

1

Introduction to Enzyme Technology

1.1

Introduction

Biotechnology offers an increasing potential for the production of goods to meet various human needs. In enzyme technology – a subfield of biotechnology – new processes have been and are being developed to manufacture both bulk and high added-value products utilizing enzymes as biocatalysts, in order to meet needs such as food (e.g., bread, cheese, beer, vinegar), fine chemicals (e.g., amino acids, vitamins), and pharmaceuticals. Enzymes are also used to provide services, as in washing and environmental processes, or for analytical and diagnostic purposes. The driving force in the development of enzyme technology, both in academia and in industry, has been and will continue to be

- the development of new and better products, processes, and services to meet these needs, and/or
- the improvement of processes to produce existing products from new raw materials such as biomass.

The goal of these approaches is to design innovative products and processes that not only are competitive but also meet criteria of sustainability. The concept of sustainability was introduced by the World Commission on Environment and Development (WCED, 1987) with the aim to promote a necessary “. . . development that meets the needs of the present without compromising the ability of future generations to meet their own needs.” This definition is now part of the *Cartagena Protocol on Biosafety to the Convention on Biological Diversity*, an international treaty governing the movements of living modified organisms (LMOs) resulting from modern biotechnology from one country to another. It was adopted on January 29, 2000 as a supplementary agreement to the Convention on Biological Diversity and entered into force on September 11, 2003 (<http://bch.cbd.int/protocol/text/>). It has now been ratified by 160 states. To determine the sustainability of a process, criteria that evaluate its economic, environmental, and social impact must be used (Gram *et al.*, 2001; Raven, 2002; Clark and Dickson, 2003). A positive effect in all these three fields is required for a sustainable process. Criteria for the quantitative evaluation

of the economic and environmental impact are in contrast with the criteria for the social impact, easy to formulate. In order to be economically and environmentally more sustainable than an existing process, a new process must be designed not only to reduce the consumption of resources (e.g., raw materials, energy, air, water), waste production, and environmental impact, but also to increase the recycling of waste per kilogram of product (Heinzle, Biwer, and Cooney, 2006).

1.1.1

What are Biocatalysts?

Biocatalysts either are proteins (*enzymes*) or, in a few cases, may be nucleic acids (*ribozymes*; some RNA molecules can catalyze the hydrolysis of RNA). These ribozymes were detected in the 1980s and will not be dealt with here (Cech, 1993). Today, we know that enzymes are necessary in all living systems, to catalyze all chemical reactions required for their survival and reproduction – rapidly, selectively, and efficiently. Isolated enzymes can also catalyze these reactions. In the case of enzymes, however, the question whether they can also act as catalysts outside living systems had been a point of controversy among biochemists in the beginning of the twentieth century. It was shown at an early stage, however, that enzymes could indeed be used as catalysts outside living cells, and several processes in which they were applied as biocatalysts have been patented (see Section 1.3).

These excellent properties of enzymes are utilized in enzyme technology. For example, they can be used as biocatalysts, either as isolated enzymes or as enzyme systems in living cells, to catalyze chemical reactions on an industrial scale in a sustainable manner. Their application covers the production of desired products for all human material needs (e.g., food, animal feed, pharmaceuticals, bulk and fine chemicals, detergents, fibers for clothing, hygiene, and environmental technology), as well as for a wide range of analytical purposes, especially in diagnostics. In fact, during the past 50 years the rapid increase in our knowledge of enzymes – as well as their biosynthesis and molecular biology – now allows their rational use as biocatalysts in many processes, and in addition their modification and optimization for new synthetic schemes and the solution of analytical problems.

This introductory chapter outlines the technical and economic potential of enzyme technology as part of biotechnology. Briefly, it describes the historical background of enzymes, as well as their advantages and disadvantages, and compares these to alternative production processes. In addition, the current and potential importance and the problems to consider in the rational design of enzyme processes are also outlined.

1.1.2

Bio- and Chemocatalysts – Similarities and Differences

Berzelius, in 1835, conceived the pioneering concept of catalysis, including both chemo- and biocatalysis, by inorganic acids, metals such as platinum, and enzymes

(Berzelius, 1835). It was based on experimental studies on both bio- and chemocatalytic reactions. The biocatalytic system he studied was starch hydrolysis by diastase (a mixture of amylases). In both systems, the catalyst accelerates the reaction, but is not consumed. Thus, bio- and chemocatalysis have phenomenological similarities. The main differences are the sources and characteristics of these catalysts. Chemocatalysts are designed and synthesized by chemists, and are in general low molecular weight substances, metal catalysts, complexes of metals with low molecular weight organic ligands, such as Ziegler-Natta and metallocene catalysts, and organocatalysts (Fonseca and List, 2004). In contrast, biocatalysts are selected by evolution and synthesized in living systems. Furthermore, enzymes (including ribonucleic acid-based biocatalysts) are macromolecules, their highly sophisticated structure being essential for their function, and notably for their regio-, chemo-, and enantioselectivity.

Due to development of gene and recombinant technologies in the past 40 years, enzymes that previously only could be obtained in limited amounts from microorganisms and tissues can now be synthesized in nearly unlimited quantities in suitable microorganisms. Further, based on the development in biochemistry, bioinformatics, and micro- and molecular biology, new tools have been developed to improve the properties of enzymes for their use in biocatalytic processes. They are rational protein design and *in vitro* evolution in combination with high-throughput screening tools. Very recently, also the *de novo* computational design of enzymes was described, but so far these show little activity in the same range as catalytic antibodies (Jiang *et al.*, 2008; Röthlisberger *et al.*, 2008).

Until the first oil crisis of 1973, the development and application of bio- and chemocatalysis occurred in – at that time – nonoverlapping fields. Biocatalysis was mainly studied by biochemists, biochemical engineers, microbiologists, physiologists, and some physical organic chemists (Jencks, 1969). It was mainly applied in the food, fine chemical, and pharmaceutical industries and medicine (see Section 1.3). Chemocatalysis was mainly studied by chemical engineers and chemists. It was applied in the production of bulk chemicals such as acids and bases, and products derived from coal and oil (fuel, plastics, etc.). This resulted for a long time in a small exchange of fundamental results between those who studied and applied bio- and chemocatalysis. The analytical description of heterogeneous catalysis, where the catalyst is located only in a part of the system, was first developed and verified experimentally for living systems in the 1920s by biochemists. Contrary to homogeneous catalysis, this description involves the coupling of the reaction with mass transfer. This applies also for heterogeneous chemocatalysis. The same description as for living systems was derived independently by chemical engineers in the end of the 1930s (see Chapter 10).

The detailed mechanism of the catalyzed reactions has now been determined for many bio- and chemocatalysts. This knowledge that is continuously increasing yields information that can be used to design improved bio- and chemocatalysts. This, however, requires a closer cooperation of those working with these catalysts. Fortunately, due to the increasing use of enzymes by organic chemists in the past decades, this cooperation has increased markedly.

1.2

Goals and Potential of Biotechnological Production Processes

Biomass – that is, renewable raw materials – has been and will continue to be a sustainable resource that is required to meet a variety of human material needs. In developed countries such as Germany, biomass covers $\approx 30\%$ of the raw material need – equivalent to ~ 7000 kg per person per year. The consumption of biomass for different human demands is shown schematically in Figure 1.1. This distribution of the consumption is representative for a developed country in the regions that have a high energy consumption during the winter. However, the consumption of energy (expressed as tons of oil equivalent per capita in 2007) showed a wide range, from 8 in the United States to 4 in Germany and the United Kingdom, 1.5 in China, and 0.5 in India (IEA, 2010).

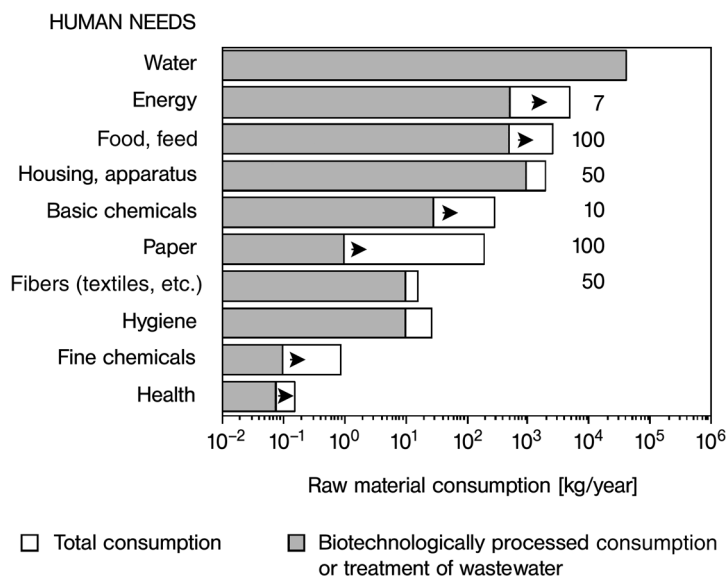


Figure 1.1 Consumption of raw materials for various human needs per person and year in Germany 1992. The water consumption is only for household use. These numbers are still valid. The energy consumption per capita has hardly changed since then. However, now (2010) 11% is derived from renewable resources (biomass, solar, water, wind) (AGEB, 2010). The arrowheads indicate the current increase in biotechnological processing of the products for different demands. For food and animal feed, only renewable raw materials (biomass) can be used; the figures to the right give the percentage for biomass of the raw

materials currently used for the production. They can, especially for energy, only increase when they do not interfere with the biomass demand for food and feed. Due to the low material demands for hygiene, fine chemicals, and health products, 0–100% of the raw materials can be biomass, depending on the product. After the use of the products, the unavoidable waste must be recycled in a sustainable manner. Besides wastewater, this results in about 1000 kg of solid waste per year (soil, building materials, plastics, sludge, etc.). Energy is measured in coal equivalents.

This is mainly due to differences in energy use for housing, transport, and the production of other material needs. In less-developed countries, although the fraction of biomass as raw material to meet human demands is higher than that in the developed countries, the total consumption is smaller.

Biomass – in contrast to nonrenewable raw materials such as metals, coal, and oil – is renewable in a sustainable manner when the following criteria are fulfilled:

- the C, N, O, and salt cycles in the biosphere are conserved, and
- the conditions for a sustainable biomass production through photosynthesis and biological turnover of biomass in soil and aqueous systems are conserved (Beringer, Lucht, and Schaphoff, 2011).

