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Review

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Lipase-Catalyzed Process for Biodiesel Production: Protein Engineering and Lipase Production

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Abstract: Biodiesel is an environment-friendly and renewable fuel produced by transesterification of various feedstocks. Although the lipase-catalyzed biodiesel production has many advantages over the conventional alkali catalyzed process, its industrial applications have been limited by high-cost and low-stability of lipase enzymes. This review provides a general overview of the recent advances in lipase engineering, including both protein modification and production. Recent advances in biotechnology such as in protein engineering, recombinant methods and metabolic engineering have been employed but are yet to impact lipase engineering for cost-effective production of biodiesel. A summary of the current challenges and perspectives for potential solutions are also provided.

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KEYWORDS: biodiesel; lipase; enzyme; transesterification; protein engineering; lipase production

Introduction

In recent years, owing to emerging economies and increasing population, the price of gasoline and diesel remains high. Based on the current consumption rate, the available supply of fossil fuels may last for <50 years (Shariff et al., 2010). In addition, increasing CO_2 emissions due to burning fossil fuels put pressure on the ecological cycle and may account for global climate change. In this context, biodiesel, as an alternative fuel from renewable sources which contain free fatty acids (FFAs) and triglycerides (TGs), can provide a partial solution to this problem.

Biodiesel, a mixture of fatty acid alkyl esters (FAAEs), can be obtained by esterification of FFAs or transesterification of TGs (Andrade et al., 2011; Meher et al., 2006). The typical chemical reactions involved in biodiesel production are shown in Figure 1. Currently, the main feedstock for biodiesel production is virgin oil such as soybean and rapeseed oil (Bart et al., 2010; Moser, 2011). For biodiesel from edible oil sources, the cost of the feedstock represents 70–80% of total biodiesel production costs (Demirbas, 2009). From this context, non-edible oils (e.g., castor bean, jatropha, pongamia, etc.), low value lipids (e.g., animal fat, waste cooking oils, etc.) and microalgae have recently attracted considerable interest (Azocar et al., 2010; Bart et al., 2010; Hama and Kondo, 2013; Lai et al., 2012; Moser, 2011; Olmstead et al., 2013; Zhang et al., 2003b). The selection of appropriate feedstock depends on the regional availability and economics. Biodiesel is a CO_2-neutral fuel since its primary feedstock originates from carbon dioxide in the air. As compared to mineral diesel, biodiesel contains very little sulfur and aromatic compounds, thus has minimal negative impact on air quality (Andrade et al., 2011; Meher et al., 2006). For these reasons, biodiesel is now widely accepted as a sustainable alternative to diesel fuel for transportation applications.

The current world supply of biodiesel comes almost exclusively from chemical-catalyzed conversion processes, using alkali catalysts such as NaOH or KOH (Kaieda et al., 1999; Meher et al., 2006; Srivastava and Prasad, 2000; Zhang et al., 2003b). The alkali-catalyzed reaction, however, produces a large amount of soap which inhibits separation between FAAE and glycerol (see Fig. 1a). In addition, the chemical process typically utilizes large quantities of water to remove alkali catalyst from the product, hence generates

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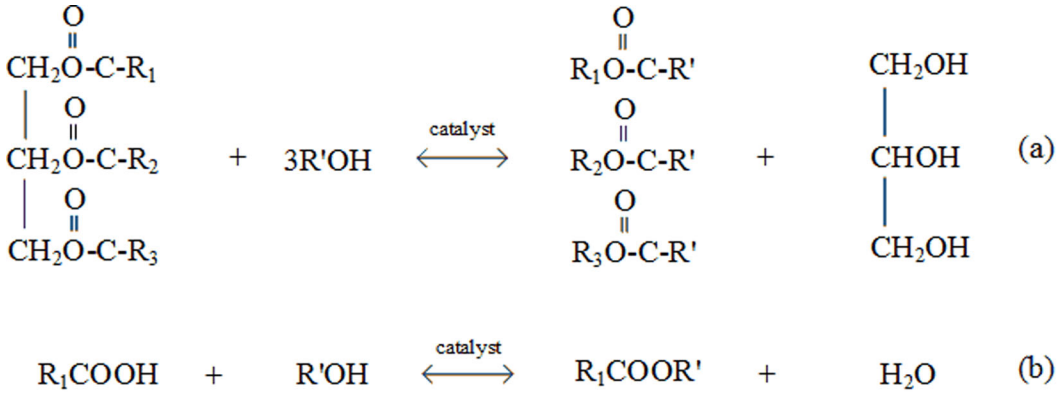

Figure 1. Typical reactions in biodiesel production, (**a**) transesterification of triglycerides (TG), and (**b**) esterification of free fatty acids (FFA).

waste water which adds a significant burden to the ecological system (Suehara et al., 2005). Furthermore, because the esterification and transesterification reactions require different operating conditions, a two-staged reaction system is generally required for the chemical process.

As compared to the chemical-catalyzed process, the enzyme (lipase)-catalyzed process does not have the above noted drawbacks. Specifically, lipases can convert both FFAs and TGs to produce FAAE without soap formation in a single reactor (Fjerbaek et al., 2009; Vasudevan and Briggs, 2008). It is, therefore, also easier to adjust for changes in the supply chain due to market fluctuations, for example, soy bean oil price increase. In addition, the enzymes immobilized in insoluble materials can be relatively easily separated from the final product, which simplifies the downstream separation steps and decreases cost. Despite its great promise, there are major challenges in adapting the lipase-catalyzed process to industrial scale, including performance, stability, recyclability, and production of lipases. Specifically, the rate of enzymatic reactions are generally low. Although lipase is potentially recyclable, it tends to lose activity after continuous operation and can be deactivated by short-chain alcohols and glycerol (Chen and Wu, 2003; Chesterfield et al., 2012; Lu et al., 2012; Salis et al., 2005; Shimada et al., 1999). The high cost of enzymes is also a barrier towards the industrial application of the enzyme-catalyzed biodiesel production processes. Improving the performance and durability of lipase and reducing its manufacturing cost thus hold the key to large-scale commercialization of lipase-catalyzed biodiesel production. Table I lists the most commonly used commercial lipases for biodiesel production.

