Organic Process Research & Development **2011,** *15,* 224–230

Enzymes and Bioconversions of Industrial, Pharmaceutical, and Biotechnological Significance

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Abstract:

Enzymes are an important part of industry due to their many favorable properties. The development of industrial enzymes has depended heavily on the use of microbial sources. Microbes are useful because they can be produced economically in short fermentations and inexpensive media. Screening is simple, and strain improvement for increased production has been very successful. In the 1980s and 1990s, microbial enzymes replaced many plant and animal enzymes. They have found use in many industries including food, detergents, textiles, leather, pulp and paper, diagnostics, and therapy. The development of recombinant DNA technology had a major effect on production levels of enzymes as their genes were transferred from native species into industrial strains. Over 50% of the enzyme market is provided by recombinant enzymes. In many cases, enzymes have also been used to carry out conversions that were synthetic in previous years. Accompanying this trend is the use of whole cells to carry out bioconversions. Bioconversions have become very important to industry especially because of the demand for single isomer intermediates.

1. Use of Enzymes in Industry

Overview. According to the International Union of Biochemistry (IUB), enzymes are divided into six classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Enzymes have become very important in industry due to their valuable properties, i.e., rapid and efficient action at low concentrations under mild pH values and temperatures, high substrate specificity, low toxicity, and ease of termination of activity. Some microbial strains produce high concentrations of extracellular enzymes. Of the hundred or so enzymes being used industrially, over half are from fungi, over one-third are from bacteria, with the remainder originating from animal (8%) and plant (4%) sources. Microbial enzymes are economical on a large scale due to inexpensive media and short fermentation cycles. Screening for the best enzymes is simple, allowing the examination of thousands of cultures in a short time. Different microbes produce somewhat different enzymes that catalyze the same reaction. This offers flexibility with respect to operating conditions in the reactor.

Over 500 commercial products are made using enzymes.[1] The industrial enzyme market reached $1.6 billion in 1998 divided into the following application areas; food, 45% (of which starch processing represented 11%); detergents, 34%; textiles, 11%; leather, 3%; pulp and paper, 1.2%. This does not include diagnostic and therapeutic enzymes. In 2000, the industrial enzyme market reached $2 billion and, in 2003, $2.3 billion. The distribution of the enzyme market in 2003 is shown in Table 1.[2] In 2006, the industrial enzyme market reached $3.7 billion. World enzyme demand grew at a nearly double-digit pace from 2003 to 2008. In 2009, the market was $5.1 billion. Some applications of bacterial enzymes are shown in Table 2, and those from yeasts and filamentous fungi can be seen in Table 3.

Table 1. **World enzyme market in 2003**

Product	USD (millions)
Detergents	789
Foods	634
Agriculture and feed	376
Textile processing	237
Pulp and paper, leather and chemicals	222

Table 2. **Some industrial applications of bacterial enzymes**

Enzyme	Source (genus)	Application
α-Amylase	*Bacillus*	Baking industry
Asparaginase	*Escherichia*	Pharmaceutical
Cellulase	*Clostridium*	Plant waste treatment
Dextranase	*Leuconostoc*	Pharmaceutical
Glucose isomerase	*Bacillus*	Fructose syrup
Oxidoreductases	*Bacillus*	Food, detergents
Penicillin amidase	*Bacillus*	Pharmaceutical
Penicillin acylase	*Escherichia*	Pharmaceutical
Protease	*Bacillus*	Laundry, detergents
Pullulanase	*Klebsiella*	Baking
Subtilisin	*Bacillus*	Pharmaceutical, detergents

Recombinant DNA. With the development of recombinant DNA methodology, it became possible to clone genes encoding enzymes from microbes and express them at levels hundreds of times higher than those produced naturally. This technology was accepted by the industrial enzyme industry because it could increase production levels and move enzyme production from strains not suited for industry into industrial strains such as species of *Aspergillus*, *Trichoderma*, *Kluyveromyces*, *Saccharomyces*, *Yarrowia*, and *Bacillus*.[3]

Pectic Enzymes. In the paper and textile industries, enzymes are being increasingly used to develop cleaner processes and reduce the use of raw materials and production of waste. An alternative enzymatic process in the manufacturing of cotton

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Published on Web 12/10/2010
10.1021/op100302x

