Journal of Molecular Catalysis B: Enzymatic 62 (2010) 1–8

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Review

Technical methods to improve yield, activity and stability in the development of microbial lipases

Zheng-Yu Shu[a,b,c], Huan Jiang[a,b,c], Rui-Feng Lin[a,b,c], Yong-Mei Jiang[a,b,c], Lin Lin[a,b,c], Jian-Zhong Huang[a,b,c,*]

[a] Engineering Research Center of Industrial Microbiology, Ministry of Education, Fujian Normal University, Fuzhou, Fujian 350108, PR China
[b] College of Life Sciences, Fujian Normal University, Fuzhou, Fujian 350108, PR China
[c] Engineering Research Center of Fujian Modern Fermentation Technology, Fuzhou, Fujian 350108, PR China

ARTICLE INFO

Article history:
Received 2 May 2009
Received in revised form 3 September 2009
Accepted 4 September 2009
Available online 10 September 2009

Keywords:
Lipase
Method
Improved yield
Improved activity
Improved stability

ABSTRACT

Lipases are ubiquitous biocatalysts that catalyze various reactions in organic solvents or in solvent-free systems and are increasingly applied in various industrial fields. In view of the excellent catalytic activities and the huge application potential, more than 20 microbial lipases have been realized in large-scale commercial production. The potential for commercial exploitation of a microbial lipase is determined by its yield, activity, stability and other characteristics. This review will survey the various technical methods that have been developed to enhance yield, activity and stability of microbial lipases from four aspects, including improvements in lipase-producing strains, modification of lipase genes, fermentation engineering of lipases and downstream processing technology of lipase products.

Contents

* Corresponding author at: College of Life Sciences, Fujian Normal University, Fuzhou, Fujian 350108, PR China. Tel.: +86 591 22868212; fax: +86 591 22868212.
E-mail addresses: shuzhengyu@gmail.com (Z.-Y. Shu), hjz@fjnu.edu.cn (J.-Z. Huang).

1381-1177/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.molcatb.2009.09.003

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) can catalyze both the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids at the interface between the insoluble substrate and water [1]. These reactions usually proceed with high chemo-, regio- and/or enantioselectivity, making lipases an important group of biocatalysts [2]. Lipases have been widely used in many industrial fields such as organic synthesis, paper manufacture, oleochemistry, dairy industry and detergents. When used as a detergent additive, more than 1000 tons of lipases are needed each year for the worldwide market [3].

Microbial lipases are currently receiving much more attention than lipases from plants and animals because of their diversity in catalytic activity, high yield and low cost production, as well as relative ease of genetic manipulation. Moreover, microbial lipases are also stable in organic solvents, do not require cofactors and possess broad substrate specificity [4]. Gupta et al. and Sharma et al. summed up the resources of the lipase-producing microbial strains and Pleiss et al. listed the gene resources of microbial lipases (http://www.led.uni-stuttgart.de/) [5–7]. So far, more than 20 microbial lipases have been realized in large-scale commercial production (Table 1).

For commercial exploitation of a specific microbial lipase, it is essential to achieve high yield, high activity and high stability. The characteristics of high yield and high activity mean that the lipase product has greater market competitiveness. High stability of the lipase product will help expand its field of application, extend its shelf half-life and prolong its use cycles. For example, a requirement for the lipases is a resistance to methanol or to ethanol in the biodiesel production catalyzed by lipases. The characteristics of high thermal stability and alkaline tolerance are a prerequisite for lipases used in the pulp and paper industry.

Biologists and chemists have made tremendous efforts to obtain a lipase hyperproducing strain, improve lipase activity, and in particular, to enhance lipase stability. The inactivation mechanism and the inactivation models of lipases under various conditions have been researched in-depth [8,9]. Various strategies to protect lipases from inactivation and to increase the operational stability and activity of lipases have been developed, including the use of stabilizing additives, chemical modification of structure, immobilization, protein engineering, medium engineering, etc. [10]. In the area of production, various commercial expression systems, including *Escherichia coli*, *Bacillus subtilis*, *Pichia pastoris*, *Aspergillus oryzae*, etc. have been successfully used in the production of lipases [11].

This review will survey the various technical methods that have been developed to enhance the yield, activity and stability of microbial lipase products themselves from four aspects, including improvements in lipase-producing strains, modification of lipase genes, fermentation engineering of lipases and downstream processing technology of lipase products. Various effect factors on the activity and stability of lipases in a specific biocatalytic reaction are not within the scope of this review.

2. Strain improvement to enhance yield and stability

2.1. Induced mutation and screening techniques of the lipase-producing strain to enhance yield

In the past few decades, although there has been flourishing progress in strain improvement by gene engineering, the desired high yield production strain can still be obtained by the rational use of conventional physical or chemical mutagenesis methods. Several physical or chemical mutagenesis methods, including UV-irradiation, γ-ray, fast neutron irradiation, neodymium-doped yttrium aluminium garnet (Nd:YAG) laser, nitrosoguanidine (NTG), diethyl sulfate and nitrous acid, have been successfully applied to breed lipase-producing microorganisms [12,13]. The mutation method of fast neutron irradiation has some notable advantages including operation simplicity and a perfect mutation effect. Mutagenesis doses and screening techniques are the key technologies in producing a desirable mutant strain. Resistance to bile salts, carbandazim, etc. [14] and high H/C diameter ratio (diameter of hydrolysis halo/diameter of cell colony) [15] are usually used as parameters to screen positive mutants. Treatment with ultraviolet (UV) light, NTG and quick neutron irradiation resulted in a mutant *Candida* sp. strain with a 92-fold improvement in lipase yield [16]. In most cases, the lipase yield can be improved by 1- to 10-fold. However, the shortcomings of the conventional breeding method of mutation induction, such as the low positive mutation rate, the long periodicity and tedious work of screening limited its widespread application.

2.2. Heterologous expression of lipase gene to enhance yield

Hundreds of lipase-producing microorganisms have been isolated from diverse environments [5,6,17–20]. However, many microorganisms are not easily cultivated in laboratory conditions or the lipase yield is too low for economic use. With the development of gene engineering technology, many microbial lipase genes have been cloned over the past few years. Using recombinant DNA technology, heterologously expressing lipase genes in commonly used industrial strains have become common practice.

Among the types of heterologous expression systems, the *P. pastoris* expression system and filamentous fungi expression system are most suitable for the production of extracellular lipase because of their powerful secretion ability and the mature fermentation technology. The expression level of lipase genes in these hosts can be hundreds of times higher than that in the native host, even greater than gram per liter [21–23]. Factors which influence lipase production in these expression systems include: codon bias, copy number of the expression cassette, A+T composition of the heterologous DNA, nature of secretion signal, endogenous protease activity, etc. [24]. In the commercial production of microbial lipase, the choice of the *P. pastoris* expression system is limited by the safety and toxicity considerations associated with the inducer of methanol. A filamentous fungi expression system is always preferred for high-level expression of recombinant microbial lipases. In Novozymes A/S Co., the recombinant *Thermomyces lanuginosus* lipase, *Candida antarctica* lipase B, etc. are produced in an *A. oryzae* expression system.

