1
History of Industrial Biotransformations – Dreams and Realities

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Throughout the history of mankind, microorganisms have been of enormous social and economic importance. Without even being aware of their existence, very early on in history man was using them in the production of food and beverages. The Sumerians and Babylonians were practising the brewing of beer before 6000 BC, references to wine making can be found in the Book of Genesis and the Egyptians used yeast for baking bread. However, knowledge of the production of chemicals such as alcohols and organic acids through fermentation is relatively recent and the first reports in the literature only appeared in the second half of the 19th century. Lactic acid was probably the first optically active compound to be produced industrially by fermentation. This was accomplished in the USA in 1880 [1]. In 1921, Chapman reviewed a number of early industrial fermentation processes for organic chemicals [2].

In the course of time, it was discovered that microorganisms could modify certain compounds by simple, chemically well defined reactions, which were further catalyzed by enzymes. Nowadays, these processes are called "biotransformations". The essential difference between fermentation and biotransformation is that there are several catalytic steps between the substrate and the product in a fermentation while there are only one or two in a biotransformation. The distinction is also in the fact that the chemical structures of the substrate and the product resemble one another in a biotransformation, but not necessarily in a fermentation.

1.1
From the "Flower of Vinegar" to Recombinant *E. Coli* – The History of Microbial Biotransformations

The story of microbial biotransformations is closely associated with vinegar production which dates back to around 2000 years BC.

Vinegar production is perhaps the oldest and best known example of microbial oxidation, which can illustrate some of the important developments in the field of biotransformations by living cells (Fig. 1.1).

Since ancient times, man has wanted to see things that are far smaller than can be perceived with the naked eye. In the 16th century this led to the construction of a magnifier

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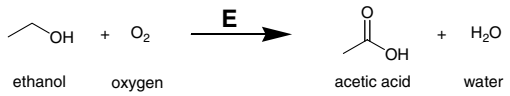


Fig. 1.1 Vinegar production.

consisiting of a single convex lens, and this, in turn, led eventually to the development of the microscope (Fig. 1.2). Antony von Leeuwenhoek (1632–1723) became the first person, or microscopist [3], to make and use a real microscope. He described microorganisms including bacteria, algae and protozoa in fresh water (Fig. 1.3). In fact he constructed a total of 400 microscopes during his lifetime. Subsequently, the compound microscope system was invented in the 17th century. This type of microscope, incorporating more than one lens, has made tremendous contributions to the progress of science. Using this microscope Hooke (1635–1703) discovered the fact that living things are composed of cells, and later on Pasteur, among others, discovered yeast fungus. The microscope has possibly had a greater impact on the development of knowledge than any other scientific instrument in history [4]. The discovery of new microscopic life was the starting point for experimental biology as a basis for the development of the biotransformations.

A prototype bioreactor with immobilized bacteria has been known in France since the 17th century. The oldest bioreactor to use immobilized living microorganisms, a so-called generator, was developed in 1823 [5, 6]. Even today, acetic acid is still known as "vinegar" if it is obtained by oxidative fermentation of ethanol-containing solutions by acetic acid bacteria [7].

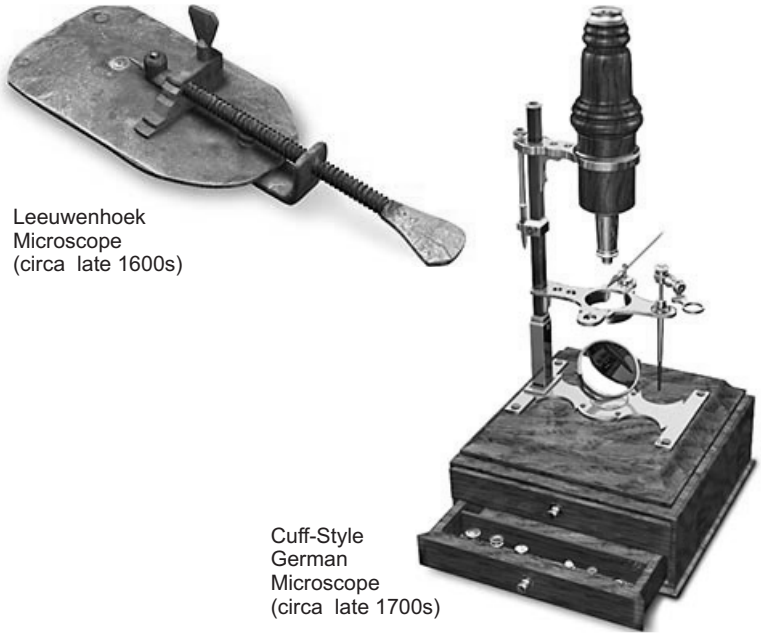


Fig. 1.2 Historical microscopes (photographs courtesy of Michael W. Davidson).

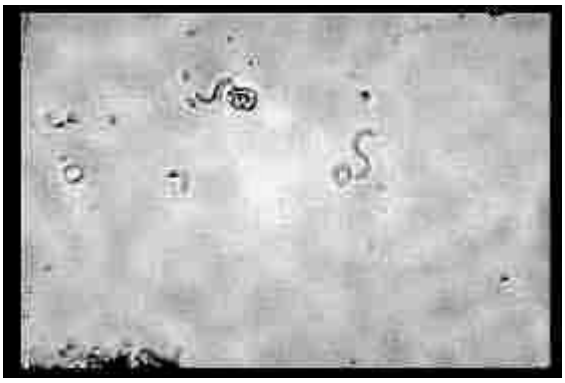
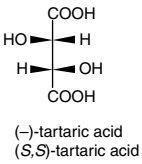


Fig. 1.3 Spiral bacteria (photograph courtesy of Michael W. Davidson).

In 1858, Pasteur [8] was the first to demonstrate the microbial resolution of tartaric acid. He performed fermentation of the ammonium salt of racemic tartaric acid, mediated by the mold *Penicillium glaucum*. The fermentation yielded (–)-tartaric acid (Fig. 1.4).



(–)-tartaric acid
(*S*,*S*)-tartaric acid

Fig. 1.4 Pasteur's product of the first resolution reaction.

This was also the first time that a method was used where the microorganisms degraded one enantiomer of the racemate while leaving the other untouched.

In 1862, Pasteur [9] investigated the conversion of alcohol into vinegar and concluded that pellicle, which he called "the flower of vinegar", "served as a method of transport for the oxygen in air to a multitude of organic substances".

In 1886 Brown confirmed Pasteur's findings and gave the causative agent in vinegar production the name *Bacterium xylinum*. He also found that it could oxidize propanol to propionic acid and mannitol to fructose (Fig. 1.5) [10].

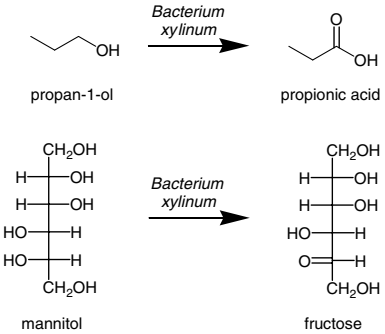
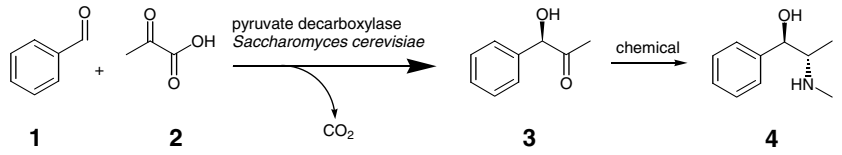


Fig. 1.5 Reactions catalyzed by *Bacterium xylinum*, the vinegar biocatalyst.

In 1897 Buchner [11] reported that cell-free extracts prepared by grinding yeast cells with sand could carry out alcoholic fermentation reactions (breaking down glucose into ethanol and carbon dioxide) in the absence of living cells. This initiated the use of resting cells for biotransformations.

In 1921 Neuberg and Hirsch [12] discovered that the condensation of benzaldehyde with acetaldehyde in the presence of yeast forms optically active 1-hydroxy-1-phenyl-2-propanone (Fig. 1.6).



1 = benzaldehyde
2 = 2-oxo-propionic acid
3 = 1-hydroxy-1-phenylpropan-2-one
4 = 2-methylamino-1-phenylpropan-1-ol

Fig. 1.6 L-Ephedrine production.

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Tag der Bekanntmachung über die Erteilung des Patents: 24. März 1932

Knoll A.-G. Chemische Fabriken in Ludwigshafen a. Rh., Dr. Gustav Hildebrandt und Dr. Wilfrid Klavehn in Mannheim

Verfahren zur Herstellung von l-1-Phenyl-2-methylaminopropan-1-ol

Patentiert im Deutschen Reiche vom 9. April 1930 ab

Racemisches 1-Phenyl-2-methylaminopropan-1-ol kann bereits nach verschiedenen Verfahren synthetisch hergestellt werden (vgl. z. B. Nagai u. Kanao, Annalen 470 [1929], S. 157; Patentschrift 469 782; Skita u. Keil, Ber. 62 [1929], S. 1142 ff.; Patentschrift 524 806).

Das racemische 1-Phenyl-2-methylaminopropan-1-ol läßt sich nach bekannten Verfahren (vgl. Nagai u. Kanao, Annalen 470 [1929], S. 157; britische Patentschrift 297 385) in seine optischen Isomeren spalten. Das bei der Spaltung entstehende l-1-Phenyl-2-methylaminopropan-1-ol ist identisch mit dem natürlichen Ephedrin und wird neuerdings therapeutisch erfolgreich verwendet.

Bisher ist jedoch kein Verfahren bekannt geworden, nach welchem l-1-Phenyl-2-methylaminopropan-1-ol auf unmittelbarem Wege dargestellt werden kann.

Es wurde nun gefunden, daß man mit guten Ausbeuten unmittelbar zum l-1-Phenyl-2-methylaminopropan-1-ol gelangt, wenn man links drehendes Phenylpropanolon (Neuberg, Biochem. Zeitschrift 115 [1921], S. 282 ff., und 128 [1922], S. 610 ff.) in Gegenwart von Methylamin der Reduktion unterwirft.

Die Bildung von l-1-Phenyl-2-methylaminopropan-1-ol war keineswegs vorauszusehen, da bekanntlich Abwandlungen von optisch aktiven Verbindungen nicht notwendig zu Verbindungen von optisch gleicher Drehungsrichtung führen müssen, sondern ebensohäufig zu solchen der entgegengesetzten Drehungsrichtung führen können (so entsteht z. B. l-Mandelsäure aus d-Benzaldehydcyanhydrin bei der Verseifung).

Ferner ist hervorzuheben, daß in der vorliegenden Erfindung eine asymmetrische Synthese vorliegt, bei welcher wiederum nicht vorauszusehen war, welche von den möglichen Konfigurationen entstehen würde. Es stand zu erwarten, daß sowohl d- oder l- oder dl-1-Phenyl-2-methylaminopropan-1-ol als auch d- oder l- oder dl-Pseudo-1-Phenyl-2-methylaminopropan-1-ol oder endlich ein Gemisch von mehreren dieser Komponenten entstehen würde. Daß bei der Reduktionskondensation des l-Phenylpropanolons mit Methylamin fast ausschließlich l-1-Phenyl-2-methylaminopropan-1-ol entsteht, war daher durchaus überraschend.

Die Erhaltung der optischen Aktivität war auch deswegen überraschend, weil auf Grund der Neubergschen Beobachtung (Biochem. Zeitschrift 128 [1922], S. 613) l-Phenylpropanolon sich bereits in verdünnt alkalischer Lösung in kurzer Zeit racemisiert. Da bei dem Verfahren der vorliegenden Erfindung die Reduktion in alkalischer Lösung stattfindet, war vorwiegend mit der Bildung von racemischen Basen zu rechnen.

