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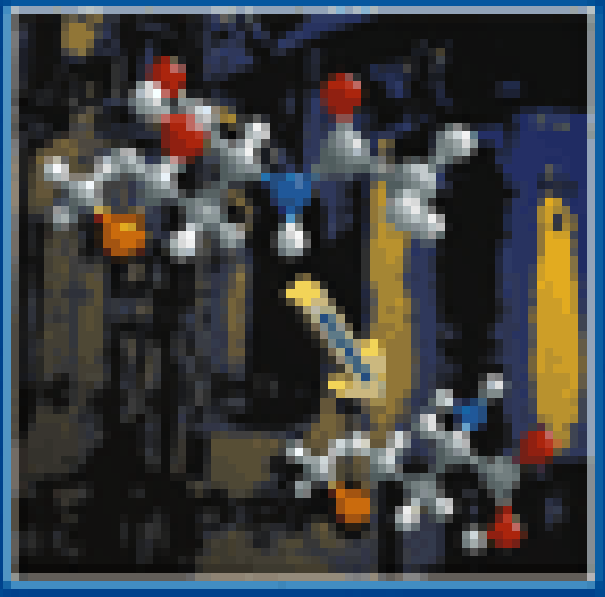
A. Liese, K. Seelbach, C. Wandrey

Industrial Biotransformations



Content

1 Introduction

The main incentive in writing this book was to gather information on one-step biotransformations that are of industrial importance. With this collection, we want to illustrate that more enzyme-catalyzed processes have gained practical significance than their potential users are conscious of. There is still a prejudice that biotransformations are only needed in cases where classical chemical synthesis fails. Even the conviction that the respective biocatalysts are not available and, if so, then too expensive, unstable and only functional in water, still seems to be widespread. We hope that this collection of industrial biotransformations will in future influence decision-making of synthesis development in such a way that it might lead to considering the possible incorporation of a biotransformation step in a scheme of synthesis.

We therefore took great pains in explicitly describing the substrates, the catalyst, the product and as much of the reaction conditions as possible of the processes mentioned. Wherever flow schemes were available for publication or could be generated from the reaction details, this was done. Details of some process parameters are still incomplete, since such information is only sparingly available. We are nevertheless convinced that the details are sufficient to convey a feeling for the process parameters. Finally, the use of the products is described and a few process-relevant references are made.

We would go beyond the scope of this foreword, should we attempt to thank all those who were kind enough to supply us with examples. Of course, we only published openly available results (including the patent literature) or used personally conveyed results with the consent of the respective authors. We are aware of the fact that far more processes exist and that by the time the book is published, many process details will be outdated. Nonetheless, we believe that this compilation with its overview character will serve the above-mentioned purpose. This awareness could be augmented if the reader, using his or her experience, would take the trouble of filling out the printed worksheet at the end of this book with suggestions that could lead to an improvement of a given process or the incorporation of a further industrial process into the collection.

Requesting our industrial partners to make process schemes and parameters more accessible did not please them very much. Even so, we are asking our partners once again to disclose more information than they have done in the past. In many instances, far more knowledge of industrial processes has been gained than is publicly available. Our objective is to be able to make use of these “well known secrets” as well. We would like to express our gratitude to all those who supplied us with information in a progress-conducive manner. Thanks also go to those who did not reject our requests completely and at least supplied us with a photograph in compensation for the actually requested information.

The book begins with a short historical overview of industrial biotransformations. Since the process order of the compilation is in accordance with the enzyme nomenclature system, the latter is described in more detail. We also include a chapter on reaction engineering to enable an easier evaluation of the processes.

The main part of the book, as you would expect, is the compilation of the industrial biotransformations. The comprehensive index will allow a facile search for substrates, enzymes and products.

We sincerely hope that this book will be of assistance in the academic as well as the industrial field, when one wants to get an insight into industrial biotransformations. We would be very thankful to receive any correction suggestions or further comments and contributions. At least we hope to experience a trigger effect that would make it worth while for the readership, the authors and the editors to have a second edition succeeding the first.

We are indebted to several coworkers for screening literature and compiling data, especially to Jürgen Haberland, Doris Hahn, Marianne Hess, Wolfgang Lanters, Monika Lauer, Christian Litterscheid, Nagaraj Rao, Durda Vasic-Racki, Murillo Villela Filho, Philomena Volkmann and Andrea Weckbecker.

We thank especially Uta Seelbach for drawing most of the figures during long nights, as well as Nagaraj Rao and the "enzyme group" (Nils Brinkmann, Lasse Greiner, Jürgen Haberland, Christoph Hoh, David Kihumbu, Stephan Laue, Thomas Stillger and Murillo Villela Filho).

And last but not least we thank our families for their support and tolerance during the time that we invested in our so called 'book project'.

2 History of Industrial Biotransformations – Dreams and Realities

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Throughout the history of mankind, microorganisms have been of tremendous social and economic importance. Without even being aware of their existence, man used them in the production of food and beverages already very early in history. Sumerians and Babylonians practised beer brewing **before 6000 B.C.**, references to wine making can be found in the Book of Genesis, and Egyptians used yeast for baking bread. However, the knowledge of the production of chemicals such as alcohols and organic acids by fermentation is relatively recent and the first reports in the literature appeared only in the **second half of the 19th century**. Lactic acid was probably the first optically active compound to be produced industrially by fermentation. It 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 substrate and product in fermentation while there are only one or two in 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.

2.1 From the "flower of vinegar" to the recombinant *E. coli* – The history of microbial biotransformations

The story of microbial biotransformations is closely connected with vinegar production which dates back to some **2000 years B.C.**

Vinegar production is perhaps the oldest and best known example of microbial oxidation which may illustrate some of the important developments in the field of biotransformations by living cells (figure 1).

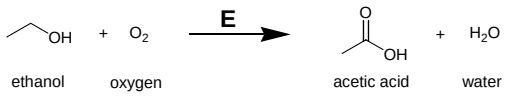


Fig. 1 Vinegar production (**E** = biocatalyst).

A prototype bioreactor with immobilized bacteria has been known in France since the **17th century**. The oldest bioreactor using immobilized living microorganisms, a so-called generator, was developed in **1823** [3,4]. Even today, acetic acid is still known as "vinegar" if it is obtained by oxidative fermentation of ethanol-containing solutions by acetic acid bacteria [5].

In **1858**, Pasteur [6] 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 (figure 2).

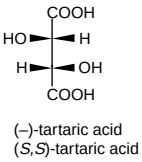


Fig. 2 Pasteur's product of the first resolution reaction.

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

In **1862**, Pasteur [7] investigated the conversion of alcohol to vinegar and concluded that the pellicle, which he called "the flower of vinegar", "serves as a transport of air oxygen to a multitude of organic substances".

In **1886**, Brown confirmed Pasteur's findings and named the causative agent in vinegar production as *Bacterium xylinum*. He also found that it could oxidize propanol to propionic acid and mannitol to fructose (figure 3) [8].

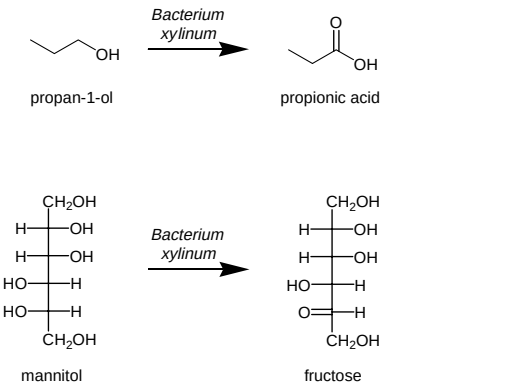
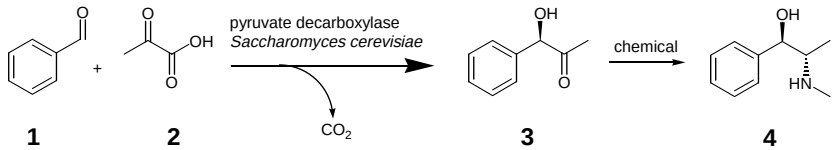


Fig. 3 Reactions catalyzed by *Bacterium xylinum*, the vinegar biocatalyst.

In **1897**, Buchner [9] reported that cell-free extracts prepared by grinding yeast cells with sand could carry out alcoholic fermentation reactions in the absence of living cells. This initiated the usage of resting cells for biotransformations.