Protein engineering has been used extensively to tailor-design lipase enzymes for improved performance and durability (Kourist et al., 2010; Singh et al., 2013). Both *rational design* and *directed evolution* approaches have been successfully applied to redesign lipases for enhanced thermostability, tolerance to organic solvents and substrate specificity (Kourist et al., 2010; Singh et al., 2013). Additionally, the production of lipase has been improved by optimal selection of host strains (Treichel et al., 2010) and utilization of metabolic engineering principles (Ramkrishna and Song, 2012; Song and Ramkrishna, 2011). The use of free enzymes has technical limitations due to difficulty of their recovery for reuse, which increases the process cost. Immobilization methods have been utilized to allow recycling of enzyme biocatalysts, which decreases cost and further improves their activity (Jegannathan et al., 2008; Tan et al., 2010). Finally, the biodiesel production process must be optimized to maximize yield of biodiesel while minimizing the process cost (Fjerbaek et al., 2009).

Several recent reviews have addressed the utilization of lipase in biodiesel production (Andrade et al., 2011; Bart et al., 2010; Fjerbaek et al., 2009; Gog et al., 2012; Hama and Kondo, 2013; Moser, 2011; Mounguengui et al., 2013; Tan et al., 2010). In this review, we specifically focus on (1) lipase redesign using directed evolution and rational design approaches, and (2) lipase production using host strain engineering and metabolic engineering techniques.

Protein Engineering

Protein Engineering Strategies

Although lipases derived from natural sources can be used in biodiesel production, they typically lack the desirable features that are suitable for industrial scale reactions. Specifically, natural lipases have maximum catalytic activities in the temperature range 30–50°C (Fjerbaek et al., 2009). At these temperatures, the transesterification reaction has low reaction rate which makes the process time-consuming and less economically competitive. Increasing the working temperature range of lipases by improving thermostability, therefore,

Table I. Biodiesel production with various commercial lipases.

Commercial name	Lipase origin	Oil	Alcohol	Alcohol/oil	Temp (°C)	Yield (%)	Refs.
Lipase AK	*Pseudomonas fluorescens*	Sunflower oil	Methanol	4.5	40	>95	Soumanou and Bornscheuer (2003)
		Sunflower oil	Iso-butanol	3	40	45.3	Deng et al. (2005)
Lipase LA201	*Thermomyces lanuginosa*	Sunflower oil	2-Propanol	3	40	72.8	Deng et al. (2005)
Lipase PS	*Pseudomonas cepacia*	Mahua oil	Ethanol	4	40	96	Kumari et al. (2007)
		Soybean oil	Methanol	7.5	35	67	Noureddini et al. (2005)
		Sunflower oil	1-Butanol	3	40	88.4	Deng et al. (2005)
Lipozyme RM IM	*Rhizomucor miehei*	Sunflower oil	Methanol	3	40	>80	Soumanou and Bornscheuer (2003)
		Sunflower oil	Ethanol	3	40	79.1	Deng et al. (2005)
		Soybean oil	Ethanol	3	60	90	Batistella et al. (2012)
Lipozyme TL IM	*Thermomyces lanuginosa*	Sunflower oil	Methanol	3	40	>60	Soumanou and Bornscheuer (2003)
		Soybean oil	Methanol	3	40	90	Du et al. (2005)
		Sesame oil	Ethanol	5	50	100	Criado and Otero (2010)
Lipopan 50BG	*Thermomyces lanuginosa*	Sunflower oil	Ethanol	3.4	20	70	Verdugo et al. (2011)
Novozym 435	*Candida antarctica*	Sunflower oil	Methanol	3	40	93.2	Deng et al. (2005)
		Sunflower oil	Ethanol	20.6	25	90	Pessoa et al. (2010)
		Soybean oil	Ethanol	14.4	25	100	Pessoa et al. (2010)
		Soybean oil	Ethanol	3	60	57	Batistella et al. (2012)
		Cotton oil	Methanol	6	50	97	Royon et al. (2007)
		Sesame oil	Ethanol	5	50	78.2	Criado and Otero (2010)
		Soybean oil	Methanol	6	40	95	Yu et al. (2010)

is a critical aspect of lipase engineering. Second, owing to degradation, natural lipases typically have limited lifetimes and have to be replaced frequently in industrial reactors. The lipase lifetimes can be further shortened when short chain solvents are used in the conversion reactions (Chen and Wu, 2003; Chesterfield et al., 2012; Lu et al., 2012; Salis et al., 2005; Shimada et al., 1999). It is therefore desirable to engineer a lipase with prolonged lifetimes by enhancing its resistance to both natural and short-chain facilitated degradation pathways. Third, the lipase-catalyzed conversion reactions require binding and unbinding of substrates to the catalytic centers and the reaction rate is typically controlled by the accessibility or diffusion barriers to the catalytic centers. Low reaction rates are generally observed in lipase-catalyzed processes. Increasing the reaction rate is thus crucial for the success of industrial lipases. Finally, most natural lipases have been evolved to target a specific type of substrate with defined chain lengths, while for industrial processes adaptability to various feedstocks with distinctive compositions is desirable. To be commercially competitive, a natural lipase has to be redesigned to possess enhanced features in all of the above-mentioned aspects.

Two major protein-engineering approaches, namely rational design and directed evolution, have been applied to improve the relevant properties listed above. Although both approaches can improve functional properties of lipases, the choice of method depends on the availability of knowledge such as the structure-function relationship of a specific lipase and high-throughput screening approaches.

The rational design of proteins requires a priori knowledge of the structure-function relation of an enzyme. Brady et al. (1990) first utilized X-ray crystallographic analysis to understand the structure of *Rhizomucor miehei* lipase. The structures of other major lipase types have been identified in recent years, including *Bacillus thermocatenulatus*, *Candida antarctica*, *Pseudomonas Cepacia*, and *Bacillus-Subtilis* (Carrasco-Lopez et al., 2008; Ericsson et al., 2008; Kim et al., 1992; Ransac et al., 1994). This information provides a concrete knowledge base to rationally select potential modification sites on lipases. The recent advances in computer-assisted protein design by molecular dynamic simulation tools have further enabled predictions of point mutation(s) on functional properties of lipases (Guieysse et al., 2008). If the structural information of a specific type of lipase is missing, the structure of a homologous enzyme can be utilized to facilitate the modification process (Bordes et al., 2009; Bornscheuer and Pohl, 2001; Kazlauskas, 2000). For example, the structure of *Burkholderia cepacia* lipase (PDB:3LIP) is illustrated in Figure 2 (Schrag et al., 1997). For most lipases, access to the active site containing a serine, histidine and aspartate triad, is shielded by a lid domain. This lid consists of α-helices, which are connected by a loop, linked to the body of a lipase. In the open and active form of the lipase, the lid moves away by rotating hinge region and makes the active site accessible to the substrate. This mobile lid region likely contributes to the stability and activity of the lipase and has been the "hot spot" for lipase engineering in recent years.

