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Highly soluble expression and molecular characterization of an organic solvent-stable and thermotolerant lipase originating from the metagenome

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ABSTRACT

A novel organic solvent-stable and thermotolerant lipase gene (designated ostl28) was cloned from a metagenomic library and overexpressed in *Escherichia coli* BL21 (DE3) in soluble form. OSTL28 contained 262 amino acids with relative molecular mass 30.1 kDa and isoelectric point 9.7. The optimum pH and temperature of the OSTL28 were 7.5 and 60 °C, respectively. OSTL28 was stable in the pH range of 4.5–9.5 and at temperatures below 65 °C. The enzyme could hydrolyze a wide range of ρ-nitrophenyl esters, but its best substrate is ρ-nitrophenyl laurate with the highest activity of 236 U/mg (54,000 U/L). The recombinant OSTL28 was highly resisted to organic solvents, especially glycerol and methanol. The metal ions, with the exception of Hg^{2+} and Ag^{+}, did not have any influence on enzyme activity, whereas non-ionic surfactants and Al^{3+} slightly activated the enzyme. These features indicate that it is a potential biocatalyst for biodiesel production.

1. Introduction

Lipases (triacylglycerol hydrolase; EC 3.1.1.3) are an important group of biotechnologically valuable enzymes distributed in a wide range of organisms from bacteria to animals [1]. They hydrolyze acylglycerides and other fatty acid esters in aqueous environments and synthesize them in non-aqueous environments [2]. In general, lipases hydrolyze long-chain acylglycerols (≥C10) [3], whereas esterases have the ability to hydrolyze ester substrates with short-chain fatty esters (≤C10). The stereo-, regio- and enantiospecific behaviours of lipase-catalyzed reactions in organic solvents have caused tremendous interest among scientists and industrialists because of their potential applications in various industries such as food, dairy, pharmaceutical, detergents, textile, biodiesel, and cosmetic, and in synthesis of fine chemicals, agrochemicals, and new polymeric materials [4–7].

Although reactions catalyzed by lipases in the presence of organic solvents have many advantages [8], enzymes, including lipases, are not stable in organic solvents. Because organic solvents may alter the structure and activity of enzymes that usually function in an aqueous environment. In order to alleviate this drawback, several strategies, such as chemical modification, immobilization, and protein engineering, have been employed for the stabilization of enzymes for use in organic solvents [2,7,9,10]. Alternatively, instead of modifying enzymes to increase their solvent stability, it would be more desirable to screen for naturally evolved solvent-tolerant enzymes for application in non-aqueous enzymatic synthesis [11].

Microbial lipases are currently receiving considerable attention due to their diversity in catalytic activity, high yield and low cost production, and relative ease of genetic manipulation. Particularly some lipases from solvent tolerant bacteria are stable in organic solvents such as *Pseudomonas aeruginosa* LST-03 [12], *Bacillus sphaericus* 205y [13], *Bacillus megaterium* [14], *Staphylococcus saprophyticus* M36 [15], *Bacillus cepacia* ST-200 [16], *Burkholderia multivorans* V2 [17] and *Pseudomonas fluorescens* P21 [5]. As mentioned above, some organic solvent-stable lipases were purified and characterized, and a few genes of organic solvent-stable lipases were cloned and expressed [18,19]. However, all these original investigations relied on pure culture analysis for determination of the organic solvent-stable lipases. In contrast, the search strategies for novel organic solvent-stable lipases can be achieved using uncultured methods.

To avoid the limitation of pure culture, metagenomics has been in the spotlight since the 1990s [20]. More than 90% of bacteria in the environment cannot be cultured using conventional methods [21,22]. The metagenomic approach, direct cloning of DNA from environmental samples and thereby accessing the potential of unculturable organisms, has proven to be a powerful tool for the isolation of novel biocatalyst genes. Current computed estimates of soil diversity are in the range of a million species per 1 g of soil [23], and metagenomic strategy has led to the discovery of quite diverse and novel enzymes for a broad range of applications [24]. Metagenome-derived enzymes include polysaccharide

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degrading/modifying enzymes, esterases, nitrilases, amidases, proteases, dehydrogenases, and oxidoreductases [25]. Many of them displayed special enzymatic characteristics and high stability under extreme conditions, and thus had potential applications in industries. Although several cold-adapted or thermostable lipase genes were cloned by metagenomic approach from different environments (compost, intertidal flat sediment and hot spring) [26–29], hitherto there have been no reports on organic solvent-stable and thermotolerant lipases obtained via metagenomic strategies.

In this study, a metagenomic library from topsoil of Jiang Han oil field of Hubei province in China was constructed for the screening of clones with lipolytic activity. One novel lipase (OSTL28) was overexpressed in *Escherichia coli* BL21 (DE3), purified and characterized. This enzyme displayed excellent tolerance of organic solvents and thermostability, and thus is considered as a good candidate of lipases for industry after further study.

2. Materials and methods

2.1. Strains, plasmids and culture conditions

E. coli TOP10 and pZErO-2 (Invitrogen, USA) were used as the host and vector for the metagenomic library construction, respectively. The pET-28a (+) (Novagen, USA) was used as an expression vector to produce the target protein. *E. coli* BL21 (DE3) was used as a host for expression of the OSTL28 gene under the control of the T7 promoter. *E. coli* transformants were grown at 37 °C in Luria–Bertani (LB) broth containing kanamycin (50 μg/mL), unless otherwise stated.

