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Preface

Hardly any other term in the field of biotechnology has been the subject of so much discussion among experts as white biotechnology at present. This term is an alias for "industrial biotechnology," an already established "heavyweight" that focuses on the production of the most diverse products (bulk and fine chemicals, enzymes, food and animal feed additives, pharmaceutically active substances and agrochemicals, auxiliary agents for process industries, etc.).

In some segments, white biotechnology has already captured leading market positions:

- In recent years the annual biotechnological production of amino acids exceeded one million tons.
- In vitamin production there have been several recent cases of a changeover from a chemical to a biotechnological synthesis process, a trend that is expected to increase.
- During the last 10 years the market volume for enzymes has increased by 50%.
- The successful launch of polylactide marked white biotechnology's breakthrough into the field of polymers and synthetics.

Today crude oil is the most important energy source and the most widely used chemical raw material. Both primary industry and polymer chemistry currently depend to a great extent on oil. However, it is only a matter of time before the world's oil reserves are depleted. Almost all studies presented to date agree that peak oil, i.e. the point in time when oil extraction reaches its highest level, will take place in the first half of the present century. The increasingly difficult development of new sources of oil have triggered initiatives worldwide to reduce national dependence on oil imports.

To summarize, there is no long-term alternative to developing a technology based firmly on renewable resources and industrial biotechnology may offer various solutions in this field. The tremendous pace of progress in the field of molecular biology has provided an unprecedented and promising launching pad for the development of further industrially relevant biocatalysts. Simultaneously, bioprocess engineering know-how is supporting efficient process development from titer plate format to shaker flasks to industrial scale. Thus, in principle, a basis exists for accelerating the development of new industrial

bioprocesses in parallel with all disciplines concerned. In this book authors from different scientific and business areas of industrial biotechnology aim to give you an overview of the state of the art and ongoing developments.

Frankfurt and Kaiserslautern, October 2006

Dieter Sell
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Raw Materials

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Abstract Industrial fermentations need raw materials that fulfill the requirements of the organism (suitable carbon and nitrogen source, minerals and specific nutrients) and that are available in a high quantity and quality. This contribution gives a comprehensive overview, including the new trends and progress of recent years. The use of feedstock based on several raw materials such as sugar, starch, inulin and lignocellulose is discussed. Biomass-based raw materials are by far the most applied feedstocks for fermentation. However, there are also raw materials for fermentations derived from the petrochemical industry. These substrates are especially hydrocarbons, alcohols and carboxylic acids. Some applications are given in this chapter.

Keywords Raw materials · Carbohydrates · Lignocellulose · Oil & fat · Glycerol

1 Introduction

Most fermentation media consist generally of carbon sources, nitrogen sources, minerals and specific nutrients; in the case of aerobic microorganisms oxygen or an oxygen source is also a key nutrient. Media composition is mostly only chemically well defined in the laboratory whereas in industrial fermentations complex media are used.

Industrial fermentations use mainly chemoorganotrophic microorganisms that meet their energy and carbon demand by metabolising organic substrates. Furthermore, the carbon source is the main component by weight in fermentation media. Hence, this chapter will focus on carbon sources for chemoorganotrophs.

There is a broad variety of fermentation raw material and feedstock as carbon source available for industrial fermentations to bioproducts and biofuels. Carbohydrates derived from sugar or starch plants are the main fermentable carbon source in industrial fermentations. Moreover, fats and oils are applied as a single carbon source or in combination with carbohydrates. Furthermore, alcohols, hydrocarbons and other organic substrates are used. In recent years lignocellulosic raw materials have come into the focus of research.

Media for industrial fermentations have already been reviewed in several papers [1–5] that discussed raw materials and nutrients in detail. Thus in this chapter, a short comprehensive overview including new the trends and progress of recent years will be given.

2 Carbohydrate-Based Raw Materials

Carbohydrates [6, 7] are by far the most used raw material and include saccharose (also called sucrose) and molasses from sugar beet and sugarcane as well as hydrolysed starch products like glucose and dextrins. There is also fructose derived from inulin and other carbohydrate sources that are used as raw material. Carbohydrate-based sources for industrial fermentations were reviewed in detail for sugars-based raw materials [8] and starch-based raw materials [9] some years ago (see also [10, 11]). For ethanol fermentation several reviews discussing suitable raw materials have been published, for example [12–16]. Lignocellulosic materials containing cellulose and hemicelluloses became the focus of research, driven by efforts to produce cheaper bioethanol. There is currently an intensive ongoing research to use lignocellulosic raw materials as source for fermentable carbohydrates (for example [17–21]). Moreover, residue and waste biomass are being considered as fermentation feedstock [49]. Some are already used (whey, pulp waste liquor) while others (lignocellulosic residues) are mostly still under investigation.

2.1 Sugar-Based Feedstock

Sugar plants are plants containing the disaccharide saccharose (also called sucrose), which consists of glucose and fructose. The term sugar is used as a common synonym for the carbohydrate saccharose in agricultural science and botany. Sugar beet and sugarcane are the main source for saccharose and saccharose-rich molasses. Other alternative plant sources for saccharose are sweet sorghum, sugar maple and sugar palms. However, only sweet sorghum has some importance and is used as a sugar feedstock source for fermentations. The saccharose contents of the main sugar plants are given in Table 1.

The world production of beet and cane raw sugar amounted to about 155 million tons in 2004/2005 (23% from sugar beet and 77% from sugar cane) [22]. Main producers are Germany, France and USA for beet sugar as well as Brazil, Australia, India, Thailand, Mexico and China for cane sugar.

Sugar beet (*Beta vulgaris*) is a temperate climate biennial root crop. It produces sugar during the first year of growth. It is sown in spring and harvested between autumn and early winter. Sugar beet is processed by extracting sliced beet cossettes with hot water (70 °C) to produce the raw sugar juice. The raw juice is purified to get the thin juice with an average sugar content of 16%. The thin juice is then concentrated in multiple steps, resulting in a thick juice with an average sugar content of 67%. The remaining wet residue of pressed, exhausted cossettes called pulp is dried, often pelleted and molassed for use as feed.

Sugarcane (*Saccharum officinarum*) is a perennial subtropical and tropical climate grass that is planted practically throughout the year in the equatorial belt; mostly in spring or late spring, or sometimes in autumn. The stalks are harvested after a minimum of 10 months or a maximum of 24 months after planting. The harvesting time depends on the sugar content and the stage of maturity. Sugar cane is harvested by hand or mechanically. The stalks are cut at the base and the leaves are removed. Afterwards, the plants develop new stalks, which allows up to eight seasons before new planting is necessary. Usually the harvesting and planting operations overlap in order to avoid storing the planting material. Sugarcane is processed by washing, chopping,

Table 1 Saccharose content of plants

Plant	Average % saccharose content
Sugar beet	16–24
Sugarcane	7–20
Sweet sorghum	7–15

and shredding the stalks. The shredded stalks are then extracted with water to produce the raw sugar juice with a sugar content of 10–15%. After clarification, the juice is concentrated by evaporation to get a thick syrup with an average sugar content of 60%. The remaining fibrous residue, called bagasse, is mostly burned for energy production.

The saccharose juice either from sugar beet or sugarcane is purified (refined) by crystallisation and centrifugation yielding 93–96% pure raw sugar syrup. Additional washing, centrifugation, and crystallisation operations yield > 99% pure white sugar. The remaining green run-off from the last crystallisations is called molasses, having still a carbohydrate content of 40–60%. The final molasses contain saccharose as well glucose and fructose.

Sweet sorghum (*Sorghum saccharatum*) is a warm climate cereal grass that is widely grown in Africa, Asia, and the Americans. After planting in spring, harvesting is similar to that of sugarcane. The sweet stems contain the saccharose. Due to an additional glucose content of 1%, crystallisation to produce white sugar is difficult. Hence, only sugar syrup is produced and used in the food industry and as feedstock for ethanol fermentation.

The different saccharose-containing fractions (raw juice, thick juice, saccharose, molasses) of the process may all serve as fermentation feedstock. Thus, saccharose of different purity is available as syrup or powder for fermentation. On the one hand, the pure character of saccharose, especially of crystallised white sugar, compared to molasses results in less interference in the fermentation process. On the other hand, saccharose is more expensive than molasses and does not contain additional nutrients such as nitrogen-sources, minerals or vitamins. Various more-or-less raw sugars of minor purity such as juices, syrups and green run-offs are available (Table 2). However some of them are only available during the harvest campaign. Thus, only crystallised sugar, refinery sugar liquids and molasses are available year around.

Beet and cane molasses are the cheapest and mostly used carbohydrate fermentation media of the sugar industry. Cane molasses accounted for approximately 80% of the world molasses production of about 48 million tons in 2004/2005 [23]. There are basically three types of molasses:

Table 2 Sugar content and purity of different feedstock from sugar beet processing [8]

Source	% Sugar	% Purity
Raw juice	13–17	89–91
Thin juice	13–17	91–93
Thick juice	61–70	> 91
Green run-off	63–70	77–90
Molasses	50–56	58–63

1. Beet or cane blackstrap molasses
2. Cane refinery blackstrap molasses
3. Cane high-test or inverted molasses

Blackstrap molasses are the final effluent during cane processing, where the mother liquor from the subsequent crystallisation steps still contains approximately 52 percent of total sugars. Beet molasses are produced by processes that are similar to those of sugarcane. Blackstrap molasses contains small amounts of complex polysaccharides and invert sugars as well as also various non-carbohydrate materials, including nitrogen-containing substances. The dark colour results from "browning reactions" (due to the Maillard reaction of the sugars with amino acids because of the heat and alkali used in processing). Blackstrap molasses is normally the cheapest sugar-based feedstock. Hence, it is most used for industrial fermentations.

Refinery blackstrap molasses is a product that differs from blackstrap molasses only in that it is the residual mother liquor that has accumulated in the recrystallisation refining of the crude cane-derived saccharose.

High-test or invert molasses contains approximately 70–75% sugar, and is produced in a manner different from that previously described as crystalline saccharose is not the desired final product. The sugarcane juice is partially inverted to prevent sugar crystallisation; that is, the sugar is partially hydrolysed to monosaccharides (either with heat and acid or enzymatically), purified, and finally concentrated to a syrup. Thus, high-test or invert molasses is strictly speaking an only moderately purified, inverted syrup that consists of glucose and fructose. It is preferred to blackstrap molasses because of the lower shipping charges on a sugar concentration basis and because of its lower levels of non-fermentable components. High-test molasses is produced only during years of sugar cane overproduction.

2.2 Starch-Based Feedstock

Starch plants contain the polysaccharide starch, which is the most abundant storage carbohydrate in the plant kingdom [24]. Starch is a mixture of the polysaccharides amylose (10–30%) and amylopectin (70–90%), both consisting of glucose units that are α-1,4-glucosidic linked together. However, amylose consists only of linear, unbranched chains of glucose, whereas amylopectin contains additionally α-1,6-linked side chains.

The most important starch sources are cereals (corn, wheat, rice), manioc, sweet potatoes and potatoes. The starch content depends on the plant species (Table 3). The world production of starch amounted to about 58 million tons in 2002 (roughly 69% from corn, 10% from manioc, 9% from sweet potatoes, 6% from wheat, 6% from potatoes, and less than 1% from other sources) [25]. Main producers are the USA, the European Union, China and Japan.

Table 3 Starch content of plants

Plant	Average % starch content
Corn	60–70
Wheat	55–70
Potatoes	12–20
Manioc	20–40
Rice	70–75
Sweet Potatoes	19–25

Corn or maize (*Zea mays*) is an annual grass. The starch is located in the grains and most commonly processed by wet milling. After steeping the grains, germ separation, milling and sieving starch is separated from the protein gluten. The starch slurry is than purified and dried. The remaining liquid from the steeping stage, called corn steep liquor, is also a fermentation feedstock.

Wheat (*Triticum aestivum*) is a moderate climate annual grass. The starch is located in the grains and processed by dry or wet milling. The wet milling process for wheat is adapted from corn wet milling. In dry milling, the grains are finely milled, the grains are separated and the wheat flour is than mixed with water. After sieving, screening, gluten separation and washing, the starch is separated by centrifugation. Further purification by sieving, screening and drying will led to pure wheat starch.

Potatoes (*Solanum tuberosum*) are moderate climate plants. Due to the non-frost-resistance of the varieties, tubers are harvested in autumn (after planting in spring). After washing, the potatoes are pulped and the starch separated from fibre and protein. Additional washing, separating and purifying processes then yield the dry starch.

Sweet potato (*Ipomoea batatas*) is a perennial crop that is cultivated throughout tropical and warm climate regions. Sweet potatoes are grown year-round in the tropics. The crop is normally harvested about 4 months after planting and harvesting may be spread over several months. Sweet potatoes are grown as an annual crop in temperate climate regions. Due to the non-frost-resistance, harvesting must take place here before the cold season. After harvesting, the starch is extracted from the tubers. The processing and refining is similar to the potato starch process.

Manioc (*Manihot esculenta*) is a tropical climate perennial plant. Twelve months after planting the tubers are harvested. Tapioca or cassava starch is extracted from the tubers of the manioc plant by washing, peeling and pulping. The separated starch milk is then centrifuged, washed, purified and dried.

Rice (*Oryza sativa*) is a subtropical and tropical climate perennial plant that is cultivated as annual crop. Rice grains are steeped and milled. The re-

sulting mash is screened and the protein separated by centrifugation. The starch slurry is then purified, concentrated and dried.

As most microorganisms are not able to metabolise starch it is further enzymatically processed to get a fermentable feedstock. This process includes the production of liquefied starch, maltodextrins, maltose, glucose and isoglucose [9].

Maltodextrins are produced by enzymatic or acid hydrolysis of starch. Starch liquefaction uses heat-tolerant α-amylase. The degree of dextrinisation (DE) depends on the conditions and enzymes used (DE = 0 describes unhydrolysed starch and DE = 100 is fully hydrolysed starch, i.e. glucose). Maltodexrins have usually a DE of 10–20.

Maltose is produced by enzymatic degradation of starch liquefacts using β-amylase, e.g. malt extract. The final maltose content depends strongly on the degree of dextrinisation of the liquefact. A maximum content of 50% maltose is achievable. Applying a debranching enzyme (e.g. pullulanase, isoamylase) to the β-amylase allows maltose contents of 75–80% to be reached.

Glucose is produced by enzymatic degradation of starch liquefacts using α-glucoamylase. Sometimes debranching enzymes are added for a more efficient hydrolysis. The glucose content of the resulting glucose syrup depends on the conditions. Using further concentration and purification methods a 99% pure glucose syrup can be produced. Crystalline glucose can be obtained from concentrated glucose syrup solutions, either in monohydrated or anhydrous form. Crystalline glucose is also named dextrose.

Hydrol is a molasses resulting from the production of crystalline glucose from corn starch. It contains approximately 60% sugar but it also contains a relatively high salt concentration that must be considered if these molasses are to be used as a raw material. Corn molasses plays only a minor role as fermentation feedstock.

Isoglucose is the common name in the sugar industry for fructose-rich fructose/glucose mixtures prepared by base-catalysed isomerisation of starch-derived glucose. In general, isoglucose is not industrially applied for fermentation. Most produced isoglucose is used as sweetener.

The steep waters of corn starch processing are concentrated to approximately 50% solids, and this corn steep liquor is used as a fermentation medium, mainly as nitrogen source. It was first extensively employed as fermentation media for the production of penicillin. Corn steep liquor contains lactic acid, amino acids, glucose and other reducing carbohydrates, salts and vitamins. The high lactic acid content results from the growth of lactic acid bacteria and of mycoderms during starch processing. Thus, corn steep liquor varies in composition from different suppliers.

Attempts have been made to use starch directly as a feedstock, especially in ethanol fermentation by yeasts. The yeast *S. cerevisiae* normally cannot directly utilise starchy materials. Amylolytic enzymes have to be added additionally and high temperatures are need to obtain a high ethanol yield from

starch feedstock. In recent years recombinant yeasts *S. cerevisiae* were developed that are able to directly use starchy materials [26, 27].

2.3 Inulin-Based Feedstock

Inulin is an oligosaccharide that mainly consists of fructose units. These biopolymers are also called fructans. Inulin-type fructans are β-2,1-linked and typically have a terminal glucose unit [28]. Plant inulins contain 6–150 fructose units. Fructans having only 3–6 fructose units are called fructooligosaccharides (FOS). Inulin is known from more than 30 000 plant species. A majority belongs to the families *Compositae* or *Liliales*. The inulin plant that is mainly industrially used is chicory, whereas Jerusalem artichoke (topinambur) and dahlia only play a minor role; blue agave is used for the tequila industry [29, 30]. The inulin content differs between the plant species (Table 4).

The world production of inulin amounts currently to about 350 000 tons. Main producers are Belgium, France, and the Netherlands as well as Chile in the near future. Furthermore, roughly 1000 ha of blue agave are planted in Mexico.

Chicory (*Cichorium intybus*) is a temperate climate biennial root crop. After planting in spring and 180–200 days of growth, harvesting of the roots takes place in autumn/early winter. Crop requirements are similar in nature but somewhat lower than those for sugar beet. The harvest can be performed with a modified sugar beet harvester. Inulin production is similar to sugar beet processing. The chicory roots are sliced and extracted with hot water. Purification of the raw inulin yields inulin juice that can be dried to produce a pure inulin powder.

Chemical or enzymatic hydrolysis of inulin and purification by ion exchangers can give fructose syrup.

Jerusalem artichoke (*Helianthus tuberosus*) is a perennial tuberous plant. The yield is higher if harvested annually. Cultivation of Jerusalem artichoke is comparable to potato production. The plant itself is frost-resistant but inulin biosynthesis is temperature-sensitive. Thus, cultivation is carried out with an annual season, with planting in spring and harvesting in autumn/early winter. Processing must start within two weeks after harvesting. The tubers are

Table 4 Inulin content of plants

Plant	Average % inulin content
Chicory	15–18
Jerusalem artichoke	14–18
Dahlia	10–12

crushed, pulped and pressed to get the extract containing the inulin. Further processing and purification gives pure inulin or inulin syrup. The low degree of polymerisation of inulin from Jerusalem artichoke is unfavourable for many applications. Thus, Jerusalem artichoke is rarely cultivated for non-food uses. In view of fermentation the degree of polymerisation is not important because only the part of fermentable carbohydrates is important. Thus, it was used for alcohol fermentation in France. Nevertheless, chicory is the plant of choice for inulin production.

Dahlia (*Dahlia spp.*) is a tuberous flower plant. There are many dahlia varieties available, but they are usually bred as flowers and not as a source of inulin. Furthermore, they are problematic in cultivation and harvesting and also dahlia has a lower inulin content and yield than chicory. Hence, tubers of dahlia (e.g. *Dahlia variabilis*) are only used to produce very pure inulin.

Blue agave (*Agave azul tequilana*) is a desert succulent requiring 7–12 years of growth to reach maturity. At this stage, the agave will have begun its final build-up to propel the central flower-bearing stalk. This penultimate burst of growth usually marks the death knell of the plant. Just before the stalk emerges harvest begins. The manufacturing process involves harvesting the stem of the agave plant and removing the leaves. Processing involves cooking the core of the agave to convert the inulin into a mixture of simple carbohydrates with fructose (about 70%) and glucose (about 20–25%) as main components. The carbohydrates are then extracted by milling and pressing giving the blue agave juice for fermentation.

In general, inulin is rarely industrially used for fermentation [13]. Most produced inulin is enzymatically hydrolysed to fructose or fructooligosaccharides for the use as sweetener. Some inulin is directly used as food ingredient.

2.4 Lignocellulosic Feedstock

Lignocellulosic biomass includes different sources that have been considered as fermentation feedstocks; see e.g. [17, 20, 31–36]:

- Agricultural residues (e.g. straw, stems, bagasse, husks as well as corn stalks, cobs and stover)
- Crops grown specifically for non-food biomass use (e.g. cereals, grasses, short rotation trees)
- Wood and woody biomass
- Biomass wastes (e.g. green waste, vegetable and fruit wastes, paper mill sludge, sorted municipal solid waste, paper)

The main sources that are considered mostly for large-scale industrial fermentations are agricultural residues, especially straw, wood and short rotation trees.

Lignocelluloses are mainly composed of cellulose (30–60%), hemicelluloses (20–40%) and lignin (10–30%). Cellulose and hemicelluloses are carbohydrate components whereas lignin is a phenolic polymer [37]. The content of the components depends on the source (Table 5) [38, 50].

Cellulose is a polysaccharide consisting of glucose units that are β-1,4-glucosidic linked together. Moreover, the secondary and tertiary structure, including intra- and intermolecular hydrogen bonding, results in amorphous and crystalline regions of the cellulose fibre.

Hemicelluloses (also called polyoses) are heteropolysaccharides consisting of short and highly branched chains of carbohydrates (hexosans and pentosans). Thus, they contain six-carbon sugars (e.g. galactose, glucose and mannose) and five-carbon sugars (e.g. xylose and arabinose). Hemicellulose from hardwoods is higher in five-carbon sugars (usually in xylose) whereas hemicelluloses from softwoods yield more six-carbon sugars. Hemicelluloses are much easier to hydrolyse than cellulose.

The composite formed by cellulose, hemicelluloses and lignin is responsible for the remarkable resistance against hydrolysis and enzymatic attacks. Thus, lignocellulose must be pre-treated and processed in order to get a fermentable feedstock and to remove the lignin [39]. Moreover, the degradation of the hydrolysis products has to be prevented (e.g. degradation reactions of monosaccharides resulting in furfural or 5-hydroxymethylfurfural). After mechanical separation and milling, lignocellulosic biomass must be hydrolysed to convert the cellulose and hemicelluloses into simple six- and five-carbon carbohydrates. The carbohydrate mixture obtained after hydrolysis contains mainly glucose and xylose as well as in minor quantities arabinose, galactose and mannose.

Hydrolysis technologies may involve a physical treatment (e.g. steam explosion, ammonia explosion, hot water, thermohydrolysis under pressure, lime), chemical methods such as hydrolysis by concentrated and dilute acid, as well as enzymatic methods whereby often chemical and enzymatic hydro-

Table 5 Average content (%) of lignocellulose components in different plants and sources

Source	Cellulose	Hemicellulose	Lignin
Hardwood	43–47	25–35	16–24
Softwood	40–44	25–29	25–31
Monocotyledon stems	25–40	25–50	10–30
Wheat straw	30	50	15
Corn cobs	45	35	15
Corn stalks	35	25	35
Bagasse	40	30	20
Low or non-lignified fibre plants	70–95	5–25	0–6

lysis are combined in consecutive steps [14, 40–42]. The liquid hydrolysates containing the carbohydrates are recovered from each stage and neutralised for fermentation.

The process whereby enzymatic hydrolysis is performed separately from the fermentation step is known as separate hydrolysis and fermentation (SHF). There are also processes of simultaneous saccharification of cellulose to glucose and fermentation (SSF), and of simultaneous saccharification of both cellulose and hemicelluloses to hexoses and pentoses, respectively, as well as co-fermentation of all fermentable carbohydrates (SSCF).

Residual cellulose and lignin left over in the solids from the hydrolysis reactors may serve as fuel for process energy production.

A number of acid-catalysed hydrolysis processes were developed at the beginning of the last century for the saccharification of wood to get fermentable carbohydrates. Two processes in particular have become important: the Bergius–Rheinau process and the Scholler–Tornesch process [40, 43]. Moreover, other processes using dilute or concentrated acids were developed and applied. The Bergius–Rheinau process is the only process that obtains crystalline glucose from wood cellulose. The processes were used in Germany in the 1930s first and later on also in other countries, e.g. USA, Sweden, the former USSR and others. Acid-catalysed hydrolysis processes were used to supply fermentable carbohydrates for the fermentation to industrial-grade ethanol, yeasts or SCP-feed. After the war, most plants were closed little by little in western countries. The saccharification of wood played a significant role only in the former USSR over decades.

The acetone–butanol–ethanol (ABE) fermentation was before, during and shortly after World War II the second largest industrial fermentation process [44, 45]. In the former USSR the ABE fermentation was also run using agricultural residues as raw material [46, 47]. Acetone and butanol were industrially produced in a plant in Dokshukino. In 1961 the feedstock was modified from pure wheat and/or rye flour hydrolysate to a mixture of flour and molasses (30 : 70). Additionally, a hydrolysate obtained from an acid-catalysed saccharification by sulfuric and phosphoric acid (3 : 1 or 4 : 1) from lignocellulosic biomass (corn stub, sunflower shells, and other agricultural waste) was used carbon source.

Research and development on the conversion of lignocellulosic feedstocks came again into focus in the 1980s and 1990s and has continued over the last 25 years. Investigations cover all processing steps: pretreatment, hydrolysis, fermentation and product separation in order to develop competitive and economic processes. Special attention is paid to the enzymatic treatment of the lignocellulosic feedstocks, the utilisation of pentoses for fermentation, and the development of optimised microorganisms.

The use of lignocellulosic biomass for fermentations is still in the research and pilot phase in contrast to raw materials derived from sugar and starch plants that are used in industrial scale. There is only one near-industrial scale

pilot plant operated by Iogen in Canada (http://www.iogen.ca), which uses straw as feedstock for the production of ethanol in a pre-commercial demonstration facility having an annual capacity of about 2.500 tons of ethanol. Based on softwood as fermentation feedstock a process development plant of an annual capacity of 200 tons of ethanol is operated by ETEK in Sweden (http://www.etek.se).

2.5 Whey

Whey is a by-product of cheese production. From 10 L of milk 1 kg cheese and 9 L whey are obtained. Cheese whey has a high biochemical oxygen content causing severe waste disposal problems. Hence, fermentation was considered as an added value outlet. The amount of raw whey annually produced worldwide is estimated to be between 40 million tons [48] and 115 million tons [49].

Whey contains about 3–8% of the disaccharide lactose and 1% milk protein. It remains after removing fat and casein from the milk. The disaccharide lactose consists of glucose and galactose. Depending on the type of cheese, sweet and sour whey is obtained. Whey is applied as fermentation media as either liquid or dried whey as well as whey permeate (Table 6). The latter is obtained after recovering the protein from the raw whey by ultrafiltration or osmolysis.

Whey has some limitations as a substrate because only some microorganisms (e.g. *lactobacilli* and certain yeasts) can utilise lactose rather than other carbohydrates. However, hydrolysis by β-galactosidase gives fermentable monosaccharides. Thus, *Lactobacillus bulgaricus* is capable of converting over 90% of the lactose in whey to lactic acid. Various lactose-fermenting yeasts (such as *Kluyveromyces, Candida, Trichosporon, Torula*) can convert the sugar to various products. This has become a commercial process for producing lactose-free whey, ethanol and single cell protein (SCP). Fermentation of whey permeate lactose directly into ethanol has had only limited commercial success, as the yields and alcohol tolerances of the organisms capable of directly fermenting lactose are low.

Whey is on one hand cheap and on the other hand perishable and sometimes inconsistent in quality. Thus, lactose may be separated from whey or

Table 6 Whey composition (content in %) [1, 50]

Constituent	Lactose	Proteins	Minerals	Fat
Dry raw whey	69–78	12–14	8–9	–
Dry whey	78	11–12	–	1–2
Dry whey permeate	75	3	12	–

whey permeate and can be used as lactose-rich fermentation media, especially for high value products, e.g. for fermentations to produce antibiotics or recombinant proteins [1, 52, 53].

Hydrolysis of whey lactose either enzymatically by β-galactosidase or chemically by acids gives fermentable monosaccharides that may be metabolised by several microorganisms. The enzyme cost in the first case and the degrading side-reaction into undesirable chemicals in the latter acid-catalysed hydrolysis are drawbacks [51].

2.6 Pulp Waste Liquor

Wood for paper production is treated either by the sulfite or sulfate process. In the sulfite process, wood is digested and cooked in liquor containing sodium, magnesium, ammonium or calcium bisulfite to get the cellulose fibre pulp and to remove the lignin. The remaining waste (called sulfite spent liquor, SSL) contains, besides lignin and sulfite, carbohydrates derived from the partial hydrolysis of cellulose and hemicelluloses. The carbohydrates consist of a mixture of hexoses and pentoses (mainly glucose, galactose, mannose, arabinose and xylose) and have a concentration of about 2–4% [12, 50]. The relative amounts of these sugars depends on the wood digestion, with soft woods being higher in hexoses and hardwoods higher in pentoses. The SSL is stripped or otherwise pre-treated to remove sulfur dioxide, sulfurous acid and furfural from the liquor. The carbohydrate concentration is adjusted to 10–12%.

Due to the huge amounts of SSL waste it was attractive to search for an added value use of this paper mill waste stream. Thus, SSL was commercially used in alcohol fermentation in the 1940s and later on in the production of protein biomass (single cell protein) for animal feed. Thus, yeasts have been used for alcohol and feed yeast production from paper mill waste. *C. utilis* is especially suitable because it has a high tolerance for sulfite and can convert both hexoses and pentoses. Two commercially established processes are the Torula and Pekilo processes. The commercial Pekilo process has been developed in Finland for the production of single-cell feed from SSL using *Paecilomyces variotii*. The first commercial Pekilo plant built at the United Paper Mills pulp mill at Jämsänkoski, Finland, had a capacity of 10 000 tons of single-cell protein annually [54]. Torula yeast (*T. utilis*) was produced from SSL by the Boise–Cascade Corp. as a high protein food extender and animal feed. An industrial ethanol plant in connection to a sulfite pulp mill is in operation at Örnsköldsvik in Sweden.

2.7 Others

There are some other carbohydrate-containing feedstocks that can be used for fermentation. Because they are seldom used in industrial fermentation to non-food products they will only be listed:

- Malt, malt extract, malt wort
- Soy flour
- Wine must
- Fruit juice, fruit residues
- Distiller's wash

Such sources are mostly really cheap but suffer in quality and contain components that may disturb the fermentation. For large-scale industrial fermentation their quantitative availability is often not sufficient.

3 Oil- and Fat-Based Raw Materials

Oil and fat consist of different triglycerides. Triglycerides are esters of glycerol that are esterified with three fatty acids. Thus, oil- and fat-based raw materials are on the one hand vegetable oil, vegetable fat and animal fat and on the other hand feedstocks derived thereof, i.e. fatty acids and glycerol. Chain lengths of the fatty acids in natural triglycerides of plants and animals vary mostly from four to 22 carbon atoms. The fatty acids are saturated (e.g. palmitic acid, stearic acid, lauric acid) or unsaturated (e.g. oleic acid, linoleic acid, linolenic acid, eruca acid). Typically, biosynthesis of plants and animals produces fatty acids that comprise only even numbers of carbon atoms. Plants from tropical areas mostly contain vegetable fats having a higher melting point (e.g. coconut fat, palm oil, palm kernel oil), i.e. they are solid at room temperature. In areas of moderate climate, the storage occurs as liquid vegetable oil.

Animal fat is mainly produced from animal residues. Animal fats are generally heat-rendered from animal tissues to separate them from protein and other naturally occurring materials. Fat rendering may be either with dry heat or with steam.

There are more than 230 plant species that store oils in their seeds, fruits, tubers/roots [55, 56]. However, the oil content and yield is quite different and there are only some oil plants that are of broad industrial importance (Table 7). Annual and perennial crops produce millions of tons of vegetable oils yearly. The world production of vegetable oils and fats was about 134 million tons in 2004/2005 [57]. The total world food and non-food consumption of major vegetable oils is currently, in descending order: soybean oil, palm oil,

Table 7 Oil content of plants

Plant	Source	Average % oil content
Oil palm	Fruits	40–65
Coconut palm	Seeds	60–70
Sunflower	Seeds	36–44
Rape	Seeds	39–43
Oil palm	Seeds	46–53
Soybean	Seeds	16–19

rape oil, sunflower oil, peanut oil, cottonseed oil, coconut oil and palm kernel oil. Producers of the three major vegetable oil plant sources are Malaysia and Indonesia for oil palms; USA, Argentina, Brazil, and China for soybeans; and the the European Union, China, India, and Canada for rapeseed.

The most important plant fruit as source of vegetable oil and fat comes from the oil palm (*Elaeis guineensis*). The olive tree (*Olea europaea*) and avocado pear (*Persea americana*) are of less importance and mainly used in the food industry.

Oil palms are tropic evergreen trees reaching a height of 10–15 m. The oil palm is monoecious and each tree changes periodically between male and female inflorescence. The oil palm can be productively harvested after 12–15 years and reaches 80 years. The stem ends in a crown of 70–100 leaves. Each leaf axil produces a blossom containing 1000–4000 fruits. Fruit flesh and kernel give different oils called palm oil and palm kernel oil. The fruits are harvested when the colour changes from black to orange and then autoclaved. After digestion and stirring at elevated temperature (95–100 °C) giving a pulp, the fruit flesh is pressed to separate the palm oil, and the nuts are separated from the pulp. The nutshells are broken and the kernels separated. Pressing or solvent extraction is used to obtain the kernel oil.

A broad range of plants are able to store reserves of vegetable oil and fat in their seeds. Soybean (*Glycine max*), rapeseed (*Brassica napus*), sunflower (H*elianthus annuus*), oil palm (*Elaeis guineensis*) and coco palm (*Cocos nucifera*) are the most important plants here. Turnip rape (*Brassica rapa*), oriental sesam (*Sesamum indicum*), cotton (*Gossypium*), flax (*Linum usitatissimum*), castor bean (*Ricinus communis*), poppy (*Papaver somniferum*), tung oil tree (*Aleurites cardata*), pea nut (*Arachis hypogaea*) and cocoa tree (*Theobroma cacao*) are only used in the food industry or/and as oleochemicals, rarely as fermentation feedstock.

The plant seeds of soybean, rape or sunflower are harvested, then pressed and extracted in order to obtain the crude vegetable oil. Historically, cold or hot extraction methods were used. These methods have been replaced by sol-

vent extraction (mainly using hexane), which gives a better oil yield. During the following processing operations, the crude vegetable oil is refined.

The coconut palm, reaching a height of 25–30 m, is a tree growing in subtropic and tropical regions. The coconut palm is male and female in the same inflorescence. The coconut palm can be productive harvested after 10–15 years and reaches 50–70 years in cultivation. Each palm produces 30–50 nuts per year, i.e. about 8000 nuts per hectare of plantation. The fruits are cut off manually at full ripeness. The husks are removed, the nuts are broken, and the meat is removed to obtain the copra after drying. The copra is pressed to produce the oil.

Jojoba oil from the jojoba plant (*Simmondsia chinensis*) is an example of a vegetable oil that is not a triglyceride. However, strictly speaking jojoba oil is a wax consisting of monoesters of alcohols and fatty acids having mostly 20 and 22 carbon atoms and being unsaturated. Jojoba oil is mostly used for technical applications. It is too expensive for fermentation.

Fatty acids are currently produced from triglycerides by hydrolysis of the ester with water at temperatures of 250 °C and pressures of 50 bar. Partial hydrolysis of triglycerides will yield mono- and diglycerides and fatty acids. When the hydrolysis is carried to completion mono-, di-, and triglycerides will hydrolyse to yield glycerol and fatty acids.

3.1 Vegetable Oil, Animal Fat, Fatty Acids

Vegetable oil and animal fat or fatty acids are often used as additional carbon sources in combination with carbohydrates and less as single carbon source. Vegetable oils are especially added in fermentations where the product contains long hydrocarbon chains or units, e.g. in the fermentative production of some antibiotics [1, 117], glycolipide-type surfactants [58, 59, 118] or polyhydroxycarboxylic acids [60, 61]. Unsaturated fatty acids have been used as a glucose supplement in amino acid fermentations [1, 62]. Hence, they are used both as a nutrient and as a biosynthetic carbon source.

Glycolipids can be produced by fermentation not only from vegetable oils but also from fatty acids [58, 63, 64, 118]. One example is the production of sophorose lipids by *C. bombicola* [65, 66]. Using vegetable oil or oleic acid as single raw material only low sophorose lipids yields are found, whereas the addition of glucose as additional carbon source resulted in higher yields and product concentrations [67].

There are also other publications concerning the production of chemicals using various types of pressed and refined vegetable oils like rapeseed, sunflower, maize, soybean and palm oil, e.g. citric acid [68, 69], oxalic acid [70] and lactones [119]. Sometimes vegetable oils are added in small quantities resulting in an auxiliary and enhancing effect (e.g. in yield [71, 120] or as an antifoaming agent [72]).

Furthermore, fermentation processes are used for the functionalisation of fatty acids, e.g. hydratisation of unsaturated fatty acids and oxidation [73, 126]. Thus, oleic acid, palmitic acid, stearic, and eruca acid are oxidised in the ω-position to the corresponding α,ω-dicarboxylic acids [74, 75].

3.2 Glycerol

Glycerol used in chemical, technical, and food applications is derived from the transesterification of vegetable oils and animal fats in the production of fatty acid, e.g. for surfactants, polymer additives and pharmaceuticals. Normally, raw glycerol is upgraded to pharmaceutical quality (refined glycerol). The world demand in the glycerol market amounted to about 520 000 tons in 2002 and can be fully fed from the transesterification to produce fatty acids [76]. However, glycerol is also a co-product from biodiesel production, which has increased dramatically during the last ten years and resulted in huge amounts of excess glycerol.

Biodiesel is a mixture of fatty acid methyl esters (FAME) made by transesterification of vegetable oil (mainly rape, canola, palm oil, soybean) or animal fat with methanol leaving glycerol as co-product. The majority of FAME is produced by a base-catalysed transesterification reaction. Due to the increased production of biodiesel, especially in Europe, the mean raw glycerol price dropped significantly from about 650–700 €/ton in 1996 to about 250–300 €/ton in 2004 and will further decrease in the future as the market trend for 2005 suggests mean prices of roughly 150–200 €/ton [76, 77]. Therefore, a feedstock that is relatively cheap and available in huge amounts is emerging as a potential fermentation substrate. Assuming a biodiesel production of more than 4 million tons in Europe in 2005 and bearing in mind that about 100 kg raw glycerol per ton of biodiesel is accumulated, more than 400 000 tons of raw glycerol are available.

Glycerol is also accessible by fermentation [121]. The microbial production of glycerol from carbohydrate carbon sources has been known for 150 years. As early as during World War I, glycerol was produced commercially. However, petrochemical routes to glycerol made the fermentation process uneconomical. Glycerol fermentation has become new interests as pathway intermediate in the production of chemicals from carbohydrate feedstock via glycerol as metabolite.

In the last decade, fermentations using glycerol as substrate have been increasingly explored. Hydrogen and ethanol production from glycerol-containing wastes discharged after a manufacturing process for biodiesel fuel using a strain of *Enterobacter aerogenes* was recently evaluated [122]. Examples are the production of polyhydroxycarboxylic acids (PHA) [60, 78–81], 1,3-propanediol (PDO) [82–85, 123, 124], 2,3-butanediol [86], succinic acid [125], 3-hydroxypropionaldehyde [87, 88] and antibiotics [117].

In the case of PHAs, the conversion of glycerol to short-side-chain PHAs (ssc-PHAs that are composed of C3 to C5 3-hydroxycarboxylic acids) like polyhydroxybutyric acid (PHB) or medium-side-chain PHAs (msc-PHAs that are composed of C6 to C16 3-hydroxycarboxylic acids) by some *Pseudomonas* bacteria has been described. The conversion of glycerol to PDO has been investigated in several studies [82, 83] using refined and raw glycerol driven by the use of PDO for the production of the polyester polytrimethylene terephthalate (PTT).

4 Other Raw Materials

Biomass-based raw materials are by far the most applied feedstocks for fermentation. However, there are also raw materials for fermentations derived from the petrochemical industry. These substrates are especially hydrocarbons, alcohols, and carboxylic acids [128–135].

Hydrocarbons including methane and alcohols, especially methanol, have been intensively investigated in the 1950s and 1960s as substrates for the production of single cell proteins (SCP) [1, 89, 90, 127], mainly for use as protein feed. Several processes were developed and brought to industrial scale. Most of the plants closed by the end of the 1980s due to increasing crude oil and raw material prices as well as to cheaper feed alternatives. Currently, only one SCP plant is in operation in western countries. It was established by Norferm, a joint venture between DuPont and Statoil, in Norway in 1998 and has a capacity of 8000 tons/year. However, the company's future is currently in question [91].

4.1 Alcohols

From the range of different alcohols, methanol [127, 130, 136] and ethanol were mainly investigated in depth as fermentation feedstock. The use of methanol and ethanol was mainly for SCP fermentation. From the point of view of carbon utilisation, methanol is one of the cheapest fermentation substrates. However, it causes some problems in fermentation (e.g. low vapour pressure, cell toxicity) and the number of microorganisms that can use methanol is small. The ICI plant converting methanol to SCP had a 3000 m^3 fermenter and produced 55 000 tons/year, but was closed down in 1985 after 6 years of operation due to increasing raw material costs and changes in the feed market [89]. Besides the use for SCP, methanol was investigated as a fermentation feedstock for chemicals and biopolymers, e.g. amino acids [92, 93], PHAs [80, 94, 95, 127], and others [99].

Ethanol was mainly used as fermentation feedstock in times when it was synthetically relatively cheaply available from ethylene. However, since ethanol is made more and more by fermentation it became uninteresting as a substrate because of cost competition and because it is more suitable and economical to ferment carbohydrate substrates to the desired products directly. The only industrial process that uses ethanol as substrate is the fermentation of acetic acid; mainly used in developing countries like India and China.

4.2 Methane

In principle, there is no difference between using methane from natural gas or biogas as carbon source. In both gases, refining may be necessary depending on the gas source and composition.

The composition of natural gas can vary widely, but the main component of natural gas is methane. A typical composition is 70–90% methane, 0–20% ethane/propane/butane, 0–8% carbon dioxide, 0–5% nitrogen, 0–5% hydrogen sulfide, and traces of other gases.

Biogas is produced by anaerobic fermentation. It is produced from organic materials (e.g. sewage or manure) when it is fermented in the absence of oxygen. The origin of the organic substrates can vary, ranging from livestock waste, harvest surplus, vegetable oil remains, to materials from household organic waste collection containers. Besides conventional materials, corn can also be fermented due to progress in fermentation methodology. Evaluation of materials for implementation in the biogas process depends on their potential attainable yield. Biogas is a gas mixture, consisting of approximately 40–75% methane, 25–60% carbon dioxide, and approximately 2% of other gases (hydrogen, hydrogen sulfide, ammonia and carbon monoxide).

In recent years, several thousand biogas plants have started operation across Europe as the inflammable biogas is a versatile source of energy that is now preferred for convertion into electricity and heat. Methane from biogas may in principle also be used as carbon source for methane-consuming microorganisms [138], however, only methane from natural gas has been used to date for fermentation. The use of methane from biogas is limited by the availability and the competitive energy use.

The highly reduced carbon in methane gives yields related to the amount of gas consumed. However, methane has found only limited use as a fermentation substrate because not too many microorganisms can utilise it. Most research was done by Shell in order to develop an SCP process. The production of PHB from methane [96, 97, 139] including biogas [98, 140] was the only intensively investigated and developed process, except for the use in protein feed production.

4.3 Hydrocarbons

This includes alkanes and other hydrocarbons from the petrochemical industry. Sources of hydrocarbons are crude oil, gas oil or refined alkanes.

The use of hydrocarbons as feedstock for fermentation was mainly forced – similar to methanol and methane – by investigations on SCP feed [89, 90]. Microorganisms for SCP production prefer alkanes with more than ten carbon atoms.

However, chemical intermediates and products, e.g. organic acids and vitamins [99], were also produced by fermentations based on hydrocarbons. Thus, alkanes were used as feedstock in the citric acid fermentation with *C. lipolytica*. However, an industrial plant producing 53 000 tons/year was closed in 1979 due to economic reasons as paraffin prices increased [100]. Besides fatty acids, *n*-alkanes were also used for the production of long-chain dicarboxylic acids [101].

5 Raw Material Availability, Costs and Economics

5.1 Availability of Biomass

Fermentation feedstocks rely mainly on carbon sources that are based on biomass and especially on plant biomass. Hence, sufficient availability of plant biomass is a prerequisite for the use of renewable resources as fermentation feedstocks.

Carbon dioxide fixation of plants by photosynthesis and its conversion to biomass, especially carbohydrates, occurs via different biochemical pathways: the three-carbon-cycle, the four-carbon-cycle or the crassulacean acid metabolism (CAM). The three pathways show significant differences in net photosynthetic assimilation. Typical C3-plants are sugar beet, wheat, barley, rice, potato, sunflower and soybean. The group of C4-plants include sugarcane, corn, sorghum and tropical grasses. CAM-plants are for example cacti and succulents. The main metabolic outcome of these pathways and hence the main component of biomass is carbohydrate.

There are various studies estimating the world biomass production. Estimates of the annual biomass production range from 140 to 220 billion tons and mostly an estimation of 170 billion tons of dry biomass is mentioned in the literature.

The net photosynthetic production of dry biomass carbon (assuming 45% carbon per ton dry biomass) was estimated in an assessment study in 1975 [102, 103]. The total biosphere production of dry terrestrial and ma-

rine biomass carbon was estimated at about 834 billion tons of standing dry biomass carbon and at about 77 billion tons of annual dry biomass carbon increase. The latter amounts to roughly 140 billion tons of dry biomass. The standing land biomass was estimated at roughly 829 billion tons of dry biomass carbon, whereas for the mean annual net land biomass production an estimate was given of 53 billion tons dry biomass carbon, i.e. roughly 100 billion tons of dry biomass. According to these studies, the annual land biomass productions are 61% from forests, 8% from cultivated area, 5% from temperate grassland, 5% from woodland and shrubland as well as 22% from other land areas.

The estimate of 220 billion tons globally available dry biomass was made in a study in 1999 [104] and relies on the annual terrestrial and marine oven-dry biomass from photosynthesis.

In a another recent study published in 1999, the annual biomass production is estimated at roughly 170 billion tons, considering that the annually available biomass consists of about 75% carbohydrates, 20% of lignin, and 5% of other organic biomass, e.g. vegetable oils, proteins, plant ingredients [105].

Biomass-based raw materials are mainly provided by agriculture and forestry. Only about 6 billion tons or 3.5% are used by humans from the total annual biomass production (referring to 170 billion tons) [106]:

- 3.7 billion tons (62%) of non-woody biomass for food
- 0.3 billion tons (5%) of non-woody biomass for non-food uses
- 2 billion tons (33%) wood for bioenergy and bioproducts

Considering only cultivated arable land, estimations were made of the potential area that may be globally available in the future for non-food uses. Of the global agricultural area of 2.8 billion hectares, roughly one third will not be necessary for food supply in 2040 (assuming a world population of 9 billion people) [107]. Hence, about 1 billion hectares can be used for the cultivation of non-food crops. This would globally result in 10–15 billion tons of annual agricultural non-woody biomass as raw materials if a yield of 10–15 tons/ha is assumed. A considerable part may be used as feedstock for fermentations, either from carbohydrate and starch plants or from agricultural lignocellulosic carbohydrate sources. Moreover, there is above all an additional raw material potential from lignocellulosic carbohydrate sources from wood and woody biomass for fermentation. In fact, considering overall land production, annual biomass production results mostly from forests.

5.2 Raw Material Costs and Fermentation Process Economics

Fermentation economics depends mainly on fermentation plant investment costs, raw material costs, maintenance costs including upstream and downstream costs, process yield and throughput. Another influence on economics

comes from the value and amount of the fermentation product. As the scale moves from specialities and fine chemicals into the segment of commodities and eventually into bulk products, feedstock prices become a more important issue. In fermentations yielding products having a high value the raw material costs are often only a minor part of the total costs.

A couple of years ago, the fermentation costs for two differently valued fermentation products – on the one hand a antibiotic and on the other hand the fine chemical citric acid – were compared based on data from ICI [108]. The antibiotic is made in a 50 tons/year production unit using 45 tons/year of sugar whereas citric acid comes from a 10 000 tons/year fermentation plant processing 17 000 tons/year of sugar. The share of sugar costs is 2.5% and 21%, respectively, assuming a low sugar price of 146 €/ton and is 8.3% and 48%, respectively, assuming a high sugar price of 511 €/ton. The comparison shows impressively the influence of the sugar price. Even in the case of a quite low sugar price, citric acid fermentation economics depends highly on the raw material price, i.e. a citric acid fermentation plant will be located in areas of cheaply available sugar, and also the fermentation may use less refined sugar or other carbohydrate sources. Table 8 gives prices for some carbohydrate sources [109, 110].

Nevertheless, the existing role of the different cost determinants mentioned above, the raw material and its price, is often a crucial point. In bulk fermentations, overall costs were often dominated by the raw material costs. Therefore, product yield and carbon atom economy and efficiency as well as microorganism productivity are important considerations.

The role of the carbon source is demonstrated in Table 9, which shows the influence of the carbohydrate feedstock on the production of a β-lactam an-

Table 8 Average prices for carbohydrate substrates

Carbohydrate substrate	Content of fermentable carbohydrate [%]	Price [$/kg hexose]
Saccharose	100	0.20–0.30
Molasses	47–53	0.15–0.25
Dextrose, monohydrate	91	0.30–0.60
Glucose, syrup 70%	70	0.30–0.60
Corn starch	85	0.25–0.60
Shredded corn	70	0.10–0.45
Lactose, edible	94	0.50–0.90
Lactose, crude	48	0.15–0.20
Dry whey	75	0.50–1.30
Whey Permeate	79	0.45–0.70
Fructose	100	1.00

Table 9 Influence of the feedstock on the production of a β-lactam antibiotic (values of glucose are set to 100)

Carbon source	Antibiotic concentration (w/v)	Cell Concentration (w/v)	Antibiotic yield per cell (w/w)
Glucose	100	100	100
Maltose	136	97	141
Fructose	151	96	157
Galactose	199	85	234
Saccharose	125	53	237

tibiotic by *Cephalosporium acremonium* [50, 111] related to cell growth and yield. Thus, a careful selection of the substrate is necessary when selecting a raw material.

However, the economics of fermentation raw materials and feedstock is rather complex and is not just a price competition between agricultural/forestry raw materials and petrochemicals. Of course, crude oil prices are a crucial factor affecting the competitiveness of fermentation processes as compared to petrochemical processes. Other important factors concerning raw materials are also:

- Requirements of the bioprocess and the microorganisms
- Security of supply concerning amount, quality, price
- Long-term availability
- Environmental regulations and demands
- Agricultural and forestry politics, international trade regulations
- Production refunds for the use of agricultural raw materials in non-food applications, including fermentations (e.g. production refunds for sugar and glucose syrups from starch when used in the chemical industry)
- Fiscal benefits and incentives for competitive uses of biomass (e.g. promotion of bioenergy, tax incentives for bioethanol)
- Environmental benefits and incentives for uses of biomass (e.g. CO_2-certificates)

5.3 Sugar Market Organisation for the Chemical Industry

The common market organisation (CMO) in the sugar sector was set up in 1968 and was revised in June 2006 [112–114, 141]. At present, the CMO for sugar is governed by Council Regulation (EC) No 1260/2001. Its essential features have been price arrangements, production quotas, arrangements for trade with third countries, and self-financing. The CMO provided for Com-

mission Regulation (EC) No 1265/2001 production refunds granted for sugar used by the pharmaceutical and chemical industries. The measure was designed to ensure that these industries can obtain Community sugar at the same price they would have to pay on the world market. The refund was fixed each month on the basis of the average refund resulting from the invitation to tender, less 64.5 € per tonne for shipping costs. This measure applied to some 500 000 tonnes of sugar and isoglucose in the EU-25. The cost of these refunds were covered by levies and is not therefore a drain on the Community budget.

Since 2006, the CMO for sugar is working under a new regime. The sugar sector is now governed by Council Regulation (EC) No 318/2006 [141]. The reform has come into force on 1 July 2006. A key element of the reform is the enlargement of alternative outlets for out-of-quota sugar [141, 142]. Furthermore, sugar beet should qualify for set-aside payments when grown as a non-food crop and should also be eligible for the energy crop aid of 45 euros/ha. The European Commission aims to improve the industrial uses of sugar for chemical products and biofuels. Industrial Sugar for the manufacture of chemical and pharmaceutical products should be available in the European Union at a price corresponding to the world price. The Commission has at its disposal different instruments to ensure the availability of sugar at competitive prices: out-of quota-sugar, production refunds, inward processing, duty free imports. Thus, the chemical and pharmaceutical industries will have access to competitive-priced sugar which should guarantee the chemical industry reasonable raw material prices. The near future will show if the aim can be reached.

The most striking feature of agricultural raw materials compared to petrochemical feedstocks is the fact that most states producing agricultural raw materials regulate their domestic agricultural markets and prices, including agricultural raw materials, to some extent. As far as fermentation feedstocks are concerned, this is especially true for the sugar market and prices, whereas starch and vegetable oil markets and prices are more related to the world market and prices. However, market regulations exist in most countries for the use of agricultural commodities in the chemical industry in order to allow access to competitively priced agricultural raw materials. Such systems include production refunds (e.g. covering the price difference between world and domestic prices), the cultivation of plants on set-aside land for industrial use, as well as subsidies and abatements [114, 115].

6 Conclusions

The biochemistry of microorganisms is based on carbon. There is a wide range of fermentation feedstocks available as carbon sources for fermenta-

tions. Carbohydrate sources from agricultural plants are the most common fermentation raw material. The nature of the carbon source used as raw material for fermentations depends first on the particular fermentation process and the requirements and productivity of the microorganisms. Within the range of suitable raw materials that meet the technological, processing and quality demands of the bioprocess, the raw material price is crucial. This demands a powerful agriculture and forestry. This will also force the integration of agricultural and chemical sites, as can be seen with the ADM complex in Decatur, Illinois (USA), where among others a corn wet mill and fermentation plants for citric acid, ethanol and lysine are located [116]. Furthermore, the range of feedstocks will be extended in the forthcoming years, and the use of lignocellulosic raw materials in particular as carbohydrate source will be a major contribution. Moreover, improving strains and microorganisms with respect to carbon atom efficiency, the acceptance of different or mixed raw materials, and the use of less refined or new feedstocks will all result in fermentation processes that are competitive and economic.

The use of biomass for fermentation has been increasing in recent years. Fermentation, and hence carbohydrate-based feedstocks, have become dominant in the production of some chemical products, e.g. ethanol and amino acids. The future will show further specialities, fine chemicals and bulk products that will be made by fermentation. The increased use and demand of agricultural raw materials for fermentation in the future will also force agriculture to provide high quality and cheap feedstocks, i.e. the cultivation and processing of industry plants has to be improved by cultivation measures, breeding, plant biotechnology and technological progress.

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Screening Systems

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Abstract Enzyme screening technology has undergone massive developments in recent years, particularly in the area of high-throughput screening and microarray methods. Screening consists of testing each sample of a sample library individually for the targeted reaction. This requires enzyme assays that accurately test relevant parameters of the reaction, such as catalytic turnover with a given substrate and selectivity parameters such as enantio- and regioselectivity. Enzyme assays also play an important role outside of enzyme screening, in particular for drug screening, medical diagnostics, and in the area of cellular and tissue imaging.

In the 1990s, methods for high-throughput screening of enzyme activities were perceived as a critical bottleneck. As illustrated partly in this chapter, a large repertoire of efficient screening strategies are available today that allow testing of almost any reaction with high-throughput.

1 Introduction

Enzyme technology for industrial application depends on enzymes that carry out selective chemical transformations and allow the desired processes to take place efficiently. Such enzymes must be discovered and optimized for each application by screening various sources of enzymes for activity. Enzyme sources suitable for discovery consist of large collections of enzyme-containing or enzyme-producing biological samples. These collections are typically of environmental origin, such as microbial strain collections [1, 2] and metagenomic libraries [3]. Alternatively, libraries of mutant enzymes are generated in the laboratory by random or directed mutagenesis from a known enzyme, and a large variety of mutagenesis methods have been developed and applied to the problem of optimizing enzyme expression and function through directed evolution [4–7]. Screening also includes process optimization with a single enzyme under changing reaction parameters such as solvents, buffers, additives, pH, and temperature.

Screening consists of testing each sample of a sample library individually for the targeted reaction. This requires enzyme assays that accurately test relevant parameters of the reaction, such as catalytic turnover with a given substrate and selectivity parameters such as enantio- and regioselectivity. Enzyme assays for screening must in addition be applicable to high-throughput, particulary in the context of enzyme discovery and engineering, where there are often thousands or millions of samples to test. Enzyme assays exist in different formats, which are adapted to the various types and sizes of enzyme sample libraries.

Here we have classified screening systems into three different classes (Fig. 1). The first class are assays applicable to test growing microbial colonies directly for enzyme activity, which may include a genetic selection step where the presence of an active enzyme is linked to cell survival. These assays have very high-throughput potential, but may be quite difficult to develop and implement. The second class concerns chromogenic and fluorogenic assays applicable in microtiterplate and microarray format. These are the most flexible and popular assays, and are also used for diagnostic and drug screening applications. Finally, the third class of assay are those relying on product detection by analytical instruments such as GC, HPLC, MS, or NMR, that have been adapted for high-throughput. Such assays require a strong commitment in resources and only allow medium throughput, but they are very rapidly adapted to any problem once the instrument is available. Instrumental assays deliver superior screening information by allowing one to work directly with the substrate of interest and they can also be used to test enantioselectivity.

Enzyme screening technology has undergone massive developments in recent years, particularly in the area of high-throughput screening and microarray methods. These developments were largely triggered by the de-

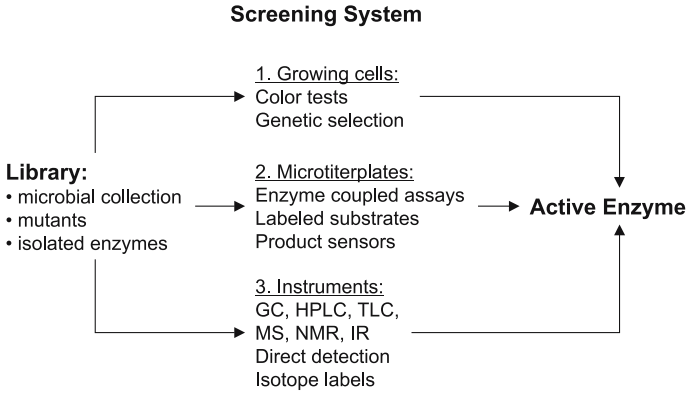


Fig. 1 Screening systems are key to find active enzyme in libraries

mands made by random mutagenesis on enzyme engineering, where high-throughput assays are indispensable tools for identifying active mutants, and by the exploration of biodiversity and the metagenome. Enzyme assays also play an important role outside of enzyme screening, in particular for drug screening, medical diagnostics, and in the area of cellular and tissue imaging. These various developments have been the subject of recent review articles and books [8–17]. Recent developments in high-throughput screening of organometallic reactions may also be of interest [18].

2 Screening and Selection of Enzyme Activities in Growing Cell Cultures

Most industrial enzymes are of microbial origin and are produced recombinantly in host cells. A number of screening systems have been adapted to identify enzyme activity directly on a living culture to allow isolation of a particular strain expressing the active enzyme of interest. Assays have also been developed to allow not only detection of enzyme activity through a signal (which is called screening) but also genetic selection of the active enzyme by linking expression of the enzyme to cell survival. The latter procedure allows, in principle, the screening of very large numbers of variants and the performance of Darwinian evolution experiments. In the context of screening variants of a pre-existing enzyme, it must be noted that the enzyme assay indicating turnover delivers a stronger signal for improvement of any parameter favoring product formation. This is the case with an improved enzyme mutant, but also if the mutant cell has a higher expression level of the desired enzyme, if protein folding is improved. These goals of enzyme expression optimization may indeed be the desired outcome during strain optimization for heterologous expression.

2.1 Agar Plate Assays

Most microorganisms can be conveniently cultured on agar in petri dishes. A single agar plate may contain thousands of different colonies, typically cells transformed with plasmids for expression of mutant enzymes. Enzyme assays based on chromogenic substrates producing an insoluble colored product can be applied either directly to agar plates or to filter paper replicas of these plates to identify colonies producing active enzymes, which can be then be picked for further culture. For example, indican (indoxyl-β-D-glucopyranoside, **1**) and related glycosides are natural products found in plants such as *Isatis tinctoria* and *Polygonum tinctorum* (Fig. 2) [19]. These plants have been used since Neolithic times to produce indigo **2** by fermentation, which results in glycoside hydrolysis and oxidation. A number of enzyme substrates have been designed following this natural product example, such as X-Gal (**3**) used to visualize galactosidase activity, and hence the lacZ reporter gene useful as a marker of plasmid incorporation or as a reporter for gene activation. Halogenation of the indole ring in these synthetic substrates

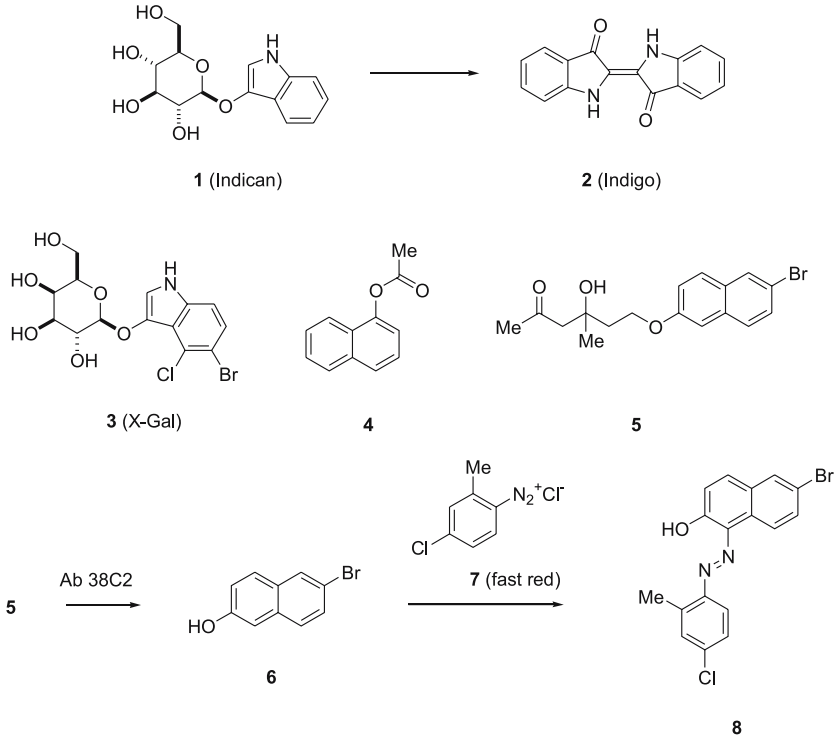
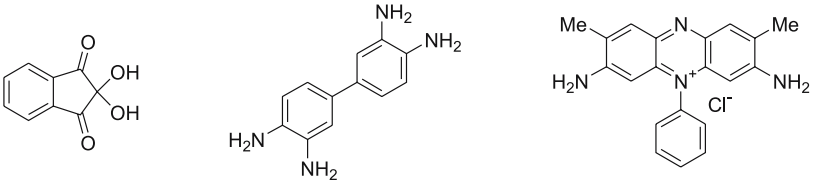


Fig. 2 Chromogenic substrates forming insoluble colored products

improves cell permeability. Indigo-type substrates are available commercially for a variety of hydrolytic enzymes, including among others phosphatases, esterases, and glycosidases [20].

Naphthols react readily with diazonium salts to form azo-dyes, and this principle is used is cytochemistry to test esterase activities in tissue samples with naphthyl acetate **4** [21]. Along the same principle, substrate **5** allows assay of aldolase antibodies in agar plates [22]. Retro-aldolization and β-elimination, are catalyzed by the antibody, which liberates bromonaphthol **6**. Reaction with the diazonium reagent **7** (fast red) then forms the insoluble bright red colored diazo dye **8**.

The examples above require the direct incorporation of the precipitating group into the enzyme substrate. Unlabeled substrates can also be used if secondary reagents are available for selective product staining. For example ninhydrin **9** (Fig. 3) was used to detect amidase activities on agar-plate colonies of bacteria expressing an enantioselective acylase cleaving a bicyclic lactam substrate [23]. Microorganisms were preselected for growth on *N*-acetyl phenylalanine as substrate, and the acylase enzyme was cloned into a bacterial host. Horse radish peroxidase (HRP) and H_2O_2 as oxidant was used to detect the naphthol product formed by hydroxylation of naphthalene by a $P450_{cam}$ monoxygenase [24, 25]. The HRP oxidation of naphthol produces highly fluorescent dimers and polymers, which can be detected directly on agar plates. 3,3′-Diaminobenzidine **10**, a colorless aromatic diamine, is rapidly oxidized by hydrogen peroxide to form a red precipitate. This assay allows one to detect enzymes producing hydrogen peroxide. Direct screening of microbial colonies on agar plates using this assay was used recently to screen mutants of a monoamine amine oxidase from *Aspergillus niger* for enantioselective oxidation of α-methyl benzylamine [26], and was also instrumental in further studies to broaden the substrate specificity of the enzyme by further mutagenesis [27]. Epoxide hydrolase activity on butane-oxide was detected in *E. coli* cultures on agar plates using safranin O (**11**) [28]. Oxidation of the 1,2-diol product by *E. coli* modified the membrane potential and lead to accumulation of the red dye in the colonies producing active enzyme, allowing for direct selection.



9 (ninhydrin) **10** (3,3'-diaminobenzidin) **11** (Safranin O)

Fig. 3 Dyes for staining reaction products on agar plates

Formation of a detectable colored product has also be linked indirectly to enzyme turnover via the so-called three-hybrid system [29]. In this system the reaction of the desired enzyme on a synthetic substrate leads to the formation of a product that acts as a bridge between a DNA-binding domain and transcription activation domain, thus forming an artificial transcription factor. This transcription factor then triggers the expression of a reporter gene, for example the green fluorescent protein, or an enzyme such as galactosidase which is then detected using an appropriate substrate [30, 31].

The above examples illustrate assays where a colored product is formed through the reaction of the enzyme and allows visual identification of the active colony. A number of agar-plate assays allow linking turnover by the enzyme to cell survival, such that only colonies expressing an active enzyme actually grow on the plate. In classical microbiology, one often uses the substrate of interest as the carbon source to detect microorganisms that might produce an enzyme capable of degrading this substrate, hopefully by performing the desired reaction, such as the hydrolysis of an ester bond. Such growth selection on the substrate is a quite primitive but effective method to discover enzymes, in particular for hydrolytic reactions.

Alternatively, one can link enzyme turnover to cell survival, eventually to perform directed evolution of biocatalysts [4–7]. The most simple example is the selection of cells expressing the enzyme β-lactamase by growth on a medium containing a β-lactam antibiotic. One can also perform genetic selection with so-called auxotrophic strains, in which a gene for production of an essential metabolite has been deleted, for example a gene for the biosynthesis of certain vitamins or amino acids. Turnover by the desired enzyme can then be selected if it restores the desired biosynthetic pathway or provides the essential metabolite by a different route, such as the hydrolysis of a synthetic precursor. For example, mutants of chorismate mutases can be genetically selected for activity in bacterial strains deficient in this enzyme, which is essential for aromatic amino acid biosynthesis [32]. Strains auxotrophic for aromatic amino acids can are used to select acylase activity in the presence of *N*-acylated aromatic amino acids as substrates. The three-hybrid system described above may also be used for genetic selection if product formation leads to transcription activation of a gene expressing the deleted function. This was elegantly demonstrated for the directed evolution of a glycosynthase by linking product formation to expression of a gene for leucine biosynthesis [33].

2.2 Fluorescence-Activated Cell Sorting

The genetic selection assays described above may also include a preselection step growing the cells in liquid culture before plating out. Assay systems have also been developed to directly identify cells expressing active enzymes in

liquid culture, based on fluorescence-activated cell sorting (FACS). For example mutants of the protease OmpA were displayed on the surface of *E. coli* cells, and a cell-surface adherent fluorogenic substrate for protease was added to the culture [34]. Cells expressing an active protease became fluorescent and could be sorted out by FACS, which allowed discovery of mutants with a 30-fold improved activity. The same technique has been used for sorting microemulsion droplets, where an enzyme gene and expression machinery are compartimentalized together with a fluorogenic substrate system [35, 36]. Both methods allow screening of very large numbers of variants ($> 10^7$).

3
High-Throughput Screening in Microtiter Plates and Microarrays

High-throughput refers to the ability to carry out a large number of experiments within a short time window. The screening and selection methods for living cells described above allow one to perform high-throughput screening if the cell cultures being tested contain a large variety of enzymes. High-throughput screening is most often realized using parallel experiments in small volume samples distributed in microtiter plate wells. This format is suitable for any collection of samples, which includes samples of living cells. It is also suitable for isolated enzymes under any variety of reaction conditions, such as for process optimization or the screening of a series of substrates or inhibitors on the same enzyme. Microtiter plates are particularly well-suited for spectroscopic reading using either UV/VIS or fluorescence plate readers. Accordingly, almost all enzyme assays suitable for microtiterplates are based on chemical systems that induce an absorbency or fluorescence change in the reaction solution upon turnover. Recent examples are discussed here using either enzyme coupled assays, labeled substrates, or indirect sensor systems. Such assays can usually be further miniaturized in microarray format, generally using fluorescence-based reporter systems, for which microarray scanners are well developed.

3.1
Enzyme-Coupled Assays

Most enzyme reactions do not produce any detectable changes in UV/VIS or fluorescence of the solution where they are taking place, typically because the substrate itself does not contain a chromophore, or the properties of its chromophores are not affected significantly by the reaction. This is particularly true for enzymes involved in basic metabolism, such as kinases, aldolases, isomerases, and hydrolases. One important exception is NAD(P)-dependent enzymes such as dehydrogenases, for which reactions the transformation of $NAD(P)^+$ into NAD(P)H, or vice-versa, is readily detectable due to an ab-

sorbency change at 340 nm and the fact that NAD(P)H is blue fluorescent, while $NAD(P)^+$ is not. The formation of NADH can also be revealed by a secondary reaction with nitroblue tetrazolium and phenazine methosulfate, which form a colored product.

