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REVIEW ARTICLE

Enzymes of industrial interest

Enzimas de interés industrial

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ABSTRACT

For many years, industrial enzymes have played an important role in the benefit of our society due to their many useful properties and a wide range of applications. They are key elements in the progress of many industries including foods, beverages, pharmaceuticals, diagnostics, therapy, personal care, animal feed, detergents, pulp and paper, textiles, leather, chemicals and biofuels. During recent decades, microbial enzymes have replaced many plant and animal enzymes. This is because microbial enzymes are widely available and produced economically in short fermentations and inexpensive media. Screening is simple, and strain improvement for increased production has been very successful. The advances in recombinant DNA technology have had a major effect on production levels of enzymes and represent a way to overproduce industrially important microbial, plant and animal enzymes. It has been calculated that 50-60% of the world enzyme market is supplied with recombinant enzymes. Molecular methods, including genomics and metagenomics, are being used for the discovery of new enzymes from microbes. Also, directed evolution has allowed the design of enzyme specificities and better performance.

Keywords: source, immovilization, industrial applications, improvement, discovery.

RESUMEN

Durante muchos años, las enzimas industriales han jugado un papel importante en el beneficio de nuestra sociedad debido a sus muchas propiedades útiles y una amplia gama de aplicaciones. Son elementos clave en el progreso de muchas industrias incluyendo alimentos, bebidas, productos farmacéuticos, diagnóstico, terapia, cuidado personal, alimento para ganado, detergentes, pulpa y papel, textiles, cuero, productos químicos y biocombustibles. Durante las últimas décadas, las enzimas microbianas han reemplazado

muchas enzimas vegetales y animales. Esto se debe a que las enzimas microbianas están ampliamente disponibles y se producen económicamente en fermentaciones cortas y medios de cultivo económicos. La detección es simple, y la mejora de las cepas para aumentar la producción ha sido muy exitosa. Los avances en la tecnología del ADN recombinante han tenido un efecto importante en los niveles de producción de enzimas y representan una forma de sobreproducir enzimas microbianas, vegetales y animales industrialmente importantes. Se ha calculado que el 50-60% del mercado mundial de enzimas es suministrado con enzimas recombinantes. Métodos moleculares, incluyendo genómica y metagenómica, han sido utilizados para el descubrimiento de nuevas enzimas de microbios. Además, la evolución dirigida ha permitido el diseño de especificidades enzimáticas y un mejor rendimiento.

Palabras clave: fuente, inmovilización, aplicaciones industriales, mejoramiento, descubrimiento.

1. SOURCE AND MARKET OF INDUSTRIAL ENZYMES

Enzymes have become very important in industry due to their valuable properties. They have a rapid and efficient action at low concentrations and can eliminate the use of high temperatures, extreme pH values, organic solvents, and at the same time, offer high substrate specificity, low toxicity, product purity, reduced environmental impact and ease of termination of activity. Enzymes are used in industries such as food, beverage, animal feed, textile, detergent and medicine (Jemli *et al.*, 2016). Since ancient times, natural enzymes have been employed to make cheese, sour dough, beer, potable spirits, vinegar, and manufactured commodities (leather, linen). In recent years, the list has expanded to include biofuels. The enzymes are derived from animals and plants but mainly from microbes. More than 75% of commercial enzymes are represented by proteases, amylases, lipases and cellulases. Industrial enzymes are divided into (a) detergent, (b) technical, (c) food, and (d) animal feed enzymes (Holmuth *et al.*, 2013)**.** The food enzymes are a major group, and they are used in the dairy, brewing, wine, juice, fat, oil and baking industries. In recent years, the use of enzymes in organic synthesis has increased; these includes hydrolases, transaminases, reductases, and oxidases.

Microbial enzymes as tools for biotechnological processes were reviewed in 2014 (Adrio & Demain, 2014) and in 2017, a new book titled "Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications" (Brahmachari *et al*., 2017) has been published.

Technical enzymes include those for textiles, leather, pulp and paper, and fuel ethanol. The food enzymes are used in the dairy, brewing, wine, juice, fat, oil and baking industries. They include amylase, xylanase, glucose oxidase, hexose oxidase, pectinase, glucanase, invertase, glucose isomerase, protease, lipase, phosphorylase, lactase, milk-clotting enzymes, animal rennet, microbial rennet and chymosin. The main sources of the industrial enzymes are fungi (e.g., *Aspergillus niger* and *Kluyveromyces lactis)* and bacteria (e.g., *Escherichia coli* and *Bacillus* species).

Cell-free systems for production of commercial products have been very useful (You & Zhang, 2012). These products include (a) bio-commodities selling from $0.3 to several

dollars per kg, (b) specialties and biomaterials at tens of dollars per kg, (c) fine chemicals at hundreds of dollars per kg, (d) pharmaceuticals at thousands of dollars per kg, and (e) protein drugs at tens of thousands of dollars per kg. Advantages of cell-free systems over living organisms include high product yield, rapid reaction rate, high product titer, more control and freedom of design, and broad reaction conditions.

As far back as the 1960s, immobilized enzymes were used to make (a) methionine by aminoacylase and (b) 9 million tons per year of fructose syrup by glucose isomerase. In 1985, acrylamide production began and reached 100,000 metric tons per year by use of immobilized nitrile hydrolase.