2.2. DNA manipulation

Routine DNA manipulations were carried out according to standard techniques. Restriction enzymes and DNA polymerase were purchased from Takara (Dalian, China). Each enzyme was used according to the recommendations of the manufacturer. DNA ligation was performed using T4 DNA ligase (Fermentas). Plasmids were prepared from *E. coli* by using a QIAGEN miniplasmid purification kit according to the manufacturer's instructions (QIAGEN Inc.). DNA fragments were isolated from agarose gels by using a QIAquick gel extraction kit (QIAGEN Inc.). Electroporation was performed with a Gene-Pulser II electroporation apparatus (Bio-Rad Laboratories).

2.3. DNA sequencing and sequence analysis

Sequencing reactions were performed using a BigDye sequencing kit according to the instructions of the manufacturer. DNA sequencing of positive plasmid clone (pZD8-1) was analyzed on ABI 377 DNA sequencer. Sequence manipulation, open reading frame (ORF) searches, and multiple alignments among similar enzymes were conducted with DNASTAR software. Database homology search was performed with BLAST program provided by NCBI. The conserved patterns of discrete amino acid sequences related enzymes were analyzed by Clustal W program.

2.4. DNA extraction from soil samples

The topsoil samples (5–10 cm) from oil field were used for the experiments. Samples were collected and stored at −80 °C until the DNA extraction was performed. Extraction of the total genomic DNA from soil was performed using with the Fast DNA® SPIN kit for soil according to the recommendations of suppliers (MP Biomedicals, USA).

2.5. Construction of genomic libraries and screening for lipase activity gene

The metagenomic library was constructed from environmental DNA isolated from oil field soil using protocols provided by the manufacturer. DNA fragments (3–8 kb) obtained after partial *Sau3*AI digestion were ligated into the *Bam*HI restriction site of the pZErO-2 vector, which had been previously digested with *Bam*HI. *E. coli* TOP10 was transformed by electroporation with the ligated fragments and plated onto Luria–Bertani (LB) agar plates containing kanamycin (50 μg/mL), 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), tributyrin (0.5%, v/v) and the fluorescent dye rhodamine B (0.001%, w/v). Orange fluorescent halos around lipase-positive *E. coli* strains could be seen when these plates were exposed to UV light of 350 nm [30]. To avoid the isolation of false-positive clones, plasmid DNA was isolated from the positive clones obtained in the initial screening and retransformed, and the new clones were examined on the same type of indicator plates for lipase activity. The 3 colonies were further tested for their abilities to hydrolyze *ρ*-nitrophenyl laurate. The only one transformant (pZD8-1) with lipase activity was obtained and reconfirmed.

2.6. Cloning and overexpression and purification of OSTL28

The putative lipase gene was amplified from the pZD8-1 plasmid by using the primers and to introduce *Bam*HI and *Hin*dIII restriction sites for cloning in the pET-28a (+). The following primers were used: fw (5′- GCCATGGCTGATATCGGATCCATGACCATTATTTTAC; the *Bam*HI cutting site is underlined) and rv (5′-TTCAAGTTCAGACTCAAGCTTTCAAAACCGCTGCGGT; the *Hin*dIII cutting site is underlined). The PCR product was digested with *Bam*HI/*Hin*dIII, and then ligated into *Bam*HI/*Hin*dIII digested expression vector pET-28a (+), and transformed into *E. coli* BL21 (DE3) (Stratagene). The *E. coli* transformed with this plasmid was plated on LB agar containing kanamycin (50 μg/mL). The transformant was grown in a 250-mL flask containing 50 mL LB medium supplemented with kanamycin (50 μg/mL) at 37 °C until the cell concentration reached OD_{600} of 0.8, and 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) of final concentration to induce target protein expression. After incubation at 30 °C for 6 h with shaking at 220 rpm, cells were harvested by centrifugation (6000 × *g* for 20 min at 4 °C) and resuspended in 50 mM Tris–HCl buffer (pH 7.5) and disrupted by sonication for 10 s in ice-water bath. The cell debris was removed by centrifugation at 10,000 × *g* for 5 min at 4 °C. The clear supernatant was collected and the recombinant OSTL28 was purified with his-tag affinity column. The supernatant was applied to a Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography column (Qiagen, Hilden, Germany), equilibrated with buffer A (10 mM Tris–HCl, 500 mM NaCl, 5 mM imidazole, pH 8) at a flow rate of 0.5 mL/min. The bound protein was eluted with buffer B (10 mM Tris–HCl, 500 mM NaCl, 500 mM imidazole, pH 8) at 4 °C. The purity of the enzyme was estimated by 10% SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel electrophoresis) in the eluted fractions. Protein concentration was determined by the method of Bradford, and bovine serum albumin (Sigma) was used as standard for calibration [31]. Enzyme samples were stored at −20 °C until further use.