Das Verfahren stellt eine neue Methode dar, um das links drehende Phenylpropanolon in Form von l-1-Phenyl-2-methylaminopropan-1-ol nutzbringend zu verwerten.

Diese unmittelbare Synthese des l-1-Phenyl-2-methylaminopropan-1-ols hat ferner den Vorzug, daß kein therapeutisch wertloses d-1-Phenyl-2-methylaminopropan-1-ol anfällt, wie es bei den bekannten Spaltungsverfahren des Racemkörpers der Fall ist.

Beispiel 1

120 g des durch Ätherauszug gewonnenen phenylpropanolonhaltigen Gärungsproduktes (vgl. Biochem. Zeitschrift 115 [1921], S. 282 ff.) läßt man ohne weitere Reinigung in eine Lösung von 10 g Methylamin in 500 ccm Äther in Gegenwart von 20 g aktiviertem Aluminium unter Rühren im Verlaufe von 2 Stunden eintropfen. Gleichzeitig läßt man 20 bis 30 g Wasser tropfenweise zufließen. Die sofort heftig einsetzende Umsetzung wird zeitweilig durch Kühlung gemäßigt. Nach beendigter Reduktion wird der filtrierten ätherischen Lösung die entstandene optisch aktive Base mit verdünnter Säure entzogen. Die Aufarbeitung erfolgt in bekannter Weise.

Man erhält das Hydrochlorid des l-1-Phenyl-2-methylaminopropan-1-ols vom F. 214°, welches die aus der Literatur bekannte Linksdrehung zeigt. Die Ausbeute beträgt je nach Art des verwendeten Ausgangsstoffes 25 bis 45 g Hydrochlorid.

Beispiel 2

360 g des in Beispiel 1 verwendeten phenylpropanolonhaltigen Ätherauszuges werden unter vermindertem Druck destilliert. 300 g der bei 100 bis 150° unter 14 mm Druck übergehenden Fraktion werden in Gegenwart von kolloidalem Platin (70 ccm 1%ige Lösung) und 85 g 33%iger Methylaminlösung der katalytischen Reduktion unterworfen. Es ist vorteilhaft, etwas Äther zuzusetzen. Nach Beendigung der Wasserstoffaufnahme wird die ätherische Lösung mit Salzsäure ausgeschüttelt und das l-1-Phenyl-2-methylaminopropan-1-ol in bekannter Weise abgetrennt.

Das Hydrochlorid schmilzt bei 214° und zeigt die aus der Literatur bekannte Linksdrehung. Die Ausbeute an Hydrochlorid beträgt 110 g.

Beispiel 3

100 g nach Neuberg (Biochem. Zeitschrift 128 [1922], S. 611) abgetrenntes l-1-Phenylpropan-1-ol-2-on werden in 200 ccm Äther gelöst, mit 75 g 33%iger Methylaminlösung versetzt und etwa eine halbe Stunde lang geschüttelt. Hierbei findet unter Wärmeentwicklung Kondensation statt. Anschließend wird in Gegenwart von 70 ccm 1%iger kolloidaler Platinlösung mit Wasserstoff reduziert.

Die Aufarbeitung geschieht nach Beispiel 2.

Das Hydrochlorid des l-1-Phenyl-2-methylaminopropan-1-ols kristallisiert aus Alkohol in derben Prismen vom F. 214 bis 216°. Der F. der freien Base liegt bei 40°.

Patentansprüche:

1. Verfahren zur Darstellung von l-1-Phenyl-2-methylaminopropan-1-ol, dadurch gekennzeichnet, daß man links drehendes l-Phenylpropan-1-ol-2-on mit Methylamin kondensiert und das Kondensationsprodukt gleichzeitig oder nachträglich mit Reduktionsmitteln, wie aktiviertem Aluminium in Gegenwart von Wasser oder Wasserstoff in Gegenwart eines Platinkatalysators, behandelt.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß man — unter Umgehung der Reindarstellung des l-Phenylpropanolons — Destillate oder Auszüge aus l-Phenylpropanolon enthaltenden Gemischen verwendet, wie sie z. B. bei der Vergärung von Zuckern oder von zuckerhaltigen Produkten in Gegenwart von Benzaldehyd entstehen.

Fig. 1.7 Knoll's patent of 1930.

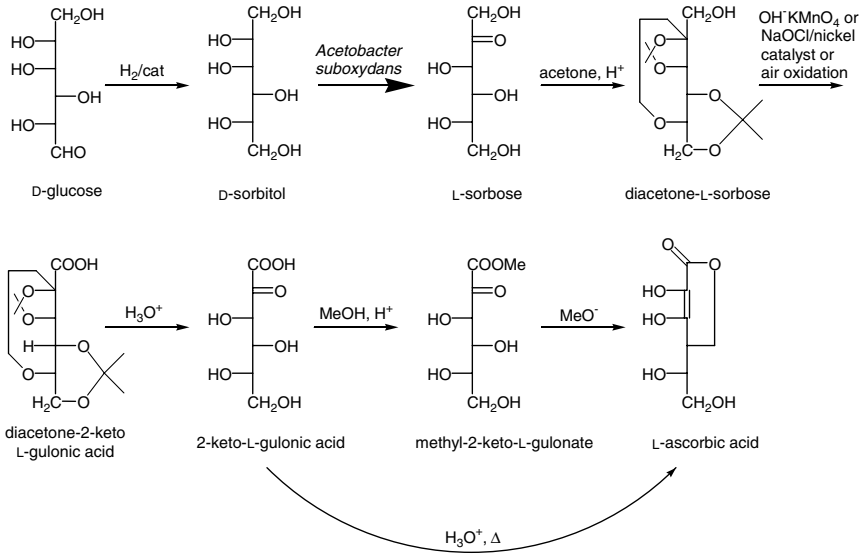

Fig. 1.8 Reichstein–Grüssner synthesis of vitamin C (L-ascorbic acid).

The compound obtained was later chemically converted into L-(–)ephedrine by Knoll AG, Ludwigshafen, Germany in 1930 (Fig. 1.7) [13].

The bacterium *Acetobacter suboxydans* was isolated in 1923 [14]. Its ability to carry out limited oxidations was utilized in a highly efficient preparation of L-sorbose from D-sorbitol (Fig. 1.8).

L-Sorbose became important in the mid-1930s as an intermediate in the Reichstein–-Grüssner synthesis of L-ascorbic acid [15].

In 1953, Peterson at al. [16] reported that *Rhizopus arrhius* could convert progesterone into 11α-hydroxyprogesterone (Fig. 1.9), which was used as an intermediate in the synthesis of cortisone.

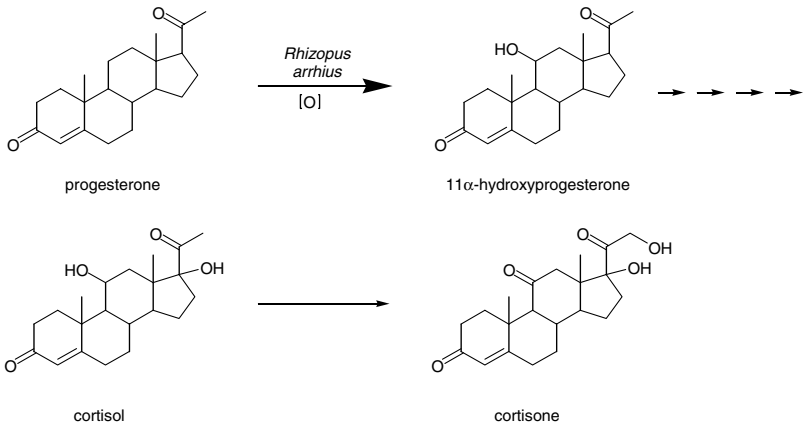

Fig. 1.9 Microbial 11α-hydroxylation of progesterone.

This microbial hydroxylation simplified and considerably improved the efficiency of the multi-step chemical synthesis of corticosteroid hormones and their derivatives. Although the chemical synthesis [17] (Fig. 1.10) from deoxycholic acid developed at Merck, Germany, was workable, it was recognized that it was complicated and uneconomical: 31 steps were necessary to obtain 1 kg of cortisone acetate from 615 kg of deoxycholic acid. The microbial 11α-hydroxylation of progesterone rapidly reduced the price of cortisone from \$200 to \$6 per gram. Further improvements have led to a current price of less than \$1 per gram [18].

In the 1950s the double helix structure and the chemical nature of RNA and DNA – the genetic code for heredity – were discovered by Watson and Crick [19]. Beadle and Tatum [20] received the Nobel Prize in 1958 for concluding that the characteristic function of the gene was to control the synthesis of a particular enzyme. They exposed the red bread mold, *Neurospora crassa*, to X-rays and studied the altered nutritional requirements of the mutants thus produced. These experiments enabled them to conclude that each gene determined the structure of a specific enzyme which, in turn, allowed a single chemical reaction to proceed. A basic hypothesis arose out of this work: one gene specifies the production of one enzyme thus the "one gene–one enzyme" hypothesis. Lederberg [21] shared the Nobel Prize with Beadle and Tatum in 1958 for his discoveries concerning genetic recombination and the organization of the genetic material of bacteria. The Beadle, Tatum and Lederberg discoveries can be regarded as milestones among the main scientific achievements of the 20th century. This led to the synthesis of recombinant DNA and gave a fillip to genetic engineering in the 1970s. In 1973, Cohen and Boyer [22] discovered recombinant DNA technology. They observed that genes from any biological species could be propagated and cloned in foreign cells by linking them to DNA molecules that posses the capacity to replicate in the intended host. Such developments quickly made the recombinant DNA technology part of industrial microbial transformations. Application of this technology for the production of small molecules began in 1983. Ensley et al. [23] reported on the construction of a strain of *E.coli* that excreted indigo, one of the oldest known dyes. They found that the entire pathway for conversion of napthalene into salicylic acid is encoded by the genes of *Pseudomonas putida*. These genes can be expressed in *E. coli*. Their results led to the unexpected finding that a subset of these genes was also responsible for the microbial production of indigo. Moreover, they have shown that indigo formation was a property of the dioxygenase enzyme system that forms *cis*-dihydrodiols from aromatic hydrocarbons. Finally, they proposed a pathway for indigo biosynthesis in a recombinant strain of *E. coli* (Fig. 1.11).

