aqueous media, and their ability to work without expensive cofactors. The reliable and quick identification of the amino acid substitutions that generate desired changes in enzyme performance remains the ultimate goal for protein engineering research.

Thermal stability can be improved by site-directed and random mutagenesis as well as by directed mutagenesis. These are now established techniques.

It has been proved very difficult to overproduce enzymes in a suitable host. Another option is to engineer a commercially available enzyme to be a better industrial catalyst. In the future, enzymes may be redesigned to fit more appropriately into industrial processes; for example, making glucose isomerase less susceptible to inhibition by the Ca^{2+} present in the starch saccharification processing stream. The amount of enzyme produced by a microorganism may be increased by increasing the number of gene copies that code for it. This principle has been used to increase the activity of penicillin G amidase in *Escherichia coli*. Site-directed mutagenesis is another approach for creating new enzymes. It involves stepwise substitution of only one or two amino acid residues out of the total protein structure.

Although a large and rapidly expending database of sequence–structure correlations is available, together with the necessary software, it is still impossible accurately to predict three-dimensional changes as a result of such substitutions. The main problem is assessing the long-range effects, including the solvent interactions on the new structure. Apparently, even quite small sequence changes may give rise to large conformational alterations and these may affect the rate-determining step in enzymatic catalysis.

Nevertheless, it is reasonable to suppose that, given a sufficiently detailed database plus suitable software, the relative probability of success will increase over the coming years and the products of protein engineering will make a major impact on enzyme technology.

5.3.1 Recombinant DNA Technology

Microorganisms isolated from diverse environments represent a source of enzymes that can be used for industrial process chemistry. Using high-throughput screening (HTS) methods, new biocatalysts can be found from these microorganisms. But many microorganisms are not easily cultivated in laboratory conditions or their enzyme yield is too low to be economically feasible. Using recombinant DNA technology, cloning the genes encoding these enzymes and heterologously expressing them in commonly used industrial strains has become a common practise.

The novel enzymes suitable for specific conditions may be obtained by genetically modifying the microorganism. Recombinant DNA technology enables the production of enzymes at levels 100-fold greater than native expression, making them available at low cost and in large quantities [36]. As a result, several important food-processing enzymes, such as amylases and lipases, with properties tailored

to particular food applications have become available. Several microbial strains have been engineered to increase enzyme yield by deleting native genes encoding extracellular proteases. Moreover, certain fungal production strains have been modified to reduce or eliminate their potential for the production of toxic secondary metabolites [37]. This approach precludes the transfer of any extraneous or unidentified DNA from the donor organisms to the production strain.

5.3.2 Protein Engineering

Although the use of recombinant DNA technology significantly lowers the cost of enzyme production, the applications for the enzymes produced are still limited. Most chemicals of industrial interest are not natural substrates for these enzymes. If a desired enzyme activity is found, the yield is often low. Moreover, enzymes are not usually stable in harsh reaction conditions, such as pH higher or lower than physiological pH 7, high temperature, or the presence of organic solvents required to solubilize many substrates. With recent advances in polymerase chain reaction (PCR) technology, site-specific and random mutageneses are readily available to improve enzyme stability in a wider range of pH values and temperatures and tolerance to a variety of organic solvents.

Since a large quantity of enzyme can be obtained by recombinant expression, X-ray crystallography can be used to facilitate the understanding of the tertiary structure of an enzyme and its substrate-binding/recognition sites. This information may assist a rational design of the enzyme, predicting amino acid changes for altering substrate specificity, catalytic rate, and enantioselectivity (in the case of chiral compound synthesis).

Two PCR-mediated methods – random or saturated site-specific mutagenesis and gene shuffling – are generally used for generating mutants, and to engineer a commercially available enzyme to be a better industrial catalyst, two different approaches are presently available: a random method called directed evolution (see Chapter 4) and a protein engineering method called rational design.

In protein engineering a protein sequence is changed to achieve a desired result, such as a change in the substrate specificity or increased stability to temperature, organic solvents, and/or extremes of pH. Many specific methods for protein engineering exist, but they can be grouped into two major categories: rational design and combinatorial methods. The rational methods, such as site-directed mutagenesis, require targeted amino acid substitutions, and therefore, require a large body of knowledge about the biocatalyst being improved, including the three-dimensional structure and the chemical mechanism of the reaction. The main advantage of rational design is that a very small number of protein variants are created, meaning that very little effort is necessary to screen for the improved properties.

The combinatorial methods, on the other hand, create a large number of variants that must be assayed; however, they have the advantage of not requiring such extensive knowledge about the protein. In addition, often non-obvious changes in the protein sequence lead to large improvements in their properties, which are

extremely hard to predict rationally, and thus, can only be identified by the combinatorial methods.

Several enzymes have already been engineered to function better in industrial processes. These include the proteinases, lipases, cellulases, α-amylases, and glucoamylases. Xylanase is a good example of an industrial enzyme that needs to be stable at high temperature and active at physiological temperatures and pH when used as a feed additive and in alkaline conditions when used for bleaching in the pulp and paper industry. One of the organisms used in the industrial production of xylanase is *Trichoderma* sp. Its xylanase has been purified and crystallized and by designed mutagenesis its thermal stability has been increased by about 15 °C. Mutational changes have increased the half-life at 65 °C from approximately 40 s to approximately 20 min, and at 70 °C from less than 10 s to approximately 6 min [38]. This has increased its thermal stability at 70 °C about 2000 times and its pH optimum has been shifted towards alkaline region by one pH unit. The most successful strategies to improve the stability of the *Trichoderma* xylanase include the stabilization of the alpha-helix region and the N-terminus.

5.4 Large-Scale Enzyme Applications

Isolated enzymes have found several applications in the fine chemical industry. Enzymes are used in the production of chirally pure amino acids and rare sugars. They are also used in the production of fructose and penicillin derivatives as well as several other chemicals. Enzymes should be considered as a part of a rapidly growing biocatalyst industry also involving genetically optimized living cells as chemical production factories. Table 5.1 summarizes major large-scale enzyme applications.

5.4.1 Detergents

Detergents were the first large-scale application for microbial enzymes. The use of enzymes in detergent formulations is now common in developed countries, with half of the detergents available containing enzymes. Dirt comes in many forms and includes proteins, starches, and lipids. The use of enzymes allows lower temperatures to be employed and shorter periods of agitation, often after a preliminary period of soaking. In general, enzyme detergents remove protein from clothes soiled with blood, milk, sweat, grass, etc. far more effectively than non-enzyme detergents.

Proteinases are still the most important detergent enzymes used to hydrolyze stains caused by proteins. Lipases decompose fats into more water-soluble compounds by hydrolyzing the ester bonds between the glycerol backbone and fatty acid. Amylases are used in detergents to remove starch-based stains. Amylases hydrolyze gelatinized starch, which tends to stick to textile fibers and bind other

Table 5.1 Application of various enzymes in important industrial sectors.

Industry	Enzyme	Application/function/role
Detergent	Protease	Removing protein stains by degrading them
	Cellulase	Loosening of cellulose fibers to easily remove dirt and color brightening
	Lipase	Removing fat stains by degrading them
Paper and pulp	Xylanase	Biobleaching
	Cellulase	De-inking of paper for recycling
	Laccases and peroxidase	Polymerizing materials with wood-based fibers
Textile	Cellulase	Bio stonewashing denim, biopolishing
	Amylase	Desizing of textiles
	Catalase	Bleach clean-up
Leather	Protease, lipase	Soaking, bating, and de-hairing of animal skin
Animal feed	Phytase	Release of phosphate
	Xylanase	Fiber solubility
Food industry		
Starch	α- and β-Amylase, pullulanase, invertase, glucose isomerase	Production of various types of syrups from starch and sucrose
	Glucose oxidase	Enhancing the storability of food by removing oxygen and glucose from the food stuff
Fruit juice	Cellulase, xylanase, pectinase	Juice clarification and juice extraction
Bakery	Xylanase	Dough conditioning
	α-Amylase	Loaf volume, shelf-life
	Glucose oxidase	Dough quality
Dairy	Renin	Protein coagulation
	Lactase	Lactose hydrolysis
	Protease and lipase	Ripening of cheese
Brewing	Glucanase	Filter aid
	Papain	Haze control
Biofuel	Cellulase and β-glucosidase	Hydrolyzing cellulosic biomass to generate glucose
	Xylanase	Hydrolyzing hemicelluloses to generate pentoses

Table 5.1 Continued.

Industry	Enzyme	Application/function/role
Personal care products	Proteinase and lipase	Contact lens cleaning
	Glucose amylase	Liberating glucose from starch-based oligomers
	Glucose oxidase	In toothpaste to convert glucose into gluconic acid and hydrogen peroxide as both act as disinfectant

stain components. Cellulases have been part of detergents since the early 1990s. Cellulase is actually an enzyme complex capable of degrading crystalline cellulose to glucose. In textile washing cellulases remove cellulose microfibrils that are formed during washing. This results in color brightening and softening of the material.

5.4.2 Food Industry

5.4.2.1 Baking

Alpha-amylases have been most widely studied in connection with improved bread quality and increased shelf-life. Both fungal and bacterial amylases are used. The amount added needs to be carefully controlled as overdosage may lead to sticky dough.

One of the motivations for the study of the effect of enzymes on dough and bread qualities comes from the pressure to reduce other additives. In addition to starch, flour typically contains minor amounts of cellulose, glucans, and hemicelluloses such as arabinoxylan and arabinogalactan. There is evidence that the use of xylanases decreases water absorption and thus reduces the amount of added water needed in baking. This leads to a more stable dough. Xylanases in particular are used in wholemeal rye baking and the dry crisps common in Scandinavia.

Proteinases can be added to improve dough-handling properties; glucose oxidase has been used to replace chemical oxidants and lipases to strengthen gluten, which leads to more stable dough and better bread quality.

5.4.2.2 Starch Hydrolysis and Fructose Production

The use of starch-degrading enzymes was the first large-scale application of microbial enzymes in food industry. Mainly two enzymes carry out conversion of starch to glucose: alpha-amylase and glucoamylase. Sometimes additional debranching enzymes like pullulanase are added to improve the glucose yield. Beta-amylase is commercially produced from barley grains and used for the production of the disaccharide maltose.

In the United States large volumes of glucose syrups are converted by glucose isomerase after Ca^{2+} (alpha-amylase needs Ca^{2+} for activity but it inhibits glucose isomerase) removal to fructose-containing syrup. This is done by bacterial enzymes, which need Mg^{2+} ions for activity. Fructose is separated from glucose by large-scale chromatographic separation and crystallized. Alternatively, fructose is concentrated to 55% and used as a high-fructose corn syrup in the soft drink industry.

5.4.2.3 Drinks and Diary

Enzymes have many applications in the drinks industry. Chymosin is used in cheese making to coagulate milk protein. Another enzyme used in the milk industry is beta-galactosidase or lactase, which splits lactose into glucose and galactose. This process is used for milk products for lactose-intolerant consumers.

Enzymes are also used in fruit juice manufacture. The addition of pectinase, xylanase, and cellulase improve the liberation of the juice from the pulp. Pectinases and amylases are used in juice clarification. Similarly, enzymes are widely used in wine production to obtain a better extraction of the necessary components and thus improve the yield. Enzymes hydrolyze the high molecular weight substances, such as pectin.

Enzymes can be used to help the starch hydrolysis (typically alpha-amylases), solve filtration problems caused by beta-glucans present in malt (beta-glucanases), hydrolyze proteins (neutral proteinase), and control haze during maturation, filtration and storage (papain, alpha-amylase and beta-glucanase).

5.4.3 Animal Feed

Enzyme addition to animal feed has been intensively used since the 1980s. Enzymes reduce viscosity, increase absorption of nutrients, liberatate nutrients either by hydrolysis of non-degradable fibers or by liberating nutrients blocked by these fibers, and reduce the amount of feces. They are added as enzyme premixes (enzyme–flour mixture) during the feed manufacturing process, which involves extrusion of wet feed mass at high temperatures (80–90 °C). This means that the feed enzymes need to be thermotolerant during the feed manufacturing but operative in the animal body temperature.

The first commercial success came from addition of beta-glucanase to barley-based feed diets. Barley contains beta-glucan, which causes high viscosity in the chicken gut. The net effect of enzyme usage in feed is increased animal weight gain with the same amount of barley, resulting in an increased feed conversion ratio. Addition of xylanase to wheat-based broiler feed has increased the available metabolizable energy 7–10% in various studies.

Another important feed enzyme is phytase, which is a phosphoesterase that liberates phosphate from phytic acid. Phytic acid is commonly present in plant-based feed materials. Supplementation with phytase results in reduced phosphorous in the feces, resulting in reduced environmental pollution. It also minimizes the need to add phosphorous to the feed. Currently phytase is considered as one

of the most potent feed enzymes, in particular that from fungal sources [29]. Usually a feed-enzyme preparation is a multienzyme cocktail containing glucanases, xylanases, proteinases, and amylases.

5.4.4 Textiles

The use of enzymes in the textile industry is one of the most rapidly growing fields in industrial enzymology. Amylases are used for desizing textile fibers. Another important group of enzymes used in the textile industry is the cellulases. Due to their ability to modify cellulosic fibers and so improve the quality of fabrics in a controlled fashion, these (neutral or acidic) enzymes offer an excellent replacement for stonewashing of blue denim garments. Eliminating the use of stones has many advantages, such as reduced damage to the washers and garments, improved handling and fewer environment problems. Enzymatic stonewashing allows up to 50% higher jean load and produces the desired look and softer finish. Neutral cellulase is the enzyme of choice for stonewashing because of its lower backstaining and broader pH profile. This latter property reduces the need for rigid pH control of the wash, resulting in a more reproducible finish from wash to wash.

Fuzz formation and pilling are common problems associated with fabric using cotton or other natural fibers; cellulases are used to digest the small fiber ends protruding from the fabric, resulting in a better finish [39].

Recently, hydrogen peroxide has been tested as a bleaching agent for textiles to replace chlorine-based chemicals, and catalase has been used to degrade the excess peroxide. Another recent approach has been the use of oxidative enzymes directly to bleach textiles. Laccase – a polyphenol oxidase from fungi – is a new candidate in this field. This is a copper-containing enzyme which is oxidized by oxygen. In an oxidized state it can oxidatively degrade many different types of molecules, including dye pigments.

5.4.5 Pulp and Paper

Intensive studies have been carried out during the last 20 years to apply many different enzymes in pulp and paper industry. Xylanases are used in pulp bleaching, where they liberate lignin fragments by hydrolyzing residual xylan. This reduces considerably the need for chlorine-based bleaching chemicals. Cellulases are used for de-inking the cellulose fibers during recycling.

In paper making, amylases are used, especially for the modification of starch, which improves the strength, stiffness, and erasability of paper. The starch suspension must have a certain viscosity, which is achieved by adding amylase enzymes in a controlled process.

The removal of pitch, a sticky substance composed of lipids present mainly in softwoods, is a special problem when mechanical pulps of red pine are used as a raw material. Pitch can be removed by lipases.

5.4.6
Leather

The leather industry uses proteolytic and lipolytic enzymes in leather processing. The use of these enzymes is associated with the structure of animal skin as a raw material. Enzymes are used to remove unwanted parts. Alkaline proteases are added in the soaking phase. This improves water uptake by the dry skins, removal and degradation of protein, dirt and fats and reduces the processing time. In some cases pancreatic trypsin is also used in this phase.

Proteases are used in dehairing and dewooling of leather, and improve its quality (cleaner and stronger surface, softer leather, less spots). Lipases are used in this phase or in the bating phase to specifically remove grease. The use of lipases is a fairly new development in the leather industry.

5.4.7
Biofuel from Biomass

Perhaps the most important emerging application of enzymes currently being investigated is the utilization of lignocellulosic biomass for the production of biofuel. Biomass represents an abundant renewable resource available to humanity. Its use is limited, however, because of the lack of a cost-effective enzyme conversion technology due to the high cost of cellulases and the lack of specificity for various lignocellulosic substrates. The strategy employed currently in bioethanol production from biomass is a multistep process which includes enzymatic hydrolysis as a crucial step. In the effort to develop efficient technologies for biofuel production, significant research has been directed towards the identification of efficient cellulase systems and process conditions, besides studies directed at biochemical and genetic improvements of the existing organisms utilized in the process [40]. Effective strategies are yet to be resolved and active research is continuing in this direction.

5.4.8
Enzyme Applications in the Chemistry and Pharma Sectors

An important issue in the pharma sector is the large number of compounds that must be tested for biological activity to find a single promising lead. Combinatorial biocatalysis has received much attention here, as it could add a level of complexity to the diversity of existing chemical libraries or it could be used to produce the libraries *de novo* [41]. An example is the use of glycosyltransferases to change the glycosylation pattern of bioactive compounds. Although only a few commodity chemicals, such as acrylamide, are currently produced by enzyme technology (annual production scale 40 000 tons), this success has demonstrated that bioconversion technology can be scaled up. Many other chemicals, including chiral compounds [2], are also produced by biocatalysis on a multi-ton scale.

5.4.9
Specialty Enzymes

In addition to large-volume enzyme applications, there are a large number of specialty applications for enzymes. These include use of enzymes in clinical analytical applications, flavor production, protein modification, and personal care products, DNA technology, and in fine chemical production. Unlike bulk industrial enzymes, these enzymes have to be free from side-activities, which emphasizes the need for elaborate purification processes.

Alkaline phosphatase and peroxidases are used for immunoassays. An important development in analytical chemistry is biosensors. The most widely used application is a glucose biosensor involving a glucose oxidase catalyzed reaction:

$$\text{glucose} + O_2 + H_2O \rightarrow \text{gluconic acid} + H_2O_2$$

Several commercial instruments are available that apply this principle for measurement of molecules such as glucose, lactate, lactose, sucrose, ethanol, methanol, cholesterol, and some amino acids.

5.4.10
Enzymes in Personal Care Products

This is a relatively new area for enzymes and the amounts used are small but it is worth mentioning as a future growth area. One application is contact lens cleaning. Proteinase- and lipase-containing enzyme solutions are used for this purpose. Hydrogen peroxide is used in disinfections of contact lenses and the residual hydrogen peroxide after disinfections can be removed by a heme-containing catalase enzyme.

Glucoamylase and glucose oxidase are used in some toothpastes, as glucoamylase liberates glucose from starch-based oligomers produced by alpha-amylase and glucose oxidase converts glucose to gluconic acid and hydrogen peroxide which both function as disinfectants. Dentures can be cleaned with protein-degrading enzyme solutions. Enzymes such as chitinase are also being studied for applications in skin- and haircare products.

5.4.11
Enzymes in DNA Technology

The DNA-modifying enzymes play a crucial role in DNA technology that has revolutionized traditional as well as modern biotechnology. They can be divided into two classes:

- Restriction enzymes: These recognize specific DNA sequences and cut the chain at these recognition sites.
- DNA-modifying enzymes: These synthesize nucleic acids, degrade them, join pieces together and remove parts of the DNA.

Restriction enzymes produce cleavage after recognizing a specific code sequence in the DNA. They are essential in gene technology. DNA polymerases synthesize new DNA chains using a model template which they copy. Nucleases hydrolyze the phosphodiester bonds between DNA sugars. Kinases add phosphate groups and phosphatases remove them from the end of DNA chain. Ligases join adjacent nucleotides together by forming phosphodiester bonds between them.

In the cell these enzymes are involved in DNA replication, degradation of foreign DNA, repair of mutated DNA, and in recombining different DNA molecules. The enzymes used in gene technology are produced like any other enzyme but their purification needs extra attention.

5.5 Conclusions

Enzymes have been employed extensively to improve the environment, protect our resources, and create new opportunities. There is hardly any area which is untouched by enzymes. The global commercial enzyme market has been steadily growing as a result of improved production efficiency as well as downstream processing, giving cheaper and more efficient enzymes. These are widely employed in industries as discussed in detail in the chapter; and there is still scope for improvement. This emphasizes the need for continued effort to discover novel enzymes with novel properties from exotic environments through screening programmes.

Clearly, enzymes will be widely used in the future and this will be reflected in the number of the enzymes available on an industrial and research scale, the variety of reactions catalyzed, and environmental conditions under which they will operate. In particular, novel applications are to be expected in the field of personal care products and breakthroughs can be expected in second-generation biofuel production from biomass, where cellulases play a key role in the conversion of biomass to sugars which are further fermented to ethanol. Increasing environmental pressures and hikes in energy prices are likely to give this application real importance.

Industrial enzyme technology is an ongoing active research field going through a phase of maturation as well as evolution. A better understanding of the previously discovered enzymes and their functional significance suggests that there are many novel applications for their catalytic activities. The established enzymes will be put to the new uses and the novel enzymes, discovered within their biological niches or produced by the design using the enzyme engineering, will be used to catalyze hitherto unexploited reactions. This is just the start of the enzyme technology era. It is expected that tools for directed evolution and molecular biology techniques coupled with the development of powerful high-throughput screening and selection methods will solve challenging problems in protein engineering and metabolic engineering in coming years.

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6
Applied Biocatalysis: An Overview

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6.1
Introduction

The concept of applied biocatalysis is based on the use of a biocatalyst to promote the chemical transformation of a given substrate into a product, in a chemo-/regio-/stereo-selective manner, under mild, controlled conditions [1]. The biocatalyst can be either an isolated enzyme or a whole cell containing the required enzyme(s). According to the nature of the biocatalyst, the transformation is usually labeled as biocatalysis or biotransformation [2]. Throughout the text the broad term "bioconversion" will be used to designate either of these conversions, unless specification is needed for the context.

Isolated enzymes and whole cells, in free or immobilized form, are currently used in the production and processing of a large array of compounds and goods, as discussed in detail in different chapters of this book. Biocatalysts are thus widely used in the production of commodity and fine chemicals, consumer care products and biopharmaceuticals; in the development of analytical and diagnostic applications; in the biodegradation of harmful compounds or waste products; in the energy sector; and in the restoration of damaged artworks [3–9]. Despite such wide dissemination, it must be pointed out that the task of developing, optimizing, and implementing an effective bioconversion system is not an easy task, since several aspects have to be looked into carefully. The selection of the biocatalyst, its nature and formulation, the characteristics of the bioconversion medium, the most favored reactor configuration are all features that have to be investigated, and are furthermore interrelated.

Once the catalytic step/pathway required has been defined, the traditional approach for the development of a given bioconversion system focuses on the properties of the biocatalyst, since it will operate under strict operational conditions and its cost may be a significant portion of the overall process costs [10], although this latter feature is often overestimated [1, 11]. Moreover, recent improvements in biocatalyst production [12, 13], combined with increased costs of raw materials, tend to further deflate the importance of biocatalysts in process costs [10]. On the other hand, our growing understanding of enzyme structures and

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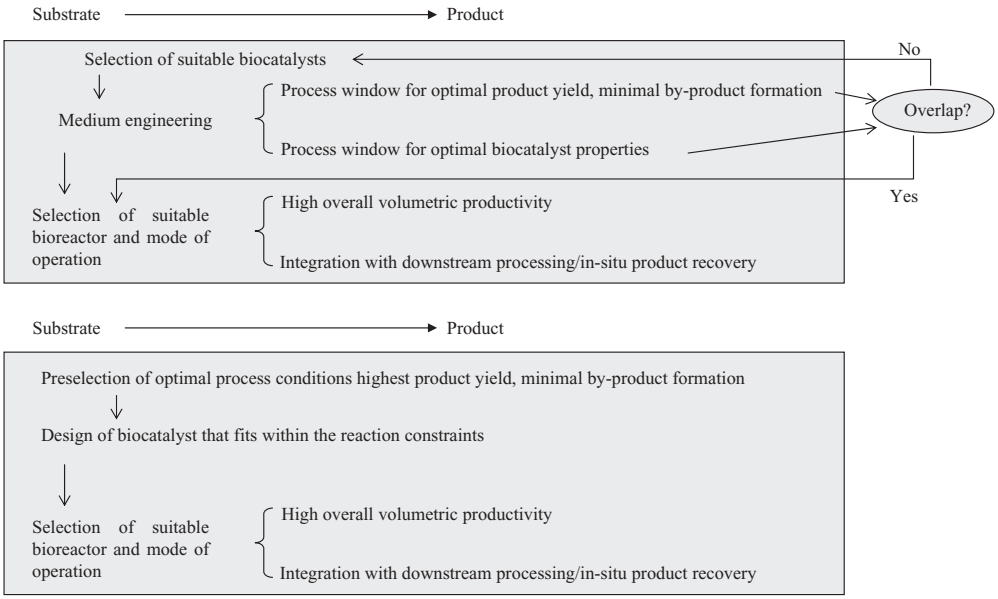

Figure 6.1 A brief outline of the main steps to be considered in the design of a bioconversion system. The conventional approach (a) concentrates on the biocatalyst properties, while the emerging new paradigm (b), conceptualized *ab initio*, is centered on the reaction constraints. Adapted from [10, 18, 19].

reaction mechanisms [14], coupled with methods for fast development of biocatalysts with enhanced features (e.g., activity, specificity, and stability, particularly under unconventional reaction conditions), affinity towards unusual substrates [15], as well as high throughput screening methods that allow the identification of new/improved biocatalysts and/or novel biocatalytic activities [16, 17], have all contributed to widen the target substrates for biocatalysis. They also allow for high substrate concentrations and hence enhanced productivity, and have paved the way for a shift in the traditional approach. That is to say, to compel the biocatalyst to fit into a biocatalytic system designed around the reaction, taking into account the thermodynamics of the reaction and the properties of substrate and products [10] (Figure 6.1).

The implementation of such a paradigm shift requires a combination of several technologies, with cost implications in the short term, but the medium- and long-term revenues are likely to be rewarding. The bioconversion system should also be designed as to ease product recovery, through *in situ* product recovery, either through continuous transfer of the product to a second liquid or solid phase [20–22], or through the formation of a second solid phase [23]. Particular consideration is also required if the biocatalytic step is part of a pathway also involving chemical catalysis. The combination of chemical and enzymatic catalysis is particularly appealing in the synthesis of polymers, since the raw materials are often

molecules with several similar functional organic groups, which are easily discernible by the regio-selective enzyme but not by a chemical catalyst, whose applications require protection and deprotection of all those groups except the one that is targeted for change. Polymerization of monomers by chemical catalysis can then take over more swiftly than the enzymatic approach [15, 24]. This combined approach has been used in the synthesis of sugar-based polymers [25], peptides [26, 27], 2′-deoxyribonucleosides [28], β-amino-acids [29], and polymeric prodrugs of profens [30].

6.2 The Design of the Bioconversion System

6.2.1 The Biocatalyst

6.2.1.1 Selection

In the selection of a suitable biocatalyst for a given bioconversion, several features have to be taken into consideration. These include the activity, selectivity, and stability to work under the required operational conditions (pH, temperature, media composition, substrate, and product concentrations). Several approaches can be considered in choosing the biocatalyst for the intended bioconversion, namely use of existing biocatalyst, genetic modification of existing biocatalysts or screening for novel biocatalysts [19, 31]. Browsing through the literature is the common approach for the selection of an existing biocatalyst. This traditional procedure can be complemented with the information gathered in databases such as BRENDA (http://www.brenda.uni-koeln.de), which gives easy assess to details on existing enzymes, such as natural substrates and products, cofactors, pathways, and inhibitors. This may contribute significantly to speeding up the selection procedure. Recently text-mining data (AMENDA and FRENDA) has been added to the repository.

It should be taken into consideration that using existing enzymes under atypical reaction conditions may uncover unexploited biocatalytic activities. This can also be achieved through genetic modification of existing biocatalysts by site-directed or random mutagenesis. Such modifications may also be made in order to increase activity and/or stability and/or selectivity.

The third approach for biocatalyst screening, browsing for new microorganisms with novel activities, takes into account the overwhelming biochemical diversity in nature. It should be pointed out that this approach is far from fully exploited since less than 10% of the microorganisms (i.e., bacteria) present in a given environment can be cultured on standard media [32]. The development of technology that allows the screening of large numbers of organisms through cheap, simple, rapid, and selective detection methods, with automation, has considerably improved this usually tedious process.

6.2.1.2 Whole Cells or Isolated Enzymes?

The use of an isolated enzyme is advised for the catalysis of a single-step reaction, provided that isolation is straightforward and cheap and the resulting enzyme is stable in a cell-free environment. It has been estimated that in order to be competitive with whole cell processes the cost of the enzyme should not exceed 5–10% of the total product cost [18]. A number of isolated enzymes in (partially) purified form have been used in bioconversion systems, such as alcohol dehydrogenases, aldolases, amylases, amyloglucosidases, catalases, cellulases, β-galactosidases (including lactases), glucose isomerases, glucose oxidases, inulinases, glycosidases (including glycosynthases), glycosyltransferases, inulinases, ketolases, laccases, lipases, pectinases, pectin methyl esterases pullulanase, penicillin G acylases, phospholypases, and trypsins [19, 33]. Removal of the enzyme from its natural environment may, however, be complex and expensive, and the isolated enzyme may be less stable and/or lose catalytic activity. Loss of catalytic activity is often reported when membrane-bound enzymes (e.g., the iron-containing oxygenases, such as cytochrome P450s and alkane hydroxylase) are involved [34, 35].

Oxidation–reduction reactions, in which cofactors such as acetyl CoA, ATP, FAD, NAD(P)H, sugar nucleotides or 3′-phosphoadenosine-5′-phosphosulfate (PAPS) are needed, may also make the use of whole cells as biocatalysts attractive, since cofactors are expensive and their regeneration is required in order to make the bioconversion system economically feasible. The use of whole cells with the help of a carbon source that provides maintenance energy for the cell as well as reducing power for cofactor regeneration [1], is usually considered more cost-effective, even though it may bring side-product formation and hamper product recovery [36]. Several chemical, electrochemical, chemo-enzymatic, and enzymatic approaches have been suggested to allow the use of enzymes in redox bioconversion systems [37, 38].

In order to be useful and practical, all these methods need to comply with certain requirements for cofactor regeneration [37]:

- Total turnover number (defined as the total number of moles of product formed per mole of cofactor during the time-course of a complete reaction) of the cofactor should be high (at least from 10^3 to 10^5).
- All materials involved (enzymes, reagents) should be easily available and manipulated, relatively cheap and stable under operational conditions and, along with by-products of the regeneration step, should not interfere with the main reaction system.
- The regeneration step should be favorable kinetically and thermodynamically.

Enzymatic methods based on a coupled reaction (Figure 6.2) are currently favored for cofactor regeneration, since they provide the best fit for those requirements, although chemo-enzymatic methods, based in the use of chemical catalysts, such as the organometallic complex (2,2′-bipyridyl)(pentamethylcyclopentadienyl) rhodium [39] and zinc/cobalt(III)sepulchrate [38] for cofactor regeneration, look promising.

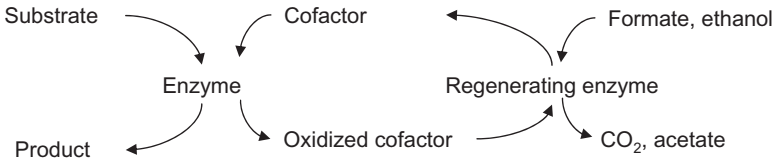

Figure 6.2 Schematics of a biocatalytic system with enzymatic cofactor regeneration. Common regenerating enzymes are alcohol dehydrogenase (ADH), formate dehydrogenase (FDH), and glucose dehydrogenase (GDH).

Compared with isolated enzymes, whole cell biocatalysts are generally faster and cheaper, and since the enzymes are protected from the external environment, they are likely to be more stable in the long term. On the hand, in a whole cell system several enzymes are present in addition to the desired enzyme, a feature that is likely to lead to by-product formation. Heat treatment of the cells to eliminate unwanted enzymatic activity can be envisaged, provided the desired enzyme endures the treatment [40–42]. Other alternatives to evade unwanted enzyme activity include the use of lyophilized cells [43]. If, however, those additional enzymes have no impact in product purity and yield, the use of whole cells is favored for the aforementioned cost and simplicity issues.

On the other hand whole cell bioconversions tend to display lower transformation rates than free enzymes, roughly by a factor of one or two orders of magnitude, due to mass transfer limitations caused by cell walls and cell membranes, which act as physical barriers and thus reduce the permeability of substrate (and product) [44–48]. These particular mass transfer limitations may alter the significance of screening trials for identification of whole cell biocatalysts, since some may be termed non-active if the reaction of interest is not detected in a suitable timeframe, even though the cell may have active enzymes. Cell permeabilization may be carried out in order to overcome such mass transfer limitations. Several approaches may be used to alter the structure of the cell wall or membrane without affecting the biocatalyst. Traditional approaches are based on physico-chemical methods, such as [20, 47]:

- heat-drying
- acetone-drying
- repeated cycles of freezing and thawing
- sonication
- addition of detergents (cetyltrimethylammonium bromide Pluronic F-68, Triton X-100)
- incubation with organic solvents (chloroform, diethyl ether, dimethyl sulfoxide, short-chain alcohols, toluene)
- NaCl stress.

When biotransformation is performed by growing cells, cell permeabilization can be performed by media manipulation through addition of compounds that tamper with the mechanisms of cell wall or membrane formation. Examples include the

use of glycine, isoniazid, D,L-norleucine and *m*-fluorophenylalanine or polycations such as protamine, polymyxin B nonapeptide, and polyethyleneimine [49–51].

These strategies for reducing mass transfer limitations are well disseminated, but they have been developed by trial-and-error, add several steps to the bioprocess, and the desired biocatalytic activity may be hampered. Permeabilizing agents are also likely to cause difficulties in downstream processing.

A different approach is based on the use of recombinant DNA technology to alter cell permeability in a predictable manner through the introduction of mutations in the proteins involved in the synthesis of cell walls or membranes [47, 48, 52]. Also using molecular engineering, the permeability barrier effect has been minimized by expressing an intracellular heterologous enzyme in the periplasmic space of a Gram-negative bacteria [53].

When multistep bioconversions are required that involve cascades of enzymatic reactions, for example in the production of large and complex molecules, whole cells are definitely the favored option since these are too complicated to perform *in vitro* [15, 28, 34]. Some representative examples of multistep bioconversions include sterol side-chain cleavage to produce steroid intermediates, the production of L-methionine from hydantoin, the production of 2′-deoxyribonucleosides, and carotenoid synthesis. Cell-free coupled enzymatic processes are relatively scarce, and the application of such biocatalytic systems is reduced to the association of two enzymes [54, 55].

The potential presented by whole cell–based biotransformation systems can also be increased through the use of metabolic engineering and directed evolution. The quantitative analysis of metabolic regulation allows the identification of bottlenecks in pathways and this paves the way for genetic modifications, targeted to optimize natural pathways. Furthermore, existing pathways can be modified in order to produce novel compounds and pathways can be pulled together [15], thus creating an almost unlimited field for applied biocatalysis.

6.2.1.3 **Immobilization of Biocatalysts**

Fundamental research on the characterization of both biocatalyst properties and a given bioconversion relies on the use of free biocatalysts suspended or dissolved in a liquid phase. Once that is established, immobilization of the biocatalyst is usually sought after, but for specific situations that require a free biocatalyst, such as in the formulation of detergents (washing powders), bread and cheese making. Some practical advantages at process level resulting from the confinement of the biocatalyst in the bioconversion system are easily anticipated. The heterogeneity of the immobilized biocatalytic system eases recovery of biocatalyst and product, allows multiple reuse of the biocatalyst, continuous mode of operation, and a wide variety of bioreactors. Immobilization provides a protective microenvironment to the biocatalyst; it may also, in the particular case of enzymes, somehow mimic their natural mode of occurrence in cells, where enzymes are often attached to cellular membranes. Immobilization can thus stabilize the structure of enzymes, and their catalytic activity. Along with these advantages, some drawbacks resulting from immobilization are reported, as summarized in Table 6.1.

Table 6.1 Advantages and disadvantages of biocatalyst immobilization.

		Main effects/Specific features
Advantages	Biocatalyst contained in the reactor	Biocatalyst reuse Continuous mode of operation Contamination of the product with the biocatalyst is avoided and *in situ* product recovery facilitated
	Definition of biocatalyst microenvironment	Biocatalyst activity and specificity can be tailored Stabilization of the biocatalyst Protection of shear-sensitive biocatalysts and interfacial inactivation minimized
	High biocatalyst concentration	Enhanced reaction rates High volumetric productivities
Disadvantages/ limitations	Empirical method	Time-consuming, case-specific process, intricate modeling and control
	Increased cost	Further material, equipment, and time required
	Loss of biocatalytic activity during immobilization	Exposure to extreme pH, high temperature, high shear or mechanical stress, toxic compounds Mass transfer limitations Active site blocked/tampered with Assess to substrate macromolecules is impaired Changes in the pH of the microenvironment
	Loss of biocatalytic activity during immobilization	Loss of the biocatalyst due to leakage/desorption; particle erosion, solubilization, or rupture due to cell growth; loss of fines (particles with diameters below 50–100 μm) in the outflow; accumulation of inhibitors/ contaminants in the microenvironment
	Operational restraints	Specific reactor configuration may be required Build-up of suspended solids Control of feed compositions

Adapted from [19, 56].

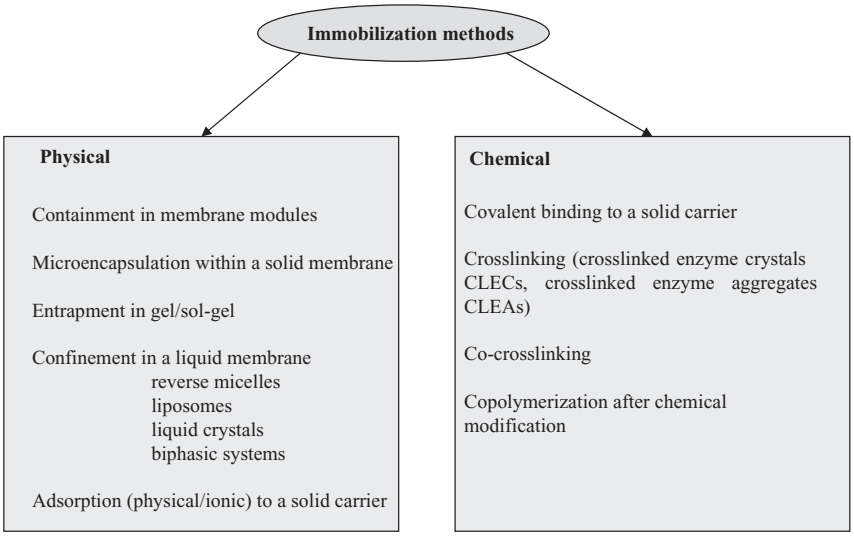

Figure 6.3 Classification of immobilization methods. Adapted from [19, 56–58].

Table 6.2 Examples of commonly used carriers for biocatalyst immobilization.

Natural polymers	Carbohydrates: Agar, agarose, alginate, chitin, chitosan, dextran, gellan, κ-carrageenan, pectin, pectates, pectinates
	Proteins: Ceratin, collagen, gelatine
Synthetic polymers	Polyacrylates
	Polyamides
	Polypropylene
	Polystyrene
	Polysiloxane
	Polyurethane
	Polyvinyls
	Silane polymers/silica gels
	Transition metal oxides

Immobilization can be achieved by physical or chemical methods (Figure 6.3), using several carriers (Table 6.2). In addition, several common materials, some of them unlikely at a first glance, can be used, such as wood chips [59], pumice stone [60], dry raisins [61], and orange peel [62]. The characteristics of the carrier play a major role in the performance of the immobilized biocatalyst and a given set of properties will be needed. These could include the following:

- Surface area: high surface area (above 100 m^2/g) [56] is often envisaged, which favors porous carriers. In the latter case the pores have to large enough to accommodate the biocatalyst so that the substrate and product can migrate easily.
- Insoluble in the reaction medium.
- Mechanical, chemical and biological stability so as to endure hydrodynamic and mechanical stress, be inert to chemicals in the reaction medium and to microbial degradation. Ease of regeneration (in the case of expensive carriers).
- Form and size of the support, since it affects the interrelated pressure drop and flow rate in column reactors, and accordingly filtration rate during recovery in repeated batch mode, as well as diffusion. The best compromise may be spherical particles of 150–300 μm diameter [56] but for nanoscale bioconversions.
- Hydrophobic/hydrophilic nature of the carrier, since it influences non-covalent immobilization and substrate/product availability and distribution.
- Nature, surface density, and distribution of functional groups.

The immobilization of a biocatalyst onto the surface of a solid carrier may require chemical modification (activation) of the biocatalyst [63] or of the carrier [56], the former often bringing with it severe loss of activity. Alternatively a coupling agent can be used as a mediator between the functional groups of biocatalyst and carrier, which may also play the role of a spacer, thereby reducing steric hindrance. In a more refined approach, recombinant DNA technology can be used to modify the biocatalyst, so that it can adsorb onto a specific carrier. These methods, or combinations thereof, are widely disseminated, and novel approaches or improved methodologies are published at a considerable rate. In particular the use of sol-gel encapsulation is gathering considerable relevance since it yields flexible and robust immobilized biocatalysts [64, 65], as well as crosslinked enzyme aggregates [58], crosslinked enzyme crystals [66], and nanoscale biocatalysis [67, 68].

In view of the nanometer scale of enzyme molecules, only these biocatalysts can benefit from the high surface area to volume ratios of nanoscale materials. These materials comprise nanoparticles made of silica, magnetite, gold, carbon nanotubes, and polymeric nanofibers. The nanostructure can combine hydrophobic and hydrophilic domains, thus easing the diffusion of hydrophobic substrates and products to/from a hydrophilic phase where the enzyme is immobilized. This approach allows an increase of more then 200-fold in the turnover number for horseradish peroxydase catalyzed reactions in heptane, as compared to free enzyme [69].

The nanoscale approach seems to provide an adequate solution to the often contradictory issues faced in the optimization of immobilized enzymes systems, namely high surface area and enzyme loading commonly bringing high mass transfer resistances within the supports. There are some drawbacks, though, in particular the dispersion of the particles in the bioconversion medium and their

Table 6.3 Effect of immobilization on biocatalyst kinetics and properties.

Conformational effects	Changes in the tertiary structure of enzymes upon binding to a solid carrier or crosslinking; enzyme denaturation resulting from the action of chemicals used in entrapment/encapsulation methods. Changes in the temperature activity profile, and in thermal, operational and storage stability as compared with the free form
Steric effects	Three-dimensional hindrances that limit the assess of the substrate
Partition effects	These occur when the carrier is charged or has a hydrophilic/hydrophobic nature unlike the bulk medium. Results in different concentrations of compounds in either side of the interface. Shift in the pH–activity profile. Similar reasoning if compounds involved are charged or have a hydrophilic/hydrophobic nature unlike the carrier
Mass transfer effects	Since substrate must migrate from the bulk solution to the active site of the immobilized biocatalyst, external and internal (diffusion) mass transfer resistance occur, the later only for porous supports. High circulation rates can minimize external mass transfer resistances. Diffusion resistance takes place along with the reaction, thus the overall reaction rate overall reaction depends on the substrate concentration and the distance from the outside support surface
Miscellaneous effects	Changes in the specificity, kinetic constants, thermal, operational and storage stability of the immobilized biocatalyst as compared with the free form

recovery, limited bioreactor configurations, and health and environmental concerns related to the handling of nanoparticles [67, 68].

Despite being extremely useful, immobilization often results in changes in biocatalyst properties (Table 6.3). These have to be carefully assessed, since they interfere in the design of the process. Regardless of the wide number of methods and supports available, it is not possible to single out one method and one support as the most suitable for all biocatalysts and applications. The selection of the support and immobilization method must take into consideration the nature and properties of the substrate, product, and biocatalyst. For instance, low molecular weight enzymes easily leak out from gel-based supports; if macromolecular substrates are use in the bioconversion system, entrapment is also not a good option, given extreme diffusion limitations; hydrophobic substrates do not partition into hydrophilic supports. Furthermore, all of the methods present advantages and drawbacks. Adsorption is simple, cheap and effective but often easily reversible by changes in pH, ionic strength or hydrodynamic conditions; covalent attachment and crosslinking assure a strong and durable immobilization, with long-term stability, but they are expensive and enzyme activity often decreases significantly upon immobilization. Entrapment, microencapsulation, and membrane reactor confinement are commonly associated with significant mass transfer resistances, particularly the former, although they are very mild methods that hardly affect

intrinsic biocatalytic. Such mass transfer resistances can be turned into an advantage in membrane reactors for the conversion of high molecular weight or insoluble substrates, such as starch, cellulose, inulin, or proteins, since the membrane retains them along with the biocatalyst, which is present in a soluble (enzyme) or suspended (cells) form, while allowing the low molecular weight products to migrate out. The selection of the most adequate immobilization conditions for a given biocatalyst and application is therefore a trial-and-error process, aimed at identifying the best compromise for retention of biocatalytic activity and operational stability.

6.2.2 The Bioconversion Medium

Once exclusively limited to application in homogeneous aqueous media, bioconversion processes have been shown to be practicable in the presence of organic solvents (water miscible or immiscible) [19, 70], ionic liquids [71, 72], supercritical fluids [73, 74], gas phase [75], aqueous two-phase systems [76], and in liquid–solid (resin assisted) media [22]. These approaches are usually designated as "non-conventional biocatalysis." Illustrative examples of recent applications are given in Table 6.4. The main reasons underlying the need for bioconversion media other then aqueous are the sparing water solubility of most substrates and/or products in such media, as well as the potential toxic or inhibitory role of those compounds on the biocatalyst, which can be reduced if biocatalyst and compounds are in different phases. In either case, if the bioconversion is carried out in aqueous media, the productivity may be severely constrained.

According to Straathof [93], for compounds with an aqueous solubility between about 0.0003 and 1 M, a second phase is needed to reach relevant productivities. Furthermore, the substrate concentration and enantiomeric purity of products have been shown to correlate inversely in several bioconversion systems, thus lowering substrate concentration in the reaction medium enhances selectivity [94]. An auxiliary second phase can thus be advantageously used, to provide a pool for compounds that are sparingly soluble in the phase where the biocatalyst remains and/or to keep their concentration in said phase under toxic/inhibitory levels.

6.2.2.1 Organic Solvents

The use of organic solvents in a bioconversion medium is by far the oldest alternative to purely aqueous systems [95–97], but this approach was only established firmly in the early 1980s [98]. The advantages and disadvantages of this approach are well established (Table 6.5).

Bioconversion systems involving organic solvents can be either homogeneous or heterogeneous, depending on whether the solvents used are water miscible (e.g., acetonitrile, dimethyl sulfoxide, ethylene glycol, glycerol, methanol, propylene glycol) or water immiscible (diisopropyl ether, ethyl acetate, methyl-*t*-butyl ether, octane, *n*-octanol, toluene). The latter systems favor process integration since they allow *in situ* product recovery and provide a pool for toxic or inhibitory

Table 6.4 Some recent examples of non-conventional bioconversion systems.

Biocatalyst	System	Reference
Crosslinked enzyme aggregates (CLEA) of penicillin acylase and polyacrylamide gel surface bound penicillin acylase	Synthesis of cephalexin in in ethylene glycol medium	[77]
α-Amylase and glucoamylase	Starch hydrolysis in PEG/dextran aqueous two-phase systems	[78]
Lipases encapsulated in microemulsion-based organogels	Esterification of lauric acid and 1-propanol in supercritical CO_2	[79]
Recombinant *Escherichia coli* containing xylene monooxygenase	Oxidation of pseudocumene to 3,4-dimethylbenzaldehyde in (2-ethylhexyl) phthalate: aqueous minimal medium	[80]
Lipase	Resolution of epoxy-enol esters in buffer: hexane	[81]
Immobilized lipase	Acylation of naringin and rutin with vinyl butyrate in ionic liquids	[82]
Saccharomyces cerevisiae	Reduction of 4-chloro acetoacetate to yield (*S*)-4-chloro-3-hydroxybutanoate in ionic liquids	[83]
Lipase	Enantioselective esterification of (±)-menthol in ionic liquids and organic solvents	[84]
Lipase immobilized onto carbon fiber	Transesterification between vinyl acetate and *n*-propanol in the gas phase	[85]
Lyophilized cells of *Rhodococcus erythropolis*	Hydrolysis of 1-chlorobutane to 1-butanol and HCl in the gas phase	[86]
Lipase	Synthesis of esters of isoamyl alcohol in supercritical carbon dioxide	[87]
Immobilized lipase and immobilized cutinase	Transesterification of (*R,S*)-2-phenyl-1-propanol with vinyl butyrate in supercritical media, ionic liquids and organic solvents	[88]
E. coli strain overexpressing cyclohexanone monooxygenase	Asymmetric Baeyer-Villiger oxidation using *in situ* substrate feeding and product removal with DOWEX Optipore L-493 resin	[89]
Mycobacterium sp. NRRL B3805	Sitosterol side-chain cleavage in dioctyl phthalate:Tris-HCl buffer	[90]
Lipase	Selective acylation of glycosides in ionic liquids	[91]
Recombinant *E. coli*	Deacylation of penicillin G to 6-aminopenicillanic acid in PEG/potassium phosphate	[92]

Table 6.5 Advantages and drawbacks of the use of organic solvents in bioconversion media.

Advantages	Enhanced solubility of non-polar substrates and products, increasing space/time yield and volumetric productivity
	Reversal of thermodynamic equilibrium, favoring synthesis over hydrolysis. Enables unlikely reactions in aqueous media (e.g., transesterification, thioesterification, aminolysis)
	Enantioselectivity can be altered depending on the organic solvent
	Unwanted water-dependent side-reactions are avoided
	Microbial contamination is reduced
	Enzyme stabilization in low water environment
Drawbacks	Biocatalyst denaturation and/or inhibition by organic solvent
	Increased complexity of the system
	Partition and mass transfer hindrances (in organic–aqueous two-liquid phase systems)
	Interfacial deactivation of enzymes (in organic–aqueous two-liquid phase systems)

substrates/products, since only minute amounts of these are present in the aqueous phase. They also enable interfacial catalysis by making available an aqueous–organic interface, which is required by some enzymes to display the intended activity (interfacial activation) as a result of conformational changes upon adsorption to said interface [93, 99, 100].

Heteregeneous systems may be further divided into micro- and macro-heterogeneous systems. In micro-heterogeneous systems, phase separation is only observed on a microscopic scale, as in the case of reverse micelles, whereas in macro-heterogeneous systems there is a visible phase separation, as occurs in two-liquid phase systems, or systems that use powdered or immobilized enzymes.

Organic solvent toxicity, a major problem associated with their use in bioconversion systems, may be ascribed to interactions with non-polar groups of the enzyme and eventual disruption of hydrophobic interactions and to competition with the protein molecule for the essential water required by the latter for proper polypeptide conformation. Solvents may ultimately strip such an essential water layer. When whole cells are involved, the deleterious action of the solvent can be ascribed to its accumulation in the cytoplasmic membrane, altering its structure and preventing the cell from carrying out key functions, such as dissipation of pH and electrical potential and inhibiting membrane protein functions, and ultimately leading to cell lysis and death [101].

Given the interaction between solvent, substrate product, and biocatalyst, several aspects have to be considered in solvent selection. A suitable solvent must be biocompatible with the biocatalyst, display high affinity to substrates and/or

products, be cheap, non-biodegradable, non-toxic to humans; have a relatively high boiling point (preferentially lower then water, though) and low volatility. It should be also taken into consideration that the organic solvent may influence the selectivity of the biocatalyst. Extensive efforts have been made to predict the biocompatibility of the solvent on the basis of the physical properties of the solvent. The Hansch parameter (log P_{oct} or π value), the logarithm of the partition coefficient in a standard octanol–water two-phase system, is the parameter most commonly used to predict biocompatibility. It suggests that the higher the log P_{oct}, the more biocompatible the solvent; most whole cells are only compatible with solvents with log P_{oct} above 4. Nevertheless, it is far from being foolproof [93].

The limitations of such a predictive model may be ascribed to the mechanisms involving biocatalyst/solvent interaction, which are far too complex for solvent biocompatibility to be predicted by a single physical parameter [102, 103]. In the design of the bioconversion system, adequate hydrodynamic conditions and phase volume ratio values have to be selected, in order to enhance mass transfer and thus increase the overall reaction rate. Particular care has to be given to avoid the emulsification of the reaction medium, which limits mass transfer. Furthermore, reactors with extra safety precautions, namely explosion-proof conception, may be required [104].

6.2.2.2 Ionic Liquids

Given the ever-increasing pressure from governmental organizations, regulatory entities, NGOs, and public opinion to protect the environment, there is a trend to develop suitable alternative solvents and technologies that may replace organic solvents held responsible for the production of hazardous wastes. Ionic liquids are salts that are in liquid state at room temperature and, unlike organic solvents, they have virtually no vapor pressure, and offer the possibility of the development of green and clean processes. As with organic solvents, water-miscible and water-immiscible ionic liquids are available.

The physical properties of ionic liquids, such as density, hydrophobicity, melting point, and viscosity can be tailored to meet the requirements of the bioconversion system by altering the nature of anions and cations. Ionic liquids furthermore have high thermal and storage stability, the ability to dissolve a wide array of compounds, from inorganic to organic and polymeric, they are also non-reactive and may be used in enantioselective and stereoselective reactions [72].

Hydrolases, such as proteases and lipases, oxidoreductases, and dehydrogenases, retain catalytic activity in the presence of ionic liquids, and it seems that all the enzymes that work in organic solvents also work in ionic liquids. The use of ionic liquids has been shown to increase enzyme activity, stability, enantio- and region-selectivity when compared to the use of organic solvents, but such a trend is not absolute [91, 94, 105]. Not all ionic liquids have been found suitable for biocatalysis. Enzymes tend to retain catalytic activity in ionic liquids containing dimethylphosphate, tetrafluoroborate (both water soluble), bis((trifluoromethyl)-sulfonyl)imide, hexafluorophosphate (both water insoluble), but not in ionic liquids containing Cl, NO_3, CF_3SO_3, trifluoroacetate, or acetate anions [106, 107].

Whole cells have also been shown to retain activity in ionic liquids containing bis((trifluoromethyl)-sulfonyl)imide, hexafluorophosphate [83]. Still, the application of ionic liquids in whole-cell biotransformations is less disseminated than in enzymatic biocatalysis.

In the selection of a suitable ionic liquid for application in a bioconversion system, some issues have to be attended. The ionic liquid should be available free of impurities that could hinder the process, non-corrosive, non-toxic, and biocompatible. Furthermore, suitable methods of product isolation, such as distillation, supercritical CO_2, column chromatography, or extraction should be applicable. Given the cost of ionic liquids, which can be two orders of magnitude more than that of common organic solvents, the feasibility of the use of ionic liquids in large scale strongly depends upon the efficient recovery of the ionic liquid and reuse [105]. In order to be used efficiently the ionic liquid must dissolve the compounds involved at least up to several hundred millimolar, and the distribution coefficients for substrates and products between ionic liquids and buffer must be above 2.0, both for extraction efficiency and to lower their concentration in the aqueous phase, hence toxicity [83].

6.2.2.3 **Two-Phase Aqueous Systems**

Two-phase aqueous systems are composed of solutions of two incompatible polymers, for instance polyethylene glycol and dextran, or a solution of water-soluble polymer with another salt solution, such as phosphate buffer or magnesium sulfate, with a low interfacial tension between the two phases. These systems provide a relatively mild environment for biocatalysts and are particularly suitable for the extraction of hydrophilic products [76]. The main problems in the design of two-phase aqueous bioconversion systems lies on the lack of suitable models for prediction of biocatalyst activity as well as biocatalyst and product partition, and its large-scale implementation has been hampered by the cost of many of the polymers used, given their purity levels. If cheaper, less pure polymers could be used without significant reduction in the productivity, aqueous two-phase systems may provide an interesting alternative for bioconversion systems involving hydrophilic compounds [78].

6.2.2.4 **Solid Resins**

Resins with high surface area added to the bioconversion media have been used to overcome limitation associated with product inhibition through *in situ* product recovery [94], or to avoid liquid–liquid extraction from whole-cell bioconversion media [108], since the product is transferred to the resin as it is formed. Resins can also be used for substrate delivery, which requires previous saturation of the resin with the substrate, prior to its incubation in the bioconversion medium to which it is delivered. Resins can also provide *in situ* substrate feeding and product recovery [89].

The underlying concept of resin-based systems resembles that of two-liquid phase systems, but it avoids the deleterious effects on the biocatalyst often encountered when organic solvents (or ionic liquids) are used, although high-resin loads

may lead to irreversible damage to the biocatalyst [94]. In the selection of a suitable solid phase, particular care has to be given to the affinity of substrate, product, and biocatalyst towards the resin. Excessive binding of the substrate to the resin may lead to its low bioavailability in the aqueous phase. Adsorption of the biocatalyst to the solid phase is also to be avoided.

6.2.2.5 **Solid–Gas Systems**

Solid–gas systems present several advantages over liquid-based systems, namely enhanced mass transfer, as a result of high diffusion coefficients and low viscosity of gases, enhanced solubility of substrates and products, eventual discard of the use of solvents, as well as minimization of occurrence of microbial contamination as result of the relatively high temperatures (e.g., 45 to 85 °C) used, low risk of by-product formation, simpler immobilization procedures, eased recovery of products (and unreacted substrates) by condensation and reduction of thermal denaturation, given the partial dehydrated condition of the biocatalyst [85, 109]. In addition these systems can provide useful information on the interactions between biocatalysts and their microenvironment, and the concomitant influence on catalytic activity and stability, since the thermodynamic activities of the different species can be fixed independently [109].

Key features in the design of the system include the strategy for creating a gas phase, the absolute working pressure, and the thermodynamic activity of water.

6.2.2.6 **Supercritical Fluids**

Interest in the use of supercritical fluids (SCFs) can be related to the growing environmental concerns with the amount of waste generated by the chemical industry, most of them organic solvents and by-products thereof. SCFs also present advantages with regard to mass transfer, since the diffusivity of substrates and product is high, the viscosity of the bioconversion medium is low, and internal mass transfer limitations, often a bottleneck in heterogeneous bioconversions, are reduced because of the high diffusivity of SCFs.

The SFC most commonly used in bioconversions, sc-CO_2, is non-carcinogenic, non-toxic, non-mutagenic, non-flammable, and thermodynamically stable, and thus presents obvious health and safety benefits. It is also environmentally friendly and recyclable. However, its non-polar nature typically favors sc-CO_2 application when hydrophobic compounds are involved. Recent research efforts have led to the development of surfactants that allow solubilization of both hydrophilic and hydrophobic chemicals, which may widen the range of applications of sc-CO_2 in biotransformations [74].

Other SCFs used in biocatalysis, such as sc-ethane [88], sc-propane, and sc-butane [73, 110], lack the "green" label [74]. On the other hand, water has lower affinity to these fluids than to sc-CO_2, so the risk of their removing essential water from the enzyme molecule is lowered [73].

In the design of a bioconversion systems based on the use of these dense gases several key issues have to be addressed. These include solvent selection, along with

the factors that influence enzyme activity/stability, which in these particular systems are pressure, pressurization/depressurization, and water activity. Such assessment is based in experimental research, since currently it is almost impossible to predict biocatalyst activity/stability in the presence of SCFs.

6.3 Bioreactors

Bioconversions are commonly carried out in basic chemical reactors, operating in batch, continuous, or fed-batch mode. The latter mode is a suitable alternative to the use of an auxiliary second phase when the substrate is toxic/inhibitory above a given concentration and/or is sparingly soluble in the aqueous phase. Careful monitoring is required because substrate limitation may occur [111]. Batch reactors are most commonly (but not exclusively) used when free biocatalysts are available. If immobilized biocatalysts are used in this mode of operation and if reuse is envisaged, the separation and recovery of the biocatalyst is mandatory, which may bring a loss of biocatalyst mass as well as loss of activity.

6.3.1 Batch Reactors

Although stirred tanks are commonly used to give an almost ideal mixture pattern and avoiding concentration (and in particular pH) and temperature gradients, the immobilization support particles are often sensitive to shear stress. This means that only durable preparations of immobilized biocatalysts should be used in stirred tank reactors. An alternative is the so-called basket reactor, where the immobilized biocatalyst particles are retained within a basket either forming the impeller blades or the baffles of the tank reactor. A more commonly used approach relies on a plug flow pattern. The plug flow reactor type, which may be a packed or fluidized bed reactor, operates in total recycle mode; this is needed if a single pass gives a low conversion yield. This feature is also particularly useful for the acquisition of kinetic data, since the reactor can be considered as a differential reactor. Furthermore, external mass transfer resistances can easily be reduced by operating at high flow rates.

6.3.2 Fed-Batch Reactors

In a fed-batch reactor reactants/media components are added continuously or in pulses. Particular care has to be given to the effect of feeding on the reaction volume, mainly on hydrodynamic condition and on the initial headspace, so as to avoid medium overflow. Control and modeling of the process is more complex then the remaining operation modes and research in this particular field dedicated to enzymatic bioreactors is rather scarce [112].

6.3.3
Continuous Reactors

The continuous operation of immobilized biocatalysts has some advantages when compared with other modes of operation, namely ease of automatic control, ease of operation, and quality control of products. Continuous reactors can be divided into two types: the continuous feed stirred tank reactor (CSTR) and the plug flow reactor (PFR). In the ideal CSTR complete mixing is achieved, therefore the degree of conversion is independent of the position in the vessel and the conditions within the CSTR are the same as those in the outlet stream, hence low substrate and high product concentrations. In the ideal PFR the conversion yield depends on the length of the vessel. However the PFR contains no mixing devices and the conditions within the reactor are thus uneven, often with temperature and velocity and concentration gradients normal to the flow direction, which is not ideal.

Selection between these two types should take into consideration kinetic and operational features. Thus, for Michaelis–Menten kinetics, the PFR is preferred to the CSTR, since it requires less biocatalyst to reach the same conversion yield. Given the characteristics of the PFR and the CSTR the former is preferred for product-inhibited systems, whereas the latter should be used for substrate-inhibited systems. However, if pH control is required, the use of a PFR is ill-advised.

Particular care has to be given to the dimensions of the immobilized biocatalyst; particles may lead to high pressure drop as well as plugging (see Table 6.5).

The use of a fluidized bed reactor (FBR), which has a mixing pattern intermediate to the CSTR and the ideal PFR, can be used with low pressure drop. Insoluble substrates and highly viscous fluids are also better processed in a FBR or in a CSTR.

The selection of a suitable reactor is not a straightforward task, it requires thorough knowledge of the requirements of a given bioconversion system.

6.4
Rationalizing and Speeding up the Development and Characterization of a Bioconversion Process

Bioconversion systems are quite complex and depend on a wide range of variables. Hence, the development of rational planning strategies to establish a rational experimental planning, coupled to fast and reliable procedures that allow the evaluation of the relevant features and characterization of the system, are required to enhance the pace of process development. Dedicated efforts and technological breakthroughs are contributing to make this goal a reality, by taking advantage of developments in computational sciences and in miniaturized systems and components.

6.4.1 Computational Methods

Developments in computational sciences are providing tools that allow the development of guidelines for a rational experimental planning, therefore allowing cost saving in time and resources. Molecular modeling and chemometrics are two computational fields that complement each other and, once gathered, contribute decisively to the development of predictive models. In molecular modeling, virtual models can be built that allow a better understanding of enzyme–substrate interactions. These are mostly based on the calculation of the free energy for the reaction, obtained from the simulation of the transition state. This is usually performed through molecular mechanics calculations, which are unable to provide precise predictions. On the other hand these calculations are not computationally demanding and provide results relatively quickly. Quantum mechanics methods are far too complex and computationally demanding to be used. An intermediate approach treats the active site and the substrate at a quantum mechanics level and the remaining system at a molecular mechanics level, but even this can be computationally too demanding.

Molecular modeling techniques are routinely used for the characterization of enzyme–substrate interactions and are being applied to the prediction of the enantioselectivity of enzymes [113]. Chemometrics enables the correlation of the measurements made on a chemical system/process to the state of the system through a series of mathematical methods, comprising multivariate statistical analysis and experimental design. The latter provides an invaluable tool to simultaneously study multiple variables and identify optimal experimental conditions, whereas the former enables the interpretation of data contained in complex systems containing a wide number of variables, by reducing its dimensionality [114].

Combining the two methods is particularly useful in the development of predictive models, namely for establishing empirical equations or parameters. This combined approach was successfully used for the identification of the amino acid residues responsible for selectivity when comparing penicillin G amidases from different sources [115, 116].

6.4.2 Microscale Processing Methods

This approach, based on the use of miniature bioreactors with volumes below 100 ml, coupled to analytical techniques and instruments able to handle a vast number of samples in a short time-frame, allows parallelization, automation, and cost reduction, both in human and material resources, of experimental trials. Such small-scale bioreactors comprise shaken vessels, such as shake flasks, test tubes, and microtiter plates, with different volumes and configurations, as well as stirred miniature bioreactors and membrane reactors. Technological developments have

made it feasible to monitor and control pH and dissolved oxygen tension on-line (at least in some cases, some of which also comprise air-flow rate control), which further enhances the potential of these devices [117–119].

Miniature bioreactors are currently used for several applications, including early stage recombinant or wild-type organism appraisal, strain improvement, and growth/bioconversion medium development [118]. Particular care has to be taken to ensure that operational conditions are such that they do not mask the output [120]. For instance, if inadvertently working with oxygen as limiting substrate while evaluating the effect of media composition on catalytic activity, the outcome of the set of experiments is likely to be illusive [121]. Reproducibility and scalability become of crucial importance if miniature bioreactors are used in the later stages of process development. In such cases it must be checked whether the "rule of thumb" methods commonly used in industry to scale from bench-top to production scale are applicable. Such methods are based on various parameters, including $k_L a$ (oxygen volumetric mass-transfer coefficient), power consumption per unit volume, agitator tip speed, constant dissolved oxygen tension, or mixing time. Although $k_L a$ and power consumption per unit volume are often taken as reference, and concomitantly significant advances have been made in the development of empirical correlation for the prediction of such parameters [122, 123], the diversity of parameters shows that there is no single criterion and furthermore not all the miniature systems are eligible to comply with all the criteria for scaling [118, 119].

6.5 Concluding Remarks

This chapter aimed to provide an overall perspective of applied biocatalysis, with particular focus on the issues that have to be addressed in the conception of a given bioconversion system, and to the relevant criteria that have to be considered for the selection of the best option to be used throughout the different stages of such a process. Some broad guidelines that assist in the overall design of a bioconversion system are provided, but it is pointed out there are no universal criteria, so each system requires detailed attention, given the intrinsic requirements. Recent technological developments have enhanced the potential of designer biocatalysts, enabling a shift in the design paradigm of bioconversion systems from using biocatalysts with properties oriented to the reaction constraints to tailoring the former to the requirements of the latter. The ability of biocatalysts to operate in media other than aqueous is increasing the use of alternative, productivity-enhancing media.

Growing environmental concerns are favoring the development and implementation of "green" bioconversion processes, using non-toxic, organic solvent-free media (supercritical fluids, ionic solvents, gas phase). Extensive work in the development of predictive models to anticipate the behavior in such media is required. The competitiveness of bioconversion systems against chemical-based processes

is also increasing, since the time-frame and resources for the development steps of bio-based processes is being drastically reduced with the use of miniaturized systems able to evaluate and analyze a wide number of variables simultaneously and hastily. The combination of molecular modeling with developments in computational sciences further helps to reduce costs and time needed to develop the models, reducing the number of experimental trials required and providing a rational basis for the engineering of biocatalysts. All these features clearly highlight current trends for establishing a more rational basis in the design and development of bioconversion systems, developing faster and more reliable screening tools and promoting the integration of complementary fields of knowledge (engineering, biology, chemistry, mathematics, and computational sciences) for more efficient process development.

