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Isolation and functional expression of a novel lipase gene isolated directly from oil-contaminated soil

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A lipase gene *SR1* encoding an extracellular lipase was isolated from oil-contaminated soil and expressed in *Escherichia coli*. The gene contained a 1845-bp reading frame and encoded a 615-amino-acid lipase protein. The mature part of the lipase was expressed with an N-terminal histidine tag in *E. coli* BL21, purified and characterized biochemically. The results showed that the purified lipase combines the properties of *Pseudomonas chlororaphis* and other *Serratia* lipases characterized so far. Its optimum pH and temperature for hydrolysis activity was pH 5.5–8.0 and 37°C respectively. The enzyme showed high preference for short chain substrates (556.3 ± 2.8 U/μg for C10 fatty acid oil) and surprisingly it also displayed high activity for long-chain fatty acid. The deduced lipase SR1 protein is probably from *Serratia,* and is organized as a prepro-protein and belongs to the GX-SXG lipase family.

Keywords: lipase, gene cloning, hydrolysis activity, meta-genomic library

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INTRODUCTION

Lipases (EC 3.1.1.3) play an important role in lipid metabolism as biocatalysts for lipolytic reactions. Many lipases have been purified and cloned from bacteria and fungi such as *Burkholderia glumae, Candida cylindracea, C. rugosa, C. albicans, C. deformans, Geotrichum candidum, Trichosporon fermentans, Pseudomonas fragi,* and *Staphylococcus aureus* (Gilbert *et al.,* 1991; Akastuka *et al.,* 1994; Lee *et al.,* 1994; Choo *et al.,* 1998; Gupta *et al.,* 2004; Kim *et al.,* 2007; Shu *et al.,* 2007; Liu *et al.,* 2008). They can catalyze lipolytic, alcoholysis, acidolysis, esterification or trans-esterification reactions under different conditions (Yan *et al.,* 2007). They exhibit broad substrate specificity and degrade acyl *p*-nitrophenyl esters, tweens, and phospholipids with positional, stereo and chain-length selectivity (Jaeger *et al.,* 1994; 1999). Lipases have been widely used in many fields including food processes, biomedical or chemical industry, leather industry and cosmetics industry as well as environment management (Jaeger *et al.,* 1994; 1998; 1999; Hanson *et al.,* 2006).

Lipases from extreme environments have recently attracted attention because they are heat/cold-tolerant, show organic solvents tolerance and special catalytic activities (Feller *et al.,* 1991; Jiang *et al.,* 2006; Liu *et al.,* 2008; Kiran *et al.*, 2008). Mazzafera *et al.* (1996) have reported that a lipase from *Serratia marcescens* isolated from soil under coffee cultivation can degrade caffeine and related methylxanthines. Several lipases (such as those from *Yarrowia lipolytica* CL180 or *Aureobasidium pullulans* HN2-3) from cold marine environment displayed high lipolytic activity (Kim *et al.,* 2007; Liu *et al.,* 2008). In salmon grease-contaminated soil, most lipases originated from the *Myroide, Arthrobacter, Bacillus* and *Serratia* genus (Ciudad *et al.,* 2008). Margesin *et al.* (1999) have found that monitoring of soil microbial lipase activity is a valuable indicator of diesel oil biodegradation in freshly contaminated soils. These results indicate that there is a great diversity of lipases in oil-contaminated environment and these lipases could be used in oil biodegradation (Jaeger *et al.,* 1999; Cervantes-González *et al.,* 2004).

In this report, we described the cloning of a lipase gene from oil-contaminated soil and the purification of recombinant his-tagged mature form of the lipase. Biochemical characterization of this lipase revealed that its properties differ markedly from those of other reported lipases. It had a high hydrolytic activity towards peanut oil without trans-esterification activity. Homology alignment showed high homology of this lipase with other *Serratia* lipases except ten nucleotide substitutions. This lipase gene probably originated from *Serratia* and the encoded enzyme could find use in environment management.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* DH5α and DH10B were used for cloning of the lipase gene. The plasmids pMD18-T and pET-32a were used as gene cloning and protein expression vectors, respectively. For the construction of expression plasmids, *E. coli* strain BL21 was used.

