



A new extracellular thermo-solvent-stable lipase from *Burkholderia ubonensis* SL-4: Identification, characterization and application for biodiesel production



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ABSTRACT

In the present study, a new lipase SL-4 from *Burkholderia ubonensis* SL-4, was purified by 80% ammonium sulphate precipitation, Q Sepharose FF anion exchange and Superdex 75 gel filtration chromatography finally leading to 68.5-fold purification and 13.34% recovery. It had a molecular mass of ca. 33 kDa and the whole gene (1095-bp) was cloned by using degenerate primers. Amino acid sequence analysis revealed that lipase SL-4 is a new member of subfamily I.2 lipases. Lipase SL-4 exhibited optimum activity toward *p*-NP myristate (C14) at pH 8.5 and 65 °C with a K_m of 0.72 mM, a k_{cat} of 391.63 s⁻¹ and a k_{cat}/K_m of 543.93 s⁻¹ mM⁻¹. It had good thermostability at 50 °C and pH 8.5, and could be activated strongly by Ca²⁺ and Mn²⁺, but inhibited by some transition metal ions and EDTA, PMSF, DTT and β-ME. Additionally, lipase SL-4 possessed non-ionic detergent stability and organic solvent stability. When preliminarily employed to catalyze soybean oil for biodiesel production, the liquid lipase SL-4 could attain a conversion ratio of 92.24% in a solvent-free system. These results demonstrate that the new thermo-solvent-stable lipase possesses an attractive potential for biotechnological applications as biocatalyst, especially for biodiesel production.

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1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are ubiquitous carboxylic ester hydrolases that can catalyze both of the hydrolysis and the synthesis of relatively long-chain acylglycerols [1,2]. Although lipases exist widely in nature and are produced by various organisms [3], extracellular bacterial lipases are of considerable importance for industrial applications, serving as food ingredients and detergents for fat degradation, as well as for fine chemical production, paper-making, drug manufacturing and biodiesel production [2], because of their diverse substrate specificities, stereoselectivities, and good stabilities against heat, various detergents and organic solvents [4,5].

According to their molecular biological and biochemical characteristics, bacterial lipolytic enzymes are classified into 14 families [6], and these lipases of family I are grouped into eight subfamilies based on phylogenetical analyses of their amino acid sequences [7,8]. Subfamily I.2 lipase have more than 80% of amino acid

sequence homologies, and they require need a molecular chaperone for secretion via the type II secretion machinery [3,9]. To date, major subfamily I.2 lipases have been characterized in species of the *Pseudomonas* and *Burkholderia* genera [10]. *Burkholderia* lipases are excellent biocatalysts, and in recent decades they have been used in a broad range of industrial applications, including drug manufacturing [11–13], detergent formulation [14], organic synthesis [12,15,16], and bioenergy production [10,17]. However, although many good lipases from *Burkholderia* species have been identified [10,12–16,18,19], the rapid development of modern biochemical industries and increasing numbers of biochemical engineering studies have shown that these lipases are far from optimal. Therefore, isolating more lipases with unique characteristics from *Pseudomonas* and/or *Burkholderia* species is urgently needed and of great importance.

With a shortage of petroleum and an increase of people's consciousness of the need to protect the environment, biodiesel fuels (fatty acid methyl esters, FAMEs) have been regarded as one of the most important substitutes for fossil diesel fuel [10,20]. Commercially, biodiesel is typically produced using chemical methods, including alkaline or acid catalysis [20]. Because of its non-toxicity, environmental friendliness, mild operating conditions, and sim-

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ple procedures for the recovery of FAMEs and the by-product glycerol, enzyme catalyzed biodiesel production by *Pseudomonas* and/or *Burkholderia* lipases has recently drawn wide attention [10,17,21,22]. Thus far, many lipases have been used for biodiesel preparation in their immobilized form, and they have exhibited high biodiesel yields of greater than 90% [23]. Nevertheless, the supports for enzyme immobilization are often expensive and/or sophisticated, and accordingly, this results in a high price and/or a complex immobilization process, which usually affect their industrial applications [20,24]. In addition, the released glycerol could bind to the supports, leading to negative effects on lipase activity and operational stability [25,26]. In contrast, liquid lipases can be produced and sold at a price that is 30–50 times lower than that of immobilized ones, making the whole process more competitive and sustainable [21]. Furthermore, compared with a solvent system, a solvent-free system is simpler to perform, has a lower cost, and is better for the environment [27]. Hence, a lipase that is highly heat stable, tolerant to organic solvents (the short-chain alcohols methanol and ethanol), and which has the potential for biodiesel conversion in a solvent-free system would be a valuable resource.

In this study, the lipase-producing *Burkholderia ubonensis* strain SL-4 was isolated from soil samples from Ma'anshan Forest Park, Wuhan, PR China ($30^{\circ}31'16.11''N$, $114^{\circ}26'32.77''E$). We report the partial purification, identification, gene cloning, and biochemical characterization of a thermo-solvent-stable lipase SL-4 from this strain. Furthermore, we tried to use liquid lipase SL-4 to transesterify soybean oil into biodiesel in a solvent-free system in order to explore a novel lipase from *Burkholderia* strains and achieve an effective biodiesel preparation by using its liquid enzyme.

2. Materials and methods

2.1. Chemicals and materials

Q Sepharose FF column (GE Healthcare, Pittsburgh, PA, USA) and Superdex 75 column (GE Healthcare, Pittsburgh, PA, USA) were purchased from GE Healthcare (Uppsala, Sweden). All *p*-nitrophenyl (*p*-NP) esters and high-performance gas chromatography (HPCG)-grade solvents were got from Sigma-Aldrich (St. Louis, MO, USA). Unstained protein molecular weight marker (*Mr* of 14.4 kDa, 18.4 kDa, 25.0 kDa, 35.0 kDa, 45.0 kDa, 66.2 kDa and 116.0 kDa) was bought from Fermentas Co. as reference (Fermentas, SM0431, Thermo Fisher Scientific, Waltham, MA, USA). All other chemicals used were of analytical grade and were commercially available from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The vector T-Vector pMD19 (Simple) (TaKaRa, Otsu, Japan) was used for gene cloning. Kits for genome extraction, plasmid extraction and gel extraction were purchased from Omega Bio-tek (Norcross, GA, USA). Restriction endonucleases, T4 DNA ligase, and Taq DNA polymerase were all got from TaKaRa. To validate the accuracy of gene insertions, DNA sequencing was performed by Invitrogen Biotechnology Company (Carlsbad, CA, USA). Reference standards of fatty acid methyl esters including methyl tridecanoate, methyl laurate, methyl palmitate, methyl heptadecanoate, methyl stearate, methyl oleate, methyl linoleate, and methyl linolenate were from the Sigma Chemical Co., Ltd. (St. Louis, MO, USA) commercially.

2.2. Sample collection, isolation of lipase producing strains

All soil samples were collected at Ma'anshan Forest Park, Wuhan, PR China ($30^{\circ}31'16.11''N$, $114^{\circ}26'32.77''E$) and processed for laboratory studies within 24 h. One gram of each sample was enriched in 10 mL of enrichment medium (basic M9 salts medium: Na_2HPO_4 12.8 g/L, KH_2PO_4 3.0 g/L, $NaCl$ 0.5 g/L, NH_4Cl 1.0 g/L, $MgSO_4$ 2 mM, $CaCl_2$ 0.1 mM) supplemented with olive oil (2%, v/v)

in 50 mL test tubes, and incubated at $37^{\circ}C$ with shaking at 200 rpm for 2 d. After twice transferring to the fresh enrichment medium, the broth was diluted and spread on the screening plates consisting of basic M9 salts culture, olive oil (2%, v/v) and rhodamine B (1% w/v, Sigma-Aldrich, Germany) for screening lipase-producing microbes. Strains having orange fluorescent zones under UV were regarded as putative lipase producers. A bacterium designated as strain SL-4 showed the largest orange fluorescent zone on the screening plates and was then inoculated into 5 mL Luria-Bertani (LB) broth with 100 mg/mL ampicillin, and incubated at $37^{\circ}C$, 200 rpm overnight for further work on lipase production.

2.3. 16S rDNA gene of strain SL-4 and identification of the strain

To identify the isolated strain SL-4, the 16S rDNA was amplified using the universal primers 27F (5'-AGAGTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCCGCA-3') as described previously [28]. The amplification of 16S rDNA gene was done in PCR Thermal cycler (TaKaRa, Otsu, Japan). The nucleotide sequence of 16S rDNA gene of strain SL-4 was used to conduct BLAST with the non-redundant database (NRDB) of NCBI genbank database. The strain SL-4 was identified by 16S rDNA sequence. Homologous sequences of the 16S sequence were obtained from the National Center for Biotechnology Information (NCBI) database using BLAST, and the phylogenetic tree was constructed by neighbor-joining algorithm using Kimura 2 parameter distance and 1000 replicates in Molecular Evolutionary Genetics Analysis 5.0 software [15,29]. The 16S rDNA sequence of strain SL-4 has been submitted to the GenBank database under the accession numbers KT360943.

2.4. Assay for lipase activity

Lipase activity was measured spectrophotometrically using *p*-nitrophenyl myristate (*p*-NPM) as substrate and formation of *p*-nitrophenol (*p*-NP) was measured at 410 nm in a spectrophotometer (UV-1600 MAPADA, Shanghai) [4,29]. In a standard assay, the total 1 mL reaction system contained 940 μ L of Tris-HCl buffer (50 mM, pH 8.0), 10 μ L of *p*-NP ester (100 mM, a 1 mM substrate final concentration), 40 μ L of ethanol, and 10 μ L of the diluted enzyme solution [4,5]. Unless otherwise stated, the pH and the temperature of the enzyme reaction were kept at 8.0 and $65^{\circ}C$ for 10 min, respectively. The reaction was stopped by chilling on ice. Control reactions without enzyme were utilized to account for any spontaneous hydrolysis of the substrates tested. All enzyme assays were performed in triplicates. One unit of lipase activity (U) was defined as the amount of enzyme releasing one micromole of *p*-NP per minute under the assay conditions.

