

## Isolation of a Novel Lipase Gene from *Serratia liquefaciens* S33 DB-1, Functional Expression in *Pichia pastoris* and its Properties

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**Abstract** A new lipase gene designated as *SLipA* was isolated from *Serratia liquefaciens* S33 DB-1 by the genomic-walking method. The cloned gene contained an open reading frame (ORF) of 1,845 bp encoding 615 amino acids with a conserved GX SXG motif. Genome sequence analysis showed that an aldo/keto reductase gene closed to the *SLipA* gene. The lipase gene was cloned into the expression vector pPICZαA and successfully integrated into the heterologous host, methylotrophic yeast *Pichia pastoris* GS115. Five transformants could be expressed as secreted recombinant proteins with the high activity on Triglyceride–Agarose plate and as candidates to produce the recombinant enzyme. A C-terminal His tag was used for its purification. The lipase activity of different transformants against substrate *para*-nitrophenyl laurate (*p*-NPL) varied from 14 to 16 U ml<sup>-1</sup>. For the substrates *para*-nitrophenyl caprate (*p*-NPC), *p*-NPL, *para*-nitrophenyl myristate (*p*-NPM), *para*-nitrophenyl palmitate (*p*-NPP), and *para*-nitrophenyl stearate (*p*-NPS), the

specific activity was shown to be preferred to long acyl chain length of *p*-NPS.

**Keywords** Cloning · Expression · Genomic-walking · Lipase · *Pichia pastoris* · *Serratia liquefaciens*

### Introduction

Lipase (EC.3.1.1.3) is one of the five international commercial enzymes with maximum output and has broad industrial applications in food agents for modification of milkfat [1], improving flour baking quality [2], and machining oils [3]. Lipase can also be used in synthesis of ideal chemicals in enantioselective transesterification [4] and as carrier for drug delivery [5]. Recently, lipase has become more attractive for biodiesel fuel production by transesterification of oils [6]. Lipases usually display exquisite chemoselectivity, regioselectivity, and stereoselectivity, making them become attractive ideal tools in synthesis of organic chemicals. Thereby, it prompts a demand for diverse lipases from different organisms to expand not only their functionalities, but also their specific applications.

As one of genus *Serratia*, *Serratia liquefaciens* is frequently referred to causing the human nosocomial infections [7]. *S. liquefaciens* strain was studied as a candidate compound for the management of hypertriglyceridemia for producing a pharmacological effect of FR177391 due to its infectivity [8]. Until now, there have been few reports on lipases from *S. liquefaciens*. In 1978, one test on the lipase and phospholipase revealed that *S. liquefaciens* exhibited strong lipase, but only a minor phospholipase activity [9]. Yu et al. isolated a strain with the highest lipolytic activity, *S. liquefaciens* S33 DB-1, from 150 oil-contacted soil

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samples [10]. The strain showed extracellular alkaline lipase activity of lipolytic olive oil within the pH 7.0–9.0 at a temperature below 45°C [10]. Further investigation elucidated its capability and optimum condition of producing large amount of extracellular alkaline lipase under the induction of oils [11]. To my knowledge, there has not been any report about cloning of lipase gene from *S. liquefaciens* and expressing of the lipase by heterologous host. So it is a necessity to clone lipase gene from *S. liquefaciens* and to produce recombinant lipase for large-scale engineering from heterologous host. In this report, we chose *S. liquefaciens* S33 DB-1 strain to clone and functionally express its lipase gene in *P. pastoris*.

## Materials and Methods

### Materials

Restriction enzymes, T4-DNA ligase, Taq polymerase, and LA PCR in vitro Cloning kit were purchased from Takara (Japan). The DNA Gel-Extraction Kit, Mini Plasmid Kit and Yeast Genomic DNA Extraction Kit were from Watson (Shanghai, China). The different lipase substrates including *para*-nitrophenyl caprate (*p*-NPC), *para*-nitrophenyl laurate (*p*-NPL), *para*-nitrophenyl myristate (*p*-NPM), *para*-nitrophenyl palmitate (*p*-NPP), *para*-nitrophenyl stearate (*p*-NPS), and bovine serum albumin (BSA) as a standard of protein concentration, were all from Sigma (St. Louis, USA). Trypton, peptone, yeast extract, and yeast nitrogen base were from Amresco (Solon, OH, USA). The antibiotic zeocin was supplied by Invitrogen (CA, USA). All reagents were of analytical grade unless otherwise stated.

### Strains, Plasmids, and Growth Conditions

*Escherichia coli* strain TOP10F' (Invitrogen, CA, USA) was used for vector construction work and *Pichia pastoris* strain GS115 (*his4*) (Invitrogen, CA, USA) for lipase expression. *E. coli* was grown in low salt LB-zeocin medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 25 mg zeocin in 1 l water, pH 7.5).

*P. pastoris* was grown in YPD medium (10 g yeast extract, 20 g peptone, and 20 g dextrose/l) or BMGY medium (10 g yeast extract, 20 g peptone, 13.4 g yeast nitrogen base, 0.4 mg biotin, 10 ml glycerol, and 100 ml 1 M K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 6.0 per l). BMMY medium (10 g yeast extract, 20 g peptone, 13.4 g yeast nitrogen base, 0.4 mg biotin, 5 ml methanol, and 100 ml 1 M K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 6.0 per l) was used for induction. For selection of transformants, YPDS-zeocin plates were used (182.2 g sorbitol, 20 g agar, 20 g dextrose, 10 g yeast

extract, 20 g peptone, 100 mg zeocin/l). MDH (13.4 g yeast nitrogen base, 0.4 mg biotin, 20 g dextrose, 15 g agar, and 4 g histidine/l) and MMH (13.4 g yeast nitrogen base, 0.4 mg biotin, 5 ml methanol, 15 g agar, and 4 g histidine/l) plates were used to confirm the Mut<sup>+</sup> (Methanol utilization plus) phenotype.

