INDUSTRIAL USE OF ENZYMES

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Summary

Enzymes have been used since time immemorial in cheese manufacturing and indirectly, via yeasts and bacteria, in food manufacturing. Isolated enzymes were first used in detergents in 1914, their protein nature was proven in 1926, and their large-scale microbial production began in the 1960s. The industrial enzyme business is steadily growing thanks to improved production technologies, engineered enzyme properties, and new application fields. Most enzymes are produced by microorganisms with GRAS-status in large biological reactors known as fermentors. Usually the production organisms—and often the individual enzyme as well—have been genetically engineered for maximal productivity and optimized enzyme properties. Large-volume industrial enzymes are usually not purified but sold as concentrated liquids or granulated dry products. Enzymes used in special applications such as diagnostics or DNA technology need to be highly purified. Isolated enzymes have found several applications in the fine chemical industry. Enzymes are used in the production of enantiomerically pure amino acids and rare sugars. They are also used in the production of fructose and penicillin derivatives as well as several other chemicals. Enzymes should be considered as a part of a rapidly growing biocatalyst industry that also involves genetically optimized living cells as chemical production factories.

1. Historical Background

Most of the reactions in living organisms are catalyzed by protein molecules called enzymes. Enzymes can rightly be called the catalytic machinery of living systems. Humankind has indirectly used enzymes since almost the dawn of history. Enzymes are responsible for the biocatalytic fermentation of sugar to ethanol by yeasts, a reaction that forms the basis of making beer and wine. Enzymes oxidize ethanol to acetic acid. This reaction has been used in vinegar production for thousands of years. Similar microbial enzyme reactions of acid-forming bacteria and yeasts are responsible for aroma-forming activities in breadmaking and for preserving activities in sauerkraut preparation.

The fermentative activity of microorganisms was only discovered in the eighteenth century and finally proved by the French scientist Louis Pasteur. The term “enzyme” comes from the Latin with the literal meaning of “in yeast.” This was due to the fact that enzymes were closely associated with yeast activity. The study of enzymes is fairly recent. The first clear recognition of enzymes came in 1833 when scientists discovered that an alcohol precipitate of malt extract contained a thermolabile substance that converted starch into sugar. They called the substance diastase. We now know that this was an enzyme that we call amylase. Sumner finally proved the protein nature of enzymes in 1926 when he was able to crystallize urease enzyme from jack beans.

Probably the first application of cell-free enzymes was the use of rennin isolated from calves’ or lambs’ stomachs in cheese making. Rennin is an aspartic protease (see *Mechanisms of Enzyme Action*) which coagulates milk protein and has been used for hundreds of years by cheesemakers. Röhm in Germany prepared the first commercial enzyme preparation in 1914. This trypsin enzyme, isolated from animals, degraded proteins, and was used as a detergent. It proved to be so powerful compared with traditional washing-powders that German housewives’ suspicions were aroused by the small size of the original package, so the product had to be reformulated and sold in

larger packages. The real breakthrough for enzymes occurred with the introduction of microbial proteases into washing powders. The first commercial bacterial *Bacillus* protease was marketed in 1959, and became big business when Novozymes in Denmark started manufacturing it and major detergent manufacturers started using it around 1965.

In 1930, in addition to cheese manufacture, enzymes were already being used in the food industry in the manufacture of fruit juice. These enzymes, known as pectinases, clarify the juice and contain numerous different enzyme activities. The major usage of microbial enzymes in the food industry started in the 1960s in the starch industry. The traditional acid hydrolysis of starch was completely replaced by alpha-amylases and glucoamylases that could almost entirely convert starch to glucose. The starch industry became the second largest user of enzymes after detergent manufacture.

The industrial enzyme companies currently sell enzymes for a wide variety of applications. The estimated value of the world enzyme market is presently about US$1.3 billion and is predicted to grow to almost US$2 billion by 2005. Detergents (37%), textiles (12%), starch (11%), baking (8%), and animal feed (6%) are the main industries, and use about 75% of industrially-produced enzymes. Enzymes are also indirectly used in biocatalytic processes involving living or dead and permeabilized microorganisms. This review concentrates on the use of isolated enzyme preparations in large-scale and specialty applications and chemical manufacturing. The use of microorganisms as biocatalysts in chemical production is, however, an interesting and growing field. The techniques of genetic, protein, and pathway engineering are making chemical production by living cells an interesting green alternative to traditional chemical processes.