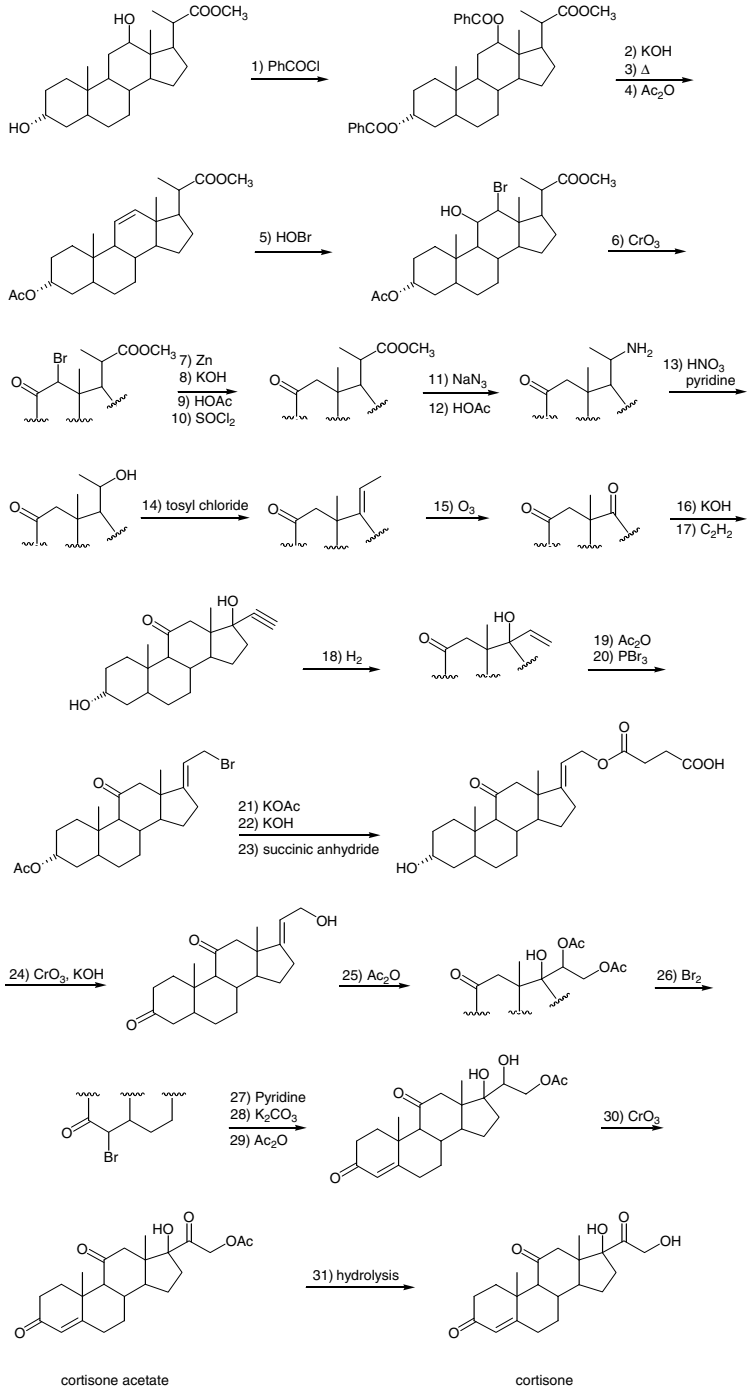

Fig. 1.10 Chemical synthesis of cortisone.

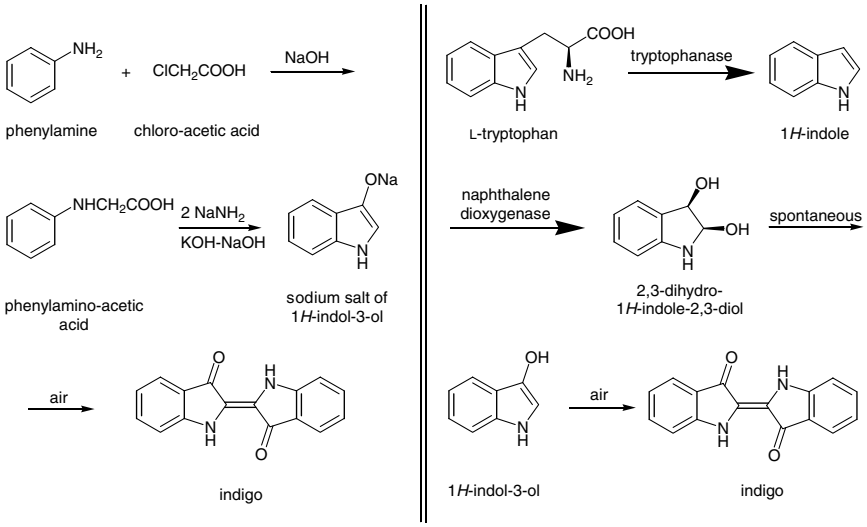

Fig. 1.11 Comparison of the chemical and biological routes to indigo.

Genencor Internationaol is developing a commercially competitive biosynthetic route to indigo using recombinant *E. coli*, which can synthesize indigo directly from glucose [24]. At the neutral pH of the fermentation the indigo precursor indoxyl yields isatin as a significant by-product. An enzyme that hydrolyzes isatin to isatic acid has been identified. After cloning and incorporating the new enzyme in the production strain, the indigo product performed equally well as the indigo produced chemically [25].

In 1984 Novozymes developed the first enzyme from a genetically modified organism for use in the starch industry – a maltogenic amylase, still marketed today under the name Maltogenase® [26].

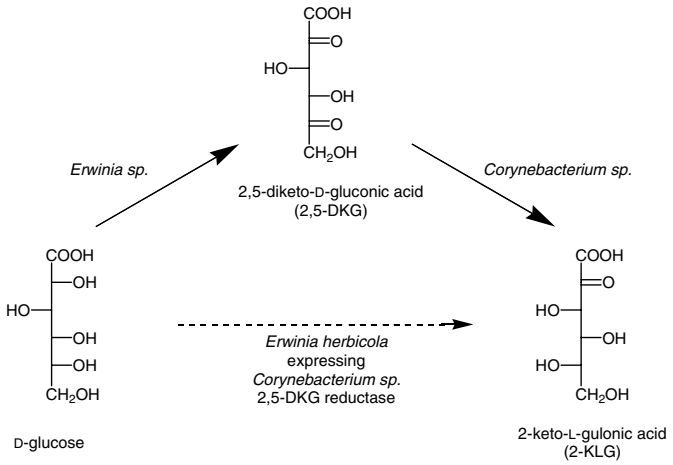

Fig. 1.12 Biosynthesis of 2-keto-L-gulonic acid.

Anderson et al. [27] reported in 1985 on the construction of a metabolically engineered bacterial strain that was able to synthesize 2-keto-L-gulonic acid (Fig. 1.12), a key intermediate in the production of L-ascorbic acid (vitamin C).

BASF, Merck and Cerestar have built a ketogulonic acid plant in Krefeld, Germany. The operation started up in 1999. They developed a new fermentation route from sorbitol directly to ketogulonic acid [28]. This method is probably similar to that described in 1966 [29].

The vision of manufacturing L-ascorbic acid directly by fermentation without the need to isolate 2-keto-L-gulonic acid (2-KLG) has remained elusive. Nevertheless, efforts to this end are ongoing at Genencor International [30].

The Cetus Corporation (Berkeley, CA, USA) bioprocess for converting alkenes into alkene oxides emerged in 1980 [31]. This bioprocess appeared to be very interesting, because of the possibility of replacing the energy-consuming petrochemical process.

There were high hopes that the development of recombinant DNA technology would speed up technological advances. Unfortunately, there is still a great deal of work to be done on the development and application of bioprocesses before the commercial production of low-cost chemicals becomes feasible [32]. The development of some of the flagship bioprocesses of today took between 10 and 20 years: the development of the acrylamide process took 20 years and the Lonza process for L-carnitine 15 years [33]. However, today even the traditional chemical companies such as Dow Chemicals, DuPont, Degussa-Hüls AG, etc., under pressure from investors and because of technological advances, are trying to use microbial or enzymatic transformations in their production processes. This is because they need to establish whether natural feedstocks can provide more advantages than crude oil. One only needs to compare the cost of a barrel of oil to that of corn starch to see that the latter is considerably cheaper [28].

Tepha Inc. (Cambridge, MA, USA) currently produces poly-4-hydroxybutyrate (known commercially as PHA4400) (Fig. 1.13) for medical applications, using a proprietary transgenic fermentation process that has been specifically engineered to produce this homopolymer. During the fermentation process, poly-4-hydroxybutyrate accumulates inside the fermented cells as distinct granules. The polymer can be extracted in a highly pure form from the cells at the end of the fermentation process. The heart of the process is the genetically engineered *Escherichia coli* K12 microorganism, incorporating new biosynthetic pathways to produce poly-4-hydroxybutyrate [34].

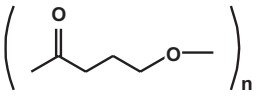

Fig. 1.13 Chemical structure of poly-4-hydroxybutyrate.

A range of polyhydroxyalkanoates with 0–24% hydroxyvalerate have been produced under the trade name of "Biopol" by Zeneca Bio Products and other manufacturers [35]. However, the polyhydroxyalkanoates production price is way above the market price of conventional plastics ($16 per kilogram for "Biopol" against $1 per kilogram for oil-derived plastics). Potentially, the production cost can be lowered by process scale-up, to around $8 per kilogram and by use of recyclable waste material as the substrates [35].

More recently, researchers from the company DSM succeeded in combining enzymatic ring opening polymerization and chemical nitroxide mediated living free radical polymerization. This genuine one-pot reaction is a method for the synthesis of block copolymers in a metal-free fashion. After proving the principle they are extending their chemo-enzymatic polymerization approach to obtain new functional polymers based on new raw materials, and to develop the technology further towards a kinetic resolution polymerization [36].

Acrylamide is one of the most important commodity chemicals with a global consumption of about 200 000 tonnes per year. It is required in the production of various polymers for use as flocculants, additives or for petroleum recovery. In conventional syntheses, copper salts are used as catalysts in the hydration of nitriles. However, this is rather disadvantageous as the preparation of the catalysts is fairly complex. In addition, it is difficult to regenerate the used catalyst and to separate and purify the acrylamide formed. Furthermore, as acrylamides are readily polymerized, their production under moderate conditions is highly desirable. In contrast to the conventional chemical process, there is no need to recover unreacted acrylonitrile in the enzymatic process, because the conversion and yield of the enzymatic hydration process are almost 100%. The removal of the copper ions from the product is no longer necessary. Overall, the enzymatic process – being carried out below 10 °C under mild reaction conditions and requiring no special energy source – proves to be simpler and more economical. The immobilized cells are used repeatedly and a very pure product is obtained. The enzymatic process, which was first implemented in 1985, is already producing about 6000 tonnes of acrylamide per year for Nitto [37, 38]. The use of a biotacalyst for the production of acrylamide may be not the first case of biotransformation being used as part of a biotechnological process in the petrochemical industry. However, it is the first example of the successful introduction of an industrial biotransformation process for the manufacture of a commodity chemical (Fig. 1.14).

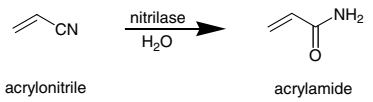

Fig. 1.14 Acrylamide synthesis.

Improvements to the production of 1,3-propanediol, a key component of an emerging polymer business, have been realized. By utilizing genes from natural strains that produce 1,3-propanediol from glycerol, metabolic engineering has enabled the development of a recombinant strain that utilizes the lower cost feedstock D-glucose [39].

Some representative industrial microbial transformations are listed in Table 1.1.

Tab. 1.1 Some representative industrial biotransformations catalyzed by whole cells.

Product	Biocatalyst	Operating since	Company
vinegar	bacteria	1823	various
L-2-methylamino-1-phenylpropan-1-ol	yeast	1930	Knoll AG,Germany
L-sorbose	Acetobacter suboxydans	1934	various
prednisolone	Arthrobacter simplex	1955	Shering AG, Germany
L-aspartic acid	Escherichia coli	1958	Tanabe Seiyaku Co., Japan
7-ADCA	Bacillus megaterium	1970	Asahi Chemical Industry, Japan
L-malic acid	Brevibacterium ammoniagenes	1974	Tanabe Seiyaku Co., Japan
D-p-hydroxyphenylglycine	Pseudomonas striata	1983	Kanegafuchi, Chemical Co., Japan
acrylamide	Rhodococcus sp.	1985	Nitto Chemical Ltd, Japan
D-aspartic acid and L-alanine	Pseudomonas dacunhae	1988	Tanabe Seiyaku Co., Japan
L-carnitine	Agrobacterium sp.	1993	Lonza, Czech.Rep.
2-keto-L-gulonic acid	Acetobacter sp.	1999	BASF, Merck, Cerester, Germany

1.2 From Gastric Juice to SweetzymeT – The History of Enzymatic Biotransformations

Enzymes had been in use for thousands of years before their nature became gradually understood. No one really knows when a calf stomach was used for the first time as a catalyst in the manufacture of cheese.