2.3. Homologous expression of lipase gene to enhance yield

Exogenous lipase genes may not be able to achieve significant overexpression or active expression in the host strain because of various constraint factors, including restriction modification system, codon bias, protein secretion mechanism, and post-translation modification. The technology of homologous expression can not only overcome the above constraint factors, but can also add the copy numbers of lipase genes. With this technology, Gerritse et al. improved the lipase yield of recombinant *Pseudomonas alcaligenes* up to 23-fold [25]. However, due to two constraint factors, a positive correlation existed between the lipase yield and the copy numbers of lipase genes only within a certain range. One constraint factor was the total amount of the secretion protein, and the other factor was the amount of the lipase molecular chaperone.

Microbial lipases are extracellular enzymes, and the translocation process is involved in many secretion proteins. Once a lipase gene is overexpressed in the host cells, a further improvement in

Table 1
Commercially available microbial lipases from manufacturers.

Manufacturer	Commercial microbial lipase
Novozymes A/S Co.	*Thermomyces lanuginosus* lipase; *Candida antarctica* lipase B; *Rhizomucor miehei* lipase; *Candida antarctica* lipase A; *Humicola lanuginose* lipase
Genencor Co.	*Pseudomonas alcaligenes* lipase; *Pseudomonas mendocina* lipase
Amano Co.	*Aspergillus niger* lipase; *Candida rugosa* lipase; *Penicillium roqueforti* lipase; *Penicillium camemberti* lipase; *Rhizopus niveus* lipase; *Burkholderia cepacia* lipase; *Pseudomonas fluorescens* lipase; *Rhizopus oryzae* lipase; *Mucor javanicus* lipase; *Rhizopus delemar* lipase; *Rhizomucor miehei* lipase
Others	*Chromobacterium viscosum* lipase; *Geotrichum candidum* lipase; *Aspergillus oryzae* lipase; *Rhizopus arrhizus* lipase; *Bacillus thermocatenulatus* lipase; *Fusarium solani* lipase; *Penicillium expansum* lipase

the yield of lipase is usually bottlenecked by the secretion ability of the host cells. Ahn et al. found that the lipase yield of recombinant *Pseudomonas fluorescens* with coexpression of the lipase gene and the corresponding secretion protein gene cluster, *tliDEFA*, was 70-fold higher than that of the control recombinant strain with only expression of the lipase gene [26]. However, too much production of the secretion protein affected the growth of the host cell because of its hydrophobicity. Therefore, a rational strategy is that the lipase gene was cloned and expressed on a high copy plasmid and the secretion protein gene cluster was cloned and expressed on a low copy plasmid.

The lipases from *Burkholderia cepacia*, *Pseudomonas aeruginosa*, *B. glumae*, etc. fold into an enzymatically active conformation in the periplasm before they are translocated through the outer membrane [27]. To achieve secretion-competent conformation, lipases require specific intermolecular chaperone proteins, the Lif proteins [28]. Gerritse et al. found that the copy numbers of the helper gene (*lif*) will become a limiting factor when the copy numbers of the lipase gene is above 10 in the homologous expression of lipase genes from *P. alcaligenes*. The lipase yield of the recombinant *P. alcaligenes* with coexpression of the lipase gene and the corresponding *lif* gene on the high copy number plasmid, pJRD215, was greatly improved compared with that of the recombinant strain with only the lipase gene on the plasmid of pJRD215 [25]. Besides the *lif* protein, the prosequence, as intramolecular chaperones, of the lipases from *Rhizopus* sp., *Fusarium heterosporum*, *Staphylococcus* sp., etc. is also essential for correct folding *in vivo* and for secretion of active lipase in host cells [29].

2.4. Construction of protease deficient strain to enhance yield and stability

Many microbial strains can not only produce extracellular lipases, but also produce various proteases at the same time, which cleave and degrade the lipases in the fermentation broth. To prevent the degradation of lipases by proteases, the protease genes of the recombinant expression strain were always knocked out. A series of protease deficient recombinant strains, including *Aspergillus niger*, *Staphylococcus cerevisiae*, *B. subtilis*, *P. pastoris*, etc. have been constructed and applied to lipase production.

In the *S. cerevisiae* expression host strain, the *KEX2* gene, which encodes the Kex2p protease, was disrupted and the intact recombinant *Rhizopus oryzae* lipase (rProROL) was produced. The T_{50} of the recombinant lipase was raised from 40 to 55 °C (The T_{50} is the temperature resulting in 50% loss of activity) [30]. In the *B. subtilis* expression host strain, the yield of lipase LipA was significantly improved when multiple protease genes were deleted. *In vivo*, the SkfA protein protects lipase LipA against proteolytic degradation. Once the *skf*A gene was deleted, the expression level of lipase LipA from wild-type *B. subtilis* greatly decreased and the intact *skf*A gene was a prerequisite for the overproduction of lipase LipA [31]. Because the lipase-producing strain can often also produce multiple proteases, it is important to analyze how the lipase was degraded by the protease and then delete the corresponding protease gene.

Although various new methods, including genome shuffling, etc. have been applied in the field of lipase-producing strain modification [32], there is a very low possibility of significantly improving lipase production in any specific method due to the complexity of lipase gene expression and lipase secretion. It is also necessary to understand the mechanism of lipase gene expression and the bottleneck of lipase secretion using two-dimensional electrophoresis before modification of the lipase-producing strain.

3. Modification of lipase gene and secretion protein gene to improve yield, activity and stability

3.1. Screening of stronger promoter to improve lipase gene expression

A fundamental factor influencing the expression of heterologous genes in host cells is the level of transcription provided by the promoter. A common strategy to increase the expression level of a specific gene is to clone, screen and select a strong promoter.

In *E. coli* expression systems, the strong promoters used most often include T7, λP_{RL}, *tac*, *lac*, *trc*, *csp*A, *ara*BAD, etc. Under the control of these strong promoters, the expression protein of the heterologous gene can comprise more than 50% of the total cell protein within a few hours of induction. However, the overexpressed protein always formed insoluble inclusion bodies and showed low catalytic activity. Some strategies have been developed to increase the functional expression level of the recombinant protein, including coexpression with various chaperone proteins, fusion of the recombinant protein with affinity tags, etc. [33]. By coexpression of chaperones, the expression level of the active *C. antarctica* lipase B was raised from 11 to 61 U/mg in the *E. coli* cytoplasm [34].

AOX1, AOX2, FLD1, ICL1, GAP, PEX8, YPT1, ACT1, ADH1, PGK1 and TDH1 are the promoters most often used in yeast expression systems. Macauley-Patrick et al. summarized the advantages and disadvantages of different promoters [35]. In the *P. pastoris* expression system, the GAP promoter was a promising alternative to the well-known AOX1 promoter. Stable productivity of *Candida rugosa* lipase reached 14 000 U/ml under the control of the GAP promoter [36]. In the *Yarrowia lipolytica* expression system, the lipase yield of *A. oryzae* reached 90 500 U/ml in fed-batch fermentation under the control of the hp4d promoter [37]. In the *S. cerevisiae* expression system, the PGK promoter resulted in the productivity of 1600 U/ml of *Rhizopus niveus* lipase [38].