Enzyme immobilization can improve enzyme activity, stability and selectivity (Mateo *et al.*, 2007). This is necessary for reuse over long time spans in industrial reactors. In addition to reuse, other improved properties are stability, activity, resistance to inhibition by reaction products, and selectivity towards non-natural substrates. Immobilization also allows simplification of reaction design and control of the reaction.

In the 1990s, one-pot multi-enzyme systems were employed to produce high-value chiral compounds for the pharmaceutical industry. In the 2000s, mixtures of at least three enzymes were employed to make research proteins and high-value antibodies. Cell-free systems for production of commercial products included low-cost enzymes such as *Bacillus* protease and amylase, *Trichoderma* and *Aspergillus* cellulases (at $5-10 per kg of dry protein), and *E. coli* recombinant proteins (at $100 per kg of dry protein, including materials, labor and capital depreciation).

Production of over 500 industrial products use enzymes (Kumar & Singh, 2013). 65% of these enzymes are used to prepare detergents, starch, leather, textiles, personal care products, pulp, and paper. 25% are used in food processing and 10% are for animal feed supplements (Rubin-Pitel & Zhao, 2006). Microorganisms produce high concentrations of extracellular enzymes and, thus, they are preferred as the source of such catalysts (Anbu et al., 2015). Screening for the best microbial enzymes is simple, allowing the examination of thousands of cultures in a short period of time. Of the two hundred or so enzymes being used industrially (Li et al., 2012), over half are from fungi, over one third are from bacteria, and a low proportion originate from animal (8%) and plant (4%) sources (Sánchez and Demain, 2011). Microorganisms used for enzyme production include around 50 GRAS fungi and bacteria (Adrio & Demain, 2014). Fungi are mainly represented by *Aspergillus*, *Mucor*, and *Rhizopus*. Bacteria are usually represented by *Bacillus subtilis*, *Bacillus licheniformis*, and various *Streptomyces* species. Micro-organisms can be cultured in large quantities in a relatively short period of time by established methods of fermentation. Microbial enzymes offer flexibility with respect to operating conditions in a reactor since different microbes produce somewhat different enzymes that catalyze the same reaction. In large-scale fermentations, microbial enzyme production is economical due to inexpensive culture media and short fermentation cycles.

More than 3000 different enzymes are known, but only about 5% are commercially used (Binod et al., 2013). Over 500 commercial products are manufactured with the use of enzymes (Johannes & Zhao, 2006). The enzyme market was $3.3 billion in 2010 (Jemli et al., 2016) and $4.9 billion in 2013 (Rawat et al., 2013). The industrial enzyme market is expected to reach $7.2 billion in 2020 (Freedonia Group, Inc., 2016). The technical group of enzymes reached $1.2 billion in 2013. The main technical enzymes are used in bulk form for manufacture of detergents, textiles, leather, pulp, paper, and biofuels (Li et

al., 2012). Additional applications include foods, animal feed, household care, fine chemicals and pharmaceuticals. Feed enzymes reached a market of $144 million in 2007. The world enzyme demand has been fulfilled by about 12 main producers and 400 minor suppliers. Three top enzyme companies produce nearly 75% of the total enzymes. They are Denmark-based Novozymes, US-based DuPont (via the May 2011 acquisition of Denmark-based Danisco) and Switzerland-based Roche. The market is highly competitive, technologically intensive and yields small profit margins (Li et al., 2012).

Feed enzymes are used to increase nutrient digestibility and to degrade unacceptable components of the feed. They are applied mainly for poultry and swine and include proteases, phytases, glucanases, alpha-galactosidases, alpha-amylases and polygalacturonases. Sales of feed enzymes are expected to reach $1.37 billion by 2020 **(**www.marketsandmarkets.com/Market-Reports/feed-enzyme-market-1157.html**)**. Recent emphasis has been on the development of heat-stable enzymes, improvement of activity, economical and rapid assays that are more reliable, and discovery of new non-starch polysaccharide-degrading enzymes.

Enzymes for food and beverage manufacture are a major part of the industrial enzyme market. Their global market was $1.5 billion in 2013. Lipases constitute a major portion of the use, especially in fats and oils. They are potentially useful as emulsifiers for foods, pharmaceuticals, and cosmetics. *Aspergillus oryzae* is used as the preferred host to produce fungal lipases, such as those from *Rhizomucor miehi, Thermomyces lanuginosus,* and *Fusarium oxysporum*.

Regarding detergent additives, proteases, lipases, oxidases, amylases, peroxidases and cellulases are included. They catalyze the breakdown of chemical bonds upon the addition of water. Some of them are active at thermophilic temperatures (ca 60 ^{o}C) and extreme pH values (9-11), in the presence of components of washing powders.

Over 60% of the worldwide enzyme market is devoted to proteases. These are involved in the manufacture of foods, pharmaceuticals, leather, detergents, silk, and agrochemicals. Their use in laundry detergents constitutes about 25% of global enzyme sales. By 2013, proteases for the manufacture of detergents had a production level of 900 tonnes per year in Europe (van Dijl & Hecker, 2013). Bacilli are very useful for enzyme production, especially *B. subtilis, Bacillus amyloliquefaciens* and *B. licheniformis.* This is due to excellent fermentation properties, high product yields (23-25 g/L) and lack of toxic byproducts. Trademarks comprise the most important enzymes used in laundry: the alkalase Biotex^{TM} from *B. licheniformis*, and the lipases Lumafast^{TM} from *Pseudomonas mendocina*, Lipomax^{TM} from *Pseudomonas alcaligenes* and Lipolase^{TM}, the first recombinant detergent lipase made by cloning the lipase from *Humicola lanuginose* into *A. oryzae* (Boel & Huge-Jensen, 1995).

