Research review paper

Industrial applications of enzyme biocatalysis: Current status and future aspects

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Abstract

Enzymes are the most proficient catalysts, offering much more competitive processes compared to chemical catalysts. The number of industrial applications for enzymes has exploded in recent years, mainly owing to advances in protein engineering technology and environmental and economic necessities. Herein, we review recent progress in enzyme biocatalysis, and discuss the trends and strategies that are leading to broader industrial enzyme applications. The challenges and opportunities in developing biocatalytic processes are also discussed.

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1. Introduction

Enzymes are the most proficient catalysts, offering much more competitive processes compared to chemical catalysts. A number of enzyme-based processes have been commercialized for producing several valuable products since the biocatalysis was first introduced almost century ago (Bruggink et al., 1998; Estell et al., 1985; Jensen and Rugh, 1987; Sedlaczek, 1988). Despite great potential of enzymes, however, their industrial applications have been hampered mainly owing to undesirable property in terms of stability, catalytic efficiency, and specificity. To overcome such shortcomings, a variety of approaches have been attempted, including screening of enzymes from natural sources, random mutations, immobilization (Dincer and Telefoncu, 2007; Elleuche et al., 2014). During 1980s and 1990s, engineering of enzymes based on structural information allowed extension of their substrate ranges, enabling the synthesis of unusual intermediates. Accordingly, the use of enzymes has been expanded to the manufacture of pharmaceutical intermediates and fine chemicals (Griengl et al.,...
Although industrial applications of biocatalysis have expanded through structure-based rational design, a lack of structural and mechanistic knowledge about enzymes has limited widespread use of enzymes. Since the mid 1990s, in vitro version of Darwinian evolution, so called directed evolution, has made a great contribution to the development of enzymes with great potential (Bornscheuer et al., 2012). Iterative cycles of random mutagenesis, followed by screening of a library enabled rapid and extensive improvement of various properties of enzyme, including stability, substrate specificity, and enantioselectivity. Directed evolution has thus made remarkable progresses in industrial applications of biocatalysis (Kumar and Singh, 2013). Recently, directed evolution tends to be merged with rational design and computational methods to enhance the efficiency of enzyme design by creating focused and smarter libraries (Bornscheuer et al., 2012). Rational design and computational methods based on the structure-function relationships are becoming popular in enzyme engineering. Structural complexity of valuable substances such as drugs and chiral intermediates requires biocatalysts with high stereo- and regio-specificity in industrial process. Accumulated knowledge on the structure-function and dynamics-function relationships are enabling de novo design of enzymes with new functions (Rothlisberger et al., 2008; Siegel et al., 2010), broadening the repertoire of enzymes.

Soaring oil prices have led industries to seek alternative sources of raw materials, such as biomasses. Furthermore, public pressure on green technologies owing to environmental issues has strongly demanded the replacement of chemical processes with cleaner, safer, and more eco-friendly biocatalytic processes (Benkovic and Hammes-Schiffer, 2003). In these regards, enzyme biocatalysis has rapidly substituted traditional chemical processes in many areas, and such replacement is expected to be more accelerated through the development of new technologies in enzyme engineering. In this review, we describe recent advances in enzyme biocatalysis for industrial applications, and discuss the trends and strategies in engineering of industrial enzymes. The challenges and opportunities of enzyme biocatalysis in future will be also discussed.

2. Biocatalysts in industrial biotechnology

2.1. Fine and bulk chemical industries

Applications of enzymes and whole cell biocatalysis for producing diverse types of chemical and biological substances have become a proven technology in chemical and pharmaceutical industries because enzyme-based processes usually lead to a reduction in the process time, number of reaction steps, and amount of waste (Woehlgenuth, 2010). In particular, enzymes provide a more powerful way of producing enantiomerically pure compounds mainly through high chemoselectivity, regioselectivity, and stereoselectivity (Nest et al., 2011). Some examples showing the contribution of biocatalysis to fine and bulk chemical fields are described below.

