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Biocatalyst Development by Directed Evolution

Meng Wang^{1,a}, Tong Si^{1,a}, and Huimin Zhao^{1,2,*}

¹Department of Chemical and Biomolecular Engineering, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801

²Departments of Chemistry, Biochemistry, and Bioengineering, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801

Abstract

Biocatalysis has emerged as a great addition to traditional chemical processes for production of bulk chemicals and pharmaceuticals. To overcome the limitations of naturally occurring enzymes, directed evolution has become the most important tool for improving critical traits of biocatalysts such as thermostability, activity, selectivity, and tolerance towards organic solvents for industrial applications. Recent advances in mutant library creation and high-throughput screening have greatly facilitated the engineering of novel and improved biocatalysts. This review provides an update of the recent developments in the use of directed evolution to engineer biocatalysts for practical applications.

Keywords

Directed evolution; Biocatalysis; Enzyme engineering; High-throughput screening

1. Introduction

Biocatalysts have been extensively studied and increasingly applied in the industrial production of bulk chemicals and pharmaceuticals (de Carvalho, 2011; Du et al., 2011; Patel, 2011; Rubin-Pitel & Zhao, 2006; Schoemaker et al., 2003; Wen et al., 2009a; Zhao, 2011). Compared to chemical catalysis, biocatalysis provides tremendous advantages such as high efficiency, high degree of selectivity (regio-, chemo-, and enantio-), and “green” reaction conditions (Hudlicky & Reed, 2009; Reetz, 2009). Notable examples include the nitrile hydratase catalyzed hydrolysis of acrylonitrile to acrylamide for use in plastics, which reached a production level of 10,000 tons per year and the large-scale D-amino acid oxidase catalyzed transformation of cephalosporin C to -keto-adipyl-7-aminocephalosporinic acid for use in antibiotics production (Wandrey et al., 2000).

Thanks to recent advances in functional genomics, numerous new enzymes have been discovered from various sources. However, in most cases, naturally occurring enzymes are not optimized for practical applications due to the difference between the cellular environment and the industrial setting. Different approaches have been taken to solve this

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*To whom correspondence should be addressed. Phone: (217) 333-2631. Fax: (217) 333-5052. zhao5@illinois.edu.

^aThese two authors contributed equally.

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problem, such as tailoring the target chemical manufacturing process to suit the biocatalyst and exploring enzyme homologs to suit the industrial process. The latter is greatly facilitated by the exponential growth of genome and metagenome sequencing data (Fernandez-Arrojo et al., 2010). Nevertheless, the most successful approach is to engineer existing biocatalysts to be compatible with the target industrial process via directed evolution. Through iterative cycles of mutation, selection and amplification, important traits (e.g. thermostability, activity, selectivity, and tolerance towards organic solvents) of biocatalysts can be optimized for industrial application.

The purpose of this review is to update the recent developments in the use of directed evolution to engineer biocatalysts for practical applications. New tools for directed evolution are briefly discussed. Recent examples of enzyme engineering via directed evolution are grouped according to different enzyme classes and discussed in detail.

2. New tools for directed evolution

A general scheme of directed evolution experiments contains four main steps (Figure 1). The first step is to choose a parent protein. Usually, a parent protein has a similar target function but is not optimal for the desired application, although recent reports have indicated that completely novel functions can be created via a combined rational design and directed evolution approach (Chen et al., 2009; Jiang et al., 2008; Smith & Hecht, 2011). In addition, an unnatural amino acid containing protein can be evolved to improve its target property (Brustad & Arnold, 2011). The second step is to create a mutant library based on the parent protein. A wide variety of methods have been developed to create a mutant library, including chemical mutagenesis, mutator strain, error-prone PCR, site-saturating mutagenesis, and DNA shuffling (Labrou, 2010; Wen et al., 2009a). Library creation is relatively easy but critical to the success of directed evolution because of our minuscule library search ability compared to the unlimited sequence space. Although conventional random mutagenesis methods such as error-prone PCR is still widely used, tremendous effort has been focused on designing and generating a smart (sequence-space restricting) library for directed evolution (Shivange et al., 2009). Unlike the random mutagenesis method, which only requires limited prior knowledge of the parent protein, the success of designing a smart library relies heavily on three major factors: (a) Understanding of the consequences of mutations on protein folding and activity; (b) Structure–function relationships of the parent protein; (c) Methods and programs for identifying ‘key beneficial’ mutations. Several methods have been developed for generating a smart library. For example, Iterative Saturation Mutagenesis (ISM) (Reetz & Carballreira, 2007) was used to improve stereoselectivity and thermostability of lipases (see lipases section for more detail) or protein ligand specificity (Chockalingam et al., 2005). Alternatively, Incorporating Synthetic Oligonucleotides via Gene Reassembly (ISOR) (Herman & Tawfik, 2007), One-pot Simple methodology for Cassette Randomization and Recombination (OSCARR) (Hidalgo et al., 2008), and Overlap-Primer-Walk Polymerase Chain Reaction (OPW-PCR) (Kumar & Rajagopal, 2008) were also developed for creating a smart library. In addition, a few computational tools have been developed to assist the design of a mutant library (Damborsky & Brezovsky, 2009). Although more prior knowledge of the parent protein is required, the smart library strategy can dramatically reduce the library size and increase the success rate of directed evolution experiments. The third step is to identify variants with improved target properties. This is the most challenging yet interesting step in directed evolution experiments. Generally, selection methods are preferred over screening methods because of their higher throughput (Seelig, 2011). However, recent advances in microfluidic methods may be able to bridge this gap (Agresti et al., 2010; Tu et al., 2011). Fluorescence activated cell sorting (FACS) has emerged as a powerful tool for directed evolution experiments due to its high sensitivity and ultrahigh-throughput (10^8 variants per day) (Yang