Twelve proteases have been approved by the FDA in the USA as therapeutic drugs for hemophilia, stroke, acute myocardial infarction, sepsis, traumatic bleeding, muscle spasms, digestive disorders, inflammation, cystic fibrosis, retinal disorders, and psoriasis (Craik et al., 2011). These proteases, at one time, were traditionally extracted from natural sources such as urine, bovine blood, and human plasma, but recently, recombinant generation in non-human sources (e.g., CHO cells, *E. coli* and *Clostridium botulinum*) is being used. The marketed proteases are mainly serine proteases except for bacterial botulinum neurotoxins which are zinc metalloproteases. Protein engineering is being used to improve the properties of proteases.

2. INDUSTRIAL APPLICATIONS OF MICROBIAL ENZYMES

According to their applications, microbial enzymes have been applied to make numerous biotechnology products and in processes commonly encountered in the production of laundry, food, and beverages, paper and textile industries, clothing, etc.

2.1 Enzymes as detergent additives

The use of enzymes as detergent additives embodies the main application of industrial enzymes. The detergent market for enzymes has grown strongly in the last 25 years. In the year 2003, it was around $0.79 billion, with proteases as the major detergent enzyme product. The detergent industry uses more than 25% of the total enzyme production.
Proteases, lipases, amylases, oxidases, peroxidases and cellulases are added to the detergents to catalyze the breakdown of chemical bonds on the addition of water. For this purpose, they must be active under thermophilic (60ºC) and alkalophilic (pH 9-11) conditions, as well as in the presence of various components of washing powders (Stoner et al., 2005). The market share of detergent proteases is estimated to be at 72% of the global detergent enzyme market (Maurer, 2015). The first detergent containing a bacterial protease was introduced in 1956, and in 1960, Novo Industry A/S introduced alcalase produced by *B. 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 (Ozaki et al., 1990). Later, Novozyme launched a detergent using a cellulase complex isolated from *Humicolla insolence* (Celluzyme®).

Certain microorganisms, called extremophiles, grow under extreme conditions such as 100 degrees C., 4 degrees 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. As early as the mid-nineties, virtually all laundry detergents contained genetically-engineered enzymes (Stoner et al., 2005). Over 31% of the enzymes used in detergents are recombinant products (McAuliffe et al., 1996).
Lipases are also used in detergent formulations for removal of lipid stains, fatty food stains and sebum from fabrics (Hasan et al., 2010). Alkaline yeast lipases are preferred because they can work at lower temperatures, as compared to bacterial and fungal (mold) lipases. Cold-active lipase detergent formulation is used for cold washing which reduces 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 (http://www.aukbc.org/beta/bioproj2/introduction. html). The basidiomycete *Pseudozyma antarctica* produces lipase for industrial use (Johnson, 2013a).

2.2 The use of enzymes in food and beverage preparation

The major application of proteases in the dairy industry is for the manufacturing of cheese. Four recombinant proteases have been approved by FDA for cheese production.

Calf rennin had been preferred in cheese-making due to its high specificity, but microbial proteases produced by GRAS microorganisms like *Mucor miehei*, *Mucor pusilis*, *B. subtilis*, and *Endothia parasitica* 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 macro peptides. Nearly 40,000 U/g of milk-clotting activity are produced by *A. oryzae* at 120 hours by solid state fermentation (Vishwanatha et al., 2009). For many years, proteases have also been used for the production of low allergenic milk proteins used as ingredients in baby milk formulas (Gupta et al., 2002).

Proteases can also be used for the synthesis of peptides in organic solvents. Thermolysin is used in this way to make aspartame**,** a sugar substitute (Oyama et al., 1981). Aspartame was sold for $1.5 billion in 2003 (Baez-Viveros et al., 2004). In 2004, the production of aspartame amounted to 14,000 metric tons. The global sugar substitute market is the fastest growing sector of the sweetener market.

Fungal alpha-amylase, glucoamylase, and bacterial glucose isomerase are used to produce "high fructose corn syrup" from starch in a business amounting to $1 billion per year. Fructose syrups are also made from glucose by "glucose isomerase" (actually xylose isomerase) at an annual level of 15 million tons per year. The food industry also uses invertase from *Kluyveromyces fragilis*, *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* for manufacture of candy and jam. Beta-galactosidase (lactase), produced by *K. lactis*, *K. fragilis* or *Candida pseudotropicalis*, is used to hydrolyze lactose in milk or whey and alpha-galactosidase from *S. carlsbergensis* is employed in the crystallization of beet sugar.

Microbial lipases catalyze the hydrolysis of triacylglycerol to glycerol and fatty acids. They are commonly used in the production of a variety of products ranging from fruit juices, baked foods, pharmaceuticals, and vegetable fermentations for 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.

Pectinases, pectin lyases and esterases reduce viscocity of pectin gels from fruits and vegetables and are used for juices preparation (apple, orange, pineapple, carrot, etc..) by reducing viscosity and improving processability (Tucker & Woods, 1995). Total liquefaction of juices can be obtained with the use of hemicellulases and amylases.