2. Enzyme Classification

To date over 2000 different enzyme activities have been isolated and characterized (see *Enzymology; Concept and Scope of Enzyme Action*)**.** The sequence information of a growing number of organisms opens the possibility of characterizing all the enzymes of an organism on a genomic level. The smallest known organism, *Mycoplasma genitalium*, contains 470 genes, of which 145 are related to gene replication and transcription. Baker's yeast has 7000 genes coding for about 3000 enzymes. Thousands of different variants of the natural enzymes are known. The number of reported three-dimensional enzyme structures is rapidly increasing. In the year 2000 the structure of about 1300 different proteins was known. The enzymes are classified into six major categories based on the nature of the chemical reaction they catalyze:

- **oxidoreductases** catalyze oxidation or reduction of their substrates;
- **transferases** catalyze group transfer;
- **hydrolases** catalyze bond breakage with the addition of water;
- **lyases** remove chemical groups from their substrates often forming a double bond;
- **isomerases** catalyze intramolecular rearrangements; and
- **ligases** catalyze the joining of two molecules at the expense of chemical energy.

Only a limited number of all the known enzymes are commercially available and even fewer are used in large quantities. More than 75 percent of industrial enzymes are hydrolases. Protein-degrading enzymes constitute about 40 percent of all enzyme sales. Proteinases have found new applications but their use in detergents is the major market. More than fifty commercial industrial enzymes are available and they are increasing steadily in number.

3. Enzyme Production

Some enzymes are still extracted from animal or plant tissues. Plant-derived commercial enzymes include proteolytic enzymes papain, bromelain, and ficin, and some other specialty enzymes such as lipoxygenase from soybeans. Animal-derived enzymes include proteinases like pepsin and rennin. Most enzymes, however, are produced by microorganisms in submerged cultures in large reactors called fermentors. The enzyme production process can be divided into the following phases:

1. Selection of an enzyme.
2. Selection of a production strain.
3. Construction of an overproducing strain by genetic engineering.
4. Optimization of culture medium and production conditions.
5. Optimization of recovery process (and purification if needed); and
6. Formulation of a stable enzyme product.

Criteria used in the selection of an industrial enzyme include specificity, reaction rate, pH and temperature optima and stability, effect of inhibitors, and affinity to substrates. Enzymes used in the paper industry should not contain cellulose-degrading activity as a side-activity because it would damage the cellulose fibers. Enzymes used in the animal feed industry must be thermo-tolerant to survive in the hot extrusion process used in animal feed manufacture. The same enzymes must have maximal activity at the body temperature of the animal. Enzymes used in industrial applications must usually be tolerant of various heavy metals and have no need for cofactors. They should already be maximally active in the presence of low substrate concentration so that the desired reaction proceeds to completion in a realistic timeframe.

3.1. Microbial Production Strains

When choosing the production strain several aspects have to be considered. Ideally the enzyme is secreted from the cell. This makes the recovery and purification process much simpler than in production of intracellular enzymes, which must be purified from thousands of different cell proteins and other components. Second, the production host should have a GRAS-status; in other words, be Generally Regarded As Safe. This is especially important when the enzyme produced by the organism is used in food processes. Third, the organism should be able to produce high amounts of the desired enzyme in a reasonable timeframe. The industrial strains typically produce over 50 grams per liter of extracellular enzyme proteins. Most of the industrial enzymes are produced by a relatively few microbial hosts like *Aspergillus* and *Trichoderma* fungi, *Streptomyces* mycelial bacteria, and *Bacillus* bacteria. Yeasts are not good producers of extracellular enzymes and are rarely used for this purpose. Most of the industrially used

microorganisms have been genetically modified to overproduce the desired activity and not to produce undesirable side-activities.