As early as 1783, Spallanzani showed that gastric juice secreted by cells could digest meat *in vitro*. In 1836, Schwan called the active substance pepsin [40]. The French scientist Payen [41] isolated an enzymatic complex from malt in 1833, naming it "diastase". Diastase, the enzyme that catalyzes the breakdown of starch into glucose, was the first enzyme to be discovered. In 1876, Kühne (Fig. 1.15) presented a paper to the Heidelberger Natur-Historischen und Medizinischen Verein, suggesting that such non-organized ferments should be called *e n z y m e s* [42]. At that time two terms were used: "organized ferment", such as the cell-free yeast extract from Büchner, and "unorganized ferment", such as the gastric juice secreted by cells. Today the terms "intracellular" and "extracellu-

lar" are used. Kühne also presented some interesting results from his experiments with trypsin. The word "enzyme" comes from Greek for "in yeast" or "leavened" [43].

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Ueber das Verhalten verschiedener organisirter und sog. ungeformter Fermente.

Ueber das Trypsin (Enzym des Pankreas).

Von **W. Kühne.**

1876

Ueber das Verhalten verschiedener organisirter und sog. ungeformter Fermente.

Sitzung am 4. Februar 1876.

Hr. W. Kühne berichtet über das Verhalten verschiedener organisirter und sog. ungeformter Fermente. Um Missverständnissen vorzubeugen und lästige Umschreibungen zu vermeiden schlägt Vortragender vor, die ungeformten oder nicht organisirten Fermente, deren Wirkung ohne Anwesenheit von Organismen und ausserhalb derselben erfolgen kann, als *Enzyme* zu bezeichnen. — Genauer untersucht wurde besonders das Eiweiss verdauende Enzym des Pankreas, für welches, da es zugleich Spaltung der Albuminkörper veranlasst, der Name *Trypsin* gewählt wurde. Das Trypsin vom Vortr. zuerst dargestellt und zwar frei von durch dasselbe noch verdaulichen und zersetzbaren Eiweissstoffen, verdaut nur in alkalischer, neutraler, oder sehr schwach sauer reagirender Lösung. Dasselbe wird durch nicht zu kleine Mengen Salicylsäure, welche das Enzym in bedeutenden Quantitäten löst, bei 40° C. gefällt, ohne dabei seine specifische Wirksamkeit zu verlieren. Wird die Fällung in Sodalösung von 1 pCt. gelöst, so verdaut sie höchst energisch unter Bildung von Pepton, Leucin, Tyrosin u. s. w. Nur übermässiger Zusatz von Salicylsäure bis zur Bildung eines dicken Krystallbreies vernichtet die enzymotischen Eigenschaften. Dies Verhalten war kaum zu erwarten, seit Kolbe und J. Müller die hemmende, selbst vernichtende Wirkung kleiner Mengen Salicylsäure auf einige Enzyme hervorgehoben hatten. Die Beobachtungen des Vortr., der ausser dem Trypsin noch das Pepsin eingehender untersuchte, stehen jedoch mit den Angaben von J. Müller, nach welchen Salicylsäure bei einem Gehalte der

Fig. 1.15 W.F. Kühne [42].

Microorganisms synthesize numerous enzymes, each one having a specific function. Intracellular enzymes operate inside the cell in a protected and highly structured environment, while extracellular enzymes are secreted from the cell, thus working in the medium surrounding the microorganism.

The commercial usage of extracellular microbial enzymes started in the West around 1890, thanks to the Japanese entrepreneur Takamine. He settled down in the USA and started an enzyme factory based on Japanese technology. The principal product was called takadiastase. This was a mixture of amylolytic and proteolytic enzymes prepared by cultivation of *Aspergillus oryzae*. In France, Boidin and Effront developed bacterial enzymes in 1913. They found that the hay bacillus, *Bacillus subtilis,* produces an extremely heat-stable α-amylase when grown in still cultures on a liquid medium prepared by extraction of malt or grain [44].

In 1892, in a study of the rate of fermentation of sucrose in the presence of yeast, the British chemist Brown found that the rate seemed to be independent of the amount of sucrose present [45]. He later suggested that this result could be explained if the invertase molecules present in the yeast formed an addition complex with sucrose [46]. This was the first time that the existance of an enzyme–substrate complex had been deduced from the kinetics of an enzyme reaction [47].

As part of his studies on sugars, in 1894 Emil Fischer [48, 49] observed that the enzyme known as emulsin catalyzes the hydrolysis of β-methyl-D-glucoside, while the enzyme known as maltase is active towards the α-methyl-D-glucoside substrate (Fig. 1.16).

This led Fischer to suggest his famous "lock-and-key" theory of enzyme specificity, which he described in his own word as follows: "To use a picture, I would say that enzyme and the glucoside must fit into each other like a lock and key, in order to effect a chemical reaction on each other" [1].

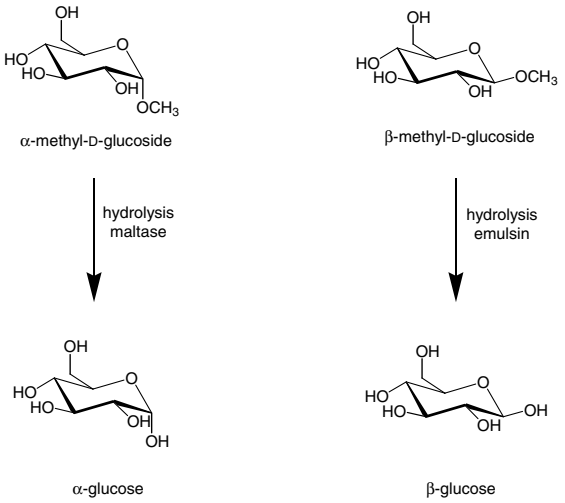

Fig. 1.16 Emil Fischer's substrates.

In 1913, the German biochemist Leonor Michaelis and his Canadian assistant Maud Leonara Menten published a theoretical consideration of enzymatic catalysis. This consideration envisaged the formation of a specific enzyme–substrate complex which further decomposed and yielded the product with the release of the enzyme. They had observed that the effect noted by Brown [45] is only observed at higher concentrations of the substrate. At lower concentrations the rate becomes proportional to the concentration of the substrate. This led to the development of the Michaelis–Menten equation to describe the typical saturation kinetics observed with purified enzymes and single substrate reactions [50]. Some years later a more general formulation of the Michaelis–Menten equation was given by Briggs and Haldane [51]. They pointed out that the Michaelis assumption that an equilibrium exists between the enzyme, substrate and enzyme–substrate complex is not always justified, and should be replaced by an assumption that the enzyme–substrate complex is not necessarily present at equilibrium but in a steady state. With the purification and crystallization of proteins in the 1920s, enzyme kinetics entered a new phase. It became possible to study the interactions between enzyme molecules and their substrate in much more detail. The British physical chemist Butler was the first to carry out kinetic studies with a pure enzyme, trypsin [52].

By 1920, about a dozen enzymes were known, none of which had been isolated [53]. Then, in 1926, Sumner [54] crystallized urease from jack bean, *Canavalia ensiformis,* and announced that it was a simple protein. He later, in 1946, received the Nobel Prize for his work with the enzyme urease, extracted from the jack bean. Urease is an enzyme that catalyzes the conversion of urea into ammonia and carbon dioxide (Fig. 1.17).

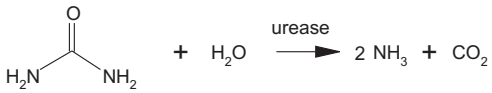

Fig. 1.17 The conversion of urea into ammonia and carbon dioxide.

Northrop and his colleagues [40] soon supported Sumner's claim that an enzyme could be a simple protein. They isolated many proteolytic enzymes, beginning with pepsin in 1930 by applying classic crystallization experiments. By the late 1940s many enzymes were available in a pure form and in sufficient amounts for investigations of their chemical structure to be carried out. Currently, more than 3000 enzymes have been catalogued [55]. The ENZYME data bank contains information related to the nomenclature of enzymes [56]. The current version contains 4309 entries. It is available through the ExPASy WWW server (http://www.expasy.org/enzyme/). Several hundred enzymes can now be obtained commercially [57].

In 1950 there was still no evidence that a given protein had a unique amino acid sequence. Lysosyme was the first enzyme to have its tertiary structure defined (Fig. 1.18), this was in 1966 with the help of X-ray crystallography [58].

However, ribonuclease A was one of the first enzymes to be prepared on a laboratory scale using organic chemistry methods. In 1969, Gutte and Merrifield synthesized its whole sequence in 11 931 steps [59].

By 1970, the complete molecular structures of several enzyme had been established and plausible reaction mechanisms could then be discussed [40].

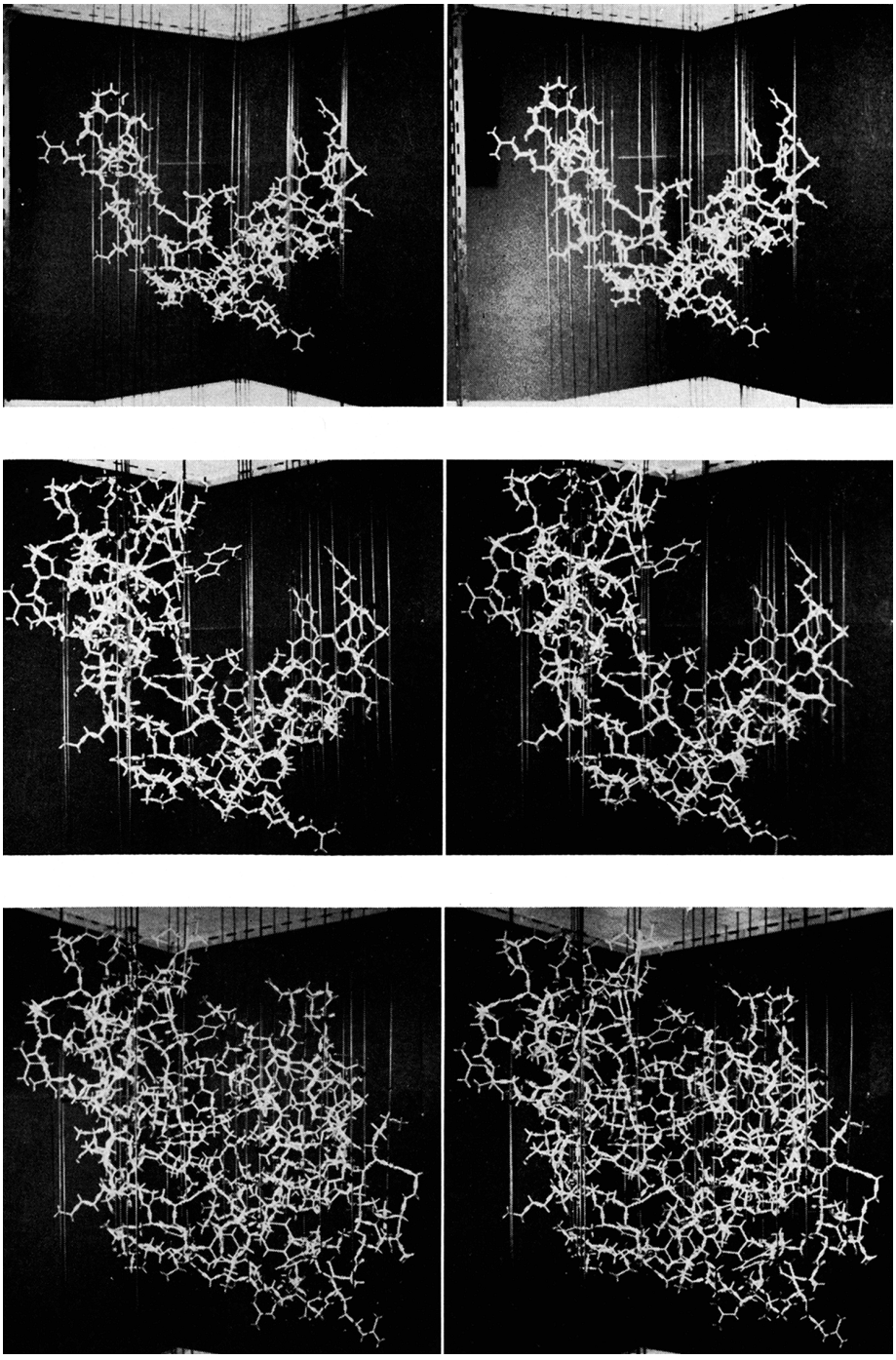

Fig. 1.18 Stereo photographs of models of part of the lysozyme molecule [58].

