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Purification and characterization of an organic solvent-tolerant lipase from *Pseudomonas aeruginosa* LX1 and its application for biodiesel production

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ABSTRACT

An organic solvent-tolerant lipase from newly isolated *Pseudomonas aeruginosa* LX1 has been purified by ammonium sulfate precipitation and ion-exchange chromatography leading to 4.3-fold purification and 41.1% recovery. The purified lipase from *P. aeruginosa* LX1 was homogeneous as determined by SDS-PAGE, and the molecular mass was estimated to be 56 kDa. The optimum pH and temperature for lipase activity were found to be 7.0 and 40 °C, respectively. The lipase was stable in the pH range 4.5–12.0 and at temperatures below 50 °C. Its hydrolytic activity was found to be highest towards *p*-nitrophenyl palmitate (C16) among the various *p*-nitrophenol esters investigated. The lipase displayed higher stability in the presence of various organic solvents, such as *n*-hexadecane, isooctane, *n*-hexane, DMSO, and DMF, than in the absence of an organic solvent. The immobilized lipase was more stable in the presence of *n*-hexadecane, *tert*-butanol, and acetonitrile. The transesterification activity of the lipase from *P. aeruginosa* LX1 indicated that it is a potential biocatalyst for biodiesel production.

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis of triacylglycerols to diacylglycerols, monoacylglycerols, fatty acids, and glycerol at the oil–water interface. In non-aqueous solvent systems, lipases can catalyze the reverse reactions, such as ester synthesis, with high regio- and stereoselectivity [1]. Although reactions catalyzed by lipases in the presence of organic solvents have many advantages [2], most enzymes, including lipases, are not stable in organic solvents, especially hydrophilic organic solvents, because of the tendency of organic solvents to strip water molecules from the enzyme surface, thereby leading to inactivation of the enzyme [3]. In order to overcome this limitation, several strategies, such as chemical modification, immobilization, and protein engineering, have been employed for the stabilization of enzymes for use in organic solvents [4]. Alternatively, it has been proposed that 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.

There have been some reports of the purification and characterization of solvent-tolerant lipases. On the one hand, lipases reported by some researchers have been characterized by a certain degree of solvent tolerance and have been applied in the synthesis of many useful products, such as biodiesel [5,6] and docosahexaenoic acid (DHA) [7]. Besides, resolutions of (±)-methyl *trans*-3-(4-methoxyphenyl)glycidate (MPGM) [8,9] and (*R*,*S*)-phenyl ethyl acetate [10] by solvent-tolerant lipases based on their good stereoselectivity have also been described. On the other hand, some researchers have isolated solvent-tolerant microorganisms producing solvent-tolerant lipases. These lipases proved to be stable at high concentrations in organic solvents [11–18]. However, it remains a challenge to meet the requirements for practical catalysis, especially in view of the broad range of substrates catalyzed by lipases. Hence, it is necessary to explore new solvent-tolerant lipases.

In the work described in this article, we have isolated a solvent-tolerant lipase-producing strain LX1, identified as *Pseudomonas aeruginosa*. The purification and characteristics of the lipase, especially its stability in the presence of various organic solvents, are reported. In addition, the lipase has been shown to be potentially useful for biodiesel synthesis based on its good solvent tolerance.

2. Materials and methods

2.1. Materials

All *p*-nitrophenyl esters examined and high-performance liquid chromatography (HPLC)-grade solvents were purchased from Sigma (St. Louis, MO, USA). DEAE-Sepharose and protein molecular

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weight markers were purchased from Amersham Biosciences (Uppsala, Sweden). All other chemicals used were of analytical grade.