Acrylamide is an important commodity chemical for synthesizing polyacrylamide used for petroleum recovery, wastewater treatment, papermaking, pesticide formulation, soil erosion protection, and gel electrophoresis (Asano, 2002; Leonova et al., 2000). Traditionally, acrylamide can be produced chemically by oxidizing acrylonitrile using copper and sulfuric acid as a catalyst at high temperature (Padmakumar and Oriel, 1999). However, both methods are known to cause several types of environmental pollution. The discovery of nitrile hydratase (EC 4.2.1.84) and its application in nitrile hydration has offered a new process for the production of acrylamide (Asano et al., 2000). Rhodococcus rhodochrous J1 overexpressing nitrile hydratase efficiently converts acrylonitrile into acrylamide at up to 45% (W/W) under mild conditions (Scheme 1). This biotransformation process produces over 650,000 t annually in Japan (Ogawa and Shimizu, 2002; Groger et al., 2012; Reetz, 2013). To further increase the productivity, Cui et al. recently engineered nitrile hydratase from Pseudomonas putida NRRL-18668, and showed improvements in thermal stability and catalytic activity of 3.5- and 1.5-fold, respectively (Cui et al., 2014). Kang et al. also reported the overexpression of nitrile hydratase from Rhodococcus rhodochrous in Corynebacterium glutamicum, and the recombinant cells resulted in a conversion yield of 93% and final acrylamide concentration of 42.5% in 6 h (Kang et al., 2014).

Glycolic acid is a C2 chemical building block that has found a wide range of applications in cosmetics, food industry and as a precursor for biopolymers (Koivistoinen et al., 2013; Panova et al., 2007). Glycolic acid can be polymerized into polyglycolic acid (PGA), which has high strength and thermo-tolerance as well as low gas permeability suitable as an ideal packaging material for food and other goods. The glycolic acid market in 2011 was $93.3 million, and the total production amount was 40 million kg. The market is expected to reach $203 million in 2018 (Koivistoinen et al., 2013). The conventional method of glycolic acid production relied on the reaction of formaldehyde and carbon monoxide through an acid catalysis at high pressure and temperature (Panova et al., 2007). An alternative method is the use of heterologous host expressing nitrilase (EC 3.5.5.1), lactoaldehyde reductase (EC 1.1.1.77), and lactoaldehyde dehydrogenase (EC 1.2.1.22) for the hydrolysis of glycolonitrile and the oxidation of ethylene glycol, followed by the conversion of glycolic acid (Chauhan et al., 2003). However, conventional chemical and biotransformation methods for glycolic acid have certain drawbacks such as high impurity. For the production of high-purity glycolic acid, Panova et al. attempted a chemo-enzymatic process using E. coli cells overexpressing nitrilase from Acidovorax facilis 72 W (Panova et al., 2007). This chemo-enzymatic process comprises the synthesis of glycolonitrile from formaldehyde and hydrogen cyanide using NaOH, followed by the conversion of glycolonitrile into ammonium glycolate by nitrilase at room temperature, which is further converted to glycolic acid by ion exchange chromatography (IEC) (Scheme 2). This process enables the productivity of more than 1 kg of glycolic acid/g dry cell weight. In addition, to further increase the catalytic activity of nitrilase, a directed evolution was attempted, resulting in a 125-fold increase (Wu et al., 2008).
1,3-Propanediol is a valuable chemical as a C3 chemical building block, and is polymerized with terephthalates used in the manufacturing of textile fiber, film, and plastic (Maervoet et al., 2011). The market for 1,3-propanediol is over 100 million tons/year, and is growing rapidly (Zeng and Bibel, 2002). Currently, the production of 1,3-propanediol is produced from glycerol by metabolically engineered Saccharomyces cerevisiae and Klebsiella pneumonia (Nakamura and Whited, 2003; Sabra et al., 2010). This bioconversion process was shown to result in a maximum yield of about 50% (Sabra et al., 2010). This bioconversion process was shown to result in the microbial fermentation process. For the economic and eco-friendly production of 1,3-propanediol, Rieckenberg et al. developed a novel biosynthetic process using glycerol dehydratase (EC 4.2.1.30) and oxidoreductase-isoenzyme (EC 1.1.1.202) (Scheme 3) (Rieckenberg et al., 2014). In this process, glycerol dehydratase converts glycerol into 3-hydroxymethylglycerol (3-HMG), which is further transformed into 1,3-propanediol by NADPH-dependent propanediol oxidoreductase-isoenzyme from E. coli. Interestingly, the conversion yield of glycerol into the target product, 1,3-propanediol, reached almost 100%.

5-hydroxymethylfurfural (HMF) is considered a promising building block because it allows diverse synthetic processes leading to various chemical compounds such as dimethylfuran (biofuel), 5,5-diformylfuran, 4,4-lactone, and 3,3-butanediol. HMF has recently been considered as a promising biofuel (Jin et al., 2012). HMF can be produced from glucose isomerase followed by fructose dehydration (Scheme 4). Traditionally, HMF is produced through the acid-catalyzed dehydration of monosaccharides such as fructose or glucose (Huang et al., 2010). It was reported that the production yield of HMF from fructose at higher than 70–100% (w/w) can be achieved using hydrochloride (Choi et al., 2011). However, fructose is less stable than glucose. For a more economical production of HMF, attempts to directly use glucose or fructose-based carbohydrates have been made, leading to the development of a process for the production of HMF through a combination of glucose–fructose isomerization using glucose isomerase (EC 5.3.1.5) followed by fructose dehydration into HMF by acid (Scheme 4). This process results in a production yield of about 63–87% (w/w) (Huang et al., 2010).