The basic idea in most enzyme-coupled assays is to detect formation of a given product by introducing additional enzymes that convert this product further until an NAD(P)-dependent enzyme reacts in one of the subsequent steps. Such an enzyme-coupled assay was recently used in a directed evolution experiment with tagatose-1,6-bisphosphate aldolase, which resulted in a 80-fold improvement in catalytic efficiency and 100-fold selective evolution in favor of fructose-1,6-bisphosphate [37]. In this case, fructose-1,6-bisphosphate (**12**) was used as the substrate, and glycerinaldehyde-3-phosphate dehydrogenase as the coupled enzyme in conjunction with NAD^+ as cofactor and phenazin methosulfate (**15**) and nitroblue tetrazolium (**16**) as chromogenic reagents. Oxidation of the glycerinaldehyde-3-phosphate product **13** by the dehydrogenase produces 3-phopsho-glycerate **14** and NADH. The NADH then reacts with oxygen, **15**, and **16** in a process involving superoxide radicals to produce a colored soluble product (Fig. 4). Bacterial colonies expressing mutant enzymes were first grown on agar plates, and then transferred to 96-well microtiterplates using a colony-picker for screening. A similar enzyme-coupled assay for sialic acid aldolase relies on the formation of pyruvate, which is detected by reduction with lactate dehydrogenase and concomitant oxidation of NADH. This assay was used recently for screening targeted saturation mutagenesis libraries of three residues of

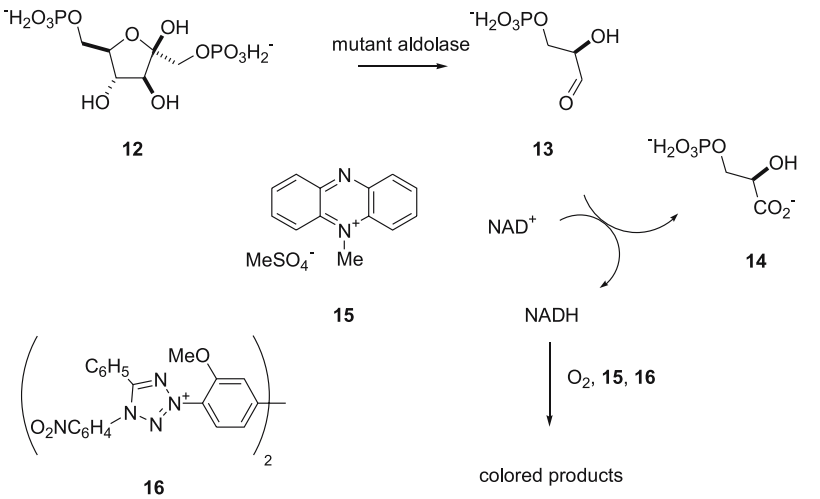


Fig. 4 Enzyme coupled assay for screening mutant tagatose-bisphosphate aldolases for altered stereospecificity, using a chromogenic detection of NADH with phenazin methosulfate (**15**) and nitroblue tetrazolium (**16**)

the active site to optimize this enzyme for conversion of unnatural sialic acid analogs [38].

A large number of enzyme-coupled systems are known for various enzymes. Examples of interest in the context of enzyme screening include the use of an acetic acid detection kit, consisting of three enzymes resulting in NAD-oxidation. This has been used to follow lipase activities on acetate esters of alcohols, in particular chiral alcohol to test kinetic resolution in high-throughput [39]. In an elegant experiment, a pair of alcohol dehydrogenases with opposite enantioselectivities was used to screen the enantiomeric excesses of chiral alcohols [40]. An antibody-catalyzed retro-aldol reaction releasing nonanal was detected by luminescence in the presence of bacteria expressing luciferase, which produces light upon oxidation of nonanal with molecular oxygen using flavin mononucleotide (FMN) as cofactor [41].

3.2 Chromogenic and Fluorogenic Substrates

The most straightforward method to obtain a UV/VIS or fluorescence signal from an enzymatic transformation is to use a synthetic labeled substrate such that the enzymatic reaction leads to a colored product. Typically, nitrophenol and umbelliferone analogs of the indoxyl substrates described above (Fig. 2) are commercially available for assaying hydrolytic enzymes such as lipases, phosphatases, glycosidases, and amidases in microtiter plates [20]. Esters and ethers of nitrophenol and umbelliferone are colorless and non-fluorescent, while the phenolates released after hydrolysis are yellow (nitrophenolate **18**) and strongly blue fluorescent (umbelliferone anion **20**). A broad range of related phenols exhibit strong absorbency and fluorescence changes upon protonation, acylation, or alkylation of the phenolate oxygen atom and can be used similarly for enzyme assays. Examples include nitrophenyl phosphate **17** to assay phosphatases, and 4-methyl-umbelliferyl-β-D-galactoside **19** to assay β-galactosidases. Furthermore, the red colored, red fluorescent resorufin and the green fluorescent fluorescein are formed by ester hydrolysis from the corresponding dibutyrates **21** and **22**, allowing assay of lipases and esterases. Anilines such as 4-nitroaniline and 7-amino-4-methylcoumarin are colored and fluorescent, respectively, while amide derivatives of these compounds are colorless and non-fluorescent. Thus, amides such as the trypsin substrate **23** and the aminopeptidase substrate **24** can be used to test acylases, amidases, and proteases. Peptidyl aminocoumarin amides have been used in combinatorial library format for protease profiling (Fig. 5) [42–46].

The above substrates for hydrolytic enzymes are problematic because they are quite unstable due to the strong leaving group ability of the phenols and aniline labels. In addition, the aromatic label is located directly at the reactive functional group, which may block the reaction of certain enzymes and does not allow structural modifications. The situation can be improved by using

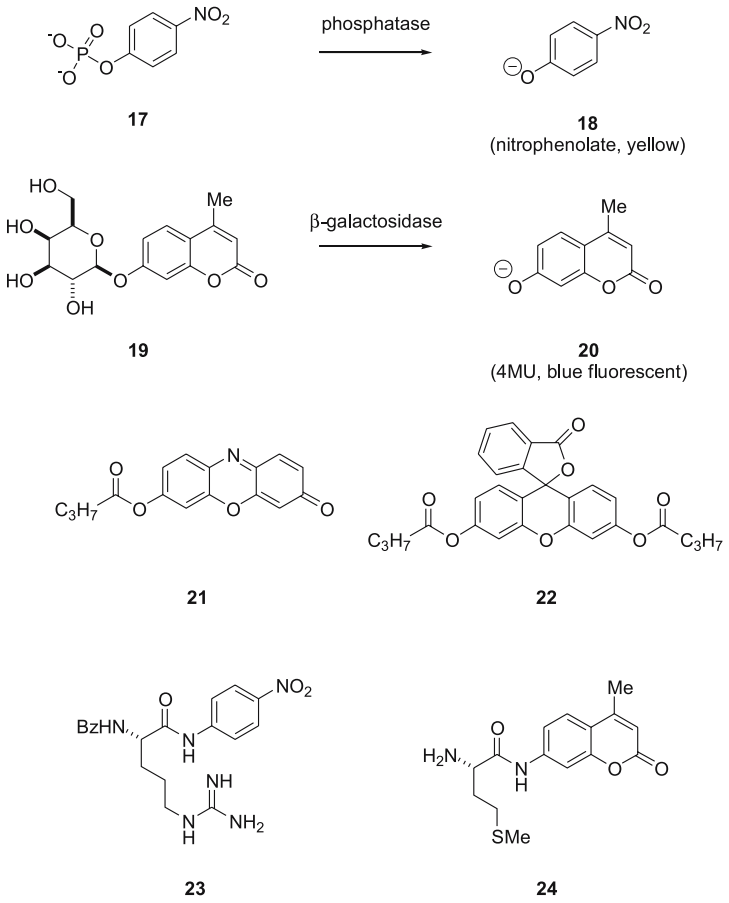


Fig. 5 Examples of commercially available chromogenic and fluorogenic ethers and esters of fluorescent and colored phenols serving as enzyme substrates

substrates which release the marker phenolate group indirectly after enzymatic turnover. For example, the chiral secondary alcohol **25** serves as a fluorogenic substrate for alcohol dehydrogenases (Fig. 6) [47]. Oxidation of the secondary alcohol produces ketone **26**, which is unstable in aqueous medium and rapidly releases the fluorescent product umbelliferone **27** by β-elimination under catalysis by bovine serum albumin [48]. β-Aryloxycarbonyl compounds of acidic (pKa ≤ 7) phenols such as umbelliferone or nitrophenol are generally fluorogenic or chromogenic by β-elimination, and a number of enzyme substrates can be formulated that produce such carbonyls as reaction products [49]. Examples include substrates for lipases and esterases [50], aldolase antibodies [51–53], transaldolases [54], and transketolases [55].

The carbonyl product suitable for chromogenic or fluorogenic β-elimination (e.g., **30**) may also be formed by oxidative cleavage of a primary 1,2-diol or

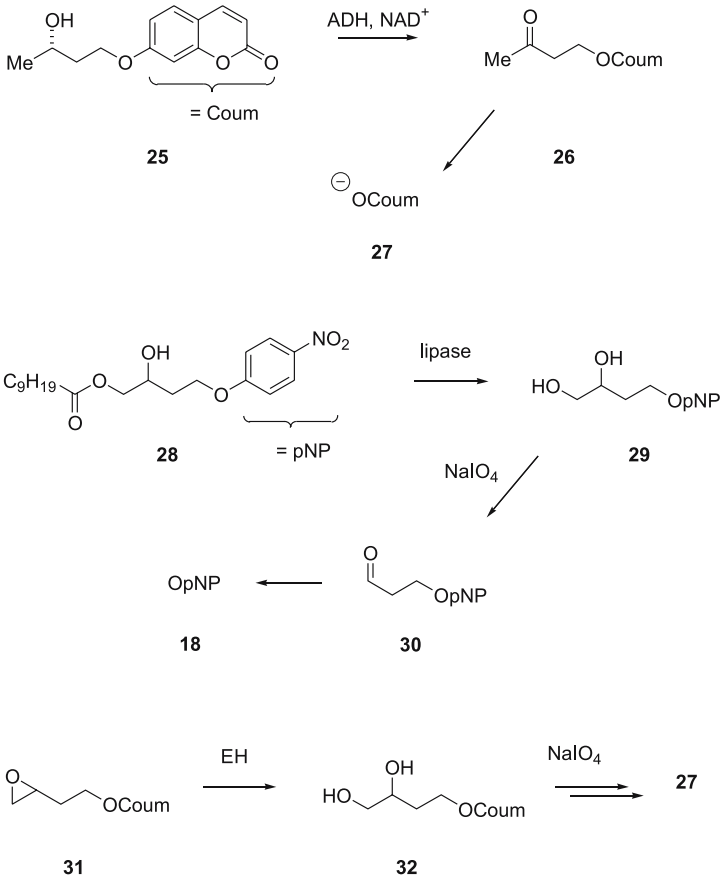


Fig. 6 Screening substrates with secondary β-elimination of the colored/fluorescent product

1,2-aminoalcohol product (e.g., **29**) by sodium periodate in situ (Fig. 6). These 1,2-diol or 1,2-aminoalcohols are themselves formed from an enzyme-labile precursor [56, 57]. Thus, mono- and diesters of 1,2-diols are used for testing esterases and lipases [59, 60], the corresponding phosphates for phosphatases, and the acyl or amino acid amides derivative of the 1,2-aminoalcohol for the detection of acylases and protease activities [61]. The periodate-coupled assay is particularly useful for the detection of lipase activities using the nitrophenyl-containing C10-ester **28** [62], which was used to isolate lipases in thermophilic microorganisms [63]. Epoxide **31** serves as a useful chiral probe for epoxide hydrolases by formation of the periodate cleavable 1,2-diol **32** and has been applied to screening of microorganism cultures [64].

Indirect release of nitrophenol and umbelliferone also occurs by spontaneous cleavage of hemiacetals (e.g., **34**) formed as primary enzyme reaction

products. For example, ester **33** is a useful fluorogenic substrate for catalytic antibodies [65], and for lipases and esterases (Fig. 7) [66]. 2-Aryloxyketones such as **35** were recently used to detect Bayer–Villigerase activity directly in cell culture [67]. The initially formed lactone **36** is spontaneously hydrolyzed in the medium to form hemiacetal **37** and finally releases umbelliferone **20**. Yet another indirect release mechanism involves the penicillin G acylase (PGA) substrate **38**, where the enzymatically released primary amine **39** cyclizes onto a nitrophenyl carbamate to release urea **40** and nitrophenol **18** [68]. The same principle is used in epoxide hydrolase assays based on glycidyl nitrophenyl carbonate **41** and related substrates by formation of a 5-membered ring carbonate; however, the carbonate function is very labile and also reacts with esterolytic enzymes [69].

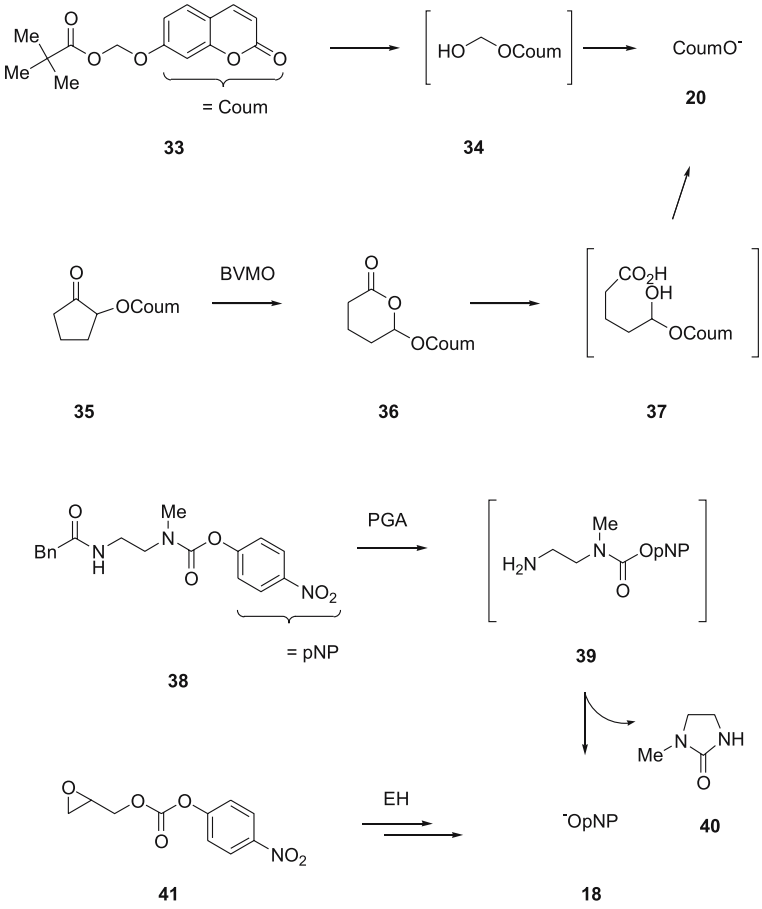


Fig. 7 Enzyme substrates with secondary release

Another important principle for fluorescence modulation in enzyme assays is the use of fluorescence resonance energy transfer (FRET) to follow the cleavage of substrates containing both a fluorophore and a quencher separated by an enzyme-labile group. The principle was initially reported for an HIV-protease assay [70], and has been used generally for proteases, for example in the context of protease-profiling experiments with solid-supported combinatorial libraries [71–73]. Useful FRET-substrates are also known for lipases [74–76], and for cellulases [77]. FRET-peptides can be used indirectly in the presence of proteases to detect enzyme activities forming these peptides as products from protease-resistant precursors, in particular phosphorylated peptides for detection of kinases and phosphatases [78].

A fluorophore or chromophore may also be released from a primary reaction product by reaction with a secondary enzyme that does not react with the initial substrate. This principle has been used to screen mutant glycosynthases in a directed evolution experiment using β-D-nitrophenyl glucoside as glycosyl acceptor, α-D-glucosylfluoride as an unnatural acceptor, and an endocellulase releasing nitrophenol only from the disaccharide product as secondary enzyme [79]. Chromogenic and fluorogenic protease substrates may also be used to screen kinases and phosphatases, using peptidases as secondary enzymes since these generally do not process phosphorylated peptides [80].

Further interesting principles for color or fluorescence modulation for detecting enzymes of synthetic interest include the formation of the blue fluorescent 6-methoxynaphthaldehyde from the corresponding alcohol, which is non-fluorescent. This chromophore was used in substrates for alcohol dehydrogenases, aldolase antibodies [53], lipases, and epoxide hydrolases [58]. Fluorogenic electrophiles such as maleimides [81] and quenching aldehydes [82] have been used to follow aldol addition reactions catalyzed by catalytic antibodies. Small UV absorbency changes upon reaction may also occur in a substrate without direct chemical modification of the chromophore. For example, the extinction coefficient at 350 nm of nitrophenyl glycidyl ether changes sufficiently upon hydrolysis to allow direct measurement of epoxide hydrolase activity on this substrate in microtiter plates [28]. Fluorescence modulation has also been used to create peptide- and protein-based fluorescent assays for kinases [83].

3.3 Chromogenic and Fluorogenic Sensors

The development of biocatalysts for fine chemistry is targeted to very specific substrates. Many experiments have shown that the use of a surrogate assay based on a labeled substrate for screening, such as chromogenic and fluorogenic substrate as discussed above, may lead to enzymes that are inactive or show poor selectivity with the application substrate. Several microtiter

plate assays have been developed to allow direct screening of reactions with unlabeled substrates. In these systems, binding or reaction of the product to a sensor system is used to trigger a color or fluorescence change in solution. The sensor generally functions by reaction with a functional group uncovered by the enzyme reaction. These sensors often require relatively high concentrations of product for returning a signal, typically in the 1–50 mM range, which is approximately 1000-fold higher than the detection limit for fluorogenic and chromogenic substrates. However, this is not a problem in industrial biocatalysis screening because only enzymatic reactions capable of preparative turnover at high substrate concentrations are of real interest, such that screening should indeed be conducted at relatively high substrate concentration.

pH-indicators represent the most simple example of versatile product sensors. They can be used to follow any reaction that induces a change in pH, typically the hydrolysis of an ester releasing a carboxylic acid. The optimal system involves the protonation of the yellow nitrophenolate **18** (Fig. 5) upon reaction. In addition, the use of the fluorogenic resorufin ester **21** (Fig. 5) as reference substrate allows combination of measurements with a pair of enantiomeric esters for accurately predicting the kinetic resolution behavior of the enzyme (E-value) [84, 85]. The method was used to profile hydrolases [86]. Nitrofurazane reagent **42** serves as fluorogenic reagents to screen vinyl ester esterification (Fig. 8). Vinyl ester cleavage produces acetaldehyde, which reacts immediately with **42** to form a hydrazone, which is fluorescent in organic solvent. This method allows screening of the synthetic activity of hydrolases [87]. Amino acids can be detected by fluorescence using the non-fluorescent Cu(II) complex of calcein **43**. The amino acid displaces Cu(II) from calcein **43**, whereby calcein regains its yellow fluorescence. This simple assay is suitable for screening acylases, amidases, and proteases [88, 89]. Enzyme substrates that release a thiol group as the product are readily detected using thiol-specific reagents such as Ellmann's reagent **46** or a recently reported dinitrobenzensulfonyl fluorescein derivative [90]. For example, thioacetyl choline **44** is hydrolyzed by acetylcholine esterase (AchE) to give thiocholine **45**, which immediately reacts with **46** by thiol exchange to give the mixed disulfide **47** and the yellow colored thiophenolate **48**. Dimedone esters such as (*S*)-**49** serve as useful chromogenic substrates for lipases due to formation of a blue-green complex of the dimedone hydrolysis product **50** with Cu(II) [91]. *Candida viscosum* lipase (CVL) is highly enantioselective for (*S*)-**49**.

The above examples can be used to follow the enzyme reaction in real time. A number of end-point assays are also possible to assay reactions by indirect product sensing, which further broadens the range of reactions accessible for screening. Amines can be assayed by reaction with chloronitrobenzofurazane to screen amidases [92]. Similarly, ammonia released by the hydrolysis of nitriles by nitrilases can be revealed by its reaction with

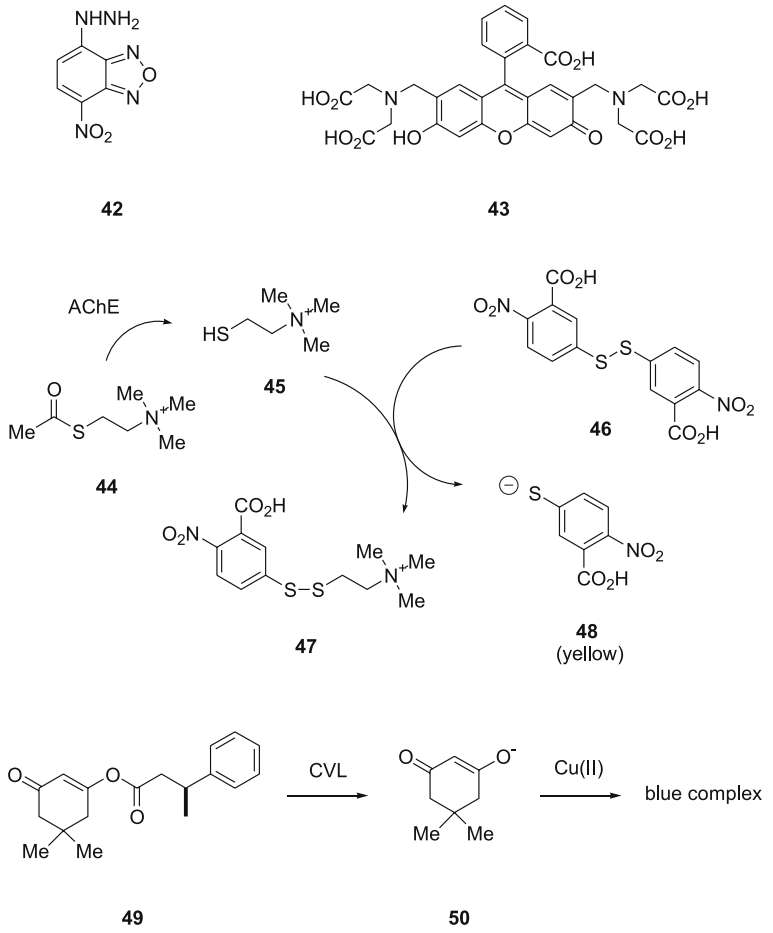


Fig. 8 Indirect product sensor that allows real-time monitoring of enzyme reactions

o-phthalaldehyde and β-mercaptoethanol to form a fluorescent isoindole derivative [93]. Amino acid amidases were screened by quantification of the released amino acids by complexation with $CuSO_4$, which forms a colored complex at basic pH [94].

1,2-Diols and 1,2-aminoalcohols can be quantified by back-titration of sodium periodate with adrenaline (**55**, Fig. 9). Adrenaline reacts quantitatively and rapidly with sodium periodate to form the deep red adrenochrome (**56**), a reaction which is does not take place if a 1,2-diol or 1,2-aminoalcohol has already consumed the periodate oxidizing agent. This provides a practical end-point assay for a variety of hydrolytic enzymes. The adrenaline assay can be used to screen the enzymatic hydrolysis of epoxides (**51**) by epoxide hydrolases, triglycerides such as tributyrin **52** by lipases, and the dephosphorylation of phytic acid (**53**) by phytases [95]. This rapid assay was used to profile

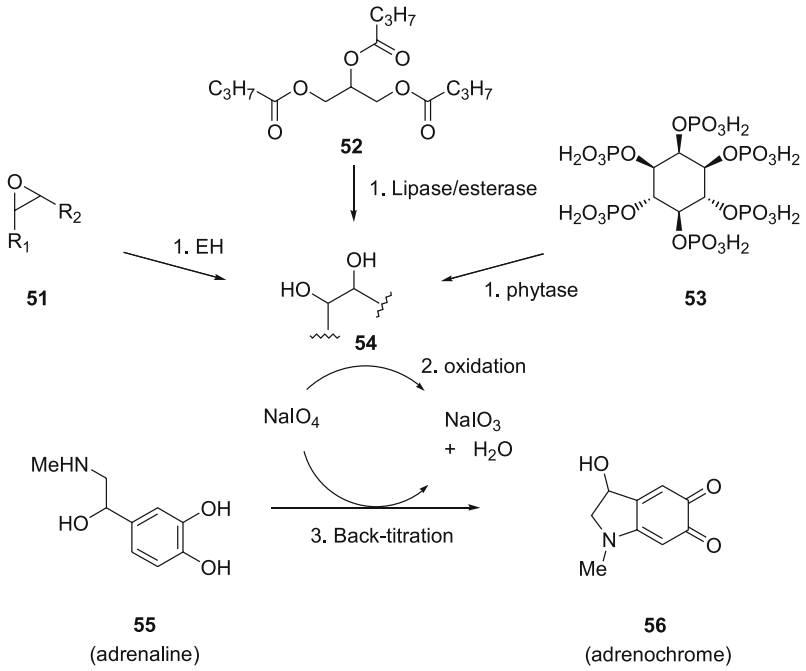


Fig. 9 Screening of enzyme reactions by endpoint quantification of reactions products with the adrenaline test

the activity of a series of microbial esterases with carbohydrate and polyol acetates [96]. Sodium periodate also decolorizes certain chromophores, and the assay was used to screen epoxide hydrolases using fluorescein as periodate reporter [97]. Epoxide hydrolases can also be screened by periodate cleavage of the 1,2-diol product by detecting the aldehyde product either directly [98], or by reaction with a chromogenic Schiff's base reagent [99]. Epoxides can be revealed by alkylation of 4-nitrobenzyl-pyridine, and the principle was used to screen epoxide hydrolases [100].

While the above examples involve rather simple reagents, enpoint quantification of reaction products has also been realized with more complex sensor systems. Catalytic reactions have been screened using product-specific antibodies to detect product formation on carrier-protein conjugated substrates [101–103], or using a competitive immunoassay to detect products in solution [104], which allows to screen for enantioselective reactions [105]. Fluorescein-containing vesicles containing synthetic multifunctional pores may be used to screen a variety of reactions, including reactions that consume ATP [106–108]. ATP can also be quantitated using luciferase and luminol [109], or using aptamer sensors [110]. Phosphate-binding fluorophores such as labeled Zn-bipyridylamines can be used to mark phosphorlyated peptide to study protein kinases [111, 112].

3.4 Microarrays

Plates of 96 wells and 384 wells are the workhorses of high-throughput screening, They offer a sufficient number of small volume reactors to screen thousands of samples in a volume of 5–200 μL each, which can be readily handled by standard pipetting robots. Liquid handling at lower volume becomes problematic due to rapid evaporation and inefficient mixing of assays and test solutions. Further miniaturization of enzyme assays can be obtained using solid supported assays.

The substrate mixing problem is elegantly solved by using silicagel plates preimpregnated with a fluorogenic substrate as the reaction medium [113]. A robotic arm is used to dispense the enzyme-containing test solutions in a volume of 1 μL per assay, which results in a homogeneously dispersed spot on the silicagel surface, on which the enzyme reacts evenly with the substrate (Fig. 10). The high-throughput potential of this method was demonstrated by profiling 40 different esterases and lipases across 35 different fluorogenic ester substrates of umbelliferone **20** or 4-methyl-umbelliferone **58** with various acyl chains (e.g., **57**), using only 50 μL of each enzyme solution, and submilligram amounts of each substrate for a total of over 7000 tests.

Further miniaturization of enzyme assays is possible with microarrays printed on glass slides. A nanospray system was used to homogeneously

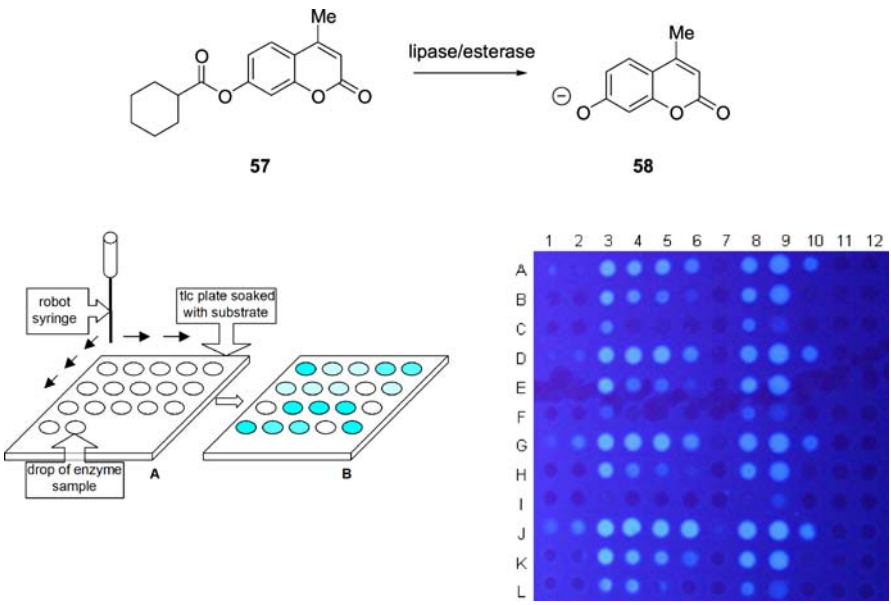


Fig. 10 High-thoughput screening with microliter reaction on silicagel plates

distribute nanodroplets of a solution containing three fluorogenic protease substrates on a microarray, on which spots of enzyme containing solutions had been previously printed, allowing high-throughput screening of enzyme inhibitors [114]. Fluorogenic substrates have also been arrayed with covalent attachment to the surface of glass slides to allow activity profiling experiments with hydrolytic enzymes [115], and with proteases using a combinatorial series of peptides [116]. Combinatorial libraries of PNA-encoded dipeptidyl-rhodamine substrates of type **59** were developed as reagents for profiling proteases (Fig. 11) [117]. Proteolysis of the acyl-rhodamine linkage produces a fluorescent product and the identity of the cleaved peptides is assigned by hybridization to a DNA-microarray printed with sequences complementary to the substrate's PNA tags at known positions, whereby the site of hybridization identifies each sequence. This format allows one to carry out the enzyme assay in solution, which is kinetically favorable, followed by microarray hybridization for determining the peptide sequences corresponding the fluorescent products formed.

Analytical methods for screening enantioselectivity have also been reported using microarray format, and they could potentially be useful for enzyme screening. Microarrays have also been used to estimate the optical purity of amino acids after covalent attachment by reaction with a pseudo-enantiomeric pair of labels bearing two different fluorophores [118]. The ratio of fluorophore can be correlated to optical purity using Horeau's method [119, 120]. Enantioselectivities of reactions with suitably derivatized substrates can also be measured by the color changes induced in a cholesteric-phase liquid crystal by doping with the reaction product in a setup suitable for high-throughput measurements [121].

Detection methods other than fluorescence can also be used to monitor enzymatic reactions at surfaces, and might be potentially useful for high-throughput screening. Direct electrochemical detection of enzyme activity is possible using substrates immobilized at the surface of electrodes. For example, the activity of the lipase cutinase was detected using a gold electrode

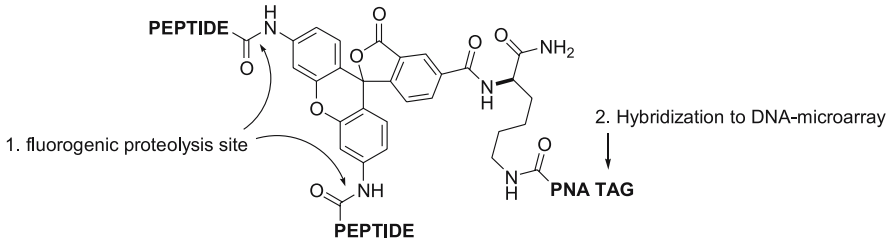


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Fig. 11 PNA-encoded substrates for protease fingerprinting

coated with a mixed self-assembled monolayer (SAM) containing a redox-inactive hydroquinone ester bearing a thiolated polyethylene glycol spacer and an alkane thiol [122]. Enzyme hydrolysis liberated the hydroquinone, which was detected using cyclic voltametry. In a similar setup, a ferrocene-modified aliphatic ester adsorbed on a SAM-coated gold electrode allowed direct monitoring of *Thermomycer lanuginosus* lipase [123]. In this case, ester bond cleavage detaches the redox-active ferrocene group from the surface, upon which the electrochemical signal decreases. Biotinylated amylopectin or pullulan immobilized on the surface of a streptavidin-coated quartz crystal was used in a quartz-crystal microbalance (QCM) to monitor the activity of *Aspergillus niger* glucoamylase [124]. The instruments detects mass changes upon enzyme–substrate binding (frequency decrease) and hydrolysis of the substrate (frequency increase).

4
Instrumental Enzyme Assays

Assays systems developed for genetic selection or for high-throughput screening by producing a selective signal can approach perfection in terms of throughput, sensitivity, and selectivity. However their development is time consuming and this often creates a bottleneck that is not affordable in the context of industrial biocatalysis. In that context it may be much more practical to develop generic high-throughput assay techniques adaptable for the general detection of any reaction product, independent of selective secondary reactions, sensors, or labels. This is possible by adapting standard chemical analytical instruments to high-throughput screening. Although instrumental assays may be limited in throughput, applying them to a well-selected focused collection of test samples or enzymes can be sufficient to solve the catalysis problem at hand.

4.1
Chromatography

The workhorse of analytical chemistry in synthetic chemistry is chromatography, be it thin layer chromatography (TLC), gas chromatography (GC), high pressure liquid chromatography (HPLC), or capillary electrophoresis (CE). Each of these methods can be adapted to high-throughput screening. Parallelization of TLC is mainly a manual handling problem, and one can readily operate thousands of parallel TLC analysis per day, as was demonstrated for screening with acridone-tagged substrates, allowing high sensitivity for product detection [125]. For GC, HPLC, and CE, autosamplers allow 10–1000 analyses per machine per day or more, depending on the analysis cycle. Even higher throughput is accessible using several instru-

ments in parallel, and this strategy has been applied in industrial biocatalysis projects.

Chromatographic analyses can be adapted for screening enantioselective reactions using chiral phases, and this has been demonstrated in several elegant studies by the Reetz group (Fig. 12). For example, a library of *Pseudomonas aeruginosa* lipase mutants created by error-prone PCR was screened for enantioselective acylation of racemic alcohol **60** with vinyl acetate by quantification of the enantiomers of the unreacted alcohol on a GC-column coated with a cyclodextrin derivative [126]. In another example, mutant cyclohexanone monoxygenases (CHMO) were screened for enantioselective oxidation of the prochiral 4-hydroxycyclohexanone **61** by resolving the product lactone **62** on chiral GC [127], as well as for enantioselective oxidation of sulfide **63** to either enantiomer of sulfoxide **64**, which were separated on a chiral HPLC column [128]. Gas chromatography was also used to characterize the enantio- and stereoselectivity of a family of 18 different alcohol dehydrogenases (ADH) from the yeast genome expressed recombinantly in *E. coli* for the reduction of β-keto ester substrates (e.g., **65**) to β-hydroxyesters (e.g., **66**) [129]. Capillary array electrophoresis (CAE), which uses bundles of 96 capillaries in parallel for gene sequencing, also allowed direct screening of enantiomers by separation of fluorescent product derivatives using cyclodextrin-containing phases, as shown for the fluorescein isothiocyanate (FITC) derivatives of several secondary amines [130]. CAE instruments allow throughputs of several thousand samples per day.

Due to its separating power, chromatographic screening offers the possibility of assaying several different substrates simultaneously. The use of

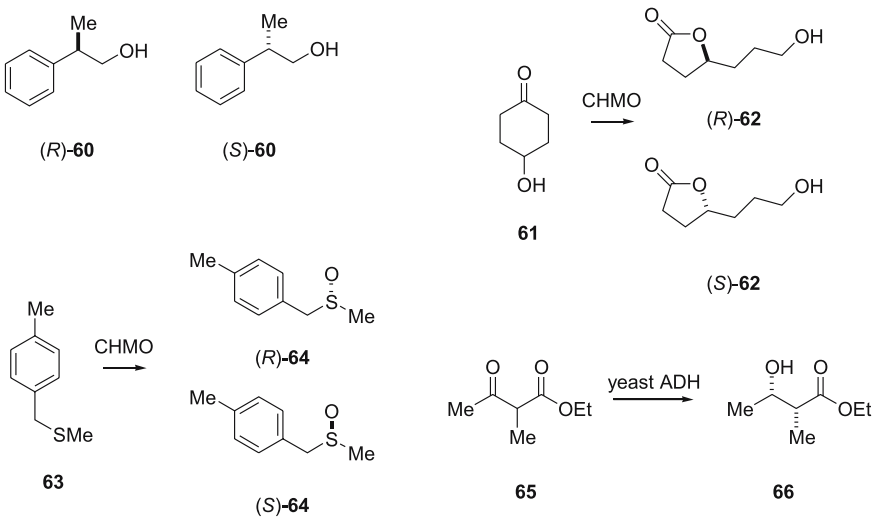

Fig. 12 Stereoselective reactions assayed by chiral-phase GC and HPLC

such substrate cocktails is particularly advantageous for enzyme profiling because the relative amounts of product formation defining the reactivity profile, or fingerprint [131, 132], can be precisely reproduced by this method. The principle was recently reported for fingerprint analysis of lipases and esterases using a cocktail of monoacyl-glycerol analogs [133], and for proteases using a cocktail of five hexapeptides [134]. A single HPLC analysis returns the activity fingerprint, which can be used for functional classification of the enzyme. Such characterization tools may prove useful for identifying novel enzymes with unusual selectivities, as well as in the area of diagnostics. Similarly, the classical APIZYM [135–137] substrate palette for microbial characterization can be formulated as a cocktail reagent, allowing 16 different enzyme reactivities to be determined in a single analysis [138]. The method is also suitable for analyzing thermophilic microorganisms and, generally, extremophiles.

4.2 Mass Spectrometry

Mass spectrometry (MS) is in principle a perfect method for screening enzymes, since almost any reaction results in a change in the molecular weight of the substrate. MS is often not quantitative, but this problem can be circumvented by using internal standards. Parallelization of MS is also possible for high-throughput, and can be adapted to almost any enzyme screening experiment.

MS was first reported as a screening method for enzymes in the case of enantioselective reactions. The enantioselectivity of reaction products can be measured by derivatization with mass-labeled pseudo-enantiomers [139, 140], and this was adapted recently to modern instruments and high-throughput screening [141]. More directly, one can use isotopically labeled pseudo-enantiomers as the enzyme substrates to screen enzymatic kinetic resolutions, typically ^{13}C-labeled acetate esters of chiral alcohols [142]. The method has been used for directed evolution of a *Bacillus subtilis* lipase for enantioselective hydrolysis of the ^{13}C-labeled pseudo-prochiral diacetoxycyclopentene substrate **67** to form chiral monoacetate **68** (Fig. 13) [143]. The method was also used to evolve *Aspergillus niger* epoxide hydrolase for improved enantioselectivity on phenyl glycidyl ether using the pseudo-racemic substrate pair **69a/b** [144]. In a similar setup, the deracemization of 3-hydroxyglutaronitrile, using an optically pure dinitrile substrate (*R*)-**70** bearing a single ^{15}N-label at one of the prochiral nitriles, was used to screen point mutants with improved enantioselectivities for formation of (*R*)-**71**, an intermediate for the synthesis of the cholesterol-lowering drug Lipitor [145].

A general MS-based screening system was recently reported for glycosynthases by quantification of nucleoside diphosphate sugar substrates using electrospray ionization mass spectrometry (ESI-MS) in negative mode [146].

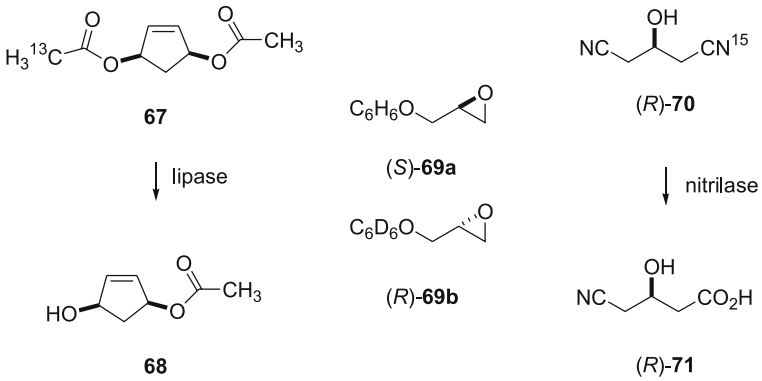

Fig. 13 MS-based enantioselectivity screens using isotopic labels

The method is particularly useful because such complex reactions are difficult to test otherwise with non-labeled substrates. MALDI-TOF MS was used for screening lipases and decarboxylases [147]. Enzyme and inhibitor screening for proteases, acetylcholine esterase, and glycosyl transferase has also been performed in chip format in very high-throughput using desorption/ionization on silicon mass spectrometry (DIOS-MS) [148], a powerful method broadly applicable in microanalytics and proteomics. Mass spectrometry was furthermore used to analyze enzyme activities on multiple substrates simultaneously using substrate cocktails. Mass-differentiated glycosides were used to profile glycosidase activities to explore the genome of plants such as *Arabidopsis thaliana*, which harbors 381 genes marked as potential glycosidases or glycosyl transferases, for novel enzymes [149]. A mixture of four aminopeptidase substrates marked with four different MS-tags was used to profile the corresponding enzymes in different bacteria [150].

4.3 NMR Spectrometry

NMR spectrometry serves to determine the structure of organic molecules one at a time. The instrument can be adapted to handle samples in series at a relatively high throughput, which makes the analysis interesting for screening. Since any chemical reaction induces a detectable change in the NMR spectrum of a substrate, the method is completely general. Instruments have been commercialized that allow the analysis of multiple samples in series [151]. The Reetz group used NMR analysis to screen enantioselective kinetic resolution of the acetate ester of 1-phenylethanol using either the ^{13}C-acetate labeled pseudo-racemic ester as substrate, or through formation of diastereomeric Mosher's esters [152]. The throughput potential for screening by NMR can be increased toup to several thousand samples per day using

chemical shift imaging (CSI) to follow 19 reactions in parallel in 19 capillaries, through which different test samples are passed. This was exemplified for the case of ^{13}C-labeled enantiomeric mixtures [153].

4.4 Infrared Radiation

Infrared thermography allows monitoring of temperature changes on the order of 0.1 °C in a sample, as might occur with an exothermic or endothermic reaction. For example, the esterification of either enantiomers of 1-phenylethanol with vinyl acetate catalyzed by an immobilized *Candida antartica* lipase was monitored using an infrared camera [154]. The reaction vessels consisted of holes of 8 mm diameter and 35 mm depth drilled in an aluminium plate. An array of 96 thermistors immersed in a standard microtiter plate, placed in a thermostatized plate holder, allowed a more sensitive detection of temperature [155]. The device was used to follow the hydrolysis of penicillin G by a β-lactamase, which is only moderately exothermic.

Infrared spectroscopy allows one to probe the presence of specific functional groups in molecules. The Reetz group has used a commercially available high-throughput Fourrier transform infrared (FTIR) instrument to monitor the enantioselectivity of hydrolysis in the ^{13}C-labeled pair of pseudo-enantiomers at up to 10 000 samples per day [156]. Each enantiomeric ester was quantified independently by measuring the intensity of stretching vibration of the ester carbonyl group. The absorption bands are located at $\nu(^{12}C=O) = 1751\ cm^{-1}$ in the unlabeled enantiomer, and $\nu(^{13}C=O) = 1699\ cm^{-1}$ in its labeled pseudo-enantiomer, and are baseline separated in the FTIR spectrum.

5 Conclusion

In the 1990s, methods for high-throughput screening of enzyme activities were perceived as a critical bottleneck. This situation was caused in large part by the advent of random mutagenesis methods for directed evolution, which multiplied demands for screening by orders of magnitude. Development of new screening methods based on chemistry, biology, and instrumentation has followed, rising to this challenge. Part of this process involved reviving and refining older methods in the context of efficient and sensitive parallel instruments. As illustrated partly in this chapter, a large repertoire of efficient screening strategies are available today that allow testing of almost any reaction in high-throughput. Nevertheless, high-throughput screening remains an active area of research allowing for surprising discoveries, and many screening strategies remain to be devised and realized.

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Industrial Enzymes

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Abbreviations

LCA	Life cycle assessments
PE	Person equivalent per year
ADD	Automatic dishwashing detergents
LAS	Linear alkyl aryl sulfonates
LOM	Launder-O-meter
TOM	Terg-O-tometer
TG	Triglycerides
FFA	Free fatty acid
MOW	Mixed office waste
ONP	Old newsprint
TMP	Thermomechanical pulp
HFCS	High fructose corn syrup
ETBE	Ethyl tertiary butyl ether
MTBE	Methyl tertiary butyl ether
FFV's	Flexible fuel vehicles
DDG	Dried distillers grains
DDGS	Dried distillers grains with solubles
SSF	Simultaneous saccharification and fermentation
FAN	Free amino nitrogen
ADA	Azodicarbonamide
DATEM	Diacetylated tartaric esters of monoglycerides
SSL	Sodium stearoyl lactylate
IARC	International Agency for Research on Cancer
NSP	Non-starch polysaccharides
MW	Molecular weight

1
General Introduction

Enzymes are the tools of nature – and using natures' own technology in modern industries is associated with many advantages. Accordingly enzymes are appreciated as the main contributors to clean industrial products and processes [1–3]. Compared to chemicals enzymes are specific in their action and work at mild conditions. Enzymes can often carry out reactions which are not even possible with conventional chemistry and thereby provide novel benefits. They are compatible with the environment i.e., they are produced from renewable resources and are fully biodegradable, and even the excess biomass from the production of enzymes is renewable as it is transferred to agricultural land as soil conditioners and fertilizers. Various industries have substituted old processes using chemicals which can cause detrimental effects on the environment and equipment with new processes using biodegradable enzymes. The number of applications in which enzymes are used are many and diverse [3, 4]. Technical enzymes represent the largest part of the market with a value of approximately US$ 1 billion in 1999 with enzymes for detergent being the largest single market for enzymes valued at around US$ 500 million [5]. The other dominating markets are baking, beverage and dairy as well as feed and paper and pulp. All these industries are traditional users of enzymes. Overall, the estimated value of the worldwide use of industrial enzymes has grown from US$ 1 billion in 1995 [6] to US$ 1.5 billion in 2000 [7].

Industrial enzymes are currently manufactured by three major suppliers, Novozymes A/S (headquartered in Denmark), Genencor International Inc. (headquartered in the US), recently acquired by Danisco A/S (headquartered in Denmark) and DSM N.V. (headquartered in the Netherlands). Novozymes A/S is the largest supplier in each of the mentioned industries with an estimated market share between 41 and 44% of the industrial enzyme market in 1999. Genencor International Inc., which operates in technical and feed segment, and DSM N.V., with focus on food and feed, had, according to the estimates of Novozymes A/S, market shares of around 21% and around 8%, respectively, that year. The rest of the market is divided among a few smaller enzyme producers, some of which produce enzymes for their own use, in the US, Canada, Europe and Japan as well as a number of local producers in China.

1.1
Introduction to Life Cycle Assessments

Like other industrial processes, the production of enzymes also consumes fossil energy, water and agricultural raw materials. So if we want to claim that enzyme technology is an advantage to the environment we need to compare the environmental load of producing enzymes with the environmental bene-

fits of using enzymes. Life cycle assessments (LCAs) allow such comparisons and are used by Novozymes to determine the magnitude of the environmental benefits provided by enzyme technology in quantitative terms.

An LCA is a methodology which enables us to compare the environmental impacts of alternative production technologies providing the same user benefit. An LCA provides a holistic view. It takes into consideration the whole life cycle of a production system, from production of raw-materials to disposal of waste. This means that an LCA study which compares enzyme technology with an alternative technology will always consider the environmental load of producing the enzymes.

Furthermore LCA studies address a wide range of environmental impacts. For each process of the production system specific data for consumption of resources and environmentally harmful emissions are collected. Based on these data the potential contribution to a number of impacts is calculated.

Novozymes LCA studies typically address the following categories of impact potentials: energy consumption, global warming, acidification, nutrient enrichment, smog formation (photochemical ozon formation). The assessments are in agreement with the ISO 14040 requirements and are based on the principles described by Wenzel et al. [8], which ensures that the comparisons are made in a standardized and transparent way. The modelling has been facilitated in SimaPro 6.0 software.

The following paragraphs in which we describe application of enzymes provide examples of the results obtained from comparing enzyme technology with conventional technology by means of LCA methodology. The results are extracted from studies that are either carried out by the Technical University of Denmark or by Novozymes, in which case they have been subject to external critical review. Normalisation of results on energy and global warming potential is based on global reference values – whereas the other impacts are normalised according to Danish reference values. Normalisation values for nutrient enrichment, acidification and smog creation are only shown for studies based on Danish or European conditions. Table 1 shows the actual normalisation references used in the LCA studies referred to in this chapter.

Table 1 Normalisation references which were used in the life cycle analysis (LCA studies)

Impact potential	Geographical coverage	Values per person Equivalent per year
Energy consumption	Europe	159 000 MJ
	World	68 200 MJ
Global warming	World	8.2 ton CO_2 equivalents
Acidification	Denmark	101 kg SO_2 equivalents
Nutrient enrichment		24.9 kg PO4 equivalents
Smog formation		20 kg ethylene equivalents

2
Application of Enzymes

2.1
Enzymes for Laundry Detergents and Automated Dishwashing

2.1.1
Laundry Detergents

The word "detergents" – originally derived from the Latin word "*detergere*" – meaning to clean/remove, represent laundry, dish wash and industrial and institutional cleaning products [9]. The first humans used fresh surface water for washing their textiles and discovered that soft water was better for cleaning due to the prevention of calcium precipitations. Later it was found that textiles were cleaned more effectively if they were trampled into the river mud and then rinsed several times. The wash method was further improved by addition of soap from oil (olive or similar) and wood ash, which served as a source of alkali for producing surface active components. In the late 1700s, new technologies for soda ash manufacturing were developed and by the end of the 1800s soap powder was popular due to convenience – one step wash. Through the sulfation of fatty alcohols, the problem with soap precipitation in hard water was reduced and the first synthetic detergent was produced around 1930 [10].

In 1913, Dr. Röhm issued a patent in which he protected the application of tryptic enzymes for washing purposes [11]. In this patent he mentioned that "*It appeared that the fabric could be cleaned in a shorter time with less exertion of strength and at a temperature far below the boiling point of water than without enzymes added. Further the fabric had a better appearance and much less soap was necessary*".

In 1958, Novo Industries A/S (today Novozymes A/S), began a research program with enzymes for washing work clothes from the meat and fish industry, which were difficult to clean by traditional methods. This work resulted in a new washing method and a more alkaline protease, Alcalase [12]. In 1963, the protease containing pre-soak product Biotex was launched in Holland and within a short time gained 20% of the Dutch market for detergents. A better compatibility of the used enzymes with the detergent systems greatly stimulated the development of other enzyme containing detergents. Today, amylases, lipases and cellulases are also commercially available and during recent year's mannanases and pectate lyases have been introduced in detergents.

The detergent enzyme market has grown strongly and was in 2000 around 0.6 billion USD (Novozymes data) with proteases as the major detergent enzyme product. Penetration of enzymes into the world market's laundry detergents is quite high with 95% in Western Europe and Japan, 70% in North America and around 50% in Latin America and Asia.

The trend in both Europe and North America during the last ~ 20 years has been towards lower wash temperatures in order to save energy, whereas Latin America and Asia have been historically low wash temperature areas. In a LCA study, the environmental impact of reducing the washing temperature was investigated [13], see also Sect. 2.1.4. By reducing wash temperatures, a strong decrease in CO_2 emissions and hence a potential beneficial contribution to reducing global warming by the reduction of electricity consumption was shown. It further appeared possible to keep the wash performance at the same level as that obtained at higher washing temperatures through the addition of small amounts of enzymes (i.e., in the mg area calculated as active enzyme), see Fig. 1. Figure 1 shows two scenarios, reducing the wash temperature from 60 °C to 40 °C and from 40 °C to 30 °C, respectively. In the 60 °C to 40 °C wash scenario, the wash performance is significantly reduced by lowering the wash temperature while the reduction is compensated by addition of mainly traditional enzymes such as proteases, amylases, and lipases. The other scenario, reducing the wash temperature from 40 °C to 30 °C, shows that the reduced wash performance at the lower temperature is significantly compensated for. Further, these results suggest that an even higher wash performance can be reached by the addition of today's optimized protease, amylase, and lipase to the detergent (please note that two different detergents have been used in the two scenarios). The addition of the enzymes itself to the detergents only give small increase to global warming as illustrated in Sect. 2.1.4.

Another ecological gain which may be realized through the addition of enzymes to the detergent is the possibility of replacing conventional chemical-based ingredients that may be harmful to the environment. This has become possible due to improved performance/cost ratio for enzymes as a result of

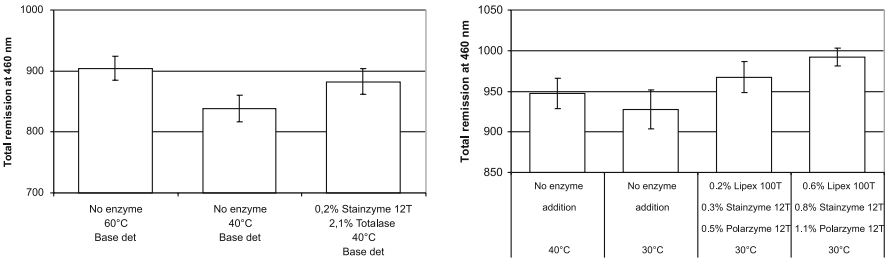

Fig. 1 Wash performance measured as the remission of the swatch after wash (the higher remission the better wash performance) on 16 stains (extended Nordic Eco-labelling test) at two different scenarios, reducing the wash temperature from 60 °C/140 °F to 40 °C/104 °F and from 40 °C/104 °F to 30 °C/86 °F, respectively. In the 60 °C/140 °F to 40 °C/104 °F scenario, an enzyme free detergent has been used whereas in the other scenario, 40 °C/104 °F to 30 °C/86 °F, a different detergent containing 0.4 wt % Savinase 8.0 T has been used

the development of more efficient enzymes, enzyme production optimization, and an industry trend toward reduced pricing [14–19].

As was mentioned in the historical section above, soap from oil belongs to the group surfactants (surface active agents) and the wood ash belongs to the group alkali, a prototype of builders. The most common surfactants used today are the anionic linear alkyl aryl sulfonates (LAS) and the nonionic alcohol ethoxylates. The most common builder has been triphosphate which, due to concern over its negative effects on the environment (e.g., eutrophication of lakes and rivers), has mainly been replaced with zeolites and polycarboxylate polymers. Other typical components in a detergent are bleaches, today typically sodium percarbonate, soil release polymers, dye transfer inhibitors, fluorescent whitening agents and perfumes.

Most of these detergent components can negatively impact the stability and efficacy of enzymes in the detergent by interacting with the whole protein structure or with the active site only. The enzyme activity will mainly be influenced negatively in the detergent by autodigestion/proteolysis, denaturation and by chemical modification. Autodigestion or proteolysis occurs when proteases either degrade other protease molecules or degrade other enzyme types added to the detergent. This effect can be reduced by keeping the detergent storage temperature low and by sterically blocking the catalytic centre of the protease molecule. Denaturation can be influenced by selection of a more stable wild-type and/or protein engineering. Chemical modification can be prevented by replacement of sensitive methionines present in the amino acid sequence of the enzyme with more resistant amino acids in the enzyme molecule, adjust the ratio between nonionic and anionic surfactants, and by optimization of the pH. For powder detergents, inert granulation material protects the enzyme from attack by other detergent ingredients during storage, and for liquid detergents, enzymes may be protected by proper formulation of the detergent as noted above and by encapsulation. Often reversible protease inhibitors such as boric acid and boronic acid derivatives are added to the liquid formulation as reversible protease inhibitors. This can prevent protease action in the undiluted, concentrated liquid detergent formulation, but will be dissociated from the enzyme when the detergent is added to the wash, thereby allowing it to carry out its task of breaking down protein soils [17, 20].

To be well suited for use in a typical detergent today, enzymes should have the following properties [21]:

- Alkaline pH optimum
- Efficacy at low wash temperatures of 20–40 °C
- Stability at wash temperatures up to 60 °C
- Stability in the presence of other detergent ingredients
- Specificity broad enough to enable the degradation of a whole class of molecules

Since the substrates for detergent enzymes are typically situated at the solution/textile interface, enzymes must first adsorb to the textile surface in order to be able to degrade the substrates by a hydrolytic reaction. Enzyme adsorption is a complex process which can be characterized by various aspects such as the kinetic rate, type of binding, adsorbed amount, and structure of the adsorbed layer and of the individual molecules therein. The tendency of the enzymes to accumulate at the interface is determined not only by the properties of the enzyme molecules and the surface but also by the nature of the solvent, the presence of other solutes (e.g., surfactants and surface-substantive polymers), pH, ionic strength and temperature.

Consumer tests, e.g., a test in which consumers use their own washing machines on their own laundry and soil to evaluate a new detergent product – are conducted to monitor if the people will really recognise the benefits of a new detergent component. Since a large number of consumers have to participate in such a test to make the results statistically meaningful, such tests are typically expensive.

A somewhat simplified evaluation is to use real soiled consumer items in commercial washing machines in special test laundries under controlled conditions. In order to reduce the variation, fresh stains can be used instead of the real items and also technical or aged stains can be introduced. To simplify further and to simulate typical machine-washing conditions in laboratory scale, it is common to use either a Launder-O-meter (LOM), which simulates typical European household washing machines or a Terg-O-tometer (TOM), which simulates vertical drum washing machines (common in USA etc).

The removal of stains as described below is the most direct cleaning effect of detergent enzymes. However, various kinds of dirt (e.g., particulate soils) may also adhere to the textile surfaces via binding to residues of proteinaceous, carbohydrate or fatty material. In cases of anchored dirt in the laundry, an enzyme may assist in removing the dirt even though it does not attack it directly through breaking down the underlying proteinaceous, carbohydrate, or fatty layer. Detergent enzymes can also have an antiredeposition effect, i.e., prevention of redeposition of dirt and colorants by degradation of sticky residues that in thin layers may cover the textile surfaces.

2.1.2 Automatic Dishwash Detergents

The first automatic dishwashing detergents (ADDs) contained almost 100% sodium tripolyphosphates (triphosphates) [22, 23]. Until the end of the 1980s, sodium tripolyphosphates remained the main ingredient but chlorine-based bleaching agents such as sodium trichloroisocyanurate were added in order to remove bleachable stains like tea and with the highly alkaline sodium metasilicate. Due to the high alkalinity, these ADDs remove very efficiently

most types of soils that swell at high pH. This effect drops drastically if the pH value of the cleaning solution falls below about pH 11.5.

In order to produce more environmentally-friendly ADDs, some manufacturers have opted to reduce the pH and bleach content of their detergents. The observed loss in cleaning performance can be partially or completely compensated by incorporating enzymes, α-amylases and proteases, into products of reduced alkalinity and without chlorine bleach. The use of enzymes in these products can even lead to an improved performance on specific soils (e.g., dried-on starch). Furthermore, the movement to less aggressive and more environmentally friendly ingredients like disilicate, triphosphate (now reduced amount) or citrate, phosphonates, polycarboxylates, non-ionic surfactants, a hydrogen peroxide source (such as sodium percarbonate), bleach activator and enzymes have even made it possible to reduce the temperature from 65 °C to today about 50 °C to 40 °C, thereby further helping to save on energy costs for water heating.

With the help of a dishwasher the washing up can be done easier and even more economically than by hand. The amount of water necessary for a cleaning program in an automatic dishwasher has been drastically reduced in the last 10–15 years from 45 L to about 13–15 L. In the cleaning cycle, water consumption has been reduced from about 10 L to about 4 L

In the United States, the use of automatic dishwashers is very common, with far more than 50% of the households owning one today. In Europe, dishwasher penetration differs dramatically from country to country. For example, in Northern Europe, the penetration is quite high at approximately 60% in Norway and Sweden. In countries like Switzerland, Germany and Austria more than 40% of households own an automatic dishwasher. However, in southern and Eastern Europe the penetration is still quite low. Thus, substantial growth has been observed, especially in the last mentioned regions.

Today in Europe, German standard methods are mainly used for evaluating dishwasher detergents. In the US, other methods are used. As the ADDs become more efficient the test methods sometimes have difficulties when discriminating between the detergents. Therefore the methods are often optimized locally and a wide number evaluation versions can often be found.

2.1.3 Overview and Latest Developments

2.1.3.1 Proteases

Proteases have been used in household detergents for more than 40 years. They are considered as a basic ingredient and are present in around 75% of the detergents world-wide. Proteases are hydrolases that catalyse the breakdown of proteins and thus enhance the cleaning of protein-based soils

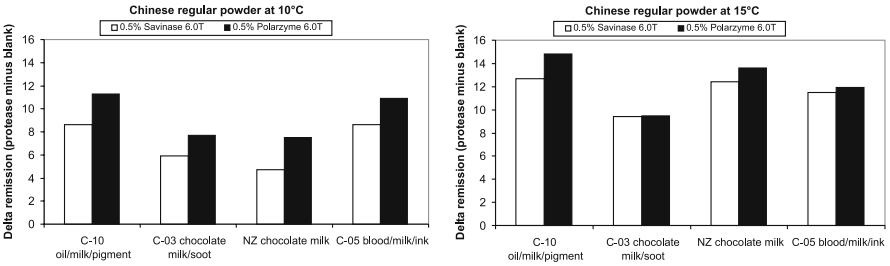

Fig. 2 Wash performance measured as the difference in remission for protease treated swatches versus swatches washed without protease of Polarzyme or Savinase in Asian wash machines using a Chinese regular powder at 10 °C/50 °F (*left*) and 15 °C/59 °F (*right*), respectively

as blood, grass, spinach, milk, milk-containing products (e.g., cocoa), egg and meat-containing soils (e.g., gravy). Proteases serve a multifunctional role in the overall cleaning process. By degradation of proteins into minor more dispersible fragments, proteases boost the performance of surfactants [24]. In addition, proteases provide whiteness benefits as illustrated in [18] and [24].

All currently used detergents proteases belong to the class of serine proteases originating from the *Bacillus* species. Intensive protein engineering programs have resulted in a broad range of commercial protease products [9, 10, 17, 22, 25–27]. In order to suit the different wash conditions and the diverse detergent products that enter the market, these protease products mainly differ in 1) their temperature or pH optima, 2) dependence on Ca and Mg ion concentration for stability and 3) compatibility with other detergent components as the bleaching system and surfactants. Examples of these are the development of proteases with improved storage stability in bleach-containing powder detergents. This was accomplished by replacing a bleach-sensitive methionine with amino acids not sensitive toward oxidation [16, 28]. A protease with improved performance on egg-stain removal was recently launched for automated dishwashers. Changes in amino acids have improved the resistance to inactivation by the ovoinhibitors contained in the egg [22, 29]. Proteases more active at lower wash temperature have also been introduced to the market [10, 30]. In Fig. 2, the performance of a recently introduced low temperature protease, Polarzyme®, is compared to one of the traditional proteases, Savinase®, at 10 °C and 15 °C respectively demonstrating more superiority in performance by Polarzyme, at the lowest wash temperature. The increased flexibility of the protease molecule resulting in higher activity at lower temperatures often leads to a decrease in stability of the molecule. There is, however, an increasing number of examples showing that the structural features involved in stability or activity can be very different and act independently [31, 32].

2.1.3.2 Amylases

Alpha-amylases have been used in detergents since the beginning of the 1970s and the demand is still increasing. One reason for the increasing demand could be that many food products today, such as chocolate pudding, ketchup, spaghetti sauce, chilli sauce, fruit puree and baby food, contain starch in different forms (modified starches) in order to get, among other things, appropriate viscosity.

Removal of starch from textile surfaces is also important since starch can form a film on the textile that can result in an increased pick-up of particulate soil after washing [33]. As a result, white laundry items turn increasingly grey after repeated wash cycles. Among the starch hydrolysing enzymes, α-amylases are responsible for the solubilization of starch. Starch consists of two glucose polymers, amylose, which is exclusively α-1-4 linked and amylopectin which in addition to the α-1-4 linkages found in amylose also contains α-1-6 branch points. The α-amylases catalyze the hydrolysis of internal α-1-4 glycosidic linkages and are thus ideally suited to cutting a starch polymer into smaller fragments, reducing the viscosity and increasing the solubility of attached starch.

The detergent α-amylases in use today derive mainly from the *Bacillus* family, have highly homologous primary amino acid sequences and a tertiary structure comprised of three domains A, B and C [17]. The active site is typically located in a cleft between domains A and B and is usually comprised of acidic amino acids such as aspartic and glutamic acids. Like many proteases, most α-amylases contain essential calcium ions, which are important in maintaining the tertiary structure of the enzyme molecule.

In 2004, a new amylase, Stainzyme®, was introduced which shows a significantly stronger performance effect in most detergent segments compared to other traditional commercial amylases. This enzyme has a broader pH and

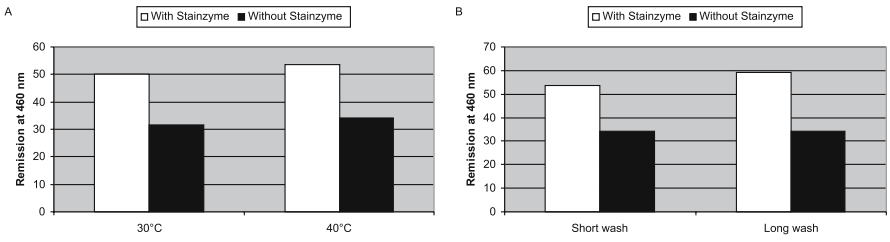

Fig. 3 Wash performance measured as average remission of the swatches after wash (the higher remission the better wash performance) on six different technical, aged stains at **A** two different wash temperatures 30 °C/86 °F and 40 °C/104 °F, respectively (short washing time) and at **B** short and long washing time (40 °C/104 °F). The trial was conducted at typical European wash conditions

temperature range as compared to earlier amylases, meaning that in a typical detergent, for example, nearly equal performance is obtained at both 30 °C as at 40 °C, see Fig. 3A. Furthermore, the washing time can also be reduced but still nearly the same wash performance will be reached, as shown in Fig. 3B.

2.1.3.3 Lipases

Lipases are glycerol ester hydrolases. These enzymes act on the carboxyl ester bonds of triacylglycerols – the "triglycerides" of fats and oils – liberating carboxylic acids and glycerol. Relevant soils present on laundry items include fatty stains from food sources and the triglyceride components of sebum [9, 16, 22].

Effective removal of these triglycerides during washing is often difficult to achieve, and this difficulty becomes more and more obvious as the wash temperature is decreased (in particular when the washing is done at temperature below the melting point of the various fatty substances). The lipase catalysed hydrolysis of these hydrophobic fats and/or oils produces compounds that have lower melting points and are more readily dispersible at low washing temperatures. Thus lipases provide a particularly relevant approach to one of the goals of "white biotechnology" – the reduction of energy consumption – by contributing to good washing performance at lower temperatures.

Mechanistically, the action of lipases are often dominated by strong surface or interfacial effects. For some lipases, the reaction involves a two step process. First the enzyme is absorbed onto the hydrophobic lipid surface; this causes conformational changes around the active site of the enzyme, which sometimes can be identified in terms of "lid opening". Then, in this absorbed and activated form, the enzyme can catalyse the hydrolysis reactions via traditional enzyme-substrate complexes.

Microbial lipases were first introduced in household detergents in 1988. A characteristic of these early "first generation" lipases was a relatively small performance benefit during the wash itself. Much of the enzyme benefit was actually first achieved during the drying step – and thus was only detected after the following wash cycle.

Since then considerable progress has been made, mainly in terms of providing lipases with stronger "first wash" effects. Protein engineering has been extensively employed to achieve these improvements. In addition some complex questions related to surfactant compatibility have been understood to a greater extent. Figure 4 shows comparisons between the earlier lipases and a current commercial variant Lipex – which is a lipase which does provide strong benefits already in the first wash.

As with several of the other classes of detergent enzyme discussed in this section, the benefits from lipase use are not restricted to the primary stain

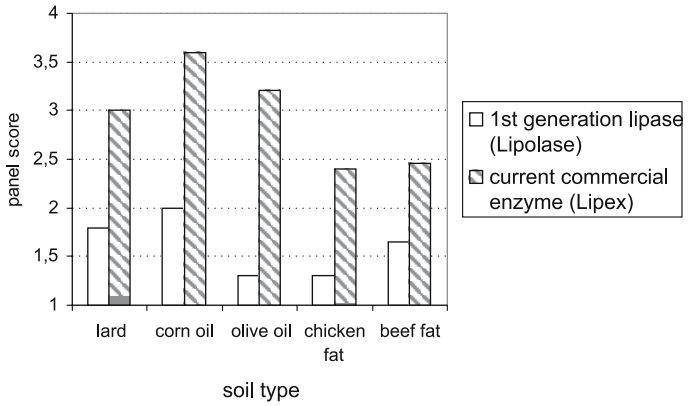

Fig. 4 Lipases now provide first wash benefits on a range of soils. Comparison of single wash performance of the first generation Lipolase and the current Lipex, both dosed at 750 LU per liter using panel score units (maximum score is 5). Test fabric: Cotton t-shirt with greasy stains. Wash conditions: American washing machine, powder detergent, 30 °C/86 °F wash

removal improvement. Lipid soil has a strong tendency to redeposit onto fabrics during the wash. Since the oily soil is frequently strongly colored by oil-soluble chromophores, the result of such reposition can be highly undesirable. Furthermore, because of the hydrophobicity of the lipid soil, it will deposit primarily onto the more hydrophobic fabrics in the wash load – normally the polyesters. This can result in relatively high soil intensities on restricted areas of the wash load. By incorporating an effective lipase in the detergent system the redeposition can be very efficiently prevented.

2.1.3.4 Cellulases

Several types of cellulases are used in laundry detergents. Cellulases can both boost cleaning performance and provide fabric care benefits [9, 16, 22].

Cleaning performance improvement is due to removal of cellulosic molecular fragments; polymeric glucans increase binding of colored soils onto the cellulose fabrics. The fabric care effects, on the other hand, are related to removal of larger cellulose fiber fragments, i.e., fragments which are large enough to be seen by eye and which influence the color shade of the fabric and/or are visible as surface fluff or pilling. These fiber fragments give the fabric a worn, aged appearance and their removal results in a "rejuvenation effect". Today the cellulases are the only class of enzymes that can provide such fabric rejuvenation benefits.

The term "cellulases" covers several classes of enzymes that, collectively, are involved with hydrolysis of cellulose. Cellulose is a linear polymer of glu-

cose, in which the individual units are all joined by beta-1,4-glucosidic bonds. The two most important classes of cellulases are the exo-acting "cellobiohydrolases" and the "endo-glucanases". In detergent applications, only the endo-glucanases (enzymes that hydrolyse in the mid of the cellulose chain and which do not result in formation of low molecular weight, soluble sugars) are able to provide the required performance enhancements.

Some fungal endo-glucanases are particularly effective at removing the visible fuzz or "pilling" from cotton fabrics during washing, thus providing the fabric rejuvenation effect. A characteristic of these "anti-pilling" enzymes is their strong affinity for insoluble cotton fibers. This is due to the presence in the enzyme molecule of a specialised polypeptide region known as the Cellulose Binding Module. The performance of these enzymes depends strongly on the characteristics of the CBM. On the other hand, the soil release and cleaning effects of cellulases do not depend on strong enzyme binding onto insoluble cellulose. Thus low affinity endo-glucanases – including some without any CBM – can be used for this purpose. The choice is based on the activity and compatibility with the detergent formulations.

2.1.3.5 Mannanases

A new enzyme class was introduced into household detergents in 2000 by inclusion of a mannanase [22, 34]. Mannanases degrade galactomannan by cleavage of the β-1.4 links between the mannose units. Galactomannan is the main constituent in guar gum and locust bean gum, which are used commonly as formulation aids in foods e.g., ice-creams, barbecue sauces, soups, dressings and drink products as well as in personal care products. The inclusion of a mannanase in a detergent provides stain removal effects on the above-mentioned stain types as well as whiteness maintenance effect by preventing reappearing stains as demonstrated in [18] and [35].

2.1.3.6 Pectate Lyases

The most recent introduction of a new enzyme class into laundry detergents is the addition of a pectate lyase in 2003. Pectate lyases degrade pectins by cutting poly-galacturonic acid with β-elimination forming a double bond. The effect of pectate lyases in detergents are removal of fruit and vegetable stains as they have pectin as a natural constituent and removal of food stains where pectin is added as a thickener e.g., marmalades [16, 34, 36]. In addition, whiteness can also be maintained with this as with several of the other enzyme classes used in household detergents. An example of the effect of adding a typical dosage level of pectate lyase to an European liquid detergent is shown in Fig. 5.