Lipase gene cloning and sequence analysis. Oil-contaminated soil was collected from the ground of a waste oil and sewage treatment area in the cafeteria of Shanghai Jiaotong University (Shanghai, China). Meta-genomic DNA of oil-contaminated soil was isolated according to the method of Gabor (2004). Crude DNA was purified by agarose gel electrophoresis using QIAEXII Gel Extraction Kit (USA). Purified DNA was then partially digested with *Sau*3AI (Promega, Mannheim, Germany) and separated by electrophoresis on

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1% agarose gel. DNA fragments of 2–5 kb were then ligated into *Bam*HI digested pMD18 (Takara, Japan) plasmid DNA using T4 DNA Ligase (Promega, USA). The recombinant plasmids were electroporated into *E. coli* DH10B to generate a meta-genomic library (Henne *et al.,* 2000; Helen *et al.,* 2005; Lee *et al.,* 2006).

The Gene Images random priming labeling kit and Gene Images CDP-Star detection kit (Amersham Pharmarcia, USA) were used for probe labeling and library screening. A fragment derived from conserved regions (GXSXG, GDSL, 200 bp) of a multiple alignment of different bacterial lipases was used as the probe to screen the DNA library. The insert of the plasmid isolated was sequenced with M13 forward and reverse primers. The entire nucleotide sequence of the lipase gene, named as *SR1* has been deposited in the GenBank database and assigned the Accession No. bankit 1162518. The putative protein and DNA sequence of the *SR1* gene was blasted on the web (http://ncbi.nlm.nih.gov). The alignment analysis was performed with ClustalW (Thompson *et al.,* 1994).

Protein expression and purification. To characterize the putative lipase encoded by the *SR1* gene, it was expressed in *E. coli* BL21. *Candida antarctica* lipase B (CALB) has been employed in organic synthesis with a broad range of applications (Anderson *et al.,* 1998). So, the *CALB* gene was used as a control. The open reading frame of the lipase gene was amplified from *pMD18-T*. The PCR product was digested with *Nco*I/*Eco*RI and subcloned into the plasmid pET32-a. The plasmid of a positive clone was then transformed into *E. coli* BL21.

Lipase activity was detected by using rhodamine B-triolein agar plates by directly applying 4 mM IPTG-induced cells to the plates with 50 mg/l ampicillin to screen for lipase protein expressing clones. Lipase activity was observed when the colonies became deeply red after 24 h of cultivation at 37°C.

A positive clone was inoculated into liquid LB with 50 mg/l ampicillin. Isopropyl-β-D-thiogalactopyranoside (final concentration 4 mM) was added when OD_{600} of cultures reached 0.6. The cells were further cultured for 3 h and harvested by centrifugation at 4000 rpm at 4°C for 10 min. The pellets were re-suspended in 50 mM Tris/HCl (pH 7.5) containing 0.5 M NaCl and 1 mM phenylmethylsulfonylfluoride and lysed by sonication. Following centrifugation at 15000 rpm at 4°C for 30 min, the supernatant was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to assess the expression of lipase protein. The supernatant of a highly expressing clone was then loaded onto a His-tag Sepharose-Fast-Flow gel column (Amersham-Pharmacia Biotech, USA) and washed with a gradient of 100 mM to 1 M wash solution to elute pure recombinant protein for activity analysis.

Analysis of enzyme activities. Trans-esterification assay of lipase protein: The trans-esterification activity towards soybean oil was assayed in a reaction containing 0.339 g of soybean oil and 0.034 g of enzyme; 3.6 ml n-heptane and 48.5 μl methanol was added to one-three volume by batch every 6 h. The reaction was performed at 37°C for 24 h with 150-rpm shaking (Freedman *et al.,* 1984).

To follow the trans-esterification reaction, the fatty acid methyl ester (FAME) products were analyzed by an Agilent 6890N gas chromatograph equipped with an HP-5 5% phenyl methyl siloxane capillary column (30 m × 0.32 mm × 0.25 μm), an automatic injector 7376 and a FID detector (Palo Alto, CA, USA). The injector and detector temperatures were 180°C and 300°C, respectively. The sample analyses were performed at least three times.

