RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2011.37.105

ScienceAsia 37 (2011): **105–114**

Production of a thermostable lipase by *Aeromonas* sp. EBB-1 isolated from marine sludge in Angsila, Thailand

Jittima Charoenpanich[a,b,*], **Sureeporn Suktanarag**[a,c], **Naruemon Toobbucha**[a]

[a] Department of Biochemistry, Faculty of Science, Burapha University, Bangsaen, Chonburi 20131, Thailand
[b] Environmental Science Program and Centre of Excellence on Environmental Health, Toxicology and Management of Chemicals (ETM-PERDO), Faculty of Science, Burapha University, Bangsaen, Chonburi 20131, Thailand
[c] Biological Science Program and Centre of Excellence for Innovation in Chemistry (PERCH-CIC), Faculty of Science, Burapha University, Bangsaen, Chonburi 20131, Thailand

*Corresponding author, e-mail: jittima@buu.ac.th

Received 29 Nov 2010
Accepted 25 Apr 2011

ABSTRACT: *Aeromonas* sp. EBB-1 isolated from marine sludge in Angsila, Thailand produced an extracellular thermophilic lipase. Maximum lipase activity of the culture medium was obtained after 15 h at 25 °C and pH 8.0 with 0.5% (v/v) olive oil as a carbon source. The enzyme was successfully purified 30-fold to homogeneity by successive ammonium sulphate precipitation, gel-permeation column chromatography, and ultrafiltration. The purified bacterial lipase possessed a relative molecular mass of 45 kDa. Highest lipase activity was determined at pH 8.0 and 37 °C with *p*-nitrophenyl palmitate as a substrate. The enzyme was stable at pH 6.0–8.0 and at temperatures of 30–80 °C. Enzyme activity decreased slightly in 50% (v/v) isoamyl alcohol, decane, or heptane. Lipase activity decreased to a half in the presence of butanol and benzene, and by more than 60% in DMSO, methanol, ethanol, and hexadecane. Lipase activity was inhibited by most of the salt ions (except Ca^{2+} ions) as well as by Tween 80, DTT, PMSF, SDS, and EDTA. Although lipase showed variable specificity/hydrolytic activity towards a number of *p*-nitrophenyl esters, it was preferentially active towards long-carbon chain acyl esters (C_{12}–C_{16}). The high temperature stability and ability to hydrolyse long length esters support the potential of this lipase enzyme as a vigorous biocatalyst for industrial applications.

KEYWORDS: alkaline lipase, thermophilic

INTRODUCTION

Widespread use of lipases or triacylglycerol hydrolases (EC 3.1.1.3) as an industrial biocatalyst has noticeably increased in biotechnological applications[1]. Most of the lipases used in industrial applications are derived from plants, animals, or microorganisms[1,2]. Microbial lipases are of major interest because of the diversity of catalytic activities, high yields, ease of genetic manipulation, rapid growth, and inexpensive culture[1]. Each lipase has a number of unique characteristics such as substrate specificity, regio-specificity, and chiral selectivity and some enzymes are important for the industrial production of free fatty acids, synthesis of useful esters and peptides[1,3]. A major requirement of a commercial lipase is its thermal stability and performance at high temperature that would increase the reaction conversion rates, substrate solubility, and also prevent contamination by microorganisms[4]. Recently, there has been a great demand for thermostable enzymes in industry, and a number of thermostable lipases from various sources have been purified and extensively characterized[5–7]. As lipase producing microbes are widely distributed in nature, there is an immense need to explore natural habitats to isolate thermally stable lipase producing microbes. The objective of this study was to isolate, identify and characterize a novel bacterium that produces a thermostable lipolytic enzyme.

MATERIALS AND METHODS

Isolation of a lipase-producing strain

Ten marine sludge samples were collected close to a fish market in Angsila, Thailand during low tide. Ten grams of each of the samples were mixed with 100 ml of sterile water. The heavy particles were allowed to settle by sedimentation and the upper phase was directly used for screening. Lipase-producing strains were preliminary screened by enrichment in 0.2× Luria-Bertani medium[8] supplemented with 0.5% (v/v) of palm oil and 1% (w/v) of gum arabic.

Cultures were maintained at 25 °C for 24 h with continuous shaking at 250 rpm. The cultures showing growth turbidity were used for further isolation by an enrichment method (7 times) under the same culture conditions. A pure culture was isolated by spreading the turbid culture (OD_{600} ranging from 0.6–1.0) onto a nutrient agar plate. The colonies were further purified to form a single colony by repeated streaking under the same conditions. Then, lipase-producing bacteria were screened by an orange fluorescence shown under UV exposure on a plate containing 1% (v/v) of substrate palm oil, 0.4% (w/v) of NaCl, 0.8% (w/v) of nutrient medium, 1% (w/v) of gum arabic, and 0.001% (w/v) of rhodamine B[9]. Subsequently, hydrolytic activity using *p*-nitrophenyl palmitate (Sigma) as a substrate was confirmed according to the method of Pencreac'h and Baratii[10]. One unit (U) of enzyme was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under assay conditions. The amount of *p*-nitrophenol was calculated from the *p*-nitrophenol (Sigma) standard curve. Protein concentration was determined spectrophotometrically according to Bradford[11] using Bio-Rad assay reagent (Hercules, USA) and bovine serum albumin as the standard. The bacterial strain that showed the highest lipase activity after 24 h cultivation was selected for further experiments.

