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ORIGINAL PAPER

A novel thermostable and organic solvent-tolerant lipase from *Xanthomonas oryzae* pv. *oryzae* YB103: screening, purification and characterization

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Abstract Thermostable lipases offer major biotechnological advantages over mesophilic lipases. In this study, an intracellular thermostable and organic solvent-tolerant lipase-producing strain YB103 was isolated from soil samples and identified taxonomically as *Xanthomonas oryzae* pv. *oryzae*. The lipase from *X. oryzae* pv. *oryzae* YB103 (LipXO) was purified 101.1-fold to homogeneity with a specific activity of 373.9 U/mg. The purified lipase showed excellent thermostability, exhibiting 51.1 % of its residual activity after incubation for 3 days at 70 °C. The enzyme showed optimal activity at 70 °C, suggesting it is a thermostable lipase. LipXO retained 75.1–154.1 % of its original activity after incubation in 20 % (*v/v*) hydrophobic organic solvents at 70 °C for 24 h. Furthermore, LipXO displayed excellent stereoselectivity (e.e.$_p$ >99 %) toward (S)-1-phenethyl alcohol in *n*-hexane. These unique properties of LipXO make it promising as a biocatalyst for industrial processes.

Keywords Biotechnology · Enzyme stability · Enzyme application · Enzyme structure · Function · Synthesis in organic solvents · Protein stability

Introduction

Lipase is a class of versatile enzyme capable of showing high enantioselectivity and broad substrate specificity simultaneously. Lipases (triacylglycerol hydrolases, EC 3.1.1.3) catalyze both the hydrolysis and the synthesis of acylglycerides and other fatty acid esters at high activity, regioselectivity, and stereoselectivity in aqueous and non-aqueous media (Gotor-Fernández et al. 2006; Tran et al. 2012). Although lipases are produced by animals, plants, and microorganisms (Franken et al. 2011; Cardenas et al. 2001), microbial lipases have an immense prospective for commercial applications (Haki and Rakshit 2003). They are widely utilized in a number of applications in various industries, including the biodiesel, pharmaceutical, detergent, cosmetic, and food industries (Dheeman et al. 2011; Dandavate et al. 2009).

Thermostable lipases usually function effectively at high temperatures, in comparison to mesophilic or cold-active lipases which show little activity at high temperature. In this respect, the thermostability of lipases is of paramount importance for any bioprocess in industry due to most of other enzymes being inherently unstable (Ebrahimpour et al. 2011; Faoro et al. 2012; Mahadevan and Neelagund 2014). Utilization of thermostable lipases in elevated temperatures can take several advantages, including increasing the reaction rates due to a decrease in viscosity and an increase in diffusion coefficient of substrates, reducing the risk of contamination by common mesophiles, and improving the process yield due to increased solubility of substrates (Haki and

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Rakshit 2003; Ebrahimpour et al. 2011; Vieille and Zeikus 2001). Therefore, discovery of novel thermostable lipases is prerequisite for industrial application.

Organic solvent-tolerant lipases offer new possibilities such as enabling the use of hydrophobic substrates and shifting of the thermodynamic equilibria in favor of synthesis (Ahmed et al. 2010; Doukyu and Ogino 2010; Lima et al. 2004). Furthermore, of particular importance has been the discovery that enzymatic stereoselectivity (substrate, stereo-, regio- and chemoselectivity) can be markedly affected by the solvent (Klibanov, 2001; Jaeger and Eggert 2002). If the lipases are intrinsically stable and active in solvents, it should make them more useful for enzymatic reaction in chemical industry.

In this work, we describe the identification, purification, and characterization of a novel thermostable and organic solvent-tolerant lipase (LipXO) from strain *Xanthomonas oryzae* pv. *oryzae* YB103. This lipase has been found stable not only at high temperature, but also in the presence of organic solvents. Furthermore, the lipase showed excellent stereoselectivity toward (S)-1-phenethyl alcohol in *n*-hexane. The characteristics of LipXO make it a potential biocatalyst for industrial biochemical processes.

Materials and methods

Materials

p-Nitrophenol (*p*NP), *p*-nitrophenyl-acetate, *p*-nitrophenyl-butyrate, *p*-nitrophenyl-caproate, *p*-nitrophenyl-octanoate (*p*NPO), *p*-nitrophenyl-decanoate, *p*-nitrophenyl-laurate (*p*NPL), *p*-nitrophenyl-myristate, *p*-nitrophenyl-palmitate, *p*-nitrophenyl-stearate, triacylglycerols (glyceryl triacetate, glyceryl tributyrate, glyceryl trihexanoate, glyceryl trioctanoate, glyceryl tridecanoate, glyceryl tridodecanoate, glyceryl trimyristate, glyceryl tripalmitate and glyceryl tristearate), (R, S)-1-phenethyl alcohol and (R)-1-phenethyl acetate were acquired from Sigma-Aldrich Co., Ltd. (Shanghai, China). All other chemicals used were of analytical grade from Guoyao Co. Ltd. (Shanghai, China).