Pectinases, and cellulases, are also used, for maceration of grapes and processing of the resulting juice to produce wine. Besides, the activity of exoglycosidases to cleave glycosides and β-glycosidases enables the release of aromatic aglycone molecules for aroma compounds. Lysozyme prevents unwanted malolatic fermentation (Gómez-Plaza et al., 2010) and laccases remove polyphenols in white wines (Kunamneni et al., 2008).

Pectinases, and cellulases also have an important role in the malting process for beer production. Other important enzymes in this industry are glucanases, used to breakdown barley, xylanases utilized for arabinoxylans degradation and proteases required to hydrolyze large chain protein molecules present in the cereal and therefore, influencing the flavors produced during fermentation (Blanco et al., 2014).

2.3 The use of enzymes in paper and textile industries

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 manufacture of cotton 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. 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. Also, variants with improved thermotolerance were produced by Gene Reassembly™ technology (Solvak et al., 2005). The best performing variant (CO14) contained eight mutations and had a melting temperature 16 ^{o}C higher than the wild-type enzyme while retaining the same specific activity at 50 ^{o}C. Optimal temperature of the evolved enzyme was 70 ^{o}C, which is 20 ^{o}C higher than 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 (Solvak et al., 2005). Furthermore, alkaline pectinases are used for the treatment of pectic wastewaters, degumming of plant bast fibers, paper making, and coffee and tea fermentations (Hondal et al., 2002).

Lipases are used 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 (Jaeger & Reetz, 1998). 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 *Bacillus* strains, which grow under highly alkaline conditions in combination with other alkaline or neutral proteases, are currently being used in this industry.

2.4 Additional applications of microbial enzymes

Enzymes are more useful than chemical synthesis for production of chemicals and pharmaceuticals. Chemical synthesis has several disadvantages: low catalytic efficiency, lack of enantiomeric specificity for chiral synthesis, need for high temperature, low pH and high pressure, and high levels of organic waste and pollution. On the other hand, enzymes use mild reaction conditions (temperature, pressure and atmospheric conditions) (Johnson, 2013b). They do not need the protection of substrate functional groups and have long half-lives of several days to months. Enzymes can be selected genetically and chemically-modified to enhance stability, substrate specificity and specific activity. Some are active in organic solvents, super-critical fluids, ionic solvents and at high pressure. They can be immobilized, and such forms of the enzymes sometimes have advantages over enzymes in solution, e.g., stability, process duration and catalytic efficiency. Although some enzymes require co-factors, various approaches, such as co-factor recycling and use of whole cells, can solve this problem. About 150 industrial processes use enzymes or whole microbial cell catalysts.

In other enzyme applications, laccases oxidize phenolic and non-phenolic lignin-related compounds as well as environmental pollutants (Rodríguez-Couto & Toca-Herrera, 2006).

They are used to detoxify industrial effluents of the paper and pulp, textile, and petrochemical industries, as medical diagnostic tools, for bioremediation of herbicides, pesticides, and explosives in soil, as cleaning agents for water purification systems, catalyst in drug manufacture, and as ingredients in cosmetics.

Enzymes are also used in a wide range of agro-biotechnological 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 animal feed ingredients and also in foods to improve phosphorous plant uptake by monogastric animals (Vohra & Satyanarayana, 2003). 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, the use of phytase in the food industry involves removal of phytic acid which acts as an anti-nutritional factor. The annual market of phytase is about $500 US million dollars. The enzyme is made by many bacteria, yeasts, and filamentous fungi. Production is controlled by phosphate. Cloning of the phytase-encoding gene *phyA* from *A. niger* var. *awamori* and reintroduction at a higher dosage increased phytase production by seven-fold (Piddington et al., 2003). Recombinant *Hansenula polymorpha* produced 13 g/L of phytase (Mayer et al., 1999). New fungal phytases with higher specific activities or improved thermostability have been identified (Haefner et al., 2005).

Hydrolytic enzymes, such as cellulase and hemicellulase, are very important for the conversion of cellulose to sugars used to prepare biofuels (Zhang, 2011; Chandel et al., 2012). Cellulases attacking corn stover yielded over 50 g/L ethanol (Lu et al., 2010), however when acting on food waste, the production was 81 g/L (Yan et al., 2010). The cost of cellulase is a major factor in the production of ethanol from biomass, amounting to about 40% of total processing costs. Filamentous fungi can produce native cellulases at very high levels (Cherry & Fidanstel, 2006). *Trichoderma* spp. and *Chrysosporium lucknowense* can produce more than 100 g of crude cellulase per liter of broth. *Trichoderma reesei* produces high levels of several endoglucanases and exoglucanases that act synergistically. This organism can produce 100 g/L of extracellular protein of which 60% is the major cellulase Cel7a (CBH I) and 20% is Cel6a (CBH II) (Shuster & Schmoll, 2010). Genencor scientists produced a strain doubled in ability to hydrolyze pretreated corn stover and sugar cane bagasse (Throndset et al., 2010). A cellulase/hemicellulase system, more than twice as active as that from *T. reesei*, was obtained from the extreme thermophilic bacterium *Caldicellulosiruptor bescil* (Kanafusa-Shinkai et al., 2013). The cellulosome contains a group of enzymes important in the conversion of cellulose to sugars. All such enzymes contain a twice-repeated dockerin sequence, not found in non-cellulosomal enzymes. *Zymomonas mobilis* can produce endogenous enzymatic activity against carboxymethyl cellulose. Linger et al., 2010) expressed genes E1 and GH12 from *Acidothermus cellulolyticus* in *Z. mobilis* and these cellulolytic enzymes were expressed as soluble, active enzymes. By fusing secretion signals native to *Z. mobilis* to the N terminals of E1 and GH12, the enzymes were found in the periplasmic space. Actually, E1 is an endo-1,4-β-glucanase and GH12 resembles the same activity.