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7
Nanobiotechnology

Rudy J. Koopmans

7.1
Setting the Stage

In the late eighteenth and early nineteenth century a major shift in technological, socioeconomic, and cultural conditions occurred, known as the Industrial Revolution. Manual labor was replaced by machinery, and goods were produced in large quantities. Distribution and transportation was facilitated by newly built canals, improved roads and railways. An increasing need for natural products stimulated investigations into alternative feedstocks and replacement materials.

In the sciences it was generally agreed that organic substances obtained from plants and animals could not be created in the laboratory; they could only be isolated and examined, and perhaps broken down into simpler substances. The reverse, the production of the complex from the simple, was beyond human competence. This was the work of a creator, or of a life force operating within living systems [1].

A little more than half a century later, in 1858, Archibald Scott Couper [2] determined the tetra-valence of the carbon atom and in 1865 Friedrich August Kekulé published his paper on the hexagonal ring of the benzene molecule [3]. By now a language for nanoscale objects (i.e., molecules) had been developed and a separate name and diagram assigned to each organic compound. Suddenly, thousands of new substances were being made in the laboratory. The science of chemistry became almost unique among the sciences in creating much of the world of materials. And the chemical industry was born.

About 150 years later at the beginning of the twenty-first century a similar scenario seems to be developing. Alternative feedstock and innovative materials are being investigated in a drive to create a more sustainable society. Nowadays a far better scientific grounding of nature exists. More sophisticated instruments are available to probe the world of molecules, the nano-world (10^{-9} m). Researchers are getting more used to thinking like nature. It is realized that structure can be built up from the nanoscale to the macroscopic scale of everyday experience, that is, "from the bottom up" [4, 5].

Materials and devices are now being built from molecular components, which assemble themselves chemically using the principles of molecular recognition. Far

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more precision and functionality can be achieved than with a "top down" assembly approach, where nano-objects are constructed from larger entities without atomic-level control. Science, however, is still trying to understand the fundamental rules of structure and functionality formation in order for technology to apply them. The challenges are legion, requiring multidisciplinary approaches as their complexity is substantial.

Although all definitions are tautological (i.e., redundant), they still may be helpful in the demarcation of boundaries between investigative disciplines to allow us to gather detailed knowledge. Thus the subject of nanobiotechnology can be viewed as the branch of nanotechnology focusing on biological and biochemical applications or uses.

Nanotechnology covers a broad field of applied sciences and technologies. The unifying theme is the control of matter on a scale smaller than one micrometer (10^{-6} m), as well as the fabrication of devices on this same length scale [6, 7].

According to the UN Convention on Biodiversity, biotechnology means: "any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use" [8].

Nanobiotechnology more specifically accesses the nanostructures and nanomachines designed by 3.8 billion years of natural selection; namely, cell machinery and biological molecules (i.e., DNA, RNA, ATP, lipids, proteins, and polysaccharides).

Exploiting the extraordinary self-organizing behaviors and functional properties of these biological molecules and cell processes is considered the path forward to accomplish many societal goals that are difficult or impossible to achieve by other means. It requires a multidisciplinary approach with substantial knowledge in the fields of biology, chemistry, medicine, mathematics, physics, ecology, psychology, sociology, and many engineering disciplines.

7.2 Industrial Perspective

Innovation, or more precisely renewal of the product offerings that will fit societal needs – the "goods" delivered to people who are willing to pay a price – is essential for making an economic activity sustainable. (Note: "Goods" can be anything that is tradable in an economic transaction ranging from feedstock and produce to services.) There is some analogy to draw with a natural selection process, in this case applied to systems, being organizations of people called companies. In companies people come together around a common cause striving to survive and perpetuate the cause. Companies will only sustain themselves if their "meme" – a unit of cultural information transferable from one mind to another – is adaptive to changing environmental requirements [9, 10].

Key challenges in this evolutionary economic process are the identification of what those goods are and how precisely they can be delivered under pressure of the constraints companies face. The "what's" are often inspired by the market and the "zeitgeist" (spirit of the times). Once upon a time it was accepted practice to

extract goods from nature and discard them after a useful life without much thought. Now the same or similar goods need to be recycled or replaced by goods having a minimal ecological footprint (defined as the land area required to provide the resources, i.e., grain, feed, wood, fish, and urban land, and absorb the emissions, that is, carbon dioxide or equivalents [11]). The "how's" relate to the resources required in terms of people and funds, the state of technology and the time for bringing the goods to the market. It encompasses the process of perpetuating the adaptive cause of the companies.

As a direction for industry, nanobiotechnology as a "how" should be viewed in this context. Today it is still mainly an academically driven activity with the exception of few start-up companies – estimated at about 150 worldwide [12]. Medical and pharmaceutical companies are the most likely early adopters. The key challenge is successful clinical trial results to forge a market position. In other sectors of industry there may be additional hurdles related to regulatory legislation and socioeconomic acceptance.

However fascinating the science may be, bringing it to a level of major economic activity will take time. How long this takes depends on the science and technological breakthroughs and the associated meme. Therefore writing on the industrial aspects of nanobiotechnology is by default limited to the imagination of today's scientists as reported in the literature. It just remains a non-exhaustive attempt to inspire researchers and any other interested party for finding a path of learning and applying the lessons of nature. Nevertheless, the research efforts are fast paced and growing as there definitely is "plenty of room at the bottom" [13].

7.3
Nanotechnology in Biology and Biochemistry

Although nanoparticles do not necessarily fall under the definition of nanobiotechnology and associated applications there is certainly some overlap. An important inroad of nanotechnology in materials innovation has come from the study of small, nanosized particle, 5 nm and larger [14, 15]. At these dimensions polymeric (synthetic) (e.g., polystyrene), ceramic (e.g., clays), or metal (e.g., silver, gold) particles can interact through intermolecular forces with other molecules comprising the living cell or biological molecules. The concept is to attach a nanosized particle to biological molecules and exploit either the functionality of the particle (e.g., silver as an antimicrobial agent) or the biological molecule (e.g., antibodies, soluble glycoproteins, i.e., biomolecules composed of a protein and a carbohydrate (an oligosaccharide)) (Figure 7.1). Several applications have been explored [16–30], including:

- fluorescent biological labels
- drug and gene delivery
- biodetection of pathogens
- detection of proteins

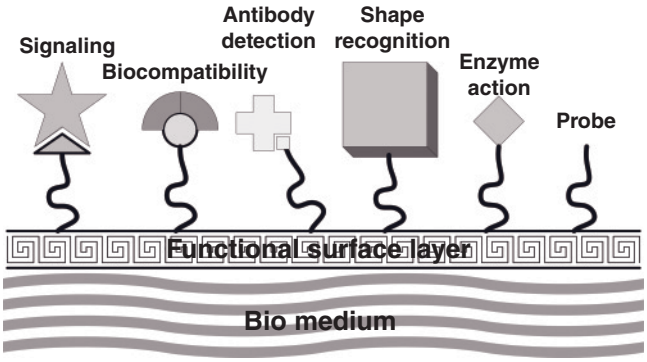

Figure 7.1 Simplified representation of possible functionalities induced through attaching nanoparticles to biomolecular substrates or from particle selective biomolecular probe, attached to bioactive surface layer.

- probing of dna structure
- tissue engineering
- tumor destruction via heating (hyperthermia)
- separation and purification of biological molecules and cells
- MRI contrast enhancement
- phage therapy (alternatives to antibiotics).

7.4 Biomimicry

Biomimicry [31] is from the Greek "bios" meaning life, and "mimesis" meaning imitation. Biomimicry is all about trying to reproduce what nature already accomplishes. It is related to nanobiotechnology as it involves understanding the structure and processes of nature. Insights at the molecular level may thus allow a bottom-up approach to imitate nature either using the same or similar building blocks. The subject has become an important area of research and the number of materials, processes, and products based on natural examples keeps expanding. For example, in the field of materials several lessons are learned from nature [32]:

- abalone-inspired ceramics
- blue mussel-inspired glues
- butterfly-inspired pigment-free color
- beetle-inspired water harvest
- microbe-inspired replacement of platinum catalysts in fuel cells
- diatom and sponge-inspired silicon manufacture
- anhydrobiosis-inspired vaccine storage.

7.4.1 Silk Fibers

Probably one of the most sought after biomaterials is spider silk. These fibers have the advantage of being both light and flexible. On a weight for weight basis it is roughly three times stronger than steel: the tensile strength of the radial threads of spider silk is about 1.1 GPa while that of steel is 0.4 GPa [33, 34]. Spiders produce many different types of silk [35] but the one most studied is the major ampullate silk, which forms the dragline and web frame. As it is considered impractical to breed spiders and harvest their silk, research has focused on understanding silk proteins and the self-assembling process of forming fibers. Using recombinant DNA technology a number of spider silk-like proteins have been produced. Besides the silk protein's primary and secondary structure the properties of the fiber are defined by the processing condition [36–41]. Using recombinant and regenerated silk proteins it has now become possible to produce strong fibers artificially. Two companies have efforts in place to produce artificial spider silk, Nexia biotechnologies Ltd (www.nexiabiotech.com) in Canada and Spintec Engineering Gmbh (www.spintec-engineering.de) in Germany. This technology could have applications in the field of medicine, as a new form of strong, tough artificial tendons, ligaments, and limbs. Spider silk could also be used to help tissue repair, wound healing, and to create super-thin biodegradable sutures for eye or neurosurgery, as well as being used as a substitute for synthetic fibers.

The remarkable properties of spider silk are because of its unique molecular structure (Figure 7.2). X-ray diffraction studies have shown that the silk is composed of long amino acid chains that form protein crystals [42]. The majority of silks (various spider and silk worm silks) also contain beta-pleated sheet crystals that form tandem repeated amino acid sequences composed of an 8–10 alanine-rich block and a 24–35 residue glycine-rich block. The resulting beta-sheet crystals crosslink the fibroins into a polymer network with great stiffness, strength, and toughness. This crystalline component is embedded in a rubbery component that permits extensibility, composed of amorphous network chains 16–20 amino acid residues long. It is this extensibility and tensile strength, combined with light weight that protects webs from wind damage and stops their anchoring points from being pulled off.

To transform the primary and secondary structures into an actual fiber the processing technology is critical. It involves ion-controlled self-assembly of individual proteins during the extrusion of a solution. The "die" shape and "extrusion rate" are critical for aligning the proteins and inducing the phase separating fiber formation (Figure 7.3).

Understanding the primary structure of spider silk has inspired scientists to reproduce the specific alanine–glycine beta-sheet–forming amino acid sequence and combine it with synthetic oligomers – polyethyleneglycol (PEG) [43, 44]. The latter prevents the complete aggregation of the beta-sheets and generates fibril structures similar to spider silk as shown in Figure 7.2. These hybrid approaches

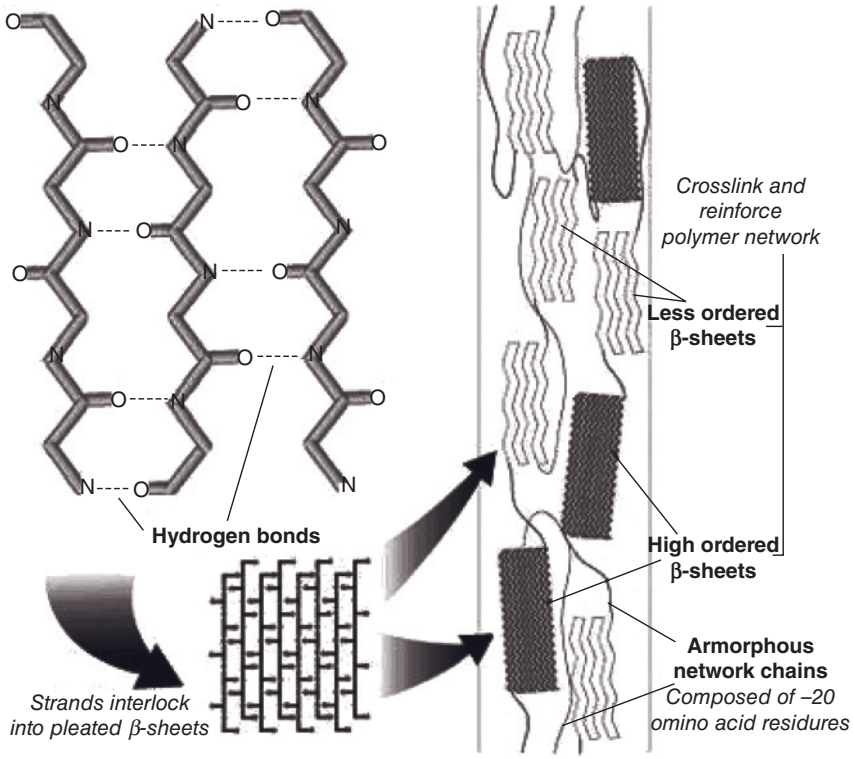

Figure 7.2 Representation of the structure of a strand of silk showing the primary amino acid residue structure and the beta-pleated sheet ultimately associating together multiple fibroin molecules. Source: (2009) http://www.scq.ubc.ca/biomimicrybimimetics-general-principles-and-practical-examples/

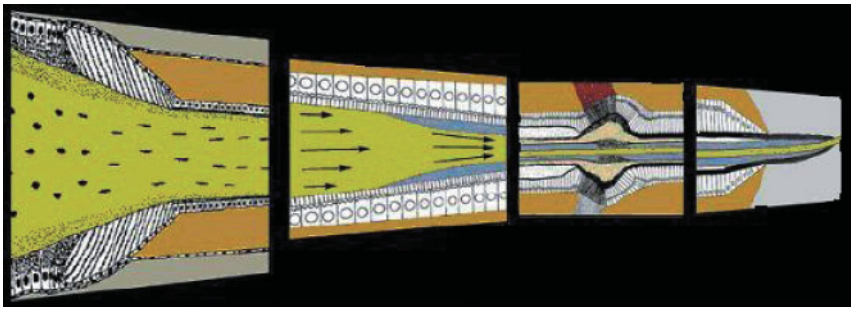

Figure 7.3 Schematic illustration of the spinning duct for production of dragline silk by orb-spider *Nephila*. It illustrates the fiber formation zone in which rapid elongational flow induces molecular alignment, phase separation, and fiber formation. Source: F. Vollrath, D. Knight, *Nature* 2001, 410: 541–548.

of combining peptides or other bioorganic oligomers with synthetic polymers can lead to rationally designed structures and tailored materials properties.

7.4.2 Gecko Adhesives

Spiderman, the Marvel Comics character created by Stan Lee and Steve Ditko in 1962, has the ability to use dragline spider silk to swing between high-rise buildings and to stick and climb any surface. These sci-fi capabilities are being turned into reality through technologies design to mimick the adhesive principles used by gecko lizards [45]. Their ability to adhere to nearly any surface relies on submicron keratin hairs that cover the soles of their feet (Figure 7.4). Each hair produces a minuscule force of ~10^{-7} N through van der Waals forces and capillary interaction but the millions of hairs acting together generate a formidable adhesion of ~10 N/cm^2 [46].

Researchers at the University of Manchester [47] and other universities have developed technologies to reproduce the shape and size of gecko hairs providing opportunities for reversible "stickiness without glue" or high-friction surfaces to support loads on smooth surfaces. For the latter the engineers at the University of California, Berkeley packed together 42 million fibers per square centimeter, each measuring a mere 20 μm long and 0.6 μm in diameter [48].

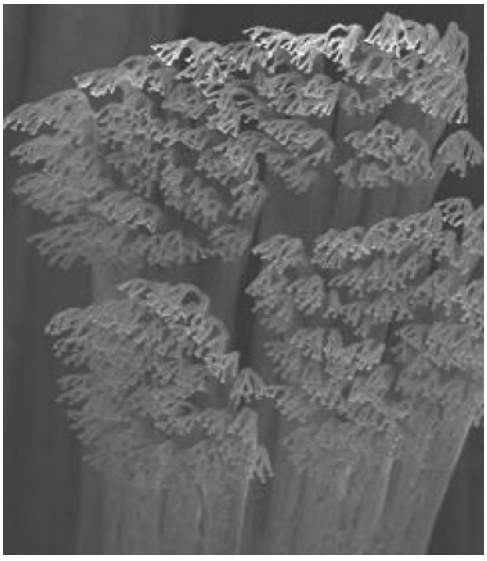

Figure 7.4 Electron microscopy analyses of the structure of *Gekko gecko* setae, indicating their formation from aggregates of proteinaceous fibrils held together by a matrix and potentially surrounded by a limiting proteinaceous sheath. Reproduced from [49].

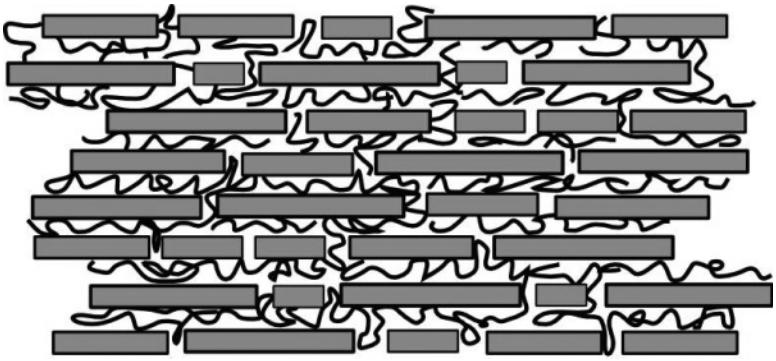

Figure 7.5 Representation of a biocomposite such as nacre, characterized by aragonite platelet layers in between organic polymers.

7.4.3
Nacre and Biomineralization

Nacre, also known as the mother of pearl, is a naturally occurring organic–inorganic composite. It is composed of hexagonal platelets of aragonite (calcium carbonate ($CaCO_3$) crystals) that are 10–20 μm wide and 0.5 μm thick, arranged in a continuous parallel lamina. The layers of platelets are separated by sheets of elastic biopolymers such as chitin, a polysaccharide, or silk-like proteins (Figure 7.5). This mixture of brittle platelets and the thin layers of elastic biopolymers make the material strong, resilient, and twice as tough as high-tech ceramics. Strength and resilience are conferred by the "brickwork" arrangement of the platelets, which inhibits transverse crack propagation and allows the material to slide under compressive force. Several research groups have mimicked nacre's structure, aiming to produce lightweight rigid composites and coatings for aircraft parts, artificial bone, and transparent abrasion resistance coatings.

In one approach, alternating layers of clay and a polyelectrolyte give a material that comes close to nacre's properties of strength and flexibility [50, 51].

Another approach takes advantage of a self-assembly process to create mineral/polymer layered structures that are optically clear but much tougher than glass. Unlike traditional top-down technologies, this evaporation-induced, low-temperature process allows liquid building blocks to self-assemble and harden into coatings that can toughen windshields, bodies of cars, airplanes, or anything that needs to be lightweight but fracture-resistant [52, 53].

Still other researchers have caused supersaturated solutions of calcium carbonate to deposit calcium carbonate films by addition of charged polypeptides [54, 55].

All these approaches are inspired by the process of biomineralization, through which living organisms produce minerals, often to harden or stiffen existing tissues. Examples include silicates in algae, carbonates in diatoms and invertebrates, and calcium phosphates (apatite) and carbonates (aragonite, calcite) in

vertebrates. These minerals typically form structural features such as sea shells and the bone in mammals, and are produced at ambient conditions without the need for elevated temperatures, pressures, and strong chemical solutions as for synthetic minerals [56, 57].

7.5 Materials and Products

7.5.1 Peptides and Proteins

Typically the term "peptide" refers to relatively short sequences of amino acid (<50) residues, and "protein" indicates polypeptides consisting of much longer amino acid sequences. The division is somewhat arbitrary, however, and relates to the approximate minimal sequence expressible in microorganisms or the maximum length that can be synthetically produced. According this convention insulin (51 amino acid residues) and the amyloid beta "protein" (39–43 amino acid residues) associated with Alzheimer's disease are peptides. Proteins tend to be the functional state in nature rather then the primary and secondary structures of the component polypeptide chains.

As living organisms are organized by proteins tremendous efforts have been spent in biochemistry and molecular biology to understand the relationship between monomer sequence (primary structure) and higher orders of organization in terms of secondary (alpha-helices and beta-sheets), tertiary (combinations of secondary structures in one molecule), and quaternary structures (assemblies of multiple molecules). Indeed by changing their conformational shape, proteins are able to perform a variety of functions, including muscle movement, molecular binding, enzymatic catalysis, metabolism, and transportation. The dynamic protein structure results from various forces encompassing charged interactions (covalent, ionic, electrostatic, and hydrogen bonds), hydrophobic interactions and dipole interactions. The latter, known as van der Waals forces include interactions like permanent dipole–permanent dipole, permanent dipole–induced dipole, and induced dipole–induced dipole (London dispersion forces) [58, 59].

Surprisingly, it was only in the 1990s that peptides and proteins were systematically investigated by physico-chemists for their enormous potential as "non-biological" building blocks for advanced chemistry and materials development beyond living organisms (e.g., [60–62]). The attraction is the potential to tune proteins in terms of structure, stereoisomerism, polarity, functionality, and function by altering the amino acid monomer sequence. However, in view of the structural complexity of proteins, and the many interactive forces, a focus on peptides seemed like a more practical approach.

In particular, small peptides (3–25 amino acid residues) are the object of much attention. They are small enough to produce synthetically and demonstrate sufficient functionality and diversity to study bottom-up molecular self-organization.

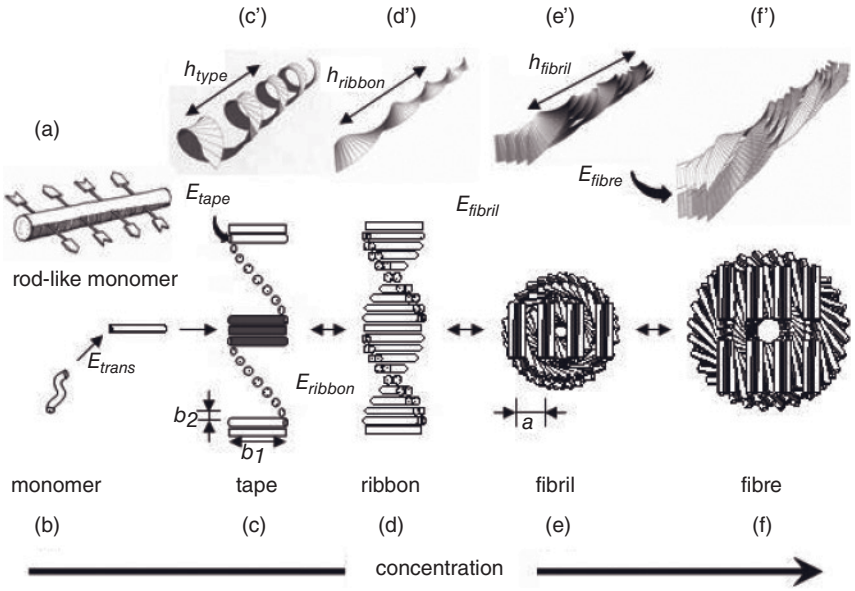

Figure 7.6 Peptides of a few amino acids (3–25) will self-assemble into various shapes depending on the boundary conditions (concentration, solvent, pH). The rod-like monomer may form tape-like beta-sheets that in turn may associate to form ribbons, fibrils, and fibers [63, 64]. Courtesy A. Aggeli, S. Scanlon.

The highly directional and tunable self-assembly capability is triggered by boundary conditions, such as concentration, pH, temperature, and electromagnetic field. It allows hierarchical layers of supramolecular structures to be built and controlled, and ultimately the creation of materials from the nano to the macro length scale (Figure 7.6).

7.5.1.1 Self-Assembling Peptide and Protein Applications

A vast array of applications of peptides and proteins as novel materials is possible. To date, several self-assembling peptides have been studied either with a focus on understanding the structure-forming mechanism or the use of these systems and mechanisms for specific applications. A few of the more industrially relevant efforts beyond medical and pharmaceutical applications relate to [65–71]:

- construction motifs
- detergents and surfactants
- molecular switches
- inks
- nanofibers
- nanowires
- nanotubes and vesicles
- functional nanocoatings and surface layers

- templates
- scaffolds
- hydrogels.

7.5.1.2 **Antimicrobial Peptides**

Another important application exploiting the self-organization capacity of peptides is found in their antimicrobial action [72–79]. Antimicrobial peptides are typically between 12 and 50 amino acid residues long. They are found in nature or are synthetically manufactured. The amino acid sequence and length is specific for the peptides to be able to fold into their final configuration as an alpha-helix or beta-sheet structure and to partition in the biological membranes, mostly lipid bilayers. The antimicrobial peptides include two or more positively charged residues provided by arginine, lysine, or, in acidic environments, histidine, and a large proportion (generally >50%) of hydrophobic residues. This arrangement depends upon the formation of a secondary structure with a hydrophobic–apolar (A)–and hydrophilic–polar (P)–side to interact with the biological membrane (Figure 7.7) [80, 81]. Such amphipathic helices can pack together forming globular structures with an exposed polar surface in aqueous media. Typically, the alpha-helices consist of seven amino acid residues allowing for a periodicity of 3.5 amino acids per turn in a sequence of AAPAAAP or PPAPPPA.

The ability to associate with membranes for permeabilization is a definitive feature of antimicrobial peptides although action on a range of cytoplasmic targets is also possible [82]. However poly-ε-lysine, which is a cationic, naturally occurring peptide of L-lysine, also shows a wide range of antimicrobial activity in very low concentrations and is stable at high temperatures [83]. The product is widely used in Japanese sushi to preserve freshness.

The development of an amphipatic nature through secondary structure formation opens options for switchable antimicrobials and reversible surfactants [84].

7.5.1.3 **Antifreeze Proteins**

Antifreeze proteins (AFPs) or ice-structuring proteins (ISPs) are a class of peptides and glycoproteins produced by certain vertebrates, plants, fungi, and bacteria that

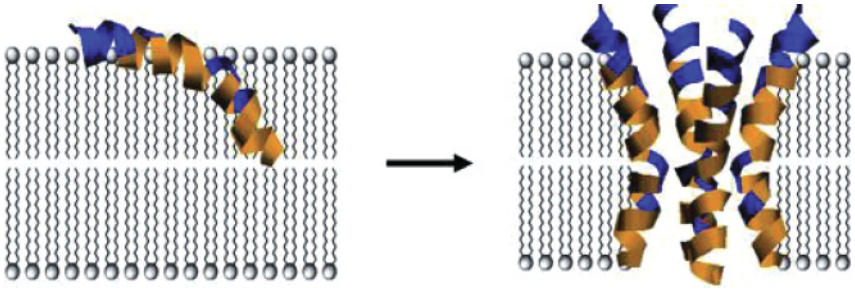

Figure 7.7 The antimicrobial action of peptides follows from the formation of amphipathic alpha-helices that permeabilize the lipid bilayer of microorganisms.

permit their survival in subzero environments. AFPs prevent ice nucleation and bind to small ice crystals to inhibit growth and recrystallization of ice that would otherwise destroy cells and organelles [85–88].

Commercially, there appear to be infinite uses for AFPs. Numerous fields would be able to benefit from protection from tissue damage by freezing. Businesses are currently investigating the use in:

- increasing freeze tolerance of crop plants and extending the harvest season in cooler climates
- improving farmed fish production in cooler climates
- lengthening shelf-life of frozen foods
- improving cryosurgery
- enhancing preservation of tissues for transplant or transfusion in medicine
- a therapy for hypothermia.

Currently two companies market AFPs extracted from natural resources: A/F Protein Inc, in the United States (www.afprotein.com/) and Ice Biotech Inc. in Canada (www.icebiotech.com/).

One recent successful business endeavor has been the introduction of AFPs into ice cream and yogurt products. The proteins are isolated from fish and replicated, on a larger scale, in yeast. Currently, the Unilever Company incorporates AFPs into some of its products, including popsicles and a new line of Breyers Light Double Churned ice cream bars. In ice cream, AFPs allow the production of very creamy, dense, reduced fat ice cream with fewer additives. They control ice crystal growth brought on by thawing on the loading dock or kitchen table which drastically reduces texture quality [89, 90].

7.5.1.4 **Biosynthetic Hybrids**

Despite the huge potential, the drawback for many of the peptide and protein-based materials is their complexity and the high cost of synthesis.

It is thought that these disadvantages can be avoided to some extent through hybrid systems. The aim is to combine the benefits of the defined secondary structure formation capacity of peptides and proteins with the excellent material properties and cheap and easy to synthesize synthetic polymers. The same principle could be applied to the use of other biomolecules such as nucleic acids, lipids, and carbohydrates.

The polymer hybrid systems consist of two components: one responsible for material properties and the other for structure formation resulting from spontaneous or induced aggregation of the biomolecule. The properties of the resulting materials are dominated by the synthetic polymer. It is anticipated that this allows for a rational targeting of structures as defined by aggregation motifs known in biological systems. Furthermore, these materials could have abilities to interact actively with living biological systems, addressing aspirations for bioadhesion, biorepulsion, biocompatibility, and specific bio-interaction issues [33, 43, 44, 91–96].

7.5.2 Polynucleotides

DNA is a nucleic acid that contains the genetic instructions for the development and functioning of living organisms. DNA is a long polymer of simple units called nucleotides, which are held together by a backbone made of sugars and phosphate groups. This backbone carries four types of molecules called bases, and it is the sequence of these four bases that encodes information. A nucleotide is a chemical compound that consists of three portions: a heterocyclic base, a sugar, and one or more phosphate groups. In most nucleotides the base is a derivative of purine (adenine, guanine) or pyrimidine (cytosine, uracil, thymine), and the sugar is a pentose (five-carbon sugar) deoxyribose or ribose. Nucleotides are the monomers of nucleic acids, with three or more bonding together in order to form a nucleic acid.

RNA is a nucleic acid polymer consisting of nucleotide monomers that acts as a messenger between DNA and ribosomes (the organelle that synthesizes proteins). RNA polynucleotides contain ribose sugars and predominantly uracil, unlike DNA, which contains deoxyribose and predominantly thymine.

It is clear that these polynucleotides complement the use of peptides and proteins. However the number of building blocks is fewer and their secondary structure is typically restricted to helices, making them far more selective but less versatile when exploiting the ability to self-assemble. Nevertheless, there is still potential for a wide range of applications [97–108], such as:

- conductive nanowires
- nano architectures and patterns
- computing
- molecular recognition devices
- catalysts
- nanodevices
- nanomachines.

Currently, polynucleotide research is mostly confined to the academic world, focusing on gaining knowledge and exploring possible structural constructs as mentioned above.

7.5.3 Lipids

Lipids are an amphiphilic class of hydrocarbon-containing organic compounds, with complicated solvation properties, giving rise to lipid polymorphism. Lipid molecules consist largely of long hydrocarbon tails and polar headgroups (e.g., phosphate-based functionality, and/or inositol (cyclic polyol, carbohydrate)-based functionality). In living organisms, lipids are used for energy storage, serve as the structural components of cell membranes, and constitute important signaling

molecules. Although the term lipid is often used as a synonym for fat, the latter is in fact a subgroup of lipids called triglycerides. Chemically, fatty acids can be described as long-chain monocarboxylic acids and have a general structure of $CH_3(CH_2)_nCOOH$. The length of the chain usually ranges from 12 to 24, always with an even number of carbons.

Glycerides are lipids possessing a glycerol (propane-1,2,3-triol) core structure with one or more fatty acyl groups, which are fatty acid–derived chains attached to the glycerol backbone by ester linkages. Glycerides with three acyl groups (triglycerides or neutral fats) are the main storage form of fat in animals and plants.

Phosphoglycerides or glycerophospholipids are important types of glyceride-based molecules found in biological membranes, such as the cell's plasma membrane and the intracellular membranes of organelles. A biological membrane is a form of lipid bilayer. Formation of lipid bilayers is an energetically favored process when the glycerophospholipids are in an aqueous environment. The polar heads of lipids orient towards the polar, aqueous environment, while the hydrophobic tails minimize their contact with water. The lipophilic tails of lipids tend to cluster together, forming a lipid bilayer (1) or a micelle (2). Other aggregations are also observed and form part of the polymorphism of amphiphile (lipid) behavior. Micelles and bilayers in an aqueous environment induce the water molecules to form an ordered "clathrate" cage around the dissolved lipophilic molecules (Figure 7.8). Clathrate cages of ordered water form around individual lipids which entails a high entropy penalty driving self-assembly into bilayers or micelles (i.e., it leads to the release of the bound water, meaning an entropy gain). This is the main driving force for self-assembly.

The self-organization polymorphism potential depends on the concentration of the lipid present in solution. Below the critical micelle concentration (CMC) the lipids form a single layer on the liquid surface and are (sparingly) dispersed in the

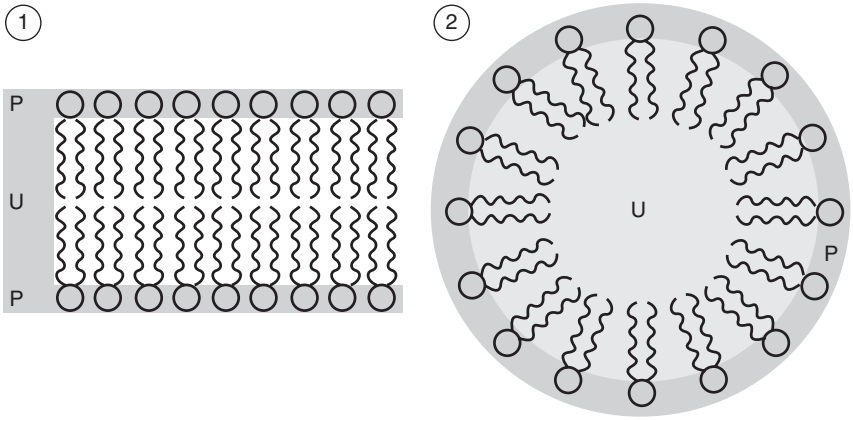

Figure 7.8 Self-organization of phospholipids. A lipid bilayer is shown on the left (1) and a micelle on the right (2) P = hydrophylic "head" oriented towards the water phase and U = hydrophobic "tail" oriented to non-water phase.

solution. At the first CMC-I, the lipids organize in spherical micelles, and at given points above this concentration, other phases are observed.

Nanobiotechnologists rely on these self-assembling properties of lipids to create nanostructures, colloid and template patterns [109–111], and to explore interaction with other biomolecules [112–114]. The versatility of lipids is predominantly based on their physico-chemistry and phase behavior but less on higher order structure formation and functionality as is the case with peptides and polynucleotides. Differently put, lipid self-assembly is mainly caused by phase separation and molecular packing, rather than being a result of specific intermolecular interactions. It is mainly entropically driven, whereas in (poly)peptides and (poly)nucleotides self-assembly is mainly enthalpically driven.

In most cases the structural integrity of lipids is limited to specific physiological boundary conditions. This may be a benefit, particularly when considering lipid micelles as a delivery vehicle for cosmetics [115], nutrients [116], and drugs [117–120].

7.5.4 Carbohydrates

Carbohydrates or saccharides (from the Greek word *sakcharon* meaning "sugar") are straight-chain polyhydroxylated aldehydes or ketones containing 3–9 carbon atoms. Carbohydrates are the most abundant biological molecules, and fill numerous roles in nature, such as the storage and transport of energy (starch, glycogen) and structural components (cellulose in plants, chitin in animals). In addition, carbohydrates and their derivatives play major roles in the functioning of the immune system, fertilization, pathogenesis, blood clotting, and cell growth.

Carbohydrates containing between about 3 and 6 monosaccharide units are termed oligosaccharides; anything larger than this is a polysaccharide. Polysaccharides, such as starch, glycogen, or cellulose, can reach many thousands of units in length. Many carbohydrates contain one or more modified monosaccharide units that have had one or more groups replaced or removed. For example, deoxyribose, a component of DNA, is a modified version of ribose; chitin is composed of repeating units of *N*-acetylglucosamine, a nitrogen-containing form of glucose.

Carbohydrates provide additional chemical complexity as they have multiple chiral centers giving rise to stereoisomers.

Starch, cellulose, amylopectin, amylose, and chitin are the most widely commercially used biopolymers today. In fact they were the first polymers to be studied and used to replace natural materials (e.g., ivory) in the novel applications that laid the foundations of the synthetic plastics industry in the late nineteenth century.

In the field of nanobiotechnology, carbohydrates have been investigated for various purposes. Carbohydrate hydrogels are biocompatible and can be used for the uptake of other biomolecules [121]. Exploiting the self-assembling characteristics of carbohydrates allows for either formation of organized nano-structures or in combination with other molecules the construction of distinct nano-structures depending on the composition of the block molecule [122]. Such approaches relate

to the use of antifreeze glycoproteins as mentioned above, which allow the carbohydrate part to be functionalized with metallic nanoparticles [123].

7.6 Processes and Devices

Nanobiotechnology comprises a multitude of science and technology approaches, devices, and systems. The aim is the understanding and construction of structures starting from atoms and molecules in order to develop useful products and functions. The various building blocks found in nature and described above demonstrate the benefit of extreme versatility to achieve precisely that. Comprehensive studies of cellular nanostructures such as photosynthetic reaction centers, ribosomes, DNA replication, mitochondria, and membrane channels have provided inspiration for the exploitation of various biological processes and devices [100, 124].

7.6.1 Nanomachines

Nanomachines can be envisioned as tiny assemblies built from single molecules and useful as tools to create complex materials, to repair tiny defects on surfaces or in living cells, or to store and retrieve information. Nanomachines are very different from macro world machines but perform related functions, such as transporting things or changing shapes.

An essential nanomachine component is a molecular motor that converts chemical energy into mechanical energy. In nature this is typically achieved through hydrolysis of ATP or via the translocation of ions through cell membranes.

Some examples of biologically important molecular motors are:

- motor proteins
- myosin – responsible for muscle contraction [125–127].
- kinesin – moves cargo inside cells away from the nucleus along microtubules [128, 129]
- dynein – produces the axonemal beating of cilia and flagella and also transports cargo along microtubules towards the cell nucleus [130, 131]
- F0F1 ATP synthase – generates ATP using the transmembrane electrochemical proton gradient inside mitochondria [132] (Figure 7.9)
- RNA polymerase – transcribes RNA from a DNA template [133]
- actin polymerization – generates forces and can be used for propulsion [134]
- topoisomerases – reduce supercoiling of DNA in the cell
- the bacterial flagellum – responsible for the swimming and tumbling of *E. coli* and other bacteria and acts as a rigid propeller that is powered by a rotary

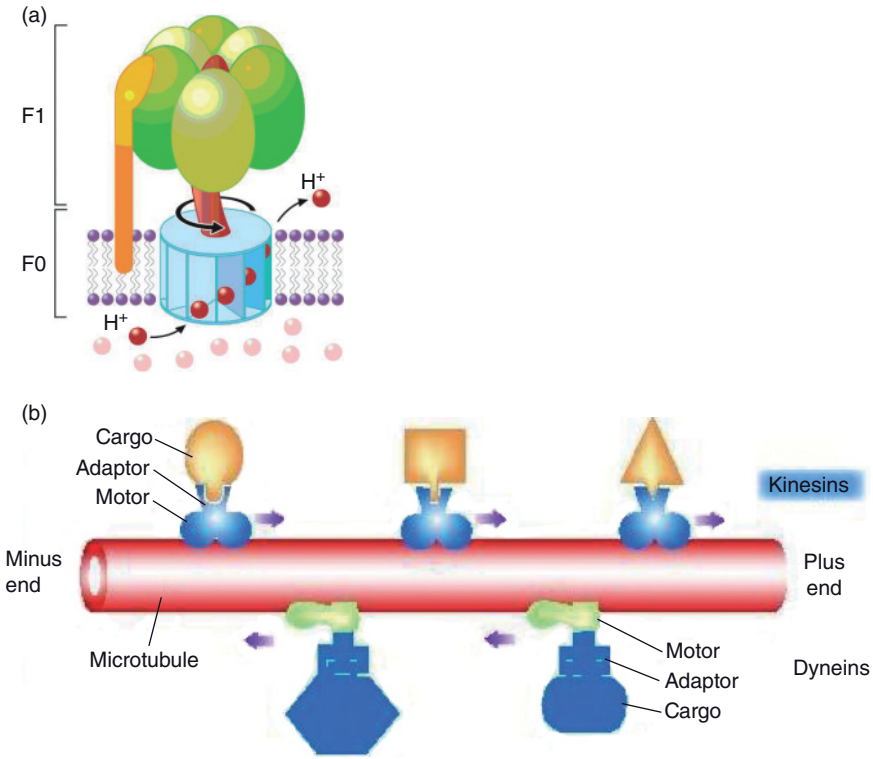

Figure 7.9 Simplified representation molecular motors providing a rotational motion, the F0F1 ATP synthase (a), and linear motion from kinesin and dynein (b).

motor. This motor is driven by the flow of ions across a membrane, possibly using a similar mechanism to that found in the F0 motor in ATP synthase.

- viral DNA packaging motors – inject viral genomic DNA into capsids as part of their replication cycle
- synthetic molecular motors – created by chemists that yield rotation, possibly generating torque.

All such molecular motors provide either rotational movement or linear, forward propulsion. They will be essential in future nanodevices such as levers, valves, pumps, pincers, and other functional parts of nanomachinery. The ever better understanding of their operation has inspired scientists to apply similar concepts and begin to design molecular machines.

Kinesins are responsible for the transport of gases or glucose as cargo about the cell by walking unidirectionally along microtubule tracks hydrolyzing one molecule of ATP at each step. The analogy with a "cargo train" on a track inspired ETH Zurich researchers to build a complete "railroad" for an application, such as

a surface-imaging method based on nanoscale probes, which are propelled by motor proteins [135–137].

Researchers at Cornell University (USA) have been able to connect a biological protein F0F1 ATP synthase-type motor to a tiny metal propeller. The device consists of an 11-nm-square motor, anchored to a 200-nm nickel post, and sports a 750-nm-long nickel propeller. The tiny bits of metal were produced using microelectromechanical systems (MEMS) processes. The energy released from the three ATP molecules needed to rotate the motor once is actually 240 piconewtons per nanometer, giving the motor a 50% efficiency rate [138, 139].

Beyond biological molecular motors, alternative approaches are underway to develop non-biological, non-peptide synthetic molecular motors that are capable of rotation with energy input. Chemists have made several attempts. A three-bladed triptycene rotor connected to a rigid helicene scaffold is able to rotate 120° in a five-step reaction sequence [140].

A 360° rotating molecular motor system activated by temperature has been developed at Groningen University (The Netherlands). It consists of a bis-helicene connected by an alkene double bond displaying axial chirality and with two stereocenters [141].

Electrochemically switchable single polymer chains are also possible candidates for such motors. Poly(ferrocenyldimethylsilane) (PFS) can reversibly be oxidized and reduced by an external potential. This leads to changes in its mechanical properties, which can be used to drive a cyclic molecular engine [142].

More recently, researchers at Rice University, Texas (USA) designed a nanodevice to solve the question of how fullerenes move about on metal surfaces; specifically, whether they roll or slide. The "car-like" molecule consists of a H-shaped "chassis" with fullerene groups attached at the four corners to act as wheels. When dispersed on a gold surface, the molecules attach themselves to the surface via their fullerene groups. Upon heating the surface to 200 °C the molecules move forward and back as they roll on their fullerene "wheels." The "nanocar" is able to roll about because the fullerene wheel fits to the alkyne "axle" through a carbon–carbon single bond. The hydrogen on the neighboring carbon is no great obstacle to free rotation. When the temperature is high enough, the four carbon–carbon bonds rotate and the car rolls about [143].

Beyond motors, the next generation of non-silica computers is being explored using DNA as an information storage molecule. In 1994, Leonard Adleman of the University of Southern California (USA) demonstrated a proof-of-concept use of DNA as a form of computation [144]. Ten years later researchers at the Weizmann Institute (Israel) constructed a DNA computer [145]. For certain specialized problems, DNA computers are faster and smaller than silicon-based computers. However, for problems which grow exponentially with the size of the problem the amount of DNA required is too large to be practical.

Double-stranded DNA or protein chains that are modified with metallic nanoclusters act as templates for the synthesis of metallic nanowires. The nanowires are used as building blocks to assemble nanodevices such as a transistor or a nanotransporter [101, 146].

7.6.2 Biosensors and Biochips

Biomolecules are highly functional. In combination with their capacity for self-assembly, very specific shapes and surfaces can be built depending on the boundary conditions. This makes them ideal for detecting and signaling specific processes, particles, and chemicals at a molecular level. The literature in this field is exploding with ideas and proof of concepts.

Typically a biosensor consists of three parts:

- a sensitive biological element (tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, peptides, lipids, carbohydrates)
- a transducer connecting and translating the sensor information to the detector
- a detector (displaying and registering in a physicochemical way, optical, piezoelectric electrochemical, thermometric, or magnetic effects).

These sensors can be immobilized on substrates or may be free floating in liquids or serum. Biosensors are also known as biochips in analogy with the semiconductor industry where miniaturization has allowed for ever more advanced and smaller sensor development.

There are many potential applications for biosensors of various types for specific research or commercial requirements. Some examples are:

- glucose monitoring in diabetes patients
- medical health-related targets
- environmental applications (e.g., the detection of pesticides and river water contaminants)
- remote sensing of airborne bacteria (e.g., in counter-bioterrorist activities)
- detection of pathogens
- determining levels of toxic substances before and after bioremediation
- detection and determining of organophosphates
- routine analytical measurement of folic acid, biotin, vitamin B_{12}, and pantothenic acid as an alternative to microbiological assay
- determination of drug residues in food, such as antibiotics and growth promoters, particularly meat and honey
- drug discovery and evaluation of biological activity of new compounds
- high throughput screening in genomics and proteomics research.

Proteins and polynucleotides are typically used as the sensor molecules while lipids and carbohydrates often provide the substrate surfaces [147–155].

7.6.3 Bioreactors

In the context of nanobiotechnology a bioreactor refers to any device or system that supports a biochemically active environment at the molecular level. In contrast to macro bioreactors ranging in size from some liters to cubic meters, the

present bioreactors consist of vesicles of nano or micron size in which chemical process may also take place. The reactors are built to exploit the self-assembly capacity of biomolecules such as lipids, surface layer proteins, and carbohydrates. In a number of other cases various biomolecular surfaces are activated to act as reactors through immobilized enzymes, polynucleotides, and carbohydrates [156–158]. The scaling down of reactors offers opportunities for high throughput research, as in the case of biosensors.

7.6.4 Catalysts

Catalysts are molecules that facilitate and accelerate chemical reactions without being consumed in the process. Catalysts are widely used in all kind of chemical processes in a variety of industries. Enzymes are the biological versions of catalysts. They are proteins that typically enable highly specific reactions and operate under mild, environmentally friendly conditions. Several natural enzymes are used in and studied for their potential application in multiple industries. Many market sectors are taking advantage of the water-based environment and specific activity of enzymes.

There are many enzymes with impressive levels of stereospecificity, regioselectivity, and chemoselectivity [159]. However, enzymes have limitations in the number of reactions they can catalyze and in their lack of stability in organic solvents and at high temperatures. In addition cofactors (helper molecules) may be required for actual catalysis to take place. Consequently, protein engineering is an active area of research and involves attempts to create new enzymes with novel properties, either through rational design or *in vitro* evolution [160, 161]. Some of that research is focused on, for example, lipases, and finding interesting enzymes in nature. Several companies are investing in such activities. For example, Diversa Inc. (www.diversa.com/) and Maxygen Inc. (www.maxygen.com/) have investigated enzyme development for medical and non-medical applications.

A lipase is a water-soluble enzyme that catalyzes the hydrolysis of ester bonds in water-insoluble, lipid substrates. The physiological role of lipases is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. Of all known enzymes, lipases have attracted the most scientific attention. In addition to their natural function of hydrolyzing ester bonds, lipases can catalyze esterification, interesterification, and transesterification reactions in non-aqueous media. This versatility makes lipases the enzymes of choice for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries. The most significant industrial applications of lipases are found in the food, detergent, and pharmaceutical sectors [162].

One particular class of enzymes, the laccases, has received much attention for its ability to oxidize both phenolic and non-phenolic lignin-related compounds as well as environmental pollutants. Laccases are multicopper oxidases (1,4-benzenediol oxidases) (i.e., dimeric, trimeric or tetrameric glycoproteins that contain four copper atoms per monomer). These enzymes are found in higher plants, fungi,

and microorganisms of wide specificity and carry out one-electron oxidation of diphenols, polyphenols, aminophenols, polyamines, lignins, and aryldiamines. Laccases can also be used as the cathode in an enzyme-catalyzed fuel cell. They perform the reduction of oxygen to water and can be paired with an electron mediator [163].

7.6.5 Scaffolds

The capacity of many biomolecules to self-assemble into higher order structures opens opportunities to build tailor-made frameworks that temporarily support the construction of more permanent structures. Particularly in the medical field several scaffold applications can be envisioned. One is tissue engineering for wound care where biocompatible scaffolds provide temporary substitutes for an extracellular matrix [164, 165]. The concepts around wound healing are various and range from use of regenerated tissue, biodegradable nanofiber structures, and hydro gels with the aim of using collagen or induce fiber formation in the wound. Scaffolds can also be used to provide the matrix for various cells, for example, stem cells that may regenerate bone or nerves. Researchers at Northwestern University (USA) have used peptide solutions that, after injection into the spinal cord or bone, self-assemble and form an environment for stem cells to regenerate nerves and bones [166–168]. Similarly the switchability from a liquid phase to a self-supporting gel using self-assembling peptides provides support for shaping biomineralization [56], and delivery of active molecules in difficult to reach places [169].

Beyond medical reconstructive applications, the same principle can be applied for drug, dyes, perfumes, and colloidal systems delivery.

7.7 Outlook

The convergence of nanotechnology and biotechnology into nanobiotechnology has spurred significant research effort. In the last few years the number of papers has been and is still growing exponentially. It is clear that many drivers encourage this process. A focus on sustainability emphasizes the need for more environmentally sound materials, processes, and devices to maintain or improve the quality of life. Looking for inspiration in nature is a logical consequence. A better understanding of biological processes combined with new tools for manipulating biomolecules has opened an avenue towards innovative goods and services. Proteomics and genomics have provided novel tools and insights in the workings of natural processes. Nevertheless, many challenges remain to be overcome before the potential of nanobiotechnology can be realized. Operating at a molecular level requires different tools and presents researchers with a new kind of physics. Self-organization of molecules and the self-assembly of higher order structures is governed and coupled to the environment of the molecular reality. To understand and to

learn to apply the physics of molecular association is a major endeavor. There exists an almost infinite number of self-organization possibilities in view of the versatility of the building blocks and the diversity of triggering mechanisms and boundary conditions. In addition, the production of tailor-made biomolecules with consistent quality in large quantities is not yet a reality. For example, the expression of proteins with recombinant DNA technology is a costly, very specific operation with significant engineering challenges relating to separation and purification. Accordingly many of the nanobiotechnology potentials are conceptually sound, innovative, and fascinating but to date restricted mainly to academic research.

Nevertheless, it worth remembering that similar considerations were expressed at the beginning of the nineteenth century about organic substances being impossible to create in the laboratory. We know better now. As an analogy, nanobiotechnology encompasses a science and technology field in its infancy with many unsolved problems and limited technological tools. Moreover, there is no guidance on what path to follow to achieve a successful industrial application except for knowing that the future is already found in nature.

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8
Downstream Processing in Industrial Biotechnology

Rajni Hatti-Kaul

8.1
Introduction

Downstream processing (DSP) implies recovery of a biotechnological product from a bioreactor and its purification to a form suitable for its intended use [1]. The products of industrial biotechnology are diverse and are normally grouped into chemicals, materials, and fuels. As the production is mainly based on fermentation technology and biocatalysis, the starting point of downstream processing often constitutes either a crude fermentation broth containing the producer cells, nutrients, metabolites, unconsumed substrate, side-products, etc. in large volumes of water or a reaction mixture containing the enzyme/whole cells (free or immobilized), reactants, solvent, water, and side-products. The main challenges in downstream processing thus involve separation of the product, which is often formed in relatively low concentrations, from a multitude of other components, especially those with similar properties as the product itself.

A conventional sequence of separations starts with the use of techniques that separate components having largest difference in physiochemical properties and ending with separation of molecules with more or less similar properties (Figure 8.1). This makes separation of the soluble product from cell mass or biocatalyst a primary recovery operation. In case the product is intracellular, for example, proteins and other biopolymers, the cells have to be disrupted to release the product followed by another solid–liquid separation step. The particle-free liquid is then processed for concentration and purification of the product using a number of unit operations, the choice of which is defined not only by properties of the product but also by the complexity of separations, scale of operation, economics, and desired purity of the product.

The importance of DSP for a process is realized by its influence on the total production costs. It is suggested that DSP typically accounts for 50–70% of total production costs for fermentation products; for bulk products the costs can be in the range of 10–50%, and up to 90% for high-purity pharmaceutical products. As biotechnological production moves into large-volume, low-priced chemicals, it

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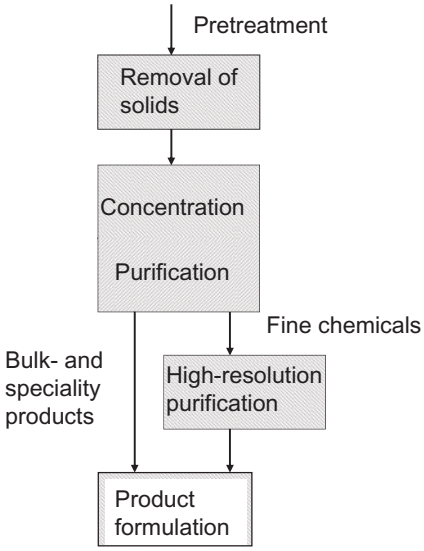

Figure 8.1 A simplified scheme for downstream processing of products of industrial biotechnology.

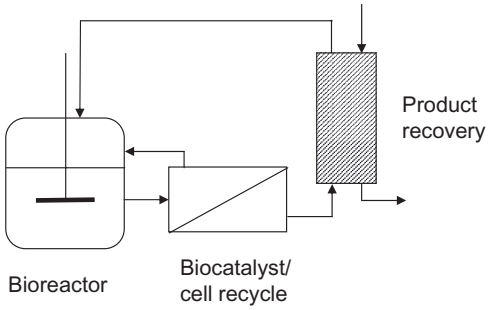

Figure 8.2 The concept of *in situ* product recovery (ISPR). The product is continuously or intermittently harvested from the bioreactor using a suitable separation technique. The biocatalyst or whole cells used in the process may be removed from the process stream and recycled prior to the product capture step.

becomes necessary to minimize costs by maximizing separation efficiency with minimal energy input and low waste generation.

It is thus important to consider the downstream processing strategy already when designing the bioprocess. An optimal choice of separation techniques is crucial. Yet another interesting aspect is the possibility of utilizing the product separation as a means of harvesting the product directly from the bioreactor (Figure 8.2). The concept of *in situ* product removal (ISPR) is attracting growing interest as a technological solution for improving the productivity of bioprocesses

that are often characterized by low yields. Increases in the yield may be expected due to overcoming inhibitory or toxic effects of the products, or minimizing product losses by degradation, or shifting unfavorable reaction equilibria [2–6]. Moreover, the number of downstream steps can be reduced, especially if the product recovery is done in a selective manner. However, for ISPR to be successful, evaluation of a separation technique for its suitability for integration with the bioreactor without any negative influence on the process (e.g., toxicity to cells or denaturation of enzymes) is an important prerequisite.

This chapter provides an overview of the unit operations used for separations in biotechnology followed by downstream processing examples of some well-known products.

8.2 Separations in Industrial Biotechnology

The various downstream processing unit operations used in biotechnology can be classified on the basis of driving force for separation as shown in Table 8.1. Many of the techniques are well known traditional unit processes and have been adapted from chemical and pharmaceutical industry and also food industry. Some techniques have been developed, however, and refined to meet the needs of the biotechnology industry. In this respect, membrane-based and chromatographic separations are worth mentioning. Developments in membrane technology have made membrane function highly versatile, allowing separation of a wide range of products based on different separation principles. Liquid chromatography is likewise based on different separation forces and is widely used in a variety of modes for resolution of both small molecules as well as macromolecules.

8.2.1 Separation of Solid Particles

Solid–liquid separation is the predominant primary unit operation in downstream processing, involving the removal of cell biomass from fermentation broth, cell debris after cell disruption in case of intracellular products, and immobilized biocatalyst, but is also used during the intermediate and final stages for separation of precipitates and crystals [7, 8]. Often the choice is made between centrifugation and filtration (and in recent past microfiltration), however a few other techniques with varying separation mechanisms have also been used to some extent.

8.2.1.1 Filtration

Filtration is the most cost effective means of separating large solid particles from a suspension. Passage of a particulate suspension through a porous filter medium results in deposition of particles and formation of a cake on the filter surface which increases in size with time. The rate of filtration at any given time is related to different parameters as:

Table 8.1 Classification of separation techniques used on the basis of specific product characteristics.

Separation basis	Separation technique	Application in downstream processing
Size	Filtration	Separation of cell biomass, debris, immobilized biocatalyst, precipitate and crystals
	Microfiltration	Separation of cell biomass
	Ultrafiltration	Concentration of proteins, desalting
	Nanofiltration/reverse osmosis	Removal of small solutes, desalting
Density difference	Centrifugation	Separation of cell biomass, debris, immobilized biocatalyst, precipitate and crystals
Volatility	(Vacuum-) distillation Gas stripping	Recovery of low molecular weight volatile products
Volatility + membrane permeation	Pervaporation Transmembrane distillation	
Solubility	Liquid–liquid extraction	Recovery of non-polar (and charged) low molecular weight products
	Supercritical fluid extraction	
	Aqueous two-phase extraction	Recovery of proteins
	Precipitation	Concentration and purification of low molecular weight products and proteins
	Crystallization	Purification and formulation of low molecular weight products and proteins
Charge	Ion exchange	Concentration and purification of charged low and high molecular weight products
	Electrodialysis	Purification of organic acids
Hydrophobicity	Reverse phase	Purification of non-polar solutes
	Hydrophobic interaction chromatography	Purification of proteins
Molecular recognition	Affinity chromatography	Selective purification of proteins
	Molecular imprinting	Separation of structurally similar compounds, enantiomers, etc.

$$\frac{dV}{dt} = \frac{\Delta pA}{\alpha\mu m + \mu r_m} \tag{8.1}$$

where V is the filtrate volume, t is the filtration time, A is the filter area, Δp is pressure drop across the filter, μ is dynamic viscosity, m is total mass of the cake, α is the average specific cake resistance, and r_m is the filter medium resistance. The mass of the cake deposited per unit area is related to the concentration of solids C in the suspension, that is, $mA = CV$.

The r_m is often negligible compared with α, which is dependent on the shape and size of the particles, size of the interstitial spaces between them, and mechanical stability of the cake. If the filter cake is incompressible, α does not vary with the pressure drop across the filter. However, the filter cakes obtained from fermentation broths are compressible, which cause decrease in filtration rates. The specific cake resistance can be reduced by increasing the cake porosity, altering the morphology, or increasing the average size of the particles [9]. Filter aids such as diatomaceous earth are widely used to improve the efficiency of filtration. The filter aids can be used as a pre-coat on the filter medium to prevent blockage of the filter by solids, and can also be added to the fermentation broth to increase the porosity of the cake. Heating the fermentation broth to denature proteins or addition of electrolytes to promote coagulation of colloids into larger, denser particles are other means of pretreatment to improve the filtration characteristics of broths.

Filtration can be operated in two different modes – at constant pressure (vacuum) where the filtration rate will progressively reduce as resistance due to the cake increases, and at constant rate where the flow rate is maintained by gradually increasing the pressure drop. Constant pressure filtration is the most common mode of filtration, where V and t are the only variables. Rearrangement of Equation 8.1 and integration from $V = 0$ to $V = V$ and $t = 0$ to $t = t$ gives:

$$\frac{dt}{dV} = \frac{\alpha\mu CV}{A^2\Delta p} + \frac{\mu r_m}{A\Delta p} \tag{8.2}$$

$$\frac{t}{V} = \frac{\alpha\mu C}{2A^2\Delta p}V + \frac{\mu r_m}{A\Delta p} \tag{8.3}$$

The terms $\alpha\mu CV/(2A^2\Delta p)$ and $\mu r_m/A\Delta p$ can be further simplified to K_1 and K_2, respectively. K_1 and K_2 are constants during constant pressure filtration and a plot of t/V versus V is thus a straight line, wherein the slope K_1 depends on the filtration pressure drop and properties of the cake and the intercept K_2 is also dependent on the pressure drop but is independent of the cake properties [10].

Large-scale filtration of fermentation broths containing fungal mycelia is often done using vacuum filters such as the rotary drum vacuum filter shown in Figure 8.3.

8.2.1.2 **Microfiltration**

The past decade has seen a dramatic increase in the use of membrane filtration for separation of particles and molecules, wherein a semi-permeable membrane

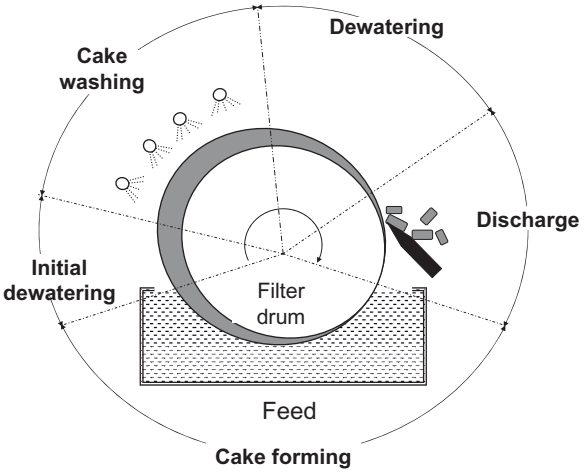

Figure 8.3 A rotary drum vacuum filter and its operation. The equipment comprises a rotating drum covered with a filter partially immersed in a tank of particulate broth. The drum is maintained under reduced internal pressure and rotated at a slow speed, picking up the biomass that is deposited as a cake on the filter surface. The continuous rotation of the drum allows dewatering, washing, and drying of the filter cake to be performed prior to its discharge from the drum surface. The filter is normally pre-coated with a filter aid.

constitutes the filter medium and the hydrostatic pressure difference between the feed and the permeate side of the membrane is the driving force for filtration. The membrane filtration processes are named according to the size of the pores in the filter: microfiltration is widely used for separation of cells such as *Escherichia coli* and yeasts during the primary recovery stages of downstream processing (Figure 8.4). Microporous membranes with a pore size range of 0.02–0.2 µm are used for separation of bacterial cells, while pores of 0.2–2 µm can be used with yeasts [12]. A microfiltration unit can in fact form an integral part of the bioreactor for retaining whole cells or immobilized biocatalysts while the product permeates through the membrane to enable an ISPR operation. Membrane filtration is described in more detail in Section 8.2.3.1.

8.2.1.3 **Centrifugation**

Centrifugation is used to separate materials varying in density under the influence of a force greater than gravity. The size range of particles that is separated by centrifugation is between 100 and 0.1 µm. For a spherical particle to be separated from a dilute suspension, the terminal velocity for separation, V_c in a centrifugal field is given by Stokes' law:

$$V_c = \frac{r\varpi^2 D_p^2(\rho_p - \rho_f)}{18\mu} \tag{8.4}$$

wherein centrifugal acceleration $r\omega^2$ (r is the radial distance from the axis of rotation and ω is the angular velocity of rotation) substitutes the gravitational accelera-

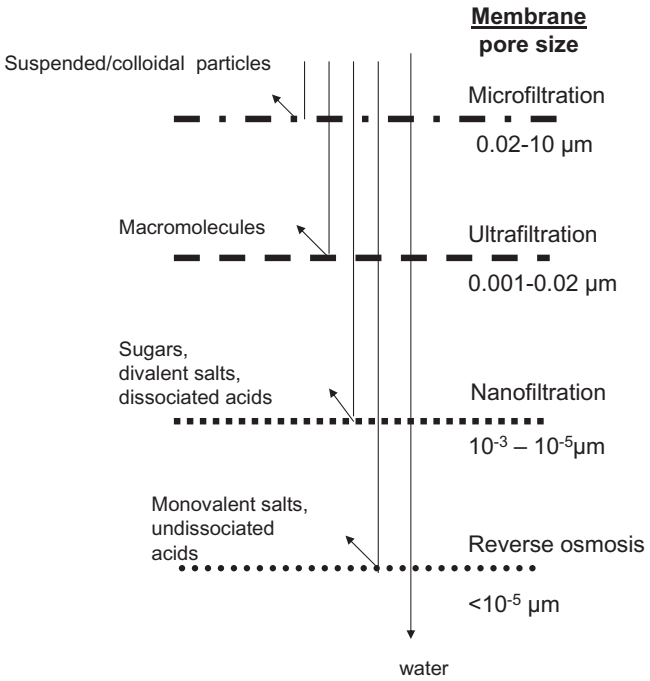

Figure 8.4 Types of membrane filtration operations used in biotechnology for separations according to size [11].

tion g that influences the sedimentation rate V_g in a gravitational field. According to Equation 8.4, particle velocity in a centrifuge is increased by increasing the particle diameter D_p, increasing the density difference between particle (ρ_p) and diameter (ρ_f), and decreasing the viscosity µ of the suspension. The ratio of velocity in the centrifuge to velocity under gravity is called the centrifuge effect or g-number and is denoted as $Z = r\omega^2/g$. Industrial centrifuges have Z factors from 300 to 16 000, while for the small laboratory centrifuge Z factors may be up to 500 000.

Centrifugation requires more expensive equipment than filtration and is also characterized by high power consumption; however the technique is effective for small particles that are difficult to filter. Centrifugation of cells from a fermentation broth produces thick, concentrated slurry containing more liquid than in a filter cake, and the supernatant is not free of cells. As in the case of filtration, pretreatment of the broth for example, by addition of flocculating agents such as polycations, inorganic salts, etc., increases the ease of cell separation by centrifugation.

Centrifugation on a large scale is often done in a continuous mode using different types of centrifuges among which disk stack and decanter or scroll centrifuges are most widely used in biotechnology (Figure 8.5). As centrifugation can be performed under sterile conditions, it can be integrated with a fermenter in an

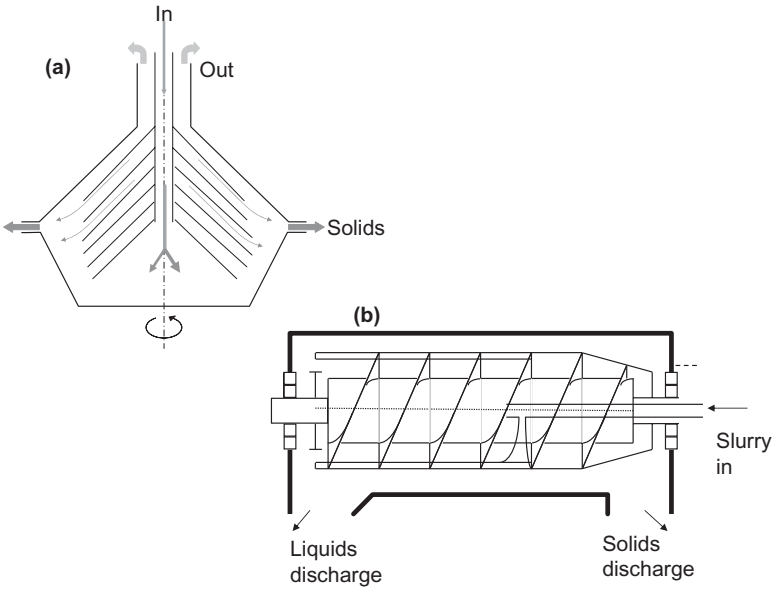

Figure 8.5 Schematic view of two types of centrifuges used in biotechnology. (a) Disk stack centrifuge contains several disks (between 30 and 200) placed at an angle of 35–50° and kept 0.4–2 mm apart, dividing the bowl into separate settling zones. The feed enters the centrifuge through a central feed pipe to the bottom of the stack. Solids settle on the surface of each disk and move towards the periphery of the bowl, while the clarified liquid moves inward and upward to the overflow pipe around the feed pipe. (b) A decanter or scroll centrifuge is used to concentrate slurries with high dry solids concentrations. Reproduced with permission from Hatti-Kaul and Mattiasson [1], Cambridge University Press). It consists of a rotating horizontal bowl tapered at one end and fitted with a helical screw that rotates at a slightly different speed. The solids deposit on the wall of the bowl and are scraped off by the screw and discharged from the narrow end of the bowl.