Selection and identification of thermostable lipase-producing strain

One hundred and twenty-seven soil samples were collected from different location of Hubei province, China. Each soil sample (0.1 g) was subjected to cultivation in a 50-mL olive oil medium (olive oil 1.0 %, $(NH_4)_2SO_4$ 1.0 %, Na_2HPO_4 0.3 %, $MgSO_4$, 0.01 %, pH 7.2) at 37 °C for 48 h with a shaking rate of 200 rpm. The samples of culture were streaked on triolein agar plates. The bacterial colonies forming transparent zones after incubation for 48 h at 37 °C were further purified by repeated streaking. After incubation in the olive oil medium, the clear supernatant, as crude extracellular lipase, was obtained by centrifugation at 8000 × g and 4 °C for 10 min. The crude intracellular lipase was obtained as follows. The harvested cells were resuspended in Tris–HCl buffer (50 mM, pH 7.2) and disrupted by sonication (240 W). The resultant cell debris was removed by centrifugation at 12,000 × g and 4 °C for 20 min, and the clear supernatant was used as intracellular lipase. The crude (intracellular and extracellular) lipase was incubated at 70 °C for 24 h, and then the remaining activity was determined at 50 °C by the colorimetric method using *p*NPO as substrate. The strain was identified based on morphological and 16S rDNA sequence by the method described previously (Hold et al. 2002).

Purification of intracellular LipXO

A 24-h growth of the isolated bacterial strain YB103 was inoculated into 100 mL medium (olive oil 1.0 %, yeast extract 1.0 %, tryptone 1.0 %, NaCl 0.5 %, $(NH_4)_2SO_4$ 1.0 %; pH 7.2) contained in 500 mL flask. The flask was incubated at 37 °C with shaking (200 rpm) for 48 h. Lipase was purified as we described previously with a little modification (Li et al. 2013). The crude intracellular lipase was ultrafiltrated using a 40 kDa cutoff centrifugal membrane filter (Millipore, USA). The resultant concentrated solution was precipitated by adding solid ammonium sulfate at saturation of 65 %, and maintained at 4 °C for 18 h. Then the solution was centrifuged at 8000 × g for 10 min. Pellet was collected and suspended in minimum volume of Tris–HCl buffer (25 mM, pH 7.2). The highly active fractions were pooled and applied on phenyl Sepharose column (75 cm × 1.25 cm, GE, USA) previously equilibrated with Tris–HCl buffer (25 mM, pH 7.2). The unbound proteins were eluted by same buffer while the bound proteins were eluted with $(NH_4)_2SO_4$ gradient from 1.4 to 0.0 M. The column was run at flow rate of 0.5 mL/min. The resultant fractions were detected for lipase hydrolytic activity by the colorimetric method using *p*NPL as the substrate, and stored at 4 °C for further study. The lipase preparations (crude and purified lipase) were electrophoresed on a SDS-PAGE gel (10 % polyacrylamide).

Biochemical characterization

Effect of temperature on lipase activity and stability

Optimum temperature of LipXO was determined by assessing the hydrolytic activity at various temperatures (4–90 °C, at the interval of 10 °C) under standard conditions (50 mM CHES buffer, pH 9.0). To study thermostability, purified lipase was incubated at various temperatures

(4, 50 and 70 °C) for up to 7 days. After the lipase solution was heated to appointed temperature, the pH value was adjusted to the desired value at this temperature on a Mettler Toledo S470-USP/EP Seven Excellence pH/Conductivity Meter (Schwerzenbach, Switzerland). Before the assay of activity, the temperature and pH of solution were adjusted to 70 °C and 9.0, respectively. Aliquots of enzyme solution were withdrawn at various time intervals, and the residual hydrolytic activity was measured by the colorimetric method using *p*NPL as the substrate at 70 °C.

Circular dichroism (CD) spectra

Circular dichroism spectroscopy was performed on a Jasco 1500 spectropolarimeter equipped with peltier-type temperature controller (Jasco, Inc., Easton, MD). Purified LipXO protein samples were prepared as described above and then diluted to 5.0 μmol/L in 50 mM CHES buffer (pH 9.0) for CD analysis in a 1.0-mm path length cell. Variable wavelength measurements of protein solutions were scanned at 25 °C from 180 to 260 nm with a scan rate 50 nm/min and data points were collected every 0.1 nm. The signal was averaged over six scans. Each spectra was acquired independently three times. Thermal denaturation experiments were conducted using 5 μmol/L protein in a 1.0 mm quartz cuvette. The protein samples were heated from 5 to 90 °C at a rate of 1.0 °C/min. Thermal denaturation profiles were displayed in molar ellipticity at 222 nm. For all experiments, 222 nm voltages were within accepted limits (<700 V at 216 nm and significantly lower for 222 nm), which allowed for monitoring of thermally induced loss of secondary structure by plotting ellipticity at 222 nm versus temperature. The results were analyzed with Standard Analysis software (JACSO) and expressed as mean residue molar ellipticity [θ].