Scheme 4. Synthesis of 5-hydroxymethylfurfural from fructose using glucose isomerase.

18.5% with ee of 98% in organic solvents (Jin et al., 2012). Based on the results, the use of organic solvents appeared to solve the problem of instability and low solubility of epichlorohydrin. However, these processes are not practically feasible at the industry scale because of a low yield and ee value owing to the substrate and product inhibition. In an effort to tackle this problem, Jin et al. attempted an intermittent feeding of the substrate in a two-phase system, achieving a 42.7% yield of (R)-epichlorohydrin and an ee value of above 99% (Jin et al., 2013). Furthermore, a great deal of effort has been made to increase the productivity, stability and enantioselectivity through the engineering and discovery of epoxide hydrase (Karbourne et al., 2006; Kotik and Kyslik, 2006; Kotik et al., 2011; Reetz et al., 2009).

Cycloextrins are cyclic α-1,4-1,4-glucans that are mainly used as a mixing agent to increase the solubility of water-insoluble compounds and the chemical stability of active ingredients in the pharmaceutical, cosmetics, food, and textile industries (Del Valle, 2004). Cycloextrins are produced from starch or starch derivatives using cyclodextrin glycosyltransferase (EC 2.4.1.19) (Bonnet et al., 2010). Although cycloextrins can easily be produced by enzymes, this process has certain drawbacks such as a low productivity, specificity, and stability (Leemhuis et al., 2010). Takada et al. identified a novel cyclodextrin glycosyltransferase from Bacillus clarkii 7364, which was shown to convert potato starch into γ-cyclodextrin, with a yield of 79% (Takada et al., 2003). However, the total yield of cycloextrins from starch was as low as 14%. As an alternative approach to improving the yield of cyclodextrin production, the use of a bi-enzyme system comprising cyclodextrin glycosyltransferase and a de-branching enzyme such as isoamylase and pullulanase has been attempted (Wang et al., 2013). During the reaction process, a de-branching enzyme was first added to hydrolyze the α-1,6-linkage amylopectin that inhibits the cyclodextrin glycosyltransferase reaction followed by the addition of cyclodextrin glycosyltransferase to catalyze the cyclization. Duan et al. reported a synchronous process based on isoamylase and α-cyclodextrin glycosyltransferase together to catalyze the reaction. This synchronous process enables an 84.6% (w/w) production yield of cyclodextrin in 24 h (Duan et al., 2013).

Scheme 5. Synthesis of (R)-epichlorohydrin from 1,3-dichloro-2-propanol using halohydrin dehalogenase and epoxide hydrase.

5.2. Pharmaceutical industry

Over the decades, the pharmaceutical substances have become increasingly complex, and public and environmental quests for green technologies have increased. Therefore, the industry is seeking low-cost, safer, and greener biocatalytic processes as alternatives to traditional chemical catalysis (Huisman and Collier, 2013; Tomsho et al., 2012). Recently, the Chemical Manufacturing Methods for the 21st Century project (CHEM21) launched in Europe, which is funded by both government...
and industry at up to €26.4 (€21.2) million for four years (Aldridge, 2013). Specific reactions that can be replaced with biocatalysis have been identified in the synthesis of pharmaceuticals, including chiral amine synthesis, stereo and regio-specific hydroxylation of complex molecules, and other redox reactions (Aldridge, 2013; Lutz et al., 2009). Some recent advances in biocatalysis for the pharmaceutical industry are described below.

One of the most successful examples in the practical application of enzymes in the pharmaceutical industry is the anti-diabetic compound, sitagliptin (Desai, 2011; Savile et al., 2010). Sitagliptin is a drug for type II diabetes that has been marketed under the trade name Januvia by Merck (Desai, 2011). Researchers at Codexis and Merck engineered R-selective transaminase (R-ATA, ATA-117) from Arthrobacter sp. for the asymmetric amination of proslagitpin ketone. By applying a substrate walking, modeling, and mutation approach, they were able to overcome the limitation of the substrate's size for the enzyme. A combination of the further directed enzyme evolution and process engineering yielded a variant that converts 200 g/L of proslagitpin ketone into sitagliptin at an enantio-purity of greater than 99.95% even in the presence of 1 M n-PnNH2, with 50% DMSO at a higher temperature than 40 °C (Savile et al., 2010) (Scheme 6). Compared with the rhodium (Rh)-catalyzed process, the biocatalytic process not only reduces the total waste and eliminates the requirement of a rare heavy metal (Rh), it also increases the overall yield by 10% and the productivity (kg/L per day) by 53% (Desai, 2011; Chislieri and Turner, 2013). Mobilization of engineered (R) selective-ATA enables the maintenance of the enzyme activity and stability in an organic solvent, simplifying the workup and allowing a repetitive use of the enzyme (Truppo et al., 2012). The use of several R- or S-selective-ATAs have been reported in a large-scale synthesis of potential drugs such as niraparib (Chung et al., 2013), an orexin receptor antagonist (Girardin et al., 2012), and Janus kinase 2 (JAK2) inhibitor (Froodsham et al., 2013; Meadows et al., 2013).