Bacterial identification

Bacterial identification was based on "API Skills Bacterial Identification Method" and also by 16S rRNA sequence analysis[12]. PCR amplification of 16S rRNA gene was done with primers designed from the conserved regions at the base positions 22 to 41 and 1066 to 1085 in 16S rRNA gene of *Escherichia coli*[13]. Chromosomal DNA (100 ng) prepared with a GF-1 Nucleic acid extraction kit (Vivantis, Malaysia) was used as a DNA template for PCR reaction. PCR was carried out with a denaturation step at 95 °C for 10 min followed by 30 cycles at 95 °C for 30 s, 60 °C for 45 s, and 72 °C for 90 s. Final extension was performed at 72 °C for 7 min. The expected PCR product (about 1.1 kb) was purified with a GF-1 Gel DNA recovery kit (Vivantis, Malaysia). The obtained 16S rRNA fragment was then ligated into T/A cloning vector (RBC, Taiwan) according to manufacturer's instructions. After transformation into *E. coli* DH5α, plasmids were extracted with a GF-1 Plasmid DNA extraction kit (Vivantis, Malaysia) and used as the template for sequencing. Similarity of nucleotide sequence was determined using BLAST (National Centre for Biotechnology Information databases, Bethesda MD, USA). The phylogenetic analysis was performed using CLUSTAL W-multiple sequence alignment software package at align.genome.jp. These sequence data have been submitted to the GenBank databases under accession no. HM 214925.

Growth curve and lipase production

Bacterial growth and lipase production were measured in nutrient medium (pH 7.0) in a 1 l flask (1% inocula). Aliquots were withdrawn at 3 h intervals to measure lipase production, pH of culture medium, and biomass. Lipase production was determined by assaying the hydrolytic activity as described above and biomass was expressed in colony-forming units per ml (CFU/ml). Statistical analyses were performed using a two-tailed t-test and $P < 0.05$ was considered statistically significant.

Physical parameters affecting the lipase production

Culture was done over pH 2–12, temperature 15–45 °C (at 5 °C intervals), and agitation 100–300 rpm (at 50 rpm intervals). Throughout the study, general culture procedures were as followed: A 5 ml inoculum from a 15 h bacterial culture was added to 100 ml of nutrient medium. The samples were taken from the culture at the late exponential phase of growth ($OD_{600} \sim 1.5$). Culture was centrifuged at 8000g and 4 °C for 10 min and the supernatant obtained was filtered through a 0.45 μm nylon membrane filter (Whatman, England) to collect cell-free supernatant to be used for enzymatic assay. Growth was monitored at an OD_{600} and lipase production was followed by assay of hydrolytic activity as described above. Experiments were done in triplicate and the activities of lipase were expressed as relative activity compared to the maximum (100% relative activity) observed value.

Effect of carbon sources on lipase production

The effect of carbon sources on the lipase production was examined in a 0.2× nutrient medium including different oils or other inducer (0.5% v/v), emulsified with gum arabic (1% w/v) at 25 °C, 250 rpm for 15 h. This study used corn, olive, palm, safflower, soybean, and sunflower oils with Triton X-100, Tween 80, tributyrin, and hexadecane as inducers. Media without the addition of oils or inducer was used as control. Enzymatic activities were determined in triplicate and reported as averages ± standard deviation.

Lipase purification

A single colony of the bacterial strain was grown in 1 l of 0.2× nutrient medium supplements with 0.5% (v/v) of olive oil at 25 °C, 250 rpm for 24 h. The

protein/lipase in the cell-free culture (10 000g, 20 min, 4 °C) was 'salted out' using ammonium sulphate (30 and 60% saturation at 10% intervals). The cell suspension was kept on ice for 30 min with gentle stirring. The precipitate was collected by centrifugation at 10 000g and 4 °C for 30 min and further resuspended in 50 mM Tris-HCl buffer (pH 8.0). The reconstituted fraction was dialysed extensively against the same buffer and then loaded on a Sephadex G-75 gel permeable chromatography column (1×65 cm; GE Healthcare Bio-Sciences AB, Sweden) equilibrated with 50 mM Tris-HCl buffer (pH 8.0). All fractions were assayed both for lipase activity and total protein (A_{280}). Fractions showing lipase activity were pooled and concentrated with a 10 kDa-cutoff ultrafiltration membrane (Millipore, Island). The concentrate was assayed for protein content as described above. The specific activity of the purified enzyme was compared with that of crude enzyme and purification factor was calculated. Finally, the insoluble materials found in the purified enzyme were discarded by filtration through 0.2 µm pore size of nylon membrane filter (Whatman, England) and the filtrate was used for purification and characterization by chromatography and electrophoretic analysis.

Determination of molecular mass

Relative molecular mass of lipase was estimated by discontinuous sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (5% stacking and 12.5% separating gel)[14], using High-Range Rainbow molecular weight markers (GE Healthcare, England) as the standard protein markers. The relative molecular mass of the native enzyme was estimated by gel permeable column chromatography as indicated before.

Effect of pH on lipase activity and stability

The effects of pH on lipase activity and stability were determined at 37 °C over a pH range of 3.0–12.0 using *p*-nitrophenyl palmitate as substrate. For optimum pH determination, reaction mixture was incubated at 37 °C for 15 min. The enzyme was incubated in 50 mM buffer at the specific pH for 6 h at 37 °C and then the residual activity was determined at pH 8.0. Buffer systems were acetate (pH 3.0–6.0), phosphate (pH 6.0–8.0), Tris-HCl (pH 7.0–9.0), and carbonate (pH 9.0–12.0).

Effect of temperature on lipase activity and stability

Thermal stability of lipase was determined by incubating the purified lipase at different temperatures (range 20–80 °C) for 6 h in a water bath and then measuring the residual activity using the activity at 37 °C as control. The half-life of the enzyme at 60 °C was monitored periodically up to 72 h.

Effect of organic solvents on the stability of lipase

Lipase solutions were mixed with equal volumes of each of the selected organic solvent to prepare the 50% organic solution, and the mixtures were consequently shaken and incubated at 37 °C for 6 h at 150 rpm. The solvent contained in the mixture was partially eliminated by evaporation at 37 °C for 5 min and the residual lipase activity was measured at 37 °C and pH 8.0 and compared to that of the control (no solvent).