Different from the rational design approach, directed evolution does not require a detailed understanding of the structural features of lipases. The most commonly adopted approaches for performing directed evolution start with error-prone polymerase chain reaction (ep-PCR) and/or DNA shuffling. The randomly mutated products then undergo a directed evolution process by imposing selection pressures on the produced constructs. The survivors of the screening process are then analyzed to reveal enhancement of desirable characteristics. In general, a typical random mutagenesis reaction creates 10^4–10^5 variants. For this

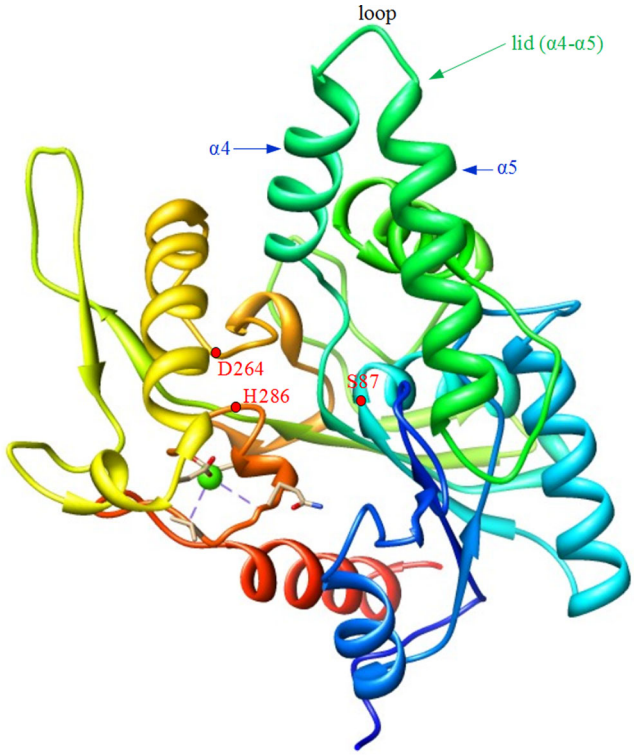

Figure 2. The structure of *Burkholderia cepacia* lipase: The lid region 118–159 (α4-loop-α5) and the catalytic triad (S87, D264, and H286) by red spheres are shown (Schrag et al., 1997).

reason, rapid and efficient high-throughput screening and selection systems are necessary to identify promising candidates. A detailed comparison of the two types of protein engineering strategies is illustrated in Figure 3. Some recent examples of lipase improvements by various protein engineering methods are listed in Table II.

In the following sections, we provide specific examples of how various protein engineering techniques has been used to improve thermostability, organic solvent stability and substrate specificity of lipases.

Thermostability

Since they can be deactivated due to thermal denaturation, thermostability is an important requirement for commercial lipases. In general, increased reaction temperature enhances the solubility of alcohols in oil, which promotes a faster transesterification reaction. Thus, enzymatic catalysts used at high temperatures could increase transesterification yield and require shorter reaction time. Several types of lipases, particularly those originating from thermophilic organisms, such as *Bacillus subtilis*, *Thermomyces lanuginose*, *Rhizopus orzae*, and *Psedomonas* sp., have been reported to have heat resistance up to 90°C (Bouzas et al., 2006; Haki and Rakshit, 2003). The current performance of lipases, however, still falls short of industrial expectations in terms of long-term thermostability.

Both rational design and directed evolution strategies have been employed to enhance thermostability. As an example of the former, Santarossa et al. (2005) identified three polar residues (T137, T138, and S141) in the lid region of cold-adapted *Pseduomonas fragi* lipase using homologous structure. They found that those residues contribute significantly to enhance thermostability of the lipases (Santarossa et al., 2005). In a directed evolution approach, Yu et al. (2012b) improved thermostability of lipase from *Rhizopus chinensis* significantly by two rounds of ep-PCR and two rounds of DNA shuffling. They found that, owing primarily to increasing the hydrophilicity and polarity of the protein surface and creating hydrophobic contacts inside the protein, the melting temperature of a variant was 22°C higher and half-lives at 60 and 65°C were 46- and 23-time longer, as compare to the parent. They also explored the relationship between lid rigidity and lipase activity by introducing a disulfide bond in the hinge region of the lid of lipase (Yu et al., 2012a). They found that, as compared to the wild-type, the cross-linked variant showed ∼11-fold increase in half-life at 60 and 7°C increase of melting temperature. Reetz et al. (2006) introduced the so-called B-factor iterative test (B-FIT) to determine the thermostabiliity of enzymes. Their approach was based on the observation that thermostability can often be related to the rigidity of the protein. A higher B-factor means that an amino acid residue has a low number of contacts with other amino acids and is considered to be more flexible and more thermo-unstable (Radivojac et al., 2004). By an iterative saturation mutagenesis of seven sets of residues from *Bacillus subtilis* lipase A with a high B-factor, they were able to shift the temperature stability ($T_{50,60}$: 50% activity after 1 h at the defined temperature) from 48°C (wild type) to 93°C (Reetz and Carballeira, 2007).

Several structural parameters contribute to the thermostability of a lipase, primarily polarity of enzyme surfaces such as the lid domain (Santarossa et al., 2005; Yu et al., 2012a). Since there are many candidate amino acid sites for possible mutations, as compared to rational design, directed evolution is generally more efficient in exploring all potential mutants. For this reason, it is the preferred strategy followed by many investigators.

Stability in Organic Solvents

Low solubility of short-chain alcohols in oil leads to lipase inactivation. Addition of organic solvent to the mixture of alcohol and oil improves the stability by enhancing the solubility as well as decreasing the viscosity of the reaction mixture. However, lipases can be denatured in organic solvents and therefore activity of the lipases can be limited in systems containing organic solvents (Brocca et al., 2003). Both directed evolution and rational design approaches have been employed to enhance stability of lipase in the presence of organic solvents.