Table 3. **Some industrial applications of enzymes from yeast and filamentous fungi**

Enzyme	Source (genus)	Application
α-Amylase	*Aspergillus*	Baking
Catalase	*Aspergillus*	Food
Cellulase	*Aspergillus*	Waste, textiles
Dextranase	*Penicillium*	Food
Phytase	*Aspergillus*	Feed
Glucose oxidase	*Aspergillus*	Food
α-galactosidase	*Saccharomyce*	Dairy
Invertase	*Kluyveromyces*	Food
Naringinase	*Penicillium*	Food
Lactase	*Aspergillus*	Dairy
Lipase	*Rhizopus, Candida*	Food
Rennin	*Mucor*	Cheese
Pectinase	*Aspergillus*	Drinks
Protease	*Aspergillus*	Baking
Xylanase	*Aspergillus*	Food, Paper

was developed based on a pectate lyase. The process for removing pectin and other hydrophobic materials from cotton fabrics is performed at much lower temperatures and uses less water than the classical method. By applying Gene Site Saturation Mutagenesis technology on DNA encoding for pectinolytic enzymes (selected from more than 100 environmental DNA libraries), single site mutants exhibiting improved thermotolerance were produced. In addition, variants with improved thermotolerance were produced by Gene Reassembly technology.[4] The best performing variant (CO14) contained eight mutations and had a melting temperature 16 °C higher than that of the wild type enzyme while retaining the same specific activity at 50 °C. The optimal temperature of the evolved enzyme was 70 °C, which is 20 °C higher than that of the wild type. Scouring results obtained with the evolved enzyme were significantly better than the results obtained with chemical scouring, making it possible to replace the conventional and environmentally harmful chemical scouring process.[4] Furthermore, alkaline pectinases are used for treatment of pectic wastewaters, degumming of plant bast fibers, paper making, and coffee and tea fermentations.[5]

Proteases. The major application of proteases in the dairy industry is for the manufacturing of cheese. Calf rennin had been preferred in cheese-making due to its high specificity, but microbial proteases produced by GRAS microorganisms like *Mucor miehei*, *Bacillus subtilis*, *Endothia parasitica*, and *Aspergillus oryzae* MTCC 5341 are gradually replacing it. The primary function of these enzymes in cheese-making is to hydrolyze the specific peptide bond (Phe105-Met106) that generates *para*-k-casein and macropeptides. Nearly 40 000 U/g bran of milk-clotting activity were produced by *A. oryzae* at 120 h by solid state fermentation.[6]

The use of enzymes as detergent additives still represents the largest application of industrial enzymes. The detergent market for enzymes has grown strongly in the past 25 years. In the year 2003, it was around $0.79 billion, with proteases as the major detergent enzyme product.

Proteases, lipases, amylases, oxidases, peroxidases, and cellulases are added to the detergents where they catalyze the breakdown of chemical bonds on the addition of water. To be suitable, they must be active under thermophilic (60 °C) and alkalophilic (pH 9−11) conditions as well as in the presence of the various components of washing powders.[7] Proteases in laundry detergents account for approximately 25% of the total worldwide sales of enzymes. The first detergent containing a bacterial protease was introduced in 1956, and in 1960, Novo Industry A/S introduced alcalase produced by *Bacillus licheniformis* (Biotex). Cellulase from *Bacillus* sp. KSM-635 has been used in detergents because of its alkaline pH optimum and insensitivity to components in laundry detergents. Later, Novozyme launched a detergent using a cellulase complex isolated from *Humicolla insolence* (Celluzyme). As early as the mid-1990s, virtually all laundry detergents contained genetically engineered enzymes.[7] Over 60% of the enzymes used in the detergent, food, and starch processing industries are recombinant products.[8]

Proteases can be used for the synthesis of peptides in organic solvents. Thermolysin is used in this way to make aspartame.[9] Aspartame sold for $1.5 billion in 2003. In 2004, the production of aspartame amounted to 14 000 tons. Proteases are also used for the production of low allergenic milk proteins used as ingredients in baby milk formulas.[10]