2.2. Screening of organic solvent-tolerant lipase-producing strains

Soil samples were collected from an oil- and chemical-contaminated environment located in Jiangsu Province, China. Toluene and cyclohexane were added to the enrichment medium (peptone 0.1%, corn steep liquor 0.18%, $(NH_4)_2SO_4$ 0.35%, KH_2PO_4 0.3%, NaCl 0.25%, $MgSO_4$ 0.1%, and sunflower oil 5%) separately, each at a concentration of 10% (v/v). The cultivation vessel was plugged with a chloroprene-rubber stopper to prevent evaporation of the organic solvent. Cultivations were conducted in 10 mL of medium in 50 mL test tubes at 30 °C with agitation at 180 rpm for 48 h. The culture was transferred to fresh medium three times. Then, samples of the cultures were diluted and spread on tributyrin agar plates (yeast extract 1.0%, tryptone 0.25%, NaCl 0.5%, tributyrin 0.5%, and agar 1.5%). Colonies that secreted a lipase hydrolyzed the tributyrin, leading to a clear zone around the colony. Colonies showing lipase activity were gathered and re-spread on Rhodamine B agar plates (yeast extract 0.1%, corn steep liquor 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, olive oil 6%, Rhodamine B 0.0024%, and agar 1.8%). The plates were incubated at 30 °C for 48 h. The microbes showing high ratios of orange fluorescent halo diameter to colony diameter under UV irradiation at 350 nm were selected as potential lipase producers. In order to isolate for the strain with wide organic solvents stability, the effect of various organic solvents at 25% (v/v) concentration on lipase activity was investigated. Strain LX1 secreting the lipase with broad organic solvent tolerance was selected and identified by means of a 16 S rDNA and Microlog Microbial Identification System (Biolog, USA).

2.3. Lipase production

Inoculum doses were prepared by transferring loopfuls of fresh *P. aeruginosa* LX1 cells into LB medium followed by incubation at 30 °C with shaking at 180 rpm. After 10 h of incubation, a bacterial inoculum of 2 mL was inoculated into 40 mL of basic medium in a 250 mL Erlenmeyer flask. The composition of the preliminary optimized medium was as follows: corn steep liquor 2%, beef extract 1%, glucose 0.5%, rapeseed oil 0.5%, K_2HPO_4 0.1%, and $MgSO_4 \cdot 7H_2O$ 0.05%. The pH was adjusted to 7.0 with 1 M NaOH. The incubations were performed at 30 °C with shaking at 200 rpm. The culture was harvested after 36 h of incubation and the supernatant was obtained by centrifugation at 12,000 × *g* at 4 °C for 20 min. The supernatants were used for purification studies and lipase assays.

2.4. Purification of the lipase

2.4.1. Ammonium sulfate precipitation

Solid ammonium sulfate was added to the culture supernatant to 20% saturation. The mixture was kept at 4 °C for 2 h under very slow stirring, and then the solid contents were centrifuged at 12,000 × *g* at 4 °C for 20 min. The precipitate was discarded, further solid ammonium sulfate was added to the supernatant up to 50% saturation, and the resulting mixture was kept at 4 °C for a further 4 h. The resulting precipitate was collected by centrifugation at 12,000 × *g* for 30 min. This precipitate was redissolved in 0.05 M sodium phosphate buffer (pH 7.0) and dialyzed overnight in the same buffer.

2.4.2. Ion-exchange chromatography

The above ammonium sulfate precipitated fraction was applied to a DEAE-Sepharose FF column (1 × 5 mL; Amersham Biosciences, Sweden) that had been pre-equilibrated with 0.01 M Tris–HCl buffer (pH 7.1). The effluent was obtained at a flow rate of 1.0 mL/min using the ÄKTA prime plus system (Amersham Biosciences, Sweden) and was monitored at 280 nm. The bound protein was then eluted by increasing the concentration of NaCl in the 0.01 M Tris–HCl (pH 7.1) buffer from 0.2 to 0.8 M with a linear gradient. Fractions that exhibited lipase activity were pooled and used for further analysis.

2.5. Measurement of lipase activity

Lipase activity was determined as described by Winkler and Stuckmann [19] with some modifications. The substrate *p*-nitrophenyl palmitate (*p*NPP) (3 mg) with final concentration of 0.3 mg/mL was dissolved in 1 mL of isopropanol and mixed with 9 mL of 0.05 M sodium phosphate buffer (pH 7.0) containing gum arabic (0.1%) and triton X-100 (0.6%). The reaction was carried out at 40 °C by adding 10 μL of appropriately diluted enzyme solution to 240 μL of substrate solution after pre-incubation for 5 min, and incubation was continued for a further 10 min. The test tubes were immersed in ice prior to measurement of the optical density at 410 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol/min of *p*-nitrophenol (*p*NP) under the standard assay conditions.

2.6. Protein determination

The protein concentrations in this study were determined by the method of Bradford [20] using bovine serum albumin as a standard.