In the enzymatic and synergistic breakdown of cellulose, the hydrolytic enzymes are important but so are the lytic polysaccharide monooxygenases (LPMOs) (Forsberg et al., 2014). They cleave glycosidic bonds of polysaccharides that are inaccessible to the hydrolases. LMPOs are produced by bacteria and fungi. Regulation of cellulolytic and hemicellulolytic enzyme production in filamentous fungi involves transcription factors such as xlnR from *Aspergillus*, which is involved in D-xylose induction of xylanolytic and cellulolytic enzymes. Other transcriptional factor include C1R-112 from *Neurospora*, ManR, McMA and C1bR from *Aspergillus*, and Bg1R from *Trichoderma*, which regulate cellulolytic and/or hemicellulolytic enzyme production.

Microbial lipases are excellent for biodiesel production, since they are stable in organic solvents, do not need cofactors, have broad substrate specificity and high enantiospecificity (Narwal & Gupta, 2013). They are produced by many microbes, e.g., *Candida antarctica*, *Pseudomonas capacia* and others. The four different methods of biodiesel production are transesterification, blending, microemulsions and pyrolysis; transesterification is the best. Yields of transesterification can reach 100%. Lipase-catalyzed transesterification of soybean oil to biodiesel was expensive due to the cost of lipase, low yields, long reaction times, and the frequent use of co-solvents which had to be removed. To reduce cost, Salum et al. (2010) used whole cells of *Burkholdia cepacia*, produced by solid-phase fermentation on a mixture of sugar cane bagasse and sunflower seed meal as nutrients. The solid material contained lipase and successfully produced biodiesel from soybean oil. The conversion was 95% in 46 hours. The use of enzyme immobilization, and especially recycling of the immobilized enzyme, reduced cost. Adsorption is the best immobilization procedure due to its simplicity, ease, use of mild conditions and low cost. The use of immobilized lipase preparations for biodiesel production has been reviewed by Zhang et al. (2012).

Another advance was devised by Takaya et al. (2011) who used a whole-cell biocatalytic system with *A. oryzae* over-expressing lipase on biomass support particles, resulting in a highly efficient biodiesel production process.

In the chemical industry, enzymes are used to replace chemical processes when they successfully compete on a cost basis. Frequently, require less energy, and yield a higher titer with enhanced catalytic efficiency. They can produce less catalyst waste and by-products, and lower volumes of wastewater streams. They can be used to produce valuable compounds such as L-amino acids. For example, L-tyrosine has been made from phenol, pyruvate, pyridoxal phosphate and ammonium chloride with a thermostable and chemostable tyrosine phenol lyase from *Symbiobacterium toebii*. The amino acid was produced at 130 g/L in 30 hours with continuous substrate feeding. About 150 biocatalytic processes are used in the chemical industry, and this number will increase with the application of genomics and protein engineering. Another example involves over-expression of genes encoding glycolytic enzymes in *Corynebacterium glutamicum*, which enhanced glucose utilization under oxygen deprivation and improved L-alanine production to 275 g/L in 72 hours (Yamamoto et al., 2012). Also, a strain of *C. glutamicum*, containing feedback-resistant enzymes threonine dehydratase and acetohydroxy acid synthetase, could produce 30 g/L of L-isoleucine (Yin et al., 2012).

Free and immobilized thermostable aspartase of *B. subtilis* YM-55-1, expressed in *E. coli*, has been used to prepare 500 mM aspartic acid (66 g/L) in a 24 h fermentation (Tajima et al., 2015). Aspartic acid is used to make the synthetic sweetener aspartame and amino

acid analogs of C4 building block chemicals, such as 1,4-butanediol, tetrahydrofuran, and gamma-butyrolactone.

Determination of the key enzymes involved in L-threonine production in *E. coli* by proteomics indicated that the isoenzyme LysC, which catalyzes the final step, was the key enzyme of the pathway from aspartate to threonine (Zhang et al., 2015). Over-expression of LysC increased the threonine titer by 30% to 130 g/L and the yield was raised from 40% to 50%.

Other useful products made from microbes are organic acids. For example, itaconic acid was produced at 45 g/L after insertion of a modified *pkfA* gene encoding 6-phospho-1-kinase from *A. niger* into *Aspergillus terreus* (Tevz et al., 2010).

Gamma-aminobutyric acid (GABA) is made by genetically-engineered *Clostridium glutamicum* at a level of 29.5 g/L from endogenous glutamic acid (Wang et al., 2015). GABA is used in pharmaceuticals and functional foods. The genetic modifications are the deletions of genes *odhA* and *pyc*. Gene *odha* encodes the E1 subunit of the 2-oxyglutarate dehydrogenase complex (OSHC) whereas gene *pyc* encodes pyruvate decarboxylase. The result was the generation of recombinant strains which accumulated GABA. One strain produced 29.5 g/L which was the highest known for GABA production.