Effects of chemicals on lipase activity

Effects of a detergent, metal ions, and inhibitors on the enzyme were determined by incubating purified lipase for 1 h at 37 °C in 50 mM Tris-HCl buffer (pH 8.0) with the selected chemicals. The residual lipase activity was compared to that of the control (no chemical added).

Substrate specificity

Substrate specificities of lipase towards *p*-nitrophenyl esters (Sigma, Germany), *p*-nitrophenyl acetate (C_2), butyrate (C_4), caprylate (C_8), caprate (C_{10}), laurate (C_{12}), myristate (C_{14}), palmitate (C_{16}), and stearate (C_{18}) were determined by assaying the hydrolytic activity of the purified lipase and comparing to the activity using *p*-nitrophenyl ester as a substrate.

RESULTS AND DISCUSSION

Isolation and identification of lipase producing bacterium

Out of the 22 lipase-producing bacteria isolates, one that showed high lipase production (6.50 ± 0.03 U/ml) and ability to grow at up to 55 °C (data not shown) was selected as a candidate of thermophilic-lipase producer. The strain was a rod shape gram negative bacterium and gave negative results in reactions with ornithine decarboxylase, urease, and tryptophane deaminase. Positive results were recorded for cytochrome-oxidase, β-galactosidase, arginine dihydrolase, lysine decarboxylase, and gelatinase. The strain could use trisodium citrate and produce indol and Voges Proskauer acetoin but without H_2S. Also, the strain could ferment or oxidize some of the substrates (glucose, manitol, and sucrose) and not the others (inositol, sorbitol, rhamnose, melibiose, amygdalin, and arabinose). In addition, the strain could produce gas from glucose and gave a negative result with

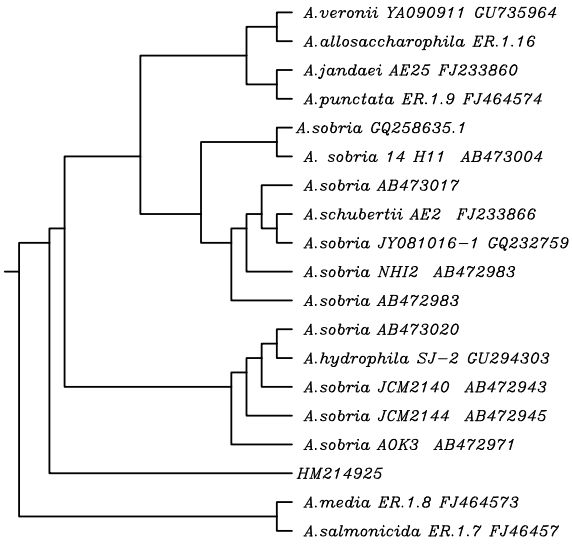

Fig. 1 Phylogenetic tree of *Aeromonas* sp. homologues to the isolate. The phylogenetic tree was constructed by the neighbour-joining method with the CLUSTAL W-multiple sequence alignment software package. The scale represents the number of nucleotide substitutions per site.

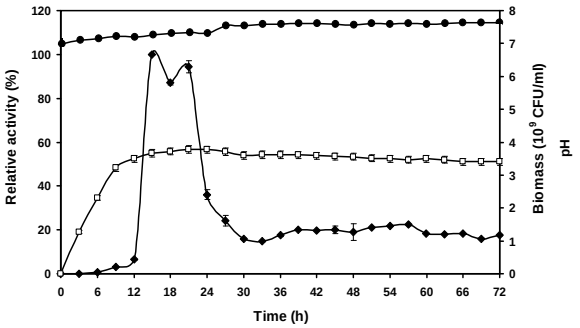

Fig. 2 Growth curve and lipase production of *Aeromonas* sp. EBB-1 in a nutrient medium (pH 7.0) at 25 °C, 250 rpm. Samples were taken at 3-h intervals for lipase production (closed diamonds) and pH (closed circles) and biomass (open squares) determinations. Error bars represent standard deviations for three measurements.

methyl red. The 16S rRNA gene sequence was 99% similar to the following *Aeromonas sobria* strains: *A. sobria* JY081016-1 (accession no. GQ232759.1), *A. sobria* LD081008A-1 (accession no. GQ205446.1), *A. sobria* NHI2 (accession no. AB472983.1), *A. sobria* AOK3 (accession no. AB472971.1), *A. sobria* JCM2144 (accession no. AB472945.1), and *A. sobria* TM4 (accession no. AB472941.1). The same confidence level (99% identity) was also found in other species including *A. veronii* strain YA090911 (accession no. GU735964.1), *Aeromonas* sp. MK2 (2010) (accession no. GU566308.1), *Aeromonas* sp. KC14 (accession no. AB472995.1), *A. hydrophila* strain SJ-2 (accession no. GU294303.1), *A. veronii* strain MRM0908 (accession no. GQ983054.1), *A. hydrophila* strain KAE20 (accession no. AB473040.1), *A. veronii* bv. veronii strain 2 T3C84 (accession no. EF 634213.1) and *A. veronii* bv. sobria strain AE33 (accession no. EF 631963.1). Although biochemical characteristics suggested the bacterial isolate as *A. sobria*, a phylogenetic tree using neighbour-joining method (Fig. 1) suggested that the isolate was close to *Aeromonas* spp., a common inhabitants of aquatic environments[15]. Hence this strain was identified as *Aeromonas* sp. EBB-1.