2.7. Estimation of the purity and molecular mass of the purified lipase by SDS-PAGE

SDS-PAGE was carried out according to the method of Laemmli [21] using a 12.5% cross-linked polyacrylamide gel and an Amersham gel electrophoresis unit. The protein bands were stained with Coomassie brilliant blue R-250 (Sigma). The molecular weight of the lipase was estimated by comparing the relative mobilities of proteins of different molecular weights as standard markers in the range 116.0–14.4 kDa (Fermentas, Germany).

2.8. Effect of pH on the activity and stability of the lipase

To investigate the optimum pH, lipase activity was determined at 40 °C at various pH values (6.0–9.0). The stability of the lipase in the range pH 4.0–12.0 was examined by incubating solutions of the enzyme for 1 h at 30 °C at different pH values, and the residual activity was measured according to the *p*NPP method described above.

2.9. Effect of temperature on the activity and thermostability of the lipase

To investigate the optimum temperature, lipase activity was measured in the range 25–60 °C in 0.05 M sodium phosphate buffer (pH 7.0). To assess the effect of temperature on lipase stability, the lipase was incubated at different temperatures (30–70 °C) for 1 h and then its residual activity was measured according to the *p*NPP method.

2.10. Effect of metal ions on the activity of the lipase

The effects of various metal ions, namely Zn^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, K^+, Fe^{2+}, Li^+, Mn^{2+}, Co^{2+}, and Ba^{2+} (1 and 10 mM), and of the metal-chelating agent ethylenediaminetetraacetic acid (EDTA) on the lipase activity in 0.05 M sodium phosphate buffer (pH 7.0) were investigated. In these experiments, the lipase was pre-incubated

Table 1
Purification of the lipase from *Pseudomonas aeruginosa* LX1.

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Supernatant of culture	721	19.8	36.4	100	1.0
Ammonium sulfate precipitation	424	6.6	64.6	58.7	1.8
DEAE-Sepharose FF	296	1.9	156	41.1	4.3

with the respective metal ions for 1 h at 30 °C, and then its residual activity was tested by the *p*NPP method.

2.11. Substrate specificity

Substrate specificities towards different *p*-nitrophenyl (*p*NP) esters were determined at 40 °C in 0.05 M sodium phosphate buffer (pH 7.0) by means of the *p*NPP method. Synthetic *p*NP esters with chain lengths between C2 and C18, namely *p*NP acetate (C2), *p*NP butyrate (C4), *p*NP caproate (C8), *p*NP decanoate (C10), *p*NP myristate (C14), *p*NP palmitate (C16), and *p*NP stearate (C18), were used as substrates at the final concentration of 0.3 mg/mL, separately.

2.12. Effect of organic solvents on the activity and stability of the lipase

The effect of organic solvents of different log *P* values (the logarithm of the partition coefficient of that solvent between *n*-octanol and water) at 25% (v/v) concentration on the stability of the purified lipase was investigated [15]. The purified lipase dissolved in 0.05 M sodium phosphate buffer (pH 7.0) was filter sterilized (using cellulose acetate membrane, 0.22 μm). Aliquots (1 mL) of the organic solvents were added to portions (3 mL) of filtrate (25%, v/v) in glass vials. The vials were sealed and the mixtures were incubated at 30 °C with shaking at 150 rpm. The residual lipase activity was determined by the *p*NPP method every day. If the residual activity was more than 50% after 10 d, half-life was taken as ">10 d". If the residual activity was less than 50% after 1 h, half-life was taken as "<1 h".

The stabilities of the immobilized lipase in the respective organic solvents (100%) were determined by incubating 10 mg of immobilized lipase in each solvent at 30 °C. The immobilized lipase was separated from the organic solvents by centrifugation at 12,000 × *g* for 10 min and the residual activity was measured using the *p*NPP method.

2.13. Immobilization of the lipase

The immobilized lipase was prepared as follows. One gram of Celite (Xilong Chemical Company, Shantou, China) was added to 5 mL of solution containing 0.4 mg of purified lipase (62.4 U), and the mixture was stirred for 1 h at 20 °C. The immobilized lipase was recovered through centrifugation, dried in a vacuum desiccator, and stored at 4 °C. The activity of the immobilized lipase was determined to be about 20 U/g by the *p*NPP method.