Glutathione (L-gamma-glutamyl-L-cysteinyl-glycine; GSH) is an antioxidant and immune booster used in foods, pharmaceuticals, and cosmetics. Fermentation appeared to be a favorable production process due to its mild conditions, high recovery and low cost. A problem, however, was that GSH inhibited GSH synthetase, resulting in low fermentation levels. This problem was solved when a novel GSH synthetase, less sensitive to GSH inhibition was discovered. An *E. coli* strain, engineered to contain this GSH synthetase, produced 11.3 g/L of GSH in-fed batch culture with a productivity of 2.06 mmoles per h when 75 mM of each of the precursor amino acids (glutamate, cysteine, glycine) were added along with glucose (Wang et al., 2016).

The bioconversion of D-sorbitol to L-sorbose by *Gluconobacter oxydans* is catalyzed by D-sorbitol dehydrogenase, encoded by the *sldh* gene. Increased expression of this gene resulted in increased bioconversion activity (Hu et al., 2015). The sorbose concentration was increased to 144 g/L at 16 hours.

Enzymes are also important in the pharmaceutical industry. They are used in the preparation of beta-lactam antibiotics such as semi-synthetic penicillins and cephalosporins (Valpato et al., 2010). Other beta-lactams include clavams, carbapenems and monocyclic beta-lactams (Tahlan & Jensen, 2013). The beta-lactam antibiotic group is extremely important, making up 60-65% of the total antibiotic market in 2010, i.e., 3×10^7 kg/year out of a total antibiotic market in of 5×10^7 kg/year. Aspartokinase is the initial enzyme of the aspartate pathway in *Streptomyces clavuligerus*, the producer of cephamycin C. This pathway leads to the production of alpha-aminoadipic acid, a precursor of cephalosporins. Incorporating multiple copies of the *ask* gene into *S. clavuligerus*, encoding aspartokinase, tripled cephamycin C production (Ozcenjiz et al., 2010). Beta-lactam synthesizing enzymes are made by fungi, streptomycetes, and unicellular bacteria. Clavulanic acid is made by *S. clavuligerus* (Paradkar, 2013). Augmentin is a combination of the semi-synthetic amoxicillin and clavulanic acid, which is used orally against bacterial infections such as sinusitis, pneumonia, and bronchitis; it had annual sales of over $2.1 billion in 2012.

Beta-lactamases are dangerous enzymes since they can destroy valuable antibiotics, such as penicillins and cephalosporins. About 1300 of these enzymes were already known by 2013 (Bush, 2013). The most dangerous are the (a) extended spectrum beta-lactamases that hydrolyze most penicillins and cephalosporins, and the (b) carbapenemases that can inactivate all beta-lactam drugs. Hospital-acquired pathogens produce multiple beta-lactamases, up to eight by a single strain. Importantly, beta-lactamase inhibitors are known, some of which have the beta-lactam core and have been approved for use (Ehmannet et al., 2012). They include clavulanic acid, tazobactam and sulbactam. Metallo-beta lactamases (MBLs) cause resistance to beta-lactam antibiotics in Gram-negative bacteria (DuToit, 2014; Meziane-Cherif & Courvalin, 2014). Therefore, antibiotic susceptibility was restored in *E. coli* possessing such an enzyme by an extract of *Aspergillus versicolor* containing aspergillomarasmine A (AMA). AMA is a peptide inhibitor of metallo-proteinases.

Another pharmaceutical of major interest is pravastatin, a member of the cholesterol-lowering statins. Statins also include the synthetic atorvastatin (Lipitor) and the semi-synthetics simvastatin (Zocor) and pravastatin (Pravachol). Simvastatin and pravastatin are variants of the naturally-occurring lovastatin and compactin. Lovastatin and compactin are produced by the molds *A. terreus* and *Penicillium citrinum*, respectively. Pravastatin can be produced from compactin, but this involves an expensive dual-step fermentation and biotransformation process. McLean et al. (2015) reprogrammed *Penicillium chrysogenum*, leading to the discovery and engineering of an enzyme involved in the hydroxylation of compactin. This led to a single-step fermentation yielding pravastatin at a concentration higher than 6 g/L.

The oleaginous yeast *Yarrowia lipolytica* is used to produce recombinant enzymes, including lipases, proteases, amylase, mannanase, laccase, and leucine aminopeptidase (Madzak, 2015). Their metabolic products include citric acid, isocitric acid, alpha-ketoglutaric acid, succinic acid, carotenoids (lycopene, beta-carotene) and polyunsaturated fatty acids (PUFAs), such as gamma-linolenic acid. This yeast can accumulate lipids at 40-90% of its dry cell weight (Tai & Stephanopoulos, 2013; Blazek et al., 2013).

Enzymes are also used to produce plastics such as polylactic acid. This plastic is made from microbial lactic acid via chemical polymerization with a heavy metal catalyst. The metal is usually tin, which can lead to environmental problems. To avoid such problems, a novel process was developed using a polyhydroxyalkanoate (PHA) synthase (Matsumoto & Taguchi, 2010; Taguchi, 2010). PHAs are biosynthetic thermoplastics and elastomers, like poly(3-hydroxybutyrate. The PHA synthase forms polylactic acid from lactic acid in a “one pot” enzymatic process. PHA synthase coming from *Pseudomonas* sp.61-3 has broad substrate specificity. The discovery of the lactate-polymerizing enzyme (LPE) came about by engineering a PHA synthase to accept the coenzyme A ester as substrate. This was a major advance for the production of “environmentally-friendly” bioplastics.

