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 sub-field 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 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 as biomass.

The goal of these approaches is to design innovative products and processes that are not only 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". 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 processes, a new process must be designed to reduce not only 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.

Biocatalysts and Enzyme Technology. K. Buchholz, V. Kasche, U.T. Bornscheuer

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1.1.1
What are *Biocatalysts*?

Biocatalysts are either proteins (*enzymes*) or, in a few cases, they 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 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, fine and bulk chemicals, fibers, hygiene, and environmental technology), as well as in 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.

1.2
Goals and Potential of Biotechnological Production Processes

Biomass – that is, renewable raw materials – has been and will continue to be a sustainable resource which is required to meet a variety of human material needs. In developed countries such as Germany, biomass covers ≈30 % of the raw material need – equivalent to ~7000 kg per person per year. The distribution of biomass across 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 coal equivalent per capita in 1999) showed a wide range, from 11.4 in the United States, to 5.5 in Germany and the UK, 0.8 in China, and 0.43 in India (United Nations, 2002). This is mainly due to differences in energy use for housing,

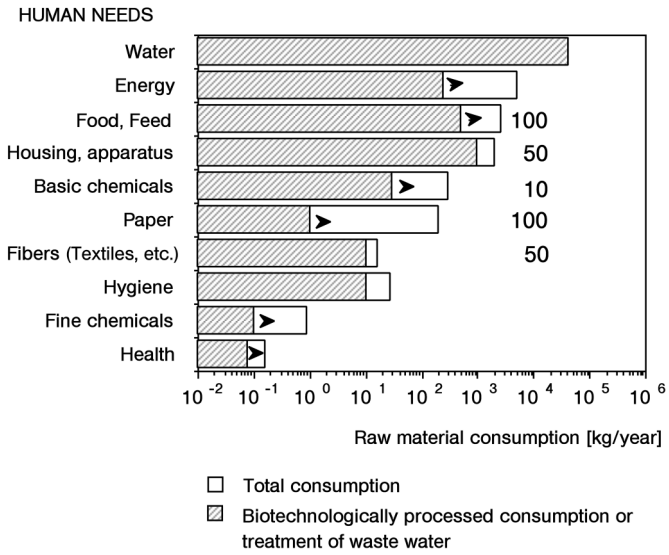


Fig. 1.1 Consumption of raw materials for various human needs per person and year in Germany 1992. The water consumption is only for household use. The arrowheads indicate the current increase in biotechnological processing of the products for different demands. For food and animal feed, only renewable raw materials (biomass) can be used, the figures to the right give the percentage biomass of the raw materials used for the production. 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 waste water, this results in about 1000 kg of solid waste (soil, building materials, plastics, sludge, etc.) that must be recycled in suitable environmental biotechnology processes. Energy is measured in coal equivalents.

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 in the developed countries, the total consumption is smaller.

Biomass – in contrast to non-renewable 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.

Currently, these criteria are not fulfilled on a global level, one example being the imbalance between the CO_2 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 – have been introduced in an attempt to counteract these developments and to reach a goal that fulfills the above criteria.

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 hu-

man 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 (Fig. 1.2). The economic importance of such processes is detailed in Table 1.1. This also involves the development of suitable equipment to obtain more sustainable processes. From the information

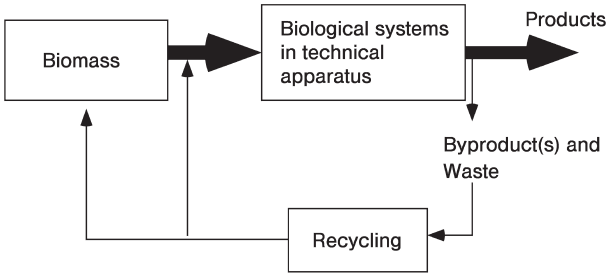


Fig. 1.2 Schematic view of an ideal sustainable biotechnological production process. Biomass as a regenerable resource is converted into desired products with minimal waste and byproduct production. The waste and byproducts must be completely recycled.

Table 1.1 Yearly production and value of biotechnologically produced products to meet human needs. All production data are from 1999–2003, values are only given where sources gives these or when they can be estimated, based on prices in the European Union (EU).