2.5. Production and partial purification of lipase

The strain SL-4 was cultivated in each of 2-L Erlenmeyer flask containing 1 L of the induced medium (1% w/v sorbitol, 1% w/v tryptone and 2% v/v olive oil on the basis of basic M9 salts medium) and incubated at $37^{\circ}C$ on a rotary shaker at 200 rpm for 96 h. The culture supernatant was collected by centrifugation at 12,000 rpm for 15 min at $4^{\circ}C$. The clear supernatant containing extracellular lipase was regarded as the crude enzyme preparation and precipitated by adding ammonium sulphate (80% saturation) at $4^{\circ}C$ overnight. The pellet thus was harvested by centrifugation at 12,000 rpm for 30 min at $4^{\circ}C$ and re-suspended in a small amount of Tris-HCl buffer (50 mM, pH 8.0) to allow the solubilization of proteins. The precipitate collected was dialyzed over night by keeping dialysis bag with four changes of the buffer overnight. The insoluble proteins were then removed by centrifugation. The supernatant was subjected to filtration using Reusable Bottle-Top Filter (DS0320-5045, Thermo Fisher Scientific Inc.) and then ultrafiltration with

a 10 kDa molecular weight (10 kDa MW) cutoff filter. The protein content and lipase activity were determined after each step. The concentrated lipase preparation was then loaded onto a Q Sepharose FF column (1.6 cm $\Phi \times$ 20 cm) equilibrated with 50 mM Tris–HCl buffer (pH 8.0). The column was washed with two bed volumes of the same buffer. The protein was eluted with the same buffer containing a linear gradient of NaCl from 0 to 1.0 M at flow rate of 1.5 mL/min and 3 mL fractions were collected each time. The fractions showing high lipase activity were pooled, and concentrated in a dialysis bag (10 kDa MW cut-off), and dialyzed against the same Tris–HCl buffer overnight. Then, the dialyzed fraction (concentrated 2 mL) was loaded onto the Superdex 75 gel filtration column (1.6 cm $\Phi \times$ 60 cm) pre-equilibrated with the buffer (20 mM Tris–HCl, 500 mM NaCl, pH 8.0). The aimed proteins were eluted, dialyzed and concentrated by 10 kDa MW cutoff filter before the concentrations were estimated by the method of Bradford with bovine serum albumin (BSA) as the standard [30]. Purified lipase was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli [31].

2.6. Identification and cloning of lipase SL-4

The Coomassie-stained band of the purified lipase SL-4 was excised from the gel and submitted to analysis of trypsin-digested protein fragments by LC/MS/MS analysis (Yanxing, China), and the sequences of the fragments were submitted to the Mascot program for possible identity matching. Since some fragments of the lipase were similar to the lipases from *Burkholderia* sp. species, the degenerate primers including the forward primer BT-F (5'-ATGGCCAGAWCGATGCGWTCCAG-3') and the reverse primer BA-R (5'-TCACRCGCCCGCGAGYTTCARCCGGT-3') were designed based on the nucleotide sequences of the *Burkholderia* sp. lipases. PCR amplification was carried out with the primers by using the genomic DNA of strain SL-4 as template. The PCR product was purified and ligated into a pMD19-T vector (Takara, Ltd.) and then sequenced by Invitrogen Biotechnology Company (Carlsbad, CA, USA). Sequence alignments of DNA and protein sequences were performed using BLASTN and BLASTP, respectively (<http://www.ncbi.nlm.nih.gov/BLAST/>). A phylogenetic analysis was conducted with MEGA 5.0 software using the neighbor-joining method [29]. A bootstrap analysis with 1000 replicates was used to estimate the reliability of the tree. A signal peptide was predicted using the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>) [3]. A multiple sequence alignment was conducted using Clustal W2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and presented using ESPript 2.2 (<http://escript.ibcp.fr/ESPript/ESPript/>) [5]. A three-dimensional structure of the target protein was constructed by SWISS-MODEL (<http://swissmodel.expasy.org/>) and presented using PyMOL 2.7 [32]. The quality of model was evaluated by PROCHECK (<http://nihserver.mbi.ucla.edu/SAVES/>) [11]. The nucleotide sequence of strain SL-4 has been submitted to the GenBank database under the accession numbers KT360944.

2.7. Substrate specificity and kinetic study

To investigate the substrate specificity of lipase SL-4, activity was determined using different *p*-nitrophenyl (*p*-NP) esters of various chain lengths (Sigma-Aldrich): *p*-NP acetate (C2), *p*-NP butyrate (C4), *p*-NP caprylate (C8), *p*-NP decanoate (C10), *p*-NP laurate (C12), *p*-NP myristate (C14) and *p*-NP palmitate (C16) as substrates as described previously [29]. A stock solution of each *p*-NP ester was prepared in acetonitrile/isopropanol (1:4, v/v) at the same mole number (100 mM). In addition, activity of the purified lipase was also measured by titration of free fatty acids released by the hydrolysis of olive oil using the pH stat method [32]. The mea-

surements were conducted in triplicates. One unit was defined as the amount of enzyme liberating one micromole of fatty acid per minute. The kinetic parameters (K_m , V_{max} , k_{cat} and k_{cat}/K_m) were determined by using different concentrations (0.1, 0.3, 0.5, 1, 2, 3 mM) of *p*-NPM as substrate according to Lineweaver–Burk plots [5,33] in 50 mM Tris–HCl (pH 8.5) at 65 °C. Lineweaver–Burk plot is the most useful expression of enzymatic kinetics that results in a smaller [S] value.

2.8. Effect of temperature and pH on the activity and stability of lipase SL-4

The optimum temperature and thermostability of the purified lipase were tested. The activity was determined by incubating the reaction mixture at various temperatures from 15 to 100 °C at 50 mM Tris–HCl buffer (pH 8.0) for 10 min. For thermostability measurement, the purified enzyme was pre-incubated in 50 mM Tris–HCl buffer (pH 8.0) at respective temperature from 50 to 90 °C with an interval of 5 °C for 0–24 h with an interval of 30 min before assay. The initial activity was taken as 100% and the residual activity after incubation of the purified enzyme at various temperatures was expressed as percentage of the initial activity, and measured under the conditions of 50 mM Tris–HCl buffer (pH 8.0) at 65 °C for 10 min.

Effect of pH was studied by assaying lipase activity in a range of pH values from 6.0 to 11.5. Buffers (50 mM) used were sodium phosphate buffer (pH 6.0–8.0), Tris–HCl buffer (8.0–9.0), Glycine–NaOH buffer (9.0–11.0), and Na₂HPO₄–NaOH buffer (11.0–11.5) [22]. For the activity test, substrate was prepared with respective buffer having specific pH and quantified accordingly. For pH stability test, the purified enzyme was pre-incubated in different buffers (pH 7.0–11.0) for 0–6 h before a quantification of each 30 min.

2.9. Effect of metal ions, inhibitors, detergents and organic solvents on enzyme activity

The effects of different metal ions, inhibitors, detergents and organic solvents on the purified enzyme activity were investigated. All the additives were prepared in 50 mM Tris–HCl buffer (pH 8.0). The effects of metal ions and inhibitors on lipase activity were studied by pre-incubating the diluted lipase with each final concentrations of 1 and 10 mM of the metal ions and/or inhibitors (listed in Table 3). Reactions containing 0.1% or 1% (v/v or w/v) of commercial detergents (listed in Table 4) were incubated at 50 °C for 30 min. Reactions containing 15% (v/v, i.e., mixing 0.45 mL of organic solvents in 3 mL of the enzyme solution) or 30% (v/v) organic solvents (listed in Table 5) were incubated at 50 °C with shaking at 200 rpm in an orbital shaker for 2 h. After these incubations, residual lipase activities were assayed in 50 mM Tris–HCl (pH 8.0) at 65 °C for 10 min. The enzyme activity determined in the buffer with no additives was set as 100%. The relative activity percentage was calculated relative to the control. In all cases, each analysis was performed in triplicates. Values are means with standard errors from the three independent experiments.

2.10. Liquid lipase SL-4 catalyzed biodiesel production

Transesterification reactions by liquid lipase SL-4 were conducted in 50 mL shaking flasks and incubated at 50 °C in a thermostat shaking bed with a stirring rate of 200 rpm for 36 h. The reaction mixtures consisted of 0.55 g soybean oil, short-chain alcohol (methanol or ethanol, 3:1 molar ratio between alcohol and oil) by added in a step, 15% water content (w/w, based on the oil weight, g) and 0.4% enzyme (w/w, based on the oil weight, g). After these reactions, the optimum short-chain alcohol (methanol) was

selected for the follow-up tests. The influence of crucial operational parameters such as molar ratio of alcohol/oil from 1:1 to 5:1, the methanol addition approach in three steps at intervals of 6 h, 8 h, 10 h and 12 h, enzyme amount from 0.1% to 0.7% (w/w, based on the oil weight, g), water content from 2% up to 35% (w/w, based on the oil weight, g), reaction temperature from 30 °C to 60 °C and reaction time up to 72 h on the biodiesel yield, was systematically studied. Except for the corresponding volume of Tris-HCl (pH 8.5) buffer instead of the free lipase, transesterification reactions in the solvent-free system under the same conditions were employed as the controls. After a certain reaction period, 50 µL samples were taken from the reaction mixture and centrifuged to obtain the upper layer. Then, 10 µL withdrawn from the supernatant and 290 µL of 1 mM heptadecanoic acid methyl ester, served as the internal standard, were precisely measured and mixed thoroughly for gas chromatography (GC) analysis for biodiesel yield according to the method reported in our previous work [10,17].

3. Results and discussion

3.1. Isolation and identification of lipase producing bacterium

A strain, designated as SL-4, formed a colony with a clear orange fluorescent zone under UV when grown at 37 °C for 3 d on the screening plates. The strain SL-4 was found to be a lipase producer with a maximum lipase activity of approximately 11.07 U/mL toward *p*-NP C14, and a specific activity of 2.16 U/mg of the total protein. When strain SL-4 was inoculated into basic M9 salts medium without olive oil, its lipase activity was very low (0.85 U/mL), suggesting that olive oil was the sole carbon source and the inducer of lipase production. Similar results have been found in *Burkholderia glumae* and *Burkholderia cepacia* S31 [9,19]. The major factor required for the expression of lipase activity has always been carbon, as lipases are by and large inducible enzymes [34]. The result was also supported by studies that showed that large amounts of inducible lipases are generally produced in the presence of a lipid source, such as olive oil [19], or triacylglycerols [34] or tweens [9].

In this work, the almost-complete 16S rDNA sequence (1523 nucleotides) of strain SL-4 was obtained. A nucleotide Basic Local Alignment Search Tool (BLASTn) search revealed that the 16S rDNA sequence has high sequence similarity to the 16S rDNA genes of other *Burkholderia* species, and it exhibited 99% sequence homology with the complete sequence of the 16S rDNA gene of *B. ubonensis* MSMB22 (GenBank: CP009488). A phylogenetic tree showed that SL-4 clustered with related *Burkholderia* species (Fig. 1). The phylogenetic analysis indicated that the newly isolated strain SL-4 was affiliated with *Burkholderia* species. Thus, strain SL-4 was identified as a member of the genus *Burkholderia* and named *B. ubonensis* SL-4.