Effect of pH on lipase activity and stability

The optimum pH of the purified lipase was investigated by measuring its hydrolytic activity at 50 °C at pH ranging from 7.0 to 12.0. The following buffers were used at 50 mM concentration; MOPS buffer for pH 6.5–7.5, Tricine buffer for pH 7.5–8.5, CHES buffer for pH 8.5–10.0, and CAPS buffer for pH 10.0–11.0. The pH stability was determined by incubating 1 mL purified lipase in the aforementioned buffers (20 mM) for 60 min at 70 °C, and its hydrolytic activity was detected in the presence of *p*NPL in 50 mM CHES buffer (pH 9.0) at 70 °C. The stability was determined as the relative activity to the control.

Effect of organic solvents on lipase stability

One milliliter of lipase solution (CHES buffer, 50 mM, pH 9.0) was mixed with 0.25 organic solvents at a final concentration of 20 % (*v/v*). The mixture was incubated at 70 °C and 200 rpm for 24 h. The residual activity was determined by the colorimetric method using *p*NPL as the substrate at 70 °C. The organic solvents used included dimethyl sulfoxide (DMSO), methanol, ethanol, acetonitrile, toluene, chloroform, *n*-hexane and *n*-heptane.

Substrate specificity

Various *p*-nitrophenyl esters containing different chain length of acyl group (C_2–C_{18}) were tested to determine the substrate specificity of the enzyme. The hydrolytic activity was assayed at 50 °C and pH 9.0 by the colorimetric method. Moreover, triacylglycerols were also selected as substrates for determination of substrate specificity. The lipase activity was measured at 70 °C and pH 9.0 by the titrimetric method the colorimetric method.

Transesterification of (R, S)-1-phenethyl alcohol

The lipase (5.0 mg/mL), (R,S)-1-phenethyl alcohol (100 mM) and vinyl acetate (100 mM) were mixed with anhydrous *n*-hexane (10 mL) in a 20-ml screw-capped vial, and the reaction mixture was shaken at 200 rpm and 70 °C for 12 h. The resulting mixture was sampled several times to determine enantiomeric excess ($e.e._p$) and conversion using GC analysis.

Analysis method

Lipase hydrolysis activity assay

In general, the reaction mixture contained 950 μL of CHES buffer (50 mM, pH 9.0), 45 μL of *p*NP-ester (40 mM) solution. After 5 min of incubation at 70 °C, the reaction was initiated by the addition of 5 μL of lipase solution. The enzyme reaction mixture was centrifuged at 12,000 × g for 30 s at room temperature, and the activity was measured at 410 nm on a spectrophotometer (Shimadzu, Japan) (Hess et al. 2008). The *p*NP-esters were beforehand dissolved in isopropanol to 40 mM. One enzyme unit (U) is defined as 1.0 μmol of *p*NP enzymatically released from the *p*NP-esters per minute at 70 °C.

Lipase activity of the samples was also determined with the pH–stat titrimetry (Glogauer et al. 2011). The reaction was carried out in a glass vessel thermostated at 70 °C containing 9.8 mL of substrate emulsion and 0.2 mL of lipase. Lipase activity was detected by the production of free fatty acids which were titrated automatically in a pH–stat titrator (Metrohm, Switzerland) with 0.05 M NaOH for 5 min. The triacylglycerol emulsion was prepared by mixing 75 mM triacylglycerol, 3 % (w/v) gum arabic, and 2.5 mM CHES buffer (pH 9.0). The solution was emulsified for 10 min and then for an additional 2 min immediately before use.

One unit (U) of lipase activity was defined as the amount of lipase liberating 1 μmol equivalent of fatty acid from triacylglycerol per min at 70 °C.

GC analysis

The optical purity and conversion were determined by GC-7890 gas chromatography (Agilent Technologies, USA) equipped with FID detector and CP7501 Chiral column (50 m × 0.39 mm, 0.25 mm) from Varian Co. Ltd. (USA). *n*-Dodecane was used as an internal standard, and N_2 was used as carrier gas. The injector, detector and column temperatures were set at 260, 250 and 110 °C, respectively. Enantiomeric excess ($e.e._p$) and conversion were calculated as described by Chen et al. (1982).

Protein

The protein content was determined by the method of Bradford using bovine serum albumin as the standard (Bradford 1976).