Currently, these criteria are not fulfilled on a global level, one example being the imbalance between the CO₂ production to meet energy requirements and its consumption by photosynthesis in the presently decreasing areas of rain forests. This leads to global warming and other consequences that further violate these criteria. International treaties – for example, the Kyoto Convention and the Convention on Biological Diversity – have been introduced in an attempt to counteract these developments and to reach a goal that fulfills the above criteria (see Section 1.1).

Only when the above sustainability criteria are fulfilled, biomass can be used as raw material to meet the human demands illustrated in Figure 1.1. The needs for human food and animal feed must be met completely by biomass, though when these needs of highest priority are met, biomass can be used to fulfill the other demands shown in Figure 1.1. This applies especially to those areas with lower total raw material consumption than for food. From this point, it also follows that a large consumption of biomass to meet energy demands is only possible in countries with a low population density and a high biomass production.

By definition, biotechnological processes are especially suited to the production of compounds from biomass as the raw material (Figure 1.2). The amount produced in, and economic importance of, such processes is detailed in Table 1.1.

This also involves the development of suitable concepts, methods, and equipment to obtain more sustainable processes. From the information provided in

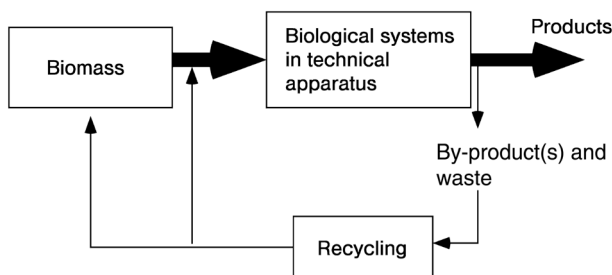


Figure 1.2 Schematic view of an ideal sustainable biotechnological production process. Biomass as a regenerable resource is converted into desired products with minimal waste and by-product production. The waste and by-products must be completely recycled.

Table 1.1 Yearly production and value of biotechnologically produced products to meet human needs.

Human need	Product (year)	World production (t year ⁻¹)	Value (×10 ⁹ euros year ⁻¹)	Production method	
				Biotechnological	Chemical
Food and feed	Beer/wine (2009)	195 000 000 (a)	≈300	F, E	
	Cheese (2009)	19 400 000 (a)	≈100	F, E	
	Baker's yeast (1992)	1 800 000	?	F	
	Vegetable oils (partly used for biodiesel) (2009)	107 000 000 (a)	?	E	+
	Vinegar (10% acetic acid)	>1 500 000*		F	
	Amino acids (2006)	3 000 000 (c)	3 (c)	F, E	+
Fine chemicals including feed and food additives	Glucose–fructose syrup	12 000 000 (g)	5	E	
	Vitamin C (2006)	80 000 (c)	1 (c)	F, E	+
	Aspartame (dipeptide) (2006)	15 000 (c)	?	F, E	+
	Citric acid (2006)	1 500 000 (c)	1.2 (c)	F	
	Herbicides, insecticides	>2 200 000*	?	E	+
	Enzymes (2010)	>10 000	2.6 (d)	F	
Basic chemicals	Products from biomass				
	Biodiesel from vegetable oils (2007)	9 000 000 m ³ (e)			
	Bioethanol (2007)	50 000 000 m ³ (e)		E, F	
	Acrylamide	>150 000*	?	E	+
	1,3-Propanediol	>100 000*		F	
	Polylactic acid	140 000 (g)		F	+
	Acetic acid (2003)	3 400 000 (b)			+

Fibers for textiles	Cotton (2008)	25 000 000 (a)	30 (a)	E	+
	Wool (2008)	2 000 000 (a)	3.5 (a)	E	+
	Linen (2008)	600 000 (a)	0.3 (a)	F, E	+
Paper	All forms (2009)	370 000 000 (a) (50% recycled)		E	+
Hygienics/detergents	Biotensides	?	?	E, F	+
	Washing powder (2003)	24 000 000 (b)		F	+
Therapeutics	Antibiotics	>60 000*	≈35 (h)	F, E	+
	Insulin (2010)	>10	10 (f)	F, E	
	Recombinant proteins (factor VIII, interferons, tPA, hormones, growth factors, etc.) (2008)	?	50 (g)	F, E	
	Monoclonal antibodies (2008)	?	20 (g)	F	
Diagnostics (estimated figures)	Monoclonal antibodies	?	>1	F, E	+
	DNA/protein chips	?	?	F, E	+
	Enzyme-based	<1	>1	F, E	+
Environment	Clean water (only Germany)	13 000 000 000	13	F	+
	Clean air/soil (soil remediation)	?	?	F	+
Comparison	All products (2009)	>1 000 000 000*	≈1900 (i)		
Chemical industry	Chemical catalysts (2009)	?	12 (j)		

All production data are from 2003 to 2010; values are only given where sources give the present data or when they can be estimated, based on prices in the European Union (EU). F = fermentation; E = enzyme technology.

Sources: (a) FAOSTAT (<http://faostat.fao.org/site/339/default.aspx>); (b) UN (2003); (c) Soetaert and Vandamme (2010); (d) Novozymes (2011); (e) FAO (2008); (f) Novo Nordisk (2011); (g) Buchholz and Collins (2010); (h) Hamad (2010); (i) CEFIC (2010); (j) Bryant (2010).

* Estimated as newer data are not available for open access.

Figure 1.1, it also follows that biotechnology has a major potential in the development of sustainable processes to meet all human needs.

Enzyme technology is a part of biotechnology that is defined in the internationally accepted Cartagena Convention (see Section 1.1) as follows:

“Biotechnology means any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use.”

with the following amendment:

“The use must be sustainable, this means the use of components of biological diversity in a way and at a rate that does not lead to long-term decline of biological diversity, thereby maintaining its potential to meet the needs and aspirations of present and future generations.”

This requires that traditional classical – as well as new biotechnological – processes must be improved and/or developed in order to be sustainable (Figure 1.2). The fundamentals needed for the development of such processes in the interdisciplinary field of biotechnology require the close cooperation of biologists, chemists, and biochemical and chemical engineers.

1.3

Historical Highlights of Enzyme Technology/Applied Biocatalysis

1.3.1

Early Developments

Applied biocatalysis has its roots in the ancient manufacture and preservation of food and alcoholic drinks, as can be seen in old Egyptian pictures. Cheese making has always involved the use of enzymes, and as far back as about 400 BC, Homer's Iliad mentions the use of a kid's stomach for making cheese.

With the development of modern natural science during the eighteenth and nineteenth centuries, applied biocatalysis began to develop a more scientific basis. In 1833, Payen and Persoz investigated the action of extracts of germinating barley in the hydrolysis of starch to yield dextrin and sugar, and went on to formulate some basic principles of enzyme action (Payen and Persoz, 1833):

- small amounts of the preparation were able to liquefy large amounts of starch,
- the material was thermolabile, and
- the active substance could be precipitated from aqueous solution by alcohol, and thus be concentrated and purified. This active substance was called *diastase* (a mixture of amylases).

In 1835, the hydrolysis of starch by diastase was acknowledged as a catalytic reaction by Berzelius. In 1839, he also interpreted fermentation as being caused by a catalytic force, and postulated that a body – by its mere presence – could, by affinity to the fermentable substance, cause its rearrangement to the products (Hoffmann-Ostenhof, 1954).

The application of diastase was a major issue from the 1830s onwards, and the enzyme was used to produce dextrin that was used mainly in France in bakeries, and also in the production of beer and wines from fruits. The process was described in more detail, including its applications and economic calculations, by Payen (1874) (Figure 1.3). Indeed, it was demonstrated that the use of the enzymes in malt (amylases, amyloglucosidases) in this hydrolytic process was more economic than that of sulfuric acid.

Lab preparations were also used to produce cheese (Knapp, 1847), and Berzelius later reported that 1 part of lab ferment preparation coagulated 1800 parts of milk, and that only 0.06 parts of the ferment was lost. This provided further evidence for Berzelius' hypothesis that ferments were indeed catalysts.

About two decades later, the distinction of organized and unorganized ferments was proposed (Wagner, 1857), and further developed by Payen (1874). These investigators noted that fermentation appeared to be a contact (catalytic) process of a degradation or addition process (with water), and could be carried out by two substances or bodies:

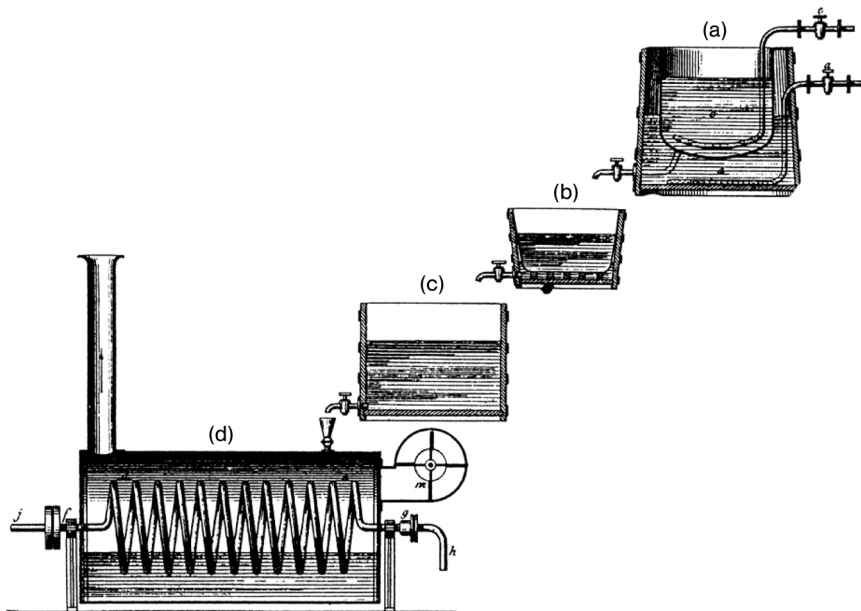


Figure 1.3 Process for dextrin production, with reaction vessel (a), filter (b), reservoir (c), and concentration unit (d).

- A nitrogen-containing organic (unorganized) substance, such as protein material undergoing degradation.
- A living (organized) body, a lower class plant, or an “infusorium,” an example being the production of alcohol by fermentation.

It is likely that the effect is the same, insofar as the ferment of the organized class produces a body of the unorganized class – and perhaps a large number of singular ferments. Consequently, in 1878, Kühne named the latter class of substances *enzymes*.