Growth curve and lipase production

Biomass of the bacterial culture increased rapidly after inoculation, reached a stationary phase in 15 h (Fig. 2), was highest at 21 h, and declined slightly after 72 h. The lipase activity could be detected at 9 h, reached a maximum value at 15 h with a gradual decrease, followed by a drastic decrease after 21 h of cultivation. This may be an interesting property because it could allow the enzyme to be harvested shortly after cultivation. Incubation periods ranging from 12 h to 16 h were found to be best suited for maximum lipase production by bacteria. An incubation period of 12 h was optimum for lipase production by *Acinetobacter calcoaceticus* and 16 h for *B. thermocatenulatus* and *Pseudomonas* sp. KB700A[16–18]. This can be explained by the quorum sensing theory stating that once cell densities reach a threshold level the expression of genes encoding extracellular enzymes and secretion systems are completely induced[19]. A drop in lipase activity after 21 h incubation might be attributed to a decline in the bacterial cell metabolism.

Effect of carbon sources and physical parameters on lipase production

Bacterial lipases are typically extracellular and are influenced greatly by nutritional and physico-chemical factors, such as temperature, pH, and dissolved oxygen concentration[20]. Since lipases are inducible enzymes that are normally produced in the presence of a lipid source[21,22], the major requirement for the expression of lipase activity has always been for the production of carbon. This is also explained in the production of lipase from *Aeromonas* sp.[23–25] In

the present study, different carbon sources significant reduced the lipase production. The best carbon source was olive oil (440 ± 13 U/ml), giving a 68-fold increase in lipase production compared to control (medium without any supplements; 6.0 ± 0.7 U/ml). Addition of Tween 80 (141 ± 12 U/ml), soybean (103 ± 2 U/ml), sunflower (51.5 ± 3.5 U/ml), and corn oils (39 ± 3 U/ml) caused increased lipase activity by as much as 22, 16, 8, and 6-fold, respectively. On the other hand, Triton X-100 reduced lipase production by 19% (5 ± 1 U/ml). Therefore, organic nitrogen medium (nutrient medium) alone was not sufficient to stimulate the bacterial lipase production. Supplementing the medium was essential for optimum lipase production of this strain.

Lipase activity in oil-supplemented medium is limited by the exposure of the lipid substrate to attack by the bacterium which increases dispersion of the substrate and enhances growth and enzyme production[26]. Among natural oils, olive oil dominantly enhanced lipase production. This might due to high content of unsaturated long-chain fatty acid (80.3% content of C18:1) compared to soybean and sunflower oils (67.5% and 53.7% content of C18:2, respectively)[27]. The Tween 80 also significantly promoted lipase production by the tested bacterial strain. This might be due to the involvement of a fatty acyl ester bond that functions as an inducer of the lipase operon[28]. Although hexadecane enhances lipase production in some bacteria[28,29], while it complete inhibits it in others[30]. This is in agreement with the conclusion of Kok et al[31] that some alkanes could repress lipase expression. Moreover, a similar observation about short-chain fatty acid repression was also reported in the regulation of lipase production[16,32].

The strain grew and efficiently produced lipase at pH 5.0 to 10.0 with a maximum activity at pH 8.0 (Fig. 3a). Above pH 10.0, the lipase production was suppressed and ceased at pH 12.0. Most microorganisms can survive within a pH range 5 to 8.5 and exhibit maximum growth rates at close to neutrality[33]. In the case of *Aeromonas* sp., growth and lipase secretion were highest at neutral to alkaline pH[34]. Thus it is of interest to note here that production of lipase at pH 5.0 seems to be an attractive characteristic of this bacterium that needs further investigation.

The bacterium that grew and produced lipase between 20 and 40 °C, exhibited maximum activity at 25 °C, and ceased at 45 °C (Fig. 3b). Low temperatures decrease lipase export to the supernatant phase and high temperatures result in enzyme denaturation[35]. Optimum growth temperature in this study is in agreement with the findings of others on the production of lipase by a bacterium of the same genus[19,20].

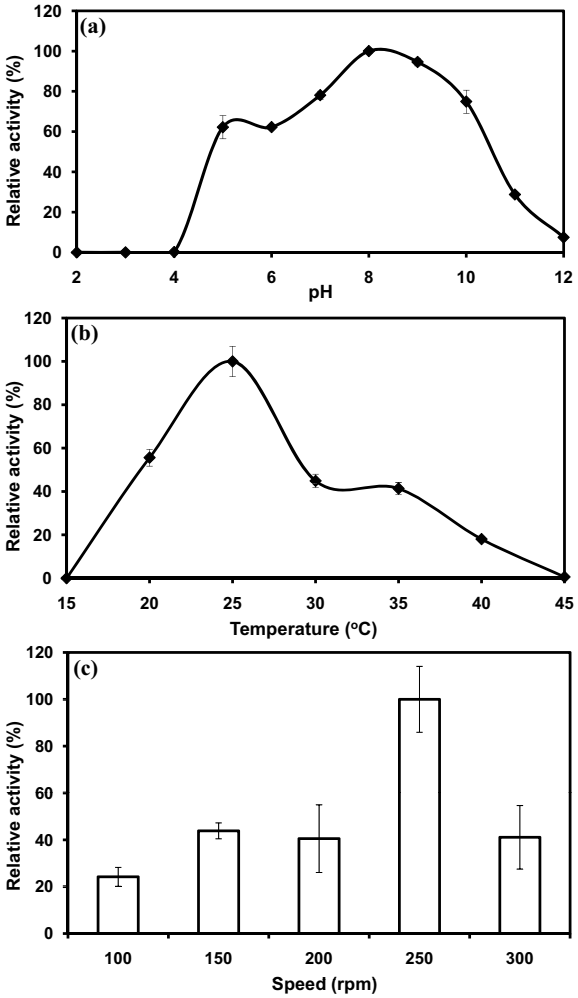

Fig. 3 Effects of (a) initial pH, (b) incubation temperature, and (c) aeration on lipase production by *Aeromonas* sp. EBB-1. The strain was cultivated in nutrient medium with optimum conditions. Percentages shown are relative to maximum activity and expressed as the average of three determinations with the standard deviations.