Results and discussion

Screening and identification of strain producing thermostable lipase

To isolating true lipase producers from microbial sources in preliminary screening plates, soil samples were incubated in the medium containing olive oil as sole carbon source (Hama et al. 2007). After cultivation at 37 °C for 48 h, the stability of intracellular and extracellular lipases from 127 soil samples was measured at 70 °C. From the strains tested, the crude intracellular lipase of strain YB103 displayed the highest stability, retaining 63 % residual activity after 24-h incubation at 70 °C for 24 h. So it was selected as the best thermostable lipase producer.

The strain YB103 was identified based on its 16S rDNA sequence (Hold et al. 2002). The BLAST search suggested a close relationship between strain YB103 (Genbank Accession No. KP313821) and the members of the *X. oryzae* pv. *oryzae* with a maximum sequence homology (99 %) and hereafter named *X. oryzae* pv. *oryzae* YB103. This strain is currently deposited in the China General Microbiological Culture Collection Center (CGMCC, Beijing, China), with the accession number CGMCC 10275.

Purification of intracellular LipXO

To exclude other intracellular proteins or enzymes which can disturb the properties of desired enzyme, the purification of enzyme was performed (Ahmed et al. 2010). In the present study, the strain *X. oryzae* pv. *oryzae* YB103 was cultivated in olive oil medium at 37 °C and 200 rpm for 48 h. The lipase hydrolytic activity (9.7 U/mL, *p*NPL as substrate) was observed after sonication disruption. The intracellular lipase from *X. oryzae* pv. *oryzae* YB103 (LipXO) was purified to homogeneity by a combined method of ultrafiltration concentration, ammonium sulfate precipitation and hydrophobic chromatography in sequence. The lipase was purified 101.1-fold with 15.7 % yield with a specific activity of 373.9 U/mg. A summary of the purification data is presented in Table 1. In electrophoretic analysis, LipXO migrated as a single band (about 58 kDa) corresponding to Coomassie Brilliant Blue staining of the purified LipXO fraction (Fig. 1). To the best of our knowledge, the known lipase from *X. oryzae* pv. *oryzae*

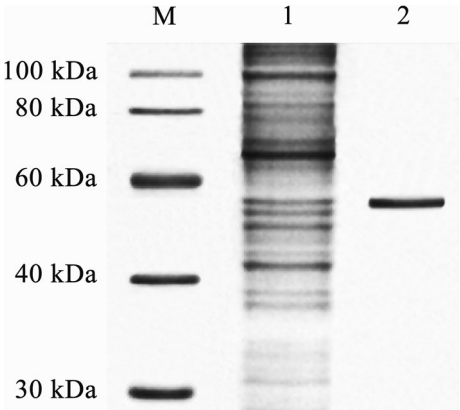


Fig. 1 SDS–PAGE of purified intracellular LipXO. *Lane 1*, intracellular protein solution (concentrated by ultrafiltration); *lane 2*, purified protein after phenyl Sepharose chromatography; *lane M*, molecular weight markers: 30, 40, 60, 80 and 100 kDa

Table 1 Summary of LipXO purification from *X. oryzae* pv. *oryzae* YB103

Purification steps	Total (units)[a]	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Culture filtrate	9743	2613.7	3.7	1	100
$(NH_4)_2SO_4$ precipitation	6561	298.6	22.0	5.9	67.3 %
Phenyl sepharose chromatography	1533	4.1	373.9	101.1	15.7 %

[a] The lipase hydrolytic activity was determined by the colorimetric method in Tris–HCl buffer (50 mM, pH 9.0) at 70 °C using *p*NPL as the substrate