2.7. Determination of molecular mass and isoelectric point

The molecular mass of the denatured protein was determined by 10% SDS-PAGE. Proteins were stained with Coomassie brilliant blue G. The molecular mass of the enzyme subunit was estimated using protein marker as standards. Rabbit muscle phosphorylase B (97,200 Da), bovine serum albumin (66,409 Da), ovalbumin (44,287 Da), carbonic anhydrase (29,000 Da) were used as the

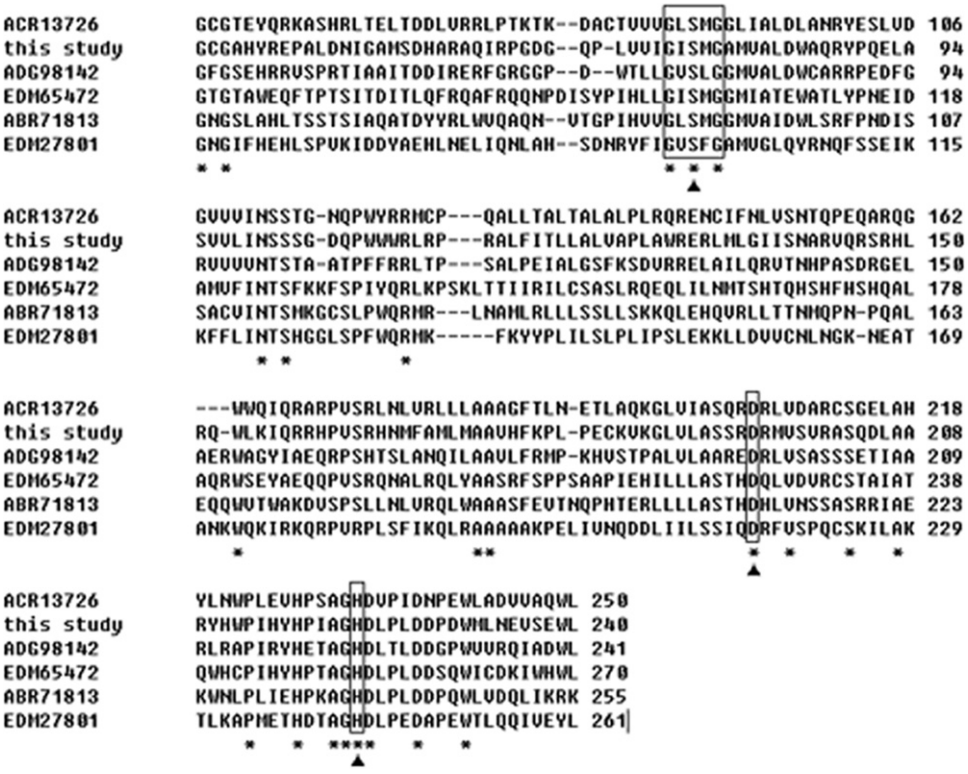

Fig. 1. Multiple amino acid sequence alignment of OSTL28. Multiple alignment of the partial Amino acid sequences containing the conserved motifs of G × S × G and putative catalytic triad resides of alpha/beta hydrolase family proteins. Except for OSTL28 (this study), other protein sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov). The accession numbers of the aligned sequences are for the following organisms: ACR13726, alpha/beta hydrolase family protein from *Teredinibacter turnerae* T7901; ADG98142, alpha/beta hydrolase fold protein from *Segniliparus rotundus* DSM44985; EDM65472, hydrolase, alpha/beta fold family from *Moritella* sp. PE36; ABR71813, alpha/beta hydrolase fold from *Marinomonas* sp. MWYL1; EDM27801, hydrolase, alpha/beta fold family protein from *Lentisphaera araneosa* HTCC2155. The alignment was carried out using the Clustal W method. The open boxes indicate amino acid resides belonging to the putative catalytic triad resides, triangles denote the active site. The same amino acid resides are marked by (*).

reference proteins. Isoelectric point (pI) was estimated by PAGE with 6.25% Ampholine (pH 3.5–10) in a gel rod (0.5 by 10 cm) using a kit for Isoelecric Focusing Calibration according to recommendations by the supplier.

2.8. Substrate specificity of OSTL28

Substrate specificity against different ρ-nitrophenyl esters was determined using ρ-nitrophenyl acetate (C2), ρ-nitrophenyl butyrate (C4), ρ-nitrophenyl caprylate (C6), ρ-nitrophenyl caproate (C8), ρ-nitrophenyl decanoate (C10), ρ-nitrophenyl laurate (C12), ρ-nitrophenyl myristate (C14), ρ-nitrophenyl palmitate (C16), and ρ-nitrophenylstearate (C18) as substrates, the activity was then measured described above at the final substrate concentration of 0.3 mg/mL separately.

2.9. Analysis of lipase OSTL28 activity

The lipase activity against ρ-nitrophenyl laurate (ρNPL) was determined by measuring the amount of ρ-nitrophenol released by lipase-catalyzed hydrolysis. The substrate (ρNPL) (3 mg) with final concentration of 0.3 mg/mL was dissolved in 1 mL of isopropanol and mixed with 9 mL of 50 mM Tris–HCl buffer (pH 7.5) containing gum arabic (0.1%, w/v) and Triton X-100 (0.6%, w/v). After preincubation for 5 min at 60 °C, the reaction was initiated by adding enzyme into the reaction mixture. The hydrolysis of substrate was performed at 60 °C for 10 min in 50 mM Tris–HCl (pH 7.5). The production of ρ-nitrophenol was monitored spectrophotometrically at 405 nm by Labsystems Dragon Wellscan MK3. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of ρ-nitrophenol per minute under these conditions. In each measurement, the effect of nonenzymatic hydrolysis of substrates was taken into consideration and subtracted from the value measured when the enzyme was added.

2.10. Kinetic assay

The purified lipase was incubated with various concentrations of ρ-nitrophenyl laurate. The final concentration ranged from 1.0 mM to 10.0 mM in Tris–HCl buffer (7.5). The kinetic constants were calculated by fitting the initial rate data into the Michaelis–Menten equation using the GraFit software (version 6; Erithacus Software Ltd., Horley, UK).