Lipase hydrolysis activity analysis. Lipase hydrolysis activity was measured according to the method described by Winkler and Stuckmann (1979). *p*-Nitrophenyl caprate was the substrate in this study. The assay mixture contained 89 μl of Tris/HCl buffer (50 mM, pH 7.5), 10 μl of substrate solution (25 mM *p*-nitrophenyl caprate in DMSO), and 1 μl of enzyme solution (0.001 mg). The reaction time was allowed to proceed for 10 min and was then stopped by adding 100 μl of ethanol. The A_{410} of liberated *p*-nitrophenol was measured with *p*-nitrophenol as a standard. One unit was defined as the mount of enzyme required to release 1 μmol of *p*-nitrophenol per min under assay conditions (Luisa-Rúa *et al.,* 1997).

The substrate specificity of the lipase towards different *p*-nitrophenyl esters (*p*NP-) was determined by a spectrophotometric method with *p*-nitrophenyl caprate, laurate, myristate, palmitate and stearate as substrates. Liberation of *p*NP was measured at room temperature by reading the absorbance at 410 nm (Schmidt-Dannert *et al.,* 1994; Zhang *et al.,* 2003).

pH and thermo-stability analysis of SR1 enzyme. The effects of pH on the recombinant lipase activity were determined by incubating the recombinant enzyme between pH 4.0–10.0. The buffers used in this study were 0.1 M citric acid/sodium citrate buffer (pH 4.0–5.5), 0.1 M potassium phosphate buffer (pH 6.0–8.0), and 0.1 M glycine/NaOH buffer (pH 8.5–10). The recombinant enzyme had been incubated in the buffer for 2 h at 37°C. After incubation, the reaction buffer was cooled on ice to determine the remaining enzyme activities. The remaining activities of the recombinant lipase were measured immediately after this treatment with the standard method as described above.

The optimal temperature for activity of the enzyme was determined at 25, 30, 35, 40, 50, 60 and 70°C in the same buffer at pH 7.5. The thermo-stability of the recombinant enzyme was tested by pre-incubating the enzyme at different temperatures for 30 min. The residual enzyme activity was measured as described above.

RESULTS

Lipase gene cloning from oil-containment soil

In order to clone the lipase gene, oil-containment soil DNA was partially digested with *Sau*3AI which resulted in fragments ranging in size from 2 to 5 kb as determined by electrophoresis in 0.4% (w/v) agarose gels. The digested DNA was then ligated with *Bam*HI-digested pMD18 vector (Takara, Japan) to generate an environmental meta-genomic DNA library with 6.07×10^5 clones. Using lipase-homologous fragment as the probe to screen this library, a clone with a 2133-bp insert containing a complete lipase gene was isolated after three rounds' of screening. By DNA sequencing, a 2133-nucleotide insert was determined (Fig. 1) and named *SR1* gene.

Sequence analysis of *SR1* gene

Bioinformatic analysis showed that the *SR1* gene contained an open reading frame from nucleotide 132 to 1977, encoding a protein of 615 amino-acids (Fig. 1). A database comparison using this amino-acid sequence revealed a significant similarity with various *Pseudomonas*