Human need	*Product*	*World production (tons year^{-1})*	*Value (10^9 Euro year^{-1})*	*Production method*	
				Biotechnology	*Chemical*
Food and feed	Beer/Wine	170 000 000	200	F,E	
	Cheese	13 000 000	100	F,E	
	Baker's yeast (1992)	1 800 000	?	F	
	Vegetable oils (refined)	25 000 000	?	E	+
	Acetic acid (10%)	1 200 000	?	F	
Fine chemicals	Amino acids	>1 000 000	>5	F,E	+
	Glucose-Fructose syrup	12 000 000	5	E	
	Vitamin C	>100 000	>2	F,E	+
	Aspartame (Dipeptide)	10 000	?	F,E	+
	Citric acid	800 000	1	F	
	Herbicides, insecticides	2 200 000	?	E	+
	Enzymes	>10 000	2	F	

Table 1.1 Continued

Human need	*Product*	*World production (tons year^{-1})*	*Value (10^9 Euro year^{-1})*	*Production method*	
				Biotech-nology	*Chemical*
Basic chemicals	Products from starch (Ethanol, etc.)	35 000 000	10	E,F	
	Acrylamide	400 000	?	E*	+
	Acetic acid	3 400 000	?		+
Fibers for textiles	Cotton	17 000 000		E	+
	Wool	1 000 000		E	+
	Linen	70 000		F,E	+
Paper		300 000 000		E	+
Hygienics/ detergents	Biotensides	?	?	E,F	+
	Washing powder	22 000 000		F	+
Therapeuticals	Antibiotics	>60 000	≈60	F,E	+
	Insulin	≈10	3	F,E	
	Other peptide hormones	?	?	E,F	+
	Recombinant proteins (Factor VIII, Interferons, tPA, growth factors, mono-clonal antibodies, etc.)	?	20	F,E	
Diagnostics	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					
Chemical industry	All products	≈1 000 000 000	≈2 000		
	Chemical catalysts	180 000	8		

F = fermentation; E = enzyme technology; * 25 % by enzyme technology

Sources: UN (2003); Hassan and Richter (2002); ifok (2003); C & EN/Datamonitor (2001); Novozymes (2004).

provided in 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 the European Federation of Biotechnology 1989 defined as:

> *"Biotechnology is the integration of natural sciences and engineering sciences in order to achieve the application of organisms, cells, parts thereof and molecular analogues for products and services."*

However, the following amendment was added:

> "*It is a clear understanding that Biotechnology is directed to the benefit of mankind by obeying biological principles.*"

Some of these principles have been outlined above. Here, we add the requirement that traditional classical – as well as new biotechnological processes – must be improved and/or developed in order to be sustainable (Fig. 1.2). The fundamentals needed for the development of such processes in the interdisciplinary field of biotechnology require the close cooperation of biologists, chemists, bioengineers, 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 18th and 19th 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 liquify large amounts of starch,
- the material was thermolabile,
- 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) (Fig. 1.3). Indeed, it was demonstrated that the use of malt in this hydrolytic process was more economic than that of sulfuric acid.

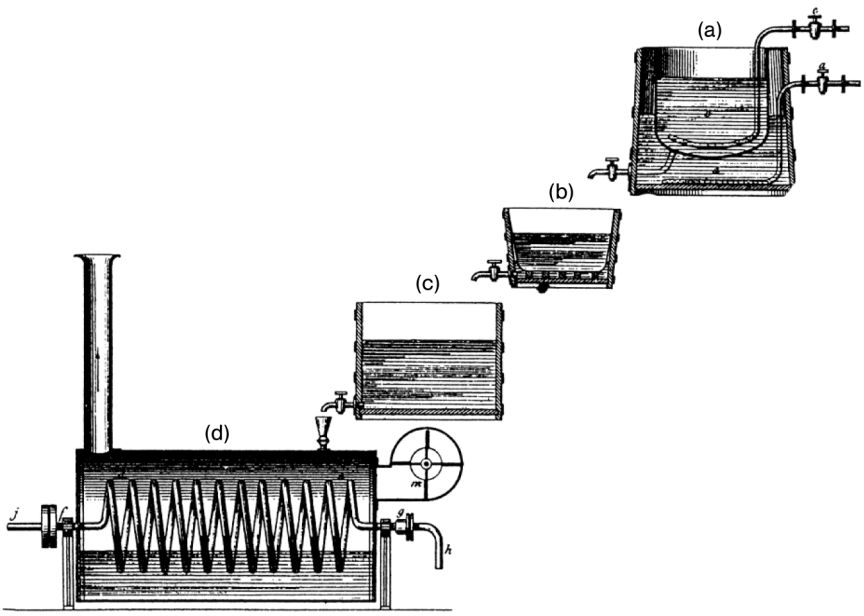


Fig. 1.3 Process for dextrin production, with reaction vessel (a), filter (b), reservoir (c), and concentration unit (d).