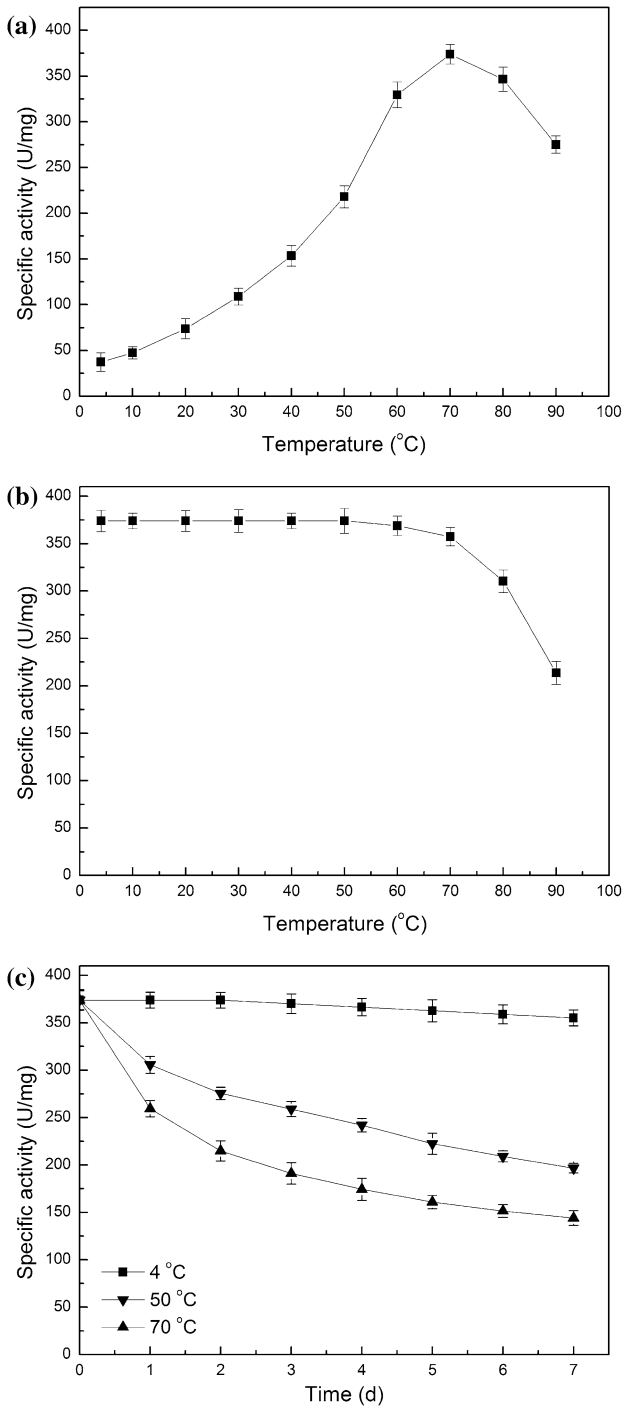


Fig. 2 Effects of temperature on the activity and stability of purified LipXO. **a** LipXO hydrolytic activity was determined at different temperatures (4–90 °C) in 50 mM CHES buffer (pH 9.0) using *p*NPL as the substrate. The lipase samples were withdrawn periodically to estimate the residual activities at 70 °C in 50 mM CHES buffer (pH 9.0) using *p*NPL as the substrate. **b** After pre-incubation of the lipase at 4–90 °C at an interval of 10 °C for 30 min, the remaining activity was determined at 70 °C in 50 mM CHES buffer (pH 9.0) using *p*NPL as the substrate. **c** Thermostability of purified LipXO for long period. The purified lipase (0.5 mg/mL) was incubated at different temperatures (*filled squares* 4 °C, *filled downward triangles* 50 °C and *filled upward triangles* 70 °C) for 7 days. *Values* represent the mean of three replicates

has not been found to possess the same molecular mass (Aparna et al. 2007). The purified enzyme preparations were stored at 4 °C and were used to study its properties.

Thermostability and optimum temperature of LipXO

LipXO showed remarkable stability at high temperature in this study. The enzyme showed optimum temperature at 70 °C with no significant decrease at 80 °C (Fig. 2a). At 60 and 90 °C, the specific activity decreased to 329.5 U/mg (88.1 %, relative activity) and 275.1 U/mg (73.6 %, relative activity) of the maximum activity, respectively. However, less activity at lower temperatures could be due to the optimum temperature needed to trigger the lid opening of the lipase (Masomian et al. 2013). This profile is similar to those of thermostable lipases from *Bacillus thermoleovorans* ID-1 and *Geobacillus* sp. (Dong-Woo et al. 1999; Abdel-Fattah 2002). To study the effect of temperature on lipase thermostability, various incubation times and temperatures have been investigated. As a result shown in Fig. 2b, LipXO even retained 57.1 % (213.6 U/mg) of its residual activity after incubation at 90 °C for 30 min. The specific activity of a lipase from *Geobacillus* sp. Iso5 was 100.8 U/mg after incubation at 90 °C for 30 min (Mahadevan and Neelagund 2014).

To comprehensively evaluate the long-term thermostability of LipXO, the lipase solution was pre-incubated at 4, 50, and 70 °C for 7 days before assay. Although good stability of bacterial and fungal lipases at high temperature for long period is rare (Haki and Rakshit 2003), LipXO retained 51.1 % (191.1 U/mg) of its original activity after incubation at 70 °C for 3 days (Fig. 2c). The hydrolytic activity of thermostable lipases from thermophilic anaerobic bacteria *Thermoanaerobacter thermohydrosulfuricus* and *Caldanaerobacter subterraneus* subsp. *tengcongensis* was 10.9 and 9.0 U/mg after incubated for 24 h at 70 °C (Royter et al. 2009). Thermostable lipase of *Fervidobacterium changbaicum* CBS-1 $HSAUP_{03}80006$ showed 11.1 U/mg at 70 °C for 9 h incubation, respectively (Cai et al. 2011). Taken together, LipXO was designated a thermostable lipase. Although some work on lipases from *X. oryzae* pv. *oryzae* has been published (Aparna et al. 2007), there are no reports on a thermostable lipase from this organism.