Neuberg and Hirsch [10] discovered in **1921** that the condensation of benzaldehyde with acetaldehyde in the presence of yeast forms optically active 1-hydroxy-1-phenyl-2-propanone (figure 4).



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

Fig. 4 L-Ephedrine production.

The obtained compound was further chemically converted into D-(–)-ephedrine by Knoll AG, Ludwigshafen, Germany in **1930** (figure 5) [11].

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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 469 783; 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 ebensogut 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 nach derwegen 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.

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Das Verfahren stellt eine neue Methode dar, um das links drehende Phenylpropanolon in Form von l-1-Phenyl-2-methylaminopropan-1-ol zutreffend 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 ausfällt, wie es bei den bekannten Spaltungsverfahren des Racemkörpers der Fall ist.

Beispiel 1

120 g des durch Ätherauszug gewonnenen phenylpropanololhaltigen 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 phenylpropanololhaltigen Ätherauszuges werden unter verminderten Druck destilliert. 300 g der bei 100 bis 150° unter 14 mm Druck übergehenden Fraktion werden in Gegenwart von kolloidalem Platin (70 ccm 1%iger 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 210 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 215°. 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. 5 Knoll's patent of **1930**.

The bacterium *Acetobacter suboxydans* was isolated in **1923** [12]. Its ability to carry out limited oxidation was used in a highly efficient preparation of L-sorbose from D-sorbitol (figure 6).

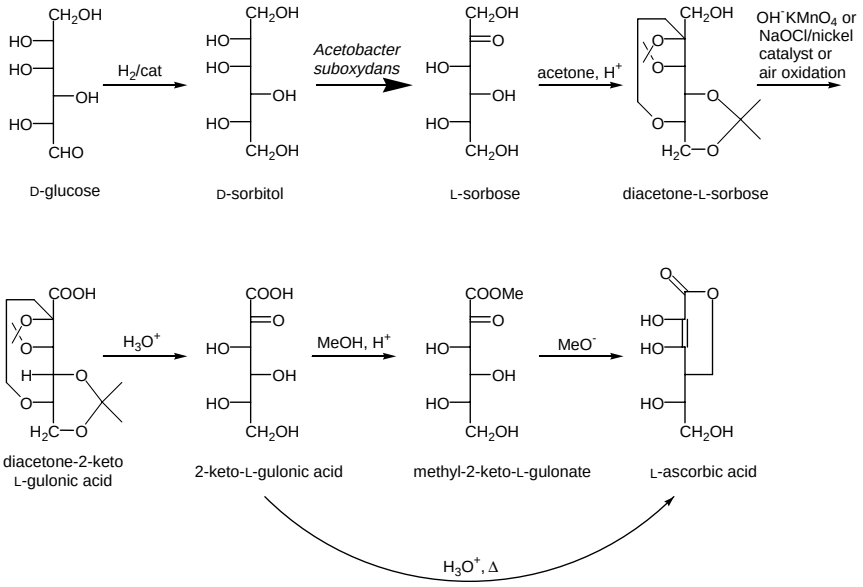

Fig. 6 Reichstein-Grüssner synthesis of vitamin C (L-ascorbic acid).

L-Sorbose became important in the **mid-1930's** as an intermediate in the Reichstein-Grüssner synthesis of L-ascorbic acid [13].

In **1953**, Peterson at al. [14] reported that *Rhizopus arrhius* converted progesterone to 11α-hydroxyprogesterone (figure 7), which was used as an intermediate in the synthesis of cortisone.

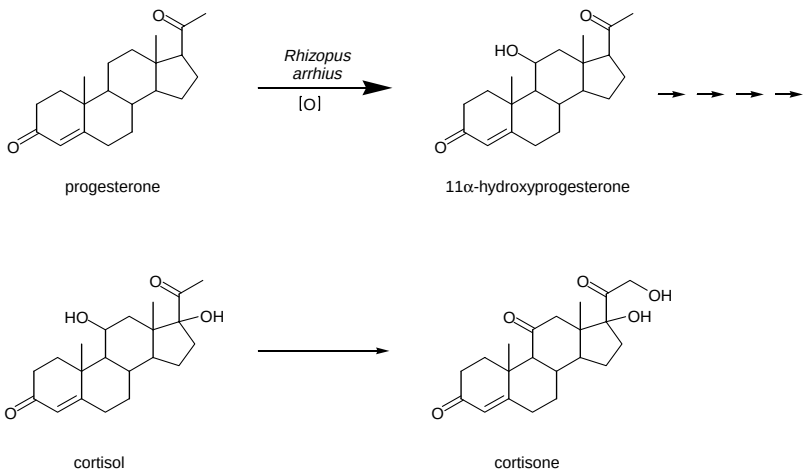

Fig. 7 Microbial 11α-hydroxylation of progesterone.

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 [15] (figure 8) from deoxycholic acid that was 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 quickly 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 [16].

In the **1950's**, the double helix structure and the chemical nature of RNA and DNA – the genetic code of heredity – were discovered. This discovery can be regarded as one of the milestones among this century's main scientific achievements. It led to the synthesis of recombinant DNA and gave a fillip to genetic engineering in the seventies'. Such developments quickly made the rDNA technology a part of industrial microbial transformations. Application of this technology for the production of small molecules began in **1983**. Ensley et al. [17] 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 naphthalene to salicylic acid is encoded by 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 showed 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* (figure 9).

Genencor International is developing a commercially competitive biosynthetic route to indigo using recombinant *E.coli* that can directly synthesize indigo from glucose [18]. Anderson et al. in **1985** [19] reported the construction of a metabolically engineered bacterial strain that was able to synthesize 2-keto-L-gulonic acid (figure 10), a key intermediate in the production of L-ascorbic acid (vitamin C).

BASF, Merck and Cerestar are building a 2-keto-L-ketogulonic acid plant in Krefeld, Germany. The start up of operation is scheduled for **1999**. They developed a new fermentation route from sorbitol directly to the ketogulonic acid [20]. This method is probably similar to the method described in **1966** [21].

The Cetus Corporation (Berkeley, California, USA) bioprocess for converting alkenes to alkene oxides emerged in **1980** [22]. This bioprocess appeared to be very interesting, thanks to the possibility of replacing energy-consuming petrochemical processes.

There were high hopes that the development of recombinant DNA technology would speed up technological advances. Unfortunately, there is still a lot left to be done about the development and application of bioprocesses before the commercial production of low-value chemicals becomes feasible [23]. However, today even the traditional chemical companies like Dow Chemical, DuPont, Degussa-Hüls AG etc., pressurized by investors and technological advances, are trying to use microbial or enzymatic transformations in production. They are doing this to see whether natural feedstocks can bring more advantages than crude oil. One only needs to compare the cost of a barrel of oil with that of corn starch to see that the latter is quite cheaper [20].