ISPR operation so that the clarified liquid can be processed for product recovery while the biomass is recycled back to the fermenter.

8.2.1.4 **Hydrocyclone**

There are examples of solid–liquid separation using a hydrocyclone (Figure 8.6), which unlike centrifuges has no moving parts and the vortex motion is performed by the fluid itself that is introduced tangentially into the upper part of the equipment, giving it a strong swirling downward movement [8]. Only a small fraction of the feed escapes through the underflow, carrying the denser particles, while most of the flow reverses its direction and goes up in an even stronger vortex motion and out through the overflow pipe, carrying the lighter particles with it.

8.2.1.5 **Flotation and Extraction**

Flotation and extraction comprise other less conventional but quite effective unit operations for removal of cells and cell debris that may be difficult to separate by

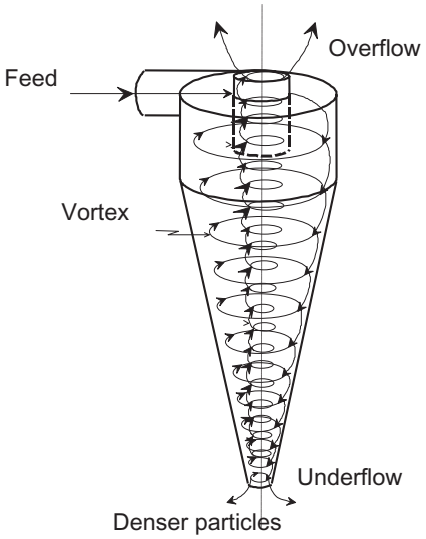

Figure 8.6 Schematic presentation of the solid–liquid separation in a hydrocyclone. The equipment comprises a conical section with an opening at the bottom and attached to a cylindrical head fitted with a tangential inlet and closed by an end-plate with an axially mounted overflow pipe [8].

centrifugation or filtration. In flotation, the particles are carried by air bubbles to the top of a flotation column where they are skimmed off in the form of froth, and this process is particularly advantageous in separating fine particles. Using gas bubbles made from a surfactant solution, or colloidal gas aphrons, allows effective separation of microorganisms from a dilute solution [13].

Extraction in aqueous two-phase systems (ATPS) allows an easy separation of particles from feeds that are highly viscous or have heterogeneous distribution of particles [14]. Such systems are generated by mixing two different polymers or a polymer and a salt above a certain concentration in water; the two phases are enriched in the respective phase components. Partitioning of particles in such systems is often one-sided, and the technique has been used for extraction of proteins directly from crude cell homogenates such that the particulate matter is enriched in the bottom phase while the target product is obtained in top phase (see Section 8.2.5.1). Such a separation can be done quite rapidly under gravity or at low centrifugation speeds.

8.2.2
Cell Disruption for Release of Cell-Associated Products

The different strategies that can be used for rupture of microbial cells include breaking the cell structure by mechanical forces, damaging preferentially the cell wall, for example, by drying or enzymatic lysis, or lysing primarily the membranes (e.g., by treatment with chemicals) [7, 15, 16]. Mechanical disruption is the most

common means of releasing intracellular products, which at industrial scale is achieved by high-pressure homogenization or by vigorous agitation with abrasives. Disruption follows a first-order process, and the extent of product release in a high-pressure homogenizer is represented by:

$$\ln\left(\frac{R_m}{R_m - R}\right) = kNP^a \tag{8.5}$$

where R_m and R are the maximal amount of product available for release and the amount released at a given time, respectively (kg product/kg cells), k is the first-order rate constant, N is the number of passages, P is the operating pressure and a the pressure exponent.

Non-mechanical disruption of cells is possible by physical, chemical, or enzymatic means. Among the traditional physical rupture techniques are desiccation followed by extraction of the microbial powder, repeated freezing and thawing of cell suspension, and osmotic shock. Exposure to high temperature can be an effective approach to cell disruption but is limited to heat-stable products.

Cell disruption/permeabilization can be achieved with different chemicals such as alkali, EDTA, solvent, detergent, antibiotic, etc., but only a few of them find applications for product release owing to issues based on cost, safety, or effect on product stability. Enzymatic lysis of cells has advantages in terms of selectivity and the mild conditions required for product release. The enzyme used extensively for lysis of bacteria is lysozyme, while for yeasts glucanase, mannanase, and protease are used. The cost of enzymes makes enzymatic lysis more expensive than mechanical disruption of cells. A combination of enzymatic/chemical and thermal treatment with mechanical disintegration has been suggested to enhance the efficiencies of the respective methods, with savings in time and energy and the facilitation of subsequent downstream processing [17]. Cell disruption is followed by a solid–liquid step to remove the cell debris.

8.2.3
Size-Based Separation of Molecules

Separations of molecules based on size are often performed during downstream processing to achieve concentration of the product, or for desalting and buffer exchange. Membranes play a predominant role for such separations. Desalting of small volumes of protein solutions is often done by dialysis using a dialysis membrane – low molecular weight solutes move from a high to a low concentration region, and at equilibrium the chemical potentials of the diffusing compounds on both sides of the membrane are equal.

8.2.3.1 Membrane Filtration

Membrane filtration using membranes with lower pore size than microfiltration is used for separation of high and low molecular weight molecules (Figure 8.4). Ultrafiltration separates polymeric solutes in the 0.001–0.02 µm range and is often used for removal of lower molecular weight solutes from macromolecules and as

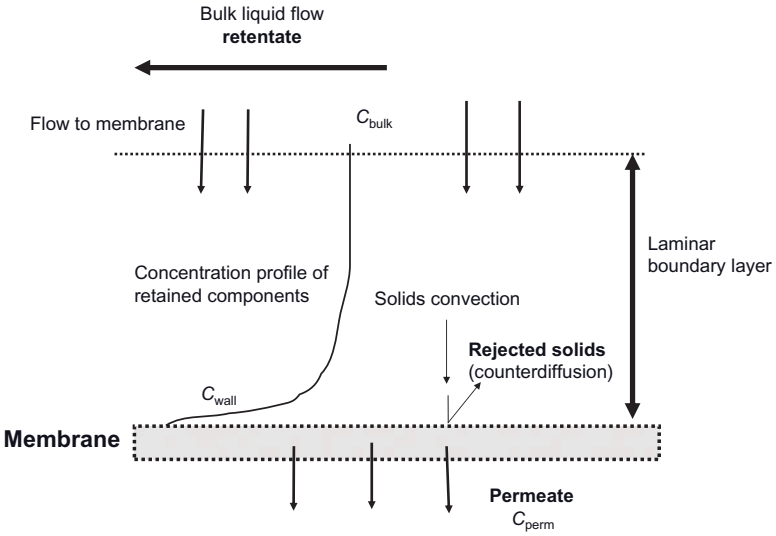

Figure 8.7 Concentration polarization during micro-/ultrafiltration. Concentration of proteins and other hydrophobic solutes increases at the membrane surface with time, resulting in a counter-diffusion to the bulk liquid and limiting the permeate flow through the membrane [10].

a means of concentrating protein solutions subsequent to clarification by microfiltration [7, 11]. The actual flux through a membrane is influenced by transmembrane pressure (Δp) besides the membrane properties and the flow properties of the liquid. However, during ultrafiltration as also in microfiltration, transmembrane pressure plays a role only to a certain extent. With time, concentration of the high molecular weight solutes at the membrane surface rises, resulting in a phenomenon known as concentration polarization, and the flux is limited by the balance of the convective flow to the membrane surface of the protein and its counter diffusion to the bulk as a result of concentration difference (Figure 8.7). The resultant membrane flux J can be derived as:

$$J = K' \ln \frac{C_{wall} - C_{permeate}}{C_{bulk} - C_{permeate}} \tag{8.6}$$

where K' is the mass transfer coefficient and is equal to D_e/δ (D_e being the effective diffusivity of solute in liquid film and δ the film thickness); C_{wall}, C_{bulk}, and $C_{permeate}$ are the protein concentration at wall, in bulk and permeate, respectively. Since $C_{permeate} < C_{bulk}$,

$$J = K' \ln \frac{C_{wall}}{C_{bulk}} \tag{8.7}$$

Hence, in the event of concentration polarization, the flux will be independent of transmembrane pressure but dependent on the ratio C_{wall} to C_{bulk}.

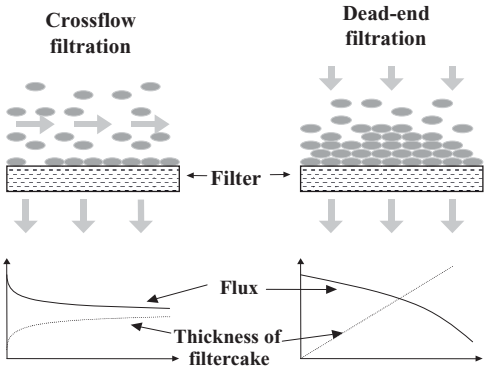

Figure 8.8 Comparison of crossflow with dead-end filtration. The flow of liquid is perpendicular to the membrane surface in crossflow filtration, leading to reduced fouling of the membrane and a lower decrease in the flux compared with dead-end filtration, where the liquid flow is perpendicular to the membrane surface.

Crossflow or tangential flow filtration has been adopted as the normal mode of membrane filtration where pressure is directed not perpendicular to the membrane surface (as in case of dead-end filtration) but parallel to it (Figure 8.8). This mode of filtration provides shear force close to the membrane surface sufficient to restrict settling of particles and reduces adsorption of hydrophobic solutes and resultant fouling of the membrane. The flux in crossflow filtration, as a function of transmembrane pressure drop, is given by:

$$J = \frac{\Delta p}{R_G + R_M} \tag{8.8}$$

where R_G and R_M are the gel and membrane resistances, respectively. R_M is constant while R_G varies with the solute concentration and tangential velocity across the membrane. It is possible to maximize the filtration rate at an optimal fluid velocity.

Both ultra- and microfiltration membranes are now available in different materials that provide good flowthrough and are easy to clean. Membrane filtration units are available in two major configurations – tubular (e.g., hollow fiber) cartridges and flat plate modules that can take the form of a plate and frame system or a spirally wound configuration. Hollow-fiber and plate and frame systems are most commonly used for processing crude feeds (Figure 8.9). These systems allow both batch and continuous processing of the feed. The hollow-fiber systems rely on laminar flow to minimize the gel layer while plate and frame systems use screens in between the membranes to generate turbulent flow and minimize fouling.

Reverse osmosis and nanofiltration are the other membrane processes that are attracting interest for removal of ionic solutes typically smaller than 0.001 µm. These techniques are not commonly used for biotechnological separations; they are, however, widely used in water purification, while nanofiltration is also used for

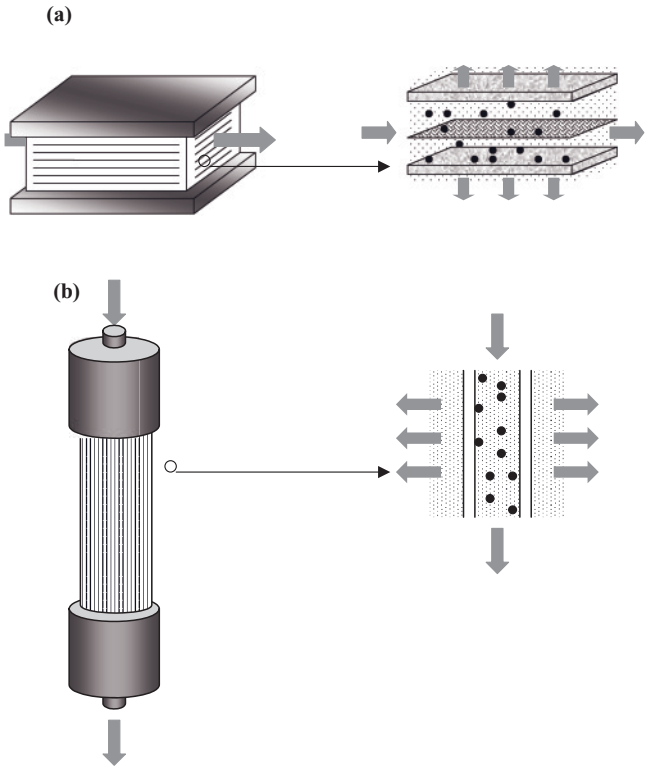

Figure 8.9 Crossflow filtration systems: (a) Plate and frame system having stacked membranes interspersed with spacers, and (b) hollow fiber cartridge comprising a bundle of membrane capillaries in a tubular module. Particles and molecules larger than the membrane pore size are retained on the membrane while smaller solutes pass through the membrane.

water softening. The two techniques are rather similar in operation, the key difference being that nanofiltration is operated at lower pressure and has lower rejection selectivity. Reverse osmosis removes the monovalent ions at 98–99% level at 200 psi, whereas removal of monovalent ions by a nanofiltration membrane varies between 50% and 90% depending on the material and manufacture of the membrane.

A pressure equivalent to or slightly larger than the osmotic pressure is used to drive the solvent and solute molecules across the membrane [12]. The solvent and solute fluxes are expressed as:

$$N_1 = K_p(\Delta p - \pi) \tag{8.9}$$

$$N_2 = C(1-\sigma)N_1 + K_{p'}\Delta C \tag{8.10}$$

where K_p and $K_{p'}$ are permeability coefficients for solvent and solute, respectively, π is the osmotic pressure, C is the average solute concentration in solution, ΔC is

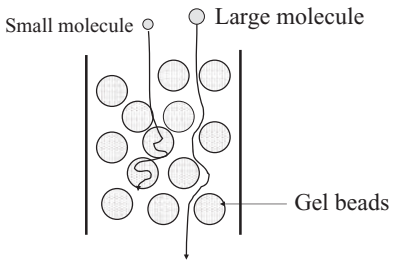

Figure 8.10 Size exclusion chromatography for separation of molecules varying in size. The stationary phase comprises a matrix with a defined pore size range to allow small molecules into the pores and delaying their passage through the column, while the larger molecules are excluded and pass unhindered through the column with the mobile phase.

the solute concentration difference across the membrane, and σ is a reflection coefficient of a solute, that is, the fraction of solute molecules retained on one side of the membrane in the presence of a solvent flux.

8.2.3.2 Size Exclusion Chromatography

Besides membrane filtration, chromatography in the form of size exclusion chromatography (also known as gel filtration) is also a size-based separation technique used often in final stages of protein purification [18]. Separation is achieved on the basis of the different time periods required by molecules of different sizes to pass through the pores of a chromatography gel matrix packed in a column. Molecules larger than the pore size of the matrix are excluded from the pores and are thus rapidly eluted from the column in the void volume (outside the particles) while the molecules of smaller size are eluted depending on their passage time through the pores (Figure 8.10). Solutes that are only partly excluded from the stationary phase elute with a volume described by the following equation:

$$V_e = V_o + K_p V_i \tag{8.11}$$

where V_e, V_o, and V_i are elution volume, void volume, and internal volume of liquid in the particle pores, respectively, and K_p is the gel partition coefficient defined as the fraction of the internal volume available to the solute. For large molecules, $K_p = 0$.

Size exclusion chromatography is limited by low capacity, that is, the sample volume is about 2–5% of the chromatography bed volume, and separation is improved by increasing the column height and at reduced flow rates. The technique further results in dilution of the product.

8.2.4 Separations Based on Product Volatility

Removal of a volatile product as vapor can be used both for concentration and as a means of its separation and purification. Such separation is often also required

for continuous removal of solvent or water generated as a by-product in a process, in which case integration with the bioreactor is required.

8.2.4.1 Distillation

Although it is energy intensive, distillation has been the method available for recovery of volatile products such as ethanol from large amounts of aqueous feed. If more than one volatile product is present in the feed, their separation is achieved by fractional distillation. Vacuum distillation is used to facilitate distillation at lower temperatures, for example, at the temperature of the fermentation process, by reducing the pressure above the liquid mixture to be distilled to less than its vapor pressure. The method has been used for recovery of ethanol directly from the fermenter and also for processing temperature sensitive materials such as β-carotene to remove solvents without undue damage.

8.2.4.2 Gas Stripping

Gas stripping is an alternative to distillation in which a gas is sparged through a bioreactor to remove the volatile product, which is then condensed and recovered [19]. The method is regarded as simple, not requiring expensive apparatus. Moreover, gas stripping does not lead to removal of nutrients and reaction intermediates, and can be used for *in situ* product removal, resulting in reduced solvent toxicity and increased productivity [20].

8.2.4.3 Membrane Distillation and Pervaporation

The potential of membranes has been extended to the separation of volatile products by membrane distillation and pervaporation, respectively [21, 22]. In both techniques the liquid feed is in contact with the upstream side of the membrane, while the permeated product is obtained as a vapor on the downstream side, which is then condensed. However, the fundamental difference between the two is the role that the membrane plays in separation (Figure 8.11).

Membrane distillation utilizes a non-wetting, microporous membrane that prevents liquid solutions from entering its pores due to surface tension forces, and acts only as a barrier for the liquid–vapor interfaces without contributing to the separation performance. Separation in membrane distillation occurs when vapor from components of higher volatility passes through the membrane pores by a convective or diffusive mechanism and are condensed on the permeate side of the membrane. The driving force for transport is the vapor pressure difference across the membrane pores, which is maintained by different means [22].

In direct contact membrane distillation, both sides of the membrane contact a liquid phase and the liquid on the permeate side is used as the condensing medium for the vapors leaving the hot feed solution. In air gap membrane distillation a stagnant air gap is present between the membrane and the condensation surface while in sweeping gas membrane distillation a cold inert gas sweeps the permeate side of the membrane carrying the vapor molecules that are condensed outside the membrane module, and in vacuum membrane distillation vacuum is

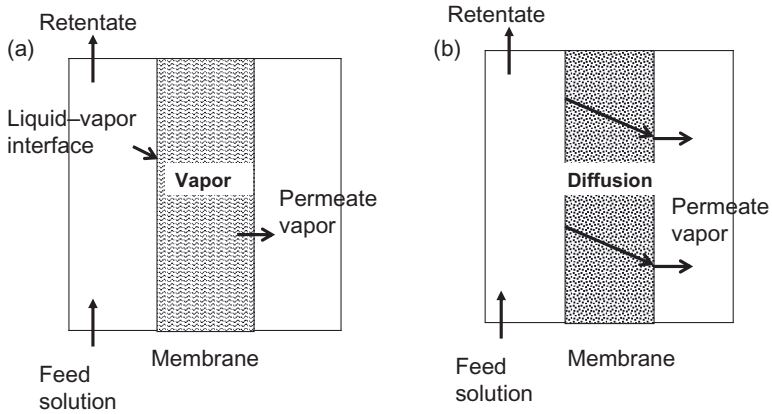

Figure 8.11 Separation of volatile products through membranes in (a) membrane distillation and (b) pervaporation. In membrane distillation, the vapor of volatile products passes through the membrane pores and is condensed on the permeate side by different mechanisms. In pervaporation, molecules in the liquid feed diffuse though the membrane pores and are converted to vapor on the permeate side of the membrane by applying vacuum or carrier gas.

applied on the permeate side which is lower then the saturation pressure of volatile molecules to be separated from the feed solution.

The hydrophobic membranes used in membrane distillation are those used for microfiltration, fabricated from polytetrafluoroethylene (PTFE), polypropylene, polyvinylidene fluoride materials, and have pore size in the range of 10 nm–1 µm. Membrane distillation has been used for removal of volatile organic compounds from dilute aqueous solutions and even separation of non-volatile components, however there has been little acceptance for large-scale applications due to several limitations related to membrane features, and energy and economic costs.

Pervaporation, on the other hand, makes use of dense membranes made of swollen homogeneous polymers that render the membranes permeable. Unlike membrane distillation, molecules in the liquid feed diffuse through the membrane and are desorbed into the vapor phase at the downstream side of the membrane either by creating a vacuum or using a carrier gas [10]. The separation is thus based on the relative solubility and diffusivity of each component in the membrane material that allows removal of even high boiling solvents. The permeate flux ϕ_m in pervaporation is given as:

$$\phi_m = \frac{DKC}{d_m}\left(1 - \frac{p_2}{p_1}\right) \tag{8.12}$$

where K is solubility constant, D is diffusion coefficient, C is concentration of the component in the membrane, d_m is membrane thickness, and p_1 and p_2 are partial pressures on retentate and permeate side of the membrane, respectively.

Pervaporation exhibits higher selectivity but much lower fluxes than membrane distillation. Depending on the permeating component, there are two modes: (i) hydrophilic pervaporation in which the water is separated from aqueous–organic mixtures by being preferentially permeated through a hydrophilic (e.g., polyvinyl alcohol) membrane, and (ii) organophilic pervaporation in which the target organic compounds are preferentially permeated from the aqueous–organic mixtures using organophilic membranes (e.g., nitrile, butadiene rubber, polydimethylsiloxane).

Pervaporation has also been rather slow in development due to the high energy consumption, low flux, insufficient selectivities, and difficult process design. However, over the years there has been a great deal of interest in applying pervaporation for selective removal of solvents during fermentation as well as for their concentration, and efforts have been made to make membranes with improved selectivity. There is also growing interest in integration of pervaporation with biocatalytic reactions for *in situ* product removal of inhibitory products [23]. Separation of azeotropic mixtures is effectively achieved by both membrane distillation and pervaporation.

8.2.5
Separations Based on Product Solubility

Separation techniques that utilize the differential solubility of molecules in two immiscible phases (i.e., extraction) or decrease in solubility under certain environmental conditions (i.e., precipitation or crystallization) are very commonly used for product concentration and purification. The main advantage of these techniques is simplicity of operation but they require the addition of auxiliary reagents that need to be recycled.

8.2.5.1 Extraction

Liquid–Liquid Extraction This is one of the most developed and extensively used unit operations in the chemical and biotechnology industry, having found applications for concentration and purification of a variety of products such as steroids, antibiotics, and organic acids. For the recovery of bioproducts, extraction implies the treatment of the aqueous phase (containing the solute) with a non-miscible organic solvent, by which the solute will distribute itself between the phases depending on the difference in its solubility in the two liquids [7, 10].

The ratio of the concentration in the two phases is called the distribution coefficient and is given by $\alpha = Y_L/X_H$, where Y_L and X_H are concentrations of the solute in the light phase and heavy phase, respectively. The distribution coefficient can be influenced by the extracting solvent (hydrophilic or hydrophobic character), the pH value (especially for weak acids and bases), temperature, salts (counterions for ionic solutes), other additives (modifiers), and to a small extent by the external pressure on the system.

Assuming that α is constant and the mass flows of the immiscible phases is conserved, a mass balance on the extracted solute would be:

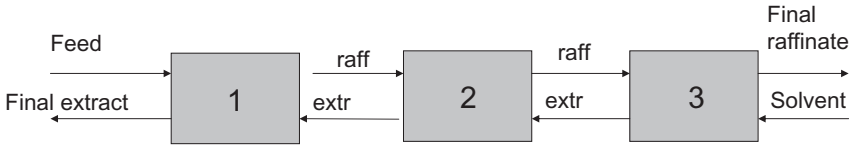

Figure 8.12 Multistage countercurrent mode of liquid–liquid extraction.

$$H(X_0 - X_1) = LY_1 \quad \text{or} \quad X_1 = X_0 - \frac{L}{H}Y_1 \tag{8.13}$$

Substituting K_D in the above equation,

$$X_1 = X_0 - \frac{L\alpha}{H}X_1 \tag{8.14}$$

$$\frac{X_1}{X_0} = \frac{1}{1+(L\alpha/H)} = \frac{1}{1+E} \tag{8.15}$$

where $E = L\alpha/H$ is the extraction factor for a solute in a defined system.

Multistage extraction is often employed to achieve maximal process efficiency, which on a large scale is commonly done in a countercurrent mode (Figure 8.12), using equipment such as centrifugal Podbielniak extractor, Delaval contactor, or Westfalia extraction-decanter. For continuous countercurrent operation, material balance on extracted solute yields:

$$R = \frac{E(E^n - 1)}{E - 1} \tag{8.16}$$

where R is the rejection ratio (i.e., the weight ratio of the solute leaving in the light phase to that in the heavy phase) and n is the number of equilibrium stages. The fraction of solute extracted becomes:

$$\%\ \text{extraction} = 1 - \frac{1}{R+1} \tag{8.17}$$

For extraction from a crude mixture, it is preferable to remove cells or other particulates to avoid the formation of emulsions at the interface. After extraction, the product is removed from the solvent either by distillation (of the product in case of high-boiling solvent or of the solvent with a low-boiling point), or by back extraction into an aqueous phase.

It may be relatively easy to extract lipophilic solutes because of their preference to distribute into the organic phase. But for extraction of less hydrophobic molecules other parameters are often taken into consideration. For charged solutes it is possible to exploit their dissociation constants to achieve an effective separation, that is, extraction can be performed below the pK_a of acids and above the pK_a of bases when they are predominantly in the unionized form as shown:

$$\text{pH-p}K_a = \log_{10}\left[\frac{\alpha^0}{\alpha} - 1\right] \text{ for acids} \tag{8.18}$$

$$\text{p}K_a\text{-pH} = \log_{10}\left[\frac{\alpha^0}{\alpha} - 1\right] \text{ for bases} \tag{8.19}$$

where α^0 is the intrinsic distribution coefficient, that is, the ratio of concentration of the unionized solute in the organic and water phases, respectively, and α is the effective distribution coefficient, taking into account the ionized component of the solute in the water phase. Differences in the pK_a of the ionizable components can be used to overcome an adverse ratio of distribution coefficients and to achieve their separation from a mixture.

Reactive extraction is yet another way to facilitate extraction, which is based on a reversible reaction between the acid and an extractant (carrier) such as an aliphatic amine or a phosphorus compound added to the solvent [24]. The complex formed is also insoluble in the aqueous phase, and hence the solute is carried to the organic phase. This method is being applied for the extraction of organic acids.

Extraction is often a method of choice whenever applicable for product recovery; however several disadvantages such as expensive solvent recovery, environmental concerns, and safety aspects need to be considered. Furthermore, the problems of emulsion formation and toxicity to the cells limit the use of solvent extraction as an ISPR technique.

Direct contact of the solvent with the cells is avoided by integrating solvent extraction with membrane permeation in a technique called perstraction. By using the membrane as a barrier between an aqueous feed and an organic solvent it is possible to transfer molecules that partition into the liquid filling the membrane pores. For example, hydrophobic membranes, which have pores filled with the organic phase, are used to extract the non-polar product from the aqueous medium.

Supercritical Fluid Extraction The disadvantages associated with solvent extraction have led to an increasing interest in and development of supercritical fluid extraction (SFE) as an alternative extraction technique. Supercritical fluids (SCFs) are materials that exist as fluids above their critical temperature and pressure. Many of the properties of SCFs (e.g., diffusivity, kinematic viscosity, and density) are intermediate between those of gases and liquids, which provide better conditions for extraction such as faster mass transport than in liquids [25]. Furthermore, the solvent properties of the SCFs are highly sensitive to changes in both temperature and pressure, and a cosolvent (e.g., ethanol) is used, which provides the opportunity to tailor the solvent strength to a given application. The SCF used for biological extractions is supercritical CO_2 because of its relatively low critical temperature (31.3 °C) and pressure (72.9 bars). There is presently great interest in using supercritical CO_2 as a "green" solvent during enzymatic transformations of hydrophobic substances.

In a typical extraction process used for product recovery the feedstock is extracted with compressed supercritical CO_2 in an extraction column. The loaded SCF is

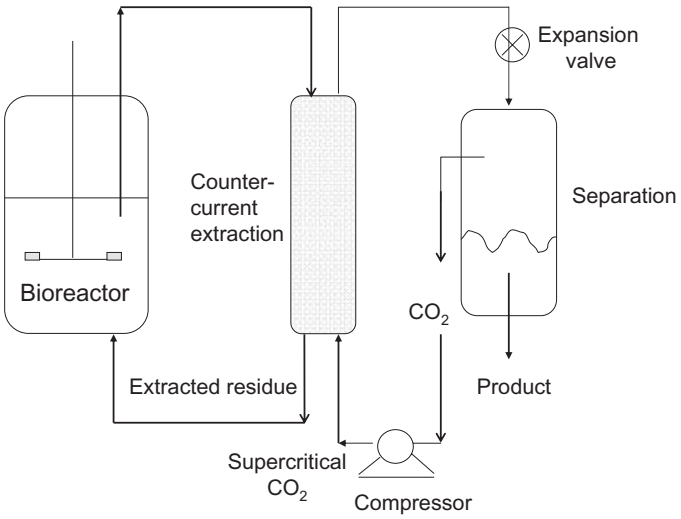

Figure 8.13 A scheme for using supercritical fluid extraction for downstream processing in industrial biotechnology. The product extracted into supercritical carbon dioxide is recovered after lowering the pressure and the CO_2 gas is recompressed and recycled [10].

then transferred to a separator where the pressure is lowered so that the liquid turns into a gas, releasing the product as a precipitate. The gas is repressurized and recycled to the extraction column (Figure 8.13).

The requirement for high-pressure equipment makes SFE more expensive than solvent extraction. Despite this, some economically viable large-scale applications exist (e.g., extraction of oils and coffee). Besides direct extraction of the feed the SCF can be used to remove traces of solvents from the product recovered by solvent extraction.

Aqueous Two-Phase Extraction While solvent and SCF extraction are limited to the recovery of small organic molecules, extraction of proteins has been performed using aqueous biphasic systems as mentioned above in Section 8.2.1 [14]. The basis of separation in an ATPS is the selective distribution between the two phases. While small molecules are more or less evenly distributed between the two phases, partitioning of macromolecules is extremely variable. This distribution is governed by a number of parameters related to properties of the phases and the solute as well as interactions between the two. The partitioning can be made more selective, for example, by manipulating the system properties to make a particular kind of interaction predominant.

ATPS are easy to use and have the potential for integrating clarification, concentration, and purification of proteins in one unit operation. Industrial-scale recovery of some enzymes is done by extraction in ATPS. Systems made of polyethylene glycol (PEG) and salt are commonly used because of their low cost. The extraction conditions are chosen such that the target protein partitions to the PEG-

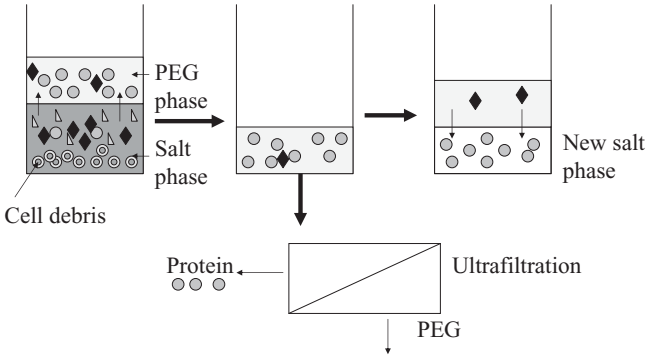

Figure 8.14 Extraction of proteins in an aqueous two-phase system made of polyethylene glycol (PEG) and salt. The cell homogenate containing the protein of interest is equilibrated with the phase components under conditions allowing the protein to partition to the PEG-rich top phase. The phases are separated and the product is recovered from the PEG phase either by ultrafiltration to remove the polymer or by a second partitioning step such that the protein partitions to the salt phase from which it is easily separated.

rich top phase while the cell particles and the majority of soluble contaminants partition to the bottom phase (Figure 8.13). After mixing the phase components with cell homogenates, phase separation is done and the protein is recovered from the upper phase either by a second partitioning step into a new salt phase or by ultrafiltration as shown in Figure 8.14.

Countercurrent Chromatography Liquid–liquid extraction can provide high-resolution separation of solutes when operated in countercurrent chromatography mode. The two immiscible liquid phases can be aqueous–organic, organic–organic, or aqueous–aqueous. One liquid phase is used as a stationary phase with which the mobile phase is contacted in different modes. The most highly developed mode of countercurrent chromatography employs a planetary motion of a helical tube wound around a drum in a centrifugal field which provides alternate zones of phase mixing and separation of the two immiscible liquids in the coil [26]. The heavy stationary phase is retained in the coil while the light mobile phase is pumped through it. The solutes to be separated are introduced into the coil with the mobile phase and are subjected to multistage partition. The technique can be adaptable for ISPR applications, where use of solvents providing selective extractions would be desirable [4]. When using the technique to perform ISPR, enzymes or whole cells may also be immobilized within the stationary phase of the coil while the product is preferentially partitioned into the mobile phase.

8.2.5.2 Precipitation and Crystallization

Precipitation and crystallization are based on liquid–solid phase transition under defined physico-chemical conditions.

Precipitation This is commonly used in downstream processing of proteins for product concentration, but can also be used to achieve a certain degree of purification. Its advantages include adaptability to continuous processing and larger scales, the wide variety of possible precipitants, and the ability to retain biological activity. Although there are several means to precipitate proteins, the most commonly used are salting out using high concentration of ammonium sulfate or sodium sulfate, and precipitation by adding solvents (such as ethanol or acetone) at low temperature.

In salting out, the added salt ions interact with water more strongly, causing the protein to precipitate due to increase in hydrophobic interactions. The solubility of proteins as a function of ionic strength of the solution is given by:

$$\log\frac{S}{S_0} = -K_s(\mathrm{I}) \tag{8.20}$$

where S is the protein solubility in solution at ionic strength I, S_0 is solubility at zero ionic strength, and K_s is the salting-out constant, which is a function of temperature and pH. The ionic strength of a solution is equivalent to

$$\mathrm{I} = \frac{1}{2}\sum C_i Z_i^2 \tag{8.21}$$

where C_i and Z_i are the molar concentration and charge, respectively, of the ionic species.

Protein precipitation on addition of organic solvent is caused by reduction in dielectric constant of the solution according to:

$$\log\frac{S}{S_0} = -\frac{K'}{D_\varepsilon^2} \tag{8.22}$$

where D_ε is the dielectric constant of the solution and the constant K' depends on temperature and the protein employed. The reduction in dielectric constant facilitates protein precipitation due to increase in electrostatic interactions between the protein molecules.

Precipitation can also be applied for nucleic acid removal during purification of a bioproduct from microbial cells. Among the lower molecular weight biological products, organic acids are precipitated as calcium salts. Organic acids may also be isolated as sparingly soluble complexes, for example, cephalexin is recovered by complexation with β-naphthol and the method adapted for *in situ* product removal [27]. Whatever the choice of precipitation technique, one needs to consider its influence on subsequent downstream processing and also handling of the waste generated.

Crystallization This is one of the most powerful purification methods available and is normally used as a final step for polishing and formulation of chemical products from a solution containing a uniform population of product molecules. Even in the case of protein products, crystallization, which was originally limited to determination of their three-dimensional structure, has now been extended to

its application as a downstream processing technique for recovery directly from relatively impure solutions and even from fermentation broths [28]. The possibility of replacing competing methods such as chromatography with a relatively simple and inexpensive technique makes crystallization an attractive purification technique, with further advantages of formulation and storage. *In situ* removal of product from a fermenter has been shown to be possible by means of an external crystallization loop [29].

Supersaturation is the driving force of crystallization, and control over supersaturation is the critical aspect of the process. Solvent evaporation is often not desirable because of the limited thermal stability of the products, hence alternative methods for removal of water have been tried. In osmotic dewatering, solvent is transported through solvent-selective membranes under pressure. Extractive crystallization is based on the tendency of certain aqueous–solvent mixtures to split into two liquid phases upon small variations in temperature so that the aqueous solute becomes concentrated in a smaller volume and eventually crystallizes, while the pure solvent is recycled.

Fractional crystallization is used to separate multicomponent mixtures, for example, organic acids, monomers, isomers, etc., into narrow fractions, ultimately leading to high purities of the selected components. A falling film mode of fractional crystallization (Sulzer Chemtech, Switzerland) involves growth of crystals on a surface cooled externally by a co-current falling film of a coolant. Heating of the tube wall induces partial melting (sweating) of crystals and the molten material is drained off. Eventually the remaining crystal layer is melted and collected as a product, while the sweat fraction is recycled for crystallization. In a similar way, the residue drained off in the first phase may be further subjected to the same procedure to give enhanced yields.

Diastereoisomeric crystallization is the predominant technique employed in the resolution of racemic mixtures, which is based on binding of a resolving agent to enantiomers to give a diastereoisomeric salt pair which can be separated easily because of difference in solubility [30]. For example, a common resolving agent used for the enantiomeric separation of ibuprofen is L-lysine; the D-ibuprofen-L-lysinate salt has much lower solubility than that of the salt of L-enantiomer and hence can easily be separated [31]. In most cases, after the desired enantiomer has been separated from the diastereomeric salt, the resolving agent is recovered and becomes available for reuse. If the remaining undesired enantiomer is re-racemized, more desired material can be recovered from the mixture. Diastereomeric crystallization can also be used to obtain higher purity (e.g., subsequent to enzymatic resolution of racemates).

8.2.6
Molecular Separations Based on Adsorption to a Solid Matrix

Adsorption of water-soluble solutes to solid sorbents through various mechanisms is a common way to separate them from a liquid feed, and provides a useful method for concentration and purification of products. In physical adsorption,

weak forces such as van der Waals forces are dominant, while ion exchange adsorption utilizes strong ionic bonds. Activated carbon is an adsorbent frequently used to decolorize fermentation broths. Ion exchange resins and other polymeric adsorbents are used for separation of small organics, for example, antibiotics, amino acids, etc., and also macromolecules. Adsorption capacity depends on the nature of the adsorbent and solute, and the physico-chemical conditions used.

Like extraction, the process of adsorption is based on an equilibrium relationship between solute concentration in the liquid phase and the auxiliary (solid) phase, which determines the extent to which a solute can be adsorbed to a surface. The equilibrium relationships have been depicted by several types of adsorption isotherms. The Freundlich isotherm is widely used in liquid–solid systems:

$$C_S^* = K_F C_L^{*(1/n)} \tag{8.23}$$

where C_L^* and C_S^* are equilibrium concentrations of the solute in liquid and solid phases, respectively; K_F and n are constants characteristic of a particular adsorption system; the dimensions of K_F are dependent on the dimensions of C_L^* and C_S^* and the value of n (which is greater than 1 if adsorption is favorable and lower than 1 if adsorption is unfavorable).

The Langmuir isotherm is based on molecular adsorption as a monolayer and is often used for determining protein adsorptions:

$$C_S^* = \frac{C_{Sm} K_L C_L^*}{1 + K_L C_L^*} \tag{8.24}$$

where C_{Sm} is the maximum loading of solute on the adsorbent. K_L is the Langmuir equilibrium constant, which depends on the strength of binding forces on the surface; its value is always greater than zero. In the case of proteins K_L depends on the isoelectric point of the protein, pH, and ionic strength of the solution.

Particulate adsorbents are often used in different types of solid–liquid contactors such as packed-bed, moving-bed, fluidized-bed or agitated vessel contactors. Packed- and moving-bed contactors are the most commonly used as they provide the largest adsorption area per unit volume. Operation of a down-flow packed-bed adsorber is shown in Figure 8.15. Even micro/ultrafiltration membranes have been derivatized with active groups to facilitate adsorption of solutes directly from crude broths; these can be later eluted and recovered in the permeate [11].

Analysis of adsorption in a packed-bed column is based on a differential mass balance of the input and output streams. The input and removal of the solute from a defined section of a packed bed occurs mainly by the liquid flow down the column, while other mechanisms could be related to local mixing and diffusion processes within the gaps between the adsorbent particles. Accumulation of the solute will occur because of adsorption into the interior and exterior surface of the adsorbent, and even in the gaps between the adsorbent particles. The analysis can be simplified by not taking into account the axial dispersion effects to give:

$$U\frac{\partial C_L}{\partial z} + \varepsilon\frac{\partial C_L}{\partial t} = -(1-\varepsilon)\frac{\partial C_S}{\partial t} \tag{8.25}$$

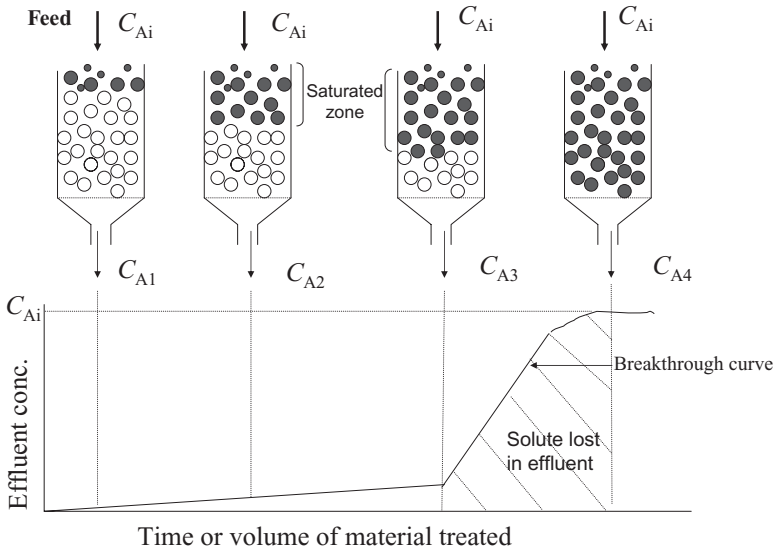

Figure 8.15 Movement of the adsorption zone in a packed-bed adsorber and corresponding breakthrough curve. The liquid feed containing the solute at concentration C_{Ai} is fed in at the top of the column packed with an adsorbent which takes up the solute, and the solution emerging from the column is depleted of the solute. As the liquid moves down the column, the region of the adsorbent bed where most adsorption occurs, the adsorption zone, moves down the column, eventually reaching the bottom of the bed. As the column gets almost saturated, the concentration of solute in the effluent starts to rise (breakpoint) and reaches a level of the inlet concentration. Reproduced with permission from Doran [9], Academic Press.

where U is the superficial velocity of the liquid, C_L is the concentration of solute in the liquid, C_S is the average solute concentration in the solid phase, z is the bed depth, t is time, and ε is the void fraction in the bed. The first term in the equation represents convective transfer of solute in the bed, the second term is the rate of change of solute concentration in liquid around the adsorbent particles, and the last term is the rate of transfer of solute from liquid to solid phase.

If the liquid content around the particles is small compared with the total bed volume, the second term can also be eliminated. Substituting $\partial C_S/\partial t = K_a(C_L - C_L^*)$ (where K_a is the overall mass transfer coefficient describing the internal and external mass transfer resistance), the equation is further simplified to:

$$U\frac{\partial C_L}{\partial z} = -K_a(1-\varepsilon)(C_L - C_L^*) \tag{8.26}$$

The value of K_a will depend on the properties of the liquid and flow conditions. The above equation highlights the importance of mass transfer in adsorption systems. Equilibrium is seldom achieved in commercial adsorption systems, performance being controlled by the overall rate of adsorption. Improvements in adsorption may be achieved by reducing the mass transfer resistance.

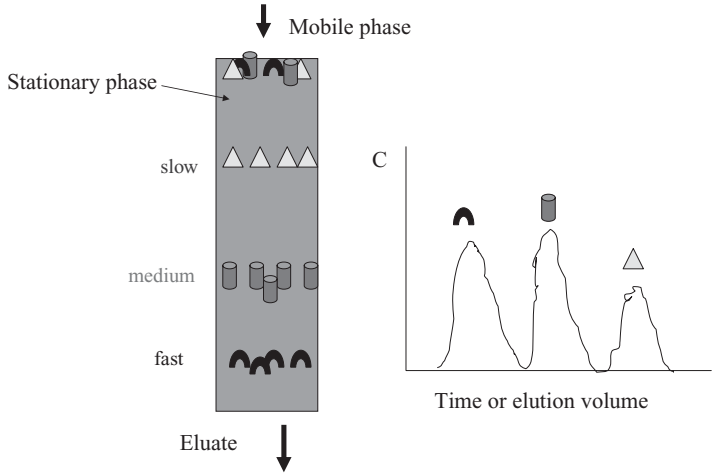

Figure 8.16 Principle of liquid chromatography. The solutes binding with different strengths to the stationary phase are eluted at different times or elution volumes from the column, by varying the composition of the mobile phase in a stepwise or a continuous mode.

Adsorption from a feed is followed by washing of the sorbent to remove loosely bound solutes prior to elution of the product of interest. The sorbent is then recycled after regeneration.

8.2.6.1 **Adsorption Chromatography**

Chromatography on a bed of adsorbent particles forms a high-resolution technique for separation of solutes and is widely used from laboratory to industrial scale for purification of most high-value products of biotechnology [7, 18]. It utilizes the differential adsorption of the molecules at the surface of a matrix derivatized to contain covalently linked functional groups (Figure 8.16). The solutes bound to the matrix with varying affinities can be eluted by using a suitable eluant. As the solutes move down the column with the flow of the eluant, they continue to adsorb to and desorb from the matrix; the weakly bound entity will be more easily desorbed and moves forward more rapidly. Hence, each component separated by chromatography has a different elution volume V_e (i.e., the volume of the eluant required to carry a solute through the column until it emerges at its maximum concentration).

Ideally the solutes should emerge from the column as narrow elution bands at times well separated from each other. However, in practice, the elution bands spread out because of diffusion effects that the solute may be subjected to in the column. The so-called zone spreading in chromatography is analyzed using the concept of theoretical plates. The chromatography column is considered to be made up of a number of segments or plates of height H, the magnitude of which is of the same order as the diameter of the adsorbent particles and within which equilibrium is supposed to exist. The lower the plate height or height

equivalent to a theoretical plate (HETP) the narrower the solute peak. HETP is expressed as:

$$H = \frac{A}{u} + Bu + C \tag{8.27}$$

where u is linear liquid velocity, A, B, and C are the effects of liquid–solid mass transfer, forward and backward axial dispersion, and non-ideal distribution of liquid around the packing, respectively. Reducing the values of A, B, and C results in a decrease in HETP.

HETP for a particular solute is related to its elution volume and width of the elution peak as it appears on the chromatogram. Provided the elution peaks are symmetrical, the number of theoretical plates N can be calculated as follows:

$$N = 16\left(\frac{V_e}{w}\right)^2 \tag{8.28}$$

w being the width at the base of the peak. The above equation applies when the sample is introduced as a narrow pulse. The length of the column L is determined by N and HETP according to:

$$N = \frac{L}{H} \tag{8.29}$$

Hence, for a given column the greater the N the greater is the number of equilibrium stages and the more efficient is the separation. In case of broad peaks, overlapping can occur even at high plate numbers. The ability of a column to separate peaks (i.e., resolution R) further determines the performance of a chromatography column:

$$R = \frac{2(V_{e1} - V_{e2})}{\frac{1}{2}(w_1 + w_2)} = \frac{\Delta t_r}{2(\sigma_1 + \sigma_2)} \tag{8.30}$$

where V_{e1} and V_{e2} are elution volumes, w_1 and w_2 are the widths of peak at base, and σ_1 and σ_2 are standard deviations for components 1 and 2, respectively; Δt_r is the distance between maxima of the two peaks represented by V_{e1} and V_{e2}. R should be greater than 1 to provide a good separation of the components.

Elution of the bound solutes can be done using the mobile phase in an isocratic (composition of the mobile phase is the same throughout separation) or non-isocratic mode (changing mobile phase composition). An alternative mode of elution is used in displacement chromatography, wherein a displacer, a substance with extremely high affinity for the stationary phase, is introduced into the column which competes for the adsorption sites and displaces the bound components from the column.

The stationary phases used in chromatography of bioproducts are made of both inorganic (e.g., silica) and organic (synthetic and natural polymer) materials. In order to have a high surface area, porous matrices are used with an average pore size of 100–300 μm. The mass transport within the particle pores is mainly by

diffusion. On the other hand, mass transfer occurs by convection rather than diffusion in the stationary phase systems based on membrane configurations. The faster adsorption kinetics and low back pressure allow the use of high flow rates and easier scale-up in membrane adsorption chromatography. Other novel stationary phases currently used are monolithic supports, which are cast as continuous homogeneous phases as opposed to individual particles packed in a column [32, 33]. Even monoliths can be used to achieve efficient separations at high flow rates.

Ion exchange chromatography is by far the most widely used technique because of its general applicability, good resolution, and high capacity. It can be used in the initial phase of downstream processing to provide both purification and volume reduction of the process fluid. Compounds are separated on an ion exchanger according to the difference in their surface charges. Anion exchangers (having positively charged groups such as diethyl aminoethyl) are used for binding negatively charged solutes and cation exchangers (having negatively charged groups, e.g., carboxymethyl) are used for binding positively charged solutes. The bound solutes are eluted either by increasing the salt concentration or altering the pH of the mobile phase.

Separation of non-polar substances is invariably done by reversed phase chromatography on stationary phases with covalently bound hydrophobic ligands (C_4, C_8, C_{18}-alkyl chains or aromatic functions). The strong interaction of the solute with the stationary phase requires its elution by the use of hydro-organic eluants. Hydrophobic interaction chromatography (HIC), which is used for purification of proteins, relies on comparatively weak hydrophobic interactions between mildly hydrophobic ligands and accessible hydrophobic amino acids on the protein surface. Adsorption in HIC can be promoted at high salt concentration while elution is facilitated by lowering the salt concentration or temperature or polarity of the medium.

Affinity chromatography, based on molecular recognition, is the most selective form of chromatography of proteins. It exploits selective binding of a protein to an affinity ligand immobilized to a matrix. The ligands may be specific to one or a group of proteins, and range from antibodies, receptors, substrate analogs, inhibitors, cofactors to metal ions, and also may be pseudo-biospecific such as synthetic dyes. The biological ligands are invariably sensitive to fouling and also more expensive. They are hence used towards the end of a purification scheme. The non-biological ligands, on the other hand, can be used early in the purification process and may help to reduce the number of steps for obtaining a pure product.

Chromatography has also been heavily exploited for chiral separations based on the different extent of interaction between the stationary phase and the different enantiomers, hence leading to different retention times and separation. There are many chiral stationary phases available but only a few, such as polysaccharide and protein-based phases, have proven to be versatile. A hybrid chromatography–crystallization process has been proposed where chromatography can provide a good initial separation of enantiomers, and it becomes more economical to combine it with crystallization for recovering the solid product in pure form (ChiralTechnics, California, USA). However, successful development of such a

hybrid process depends on the design of both the chromatographic column and the crystallization system.

8.2.6.2 Continuous Chromatography

Chromatography has conventionally been operated in a packed-bed mode employing sequential passage of feed, washing buffer, and eluant through the column. For isolation of the target product from unclarified feed, fluidized/expanded bed adsorption chromatography is applied [34]. Here the feed is pumped in an upward direction through a bed of adsorbent particles packed in a column at a flow rate that results in fluidization of the bed, leading to space between the adsorbent beads, hence allowing particulate matter to pass through. Expanded beds are more stable than fluidized beds because of the distribution of particle sizes that move up to different levels, and allow separation similar to a packed chromatographic column.

In continuous chromatography the adsorbent and the eluant move in opposite directions with respect to the point of sample introduction. This is achieved either with a moving bed, moving column, or simulated moving bed mode. The movement of the bed and the liquid flow can be perpendicular or opposite with respect to each other. A potential application of continuous chromatography seems to be as an ISPR operation for harvesting and separation of products from continuous fermentations or enzymatic processes.

Simulated moving bed technology (SMB) is a multi-column continuous chromatographic separation technique involving countercurrent movement of a liquid and a stationary phase packed in columns [35]. In spite of its large-scale application for almost 40 years in the petroleum industry, its potential has been only relatively recently recognized in the fine chemicals, cosmetics, and pharmaceutical industries. SMB has been used for the separation of sugars, amino acids, proteins, racemic mixtures, and for desalting, and can also be useful for separation of enantiomers.

As shown in Figure 8.17, a classical moving bed in SMB is divided into four zones: zone I where more firmly bound product must be completely desorbed, zone II where less firmly bound product must be completely desorbed, zone III where firmly bound product must be completely adsorbed, and zone IV where less firmly bound product must be completely adsorbed. A typical SMB consists of 4–24 columns distributed within the different zones and that are connected in an annular alignment by multiway valves. The important parameters to be considered in a SMB operation are the flow rates of the various flow streams, number of columns, column length and diameter, particle size, the shift period of the columns, and the feed concentration. SMB provides a means of increasing the productivity of chromatography, with reduction in the eluant consumption and product dilution. The limitations of the technique are, however, that the feed comprising a multicomponent mixture can only be divided into two product streams, each of which can have more than one compound. While isocratic mode is the most common way to run the system, various kinds of gradient, for example, solvent, salt, pressure, allow several-fold minimization of eluent and resin volumes.

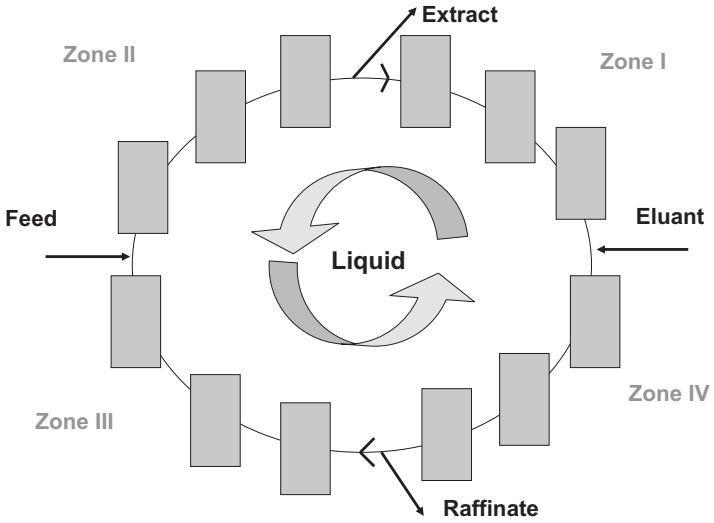

Figure 8.17 Simulated moving bed chromatography. The classical moving bed consists of four different zones. The feed mixture is introduced into the system between zones II and III and transported with the mobile phase into zone III, where the compounds having higher affinity to the sorbent are adsorbed and transported with the stationary phase to zone I where they are desorbed by a mixture of fresh eluant introduced between zones I and IV. The less adsorbed components in zone III are transported by mobile phase to zone IV where they are adsorbed and transported with the stationary phase to zone II for desorption. The different adsorption and desorption events are controlled by the flow rates adjusted by means of 3 or 5 external pumps and the column switch times, and the flow rates may vary from zone to zone. The internal flow of the mobile phase is maintained using a recycling pump that is often placed between the first and the last column.

The SMB concept has been used to design a simulated moving bed reactor (SMBR), in which the enzyme is simply added throughout the SMB unit either in an immobilized form or to the mobile phase in a soluble form. This allows the starting material to be continuously converted in all sections of the SMBR [36].

Continuous annular chromatography (CAC) is the only chromatography that allows continuous separation of multicomponent mixtures [37]. In CAC the stationary phase occupies an annular space formed between two concentric cylinders, one covering the other. The annular bed is rotated around its vertical axis past a fixed port through which the sample is continuously fed. The elution buffer percolates downwards through the rest of the annulus. With time, helical component bands develop from the feed-point to different points at the bottom of the annular column (Figure 8.18), the slopes of which are dependent upon elution velocity, rotational speed, and the distribution coefficient of the component between the fluid and the adsorbent phase. The stronger the adsorption of a solute the farther away from the point of sample introduction it will appear at the bottom of the bed. Non-isocratic elution can also be used for separation in CAC.

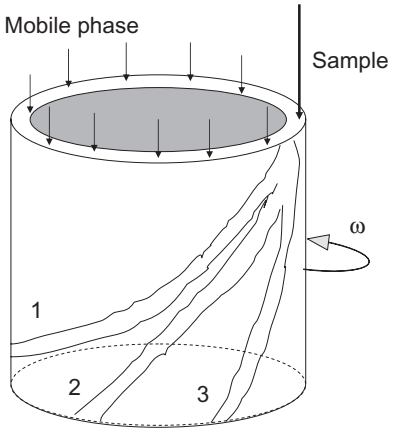

Figure 8.18 Continuous annular chromatography. The sample is introduced at a fixed point on to a rotating annular bed of stationary phase, while the mobile phase is passed over the remaining space. Components 1, 2, and 3 present in the sample are separated based on their distribution coefficient between the stationary and mobile phase, and are eluted at different points from the column [37].

8.2.7
Molecularly Imprinted Materials for Selective Product Capture

Molecular imprinting is a technology for introducing selective recognition sites, in the form of an imprint of the target molecule, in a highly crosslinked polymer matrix, hence providing a structure with extremely high selectivity for separation [38]. For preparation of the molecular imprinted polymers (MIPs), the template (target molecule) is associated with functional monomers in a solvent prior to adding the crosslinker and polymerization initiator. After polymerization, the template is washed out from the polymer network leaving an imprint of the template structure (Figure 8.19). This allows specific recognition of the target molecule and hence discriminates between closely related molecules in a mixture. MIP production can be done using covalent or non-covalent approaches, although the latter is preferred because of its simplicity. In the former approach the template is chemically derivatized with molecules containing polymerizable groups using reversible covalent bonds. In the non-covalent approach the template assembles with the functional monomers using different interactions like van der Waals, hydrophobic, or electrostatic. MIPs have been used for chromatographic separation of a wide group of product categories, including structurally similar compounds, enantiomers, isomers, etc. [39, 40]. It has also been used as an ISPR technique, for example, for recovery of secondary metabolites produced by fermentation [41] and enzymatic synthesis of aspartame. The technique has been employed more at an analytical scale; there is, however, great interest in larger scale applications, but this requires improvements in the binding capacity and costs of the MIPs.

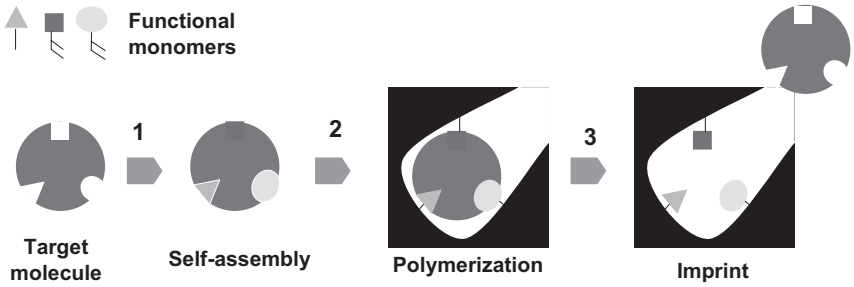

Figure 8.19 Synthesis of molecular imprinted polymer by a non-covalent approach. The different steps involve: (1) self-assembly of target molecule (template) with functional monomers, (2) polymerization in the presence of a crosslinker, and (3) extraction of the template from the imprinted polymer network. Courtesy: Mathieu Lenoir, Department of Biotechnology, Lund University.

8.2.8
Membrane Separation of Ionic Solutes: Electrodialysis

Electrodialysis is used to separate ionized compounds from non-ionized compounds by ion exchange membranes under the influence of a potential gradient. Membranes with fixed charged groups selectively allow the passage of oppositely charged ions and reject ions of the opposite charge, allowing concentration, removal, or separation of electrolytes. Bipolar membrane electrodialysis or "water splitting" has been developed for converting aqueous salt solutions into acids and bases and has been applied for recovery of organic acids from fermentation medium without the addition of chemicals. The principle is based on the use of bipolar membranes in which an anion- and a cation-permeable membrane are laminated together. Orienting such a composite structure such that the cation exchange layer faces the anode and imposing a potential field across the membrane it is possible to split water into H^+ and OH^- ions and cause the ions to migrate toward the electrode of opposite charge. This results in the production of acidic and basic solutions at the surfaces of the bipolar membranes. Placing multiple bipolar membranes along with anion and cation exchange membranes in an electrodialysis stack in between a single pair of electrodes it is possible to convert aqueous salt solutions into acids and bases (Figure 8.20). The disadvantage of bipolar membranes is their intolerance to multivalent cations, such as calcium and magnesium, that form insoluble hydroxides at the critical interface of the bipolar membranes where the ions separate. Hence the removal of these ions is necessary to prevent fouling of the bipolar membranes.

8.2.9
Chiral Separations Using Membranes

Besides chromatography, membranes offer a potential scalable technology for chiral separations [42]. Use can be made of an enantioselective membrane to allow

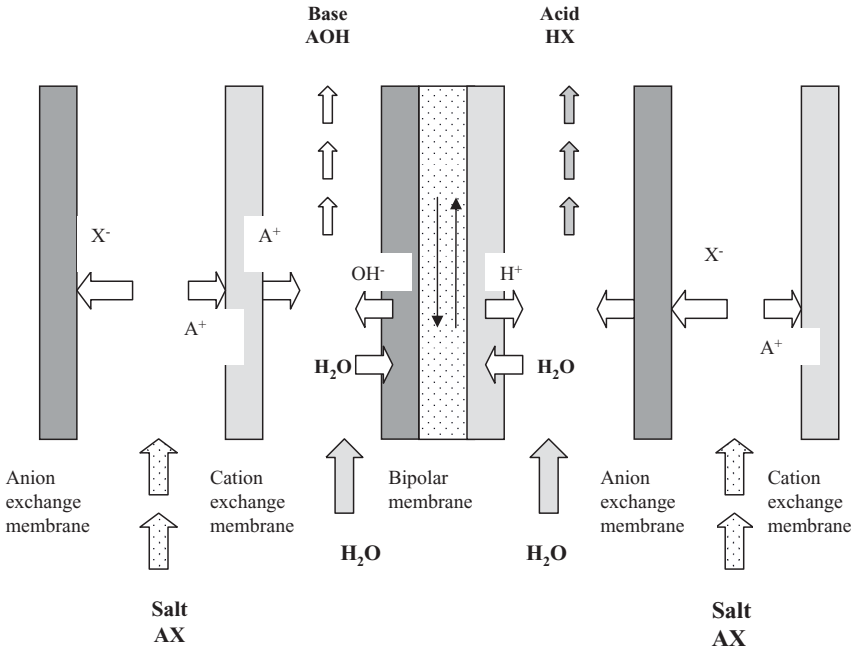

Figure 8.20 Bipolar membrane electrodialysis. A bipolar membrane located between an anion and a cation exchange membrane results in splitting of water to H^+ and OH^- ions which are used in an electrodialysis stack to combine with anions and cations of the salt to produce acids and bases.

selective transport of one of the enantiomers of a racemic mixture or a non-selective membrane to facilitate an enantioselective process.

An enantioselective membrane may be a dense polymer or a liquid (that itself is chiral or has a chiral additive). A liquid membrane can be designed to contain an enantiospecific carrier that selectively forms a complex with one of the enantiomers of a racemic mixture in the feed solution, transports it across the membrane, and releases it on the other side. Although high selectivities can be obtained in such systems, the liquid membranes lack stability over long periods of time and are expensive to run on a large scale.

The enantioselective polymer membranes can be made up of a non-selective porous support coated with a thin layer of an enantioselective polymer, or an ultrafiltration membrane with an immobilized chiral component, or molecular imprinted polymers (as described above). As the desired optical purity is often not achieved in a single step, a cascade of chiral membrane units can be used.

8.2.10
Drying/Solvent Removal

The removal of residual water or solvent is normally used during the final stages for drying of the product to prepare it for storage, but may also be required during

the initial stages of downstream processing as a means of concentration of product from dilute solutions. A variety of drying methods and equipment are available and are classified according to the mode of heat transfer, for example, conduction (i.e., through contact with a heated surface), convection (e.g., by spraying into a hot dry gas) and radiation, or a combination of these. Various additives may be added to the product prior to drying for maintaining stability, improving product solubility, etc.

Drying of smaller quantities of products may be done in a chamber dryer where the product is placed on shelves and transfer of heat takes place partly by contact and partly by convection. On a larger scale, batchwise drying in many contact dryers is facilitated using mechanically moved layers for providing uniform thermal stress on the material being dried, high throughput, and possibility of developing a continuous process. In rotary drum driers, water is removed by heat conduction over a thin film of solution on the steam-heated surface of a rotating drum.

Spray-drying is the most important example of a convective drying method for bioproducts, and involves generation of aerosol of tiny droplets by means of a nozzle or rotating disk, and directing it into a stream of hot gas (150–250 °C). Evaporation proceeds rapidly, leaving behind solid product particles.

Freeze-drying or lyophilization represents one of the least harsh methods and is used for drying of pharmaceutical products. The drying principle is based on sublimation of the liquid from the frozen material.

8.3 Examples of Downstream Processing of Different Product Groups

8.3.1 Alcohols

Ethanol and butanol (acetone–butanol) are the alcohols produced by fermentation under anaerobic conditions. Both alcohols have attracted interest not only for application as a biofuel but also as platform molecules for production of other chemicals. Ethanol is currently a major product worldwide (about 9 billion gallons during 2006), and increase in production is expected during the coming years in many countries. The raw materials used vary depending on the region, for example, sugar cane is used in Brazil while in the United States it is mainly corn starch.

The industrial processes for ethanol production normally use distillation as the main recovery step. About 40% of the total energy needed for corn-to-ethanol conversion is attributed to distillation costs. Prior to distillation, the liquid (beer) from the fermentation is heated and sent through a degasser drum to flash off the vapor containing primarily ethanol and water with some residual carbon dioxide. The ethanol and water vapors are then condensed and recombined with the liquid stream that is fed into the distillation column, while any uncondensed vapor is combined with the CO_2 produced during fermentation and sent through a CO_2

scrubber. During distillation about 90% of the ethanol is removed as a 40% by weight ethanol/water vapor. Subsequent recovery of ethanol from the beer column distillate is achieved by a combined action of rectifier, stripper, and molecular sieves. Over 99% of the ethanol goes out from the top of the rectifier as distillate. The remaining bottom product is fed to the stripping column to remove additional water and the ethanol distillate from the stripping is recombined with the feed to the rectifier. The distillate of the rectifier, containing primary ethanol, is fed to the molecular sieves, which catch the last traces of water giving 99.6% pure ethanol [43]. The fuel ethanol is produced after mixing the refined ethanol with approximately 5% denaturant (gasoline).

The residue from the bottom of the beer column, containing significant amount of water and non-fermentable material such as protein, oil, fibers, and unconsumed chemicals during fermentation (amounting to 15% solids) is fed to the stillage tank, centrifuged to remove over 80% water giving wet distiller's grain at 37% solids. From the liquid product, known as thin stillage, part of the water is recycled back for the starch liquefaction process while the remaining is fed to the evaporator. The concentrate from the evaporator is mixed with the wet distillers grain and dried in rotary drum dryers.

In the Biostil®2000 process of ethanol production (Chematur Engineering, Sweden) (Figure 8.21), the alcohol is continuously removed from the fermentation process, maintaining the alcohol concentration at 4.5% by weight. The fermentation broth is fed to centrifugal separators for separation of yeast that is recycled back to the fermenter, while the clarified beer is processed as above. Most of the ethanol depleted beer (weak beer) after distillation is pumped through the regenerative heat exchanger and a trim cooler prior to being recycled back to the fermenter.

Pervaporation has been proposed as an alternative means for ethanol recovery. A proposed downstream scheme is to remove the alcohol from the fermentation broth by pervaporation, concentrate it to ~80% by distillation, and finally use pervaporation to prepare pure ethanol. Various studies have indicated the potential of pervaporation to improve process economics, but propose improvements in membrane performance and costs [44]. For ethanol recovery from the crude broth, the available pervaporation membranes have low selectivity and deteriorate with time due to adsorption of other components such as acids and glycerol. Modification of the membranes has been shown to improve the performance [45].