3.2. Partial purification, identification and sequence analysis of lipase SL-4

The extracellular lipase from *B. ubonensis* SL-4 was purified by Q Sepharose Fast Flow (FF) and Superdex 75 gel filtration chromatography (Fig. 2). Table 1 summarizes the purification procedure. Lipase SL-4 was eluted as fractions from the Q Sepharose FF column with a 25.5-fold purification and a 33.90% yield, and it showed a specific activity of 135.28 U/mg. Then, Superdex 75 gel filtration chromatography recovered 13.34% of the total activity with a 68.5-fold purification, and the specific activity was 362.82 U/mg. This final recovery (yield) of lipase SL-4 was lower than those of lipases from *Staphylococcus aureus* (20%) [1], *Burkholderia* sp. HY-10 (30%) [3], *Staphylococcus* sp. CJ3 (32%) [28], *Pseudomonas aeruginosa* LX1

(41.1%) [22] and *P. aeruginosa* PseA (51.6%) [33], higher than those of lipases from *Burkholderia* sp. EQ3 (0.8%) [16], *B. multivorans* V2 (0.96%) [15], *Burkholderia* sp. GXU56 (3.9%) [12], *B. cepacia* ATCC 25416 (4.8%) [14] and *Pseudomonas* sp. AKM-L5 (7.3%) [35], and similar to that of lipase YCJ01 from *B. ambifaria* YCJ01 (12.5%) [13]. According to SDS-PAGE analysis, the purified lipase SL-4 was homogeneous with an apparent molecular mass of 33–35 kDa (Fig. 2), which was similar to the molecular mass of lipase LipA from *B. cepacia* G63 (33 kDa) [10], lipase LipA from *B. cepacia* ATCC 25416 (33 kDa) [14], lipase HY-10 from *Burkholderia* sp. HY-10 (33.5 kDa) [3], lipase YCJ01 from *B. ambifaria* YCJ01 (34 kDa) [13], lipase GXU56 from *Burkholderia* sp. GXU56 (35 kDa) [12] and lipase S31 from *B. cepacia* S31 (about 35 kDa) [19], and slightly lower than cholesterol esterase CheS from *B. cepacia* ST-200 (37 kDa) [18], even apparently lower than lipase BMV2 from *B. multivorans* V2 (~44 kDa) [15].

Internal fragments of the SL-4 lipase were identified by trypsin digestion and sequencing. These sequences including GEQLLAQVK (fragment 1), VNLVGHSQGGLSSR (fragment 2), YVAAVAPELVASVT-TIGTPHR (fragment 3) and WNHVDEINQLLGVR (fragment 4), showed significant homology to those of lipases from other *Burkholderia* species. Based on the sequences of the lipases from the other *Burkholderia* species, the entire SL-4 gene was successfully cloned. The full-length open reading frame (ORF) of the lipase consisted of 1,095-bp that encodes 364 amino acid residues, and the mature lipase consisted of 320 amino acid residues with a theoretical molecular weight of 33.10 kDa and an isoelectric point (pI) of 5.36. The molecular weights estimated from the purified enzyme and the deduced amino acid sequence were similar to those of other subfamily I.2 lipases [3,10–16,19].

Database searching and a phylogenetic analysis revealed that lipase SL-4 could be grouped into subfamily I.2 (Fig. 3) and that it showed 88% sequence identity to lipase YCJ01 from *B. ambifaria* YCJ01 [13], as well as lipases from *B. cepacia* S31 [19] and *B. cepacia* [11], 86% identity to LipA from *B. cepacia* ATCC 25416 [14], and 81% identity to a lipase from *Burkholderia* sp. HY-10 [3]. Similar to the above-mentioned subfamily I.2 lipases, lipase SL-4 was secreted into the external medium through the type II secretion machinery [3], which is a two-step secretion process required for passage through the inner and outer membranes of Gram-negative bacteria [7]. A multiple sequence alignment (Fig. 4) showed that lipase SL-4 contained a putative catalytic triad of Ser₁₃₁-His₃₃₀-Asp₃₀₈ with Ser₁₃₁ in a consensus G-X1-S-X2-G pentapeptide, two Asp residues (Asp₂₈₆ and Asp₃₃₂) involved in Ca²⁺-binding, and two Cys residues (Cys₂₃₄ and Cys₃₁₄) that may form a disulfide bond. The disulfide bond and the calcium-binding site are thought to be important for lipase stability [9,15]. Like other subfamily I.2 lipases [3,10,13,14], lipase SL-4 also contains N-terminal signal sequence of 40 amino acids with a cleavage position between Ala₄₀ and Ala₄₁, as predicted by SignalP 3.0 software. However, the N-terminal amino acid sequencing results showed that the carboxyl terminal of Pro₄₄ was the last cleaved site of the secreted enzyme (data not shown). Similar to a lipase from *Burkholderia* sp. HY-10 [3], we could not exclude the possibility that the secreted polypeptide was modified by a second proteolysis event while it folded into its active conformation with the assistance of its chaperone and formed a disulfide bond via the disulfide bond formation system (Dsb) in the periplasm.

By molecular structural modeling, we found that the crystal structure (PDB: 2nw6) [11] of a lipase from *B. cepacia* lipase (BCL, formerly called *P. cepacia* lipase) was the most appropriate template for homology modeling. As validated by the online program PROCHECK, 87.0% of the residues in the modeled structure were in most favored regions, 11.9% of the residues were in additional allowed regions, 0.7% of the residues were in generously allowed regions, and only 0.4% out of 319 amino acids were in disallowed regions. These results indicated that the model is satisfactory. Like BCL in the open conformation, the final structure of lipase SL-4 con-

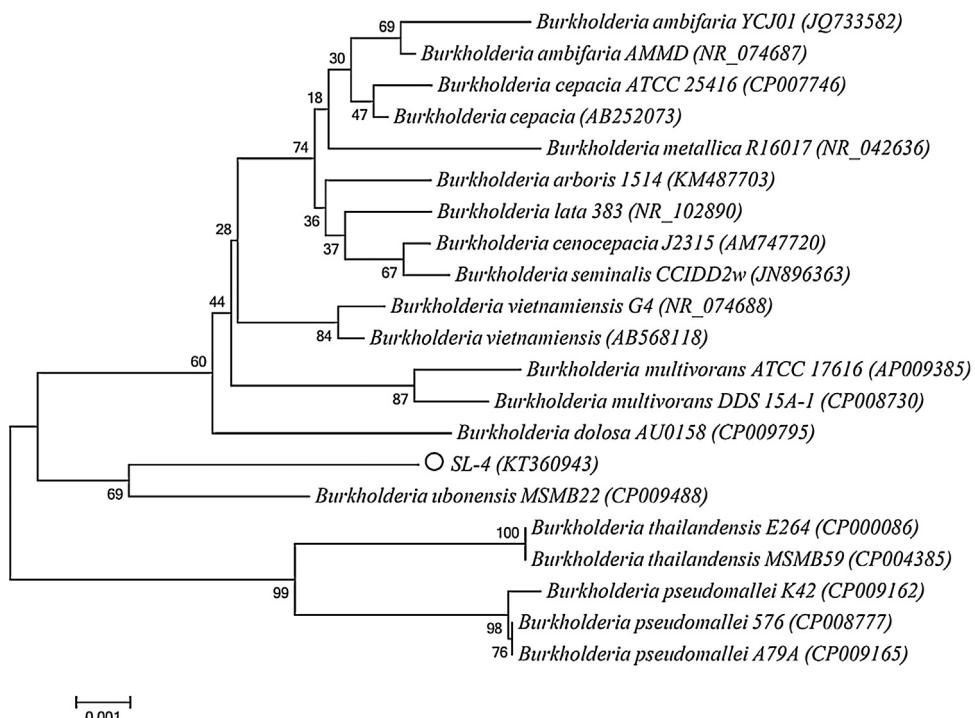


Fig. 1. Phylogenetic tree derived from 16S rDNA gene sequence of strain SL-4 and these sequences of *Burkholderia* species obtained by NCBI BLAST(*n*) analysis, numbers in the parenthesis indicate GenBank accession numbers of corresponding sequence. The strain SL-4 was marked with an empty circle (○). The values at nodes indicate the bootstrap percentage of 1000 replications. The tree was constructed using neighbor joining algorithm with Kimura 2 parameter distances in MEGA 5.0 software.

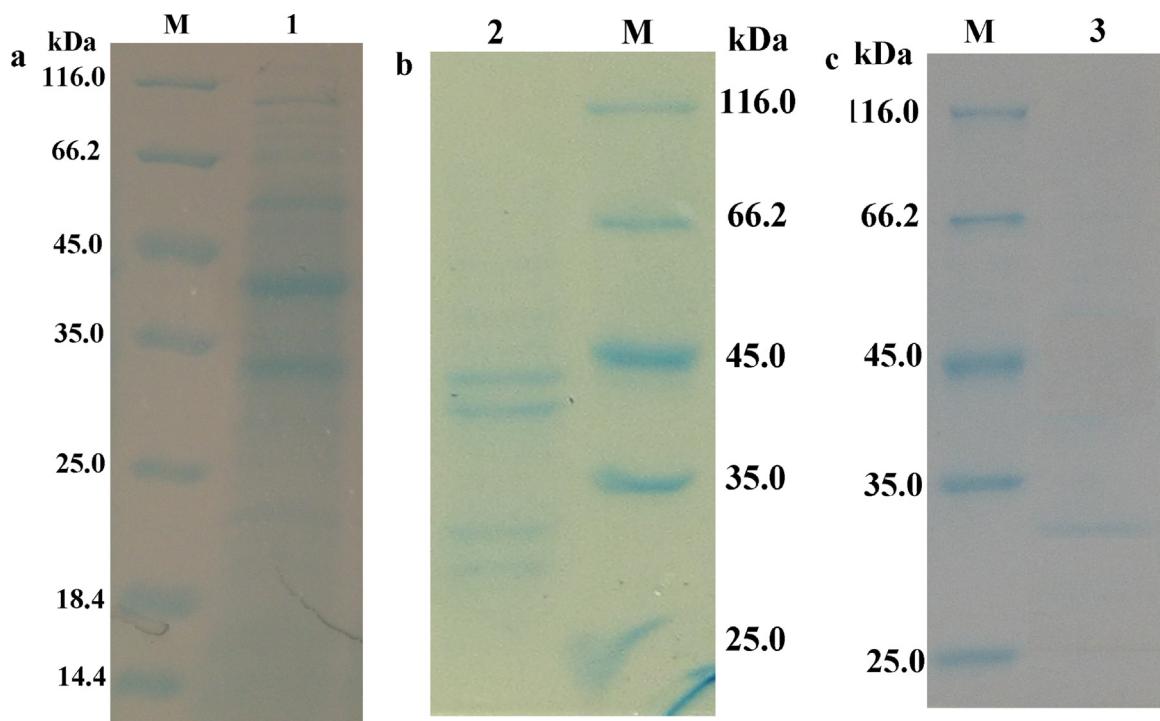


Fig. 2. SDS-PAGE analysis of lipase SL-4 (LSL-4) from *Burkholderia ubonensis* SL-4 at each stages of purification. Lane 1 (a): culture supernatant; lane 2 (b): crude enzyme after Q-Sepharose FF chromatograph; lane 3 (c): LSL-4 purified by Superdex 75 gel filtration chromatography; M: unstained protein molecular weight markers (Fermentas, SM0431).

tains 11 α -helices and 11 β -sheets, which are composed of three common domains, named as the catalytic domain, the α/β domain, and the regulatory domains (Fig. 5). The catalytic triad consists of Ser₁₃₁, His₃₃₀, and Asp₃₀₈, marked in blue, yellow, and magenta spheres, respectively, which are located on the loops between β 5-