Optimum production of lipase by *Aeromonas* sp. EBB-1 was facilitated by continuous shaking at 250 rpm and was lower at lower and higher speeds (Fig. 3c). The lower cell growth and lipase production at 100–200 rpm suggested limitation of dissolved oxygen. In addition, the decrease in lipase production at 300 rpm might have been related to cell removal from the culture and the formation of foam resulting from mechanical and/or oxidative stress[36].

Table 1 Summary of lipase purification from *Aeromonas* sp. EBB-1.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude lipase	6500	579	11.2	100	1.0
40–50% $(NH_4)_2SO_4$ precipitation	5349	23.6	226.7	82.3	20.2
Sephadex G-75	4323	14.8	292.1	66.5	26.1
Ultrafiltration	2132	6.4	333.1	32.8	29.7

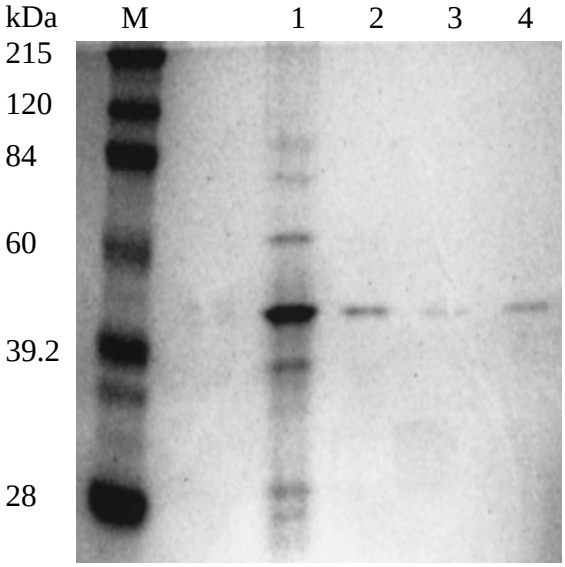

Fig. 4 SDS-PAGE analysis of the purified *Aeromonas* sp. EBB-1 lipase. Lane M: protein marker; lane 1: cell-free supernatant; lane 2: pooled lipase fraction from 40–50% ammonium sulphate precipitation; lane 3: pooled lipase fraction from Sephadex G75-column chromatography; lane 4: pooled lipase fraction from ultrafiltration.

Enzyme purification

An extracellular lipase from *Aeromonas* sp. EBB-1 was purified to homogeneity by ammonium sulphate precipitation, Sephadex G-75 column chromatography and ultrafiltration with a total yield of 32.8% and 29.7-fold purification (Table 1). The pool of the lipase fractions collected from the last purification step produced a single protein band in the SDS-PAGE with a relative molecular mass of 45 kDa (Fig. 4). The molecular mass of the enzyme was smaller than that determined for other lipases in the same genus: 97 kDa for *A. sobria* LP004[37], 67 kDa for *A. hydrophila*[38] and 50 kDa for *Aeromonas* sp. LPB4[25].

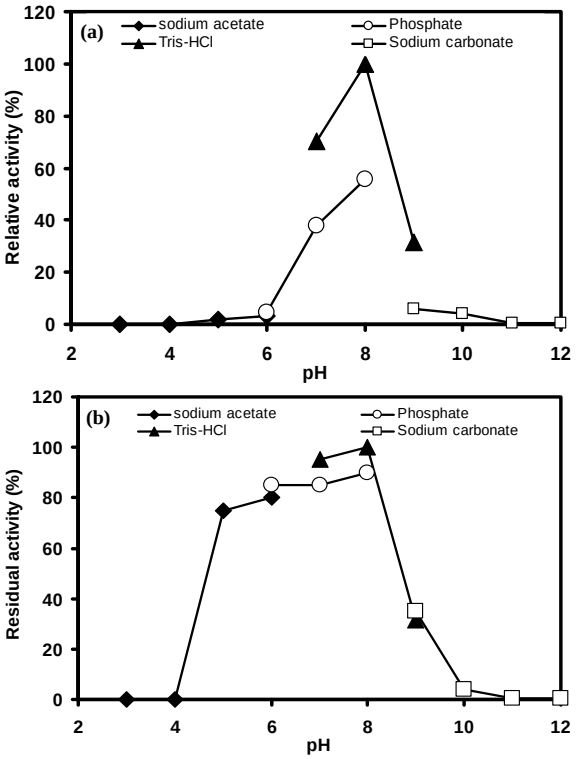

Fig. 5 Effect of pH on (a) lipase activity and (b) stability. The activity was determined by incubating the reaction mixture in 50 mM buffer of a specific pH at 37 °C using *p*-nitrophenyl palmitate as the substrate. Buffer systems used were acetate (pH 3.0–6.0; closed diamonds), phosphate (pH 6.0–8.0; open circles), Tris-HCl (pH 7.0–9.0; closed triangles) and carbonate (pH 9.0–12.0; open squares). The pH stability was studied by measuring the residual activities after 6 h incubation compares with control (100% relative activity) at pH 8.0.

Effect of pH and temperature on lipase activity and stability

Lipase secreted from this strain exhibited maximum hydrolytic activity towards *p*-nitrophenyl palmitate at pH 8.0 (Fig. 5a). More than 80% residual activity remained after 6 h incubation between 5.0 and 8.0 pH, while at pH $>$ 8.0 it was drastically reduced (Fig. 5b). At an optimum pH 8.0 and stability under weak acidic conditions, the enzyme revealed an acidophilic characteristic different from other bacterial lipases[35,39]. The lack of lipase activity at a strongly acidic pH might be due to Ca^{2+} loss and its coordinating role at the active site of the enzyme[40].