Enzymes are also involved in the preparation of chiral medicines, i.e., complex chiral pharmaceutical intermediates. For example, esterases, proteases, lipases, and ketoreductases are used to prepare chiral alcohols, carboxylic acids, amines and epoxides.

3. IMPROVEMENT OF ENZYME PRODUCTION AND CATALYTIC PROPERTIES

Natural enzymes are often unsuitable as industrial biocatalysis and need modifications for industrial use. These modifications include improved stability (i.e., thermostability, oxidative stability, organic solvent tolerance, pH stability, and substrate specificity). The production strains are usually modified by genetic manipulation to gain improved properties including high production level. Tailor-made biocatalysts are made from wild-type enzymes by protein engineering (Bornscheuer, 2013). Goals of protein engineering include improving substrate specificity, altering regio- and stereo-selectivity, increasing reaction rate, and eliminating substrate inhibition and product inhibition. Improvements in tolerance to pH, heat, solvents, reactive components, and high substrate concentrations are also included. In addition, improvement in expression and folding of active proteins, simplification of purification, and attaining better storage stability are visualized. Protein engineering can be carried out by rational design or by directed (molecular) evolution. The latter is also known as molecular engineering (Yang et al., 2014). Rational design requires the 3D structure of the protein whereas directed evolution only requires functional expression of the gene to produce the protein. The gene is subjected to random mutagenesis, e.g., by error-prone polymerase chain reaction (PCR) or gene shuffling. As far back as 2003, approximately 90% of industrial enzymes were recombinant forms (Cherry & Finanstef, 2003). Large enzymes (over 100 kd) are usually expressed in eukaryotes while small enzymes (<30 kD) are expressed in prokaryotes (Liu et al., 2013). With the introduction of recombinant DNA technology, it has been possible to clone genes encoding enzymes from microbes and express them at levels tens and hundreds of times higher than those produced by unmodified microorganisms. For example, the Pfenex strain of *Pseudomonas fluorescens* can produce 20 g/L of recombinant protein (DePalma, 2013). Also, the plant-derived hydroxynitrile lyase was produced in *Pichia pastoris* (reclassified as *Komagataella pastoris)* at over 20 g/L (Hasslacher et al., 1997). Because of these elevated levels, the enzyme industry rapidly accepted the technology and moved enzyme production from strains not suited for industry into industrial strains (Galante & Formantici, 2003).

Directed evolution of proteins is based on the principle of natural evolution processes such as random mutagenesis and genetic recombination. This leads to large mutant libraries (10^6 to 10^9 mutants), which are subject to high-throughput screening. Rational design, on the other hand, requires information on enzyme structure-function relationships to allow modification of one or more amino acids.

Semi-rational design combines directed evolution and rational design and has become popular. Directed evolution includes 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 the combination of rational design with directed evolution (Yuan et al., 2005; Siehl et al., 2005; Bershstein & Tewfic , 2008; Reetz , 2009). It is used for tailoring of enzymes and changing substrate specificity (Kumar & Singh, 2013). Directed evolution involves iterative mutagenesis and screening or selection, i.e., repeated rounds of generation of random gene libraries, expressing the genes in a suitable host, and screening the libraries for the property desired. Improved

properties include enantioselectivity, catalytic efficiency, rate, stability, pH-activity profile, functionality in organic solvents, product inhibition, and substrate specificity. It has markedly increased the activity, stability, solubility, specificity, and enantioselectivity of enzymes (Boettcher & Bornscheuer, 2010). For example, it increased the activity of glyphosate-N-acetyltransferase 10,000-fold and, at the same time, thermostability was raised by 5-fold.

Certain enzymes face problems such as poor stability, substrate/product inhibition, narrow substrate specificity, or enantioselectivity. To solve these problems, genetic modification is often carried out using recombinant DNA techniques. Genetic modification and recombinant DNA technology are thus very important. Protein engineering is used to change protein sequence, rationally or combinatorially. Rational methods include site-directed mutagenesis to target amino acid substitutions, thus requiring knowledge of the 3-dimensional structure and chemical mechanism of the reaction. A small number of variants are produced which are then screened. Combinatorial methods, on the other hand, create a large number of variants for screening, but do not require extensive knowledge about the enzyme. In some cases, industrial production of enzymes has been increased by 100-fold (Singhania et al., 2010). Modification of the enzyme is carried out by (a) rational redesign of the biocatalyst, and/or by (b) combinatorial methods in which the desired functionality is searched for in randomly generated libraries. The rational design approach is carried out by site-directed mutagenesis to target amino acid substitutions. It often fails but successes have been achieved (Beppu, 1990; Van den Burg et al., 1998). Combinatorial methods include directed evolution, which does not require extensive knowledge about the enzyme. Here, a large number of variants are created for screening for catalytic efficiency, enantio-selectivity, solubility, catalytic rate, specificity and enzyme stability. It is rapid and inexpensive. It includes a range of molecular biological methods which allow the achievement of genetic diversity, mimicking mechanisms of evolution in nature. Random mutagenesis of the protein-encoding gene is carried out by various techniques such as (a) PCR, (b) repeated oligonucleotide directed mutagenesis, or (c) by action of chemical agents. Error-prone PCR introduces random point mutations in a population of enzymes. Molecular breeding techniques, such as DNA shuffling, allow *in vitro* random homologous recombination, usually between parental genes with homology above 70% (Ness et al., 2000). After cloning and expression, a large collection of enzyme variants, i.e., about 10^4 to 10^6, is generated and subjected to screening or selection. Improvement of enzymes and whole cell catalysis has been reviewed by de Carvalho (2011). As a result of such improvement, certain enzymes have achieved large markets. For example, the Taq DNA polymerase isolated from *Thermus aquaticus* had sales of $500 million in 2009.