Lipases. Lipases are commonly used in the production of a variety of products ranging from fruit juices, baked foods, pharmaceuticals, and vegetable fermentations to dairy enrichment. Fats, oils, and related compounds are the main targets of lipases in food technology. Accurate control of lipase concentration, pH, temperature, and emulsion content is required to maximize the production of flavor and fragrance. The lipase mediation of carbohydrate esters of fatty acids offers a potential market for use as emulsifiers in foods, pharmaceuticals, and cosmetics. Another application of increasing importance is the use of lipases in removing pitch (hydrophobic components of wood, mainly triglycerides and waxes). A lipase from *Candida rugosa* is used by Nippon Paper Industries to remove up to 90% of these compounds.[11] The use of enzymes as alternatives to chemicals in leather processing has proved successful in improving leather quality and in reducing environmental pollution. Alkaline lipases from strains which grow under highly alkaline conditions in combination with other alkaline or neutral proteases are currently being used in this industry. Lipases are also used in detergent formulations for removal of lipid stains, fatty food stains, and sebum from fabrics.[12] Alkaline yeast lipases are preferred because they can work at lower temperatures as compared to bacterial and mold lipases. Cold active

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lipase detergent formulation is used for cold washing which reduces the energy consumption and wear and tear of textile fibers. It is estimated that every year, about 1000 tons of lipases are added to approximately 13 billion tons of detergents.

Yeast Enzymes. Enzymes produced by yeasts useful in food industry include invertase from *Kluyveromyces fragilis*, *Saccharomyces carlsbergensis*, and *Saccharomyces cerevisiae* for candy and jam manufacture; β-galactosidase (lactase) from *K. fragilis* or *Kluyveromyces lactis* for hydrolysis of lactose from milk or whey; and α-galactosidase from *S. carlsbergensis* for crystallization of beet sugar.

Laccases. Laccases oxidize phenolic and nonphenolic lignin-related compounds as well as environmental pollutants.[13] They are used to detoxify industrial effluents of the paper and pulp, textile, and petrochemical industries; as a medical diagnostic tool; for bioremediation of herbicides, pesticides, and explosives in soil; as a cleaning agent for water purification systems; as a catalyst in drug manufacture; and as ingredients in cosmetics.

Phytases. Enzymes are also used in a wide range of agrobiotechnological processes, and the major application is the production of feed supplements to improve feed efficiency. A recent advance in feed enzymes involves the application of phytases in agriculture as an animal feed ingredient and also in foods to improve plant phosphorus uptake by monogastric animals.[14] Phytate phosphorus is often unavailable to farm animals and chelates valuable minerals. Phytase allows liberation of phosphorus from plant feedstuffs which contain about 2/3 of their phosphorus as phytate. Hydrolysis of phytate prevents its passage via manure into the soil where it would be hydrolyzed by soil and water microbes causing eutrophication. Therefore, its use in the food industry is to remove phytic acid which acts as an antinutritional factor. Its annual market is about $500 million. The enzyme is made by many bacteria, yeasts, and filamentous fungi. Production is controlled by phosphate. Cloning of the phytase-encoding gene *phyA* from *Aspergillus niger* var. *awamori* and reintroduction at a higher dosage increased production 7-fold.[15] Recombinant *Hansenula polymorpha* produced 13 g/L of phytase.[16] New fungal phytases with higher specific activities or improved thermostability have been identified.[17]

Extremozymes. Certain microorganisms called extremophiles grow under extreme conditions such as 100 °C, 4 °C, 250 atm, pH 10, or 5% NaCl. Their enzymes that act under such extreme conditions are known as extremozymes. One such enzyme called Cellulase 103 was isolated from an alkaliphile and commercialized because of its ability to break down microscopic fuzz of cellulose fibers which trapped dirt on the surface of cotton textiles. It has been used for over 10 years in detergents to return the "newness" of cotton clothes even after many washings.