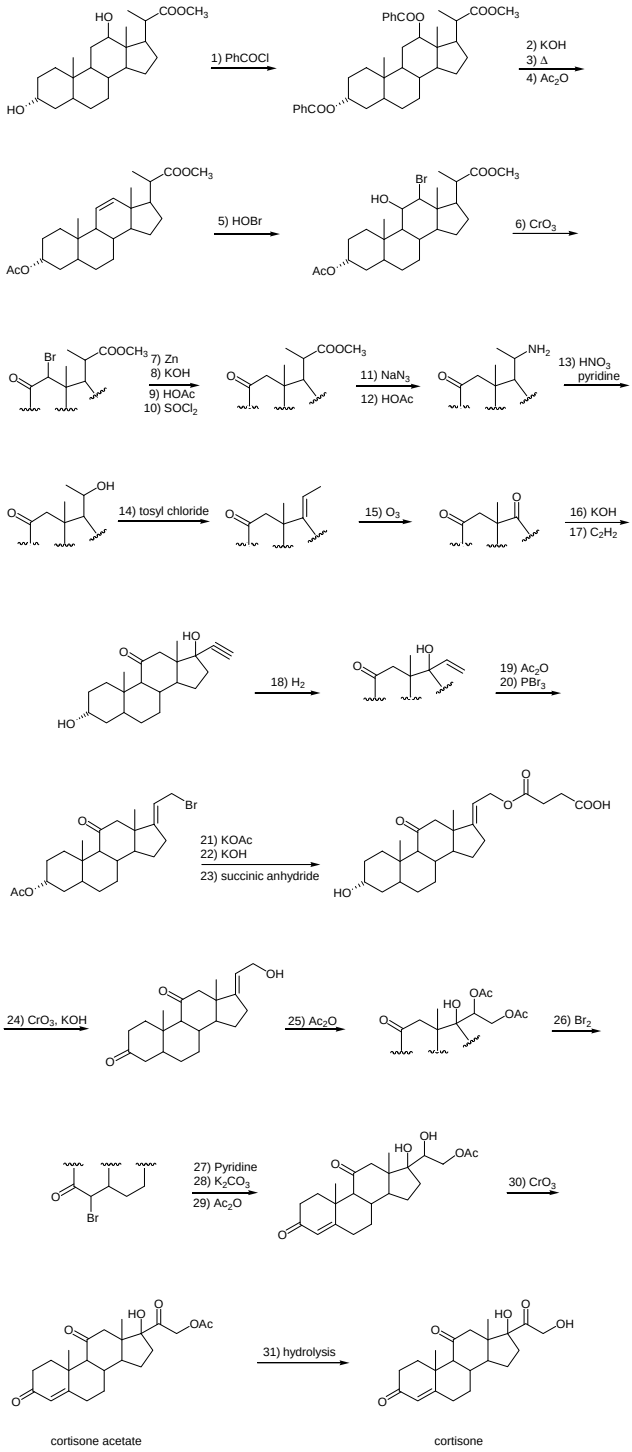

Fig. 8 Chemical synthesis of cortisone.

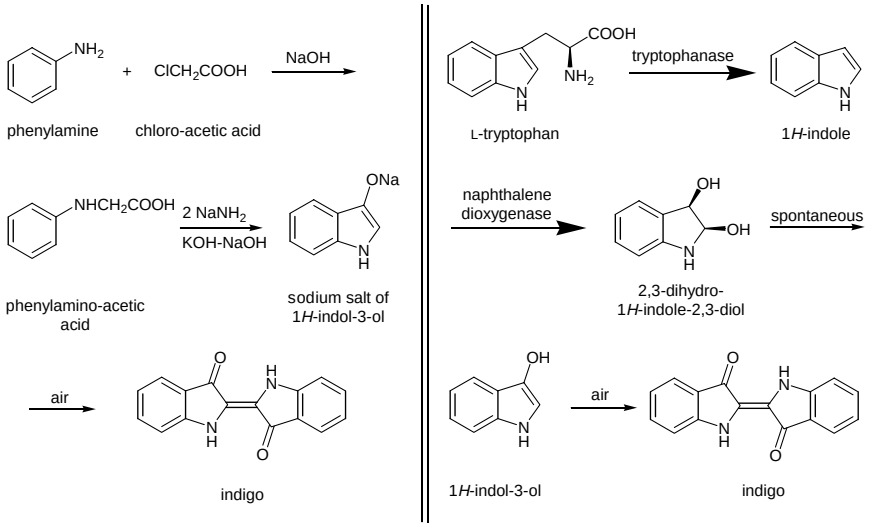

Fig. 9 Comparison of chemical and biological routes to indigo.

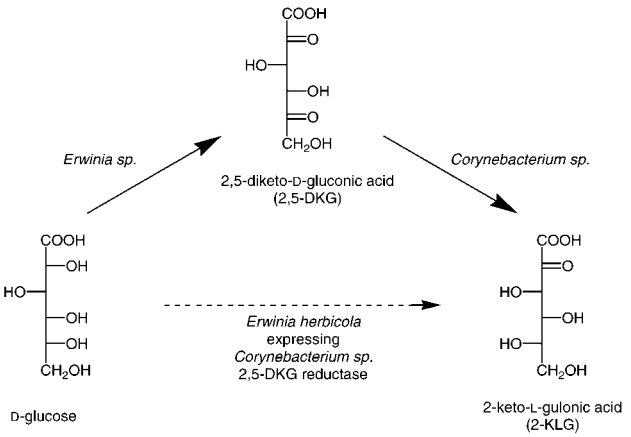

Fig. 10 Biosynthesis of 2-keto-L-gulonic acid.

Acrylamide is one of the most important commodity chemicals. Its global consumption is about 200,000 tonnes per year. It is used in the production of various polymers for use as flocculants, additives or for petroleum recovery. In conventional synthesis, copper salts are used as catalysts in the hydration of nitriles. However, this is rather disadvantageous as the preparation of the catalysts is quite complex. Additionally, it is difficult to regenerate the used catalyst and separate and purify the formed acrylamide. Furthermore, since 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 pro-

cess 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 tons of acrylamide per year for Nitto [24,25]. The use of biocatalyst for the production of acrylamide may not be the first case in which biotransformation as a part of biotechnology was used in the petrochemical industry. However, it is the first successful example of the introduction of an industrial biotransformation process for the manufacture of a commodity chemical (figure 11).

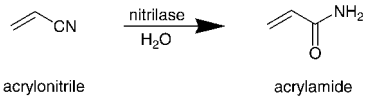

Fig. 11 Acrylamide synthesis.

Some representative industrial microbial transformations are listed in Table I.

Table I: 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	Schering 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, Cerestar, Germany

2.2 From gastric juice to SweetzymeT – The history of enzymatic biotransformations

Enzymes were in use for thousands of years before their nature was gradually understood. No one really knows when the calf stomach was used as a catalyst for the first time 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 [26]. In **1876**, Kühne (figure 12) 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** [27]. At that time two terms were used: "organized ferment" such as cell-free yeast extract from Büchner, and "unorganized ferment" such as gastric juice secreted by cells. Today the terms "intracellular" and "extracellular" are used. Kühne also presented some interesting results from his experiments with trypsin. The word "enzyme" comes from Greek for "in yeast" or "leavened" [28].

Microorganisms synthesize numerous enzymes, each having its own 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 United States 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 [29].

In **1894**, Emil Fischer [30,31] observed in his studies of sugars that the enzyme called emulsin catalyzes the hydrolysis of β-methyl-D-glucoside, while the enzyme called maltase is active towards the α-methyl-D-glucoside as substrate (figure 13).

This led Fischer to suggest his famous "lock–and-key" theory of enzyme specificity, which he would describe in his own words 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].

In **1913**, Michaelis and 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. This led to the development of the famous Michaelis-Menten equation to describe the typical saturation kinetics observed with purified enzymes and single substrate reactions [32].

By **1920**, about a dozen enzymes were known, none of which had been isolated [33]. Then, in **1926**, Sumner [34] crystallized urease from jack bean, *Canavalia ensiformis,* and announced that it was a simple protein.

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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. 12 W. F. Kühne [27].

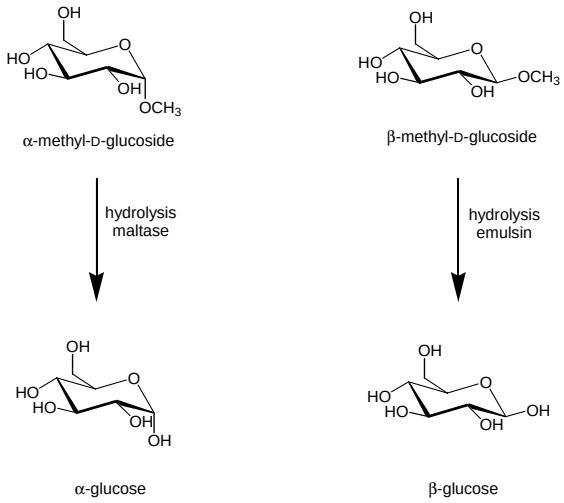

Fig. 13 Emil Fischer' s substrates.

Northrop and his colleagues [26] 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 classical crystallization experiments. By the late **1940s** many enzymes were available in pure form and in sufficient quantity for investigation of their chemical structure. Currently, more than 3,000 enzymes have been catalogued [35]. The ENZYME data bank contains information related to the nomenclature of enzymes [36]. The current version contains 3,705 entries. It is available through the ExPASy WWW server (http://www.expasy.ch/). Several hundreds of enzymes can be obtained commercially [37].

In **1950**, there was still no evidence that a given protein had a unique amino acid sequence. Lysosyme was the first enzyme whose tertiary-structure (figure 14) was defined in **1966** with the help of X-ray crystallography [38].