8.3.2 Organic Acids

Organic acids constitute an important group of chemicals produced by fermentation. While acetic and citric acid may be regarded as traditional products, interest in fermentative production of lactic and succinic acid as platform chemicals has increased dramatically during the past decade.

Several methods are applicable for the recovery of organic acids; however, the traditional means of their recovery as calcium salts is still widely practiced [46–49].

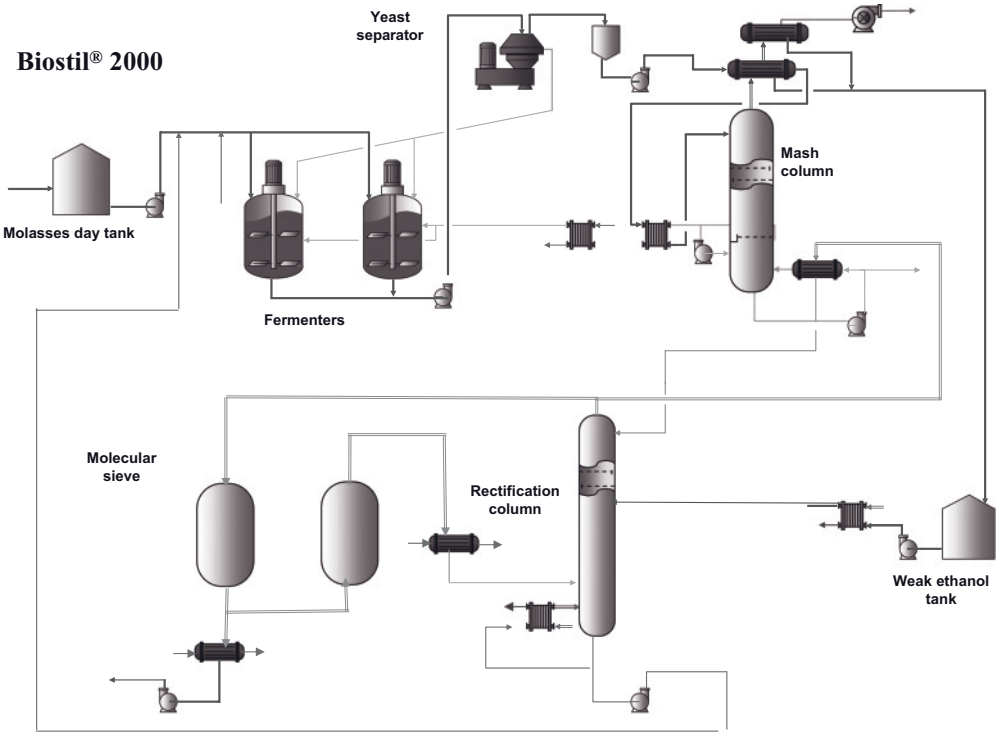

Figure 8.21 The Biostil®2000 process of ethanol production. Ethanol is continuously recovered from the fermentation broth to maintain a low concentration in the fermenter. The yeast cells are separated by centrifugation and recycled back to the fermenter, while the clarified broth (beer) is sent to the distillation (mash) column where ethanol is removed as a ~40% ethanol/water vapor (weak ethanol). Subsequent recovery of pure ethanol from the distillate is achieved by rectification and stripping, and last traces of water are removed by molecular sieves. Courtesy: Chematur Engineering, Karlskoga, Sweden.

Excess calcium hydroxide/carbonate is added to neutralize the acid during fermentation, and the calcium salt of the acid can be obtained as a precipitate or maintained in solution. The latter helps to easily separate the product from the cells and other particulate matter. At the end of fermentation, the broth is filtered to remove the solids, carbon-treated, evaporated, and acidified with sulfuric acid to convert the salt into the free acid and the calcium sulfate formed is filtered off. The filtrate can be further purified using carbon columns and ion exchange and evaporated to produce technical grade product. The technical grade lactic acid is converted to the high-purity, heat-stable product by esterification with methanol or ethanol, recovering the ester by distillation, followed by hydrolysis with water, evaporation, and recycling of the alcohol. A major drawback of this process is the large amounts of calcium sulfate generated as the by-product.

Liquid–liquid extraction using a hydrophobic tertiary amine extractant (Alamine® 304-1) is a classical method of recovering organic acids from fermentation broths. The loaded solvent is stripped with hot water. This permits the recovery of free acid rather than the corresponding salt. The water is evaporated to yield crystalline acid [50]. The regenerated amine solvent mixture is recycled. This method has been applied to all the organic acids and has been considered as an effective and economic purification method. More recently, an integrated succinic acid recovery process composed of reactive extraction, vacuum distillation, and crystallization has been developed, giving succinic acid purity of 99.8% and yield of about 73% from a fermentation broth of *Mannheimia succiniciproducens* [51]. The vacuum distillation helps in the removal of acetic acid from the product.

A further development of extraction using tertiary amine/carbonate technology has been reported. Lactic acid, produced as sodium lactate, is extracted from the concentrated broth with a tertiary amine–solvent mixture under CO_2 pressure, giving a precipitate of sodium bicarbonate and an amine–lactic acid extract. While lactic acid is obtained by back-extraction with hot water at 140 °C and 100 psig, the sodium bicarbonate is heated to produce sodium carbonate and CO_2, which are recycled into the process.

Electrodialysis has attracted a lot of attention for lactic acid production that does not produce a salt waste [52]. The fermentation broth after microfiltration is fed to the electrodialysis unit. A process configuration called the "double ED" process has been developed that uses a desalting electrodialysis unit to remove the multivalent cations and concentrate the lactate salt, followed by a "water-splitting" electrodialysis unit with bipolar membranes where the ionic species are converted to their equivalent acid and base forms and separated. Sodium lactate is converted to lactic acid which is enriched as the process proceeds. Sodium ions are transported across the cation membrane and associate with the hydroxyl ions to form sodium hydroxide which is recycled for fermentation. This enables the process to operate efficiently and economically. A similar method has been developed for recovery of pure succinic acid that is subsequently passed through the evaporative crystallizer to produce very pure crystals [53, 54].

8.3.3 Amino Acids

Amino acids were among the first products to be made by fermentation on an industrial scale. In a typical downstream process for amino acid recovery, separation of the cells by filtration or centrifugation is followed by decolorization of the clarified broth prior to passage over an ion exchange column. The bound amino acid is eluted and crystallized (after evaporation) followed by further conditioning by drying and sieving. Some amino acids (e.g., L-threonine) are produced in very high concentrations with very low amounts of by-product, and may be directly crystallized without the need of an ion exchange step.

8.3.4 Enzymes and Proteins/Peptides

Proteins comprise a diverse group of macromolecules differing in composition, size, surface properties, and function; these differences are exploited in the separation of proteins from each other. Among the commercially available proteins are bulk enzymes (e.g., amylases, xylanases, proteases, and lipases), diagnostic enzymes (such as glucose oxidase, peroxidase, urease), and therapeutic proteins (e.g., monoclonal antibodies, human growth hormone, insulin). The applications of the proteins determine the degree of purification required; the bulk enzymes are relatively crude, concentrated preparations formulated to meet the requirements for activity and stability, while therapeutic proteins require extremely high levels of purity (>99%).

Currently, large-scale production of proteins is mainly achieved using a variety of recombinant hosts including bacteria, yeasts, fungi, and mammalian cells. The production host often determines the choice of the solid–liquid separation method and the eventual cell lysis method to be used. Extra-/intracellular or periplasmic location of the target product are also considerations dependent on the host. Excretion of proteins into the extracellular medium is often preferred in order to avoid the harsh cell disruption step, and also because fewer contaminants need to be dealt with during purification. Bulk enzymes are invariably produced extracellularly; after clarification of the broth the enzymes are concentrated by precipitation or ultrafiltration and formulated often as liquid products.

Intracellular production of proteins in *Escherichia coli* can result in their expression as insoluble inclusion bodies, which has the advantage of the protein being quite pure. The recovery of the active protein, however, requires solubilization of the aggregated protein under denaturing conditions followed by refolding of the solubilized protein by slow removal of the denaturant [7].

High-resolution purification of proteins is normally done using different chromatographic procedures depending on their surface properties and/or molecular recognition characteristics. The matrices used for chromatography of proteins are normally based on hydrophilic gels such as agarose. Purification of therapeutic proteins requires a number of polishing steps to remove all the contaminants arising from the production host (e.g., DNA, viruses, endotoxins, and host cell proteins), culture medium, and also degraded and aggregated variants of the product. Selective purification of the protein from a clarified crude solution is made possible by an affinity tag genetically fused to the protein. A stretch of histidine residues is one of the most commonly used tags for enabling purification of the recombinant protein by immobilized metal ion affinity chromatography.

Use of aqueous two-phase systems or expanded bed chromatography has also been used to recover proteins directly from unclarified feedstocks. This in turn allows reduction in the number of downstream steps.

8.3.5 Antibiotics

Like amino acids, antibiotics were some of the earlier successful products from biotechnology. The main separation technique for their purification is either liquid–liquid extraction or adsorption. Penicillin, tetracycline, erythromycin, and bacitracin are the examples of antibiotics purified by extraction. Penicillin and tetracycline are extracted at low pH while erythromycin extraction is performed at high pH, using a solvent like butyl acetate. Taking the example of penicillin, downstream processing starts with filtration to separate the fungus, *Penicillium notatum* or *P. chrysogenum*, and the filtrate is cooled down to 0–4 °C prior to addition of ammonium sulfate or tannic acid to induce protein precipitation. The pH of the clear filtrate is lowered to below the pK_a of penicillin followed by extraction using a solvent (butyl acetate) to broth ratio of 0.1. The penicillin-containing solvent is treated with activated carbon and then back-extracted with aqueous phosphate buffer (pH 5–7.5) at a buffer to solvent ratio of 0.1–0.2. This process can be repeated and eventually the antibiotic is crystallized either from aqueous or solvent phase in a stirred tank reactor. The crystals are separated by filtration, washed with relatively volatile solvents to remove residual impurities, dried under vacuum, and finally with hot air. The solvent used for extraction is recovered by distillation and recycled.

8.3.6 Carotenoids

There has been an increasing interest over the past 10–20 years in developing microbial sources for industrially important carotenoids for use in foods, feeds, pharmaceuticals, and cosmetics [55]. Improvements in yields from microbial sources by mutagenesis or processes based on engineering of carotenoid biosynthesis pathways are also being developed. Microbial carotenoid products from *Dunaliella* sp., *Haematococcus pluvialis*, and *Phaffia rhodozyma* are produced commercially.

Although whole biomass can be used as a source of carotenoids, for applications in foods and health the carotenoids need to be extracted. Different means of recovery and purification of carotenoids from algal cultures have been applied. Solvent extraction from a ground, dried biomass is a common means for recovery of carotenoids. In a commercial process of astaxanthin production by *H. pluvialis*, the carotenoid accumulated in the algal cysts is released by grinding the dried cysts at –170 °C in the presence of antioxidants. Supercritical carbon dioxide extraction has been found to be an effective means for quantitative recovery of astaxanthin and other carotenoids from ground, freeze-dried, and crushed *H. pluvialis* [56].

Countercurrent chromatography has resulted in high recovery and purity of zeaxanthin from a crude extract obtained by solvent extraction of saponified microalga, *Microcystis aeruginosa* [57].

8.3.7
Biosurfactants

Biosurfactants are surface-active molecules that are produced by a variety of microorganisms. It is also possible to produce sugar-based biosurfactants (e.g., sugar esters and alkyl glycosides) using enzymatic synthesis. Although industrial production is still limited, biosurfactants have been found interesting for a variety of applications such as in environmental remediation, enhanced oil recovery, cosmetics and pharmaceutical formulations because of their biodegradability, low toxicity, and higher stability at high pH. Several methods for recovery of biosurfactants have been reported, such as acid precipitation, solvent extraction, ammonium sulfate precipitation, crystallization, and centrifugation [58]. Some other unconventional startegies that take advantage of the surface activity and their ability to form micelles and/or vesicles, and that are applicable for large-scale continuous recovery of biosurfactants from culture broths have also been reported. These include foam fractionation, ultrafiltration, adsorption on polystyrene resins, and ion exchange chromatography.

8.3.8
Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) represent a complex class of storage polyesters that are deposited as insoluble intracellular inclusions in a number of microorganisms [59]. PHAs possess material properties similar to various synthetic thermoplastics and elastomers currently in use (from propylene to synthetic rubber), but have the advantage of being non-toxic, biocompatible, and biodegradable [60]. The microorganisms that produce high amounts of PHAs include *Alcaligenes latus*, *Azotobacter vinelandii*, and *Cupriavidus necator* (formerly called *Ralstonia eutropha*), but commercial production of the polymer is done using recombinant *Escherichia coli*. Poly (3-hydroxybutyrate) (PHB) recovery has been attained by diverse procedures such as mechanical cell disruption, physical, chemical and enzymatic lysis of microbial cells, and combinations of different methods [17, 61, 62]. Solvent extraction is known to lead to suitable PHB purification with negligible reductions in its molecular weight [63], but this procedure is expensive, creates disposal problems, and changes the morphology of polymer granules. Chemical disruption of cells to release PHB from biomass is considered as one of the most efficient and economical options [64]. Polymer extraction by cell digestion with aqueous NH_3, NaOH, or KOH from *A. vinelandii* and recombinant *E. coli*, respectively, results in high recovery of pure polymer [64, 65].

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9
Industrial Biotechnology in the Chemical and Pharmaceutical Industries

Maurice C.R. Franssen, Manfred Kircher, and Roland Wohlgemuth

9.1
Introduction

The products of the chemical and pharmaceutical industries have contributed at the micro- and macroeconomic level not only to cost savings, but also to enormous progress in the quality of life, everyday health, nutrition, and protection of the environment in many areas worldwide. Global initiatives such as, for example, the United Nations Environment Program, the World Summit on Sustainable Development in Johannesburg, and the Global Product Strategy and Responsible Care program of the International Council of Chemical Associations (ICCA) are aimed at minimizing significant adverse effects of the use and production of chemicals on the environment and human health.

These goals require continuous improvements on many levels, but it is clear that a more selective reaction methodology and a move from stoichiometric to catalytic reactions will have a fundamental and positive influence. Catalytic process technologies instead of classic organic syntheses, which require auxiliary reagents in stoichiometric amounts, minimize the amount of waste per kilogram of product and environmental, health, and safety issues are improved by avoiding the use of toxic organic solvents. The minimization of waste in relation to product is the goal of both green chemistry and white/industrial biotechnology where nature's catalysts are leading the way in industrial process designs. Since biocatalysts are easily degradable and non-toxic, procedures using biocatalytic tools have not only found their way into industrial large-scale production in the chemical and pharmaceutical industries, but are also finding increased application in the research and development phase. An overview of some of these processes will be given in this chapter.

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9.2
Biocatalytic Processes: Scientific and Technological Perspectives

9.2.1
Beta-Lactam Antibiotics

9.2.1.1 The Nucleus

Beta-lactam antibiotics are bicyclic compounds produced by fungi of the genera *Penicillium* and *Cephalosporium*. There are two classes: penicillins and cephalosporins (Figure 9.1) where the difference between the two resides in the nucleus: penicillins have a 6-aminopenicillanic acid (6-APA) nucleus whereas cephalosporins have a 7-aminocephalosporanic acid (7-ACA) or a 7-aminodeacetoxycephalosporanic acid (7-ADCA) nucleus. The first commercially available β-lactam antibiotics were penicillin G (PenG) and penicillin V (PenV) [1].

With the appearance of increasing microbial resistance since the late 1950s, the quest for active derivatives became evident. Since the activity resides in the β-lactam part, a method had to be found to replace the side-chain, and isolation of the β-lactam nucleus was at that time an unavoidable step. Chemically, this is a formidable task because although both the β-lactam and the connection between the side-chain and the nucleus are amide linkages, the β-lactam is by far the most reactive one because of the ring strain. At Gist-Brocades (now DSM Anti-Infectives, Delft, The Netherlands), a four-step chemical process for the selective cleavage of the side-chain of penicillin G was developed which consisted of a protection–deprotection step, extensive use of hazardous chemicals such as PCl_5, and cooling to low temperatures. Although a chemical masterpiece, the environmental burden of this process was considerable because of the toxic waste and

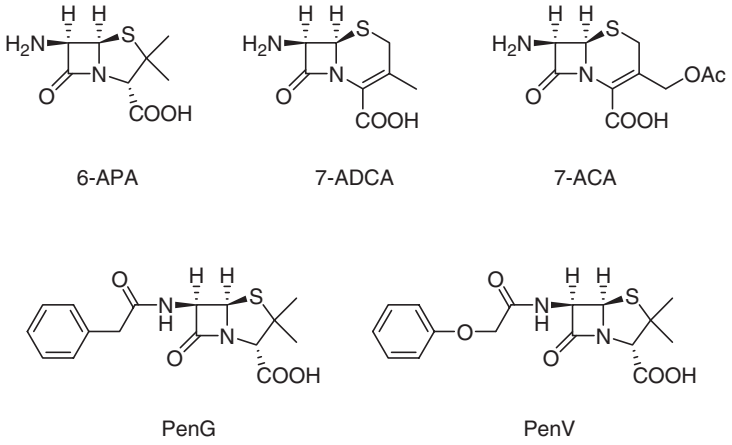


Figure 9.1 Structure of some β-lactam antibiotics and their nuclei. 6-APA, 6-aminopenicillanic acid; 7-ADCA, 7-aminodeacetoxycephalosporanic acid; 7-ACA, 7-aminocephalosporanic acid; PenG, penicillin G; PenV, penicillin V.

solvents and the high energy costs. It is no surprise then that a search for a selective enzymatic process was started.

As early as 1960 four companies (Bayer, Beecham, Bristol, and Pfizer) independently announced the discovery of an enzyme that could split penicillin G into 6-APA and phenylacetic acid. It took some time before this process became competitive, but the biocatalytic process predominated from the moment that robust and immobilized enzymes came on the market. Most companies use the penicillin acylase from *E. coli*. The world market for 6-APA is now around 35 000 tonnes per year. The most important advantage of this process is its eco-efficiency.

Cephalosporins enjoy increased importance because they have stronger antibacterial activity, especially against penicillin-resistant strains. Furthermore, they are better tolerated by people who are allergic to penicillins. A change in side-chains was necessary because of the low biological activity of the primary fungal products. The side-chains in cephalosporins are different from those in penicillins (usually adipic acid derivatives) and their removal is not trivial. Therefore, elaborate chemical ring expansion routes from penicillins, mainly penicillin G, were developed, followed by the known chemical or enzymatic removal of the side-chain to give 7-ADCA and 7-ACA. DSM now has a direct fermentative process for cephalosporins based on genetically engineered *Penicillium chrysogenum* followed by an acylase-mediated hydrolysis of the adipic side-chain to give 7-ADCA.

Greatly reduced production costs and substantial reductions in reagent and solvent usages are the biggest advantages of such a process (see ref. [2] for a history of this process).

9.2.1.2 **The Side-Chains**

A wide variety of side-chains for β-lactam antibiotics have been developed over the years, too many to be mentioned here. The most important ones (Figure 9.2) are D-phenylglycine (D-PG) and D-hydroxyphenylglycine (D-HPG). Table 9.1 gives an overview of the products and their markets.

The side-chains PG and HPG only lead to active antibiotics when the enantiopure D-isomer is coupled to the β-lactam nucleus. Chemical synthesis of the racemate is trivial, but the individual stereoisomers have to be obtained by separation. This was originally done by diastereomer crystallization, which means that a chiral acid (e.g., camphorsulfonic acid) is added to the racemate, forming diastereomeric

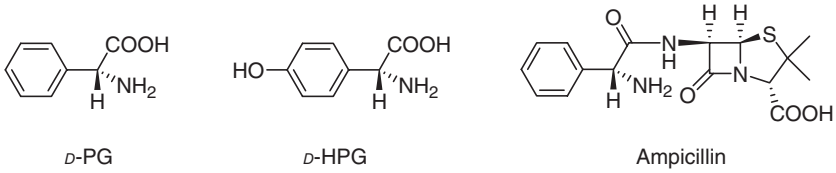
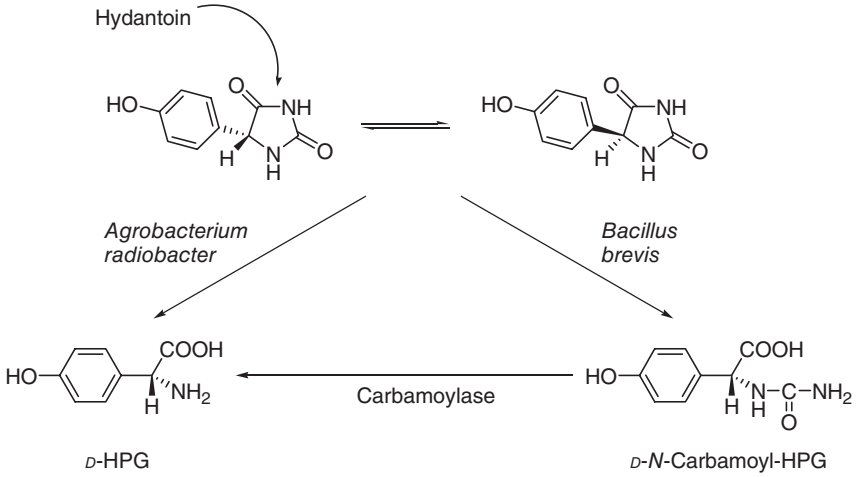


Figure 9.2 Important side-chains of semi-synthetic β-lactam antibiotics and the structure of such an antibiotic. D-PG, D-phenylglycine; D-HPG, D-hydroxyphenylglycine.

Table 9.1 Some semi-synthetic antibiotics and their markets.

Side-chain	Nucleus	Product name	Introduced to the market	Estimated world market (tonnes per year, 2006)
D-Phenylglycine	6-APA	Ampicillin	1961	5 000
	7-ADCA	Cephalexin	1970	4 000
D-Hydroxyphenylglycine	6-APA	Amoxicillin	1972	16 000
	7-ADCA	Cefadroxil	1977	1 000

6-APA, 6-aminopenicillanic acid; 7-ADCA, 7-aminodeacetoxycephalosporanic acid.

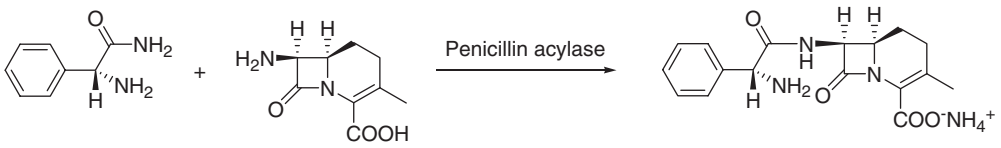


Scheme 9.1 Hydantoin process for the production of D-hydroxyphenylglycine.

salts, of which only one crystallizes. This is still the dominating process for D-PG production [2], although enzymatic processes have been described [3].

D-HPG, on the other hand, is only made by an enzymatic process via a hydantoin (Scheme 9.1) [2]. The hydantoin process has the advantage that the substrate spontaneously racemizes under the reaction conditions, avoiding the chemical racemization of the undesired isomer, leading to much higher production efficiencies and improved product quality.

Initially two companies have commercialized the hydantoin process for D-HPG: the Japanese firm Kanegafuchi (now Kaneka) and the Italian company Recordati. In the Japanese route, only one carbon–nitrogen bond is broken by the hydantoinase in *Bacillus brevis*, which means that the remaining urea part has to be removed by another enzymatic step. The Recordati microorganism splits both bonds, so this is a one-step process. Recently, Kaneka has also introduced a D-carbamoylase into their process.



Scheme 9.2 Enzymatic formation of semi-synthetic β-lactam antibiotics.

9.2.1.3 **Enzymatic Semi-Synthesis**

New β-lactam antibiotics were originally made by adding new side-chains to the fungal fermentation broth, but that had limited success because of the selective uptake of these compounds by the fungi. For the chemical coupling of side-chain and nucleus, two processes have been developed, both of which suffer from disadvantages such as deep cooling (Dane salt method) and reactive chemicals (Dane salt and Dane anhydride method). Recent research, initially at NOVO, Denmark, and completed by DSM and the universities of Delft, Groningen, Nijmegen, and Wageningen in the Netherlands has led to the development of an enzymatic coupling process, using the same or similar penicillin acylase as was used for the removal of the side-chain in the fungal β-lactam antibiotic (see Scheme 9.2). For thermodynamic reasons, the D-(H)PG amide is used instead of the free acid.

A production plant for cephalexin based on this reaction was opened by DSM Chemferm in Barcelona (Spain) in the late 1990s. The new process has clear environmental benefits as well as improved product quality and product stability [4]. Overall, compared with the traditional chemical routes, the combination of direct fermentation of the 7-ADCA structure together with the biocatalytic side-chain replacement has led to improvements of 50% or more on important parameters such as solid waste formation, emissions to air, energy need, toxicity, and risk potential and consumption of reagents and solvents [4, 5].

9.2.2
Chiral Building Blocks

A collection of case studies have shown that the biocatalytic asymmetric synthesis of many important building blocks is not only successful on the research scale but can also compete successfully on an industrial scale with classical stoichiometric approaches [6, 7]. Biocatalytic production using hydrolases is well established globally and at Sigma-Aldrich more than 100 biocatalytic reactions are routinely performed on an industrial scale. The development and scale-up of other enzymatic reaction types, such as oxidation reactions, has been of great interest, since chemical oxidations using a combination of reactive oxidants and flammable solvents at production scale is a major limitation.

The first large-scale biocatalytic Baeyer-Villiger oxidation was established [8–10] and replaced the classical oxidants with air and flammable solvents with

water. This not only lowered the safety risks of this classical oxidation reaction, but also meant it could be performed in a highly stereospecific way, giving access to useful chiral lactones as pharmaceutical intermediates. The benefits of biocatalytic production routes lie not only in the way a product is manufactured but also in the knowledge acquired about the routes not chosen. Thus a network of bridges between stoichiometric, catalytic, and biocatalytic reactions is created upon which new productions can be based [11].

9.2.3 Building Blocks for Polymers

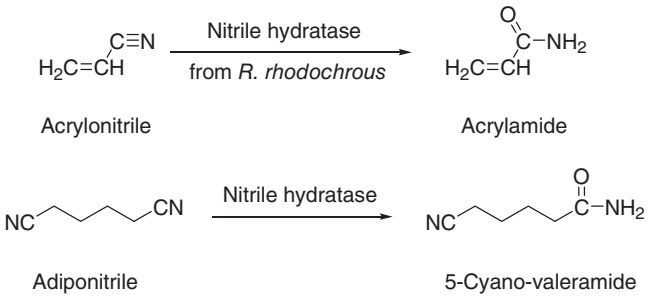
Most building block polymers are bulk chemicals, which means that they are produced in very high volume. Production costs and product specification are key parameters. This is a difficult area to penetrate for industrial biotechnology, not only because of the large volume (huge amounts of enzymes or cells have to be produced) but also since the development costs have to be recouped and the margins are small in bulk chemistry. Nevertheless, a few remarkable successes have been reported over the last two decades.

Acrylamide is a building block for polyacrylamide, which is widely used in the laboratory for separation in chromatography and electrophoresis or as a water-soluble thickener in wastewater treatment and paper making. The monomer is made by the addition of one molecule of water to acrylonitrile, which in turn is made from naphtha-derived propene and ammonia. The conversion of acrylonitrile to acrylamide has been done for many decades using a copper-based catalyst at 80–140 °C. Although the process is efficient, it produces toxic wastewater containing copper and HCN. The high temperature used leads to undesired polymerization of acrylamide which makes it necessary to purify the product.

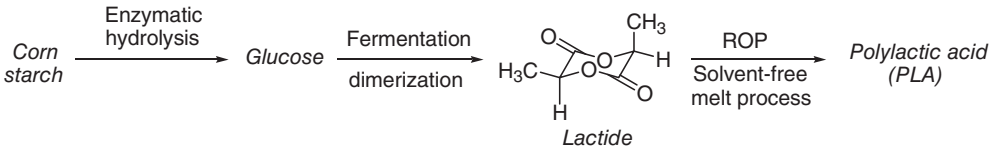
In the 1970s, in the laboratory of Professor Hideaki Yamada in Kyoto (Japan) some microorganisms were found which were able to grow on acrylonitrile. They hydrolyzed the substrate to acrylamide and subsequently to acrylic acid, which was then further metabolized. After optimizing the strain and knocking out the second hydrolytic enzyme, microorganisms were obtained which could selectively convert acrylonitrile to acrylamide (see Scheme 9.3). Initially *Pseudomonas chloraphis* was used, but later optimized *Rhodococcus rhodochrous* became the standard. The process runs at much lower temperature than the chemical process (0–15 °C), avoiding spontaneous polymerization and subsequent purification. The latter factor appeared to be the key success factor in economic terms. Both the substrate and the product are reactive compounds but the cell-free extract tolerates up to 500 g/l of substrate when immobilized in polyacrylamide (its polymerized product!).

The enzymatic process was first commercialized by Nitto Chemicals (now part of Mitsubishi Rayon) but it nowadays runs at many companies around the world, including SNF (France) and Evonik Industries (Germany), in a global volume of more than 250 000 tonnes per year.

A related process has been commercialized by Dupont. In this case, a nitrile hydratase converts one of the nitrile groups of adiponitrile, giving 5-cyanovaleramide



Scheme 9.3 Use of nitrile hydratase for the production of base chemicals.



Scheme 9.4 Cargill-Dow's process for polylactic acid.

(see Scheme 9.3), which is a starting material for the synthesis of the herbicide azafenidin. The process runs on a multi-tonne scale. The key advantage of this process is its selectivity: the reaction produces the monoamide with 96% selectivity at 97% conversion [12–15].

Cargill and Dow Chemical have joined forces to commercialize their finding that polylactic acid, a polymer with great commercial potential, can be produced using industrial biotechnology. The process is shown in Scheme 9.4. In the first step, corn starch is hydrolyzed by glucoamylase and pullulanase to give glucose syrup, perhaps the largest, oldest and most established industrial biotechnology process on earth! Glucose is then anaerobically fermented by optimized microorganisms into L-lactic acid, which is chemically dimerized to the lactide (see Scheme 9.4). A little bit of D-lactic acid is also obtained, leading to some D,L-lactide, which has to be removed by distillation. The polymerization of the lactide is done chemically.

Polylactic acid (PLA) has properties comparable to those of polyethylene and polypropylene. It is less heat resistant but much more biodegradable. The joint venture NatureWorks (now owned by Cargill) has a PLA plant in Blair (Nebraska) since 2002, with a production capacity of 140 000 tonnes per year (Figure 9.3).

Another building block for plastics is 1,3-propanediol, made by DuPont from glucose in a fermentative process. The microorganism was heavily optimized by pathway engineering. Coupling of 1,3-propanediol with terephthalic acid gives a polymer called Sorona™, which is spun into fibers. Current use is in clothes and carpeting, where it is appreciated for its enhanced softness, dyeability, and stretch.

Figure 9.3 NatureWorks plant, Blair, Nebraska, USA.

1,3-Propanediol produced via fermentation has a lower cost of manufacture than that produced via the competing chemical processes, and uses a renewable feedstock [16].

Up to now the polymerization process has been done chemically, but research on enzymatic polymerization is going on. Papers by the groups of Gross [17] and Heise [18] have shown that lipases, especially immobilized *Candida antarctica* lipase B (Novozym 435) is an excellent catalyst for the polymerization of lactones. Enantioselective polymerization with *in situ* resolution has also been reported [19].

9.2.4 Fine Chemicals: Statins

Increased cholesterol levels are a growing concern to the health of the human population and it is no wonder that a cholesterol-lowering medicine like atorvastatin (Lipitor™, Figure 9.4) is the best-selling drug at the moment worldwide [6]. Statins are inhibitors of HMG-CoA reductase, an essential enzyme in the biosynthesis of cholesterol. They consist of a core of several rings which may be annellated (e.g., lovastatin) or substituted with other rings (e.g., atorvastatin). In all cases there is a functionalized enantiopure β-hydroxy acid attached to the core as a side-chain. Because of the huge market potential of the statins, many different companies have commercialized routes to the enantiopure side-chains.

Codexis has developed a process for the preparation of the first chiral center in the statin side-chain. The second one can be easily introduced by asymmetric induction using the first chiral center. The Codexis process consists of a two-step enzymatic cascade (Scheme 9.5a). In the first step, the commercially available 4-chloro-3-ketocaproic acid ethyl ester is regio- and stereoselectively reduced by the

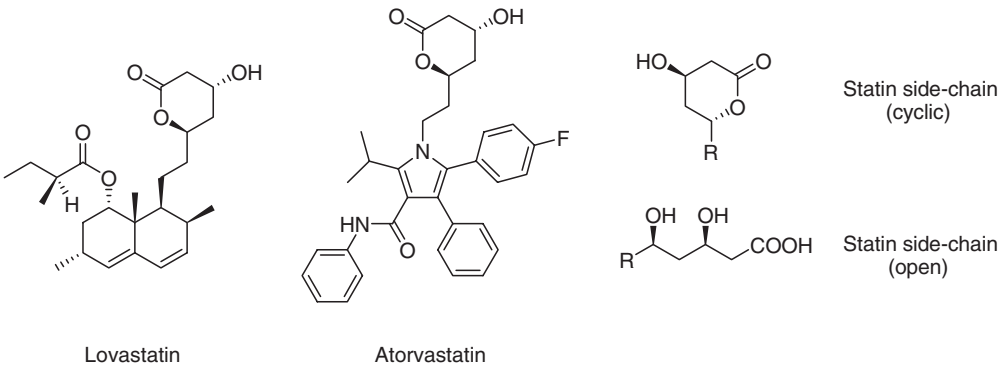
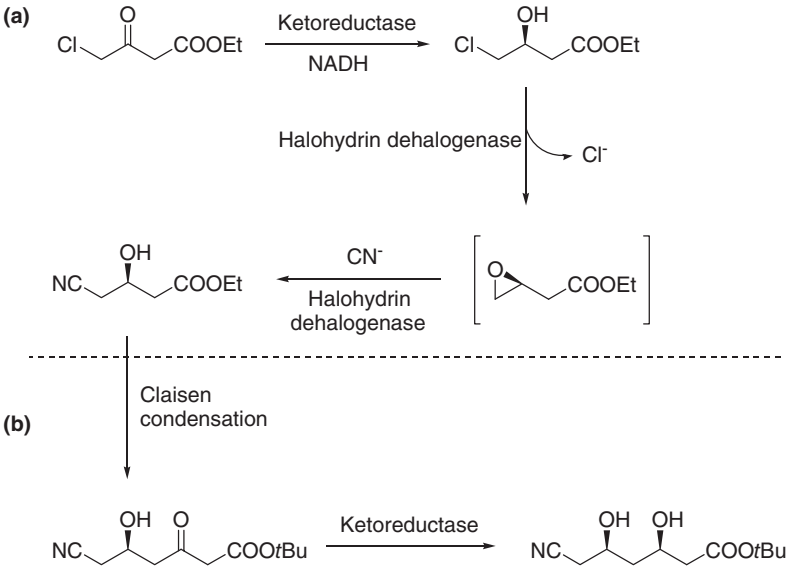
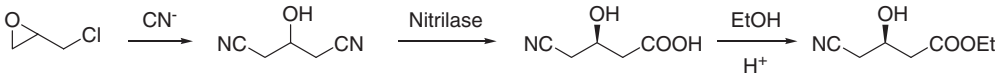


Figure 9.4 Two important statins and their side-chains.



Scheme 9.5 Codexis processes for the statin side-chain.

ketoreductase from *Candida magnoliae*, cloned into *E. coli* [20]. The cofactor NADH was recycled using optimized glucose dehydrogenase. The ketoreductase had to be genetically optimized in order to meet the desired specifications: a substrate tolerance of more than 150 g/l, a substrate/enzyme ratio of >100, an enantiomeric excess (ee) of >99.5%, a cofactor turnover frequency of >20 000 and a reaction time of less then 10 h. In the second step, a halohydrin dehalogenase from *Agrobacterium radiobacter* expressed in *E. coli* was used to form an epoxide and subsequently open it with cyanide ion. This enzyme is not a very good cyanation catalyst, so it was also optimized by directed evolution. The criteria to be met were in this case:



Scheme 9.6 Diversa process for the statin side-chain.

complete conversion of at least 100 g/l substrate, a volumetric productivity of >20 (g product/l.h.g enzyme), a simple product isolation procedure and simple enzyme formulation process. An enzyme with 37 mutations having a ~4000-fold improved volumetric productivity was eventually obtained [21]. The whole process now runs at cubic meter scale, producing tonnes of product.

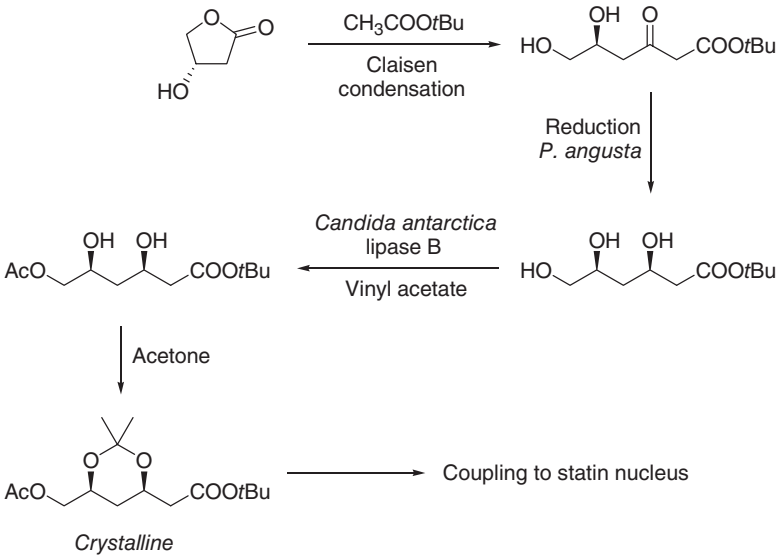
The key advantage of the enzymatic cyanation reaction is that the reaction is clean and no difficult-to-remove by-products are formed. The typical by-products in the chemical cyanation of vicinal hydroxynitriles cause degradation of the product if special precautions are not taken [22]. The Codexis process won the Presidential Green Chemistry Award in 2006.

Recently, the second chiral center of the statin side-chain has been introduced biocatalytically using another ketoreductase (Scheme 9.5b). Several ketoreductase genes were synthesized and optimized by directed evolution. A twenty-fold increase in activity was found after five rounds, but this was still not good enough. Using a model built on existing structures, a combinatorial library of semi-synthetic genes with active-site mutations was constructed. This yielded an enzyme which is able to work with substrate concentrations over 300 g/l at low enzyme loading, giving 99.3% conversion and >99.9% diastereomeric excess (de). The work-up is easy and the process needs much less organic solvents then the chemical process (3.2 versus 22.2 l/kg). The process now runs on a cubic meter scale in India.

Diversa constructed a successful route for the same building block for statins as that initially produced by Codexis (Scheme 9.6). Their approach starts from racemic epichlorohydrin, which when treated with an excess of cyanide, gives a prochiral dinitrile. Selective hydrolysis by a nitrilase and subsequent esterification gives the enantiopure (*R*)-4-cyano-3-hydroxy-butanoic acid [23, 24]. The nitrilase was optimized by directed evolution and completely converts the substrate at substrate concentrations greater than 360 g/l [25]. A key advantage of this process is the low cost of the starting material epichlorohydrin, which costs only 2€/kg. Another driver for this process was the availability of a large collection of nitrilases at Diversa, one of which is ideally suited for this application.

A similar product (with chloride instead of cyanide) has been developed by Kaneka since 2001 and is made by enzymatic reduction of the corresponding ketone. However, this approach requires that the difficult cyanation chemistry is late in the synthetic scheme.

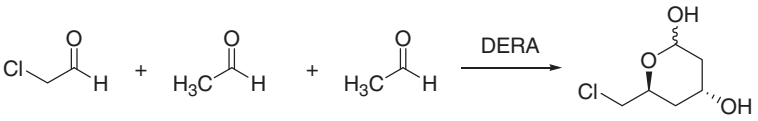
Just like Codexis, Avecia (now NPIL Pharmaceuticals) also developed a process using a ketoreductase (Scheme 9.7). The starting material is enantiopure (*S*)-3-hydroxybutyrolactone, which is commercially available. This compound is subsequently coupled to *tert*-butyl acetate by a Claisen condensation. The product is then stereoselectively reduced by the ketoreductase from *Pichia angusta*, giving >96%



Scheme 9.7 Avecia process for the statin side-chain.

conversion, >99% de and >99% ee [26]. The development of the process for the chiral triol from 1 g to 1 tonne was done in less than a year. This compound is now produced by NPIL Pharmaceuticals on 70 000 l scale, giving tonnes of product per year. For the coupling to the statin nucleus, some protective group chemistry has to be done. The secondary hydroxyls have to be protected in order to facilitate selective coupling of the primary hydroxyl group to the nucleus. However, protection by acetone leads to the wrong acetonide, so the primary hydroxyl group was first blocked by the regioselective action of a lipase (*Candida antarctica* lipase B) and vinyl acetate. This reaction is fast (<5 h for full conversion) and highly regioselective. Subsequent treatment with acetone gives the suitably protected side-chain. Recycling of the enzymes used is not necessary since they are cheap enough. The main advantages of this route are the extremely high stereoselectivity of the reduction achieved by *Pichia angusta*, the highly regioselective protection of the triol and the crystalline nature of the acetyl acetonide that permits purification by crystallization.

DSM embarked on a route based on aldolases. It had already been shown by Professor Chi-Huey Wong (Scripps, San Diego, USA) that 2-deoxyribose 5-phosphate aldolase (DERA) is able to couple three aldehydes in a consecutive and highly stereoselective fashion. The selectivity of each step is less then 98%, but the minor stereoisomer is not accepted as a substrate in the second aldol reaction, leading to an exceptionally high de [45]. In order to make the statin side-chain, two molecules of acetaldehyde have to be coupled to one molecule of chloroacetaldehyde (Scheme 9.8). This poses a problem since the wild-type enzyme is strongly inhibited by chloroacetaldehyde at industrially relevant concentrations. Random



Scheme 9.8 DSM process for the statin side-chain.

mutagenesis of DERA led to a library of 10 000 clones which were screened by GC-MS-MS for chloroacetaldehyde resistance and formation of the final product. The best mutants were analyzed, combination of mutations proved to be beneficial. In the end, a double mutant was obtained which was even activated by chloroacetaldehyde.

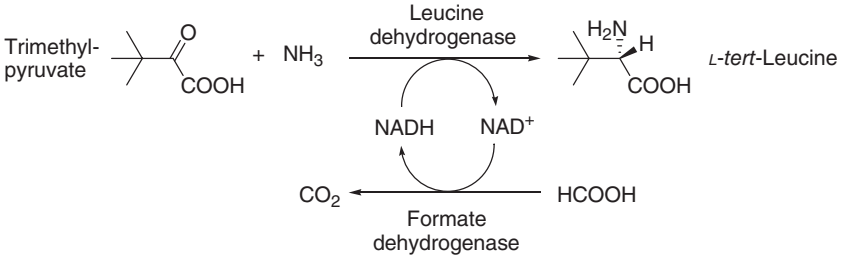
The process has the advantage that two chiral centers are formed in one step from very cheap achiral material, giving rise to a 100% yield/100% ee concept in theory. The fact that DSM has a strong position in aldolases was a key factor in the development of this process, which was scaled up to industrial scale.

9.2.5 Amino Acids

Amino acids are extremely important products in various branches of industry, especially in the food and flavor industry. L-Glutamate is a very important taste enhancer (worldwide production is 1.7 million tonnes per year) [6], L-phenylalanine is a building block for the artificial sweetener aspartame (10 000 tonnes per year in 2004) [28], L-aspartic acid is an acidulant and a building block for aspartame (17 000 tonnes per year in 2004) [www.ajinomoto.com], and L-lysine is used as a food and feed additive (700 000 tonnes per year in 2004) [28]. For some amino acids there are fermentation processes (e.g., L-aspartic acid), for others there are processes based on enzymatic kinetic resolution (e.g., L-phenylalanine) or enzymatic asymmetric synthesis (L-aspartic acid). An interesting interconversion process is done by Tanabe in Japan, where L-aspartic acid is converted into L-alanine by an aspartate-β-decarboxylase.

Amino acids like D-phenylglycine and D-*p*-hydroxyphenylglycine are very important for the pharmaceutical industry, as has been discussed earlier in this chapter. Two smaller but still interesting amino acids are L-*tert*-leucine and D-valine.

L-*tert*-Leucine is an unnatural amino acid used as a chiral ligand in transition metal complexes for catalytic reduction reactions in pharmaceutical industry. The market size is less then 10 tonnes per year and the compound is used for high-value products. Product purity is the key factor, allowing a state-of-the-art process developed by Evonik (Scheme 9.9). The process is based on a reductive amination of trimethylpyruvate by leucine dehydrogenase in the presence of ammonia and NADH, giving enantiopure L-*tert*-leucine in quantitative yield. The cofactor NADH is recycled by formate dehydrogenase, using formic acid as electron donor giving harmless carbon dioxide as coproduct. The NADH was coupled to polyethylene



Scheme 9.9 Process for L-*tert*-leucine by Evonik.

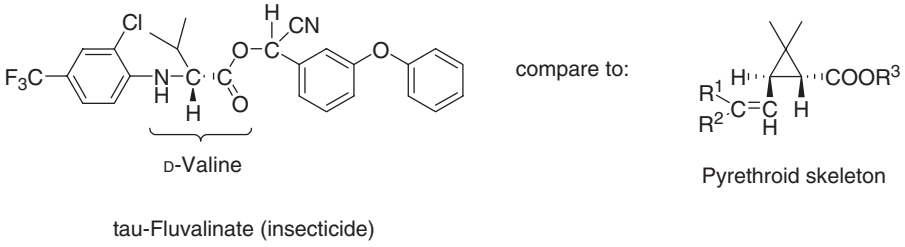


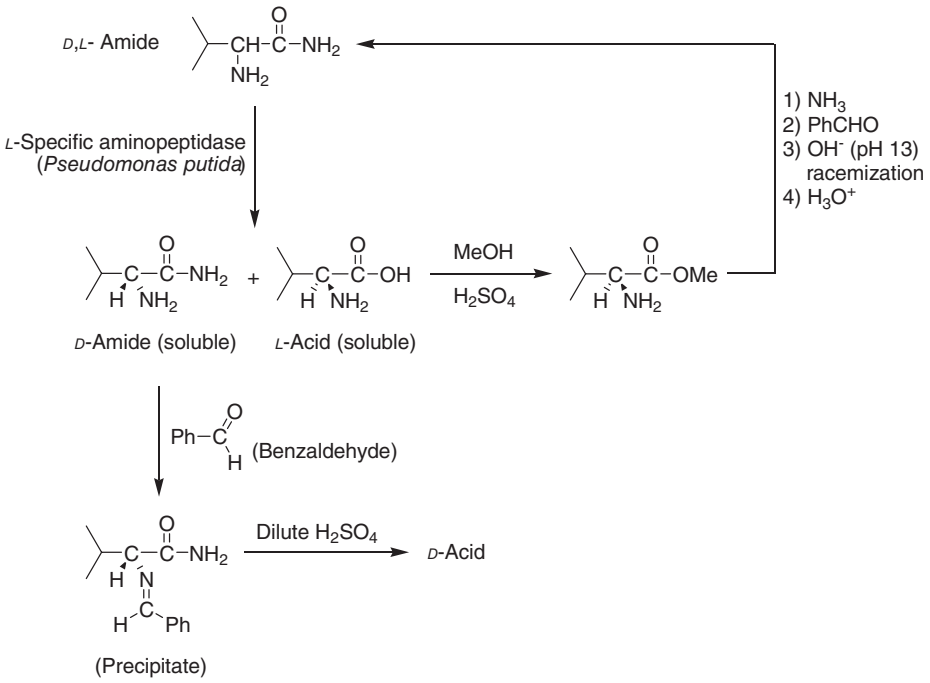
Figure 9.5 Structures of tau-fluvalinate and pyrethoids.

glycol in order to use it inside an ultrafiltration membrane reactor. High space–time yields and turnover numbers of 125 000 were obtained [28].

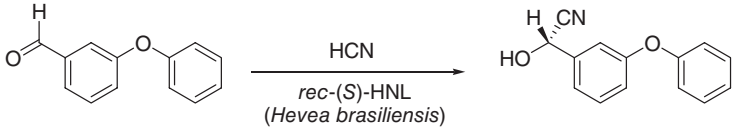
D-Valine is a constituent of tau-fluvalinate, an insecticide of the pyrethroid class (Figure 9.5) [29]. Pyrethrin is a natural product occurring in *Chrysanthemum* flowers. Natural pyrethrin is relatively easily detoxified by enzymes inside the insect, so synthetic variants have been made (pyrethroids) which are more stable. In addition, they are more effective and less toxic to mammals than natural pyrethrin. Tau-fluvalinate is the most important pyrethroid. It contains two chiral building blocks, D-valine and a chiral cyanohydrin, but it is marketed as a mixture of diastereomers, with only the stereochemistry in the amino acid part fixed [29].

D-Valine can be made by DSM using, for example, their amidase or hydantoinase technology. The general scheme of the amidase process is given in Scheme 9.10 [3]. The chemically synthesized racemic amide is resolved by the L-specific aminopeptidase from *Pseudomonas putida* [30]. The unreacted D-amide is precipitated as its Schiff base, while the L-acid product can be racemized and reconverted into the amide if required by process economics. This aminopeptidase is extremely versatile since it accepts a wide variety of amino acid side-chains. However, at the α-carbon there should be a hydrogen atom. For more hindered substrates the *Mycobacterium neoaurum* aminoamidase [31] and the *Ochrobactrum anthropi* amidase [32, 33] have been developed by DSM.

The second chiral building block can also be synthesized in enantiopure form by biocatalysis, for example, by DSM ES/IM in Linz, Austria (Scheme 9.11). The



Scheme 9.10 DSM process for enantiopure amino acids.

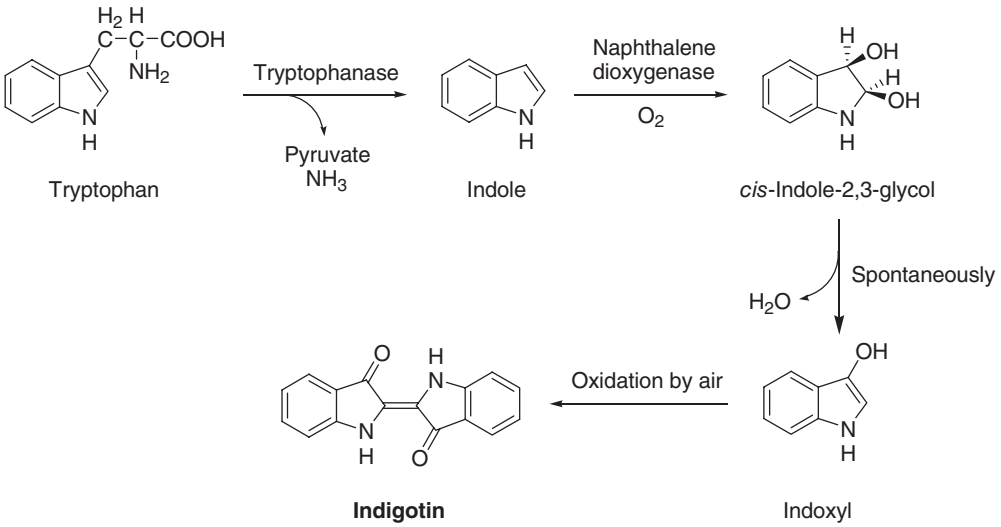


Scheme 9.11 DSM process for *S*-cyanohydrins.

enzyme used is the *S*-hydroxynitrile lyase from *Hevea brasiliensis* (the rubber tree), cloned and overexpressed in the yeast *Pichia pastoris*. This versatile enzyme accepts both aliphatic and aromatic substrates at molar concentrations. The other enantiomer can be made using the *R*-hydroxynitrile lyase from almonds (*Prunus amygdalus*). The key advantage is the fact that asymmetric synthesis is performed, that is, the theoretical yield is 100%.

9.2.6 Indigotin

Not every biotechnological process is a success, even when the benefits, for example, a lower environmental burden, are clear. There are presumably more of these cases but indigotin is at least the best documented one. Indigotin is an intensely



Scheme 9.12 Amgen process for indigotin.

blue compound, well-known for the dyeing of denim (indigo dye), which has become an essential component of the lifestyle for large groups of people in the western world. The production is around 80 000 tonnes per year. Originally indigotin was a natural compound derived from plants as "true indigo" (*Indigofera tinctoria*) and woad (*Isatis tinctoria*) [www.answers.com/topic/indigo-3008]. Industrial production since its discovery in the early twentieth century is based on a four-step chemical sequence (the so-called Heumann process), starting from anthranilic acid which is obtained by a mercury-catalyzed oxidation of fossil-derived naphthalene. The process also involves toxic and potential carcinogenic nitrobenzene for extraction. By accident, researchers at Amgen found indigotin production when they put the genes for naphthalene oxidation into *E. coli*. Their aim was to construct a pathway for salicylic acid (building block for aspirin). However, it appeared that *E. coli* produces indole when grown on glucose, from breakdown of tryptophan (Scheme 9.12). The indole is oxidized by the new enzyme naphthalene dioxygenase into indoxyl, which oxidatively dimerizes upon exposure to air to give indigotin.

Although the original production level was only about 25 mg/l per day this was strongly optimized by the scientists at Amgen. However, this beautiful process was regarded as highly competitive by the indigotin-producing chemical industry, and they decided to lower the price of indigotin significantly in order to force the new producer out of business. They were able to do that because indigotin plants were fully depreciated, so that only operating costs had to be covered [34]. Amgen (which sold their process to Eastman-Kodak) was not able to recover the investments and the process was abandoned. Genencor now owns the rights for the biotech process and is still working on it, but to date there is still no full-scale biotechnological plant for indigotin.

The crystal structure of naphthalene dioxygenase was recently published [35]. It appears to have a broad substrate specificity paired to a good stereoselectivity and this will undoubtedly boost further research towards the application of this enzyme.

9.3 Biocatalytic Processes: Business and Commercial Perspective

Today the chemical industry produces 27 million tonnes of organic chemicals such as polymers, pharmaceuticals, fine and specialty chemicals, and more for all segments of our daily life worth 2000 billion euros worldwide (Figure 9.6). Most products are produced by chemical synthesis starting from simple chemical building blocks produced from fossil oil [27].

Industrial biotechnology has been well established in the chemical industry for decades. However, it contributes only 50 billion euros or 2% to the chemical industry's sales volume of 2000 billion euros, mainly in the three segments pharmaceuticals, fine and specialty chemistry as well as detergent and hygiene products. Sugar extracted directly from agricultural biomass such as sugar cane or sugar beet or produced via enzymatic hydrolysis from corn starch is the dominant carbon source.

Typical product examples are amino acids such as L-lysine, L-threonine, and L-tryptophan produced by fermentation or D-amino acids as well as derivatives such as L-*tert*-leucine provided by combined chemical synthesis and enzymatic catalysis (Rexim, France). Examples of companies specializing in enzymatic routes to enantiomerically pure molecules include Codexis and Bayer Health Care. It is necessary for these applications to combine chemical synthesis with bioprocesses as, for example, in the production of the active pharmaceutical ingredient Miglitol, an inhibitor of intestinal α-glucosidases effective for the oral treatment of diabetes (Scheme 9.13).

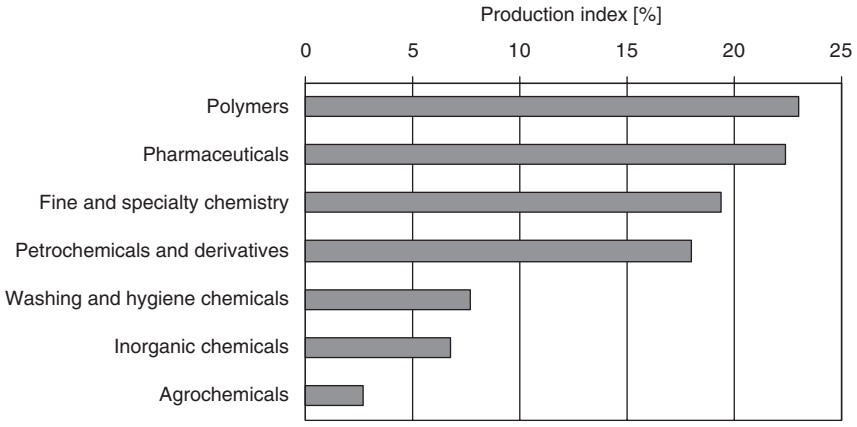
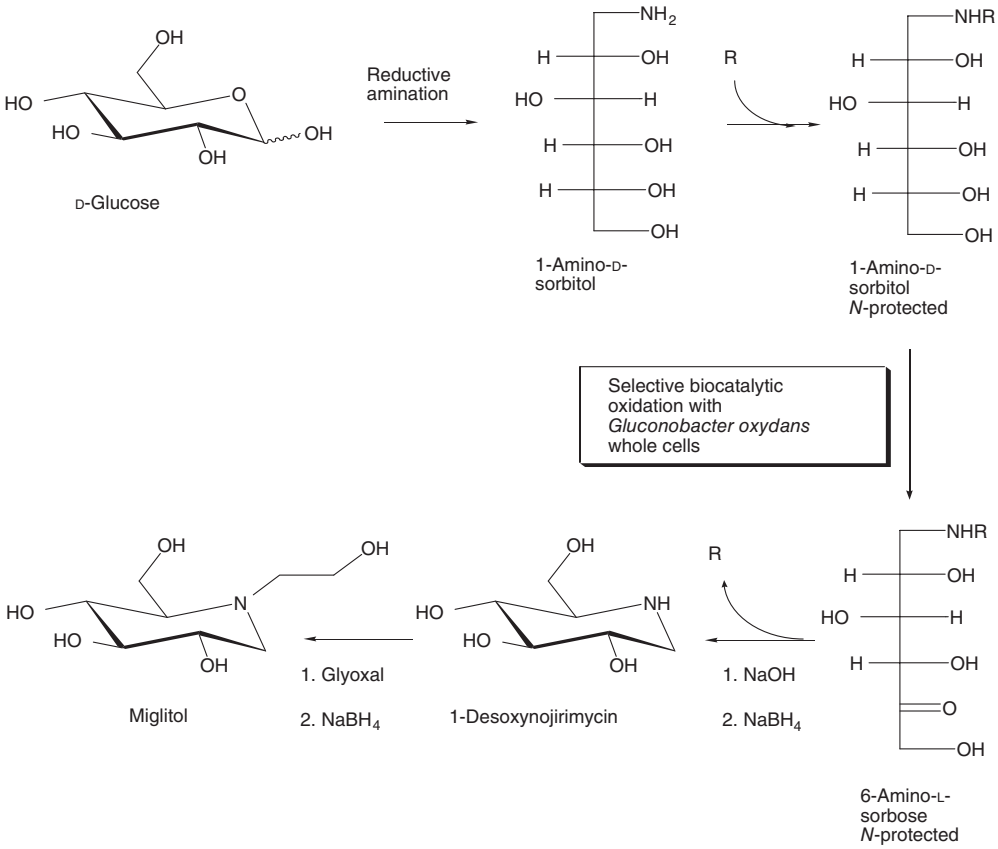


Figure 9.6 The chemical industry's production index (Germany).



Scheme 9.13 Combined synthetic/biocatalytic process to Miglitol (Bayer Health Care).

Scheme 9.14 Compatible solutes ectoin (left) and OH-ectoin (right).

Compatible solutes, a group of protective molecules synthesized by halophilic bacteria, are produced by whole cell catalysis by Bitop, Germany. Most relevant are ectoine and OH-ectoine–both marketed as functional ingredients for skincare applications (Scheme 9.14).

Brain Biotech, Germany launched a new skincare product for skin revitalization containing recombinant tissue inhibitor of metalloproteinase-2. The market

success of such products is predominantly based on its function as a highly specific pharmaceutical building block or cosmetic agent with a nutritional or biomedical function. The value is in the function. This type of value is what investors are looking for. The venture capital financed exit of Julich Fine Chemicals into Codexis is only one of many examples. In the biopharmaceutical field but also in the biomedical, cosmetic, and nutrition markets many examples are well known; for example, Senomyx developing functional products for the food additive market. Functional products of this type mostly have a limited market size and the costs of production are not decisive for market success.

Nevertheless, big-volume products are also well established, such as feed-additive amino acids (>1 million tonnes per year; Evonik, ADM, Ajinomoto), vitamins (DSM), and detergent enzymes (Novozymes, Henkel). All these products are produced by processes of industrial biotechnology because it offers the only technical choice. Chemistry derived from petrol-based starting materials does not provide the large-scale methods for producing enantiomerically pure molecules or complex amino acids. However, if there is a chemical route available or developable in a short time period, timelines in the chemical industry tend to favor chemical synthesis because of the large reaction knowhow acquired over the years and the predictability of the required time per synthetic step.

There are, however, a number of products which are not easy to prepare from commonly available starting materials or the commonly available reaction methodology is not selective enough. It is in those cases where biocatalytic processes have found their place in the chemical industry and many companies in the fine and specialty chemicals area are making increasing use of biocatalysts.

Beside the fact that industry is in general much more familiar with chemical synthesis and has depreciated production plants available, there is a simple reason for the prevalence of chemical synthesis: chemistry's most relevant feedstock, carbon-source mineral oil, has so far been unbeatably cheaper than biotechnology's preferred carbon sources from biorenewables.

The cost of carbon is crucial for the production cost of many chemicals: In 2004 – at an oil price of lower than $50/barrel – the product-specific hydrocarbon cost varied between 42% (polystyrene), 49% (PET resin), and 79% (butadiene rubber). Whenever chemical synthesis and industrial biotechnology are compared from a technical view, the costs of feedstock carbon are decisive, but socio-economical aspects such as product acceptance by customers, ecological and changing economical boundary conditions may make biorenewables more competitive.

However, the cost of carbon sources is changing – slowly over a scale of years but dramatically in recent months. The cost of oil is increasing to never expected levels. Since 2004 the markets have registered an increase of 50%. The rising cost of oil has had a significant impact on the competitiveness of biorenewable carbon sources: whereas in 1998 the cost per barrel of mineral oil was US$17 versus US$88 for soy bean oil, the relation has changed today to US$72 versus US$78, showing that fossil oil has lost its traditional cost advantage.

With biorenewable feedstocks becoming competitive their processing technologies gain new relevance too. Biotechnology is no longer limited to products where

it is the only mean of production. The decision for biotechnological processes is determined by the most competitive carbon source. If it is an agricultural plant-based carbon source, biotechnology is the preferred process.

This changing economic environment is already favouring innovative new products and new processes. Cargill's polylactic acid is an example of a new polymer based on biorenewable feedstocks. Lactic acid is produced by fermentation based on sugar and further on polymerization by chemical processes. Combining biotechnological and chemical process steps may represent a prototype for the future chemical industry. Cargill have already invested in a 140 000 tonnes per year plant. Since 2004 Evonik have produced the antiknocking agent ethyl *tert*-butyl ether with a capacity of 250 000 tonnes per year using bioethanol as feedstock. DuPont Engineering Polymers produces Bio-PDO (propanediol) as a starting material for Sorona and other hybrid polymers.

These are early examples where biotechnological processes have led to products giving an alternative to their petrochemical counterparts. More processes are in the pipeline. In contrast to the products mentioned before, the functionality of monomers such as lactic acid or of products like PDO and ETBE is rather low. Their market success depends on the cost of production and the decisive competitive factor is the cost-efficiency of the process. From the investor's point of view the process may not be seen as worth investing in. However, this position may change when investors realize the huge market volume of biotechnological substitutes of traditional petrochemical products.

On the other hand convincing traditional biotech investors may take time because here the business model is different to the former one: now we are speaking about low-value products for high-volume markets. Attention from investors will increase when the financial world realizes biotechnology's platform character. Basic intermediates for the chemical industry such as lactic acid, 1,3-propanediol, succinic acid, 3-hydroxypropionic acid, *n*-butanol, itaconic acid, propylene glycol, isosorbide, and levulinic acid are all produced by microbial primary metabolism pathways. Optimizing one pathway for industrial purposes may be costly for the first product, but once established in the chemical industry the very same pathway may deliver after only few modifications another high-volume product and so on. Consequently biological production systems for cost-efficient processes for simple industrial compounds gain an immense value. This combination will draw capital into industrial biotechnology, accelerating the development of more biotechnological processes for the chemical industry.

9.4
Safety, Health, and the Environmental Perspective

In this world with its increasing human population, it is unavoidable that industrial activities will be close to places inhabited by humans. People are thus becoming more vulnerable to safety and health issues connected to these industrial production activities. Safety and health issues are determined by the toxicity and

stability of chemicals, auxiliaries, intermediates, products, and side-products. In addition, narrow stability and error-tolerance limits of certain reactions have to be taken into account. Bioprocesses make an important contribution to fundamental waste reduction and safety, health and energy improvements by utilizing nature's catalysts, thereby avoiding highly reactive and non-selective chemicals [36].

At the environmental interface, biocatalysis and biotransformations have been used to convert toxic by-products and waste into environmentally compatible materials [37], but there is an urgent need to integrate environmental aspects into methods of synthesis. Selective and scalable biocatalytic tools are beginning to be incorporated into the planning of synthetic routes from starting material to product and are key to sustainable global economic growth with an environmental bonus [38]. It is important not only to solve environmental issues by end-of-the pipe solutions, but to avoid waste and achieve waste reduction by selective reactions and by avoiding complicated protection–deprotection schemes with side-reactions. Thus the design and practical implementation of high yield and low waste reactions at the microenvironmental level translates into tremendous improvements at the macroenvironmental level.

9.5 Outlook

Industrial biotechnology is providing us with chemicals, materials, fuels, and pharmaceuticals in a sustainable way. Several successful large- and small-scale processes have been mentioned in this chapter and it should be stressed that these are just examples. The list of processes given is by no means exhaustive; there are numerous other applications, especially in fine and specialty chemistry. Biotechnology plays an important role in these sectors because of the stringent purity issues, so the well-known selectivity of enzymes is fully exploited there. In addition, the value of these products is in their function – for example, in the case of a pharma building block in the specific binding to a receptor or in case of a catalytic enzyme in its specific reaction.

Looking at the value chain – starting from raw materials to intermediates up to the consumer product – functional products are positioned high. The value of such molecules depends much more on their contribution to the performance of the final product than on the cost of production (COGS; cost of goods and services). Therefore the margins in this sector are higher than in others, allowing the development costs of (optimized) biocatalysts to be recouped even though the production volume in specialty chemistry might be only a few tonnes annually. An example of a product with a limited market and production volume is L-*tert*-leucine by Evonik.