2.11. Effect of pH and temperature on activity and stability of OSTL28

The optimum pH of OSTL28 was measured using ρ-nitrophenyl laurate as a substrate at 60 °C. The buffers (at a final concentration of 50 mM) used for the measurement were as below: citric acid–NaOH buffer (pH 3.5–5.5); potassium phosphate buffer (pH 5.0–7.0); Tris–HCl buffer (pH 6.5–9.0), glycine–NaOH buffer (pH 8.5–10.0). Overlapping pH values were used to verify that there were no buffer effects on substrate hydrolysis. The optimum temperature was determined analogously by measuring esterase activity at pH 7.5 in the temperature range of 30–80 °C. The pH stability was tested after incubation of the purified enzyme for 24 h at 30 °C in the above different buffers. Temperature stability was measured by preincubation of the purified enzyme for different time in 50 mM Tris–HCl buffer (pH 7.5) at different temperatures.

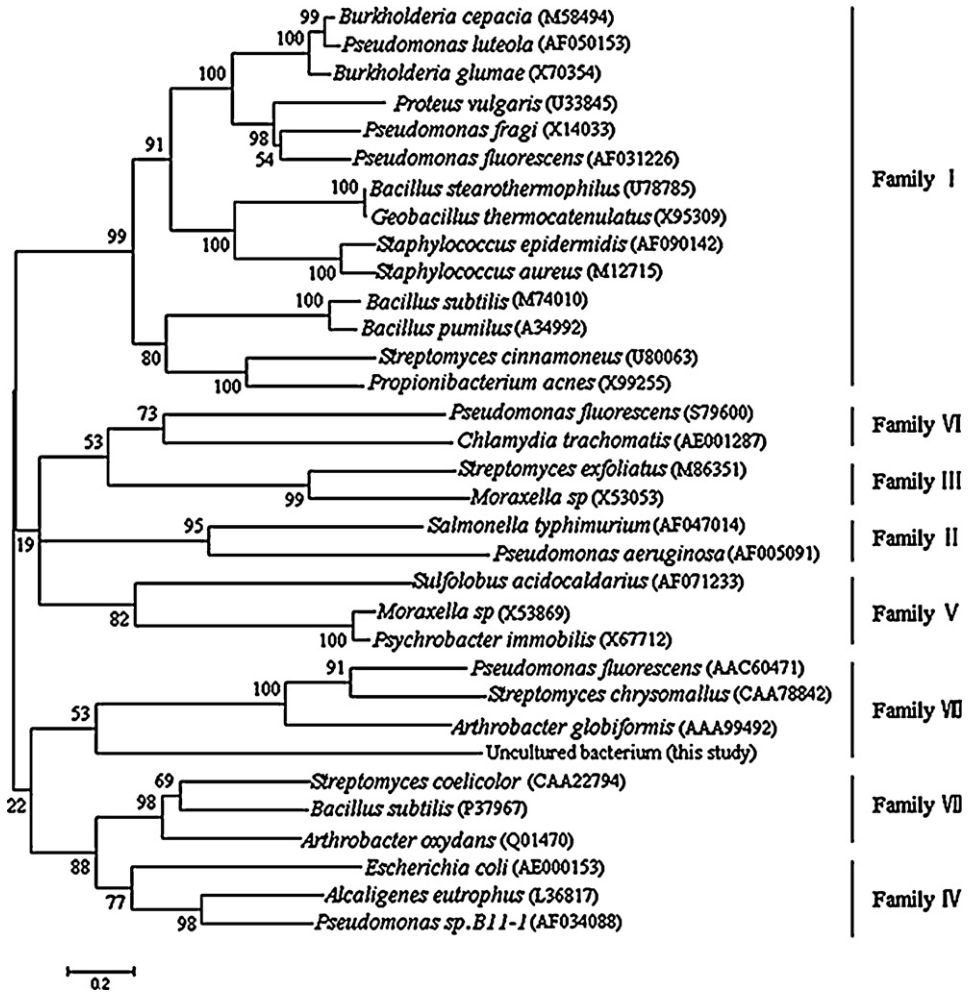

Fig. 2. Phylogenetic analysis of OSTL28, its homologues and other lipases. Phylogenetic relationship of OSTL28 and lipase/esterase proteins of 8 different families was performed using the program MEGALIGN (DNASTAR, Madison, WI). Except for OSTL28, the other protein sequences for previously identified families of bacterial lipolytic and esterolytic enzymes were retrieved from GenBank (http://www.ncbi.nlm.nih.gov). The numbers at node indicate the bootstrap percentages of 1000 resamples. The units at the bottom of the tree indicate the number of substitution events.

2.12. Effect of various reagents on activity and stability of OSTL28

The effects of various chemicals on OSTL28 activity were investigated by preincubating for 24 h at 30 °C, containing 1% (v/v) of the detergents (Triton X-100, Tween 80 and SDS) and 1 mM of $CaCl_2$, $CdCl_2$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, $MnCl_2$, $AgNO_3$, $HgCl_2$, $AlCl_3$, EDTA, 1,10 phenanthroline, phenylmethanesulfonyl fluoride (PMSF). The residual activity was then measured described above and expressed as a percentage of the activity obtained in the absence of the added compound.