& Withers, 2009). Variant proteins can be expressed intracellularly to perform a particular function to generate a fluorescence signal from either the fluorescent product or protein like green fluorescent protein (GFP). Variant proteins can also be displayed on cell surface to facilitate the catalysis of a desired reaction. For example, Agresti and coworkers developed an ultrahigh-throughput screening system for directed evolution using drop-based microfluidics (Agresti et al., 2010). The substrate and a single yeast cell displaying a variant protein on its surface are confined to an aqueous drop, which is then sorted by fluorescence activated cell sorting (FACS). As proof of concept, $\sim 10^8$ variants of horseradish peroxidase were screened in only 10 hours, using only 150 L of total reagent volume. Several significantly improved variants, some of which approached the diffusion-limited efficiency, were identified. The last step is to repeat the entire process until the desired function is achieved or until no further improvement is possible.

Usually, one round of variant creation and selection/screening spans from weeks to months, which is a bottleneck in directed evolution experiments. But recently, an elegantly designed directed evolution system may have overcome this bottleneck (Esvelt et al., 2011). The so-called phage-assisted continuous evolution (PACE) system contains a fixed-volume vessel to which uninfected *E. coli* cells are continuously supplied and a mixture of infected and uninfected *E. coli* cells are continuously removed. At the same time, a phage population encoding the target gene is replicating in the same vessel. The infection ability of phage is linked to the function of the target gene. In other words, the maintenance or enrichment of a particular phage in the vessel is dependent on the improved function of the target gene. Therefore, one phage replication/infection cycle equals to one cycle of traditional mutation/selection, and those cycles happen continuously without human intervention. As proof of concept, in a few days the researchers were able to identify a T7 RNA polymerase variant that can recognize the T3 promoter, a novel DNA sequence.

3. Examples

3.1. Oxidoreductases

3.1.1. Oxidases—Non-specific oxidases, such as laccases and peroxidases, have great potential in many industrial processes such as paper pulp bleaching and functionalization, bioremediation, and textile industries (Demarche et al., 2011). Several traits of this class of enzymes have been improved through directed evolution to be more compatible with current industrial processes (Gupta & Farinas, 2010; Liu et al., 2011b; Ribitsch et al., 2010). For example, García-Ruiz and coworkers generated a mutant library of both a laccase from basidiomycete PM1 and a peroxidase from *Pleurotus eryngii* via Mutagenic StEP (Staggered Extension Process) (Zhao et al., 1998) followed by *in vivo* DNA shuffling and IvAM (In vivo Assembly of Mutant) libraries with different mutational spectra. After high-throughput screening using 96-well plates, the thermostability of laccase and peroxidase was improved by 3-fold and 10-fold, respectively (García-Ruiz et al., 2010). In another example, a laccase from *Pleurotus ostreatus* was evolved to have up to 4-fold higher half-life in acidic pH (Miele et al., 2010), which is preferred in industrial applications.