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:

- 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 iden-

tity. 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 Liebing 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 onwards, Emil Fischer elaborated on 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 amongst the agents which 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, 1979).

A few years after Fischer's initial investigations, Eduard Buchner published a series of papers (1897, 1898) which signalled 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 which 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 – which 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 onwards (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 (Sumner and Myrbäck, 1950). This principle was subsequently 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 dialysable 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 and Myrbäck, 1950).

Despite these advances, the number of new applications of enzymes remained very small. In the USA, 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 "Amyloprocess". This comprised cooking of the cereals, inoculation with a mold which 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 20th 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 USA (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) which 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 pancreas 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 tradename Oropon, followed by 53 tons and 150 tons in the subsequent years. In 1913, the company (Fig. 1.4) was employing 22 chemists, 30 other employees, and 48 workers (Trommsdorf, 1976). The US-based subsidiary – today's Röhm and Haas Company – was founded in 1911. The example of Rohm's company 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 pancreas 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 enzy-

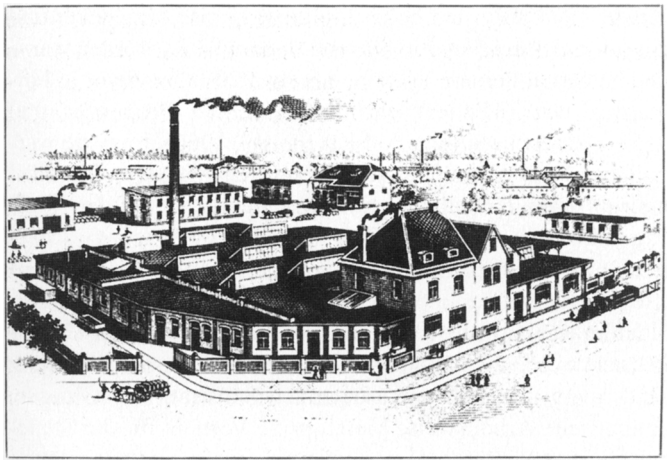


Fig. 1.4 The factory of the Röhm & Haas Company, Darmstadt, Germany, 1911.

matic 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 sugar-cane or sugar beet was the main sweetener used, an alternative raw material was that of starch, which is produced in large quantities from corn (mainly in the USA). 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 to the introduction of immobilized glucose isomerase as a biocatalyst. 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 last 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 Katzir's co-workers 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 (Carleysmith and Lilly, 1979, together with Beecham Pharmaceuticals, UK, and G. Schmidt-Kastner, Bayer, Germany).

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 ~500 kg HFCS kg^{-1} immobilized enzyme product (in 1975) to ~15 000 kg kg^{-1} (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 $1 million until 1965. However, with the appearance of the detergent proteases, the use of enzymes increased dramatically, and during the late 1960s, everybody wanted Biotex, the protease-containing detergent. At the same time, an acid/enzyme process to produce dextrose using glucoamylase was used increasingly in starch processing. As a consequence, by 1969 – within only a four-year period – Novo's enzyme turnover exceeded US$ 50 million annually, and in 2003 Novozymes' turnover was approximately US$ 1000 million. The present global market is estimated to be around 2 billion € (Novozymes, 2004) (Fig. 1.5a), and this has been reflected in the increased employment within the enzyme producing industry (Fig. 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.

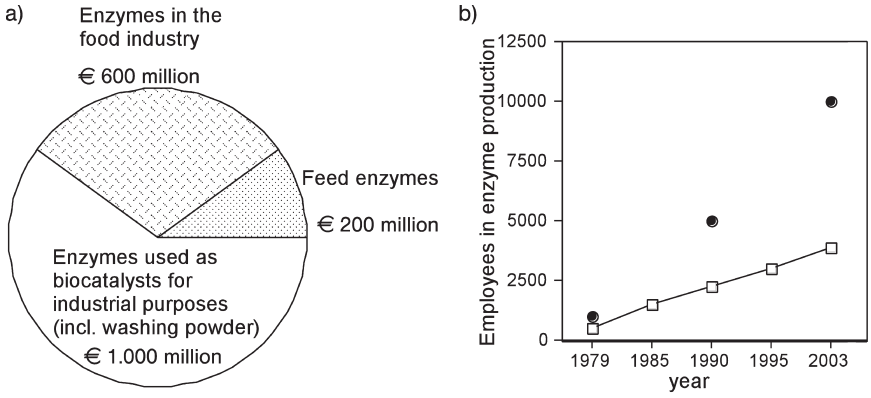


Fig. 1.5 Current market for enzymes for different purposes (a) and the increase in the application of enzymes reflected in the number of employees in the enzyme-producing industry (b) (for Novozymes (□) from yearly reports; worldwide (●) estimated).