Directed Evolution. Strategies for directed evolution of enzymes[18−21] include DNA shuffling, whole genome shuffling, heteroduplex, random chimeragenesis of transient templates, assembly of designed oligonucleotides, mutagenic and unidirectional reassembly, exon shuffling, Y-ligation-based block shuffling, non-homologous recombination, and combining rational design with directed evolution. Directed evolution has increased the activity, stability, solubility, and specificity of enzymes. It increased the activity of glyphosate-*N*-acetyltransferase 10 000-fold and, at the same time, its thermostability by 5-fold. A number of new and useful enzymes have been obtained by metagenomics.[22]

2. Enzymatic Conversions

General. Bioconversion is becoming essential to the fine chemical industry in that their customers demand single isomer intermediates.[23] Since the entire 1998 worldwide market for fine chemicals was $50 billion, the segments for these customers, i.e., $25 billion for pharmaceuticals and $10 billion for agrochemicals, are a major portion. In many cases, biocatalysis has replaced chemical catalysis because it (i) has higher enantioselectivity and higher regioselectivity in aqueous solution; (ii) does not require protection and deprotection of functional groups; (iii) has better stability; (iv) operates under milder conditions, i.e., avoidance of high and low temperatures; and (v) has greater efficiency and (vi) higher product yields, e.g., 90 to 100%.[24] Out of 38 major asymmetric synthetic processes of the chemical industry, 22 use biocatalysis.[25] Most use organic solvents as part or all of the medium.[26,27]

About 150 biocatalysis processes are used in industry, the majority of which utilize isolated enzymes, immobilized or free, rather than cells.[28] Enzymes have been used, e.g., lipases, for many organic chemical reactions. Such enzymes are stable, easy to handle, and available commercially and do not require cofactors. They can hydrolyze esters in water and can esterify in organic solvents, usually enantioselectively. Use of oxidoreductases suffered earlier due to problems of enzyme stability and cofactor regeneration. These problems have been resolved by combining enzyme immobilization with more efficient and cheaper ways to regenerate the NADPH cofactor, especially in the case of Baeyer−Villigerases and other cofactor-requiring enzymes. Baeyer−Villigerases are enzymes that can carry out Bayer−Villiger oxidations involving C−C cleavage and insertion of an oxygen atom next to an alicyclic or aromatic keto group. Table 4 lists some industrially important enzymatic conversions.

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Table 4. **Some examples of industrial enzymatic conversions**

Enzyme	Source (genus)	Application
Glucose isomerase	*Streptomyces*	fructose syrup
Hydroxymethyl transferase	*Klebsiella*	L-serine
Lipases		
Nitrilo hydrolase	*Rhodococcus*	acrylamide
Phenylalanine dehydrogenase	*Rhodococcus*	L-phenylalanine
Phenylalanine aminotransferase	*Paracoccus*	L-phenylalanine
Serine hydroxymethyl transferase	*Klebsiella*	L-serine
Tyrosine phenol lyase	*Symbiobacterium*	L-tyrosine

Enzyme Immobilization is an advantageous and useful tool for industrial enzyme applications for the following reasons. (i) It permits reuse of enzymes, (ii) it is ideal for continuous operations, (iii) the product is enzyme-free, (iv) it reduces effluent disposal problems, and (v) it usually improves enzyme stability. Figure 1 shows various types of enzyme immobilization.

Promiscuity. Some enzymes are very promiscuous and can form many products. This can be very important in the search for new enzyme-catalyzed reactions. One example is a sesquiterpene synthase which is known to produce 52 sesquiterpenes from farnesyl diphosphate via various cyclization reactions. The enzyme is γ-humulene synthase from *Abies grandis* (grand fir).[29]

Important Developments. An enzymatic conversion of phenol, pyruvate, pyridoxal phosphate, and ammonium chloride to L-tyrosine using a thermostable and chemostable tyrosine phenol lyase yielded 130 g/L of L-tyrosine in 30 h with continuous feeding of substrate.[30] The enzyme came from *Symbiobacterium toebii*.

The replacement of costly steps of chemical synthesis by enzymatic conversions has been achieved in the production of blockbuster drugs such as simvastatin (Zocor), atorvastatin (Lipitor), pregabalin (Lyrica), paroxetine (Paxil), and levetiracetam (Keppra). For example, Codexis developed four enzymes and lowered the cost of two intermediates for producing atorvastatin (Lipitor). The work eliminated boron reagents, reduced solvents by 85%, decreased waste by 60%, increased the yield of intermediate ATS-7, and achieved greater stereopurity.[31]