*S. liquefaciens* S33 DB-1 was kindly supplied by Dr. Wenxin Zou (Department of Biology, Nanjing University, Nanjing, P R China).

Plasmid pGEM T-easy vector (Promega, Madison, WI, USA) and pPICZαA vector (Invitrogen, CA, USA), were used for cloning and expression, respectively.

### Gene Amplification and Sequencing

Genomic DNA was isolated from *S. liquefaciens* S33 DB-1 with bacterial genomic DNA isolation kit from Watson (Shanghai, China) according to the manufacturer's protocol and was used as templates for PCR amplification of the conserved region of lipase from *S. liquefaciens*. Two degenerate oligonucleotide primers, SILipF (5'-AT(C/T)GC(A/C/T/G)TTTCGCGGCACCAG-3'), SILipR (5'-TCGTTGAA(G/A)(A/C/T/G)T(G/A)ACGATGTTGT-3'), were designed according to the conserved sequences of other lipase genes and used for the amplification of the core cDNA fragment of *SILipA* by standard gradient PCR amplification (from 52 to 60°C) on ThermoHyaid PX2 Thermocycler (Hyaid, UK). Subsequently, amplified at the annealing temperature of 56°C, the core fragment was subcloned into pGEM T-easy vector (Promega, Madison, WI, USA), and transformed into *E. coli* strain DH5α followed by sequencing. The core fragment was subsequently used to design the gene-specific primers for cloning the full-length DNA of *SILipA* by genomic walking.

LA PCR™ in vitro Cloning Kit (TaKaRa, Japan) was used to clone the 3'- and 5'-flanking regions of *SILipA* gene. The genomic DNA was completely digested by an appropriate enzyme *Bam*HI, and the digested DNA fragments were ligated to the *Bam*HI cassettes according to the manufacture's instructions. The ligated DNA fragments with *Bam*HI cassettes were used as the templates for amplification of 3'- and 5'-flanking regions of *SILipA* gene.

For the amplification of 3'- and 5'-flanking regions of *SILipA* gene, two 3'- and 5'-gene-specific primers were designed. Cassette primers (C1 and C2) were provided by the kit. For the first amplification of 3'-flanking region of *SILipA* gene, SILip3-1 (5'-TGCTGGCCGGTTTCGGCCC GAATGGCTATGCC-3') and Cassette primer C1 (5'-HOGTACATATTGTCGTTAGAACGCGTAATACGACT CA-3') were used. For the nested amplification of 3'-flanking region, primers SILip3-2 (5'-CCAACATATGTC GCCTTTGCTTCTCCTACCCAA-3') and Cassette primer

C2 (5′-HOCGTTAGAACGCGTAATACGACTCACTA TAGGGAGA-3′) were used with the diluted products of the first amplification as templates. Two nested specific primers SILip5-1 (5′-GGCATAGCCATTCGGGCCGAAA CCGGCCAGCA-3′) and SILip5-2 (5′-AGAGATTCGCG CGGGCCGCTGGTGCCGCGAAA-3′) were used for nested PCR amplification of the 5′-flanking region of *SLLipA* gene with Cassette primer C1 and C2. The first and nested PCRs were carried out under the conditions described in the protocol (LA PCR™ in vitro Cloning Kit, User Manual, TaKaRa): 30 cycles (30 s at 94°C, 2 min at 55°C, 2 min at 72°C). The nested 3′- and 5′-flanking region products were purified and subcloned into pGEM T-easy vector followed by sequencing.

By aligning and assembling the sequences of the core fragment, 3′- and 5′-flanking region products on Contig Express (Vector NTI Suite 8.0), the coding sequence of *SLLipA* gene was deduced, amplified by using genome DNA as the template and a pair of primers, SILipF-ORF (5′-AT GGGAACTCTTAATTATCAAGGT-3′) and SILipR-ORF (5′-GGCCAGTACCACTTGGCCGTCCG-3′). The amplified product was subsequently cloned into pGEM T-easy vector (Promega, Madison, WI, USA), transformed into *E. coli* strain DH5α followed by sequencing. The PCR amplification and sequencing of the ORF of *SLLipA* gene were repeated thrice to avoid PCR errors.

#### Recombinant Vector Construction and Transformation

A pair of primers containing *Pst*I and *Not*I digesting sites, SILipA-F-*Pst*I (5′-AACTGCAGAACCAATGGGAATCT TTAATTATCAAGGT-3′) and SILipA-R-*Not*I (5′-AAG GAAAAAGCGGCCGCGCCAGTACCACTTGGCCG TCCG-3′), were designed and used to amplify the ORF region using the cloned ORF fragment as the template. PCR product was digested with *Pst*I and *Not*I, and ligated into the vector pPICZαA that was digested with the same enzymes, to generate the expression vector pPICZαA-*SLLipA*. After transformation of *E. coli* TOP10F′ with pPICZαA-*SLLipA*, zeocin-resistant clones were selected and analyzed by sequencing to confirm the correct fusion of *SLLipA* coding gene to the α-factor secretion signal.