P450 monooxygenases have received extensive attention due to their capability to catalyze a broad range of interesting reactions with high selectivity. However, the application of P450 monooxygenases is hampered by their low catalytic efficiency, low stability, and the need for a complex electron-transfer system. Recent advances have shown that different traits of P450, such as activity and selectivity, can be improved through directed evolution (Fujii et al., 2009; Kabumoto et al., 2009; Koch et al., 2009). A combination of site-specific mutagenesis and error-prone PCR was applied to produce 7800 variants of P450 CYP116B3 from *Rhodococcus ruber*. After 4 rounds of directed evolution, the dealkylation activity was improved by 240-fold (Liu et al., 2010a). Improving or altering selectivity is another

interesting aspect of P450 engineering. Recently, our group developed an efficient high-throughput enantiomeric excess (*ee*)-screening method and reported the inversion of enantioselectivity of the P450_{pyr} monooxygenase by directed evolution (Figure 2), which can provide desirable enantiomers of important pharmaceutical intermediates (Tang et al., 2010).

Baeyer–Villiger Monooxygenases are another important class of oxidases which can catalyze asymmetric ring expansion. Phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* has been extensively engineered by the Reetz group due to its high thermostability and stability in organic solvents. Based on the crystal structure of PAMO, appropriate sites were identified for saturation mutagenesis. As a result, PAMO was evolved to catalyze the kinetic resolution of novel substrates, such as 2-aryl and 2-alkylcyclohexanones, with very high enantioselectivity (*E*-value>200) (Reetz, 2009; Reetz & Wu, 2009; Wu et al., 2010).

3.1.2. Dehydrogenases—Enantiomerically pure alcohols are important building blocks for pharmaceuticals, agriculture, and fine chemicals. General methods for biosynthesis of enantiomerically pure alcohols are asymmetric reduction or kinetic resolution of racemic compounds. For practical applications, high activity and selectivity of biocatalysts are required. In order to reduce the product inhibition of aminoalcohol dehydrogenase (AADH) from *Rhodococcus erythropolis* MAK154, Urano and coworkers screened a mutant library using a colorimetric assay in the presence of high concentrations of the product *d*-pseudoephedrine. As a result, a variant AADH carrying a double mutation was obtained, which showed higher activity in the presence of up to 100 mg/mL *d*-pseudoephedrine (Urano et al., 2011). In order to perform kinetic resolution of *DL*-propargylglycine, phenylalanine dehydrogenase was engineered by Chen and coworkers via directed evolution (Chen & Engel, 2009). A variant with 7.4-fold higher activity and 612-fold higher selectivity was identified after screening around 10,000 transformants.

Sugars of *L*-conformation including *L*-glucose, *L*-galactose and *L*-ribose are essential building blocks for pharmaceuticals. NAD-dependent mannitol-1-dehydrogenase (MDH) from *Apium graveolens* has unique applications in *L*-sugar synthesis due to its regio-selectivity. Extensive directed evolution of MDH was performed by Woodyer and coworkers to improve its activity and thermostability (Christ et al., 2010; Woodyer et al., 2010). A recombinant *E. coli* strain was developed to produce *L*-ribose, *L*-galactose and *L*-gulose at 17 g/L/d, 4.6 g/L/d and 0.90 g/L/d, respectively.

Molecular hydrogen can be generated by hydrogenases using protons and electrons. This class of enzymes has drawn great interest due to its potential applications in renewable energy production. However, low activity and high sensitivity to oxygen inactivation make them unsuitable for practical applications. Stapleton and Swartz have developed a reproducible, albeit complicated, *in vitro* screening protocol for engineering hydrogenases via directed evolution (Stapleton & Swartz, 2010). As proof of concept, a variant [Fe-Fe] hydrogenase HydA1 with ~4-fold improved activity was identified.

3.1.3. Reductases—Directed evolution has also been applied to other classes of oxidoreductases to improve their thermostability (Imamura & Shigemori, 2010) and activity (Suzuki et al., 2010). For instance, Cu-containing nitrite reductase (NiR) from *Alcaligenes faecalis* S-6 was evolved to have better activity towards 3, 3'-diaminobenzidine (DAB) (MacPherson et al., 2010). After screening 20,000 colonies, a double mutant M150L/F312C was identified to have 5.5-fold activity improvement when using dioxygen as the electron acceptor. However, when nitrite was used as an electron acceptor, variant M150L was identified as the best variant (8.5-fold improvement).