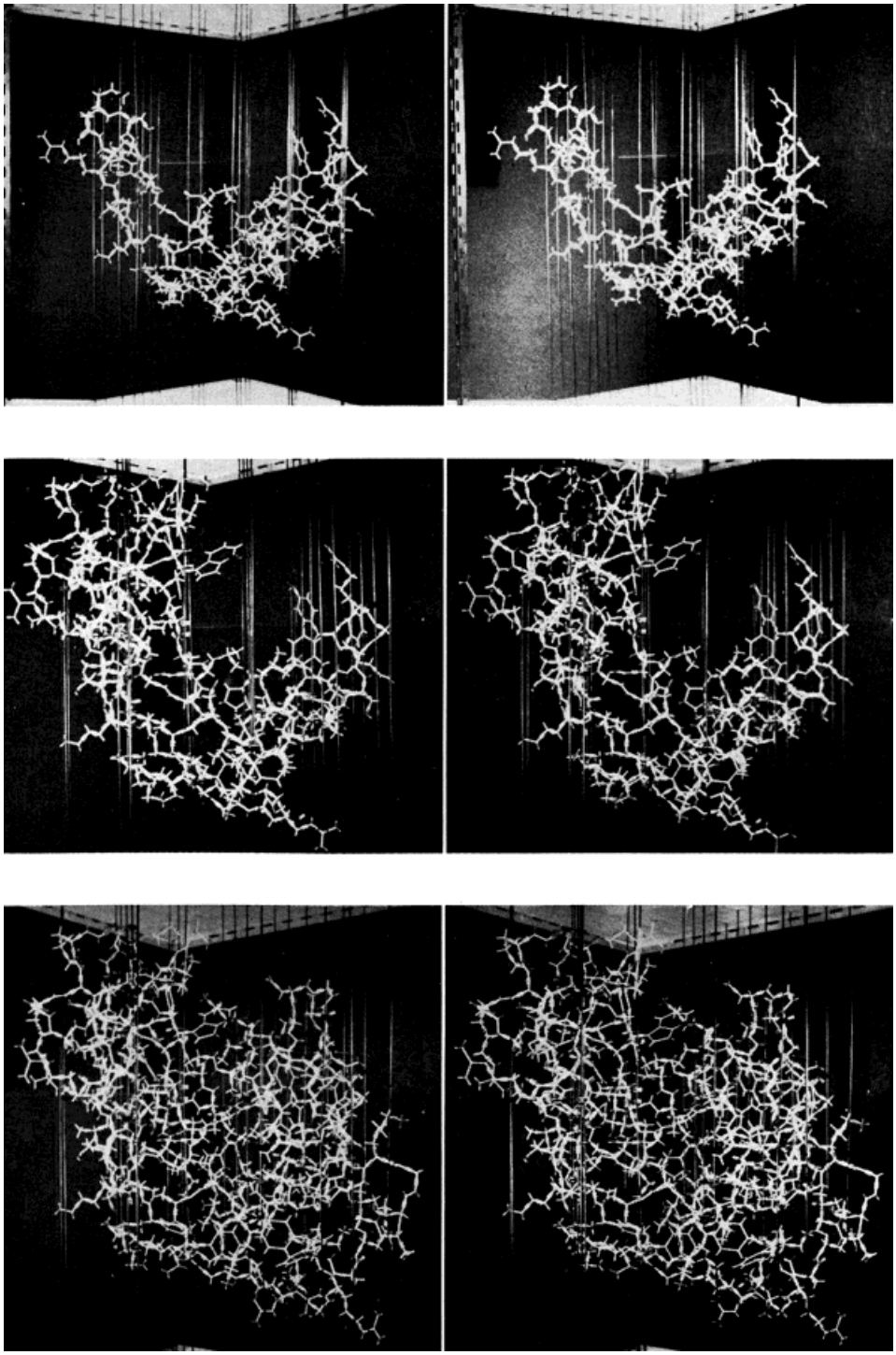

Fig. 14 Stereo photographs of models of part of the lysozyme molecule [38].

Further, ribonuclease A was one of the first enzymes made on a laboratory scale by organic chemistry methods. In **1969**, Gutte and Merrifield synthesized its whole sequence in 11,931 steps [39].

By **1970**, the complete molecular structures of several enzymes had been established and plausible reaction mechanisms could be discussed [26].

Hill (**1897**) was the first to show that the biocatalysis of hydrolytic enzymes is reversible [40].

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 [41].

While the first benefit for the industry from the microbiological development had come early, the investigations with isolated enzymes hardly influenced the industry at that time. Consequently, industrial enzymatic biotransformations have a much shorter history than microbial biotransformations in the production of fine chemicals.

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 as the preferred reagent was unavailable at that time (figure 15) (42).

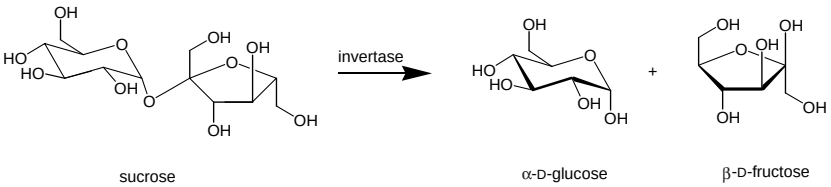

Fig. 15 Inversion of sucrose by invertase.

Yeast cells were autolysed 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 used for syrup decolorisation. The scale of 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 since the limiting factor was microbial contamination or loss of decolorising 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-hydrolysed material. This is the reason why the immobilized enzyme was abandoned once the acid became available again [42].

Industrial processes for L-amino acid production based on the batch use of soluble aminoacylase were already in use in **1954**. However, like many batch processes with soluble enzymes, they had their disadvantages such as higher labor costs, complicated product separation, low yields, high enzyme costs and non-reusability of 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 (figure 16). This was the first full scale industrial use of an immobilized enzyme. The most important advantages are the relative simplicity and ease of control [44].

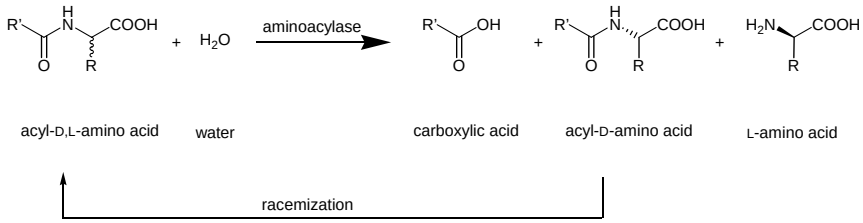

Fig. 16 L-Amino acid production catalyzed by aminoacylase.

In a membrane reactor system developed at Degussa-Hüls AG in Germany in **1980** [45], native enzymes, either pure or of technical grade, are used in homogeneous solution for the large scale production of enantiomerically pure L-amino acids (figure 17).

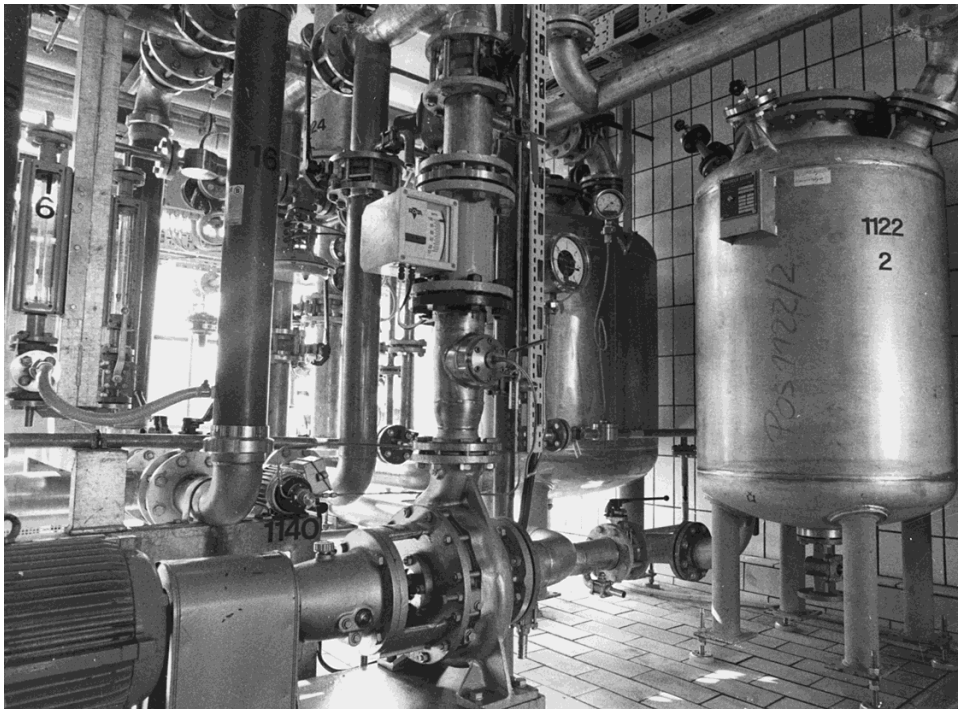

Fig. 17 Enzyme membrane reactor (Degussa-Hüls AG, Germany).