The plasmid was linearized with *Sac*I and electroporated by Bio-Rad MicroPulse (Hercules, CA, USA) into electrocompetent *P. pastoris* cells (GS115) before the host cells were spread on YPDS-zeocin plates and incubated at 30°C for 2 days. MDH and MMH plates were used to confirm the Mut<sup>+</sup> phenotype for metabolizing methanol as the sole carbon source to induce expression of the alcohol oxidase gene (*AOX1*) that was used to drive expression of the gene of interest encoding the desired heterologous protein [12, 13].

Genomic PCR was used to determine whether the heterologous gene integrated into the yeast genome. The PCR template genomic DNA was isolated according to the procedure of Yeast Genomic DNA Extraction Kit (Watson, Shanghai, China).

#### Enzyme Screening

Screening of *P. pastoris* transformants for enzyme secretion was performed using Rhodamine–Triglyceride–Agarose Assay following the procedure [14] to drop 5 μl supernatants after induction by methanol into the Rhodamine–Triglyceride–Agarose plate by a pricker. Enzyme in supernatants hydrolyzed triglyceride to form a clear transparent halo around the pinhole by incubation at 37°C for about 8 h. The SILipA clones with high hydrolyzing activity toward triglyceride were confirmed according to the size of transparent halo on the agar plate by addition of rhodamine B.

#### Expression of Recombinant Lipase and Cultivation

Transformants with large hydrolytic cycle were inoculated in 50 ml BMGY medium in a 500 ml baffled flask to an optical density (OD) of 3–4 at 30°C with 250-rpm agitation. The cells were harvested and resuspended in 50 ml BMMY medium to reach OD1.0 for inducing gene expression, growing at 30°C with 250-rpm agitation. Methanol was added to the culture medium to a final concentration of 0.5% every 24 h for the next 3 days to maintain induction. An aliquot of the expression culture was harvested at different time point to analyze expression levels and the optimal time of post-induction.

The culture was chilled rapidly to 4°C, and then the cells were removed by centrifugation. The crude enzyme was precipitated from the culture supernatants by 10% trichloroacetic acid, washed with ice acetone, lyophilized at –50°C and then analyzed by SDS-PAGE [15]. The collected total protein samples of different time courses were analyzed by SDS-PAGE (Bio-Rad, Mini-PROTEAN) on a 10% polyacrylamide gel. The band intensities were determined by Coomassie Blue-stained gel.

#### Purification of Protein

The purification of protein was carried out using Pro-Bond™ Purification System (Invitrogen, CA, USA) according to the manufacturer's instructions. The precipitated and lyophilized total protein sample from 50 ml culture supernatants was resuspended in 8 ml Native

Binding Buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 0.5 M NaCl), which was then added to the previously equilibrated purification column packed with ProBond™ nickel-chelating resin. The purified protein was eluted with 8 ml Native Elution Buffer (250 mM imidazole, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, and 0.5 M NaCl). The eluted fraction was concentrated to analyze with SDS-PAGE. Protein concentration was determined by the method of Bradford with BSA as a standard [16].

#### Enzyme Activity and Specificity Assay

Lipase activity was measured by a spectrophotometric method with *p*-NPL as substrate [17]. The assay mixture contained 44.5 mM Tris-HCl buffer, pH 7.5, and 2.5 mM substrate solution *p*-NPL that was dissolved in dimethyl sulfoxide (DMSO) before use, and 10 μM of enzyme solution for 10 min at about 25°C room temperature. The enzyme reaction was stopped by adding equal volume of ethanol. The A<sub>405</sub> of liberated *p*-nitrophenol was measured with *p*-nitrophenol as a standard. One unit was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per min under assay conditions [18].

Substrate specificity of SILipA by a spectrophotometric method toward different *p*-nitrophenyl esters (*p*NP-) was determined with *p*-NPC, *p*-NPL, *p*-NPM, *p*-NPP and *p*-NPS as substrates. Cleavage of *p*NP- was measured at room temperature, and reading the corresponding absorbance at 405 nm [19].

## Results and Discussion

#### Cloning and Analysis of Lipase Gene from *S. liquefaciens* S33 DB-1

On the basis of conserved region of other lipase sequences, two degenerate oligonucleotide primers (SILipF and SILipR) were designed and used for gradient PCR amplification of the core cDNA fragment of lipase from *S. liquefaciens* S33 DB-1. An approximately 500 bp product was amplified and subcloned, followed by sequencing. DNA sequence BLAST search revealed this 461 bp DNA fragment shared over 80% homology with *S. marcescens* lipase gene, implying that it was probably a partial sequence of lipase gene. Thus this fragment was used to design gene specific primers for both 3′- and 5′-flanking regions of *SILipA*.