Progress in the knowledge of soluble ferments (enzymes) remained slow until the 1890s, mainly due to a scientific discussion where leading scientists such as Pasteur denied the existence of “unorganized soluble ferments” that had no chemical identity. Consequently, the subject of enzymatic catalysis remained obscure, and was considered only to be associated with processes in living systems. In the theory of fermentation, a degree of mystery still played a role: Some *vital factor*, “*le principe vital*,” which differed from chemical forces, was considered to be an important principle in the chemical processes associated with the synthesis of materials isolated from living matter. But Liebig and his school took an opposite view, and considered fermentation simply to be a decay process.

In 1874, in Copenhagen, Denmark, Christian Hansen started the first company (Christian Hansen’s Laboratory) for the marketing of standardized enzyme preparations, namely, rennet for cheese making (Buchholz and Poulson, 2000).

1.3.2

Scientific Progress Since 1890: The Biochemical Paradigm; Growing Success in Application

From about 1894 onward, Emil Fischer elaborated the essential aspects of enzyme catalysis. The first aspect was *specificity*, and in a series of experiments Fischer investigated the action of different enzymes using several glycosides and oligosaccharides. For this investigation, he compared invertin and emulsin. He extracted invertin from yeast – a normal procedure – and showed that it hydrolyzed the α -, but not the β -methyl-D-glucoside. In contrast, emulsin – a commercial preparation from Merck – hydrolyzed the β -, but not the α -methyl-D-glucoside. Fischer therefore deduced the famous picture of a “lock and key,” which he considered a precondition for the potential of an enzyme to have a chemical effect on the substrate. In this way, he assumed that the “geometrical form of the (enzyme) molecule concerning its asymmetry corresponds to that of the natural hexoses” (sugars) (Fischer, 1909).

The second aspect referred to the protein nature of enzymes. In 1894, Fischer stated that among the agents that serve the living cell, the proteins are the most important. He was convinced that enzymes were proteins, but it took more than 20 years until the chemical nature of enzymes was acknowledged. Indeed, Willstätter, as late as 1927, still denied that enzymes were proteins (Fruton, 1976). A few years after Fischer’s initial investigations, Eduard Buchner published a series of papers (Buchner, 1897, 1898) that signaled a breakthrough in fermentation and enzymology. In his first paper on alcoholic fermentation without yeast cells, he stated, in a

remarkably short and precise manner, that “. . . a separation of the (alcoholic) fermentation from the living yeast cells was not successful up to now.” In subsequent reports, he described a process that solved this problem (Buchner, 1897), and provided experimental details for the preparation of a cell-free pressed juice from yeast cells that transformed sugar into alcohol and carbon dioxide. Buchner presented the proof that (alcoholic) fermentation did not require the presence of “. . . such a complex apparatus as is the yeast cell.” The agent was in fact a soluble substance – without doubt a protein body – that he called *zymase* (Buchner, 1897). In referring to the deep controversy on his findings and theory, and in contradiction to the ideas of Pasteur (see above), Buchner insisted that his new experimental findings could not be disproved by older theories.

After a prolonged initial period of about a hundred years, during which time a number of alternative and mysterious theories were proposed, Buchner’s elaborate results brought about a new biochemical paradigm. It stated – in strict contrast to the theories of Pasteur – that enzyme catalysis, including complex phenomena such as alcoholic fermentation, was a chemical process not necessarily linked to the presence and action of living cells, nor requiring a vital force – a *vis vitalis*. With this, the technical development of enzymatic processes was provided with a new, scientific basis on which to proceed in a rational manner.

The activity in scientific research on enzymes increased significantly due to this new guidance, and was reflected in a pronounced increase in the number of papers published on the subject of soluble ferments from the mid-1880s onward (Buchholz and Poulson, 2000). Further important findings followed within a somewhat short time. In 1898, Croft-Hill performed the first enzymatic synthesis – of isomaltose – by allowing a yeast extract (α -glycosidase) to act on a 40% glucose solution (Sumner and Somers, 1953). In 1900, Kastle and Loevenhart showed that the hydrolysis of fat and other esters by lipases was a reversible reaction, and that enzymatic synthesis could occur in a dilute mixture of alcohol and acid. Subsequently, this principle was utilized in the synthesis of numerous glycosides by Fischer and coworkers in 1902, and by Bourquelot and coworkers in 1913 (Wallenfels and Diekmann, 1966). In 1897, Bertrand observed that certain enzymes required dialyzable substances to exert catalytic activity, and these he termed *coenzymes*.

The final proof that enzymes were in fact proteins was the crystallization of urease by Sumner in 1926, and of further enzymes (e.g., trypsin) by Northrup and Kunitz in 1930–1931. In all known cases, the pure enzyme crystals turned out to be proteins (Sumner, 1933).

Despite these advances, the number of new applications of enzymes remained very small. In the United States, J. Takamine began isolating bacterial amylases in the 1890s, in what was later to become known as Miles Laboratories. In 1895, Boidin discovered a new process for the manufacture of alcohol, termed the “amylprocess.” This comprised cooking of the cereals, inoculation with a mold that formed saccharifying enzymes, and subsequent fermentation with yeast (Uhlig, 1998). The early applications and patents on enzyme applications in the food industry (which numbered about 10 until 1911) have been reviewed by Neidleman (1991).

At the beginning of the twentieth century, plant lipases were produced and utilized for the production of fatty acids from oils and fats, typically in the scale of 10 tons per week (Ullmann, 1914). Likewise, in the chill proofing of beer, proteolytic enzymes have been used successfully since 1911 in the United States (Tauber, 1949). Lintner, as early as 1890, noted that wheat diastase interacts in dough making, and studied the effect extensively. As a result, the addition of malt extract came into practice, and in 1922 American bakers used 30 million pounds (13.5×10^6 kg) of malt extract valued at US\$ 2.5 million (Tauber, 1949).

The use of isolated enzymes in the manufacture of leather played a major role in their industrial-scale production. For the preparation of hides and skins for tanning, the early tanners kept the dehaired skins in a warm suspension of the dungs of dogs and birds. In 1898, Wood was the first to show that the bating action of the dung was caused by the enzymes (pepsin, trypsin, lipase) that it contained. In the context of Wood's investigations, the first commercial bate, called Erodine, was prepared from cultures of *Bacillus erodiens*, based on a German patent granted to Popp and Becker in 1896. In order to produce Erodine, bacterial cultures were adsorbed onto wood meal and mixed with ammonium chloride (Tauber, 1949).

In 1907, Röhm patented the application of a mixture of pancreatic extract and ammonium salts as a bating agent (Tauber, 1949). Röhm's motivation as a chemist was to find an alternative to the unpleasant bating practices using dungs. Although the first tests with solutions of only ammonia failed, Röhm was aware of Buchner's studies on enzymes. He came to assume that enzymes might be the active principle in the dung, and so began to seek sources of enzymes that were technically feasible. His tests with pancreatic extract were successful, and on this basis in 1907 he founded his company, which successfully entered the market and expanded rapidly. In 1908, the company sold 10 tons of a product with the trade name Oropo, followed by 53 and 150 tons in the subsequent years. In 1913, the company (Figure 1.4) was employing 22 chemists, 30 other employees, and 48 workers (Trommsdorf, 1976). The US-based company – later the Rohm and Haas Company (now

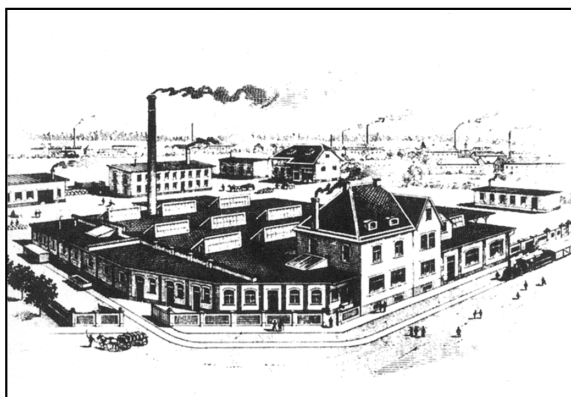


Figure 1.4 The factory of the Rohm and Haas Company, Darmstadt, Germany, 1911.

subsidiary of Dow) – was founded in 1911. The example of Röhm's company (now a part of Evonik, Germany) illustrates that although the market for this new product was an important factor, knowledge of the principles of enzyme action was equally important in providing an economically and technically feasible solution.

This success of the enzymatic bating process was followed by new applications of pancreatic proteases, including substitution therapy in maldigestion, desizing of textile fibers, wound treatment, and the removal of protein clots in large-scale washing procedures. During the 1920s, however, when insulin was discovered in pancreas, the pancreatic tissue became used as a source of insulin in the treatment of diabetes. Consequently, other enzyme sources were needed in order to provide existing enzymatic processes with the necessary biocatalysts, and the search successfully turned to microorganisms such as bacteria, fungi, and yeasts.

1.3.3

Developments Since 1950

Between 1950 and 1970, a combination of new scientific and technical knowledge, market demands for enzymes for use in washing processes, starch processing, and cheaper raw materials for sweeteners and optically pure amino acids stimulated the further development of enzyme technology. As a result, an increasing number of enzymes that could be used for enzyme processes were found, purified, and characterized. Among these were penicillin amidase (or acylase), used for the hydrolysis of penicillin and first identified in 1950, followed some years later by glucose isomerase, which is used to isomerize glucose to the sweeter molecule, fructose. With the new techniques of enzyme immobilization, enzymes could be reused and their costs in enzyme processes reduced. Although sucrose obtained from sugarcane or sugar beet was the main sweetener used, an alternative raw material was starch, which is produced in large quantities from corn (mainly in the United States). Starch can be hydrolyzed to glucose, but on a weight basis glucose is less sweet than sucrose or its hydrolysis products, glucose and fructose. As glucose isomerase can isomerize glucose to fructose, starch became an alternative sweetener source. The process was patented in 1960, but it lasted almost 15 years until the enzyme process to convert starch to glucose–fructose syrups became industrialized. This was in part due to an increase in sucrose prices and due to the introduction of immobilized glucose isomerase as a biocatalyst. It is interesting to note that scientists working in this field discussed the political and social consequences for the main sugar-producing countries, before this process was introduced on an industrial scale (Wingard, 1974). Although European engineers and scientists had contributed strongly to the development of this process, it is applied only minimally in Europe (~1% of world production) due to the protection of sucrose production from sugar beet.