Hill (1897) was the first to show that the biocatalysis of hydrolytic enzymes is reversible [60].

Pottevin (1906) went further and demonstrated that crude pancreatic lipase could synthesize methyl oleate from methanol and oleic acid in a largely organic reaction mixture [61].

While the first benefits for industry from the microbiological developments had come early on, investigations with isolated enzymes had hardly any influence on the industry at that time. Consequently, industrial enzymatic biotransformations for the production of fine chemicals have a much shorter history than microbial biotransformations.

Invertase was probably the first immobilized enzyme to be used commercially for the production of Golden Syrup by Tate&Lyle during World War II, because sulfuric acid, the preferred reagent, was unavailable at that time (Fig. 1.19) [62].

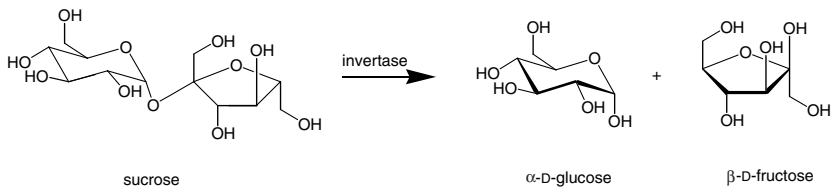

Fig. 1.19 Inversion of sucrose by invertase.

Yeast cells were autolyzed and the autolysate clarified by adjustment to pH 4.7, followed by filtration through a calcium sulphate bed and adsorption into bone char. A bone char layer containing invertase was incorporated into the bone char bed, which was already being used for syrup decolorization. The scale of the operation was large, the bed of invertase–char being 60 cm deep in a 610 cm deep bed of char. The preparation was very stable as the limiting factor was microbial contamination or loss of decolorizing power rather than the loss of enzymatic activity. The process was cost-effective but the product did not have the flavor quality of the acid-hydrolyzed material. This is the reason why the immobilized enzyme method was abandoned once the acid became available again [63].

Industrial processes for L-amino acid production based on the batch use of soluble aminoacylase were in use by 1954. However, like many batch processes with a soluble enzyme, they had their disadvantages, such as higher labor costs, complicated product

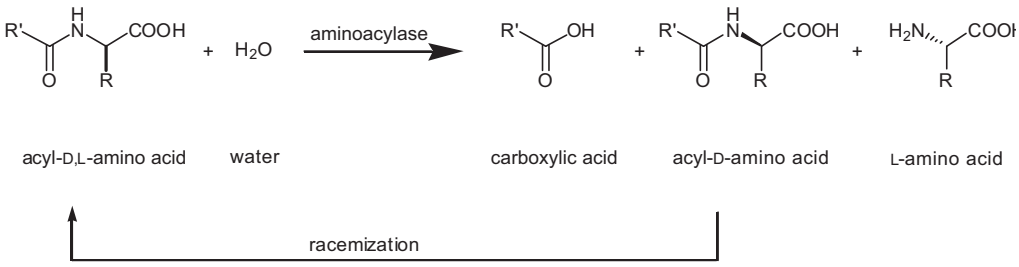

Fig. 1.20 L-Amino acid production catalyzed by aminoacylase.

separation, low yields, high enzyme costs and non-reusability of the enzyme. During the mid-1960s the Tanabe Seiyaku Co. of Japan was trying to overcome these problems by using immobilized aminoacylases. In 1969, they started the industrial production of L-methionine by aminoacylase immobilized on DEAE–Sephadex in a packed bed reactor (Fig. 1.20). This was the first full scale industrial use of an immobilized enzyme. The most important advantages are relative simplicity and ease of control [64].

In a membrane reactor system developed at Degussa-Hüls AG in Germany in 1980 [65], native enzymes, either pure or of technical grade, are used in homogeneous solution for the large scale production of enantiomerically pure L-amino acids (Fig. 1.21).

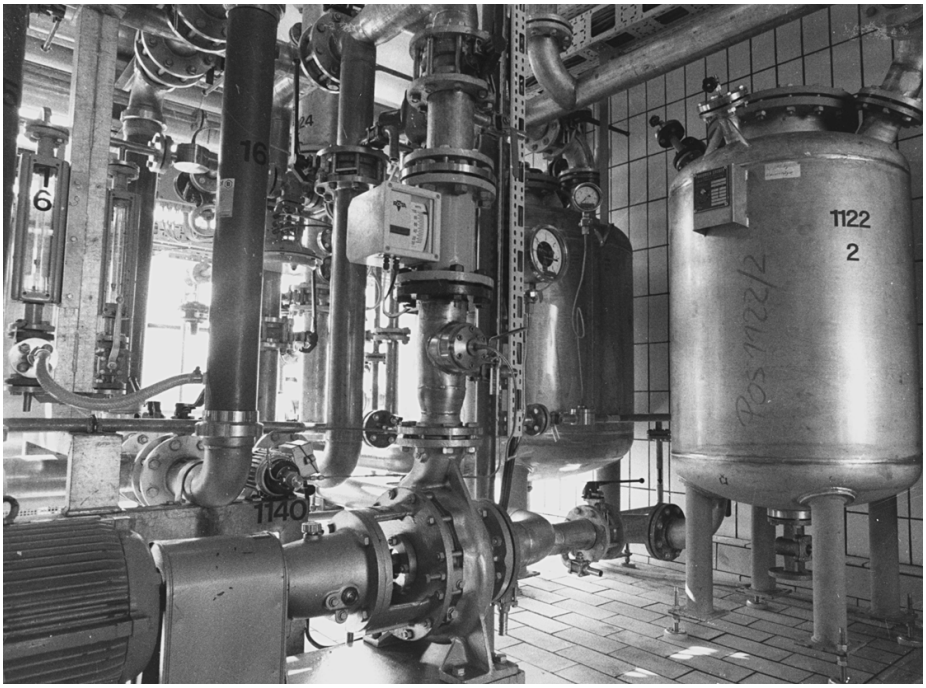

Fig. 1.21 Enzyme membrane reactor (Degussa-Hüls AG, Germany).

A membrane reactor is particularly suitable for cofactor-dependent enzyme reactions, especially if the cofactor is regenerated by another enzyme reaction and retained by the membrane in a modified form [66]. There are several advantages to carrying out biocatalysis in membrane reactors over heterogeneous enzymatic catalysis: there are no mass transfer limitations, enzyme deactivation can be compensated for by adding a soluble enzyme and the reactors can be kept sterile more easily than with immobilized enzyme systems. The product is mostly pyrogen free (a major advantage for the production of pharmaceuticals), because the product stream passes through an ultrafiltration membrane. Scale-up of membrane reactors is simple because large units with increased surface area can be created by combining several modules.

The enzymatic isomerization of glucose to fructose (Fig. 1.22) represents the largest use of an immobilized enzyme in the manufacture of fine chemicals.

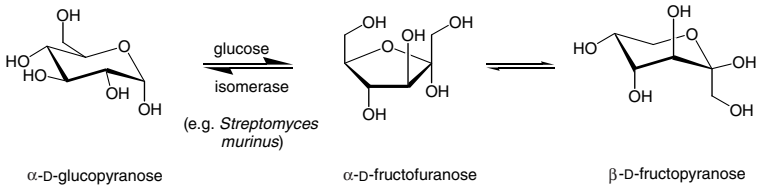

Fig. 1.22 Isomerization of glucose to fructose.

The production of high-fructose syrup (HFCS) has grown to become a large-volume biotransformation [67]. While sucrose is sweet, fructose is approximately 1.5-times sweeter and consequently high quality invert syrups (i.e., hydrolyzed sucrose) can be produced. Invert syrups contain glucose and fructose in a 1:1 ratio. However, it took the food industry a long time to become acquainted with the potential of glucose isomerase in the production of high quality fructose syrups from glucose. Again, the Japanese were the first to employ soluble glucose isomerase to produce high quality fructose syrups, in 1966. At the beginning of 1967, the Clinton Corn Processing Company, IA, USA, was the first company to manufacture enzymatically produced fructose corn syrup [67]. The glucose-isomerase catalyzed reversible reaction gave a product containing about 42% fructose, 50% glucose and 8% other sugars. For various reasons, economic viability being the most important among them, the first commercial production of fructose syrups using glucose isomerase immobilized on a cellulose ion-exchange polymer in a packed bed reactor plant only started in 1974. It was initiated by Clinton Corn Processing [64]. In 1976, Kato was the first company in Japan to manufacture HFCS in a continuous process as opposed to a batch process. In 1984, it became the first company to isolate crystalline fructose produced in this process by using an aqueous separation technique.

The glucose isomerase Sweetzyme T, produced by Novo, Denmark, is used in the starch processing industry in the production of high fructose syrup. The key to its long life is immobilization. The enzyme is chemically bound to a carrier, making the particles too large to pass through the sieve at the bottom of isomerization columns. Sweetzyme T is packed into columns where it is used to convert glucose into fructose. The record for the longest lifetime of a column is 687 days, held by a Japanese company, Kato Kagaku in Kohwa near Nagoya. The reaction conditions are pH 7.5 and T = 55 °C. Although enzyme activity is reduced at this temperature, its stability and productivity are considerably improved [68].

The engineers from Kato used to say: "The better the substrate you put in, the better the results you get out". Each column at Kato contains 1800 kg of Sweetzyme T. The column needs to be changed when the flow rate decreases to about 10% of the initial value. Sweetzyme T displays a linear decay curve under steady state operating conditions. With regard to productivity, the yield from the record-breaking column was 12 000 kg of fructose syrup (containing 42% fructose) (dry substance) per kg of Sweetzyme T. The normal column productivity was 8000–10 000 kg per kg of enzyme. Thus the 687-day record for Sweetzyme T is also a world record in the starch industry [68] (Fig. 1.23).