3.2. Transferases

Complex carbohydrates are a major class of biological polymers which provides both structural and storage materials for all living organisms. In addition, carbohydrates such as glycans in the form of glycoconjugates play an important role in different biological processes. The structures of carbohydrates are almost unlimited due to their variations in length, sequence, and anomeric linkage (Kittl & Withers, 2010). Because of the importance and complexity of carbohydrates, their generation has drawn great interest. Conventional chemical synthesis either cannot provide precise regio- and stereo-selectivity or requires extensive protection/deprotection steps. On the other hand, enzymatic glycosylation can provide the required high regio- and stereo-selectivity without protection/deprotection steps. Both glycosyltransferases and glycosidases have been extensively explored for carbohydrate synthesis (Wang & Huang, 2009). In addition, directed evolution was used to overcome the problems of low activity and product hydrolysis (De Groeve et al., 2009; De Groeve et al., 2010; Kittl & Withers, 2010; Moretti et al.). For example, Yang and coworkers developed an FACS-based ultrahigh-throughput screening method for directed evolution of sialyltransferases (Yang et al., 2010). Error-prone PCR was used to generate a library of $>10^7$ β -1,3-galactosyltransferase CgtB variants. After library screening, a variant CgtB with 300-fold increased activity was identified. In another example, β -glycosidase from *Thermus thermophilus* was also engineered through a newly developed digital imaging-based high-throughput screening method to obtain a 70-fold improvement in transglycosidase/hydrolysis activity ratio. The yield of trisaccharides synthesis was increased to 76% (Kone et al., 2009).

Glycosyltransferases and glycosidases have also been engineered to synthesize glycoconjugates. For instance, glycosphingolipid-hydrolyzing enzyme endoglycoceramidase II (EGC) was engineered via site-directed mutagenesis and directed evolution with an ELISA-based screening strategy to broaden its substrate scope and improve its catalytic efficiency. The success of identifying a variant with high activity towards a previously inactive substrate demonstrates the general utility of this method (Hancock et al., 2009).

Other classes of transferases have also been evolved to improve their properties for practical applications. Acyltransferase LovD from the lovastatin biosynthesis pathway was engineered via directed evolution to improve its activity by 11-fold (Gao et al., 2009). Another great example of transferase engineering was presented by Savile and coworkers (Savile et al., 2010) (Figure 3). A transaminase with marginal activity toward a previously inactive substrate for the synthesis of a chiral amine was initially created by rational design, which was subject to further directed evolution. After 11 rounds of evolution, all the enzymatic traits that are required in the industrial process were improved. The final variant carrying 27 mutations can convert 200 g/L prositagliptin ketone to sitagliptin of $>99.95\%$ ee with a 92% yield (Savile et al., 2010). This extraordinary example demonstrates the power of directed evolution in improving biocatalysts for industrial applications.

3.3. Hydrolases

Hydrolytic enzymes are the most widely used biocatalysts in organic synthesis and industrial applications, which perform a wide range of bioconversions, such as hydrolysis, transesterification, esterification, alcoholysis, acidolysis, and aminolysis. Thus intensive engineering endeavors have been taken to improve the properties of hydrolases.

3.3.1. Lipases—Lipases constitute the majority of industrial enzymes. While traditional directed evolution methods still prove to be a powerful strategy to increase the robustness towards heat or organic solvents (Kawata & Ogino, 2009; Khurana et al., 2011), new efforts are focused on maximizing the quality of mutant libraries, defined in terms of the frequency

of superior mutants (hits) in a given library and the actual degree of catalyst improvement. To address such a challenge, iterative saturation mutagenesis (ISM) was used to provide a means to accelerate the improvement of stereoselectivity and thermostability (Nair & Zhao, 2008; Prasad et al., 2011; Reetz et al., 2006)

In the ISM process, appropriate sites in the protein comprising one or more amino acid positions, are first randomized with formation of focused libraries. The gene of a given hit then serves as a template for performing saturation mutagenesis at the other remaining sites, and the process is repeated until the desired catalyst quality is achieved or no further improvement is possible.

When addressing stereoselectivity and/or substrate scope, sites aligning the complete binding pocket are considered in a process termed combinatorial active site saturation test (CAST). For example, the lipase A from *Pseudomonas aeruginosa* (PAL) was used as a catalyst in the hydrolytic kinetic resolution of 2-methyldecanoic acid *p*-nitrophenyl ester. As shown in Figure 4, when subject to iterative CASTing, one form of ISM, better results were obtained ($E = 594$ (*S*) versus $E = 51$ (*S*)) (Reetz et al., 2010a) compared to all previous systematic efforts utilizing error-prone PCR, saturation mutagenesis, and/or DNA shuffling while requiring much smaller mutant libraries (10,000 versus 50,000 transformants). CAST was also tested as a means to broaden the substrate scope of this lipase (Prasad et al., 2011). Highly active and enantioselective (E up to 436) variants were identified using more bulky 2-phenylalkanoic acid esters as substrates. Another effort to broaden the substrate scope was performed on *Candida antarctica* lipase A (CalA) (Engstrom et al., 2010). Based on the crystal structure of CalA, residues with side chains pointing toward the substrate cavity were selected for the CASTing libraries. The E values of selected variants for 7 α -substituted *p*-nitrophenyl esters were 45–276, a large improvement compared to 2–20 for the wild type enzyme. In addition, a reversed (*R*)-selectivity of up to 99% *ee* was also achieved.