Industrial biotechnology has also successfully penetrated the polymers and materials sector. Polylactic acid (PLA) is a nice example of a new product which has some features in common with the existing polyethylene and polypropylene. However, it also demonstrates the impact that the cost of carbon sources is having

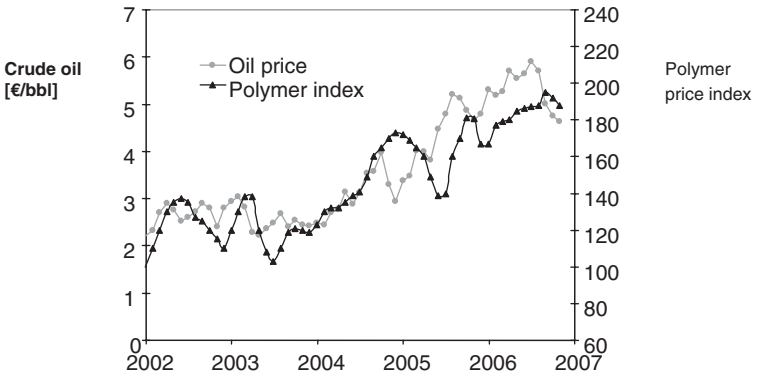


Figure 9.7 Correlation of market prices of crude oil and polymers [41].

Table 9.2 Production volume and prices of basic carbon feedstocks [39, 40].

		Production (million tonnes per year)	Price (€/t)	C	H	O	Carbon (€/t C)
Petrochemicals							
Ethylene	C_2H_4	110	785	86%	14%		917
Propylene	C_3H_6	75	785	86%	14%		917
Benzene	C_6H_6	45	610	92%	8%		661
Biorenewables							
Glucose	$C_6H_{12}O_6$	143	300	40%	7%	53%	750
Bioethanol	C_2H_6O	36	365	52%	13%	35%	700

on the competitiveness of biotechnology in the field of bulk polymers of low functionality. Here the achievable market price is directly dependent on COGS as is demonstrated by the correlation between the price of crude oil and the index of petrochemical polymers.

Since 1998 the price of oil has increased by about a factor of 5 as shown in Figure 9.7. These rising costs have a crucial impact on the competitiveness of products based on biorenewables – among them biotechnological polymers.

Only recently have biorenewables become competitive to platform petrochemicals like ethylene and benzene based on the cost of carbon (Table 9.2).

However, investments in industrial biotechnology are long term and need long-term competitiveness of the feedstock base as well. In the long term the cost of biorenewables will be driven by the energy markets because of their dominance. At the moment 93% of crude oil globally goes into energy production and only 7% is consumed in the chemical industry. The increasing impact of the energy market on the price of biorenewables is already becoming clear. The significant increase in bioethanol production drives the market demand for its feedstock

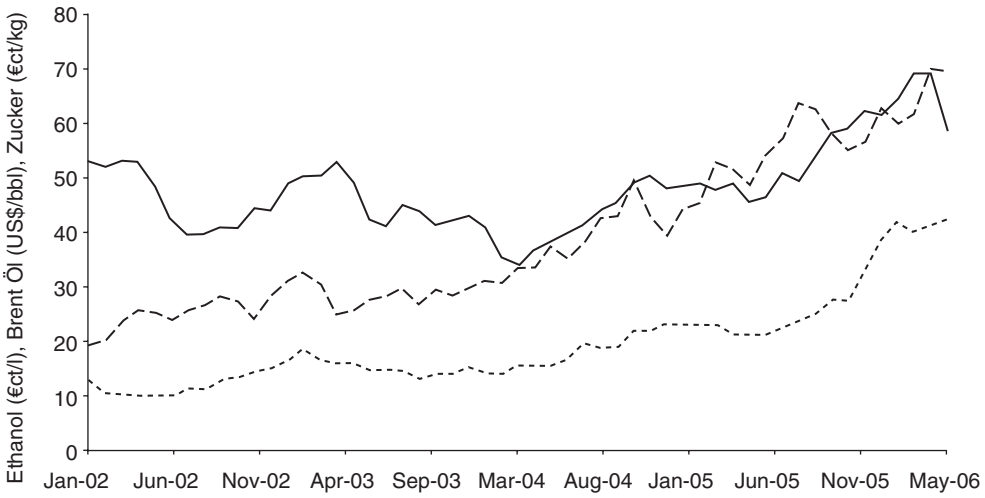


Figure 9.8 Development of prices of oil, ethanol, and sugar, January 2002 to May 2006. Solid line, ethanol; dashed line, oil; dotted line, sugar. Cost of ethanol in Europe based on dehydrated ethanol produced in Brazil plus transport (€45/m^3) and EU customs duty (€192/m^3).

sugar, leading to a correlation between sugar, ethanol, and oil prices. Figure 9.8 shows the price development of sugar in parallel to bioethanol and oil [40], and because the cost of oil and sugar are correlated by 85%, corn follows the rising cost of sugar (http://futures.tradingcharts.com).

Because of these correlations it is possible to calculate the cost at which oil biorenewables and the products based on them will be competitive. Because of its different energy content (gasoline 32 MJ/l; ethanol 21 MJ/l) the gasoline-based energy equivalent is at US$60/barrel of oil or €400/t ethanol. With the bioethanol process economics prevailing today this limits the maximum carbon cost for the production of bioethanol to €238/t [41].

Are biotech-based polymers like PLA competitive in feedstock markets as described? Based on published data on the economics of the PLA process the yield of the lactic acid monomer is 90%, that of polymerization 80%. About 40–50% of total COGS of PLA is related to lactic acid, about 30% is related to the carbon source sugar [42, 43].

Starting from these and public market data the sugar cost limiting profitability can be estimated: at a sales price of about €1100/t PLA sugar should not exceed €210/t – this is slightly lower than the current market price [41]. However the price of the competing petrochemical polymer PET is in the same range – giving PLA no cost advantage to petrochemistry.

Because of the lack of significant cost benefits, industrial biotechnology so far is established only in those fields where it is the only technical option and impressive examples have been described. Although the future is always different from that

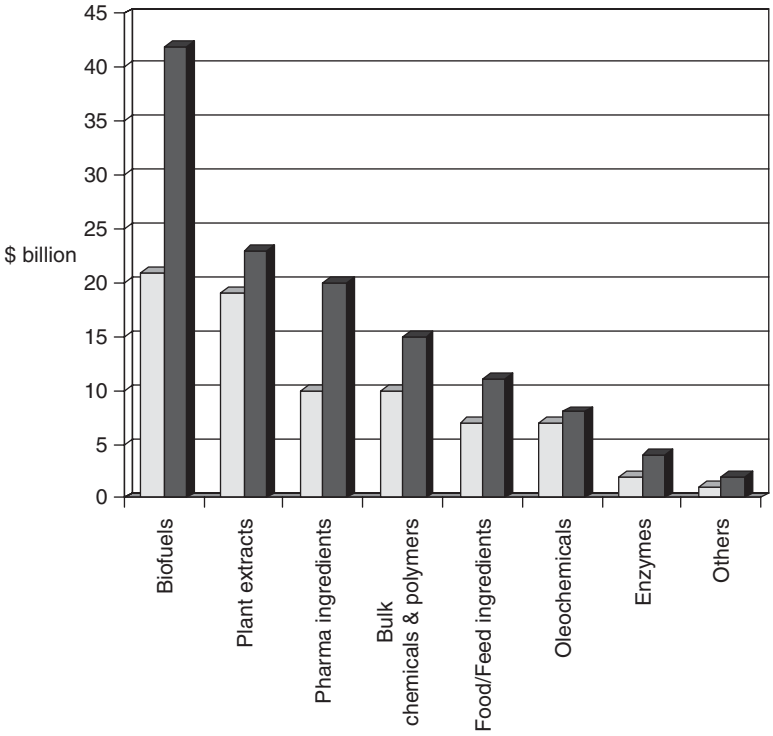
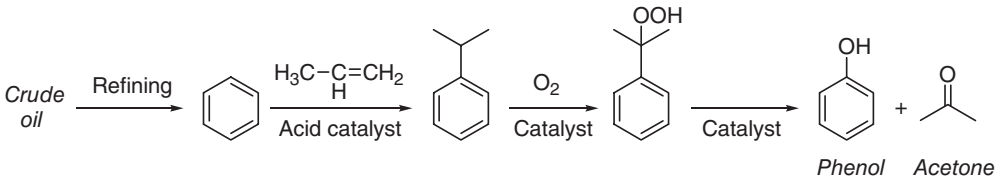


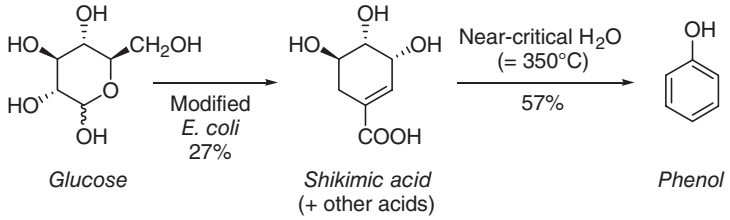
Figure 9.9 Market segments of industrial biotechnology 2005 (light) and 2010 (dark) [48].

predicted and can also depend on novel discoveries which can completely change the market boundary conditions, it is interesting to note that McKinsey estimated the total sales volume of industrial biotechnology to be US$77 billion in 2006 as shown in Figure 9.9 [49]. This sales volume represents a niche of only about 5% when looking at the whole industry. Therefore it should be kept in mind that fossil-derived products may not necessarily be replaced with the same bio-based compounds: a new compound with better performance than the fossil-derived one is needed! Thus improved performance or functionality of the final product, unique technology platforms and safety, health and environmental improvements are economic justifications for investments in industrial biotechnology.

Despite the complex and changing interrelations between feedstock and product markets it is important that industrial biotechnology leaves behind its 5% sales volume niche in the chemical industry. This is in our view the biggest challenge and at the same time has breakthrough potential typical of economic or technological transitions. This will happen only if biotechnology provides competitive processes to bulk chemistry. Many millions of tonnes of bulk chemicals are produced every year and it is not an easy task to replace these processes by clean bioprocesses. The replacement of the chemical process for acrylamide has shown how powerful



Scheme 9.15 Current production process for phenol.



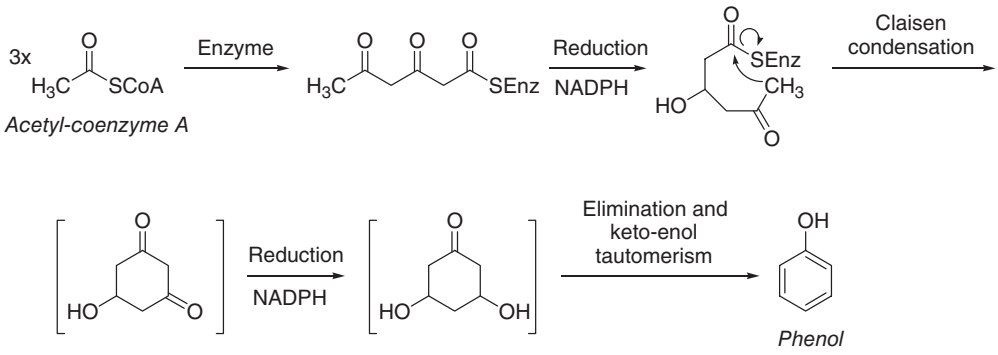
Scheme 9.16 Frost route for phenol.

the contribution of industrial biotechnology can be on a very large scale, but the feedstock there (acrylonitrile) is still fossil-derived. More focus on research towards bio-based "green" production methods of bulk chemicals is definitely required. Significant improvements in biofeedstocks (e.g., lignocellulosic carbon sources), biocatalysts and bioprocesses may give industrial biotechnology another push. A (partly theoretical) example, given below, deals with the production of phenol.

Phenol is a real commodity, produced at 8.9 million tonnes per year and increasing 6% per year. It is used as a building block for phenolic resins and as a starting material for pharmaceuticals such as aspirin. Although phenol can be obtained from coal tar, the most important production method is based on fossil-derived benzene and is given in Scheme 9.15. In the first step, benzene is reacted with propene by the action of an acid catalyst, producing cumene. The latter is catalytically oxidized and the peroxide is subsequently decomposed to give phenol and acetone, which is sold as a coproduct. There is a fear that the market for phenol may increase so much that there will be a large surplus of acetone which will influence the economics of the whole process.

The group of John Frost at Michigan State University has devised a process for phenol from a bio-based feedstock (Scheme 9.16) [46]. They cloned the biochemical pathway for the conversion of glucose to shikimic acid into *E. coli*, and got a 27% yield (on a molar basis) of shikimate by this modified *E. coli* strain. The shikimate was subsequently converted to phenol in one step using near-critical water in 57% yield. Unfortunately the second step is energy-intensive, involving heating to 350 °C and high-pressure equipment, but if this could be avoided this process would be very elegant and efficient.

Taking the Frost group approach one step further, one could even construct a complete biosynthetic route for phenol. A theoretical route based on polyketide



Scheme 9.17 Theoretical biosynthetic route for phenol.

biosynthesis is given in Scheme 9.17. Phenolic compounds are abundant in the plant kingdom so one would expect that such a battery of enzymes can be found somewhere in plants or can be modified in such a way that they selectively perform the desired reaction.

The real challenge, however, is not to produce phenol in this or any other biochemical way, but to produce phenol on a multi-million tonne scale for, let us say, one euro per kilogram. This means that we need microorganisms with extremely high productivity, combined with high stability and tolerance for this very toxic product. We also need excellent downstream processing techniques to get phenol efficiently out of a (not too dilute!) aqueous stream. This will be the real challenge in the decades to come.

For the sake of completion: it has been reported [41] that it is possible to pyrolyze biomass and use the resulting oil, which contains many phenols, as an additive to "real" phenol in the production of phenol–formaldehyde resins. The lack of a purification stage reduces the energy needed, and the gases and charcoal also produced can be used as fuel for heating the biomass. Cost savings are claimed to be as high as 25%.

When we devise new routes for bulk chemicals, we must look carefully at the starting material to be used. More specifically, many important bulk chemicals (caprolactam, acrylamide, pyridine, etc.) contain one or more nitrogen atoms. The conversion of molecular nitrogen to ammonia, which is the feedstock for all nitrogen-containing compounds in the (petro)chemical industry, is very energy-intensive. Conversely, biomass contains a lot of nitrogen which has been fixed by nature in the form of amino acids, proteins, and nucleic acids. Proteins in particular comprise a large proportion of the biomass and can be conveniently isolated and it would be very important to consider their constituents (amino acids) as a feedstock for the bio-based production of nitrogen-containing bulk chemicals [47].

In conclusion, the perspectives for industrial biotechnology in the chemical and pharmaceutical industry and for the partnership of the involved sciences of biology, chemistry, and engineering are excellent today and continue a successful tradition

of interactions in the past century which has led to enormous contributions to the quality of human life [44].

Acknowledgments

We would like to thank all the industrial scientists who were willing to share with us the information on many processes mentioned in this chapter. In particular, we are indebted to Dr. Gjalt Huisman (Codexis), Dr. Robert Holt (NPIL Pharma), and Prof. Dr. A. Bruggink.

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10
Industrial Biotechnology in the Food and Feed Sector

Pierre Monsan and Michael J. O'Donohue

10.1
Introduction

Since the very beginning of human history, living systems and their extracts have been used on a fully empirical basis to solve one of humanity's most basic needs: how to produce and store food. Cheese and beer production are two examples of our earliest progress in this area. In the case of cheese, a biodegradable product, milk, is transformed into a stable, storable, and tasty derivative. Later discoveries, notably by Louis Pasteur, who demonstrated the role of microorganisms in food and beverages and the characterization of digestive enzymes, marked a major turning point in humanity's approach to food production and storage. Since the second half of the twentieth century, the rational use of enzymes and microbial strains in a wide range of food and feed applications has given birth to a new discipline known as "biochemical engineering."

More recently, the introduction of genetic engineering has opened the way to the design of improved biocatalysts for the transformation of agricultural raw materials. The fact that no exogenous DNA is present within enzyme preparations allows the use of such molecular engineering approaches for increasing enzyme catalytic efficiency. This is important because public concern with regard to genetically modified organisms (GMOs) strongly hampers the possibility of innovation in many fields. In this respect it must be underlined that even if the food and feed sectors are traditionally among the main application areas of biotechnology, the development of new ingredients and processes is becoming increasingly difficult because of tight regulatory constraints.

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10.2 Food Applications

10.2.1 Starch Transformation

In plants, energy derived from photosynthesis is principally stored in the form of starch, which in turn is the principal source of energy in human and animal diets. Schematically, starch contains two polymers:

- amylose, which is linear, composed of α-1,4-osidic linkages, and generally a minor starch component (about 25–33%)
- amylopectin, which presents a higher degree of polymerization than amylose and which presents a similar structure but contains α-1,6 branching linkages.

Starch is used to produce food extenders and sugars syrups such as maltodextrins, glucose, dextrose (purified glucose), fructose, maltose, and hydrogenated derivatives (e.g., sorbitol, mannitol). The main sources of starch are corn, potato, wheat, barley, rice, cassava, and sorghum [1].

The first industrial process for starch processing achieved hydrolysis using acid catalysis. The reaction was performed on starch slurry (30–40% dry solids) adjusted to pH 1.5–2.0 using hydrochloric acid. Hydrolysis was completed at 140–150 °C over a 5–8 min period. However, in more recent developments, to avoid the generation of undesirable by-products resulting from carbohydrate oxidation and salt content, an endo-hydrolyzing, depolymerizing α-amylase from *Bacillus amyloliquefaciens* was introduced. To start with, this enzymatic liquefaction step was achieved at pH 6.0–6.5 for 5–8 min at 85 °C. Then, to promote starch gelatinization, the reaction mixture was heated at 140 °C for 5 min, then cooled to 85 °C in order to be able to introduce a second batch of enzyme. This second enzyme addition allowed the completion of starch liquefaction.

This process was improved by the introduction of new thermostable α-amylases from *Bacillus licheniformis* or *Bacillus stearothermophilus*. These can be added directly to the starch slurry at pH 5.8–6.2 in a jet-cooker that is operated at 103–107 °C for 5–10 min. Consequently, the necessity for the 140 °C heat treatment is eliminated. To pursue dextrinization, the liquefied starch is then cooled to 95 °C and calcium ions are added in order to avoid α-amylase denaturation (around 10 ppm). Depending on products that are targeted (dextrose, i.e., pure D-glucose, maltose, maltodextrins) a degree of hydrolysis described by a DE value (dextrose equivalent: ratio of the reducing power, expressed as dextrose, to the total potential reducing power corresponding to the dry matter) of 12–30 is obtained.

The degree of hydrolysis can be increased to produce dextrose ("saccharification step") using the combination of an exo-hydrolyzing enzyme, glucoamylase (amyloglucosidase) from *Aspergillus niger*, which is able to hydrolyze α-1,4 and α-1,6 glucosidic linkages (the latter is hydrolyzed at a slower rate) and a debranching enzyme specific for the hydrolysis of α-1,6 linkages, such as pullulanase. After 40–72 h at pH 4.2–4.6 and 60 °C, a DE of 97–99 is attained, corresponding to 96–97% D-glucose.

Maltose syrups containing 50–60% maltose can be obtained from maltodextrins by using a maltogenic α-amylase from *Aspergillus oryzae* or a β-amylase from plant or microbial origin.

One of the main applications of dextrose syrups is the production of high fructose content syrups (HFCS) by enzymatic isomerization of glucose into fructose (isoglucose or levulose) using glucose isomerase. Because of the equilibrium of this reaction, syrups containing 42% fructose are obtained directly, but syrups containing 55% fructose (used as sucrose substitutes in soft drinks) require the chromatographic separation of glucose and fructose on calcium or potassium ion exchange resins to yield a 90% fructose solution. Various sources of glucose isomerase are available to produce HFCS: *Acrobacter*, *Actinoplanes*, *Arthrobacter*, *Bacillus*, *Streptomyces*. They are operated as immobilized enzymes in continuous column reactors.

A key achievement in this area was the development of industrial-scale crystallization of glucose isomerase, which yields a highly pure preparation of the enzyme. Because glucose isomerase is highly sensitive to inhibition by calcium ions and needs magnesium ions for stabilization, it is essential to remove excess calcium ions from dextrose syrups by ion exchange chromatography and to add magnesium before the isomerization step. However, more recent developments in molecular enzyme engineering have solved this problem by creating a calcium-independent α-amylase [2, 3]. To achieve this, the fine details of calcium binding to *B. licheniformis* α-amylase have been elucidated [4].

A major evolution in the field of enzyme-catalyzed starch hydrolysis is the development of a raw starch-hydrolyzing α-amylase, which efficiently hydrolyzes raw starch granules. This enzyme, from *Anoxybacillus contaminans*, contains four domains, including a starch-binding domain, which is not the case for traditional starch-liquefying α-amylases [5]. Such starch-binding domains are more usually found in CGTases, maltogenic α-amylase, and glucoamylase. This original 4D α-amylase is able to catalyze the hydrolysis of starch at a temperature below the gelatinization temperature, thus avoiding the usual high temperature treatment. At 60 °C, in combination with a glucoamylase from *Aspergillus niger*, 99% liquefaction can be obtained, corresponding to 95% dextrose (instead of 96.3–96.5% in a traditional process). The corresponding new process for starch hydrolysis is highly promising, especially within the context of bioethanol production from starch by alcoholic fermentation.

Similarly, it has been demonstrated that the addition of the starch-binding domain derived from the glucoamylase of *A. niger* to a glucoamylase from an amylolytic strain of *Saccharomyces cerevisiae* (var. *diastaticus*) results in a chimeric enzyme which hydrolyzes insoluble starch [6].

10.2.2 Dairy Industry

Milk transformation into cheese and various processed food products is an intrinsically biological process involving enzymes and microbes that provides an

efficient way to store this liquid raw material. Initially, such transformations were achieved in a totally empirical way, until humans began to master them. Today, industrial milk transformation processes are finely tuned to provide products with constant organoleptic characteristics from a variable raw material.

The key industrial players in this field are Chr. Hansen, Danisco Rhodia, and DSM. The lactic acid bacteria starter cultures produced by these companies are widely used. Importantly, so far these microbial strains have not undergone any genetic engineering.

10.2.2.1 Milk-Clotting Enzymes

κ-Casein, which represents about 15% of milk caseins, is located at the periphery of milk micelles. The specific hydrolysis of the Phe105–Met106 peptide bond in κ-casein by rennet results in destabilization and aggregation of the micelles, facilitated by calcium ions [7], leading to the formation of a gel structure that is cut to yield the curd for cheese making. Curd formation is an excellent illustration of the complexity of biotechnology. It is very difficult to obtain accurate market data for this field. However, three types of enzymes compete for this market and their use is highly variable depending on local tradition, cost considerations, and consumer pressure [8]:

- animal rennet, which is a mixture of chymosin and pepsin extracted mainly from calf stomach (abomasum)
- microbial rennet, which is an extracellular proteolytic preparation obtained from fungal cultures (*Cryphonectria parasitica*, *Mucor pusillus* Lindt, *Rhizomucor miehei*)
- recombinant chymosin, which is a product of genetic engineering and is expressed by *Aspergillus niger*, *Aspergillus oryzae*, *Escherichia coli*, or *Kluyveromyces marxianus* var. *lactis*.

These proteases are all aspartic endoproteases [9]. Tentative figures for their use are given in Table 10.1. However, these are subject to significant variation, as previously stated. At the end of the 1990s in the United Kingdom, recombinant chymosin accounted for 70% of the market. However, because of the rise in anti-GMO feeling, consumer pressure has caused this figure to fall to approximately 30%. In France, the use of recombinant chymosin is very limited and microbial rennet accounts for less than 15% of the market.

Table 10.1 Milk-clotting enzymes market shares.

	Europe	North America and Australia	Rest of the world
Animal rennet	55–60%	10%	10%
Microbial rennet	30%	60%	45%
Recombinant chymosin	10–15%	40%	45%

10.2.2.2 Cheese Ripening and Flavor

Proteases and lipases are used to develop cheese flavors in enzyme-modified cheese (EMC) [10]. These spray-dried products are obtained from short-ripened cheese to which enzymes and additives are added [9].

10.2.2.3 Lipase

Lipases catalyze the hydrolysis of fat triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. The release of free fatty acids is particularly important in blue and Italian type cheeses [11], where they are responsible for the typical sharp and piquant flavor of these products. Lipases from *Rhizomucor miehei* and *Aspergillus* sp. are used for this purpose [9]. These enzymes are specific for the hydrolysis of fatty acids located at the 1,3-position of triglycerides.

10.2.2.4 Proteases

Due to their very well-balanced amino acid composition, milk and whey proteins are ideal starting materials for the production of hydrolysates for:

- enteral and parenteral nutrition
- infant nutrition
- health and fortifying sports drinks
- dietetic foods.

To make milk or whey protein hydrolysates various endo- and exo-acting proteases are employed. These are either of animal origin (pepsin, pancreatic trypsin, and chymotrypsin), plant origin (papain, bromelain), or microbial origin (*Bacillus subtilis, Bacillus licheniformis*) [9]. In addition to their use as proteases, microbial aminopeptidases can be employed to specifically remove the terminal hydrophobic amino acids that are responsible for the bitter taste of peptides.

10.2.2.5 Lysozyme

As well as being present in human tears, lysozyme is also found in hen egg white. This "natural antibiotic" catalyzes the hydrolysis of the β-1,4 linkage between *N*-acetylmuramic and *N*-acetylglucosamine present in the cell wall of Gram-positive bacteria. Lysozyme is used in the dairy industry to avoid the growth of gas-producing butyric acid bacteria, particularly *Clostridium tyrobutyricum*, and prevent "late blowing" in cheese [12, 13].

10.2.2.6 Transglutaminase

The transacylation reaction between the γ-carboxyamide group of glutamine residues and the ε-amino group of lysine residues is catalyzed by transglutaminase. This results in the crosslinking of proteins and their three-dimensional texturation. This reaction is of interest in many food applications, particularly in fresh cheese making and in cream whipping [14, 15].

10.2.2.7 β-Galactosidase

The hydrolysis of lactose, the main carbohydrate present in milk produced by mammals, is achieved using β-galactosidase, also called "lactase." This enzyme

catalyzes the splitting of the β-1,4-osidic linkage between D-galactopyranose and D-glucopyranose, the two sugars that form lactose. Lactose intolerance, common in Asian and African populations, results from a deficiency in β-galactosidase in the digestive tract. In affected people this condition leads to bloating, diarrhea, and flatulence [16]. Fungal lactases are characterized by an acidic optimal pH, while yeast and bacterial enzymes have a neutral one. The latter are used for the production of low-lactose milk for nutritional purposes, as well as for the hydrolysis of lactose in "sweet whey." In the case of "acid whey," which contains lactic acid, fungal lactases are preferred [9].

Due to the limited solubility of lactose, another application area for lactases is the prevention of lactose crystallization, which gives rise to a "sandy" texture in ice creams.

The commercial β-galactosidase from *Kluyveromyces fragilis* (Lactozym™) has been covalently immobilized onto cellulose beads via epichlorhydrin coupling. The advantage of this modification is that the enzyme can be operated in a fluidized bed reactor that allows more than 90% hydrolysis of whey lactose over a 5 h period (in contrast to 48 h when the enzyme is operated in continuous batch mode). Similarly, within 5 h the immobilized β-galactosidase will hydrolyze 60% of the lactose in milk [17].

Lactose can also be used as a starting material to produce lactosucrose. This is achieved by the transfer of a β-D-fructofuranosyl unit from sucrose onto the α-anomeric position of the D-glucopyranosyl moiety of lactose. Lactosucrose is a non-digestible artificial sweetener with prebiotic bifidogenic properties [18]. This reaction can be catalyzed by a β-fructofuranosidase from *Arthrobacter* sp. K-1 using a simulated moving bed reactor [19].

A new process for the hydrolysis of lactose skimmed milk has been suggested. This involves the use of a membrane hollow-fiber reactor. Skimmed milk is circulated in the lumen of the fiber, while the β-galactosidase solution is circulated abluminally. The main problem encountered is microbial growth in the enzyme solution. However, a conversion rate of 78.11% can be achieved in a hollow-fiber reactor with a membrane area of 4.9 m^2 using a skimmed milk flow rate of 9.9 l/h, an enzyme activity of 120 U/ml, and a temperature of 23 ± 2 °C [20].

10.2.3 Baking Industry

10.2.3.1 Amylases

Fungal α-amylase from *Aspergillus oryzae* is the most widely used enzyme in baking [9]. It is used to supplement the α-amylase activity found in flour. The main effect is to reduce dough viscosity during initial starch gelatinization. In addition, limited starch degradation improves maltose production by endogeneous β-amylase, and thus yeast fermentation and carbon dioxide production. This enzyme is characterized by limited thermostability that facilitates its inactivation after initial starch gelatinization at 70–80 °C and prevents excessive starch hydrolysis.

Overall, the consequences of amylase action are increased bread volume and more homogeneous crumb structure.

Another target for α-amylase use is the increase of shelf-life of baked products through its anti-staling effect. Staling is mainly due to the retrogradation of amylopectin side-chains [21, 22]. To procure an anti-staling effect it is necessary to use a thermostable α-amylase preparation that can operate after starch gelatinization. To this end, the thermostable endo-amylase from *Bacillus amyloliquefaciens* has been used [9]. A significant improvement in the area of anti-staling was the introduction of the maltogenic amylase from *Bacillus stearothermophilus* (Novamyl®). This enzyme is an exo-amylase that shortens amylopectin side-chains and releases maltooligosaccharides [23, 24]. It reduces amylopectin retrogradation without weakening the amylose network necessary for obtaining optimal crumb structure [25].

10.2.3.2 **Xylanases**

Insoluble arabinoxylans present in flour are involved in the disruption of the stability of the gas cells in the dough, while soluble arabinoxylans have positive functional properties, particularly the maintenance of moistness in baked products, which is necessary for good shelf-life performance [24, 26]. Therefore, xylanases used in baking must be highly specific for the hydrolysis of the β-1,4 xylosidic linkages in insoluble arabinoxylans, in order to avoid degration of soluble ones. Appropriate xylanases provide an improved crumb structure, increased volume, and good dough-processing abilities [27, 28].

10.2.3.3 **Oxidases**

Gluten, the protein fraction of flour, plays a key role in the structure and stability of the envelope of gas cells produced during dough fermentation. Because disulfide bridges between gluten molecules are very important to strengthen this protein network, the use of oxidants is widespread in baking [9, 24]. They improve stability against dough stress, bread volume, and crumb structure. In order to avoid the use of undesirable chemical oxidants such as bromate and azodicarbonamide (ADA), oxidative enzymes that generate hydrogen peroxide can be used. In the presence of hydrogen peroxide, endogeneous glutathione dehydrogenase oxidizes glutathione and thus prevents the formation of disulfide bridges between gluten molecules [29]. The hydrogen peroxide-producing oxidases that are mainly used for this purpose are:

- glucose oxidase from *Aspergillus niger*, which oxidizes glucose into gluconolactone in the presence of oxygen
- hexose oxidase from *Chondrus crispus*, a carrageenan seaweed, which broadly oxidizes glucose, galactose, maltose, etc. [30]
- lipoxygenase from soybean or bean flour which oxidizes unsaturated fatty acids containing *cis-cis*-1,4-pentadiene groups and has a whitening effect on bread crumb through the reaction of resulting fatty acid hydroperoxide with carotenoids in the dough [9]

- sulfhydryl oxidase from *A. niger* [31]
- peroxidases, which oxidize a variety of compounds in the presence of hydrogen peroxide [9, 32]
- polyphenol oxidases, which oxidize diphenolic groups to quinines [9].

10.2.3.4 **Phospholipase**

The natural emulsifier used in cake production is egg yolk. This is because egg yolk contains lecithin (2.8% of egg's weight), the major constituent of which is phosphatidylcholine. Because lysophosphatidylcholine has a stronger emulsifying power than phosphatidylcholine, phospholipase A2 from *Aspergillus niger* (CakeZyme™) has been developed by DSM [33] to catalyze this regiospecific hydrolysis. This results in 20% reduction in egg use in cake recipes. The corresponding weight loss (75% less egg use) can be compensated for by adding an appropriate quantity of water. The use of phospholipase also results in increased cake volume. This is because lysolecithin complexes to the amylose fraction of starch, which may result in delayed crumb setting. Likewise, phospholipase treatment procures improved crumb structure and softness, as well as a longer shelf-life, because of increased moisture retention.

10.2.3.5 **Dextransucrase**

Sourdough contains a variety of lactic acid bacteria, among which some present a dextransucrase activity. This enzyme is a transglucosidase which catalyzes the synthesis of a homoglucan polymer, dextran (see Section 10.3.2.4), from sucrose. Dextran addition to bakery products has been proved to have a positive effect on bread volume and crumb softness. The company Puratos has isolated *Leuconostoc mesenteroides* LMGP-16878 strain from panettone sourdough and has developed a process to obtain high molecular weight dextran as a new functional ingredient for bakery products [34, 35].

10.2.4 **Beer-Making Industry**

10.2.4.1 **Malting**

One of the key features of brewing is the malting process, which is divided into three stages: steeping, germination, and kilning. The aim of malting is to promote optimal hydrolysis of the cell walls in barley kernels in order to provide a quality fermentable extract upon mashing. Therefore, in malting as well as amylases and proteinases, endogenous glucanase and pentosan-hydrolyzing activities are essential. However, due to the natural variability of barley, the addition of adjuncts (other starch-based cereals), and the process steps that lead to enzyme inactivation, it is common during mashing to reinforce glucanase and xylanase activities through the addition of exogenous enzymes in order to optimize wort extraction and achieve full β-glucan hydrolysis [36]. This is important for the final product

quality because the presence of β-glucan contributes to a phenomenon called chill haze, the industry term for the cold-induced appearance of gelatinous precipitates in beer [37].

10.2.4.2 Prevention of Chill Haze in Beer

The interaction of proteins with polyphenols during beer production results in the formation of a haze, particularly during cold storage. This interaction mainly results from hydrophobic effects [38]. One way to handle this problem is to absorb haze-active proteins onto solid supports such as silica [39], which is of interest for beers having moderate malt contents and limited shelf stabilities [40], or polyvinyl pyrrolidone (PVPP). An alternative remedy is to use proteases. Similarly, papain has been used to prevent chill haze by hydrolyzing proteins into peptides [41].

Alternatively, based on the observation that the proteins involved in this phenomenon are rich in proline [42, 43], DSM Food Specialties has developed a proline-specific protease, Brewers Clarex™, for this purpose [44]. This acidic extracellular prolyl endoprotease from *Aspergillus niger*, which belongs to the prolyl oligopeptidase family [45], was initially developed for the removal of bitter taste from protein hydrolysates [46]. Interestingly, as beer foam-active proteins are characterized by low proline content, unlike papain, treatment with this enzyme does not affect foam stability [47]. Moreover, it is noteworthy that enzyme-treated beers contain approximately 200 mg/l polyphenol, while PVPP-treated beer contains approximately 125 mg/l, which is of nutritional interest due to the antioxidant effect of polyphenols.

10.2.5 Fruit Processing

10.2.5.1 Pectinases

Enzymatic processing of fruits is accomplished using a wide variety of enzyme types. However, although the exact enzyme requirement will be determined by the fruit type and the nature of the processing step, pectinases are the major workhorses [9]. Commercial pectinases, usually produced by aspergilli [48], are an array of different enzymes that bring about the hydrolysis of pectin, a generic term for a mixture of complex polysaccharides whose structures vary according to their botanical origin (Figure 10.1) [49]. The main pectinolytic enzymes are polygalacturonases (EC 3.2.1.15). These endo-enzymes attack the main chain and split the β-1,4 bonds that link galacturonic acid residues. A wide variety of these enzymes exist, some being more or less sensitive to methylation of the substrate. Other endo-acting enzymes are α-L-arabinanases (EC 3.2.1.99) which depolymerize the arabinose-containing ramifications in the hairy regions.

Exo-acting enzymes include exopolygalacturonases (EC 3.2.1.67), L-arabinofuranosidases (EC 3.2.1.55), feruloyl esterases (EC 3.2.1.73), and pectin methylesterases (EC 3.2.1.11). These latter remove methoxyl groups on pectin and

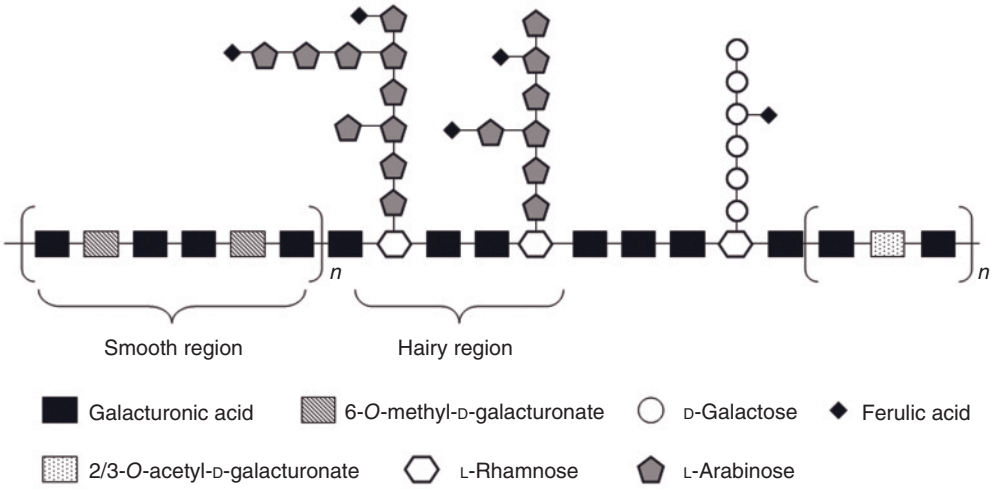

Figure 10.1 Schematic representation of pectin structures. Both hairy and smooth pectin structures are shown.

generate methanol acidic pectin and methanol. The enzymes listed so far are all hydrolases, but pectin lyases (EC 4.2.2.10) are also commonly found in fungal preparations. These endo-enzymes depolymerize the main galacturonan chain through a β-elimination reaction that produces oligo-uronides.

10.2.5.2 Enzymatic Maceration of Fruit

Enzymatic maceration of fruits is a well-established technology in fruit processing. In this process, degradation of plant cell walls by a combination of exogenous polygalacturonases, pectin methylesterases, pectin lyases, and cellulases maximizes juice extraction. Likewise, this treatment lowers the viscosity of the pulp and reduces pomace (the solid waste obtained after pressing) volume. For certain fruits that contain significant amounts of starch (e.g., apples and pears), other enzyme activities such as amylase are essential to obtain satisfying results and to reduce haze, which is caused by the presence of polysaccharides [50].

Unlike clarified apple juice, orange juice is sold as a cloudy beverage. Therefore, modern processing and juice-conditioning steps should promote and stabilize cloudiness. At the time of pressing, orange pectins are less methylated than apple pectins, because oranges naturally contain a much higher level of endogenous pectin methylesterase. Unfortunately, in its acidic form pectin will interact with calcium ions to form calcium pectate, which leads to the precipitation of the cloud particles that are formed from pectin and proteins. To avoid this, juice can be heated to thermally inactivate the pectin methylesterase. However, this approach leads to undesirable modifications to juice quality. Alternatively, the juice can be

frozen to cold inactivate the enzyme. However, transportation of frozen juice leads to inevitably higher costs [50].

10.2.5.3 Grapes and the Wine Industry

Grapes constitute the world's top fruit production (60 million tonnes per year) and most grapes are used for wine production. Although wine making is one of the oldest biotechnologies, it is only recently that a better understanding of the biochemical processes involved has fueled the development of enzyme technology. Among the targets for modification by exogenous enzyme, particular focus has been put on carbohydrates and glycosylated secondary metabolites.

Exo-acting carbohydrases (glycosidases) that act on glycosylated secondary metabolites can be used as aroma enhancers in wine making. These enzymes, generally α-L-arabinofuranosidases, β-D-glucosidases or α-L-rhamnosidases, sequentially hydrolyze the glycone moiety, which is often a diglycoside, and ultimately release the volatile aglycone moiety [51, 52]. Commercial examples of such enzymes are Novarom Blanc (Novozymes A/S, Bagsvaerd), a pectinase preparation that contains significant glycosidase side-activities and Lallzyme beta (Lallemand Inc, Montreal), also a blend of pectinases, that contains α-L-arabinofuranosidase, β-D-glucosidase, β-D-apiosidase, and α-L-rhamnosidase.

An alternative to the addition of exogenous glycosidases during wine preparation is the use of GMO strains. Likewise, yeast strains for wine making have been genetically modified using genes encoding various glycosidases. Although these strains have been shown to produce wines with superior aromatic properties [53], none of them have been put to commercial use so far.

10.3 Food and Feed Applications

10.3.1 Probiotics

The United Nations Food and Agriculture Organization and the World Health Organization define probiotics as "live organisms, which, when administered in adequate amounts, confer a health benefit on the host" [54]. The human body contains about ten times more microbial cells than human ones. Following birth, the human gut is progressively colonized by microbial strains, starting with the mother's vaginal flora. Our knowledge and understanding of the role of bacteria in the digestive tract is still very limited, even if molecular biology approaches such as metagenomic studies provide a deeper insight into this area. It appears that the gut microbiota is relatively stable in an individual, but significantly different from one individual to another.

More and more published scientific papers have discussed the role of the gut microbiota in human health problems such as obesity [55] and metabolic disorders (diabetes, cardiovascular diseases) [56]. In addition, microbial stimulation plays a

key role in the development of the human immune system, as microorganisms interact with both the innate and the acquired immune systems [57].

Probiotics, mainly lactobacilli and bifidobacteria originating from the intestinal content of healthy humans, have been selected for their specific properties in extensive screening procedures. They are available to the consumer as powders or tablets, but most commonly as milk-based products [58]. Among the beneficial outcomes for human beings, as well as for the animal host, the following can be cited [57]:

- anti-infectious properties
- immunomodulatory effects
- enhanced barrier functions
- metabolic effects
- alterations of intestinal mobility or function.

Examples of target disorders are:

- diarrhea, including that caused by rotavirus in children [59, 60] or *Clostridium difficile* in elderly patients [61] or other causes in infants [62, 63]
- pouchitis [64, 65]
- irritable bowel syndrome [66]
- bladder cancer using *Lactobacillus casei* [67]
- urogenital infections [67]
- *Clostridium difficile* infection using *Saccharomyces boulardii* [68]
- atopic eczema using *Lactobacillus rhamnosus* and *Lactobacillus reuteri* [69].

In the case of animal husbandry, the use of probiotics has recently been reinforced by the European Union's decision to prohibit the use of antibiotics as livestock growth stimulators. Resistance to salmonella colonization of newly hatched chicks resulting from the administration of gut contents from healthy adult birds was demonstrated as early as 1973 [70]. This concept is known as competitive exclusion.

Because of the broad diversity of probiotic products present on the market, there is still an important need for carefully designed, randomized, and placebo-controlled clinical trials. It is also necessary for each probiotic strain to decipher the specific molecular mechanisms involved, to strengthen the corresponding claims. The main companies involved in the probiotics market are BioGaia Biologics, Chr. Hansen, ConAgra Functional Foods, Danisco, Danone, Institut Rosell (Lallemand), Lifeway Foods, Natren, Nestlé, Seven Seas, Stonyfield Farm, and Yakult.

Intestinal lactobacilli and bifidobacteria have been subjected to genetic modifications. This development has been driven not only by the desire to better understand the role of these two groups of bacteria in the intestine, but also to enhance their existing probiotic characteristics (immunostimulation, vitamin synthesis, colonization determinants) and to develop novel properties (oral vaccine development, production of antimicrobials, digestive enzymes, antibodies, cytokines) [71, 72]. However, *in vivo* gene transfer and public acceptance of GMOs is a key concern at this level.

10.3.2 Prebiotics

An alternative or complementary approach to the use of probiotics is to directly feed the key microbial components of the intestinal flora. For this purpose, non-digestible carbohydrates have been developed. Initially designed as low-calory food ingredients ("soluble fibers"), such non-digestible oligosaccharides have been shown to be partially and specifically metabolized by components of the intestinal flora. This resulted in the concept of "prebiotics," originally developed in Japan and defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health" [73].

Prebiotics share the property of partially or totally resisting attack by the digestive enzymes of humans and animals. Likewise, they are not directly metabolized by the host and can reach the colon, where they can act as specific substrates for the microbial flora. They can also interact with the carbohydrate receptors present at the surface of either microbial or epithelial cells, affecting cell adhesion and immunomodulation [74]. Non-digestible oligosaccharides are obtained by different routes:

- extraction from plant sources: fructooligosaccharides, α-galactooligosacharides
- controlled enzymatic hydrolysis of plant polysaccharides: fructooligosaccharides, xylooligosaccharides
- enzymatic synthesis: fructooligosaccharides, α-glucooligosaccharides, β-glucooligosaccharides, β-galactooligosaccharides.

10.3.2.1 Inulin

Inulin is a linear fructose polymer containing β-2,1 linkages and a sucrose unit at the non-reducing end. It is used by several plants for energy storage, particularly chicory (*Cichorium intybus*) and Jerusalem artichoke (*Helianthus tuberosus*). It can be hydrolyzed totally to produce fructose, by using an exo-inulinase alone or in combination with an endo-inulinase, or to produce fructooligosaccharides by using an endo-inulinase alone [75–78]. The key interest of this fructan is that it contains osidic linkages which cannot be hydrolyzed by human and animal digestive enzymes. Inulin and the inulin oligosaccharides obtained by controlled enzymatic hydrolysis can thus be regarded as "prebiotics" [79, 80]: such non-digestible carbohydrates are specifically metabolized by components of the intestinal microflora, bifidobacteria, resulting in positive health effects [81].

The concept of "functional foods" has been developed during the past 15 years, with the understanding of the importance of the intestinal flora in digestive problems. The positive effect of such dietary fibers has been demonstrated on the stimulation of the intestinal microflora [82], mineral absorption [83], lipid metabolism, colonic carcinogenesis, and immune functions of the digestive tract.

Prebiotic inulin and derived fructoligosaccharides are marketed by various companies, particularly Orafti, a Südzucker division, under the trade name of Beneo®.

Similar fructooligosaccharides are also present in various plants [84], with particularly high levels in onions [85] and asparagus [86].

10.3.2.2 Fructooligosaccharides

Fructooligosaccharides containing β-D-fructofuranose units linked by 2,1-osidic linkages are also obtained by enzymatic synthesis from sucrose, using a fructosyl transferase from *Aspergillus niger* or *Aureobasidium pullulans* [87, 88]:

$$\underset{\text{sucrose}}{n\ \text{Glc-Fru}} \xrightarrow{\text{fructosyltransferase}} \text{Glc-(Fru)}_n + (n-1)\ \text{Glc}$$

A series of fructooligosaccharides of increasing degree of polymerization are thus obtained (Figure 10.2). These products are marketed by Beghin-Meiji, a joint venture between Tereos and Meiji Seika, under the trade names Actilight® and Profeed®.

10.3.2.3 Galactooligosaccharides

α-Galactooligosaccharides are produced by extraction from plant sources, particularly soybean [89]. They consist of a series of α-1,6-galactosyl derivatives of sucrose (Figure 10.3): raffinose, stachyose, and verbascose, respectively, containing 1, 2

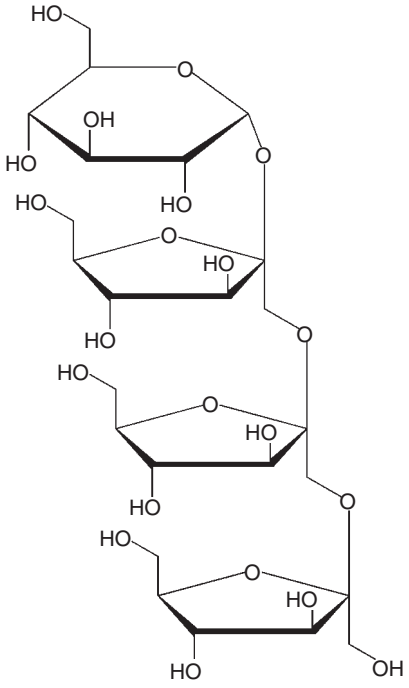

Figure 10.2 Structure of nystose, an α-1,2-linked fructofuranosyl tetrasaccharide with a terminal glucosyl moiety. From [74].

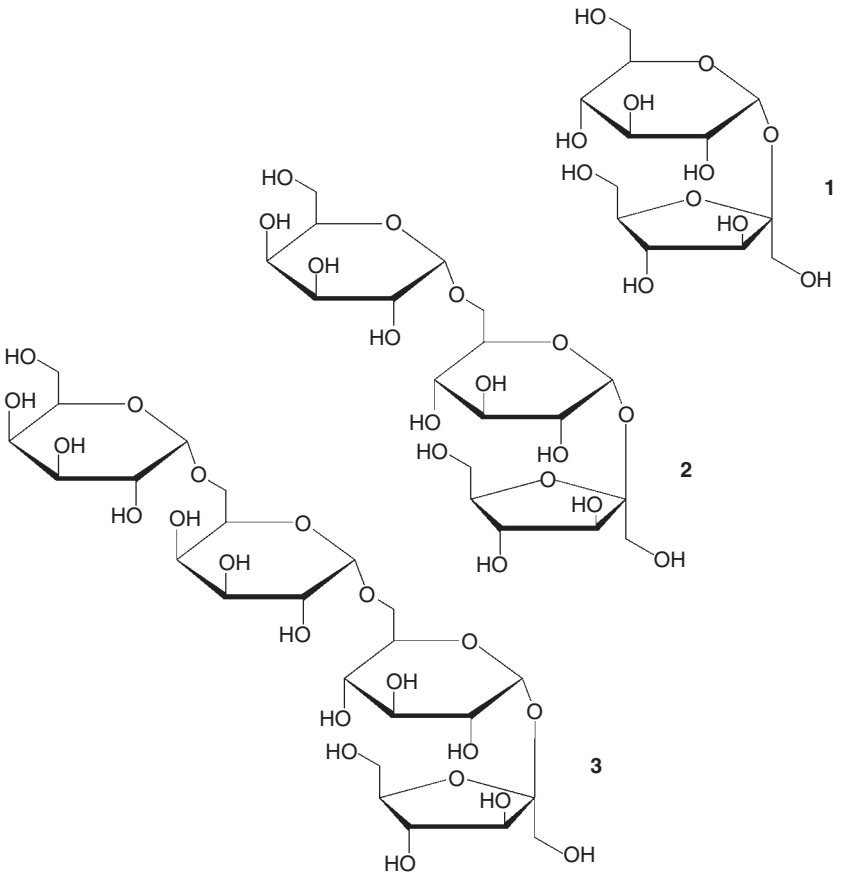

Figure 10.3 Structure of the trisaccharide raffinose (2) and the tetrasaccharide stachyose (3) α-1,6-linked derivatives of the disaccharide sucrose (1). From [74].

and 3 α-D-galactosyl residues. These α-galactooligosaccharides are used as prebiotics in Japan. They are also responsible for flatulence problems following consumption of beans.

β-Galactooligosaccharides are obtained from the galactosyltransferase activity of a β-galactosidase, from *Aspergillus oryzae* for example [90], onto lactose, contained in whey, a dairy by-product, according to the following reaction scheme:

$$\underset{\text{lactose}}{\beta\text{-D-Gal-1,4-D-Glc}} \xrightarrow{\beta\text{-galactosidase}} (\beta\text{-D-Gal})_n\text{-D-Glc} + (n-1)\ \text{D-Glc}$$

These products contain β-1,6-D-galactosyl linkages, in addition to the β-1,4 linkage of lactose, and are known as transgalactooligosaccharides (TOS).

The composition of the product developed by Borculo Domo Ingredients, Lactifit®, contains oligosaccharides up to a degree of polymerization (DP) of 8

Table 10.2 Composition of Lactifit® from [91].

Component	% w/w dry matter
Transgalactooligosaccharides	60%
DP2	33%
DP3	39%
DP4	18%
DP5	7%
DP6–8	3%
Lactose	20%
Glucose	19%
Galactose	1%

(Table 10.2) [91]. They are not digested and they selectively stimulate the growth of bifidobacteria and decrease the toxicity of the colon contents [92].

10.3.2.4 Glucooligosaccharides

Isomaltooligosaccharides are D-glucopyranosyl oligomers containing mainly α-1,6 linkages [93]. They are produced from starch hydrolysates by treatment with an α-transglucosidase from *Aspergillus* sp., as α-1,6 linkages are thermodynamically more stable than initial α-1,4 ones [94]. In this case, maltose and maltodextrins present in the starch hydrolysate act as both D-glucopyranosyl unit donor and acceptor. Isomaltooligosaccharides are one of the main prebiotic products on the Japanese market, produced, for example, by Hayashibara [95].

Isomaltooligosaccharides can also be obtained by the combined action of glucoamylase and pullulanase on starch hydrolysates. Such a process has been developed by the Japanese company Showa Sangyo [96]. Another production technique is to operate a neopullulanase enzyme from *Bacillus stearothermophilus* modified by site-directed mutagenesis to catalyze a transglucosylation reaction [97].

An alternative route to glucooligosaccharide synthesis is to use the transglucosylation reaction catalyzed by glucansucrases, using sucrose as D-glucosyl residue donor:

$$\underset{\text{sucrose}}{n\ \text{Glc-Fru}} \xrightarrow{\text{Glucansucrase}} (\text{Glc})_n + (n-1)\ \text{Glc}$$

In the presence of sucrose alone, high molecular weight glucan polysaccharides are obtained with a wide variety of structures [98], according to glucansucrase regioselectivity [99]:

- dextran, containing more than 50% α-1,6 linkages: dextransucrase
- mutan, containing more than 50% α-1,3 linkages: mutansucrase
- alternan, containing alternating α-1,3 and α-1,6 linkages: alternansucrase
- amylose, containing 100% α-1,4 linkages: amylosucrase.

When an efficient acceptor is added to the sucrose reaction medium, like maltose or isomaltose for example, low molecular weight oligosaccharides are synthesized [100].

Cargill is using alternansucrase to produce from sucrose and maltose a sweetening prebiotic mixture, Xtend™ Sucromalt [101] containing both residual fructose and alternanoligosaccharides. Alternansucrase can be produced from *Leuconostoc mesenteroides* NRRL B-1355, from which the corresponding encoding gene was isolated [102, 103]. *Penicillium* spp. is also a potential source for this enzyme [104]. The efficiency of the corresponding oligosaccharides for controlling enterical bacterial pathogens has been demonstrated [105]. Interestingly, fully active truncated forms of alternansucrase, deleted in the C-terminal domain ("glucan-binding domain") have been efficiently designed [106].

The dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299 catalyzes the synthesis of dextran polymers containing 27–35% α-1,2 branch linkages, and is mostly insoluble and associated to the cell outer layers [94, 98]. This enzyme maintains its specificity when maltose is used as acceptor in the presence of sucrose, and two main families of products are obtained [107, 108]:

- containing only α-1,6 linkages in addition to the α-1,4 linkage of maltose (which is located at the reducing end)
- containing 1 to 4 α-1,6 linkages and an α-1,2 linkage at the non-reducing end (Figure 10.4).

The presence of such α-1,2 linkages results in a very high resistance to the attack of human and animal digestive enzymes [109]. They are not metabolized by germ-free rats [110] and they are specifically metabolized by bifidobacteria, lactobacilli, and mainly bacteroides [74]. They have also been demonstrated to induce a broad range of glycolytic enzymes, without any significantly increased side-production of gases, and thus without any detrimental effect [111].

Field trials with calves have demonstrated that the administration of 0.15% of such glucooligosaccharides in the feed results in a 20% reduction of veterinary

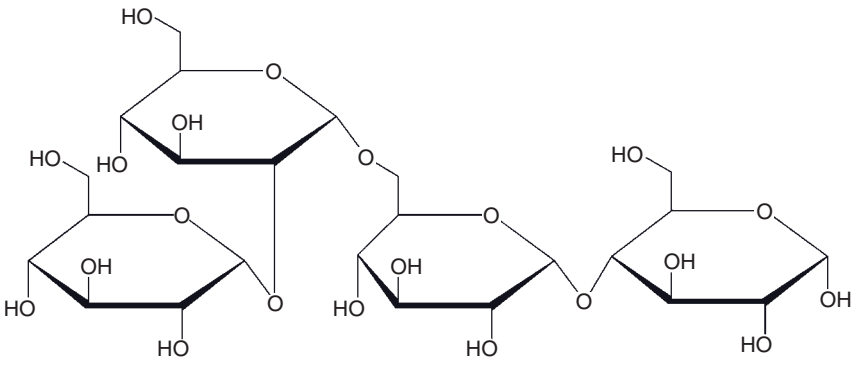

Figure 10.4 Structure of an α-1,2-linked glucospyranosyl tetrasaccharide (GOS). From [74].

costs [74]. Unfortunately, this product has never been tested in human trials and thus only approximately 60 tonnes per year are produced, essentially for dermocosmetic applications [112].

Besides their prebiotic properties, glucooligosaccharides containing α-1,2 linkages have been proved to prevent the development of type 2 diabetes in over-fed mice [113]. This very interesting property is currently the focus of intense study. Surprisingly, unlike other glucansucrases in the glycoside hydrolase family 70, the α-1,2 bond-forming dextransucrase from *L. mesenteroides* NRRL B-1299 possesses two catalytic sites, instead one [114]. In common with other family 70 members, this enzyme is characterized by a catalytic site located in the N-terminal part of the enzyme that catalyzes the formation of α-1,6 linkages (dextran synthesis). However, a second catalytic site, located in the C-terminal region, is highly specific for the synthesis of α-1,2 linkages. This activity leads to the formation of α-1,2-ramified dextran.

Using enzyme engineering techniques, the native gene encoding the dextransucrase from *L. mesenteroides* NRRL B-1299 has been N-terminally truncated. This has allowed the design of a new enzyme that only has one catalytic domain that displays α-1,2 bond-forming activity. The deployment of this enzyme on dextran allows the tailored grafting of ramifications that lead to the formation of novel dextran derivatives [115].

10.3.2.5 **Resistant Starch**

For many years, it was generally considered that starch was fully digested into glucose and thus metabolized. However, more recent studies have demonstrated that a significant fraction of starch is not attacked by digestive enzymes [116, 117]. This "resistant starch" [118] reaches the large intestine where it is metabolized by the bacterial flora. In this respect, resistant starch can be regarded as an excellent prebiotic compound [119–121]. The term "resistant starch" originally described the residual intact starch fraction that is obtained after exhaustive *in vitro* α-amylase and pullulanase treatment [122]. However, a more accurate definition is the fraction of dietary starch which escapes digestion in the small intestine, corresponding to the difference between total starch and the sum of rapidly digestible starch and slowly digestible starch [122].

Four types of resistant starch (RS) can be distinguished [118]:

- **RS_1**: physically inaccessible starch such as that found in whole unprocessed grains and seeds, legumes. It is heat stable and used as an ingredient in conventional foods.
- **RS_2**: starch in a granular form (raw starch granules), such as the starch in uncooked potato or green banana. It is slowly and incompletely digested in the small intestine.
- **RS_3**: mainly retrograded amylose, formed after cooking and cooling, such as in bread, cornflakes, and cooked and chilled potatoes. It is the most resistant starch fraction.

- **RS_4:** obtained by chemical modification of starch, such as distarch phosphate ester.

Processing techniques greatly influence resistant starch formation. Highly processed cereal flours contain much lower levels of resistant starch (approximately 1.5% w/w) than beans, for example (approximately 60% w/w). Techniques such as extrusion followed by cooling [123], steam cooking of legumes [124], autoclaving of wheat starch [125], parboiling of rice [126], baking [127], and pyroconversion of Lima bean (*Phaseolus lunatus*) [128] increase the resistant starch content of foodstuffs.

Enzymatic treatments can also be applied to prepare resistant starch. The action of α-amylase on crude starch allows the degradation of the non-resistant starch fraction and thus results in a starch preparation that is enriched in resistant starch [129]. Similarly, the action of a thermostable α-amylase on isolated pea starch produces a fraction containing 70% w/w resistant starch [130]. Another possibility is to use a debranching enzyme to hydrolyze α-1,6 linkages in amylopectin. This treatment generates amylase, such that upon heating granular resistant starch is formed retrogradation reaction [131]. A similar approach involves the action of a pullulanase on low amylose starches, such as rice starch and rice flour [132].

Ultrapure amylose chains can be directly synthesized from sucrose using a transglucosidase, such as the amylosucrase from *Neisseria polysaccharea* [133, 134]. This enzyme catalyzes the following reaction:

$$\underset{\text{sucrose}}{n\ \text{Glc-Fru}} \xrightarrow{\text{amylosucrase}} \underset{\text{amylose}}{(\alpha\text{-1,4-D-Glc})_{n-1}\text{-D-Glc}} + n\ \text{D-Fru}$$

Amylosucrase belongs to family 13 of the glycoside hydrolase classification system [135], which is otherwise known as the "α-amylase family". Its three-dimensional structure has been determined and so far this is the only available structure of a glucansucrase. The mechanism of action of amylosucrase has been elucidated at the molecular level [136] and the products of elongation of α-glucans [137] and the amylose products [138] have been characterized.

The company Südzucker is presently developing resistant starch production using amylosucrase. The product brand name is NEO-amylose® [139].

To acquire the beneficial health benefits of resistant starch, the recommended daily consumption is 20 g. Legumes such as black beans contain a high resistant starch content (63% w/w). It has been demonstrated that resistant starch consumption promotes lipid oxidation. The replacement of 5.4% of total dietary carbohydrate with resistant starch increased post-prandial lipid oxidation in a sample of 12 study subjects by 23%, and therefore could decrease fat accumulation in the long term [140]. However, this study also revealed that there may be a maximal effect of resistant starch addition to the diet and that the addition over this threshold confers no metabolic benefit or change from a 0% resistant starch meal.

The production of a wide variety of β-glycooligosaccharides has been described [141]. Most of them are not competitive from a commercial point of view, even if their potential interest was demonstrated at laboratory scale. The action of a β-

glucosidase on concentrated glucose solutions (70%, w/w) results in the synthesis of β-glucooligosaccharides [142], mainly di- and trisaccharides containing β-1,6 (gentiobiose and gentiotriose) and β-1,4 (cellobiose and cellotriose) linkages.

Enzymatic or thermochemical hydrolysis of plant xylans can be used to produce β-xylooligosaccharides [143, 144], which are also naturally found in bamboo shoots [145]. However, when endoxylanases are used, the extent of hydrolysis is highly influenced by the presence of arabinofuranosyl side-chains in the xylan polymer [146]. Presently, the commercial distribution of xylooligosaccharides (XOS) as food additives is limited to Asia, where Suntory Ltd sells several XOS products. Research is being conducted, however, on the prebiotic effects of XOS and several studies have shown that they positively influence the growth of bifidobacteria and possibly lactobacilli [147, 148].

10.4 Feed Applications

The use of exogenous enzymes in animal husbandry to improve the nutritional value of feeds is widespread and has now been practiced since the 1980s. A range of both carbohydrate-hydrolyzing enzymes (carbohydrases) and phytases are currently used and approximately 40 enzyme products have obtained definitive European Union approval. The overall feed enzyme market is estimated to be worth €270 million.

10.4.1 Phytate Hydrolysis

Phytic acid or myo-inositol hexakisphosphate (IP6) is a widespread heat and acid-stable component of most plant-derived feed and foodstuffs, including cereal grains, nuts, and seeds (Figure 10.1). IP6 is a strong chelator of various cations including Mg^{2+}, Ca^{2+}, Fe^{2+}, and Zn^{2+}, which are important nutritional elements in human and animal diets. Because of this, and the fact that IP6 can form complexes with proteins and metals, IP6 is often considered to be an antinutritional agent for both animals and human beings. However, IP6 is also an abundant source of phosphate, a crucial component of many important biological molecules and therefore an essential compound for all animals, including human beings. Unfortunately, while ruminants can mobilize the phosphate stored in IP6 through microbial activity in their digestive tracts, poultry, pigs, and humans (among others) only make very poor use of it because they lack the appropriate enzymes. There are two major consequences of this relative deficiency. First, the phosphate stored in IP6 is unavailable to poultry and pigs, which means that, in the case of animal husbandry, it is desirable to supplement diets with an alternative phosphate source. Second, because IP6 remains undigested, intensive rearing of pigs and poultry is an important source of pollution, since the associated phosphate-rich waste leads to water pollution and eutrophication.

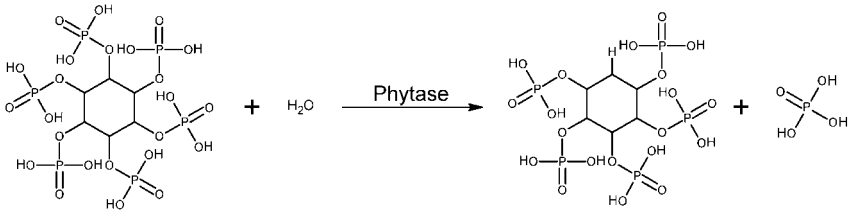

Figure 10.5 The generic reaction catalyzed by phytases.

Accounting for 60–70% of the enzyme market, phytases constitute the single most important feed enzyme category. Phytases or myo-inositol hexakisphosphate phosphohydrolases are specialized phosphatases that catalyze the sequential dephosphorylation of IP6. Phytases have been isolated from a variety of sources including plants, animals, and, in particular, microorganisms [149–154]. The IUBMB enzyme classification includes three groups of phytases (EC 3.1.3.8, 26, and 72), but in the literature enzymes EC 3.1.3.8 and 26 are generally cited as the main phytase groups; EC 3.1.3.2 (a wide specificity acid phosphatase) has also been given the name phytase.

The first phytase to be commercialized was a histidine acid phosphatase from *Aspergillus niger* NRRL3135, but more recent arrivals on the market are usually of bacterial origin. Currently the phytase market has an approximate value of €150 million [155] and is dominated by three principal enzyme preparations: Natuphos (BASF, Ludwigshafen) a 3-phytase (EC 3.2.1.8) produced by *Aspergillus niger* CBS 114.94, Phyzyme (Danisco Animal Nutrition) a recombinant 6-phytase (EC 3.2.1.26) originally from *Escherichia coli*, and Ronozyme (DSM, Basel), a recombinant 6-phytase (EC 3.2.1.26) originally from *Peniophora lycii* [156].

Incorporated into poultry feed, phytases lead to decreased total phosphate content in excreta, increased egg production and egg mass, and increased bone mineral content and animal body weight. Likewise, by decreasing the need for supplemental phosphate, exogenous incorporation of phytase into pig diets leads to lower total and water-soluble phosphate concentration in excreta and improves bone strength.

In terms of biotechnology, phytases have been the object of much research, mainly aimed at the production of thermotolerant enzymes. Two major approaches have been adopted: screening biodiversity for naturally thermostable phytases [157–161] and protein engineering [162–164].

Advances in biotechnology have also given rise to attempts to engineer animals and plants [165] to produce phytase. Likewise, Golovan *et al*. described the production of an *E. coli* phytase in the salivary glands of pigs [166]. Although this study provided an elegant demonstration of the effectiveness of this strategy to reduce both inorganic phosphate requirements and fecal phosphate content, it is unclear at present whether the ethical issues surrounding this approach will allow future implementation [167].

10.4.2 Carbohydrate Hydrolysis

Non-starch polysaccharides (NSPs) are a heterogeneous group of polymers that, taken together, constitute a highly abundant source of nutritional fiber. In animal feed, three main types of NSP are of particular importance: (i) β-1,3-1,4-glucans, which are frequent in both the envelope and endosperm of grains such as barley, (ii) arabinoxylans (AXs), also widespread in cereals, and (iii) mannans (especially galactomannans) that are widespread in the seeds of leguminous plants and are important component of guar and soya meal [168].

When used to treat animal feed, the primary effect of carbohydrases is to reduce the viscosity of digesta [169]. However, the underlying consequence of this action is a reduction in the incidence of wet litter and an increase in nutrient digestibility [170, 171]. In broiler chickens enzyme treatment of a wheat-based diet increases the availability of metabolizable energy [172, 173] and, in the case of laying hens, improves laying performance [174].

Within the context of the European ban of antibiotic growth promoters in animal feed, it is important to note that carbohydrases also exert beneficial effects at the interfaces between the diet, the intestinal microflora, and enteropathogens [175].