By 3′- and 5′-genomic walking for amplification, the 1,719 and 627 bp nested PCR products were respectively obtained. Sequencing results revealed that the products

were the 562 bp 3′-flanking region and 178 bp 5′-flanking region of *SILipA*, respectively. After assembling and analyzing the two sequences and the core fragment on Contig Express (Vector NTI Suite 8.0), ORF Finding analysis on NCBI showed that the genomic fragment contained a 1,845 bp ORF, encoding a protein of 615 amino acids with a calculated molecular mass of 64.85 kDa and the predicted isoelectric point of 4.31 (<http://cn.expasy.org/tools/protparam.html>). A Shine-Dalgarno sequence, AAG-GAA, was found six bases upstream of the ATG start codon (Fig. 1). Confirmed by amplifying and sequencing, ORF of the sequence was analyzed in NCBI for BLAST searching (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the result showed that *SILipA* shared 74% homology with *SmLipA* (Acc. No. DQ841349). Moreover, the previous research reported that *S. proteamaculans* and *S. liquefaciens* had been thought to be synonymous on the basis of DNA relatedness [20]. The *SILipA* ORF sequence has been deposited at GenBank under accession number EF202840. In addition, sequence analysis by PSI-BLAST searching (<http://www.ncbi.nlm.nih.gov/BLAST/>) showed that there was a putative aldo/keto reductase gene in the reverse complemented sequence from 2,085 to 2,588 at 61 bases downstream from the stop codon of 1,845 bp *SILipA* ORF.

Analysis of the deduced amino acid sequence for the reverse complemented sequence from 2,085 to 2,588 by PSI-BLAST searching (<http://www.ncbi.nlm.nih.gov/BLAST/>) revealed that it coded partial aldo/keto reductase near C-terminus, suggesting that the gene sequence of *SILipA* lipase was flanking an aldo/keto reductase gene in the genome of *S. liquefaciens* (Fig. 1).

Analysis of the deduced amino acid sequence of *SILipA* for PSI-BLAST searching (<http://www.ncbi.nlm.nih.gov/BLAST/>) revealed that *SILipA* shared 87% sequence identity with the lipase (Acc. No. ZP\_01534977) from *S. proteamaculans* 568 and about 70% sequence identity with the lipase (Acc. No. ABI83633) from *S. marcescens*. It suggests that *SILipA* belongs to the lipase family (Fig. 2). Simultaneously, *SILipA* had 60% lower identity with other lipases, putative lipases from *Pseudomonas fluorescens* (Acc. No. AAT48728), and other *Pseudomonas* sp.

According to the sequence conservation characteristic, lipases could be divided into GX SXG and GDSL families. The GX SXG lipases are usually named as “classical” lipases, and exist in the majority of bacteria and eukaryotes. Serine residue of the highly conserved Gly-X-Ser-X-Gly motif is a key element of the active site of fatty acid-deesterifying lipases [21, 22]. The amino acid sequence derived from *SILipA* nucleotide sequence contained GX SXG motif. On the basis of characteristics possessed by GX SXG family of lipases, *SILipA* was predicted to belong to GX SXG family.

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tcaaacacacacatttttgcgatcaaatgctcattctcaataattagtagtaataa 58
tcattctaccagatgctcagctcctcagaccagctggcatttggcgtactctgttcaactaca 118
ctcgctgcaacagtagtaccacaagatttaaccggcgcaatgcagcaaatctcaaaaggaactgat 178
ATGGAATCTTTAATATCAAGGTCTCGATGAGGCGAAATCCAAAACGTTATTTACCGAC 238
M G I F N Y Q G L D E A K S K T L F T D
GCCATGGCGATTTCCACCTACGCCATATGCAATATTGATAATGGTTTGACGAGGGCTAT 298
A M A I S T Y A Y H N I D N G F D E G Y
CACAGCTCCGGTTTTGGCCTGGCTTGCCTTCCAGCTGGTGAACCGCGCTGATAGGCAGC 358
H S S G F G L G L P F T L V T A L I G S
ACGCAATCTCAGGGTGGCCTACCCGGTATTCCTGGAATCCCGACTCGGAACAAGCGCGC 418
T Q S Q G G L P G I P W N P D S E Q A A
CTGGCAGCGGTAATAACCGCGGCTGGTCAATGATAAGCGGACGACCACTAGGCTATCAG 478
L A A V N N A G W S L I S A D Q L G Y Q
GGCAAAACGGATGCCCGGGCACCTATTACGGTGAACGTTCCGGTACACACGGCCAGCAG 538
G K T D A R G T Y Y G E T F G Y T T A Q
GCTGAAGTGTGGGAAAATACGACAGCGAGGTAATCTCACCGGCTACGGCATTGCTTTT 598
A E V L G K Y D S E G N L T G I G I A F
CGCGGCACAGCGCGCCCGCGCAATCTGTATCACCAGTACCATCGCGGATTTGGTCAAT 658
R G T S G P R E S L I T D T I G D L V N
GACCTGCTGGCGGTTTCGGCCGGAATGGCTATGCCGATAACTACAGTCTGAAGCCCTTC 718
D L L A G F G P N G Y A D N Y S L K A F
GGCACCTGCTGGAGGATGTGGCAAATTTCGCCACGGCGCACGGGCTGAGCGGGATGAC 778
G T L L E D V A K F A Q A H G L S G D D
ATTACCATCAGTGGCCATAGCCTGGCGGCGCTGGCCGTGAACAGTATTGGCGCGCTCAGC 838
I T I S G H S L G G L A V N S M A A L S
GACGGCAATTGGGGGGCTTCTATGCGCAGTCCAACATGTGCGCTTTGCTTCTCTACC 898
D G N W G G F Y A Q S N Y V A F A S P T
CAATACGAAACCGGGCGCAAGTATTACCGTGGTACGAAATGACCCGATTCGCG 958
Q Y E T G G K V I N V G Y E N D P V F R
CGCTTGGACGGTACAACCGCGCACCTGCAACGCTGGCGTACATGACGACCCGCAAGC 1018
A L D G T T R T S A T L G V H D A P Q D
TCCGCCACCAACACATCGTTAATTTCAACGACCACTATGCCTCCGCGCCTGGAATATT 1078
S A T N N I V N F N D H Y A S A A W N I
CTGCCCTTCTGATCCTCAATATCCAACTGGTATCTCACCTGGCGCTTCTTTATCAG 1138
L P F S I L N I P T W L S H L P F F Y Q
GACGGGTGATGGCGGTTTGAATTCAGAGTCTACTCGCTGACGAGCAAGACTCGACG 1198
D G L M R V L N S E F Y S L T S K D S T
GTGATCGTTTTCCAACTTTCTGACGTACACCGCGGCAATACCTGGTGGAAAGACCTCAAC 1258
V I V S N L S D V T R G N T W V E D L N
CGCAACCGGAGCAACACAGCGGCCCAACCTTTATTTGTCGGCAGCGAGGCAATGACCTT 1318
R N A E Q H S G P T F I V G S D G N D L
ATCAAGGGCGGCACTGGAATGATTATCTCGAGGGCGTCCCGCAATGACACATTCGCT 1378
I K G G T G N D Y L E G R A G N D T F R
GACGATGGCGGTTTTAATATTATTTCCGGCGGTGAAGGCCATAACACCCCTGGATTTGCGAC 1438