The effect of temperature on the stability of LipXO was also determined by CD spectra. The thermal denaturation process was followed at 222 nm by CD in the range 30–95 °C (Fig. 3). Wavelength at 222 nm was set to detect α-helical to random coil transition, as they exhibited characteristic signals at this wavelength (Leow et al. 2007). The denaturation temperature (T_m) is the temperature where half of the protein becomes denatured and is an estimate of protein stability (Reed et al. 2014). T_m of the enzyme was 78.8 °C. This datum confirms the very high

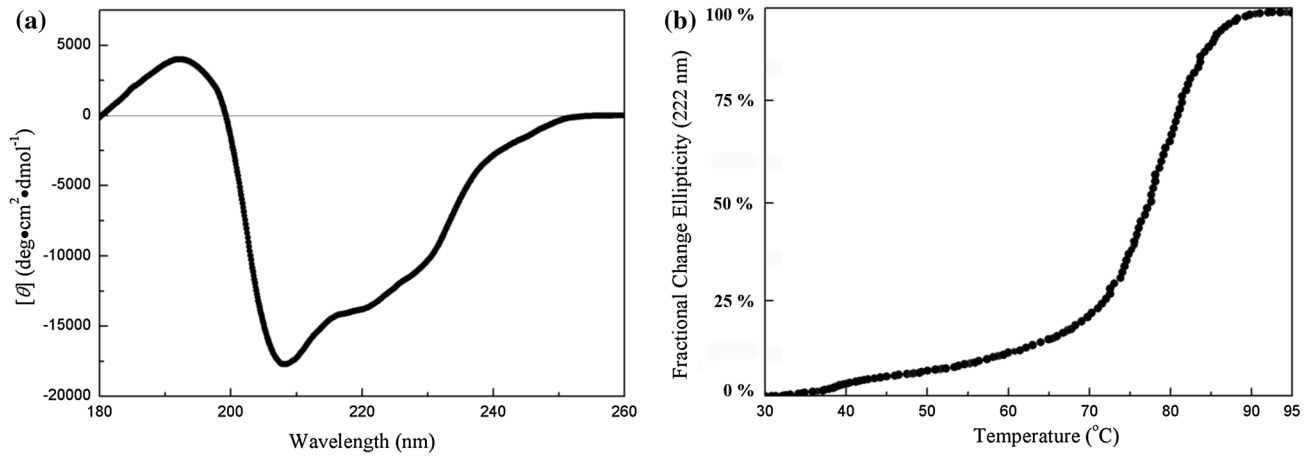


Fig. 3 CD spectra of LipXO protein. **a** The CD spectra from 180 to 260 nm of protein. **b** Plots of 222 nm CD intensities as a function of temperature. The thermal denaturation of LipXO was monitored by following the ellipticity at 222 nm from temperatures 30–95 °C

thermal stability of LipXO inferred from enzymatic activity measurements. Lipase has a hydrophobic β-sheet core surrounded by a hydrophilic α-helix surface (Carrasco-López et al. 2009). The catalytic residues of lipase were located in the hinge between β-sheet and α-helical protein domains (Nardini et al. 2000). Although CD spectra indicated that the lipase lost all its α-helical structure (Kirwan and Hodges 2014), LipXO might still have the β-sheet core structure (Khusainov et al. 2015). Thus, the results of CD spectra and thermostability suggested that the lipase could show catalytic ability in spite of losing almost all α-helical structure.

Effect of organic solvents on lipase stability

LipXO displayed high stability in all kinds of the hydrophilic and hydrophobic organic solvents tested in this study (Fig. 4). The effects of organic solvents on the activities of LipXO were determined by measuring residual activity after incubation in 20 % (*v/v*) solvents at 70 °C for 24 h. Although high stability of microbial lipases in hydrophilic solvents is rare (Doukyu and Ogino 2010; Zhao et al. 2008), the purified LipXO showed very stable in hydrophilic solvents with low log*P* values. It is noteworthy to point out that >100 % activity remained stable in presence of 20 % (*v/v*) methanol at 70 °C for 24 h, indicating LipXO a potential catalyst for chemical industry. Moreover, the high stability was observed in the presence of *n*-hexane with a 54.1 % (400.0 U/mg) increase in its activity compared to the control (259.5 U/mg, without *n*-hexane). Specific activity of LipXO increased to 294.6 U/mg (113.5 % of its original activity) after incubation in 20 % (*v/v*) *n*-heptane at 70 °C for 24 h. On the other hand, 194.8 U/mg and 250.1 U/mg of lipase activities were observed in the presence of 20 % (*v/v*) chloroform and toluene,