"Central del Latte" of Milan, Italy, was the first company to commercially hydrolyze milk lactose with immobilized lactase using SNAMprogetti technology [69]. An industrial

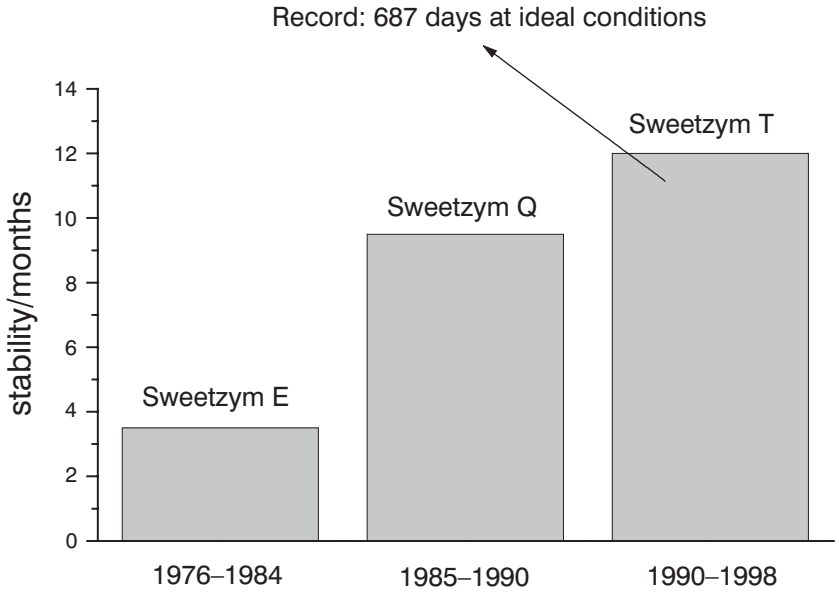

Fig. 1.23 Improved biocatalyst stability by biocatalyst engineering at NOVO.

plant with a capacity of 10 tons per day is situated in Milan. The entrapped enzyme is lactase obtained from yeast and the reaction is performed batchwise at low temperature. Lactase hydrolyzes lactose, a sugar with poor solubility properties and a relatively low degree of sweetness, to glucose and galactose (Fig. 1.24).

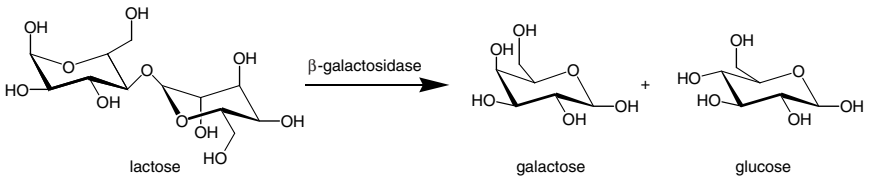

Fig. 1.24 *β*-Galactosidase catalyzed hydrolysis of lactose to galactose and glucose.

After the processed milk reaches the desired degree of hydrolysis of lactose, it is separated from the enzyme fibers, sterilized and sent for packing and distribution. SNAMprogetti's process facilitates the manufacture of a high-quality dietary milk at low cost. This milk has remarkable digestive tolerance, pleasant sweetness, unaltered organoleptic properties and a good shelf-life. It does not contain foreign matter. The industrial plant is shown in Fig. 1.25.

Penicillin G, present in *Penicillium notatum* and discovered by Fleming in 1929, revolutionized chemotherapy against pathogenic microorganisms. Today, *β*-lactam antibiotics such as penicillins and cephalosporins are used very widely. Thousands of semisynthetic *β*-lactam antibiotics are being synthesized to find more effective compounds. Most of

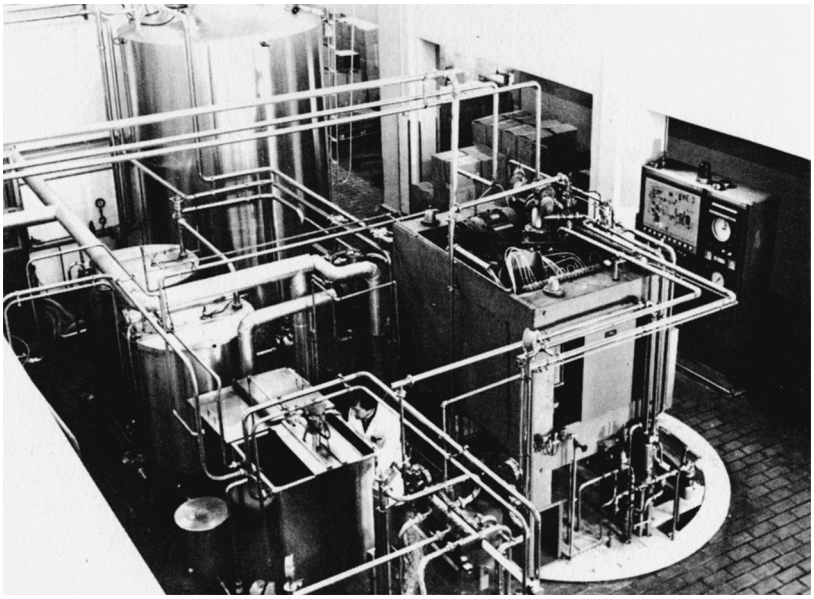

Fig. 1.25 Industrial plant for processing low-lactose milk [69].

these compounds are prepared from 6-aminopenicillanic acid (6-APA), 7-aminocephalosporanic acid (7-ACA) and 7-amino-desacetoxycephalosporanic acid (7-ADCA).

At present, 6-APA is mainly produced either by chemical deacylation or by enzymatic deacylation using penicillin amidase from penicillin G or V. This process, which exemplifies the best known usage of an immobilized enzyme in the pharmaceutical industry, has been used since around 1973 (Fig. 1.26). Several chemical steps are replaced by a single enzymatic reaction. Organic solvents, the use of low temperature (–40 °C) and the need for totally anhydrous conditions, which make the process difficult and expensive, were no longer necessary in the enzymatic process [70].

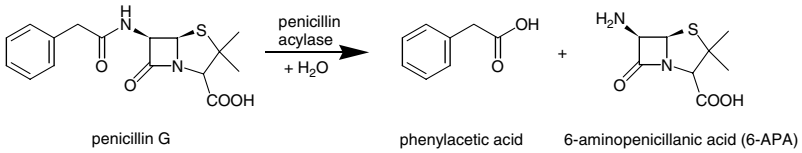

Fig. 1.26 Enzymatic synthesis of 6-aminopenicillanic acid (6-APA).

For many years enzymatic 7-ACA production was nothing but a dream. This changed in 1979, when Toyo Jozo, Japan, in collaboration with Asahi Chemical Industry, also of Japan, successfully developed the industrial production of 7-ACA by a chemoenzymatic two-step process starting from cephalosporin C (Fig. 1.27).

The chemical process requires highly purified cephalosporin C as a raw material. A number of complicated reaction steps are carried out at –40 to –60 °C, and there is a

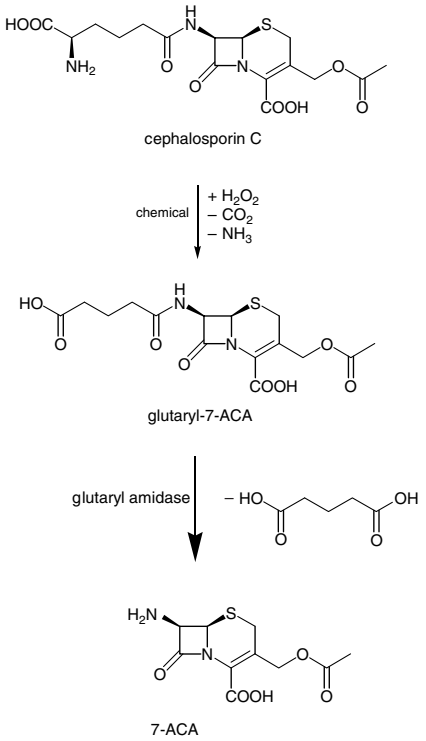

Fig. 1.27 Two-step process for production of 7-ACA from cephalosporin C.

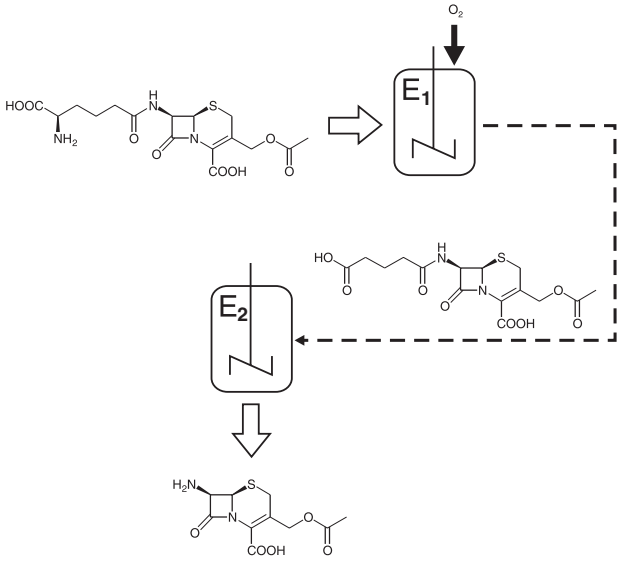

Fig. 1.28 Flow-scheme for the production of 7-ACA. Production carried out at Asahi Chemical Industry (**E**$_1$ = D-amino acid oxidase; **E**$_2$ = glutaryl amidase).

long reaction time. Furthermore, hazardous reagents, such as phosporus pentachloride, nitrosyl chloride and pyridine are used in this process and the subsequent removal of such reagents causes significant problems. Therefore, the development of an enzymatic process was a dream for a long time. In the enzymatic process, liberated glutaric acid reduces the pH and inhibits glutaryl-7-ACA amidase, the enzyme that catalyzes the deacylation of cephalosporin C. Because of this change in pH the reaction rate is decreased, necessitating strict pH control during the reaction process. For these reasons, a recircu-

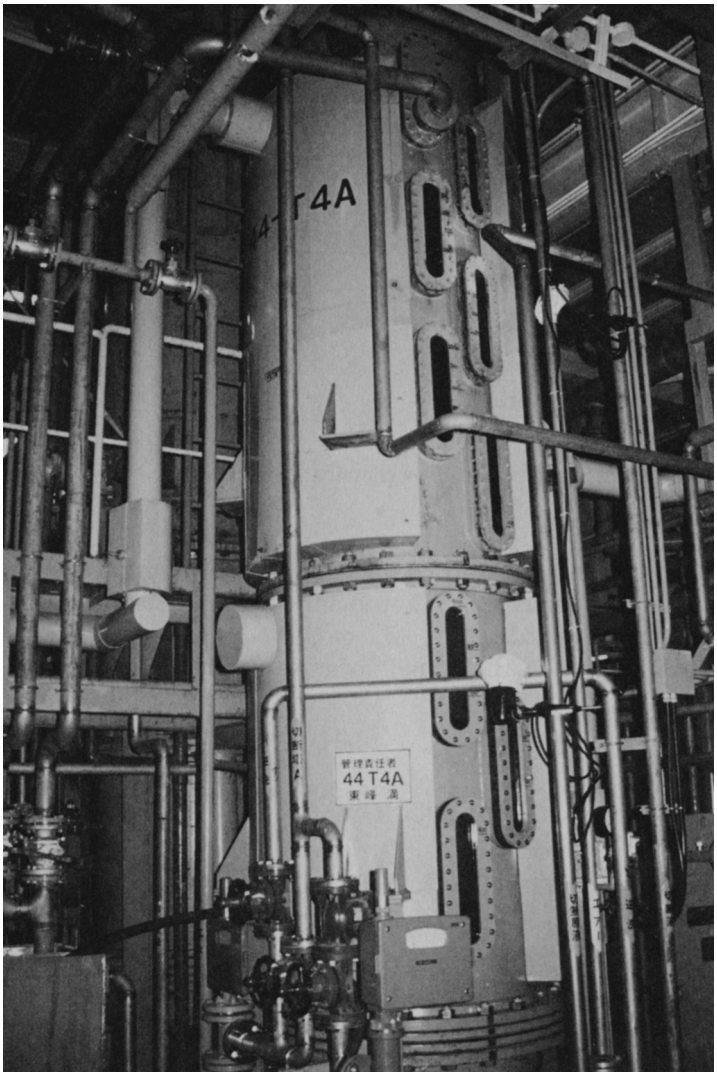

Fig. 1.29 Bioreactor plant for the production of 7-ACA carried out at Asahi Chemical Industry (reprinted from Ref. [71], p. 83, courtesy of Marcel Dekker Inc.).

lating bioreactor with immobilized glutaryl-7-ACA amidase and an automatic pH controller were designed for the production of 7-ACA. The bioreactor for industrial 7-ACA production is shown in Figs. 1.28 and 1.29. The process has been in operation at Asahi Chemical Industry since 1973. It is reported that about 90 tons of 7-ACA are produced in this way annually [71].