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D D G G F N I I S G G E G H N T L D L Q
CATGCATTGAAAAACACCGAGGTCGCGTACGACGGCAATACGCTATTTCGCCGATGCT 1498
H A L K N T E V A Y D G N T L Y L R D A
GACGGTGGCATCACCTGGTAACTCGATCGGCACGCTGAAAAGCAAAGAGTCTGCTACTG 1558
D G G I T L A N S I G T L K S K E S S L
TTGATTTTACCAAAAGAGGTGATCACCAGGTGACCGACAACCGGCTGCTTTCCACCAAG 1618
L I F T K E V D H Q V T D N G L L S T K
GGCGTGAACCGCTATGCCAGTTTCGGCAAACGGCACCGCGGATGACCTTCTGACTGCT 1678
G L T A Y A S S A N G T A A D D V L T A
AAAGAGTCCGGCTCTGGCTCTTGGTTTGGAGGGAATGACCGAGCTGTTTGGCGGCAAA 1738
K E S G S W L F G L E G N D Q L F G G K
GGCAATGACGTGTTTCGTCGGCGCGCGGTAACGACGTTATGCACAGCCAGGTTGGCAGC 1798
G N D V F V G G A G N D V M H S Q G G S
AATACCTCTGTTTCAGTGGCGACTTCGGTCAAGTCTGATTTACGGTTATCAGGCGCAG 1858
N T F L F S G D F G Q D L I Y G Y Q A Q
GATAAGCTGGTGTATTCGGTACCGAAGGCGAGCTCGGGCGGTAATATTCGGCATTTT 1918
D K L V F I G T E G S S S G G N Y R D F
GTTTCCGAGGTGAACGACAACTGGTGTAACTTTGCGCGCAGTACGGTCACTTTAGT 1978
V S E V N D N L V F N F G G S T V T L V
GGGATCGGATTCGATAGCCTGTCGGACGGCAAGTGGTACTGGCC704acaccggggcat 2038
G I G F D S L S D G Q V V L A *
taacgtataaacggggcgccggaggtccocgttttatcgtgocgctTTAAGTACGTC 2096
* P V D
GTAACCCAGCGCGCTTTGGCGATCGGGAACCACTGTTGACGATCCAGCGCCAAATGCTG 2156
Y G L A A K R I R F W Q Q R D L A L Q Q
CGCCTTACGCGCGCAACGACCGGTTCAATCTTCCCGGACCGGATATCGGCAACGGTGC 2216
A K L A S R V R E I K G S G I P L P A
CGACGGCAGTCCGATCACCCAGGCGTAGACCACTGTCGATAGTGTGGGCCCTATTTTC 2276
S P L R M V W A Y V V Q E I T D A G I E
CTGCGCCACCCTGCAACTCATCGCGAGCGGCTGGAATCTCGCGTTCGCTGAACAGACG 2336
Q A V A Q L E D R L P Q F E A D S F L R
TCCGCCCCAGACAAAGACCGCCATCGGCTGATGCGCACTGCTGGCAGTACTCCAG 2396
G G G L C S W A M P K I R L Q Q C Q D L
CGATCCATCCAGGACTACTCGGCTGGTGAATCGGTGAATCTCCAGTTGATGGTCAACGAG 2456
S G D L I S P Q H I P S I E L Q N T V L
CGAATGGCAGCGCGACTCGACCAAGTAAATTCGCGCGGTGTGAAGTGGAAACGCC 2516
S F P L R S Q L L T F Q A P T F N S V G
GAAGTGGCGCACTTGGCCATTTGGTGCAGAGACACAAAGCCCTCGGCGACCTCATCGG 2576
F H R V K G S Q H L S V F A E A V E D A
ATCCATCAGCGG 2588
D M L P

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**Fig. 1** Nucleotide sequence of a 2.5-kb DNA fragment from *S. liquefaciens* (SI) containing both the ORF coding for lipase and the partial CDS for aldo/keto reductase. The start codon (ATG) for lipase was underlined in bold and the stop codon (TGA) was underlined *italically* in bold. The putative ribosome-binding site was

