ISSN : 0974 - 7435 Volume 11 Issue 1

BioTechnology
An Indian Journal
Review

BTAIJ, 11(1), 2015 [05-13]

Cloning and expression of lipase with microbial origin

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ABSTRACT

Lipases are ubiquitous in nature and have several potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries. The growing demand for lipases has aroused more attention to prospecting for novel lipases with established technical applications. Herein, cloning and expression of lipase genes with microbial origin is highlighted.

KEYWORDS

Microbial lipase;
Gene cloning;
Expression.

INTRODUCTION

Lipases (E.C.3.1.1.3) are an important group of enzymes. Lipases not only catalyze the hydrolysis of water-insoluble triacylglycerol into diacylglycerol and monoacylglycerol, free fatty acids and glycerol, but accelerate other reactions, such as esterification, interesterification, aminolysis, acidolysis and alcoholysis in immiscible or anhydrous solvents[1]. The multifaceted properties make lipases find usage in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries[2].

Lipases can be sourced from nature ubiquitously and occur in various animals, plants and microorganisms. However, microbial lipases are used more widely than lipases derived from animals and plants due to their catalytic versatility, high yield and ease of genetic engineering, rapid growth of microorganism and good stability especially in the extreme condition such as low and high temperature, extreme pH and high salt and organic solvent concentration[3]. A variety of bacteria, actinomycetes and fungi were reported to produce lipase by several researchers[4-12]. All the lipases are members of α/β hydrolase superfamily, which shows a common architecture formed by α helices and β strands. In the Lipase Engineering Database (LED) (http://www.led.uni-stuttgart.de), sequences of 92 microbial lipases and homologous serine hydrolases were assigned to 32 homologous families and 15 superfamilies[13].

Since microbial lipase was found in 1901 for the first time, the researches on the screening, optimization of fermentation conditions, purification and characterization, cloning and expression, molecular engineering, immobilization and application of microbial lipase have been well documented[14-24]. The present review focuses on cloning and expression of lipase genes with microbial origin.

CLONING OF LIPASE GENES

With the development of molecular biology, the researches on lipase gene are advanced rapidly. Lipase genes from tens of kinds of microbial genus were cloned

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TABLE 1 : Pros and cons of three methods for lipase gene cloning

Methods	Benefits	Drawbacks
direct amplifying	Quickest and easiest method, Lower cost and less labour	Dependent on the known lipase sequence, low possibility of novel gene discovery, inaccessible to of unculturable organisms
construction of genomic DNA library	high possibility of novel gene discovery, independent on the known lipase sequence	A complicated method, inaccessible to unculturable organisms
metagenomic approach	high possibility of novel gene discovery, accessible to unculturable organisms, high potential	Most complicated and laborious method, low efficiency

TABLE 2 : Genes cloning of lipases from bacteria and actinomycetes

Gene	Microorganism	length	Reference
lip9	*Pseudomona aeruginosa* LST-03	933 bp	[29]
P.fragi lipase gene	*Pseudomonas fragi* IFO-12049	1178 bp	[30]
lipase K80	*Proteus vulgaris* K80	861 bp	[31]
lipA	*Actinetobacter calcoaceticus* RAG-1	1017 bp	[32]
lipA	*Burkholderia cepacia* ATCC 25416	1095 bp	[33]
lipA	*Pseudomonas fluorescens* 26-2	1908 bp	[34]
lipA	*Serratia marcescens* ECU1010	2218 bp	[35]
B. subtilis Lipase gene	*Bacillus subtilis* IFFI10210	742 bp	[36]
BS-lip	*Bacillus sphaericus* 205y	1560 bp	[37]
lipase L62	*Staphylococcus haemolyticus* L62	2136 bp	[38]
SWL2	*Staphylococcus warneri* 863	2199 bp	[39]
S. rimosus lipase gene	*Streptomyces rimosus* R6-554W	1068 bp	[40]
SCO1725, SCO7513	*Streptomyces coelicolor* M145	807 bp, 888 bp	[41]

and sequenced. A method for the cloning of lipase genes is involved in directly amplifying from genomic DNA of microorganism[25]. As an alternative way, lipase gene can be identified by constructing a genomic DNA library, which is applied for a direct selection[26]. The metagenomic approach, direct cloning of genes from environmental samples and thereby accessing the potential of unculturable organisms, has become a potential method to obtain novel lipase genes[27]. Pros and cons of three methods for lipase gene cloning were summarized in TABLE 1.