4. DISCOVERY OF NEW ENZYMES

Screening of natural microbes for enzymes suffers from the fact that less than 1% of the microbes inhabiting the biosphere can be cultivated in the laboratory by standard techniques. Genomics, metagenomics, proteomics, and recombinant DNA technology are

now employed to facilitate the discovery of new enzymes from microbes in nature and to create or evolve improved enzymes.

A number of new and useful enzymes have been obtained by metagenomics (Ferrer et al., 2007). Metagenomic screening (Ferrer et al., 2011) involves preparation of a genomic library from environmental DNA and the systematic screening of the library for open reading frames potentially encoding novel enzymes (Uchiyama & Miyazaki, 2009; Gilbert & Dupont, 2011). Metagenomic screening of particular habitats (arctic tundra, cow rumen, volcanic vents, marine environments and termite guts) has yielded enzymes such as lipase, oxidoreductase, amidase, amylase, nitrilase, decarboxylase, epoxide hydrolase and beta-glucosidase. Although *E. coli* has been the usual host for the screening of foreign genes, the system has been improved by the use of alternative hosts and expression systems such as *Streptomyces lividans*, *Pseudomonas putida* and *Rhizobium leguminosarum*.

Genome mining involves exploring genome sequence databases for genes encoding new enzymes. An example of a useful database is the NCBI database (NCBI Microbial Genomes, 2013) which includes more than 2,000 genome sequences and draft assemblies. Two methods are used for the discovery of new enzymes. One of these, i.e., genome hunting, involves the search for open reading frames in the genome of a particular microbe. Those sequences that are annotated as putative enzymes are subjected to subsequent cloning, over-expression and activity screening. A second approach called data mining is based on homology alignment among all sequences deposited in databases. Using bioinformatic tools such as BLAST, the search for conserved regions between sequences yields homologous protein sequences that are then considered candidates for further study.

Enzyme evolution was used by the Codexis Corporation, in cooperation with the Pfizer pharmaceutical corporation, to produce ®-2-methylpentanol, an important intermediate for the manufacture of pharmaceuticals and liquid crystals (Gooding et al., 2010). Enzymatic processes have also been developed by Codexis to replace and improve chemical transformations for production of sitagliptin (Saviole et al., 2010), montelukast (Singulair) (Liang et al., 2009), and sulopenem (Liang et al., 2009b).

Extremophiles can survive under extreme conditions. These include temperature (-2 to 12 ^{o}C, 60 to110 ^{o}C) pressure, radiation, salinity (2-5 M NaCl) and pH (<2, >9). They contain extremely stable enzymes. Genera such as *Clostridium*, *Thermotoga*, *Thermus* and *Bacillus* contain extreme thermophiles growing at 60-80 ^{o}C, whereas hyper-thermophiles are members of *Archea*, e.g., *Pyrococcus*, *Methanopyrus*, and *Thermococcus*. An example of an extremely useful enzyme is the Taq DNA polymerase from the thermophile *T. aquaticus*, which had sales of $500 million in 2009 (De Carvalho, 2011). Industry already uses thermophilic cellulases, amylases, and proteases.

Psychrophiles are already supplying cold-active enzymes such as proteases, amylases, and lipases for future development of detergents to reduce wear and tear of textile fibers. Cold-active cellulases and xylanases are of interest in the pulp and paper industry and for production of second generation biofuels via saccharification of pre-treated lingo-cellulosic biomass. They are also potentially useful for extraction and clarification of fruit juices, improvement of bakery products, bioremediation of waters contaminated with hydrocarbons or oils, and polishing and stone-washing of textiles. Halophilic xylanases,

proteases, amylases, and lipases have been isolated from halophiles such as species of *Halobacillus*, *Halobacterium* and *Halothermothrix* (Van den Burg, 2003).
Also of interest are microbes surviving under extreme pH conditions which could be useful for isolation of thermoalkiphilic proteases and lipases for use as additives in laundry and dishwashing detergents (Shukla et al., 2009).

5. 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. They 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 for the ability to recycle cofactors, and engineering enzymes to function in various solvents with multiple activities, are important technological developments, which will steadily create new applications.
The industrial enzyme market will grow steadily mainly due to improved production efficiency resulting in cheaper enzymes, new application fields, new enzymes from screening programs, and by engineering properties of traditional enzymes. Tailoring enzymes for specific applications will be a future trend with continuously improving tools, further understanding of structure-function relationships, and increased searching 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 or butanol by fermentative organisms has been a major topic of study for years. Increasing environmental pressures and energy prices will make this application a real possibility in the future.
Enzymes should never be considered alone but rather as a part of a biocatalyst technology. Recent developments in the fields of genetic engineering and protein chemistry are bringing ever more powerful means of analysis to bear on the study of enzyme structure and function, that 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 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 a second explosive expansion to the current multibillion dollar enzyme industry.

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