Table 1.2 Products produced in quantities larger than 1000 t year^{-1} by different companies with enzymes as biocatalysts. Some other products produced in the range 10 to 1000 t year^{-1} in recently developed enzyme processes are also included.

Product	*Enzyme*	*Free or immobilized enzyme*	*Companies*
> 10 000 000 t a^{-1}			
HFCS	amylase	free	several
	glucoamylase	free	
	glucose isomerase	immobilized	
Ethanol (gasoline additive)	amylase, glucoamylase	free free	several
> 10 000 t a^{-1}			
Acrylamide	nitrilase	immobilized cells	Nitto, DSM
6-Aminopenicillanic acid (6-APA)	penicillin amidase	immobilized	several
Cacao butter	lipase	immobilized	Fuji Oil, Unilever
Isomaltulose	sucrose mutase	in immobilized cells	Südzucker
Lactose-free milk or whey	β-galactosidase	free or immobilized	several
> 1000 t a^{-1}			
7-Aminocephalosporanic acid (7-ACA)	(*R*)-amino oxidase	immobilized	several
	glutaryl amidase	immobilized	
7-Aminodesacetoxycephalosporanic acid (7-ADCA)	glutaryl amidase (modified (?))	immobilized	DSM
(*S*)-Aspartic acid	aspartase	immobilized (?)	Tanabe
Aspartame	thermolysisn	immobilized	Toso, DSM
(*S*)-Methoxyisopropyl amine	lipase	immobilized	BASF
(*R*)-Pantothenic acid	aldolactonase		Fuji chem. Ind.
(*R*)-Phenylglycine	hydantoinase, carbamoylase	immobilized	several
(*S*)-Amino acids	aminoacylase	free	Degussa, Tanabe
1000 > 10 t a^{-1}			
Amoxicillin	penicillin amidase	immobilized	DSM
Cephalexin	penicillin amidase	immobilized	DSM
(*S*)-DOPA	β-tyrosinase	immobilized	Ajinomoto
Human insulin	carboxypeptidase A	free	Aventis
	lysyl endopeptidase	free	several
	trypsin	free	BASF
Sterically pure alcohols and amines	lipase	immobilized	BASF
(*R*)-Mandelic acid	nitrilase	immobilized	BASF

The recent development of techniques of site-directed mutagenesis, gene shuffling and directed evolution have opened the perspective of modifying the selectivity and specificity of enzymes (see Chapter 2, Section 2.11, and Chapter 3).

More detailed accounts on the scientific and technological development can be found in articles by Sumner and Myrbäck (1950), Sumner and Somers (1953), Ullmann (1914), Tauber (1949), Neidleman (1991), by Turner (Roberts et al., 1995), and Buchholz and Poulsen (2000). 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 (Fig. 1.6). When more enzymes and cosubstrate regeneration are required, fermentation processes with living cells are more effective than processes with isolated enzymes. These processes will not be dealt with here, except for their application in environmental biotechnology (see Chapter 7). For enzyme processes which utilize few enzymes (≤3) without any cosubstrate (ATP, NADH) regeneration, those with isolated enzymes or enzymes in 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 stabilty 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.
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 technological 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 fulfill these criteria better. This is illustrated by the first two large-scale enzyme processes, namely the hydrolysis of penicillin (Fig. 1.7) and the production of glucose-fructose syrup from starch (Fig. 1.8). Both processes were developed some 30 years ago, and both replaced purely chemical processes that were economically unfavorable, and not sustainable.