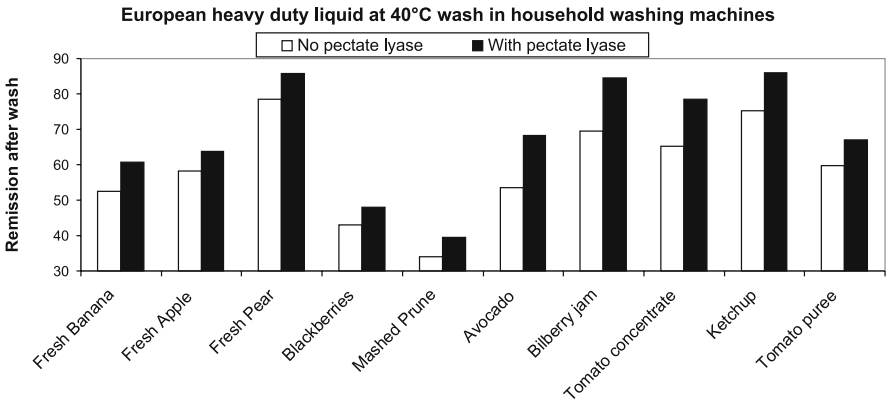

Fig. 5 Wash performance measured as remission of the swatches after wash (the higher remission the better wash performance) of an EU liquid detergent either without or with addition of pectate lyase

2.1.3.7 Oxidoreductases

In the recent years, many patents concerning different types of oxidoreductases have been found in the literature although no commercial product has appeared. Examples of these are peroxidases, laccases, phenol oxidases, haloperoxidases, lipoxygenases, choline oxidases, glucose oxidases. In a laundry context, oxidoreductases may provide effects as bleaching of stains, dye transfer inhibition and disinfection [9, 16, 18]. An enzymatic bleaching system is attractive due to the possibility of usage also in liquid detergent formulations (current bleaching systems can only be used in powders), their possible better performance compared to current bleaching systems at lower wash temperatures and the disinfection potential that increases in importance due to the move towards lower wash temperatures. The environmental benefits of an enzymatic bleaching system are the increased possibility of washing at lower temperatures and a much lower impact on the environment than current bleaching systems. Despite these benefits, no oxidoreductase enzymes have been incorporated into laundry detergents to date.

2.1.4 Environmental Benefits of Enzymes in Detergents

The typical benefits of addition of enzymes to detergents are cleaner clothes, more environmental friendly detergent formulations in combination with energy savings due to lower washing temperatures. The development of new

improved detergents has been going on for many years and has been driven by development of new efficient enzymes as well as of improved surfactants. This co-development makes it difficult to quantitative the environmental benefits of the enzyme technology alone. Table 5 shows LCA on enzymes for textile production.

An LCA study [13] based on the principles described by Wenzel [36] and conducted according to the principles within the ISO 14040-series on LCA has been carried out to analyse the consequences of reducing laundry wash temperature in terms of wash performance and in terms of environmental implications due to increased enzyme application and reduced electricity requirement for the wash process. The wash performance evaluation and environmental assessment refer to Danish average conditions.

Two scenarios were addressed, reducing the wash temperature from 60 °C to 40 °C with an enzyme-free detergent and reducing the wash temperature from 40 °C to 30 °C with a detergent containing a traditional protease. Table 2 shows the yearly reduction of potential environmental impact with reducing the wash temperature from 40 °C to 30 °C. In order to get a better understanding of the values, the actual reductions are calculated to number of people equivalent which indicates the number of people generating the same environmental load annually on average. "CO_2 equivalents" is a standard measure for potential contribution to global warming, calculated as a sum of contributions from various gasses emitted from the considered processes (CO_2, CH_4, N_2O etc.) taking into account their individual strength as greenhouse gases.

By reducing the wash temperature, a strong decrease in CO_2 emission and hence potential contribution to global warming by the reduction of electricity consumption, was shown, see Fig. 6, while it was possible to keep the wash performance at the same level by the addition of enzymes as illustrated in Fig. 1 in the introduction Part 2.1.2.1. The addition of the enzymes itself to the

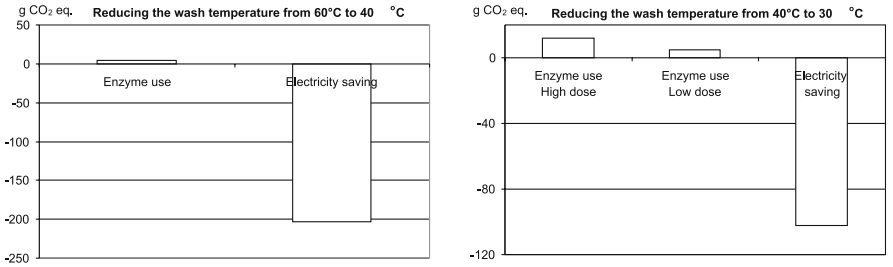

Fig. 6 Reduction of electricity consumption, calculated as g CO_2 equivalents, by decreasing the wash temperature from 60 °C/140 °F to 40 °C/104 °F and 40 °C/104 °F to 30 °C/86 °F respectively, and the contribution from the enzymes when added to the detergent in order to compensate for the lower wash performance at the lower wash temperatures

Table 2 The yearly reduction of potential environmental impact per 5 million people by reducing the washing temperature from 40 °C/104 °F to 30 °C/86 °F (Danish average conditions). PE (People Equivalent) indicates the number of people generating the same environmental load annually on average. "CO_2 equivalents" is a standard measure for potential contribution to global warming, calculated as a sum of contributions from various gasses emitted from the considered processes (CO_2, CH_4, N_2O etc.) taking into account their individual strength as greenhouse gases

Reduction of potential environmental impact per 5 million Danes (annual basis – 40 °C washes)	Energy consumption	Global warming	Acidification	Nutrient Enrichment	Smog formation
Actual reductions	550 million MJ	42 000 ton CO_2 equiv.	35 ton SO_2 equiv.	4.2 ton PO_4 equiv.	3.1 ton C_2H_2 equiv.
Number of people equivalents (PE)	8100 PE	5100 PE	350 PE	170 PE	150 PE

detergents only give a small contribution to global warming (Fig. 6) because the quantity applied is very small (~ 1 g enzyme product per wash or only mg active enzyme per wash).

The environmental profile of detergents has also strongly been improved during the last 10–15 years due to the introduction of compact detergents in the early 1990s containing less of ingredients that are harmful to the environment due to slow biodegradability and/or toxicity. In order to increase the compactness, products like enzymes with high weight-efficiency, to do more with less, had to be developed [15]. Successful partial replacement of surfactant or builder with protease is described in [14].

These results illustrate that the recent developments in detergent technology offer significant opportunities for society to save energy and reduce the environmental load of washing. For the reduction of energy consumption due to reduced wash temperatures the main potential is, however, only realised if consumers are willing to accept low temperature washing.

2.2 Enzymes for Production of Textiles

2.2.1 Introduction

The textile industry was one of the first to benefit from targeted use of biotechnology. The use of malt extracts for starch degradation was known already in the 1830s and commercial products based on malt extracts were in use by the early 1900s for removing starch size from woven fabrics. The first

commercial enzyme produced in 1952 by microbial fermentation was an amylase, used for textile desizing. Denim finishing with cellulases emerged in the 1980s, radically changing the conventional process by reducing the need for pumice stones, and creating the largest market segment for enzyme technology in textiles.

World demand for manufactured fibers is forecast to increase 5.4% per year to 44.3 million metric tons in 2005, where an average of US$ 275 worth of process chemicals are used per ton of fiber [38]. About 40% of the chemical value is in the dyes and colorants, with the remaining value divided among emulsion polymers, surfactants, silicones, gums, starches, solvents, oils, waxes, and enzymes. Biocompatible enzyme technology supports the overall industry trend toward shorter process time (leading to higher productivity) and milder process conditions, as well as delivering cost-effective innovation and providing compliance with regulations. Table 3 shows the main enzymes used in commercial textile processing.

2.2.2
Current Applications of Enzymes for Textile Processing

Enzymes facilitate the removal of natural or applied impurities and help modify the physical properties of textiles (Table 3). Enzymes are applied during the preparation, dyeing, and finishing ("wet process") stages of textile production, during which the fibers are exposed to water. Many conventional wet process steps involve the use of high concentrations of harsh chemicals, such as caustic soda. Although these chemicals are broad acting, effective, and low cost, when the energy, water consumption, and resulting fiber properties for the whole process are considered, the more selective enzymatic processes can and do offer significant advantages (Table 4). In some cases, enzymes are able to provide benefits that are not even possible with conventional chemistry (e.g., biopolishing, which can lead to higher value end products), though usually it is savings in total process cost that drive the use of textile enzyme technology.

2.2.2.1
Cotton Preparation and Processing

Cotton is the dominant natural fiber used for apparel and home furnishings. Cotton fibers are individual cells that grow outward from the cotton seed. The outer primary cell wall contains waxes and hemicellulosics that give cotton a hydrophobic surface. The interior of the fiber is almost pure cellulose. Harvested fibers are mechanically cleaned to remove plant debris, then spun into yarns and made into fabrics. During fabric manufacture, the yarns may be coated with a sizing agent to provide strength on weaving machines. These dry process steps result in greige fabrics that are stiff, hydrophobic, and may still contain small fragments of plant matter. The goal of subsequent desizing,

Table 3 Main enzymes used in commercial textile processing

Target fiber	Process stage	Main enzyme	Typical enzyme Treatment conditions, pH/T/t[a]
Cotton	Scour	Pectinase	pH 8–9/45–65 °C/10–30 min
	Bleach	Glucose oxidase[b]	pH 5–7/30–50 °C/1–4 h
	Depilling and softening	Cellulase	pH 5–8/30–60 °C/30–60 min
	Denim abrasion		
Lyocell	Defibrillation		
Flax	Retting	Pectinase[b]	pH 5–9/40–50 °C/4–24 h
Ramie	Preparation/degumming	Pectinase/xylanase	pH 8–9/50–60 °C/1–2 h
Wool	Scour	Lipase	pH 7–9/20–70 °C/10–30 min
	Softening	Protease	pH 7–9/40–50 °C/30–60 min
Silk	Degumming	Protease	pH 8–9/50–60 °C/0.5–2 h
Polyester	Oligomer removal	Cutinase[b]	pH 7–10/40–80 °C/0.5–10 h (sometimes > 300 h)
	Surface modification		
Target auxiliary	**Process stage**	**Main enzyme**	**Typical enzyme Treatment conditions, pH/T/t[a]**
Starch	Desize	Amylase	pH 5–10/20–115 °C/0.2–16 h
Tallow	Desize	Lipase	pH 7–9/20–70 °C/10–30 min
Peroxide	Bleach clean-up	Catalase	pH 6–7/30–60 °C/10–20 min
Dye	Reactive dye rinse	Peroxidase	pH 6–9/40–80 °C/10–30 min
	Denim decolorization	Laccase	pH 4–6/60–70 °C/15–30 min

[a] T = temperature; t = time
[b] Some evidence of (pre-)commercial application

scouring, and bleaching wet process steps is to make the fabric uniform and wettable, with minimal imperfections and the required degree of whiteness prior to dyeing and finishing [39]. Table 3 shows the commercial benefits of bioscouring with enzymes.

By volume, half to three quarters of textile sizing agents used are based on starch. Amylase degradation of the α-1,4-glucose linkages facilitates removal of starch sizes. This enzymatic desizing can be carried out in all relevant process configurations and equipment (e.g., cold-pad-batch, continuous pad-steam) due to the broad range of commercially available amylases [40, 41]. Although not a major application, lipases can assist desizing when animal fats (tallow) are present.

Conventional scouring uses high concentrations of sodium hydroxide and surfactant to remove the waxes and hemicelluloses in the primary cell wall layer of cotton. Both cellulases and certain types of pectinases are able to give

Table 4 Commercial benefits of bioscouring with enzymes

Case study	Jinbao Mill [49]	Korean NCPC study [48]
Type of goods	Linen and blends	Dark shade cotton knit
Process comparison	Enzymatic preparation versus conventional desize-scour-bleach	Enzymatic scouring versus conventional alkaline scour
Equipment	Pad-batch (existing equipment)	Jet (existing equipment)
Enzyme type	Amylase and pectate lyase	Pectate lyase
Quality and versatility benefits	• Flexible incubation time (2–48 h) without fabric damage. • Time, water,and energy savings and reduced fabric shrinkage due to fewer process steps. • Improved side-to-side and end-to-end uniformity of dyeing. • Softer fabric handle and smoother appearance. • Improved operator working conditions. • Effluent recycling is possible.	• 8–10 tons of wash water saved per ton fabric production. • Higher fabric strength and softer fabric feel. • Less weight reduction. • Safer and easier handling for operators. • Water pollution reduction by 50% for total organic pollution, 80% for total dissolved solids, 50% for effluent color, and 3–4 unit process pH reduction.
Cost benefits	Cost of pre-treatment reduced to about 50%, with chemical costs halved and savings in energy. Production capacity increased by about 30% due to fewer process steps.	4% savings in total production costs.
Summary	The mill has changed over to enzymatic preparation process due to major benefits achieved in cost savings and improved quality.	A mill participating in the study decided to use enzymatic scouring for 30% of their production.

a scouring effect [42, 43]. A particularly effective pectinase (alkaline pectate lyase) was discovered by observing that selective degradation of a small component in the primary cell wall was key to providing efficient wax removal, and the needed wettability [44–46]. Commercial acceptance of this bioscouring technology is increasing due to demonstrated cost benefits (Table 4). The selectivity of this enzyme technology also results in reduced environmental load [48, 49].

About 70% of cotton fabrics go through the last preparation stage, which is bleaching. In this step it is important to obtain a high whiteness for full white and light shade fabrics. Conventional bleaching is carried out with alkaline hydrogen peroxide. Although experimental evidence shows that enzymatic bleaching with glucose oxidase (using glucose, starch, or cellulose as the sub-

strate) to produce in situ peroxide can lead to desired fabric whiteness [50], finding a truly robust and cost effective enzyme-based bleaching system is still needed. Bleach activator chemistry [51] in combination with enzyme treatment may offer some possibilities, though reducing the bleaching pH will be important.

For a portion of woven fabric production, the final stage of cotton preparation is mercerization. Very high (20–25%) concentrations of caustic soda are used to alter the crystal morphology of cotton, giving a rounder cross-section and more lustrous, easily dyeable fiber. As yet there is no enzymatic solution to deliver this effect.

Biopolishing is a treatment in which cellulase is used to remove small fuzz and fibrils from the surface of the fabric. This versatile treatment can be carried out as a separate step or in combination with other enzymatic preparation processes on cellulose-containing (cotton, viscose rayon, lyocell) yarns, fabrics and garments to reduce the pilling (or fibrillation) tendency of the fabric and provide a softer feel [52, 53]. Frequently, improvements in dye uptake are also observed. Enzyme inactivation is needed after treatment with cellulase to stop the hydrolysis before damage occurs. Typical inactivation is carried out by raising the liquor pH to 10 with sodium carbonate and heating at 80 °C for 10 minutes. The cotton textile industry trend is to increase productivity, and reduce costs, often by seeking reductions in the number of process steps. Step-reductions give the industry reduced energy, labor and waste discharge costs. With rapid advances in enzyme technology, it is increasingly possible to create mixtures of key enzyme activities to achieve one-bath processing (e.g., amylase and pectinase combined to give one-step desize and scour). The ultimate goal of this effort is to develop a one-bath process for full cotton preparation and dyeing.

2.2.2.2 Denim Processing

Cotton denim constitutes a large sub-segment of the cotton processing industry. This segment is fashion-driven and cost competitive. The introduction of cellulases in the 1980s truly revolutionized denim garment processing [54, 55]. The original stonewashing technology relied on tumbling garments with pumice stones to give the characteristic salt-and-pepper blue appearance of denim blue jeans. Cellulases, which hydrolyze the β-1,4-linkages in cellulose, were found to give similar abrasion effects (Fig. 1). Whereas processing with stones is highly labor intensive and damaging to equipment, using formulated cellulases at dose levels around 1–3% on weight of fabric reduces the denim to stone ratio from 1 : 1 to 1 : 0.25 or less. Although concerns about garment strength of cellulase-treated denim are sometimes raised, a recent multiple wash home laundry study demonstrated that the physical properties of stonewashed and enzyme washed denim jeans are similar [56]. Many

Fig. 7 Enzyme treatments in denim process: (*left*) amylase desized, (*center*) cellulase abrasion, and (*right*) laccase/mediator decolorization

different cellulases are known [57] and product formulations are continuously developed for the denim segment as fashion and production preferences change.

A more recent development in denim finishing came with the introduction of laccase for indigo decolorization [58]. This technology uses a chemo-enzymatic approach to selectively remove indigo dye from denim fabric leading to a fabric-safe decolorized or "washed-down" look (Fig. 7). The treatment can also be used to minimize transfer of indigo to the white fibers (i.e., prevent backstaining), leading to a better contrast fabric pattern.

2.2.2.3 Polyester Treatment

To date, greater than 95% of all work on enzymes for textiles has focused on natural fibers, especially cotton. The main reason is that synthetic fibers are not natural substrates for "wild type" enzymes. Many new petrochemical-based fiber types were developed in the period from 1940–1980 [59]. Although manmade fibers have the right chemical functionality (e.g., ester function in polyester or amide function in nylon), the monomer composition and morphology of synthetic substrates is significantly different from native substrates. Nevertheless, with polyester fiber commanding 50% of global fiber production, there is a clear interest in finding ways to apply enzyme technology in this segment. Beneficial effects have been demonstrated in the lab, including oligomer removal [60, 61], surface modification [62], and biopolishing [63, 64], though typically with long treatment times. The current challenge is to make treatments cost-effective. As historically has been the

case, a combination of enzyme improvements and process modifications will hold the key to success.

2.2.2.4 Bast Fiber Processing

Even before the special catalytic role of enzymes was understood, textiles were treated with microorganisms to improve properties. Flax dew-retting is the oldest of these microbial processes, and, after millennia, is still in use today. During dew-retting flax fiber is separated from non-fibrous tissues in the stems by the action of enzymes from indigenous soil fungi. Dew-retting produces high quality fiber used for linen, but the process is limited to certain geographic climates and requires several weeks idle incubation time on agricultural fields [65]. Alternative methods such as water-retting, chemical retting, and steam explosion have been tested, but with limited commercial success.

For at least a decade, it has been known that pectinases from dew-retting organisms are essential for effective retting. However, early enzyme products included side activities, such as cellulase, that caused excessive fiber strength loss. In a variation on the batch-wise water-retting process, a new spray enzyme retting treatment, involving crimping fiber flax followed by spraying or brief immersion with a selection of pectinase-containing enzyme preparations and chelators (e.g., EDTA), gave effective retting for a short length grade of fiber [66]. When properly controlled good fiber yield and strength are obtained [67]. Pectinases have also been used successfully during treatment of long fiber flax during linen processing to reduce costs and water pollution [68]. A combination of xylanase and pectate lyase was combined with alkaline chemical treatment steps to achieve industry relevant ramie degumming, with residual gum levels as low as 1.5% [69].

2.2.2.5 Wool and Silk Processing

Silk and wool are both protein-based fibers, easily damaged by alkaline treatment. Silk fiber (fibroin) is composed of polypeptides with a high glycine and alanine content that form hydrogen-bonded antiparallel β-pleated sheet morphology. The amino acid composition of wool fiber is much more mixed, also including a large number of cystine units that contribute stability to the α-helical morphology through disulfide bonds [70]. During silk degumming, protease enzymes mildly remove the sticky protective surface protein (sericin) prior to further processing. Proteases are also used to give a "peach-skin" type effect on silk fabric or are used to soften wool fibers to improve comfort [71].

Proteases have been tested to improve the shrink-resistance of wool as an alternative to the conventional chlorination processes, which cannot be used in some regions due to environmental concerns. In one process, an alkaline

peroxide pretreatment bath containing several chemical auxiliaries followed by a protease treatment in the presence of sodium sulfite gave very good shrinkage control (< 2%) with soft fabric texture and minimal strength and weight loss [72]. Another method used steric-hindered PEG-modified protease to treat wool, giving some shrinkage improvement [73]. A number of other enzymes have also been tested for their ability to give cross-linking effects on wool, such as transglutaminases [74], tyrosinase and peroxidase [75], and laccase [76], though further work is needed to give commercially relevant effects. The selectivity and mildness of enzyme treatments allows wool and silk to be processed with other fibers, like cotton and ramie, in blends.

2.2.2.6 Dyeing and Finishing

Fabrics are often bleached with hydrogen peroxide prior to dyeing and finishing. Residual hydrogen peroxide must be removed to obtain the most efficient dyeing. Repeated water washes or chemical reducing agents are traditionally used, but now it is common practice to apply catalase enzymes which decompose hydrogen peroxide to oxygen and water. Significant process savings are possible because the treatment is fast, mild, and dyeing can be carried out in the same liquor and equipment as the catalase treatment. In one study at an Egyptian textile mill, measured benefits were 24% reduction in energy consumption, 50% reduction in water consumption, and 33% reduction in processing time [77]. This treatment also quickly stops the bleaching reaction to prevent fiber damage.

Several enzymatic techniques for dyeing based on use of the redox enzymes laccase and peroxidase have been tested, but none are yet commercial. They include mild re-oxidation of vat and sulfur dyes [78], dye discharge printing [79], and (in situ) dye synthesis [80, 81]. Work with laccase has demonstrated the technical possibility of producing trichromatic dyes for wool [82]. The removal of reactive dyes using a chemo-enzymatic peroxidase-based technology has been possible in commercial systems [83]. In this case, the selective removal of unfixed dyestuff reduces the number of rinse cycles needed after dyeing. Variations on this technology have also been demonstrated for disperse dye after-clearing using laccases [84] or esterases [85] and using immobilized peroxidase to treat textile dye effluent [86].

2.2.2.7 Textile Process Water

Over 10 000 dyes with a total annual production in excess of 7×10^5 metric tons worldwide are commercially available, and typically 5–10% of this amount is discharged in industrial effluents [87]. Dyestuffs present in textile industry wastewater can cause problems in treatment plants since these

compounds are hard to degrade by biological means. This means that even after waste treatment, dye color can remain in the water, as well as increased chemical oxygen demand and potential toxic effects.

Broad evidence shows that enzymes secreted by different white-rot fungi are capable of decolorizing a range of textile effluents, though some dyestuffs are clearly more susceptible than others and a chemical mediator is sometimes needed together with the enzyme [88, 89]. The decolorization rate of white-rot fungi was observed to be related to their production of extracellular lignin degrading enzymes – laccase, lignin peroxidase, and manganese-dependent peroxidase [90, 91]. Gübitz and colleagues have conducted a number of detailed mechanistic studies using laccases from *Trametes hirsuta* and *Sclerotium rolfsii* and a range of commercial and model dye compounds to show that electron-withdrawing substituents tended to diminish reaction rates and electron-donating group enhanced dye susceptibility to enzymatic oxidative degradation [92–94]. Chemical mediators were able to broaden laccase ability to act on substrates of higher redox potential, though some dyes could be decolorized directly by laccase. Intracellular NADH-dependent azoreductases from *Bacillus* working in oxygen-free environments have also been shown to degrade a number of azo dyes, especially *o*-nitro substituted [95].

2.2.3
Future Opportunities

Enzyme technology has clearly had a major beneficial impact on the textile industry. A wide variety of enzyme activities are both available and useful for textile processing. Future challenges include total color removal from effluent, modification of synthetic substrates [96], such as nylon [97], acrylic [98] and polyvinyl alcohol [99], bleaching during cotton preparation, mercerization, and covalent chemistry leading to such effects as cross-linking, crease-resistance, functional fabrics, dye synthesis, and formation of new fiber types. Supporting continued work in these areas, the research community has established international technical venues to promote knowledge-sharing and speed development [88].

2.2.4
Environmental Benefits of Enzymes for Production of Textiles

The environmental benefits of enzyme technology in the textile industry mainly built on less consumption of energy, water and chemicals and less waste water treatment. Enzyme technology may also represent a more gentle treatment and thus improve the wearing qualities of the fabric.

A typical example is the use of Scourzyme for removal of waxy impurities of cotton fabric. An LCA study compared the enzymatic treatment with the traditional chemical method, which is based on treatment with sodium

Table 5 LCA on enzymes for textile production

Reduction of potential environmental impact Per 1000 kg cotton fabric impact	Energy consumption	Global warming	Acidification	Nutrient enrichment	Smog formation
Actual reductions	7700 MJ	500 kg CO_2 equiv.	6 kg SO_2 equiv.	4 kg PO_4 equiv.	0.5 kg C_2H_2 equiv.
Number of people equivalents (PE)	0.11 PE	0.06 PE	–	–	–

hydroxide and surfactant at high temperature. The study is based on Chinese conditions and the results are shown in the Table 5 for the production of 1000 kg cotton fabric.

The results show that particularly energy reductions and the reduced pollution of the aquatic environment are important. Reductions in energy use can translate either to reduced cost or increased productivity, which are tangible benefits to the textile mills. As establishment and enforcement of environmental regulations increase, resulting in textile mills paying fees or fines, the pollution reduction aspect also becomes a more important driver globally.

2.3 Enzymes in Pulp & Paper Industry

2.3.1 Introduction

Over the last two decades the application of enzymes in the pulp and paper industry has increased dramatically. Enzyme applications are no longer limited to starch modification for coatings and bleach boosting. Many new enzyme applications are now in regular commercial use. To name a few, lipase for pitch control, esterase for stickies removal, cellulases for fiber modification and pectinases for charge control. In certain cases, enzyme technologies have not only become an integral part of the chemical solutions, but have also been accepted as the preferred solution by many pulp and paper mills around the world.

2.3.2 Overview

Enzymes have already been tested in virtually every part of the pulp and paper mill from debarking to the finished products. The commercial applica-

Table 6 Enzyme applications in the pulp and paper industry

Process flow	Application	Enzyme class
Pulp furnish	Bleach boosting	Xylanase
	Strengthening and refining	Cellulase, xylanase, laccase
	Deinking	Amylase, cellulase, lipase
	Drainage improvement	Cellulase and xylanase
	Starch conversion	Amylase
Process equipment	Pitch control	Lipase
	Stickies control	Esterase
	Cleaning	Protease, lipase, amylase, cellulase
	Peroxide quenching	Catalase
Process water	Cationic demand reduction	Pectinase
	Color removal	Laccase, (peroxidase)

tion of enzymes has also become more diversified. Certain classes of enzymes are optimized for treatment of pulp furnish, whereas others are developed for equipment cleanup and water treatment. Table 6 summarizes most of the commercial enzyme applications in the pulp and paper industry.

2.3.3 Current Applications

2.3.3.1 Esterase for Stickies Control

Stickies are common problems for most of the mills using recycled paper and paperboard. Stickies can originate from pressure sensitive adhesives, hot melts, latex coatings, and synthetic toner ink binders. Often stickies are found to contain a significant amount of polyvinylacetate or acrylate esters that are potential enzyme substrates.

The stickies can cause deposit problems on the process equipment, machine runnability problems, breaks, and unexpected shut-downs. Esterases have emerged as one of the most effective solutions for stickies control [100]. The esterase breaks down the stickies into smaller, less tacky particles, which then can be removed by the DAF system. Figure 8 illustrates the effectiveness of an esterase before and after enzyme treatment of a sticky material (Courtesy of Buckman Laboratories 2005).

2.3.3.2 Lipases for Pitch Control

Pitch deposit can cause frequent shutdowns and inferior pulp quality. For mechanical pulps triglycerides (TG) have been identified as a major cause of

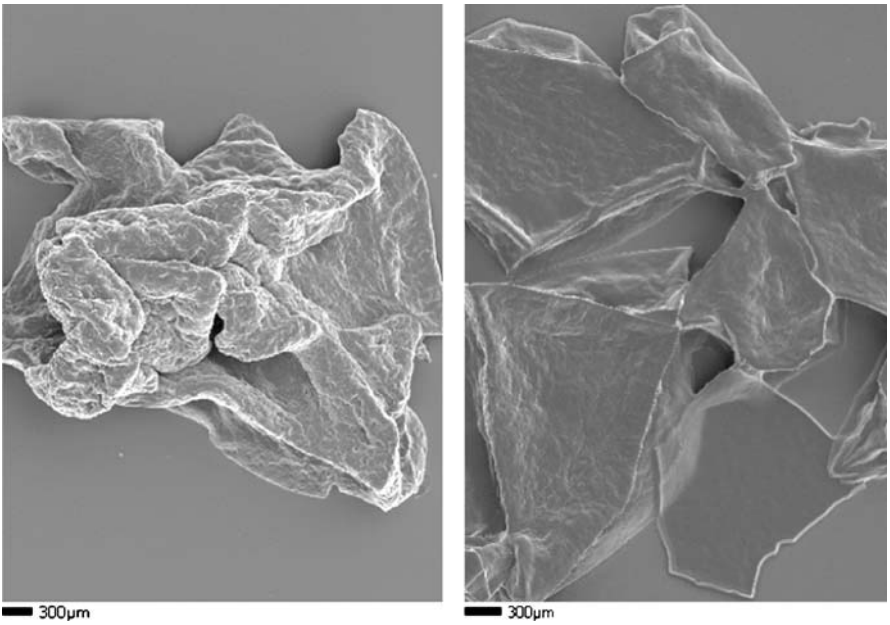

Fig. 8 SEM pictures of a sticky material before (*left*) and after (*right*) esterase treatment

pitch deposit. Among all the available technologies for pitch control, an enzymatic solution appears to be the most effective. Jujo Paper in Japan pioneered the use of lipases for pitch control in ground wood pulp. The lipase enzyme can degrade the triglyceride into glycerol and free fatty acids (FFA's), which can then be washed away from the pulp or fixed onto the fibers by use of alum or other fixatives.

Lipase treatment can significantly reduce the level of pitch deposition on the paper machine and the number of defects on the paper web [101]. In case that the fatty acids generated by the lipase are fixed back on the fibers by the addition of alum, lipase treatment can also prevent the accumulation of pitch in the recycled wastewater system.

At Nanping Paper mill, not only did the use of lipase solve the pitch problem and increase paper machine speed, but it also allowed the mill to use up to 50% fresh logs without seasoning, which in turn improved pulp brightness [102]. Lipase treatment of a TMP Norway spruce also led to significant improvement in tensile strength [103].

Recent developments in the lipase area has furthermore resulted in the commercial availability of engineered lipases with improved activity at high temperature (i.e., at 80–90 °C) thus showing optimal performance in TMP mills [104], Fig. 9.

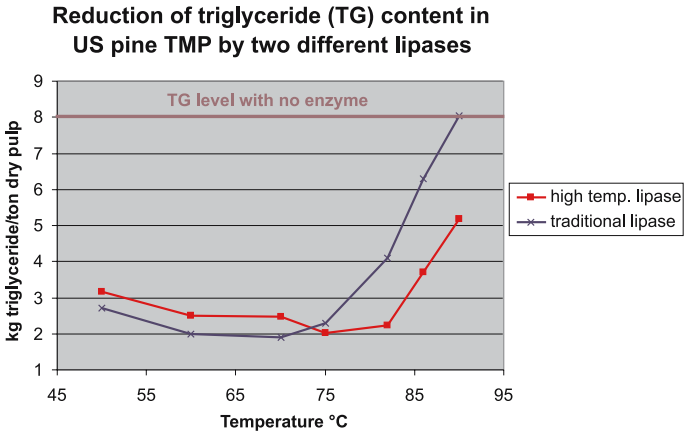

Fig. 9 Reduction in triglyceride (TG) level in US pine TMP pulp by the application of two different lipases at different temperatures. TG level without lipase treatment is 8 kg/ton dry pulp

2.3.3.3 Cellulase, Amylases and Lipases for Deinking

Recycled fiber is one of the most important fiber sources for tissue, newsprint and printing paper. Enzymatic deinking represents a very attractive alternative to chemical deinking. The most widely used enzyme classes for deinking are cellulase, amylase and lipase.

A significant part of the mixed office waste (MOW) furnish contains starch as a sizing material. Amylase can effectively degrade starch size and release ink particles from the fiber surface. Different from amylases, cellulases function as surface cleaning agents during deinking. They defibrillate the microfibrils attached to the ink and increase deinking effciency. Lipase was also shown to be very effective for deinking of MOW in a broad pH range [105]. It attacks the ester constituents of the toners and the sizing material in the paper furnish and releases the ink particles from the fiber.

A comprehensive study on old newsprint (ONP) deinking with various enzymes showed that cellulase and xylanase acted synergistically for ink removal [106]. The increase in environmental awareness has resulted in the development of printing inks based on vegetable oils. It has already been demonstrated that using lipases for deinking of vegetable oil based newspaper could achieve remarkable ink removal and brightness improvement [107].

Enzymatic deinking has already been implemented in many mills. However, for each mill, specific combinations of enzymes and surfactants have to be carefully studied to ensure that the enzyme is compatible with all of the existing process chemicals.

2.3.3.4 Bleach Boosting by Xylanases

In the bleaching of Kraft pulps a treatment with a xylanase can significantly reduce the consumption of bleaching chemicals or increase the resulting brightness levels. [108], Fig. 10. Xylanases degrade lignin-carbohydrate complexes and precipitated xylan on fibre surfaces, thereby increasing the effect of the following traditional bleaching. Application of xylanases allow Kraft pulp mills to take advantage of bleach boosting without the need for major investments in equipment or major process changes. Xylanase treatment can be carried out in washed pulps after kraft pulping or after e.g., an oxygen prebleaching. Xylanases specifically hydrolyse xylan, thus selectively removing compounds that limit the pulp bleachability. The treatment is specific without negative effects on pulp strength.

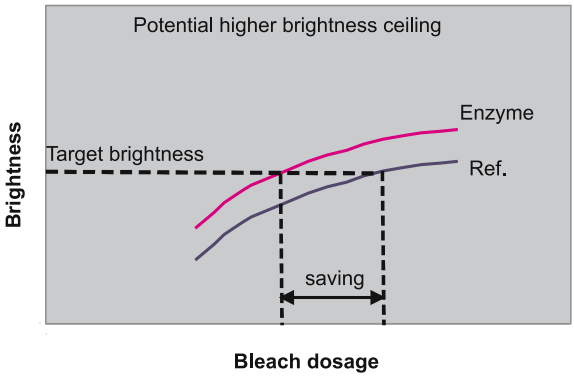

Fig. 10 Brightness as a function of bleach dosage for a xylanase treated pulp versus a non enzyme treated (ref.) kraft pulp

2.3.3.5 Cationic Demand Reduction by Pectinases

The pectin content of different wood species varies between 1–3%. During the alkaline bleaching processes, pectin is demethylated and dissolved in the process water thereby becoming part of the dissolved and colloidal substances [109]. Since pectic acids are negatively charged at the alkaline conditions, they interfere with the cationic retention and drainage aid, increase deposit formation, reduce sheet brightness and reduce paper strength [110]. Such negatively charged polymers are often referred to as "anionic trash" and result in an increasing "cationic demand" of the pulp stock.

Pectinase can effectively degrade the pectic acid into small oligomers (DP < 6) and reduce cationic demand of TMP pulp by more than 40% [111]

and thereby lead to a significant reduction in cationic polymer consumption.

A mill trial in Canada showed pectinase treatment reduced the cationic demand of TMP filtrates by up to 60% [112]. Comparison of a commercial pectinase mixture with two purified polygalacturonases indicated that the multi-component commercial enzyme product was still the most effective [113].

2.3.3.6 Enzymes for Fiber Modification

Fiber modification is one of the most promising growth areas for enzyme applications in the pulp and paper industry. Increasingly, more and more low-quality recycled fiber and mixed wood furnishes are being used by the pulp and paper industry to reduce raw material costs. Consequently, many mills are struggling to meet their quality targets. Due to the highly specific nature of enzymes, they are one of the most effective means to upgrade fiber quality.

Historically, cellulases were perceived as having negative impact on paper strength and yield. However, remarkable improvement in dry tensile and tear strength of bleached kraft pulps were observed after the treatment by a few selected cellulases [114]. In addition, significant improvement in wet tensile strength was also attained. There seems to be a correlation between strength gain and the increase in reducing end groups on the pulp surface generated by cellulases. Other than the fibrillation effect of cellulases, it is likely that part of the strength improvement is due to the random cross-linking between the aldehyde groups at the reducing ends of cellulose polymer and the hydroxyl groups on the cellulose backbone. As illustrated in Fig. 11 one possible mechanism for the increasing fiber bonding and improved dry and wet strength of the paper sheet could be the formation of hemiacetal group linking the fibers together.

Refining is one of the key steps in the paper-making processes to develop strength. The cellulosic fiber for most of the paper products has been refined at least once before it gets on the paper machine. An earlier study on treatment of a chemical pulp by a crude xylanase showed extensive external fibrillation and decreased energy demand for refining [115]. This work was confirmed by others using several commercially available xylanases [116]. Cellulases have also been studied extensively for chemical pulp refining [117]. It seems that cellulase treatment could reduce the refining intensity needed to reach the same strength or freeness target. It should be noted that enzyme treatment could fundamentally change the well defined refining curve [118]. Because enzymatic refining expanded the scope of mechanical refining with additional biochemical modifications of cellulosic fiber, the tear, tensile, and freeness relationship will no longer be the same. Therefore, particular care

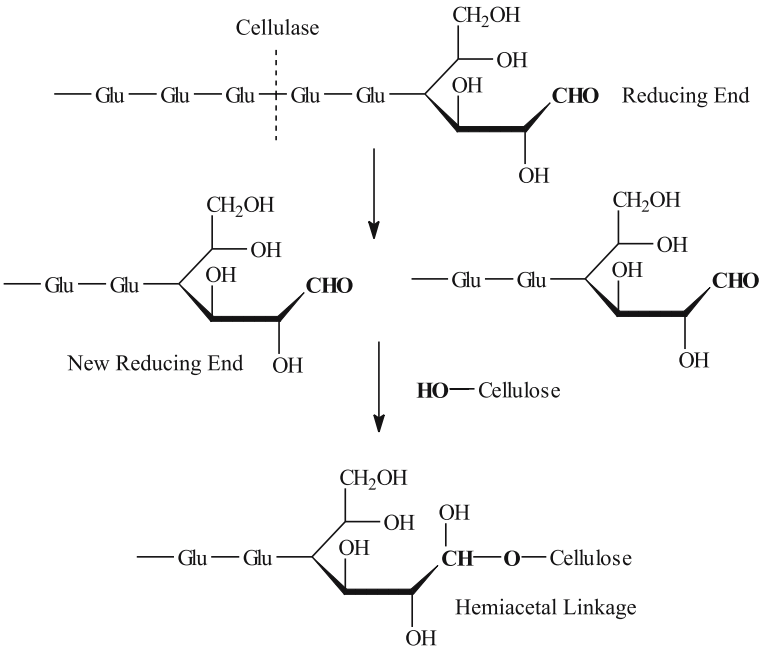

Fig. 11 Proposed mechanism for strength development by cellulases

has to be taken to balance the enzyme treatment and refine intensity to ensure proper fiber quality.

2.3.3.7 Drainage Improvement

Recycled fibers, mechanical pulps, and some highly refined chemical pulps sometimes have poor drainage properties due to the presence of relatively high fines content and colloidal materials. Quite often, poor drainage can lead to higher drying energy consumption, slower paper machine speed and lower productivity. Given the right conditions, enzyme treatment is one of the most effective and versatile technologies to improve drainage and production rate without any capital investment. Significant improvement in drainage was reported by cellulase and hemicellulase treatment of ONP, OCC, and virgin fibers [119, 120]. Enzyme treatment of a highly refined bleached kraft pulp did not change either fiber length or fines content of the pulp. It was proposed that the true mechanism of enzymatic drainage improvement is the removal of the polysaccharide colloidal gel material on the fiber surface rather than fines reduction [121]. Currently, this technology is already being used by several mills to increase the production rate [122] (Paice and Zhang 2005). It is

likely that enzyme will play a more prominent role on the wet end of the paper machine in the near future.

2.3.3.8 Starch Modification

Starch is used as coating and surface sizing material for different types of paper. The coating improves printability and makes the sheet more resistant to abrasion. The raw starch needs to be partly degraded to lower viscosity of the solutions applied to the paper surface. The use of a specific amylase is a nice tool to obtain the desired degree of degradation compared to the alternative use of unspecific oxidation reagents like hydrogenperoxide, persulphates, etc. The starch modification is carried out directly in the mill either in batch processes or in continuous jet-cooker-based processes. The inactivation of the amylase when the desired degree of degradation is reached is, of course, of utmost importance.

2.3.3.9 Cleaning

In boil-out processes for cleaning of equipment and tubings enzymes can be applied to improve removal of organic material and depress the growth of microbes. For the cleaning processes proteases, amylases, lipases and cellulases can be applied.

2.4 Enzymes in Starch Applications

2.4.1 Introduction

Starch is the second most available carbohydrate source (after cellulose) on earth and found in crops such as rice, wheat, potatoes, corn and tapioca. The total world production in 2001 was 48 million tons of which more than 70% came from corn.

Starch consists of chains of glucose molecules, which are linked together by $\alpha(1 \rightarrow 4)$ and $\alpha(1 \rightarrow 6)$ glycosidic bonds The two major parts of starch are amylose (20–30%), essentially linear $\alpha(1 \rightarrow 4)$ glucan chains and amylopectin (70–80%), a branched molecule containing 4–5% $\alpha(1 \rightarrow 6)$ linkages. Amylopectin forms a characteristic cluster structure.

Starch is organized in microscopic granules (granular starch), which are insoluble in water at room temperature. When analyzing the starch granule using small X-ray scattering it is found that it contains highly crystalline areas as well as more amorphous areas [123]. The highly crystalline areas are what

French [124] proposed to be the clusters in the amylopectin fraction consisting of highly ordered glucan double helices.

The major part of the industrially produced starch is used as starch or chemically modified starch in food and technical industries. The rest (about 35%) is modified with enzymes to syrups or, to a minor extent, enzyme modified starches. The syrups are high fructose corn syrups (HFCS) (worldwide production 12 million tons of which 8–9 million tons are consumed in the US) and glucose and various maltose syrups (worldwide production 7 million tons).

After a steeping process where the corn kernel is separated into its components (starch, gluten, germ oil, and fibre) the production of HFCS from the starch fraction comprises three enzymatic process steps: liquefaction (α-amylase), saccharification (glucoamylase and a debranching enzyme), and isomerization (glucose isomerase) [125].

The process was introduced commercially in 1973 with the launch of a bacterial α-amylase that could work at temperatures above 100 °C [126], and has not changed much over the years.

The world's leading producers of syrups are: Cargill/Cerestar (US/France), Tate and Lyle (UK), Archer Daniels Midland (US), and Roquette (France).

2.4.2 Overview

Typical process conditions for production of HFCS from starch are given in Table 7.

Table 7 Typical process conditions for the production of high fructose corn syrup

Process	Temperature (°C)	Dry substance content (%)	pH	Process time (hr)
Jet cooking/dextrinization	105/95	30–35	5.2–5.6	0.1/1–2
Saccharification	60	30–35	4.3–4.5	25–50
Isomerization	50–60	40–50	7–8	0.3–3

The enzymes which are used in the starch industry are the following:

2.4.2.1 Bacterial α-Amylases

Bacterial α-amylases (EC 3.2.1.1) catalyze the hydrolysis of internal α-1,4 glycosidic bonds. This reduces the viscosity, which is necessary for further processing. The degree of hydrolysis is measured by the dextrose equivalent

(DE), a measure of the reducing power relative to glucose. DE after liquefaction is typically 8–12.

During the last 10 years, protein engineering techniques have improved the application performance of the α-amylases considerably [127, 128]. The addition of Ca^{++} to the starch slurry for stabilization of the amylase is no longer necessary, the operating pH during liquefaction has been lowered (reducing costs of ion exchange), and it is no longer necessary to inactivate the α-amylase before saccharification (the specificity of the bacterial α-amylase has been changed, so it does not form panose, if active during saccharification).

2.4.2.2 Glucoamylase

Glucoamylase (EC 3.2.1.3) is an exo-amylase that is added to the partly hydrolyzed starch after liquefaction. Glucose units are removed in a stepwise manner from the non-reducing end of the molecule. The rate of hydrolysis depends on the chain length: maltotriose and, in particular, maltose being hydrolyzed at a lower rate than higher oligosaccharides.

Furthermore, α-1,6 linkages are broken down more slowly than α-1,4 linkages, but eventually almost complete conversion of starch into glucose is possible. Application parameters are 60–63 °C and pH 4.3–4.5.

2.4.2.3 Pullulanase

Pullulanase (EC 3.2.1.41). Industrially used pullulanases are heat stable enzymes that act simultaneous with glucoamylase during saccharification. Pullulanases catalyze the hydrolysis of the α-1,6 linkages in amylopectin, and especially in partially hydrolysed amylopectin.

2.4.2.4 Glucose Isomerase

Glucose isomerase (EC 5.3.1.5). Glucose does not match the sweetness of sucrose and part of the glucose is, therefore, converted to the more sweet fructose by the enzyme glucose isomerase. The equilibrium conversion under industrial conditions is 50% making chromatographic separation necessary in order to obtain the industrial product 55% fructose, which has sweetness similar to sucrose.

Glucose isomerase is used industrially as an immobilized enzyme. In order to avoid clogging of the enzyme bed very pure syrup is required. Ca^{++} is an inhibitor of the enzyme and has to be removed (even with the developments in α-amylase stabilization the original content of Ca^{++} in the corn is too high

for the glucose isomerase). Prior to isomerization the syrup is, therefore, filtered, ion exchanged and usually also carbon treated. Mg^{++} is added as an activator for the GI after the purification step.

Commercial immobilized glucose isomerase preparations have half-lives between 100 and 200 days. Most columns, therefore, last for more than one year. Productivities are typically around 15 tons syrup dry substance/kg immobilized enzyme.

Interesting developments in the early 90s were on column loading and use of ion exchange resins as carrier material (with reuse) [129]. Most commercial plants are however now back to the original column set-up with one time use of the immobilized GI-particles based on glutaraldehyde cross-linked cell material.

2.4.2.5 Fungal α-Amylase

Depending on the maltose content, maltose syrups are made (after liquefaction to 10 DE with α-amylase) by fungal α-amylase (EC 3.2.1.1) (50–55% maltose), by fungal α-amylase, maltogenic amylase (EC 3.2.1.133) [130], and pullulanase (55–65%), by β-amylase (EC 3.2.1.2) and pullulanase (70–80%), and by β-amylase, maltogenic amylase, and pullulanase (> 80%) all at 30% DS. Properties and production methods of high maltose syrups, maltodextrins, and oligosaccharides are described in [131].

Confectionary syrups and speciality syrups (brewing syrups) are made from liquefied starch (thinned by acid treatment or α-amylase) followed by treatment with fungal amylase or combinations of fungal amylase and glucoamylase.

2.4.3 Latest Developments

2.4.3.1 Steeping

The advantage of the wet milling process compared to the dry milling process predominantly used in the new fuel alcohol plants is the separation of the corn kernel into valuable by-products (gluten, germ oil, fibres) in addition to starch. Disadvantages are the long steeping times (up to 48 hours) and the use of SO_2. Enzymatic milling [132, 133] has been developed that uses proteases to significantly eliminate/reduce these disadvantages of the steeping process.

Research is also in progress on making by-product recovery possible in the dry milling process with e.g., pre-treatment with anhydrous ammonia [134].

2.4.3.2 Resistant Starch

It is possible through a combined heat-treatment and acid or enzymatic hydrolysis to produce starches that are not digestible (called RS – resistant starch) or slowly digestible (SDS – slowly degradable starch).

Food made from RS instead of normal starch has created strong interest in the US, where a large part of the population see the consumption of carbohydrate containing foods as a major contributor to obesity (Atkins' diet). All over the world there is great interest in producing SDS and building up knowledge about the health aspects of the consumption of foods containing SDS. This is because these types of food are beneficial for people suffering from type II diabetes or hyperlipidemia (increased levels of lipids in the blood), but also because healthy people see a positive effect on a number of physiologic factors through a slower degradation of starch in the stomach/intestine [135].

Through production of RS and SDS, one will typically attempt to increase the level of amylose. Amylose is a linear molecule and crystallizes (also called retrogradation) easier than amylopectin. The crystals are built up of double helices, which are almost completely inaccessible for the amylases.

Products on the market are based on high amylose starches or enzymatically debranched amylopectin [136].

2.4.3.3 Specialty Starches

Enzymatically treated starches other than resistant starches are commercially available. Examples are cyclodextrins [137], starch emulsifiers [138] and various oligosaccharides made from starch (e.g., trehalose [139]).

There is increased interest in engineering novel glucose polymers [140]. The action of branching enzyme (EC 2.4.1.18) [141] increases the number of α-1,6 linkages in both amylose and amylopectin. It has been reported that the action of branching enzyme on amylose creates glycogen-like molecules [141], while the action on amylopectin creates dextrins highly soluble in water [140]. The new glucose polymers are of interest for use in foods [142].

Amylomaltase (EC 2.4.1.25) [143, 144] transfers α-1,4 linked glucans to e.g., the 4-OH group of glucose or other α-1,4 linked glucans. The enzyme can produce large cyclodextrins (DP larger than 9) from amylose [138] as well as thermoreversible gels from amylopectin [144].

2.4.3.4
Enzymatic Hydrolysis of Starch below the Gelatinization Temperature

Several research papers and patents describe the discovery of amylolytic enzymes effective in raw starch hydrolysis (hydrolysis of granular starch at temperatures below the initial gelatinization temperature of granular starch) [145].

It is also evident that the presence of a starch binding domain (SBD) on the enzyme facilitates a disruption of the starch granule and subsequent a faster breakdown of the starch [146].

The previously described enzymes for raw starch hydrolysis have however not been as efficient as the traditional starch jet cooking process in solubilizing the starch [147]. Recently however new α-amylases have been found [148] that are very effective in hydrolyzing raw starch. These enzymes are likely to find applications both in the starch processing industry as well as the dry milling fuel ethanol industry.

2.5
Enzymes for Production of Bioethanol

2.5.1
Introduction

Bioethanol is ethanol derived through fermentation of sugar contained in agricultural feedstocks, such as sugar crops, grains, or cellulosic materials. Though fermentation of sugar is the primary route for beverage ethanol used by mankind for at least 2000 years, the name bioethanol today is commonly applied to the growing production and use on a major scale of ethanol for motor vehicle fuel.

Bioethanol and its derivative, ETBE (ethyl tertiary butyl ether), are oxygenated fuels which can be used in gasoline blends for most gasoline-powered engines. Ethanol can be blended up to 10% by volume (E10) for standard gasoline engines, and up to 85% by volume (E85) for slightly modified engines in flexible fuel vehicles (FFV's). Both ethanol and ETBE are used as high-octane, oxygenated, fuel enhancers in place of fossil-based methanol and MTBE (methyl tertiary butyl ether). Use of MTBE has been banned throughout the USA due to groundwater pollution from leaking underground storage vessels; over the last five years ethanol has essentially replaced MTBE in markets where reformulated gasoline is sold. For more information on fuel ethanol use and FFV's the reader is referred to the Web site of the US Renewable Fuels Association www.ethanolrfa.org.

Recently the use of ethanol has been promoted by national governments as a fuel extender (extending the petroleum resources by large scale use) or as a means of meeting Kyoto protocol agreements. An example of this is the

energy bill passed in the USA in 2005, which mandated the use of renewable fuels such as ethanol at an increasing minimum level for the next seven years.

Bioethanol can be produced from nearly any readily available sugar or starch based crop, though globally, the dominant substrates are sugar cane (Brazil) and corn (US, Canada and China). Grain sorghum is also used in the Western USA. Wheat, barley, and sugar beets are used in Northern Europe and sweet sorghum in Southern Europe. In Western Canada, wheat and barley are used.

The most abundant material on the earth from which to produce bioethanol is lignocellulosic biomass. Research on the conversion of cellulose to ethanol has intensified in recent years; however, feasible large scale manufacturing of biomass ethanol has yet to become a reality. The economic use of cellulosic substrates for ethanol production has posed a great challenge, due to the difficulty of breaking down these complex substrates into simple sugars.

2.5.2 Overview

2.5.2.1 Major Drivers for Fuel Ethanol Growth

The world's production of bioethanol in 2004 was approx. 8.2 billion gallons (2.2 million m^3) and was distributed as shown in Table 8. This figure has been growing by around 10% annually, driven by the factors mentioned above, phase out of MTBE, agricultural and energy policies, as well as the steadily increasing price of crude oil. Table 8 summarises total world estimate of ethanol production in 2004.

Table 8 Total world estimate of ethanol production in 2004 (Source: Novozymes estimates)

Geographical area	Volume, million gallons ($1000\ m^3$)	Estimated annual growth, % 2004–2005
North America	3800 (1005)	10%
Europe	125 (33)	60%
China	100 (27)	35%
Brazil	4100 (1090)	5%
Other	500 (130)	10%
Total	8625 (2282)	8–10%

As further illustrated in Fig. 12, by far the main production of bioethanol today occurs in the USA and Brazil.

2.5.2.2 Ethanol Production from Grain/Starch

The primary application of white biotechnology in the manufacture of bioethanol is in the development and application of enzymes for conversion of starch based crops. The primary growth segment in the industry is in the dry-mill ethanol plants, where whole grain is ground and fermented, and the

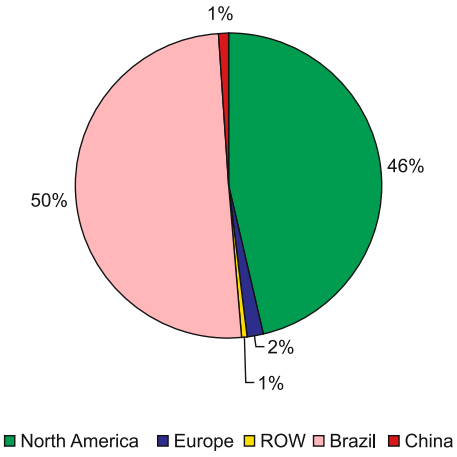

Fig. 12 Distribution of ethanol production by region in 2004 (Source: Novozymes)

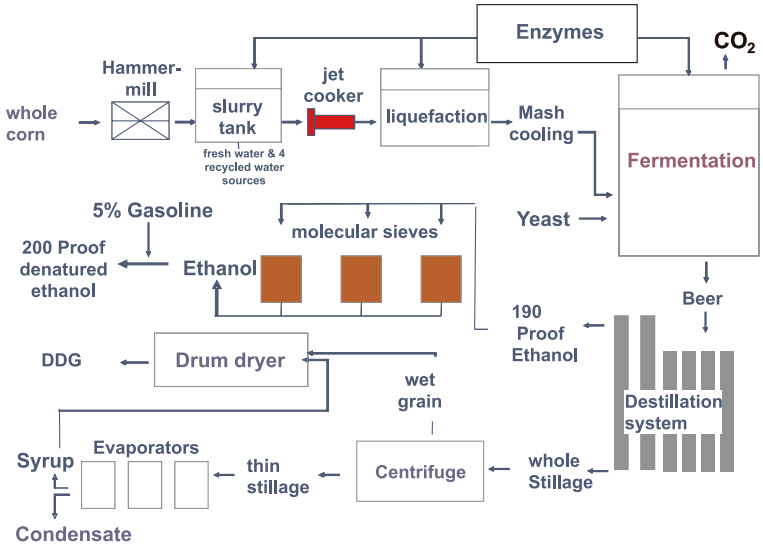

Fig. 13 Process overview of dry-mill ethanol production

residual fiber and protein is sold as an animal feed product: Dried distillers grains (DDG), dried distillers grains with solubles (DDGS) or wet distillers grains.

2.5.2.3 Liquefaction and Pre-Hydrolysis

After grinding, the grains are cooked at a temperature of 80–120 °C for 1–2 hrs to solubilize the starch and allow enzymatic breakdown. Alpha-amylases are used in this stage to break down the starch into dextrins, and reduce the viscosity of the starch solution. If the grains contain large amounts of soluble beta-glucans or xylans such as are often found in wheat, barley, or rye, additional enzymes may be added prior to liquefaction for the purpose of viscosity reduction.

2.5.2.4 Simultaneous Saccharification and Fermentation

Following liquefaction, the cooked "mash" is cooled to fermentation temperature of 30–35 °C and enzymes and yeast are added to commence fermentation. Glucoamylase enzymes are used to create glucose from the dextrins to be fermented to ethanol by the yeast. Generally, the objective of SSF is to add glucoamylase in the appropriate dose so that glucose generation by the enzyme and consumption by the yeast is somewhat balanced; however, a perfect balance is rarely achieved in practice.

Typically, urea, ammonia, or other nitrogen source is added to the front of the process for the purpose of yeast nutrition. Proteases may also be added in this step with the objective of free amino nitrogen (FAN) generation for yeast, as well as releasing starch bound in protein complexes.

2.5.2.5 Ethanol Recovery and Water Recycling

After 48–72 hours the fermentation is complete and the ethanol is recovered, first by azeotropic distillation, followed by molecular sieve drying. The residual fiber, starch, and protein are recovered by centrifugation, and are typically dried. The water recovered from the fermentation is recycled to the front of the process as "backset". Most modern dry-mill ethanol (Fig. 13) plants have no process wastewater discharge, with the only emissions being CO_2 and water vapor. Table 9 gives a summary of enzyme use in dry-mill fuel ethanol production.

Table 9 Summary of enzyme use in dry-mill fuel ethanol production

Enzyme	Step	Purpose
Alpha-amylase	Liquefaction	Hydrolyze starch to dextrins and reduce solution viscosity
Beta-glucanase, cellulase, xylanase	Pre-liquefaction	Reduce viscosity
Glucoamylase	Simultaneous saccharification and fermentation	Provide glucose for yeast
Protease	Fermentation	Provide nutrients for yeast and improve starch accessibility

2.5.3 Latest Developments

In 2005 there are three major technology developments occurring in the U.S. bioethanol industry.

2.5.3.1 Raw Starch Hydrolysis

As mentioned above the process of converting starch into ethanol has conventionally been carried out by high-temperature liquefaction of the starch followed by enzyme hydrolysis.

Conversion of the starch below gelatinization temperature (raw starch hydrolysis or RSH) is potentially advantageous because of the potential for energy savings, reduction in Maillard products, and reduced viscosity of a granular starch suspension. Though this process is used in several traditional beverage alcohol fermentation processes, its use in commercial bioethanol production has been hampered by high enzyme dose and low process efficiency in the conversion of starch. During the last 3–4 years significant advances in the enzyme efficiency for conversion of raw starch have been made, along with process advances to take advantage of the energy savings and reduce the threat of bacterial contamination. This process has advanced to the state that as of this writing there are eight dry-mill ethanol facilities operated in the US by the Broin companies that utilize this technology [149].

2.5.3.2 Fractionation

In the last 10–15 years the bioethanol industry in the U.S. has followed growth of the dry milling concept – where the primary product is ethanol, and co-

products are CO_2 and animal feed (DDG). DDG is subject to wide price variations related to local demand. In order to improve the value and variety of co-products, many producers are investigating strategies for separation and processing of the different corn fractions; this "biorefinery" approach is similar to the business model of the large integrated wet-mills. Such an approach could create new co-products with higher, more predictable value, as well as new markets for the feed products. In the broadest sense, the sugars and proteins produced in the biorefinery could be basic feedstocks for all types of industrial processes, similar to today's oil refineries. Both dry fractionation [150] and aqueous-enzymatic approaches [151, 152] are currently in development.

2.5.3.3 Lignocellulosic Biomass

Significant improvement has been made in the area of cellulases for biomass conversion, where DOE funded research at major enzyme producers has resulted in a dramatic reduction in the cost of cellulase application [153, 154].

Key challenges remaining are the efficient liberation and conversion of C5 sugars, collection and storage of biomass, and relatively low solids processing leading to low ethanol titers in fermentation. Integration of the unit operations in a demonstration facility is a major focus of several projects starting up in the 2006–2007 time frame. Process integration to solve these and other challenges is viewed as the most pressing need for moving biomass ethanol closer to reality [155].

2.5.4 Environmental Benefits of Enzymes for the Production of Bioethanol

The development of the bioethanol technology has to a large extent been driven by the environmental problems caused by oxygenation agents such as MTBE. Another driver has been the wish of independent supplies of fuel. However, the fact that a petrochemical product is substituted by an agricultural product has a rather broad range of environmental consequences.

Large efforts are carried out to develop a technology based on agricultural waste; however, the majority of the current production of bioethanol is still based on starch, first and foremost from corn.

As a consequence of this an LCA study was carried out comparing the environmental load of driving 1 mile on gasoline and bioethanol, respectively. The bioethanol production was based on corn grown and processed in US and on the assumption that unlimited land for biomass production is available. The results are shown in Table 10 for driving 1,000 miles.

The results show that the current technology to produce ethanol from starch for use in car-driving offers both advantages and disadvantages to the

Table 10 LCA on enzymes for bioethanol production

Change of potential environmental impact
For driving 1000 miles on bioethanol versus gasoline
(Figures in brackets indicate increased environmental load)

	Energy consumption	Global warming	Acidification	Nutrient enrichment	Smog formation
Actual reductions	2800 MJ	170 kg CO_2 equiv.	(0.9 kg) SO_2 equiv.	(1.6 kg) PO_4 equiv.	0.4 kg C_2H_2 equiv.
Number of people equivalents (PE)	0.05 PE	0.02 PE	–	–	–

environment. The advantages are first and foremost related to a reduction of consumption of fossil fuels and emission of fossil CO_2.

The disadvantages are related to the farming process. Bioethanol production uses land and gives rise to emission of nutrients to the environment. It is difficult to balance these impacts against the benefits of saving fossil fuel and limiting contributions to global warming. Specific considerations of local conditions and alternative uses of the land are required to do so.

2.6 Enzymes in Food

2.6.1 Introduction

The food applications of enzymes represent a wide and highly diverse field including baking, dairy, juice, vegetable processing and meat. The enzymes are used to obtain a number of benefits like more efficient processes leading to reduced use of raw materials, improved or consistent quality, replacement of food additives, and avoidance of potential harmful by-products in the food. Due to the diversity of the area this chapter can only present representative examples of enzyme applications.

2.6.2 Overview

The uses of enzymes for food application are summarized in Table 11.

Table 11 Overview of use of enzymes in food applications

Application	Advantages	Used enzymes
Baking and Snacks		
Shelf life extension	Increase shelf life and improve quality by retain the soft, elastic and moist.	Exo and endo-amylases Xylanases
Dough conditioning	Ensuring uniform good bread volume and good dough handling properties	Xylanases.
Flour correction	Ensuring uniform yeast fermentation	Endo amylase.
Dough strengthening	Improved volume, finer crumb and robustness towards stress on the dough during processing. Emulsifier replacement	Lipases, broad specificity
Gluten strengthening	Improved volume and robustness in dough processing. Replacement of oxidants like bromate and ADA.	Oxidases
Acryl amide prevention	Avoid formation of acryl amide during the Maillard processes	Asparaginase
Dairy		
Cheese clotting	Formation of the cheese curd	Rennets, Chymosin Specific proteases
Cheese yield enhancement	Increasing the yield of protein and fat beyond what the rennet can provide	Phospholipases Transglutaminase
Hydrolyse lactose	Enables milk consumption by lactose intolerant consumers, and increases sweetness.	Lactase
Low allergenic baby milk formulas	Hydrolyse milk proteins to reduce risk of allergic reactions to milk	Endo-proteases
Flavour improvement	Enhanced cheese ripening Enzyme modified cheese, cheese flavour	Exopeptidases, endoproteases, lipases
Juice & vegetable processing		
Mash treatment	Improve the yield of juices with addition of enzyme to the mash	Pectinase mixtures.
Clarification	Prevent haziness in the juice	Pectinase mixtures,
Fruit Firming	Firming the surface of fruit pieces to resemble fresh fruit, e.g. for yogurt or pies	Pectin Methyl Esterase
Meat and proteins		
Protein hydrolyzates	Hydrolysing proteins like soy and gluten e.g. for the savoury flavours,	Exo-peptidase and endoprotease mixtures
Meat processing	e.g. Improved texture of sausages, Extraction of flavour and protein	Transglutaminase Endo- and exo-peptidase
Restructured meat	Restructuring meat from trimmings	Transglutaminase

2.6.2.1 Baking

Bread becomes progressively harder, drier and less elastic over time. Amylases are used not only to limit this staling process both in order to provide a better eating quality, but also to prolong the shelf life and thereby leading to reduced stale returns and savings in the logistical system.

Amylases used for antistaling need to be sufficiently thermostable to be able to work after the starch is gelatinized. The most ideal amylases are exoacting amylases which reduces the length of side branches amylopectin in order to prevent the retrogradation of amylopectin, and with a minimum of degradation the backbone in order to keep the bread crumb elastic. Thus the maltogenic exo-amylase from *Bacillus stearothermophilus* (Novamyl®) [156] enjoys widespread use in baking industry. This amylase is often used in combination with endo-amylases in order to provide softer crumb, which is obtained on the expense of a less elastic crumb. Such amylases would, if used alone, lead to an unacceptable gummy crumb, due to a too extensive degradation of amylopectin. Table 13 shows an LCA of the use of Novamyl for white pan bread in US.

Xylanases also contributes to retaining a soft and moist crumb, even though xylanases are primarily used as dough conditioners to ensure a good volume and good dough processing abilities. Good baking xylanases have a high specificity for the insoluble arabinoxylans which otherwise disrupts the stability of the gas cells in the dough, while only providing a minimal degradation of the soluble arabinoxylan to retain the good functional properties of the this arabinoxylan fraction [157] including the moistness keeping properties needed for a long shelf life.

The gas cells in the dough are surrounded by the gluten network, which provides stability for the growing gas cell during fermentation. Chemical oxidants like bromate, ADA (azodicarbonamide) and ascorbic acid have a widespread use in strengthening the gluten network. The result is stability against dough stress, increased bread volume and improved crumb structure. Due to health concerns bromate and ADA are not allowed in many countries, it is therefore not surprising that glucose oxidase, typically from *Aspergillus niger*, and more recent hexose oxidase from the red algae *Chondus crispus* [158] are enjoying an increased used for gluten strengthening, even though it is difficult to obtain the same strong oven spring as with bromate. The oxidases are believed to work via generation of hydrogen peroxidase, which via the endogenous glutathione dehydrogenase oxidizes glutathione, and thereby prevents the glutathione to form S – S bridges to gluten which otherwise would form less gluten macropolymer [159]

2.6.2.2 Dairy

Dairy represents the most classical enzyme application as rennets (including chymosin) have been used for milk clotting since ancient times. The enzyme acts via a specific cleavage of the hydrophilic glucomacroprotein from kappa-casein, resulting in aggregation of the remaining less hydrophilic casein micelle. In the aim of higher cheese yields (amount of cheese per quantum milk) chymosin has been produced with increasing purity to avoid unspecific hydrolysis by other proteases than chymosin, which would otherwise lead to a yield loss. This development has been strongly facilitated by the use of cloned chymosin over-expressed in e.g., *Aspergillus niger* and purified by large scale chromatography.

The use of lactase for production of low lactose milk represents another significant dairy application. Typically lactase from the yeast *Klyveromycis lactis* is used to make the milk suitable for people with lactose intolerance. By hydrolyzing the lactose to glucose and galactose the milk becomes digestible also for the approx. 70% of the world population that lacks the lactase in their digestive tract. Further, the sweetness of the milk is increased.

Proteases are used for production of low allergenic milk proteins used as ingredients in baby milk formulas. The purpose of hydrolyzing the proteins is to eliminate allergic reaction that can be caused by intact milk proteins. Low allergenic baby milk formulas has been on the market in more than 60 years.

Other enzyme applications for dairy play a relative minor importance; this includes the use of exo-peptidases for the development of cheese flavors including accelerated cheese ripening. Some years ago transglutaminase was predicted to find widespread use within dairy; however, this enzyme has surprisingly only found a limited use. Transglutaminase crosslink's proteins like casein through the formation of (ε-(γ-glutamyl)-lysine links. The dairy opportunities of transglutaminase includes thickening of low-fat, medium-solid yogurt to obtain the same fatty texture and medium fat yoghurt [160]. Acid stable milk, increased cheese yield and texturizing in ice-cream are other examples of transglutaminase opportunities.

2.6.2.3 Meat and Protein Applications

In contrary to dairy, transglutaminase has found significant applications in the meat industry especially in East Asia based on the transglutaminase from *Streptoverticillium mobarence*, which is well suited since it is calcium-insensitive, which is important as calcium-complexing polyphosphates are widely used in the meat industry as meat binders. The meat applications include texture improvements for sausages & surimi and production of restructured meat, where low cost trimming are glued together at 5 °C.

Proteolytic enzymes represent an alternative for acid hydrolysis for production of protein hydrolysates for soups, savory flavours as ingredients in emulsified meat products and in brines for marinated products. Alternatives for acid hydrolysis are desirable as low levels chlorinated by-products like mono-and di-chlorpropanols can be formed in acid hydrolysis. A mixture of proteolytic enzymes from *Aspergillus oryzae* (Flavourzyme™) including endo-proteases, mono-, di- and tri-amino-peptidases and carboxy-peptidases have been demonstrated useful for production of hydrolysates of soy, gluten, meat and gluten with a degree of hydrolysis of 40–60% depending on the protein source. The content of prolin and glycin as well as the solubility of the protein source are the limiting factors for reaching high degrees of hydrolysis.

2.6.2.4 Juice and Vegetable Applications

Pectinolytic enzymes have been applied in juice processing for several decades. The enzyme preparations contain a large number of enzyme components needed for a degradation of the fruit cell wall. This include pectinases, arabinanases, galactanases, endoglucanases and a range of glycosidases. The enzymes are used for the processing of fruits to clear juices at temperatures up to 45–55 °C. Benefits are increased press capacity, improved juice yield (reduction of waste) and easier clarification and filtration of the juice. The enzymes can also be used in vegetable processing, e.g., for an optimized extraction of beta-carotene from carrots. Enzymes from *Aspergillus aculeatus* (Pectinex™ Ultra) are in particular efficient as they contain rhamnogalacturonan degrading enzymes, which are important for a total disintegration of the cell wall. More recently cloned pectinases like a thermo- and acid-stable pectin lyase have been introduced. Used alone it allows for a more controlled maceration of the cell wall. It is also used for spiking multicomponent pectinases thus making the treatment more efficient and economic

2.6.3 Latest Developments

Emulsifiers like DATEM (diacetylated tartaric esters of monoglycerides) and SSL (sodium stearoyl lactylate) are used extensively in baking to provide dough strengthening with increase in volume, better stability to mechanical stress on the dough, and a fine, uniform crumb structure. It has recently been found that certain lipases [161] fully or partially can replace the emulsifiers (Fig. 14), these lipases have simultaneous activity on triglycerides, galactolipids and phospholipids. The lipases convert the diacylated phospholipids and galactolipids into the corresponding monoacylated lipids, which stabilize the interface around the gas cells by formation of a stable lipid monolayer like DATEM and SSL, which are also monoacylated polar lipids. The develop-

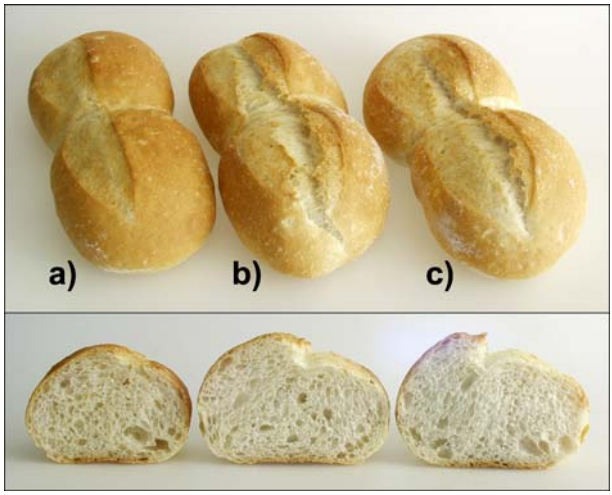

Fig. 14 Crusty rolls lip made **a** without enzyme, **b** with 0.3% DATEM and **c** lipase (30 ppm Lipopan® F). Figure from Novozymes A/S, Denmark

ment of such lipases does therefore represent another step towards industrial bread baking based on biotransformantion of flour components rather than chemical synthesized additives.