In other expression systems, strong promoters have also been screened, selected and applied to lipase production. In the *R. oryzae* expression system, the protein yield was directly correlated with the choice of promoter with *pdc*A > *amy*A > *pgk*1 [39]. In the *B. subtilis* expression system, the lipase yield was increased by 100-fold under the control of the strong promoter from the *B. subtilis* strain A.S.l.1700 [40]. In the *Lactococcus lactis* expression system, the

recombinant lipase reached up to 30% of the total cellular proteins under the control of the inducible promoter P*nisA* [41]. In *P. aeruginosa*, the modified *arc* promoter led to a 30-fold increase in lipase yield [42].

In addition to the above known strong promoters, multiple gene-promoter shuffling technology has also been applied to screen and select new strong promoters in industrial microbial breeding [43]. On the other hand, there have been reports on the constitutive promoter applied in the heterologous expression of microbial lipases, however, strong constitutive promoters were not a good choice for the overexpression of lipase due to cell toxicity of lipases. The expression level of lipase genes under the control of a strong promoter cannot exceed the maximum secretion capacity of the host cell.

3.2. Codon optimization to improve lipase gene expression

Lipase genes are often difficult to express in heterologous host cells because different microbial strains show different codon usage bias, and lipase genes may contain codons that are rarely used in the desired host. It is necessary to redesign lipase gene sequences to maximize their expression levels in heterologous host cells. *C. rugosa* displays a non-universal codon usage, in which codon CUG, a universal codon for leucine, is read as serine [44]. After having converted the 19 non-universal CTG-serine codons into universal TCT-serine codons, the *lip1* gene of *C. rugosa* was functionally expressed in *P. pastoris* and the expression level reached 253.3 ± 18.8 U/ml [45]. On the basis of this result, the G + C content of the *lip1* gene fragment was decreased from 63% to 42%, which further improved the production level of the recombinant LIP1 from 33 to 152 mg/l [46]. With this same method, the *lip2* gene, *lip3* gene and *lip4* gene from *C. rugosa* were also functionally expressed in *P. pastoris* and the production yield of the *lip3* gene was improved by 69-fold [46].

3.3. Construction fusion gene to enhance secretion level

Once the lipase genes are functionally overexpressed *in vivo*, the secretory capacity of the host cells will directly affect the production of lipases. The level of microbial lipase secretion is involved in many factors, including signal sequences, fusion tag, molecular chaperones, Dsb-proteins, as well as a variety of periplasmic proteases [27]. In the *S. cerevisiae* expression system, the signal peptide from the *Kluyveromyces lactis* killer toxin replaced the endogenous leader sequence from the *lip1* gene of *C. rugosa*, which resulted in a yield of over 1 g/l [47]. Ahn et al. connected the cellulose-binding domain (CBD) from *Trichoderma harzianum* endoglucanase II (THEG) to the N-terminal of *Bacillus stearothermophilus* L1 lipase. The CBD enhanced the protein-folding stability in the ER and escape of the protein from the ER, which resulted in a 7-fold increase in the secreted fusion protein from *S. cerevisiae* [48]. In the *E. coli* expression system, the expression level of recombinant *C. antarctica* lipase B in *E. coli* cytoplasm rose from 2 to 11 U/mg when the lipase gene was fused with the encoding gene of thioredoxin [34].

3.4. Directed evolution of secretion protein to enhance secretion level

Lipase from *P. fluorescens* is secreted into the culture medium through an ATP-binding cassette (ABC) transporter, which is encoded by three genes, *tliD*, *tliE* and *tliF* [49]. Among the three component proteins of the ABC transporter, the secretion ability of the TliD determines the secretion level of the recombinant lipase from the host strain [50]. Random point mutations were introduced into the *tliD* gene and the mutation library of the *tliD* gene was introduced into *E. coli* and coexpressed with the lipase gene. One variant showed a 3.2-fold increase in the secretion level of recombinant lipase from *E. coli* [51].

3.5. Improvements in activity and stability by protein engineering

Rational design or directed evolution has become a powerful tool to produce suitable lipase with high catalytic activity and stability in an industrial environment. Based on the known three-dimensional information, the lipase molecule can be customized in a rational design method. With this method, the space of the active site of *C. antarctica* lipase B was enlarged by the site-directed mutation of W104H and W104A [52,53] and its enantioselectivity was improved by the site-directed mutation of S47A [54]. However, only 28 three-dimensional structures of microbial lipases have been solved. Directed evolution, such as error prone PCR and DNA shuffling, represents an alternative approach when no structural and functional knowledge of the respective lipases can be obtained. Among the four steps in the whole process of directed evolution [55], the methods for mutagenesis are relatively mature in technology [56]. To achieve successful directed evolution of lipases, establishment of an appropriate expression system is a prerequisite and the design of a high-throughput screening system is a major challenge.

Among the various expression systems, *E. coli* is still the most frequently used prokaryotic expression host for heterologous proteins. Much effort has been made to obtain active expression of lipase in *E. coli* cytoplasm and secrete the recombinant lipase into the culture media. The recombinant *Geobacillus* sp. T1 lipase and *Pseudomonas* sp. MIS38 lipase were secreted into the culture media by coexpression of lipase with the secretion protein [57,58]. Cell lysis is an alternative method to release recombinant lipase into the culture media under the control of the heat-inducible promoter or the UV-inducible promoter [59,60]. Active expression and secretion of recombinant lipases simplify the following screening procedure of the mutation library.

Various novel high-throughput screening methods for the mutation library of microbial lipases have been designed in recent years. Every screening method has its practical application [55] and the adopted screening method varies with the catalytic activity of the lipases. Using an NMR-based approach or FTIR spectroscopy-based approach for high-throughput screening of lipase enantioselectivity, the screening efficiency reached up to 1400 mutations per day and 20 000 mutations per day, respectively [61,62].

Progress in lipase protein engineering in recent years is summarized in Table 2. A series of superior lipases with modified substrate specificity, enhanced thermo- and solvent stability, or improved enantioselectivity were achieved by protein engineering. However, the existing methods of directed evolution have been limited to modifying the activity of existing lipases. The challenge to create novel lipase with new catalytic activity by rational design combined with directed evolution still exists [108].

4. Improvement yield and stability by fermentation engineering

Microbial lipases are mostly produced by submerged culture. As an inducible extracellular enzyme, lipase production is greatly influenced by the type and concentration of lipid sources such as oils or other inducers [109]. In addition to the lipidic inducers, other nutritional and physico-chemical factors, such as substrates, inorganic salts, the C/N ratio of the medium, temperature, pH and dissolved oxygen concentration, also have important effects on lipase yield [4,5,18,110]. In the solid-state fermentation process of microbial lipases, the moisture of the culture medium is an

Table 2
Modification of microbial lipases by protein engineering.