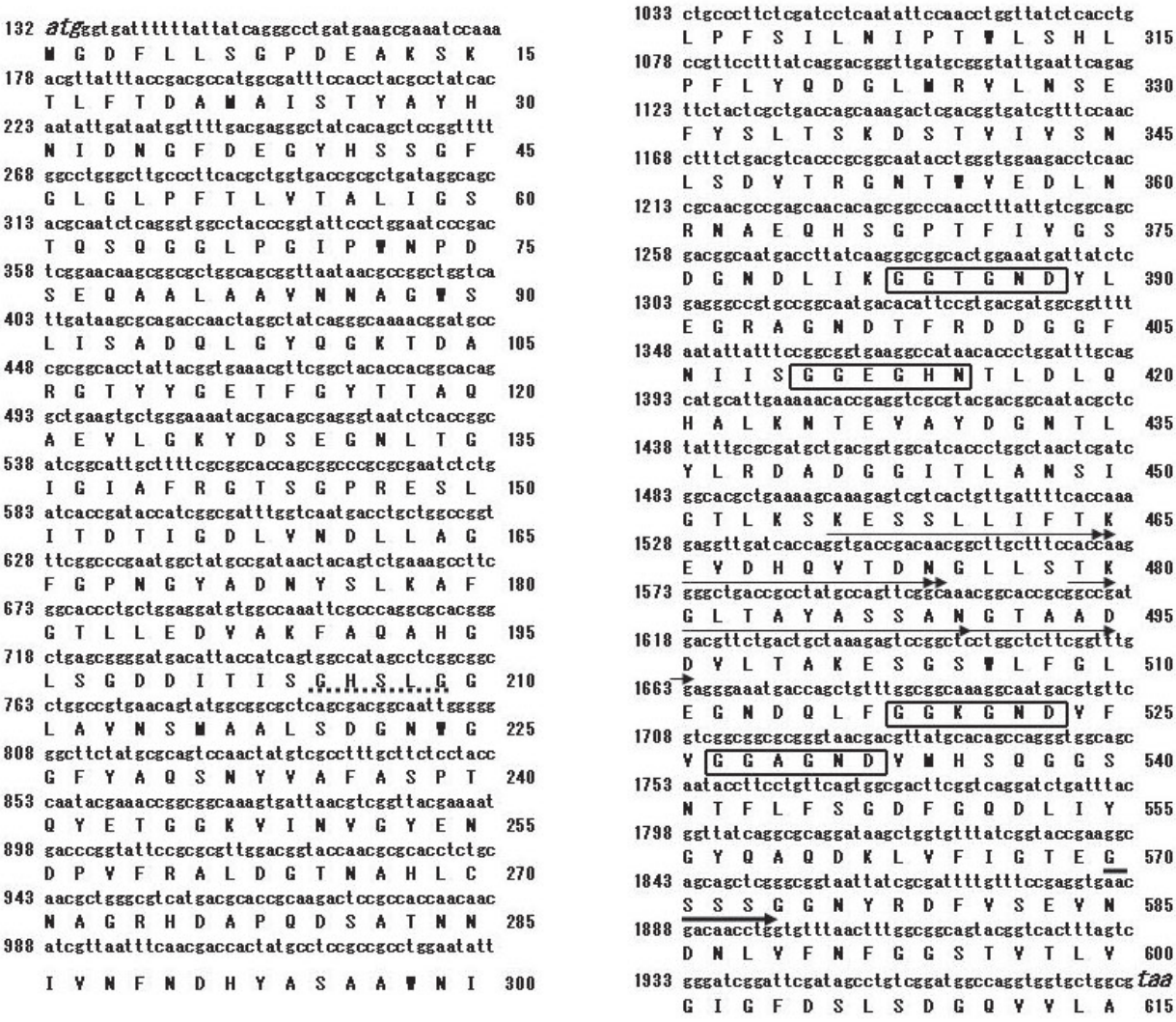

Figure 1. Full-length DNA sequence and deduced amino-acid sequences of SR1 lipase protein
Nucleotide positions are given on the left side of the sequence in the 5′ to 3′ orientation. The start codon ATG and the stop codon TAG are in italics. The deduced amino-acid sequence is shown beneath the nucleotide sequence and the amino acids are numbered on the right side. The motif G×S×G is underlined **....** Another GSSSG similar to the motif G×S×G is underlined with a thick arrow. Glycine-rich consensus motifs are boxed. A putative charged amphipathic region is double-arrowed. The sequence of *SR1* lipase gene has been deposited at GenBank (Acc. No. bankit1162518).

and *Serratia* lipases. The highest overall identities were found with the lipase ZP_01534977 (*Serratia proteamaculans)*, NP_929565 (*Photorhabdus luminescens sub* sp.), and AAD22743 (*Pseudomonas chlororaphis*), sharing 98%, 95% and 90% identity, respectively. Compared with our previously reported *SLlipA* gene from *Serratia liquefaciens* S33 DB-1, the *SR1* gene has six mutations at its N-terminal and four nucleotides mutations at its C-terminal (Yao *et al.,* 2008).

Direct alignment with other lipases revealed that SR1 lipase has no signal peptide at its N-terminus. The enzyme has four glycine-rich repeated motifs. This motif is always present in the RTX toxin family and in the proteins secreted by the class I secretion pathway (Li *et al.,* 1995). The amino-acid sequence derived from the nucleotide sequence contained the lipase-specific active-site consensus, Gly-X-Ser-X-Gly, found in the majority of bacterial and eukaryotic lipases. The Ser residue of this motif is the key element of the active site of fatty acid-esterification lipases. Based on these characteristics, the SR1 lipase was predicted to belong to the GXSXG family of lipases (Fig. 2).