In 2002 it was found that acrylamide is formed in several starch containing products including, French fries, potato chips, biscuits and snacks. Shortly after it was discovered that acrylamide formation is through a Maillard reaction between reducing sugars and asparagines [162]. Acrylamide is a known carcinogen in rats and classified as probably carcinogenic to humans by the International Agency for Research on Cancer (IARC) [163], and is consequently undesirable in food products. Conversion of asparagine to aspartic acid using asparaginase reduces the acrylamide content almost completely in most dough based products and partially in products based on cut or sliced potato products like French Fries. Cloned *Aspergillus* asparaginases, which are expected to become commercial within a short time, have been demonstrated to effectively reducing the acrylamide content as demonstrated in Fig. 15.

It is desirable to get a high cheese yield in order to minimize the loss into the less valuable side product whey. It has recently been discovered that certain phospholipases can be used to improve the cheese yield with 1–3%, including increased yield of both fat and proteins, when used in combination with chymosin. The phospholipase A1 from *Fusarium venenatum* (YieldMax™ PL) has been found especially efficient as significant yield improvements can be obtained with no changes to cheese functionalities such as taste, browning and stretchability. The most pronounced effect is seen in Mozzarella production as the phospholipase both increases the yield during

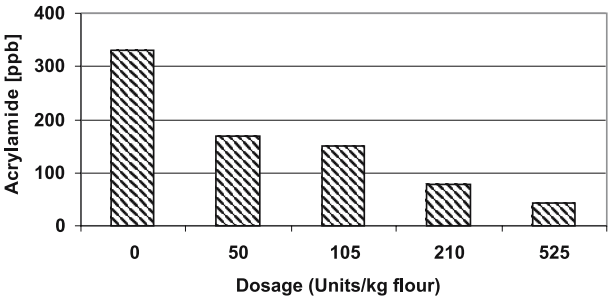

Fig. 15 Reduction of the acrylamide content in semi-sweet biscuits by *Aspergillus oryzae* asparaginase, cloned and over-expressed in *Aspergillus oryzae*. Data from Novozymes A/S, Denmark

Table 12 Yield increase in two types of pasta filata cheeses; low moisture part skim Mozzaralla and Provonole by the phospholipase YieldMax™ PL. Data provided from Chr. Hansen A/S, Denmark

	Normal process	With Phospholipase
Mozzarella (part skim, ≈ 40 +)	1 kg	1.018 kg (+ 1.8%)
Provolone (whole milk, ≈ 50 +)	1 kg	1.030 kg (+ 3%)

clotting and also minimizes the loss of fat in the stretcher, see Table 12. The mechanism for this effect is still not understood; however, it might be related to an increased interaction between the fat-globules and the casein structure.

2.6.4 Environmental Benefits of Enzymes for Baking

The life cycle analysis (LCA) of food technology have shown that the better utilisation of agricultural raw materials caused by the application of enzymes have significant environmental benefits.

The use of a maltogenic amylase to expand the shelf life of bread has provided the industrial bakeries new opportunities for changing their production and delivery set-up and has thereby allowed the industry to save both money and energy. However, the fact that there is less waste bread also means that the efficiency of utilisation of agricultural raw materials is improved.

To investigate this aspect an LCA study which exclusively addressed the reduction of waste bread was carried out. The study compared the environmental impact of increasing the dosage of Novamyl® from 14 g to 28 g per 1,000 bread consumed. The study was based on US conditions, as this is where the technology has so far had it greatest impact.

Table 13 LCA of the use of Novamyl for white pan bread in US

	Energy consumption	Global warming	Acidification	Nutrient enrichment	Smog formation
Actual reductions	532 MJ	54 kg CO_2 equiv.	0.3 kg SO_2 equiv.	0.3 kg PO_4 equiv.	0.1 kg C_2H_2 equiv.
Number of people equivalents (PE)	0.008 PE	0.007 PE	0.003 PE	0.01 PE	0.005 PE

The study illustrates that there are significant environmental gains to go for as a consequence of a better utilisation of agricultural raw materials. These gains should be added to the energy savings realised by the bakeries due to the possibilities of changing the production and distribution setup. The incentive of the bakeries to adopt the enzyme technology has been their own energy savings. This story illustrates that there is a significant additional gain to society from the bakeries doing so.

2.7 Enzymes as Additives to Animal Feed

2.7.1 Introduction

The use of microbial enzymes as feed additives is a young technology, compared to the use of enzymes in most other industries. Only in 1987, the first commercial microbial enzyme product targeting animal feed was launched by Finfeeds. This was a traditional fermented mixture of cell wall degrading enzymes from the strain *Trichoderma*, until then widely used for production of enzymes for the pulp & paper industry. Since then, increasingly specialized development of enzymes for the animal feed industry has taken place, and today a majority of enzyme suppliers worldwide have enzymes for feed in the product range. Today enzymes for animal feed make up approximately 10% of the market for industrial enzymes [164].

As a consequence of the continued intensification of livestock production in the industrially developed countries, this industry is subject to increasing environmental regulation, primarily concerning effluents from the animal husbandry.

This industry, as largely all other industries is dealing with the challenge of optimizing the production efficiency, and yet at the same time reducing the impact on the environment. The response to such a challenge is often "white biotechnology" [165] such as enzymes. Enzymes offer a proactive and cost-efficient way to cope with environmental impact of effluents. By addition of enzymes to the diet of the animals, the digestion of certain raw materials can

be enhanced. Not only does this provide an improved production economy (increased utilization of raw materials and a reduced need for nutritional additives), but at the same time it enables reduction of polluting nutrients in the effluents. As an example, a life cycle analysis (LCA) showing the global environmental impact of the use of phytase is presented later in this chapter.

This chapter gives a short overview of the most commonly used enzyme types in the animal feed industry, and the financial and environmental benefits of their use.

2.7.2 Overview

The use of enzymes makes it possible to reduce the need for adding supplementary nutrients to the diet, or to use lower grade raw materials without loss of production performance. As a matter of mass balance, an improved utilization of the nutrients will reduce the amounts of effluent from the production. For compounds like nitrogen and phosphorus, which have increasingly gained public attention, enzymes currently represent one of the best opportunities for reducing the outlet into the environment. An overview of the commonly used feed enzymes and their functions and benefits is given in Table 14.

2.7.2.1 Registration of Feed Enzymes

Whenever considering the development of enzymes for animal feed, one must be aware of the extensive regulatory requirements associated with registration of feed additives, especially in the European Union (EU). Enzymes used as processing aids in food or feed production are subject to strict regulatory requirements (product composition, safety, stability etc), but to register an enzyme as an additive for animal feed is even more complicated and a.o. requires proof of efficacy. The EU commission has established quite detailed guidelines for the efficacy documentation required to obtain EU approval of an enzyme product for animal feed. Among the requirements is that statistically significant effects of the product must be documented in three independent performance trials performed in at least two different member countries – per species (pigs, chicken, ducks, turkeys etc.) and category (e.g., sows, piglets, growing/fattening pigs etc.) [166]. After submission of the registration dossier the approval process may take up to several years. Thus, the registration demands can constitute a considerable barrier towards the introduction of new feed enzymes in many countries.

Table 14 Overview of enzymes commonly used for animal feed, their functions and main benefits

Enzyme	Feed substrate	Function	Production benefits	Environmental benefits
Phytases	All plant feedstuffs	Release of phosphate from phytate	Enhanced utilization of phosphate- and calcium in plant material. Reduced concentration of phosphorus in litter.	
β-glucanases	Barley, oats	Viscosity reduction	Enhanced digestion and utilization of nutrients. Degradation of anti-nutritional polysaccharides.	Improved utilization of nutrients naturally present in the feed raw materials, and consequently a decreased the need for adding supplementary nutrients to animal diets.
Xylanases	Wheat, wheat bran, rye, triticale	Viscosity reduction, plant cell wall degradation	Enhanced digestion and utilization of nutrients. Degradation of anti-nutritional polysaccharides.	Reduced amount of nutrients in the effluents from the production, and consequently reduced release of nutrients into the environment.
Galacto-sidases, galactanases	Grain legumes, Lupins	Viscosity reduction	Enhanced digestion and utilization of nutrients. Degradation of anti-nutritional polysaccharides.	Overall beneficial life-cycle balance compared to the use of non-catalytic additives (so far documented for phytase. See life-cycle analysis in this chapter).
Proteases	Any protein containing raw material	Hydrolysis of protein	Enhanced digestion and utilization of proteins. Degradation of anti-nutritional peptides.	
Lipases	Diets rich in lipids	Hydrolysis of lipid	Support for lipid digestion in young animals.	
Amylases	Diets rich in starch	Hydrolysis of starch	Support for starch digestion in young animals.	

2.7.2.2 Enzymes in Swine and Poultry Production

Swine and poultry production is the largest in the industry, utilizing approximately 65–70% of the industrially produced feed world wide [167]. Despite the anatomical differences of these species, the upper gastrointestinal tracts of monogastric animals have resembling limitations, and the range of enzyme products offered for pigs and poultry are similar in their functions. The enzymes sold today are mainly phytases and plant cell wall degrading enzymes, but other types of feed enzymes, such as lipases, proteases and amylases, are also present in the market, mainly outside of Europe.

Phytases

Monogastric animals such as swine and poultry have an insufficient capability to degrade phytate (myo-inositol hexakisphosphate), which is the main phosphorus (P) storage compound in plant feed stuffs and constitutes 60–70% of the total plant phosphorus. The insufficient phytate utilization in monogastrics necessitates supplementation with inorganic phosphates to industrial feeds in order to meet phosphorus demands during growth. Without P-supplementation, the growth performance of animals is seriously impaired, and bone mineralization is compromised. Inorganic feed phosphates, however, can be expensive and the excretion of undigested P from phytate or inorganic phosphate can lead to eutrophication of fresh water reservoirs in regions of intensive livestock production. The concept of applying microbial phytase in order to increase phytate-P utilization has been investigated for more than 30 years. Pioneering research carried out by Jongbloed, Kemme and others in the Netherlands provided documentation of the beneficial effects of microbial phytase in pig production [168–172]. It was shown that phytase improved overall P-digestibility by 25–30%, resulting from around 50% degradation of the cereal phytate.

Besides the improved phosphorus digestibility, it was also found that phytase application resulted in an improved digestibility of calcium and amino acids. The explanation for these effects lies in the chemical properties of the phytate molecule: The hexakisphosphate anion is a strong chelator of not only positively charged divalent cations such as Ca^{2+}, Mg^{2+} and Zn^{2+}, but also of amino acids via the positively charged amino groups. Thus, by degrading phytate a whole spectrum of nutrients can be released, thereby improving overall animal performance [173, 174]. However, only the effects of phytase on P- and Ca-digestibility are substantial while effects on digestibility of amino acids and metabolizable energy are comparatively small. Generally, application of phytase at their recommended dose allows replacement of up to 1 g total P and 0.8 g Ca per kilogram feed.

The groundbreaking research work was first commercialized by the Dutch company Gist Brocades (GB) in the mid nineties. GB launched the *Aspergillus niger* 3-phytase, and application of this enzyme allowed feed producers to significantly reduce the amount of inorganic feed phosphates, calcium and amino acids added to the diets. Thus, phytase application did not only help to reduce the overall P-excretion through animal manure but it also offered a means of substantial cost-saving for the livestock producers. The commercial success of phytase in the Netherlands had dynamic repercussions within the White Biotech industry community: Several other European enzyme producers such as Novo Nordisk/Novozymes (Denmark), Finnfeeds/Danisco (Finland and Denmark), and Primalco/Roehm (Germany) launched their own versions of *Aspergillus* phytase products in the European markets. The product of GB, NATUPHOS® (now manufactured by BASF), developed into a global sales success, only rivaled by the equally successful RONOZYME® P(CT), produced by Danish Novozymes (former Novo Nordisk) and distributed worldwide by DSM Nutritional Products (former Roche Vitamins). While the biological efficacy of NATUPHOS (*A. niger* phytase) and RONOZYME P (6-phytase from *Peniophora lycii*) are equal at their commercially recommended dosages, the RONOZYME phytase granulate has the advantage of the CT (thermostable) formulation which allows the product to be used in the production of pelleted feeds, where processing temperatures up to 90 °C are applied.

Phytase as a White Biotechnology success story has also inspired numerous Chinese and Indian producers to embark on commercialization of this technology in the world's fastest developing feed markets. In China, more than 30 phytase producers are fighting aggressively for market share, constantly seeking to improve efficacy and granulation quality.

Plant Cell Wall Degrading Enzymes (NSP Enzymes)

Feed cereals (primarily wheat, barley, oats and rye) have a high content of indigestible cell wall material, the so-called dietary fiber fraction, consisting mainly of non-starch polysaccharides (NSP), such as arabinoxylans and β-glucans. The soluble NSP's are considered anti-nutritional because of their ability to increase the viscosity of the intestinal contents, which has adverse effects on nutrient absorption through the intestinal cell wall [175, 176]. A too high viscosity in the intestines further leads to unhygienic wet litter ("sticky droppings") [177]

By the catalytic action of NSP-degrading enzymes (e.g., arabinoxylanases and β-glucanases), the undesirable effect of the soluble NSP's can be eliminated, thereby decreasing the sensitivity of the animal to NSP-containing raw materials. This in turn allows for an increased digestibility of nutrients, increased feed intake and growth [178, 179], and the wet litter can be prevented [180]. In addition to the evident viscosity effects of NSP enzymes,

some scientists propose an encapsulation theory, in which nutrients encapsulated in cell wall polysaccharides are released through the action of NSP enzymes [181].

Another, recently recognized, aspect of cell wall degrading enzymes is their documented effect on the composition of the intestinal flora. By allowing the release of nutrients in the upper intestinal tract, fewer nutrients become available for the microflora in the lower part of the intestine, which in turn may have a positive influence on the health and well-being of the animal. Supplementation of xylanase and β-glucanase to a wheat/barley diet was shown to significantly reduce the facultative anaerobic bacteria and *E. coli* in the intestine of broiler chickens [182], and positive effects on the intestinal flora of weaned pigs have also been demonstrated [183].

In addition to the production benefits described above, both the increased utilization of nutrients and an increased growth (shorter production period) have obvious beneficial environmental impact, because of the reduced effluent per animal produced.

2.7.2.3 Enzymes for Aquaculture

Also in fish farming, environmental concern is causing the authorities in many countries to place restrictions on the management of waste [184]. Like other animals, fish require phosphorus in their feed, and not all of it is digested. Thus, also for this species the phosphorus content of the effluent may become critical with intensive farming. It has been shown that the addition of phytase to plant-meal-based diets can increase the utilization of plant phosphorus and reduce phosphorus waste from salmonids [185], and phytases such as RONOZYME® P 20 000 L are registered and commercially available for fish.

Examples of physiological effects of other enzyme types, such as lipase have been reported [186], but financial or environmental benefits still remain to be demonstrated.

2.7.2.4 Enzymes for Ruminants

Enzymes for ruminants, particularly for beef cattle and dairy cows, are commercially available. These are mainly mixtures of carbohydrate degrading enzymes, which are claimed to improve production results. Literature shows many examples of positive effects of such enzyme products, resulting in increased body weight of beef cattle or milk yield of dairy cows [187], but also many indications of the opposite [188, 189]. The inconsistency of the enzyme response in ruminants is not surprising when taking into consideration the variability of diets, breeds and age of cows and not least their

sophisticated digestion system. Ruminants are characterized by their rumen, a complex fermentation system, employing a variety of anaerobic bacteria, fungi and protozoa, acting synergistically to degrade feed material and produce the fatty acids which are necessary for producing milk [190]. Thus, ruminants have their own substantial supply of microbial enzymes and are very well adapted to the traditional fiber-rich diets. However, it is not an unrealistic assumption that with the ongoing demand for higher-yielding animals and corresponding adaptations to the diet, supplementary enzymes may play a bigger role in the future.

Literature reports examples of positive effects of phytase supplementation on the phosphorus digestibility in ruminants [191]. Despite this, effects with significant impact on production economy have so far not been reported. Studies with other types of enzymes, e.g., proteolytic enzymes, have also been conducted but with limited success [192].

2.7.3 Latest Developments

Recent development has been concentrated on enzymes for the well-established application areas (phosphorus release and NSP degradation). An important research area has been the enzyme stability during high temperature processing of feeds. Improvements have primarily been obtained via optimized product formulations and through protein engineering on existing enzymes to further optimize their thermal stability. Also, the availability of mono-component enzymes for plant cell wall degradation has improved the possibility for securing consistent thermal stability and enzymatic activity in the application. In addition, bacterial sources of enzymes have been taken into commercial use, and new expression systems are emerging.

2.7.3.1 Processing Stable Products

To minimize the risk of microbial contamination of animal feed, heat processing is recommended. In most feed mills, a large proportion of the feed is processed into pellets. The pelleting process typically involves a heat treatment (80–90 °C) for 30–90 seconds, depending on equipment and stability requirements. Enzyme products that do not endure elevated temperatures must be sprayed onto the feed pellets in a later processing step, with the potential risk of a less even distribution of the enzymes in the feed. Thus, pelleting-stable enzymes are obviously attractive, which has caused enzyme manufacturers to focus on this parameter in their development of new enzymes. An example of such development is the CT ("Coated Thermostable") formulation technology, now commercially used in e.g., RONOZYME® WX (CT) and RONOZYME® P (CT). While the stability of RONOZYME P (CT)

is still unsurpassed, recent developments in phytase technology include application of protein engineering to increase the intrinsic stability of phytase enzymes. A recent example is the Quantum® phytase, a modified *E. coli* phytase developed by the California-based company Diversa in a joint venture with the Swiss company Syngenta.

2.7.3.2 Mono-Component Enzymes

The majority of the NSP-products supplied for the feed industry are multi-component products, containing the whole spectrum of enzymes produced by the originating microorganism, of which some may work synergistically in degrading certain cell-wall polysaccharides. With more knowledge of enzyme mechanisms and digestive physiology, it has become possible to identify and isolate the core enzyme activities of traditional multi-component products. By means of microbial cloning, such pure enzyme preparations have been made commercially available for technical applications. Where for a classic multi-component product characteristics like stability can vary between the various enzymes in the preparation, the physical characteristics of a mono-component product are well defined, and e.g., processing stability can be ensured. An example of such a product is RONOZYME® WX (CT), which is a pelleting stable mono-component xylanase. It is imaginable to combine such selected mono-component activities to produce tailor made multi-component products with good pelleting stability of all the active components.

2.7.3.3 Bacterial Enzymes

Traditionally, industrial enzymes developed for animal feed have originated from fungal microorganisms. In recent years, researchers have sought alternative donors of enzymes for this application. Two recently launched bacterial phytases, Phyzyme® (a wild type of *E. coli* phytase developed by Danisco) and Quantum® phytase (described above) are the so far most visible commercial results of this research.

2.7.3.4 Plant Expression

An alternative route to improved utilization of plant phosphorus has been taken by the "Green Biotechnology" [193] where two approaches have evolved. The first concept aims at using mutational breeding technology to create plant varieties with a low content of phytate while keeping the total P-level in the seed unchanged. Research in this area has been pio-

neered by Victor Raboy and co-workers with the US Department of Agriculture [194, 195]. Low phytic acid (LPA) lines of barley [196, 197], maize [198], soybean [199] and rice [200] have been developed but one barrier to commercialization is the lower yields exhibited by these variants in comparison to commercial elite lines.

The second "green" approach towards improved P-utilization lies in the use of genetic transformation of plants with microbial phytase genes [201]. By over-expressing a microbial phytase gene in plants the level of phytase can be increased [202], thereby eliminating the need for exogenous phytase. Although technologically feasible, at least two issues seem to slow down commercialization of this approach: First, the need for safe biological containment of genetically engineered plants has sparked serious political debates in several countries. This debate is reflected in increasingly stringent regulations. Secondly, a mechanism to prevent unlawful propagation of transgenic seeds is needed.

Taken together, solving the phytate/phytase challenge by means of Green Biotechnology seems to require more profound changes in acceptance, safety management, production economy, and intellectual property rights than the contained and controlled application of the White Biotech approach.

2.7.4 Environmental Benefits of Enzymes as Additives to Animal Feed

The major enzyme application in the animal feed industry is the use of phytase, which releases the phosphate bound in the grain and thus diminishes the need for addition of inorganic phosphate to the feed. Phytase was developed for use in animal feed based on the assumption that the substitution of inorganic phosphate with phytase would offer a significant environmental advantage.

To check this assumption, an LCA study has been carried out on the use of phytase for intensive pig farming. The study builds on Danish conditions, which means that the average phosphate binding capacity for agricultural soils has been assumed to be 95%. The results are shown in the Table 15.

Most significant is the reduction of the pollution of the aquatic environment with phosphate, which in Denmark due to the intensive pig farming is a major problem. It can be calculated that the effect of using phytase for all the pigs in Denmark (23 million) reduces the emission of P to the aquatic environment with 260 t P. This corresponds to approximately 25% of the diffuse emission of P from Danish agriculture. Another significant benefit of phytase is the saving of the consumption of phosphate, which is an essential, but limited resource. The phosphate saving from feeding all Danish pigs phytase corresponds to the annual consumption of phosphate from 1 million people.

Most other enzyme applications within animal feed address the efficiency of digestion of the grain nutrients. It is expected that such efficiency gains will

Table 15 LCA on phytase in animal feed

Reduction of potential environmental impact Per 1000 pigs	Energy consumption	Global warming	Acidification	Nutrient enrichment	Smog formation
Actual reductions	13 000 MJ	1100 kg CO_2 equiv.	33 kg SO_2 equiv.	50 kg PO_4 equiv.	0.44 kg C_2H_2 equiv.
Number of people equivalents (PE)	0.20 PE	0.13 PE	0.32 PE	2.0 PE	0.022 PE

drive significant environmental benefits – just as it has been demonstrated for the food industry.

2.8 Enzymes in Organic Synthesis

2.8.1 Introduction

An important parameter to consider when planning an organic synthesis process is the E-factor. This factor measures the efficiency of the process in terms of kg waste per kg product. Bulk chemicals have typically an E-factor of < 1 to 5, – fine chemicals have E-factors between 5 and 50. For certain pharmaceutical products the E-factor might even go up to 100.

White biotechnology processes have already demonstrated that they offer qualified alternatives to traditional chemical synthesis processes. DSM provided data describing Cephalexin production from from 1975 to 1985, which was produced in a ten-step process with conventional chemistry and a waste stream of 30–40 kg per kg final product. In 1985, the E-factor was reduced to 15, as a result of a long optimization process, and the introduction of recycling. After the introduction of enzymatic synthesis the E-factor dropped to < 10 in 1995. The goal is a reduction down to an E-factor of 2–5 by introduction of engineered microorganisms. Biochemie (Novartis) have reported a similar success story when producing 7-ACA. The E-factor has been reduced from 31 to 0.3 by changing to White Biotech processes [203]. To illustrate the potential of White Biotechnology in syntheses of performance and fine chemicals, we have chosen to describe two examples in more details. For review the reader is referred to Schäfer et al. 2003 [204].

Beta-lactam antibiotics has been selected as the first example in Sect. 2.8.3 as this industry segment has been the most important playing field for White Biotechnology over the last 30–40 years. Production of polymers has been

chosen as the second example to illustrate issues in a potential very big future industry segment for White Biotechnology in Sect. 2.8.4.

2.8.2 Overview

A variety of different enzymatic activities have been explored within organic synthesis. Most enzymes used are various hydrolases. Especially lipases have found use in a number of different applications. As these enzymes are designed in nature to operate at an oil-water inter-phase they are, generally, very compatible with organic solvents. Furthermore, lipases catalyze a variety of different reactions being very different from the reaction they are designed for in nature which is the hydrolysis of triglycerides. One particular lipase, the B-component from the yeast *Candida antarctica*, has in numerous applications been shown to be a particularly efficient enzyme catalyzing a great number of different reactions including both regio- and enantioselective syntheses and even reactions involving both sulfur and nitrogen based nucleophiles [205]. Other hydrolases of significant importance within organic synthesis are nitrilases, esterases, amidases, peptidases, and hydantoinases. A few oxidoreductases have also gained industrial importance but this potential of this enzyme-class is, as outlined below, still in the early phase of exploitation

2.8.3 Example: White Biotechnology and Synthesis of Beta-Lactam Antibiotics

All production of beta-lactam antibiotics (in 2005 more than 50 000 tons beta-lactams were produced per year) starts with a fermentation process. Just three products: Penicillin G, Penicillin V, and Cephalosporin C are the starting points for all semi-synthetic beta-lactam antibiotics. Initially the product spectrum was increased by adding various precursors to the culture broth, but it became soon clear that the productive microorganisms only accepted aliphatic or aryl aliphatic carboxylic acid side-chains.

The base beta-lactams (6-APA, 7-ADCA, and 7-ACA) were discovered around 1960. New product discoveries thereafter centered around semi-synthetic beta-lactams constructed from base beta-lactams (produced by fermentation, and hydrolysed), and chemical synthesized side-chains (from petro-chemical sources).

It is interesting to note that as early as 1960 an enzymatic hydrolysis of Penicillin G to 6-APA was reported by four companies (Bayer, Beecham, Bristol-Myers, and Pfizer). Bayer and Beecham cooperated in developing an industrial process, and succeeded around 1968, where after they for several years had enzymatic and chemical hydrolysis processes running in parallel for more than ten years, before finally the chemical route was abandoned.

Bristol-Myers and Novo Industry (now Novozymes) developed an enzymatic industrial process for making 6-APA from Pen V around 1980 utilising a Penicillin V acylase from Fusarium oxysporum. For splitting Cephalosporin C to 7-ACA a two enzyme process was developed by Biochemie (now Novartis) in the 1970s. A D-Amino acid oxidase catalyses the oxidative deamination of the adipoyl side chain group to the alpha-keto adipoyl derivative. This looses carbon dioxide in the presence of oxygen and the resulting glutaryl derivative is hydrolysed by a glutaryl acylase to 7-ACA.

Several companies thereafter continued striving to develop enzyme processes for making the semi-synthetic beta-lactam products – initially without luck.

The enzymatic synthesis functions initially well, but when product yield rose to 70–75%, the hydrolysis rate of the semi-synthetic beta-lactam became larger than the synthesis rate. Furthermore the activated side chain needed to drive the synthesis reaction was also hydrolysed by the enzyme used.

The breakthrough came around 1989 at Novozymes when the enzymatic process catalysed by the E. coli Penicillin G acylase was tried in supersaturated conditions. In an aqueous process where 6-APA initially was present as crystals, together with D-phenylglycine activated as amide also present a crystals the immobilised Pen G acylase created a yield of around 90% conversion as the formed Ampicillin was not hydrolysed because it reached the saturation point and started crystallising. This process was thereafter optimised during a couple of years. The best reactor turned out to be a standard tank with a sieve-bottom, as it allowed the immobilised enzyme to stay in the reactor, when the solution including the substrate and product crystals were purged. A centrifuge would return the solution back to the reactor after the crystals were separated. More substrate crystals were added to the reactor and the reaction could continue up to seven times before the ion-strength of the solution became too high. This process allowed the yield to rise up in the mid-nineties. The substrate and product crystals were separated by a pH adjustment. The left-over substrate returned to the reactor, and the product re-crystallised. Crystallising beta-lactams is much easier in water than in methylene chloride traditionally used. Surprisingly, the purity of the resulting semi-synthetic beta-lactams turned out to be purer (more than 99%) than the USP-standards (98.9% purity), which were considered the highest obtainable purity. Standard semisynthetic beta-lactams produced chemically are typically 97% pure. Another surprise was that the semi-synthetic beta-lactams produced by the enzymatic process were without the normal beta-lactam off-taste. Accordingly, it was concluded that the off-taste which is disliked by most users of beta-lactam antibiotics is not a characteristic of the beta-lactam itself, but rather a characteristic of by-products which are present in chemically synthesised beta-lactam products.

This enzymatic synthesis process was scaled up to pilot scale at Novozymes and then acquired by Gist-brocades (now DSM) at the end of 1994. Up-scaling

to industrial scale took place in the second half of the 1990s. Today significant quantities of Cephalexin, Amoxicillin and Ampicillin are produced by such enzymatic routes primarily by DSM.

An interesting possibility is that the enzymatic splitting process, and the enzymatic synthesis process are both performed as aqueous processes. A combination of the two processes into one aqueous process is definitely a possibility with further yield increases as result if the intermediate base beta-lactam product is eliminated as a recovered product. Such a combination process is, however, not yet scaled up to industrial scale [206, 207].

2.8.4 Example: White Biotechnology and Synthesis of Polymers

In nature, living organisms are constantly producing different macromolecules for their metabolic needs. These macromolecules, such as polysaccharides, proteins, or polyesters are essential to survival of the organisms. There is still a long jump from such macromolecules to polyester polymers as they are typically produced in industry today.

Raw materials for making polyester polymers are all derived from crude oil. Polyesters are typically produced utilising Ziegler-Natta catalysts (which contains transition metals from group 4 to 7, and an organometallic compound of a metal from groups 1 to 3 of the periodic table) at process temperature around 200–250 °C. There seems at present to exist a growing interest for evaluating process alternatives. The development of the crude oil price the next 10–20 years is quite unpredictable and prices might go significantly higher than the 60 USD/barrel of today.

What does an enzymatic process alternative offer? A gradual change from the chemical processes/crude oil derived substrates to enzymatic processes/crude oil derived substrates is a realistic alternative in the near future, before the possible further change to white biotechnology polymers (produced by combined fermentation and enzyme processes).

Enzymes as alternatives to Ziegler-Natta catalysts offer a reduction of process temperature from 200–250 C to 60–110 °C, which may reduce costs for heating, as well as reduce process time for heating up and cooling down.

New product properties like a narrow MW distribution, and new products which has not been possible to produce by conventional chemistry might be possible to make. Ziegler-Natta catalysts tend to stay in the final product and although used only in ppm-quantities Ni might cause allergic reactions for certain individuals.

Examples of enzyme applications are:

1. polycondensation of hydroxy acids or dicarboxylic acids with diols,
2. polymerisations of cyclic diacid anhydrides with diols or oxirans,
3. polymerisations of polyanhydrides with diols,

4. transesterification of polycaprolactone and poly(alkylene dicarboxylate)s,
5. ring-opening polymerisation of lactones,
6. copolymerisation of lactones with aliphatic polyesters,
7. polycondensation of dialkyl carbonate and diol.

Although many synthetic reactions catalysed by enzymes have appeared in synthetic organic chemistry, relatively few examples of industrial polymerisation reactions have been reported so far. Perhaps the earliest large-scale synthesis of polyesters using enzymes was reported by Binns et al. [208]. Condensation of adipic acid and 1,6-hexanediol was successfully scaled-up to a 0.5 tonne level. Constant removal of water from the Novozym 435-catalysed polymerisation process is crucial since it is apparent that without the consequent shift in equilibrium, the reaction will not go to completion [209, 210].

Besides using lipases for polymerisation reactions lipases of course also have industrial potential in depolymerisation (recycling) processes.

2.8.5 Environmental Benefits of Enzymes for Organic Synthesis

When enzymes substitute conventional catalysts the typical environmental benefits are lower processing temperatures, fewer processing steps and less waste. Typically the yield and purity is also higher.

As an example is presented the LCA study of the production of fatty acid esters by means of Novozymes 435. The conventional process used for comparison is a process based on tin catalysis. The study builds on conditions at a German manufacturer of fatty acid esters who operates both types of processes. Table 16 shows the results.

The enzymatic process is superior to the tin catalysed process for all the considered impact categories. Major reasons for the reduced environmental impact are savings of electricity for heating and substitution of the tin catalyst. The environmental benefits of using the enzyme are assumed to be

Table 16 LCA on enzymes in organic synthesis

Reduction of potential environmental impact Per 1 ton of fatty acid ester produced	Energy consumption	Global warming	Acidification	Nutrient enrichment	Smog formation
Actual reductions	2600 MJ	190 kg CO_2 equiv.	1.9 kg SO_2 equiv.	0.1 kg PO_4 equiv.	0.07 kg C_2H_2 equiv.
Number of people equivalents (PE)	0.038 PE	0.02 PE	0.02 PE	0.005 PE	0.004 PE

smaller if the alternative process is based on acid catalysis and heat derived directly from gas or oil. Nevertheless the study illustrates that enzyme technology holds great potential as the environmentally friendly alternative in organic synthesis.

3
Technologies for Discovery of Enzymes in Brief

3.1
Introduction

Discovery of industrial enzymes is a multidisciplinary effort involving a wide array of different technologies. As each screening project is uniquely designed to solve unique application problems it is not possible to define per se which method is the most important one, but it is obvious that major players have to master a variety of technologies which often in combination lead to the solution. Nature holds a wonderful diversity of organisms and the corresponding wealth of enzymes. For a variety of applications even nature's assortment faces some limitations. It is the challenge for scientists to optimise the natural enzymes and to generate additional and "artificial" diversity to tailor-make enzymes for a given application. Finally, many more enzymes have already been made available as products for several applications. For all approaches it is important to stress that it is not the *broadest* possible diversity, but rather the highest possible *quality* of diversity which will lead to the ultimate goal, namely a novel product addressing the exact and specific demands of an industrial enzyme application. In this respect selection/deselection via perfectly designed assays is of utmost importance indicating the significance of linking process understanding to biochemistry. Natural diversity approaches and optimisation strategies are complementing routes and both are equally important to develop a high-quality diversity of enzymes.

3.2
Diversity Input to a Screening Program

One of the main questions which has to be answered at the start of each discovery initiative is "where to look for diversity" [211]. There are various potential sources as input to screening programs basically divided into (a) natural protein diversity and (b) manmade diversity, which are comprehensively reviewed in [212] and in [213]. Here we will summarize some basic principles.

The challenge is that Nature's diversity virtually is infinite and independent which strategy is used all screening efforts face a limitation as we are only scratching the surface. Isolated microorganisms, bacteria, fungi and archaea,

which are stored in culture collections, single strains described publicly and which are known to catalyze a certain reaction, or complex environmental libraries without the need to culture the corresponding microbes [214] comprise the biological material which is used as starting material.

Several strategies can be followed in order to optimise the properties of enzymes found from nature. A simple way to look at protein optimization technologies is to divide the field into rational protein engineering and molecular evolution. Rational protein engineering is based on the ability to create protein variants with designed and deliberate amino-acid alterations at any desired position provided the capability of precise probing of structure-function relationships in proteins [215].

The basic principle of molecular evolution is to carry out the more or less random introduction of mutations thereby generating DNA libraries consisting of thousands or even millions of variant genes. The DNA variation is expressed into protein diversity in a variant library where the tight connection between the variant protein and its encoding gene is necessary to reveal the identity of improved proteins isolated from a high through put screening approach [216].

3.3 Diversity Output: How to Screen

After the question "where to screen" has been answered, the next question is "how to screen" [211]. There are several possibilities and the most important and generally preferred route is via functional screening assays. This allows one to screen libraries of microorganisms, clones and/or variants for a wanted enzyme activity. The approach requires that the problem that shall be solved by an enzyme can be translated to a biochemical assay which ideally can be screened in high throughput as the input material offers up to millions of variations. This is often a real challenge to biochemists as the biophysical matrix in the target application is often very complex and it can hardly be decided upfront which criteria are the most important ones. Accordingly, hypotheses are often built in the initial steps of screening. Ideally, functional screening procedures help to select the best performers of a given library. The best candidates might be chosen as product candidates directly or alternatively form the starting point for the next round of the repeated directed evolution cycle depending whether a performance gap still exists. In this round enzymes from natural diversity or protein engineered variants might be included if they show beneficial characters for a given application.

For delivery of monocomponent wild type enzymes for application testing cloning of genes and expression of proteins is an important step. In most cases a technique called expression cloning is used which is an effective means of isolating a gene from a gene library based on its encoded activity. Monocomponent enzymes produced by recombinant DNA technol-

ogy are preferred in small-scale applications to clearly refer measured effects to a given protein. Sequence based approaches also described as molecular screening complements functional screens. This is based on similarities between enzyme-encoding gene sequences [217, 218]. Sequence information from a set of related enzyme genes is used to identify evolutionarily conserved regions and to design PCR primers to screen and to clone additional genes from other organisms. Using this method, a number of genes homologous to the initial gene sequences can quickly be identified. The limitation of the method is that enzyme variants rather than totally novel enzymes are detected. The advantages, on the other hand, are that this method is not dependent on growing the strains in the lab or active expression of a protein, which actually makes this an interesting option for screening of metagenomic libraries. Secretome studies [219], transriptomics and proteomics gain more importance as screening tools [220]. All these approaches have drastically increased the amount of data available in public databases. Additionally, hundreds genome projects have led to an explosion of data. The current status on established genomes and those underway can be obtained by visiting the home page of the TIGR institute (http://www.tigr.org/), or the home page of the DOE Joint Genome Institute (http://genome.jgi-psf.org/). As a consequence relatively new disciplines, genomics and bioinformatics, have developed during the last years and those disciplines are considered key for future developments.

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Building Blocks

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Abstract This contribution illustrates the versatility of fundamental approaches in industrial biotransformations. The applicability of biotechnology in organic synthesis on an industrial scale is discussed, followed by an overview of historical development and future progress. This chapter depicts three different approaches for the use of biocatalysts in production processes: non-chiral synthesis, asymmetric synthesis, and racemic and dynamic resolution. Applications for whole cells and isolated enzymes as catalysts are introduced. Finally, critical but optimistic conclusions are given.

Keywords Building blocks · Non-chiral synthesis · Asymmetric synthesis · Racemic resolution

1 Introduction

Based on the analysis of technology, market trends, and current R&D activities, McKinsey and Festel (in their very optimistic studies [1, 2]) estimate that biotechnology can be applied to the production of 10–20% of all chemicals by the year 2010. However, the rate at which biotechnology processes

are introduced and used in different chemical markets varies. To prepare such products as fine chemicals, for example, sugars are converted by tailor-made microorganisms, enzymes, or chemico-physical treatment [3]. These may come from degradation of raw materials, including byproducts from agricultural sources and households. Typical products of industrial biotransformations include enzymes, vitamins, flavors, and fine chemicals such as chiral building blocks for the pharmaceutical industry [4]. These biochemicals may be divided into bulk chemicals and fine chemicals according to the amount produced and the price per ton.

This chapter illustrates the versatility of fundamental approaches in industrial biotransformation and is not expected to give a complete overview. For further information the reader is referred to recent literature [5–11]. For a clearer picture the section on synthesis (Sect. 4) has been subdivided into the three different approaches: non-chiral synthesis (Sect. 4.1), asymmetric synthesis (Sect. 4.2), and racemic and dynamic resolution (Sect. 4.3). These examples are once again subdivided into the application of whole cells or isolated enzymes as catalyst.

2
Biocatalysis and Chemical Building Blocks

The economics of specific processes are important for the success of biocatalytic steps in the chemical industry. A biocatalytic step will always be compared with the conventional organic synthesis. Only if the biocatalytic step is saving money, by higher selectivity and lower process costs compared to established chemical processes, will it prevail. However, the target will not be the replacement of a single chemical step by a biocatalytic one, rather the target will be the total redesign of the synthesis route to comprise a sequence of chemical and biocatalytic steps. This was successfully demonstrated by DSM in a total redesign of the synthesis route to cephalexin. The original process started with the fermentation of *Penicillinum chrysogenum* yielding penicillin G. This was followed by eight chemical steps yielding the final antibiotic cephalexin. In the present process, the latter classical route was replaced by two biocatalytic and one chemical step. In the near future this synthesis will be totally redesigned so that the product cephalexin is directly obtained from one fermentation step starting from a carbohydride source. The sequence of three biotransformations in series will be carried out in the fermented cells, integrating one expandase and two acylase steps. This demonstrates the enormous power introduced by industrial biotechnology into the synthesis of chemicals.

The difference between a biocatalytic processes and a conventional chemical process is the biocatalyst. Enzyme kinetics, stability under technical conditions, and features that derive from its role in the cell's physiology – growth,

induction of enzyme activity, use of metabolic pathways in multistep reactions – have strong influence on the industrial process. The requirements and issues of the remaining factors of biocatalysis in organic solvents are comparable to standard organic chemical processes. Therefore, more experience in application of enzymes will lead to an easier access to further biocatalytic steps. The selectivities of enzymes are generally high, but there are problems concerning substrates in that there is often a limited substrate spectrum and limitations in the steric demand and electronic properties of different starting materials. In addition to high selectivity, high activity is also important because on the industrial scale high space–time yields are desired. Solvent tolerance, substrate versatility, and temperature stability can also now be improved by the methods of molecular biology.

The challenge is to develop new biocatalytic processes that are useful in organic synthesis. For this purpose biocatalysts must be identified and processes must be set up. Also, many different parameters must be optimized by the specialists of these particular fields (Fig. 1); An efficient industrial process will be facilitated by overcoming the limiting aspects of the biocatalytic process. In addition to the examined process and its reactants and products,

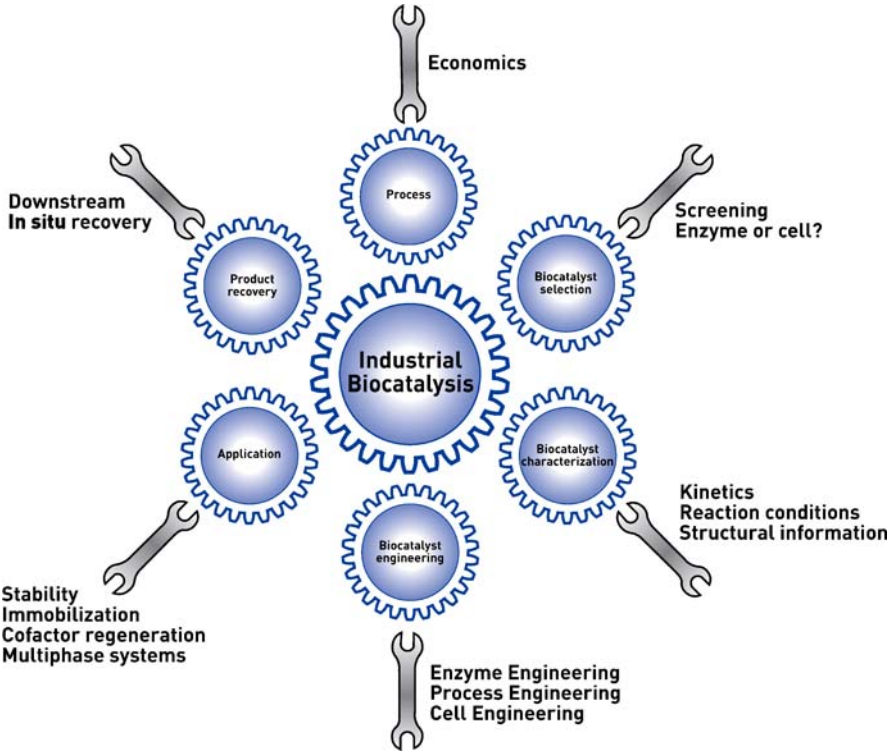


Fig. 1 Biocatalysis cycle

novel reactions can be found during process and enzyme engineering [12]. A highly fascinating new approach is to make use of the promiscuity of enzymes, as described by Hult et al. [13–17].

Analysis of industrial biotransformations has shown a general tendency towards natural compounds or their derivatives; however, there is no one prevailing class of natural compounds. Biotransformations leading to carbohydrate and fat derivatives are mostly found in the food sector, whereas the remainder of the compounds are mostly applied in pharmaceutical and agricultural sectors. All in all, taking into account the number of processes used, industrial biotransformations are most commonly used in the pharmaceutical sector. Only a small number of products are made in bulk, perhaps indicating that biotransformations are particularly valuable in the fine chemicals segment. However, a new drive in the development of biotransformation applications in bulk chemical production is developing at present. The field of renewable resources plays an especially important role in this area.

Chiral configuration is a key issue. Chirality originates for most of the enantiomerically pure products directly from the precursors. The enantiomeric purity is facilitated only by a small number of biotransformations, using both kinetic resolution and asymmetric synthesis. In the majority of all cases kinetic resolutions involve hydrolases, and asymmetric syntheses involve oxidoreductases as well as lyases. The majority of redox biotransformations are carried out using metabolizing cells containing the complete kit of enzymes, including the oxidoreductases. Whole cells are also used in many non-redox biotransformations where the cells are not metabolizing and only one or two key enzymes are active. In general, whole cells are more popular than isolated enzymes [18]. One reason for this is the additional labor and cost required for the production of isolated enzymes, e.g., cell disruption and downstream purification.

3 History and Development

For industrial application of biotechnological synthesis methods, two production types have to be differentiated. Firstly, organic building blocks can be produced using either fermentation or whole cell technology. Secondly, synthesis can be carried out using isolated enzymes. In both cases the final products can be distinguished as achiral or chiral. Chiral products, in turn, can be generated via racemic resolution or through direct asymmetric synthesis.

Historically the use of whole cells is by far the oldest technique, for example in the production of cheese or beer. It must be noted, though, that when producing food using whole cells, there is no fundamental knowledge implemented. Production is merely based on trial and error. One product

that has built a bridge between unknowing application and targeted production is the synthesis of penicillin, due to available knowledge concerning the existence of microorganisms. Nonetheless, new production methods are preferably implemented if they are controllable and predictable. In most cases profound understanding avails. Therefore, development during the 20th century has moved towards using single enzymes, since it has been easier and faster to develop a greater understanding of the reaction mechanisms and kinetics of these processes. Thus, the deep knowledge of one single biocatalyst has led to its preferred implementation over the very complex biochemical synthesis methods using whole cells. This way, defined reactions can be steered by using a limited amount of components. In the meantime, detailed knowledge of the catalytic reaction, in combination with the structural details of the protein, leads to the standardized evolution and further development of proteins and enzymes.

Both asymmetric syntheses and racemic resolution, using individual enzymes, are applications that can only be used for producing expensive building blocks, due to the high costs of the preceding biocatalyst purification and its limited stability. Non-chiral products are rarely produced via biotransformation (catalyzed by individual enzymes) since their production must always compete with standard chemical syntheses. Here it must be mentioned that, especially for chiral products, the yield and selectivity of a reaction have a crucial impact on the cost of the final product. Thus, when forming a racemate with 100% conversion, a yield of only 50% relative to the desired enantiomer can be obtained. In the case of only 50% conversion of the reactant, a maximum of 25% of the desired enantiomer can be produced. Continuing this observation towards a selectivity of 100%, the formation of only one enantiomer, a conversion of 100% also leads to a yield of 100%, whereby a conversion of only 50% remarkably leads to a yield of 50%. This brief discourse illustrates why production costs can be much higher when a reaction requires a selectivity for one isomer or enantiomer relative to the costs for achiral products. In turn, this also explains the low number of achiral biocatalytic reactions. Thus, bulk chemicals, which must be produced in a large amount at low cost, can hardly be synthesized using enzymatic transformation. Nonetheless, their synthesis can also be carried out using biotechnology at competitive prices when implementing fermentation techniques.

This is a tendency that has been increasingly observed over the past 20 years and can be further accelerated through increasing knowledge of synthesis routes, metabolic networks integrating thermodynamics and kinetics and their interaction within the cell (system biology). Thus, the use of whole cells (resting, immobilized, etc.) is implemented parallel to classical fermentation for synthesizing various building blocks. Lately, this tendency has been further accelerated by new knowledge about direct metabolite concentration determination within the cell, as well as the possibility of direct intervention into metabolic processes. This development originates in the formation

of so-called designer bugs, which have an optimized metabolism designed for a specific synthesis route, much like the evolutive optimization of single proteins. Here, one enzymatic step is not followed by the next, but rather the complex biochemical equilibrium of the cell is shifted towards the desired product as far as possible. Selectivity and yield of the enzymatic biocatalysis are combined with the stability and capability of cofactor regeneration of the single cell.

4
Biotechnological Synthesis

4.1
Non-chiral Synthesis

Biological catalysts, proteins, were already used by men long before Paracelsus and Oswald conducted their investigations, without anybody even being aware of their existence in the production of food and beverages. These were the first large-scale non-chiral syntheses. Even before 6000 BC the Sumerians and Egyptians practiced beer brewing, and the Egyptians used yeast for baking bread. There are even references to wine making to be found in the Book of Genesis. However, the utilization of biocatalysts for the production of defined chemicals such as alcohols and organic acids by fermentation only surfaced in the second half of the 19th century. In due time it was discovered that microorganisms can be used to modify certain compounds by simple, chemically well-defined reactions. Nowadays, there are only a limited number of biotransformations known that lead to non-chiral bulk chemicals. The only examples that were successful in the past and will be successful in the future are those where the new biocatalytic routes are economically more attractive than the established ones. Examples in this area are acrylamide, high fructose corn syrup (HFCS), and nicotineamide.

In this contribution we will only address biotransformations, where there are only a limited number of catalytic steps between the substrate and product, in contrast to fermentation where there are several catalytic steps. Additionally, in the biotransformations addressed here there is the special distinction that the chemical structures of the substrate and the product resemble one another. This is not necessarily so in fermentation. However, according to the study of McKinsey and Company, industrial biotransformations will have a significant influence on the production of bulk products and polymers in the future [1, 2]. Additional non-chiral large scale products with a high market volume are those from the food, feed, and agricultural industries. From the viewpoint of sustainable development, meaning that biotransformations start from renewable starting materials, a new area of research is opening up. Here, the implementation of process engineering at an early research

and development state is crucial. Nowadays, the application of biotransformations is still mainly focused on the direct transformation of a starting material to a defined product. In the future, degrading biotransformations will play a more important role (e.g., breaking down starch or lignocellulose) in view of freeing starting materials for the synthesis of small molecules in a biorefinery. Also, the synthesis of paint components or the synthesis of new biomaterials will be new topics for the future.

4.1.1 Non-chiral Products Synthesized by Whole Cells

The biocatalytic synthesis of acrylamide **2** is the first example of an industrial biotransformation in the petrochemical industry (Fig. 2) and of the successful enzymatic manufacture of a bulk chemical (30 000 t a^{-1}). The strain *Rhodococcus rhodochrous* J1 produces two kinds of nitrile-converting enzymes, nitrilase (EC 3.5.5.1) and nitrile hydratase (EC 4.2.1.84), and is used for acrylamide production by Nitto Chemical Industry Co. [19, 20]. Acrylamide is an important commodity monomer used in coagulators, soil conditioners, and stock additives for paper treatment and paper sizing, as well as for adhesives, paints, and petroleum recovering agents. Because it is unstable and polymerizes easily the process is carried out at a low temperature of 5 °C, but shows a conversion of 100%.

Nitrile hydratases catalyze the formation of nitriles via addition of water to amides. The enzyme has been found in different bacteria and belongs to the enzyme class of lyases. Nitrile hydratases belonging to lyases are not to be confused with the nitrilases belonging to hydrolases, which convert nitriles to the corresponding carboxylic acids. Various amides are produced by immobilized whole cells of *Pseudomonas chlororaphis* and *Rhodococcus rhodochrous* from nitriles as adiponitrile **3**. Thus, 5-cyano-valeramide **4** is synthesized by DuPont using *Pseudomonas chlororaphis* B23 cells, which are immobilized in calcium alginate beads (Fig. 3). The criterion for the selection of the strain

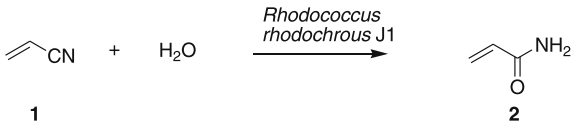


Fig. 2 Biotransformation to produce acrylamide by *Rhodococcus rhodochrous* J1

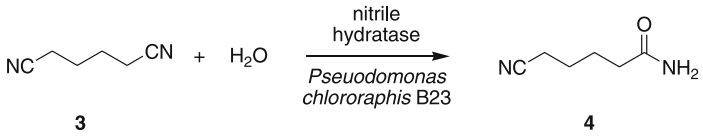


Fig. 3 Production of 5-cyano-valeramide **4** by addition of water to adiponitrile **3**

was the absence of any amidase activity, which would lead to further hydrolysis of the amide to the carboxylic acid. This biotransformation was chosen over the chemical transformation because of the higher conversion, selectivity, and production of higher amounts of product per catalyst weight, as well as less waste [21, 22].

Due to the mutagenic and thermal properties of the reactant, the chemical synthesis starting at the di-*N*-oxide is considered unsuitable for scale-up. Nevertheless *P. putida* is able to catalyze the biotransformation of 2-methylquinoxaline **5** applying benzylalcohol as inducer and sole carbon source (Fig. 4). This reaction is sensitive to the accumulation of substrate and benzylalcohol and performs poorly at substrate concentrations above 1.5 g L^{-1} and benzyl alcohol concentrations above 1 g L^{-1}.Therefore, addition of substrate and benzyl alcohol must be carefully controlled to achieve high yields. Thus, this biotransformation illustrates the possibility of overcoming the limitations of bioprocesses by reaction engineering. Quinoxaline and piperazine, as compounds with two nitrogen atoms, are better substrates for *P. putida* than quinoline derivatives. This process yields a product concentration of more than 10 g L^{-1}, which is a commercially feasible synthesis. The product is a building block for a broad variety of biologically active compounds [23].

The first commercial production of Xolvone (1,5-dimethyl-2-piperidone) **11** employed direct hydrogenation of 2-methyl glutaronitrile **10** in the presence of methylamine. In this process, a mixture of 1,3- and 1,5-dimethyl-2-piperidones is produced. The following example illustrates the combination of a biocatalytic step and a chemical step, where the chemoenzymatic process (shown in Fig. 5) is scheduled to replace the current chemical process.

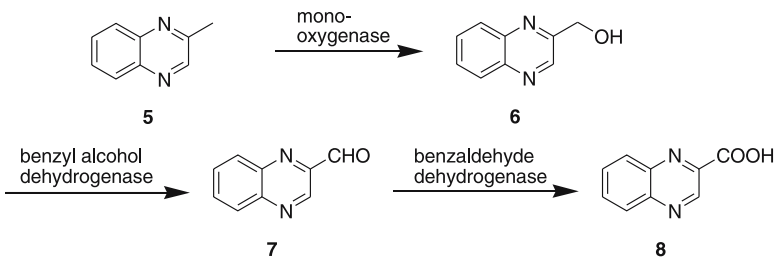


Fig. 4 Bioconversion of 2-methylquinoxaline **5** by *P. putida*

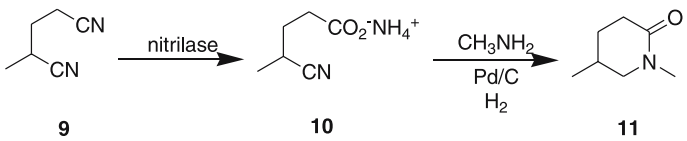


Fig. 5 Production of Xolvone **11** using a chemo-enzymatic pathway

This process produces a single geometric isomer of dimethyl-2-piperidone with a higher boiling point than the mixture of geometric isomer produced in the chemical process. The chemoenzymatic pathway (nitrilase, EC 3.5.5.1) shows a higher yield and less byproduct formation, resulting in a productivity of 3500 g product per gram biocatalyst. The volumetric productivity was increased by using immobilized *E. coli* transformant in alginate. Xolvone is not flammable, is completely miscible with water, has a good toxicological profile, and is readily biodegradable. Therefore it is a good precision cleaning solvent and is used in a variety of industrial applications, including electronics cleaning, photoresist stripping, industrial degreasing, metal cleaning, and resin cleanup. It is also used in the formulation of inks and industrial adhesives, and as a reaction solvent for the production of polymers and chemicals [24, 25].

4.1.2
Enzymatic Pathways to Produce Non-chiral Products

The following process demonstrates the utilization of a free enzyme within the production of a large quantity chemical. The nicotinamide synthesis carried out by Lonza on an industrial scale is based on the non-selective nitrile hydratase (EC 4.2.1.84) from *Rhodococcus rhodochrous*. In this biotransformation, 3-cyanopyridine **12** is converted to nicotinamide **13** (also called vitamin B3), which is used as a vitamin supplement for animal feed. The continuous process is carried out on a scale of 3000 t per year (Fig. 6).

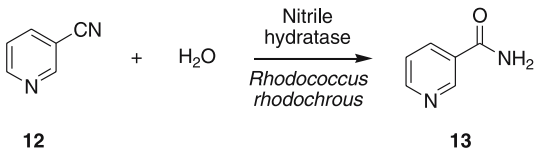


Fig. 6 Synthesis of nicotinamide **13** applying nitrile hydratase from *Rhodococcus rhodochrous*

4.2
Asymmetric Synthesis

In principle there are two different approaches for producing chiral centers: asymmetric synthesis and racemic resolution. From the viewpoint of reaction engineering, the major difference is the maximum reachable yield. In the case of asymmetric synthesis, stating that the enzyme is totally enantioselective, 100% yield and 100% enantiomeric excess is possible. This is true for asymmetric reductions as well as for asymmetric C – C couplings. However, in a classical kinetic resolution, in contrast to the asymmetric synthesis

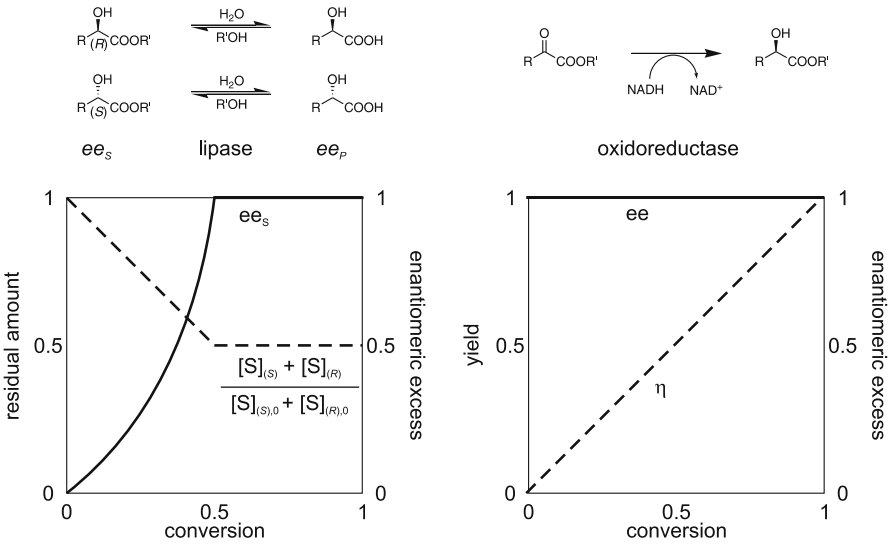


Fig. 7 Comparison between lipase catalyzed hydrolysis and oxidoreductase catalyzed reduction. Enantiomeric excess and residual amount respectively yield vs. conversion

starting from a racemate, only a maximum yield of 50% reaching 100% enantiomeric excess is possible in an ideal case (Fig. 7). The selected route depends on the cost and availability of the starting materials as well as on the enantioselectivity of the catalyst.

4.2.1 Asymmetric Products Synthesized by Whole Cells

In nature you find many synthesis pathways, which are mostly reaction sequences. In this way cells are able to synthesize chemicals with high selectivity and in a well regulated manner. The intermediates produced can be immediately converted by other enzymes so that no concentrations are reached that could be inhibiting or denaturating for the cell. All these reaction sequences must follow the principle of Le Chatelier, which means that the conversion and therewith the removal of products lead to new synthesis of these products. If the final product is not removed or further converted, no new product is synthesized and the whole synthesis pathway stops reaching thermodynamic equilibrium.

The production of maleic acid illustrates the versatility of enzymatic synthesis. Manufacturing of both pure enantiomers is possible by applying the complementary biocatalysts malease and fumarase. Maleases (EC 4.2.1.31) belong to the enzyme class of lyases and have been found in many bacteria, yeasts, and fungi but also in plants and animals. *Pseudomonas pseudoalcaligenes* is used in a commercial process of DSM to produce D-malic acid

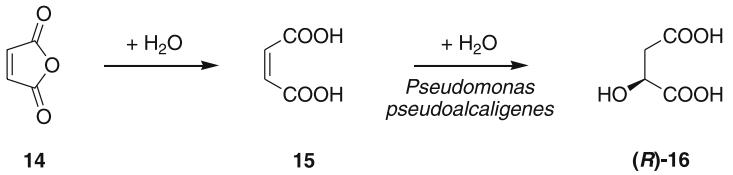


Fig. 8 Commercial process to produce D-malic acid ((*R*)-16)

(Fig. 8). It was identified by thorough microbial screening [26]. D-Malic acid ((*R*)-16) is a chiral 2-hydroxy acid useable as chiral synthon, as resolving agent, or as a ligand in asymmetric synthesis. Instead of maleic acid, the cheaper maleic anhydride **14** can be used since hydrolysis in situ occurs. Optimization of this process is still a matter of research. In comparison to this process, the enantioselective addition of water to the double bond of prochiral fumaric acid catalyzed by fumarase (EC 4.2.1.2) can be seen. Tanabe uses immobilized brevibacteria or fungi to produce L-malic acid in that way.

To produce amino acids, their isolation as components of natural protein-containing materials is done by extractive processes. Alternative conventional organic chemical and biotechnical methods allow the targeted synthesis of amino acids. In principle all proteinogenic amino acids are accessible by fermentation methods. Economic reasons are responsible for the production of the high-turnover chiral products L-glutamic acid, L-lysine, and L-threonine by fermentation processes. By using modern production strains, glutamic acid and lysine concentrations of more than 160 g L^{-1} are reached [27]. The secretion of the amino acids results from an active transport across the cell membrane [28]. The first steps in the bacterial synthesis of the amino acids mentioned before are catalyzed by the same enzymes. To produce threonine a further understanding of amino acid biosynthesis will be helpful for improvement in production strains. Today, genetically modified *E. coli* or *Serratia marcescens* allow end concentrations of L-threonine of 100 g L^{-1} [29, 30]. For the industrial production of L-threonine *E. coli* strains containing an increased copy number of the threonine operon seem to be most successful [31].

Oxidoreductases (EC 1) represent a versatile class of biocatalysts for specific reduction, oxidation, and oxyfunctionalization reactions. They depend on cofactors like NAD^+ or $NADP^+$ to supply or withdraw redox equivalents released during the catalytic process. Amino acid dehydrogenases (deaminating amino acid oxidoreductases, EC 1.14) are used also for enantioselective biotransformations on an industrial scale. It is also possible to convert non-natural compounds if the substrate specifity of an enzyme is low enough. However, the enantioselectivity still needs to be high enough for application. In addition to ammonia and the respective 2-oxocarboxylic acid, amino acid dehydrogenases (EC 1.4) need a cosubstrate, which supplies the hydride ions

to reduce the intermediate imine [32]. NADH and NADPH are suitable cosubstrates. The oxidized cosubstrates (e.g., NAD^+) must be regenerated in a further redox reaction because of their high costs.

The Mercian Corporation produces L-piperidine-2-carboxylic acid ((*S*)-20) from L-lysine (L-17) using an aminotransferase (EC 2.6.1) and a dehydrogenase in whole, recombinant *E. coli* cells (Fig. 9). The aminotransferase, transferring a nitrogenous group, belongs to the class of transaminases. L-Lysine is deaminated to 5-formyl-2-methylpentanoic acid (L-18) by L-lysine aminotransferase derived from *Flavobacterium lutescens*. 5-Formyl-2-methylpentanoic acid is dehydrated to 2,3,4,5-tetrahydropyridine-2-carboxylic acid **19** [33–35]. This process exemplifies the possibility of transamination followed by reduction utilizing two different biocatalysts in a reaction sequence. (*S*)-20 is a building block for a number of pharmaceutical products, such as the local anesthetic Bupivacaine (AstraZeneca).

Other enzymes like tyrosinphenol lyase (EC 4.1.99.2) are pyridoxal phosphate dependent multifunctional enzymes and catalyze the reversible reaction of phenol, pyruvate, and ammonia to tyrosine. This enzyme is used by the Ajinomoto Co. to produce 3,4-dihydroxy-L-phenylalanine **23**, also called L-DOPA. They apply catechol **21** instead of phenol (Fig. 10) and acetic acid **22**. The tyosinphenol lyase is derived from *Erwinia herbicola*. To carrying out the production process, the cells are first prepared by cultivation in L-tyrosine-containing medium to induce tyrosinphenol lyase. After harvesting the cells by centrifugation they are transferred to the reactor. The

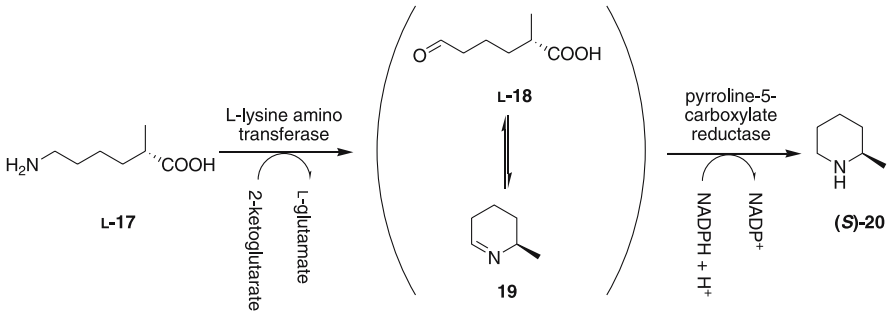


Fig. 9 Production of L-piperidine-2-carboxylic ((*S*)-20) from L-lysine (L-17)

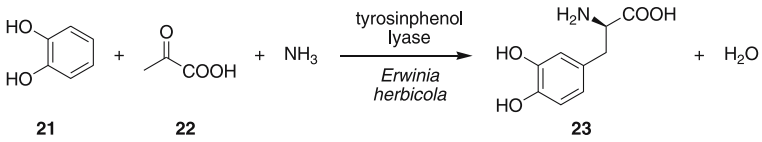


Fig. 10 Ajinomoto Co. produces L-DOPA using suspended cells of *Erwinia herbicola* cells in a fed batch reactor

one-step biotransformation produces L-DOPA from catechol, showing higher economic efficiency than the established chemical route [36]. This example demonstrates the implementation of resuspended cells within an industrial bioprocess. More than 125 tons of L-DOPA are supplied by biotransformation per year and are used in the treatment of Parkinson's disease. This progressive disease is caused by a lack of L-dopamine and its receptors in the human brain. L-DOPA is applied in combination with dopadecarboxylase inhibitors. This ought to avoid formation of L-dopamine outside the brain, enabled by the blockade of the blood–brain barrier towards L-dopamine.

Another class of lyases is represented by tryptophan synthase (4.2.1.20). This lyase has been found in a broad range of organisms. The α-subunit catalyzes the conversion of 1-(indol-3-yl)-glycerol 3-phosphate to indole and glyceraldehyde 3-phosphate, whereas each β-subunit has a pyridoxal phosphate-containing active site. Here the indole **25** and serine **24** are converted to tryptophan **26** (Fig. 11). For commercial purposes the enzyme is used to produce L-tryptophan as a pharmaceutical active ingredient for parenteral nutrition and as an active ingredient in sedatives, narcoleptics, antidepressants, and food additives. Amino, Germany, uses *E. coli* in suspended whole cells to produce L-tryptophan in a fed batch reactor. Based on indole a yield of more than 95% is obtained and 30 t per year are produced [37–39].

Transaminases (EC 2.6.1) show one major drawback in reaching an equilibrium conversion of only about 50% with respect to the amino donor. NSC Technologies, Monsanto, has patented a fermentation process for the production of D-amino acids by whole cells [40]. The growth medium is supplemented with a cheap L-amino acid (L-29), e.g., L-aspartate, as an amino group donor for the L-amino acid transaminase (EC 2.6.1.57). Using amino acid racemase the amino-group donor is also accessible from cheap racemic mixtures of amino acids. This process demonstrates how to overcome limitations given by the reaction equilibrium of biotransformations. Figure 12 illustrates an example for the production of D-phenylalanine (D-27) starting from endogenously produced L-phenylalanine or from racemic phenylalanine added to the medium. First an L-amino acid deaminase generates the α-keto acid phenylpyruvate **28** from the L-phenylalanine. The D-phenylalanine is not converted due to the fact that the metabolism of the cells is modi-

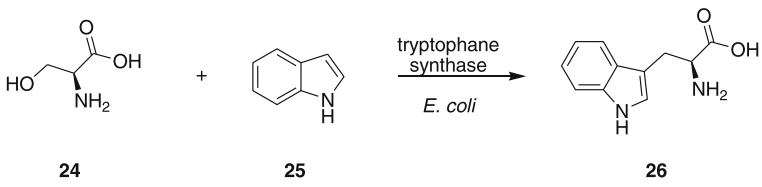


Fig. 11 Commercial process run at Amino, Germany, producing L-tryptophan **26** using *E. coli* in suspended whole cells

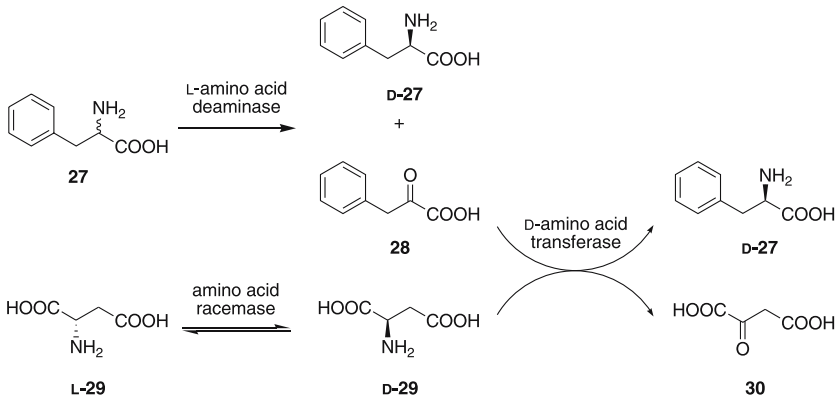


Fig. 12 Both L-amino acid deaminases and D-amino acid aminotransferases are non-specific, enabling the production of D-alanine, D-leucine, D-glutamic acid, and D-tyrosine from the corresponding L-amino acids

fied in such a way that no functional D-amino acid deaminases are produced. The amino group of an inexpensive D-amino acid like asparagine is transferred to the phenylpyruvate using D-amino acid aminotransferase. Hereby D-phenylalanine and 2-oxo-succinic acid **30** are formed. The byproduct 2-oxo-succinic acid decarboxylates to pyruvic acid and CO_2. Pyruvic acid itself is also an α-keto acid and is converted by dimerisation using acetolactate synthase to acetolactate. This undergoes spontaneous decarboxylation to acetoin and can be thus be easily removed. As both L-amino acid deaminases and D-amino acid aminotransferases are non-specific, this approach is applicable in the production of D-alanine, D-leucine, D-glutamic acid, and D-tyrosine from the corresponding L-amino acids [41, 42]. In general, amino acids are used as food additives and in medicine in the form of infusion solutions.