1.3 From Wine Bottle to a State-of-the-Art Facility – The History of Biochemical Engineering

Processing of biological materials and using biological agents such as cells, enzymes or antibodies are the central features of biochemical engineering. The scope of biochemical engineering has grown from simple wine-bottle microbiology to the industrialization of the production of antibiotics, chemicals, proteins, etc. Success in biochemical engineering requires integrated knowledge of the governing biological properties and principles and of engineering methodology and strategy. The objective of biochemical engineering could be formulated as the advancement of the scientific fundamentals of engineering for a rational, sustainable and safe approach to the development of products, processes and services in the chemical, pharmaceutical and life science industries. The aim is to provide tools for the efficient development of novel know-how [72] (Figs. 1.30 and 1.31).

In the 1940s complementary developments in biochemistry, microbial genetics and engineering ushered in the era of antibiotics, which brought tremendous relief to suffering and mortality of mankind. This period marks the birth of biochemical engineering

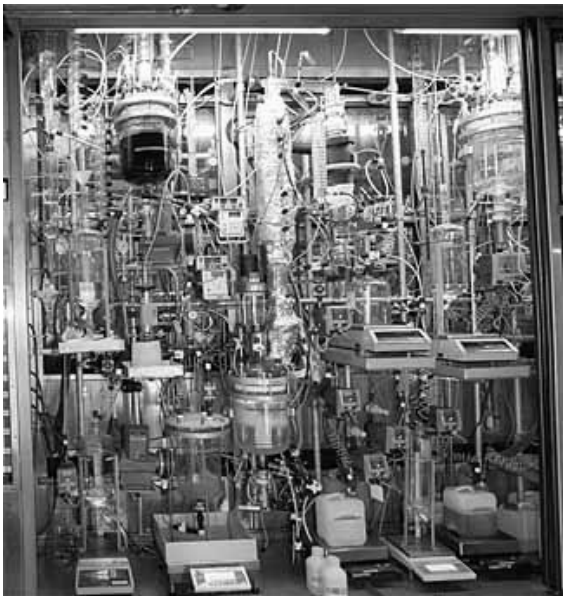

Fig. 1.30 Mini-plant at BASF (photograph courtesy of Prof. Bernhard Hauer/ZH/BASF-AG/BASF).

Fig. 1.31 Bioreactor at LONZA.

[73]. The issues of scale-up, mixing and oxygenation had never before been encountered with such urgency and criticality as that for the production of antibiotics late into and right after the Second World War. This can be marked as the genesis phase of modern biochemical engineering.

During the 1960s, the increased tempo in the cross-fertilization and integration of diverse fields of knowledge gave rise to two new interdisciplinary branches of technology: biomedical engineering, which encompasses practically all the engineering disciplines and the medical sciences, and biochemical engineering [74, 75]. Biochemical engineering is a multidisciplinary area bordering biology, chemistry and engineering sciences. As a border area, biochemical engineering plays an ever increasing role in biotechnological developments. This is demonstrated by examples ranging from substrate preparation and bioconversion to downstream processing [76] (Fig. 1.32).

Biochemical engineering has been recognized as a scientific discipline in its own right for at least three decades, but it is often misunderstood in that it is thought to be synonymous with bioprocessing development [77]. The term biochemical engineering science [78] was introduced to distinguish between the study of biochemical engineering and its application in industry [79]. Biochemical engineering science represents the fundamental research into all aspects of the interactions between engineering and other disciplines necessary to underpin the development of industrial-scale biologically based processes [78].

Bioreactor mixing and kinetics issues, the unstructured kinetic models, the emergence of whole-cell biotransformations and bioreactor instrumentation and control defined the

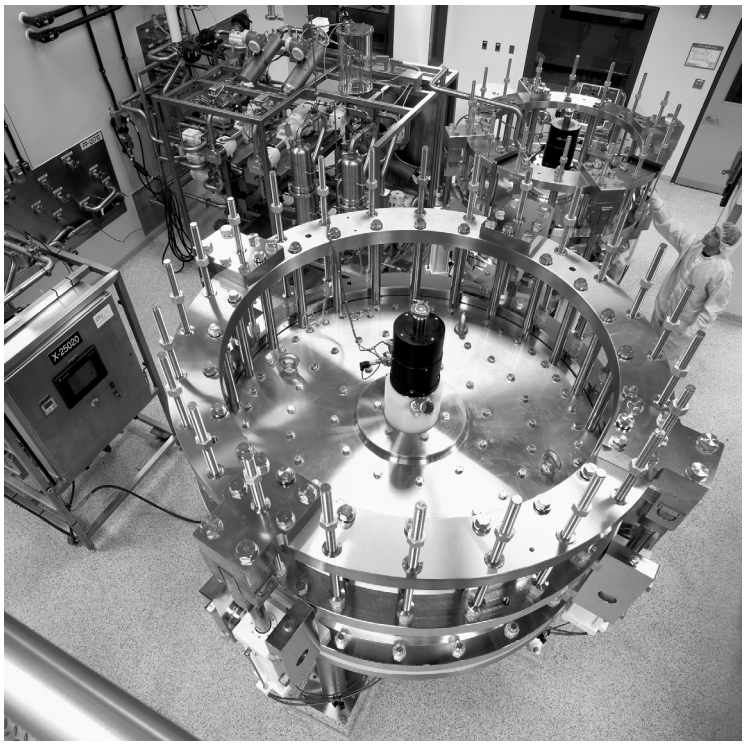

Fig. 1.32 Down-stream processing at LONZA – the purification step.

1960s. The 1970s were defined by the development of enzyme technology (including, most critically, enzyme immobilization technologies [80]), biomass engineering, single-cell protein production and bioreactor design [81], operation and control innovations. The period from about 1960 to 1980 can be defined as an evolutionary phase for biochemical engineering. The next 20 years (1980–2000) could be called the 1st step-change (growth) phase (or how-much-biology-can-an-engineer-handle phase) in biochemical engineering. As a result of this phase, biochemical engineering will never be like anything in the past ever again. The emergence of the recombinant DNA technology, the hybridoma technology, cell culture, molecular models, large-scale protein chromatography, protein and DNA sequencing, metabolic engineering and bioremediation technologies changed biochemical engineering in a drastic and profound way. New problems, new ways of thinking, the prominence and challenges of new biotechnology, everything now seems so different, the world of biochemical engineering and biology has been turned upside down [82].

The idea that understanding the secrets of cells lies in understanding the complex networks that a cell uses is now gaining acceptance. The bioprocesses suffer from a lack of robustness to disturbances of the individual steps and the responses to perturbation in a single step or in multiple steps are often detrimental to the system. Cells, however, have an amazing ability to withstand disturbances and their responses are rapid and effective.

Understanding these features and adapting them for engineered processes would enable better process control and might lead to novel methods for producing new chemicals using cell-derived catalysts [83].

Four technological advances, which had a major impact on enzymatic biotransformations, were required for the acceptance of enzymes as "alternative catalysts" in industry [84]. These advances have resulted from a greater understanding of biochemical engineering leading to changes in fermenter operation, such as controlled feeding of nutrients and improved oxygen transfer, or enabling technologies and tools, such as on-line sensors and computer simulation.

1. *The first technological advance* was the development of large-scale techniques for the release of enzymes from the interior of microorganisms [85]. Although the majority of industrial purification procedures are based on the same principles as those employed on a laboratory scale, the factors under consideration while devising industrial-scale purification regimes are somewhat different. When isolating enzymes on an industrial scale, for commercial purpose, a prime consideration has to be the cost of the production in relation to the value of the end-product. Therefore, techniques used on a laboratory scale are not always suitable for large-

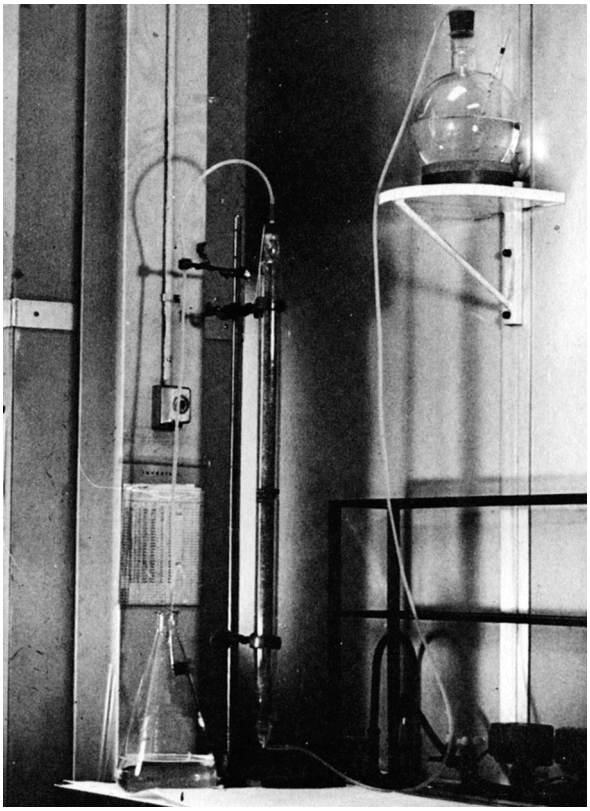

Fig. 1.33 Historical invertase column [69].

scale work [86]. Production and isolation of an intracellular microbial enzyme are fairly expensive. The cost of using a water-soluble protein as a catalyst for biotransformations can be justified only by its repeated use [87].

2. *The second technological advance* was the development of techniques for the large-scale immobilization of enzymes. As mentioned earlier, the first enzyme immobilized in the laboratory was invertase, adsorbed onto charcoal in 1916 [88]. However, only after the development of immobilization techniques on a large scale had occurred in the 1960s, were many different industrial processes using immobilized biocatalysts established. The historical invertase column that has been in operation on a laboratory scale since 1968 is shown in Fig. 1.33.

It was shown that by increasing the concentration of sucrose, the efficiency of the fiber-entrapped invertase (which hydrolyzes sucrose) can be increased. This occurred because the substrate, which is an inhibitor of the enzyme, could not reach high concentration levels inside the microcavities of the fibers due to diffusion limitations [69].

Table 1.2 lists some industrial biotransformations performed by isolated enzymes.

Tab. 1.2 Selected historical, industrial applications of isolated enzymes.