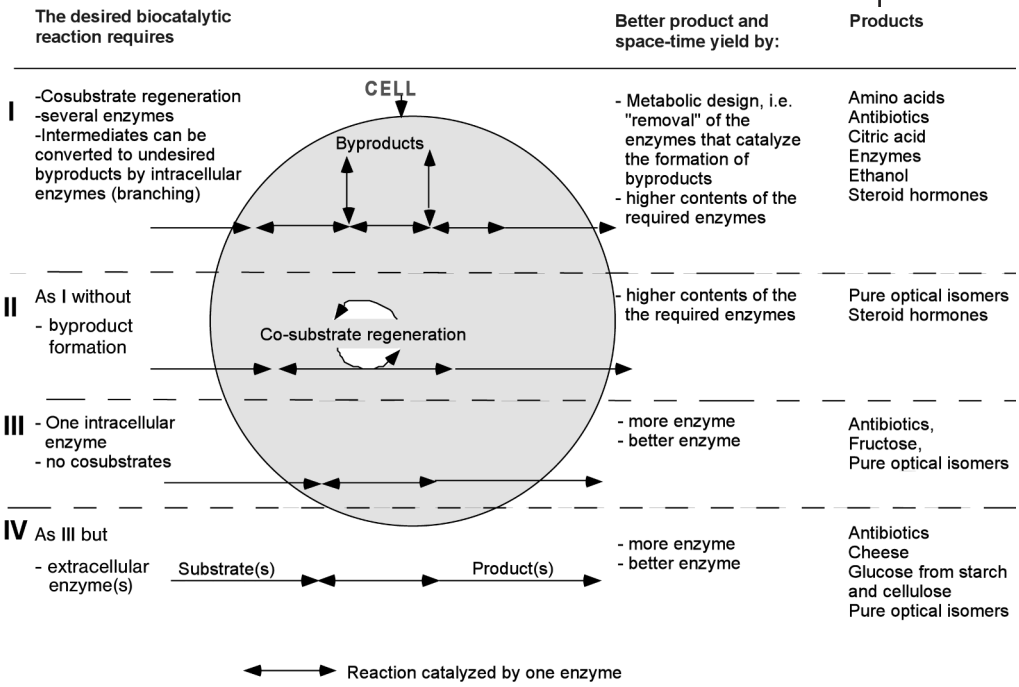
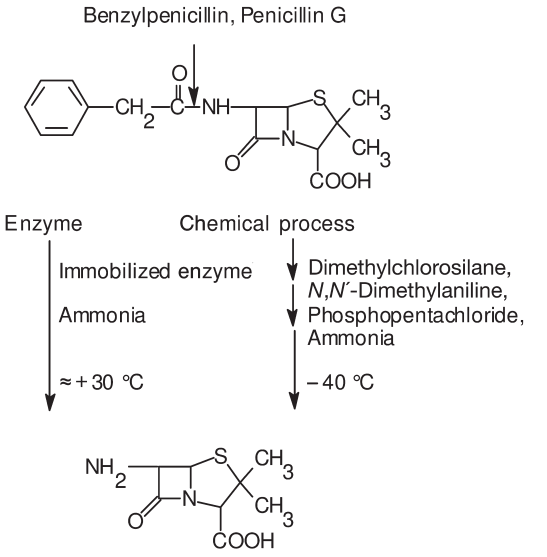


Fig. 1.6 Classification of biocatalytic processes with enzymes as biocatalysts. I and II must be performed with enzymes in living cells; III can be performed with enzymes in dead cells or as IV with isolated enzymes. Processes II–IV will be covered in this book. Process I will only be treated in connection with waste water and exhaust air treatment with immobilized cells (see Chapter 7).

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 considerable reduction in waste which in turn reduces the processing costs. 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 byproducts, 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 of enzymes as biocatalysts may be limited by their biological and chemical properties (see Chapter 2).



Required for the production of 500 t 6-APA

For hydrolysis:

1000 t	penicillin G	1000 t	penicillin G
45 t	ammonia	300 t	dimethylchlorosilane
≈ 1 t	immobilized enzyme	800 t	*N*,*N*-Dimethylaniline
10000 m^3	water	600 t	phosphopentachloride
		160 t	ammonia
		4200 m^3	dichloromethane
		4200 m^3	*n*-butanol

For downstream processing:

acetone	hydrochloric acid
ammonium bicarbonate	butylacetate
	acetone

Fig. 1.7 Comparison of the old (chemical) and the new (enzyme) process for the hydrolysis of penicillin G. The product, 6-aminopenicillinanic acid (6-APA), is used for the synthesis of semisynthetic penicillins with side chains other than phenylacetic acid. In the enzyme process, the byproduct phenylacetic acid can be recycled in the production of penicillin by fermentation (from Tischer, 1990).