Another example of a chiral amine synthesis is boceprevir (Li et al., 2012), which is a clinically used drug for chronic hepatitis C infections under the trade name Victrelis by Merck. In the synthesis of boceprevir, an efficient and enantio-pure desymmetrisation of a bicyclic proline intermediate is highly required. Codexis and Merck have employed monoamine oxidase (MAO) from Aspergillus niger for the asymmetric amine oxidation of the intermediate (Scheme 7). Although the activity, solubility, and thermal stability of the enzyme were sufficiently improved to sustain the manufacturing process through protein engineering, an irreversible product inhibition remained a challenge. However, this problem was successfully solved through the trapping of an imine product by the addition of bisulfite, which demonstrates the importance of process engineering in the industrial application of a biocatalyst. Compared with the resolution method, the biocatalytic process not only increases the product yield by 150%, but also reduces the use of raw materials by 59.8%, the consumption of water by 60.7%, and the overall process waste (E factor) by 63.1% (Li et al., 2012). Although the process has yet to be scaled up to the industrial scale, the same asymmetric amine oxidation by MAO is currently used in the synthesis of another drug for hepatitis C infection, i.e., telaprevir (Znabet et al., 2010). The anti-asthmatic drug, montelukast, was developed and marketed by Merck (Liang et al., 2009). A key intermediate in the synthesis of montelukast was asymmetrically reduced using chlorodipinocampheylborane (DIP-Cl). Based on a previously developed variant of KRED that showed activity for this bulky intermediate (Bornscheuer et al., 2012; Liang et al., 2009), Codexis focused on enhancing the activity and stability of the enzyme for replacing the chemical catalyst, DIP-Cl. Combined with a directed evolution and process optimization, the engineered KRED exhibits a high enantioselectivity (>99.9%) and stability even in the presence of ~70% organic solvent at 45 °C (Scheme 8). The biocatalytic process is currently operated on a ~200 kg scale substrate, replacing a hazardous DIP-Cl catalyst. The most intriguing point in the engineering of KRED is increasing the enzyme stability even at a high organic solvent concentration and temperature. Because of the low solubility of the substrate in water, the high organic solvent concentration and temperature are necessary. Based on the correlation between the thermo stability and solvent tolerance (Huisman et al., 2010), researchers at Codexis primarily screened enzyme mutants with increased thermal stability followed by a screening for solvent tolerant mutants (Huisman et al., 2010).

Another example involving KRED is the synthesis of hydroxyxnitrile, which is a key intermediate for atorvastatin, using a multi-enzyme process (Ma et al., 2010). Atorvastatin is a member of the statin family that lowers cholesterol by blocking the cholesterol synthesis in the liver, and is currently marketed by Pfizer under the trade name Lipitor (Ma et al., 2010). Using pre-evolved enzymes, Codexis developed a two-step process composed of three enzyme steps including halohydrin dehalogenase (HDDH), glucose dehydrogenase (GDH), and KRED (Scheme 9). In this process, KRED is involved in the first step of ethyl-4-chloroacetacetate reduction coupled with GDH for cofactor regeneration. Advances in protein engineering technology have enabled a large-scale synthesis of hydroxyxnitrile intermediates. This multi-enzyme process has been proven to be not only environmentally attractive, but also economically viable compared to a traditional chemical process.