2.13. Effect of organic solvents on stability of OSTL28

The effect of various organic solvents at 25% (v/v) concentration on the stability of OSTL28 was investigated. The reaction mixture in screw capped tubes was incubated at 30 °C under shaking condition (150 rpm). After 24 h, the remaining activity was measured as described above. Remaining activity was assayed under the standard condition and expressed as the lipase activity with distilled water added to the reaction mixture instead of solvents.

3. Results and discussion

3.1. Cloning, and sequence analysis of novel lipase OSTL28 gene

Approximately 83,000 transformants of *E. coli* TOP10 containing the pZErO-2-based metagenomic DNA library were generated. Restriction analysis of 100 randomly selected clones showed that the average insert size was about 3.9 kb. The only one lipase-positive strain from 83,000 transformants was selected, as indicated by an orange fluorescent halo surrounding the colony, and could hydrolyze tributyrin. Nucleotide sequence analysis of the about 4.9 kb DNA insert in this clone showed the presence of an open reading frame (789 bp) encoding a full-length lipase gene (ostl28). The nucleotide sequence data reported here was submitted to GenBank with the accession number HQ204320. The DNA length of ostl28 is smaller than others derived from the metagenome [28,32]. In some respects, the lipase gene ostl28 is easier to be manipulated in follow-up experiments.

The lipase encoding by ostl28 gene contained 262 amino acids with a predicted molecular mass of 30.1 kDa. The putative amino acid sequence of OSTL28 was used to perform a BLAST program provided by the National Centre for Biotechnology Information and Swissprot databases. This search showed the moderate identity

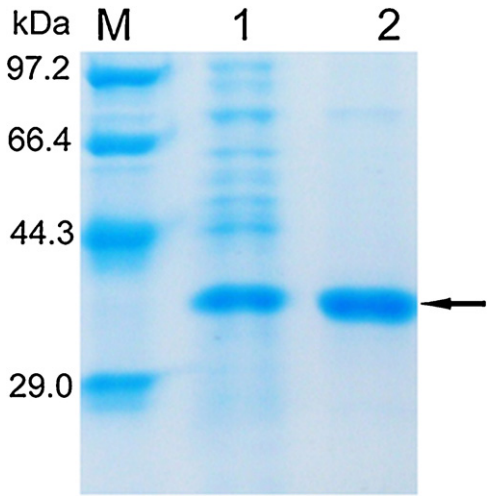

Fig. 3. Purification of OSTL28. SDS-PAGE analysis of the purified His6-tagged mature OSTL28. Arrowhead indicates purified target protein (lane2); supernatant of *E. coli* BL21 (DE3) cell lysates (lane 1); protein markers (lane M) stained with Coomassie blue.

(<50%) between OSTL28 and other esterases/lipases, the highest with alpha/beta hydrolase family protein from *Teredinibacter turnerae* (101/249, 40% identity), followed by alpha/beta fold family protein from *Marinobacter algicola* (102/248, 41% identity), alpha/beta fold family hydrolase from *Saccharophagus degradans* 2-40 (95/233, 40% identity), and alpha/beta fold family from *Moritella* sp. PE36 (92/249, 36% identity). Multiple alignments of the deduced amino acids of OSTL28 with the homologous proteins are presented in Fig. 1. The putative protein contained the conserved active site motif of the pentapeptide G × S × G found in most bacterial and eucaryotic serine hydrolases (residues from 74 to 78) with a serine acting as the catalytic nucleophile, a conserved aspartate or glutamate and a histidine, together constituting a catalytic triad sequence (Ser 143, Asp195, His 222). An unrooted phylogenetic tree based on the amino acid sequences was constructed in order to further verify the evolutionary relationship of the OSTL28 protein to other known lipase/esterase proteins, and 32 bacterial lipase/esterase proteins representing 8 different families were selected for the phylogenetic tree analysis. As shown in Fig. 2, the OSTL28 protein belongs to Family VIII.

3.2. Heterologous expression and purification of OSTL28

The PCR product of ostl28 gene was purified and digested with *Bam*HI/*Hin*dIII to ligate it into a T7 RNA polymerase derived *E. coli* expression vector of pET 28a (+) and was expressed in *E. coli* BL21 (DE3) at 30 °C with 0.5 mM IPTG induction for 8 h. The cells were harvested and disrupted by sonication in ice-water bath. The recombinant OSTL28 was expressed in soluble form with no inclusion bodies in Fig. 3. The highest expression level of OSTL28 (OD_{600} = 2.8) was about 0.23 mg/mL and its content in total soluble protein reached up to 42.7% according to Quantity One software (Bio-Rad laboratories Inc., Hercules, USA) for protein band visualization.

In recent years, a variety of lipase-encoding genes from different species have been cloned and sequenced. Unfortunately, heterogeneous expressions of many lipases are hampered by the fact that a lipase chaperone is necessary for correctly folding to the active form [33]. In contrast, we assume that OSTL28 exhibits an intrinsic folding capability in vitro without chaperon protein.