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 pancreas insufficiencies, these peptidases may be transported from the pancreas directly into the bloodstream

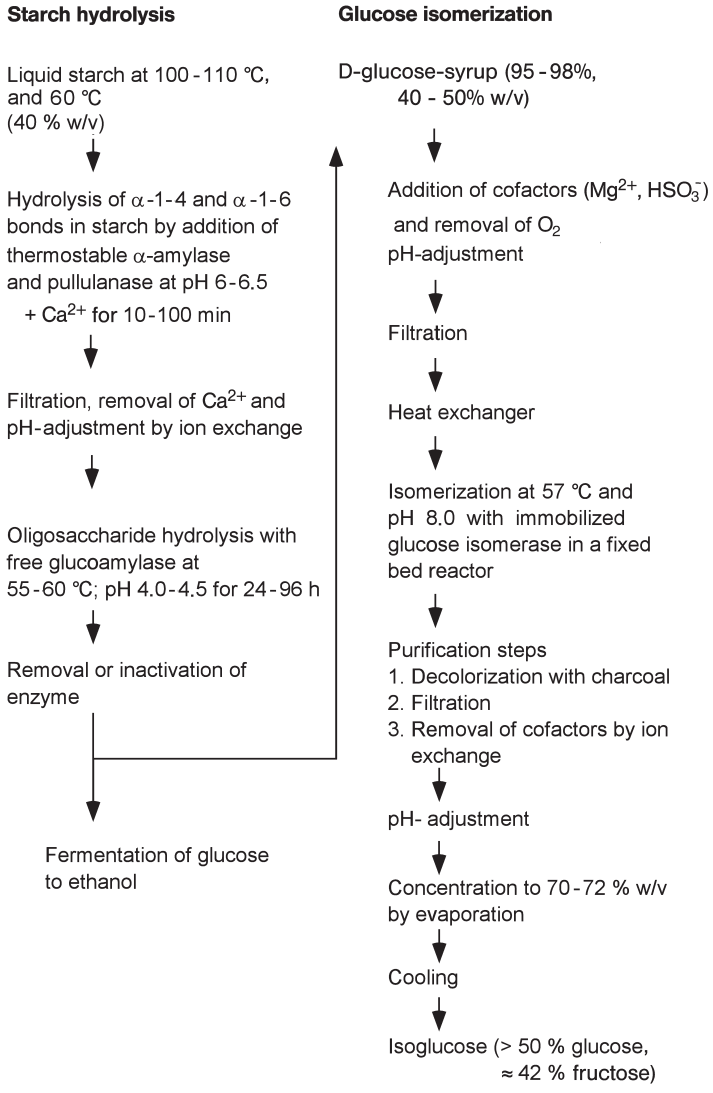


Fig. 1.8 The enzyme process for the hydrolysis of starch to glucose, and the isomerization of glucose to fructose. (w/v = weight per volume) (see Chapters 5, 6, and 9).

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 in-

duce 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. For example, enzymes used in washing powders are dried as large particles covered with a wax layer; consequently, the size of the particles is so large (>100 μm) that they cannot be inhaled into the lungs. 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.

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 are 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 (Fig. 1.9):

1. *Equilibrium-controlled processes*: the desired product concentration or property has a maximum at the end-point of the process (B in Fig. 1.9); the chemical equilibrium is independent of the properties of the catalyst (enzyme), but is dependent upon 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 Fig. 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.

In both cases the time to reach the maximum product concentration or property depends on the properties and amount of enzyme used, and of the catalyzed process

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

Advantages:	• Stereo- and regioselective • Low temperatures (0–110 °C) required • Low energy consumption • Active at pH 2–12 • Less byproducts • Non-toxic when correctly used • Can be reused (immobilized) • Can be degraded biologically • Can be produced in unlimited quantities
Disadvantages:	• Cells and enzymes are – unstable at high temperatures – unstable at extreme pH-values – unstable in aggressive solvents – inhibited by some metal ions – hydrolyzed by peptidases • Some enzymes – are still very expensive – require expensive cosubstrates • When inhaled or ingested enzymes are, as all foreign proteins, potential allergens

(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 (up to 1 M) that are much higher than those in living systems (≤0.01 M). At enzyme concentrations used in enzyme technology, the formation of undesired byproducts in uncatalyzed bimolecular reactions cannot be neglected.