With a growing number of enzymes available for the synthesis of pharmaceutical compounds, attempts at developing a "one-pot" processes based on multi-enzyme cascade reactions are also increasing (Oroz-Guinea and Garcia-Junceda, 2013; Ricca et al., 2011; Shin et al., 2013; Simon et al., 2013). Compared to a traditional chemical process and a single-enzyme process, a one-pot process is highly enantioselective and efficient by circumventing the need for multiple steps. Many kinds of cascade reactions involving ATA have been...
reported (Simon et al., 2013), and most of them are coupled with redox enzymes that conduct a recycling of cofactors. Recently, a one-pot cascade process for the synthesis of chiral 2,5-disubstituted pyrrolidines was reported with a combination of existing ATA and MAO variants (O’Reilly et al., 2014) (Scheme 10A). Another advantage of a one-pot reaction with a multi-enzyme cascade will be the use of inexpensive achiral molecules as starting materials. A recent study demonstrated the synthesis of nor-pseudoephedrine (NPE) and norephedrine (NE) from simple materials such as benzaldehyde and pyruvate through a combination of ATA and acetohydroxyacid synthase I (AHAS-I) (Sehl et al., 2013) (Scheme 10B). Using (R) or (S)-ATA, the stereoisomers of NPE and NE were synthesized with high enantiopurity (>99%). Moreover, pyruvate, which is a byproduct of the ATA reaction, was recycled in an AHAS-I reaction as a substrate. In addition to a cascade reaction, artificial multi-enzyme networks connected through redox-recycling have been reported (Tauber et al., 2013) (Scheme 10C).

As the structural complexity of pharmaceutical compounds increases, the demand for biocatalysts in carbon-carbon bond formation, such as aldolase, is also increasing (Windle et al., 2014). Aldol reactions are useful because they can provide key intermediates of pharmaceuticals from simple building blocks. As exemplified in the synthesis of a key intermediate in statin drugs, (3R,5S)-6-chloro-2,4,6-trideoxyhexopyranoside can be produced from simple molecules such as chloroacetaldehyde (CAA) and acetaldehyde using engineered 2-deoxy-ribose-5-phosphate aldolase (DERA) (Jennewein et al., 2006) (Scheme 11). Protein engineering enables a broader substrate range as well as the increased stability, activity, and stereoselectivity of the aldolases (Althoff et al., 2012; Baker and Seah, 2011; Cheriyan et al., 2012; Giger et al., 2013; Zandvoort et al., 2012). Accordingly, the synthetic utility of aldolases is also substantially increased (Windle et al., 2014).

2.3. Food industry

In the food industry, biocatalysis has been used to produce raw materials and final products for a long time (Fernandes, 2010). However, most uses of biocatalysis have focused on hydrolytic reactions for debranching, improving the solubility, and clarification. With the increasing request for nutritional aspects, a significant amount of attention has been paid to the functionality of foods beyond the primary function of nutrient supply. A recent trend in the food industry is to develop functional foods such as prebiotics, low-calorie sweeteners, and rare sugars (Akoh et al., 2008).

Prebiotics are a dietary substance composed of non-starch polysaccharides and oligosaccharides, including inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), lactulose, and breast milk oligosaccharides. However, most of them are not digested well by human enzymes (Figueras-González et al., 2011), and ingredients that selectively promote the growth of intestinal microorganisms have yet to be elucidated. According to a Global Industry Analysis (GIA) report, by 2015, the prebiotic market will reach nearly $225 million and $1.12 billion in the USA and Europe by 2015, respectively (Panesar et al., 2013). With the increasing demands for prebiotics, the food industry has become interested in...
the use of enzymes for high-production yields at low cost and using simple processes. Difructose anhydride (DFA) III is a non-cariogenic sweetener and non-digestible disaccharide that promotes the absorption of calcium, magnesium, and other minerals in the intestine (Haraguchi et al., 2006). DFA III is produced from inulin by the exo-acting inulin fructotransferase (EC 4.2.2.18) from Arthrobacter ureafaciencs (Scheme 12A). Since then, inulin fructotransferase from Arthrobacter sp. and other bacteria has been identified (Hang et al., 2011; Kikuchi et al., 2009). However, the industrial use of DFA III was limited by a low thermal stability and expensive inulin (Hang et al., 2013). A great deal of effort has been made to isolate heat-stable inulin fructotransferase from various microorganisms and to develop a novel process using a cheap substrate. Recent reports have shown a novel inulin fructotransferase Arthrobacter pascens T13-2, Arthrobacter sp. L68-1, and other Nonomuraea species, stable up to 70–80 °C after 1 h of heat treatment (Haraguchi et al., 2006; Pudjiraharti et al., 2011). Fructooligosaccharides (FOS) as a prebiotic can be synthesized from sucrose using fructosyltransferase (EC 2.4.1.19). Inulin has a similar fructofuranosidic linkage to FOS, which is the smallest substrate for inulin fructotransferase. The utilization of sucrose as a substrate to produce DFA III, resulting in about a 10% (w/w) yield, was attempted through a coupled enzyme reaction as a novel approach (Scheme 12B) (Hang et al., 2013).