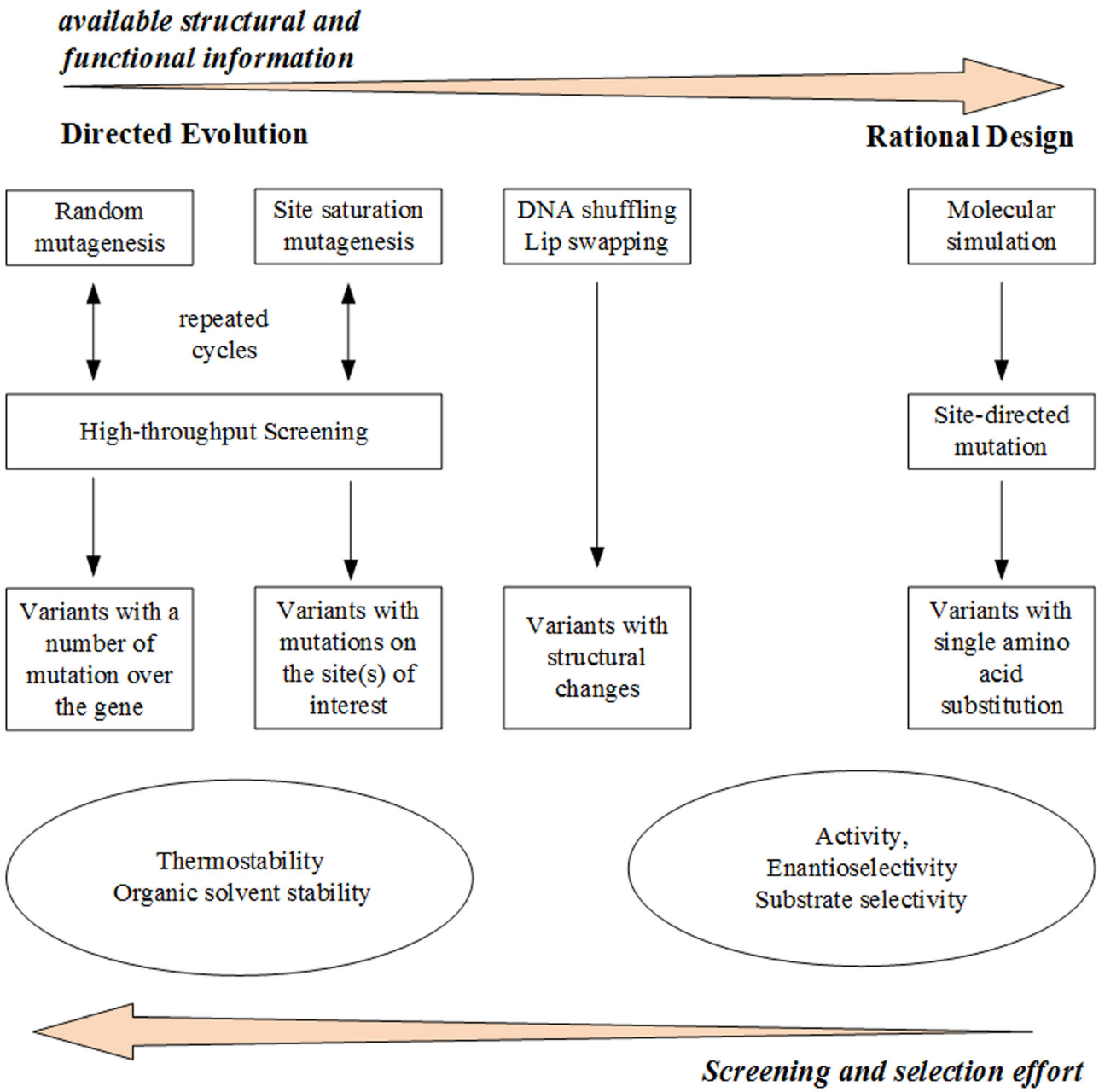

Figure 3. Schematic of strategies to select protein engineering methods.

By means of ep-PCR, mutants of *Psedomonas aeruginosa* LSt-03 lipase have exhibited higher half-life in solvents such as dimethyl sulfoxide (DMSO), cyclohexane, n-octane, and n-decane (Kawata and Ogino, 2009). The structural analysis of these variants revealed that a large fraction of mutations were located on the enzyme surface. Based on this information, they employed a site-directed mutagenesis method (Kawata and Ogino, 2010). Five mutations (S155L, G157R, S164K, S194R, and D209N) were identified to improve stability of lipase in the presence of organic solvents by inducing structural changes which led to an improved packing of the hydrophobic core. Recently, it has been suggested that the loops located on the surface of *Bacillus subtilis* lipase play a critical role in tolerance to organic solvents such as DMSO (Yedavalli and Rao, 2013). They screened ~18,000 clones, based on site saturation mutagenesis of all 91 amino acids in the loop region and found that a variant has eight times higher catalytic turnover in 60% DMSO.

Table II. Examples of enzymes improved by protein engineering techniques.

Target	Enzyme	Mutation methods	Refs.
Thermostability	*Candida antarctica lipase B*	Directed evolution (site saturation mutagenesis)	Peng (2013)
	Aspergillus niger	Directed evolution (iterative saturation mutagenesis)	Gumulya and Reetz (2011)
	Bacillus sp.	Directed evolution (ep-PCR)	Khurana et al. (2011)
	Bacillus subtilis	Directed evolution (iterative saturation mutagenesis)	Gumulya and Reetz (2011)
	Bacillus subtilis	Directed evolution (site saturation mutagenesis)	Ahmad and Rao (2009)
	Bacillus subtilis lipase A	Directed evolution (ep-PCR)	Ahmad et al. (2008)
	Bacillus subtilis lipase A	Directed evolution (iterative saturation mutagenesis)	Augustyniak et al. (2012)
	Candida antarctica lipase B	Rational design	Kim et al. (2010)
	Candida antarctica lipase B	Directed evolution (ep-PCR)	Zhang et al. (2003a)
	Fervidobacterium changbaicum	Rational design	Li et al. (2012)
	Geobacillus sp.	Directed evolution (ep-PCR and site saturation mutagenesis)	Shih and Pan (2011)
	Geobacillus sp.	Rational design	Wu et al. (2010)
	Pseudomonas aeruginosa	Directed evolution (iterative saturation mutagenesis)	Reetz et al. (2010a)
Solvent tolerance	*Bacillus subtilis*	Directed evolution (site saturation mutagenesis)	Yedavalli and Rao (2013)
	Bacillus subtilis	Directed evolution (iterative saturation mutagenesis)	Reetz et al. (2010b)
	Pseudomonas aeruginosa	Rational design	Kawata and Ogino (2010)
Catalytic activity	*Burkholderia cepacia*	Rational design	Ema et al. (2012)
	Bacillus thermocatenulatus	Rational design	Karkhane et al. (2009)
	Candida antarctica lipase B	Rational design	Skjot et al. (2009)
	Candida rugosa LIP4	Rational design	Hung et al. (2011)
	Rhizopus delemar	Rational design	Joerger and Haas (1994), Klein et al. (1997)
Substrate selectivity	*Candida antarctica lipase B*	Rational design	Hamberg et al. (2012)
	Pseudomonas fragi	Rational design	Santarossa et al. (2005)
	Rhizopus delemar	Rational design	Joerger and Haas (1994), Klein et al. (1997)