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. The stages 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 (150–200 g L^{-1}), which is lower than 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

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-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 • energy • desizing of fibers • cheese ripening • malting in beer production	 Sterile filtration of plant extracts; Low temperature washing powder Desizing with enzymes Increase rate of process with enzymes
	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 (sweeteness)	Glucose isomerization to glucose-fructose syrup
Utilization of new regenerable sources of raw materials	Utilization of wastes from food and wood industry (whey, filter cakes with starch and protein from vegetable oil production, cellulose)	Drinks from whey Ethanol, biodiesel Animal feed
Reduction of environmental impact	Reduction of non-recyclable waste	Penicillin-, Cephalosporin C hydrolysis, leather production, paper bleaching
	Waste recycling	Utilization of whey

properties of the biocatalyst. Its selectivity (ratio of hydrolysis to synthesis rates) must be high in order to minimize the formation of byproducts (oligosaccharides). 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 endpoint of the process within a given time. When other constraints have been identi-

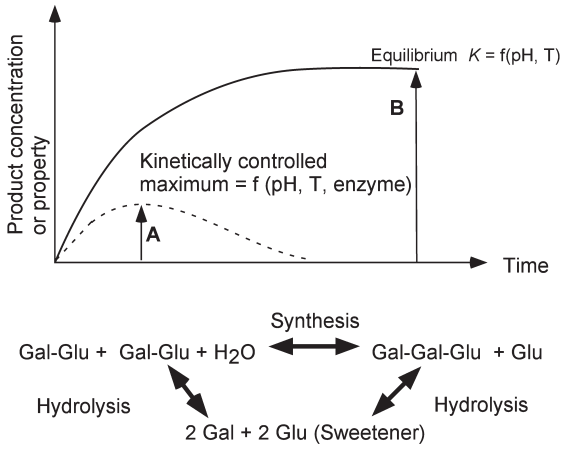


Fig. 1.9 Time-dependence (progress curves) of equilibrium- (solid line) and kinetically (broken line) controlled processes catalyzed by enzymes. The suitable end-points of these processes are those where the maximum product concentration or property is achieved – that is, A for the kinetically and B for the equilibrium-controlled process. Such processes are illustrated with the hydrolysis of lactose in milk or whey. [Whey is an inevitable byproduct 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 galactosyl-glucose), 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 are 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 tetra-saccharides. When lactose is consumed, these oligosaccharides are hydrolyzed. This also illustrates the formation of undesired byproducts in enzyme-catalyzed processes. The oligosaccharides are byproducts 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, byproduct formation is due to the hydrolysis to monosaccharides (Illanes et al., 1999; Bruins et al., 2003). The formation of the byproducts must be minimized by selecting suitable process conditions and biocatalysts.

fied, 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 as a function of pH and *T* is only defined by the catalyzed reaction. When this maximum is outside the process window, the enzyme process can only be improved by screening for a better biocatalyst or changing its properties by recombinant methods, so that the process can be carried out at pH- and *T*-values where this maximum can be reached.

In order to reduce the enzyme costs, the enzyme production can be improved (see Chapter 4) or the enzymes used in a re-usable 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 6 and 7). In these systems, the kinetics differ from those of