The optimum temperature of the secreted lipase was 37 °C (Fig. 6a), retaining more than 90% of initial activity after 6 h incubation at 30–80 °C (Fig. 6b).

Lipase retained 100% activity at 60 °C for 3 h and the half-life was 36 h (Fig. 6c). Previously, lipase from *Aeromonas* sp. was found to be stable below 40 °C[23,25] and bacterial lipases generally have temperature optima in the range 30–60 °C[41,42]. Thus this enzyme seems to have considerably higher thermostability and is therefore appropriate for industrial use[4].

Effect of organic solvents on the stability of lipase

The enzyme retained more than half of its original activity in the presence of isoamyl alcohol ($\log P_{\mathrm{o/w}} = 1.3$; 90% relative activity), benzene ($\log P_{\mathrm{o/w}} = 2.0$; 58%), hexane ($\log P_{\mathrm{o/w}} = 3.5$; 72%), heptanes ($\log P_{\mathrm{o/w}} = 4.0$; 78%), and decane ($\log P_{\mathrm{o/w}} = 5.6$; 85%) while in hexadecane ($\log P_{\mathrm{o/w}} = 8.8$) residual activity was only 28%. Perhaps hydrophobic solvents hinder efficient interaction between enzymes and substrates[43]. Also, it has been reported that alkanes/solvents such as decane and hexadecane repress lipase activity[31]. In contrast, hydrophilic solvents like DMSO ($\log P_{\mathrm{o/w}} = -1.22$) and short-chain alcohols severely decreased lipase activity: DMSO (38% relative activity), ethanol (19%), methanol (39%), butanol (50%). It is possible that hydrophilic solvents are able to dissolve enzyme more efficiently thus resulting in high degree of inactivation[44] and short-chain alcohols have low solubility in oils forming a new liquid phase at moderate concentrations thus leading to enzyme inactivation[45]. Recently, lipases are increasingly being used in reactions containing water-immiscible organic solvents and small amounts of water[31,44,46]. Thus the stability of the bacterial lipase in hydrophobic solvents as well at elevated temperatures makes the enzyme suitable for applications in organic synthesis as well as detergents.

Effects of chemicals on lipase activity

Lipase from *Aeromonas* sp. EBB-1 was very sensitive to Zn^{2+} (39% relative activity), SDS (28%), and EDTA (19%) but the activity was 29% enhanced by Ca^{2+}. The effect of EDTA on enzyme activity demonstrated that lipase is a metalloenzyme that is inconsistent with the requirement of Ca^{2+} located in a substrate binding pocket[47]. An increase of lipase activity was caused by the complex action of calcium ions on the liberated fatty acids and enzyme structure. Calcium ions bound to the protein rather than having a catalytic role can stabilize the structure preventing heat denaturation and protease attack[47]. Surfactants like SDS do not inhibit the enzyme production directly but inhibit lipase activity by changing the conformation of lipase or the interfacial properties[48]. Addition of

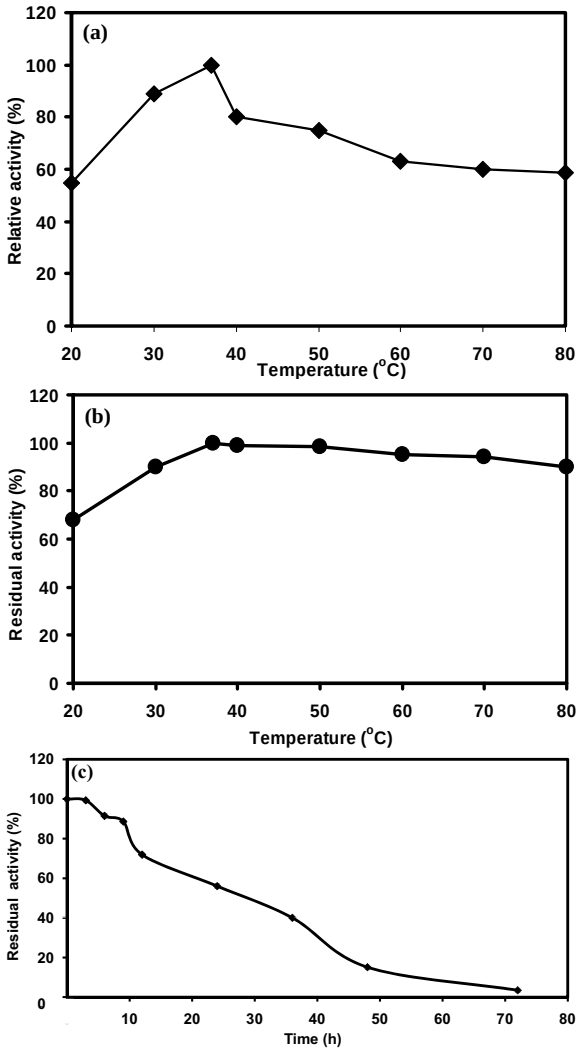

Fig. 6 Effect of temperature on (a) lipase activity and (b) stability, and (c) stability profile of the enzyme at 60 °C. The activity was measured by incubating the purified enzyme in the temperature range of 20–80 °C in a thermostatically controlled water bath. The thermal stability of lipase was measured by pre-incubating lipase solution at different temperature for 6 h, after which the residual activity was calculated comparing with control (100% of relative activity) at 37 °C.