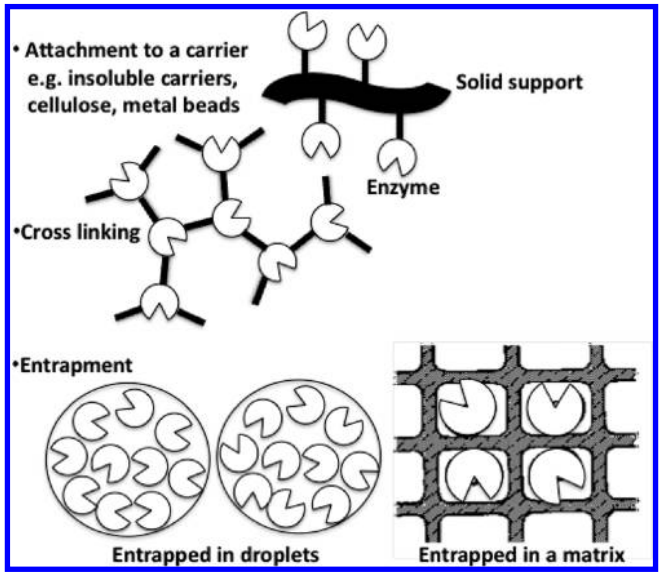

Figure 1. **Types of immobilization.**

"Fructose syrup" produced by bacterial glucose isomerase (also known as xylose isomerase) from glucose is at a level of 15 million tons/year. The enzyme is also used in conjunction with fungal α-amylase and glucoamylase to convert starch to mixtures of glucose and fructose known as "high fructose corn syrup" in a $1 billion business.[32]

Acrylamide is made from acrylonitrile by immobilized nitrile hydrolase from bacteria at 100 000 tons/year.[33,34]

Requirements for Industrial Usage. Successful application of enzymatic processes in the chemical industry depends mainly on cost competitiveness with the existing, and well established, chemical processes, and a significant increase in the use of biocatalysts for chemical synthesis has been observed within recent years. Lower energy demand, increased product titer, increased catalyst efficiency, less catalyst waste and byproducts, and lower volumes of wastewater streams are the main advantages that biotechnological processes have compared to the well established chemical processes.

Obtaining enantiomerically pure intermediates and products efficiently and economically is of great importance in the pharmaceutical and chemical industries. Esterases, lipases, and proteases have been widely applied in the preparation of enantiopure compounds from racemic pairs, prochiral or meso compounds, or diastereomeric mixtures. The targets of pharmaceutical compounds such as cell-surface receptors or enzymes are chiral biomolecules. Often, just one of the two isomers of a given compound exerts the desired effect forcing the FDA to require the evaluation of both forms. Some examples are amoxicillin (an antibiotic) and captopril (an inhibitor of the angiotensin-converting enzyme [ACE]). The worldwide sales volume for single enantiomer drugs already exceeds $100 billion.

Enzymes in Organic Solvents. Many chemicals and polymers are insoluble in water, and its presence leads to undesirable byproducts and degradation of common organic reagents. Although switching from water to an organic solvent as the reaction medium might suggest that the enzyme would be denatured, many crystalline or lyophilized enzymes are actually stable and retain their activities in such anhydrous environments. Enzymes function normally in hydrophobic environments such as in a membrane. These environments are much lower in their contents of water than the 55 M concentration in aqueous environments. It is thus not surprising that some enzymes function best in organic media.[35] Application of enzymes in several industrial bioconversions has been broadened by the use of organic solvents replacing water, an important development in enzyme engineering. From a bio-

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technological perspective, there are many advantages of employing enzymes in organic as opposed to aqueous media. Organic media have been used in oxidations of steroids, epoxidations, hydroxylations, alcohol dehydrogenation, phenolic polymerization, lignin depolymerization, ester synthesis, peptide synthesis, and resolution of racemic mixtures.