Expression and purification of SR1 lipase

The open reading frame encoding mature SR1 lipase was amplified and cloned into *pET-32a.* The expression plasmid *pET-32a* containing the *SR1* gene was then transformed into *E. coli* BL21 strain to express the SR1 protein. The positive clones were confirmed by PCR. One hundred and twenty-eight positive clones were then inoculated into a selective medium to characterize SR1 lipase expression. The clones expressing active SR1 lipase were determined by a red halo on Rhodamine-Triglyceride-Agarose plate by adding 1 μl of supertant.

One clone which became deeply red after incubating for 24 h at 37°C was used in the subsequent analysis of enzyme activities (Fig. 3). After inducing with 4 mM IPTG for 4 h, supernatants from different clones and a control (a clone with *pET-32a*) were used in protein analysis. Total proteins extracted from induced LB

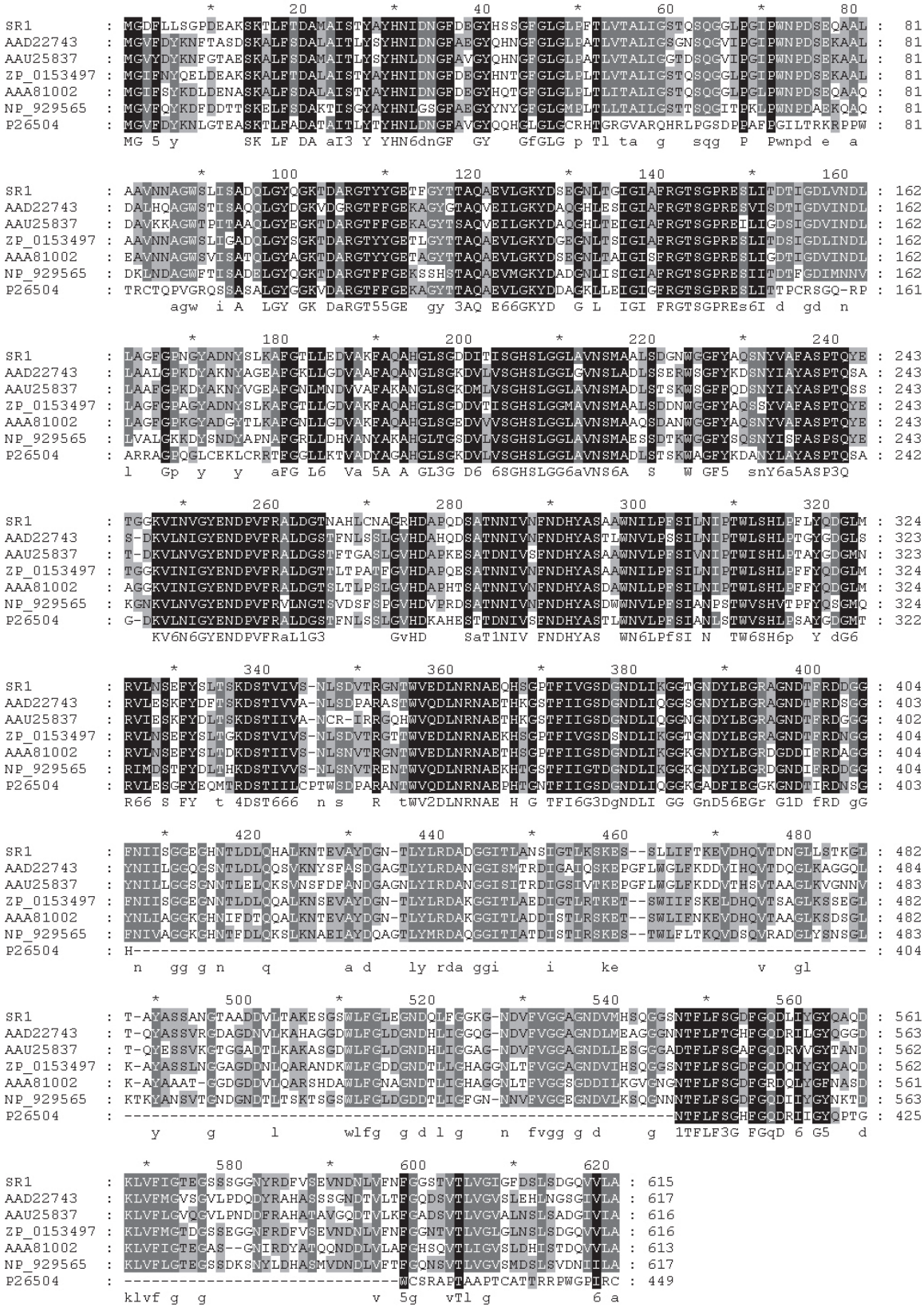

Figure 2. Alignment of amino-acid sequences of lipases from different bacterium and fungal accessions
The predicted amino-acid sequence of SR1 lipase was aligned with lipase polypeptide sequences from *Serratia proteamaculans* (ZP_01534977), *S. marcescens* (AAA81002), *Photorhabdus luminescens sub* sp. (NP_929565), *Pseudomonas chlororaphis* (AAD22743), *P. fluorescens* (AAU258370), *Psychrobacter* sp. *PRwf-1* (ZP_01271540) using the Clustal multiple alignment program. Gaps to optimize alignments are