It has been reported that surface properties of lipases such as hydrophobicity and charge distribution are dominant factors to render lipase stability in organic solvents (Chakravorty et al., 2012). Since most hydrophilic and hydrophobic residues face towards the core and the surface, respectively, a change in surface hydrophobicity would influence lipase contact with solvents. Therefore, modifications of surface residues of lipases, particularly in the loop region, can lead to improved enzyme stability in organic solvents. For this, similar to the case of thermostability, it appears that directed evolution may be more efficient than the rational design method.

Catalytic Activity and Substrate Specificity

The common feedstock for biodiesel production is long-chain carboxylic acids (Knothe, 2005). Thus, lipases with high selectivity for long-chain fatty acids have valuable applications for biodiesel production. In this section, we focus our discussion on improving the specificities for long-chain substrates.

Based on *Candida rogusa* LIP2 crystal structure, two residues located in the substrate-binding site were identified and considered for saturation mutagenesis to examine the effect of these amino acids on substrate specificity (Yen et al., 2010). Two mutant variants of the same position (L132A and L132I) showed a shifted specificity from short- to medium/long-chain length triglycerides, indicating that the specific position has a major impact on substrate specificity (Yen et al., 2010). Using the same approach, Hidalgo et al. (2008) also extended the substrate scope of an arylesterase from *Pseudomonas fluorescens* towards long-chain fatty acid esters. They randomized several cassettes in the gene by a PCR technique and found that the decisive amino acid exchange occurred at the entrance of the active site.

It was also reported that modifications of the lid region can result in changes in the substrate selectivity (Boersma et al., 2008; Fernandez et al., 2008; Santarossa et al., 2005; Skjot et al., 2009). Santarossa et al. (2005) observed that the chain length preference of the lipase from *Pseudomonas fragi* was changed by exchanging polarity in the lid region (Santarossa et al., 2005). Substitutions at Glu87 and Trp89 in the lid region have also been reported to alter the activity of the lipase from *Humicola lanuginose* (Martinelle et al., 1995). By swapping a lid domain of *Candida rugusa*, the absence of the chain length specificity was observed (Brocca et al., 2003; Secundo et al., 2004).

In summary, various efforts to improve catalytic activity for long chain substrates have been made. Particularly, a lid region in lipases interacts with the substrates, leading to an open form that participates in substrate binding and recognition. Therefore, designing regions of lid and substrate-binding site has proven to be effective to modify the activity of lipase, suggesting that site-directed mutagenesis method is an efficient approach. However, a better understanding of the sequence-structure relationship is needed for further improvement.

Current Challenges and Perspectives in Protein Engineering

Since specific structural domains have been recognized and linked to the catalytic activity and substrate specificity of lipases (Boersma et al., 2008; Brocca et al., 2003; Fernandez et al., 2008; Hidalgo et al., 2008; Martinelle et al., 1995; Santarossa et al., 2005; Secundo et al., 2004; Skjot et al., 2009; Yen et al., 2010), rational design is found to be more efficient in designing these types of lipase specificities. Nevertheless, properties such as thermostability and solvent-tolerance are commonly affiliated with the global folding of the protein (Chakravorty et al., 2012; Kawata and Ogino, 2010; Santarossa et al., 2005; Yedavalli and Rao, 2013; Yu et al., 2012a,b). Introducing a single mutation or altering a single structural domain is thus unlikely to significantly affect the global folding and stability. Although modern molecular simulation tools can provide insightful suggestions regarding the modifications sites, the agreement between simulation predictions and experimental outcomes are not always satisfactory. Because of the lack of rational selection guidelines for optimizing global folding under different conditions, directed evolution is thus considered to be the most promising approach for improving thermostability and solvent tolerance.

More recent developments have focused on making smaller libraries ($<$100). This approach refers to the randomization of all amino acids at a defined position or to the simultaneous randomization of two or more positions in an enzyme. In this case, the sequence libraries become smaller and hence faster to screen. In this context, the key to success lies rather in the efficient combination of directed evolution and rational protein design approaches, as suggested previously (Morley and Kazlauskas, 2005).

Despite great advances of engineered lipases in recent years, the existing lipases still lack sufficient stability for long-term continuous operation to be economically feasible to compete with alkali catalysts. Although modern protein engineering techniques can be used to improve some particular aspect of lipase performance, most technical challenges are affiliated with the stability of the enzyme that requires global optimization of the overall protein structures. A systematic screening approach incorporating all process considerations, such as active temperature range, organic solvent stability, catalytic activity, substrate selectivity etc., will thus be required to redesign lipases for commercial scale biodiesel production.

Lipase Production

Lipases are ubiquitous in nature and found in plants, animals and microorganisms. Among them, microbial lipases are the most commonly used in industrial applications due to their selectivity, stability and broad substrate specificity. In spite of improvements in lipase properties in recent years, the high manufacturing cost of lipases is still the major roadblock for commercialization of lipase-catalyzed biodiesel production processes. In this section, we discuss the recent advances in lipase production using host strain and metabolic engineering techniques.

Host Strain Selection

Production of functional lipases using heterologous approaches is the most promising strategy to lower the cost of lipases (Valero, 2012). Many different species have been developed into efficient host strains for heterologous expression of lipases in the past decade, as summarized in Table III. Below, we provide a general overview of the most commonly used expression hosts for the enhanced production of recombinant lipases.