The benefits of organic media are (i) increased solubility of nonpolar substrates, (ii) shift of thermodynamic equilibria to favor synthesis over hydrolysis, (iii) reduction of aqueous side reactions, (iv) elimination of the need to immobilize in cases where enzymes are insoluble in organic solvents, (v) ease of enzyme recovery by simple filtration, (vi) adsorption onto a nonporous surface (e.g., glass beads) that can be done if immobilization is desired and there is no leaching in organic media, (vii) ease of product recovery from low boiling solvents, (viii) enhanced thermostability since water is required to inactivate at high temperatures, (ix) lack of problems from contaminating microbes, (x) potential to use enzymes directly in a chemical process, (xi) alteration of substrate specificity, and (xii) small reaction volumes. Other potential advantages include (xiii) possible increases in activity (although enzymes usually show lower catalytic activities in organic than in aqueous solution) and (xiv) the possibility of carrying out reactions unfeasible in water.[36] Yeast lipases have been used to catalyze butanolysis in anhydrous solvents to obtain enantiopure 2-chloro- and 2-bromo-propionic acids that are used for the synthesis of herbicides and pharmaceuticals. Another lipase is used in a stereoselective step, carried out in acetonitrile, for the acetylation of a symmetrical diol during the synthesis of an antifungal agent. Enzymatic resolution of racemic amines by esterases or lipases in organic solvents is conducted by BASF on a multiton scale. These enantiopure amines may find use as inhibitors of monoamine oxidase in the treatment of diverse neurological disorders such as Parkinson's and Alzheimer's diseases.

Further Developments. An enzyme process using phenylalanine ammonia lyase from *Rhodotorula rubra* produces 59 g/L L-phenylalanine from *trans*-cinnamic acid with a yield of 90%.[37] Other enzymatic conversions include (i) phenylpyruvate to L-phenylalanine by phenylalanine dehydrogenase from *Rhodococcus* sp. in 95% yield and a rate of 456 g/L/d[38] and (ii) L-phenylalanine aminotransferase from *Paracoccus denitrificans* converting phenylpyruvate to 122 g/L of L-phenylalanine with a 92% yield.[39] A crude enzyme preparation containing serine hydroxymethyl transferase from *Klebsiella aerogenes* converts glycine plus formaldehyde to 160 g/L L-serine.[40] By development of a feedback control system for formaldehyde feeding, the titer was increased to 450 g/L.[41]

Table 5. **Advantages and limitations of cell immobilization**

Advantages
• Unlimited number of microbes available for use
• No enzyme isolation and purification are required
• Multienzyme complex reactions are available
• Cofactor regeneration in native system
• Application of anchorage-dependent cells (e.g., mammalian cells)
• Syntrophic mixed cultures
• The processes are usually robust
Limitations
• Insufficient stability, low resistance
• Mass transfer limitation to the cells
• Side reactions are common with product degradation
• Byproducts from cell lysis or toxic metabolites

3. Cellular Bioconversions

Use of Whole Cells for Conversions. Microorganisms are extremely useful in carrying out biotransformation processes including the coupling of reactions in which a compound is converted into a structurally related product by one or a small number of cellular enzymes working in series.

In deciding whether to use an enzyme or whole cells to catalyze a bioconversion, the following are important.[42] Isolated enzymes can often be obtained from commercial sources, the process can be implemented simply and in a controlled manner, the enzyme medium is usually simple facilitating product purification, and the process requires simple equipment. However, they may need cofactors, and the enzymes could be costly especially if not commercial. The benefits of whole cells are the almost unlimited number of microbes available for use, the cofactors and their cycling are internal, and the processes are usually robust. However, the organisms have to be cultivated, side reactions are common, and purification involves cell removal and product extraction from a possibly complex medium. Often, immobilized cells are used. Some advantages and disadvantages of cell immobilization are listed in Table 5.

Cellular Bioconversion Processes. Whole-cell bioconversions have replaced chemical synthesis in producing L-sorbose, L-malic acid, D-aspartic acid, and 2-keto-L-gulonic acid.[43] One of the most successful examples of biocatalytic production of a commodity chemical is the bioconversion of acrylonitrile to acrylamide. Using *Rhodococcus rhodochrous* J1 or *Pseudomonas chloraphis* B23, about 20 000 tons of acrylamide are annually produced for use as a flocculant, a component of synthetic fibers, a soil conditioner, and a recovery agent in the petroleum industry. This development was the first example of the use of a biological process to compete with a chemical process in the petrochemical industry. The Japanese bioconversion process is carried out at 10 °C and yields 100% conversion. The cells can be used many times. The titer is 656 g/L after 10 h, and the productivity is higher than 7 kg of acrylamide per g dry cell weight. Acrylic acid acts as an inducer. The bioconversion competes well with chemically synthesized acrylamide.[44] It is cheaper due to the elimination of steps recovering unreacted acrylonitrile and removal of copper ions.