2.14. Production of biodiesel catalyzed by the lipase

Soybean oil (5 mmol, ~4.5 g), methanol (15 mmol, 600 μL) and water (0.27 g) were dissolved in *tert*-butanol (5 mL), and then immobilized lipase (1 g) was added. The transesterification reactions were carried out at 30 °C at 180 rpm. Aliquots (100 μL) of the reaction medium were withdrawn and diluted with *n*-heptane for GC analysis.

The methyl ester contents of the reaction mixtures were measured on a gas chromatograph (Agilent Technologies Model 6890N) equipped with an SE-30 capillary column (0.33 μm × 0.25 mm × 30 m; Agilent). Nitrogen was used as carrier gas at a flow rate of 1.0 mL/min. The column temperature was kept at 200 °C for 1 min, raised to 240 °C at 4 °C/min, and then maintained at 240 °C for 6 min. The injector and detector temperatures were both set at 280 °C. Methyl esters of palmitic, stearic, oleic, and linoleic acids were purchased from Sigma as standards.

3. Results and discussion

3.1. Selection of the most potentially solvent-tolerant lipase producer

The solvent-tolerant microbes were found to be good sources of solvent-stable enzymes, especially extracellular enzymes [17,18]. By the screening method described above, 123 strains of solvent-tolerant lipase producers were obtained. Of these, six showed a high ratio of orange fluorescent halo clear zone diameter to colony diameter on Rhodamine B agar plates. These strains were belonged to *P. aeruginosa*, *Pseudomonas stutzeri*, *Acinetobacter junii*, *Burkholderia ambifaria* and *Burkholderia cepacia*. The crude lipase secreted by strain LX1 among the six strains was found to be quite stable in the presence of hydrophilic organic solvents and showed higher activity in the preliminarily optimized medium. Organic solvent-tolerant lipase producer strain LX1 was identified as *P. aeruginosa* on the basis of 16S rDNA and by means of a Biolog Automated Micro-Station System. Strain LX1 was deposited in the China Center for Type Culture Collection (Wuhan, China) with the accession number CCTCC M 209221.

3.2. Purification of the lipase from P. aeruginosa LX1

The extracellular lipase from strain LX1 was purified by ammonium sulfate precipitation and DEAE-Sepharose FF anion-exchange chromatography (Table 1). A single band was obtained for the lipase by SDS-PAGE analysis with about 4.3-fold purification and 41.1% recovery. The lipase was thus purified simply and with higher recovery. Ogino et al. [15] reported purification of the lipase from *P. aeruginosa* LST-03 by four successive steps with 12.6% recovery. Relative low purification yields of 2.1% for the lipase from *P. aeruginosa* MTCC 5113 [9] and 16% for the lipase from *P. aeruginosa* San-ai [22] have also been reported. Rahman et al. [17] succeeded in achieving a higher recovery of 52% of the lipase from *Pseudomonas* sp. strain S5 using affinity chromatography in combination with ion-exchange chromatography.

The result of SDS-PAGE of the purified lipase from strain LX1 is shown in Fig. 1. The purified lipase was homogeneous and its molecular mass was estimated to be 56 kDa. The reported solvent-tolerant lipases from the *Pseudomonas* genus are summarized in Table 2. It was shown that the molecular masses of the solvent-tolerant lipases mainly fall into two groups, 30–45 and 50–60 kDa. Therefore, the present lipase could be related to the higher molecular mass lipases.

3.3. Effect of pH on the activity and stability of the lipase

The optimal pH for the lipase activity was 7.0 (Fig. 2). The lipase activity of strain LX1 in pH 8.0 buffer was only 47% relative to that in pH 7.0 buffer. To the best of our knowledge, the optimal pH values of

Table 2
Molecular mass, temperature, and pH properties of some solvent-tolerant lipases from different microorganisms.