Enzyme immobilization was first introduced to enable the reuse of costly enzymes. Some of the initial attempts to do this were described during the early parts of the past century (Hedin, 1915), but the enzymes when adsorbed to charcoal proved to be very unstable. Around the time of 1950, several groups began to

immobilize enzymes on other supports (Michel and Evers, 1947; Grubhofer and Schleith, 1954; Manecke 1955, cited in Silman and Katchalski, 1966). Georg Manecke was one of the first to succeed in making relatively stable immobilized systems of proteins on polymer carriers, and although he was granted a patent on his method he could not convince industry of the importance to further develop this invention. Rather, it was a group of chemists working with Ephraim Katchalski-Katzir in Israel who opened the eyes of industry to the world of immobilized enzymes (among Katchalski-Katzir's coworkers were Klaus Mosbach and Malcolm Lilly who later made important contributions to establish enzyme technology). The first industrial applications of immobilized enzymes, besides the isomerization of glucose to fructose to produce high-fructose corn syrups (HFCS), were in the production of optically pure amino acids (Tosa *et al.*, 1969) and the hydrolysis of penicillin G (Carleymith and Lilly, 1979, together with Beecham Pharmaceuticals (now Glaxo SmithKline, UK), and G. Schmidt-Kastner (Bayer, Germany), in cooperation with the penicillin producer Gist Brocades (now DSM, The Netherlands)).

Even today, the largest immobilized enzyme product in terms of volume is immobilized glucose isomerase. As these products were introduced, they became more efficient, and stable biocatalysts were developed that were cheaper and easy to use. As a result, the productivity of commercial immobilized glucose isomerase increased from $\sim 500 \text{ kg HFCS kg}^{-1}$ immobilized enzyme product (in 1975) to $\sim 15\,000 \text{ kg HFCS kg}^{-1}$ immobilized enzyme product (in 1997) (Buchholz and Poulson, 2000).

During the 1960s, enzyme production gained speed only in modest proportions, as reflected by the growing sales of bacterial amylases and proteases. Indeed, the annual turnover of the enzyme division of Novo Industri (now Novozymes), the leading enzyme manufacturer at the time, did not exceed \$1 million until 1965. However, with the appearance of the detergent proteases, the use of enzymes increased dramatically, and during the late 1960s, everybody wanted Biotex, the protease-containing detergent. At the same time, an acid/enzyme process to produce dextrose using glucoamylase was used increasingly in starch processing. As a consequence, by 1969 – within only a 4-year period – Novo's enzyme turnover exceeded US\$ 50 million annually, and in 2009 Novozymes' turnover was approximately US\$ 1400 million. The present global market is estimated to be around 2.5 billion € (Novozymes, 2011) (Figure 1.5a), and this has been reflected in the increased employment within the enzyme-producing industry (Figure 1.5b). The main industrial enzyme processes with free or immobilized enzymes as biocatalysts are listed in Table 1.2.

The introduction of gene technology during the 1970s provided a strong impetus for both improved and cheaper biocatalysts, and also widened the scope of application. Productivity by recombinant microorganisms was dramatically improved, as was enzyme stability, and this led to a considerable lowering of prices and improvements in the economics of enzyme applications. Today, most of the enzymes used as biocatalysts in enzyme processes – except for food processing – are recombinant. The recent development of techniques of site-directed mutagenesis, gene shuffling, and directed evolution has opened the perspective of modifying the selectivity and specificity of enzymes (see Section 2.11 and Chapter 3).

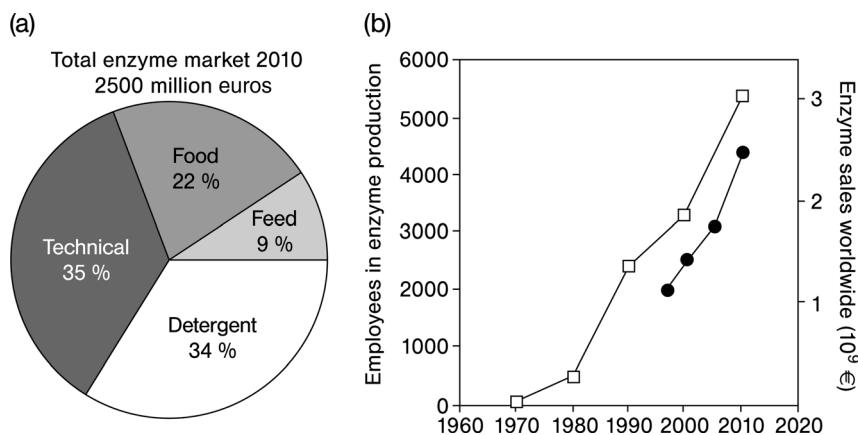


Figure 1.5 (a) Market for enzymes used as biocatalysts for different purposes (2010), and (b) the increase in the application of enzymes reflected in the number of employees in the industry producing enzymes for biocatalytic purposes and their worldwide sales since 1970. Number of Novozymes employees that has about 50% of the world market for such enzymes (squares), and value of their

worldwide sales (filled circles) (Novozymes yearly reports, last one from 2010). The value of the world production of technical enzymes is much larger than that shown in (a), as many companies that use enzymes as biocatalysts produce them in-house in order to have a safe and stable enzyme supply and/or protect their proprietary knowledge.

More detailed accounts on the scientific and technological development can be found in articles by Sumner and Somers (1953), Ullmann (1914), Tauber (1949), Neidleman (1991), Roberts *et al.* (1995), Buchholz and Poulson (2000), and Buchholz and Collins (2010). A profound analysis of the background of biotechnology and “Zymotechnica” has been presented by Bud (1992, 1993).

1.4

Biotechnological Processes: The Use of Isolated or Intracellular Enzymes as Biocatalysts

Biotechnological processes use one or more enzymes with or without cofactors or cosubstrates as biocatalysts (Figure 1.6). When more enzymes and cosubstrate regeneration (ATP, NADH) are required, fermentation processes with living cells are more effective than processes with isolated enzymes. In environmental biotechnology, mixed living cultures are mainly used as biocatalysts (see Chapter 9). Now cells can be designed where enzymes have been deleted that catalyze side reactions, avoiding or reducing by-product formation, and/or enzymes that catalyze consecutive reactions that reduce the product yield (Figure 1.6, I) (Keasling, 2010). Uses of such designed cells to produce desired products in fermentations will be covered in Chapter 5. For enzyme processes, which utilize few enzymes (≤ 3) without any cosubstrate regeneration, those with isolated enzymes or enzymes in

Table 1.2 Products produced in quantities larger than 1000 t year⁻¹ by different companies with enzymes or cells as biocatalysts.

Product	Enzyme/designed cells	Free or immobilized isolated enzyme or cell	Companies
>10 000 000 t a⁻¹			
HFCS	Amylase Glucoamylase Glucose isomerase	Free Free Immobilized	Several
Ethanol (gasoline additive)	Amylase Glucoamylase Hemicellulases	Free Free Free	Several
>100 000 t a⁻¹			
Acrylamide	Nitrilase in resting cells	Immobilized resting cells	Nitto, DSM
Cocoa butter	Lipase	Immobilized	Fuji Oil, Unilever
Isomaltulose	Sucrose mutase in dead cells	Immobilized dead cells	Südzucker, Cerestar
Polylactic acid	Designed cells		Cargill-Dow Polymers LLC
1,3-Propanediol	Designed cells		DuPont
Transesterification of fats and oils	Lipase	Immobilized	Several
>10 000 t a⁻¹			
6-Aminopenicillanic acid	Penicillin amidase	Immobilized	Several
Lactose-free milk or whey	β-Galactosidase lactase	Free or immobilized	Several
>1000 t a⁻¹			
Acarbose	Selected high-yield cell strain after mutagenesis	Immobilized living cells	Bayer
7-Aminocephalosporanic acid (7-ACA)	Engineered cephalosporin C amidase	Immobilized	Novartis
7-Aminodesacetoxycephalosporanic acid (7-ADCA)	Engineered glutaryl amidase	Immobilized	DSM
(S)-Aspartic acid	Aspartase	Immobilized (?)	Tanabe
Aspartame	Thermolysin	Immobilized	Toso, DSM
(S)-Methoxyisopropylamine	Lipase	Immobilized	BASF
(R)-Pantothenic acid	Aldolactonase		Fuji Chemical Industry Co., Ltd
(R)-Phenylglycine	Hydantoinase, carbamoylase	Immobilized	Several
(S)-Amino acids	Aminoacylase	Free	Degussa, Tanabe

1000 to >10 t a⁻¹

Amoxicillin	Penicillin amidase	Immobilized	DSM
Cephalexin	Penicillin amidase	Immobilized	DSM
(S)-DOPA	β -Tyrosinase	Immobilized	Ajinomoto
Insulin	Carboxypeptidase B	Free	Novo Nordisk
	Lysyl endopeptidase	Free	Sanofi
	Trypsin	Free	Eli Lilly
Sterically pure alcohols and amines	Lipase	Immobilized	BASF
(R)-Mandelic acid	Nitrilase	Immobilized	BASF

Some other products produced in the range 10–1000 t year⁻¹ in recently developed enzyme processes are also included.

(either) dead (or living) cells have the following advantages compared with fermentations:

- Higher space–time yields can be obtained than with living cells; smaller reactors can then be used, reducing processing costs.
- The risk that a desired product is converted by other enzymes in the cells can be reduced.
- The increased stability and reuse of immobilized biocatalysts allows continuous processing for up to several months.

For such enzyme processes:

- 1) The required intra- or extracellular enzyme must be produced in sufficient quantities and purity (free from other disturbing enzymes and other compounds).
- 2) Cells without intracellular enzymes that may disturb the enzyme process must be selected or designed.
- 3) The enzyme costs must be less than 5–10% of the total product value.

Tables 1.1 and 1.2 provide information about important enzyme products and the processes in which they are produced. In many cases, enzyme and chemical processes are combined to obtain these products. This will also be the case in the future when both processes fulfill the economic and sustainability criteria listed above. When this is not the case, new processes must be developed that better fulfill these criteria. This is illustrated by the first two large-scale enzyme processes, namely, the hydrolysis of penicillin (Figure 1.7) and the production of glucose–fructose syrup from starch (Figure 1.8). Both processes were developed some 30 years ago, and the first replaced a purely chemical process that was economically unfavorable, and less sustainable, compared with the enzyme process (Tischer, 1990; Heinzle, Biewer, and Cooney, 2006).