Cloning of lipase genes from bacteria and actinomycetes

To date, a large number of lipase genes from bacteria and actinomycetes were cloned (TABLE 2). Lipases from *Pseudomonas* genus represent a kind of thoroughly studied enzymes. *Pseudomonas* lipases were divided into three groups according to their amino acid sequences, which belonged to the subfamily I.1, I.2 and I.3[28]. Subfamily I.1 included lipase from *Pseudomonas aeruginosa*, *Pseudomonas fragi*, *Proteus vulgaris* etc. Subfamily I.2 contained lipase from *Burkholderia glumae*, *Chromobacterium viscosum*, *Burkholderia. cepacia* and *Pseudomonas luteola*. Subfamily I.3 included lipase from *Pseudomonas fluorescens and Serratia marcescens*. Ogino *et al.* devised one pair of primers to obtain the genes of the LST-03 lipase and lipase-specific foldase. LST-03 lipase was unique in organic solvents tolerance[29]. A lipase gene was identified from *Pseudomonas fragi* IFO-12049 using the expression library. The amino acid sequence of the lipase deduced from the nucleotide sequence indicated that the lipase was composed of

277 amino acid residues[30]. The gene for lipase K80 from *Proteus vulgaris* K80 was cloned in *Escherichia coli*. The deduced primary structure of the lipase gene had 46.3% identity to the lipase from *Pseudomonas fragi*. Lipase K80 was fairly stability over a wide pH range from 5 to 11 and had optimal activity at pH 10, suggesting it was an alkaline enzyme[31]. Genes encoding the lipase (LipA) and lipase chaperone (LipB) from *Acinetobacter calcoaceticus* RAG-1 were cloned and then sequenced. The deduced amino acid sequences for the lipase and its chaperone were found to encode mature proteins of 313 aa and 347 aa, respectively. Phylogenetic analysis showed that LipA belonged to a previously described family of *Pseudomonas* and *Burkholderia* lipases[32]. The lipase from *Burkholderia cepacia* ATCC 25416 found application in detergent and leather industry for its peculiarities of high alkaline and thermal stability. The lipase gene (*lipA*), lipase chaperone gene (*lipB*), and native promoter upstream of *lipA* from *Burkholderia glumae* ATCC 25416 were cloned. The lipase was tolerant of various ionic and non-ionic surfactants as well as oxidant H_2O_2 and showed good stability in the presence of low- and non-polar solvents[33]. Yang *et al.* successfully cloned a lipase gene from *Pseudomonas fluorescens* 26-2. The multi-alignment of the putative amino acid and the secondary structure prediction revealed the lipase belonged to subfamily I.3. The lipase exhibited a high optimal temperature (50°C) and a good transesterification capacity (83.8%) from soybean oil to fatty acid methyl esters, which make it a useful biocatalyst for production of biodiesel[34]. Using forward primer (52 CCGCATACCAATAACG TTTCATCA32) and reverse primer (52 CAGCAGTGGT TCCGCCTTCGCAAG32), *lipA* gene was amplified, which shares 94–96% identities on DNA level and 96–98% identities on amino acid level with those of *S. marcescens* Sr41, *S. marcescens* SM6 and *S. marcescens* ES-2, respectively. The lipase showed good enantioselectivity for the kinetic resolution of ketoprofen ethyl ester, with an ee(p) of 91.6% and E-value of 63 obtained at 48.2% conversion[35]. *Bacillus subtilis* lipase has potential for application in food and chemistry industry. A mature lipase gene from *Bacillus subtilis* strain IFFI10210 was cloned into the plasmid pBSR2 and transformed into *B. subtilis* A.S.1.1655. Lipase gene from *B. subtilis* IFFI10210 was the same as that of previously reported lipase gene from *B. subtilis* 168[36]. *BS-lip* gene was screened from a genonic library and the gene was a novel gene distinct from other lipase gene in Family I.4 and I.5. The crude lipase showed a slight (10%) increase in activity incubated for 30 min in 25% (v/v) n-hexane at 37°C and only lost 10% of its activity after a similar period incubation in 25% (v/v) p-xylene[37]. A gene encoding the lipase of *Staphylococcus haemolyticus* L62 was cloned in *Escherichia coli*. The mature lipase had 49–67% amino acid sequence homology with other staphylococcal lipases. However, it had less than 30% homology with all other bacterial lipases[38]. A gene encoding an extracellular lipase was identified in *Staphylococcus warneri* 863. The deduced lipase has significant similarity to other staphylococcal lipases. It had a high preference to hydrolyze short chain substrates and it showed activity towards phosphoesters[39]. Vujaklija *et al.* reported the cloning and sequencing of lipase gene from *Streptomyces rimosus* R6-554W. This lipase, which belonged to family II of lipolytic enzymes, showed no overall amino acid sequence similarity to other lipases in the GenBank[40]. Using chromosomal DNA from *Streptomyces coelicolor* M145 as template, SCO1725 and SCO7513 lipase genes were amplified by PCR. Subsequently, the cloning of these two lipase genes into protoplasts of *S. lividans* mutant strain 10-164 was carried out. Both lipases hydrolyzed mid- to long-chain substrates and were active at alkaline pH[41]. For directly amplifying lipase gene from genomic DNA of bacteria and actinomycetes, devising suitable primers is of importance. If bacteria and actinomycetes were identified, it is possible to devise primers according the homologous sequences in the data bank. However, if lipase genes are obtained by constructing a genomic DNA library, the process is laborious and time consuming. Construction of library with suitable size and high-throughput screening are key points during the process. Fortunately, the possibility of identification of novel lipase by the method is higher as compared with direct amplification of lipase gene from microbial genome.