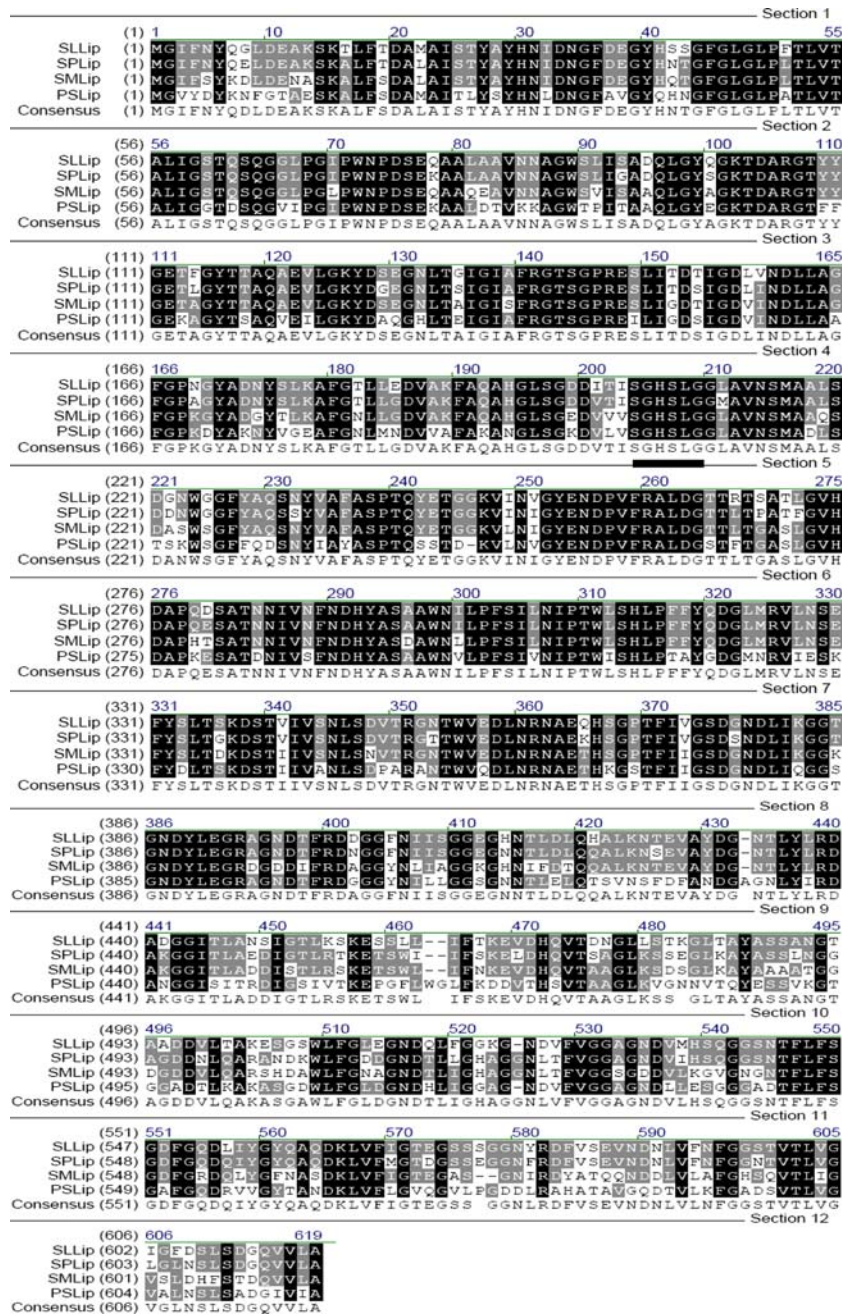
doubly underlined in bold. The reverse complemented sequence was from 2,085 to 2,588 for coding partial aldo/keto reductase. The sequential coding was showed in arrowhead. The sequence data has been deposited at GenBank under the accession number EF202840

## Transformation in *P. pastoris* and Selection of Positive Transformants

For secretive expression, the gene sequence encoding SILipA was directly fused in frame to the  $\alpha$ -factor prepro signal sequence from *Saccharomyces cerevisiae* to target the protein to the secretory pathway, and placed under the control of methanol inducible alcohol oxidase gene (*AOX1*) [23, 24]. One hundred positive transformants were selected on solid selective medium (YPD containing zeocin), confirmed by genomic PCR, and induced by BMMY medium. Five transformants of expressing active lipase were determined by transparent halo on Rhodamine–Triglyceride–Agarose plate performed by adding 5  $\mu$ l induced culture supernatant (Fig. 3). The culture supernatants of positive lipase colony could hydrolyze triglyceride, and a transparent halo appeared in the triglyceride-containing plate.

The expression system of recombinant proteins using pPIC and pPICZ in *P. pastoris* has many advantages for scale-up expression of recombinant proteins [23, 25–29]. *P. pastoris*, a methylotrophic yeast, could metabolize methanol as its sole carbon source, which was a major advantage to induce the expression of heterologous proteins. There are generally two approaches to add methanol for screening positive colony on plate containing emulsified triglyceride. One was to drop methanol onto the lid of plate, and then invert the plate to induce protein expression of positive colony at 28°C [14, 30, 31]. Another classical approach is to add the culture supernatant after induction by methanol into the hole of plate to observe at 37°C. One disadvantage of the former approach was that the colony could be diluted by volatilization of methanol. In this study, we used the latter approach to screen positive colony using 5  $\mu$ l of the induced culture supernatant.