Microorganism	Optimum temperature (°C)	Optimum pH	pH stability	Molecular mass (kDa)	References
Pseudomonas aeruginosa LST-03 (lip9)	37	6.0	5.0–8.0	27.1	[15,16]
Burkholderia cepacia strain G63	70	–	–	33	[5]
Pseudomonas aeruginosa LST-03 (lip3)	35	6.0–7.0	5.0–8.0	34.8	[13]
Pseudomonas aeruginosa LST-03 (lip8)	30	7.0	–	41	[14]
Burkholderia multivorans V2	45	8.0	–	44	[23]
Pseudomonas monteilii TKU009	40	7.0	8.0–11.0	44	[6]
Pseudomonas aeruginosa San-ai	70	11.0	4.0–11.5	54	[22]
Pseudomonas fluorescens JCM5963	55	9.0	5.0–9.5	55	[24]
Pseudomonas aeruginosa MTCC 5113	–	–	–	59.4	[9]
Pseudomonas aeruginosa PseA	40	8.0	6.0–8.5	60	[18]
Pseudomonas sp. strain S5	45	9.0	6.0–9.0	60	[17]
Pseudomonas aeruginosa LX1	40	7.0	4.5–12.0	56	This article

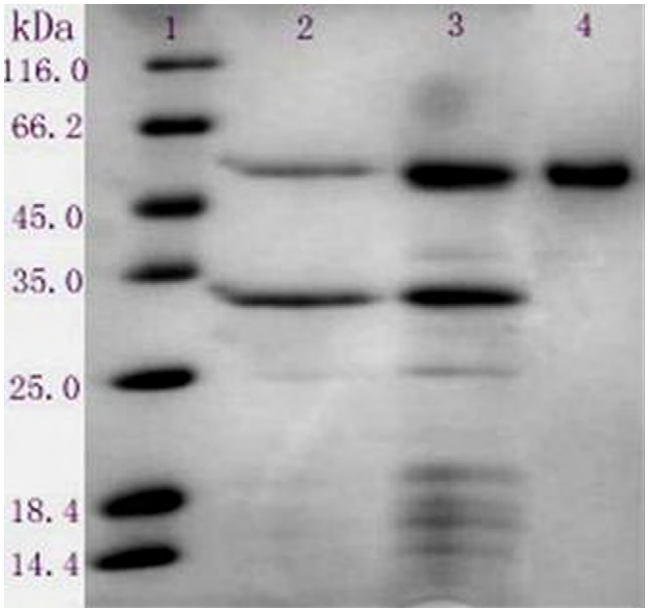

Fig. 1. SDS-PAGE analysis of the purified lipase from *Pseudomonas aeruginosa* LX1. Lane 1, protein molecular weight marker; lane 2, supernatant; lane 3, lipase concentrated by ammonium sulfate precipitation; lane 4, lipase purified by DEAE-Sepharose FF.

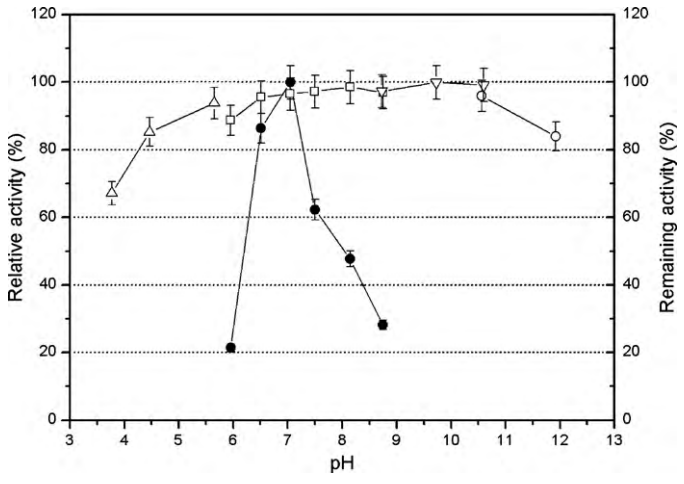

Fig. 2. Effect of pH on activity and stability of the lipase. The activities (●) are shown as values relative to that measured in 0.05 M sodium phosphate buffer pH 7.0 (taken as 100%). The stability of the lipase was incubated at 30 °C for 1 h in the following buffer systems: 0.05 M citric acid/sodium citrate (pH 3.0–6.0) (△), 0.05 M Na_2HPO_4/KH_2PO_4 (pH 6.0–8.5) (□), 0.05 M Gly/NaOH (pH 8.6–10.6) (▽), and 0.05 M Na_2HPO_4/NaOH (pH 10.0–12.0) (○). The remaining activities of the lipase are shown as values relative to that before incubation (taken as 100%). The composition of the reaction mixture was 0.1 mg/mL of lipase and 0.3 mg/mL *p*NPP with different buffers.

hitherto reported solvent-tolerant lipases with molecular masses in excess of 50 kDa from the *Pseudomonas* genus have been above 8.0 (Table 2). The lipase from strain LX1 showed good stability in the broad pH range 4.5–12.0 for 1 h (Fig. 2) and retained 83.9% of its original activity after treatment with pH 12.0 buffer. The lipase from *P. aeruginosa* San-ai showed the similar pH stability, but with quiet different of optimum pH 11 [22]. These differences suggest that the lipase from *P. aeruginosa* LX1 might be a novel pH and solvent-tolerant lipase.