Using cheap cosubstrates to generate easily separable coproducts is essential for the design of an economically attractive industrial process utilizing NAD(P)H-requiring oxidoreductases. In the case of the production of (2*R*,5*R*)-hexanediol **33** from 2,5-hexanedione **31**, glucose acts as this cosubstrate (Fig. 13). Using whole cells from *Lactobacillus kefiri* circumvents the application of additional cofactors. The applied biocatalyst is the genetically unmodified wild-type microorganism and the starting material 2,5-hex-

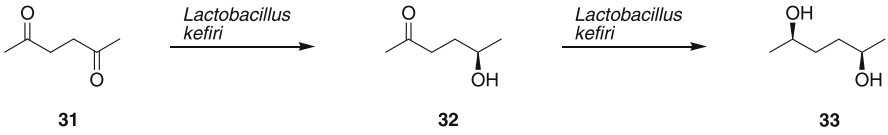


Fig. 13 Process of Jülich Chiral Solutions to produce (*2R*,*5R*)-hexanediol **33**

anedione is routinely available from diketene chemistry. The product is not only an auxiliary for organic synthesis but also an important building block for chiral ligands with phospolane structure [43–45]. Additionally, the resolution of hexanediol is possible (see Sect. 4.3.2).

The diastereomer (2*S*,5*S*)-hexanediol can also be synthesized in a biocatalytic way using baker's yeast to convert the 2,5-hexanedione.

Enantiomerically pure α-halo alcohols are used as starting material by the Kaneka Corporation using dehydrogenases in whole cells to produce (*R*)- and (*S*)-styrene oxides. Bristol Meyers Squibb prepare chlorohydrin ((*S*)-35) from methyl-(3*S*)-3,4-epoxybutyrate **36**. The (*S*)-chlorohydrin is available showing 99% ee on a multikilogram scale. It is produced from methyl 4-chloro-3-oxobutanoate **34** by reduction, applying whole cells from *Geotrichum candidum* utilizing its dehydrogenase (Fig. 14) [46]. The regeneration of the cofactor NADPH can – as shown exemplarily in this process – be ensured by the metabolism of the cell. (*S*)-4-Chloro-3-hydroxybutanoic acid methyl ester is used as chiral building block to synthesize a cholesterol antagonist **37**, inhibiting the hydroxylmethyl glutaryl CoA (HMG-CoA) reductase.

In contrast to chemocatalyzed carboligations, very high enantiomeric purities are reached utilizing lyases as biocatalysts. For example, pyruvate decarboxylase (EC 4.1.1.1) depends on thiamine diphosphate and magnesium ions as cofactors and is found in many types of yeast, fungi, plants, and some bacteria. This enzyme catalyzes the enantioselective carboligation of two aldehyde molecules resulting in a 2-hydroxyketone. The carboligation of acetaldehyde and benzaldehyde **38** is one of the oldest biotransformations applied on industrial scale. This stereoselective acyloin condensation to form (*R*)-hydroxy-1-phenylpropanone ((*R*)-39) is part of the production of the sympathomimetics (1*R*,2*S*)-ephedrin **40** and (1*R*,2*R*)-pseudoephedrine **41** [47–49]. For this reason (*R*)-hydroxy-1-phenylpropanone is an important building block in the synthesis of fine chemicals. The combination of biotech-

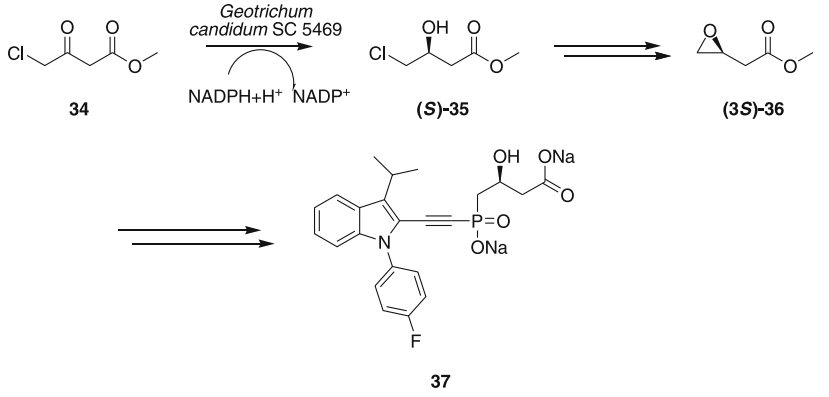


Fig. 14 Synthesis of a cholesterol antagonist **37** inhibiting HMG-CoA reductase

nological and chemical steps enables the production of various drugs having α- and β-adrenergic properties such as ephedrine, norephedrine, and pseudoephedrine. A route to ephedrine and pseudoephedrine by reductive methylamination of (*R*)-hydroxy-1-phenylpropanone was established at Knoll [50]. The extraction of (1*R*,2*S*)-ephedrine from natural raw materials is not competitive to its biotechnological/chemical synthesis. The fermentative process is performed by fermenting *Saccharomyces cerevisiae* cells in a fed batch reactor by BASF and Krebs Biochemicals&Industries.

It was found that fermenting yeast, containing the pyruvate decarboxylase, catalyzes the stereoselective acyloin condensation of benzaldehyde and endogenous acetaldehyde to form (*R*)-1-hydroxy-1-phenylpropanone [51]. Unlike whole cell biotransformation processes, using isolated pyruvate decarboxylase circumvents side-product formation caused by the various enzymes in whole cells. This example may demonstrate the advantages and disadvantages regarding the number of possibly active enzymes within a process. Thus, such enzymatic biotransformations lead to product concentrations up to 100 g L^{-1} of (*R*)-L-hydroxy-1-phenylpropanone.

Alternatively, Fuji Chemical Industries demonstrates a biocatalytic reaction carried out using different microorganisms (Fig. 15). Chemically synthesized 2-methyl-amino-1-phenylpropanone **42** is reduced enantioselectively to pseudodephedrine. While carrying out the dynamic, kinetic resolution the unconverted enantiomer is racemized in situ.

Bacterial mono- or dioxygenases can be used in hydroxylation processes to catalyze bond formation between one or both oxygen atoms of O_2 and non-activated carbon atoms. These hydroxylations are usually regio- but not stereoselective [52–55]. To provide hydroxycarboxylic acids using microbial

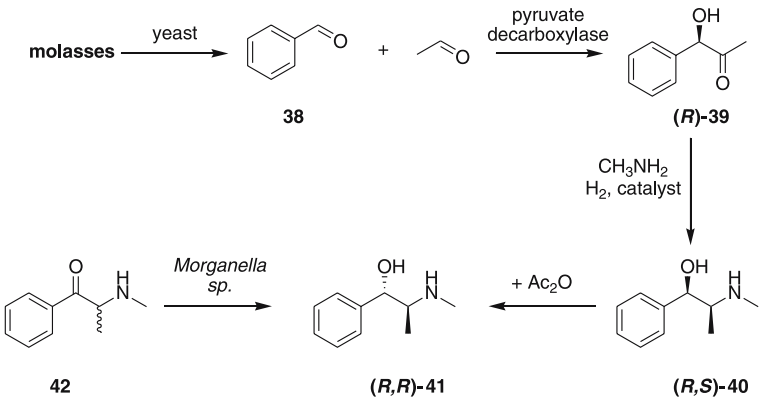


Fig. 15 Stereoselective acyloin condensation of benzaldehyde **38** and acetaldehyde leading to pseudoephedrine **41**. Fuji Chemical Industries synthesizes pseudoephedrine by kinetic resolution of ephedrone **42**

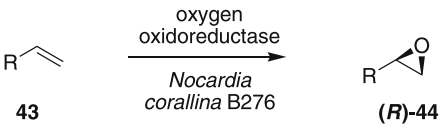


Fig. 16 Monooxygenase from *Nocardia corallina* catalyzes the epoxidation of terminal alkenes

hydroxylations, sequential α,β-dehydration and hydrolysis steps are often involved.

The epoxidation of terminal and subterminal alkenes **43** is an example of the application of alkene monooxygenases (Fig. 16) on an industrial scale. The stereospecific epoxidation using oxygen oxidoreductase from *Nocardia corralina* B276 yields predominately the (*R*)-enantiomer **44** and can be carried out in a conventional fermentation system. By dividing the substrates into three classes, depending on the chain length, three reaction processes can be differentiated:

1. Short chain, gaseous epoxides (C_3–C_5) are very toxic to cells and product recovery is complicated. The rate of aeration during fermentation has to be raised to extract the short chain toxic epoxides. The very low amounts of epoxides in the gas phase can be recovered by a special solvent extraction system.
2. Applying C_6–C_{12} alkenes, a two-phase system with a non-toxic solvent is used. The addition of this solvent has two effects. First the concentration of the inhibiting epoxide is lowered and second the product can be extracted continuously.
3. For C_{13}–C_{18} alkenes, growing cells are applied since the products are less toxic than in the case of shorter chain epoxides where resting cells are used. Using these long chain alkenes, limiting of components in the medium during growth is advantageous [56–59].

The selective assimilation of (*R*)-2,3-dichloro-1-propanol ((*R*)-45) using *Pseudomonas sp.* (OS-K-29) leads to the *S*-enantiomer (Fig. 17) [60–62]. The *R*-enantiomer is obtained by degrading the *S*-enantiomer using *Alcaligenes sp.* (DS-K-S38) [63–65]. Basic conditions lead to the chiral epichlorohydrins ((*S*)- or (*R*)-46). 3-Chloropropane-1,2-diol **47** can be produced in a similar way. Afterwards, the enantiomers are converted into (*R*)-glycidol ((*R*)-48) and (*S*)-glycidol ((*S*)-48), respectively. This example illustrates that not only the synthesis of one enantiomer leads to an enantiomeric pure product but also the selective degradation of the unwanted one.

Racemic 6-hydroxynorleucine **49** is produced by hydrolysis of commercially available 5-(4-hydroxybutyl)hydantoin. The 2-keto-6-hydroxyhexanoic acid is converted further to L-6-hydroxynorleucine using amino acid dehydrogenase (EC 1.4.1.2) and beef liver glutamate dehydrogenase (EC 1.4.1.5) (Fig. 18). Within this process the D-amino acid oxidase has been used to

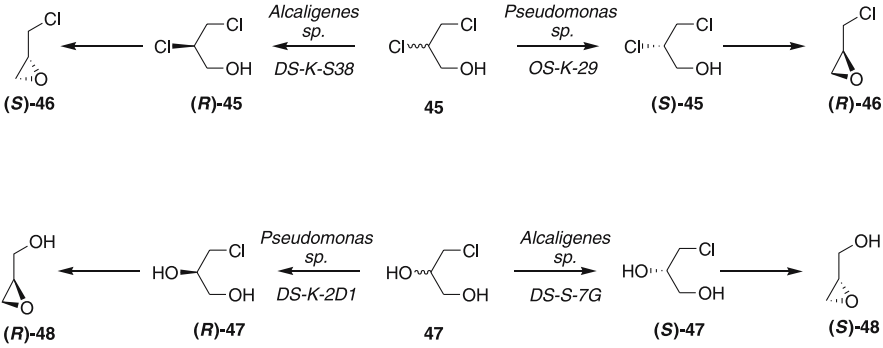


Fig. 17 Daiso produces chiral epichlorohydrin and glycidol by fermentation

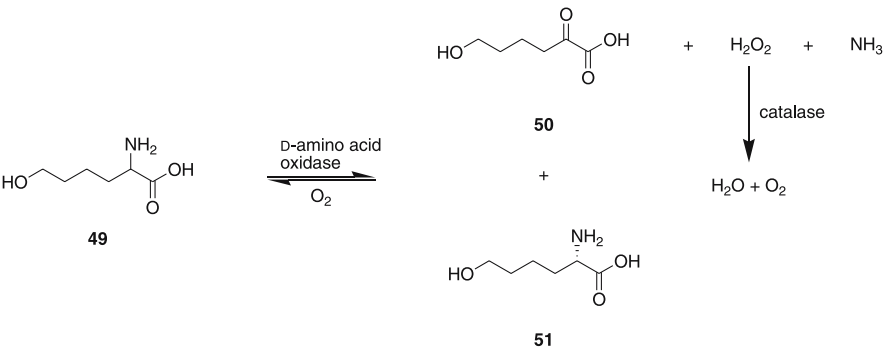


Fig. 18 D-Amino acid oxidase converts the D-amino acid to a ketoacid **50**, leaving the L-enantiomer unconverted

convert the D-amino acid to a ketoacid **50**, leaving the L-enantiomer unconverted. The stoichiometrically formed coproduct H_2O_2 is degraded by catalase to prevent deactivation of D-amino acid oxidase. This enantiomer, L-6-hydroxynorleucine, can be isolated by ion exchange chromatography. L-6-Hydroxynorleucine **51** is a chiral intermediate useful for the synthesis of vasopeptidase inhibitors, which are in clinical trial. Additionally, they are building blocks for the synthesis of C-7 substituted azepinones as potential intermediates for other antihypertensive metalloproteinase inhibitors [66–69].

The Kaneka process shown in Fig. 19 is a biochemical transformation taking place via a reaction sequence in a whole cell. This three-step reac-

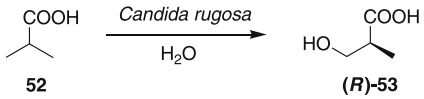


Fig. 19 The Kaneka process produces (*R*)-β-hydroxy-isobutyric acid ((*R*)-53) by β-hydroxylation

tion initially starts by dehydrogenating the aliphatic acid 2-methylpropionic acid **52** to an α,β-unsaturated acid. In a subsequent step the formed α,β-unsaturated acid is enantioselectively acylated and afterwards hydrated to give (*R*)-β-hydroxy-isobutyric acid ((*R*)-53), which is used as a building block in the synthesis of captopril, an ACE inhibitor [70].

4.2.2 Asymmetric Products Synthesized by Isolated Enzymes

Many substances can be isolated from suitable raw materials or are produced in biocatalytic or chemical processes. Furthermore, fermentation processes are available for, e.g., naturally occurring carboxylic acids, especially for quantities of more than 1000 t per year. To obtain the desired carboxylic acid, glucose or cheap industrial products such as molasse are used as carbon source. Nevertheless, the focus in this section will be on the rapid development of different biotransformations utilizing isolated enzymes as biocatalysts.

However, often substrates, products and/or solvents that are required may be toxic for the cells. In general, the product concentration in whole cell biotransformations is also pretty low. If recombinant microorganisms are employed the genetic stability during cultivation has to be observed carefully. If isolated enzymes are applied, the purification may cause additional costs but, in contrast to biochemical characterization, it is not necessary to purify the protein to homogeneity. On the contrary, the remaining protein content in the partly purified extract may increase its stability. The major advantage of purified enzymes over whole cells is that side reactions might be more easily avoided and substrates that are toxic for the cell, or which may not be able to enter the cell, can be converted.

Oxidoreductases belong to the class of enzymes catalyzing oxidoreduction reactions. The substrate being oxidized is regarded as a hydrogen donor, which is NAD^+ in the following example. The systematic name is based on the name of donor followed by the name of acceptor ending with oxidoreductase, whereas the commonly used name is dehydrogenase or reductase. Oxidase is only used in cases where oxygen is the acceptor [71].

The chemical production of (*R*)-3-(4-fluorophenyl)-2-hydroxy propionic acid **56** shows either low yields or low stereospecifity (Fig. 20). Using D-lactate dehydrogenase (EC 1.1.1.28) from *Leuconostoc mesenteroides* the process leads to a much higher yield of up to 88% and an enantiomeric excess of more than 99.9%. The starting material sodium-3(-4-fluorophenyl)-2-oxopropanoate **54** is synthesized from fluorobenzaldehyde and the hydantoin upon condensation and saponification. The cofactor NAD^+ is regenerated by the formate dehydrogenase from *Candida boidinii*, which oxidizes formate to carbon dioxide. This is an example of the in situ cofactor regeneration by an enzyme-coupled reaction. Afterwards the enantiomerically pure hydroxy

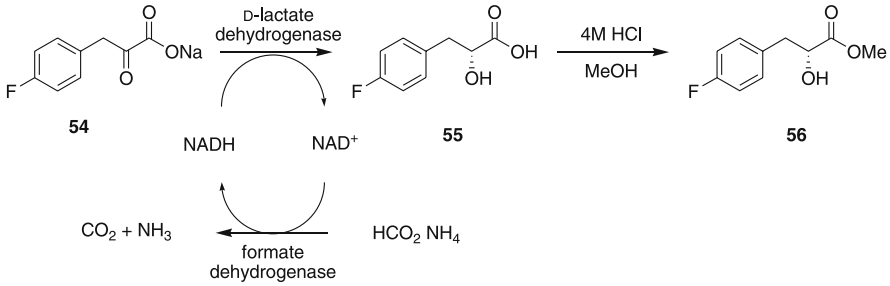


Fig. 20 Synthesis of (*R*)-3-(4-fluorophenyl)-2-hydroxy propionic acid – a building block of the rhinovirus protease inhibitor Rupintrivir

acid **55** is methylated for integration in the chemical synthesis route of the protease inhibitor. The product is a building block for the synthesis of Rupintrivir, an example of a rhinovirus protease inhibitor [72, 73].

Using a keto-reductase, ethyl-(*S*)-4-chloro-3-hydroxybutyrate **58** is synthesized in a redox reaction (Fig. 21). Carrying out the conversion $NADP^+$ is regenerated by glucose dehydrogenase, which later oxidizes glucose to gluconic acid. The reaction shows an enantiomeric excess of more than 99.9% and represents the first step within the synthesis of ethyl-(*R*)-4-cyano-3-hydroxybutyrate **59** used by Codexis. The second step of this synthesis is a cyanation, applying halohydrin dehalogenase. By means of gene shuffling the rate, stability, and product tolerance was significantly improved, indicating the fascinating opportunity of enhancement of enzymes by means of molecular biology. The optical purity can be retained (ee > 99.9%) and a yield of more than 90% can be obtained. The hydroxy nitrile ethyl-(*R*)-4-cyano-3-hydroxybutyrate is an important building block for the production of atorvastatin [14–17].

6-Benzyloxy-(3*R*,5*S*)-dihydroxy-hexanoic acid ethyl ester **63** is a key chiral building block for anticholesterol drugs that act by inhibition of hydroxy methyl glutaryl coenzyme A (HMG-CoA) reductase. The shown biotransfor-

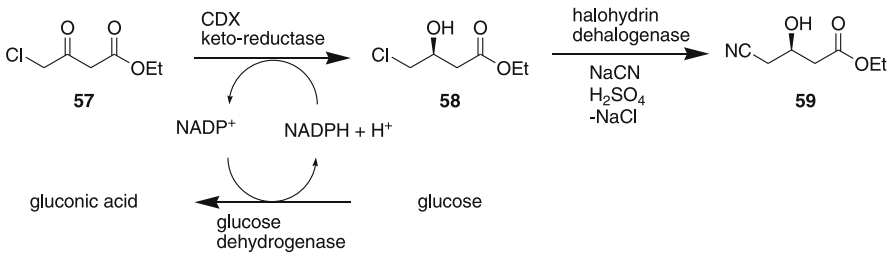


Fig. 21 Synthesis of ethyl-(*R*)-4-cyano-3-hydroxybutyrate **59** – a building block for atorvastatin

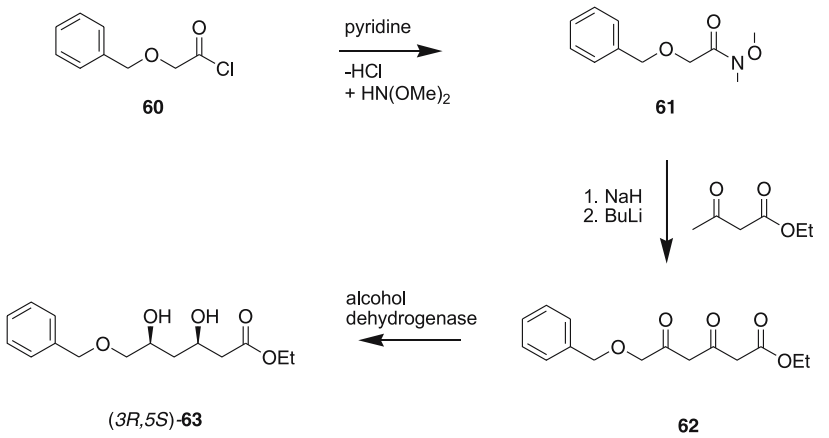


Fig. 22 Bioreduction for production of 6-benzyloxy-(3*R*,5*S*)-dihydroxy-hexanoic acid ethyl ester **63**

mation is an alternative to the chemical synthesis via the chlorohydrin and selective hydrolysis of the acyloxy group. This chemical synthesis reaches an overall yield of only 41% after final fractional distillation. In contrast, the biotransformation leads to a yield of 92%. This bioreduction, illustrated in Fig. 22, can be carried out with whole cells as well as with cell extract. The cofactor regeneration is facilitated by addition of glucose dehydrogenase, glucose, and NAD^+ to the reaction medium [74–77].

Hydroxynitrile lyases (HNL) (EC 4.1.2) catalyze the stereoselective addition of hydrogen cyanide to aldehydes and ketones. The cleavage of cyanhydrins is also a potential catalytic step using the corresponding reaction conditions [78–87]. α-Hydroxycarboxylic acids are produced by hydrolysis of chiral cyanhydrins using hydrochloric acid, whereas this reaction strategy affords quantitative conversion of the aldehyde into the product. Their ability to cleave HCN from hydroxynitriles, probably part of a plant defense system against carnivores, is synthetically applied to synthesize chiral hydroxyl nitriles. Certain chiral aromatic α-hydroxycarboxylic acids are also produced in industrial synthesis using HNL processes. Derivatives of chiral mandelic acid are used as building blocks in diverse syntheses and as racemate resolving agents.

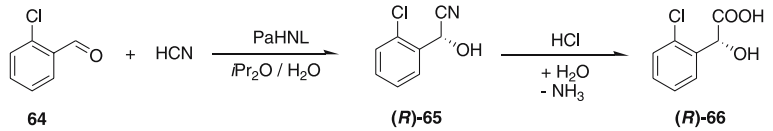


Fig. 23 (*R*)-*o*-Chloromandelic acid ((*R*)-66) can be produced using a HNL-based production process

(*R*)-*o*-Chloromandelic acid ((*R*)-66) is an intermediate in the synthesis of the antidepressant and platelet-aggregation inhibitor Clopidogrel. It can be produced using a HNL-based production process (Fig. 23) [88–95]. To carry out this synthesis, hydrocyanic acid is coupled enantioselectively to *o*-chlorobenzaldehyde **64** in micro-aqueous or biphasic systems [96]. The catalyst mandelonitrile lyase from *Prunus amygdalus* (PaHNL; EC 4.1.2.10) is used in almond-flour extract form or immobilized on Avicel microcrystalline cellulose, whereby a use of several months is possible, depending on the solvent employed. This illustrates that it is not compulsory to use a pure protein for biotransformation but that the application of an extract is also possible. The hydrolysis of (*R*)-*o*-chloromandelonitrile ((*R*)-65), resulting in the corresponding carboxylic acid, is done without undergoing racemization. The crystallization of diastereomeric ammonium salts, the use of enzymatic acylation and deacylation, or the application of transesterifications are possible strategies for carrying out a racemate resolution. The great advantage of these reaction sequences is the theoretical yield of 100%.

Processes to produce (*S*)-cyanohydrins and (*S*)-hydroxycarboxylic acids catalyzed by HNLs have become economically attractive applying recombinant enzyme preparations in *E. coli* [97–102]. The chemistry of (*S*)-oxynitrilase-catalyzed hydrocyanation in biphasic solvent systems consisting of an aqueous phase and a water-immiscible phase has been intensively investigated [103–107], demonstrating the applicability of these enzymes under unnatural conditions. (*S*)-Oxynitrilase from *Hevea brasiliensis*, cloned and overexpressed in a microbial host organism, has been used. In the presence of this biocatalyst the desired products were obtained at high enantioselectivities [108]. This oxynitrilase-catalyzed hydrocyanation has already been extended to a commercial process. For the production of (*S*)-*m*-phenoxybenzaldehyde cyanohydrin ((*S*)-68), DSM established an enzymatic hydrocyanation process (Fig. 24) on an industrial scale based on an efficient

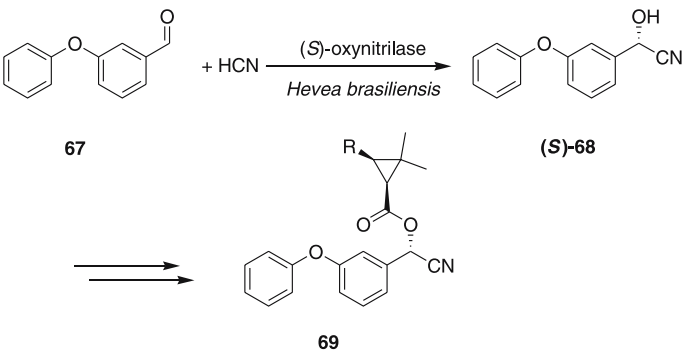
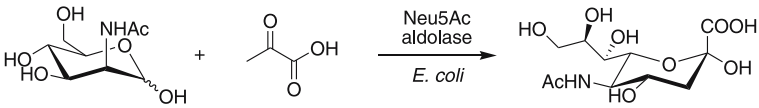


Fig. 24 Production of (*S*)-*m*-phenoxybenzaldehyde cyanohydrin ((*S*)-68) by an enzymatic hydrocyanation process established by DSM



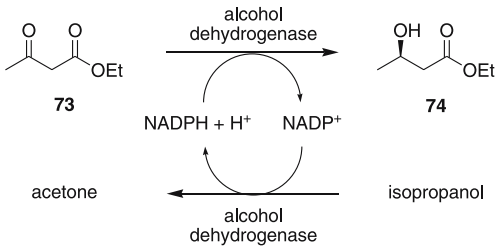
70 71 72

Fig. 25 Production of *N*-acetylneuraminic acid **72** in a one-pot biotransformation

protocol by Griengl et al. [109–114]. The cyanohydrin is a building block for the production of pyrethroids **69**.

N-Acetyl-D-neuraminic acid aldolases (Neu5Ac-aldolase; EC 4.1.3.3) have been characterized from many organisms. In vivo Neu5Ac-aldolase catalyzes the reversible aldole reaction of *N*-acetyl-D-mannosamine **70** and pyruvate **71** to *N*-acetyl-5-amino-3,5-didesoxy-D-glycero-D-galacto-2-nonulosonic acid **72**. The amino sugar neuraminic acid is the aldol condensation compound of pyruvic acid and *N*-acetyl-D-mannosamine. Nitrogen- and oxygen-substituted *N*-acyl derivatives belong to a family of unique 9-carbon monosaccharides called sialic acids. They represent terminal sugars of cell surface glycoproteins, especially in animal tissues and blood cells. Their tasks involve cell adhesion, recognition, and interaction. Furthermore, an industrial process using recombinant Neu5Ac-aldolase from *E. coli* in an immobilized form has been established for the synthesis of Neu5Ac on a multiton scale (Fig. 25) [115]. This example demonstrates the high regioselectivity of biocatalysts in contrast to classical chemical syntheses. This plays an important role, especially in the field of carbohydrate chemistry, omitting protecting group chemistry.

The free enzyme alcohol dehydrogenase is applied by Jülich Chiral Solutions and Wacker Chemie to the production of (*R*)-ethyl-3-hydroxybutyrate **74**. Ethyl acetoacetate **73** is converted by the enzyme obtained from *Lactobacillus brevis* through an asymmetric reduction. During the process, the coproduct acetone is removed by continuous stripping (Fig. 26). This provides several benefits. First, the equilibrium of this reaction is shifted to



73 74

Fig. 26 Process to synthesize (*R*)-ethyl-3-hydroxybutyrate **74** by alcohol dehydrogenase from *Lactobacillus brevis*

the product side, which enables the complete conversion of the reactants. Later, low-boiling solvents for extraction become applicable and a continuous reuse of the aqueous phase in the reactor vessels is possible. These biotransformations exemplify a substrate-coupled reaction in comparison to the enzyme-coupled reaction shown before (Fig. 20). The product (*R*)-ethyl-3-hydroxybutyrate is obtained with a yield of 96% and more than 99.8% ee. It is a chiral β-hydroxyester and is an important intermediate for organic synthesis. β-Hydroxyester are widely used as building blocks for pharmaceutical and agrochemical products as well as for fragrances [116–118].

7-Aminocephalosporanic acid (7-ACA) **79** is a building block for the synthesis of semisynthetic cephalosporin antibiotics. This enzymatic process utilizes immobilized enzymes, starting at the deamination of cephalosporin C **75** forming α-ketoadipyl-7-ACA **76** (Fig. 27). This step is catalyzed by a carrier-fixed D-amino acid oxidase (DAO; EC 1.4.3.3) in the presence of oxygen. Afterwards, the reaction conditions lead to an oxidative decarboxylation of the α-keto intermediate forming glutaryl-7-ACA **77**. The final product 7-ACA is synthesized by a carrier-fixed glutaryl amidase (EC 3.1.1.41) hydrolyzing the glutaryl-7-ACA. The produced cephalosporin belongs to the β-lactams, representing medicinal antibiotics synthesized by bacteria and fungi [119].

Cyclodextrins **81** can be produced by cyclodextrin glycosyltransferases (EC 2.4.1.19) as a mixture of α-, β-, and γ-cyclic oligosaccharides (Fig. 28). This process is characterized by many byproducts within the reaction mixtures and inhibition of the enzyme by increasing cyclodextrin concentration. These challenges have been overcome by separation of the cyclodextrins from the reaction media. The separation is carried out by selective adsorp-

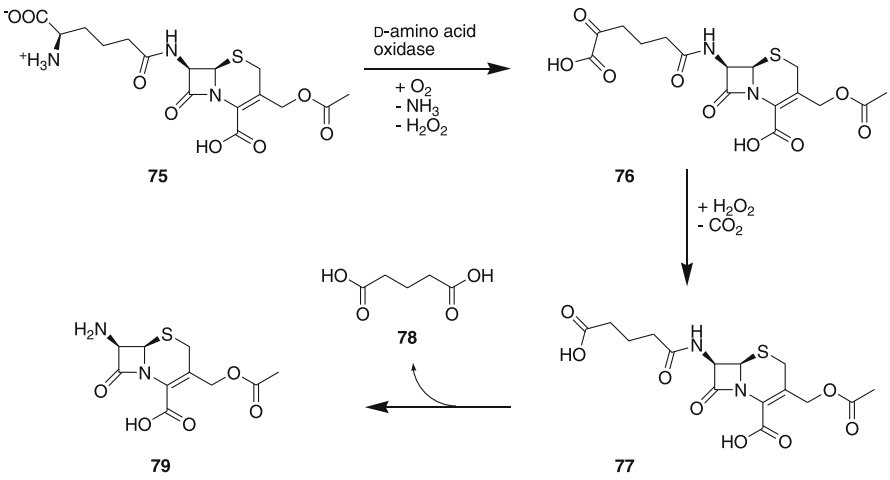


Fig. 27 Immobilized enzymes for deamination of cephalosporin C **75** forming α-ketoadipyl-7-ACA **76**

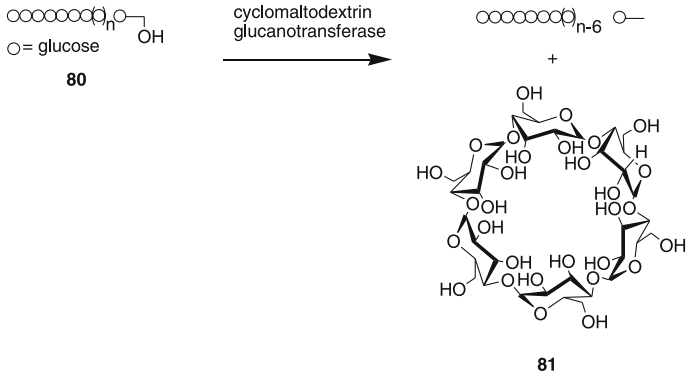


Fig. 28 Cyclodextrin glycosyltransferases produces a mixture of α-, β- and γ-cyclic oligosaccharides, which are separated by selective adsorption

tion of α- and β-cyclodextrins on chitosan beads with appropriate ligands. α-Cyclodextrins selectively interact with stearic acid, and β-cyclodextrins with cyclohexanepropanamide-*n*-caproic acid. The adsorption selectivity is almost 100%. In the case of the β-cyclodextrins a capacity of 240 g L^{-1} gel bed is reached. Thus, the establishment of an economical cyclodextrin production was possible. Because at 30 °C almost no cyclodextrins are formed during circulation, the temperature is lowered before entering the adsorption column. Before re-entering the main reactor the temperature of the solution is again adjusted to 55 °C by using the energy of the reaction solution

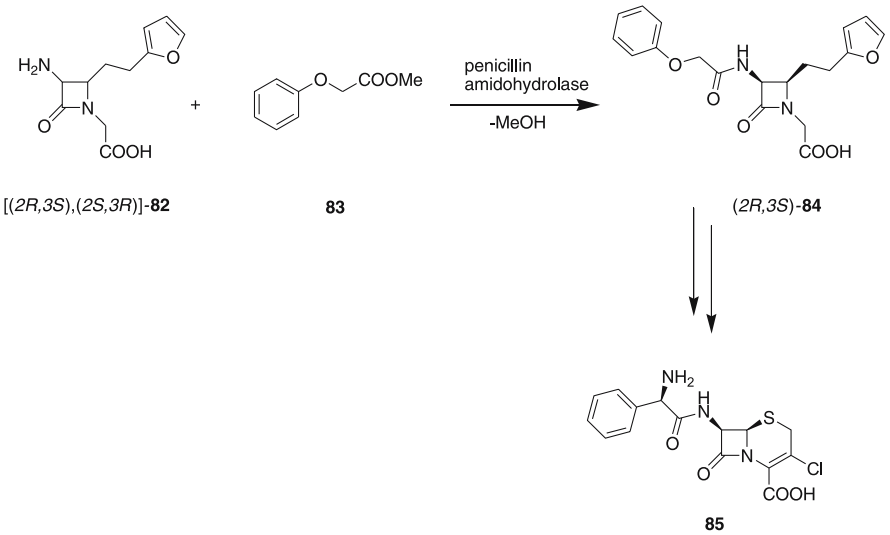


Fig. 29 Chemoenzymatic reaction strategy for the production of the carbacephalosporin antibiotic loracarbef **85**

leaving the reactor. This process exemplifies that byproducts are also formed in enzymatic processes, which consequently operate with limited selectivity. However, subsequent separation procedures overcome this limitation and turn such processes into competitive ones. Cyclodextrins serve as molecular hosts and are used in the food industry for capturing and retaining flavors. They are also used in the formulation of pharmaceuticals [120–122].

The chemical resolution of the racemic azetidinone **82** gives low yields. Because of this a biotechnological pathway for its production may be successful. The Lilly process shows that the penicillin G amidase (EC 3.5.1.11) acylates the 3-amino function with the methyl ester of phenoxyacetic acid **83** (Fig. 29). Notwithstanding, the penicillin G amidase does not hydrolyze the phenoxyacetyl side-chain of penicillin V. The example shown here illustrates that the substrate spectrum is not as limited as thought. The acylation occurs using methyl phenylactetate (MPA) or methyl phenoxyacetate (MPOA) as the acylating agents, whereby the enzyme displays similar enantioselectivity with MPA or MPOA. It is immobilized on Eupergit to produce the (2*R*,3*S*)-azetidinone. This a key building block in the synthesis of loracarbef **85**, a carbacephalosporin antibiotic [123, 124].

4.3 Racemic Resolution

Especially for chiral products, the yield and selectivity of a reaction have a crucial impact on the cost of the final product. However, utilizing different kind of tricks, it is possible to turn a classical kinetic resolution with a maximum of 50% yield into a new one with 100% yield (Fig. 30). Here one must differentiate whether the target compound is the slower non-converted enantiomer of the starting material or the predominately formed product enantiomer. In the latter case, a dynamic resolution must be used. The trick lies in the fast isomerisation of the starting material enantiomers. Here it is important that $k_{rac} > k_1$ is given. Otherwise the loss in yield has to be taken into account. Isomerization of the starting material enantiomers can be introduced either by enzymes like racemases or isomerases or alternatively by chemicals like pyridoxal-5-phosphate, or by special reaction conditions (e.g., low pH in the case of hydantoins or azlactones). Another often-used trick, also on large scale, is the inversion of the wrong enantiomer by chemical

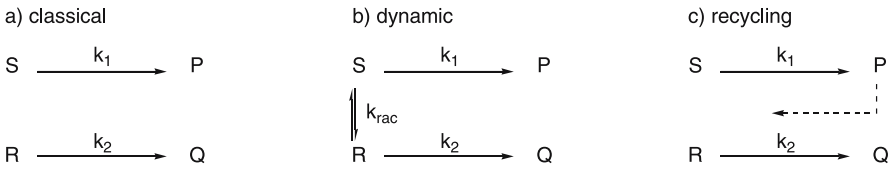


Fig. 30 Methods for kinetic resolution of racemates

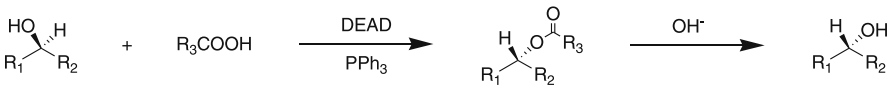


Fig. 31 Inversion of a chiral alcohol by the Mitsunobu reaction [125]

means (Mitsunobu reaction (Fig. 31) or sulfation in a subsequent reaction step to the biotransformation). In the next section, different examples are given discussing these different technologies.

4.3.1 Racemic Mixtures Resolved by Whole Cells

Enantioselective pantolactone hydrolases (EC 3.1.1.25) are useful for the resolution of the amide pantolactone **86**. This special case of ester hydrolysis produces D-pantolactone, which is required as a precursor of D-pantothenic acid, also called vitamin B5 (D-88) (Fig. 32). D-Pantolactone is used as feed additive in pig and poultry breeding. Both D- and L-pantolactones are used as chiral building blocks in chemical synthesis. Process development was done in the 1990s by researchers at the Daiichi subsidiary of Fuji Chemical Industries (now Daiichi Fine Chemical Co.) in cooperation with Yamada and coworkers [126–128]. For the synthesis, whole cells are immobilized in calcium alginate beads and used in a fixed bed reactor. The immobilized cells retain more than 90% of their initial activity even after 180 days of continuous use. At the end of the reaction L-pantolactone is extracted and reracemized to

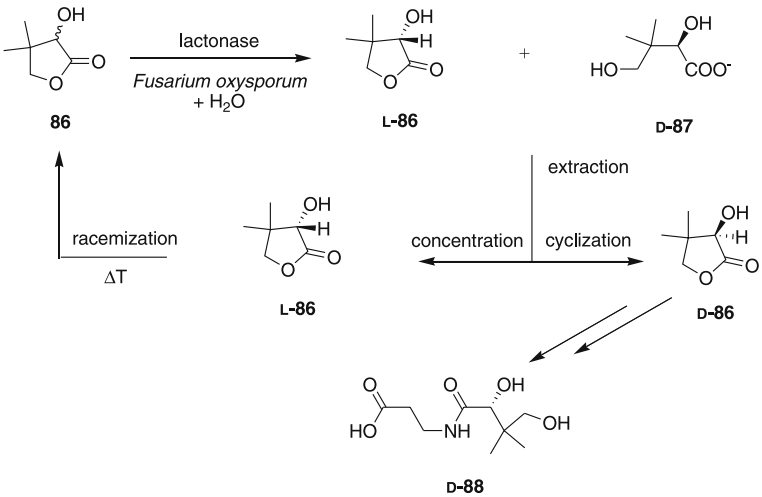


Fig. 32 Enantioselective pantolactone hydrolase is used for the resolution of pantolactone **86**

D,L-pantolactone that is recycled into the reactor. The D-pantoic acid is chemically lactonized to D-pantolactone [129–131]. The enzyme is highly selective and extremely stable in the form of immobilized fungal mycelia.

4.3.2
Racemic Mixtures Resolved by Isolated Enzymes

Although penicillin G acylase (PGA; EC 3.5.1.11) has traditionally been used to hydrolyze penicillin G it has also been used to resolve other amino compounds. Within the process shown, the racemic ethyl 3-amino-5-(trimethylsilyl)-4-pentynoate **89** is acylated prior to the deacylation by the enzyme PGA (Fig. 33). Acylation and deacylation can be performed by the same enzyme at different reaction conditions, allowing the discrete production of each enantiomer in the same reactor. That way, on a scale up to 70 L, 25 cycles were shown to be plausible. The synthesized β-amino acid, (*S*)-ethyl-3-amino-4-pentynoate, is a chiral synthon used in the synthesis of Xemilofiban hydrochloride, an anti-platelet agent [132, 133].

Often biotransformations are used to carry out a racemic resolution of the racemic product of a non-selective chemical equilibrium reaction. Such

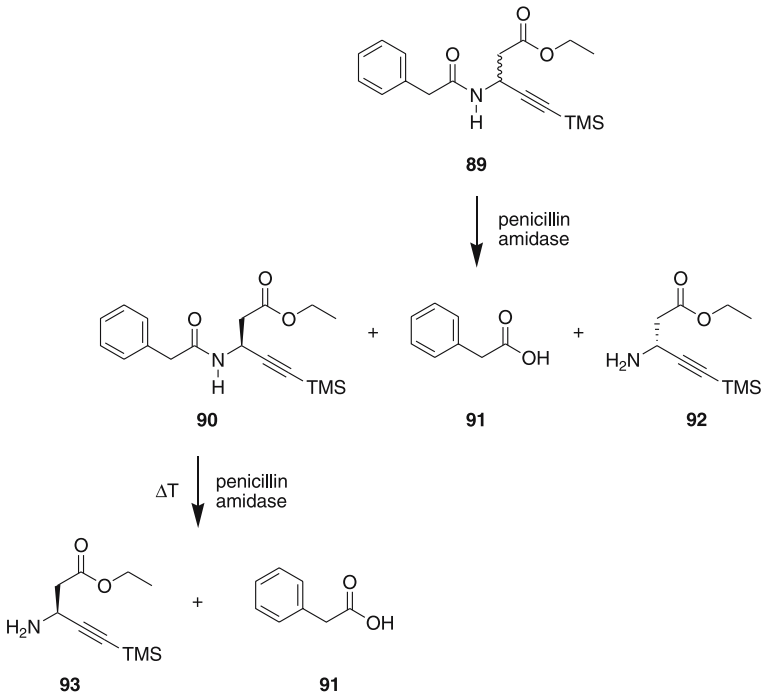


Fig. 33 Acylation and deacylation of ethyl 3-amino-5-(tri-methylsilyl)-4-pentynoate **89**

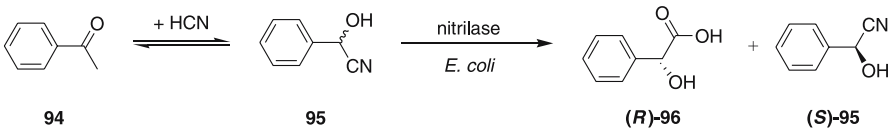


Fig. 34 Nitrilase process: Enantioselective hydrolysis of mandelonitrile **95** to (*R*)-mandelic acid ((*R*)-96)

types of conversions have been applied economically to the synthesis of (*R*)-mandelic acid. The synthetic route to hydroxy carboxylic acids is based on a two-step process using nitrilase (EC 3.5.5.1) from *E. coli* (Fig. 34). At first, a classical chemical hydrocyanation of the aldehyde **94** gives racemic cyanohydrin **95** and its derivatives. Afterwards, the cyanohydrins are subsequently hydrolyzed stereoselectively to the desired (*R*)-mandelic acids ((*R*)-96) [134, 135]. The hydrocyanation of aldehydes described above may also be carried out using hydroxynitrile lyases in an asymmetric manner, which is to date used in the synthesis of pyrethroids.

Racemic mixtures of hydantoins **97** are derived by chemical synthesis. The corresponding enantiomerically pure carbamoyl derivatives **98** can be obtained by using specific natural hydrolases. Specific hydrolases to hydrolyze the carbamoyl derivates also exist, allowing the synthesis of enantiomerically pure amino acids **99** from the racemic hydantoins [136]. The racemization of hydantoins happens spontaneously at pH values above 8 and the rate of racemization is dependent on the side chain of the amino acid. The dynamic resolution can be accelerated by using a racemase (Fig. 35).

The enantioselectivity of D-hydantoinase (EC 3.5.2.2) from *Arthrobacter sp*. DSM 9771 could be reversed by directed evolution [137]. In this way the synthesis of L-amino acids becomes technically possible, previously limited by the poor enantioselectivity of L-hydantoinases. The combination of the reversed D-hydantoinase and a carboxylase as well as a racemase is used on a pilot scale to produce L-*tert*-leucine at 100 kg-levels [11].

Aminotransferases (EC 2.6.1.1) transfer amino groups of amino acids to 2-oxocarboxylic acids or ketones. Carrying out a kinetic resolution, the prochiral ketone **100** accepts an amino group from the amino group donor isopropylamine **101**. To resolve the amine **102** the (*S*)-enantiomer is converted by the same transaminase into the ketone **100**. Propionaldehyde is converted to propionamine, working as amine-group acceptor (Fig. 36). The applied transaminases accept many different aliphatic and aromatic ketones and amines as substrate. In the case of α-ketocarboxylic acids working as amine-group acceptor, amino acids are formed. The limiting factor is the hydrophobic character of the substrate since the reaction must be carried out either in aqueous solution or in a mixture of organic solvent and water. The racemic resolution leads to a product mixture that can only be separated using laborious methods [11, 138].

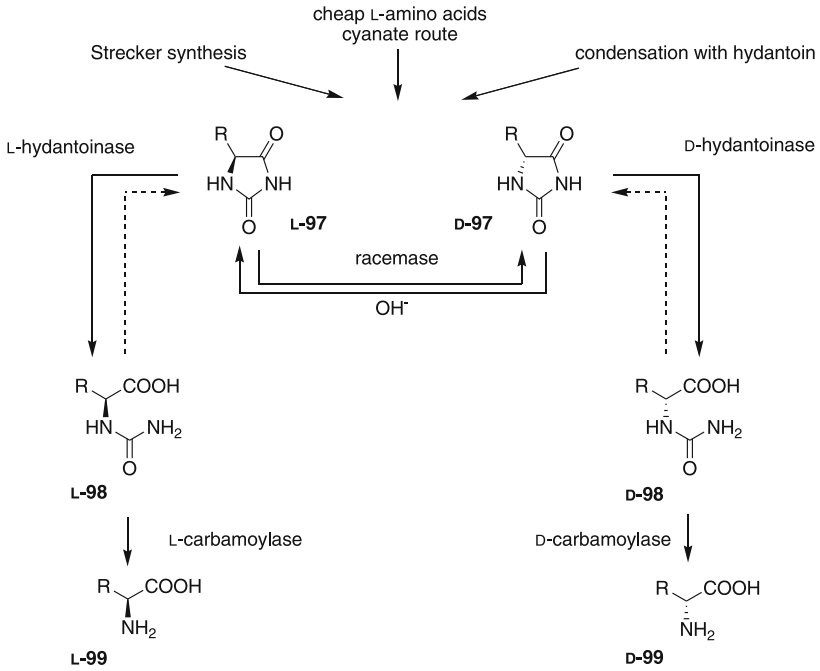


Fig. 35 Carbamoyl derivatives **98** can be obtained using specific natural hydrolases

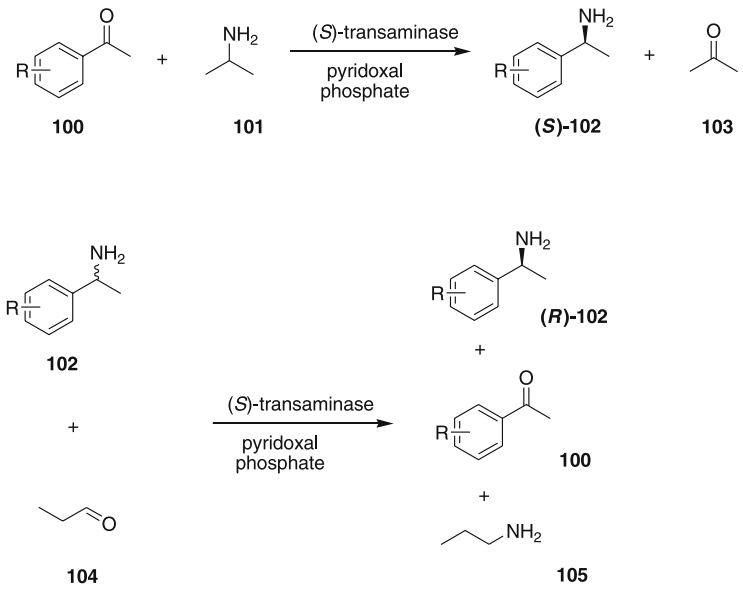


Fig. 36 Aminotransferases transfer amino groups of amino acids to 2-oxocarboxylic acids resulting in asymmetric pure amines

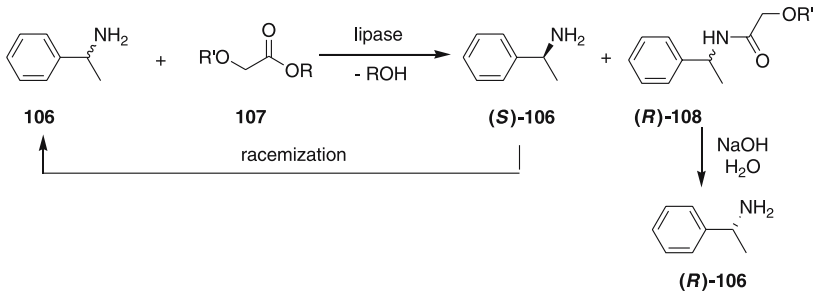


Fig. 37 Selective acylation of (*R*)-1-phenylethylamine ((*R*)-106) patented by BASF

The combination of hydrolases and acylating agents is an established technology (Fig. 37). BASF has commericialized a selective acylation of (*R*)-1-phenylethylamine ((*R*)-106). In this process (*S*)-1-phenylethylamine is produced from the racemic mixture yielding also the amide (*R*)-108 [139–143]. (*S*)-1-Phenylethylamine is separated by extraction or distillation. Afterwards, the amide (*R*)-108 is hydrolyzed giving (*R*)-106. The solvent mixture used for basic hydrolysis enables almost no racemization and quantitative yield [144, 145]. A wide variety of amines can be resolved such as aryl alkyl amines, alkyl amines, or amino ethers showing the broad substrate tolerance of the catalyst. Additionally, the ability to recycle undesired enantiomers by racemization [146–153] and the recovery of the acylating agent [154, 155] are benefits of the process. Some compounds are resolved by BASF on a multiton scale. Biocatalysts that can be applied are *Subtilisin carlsberg, Burkholderia plantarii, Candida antarctica* lipase B (EC 3.1.1.3), and penicillin acylase (EC 3.5.1.11).

Enantiomerically pure 2,5-hexanediol is an important building block in the synthesis of chiral acetals [156]. The direct asymmetric synthesis has already been illustrated (Fig. 13). Here the starting material, a 1 : 1 racemic/meso mixture, is acylated by a lipase (Fig. 38). Ideally, the enzymatic esterification resolves the mixture into 25% diol ((2*S*,5*S*)-111), 50% monoester ((2*R*,5*S*)-112), and 25% diester ((2*R*,5*R*)-113). Good results were found using Chirazyme L2 with vinyl propionate in heptane. A selective partition exists between the diol and the esters into water and heptane, allowing almost complete transfer of the diol to the water. A heptane mixture of the (*R*,*S*)-monoester and (*R*,*R*)-diester remains. This is treated with triethylamine and methanesulfonyl chloride resulting in the monomesylate, leaving the propionate ester functions untouched. Hydrolyzing the diester mixture to the (*R*,*R*)-diol was done using KOH in methanol. In contrast to the propionate ester, which is hydrolyzed under retention, the mesylated ester is hydrolyzed under inversion, yielding in both cases the *R*-hydroxy function. Neutralization and evaporation complete the synthesis step for the chemical inversion. (2*R*,5*R*)-Hexanediol was achieved showing 99% ee and 99% de as

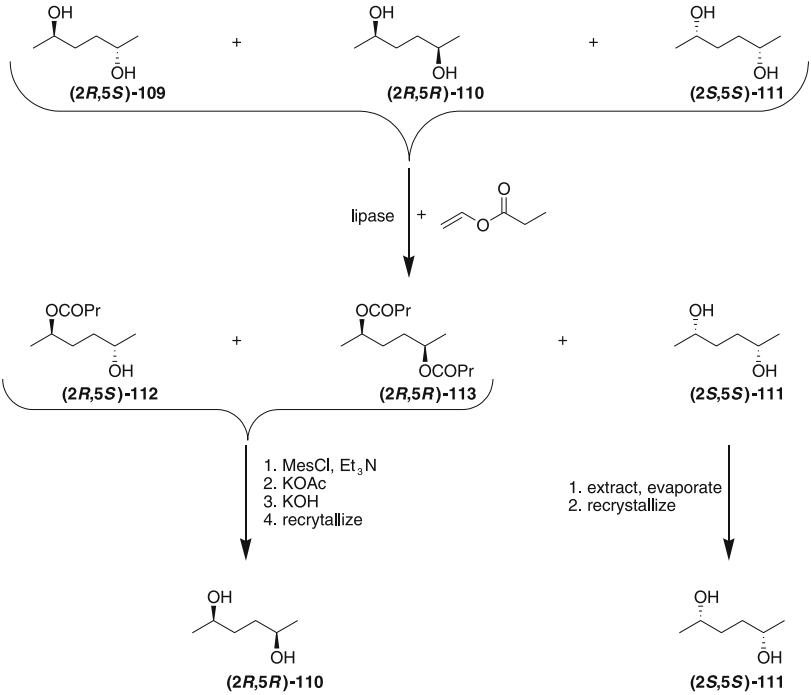


Fig. 38 Production of enantiomeric pure 2,5-hexanediols

well as (2*S*,5*S*)-hexanediol with 99% ee and 99% de, scaled up to 40 kg input batches.

At Degussa, the synthesis of (*S*)-*tert*-leucine is carried out as an asymmetric reductive amination of the prochiral keto acid utilizing a dehydrogenase and integrating cofactor recycling. Alternatively, Chiroscience uses the hydrolase-catalyzed classical resolution of azlactones **113** (Fig. 39). These azlactones easily racemize. They are prepared by cyclodehyration of an *N*-acylated amino acid **114** using acetic anhydride. The opening of the ring with water can also be achieved non-enzymatically, but this reaction is very unselective and uses an alcohol in a water-free system. Even if the process is formally described as a kinetic resolution it is ultimately a deracemization, whereby the azlactone is reracemized in situ, resulting in an overall yield of 90% for the chiral amino acid. Additionally, a high substrate concentration promotes racemization of the (*R*)-lactone and improves yield and enantiomeric excess. The substrate can also be synthesized via the glycine azlactone. This methodology can also be applied to other *tert*-alkyl glycine derivatives.

The concentration of the substrate could be increased to 20% and the reaction completed within 24 h. The cleavage by acid hydrolysis leads to

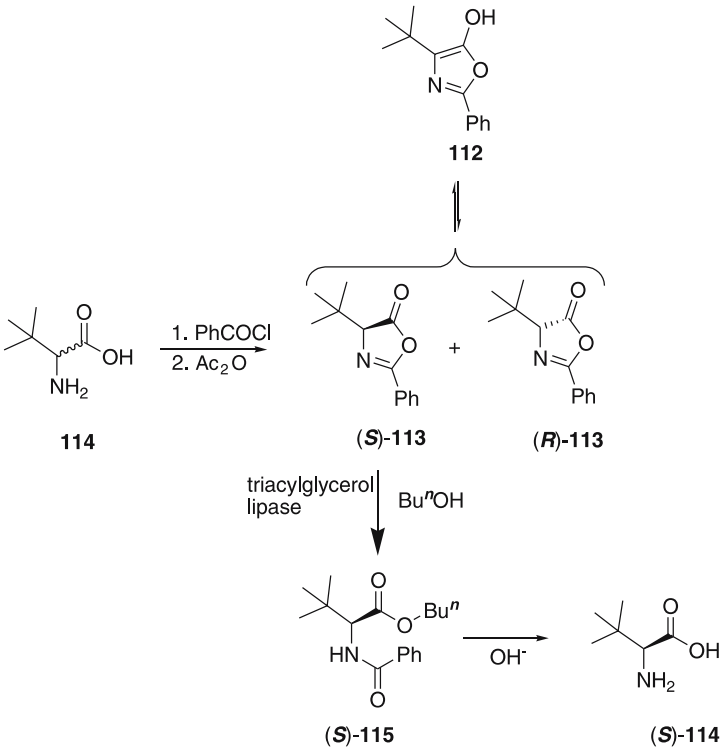


Fig. 39 Hydrolase-catalyzed dynamic resolution of azlactones

partial racemization caused by transient recyclization to the stereochemically labile azlactone. The ester amide **115** is hydrolyzed to the amino acids utilizing potassium hydroxide. Cleavage by peptidases is disfavored because of its bulky nature, resulting in peptides of improved metabolic stability. In doing so the product is useful as a lipophilic, hindered component of peptides. The amino acids are also useful building blocks for a number of chiral auxiliaries and ligands where the presence of the bulky *tert*-butyl group makes these compounds particularly effective for asymmetric synthesis [157, 158].

The (*S*)-alcohol (*S*)-117 is used as an intermediate in the synthesis of pyrethroids, which are used as insecticides. They show excellent insecticidal activity and a low toxicity in mammals. Subsequent to the lipase catalyzed hydrolysis, the cleaved alcohol (*R*)-117 is sulfonated in the presence of the acylated compound using methanesulfonyl chloride (Fig. 40). The hydrolysis of the sulfonated enantiomer (*R*)-118 in the presence of small amounts of calcium carbonate takes place under inversion of the chiral center. In contrast, hydrolysis of the acylated enantiomer is carried out under retention of the chiral center. This achieves an enantiomeric excess of 99.2% and

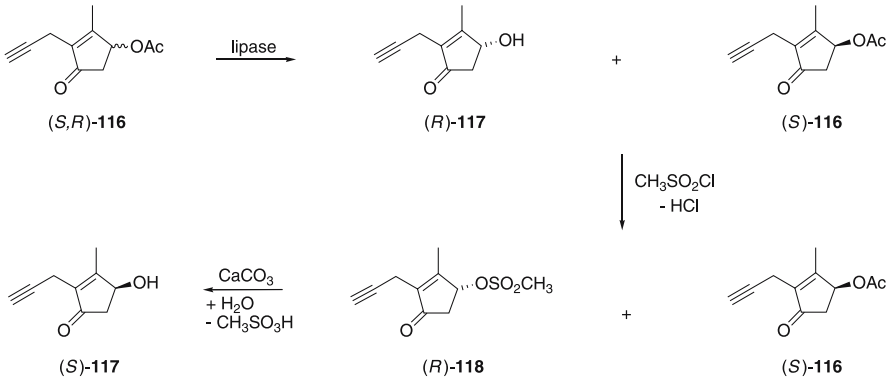


Fig. 40 Hydrolysis taking place under inversion of the chiral center

a very high yield regarding the (*S*)-alcohol [159]. This is a very efficient additional technology complementing the mesylation applied in the case of 2,5-hexanediol.

The introduction of novel racemases (EC 5.1.1), which were found by screening, led to the optimization of the L-acylase (EC 3.5.1) process (Fig. 41). Several hundred tons of methionine **120** are produced per year using this process. The enzymatic transformation is carried out within an enzyme–membrane reactor. This novel process has a higher potential than the classical resolution process because a dynamic resolution is applied. The *N*-acylamino acid racemase accepts various industrially important aromatic as well as aliphatic *N*-acylamino acids as substrate [160, 161].

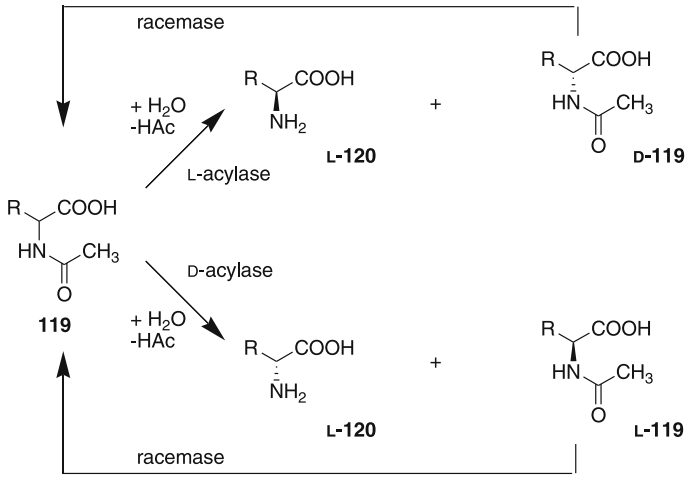


Fig. 41 L-Acylase process within an enzyme–membrane reactor

5 Conclusion and Outlook

In the first half of the 20th century organic chemistry developed methods to produce many products from oil using chemical catalysts, high temperatures, and high pressure. These petroleum-based consumer goods may be replaced by biotechnological products. Within this chapter different processes have been introduced that include at least one biocatalyzed step. For a wide range of applications "white biotechnology" has potentially large benefits:

- Less production costs
- Reduction of reaction steps
- New products and applications
- Less energy consumption
- Reduction of separation and purification steps
- Better yield on (renewable) raw materials
- Reduction of emissions
- New business areas

In contrast to these economic and environmental benefits there are several hurdles and challenges to be dealt with. The relative young white biotechnology has to compete with a mature oil-based chemical industry, meaning that biotechnological processes per se are not superior to classical chemistry. If a biotechnological product is more expensive to produce than products based on classical synthesis routes, it must be taken into account that it will not be successful on the market (Fig. 42). A biotechnical production is not an end

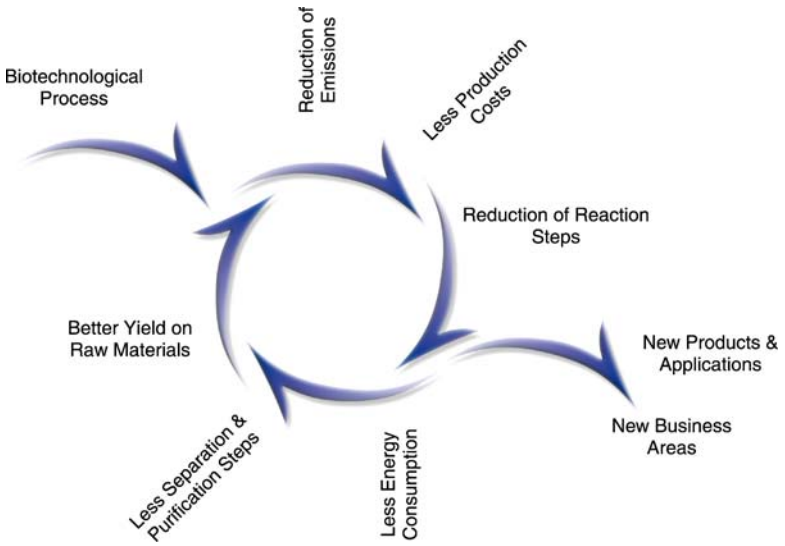


Fig. 42 Possible economical advantages of biotechnological processes

in itself but only one tool among many others. In each individual case a decision must be made about which tool to choose. This decision will be based on economic and ecological considerations, whereas ecological factors given by state and society only indirectly influence the market potential. Incidentally, switching to a novel process takes time and money, which is valid both for chemical and for biotechnological synthesis.

White biotechnology uses living cells (from yeast, molds, bacteria, and plants) as well as isolated enzymes to synthesize products, requires less energy, and creates less waste during production. However, the source "Nature" does not inevitably mean an environmentally friendly product. The production of an insecticide by a lipase-catalyzed process represents a conspicuous example. Likewise, biocatalyzed production cannot be equated with biological pathways concerning the production of the greenhouse gas CO_2. Only if the production of chemical building blocks is based on renewable resources and not on fossil sources is a CO_2-neutral process reached. Also, the production of energy from renewable resources and biomasses by turning, e.g., starch into ethanol, may not be the most environmentally nor economically efficient method. The use of plant protecting agents, irrigation, and machineries might consume more energy than can be allocated. Frequently the aqueous phase, which is often the basis for biocatalytic synthesis, is commended. However, this aqueous phase causes the need for large amounts of organic solvents because the products often need to be extracted in the course of downstream processing.

Today, modern biology allows precise modifications of the genetic information and improvements in enzyme properties. This allows shorter timelines for the development of processes and potentiates the production of new products. The new approaches using improved production strains and enzymes lead to benefits for the synthesis of building blocks in the fine chemical industry. Linkage of fermentation steps with chemical downstream processing steps can lead to successful production of bulk chemicals, starting from cheap renewable raw materials. In analogy to classical chemical production also processes in the field of white biotechnology will be enhanced by process development and optimization. Whenever there is an economical advantage, biotechnological processes will replace chemical processes. The number of these processes will increase through the development of new biocatalysts and through broadly educated scientists that have learned to speak the scientific languages of the different disciplines that are involved in designing industrial processes.

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Biorefineries – Multi Product Processes

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Abstract The development of biorefineries represents the key for access to an integrated production of food, feed, chemicals, materials, goods, and fuels of the future [1]. Biorefineries combine the necessary technologies of the biogenic raw materials with those of intermediates and final products. The main focus is directed at the precursors carbohydrates, lignin, oils, and proteins and the combination between biotechnological and chemical conversion of substances. Currently the lignocellulosic feedstock biorefinery, green biorefinery, whole corn biorefinery, and the so-called two-platform concept are favored in research, development, and industrial implementation.

Keywords Biobased industrial products · Biogenic raw material · Biorefineries · Green biorefinery · Lignocellulosic feedstock biorefinery · Two-platform concept · Whole-crop biorefinery

1 Introduction

Sustainable economic growth requires adequate resources of raw materials for industrial production. Today's most frequently used industrial raw material petroleum, is neither sustainable, because it is limited, nor environmentally friendly. While the economy of energy can be based on various alternative raw materials, such as wind, sun, water, biomass, as well as nuclear fission and fusion, the economy of substances fundamentally depends on biomass, in particular the biomass of plants. Special requirements are placed on both the substantial converting industry as well as research and development regarding the efficiency of raw materials and product lines as well as sustainability. "The development of biorefineries represents the key for the access to an integrated production of food, feed, chemicals, materials, goods, and fuels of the future" [1].

Whereas great successes regarding research and development in the young field of biorefinery system research are most notable in Europe and Germany [2–4], significant industrial developments have for the first time been pushed for by the President [5] and Congress [6] of the United States. In the United States of America it is expected by 2020 that at least 25 percent (compared to 1995) of organic carbon-based industrial feedstock chemicals and 10 percent of liquid fuels are derived from a biobased product industry. This would mean that more than 90 percent of the consumption of organic chemicals in the US and up to 50 percent of liquid fuel needs would be covered by biobased products [1]. The Biomass Technical Advisory Committee (BTAC) of the USA in which leading representatives of industrial companies, such as Dow Chemical, E.I. du Pont de Nemours, Cargill Dow LLC, Genencor International Inc. as well as corn growers associations and the Natural Resources Defense Council are involved and which acts as an advisor to the US government, has made a detailed step-plan for the targets of 2030 regarding bioenergy, biofuels, and bioproducts [7, 8] (Table 1).

Research and development are necessary to:

(a) increase the scientific understanding of biomass resources and improve the tailoring of those resources;
(b) improve sustainable systems to develop, harvest, and process biomass resources;
(c) improve efficiency and performance in conversion and distribution processes and technologies for a host of products development from biobased products; and
(d) create the regulatory and market environment necessary for increased development and use of biobased products.

The Biomass Advisory Committee has established specific R&D objectives for feedstock production research. Target crops should include oil and cellulose-producing crops that can provide optimal energy content and usable plant

Table 1 The U.S. national vision goals for biomass technologies by the Biomass Technical Advisory Committee

Year	Current	2010	2020	2030
BioPower (BioEnergy) Biomass share of electricity & heat demand in utilities & industry	2.8% (2.7 quads)	4% (3.2 quads)	5% (4.0 quads)	5% (5.0 quads)
BioFuels Biomass share of demand for transportation fuels	0.5% (0.15 quads)	4% (1.3 quads)	10% (4.0 quads)	20% (9.5 quads)
BioProducts Share of target chemicals that are biobased	5%	12%	18%	25%

components. Currently, however, there is a lack of understanding of plant biochemistry as well as inadequate genomic and metabolic information on many potential crops. Specifically, research to produce enhanced enzymes and chemical catalysts could advance biotechnology capabilities.

In Europe there are current regulations regarding substitution of not-renewable resources by biomass in the area of biofuels for transportation [9] beside the "Renewable Energy Law" [10]. According to the EC-Directive "On the promotion of the use of biofuels" the following products are considered as "biofuels":
(a) "bioethanol",
(b) "biodiesel",
(c) "biogas",
(d) "biomethanol",
(e) "biodimethylether",
(f) "bio-ETBE (ethyl-tertiary-butylether)" on the basis of bioethanol,
(g) "bio-MTBE (methyl-tertiary-butylether)" on the basis of biomethanol,
(h) "synthetic biofuels",
(i) "biohydrogen",
(j) pure vegetable oil.
Member States of the EU are requested to define national guidelines for a minimal amount of biofuels and other renewable fuels (with a reference value of 2% by 2005 and 5.75% by 2010 calculated on the basis of energy content of all petrol and diesel fuels for transport purposes).

Today there are no guidelines concerning "biobased products" in the European Union or in Germany. However, after passing directives for bioenergy and biofuels such decisions are on the political agenda. The directive of "biofuels" already includes ethanol, methanol, dimethylether, hydrogen

and biomass pyrolysis which are fundamental product lines of the future biobased chemical industry. A recent vision paper published by the Industrial Biotechnology section of the European Technology platform for Sustainable Chemistry foresaw up to 30% of raw materials for the chemical industry coming from renewable sources by 2025 [11]. Recently, the European Commission and US Department of Energy have come to an agreement to cooperate in this field [12].

2 Raw Material Biomass

Nature is a permanently renewing production chain for chemicals, materials, fuels, cosmetics, and pharmaceuticals. Many of the currently used biobased industry products are the result of a direct physical or chemical treatment and processing of biomass, such as cellulose, starch, oil, protein, lignin, and terpene. On the one hand one has to mention that due to the help of biotechnological processes and methods feedstock chemicals are produced such as ethanol, butanol, acetone, lactic acid and itaconic acid as well as amino acids, e.g. glutaminic acid, lysine, tryptophan. On the other hand, currently only 6 billion tons of the yearly produced biomass, $1.7–2,0 \times 10^{11}$ tons, are used, and only 3.0 to 3.5 percent of this amount is used in the non-food area, such as chemistry [13].