Bacteria

For a variety of reasons *Escherichia coli* remains the most popular expression host for recombinant protein expression. *E. coli* is more adaptable for genetic manipulation and also has high transformation efficiency and rapid growth rates. This prokaryotic host has been used for expressing a variety of lipase originated from bacteria (Akbari et al., 2010), yeast (Jung et al., 2011), and filamentous fungi (Di Lorenzo et al., 2005). Due to the lack of proper folding mechanisms, however, *E. coli* system typically results in intracellular accumulation of inactive or insoluble inclusion bodies. A number of methods have been employed to circumvent this limitation. For example, active form of Lipase B from *Candida antarctica* (CalB), one of the most widely produced enzymes in biocatalysis industries, can be expressed in *E. coli* by changing the reaction medium or modifying the lipase (Blank et al., 2006; Narayanan and Chou, 2009). Fusion of lipases with a polycationic amino acid tag can also increase the solubility of expressed proteins in *E. coli* (Blank et al., 2006). In addition, some types of lipases require the formation of specific disulfide bonds to facilitate the folding of functional proteins. This issue can be addressed by using a specialized *E. coli* Origami (DE3) strain or co-expression of the Dsb-family protein (e.g., DsbA) where disulfide bond formation is involved (Di Lorenzo et al., 2005; Xu et al., 2008b).

Yeasts

Yeasts offer a number of advantages as expression systems for complex proteins, including strong growth capacity, allowing disulfide bond formation, easy genetic manipulation, and post-translational processing of proteins (Darvishi, 2012; Shockey et al., 2011).

Saccharomyces cerevisiae is nonpathogenic and has been used as a host for heterologous lipase production for some time (Yu et al., 2007). Shockey et al. and Darvishi transformed *Yarrowia lipolytica* lipase 2 (LIP2) gene into *S. cerevisiae* with PEX11 promoter. They successfully generated *S. cerevisiae* strains that secrete active Lip2 lipase (Lip2p) into the growth media (Darvishi, 2012; Shockey et al., 2011). Although *S. cerevisiae* expression system allows

Table III. The host strains used for heterologous lipase production.

Host strains	Source		Refs.
	Genus	Species	
Bacteria			
Escherichia coli	*Candida*	*C. antarctica* (LipB)	Jung et al. (2011)
	Candida	*C. antarctica* (LipB)	Ericsson et al. (2008)
	Candida	*C. antarctica* (LipB)	Blank et al. (2006)
	Candida	*C. antarctica* (LipB)	Larsen et al. (2008)
	Candida	*C. antarctica* (LipB)	Liu et al. (2006)
	Pseudozyma	*P. antarctica* (PalB)	Narayanan and Chou (2009)
	Aspergillus	*A. fumigatus* (AFL1-1)	Shangguan et al. (2011)
	Rhizopus	*R. oryzae* (ROL)	Di Lorenzo et al. (2005)
	Ralstonia	*R. solanacearum* (LipA and LipB)	Quyen et al. (2005)
	Ralstonia	*R. solanacearum* (LipA and LipB)	Quyen et al. (2012)
	Psychrobacter	*Psychrobacter* sp.	Lin et al. (2010)
	Pseudomonas	*Pseudomonas* sp.	Akbari et al. (2010)
	Pseudomonas	*P.aeruginosa* (LipA and LipB)	Wu et al. (2012)
	Geobacillus	*G. thermoleovorans*	Abde-Fattah and Gaballa (2008)
	Bacillus	*B. subtilis*	Shi et al. (2010)
	Bacillus	*B. subtilis* (LipA and LipB)	Detry et al. (2006)
Bacillus subtilis	*B. subtilis* A.S.1.1 655	*B. subtilis* IFFI10210	Ma et al. (2006)
	Proteus	*P. vulgaris* (PVL)	Lu et al. (2010)
	Acinetobacter	*Acinetobacter* sp. (LipA)	Han et al. (2003)
Yeasts			
Saccharomyces cerevisiae	*Yarrowia*	*Y. lipolytica* (Lip2)	Darvishi (2012)
	Yarrowia	*Y. lipolytica* (Lip2)	Shockey et al. (2011)
	Yarrowia	*Y. lipolytica* (Lip2)	Yu et al. (2007)
	Yarrowia	*Y. lipolytica* (LIPY7 and LIPY8)	Song et al. (2006)
	Candida	*C. antarctica* (LipB)	Suen et al. (2004)
	Candida	*C. antarctica* (LipB)	Zhang et al. (2003a)
	Pseudomonas	*P. fluorescens*	Jiang et al. (2008)
Pichia pastoris	*Rhizopus*	*R. oryzae* (ROL)	Arnau et al. (2010)
	Rhizopus	*R. oryzae* (ROL)	Guillen et al. (2011)
	Rhizopus	*R. oryzae* (ROL)	Resina et al. (2004)
	Rhizopus	*R. oryzae* (ROL)	Cos et al. (2005)
	Rhizopus	*R. oryzae* (ROL)	Surribas et al. (2007)
	Rhizopus	*R. chinensis* (RCL)	Yu et al. (2009)
	Candida	*C. antarctica* (LipB)	Ferrer et al. (2009)
	Candida	*C. antarctica* (LipB)	Larsen et al. (2008)
	Candida	*C. parapsilosis*	Brunel et al. (2004)
	Candida	*C. antarctica* (LipB)	Eom et al. (2013)
	Candida	*C. Antarctica* (LipB)	Vadhana et al. (2013)
	Candida	*C. antarctica* (LipA)	Yang et al. (2012)
	Candida	*C. antarctica* (LipA)	Liu et al. (2012)
	Candida and *Rhizomucor*	*C. antarctica B (CALB)* and *R. miehei (RML)*	Jin et al. (2013)
	Galactomyces	*G. geotrichum* (BT107)	Fernandez et al. (2006)
	Malassezia	*M. globosa* (Lipase SMG1)	Wang et al. (2012)
Hansenula polymorpha	*Candida*	*C. antarctica* (LipB)	So-Young et al. (2007)
Yarrowia lipolytica	*Candida*	*C. antarctica* (LipB)	Emond et al. (2010)
	Yarrowia	*Y. lipolytica* (LIP2)	Cambon et al. (2010)
Fungi			
Aspergillus niger	*Thermomyces*	*T. lanuginosus*	Prathumpai et al. (2004)
Aspergillus oryzae	*Fusarium* and *Aspergillus*	*F. heterosporum* (FHL) and *A. oryzae* (LipB)	Adachi et al. (2011)
	Candida	*C. antarctica* (LipB)	Adachi et al. (2013)
	Candida	*C. antarctica* (LipB)	Tamalampudi et al. (2007)
	Fusarium	*F. heterosporum*	Hama et al. (2007)
Trichoderma reesei	*Aspergillus*	*A. niger*	Qin et al. (2012)
	Penicillium	*P. allii* (LipPA)	Bradner et al. (2003)

genetic manipulation and high-level of heterologous protein expression, it also has several drawbacks such as poor plasmid stability, low secretion capacity, difficulty in scale-up, and hyper-glycosylation.