Galacto-oligosaccharides are health promoting ingredients that show prebiotic properties, but are poorly digestible sugars (Rastall, 2013). Additionally, many other health benefits have been reported, including an improvement in defecation, the stimulation of mineral absorption, colon cancer prevention, and protection against certain pathogenic bacterial infections (Barile and Rastall, 2013). The production of galacto-oligosaccharides was achieved through an enzymatic reaction of lactose with β-galactosidases from various microbial, yeast, and fungal sources, leading to the structural diversity of galactooligosaccharides (Rodriguez-Colinas et al., 2011; Vera et al., 2012). The basic structure of galacto-oligosaccharides contains a lactose (galactose-glucose) backbone at the reducing end, which is expanded up to six galactose residues (Scheme 13). In general, production yield of galacto-oligosaccharides using β-galactosidase reached around 30–35% (w/w) (Park and Oh, 2010). To increase the production yield, a great deal of effort has focused on the isolation and application of thermostable β-galactosidase from thermophilic microorganisms such as Geobacillus stearothermophilus, Pyrococcus furiosus, S. solfataricus, T. maritima, and Thermus sp. because thermostable enzymes lead to a high reaction velocity, lower contamination, and high lactose solubility at high temperature (Bruins et al., 2003; Placier et al., 2009).

Like GOS, fructo-oligosaccharides (FOS) are used as an artificial sweetener and dietary fiber with low caloric levels, promoting the growth of Bifidobacterium in the human colon. In addition, it has an important role in the stimulation of calcium and magnesium absorption, and a lowering of the cholesterol, phospholipid, and triglyceride levels in human serum (Moore et al., 2003; Sanchez et al., 2010). FOS is produced from sucrose by enzymes showing transfructosylation.

Scheme 12. Synthesis of di-fructose anhydride (DFA) III from inulin using inulin fructotransferase (A), and a coupled enzyme reaction (B).

activity. Such enzymes are β-fructofuranosidase (EC 3.2.1.26) and fructotransferase (EC 2.4.1.9), which originate from fungi and bacteria (Hågg and Woodams, 1996; Silva et al., 2013). The reaction of FOS by enzymes is one D-glucose unit (G) and one fructose units (F) in each sucrose (GF) bound together by β (2 → 1) glycosidic linkages (GF + GF → GFn−1 + GFn−1) (Scheme 14). The production yield of FOS at an industrial scale was reported to reach 55–60% (w/w) based on the initial sucrose concentration (Mussatto and Teixeira, 2010). A further increase in yield was shown to be difficult because a high level of glucose was also produced during the reaction, inhibiting the transfructosylation activity. To solve this problem, additional glucose oxidase was added to the reaction mixture in an attempt to convert glucose into gluconic acid, which resulted in a production yield of up to 90–98% (w/w) (Lin and Lee, 2008). As an approach to remove glucose in a reaction mixture, glucose dehydrogenase and calcium carbonate were simultaneously used to precipitate the gluconic acid (Sheu et al., 2013).

2.4. Cosmetic industry

A variety of the ingredients used in the cosmetic industry are produced from petrochemical-based raw materials (Turner, 2012). Recently, however, the cosmetic industry has faced a challenge because of increasing consumer demands for natural and eco-friendly cosmetics (Ansorge-Schumacher and Thum, 2013). Accordingly, the cosmetic industry promotes basic research and eco-friendly processes using enzymes for developing more effective cosmetic products.

Arbutin is the most common skin-lightener, and is known to inhibit melanogenesis without causing melano-cytotoxicity (Hori et al., 2004). As an enzymatic approach to producing arbutin, various enzymes have been used, including α-amylase, α-glucosidase, transglucosidase, sucrose phosphorylase, and dextrosucrase (Wang et al., 2006). Most enzymatic processes, however, have certain drawbacks such as a high substrate cost and low conversion yield (Seo et al., 2009). Recently, a high production yield was achieved using amylosucrase (EC 2.4.1.4), which belongs to glycoside hydrolase family 13 that catalyzes the synthesis of amylose-like glucans from sucrose (Seo et al., 2012). Amylosucrase from Deinococcus geothermalis was shown to catalyze a glycosyltransferase reaction using sucrose and hydroquinone as a donor and an acceptor, respectively. The maximum conversion yield of α-arbutin was higher than 90% in the presence of 0.2 mM ascorbic acid.

Emollient esters are multi-functional oлеochemicals that are widely used in cosmetic products owing to their moisturizing property. Emollient esters such as myristyl myristate were conventionally produced using tin oxalate as catalyst at a high temperature through transesterification of vegetable oils and alcohols (Veit, 2004; Paravindov and Hanefeld, 2011). An esterification reaction was carried out without a solvent in the presence of equal amounts of reactants at 75 °C using Novozym 435 lipase, and a space time yield of 6,731 g d−1 L−1 was achieved for myristyl myristate (Hilterhaus et al., 2008).