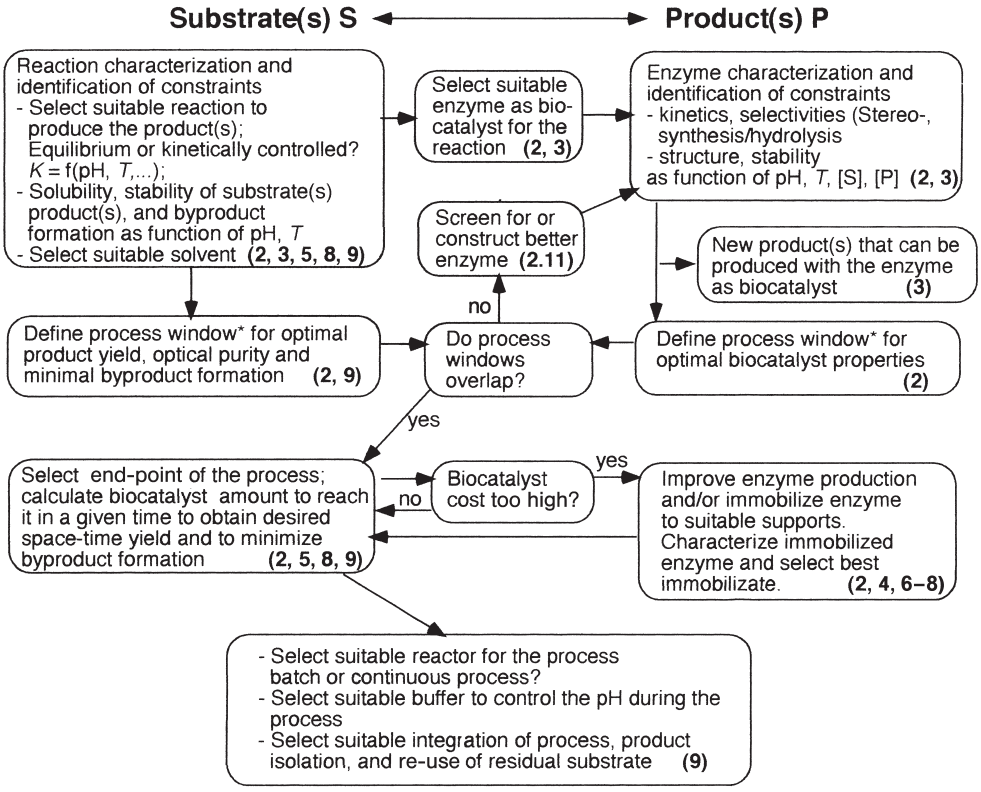


Fig. 1.10 Steps to consider in the design of an enzyme process to produce existing or new products (bold numbers refer to chapters in this book). *Process window = the range in a pH-*T*- (or pH-[S]-, pH-[P]-, *T*-[S]-, *T*-[P]-) plane where the reaction can be carried out with a given yield or optical purity, and where the properties (activity, selectivity, stability) of the biocatalyst are optimal.

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 8).

Once the process conditions and its end-point have been chosen in the process window, the enzyme costs per kg product are influenced by the type of reactor (batch, or continuous stirred tank or fixed-bed reactor) selected to carry out the process (see Chapter 9). There, the procedure to design an enzyme process (summarized in Fig. 1.10), will be illustrated in more detail as case studies for the design of a classical (HFCS production) and newer (7-ACA production) enzyme process.

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 value of the enzymes used as biocatalysts for different applications are shown in Figure 1.5. Besides industrial uses, 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). Another advantage of the recombinant production of these enzymes is that possible contamination with pathogenic human viruses (HIV, herpes) can be avoided.

The large number of new enzyme processes (>100) introduced during the past 30 years has recently been reviewed in detail (Liese et al., 2000). The type of process used, the compounds produced, and the enzymes used for these processes have been analyzed statistically (Straathof et al., 2002). These data show that hydrolases, lyases and oxidoreductases are used in two-thirds of all processes, while only about 1 % of the about 3000 known enzymes are used in larger amounts for enzyme technological and therapeutic purposes. During the past 10 years, the three-dimensional structures and detailed mechanisms of the reactions that they catalyze have been determined for many of the enzymes seen 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 enzyme (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, yield in the fermentation), and this is a task for the micro- or molecular biologist. The methods by which this problem may be solved is either to screen for better enzymes in nature, or to promote molecular *in vitro* evolution (see Chapter 2, Section 2.11). 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 1.8. The number of processing steps can be reduced when the enzymes used function at the same pH-value and have the

same requirements for metal ions, but this problem has not yet been sufficiently solved.

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

- The production of optically pure therapeutics and 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 (Collins et al., 1992; Breuer et al., 2004).
- The synthesis of antibiotics (Bruggink, 2002).
- Paper production or recycling to reduce waste and energy consumption (Bajpai, 1998).
- The regio- and stereoselective synthesis of oligosaccharides for food and pharmaceutical purposes.
- The selective glycosylation of peptides, proteins and other drugs.
- Environmental biotechnology.

1.7 Exercises

1. How was it shown that enzymes can act as catalysts outside living cells? In which enzyme process was this knowledge first applied?
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?
3. Explain the relevance of Figure 1.9 for enzyme technology. Which system properties must be known in addition to the properties of the biocatalyst to improve the yields of these processes?
4. Which properties of the enzyme and the catalyzed process must be known to minimize byproduct formation in the production of oligosaccharides from lactose, as shown in Figure 1.9? (Hint: Use Figure 1.10 to answer this question).
5. Test whether Figure 1.1 is in agreement with your consumption pattern.
6. How can the allergic and toxic risks due to enzymes be avoided in enzyme technology?

Literature

Overview on Enzyme Technology

The following books give an overview on 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:

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Internet resources for enzyme technology see Appendix I (p. 419)