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Screening of microorganisms as catalysts for the hydration of adiponitrile led to the isolation of several bacteria with nitrile hydratase activity that regioselectively produced 5-CVAM (5-cyanovaleramide), a starting point for the synthesis of azafenidin, a herbicide, from adiponitrile with a 93% yield and 96% selectivity.

Production of nicotinamide (vitamin B3) using a bioconversion process is currently being employed at Lonza's facilities in Guangzhou, China. The process involves immobilized cells of *R. rhodochrous* J1 and reaches 100% conversion whereas the chemical process produces 4% of a nicotinic acid byproduct.

Vanillin is utilized in personal care products and in foods with an annual production of 12 000 tons, all synthetic. Natural vanilla comes from the orchid plant but can only supply 1% of the market. The more desirable natural vanillin is produced at less than 120 tons.[45] The synthetic compound sells for <$15/kg whereas the natural form has a price varying between $1200 and $4000/kg. Bioconversion of ferulic acid to vanillin had not been successful due to the toxicity of vanillin and its degradation to vanillyl alcohol and vanillic acid. However, new processes have been developed using a variety of bacteria. The natural compound can be made from ferulic acid by a number of bacteria or enzymatically by feruloyl-CoA synthetase plus enoyl CoA hydratase/aldolase. Cloning of the genes encoding these two enzymes into *Escherichia coli* yielded 1.1 g/L in 48 h from 0.2% (w/v) ferulic acid. A strain of *Bacillus pumilis* converted 10 g/L isoeugenol to 3.75 g/L vanillin with a molar yield of 40% in a 150 h batch process.[46] Bioconversion of 50 g/L isoeugenol to vanillin by *Bacillus fusiformis* resulted in a titer of 8 g/L.[47] In another study, *Pseudomonas putida* converted isoeugenol to 16 g/L vanillin with a molar yield of 71% in 24 h in the presence of DMSO. 10% DMSO was used to dissolve the isoeugenol in water. A process developed by Hua et al.[48] uses *Streptomyces* sp. V-1 and an adsorbent resin (DMM 11) to capture the vanillin. As a result, 45 g/L of ferulic acid were converted to 19 g/L vanillin. Recombinant *E. coli* was able to convert ferulic acid to 5 g/L vanillin in 24 h with an 86% molar yield.[49] Another recombinant strain of *E. coli* containing the isoeugenol monooxygenase gene from *P. putida* converted isoeugenol to vanillin with a conversion yield of 81% and a titer of 28 g/L vanillin in a 6 h bioconversion.[50]

A bioconversion of ribitol to L-ribulose by *Gluconobacter oxidans* resting cells occurs at 15.7 g/L/h and yields a complete conversion of 300 g/L ribitol to L-ribulose in 30 h.[51]

Bioconversion of 50 g/L DL-5-indolylmethyl hydantoin to 43 g/L of L-tryptophan (molar yield of 97%) was done with *Flavobacterium* sp. Such cells can also work on 5-substituted hydantoins of other amino acids.[52] 180 g/L of L-tryptophan were produced in 8 h from indole and serine by *E. coli* cells containing a stabilized plasmid encoding trp synthase. The inducer was 3-indoleacrylic acid.[53]

L-Phenylalanine can be made from phenylpyruvate using immobilized cells of *E. coli* with a molar yield of 98% and a titer of 30 g/L.[54] The enzyme is aspartate-phenylalanine transaminase. The cells have a half-life of over 8 months at 37−41 °C. In another bioconversion process, a constitutive mutant of *Corynebacterium equi* converts α-acetamidocinnamic acid to L-phenylalanine in over a 99% molar yield and a titer of 33 g/L.[55]

A bioconversion of 3-chloro-L-alanine to L-cysteine is carried out with *Bacillus sphaericus* cells containing *O*-acetylserine sulfhydrylase in 80−85% yield and a titer of 70 g/L.[56]

Glucosamine synthase (GlmS) in *E. coli* produces glucosamine-6-phosphate from fructose-6-phosphate. The glucosamine-6-phosphate is dephosphorylated, and the glucosamine is excreted by the cells. Gene *glmS* was improved by directed evolution. The best mutant yielded 8.6 g/L of glucosamine as compared to 0.3 g/L by the wild type at 24 h. The mutant continued to produce glucosamine for 72 h reaching 17 g/L, about a 60-fold improvement.[57]

Immobilized Cells. Continuous production of NADPH from NADP+ can be carried out with immobilized cells of *G. suboxydans* containing glucose dehydrogenase.[58] Conversion is 73%, and 73 g/L product is made after 1 h.