designated by dashes (-). Asterisks (*) indicate consensus amino-acid identity among all organisms. Dots (. or :) indicate positions of conservative amino acid replacement. Black blocks show that these amino-acids were extremely conserved in all the proteins. Gray blocks show conserved amino acids. The numbers at the top or at the right column refer to the position in the amino-acid sequence.

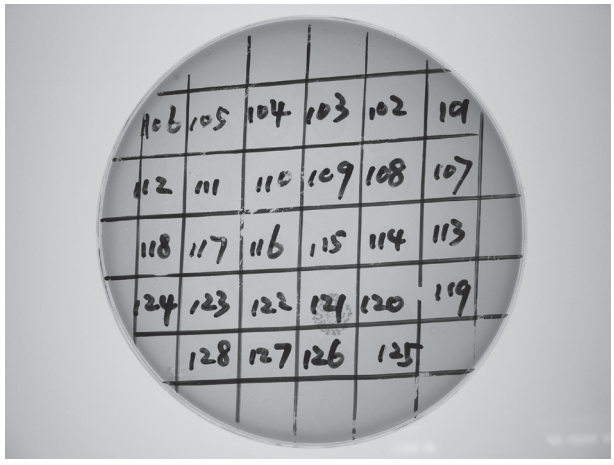

Figure 3. Rhodamine B-triglyceride agar plate showing different recombinant clones
Five microliter of culture supernatant (sterile filtered) induced by IPTG was poured into each preformed hole, and the plate was incubated at 37°C overnight. Deeply red halo (No.121) indicates the production of recombinant lipase.

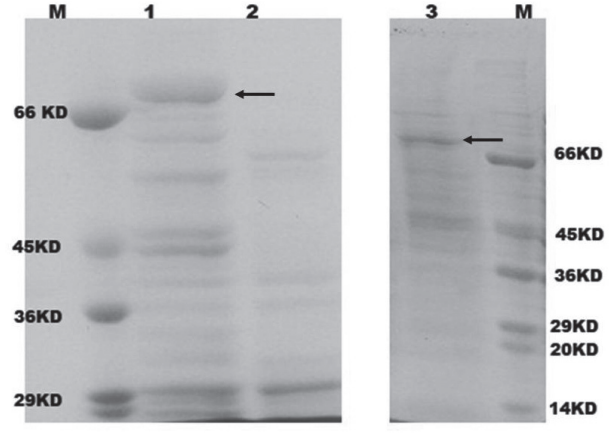

Figure 4. SDS/PAGE analysis of total secreted protein after induction with 4 mM IPTG
Culture supernatants were harvested at various time intervals, precipitated with 10% trichloroacetic acid, washed with acetone, freeze-dried and then analyzed by SDS/PAGE. The band intensities of Coomassie Blue-stained gel is shown. Lane M, middle molecular mass protein markers (Watson, Shanghai, China). Lanes 1, 3, 4 h after induction with 4 mM IPTG. Lane 2, before induction.

were separated on a 15% SDS/PAGE gel. The results showed that those plasmids (containing *SR1* gene) encoded the His-tagged mature lipase with a calculated molecular mass of 65 kDa (Fig. 4).