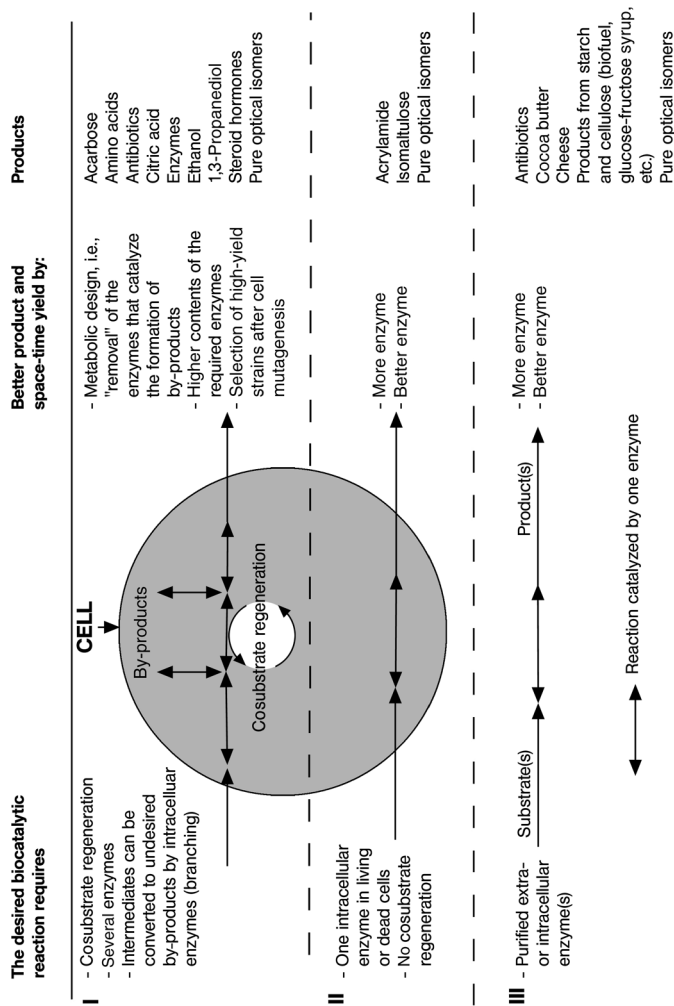


Figure 1.6 Classification of biocatalytic processes with enzymes as biocatalysts. I must be performed with enzymes in living cells, II can be performed with enzymes in living or dead cells, and III with isolated enzymes. For I now mainly designed cells are used, and for II high-yield cells for one-step reactions that do not require cosubstrate regeneration are used. Processes I–III will be covered in this book.

1.5

Advantages and Disadvantages of Enzyme-Based Production Processes

In Figures 1.7 and 1.8, the enzyme processes for the hydrolysis of penicillin and the production of glucose–fructose syrup are compared with previously used procedures that had the same aims. In the case of penicillin hydrolysis, the chemical process uses environmentally problematic solvents and toxic compounds, leading to toxic wastes that are difficult to recycle. The process is, therefore, not sustainable. The enzyme process is more sustainable than the previous process, and leads to a

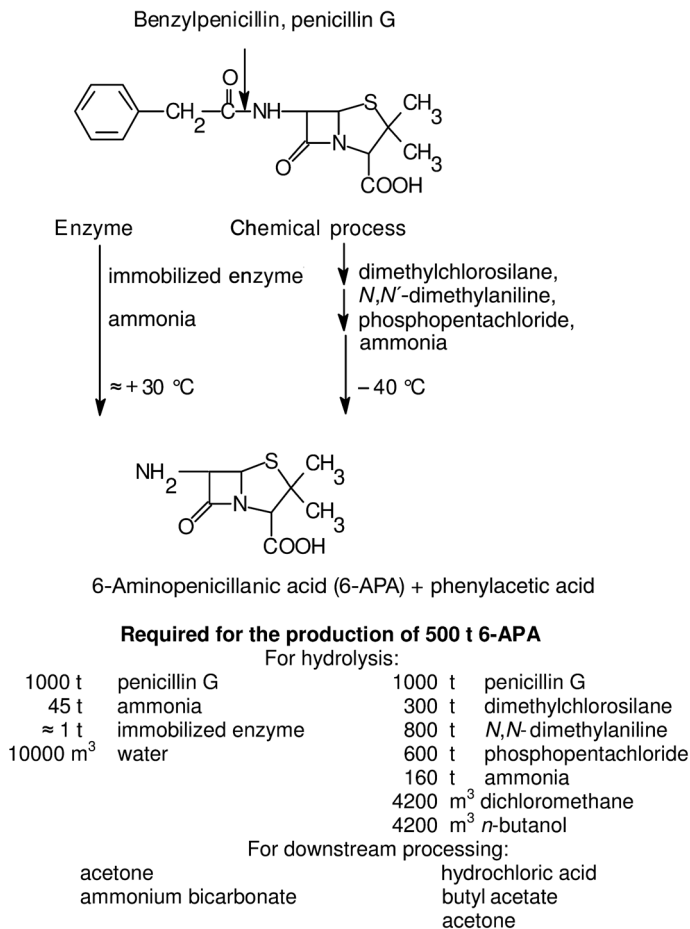


Figure 1.7 Comparison of the old (chemical) and the new (enzyme) process for the hydrolysis of penicillin G. The product, 6-aminopenicillanic acid (6-APA), is used for the synthesis of semisynthetic penicillins with side

chains other than phenylacetic acid. In the enzyme process, the by-product phenylacetic acid can be recycled in the production of penicillin by fermentation (from Tischer, 1990).

Starch hydrolysis

Liquid starch at 100–110 °C,
and 86 °C
(40 % w/v)

Hydrolysis of α -1-4 and α -1-6
bonds in starch by addition of
thermostable α -amylase
at pH 6–6.5
+ Ca^{2+} for 10–100 min

Filtration, removal of Ca^{2+} , and
pH adjustment by ion exchange

Oligosaccharide hydrolysis with
free glucoamylase at
55–60 °C; pH 4.0–4.5 for 24–72 h

Removal or inactivation of
enzyme

Fermentation of glucose
to ethanol

Glucose isomerization

D-glucose syrup (95–98%,
40–50% w/v)

Addition of cofactors (Mg^{2+} , HSO_3^-)
and removal of O_2
pH adjustment

Filtration

Heat exchanger

Isomerization at 57 °C and
pH 8.0 with immobilized
glucose isomerase in a fixed-
bed reactor

Purification steps

1. Decolorization with charcoal
2. Filtration
3. Removal of cofactors by ion
exchange

pH adjustment

Concentration to 70–72 % w/v
by evaporation

Cooling

Isoglucose (> 50 % glucose,
≈ 42 % fructose)

Figure 1.8 The enzyme process for the hydrolysis of starch to glucose, and the isomerization of glucose to fructose (w/v = weight per volume) (see Chapters 7, 8, and 12).

considerable reduction in waste that in turn reduces the processing costs (Tischer, 1990; Heinzle, Biwer, and Cooney, 2006). In this process, the product yield could also be increased to >95%. The hydrolysis of starch and isomerization of glucose cannot be performed chemically at reasonable cost, as each would result in lower yields, unwanted by-products, and the considerable production of waste acids. These processes illustrate some of the advantages of enzyme processes compared with alternative processes (Box 1.1). However, it must be remembered that the use

Box 1.1: Advantages and disadvantages of cells and enzymes as biocatalysts in comparison with chemical catalysts.

Advantages	<ul style="list-style-type: none"> • Stereo- and regioselective • Low temperatures (0–110 °C) required • Low energy consumption • Active at pH 2–12 • Less by-products • Nontoxic when correctly used • Can be reused (immobilized) • Can be degraded biologically • Can be produced in unlimited quantities
Disadvantages	<ul style="list-style-type: none"> • Cells and enzymes are <ul style="list-style-type: none"> – unstable at high temperatures – unstable at extreme pH values – unstable in aggressive solvents – inhibited by some metal ions – hydrolyzed by peptidases • Some enzymes <ul style="list-style-type: none"> – are still very expensive – require expensive cosubstrates • When inhaled or ingested, enzymes are, as all foreign proteins, potential allergens

of enzymes as biocatalysts may be limited by their biological and chemical properties (see Chapter 2).

Enzymes are proteins that are essential for living systems and, in the right place, they catalyze all chemical conversions required for the system's survival and reproduction. However, in the wrong place, they can be harmful to an organism. Peptidases from the pancreas are normally transported into the intestine where they are necessary for the digestion of proteins to amino acids. The amino acids are transported into blood vessels and distributed to different cells, where they are used for the synthesis of new proteins. Under shock situations or pancreatic insufficiencies, these peptidases may be transported from the pancreas directly into the bloodstream where they may cause harmful blood clotting. To prevent this from occurring, the blood contains inhibitors for pancreatic peptidases.

Enzymes are normal constituents of food and, as with all orally ingested proteins, they are hydrolyzed in the stomach and intestine. However, if enzymes or other proteins are inhaled as small particles or aerosols in the lungs, they can be transferred directly into the bloodstream. There, they are recognized as foreign proteins and induce an immune reaction – that is, the production of antibodies against them. This may also lead to enzyme or protein allergies. These risks must be considered in the production and use of enzymes and other proteins, and simple measures can be taken to minimize

them. The enzyme-producing companies neglected this, when they introduced enzymes in detergents for household use around 1970. The number of allergy cases increased rapidly among the employees and users, due to the inhalation of the small enzyme particles. Fortunately, the companies reacted rapidly and covered the enzymes used in washing powders in a drying process with a wax layer, yielding particles so large ($>100\ \mu\text{m}$) that they cannot be inhaled into the lung. This reduced the number of new allergy cases rapidly. Likewise, when enzymes are used in the liquid phase, aerosol formation must be prevented.

Some proteins (enzymes) can also be transferred from the digestive tract into the bloodstream and cause allergies. This applies to proteins that are digested very slowly in the stomach and intestine (Fuchs and Astwood, 1996; Jank and Haslberger, 2003). The slow digestion has been correlated with a high thermal stability, and enzymes used on an industrial scale as biocatalysts should therefore be rapidly hydrolyzed by peptidases in the digestive system in order to minimize the allergy risk. This applies especially to enzymes that cannot easily be used in closed systems, and particularly those used in food processing. Regulations that control the use of enzymes as biocatalysts are given in Chapter 6.