The recombinant OSTL28 was purified by Ni-NTA chromatography. SDS-PAGE of the purified protein indicated a single protein band with molecular mass of about 34 kDa, which was higher than

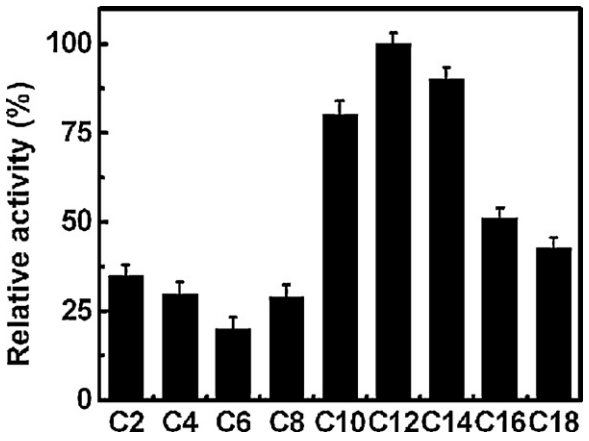

Fig. 4. Substrate specificity of OSTL28. Substrate specificity of OSTL28 for ρ-nitrophenyl esters of various fatty acids. This lipase displayed the highest activity of 236 U/mg (54,000 U/L) with ρ-nitrophenyl laurate (C12) as a substrate, and the activity was taken as 100%.

Table 1
Kinetic characterization of OSTL28.

Substrate	K_m (μM)	k_{cat} (S^{-1})
ρ-Nitrophenyl acetate	69.8 ± 3.5	41.7 ± 2.9
ρ-Nitrophenyl butyrate	74.4 ± 2.9	29.6 ± 1.2
ρ-Nitrophenyl caprylate	130.2 ± 7.1	10.2 ± 0.8
ρ-Nitrophenyl caproate	76.1 ± 4.3	34.4 ± 1.2
ρ-Nitrophenyl decanoate	13.6 ± 3.0	68.9 ± 5.1
ρ-Nitrophenyl laurate	5.1 ± 0.5	99 ± 4.6
ρ-Nitrophenyl myristate	8.3 ± 0.2	88.2 ± 3.2
ρ-Nitrophenyl palmitate	45.9 ± 2.7	58.8 ± 2.2
ρ-Nitrophenyl lstearate	57.3 ± 3.9	43.4 ± 4.5

that of putative OSTL28 protein due to N-terminal fusion peptide of 32 amino acids (about 4 kDa) in Fig. 3. This result is in agreement with the calculated molecular mass of the predicted amino acid sequence (30.1 kDa). The relative molecular mass of native enzyme estimated by gel filtration on a calibrated column of Sephacryl 200 HR was 30 kDa. Hence, it is assumed that OSTL28 is a monomer. The pI value was estimated to be 9.7.

3.3. Biochemical characterization of recombinant lipase OSTL28

3.3.1. Substrate specificity and activity of OSTL28

The substrate specificity toward ρ-nitrophenyl esters of various fatty acids is shown in Fig. 4. Recombinant lipase OSTL28 showed the highest activity with ρ-nitrophenyl laurate (C12) among the ρ-nitrophenyl esters examined, but it exhibited very low levels of activity toward ρ-nitrophenyl esters with acyl groups shorter than C8 or longer than C16, similar finding was reported by other lipases. For example, the highest hydrolytic activities of thermostable lipases from the extreme thermophilic anaerobic bacteria *Thermoanaerobacter thermohydrosulfuricus SOL1* and *Caldanaerobacter subterraneus* [34], different from esterases that can only catalyze a variety of hydrolytic or synthetic reactions of short-chain fatty acid esters. From these results, it is very clear that OSTL28 is a true lipase.

Moreover, when acyl chain lengths were 12, its k_{cat} and K_m values was 99 S^{-1} and 5.1 μM, respectively. When acyl chain lengths were between 6 and 12, its k_{cat} values increased, while K_m values decreased with increasing acyl chain length. In contrast, when acyl chain lengths were in the range of 14–18, the k_{cat} values decreased, whereas K_m values remained constant with increasing acyl chain length (Table 1). This kind of behavior characterizes that OSTL28

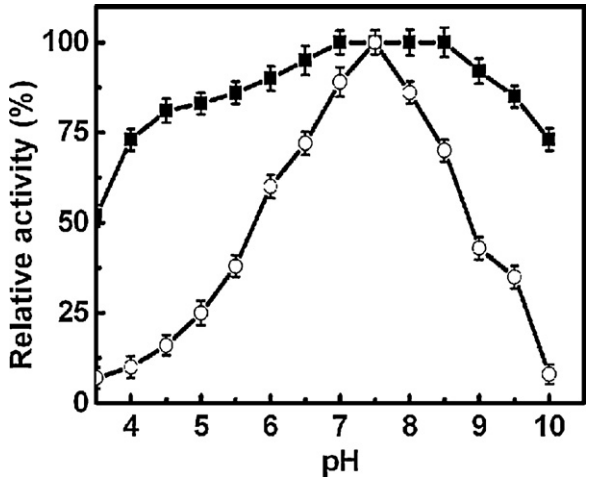

Fig. 5. Effect of pH on enzyme activity. Effect of pH on activity (○) and stability (■) of recombinant OSTL28.

displayed the highest catalytic efficiency toward ρ-nitrophenyl laurate (C12).

At 60 °C, this lipase displayed the highest activity of 236 U/mg (54,000 U/L) with ρ-nitrophenyl laurate (C12) as a substrate, while 189 U/mg (ρ-nitrophenyl decanoate), 210 U/mg (ρ-nitrophenyl myristate), and 117 U/mg (ρ-nitrophenyl palmitate). It was much higher than lipases from *T. thermohydrosulfuricus* SOL1 and *C. subterraneus* [34], but slightly lower than low-temperature lipase from *Psychrotrophic Pseudomonas* sp. Strain KB700A with ρ-nitrophenyl palmitate as substrate [35]. These above showed that the inducible lipase displayed high activity in heterogeneous hosts.