Triton X-100 and Na^{+} had little effect on activity (98% and 99% relative activity, respectively) but a little antagonistic effect on lipase activity was found by the action of Ba^{2+} (90%), Mn^{2+} (95%), Ni^{2+} (84%), Tween80 (89%), DTT (92%), and PMSF (80%). The presence of K^{+}, Li^{+}, Ag^{+}, and Mg^{2+} ions significantly reduced the lipase activity to 70%, 79%, 72%, 65%,

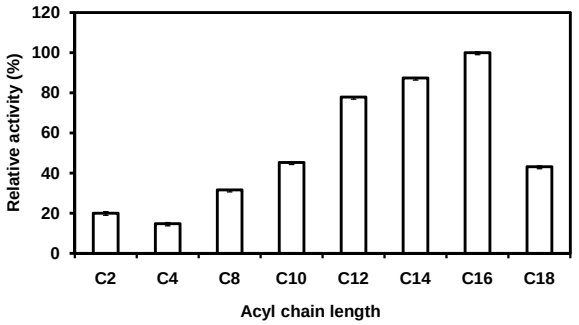

Fig. 7 Substrate specificity towards esters of *p*-nitrophenyl. Percentages shown are relative to maximum activity (C_{16}). Results reported as averages ± standard deviation.

respectively. Consistent with previous reports[48–50], the enzyme retained half of its original activity in the presence of Fe^{2+} (52%) or Hg^{2+} (54%). The 20% inactivation of lipase activity in the presence of PMSF revealed that the bacterial lipase had an active serine residue in its catalytic site thus giving it a property of serine protease[47]. The small effect of DTT on lipase activity indicated that the active site may lack a disulphide bond which is important for catalytic function[34,47]. The inhibition of lipase activity by Zn^{2+}, Hg^{2+}, and Ag^{+} may indicate the presence of thiol-containing amino acid residues in the enzyme catalytic centre[47], while other ions may affect the ability of the salts to react with free fatty acids adhering to the oil droplets and decreasing their surface area to react with the enzyme[48].

Substrate specificity

The purified EBB-1 lipase showed a variable specificity/hydrolytic activity towards *p*-NP esters of different carbon chain length (Fig. 7). Among the substrates, the highest activity was found with *p*-NP palmitate (C_{16}). Long-chain (C_{12} and C_{14}) esters were more efficiently hydrolysed than other esters. Such a preferential specificity towards longer carbon chain length substrates has been reported for a lipase from *Aeromonas* sp.[25,38]

The few reports on lipases from *Aeromonas* sp. indicate stability at moderate temperature. For example, lipase from *A. sobria* LP004 showed thermostability $< 40\,^{\circ}C$ under alkaline conditions[37]. Lipase from psychrotrophic *Aeromonas* sp. LPB 4 is stable at temperatures $< 50\,^{\circ}C$[25]. Thus, to the best of our knowledge, this is the first report on the production of thermostable lipase by *Aeromonas* sp. This unique property makes the enzyme attractive for various biotechnological applications.

Acknowledgements: This study was partly supported through J.C. by the Centre of Excellence on Environmental Health, Toxicology and Management of Chemicals (ETM-PERDO). We are grateful for financial support to S.S. from the Centre of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education. This work is linked with Asian Core Program on capacity building and development of microbial potential and fermentation technology towards new era. We thank F. W. H. Beamish for editorial assistance.

REFERENCES

1. Hansan F, Shah AA, Hameed A (2006) Industrial applications of microbial lipases. *Enzym Microb Tech* **39**, 235–51.
2. Fang Y, Lu Z, Lv F, Bie X, Liu S, Ding Z, Xu W (2006) A newly isolated organic solvent tolerant *Staphylococcus saprophyticus* M36 produced organic solvent-stable lipase. *Curr Microbiol* **53**, 510–5.
3. Dordick JS (1989) Enzymatic catalysis in monophasic organic solvents. *Enzym Microb Tech* **11**, 194–211.
4. Haki GD, Rakshit SK (2003) Development in industrially important thermostable enzyme: a review. *Bioresource Tech* **89**, 17–34.
5. Namboodiri VMH, Chattopadhaya R (2000) Purification and biochemical characterization of a novel thermostable lipase from *Aspergillus niger*. *Lipids* **35**, 495–502.
6. Li H, Zhang X (2005) Characterization of thermostable lipase from thermophilic *Geobacillus* sp. TW1. *Protein Expr Purif* **42**, 153–9.
7. Kanwar SS, Ghazi IA, Chimni SS, Joshi GK, Rao GV, Kaushal RK, Gupta R, Punj V (2006) Purification and properties of a novel extracellular thermotolerant metallolipase of *Bacillus coagulans* MTCC-6375 isolate. *Protein Expr Purif* **46**, 421–8.
8. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (2nd edn, Cold Spring Harbor Laboratory.
9. Kouker G, Jaeger KE (1987) Specific and sensitive plate assay for bacterial lipases. *Appl Environ Microbiol* **53**, 211–3.
10. Pencreac'h G, Baratti JC (1996) Hydrolysis of *p*-nitrophenyl palmitate in *n*-heptane by the *Pseudomonas cepacia* lipase: A simple test for the determination of lipase activity in organic media. *Enzym Microb Tech* **18**, 417–22.
11. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing, the principle of protein-dye binding. *Anal Biochem* **72**, 248–54.
12. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.
13. Precigou S, Wieserl M, Pommares P, Goulasl P, Duran R (2004) *Rhodococcus pyridinovorans* MW3, a bac-