Pichia pastoris is the most commonly used host for producing various lipases. It presents several advantages over other hosts, including a highly-regulated promoter of the alcohol oxidase (*AOX*). It can be grown to extremely high

cell density in minimal medium of eukaryotic origins, and has low levels of proteasome secretion and post-translational modifications of proteins. More importantly, it has the ability to efficiently secrete heterologous protein hosts (Arnau et al., 2010; Guillen et al., 2011). For example, *Rhizopus* sp. lipase does not express in *E. coli* due to the lack of necessary proteases to process fungal maturation signals. However, it can be successfully expressed in *P. pastoris host* (Cos et al., 2005; Surribas et al., 2007). *P. pastoris* is commonly used to decrease the process cost since it can secrete heterologous target protein extracellularly with small amounts of contaminating proteins (Yu et al., 2009). Among different yeast strains, *P. pastoris* is considered the most promising host for heterologous lipase production, particularly from eukaryotic sources.

Utilization of non-conventional yeasts, such as *Hansenula polymorpha* and *Yarrowia lipolytica*, has also been studied (Cambon et al., 2010; Emond et al., 2010; So-Young et al., 2007). These strains show distinctive performance depending on the type of heterologous protein. They are thus not generally considered as universal host candidates for lipase production. Among these non-conventional strains, *Y. lipolytica* appears to be a more attractive alternative host due to its high yields of lipase (Madzak et al., 2004) and has recently been adapted for the production of CalB (Emond et al., 2010).

Fungi

Fungi such as genera *Mucor*, *Rhizopus*, *Geotrichum*, *Rhizomucor*, *Aspergillus* and *Penicillium*, are the major lipase-producing sources. As compared with bacteria and yeasts, the filamentous fungi hosts are considered as a supplementary approach. Filamentous fungi have several advantages including higher plasmid copy number, plasmid stability and higher ability to secrete extracellular proteins as compared to other heterologous hosts. Among different fungi species, *Aspergillus* sp. and *Trichoderma* sp. are widely used for lipase production in industrial applications (Adachi et al., 2011, 2013). Prathumpai et al. (2004) reported two recombinant strains of *Aspergillus niger* producing a heterologous lipase from *Thermomyces lanuginosus* using the TAKA amylase promoter from *Aspergillus oryzae*. The most studied filamentous fungi host is *Aspergillus oryzae*, for example, CalB with high esterification activity has been heterologously produced by *Aspergillus oryzae* and immobilized for whole-cell biocatalyst for enzymatic biodiesel production (Prathumpai et al., 2004). In addition, *Trichoderma reesei* has drawn attention for recombinant protein production in recent years using *cbh1* promoter (Wang and Xia, 2011), hence is considered an alternative host for recombinant lipase production.

Host Strain Engineering

In order to use lipases in industrial applications, large production of lipases is required and a number of approaches have been utilized for this purpose. In this section, we review the various processes and metabolic engineering techniques to maximize lipase productivity.

Genetic Manipulation of Host Strains

Commercial lipases are generally obtained from microorganisms that produce a wide variety of extracellular lipases. Thus, different approaches must be applied to optimize extracellular lipase production from various microorganisms. Earlier studies on the production of free-type lipase focused primarily on the performance in batch cultures where optimization of medium and operating conditions are the main parameters (Marcin et al., 1993; Ohnishi et al., 1994; Rapp, 1995; Shimada et al., 1992). Recently, significant increase of lipase production has been achieved by fed-batch fermentation process. Using two different schemes, Fickers et al. (2009) obtained increased production of lipase Lip2 from *Y. lipolytica* mutant-strain *LgX*64.81. Show et al. developed an extractive fermentation method where cell cultivation and downstream processing for the enhanced production of extracellular *Burkholderia cepacia* lipase could be simultaneously obtained through a two-phase system fermentation using a specific thermo-separating reagent (Show et al., 2012). Solid state fermentation introducing sugarcane bagasse as a support and impregnated with a liquid medium was also used to enhance lipase production. Due to the possibility of using agro-industrial residues or by-products as substrate as well as support, it is possible for solid-state fermentation to decrease the final cost of the enzyme (Rodriguez et al., 2006). The major limitations of wild-type lipase production are relatively low productivity and high cost. In addition, wild-type lipase enzymes typically lack optimal specificities and desirable catalytic properties for industrial feedstock.

To meet the standards of quantity and manipulation of industrial processes, cloning and expression of the recombinant lipase genes are the most promising approaches to obtain large amounts of pure lipases. Thus the improvement of the recombinant lipase expression and secretion has been attractive to investigate.

Promoter optimization is a commonly used strategy which significantly enhances the production of lipases. For large scale production of lipase from *Y. lipolytica*, strong constitutive promoters such as *XPR2*, *TEF*, and *RPS7* (Muller et al., 1998) and inducible promoters such as *ICL1*, *POT1*, and *POX2* (Madzak et al., 2004) have been developed. However, *Y. lipolytica* was not considered as an ideal host because the "perfect" inducible promoter is absent. Heterologous expression of protein in *E. coli* system is known for its intracellular accumulation of inactive or insoluble inclusion bodies. Xu et al. (2008a) co-expressed *Pseudozyma antarctica* lipase B (PalB) in *Escherichia coli* with several periplasmic folding factors, such as DegP, FkpA, DsbA, and DsbC. The presence of these folding factors can rescue unstable and inactive PalB (inclusion bodies). Consequently, functional PalB expression in both cytoplasm and periplasm increased significantly.

The ABC transporter protein is typically used for importing and exporting a wide variety of substrates, such as ions, sugars, and amino acids (Gentschev and Goebel, 1992). It is an inner membrane protein composed of an N-terminal membrane domain with 6–8 trans-membrane segments and a C-terminal

ATPase domain. Engineered ABC transporter, consisting of TliD, TliE and TliF, has been used to facilitate the secretion of a thermostable lipase (TliA) in *E. coli* (Eom et al., 2005). By co-expressing TliA with mutated TliD, the secretion levels of TliA lipase was increased by approximately threefold while the expression level of the transporter proteins remained almost unchanged, indicating that engineered transporter proteins can facilitate the secretion of lipases. Cell surface display is a technique to express target proteins fused to an anchoring motif on the surface of various host cells (Chen and Georgiou, 2002). Baek et al. (2010) developed a cell surface display system using *E. coli* OmpC as an anchoring motif to enhance expression of the *Pseudomonas fluorescens* SIK W1 lipase TliA. Cell surface display of lipase appears to stress the cell due to its more heterologous protein "burden" (Bentley et al., 1990), but this system can substantially improve protein production in prokaryotic and eukaryotic host cells. However, the exact mechanism that leads to the improvement remains elusive.