Outcome	Target	Method	Mutation site	Reference
Modified the enantioselectivity	*Candida antarctica* lipase B	Site-directed mutagenesis	S47A	[63]
	Geotrichum candidum lipase	Rational recombination of the two isozyme genes; site-directed mutagenesis	I-348-II-406-I, I357A and L358F	[64]
	Rhizopus oryzae lipase	Site-directed mutagenesis	L258A, L258S and L258F	[65]
	Candida antarctica lipase B	Site-directed mutagenesis	W104A	[52,66]
	Burkholderia cepacia lipase	Single-molecule PCR amplification	L17F, F119L, L167G and L266V	[67]
	Pseudomona aeruginosa lipase	Error prone PCR and DNA shuffling	M16L, A34T, etc. 11 amino acid substitutions	[68]
	Pseudomona aeruginosa lipase	Combinatorial multiple-cassette mutagenesis	D20N, S53P, S155M, L162G, T180I and T234S	[69]
	Pseudomona aeruginosa lipase	Error prone PCR and saturation mutagenesis	S149G, S155F, V47G, V55G and S164G	[70]
	Bacillus subtilis lipase A	Error prone PCR and saturation mutagenesis	I22V, Q60L, Q60R, Y49C and N50S	[71]
	Candida antarctica lipase B	DNA shuffling	Chimeras containing DNA elements from two to three parents and no point mutations were found	[72]
	Candida antarctica lipase B	Site-directed mutagenesis	S47A, T42V and S47A	[54]
	Pseudomona aeruginosa lipase	Error prone PCR	S155F, L162G and S155F; L162G, S53P and S155F	[73]
	Bacillus subtilis lipase A	Multiplex-PCR-based recombination	N18Q and Y49V	[74]
	Burkholderia cepacia lipase	Single-molecule PCR amplification	L17F, F119I, L167G and L266V; L17F, L167G and L266I	[75]
	Candida antarctica lipase B	Site-directed mutagenesis	T103G	[76]
	Yarrowia lipolytica lipase	Not mentioned	V232A	[77]
	Pseudomonas fragi lipase	Site-directed mutagenesis	T137V, T138N, T137V and T138N; T137V and S141G; T137V, T138N and S141G	[78]
	Rhizomucor miehei lipase	Site-directed mutagenesis	F94G	[79]
	Rhizopus delemar lipase	Site-directed mutagenesis	V209W and F112W; F95D and F214R	[80]
Modified the chain length selectivity	*Candida rugosa* lipase lip4	Site-specific saturation mutagenesis	A296I, V344Q and V344H	[81]
	Candida rugosa lipase lip1	Site-directed mutagenesis	P246F, L413F, L410W, L410F and S300E; L410F and S365L	[82]
	Acinetobacter sp. lipase	Error prone PCR	S21F, A102G, S103F, D299E and N300H	[83]
	Thermomyces lanuginosa lipase	Cassette mutagenesis	S83T	[84]
	Rhizopus delemar lipase	Site-directed mutagenesis	F95D, F112W and V209W	[85]
	Burkholderia cepacia lipase	Site-directed mutagenesis	L167V, F119A and L167M	[86]
	Rhizomucor miehei lipase	Site-directed mutagenesis	F94R	[87]
Expanded the range of substrate acceptance	*Pseudomonas aeruginosa* lipase	Combinatorial active-site saturation test	M16A and L17F, M16G, M16G and L17F	[88]
	Candida antarctica lipase B	Circular permutation	A283/A283-KRPRINSP	[89]
Improved the hydrolysis activity	*Pseudomonas fluorescens* lipase	Error prone PCR	N8S, S14G and W72R	[90]
	Rhizopus oryzae lipase	Error prone PCR	V95D, I53V, P96S and F196Y; Q128H and Q197L	[91]
	Penicillium expansum lipase	Site-directed mutagenesis	R182K	[92]
Improved the amide-hydrolysis activity	*Pseudomonas aeruginosa* lipase	Error prone PCR and site-directed mutagenesis	F207S and A213D	[93]
Improved the phospholipase activity	*Bacillus thermocatenulatus* lipase	Error prone PCR	H15P and L184P	[94]
	Staphylococcus aureus lipase	Error prone PCR and DNA shuffling	L179F, K282E, K300N, S358T, etc. six amino acid substitutions	[95]
Improved the catalytic activity for aldol reactions	*Candida antarctica* lipase B	Site-directed mutagenesis	S105A	[96]
Improved the methanolysis activity	*Rhizopus oryzae* lipase	Error prone PCR	K138R	[91]
	Fusarium heterosporum lipase	Site-directed mutagenesis	R275A	[97,98]
	Rhizopus oryzae lipase	Site-directed mutagenesis	R29A	[98]
	Pseudomonas fragi lipase	Site-directed mutagenesis	T137V, T138N, T137V and T138N; T137V and S141G; T137V, T138N and S141G.	[78]
	Candida antarctica lipase B	Site-directed mutagenesis	T103G	[99]
Improved the thermostability	*Candida antarctica* lipase B	DNA shuffling	Chimeras containing DNA elements from two to three parents and no point mutations were found	[72]
	Rhizopus arrhizus lipase	Error prone PCR and DNA shuffling	E190V	[100]
	Candida antarctica lipase B	Error prone PCR	A281E and V221D	[101]
	Penicillium expansum lipase	Site-directed mutagenesis	K55R	[102]
	Bacillus subtilis lipase	Error prone PCR and Site-directed mutagenesis	N66Y and A132D; N66Y, A132D and L114P	[103]
	Candida antarctica lipase B	Error prone PCR	N317Y	[104]
	Penicillium camembertii lipase	Site-directed mutagenesis	Y22C and G269C	[105]
	Bacillus subtilis lipase	Error prone PCR	A15S, F17S, A20E, N89Y, G111D, L114P, A132D, I157M and N166Y	[106]
Improved the stability in organic solvents	*Pseudomonas* sp. lipase	Error prone PCR	F146L, I289T and V304A	[107]
Improved the resistance to oxidative degradation	*Candida antarctica* lipase B	Site-directed mutagenesis	M72L	[53]

important factor affecting lipase production [111]. Under optimum fermentation conditions, the maximum yield of *Rhizopus homothallicus* lipase reached 826 U/g DM in solid fermentation, which can be compared with the highest value obtained in submerged fermentation [112]. The thermal stability and the specific activity of *R. homothallicus* lipase are higher when produced in a solid-state fermentation process than in a submerged fermentation process [113]. In the continuous fermentation of microbial lipases, lipase activity in the medium is inversely related to the dilution rate. The productivity of *C. rugosa* lipase and *Bacillus* sp. IHI-91 lipase increased by 50% compared with that in batch fermentations and depended on the proper dilution rate [114,115]. Other fermentation processes, such as the fed-batch fermentation process and the repeated fed-batch fermentation process have also been used in many cases and have achieved good results [116]. In the continuous fermentation process or repeated fermentation process, the cells are always immobilized on various solid supports and lipase productivity was also associated with the mass transfer ability of various support materials [117]. Although the submerged fermentation technology is still the mainstream production technology in the commercial production of microbial lipases, the solid-state fermentation technology possesses several biotechnological advantages, such as higher fermentation productivity, higher product stability and lower catabolic repression and has been developed as an attractive alternative to the submerged fermentation technology in recent years [118–120].