1.6

Goals and Essential System Properties for New or Improved Enzyme Processes

1.6.1

Goals

The advantages detailed in Box 1.1 are not sufficient alone for the industrial use of enzyme processes. Sustainability goals, derived from the criteria outlined in Section 1.1, must also be considered (Table 1.3).

Enzyme processes have become competitive and have been introduced into industry when they attain these goals better than alternative processes. This, however, also requires that these goals be quantified such that the amount of product and by-products (or waste) produced with a given amount of enzyme in a given time must be determined. For this aim, enzyme processes – as with all catalyzed chemical processes – can be divided into two categories (Figure 1.9):

- 1) **Equilibrium-controlled processes:** the desired product concentration or property has a maximum at the end point of the process (B in Figure 1.9); the chemical equilibrium is independent of the properties of the catalyst (enzyme), but is dependent on pH and temperature.
- 2) **Kinetically controlled processes:** the desired product concentration or property (such as fiber length or smoothness in textiles or paper) reaches a maximum (A in Figure 1.9), the concentration or properties of which depend on the properties of the catalyst (enzyme, see Chapter 2), pH, and temperature. The process must be stopped when the maximum is reached.

Table 1.3 Economic and environmental sustainability goals that can be realized in enzyme processes (modified from Uhlig, 1998).

Goals	Means to achieve the goals	Products/processes
Cost reduction	Yield increase	Penicillin and cephalosporin C hydrolysis
	Biocatalyst reuse and increased productivity by immobilization	Glucose isomerization
	Better utilization of the raw material	Isomaltulose production
		Juice and wine production
	Reduction of process costs for	
	• Filtration	Sterile filtration of plant extracts
	• Energy	Low-temperature washing powder
	• Desizing of fibers	Desizing with enzymes
	• Cheese ripening	Increase rate of process with enzymes
	• Malting in beer production	
	Reduction of residence time in starch processing	
Improvement of biological properties and quality	Produce only isomers with the desired biological property	Racemate resolution
	Improved preservation of foods	Juice concentrates
	Improvement of technical properties	Protein modification, flour for baking, transesterification of vegetable oils, biodiesel
	Improved taste (sweetness)	Glucose isomerization to glucose–fructose syrup
Utilization of new regenerable sources of raw materials	Utilization of wastes from food and paper production (such as whey, filter cakes from vegetable oil production, waste water)	Drinks from whey
		Animal feed
		Biofuels (Biodiesel, Biogas Ethanol)
Reduction of environmental impact	Reduction of nonrecyclable waste	Penicillin and cephalosporin C hydrolysis, leather production, paper bleaching
	Waste recycling	Utilization of whey

In both cases, the time to reach the maximum product concentration or property depends on the properties and amount of enzyme used, and on the catalyzed process (endo- or exothermal, pH and temperature dependence of equilibrium constants, solubility and stability of substrates, products, etc.). This must be considered in the rational design of enzyme processes. Another difference to consider is that in these processes the enzymes are used at substrate concentrations

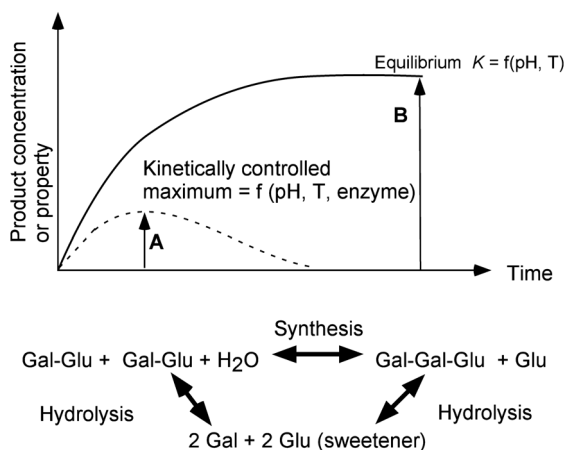


Figure 1.9 Time dependence (progress curves) of equilibrium- (solid line) and kinetically (broken line) controlled processes catalyzed by enzymes. The suitable end points of these processes are those where the maximum product concentration or property is achieved – that is, A for the kinetically and B for the equilibrium-controlled process. Such processes are illustrated with the hydrolysis of lactose in milk or whey. Whey is an inevitable by-product in cheese production, where mainly protein and fats are precipitated in the milk by addition of a “coagulating” enzyme (chymosin or rennin, a carboxyl acid peptidase, EC 3.4.23.4). The remaining liquid phase (whey) contains ~5% sugars (mainly lactose, a disaccharide galactosylglucose), 1% protein, 1% amino acids, and 1% ions (Ca^{2+} , Na^+ , phosphate ions, etc.). Previously, with mainly small dairies, whey was used as a feed, or condensed to various sweet local products. Today, in large cheese-producing dairies, up to 10^7 tons of whey is formed each year that

cannot be used as before. The main content lactose cannot be used as a sweetener as a part of the population cannot tolerate lactose (this fraction is higher in parts of Asia and Africa). When it is hydrolyzed, its sweetness is increased. The enzyme β -glucosidase that catalyzes the hydrolysis of lactose also catalyzes the kinetically controlled synthesis of tri- and tetrasaccharides. When lactose is consumed, these oligosaccharides are hydrolyzed. This also illustrates the formation of undesired by-products in enzyme-catalyzed processes. The oligosaccharides are by-products in the equilibrium-controlled hydrolysis of lactose. On the other hand, in the kinetically controlled synthesis of the oligosaccharide that can be used as prebiotics, by-product formation is due to the hydrolysis to monosaccharides (Illanes, Wilson, and Raiman, 1999; Bruins *et al.*, 2003). The formation of the by-products must be minimized by selecting suitable process conditions and biocatalysts.

(up to 1 M) that are much higher than those in living systems (≤ 0.01 M). At the substrate and product concentrations used in enzyme technology, the formation of undesired by-products in catalyzed and uncatalyzed bimolecular reactions cannot be neglected (see the next section and Case Study 3 in Chapter 12).

1.6.2

Essential System Properties for Rational Design of an Enzyme Process

The steps to be considered for the design of enzyme processes that are within the scope of this book may be illustrated based on the equilibrium-controlled

hydrolysis of the substrate lactose to the products glucose and galactose, as shown in Figure 1.9. This process was developed to reduce the lactose content in milk products so that those who suffer from lactose intolerance can consume them. It can also be used to increase the sweetness of products derived from whey (Illanes, Wilson, and Raiman, 1999). The steps are summarized in Figure 1.10.

A high substrate content is favorable in order to reduce downstream processing costs. In milk, the lactose content cannot be changed, but in whey it can be increased by nanofiltration. The upper limit is given by the solubility ($150\text{--}200\text{ g l}^{-1}$), which is lower than that for other disaccharides such as sucrose. As both substrates and products have no basic or acidic functional groups, the equilibrium constant should not depend on pH, but on the temperature. This dependence must be known in order to select a suitable process temperature (T), though the selection also depends on the properties of the biocatalyst. Its selectivity (ratio of hydrolysis to synthesis rates) must be high in order to minimize the formation of by-products (oligosaccharides) in a kinetically controlled process. In addition, its catalytic properties and stability as a function of pH and temperature must also be known in order to calculate the amount of biocatalyst required to reach the end point of the process within a given time.

When other constraints have been identified, a process window in a pH– T plane can be found where it can be carried out with optimal yield and minimal biocatalyst costs. The maximal yield of an equilibrium-controlled process as a function of pH and T is only defined by the catalyzed reaction. When this maximum is outside the optimal process window of the enzyme, the process can be improved as follows: either by screening for a better biocatalyst or by changing the properties of the enzyme by protein engineering, so that the process can be carried out at pH and T -values where this maximum can be reached (see Section 2.11 and Chapter 3).

In order to reduce the enzyme costs, the enzyme production can be improved (see Chapter 6) or the enzymes used in a reusable form. This can be achieved by their immobilization to porous particles that can easily be filtered off at the end of the process (see Chapters 8 and 11). In these systems, the kinetics differ from those of systems with free enzymes, as the mass transfer inside and to and from the particles with the biocatalyst causes the formation of concentration and pH gradients that influence rates and yields (this topic is dealt with in Chapter 10).

Once the process conditions and its end point have been chosen in the process window, the enzyme costs per kilogram of product are influenced by the type of reactor (batch, continuous stirred tank, or fixed-bed reactor) selected to carry out the process (see Chapter 11). The procedure to design an enzyme process (summarized in Figure 1.10) will be illustrated in more detail as case studies for the design of classical (HFCS production) and newer (7-ACA production by direct hydrolysis of cephalosporin C biofuel production from biomass, and lipitor side chain synthesis) enzyme processes (see Chapter 12).