The basis reaction of the biomass is photosynthesis according to:

$$n\mathrm{CO_2} + n\mathrm{H_2O} \Rightarrow (\mathrm{CH_2O})_n + n\mathrm{O_2}\ .$$

Industrial utilization of raw materials for the energetic and material-converting industry coming from agriculture, forestry and from landscape care is still in the early stages.

The majority of biological raw materials are produced in agriculture, forestry, and by microbial systems. Forestry-derived plants are excellent raw materials for the paper and cardboard industry, the construction and chemical industry. Field fruits represent an organic chemical pool, from which fuels, chemicals and chemical products as well as biomaterials are produced (Fig. 1) [14]. Waste biomass and biomass of nature and landscape cultivation are valuable organic reservoirs of raw material and must be used in accordance to their organic composition. During the development of biorefinery systems the term “waste biomass” will become obsolete in the medium-term [4]. Because of low cost, plentiful supply, and amenability to biotechnology, carbohydrates appear likely to be the dominant source of feedstocks for biocommodity processing. Starch-rich and cellulosic materials each have important advantages in this context. Corn is by far the dominant feedstock for biological production of commodity products today. Advantages over cellulosic materials include much larger ultimate supply, lower purchase cost and

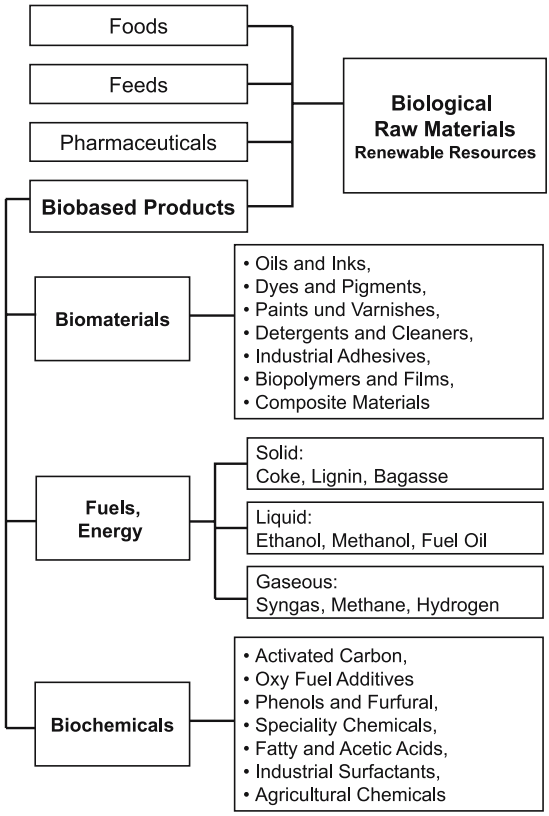

Fig. 1 Products and product classes based on biological raw materials [17]

lower anticipated transfer cost, less erosity, and lower inputs of chemicals and energy required for production [15]. Recently, the goal of the US Department of Agriculture and the US Department of Energy has been the additional supply of 1 billion tons of biomass for a prize of 35 US-Dollar per ton per year for industrial chemical and biotechnological utilization, without the restriction of today's applications of biomass from agriculture and forestry [16].

3
Principles of Biorefineries

3.1
Fundamentals

Biomass has—like petroleum—a complex composition. Its primary separation into main groups of substances is appropriate. Subsequent treatment and

processing of those substances leads to a whole palette of products. Petrolchemistry is based on the principle of generating from hydrocarbons simple-to-handle and well-defined chemically pure elements in refineries. In efficient product lines, a system based on family trees has been built, in which basic chemicals, intermediate products, and sophisticated products are produced. This principle from petroleum refineries must be transferred to biorefineries. Biomass contains the synthesis performance of Nature and has a different C : H : O : N-ratio than petroleum. Biotechnological conversion will become, beside the chemical, a big player in the future Fig. 2.

Thus, biomass can already be modified within the process of genesis in such a way, that it is adapted to the purpose of subsequent processing and particular target products have already been formed. For those products the term "precursors" is used.

Plant biomass always consists of the basic products carbohydrates, lignin, proteins and fats, besides various substances such as vitamins, dyes, flavors and aromatic essences of mostly different chemical structure. Biorefineries combine the essential technologies between biological raw materials and the industrial intermediates and final products (Fig. 3).

A technically feasible separation operation, which would allow a separate use or subsequent processing of all these basic compounds, exists up to now only in the form of an initial attempt. Assuming that out of the estimated annual production of biomass by biosynthesis of 170 billion tons 75 percent are carbohydrates, mainly in the form of cellulose, starch, and saccharose, 20 percent lignin and only 5 percent other natural compounds such as fats (oils), proteins and various other substances [20], attention should firstly be focused on an efficient access to the carbohydrates, their subsequent conversion to chemical bulk products and corresponding final products. Glucose, accessible by microbial or chemical methods from starch, sugar or cellulose,

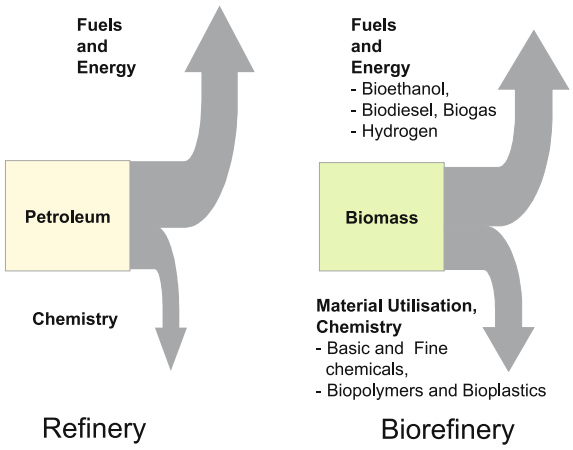

Fig. 2 Comparison of the basic-principles of petroleum-refinery and biorefinery [18]

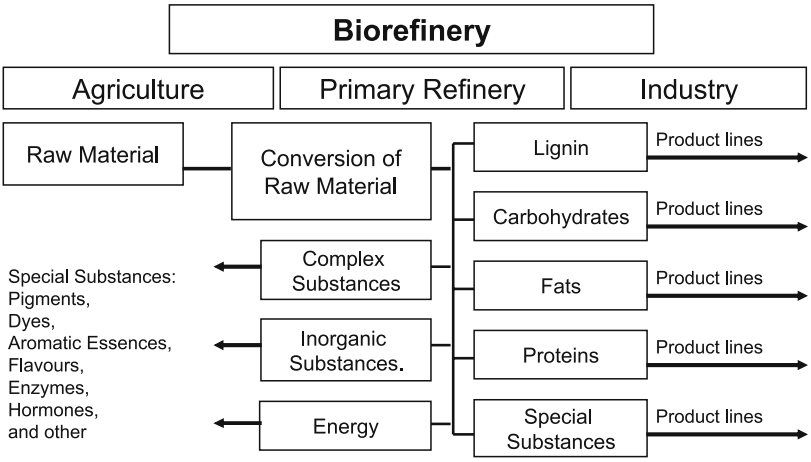

Fig. 3 Providing code-defined basic substances via fractionation for the development of relevant industrial product family trees [17, 19]

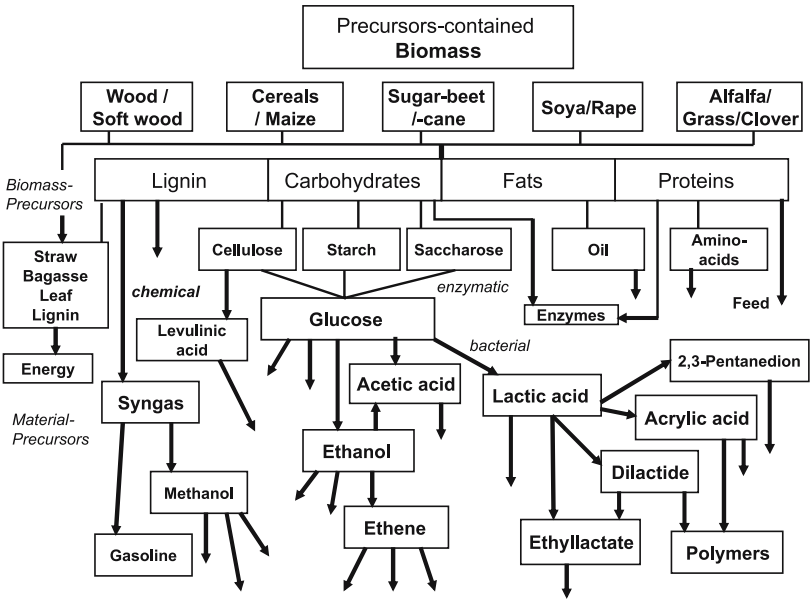

Fig. 4 A possible biorefinery rough-scheme for precursors-containing biomass with preference of carbohydrate line. acc. to [17, 19]

is among other things predestined for a key position as a basic chemical, because a broad palette of biotechnological or chemical products is accessible from glucose. In the case of starch the advantage of enzymatic compared to chemical hydrolysis has already been realized [21, 22].

In the case of cellulose this has not yet been realized. Cellulose-hydrolyzing enzymes can only act effectively after pre-treatment to break up the very stable lignin/cellulose/hemicellulose composites [23]. These treatments are still mostly thermal, thermo-mechanical, or thermo-chemical and require a considerable input of energy. The arsenal for microbial conversion of substances out of glucose is large, the reactions are energetically profitable. It is necessary to combine the degradation processes via glucose to bulk chemicals with the building processes to their subsequent products and materials (Fig. 4).

Among the variety of possible microbial and chemical products accessible from glucose, in particular lactic acid, ethanol, acetic acid, and levulinic acid are favorable intermediates for the generation of industrially relevant product family trees. Here, two potential strategies are considered: first, the development of new, possibly biologically degradable products (follow-up products of lactic and levulinic acid) and secondly, the entry as intermediates into conventional product lines (acrylic acid, 2,3-pentanedione) of petrochemical refineries [17].

3.2 The Role of Biotechnology

The application of biotechnological methods will be of high importance for the development of biorefineries for the production of basis chemicals, intermediate chemicals, and polymers [24–26]. The integration of biotechnological methods must be managed intelligently in respect to the physical and chemical conversions of the biomass. Therefore, the biotechnology cannot remain limited to glucose from sugar plants and starch from starch-producing plants (Fig. 5).

A main goal is the economical coverage of biomass containing lignocellulose and provision of glucose in the family tree system. Glucose is a key chemical for microbial processes. The preparation of a large number of family tree-capable basis chemicals is shown in the following sections. A variety of

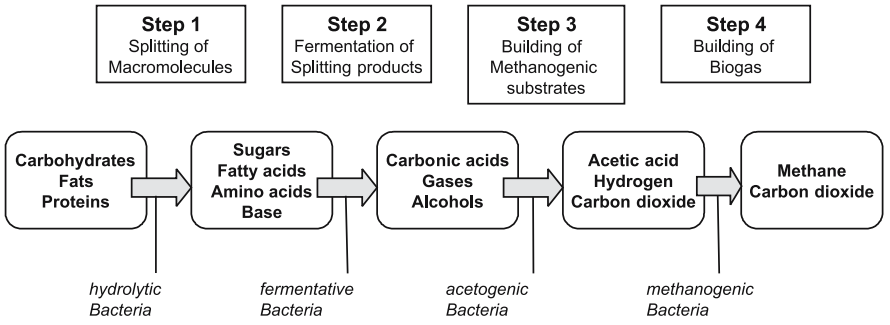

Fig. 5 Simplified presentation of a microbial biomass-breakdown regime [19]

microbial and chemical sequence products that are possibly accessible from glucose can be developed into a product family tree, such as (C-1)-chemicals methane, carbon dioxide, methanol; (C-2)-chemicals ethanol, acetic acid, acetaldehyde, ethylene, (C-3)-chemicals lactic acid, propandiol, propylene, propylene oxide, acetone, acrylic acid, (C-4)-chemicals diethylether, acetic acid anhydride, malic acid, vinyl acetate, *n*-butanol, crotone aldehyde, butadiene, 2,3-butandiol, (C-5)-chemicals itaconic acid, 2,3-pentane dione, ethyl lactate, (C-6)-chemicals sorbic acid, parasorbic acid, citric acid, aconitic acid, isoascorbinic acid, kojic acid, maltol, dilactide, (C-8)-chemicals 2-ethyl hexanol (Fig. 6).

Currently, guidelines are developed for the fermentation section of a biorefinery. The question, what represents an efficient arrangement of the technological design for the production of bulk chemicals, needs an answer.

Considering the manufacture of lactic acid and ethanol, the technological basic operations are very similar. The selection of biotechnologically based products from biorefineries should be done in a way that they can be produced from the substrates glucose or pentoses. Furthermore, the fermentation products should be extracellular. Fermentors should have either batch, feed batch, or CSTR design. Preliminary product recovery should require steps like filtration, distillation, or extraction. Final product recovery and purification steps should possibly be product unique. In addition, biochemical and chemical processing steps should be advantageously connected.

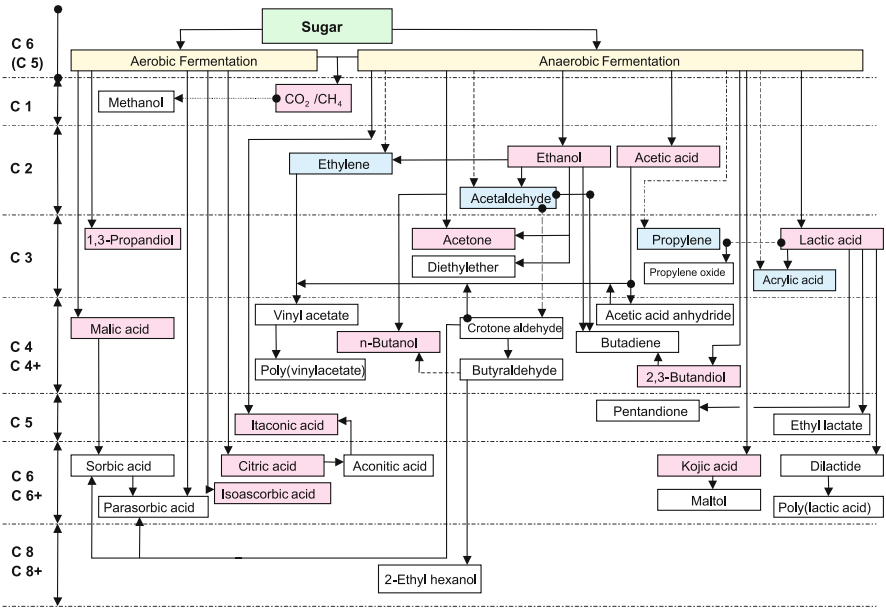

Fig. 6 Biotechnological sugar-based product family tree (cut out)

Unresolved questions for the fermentation facility include: (a) whether or not the entire fermentation facility can/should be able to change from one product to another; (b) if multiple products can be run in parallel, with shared use of common unit operations, (c) how to manage scheduling of unit operations, and (d) how to minimize in-plant inventories, while accommodating necessary change-overs between different products in the same piece of equipment [28].

3.3 Building Blocks, Chemicals and Potential Screening

A team from PNNL and NREL submitted a list of 12 potential biobased chemicals [29]. Key areas of the investigation were biomass-precursors, platforms, building blocks, secondary chemicals, intermediates, products and uses (Fig. 7).

The final selection of 12 building blocks began with a list of more than 300 candidates. The shorter list of 30 potential candidates was selected using an iterative review process based on the petrochemical model of building blocks, chemical data, known market data, properties, performance of the potential candidates, and the prior industry experience of the team at PNNL and NREL. This list of 30 was ultimately reduced to 12 by examining the potential markets for the building blocks and their derivatives and the technical complexity of the synthesis pathways.

The reported block chemicals can be produced out of sugar via biological and chemical conversions. The building blocks can be subsequently converted

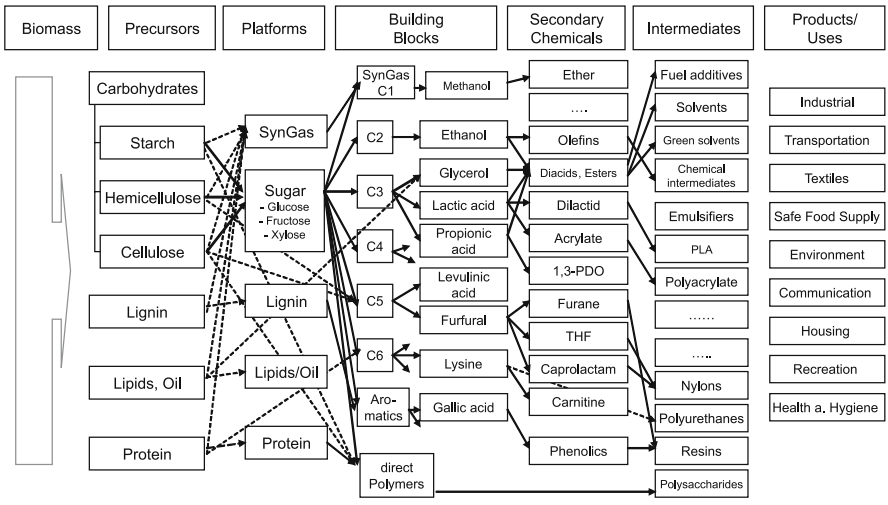

Fig. 7 Model of a biobased product flow-chart for biomass feedstock, cut out [29]

to a number of high-value biobased chemicals or materials. Building block chemicals, as considered for this analysis are molecules with multiple functional groups that possess the potential to be transformed into new families of useful molecules. The 12 sugar-based building blocks are 1,4-diacids (succinic, fumaric and malic), 2,5-furan dicarboxylic acid, 3-hydroxy propionic acid, aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxybutyrolactone, glycerol, sorbitol, and xylitol/arabinitol [29].

A second-tier group of building blocks was also identified as viable candidates. These include gluconic acid, lactic acid, malonic acid, propionic acid, the triacids, citric and aconitic; xylonic acid, acetoin, furfural, levuglucosan, lysine, serine, and threonine. Recommendations for moving forward include examining top value products from biomass components such as aromatics, polysaccharides, and oils; evaluating technical challenges in more detail related to chemicals and biological conversions; and increasing the suite of potential pathways to these candidates. From Syngas no further down select products were undertaken. For the purposes of this study hydrogen and methanol comprise the best near-term prospects for biobased commodity chemical production because obtaining simple alcohols, aldehydes, mixed alcohols and Fischer–Tropsch liquids from biomass is not economically viable and requires additional development [29].

3.4 Model Building Blocks

3.4.1 Example Succinic Acid

Succinic acid, derived from the fermentation of glucose, has a speciality chemical market in industries producing food and pharmaceutical products, surfactants and detergents, green solvents and biodegradable plastics, and ingredients to stimulate animal and plant growth. As a bifunctional compound, fermentation-derived succinate has the potential to supply over 2.7×10^8 kg industrial products/year including: 1,4 butandiol, tetrahydrofuran, γ-butyrolactone, adipic acid, *N*-methyl-pyrrolidone and linear aliphatic esters [30, 31]. In the last decade numerous patents have been issued on anaerobic production of succinic acid, microorganisms for such fermentations, and methods for purification, including both separations from cellular biomass and salt splitting [32–35]. Figure 8 shows important succinic acid-based intermediate chemicals that are currently in the research and development or demonstration stage. 2-Pyrrolidone (**path *s***) can be produced by direct hydrogenation of aqueous diammonium succinate with hydrogen in the presence of an active metal catalyst [36]. A mixed product of 2-pyrrolidone and *N*-methyl pyrrolidone (**path *s*** and **path *r***) can be produced from the conversion of diammonium succinate in the presence of methanol and an active

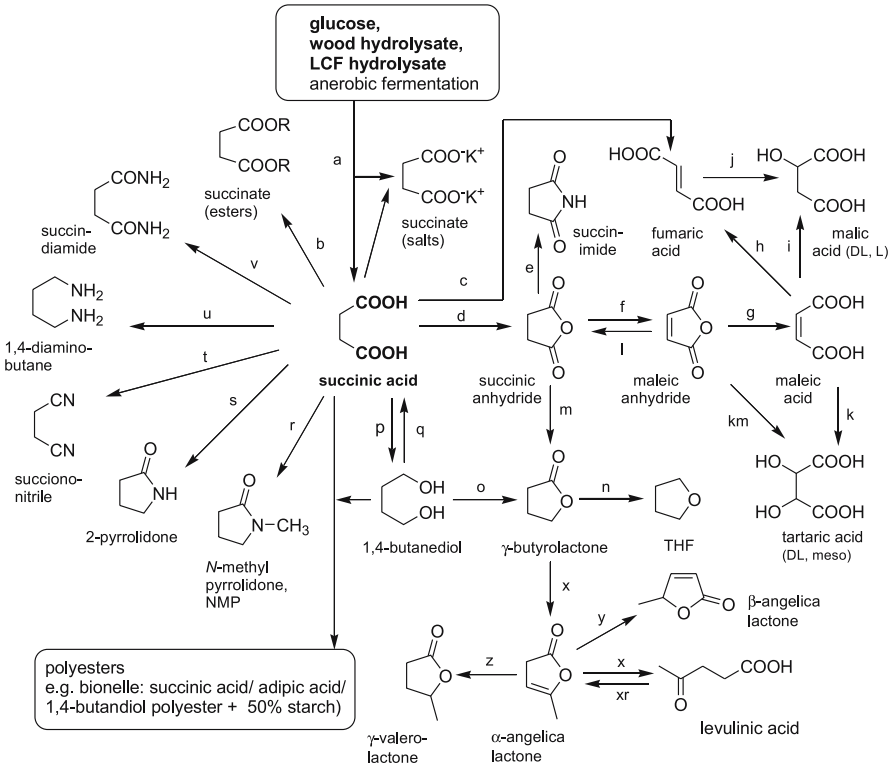

Fig. 8 Product family tree based on succinic acid

metal catalyst [31]. *N*-methyl pyrrolidone has been recommended as a replacement for methylene chloride as a solvent [30].

Butandiol (**path *p***) is an important industrial solvent and raw material for polybutylene terephthalate resins. γ-Butyrolactone (**path *p*** and **path *o***) is a chemical intermediate and ingredient of paint removers and textile products. Tetrahydrofuran from conversion of γ-butyrolactone (**path *n***) is a solvent and key ingredient of adhesives and printing inks. Linear aliphatic esters, such as succinate esters, are important compounds used in making resin and plastics. L-malic acid from the conversion of fumaric (**path *j***) acid by fumarase [37, 38] is applied as a building block for biodegradable polymers [39]. α-Angelica lactone, levulinic acid as well as γ-valerolactone (**paths *x,y,z***) are important building blocks for making solvents, biopolymers, fuels, and miscellaneous organics [40, 41].

3.4.2 Example Lactic Acid

Lactic acid was first produced worldwide from glucose or pure starch using fermentative methods [42]. First efforts aimed at developing bioconversion processes for the production of lactic acid directly from agricultural starchy feedstocks were published by Xiaodong et al. [43]. During the last few years, starchy hydrolyzates obtained from agricultural resources like corn or barley [21, 44], cassava [45], wheat [46], rye [47], potatoes [22], and sago [48] have also been tested for their suitability as substrates for lactic acid fermentation.

Lactic acid bacteria need besides the carbon source also a source of nitrogen and other nutrients, and a phosphorus source. The latter is available when inorganic phosphate salts are added to the medium. Lactic acid bacteria also need a series of nitrogen-containing nutrients (amino acids, peptides etc.) for growth and therefore, the medium has to be supplied by complex protein hydrolyzates (yeast extract, peptone etc.). The protein extracts mentioned are very expensive and their substitution by low-priced nutrient extracts is necessary when a large-scale production is planned. A useful combination of green biomass processing for the production of fodder pellets and the utilization of the pressed juice for lactic acid fermentation was described by [49, 50]. The use of date juice together with different nitrogen sources as a substrate for lactic acid production was investigated by [51].

In recent years, new lactic acid production processes consisting of the operations of enzymatic hydrolysis of starch [52–55] and cellulose [56–60], fermentation processes [42, 61–65], lactic acid separation and product purification [66–71] have been developed.

Lactic acid has the potential to become a commodity chemical. High rates of growth are expected, the volume of lactic acid sequence products rise to 1.36–1.80 million tons in the year alone for the US market [72]. Chemical sequence products are propylene glycol, propylene oxide, epoxides. Propylene oxide is a starting material for the production of polyester, polycarbonates, and polyurethanes. Further chemical sequence products are acrylic acid as a monomer for polyacrylic acid and resins as well as alkyllactates for application as "green solvents". Furthermore, enantiomeric forms of lactic acid, its salts and esters are applied in drugs, pharmaceuticals, and agrochemicals. Classical areas of application include so-called consumption lactic acid, for example, in the food and agricultural industries as well as in technical processes, for example, tanneries, textile industry, chemical industry [73, 74].

Enormous growth is expected for polymeric materials of lactic acid, poly(lactic) acid [75–79]. Polylactide is a versatile thermoplastic, which is processed in a manifold way [80]. They are processed for spin fibers, melt spin fibers, extrusion foils, injection moulding, thermoforming plates, ex-

trusion coating of paper and board and other applications. They are fully biodegradable and compost-able and do not disturb the normal process of biodegradation in compost.

Especially for the market segments of food packaging, food service, for example, one-way-consumption articles and performance-products for agriculture as well as fibers for textiles they are an interesting material.

For the USA a market volume of 140 000–900 000 tons per year for biodegradable polymers on the basis of lactic acid was estimated in the year 1993 (Batelle, SRI, Cargill). Since then greater effort has been placed on building larger industrial capacities. The company Cargill Dow LLC has built a commercial production facility for polylactide (PLA) in Blair, Nebraska, USA. The Blair facility started its operations in late 2002 and has a maximum capacity of 140 000 metric tons of PLA per year [81]. The establishment of further capacities by the company shall follow within the next 10 years up to a capacity of 450 000 metric tons in Asia and Europe. So it is expected that the price will decrease within the next years to the level of petrochemical-based thermoplastics [82].

The technology for the production of polylactide starts from the classical variant of lactic acid fermentation (primary production of anorganic salts of lactic acid), further steps of production of lactic acid and then oligomeric

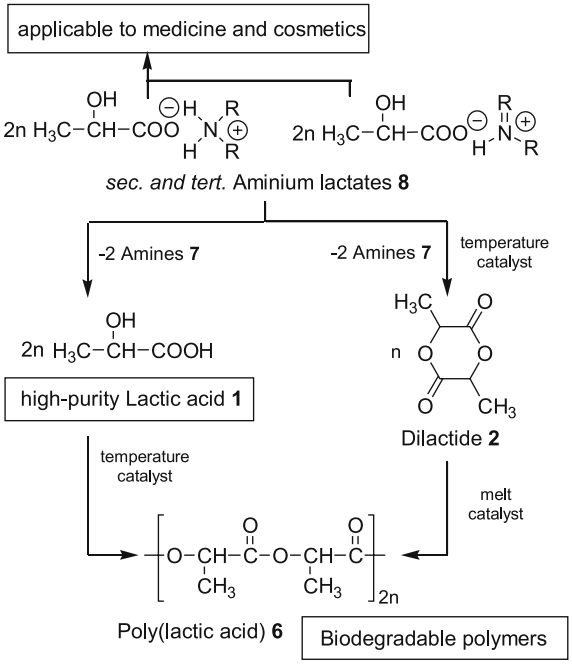

Fig. 9 Principle of procedure of extremely pure lactic acid **1** and of dilactide **2** based on aminium lactates **8** [19]

lactic acid, further synthesis of cyclic diesters of lactic acid, from which is accessible the polylactide by ring opening [79]. A new principle of technology has been patented and is shortly drafted in Fig. 9 [75, 76].

Aminium lactate can also be used as a starting material for the biotechnological and chemical conversion (synthesis of dilactide). Amium lactates have low melting points, good crystallinity and good thermal or hydrolytic dissociation. Amines have good solubility in water, a pH-value within the basic area, have sufficient thermal-, acid- and catalyst-stability for recycling after the process of cyclization, are ecologically safe and have economical availability. As an example piperazine dilactate is a good investigated model substance [74–76, 83].

4
Biorefinery Systems

4.1
Background

Biobased products are prepared for a useable economical use by a meaningful combination of different methods and processes (physical, chemical, biological, and thermal). It is therefore necessary that biorefinery basis technologies have to be developed. For this reason a profound interdisciplinary cooperation of the various compartment disciplines in research and development is inevitable. It appears to therefore be reasonable to refer to the term biorefinery-design. Biorefinery design means: Bringing together well sounded scientific and technological basics, with practice-near technologies, products and product lines inside biorefineries. The basis conversions of each biorefinery can be summarized as follows:

In the first step, the precursor containing biomass is separated by physical methods. The main products (M_1–M_n) and the by-products (B_1–B_n) will subsequently be subjected to microbiological or chemical methods. The follow-up products (F_1–F_n) of the main and by-products can furthermore be converted or enter the conventional refinery (see Fig. 4).

Currently four complex biorefinery systems are being investigated in research and development:

1. The “Lignocellulosic Feedstock Biorefinery” using “nature-dry” raw material such as cellulose-containing biomass and wastes.
2. The “Whole-Crop Biorefinery” uses raw material such as cereals or maize.
3. The “Green Biorefineries” using “nature-wet” biomasses such as green grass, alfalfa, clover, or immature cereal [17, 19].
4. The “Biorefinery Two-Platform Concept” includes the sugar platform and the syngas platform [29].

4.2 Lignocellulosic Feedstock Biorefinery

Among the potential large-scale industrial biorefineries the lignocellulose feedstock (LCF)-biorefinery will most probably be pushed through with highest success. On the one hand the raw material situation is optimal (straw, reed, grass, wood, paper-waste etc.), on the other hand conversion products have a good position in the traditional petrochemical as well as in the future biobased product market. An important point for utilization of biomass as chemical raw material is the cost of the raw material. Currently the costs for corn stover or straw are: 30 USD/ton; for corn 110 USD/ton (3 USD/bushel) [84].

Lignocellulose materials consist of three primary chemical fractions or precursors: (a) hemicellulose/polyoses, a sugar-polymer of predominantly pentoses; (b) cellulose, a glucose-polymer; and (c) lignin, a polymer of phenols (Fig. 10).

The lignocellulosic biorefinery-regime has a distinct ability for genealogical trees. The main advantage of this method is the fact that the natural structures and structure elements are preserved, the raw materials have also a low price, and large product varieties are possible (Fig. 11). Nevertheless, there is still development and optimization demand for these technologies, for example, in the field of separation of cellulose, hemicellulose, and lignin as well as lignin utilization in the chemical industry.

In particular furfural and hydroxymethylfurfural are interesting products. Furfural is the starting material for the production of nylon 6,6 and nylon 6. The original process for the production of nylon-6.6 was based on furfural. The last of these production plants was closed in 1961 in the USA for economic reasons (the artificial low prize of petroleum). Nevertheless, the market for nylon 6 is huge.

However, there are still some unsatisfactory parts within the LCF, such as utilization of lignin as a fuel, adhesive, or binder. Unsatisfactory because the lignin scaffold contains considerable amounts of mono-aromatic hydrocarbons, which, if isolated in an economically efficient way, could add a significant value increase to the primary processes. It should be noted that there are obviously no natural enzymes to split the naturally formed lignin into basic monomers as easy as this is possible for the also naturally formed polymeric carbohydrates or proteins [86].

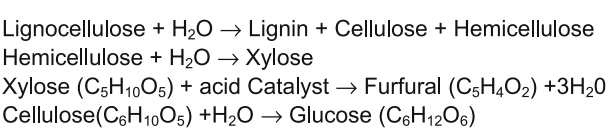

Fig. 10 A possible general equation of conversion at the LCF-Biorefinery

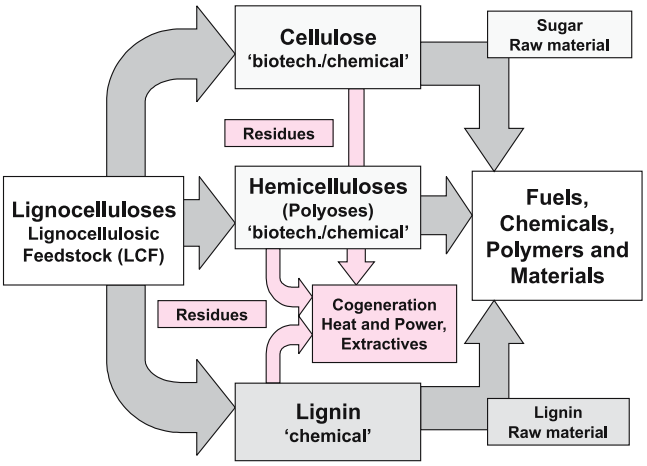

Fig. 11 Lignocellulosic feedstock biorefinery [85]

An attractive accompanying process to the biomass-nylon-process is the already-mentioned hydrolysis of the cellulose to glucose and the production of ethanol. Certain yeasts give a disproportionation of the glucose-molecule during their generation of ethanol to glucose which practically shifts its entire reduction ability into the ethanol and makes the last one obtainable in 90% yield (w/w; in regard to the formula turnover).

On the basis of recent technologies a plant was conceived for the production of the main products furfural and ethanol from LC-feedstock for the area of West Central Missouri (USA). Optimal profitability can be reached with a daily consumption of about 4360 tons of feedstock. Annually, the plant produces 47.5 million gallons of ethanol and 323 000 tons of furfural [28].

Ethanol may be used as a fuel additive. Ethanol is also a connecting product for a petrochemical refinery. Ethanol can be converted into ethene by chemical methods. As is well-known for petrochemically produced ethene, it is the starting point for a whole series of large-scale technical chemical syntheses for the production of important commodities such as polyethylene or polyvinylacetate. Further petrochemically produced substances can similarly be manufactured by microbial substantial conversion of glucose, such as hydrogen, methane, propanol, acetone, butanol, butandiol, itaconic acid, succinic acid [30, 31, 87]. DuPont has entered a 6-year alliance with Diversa in a biorefinery to produce sugar from husks, straw, stovers and develop a process to co-produce bioethanol and value-added chemicals, such as 1,3-propandiol [88]. Through metabolic engineering an Escheria coli K12 microorganism produces 1,3-propandiol (PDO), in a simple glucose fermentation process developed by DuPont and Genencor. In a pilot plant operated by Tate & Lyle, the PDO yield reaches 135 gl^{-1} at the rate of 4 $gl^{-1}\,h^{-1}$. PDO is used for the production of PTT (polytrimethylene-terephthalate), a new

polymer which is used for the production of high quality fibers branded Sorona [89]. Production is predicted to reach 500 kt per year by 2010.

4.3 Whole-Crop Biorefinery

Raw materials for the "Whole-Crop Biorefinery" are cereals, such as rye, wheat, triticale, and maize (Fig. 12). The first step is the mechanical separation into corn and straw, whereas the portion of corn is approximately 10% (w/w) and the portion of straw is 90% (w/w) [90]. Straw means a mixture of chaff, nodes, ears, and leaves. The straw represents a LC-feedstock and may further be processed in a LCF-biorefinery regime.

On the one hand there is the possibility of separation into cellulose, hemicellulose, lignin, and their further conversion within separate product lines which are shown in the LCF-biorefinery. Furthermore, the straw is a starting material for the production of syngas via pyrolysis technologies. Syngas is the basic material for the synthesis of fuels and methanol (Fig. 13).

The corn may be either converted into starch or directly used after grinding to meal. Further processing may be carried out in the four directions (a) breaking up, (b) plasticization, (c) chemical modification, or (d) biotechnological conversion via glucose. The meal can be treated and finished by extrusion into binders, adhesives, and fillers. Starch can be finished via plasticization (co- and mix-polymerization, compounding with other polymers), chemical modification (etherification) into carboxy-methyl starch; esterification and re-esterification into fatty acid esters via acetic starch; splitting reductive amination into ethylene diamine a.o. and, hydrogenative splitting into sorbitol, ethyleneglycol, propyleneglycol, glycerin [14, 91, 92].

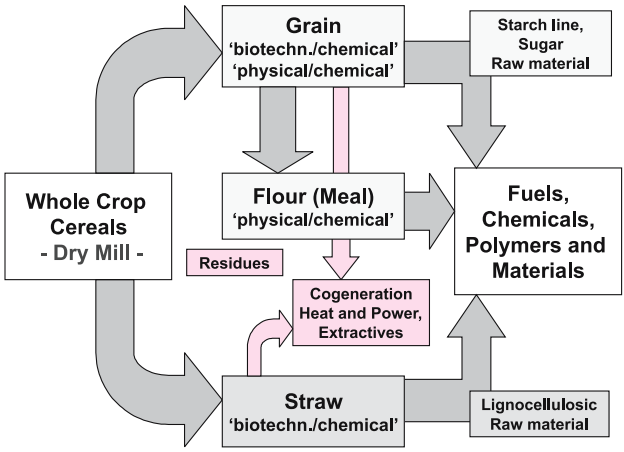

Fig. 12 Whole-crop biorefinery based on dry milling [85]

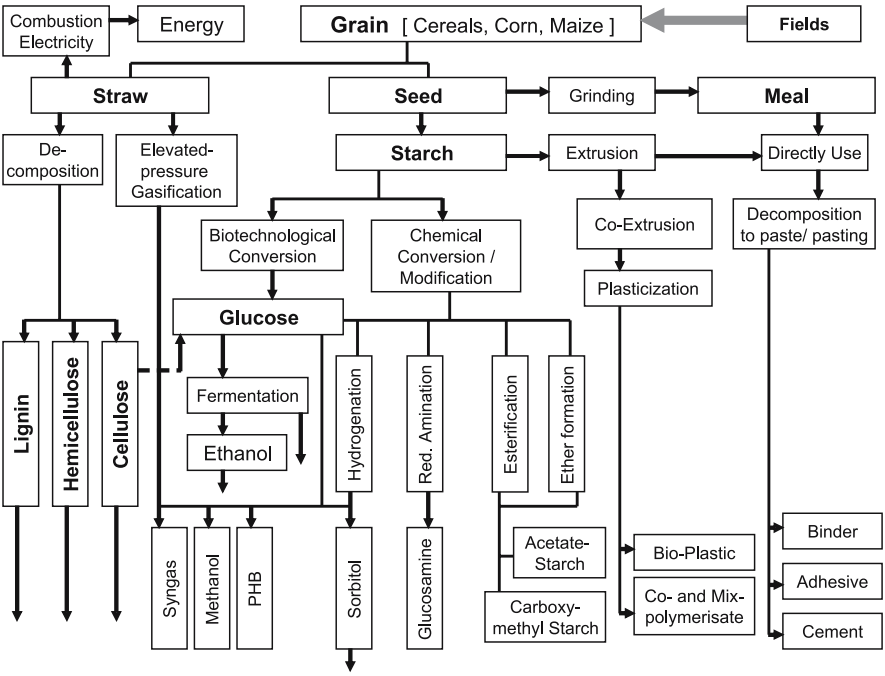

Fig. 13 Products from whole-crop biorefinery [17, 19]

Furthermore, starch can be converted by biotechnological methods into poly-3-hydroxybutyricacid in combination with the production of sugar and ethanol [93, 94].

Biopol, the copolymer poly-3-hydroxybutyrate/3-hydroxyvalerte, developed from ICI is produced from wheat carbohydrates by fermentation using *Alcaligenes eutrophus* [95].

An alternative to traditional dry fractionation of mature cereals into sole grains and straw was developed by Kockums Construction Ltd (Sweden), later called Scandinavian Farming Ltd. In this whole-crop harvest system whole immature cereal plants are harvested. The whole harvested biomass is conserved or dried for long-term storage. When convenient, it can be processed and fractionated into kernels, straw chips of internodes, and straw meal (leaves, ears, chaff, and nodes), (see also Green Biorefinery).

Fractions are suitable as raw materials for the starch polymer industry, feed industry, cellulose industry, particle board producers, gluten for the chemical industry, and as a solid fuel. Such a dry fractionation of the whole crop to optimize the utilization of all botanical components of the biomass has been described [96, 97]. A biorefinery and its profitability is described in [98].

An expansion of the product lines to grain processing represents the "whole-crop wet mill-based biorefinery". The grain is swelled and the grain germs are pressed, whereas high valuable oils are generated.

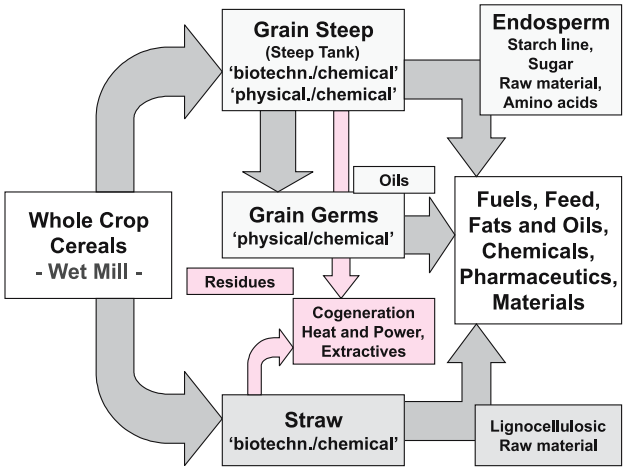

Fig. 14 Whole-crop biorefinery, wet-milling [85]

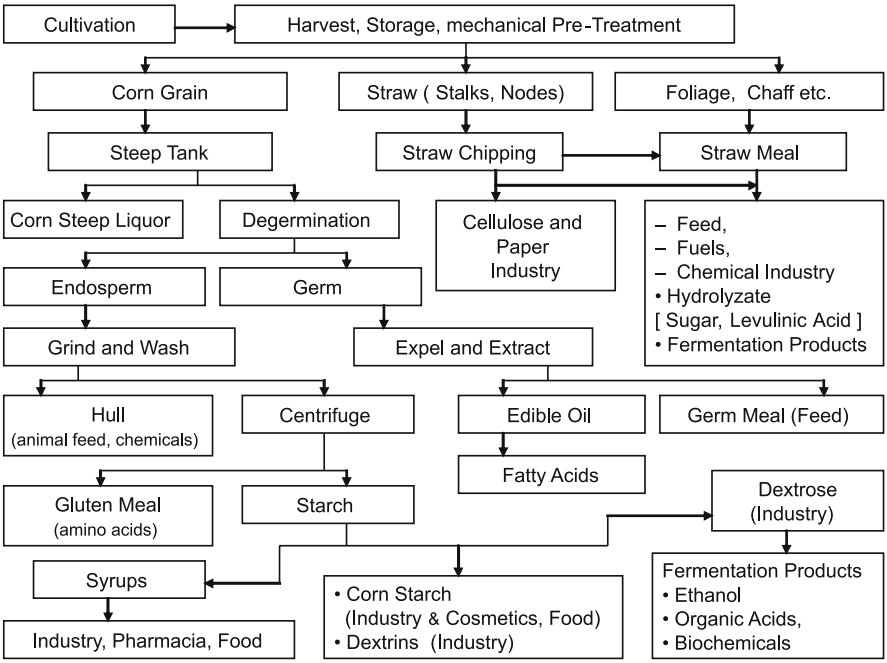

Fig. 15 Products from whole-crop wet mill-based biorefinery

The advantages of the whole-crop biorefinery based on wet milling is that the receipt of the natural structures and structure elements like starch, cellulose, oil, and amino acids (proteins) are kept to a high extent, and well-known basis technologies and processing lines can still be used. High raw material costs and the, for an industrial utilization necessary, costly source technologies are the disadvantages. Otherwise many products generate valuable prizes, for example in pharmacy and cosmetics (Figs. 14 and 15).

However, the basic biorefinery technology, the corn wet mills used 11 percent of the US corn harvest in 1992, made 7.0 billion of products, and employed almost 10 000 people [1].

Wet milling of corn yields corn oil, corn fiber, and corn starch. The starch products of the US corn wet milling industry are fuel alcohol (31 percent), high-fructose corn syrup (36 percent), starch (16 percent), and dextrose (17 percent). Corn wet milling also generates other products (e.g. gluten meal, gluten feed, oil) [99]. An overview of the product range is shown in Fig. 15.

4.4 Green Biorefinery

Often the economy of bioprocesses is still the problem because in the case of bulk products the price is affected mainly by raw material costs [100]. The advantages of the green biorefinery are a high biomass profit per hectare and a good coupling with the agricultural production, whereas the price segment of the raw materials is still low. On the one hand simple basis technologies can be used and provide a good biotechnical and chemical potential for further conversions (Fig. 16).

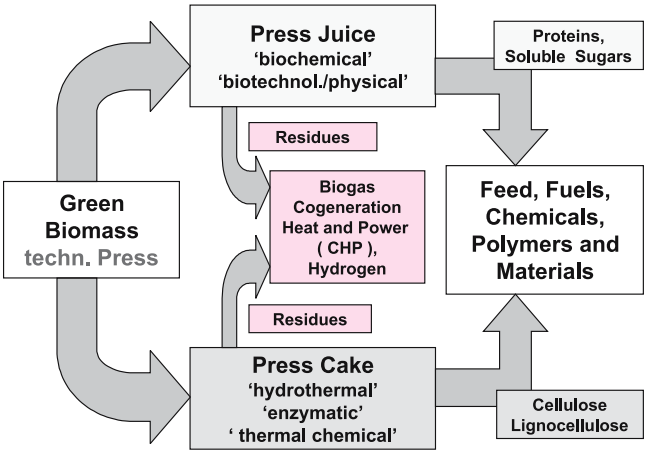

Fig. 16 A system "green biorefinery" [85]

On the other hand a fast primary processing or the uses of preservation methods like silage or drying are necessary, both for the raw materials and the primary products. However, each preservation method changes the content materials.

Green biorefineries are also multi-product-systems and act regarding to their refinery-cuts, -fractions, and -products in accordance with the physiology of the corresponding plant material.

Green biomass is represented predominantly by green crops, for example grass from cultivation of permanent grass land, closure fields, nature preserves or green crops, such as lucerne, clover, immature cereals from extensive land cultivation. Thus, green crops represent a natural chemical factory and food plant.

Green crops are primarily used today as forage and a source of leafy vegetables. A process called wet-fractionation of green biomass, green crop fractionation, can be used for simultaneous manufacturing of both food and non food items [101].

Scientists in several countries developed green crop fractionation in Europe and elsewhere [102–104]. Green crop fractionation is now studied in about 80 countries [101]. Several hundreds of temperate and tropical plant species have been investigated for green crop fractionation [104–106]. However, more than 300 000 higher plant species are left for future investigations. For reviews see: [2, 102, 103, 107–110].

Green biorefineries can, by fractionation of green plants, process from a few tonnes of green crops per hour (farm-scale process) to more than 100 tonnes per hour (industrial-scale commercial process).

The careful wet-fractionation technology is used as the first step (primary refinery) to isolate the content-substances in their natural form. Thus, the green crop goods (or humid organic waste goods) are separated into a fiber-rich press cake (PC) and a nutrient-rich green juice (GJ).

Beside cellulose and starch, the press cake contains valuable dyes and pigments, crude drugs, and other organics. The green juice contains proteins, free amino acids, organic acids, dyes, enzymes, hormones, other organic substances, and minerals. In particular, the application of the methods of biotechnology is predestinated for conversions, because the plant water can simultaneously be used for further treatments. In adding the lignin-cellulose composite of the PC are not so strongly as at lignocellulose-feedstock materials. Starting from green juice the main focus is directed to products such as lactic acid and the corresponding derivatives, amino acids, ethanol, and proteins. The press cake can be used for production of green feed pellets, as raw material for production of chemicals, such as levulinic acid, as well as for conversion to syngas and hydrocarbons (synthetic biofuels). The residues of substantial conversion are suitable for the production of biogas combined with the generation of heat and electricity (Fig. 17). Reviews of green biorefinery concepts and goals are published in [2, 3, 74, 85].

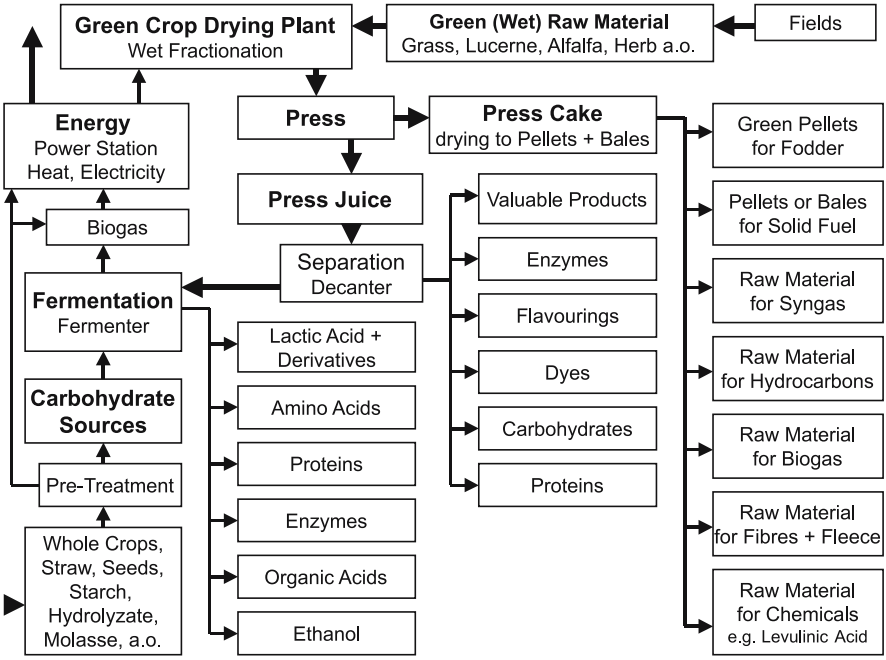

Fig. 17 Products from a green biorefinery. A system green biorefinery combined with a green crop drying plant [17, 19]

4.5 The Two-Platform Concept

The "two-platform concept" means firstly that biomass consists on average of 75 percent of carbohydrates which can be standardized over a "intermediate sugar platform" as a basis for further conversions and secondly the biomass can be converted thermochemically to syngas as a basis for further products.

The "sugar platform" is based on biochemical conversion processes and focuses on the fermentation of sugars extracted from biomass feedstocks.

The "syngas platform" is based on thermochemical conversion processes and focuses on the gasification of biomass feedstocks and by-products from conversion processes [29, 102, 111]. In addition to the gasification other thermal and thermochemical biomass conversion methods have been described: hydrothermolysis, pyrolysis, thermolysis, burning. The application is according to the water content of the biomass [113].

The gasification and all thermochemical concepts concentrate on the utilization of the precursor carbohydrates as well as their carbon and hydrogen content. The proteins, lignin, oils and lipids, amino acids and other ingredi-

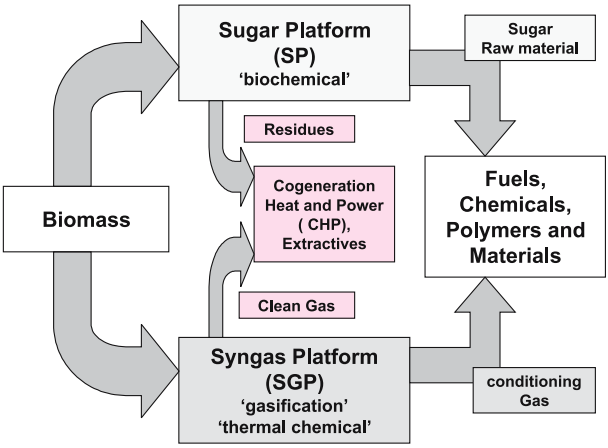

Fig. 18 Sugar platform and syngas platform [112]

ents as well as *N*- and *S*-compounds occurring in every biomass are not taken into account in this case (Fig. 18).

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Enabling Technologies: Fermentation and Downstream Processing

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Abstract Efficient parallel tools for bioprocess design, consequent application of the concepts for metabolic process analysis as well as innovative downstream processing techniques are enabling technologies for new industrial bioprocesses from an engineering

point of view. Basic principles, state-of-the-art techniques and cutting-edge technologies are briefly reviewed. Emphasis is on parallel bioreactors for bioprocess design, biochemical systems characterization and metabolic control analysis, as well as on preparative chromatography, affinity filtration and protein crystallization on a process scale.

Keywords Bioprocess design · Parallel bioreactors · Stoichiometric network analysis · Enzyme kinetic models · Metabolic control analysis · Preparative chromatography · Affinity filtration · Protein crystallization

Abbreviations

a_n, b_n	linear coefficients for the Takagi–Sugeno model
c	solute concentration
c^*	solute concentration at saturation
d_p	mean particle diameter
e	enzyme level
h_{ij}	logarithmic gain
k,k_i	kinetic constants or mass transfer coefficient
n	empirical exponent of Eq. 31
n_I	number of internal metabolites
n_P	number of metabolic products
n_R	number of reactions
n_S	number of substrates
n_X	number of biomass constituents
q	adsorbed protein concentration
q_m	maximum adsorbtion capacity of adsorbent
s	time variable
t	time variable
u	superficial fluid velocity
v_{max}	maximal substrate specific reaction rate
y	coordinate
$CCC_k^{S_i}$	concentration control coefficients
$FCC_k^{J_i}$	flux control coefficients
G	material property function
J_i	steady-state flux I
K_d	dissociation constant
K_M	Michaelis–Menten saturation constant
L	bed depth of column
MCA	metabolic control analysis
MFA	metabolic flux analysis
N	number of nuclei or mass of crystals per unit volume
S	substrate concentration
X_j	reaction specie j
$\underline{\mathbf{A}}$	substrate stoichiometric matrix
$\underline{\mathbf{B}}$	products stoichiometric matrix
$\underline{\mathbf{C}}^{J0}$	flux control coefficients matrix
$\underline{\mathbf{C}}^{X0}$	concentration control coefficients matrix
$\underline{\mathbf{E}}_x$	elasticity matrix
$\underline{\mathbf{E}}_c$	elasticity matrix
$\underline{\boldsymbol{\Gamma}}$	biomass constituents stoichiometric matrix

$\underline{\mathbf{G}}$	internal metabolites stoichiometric matrix
$\underline{\mathbf{S}}$	substrates concentration vector
$\underline{\mathbf{P}}$	products concentration vector
$\underline{\mathbf{X}}$	biomass constituents concentration vector
$\underline{\mathbf{I}}$	internal metabolites concentration vector
$\underline{\mathbf{0}}$	zero vector
$\underline{\boldsymbol{\nu}}$	vector of kinetic rates
$\boldsymbol{A}_\mathrm{n}$, $\boldsymbol{B}_\mathrm{n}$	fuzzy sets
α_ji	stoichiometric coefficient
β_ji	stoichiometric coefficient
γ_ji	stoichiometric coefficient
g_ji	stoichiometric coefficient
δ_ij	Kronecker delta
Δp	pressure drop in packed bed
ε	void fraction of packed bed
$\varepsilon_\mathrm{i}^\mathrm{k}$	elasticity coefficient
ϕ_An	fuzzy-logic membership function
$\dot{\gamma}$	local shear rate
λ	material property constant
μ	dynamic viscosity
τ	local shear stress function

1 Introduction

Fermentation science and engineering is mature compared to the emerging biotechnological fields like the "omics" tools (genomics, transcriptomics, proteomics, metabolomics, ...) and metabolic engineering. Nevertheless, the combination of a better understanding of bioreactor performance and improved equipment design with the increasing knowledge of cellular physiology and nutrition will result in enhanced fermentation processes in the near future. Fermentation engineering on an industrial scale will thus profit from advances in process modeling and control if the increasing knowledge of cellular physiology can be integrated. The modern "omics" tools will lead to more and more high-performance production strains. Fermentation engineering has to keep pace with this development. There is a pressing need for highly parallel cultivation systems in order to shorten process development times and to converge the abilities of the newly designed strains into optimized fermentation processes. The state-of-the-art in this field is outlined ("Bioprocess Design").

The basis of a purposeful metabolic engineering is the metabolic analysis of the respective biological system. Meanwhile, the established tools of metabolic flux analysis (MFA) and metabolic control analysis (MCA) are more and more combined with experimental data making use of the "omics" tools. The overall goal is to explain and predict complex behaviors of bi-

ological systems (systems biology). Some aspects of this field are outlined ("Metabolic Process Analysis"). Crucial in this respect is that the information about cellular functioning should be obtained for conditions as in industrial production processes which involve instationary growth and product formation, high biomass and product concentrations, different types of cell stress, fluctuations in nutrient concentrations (DO and other substrates), mixed substrate utilization (originating from complex substrates and biomass hydrolysis) and other typical reaction conditions on a technical scale. These conditions have to be recreated in a very controlled manner on laboratory or sub-laboratory scale.

With respect to downstream processing of bioproducts, this is a totally different situation when compared to fermentation science. A technology bottleneck is observed due to the lack of academic interest in downstream processing of bioproducts within the past decade. In part this may be due to the highly product specific point of view in this field. There is a considerable lack of dealing with downstream processing in a more generic way. Innovative downstream processing techniques (using for instance affinity separation processes, new biocompatible solvents like ionic liquids or protein crystallization) as well as process integration (the bioreactor is regarded as a unit with both upstream and downstream processing) are on the agenda of future research in this field. Some considerations to this topic are summarized in ("Downstream Processing of Bioproducts").

2 Bioprocess Design

Bioprocess design can be divided into three basic tasks. First, primary screening aims at the selection of biocatalysts with above-average productivity. The number of potential biocatalysts increases rapidly as a result of sequencing of unknown genomes, screening of existing biocatalyst libraries and the culturing of yet uncultivable organisms. Even more biocatalyst variety can be created by readily available genetic and metabolic engineering tools. Consequently, reaction systems for primary screening require enormous throughput to enable efficient screening of biocatalyst candidates. Thereby, suboptimal reaction conditions are accepted for the benefit of high throughput.

The candidates selected during primary screening are further examined during secondary screening which aims at the optimization of reaction conditions such as temperature, pH, medium composition, substrate or precursor concentration, operation mode and feeding profile. An early start of the secondary screening concurrently to primary screening favors the consideration of encountered problems during primary screening. Moreover, the more biocatalysts that enter secondary screening, the higher is the probability of a successful process design. Although high throughput during secondary

screening is beneficial, more importance needs to be attached to comparable reaction conditions as prevail in larger scale production reactors to facilitate scale-up, the last step in bioprocess design.

While primary and secondary screening are mostly carried out with simple batch reactors such as shake flasks and microtiter plates, scale-up aims at developing a reliable process with satisfying productivity in controlled stirred-tank reactors. Since the transfer of process information from the shaken to the stirred reaction system is often problematic, the bottleneck in bioprocess design can be attributed to secondary screening. More efficient process design could be accomplished with a highly parallel reaction system for cultivations under controlled production process conditions.

2.1 Reaction Systems

2.1.1 Stirred-Tank Reactors at Laboratory Scale

Modern bioreactors for microbial fermentation are usually equipped with at least three standard Rushton turbines on a common shaft. Impeller speed can be controlled at values of up to $1500\,\mathrm{min}^{-1}$ in laboratory-scale reactors thereby enabling a volumetric power input of up to $10\,\mathrm{W\,L}^{-1}$. Sterile gas supply can be controlled by digital mass flow controllers so as to reach oxygen transfer rates of up to $200\,\mathrm{mmol\,L}^{-1}\mathrm{h}^{-1}$ when air is used for aeration. The combination of several mass flow controllers can be used to control the oxygen partial pressure in the inlet gas flow to further improve oxygen transfer. Bioreactors are usually equipped with online monitoring of dissolved oxygen (DO) and pH by sterilizable electrodes. Reaction volume as well as substrate and titration agent consumption rates can be measured online or calculated gravimetrically. Exhaust gas analyzers are available for measuring oxygen and carbon dioxide content online to gain information about the metabolic state of the culture. Furthermore, several computer-controlled pumps are usually available for the automated addition of feed solution, inducing agent and acid or base for pH control.

Fed-batch operation mode is the method of choice for modern biotechnological production processes with recombinant host strains. This is because metabolic activity can be adequately controlled by application of a suitable feeding strategy. Overfeeding of a culture can lead to a carbon uptake exceeding the capacity of the central metabolic pathways and thus to the formation of by-products which interfere with cell growth and productivity. Metabolic activity can be controlled by the adapted feeding of the culture. Several strategies can be applied to control the feed flow. First, DO or pH auxostat operation ensures that sufficient oxygen partial pressure or constant pH, respectively, prevails in the medium during feeding to avoid by-product for-

mation. Second, sufficient oxygen transfer to the medium is crucial to avoid by-product formation due to anaerobic growth. In order to reach high cell densities, oxygen uptake is usually reduced by limiting the growth rate of the culture. This may be achieved by the use of defined media, the reduction of growth temperature and by the application of exponential feeding strategy which limits the growth rate to a preset value. Thereby, cell densities of over $100\,g\,L^{-1}$ can be routinely achieved. Maximum cell densities of $\sim 200\,g\,L^{-1}$ were reported [1].

An example of a recombinant fed-batch bioprocess is the production of tilapia insulin-like growth factor-2 (IGF-2) with *E. coli* BL21. The initial medium contained glucose and yeast extract. During fed-batch, pH-auxostat operation with a feed solution containing glucose and yeast extract yielded a maximum cell concentration of $183\,g\,L^{-1}$ dry cell mass after 30 h. The pH auxostat operation was able to keep the glucose concentration below $1.5\,g\,L^{-1}$ resulting in an acetate concentration of below $1\,g\,L^{-1}$ throughout the cultivation. At the end of the induced production process, the $156\,g\,L^{-1}$ dry cell mass produced $9.7\,g\,L^{-1}$ IGF-2 [2]. Another example applying the methylotrophic yeast *Pichia pastoris* is the recombinant production of angiostatin under the control of the methanol inducible AOX1 promoter. After batch growth in a defined medium with glycerol as the sole carbon source, a stepwise increasing constant glycerol feed rate of $2.3–11.1\,g\,h^{-1}$ was applied. A final cell concentration of $148\,g\,L^{-1}$ producing $108\,mg\,L^{-1}$ angiostatin was reached [3].

All these examples are the result of bioprocess design studies carried out in laboratory scale bioreactors. A skilled person is able to conduct 2–8 cultivations per month depending on bioprocess time with a single bioreactor system.

2.1.2 Parallel Stirred-Tank Reactors

To increase throughput in bioprocess development, stirred tank reactors with a working volume of 200–500 mL have been parallelized into units of 4–16 reactors each of which is equipped with separate measuring probes and control devices (Sixfors, Infors AG, Bottmingen Switzerland; Biostat Q, Sartorius BBI, Goettingen, Germany; Stirrer-pro, Dasgip AG, Jülich, Germany). Only little information about their biochemical engineering process parameters such as oxygen transfer and power input is available. The cost- and labor-intensity of the operation of these units is similar to an equivalent number of individual bioreactors, so no substantial benefit in terms of throughput can be achieved.

2.1.3 Shake-Flasks

The most widely used parallel reaction system in biotechnology is the shake flask. Several dozens of them can be simultaneously incubated in rotary

shakers. The rotary movement intensifies mixing and surface aeration. A sterile closure prevents contamination of the culture from the surrounding gas phase. The main disadvantages of the shake flask are the reaction engineering differences compared to the stirred tank reactor. The liquid inside a shaking flask comprises the bulk liquid and a thin liquid layer distributed at the inner flask wall orientated opposite the centrifugal force induced by the rotational movement. The thin liquid film contributes more to the overall oxygen transfer than the bulk of the liquid [4]. Since overall oxygen transfer is proportional to the surface to volume ratio, oxygen transfer can be increased by decreasing the filling volume or increasing the eccentricity or shaking speed. The specific oxygen transfer coefficient $k_L a$ was measured by various methods [5–7]. The most comprehensive study applied the steady-state sulfite method [8]. The $k_L a$ values of shake flasks with different nominal volumes, a relative filling volume of 4% at an eccentricity of 5 cm were modelled with a prediction accuracy of ±30%. The corresponding model $k_L a$-values are represented in Fig. 1A as a function of shaking speed. Increasing shaking speed and a smaller shake flask size lead to a higher $k_L a$-value at a constant relative filling volume.

The highest reported $k_L a$-value of 0.157 s^{-1} was measured in a 50 mL shake flask at a shaking speed of 450 min^{-1} and an eccentricity of 7 cm with a filling volume of 2 mL.

The power input in shake flasks of smaller nominal volume is lower than in their bigger counterparts [9]. The comparison of the $k_L a$-value in flasks of different nominal volume shows that higher $k_L a$-values in the smaller shake flasks are not a result of higher power input, thus the volumetric power input is not a valid scaling criterion among shake flasks of varying nominal volume (Fig. 1B).

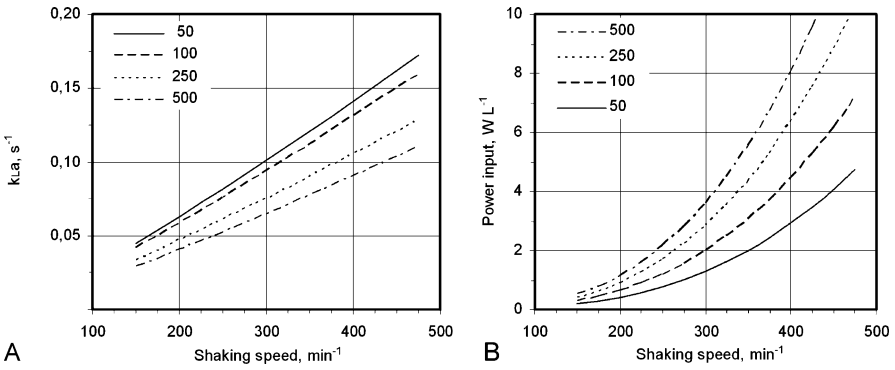

Fig. 1 **A** Specific oxygen transfer coefficient and **B** volumetric power input as a function of rotational speed in shake flasks of variable nominal volume at a relative filling volume of 4% of the nominal volume and an eccentricity of 5 cm. Correlations taken from [5, 6]

The operation mode in the classic shake flask is restricted to batch operation. Sampling is carried out manually and all process analytics are therefore conducted offline. The parallel operation of up to 16 so-called "instrumented" shake flasks with pH control in fed-batch mode was enabled by the integration of steam-sterilizable pH electrodes into the flasks and connecting a syringe pump via a controlled multi-valve system to the shake flasks [10]. The resulting intermittent feeding mode was used to cultivate *Escherichia coli* in a complex medium with glucose as the sole carbon source and pH control. The mean cell concentration of nine parallel shake flasks reached $5.1 \pm 0.64\ g\ L^{-1}$ in a process time of 12 h.

Further parallelization to more than 16 units seems difficult on the basis of the used technology.

2.1.4 Microtiter Plates

Even more parallel cultivations than in shake flasks can be carried out in microtiter plates. Various microtiter plate formats with 24, 48 or 96 wells are used for microbial cultivation. Because of the shaking motion, microtiter plates are characterized by the same reaction engineering disadvantages as shake flasks. High oxygen transfer rates can only be achieved by adjusting a high surface to volume ratio due to the formation of a vortex during shaking. Applying common shaking speeds of $300\ min^{-1}$ and an eccentricity of 25 mm, the high surface tension of aqueous media prevents the formation of a vortex and thus enhancement of oxygen transfer (Fig. 2A). A microtiter plate with 96 wells allows a maximum $k_L a$-value of $\sim 0.04\ s^{-1}$ independent of the applied eccentricity at specific maximum shaking speeds of $300–1000\ min^{-1}$. Further increase of shaking speed leads to spilling of the liquid medium out of the wells [11]. Deepwell microtiter plates allow higher shaking speeds and thus reach higher $k_L a$-values. Plates with round-shaped wells reach a $k_L a$-value of $0.15\ s^{-1}$ at $800\ min^{-1}$ (Fig. 2B). Deepwell plates with square-shaped wells break the circular motion of the liquid and even reach a $k_L a$-value of $0.23\ s^{-1}$ at $700\ min^{-1}$ because their corners act as baffles [12]. Microtiter plates with 48 round-shaped wells were examined at eccentricities of 3 and 25 mm. Interestingly, the smaller eccentricity allows a $k_L a$-value of $0.44\ s^{-1}$ since even at a shaking speed of $1450\ min^{-1}$ no spilling occurs [13].

Because of the high surface to volume ratio, evaporation in microtiter plates usually needs to be minimized. Different oxygen permeable adhesive tapes have been characterized with regard to their oxygen transfer and water retention capacity [14] allowing the choice of a sealing tape which meets the individual requirements for a given experiment. A commercially available cultivation system [13] reduces the evaporation in a deepwell microtiter plate by a cover layer consisting of pierced silicon and cotton wool. With an initial

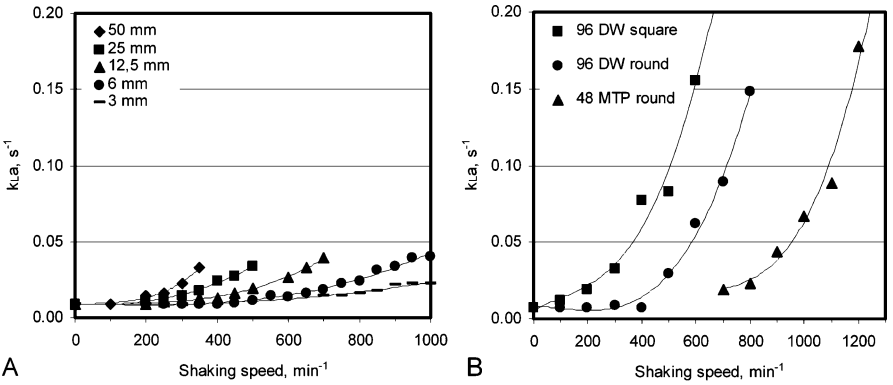

Fig. 2 **A** $k_L a$ as a function of shaking speed in a conventional round-shaped 96 well microtiter plate shaken with various eccentricities from 3–50 mm [8]. **B** $k_L a$ as a function of impeller speed for square-shaped and round-shaped deepwell (DW) microtiter plates with 96 wells [9] and round-shaped microtiter plates with 48 wells [10]. Further increase of shaking speed resulted in spilling of liquid out of the wells

reaction volume of 750 μL, the evaporation was decreased to less than 10 μL per well per day at 25 °C and a shaking speed of 300 min^{-1}.

Measurement of pH and DO concentration in microtiter plates was technically realized with fluorescence sensors immobilized at the bottom of the wells. The measurement of both parameters inside a microtiter plate photometer requires the disruption of the shaking movement and thus oxygen transfer and mixing. Microtiter plate photometers with integrated shakers are commercially available but they are characterized by limited shaking speeds and eccentricities so as to reach $k_L a$-values of $\sim 0.03\ s^{-1}$ [15].

To circumvent the problem of simultaneous shaking and measurement of fluorescence, a fluorescence detection system for pH and dissolved oxygen concentration was developed in the format of a microtiter plate (Sensordish Reader, Presens, Regensburg, Germany). The cultivation plate and the sensor dish can both be mounted on a shaker and thus simultaneous measurements without disruption of shaking is possible.