α 3, β 11- η 2, and α 9- α 10, respectively, and are clustered together at the bottom of the active site. A lid (labeled in red) in form of α -helix comprising residues from 178 to 194, which corresponds to the well-known lid of lipases [11], assumed an open conformation (Fig. 5). In addition, a calcium ion (Ca^{2+}), two Asp residues

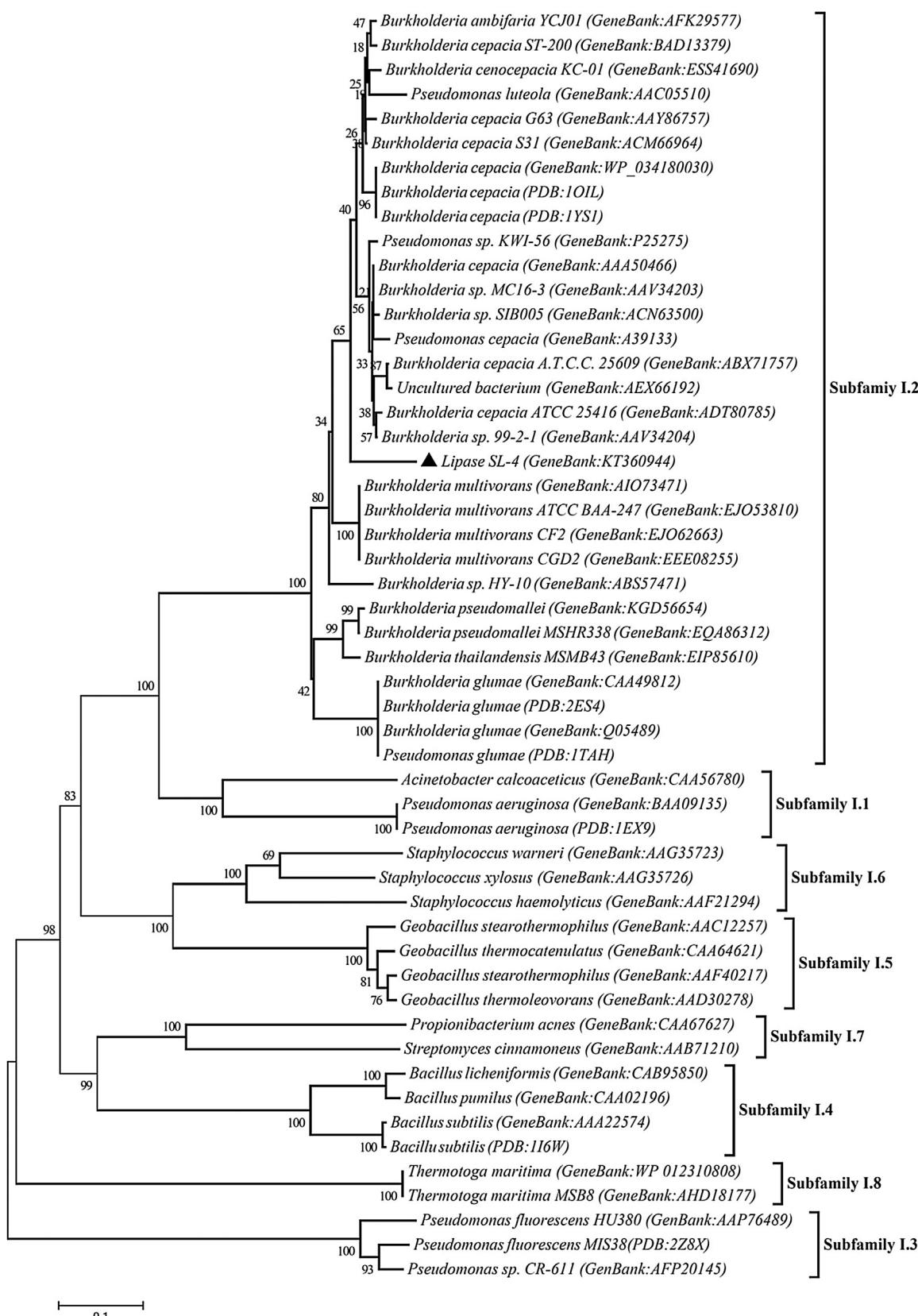


Fig. 3. Phylogenetic analysis of lipase SL-4 and other closely related lipases. The phylogenetic analysis was performed by the neighbor-joining method with *p*-distance using MEGA 5.0. Lipase SL-4 in this study was marked with a black uptriangle (\blacktriangle). The values at nodes indicate the bootstrap percentage of 1000 replications. The lengths of the branches show the relative divergence among the reference lipase amino acid sequences and scale bar indicates the amino acid substitutions per position. Genbank accession numbers are shown in brackets after each species name.

Table 1Purification of lipase from *Burkholderia ubonensis* strain SL-4.^a

Purification steps	Volume (mL)	Lipase activity (U/mL)	Total (Units)	Protein content (mg/mL)	Total protein (mg)	Specific activity (U/mg protein)	Fold purification	Recovery (%)
Culture supernatant	980	11.07	10848.60	2.09	2048.20	5.30	1	100
80% Ammonium sulphate precipitation	65	83.22	5409.11	2.27	147.47	36.68	6.92	49.86
Q Sepharose FF	15	245.21	3678.19	1.81	27.19	135.28	25.52	33.90
Superdex 75 gel filtration chromatography	10	144.77	1447.65	0.40	3.99	362.82	68.46	13.34

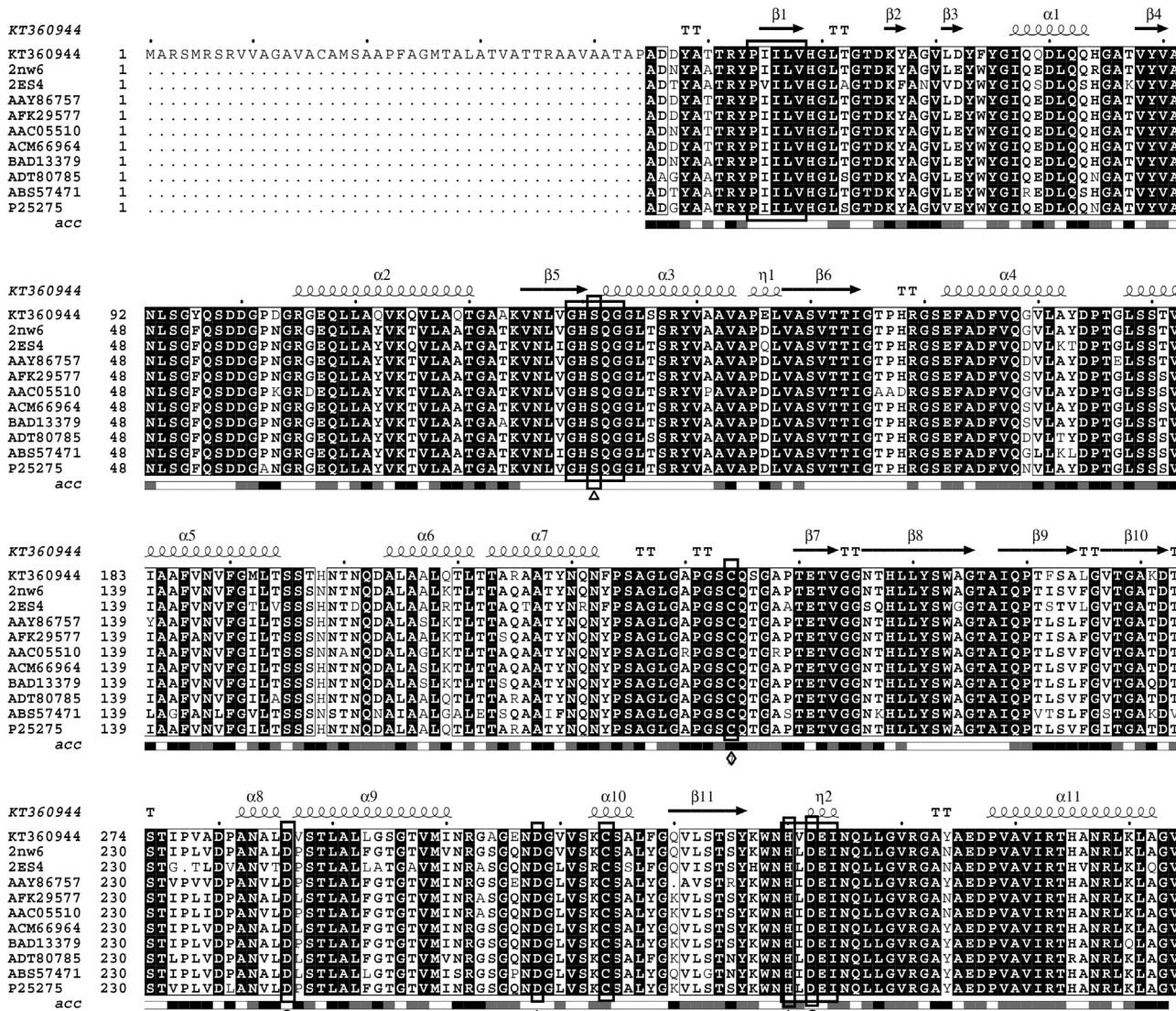
^a Lipase activity was determined with *p*-nitrophenyl myristate as substrate.

Fig. 4. Multiple sequence alignment between lipase SL-4 and other subfamily I.2 lipases. KT360944, lipase SL-4 from *Burkholderia ubonensis* SL-4; 2nw6, BCL from *B. cepacia*; 2ES4, LipA from *B. glumae*; AAY86757, LipA from *B. cepacia* G63; AFK29577, lipase YCJ01 from *B. ambifaria* YCJ01; AAC05510, lipase from *P. luteola*; ACM66964, lipase S31 from *B. cepacia* S31; BAD13379, cholesterol esterase ST-200 from *B. cepacia* ST-200; ADT80785, LipA from *B. cepacia* ATCC 25416; ABS57471, LipA from *Burkholderia* sp. HY-10; P25275, lipase from *Pseudomonas* sp. KWI-56. Sequence alignment was performed with Clustal W2 and visualized using Escript 2.2. The alpha helix, beta sheet, random coil and beta turn are identical to α , β , η and T , respectively. Empty uptriangles (Δ) represent putative catalytic residues at the corresponding positions of Ser₁₃₁, His₃₃₀, and Asp₃₀₈. Putative Ca²⁺-binding Asp residues (Asp₂₈₆ and Asp₃₃₂) are marked with empty circles (\circ). The two Cys residues (Cys₂₃₄ and Cys₃₁₄) comprise a potential disulfide bond are labeled with empty diamonds (\diamond).

(Asp₂₈₆ and Asp₃₃₂) involved in Ca²⁺-binding, and two cysteine residues (Cys₂₃₄ and Cys₃₁₄) forming the disulfide bridge are presented in light-blue, yellow-orange, red, purple-blue, and limon spheres, respectively.