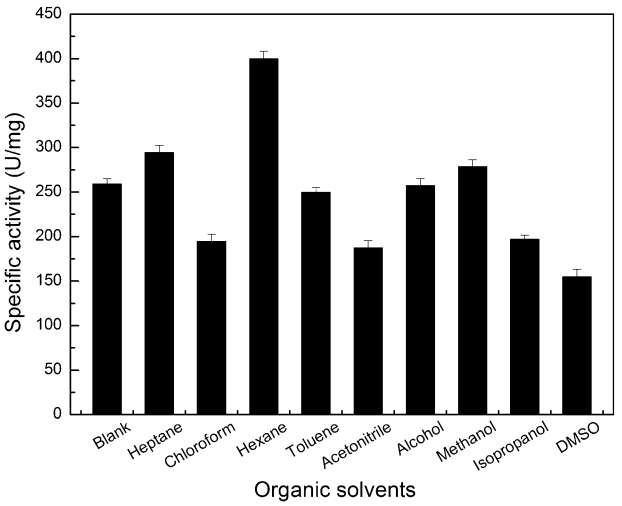


Fig. 4 Effect of organic solvents on LipXO activity. After incubating the enzyme for 24 h in different organic solvents, the residual activity was determined in 50 mM CHES buffer (pH 9.0) at 70 °C using *p*NPL as the substrate

respectively. Ogino et al. also reported very low activity of *Pseudomonas aeruginosa* LST-03 lipase in the presence of 25 % (*v/v*) solvents (Ogino et al. 2004). Thus, LipXO was an organic solvent-tolerant lipase.

Effect of pH on lipase activity and stability

LipXO turned out to be an alkaline lipase. The purified lipase exhibited high hydrolytic activity at the range of pH 6.5–11.0 with the maximum activity at pH 9.0 (Fig. 5a). At alkaline condition (pH 8.0–11.0), higher than 81.0 % of relative activity was detected compared to that at optimum pH 9.0. In pH stability experiment, the purified lipase showed good stability after 60-min incubation at pH 6.5–11.0, and

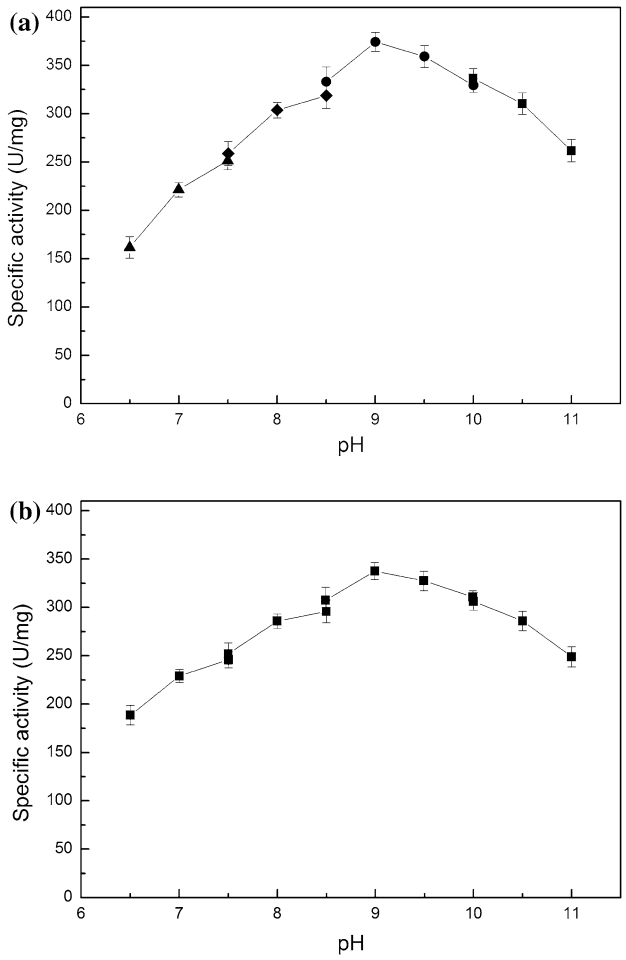


Fig. 5 Effects of pH on the purified LipXO. **a** The lipase activity was detected in different buffers with varying pH values at 70 °C using *p*NPL as the substrate. *Filled triangles* 50 mM MOPS buffer (pH 6.5–7.5), *filled diamonds* 50 mM Tricine buffer (pH 7.5–8.5), *filled circles* 50 mM CHES buffer (pH 8.5–10.0) and *filled squares* CAPS buffer (pH 10.0–11.0). **b** The lipase was pre-incubated at 70 °C in 20 mM buffers with different pH values for 60 min, and the residual activity was determined at 70 °C in 50 mM CHES buffer (pH 9.0) using *p*NPL as the substrate