A membrane reactor is particularly well suited for cofactor-dependent enzyme reactions, especially if the cofactor is regenerated by another enzyme reaction and retained by the membrane in modified form [46]. There are several advantages of carrying out biocatalysis in membrane reactors over heterogeneous enzymatic catalysis: there are no mass transfer limitations, enzyme deactivation can be compensated for by adding soluble enzyme and the reactors can be kept sterile more easily than immobilized enzyme systems. The product is mostly pyrogen free (major advantage for the production of pharmaceuticals), because the prod-

uct 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 (figure 18) represents the largest use of an immobilized enzyme in the manufacture of fine chemicals.

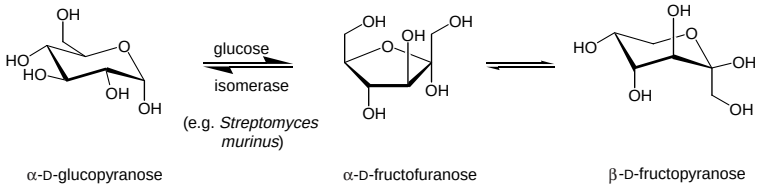

Fig. 18 Isomerization of glucose to fructose.

High-fructose corn syrup HFCS has grown to become a large-volume biotransformation product [47]. While sucrose is sweet, fructose is approximately 1.5 times sweeter and consequently high quality invert syrups (i.e. hydrolyzed sucrose) may be produced. Invert syrups contain glucose and fructose in a 1:1 ratio. However, the food industry needed a long time to become acquainted with the glucose isomerase potential to produce 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**, Clinton Corn Processing Company, Iowa, USA, was the first company to manufacture enzymatically produced fructose corn syrup [47].The glucose-isomerase catalyzed reversible reaction gave a product containing about 42 % of fructose, 50 % of glucose and 8 % of other sugars. Due to various reasons, economic viability being the more 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 started only in **1974**. It was initiated by Clinton Corn Processing [44]. 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 run out through the sieve at the bottom of the 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 called Kato Kagaku in Kohwa near Nagoya. The reaction conditions are pH 7.5 and T = 55 °C. Though enzyme activity is reduced at this temperature, its stability and productivity are considerably improved [48].

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 1,800 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)/ kg of Sweetzyme T. The normal column productivity was 8,000–10,000 kg / kg enzyme. The 687 days' record for Sweetzyme T is also a world record in the starch industry [48] (figure 19).

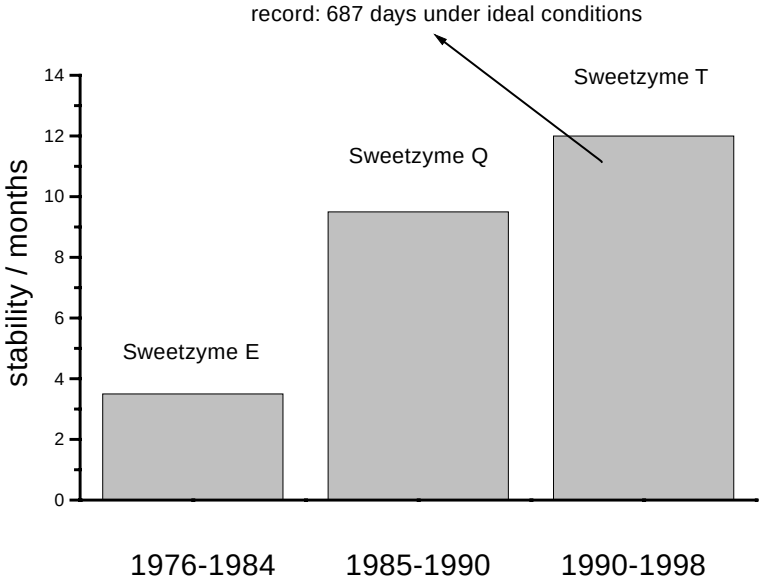

Fig. 19 Improved biocatalyst stability by biocatalyst engineering at Novo.

"Central del Latte" of Milan, Italy, was the first company which commercially hydrolyzed milk lactose with immobilized lactase using SNAMprogetti technology [49]. An industrial 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 hydrolyses lactose, a sugar with poor solubility properties and a relatively low degree of sweetness, to glucose and galactose (figure 20).

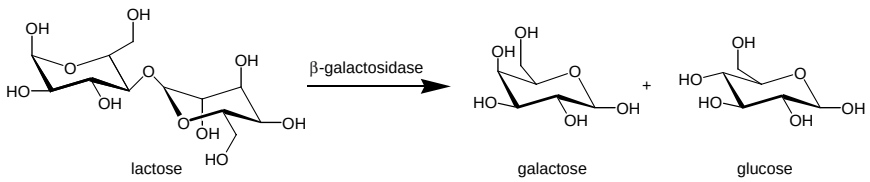

Fig. 20 β-Galactosidase catalyzed hydrolysis of lactose to galactose and glucose.

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 enables the manufacture of a high-quality dietary milk at low cost. This milk has a remarkable digestive tolerance, pleasant sweetness, unaltered organoleptic properties, and good shelf-life. It does not contain foreign matter. The industrial plant is shown in figure 21.

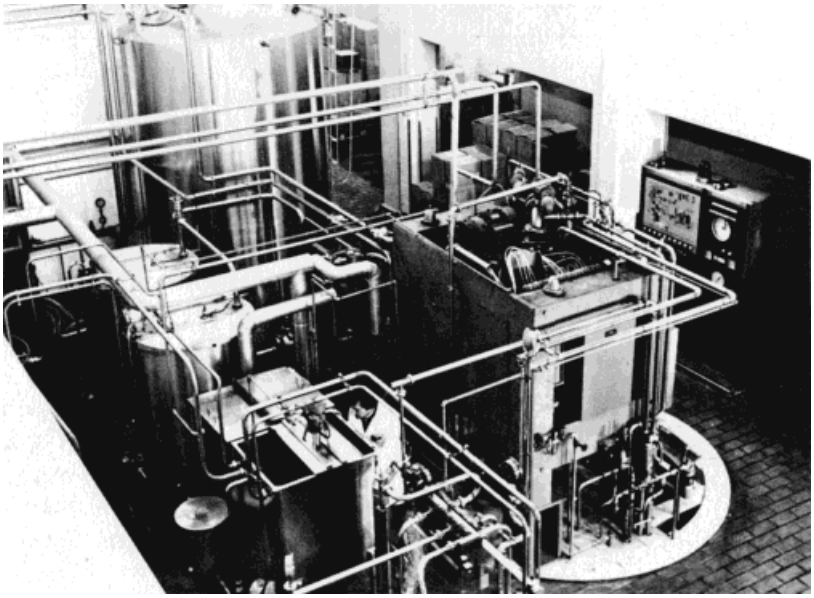

Fig. 21 Industrial plant for processing low – lactose milk [49].

Penicillin G, present in *Penicillum notatum* and discovered by Fleming in **1929**, revolutionized chemotherapy against pathogenic microorganisms. Today, β-lactam antibiotics such as penicillins and cephalosporins are very widely used. Thousands of semisynthetic β-lactam antibiotics are being synthesized to find more effective compounds. Most of 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, is being used since around **1973** (figure 22). Several chemical steps are replaced by a single enzymatic reaction. Organic solvents, the use of low temperature (-40 °C) and the need for absolutely anhydrous conditions, which made the process difficult and expensive, were no longer necessary in the enzymatic process [50].

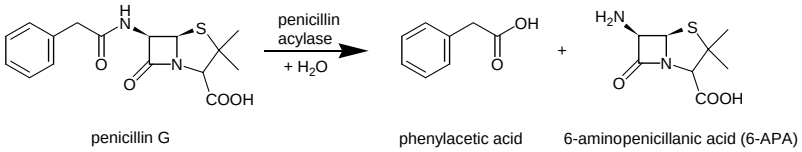

Fig. 22 Enzymatic synthesis of 6-aminopenicillanic acid (6-APA).