3.3. Effect of temperature and pH on lipase SL-4 activity and stability

The optimum temperature of the purified lipase SL-4 was 65 °C (Fig. 6a), which was similar to those of lipases from *Burkholderia* sp. HY-10 (60 °C) [3], *B. cepacia* ATCC 25416 (60 °C) [14], *B. cepa-*

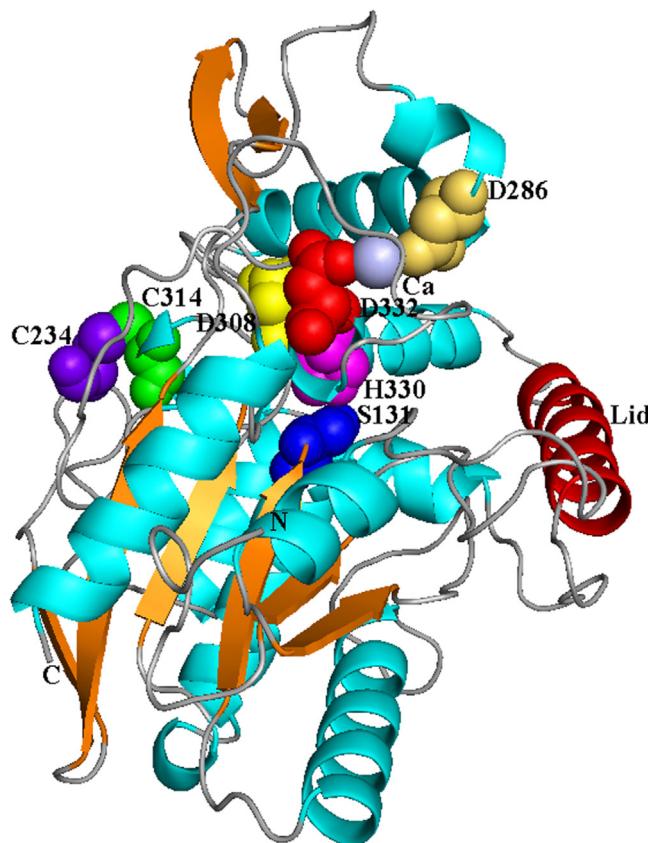


Fig. 5. The stereo view of three-dimensional (3D) model of lipase SL-4 in an open conformation. The α -helix, β -sheet, random coil and beta turn are in cyan, orange, and gray, respectively. The catalytic triad (Ser_{131} , Asp_{308} and His_{330}) are shown in blue, yellow and magenta spheres, respectively. The calcium ion (Ca^{2+}) and two Asp residues (Asp_{286} and Asp_{332}) involved in Ca^{2+} -binding are in light-blue, yellow-orange and red spheres, respectively. The two cysteine residues (Cys_{234} and Cys_{314}) forming the disulphide bridge are in purple-blue and limon spheres, respectively. The α -helical lid (residues 178–194) was indicated in red. N and C denote the N and C termini, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cia G63 (70 °C) [10] and *B. cepacia* S31 (70 °C) [19], and apparently higher than those of *Burkholderia* sp. GXU56 (40 °C) [12], *B. cepacia* ST-200 (45 °C) [18], and other *Pseudomonas* lipases (35–55 °C) [34]. Its activity increased from 10.82% to the maximal activity at temperatures ranging from 15 to 65 °C, and decreased gradually to 23.05% at 100 °C. Its half-life was 17.5 h at 60 °C and 13.5 h at 65 °C (Fig. 6b). Lipase SL-4 retained 62.34%, 61.24% and 56.02% of its activity after treatment at 60 °C, 65 °C and 70 °C, respectively for 12 h. In contrast, the highly thermostable lipase LipM from *Pseudomonas moraviensis* M9 retained 98.2%, 91% and 66.3% of its activity after incubation at 60 °C, 65 °C and 70 °C, respectively, for 12 h [5]. The thermal stable lipase G63 from *B. cepacia* G63 retained 86.1% of its activity after incubation at 70 °C for 10 h [10]. However, lipase GXU56 from *Burkholderia* sp. GXU56 was stable below 45 °C, retaining 96.3% of its initial activity after incubation at 30 °C for 2 h [12], and the thermophilic lipase S31 from *B. cepacia* S31 kept 85% of its activity after incubation at 55 °C for 12 h [19]. Compared with the former two lipases, lipase SL-4 still retained 86.04% of its activity after incubation for 12 h at 55 °C, even kept over 70% of its activity after exposure at 55 °C for 24 h, and more than 90% of its activity after incubation at 50 °C for 24 h. Thus, it can be seen that the thermostability of lipase SL-4 is slightly weaker than lipases LipM and G63, but much better than lipases GXU56 and S31.

Lipase SL-4 exhibited maximum activity at pH 8.5, which was the same or similar to the optimal pHs of other *B. cepacia* lipases, such as *Burkholderia* sp. HY-10 (pH 8.5) [3], *Burkholderia* sp. GXU56 (pH 8.0) [12] and *B. cepacia* S31 (pH 8.5–9.0) [19]. It retained more than 50% of its maximal activity in a pH range from 7.5 to 10.5, indicating that it is well adapted to a wide pH range thus it is an

alkaline lipase (Fig. 6c), which confirms the its alkaline nature of the enzyme [36]. Regarding pH stability (Fig. 6d), lipase SL-4 retained nearly 79.61% and 94.02% of its activity at pH 8.0 and 8.5, respectively, after incubation for 6 h. After incubation at pH 7.5, 9.0 and 9.5 for 1 h, the residual activities of lipase SL-4 were 68.39%, 87.09% and 88.03%, respectively. However, after incubation at lower or higher pH, the enzyme activity was greatly reduced to less than 50% of its initial activity. Although the pH stability of lipase SL-4 was slightly inferior to that of a highly alkaline lipase from *B. cepacia* ATCC 25416 (pH 9.0–11.5) [14], it is comparable or superior to those of other reported alkaline-tolerant lipases from *Burkholderia* sp. GXU56 (pH 8.0–8.5) [12], *P. aeruginosa* PseA (pH 8.0–8.5) [33], *B. cepacia* S31 (pH 8.0–9.0) [19], *B. ambifaria* YCJ01 (pH 8.0) [13] and *Staphylococcus* sp. CJ3 (pH 8.0–9.0) [28].

3.4. Substrate specificity and kinetic parameters

As shown in Table 2, lipase SL-4 exhibited activity on a wide range of substrates including *p*-NP esters with acyl fatty acid chain lengths ranging from C2 to C16. With an increase in the C-chain length of the *p*-NP substrate, the hydrolytic activity of lipase SL-4 increased, and it was shown to prefer medium- and long-chain fatty acid substrates, and its highest activity was 362.82 U/mg towards *p*-NP myristate (*p*-NPM, C14) at 65 °C after 10 min in Tris-HCl (pH 8.5). Moreover, the activity of lipase SL-4 toward olive oil was 893.11 U/mg. Based on its substrate preference, it can be concluded that lipase SL-4 is a true lipase [7,34].

The lowest K_m (0.72 mM) of lipase SL-4 indicates the high affinity of this enzyme for *p*-NPM. However, specific activity does not

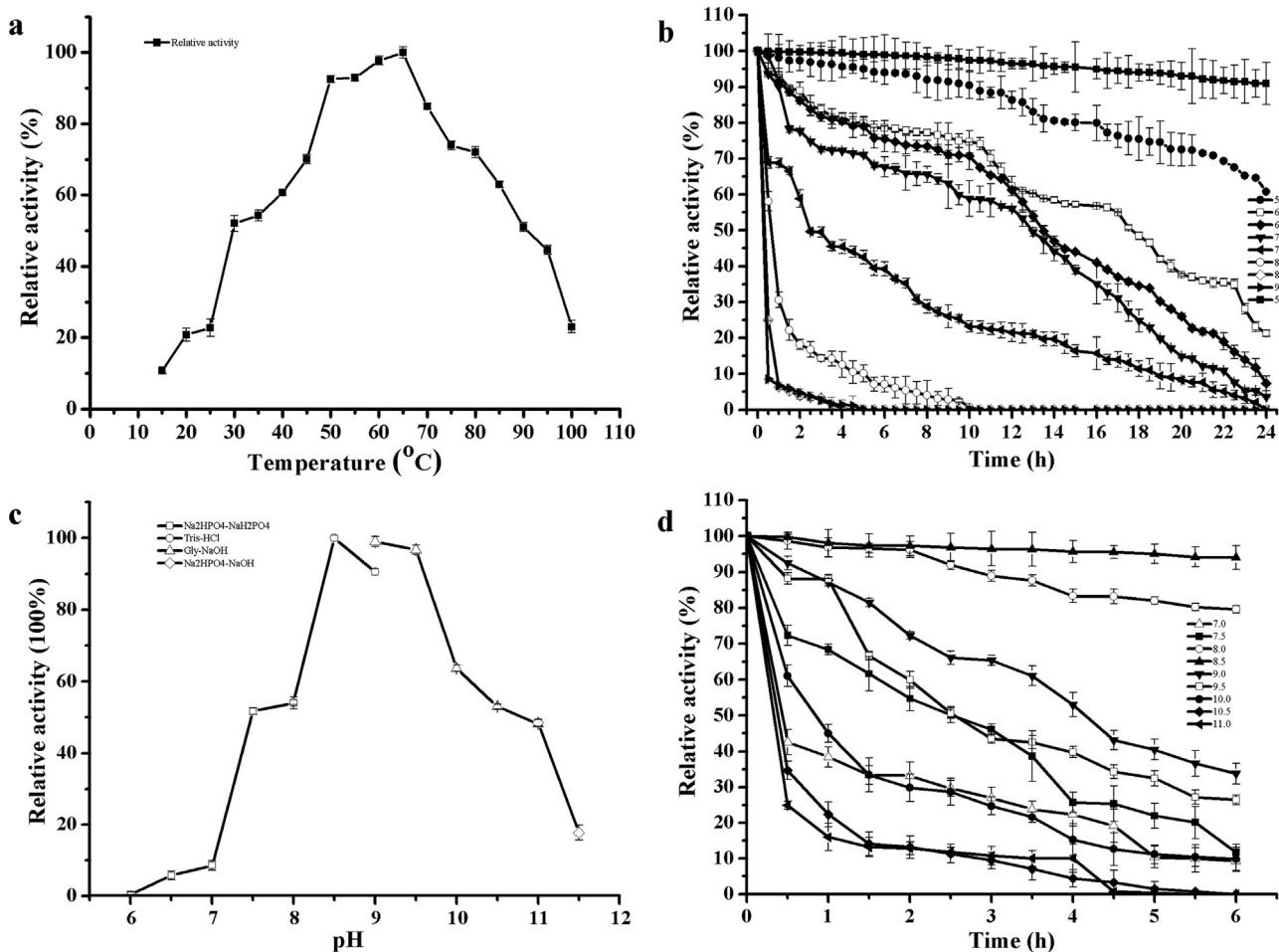


Fig. 6. Effect of temperature and pH on lipase SL-4 activity and stability. (a) Effect of temperature on the activity of lipase SL-4; (b) Thermal stability of this lipase. The enzyme was incubated at 50 °C (■), 55 °C (●), 60 °C (□), 65 °C (◆), 70 °C (▼), 75 °C (▲), 80 °C (○), 85 °C (◊) and 90 °C (►) for the indicated time; (c) The pH profile of the purified lipase SL-4. Lipase SL-4 activity was measured at different pH values. The enzyme activity in Tris-HCl (50 mM, pH 8.5) was taken as 100%. Buffers used (final concentration 50 mM) were Na₂HPO₄-NaH₂PO₄ buffer (pH 6.0–8.0), Tris-HCl buffer (pH 8.0–9.0), Glycine-NaOH buffer (pH 9.0–11.0) and Na₂HPO₄-NaOH buffer (pH 11.0–11.5); (d) pH stability of lipase SL-4. The enzyme was incubated at Na₂HPO₄-NaH₂PO₄ buffer (pH 7.0, △), Na₂HPO₄-NaOH buffer (pH 7.5, ■), Tris-HCl buffer (pH 8.0, ○), Tris-HCl buffer (pH 8.5, ▲), Tris-HCl buffer (pH 9.0, ▼), Glycine-NaOH buffer (pH 9.5, □), Glycine-NaOH buffer (pH 10.0, ●), Glycine-NaOH buffer (pH 10.5, ◆), and Glycine-NaOH buffer (pH 11.0, ▲) for the indicated time. The residual activity was measured by a standard assay. The values represent the means of three independent experiments (mean ± standard error).