Cloning of lipase genes from fungi

In the past few decades, tens of lipase genes from fungi have been cloned, the sizes of which usually were

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above l000bp (TABLE 3). Several short introns were found in some lipase genes, while some other lipase genes had no introns. Some important lipase-producing fungal genera include *Candida, Aspergillus, Rhizopus, Penicillium,* etc[42]. An important step for cloning lipase from fungi is extracting mRNA for cDNA production. The approximate 800 bp DNA fragment was successfully cloned from the total RNA of *C. antarctica* ZJB09193, which encoded a putative protein of 317 amino acids with a molecular mass of 37 kDa[43]. The lipases from *Candida cylindracea* had a relaxed specificity as comparison with other lipases, rendering them versatile for a variety of industrial applications. Lotti *et al.* reported the molecular cloning and characterization of three genes from *Candida cylindracea*. Multiple alignments indicated the lipases from *Candida cylindracea* were very weakly closed to most of other lipases, except the two lipase isoforms from *G. candidurn*[44]. Owing to their safety, crude *Candida rugosa* lipases have been utilized to synthesize food flavor enhancer in Japan and U.S.. Xu *et al.* cloned a novel lipase gene (lipJ08) from *Candida rugosa* ATCC14830, along with the already reported five lipase genes (lip1-lip5). Their study proved the cloned *lipJ08* to be a novel CRL isoform[45]. *Yarrowia lipolytica* lipase family was unique in substrate. There were some researches on LIP2, LIP7 and LIP8 among eight members of family. Especially, LIP2 was widely used for chiral resolution. Zhao *et al.* cloned cDNA sequences of LIP4 and LIP5 from *Yarrowia lipolytica* CGMCC 2.1405 by reverse transcription PCR. Both of these two lipases had a low homolog with LIP4[46]. By screening a genomic library, a triacylglycerol lipase gene (provisionally designated tglA) was successfully identified by Toida *et al*.. Although the lipase differed from cutinases from fungi in the substrate specificity, it shared homolog to those cutinases[47]. Using poly(A)-selected mRNA from *Rhizopus delemar* ATCC34612, the construction of a λgt11 cDNA library was achieved in *E.coli*. The LIP cDNA encoded a putative preprolipase consisting of a 26-aa signal sequence, a 97-aa propeptide, and a 269-aa mature enzyme. The enzyme exhibited *sn*-1 and *sn*-3 stereospecific, but not *sn*-1 stereospecific[48]. *Rhizopus oryzae* lipase was widely used as catalyst for the enzymatic production of biodiesel. A lipase from *Rhizopus oryzae* DSM 853 (ROL) was cloned from a chromosomal gene bank and then sequenced. The lipase gene had an open reading frame, which encoded a protein of 392 amino acids. The CAAT box (bases 734-737) and TATA box (bases790-797) were found[49]. *Rhizopus niveus* lipase (RNL) had 1, 3-positional specificity, which was applied to produce cocoa butter substitute. Kugimiya *et al.* were succeeded in obtaining complementary DNA encoding RNL by constructing cDNA library from *R. niveus* IF04759[50]. Zhang *et al.* successfully cloned the complete gene (PG37 lipI) from *Penicillium cyclopium* PG37. The lipase gene encoding an alkaline lipase was 2020 bp long with 632 bp of the 5' flanking promoter region and 1388 bp of the downstream fragment that contained 6 exons and 5 short introns[51]. A gene (mdlA) encoding mono- and di-