3.4. Effect of temperature on the activity and thermostability of the lipase

The optimal temperature for the lipase activity was observed to be 40 °C (Fig. 3), in agreement with data for most other lipases from the *Pseudomonas* genus (Table 2). The lipase was stable below 50 °C and retained 64% of its activity after incubation at 60 °C for 1 h (Fig. 3). The half-life of the lipase from *Pseudomonas* sp. strain S5 [17] at 50 °C was 1 h, but the lipase from *P. aeruginosa* LST-03 [15] showed lower stability at 40 °C with a half-life of just 10 min.

3.5. Effect of metal ions on the activity of the lipase

The lipase activity was found to be significantly stimulated in the presence of Ca^{2+}, Ba^{2+}, and Mg^{2+} (Table 3). Many lipases have been found to display enhanced activity in the presence of Ca^{2+}

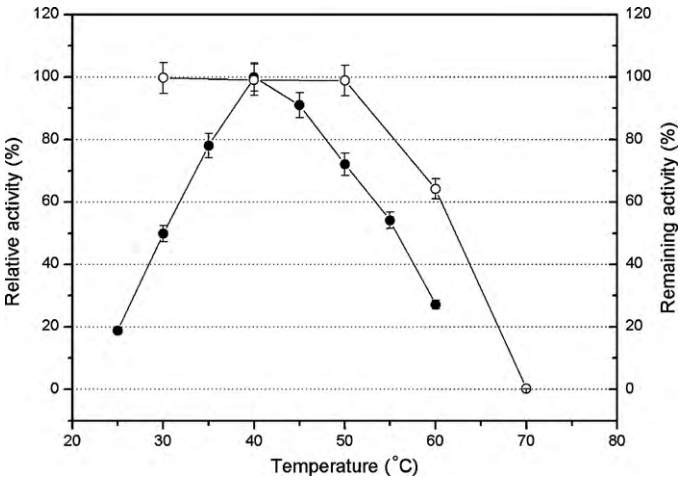

Fig. 3. Effect of temperature on activity and thermostability of the lipase. The activities (●) were shown as values relative to that measured at 40 °C (taken as 100%). The thermostability of the lipase was incubated in 0.05 M sodium phosphate buffer (pH 7.0) at various temperatures for 1 h. The remaining activities (○) of the lipase are shown as values relative to that before incubation (taken as 100%). The composition of the reaction mixture was 0.1 mg/mL of lipase and 0.3 mg/mL *p*NPP incubated at different temperature.

Table 3
Effects of various metal ions and EDTA on activity of the lipase.

Metal ions	Relative activity (%)	
	1 mM	10 mM
Control	100	100
Zn^{2+}	13.8	4.5
Ca^{2+}	121	140
Mg^{2+}	115	106
Cu^{2+}	62.4	18.2
K^{+}	88.1	105
Fe^{2+}	65.1	10.3
Li^{+}	99.3	103
Mn^{2+}	107	78.7
Co^{2+}	93.2	72.7
Ba^{2+}	120	149
EDTA	121	112

The lipase was incubated with various metal ions (1 and 10 mM) in 0.05 M sodium phosphate buffer (pH 7.0) at 30 °C for 1 h. The activities are shown as values relative to that measured without addition of any metal ions (control).

[12,17,18,23,24]. A possible explanation for this phenomenon is that Ca^{2+} binds to the active site of the lipase and changes the conformation of the protein [17]. Conversely, Fe^{2+}, Cu^{2+}, and Zn^{2+} ions significantly inhibited the activity of the lipase. The activity was not inhibited but activated by EDTA at concentration of 1 and 10 mM, thus suggesting that the lipase is not a metalloenzyme.