A fully automated cultivation system with 16 parallel microtiter plates with 48 wells has been developed for batch cultivation [16]. The microtiter plates are automatically inoculated by a liquid-handling system, sealed and then incubated on shakers. Automated fluorescence measurements were carried out in a microtiter plate reader. The fluorescence signal at 370/460 nm (NADPH) was taken as a measure for cell concentration. Cultivation with *E. coli* JM 101 in Terrific Broth reached the equivalence of 10 units optical density in a single beam photometer within 36 hours. The standard deviation of cell concentration of all 768 cultivation was determined to ~ 6%.

A technical solution for the substrate addition to shaken microtiter plates has not been realized, yet. Regarding the necessity of a sterile closure to

ensure monoseptic conditions and to reduce evaporation, the realization of fed-batch cultivations in microtiter plates seems difficult.

2.1.5 Stirred-Tank Reactors at Milliliter Scale

In the course of further miniaturization several small-scale bioreactors were developed. A stirred tank reactor with a diameter of 16 mm which is geometrically similar to lab-scale reactors was developed [17, 18]. Three Rushton turbines with a diameter of 7 mm placed on a common shaft are driven by an electric motor allowing an impeller speed of 15 000 min^{-1}. Pure surface aeration only permits a k_La-value of $\sim 0.01\ s^{-1}$. Air sparging through a miniature sparger with a volumetric air flux corresponding to 1 reactor volume per minute resulted in a maximum k_La-value of $0.11\ s^{-1}$. CFD simulations predict an energy dissipation rate of $\sim 4\ W\ L^{-1}$ at an impeller speed of 2500 min^{-1}. A batch cultivation of *E. coli* without pH control reached a cell concentration of $1.6\ g\ L^{-1}$ in the miniature bioreactor. The time profile of the cultivation was shown to be equivalent to a reference cultivation in a 20 L stirred tank bioreactor. The incorporation of fiberoptic probes enables the monitoring of pH and DO concentration. Cell concentration is estimated from turbidity in the undiluted broth which will putatively lead to a narrow measuring range. Parallelization of this miniature bioreactor might prove to be difficult with regard to the connection of individual motors. To date, no technical solution for substrate or titration agent addition was proposed.

Another approach consists of a miniature stirred tank bioreactor with a diameter of 20 mm and a working volume of 8–15 mL [19]. The reactor is equipped with a magnetically driven gas-inducing impeller which introduces gas bubbles into the reaction medium by sucking gas through a hollow shaft. Therefore, separate sparging and thus controlling of a gas volume flow is obsolete. The impeller was characterized by a maximum k_La-value of $> 0.4\ s^{-1}$. Within a volume range of 8–12 mL, the k_La-value can be maintained above $0.2\ s^{-1}$ thus allowing a volume gain of 50% during fed-batch cultivation. Power input into the medium was estimated by single phase computational fluid dynamics (CFD) simulations. At an impeller speed of 2800 min^{-1} the power input of the gas-inducing impeller reached $21.9\ W\ L^{-1}$ corresponding to a power number N_P of 3.7. Up to 48 milliliter bioreactors can be operated in parallel in a reaction block which contains heat exchangers, a head-space reflux cooler and a sterile gas cover (Fig. 3). This cover is accessible for manual pipettes or the tips of liquid-handling systems. Sterility can be maintained by a constant convective gas volume flow from the headspace of the bioreactors to the surrounding atmosphere. A liquid-handling system, a microtiter plate photometer and a microtiter plate washer enable automation of fed-batch cultivation with atline analytics for turbidity and pH measurements. An *E. coli* fed-batch cultivation with pH control in a mineral medium

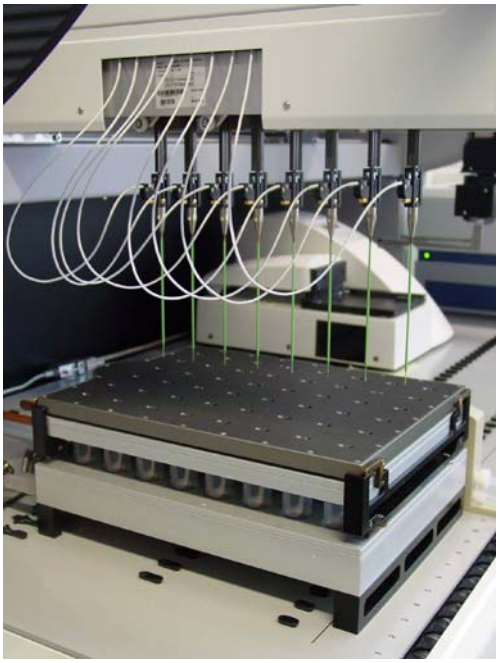

Fig. 3 Photograph of a bioreaction block with 48 stirred-tank reactors on a 10 milliliter-scale at the working-table of a liquid handler

reaching a cell concentration of 20.5 g L^{-1} was shown to be equivalent with regard to growth rate μ and biomass yield Y_{XS} to a reference cultivation in a 3 L laboratory-scale stirred tank reactor [21]. Online analytics for dissolved oxygen concentration have been developed by integration of fluorescence sensors inside the miniature bioreactors. The reproducibility of individual cultivations was shown by pH-controlled fed-batch cultivation with *E. coli* (Fig. 4). Atline pH measurements were characterized by a standard deviation of < 1.1% throughout the process. The deviation of the pH measurements from the setpoint of 6.8 is a result of intermittent pH control, i.e. the adjustment of the pH value every 60 minutes during batch and every 20 minutes during fed-batch growth. The process reached a mean cell concentration of 36.9 ± 0.9 g L^{-1} within a process time of 14.6 h.

Another system which is commercially available (Cellstation, Fluorometrix, Baltimore, USA) offers 12 parallel stirred tank reactors with a nominal volume of up to 35 mL. The agitation rate can be adjusted individually in the bioreactors in the range of 10–1000 min^{-1}. Fiberoptic sensors inside the bioreactors [22, 23] are sequentially read out to monitor pH, dissolved oxygen concentration and optical density online. No detailed information about oxygen transfer, power input or automated sampling or feeding is available.

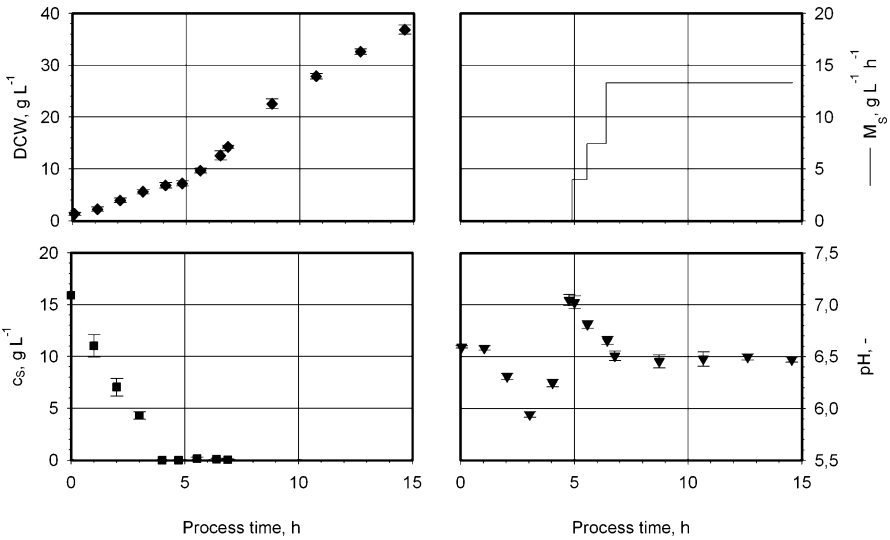

Fig. 4 Parallel, automated fed-batch cultivation of *Escherichia coli* K12 in seven milliliter-scale bioreactors in a mineral medium with glucose as the sole carbon source. Time course of dry cell weight concentration (DCW), substrate concentration c_S, feeding mass flow M_S and pH during the process time of 14.6 h

2.2 The Perspective of Parallel Bioprocess Design

The bottleneck in bioprocess design is located at the stage of secondary screening, i.e. the optimization of reaction conditions. Since no reliable high-throughput reaction system for secondary screening is commercially available, bioprocess design still involves batch cultivations in shake flasks or microtiter plates in batch operation and cost- and labor-intensive cultivations in laboratory-scale stirred tank reactors for the development of fed-batch processes. The main limitations of the shake flask and the microtiter plate have been shown not to be oxygen transfer nor power input but the limited possibility of controlling pH, conducting fed-batch processes and monitoring of important process parameters online. "Instrumented" shake flasks provide pH control and the possibility to perform fed-batch processes and thus can be used for more efficient process design. Nevertheless, further parallelization to more than 16 units seems to be a tedious technological challenge. Several reported approaches either offer only a restricted number of parallel miniature bioreactors or still lack one or more of the technological features for online monitoring of important process parameters, automated substrate addition or sampling. The highest number of parallel-operated bioreactors at milliliter scale is achieved by integrating 48 stirred-tank reactors in a bioreaction block (H+P-Labortechnik, Oberschleissheim, Germany). Since an oxygen transfer

coefficient $k_L a$ and a power input exceeding the values reported for laboratory stirred tank reactors as well as a technical solution for automated substrate addition and sampling was reported, this parallel bioreactor system will enable faster bioprocess design in the near future.

3 Metabolic Process Analysis

3.1 Biochemical Systems Characterization

The essential backbone of a biochemical system characterization is the estimation of conversion rates of substrates into various desired products and by-products. Modulated mainly by the capabilities of the cells, substrates are routed into products through a complex interconnected network of metabolic pathways. The rate by which every reaction constituent may change is limited by kinetic and regulation mechanisms. Thus, the problem of characterization consists mainly of establishing a proper description of the conversion dynamics considering the metabolic constraints.

While the stoichiometry of microbial systems is usually well known and forms the base of structure-oriented analysis [24–28], the dynamic modeling requires the identification of numerous kinetic mechanisms and parameters. These are not only difficult to elucidate and estimate, but particularly laborious to support with in vivo experiments. To date, this dynamical characterization is still a critical current challenge, especially if large-scale reaction networks are taken into account. However, once the model of the biochemical system is validated and regarded as satisfactory, it is straightforward to consider it for identification, predictive design and optimization of product targets in medical drug discovery and biotechnological industrial processes.

3.1.1 Stoichiometric Network Analysis

Stoichiometry essentially deals with changes in composition that may take place by reaction [29] and helps to formulate constraints on the rates by which the various components in a given system are allowed to vary. It delivers its information in the form of stoichiometric coefficients, which are the proportion in which the biochemical species involved react with each other.

Consider n_S substrates, n_P metabolic products, n_X biomass constituents and n_I internal metabolites of a system. To properly analyze the metabolic pathways contained in the biochemical reaction network, it is necessary to specify the stoichiometry for the whole set of reactions n_R [29, 30]. Taking

into account a matrix $\underline{\mathbf{A}}$, where the m rows correspond to the number of species and n to the number of reactions in which these species are involved, the elements a_{ij} (for $i \in \{1, ..., m\}$ and for $j \in \{1, ..., n\}$) of the matrix would represent the stoichiometric coefficients of the specie i in the reaction j. In that way, any biochemical reaction network can be mathematically described by:

$$\sum_{i=1}^{n_S} \alpha_{ji} S_i + \sum_{i=1}^{n_P} \beta_{ji} P_i + \sum_{i=1}^{n_X} \gamma_{ji} X_i + \sum_{i=1}^{n_I} g_{ji} I_i = 0 \,, \tag{1}$$

being α_{ji}, β_{ji}, γ_{ji} and g_{ji} the corresponding stoichiometric coefficients (positive in the case of products, negative for educts and null for no participation). In matrix notation:

$$\underline{\underline{\mathbf{A}}}\,\underline{\mathbf{S}} + \underline{\underline{\mathbf{B}}}\,\underline{\mathbf{P}} + \underline{\underline{\boldsymbol{\Gamma}}}\,\underline{\mathbf{X}} + \underline{\underline{\mathbf{G}}}\,\underline{\mathbf{I}} = \underline{\mathbf{0}} \,, \tag{2}$$

where $\underline{\underline{\mathbf{A}}}$ (n_R, n_S), $\underline{\underline{\mathbf{B}}}$ (n_R, n_P), $\underline{\underline{\boldsymbol{\Gamma}}}$ (n_R, n_X) and $\underline{\underline{\mathbf{G}}}$ (n_R, n_I) are the stoichiometric matrices, $\underline{\mathbf{S}}$ (n_S, 1), $\underline{\mathbf{P}}$ (n_P, 1), $\underline{\mathbf{X}}$ (n_X, 1) and $\underline{\mathbf{I}}$ (n_I, 1) the concentration vectors and $\underline{\mathbf{0}}$ (n_R, 1) a zero vector.

The stoichiometric analysis of reaction networks considers mainly the internal metabolites, such that the concentrations and rates of substrates S and products P are assumed to be fixed or known. The analysis is accomplished with the stoichiometric matrix of internal metabolites $\underline{\underline{\mathbf{G}}}$ and its consequences are extended further to the dynamics of the system [31, 32]. Applying a mass balance to each species in the metabolic network and neglecting any dilution effect due to the growth of cells (justified by the much slower cell growth dynamics compared to the dynamics of the internal metabolites) one obtains:

$$\mathrm{d}\underline{\mathbf{I}}/\mathrm{d}t = \underline{\underline{\mathbf{G}}}^{\mathrm{T}}\,\underline{\boldsymbol{\nu}} \,, \tag{3}$$

where $\underline{\boldsymbol{\nu}}$ (n_R, 1) is the vector of rates (fluxes) of the n_R reactions. The importance of the $\underline{\underline{\mathbf{G}}}$ matrix consists of its invariant property, i.e. it is not a function either of time nor of kinetics and species concentrations. Therefore, its establishment completely describes the architecture and topology of the system, because it contains key information concerning how the species are linked through reactions within the metabolic network.

Several approaches have been developed under the concept of stoichiometric network analysis, most of them centered on concepts deriving from linear algebra [29, 30, 32–36]: metabolic flux analysis, flux balance analysis, extreme pathway analysis, elementary flux modes, etc. Some of the strengths and limitations of these techniques are examined and compared with each other in [37, 38].

3.1.2 Enzyme Kinetic Models

3.1.2.1 Classical Approaches

An ideal method for establishing enzyme kinetic models is based on the description of the physical understanding of the biochemical system via a series of mass balances. This procedure can lead to simplified or highly complex models, depending on the grade of the understanding. In most cases, the complexity of the rate model is dramatically increased while improving the comprehension of the interactions involved in the bioprocess [39].

Well-known examples of simple enzymatic rate laws are the Michaelis–Menten, Hill, Koshland–Nemethy–Filmer and Monod–Wyman–Changeux models [40]. Most of them are simplified making use of some hypothesis, like the so-called rapid equilibrium or the assumption of pseudo-steady-state equilibrium for some enzyme-substrate complex. Commonly the rate expression is a linear function of the enzyme concentration, but a nonlinear function of the concentration of all species involved in the reaction. Such formulations, especially the Michaelis–Menten rate law have proven to satisfy exceptionally well numerous experimental data for in vitro kinetics:

$$v(S) = \frac{v_{\max} S}{K_M + S}, \tag{4}$$

being $v_{\max}$ the maximal substrate specific reaction rate and K_M a parameter referred to as the saturation constant, which is sometimes interpreted as the affinity of the enzyme to the substrate S, i.e. the lower K_M the better the enzyme is adapted to react. Nevertheless, although widely used as the first choice, a generalization of the late approach for the in vivo enzyme kinetics of fully interconnected reaction systems may suffer some drawbacks due to the validity of the assumptions under which it was derived (e.g. pseudo-steady-state premise).

3.1.2.2 Black-Box Approaches

Making accurate mechanistic descriptions is very difficult considering the high dimensionality and the nonlinearity of these systems that furthermore operate on different time scales. For this reason, simplified generalized enzyme kinetics representations have been proposed such as the power law approximation [41]. This is mathematically based on a Taylor linearization about an operating point in the logarithmic space, that is:

$$\text{Log (reaction rate)} = f\ (\text{Log (reaction species)}, \text{Log (co-factors)}, \ldots)\,. \tag{5}$$

Per analogy, it resembles the generalization of the rate laws for elemental chemical reactions and takes the form:

$$v(X_j) = k \prod_{j=1}^{J} X_j^{h_{ij}} , \tag{6}$$

where k is a logarithmic kinetic constant, X_j each variable affecting the rate law and h_{ij} the so-called logarithmic gain, which is bigger than zero in the case of a positive effect and smaller than zero for a negative influence on the rate.

3.1.2.3 Heuristic Approaches

When considering the case of a biochemical system just partially understood, but for which some a priori knowledge or valuable qualitative data is available, it is very advantageous to incorporate this information explicitly into a proper model. Lee et al. [42] proposed a combination of formal enzyme kinetics with fuzzy-logic approaches as an alternative method to integrate qualitative knowledge. Fuzzy models were developed as a simple and generic solution for complex tasks without a complicated mathematical form [43]. Their functioning is based on inference mechanisms or reasoning which makes use of expert understanding of the system behavior. Applied to biochemical systems, the development of fuzzy-logic approaches could be viewed as an intermediate evolutionary step between partial empirical towards full mechanistic modeling.

The fuzzy-logic technique can be employed for general kinetic reaction rate modeling using the Takagi–Sugeno approach [44]. This consists of a series of linear approximations of the variable reaction rate (v) as a function of the variable S belonging to the fuzzy set $\boldsymbol{A}_n$ such that:

$$\text{If } S \in \boldsymbol{A}_n \quad \text{then} \quad v_n(S) = a_n S + b_n , \tag{7}$$

where v_n is the linear approximation of the reaction rate for the associated fuzzy set $\boldsymbol{B}_n$, being a_n and b_n linear coefficients.

The entire range of the reaction rate variable v, can be calculated from the independent concentration variable S, for all membership functions, $\phi_{An}(S)$ from:

$$v(S) = \frac{\sum_{n=1}^{N} \phi_{An}(S) v_n}{\sum_{n=1}^{N} \phi_{An}(S)} = \frac{\sum_{n=1}^{N} \phi_{An}(S)(a_n S + b_n)}{\sum_{n=1}^{N} \phi_{An}(S)} , \tag{8}$$

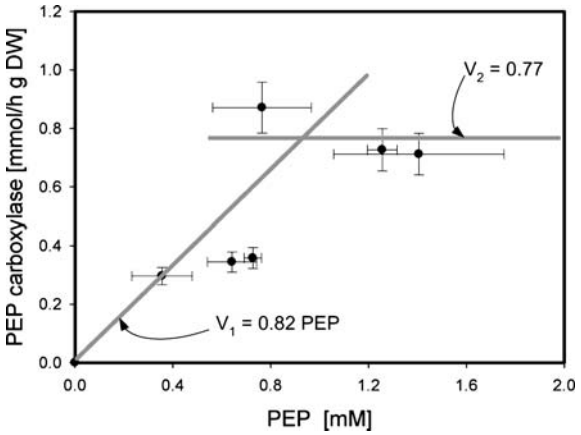

Fig. 5 Takagi–Sugeno function approximation for the phosphoenolpyruvate carboxylase reaction rate

and expanding,

$$v(S) = \left(\frac{\sum_{n=1}^{N} \phi_{An}(S) a_n}{\sum_{n=1}^{N} \phi_{An}(S)} \right) S + \left(\frac{\sum_{n=1}^{N} \phi_{An}(S) b_n}{\sum_{n=1}^{N} \phi_{An}(S)} \right) . \tag{9}$$

Equation 9 represents the fuzzy local linear model approximation of any nonlinear reaction rate variable. Figure 5 depicts the Takagi–Sugeno approach for the phosphoenolpyruvate carboxylase reaction rate [45, 46].

3.2 Metabolic Flux Analysis

Estimation of the fluxes for intracellular metabolites forms the base of metabolic flux analysis. This is accomplished using several measured exchange fluxes, the stoichiometry of the system and the mass balances around the intermediates considered. Along with the intracellular metabolite concentrations, fluxes conform to the minimum set of information required to describe cell physiology [30]. The output is a metabolic flux map showing the distribution of fluxes through all the reactions, commonly with a unitary or percentage base. Figure 6 depicts the metabolic flux analysis for a substrate-limited *Escherichia coli* cultivation with glucose and fructose [45, 46].

The essence of the metabolic flux analysis is the steady-state assumption for the mass balance of internal metabolites (Eq. 3):

$$\mathrm{d}\underline{\mathbf{I}}/\,\mathrm{d}t = \underline{\underline{\mathbf{G}}}^{\mathrm{T}}\,\underline{\boldsymbol{v}} = \underline{0}\,. \tag{10}$$

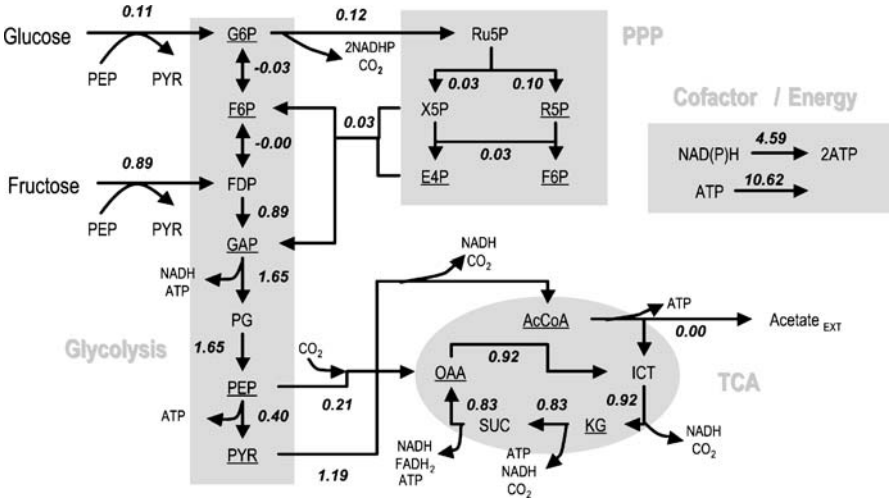

Fig. 6 Metabolic flux analysis of a carbon-limited multi-substrate cultivation of *E. coli* on a mixture of 11 to 89%, glucose to fructose, respectively. $D = 0.125\ 1/h$, $S_{Total} = 33\ g/L$, 37 °C. Biomass precursors are *underlined*

The stoichiometry matrix $\underline{\underline{\mathbf{G}}}^T$ and the reaction rate vector $\underline{\boldsymbol{\nu}}$ are then partitioned into measured and unknown parts, such that:

$$[\underline{\underline{\mathbf{G}}}_m^T | \underline{\underline{\mathbf{G}}}_c^T][\underline{\boldsymbol{\nu}}_m / \underline{\boldsymbol{\nu}}_c] = \underline{\underline{\mathbf{G}}}_m^T \underline{\boldsymbol{\nu}}_m + \underline{\underline{\mathbf{G}}}_c^T \underline{\boldsymbol{\nu}}_c = \underline{0}\,, \tag{11}$$

where the subindex "m" denotes a measured variable and "c" and unknown variable, respectively. From this last equation, the unknown reaction rate vector can be calculated from:

$$\underline{\boldsymbol{\nu}}_c = -\,(\underline{\underline{\mathbf{G}}}_c^T)^{-1} \underline{\underline{\mathbf{G}}}_m^T \underline{\boldsymbol{\nu}}_m\,. \tag{12}$$

Nevertheless, to solve Eq. 12, the $\underline{\underline{\mathbf{G}}}_c^T$ matrix must be fully-ranked, i.e. it has to be nonsingular (invertible), being the number of metabolite balances at least equal to the number of unknown rates, but presenting no linear dependencies on it. Some application examples can be found on [47–57].

In case of redundant measurements (more linear independent balances than unknown variables), these can be used for test of consistency and data reconciliation (gross error determination) [30]. For this purpose, elemental balances may also be taken into account for stoichiometry considering that the elements by which the species of a system are composed, cannot be generated or destroyed. As a rule it can be stated that at least the carbon balance must be close to about 5% before it makes sense to go ahead with further details of modeling.

3.3 Metabolic Control Analysis

With the tremendous advances in the field of molecular biology methods combined with the goal of generating valuable new product or alternative production systems, the recombinant DNA technology focused its potential on the assembly of new genes, which usually do not exist in nature, as well as on the over expression of so-called "rate-limiting" enzymes. However, the single rate-limiting concept embodies some vagueness in its definition and has been shown not to be valid, or at least not always, specifically while considering large-scale reaction networks. This fact has led to the development of mathematical methods to quantify the influence of single regulatory mechanisms and enzymes besides the estimation of the sensitivity to changes in a biochemical reaction network.

Known today as Metabolic Control Analysis (MCA), a mathematical analysis framework was developed by Kacser and Burns [58] and Heinrich and Rapoport [59]. They attempted to correlate global properties of the biochemical reaction system to the individual properties of its components, particularly to the enzymes. The method considers only the analysis of infinitesimal changes on a system under the steady-state assumption. Its main challenge is the estimation of the extent at which the material flux through the whole system is regulated (controlled) by the activity of a single enzyme (reaction).

3.3.1 Local and Global Sensitivities

Taken into account in a kinetic model, each enzyme belonging to a reaction system is related with specific metabolites (substrate, products and effectors). The rate of the enzyme, i.e. the rate at which input metabolites are processed to form the output metabolites is a local property, since they apply to enzymes isolated from the system. In contrast, metabolite concentrations and steady-state fluxes are global system properties, because any change in them would exert an specific effect on single steps in a metabolic pathway, mainly affecting the enzymes catalyzing those reactions.

Under the MCA framework, local sensitivities are characterized by the elasticity coefficient (ε_i^k), which describes the sensitivity of an enzymatic reaction rate to small changes in a metabolite concentration:

$$\varepsilon_i^k = \left(\frac{S_i}{\nu_k} \frac{\Delta \nu_k}{\Delta S_i} \right)_{\Delta S_i \to 0} = \frac{S_i}{\nu_k} \frac{\partial \nu_k}{\partial S_i} = \frac{\partial \ln \nu_k}{\partial \ln S_i} . \tag{13}$$

According to the definition by Kacser and Burns [58], the flux control coefficients ($FCC_k^{J_i}$) are defined as the fractional change in the steady-state flux (J_i) through the system divided by the fractional change in enzyme activity of

enzyme (E_k) for an infinitesimal change. However, a more general definition based on the enzymatic reaction rate (ν_k) instead of the activity is given as the sensitivity of a steady-state flux to small changes in other enzymatic reaction rates:

$$FCC_k^{J_j} = \left(\frac{\nu_k}{J_j}\frac{\Delta J_j}{\Delta \nu_k}\right)_{\Delta \nu_k \to 0} = \frac{\nu_k}{J_j}\frac{dJ_j}{d\nu_k} = \frac{d\ln J_j}{d\ln \nu_k} . \tag{14}$$

Finally, the concentration control coefficients ($CCC_k^{S_i}$), also of global nature, describe the sensitivity of a steady-state metabolite concentration (S_i) to small changes in given enzymatic reaction rates:

$$CCC_k^{S_i} = \left(\frac{\nu_k}{S_i}\frac{\Delta S_i}{\Delta \nu_k}\right)_{\Delta \nu_k \to 0} = \frac{\nu_k}{S_i}\frac{\frac{dS_i}{dp_k}}{\frac{d\nu_k}{dp_k}} = \frac{d\ln S_i}{d\ln \nu_k} , \tag{15}$$

where p_k represents any parameter acting on the enzymatic rate ν_k.

3.3.2 Properties of the Control Coefficients

The power of MCA lies in the fundamental relationship between the local and global sensitivities. Consider the combined effect of all changes in local rates in a biochemical reaction network and their influence on the system global variables, i.e. metabolite concentrations and steady-state fluxes. The summation theorem for flux control coefficient is given by:

$$\sum_k FCC_k^{J_j} = 1 . \tag{16}$$

This can be interpreted as the property of enzymes to share control of flux over the entire biochemical reaction network and might not lie in a single "rate-limiting" step. To achieve this control sharing, some of the enzymes increase metabolite concentrations while others decrease them. This property is expressed by the summation theorem for concentration control coefficient:

$$\sum_k CCC_k^{S_j} = 0 . \tag{17}$$

Finally, the relationship between global and local sensitivities is conjugated in the connectivity theorem for flux control coefficients and elasticities:

$$\sum_k FCC_k^{J_j}\varepsilon_i^k = 0 , \tag{18}$$

and in the connectivity theorem for concentration control coefficients:

$$\sum_k CCC_k^{S_i}\varepsilon_j^k = -\delta_{ij} , \tag{19}$$

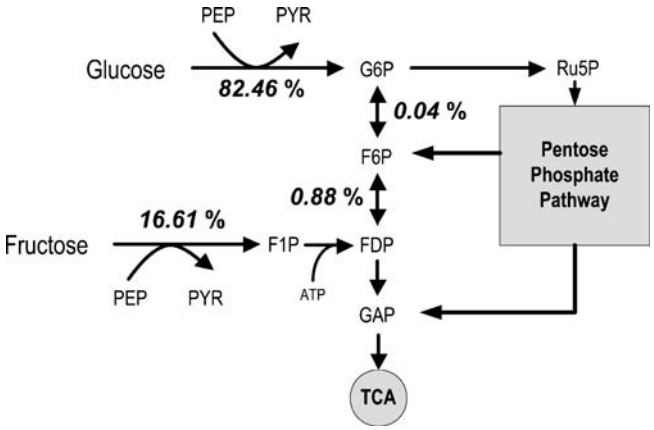

Fig. 7 Metabolic control analysis of multi-substrate cultivation of *E. coli* growing on a mixture of glucose/fructose. The flux control coefficients are given as a percentage. The phosphotransferase systems (PTS) share major control of the growth

where δ_{ij} is the Kronecker delta ($\delta_{ij} = 1$ if $i = j$; $\delta_{ij} = 0$ if $i \neq j$).

Both connectivity theorems express that the net result of any local event may affect the whole system and may not be considered as an isolated change. Through the estimation of the flux control coefficients, MCA gives a clear guideline as to which enzymes need to be modified, that is, the site for a target-oriented genetic manipulation which would exert major control on the biochemical system. Key features concerning the algebra of MCA, as well as applications to some biochemical systems are discussed in detail in [24, 29, 30, 49–68], while [69–72] discuss the influence of experimental errors on the flux control coefficient estimation from measured transient metabolite concentrations [73, 74].

Figure 7 depicts the main sites exerting control (FCC given in percentage) for a substrate-limited *Escherichia coli* cultivation with glucose and fructose considering a phosphotransferase transport system for both substrates [44, 45].

3.3.3 Alternative Frameworks

3.3.3.1 Biochemical System Theory

The first mathematical analysis framework for biological reaction networks reported is contained in the Biochemical Systems Theory (BTS) [40, 75–77]. Main premise is the dynamics formulation of the system with “degradation” and “productions” terms imbedded in a set of differential equations. The bal-

ance for the dependent variable X_i ($i = 1, 2, ..., N$) takes the form:

$$\frac{dX_i}{dt} = v_i^+(X_1, X_2, ..., X_N, X_{N+1}, ..., X_{N+M}) - v_i^-(X_1, X_2, ..., X_N, X_{N+1}, ..., X_{N+M}) , \quad (20)$$

describing the term v_i^+ as all effects augmenting the production of X_i, while the term v_i^- describes all effects diminishing the production of X_i, where the independent variables X_i ($i = 1 + N, 2 + N, ..., M + N$) do not change for a given operating point, but may differ from one experiment to another. Considering a power-law representation the S-system is given by:

$$\frac{dX_i}{dt} = \alpha_i \prod_{j=1}^{N+M} X_j^{h_{ij}} - \beta_i \prod_{j=1}^{N+M} X_j^{l_{ij}} \quad i = 1, 2, ..., N , \quad (21)$$

where α_i, β_i and h_{ij}, l_{ij} are the corresponding kinetic constants and logarithmic gains, respectively. Considering steady-state operation for the S-system representation and applying a logarithmic transformation to the variables:

$$\sum_{j=1}^{N+M} h_{ij} \ln(X_j) - \sum_{j=1}^{N+M} l_{ij} \ln(X_j) = \ln(\beta_i/\alpha_i) , \quad (22)$$

and finally defining the coefficients $a_{ij} \equiv h_{ij} - l_{ij}$ and $b_i \equiv \ln(\beta_i/\alpha_i)$ and separating dependent (sub-index "D") from independent variables (sub-index "I"), Eq. 22 can be rewritten in matrix form:

$$\ln(\boldsymbol{X})_D = (\underline{\underline{\mathbf{A}}}_D)^{-1} \underline{\boldsymbol{b}} - (\underline{\underline{\mathbf{A}}}_D)^{-1} \underline{\underline{\mathbf{A}}}_D \ln(\boldsymbol{X})_I = \underline{\underline{\mathbf{M}}}\, \underline{\boldsymbol{b}} + \underline{\underline{\mathbf{L}}} \ln(\boldsymbol{X})_I . \quad (23)$$

The elements of matrix $\underline{\underline{\mathbf{M}}}$ are known as sensitivities and those of matrix $\underline{\underline{\mathbf{L}}}$ as logarithmic gains, $L(X_j, X_k)$. Sensitivities and the logarithmic gains relate local properties of a given metabolic pathway to its overall systemic properties [40, 75–79] in a similar fashion as elasticities and flux control coefficients do in MCA [80].

3.3.3.2 The Linlog Approach

Derived from a nonmechanistic rate equation, but analogous to the thermokinetic approach [81, 82], the linlog framework is an extension of the MCA [83–85]. Any enzymatic reaction rate is represented by the sum of the logarithms of the species concentrations:

$$v(X_i) = e \left(k_0 + \sum_{i=1}^{I} k_i \ln(X_i) \right) , \quad (24)$$

where e is the enzyme level, k_i ($i = 1, 2, ..., I$) kinetic constants and X_i all metabolites involved in the reaction. The sign of the coefficients k_i de-

pends on the influence the correspondent metabolite X_i on the reaction rate: negative for decreasing and positive for augmenting effects. Considering a steady-state as reference, and taking into account the definition of the elasticity coefficient (Eq. 13), the general linlog approximation for a biochemical system of n_R reactions, m_x internal metabolites and m_c external metabolites is:

$$\underline{v}/\underline{J}^0 = [\underline{e}/\underline{e}^0](\underline{\mathbf{i}} + \underline{\underline{\mathbf{E}}}_x^0 \ln(\underline{X}/\underline{X}^0) + \underline{\underline{\mathbf{E}}}_c^0 \ln(\underline{c}/\underline{c}^0)) \,, \tag{25}$$

where the 0 super-index alludes the steady-state reference, $\underline{v}/\underline{J}^0$ the vector sized (n_R,1) of the relative flux levels, $[\underline{e}/\underline{e}^0]$ the matrix sized (n_R, n_R) of the relative enzyme levels, $\underline{\mathbf{E}}_x^0$, $\underline{\mathbf{E}}_c^0$ the (n_R, m_x), (n_R, m_c) sized elasticity matrices, and $\ln(\underline{X}/\underline{X}^0)$, $\ln(\underline{c}/\underline{c}^0)$ the (1, m_x), (1, m_c) sized vector for the internal and external metabolites, respectively. The relation deriving the flux and concentration coefficients is given by [81]:

$$\underline{\underline{\mathbf{C}}}^{J0}[\underline{v}/\underline{J}^0][\underline{e}^0/\underline{e}] = \underline{\mathbf{i}} + \underline{\underline{\mathbf{C}}}^{J0}\underline{\underline{\mathbf{E}}}_c^0 \ln(\underline{c}/\underline{c}^0) \,, \tag{26}$$

and:

$$\underline{\underline{\mathbf{C}}}^{X0}[\underline{v}/\underline{J}^0][\underline{e}^0/\underline{e}] = -\ln(\underline{X}/\underline{X}^0) + \underline{\underline{\mathbf{C}}}^{X0}\underline{\underline{\mathbf{E}}}_c^0 \ln(\underline{c}/\underline{c}^0) \,. \tag{27}$$

3.4
The Perspective of an Integral Approach: "Omics" and Systems Biology

System biology is the integration of experimental and computational approaches to achieve the overall goal of explaining and predicting complex cellular behaviors of biological systems [86]. The approach is based on examining complete annotated genome sequences for different microorganisms towards the understanding of their related phenotype [87–89]. Its focus lies in predicting metabolic potentials using databases and bioinformatics and is accomplished by conciliating experimental data from transcriptomics, proteomics and metabolomics [90–93].

However, identifying, enlisting and establishing interconnections between genes and proteins is not sufficient to understand the complexity of a biochemical system, which, besides structure, also possess dynamic and regulatory properties [94]. This last fact is crucial mainly because many annotations are still hypothesis and are not fully backed up by experimental evidence [95]. Nevertheless, the major challenge of system biology remains the reduction of cost-related issues and efforts in the search for optimal applications exploding the potential of *insilico* cells.

4 Downstream Processing of Bioproducts

In the past two decades, downstream processing of bioproducts has developed as an important independent discipline within the field of biochemical engineering. Many textbooks cover the field of bioseparations in a comprehensive way [96–104]. However, according to the late review of Lightfoot and Moscariello [105], in practice "there is still a large degree of empiricism in our designs". Hence, there is still a lot of work to be done in order to "include more quantitative understanding" and refine processes and equipment.

In order to be able to differentiate the broad class of bioproducts with regard to their sizes, Harrison et al. [104] presented a classification (Table 1). As can be seen, small biomolecules like sugars, organic acids, amino acids, and vitamins have a molecular weight in the order of 30–600 Daltons and a typical radius of 0.5–2 nm. The separation and purification methods of small biomolecules have been established in the past to a certain extent, however, there are still quite exciting new applications being developed such as extraction of bioproducts using novel types of solvents and integrating bioreaction and extraction. One example of a novel solvent is supercritical carbon dioxide ($scCO_2$). This substance has advantageous properties as an extractor due to enhanced mass transfer of the solute in the $scCO_2$ and due to reduced transport limitations of $scCO_2$ into porous solids because of the lack of surface tension [106]. In addition, $scCO_2$ is generally recognized as a safe (GRAS) compound. A second example of novel solvents with superb properties are ionic liquids (ILs) which exhibit no vapor pressure and, in many cases, low viscosity at room temperature. Such compounds can be applied in a favorable way to fine chemical synthesis or bioreactions as a substitution of conventional volatile organic solvents [107]. Several examples of successful applications of ILs for integrative reactive extrac-

Table 1 Differentiation of bioproducts with regard to their sizes (adopted from [104])

Bioproduct	Examples	Molecular weight	Typical radius
Small molecules	Sugars, organic acids, amino acids, vitamins	30–600 Da	0.5–2 nm
Large molecules	Proteins	10^3–10^6 Da	3–10 nm
	Polysaccharides	10^4–10^7 Da	4–20 nm
	Nucleic acids	10^3–10^{10} Da	2 nm–1 μm
Particles	Ribosomes, viruses		25–100 nm
	Bacteria, yeasts		1–5 μm
	Animal cells		≥ 10 μm

tion bioprocesses have been reported either using enzymes [108] or whole cells [109] as the biocatalyst. The above-mentioned applications represent a promising field and further research efforts are underway in order to enhance the understanding and the applicability of the involved phenomena and processes.

According to Table 1, proteins represent the smallest category of the macromolecular bioproducts with a molecular weight in the range of 10^3 – 10^6 Daltons and a typical radius of 3 – 10 nm. From experience, it is well known that the downstream processing of the economically interesting broad class of proteins is often by far the most cost-intensive step of a bioprocess. Typically, the bioseparation and purification of proteins may cost in excess of 90% of the total costs [110]. Thus, it is obvious that bioseparations play a major role in the production process of macromolecular bioproducts. Besides the significance of the costs, other criteria are of importance, i.e. product quality and purity, stability, and the type of formulation of the final product. The following problems are commonly encountered with the downstream processing of proteins:

(i) The products to be purified belong to a quite heterogeneous class of substances which are often difficult to characterize;
(ii) the demanded degree of purity is usually extraordinarily high;
(iii) in any case, multiple-step downstream processes are required yielding rather low product yields;
(iv) the scale-up of the processes is often difficult;
(v) as a result, many downstream processes lack the required robustness for large-scale industrial applications.

Therefore, continuing efforts of research and development are required to overcome these problems.

4.1 Current Challenges in Downstream Processing of Macromolecular Bioproducts

There exists quite a number of challenges in the field of downstream processing of bioproducts ranging from the early steps of cell separation and disruption, followed by purification steps using various methods such as filtration, extraction, precipitation, and chromatography, and finally polishing of the products by crystallization and drying. The present paper focuses on three specific fields of downstream processing:

(i) Preparative chromatography on process-scale;
(ii) large-scale affinity filtration for the isolation of proteins and;
(iii) process-scale protein crystallization.

Each of these three examples represent a case where improved understanding of downstream processing will enable an efficient and cost-proficient industrial-scale bioprocess.

4.1.1 Preparative Chromatography on Process Scale

Packed-bed chromatography is often used in bioseparation operations in spite of the fact that this process is highly mass transport limited. As a result, low throughput and high costs of operation are encountered. In addition, conventional packed-bed chromatography cannot be operated continuously and is difficult to scale-up. Consequently, chromatography alternatives such as membrane filtration using monoliths, aqueous two-phase extraction, three-phase partitioning, and others were sought and discussed in the literature [111]. Another alternative is the expanded-bed adsorption process (EBA) which has been examined thoroughly in the past years. However, this process did not enter the industrial practice as expected due to various reasons such as the low lifetime of the fluidized carrier particles [112]. Other concepts pursued the development of continuous chromatography processes such as the simulated moving-bed technology (SMB) [113] or the preparative continuous annular chromatography (P-CAC) [114]. Again, these operations did not supersede packed-bed chromatography in a significant way since in many cases, the operational stability and reproducibility was low (e.g. so-called peak wobbling with the P-CAC process) and the costs were relatively high. Therefore, on process-scale, packed-bed chromatography is still the dominating bioseparation operation with column diameters as large as 2.6 m and typical height-to-diameter ratios as small as 0.15.

Here, many questions come into mind such as the radial distribution of fluid, the influence of heat dissipation inside the column, the performance and reproducibility of the column packing procedures, the intra-particle biomolecule uptake kinetics, the compressibility of visco-elastic gel beads, and the sudden unexpected collapse of gel bead beds during operation. Some of these problems were resolved in the past such as the enabling of a rather uniform radial distribution of fluid by using sophisticated fractal distributors [115] or other devices. The influence of disadvantageous radial temperature profiles in large columns due to heat dissipation effects was studied by Arlt and co-workers using CFD methods [116]. Here, the superposition of a suitable temperature profile at the column inlet was proposed which compensated the heat dissipation effects to some extent. On the other hand, despite some progress, column packing procedures still depend on empiricism to a large extent and need to be optimized in order to prevent voids [117]. The intra-particle uptake kinetics of proteins like BSA and IgG 2a were elucidated by using confocal laser scanning microscopy methods [118]. Here, in-situ quantitative investigations of the adsorption dynamics of single- and two-component mixtures of proteins within a single particle were performed. A significant and largely unresolved problem is the behavior of compressible visco-elastic gel chromatography media during operation. Such materials are highly cross-linked polymers or copolymers either based on natural sub-

stances such as agarose and dextran or based on artificial substances such as methacrylates, acrylamides and styrene-divinylbenzenes. With these media, an unforeseeable increase of the pressure drop in the chromatography column with time was observed in some cases. Associated with this pressure drop increase was the phenomenon of the sudden unexpected collapse of gel bead beds. Here, only a few investigations have been performed so far. Ladisch described the compression behavior of a styrene-divinylbenzene gel-type ion exchange resin in two 60 cm long columns with inner diameters of 6 and 8 mm based on their own work dating back to 1978 [103]. Both columns were packed with resin (particle size 20–30 μm) from one single batch using the slurry method in the same manner. Surprisingly, during operation at a flow rate of 0.5 ml min^{-1}, the 6 mm column had a pressure drop of about 10 bar while the 8 mm column operated at a much higher pressure drop of about 249 bar. Thus, an approx. 25-fold increase in pressure drop was observed with the 8 mm column compared to the 6 mm column at identical flow conditions. Examinations revealed that there was no void volume formation at the inlets or outlets of either columns. However, scanning electron microscopy of the unpacked beds showed that some of the resin near the outlet of the 8 mm column had been compacted while the resin near the outlet of the 6 mm column appeared normal. This phenomenon was modelled using the empirical Blake–Kozeny correlation for two columns in series each having a different void fraction. The Blake–Kozeny correlation describes the pressure drop Δp in packed beds and is a simplification of the well-known Ergun equation for the case of laminar flow

$$\frac{\Delta p}{L} = \frac{150\ \mu}{d_p^2} \frac{(1-\varepsilon)^2}{\varepsilon^3} u\,, \tag{28}$$

where L is the bed depth, μ is the dynamic viscosity, d_p is the mean particle diameter, ε is the void fraction, and u is the superficial fluid velocity. In a later work, pressure-flow relationships for packed beds of agarose-based chromatography media were examined [119]. Again, the empirical Blake–Kozeny correlation was utilized in order to describe the influence of the column aspect ratio upon the pressure drop. As a result, pressure drops and flow velocities could be predicted based upon just a few experiments. However, the application was limited to columns with an internal diameter of less than 5 cm and with less than 250 ml of medium.

It is obvious that further work in this area is necessary to gain more understanding of the complex processes taking place inside large-scale columns with low aspect ratios. Instead of empirical equations, a more rigorous modelling of the flow through packed beds using Navier–Stokes equations is believed to be advantageous. These equations can be supplemented in a suitable way in order to take into account the specific rheological processes taking place inside the columns. Hence, the application of experiences from the field of rheology is proposed. The transient flow of a fluid in a cavity can gener-

ally be characterized by the local shear stress function $\tau(y,t)$ inside the fluid which is proportional to the local shear rate $\dot{\gamma}(y,t)$ at the actual time t for newtonian fluids. From rheology of non-newtonian fluids, it is known that τ is dependent upon the deformation history described by the function of the local shear rate $\dot{\gamma}(y,t-s)$. According to Böhme [120], it follows for linear visco-elastic media such as gel-type chromatography resins

$$\tau(y,t) = \int_0^\infty G(s) \cdot \dot{\gamma}(y,t-s)\,. \tag{29}$$

This convolution integral incorporates a material property function $G(s)$ which weighs the influence of deformations in the past upon the actual shear stress. Experimental investigations revealed that the influence of past events was smaller for those events which were further back in time. As a matter of fact, the function $G(s)$ decreased monotonously with time for many real fluids. Hence, these fluids were named "fluids with fading memory". In many cases, $G(s)$ is represented by a simple exponential function

$$G(s) = G(0) \cdot e^{-2/\lambda}\,, \tag{30}$$

where $G(0)$ and λ are material property constants. Equation 29 inserted into Eq. 28 and formulated in the differential form represents the stress-strain relation of a so-called Maxwell body which consists of a mere viscous portion with the viscosity $\eta_0 = G(0) \cdot \lambda$ and a mere elastic portion with the elasticity modulus $G(0)$. The function $G(s)$ can be easily determined from relaxation experiments. For nonlinear visco-elastic media, Eq. 28 may be expanded to include nonlinear functions [120].

The described strategy of rigorous modelling taking into account the rheology of compressible chromatography media can be deployed in order to be able to forecast the operational stability of columns and to achieve an improved design of large-scale chromatography processes. However, it is believed that a collaboration of researchers and practitioners from chemical/pharmaceutical industry, resin manufacturers, process equipment manufacturers, and academics is necessary in order to achieve the above-mentioned goals.

4.1.2
Large-Scale Affinity Filtration for the Isolation of Proteins

Traditional bioseparation methods such as conventional chromatography are usually limited by small mass transfer coefficients and generally exhibit low specificities. Affinity separations, on the other hand, may achieve under favorable circumstances a highly specific adsorption of target biomolecules under the conditions of minimized mass transfer limitations and subsequently optimized product elution. The requirements for this are suitable

carrier materials and affinity ligands which interact specifically and reversibly with the substances to be purified in a reproducible manner. In addition, high bioseparation capacities are demanded. These requirements cannot be met easily and thus, affinity separations represent another challenging field of downstream processing of bioproducts. Affinity chromatography methods using a wide variety of ligands are discussed thoroughly in the literature [103], however, information on scale-up and operational stability are scarce. In addition, diffusion limitations are still associated with most chromatographic packing. Therefore, nonchromatographic affinity technologies have been investigated such as membrane affinity filtration [121]. However, affinity filtration methods have not been studied much in the past. On the other hand, these methods are promising since they represent a highly desirable integrated recovery process. This integrated approach proved to be useful for the initial step in downstream processing [122]. Kula and coworkers published first studies on the application of affinity microfiltration membranes for the isolation of enzymes from crude extracts [123]. Here, cost-efficient dye ligands were utilized such as Cibacron blue F3G-A. Such dyes mimic the structure of enzyme cofactors and other prosthetic groups. Thus, a high-resolution purification technique was achieved. It was shown in a further work that these membranes had favorable characteristics compared to packed beds such as high surface area, high solute throughput, mechanical strength, a highly porous structure, and low pressure drop [124]. However, future investigations are necessary in order to enhance the coupling of the ligands to the membranes and to improve the capacity of the membrane stacks as well as to reduce the costs of the ligands.

A different approach was investigated by He and Sun where multiple stage slurry bioreactors and dead end microfiltration were employed instead of membranes [125]. The aim was purification of lysozyme from chicken egg white. Again, Cibacron blue was utilized as the ligand. The dye was immobilized on a size exclusion chromatography gel resin based on an ethylene glycol/methacrylate copolymer with particle sizes ranging from 40–60 μm. This affinity adsorbent was suspended in three slurry reactors in series each equipped with a cylindrical dead end stainless steel filter with 1 μm pores in order to retain the affinity beads. A mathematical model was developed which took into account the adsorption and elution kinetics. In the simplest case at equilibrium conditions, the adsorption kinetics for a single protein was described by the well-known Langmuir equilibrium equation

$$q = \frac{q_m \cdot c}{K_d + c}, \tag{31}$$

where q is the adsorbed protein concentration, q_m is the maximum adsorption capacity of the adsorbent, c is the concentration of the protein, and K_d is the dissociation constant. It was shown that the three-stage affinity system increased the recovery yield of lysozyme from chicken egg white from

61 to 96% compared to the single-stage process. The calculated adsorption isotherms agreed well with the measurements. However, it was mentioned that the scale-up of the utilized dead end microfiltration system was difficult.

Romero and Zydney investigated a highly specific affinity ultrafiltration system for chiral separations [126]. The objective of this study was the examination of a multi-stage affinity diafiltration process for the separation of D- and L-tryptophan which used tangential flow filtration modules. Such modules can be parallelized for large-scale commercial applications. A two-stage laboratory-scale system was used in the experiments. Bovine serum albumin (BSA) was utilized as the affinity macroligand which acted as a stereoselective binding agent. This was due to the fact that L-tryptophan bounds predominantly to one site on BSA in a highly stereo-specific manner. Thus, the concentration of L-tryptophan was higher in the retentate and the concentration of D-tryptophan was higher in the filtrate. The schematic of the experimental set-up is presented in Fig. 8. The experiments were accompanied by model calculations which accounted for competitive binding interactions between the racemic tryptophan solution and the BSA ligand. The experiments gave purification factors of more than 20 at a yield greater than 90%. In addition, a good agreement of model predictions and experimental data was obtained. However, it was stated that the overall optimization of the two-stage cascade system is quite complex and future work is necessary.

An innovative affinity filtration process was developed by Vogel et al. where a specifically designed rotating disk filter comprising an affinity membrane stack was employed [127]. This process called controlled shear affin-

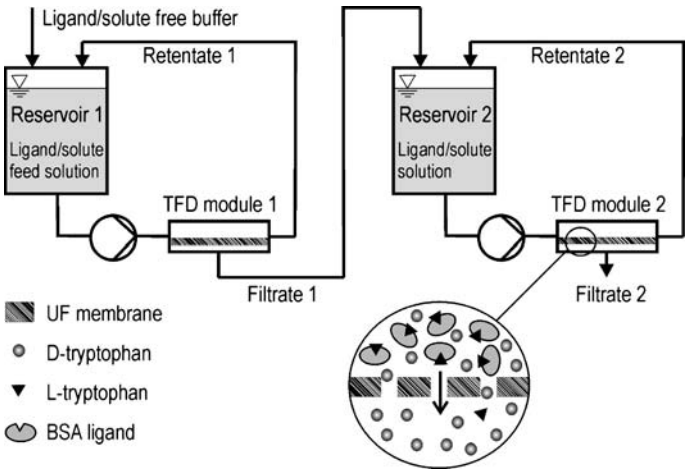

Fig. 8 Schematic of a two-stage affinity tangential flow diafiltration (TFD) process for the separation of D- and L-tryptophan with bovine serum albumin (BSA) as the affinity macroligand (adopted from [126])

ity filtration was used for the integrated cell separation and isolation of mammalian cell products. In the experiments, *t*PA was selectively adsorbed using lysine as the affinity ligand. Microfiltration Nylon membranes (0.45 μm pores) were coated with dextran, activated with bisoxiran, and the lysine was then coupled via its α-amino group at pH 9. Thus, a robust small molecular-weight affinity ligand was obtained. Because of the decoupling of shear force and pressure generation by the rotating disk filter, the viability of the shear sensitive mammalian cells could be maintained by precise control. The yield of *t*PA in the concentrated particulate-free eluate was 86% while 95% of the bulk protein was removed. A continuous operation could be realized with two systems in a tandem configuration.

Recent progress in the development of improved affinity filtration processes was achieved using monolithic supports (BIA Separations, Ljubljana, Slovenia). Contrary to packed-bed systems, these supports based on a highly cross-linked porous monolithic polymer offer low backpressure even at high flow rates. Monolithic supports have a well-defined, bimodal pore-size distribution providing improved flow characteristics. They are characterized by flow independent resolution separations and a flow independent binding capacity. Such monolithic disk-type sorbents were utilized by Vlakh et al. for affinity separation of recombinant *t*PA [128]. In this special case, the monolithic disks were modified by direct solid phase peptide synthesis in order to obtain peptidyl ligands complementary to recombinant *t*PA. The direct peptide synthesis was made possible by using monolithic disks based on a copolymer of glycidyl methacrylate and ethylene dimethacrylate. The resulting affinity sorbents were proposed to be used for the semi-preparative isolation of recombinant *t*PA from crude cellular supernatant. However, no large-scale applications have been reported so far. Therefore, it can be deduced that much future work is required in order to design and operate robust large-scale affinity filtration systems for the isolation of proteins from crude extracts.

4.1.3
Process-Scale Protein Crystallization

Crystallization is an attractive method for the downstream processing of proteins for several reasons:

(i) Products at a very high purity, e.g. 99.9%, are formed;
(ii) crystallization represents a combination of a purification step and a polishing step;
(iii) significant cost reductions may be achieved since crystallization may replace one or more chromatography steps;
(iv) crystallization is a relatively cheap bioseparation method compared to affinity separations since costly substances such as ligand-coupled adsorbents are avoided; and

(v) protein crystals represent an optimum type of formulation compared to liquid and lyophilized products since they can be stored for a longer time without significant loss of activity and do not exhibit limited delivery options for therapeutical use.

Crystallization is a process similar to precipitation. The latter bioseparation method has been used successfully for a long time for many applications, however, crystallization exhibits several advantages. These are: (i) the crystal sizes are larger thus facilitating further purification steps such as filtration; (ii) the product morphology is more defined; (iii) the required degree of supersaturation is lower; (iv) the growth rates are within a wider range; and (v) the process is better controllable [104]. On the other hand, the nucleation rates are often low compared to precipitation. In addition, many proteins are difficult to crystallize or do not crystallize at all. Here, very little systematic knowledge exists. For example, it is known that a higher degree of glycosylation rather hinders the crystallization process. Therefore, until recently (2004), the only crystalline protein being produced on a large-scale basis was recombinant insulin [129]. However, there is a need for more process-scale crystallized proteins on the market due to their significant benefits, especially for biopharmaceuticals.

The process of crystallization consists of two steps, nucleation and subsequent crystal growth. Nucleation can be differentiated into primary nucleation and secondary nucleation. Primary nucleation occurs in the absence of crystals and secondary nucleation takes place when crystal seeds exist in the solution. Primary nucleation can be either homogeneous or heterogeneous. In the case of homogeneous nucleation, no foreign particles exist in the solution while heterogeneous nucleation occurs when foreign particles initiate nucleation. Crystal growth is the process where product molecules are being added to an existing crystal surface. A typical phase diagram of protein crystallization showing the different zones and types of solid phases is given in Fig. 9 [130].

For the occurrence of nucleation, a certain level of supersaturation has to exist in order to cross the solubility curve. Then, the level of supersaturation has to be increased further in order to enter the nucleation region (first horizontal dotted arrow on the upper left of Fig. 9). A favorable strategy is depicted by the dotted arrows in Fig. 9 since with this strategy, large crystals can be obtained. Both processes, nucleation or crystal growth, are usually described by simple empirical power law expressions of the form

$$\frac{\mathrm{d}N}{\mathrm{d}t} = k(c - c^*)^n \,, \tag{32}$$

where N is either the number of nuclei per unit volume or the mass of crystals per unit volume, k is a rate constant or a mass transfer coefficient, c is the actual solute concentration, c^* is the solute concentration at saturation, and n is an empirical exponent [104]. The term $(c - c^*)$ is the supersaturation. It is ob-

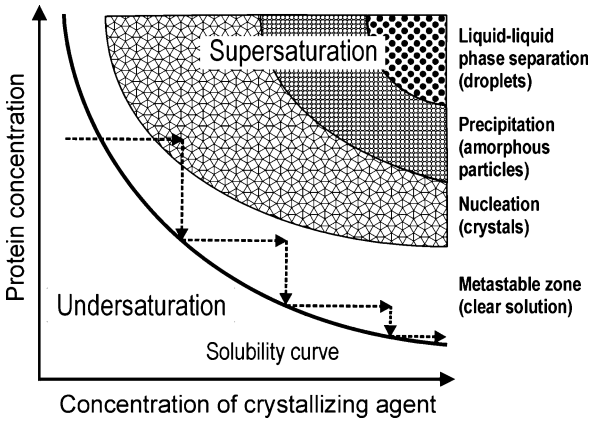

Fig. 9 Typical phase diagram of protein crystallization. The *dotted lines with arrows* outline one possible strategy to obtain large crystals (adopted from [130], p 102)

vious that this approach is only able to describe the crystallization process in a rough overall manner. Here, significant refinements are necessary in order to be able to take important criteria into account such as particle-particle interactions and more realistic kinetics of nucleation and crystal growth. As mentioned by Harrison et al. [104], "a key strategy for crystallization is to move the system very slowly to a state of minimum solubility of the desired protein until a limited degree of supersaturation is reached". This statement confirms the fact that the crystallization kinetics have to be elucidated in more detail. In the early work on protein crystallization of McPherson in 1985 [131], it was pointed out that the adjustment of high protein concentrations in the range of 10 to 100 mg ml^{-1} and seeding with small crystals of the desired protein are often beneficial. Ten years later, Judge et al. [132] demonstrated that following the right strategy, a protein (ovalbumin) was successfully crystallized from an impure solution with two other contaminant proteins present (conalbumin and lysozyme). This strategy was the same as given above (Fig. 9), keeping the supersaturation in the metastable region at all times to avoid unwanted nucleation after the initial nucleation. Judge et al. reported that under these conditions, the two other proteins did not affect the growth rate of ovalbumin crystals. During the 1990s, the rather fundamentally oriented research on protein crystallization gained more impetus. Hence, the above-mentioned beneficial effect of using seed crystals was studied at a fundamental level by Paxton et al. in 1999 [133]. Here, mineral substances such as apophyllite, topaz, and magnetite with either good or poor lattice matching to lysozyme were employed as heterogeneous nucleants for the crystallization of lysozyme from a solution with conalbumin as a contaminant. It was shown that good lattice matching had a beneficial effect on the purity of the recovered lysozyme crystals. In 2000, Vekilov and

Alexander [134], published a thorough fundamental review on the dynamics of layer growth in protein crystallization. It was concluded that unsteady dynamics of layer growth are of significance for the quality of the protein crystals and have numerous other implications. In other work, the significance of the type of surface on which the crystallization takes place was investigated. Nanev and Teneka [135] studied the heterogeneous nucleation of lysozyme on surfaces rendered hydrophobic (e.g. by means of hexamethyldisilazane) and on untreated glass surfaces using a molecular approach. This investigation with a double-(thermal)-pulse technique enabled the detachment of the nucleation process from the growth stage. In this basic paper, the numbers of lysozyme molecules required to form critical nuclei were quantified for the two different surfaces. Another fundamental work on this subject was published by Paxton et al. in 2001 [136] where the influence of vessel surfaces on the nucleation of lysozyme crystals was investigated experimentally and theoretically. It was shown that silanized low-protein-binding polystyrene surfaces were conducive for heterogeneous nucleation. These silanized compounds were similar to those used in chromatography stationary phases for protein separations. The results showed that surface treatment can have a significant effect on the crystallization kinetics. A second basic review dealing with the modelling of protein crystal growth for structure determination was published in 2001 by Kierzek and Zielenkiewicz [137]. In this work, the kinetics of protein-protein association and microscopic models of protein crystallization were addressed. However, it was clearly stated that there still are no satisfactory models of the protein crystallization process. The reason for this is that the physico-chemical aspects of this extraordinarily complex process are not understood. Another significant problem is the tremendously wide ranges of the time and size scales of any reasonable microscopic simulation.

In addition to the ongoing fundamental research, current work on protein crystallization focuses therefore on applied issues such as the development of robust industrial-scale crystallization processes. Contrary to the crystallization of proteins for structure determination, industrial processes need to be fast and reproducible. Furthermore, crystal stability and an appropriate particle size distribution are important criteria. In the work of Schmidt et al. [138], the above-mentioned strategy of keeping the process in the metastable zone (Fig. 9) was followed. It was shown that effective protein crystallization even from impure product solutions was possible by imposing controlled heterogeneous nucleation via suitable seeding strategies using the above strategy. Besides the protein and crystallizing agent concentrations and the kind of crystallizing agent, other parameters play a major role such as temperature, pH value, and additives or buffers. An interesting result of the work of Schmidt et al. was that different morphologies of crystallized proteins were obtained by variation of these parameters, sometimes even when an identical crystallizing agent was used. This is shown in Fig. 10. As already

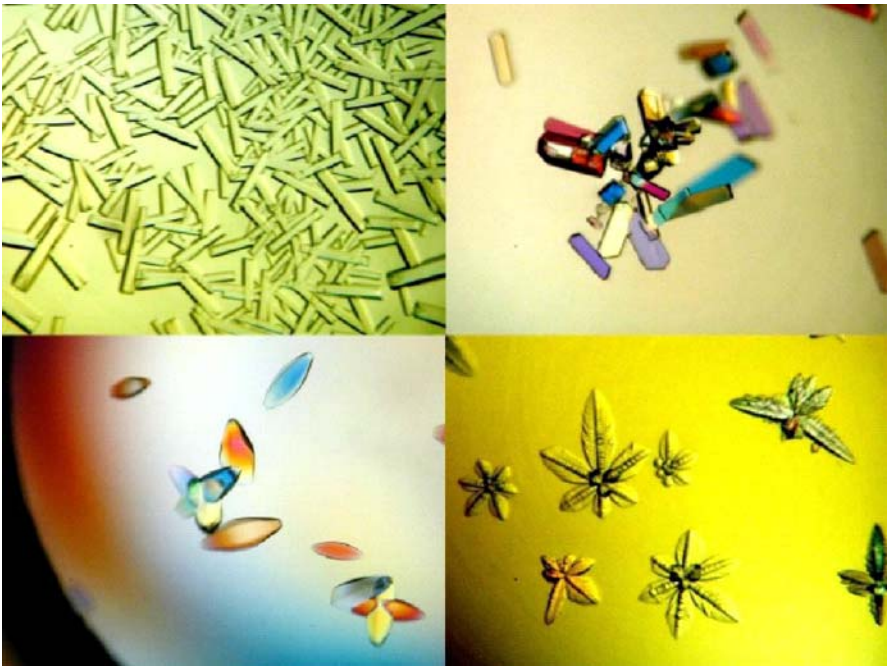

Fig. 10 Different morphologies of crystallized proteins (from [138], with permission from Wiley)

mentioned, different morphologies are relevant for the choice and/or efficiency of the further purification steps such as filtration. Another finding of Schmidt et al. was that impure solutions led to a shift and a reduction of the optimum crystallization region within the multidimensional space of the process parameters. This may result in a less robust process with decreased yields. On the other hand, the high specificity of the crystallization process was demonstrated by using different crystallizing agents for the efficient separation of enzyme isoforms. It was shown that crystallization represents an attractive method for protein purification. However, this technology must be adapted to each application individually and many parameters have to be optimized. Here, the future application of the scaled-down technique is believed to be beneficial [138].

4.2 The Perspective of Downstream Processing: Process Integration

Besides the above-mentioned areas of downstream processing, several other operations are currently being intensively investigated and will be the subject of future research. Among others, the following processes are noteworthy since they represent advantageous integrated approaches. Lali et al. [139]

reported an innovative process of affinity precipitation which utilized a reversibly soluble/insoluble polymer system based on carboxymethyl cellulose, PEG-4000, and $CaCl_2$. Cibacron blue was coupled to this so-called heterobifunctional carrier. With such a system, the affinity for the target protein and the precipitation process could be controlled quantitatively. Hence, lactate dehydrogenase was purified successfully from a crude porcine muscle extract in a one-step operation. The purification factor was 18–23 at a yield of 44–74%. Another currently interesting downstream process is protein refolding. This step is required in order to recover high value proteins being trapped in inclusion bodies, particularly under high protein expression levels. During the recovering process, chaotropic conditions lead to biologically inactive unfolded proteins. The refolding of these proteins is typically carried out by dialysis or dilution. However, these processes often lead to the unwanted formation of aggregated byproducts and are therefore inefficient. In a novel integrated approach, Ferré et al. [140] proposed a combined process of protein refolding and on-line EBA capture as two uncoupled events, thus allowing the individual optimization of each sub-process. With this arrangement, continuous and efficient refolding of human β_2-microglobulin was achieved without the formation of aggregated folding byproducts. However, the operating lifetime of the Streamline DEAE ion exchange medium used in the EBA column was not reported. Schlegl et al. [141] followed a different approach for the continuous refolding of proteins by using a combination of a P-CAC system and an ultrafiltration system for recirculation of aggregated proteins produced during the refolding process. This integrated system was tested with bovine α-lactalbumin as the model protein. Superdex 75 PrepGrade was used as the size-exclusion medium. It was shown that the yield could be enhanced compared to the batch process. Under the assumption that the aggregates can be redissolved and recycled into the feed stream in a quantitative manner, a refolding yield close to 100% was predicted.

Another innovative integrated process was described by Leisola et al. [142]. Here, simultaneous catalytic reaction and separation of inhibiting compounds from the reaction were carried out by using novel cross-linked enzyme crystals in a packed-bed column. With this approach, significantly enhanced enzymatic reaction rates due to very high local enzyme concentrations were obtained. Furthermore, the simultaneous partial separation of inhibiting compounds due to altered product ratios in the outgoing stream resulted in an increase of the catalysis rate. This integrated system was tested with equilibrium catalytic reactions like isomerizations and epimerizations using cross-linked xylose isomerase. It was shown that cross-linked enzymes can be used to shift the reaction equilibrium and to overcome product or substrate inhibition. Such a process can be useful for many specific separations and biosynthetic reactions and provide a viable alternative to current methods.

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Future Aspects of Bioprocess Monitoring

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Abstract Nature has the impressive ability to efficiently and precisely control biological processes by applying highly evolved principles and using minimal space and relatively simple building blocks. The challenge is to transfer these principles into technically applicable and precise analytical systems that can be used for many applications. This article summarizes some of the new approaches in sensor technology and control strategies for different bioprocesses such as fermentations, biotransformations, and downstream processes. It focuses on bio- and chemosensors, optical sensors, DNA and protein chip technology, software sensors, and modern aspects of data evaluation for improved process monitoring and control.

Keywords Biosensors · Microarray technologies · Process control · Process monitoring · Software sensors

1 Introduction

An increasing variety and number of sensing systems are used both in academia and industry for the monitoring and control systems of different biotechnological processes such as fermentations, biotransformations, and downstream processes. For example, to obtain the desired product from the biochemical reaction network of the biological system used in a fermentation, the complex interactions of the overall system must be understood and controlled. The most widely used sensors in biotechnology are those quantifying the physical environment of a bioreactor. Temperature, pressure, liquid level, foam, power input, and impeller speed are monitored in most bioreactors. Less common, but often important, are sensors of viscosity, liquid velocity, and entrained gas quantities. However, during the operation of a bioreactor, substrates are consumed, products and metabolites are formed, and the pH (proton concentration) may change. During batch or fed-batch processes, the levels of these chemicals change substantially, while they are maintained within a narrow range in chemostats. Devices for these measurements are di-

verse, and include electrodes, optical (absorbance and fluorescence) sensors, chromatographs, and biosensors.

In the last few years, the application of software sensors and different chip technologies has become more and more important in the development of control and monitoring strategies of different bioprocesses. In a bioreactor, the cells constitute a biological system that is surrounded by physical and chemical environments. The interactions between these three compartments of the bioreactor are complex, especially when the multiphase aspects of the system are considered. Thus, a whole range of different sensing systems must be applied to such a process. The data obtained from these sensors must be evaluated using rapid strategies to enable their use not only for monitoring but also for efficient process control. There have been many exciting developments relating to bioprocess monitoring from academic research groups. However, most of these new sensing systems and control strategies have not been implemented in industrial processes, mainly because of continuing problems with poor stability, sterilization issues, and inaccuracy. In this chapter, new and future developments, both in sensor technology and control strategies, will be described, with a view toward their ultimate incorporation into practical systems.

2
Bio-, Chemo-, and Optical Sensors

Central to the concept of bioprocess monitoring are the sensors with which measurements are made. Nature has developed very efficient, highly precise methods of controlling biological processes. The challenge is to transfer these principles into technically applicable and precise analytical systems that can be used for many applications. Although this goal has not yet been met, it is nonetheless true that an impressive range of sensing devices has been developed over the years for measurement of chemical, biological, and physical parameters of both up- and downstream bioprocesses. These devices are conveniently categorized as biosensors, chemosensors, or optical sensors, depending upon their detection principle.

In addition to the measurement of traditional bioprocess parameters such as substrate, product, and biomass concentrations, there will be an increasing need for new measurements. For example, as our design of therapeutic proteins becomes more sophisticated, it will be important to have measurements of particular glycosylation patterns. A drug with multiple chiral centers is another case of a complex analyte. Other motivations for sensors will come from systems biology: although sensors for some compounds of the metabolome have been described or even commercialized, these usually cover only some of the extracellular metabolome and only a small fraction of the range of intracellular compounds. On-line sensors

of the transcriptome and proteome may also be desired, as described in Sect. 2.1.

Regardless of the sensor type, target analyte, or application, sensors are expected to be sensitive, selective, and specific. Continuous signals and rapid responses to analyte changes are desirable, especially for process control purposes. An additional requirement, less recognized but perhaps more important, is for robustness and the ability to function in real bioprocess environments for extended periods. This includes the need for asceptic measurements, either by sterilization of the sensor or by incorporation of a sterile sampling device. Although perceived as less glamorous than the invention of a new sensing technique, creative ideas are also needed to solve practical problems so that new sensors can be commercialized and implemented.