**Fig. 2** Multiple alignment of amino acid sequences of *S. liquefaciens* lipase (SLLip, SPLip, SMLip, PSLip) and other bacterial lipases. Lipase (Acc. No. ZP\_01534977) from *Serratia proteamaculans* 568 (SPLip), lipase (Acc. No. AB183633) from *Serratia marcescens* (SMLip), and lipase (Acc. No. AAT48728) from *Pseudomonas fluorescens* (PFLip) were compared using CLUSTALW program. The identical amino acids were showed in white with black background and the conserved amino acids were showed in white with gray background. The conserved active-center sequence of lipases, -Gly-X<sub>1</sub>-Ser-X<sub>2</sub>-Gly-, was underlined

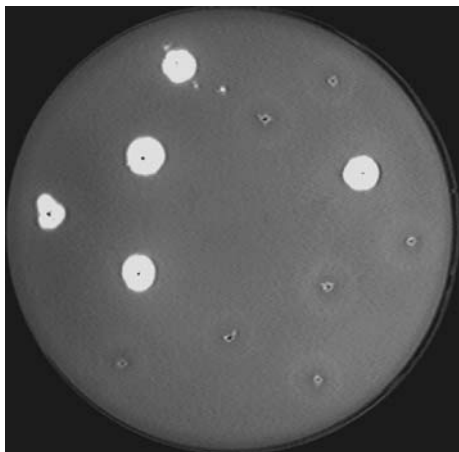


Cell Cultivation and SDS-PAGE

Transformants possessing lipolytic activity, as indicated by the formation of a clear halo on triglyceride plates, were selected and grown in shaking flasks containing 50 ml BMGY medium. After the cultures reached an OD<sub>600</sub> of 3–4, they were centrifuged and resuspended in 50 ml BMMY medium for induction of lipase expression. The cell density and lipase activity were measured as a function of time after induction with 0.5% methanol (Fig. 4). At the start of induction phase, a high increase of cell density was measured. There is a lag in activity following the rapid

increase in cell density, probably due to the remaining glycerol from the cell pellet. After 12 h of post-induction, the lipolytic activity of supernatants was determined. The lipase activity increased steadily, even after 84 h of induction with methanol. The lipase activity of different clones against substrate *p*-NPL varied between 14–16 U ml<sup>-1</sup> in batch after induction of 48 h. No lipase activity was found before induction and in the negative control.

SDS-PAGE analysis of different time-course concentrated samples showed a protein band near the predicted size of 68 kDa with 6× His-tag and c-myc epitope. The

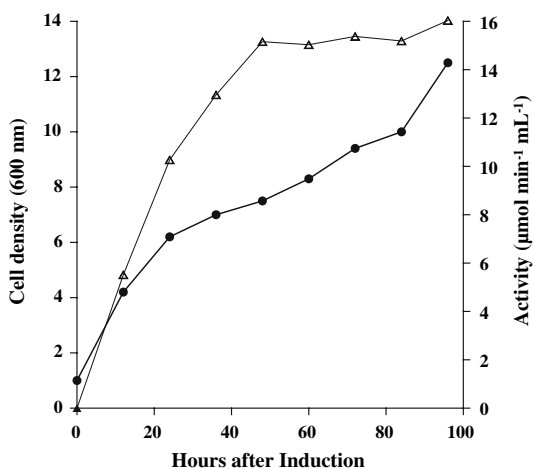


**Fig. 3** Rhodamine B-triglyceride agar plate showing different recombinant clones. A 5- $\mu$ l volume of culture supernatant (sterile filtered) induced by methanol was poured into each preformed hole, and the plate was incubated at 30°C overnight. Transparent halos around the holes (strains labeled A, B, C, D, and E) indicate the production of recombinant lipase

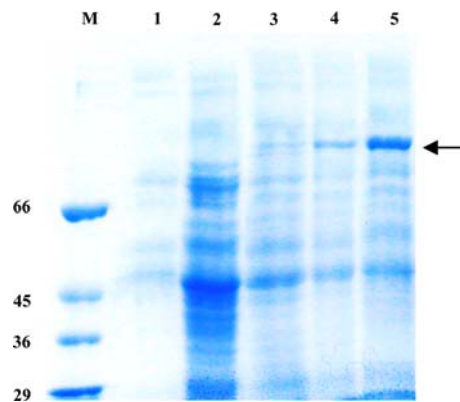
protein band became thicker from 48 to 72 h (Fig. 5), demonstrating that the secreted recombinant protein was stable in the medium. The arrow shows the position of the expressed lipase protein.