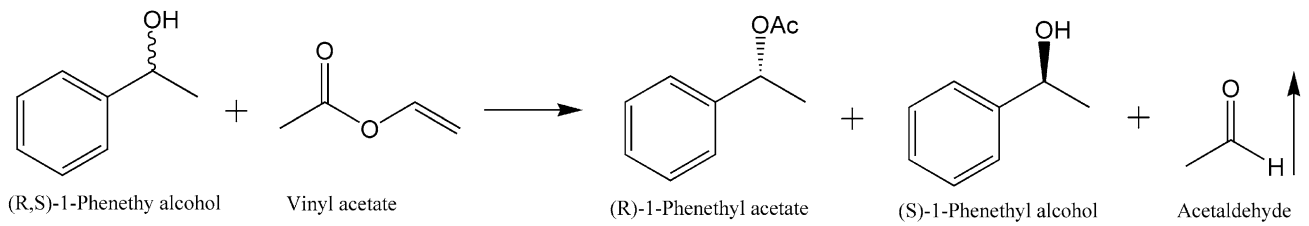
the lipase even retained 74.1 % (249.0 U/mg) of residual activity at pH 11.0 (Fig. 5b). The specific activity of thermostable lipases from *Staphylococcus aureus* and *Geobacillus* sp. Iso5 was 39.2 U/mg and 37.9 U/mg at pH 11.0, respectively (Sarkar et al. 2012; Mahadevan and Neelagund 2014). The high activity and stability of LipXO over a wide alkaline pH suggests its usefulness in a range of industrial applications, such as the synthesis of biopolymers and biodiesel and the production of pharmaceuticals, agrochemicals, cosmetics, and flavor (Abdelkafi et al. 2009; Ahmed et al. 2010).

LipXO stereoselectivity

Stereoselectivity is one of the important characteristics exhibited by lipases, which is controlled by molecular properties of the enzyme (Colombo and Carrea 2002). Stereospecific lipases can catalyze the transesterification of S- or R-configuration secondary alcohol to form corresponding ester. The stereoselectivity of purified LipXO was investigated by transesterification of 1-phenethyl alcohol with vinyl acetate as acyl donor in *n*-hexane at 60 °C for 12 h (Scheme 1). After biocatalytic reaction, products and substrates were analyzed by chiral GC. The comparison, between the chromatograms before reaction and after 12 h transesterification, indicated that LipXO catalyzed transesterification of (R, S)-1-phenethyl alcohol to (R)-1-phenethyl acetate as main product. The reaction gave the corresponding (R)-ester product with 99 % enantiomeric excess (49 % conversion) and >200 enantioselectivity (E). However, several lipases from the *Candida antarctica* (Lee and Dordick 2002) and *Burkholderia cepacia* (Lee and Kim 2011), possessed low and moderate E (four and 101, respectively) toward (R, S)-1-phenethyl alcohol. These results indicate that LipXO was a stereoselective enzyme.

Substrate specificity of LipXO

True lipases attack ester substrates that contain long-chain fatty acids (Glogauer et al. 2011; Reyes-Duarte et al. 2005). The substrate specificity of the purified LipXO was determined using *p*-nitrophenyl esters and triacylglycerols of varying acyl chain lengths at 70 °C and pH 9.0. The lipase demonstrated wide substrate specificity towards both classes of ester substrate (from C_2 to C_{18}) with preference to *p*NPL (C_{14}, 373.9 U/mg) and glyceryl trimyristate (C_{14}, 296.4 U/mg) (Fig. 6). The relatively high activities were



Scheme 1 Enantioselective transesterification of (*R*, *S*)-1-phenethyl alcohol catalyzed by purified LipXO in *n*-hexane

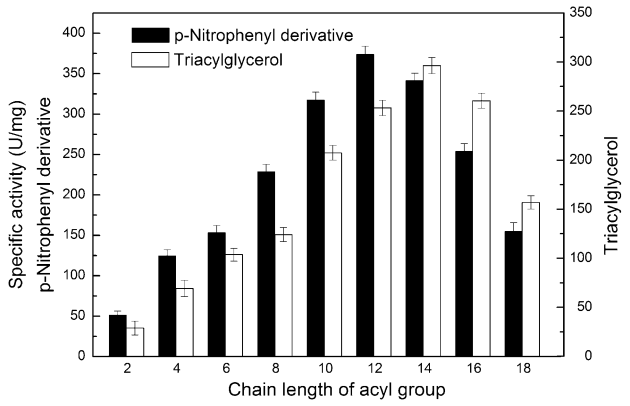


Fig. 6 Substrate specificity of purified LipXO. The lipase activities toward various *p*NP-esters and triacylglycerols were determined at 70 °C in 50 and 2.5 mM CHES buffer (pH 9.0), respectively

observed on substrates with acyl chain length from C_{10} to C_{16}, indicating LipXO showed preference to medium and long acyl chain lengths. Therefore, the lipase is a true lipase but not an esterase (Arpigny and Jaeger 1999; Reyes-Duarte et al. 2005).

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