In the fermentation production of microbial lipases, some strains produced significant amounts of proteinase, which resulted in the degradation of lipases. The yield and stability of the lipase increased with inhibition of proteinase activity. The yield of the recombinant *C. antarctica* lipase B was increased to 2 g/l (2-fold increase) in the culture supernatant after the protease activity was inhibited by pH control and low temperature [22,121]. Compounds, such as β-cyclodextrin and gum Arabic, can greatly improve the stability of *Acinetobacter calcoaceticus* lipase in the fermentation broth. Furthermore, these compounds can also enhance lipase secretion and improve lipase yield [122]. Kamiya et al. found that the *Streptoverticillium* transglutaminase can mediate the cross-linking of contaminating proteins in the fermentation broth and improve the half-life of the crude lipase from *Rhizopus* sp. by more than 10-fold [123].

5. The down stream process technology to enhance activity and stability

5.1. Effect of additives on activity and stability

In the production process of lipase preparations from fermentation broth to dry powder, two methods are always used for dehydration. One method is spray-drying, and the other method is freeze-drying. The spray-drying method always causes lipase unfolding by thermal denaturation. Milk powder and maltodextrin showed excellent protective effects on lipase activity during spray-drying and the activity recovery yield even reached as high as 100%. Lipase powder mixed with the above protective additives, calcium chloride, gum Arabic, etc., can be stored for over 18 months at 4 or 20 °C without loss of activity [124,125]. While in the lyophilization production process, both the random structure and the α-helical structure of the enzymes were always partially converted to β-sheet structure, which resulted in conformation change and loss of enzyme activity. The additions of potassium chloride and 18-crown-6 before freeze-drying of the *Humicola lanuginose* lipase fermentation broth increased the activity up to 46-fold and 41-fold compared to crude powder, respectively [126], while trehalose afforded a superior stabilization effect on *H. lanuginosa* lipase during lyophilization [127]. Other cryoprotectants and lyoprotectants, such as sugar and cyclodextrins, can also prevent structural damage to lipases in the two stages of the lyophilization process. The effect of various additives on lipase activity and stability during lyophilization is determined by the type and the concentration of the additives, lyophilization time, water content, etc.

5.2. Chemical modification technology to enhance activity and stability

As a powerful complementary approach to site-directed mutagenesis and directed evolution for customizing lipases, chemical modification methods allow an almost unlimited alteration of amino acid side chain structures and endow lipases with useful new functions. Chemical modification approaches include cross-linking with bi-functional reagents, chemical modification with monofunctional polymers and the introduction of small moieties [10]. A self-immobilized spherical particle of *P. fluorescens* lipase, formed by the cross-linking method, showed superior activity in organic solvent compared to the original free lipase. During the cross-linking process, addition of tributyrin to the reaction mixture increased the activity of *P. fluorescens* lipase up to 202-fold [128]. The thermal stability of *C. antarctica* lipase B, chemically modified with oxidized polysaccharide, was improved 9.3-fold [129]. The enantioselectivity of *Candida antarctica* lipase B, aminated by ethylenediamine, was increased more than 50-fold [130]. The activity and the stability of the modified lipases are dependent upon the applied procedure, the selected support and the added additives.

5.3. Immobilization technology to enhance activity and stability

Two obvious developmental trends in lipase immobilization exist, one is the application of new supports and the other is the exploitation of new immobilization approaches. Among various immobilization supports, nano-materials and porous supports with a large specific surface area have achieved excellent results. The long-term stability of immobilized lipases on γ-Fe_2O_3 magnetic nanoparticles or carbon nanofiber was in the region of months [131,132]. Reetz and Jaeger entrapped lipases in nanoporous sol–gel glass and the activity of the lipases was enhanced up to 88-fold [133]. As an interfacial enzyme, lipase was greatly activated when hydrophobic materials were used as immobilization supports or detergents were added to the immobilization-reaction mixtures during the immobilization process [134]. In addition to activity and stability, the enantioselectivity of lipases can also be modulated by immobilization processes [135]. The effect of immobilization on lipase properties was determined by support type, immobilization conditions, immobilization methods, etc. [136]. In respect of the new approaches for lipase immobilization, cell surface display technology enables the lipases to be self-immobilized on the cell surface and is a new alternative approach to immobilize lipases on solid supports [137,138]. The hydrolysis activity and the esterification activity of *R. oryzae* lipase immobilized on the cell surface of *S. cerevisiae* were increased up to 4.4×10^4-fold and 3.8×10^4-fold, respectively [139].

6. Concluding remarks

In addition to the modification of existing lipase-producing strains and lipase genes, new microbial lipase sources can be obtained from completely sequenced genomes, non-culturable organisms and extremophiles [20,140–141]. These new lipase sources, together with *in vitro* directed evolution, provide the chemical industry with an unprecedented opportunity to identify an ideal process-compatible lipase with high activity and high stability.

The faster the green chemistry industry develops, the larger the demand for lipases will be. The large-scale application of lipases in the chemical industry is bottlenecked by the stability and catalytic activity of lipases. Various technical methods to improve yield, activity and stability of microbial lipases are highly dependent on the particular production conditions. No single strategy will suit all cases; however, the four aspects to improve lipase yield, activity and stability will bring useful clues to specific situations, as evidenced by our selected examples in this review. Moreover, these complementary strategies will hopefully alleviate the bottleneck in lipase development. For biochemists and chemists, the identification of lipases with high yield, high activity and high stability is a goal worth pursuing.

Acknowledgement

This work was supported by the National High-Technology Project ("863" Project) of P.R. China (No. 2007AA100703), the National Natural Science Funds of P.R. China (No. 30870545) and the Natural Science Funds for Distinguished Young Scholar of Fujian Province (No. 2009J06013).