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 Japan, developed and succeeded in the industrial production of 7-ACA by a chemoenzymatic two-step process starting from cephalosporin C (figure 23):

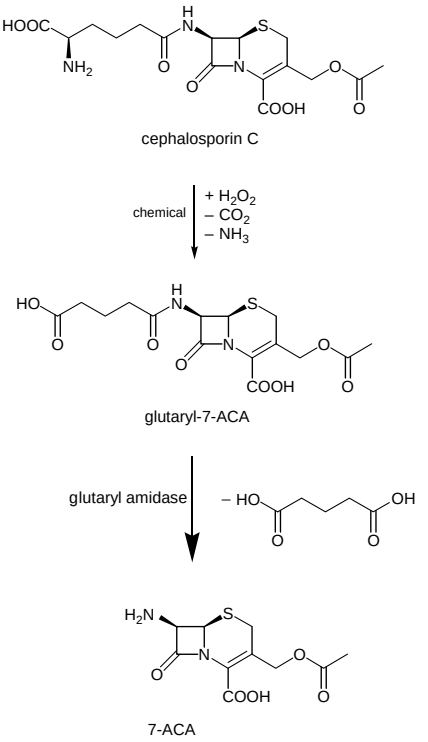

Fig. 23 Two-step process of 7-ACA production from cephalosporin C.

The chemical process requires highly purified cephalosporin C as raw material. A number of complicated reaction steps are carried out at –40 °C to –60 °C, and the reaction time is long. Furthermore, hazardous reagents, such as phosphorous pentachloride, nitrosyl chloride and pyridine are used in this process. The 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 the glutaryl-7-ACA amidase, the enzyme that catalyzes the deacylation of cephalosporin C. Because of this change in pH the reaction rate is decreased, requiring strict pH control during the reaction process. For these reasons, a recirculation bioreactor with immobilized glutaryl-7-ACA amidase and an automatic pH controller were designed for the 7-ACA production. The bioreactor for industrial 7-ACA production is shown in figures 24 and 25. The process has been in operation at Asahi Chemical Industry since **1973**. It is reported that about 90 tons of 7-ACA are thus produced annually [51].

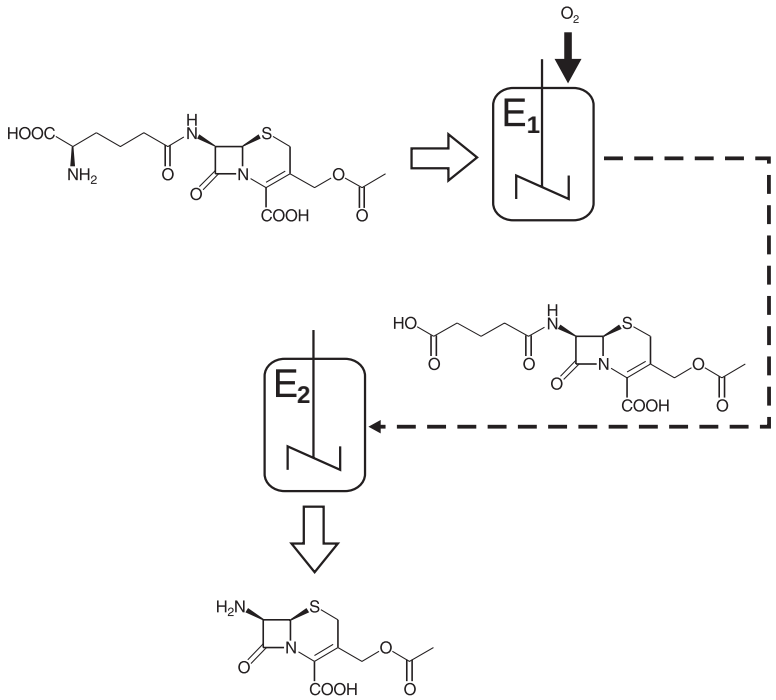

Fig. 24 Flow scheme for the production of 7-ACA. Production carried out at Asahi Chemical Industry. (**E_1** = D-aminoacid oxidase; **E_2** = glutaryl amidase).

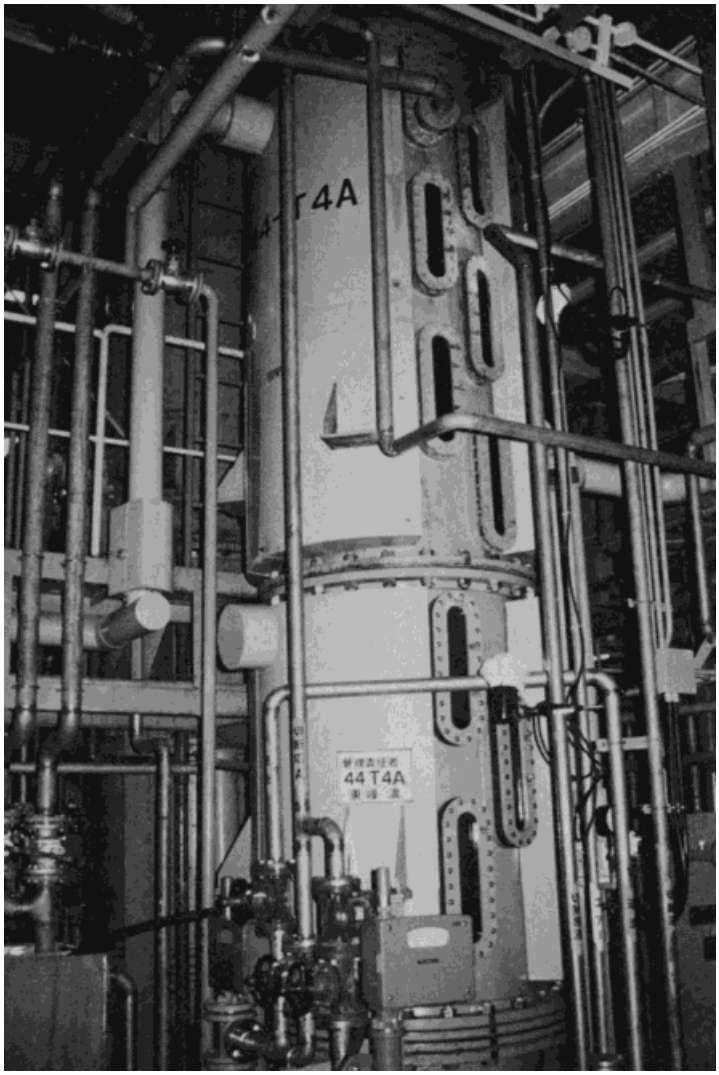

Fig. 25 The bioreactor plant for 7-ACA production carried out at Asahi Chemical Industry (Reprinted from Ref. [51], p. 83 by courtesy of Marcel Dekker Inc.).

Four technological advances, having major impact on enzymatic biotransformations, were required for the acceptance of enzymes as 'alternative catalysts' in industry [52].

The first technological advance was the development of large-scale techniques for the release of enzymes from the interior of microorganisms [53]. Although the majority of industrial purification procedures are based on the same principles as those employed at laboratory scale, the factors under consideration while devising industrial scale purification regimes are somewhat different. When isolating enzymes on an industrial scale for commercial purposes, a prime consideration has to be the cost of production in relation to the value of the end prod-

uct. Therefore, techniques used on a laboratory scale are not always suitable for large scale work [54]. Production and isolation of an intracellular microbial enzyme are quite expensive. The costs of the usage of water-soluble protein as catalyst for biotransformations can be justified only by its repeated use [55].

The second technological advance was the development of techniques for large-scale immobilization of enzymes. As mentioned earlier, the first enzyme immobilized in the laboratory was invertase, adsorbed onto charcoal in the year **1916** [56]. However, only after the development of immobilization techniques on a large scale occured in the **1960s**, many different industrial processes using immobilized biocatalysts have been established. The historical invertase column operating since **1968** on a laboratory scale is shown in figure 26.