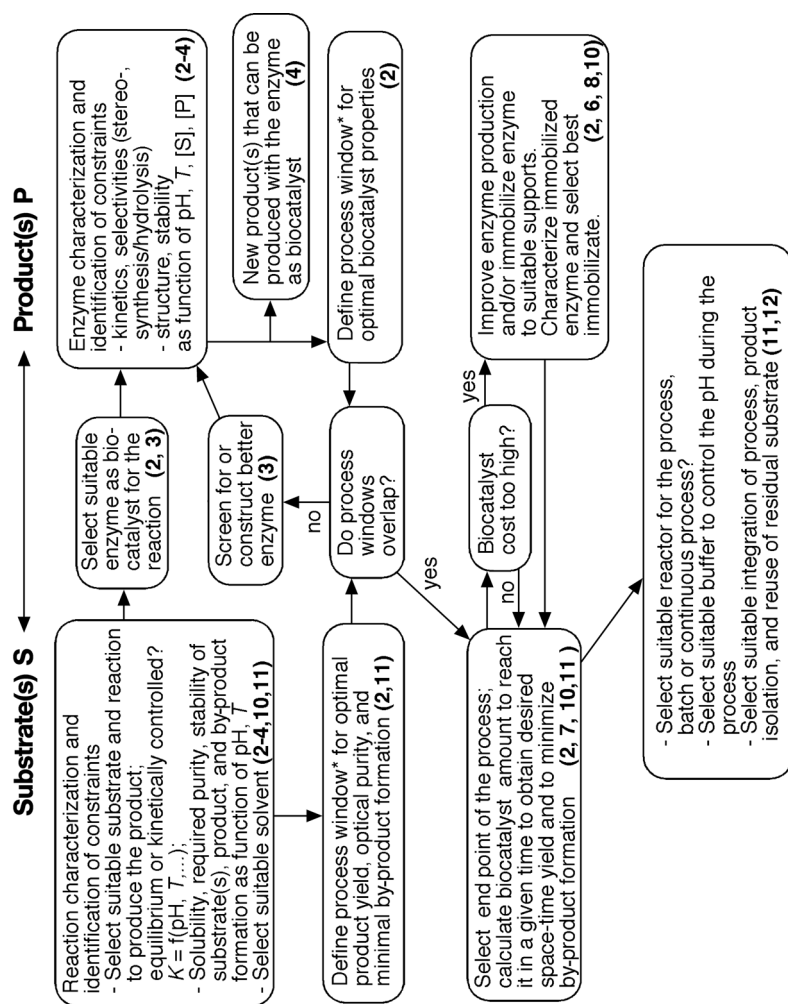


Figure 1.10 Steps to be considered in the design of an enzyme process with isolated enzymes or an enzyme in dead cells (processes II and III in Figure 1.6) to produce existing or new products (bold numbers refer to chapters in this book). *Process window = the range in a pH–T- (or pH–[S], pH–[P], T–[S], T–[P]) plane where the reaction can be carried out with a given yield or optical purity, and where the properties (activity, selectivity, stability) of the biocatalyst are optimal. For multienzyme processes with living cells (process I in Figure 1.6), the optimal process design requires metabolic engineering, and other boundary conditions than those above must be considered. With optimal metabolic engineering, almost quantitative conversion of the substrate(s) to the desired product can be obtained. Due to the many reactions involved, the time course of the reactions cannot be described as quantitatively as the processes above. The design of these processes will be covered in Chapter 5.

1.6.3

Current Use and Potential of Enzyme Technology

Current amounts of products obtained with industrial biotechnical and enzyme processes are detailed in Tables 1.1 and 1.2, respectively. The values of the enzymes used as biocatalysts for different applications are shown in Figure 1.5. Besides industrial applications, many enzymes are used for analytical purposes, mainly in diagnostics, though on a weight basis less than 1‰ of all produced enzymes are used for these applications. Some enzymes are produced in increasing amounts for therapeutic purposes; this applies especially to recombinant enzymes such as factor VIII, tPA, and urokinase that cannot be produced in sufficient amounts from natural sources (blood serum or urine) (Buchholz and Collins, 2010, Section 7.5.6). Another advantage of the recombinant production of these enzymes is that possible contamination with pathogenic human viruses (HIV, herpes) can be avoided.

A large number of new enzyme processes (>100) introduced during the past 30 years have recently been reviewed in detail (Liese, Seelbach, and Wandrey, 2006). The type of process used, the compounds produced, and the enzymes used for these processes have been analyzed statistically (Straathof, Panke, and Schmid, 2002). These data show that hydrolases, lyases, and oxidoreductases are used in two-thirds of all processes, while only about 1% of the about 4000 known enzymes are used in larger amounts for enzyme technological and therapeutic purposes. During the past 30 years, the three-dimensional structures and detailed mechanisms of the reactions that they catalyze have been determined for many of the enzymes considered to be important in enzyme technology. This information allows a more rational improvement of their properties that is essential for their application. Based on the above discussion and on the information shown in Figure 1.5b, the number of new enzyme processes is expected to increase further during the next few decades. The rational and sustainable design of these processes – and the improvement of existing processes – requires the interdisciplinary cooperation of (bio)chemists, micro- and molecular biologists, and (bio)chemical engineers. The (bio)chemist must determine the mechanism and properties of the catalyzed process, the kinetics of the enzyme-catalyzed process, and other relevant properties of substrate, product, and free and immobilized enzymes (stability, solubility, pH and temperature dependence of equilibrium constants, selectivities), and select the suitable support for the immobilization together with the engineer. This also provides information about the properties of the enzyme that should be improved (specificity, selectivity, pH optimum, stability, metal ion requirement, fermentation yield), and this is a task for the micro- or molecular biologist. The method by which this problem may be solved is either to screen for better enzymes in nature or to promote molecular *in vitro* evolution (see Section 2.11 and Chapter 3). Finally, the engineer must use this knowledge to scale up the process to the production scale. In improving the latter procedure, however, the engineer will also identify problems that must be solved by the

(bio)chemists and micro- and molecular biologists, and this is illustrated in Figure 1.6. The number of processing steps can be reduced when the enzymes used can be applied at the same pH value and have the same requirements for metal ions, but this problem has not yet been sufficiently solved. For an exception, see glucose isomerization in Section 8.4.1.

Fields where large amounts of enzymes will be required in order to realize more sustainable new enzyme processes to meet human needs include the following:

- The production of optically pure fine chemicals. It is expected that, in future, only the isomer with desirable biological activity will be approved for use by regulatory authorities. Many pharmaceuticals and fine chemicals are still provided only as racemates, the resolution of which for any process has a maximal yield of 50%. For a sustainable process, the other 50% must be racemized, and to solve this problem the rational integration of chemical and enzyme processes is required in the development of dynamic kinetic resolution processes or asymmetric synthesis (Breuer *et al.*, 2004; Bornscheuer *et al.*, 2012).
- The synthesis of antibiotics (Bruggink, 2001).
- The synthesis of pharmaceutical intermediates (building blocks) (Pollard and Woodley, 2006).
- Paper production or recycling to reduce waste and energy consumption (Bajpai, 1999; Schäfer *et al.*, 2007; Aehle, 2007, Section 5.2.6).
- The regio- and stereoselective synthesis of oligosaccharides for food and pharmaceutical purposes (Seibel and Buchholz, 2010).
- The selective glycosylation of peptides, proteins, and other drugs (Pratt and Bertozzi, 2005; Wong, 2005).
- In the modification of lipids, fats, and oils (*trans*-fatty acid-free fats/oils, diglyceride oils, phospholipid reduction) (Biermann *et al.*, 2011).
- Environmental biotechnology (Jördening and Winter, 2005).
- The synthesis of biofuels from biomass (see Case Study 2 in Chapter 12).
- For the production of bulk products from biomass in biorefineries (Busch, Hirth, and Liese, 2006; Kamm and Kamm, 2007).

The latter two will become of increasing importance as a shift from limited fossil to renewable bio-based raw materials is required in the future.

Exercises

- 1.1 How was it shown that enzymes can act as catalysts outside living cells? In which enzyme process was this knowledge first applied?
- 1.2 How can the process in Figure 1.8 be improved by a reduction in the number of processing steps? What must be done to achieve this? (*Hint*: See Case Study 1 in Chapter 12.)
- 1.3 Explain the relevance of Figure 1.10 for enzyme technology. Which system properties must be known in addition to the properties of the biocatalyst to improve the yields of these processes?

- 1.4 Which properties of the enzyme and the catalyzed process must be known to minimize by-product formation in the production of oligosaccharides from lactose, as shown in Figure 1.9? (*Hint*: Use Figure 1.10 to answer this question.)
- 1.5 Test whether Figure 1.1 is in agreement with your consumption pattern.
- 1.6 How can the allergic and toxic risks due to enzymes be avoided in enzyme technology?

Literature

Overview of Enzyme Technology

The following books give an overview of enzyme technology from the point of view of the biotechnological and chemical industry (enzyme producers and users). Besides the established and new applications of free and immobilized enzymes, some also cover health, legal, and economic aspects of enzyme technology.

- Aehle, W. (ed.) (2007) *Enzymes in Industry*, 3rd edn, Wiley-VCH Verlag GmbH, Weinheim.
- Atkinson, B. and Mavituna, F. (1991) *Biochemical Engineering and Biotechnology Handbook*, 2nd edn, Stockton Press, New York.
- Bommarius, A.S. and Riebel, B. (2004) *Biocatalysis*, Wiley-VCH Verlag GmbH, Weinheim.
- Kirst, H.A., Yeh, W.-K., and Zmijewski, M.J., Jr. (eds) (2001) *Enzyme Technologies for Pharmaceutical and Biotechnological Applications*, Marcel Dekker, New York.
- Liese, A., Seelbach, K., and Wandrey, C. (eds) (2006) *Industrial Biotransformations*, 2nd edn, Wiley-VCH Verlag GmbH, Weinheim.
- Soetaert, W. and Vandamme, E. (eds) (2010) *Industrial Biotechnology*, Wiley-VCH Verlag GmbH, Weinheim.
- Uhlig, H. (1998) *Industrial Enzymes and Their Applications*, John Wiley & Sons, Inc., New York.
- Whitaker, J.R., Voragen, A.G.J., and Wong, D.W.S. (eds) (2003) *Handbook of Food Enzymology*, Marcel Dekker, New York.

General Books and Encyclopedias on Bio- and Chemocatalysis

- Anonymous (2009) *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH, Weinheim.

- Jencks, W.P. (1969) *Catalysis in Chemistry and Biochemistry*, McGraw-Hill, New York.

Historical Development

These articles and books cover the historical development of biotechnology and enzyme technology.

- Buchholz, K. and Collins, J. (2010) Chapters 1–4, in *Concepts in Biotechnology – History, Science and Business*, Wiley-VCH Verlag GmbH, Weinheim.
- Bud, R. (1992) The zymotechnic roots of biotechnology. *Br. J. Hist. Sci.*, **25**, 127–144.
- Bud, R. (1993) *The Uses of Life: A History of Biotechnology*, Cambridge University Press.
- McLaren, A.D. and Packer, L. (1970) Some aspects of enzyme reactions in heterogeneous systems. *Adv. Enzymol.*, **33**, 245–303.
- Mosbach, K. (ed.) (1976) *Immobilized Enzymes, Methods in Enzymology*, vol. **44**, Academic Press, New York.
- Mosbach, K. (ed.) (1987) *Immobilized Enzymes and Cells, Methods in Enzymology*, vols. **135–137**, Academic Press, New York.
- Silman, I.M. and Katchalski, E. (1966) Water insoluble derivatives of enzymes, antigens and antibodies. *Annu. Rev. Biochem.*, **35**, 873–908.

International Treaties that Influence the Application of Biotechnology and Enzyme Technology

- Cartagena Protocol on Biosafety, <http://bch.cbd.int/protocol/text/>.

References

- AGEB (2010) Energieverbrauch in Deutschland, Daten für das 1. Quartal, www.ag-energiebilanzen.de.