References

[1] R.D. Schmid, R. Verger, Angew. Chem. Int. Ed. 37 (1998) 1608–1633.
[2] V. Gotor-Fernández, R. Brieva, V. Gotor, J. Mol. Catal. B: Enzym. 40 (2006) 111–120.
[3] F. Hasan, A.A. Shah, A. Hameed, Enzyme Microb. Technol. 39 (2006) 235–251.
[4] K.E. Jaeger, M.T. Reetz, Trends Biotechnol. 16 (1998) 396–403.
[5] R. Gupta, N. Gupta, P. Rathi, Appl. Microbiol. Biotechnol. 64 (2004) 763–781.
[6] R. Sharma, Y. Chisti, U.C. Banerjee, Biotechnol. Adv. 19 (2001) 627–662.
[7] J. Pleiss, M. Fischer, M. Peiker, C. Thiele, R.D. Schmid, J. Mol. Catal. B: Enzym. 10 (2000) 491–508.
[8] T. De Diego, P. Lozano, S. Gmouh, M. Vaultier, J.L. Iborra, Biomacromolecules 6 (2005) 1457–1464.
[9] M. Noel, D. Combes, Enzyme Microb. Technol. 33 (2003) 299–308.
[10] P. Villeneuve, J.M. Muderhwa, J. Graille, M.J. Haas, J. Mol. Catal. B: Enzym. 9 (2000) 113–148.
[11] C. Schmidt-Dannert, Bioorg. Med. Chem. 7 (1999) 2123–2130.
[12] T.W. Tan, M. Zhang, B.W. Wang, C.H. Ying, L. Deng, Process Biochem. 39 (2003) 459–465.
[13] X.G. Gao, S.G. Cao, K.C. Zhang, Enzyme Microb. Technol. 27 (2000) 74–82.
[14] J.G. Sandana Mala, N.R. Kamini, R. Puvanakrishnan, J. Gen. Appl. Microbiol. 47 (2001) 181–186.
[15] P. Fickers, J.M. Nicaud, J. Destain, P. Thonart, Appl. Microbiol. Biotechnol. 63 (2003) 136–142.
[16] Y. Zhou, P. Zhang, Q.Y. Meng, T.W. Tan, Chin. J. Microbiol. 28 (2001) 81–85.
[17] Z.Y. Shu, R.F. Lin, H. Jiang, Y.F. Zhang, M.Z. Wang, J.Z. Huang, J. Biosci. Bioeng. 107 (2009) 658–661.
[18] H. Treichel, D. de Oliveira, M.A. Mazutti, M.D. Luccio, J.V. Oliveira, Food Bioprocess Technol. 4 (2009), doi:10.1007/s11947-009-0202-2.
[19] B. Joseph, P.W. Ramteke, G. Thomas, Biotechnol. Adv. 26 (2008) 457–470.
[20] M. Salameh, J. Wiegel, Adv. Appl. Microbiol. 61 (2007) 253–283.
[21] L. Brunel, V. Neugnot, L. Landucci, H. Boze, G. Moulin, F. Bigey, E. Dubreucq, J. Biotechnol. 111 (2004) 41–50.
[22] M. Jahic, F. Wallberg, M. Bollok, P. Garcia, S.O. Enfors, Microb. Cell Fact. 2 (2003) 6–10.
[23] S. Yamaguchi, K. Takeuchi, T. Mase, A. Matsuura, Biosci. Biotechnol. Biochem. 61 (1997) 800–805.
[24] R. Daly, M.T. Hearn, J. Mol. Recognit. 18 (2005) 119–138.
[25] G. Gerritse, R.W. Hommes, W.J. Quax, Appl. Enviro. Microbiol. 64 (1998) 2644–2651.
[26] J.H. Ahn, J.G. Pan, J.S. Rhee, Appl. Environ. Microbiol. 67 (2001) 5506–5511.
[27] F. Rosenau, K.E. Jaeger, Biochimie 82 (2000) 1023–1032.
[28] F. Rosenau, J. Tommassen, K.E. Jaeger, ChemBioChem 5 (2004) 152–161.
[29] S. Takahashi, M. Ueda, A. Tanaka, Appl. Microbiol. Biotechnol. 55 (2001) 454–462.
[30] S. Takahashi, M. Ueda, A. Tanaka, Appl. Microbiol. Biotechnol. 52 (1999) 534–540.
[31] H. Westers, P.G. Braun, L. Westers, H. Antelmann, M. Hecker, J.D. Jongbloed, H. Yoshikawa, T. Tanaka, J.M. van Dijl, W.J. Quax, Appl. Environ. Microbiol. 71 (2005) 1899–1908.
[32] J. Lin, B.H. Shi, Q.Q. Shi, Y.X. He, M.Z. Wang, Chin. J. Biotechnol. 23 (2007) 672–676.
[33] H.P. Sørensen, K.K. Mortensen, Microb. Cell Fact. 4 (2005) 1.
[34] D. Liu, R.D. Schmid, M. Rusnak, Appl. Microbiol. Biotechnol. 72 (2006) 1024–1032.
[35] S. Macauley-Patrick, M.L. Fazenda, B. McNeil, L.M. Harvey, Yeast 22 (2005) 249–270.
[36] W. Zhao, J.W. Wang, R.Q. Deng, X.Z. Wang, J. Ind. Microbiol. Biotechnol. 35 (2008) 189–195.
[37] J.M. Nicaud, C. Madzak, P. van den Broek, C. Gysler, P. Duboc, P. Niederberger, C. Gaillardin, FEMS Yeast Res. 2 (2002) 371–379.
[38] M. Kohno, M. Enatsu, M. Yoshiizumi, W. Kugimiya, Protein Expr. Purif. 15 (1999) 327–335.
[39] J.A. Mertens, C.D. Skory, A.S. Ibrahim, Arch. Microbiol. 186 (2006) 41–50.
[40] J.S. Ma, Z.M. Zhang, B.J. Wang, X.J. Kong, Y.G. Wang, S.G. Cao, Y. Feng, Protein Expr. Purif. 45 (2006) 22–29.
[41] S. Drouault, G. Corthier, S.D. Ehrlich, P. Renault, Appl. Environ. Microbiol. 66 (2000) 588–598.
[42] H.V. Winteler, B. Schneidinger, K.E. Jaeger, D. Haas, Appl. Environ. Microbiol. 62 (1996) 3391–3398.
[43] C.F. Lu, T. Jeffries, Appl. Environ. Microbiol. 73 (2007) 6072–6077.
[44] I. Alberghina, M. Lotti, J. Mol. Cat. B: Enzym. 3 (1997) 37–41.
[45] S.W. Chang, C.J. Shieh, G.C. Lee, J.F. Shaw, Appl. Microbiol. Biotechnol. 67 (2005) 215–224.