Table 2
Substrate specificity and kinetic parameters for lipase SL-4.^a

Substrate	Specific activity (U/mg)	K _m (mM)	k _{cat} (s ⁻¹)	k _{cat} /K _m (s ⁻¹ mM ⁻¹)
p-NP acetate (C2)	73.14 ± 2.89	3.89 ± 0.09	166.56 ± 0.16	42.82 ± 0.35
p-NP butyrate (C4)	167.06 ± 2.13	3.77 ± 0.25	287.61 ± 0.34	76.29 ± 0.24
p-NP caprylate (C8)	203.53 ± 3.92	2.94 ± 0.53	235.98 ± 0.42	80.27 ± 0.31
p-NP decanoate (C10)	241.03 ± 4.54	1.30 ± 0.24	220.88 ± 0.21	169.91 ± 0.53
p-NP laurate (C12)	303.65 ± 3.91	0.77 ± 0.12	259.25 ± 0.18	336.69 ± 0.26
p-NP myristate (C14)	362.82 ± 2.46	0.72 ± 0.84	391.63 ± 0.22	543.93 ± 0.51
p-NP palmitate (C16)	86.22 ± 3.53	2.36 ± 0.55	351.72 ± 0.33	149.03 ± 0.48

^a Values are means ± SD from three independent experiments.

provide an accurate model for determining enzyme efficiency, especially for industrial applications [1]. Usually, a physiological efficiency value, such as k_{cat}/K_m, provides a useful model for this purpose [4,8]. In addition, the catalytic efficiency (k_{cat}/K_m) of lipase SL-4 increased gradually with the increasing chain length from C2 (42.82 s⁻¹ mM⁻¹) to C14 (543.93 s⁻¹ mM⁻¹), and then decreased from C14 to C16 (149.03 s⁻¹ mM⁻¹). According to Maharan and Ray [35], with an increase in substrate concentrations, the rate of reaction increases until the active sites of the enzyme are filled, at which point the maximum rate of reaction is reached. A lower K_m

and higher k_{cat}/K_m at such a test temperature are of great biotechnological and economic importance [1,35].

3.5. Effects of metal ions, inhibitors, detergents and organic solvents on lipase SL-4

Lipase SL-4 activity was significantly stimulated in the presence of Ca²⁺ and Mn²⁺ (Table 3). Many lipases, such as lipases from *P. aeruginosa* LX1 [22], *Pseudomonas* sp. DMVR46 [36], *B. cepacia* ATCC 25416 [14] and *B. multivorans* V2 [15], have been found to display enhanced activity in the presence of Ca²⁺. Similar to lipase LipZ01 from metagenome [14], Ca²⁺ or Mn²⁺ binding to some sites of lipase

Table 3Effects of some metal ions and inhibitors on the activity of lipase SL-4.^a

Additives	Relative activity (%) at different concentration	
	1 mM	10 mM
Control	100 ± 1.05	100 ± 1.05
Metal ions		
Li ⁺	100.55 ± 1.91	109.71 ± 2.13
Na ⁺	103.74 ± 2.18	110.22 ± 1.86
K ⁺	99.45 ± 1.80	107.73 ± 2.40
Mg ²⁺	97.80 ± 2.11	80.03 ± 1.52
Ca ²⁺	113.77 ± 1.13	163.69 ± 3.36
Mn ²⁺	101.16 ± 4.43	152.45 ± 4.61
Fe ²⁺	100.83 ± 2.18	77.54 ± 2.70
Co ²⁺	95.56 ± 2.04	82.81 ± 2.16
Ni ²⁺	106.93 ± 2.26	83.92 ± 2.06
Cu ²⁺	96.95 ± 1.43	65.34 ± 1.88
Zn ²⁺	97.78 ± 1.84	38.71 ± 2.17
Sr ²⁺	98.34 ± 2.04	110.54 ± 1.28
Ba ²⁺	103.33 ± 2.0	96.95 ± 1.72
Pb ²⁺	79.48 ± 1.55	68.77 ± 1.29
Al ³⁺	75.87 ± 1.40	11.54 ± 1.94
Fe ³⁺	95.84 ± 1.76	29.00 ± 1.61
Inhibitors		
EDTA	70.60 ± 1.52	44.26 ± 1.67
PMSF	67.28 ± 0.60	42.87 ± 1.21
DTT	70.05 ± 0.97	33.44 ± 1.35
β-ME	65.61 ± 1.13	28.73 ± 1.25

^a Values are means ± SD from three independent experiments.**Table 4**Effects of various detergents on the activity of lipase SL-4.^a

Detergents	Relative activity (%)	
	0.1%	1%
Control	100 ± 1.35	100 ± 1.35
Tween-20	117.47 ± 1.08	94.73 ± 1.71
Tween-40	124.68 ± 1.99	110.26 ± 1.25
Tween-60	162.40 ± 1.82	95.29 ± 1.73
Tween-80	115.53 ± 1.29	60.62 ± 1.36
Triton X-100	152.41 ± 0.96	69.22 ± 2.25
SDS	32.61 ± 2.10	4.05 ± 1.35
CTAB	126.62 ± 1.53	36.51 ± 1.27

^a Values are means ± SD from three independent experiments.**Table 5**Effects of various organic solvents on the activity of lipase SL-4.^a

Organic solvents	Log P	Relative activity (%) at different concentrations	
		15%	30%
Control		100 ± 1.02	100 ± 1.02
Glycerol	-3.03	194.27 ± 1.75	131.10 ± 2.15
Ethylene glycol	-1.4	203.01 ± 2.79	156.37 ± 1.92
Dimethylsulfoxide	-1.3	224.64 ± 1.70	144.46 ± 2.45
Dimethylformamide	-1.0	100.55 ± 1.95	86.97 ± 2.34
Isopropanol	-0.77	146.89 ± 1.80	28.57 ± 1.59
Methanol	-0.76	146.89 ± 3.10	118.22 ± 2.56
Acetonitrile	-0.33	92.47 ± 2.31	57.97 ± 1.76
Ethanol	-0.24	94.41 ± 1.62	92.95 ± 2.12
Acetone	-0.23	167.78 ± 1.54	51.41 ± 1.89
Tetrahydrofuran	0.49	30.03 ± 3.82	27.60 ± 2.48
tert-Butanol	0.8	115.79 ± 2.48	29.06 ± 2.10
n-Butanol	0.8	21.04 ± 1.44	16.42 ± 1.53
1-Hexanol	1.8	31.00 ± 2.64	21.53 ± 2.81
Chloroform	2.0	102.92 ± 1.58	68.90 ± 1.50
Isooctanol	2.55	31.49 ± 2.57	27.84 ± 1.66
Xylene	3.1	105.10 ± 1.24	49.95 ± 1.76
n-Hexane	3.5	102.19 ± 2.03	87.12 ± 1.83
n-Heptane	4.0	113.36 ± 1.36	108.99 ± 1.20
Isooctane	4.7	95.14 ± 1.49	85.67 ± 1.95

^a Values are means ± SD from three independent experiments.

SL-4 may change its conformation, and may even be necessary for its catalytic activity [14]. Additionally, Li⁺, Na⁺, K⁺, Sr²⁺ and Ba²⁺ had no significant influence on the activity of lipase SL-4 (96–110% of its initial activity). Similar to LipC12, as reported by Wang et al. [14], Mg²⁺, Pb²⁺ and Al³⁺ also inhibited the activity of Lipase SL-4 (<80% of its original activity). A final concentration of 1 mM of transition metal ions, such as Fe²⁺, Fe³⁺, Co²⁺, Ni²⁺, Cu²⁺ and Zn²⁺, slightly reduced its activity (95–98% of its original activity), whereas a 10 mM concentration dramatically reduced its activity (29–84% of its original activity). This coincides with the results of Rahman et al. [37], who reported that a 10 mM concentration of transition metal ions, such as Fe²⁺, Fe³⁺, Co²⁺, Ni²⁺, Cu²⁺ and Zn²⁺, could increase the interaction of ions with charged side chain groups of surface amino acids, which might markedly affect the ionization of some amino acid residues, thus rendering lipases less stable as a result of ion toxicity.

Lipase SL-4 was strongly affected by 1 mM or 10 mM concentrations of the chelating agent ethylenediaminetetraacetic acid (EDTA), indicating that it is a metalloenzyme. Many lipases are generally serine hydrolases [8,14,15], in which serine is an essential residue for catalytic activity. Yang et al. [32] also reported similar results. The addition of the serine proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF) at 1 mM or 10 mM concentrations had an inhibitory effect on lipase SL-4 activity, which further confirmed that lipase SL-4 is a serine lipase. The reducing agents dithiothreitol (DTT) and beta-mercaptoethanol (β -ME) can reduce the disulfide bonds of proteins and prevent the formation of intra- or intermolecular disulfide bonds between cysteine residues [4,5]. Both DTT and β -ME had significant effects on the activity of lipase SL-4 at final concentrations of 1 mM or 10 mM. Thus, the results proved that there are cysteine residues and disulfide bond(s) in the mature lipase SL-4.