3.2. Enzyme Production by Microbial Fermentation

Once the biological production organism has been genetically engineered to overproduce the desired products, a production process has to be developed. The optimization of a fermentation process covers media composition, cultivation type, and process conditions. This is a demanding task and often involves as much effort as the intracellular engineering of the cell. The bioprocess engineer asks a whole range of questions such as whether the organism in question is safe or are extra precautions needed; what kind of nutrients the organism needs and what is their optimal/ economical concentration; how the nutrients should be sterilized; what kind of a reactor is needed (mass transfer, aeration, cooling, foam control, sampling); what needs to be measured and how the process is controlled; how the organism is cultivated (batch, fed-batch, or continuous cultivation), what the optimal growth conditions are, what the specific growth and product formation rate are, and what the yield and volumetric productivity are; how to maximize cell concentration in the reactor, whether the product is secreted out from the cells, how to degrade the cell if the product is intracellular, whether some of the raw materials or products inhibit the organism; and, finally, how the product is to be recovered, purified, and preserved. A typical enzyme production scheme is shown in Figure 1.

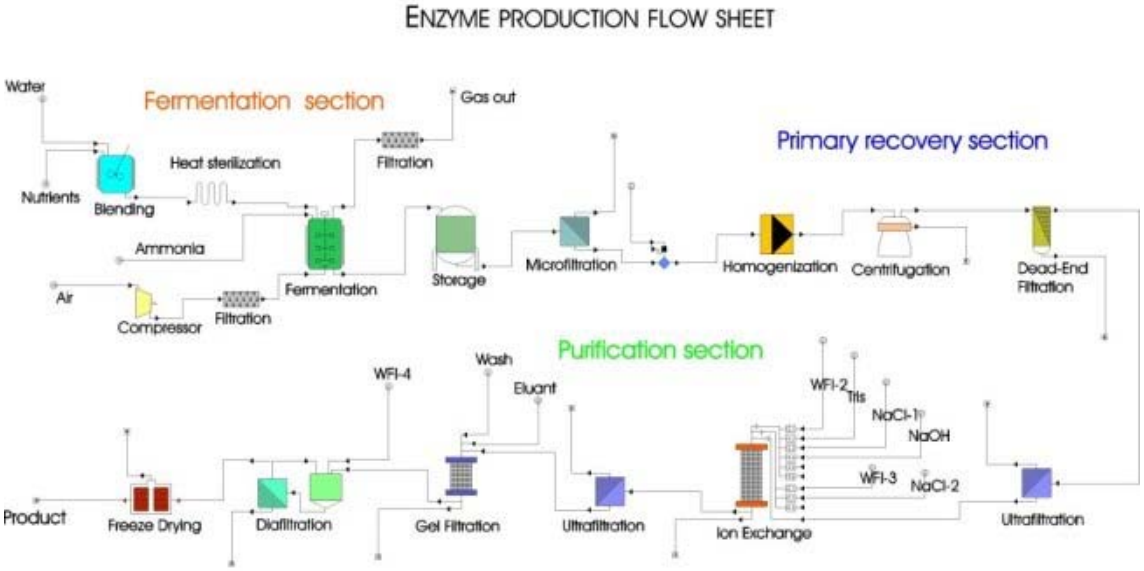

Figure 1. A typical enzyme production scheme. Large volume industrial enzymes are usually not purified. Their recovery is often finalized by an ultrafiltration step. Speciality enzymes need more purification.

The large-volume industrial enzymes are produced in 50–500 cubic meter fermentors. The extracellular enzymes are often recovered by ultrafiltration after the cells have been removed by vacuum drum filtration, separators, or microfiltration. If needed the purification is carried out by ion exchange or gel filtration. The final product is either a

concentrated liquid with the required preservatives such as salts or polyols, or alternatively it is granulated to a non-dusty dry product. Enzymes are proteins, and like any protein they can cause—and have caused in the past—allergic reactions. Protective measures are therefore necessary in both their production and their application.