[46] S.W. Chang, G.C. Lee, J.F. Shaw, J. Agric. Food Chem. 54 (2006) 815–822.
[47] F. Fusetti, S. Brocca, D. Porro, M. Lotti, Biotechnol. Lett. 18 (1996) 281–286.
[48] J. Ahn, E. Choi, H. Lee, S. Hwang, C. Kim, H. Jang, S. Haam, J. Jung, Appl. Microbiol. Biotechnol. 64 (2004) 833–839.
[49] J.H. Ahn, J.G. Pan, J.S. Rhee, J. Bacteriol. 181 (1999) 1847–1852.
[50] P. Delepelaire, J. Biol. Chem. 269 (1994) 27952–27957.
[51] G.T. Eom, J.K. Song, J.H. Ahn, Y.S. Seo, J.S. Rhee, Appl. Environ. Microbiol. 71 (2005) 3468–3474.
[52] A.O. Magnusson, J.C. Rotticci-Mulder, A. Santagostino, K. Hult, ChemBioChem 6 (2005) 1051–1056.
[53] S. Patkar, J. Vind, E. Kelstrup, M.W. Christensen, A. Svendsen, K. Borch, O. Kirk, Chem. Phys. Lipids 93 (1998) 95–101.
[54] D. Rotticci, J.C. Rotticci-Mulder, S. Denman, T. Norin, K. Hult, ChemBioChem 2 (2001) 766–770.
[55] C. Montiel, I. Bustos-Jaimes, Curr. Chem. Biol. 2 (2008) 50–59.
[56] M.T. Reetz, K.E. Jaeger, Topic Curr. Chem. 200 (1999) 31–57.
[57] R.N. Rahman, T.C. Leow, M. Basri, A.B. Salleh, Protein Expr. Purif. 40 (2005) 411–416.
[58] C. Angkawidjaja, D.J. You, H. Matsumura, Y. Koga, K. Takano, S. Kanaya, Acta. Cryst. 63 (2007) 187–189.
[59] S. Li, L. Xu, H. Hua, C. Ren, Z. Lin, J. Biotechnol. 127 (2007) 647–652.
[60] L. Xu, S. Li, C. Ren, Z. Cai, Z. Lin, Biotechniques 41 (2006) 319–323.
[61] M.T. Reetz, A. Eipper, P. Tielmann, R. Mynott, Adv. Synth. Catal. 344 (2002) 1008–1016.
[62] P. Tielmann, M. Boese, M. Luft, M.T. Reetz, Chem. Eur. J. 9 (2003) 3882–3887.
[63] D. Rotticci, Understanding and engineering the enantioselectivity of *Candida antarctica* lipase B towards *sec*-alcohols (thesis), Royal Institute of Technology, Sweden, 2000.
[64] M. Holmquist, P. Berglund, Org. Lett. 1 (1999) 763–765.
[65] H. Scheib, J. Pleiss, P. Stadler, A. Kovac, A.P. Potthoff, L. Haalck, F. Spener, F. Paltauf, R.D. Schmid, Protein Eng. 11 (1998) 675–682.
[66] A.O. Magnusson, M. Takwa, A. Hamberg, K. Hult, Angew. Chem. Int. Ed. Engl. 44 (2005) 4582–4585.
[67] Y. Koga, K. Kato, H. Nakano, T. Yamane, J. Mol. Biol. 331 (2003) 585–592.
[68] D.X. Zha, S. Wilensek, M. Hermes, K.E. Jaeger, M.T. Reetz, Chem. Commun. 24 (2001) 2664–2665.
[69] M.T. Reetz, S. Wilensek, D.X. Zha, K.E. Jaeger, Angew. Chem. Int. Ed. 40 (2001) 3589–3591.
[70] K. Liebeton, A. Zonta, K. Schimossek, M. Nardini, D. Lang, B.W. Dijkstra, M.T. Reetz, K.E. Jaeger, Chem. Biol. 7 (2000) 709–718.
[71] S.A. Funke, A. Eipper, M.T. Reetz, N. Otte, W. Thiel, G. Van Pouderoyen, B.W. Dijkstra, K.E. Jaeger, T. Eggert, Biocatal. Biotrans. 21 (2003) 67–73.
[72] W.C. Suen, N. Zhang, L. Xiao, V. Madison, A. Zaks, Protein Eng. Des. Sel. 17 (2004) 133–140.
[73] M. Bocola, N. Otte, K.E. Jaeger, M.T. Reetz, W. Thiel, ChemBioChem 5 (2004) 214–223.
[74] T. Eggert, S.A. Funke, N.M. Rao, P. Acharya, H. Krumm, M.T. Reetz, K.E. Jaeger, ChemBioChem 6 (2005) 1062–1067.
[75] R. Kato, H. Nakano, H. Konishi, K. Kato, Y. Koga, T. Yamane, T. Kobayashi, H. Honda, J. Mol. Biol. 351 (2005) 683–692.
[76] J. Ottosson, J.C. Rotticci-Mulder, D. Rotticci, K. Hult, Protein Sci. 10 (2001) 1769–1774.
[77] M. Cancino, P. Bauchart, G. Sandoval, J.M. Nicaud, I. André, V. Dossat, A. Marty, Tetrahedron: Asymmetry 19 (2008) 1608–1612.
[78] G. Santarossa, P.G. Lafranconi, C. Alquati, L. DeGioia, L. Alberghina, P. Fantucci, M. Lotti, FEBS Lett. 579 (2005) 2383–2386.
[79] D.J. Gaskin, A. Romojaro, N.A. Turner, J. Jenkins, E.N. Vulfson, Biotechnol. Bioeng. 73 (2001) 433–441.
[80] R.R. Klein, G. King, R.A. Moreau, M.J. Haas, Lipids 32 (1997) 123–130.
[81] L.C. Lee, Y.T. Chen, C.C. Yen, T.C. Chiang, S.J. Tang, G.C. Lee, J.F. Shaw, J. Agric. Food Chem. 55 (2007) 5103–5108.
[82] J. Schmitt, S. Brocca, R.D. Schmid, J. Pleiss, Protein Eng. 15 (2002) 595–601.
[83] S.J. Han, J.H. Chung, C.S. Cheong, I.Y. Chung, Y.S. Han, Enzyme Microb. Technol. 35 (2004) 377–384.
[84] S. Danielsen, M. Eklund, H.J. Deussen, T. Gräslund, P.A. Nygren, T.V. Borchert, Gene 272 (2001) 267–274.
[85] R.D. Joerger, M.J. Haas, Lipids 29 (1994) 377–384.
[86] J.H. Yang, Y. Koga, H. Nakano, T. Yamane, Protein Eng. 15 (2002) 147–152.