Non-ionic detergents, including Tween-20, Tween-40, Tween-60, Tween-80 and Triton X-100, showed different activation effects on lipase SL-4 at a final concentration of 0.1% (v/v) (Table 4). The stimulatory effect of these non-ionic detergents on lipases was also observed by Dandavate et al. [15], Zheng et al. [4] and Yao et al. [13], and it may be due to a change in the conformation of the enzyme associated with the binding of these hydrophobic agents, which makes the active site more accessible to the substrate [4]. However, when the final concentration of these non-ionic detergents (except for Tween-40) was increased to 1% (v/v), the residual activity of lipase SL-4 decreased to 60–95% of its initial activity. According to Glogauer et al. [29], the long acyl ester chains, with different compositions, of these non-ionic detergents could act as competitive inhibitors and compete with the original substrates to different degrees [32]. In contrast, both 0.1% (w/v) and 1% (w/v) sodium dodecyl sulfate (SDS) drastically inhibited lipase SL-4, which is consistent with the results of Glogauer et al. [29], Wang et al. [14], Yao et al. [13], and Yang et al. [32]. This drastic loss of activity may result from the disruption of the structure or even three-dimensional conformational changes of lipase SL-4 upon exposure to the anionic surfactant SDS [6]. Similar to Glogauer et al. [29] and Wei et al. [12], we also found that lipase SL-4 was distinctly active in the presence of 0.1% (w/v) of the cationic surfactant cetyl trimethylammonium bromide (CTAB), but it was severely inactive when the CTAB concentration increased to 1% (w/v). Therefore, the fairly good stability of lipase SL-4 in the presence of these non-ionic detergents, coupled with its alkaline nature, demonstrates that it has great potential in detergent formulations.

The stability and activity of enzymes in organic solvents have received great attention in recent decades, as lipases are used in a variety of biotechnological fields, such as catalyst in organic synthesis, biotransformations and the optical resolution of chiral compounds [1,15,38]. As shown in Table 5, the residual activity of lipase SL-4 was stable/active (92–147% of its initial activity) in

the presence of 15% (v/v) isopropanol, acetonitrile, acetone, *tert*-butanol, chloroform and xylene, while it was severely reduced (28–70% of its original activity) by a 30% (v/v) concentration of these solvents. Additionally, the organic solvents tetrahydrofuran, *n*-butanol, 1-hexanol and isoctanol strongly inhibited the activity of lipase SL-4 at 15% or 30% (v/v) concentrations. However, obviously different from lipases from *P. aeruginosa* LX1 [22], *Burkholderia* sp. S31 [19] and *S. aureus* [1], lipase SL-4 was active/stable (85–225% of the original activity) in the presence of polar (water-miscible) solvents ($\log P < 0.3$), such as glycerol, ethylene glycol, dimethylsulfoxide (DMSO), dimethylformamide (DMF), methanol and ethanol, as well as non-polar hydrophobic (water-immiscible) solvents ($\log P > 2.0$), such as *n*-hexane, *n*-heptane and isoctane. The activation of the lipase could be explained by the interaction of organic solvents with hydrophobic amino residues present in the lid that covers the catalytic site of the enzyme, thereby maintaining the lipase in its open conformation [36]. Although solvent stability has been reported for other *Burkholderia* sp. lipases [10,13,14,19], stability/activation in glycerol, ethylene glycol, DMSO, DMF, methanol and ethanol has rarely been observed [33]. Moreover, the short-chain alcohols methanol and ethanol have been widely used in transesterifications [10,21,22,39]. Thus, tolerance to methanol, ethanol and glycerol is a highly desirable feature for biodiesel production [19]. These aforementioned water-immiscible solvents allowed lipase SL-4 to remain stable (over 85% of its initial activity). This may be because these hydrophobic solvents leave the hydration shell of the protein intact, thereby resulting in only a small redistribution of water, which preserves the native protein structure and decrease the side reaction in the liquid phase [19,38]. Therefore, these organic solvent-tolerant characteristics of lipase LS-4 are particularly significant for organic synthesis, especially for biodiesel production via transesterification.

3.6. Biodiesel production from soybean oil

3.6.1. Effect of acyl acceptor type on transesterification

Biodiesel is a mixture of fatty acid alkyl esters obtained by the transesterification of triglycerides (TAGs) of vegetable or animal origin with methanol or ethanol [39–41]. Because of the aforementioned tolerance of lipase SL-4 toward methanol and ethanol, transesterification between soybean oil and either of the two acyl acceptors was performed using this lipase as a potential biocatalyst. As shown in Fig. 7 a, using methanol and ethanol, the conversion rates of soybean oil to FAMEs/fatty acid ethyl esters (FAEEs) were 44.95% and 30.65%, respectively, illustrating that methanol is better than ethanol for lipase SL-4-mediated transesterification for biodiesel production. Additionally, because the price of ethanol is considerably higher than that of methanol, researchers have taken into account the cost of biodiesel yield, and chosen methanol as an acyl acceptor for enzymatic catalysis [10,38,41].

3.6.2. Effect of molar ratio of methanol to oil on biodiesel production

The stoichiometric ratio for the transesterification reaction requires only 3 mol of alcohol and 1 mol of TAGs to yield 3 mol of fatty acid ester and 1 mol of glycerol. A higher molar ratio of alcohol to TAGs often promotes the transesterification reaction [23]. However, methanol serves as reaction substrate for biodiesel production, and it is harmful to proteins in excessive proportions [41], in particular when it is insoluble in the reaction mixture (it forms an emulsion, and the size of the droplets depends on the intensity of stirring) [17]. Therefore, the methanol concentration (molar ratio of methanol to oil) was varied from 1:1 to 5:1 to investigate its effect on the biodiesel yield. As seen in Fig. 7b, with an increasing molar ratio of methanol to oil from 1:1 to 4:1, the biodiesel yield increased from 20.74% to 46.65% of the maximal conversion

rate. The optimal molar ratio of methanol to oil (4:1) for lipase SL-4 was lower than that of the soluble lipase NS81006 (6.6:1) [24], but was higher than those of a recombinant *Rhizomucor miehei* lipase (3:1) [41] and a liquid recombinant *Candida rugosa* lipase 2 (3:1) [20]. However, similar to the aforementioned three lipases, higher methanol proportions (methanol/oil ratio from 4.5:1 to 5:1) may cause irreversible denaturation of lipase SL-4 [42], as evidenced by the reduction in the conversion rates to 37.11% and 25.43%, respectively. These results are similar to previous reports that showed that methanol is insoluble in oil at high concentrations, thereby making enzymes unstable and inactive, and that an excess of methanol resulted in low biodiesel yield [17,23,26,27].

3.6.3. Effect of the methanol addition approach on the biodiesel production

It can be seen from the above experiments that methanol had a strong inhibitory effect on the liquid lipase SL-4 during transesterification, as previously found for the solvent-free transesterification of vegetable oils [17,43]. Normally, a stepwise addition of alcohol is conducted to decrease the deactivation of the enzyme and increase its longevity [38,43]. Some researchers have added methanol into the transesterification system in three steps to avoid the deactivation of enzyme and obtain better yields [17,23,41,43]. The effect of the methanol addition approach (three steps at intervals of 6 h, 8 h, 10 h and 12 h) on the methanolysis of soybean oil is shown in Fig. 7c. When methanol was added in three steps at intervals of 6 h, 8 h, 10 h and 12 h, biodiesel yields obviously increased to 55.95%, 58.89%, 69.32% and 75.58%, respectively, after an 18-h reaction; after a 36-h reaction, 61.21%, 74.91%, 83.93% and 81.91% biodiesel yields were respectively obtained. The biodiesel yields following the addition of methanol in three steps were much better than those for the one-step addition. This is similar to a previous report in which the FAME yield increased significantly with a three-step protocol of methanol addition [17,23]. Additionally, there were slight increases (5.26% and 6.33%) of biodiesel yields when the reaction time increased from 18 h to 36 h after the methanol was added in three steps at intervals of 6 h and 12 h, respectively, while significant increases of 16.02% and 14.61% at intervals of 8 h and 10 h, respectively. The obvious difference among the four results in a solvent-free system could indicate that the enzyme was rapidly inactivated or inhibited by the addition of methanol at short intervals of 6 h, while a long interval of 12 h resulted in less enzyme inactivation or inhibition than the 8-h and 10-h intervals of methanol addition. Although an excess of methanol is a prerequisite for high transesterification yield, a large excess of methanol is detrimental for lipase activity [23,41]. Possible reasons for these results might be that the amount of methanol added to the reaction mixtures was either too high or too low for effective transesterification, which directly caused a loss of lipase activity or insufficient substrate binding, eventually leading to a relatively low biodiesel yield.

3.6.4. Effect of enzyme amount on biodiesel production

The transesterification reactions were conducted at different concentrations of soluble lipase SL-4 to investigate the effect of the amount of lipase SL-4 on the methanolysis of soybean oil for biodiesel production in a solvent-free system (Fig. 7d). Lipases are kind of interfacial enzymes in that lipase catalysis is performed at the biphasic liquid–oil interface [26]. The biodiesel yield increased from 40.89% to 87.03% as the amount of enzyme increased from 0.1% to 0.5%. However, further increases in the lipase quantity from 0.6% to 0.7% at the interface slightly decreased the transesterification rate from 83.88% to 80%, respectively. These results indicated that the maximum adsorptive capacity of liquid lipase SL-4 at the interface in the solvent-free system might be 0.5%, and that the transesterification rates could not be obviously improved by increasing the enzyme concentration (>0.5%). This leveling-