Table 1. Spectrophotometric assay of substrate activity and specificity toward different *p*-nitrophenyl esters (*p*NP-).

Enzyme (U/μg)	Substrate[a]				
	p-NPC$_{10}$	*p*-NPC$_{12}$	*p*-NPC$_{14}$	*p*-NPC$_{16}$	*p*-NPC$_{18}$
CALB	20.2±1.0	19.3±0.8	20.2±1.2	19.6±0.7	18.1±1.1
SR1	556.3±2.8	359.6±1.6	325.4±3.0	88.9±1.5	26.0±1.1
S33DB1[b]	14.4±0.5	19.8±0.6	27.6±0.3	17.3±0.4	36.1±0.4

[a]Each value is the average of three experiments; [b]This data refers to the paper (Yao *et al.*, 2006).

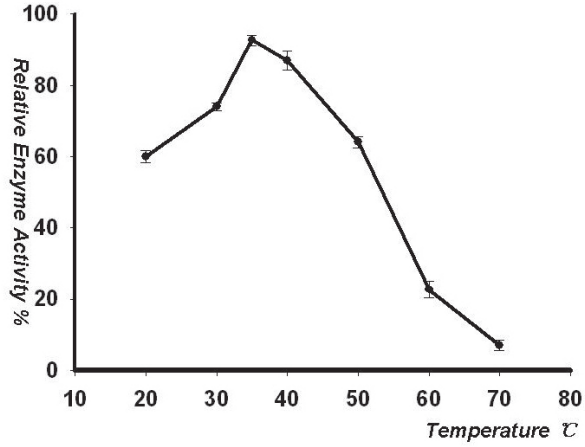

Figure 5. Effect of temperature on hydrolytic activity of the recombinant lipase
Temperature stability of the recombinant lipase was tested by pre-incubating the enzyme at different temperatures (20, 30, 35, 40, 50, 60 and 70°C) for 30 min, the residual activity was then measured as described in Material and Method. Non-incubated sample was used a reference to calculate the residual activity. Data are given as means ±S.E., n=3.

Hydrolysis and trans-esterification by the lipase

A spectrophotometric assay of enzyme activity and specificity toward different *p*-nitrophenyl esters by purified SR1 lipase was performed. The result showed that SR1 hydrolytic abilities were different for different acyl-chain length of *p*NPS. When the acyl chain length of the *p*-nitrophenyl esters increased from C_{10} to C_{18}, the activity decreased accordingly. The hydrolytic activity of SR1 was 556.30 ± 2.8 U/μg when *p*NPC10 was used as the substrate, and 25.99 ± 1.1 U/μg for *p*NPC18 substrate (Table 1). Compared with control CALB and SSDB1, hydrolytic activity of SR1 was about 28 and 40 times higher, respectively, when *p*NPC10 was used as the substrate. This result suggested that the crude recombinant lipase has potential application in digestion of lipids especially medium-chain length fatty acids.

pH and thermo-stability of SR1 lipase

The thermo-stability was investigated by pre-incubating the purified enzyme in the same buffer as described in materials and methods for 30 min at 20°C to 70°C and then the mixture was used to determine its hydrolytic activity. The purified recombinant lipase activity was the highest at 35°C–40°C (Fig. 5). As shown in Fig. 5, the residual lipase activity still amounted to 97% and 95% of the control after treatment at 20°C and 30°C for 30 min, respectively. Figure 5 also reveals that the enzyme was inactivated rapidly at temperatures higher than 50°C and was inactivated totally at 70°C within 30 min.