3.6. Substrate specificity

The substrate specificity showed that *p*-nitrophenyl palmitate (C16) was most efficiently hydrolyzed by the lipase (Fig. 4). The lipase from *P. aeruginosa* PseA [18] showed similar substrate specificity to ours. However, the highest hydrolytic activities of the lipases from *P. fluorescens* JCM5963 [24] and *P. monteilii* TKU009 [6] were seen with *p*NP caproate (C8) and *p*NP myristate (C14), respectively.

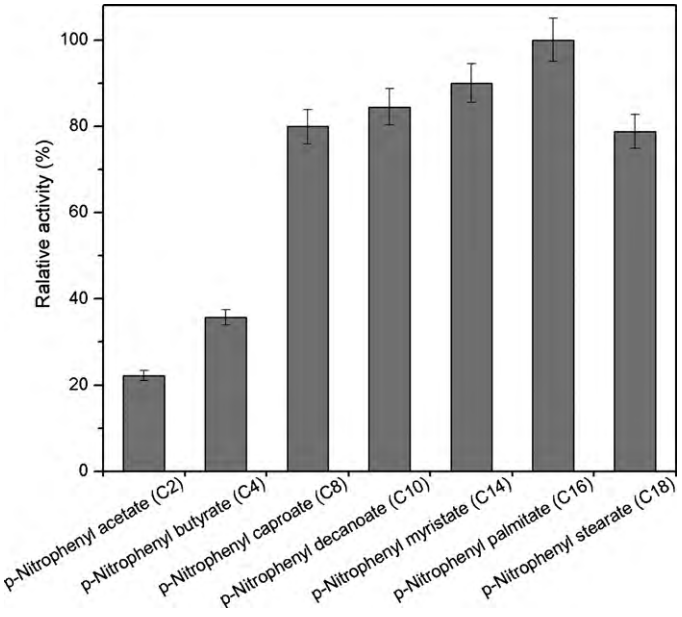

Fig. 4. Substrate specificity of the lipase towards different *p*-nitrophenyl esters. The activities are shown as values relative to the maximum activity measured towards *p*-nitrophenyl palmitate (taken as 100%). The composition of the reaction mixture was 0.1 mg/mL of lipase and 0.3 mg/mL synthetic *p*NP esters.

Table 4
Effects of organic solvents on activity and stability of the lipase.

Organic solvents	Log *P*	Residual activity (%)	
		Purified lipase	Immobilized lipase
Control[a]	–	56.3 (2 d)[b]	100 (>10 d)
n-Hexadecane	8.8	115 (>10 d)	100 (>10 d)
Isooctane	4.7	105 (>10 d)	52.4 (1 h)
n-Hexane	3.5	97.1 (10 d)	81.3 (1 d)
tert-Butanol	0.18	30.8 (1 d)	99.1 (>10 d)
Acetone	−0.23	84.0 (4 d)	80.2 (1 d)
Ethanol	−0.24	62.0 (2 d)	58.0 (1 h)
Acetonitrile	−0.34	14.6 (1 d)	91.8 (>10 d)
Methanol	−0.76	60.1 (2 d)	2.2 (<1 h)
DMF	−1.0	93.3 (5 d)	2.8 (<1 h)
DMSO	−1.35	90.3 (5 d)	1.4 (<1 h)
Glycerol	−1.76	111 (>10 d)	3.4 (<1 h)

The purified lipase was incubated with various solvents (25%, v/v) at 30 °C for 48 h with shaking at 150 rpm. The immobilized lipase was incubated in 100% of each solvent at 30 °C for 1 h with shaking at 150 rpm.

[a] The activities of the purified and immobilized lipase in the absence of organic solvents were taken as controls.

[b] The numbers in brackets are the half-lives of the lipase in the various solvents.

3.7. Effect of organic solvents on the activity and stability of the lipase

As shown in Table 4, more than 90% of the lipase activity was maintained for 2 d in the presence of *n*-hexadecane, isooctane, *n*-hexane, DMSO, DMF, or glycerol compared to the control. Furthermore, *n*-hexadecane and glycerol even increased the lipase activity to 115% and 111%, respectively. The activation of lipase could be explained that organic solvent molecules could interact with hydrophobic amino acid residues present in the lid that covers the catalytic site of the enzyme, thereby maintaining the lipase in its open conformation and conducting to catalyze [25]. Besides, the lipase showed longer half-lives in these organic solvents except acetonitrile and *tert*-butanol than in the absence of an organic solvent. The longer stability of the lipase in organic solvents was useful for catalysis.