Product	Biocatalyst	Operating since	Company
L-amino acid	amino acylase	1954, 1969	Tanabe Seiyaku Co., Ltd., Japan
6-amino penicillanic acid	penicillin acylase	1973	SNAMProgetti and many others[a]
low-lactose milk	lactase	1977	Central del Latte, Milan, Italy (SNAMProgetti technology)
7-amino-cephalosporanic acid	D-amino acid oxidase	1979	Toyo Jozo and Asahi Chemical Industry, Japan

a Beecham, Squibb, Astra Lakenedal, Bayer, Gist Brocades, Pfizer, Bristol Myers, Boehringer Mannheim, Biochemie, Novo.

The first Enzyme Engineering Conference was held at Hennicker, NH, USA, in 1971. The term "immobilized enzymes" describing "enzymes physically confined at or localized in a certain region or space with retention of their catalytic activity and which can be used repeatedly and continuously" was adopted at this conference [89].

3. *The third technological advance* was the development of techniques for biocatalysis in organic media. The usage of very high proportions of organic solvents to increase the solubility of reactants was examined in 1975 for the reaction with isolated cholesterol oxidase to produce cholestenone [90]. The enzymatic synthesis was believed to be incompatible with most organic syntheses carried out in non-aqueous media. This changed after Klibanov [91] recognized in 1986 that most enzymes could function fairly well in organic solvents. Since that time various processes involving an organic phase have been established in industry (Table 1.3).

Tab. 1.3 Industrial biotransformations involving reactants that are poorly water soluble.

Process	Biocatalyst	Operating since	Company
fat interesterification	lipase	1979, 1983	Fuji Oil, Unilever
ester hydrolysis	lipase	1988	Sumitomo
transesterification	lipase	1990	Unilever
aspartame synthesis	thermolysin	1992	DSM
acylation	lipase	1996	BASF

4. *The fourth and most recent technological advance* is recombinant DNA technology. This technology is only now being widely used for biotransformation.

   In general, microorganisms isolated from nature produce the desired enzyme at levels that are too low to provide a cost-efficient production process. Consequently, a modification of the organism would be highly desirable for process development. Currently, there are three principal approaches available for strain improvement. The first one, *direct evolution* [92], i.e., improvement by mutation and selection, has been successfully used in many industrial microbiological fields for many years. In 1978, Clark showed that evolution processes can be performed on a laboratory scale. Microevolution occurring in bacterial cultures grown in a chemostat gives rise to altered enzyme specificity enabling microorganisms to degrade some unusual synthetic organic compounds. Successive mutation steps could be responsible for the evolution of new enzymatic specificities. The rate of production of existing enzymes and the expression of previously dormant genes are also typically affected by this event [93]. The second method is *hybridization*. This involves modification of the cellular genetic information by transference of DNA from another strain. The third method is *recombinant DNA technology*, whereby genetic information from one strain can be manipulated *in vitro* and then inserted into the same or a different strain.

   Recombinant DNA technology has dramatically changed enzyme production, because enzymes are synthesized in cells by the normal protein synthesis methods [94, 95]. The 5–10 year period required for classical enzyme development can be reduced to 1–2 years. Protein engineering, in combination with recombinant expression systems, allows new enzyme variants to be plugged in and to very quickly be at manufacturing levels [96]. Novel microbial catalysts, together with recent advances in molecular biology, offer scientists an opportunity to evolve selected genes rapidly and considerably improve bacterial biocatalysts [97]. For example, a method for the rapid generation of thermostable enzyme variants has been developed [98, 99]. This is achieved by introducing the gene coding for a given enzyme from a mesophilic organism into a thermophile. Variants that retain the enzymatic activity at higher growth temperatures of the thermophile are selected. This can be accomplished by constructing the artificial environment

in which only the evolutional adaptation of the enzyme can permit cell growth. This strategy can be readily extended to the general method of screening mutant enzymes. Another example is random mutation, developed as a method for the highly efficient generation of mutant enzymes. The cloned gene coding for a given enzyme can be mutated either chemically or enzymatically *in vitro*. The mutant enzymes can be readily screened because mutant genes can be separated from intact genes. Various mutant enzymes which have changes in their properties, such as substrate specificity, thermal stability and coenzyme selectivity, have been isolated by this technique. These methods do not require predictive strategies, unlike, for example, site-directed mutagenesis. It is hoped that in the course of time that enzymes can be made which are excellent catalysts, fulfilling all the requirements for industrial use. This research field is referred to as *biocatalyst engineering* [32].

In parallel with developments in genetic engineering have come improvements in biochemical engineering that have yielded commercial benefits in reactor and fermenter design and operation, improved control techniques and downstream separation. These have resulted in more rapid delivery of new products [100] to the marketplace.

1.4 Advantages of Biotransformations Over Classical Chemistry Enzymes are proteins, things of beauty and a joy forever [40]

Biocatalysis is a relatively green technology. Enzyme reactions can be carried out in water at ambient temperature and neutral pH, without the need for high pressure and extreme conditions, thereby saving energy normally required for processing. Biocatalysis has proven to be a useful supplementary technology for the chemical industry, allowing, in some cases, reactions that are not easily conducted by classical organic chemistry or, in other cases, allowing reactions that can replace several chemical steps. Today, highly chemo-, regio- and stereoselective biotransformations can simplify manufacturing processes and make them even more economically attractive and environmentally acceptable [101].

Both new discoveries and incrementalism describe how the industrial enzyme business changed during 1996. Enzymes have competed well with chemical methods for resolution but not with synthesis. Ibuprofen, phenylethylamine and acrylamide are commonly cited as compounds prepared using enzyme-based chiral processes. There is also an unconfirmed suspicion that the fat substitute Olestra, because of some of its structural features, may require enzymatic steps in its synthesis. The outlook for industrial enzymes is positive. The suppliers have extensive portfolios of promising new enzymes in their product pipelines. The range of customers considering the utilization of enzymes as replacements for conventional chemical methods, appears to be growing. New niche applications continue to be discovered in otherwise mature segments [102]. It appears that enzyme-based processes are gradually replacing conventional chemical-

based methods, e.g., the use of enzymes as catalysts provides a totally new method of polymer synthesis; most of these polymers are otherwise very difficult to synthesize by conventional chemical catalysts [36]. Finally, the latest literature on enzymology suggests that other biocatalysts will add to future sales, both in established and in new markets. The enzyme "nitrogenase", which converts dinitrogen into ammonia, a basic chemical compound, has been discovered recently [103]. Dream reactions of organic chemists might come true in the future, with the use of biocatalysts where functional or chiral groups are introduced into molecules by utilizing H_2, O_2 or CO_2. Recently Aresta reported on a carboxylase enzyme that utilizes CO_2 in the synthesis of 4-hydroxybenzoic acid starting from phenyl phosphate [104]. The process of carbon dioxide fixation can be carried out successfully with a pure stream of carbon dioxide in bioreactor [105]. In order to make the bioprocess feasible the enzyme D-Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) was recently immobilized [106]. Also, the novel trickling spray reactor employing immobilized carbonic anhydrase, which enables concentration of CO_2 from the emission stream was developed [107] Carbonic anhydrase is one of the fastest enzymes that make fast mass transfer from gas phase to aqueous phase. The single one-step chemical process shown in Fig. 1.34 has the possibility of retaining carbon in the bound form for a very short period of time, therefore the biocatalytic fixation process, just as the basic process in plants, could be the answer to atmospheric pollution. A large number of commercially useful substances, which could be made from fixed carbon, also require the bioprocess of carbon dioxide fixation.

Fig. 1.34 The single one-step chemical process for the fixation of carbon dioxide.

Although the production of D-amino acids is currently of great interest, there has been no known industrial manufacture of D-amino acids except for D-*p*-hydroxyphenylglycine and D-phenylglycine. At present chemical methods are not suitable for the large-scale production of D-amino acids due to the low yield and high cost. Most L-amino acids are efficiently manufactured by fermentation, but D-amino acids are rarely produced by fermentation, apart from a few exceptions, because it is difficult to obtain high optical purity and productivity. Enzymatic methods are most plausible for the industrial manufacture of D-amino acids with respect to the optical purity and productivity. D-Amino acids such as D-*p*-hydroxy phenylglycine and D-phenylglycine are produced from D,L-hydantoins. From an industrial point of view, the availability of cheap starting materials and the development of suitable biocatalysts are most important. The number of substrates that are available on an industrial scale is limited. Based on these criteria, synthetic intermediates of D,L-amino acids and L-amino acids produced by biotransformations would be the most important starting materials for the production of D-amino acids. The enzymatic production of D-amino acids is classified into three categories based on the starting materials [108]:

1. D,L-amino acids (D-amino acylase);
2. synthetic intermediates (D,L-hydantoin:D-hydantoinhydrolase, D,L-amino acid amides:D-amidase);
3. prochiral substrates (α-keto acids, L-amino acids; D-transaminase and amino acid racemase).

The fed batch process [109] used in the production of L-DOPA, giving a final product concentration of 110 g L^{-1}, has many advantages over the classical chemical process, such as a single reaction step, water as the only reaction by-product, no need for optical separation, a shorter production cycle of three days, simple down-stream processing and process sustainability. L-DOPA is a metabolic precursor of dopamine, a very important drug in the treatment of Parkinson's disease.

It is difficult to assess directly the true commercial value of biocatalysis, because the real value of the products made using the biocatalysts must be taken into account. Of course, its major advantages lies in stereoselective reactions. A good example of the technological power and commercial potential is the aforementioned stereoselective hydroxylation of steroids.

In comparison with fermentation processes, fewer side-products are formed in enzymatic biotransformations, complex expensive fermenters are not required, aeration, agitation and sterility need not necessarily be maintained and the substrate is not diverted into the formation of a *de novo* cellular biomass [70]. Isolated biocatalysts are especially useful if the reaction they catalyze is about to be completed, if they are resistant to product inhibition, and if they are active in the presence of low concentrations of the substrate (such as in detoxification reactions where pollutants are present in the waste stream). "One-pot" multi-enzyme reactions are much more feasible than a combined use of several chemical catalysts or reagents, especially as the latter often have be used in reactors made of special resistant materials that can tolerate extreme conditions, such as the use of concentrated acids under elevated temperatures and pressures [70].

Silicatein is an enzyme that has purified the glassy skeletal elements of a marine sponge. It was previously shown to be capable of catalyzing and structurally directing the hydrolysis and polycondensation of silicon alkoxides to yield silica and silsesquioxanes at low temperature and pressure and neutral pH, and of catalyzing and templating the hydrolysis and subsequent polycondensation of a water-stable alkoxide-like conjugate of titanium to form titanium dioxide. Although biocatalysis has been used for a long time to produce compounds and materials based on carbon, these observations are the first to have extended this approach to valuable inorganic materials such as titanium dioxide [110].

It is no longer the case that biotransformations are relevant only to high added-value products such as pharmaceuticals. Bulk chemicals, including polymers [35, 36], may incorporate biotransformations, such as the conversion of methane into methanol (Chevron Research and Technology and Maxygen), the conversion of sugars into 3-hydroxypropionic acid (Cargill Inc., USA) [111] or the dehalogenation step in Dow's alkene oxide process. The next generation of bioprocesses will target large volume chemicals and polymers and will compete directly with petroleum-based products. Biotransformations are becoming competitive with conventional routes, but industry experts believe that further

improvements in enzymatic catalysis and fermentation engineering may be required before many companies are prepared to announce large-scale bioprocessing plants for world-wide production. Bioprocessing proponents envisage a future in which microorganisms are replaced by purified enzymes, synthetic cells or crop plants [112].

Today, both the academic and the industrial community see biocatalysis as a highly promising area of research, especially in the development of sustainable technologies for the production of chemicals and for the more selective and complex active ingredients in pharmaceuticals and agrochemicals [113–116].

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