B-FIT method, another form of ISM, was used to evolve proteins with high thermostability. Saturation mutagenesis was performed iteratively at sites displaying high B-factors available from the X-ray protein crystallography data. This approach rests on the notion that residues with high B-factors have high flexibility, and that appropriate mutations will lead to enhanced rigidity and therefore to higher thermostability. A lipase from *Bacillus subtilis* (BSL) was subjected to directed evolution by the B-FIT method. Using the hydrolysis of *p*-nitrophenyl caprylate as a model reaction, the best variant was shown to have a 45 °C increase in T_{50} to 93 °C. Such thermostable variants also showed markedly improved robustness toward hostile organic solvents (Reetz et al., 2010b).

3.3.2. Esterases—Esterases are estimated to represent approximately 8% of all enzymes used in biocatalysis (Schmidt et al., 2009). Mutagenesis methods and high throughput screening methods for esterases were reviewed recently (Schmidt et al., 2009). Efforts have been taken to improve the enantioselectivity of esterases to enable kinetic resolution of optically active tertiary alcohols (Kourist & Bornscheuer, 2011; Kourist et al., 2008). The recent notable example is the complete inversion of enantioselectivity towards tertiary alcohols of a *Bacillus subtilis* esterase (Bartsch et al., 2008). A double mutant was identified with an E -value of 70 towards a tertiary alcohol from a saturation mutagenesis library covering three residues.

There are two strategies to obtain thermostable variants. One strategy is to increase the thermostability based on highly active parents. For example, directed evolution with the B-FIT strategy generated a variant of the *Pseudomonas fluorescens* esterase with a 9 °C increased melting point without compromising catalytic properties (Jochens et al., 2010). The other strategy is to increase the activity of enzymes from thermophilic origins. The

promiscuous acylaminoacyl peptidase from the hyperthermophilic *Aeropyrum archaeon* pernix K1 (apAAP) exhibited low esterase activity. A variant (R526E) was identified by saturation mutagenesis on a conserved residue in the active site, which essentially switched this peptidase into an esterase (Wang et al., 2006). Further mutagenesis by error-prone PCR and saturation mutagenesis revealed a variant with up to 280-fold increased esterase activity on a more bulky substrate while maintaining high thermostability (Liu et al., 2011a). In another example, the thermostable lactonase from *Sulfolobus solfataricus* (SsoPox) hydrolyzes neurotoxic organophosphorus compounds at a lower rate than its natural substrate lactones. A directed evolution strategy identified a variant with a single mutation W263F with improved phosphotriesterase activity against paraoxon (Merone et al., 2010).

3.3.3. Epoxide hydrolases—Chiral epoxides and diols are important building blocks for manufacturing fine chemicals and pharmaceuticals. The epoxide hydrolases catalyze the hydrolytic ring opening of epoxides which enables hydrolytic kinetic resolution of racemic epoxides to prepare optically pure vicinal diols. Examples and reviews for the engineering of epoxide hydrolases with better enantioselectivity can be found in literature (Widersten et al., 2010).

The epoxide hydrolase from *Aspergillus niger* (ANEH) was used in the hydrolytic kinetic resolution of racemic glycidyl phenyl ether. Variant LW202 with nine mutations was identified through ISM and showed a 25-fold increase in enantioselectivity relative to the wild type enzyme (Reetz et al., 2006). Recently, the basis of the large increase in enantioselectivity ($E = 4.6$ versus $E = 115$) was revealed by kinetic data, molecular dynamics calculations, molecular modeling, inhibition experiments, and X-ray structural work (Reetz et al., 2009). Dramatic differences were observed in the active site with productive positioning of the preferred (*S*)- but not the (*R*)-enantiomer.

In contrast to most epoxide hydrolases, the limonene epoxide hydrolase from *Rhodococcus erythropolis* DCL 14 (LEH) has an unusual structure and catalyzes the hydrolysis of epoxides by a rare one-step mechanism rather than the usual two-step sequence. The substrate scope of the wild type LEH in terms of acceptable stereoselectivity is highly restricted, which hampers its application in synthetic chemistry. From less than 5000 transformants, directed evolution using ISM produced variants that catalyze the desymmetrization of cyclopentene-oxide with improved enantioselectivity towards (*R,R*)-diol (14% to 80% *ee*) or inverted enantioselectivity toward (*S,S*)-diol (93% *ee*) formation (Zheng & Reetz, 2010). The evolved variants also showed broader substrate scope with higher stereoselectivity than the wild type enzyme.