The use of exogenous carbohydrate-hydrolyzing enzymes, or carbohydrases, mainly concerns monogastric animals such as poultry, and, to a lesser extent, pigs. Currently, several enzyme types are employed:

- **Endo β-1,4 xylanases, more commonly known as xylanases (EC 3.2.1.8):** These depolymerize arabinoxylans and are the major active ingedients of commercial preparations such as Natugrain (BASF, Ludwigshafen, Germany) that are used for the improvement of turkey diets.
- **1,4-β-d-Glucan 4-glucanohydrolases (EC 3.2.1.4), 1,3-β-d-glucan 3-glucanohydrolases (EC 3.2.1.39) and, in particular, 1,3-1,4-β-glucanases (EC 3.2.1.73) or lichenases:** These are the major glucan-hydrolyzing enzymes that act on 1,3-1,4-β-glucans such as those found in cereal grains, especially barley (*Hordeum vulgare*) and oats (*Avena sativa*). In feed applications, glucanases (associated with xylanases) are the active ingredients of products such as Endofeed and Endofeed W (GNC Bioferm Inc., Saskatoon, Canada) that are used to improve diets based on oats/barley and wheat/triticale/rye respectively.
- **β-D-Mannanases (mannan endo-1,4-β-mannosidases, EC 3.2.1.78):** The commercial use of these enzymes is restricted to the animal feed sector, where preparations such as Hemicell (ChemGen Corp) are used to raise the metabolizable energy potential of soya and guar meals [176–178].

Many feed enzyme products are in fact a combination of the above enzyme types. One illustration of this is Rovabio™ Excel (Adisseo, Anthony, France) a cocktail from *Penicillium funiculosum* culture (Rovabio™ Excel), which is a mixture of endo-1,4-β-xylanases, endo-1,3(4)-β-glucanases, pectinases, and mannanases. *In vitro*

studies using a gastrointestinal model (TNO dynamic, multicompartmental system of the stomach and small intestine: TIM-1) have shown that this preparation is particularly efficient for the solubilization of insoluble wheat arabinoxylans [179].

With regard to the improvement of feed carbohydrases, the main criteria have been reviewed [170, 175, 180, 181]. These include the development of more sensitive and accurate analytical assays, better substrate identification and characterization, further development of thermotolerant enzymes (to limit denaturation during pellet production) and greater understanding of the final products of enzymatic hydrolysis in order to understand and document their beneficial effects on the intestinal microflora.

10.4.3 Amino Acid Production

The biotechnological production of amino acids targets the food (32% in 2004), feed (56%), and fine chemicals (12%) markets [182] and corresponds to a total value of US$4.5 billion in 2004. Amino acids are the building blocks of proteins. As their L-form is needed in most cases, microbial production is generally the best choice. The only exception is methionine, which is chemically synthesized as a D,L racemic mixture from acrolein, hydrocyanic acid, methyl mercaptan, and ammonia. In fact, humans and animals possess an oxidase/transaminase enzymatic system able to transform D-Met into L-Met. The amino acid balance is vital for both human and animal nutrition, as nine of them are essential (i.e., they cannot be synthesized in the metabolism of higher animals): lysine, threonine, methionine, tryptophan, leucine, isoleucine, valine, histidine, and phenylalanine [183]. Three amino acids are of interest for food applications: L-glutamic acid, L-aspartic acid, and L-phenylalanine. L-Glutamic acid is the most produced amino acid (1.5 million tons per year). Its manufacture, in the form of monosodium glutamate (MSG), is achieved using *Corynebacterium glutamicum*. The resultant MSG is used as a flavor enhancer [184]. Metabolic engineering of glutamate biosynthesis by *Clostridium glutamicum* has been investigated [185] and the genome of this microorganism of outstanding industrial interest has been fully sequenced [186]. L-Aspartic acid and L-phenylalanine are used for the synthesis of the intense sweetener Aspartame (L-aspartyl-L-phenylalanyl methyl ester). L-Aspartic acid is produced using the enzyme aspartase, which catalyzes the addition of ammonia to fumaric acid [187], while L-phenylalanine is obtained from cultures of *E. coli*.

Aspartame, which is presently produced at the scale of about 15 000 tons per year, can be obtained by direct enzymatic coupling of *N*-formyl protected aspartic acid with L-phenylalanine methyl ester catalyzed with thermolysin in a both regio- and stereospecific reaction [188].

With regard to animal feed, the principal amino acids used are L-lysine, D,L-methionine, L-threonine, and L-tryptophan. Production of L-lysine, the first limiting amino acid for pig breeding and the second, after methionine, for poultry, amounted to 850 000 tons in 2005 [182] using *Corynebacterium glutamicum* [189]. As already mentioned, this strain has undergone metabolic engineering in order

to procure improvements in performance [190]. L-Threonine was the first amino acid to be produced using a recombinant *Escherichia coli* strain [191]. The present production is level is approximately 80 000 tons per year. L-Tryptophan is also produced to 10 000 tons per year using recombinant *E. coli* and *C. glutamicum* strains [192].

For other amino acids and amino acid analogs, enzymatic reactions are often employed [193]. Pioneering work in this area was done by I. Chibata at Tanabe Seiyaku, Japan, using L- or D-acylase for the transformation of a D,L-*N*-acetyl amino acid into L- or D-amino acid respectively, with parallel racemization of the unmodified isomer [194]. Degussa operates the acylase from *Aspergillus oryzae* to produce several hundreds of tonnes of L-methionine and L-valine. The enzyme is used in continuous membrane reactor in order to minimize enzyme consumption [195].

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11
Industrial Biotechnology in the Paper and Pulp Sector

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11.1
Introduction

The modern forest industry is striving to combine sustainability with productivity and profitability. Presently, one of the aims is to increase the value of fiber products through research and development, in order to create new products and to increase applications and new fields of use for the raw materials. The general challenges of the industry are related to the decline of the traditional forest industry and paper-making businesses, calling for new growth opportunities and higher return of investments. Since the 1980s, pressure from environmental impacts because of the need to improve process performances and decrease discharges to the environment has been directed towards improving the quality and availability of raw materials, cost of energy, and value-adding production chains. The present chains include the fiber and the bioenergy chains, to be complemented in future with new ones, such as the chemical and biofuels chains.

Research on biotechnology in the pulp and paper industry has been active for many decades. During the last 20 years, basic discoveries in the field include initial reports on lignin-degrading enzymes, the application of xylanases in bleaching, lipases for pitch removal, enzymatic deinking, modification of fiber surfaces, and the combination of laccase with mediators for lignin removal. The development of microbial treatments of wood chips for biopulping purposes or for the management of pitch problems are currently interesting options for improving the raw material quality. In more recent years, the field has also used biotechnology to modify the structure of wood and other plant materials, for example, by altering the structure of lignin. Genetic engineering has opened up a wide range of possibilities to improve the growth and properties of the forest raw materials, both directly by introducing new genes or by enhancing the understanding of the plant functions through genomic research combined with conventional breeding. Fast-growing trees with, for example, lower lignin content or altered lignin structural properties, could provide significant practical benefits. The removal of lignin from the wood cell walls is the most capital intensive and environmentally problematic

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step in wood processing for pulp and paper. Thus, reduction of lignin content in trees could provide both economic and environmental benefits.

Any increase of efficiency that allows production of more wood and wood products from less land would help to conserve natural forests and reduce the environmental impact of processing wood into pulp and paper products. There is still a long way, however, to the practical exploitation of these improvements. The future exploitation of genetically modified forest trees will depend on the answers given to the scientific and ethical questions, as well as on the dialog and consensus reached between industry and the public. However, basic understanding on the biosynthetic mechanisms of wood components and fibers will help to improve fiber quality even by traditional breeding technologies already used in forestry.

There have been high expectations for the emergence of new, cutting-edge technologies based on biotechnology in the pulp and paper industry. The implementation of economically and technically viable biotechnical stages or treatments to mill-scale operation has, however, proved to be difficult. Biotechnology applications competing with mechanical and/or chemical operations must overtake their performance and result in economic benefits without changing the product quality in order to be accepted. It seems that the most likely future applications of biotechnical methods will be found in the fields of specialty products, targeted modification of the fibers and controlling the safety of products.

Successful introduction of enzymes into the pulp and paper processes requires that at least the following criteria should be fulfilled:

- clear economical benefits should be obtainable from the enzyme stage
- the quality and processing of fiber material should be maintained or improved
- no unwanted changes in the ability of the process to run should occur
- a suitable enzyme preparation should be available in large quantities and at a reasonable price for mill-scale trials and thereafter rapidly for industrial use.

The use of different enzymes in fiber modification or fiber engineering, in addition to their use as process aids, is an interesting and potential field of application for both chemical and mechanical pulps (Figure 11.1). By the targeted modification of fiber surface by enzymatic or combined enzymatic and chemical treatments, improved fiber properties or completely new fiber characteristics can be created. Fiber engineering could be used both to improve the paper- and board-manufacturing properties of pulp fibers and for modification of fibers suitable for non-paper applications.

Non-renewable energy sources predominantly relied upon today will not be available in the future in the quantities currently used. Thus, it is necessary to consider expanding the use of plant biomass, and in particular abundantly available forest raw materials. The biorefinery concept is analogous to the petroleum refinery concept whereby a single feedstock is fractionated and converted into a multitude of commodity products. Forest raw materials have significant potential to reduce greenhouse gas emissions by conversion of forest materials in biorefineries into liquid fuels, chemicals, and other products. Industrial biotechnology will also play a vital role in these future applications (Figure 11.1).

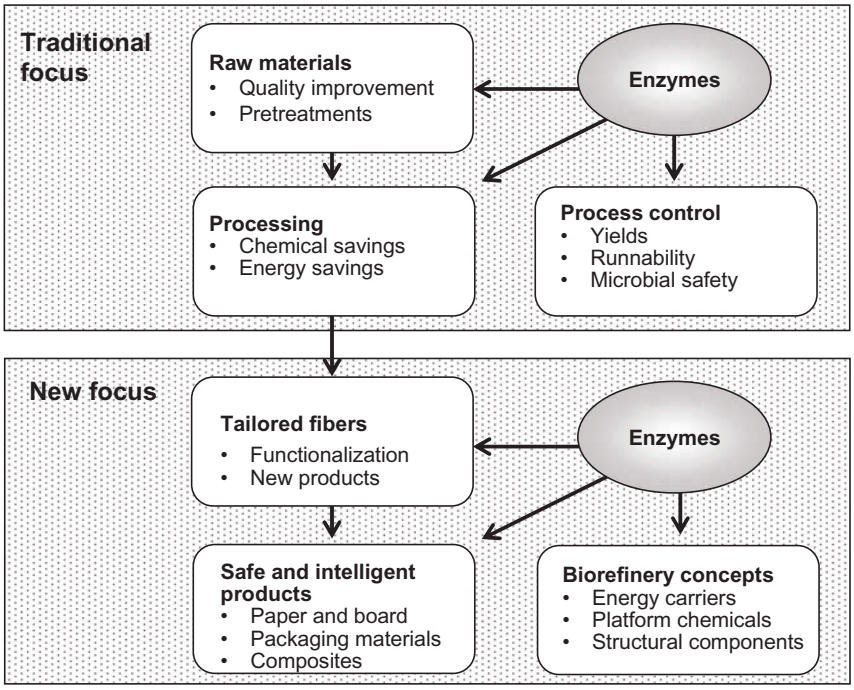

Figure 11.1 Traditional and new focuses for enzymatic applications in the pulp and paper industry.

11.2
Enzymes for the Pulp and Paper Industry

Because of the complex chemistry involved in wood materials, a number of different enzymes can be used to improve pulp and paper processes. Cellulases have been studied intensively for over two decades and the reaction mechanisms of many enzyme proteins have been revealed on a molecular level. The enzymology of hemicellulases is also well established. Recently, increased understanding of the detailed mechanisms of oxidative enzymes has been gained. The concept of using an oxidative enzyme with electron-transferring mediator molecules has opened up new possibilities for enzyme-based oxidation and delignification technologies. The prices of commercial hydrolases have decreased several fold over the last two decades. Further functional improvements are expected, especially for enzymes active at extreme conditions. Novel thermophilic and alkalophilic enzymes have already been developed for the harsh process conditions (i.e., high or low pH and/or high temperature) typically prevailing in pulp and paper manufacturing processes.

11.2.1 Cellulases

Cellulose is the main carbohydrate in lignocellulosic materials. It is a chemically simple homopolymer, consisting of β-1,4-linked anhydroglucopyranoside units. In plant fibers, and consequently in cellulose pulps, individual cellulose chains are oriented in a parallel alignment, forming cellulose I, and the tightly bound cellulose chains form elementary fibers that are further aggregated to form larger fibrillar structures [1, 2]. Cellulose contains ordered crystalline and less ordered amorphous regions. In wood fibers the winding direction of cellulose microfibrils varies in different cell wall layers giving the fiber its unique strength and flexibility.

Crystalline cellulose is the most resistant polysaccharide in lignocellulosic materials and requires the concerted action of several enzymes, which can be classified into endo-β-1,4-glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). Cellobiohydrolases releasing cellobiose units sequentially from the ends of cellulose chains are the key enzymes in total hydrolysis of crystalline cellulose. They constitute about 80% of the total cellulolytic proteins secreted by the filamentous fungus *Trichoderma reesei*, which has one the most extensively studied and industrially applied cellulolytic systems [3]. Endoglucanases act mainly on the amorphous regions of the cellulose fiber whereas β-glucosidases hydrolyze cellobiose further to glucose.

Cellobiohydrolases and endoglucanases of fungal origin usually have a multidomain structure composed of a large catalytic module connected by a linker peptide to a small cellulose-binding domain (CBD) or carbohydrate-binding module CBM) [4, 5]. The CBD is needed for the full activity of these enzymes on crystalline substrates. It has been shown that the ability of cellobiohydrolases to degrade crystalline cellulose clearly decreases when the CBD is absent [6]. This property has been also explored in fiber modification in order to limit the unwanted degradation of cellulosic fibrils. At present, glycosyl hydrolases are grouped according to their sequence similarity and the protein fold of their catalytic domains into more than 70 families (http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html).

Traditionally, commercial cellulases have been composed of multicomponent enzyme mixtures produced mainly by fungal strains. These have previously caused problems in industrial use due to their unspecificity and high degradative power, which are detrimental to pulp strength properties. Modern biotechnology has provided efficient tools able to discover new proteins and produce them efficiently at an industrial scale. This has resulted in commercially available monocomponent cellulase preparations designed for specific process applications. Monocomponent enzymes marketed for other applications (e.g., endoglucanases for textile processing) can also find uses in pulp and paper processing. Typically the commercial cellulases are of fungal origin (from e.g., *Trichoderma*, *Humicola*, *Acremonium*) and are usually produced in genetically modified fungal (e.g., *Trichoderma* or *Aspergillus*) strains.

11.2.2 Hemicellulases

The two most common wood hemicelluloses are xylans and glucomannans. Hardwoods contain mainly xylan, which is composed of β-D-xylopyranosyl units with 4-*O*-methyl-α-D-glucuronic acid and acetyl side-groups. 4-*O*-methylglucuronic acid is linked to the xylan backbone by *O*-(1,2) glycosidic bonds and the acetic acid is esterified at the C-2 and/or C-3 hydroxyl group. In softwood, glucomannan is the major hemicellulose. About a third of softwood hemicellulose is arabino-4-*O*-methylglucuronoxylan, in which the xylan backbone is substituted at C-2 and C-3 with 4-*O*-methyl-α-D-glucuronic acid and α-L-arabinofuranosyl residues, respectively. Softwood galactoglucomannan has a backbone of β-(1,4)-linked β-D-glucopyranosyl and β-D-mannopyranosyl units which are partially substituted by α-D-galactopyranosyl and acetyl groups. The distribution of carbohydrates in the wood fibers varies depending on the tree species and growing conditions [7].

The amount and composition of hemicellulose components is drastically altered in kraft cooking. All acetyl groups are degraded and most of the glucuronic acid groups in xylan are converted to hexenuronic acid groups [8]. Softwood glucomannan is deacetylated and a considerable part is solubilized in the highly alkaline and hot cooking liquor [7]. Thus, in softwood kraft pulp the relative amount of xylan is increased during pulping.

Endoxylanases (EC 3.2.1.8) catalyze the random hydrolysis of β-D-1,4-xylosidic linkages in xylans. Most xylanases belong to the two structurally different glycosyl hydrolase groups (families 10 and 11). Some xylanases have been reported to contain either a xylan-binding domain [9] or a cellulose-binding domain [10, 11]. Some of the binding domains have been found to increase the degree of hydrolysis of fiber-bound xylan whereas others have not. Neither xylan- nor cellulose-binding domain was found to have any significant role in the action of xylanases on pulp fibers [12]. Most of the xylanases characterized are able to hydrolyze different types of xylans showing only differences in the spectrum of end-products. A number of enzymes produced by extremophilic organisms have been characterized, but surprisingly few have reached commercial use, obviously because of problems related to their efficient production in heterologous host strains.

Endomannanases (EC 3.2.1.78) catalyze the random hydrolysis of β-D-1,4 mannopyranosyl linkages within the main-chain of mannans and various polysaccharides consisting mainly of mannose, such as glucomannans, galactomannans, and galactoglucomannans. Mannanases also seem to be a more heterogeneous group of enzymes than xylanases. The mannanase of *Trichoderma reesei* has been found to have a multidomain structure similar to that of several cellulolytic enzymes; that is, the protein contains a catalytic core domain which is separated by a linker from a cellulose-binding domain [13, 14]. The CBD has been found to increase the action of *T. reesei* mannanase on fiber-bound glucomannan even though the catalytic domain is able to efficiently degrade crystalline mannan [15]. The hydrolysis yield of glucomannans depends on the degree of substitution as well as on the

distribution of the substituents [16]. The hydrolysis of glucomannans is also affected by the glucose-to-mannose ratio.

The side-groups connected to xylan and glucomannan main-chains can be cleaved by α-glucuronidase (EC 3.2.1.131), α-arabinosidase (EC 3.2.1.55), and α-galactosidase (EC 3.2.1.22). Acetyl substituents bound to hemicellulose are removed by esterases (EC 3.1.1.72) [17]. Most of the side-group cleaving enzymes are able to act only on oligomeric substrates produced by the backbone depolymerizing endoenzymes, that is, xylanases and mannanases. Only a few enzymes are capable of attacking intact polymeric substrates. Even most accessory enzymes of the latter type, however, prefer oligomeric substrates.

Covalent linkages connecting lignin and hemicellulose in plant cell walls include ester linkages between lignin alcohols and carboxyl groups of uronic acids in glucuronoxylans. Recently, evidence for the existence of carbohydrate esterases that may hydrolyze these linkages has been gained [18]. The substrate specificity of this glucuronoyl esterase, attacking exclusively the esters of 4-*O*-methyl-D-glucuronic acid, is distinct from those of other carbohydrate esterases, such as acetyl xylan esterase, feruloyl esterase, and pectin methyl esterase.

11.2.3
Transferases

Xyloglucan-specific endoglucanases comprise a new class of polysaccharide-degrading enzymes, which can attack the glucan backbone also at substituted glucose residues [19]. Xyloglucan endo-transglycosylase (XET, EC 2.4.1.207) is able to transfer a high molecular weight portion from a donor xyloglucan to a suitable acceptor such as a xyloglucan-derived nonasaccharide. A xyloglucanase from *Aspergillus niger* has been shown to be active against several β-glucans, but has the highest activity against tamarind xyloglucan [20]. A plant-specific enzyme believed to be responsible for the modification of xyloglucan in the cell wall through endo-hydrolysis and glycosyl transferase activities has also been characterized [21, 22]. Because of the inherent ability of XET to catalyze transglycosylation rather than hydrolysis, this enzyme has potential for fiber modification [23].

11.2.4
Lignin-Modifying, Oxidative Enzymes

Lignin is an aromatic, amorphous, heterogeneous polymer forming an integral part of the cell wall, embedded in a carbohydrate polymer matrix of cellulose and hemicellulose. Lignin comprises three phenylpropanoid units: guaiacyl, syringyl, and hydroxyphenyl units. The exact composition of lignin varies widely with tree species, but as a rule, softwoods contain mainly guaiacyl units whereas hardwoods also contain syringyl-building blocks. It has been suggested that both the chemical and three-dimensional structure of lignin is strongly influenced by the polysaccharide matrix. Unlike cellulose and hemicellulose, lignin is not susceptible to a hydrolytic attack of enzymes. Lignin-modifying enzymes have been intensively

studied for more than 30 years, and research has resulted in the identification of the major enzyme systems thought to participate in delignification.

The array of extracellular peroxidises and oxidases thought to be involved in lignin degradation include lignin peroxidases (LiP, EC 1.11.1.14), manganese peroxidases (MnP, EC 1.11.1.13), versatile peroxidases (VP, EC 1.11.1.16), laccases (EC 1.10.3.2), as well as H_2O_2-producing enzymes, such as aryl alcohol oxidase (AAO, EC 1.1.3.7) and glyoxal oxidase (EC 1.1.3.–) produced by lignin-degrading white-rot and litter-decomposing fungi in different combinations [24]. Despite the completely different protein architecture, the peroxidase and laccase type enzymes share significantly similar properties with respect to the reducing substrates. However, due to the differences between the three-dimensional structures, prosthetic groups, and electron acceptors, peroxidases and laccases operate with very different catalytic mechanisms.

Detailed structure–function analysis has greatly increased our knowledge on lignin-modifying enzymes and of their mode of action on aromatic compounds. However, information on how these enzymes act on polymeric lignin is still incomplete.

Because of the non-specific nature of catalysis by lignin-modifying enzymes their biotechnical applications are seen as highly promising. *In vitro*, the oxidative reactions of these enzymes lead mainly to polymerization of lignin. Purified ligninolytic enzymes have, however, been shown to cause limited delignification provided that additives, such as veratryl alcohol and H_2O_2 for LiP [25], manganese, H_2O_2, organic acids and surfactants for MnP [26, 27] and mediators for laccases are supplemented. Because of the promising results, laccases and manganese-dependent peroxidases have recently been the most extensively studied groups of enzymes in the area of lignin degradation.

11.2.4.1 **Peroxidases**

Lignin-degrading peroxidases – LiP, MnP, and VP – are structurally related heme proteins which oxidize their substrates with H_2O_2 as electron acceptor through a one-electron oxidation mechanism, analogous to all other peroxidases. LiPs are capable of oxidizing various substrates including veratryl alcohol, and both phenolic and non-phenolic lignin substructures. LiP-catalyzed reactions produce phenoxyl and aryl cation radicals. Cation radical formation in non-phenolic lignin structures causes several unspecific reactions, resulting eventually in ring cleavage [28]. LiPs have been characterized from several white-rot fungi. The three-dimensional structures of LiPs from *Phanerochaete chrysosporium* are available [29, 30]. Attempts to overproduce LiP in heterologuous hosts have mostly been unsuccessful and this has hindered the commercial exploitation of the enzyme.

Manganese-dependent peroxidase (MnP) oxidizes Mn^{2+} to Mn^{3+}. Chelation of Mn^{3+} by organic acids is necessary in order to stabilize the ion and to promote its release from the enzyme. The Mn^{3+}-chelate can oxidize various phenolic substrates, carboxylic acids, and unsaturated lipids. When Mn^{3+}-chelate oxidizes lignin phenolic substructures, phenoxyl radicals are formed and a variety of unspecific reactions take place, resulting in products similar to those released by LiPs (as

reviewed in ref. [31]). Interestingly, MnP has been shown to promote peroxidation of unsaturated lipids without added H_2O_2. In lipid-mediated peroxidation reactions MnP is able to oxidize even the non-phenolic lignin substructures [32]. MnP has been detected in most of the lignin-degrading fungi studied thus far [33, 34], suggesting a crucial role in lignin decomposition.

Versatile peroxidase has been recently described as a new family of ligninolytic peroxidases [35]. So far, VP seems to be produced by just a few fungi, including *Pleurotus*, *Bjerkandera*, and *Lepista*. The most noteworthy aspect of VP is that it combines the substrate-specificity characteristics of the other fungal peroxidase families. It is thus able to oxidize a variety of (high and low redox potential) substrates including Mn^{2+}, phenolic and non-phenolic lignin dimers, α-keto-γ-thiomethylbutyric acid (KTBA), veratryl alcohol, dimethoxybenzenes, different types of dyes, substituted phenols, and hydroquinones. It has been suggested that the catalytic properties of the new peroxidases are a result of a hybrid molecular architecture combining different substrate-binding and oxidation sites [36, 37].

11.2.4.2 **Laccases**

Laccases are common enzymes in nature. They are presumably the most commonly occurring oxidoreductases in white-rot fungi and are also found widely in other fungi, some bacteria, as well as in plants and insects. The plant laccases are reported to have an important role in wound response and lignin biosynthesis, whereas in fungi laccases are involved in lignin degradation and have several other functions including pigmentation, fruiting body formation, sporulation, as well as pathogenesis [38–40].

Laccases are copper metalloenzymes containing four copper atoms in their active site. They have a surprisingly broad substrate specificity range and catalyze the oxidation of various phenolic compounds, aromatic amines, and even some inorganic molecules. Laccases oxidize their substrates with a one-electron removal mechanism and use molecular oxygen as the terminal electron acceptor. The ability of laccases to oxidize lignin is limited to phenolic subunits. Phenolic radicals are formed in lignin and they undergo further unspecific reactions, which can lead to aryl-C_α–cleavage, demethoxylation, and polymerization reactions [41].

Several complete laccase structures are presently available [42–44], helping our understanding of the catalysis. The detailed mechanism with which laccases oxidize their substrates is, however, still not fully understood. Following the discovery of the mediator concept in the early 1990s [45, 46], laccases have been intensively studied for the delignification of kraft pulps. Laccases are commercially available for industrial-scale applications, mainly for denim bleaching.

11.3
Enzymes as Process Aids

Wood fibers are composed mainly of cellulose, hemicellulose (i.e., xylan and glucomannan), lignin, and extractives. No major chemical changes in the fiber com-

ponents occur in mechanical pulping, whereas in chemical pulping, either kraft (sulfate) or sulfite cooking, about 90% of lignin is removed from the fibers. The hemicellulose components are also extensively modified through dissolution, partial degradation, and re-deposition [47]. Consequently, the chemical compositions and structures of mechanically and chemically treated fibers are different. The more open structure of chemical fibers compared with mechanical fibers renders them more susceptible to the action of macromolecular enzymes. Even in chemical pulps, however, the enzymatic action is limited to accessible surfaces, that is, to fines and to the outermost surface and accessible pores of long fibers [48]. Therefore, enzymatic treatments of fibers can be considered to be surface-specific modification methods.

Concepts for using enzymes in pulp and paper processing have been developed over the last 20 years. The targeted benefits in various applications are described in Table 11.1. The major applications are described below.

11.3.1 Pulping

11.3.1.1 Chemical Pulping

In chemical pulping, wood fibers are separated from each other in order to render them suitable for the paper-making process. In the pulping process, the lignified middle lamella located between the wood fibers is solubilized by various chemicals. Today, the predominant pulping method is the kraft process, combining high alkalinity (pH 12–14), sulfidity, and high temperature (165–170 °C). Extensive modification of hemicelluloses takes place during the pulping process. During conventional kraft cooking, part of the hemicelluloses is first solubilized in the cooking liquor. In the later phases, when the alkalinity of the cooking liquor decreases, part of the solubilized xylan is relocated onto the cellulose fibers. In softwoods, most of the glucomannan is dissolved and degraded during kraft pulping. Thus, the relative amount of xylan is increased in pine kraft pulp compared with that in pine wood. The chemistry of the residual xylan also differs from the native xylan in pulp as part of the glucuronic acid is converted to hexenuronic acid during kraft pulping.

In addition to xylan, lignin is also partially readsorbed on the fibers. Lignin has been reported to be linked to hemicelluloses, forming lignin–carbohydrate complexes. Furthermore, hemicelluloses seem to physically restrict the passage of high molecular mass lignin out of the pulp fiber cell wall. The removal of hemicelluloses, especially xylan, can thus be expected to enhance the extractability of the dark brown–colored residual lignin from pulps in the bleaching process.

Various methods to increase the diffusion of cooking chemicals in wood have been studied to improve the efficiency of the chemical pulping process. Impregnation of chemicals into wood and removal of dissolved lignin are governed by diffusion and sorption phenomena, by the porosity and structure of the cell wall matrix, as well as by the molecular size of the extractable molecules. The low porosity of wood chips limits the exploitation of macromolecular enzymes for

Table 11.1 Applications of enzymes in pulp and paper processing.

Application	Technical or process benefit	Enzyme	Challenges
Wood (chip) pretreatment	Energy saving in debarking	Cellulases Pectinases Mixtures	Penetration of enzymes into the cambial layer
Mechanical pulping	Energy saving in TMP refining Increased flexibility of fibers	Cellobiohydrolase Xylanases	Penetration of enzymes into chips
Chemical pulping	Decreased consumption of chemicals Decreased energy consumption Specialty products/ high-density paper	Endoglucanase Mixed enzymes Endoglucanases	Preservation of strength properties Process control
Bleaching–mechanical pulp	Chemical savings Prevention of brightness reversion	Laccase + mediator	Costs vs. efficiency
Bleaching–chemical pulp	Higher final brightness Chemical savings	Xylanases Laccase + mediator	Limited effect of xylanase Efficiency and biodegradability of mediators, costs of the treatment
Paper making	Drainage improvement Smoother paper machine running	Endoglucanase Hemicellulases	Process control
Deinking	Increased release of ink particles Increased release of ink particles	Mixed cellulases Amylases	Process control
DCS in process water	Decreased pitch problems Increased paper machine runnability	Lipase Esterases Laccase Mannanase	Process control
Biofilms (proteins and polysaccharides)	Slime control	Esterases and chemical additives	Versatility of slime components

DCS, dissolved and colloidal substances; TMP, thermomechanical pulp;

enhancing the impregnation of wood chips by chemicals. It has been reported, however, that enzymes, including hemicellulases, pectinases, and cellulases have been able to increase the diffusivity of sodium hydroxide in southern pine sapwood [49]. This result was attributed to the dissolution of pit membranes, which are the main resistance to flow of liquids in wood. After acetone extraction and enzyme treatment the pulps were reported to be more uniform, to have higher viscosity and yields, and lower rejects [50].

11.3.1.2 **Mechanical Pulping**

Mechanical pulps, characterized by high yield (up to 95%), are used to produce wood-containing paper grades with high bulk and good optical properties and printability. Today, the major challenge of mechanical pulping is to reduce the refining energy consumption in this energy-intensive process. The issue of energy usage has been accentuated following the recent increase in energy prices and environmental concerns. It is thus a key issue when developing new technical processes for high-yield pulping. One way of reducing refining energy is to modify the raw material by biotechnical means prior to refining. Various biotechnical options for energy saving in mechanical pulping are shown in Figure 11.2.

Currently, the main biotechnical applications related to mechanical pulping are energy-saving biopulping of wood chips with fungi [51, 52], microbial reduction of pitch components [53], and enzyme-aided refining of coarse mechanical pulp fibers [54–57]. An enzymatic process based on the treatment of coarse mechanical pulp or rejects using monocomponent cellulases was developed in the 1990s [55, 58] and this method is currenly being implemented for industrial use. The method was successfully verified at an industrial scale resulting in energy savings of about 15% in reject refining, which corresponds to 5–8% of total energy consumption [56]. No evident morphological modifications in the coarse and rigid TMP (thermomechanical pulp) fibers were induced during a short incubation by cellulase mixture or CBH I [53]. The action of CBH I seemed to induce decreased interfibrillar cohesion inside the fiber wall and resulted in loosening and unraveling of the fiber structure. The pulp quality and optical properties were maintained and

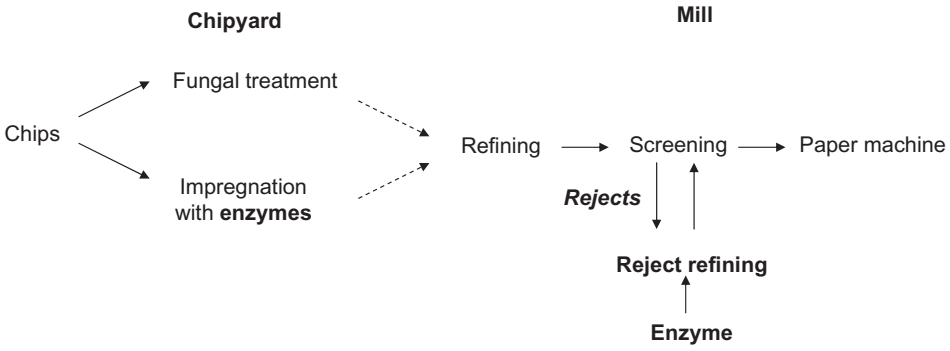

Figure 11.2 Biotechnical treatments for energy saving in mechanical pulping.

no negative effects on the subsequent paper-making process were observed after CBH I treatment. Intensification of refining by enzymatic pretreatment was also obtained in trials in which agrofibers or recycled fibers were used as raw material [54, 57].

Recently, promising results have also been obtained by pretreatment of wood chips with selected enzymes to reduce refining energy [59, 60]. The potential energy savings are higher when enzymatic pretreatment is applied to wood chips prior to the primary refining step rather than with treatment of reject pulps. For efficient modification of wood components in chips by enzymes, however, an impregnation step, in which enzymes are introduced into the inner parts of the chips, is necessary. Energy savings between 10 and 25% as compared with conventional TMP processes have been obtained in trials in which softwood or hardwood chips were treated with pectinases, cellulases, or xylanases [59–61]. TEM (transmission electron microscopy) studies of hardwood chips indicate fundamental changes in fiber separation and refining mechanisms compared with conventional TMP. Thus, significant energy savings are possible by treating chips with enzymes prior to refining.

Lipases hydrolyzing triglycerides to glycerol and free fatty acids can be used to reduce pitch problems originating from lipophilic extractives, especially in mechanical pulps. A commercial lipase preparation, Resinase (Novozymes), has been used industrially to improve the quality of groundwood pulps in Japan [62]. Triglycerides from various types of pulps have been reported to be efficiently hydrolyzed by lipases, reducing stickiness and pitch problems, allowing savings in the consumption of additives and surface-active chemicals, and increasing the tensile strength of the pulp [62–65].

In addition to hydrolytic enzymes, oxidative enzymes, especially laccases, have been used to modify the composition and structure of lipophilic and hydrophilic extractives [66, 67]. Laccase treatment polymerizes lignans to fibers and could also slightly modify lipophilic extractives. A laccase-mediator system (LMS) has also been shown to be effective for removal of sterols [68]. Various pulps were treated with laccase in the presence of 1-hydroxybenzotriazole (HBT) as the redox mediator. Most of the lipophilic compounds, including free and conjugated sitosterol, fatty and resin acids and triglycerides, were efficiently removed by this treatment. Simultaneously, significant improvement of pulp brightness and decreased kappa number were observed in the LMS-treated pulps [69].

11.3.2 Bleaching

Bleaching is used to improve the color and brightness properties of mechanical and chemical pulps. In mechanical pulps, a high yield is important and therefore, lignin-preserving bleaching is carried out by hydrogen peroxide and/or dithionite. In chemical pulps, bleaching aims at the total removal of the residual lignin present in the pulp after cooking, without decreasing the molecular weight of the cellulose. Lignin in unbleached pulps typically represents only about 1% of the dry

weight. During pulping, however, lignin is chemically modified and condensed into poorly degradable and highly colored structures. In the bleaching processes, lignin is sequentially degraded and extracted in several phases using oxygen, ozone, or peroxides and chlorine dioxide as bleaching agents [1].

Enzymes can be used to improve the bleaching process indirectly or directly. In the indirect method the bleachability of pulps is improved through the action of xylanases or other enzymes affecting the extractability of lignin, and the effect is limited. The most promising direct enzymatic bleaching system is the laccase-mediator system, which acts directly on lignin.

11.3.2.1 **Xylanase-Aided Bleaching**

Xylanase-aided bleaching of chemical pulp is the most widely used and best-established biotechnical application in the pulp and paper industry. Xylanase is used as a bleaching aid and the xylanase stage prior to subsequent chemical bleaching stages enhances the extractability of lignin in subsequent bleaching stages. Several alternative and possibly concurrent mechanisms have been proposed to be involved in xylanase-aided bleaching. The enhanced leachability of fiber-bound lignin by xylanase treatment has been suggested to be a result of hydrolysis of reprecipitated xylan or removal of xylan from the lignin–carbohydrate complexes in fibers [70, 71]. Removal of xylan by xylanases from softwood kraft fibers has been reported to uncover surface lignin [72]. The action of xylanases on both reprecipitated and lignin–carbohydrate xylan suggests that it is probably not only the type but also the location of the xylan that is important in xylanase-aided bleaching.

Because of its double bond–containing structure, hexenuronic acid increases the consumption of bleaching chemicals and permanganate, increasing the apparent kappa number of pulp [73]. The structure is quite labile, and is easily degraded under acid conditions so this property has been exploited in the industrially used removal method. In spite of extensive research efforts, no enzyme capable of removing this compound from the xylan backbone has yet been described. Recently, a side activity in a commercial lipase was claimed to reduce the kappa number and hexenuronic acid content in softwood kraft pulps but the details of this process remain to be elucidated [74]. The partial removal of hexenuronic acid–substituted xylan by xylanase treatment results in a slightly lower kappa number. This modest effect is due to the relatively small amount of xylan being removed during treatment.

Xylanase-aided bleaching is compatible with different types of kraft pulps. Mannanases have also been reported to be effective in enhancing bleachability, but in effect depends on the pulp type used [75]. Both fungal and bacterial xylanases have been reported to act on pulp xylan and result in enhanced bleachability [76]. It has been proposed, however, that some xylanases from Family 11 could be more effective in bleach boosting than the Family 10 xylanases [77]. The binding of xylanases to fibers by cellulose- and xylan-binding domains (CBD and XBD) has so far not been shown to have any significant role in the bleach-boosting efficiency of xylanases [78].

The enhanced bleachability obtained by using xylanases can be used to reduce bleaching chemical consumption or increase final pulp brightness. The benefits obtained by enzymes are dependent on the type of bleaching sequence used and on the residual lignin content of the pulp. In the 1990s, the xylanase stage was used to reduce the environmental load of chlorine-based bleaching chemicals. An average reduction of 25% in active chlorine consumption in prebleaching or a reduction of about 15% in total chlorine consumption resulted to 15–20% reduction of chlorinated compounds, measured as AOX, in the bleaching effluents.

Today, xylanases are used industrially both in elemental chlorine-free (ECF) and totally chlorine-free (TCF) sequences. In ECF sequences, the enzymatic step is often implemented because of the limiting chlorine dioxide production capacity. The use of enzymes allows bleaching to higher brightness values when chlorine gas is not used. In TCF sequences, the advantage of the enzymatic step is that it allows improved brightness, maintenance of fiber strength, and savings in bleaching costs [79].

As of 2007, about 20 mills in Northern America and Scandinavia use enzymes in kraft pulp bleaching. The chemical bleaching process conditions, high alkalinity, and high temperature set specific requirements for enzymatic treatment. Today, there are new enzyme products available that act at both high pH and temperature (pH 10 and 90–100 °C). The approximate price of xylanase treatment in 2007 was less than US$2 per ton of pulp.

11.3.2.2 **Delignifying Laccase-Mediator System for Direct Bleaching**

Direct bleaching of chemical pulps by enzyme-based oxidation systems has been under extensive research since the discovery of lignin-oxidizing enzymes in the early 1980s. Today, the most advanced direct delignification method is the laccase-mediator system (LMS). In the LMS concept, laccase is used to oxidize a chemical mediator that can further oxidize and degrade the fiber-bound lignin. In the first reported studies, the common substrate of laccases, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT) were used as mediators [80, 81]. Later, several other mediators including *N*-hydroxyacetanilidine (NHA) or a slowly NHA-releasing precursor derivative of NHA, violuric acid and TEMPO have been studied [82–84]. The most effective mediators usually contain N-OH functional groups.

To be economically feasible for commercial use, the mediators should be specific, environmentally acceptable, and biodegradable. Therefore, a number of new mediators with great structural variety have been discovered. Naturally occurring mediators among lignin-derived phenols which could provide environmental or economic advantages have been searched [85]. The tested compounds include acetovanillone, vanillin, syringaldehyde, acetosyringone, 2,4,6-trimethylphenol, *p*-coumaric acid, sinapic acid, cinnabarinic acid, and ferulic acid. Of these, syringaldehyde and acetosyringone were found to be most efficient in the LMS for both delignification and degradation of lipophilic extractives [86, 87]. In addition to nitrogen-based mediators, inorganic mediators, such as transition metal complexes, containing preferably molybdenum, have been successfully tested for

laccase-mediator bleaching [88, 89]. The mechanisms of laccase-mediated delignification of pulps have been studied intensively [90–93].

The degree of delignification after alkaline extraction is reported to be high, up to 40% [94]. The LMS system has been shown to be able to replace either the oxygen delignification or the ozone stage [93, 95]. Further improvements of bleachability can be obtained by combining xylanase treatment and LMS bleaching in sequence [96, 97]. Combination of LMS with xylanase treatment in a single stage has been found to be ineffective, apparently due to the inactivation of xylanase by the mediator [98]. In addition to LMS, enzyme-assisted oxidative systems, such as the hydrolase-mediated oxidation system (HOS) and other chemo-enzymatic slow-release systems have also been proposed for bleaching of chemical pulps [99, 100].

11.3.3 Paper Making

In paper making, paper is formed from pulp, various chemicals, and pigments in the paper machine. Following stock preparation, the paper machine normally contains web formation, pressing, drying, sizing, and calendering unit operations. Large amounts of water are needed in the formation of uniform paper web from the pulp slurry in the paper machine. High efficiency is required from all unit operations due to the high speed of production.

Enzymes can be used to modify the paper-making properties of chemical, mechanical, and recycled pulps. Use of enzymes to reduce the need for refining and to increase drainage (i.e., water removal) of chemical pulps are potential applications exploiting the ability of enzymes to modify the surface of pulp fibers. The effects of individual cellulases on the properties of unbleached or bleached kraft pulp have been studied in detail [97, 101]. *T. reesei* cellobiohydrolases (CBH) have been found to have only a modest effect on pulp viscosity, whereas the main endoglucanases (EG), especially EG II, dramatically decrease the viscosity and thus the strength properties after refining. Treatment of pine kraft pulp with *T. reesei* endoglucanases EG I and EG II has been reported to enhance the beatability considerably but at the same time the strength properties of the pulp were impaired. The decreased viscosity is presumably because the endoglucanases attack the amorphous regions of the cellulose, especially in the defects and irregular zones of the fibers [101].

The positive effect of *T. reesei* CBH I on beatability and thus on the development of the binding properties of ECF-bleached spruce kraft pulp in refining has also been reported [102]. Commercial monocomponent cellulases and cellulase–hemicellulase mixtures are available to improve the beatability of the pulp, and the drainage and running of the paper machine. These enzyme applications are suitable for certain specialty paper grades or for occasional special needs in the paper mills. The successful use of cellulase-containing preparations needs special attention with regard to enzyme selection, enzyme treatment conditions, and process control.

The minimization of water usage and effluent discharge is a constant challenge in paper manufacture because of environmental and legislative requirements to

reduce freshwater consumption. A major problem in reducing the water usage is the accumulation of dissolved and colloidal substances (DCS) in the process waters. The DCS consist mainly of hemicelluloses, pectins, dispersed wood resin, lignans, and dissolved lignin. The DCS content and composition of process waters affects the running of paper machines and paper quality, and also determines the efficiency needed in the purification of process water.

Pectinase treatment has been reported to decrease the cationic demand of white water up to 60% [103–106]. Consequently, savings in cationic chemicals (alum, retention aids, strength agents, starch) and improved running of paper machines in mill-scale trials with pectinase have been obtained [107, 108]. Mannanases can be used to degrade glucomannans, stabilizing the colloidal resin in mechanical pulp process waters [109]. As a result of enzymatic treatment, the resin particles have been shown to be destabilized and attached onto fibers as single particles. The chemical structure of galactoglucomannan present in TMP water can be further modified with acetyl glucomannan esterase, which is able to cleave acetyl groups from polymeric glucomannan [5]. The deacetylation of soluble glucomannan has been found to result in decreased solubility and subsequent adsorption of glucomannan onto the fibers [110].

11.3.4
Recycled Paper and Deinking

The use of recovered paper is increasingly used for newsprint, tissue paper, and higher grades of graphic papers. The recovered paper must be deinked, that is, repulped and cleaned of dirt and ink before it can be reused in paper making. In deinking, ink particles are detached and removed from the fibers by a combination of mechanical and chemical actions. Enzymes, especially amylases and cellulases, have been shown to be efficient in improving ink detachment from both coated and uncoated recovered paper grade fibers. The use of cellulase and hemicellulase mixtures as well as amylases in deinking has been studied on laboratory, pilot, and mill scales [111–116].

The positive effect of enzymes in enhancing ink detachment can be obtained by two basic approaches: by the enzymatic liberation of ink particles from fiber surfaces by carbohydrate-hydrolyzing enzymes such as cellulases, hemicellulases, or pectinases, or by the hydrolysis of the ink carrier or coating layer. It is thought that in enzyme-aided deinking the enzymatic hydrolysis of the ink carrier, starch coating, or fiber surface liberates ink particles that are large enough to be removed by flotation deinking. The use of enzymes in deinking of recovered paper is one of the most useful enzymatic applications in the pulp and paper industry and is already used on the mill scale.

The quality of recycled paper is also affected by “stickies,” occurring in the paper-making process. These can appear as spots or cause pick-outs and decrease paper machine efficiency because of the additional cleaning downtimes caused. Stickies can contain wood pitch, oils, sizing agents, antifoam chemicals, or polyvinylacetate (PVA), a material widely used for self-stick labels. A commercial

enzyme preparation containing esterase that attacks PVA has been developed and is currently being used in mill scale for stickies control [117].

11.3.5 Slime Control

Enzymes are potential agents for biofilm control and can act against different components of paper machine slimes, such as proteins, lipids, carbohydrates, or pitch compounds. Their effect can be boosted by synergistic treatments with mixtures of polysaccharide-degrading enzymes together with efficient proteases and lipases or with chemical agents that loosen the structure of biofilm. The major problem with the use of specific hydrolytic enzymes is the variability in the composition of slimes and slime polysaccharides [118].

Several hydrolytic enzymes have been reported to show biofilm-degrading ability. However, the most successful industrial products for slime and stickies control are based on mixtures of several enzyme activities, the composition of which is experimentally optimized even on a case-by-case basis. The activities with practical importance include proteases, possibly combined with other activities [119–121].

11.3.6 Other Applications

The formation of calcium oxalate crusting is a current problem in the pulp and paper industry. Oxalic acid and calcium occur in wood but oxalic acid is also formed from wood polymers during oxidative bleaching. A higher degree of system closure causes accumulation of oxalic acid and calcium, increasing the risk of calcium oxalate incrusts in the pipes, filters, and heat exchangers. Removal of oxalate with oxalate-degrading enzymes offers a way to solve the problem. Novel oxalate-degrading enzymes, such as oxalate oxidase and oxalate decarboxylase, have been tested for this application. Major problems are, however, caused by the inhibitors present in the filtrates, including iron, calcium, chelating agents, and extractives [122].

11.4 Enzymes for Product Design

11.4.1 Enzymatic Fiber Engineering

The specificity of enzymes makes them especially suitable for engineering of both chemical and mechanical pulps. Targeted modification of fiber surfaces by enzymatic or combined enzymatic and chemical treatments can be used to improve fiber properties or to create completely new fiber characteristics for

various applications. Not surprisingly, this is one of the fastest growing areas in the fiber-based industry. Fiber modification also reflects the trend of using biotechnical means for product design rather than for process improvement.

11.4.2 Functionalized, Value-Added Fibers

New perspectives in the use of wood or non-wood fibers are being opened up by introducing value-added properties to fibers. An interesting option is the targeted modification of fiber surfaces via enzymatic radicalization by oxidative enzymes. In principle, cellulose, hemicelluloses, and lignin would be the main target polymers in fiber material through which a functional component can be bound to fiber material. Currently, one of the most promising chemo-enzymatic methods is based on the use of lignin as a bonding matrix for designed attachment of novel functional groups to pulp [123–126]. Oxidative enzymes, such as laccases or peroxidases, activate the surface lignin of lignin-containing fibers. The primary reaction of laccase and other phenoloxidases is the formation of phenolic or cationic radicals [127, 128]. Because of the high reactivity of these radicals either with each other or with a secondary substrate, reactions such as polymerization, depolymerization, co-polymerization, and grafting can occur. This radicalization (i.e., activation of the fiber surface) is the first step of fiber functionalization when aiming at the introduction of value-added properties to fibers.

After enzymatic radicalization, specific chemical components can be grafted on to tailor the fiber properties. Radical-based activation of isolated lignin and surface lignin of fibers has previously been exploited in fiber board manufacture [129–132]. Enzymatic functionalization of fiber surfaces, however, offers many other possibilities. By choosing the compound to be bound, a variety of functional properties can be introduced; for example, modified charge, hydrophobicity, antimicrobiality, or conductivity, giving options to improve existing paper-making properties or creating completely new fiber properties [123–126]. The benefit of enzyme-based method, as compared to purely chemical methods, is the surface specificity and preservation of important chemical and physical fiber properties, such as strength. Various lignin-containing fibers (unbleached kraft, bleached, and unbleached CTMP and TMP) can be modified by this novel method.

Another interesting approach in fiber modification is the attachment of functional groups to cellulose surfaces using chemically or enzymatically modified xyloglucans as carriers [133, 134]. In this approach the xyloglucan oligosaccharides (XGO) are first modified and then joined to polymeric xyloglucan (XG) using the xyloglucan endo-transglycosylase (XET) enzyme. As xyloglucans have a naturally high affinity to cellulose, surface-specific modification of the fibers is achieved by absorption of the modified xyloglucans onto the fiber surface. XET catalyzes the cleavage and religation of xyloglucan chains. In the first step the enzyme catalyzes the cleavage of a glycosidic bond with subsequent formation of a covalent glycosyl–enzyme intermediate. In the breakdown of the glycosyl–enzyme intermediate the glycosyl moiety is transferred to a carbohydrate acceptor [134, 135].

The affinity of xyloglucans to cellulose is high and largely independent of pH, binding temperature, molecular weight, and precise sugar composition of xyloglucan. Adhesion of xyloglucan has been used successfully to increase paper sheet strength. The high affinity of xyloglucan also enables attachment of chemical moieties on cellulose surfaces in various conditions without disruption of the individual fiber or fiber matrix. The method allows the incorporation of a wide range of chemical groups to the fiber surface via the xyloglucans. It has been envisioned that this method could be used to develop novel, high-performance paper and paper-making materials [133].

11.5 Biorefinery Concepts

An integrated biorefinery is an overall concept where biomass feedstocks are converted into a spectrum of valuable products. Biorefineries combine and integrate various technologies, potentially also including biotechnical process steps, in order to utilize all components in the biomass. The primary objective of an advanced biorefinery is to increase the availability and use of bioenergy and bio-based products by implementing innovative, environmentally sound, and cost-effective production technologies for a variety of products. Lignocellulosic raw materials (wood and annual plants) provide an extensive source not only for present fiber products, but also for a large number of intermediates, specialty chemicals, and fuels (Figure 11.3).

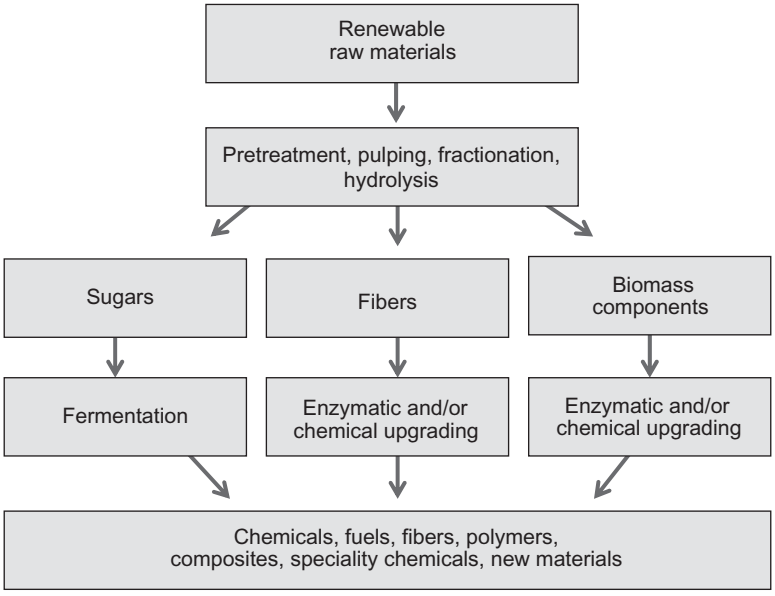

Figure 11.3 Upgrading of biomass.

Bioconversion and biorefining are gathering momentum as commercial processes, and could soon take their place alongside traditional pulping operations in the conversion of lignocellulosic feedstocks into higher value products. The production of fuel ethanol from cellulosic feedstock is of increasing interest for environmental, political, and economic reasons. The limited supply of fossil fuels has resulted in significant research efforts and recent achievements in promoting renewable energy sources. Switching to renewable fuels would allow human activities to become carbon neutral by recycling carbon from plants and would reduce carbon dioxide emissions, potentially reducing global warming. The use of lignocellulosic biomass for the production of ethanol, for example, is a necessary development from the current use of agricultural crops (i.e., starch-based substrates). The biomass feedstock would primarily be produced from agricultural, forestry or wood-based waste products or from dedicated energy plants. After the hydrolysis of biomass feedstock into carbohydrates (typically glucose and pentose sugars), these can be fermented into ethanol or other energy carriers.

Chemical products derived from lignocellulosic polymers and components can be used for a large number of applications. The target is to develop methods and applications for both large-volume and special-use products prepared from the main wood polymers, cellulose, hemicelluloses, wood extractives, and lignin. The properties of hemicelluloses can be exploited or modified and used for films, coatings, nutraceuticals, fiber products, or other value-added products. New and improved cellulose-based chemicals with specific properties could be developed with enzyme-aided methods. Wood extractives could be used as additives in different applications in the pulp and paper, food, pharmaceutical, and cosmetics industries. The most promising applications of lignin derivatives include adhesives, antioxidants, dispersing agents, and intermediate chemicals. Value-adding modifications for compounds present in bark and other residue materials will also be established. This approach will lead to new and advanced processes and technologies to convert wood into high-value added, CO_2-neutral, "green," and biodegradable products.

11.6 Conclusions

Today, enzymes are used in the pulp and paper industry as process aids, leading to savings in chemical or energy consumption. The well-established applications include enzyme-aided bleaching, deinking, refining, and pitch control. In general, the implementation of biotechnical process stages to mill-scale operation has been slow. In order to be accepted at mill scale, enzymatic applications must outperform traditional chemical methods and result in economical benefits without changing the product quality. It seems that new applications of enzymes in pulp and paper manufacture will be found in the field of targeted modification of fibers for specialty products. Lignocellulosic raw materials (wood and annual plants) provide, however, an increasingly important source not only for present fiber products, but

also for a large number of intermediates, specialty chemicals, and fuels. Thus, it is a challenge for the biotech industry to develop more efficient biocatalysts for these applications. The knowledge generated on lignocellulosic enzymes, presently applicable for textile, detergent, feed as well as pulp and paper industries, is applicable also for these new targets.

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12
Biofuels: Production and Applications

Alexandre Rojey and Frédéric Monot

12.1
A Renewed Interest in Biofuels

Biofuels are currently benefitting from renewed interest worldwide. They are considered as a promising option for reducing the dependence of energy-consuming countries on oil imports. At the same time, they can help to curb CO_2 emissions and thus provide an answer to the challenge of global climate change.

The development of biofuels is also driven by the positive experience of their wide use in Brazil, the United States, and to a lesser extent in Europe. High crude oil prices also make biofuels more competitive.

Therefore, ambitious development goals are being set up in most industrial countries. The United States and the European Union plan to bring biofuels production from a marginal level around 1% of engine fuels to a significant penetration in the engine fuels market, above 10%.

The question which arises is then to know whether enough biomass resources are available and whether the technology is ready for such a rapid progression. Furthermore, will it be possible to ensure that such production levels are sustainable?

These are the main questions which are considered in this chapter.

12.2
Present Conversion Pathways

At present two main types of biofuels are used: ethanol, in spark-ignition engines and vegetable oil methyl esters (VOME) in diesel engines. Ethanol is currently made from two types of crop (Figure 12.1): sugar-producing plants (sugar cane, sugar beet) and starch-producing amylaceous plants (wheat, corn). Glucose is obtained from starch through hydrolysis in the presence of enzymes (amylase and amyloglucosidase).

Ethanol is produced from sugar by fermentation. It can be used pure, blended with motor fuels, or in its ether form, ETBE, produced by reaction with refinery

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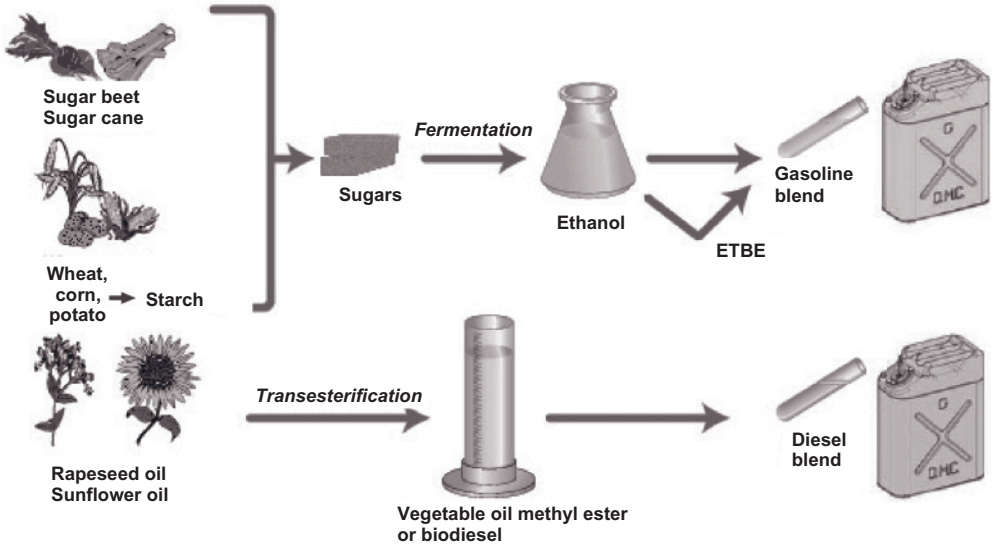

Figure 12.1 Present conversion pathways.

or petrochemical isobutene. Although ethanol can be blended with gasoline at low concentrations, between 5 and 10%, and used in an engine without major adaptations, some difficulties can arise due to its comparatively high vapor pressure and tendency to demix in the presence of water. In Europe, therefore, ethanol is mainly used in the form of ETBE in order to avoid these drawbacks.

Ethanol can also be used at high concentrations (typically 85%, in E85) in specially adapted engines. It is even possible to use both E85 and a standard gasoline or a blend of these two fuels in any proportion, in a flex-fuel vehicle (FFV). In such a vehicle, the engine is adjusted automatically according to the fuel composition being used.

Ethanol is the biofuel most widely used at present. Global production of ethanol motor fuels reached 27 million tonnes (18 Mtoe) in 2005, almost double the level in 2000. Production is concentrated in Brazil and in the United States. In 2005, Europe produced about 750 000 tonnes of ethanol for motor fuels and imported 200 000 tonnes to cover consumption. The bulk of production took place in Spain, Sweden, Germany, and France [1].

In Europe, due to the high share of diesel engines, biodiesel, produced by the transesterification of vegetable oils, has been favored. Total worldwide production of biodiesel remains low compared with that of ethanol, amounting to around 4 Mtoe in 2005. Close to 90% is produced in Europe.

Liquid biofuels in the EU 25 amounted to 2040 ktoe in 2004 or about 0.7% of the market. Biodiesel from rapeseed predominates, with production of almost 2 million tonnes in 2004, mainly in Germany, France, and Italy.

Other transport fuels are currently produced at low market volumes, for example, biogas in Sweden or pure vegetable oil in Germany.

Table 12.1 Production cost for biofuels as compared with oil-derived gasoline and diesel fuel.

	EtOH Europe	EtOH Brazil	EtOH USA	VOME Europe	Gasoline US$60/bbl	Diesel US$60bbl
€/l	0.4–0.6	0.2	0.3	0.35–0.65	0.32	0.36
€/GJ	19–29	10	14	10.5–20	9	10

EtOH, ethanol; VOME, vegetable oil methyl ester.
From IFP/IEA Data December 2006 [1].

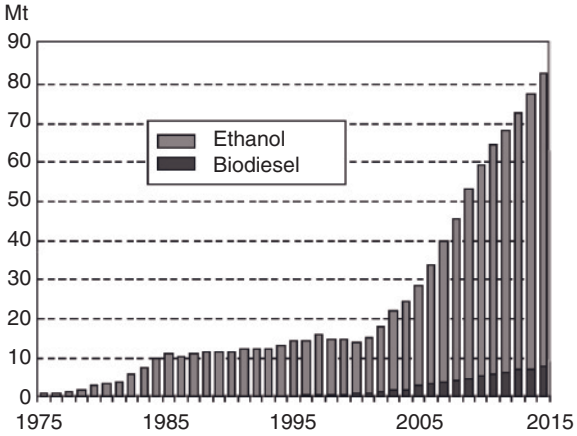

Figure 12.2 Trends in world biofuel production. From F.O. Light, Christoph Berg, World Biofuels, Seville, 2006 [2] .

Although production levels are already quite significant, biofuels still represent a small share, slightly higher than 1% of the total energy consumed for road transportation. If a significant contribution is expected from biofuels, they must move up from an additive position (a few percent) to a substantial blending fraction of the transport fuel consumption (more than 10%).

In economic terms, because of recent increases in oil prices, biofuels are starting to become more competitive, but still require public support as shown in Table 12.1. Ethanol produced from sugar cane in Brazil is the most competitive biofuel, with a cost quite close to that of gasoline. If, in the future, the price of oil increases further as is generally expected, biofuels might become directly competitive on a purely economic basis.

A rapid increase in the production of biofuels is expected in the coming years as shown in Figure 12.2. In the United States the goal is to reach a share of 4% by 2010 and 20% by 2030. The European Union has established an initial goal of 5.75% for the biofuels share by 2010. The biofuels share is expected to increase in the future, as reflected in Biofrac's vision, targeting a substitution rate of 25%

by 2030 [2]. In order to support such an evolution, the EU has decided to implement an ambitious regional strategy designed to further encourage the development and production of biofuels. The Council decision taken on March 8–9, 2007 concerning the long-term strategy for the development of renewable energy sources (RES) in the EU, mentions that out of the agreed 20% goal for RES by year 2020, 10% should be derived from biofuels.

12.3 Biodiesel Production from Vegetable Oils and Fats

12.3.1 Esterification Processes

Biodiesel is presently produced from oil-seed crops (rapeseed, sunflower, and soybean), converted into methyl esters (fatty acid methyl ester or FAME).

The direct use of vegetable oils as biodiesel fuel has been considered in the past and is sometimes still now. The properties of vegetable oils are not well suited for such a direct use, however, being too heavy, too viscous and not stable enough. When used in internal combustion engines they lead to deposits, to poor performance and possibly also mechanical failure [3].

Vegetable oils are predominantly triacylesters of glycerol (triglycerides). By reaction with methanol through a transesterification reaction, they are transformed into methylesters and glycerol. Methylesters have the required properties for use in diesel engines.

It is anticipated that future biodiesel processes will use a broader range of renewable oil feedstock. Palm oil and not yet very well explored renewable high-yield non-food feedstocks, such as jatropha, different types of algae, as well as further industrial waste streams containing triglycerides will gain market shares.

At present, the most commonly used technology for biodiesel production is based on homogeneous catalysis. Various catalysts are used: basic (e.g., hydroxides, amines) or acidic (e.g., mineral, sulfonic, zeolites, resins), titanium alcoolates, or metal oxides, and processes can be batch or continuous. Continuous processes are best suited for large capacity plants, over 100 000 tons per year.

Vegetable oil, methanol in excess, and the catalytic solution are introduced in reactors in series at low pressure (<3 bars) and at a moderate temperature (45–90 °C). The purity of the glycerol co-produced is within the range 80–90%. This means that it requires a further purification for most applications in the chemical industry.

A new technology called the Esterfip-H process, which uses a heterogeneous zinc aluminate catalyst, has been developed by IFP [3] and is being marketed by Axens. The process, which operates on a continuous basis, achieves a high yield and a higher purity glycerol, making it suitable for many applications in the chemical industry. The process flowsheet is shown in Figure 12.3. Two industrial plants are operating, one in France and one in Sweden, and six others are under construction, with a cumulative capacity of 1.3 million tons per year.

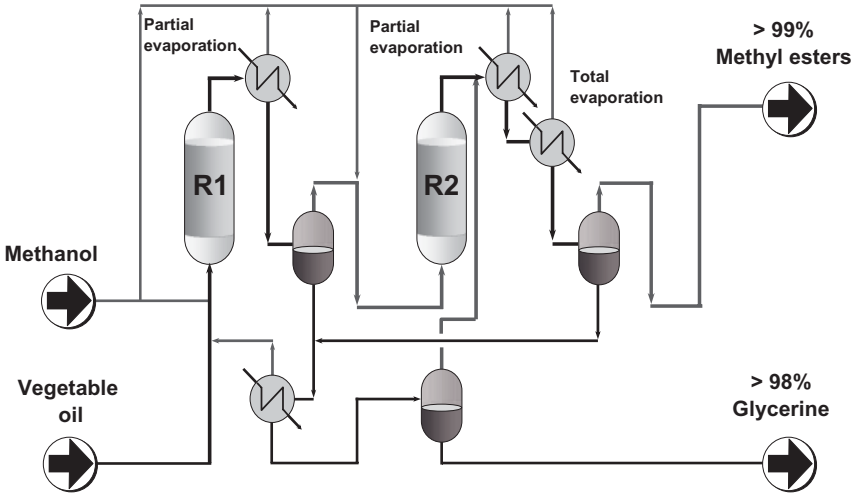

Figure 12.3 Heterogeneous catalytic process for producing fatty acid methyl ester.

Table 12.2 Main characteristics of gasoil, rapeseed oil, and fatty acid methyl ester (FAME).

Characteristics	Gasoil	Rapeseed oil	FAME
Specific weight (kg/m^3)	820–860	920	880–885
Viscosity at 40 °C(mm^2/s)	2–4.5	30.2	4.5
Cetane index	>49	35	50
Flash point (°C)	≥100	Decomposition above 320 °C	170–180

New developments are under way for producing ethylesters (fatty acid ethyl ester or FAEE) using a transesterification process in the presence of ethanol instead of methanol. The advantage is that FAEE can be produced from biomass only, whereas in the FAME process, methanol is generally produced from natural gas.

The transformation of vegetable oils into FAEE is more difficult to achieve than the transformation into FAME. It is especially difficult in the case of a process using a homogeneous catalyst because in the presence of glycerol and the catalyst the reverse reaction tends to occur during the distillation process. This makes the use of a heterogeneous catalysis appear to be more promising. Such a technology is under development but is not yet commercial.

12.3.2
Properties of Vegetable Oil Esters

Table 12.2 compares the main characteristics of standard diesel fuel derived from oil, a pure vegetable oil, and FAME produced from rapeseed oil. This shows that

FAME has properties similar to those of gasoil, in terms of specific weight, viscosity, and cetane index, which is not the case of rapeseed oil. Vegetable oil esters which are used as biofuels have to comply with specifications. In the European Union, they are defined by the norm CEN 14214, but each country has its own specifications, which define a range of properties (specific weight, viscosity), the maximum level of impurities, and the stability towards oxidation. Only products that comply with these specifications can be safely used in internal combustion engines.