[87] S.W. Oh, D.J.H. Gaskin, D. Young Kwon, E.N. Vulfson, Biotechnol Lett. 23 (2001) 563–568.
[88] M.T. Reetz, M. Bocola, J.D. Carballeira, D.X. Zha, A. Vogel, Angew. Chem. Int. Ed. Engl. 44 (2005) 4192–4196.
[89] Z. Qian, S. Lutz, J. Am. Chem. Soc. 127 (2005) 13466–13467.
[90] H.C. Jung, S. Ko, S.J. Ju, E.J. Kim, M.K. Kim, J.G. Pan, J. Mol. Catal. B: Enzym. 26 (2003) 177–184.
[91] H. Shibamoto, T. Matsumoto, H. Fukuda, A. Kondo, J. Mol. Catal. B: Enzym. 28 (2004) 235–239.
[92] C.M. Wang, S.L. Cai, Y.Z. Wu, L. Lin, Chin. J. Biotechnol. Bull. 4 (2007) 165–168.
[93] R. Fujii, Y. Nakagawa, J. Hiratake, A. Sogabe, K. Sakata, Protein Eng. Des. Sel. 18 (2005) 93–101.
[94] I. Kauffmann, C. Schmidt-Dannert, Protein Eng. 14 (2001) 919–928.
[95] M.D. Van Kampen, M.R. Egmond, Eur. J. Lipid Sci. Technol. 102 (2000) 717–726.
[96] C. Branneby, P. Carlqvist, A. Magnusson, K. Hult, T. Brinck, P. Berglund, J. Am. Chem. Soc. 125 (2003) 874–875.
[97] T. Nagao, Y. Shimada, A. Sugihara, Y. Tominaga, J. Biosci. Bioeng. 89 (2000) 446–450.
[98] T. Nagao, Y. Shimada, A. Sugihara, Y. Tominaga, J. Mol. Catal. B: Enzym. 17 (2002) 125–132.
[99] S.A. Patkar, A. Svendsen, O. Kirk, I.G. Clausen, K. Borch, J. Mol. Catal. B: Enzym. 3 (1997) 51–54.
[100] W.N. Niu, Z.P. Li, D.W. Zhang, M.R. Yu, T.W. Tan, J. Mol. Catal. B: Enzym. 43 (2006) 33–39.
[101] N.Y. Zhang, W.C. Suen, W. Windsor, L. Xiao, V. Madison, A. Zaks, Protein Eng. 16 (2003) 599–605.
[102] S.L. Cai, J.H. Lin, C.M. Wang, L. Lin, Chin. J. Biotechnol. 23 (2007) 677–680.
[103] P. Acharya, E. Rajakumara, R. Sankaranarayanan, N.M. Rao, J. Mol. Biol. 341 (2004) 1271–1281.
[104] M. Chodorge, L. Fourage, C. Ullmann, V. Duvivier, J.M. Masson, F. Lefèvre, Adv. Synth. Catal. 347 (2005) 1022–1026.
[105] S. Yamaguchi, K. Takeuchi, T. Mase, K. Oikawa, T. McMullen, U. Derewenda, R.N. McElhaney, C.M. Kay, Z.S. Derewenda, Protein Eng. 9 (1996) 789–795.
[106] S. Ahmad, M.Z. Kamal, R. Sankaranarayanan, N.M. Rao, J. Mol. Biol. 381 (2008) 324–340.
[107] H. Nakano, Y. Ide, T. Tsuda, J. Yang, S. Ishii, T. Yamane, Ann. N. Y. Acad. Sci. 864 (1998) 431–434.
[108] H.S. Park, S.H. Nam, J.K. Lee, C.N. Yoon, B. Mannervik, S.J. Benkovic, H.S. Kim, Science 311 (2006) 535–538.
[109] D. Papaparaskevas, P. Christakopoulos, D. Kekos, B.J. Macris, Biotechnol. Lett. 14 (1992) 397–402.
[110] T. Kar, F. Delvigne, M. Masson, J. Destain, P. Thonart, J. Ind. Microbiol. Biotechnol. 35 (2008) 1053–1059.
[111] S.Y. Sun, Y. Xu, D. Wang, Bioresour. Technol. 100 (2009) 3152–3156.
[112] J.A. Rodriguez, J.C. Mateos, J. Nungaray, V. González, T. Bhagnagar, S. Roussos, J. Cordova, J. Baratti, Process Biochem. 41 (2006) 2264–2269.
[113] J.C. Mateos Diaz, J.A. Rodríguez, S. Roussos, J. Cordova, A. Abousalham, F. Carriere, J. Baratti, Enzyme Microb. Technol. 39 (2006) 1042–1050.
[114] J.L. Montesinos, E. Dalmau, C. Casas, J. Chem. Technol. Biotechnol. 78 (2003) 753–761.
[115] P. Becker, I. Abu-Reesh, S. Markossian, G. Antranikian, H. Märkl, Appl. Microbiol. Biotechnol. 48 (1997) 184–190.
[116] M.A. Gordillo, J.L. Montesinos, C. Casas, F. Valero, J. Lafuente, C. Solà, Chem. Phys. Lipids 93 (1998) 131–142.
[117] X.H. Yang, B.W. Wang, F.N. Cui, T.W. Tan, Process Biochem. 40 (2005) 2095–2103.
[118] R.R. Singhania, A.K. Patel, C.R. Soccol, A. Pandey, Biochem. Eng. J. 44 (2009) 13–18.
[119] G. Viniegra-González, E. Favela-Torres, C.N. Aguilar, S.J. Rómero-Gomez, G. Díaz-Godínez, C. Augur, Biochem. Eng. J. 13 (2003) 157–167.
[120] L.R. Castilho, C.M.S. Polato, E.A. Baruque, G.L. Sant'Anna, D.M.G. Freire, Biochem. Eng. J. 4 (2000) 239–247.
[121] M. Jahic, M. Gustavsson, A.K. Jansen, M. Martinelle, S.O. Enfors, J. Biotechnol. 102 (2003) 45–53.
[122] D.A. Martinez, B.C. Nudel, Can. J. Microbiol. 48 (2002) 1056–1061.
[123] N. Kamiya, T. Ogawa, T. Nagamune, Biotechnol. Lett. 23 (2001) 1629–1632.
[124] P. Fickers, M. Ongena, J. Destain, F. Weekers, P. Thonart, Enzyme Microb. Technol. 38 (2006) 756–759.
[125] W.A.M. Alloue, J. Destain, K. Amighi, P. Thonart, Process Biochem. 42 (2007) 1357–1361.
[126] M. Persson, I. Mladenoska, E. Wehtje, P. Adlercreutz, Enzyme Microb. Technol. 31 (2002) 833–841.
[127] L. Kreilgaard, S. Frokjaer, J.M. Flink, T.W. Randolph, J.F. Carpenter, J. Pharm. Sci. 88 (1999) 281–290.
[128] D. Brady, J. Jordaan, C. Simpson, A. Chetty, C. Arumugam, F.S. Moolman, BMC Biotechnol. 8 (2008) 8.
[129] K.S. Siddiqui, R. Cavicchioli, Extremophiles. 9 (2005) 471–476.
[130] J.M. Palomo, G. Fernández-Lorente, J.M. Guisán, R. Fernández-Lafuente, Adv. Synth. Catal. 349 (2007) 1119–1127.
[131] A. Dyal, K. Loos, M. Noto, S.W. Chang, C. Spagnoli, K.V. Shafi, A. Ulman, M. Cowman, R.A. Gross, J. Am. Chem. Soc. 125 (2003) 1684–1685.
[132] K.M. de Lathouder, T. Marques Fló, F. Kapteijn, J.A. Moulijn, Catal. Today 105 (2005) 443–447.
[133] M.T. Reetz, K.E. Jaeger, Chem. Phy. Lip. 93 (1998) 3–14.
[134] Y.M. Zhang, L.F. Zhao, J. Li, H.D. Zhang, L.Y. Zheng, S.G. Cao, L. Can, Biochem. Biophys. Res. Commun. 372 (2008) 650–655.
[135] J.M. Palomo, G. Fernández-Lorente, C. Mateo, R. Fernández-Lafuente, J.M. Guisan, Tetrahedron: Asymmetry 13 (2002) 2375–2381.
[136] C. Mateoa, J.M. Palomoa, G. Fernandez-Lorentea, J.M. Guisan, R. Fernandez-Lafuente, Enzyme Microb. Technol. 40 (2007) 1451–1463.
[137] H. Wernérus, S. Ståhl, Biotechnol. Appl. Biochem. 3 (2004) 209–228.
[138] A. Kondo, M. Ueda, Appl. Microbiol. Biotechnol. 64 (2004) 28–40.
[139] S. Shiraga, M. Kawakami, M. Ishiguro, M. Ueda, Appl. Environ. Microbiol. 71 (2005) 4335–4338.
[140] H. Kwoun Kim, Y.J. Jung, W.C. Choi, H.S. Ryu, T.K. Oh, J.K. Lee, FEMS Microbiol. Lett. 235 (2004) 349–355.
[141] F. Hårdeman, S. SjÖling, FEMS Microbiol. Ecol. 59 (2007) 524–534.