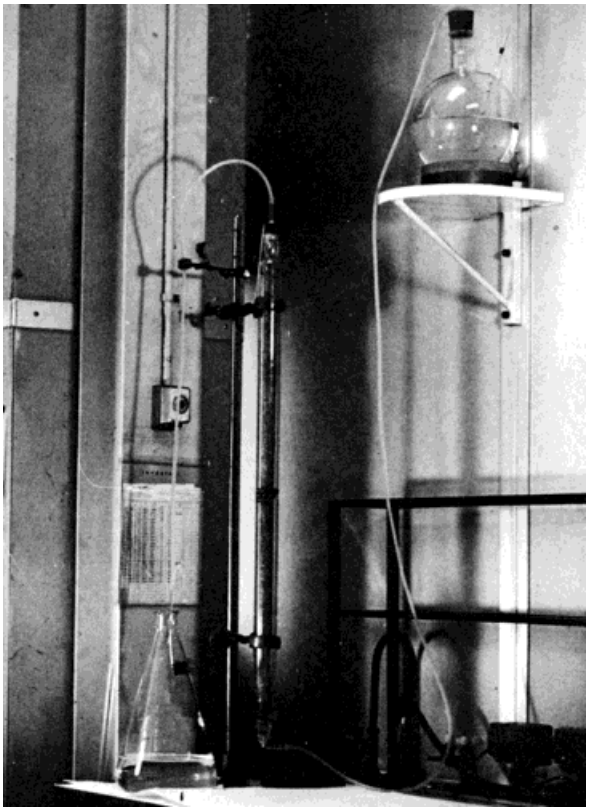

Fig. 26 Historical invertase column [49].

It was shown that by increasing the concentration of sucrose, the efficiency of the fiber-entrapped invertase (which hydrolyses 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 owing to diffusion limitations [49].

Table II lists some industrial biotransformations performed by isolated enzymes.

Table II: Selected historical, industrial applications of isolated enzymes.

Product	Biocatalyst	Operating since	Company
L-amino acid	aminoacylase	1954, 1969	Tanabe Seiyaku Co. Ltd., Japan
6-aminopenicillanic acid	penicillin acylase	1973	SNAMProgetti and others*
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

* Beecham, Squibb, Astra Lakenedal, Bayer, Gist-Brocades, Pfizer, Bristol Myers, Boehringer Mannheim, Biochemie, Novo, Hindustan Antibiotics

The first Enzyme Engineering Conference was held at Hennicker, New Hampshire, 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 [57].

The third technological advance was the development of techniques for biocatalysis in organic media. The usage of very high proportions of organic solvents for increasing the solubility of reactants was examined in **1975** in the reaction with isolated cholesterol oxidase to produce cholestenone [58]. The enzymatic synthesis was believed to be incompatible with most organic syntheses carried in nonaqueous media. This changed after Klibanov [59] recognized in **1986** that most enzymes could function quite well in organic solvents. Since that time different processes involving an organic phase have been established in industry (Table III).

Table III: Industrial biotransformations involving poorly water-soluble reactants.

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

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

Generally, microorganisms isolated from nature produce the desired enzyme at levels which are too low to offer a cost-effective production process. Consequently, the 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* [60], i.e. improvement by mutation and selection, has been successfully used in many industrial microbiological fields for many years. In **1978**, Clarke showed that evolution processes can be performed on a laboratory scale. Microevolution occuring in bacterial cultures grown in the chemostat gives rise to altered enzyme specificity, enabling microorganisms to degrade some unusual synthetic organic compounds. Successive muta-

tional 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 [61]. The second method is *hybridization*. It 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 another strain.

Recombinant DNA technology has dramatically changed enzyme production, because enzymes are synthesized in cells by the normal protein synthesis methods [62,63]. A 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 to plug in a new enzyme variant and to be very quick at manufacturing levels [64]. Novel microbial catalysts, together with recent advances in molecular biology, offer scientists an opportunity to rapidly evolve selected genes and considerably improve bacterial biocatalysts [65]. For example, a method for the rapid generation of thermostable enzyme variants has been developed [66,67]. This is done 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 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 have a change in 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 course of time they will make enzymes excellent catalysts fulfilling all the requirements for industrial use. This research field may be referred to as **biocatalyst engineering** [23].

2.3 Advantages of biotransformations over classical chemistry

Enzymes are proteins, things of beauty and a joy forever [26]

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 process energy. Biocatalysis has proven to be a useful supplementary technology for the chemical industry, allowing, in some cases, reactions which are not easily conducted by classical organic chemistry or, in other cases, allowing reactions which can replace several chemic-

al steps. Today, highly chemo-, regio- and stereoselective biotransformations can simplify manufacturing processes and make them even more economically attractive and environmentally acceptable [68].

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 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 for 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 a replacement to conventional chemical methods, appears to be growing. New niche applications continue to be discovered in otherwise mature segments [69]. It appears that enzyme-based processes are gradually replacing conventional chemical-based methods. Finally, the latest literature on enzymology suggests that other biocatalysts will add to future sales, both in established and new markets. The enzyme "nitrogenase", converting dinitrogen to ammonia, a basic chemical compound, has been discovered recently [70]. Dream reactions of organic chemists might become true in the future, with biocatalysts where functional or chiral groups are introduced into molecules by utilizing H_2, O_2 or CO_2. Recently Aresta reported of a carboxylase enzyme that utilizes CO_2 in the synthesis of 4-hydroxybenzoic acid starting from phenyl-phosphate [71].

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. Chemical methods are not suitable for large scale production of D-amino acids at the moment due to low yield and high cost. Most L-amino acids are efficiently manufactured by fermentation, but D-amino acids are hardly 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 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, 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 [72]:

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 [73] used in the production of L-DOPA, having a final product concentration of 110 $g \cdot L^{-1}$, 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, 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 Parkinsonism.

It is difficult to directly assess 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 advantage lies in stereoselective reactions. A good example of its technological power and commercial potential is the aforementioned stereoselective hydroxylation of steroids.

In comparison to fermentation processes fewer side-products are formed in enzymatic biotransformations, complex and expensive fermenters are not required, aeration, agitation and sterility need not necessarily be maintained and the substrate is not diverted into the formation of *de novo* cellular biomass [50]. 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 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 to be used in reactors made of special resistant materials to tolerate extreme conditions, such as the use of concentrated acids under elevated temperatures and pressures [50].

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3 Enzyme Classification

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3.1 The Enzyme Nomenclature

In early times of biochemistry there were no guidelines for naming enzymes. The denomination of newly discovered enzymes was given arbitrarily by individual workers. This practice had proved to be inadequate. Occasionally two different enzymes had the same name while in other cases two different names were given to the same enzyme. Furthermore, there emerged denominations which provided no clue about the catalyzed reaction (e.g. catalases, or pH 5 enzyme).

With the great progress experienced by biochemistry in the 1950's, a large number of enzymes could be isolated and characterized. By this time it became evident that it was necessary to regulate the enzyme nomenclature. So, the International Union of Biochemistry and Molecular Biology (IUBMB), formerly International Union of Biochemistry (IUB), set up in consultation with the International Union for Pure and Applied Chemistry (IUPAC), an Enzyme Commission in charge of guiding the naming and establishing a systematic classification for enzymes. In 1961, the report of the commission was published. The proposed classification was used to name 712 enzymes. This work has been widely used as a guideline for enzyme nomenclature in scientific journals and textbooks ever since. It has been periodically updated, new entries have been included or old ones have been deleted, while some other enzymes have been reclassified. The sixth complete edition of the Enzyme Nomenclature (1992) contains 3196 enzymes [1]. Five supplements to the Enzyme Nomenclature with various additions and corrections have been published until today [2,3,4,5,6] signaling the constantly growing number of new enzyme entries. An update documentation of the classified enzymes is available on the ENZYME data bank server [7,8].

The Enzyme Nomenclature suggests two names for each enzyme, a **recommended name** convenient for every day use and a **systematic name** used to minimize ambiguity. Both names are based on the nature of the catalyzed reaction. The recommended name is often the former trivial name, sometimes after little change to prevent misinterpretation. The systematic name also includes the involved substrates. This taxonomy leads to the classification of enzymes into six main classes (Table 1).

Table 1: The main enzyme classes

Enzyme class	Catalyzed reaction
1. Oxidoreductases	oxidation-reduction reactions
2. Transferases	transfer of functional groups
3. Hydrolases	hydrolysis reactions
4. Lyases	group elimination (forming double bonds)
5. Isomerases	isomerization reactions
6. Ligases	bond formation coupled with a triphospate cleavage

As the systematic name may be very extensive and uncomfortable to use, the Enzyme Commission (EC) has also developed a numeric system based on the same criteria, which can be used together with the recommended name to specify the mentioned enzyme. According to this system, each enzyme is assigned a four-digit EC number (Table 2). The first digit denotes the main class that specifies the catalyzed reaction type. These are divided into subclasses, according to the nature of the substrate, the type of the transferred functional group or the nature of the specific bond involved in the catalyzed reaction. These subclasses are designated by the second digit. The third digit reflects a further division of the subclasses according to the substrate or co-substrate, giving origin to sub-sub-classes. In the fourth digit a serial number is used to complete the enzyme identification.