Gene modification that makes the genes adaptable for expression in the recombinant host cells has also been utilized. Chang et al. performed codon optimization on the lip3 gene and improved the lipase yield by 50- to 70-fold (Chang et al., 2006). Yaver et al. employed a restriction enzyme-mediated integration (REMI) as a mutagen to generate insertion mutant libraries in a recombinant *Aspergillus oryzae* strain expressing *Thermomyces lanuginosus* lipase (Yaver et al., 2000). They found that the disruption of palB gene can result in increased lipase expression, while complementation of palB leads to a decrease in lipase production. These results demonstrated that genetic modifications can be used to efficiently modulate the expression of heterologous proteins (Yaver et al., 2000).

Although improvement of lipase production can be achieved from the recombinant lipase, the recombinant protein yield is limited by many post-translational events, such as disulfide bond formation, solubility, misfolding, secretion, proteolysis, and even the toxicity to host cells (Makrides, 1996). Thus, genetic and metabolic engineering can play a crucial role to improve production of the recombinant lipase by overcoming these limitations.

Process Considerations to Improve Lipase Production

Lipase production can also be improved by the efficient and convenient techniques of scale-up fermentation. A significantly enhanced production of *Candida rugosa* lipase in the constitutive recombinant *Pichia pastoris* was achieved by Zhao et al. (2008) in both laboratory and pilot scales by optimizing the fermentation conditions. In this study, fermentation was scaled up from 5 to 800 L using the exponential feeding, which was combined with pH-stat strategy and a two-stage fermentation strategy, which enables an excellent balance between the expression of recombinant lipase and the growth of host cells. They obtained the highest lipase activity of approximately 14,000 IU mL^{-1} and cell wet weight of 500 g L^{-1} at the 800 L scale. In large scale fermentation of recombinant lipases, the cell growth rate can be effectively controlled by tuning cell lyses and proteolytic sensitivity of the lipases (Narayanan and Chou, 2009).

Improvement of Lipase Production by Metabolic Engineering

Metabolic engineering has been of considerable interest in improving biofuels production (Atsumi and Liao, 2008; Lee et al., 2008). It has also been used in improving the production of therapeutic proteins (Dyer et al., 2002; Jorda et al., 2012). However, the application of metabolic engineering to lipase production has been relatively scarce. An exception is in the recent work of Son et al. who were concerned with the extracellular production of lipase by metabolic engineering of *P. fluorescens*, which possesses a secretion system that allows the secretion of a thermostable lipase enzyme (Son et al., 2012). The wild-type organism secretes a lipase that is, however, hydrolyzed. The degradation of the recombinant protein produced varied depending on the type of culture media and aeration. Son et al. deleted the endogenous lipase (TilA) and protease genes SIK W1 of *P. fluorescens* using the targeted gene knockout method. The deletion mutant of *P. fluorescens* secreted recombinant lipase (TilA) in a fusion form at high levels without degradation irrespective of growth conditions. It is apparent that experimental work such as that of Son et al. together with quantitative metabolic engineering offers considerable scope for further work in this direction.

This review has covered various host strains for the production of lipase. Regardless of the hosts adopted, the application of metabolic engineering represents a fruitful direction for increasing the production rate of lipase. In this connection, the use of constraint-based approaches, which have focused on increasing yield of metabolic products, has taken precedence over the more reasonable dynamic approaches for increasing productivity. Towards this end, cybernetic models (Ramkrishna and Song, 2012; Song and Ramkrishna, 2011) have potential for success because of their focus on dynamics and capacity for accounting of regulatory processes in metabolism. The facility to account for regulatory processes also makes such dynamic models more attractive for optimization of process conditions towards maximizing lipase productivity.

Current Challenges and Perspectives in Lipase Production

Among some 4,000 enzymes known to date, lipase is recognized as one of the ubiquitous enzymes of considerable industrial potential. Currently, commercial lipases are generally obtained from microorganisms that produce a wide variety of extracellular lipases. The global demand of commercial enzymes, about 75% of which are hydrolytic enzymes (including lipases), is expected to rise by about 5% in the next decade. Lipase demand in China increased greatly since 2003 as a turning point, and the production capacity has increased by ~10% annually. In 2010, the manufacturing capacity of lipases has reached about 2,500 tons. However, the

current supply of lipases falls short to meet the increasing demand.

This challenge can be addressed by screening for novel lipase-producing microorganisms and performing metabolic engineering. In addition, the development of new lipases production processes, by utilizing submerged fermentation, synthesized operation modes, new high-efficiency bioreactors, and mathematical and statistical optimization models, is also considered to be an effective approach for enhancing lipase production. Among these, high-throughput screening methods and synthetic biology are more likely to improve the productivity of lipase production. Furthermore, recent advances in screening techniques have enabled fast identification of high-yield microbes. Synthetic biology, on the other hand, can extend and modify the behavior of organisms for better lipase productivity. Application of synthetic biology for lipase production is expected to surpass traditional engineering techniques by blending the best features of natural and artificial microbial systems with rational designs that are extensible, comprehensive, and efficient.

Concluding Remarks

Lipase-catalyzed biodiesel production from renewable sources has several advantages over the conventional chemical-catalyzed process, including lower environmental concerns and energy consumption. The low stability and high cost of lipase, however, have been the main hurdles for the industrialization of lipase-catalyzed biodiesel production. In this context, protein engineering and improved lipase production system along with the optimized metabolic process are essential to address the challenges noted above. Both rational design and directed evolution techniques have been successfully used to engineer lipase enzymes for enhanced performance. Advances in modeling and computational tools for sequential and structural analysis as well as screening systems will further facilitate development of high-performance lipases. In addition, optimization of lipase production systems can increase productivity while decreasing product cost. For large-scale commercialization of lipase-catalyzed process, enzyme immobilization and optimization of the process will be also required, which can further decrease total product cost. It is concluded that a concerted research program which combines lipase engineering and metabolic engineering for high lipase productivity, and reaction engineering for process intensification, is likely to yield promising outcome for widespread application of the lipase-catalyzed biodiesel production process.

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