2.5. Textile industry

In the textile industry, prior to conversion into fabric and yarn, cotton undergoes various processes including refining, bleaching, dyeing, and polishing (Queiroga et al., 2007). These processes consume large amounts of energy, water, and resources, discharging huge amounts of waste. For the development of cleaner processes, the use of enzymes is rapidly growing. Typical examples include the staining of jeans using cellulase from Trichoderma viride, and a bio-carbonization process in the case of wool (Yachmenev et al., 2002). Cellulase and protease are used in the polishing step for clear dyeing, the improvement of color and surface vividness, and resistance to wrinkles (Dincer and Télenouc, 2007; Silva et al., 2005).

2.6. Pulp and paper industries

In the pulp and paper industries, xylanase and laccinase are used to enhance the quality of the pulp by removing lignine and hemicelluloses, which are typical impurities (Maijala et al., 2008). In pulp production, laccase is also employed for degrading the pitch in wood, the presence of which causes a serious problem in the manufacturing process. The recycling of printed papers such as newspaper using cellulase was also developed (Patrick, 2004). In the paper making process, lignin causes a dark color, and the removal of lignin is required for making bright paper. The chemical pulping process requires the addition of a large amount of alkali chemicals and chlorine (Pu et al., 2005). The use of laccase was shown to avoid elemental chlorine, and significantly reduces the amount of waste that causes ozone depletion and acidification, as well as high energy consumption.

3. Trend and strategy in enzyme engineering for industrial applications

To further expand the industrial use of enzymes, catalytic and biophysical properties of enzymes, such as catalytic efficiency, substrate specificity, and stability, should be satisfied. Even though it would be arguable, stability of enzymes against heat and organic solvents are usually considered most critical owing to harsh industrial process. Thermo-stability is often related with tolerance against organic solvents and destabilizing mutations (Huisman et al., 2010; Reetz et al., 2010; Vazquez-Figueroa et al., 2008), and enhancing thermo-stability is a prerequisite for industrial applications (Bloom et al., 2006). Current trend in engineering thermo-stability is to combine the structure-based rational design and computational methods in conjunction with directed evolution. The focused-directed evolution decreases a library size, but increasing a success rate comparable to random mutagenesis. For instance, directed evolution of Bacillus subtilis lipase guided by a temperature value (B-FIT) approach was shown to result in almost 500-fold increase in half-life of enzyme at 55 °C (Reetz et al., 2006a). B-FIT targets amino acids with high temperature factor (B-factor) in crystal structure, indicating high thermal flexibility (Reetz et al., 2006a; Reetz and Carballeira, 2007). DNA shuffling via SCHEMA effectively generated a thermostable fungal class II cellobiohydrolases (CBH II) through the screening of only 48 variants (Heinzelman et al., 2009). SCHEMA is a computational method that estimates the structural disruption of enzyme after DNA recombination (Silberg et al., 2004). Extremophiles have been considered useful sources of industrial enzymes with high stability against heat, salts, and pH (Elleuche et al., 2014; Illanes et al., 2012; Kazlauskas and Bornscheuer, 2009). Enzymes from extremophiles were revealed to have compact structure and a number
of charge interactions compared to mesoporous counterparts, and such structural and functional analyses of extreme-enzymes have provided some insight into the design of enzymes with high stability. Even though the functional expression of such enzymes remains a challenge (van den Burg, 2003), they can be used as starting templates for engineering the stability of enzymes.

As for catalytic property of enzymes, focused-directed evolution has been widely applied to increase the catalytic activity or to alter the substrate and product specificities (Evran et al., 2012; Kumar and Singh, 2013; Sterner, 2011). Directed evolution based on the mechanism of phenylalanine ammonia lyase (PAL) reaction resulted in a 15-fold higher activity and decreased substrate inhibition compared to the wild-type enzyme (Bartsch and Bornscheuer, 2010). Enantioselectivity of epoxide hydrolase was improved by directed evolution along with iterative CASTIng (Reetz et al., 2006b). CASTIng (Combinatorial Active-site Saturation Testing) was shown to be effective for systematic design and screening of a focused library around the binding pocket of enzymes (Reetz et al., 2006b). ProSAR is a strategic computational method that statistically analyzes the sequence-activity relationship of proteins (Fox et al., 2007). Directed evolution in conjunction with ProSAR, was successfully used in the development of ketoreductase (KRED) and R-selective transaminase (ATA-117) to enhance the catalytic activity and enantioselectivity toward industrially relevant substrates (Savile et al., 2010; Liang et al., 2009; Huisman et al., 2010). To use the focused-directed evolution, high-throughput screening (HTS) system with high sensitivity and efficiency is crucial (Acker and Auld, 2014; Kazlauskas, 2008). In this regard, genetic circuitry would be one of the prominent selection methods. Recently, several genetic circuits have been developed to directly measure the activity of enzyme variants based on the expression levels of reporter genes, and effectively used for directed evolution of enzymes (Choi et al., 2014a, 2014b; Jeong et al., 2012; Kim et al., 2010).