On the other hand, the activities of the immobilized lipase in 100% DMSO, DMF, or glycerol were drastically reduced after 1 h, although the immobilized lipase was stable in *n*-hexadecane, *tert*-butanol, and acetonitrile (half-lives >10 d). The activities of the free lipase in *tert*-butanol and acetonitrile after 48 h were, however, only 30.8% and 14.6%, respectively. The results indicated that immobilization of the lipase could not only enhance its stability but also significantly change its solvent stability. Similar results were reported by Bhushan for lipase immobilized on synthetic beaded macroporous copolymers, whereby the enzyme was rendered almost inactive in DMSO and DMF [26]. In contrast, Costas reported that the activity of lipase immobilized in pectin microspheres was enhanced by DMSO and glycerol [27]. These results indicate that different supports might shift the solvent stabilities of the immobilized lipases.

3.8. Application of the lipase for biodiesel production

Considering that the immobilized lipase was stable in *tert*-butanol and that the active site of the lipase seemingly better accommodated long-chain substrates, the lipase was deemed likely to be very suitable for biodiesel production. The content of methanol and by-product glycerol in the reaction mixture was limited. *tert*-Butanol was used as the solvent to reduce the influence of methanol and glycerol on the immobilized lipase. The *tert*-butanol is a tertiary alcohol and not a substrate for most of the lipases [28,29]. In addition, it was more convenient to separate biodiesel and solvent since the solidifying point of *tert*-butanol is

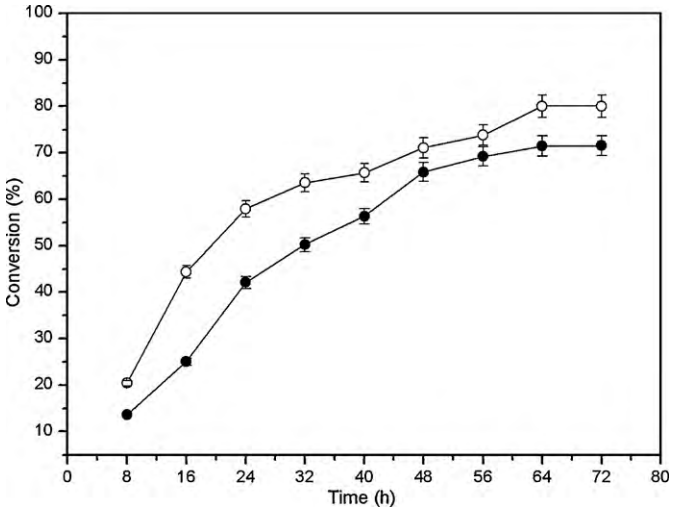

Fig. 5. Time course of biodiesel production catalyzed by the lipase from *Pseudomonas aeruginosa* LX1. Soybean oil (4.5 g) and methanol were taken in the ratio 1:3 mol mol^{-1} in a screw-capped vials dissolving in 5 mL of *tert*-butanol. Immobilized LX1 lipase (1 g, ~20 U) or free LX1 lipase (~20 U) was added to the reaction mixture and incubated at 30 °C at 180 rpm. The production methyl esters were measured by GC.

high (25 °C). Methyl esters of palmitic, linoleic, oleic, and stearic acids constituted biodiesel and were detected by GC at 8.9, 11.8, 12.0, and 12.5 min, respectively. Biodiesel production with 71.5% and 80% yields were achieved by free and immobilized lipase (Fig. 5), respectively. Immobilized lipases were known to give better transterification activities in the organic solvents [30]. This might be due to larger surface area of the immobilized lipase. However, free lipases were known to have mass transfer problem since these form aggregates in low water media [31].

4. Conclusion

In this study, an organic solvent-stable lipase from the organic solvent-tolerant *P. aeruginosa* strain LX1 was purified by a simple purification procedure with 41.1% recovery. The lipase with the molecular mass of about 56 kDa showed extreme stability in the broad pH range 4.5–12.0. The free and immobilized lipase exhibited good stability and activation in the presence of some organic solvents. The immobilized lipase was found to catalyze biodiesel synthesis in *tert*-butanol with higher yield. These results make the solvent-tolerant lipase more potentially valuable for biotechnological applications in non-aqueous catalysis.

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