Table 2: Constitution of the four-digit EC number

EC number EC (i).(ii).(iii).(iv)	
(i)	the main class, denotes the type of catalyzed reaction
(ii)	sub-class, indicates the substrate type, the type of transferred functional group or the nature of one specific bond involved in the catalyzed reaction
(iii)	sub-subclass, expresses the nature of substrate or co-substrate
(iv)	an arbitrary serial number

As an example, aminoacylase (*N*-acyl-L-amino-acid amidohydrolase, according to the systematic nomenclature), an enzyme used in the industrial production of L-methionine, has the classification number EC 3.5.1.14 (see process on page 300). The first number (i = 3) indicates that this enzyme belongs to the class of hydrolases. The second number (ii = 5) expresses that a carbon-nitrogen bond is hydrolyzed and the third number (iii = 1) denotes that the substrate is a linear amide. The serial number (iv = 14) is needed for full classification of the enzyme.

As the biological source of an enzyme is not included in its classification, it is important to mention this together with the enzyme number for full identification. So the enzyme used in the production of "acrylamide" should be mentioned as "nitrilase (EC 4.2.1.84) from "*Rhodococcus rhodochrous*" (see process on page 362).

An important aspect concerning the application of the enzyme nomenclature is the direction how a catalyzed reaction is written for purposes of classification. To make the classification more transparent the direction should be the same for all enzymes of a given class, even if this direction has not been demonstrated for all enzymes of this class. Many examples for the use of this convention can be found in the class of oxidoreductases.

A further implication of this system is the impossibility of full classification of an enzyme if the catalyzed reaction is not clear. Complete classification of the enzymes only depends on the natural substrates. Non-natural substrates are not considered for the classification of the biocatalyst.

Finally, it is important to emphasize that the advantageous influence of the enzyme classification is not limited to biochemistry's enzyme nomenclature. It is also very beneficial for organic preparative chemists because it facilitates the choice of enzymes for synthetic applications. Since the classification of the enzymes is based on the catalyzed reactions it helps chemists to find an appropriate biocatalyst for a given synthetic task. An analogous nomenclature for chemical catalysts has not been set up until today.

The number of existing enzymes in nature is estimated to reach the 25,000 mark [9]. It is one essential part of biochemistry and related sciences to try to find and identify them. The scientist isolating and characterizing a new enzyme is free to report the discovery of that "new" biocatalyst to the Nomenclature Committee of the IUBMB and may form a new systematic name for this enzyme. An appropriate form to draw the attention of the editor of the Enzyme Nomenclature to enzymes and other catalytic entities missing from this list is available online [10].

3.2 The Enzyme Classes

The following part of this chapter aims at giving a compact overview of the six main enzyme classes and their subclasses. Since the industrial bioprocesses and biotransformations illustrated in the following chapters of the book are divided according to the involved enzymes and their classes, this short survey should provide the reader with the most important information on the enzyme classes.

The six main enzyme classes are resumed separately by giving a general reaction equation for every enzyme subclass according to the Enzyme Nomenclature. The reaction equations are picturized in a very general manner pointing out just the most important attributes of the catalyzed reactions. The authors would like to emphasize that no attempt has been made to provide a complete summary of the reactions catalyzed by the enzymes listed in the Enzyme Nomenclature. The reaction schemes have been elaborated to give reaction equations being as general and clear as possible and as detailed as necessary.

An important point that needs to be considered in this context concerns the enzymes classified as EC (i).99 or EC (i).(ii).99. These enzymes are either very substrate specific and therefore cannot be classified in already existing enzyme subclasses (or sub-subclasses) or a substrate of these enzymes has not been completely identified yet.

For instance, in the enzyme main class EC 5.(ii).(iii).(iv) (isomerases), the EC number 5.99 only describes "other isomerases" that cannot be classified within the other existing subclasses EC 5.1 to EC 5.5. It is important to point out that the enzymes classified with a 99-digit have not been considered in the reaction equations unless stated explicitly. The catalyzed reactions of these enzymes differ exceedingly from those of the other enzymes in the same main division.

The following short remarks on the generalized reaction schemes should help the reader to understand the illustrated enzyme catalyzed reactions:

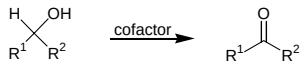
1. Each main enzyme class is introduced by a short paragraph giving a general idea of the respective enzymes.
2. By generalizing nearly all catalyzed reactions of one enzyme subclass to only one or a few reaction equations, some details of the single reactions had to be neglected, e.g. specification of the cofactor, reaction conditions (pH, temperature), electric charge or stoichiometry. Correct protonation of the substrates and products depending on the pH value of each reaction mixture has not been taken into consideration as well. Also, the enzyme itself does not appear in the reaction schemes of this chapter.
3. If the catalyzed reaction leads to a defined equilibrium, only one direction of this reaction is considered according to its direction in the Enzyme Nomenclature. In consequence, no equilibrium arrows are used in any reaction scheme of this chapter.
4. Enzymes of a given subclass may show some frequently appearing common properties or some very worthwhile uniqueness. These qualities are taken into account by additional comments below the reaction schemes.

EC 1 Oxidoreductases

The enzymes of this first main division catalyze oxidoreduction reactions, which means that all these enzymes act on substrates through the transfer of electrons. In the majority of the cases the substrate that is oxidized is regarded as hydrogen donor. Various cofactors or coenzymes serve as acceptor molecules. The systematic name is based on *donor:acceptor oxidoreductase*.

Whenever possible the nomination as a *dehydrogenase* is recommended. Alternatively, the term *reductase* can be used. If molecular oxygen (O_2) is the acceptor, the enzymes may be named as *oxidases*.

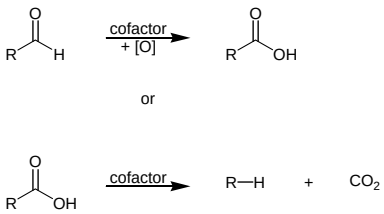
EC 1.1 Acting on CH-OH group of donors



R^1 = hydrogen, organic residue
R^2 = hydrogen, organic residue, alcoxy residue

The sub-subclasses are defined by the type of cofactor.

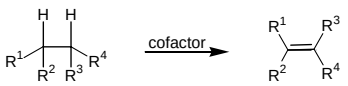
EC 1.2 Acting on aldehyde or oxo group of donors



R = hydrogen, organic residue

Analogous with the first depicted reaction, the aldehyde can be oxidized to the respective thioester with coenzyme A (CoA). In the case of oxidation of carboxylic acids, the organic product is not necessarily bound to hydrogen as suggested in the figure. It can also be bound to the cofactor. The sub-subclasses are classified according to the cofactor.

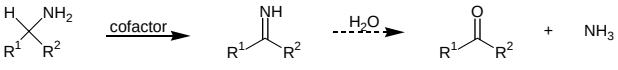
EC 1.3 Acting on the CH-CH group of donors



$R^{1,2,3,4}$ = hydrogen, organic residue

In some cases the residues can also contain heteroatoms, e.g. dehydrogenation of *trans*-1,2-dihydroxycyclohexa-3,5-diene to 1,2-dihydroxybenzene (catechol). Further classification is based on the cofactor.

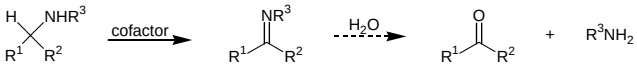
EC 1.4 Acting on the CH-NH_2 group of donors



$R^{1,2}$ = hydrogen, organic residue

In most cases the imine formed is hydrolyzed to give an oxo-group and ammonia (deaminating). The division into sub-subclasses depends on the cofactor.

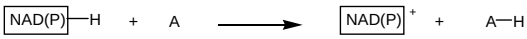
EC 1.5 Acting on the CH-NH group of donors



$R^{1,2}$ = hydrogen, organic residue
R^3 = organic residue

In some cases the primary product of the enzymatic reaction may be hydrolyzed. Further classification is based on the cofactors.

EC 1.6 Acting on NAD(P)H



A = acceptor

Generally enzymes that use NAD(P)H as reducing agent are classified according to the substrate of the reverse reaction. Only enzymes which need some other redox carrier as acceptors to oxidize NAD(P)H are classified in this subclass. Further division depends on the redox carrier used.