For the epoxide hydrolase from *Agrobacterium radiobacter*, site-saturation mutagenesis on F108 near the substrate-binding pocket yielded five different variants with 3- to 7-fold improved enantioselectivity. Additionally, 7-fold and 150-fold improved activity were obtained towards cis-2,3-epoxybutane (F108C) and cyclohexene oxide (F108A), respectively, with excellent enantioselectivity (>99% *ee*) (van Loo et al., 2009).

3.3.4. Biomass-degrading enzymes—Lignocellulosic biofuels provide a renewable and sustainable substitute or complement to fossil fuels (Lynd et al., 2002). The high cost of cellulases, mainly due to the fact that large amounts of cellulases are required to breakdown cellulose to fermentable sugars, is the major obstacle for economic production of cellulosic biofuels (Merino & Cherry, 2007). To address such challenges, two protein engineering strategies were proposed (Wen et al., 2009): (a) improving properties of individual cellulases; (b) optimizing the enzyme cocktail for maximized synergy.

Directed evolution achieved only moderate success on improving individual cellulases, mainly due to the difficulties in devising high throughput screening methods on activities towards the insoluble cellulosic substrates (Zhang et al., 2006). Cellulose hydrolysis involved the concerted actions of three major types of cellulases: exoglucanases, endoglucanases (EG), and β -glucosidases (BGL). Engineering efforts were mainly focused on the latter two, whose activities can be assayed in a high throughput manner with the help of artificial substrates that were soluble or chromogenic. For endoglucanases, carboxymethyl-cellulose (CMC) was widely used as a model substrate. A petri dish plate assay was performed where the hydrolysis of CMC can be visualized by the clear zone after staining with Congo Red. The size of the zone can be correlated to the enzymatic activity. Based on such assay, endoglucanase variants with improved activity or thermal/pH tolerance were identified by screening libraries created by error-prone PCR and/or DNA shuffling (Liang et al., 2011a; Liu et al., 2010b; Nakazawa et al., 2009a; Nakazawa et al., 2009b). For β -glucosidases, chromogenic analogues of the natural substrate cellobiose are used for screening in a 96-well plate or by FACS (Hardiman et al., 2010; Pei et al., 2011). On the contrary, few examples of directed evolution on exoglucanases existed due to the lack of a reliable screening method. The efforts are then more focused on the semi-rational library creation with higher possibility of positive hits. A series of chimeric fungal class II cellobiohydrolases (CBH II) has been created by structure-guided recombination of three homologous fungal CBH II (Heinzelman et al., 2009). Chimeras were constructed with predicted stabilizing blocks based on SCHEMA/RASPP algorithm, and evaluated by their hydrolytic activity towards insoluble phosphoric acid swollen cellulose (PASC). Out of a small set of chimeras, thermostable variants were identified with between 7 and 15 °C increase in temperature optimum (Heinzelman et al., 2009).

The major problem with most directed evolution experiments on cellulases was that artificial substrates were used to facilitate the high throughput assay. The beneficial hits from the screening did not necessarily show improved hydrolysis of natural substrates. For example, β -glucosidases variants with enhanced activity towards a substrate analogue showed only marginally improved hydrolysis of the natural substrate cellobiose (Hardiman et al., 2010). One *Bacillus* endoglucanase variant with 2.68-fold increased activity towards CMC had no activity on a crystalline substrate Avicel or other celluloses (Chakiath et al., 2009). Thus high throughput screening on natural substrate is highly desired, but also more difficult to carry out (Zhang et al., 2006). The technical challenges were due to the substrate heterogeneity, time-consuming quantification of sugar products, and evaporation control in a small volume for prolonged incubation (Decker et al., 2009). Recently, researchers tried to address these challenges during the development of various automated microplate platforms that can evaluate the enzymatic hydrolysis of lignocellulose in a high throughput manner (Chundawat et al., 2008; Navarro et al., 2010; Song et al., 2010). For example, with the help of Multiscreen® Column Loader, a fine grinded powder of wheat straw (<0.5 mm) was distributed into 96-well microtiter plates with high uniformity and reliability. Xylanase-mediated hydrolysis of wheat straw was monitored by a miniature dinitrosalicylic acid (DNS) assay in a 96-well plate for the quantification of released reducing sugar. Such system allowed high throughput screening of mutated endo- β -1,4-xylanase library from *Thermobacillus xylanilyticus*, and identified a xylanase variant with 74% increase in hydrolytic activity (Song et al., 2010). These automated high throughput systems would greatly facilitate future protein engineering on biomass-degrading enzymes or enzyme cocktails.