2.1 Biosensors

Biosensors – sensing systems with biological detection elements – have the capacity to detect substances in a sensitive and selective manner. Biosensors always consist of three parts: a biological detection component, a signal transducer, and an electrical or visual selection unit. The majority of biosensors developed to date have used an antibody or enzyme (purified or in the form of whole cells) as the biocomponent, and have employed optical, amperometric, or piezo transducers. Examples of these are described in many excellent reviews [1–5].

One of the major trends of emerging biosensor technology is the use of new biocomponents such as aptamers and carbohydrates. Since each class of biocomponent has different advantages and analyte ranges, the identification of new biosensor detection elements is of great interest. A biocomponent class with great promise is that of the aptamers, short RNA or DNA oligonucleotides with specific three-dimensional conformations that allow them to bind a wide range of small and large molecules [6–11]. Aptamers can be synthesized in a combinatorial process and screened for their binding affinity and specificity toward an analyte, which can be a protein, carbohydrate, small molecule drug, or another class of molecules. As biosensor components, aptamers are exciting because they are typically more sensitive and have higher binding affinities than antibodies, and most transducers developed for antibody (immuno) sensors can be adapted for aptamers.

With the growing interest in carbohydrates and glycoproteins has come the awareness that carbohydrates themselves can be a new class of biosensor biocomponent. This is less surprising when one considers that carbohydrates play an important role in the cell surface receptors that recognize a variety of molecules [12]. To date, the main analytes for carbohydrate-based biosensors are glycolipids (lectins) [12–16]. Not surprisingly, lectins have also been used as biocomponents in biosensors for carbohydrates (as well as for glycol-

ipids) [14, 16, 17]. In both cases, a transducer technology suited for detection of binding, such as surface plasmon resonance, can be used.

2.2 Chemosensors

Chemical sensors, or chemosensors, are similar to biosensors but are based on an abiotic detection principle. Widely employed examples are electrochemical sensors for pH and dissolved oxygen, but chemosensors based on many other detection schemes have also been described. In comparison with biosensors, chemosensors are perceived to have advantages in robustness (including sterilizability) and active lifetime, but generally lack the sensitivity and specificity of biosensors. This may change in the future, particularly with the continued development of chemosensors based on smart materials, biomimetic detection, and molecularly imprinted materials.

A smart material has one or more properties that change significantly in response to a stimulus [18]. The property that changes might be viscosity, volume, or conductivity, and the material might be a ceramic, an alloy, or a polymer. Smart polymers in particular have been used in chemosensors [19–22] and certain of these materials are known to undergo large shifts in volume, electroconductivity, and optical properties in response to chemicals (including protons). Interesting transducers such as holograms, pressure sensors, and microcantilevers are used to detect these property changes. Examples of smart polymer chemosensors include those for pH [23], carbon dioxide [24], glucose [25, 26], and organic vapors [27] based on polymer swelling, and another for organic vapors that detects shifts in a conducting polymer's electrical properties [28].

A second trend for chemosensors is the use of biomimetic materials, in which a synthetic molecule is developed to mimic a biological detection element (enzyme or antibody). The goal is to develop recognition elements that are as sensitive and specific as a biocomponent but with much greater thermostability and lifetime. Examples of sensors based on biomimetic enzymes include those for ethanol [29] and dopamine [30]. Additionally, an artificial binding element developed for glycoproteins [31] has been reported that could readily be adapted into a chemosensor.

One of the drawbacks to many chemosensors is their lack of specificity. For example, there are minimal differences in the responses of the organic vapor sensors described above to similar organics. A strategy to develop more specific chemosensors (whether based on smart materials or not) is that of molecular imprinting [32–36]. Molecular imprinting refers to the generation of specific recognition sites by forming the material in the presence of the analyte. Both biomimetic enzymes and antibodies can be produced in this way [37]. Using this imprinting approach, a chemosensor was developed for fructosylamines based on biomimetic enzymes [38]. Chemosensors based on

molecularly imprinted biomimetic antibodies are more common, and the list of analytes includes acetaldehyde [39], amino acids (including enantiomeric specificity) [40–42], and polycyclic aromatic hydrocarbons [43].

2.3 Bio- and Chemosensor Arrays

An important development for both bio- and chemosensors is the concept of array sensors, in which a number of different detection elements are present, each providing a signal. The set of signals can then be interpreted to provide multianalyte measurements and even analyte identification. Such capability is very important in bioprocess monitoring, since multiple analytes are present in nearly every measurement scenario. For example, it would be of interest to have an array sensor in a purified product stream to measure not only the concentration of the desired product but also of any known contaminants. An important requirement of an array sensor is a chemometric algorithm to interpret the signal set. With effective chemometric analysis, array sensors can be shown to be highly effective at resolving analyte mixtures, even if each individual sensor is only moderately specific and the analyte range of one sensor overlaps with that of another. Sensor arrays can be viewed as imitations of the arrays of receptors in organisms, and thus many of the first sensor arrays were termed either "artificial nose" (for gas-phase sensing) or "artificial tongue" (for liquid-phase measurements) [44–50]. There are several reports of the application of these arrays and others for bioprocess monitoring [44, 46, 48–51]. In other bioprocess-related applications, sensor arrays have been used to estimate bacterial growth rate [52] and detect infection in a mammalian cell culture [53].

2.4 Nanoscale Bio- and Chemosensors

Owing to the characteristics of their transducers, bio- and chemosensors have historically been small, and there have been efforts to reduce this size further for certain applications (e.g., in vitro measurements). However, the emphasis has been only on reducing size to fit into a certain physical space, and not for altering the capabilities of the sensor itself. Nanotechnology – development and analysis of devices with design features in a length scale of 1–100 nm – has the potential to produce new types of sensors and to lower detection limits, in addition to yielding extremely small sensing elements [54–57]. The development of nanobio- and nanochemosensors is still in its infancy but the rate of growth in this field is tremendous and commercial products are already appearing. Much of the research on nanosensors is targeted at medical sensing and diagnostics [58, 59], but nanosensors have been developed for analytes of interest to bioprocessing [56], including glu-

cose [60, 61], lactate [62], and hormones [63]. It is interesting to note that many of the developments in nanosensor technology involve combinations of themes already mentioned above: molecular imprinting, smart materials, and arrays.

2.5 Optical Sensors

Optical sensors have become more and more important for bioprocess monitoring. The term "optical sensors" is used here to encompass all analytical methods that are based on interactions of light with matter. Optical sensors offer the advantages of non-invasive, non-destructive, continuous, and simultaneous multianalyte monitoring. Their use is ideal for monitoring and control of bioprocesses because culture contamination can easily be avoided. Optical systems do not interfere with metabolism and thus in vivo measurements are possible. This can yield intracellular information that is difficult to obtain with other methods. Sampling, which always carries a risk of contamination, and time-consuming downstream modifications are seldom necessary for optical sensor systems, and typically no analyte is consumed. At this time, no general optical detection system has been developed. Since modern bioprocesses are extremely complex and differ greatly from process to process (e.g., fungal antibiotic production vs. mammalian cell cultivation), appropriate analytical systems must be set up from different basic modules, designed to meet the special demands of each particular process. An impressive variety of analytical systems has been produced for different applications in biotechnology. However, more research and development efforts are required to meet the special demands of modern and future bioprocessing – which means not only fermentation but also screening and downstream processing. Advances will likely proceed in two directions: adaptation of existing systems and evolution of new procedures or devices. Both are necessary to achieve efficient monitoring and to enable the necessary degree of control. Most optical sensors are based on the analytical principles of UV spectroscopy, IR spectroscopy (NIR and FTIR), Raman spectroscopy, fluorescence spectroscopy, and pulsed terahertz spectroscopy (PTS).

In this section, some of the newest developments in the application of these sensor types in biotechnology are described.

2.5.1 Application of UV Spectroscopy

UV spectrophotometers, especially those with high resolution, are very suitable for use in on-line monitoring of fermentation and downstream processes. New UV spectrometers have been developed in the last few years [64], capitalizing on recent advances in electronics and especially in the develop-

ment of new light detectors. One main advantage of these new devices is the use of charge-coupled devices (CCDs) or photodiode arrays to replace the rather expensive photomultipliers in conventional spectrometers. These instruments not only offer excellent performance in the UV range, but are useful for wavelengths up to the near IR. Because of their unique combination of outstanding sensitivity, high scanning speed, compactness, low cost, and especially their robustness, these detectors are also often used in industrial applications. The development of a high resolution UV spectrometer that is suitable for the on-line monitoring of different kinds of bioprocesses is described by Noui et al. [64]. In this application, a deuterium light source was used to produce a light beam with an effective spectral radiation range of 185–400 nm. With this instrument, one is able to measure a continuous spectrum and this technique allows the simultaneous detection of several analytes.

Another approach to sensing focuses not on detection of specific target analytes but rather on integrated parameters. This is especially useful in complex media, including classical culture media, as well as in domestic and/or industrial wastewater treated by biological processes. The on-line and/or in situ determination of some substances is possible, but expensive, as sample collection and pretreatment are often necessary with strict rules of sterility. Pons et al. [65] described more global methods based on UV spectroscopy that can be used to rapidly detect "accidents" such as the appearance of an undesirable by-product in a fermentation broth or of a toxic substance in wastewater. These methods combine a "hard" part, for sensing, and a "soft" part, for data treatment. An important note about the use of UV and other spectroscopic techniques is that efficient data evaluation is necessary since a simple analysis of the data is often not well-suited to the complexity of the processes. Thus, the use of mathematical procedures such as neural networks or partial least squares (PLS) is recommended [64, 66, 67].

2.5.2 Application of NIR and FTIR Spectroscopy

Near infrared (NIR) spectroscopy enables detailed monitoring of substrates and products in many important types of industrial and food bioprocesses in order to document progress, ensure comparable end-product quality, and to provide data for modeling, optimization, and process control. Like most optical techniques, NIR can be used for on-line/in-situ sensing. Examples include the report by Jorgenson et al. [68], who described a newly developed modification of multivariate statistical process control charts based on on-line NIR. This enabled simple, efficient identification of abnormal fermentation runs, even at an early stage of the fermentation, which is critical for industrial production monitoring. Sorensen [69] has developed prediction models for determination of lactic acid, acetic acid, pH, NH_3, and ethanol in grass and

corn silages by in-situ NIR spectroscopy. The application of on-line NIR spectroscopy to measure simultaneously the concentration both of substrates and products of acetic fermentation (ethanol, acetic acid, and biomass concentrations) was reported by Garrido-Vidal et al. [70].

NIR has also been used effectively in an in-line mode. For example, the NIR technique was applied to the measurement of concentrations of ethyl acetate and acetoin, two compounds of importance for the sensory characterization of commercial vinegar. The analyzed samples were taken directly from the industrial process of a vinegar producer and their NIR spectra were measured in order to serve as the basis for the subsequent development of separate PLS regression models for each studied response. Other examples of the application of in-line NIR monitoring in food biotechnology include fermentation of *Lactobacillus casei* [71], wine [72], and yogurt [73] fermentation. The application of NIR in-line to monitor and control an industrial fermentation process was investigated by Tosi et al. [74]. The determination of biomass, glucose, and lactic and acetic acids during fermentations of *Staphylococcus xylosus* was performed by an interactance fiber optic probe immersed in the culture broth and connected to a NIR instrument. PLS calibration models using second derivative NIR spectra in the 700–1800 nm region gave satisfactory predictive models for all parameters of interest. Batch, repeated batch, and continuous fermentations were monitored and automatically controlled by interfacing the NIR to the bioreactor control unit. Thus, NIR seems to be one of the most promising spectroscopic techniques for the future of bioprocess control and modeling [75].

In addition to NIR-based technologies, FTIR has also been applied to bioprocess monitoring. For an example, a novel attenuated total reflection-FTIR sensor was used for continuous on-line monitoring of chlorinated aliphatic hydrocarbons in a fixed-bed bioreactor. The sensor was based on an ATR internal reflection element coated with an extracting hydrophobic polymer, which prevented water molecules from interacting with the IR radiation [76]. FTIR spectroscopy, coupled to sequential injection analysis (SIA) was employed by Kansiz et al. [77] for the on-line monitoring of an acetone–butanol fermentation by simultaneously determining acetone, acetate, butanol, butyrate, and glucose from the mid-IR spectra of the samples.

2.5.3 Application of Raman Spectroscopy

Raman spectroscopy is based on the phenomenon of shifted wavelength scattering of molecules excited with monochromatic light due to inelastic collisions of photons with the molecule. The application of Raman spectroscopy to bioprocess analysis was made possible by the development of adjustable lasers. In principle, it is suitable for multi-analyte monitoring of complex industrial bioprocesses on-line [78, 79] as well as for performing differentiated

measurements of certain parameters, as demonstrated by Lee [81] and Shaw et al. [80].

The simultaneous estimation of glucose, acetate, formate, lactate, and phenylalanine concentrations in *Escherichia coli* cultivations using in situ Raman spectroscopy was recently reported [81]. Attenuation due to light scattering from air bubbles and biomass was corrected by internally referencing the least-squares estimated concentrations to the estimated water concentration. The estimation accuracy of this system was limited by errors in the physical model for the system, rather than by measurement noise.

Challenges for the application of Raman spectroscopy to bioprocess monitoring lie in the strong fluorescence activity of many biological molecules, which often overlay the Raman scattering bonds. One solution to this is "shifted subtracted Raman spectroscopy"; using this approach, the resulting spectra have much lower apparent noise and fluorescence due to the cancelation of irregularities in the detector response [82]. These new developments will enable the application of Raman spectroscopy to a wider range of processes in the future.

2.5.4 Application of Fluorescence Spectroscopy

Since many important biomolecules fluoresce, it is not surprising that fluorescence spectroscopy has been applied to bioprocess monitoring for several decades. A promising new version of the classic technique is two-dimensional (2-D) fluorescence spectroscopy, in which a range of excitation and emission wavelengths are scanned. In comparison to traditional NAD(P)H fluorescence techniques, this method provides much more analytical information with a single measurement. With 2-D fluorescence, all fluorophores present in a sample can be monitored simultaneously. Recently, a non-invasive 2-D fluorescence sensor was developed for the measurement of the whole cell biotransformation of L-serine and indole to tryptophan [83] and for industrial downstream processing of sugar beet molasses [84].

2.5.5 Pulsed Terahertz Spectroscopy

The new terahertz technique, pulsed terahertz spectroscopy (PTS), can be expected to lead to new analytical tools for bioprocess monitoring. Terahertz radiation lies on the electromagnetic spectrum between microwaves and infrared at a frequency of more than a trillion cycles per second. It has tremendous potential for applications in chemical and biological identification [85]. The first use of pulsed terahertz spectroscopy to examine low frequency collective vibrational modes of biomolecules has already been reported [86, 87]. Those results demonstrate that PTS is a viable technique for time-resolved

measurements of protein folding. Thus, it could become a very useful tool for bioprocess monitoring, since the correct folding of recombinant proteins is one of the most important factors in the pharmaceutical industry.

3
DNA and Protein Microarray Technology

DNA and protein microarrays ("biochips") can be used for identifying gene expression at the level of mRNA and protein. Biochips have a high degree of multiplicity (thousands of genes assayed simultaneously), which gives them a great advantage over classic molecular biological methods [88]. While conventional methods are limited to the examination of single genes, a DNA chip experiment delivers a complete gene expression pattern of the cell. Thus, biochips can significantly simplify and accelerate a number of long and expensive diagnostic methods. Gene expression analysis across various biological conditions, cell cycle states, tissues, and subjects may help identify differentially expressed genes [89]. This type of information is valuable in the investigation of biological processes and functional disorders, allowing investigators to profile complex diseases and discover novel disease-related genes. Applications of this technology have included gene expression monitoring [90], mutation detection [91–93], clone mapping, drug development [94], tailored therapeutics, SNP research [95], GMO detection [95], and high throughput screening, among others. They have a profound impact on biological research [90], industrial production [90], medicine, diagnostics, environmental research [96], bioprocess optimization [97, 98], and pharmacology [94]. Thus far, biochips have been used only as assays, but in the future it is likely that they will be transformed for bioprocess monitoring.

3.1
DNA Microarrays

3.1.1
DNA Microarray Fundamentals

To fully understand biological systems, the measurement of mRNA levels corresponding to as many as 30 000 genes will be necessary. An ideal tool for such measurements is DNA microarray technology [99], a high throughput method in which amino-modified oligonucleotides or PCR products are arrayed on silylated microscope slides. These microscope slides, containing many immobilized DNA samples ("targets"), are normally hybridized with fluorescently labeled cDNA probes (Fig. 1). Already important in biological research, DNA microarray technology will continue to gain importance, developing into a key technology of the 21st century [100–102].

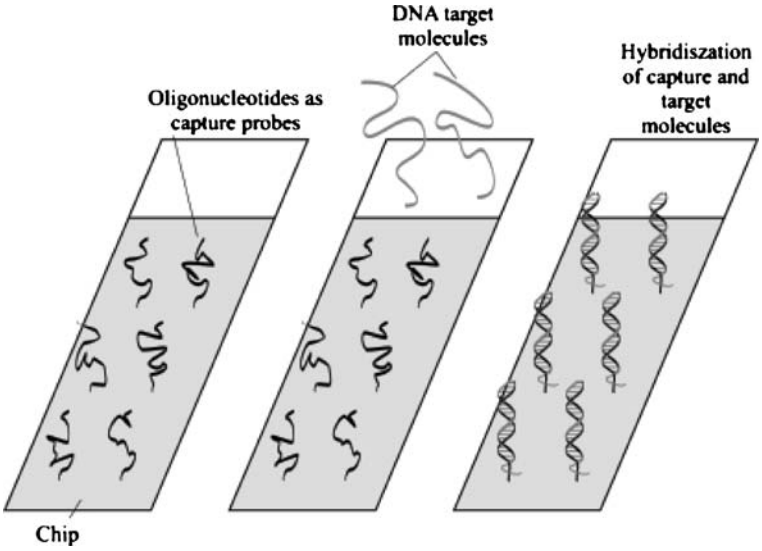

Fig. 1 Interaction of labeled target moelcules with probe molecules on a glass array

DNA microarrays are fabricated with high-speed robots by depositing probes (cDNAs, oligonucleotides, PCR products) with known identity as small spots on a specific and known site onto glass substrates. Due to the small size of spots on the array, tens of thousands of different DNAs can be immobilized on a single slide. There are three primary technologies used presently in automated microarray fabrication: photolithography, ink-jet printing, and contact printing, along with derivatives of each. Each of these technologies has specific advantages and disadvantages in microarray manufacturing. The photolithographic approach, commercialized by Affymetrix, relies on the in-situ synthesis of 25-mer oligonucleotides using photomasks. Thus, each probe is individually synthesized on the chip surface. In contrast, the ink-jet and contact printing methods attach presynthesized DNA probes to the chip surface. While the in-situ probe synthesis method requires sophisticated and expensive equipment, the contact and non-contact dispensing methods made DNA chips affordable for academic research laboratories. Since 1996, many commercial DNA printers have become available and spotted glass slide DNA arrays are currently the most popular format for gene expression profiling experiments.

In normal use, the mRNA from test and control samples are labeled with different fluorophors, mixed, and allowed to hybridize with the immobilized DNA targets, providing a comparative analysis of differentially expressed genes. Comparison of the binding efficiencies between the two samples provides an easy and efficient survey of gene transcript level changes for numerous genes in a single experiment.

RNA isolation is one of the most important steps in a DNA microarray experiment. Low quality RNA leads to poor hybridization results. Single-stranded cDNA is then synthesized enzymatically from total RNA using oligo-d(T) primers or random primers (bacteria). To exclude interference within the labeling reaction and during hybridization, treatment of the isolated RNA with DNase I is strongly recommended. During reverse transcription, a fluorescently labeled nucleotide is incorporated into the nascent cDNA. Subsequently, the template RNA is degraded by chemical treatment and the cDNA is separated from primers, unincorporated nucleotides, and the RNA debris. The two sets of differently labeled cDNAs can then be combined and cohybridized to the same array under stringent conditions. After hybridization, the unbound and non-specifically bound cDNA is removed by stringent washing from the array. After washing and subsequent scanning of the array with a confocal array scanner (Fig. 2), the fluorescence intensity of each individual spot is determined, normalized, and the expression ratio of each gene on the array determined. Data normalization is performed using non-linear regression procedures.

Since DNA microarrays allow simultaneous measurement of thousands of interactions between mRNA-derived target molecules and genome-derived probe molecules, large amounts of raw data are produced. Due to this large amount of data, bioinformatic solutions must be developed [103, 104].

Parallel gene analysis with DNA chips provides a rapid and efficient method for large-scale and high throughput applications. To date, DNA microarrays have been used primarily in biomedical research. Examples in-

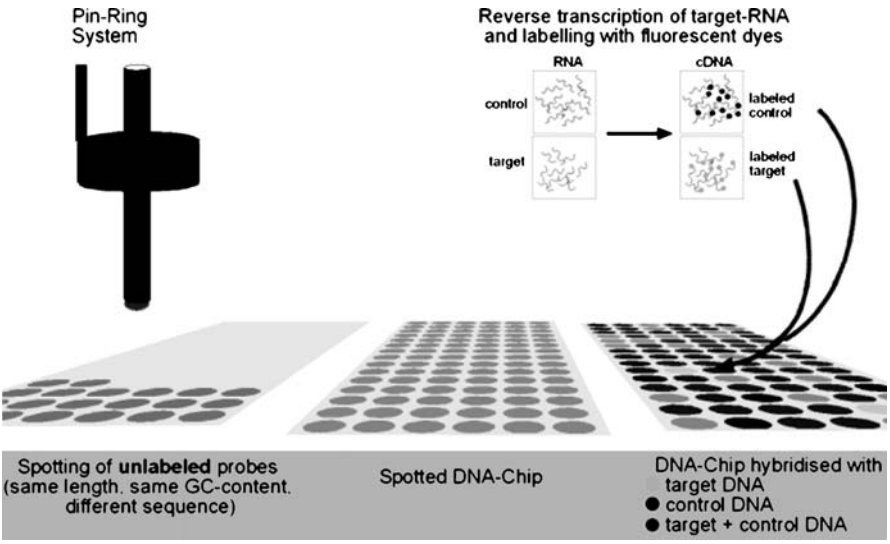

Fig. 2 Gene expression analysis through microarray technology

clude monitoring changes in gene expression in response to drug treatments, screening for mutations and sequence variations in genomic DNA, and providing insights into fundamental biological processes of human diseases at the molecular level. However, mRNA expression level studies are also important tools for the analysis of bioprocess cultivation conditions. They can be used to check for changes in gene expression during long-term culture of cells (e.g., in intensive reactor systems).

3.1.2 Gene Expression Analysis in Bioprocess Research

In the past few years, the complete DNA sequences of a number of different microorganisms have been determined and can be exploited to optimize strains as well as recombinant protein production. Based on this sequence information, DNA microarrays can be used for bioprocess applications such as strain optimization and the optimization of recombinant protein production conditions. Strain optimization on the basis of genome-wide mRNA levels in wild type and mutant strains allows one to correlate strain performance with genetic changes.

Microarray analysis can also help identify previously unknown genes required for recombinant protein production. Recombinant protein expression exerts a metabolic burden on the host cell, triggering various stress response mechanisms. DNA microarray analysis allows an investigation on a genome-wide scale, which enables qualitative and quantitative characterization of the burden on host cell metabolism. Thus, a better understanding of the impact of recombinant protein production can be achieved using the generated "snap shot" of the actual gene expression profile.

3.1.3 Gene Expression Analysis: Problems and Pitfalls

A significant challenge for the application of DNA microarrays is the lack of standards for comparing and exchanging the resulting large expression data sets. Different algorithms, different software packages, and different experimental approaches make it nearly impossible to compare data. MIAME (minimum information about a microarray experiment) standards [105] are the prerequisite to the worldwide comparability of gene expression data and there are several URLs where these standards are available. The ANOVA approach makes no assumptions about whether there are many or only a few differentially expressed genes, since it tests each gene independently of the others. A related advantage of the ANOVA approach for two-color arrays is that it does not assume that the dye effect is the same for each gene (or each gene at a particular expression level). Many technical aspects concerning microarray production and laboratory usage have been addressed in

great detail, but it still remains crucial to establish this technology in new research fields. Although the classic, relative expression level experiment is largely standardized, there are various pitfalls within the hybridization step and in the design and analysis of microarrays. Without sound quality control, experimental microarrays may produce useless or, even worse, misleading results.

3.2 Protein Microarrays

3.2.1 Proteome Analysis

Although mRNA analysis is valuable, gene expression level alone is not sufficient to predict the expression of a protein. In contrast to the genome, which is determined by the sequence and the type and number of its nucleotides, and is therefore static, the proteome is tremendously dynamic and is influenced by a variety of parameters. Not all genes are switched on at the same time in a cell, and the sensitive balance between protein synthesis and protein degradation can vary widely under different metabolic or environmental conditions. This sensitive dependence of the proteome to cultivation and other parameters presents the possibility of using specific small changes of the protein expression pattern as a sensitive biosensor.

Unfortunately, such data cannot be estimated from DNA microarrays since no strict connection between the amount of mRNA and the amount of protein exists. Differing degrees of mRNA stability, protein degradation, posttranslational modifications and others make this connection difficult to establish, and thus direct measurement of protein levels is important. One attractive method is the use of protein microarrays. The development of protein microarrays shares the principles of miniaturization and parallel analysis with DNA microarrays. In theory, protein microarray technology allows the analysis of protein expression and interaction on a whole cell (or tissue) level [106, 107]. However, the arraying of proteins is more difficult than the arraying of DNA because the correctly folded conformations of the immobilized proteins must be maintained, the chemistry of coupling the proteins to surfaces is more complex, and the detection signals are weaker. The fabrication of protein arrays has therefore lagged in development [108].

3.2.2 Fabrication of Protein Microarrays

Protein microarrays are miniaturized systems in which proteins are used either as capture probes or as targets that can be bound by specific capture

probes [109–111]. Different proteins such as antibodies, antigens, peptides, receptors and enzymes can be bound in several formats (modified glass surfaces, hydrogel, membranes, nanoplates, microfluidic chips).

Protein microarrays consist of a solid support, e.g., glass or synthetic material with a modified or coated surface. Using special printers (pin-based or ink-jet printing heads) capture probes (e.g., antibodies), are transferred to this surface in the form of microspots (< 200 μm). Every microspot contains only one kind of capture probe. These immobilized capture probes bind their specific target molecules from a complex solution. Today, the detection of immobilized antigens with antibodies is still the most common application. The binding of sample and target molecules can be detected by different methods based on fluorescence, chemiluminescence, or radioactivity. Alternatively, protein chips for direct measurement of protein mass by MALDI-TOF mass spectrometry are available.

3.3 Prospects for Microarrays in Bioprocess Monitoring

The advantages of microarray technology make adaptation to the area of biosensing highly attractive. Although the use of both DNA and protein microarrays to date has been limited to assays for research, at least two means by which microarrays can be applied to bioprocess monitoring can be envisioned:

- In an off-line format, in which the signals of on-line/in-line bioprocess sensors are correlated to the data from DNA or protein microarrays (e.g., with neural networks) to provide more depth to other sensor measurements.
- As part of an in-line sensor system, in which bioprocess samples are automatically removed, pretreated (e.g., DNA extraction), hybridized/bound to the array targets, and analyzed. While this would be a complex system, the pace of developments in lab-on-a-chip technology suggests that such capabilities will soon be available.

In both cases, further developments in microarray technology are required for practical implementation. For example, proteins and antibodies that are used as capture molecules on protein microarrays denature rapidly on glass surfaces. This might be overcome through the development of new capture probes like aptamers or PNAs (Fig. 3). Aptamers are optimized oligonucleotide sequences generated with a 30–40 base stretch in the middle to bind proteins. Aptamer technology combines the advantage of arraying DNA with the specification of antigen–antibody interaction and shows potential for proteome analysis. The use of peptide nucleic acids (PNA) as capture probes leads to the stabilization and flexibility of the sensor.

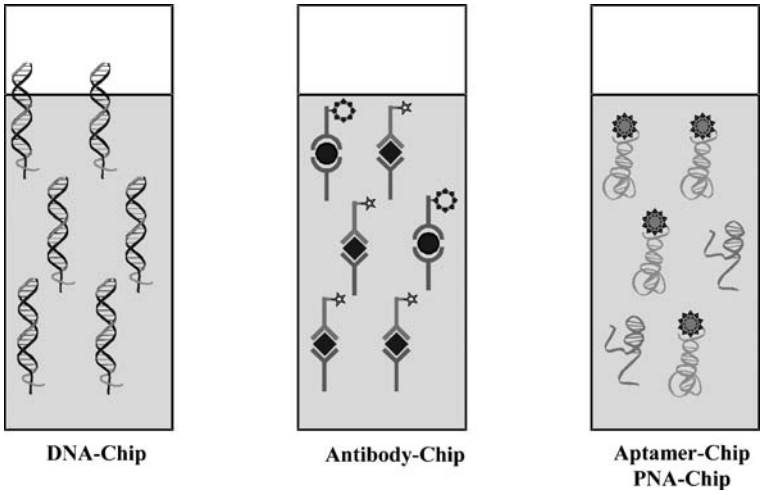

Fig. 3 Comparison of DNA, antibody, PNA, and aptamer chips

4 Software Sensors

4.1 Software Sensor Concepts

The best way to get process information from a key component is to have an appropriate on-line sensor, possessing the desired degree of specifity. But as described earlier in this chapter, such sensors are available only for a few on-line analytes. In most cases, it would not be possible for process engineers to react within an appropriate time to process fluctuations caused by variations in substrate or product concentration.

A very promising method to get on-line access to important key components of bioprocesses is the use of *software sensors*. In general, a software sensor supplies the estimation of the missing measurements by using an appropriate model that relates the desired variable with other physical or chemical measurements that are correlated to it in any way (Fig. 4) [112–115]. Generally, software sensors are solutions of inverse problems. In a complex biological system, the quantities that are normally easiest to measure are the variables, not the parameters. In the case of metabolism, the usual parameters of interest are the enzymatic rate and affinity constants, which are difficult to measure accurately in vitro and virtually impossible in vivo [116–119]. However, to describe, understand, and simulate the system of interest we need knowledge of these parameters. In other words, one must go backwards from variables such as fluxes and metabolite concentrations, which are relatively easy to measure, to the parameters.

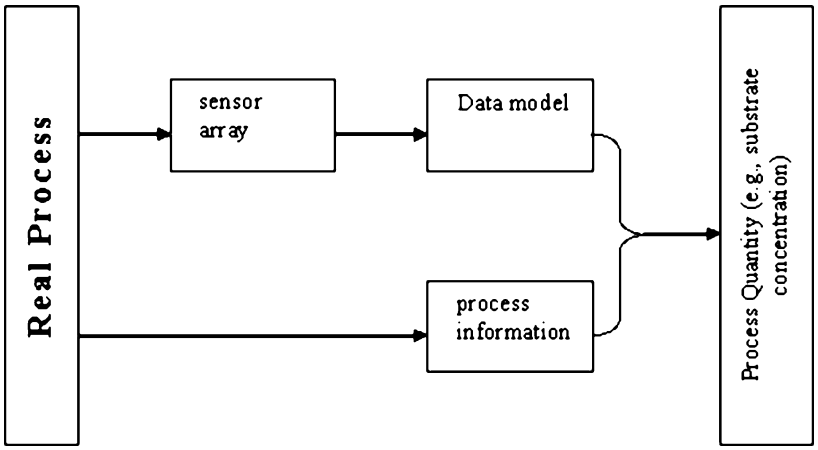

Fig. 4 General principle of a software sensor. From a process, different measuring values are determined and mapped with a data model and/or process information to a specific substrate concentration

In other words, when using software sensors, there must always be a model available that reliably relates the measured variable with the target variable or parameter of interest. Normally, measured variables are easily determined effects that are caused and influenced by the target. It is the special objective of the software sensor to reach a maximal degree of generalization. However, it is very difficult – or perhaps impossible – to achieve or furthermore to prove this claim. This leads to the basic question: Is the available information representative enough to generate a model for the accurate estimation of the quantity of interest? Consequently, the development of software sensors is often restricted to a specific application; transfer to other applications or conditions requires considerable modification.

A software sensor consists of a measuring part and a modeling part. The relative importance of each depends on the specificity, causality, and singularity of the hardware sensor in use for the desired analyte concentration. If the specificity and the relation of measured value to control quantity are high, the model part is less important, and vice versa. If different measurement devices provide the same process information, a filtering model must first be applied to eliminate/minimize redundancies or measurement errors. Typical approaches to this kind of data model include chemometric and artificial neural network models.

4.2 Applications of Software Sensors

It should be noted that approaches that use only one measurement variable for observation of a process target (e.g., carbon dioxide as a quantity to

estimate yeast growth), are actually more of a calibration method than a software sensor (by analogy to a temperature sensor, which does not measure the temperature itself but a related quantity like the resistance or voltage). Thus, software sensors utilize multiple inputs and a relatively complex process model. Spectroscopic methods represent an ideal illustration of such multiple-input sensors [120–123]. Their common property is the detection of the level of absorption or fluorescence at different stimulation frequencies. From this, a set of information is generated that possesses more information than the measurement at one specific frequency. If these data are used as inputs to an appropriate model, the accurate determination of the desired process quantity is possible. Since rapid progress is being made toward cheaper and more robust optical measuring devices, this technique is more than a convenient example, and we can expect to find increased use of on-line optical devices with both hardware and software sensor elements. Another reason for the high potential of such systems is the availability of microcontrollers in which complex evaluation algorithms can be integrated. These algorithms are necessary to eliminate matrix effects and interferences, and may eliminate the need for on-line sample pretreatment devices. In addition to spectroscopic methods, there are also some types of software sensors that use different process sensors to build the estimation model. For example, to predict the diacetyl concentration during yeast fermentation, sensors for temperature, carbon dioxide, gravity, pH, and optical density were input to an appropriate model [133]. Many similar applications have been reported.

In most cultivations and other batch processes, the situation is even more complicated since events develop in time. The estimation of the physiological state of a culture involves more than one (measurable) variable at a time and the recent history of this set of individual signal trajectories is important. Consequently, physiological state estimation requires recognition of complex patterns. Various algorithms used for this purpose to build up the model have the common feature that it is not always the present values alone that are evaluated; instead, the recent history of signal trajectories must be used. Although some researchers define software sensors only on the basis of neural networks, a broader point of view should be adopted since software sensor models have also been obtained using regression or correlation techniques, fuzzy logic models, first principle models, and combinations of all of these [124–127].

In some cases, the data describing the actual state and its recent history are compared with reference patterns, which are data from historical experiments or runs that an expert has associated with a typical physiological state. A physiological state is recognized either if the actual data constellation matches any one of the reference sets best (in this case, there is always an identification made) or if the match exceeds a predefined degree of certainty (in this case, it can happen that no identification or association is possible if the preselected threshold value is not reached). The direct association with

reference data needs normalization (amplitude scaling) and, probably, frequency analysis in order to eliminate dependencies on (time) shifts, biases, or drifts. In other cases, the data trajectories are translated into trend-qualities via shape descriptors. These combinations of trends of the trajectories of various state variables and/or derived variables define a certain system state. The advantage of this definition is that the association is no longer dependent on time or the actual numerical values of variables and rates [128].

4.3 Trends in Software Sensing

Historically, the provision of sensors and the development of a process control strategy have been treated as two separate areas in engineering science. The sensor is expected to give accurate information to the control unit, where these data are further used to determine proper control action. However, in the future of bioprocess engineering, this delineation must be abandoned. Since software sensors entail some loss of general applicability to other processes, the flow of information from the process to the sensor device is the only way to guarantee the appropriate quality of the sensor data. By including the modeling structure in the sensor data processing, the information becomes more robust against noisy measurement data, outliers, or changes in the intrinsic behavior.

Based upon these considerations, the actual trend in sensor processing and information retrieval can be seen to represent data-driven approaches and interpretations [129]. With even a brief look at successful applications of artificial neural networks or other chemometric approaches, the evolution of these trends is obvious [130]. However, the more that raw and non-specific data are used to build up data models, the more loss there is of the property that single signals provide direct access to process information. If an important message is hidden in a set of sensor signals, it is difficult to decide manually whether a signal is representative of the process, whether it changes its behavior, or whether it is a pure outlier [131]. Basically, data models possess no structure that can help with interpretation. They use the data to feed a learning or estimation algorithm, and they cannot determine whether the incoming data set is representative of the process behavior or not. The quality of the collected data is strongly dependent on the behavior of the model and, thus, so is the success of the model. It is not important to have as many data sets as possible. Instead, a representative amount that spans the whole quantity space of the variables is desirable [132]. It would be optimal that more data are available in those regions where a high sensitivity exists, while fewer data are needed where the output is nearly constant. The time cost of sensor modeling of data-driven devices changes from real modeling to data pretreatment before modeling. For on-line applications in particular, there is rarely a decision point to determine whether the new incoming data should be used

for training or not. This field of an automatic decision maker for handling only appropriate data and rejecting the others is of special interest for further scientific approaches.

Another future aspect concerning software sensors concerns the model used in the sensing devices. Although there are drawbacks in using poor data models (as described above), they are necessary to build up causal relationships or to eliminate redundant information. In general, there are no alternatives to that approach, which means that any available detailed process information would have been discarded. Recently, some new developments have been described that allow the development of hybrid structures using data modeling and process modeling approaches, simultaneously. They work neither in parallel nor in sequence, but rather interactively to reach an appropriate filtering result using data modeling approaches, and also to be more generalized due to the system information [133].

Concerning the hardware sensing devices themselves, it is to be expected that more robust and new systems will be integrated with soft-sensing principles, but the main focus of future work will have the goal of directing more information from the process using bioinformatic principles. These high-potential approaches should reveal new causalities from system state information to measurable process quantities. This consideration also includes the development of sensor arrays (e.g., electronic noses and tongues). It can be expected that new materials or modifications of existing ones can help to build up enhanced array configurations with new analytical possibilities. As noted in Sect. 1, advances in micro- and nanoscaled structures will lead to sensing cells that can be integrated into ever smaller volumes and that can carry ever more process information for more evaluation.

5 Flow Injection Analysis

For bioprocess monitoring, flow injection analysis (FIA) remains one of the most important analytical techniques. FIA allows almost any sensor to be used and thus many different substrates, metabolites, and products can be measured. Zaydan et al. [134], Ferreira et al. [135], Arndt and Hitzmann [136], Inaba et al. [137], Bracewell et al. [138], Stefan et al. [139], Rocha and Ferreira [140], Rhee et al. [141], Klockewitz et al. [142], and Nandakumar et al. [143] give many examples of its application for bioprocess monitoring and control. However, the application of FIA systems in industry is still not very common. One reason is the required maintenance work, which is not appropriate in many industrial environments. Furthermore, in bioprocess engineering, the complex sample solution with all the metabolites, proteins, and cells can significantly influence the detection reaction in the FIA system. For example, proteases, often excreted by cells, can inactivate a biosensor com-

ponent of a FIA system. Other compounds in the sample solution can affect the correlation of the analyte concentration with the detected variable because many measurements are indirect. If, for instance, pH changes are used to determine an analyte concentration, then a change in buffer capacity will influence the calibration model significantly. Compounds in the culture broth can also be deposited on the sensor and change its characteristics; moreover, they can even block flow in the system, especially in the sample module and the valves. Schügerl [144] gives many examples of the faults and requirements of FIA systems when they are applied to bioprocess monitoring and control.

The optimization of flow analysis systems is carried out by improving the detector, the manifold, the chemical environment, and other features to improve stability, sensitivity, selectivity, and robustness of the whole system. However, for a more advanced improvement of the overall hardware, one must also consider the optimization of the system related to data evaluation methods (i.e., software, methods).

In this section, various evaluation techniques for flow analysis signals are presented using practical examples. In addition to multivariate evaluation techniques and theoretical models, expert systems will be presented, and their potential for the evaluation of flow analysis signals will be discussed. Many of these techniques can also be applied to the evaluation of other process analyzers.

5.1
Robust and Reliable Evaluation of FIA Measurement Signals

To evaluate the FIA measurement signal, theoretical considerations have shown that, in the linear range, the signals can be modeled as a combination of an ideal plug flow reactor with an ideal stirred tank reactor. The resulting convolution integral gives the so-called exponential modified Gaussian (EMG) function:

$$h_{\mathrm{PFST}}(t) = \frac{h_0}{\tau\sigma\sqrt{2\pi}} \int_0^t \mathrm{e}^{-\left(\frac{t'-t_\mathrm{R}}{\sqrt{2}\sigma}\right)^2} \mathrm{e}^{-\left(\frac{t-t'}{\tau}\right)} \mathrm{d}t' \tag{1}$$

$$\approx \frac{h_0}{\tau} \mathrm{e}^{-\frac{(t-t_\mathrm{R})}{\tau}+\frac{\sigma^2}{2\tau^2}} \left(1 + erf\left(\frac{t-t_\mathrm{R}}{\sqrt{2}\sigma} + \frac{\sigma}{\sqrt{2}\tau}\right)\right).$$

Here $h_{\mathrm{PFST}}(t)$ is the signal height of this model at time t, t_R is the retention time, h_0 is a factor representing the signal height and which is proportional to the analyte concentration, σ is the standard deviation of the Gauss function, τ is the hydrodynamic residence time of the ideal stirred tank reactor, t_R is the retention time, and *erf* is the error function [145]. As an alternative to this model, a tanks-in-series-model can also be used, which describes the typical

FIA signal as:

$$h_{\mathrm{TIS}}(t) = h_0 \left(\frac{t - t_{\mathrm{R}}}{\tau} \right)^{N-1} \frac{1}{(N-1)!} \mathrm{e}^{-\frac{t-t_{\mathrm{R}}}{\tau}} . \tag{2}$$

In this expression, τ is the hydrodynamic residence time of one individual ideal stirred tank reactor, and N is the number of tanks. By fitting one of these models to a measurement signal, all measurement points are considered and the robustness of the overall evaluation is improved significantly [146]. The inspection of the values of the model parameters τ, t_{R}, σ, and N gives an efficient fault analysis. If their values are constant during the process, then the measurements can be deemed trustworthy.

Another method of signal analysis can be performed by the projective reference evaluation (PRE) method. As can be seen in Fig. 5, the array of all measurement points can be arranged as a vector $\vec{m}$. Then one can relate a measurement to a reference signal by:

$$\vec{m} = \lambda \vec{r} + \vec{e} . \tag{3}$$

Here, λ is a proportionality constant, $\vec{r}$ is the reference measurement performed under the same condition as $\vec{m}$, and $\vec{e}$ is the error vector of this model. λ can be calculated by the least squares method:

$$\lambda = \frac{\vec{m}\vec{r}}{\vec{r}\vec{r}} . \tag{4}$$

If $\vec{e}$ is calculated and all of its components that are large (more than twice the standard deviation of all values) are removed from the calculation of λ, then λ is proportional to the analyte concentration. Using this evaluation method, even faulty measurement signals can be quantified reliably. In Fig. 6, an undistorted measurement as well as three different distorted signals can be seen. The distortions have been introduced intentionally to test the evaluation method. This procedure has been carried out with signals from different FIA systems using different analyte concentrations.

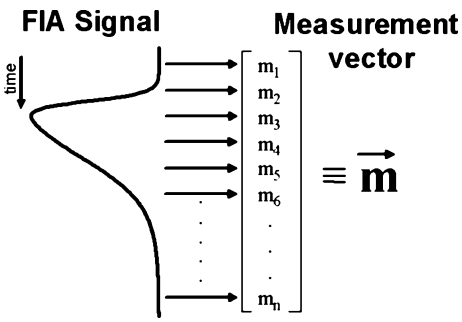

Fig. 5 Representing a FIA measurement signal as a vector

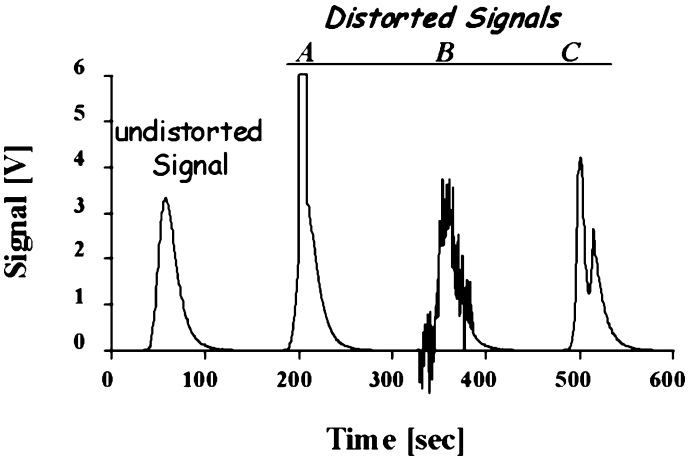

Fig. 6 An undistorted measurement signal as well as three different distorted signals used for the robust evaluation by the projective reference method

The average errors as well as the maximal errors calculated can be seen in Table 1. Although the distortions are significant, the highest maximal error is just 4%, which demonstrates the potential of this simple and fast-to-perform evaluation method. The linear range of the evaluation can also be extended by this method, as can be seen in Fig. 7. Therefore, not only the robustness and reliability is increased by this method, but also the sensitivity of the FIA system.

Since all of the measurement points of the peak-shaped signal are proportional to the analyte concentration, principal component analysis (PCA) and partial least squares (PLS) can be used to improve the evaluation. These data

Table 1 Measurement errors

Analyte		Distortion A	B	C
Glucose (3 measurements each)	Mean error [%]	1.0	0.5	0.8
0.5, 1.0, 1.25, 1.5, 1.75, 2.0 g/L	Max error [%]	3.1	2.3	2.2
Enzyme cartridge, oxygen electrode	Standard dev. [g/L]	0.01440	0.0170	0.0093
Urea (10 measurements each)	Mean error [%]	1.8	0.6	1.7
0.16, 0.31, 0.63, 1.25 g/L	Max error [%]	3.2	0.7	3.2
Enzyme field effect transistor	Standard dev. [g/L]	0.0066	0.0039	0.0064
Penicillin (3 measurements each)	Mean error [%]	1.4	1.1	1.5
1, 2, 3, 4, 5, 6 g/L	Max error [%]	4.0	3.0	3.8
Enzyme optode	Standard dev. [g/L]	0.040	0.027	0.042

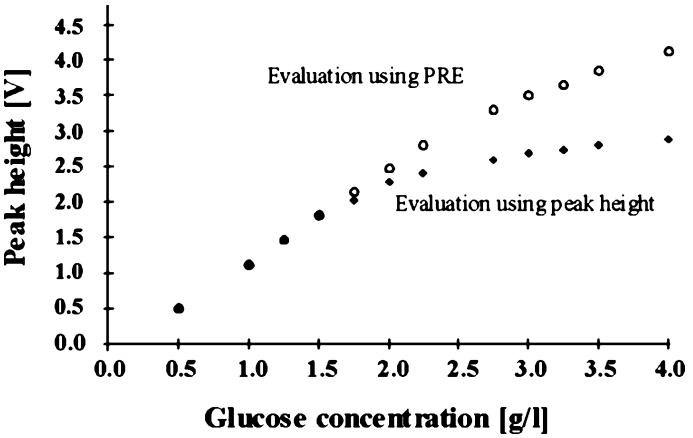

Fig. 7 Using the projective reference evaluation (PRE), the linear range can be extended and therefore the sensitivity of the FIA system as well

compression methods are appropriate if one has to deal with collinearity. In the linear range of FIA systems, the measurement peak represents collinear individual measurements. When both PCA and PLS are applied to a FIA data set, one can see [147] that one principal component can be identified, that most information is around the peak maximum, and that PLS has on average a 3.6% smaller error of prediction than PCA. Although these procedures can extend the range of evaluation beyond that obtained using peak height only, the multivariate calibration procedure requires more standards than univariate techniques. If the measurements are very noisy, an averaging of the measurement information should be performed.

Saurina and Hernandez-Cassou [148] present different chemometric calibration strategies for FIA systems and relate how these techniques can be used to enhance the analytical capacities of conventional systems. Tryzell and Karlberg [149] presented a comparison of various multivariate and univariate techniques. For a two-line gas-diffusion FIA system used to determine iron(II) and ammonium levels, they concluded that PLS regression provided the best overall solution to problems with matrix variations and large concentration ranges, but that in a given situation it is not always superior to the best of the various univariate approaches.

Wu and Bellgardt [150] presented an extended Kalman filter for peak evaluation. The concentration of the analyte and the offset of the baseline are estimated as time-variable parameters by a Kalman filtering procedure. They concluded that good estimates of the measured values are already obtained shortly after the start of the peak and that therefore the measuring dead-time of the FIA system can be reduced by the use of this method.

A knowledge-based system for a rapid detection and diagnosis of faults in FIA systems was proposed by Hitzmann and Brandt [151]. The authors re-

port that transferring the knowledge of an experienced operator into the form of a knowledge-based system and combining it with information received on-line from the recorded detector signal enables automatic operational supervision of FIA systems, which is mandatory if the FIA system is to be used as a process analyzer. This was illustrated by the supervision of an on-line multichannel FIA system for the monitoring of glucose, maltose, polysaccharide, ammonium, and alkaline protease levels [152].

Alvares-Ribeiro and Machado [153] proposed the Youden and Steiner's ruggedness test as a useful technique to ascertain that an optimized system worked properly.

5.2 Increasing the Selectivity of FIA Measurements

Data evaluation methods can also be used to improve the selectivity of FIA measurements. As an example, measurements of penicillin obtained from an enzyme–FET/FIA system are presented in Fig. 8. Enzyme–FET biosensors are based on enzymes that convert the analyte to an acidic or basic product. The resulting pH shift, which can be used to calculate the analyte concentration, is measured by a field effect transistor (FET). As can be seen in Fig. 8, the signal height changes significantly with the pH of the sample solution. Therefore, an evaluation based on the height of the signal is not possible. However, there is also information in the shape of the signal, especially in the leading side of the peak. This characteristic change in the shape of the measurements can be utilized by the data evaluation method to determine the analyte concentration selectively. Here PCA and PLS can be applied as well as artificial neural networks (ANN).

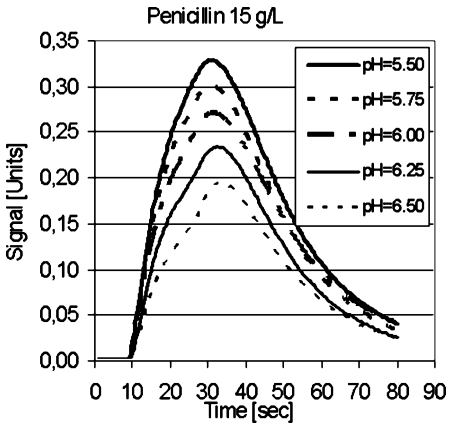

Fig. 8 FIA measurements of penicillin using an enzyme–FET biosensor of samples with different pH values

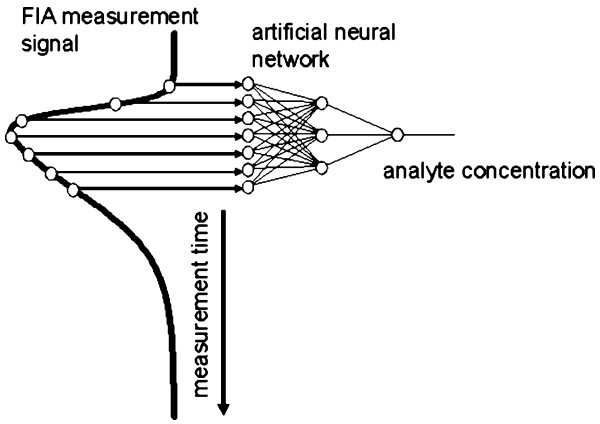

Fig. 9 Evaluation of the raw measurement by using artificial neural networks

As presented in Fig. 9, the raw measurement points of the peak-shaped signal can be used as input nodes of the ANN. The characteristic shaped measurement signal can be used as an input pattern, which can be related to a desired output pattern (the analyte concentration) by adjusting the weighting factors of the ANN. After the network is trained, it can be used to evaluate new measurements. Kullick and Hitzmann [154] demonstrated that for evaluation of the penicillin measurements by ANN, the mean error of both the training and the test sets was 3%.

ANNs have been used to analyze FIA signals in a variety of applications [155–157] and is a reliable method for extending the overall application of this analytical technique. PCA and PLS can also be applied as alternatives to ANN [148, 158, 159].

5.3 Increasing the Stability of FIA Measurements

The stability of measurements is especially important if they are performed on-line and used for control applications. During the control phase, calibration of the FIA system is impossible. A solution to this problem was proposed by Schöngarth and Hitzmann [160]. They presented a multiple injection FIA system complemented with a multivariate evaluation procedure. A standard, the sample, and another standard solution are injected in rapid succession. Due to dispersion, partial mixing of the solutions occurs. However, the overlapping measurement signals can be deconvoluted reliably by employing PLS. The time lost by the rapid threefold injection and the signal evaluation is minimal. Any change in the sensor sensitivity can be detected by this method.

Knowledge about the process under consideration can be used to reduce the noise level in the measured analyte concentration obtained by the FIA

system. This process knowledge can be provided by a Kalman filter, or, since the process model is usually non-linear, an extended Kalman filter [146, 161]. Arndt and Hitzmann [136] presented the combination of a FIA measurement and an extended Kalman filter to reduce the noise of glucose measurements. They did not use a sampling system to obtain cell-free samples and had to perform two individual FIA measurements to determine the glucose concentration. Therefore, the measurement noise was twice that of typical glucose measurements and had to be reduced by the Kalman filter. However, the filtered glucose data could be used to estimate both the biomass concentration and the growth rate. Based on this, a substrate controller could be implemented, which enabled the glucose concentration during yeast cultivations to be controlled at various set points.

5.4
Future Developments in FIA-Based Monitoring and Control

In future application, combinations of all these advanced evaluation techniques will arise and will, therefore, extend the application of flow injection analysis for bioprocess monitoring and control. Not only will the signal evaluation be improved due to intelligent software systems but also the development of FIA systems themselves can be promoted. To develop and optimize the FIA system, experimental design methods will be applied [162–165]. The joint use of mathematical models and optimization algorithm will improve FIA system development for bioprocess monitoring and control.

6
New Control Strategies

Batch or fed-batch suspension culture is still the primary mode of operation in industrial fermentations. Batch cultivation is characterized by the growth of microorganisms without supply of additional substrate after inoculation. The final cell density or biomass concentration is limited by the initial substrate concentration or accumulation of inhibitory metabolites. To overcome these growth limitations, substrates can be added to the fermentation broth during cultivation either stepwise or continuously (fed-batch mode). Different fed-batch strategies are in use, such as maintaining constant or predefined growth rates, maintaining a constant substrate concentration, substrate-limited fed-batch, or pO_2-controlled feeding.

A process of increasing industrial importance is the large-scale production of monoclonal antibodies or therapeutics (EPO, TPA, F VIII, etc.) by mammalian cells [166, 167]. The cells (hybridoma, NS0, rec. CHO, etc.) used in such processes typically exhibit low growth and production rates, and are notoriously sensitive to adverse environmental conditions. Again, the fed-

batch technique is the state of the art; however, existing control concepts are still insufficient. The direct transfer of available control strategies previously established in bioprocess engineering is limited because of changes in cell metabolism during cultivation and the complexity of the cell metabolism itself [168, 169]. In addition, the use of analytical equipment for on-line measurements of relevant variables is problematic because of the complexity of cell growth and insufficient measurement accuracy.

With current technology, only a few state variables, such as the dissolved oxygen concentration, can be measured on-line. Other variables, like substrate concentrations or cell concentrations, can be measured off-line, whereby the results are available for control purposes only after some delay. The results of open-loop control implementations are therefore often not optimal. The control of the feed rate proportional to the oxygen uptake rate of the cells, for example, does not take changes in the ratio between substrate uptake and oxygen consumption rate into account. It can be used to supply the cells with substrates but does not offer optimized control. Providing nutrient feed by linear feed profiles determined via detailed characterization of the cell line or improving a given feed profile by "trial and error" is time-expensive during implementation and lacks transferability to different cell lines. In addition, the performance of two subsequent cultivations is often quite different with respect to the duration of the lag-phase, the growth rates, and the substrate consumption rates. The application of determined feed profiles cannot be adapted to such circumstances. A controller that is intended to to achieve optimized process operation must account for all these difficulties. This results in sophisticated requirements towards adaptability and flexibility of the applied process control strategy.

The model predictive control (MPC) paradigm permits optimal feeding strategies to be implemented in a feedback form, providing that the process states can be measured or can be estimated from on-line measurements [170, 171]. According to the MPC paradigm, the optimal feed may be recomputed on-line each time new state measurements or state estimates become available. In this way, problems associated with uncertain initial conditions may largely be overcome. The use of feedback may also be expected to introduce some measure of robustness with respect to model inaccuracies. Nonetheless, the success of such feed control strategies in practice is liable to depend crucially on the general quality of the model and the ability to identify its parameters from only a few on-line measured data.

6.1 The Open-Loop Feedback Optimal (OLFO) Controller

The open-loop feedback optimal (OLFO) controller is an adaptive model-based controller [172–176]. The controller consists of an identification part, which estimates the states and parameters of the process model using the

available experimental data, and an optimization part, which calculates an optimal control feed trajectory based on the identified model status and a suitable optimization criterion (Fig. 10). At the start of cultivation, a pre-calculated feed trajectory is used that is computed using model parameters obtained from previous experiments and the current initial state data. After a certain time, when new data become available, the combined identification and optimization process is reiterated in order to obtain an updated feed trajectory, which is subsequently passed to the process control system.

Two approaches will be discussed here: a slightly modified OLFO strategy for fed-batch cultures with controlled substrate concentration [177], and an OLFO strategy for fed-batch cultures with optimized time–space yield [178]. Both cases involve the hybridoma cell line IV F 19.23, producing monoclonal IgG antibody against penicillin G amidase [179], which was cultivated in a stirred bioreactor (maximum working volume 1.8 L) [178].

The performance of an OLFO controller depends on the model applied in the identification and the optimization parts. There are three requirements for the application of a model to ensure the operation of the controller in practice. First of all, the model must adequately describe the cultivation (exponential phase, apoptotic phase). Second, the model has to have the ability to fit varying cultivation courses by adaptation of its parameters from only a few on-line measured data [180, 181]. Finally, there is a special characteristic required: on the one hand, only a good prediction quality permits the proper calculation of future feed profiles while, on the other hand, this model still has to allow the fast and safe identification of its parameters.

An unstructured model, consisting of 18 equations and 15 model parameters, was used to describe the cultivation process [177]. The model equations

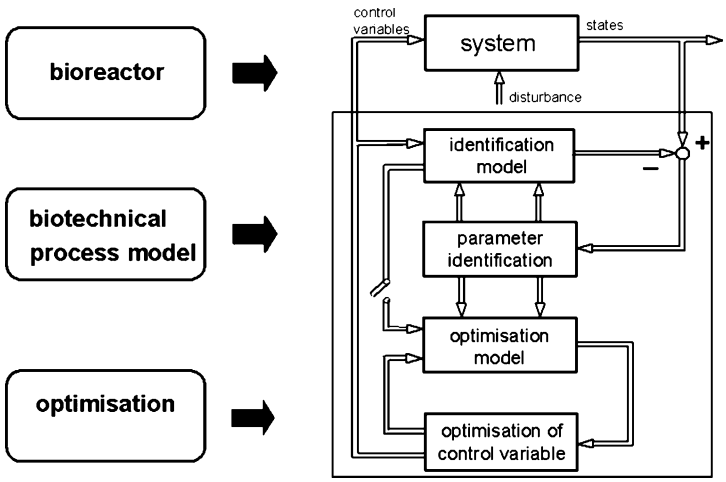

Fig. 10 Structure of open-loop feedback optimal (OLFO) control

were separated into nine coupled differential equations (description of viable and total cell concentration and all major substrate and metabolite concentrations) and nine kinetic equations (description of growth and death kinetic as well as substrate uptake and metabolite production kinetics). For identification of the model parameters, the simplex algorithm by Nelder and Mead was used.

6.2 OLFO Strategy for Fed-Batch Cultures with Controlled Substrate Concentration

The aim of this example of an OLFO-controlled fed-batch experiment was to maintain low levels of the two main substrates, glucose and glutamine, during fed-batch mode (Fig. 11, data from [177]). This is an important aspect due to the fact that the cell-specific production rates of the toxic metabolites, ammonia and lactate, increase with higher concentrations of glucose and glutamine. By maintaining low levels of glucose and glutamine, the time span and the efficiency of cell growth can be improved.

During the OLFO-controlled course of the experiment, the model parameters were adjusted at intervals of 8–16 h. The feed rates of a glucose/amino acid feed and a glutamine feed were calculated for the prediction hori-

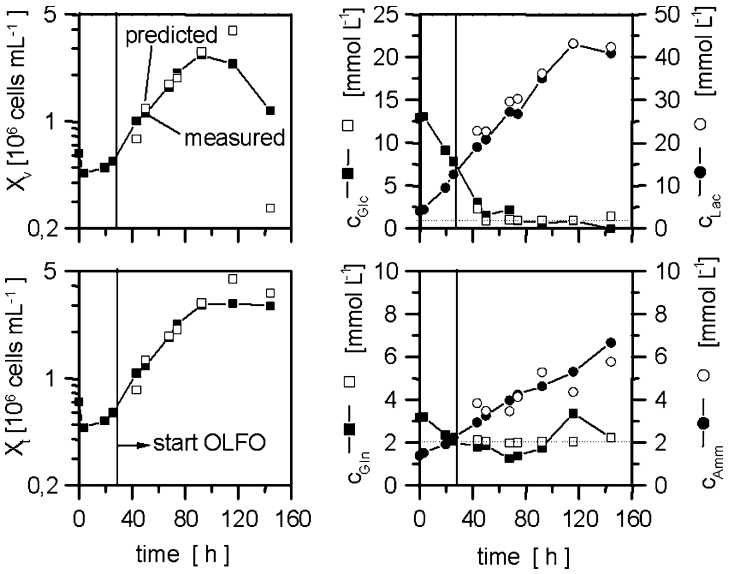

Fig. 11 Fed-batch culture of a hybridoma cell line performed with an OLFO strategy for control of glucose and glutamine concentration. Time course of viable cell concentration X_v, total cell concentration X_t, concentration of glucose c_{Glc}, glutamine c_{Gln}, lactate c_{Lac} and ammonia c_{Amm}. Comparison of measured (*filled symbols*) and predicted (*open symbols*) parameters (data from [177])

zon (8–16 h) to maintain glucose and glutamine concentrations at 1 and 2 mmol L^{-1}, respectively.

The OLFO controller maintained low substrate levels, thereby enabling enhanced cell growth and process efficiency. Important state variables like the concentrations of major substrates (glucose, glutamine) and the concentrations of toxic metabolites (lactate, ammonia) were predicted quite well. In the case of ammonia and glutamine, the measured data were only available after the experiment was been completed. Therefore, no direct feedback to the process, its corresponding model parameters, and the prediction of these concentrations was possible. In spite of this obstacle, the predicted courses of these two concentrations were within a satisfactory range. The course of glutamine and ammonia is of great importance for the process because glutamine is a major substrate, which is metabolized to ammonia at different rates according to its concentration. Ammonia inhibits cell growth at concentrations above 5 mmol L^{-1}, and this eventually led to the end of the cultivation. The final concentration of monoclonal antibodies was 41 mg L^{-1}.

6.3 OLFO Strategy for Fed-Batch Cultures with Optimized Time–Space Yield

Extension of the OLFO concept for optimization of a quality factor time–space requires methods for the calculation of feed trajectories. This was accomplished by defining an optimal control problem with side conditions [178]. The time–space yield of cells produced per unit volume and unit time was chosen as the quality factor. The optimization algorithm had to calculate the feed rates of a glucose/nutrient feed and glutamine feed to achieve the maximum time–space yield. In this case, the side conditions were required to consider minimal and maximal pump rates as well as the maximal culture volume. Furthermore, tolerated ranges for the concentrations of glucose, glutamine, ammonia, and lactate were defined. As the model did not include terms for metabolite inhibition, the concentrations of ammonia and lactate were simply maintained below inhibitory values. For optimization of the feed profiles, the collocation method was used and implemented in the software DIRCOL [182].

An experiment performed with the described optimization strategy is shown in Fig. 12. During cultivation, samples were taken twice per day to determine the concentrations of cells, glucose, and lactate. After each sampling, the OLFO algorithm was implemented, the model parameters were identified, and new feed profiles were calculated. This was repeated six times until the controller determined that the cells had started to undergo apoptosis and the feed rates were set to zero.

The measured values of glucose and glutamine concentration coincided very well with the corresponding predicted profiles. This was especially important for glutamine, as the samples were analyzed for glutamine only after

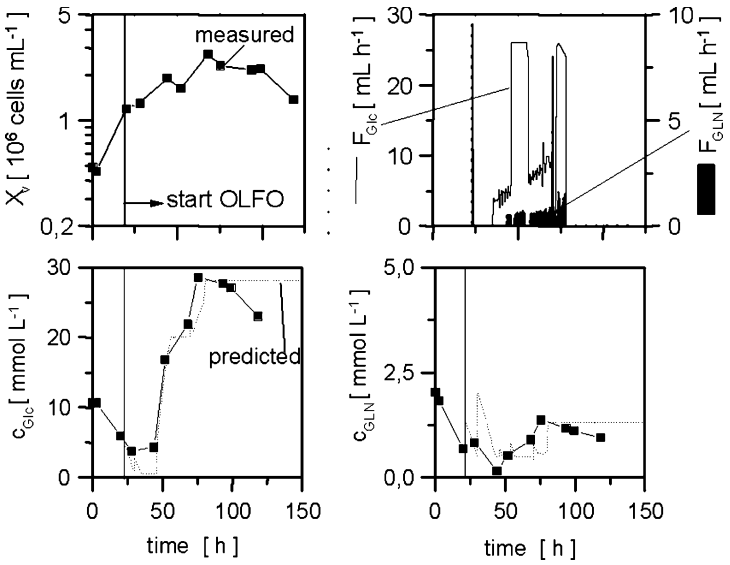

Fig. 12 Fed-batch culture of a hybridoma cell line performed with an OLFO strategy for optimization of time–space yield. Time course of viable cell concentration X_v, concentration of glucose c_{Glc}, glutamine. c_{Gln}, feed rate for nutrient-concentrate-feed F_{Glc} and glutamine F_{Gln}. Comparison of measured (*filled symbols*) and predicted parameters (*dotted line*) [178]

the experiment. The maximal concentrations of ammonia and lactate remained at 4 mmol L^{-1} and 28 mmol L^{-1}, respectively, and were therefore below the set maximum concentrations (4 and 40 mmol L^{-1}, respectively). The final antibody concentration was 57 mg L^{-1}, significantly higher than the concentration of the experiment shown in Fig. 11 (41 mg L^{-1}). The time–space yield of antibody was 70% higher (0.483 mg L^{-1} h^{-1} to 0.285 mg L^{-1} h^{-1}).

6.4 Evaluation of Controller Performance

The OLFO controller maintained predetermined substrate levels, thereby enabling enhanced cell growth and process efficiency. The experiments also demonstrated the high quality of the on-line prediction of important state variables such as the concentrations of major substrates (glucose, glutamine) and the concentrations of toxic metabolites (lactate, ammonia). The comparison of predicted and measured concentrations was very satisfactory, even when measured data were only available after the experiments (glutamine and ammonia).

Table 2 presents cell and antibody concentration data from the experiments discussed here in comparison to conventional control strategies

Table 2 Comparison of cell and product yield for different feeding strategies applied during fed-batch cultures of a hybridoma cell line

Strategy	Max. cell conc. [10^6 cells mL^{-1}]	c_{MAb}/c_{MAb_batch} [a]
Fixed profiles [b]	1.8–3.6	0.7–1.25
OUR [c]	2.8	1.5
"A priori" [d]	3	1
OLFO control [e]	2.7	2.9
OLFO opt. [f]	2.7	4.0

[a] For a batch culture performed in parallel at 2×10^6 cells mL^{-1}
[b] Fixed, predefined feed trajectories, results depending on the predefined trajectory [186]
[c] Control based on oxygen uptake rate (OUR)/substrate uptake rate [186]
[d] Determination of optimized feed trajectory based on a kinetic model [168]
[e] c_{Glc} controlled at 1 mmol L^{-1}, c_{Gln} controlled at 2 mmol L^{-1} [177]
[f] Optimized time–space yield (cells) [178]

(adapted from [183]). An evaluation of the data must take into account the fact that the experiments were performed with the same cell line over a period of several years, and therefore changes in the physiology of the cells cannot be excluded. It was confirmed in batch experiments that the growth characteristics (growth rate, maximum cell concentration, cell specific uptake rates for glucose, glutamine and amino acids, and specific production rates for ammonia and lactate) showed no significant changes during this time (data not shown). In addition, it was shown that these parameters were the same in the different media used [184]. Since the specific productivity for monoclonal antibodies varied significantly with time, Table 2 displays the antibody concentration related to the concentration obtained in a batch experiment performed in parallel to the respective fed-batch culture.

Data from the cultivations in Table 2 confirm that the success of fixed feed trajectories depends on the combination of the best trajectory with adequate and reproducible culture conditions. With respect to the maximum cell concentration, the concept of fixed feed trajectories shows the highest results in the case of the best combination, but in the case of a bad choice for the feed trajectories, the cell concentration was in the same range as for a batch experiment. For the other quantities, the cell concentration was in the same range, showing no significant difference between the applied feeding strategies. However, the product yield (related to antibody concentration) was two to three times higher for the OLFO experiment with controlled substrate concentrations, and up to four times higher for the OLFO experiment with optimized time–space yield. The main reason for the higher productivity is thought to be the prolonged duration of the culture, which was possible due to the adaptive character of the OLFO strategy.

A recommendation based on the comparison of different control strategies would favor fixed feed trajectories for an established process with a well characterized and stable production cell line. An adaptive, model-based control strategy could be the method of choice during cell line development or for rapid production of small amounts of product for clinical trials. This is due to its universal character and because it does not require intensive process development. Instead, only a few batch experiments are required to adapt the kinetic model. Since the OLFO controller can detect changes in the cell metabolism during a culture, it can adapt the model parameters and the feed trajectories.

These studies illustrate that the adaptive, model-based OLFO controller is a valuable tool for the control of fed-batch hybridoma cell cultures. Using an unstructured process model and an OLFO strategy provides automated, user-guided process control and various possibilities for process fine tuning. Due to its universal character, it can be transferred to different cell lines or adapted to different conditions (e.g., enhanced fed-batch cultivation using dialysis [185]). Sophisticated optimization criteria [178] can be implemented.

7 Conclusion

In the past few years, the basis for the application of biotechnological processes has improved, both in research and in industrial production. New experimental tools (screening methods and metabolic engineering), global analysis methods (genomics, proteomics, and metabolomics), and bioinformatics tools are increasingly available. These new methodologies make it possible to reduce the time required to develop and establish new industrial biotechnological products and processes; this was hitherto one of the major drawbacks of biotechnological, as opposed to chemical, processes. To be able to improve already existing processes, or to establish new processes in industry, a better understanding of the behavior of microorganisms, cells, and enzymes is necessary. Using innovative bioanalytical systems, such as the sensors described here together with new control strategies, can help to fulfil these goals.

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