In an attempt to explore the use of enzymes in non-natural reactions, much effort has been made to create enzymes with new catalytic functions based on rational design and computational methods in conjunction with directed evolution (Lee et al., 2009; Park et al., 2006; Song and Tezcan, 2014; Sterner et al., 2008). At the same time, computational modeling approaches, such as calculation of the free energy perturbation, substrate docking simulation, molecular dynamics (MD) and hydrogen bond energy calculation, have been employed in a rational design (Krieger et al., 2002; Schwab et al., 2008; Bommarius et al., 2006; Illanes et al., 2012; Kazlauskas and Lutz, 2009; Khare et al., 2012). In particular, MD simulations can predict unstable residues useful for altering either activity or thermo-stability. For instance, thermo-stability of xylanase was significantly improved without the expense of the enzyme activity through optimization of unstable residues predicted by MD simulations (Bhabha et al., 2011; Joo et al., 2011; Lee et al., 2010). Choi et al. reported a rational design of ornithine decarboxylase with enhanced catalytic activity using substrate docking and MD simulations (Choi et al., 2014a, 2014b). Considering the importance of dynamics in enzyme reactions, MD simulation will provide valuable information about the flexibility–function relationship of enzymes, which has not been possible by crystal enzyme structures. A novel NMR experiment in conjunction with mutagenesis has been applied to the study of enzyme catalysis (Doucet, 2011). Like MD, this approach will provide some insight into the flexibility–function relationship for understanding the effect of global networks of flexible residues on the activity and stability of enzymes (Kim et al., 2013; Seo et al., 2014). Ultimate goal in rational design of industrial enzymes is to generate enzymes with new and robust catalytic functions for industrial process. De novo design of industrial enzymes at this stage still remains elusive, but some studies have shown notable successes by Rosetta method (Rothlisberger et al., 2008; Siegel et al., 2010). Rosetta is the most advanced computational approach that applies quantum mechanics to computational design of novel enzymes based on existing scaffold (Das and Baker, 2008).

Multi-enzyme process offers some advantages, such as easy process control and monitoring, a high reaction rate, easy scale-up and low toxic by-products (Rollin et al., 2013). However, multi-enzymes process has certain obstacles yet to be overcome for industrial application, such as the requirement of a large amount of enzymes and low intermediate concentration. To enhance the reaction rate and conversion yield, the concept of substrate channeling has been attempted (Zhang, 2011). The channeling of intermediates into next-stage enzymes can allow the design of an efficient synthetic pathway without the loss of intermediates, consequently reducing the amount of enzymes in the reaction system. Typically, co-localization of the relevant enzymes in proximity of the proteins using unnatural amino acids was attempted (Seo et al., 2011). The use of scaffold molecules to induce the binding of enzymes, including DNA, RNA and proteins, has led to higher product yield (Fu et al., 2014).

4. Conclusions

Over the past decades, enzyme-based processes have continuously substituted traditional chemical processes in many areas, especially fine chemical and pharmaceutical industries. Owing to the development of new technologies in enzyme engineering as well as economic pressure and public concern about environmental pollution, such replacement will be more accelerated. Therefore, it would be a great chance for researchers to explore new applications and technologies in enzyme engineering. Current trend in enzyme engineering based on the focused-directed evolution in conjunction with computational methods will continue and even accelerate. Computational algorithms for systematic approach, such as ProSAR, will be more optimized for easy applications. New algorithms analyzing the sequence-function relationship will be explored for generating more systematic and diverse libraries. One of the most challenging problems in enzyme engineering is the lack of general rules in prioritizing enzyme properties to be improved and selecting proper methods. Wrong choice in certain engineering step could jeopardize a whole project. Accumulation of successful stories in enzyme engineering will provide proper rules in choice. To challenge the rational design of de novo enzyme with desired property, mechanistic knowledge on the structure-function and dynamics-function relationships should be further advanced to improve the algorithm for computational enzyme design. With the developed technologies, designer enzymes will be more easily created and industrially applied.

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