TABLE 3 : Genes cloning of lipases from fungi

Gene	Microorganism	length	Reference
lipase gene	*Candida antarctica* ZJB09193	~800bp	[43]
LIP3, LIP4, LIP5	*Candida cylindracea* ATCC 14830	1855bp, 1785bp, 812bp	[44]
lipJ08	*Candida rugosa* ATCC14830	1650 bp	[45]
LIP4, LIP5	*Yarrowia lipolytica* CGMCC 2.1405	1221bp, 1113bp	[46]
L3	*Aspergillus oryzae* RIB128	1025 bp	[47]
LIP	*Rhizopus delemar* ATCC34612	1287 bp	[48]
ROL	*Rhizopus oryzae* DSM 853	1441 bp	[49]
RNL	*Rhizopus niveus* IF04759	1000 bp	[50]
PG37 lipI	*Penicillium cyclopium* PG37	1480 bp	[51]
mdlA	*Penicillium camembertii* U-150	2038bp	[52]
PEL	*Penicillium expansum* PF898	1138bp	[53]

acylglycerol lipase from *Penicillium camembertii* U-150 was cloned. There was a significant difference in substrate specificity between the lipase and triacylglycerol lipases from *Mucor miehei* and *Humicola lanuginose*[52]. Bian *et al.* cloned lipase gene from *Penicillium expansum* PF898. PEL was justified for its novelty in that low sequence identities (<30%) was observed between PEL and all other known lipases and high percentage of hydrophobic residues was found in the N-terminal region of PEL[53]. For cloning lipase gene from fungi, primer designing for amplification through PCR is primary. If N-terminal sequence is known, degenerate primers are often used for PCR. Another critical step for cloning fungal lipase is extracting mRNA for cDNA production. The cell wall structure and endogenous RNase activity in filamentous fungi often interfere with the extraction process. Another difficult issue is how to separate RNA of interest from the highly complex mixture of proteins, polysaccharides and other mycelial debris. The most efficient method uses TRIzol reagent and CTAB for mRNA extraction from fungi. TRIzol reagent is a monophasic solution of phenol and guanidine isothiocyanate that has been extensively used for the isolation of total RNA from cells and tissues with good results, while cetyl trimethylammonium bromide (CTAB) can provide a slight improvement on the total RNA yield.

Cloning of lipase genes from un-culturable microorganisms

In nature, culturable microorganisms account for less than 1% of total microorganisms. Many lipases with unique properties can not be obtained because their hosts are uncultured. A metagenomic approach is employed to solve the problem. Usually, function-based strategy and sequence-based strategy are two effective methods for fishing out lipase gene from the metagenomic library. Ranjan *et al.* constructed a metagenomic library of pond water microbial assemblage. They identified a lipolytic protein with high similarity to yet uncharacterized α/β hydrolase protein family abh_upf0017, which was highlighted in the research[54]. According to Lee *et al.*, *lipG* was cloned from a Korean tidal flat metagenomic library, which encoded a lipolytic enzyme. Based on phylogenetic tree analysis, LipG did not belong to any of the known lipase families[55]. Using genome-walking method, a novel lipase gene (*lipC12*) was obtained from environmental DNA. The new lipase had a high specific activity against long chain triacylglycerols and showed good stability in water-miscible organic solvents and at high salt concentrations[56]. These studies suggested that the metagenomic approach showed powerful potential in expanding our knowledge of lipase diversity. Some novel lipase genes with low similarity to the known lipase genes were discovered by using the method.