On the other hand, the complexity of lignocellulosic materials requires not only individual, but the synergistic actions of a collection of complementary cellulases for optimal degradation (Merino & Cherry, 2007). The ratios and combinations of cellulases greatly affected the hydrolysis efficiency. Substrates from different sources or with different

pretreatment also required distinct enzyme cocktails (Merino & Cherry, 2007). Due to our limited understanding of the complex interactions between enzyme mixture and insoluble substrates, directed evolution seemed an appealing strategy to tackle such complicated systems. Recently, *Saccharomyces cerevisiae* strains with optimized ratio of three types of cellulases (CBH, EG, BGL) displayed on cell surface were developed (Yamada et al., 2010). Library of yeast strains with different integrated copy numbers of cellulases were constructed by cocktail delta-integration. Fast growers selected from PASC-containing plates showed about seven-fold increase in PASC degradation compared to the single-copy integration. More importantly, the author showed that the ratio between the cellulases were more important than the total copies integrated. This result underlined the importance of synergy engineering, which might reduce the amount and cost of enzymes needed for cellulose hydrolysis. Another potential platform for synergy engineering is the multi-enzyme cellulolytic complex called cellulosome (Bayer et al., 2004). Cellulosome composed of a cell-surface-displayed scaffoldin, which can tether different cellulases into the complex through high-affinity dockerin-cohesin interactions. In addition to the enzyme-enzyme synergy observed in the free cellulase system, enzyme proximity, enzyme-substrate and enzyme-microbe interactions all contributed to the enhanced cellulolytic hydrolysis efficiency by cellulosomes (Bayer et al., 2007). Recently, a recombinant cellulosome was successfully expressed in *Saccharomyces cerevisiae* through cell surface display (Lilly et al., 2009; Wen et al., 2010). Though not yet reported, directed evolution on the individual component or the whole complex can be readily performed on such recombinant systems for synergy engineering.

3.4. Other classes of enzymes

3.4.1. Lyases—In the case of lyases, directed evolution has proven to be a successful method when a high-throughput screening assay is available. Activity-based screening can be performed when the concentrations of products or cofactors, or the pH can be readily monitored spectrometrically (Khersonsky et al., 2010; Khersonsky et al., 2011; van Herk et al., 2009). Solubility (Asano et al., 2011) and thermostability (Rha et al., 2009) can also be the targeted properties based on an activity assay. It is also noteworthy that co-improvement can be achieved when the beneficial mutations aiming at different properties are spatially distant from each other (Rha et al., 2009). Activity screening of a random library of tyrosine phenol-lyase from *Symbiobacterium toebii* with or without heat treatment identified variants with improved thermostability or activity. The library combining the selected mutations was constructed by the staggered extension process *in vitro* recombination method (Zhao et al., 1998), from which variants of up to 3-fold improved activity and up to 11.2 °C increase in T₅₀ were obtained.

When high quality protein structure information is available, ISM can accelerate the process or improve the results of directed evolution. Based on a refined inhibitor-bound structure of arylmalonate decarboxylase (AMDase) from *Bordetella bronchiseptica*, five key active-site residues were identified and divided into two groups, which were separately mutated using the ISM strategy. Variants were selected with up to 50-fold improved activity on a range of α -aryl and α -alkenyl malonates (Okraśa et al., 2009). Three rounds of structure-guided directed evolution were performed on an (*S*)-selective AMDase variant G74C/C188S (Miyachi et al., 2011). The first screening focused on Ser188, whose hydroxyl group was believed to have unfavorable interactions with the substrate. The second and third rounds focused on residues in the hydrophobic pocket, which is involved in destabilization of the pro-*R* carboxylate. Activity improvements of up to 920-fold were achieved while the (*S*)-enantioselectivity was maintained or increased to 99% *ee*.

3.4.2. Isomerases, ligases, and transporters—For isomerases, error-prone PCR was performed on a metallic L-arabinose isomerase from *Escherichia coli* and a non-metallic tagatose-6-phosphate isomerase from *Staphylococcus aureus* (SATI), respectively (Kim et al., 2010). A SATI variant with 190% increased isomerization activity toward the unnatural substrate galactose was obtained. The authors also compared the results between the metallic and non-metallic enzymes, and found that the latter was a better template for directed evolution.