The properties of vegetable oil esters vary according to the vegetable oil used. Esters derived from soybean and sunflower oils contain comparatively high levels of unsaturated molecules (characterized by a high iodine index), and in the European Union these have to be blended with rapeseed oil methylester. Esters derived from palm oil have a high pour point and cannot be directly used for the European market. These properties can be improved by crystallization and filtering.

12.3.3 Hydrogenation Processes

Another way to transform triglycerides contained in vegetable oils is through hydrogenation. Catalytic hydro-treatment of vegetable oils at 300 °C makes it possible to obtain a mixture of *n*-paraffins, propane, and water. All unsaturated components can be eliminated and it is therefore possible to obtain a high-quality biodiesel from a wide range of feedstock, including animal fats. No glycerol is produced, which means that no specific outlet is needed for glycerol as a co-product, but the propane value is comparatively low.

The process requires hydrogen (around 3.6% by weight of the feedstock, in the case of rapeseed oil), which means that it is best suited for units operated in refineries. Thus, it is possible to consider either a co-processing operation for converting mixtures of oil and biomass-derived products or units dedicated to the conversion of biomass products.

Extensive development has been carried out by Fortum and Neste Oil in Finland. As the quality of the products achieved is very similar to that of products obtained in a Fischer-Tropsch process, the process developed by Neste Oil has been called NextBtL, as a reference to the BtL process described later in this chapter. Nevertheless, the basic conversion process is quite different [8].

This pathway not only yields diesel fuel of very high quality, but also allows some flexibility in the choice of the feedstock (palm, soybeam oil, and even animal fats). Hydrogen consumption increases the cost of production, and therefore the technology is best suited for the conversion of saturated oils and fats, such as palm and copra oils, and also animal fats.

Several plants producing around 200 000 tonnes of biofuel per year are under construction in Europe.

12.4
Ethanol and ETBE Production

12.4.1
Ethanol Production from Sugar and Starch

When starting from sugar or starchy plants, the first step is to extract the sugar (Figure 12.4). In the case of the sugar cane, this is done by grinding and pressing, in order to separate a sugar solution from bagasse, which is the lignocellulosic harvested part of the plant. The bagasse can be used as a fuel, which helps to improve the energy and carbon balance. In the case of sugar beet, the sugar is extracted by hot water through a diffusion process. The separated pulp is used for animal feeding while the extracted sugar, saccharose, can be directly sent to the fermentation step.

Starch separated from cereal grains has to be hydrolyzed in order to obtain glucose. In the past this was done in presence of chlorhydric acid, but nowadays it is achieved in the presence of enzymes, which increases the glucose yield and improves the economics (especially in the dry milling process). During a first step, the starch suspension is liquefied at 105 °C in the presence of α-amylase. During a second step at 60 °C and at a pH around 5, glucose is produced by using a second enzyme, glucoamylase.

After concentration, the sugar solution is sent to the fermentation section. The basic stoiechiometric reaction is the following:

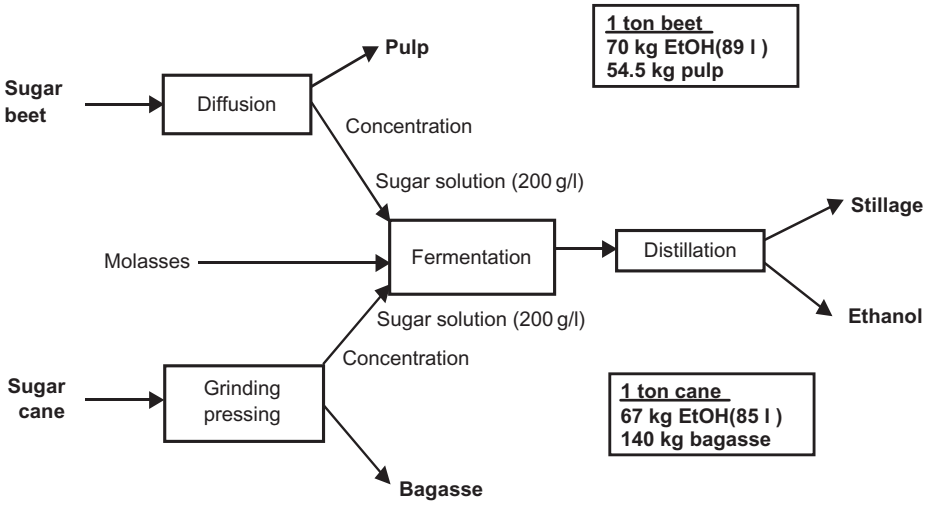

Figure 12.4 Production of ethanol from sugar plants.

Transforming sugars into ethanol by fermentation has been carried out for many years. Many microorganisms, yeasts, and bacteria, can be used. Yeasts of the *Saccharomyces* genus (especially *Saccharomyces cerevisiae*) are the most suitable for achieving a high yield. They are robust and very stable in industrial, non-sterile conditions. Among bacteria, *Zymomonas mobilis* is well adapted, but it also exhibits some drawbacks, such as the need to use sterile conditions for fermentation and the difficulty of separating it, due to its small size.

The fermentation step is now often performed in a continuous way, in a cascade of stirred reactors. Yeasts can be recovered through a filtration or centrifugation step and recycled at least partly, although the use of dry or concentrated suspensions of yeasts is an alternative of growing interest.

Ethanol concentration at the exit of the cascade is generally higher than 10% v/v. Conversion yields can amount to around 92% of the theoretical yield.

The fermentation process is exothermic and liberates 1.2 MJ/kg of produced ethanol.

12.4.2
ETBE Production

In Europe, ethanol is widely used in a derivative form, the ethyl-tertiary-butyl-ether (ETBE). ETBE is produced by reacting isobutene with ethanol and is used to avoid some of the drawbacks already mentioned concerning the use of ethanol in the gasoline pool.

The reaction is reversible and exothermic. It is operated at a comparatively low temperature (below 100 °C), in the presence of an ion exchange resin, such as an acid reticulated sulfonic polystyrene resin, in the presence of an excess of ethanol, in order to improve the conversion yield.

At the exit of the reaction zone, ETBE is separated by distillation. ETBE is recovered at the bottom of the column. At the top of the column, ethanol is separated together with light hydrocarbons with which it forms azeotropes.

This design can be improved by using a catalytic column. Catalyst is placed in the upper section of the distillation column in order to increase the conversion yield. Various technologies are available from CdTech, UOP, and IFP/Axens.

12.4.3
Properties of Ethanol and ETBE

The main properties of ethanol and ETBE are summarized in Table 12.3. Ethanol has a comparatively high octane number and a density close to the density of gasoline. As already mentioned, the main drawbacks are its high miscibility with water which can lead to phase separation, and its high vaporization latent heat, which can make engine start-up more difficult in winter. The properties of ETBE are much closer to those of standard gasoline and it can be easily incorporated in a gasoline pool.

Table 12.3 Main properties of ethanol and ethyl-tertiary-butyl-ether (ETBE).

	Ethanol	ETBE	Gasoline (standard)
Density (g/mol)	46.07	102	102.5
Volumetric mass (kg/m^3)	794	750	735–760
Vaporization latent heat (kJ/kg)	854	321	289
Boiling point (°C)	78.4	72.8	30–190
Low heating value (kJ/kg)	26 805	35 880	42 690
RON	111	117	95
MON	92	101	85

RON, Research Octane Number; MON, Motor Octane Number.
From Ballerini *et al.* [3].

12.5 The Need for New Developments

The large increase planned for the penetration of biofuels raises some difficult issues. The first is the availability of the land needed to produce the required biomass and the competition with food. In the European Union, reaching the 5.75% objective for 2010 implies a production of 16.6 million tonnes of biofuels (5.6 Mtoe of bioethanol and 11 Mtoe of biodiesel). Attaining this output will require about 82 million tonnes of biomass and a land area of about 13.8 Mha. This is to be compared with the arable land area, which amounts to 115 Mha, of which 8.3 Mha is left fallow. These figures show that already in 2010 the fallow land area will not be large enough to produce the needed quantities of biofuels and that a competition arises with food applications if these biofuels quantities are to be produced in Europe. Looking further ahead, a substitution of 10% of gasoline and diesel produced in the European Union and in the United States would require using around 40% of arable land in these regions [5]. If biofuels penetration has to exceed 10%, clearly, new sources of biomass have to be considered.

The situation becomes very different if lignocellulosic biomass can be used for producing biofuels. In such a case, much more biomass becomes available. This issue is discussed in Section 12.6.

A second issue is the sustainability of the processes considered. Different sustainability criteria have to be considered. A first one is the CO_2 balance on a life-cycle basis, from biomass production to the final use of biofuels in the internal combustion engine. If fossil fuels are used to supply energy to the conversion process, the overall gain may be small or even negative in some cases. It is therefore very important to assess properly the overall gain in CO_2 emissions. The contribution of other greenhouse gases, such as nitrogen protoxide (N_2O) as a result of the use of nitrate fertilizers, also has to be included in the balance.

Other important issues also have to be taken into account, especially in relation to biomass production. It is important that biomass production does not lead to

massive deforestation or the loss of soil nutrients. This sustainability issue is discussed in Section 12.9.

This means that new processes are required for the development of second-generation processes, which can use lignocellulosic biomass as a feedstock and help to improve the sustainability of the biofuels option.

12.6 Lignocellulosic Biomass Resources

Conversion of lignocellulosic biomass makes it possible to mobilize large quantities of straw and wood which are not currently exploited for energy. In Europe, 41.3 million tonnes of cereal straw and oilseed straw as well as 164 million tonnes of wood could be used to produce around 33 Mtoe of biofuels a year. The total amount of crop residues (including cereals, oilseeds, sugar beets, and potatoes) is around 490 million tonnes in Europe (EU 25) and 5 billion tonnes worldwide.

Forestry waste wood represents a total volume which is nearly equal to the amount of wood which is exploited. It amounts to around 2.3 billion tonnes of dry material worldwide, that is, 1 Gtoe (0.43 toe/tonne dry material).

The quantity of lignocellulosic biomass potentially available is therefore quite large. Production of biofuels does not compete in this case with food applications. Collecting the biomass and organizing the appropriate logistics represent the main difficulty. There may be also a competition with other applications: direct use of biomass in a boiler for a direct energy production, applications in the paper-pulp industry, use for materials and chemicals production.

Although they require more land, short-rotation crops (*Miscanthus*, short-rotation coppices, etc.) offer a good potential. Furthermore, it seems possible to use land that is not well suited for agricultural purposes. The amounts of water, fertilizers, and pest control products can be reduced and therefore such a production of biomass could become attractive in the future. Its future potential remains difficult to assess.

12.7 Production of Ethanol from Lignocellulosic Biomass

12.7.1 Overall Conversion Scheme

The lignocellulosic biomass is composed of three polymers: cellulose, hemicelluloses, and lignin. Cellulose is a linear homopolymer of D-glucopyranose units. As in starch, glucose is the basic structural element. Glucose can be recovered by hydrolysis, but hydrolysis is much more difficult to perform than in the case of starch because the linkages are more stable [9]. Furthermore, because of the formation of hydrogen bonds between parallel chains, cellulose molecules form a rigid structure in fibrils, which is difficult to break. This is the main limitation of

Table 12.4 Composition of lignocellulosic biomass.

Biomass	Lignin (%)	Cellulose (%)	Hemicelluloses (%)
Softwood	27–30	35–42	20–30
Hardwood	20–25	40–50	20–25
Wheat straw	15–20	30–43	20–27

the conversion process. Hemicelluloses sometimes contain large pentose contents, which cannot be used directly by conventional *Saccharomyces* strains for the production of ethanol. Lignin cannot be converted into ethanol.

The proportion of these three fractions present depends upon the plant used, as shown in Table 12.4. These three fractions are mingled in a single structure, limiting the access of enzymes to the cellulose. It is therefore necessary to first break the matrix formed by these three polymers by a pretreatment step. During pretreatment, it is necessary to avoid thermal degradation of sugars into compounds that may be toxic for the yeasts used for fermentation or that may exert an inhibiting effect on the enzymes.

After the pretreatment step, cellulose is converted to glucose in presence of enzymes. Ethanol produced from glucose by fermentation undergoes distillation and final purification.

12.7.2 Biomass Pretreatment

The pretreatment step aims at making the cellulose accessible to enzymes. In certain cases, it separates the cellulose fraction from the hemicellulose fraction, which can be extracted in the liquid phase. Two main pretreatment processes are currently used:

- In the case of acidic prehydrolysis, the biomass is treated in the presence of a dilute acid solution (containing 0.3–2% of sulfuric or chlorhydric acid), at a moderate temperature during around 15–20 min. Hemicelluloses are almost completely hydrolyzed. A small fraction of lignin is transformed too. Processes including a second step operated at higher temperatures (240 °C) and shorter residence times (2–3 min) are being investigated in order to improve the quality of the separation.
- Steam explosion consists in bringing the material to a high pressure (15–23 bars) and high temperature (180–240 °C) in the presence of steam for a short time and then suddenly expanding the steam, in order to break the structure of the lignocellulosic matrix. During the high-temperature heating phase, partial hydrolysis of hemicelluloses (especially in mild acid conditions) and fusion of lignin occurs. A recondensation of lignin occurs during the rapid expansion phase. The short residence time limits the quantity of the degradation products.

12.7.3
Enzymatic Hydrolysis

The enzymatic hydrolysis of cellulose is the main development area at present. Three types of enzymes are required to achieve complete hydrolysis of cellulose into glucose (Figure 12.5):

- endo-glucanases break cellulose chains in a random fashion
- cellobio-hydrolases liberate glucose dimers at both ends of a cellulose chain
- β-glucosidases produce glucose from short oligomer chains.

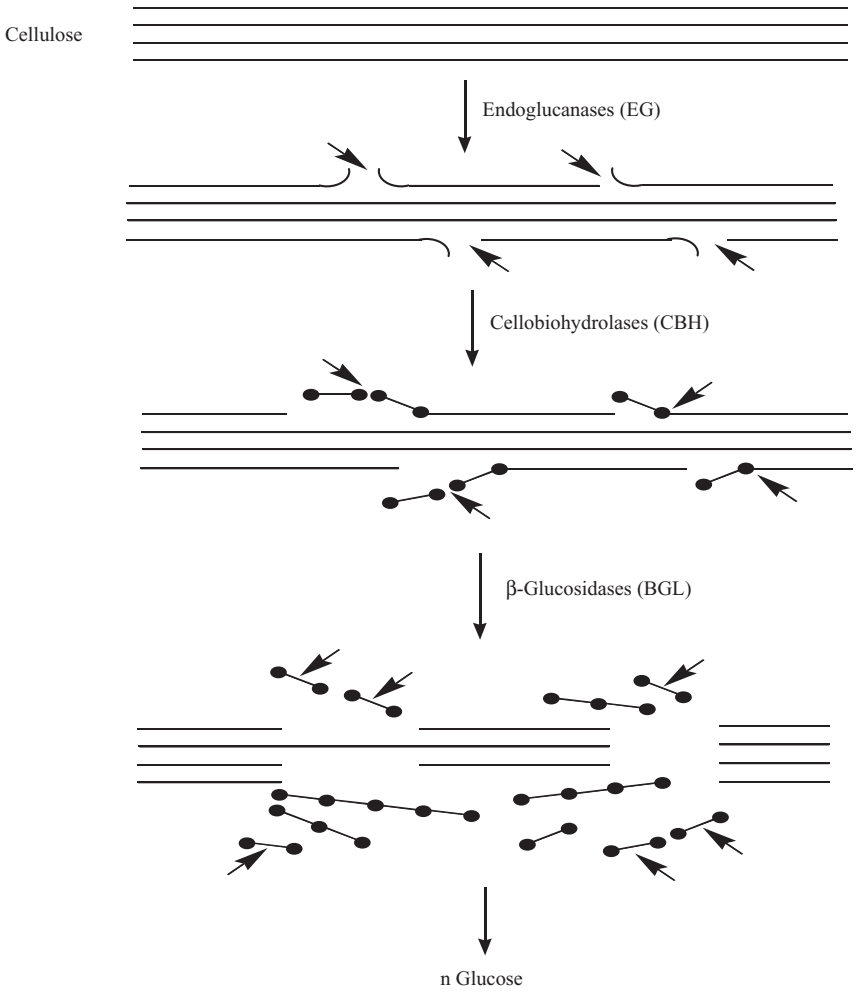

Figure 12.5 Action of cellulolytic enzymes on cellulose microfibers.

Cellulases are typically produced by fungi of the *Trichoderma* type. This is currently a very active area of research.

12.7.4 Fermentation of Glucose and Pentoses

Glucose obtained by hydrolysis can be converted into ethanol by the same type of process as used in the case of glucose obtained from starch. The main differences arise from a lower initial glucose concentration and the presence of some contaminants (such as furfural, hydroxy-methyl furfural and acetic acid) coming from the pretreatment step, which may have an inhibitory action.

By converting glucose into ethanol, only cellulose is used, which represents in most cases less than 50% of the initial feedstock, as shown in Table 12.4.

Research is therefore going on into the development of processes able to convert pentoses obtained from hemicelluloses into ethanol. Yeasts have been genetically modified in order to include appropriate genes. The productivity obtained was apparently too low for large-scale applications but very recent breakthroughs suggest that possible industrial uses could come next. Other attempts have been made at the genetic modification of bacteria, but no industrial applications are yet available [7].

The acetone-butanol-ethanol process (ABE) has been known for many years but productivity and yields were lower than those for ethanol production processes. However, biobutanol has advantages as a biofuel compared to ethanol: lower vapor mixture, low water solubility, and higher volumetric heat of combustion. It can therefore be more easily incorporated in a gasoline pool than ethanol. BP and Dupont have recently formed a new venture in order to develop biobutanol manufacturing and marketing [8].

12.8 Production of Biofuels Through the Thermochemical Pathway

12.8.1 Present Status

The thermochemical pathway is characterized by the use of high-temperature (500–1500 °C) transformations, such as pyrolysis or gasification. It can be applied to the convertion of any lignocellulosic feedstock.

Synthesis gas formed by a mixture of carbon oxide and hydrogenation is produced by gasification in presence of oxygen. Syngas can then be converted to liquid fuels by using a Fischer-Tropsch process which produces waxes, followed by a hydrocracking stage for transforming waxes into motor fuels as shown in Figure 12.6.

Such a process for producing synthetic fuels from biomass is often called the BtL process (for biomass-to-liquids) in analogy to the process used for pro-

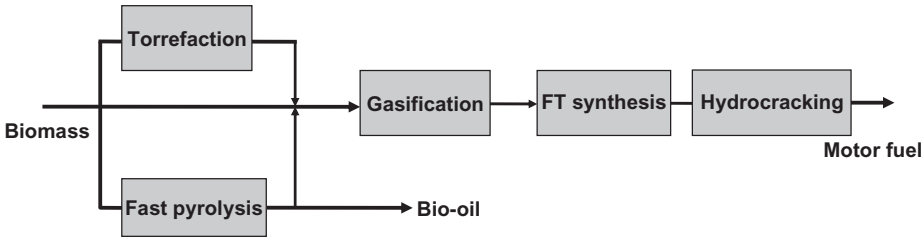

Figure 12.6 The thermochemical pathway for producing liquid fuels from biomass.

Table 12.5 Comparison between different pyrolysis conditions.

	Heating velocity	Temperature	Main product yield (weight %)	Energy yield
Slow pyrolysis	<50 °C/min	500 °C	Charcoal (<35%)	60%
Fast pyrolysis	>100 °C/s	500 °C	Bio-oil (50–80%)	75%
Pyro-gasification	>100 °C/s	>800 °C	Gas (>70%)	90%

ducing synthetic fuels from natural gas (gas-to-liquids – GtL) or from coal (coal-to-liquids – CtL).

Pyrolysis and/or torrefaction can be used for biomass pretreatment before gasification, for transforming the biomass into a homogeneous phase, which can be more easily injected into a gasifier. It is also possible to transform the biomass locally, in order to make it easier to transport the product to the gasifier.

Fast pyrolysis can be used to produce a bio-oil. Such a bio-oil cannot be used directly as it contains many products which are not desirable in a biofuel (e.g., phenols, sugars, alcohols, organic acids, and aromatic compounds).

Research is being carried out to find a method of converting such "bio-oils" into motor fuels by hydrogenation. For the time being, such a "direct" conversion pathway has not yet been proven to be feasible, because of the large quantity of hydrogen which is needed and the nature of the products obtained.

12.8.2 Pyrolysis and Torrefaction

Pyrolysis uses heat to break biomass down into three phases: solid (coal), liquid (bio-oil), and gas (mostly carbon dioxide, carbon monoxide, hydrogen and methane). The proportion of the three phases thus obtained depends upon the temperature and residence time (or heating velocity). Typical yields obtained for different heating velocities are listed in Table 12.5.

Slow pyrolysis, which is the better-known process, yields a solid (charcoal). Fast pyrolysis is presently the preferred option for the BtL pathway. It involves maintaining a temperature of 500 °C for a few seconds.

Bio-oil and/or the liquid–solid mixture obtained by fast pyrolysis can be easily transported and injected in the gasifier. The gas which is produced can be used to supply the energy needed for pyrolysis. Different technologies have been developed at pilot scale using a fluidized bed (Dynamotive), a circulating bed (Ensyn, VTT), a rotating cone (Twente University, BtG), a cyclone (TNO), a double screw (FZK), or an ablative reactor.

Torrefaction is operated at conditions which are those of an ultimate drying step. Temperature is increased progressively up to a final plateau between 240 and 300 °C. The residence time varies between a few minutes and an hour, according to the temperature. Because it is carried out at temperatures much lower than those used in pyrolysis, the process is much less energy intensive.

Torrefaction reduces the mechanical strength of lignocellulosic biomass. As a result it becomes much easier to grind and it is thus possible to obtain a finely divided solid, suitable for certain gasification technologies.

12.8.3
Production of Synthesis Motor Fuels from Biomass

At high temperatures, biomass first undergoes a decomposition process. Then, in presence of oxygen or water, it is oxidized and forms a mixture of carbon oxide, carbon dioxide, and hydrogen. High temperatures, between 1200 and 1300 °C, are required to eliminate tars or acidic compounds from the synthesis gas thus obtained. This means that the presence of oxygen is required, in order to reach high temperatures. Entrained-flow gasifiers seem to be best adapted for large-scale applications.

The first entrained-flow gasifier for coal gasification was developed by Koppers about 50 years ago. The coal was fed to the gasifier together with oxygen through pairs of diametrically opposed burners at the bottom of the gasifier and the synthesis gas was produced at the top. The Shell Coal Gasification Process and the Prenflo process are derived from this initial concept. They operate at a high pressure (30–40 bars) and with a high efficiency [9].

An entrained-downflow reactor was developed in Germany first by Noell and later by Future Energy GmbH. The concept is illustrated in Figure 12.7. After a quench with water, synthesis gas and ashes are recovered at the bottom of the reactor. Ashes are obtained in liquid phase (slag).

Such a reactor can be directly used for gasifying biomass as long as it can be operated in non-slagging conditions within the reactor. Slag and molten biomass ash can dissolve the refractory layer. The feedstock should be therefore low in ash content. Such a condition can be met with bio-oil, but is more problematic in presence of char [9].

Synthesis gas has to be further treated before being sent to the Fischer-Tropsch synthesis section. Solid particles have to be eliminated by filtration. Acid contami-

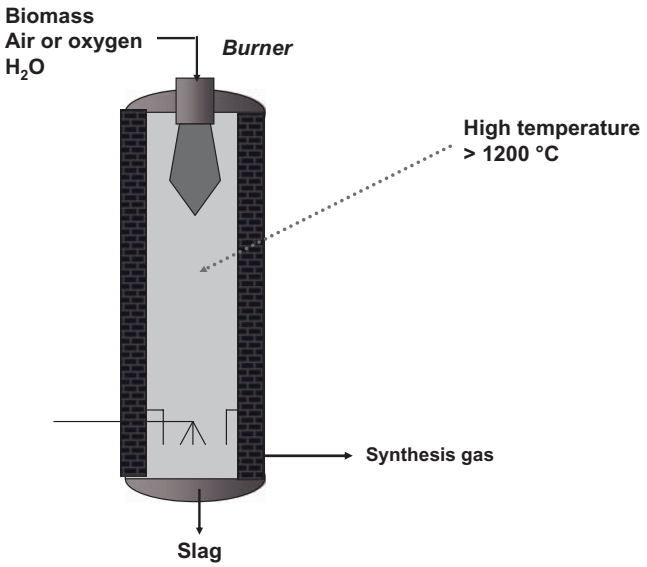

Figure 12.7 Entrained-downflow reactor.

nants such as HCl, HF, HCN, H_2S, and COS are removed through solvent wash. It is also necessary to separate CO_2. A final purification step is achieved by using reactive solid phases.

The hydrogen-to-carbon oxide ratio in the synthesis gas sent to the Fischer-Tropsch section also has to be adjusted. A shift-conversion step during which carbon oxide is reacted with water can be used for that purpose.

Fischer-Tropsch synthesis was first developed on an industrial scale during World War II. It was then used on a large scale by Sasol in South Africa to produce motor fuels from coal as a result of the embargo imposed on South Africa at that time. Various technologies have been developed (Shell, Statoil, Sasol, Exxon, IFP/ENI).

The Fischer-Tropsch synthesis process requires a catalyst. Two types of catalysts are used: iron based and cobalt based. The most recent processes use cobalt catalysts, which favor the formation of long-chain hydrocarbons (waxes). A mild hydrocracking step is then needed to obtain motor fuels, diesel fuel, and kerosene.

The diesel fuel produced through this process is of very high quality. It contains no sulfur or nitrogen, no aromatics and has a high cetane number (around 70).

12.9 Biorefineries

The growth of the biofuels market should lead in the future to the development of more complex and more integrated production systems. Such "biorefineries" will be able to produce a wide range of fuels and by-products from diverse feed-

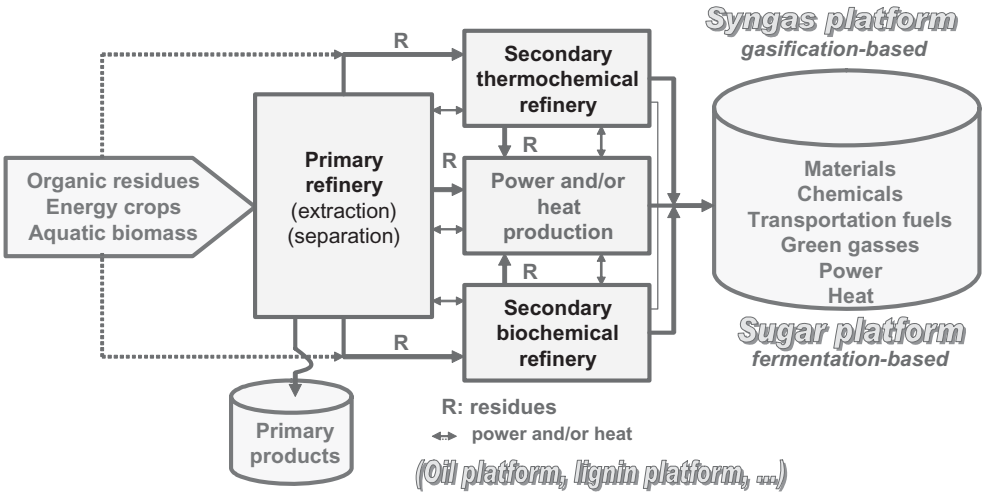

Figure 12.8 Biorefinery integrationg biochemical and thermochemical processes. From Biofrac Vision report [2].

stock, whereas presently a single biofuel is produced in a standalone unit from a single feedstock (typically FAME from rapeseed oil).

In such a biorefinery it will be possible to consider an integration of thermochemical and biochemical processes as shown in Figure 12.8. It will also be possible to integrate biomass transformation in an oil refinery and to co-process oil and biomass, or to combine biofuels production with other biomass transformations, such as sugar factories or pulp and paper mills.

Some relatively simple biorefineries already exist (e.g., sugar/ethanol plants, oil seeds crushing/transesterification plants, pulp and paper mills, biodiesel unit integrated into oil refinery), but many alternatives remain to be explored.

The polygeneration of energy, hydrogen, and biofuels is also an attractive possibility in the case of the thermochemical transformation of biomass through synthesis gas production.

12.10 Biofuels and Sustainability

Sustainability is a crucial issue, as both the advantages and potential drawbacks for the environment are often debated. The main concerns that have been expressed are the competition with food applications and a possible negative impact on the environment. Quite clearly, a large penetration of biofuels is possible only if biofuels can be produced in a sustainable way.

The development of conversion processes from lignocellulosic biomass is very important because it makes it possible to avoid competition with food applications.

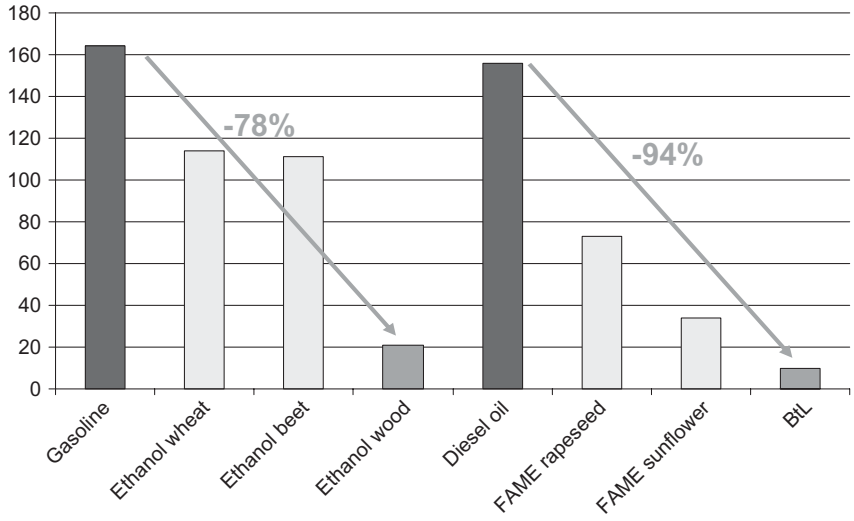

Figure 12.9 CO_2 emissions per unit energy for different biofuels. From JRC/EUCAR/CONCAWE 2006 [10].

It also makes it possible to improve the CO_2 balance on a well-to-wheel basis, as shown in Figure 12.9 [10]. Information about this essential topic can be found also elsewhere [11–14]. By producing biofuels from lignocellulosic biomass, it will become possible to reduce global CO_2 emissions per unit energy from 75% to more than 90% [15, 16].

In addition, other criteria have to be taken also into account. Biomass has to be produced in a sustainable way, avoiding a permanent soil damage, or any transformation which might have a negative impact on the environment, such as deforestation.

Other critical issues involve water consumption and possible competition for water resources, use of fertilizers, and pest control techniques, which may harm the environment. Therefore, it will be important in the future to introduce a certification system, enforcing compliance with such sustainability criteria.

12.11 Conclusion

During the present transition period, biofuels represent an attractive option both for reducing the dependence of energy-consuming countries on oil imports and for improving the CO_2 balance.

A large growth of biofuels production requires the development of innovative conversion processes for transforming lignocellulosic biomass. Such processes could also help to increase the environmental benefits of biofuels, but public

support will be needed to develop these new pathways as important technical and economic challenges remain.

In order to control the impact upon the environment, it will be necessary to develop rigorous assessment methods for sustainability criteria and to create appropriate regulatory frameworks for implementing their application.

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13
Environmental and Economic Aspects of Industrial Biotechnology

Barbara G. Hermann, Veronika Dornburg, and Martin K. Patel

13.1
Introduction

Chemicals and fuels are currently produced almost exclusively from petrochemical feedstocks derived from crude oil and natural gas. Producing the same or functionally equivalent chemicals and fuels from renewable resources can decrease the environmental impact, for example, emissions of greenhouse gases. In the light of surging oil prices as well as long-term emission targets, for example, the EU target of 15–30% reduction of CO_2 emissions by 2020, industrial biotechnology (also called "white biotechnology") applied to produce bio-based materials or fuels could contribute significantly towards achieving emission targets as well as towards reducing dependence on expensive fossil resources.

There are already many applications of biotechnology for the production of fine chemicals and specialty chemicals – an area which is expected to expand considerably in the short to medium term. In comparison, there is more uncertainty about when, how, and to what extent biotechnology will also play a role in the production of bulk chemicals.

In recent years, important steps have been made in research institutes, companies, and policy, with the goal of developing and applying industrial biotechnology in the production of chemicals and fuels, leading to high expectations in this field. New perspectives and opportunities have been opened by recent progress, due primarily to increased productivities and yields of fermentation. Further substantial progress in this area is expected, especially related to genetically modified microorganisms.

While there is a strong drive behind these developments, there has so far only been very little quantitative information available in the public domain on the current and future economic, environmental, and social implications of industrial biotechnology. Of the studies that have been published, almost all have focused on a single product, predominantly fuel ethanol [14, 34] but also on (poly) lactic acid [4, 43], and polyhydroxyalkanoates [2, 15]. However, these studies are not easily compared because of differing assumptions and methodologies. Furthermore, future improvements in technology are disregarded although they may

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substantially reduce the environmental, economic, and social impacts. To overcome these problems, we present and apply a generic approach that allows the environmental and economic impacts to be estimated based on the inputs of feedstock and energy to the process [11, 27].

The selection of products in this chapter was based on three criteria: (i) the feasibility of production by fermentation, (ii) available information on the stoichiometry of this process, productivity, and concentration of fermentation broth, and (iii) the potential to be sold in bulk quantities in the medium or long term (see [11, 27] for details). The following products were studied: 1,3-propanediol (PDO), acetic acid, adipic acid, butanol (from the acetone–butanol–ethanol [ABE] process), ethanol, lactic acid, polyhydroxyalkanoates (PHA), and succinic acid. In addition, four products that are formed by subsequent chemical conversion of these products were also considered: ethyl lactate (EL), ethylene, polylactic acid (PLA), and polytrimethylene terephthalate (PTT).

13.2 Methodology

13.2.1 Generic Approach

13.2.1.1 Methodological Background

A generic approach is a method allowing standardized comparisons between different processes based on a limited number of components. Many calculations can then be carried out based on a limited number of input data and an underlying database. This generic approach allows an ex ante estimation of the economic viability as well as the environmental effects of biotechnological processes for which pilot plant data do not yet exist or for which process data are not publicly available. This method can be applied to processes representing the current state-of-the-art as well as future technology. The results from this generic approach can then be compared to results for industrial biotechnology products calculated using industry data and/or petrochemical equivalents.

The first step of the generic approach is the preparation of a process flow diagram of the bioprocess, which converts fermentable sugar to the target industrial biotechnology chemical. Following the principle proposed by Landucci and Lynd [16, 21], these process flow diagrams contain standard modules (e.g., fermentation, ultrafiltration, evaporation). For each process flow diagram representing one production route, the mass balance containing the quantities of all inputs and outputs at the level of unit processes can be determined (see Sections 13.2.1.2 and 13.2.1.3). On this basis, an environmental assessment (see Section 13.2.2) can be performed and the costs related to all inputs and the investment costs can be estimated and the overall production costs calculated (see Section 13.2.3).

In order to ensure the comparability of the results, a common database for process inputs must be used for all calculations. This database consists of environmental

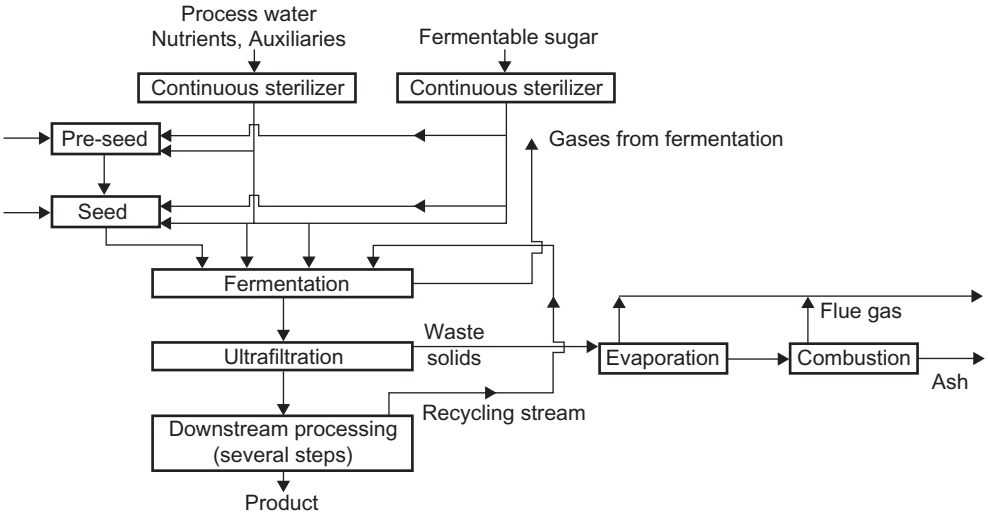

Figure 13.1 Simplified flowsheet as used in the generic approach.

background data on the global warming potential and non-renewable energy use as well as the market prices for chemicals, auxiliaries/utilities and fermentable sugar (see Sections 13.2.3.1 and 13.2.3.2). The functional unit was one tonne[1)] of organic chemical at the factory gate. This functional unit is then easily compared to the current petrochemical production of the same chemical or a functional equivalent.

13.2.1.2 Process Design of Industrial Biotechnology Routes

Separate process flow diagrams were made for current and future technologies, both with respect to the fermentation processes and downstream processing for product separation and purification. All process flow diagrams (prepared at the level of unit processes) consisted of the following sections (Figure 13.1): seed and inoculum trains (provision of microorganism), fermentation (conversion of fermentable sugar to the target product and by-products), filtration (removal of solid by-products) and downstream processing (several steps, to purify the target product). The material inputs to the system were fermentable sugar, water, nutrients, auxiliary substances, and utilities such as electricity and steam. The outputs were the target product as well as solid waste and wastewater.

13.2.1.3 Technology Assumptions for Industrial Biotechnology Routes

When preparing the mass balance, the mass flows of all compounds were estimated based on the following key parameters: yields, productivity, and broth concentration of the fermentation step (Table 13.1). The broth concentration[2)]

1) All tonnes referred to in this article are metric tonnes (ca. 1.102 short tonnes).
2) Broth concentration is defined as the mass of product relative to the total volume of the fermentation broth (in g/l).

Table 13.1 Type of fermentation and key data on concentration, productivity, and yields of fermentation for current and future technology.

Product	Type of fermentation			Concentration (g/l)	Productivity g/(l*h)	Yield g product/g glucose	Ref.
	Today/ future	Aerobic/ anaerobic	Batch/ continuous				
Acetic acid	Today	Anaerobic	Batch	18	0.15	0.50	[12, 20]
	Future	Anaerobic	Continuous	50	15	0.90	
Adipic acid	Today	Aerobic	Batch	20	0.42	0.17	[23]
	Future	Aerobic	Continuous	40	10	0.47	
Butanol	Today	Anaerobic	Continuous	20	0.36	0.42	[5, 8, 30]
	Future	Anaerobic	Continuous	45	15	0.50	
Ethanol	Today	Anaerobic	Continuous	100	2.20	0.46	[3, 10, 21]
	Future	Anaerobic	Continuous	130	50	0.47	
Lactic acid	Future	Anaerobic	Continuous	180	20	0.95	
PDO	Today	Aerobic	Both	100	1.67	0.41	[36]
	Future	Aerobic	Continuous	100	15	0.54	
PHA	Today	Aerobic	Batch	150	3.00	0.35	[2]
	Future	Aerobic	Continuous	150	10	0.43	
Succinic acid	Today	Anaerobic	Batch	80	1.8	0.88	[19, 38]
	Future	Anaerobic	Continuous	150	15	1.01	

determines the amount of water in the broth and therefore influences the energy required in downstream processing. Together with productivity[3)] it determines the residence time as well as the size of the fermentation equipment. The yield[4)] influences not only the required input of fermentable sugar but also the amount of waste biomass produced. Waste biomass is separated from the product stream by means of an ultrafiltration step that immediately follows fermentation. The waste biomass is dried and is then burned for steam production. Process water is recycled wherever possible in order to avoid excessive consumption.

As part of the generic approach, assumptions were made for the key parameters of current and future technology regarding fermentation and downstream processing (Table 13.1).

1) For current technology, both continuous and batch processes were assumed and calculations were based on data from industrial units, pilot plants, or laboratory experiments. For current fermentation technology, the values of yields, broth concentration and productivity in Table 13.1 were based on published data. Any fermentable sugar not converted to the target product was split between by-products, waste biomass and CO_2 emissions according to published data [2, 3, 5, 8, 10, 12, 19–21, 23, 30, 36, 38, 39]. Regarding lactic acid, generic calculations were only carried out for future technology because

3) Productivity is defined as the mass of the product divided by the volume of fermentation broth per unit of time needed to produce this amount (in g/[l*h]).

4) Yield is defined as the mass of the product divided by the mass of the fermentable sugar (in g/g).

industrial data for producing lactic acid according to today's technology were available [43].

2) For future technology, only continuous processes were assumed and calculations assume two to three decades of successful research and development (R&D, see Table 13.1), representing a possible upper level of technological feasibility. For future fermentation processes, we assumed a yield of 90 mol-% of the maximum theoretical yield. The remaining fermentable sugar (10 mol-%) is converted into waste biomass and CO_2 only, with an assumed carbon ratio of 1 : 2 for aerobic and 1 : 1 for anaerobic processes.[5] This implies that pathways to by-products can be suppressed, which will most likely require genetic modification of the microorganism. The estimates of future productivities were based on the experience from large-scale plants producing citric acid and ethanol as two representatives of advanced aerobic and anaerobic fermentation processes. Their future upper productivity levels were estimated by experts to reach 10 g/l per h for citric acid[6] and 50 g/l per h for ethanol.[7] Productivity of the other chemicals was assumed to approach the horizon values of citric acid for aerobic and ethanol for anaerobic fermentation, representing a comparable level of ambition. Future broth concentrations of continuous processes were estimated to be in the range of today's end-of-batch values because of interactions between productivity and concentration:

   - Future technology will rely on continuous fermentation at the point of maximum productivity of the microorganism with *in situ* removal of the product. The maximum productivity for current processes occurs at approximately half the maximum batch concentration.
   - For future technology, the broth concentration corresponding to this maximum productivity was assumed to be increased by a factor of 2, thereby resulting in broth concentrations in the range of current batch fermentation. The values assumed for these key technical parameters were critically reviewed by experts from industry and academia [27] and represent the technical potential after 20–30 years of R&D. However, predicting future key technical parameters always involves uncertainty.

Table 13.2 presents an overview of the types of downstream processes that were used to separate the fermentation products.[8] The types of downstream processing for today's technology were derived from literature.

5) This estimate is based on detailed calculations on the carbon splitting of 4 anaerobic and 4 aerobic fermentation processes. The aerobic processes have ratios around 1 : 2. For anaerobic processes the spread is much larger; since less metabolic CO_2 should be formed than for aerobic processes we assume a ratio of 1 : 1.

6) Productivities as high as 5 g/(l*h) have been reported [32], so an increase by a factor of two resulting in 10 g/(l*h) appears feasible within 20–30 years.

7) Some authors [17, 31] reported productivities even higher than 50 g/(l*h), but with current productivities around 2 g/(l*h), an increase beyond 50 g/(l*h) on an industrial scale appeared unlikely within 20–30 years.

8) Polylactic acid, polytrimethylene terephthalate, ethylene and ethyl lactate do not appear in Table 13.2 because they are derived by chemical conversion from one of the industrial biotechnology products listed in this table.

Table 13.2 Types of downstream processing used for separation in today's and future technology for the industrial biotechnology products.

	Today's technology	Future technology
Crystallization	Succinic [38], Adipic [46]	Succinic, Adipic
Distillation	PDO [36], Ethanol [10], ABE [30]	Ethanol, ABE
Electrodialysis	Succinic [48], Acetic [9]	Auccinic, Lactic, Acetic, Adipic
Enzymes	PHA [40]	PHA
Extraction	Acetic [47], PHA [40]	Acetic[1)]
Gas stripping	ABE [5]	ABE
Pervaporation	Ethanol [18]	PDO, Ethanol, ABE

1) For acetic acid future technology, there are not only flowsheets on extraction and electrodialysis but also one that combines both of these separation technologies.

Assumptions for future technology separation processes were based on a number of considerations:

- Precipitation was not considered viable for large-scale production because it involves the use of large amounts of chemicals and leads to low-value by-products such as gypsum and/or wastewater with high salt loads.
- Extraction and adsorption were considered acceptable future options because of the potential use of "green solvents" with clearly lower environmental impacts (e.g., in terms of carcinogenic and toxic effects) compared with current solvents.
- Membrane processes such as pervaporation, electrodialysis, and ultrafiltration were taken into account due to their (expected) low energy use. However, a significant amount of R&D will often still be necessary to put these membrane processes into use on an industrial scale.
- Today's as well as future technology assumed single-step evaporation up to a water–product ratio of 5 : 1 for evaporation processes; for larger proportions of water, double-effect evaporation was assumed and increased investment costs were accounted for.

13.2.1.4 Energy Use

The process energy for the system covered in the generic approach was determined by multiplying the mass and volume throughputs by the estimated specific energy use for each process step. The specific process energy as shown in Table 13.3 was estimated based on the literature, then calibrated (for a detailed description of the procedure see [27]).

13.2.2 Environmental Impacts Methodology

We analyze the environmental performance of producing bulk chemicals from biomass using industrial biotechnology considering current and future (2030)

Table 13.3 Key data on specific energy use of unit processes in fermentation and downstream processing [27].

Unit processes	Amount	Unit
Fermentation		
Sterilization	0.100	kg steam/kg $\text{medium}_{\text{fermentation}}$
Agitation	0.500	kW power/m^3 $\text{volume}_{\text{fermentation}}$
Agitation and aeration	3.000	kW power/m^3 $\text{volume}_{\text{fermentation}}$
Downstream processing		
Membrane filtration		
Microfiltration	2.000	kWh power/m^3 permeate
Ultrafiltation	5.000	kWh power/m^3 permeate
Diafiltration	5.000	kWh power/m^3 permeate
Nanofiltration	7.000	kWh power/m^3 permeate
Reverse osmosis	9.000	kWh power/m^3 permeate
Electrodialysis	0.100	kWh power/equivalent
Evaporation of water, single stage	1.200	kg steam/kg evaporated
	0.040	kWh power/kg evaporated
Evaporation of water, multi-stage	0.500	kg steam/kg evaporated
	0.005	kWh power/kg evaporated
Distillation	1.3*product's heat of evaporation	kg steam/kg evaporated

technology and then compare the results with those for the production of bulk petrochemicals. We focus exclusively on the use of the selected products as chemicals and exclude their use as fuels and animal feed.

13.2.2.1 System Boundaries

We draw from the environmental assessments carried out for the systems cradle-to-factory gate and cradle-to-grave in Patel *et al*. [27]. To evaluate the environmental effects of these chemicals, an assessment across the whole life cycle (cradle to grave) of the products is necessary and we therefore only present results for the latter. Five subsystems were distinguished when modeling the life cycle (Figure 13.2):

1) extraction of non-renewable energy resources such as crude oil,
2) agricultural production and biomass pretreatment,
3) bio-process (the actual production process),
4) process waste management, and
5) post-consumer waste management.

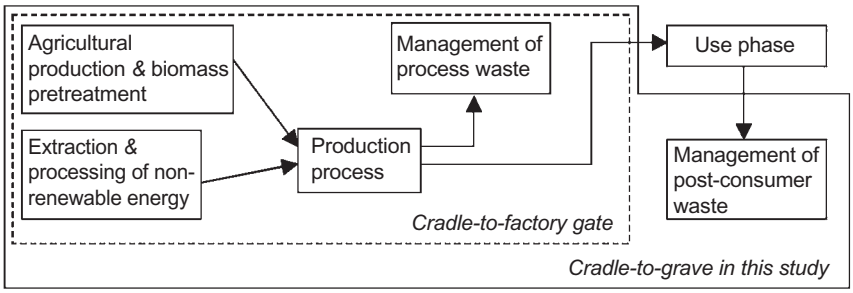

Figure 13.2 Subsystems and system boundaries considered within the environmental assessment for bio-based and petro-based chemicals.

The use phase was excluded because it is usually identical for comparable bio-based and petrochemical products (e.g., a given plastic component in a passenger car); as a further argument, bulk chemicals usually do not lead to emissions during this phase.

13.2.2.2 **Allocation and System Expansion**

For all multifunctional processes resulting in more than one product, a method must be chosen that allows the environmental impact to be expressed relative to the functional unit chosen. The two most commonly used allocation methods to this end are system expansion and partitioning. Typical system expansion accounts for a co-product by expanding the system analyzed to include also the production of the co-product by alternative means. This approach changes the functional unit to one tonne of desired chemical plus amount x of co-product. In order to carry out system expansion but limit the functional unit to one tonne of desired chemical we introduce credits representing the avoided impacts related to the manufacture of co-products (see [44]). System expansion is used when the co-product (e.g., electricity from bagasse) can also be produced by a standalone process (e.g., electricity from coal). Partitioning, on the other hand, has to be used when there is no such production process, such as corn stover (which can only be produced in conjunction with corn).

Partitioning is mostly done by dividing the overall environmental impact either according to the mass ratio or the economic value of the products. For this study, allocation and system expansion were relevant for the production of fermentable sugar, the joint production of materials and energy, and the joint production of several chemicals in the fermentation step. For example, the energy produced from burning waste biomass originating from the bioprocess resulted in an energy credit that was deducted from the inputs of non-renewable energy because this energy displaces the use of non-renewable energy, for example, electricity from the grid. System expansion was also used to deal with co-products of fermentation: all inputs to fermentation were added up and credits were introduced for the co-products (e.g., acetone and ethanol in the case of ABE). These credits were equal to the petrochemical production of these co-products because industrial biotechnology

co-products were assumed to displace chemically identical petrochemicals. Partitioning was only used in the production of fermentable sugars (see Section 13.2.2.3).

13.2.2.3 Production of Fermentable Sugar

Three types of fermentable sugar have been considered for environmental analysis: glucose from corn starch, sucrose from sugar cane, and fermentable sugars from lignocellulosics. Glucose ($C_6H_{12}O_6$) from corn is produced using enzymes to hydrolyze corn starch. The data set was derived from refs [41, 42]. For agricultural production, the environmental impacts were split between corn and corn stover removed from the field through an economic allocation using a price ratio of 4 : 1 for corn compared to stover. Fermentable sugars from corn starch were assumed to stem from a modern corn wet mill by hydrolysis of starch to dextrose. We relied on a detailed mass-based co-product allocation (see also [42]) that took into account the subprocesses that were required for the co-products of fermentable sugar from corn. After accounting for co-products the net input is 1.06 kg of dry corn for 1 kg of glucose.

Milling of sugar cane results in sucrose ($C_{12}H_{22}O_{11}$) and bagasse. The data set was calculated from refs [6, 22]. Bagasse is burned to generate energy because it is a low-value product with limited use. Therefore, we used the total factory input of sugar cane as the starting point in the default allocation and assigned credits for energy produced from bagasse. Thus 8.7 kg of wet sugar cane are required to produce 1 kg of sucrose.

Fermentable sugars from woody biomass, also referred to as C5/C6 sugars, are typically a mixture of glucose, xylose, and smaller quantities of other sugars obtained through depolymerization of cellulose and hemicellulose. We assumed the production of C5/C6 sugars from corn stover and derived the data set from ref. [1]. The main purpose of the cultivation of corn has been and will be the production of starch from corn kernels. The default case for allocating inputs for agricultural production to corn stover is therefore an economic allocation using a price ratio of 4 : 1 for corn compared to stover. The production of 1 ton of fermentable sugar requires 1.79 ton of corn stover.

13.2.2.4 Life Cycle Inventory

Each input to and output from the modeled processes was characterized by its calorific value (HHV in GJ/t product), the cumulative energy use for its production, its embodied carbon, the cumulative greenhouse gas (GHG) emissions of the inputs, and the land use required for agricultural production. The data on petrochemicals used as auxiliaries represent current technology and stem from industry [37] and from our own calculations based on ref. [28]. These data sets were then used to calculate the cumulative GHG emissions, non-renewable energy, and land use by adding up the respective data of all process inputs.

13.2.2.5 Environmental Indicators

A full life cycle assessment (LCA) includes calculating a range of environmental impacts, among others acidification, eutrophication, particulate emissions, human

toxicity, and environmental toxicity. However, the study presented here is a prospective environmental assessment dealing with future processing routes and therefore uses proxies for the overall environmental impact: non-renewable energy use (NREU), GHG emissions, and land use (LU); energy and land may become scarce resources. We calculated non-renewable energy use, GHG emissions, and land use for all industrial biotechnology products. These indicators are good proxies for the overall environmental impact.

NREU represents a straightforward and practical approach because many environmental impacts are related to energy use [13]. NREU encompasses fossil and nuclear energy and was expressed in terms of higher heating value (HHV), also called the gross calorific value. In line with LCA methodology, the NREU values reported here represent the cumulative energy demand for the system from cradle to grave.

Greenhouse gas emissions are of growing importance because of the increasing attention paid to the greenhouse effect in the policy arena, by companies, and by the public. GHG emissions were calculated in CO_2 equivalents and consist of GHG emissions from the system in the form of CO_2 or CH_4 as well as nitrous oxide (N_2O) from fertilizer use in biomass production. CO_2 emissions from renewable carbon extracted from the atmosphere during plant growth were excluded.

Land use refers to agricultural land use only and will be of increasing importance in the future because of the growth of land requirements for bio-based energy, liquid bio-fuels, bio-based chemicals, and food and feed production. We neglected the land requirements for industrial plants, for transportation infrastructure, and for waste management because they are small compared with agricultural land use and are comparable for bio-based and petrochemical products.

13.2.3
Process Economics

The economic analysis for industrial biotechnology chemicals was performed for an assumed plant capacity of 100 kt/year, which was a compromise in view of economies of scale on the one hand and transport costs for bio-feedstocks on the other. This scale was considered representative for a industrial biotechnology plant, but larger scales are also possible and a sensitivity analysis was carried out to assess the influence of plant size on economic viability. Petrochemical processes were calculated for current plant sizes, which can be clearly larger than 100 kt, depending on the product.

All cost calculations were based on investments for building a new plant in Western Europe, with calculations carried out in $€_{2000}$. The investment (total fixed capital, TFC) and labor requirements were estimated by DSM by applying their so-called Functional Unit Method [35]. These calculations were carried out for each generic industrial biotechnology route based on the individual product flow sheet, mass and energy balance (see [27] for individual numbers). We used market prices for petrochemical feedstocks and auxiliaries, the prices of fermentable sugar were set exogenously (see Section 13.2.3.1). The procedure for the economic assessment (see Figure 13.3) is in line with standard business economics: First, variable costs

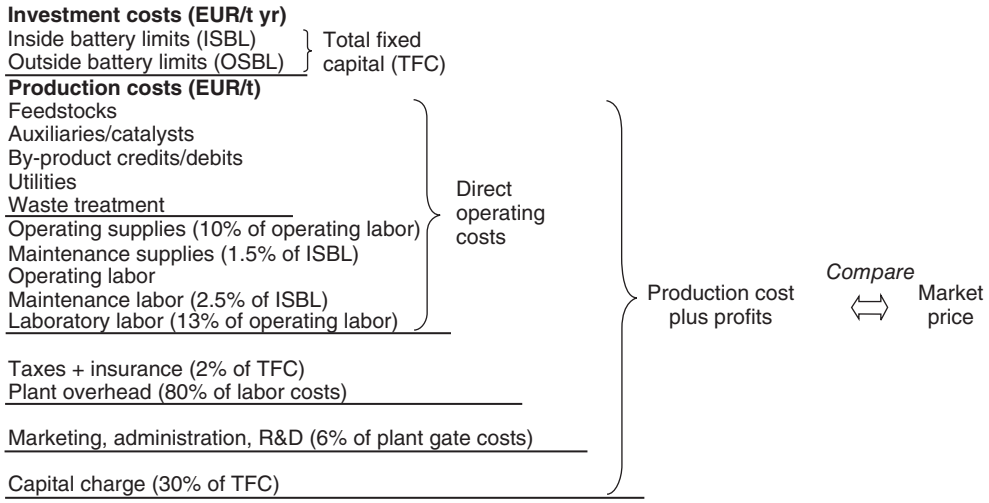

Figure 13.3 Procedure to calculate production costs plus profits (PCPP).

(feedstock, auxiliaries/catalysts, by-products, utilities, waste treatment) and fixed costs (supplies, labor) were added to obtain the total direct operating costs. Second, taxes, insurance fees, and plant overheads were added to this figure as well as an allowance for marketing, administration, and R&D. And finally, the so-called capital charge, representing the total of depreciation and profits, was added. The final result is the production cost plus profits (PCPP; also known as profited production cost) which is a proxy for the market price. The capital charge was calculated by multiplying the total fixed capital with a fixed percentage. In consultation with industry experts, a capital charge of 30% was used, partially accounting for contingency. Value-added tax was not included in the calculations.

A given industrial biotechnology product was considered economically viable if its PCPP was lower than the market price or the PCPP of its petrochemical counterpart. The real market price of the industrial biotechnology product may be higher or lower than its PCPP depending on demand and supply:

- The PCPP of an industrial biotechnology product is usually substantially lower than its market price if the industrial biotechnology product is new on the market and if it is used for niche applications. Possible reasons are that the profit made is higher, the real capacity of the production facilities is clearly lower than 100 kt/year, the process is not optimized and/or continuous operation cannot be ensured.
- The PCPP of the industrial biotechnology product can also be higher than the market price. This is in particular the case if the industrial biotechnology product is chemically identical with a petrochemical product that has been manufactured for decades via an established production route. Such petrochemical processes can be economically superior due to the advantageous economies of scale and/or production in depreciated plants.

13.2.3.1 Prices of Fermentable Sugars

Fermentable sugar was the feedstock of industrial biotechnology processes and its price influenced the economics of industrial biotechnology products. Fermentable sugar may be raw or refined and consists of biomass-derived readily fermentable carbohydrates such as sucrose, hydrolyzed starches or pretreated and hydrolyzed lignocellulose, which is still an emerging technology. In order to account for variations in fermentable sugar prices both in the near and longer term future and for world regions, calculations were carried out for four price levels of fermentable sugar. The lowest price of €70/t represents local sugar prices in Brazil[9]: due to good climatic conditions and the availability of very cheap labor, the production cost of fermentable sugar from sugar cane was at its lower boundary. The high sugar price of €200/t represents a 10-year average of world raw sugar (contract 11) as traded at the New York Board of Trade [24]. An intermediate price was chosen at €135/t. An extreme level at €400/t represents a 10-year average of US domestic raw sugar (contract 14) as traded at the New York Board of Trade [25].[10]

The price ranges for a tonne of fermentable sugar were exogenous inputs to our calculations, that is, we do not perform economic analyses for different combinations of feedstock types with technologies for producing fermentable sugar.

13.2.3.2 Prices of Utilities and Auxiliaries

For the economic assessment we assume a default stock market crude oil price of US$70 per barrel (€12/GJ) and a natural gas price of €8.5/GJ. This corresponds to an estimated electricity price of €17/GJ_e and a steam price of €25/t (€11.9/GJ). Economic credits were introduced in order to account for the avoided production of heat and power if energy was recovered. Enzyme and membrane prices were assumed to decrease in the future because of their large-scale production. Enzyme prices for current technology were set at €100/kg and were assumed to decrease by a factor of 10 for future technology; the prices of high-quality membranes were set at €100/t of product and were assumed to decrease by a factor of two.

13.3 Overall Results

13.3.1 Results of Environmental Analysis

The environmental impacts of the processes studied depend to a large extent on the productivities, yields, and concentrations assumed for the fermentation stage [27].

9) Based on an average sugar price of BRL250 for the 2003/2004 season [26] and an exchange rate of 1 BRL = 0.29€ for the same time frame.

10) These price levels were based on raw sugar prices. However, if the microorganism is sensitive to impurities then refined sugar will be used for fermentation. World refined sugar as traded at the London Stock Exchange has been traded for an average price of ca. 250€/t during the last 10 years [33] and thus within the range of prices considered.

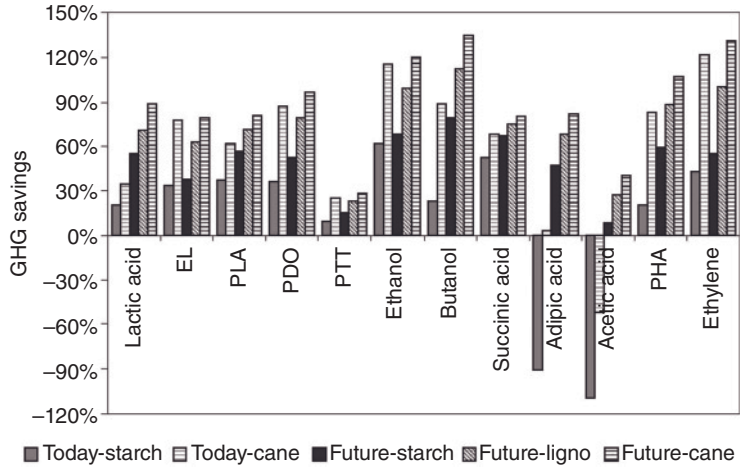

Figure 13.4 Greenhouse gas (GHG) emission savings per tonne of industrial biotechnology chemical compared with their petrochemical counterparts for current and future technology, system from cradle to grave. EL, ethyl lactate; PLA, polylactic acid; PDO, 1,3-propanediol; PTT, polytrimethylene terephthalate; PHA, polyhydroxyalkanoates.

In order to assess the long-term potential of industrial biotechnology, we chose future parameter values that are expected to be reached after 20–30 years of successful research and development (horizon values). In this paper we present results only for GHG emissions, but results for NREU show the same pattern (see [27]). Figure 13.4 shows a product-by-product analysis of average GHG savings in industrial biotechnology products compared with their petrochemical equivalents. Each bar in Figure 13.4 represents the arithmetic mean across several industrial biotechnology production routes for the same chemical. Figure 13.4 shows that the products with the highest relative savings are ethanol, butanol, and ethylene, and acetic acid and PTT have lowest savings. Differences between best cases and arithmetic means were 7–20% in GHG savings. Figure 13.4 shows that almost all products promise GHG savings for current technology.

For PHA and adipic acid this depends on the source of fermentable sugar. Acetic acid offers no savings using current technology because of low broth concentration and low productivity in fermentation as well as high utility use in downstream processing because of the difficulty of separating acetic acid from water (azeotropic mixture). GHG savings for PTT are low because this polymer is made from PDO and purified terephthalic acid, with the latter being produced from petrochemical feedstocks. GHG savings for sugar cane as the source of fermentable sugar are clearly higher than for corn starch because of the co-production of significant amounts of electricity which can be exported (see Section 13.2.2.3).

Figure 13.4 shows that in order to maximize savings in greenhouse gas emissions for industrial biotechnology products sugar cane is favored over lignocellulosics, which in turn is preferable to corn starch as a source of fermentable sugar.

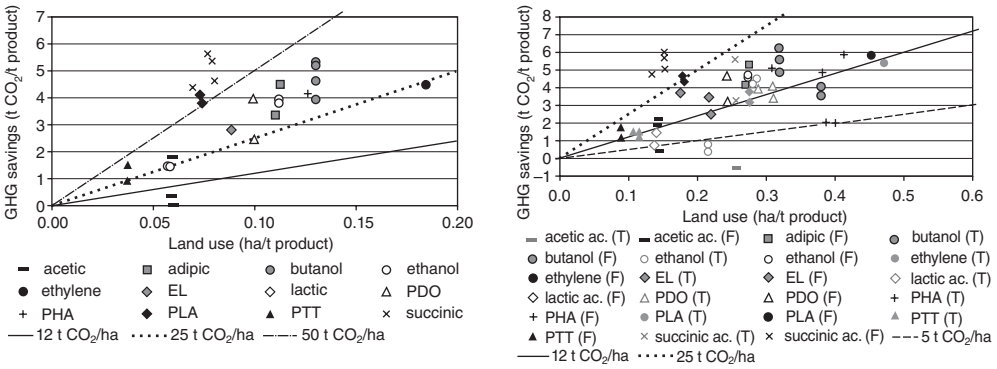

Figure 13.5 Greenhouse gas (GHG) savings versus land use per tonne of chemical for the system from cradle to grave. Fermentable sugar from lignocellulosics, future technology (left) and sugar cane, current (T) and future (F) technology (right). Straight lines are iso-lines representing CO_2 savings per hectare.

In temperate climates such as Europe and North America where sugar cane is not available from domestic production, lignocellulosics should be the preferred future feedstock.

In some of the industrial biotechnology chemicals made from lignocellulosics and sugar cane, the savings are greater than 100% (Figure 13.4) because the energy credits from co-combustion of waste biomass or from side-streams of agricultural production were larger than the NREU for the industrial biotechnology process chain. On average, GHG savings for future industrial biotechnology technology are 25–35% higher than for current industrial biotechnology technology. This shows that technological progress can further enhance the environmental advantage of industrial biotechnology products over their petrochemical equivalents.

The low GHG emissions of a process may be due to (i) high product yield from fermentation or (ii) low product yield from fermentation combined with large energy credits from subsequent combustion of co-produced biomass. In the second case, inefficient fermentation processes require considerably more land for biomass production than does efficient fermentation. If land availability becomes limited, GHG savings should be maximized for a given amount of land or, alternatively, land use should be minimized for a certain amount of GHG to be saved. Figure 13.5 therefore simultaneously analyzes GHG savings in all industrial biotechnology routes relative to the petrochemical route and land use per tonne of chemical.

Figure 13.5 shows that there is a relationship between the type of chemical and the amount of land use for its production from sugar cane. For the production of one tonne of carboxylic acid 0.1–0.2 ha of land is required, whereas the alcohols are in the range of 0.25–0.35 ha/t. For PTT, land use and GHG savings are low because only a part of this polymer is produced from bio-based feedstocks. Putting GHG savings and low land-use first, succinic acid, PLA, and butanol are the most attractive. Our results compare well with data from publications on individual

products [2, 4, 14, 15, 34, 43] when accounting for differences in allocation. Producing fuel ethanol from sugar cane results in savings of 10–16 t $CO_{2,eq}$/ha [27]. Several industrial biotechnology chemicals show CO_2 savings per hectare above 16 t $CO_{2,eq}$ and are therefore preferable from the point of view of CO_2 mitigation.

Figure 13.5 only shows future technology for lignocellulosics because the commercial production of fermentable sugars from lignocellulosics is not yet possible on a large scale. The comparison of figures for sugar cane and lignocellulosics in Figure 13.5 shows that land-use efficiency in terms of CO_2 savings per hectare is much better for corn stover than for sugar cane. Converting corn stover to chemicals using future technology almost always results in CO_2 savings above 25 t/ha. Biomass for electricity use saves approximately 12 t $CO_{2,eq}$/ha for whole crop wheat [7] and using lignocellulosics for fuel ethanol production saves 2–7 t $CO_{2,eq}$/ha [29].[11] Putting CO_2 savings first, this implies that most chemicals are preferred over bioenergy if using sugar cane as feedstock and almost all chemicals are preferred if using corn stover.