4. Protein Engineering

Often enzymes do not have the desired properties for an industrial application. In such a case one can try to find a better enzyme from nature. For over 20 years there has been an extensive search for new enzyme variants in organisms that grow in extreme conditions, but this has met with relatively little success. Sometimes a desired property, such as extreme thermostability, has been found, but other problems have surfaced. The enzyme may not be functional in the desired temperature. It may also prove very difficult to overproduce the enzyme in a suitable host. Another option is to engineer a commercially available enzyme to be a better industrial catalyst. Two different approaches are currently available: a random method called directed evolution, and a protein engineering method called rational design. Table 1 summarizes some of the reasons why industrial enzymes need to be modified, and Table 2 describes some of the requisite tools used in modification work.

Enzyme	**Industry**	**Need**
xylanase	feed	temperature stability
		acid activity
	pulp and paper	temperature and alkali stability higher pH-optimum
glucoamylase	starch	higher pH-optimum
glucose isomerase	fructose	substrate specificity acid stability thermo stability
proteinase	detergent	thermostability
		alkali stability
		oxidative stability

Table 1. Change in enzyme characteristics by protein engineering

Target	**Method**
protein structure	crystallization
	x-ray crystallography
	NMR
modeling and simulation	computational methods
gene	plasmids expression systems
	targeted mutagenesis PCR

	DNA shuffling
	random mutagenesis

Table 2. Tools in protein engineering

Several enzymes have already been engineered to function better in industrial processes. These include proteinases, lipases, cellulases, α-amylases, and glucoamylases. Xylanase is a good example of an industrial enzyme that needs to be stable in high temperature and active in physiological temperatures and pH when used as a feed additive, and in alkaline conditions when it is used for bleaching in the pulp and paper industry. One of the industrial production organisms of xylanases is *Trichoderma* fungus. Its xylanase has been purified and crystallized. By designed mutagenesis its thermal stability has been increased about 2000 times at 70 ^{o}C and its pH-optimum shifted towards the alkaline region by one pH-unit. The three-dimensional structure of a xylanase enzyme is shown in Figure 2. The known structure of an enzyme is used to design and simulate mutations. The most successful strategies to improve the stability of the *Trichoderma* xylanase include the stabilization of the alpha-helix region and the N-terminus.

Figure 2. Three-dimensional structure of a *Trichoderma* xylanase II. This enzyme is used in baking to improve bread quality, in animal feed to improve digestibility of feed, in cellulose pulp bleaching to reduce the use of chlorine chemicals and in fruit juice manufacturing to facilitate juice extraction and clarification. The two active center glutamates and the one alpha helix are shown in green.

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Biographical Sketches

Matti Leisola obtained his D.Sc. (Tech) in biotechnology in 1979 at Helsinki University of Technology (HUT), where he has been a Professor of Bioprocess Engineering since 1997. He served as a research scientist at ETH-Zürich from 1981 to 1988, and as a research director of Cultor Ltd from 1990 to 1997, where he was also a board member of Finfeeds International. He is a member of several national scientific societies and a vice-president of the International Society of Rare Sugars. He has published over 80 scientific papers on biocatalysis.

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Ossi Pastinen is a Senior Research Scientist at HUT. After qualifying in chemistry at Finland's Jyväskylä University, he spent 14 years as an industrial scientist at Cultor Ltd, specializing in chemical and enzymatic catalysis. He gained his Ph.D. in enzymatic catalysis in 2000.

Ossi Turunen is a Senior Research Scientist at HUT. He was awarded his Ph.D., with his thesis on the molecular and cell biology of ezrin, at the University of Helsinki in 1998, since when he has worked at the Helsinki University of Technology, specializing in the protein engineering of industrial enzymes.

Hans Schoemaker is a Senior Research Fellow at DSM, where he started his industrial career in 1979. The Ph.D. he was awarded that year was for model studies in alkaloid synthesis at Amsterdam University, where he has been a part-time professor since 1994. His research interests include amino acid/peptide chemistry, applied biocatalysis, and enzyme mechanisms. He has published over 60 papers on biocatalytic conversions and on the mechanism of lignin biodegradation.