Permeability often needs to be increased for the successful operation of a bioconversion. This often involves the addition of detergents and surfactants. An efficient conversion of adenine to ATP using manganese-excess *Brevibacterium ammoniagenes* is dependent upon modification of permeability with polyoxyethylene stearylamine, a cationic detergent.[59] Adenine at 3.5 g/L was converted to 13 g/L of $NaATP \cdot 3H_20$/mL, an 83% molar yield. Permeability can also be increased by the use of both a surfactant and an organic solvent. In the above process using intact cells of *B. ammoniagenes*, ATP production was only a trace in the absence of additive, 6 g/L with polyoxyethylene stearylamine, and 10 g/L with xylene. Addition of both raised production to 17 g/L.[60] Detergents such as sodium lauryl sulfate, Triton X-100, bile acid, bile extract, or deoxycholate stimulate the immobilized *B. ammoniagenes* bioconversion of fumarate to malate by 5- to 7-fold.[61] Bioconversion of 50 mg of $XMP \cdot Na_2 \cdot 7H_20$ to 43.5 mg of $GMP \cdot Na_2 \cdot 0.7H_2O$ in 22 h by dead cells of *B. ammoniagenes* was dependent on the

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surfactant polyoxyethylene stearylamine.[62] Other requirements included phosphate, Mg, glucose, and air to produce the ATP needed for the reaction. A temperature of 42 °C was required to eliminate further conversion of GMP to GDP and GTP. Whole-cell biocatalysis can be markedly improved by outer membrane mutations which increase permeability.[63]

Simultaneous Hydrolysis and Bioconversion. D-Galacturonic acid (obtained from plant pectin) can be converted into keto-deoxy-L-galactonate with potential use as a chelator, clarifier, preservative, and plastic precursor. This bioconversion was achieved with *A. niger* Δ*gaaC* which was also able to produce keto-deoxy-L-galactonate directly from pectin or polygalacturonate, demonstrating the feasibility of simultaneous hydrolysis and bioconversion. Although keto-deoxy-L-galactonate accumulated intracellularly, concentrations above 12 g/L were exported to the culture supernatant.[64]

4. Concluding Remarks

Microbial enzymes have long been used by industrial product makers as major catalysts to transform raw materials into end products. Over 500 commercial products are made using enzymes. Enzymes are economically produced by different microorganisms and are quickly broken down when they have done their job.

New technical tools to use enzymes as crystalline catalysts, to recycle cofactors, and to engineer enzymes to function in various solvents with multiple activities are important technological developments, which will steadily create new applications.

The industrial enzyme market continues to grow steadily due to improved production efficiency resulting in cheaper enzymes, new applications, new enzymes from screening programs, and the engineering of traditional enzymes. Tailoring enzymes for specific applications will be a future trend with continuously improving tools, understanding of structure−function relationships, and increasing the search for enzymes from exotic environments. New applications are to be expected in the field of textiles and new animal diets such as ruminant and fish feed. It can be expected that breakthroughs in pulp and paper applications will materialize. The use of cellulases to convert waste cellulose into sugars and further to ethanol by fermentative organisms has been a major study topic for years. Increasing environmental pressures and rising energy prices will make this application a real possibility one day.

Enzymes should not be considered alone but rather as a part of a biocatalyst technology. Whole cell catalysts, an increased ability to engineer metabolic pathways, and a combination of specific biocatalytic reactions with organic chemistry form a basis to develop new technologies for the production of chemicals. Recent developments in genetic engineering and protein chemistry are bringing ever more powerful means of analysis to bear on the study of enzyme structure and function. This will undoubtedly lead to the rational modification of enzymes to match specific requirements and to the design of new enzymes with novel properties. Techniques such as protein engineering, gene shuffling, and directed evolution[65] will enable the development of enzymes better suited to industrial environments. These tools will also allow the synthesis of new biocatalysts for completely novel applications, resulting in the production and commercialization of new enzymes, thus seeding an explosive expansion of the current enzyme industry.

Received for review November 10, 2010.

OP100302X

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