EXPRESSION OF LIPASE GENES

The traditional method for lipase preparation is involved in the extraction of fermentation product by directly using lipase-producing strains. Owing to the pathogenicity of strains and/or the requirement of higher lipase productivity, lipase genes are often over-expressed in heterogeneous host[21]. Furthermore, another useful approach to utilize lipase for biocatalysis is to display lipase on the surface of cells. Pros and cons of three methods for lipase expression were summarized

TABLE 4 : Pros and cons of three methods for lipase expression

Methods	Benefits	Drawbacks
Expression in procaryotic hosts (e.g. *E.coli*)	Very high expression level, low production cost,ease of purification procedure,	Formation of inclusion bodies, no posttranslational processing and modification
Expression in eucaryotic hosts (e.g. *Pichia pastois*)	Moderate high expression level, ow production cost, ease of purification procedure, some posttranslational processing and modification	Requirement of methanol to induce gene expression
Cell surface display	Production of biocatalysts without the extraction and purification of enzymes, a useful tool for the engineering of lipase	Overwhelming numbers of false positives, a complicated method

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in TABLE 4.

Over-expression of lipase genes in heterogeneous host

In recent years many lipase genes have been expressed in heterogeneous host (TABLE 5). Lipase genes from *Pseudomonas fluorescens* JCM5963[23], *Pseudomonas fragi* IFO-12049[30], *Serratia marcescens* ECU1010[35], *Bacillus subtilis* IFFI10210[36], *Streptomyces rimosus* R6-554W [40] and *Chromobacterium viscosum*[57] were expressed in *E.coli* host. The expression level of recombinant lipase usually made up 30-50 percent of total protein in the cells due to strong promoter in expression vector[58]. However, overexpression of lipase in *E.coli* usually resulted in the formation of inclusion body. In addition, a correct folding of lipase structure requires the precise formation of disulfide bond for most of lipase. Nevertheless, the accurate folding of lipase conformation is difficult in *E.coli*. Hence, a refolding procedure in vitro is necessary to obtain active lipase. Except for *E.coli* as a host, *Pichia pastrois* is another potent host used for lipase expression. *Pichia pastrois* expression system was developed in the 1980s, which was used successfully in the expression of some lipase genes[59-66] (TABLE 5). *Pichia pastrois* expression system is advantageous over *E.coli* and *Saccharomyces cerevisiae* expression system due to high density cultivation, low glycosylation level, ease of purification procedure of target protein, low production cost, posttranslational processing and modification, strong promoter of alcohol oxidase in *P. pastrois* and high expression level. As shown in TABLE 5, most of lipase genes were expressed in the form of secreted protein in *Pichia pastois*. Although different host strains and expression vectors were used and different expression level was observed, all of the recombinant lipases in Table 3 were active. These examples demonstrated potential of *Pichia pastois* for lipase expression.

Display lipase on the surface of cells

Surface display allows the exogenous protein, which is fused with the anchor protein, displaying on yeast, mammalian and bacterial cells surface. Nowadays, the technology has been widely used in expression of functional protein. For lipase displaying on cells, the method enables us to produce biocatalysts without the extraction and purification of enzymes and with reusability, which helps to drive down the product cost and makes the enzymatic process economically viable. Phage display appeared in the 1980s as the first cell surface displaying technology[67] and then Charbit established bacterial cell surface display in 1986[68]. However, bacterial cell surface display system has drawbacks for the expression of eukaryotic proteins due to the requirement of post-translational of these proteins such as