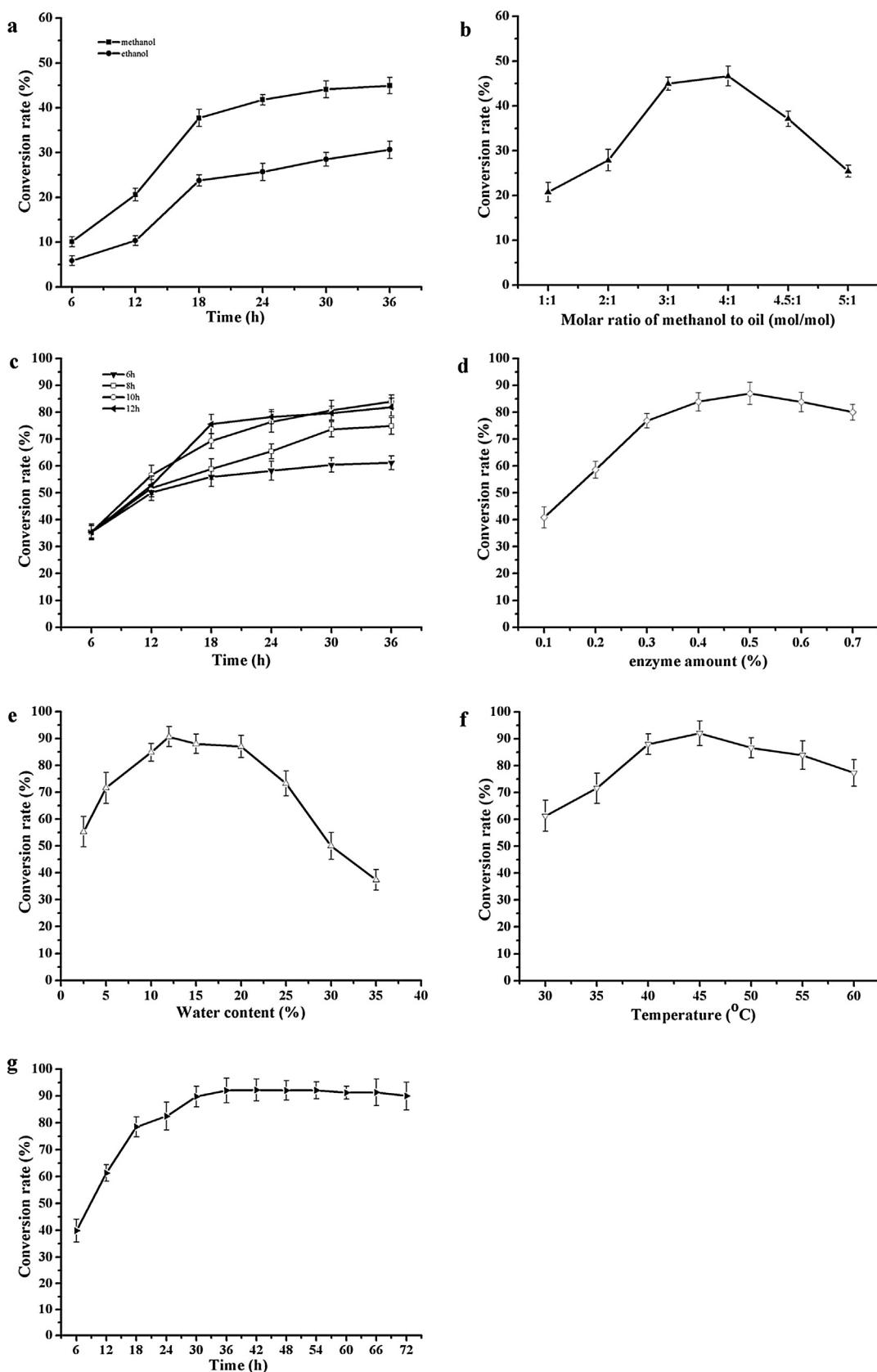


Fig. 7. Optimized conditions for preparation of biodiesel by lipase SL-4. (a) Effect of acyl acceptor type on fatty acid methyl (■)/ethyl (●) esters production catalyzed by free lipase SL-4 from soybean oil. Reaction conditions: soybean oil 0.55 g, short-chain alcohol/oil 3:1 (mol/mol), water content 15% (w/w, based on the oil weight, g), enzyme content 0.4% (w/w, based on the oil weight, g), 50 °C, 200 rpm, 36 h, short-chain alcohol was added in one step; **b**: Effect of molar ratio of methanol to oil (\blacktriangle) on biodiesel production catalyzed by free lipase SL-4 from soybean oil. Reaction conditions: soybean oil 0.55 g, water content 15% (w/w, based on the oil weight, g), enzyme content 0.4% (w/w, based on the oil weight, g), 50 °C, 200 rpm, 36 h, 1 time methanol was added; **c**: Effect of the methanol addition approach on biodiesel production catalyzed by free lipase SL-4 from soybean oil. Reaction conditions: soybean oil 0.55 g, methanol/oil 4:1 (mol/mol), water content 15% (w/w, based on the oil weight, g), enzyme content 0.4% (w/w, based on the oil weight, g), 50 °C, 200 rpm, 36 h, methanol was added in three steps at intervals of 6 h (▼), 8 h (□), 10 h (○) and 12 h (◀); **d**: Effect of the enzyme amount

off behavior of transesterification at higher lipase amounts is very typical, and it has also been reported by other researchers [17,26,44]. With increasing enzyme amounts, the reaction would likely become limited by the maximum adsorptive capacity of the interface, meaning that the interfacial area is too small to allow all of the enzymes to effectively adsorb to it [42]. Moreover, the addition of more lipase is not economical and practical because the matrix and lipase together make the solution extremely viscous, thus, increasing amounts of enzyme will not lead to further increases in biodiesel production [44].

3.6.5. Effect of water content on the biodiesel production

Water may be a “lubricant” that maintains enzymes in their active conformation [23]. During the transesterification reaction, water is necessary to maintain the enzyme configuration and to increase the available interfacial area between water and oil [20]. With increasing water addition, the amount of water available for oil to form oil–water droplets increases, which helps to maintain lipase activity [17]. The reaction rate can be reduced by a water content that is either too low, which results in a decreased contact area between the lipase and substrate, or too high, which results in a dilution of the lipase concentration [41]. In addition, it was found that the highest catalytic activity of enzyme could be achieved at the optimum water content, which depends on the enzyme, the reaction medium composition, the feedstock oil and the type of enzyme-catalyzed reaction [20,23]. Therefore, appropriate water content is an essential parameter in enzymatic biodiesel synthesis. Thus, the optimal water content (quality ratio of water to oil) for biodiesel production was determined (Fig. 7e). By increasing the water content from 2.5% to 12%, the biodiesel yield increased from 55.32% to 90.66%, while decreased slightly from 88% to 87.03% when the water content increased to 20%, and decreased substantially to 37.36% at a water content of 35%. The optimal water content (12%) of lipase SL-4 is slightly higher than those of *P. aeruginosa* 42A2 lipase (10%) [21] and soluble *Aspergillus niger* lipase NS81020 (10%) [26], and obviously lower than that of liquid recombinant *C. rugosa* lipase 2 (30–50%) [20]. The subsequent decrease in yield at higher water contents may be caused by diffusive limitations of the substrates. Furthermore, the presence of excessive water can bring about the accumulation of catalysts, which increases the biodiesel viscosity and reduces the reaction efficiency [23]. More water could also increase the hydrolysis of FAMEs and decrease the biodiesel conversion rate [45]. Based on this analysis, 12% water content was used in the following experiments.

3.6.6. Effect of reaction temperature and time on biodiesel production

The optimum temperature for liquid lipase SL-4 in the solvent-free system was 45 °C, with a biodiesel yield of 92.05% (Fig. 7f), and this temperature was fixed for the next experiment. It was observed that there was an increase in the biodiesel yield when the temperature was gradually increased from 30 to 45 °C because higher temperatures accelerate molecular diffusion [23]. Biodiesel conversion at temperatures from 40 to 50 °C was slight floating (88.01%, 92.05% and 86.66%, respectively) indicating that liquid lipase SL-4 was thermotolerant between 40 °C and 50 °C in the solvent-free system for the transesterification reaction. However,

increasing the temperature to 60 °C decreased the biodiesel yield to 77.32%. These results indicated that, as noted by Yan et al. [46], a temperature increase increases the rate of the transesterification reaction, but temperatures that are too high decreases the enzyme stability. Numerical studies revealed that the optimum temperature for enzymatic transesterification results from the interplay between the operational stability of the biocatalyst and the rate of transesterification, the alcohol/oil molar ratio, the type of methanol addition, the moisture concentration, and the thermostability of the enzymes [17]. As shown in Fig. 7g, the reaction proceeded at a fairly rapid rate up to 18 h (78.51% FAME yield), and that the FAME yield increased (82.52–92.24%) at 24–42 h, and finally stabilized (92.24–90%) at 42–72 h. After a 42-h reaction, the FAME yield reached its maximum of 92.24%. Then, the FAME yield almost remained the same, and even decreased slightly at 72 h. The partial inactivation of the enzyme by methanol and temperature might have contributed to the deceleration of the reaction.

Under the abovementioned optimal conditions for methanolysis of soybean oils in the solvent-free system, the liquid lipase SL-4 from *B. ubonensis* SL-4 attained a biodiesel yield of 92.24% at 42 h. This biodiesel yield is higher than those of lyophilized lipase G63 from *B. cepacia* G63 (87.8% after 72 h) [10], soluble lipase NS81020 from *A. niger* (88.7% after 36 h) [47], a whole cell of *Rhizopus oryzae* IFO4697 (<80% after 72 h) [43], immobilized lipase from *P. aeruginosa* LX1 (80% after 72 h in a tert-butanol system) [22] and a whole cell of *R. miehei* lipase displaying *Pichia pastoris* (83.14% after 72 h in isoctane) [23], and it was slightly lower than those of the soluble lipase NS81006 from *Aspergillus oryzae* (93.6% after 36 h) [24], liquid recombinant *C. rugosa* lipase 2 (95.3% after 48 h using *Jatropha curcas* seed oil) [20], and soluble Novozymes (96% after 24 h) [39]. This demonstrated that the purified lipase SL-4 is a very good biocatalyst for biodiesel production.

4. Conclusion

A novel extracellular lipase SL-4 from a newly isolated bacterium *B. ubonensis* SL-4 exhibited optimum activity toward p-NPC14 at pH 8.5 and 65 °C with a K_m of 0.72 mM, a k_{cat} of 391.63 s⁻¹ and a k_{cat}/K_m of 543.93 s⁻¹ mM⁻¹. It exhibited good thermostability with 86.04% residual activity after incubation at 55 °C for 12 h, even 70% residual activity after incubation at 55 °C for 24 h, and more than 90% activity after incubation at 50 °C for 24 h, and pH stability with nearly 79.61% and 94.02% residual activity at pH 8.0 and 8.5, respectively, after incubation for 6 h. Additionally, lipase SL-4 possessed non-ionic detergent stability in the presence of Tween-20, Tween-40, Tween-60, Tween-80 and Triton X-100, and good organic solvent stability in the presence of glycerol, ethylene glycol, DMSO, DMF, methanol, ethanol, *n*-hexane, *n*-heptane and isoctane. When preliminarily employed to catalyze soybean oil for biodiesel production, the liquid lipase SL-4 attained a conversion ratio of 92.24% in a solvent-free system. This study indicated that such a thermostable, solvent-tolerant and alkalescent-stable lipase possesses great potency as a biocatalyst in detergents formulation, the organic synthesis industry and transesterification reactions, especially for biodiesel production.

(◊) on biodiesel production catalyzed by free lipase SL-4 from soybean oil. Reaction conditions: soybean oil 0.55 g, methanol/oil 4:1 (mol/mol), water content 20% (w/w, based on the oil weight, g), 50 °C, 200 rpm, 36 h, methanol was added in three steps at intervals of 10 h; (e) Effect of the water content (Δ) on the biodiesel yield catalyzed by free lipase SL-4 from soybean oil. Reaction conditions: soybean oil 0.55 g, methanol/oil 4:1 (mol/mol), enzyme content 0.5% (w/w, based on the oil weight, g), 50 °C, 200 rpm, 36 h, methanol was added in three steps at intervals of 10 h; (f) Effect of temperature (▼) on the biodiesel yield catalyzed by free lipase SL-4 from soybean oil. Reaction conditions: soybean oil 0.55 g, methanol/oil 4:1 (mol/mol), enzyme content 0.5% (w/w, based on the oil weight, g), water content 12% (w/w, based on the oil weight, g), 200 rpm, 36 h, methanol was added in three steps at intervals of 10 h; (g) Effect of time (►) on the biodiesel yield catalyzed by free lipase SL-4 from soybean oil. Reaction conditions: soybean oil 0.55 g, methanol/oil 4:1 (mol/mol), water content 12% (w/w, based on the oil weight, g), enzyme content 0.5% (w/w, based on the oil weight, g), 45 °C, 200 rpm, methanol was added in three steps at intervals of 10 h.

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