The purified recombinant lipase activity was measured at various pHs in buffers with the same ionic concentrations. pH stability was tested by a 2-h pre-incubation of the crude recombinant lipase in appropriate buffers that had the same ionic concentrations at different pH values ranging from 4.0 to 10.0 at 37°C. Our results (Fig. 6) show that the maximum activity was observed at pH 7.5–8.0. The lipase activity decreased immediately when the pH

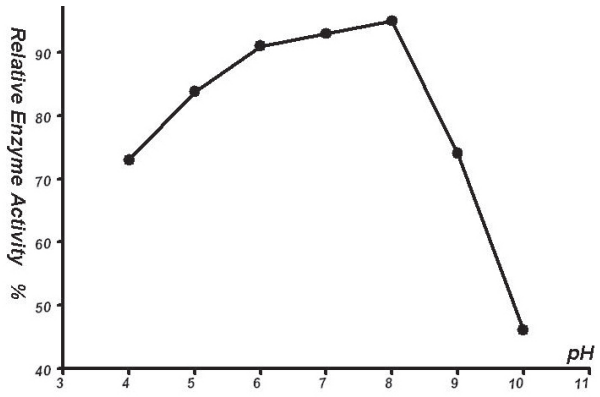

Figure 6. Effect of pH on hydrolytic activity of the recombinant lipase

pH Stability was tested by a 2-h pre-incubation of the recombinant enzyme in appropriate buffers that had the same ionic concentrations at different pH values ranging from 4.0 to 10.0 at 37°C. The remaining activity was measured immediately after this treatment with the standard method as mentioned in the text. Values are given as mean ± S.E., n = 3.

exceed 8.0, while the enzyme was stable in the pH range from 5.5 to 8.0.

DISCUSSION

The aim of this study was to clone a lipase with a high hydrolytic activity to degrade oil in the contaminated niche. Since in oil-contaminated soil a large microbial diversity is encountered, it is difficult to obtain pure microorganism cultures (Jiang *et al.,* 2006). Meta-genomic library was then constructed and used to screen lipase gene (Helen *et al.,* 2005). The *SR1* gene encoding an extracellular lipase cloned from the meta-genomic library was composed of 2133 bp in length while the ORF was consisted of 1845 bp. The predicted protein consisted of 615 amino acids with a calculated molecular mass of 65 kDa. Nucleotide sequence alignment showed that *SR1* gene has 98% identity with *S. marcescens* lipase except ten single-nucleotide substitutions. The amino- acid sequence was also found to be more closely related to *S. marcescens* than to the lipases from other bacteria like *Pseudomonas* (not shown). In this study, we also cloned five lipase genes from *Pseudomonas* (four lipase genes) and *Serratia,* besides the *SR1* gene. These genes are the same as those lipase genes described earlier (Gao *et al.,* 1998; Jinwal *et al.,* 2003; Gangwar *et al.,* 2009).

Sequence analysis showed that the SR1 protein lacks an N-signal peptides and has ABC-type secretion system. Compared with other reported lipases from *Serratia*, the SR1 protein has similar structure including glycine-rich motif and aspartate boxes which contributed to its extracellular secretion. Sequence analysis also showed that there is only one cysteine in the SR1 protein, which is similar to other reported lipase (Li *et al.,* 1995). Cysteines are often involved in the formation of disulfide bonds in proteins, and proteins without cysteines are generally more flexible because of the lack of disulfide bonds (Li *et al.,* 1995). This characteristic is important for protein secretion of extracellular bacterial proteins, which also readily allow the conformational change that accompanies interfacial activation.

The SR1 protein had a high hydrolytic activity without a trans-esterfication activity. SR1 had not significant chain-specificity, although it had a preference for short chain substrates (C10–C12 fatty acid oil). It also displayed high hydrolytic activity for long-chain fatty acids. Compared with our previously reported SLLipA from *Serratia liquefaciens* S33 DB-1, the SR1 protein had a much higher hydrolytic activity for fatty acids of different length acyl. This characteristic may result from continued selection pressure, favoring mutations enhancing the catalytic efficiency of the lipase enzyme in the contaminated environment. Optimal pH and temperature of the recombinant SR1 lipase was 5–8°C and 35–40°C, respectively. This may be due to the environment from which the gene was isolated. In the Southern China like Shanghai the soil is acidic and temperature is always high (20°C–40°C). Several lipases which have been isolated from soil recently favor acidic or alkaline environment, depending on the soil pH. Their catalytic characteristics may accelerate the process of fatty acid degradation in the soil (Lee *et al.,* 2004; Ni *et al.,* 2007).

In conclusion, we reported a lipase gene encoding an enzyme with non-specific hydrolysis activity. It could be applied as lipase biosensor for digestion of lipids in the food and medicine, and for oil-contamination treatment. The biological source of the *SR1* gene will be determined in a future study.

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