3.3.2. Effect of pH and temperature on lipase activity and stability

The effect of pH and temperature over recombinant lipase OSTL28 activity was determined. The enzyme showed an optimal activity at approximately pH 7.5 (Fig. 5). corresponding to that of other bacterial lipases such as *P. aeruginosa* LST-03 (Lip3) (pH 7.0) [18], *P. aeruginosa* LX1 (pH 7.0) [36], and *Geobacillus stearothermophilus* strain-5 (pH 8.0) [37], while the optimum pH values for the lipase activities of *Staphylococcus aureus* and *Acinetobacter* sp. EH28 were 9.5 and 10, respectively [38,39]. OSTL28 was found to be stable in the pH range of 4.5–9.5, and retained more than 80% of maximal activity (Fig. 5). Therefore, the high activity and stability of OSTL28 at broad pH conditions showed its usefulness in a range of biotechnological applications.

As shown in Fig. 6a, the optimal temperature for recombinant lipase OSTL28 is 60 °C, equal to that of *G. stearothermophilus* strain-5 lipase (60 °C) [37], lower than those recorded from *Thermoanaerobacter thermohyfrosulfuricus* (75 °C) [34] and *Thermosyntropha lipolytica* (96 °C) [1], and higher than those reported from *P. aeruginosa* LST-03 (37 °C) and *P. aeruginosa* LX1 (40 °C) [12,36]. The enzyme was fully stable below 55 °C, and the total activity retained more than 60% after 5 h incubation at this temperature (Fig. 6b). Interestingly, even incubating at 65 °C for 2 h, it still retained as much as 50% of its total enzyme activity, in comparison with thermostable lipases from other sources such as *Amycolatopsis mediterranei* DSM 43304 and *Burkholderia cepacia* [40,41], worse than lipases from *T. lipolytica* [1] and *T. thermohyfrosulfuricus* [34]. According to these results, OSTL28 could be classified in range of thermotolerant or moderately thermostable lipases. According to research that most of the industrial processes operate at a temperature exceeding 45 °C, lipase should be active and stable at a temperature around 50 °C [42]. At 45 °C, LipA and LipB from *T. lipolytica* had no activity, and the lipase from *T. thermohyfrosulfuricus* exhibited less than 30% activity [1,34]. While the novel lipase

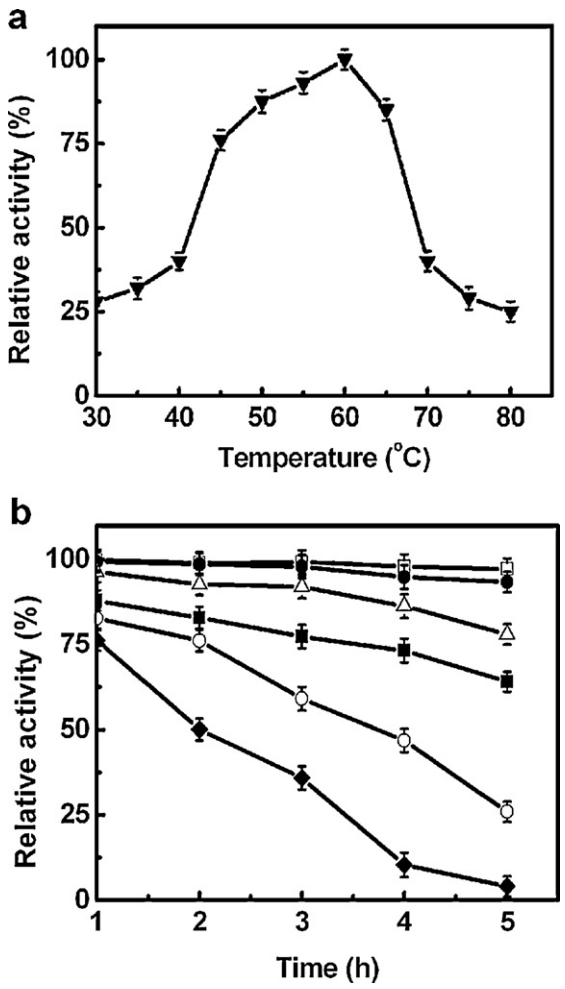

Fig. 6. Effect of temperature on enzyme activity. (a) Effect of temperature on activity (▼) of recombinant OSTL28. (b) Effect of temperature on stability of recombinant OSTL28. The purified enzyme was preincubated at 30 °C (□), 40 °C (●), 50 °C (△), 55 °C (■), 60 °C (○) and 65 °C (♦) for 1 h, 2 h, 3 h, 4 h and 5 h.

Table 2
Effect of the various substance on activity of OSTL28.

Substance	Relative activity (%)
None	100
1 mM $CdCl_2$	113 ± 5.1
1 mM $CaCl_2$	115 ± 5.6
1 mM $AlCl_3$	157 ± 6.8
1 mM $MnCl_2$	92 ± 4.3
1 mM $MgCl_2$	94 ± 4.2
1 mM $CuSO_4$	92 ± 4.1
1 mM $ZnSO_4$	93 ± 4.1
1 mM $AgNO_3$	8 ± 0.3
1 mM $HgCl_2$	6 ± 0.2
10 mM EDTA	96 ± 4.5
10 mM 1,10 phenanthroline	92 ± 4.1
1 mM PMSF	95.8 ± 3.1
SDS (1%, w/v)	5.8 ± 0.1
Triton X-100 (1%, w/v)	114 ± 4.8
Tween 80 (1%, w/v)	123 ± 5.3

OSTL28 had 75% activity. So this lipase OSTL28 is suitable for application at this condition.