Table 13.4 quantifies GHG savings potential assuming full substitution of the petrochemical equivalents and based on world production capacities in the years 1999/2000 [45]. The total saving potential for the future according to Table 13.4 (485 Mt $CO_{2,eq}$ for corn starch) disregards growth of the chemical industry. The future saving potential is even higher if lignocellulosics (787 Mt $CO_{2,eq}$) or sugar cane (991 Mt $CO_{2,eq}$) are used as feedstock. For comparison, GHG emissions from current technology production of petrochemical equivalents lead to 843 Mt $CO_{2,eq}$ for the same installed capacity and system boundaries. This shows that the potential GHG savings for current technology and corn starch as feedstock already reach 45%.

13.3.2 Results of Economic Analysis

Production costs plus profits (PCPPs) were calculated for all industrial biotechnology products using the methodology described above (see Figure 13.3). Table 13.5 shows the results for the generic approach. The petrochemical PCPPs in Table 13.5 refer to benchmark substances that are chemically identical with the bio-based compounds, unless indicated otherwise. In the case of lactic acid, no petrochemical benchmark can be given because it is already produced from bio-based feedstocks via industrial biotechnology today, the comparison was therefore made with current industrial practise. Table 13.5 shows that for current technology, two-thirds of the industrial biotechnology products are economically viable for low sugar prices. PDO, PTT, PLA, succinic acid, ethyl lactate, and ethanol are economically viable even for very high sugar prices (€400/t).

These findings are more directly visible from Figure 13.6, which shows the ratio of PCPPs of the current technology industrial biotechnology products compared with their petrochemical counterparts. Values below 100% indicate that the

11) GHG savings from ethanol here are ca. 33 t CO2,eq/ha because of the reference system: chemical ethanol replaces ethanol produced via ethylene, whereas fuel ethanol replaces gasoline.

Table 13.4 Potential worldwide annual production and best-case GHG savings of the 15 industrial biotechnology products, using corn starch as feedstock, system from cradle to grave.

Product		GHG savings	Installed world capacity [48]	Annual GHG savings
		($t CO_2/t$)	(kt/yr)	($kt CO_2/yr$)
Acetic acid	Today	–2.4	8 300	N/A
	Future	1.2		9 570
Adipic acid	Today	–5.2	2 400	N/A
	Future	3.3		7 880
Butanol	Today	1.2	2 460	3 040
	Future	3.9		9 610
Ethanol[a)]	Today	2.7	2 600	6 970
	Future	2.7		7 080
Ethyl lactate	Today	1.3	1 200	1 580
	Future	1.9	Ethyl acetate	2 220
Ethylene	Today	1.9	100 000	191 050
	Future	2.5		245 710
Succinic acid	Today	4.5	1 350	6 070
	Future	5.0	Maleic anhydride	6 780
1,3-Propanediol	Today	1.8	No data	N/A
	Future	2.9		N/A
Polyhydroxyalkanoates	Today	2.9	57 000	162 730
	Future	2.8	Polyethylene	159 640
Polylactic acid	Today	2.3	11 100	25 150
	Future	3.3	PET	36 500
Total	Today			396 600
	Future			485 000

a) We only consider installed capacity of petrochemical ethanol production because bio-based ethanol is mostly used as fuel, whereas the ethanol used in the chemical industry predominantly stems from petrochemical production processes.

Table 13.5 Production cost plus profits (€/t) of industrial biotechnology products for varying sugar prices and current and future technology according to the generic approach and the petrochemical benchmark (for US$70/barrel crude oil).

Production cost plus profits (€/t)

Product	Technology	Bio-based, function of fermentable sugar price				Petrochemical
		€70/t	€135/t	€200/t	€400/t	
ABE	Today	1070	1270	1480	2110	780
	Future	240	410	580	1110	*n*-butanol
Acetic acid	Today	2510	2650	2790	3220	510
	Future	900	980	1060	1300	
Adipic acid	Today	2920	3320	3720	4940	1320
	Future	1130	1280	1430	1880	
Ethanol	Today	520	670	820	1300	1630
	Future	420	570	720	1170	
Lactic acid	Today[a)]	850	930	1010	1260	–
	Future	520	600	680	910	
PDO	Today	740	910	1080	1590	2340
	Future	460	590	720	1130	
PHA	Today	1390	1610	1840	2520	2150
	Future	1460	1630	1800	2310	*PE*
Succinic acid	Today	800	880	960	1200	1560
	Future	550	620	680	890	Maleic anhydride
Ethyl lactate	Today	1200	1330	1460	1860	1890
	Future	890	1010	1140	1520	Ethyl acetate
Ethylene	Today	1090	1360	1630	2460	1510
	Future	920	1180	1440	2240	
PLA	Today[a)]	1500	1610	1710	2030	2160
	Future	1210	1310	1410	1710	PET
PTT	Today	1660	1720	1790	1990	2300
	Future	1550	1600	1650	1810	

a) Process data was taken from industry sources.

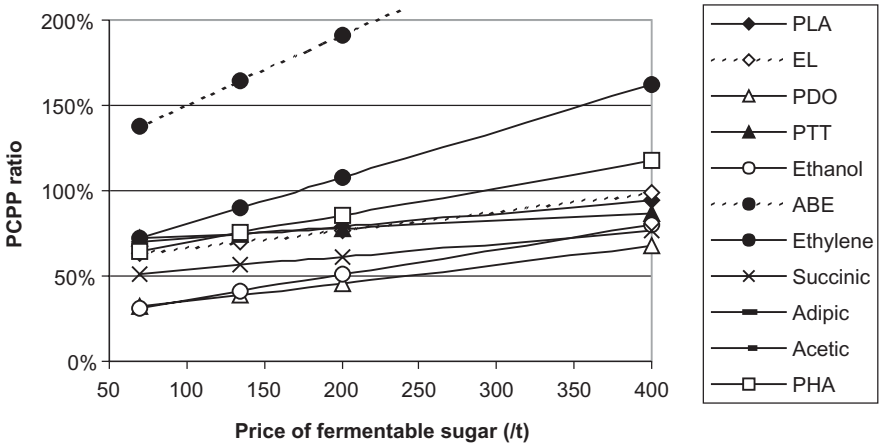

Figure 13.6 Economic viability of today's industrial biotechnology technology: ratio of production cost plus profits (PCPP) of the industrial biotechnology product to its petrochemical counterpart for today's technology as a function of the sugar price level (for US$70/barrel crude oil). EL, ethyl lactate; PLA, polylactic acid; PDO, 1,3-propanediol; PTT, polytrimethylene terephthalate; PHA, polyhydroxyalkanoates; ABE, acetone–butanol–ethanol.

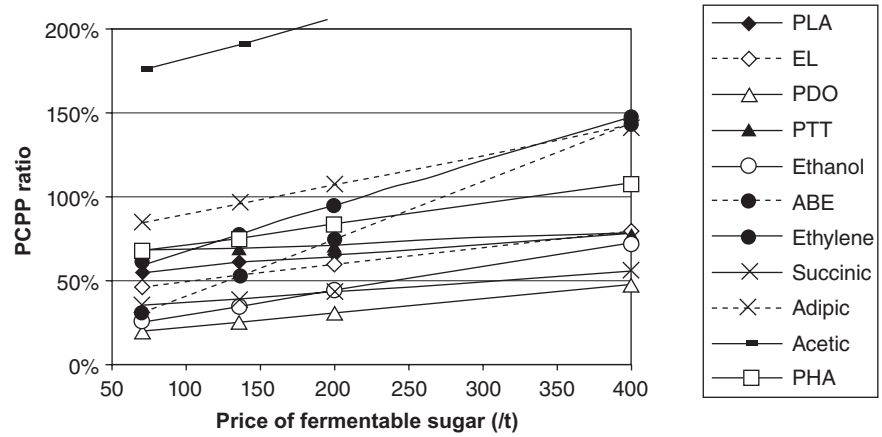

Figure 13.7 Economic viability of future industrial biotechnology technology: ratio of production cost plus profits (PCPP) of the industrial biotechnology product compared to its petrochemical counterpart for future technology as a function of the sugar price (for US$70/barrel crude oil).

production costs are lower for the industrial biotechnology product, while values above 100% represent cases in which the production of the industrial biotechnology product is more expensive than its petrochemical counterpart. The PCPP ratio of acetic acid ranges from 500% to 630% and therefore lies outside of the range of Figure 13.6. By analogy, Figure 13.7 shows the ratios of PCPP for future

Table 13.6 Viability of industrial biotechnology chemicals for four price levels of fermentable sugar for current and future technology (crude oil price: US$70/barrel).

Sugar price (€/t)	Today	Future
400	Ethanol, PDO, succinic acid, PTT, PLA, and ethyl lactate	Same as today
200	Ethanol, PDO, succinic acid, PTT, PLA, and ethyl lactate	ABE, ethylene, ethanol, PDO, succinic acid, PTT, PLA and ethyl lactate
135	Ethylene, ethanol, PDO, succinic acid, PTT, PLA, and ethyl lactate	Adipic acid, ABE, ethylene, ethanol, PDO, succinic acid, PTT, PLA and ethyl lactate
70	Ethylene, ethanol, PDO, succinic acid, PTT, PLA, and ethyl lactate	Adipic acid, ABE, ethylene, ethanol, PDO, succinic acid, PTT, PLA and ethyl lactate

technology industrial biotechnology products to the (current technology) petrochemical. Almost all products researched offer economic savings for a sugar price of €70/t, with the exception of acetic acid.

In conclusion, technological progress can contribute significantly to improve the economic viability of industrial biotechnology products: across all sugar prices, the PCPPs of products that are directly obtainable from fermentation (e.g., lactic acid) are 20–40% lower than for today's technology; for products which require a chemical conversion after fermentation (e.g., PLA), technological progress in the fermentation step still reduces the PCPP by approximately 15–20%. This results in many more products becoming economically viable in the future, even at rather high sugar prices. Table 13.6 summarizes the viability of current and future industrial biotechnology chemicals for four sugar prices.

13.4 Conclusions

In this chapter we presented and applied a generic approach which allows the systematic evaluation of present and future production routes of bio-based chemicals from industrial biotechnology, based on available data and consistent assumptions on future (bio)technology developments. Even at present, bio-based bulk chemicals from industrial biotechnology offer clear savings in non-renewable energy use and GHG emissions with current technology compared to conventional petrochemical production. Substantial further savings are possible for the future by improved fermentation and downstream processing.

Of all feedstocks, sugar cane is to be favored over lignocellulosics, which in turn is preferable to corn starch in terms of energy use and GHG savings. The products

with the highest savings are butanol (from ABE process), ethanol, ethylene, PDO, and PHA.

In general, a large number of industrial biotechnology chemicals are economically viable compared to their petrochemical equivalents. Economic competitiveness depends to a large extent on the prices of oil and sugar. For a crude oil price of US$70/barrel the following products are economically viable for current technology: 1,3-propanediol, polytrimethylene terephthalate, polylactic acid, succinic acid, ethyl lactate, ethylene, and ethanol. Comparing current to future technology, production costs plus profits for products directly obtained from fermentation are 20–40% lower and for products that require a chemical conversion step after fermentation 15–20% lower (for a crude oil price of US$70/barrel and across all sugar prices). This shows that technological progress can contribute significantly to improved economic viability of industrial biotechnology chemicals. For future technology, all studied products except for acetic acid are economically viable at fermentable sugar prices of €70–135/t. All other products improve in economic competitiveness.

From a policy perspective, environmental advantages make the production of bio-based bulk chemicals using industrial biotechnology desirable on a large scale, because savings of more than 100% in non-renewable energy use and greenhouse gas emissions are already possible at the current level of biotechnology. This builds a strong case for the production of bio-based bulk chemicals using industrial biotechnology considering the economic advantages of 1,3-propanediol, polytrimethylene terephthalate, polylactic acid, succinic acid, ethyl lactate, ethylene, and ethanol for current technology and of all products except acetic acid for future technology (see [11] for other oil prices). As a consequence, using industrial biotechnology to produce bio-based chemicals can contribute significantly to reducing climate change and the depletion of fossil energy. It is therefore a key strategy for sustainable development of the chemical industry. A large-scale introduction of industrial biotechnology–based production of economically viable bulk chemicals is therefore desirable for those products whose environmental impacts are smaller than those of current petrochemical production routes. Under these conditions, industrial biotechnology could become the center of attention for the chemical industry as well as for policy-makers.

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14
Societal Issues in Industrial Biotechnology

Patricia Osseweijer, Klaus Ammann, and Julian Kinderlerer

14.1
Introduction

Biofuels have become a hot topic in recent years. The burning of fossil oils has been blamed for their contribution to global warming and the price of oil has increased rapidly. So the search for alternatives is on. The media report almost every day about some issues related to biofuels, voicing supporters and opponents of this new application of industrial biotechnology. The debate particularly questions whether biofuels can indeed reduce greenhouse gases and whether we have enough land to grow the necessary biomass. It questions the impact on both Western societies and developing countries. Some commentators fear that we risk our food availability for a growing world population, that food prices will rise problematically, while others are concerned about the destruction of rainforests. In this climate of controversy politicians struggle to develop policy measures to reduce the dependency on oil-producing countries, to raise sustainability, and to gain environmental benefits.

What does this strong debate on biofuels mean for the development of industrial biotechnology? What have we learned from earlier public debates about biotechnology and how can we apply those lessons to support a further development and implementation of industrial biotechnology?

Modern biotechnology has long been viewed as a key technology promising better quality of life for all world citizens. Its development, however, has been accompanied by concern and criticism about the methods it uses. As early as 1992 countries discussing the state of the world in the twenty-first century indicated that biotechnology had the potential to enable "the development of, for example, better health care, enhanced food security through sustainable agricultural practices, improved supplies of potable water, more efficient industrial development processes for transforming raw materials, support for sustainable methods of deforestation and reforestation, and detoxification of hazardous wastes" (Agenda 21, chapter 16; http://earthwatch.unep.net/agenda21/16.php).

There were early technical concerns about, for example, the use of antibiotic markers in the development of transgenic crops and moral issues about the

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principles of genetic engineering leading to the charges of "playing God" and "patenting life." Later there were worries about the potential risks and opportunities for consumer choice in genetically modified (GM) food. Indeed "Frankenstein" food became so much of an issue that many supermarkets banned GM-containing products from their shelves. The public debate ultimately resulted in an effective moratorium on GM crops since 1999, which is presently slowly lifting [1]. Are these concerns relevant for the development of industrial biotechnology?

Wine, beer, bread, and cheese represent some of the centuries-old examples of industrial biotechnology which are of course widely accepted. Strains used for their production were optimized over the years, using many different techniques, including selective breeding, induced mutations by irradiation and, more recently, genetic modification. However when these modern techniques were used to produce chymosin to make cheese, for example, this was not met everywhere with great enthusiasm in spite of the fact that it would replace the use of stomachs from slaughtered newborn calves. It also seems paradoxical that modern durum wheat traits, used to produce pasta worldwide, resulting from radiation mutation breeding, seem to be accepted by a majority of producers and consumers. although considered in molecular science as a much more untargeted breeding method with unknown, random impacts on the genome. The implication is that *in situ* modification is acceptable, but inserting an infinitesimally small portion of DNA derived from another organism is unacceptable.

Within a changing society which pushed for a demand-driven economy these examples induced caution within most biotechnology companies, but even more so within the process and retail industries that use these ingredients for their products.

While industrial biotechnology aims to deliver sustainable solutions for production of consumer goods, energy, pharmaceuticals, and environmental applications it is also highly likely that these latest applications will be frowned on by at least a section of our communities as already shown above for biofuel applications. Companies are therefore hesitant to introduce these products or even develop them due to anticipated negative consumer responses stimulated especially by activist non-governmental organizations (NGOs). Policy-makers struggle to find a balance between promoting and ensuring sustainable development and anticipated public resistance.

Representation and interpretation of scientific information, methods of communication and public interaction, and ethical, legal, economic and safety issues are important elements in public opinion forming. Also strongly indicated is that timely and adequate, proactive and interactive communication initiatives help to introduce novel, socially beneficial applications. Dialog between all the main stakeholders during the early stages of development has therefore been promoted as a crucial key to realizing the potential offered by innovations in industrial genomics.

Before we can engage in effective, early engagement and interaction with the wider society we need to understand the issues at stake and identify the relevant stakeholders in such as farming, (chemical) industry, retailing, transport, local

government, and regional development. We therefore need to investigate and unravel the societal implications of a more bio-based economy and to understand the possible societal issues so we can prepare for a generally accepted implementation that is likely to be successful.

This chapter therefore explores the impact of a biomass economy and the controversy about the resulting societal issues. It takes lessons from the GM debates to propose some advice on what academia and industry may do to further a sustainable introduction of industrial biotechnology that is acceptable to most in civil society.

14.2 The Impact of Industrial Biotechnology

Before we can say anything about societal issues of industrial biotechnology we need to explore its possible impact on our society. For this we use the definition of industrial biotechnology of the European Platform on Sustainable Chemistry (Suschem)[1]:

> Industrial Biotechnology is the application of biotechnology for the processing and production of chemicals, materials and fuels. It uses enzymes, micro-organisms and cell lines to make products in sectors such as chemistry, pharma, food and feed, paper and pulp, textiles and energy, materials and polymers.

With this approach industrial biotechnology aims to provide a more sustainable production of consumer goods, energy sources, and pharmaceuticals, for example, the replacement of mineral oil with biomass for feedstocks. The reduction of carbon dioxide emissions is claimed to help alleviate global warming while the application of biotechnology in production processes should demonstrably reduce the use of both energy and water and the production of unwanted by-products and waste water.

14.2.1 How Does This Influence Our Society?

First and importantly, the replacement of oil with biomass will have an impact on our economies and global trading relations. The increased production of fine chemicals and pharmaceutical ingredients by yeast, fungi, and bacteria has already greatly increased the demand for sugar with consequent increase in prices. However, when bulk chemicals, including biofuels such as bioethanol, are produced in this way, the demand for sugars and plant oils will increase much further (as demonstrated by the dependence of the sugar price on the oil price[2] [2]). As

1) Suschem brochure 2006 downloadable from website: suschem.org.
2) http://www.europabio.org/facts_white.htm for several relevant reports (downloadable).

shown in other chapters of this book, scientific research presently focuses on the use of other biomass materials such as feedstocks for fermentation processes, including household and agricultural waste materials. Although this increases the usage per unit of biomass produced, it still requires a huge agricultural input with major effect on the global trade market and hence on local economies.

Second, the bulk production of energy, chemicals, and materials through biomass will change our landscapes. Wastelands, recreation grounds, forests, and perhaps even oceans and deserts may be considered as additional producers of biomass. Small local biorefineries may replace the old concepts of water-tower and gas-station from the early twentieth century. It will necessitate a change in our transport infrastructure and require a number of large- and small-scale "biorefineries," the latter close to the location of biomass production to minimize transport costs.[3)]

Third, the transition of industries to other production processes will affect the entire production chain, which will change skill needs and employment opportunities. All changes will directly or indirectly influence local communities. Farmers may have a larger market for their crops, citizens may be forced to recycle domestic waste biomass, politicians may need to provide for new incentives balancing economy against environment, industry managers may have to choose when and how (and on what scale) to invest in new production facilities. Oil countries may also need to find alternative incomes, while developing countries may have difficult choices for either food production or biomass export. Furthermore, societies everywhere may have to fully support the use of GM crops at least in coexistence, as it is very likely that only GM crops will deliver the required amount of biomass with the lowest impact on food supplies, water, and nutrient use and the lowest burden on the environment.

14.2.2
What Are the Political, Industrial, Economic, and Scientific Drivers and Obstacles?

Recent concerns about global warming have resulted in a series of reports and international agreements. The most famous one is the Kyoto Protocol, set up in 1997 and ratified in 2005 after the signature of Russia. With their ratification countries worldwide commit to reduce their emissions of greenhouse gases considerably with an average of 5.2% for 2008–2012 in relation to the levels in 1990. The United States have not signed the agreement. Post-Kyoto agreements in general aim for higher reductions. However that is not the only driver for a bio-based economy.

The main reasons for governments encouraging the implementation of a bio-based society to replace fossil fuels are:

1) to increase industrial competitiveness and innovation

2) to reduce environmental and atmospheric pollution

3) Biofuels for Transportation. Global potential and implications for sustainable agriculture and energy in the 21st century (Worldwatch Institute) *06/2006*, available from http://www.europabio.org/facts_white.htm.

3) to replace the rapidly depleting fossil fuels for which world demands are increasing because of economic growth in rapidly developing societies such as China and India and a growing world population

4) to replace fossil fuels the use of which increases the emission of greenhouse gases which are seen as a major contributor to global warming

5) to decrease dependency on oil-producing countries.

Further reasons for national communities may include new outlets for national farmers (in Europe) or new export opportunities (for developing countries). Industries are driven by the growing oil prices and by government incentives, but this is not a simple equation as sugar prices are now linked to oil prices and most governments have not yet decided which incentives to implement. The report of McKinsey [2] indicates that the development of a bio-based society depends on:

- fuel prices
- feedstock prices
- government regulation
- availability of conversion technologies (innovation).

Scientific challenges are detailed in other chapters of this book and include the development of novel biocatalysts for production processes, and the development of second-generation biofuels and bulk chemicals by improved process conditions, microorganisms, and enzyme specificity. These developments are mainly driven by (inter)national research programmes, following the advice of road-mapping exercises prepared by experts in biotechnology from industry and academia (EuropaBio, BIO, OECD, EU, technology platforms such as Suschem and EPOBIO, etc.[4]). Industrial challenges are the uncertainty of market opportunities and regulation, expenditure of R&D and innovative opportunities. McKinsey calculated that the industrial biotechnology market comprises 7% of the overall chemical market, equaling €77 billion in 2005. It is expected that this will grow to 10% in 2010 (€125 billion). They also calculated (on a conservative assumption) that the present feedstock supply will be enough to replace about 50% of the required transport oil. This figure is highly debatable though as it includes a number of uncertain assumptions.

14.3
Public Perceptions of Industrial Biotechnology

As already indicated in the introduction to this chapter, the development of a bio-based society is presently a topic of heated debate in the media. Strong opinions are voiced aiming to influence the political decision-making process. On October 26, 2007 the United Nations expert Jean Ziegler even requested a moratorium on

4) http://www.europabio.org/facts_white.htm for several relevant reports (downloadable).

the implementation of biorefineries to provide time for the second-generation biofuels to fully develop.[5] The question is, will this also have an impact on public support for the full development of the broader field of industrial biotechnology?

What is known about the present support for industrial biotechnological applications and what is the impact of public perceptions on political decision-making? Can we compare it with the GM food debate which took place around the turn of the century and which so dramatically influenced the implementation of GM food products in Europe?

14.3.1 What Is the Present Public Perception of Industrial Biotechnology?

In Europe, a number of surveys have been funded by the European Commission to measure public perceptions of life sciences and emerging technologies held repeatedly in a similar format over a number of years (1991, 1993, 1996, 1999, 2002, and 2005[6]) These studies cover all European Member States with a systematic sampling of about 1000 respondents by face-to-face interviews, with the later studies enabling comparison between Europe, the United States and Canada.

The latest study carried out in 2005 included a number of questions related to industrial biotechnology about biofuels (defined as "The development of special crops that can be turned into ethanol as a substitute or additive for petrol and for biodiesel") and bio-plastics (defined as "Another industrial use of crop plants is the manufacture of bio-plastics. These, as it is claimed, will be less environmentally damaging as they can be easily recycle and are bio-degradable"). It was found that 77% of European citizens supported the view that governments should support research on bio-plastics, with 71% agreeing to tax incentives. A smaller majority of 57% would (or probably would) be prepared to pay a little extra for bio-plastics. (Figure 14.1).

Figure 14.2 shows that 71% also agreed definitely or probably with the provision of tax incentives to biofuel companies. A much smaller group of 47%, however, was willing to pay more for a car designed to run on biofuels and even fewer (41%) were prepared to pay a little more for biofuels.

The Eurobarometer researchers [3] concluded that in general there is support for developments in industrial biotechnology. However, we argue that there is reason for caution.

If we compare this with the levels of support for GM food in 1996 we see a similar level of support. Although the support per country varied considerably, the average "outright support" and "risk tolerant" support for GM food was more than

5) The United Nations Special Reporter on the right to food [Jean Ziegler] called on Friday for a five-year moratorium on biofuels, saying it was a "crime against humanity" to convert food crops to fuel. Reuters, 26-10-2007.

6) Eurobarometer reports at http://ec.europa.eu/public_opinion/index_en.htm.

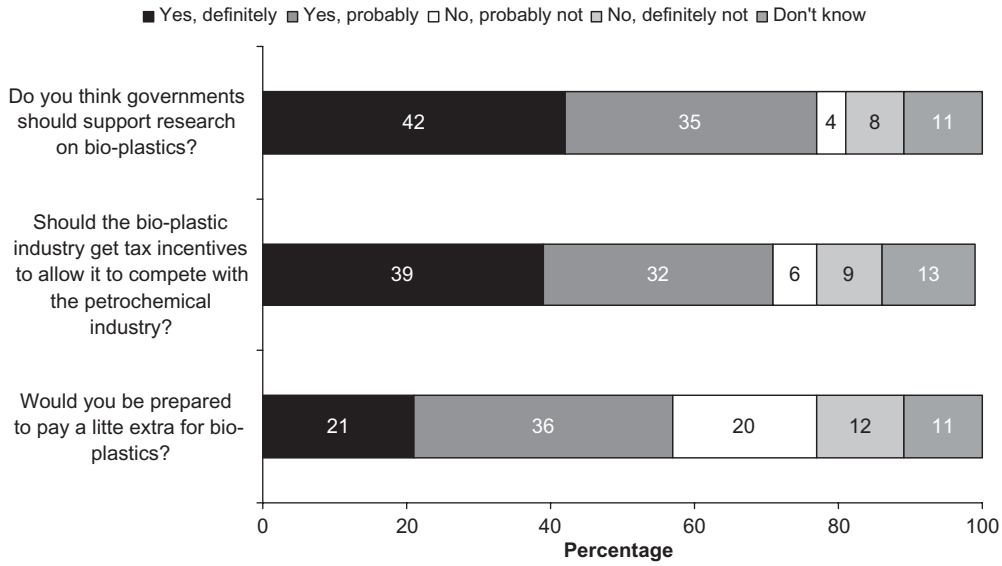

Figure 14.1 Average European support for bio-plastics, defined as "Another industrial use of crop plants is the manufacture of bio-plastics. These, as it is claimed, will be less environmentally damaging as they can be easily recycled and are bio-degradable." Gaskell *et al.* [3], Eurobarometer Studies available at: http://www.ec.europa.eu/research/press/2006/pdf/pr1906_eb_64_3_final_report-may2006_en.pdf.

60%. With the GM food controversy growing from 1996 to 1999 and issues presented emotionally in the media, this support reduced to 47% in 1999. When the media reports were fading this returned to 53% in 2002. But then it declined again to even below the levels of 1999 (Figure 14.3). Will the support for industrial biotechnology also decline now that the media covers it emotionally? What have we learned from the GM food debate? Let us start with a consideration of how public perception impacts on technology development.

14.3.2 What Is the Impact of Public Perception to Policy Development?

When the first shipment of GM soybeans entered Europe in 1996 the initial public support for GM food started to decline rapidly after heavy criticisism by NGOs such as Greenpeace. It led to questions about health and environmental impacts accompanied by protests, boycotts, and increased research on risk. In 1999 Europe was faced with a *de facto* moratorium on the commercialization of GM crops and foods. Arguably, there are several reasons for this political halt. The most direct consequence, however, was that food companies took measures to alter the composition of their products to avoid GM.

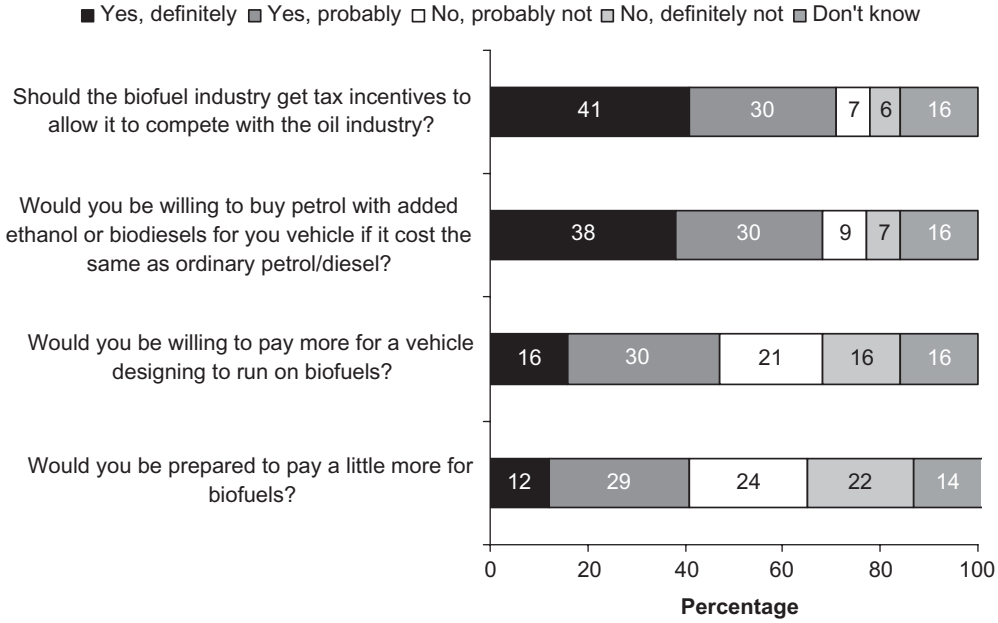

Figure 14.2 Average support by EU citizens for biofuels, defined as "the development of special crops that can be turned into ethanol as a substitute or additive for petrol and for biodiesel." Gaskell *et al.* [3], Eurobarometer Studies available at: http://www.ec.europa.eu/research/press/2006/pdf/pr1906_eb_64_3_final_report-may2006_en.pdf.

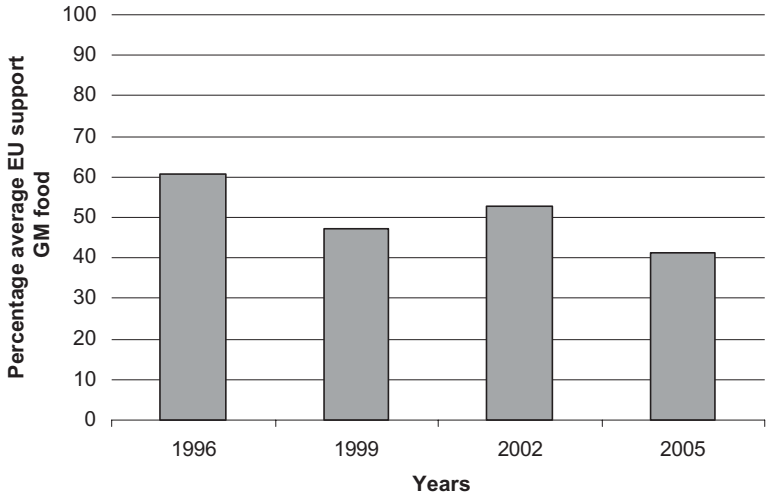

Figure 14.3 Average of EU countries respondents with "outright support" and "risk tolerant support" for GM food. After Gaskell *et al.* [3], Eurobarometer Studies available at: http://www.ec.europa.eu/research/press/2006/pdf/pr1906_eb_64_3_final_report-may2006_en.pdf.

14.3.3
Industrial Reactions to Labeling of GM Food Products

Public unease, alarmist media coverage, opportunist campaigning by some NGOs, and unwise practices by some industrial sectors have all been suggested to have played a part in the rise of public hostility to this new technology. Support for organic farming together with growing opposition to "globalization" and fear of market dominance by large multinational companies have also been mentioned as causes for the decline of public support. In addition, the coincidental emergence of bovine spongiform encephalopathy (BSE) and other food scares, although unrelated to biotechnology, were blamed for confusing the public [4]. The public scare provided the incentive for political measures in regulation. It was indeed seen as a political necessity at European and national levels to strengthen the existing Directives and increase legislation which would introduce the labeling of GM foods.

Directive 90/229, adopted in 1990, on the contained use of genetically modified microorganisms for research and non-marketing purposes was modified in 1998 by Directive 98/81. Among other things it introduced "a requirement for Member States to ensure labeling and traceability at all stages of the placing on the market of the GMO". This measure was adopted to provide consumer information as a basis for informed choice, and to enable any problems to be traced back to their source [4]. Measures were developed for authorization, traceability, and labeling of GMOs, as well as food and feed produced from GMOs, resulting in Regulation 1829/2003 1830/2003 which was enforced from April 2004. However, with the introduction of labeling, the majority of food producers changed their ingredients to non-GM, resulting in the present lack of choice for consumers.

At the moment less than 0.5% of the foods in European supermarkets are labeled to contain GM ingredients or are made with the help of GM techniques. Furthermore a number of European countries do not sell any GM products (among others, Greece, Sweden, Slovenia, Germany and Poland, and Switzerland).[7] These measures by food producers directly influenced the providers of food ingredients, who lost their European markets for anything that needs labeling and are now hesitant to introduce novel products based on the latest genomics research.

There is no rationale for this effect. Labeling was introduced to give consumers the choice to buy either GM or non-GM products. So why did the big food manufacturers replace the GM ingredients with non-GM ingredients? The non-GM ingredients were not cheaper, there were no indications that GM ingredients were more risky for consumers health[8] [5–7] and legislation provided a trustworthy information system with appropriate authorizations in place for control. It therefore seems that the food companies were either alarmed by the negative perception studies of 1999 which they perceived were likely to badly affect their sales or they were wary of campaigns by environmental organizations which could affect their image and thus their sales.

7) "Consumerchoice" final report available at http//www.KcL.ac.uk/schools/biohealth/research/nutritional/consumer/choice/download.html.
8) ENTRANSFOOD final report available at http://www.entransfood.nl/.

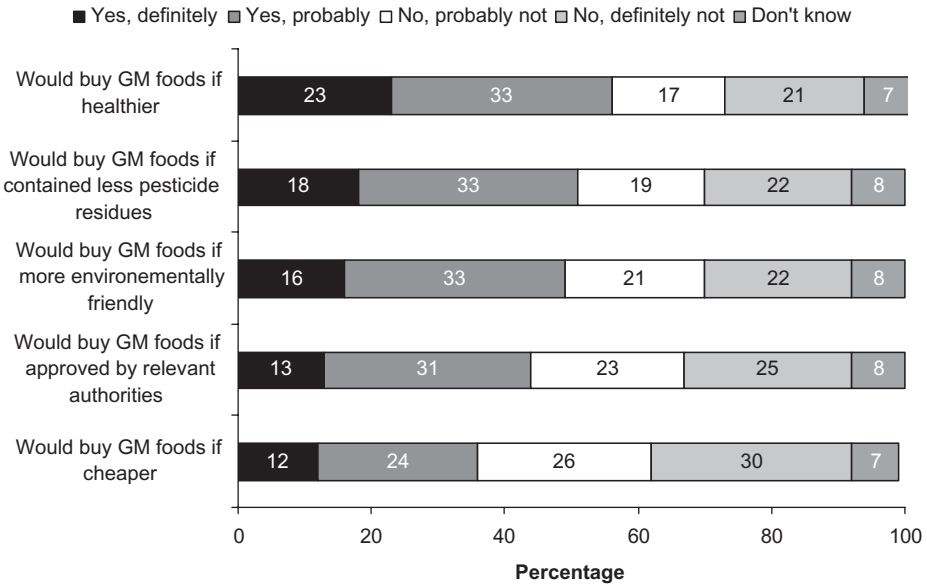

Figure 14.4 Percentage of European citizens willing to buy GM food products with particular characteristics. Gaskell *et al.* [3], Eurobarometer Studies available at: http://www.ec.europa.eu/research/press/2006/pdf/pr1906_eb_64_3_final_report-may2006_en.pdf.

It can be strongly argued that a reaction towards negative public perceptions does not provide a satisfactory answer. First, a number of social scientists had already pointed out repeatedly that public perception studies are studies of the respondents' attitudes and that they cannot be extrapolated to behavior [8–12]. More recently this has been supported by a number of studies on actual buying behavior of consumers presented with GM food products [13, 14].

Second, the results of the latest Eurobarometer survey in 2005, but also other (national) studies still showed considerable support for GM food products (Figure 14.4) [3, 15, 16]. In 2005 43% of the EU population supported GM food products. If GM food products were healthier, then even 56% would definitely or probably buy these products. Interestingly, the result for cheaper foods showed that a mere 56% would not or probably not buy GM foods if they were cheaper. This latter result further supports the point that opinion surveys cannot be taken at face value for behaviors. Gaskell *et al.* [3] suggested that some respondents reacted as citizens rather than consumers, as economics indicate that price is a key determinant in people's choices.

A further interesting point is that price is the only fact consumers can actually ascertain themselves directly. For all the other categories they need to trust some organization (authority, industry, medical research) for the information provided with the food. Whatever the reason, this highlights the doubts about opinion surveys as indicators of actual behavior.

Third, and perhaps most convincingly, the sales of products labeled as GM remained constant (orally confirmed by large supermarket chain). Although there

was no reduction in sales, food companies continue to replace the GM ingredients of the products with non-GM. For example, in the Netherlands there were more than 120 products with GM ingredients with (voluntary) labels in 1999. By 2007 this number had been reduced to 19 [53].

These considerations strongly suggest that food companies and supermarkets replaced their GM products because they were afraid of emotional actions by environmentalists groups rather than being influenced by fewer people buying GM food or trying to protect people from hypothetical risks. The food companies responded to representation from a minority of consumers and the attendant media publicity, and this resulted in an effective halt to the development of cheaper, more sustainable or healthier food products.

What can be done to facilitate a sustainable and accepted introduction of applications from industrial biotechnology? From the perception studies and the reactions of scientists, industries, and governments we also learned some lessons about communication.

14.3.4
Development of Public Interaction

Looking back over the years starting from the first Eurobarometer survey in 1991 on biotechnology we can assess how the results influenced politicians, scientists, and industries. One of the outcomes of these first Eurobarometer studies showed that the public throughout Europe had very little knowledge about biotechnology and indicated that knowledge was linked to support. As a result politicians and biotechnology scientists, aiming to increase the support for biotechnology applications in the early 1990s, started information and education campaigns to educate the public. This one-way communication was based on the belief that if more information were made available then the public would understand the potential benefits and increase their support for biotechnology. This is a good example of the so-called "deficit model" of science communication. However it soon emerged that the brochures, leaflets, lectures, education programmes, etc. did not necessarily increase support [17–20]. It became clear that more information tended to lead to further polarization of opinion, whether positively or negatively.

For the 1996 Eurobarometer study on biotechnology a group of science communication experts were invited to help in getting a clearer understanding of public perception. The social scientists chose "perceived use," "risk," and "moral acceptability" as determinants of public support. People were asked whether they thought each of six biotechnology applications were useful, risky, morally acceptable and if they should be encouraged. The results led to the conclusion that usefulness is a precondition of support and in no case is a "not useful" application given support. For example, GM food products that are similar to "normal" food products but have a lower production cost are not likely to be accepted. People will accept some risk if the application is useful and morally acceptable. For instance, GM foods containing an important vaccine or new medicines produced by yeast are likely to be accepted. Moral concerns, however, acted as a veto regardless of

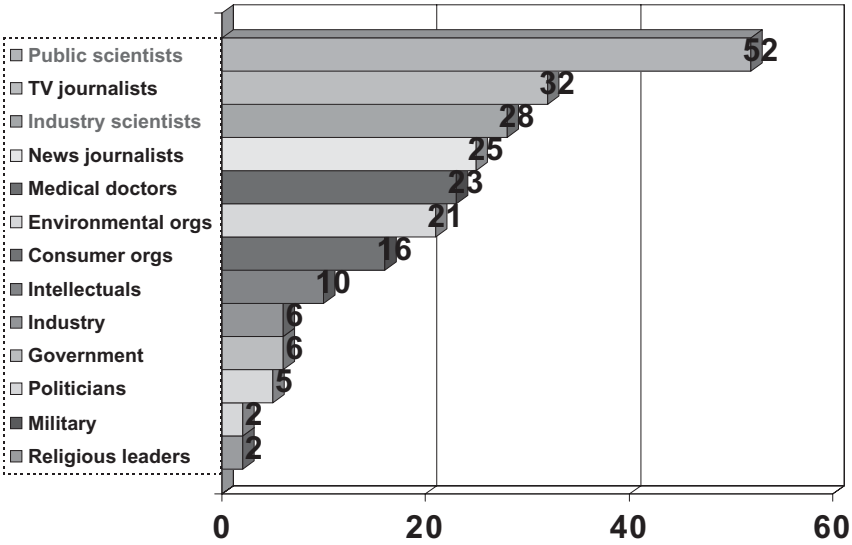

Figure 14.5 Responses of European citizens to "Who is best qualified to explain science and technology impacts on society?" from Gaskell *et al.* [3], Eurobarometer Studies available at: http://www.ec.europa.eu/research/press/2006/pdf/pr1906_eb_64_3_final_report-may2006_en.pdf.

views on risk and use. This is shown by the reluctance displayed about the production of medicines by transgenic animals. A main lesson from the study was the conclusion that "if risk is less significant than moral acceptability, then public concerns are unlikely to be alleviated by technically based reassurances and other policy initiatives dealing solely with risks" [19].

The emphasis on communication certainly shifted to show the benefits of new technology and increased the research on risk assessment, risk communication, and risk perception [21–24]. Adams showed that when risks cannot be controlled by individuals and are vague, for example as a result of scientific uncertainty, confidence decreases and an increased demand for regulation is provoked. So who are the most trusted organizations for providing information on the impact of science and technology?

As Figure 14.5 shows, the European public finds public scientists the most qualified to explain science and technology impacts on society and scientists working in industry only slightly less so. By now it had also become widely recognized that acceptance could not be achieved by simply providing information alone. Scientists had not only a role to play but also had to listen to, understand and respond to actual public concerns. The reaction of social scientists, politicians, and industry was to redevelop models of communication, as characterized by the development of a dialog model called the Mode-2 model and the upstream engagement model for communication [25–28]. Discursive models of communication have been advocated by pioneers such as Churchman [29] and Rittel, and in the last few years those approaches have been revived [30, 31].

Government committees advising on policy measures for technology development started to suggest the involvement of scientists [32–35]. The pressure on scientists to be involved in public communication was further increased by requirements for dissemination and public communication in (inter)nationally funded research projects. Increasingly, project criteria include the dissemination of results to broader audiences, followed by active involvement of stakeholders and demands for public dialogs. These forms of "proactive" communication are now seen as crucial for the implementation of novel technologies.

14.4 Societal Issues in Industrial Biotechnology

The lessons from the biotechnology debate are clear; scientists need to be involved in public communication and such communication needs to address societal issues, involve the stakeholders, etc. Several specially funded projects have been carried out over the years to explore the role of scientists, media, and industry and discuss "best practice" [20, 36]. Training courses have been developed[9] [37] and curricula for future scientists revisited. But what are the criteria for these novel forms of communication?

14.4.1 Criteria for Communication

Many academics and industrialists have concluded that biotechnology scientists need to increase their involvement in public communication to achieve greater public support. However, there are other, more urgent reasons to pay attention to communication. Independent of this wish for increased acceptance, these reasons are derived directly from the principles of a democratic society. Public involvement in decision-making processes requires public information and the social contract between society and scientific institutes demands accountability. Based on these arguments posed by present developments in society and biotechnology with its important potential impact, a set of evaluation criteria for public communication may be derived [20].

Political agendas and decisions are subject to voters' opinions. It is necessary therefore that scientists are accountable to the public about their science and their reasons for doing it so that informed decisions can be made. Scientists also have a moral imperative to communicate with the public as only they have understanding at an early stage of the possible impacts of their science for society, which they need to provide for the joint decision-making process. The complexity of novel

9) A number of courses aimed at scientists and industrialists were developed, such as those by the European Task Force on Public Perception (EU Advanced Course on Bioethics and Public Perceptions; later adapted to the Kluyver Centre Advanced Course on Strategic Communication in Biotechnology); Netherlands Centre for Society and Genomics; European Molecular Biology Organisation; Wellcome Trust.

technologies often leads people to reject new technologies but as people in a democratic system need to be able to weigh up the pros and cons themselves there is also a social obligation on scientists to provide this understanding. Furthermore, if scientists are contracted by society to develop the solutions for tomorrow's challenges, then society needs to be able to trust them. Trust acts as a summing device when full understanding is not possible. This is the general situation for modern technologies, and especially for the complexities of biotechnology. Trust is based on confidence and knowledge which is claimed to be maintained by inclusivity, transparency, and information. This relates to both factual information and emotional feelings.

A component of the contract and trust is accountability [38]. Scientists are contracted and paid for their work by society via taxation and government. They are accountable to society for the uses and outcomes of that payment. The social need for scientists to be accountable, and thereby maintain trust, is an imperative which follows from the contract between society and science.

There are also economic reasons for scientists to communicate with the public. The first relates to the fact that the generation of wealth for the functioning of modern societies wholly depends on science and technology. Biotechnology has been promoted as a major generator of wealth. In order to allow society to make informed decisions about the contribution which biotechnology may make to wealth generation, scientists need to explain its economic impact, that is, its benefits, and its costs, to society. This also includes explanation of the costs and benefits to society if a technology that is scientifically feasible is not pursued. The second economic reason is that scientists have to explain why society must return some of the wealth generated by science to science if science and wealth generation is to continue. As society pays for the publicly funded universities and research institutes, it is in the interest of all academics to communicate about their work. Society decides on the amount and distribution of public funding based on this information. However, with competing calls on limited public funds it is in the biotechnologists' own interest, as with the members of all academic disciplines, to communicate effectively.

The foregoing discussion is based on an idealized view of democracy with full public involvement in the decision-making process. However, the reality in democratic societies is that most people are simply not interested in participating in decision making, which is left to the elected representatives and their staff. They in turn tend to be influenced by communicated opinions and perceived public perceptions while subjected to often intense lobbying by special interest groups, although they are finally answerable to the electorate. Therefore the fact remains that the "silent majority" of the public at large is informed. In order to reach this "silent majority," public communication activities need to stimulate the interest of the public. Because different groups of people have different competing interests and concerns it is also necessary to know and understand their differing interests and concerns. These are not only related to the scientific and technological information, but also importantly to (bio)ethical, safety, social, and legal issues. Scientists need to be able to understand and respond to these issues.

Following from the democratic contract of science with society, these social, moral, and economic reasons dictate that scientists inform and participate in the

Table 14.1 Criteria for communication by scientists derived from the social, moral, and economic reasons for communication as partners in a contract between science and society [20].

Criteria for public communication by scientists to inform the decision-making process:
Explain science
Explain impact
Build trust
Listen and respond to ethical, legal and social concerns
Interest as many as possible
Adapt to changes in society

democratic decision-making process, which includes interaction with the public. As in any contract, good performance is in the interest of the performer. It is argued that communication is an implicit task for scientists, therefore it is in their own interest to do this effectively and it is in the interests of academic institutions to facilitate and organize this process.

From the above-mentioned arguments it can be concluded that public communication relates to:

- the availability of knowledge (information on scientific data; information on potential impact of the implementation of derived technologies in society and information on how judgments are made or can be influenced);
- the availability of skills for interaction; and
- the availability of attitude (to encourage public interest and respond to public interests and concerns).

These requirements lead to the criteria for communication by scientists summarized in Table 14.1.

14.4.2 Novel Approaches to Communication

The application of these criteria for science communication asks for novel forms of communication. Importantly the interaction should be mutual (or two-way) which requires preparedness to listen and understanding of each other's arguments by both sender and receiver. This is not easy to materialize, especially when we wish to create a solution-oriented dialog. Research on novel forms of communication is therefore looking for models with specific attention for discourse (for example focusing on respecting the symmetry of ignorance which is suggested to lead to systematic stepwise learning dynamics [39, 40] and on methods to increase participation of stakeholders [41–43] and of reaching the "uninterested" public majority through entertainment and emotion [44, 45]).

There is no doubt that the transition towards a bio-based society is a very complex design problem, which requires more knowledge than any one single

person can possess and creativity to reach reconciliation of views. As we also strive for changes in consumer behavior, it is important that we combine programmes for sustainable technology and product development with programmes focusing on changes in attitudes and behavior [46] and hence in communication. The following case study of the Kluyver Center for Genomics of Industrial Fermentation will give an example of such an approach.

14.4.3 Three International Workshops Identifying Future Issues in Industrial Biotechnology: A Case Study

One of the most important and perhaps difficult challenges for politicians nowadays is the understanding of ethical, legal, and social concerns in society. In order to fully appreciate the relevant societal issues for applications of industrial biotechnology we need to understand the value systems in our (changing) society, identify present and future stakeholders, and unravel the public and political issues into regulatory, ethical, economic, and safety issues. We also need to understand the roles and responsibilities of all stakeholders so that we can define which organizations can be held responsible for addressing these issues.

The Dutch public–private partnership "Kluyver Center for Genomics of Industrial Fermentation"[10] has carried out a series of three international workshops to identify, understand, and analyze the possible future societal issues in industrial biotechnology. The workshops form part of the Center's program on genomics and society and were aimed to inform the development of novel communication activities (for a full account, see ref. [47]). The workshops brought together 25 experts from different disciplines and affiliations (such ethics, microbiology, food sciences, risk perception, cultural management from academia, industry, government, European Commission, etc.) and also aimed to develop a coordinated strategy for public dialog. The first meeting explored the scientific trends in industrial biotechnology and their linked societal issues. The second aimed to identify the organizations involved and responsible for addressing these public concerns. The third and last meeting set out to suggest novel ways of communication and recommend a joint agenda for this approach.

In their first meeting in 2004, the expert group related scientific trends such as healthy and personalized foods, novel bio-based materials and biofuels with political incentives for industrial biotechnology, concerns about overregulation and the public's low awareness but known acceptance of the contained use of microorganisms. On this basis they identified the following "future" issues:

- Safety, including questions such as those related to contamination of food products by plants producing pharmaceuticals in coexistence with food crops
- Land-use with the possible food–energy conflicts, the rise of food prices and the loss of rainforests

10) A government-funded Center of Excellence, see http://www.kluyvercentre.nl for more information.

- Energetics and eco-efficiency questioning the evidence presented on this complex matter leading to concerns of trust
- Environmental pressure, including concerns on biodiversity; soil depletion, water constraints, and mono-cultures
- Economic feasibility with respect to the dependence on oil and linked sugar prices and resulting uncertainty for industrial investment.

The second meeting in 2005 identified the main barriers as preparedness for action; economic interests of stakeholders; coordination of agendas, and clarity on regulations and incentives. The participants recommended clarifying the notion of sustainability and searching for new ways of interaction to interest the public. Additionally they recommended building trust by showing responsibility (and preparedness for action) and the involvement and training of young scientists in dealing with this.

Although many of the above-mentioned issues were viewed worthy of further exploration, the group decided to focus on biofuels and sustainability in their final meeting in 2006. They took sustainability as the "core value" and proposed a joint agenda for key stakeholders, with the aim of reducing the use of energy and fossil sources while increasing the use of sustainable sources such as biomass. (Figure 14.6). This consensus approach would bring a single message to the public, underlined by a joint agreement, but at the same time would allow organizations to keep true to their interests, shareholders, or constituencies. With sustainability as a core value, industries and academia could focus on the increase of innovation by using industrial biotechnology. NGOs could stress the importance of reduction of energy use and pollution. And governments could develop measures to stimulate both the increase of innovation and the decrease of energy use and pollution.

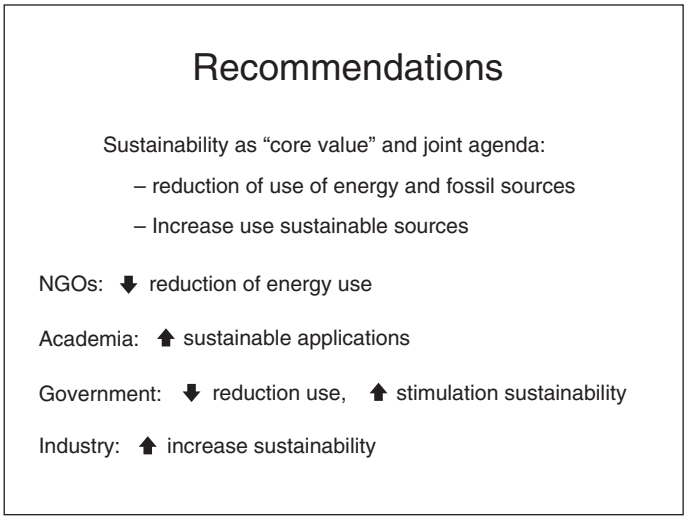

Figure 14.6 Recommendations of the international expert group of the Kluyver Center workshop on future issues in industrial biotechnology, Brussels, June 2006.

It was recognized that the adoption of this joint agenda would need further discussion with the stakeholders. Therefore it was proposed that "neutrally based" organizations such as local governing bodies and the European Commission would hold stakeholder meetings. These meetings should aim to openly discuss economic interests, values, and trust relations in order to increase understanding of differing viewpoints and decrease the development of wrong perceptions. The experts further recommended that politicians should focus on the removal of bottlenecks with a view to create uniform regulation. They should also focus on the development of clear incentive procedures. Last but not least, it was recommended that research on the development of novel forms of public communication should be increased with special attention on increasing the level of citizen involvement and responsibility. It is interesting to see that these predictions of the possible future issues of this expert group in June 2006 are the ones presently discussed in the media (Autumn 2007). But what do they entail?

14.4.4 Further Analysis of the Identified Societal Issues Related to Industrial Biotechnology

The first issue, safety, is a well-known phenomenon of our present-day risk-averse society. Although it presents itself as a rational and reasonable concern it is actually something much more than that. To begin with, many scientists claim that there is no known rational scientific basis for concern. They argue that fermentation is something that has been used for centuries and the application of GM techniques provides a more precise method than any previous technique used to improve the microorganisms. So far the many studies on risk assessment have not shown any significant risk from modern industrial engineering biotechnology where the regulated precautionary actions are followed. Neither have we witnessed any great accident since the introduction of industrial GM microorganisms some 30 years ago. Furthermore, there is firm and stringent legislation. The safety of GMOs used in industrial biotechnology depends on the characteristics of the organism and its interaction with the environment into which it is (accidentally) released. Safety legislation generally requires risk analysis that can identify and evaluate potential adverse effects of the GMO(s). Host organisms are chosen for their ability to produce the desired product but also for their inability to grow outside the production unit. If GM (micro) organisms are released for applications in the environment, further safety measures are required to minimize human health and environmental adverse effects.

The use of the precautionary principle has enforced a very stringent approach to safety in Europe. A definition of precaution is provided in the UNESCO document *The Precautionary Principle*, published by the World Commission on the Ethics of Scientific Knowledge and Technology (COMEST) in 2005. The Precautionary Principle United Nations Educational Scientific and Cultural Organization. Printed in France SHS-2005/WS/21 cld/d 20/5/:

When human activities may lead to morally unacceptable harm that is scientifically plausible but uncertain, actions shall be taken to avoid or diminish that harm.

Morally unacceptable harm refers to harm to humans or the environment that is

- threatening to human life or health, or
- serious and effectively irreversible, or
- inequitable to present or future generations, or
- imposed without adequate consideration of the human rights of those affected.

The judgment of plausibility should be grounded in scientific analysis. Analysis should be ongoing so that chosen actions are subject to review.

Uncertainty may apply to, but need not be limited to, causality or the bounds of the possible harm.

Actions are interventions that are undertaken before harm occurs that seek to avoid or diminish the harm. Actions should be chosen that are proportional to the seriousness of the potential harm, with consideration of their positive and negative consequences, and with an assessment of the moral implications of both action and inaction. The choice of action should be the result of a participatory process.

Companies, and increasingly governments, are now requesting deregulation for certain applications including industrial biotechnology, as the present situation is viewed as disadvantaging economic growth. Many supporters of biotechnology point out that the required risk assessments do not include a comparative risk assessment to existing processes, products, or practices. Additionally there is debate among regulators about the abolition of regulation on processes using GM techniques where the product does not contain any GM. Others claim that including an assessment of the potential benefits of the proposed innovation would create an incentive for beneficial innovation.

The request for deregulation stands on a sensitive level with the identified necessity for maintaining trust. Risk perception studies, such as those by Adams ([22], see also [48]) have shown that concerns increase and become less rationally based when people are unfamiliar with the actual risk of a technology or material and when they have no control themselves over its use. It is argued, therefore, that the public concerns related to safety are more likely to spring from an issue of control. It is clear that any scientific uncertainty expressed in the public domain will increase the level of public unease and, indeed, the demand for regulation. But regulation needs to be controlled by someone and that is also why maintaining and building trust has been mentioned as a crucial factor in technology innovation.

O'Neill has pointed out, however, that although an increase in regulation and control mechanisms will undoubtedly raise the trustworthiness of the system, it

will not necessarily increase trust in the people who are implementing the novel technology [49]. We urgently need to further understand this relationship and find new ways to deal with scientific uncertainty and with emotive public reactions. We also need to find ways which will build or maintain public trust not only in the scientists who develop the technology (and who already are trusted by the public, see Figure 14.5), but also in those who are responsible for regulating and controlling its uses in society.

The second issue, land-use, is probably the one that presently creates the most hype. Recent media reports include emotive terms such as "disgrace," "crime against humanity," and "food robbers" in the attack of the production of biomass for non-food materials (usually biofuels). Interestingly, the articles that are positive towards biofuel development are less emotive, perhaps with the exception of Al Gore and his supporters in their claims about the use of these technologies against global warming. In essence this "land issue" is an economic one: land owners have to decide on the basis of returns on investment what they will grow. Their choices may influence food prices, for example if they decide to grow non-food energy crops. However, it will be hard to disentangle the effect of land-use from the overall effect of an increasing demand in biomass.

A more emotively expressed area in this issue of land-use is the loss of rainforests and the choices made by poor farmers in developing countries to grow bioenergy crops rather than food crops. As some point out, this may result in more local food crises in already struggling countries but also in a higher income which may enable them to import foods. It is unclear how the economy will develop and what will work best for whom.

It is interesting, though, to see how this issue on the use of land is linked to an ethical concern of much broader underlying value. While the increase of safety is often sought by people aiming for a higher level of individual autonomy and choice, the issue of land-use is actually used to support an ideology for all. The ideology includes moral values, linked to a view on the natural world but also to values of democracy, equity for people from developed and less-developed countries, and freedom of handling in less-developed countries.

Although their intentions may be very well meant, it is those from Western societies without any land themselves who are usually most concerned with these moral issues of land-use. And their well-meant moral values may differ from those of people living in developing countries, leading to accusations of "neo-colonialism." Some countries have tried to develop regulations to control the sustainable use of our global land (Cramer Report, 2007)[11]. However it is necessary to realize that people from Western societies are generally in the highest level of "Maslow's pyramid", their basic needs for water, food, housing, healthcare, schooling, and employment are fulfilled. This is not the case in developing countries were sometimes even basic requirements such as food and housing are not yet met. People in these circumstances are not able to concern themselves with issues

11) Project group "Sustainable Production of Biomass" (2007). Testing framework for sustainable biomass. Senternorem, The Hague, Netherlands.

related to "luxury" problems for next generations, such as loss of rainforest or global warming [50].

It is likely, therefore, that consensus will be difficult to achieve as those most willing to enforce it are generally in a position to be able to afford this, while those in developing countries have more urgent needs to fulfill which may prove counterproductive.

The third issue is energetics or eco-efficiency. Presently many impact studies are performed to calculate the ecological footprint in terms of energy and materials produced versus energy and materials used in a global setting. These models aim to predict the best crops for a certain desired product produced in the best (most sustainable) way. Because much of the data needed for the calculation are uncertain and the number of variables included in the calculation differ, the models produce very different outcomes. These results are seriously questioned by scientists, industrialists, and NGOs in (industrial and agricultural) biotechnology who relate to these models as predictors for research investments. This issue therefore relates to the uncertainty of evidence and scientific inquiry. Since the results of these models are often used in public interaction as "proof" of a certain viewpoint, the issue of scientific uncertainty and factual evidence is actually magnified. This undermines public trust in scientists for their ability to produce "rational facts."

The heated debate about the validity of the data may also be perceived by the public in a different way, that is, that parties in debate select and use the "facts" which most suit them because they have an (economic) interest which they wish to advance. Such a perception may further decrease the trust relationships and increase public unease with the technology. The effect of scientific uncertainty of evidence, scientific inquiry, trust, and stakeholder perceptions of interests on public unease and technology development needs further study.

The fourth issue, environmental pressure, for example for water and soil depletion, looks like another scientific issue. It could be solved as soon as we know how to work the land in such a way that we do not deplete our soil and use too much water and prevent the loss of biodiversity. For the moment this is again a concern of scientific uncertainty on the best way to handle this issue in the short term while solutions are developed for long-term and higher demands. The abolition of tillage and introduction of drought-resistant crops are used as possible solutions, but some fear that there will never be enough water to produce the total amount of crops needed for a biomass economy. This issue further relates to biodiversity, which is a concern for many years related to GM crops and industrial agriculture.

Large agricultural practises using monocultures and herbicides and pesticides are often seen as a threat to the diversity of our global plant (and linked animal) kingdom. Diversity is needed as a source of traits (DNA) for future applications in crops or for pharmaceutical products. Areas rich in diversity of species include rainforests, but also areas in extreme environmental conditions are viewed as important providers of genetic material. Presently seed-banks have been created to maintain the traits of rare or nearly extinct sources. However, it is clear that the

in situ maintenance would be preferable as it would also allow for further evolution and creation of new characteristics. Reduction of herbicides and pesticides by using GM crops and a transition to no-tillage practises may also help to maintain biodiversity.

The issue of biodiversity and environmental pressure, however, is not only scientific but is also often related to a deeper underlying view of nature. The arguments used in the heated debates on the supposed loss of monarch butterflies in GM cornfields [51] indicate that those concerned for biodiversity are often refusing scientific solutions, but propose to go back to the "original, natural way" of producing crops (such as in organic farming practices). These views often become emotive in heated debate and lose their science-based rationality [52].

The fifth and final issue presented here is economic feasibility. This clearly is an economic issue for the industry involved and not so much a public issue. It refers to the difficulty of industries to convert to sustainable industrial biotechnology production processes. In order to achieve this, industries need to invest in innovation, manpower, and equipment but they have to decide on these matters in an environment of uncertainty. Oil and feedstock prices fluctuate wildly, innovations are still in development (such as second-generation biofuels), while governmental incentives are not clarified and regulations are still being discussed. Although industries are resourceful in creating ways of balancing these uncertainties against their shareholder values, it is clear that clarification of regulation and decisions on incentives will help to speed up the introduction of sustainable processes.

14.4.5
Other Relevant Studies and Committee Reports

Since the 1980s a whole industry of governmental, intercontinental, multidisciplinary, and multi-stakeholder committees has evolved. It reflects a change in democratic decision-making as many involve more parties in the discussion such as representatives of consumer and patient organizations, NGOs, lay people, etc. Several of these committees have produced very interesting reports, such as the UNESCO report on the precautionary principle (2005) and the Netherlands COGEM[12] report "Towards an integrated framework for the assessment of social and ethical issues in modern biotechnology" (2003). Both provide clear definitions and/or procedures for evaluation of the state of the art of governing implementation of biotechnology in society. Other studies have delivered high-profile recommendations (such as the EU-US Consultative Forum[13], 2000). The recent Cramer Report[12] provides guidelines for sustainable development of biofuels, aiming to avoid the use of rainforests and the use of other less sustainable methods.

As the players in the discursive process are extending, it is important to have such sources of information available. These studies and reports will also help us

12) Commissie Genetische Modificatie (COGEM) (2003) Towards an integrated framework for the assessement of social and ethical issues in modern biotechnology.

13) Anonymous. The EU-US Biotechnology Consultative Forum: Find Report, December 2000.

to understand the social practices around the globe and provide useful suggestions for the implementation of global sustainability. It is also important, however, to acknowledge that different stakeholders use different reports, presenting different views or even "facts." The choices between sources of information (and trust given to these sources) may play a crucial role in the discussion and needs further investigation.

14.5 Conclusions and Discussion: A Joint Agenda for the Smooth Introduction of Acceptable Sustainable Industrial Biotechnology

We have presented an account of what experts believe the impact will be of industrial biotechnology to our society. We have also showed what (European) citizens think they may support, relying primarily on the Eurobarometer surveys, and have indicated the possible social concerns that may arise from these developments. We have drawn some lessons from the GM food debate which we belief give reason for caution for an overoptimistic view to the acceptability of industrial biotechnology. These lessons taught us that more knowledge does not necessarily result in more support for a developing technology. They also gave us more insight in the risk issue and showed that risk can be overridden by moral values. Through the evidence that European citizens do not disapprove of GM foods we argued that a rational approach (in this case to provide informed choice) can sometimes be overtaken by emotional fear.

Finally the perception studies showed that scientists are one of the most trusted professionals by the public. On this basis, but also on the argument that our democratic society has a contract with scientists for which they are accountable we argued that scientists have an important role to play in public interaction on the implementation of novel technologies derived from science.

The main argument for caution in the introduction of a bio-based society is that the present debate is not based on rationality of reason and that such emotive context may easily result in equally irrational reactions from politics and industries. However, we have also pointed to the lessons learned from improved involvement of all stakeholders in and responsibility for novel forms of public interaction. This requires preparedness for action, and a preparedness to listen to the arguments and take action on concerns. It also necessitates a reconciliation of interests of all parties, which can be done if all parties adopt sustainability as a core value. This is a challenge for the public because sustainability is not something with a direct impact on the individual. And for many it relates directly to a certain view of the world. In contrast, many novel developments in healthcare are often embraced as direct improvements of people's quality of life.

It is doubtful whether new applications for the environment (and hence for future generations) will be received equally positive by all.

14.5.1 Hurdles and Challenges

Politicians are being challenged to come up with the right incentives and regulation, but they are dependent on trustworthy scientific evidence that supports their action. Unfortunately it is just this scientific evidence that is presently so much at stake in the debate. And the debate increasingly ranges from rational to emotive. Taking a position leads to polarization and political inertion. In order to reconcile different views it is important to find common aims. The experts who came together to discuss future issues in industrial biotechnology concluded that "sustainability" could be taken as a core value. They recommended the development of a joint agenda for all stakeholders involved, taking this notion of sustainability as a core value. However, we conclude that in addition to this core value, we need to make sure that plans also address the basic needs of food, health, housing, and employment.

In exploring the issues we have seen that personal views may lead to different positions, which are often not brought into the discussion, and may give rise to emotional claims. This necessitates a willingness to come together and discuss a way to reconcile positions and views.

It is good to see that this view is shared by several multi-stakeholder organizations such as the European Platform on Sustainable Chemistry together with the European trade organization EuropaBio, the Directorate Science of the European Commission, the Working Party on Biotechnology of the OECD and the World Wide Fund. They have a challenging time ahead.

14.5.2 Recommendations for Further Studies

As argued in the above text an understanding of public concerns is crucial and encompasses a much broader understanding involving values, economic interests, dealing with uncertainty, trust, and responsibility. We showed that although safety issues can represent a demand for individual autonomy, the land-use issue may represent a deeper underlying ideology for global governance. These values are undoubtedly related to different views on the relation between humans and nature, which can be controversial. The question is whether these controversial views on governance and autonomy are held by the same people and whether discussing these underlying values could help in the search for acceptable solutions for sustainable development. With this understanding we need to develop novel forms of interaction with society.

14.5.3 What Does It Mean for Citizens?

A bio-based society will change the landscape, political powers, and our national incomes – all factors with which citizens will need to come to terms. But as argued

above, a joint agenda for increased sustainability also depends on a decrease of energy and material use. This requires a responsibility and change of lifestyle for all and a re-evaluation of everything we do (holidays, sports), use (traveling, packaging, etc.), and eat. In that sense it requires that sustainability will become a moral value.

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