#### Purification of Protein

SILipA was expressed with 6 $\times$ His-tag, which was a most convenient way to purify the recombinant protein. ProBond™ nickel-chelating resin used the characteristics of chelating ligand iminodiacetic acid (IDA) that could both



**Fig. 4** Time course study of SILipA production. The cell density (●) measured as a function of cultivation time at 600 nm. The lipase activity ( $\Delta$ ) during cultivation in  $\mu$ mol min<sup>-1</sup> mL<sup>-1</sup> using *p*-nitrophenyl laurate as substrate. The results are the average of duplicate assays performed in two independent experiments

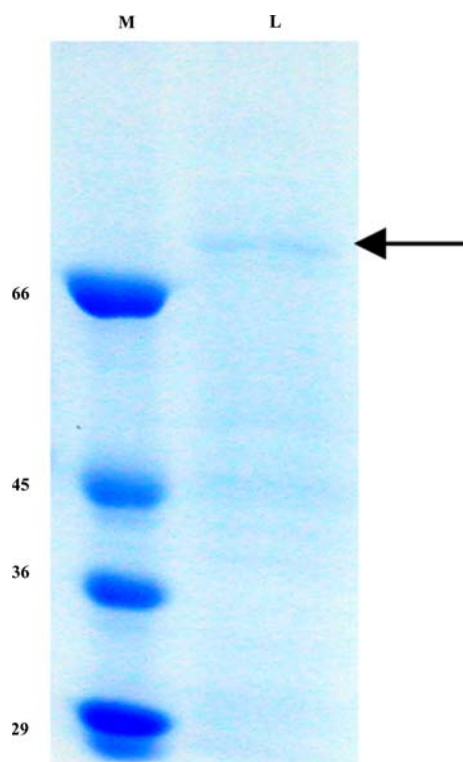


**Fig. 5** SDS-PAGE analysis of total secreted protein after induction with 0.5% methanol. Culture supernatants were harvested at various time intervals, precipitated by 10% trichloroacetic acid, washed with acetone, freeze-dried, and then analyzed by SDS-PAGE. The band intensities of Coomassie Blue-stained gel were determined. Lane numbers, 2, 3, 4, and 5 indicate induction time (h) 24, 48, 72, and 96, respectively, after induction by 0.5% of methanol. Lane 1, before induction; Lane M, middle molecular weight protein markers (Watson, Shanghai, China)

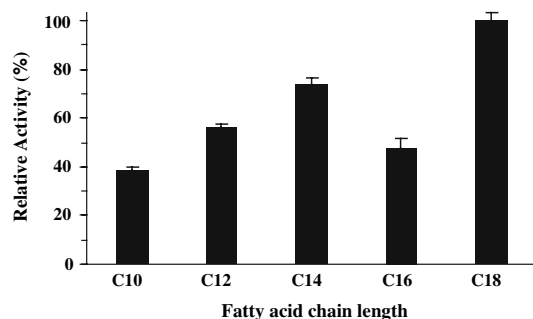
bind Ni<sup>2+</sup> ions by three coordination sites and highly cross-link agarose matrix. On the basis of high affinity and selectivity of ProBond™ nickel-chelating resin for 6 $\times$  His-tagged recombinant fusion protein, SILipA recombinant protein was purified using the ProBond™ nickel-chelating resin. The crude protein was loaded onto a Ni<sup>2+</sup>-IDA column to remove most of the contaminants. The eluted fractions contained almost one single band with a size of approximately 68 kDa, which referred to the size of the cloned SILipA with fusion of the His tag and c-myc epitope. The molecular mass of purified protein was lower than that of crude protein, which was probably caused by the removal of many modifications from protein in the process of purification. After concentrating the protein preparations, the actual concentration of the purified lipases was determined by measuring the absorbance at 595 nm. The purified protein was analyzed by SDS-PAGE (Fig. 6). The arrow shows the position of the expressed lipase protein.

#### Enzymic Specificity Activity Assays

Lipase activity and substrate specificity were usually measured by a spectrophotometric method with *p*-nitrophenyl esters as substrates [17]. Substrate activity and specificity of recombinant enzyme toward various *p*-nitrophenyl esters differing in chain length of the acyl moiety from C10 to C18 was studied in this report. As shown in Fig. 7, the recombinant lipase preferred middle acyl group *p*-NPL (C12) and *p*-NPM (C14) to *p*-NPC (C10), but showed the highest activity toward *p*-NPS



**Fig. 6** SDS-PAGE analysis of the purified SILipA protein (lane L). Lane M showed middle molecular weight protein markers (Watson, Shanghai, China)



**Fig. 7** Relative specific activity of lipase toward *p*-nitrophenyl esters. The substrates *para*-nitrophenyl caprate (*p*-NPC), *para*-nitrophenyl laurate (*p*-NPL), *para*-nitrophenyl myristate (*p*-NPM), *para*-nitrophenyl palmitate (*p*-NPP), and *para*-nitrophenyl stearate (*p*-NPS) were respectively denoted with the acyl chain length of C10, C12, C14, C16, and C18. Each value is the average of three experiments with standard deviation  $\leq 10$

(C18). The recombinant lipase shows similarities to *Streptococcus* and *Bacillus* lipases, in preference, long chain substrates to short chain substrate [32]. The lipase exhibits preference to long chain substrate, which will make the enzyme an excellent candidate for biocatalytic applications, such as synthesis of specific chemicals and food agents for improving flavor.

With methanol as substrate, the recombinant lipase SILipA did not have transesterification activity against plant oil in the non-polar organic solvent of *n*-heptane, suggesting that SILipA preferred cleavage of esters to transesterification (data not shown).

## Conclusions

This is the first report on cloning and expression of a *S. liquefaciens* lipase in *P. pastoris*. The useful characteristics of this enzyme are that these show preference to long chain *p*-NPS substrate, which makes the lipase a specific candidate for biocatalytic applications, such as synthesis of specific chemicals and food agents for improving flavor. Further investigations of SILipA including methanol feeding rates and non-toxic methanol concentration are required to optimize the product yield.

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