3.3.3. Effect of metal ions and surfactants on OSTL28

The effect of various reagents is shown in Table 2. The enzyme activity of OSTL28 was obviously promoted by 1 mM Al^{3+} (157%). The presence of Cu^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Cd^{2+} and Ca^{2+} did not have

Table 3
Effect of the various solvent on activity of OSTL28.

Solvent	Log *P*	Relative activity (%)
None	0	100
Glycerol	−1.76	148 ± 6.3
DMSO	−1.35	91 ± 4.6
DMF	−1	68 ± 3.8
Methanol	−0.76	92 ± 1.3
Ethanol	−0.24	64 ± 3.2
Acetone	−0.23	57 ± 3.1
Tert-butanol	0.18	39 ± 2.1
Benzene	2	68 ± 3.3
Toluene	2.5	76 ± 4.2
Petroleum ether	3	105 ± 4.5
n-Hexane	3.5	127 ± 6.1
n-Dodecane	6.6	113 ± 5.4
n-Tetradecane	7.6	113 ± 5.1
n-Hexadecane	8.8	118 ± 5.8

any influence on enzyme activity at 1.0 mM, while the presence of Hg^{2+} and Ag^{+} caused complete inhibition at 1.0 mM. Although there are many reports describing the activation effect of Ca^{2+} on enzyme activity due to conserved calcium-binding site formed by two conserved aspartic acid resides near the active-site in numerous lipases [36]. To our knowledge, this is the first report on the activation effect of Al^{3+} on lipase activity.

Ionic surfactant SDS (1%, w/v) showed complete inhibition, whereas a slight increase of lipase activity was observed by non-ionic surfactant Triton X-100 (114%) and Tween 80 (123%) at the concentration of 1% (w/v) due to enhancement of substrate accessibility. The metal chelating agents EDTA and 1,10-phenanthroline at the concentration of 1 mM had no significant effect on the enzyme activity (96%, 92%), suggesting that the enzyme was not a metalloenzyme. Interestingly, the lipase activity is not affected by 1 mM phenylmethylsulfonyl fluoride (PMSF), suggesting it may possess a lid structure, which could eliminate the inhibition effect, similar result was reported by other esterases/lipases [43].

3.3.4. Stability of OSTL28 in solvents

In present study, effects of various solvents on the enzyme activity were examined by preincubating the enzyme with solvents (25%, v/v) in 50 mM Tris–HCl buffer (pH 7.5) at 30 °C, 150 rpm for 24 h and then measuring the residual activities of the enzyme under standard condition. The results are shown in Table 3. The highest activity (148%) compared to that of the control was observed in glycerol. In contrast, lipase activity from *P. aeruginosa* LST-03 and *Bjerkandera adusta* R59 were inhibited by glycerol [12,44]. More than 90% of its maximal activity was retained in presence of dimethyl sulfoxide (DMSO) and methanol, whereas residual activity was 68, 64, and 57% in the presence of dimethylformamide (DMF), ethanol and acetone, respectively. The enzyme activity was slightly increased by 10–27% in the presence of *n*-hexane, *n*-decane, *n*-tetradecane, and n-hexadecane, similar to that of lipases from *B. adusta* R59 [44] and different from lipases from *G. stearothermophilus* and *S. saprophyticus* M36 [15,37]. The 39–76% of the original activity was found in the presence of tert-butanol, benzene, and toluene. The same behavior was found with lipases from *P. aeruginosa* LST-03 and *P. fluorescens* P21 [5,12]. Thus, these results showed that OSTL28 has distinct stabilities against various organic solvents which were dependent on the nature of the solvent and its structure. Stability of lipase in organic solvents is important criterion for biocatalyst used in organic synthesis, but most enzymes (including lipases) are not stable in the presence of organic solvents, especially polar organic solvents which are highly toxic to most enzymes. In fact, there are some difficulties in comparing the stable property of various lipases in the presence of organic solvents because of the differences in experimental conditions such as incubation time and temperature, shaking speed, concentration and type of organic solvents in reaction mixture [6]. Lipase-catalyzed methanolysis has attracted considerable attention [16,45,46] as it is considered to be an effective mean of circumventing drawbacks involved in the chemical processes. Unfortunately, lipase such as Novozym 435 is deactivated when more than one-third stochiometric amount of methanol present in the reaction [16,45,47]. However, high stability in the presence of glycerol and methanol (25%, v/v) was an excellent feature of OSTL28 for biodiesel production.

4. Conclusions

In this study, a novel lipase OSTL28 has been cloned and characterized by metagenomic approach. Characteristics of the purified enzyme described here differ from those reported hitherto in at least one of following aspects: molecular mass, pI, pH and temperature optima, substrate specificity and the tolerance to organic solvents. These differences of the isofunctional enzymes suggest diversity in evolution and a spread of lipase genes among different microorganisms. Given its high tolerance to glycerol and methanol, accessibility to non-ionic surfactant, overexpression in soluble form, and broad pH range and thermostability, OSTL28 is a promising candidate for application in nonaqueous biocatalysis, especially biodiesel production. In addition, this study also demonstrates that the metagenomic approach is a useful tool to expand our knowledge of enzyme diversity, especially for bacterial lipases.

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