TABLE 5 : Overexpression of lipase genes in heterogeneous host

Lipase	Host and expression vector	Reference
rPFL	*E. coli* BLP/pET28b	[23]
P.fragi lipase	*E. coli* JM83/ pUC9	[30]
lipA	*E. coli* BL21 (DE3)/ pET24a (+)	[35]
B. subtilis Lipase	*B. subtilis* A.S.1.1655/pBSR2	[36]
SCO1725, SCO7513	*msiK*⁻/pIAF933	[41]
The synthetic gene for the mature lipase	*E. coli* BL21 (DE3)/pET20b(+)	[57]
Candida parapsilosis lipase	*P. pastoris* GS115/pPIC9K	[59]
lipB52	*P. pastoris* KM71/pPIC9K	[60]
BTL2 lipase	*P. pastoris* GS115/pGAPZαC	[61]
ROL	*P. pastoris* PFLD1/pPICZFLDα	[62]
CALB	*P. pastoris* SMD1168/YpDCC541	[63]
lip4	*P. pastoris* X-33/pGAPZαC	[64]
human pancreatic triglyceride lipase	*P. pastoris* GS115/pHO1,PHIL-S1	[65]
proRCL	*P. pastoris* GS115/pPIC9K	[66]

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TABLE 6 : Display lipases on the surface of cells

Lipase	Host and anchoring motif	Reference
RML variants	*P. pastoris*/Flo1p	[69]
TliA	*P. putida* GM730/INP	[70]
LipB52	*P. pastoris* KM71/ FLO	[71]
B. subtilis lip	*B. subtilis* /CWB_c	[72]
P. fluorescens SIK W1 lipase	*E. coli*/OprF	[73]
P. fluorescens SIK W1 lipase	*P. putida* KT2442 /OprF	[74]
Lip2	*S. cerevisiae* /Cwp2	[75]
Lip7 and Lip8	*S. cerevisiae* /Aga2 of a-agglutinin	[76]
Lip A	*S. cerevisiae* /Pir4	[77]
CALB	*P. pastoris*/alpha-agglutinin and Flo1p	[78]

glycosylation, phosphrylation and so on. Fortunately, *S. cerevisiae* and *P. pastoris* are good candidates for display of eukaryotic proteins. Furthermore, the main focus for displaying lipase on the surface of cells is the suitable selection of anchoring motif. There are some successful examples of displaying of lipases with microbial origin on the cell surface[69-78] (TABLE 6). LipB52 was expressed under the control of the AOX1 promoter and displayed on the cell surface of *Pichia pastoris* KM71 with FLO, which was confirmed by the confocal laser scanning microscopy[71]. Kobayashi *et al.* fused *B. subtilis* lipase B to a small cell wall-binding domain of the *Bacillus subtilis* peptidoglycan hydrolase CwlC and the fusion gene was then localized on the cell wall of *B. subtilis*[72]. Lee *et al.* developed a new cell surface display system in *Pseudomonas putida* KT2442 using OprF, an outer membrane protein of *Pseudomonas aeruginosa*, as an anchoring motif to display *Pseudomonas fluorescens* SIK W1 lipase gene [74]. Liu *et al.* were succeeded in displaying Lipase Lip2 from *Yarrowia lipolytica* on the cell surface of *Saccharomyces cerevisiae* using Cwp2 as an anchor protein[75].

FUTURE OUTLOOK

Although a wide variety of lipases have been discovered, industrial applications demand lipase with higher activity, stability and specificity. To obtain industrially tailored lipase, novel gene of lipase with unique nature should be explored. In previous studies, lipase genes were mainly from ordinary niches. Therefore, new lipase genes should be sourced from extremophiles in various extreme environments[79], endophytes[80], non-culturable organisms and metagenome from environmental samples[27]. As for the expression of lipase, eukaryotic system such as yeast and filamentous fungi expression system are the most suitable for the production of secreted lipase owing to their potent secretion ability and mature fermentation technology among heterologous expression systems[81]. Furthermore, cell surface display technology not only provides lipase for use in whole cell biocatalysis without membrane barrier, but supplies a useful tool for the engineering of lipase. Lipase mutant libraries displayed on cell surface were applied for screening with fluorescence-activated cell sorting, which has emerged a powerful technique for the high-throughput screening of enzyme library over 10^9 variants[82].

ACKNOWLEDGEMENTS

The financial support from the National Natural Science Foundation of China is thankfully acknowledged (Project ID: 31360216).

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