- Bajpai, P. (1999) Applications of enzymes in the pulp and paper industry. *Biotechnol. Prog.*, **15**, 147–157.
- Beringer, T., Lucht, W., and Schaphoff, S. (2011) Bioenergy production potential of global biomass plantations under environmental and agricultural constraints. *GBC Bioenergy*. doi: 101111/j.1757-1707.2010.01088x.
- Berzelius, J.J. (1835) Einige Ideen über eine bei der Bildung organischer Verbindungen in der lebenden Natur wirksame aber bisher nicht bemerkte Kraft. *Jahresber. Fortschr. Chem.*, **15**, 237–245.
- Biermann, U., Bornscheuer, U.T., Meier, M.A.R., Metzger, J.O., and Schäfer, H.J. (2011) New developments for the chemical utilization of oils and fats as renewable raw materials. *Angew. Chem., Int. Ed.*, **50**, 3854–3871.
- Bornscheuer, U.T., Huisman, G.W., Kazlauskas, R.J., Lutz, S., Moore J.C., and Robins K. (2012) Engineering the third wave of biocatalysis. *Nature*, **485**, 185–194.
- Breuer, M., Ditrich, K., Habicher, T., Hauer, B., Kefeler, M., Stürmer, R., and Zelinski, T. (2004) Industrial methods for the production of optically active intermediates. *Angew. Chem., Int. Ed.*, **43**, 788–824.
- Bruggink, A. (ed.) (2001) *Synthesis of β -Lactam Antibiotics*. Kluwer Academic Publishers, Dordrecht.
- Bruins, M.E., Strubel, M., van Lieshout, J.F.T., Janssen, A.E.M., and Boom, R.M. (2003) Oligosaccharide synthesis by the hyperthermostable β -glucosidase from *Pyrococcus furiosus*: kinetics and modelling. *Enzyme Microb. Technol.*, **33**, 3–11.
- Bryant, R. (2010) Europe's place within the global fine chemical industry. *Chim. Oggi*, **28**, 14–16.
- Buchholz, K. and Poulson, P.B. (2000) Overview of history of applied biocatalysis, in *Applied Biocatalysis* (eds A.J.J. Straathof and P. Adlercreutz), Harwood Academic Publishers, Amsterdam, pp. 1–15.
- Buchner, E. (1897) Alkoholische Gärung ohne Hefezellen. *Ber. Dtsch. Chem. Ges.*, **30**, 117–124.
- Buchner, E. (1898) Über zellfreie Gärung. *Ber. Dtsch. Chem. Ges.*, **31**, 568–574.
- Busch, R., Hirth, T., and Liese, A. (2006) The utilization of renewable resources in German industrial production. *Biotechnol. J.*, **1**, 770–776.
- Carleysmith, S.W. and Lilly, M.D. (1979) Deacylation of benzylpenicillin by immobilised penicillin acylase in a continuous four-stage stirred-tank reactor. *Biotechnol. Bioeng.*, **21**, 1057–1073.
- Cech, T.R. (1993) Catalytic RNA: structure and mechanism. *Biochem. Soc. Trans.*, **21**, 229–234.
- CEFIC (European Chemical Industry Council) (2010) *The European Chemical Industry in a Worldwide Perspective*, CEFIC, Brussels.
- Clark, W.C. and Dickson, N.M. (2003) Sustainable science: the emerging research program. *Proc. Natl. Acad. Sci. USA*, **100**, 8059–8061.
- FAO (2008) *Biofuels: Prospects, Risks and Opportunities*, FAO, Rome.
- Fischer, E. (1909) *Untersuchungen über Kohlenhydrate und Fermente*, Springer, Berlin.
- Fonseca, M.H. and List, B. (2004) Combinatorial chemistry and high-throughput screening for the discovery of organocatalysts. *Curr. Opin. Chem. Biol.*, **8**, 319–326.
- Fruton, J.S. (1976) The emergence of biochemistry. *Science*, **192**, 327–334.
- Fuchs, R.L. and Astwood, J. (1996) Allergenicity assessment of foods derived from genetically modified plants. *Food Technol.*, 83–88.
- Gram, A., Treffenfeldt, W., Lange, U., McIntyre, T., and Wolf, O. (2001) *The Application of Biotechnology to Industrial Sustainability*, OECD Publications Service, Paris.
- Hamad, B. (2010) The antibiotics market. *Nat. Rev. Drug Discov.*, **9**, 675–676.
- Hedin, S.G. (1915) CT Kap. 4, in *Grundzüge der Physikalischen Chemie in ihrer Beziehung zur Biologie*, J.F. Bergmann Verlag, Wiesbaden.
- Heinze, E., Biwer, A., and Cooney, C. (2006) *Development of Sustainable Bioprocesses*, John Wiley & Sons, Inc., New York, pp. 105–107.
- Hoffmann-Ostenhof, O. (1954) *Enzymologie*, Springer, Wien.
- IEA (International Energy Agency) (2010) *Key World Energy Statistics 2009*, IEA, Paris, www.iea.org.

- Illanes, A., Wilson, L., and Raiman, L. (1999) Design of immobilized enzyme reactors for the continuous production of fructose syrup from whey permeate. *Bioprocess Eng.*, **21**, 509–551.
- Jank, B. and Haslberger, A.G. (2003) Improved evaluation of potential allergens in GM food. *Trends Biotechnol.*, **21**, 249–250.
- Jiang, L., Althoff, E.A., Clemente, F.R., Doyle, L., Röthlisberger, D., Zanghellini, A., Gallaher, J.L., Betker, J.L., Tanaka, F., Barbas, C.F., 3rd, Hilvert, D., Houk, K.N., Stoddard, B.L., and Baker, D. (2008) *De novo* computational design of retro-aldol enzymes. *Science*, **319**, 1387–1391.
- Jördening, H.-J. and Winter, J. (2005) *Environmental Biotechnology*, Wiley-VCH Verlag GmbH, Weinheim.
- Kamm, B. and Kamm, M. (2007) Biorefineries – multi product processes. *Adv. Biochem. Eng. Biotechnol.*, **105**, 175–204.
- Keasling, J.D. (2010) Manufacturing molecules through metabolic engineering. *Science*, **330**, 1355–1358.
- Knapp, F. (1847) *Lehrbuch der chemischen Technologie*, F. Vieweg und Sohn, Braunschweig.
- Neidleman, S.L. (1991) Enzymes in the food industry: a backward glance. *Food Technol.*, **45**, 88–91.
- Novo Nordisk (2011) Annual Report for 2010, Novo Nordisk, Copenhagen, www.novonordisk.com.
- Novozymes (2011) Annual Report for 2010, Novozymes, Copenhagen, www.novozymes.com.
- Payen, A. (1874) *Handbuch der technischen Chemie*, vol. II (eds F. Stohmann and C. Engler), E. Schweizerbartsche Verlagsbuchhandlung, Stuttgart, p. 127.
- Payen, A. and Persoz, J.F. (1833) Mémoire sur la diastase, les principaux produits de ses réactions, et leurs applications aux arts industriels. *Ann. Chim. Phys., 2me Sér.*, **53**, 73–92.
- Pollard, D.J. and Woodley, J.M. (2006) Biocatalysis for pharmaceutical intermediates: the future is now. *Trends Biotechnol.*, **26**, 66–73.
- Pratt, M.R. and Bertozzi, C.R. (2005) Synthetic glycopeptides and glycoproteins as tools for biology. *Chem. Soc. Rev.*, **34**, 58.
- Raven, P.H. (2002) Science, sustainability, and the human prospect. *Science*, **297**, 954–958.
- Roberts, S.M., Turner, N.J., Willets, A.J., and Turner, M.K. (1995) *Biocatalysis*, Cambridge University Press, Cambridge, p. 1.
- Röthlisberger, D., Khersonsky, O., Wollacott, A.M., Jiang, L., DeChancie, J., Betker, J., Gallaher, J.L., Althoff, E.A., Zanghellini, A., Dym, O., Albeck, S., Houk, K.N., Tawfik, D.S., and Baker, D. (2008) Kemp elimination catalysts by computational enzyme design. *Nature*, **453**, 190–195.
- Schäfer, T., Borchert, T.W., Nielsen, V.S., and Skagerlind, P. (2007) Industrial enzymes. *Adv. Biochem. Eng. Biotechnol.*, **105**, 59–131.
- Seibel, J. and Buchholz, K. (2010) Tools in oligosaccharide synthesis: current research and application. *Adv. Carbohydr. Chem. Biochem.*, **63**, 101–138.
- Straathof, A., Panke, S., and Schmid, A. (2002) The production of fine chemicals by biotransformations. *Curr. Opin. Biotechnol.*, **13**, 548–556.
- Sumner, J.B. (1933) The chemical nature of enzymes. *Science*, **78**, 335.
- Sumner, J.B. and Somers, G.F. (1953) *Chemistry and Methods of Enzymes*, Academic Press, New York, pp. XIII–XVI.
- Tauber, H. (1949) *The Chemistry and Technology of Enzymes*, John Wiley & Sons, Inc., New York.
- Tischer, W. (1990) Umweltschutz durch technische Biokatalysatoren, in *Symposium Umweltschutz durch Biotechnik*, Boehringer Mannheim GmbH.
- Tosa, T., Mori, T., Fuse, N., and Chibata, I. (1969) Studies on continuous enzyme reactions. 6. Enzymatic properties of DEAE-Sephadex aminoacylase complex. *Agric. Biol. Chem.*, **33**, 1047–1056.
- Trommsdorf, E. (1976) *Dr. Otto Röhm – Chemiker und Unternehmer*, Econ, Düsseldorf.
- Ullmann, F. (1914) *Enzyklopädie der technischen Chemie*, vol. 5, Urban und Schwarzenberg, Berlin, p. 445.
- UN (United Nations) (2003) *Industrial Commodity Statistics Yearbook 2001*, United Nations, New York.
- Wagner, R. (1857) *Die chemische Technologie*, O. Wiegand, Leipzig.

Wallenfels, K. and Diekmann, H. (1966) Glykosidasen, in *Hoppe-Seyler/Thierfelder Handbuch der physiologisch- und pathologisch-chemischen Analyse*, vol. 6, Springer, Berlin, pp. 1156–1210.

WCED (World Commission on Environment and Development) (1987) *Our Common Future*, Oxford University Press, Oxford.

Wingard, L. (1974) Enzyme engineering a global approach, *New Scientist*, **64**, 565–566.

Wong, C.H. (2005) Protein glycosylation: new challenges and opportunities. *J. Org. Chem.*, **70**, 4219–4225.

Internet Resources for Enzyme Technology

See Appendix A.