Compartmentalized directed evolution of a RNA ligase was performed to increase its neomycin resistance (Paegel & Joyce, 2010). Briefly, a microfluidic emulsification circuit was devised that generates uniform water-in-oil droplets with high throughput (10^7 – 10^8 droplets per hour). Each molecule in the population had the opportunity to undergo 10^8 -fold selective amplification. Then the progeny RNAs were harvested and used to seed new compartments. During five rounds of this procedure, the coding sequences acquired mutations that conferred resistance to neomycin and caused some enzymes to become dependent on neomycin for optimal activity.

Cellular transport systems, such as sugar transporters and efflux pumps, play an important role in many biological processes. However, they are rarely targeted for directed evolution. It is partly because they are membrane proteins and have no use in single step biotransformation, but may greatly increase the efficiency of a cell factory. Recent examples of efflux pump engineering may pave the way for directed evolution of this class of proteins. Cellular export systems, such as efflux pumps, provide a direct mechanism for reducing biofuel toxicity (Dunlop et al., 2011). Thus, engineered efflux pumps with superior performance are desired for improved biofuel production. Directed evolution of active hybrid pumps was achieved by challenging a library of mutated/shuffled TolC variants to adapt to the noncognate *Pseudomonas MexAB* translocase, and confer resistance to the efflux substrate novobiocin (Bokma et al., 2006).

4. Future prospects

Over the past decades, thanks to inherent advantages such as high efficiency, high selectivity, and “green” conditions, biocatalysis has become a great addition to traditional chemical processes for production of bulk chemicals and pharmaceuticals. In order to engineer biocatalysts to be more suitable for industrial processes, many traits such as thermostability, substrate specificity, and tolerance to organic solvents have been successfully improved by directed evolution. Two general trends can be spotted for the development of directed evolution. One is focus on designing, generating and screening of smart (sequence-space restricting) libraries. Although it relies highly on prior knowledge of the target protein, the smart library strategy can significantly reduce the library size and increase the success rate of directed evolution experiments. On the other hand, development of ultrahigh-throughput screening methods based on drop-based microfluidics and FACS has greatly enhanced the capability of screening large libraries. Although these two trends seem as in opposite directions at first glance, they are actually intertwined and complementary to each other. Novel ultrahigh-throughput methods will enable us to perform directed evolution of a target protein with limited knowledge more efficiently, which will in turn provide more insights of the target protein and “hot spots” for designing smart libraries to further improve the desired property for industrial applications. Due to the rapid development of library design and high throughput screening/selection tools, directed evolution has become the most powerful and commonly used tool in protein engineering. Naturally occurring enzymes can be routinely engineered by directed evolution, while the combination of rational design and directed evolution makes the creation of completely novel enzymes possible. Furthermore, as a critical tool in the rapidly developing field of

synthetic biology, directed evolution will be increasingly used for the engineering of pathways, genetic circuits, and genomes (Khalil & Collins, 2010; Liang et al., 2011b). Therefore, directed evolution will continue to provide tailor-made individual enzymes for single-step transformations and multi-enzyme pathways for multi-step catalysis, which will eventually lead to the development of highly complex and efficient cell factories for synthesis of value-added bioproducts.

5. Conclusions

Biocatalysts have been increasingly used in practical synthesis of chemicals. As one of the key enabling technologies in biocatalyst development, directed evolution has been demonstrated to be highly effective in altering or improving almost all kinds of protein properties for all six different classes of enzymes. Continuing development of new library creation methods and high throughput screening methods will make directed evolution even more powerful, which will lead to broader applications of biocatalysis in pharmaceutical, agriculture, food, chemical, and energy industries.

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Figure 1.
General scheme of directed evolution.

Figure 2.

Inverting the enantioselectivity of P450pyr. After three rounds of directed evolution, (S)-specific wild type P450pyr was evolved to be highly (R)-specific.

Figure 3.

Enzyme engineering of a transaminase for synthesis of sitagliptin. After 11 rounds of directed evolution, all the enzymatic traits that are required in the industrial process, including elevated substrate concentration, usage of 50% DMSO, increased pH to 8.5, and enhanced thermostability, were significantly improved.

Figure 4. Directed evolution of *P. aeruginosa* lipase (PAL) with improved stereoselectivity. (a) Hydrolytic kinetic resolution of chiral ester and comparison of results from the traditional method and ISM; (b) ISM system of three grouped sites A, B, C; Red line defines the best evolution pathway in the study.