terium producing a nitrile hydratase. *Biotechnol Lett* **26**, 1379–84.
14. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–5.
15. Austin B, Adams C (1996) Fish pathogens. In: Austin B, Alwegg M, Gosling PJ, Joseph S (eds) *The Genus Aeromonas*, Wiley, Chichester, pp 198–243.
16. Mahler GF, Kok RG, Cordenons A, Hellingwerf KJ, Nudel BC (2000) Effects of carbon sources on extracellular lipase production and *lipA* transcription in *Acinetobacter calcoaceticus*. *J Ind Microbiol Biotechnol* **24**, 25–30.
17. Rashid N, Shimada Y, Ezaki S, Atomi H, Imanaka T (2001) Low-temperature lipase from psychrotrophic *Pseudomonas* sp. strain KB700A. *Appl Environ Microbiol* **67**, 4064–9.
18. Schmidt-Dannert C, Luisa RM, Schmid RD (1997) Two novel lipases from the thermophilic *Bacillus thermocatenulatus*: Screening, purification, cloning, overexpression and properties. *Meth Enzymol* **284**, 194–219.
19. Swift S, Throup JP, Williams P, Salmond GP, Stewart GS (1996) Quorum sensing: a population-density component in the determination of bacterial phenotype. *Trends Biochem Sci* **21**, 214–9.
20. Brune AK, Gotz F (1992) Degradation of lipids by bacterial lipases. In: Winkelman G (ed) *Microbial Degradation of Natural Products*. VCH, Weinhein, pp 243–66.
21. Lotti M, Monticelli S, Monstesinos JL, Brocca S, Valero F, Lafuente J (1998) Physiological control on the expression and secretion of *Candida rugosa* lipase. *Chem Phys Lipids* **93**, 143–8.
22. Ghosh PK, Saxena RK, Gupta R, Yadav RP, Davidson WS (1996) Microbial lipases: production and applications. *Sci Progr* **79**, 119–57.
23. Lotrakul P, Dharmsthiti S (1997) Lipase production by *Aeromonas sobria* LP004 in a medium containing whey and soybean meal. *World J Microbiol Biotechnol* **13**, 163–6.
24. Dharmsthiti S, Luchai S (1998) Production and immobilization of lipase from *Aeromonas sobria* harboring a heterogous gene. *J Ferment Bioeng* **86**, 335–7.
25. Lee HK, Ahn MJ, Kwak SH, Song WH, Jeong BC (2003) Purification and characterization of cold active lipase from psychrotrophic *Aeromonas* sp. LPB4. *J Microbiol* **41**, 22–7.
26. Ota Y, Suzuki M, Yamada K (1968) Lipids and related substances inducing the lipase production by *Candida paralipolytica*. *Agr Biol Chem* **32**, 390–1.
27. Guo A, Petrovic Z (2005) Vegetable oils-based polyols. In: Erhan SZ (ed) *Industrial Uses of Vegetable Oils*, AOCS Press, pp 110–30.
28. Boekema BKHL, Beselin A, Breuer M, Hauer B, Koster M, Rosenau F, Jaeger KE, Tommassen J (2007) Hexadecane and Tween 80 stimulate lipase production in *Burkholderia glumae* by different mechanisms. *Appl Environ Microbiol* **73**, 3838–44.
29. Kanwar L, Gogoi BK, Goswami P (2002) Production of a *Pseudomonas* lipase in n-alkane substrate and its isolation using an improved ammonium sulfate precipitation technique. *Bioresource Tech* **84**, 207–11.
30. Uttatree S, Charoenpanich J (2011) Nutritional requirements and physical factors affecting the production of organic solvent-stable lipase by *Acinetobacter baylyi*. *Chiang Mai Univ J Nat Sci* **10**, 111–24.
31. Kok RG, Nudel CB, Gonzalez RH, Nugteren-Roodzant IM, Hellingwerf KJ (1996) Physiological factors affecting production of extracellular lipase (LipA) in *Acinetobacter calcoaceticus* BD413: fatty acid repression of *lipA* expression and degradation of LipA. *J Bacteriol* **178**, 6025–35.
32. Gilbert EJ, Drozd JW, Jones CW (1991) Physiological regulation and optimization of lipase activity in *Pseudomonas aeruginosa* EF2. *J Gen Microbiol* **137**, 2215–21.
33. Stolp H, Starr MP (1981) Principles of isolation and conservation of bacteria. In: Starr MP, Stolp H, Truper HG, Balows A, Schlegel HP (eds) *The Prokaryotes*, Springer-Verlag, Heidelberg, pp 135–75.
34. Dharmsthiti S, Pratuangdejkul J, Theeragool GT, Luchai S (1998) Lipase activity and gene cloning of *Acinetobacter calcoaceticus* LP009. *J Gen Appl Microbiol* **44**, 139–45.
35. Barbaro SE, Trevors JT, Inniss WE (2001) Effect of low temperature, cold shock, and various carbon sources on esterase and lipase activities and exopolysaccharide production by a psychrotrophic *Acinetobacter* sp. *Can J Microbiol* **47**, 194–205.
36. Liu IL, Tsai SW (2003) Improvements in lipase production and recovery from *Acinetobacter radioresistens* in presence of polypropylene powders filled with carbon sources. *Appl Biochem Biotechnol* **104**, 129–40.
37. Lotrakul P, Dharmsthiti S (1997) Purification and characterization of lipase from *Aeromonas sobria* LP004. *J Biotechnol* **54**, 113–20.
38. Anguita J, Aparicio LBR, Naharro G (1993) Purification, gene cloning, amino acid sequence analysis, and expression of an extracellular lipase from an *Aeromonas hydrophila* human isolate. *Appl Environ Microbiol* **59**, 2411–7.
39. Gupta R, Gupta N, Rathi P (2004) Bacterial lipases: an overview of production, purification and biochemical properties. *Appl Microbiol Biotechnol* **64**, 763–81.
40. Pratuangdeikul J, Dharmsthiti S (2000) Purification and characterization of lipase from psychrophilic *Acinetobacter calcoaceticus* LP009. *Microbiol Res* **155**, 95–100.
41. Lesuisse E, Schanck K, Colson C (1993) Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme. *Eur J Biochem* **216**, 155–60.
42. Wang Y, Srivastava KC, Shen GJ, Wang HY (1995)

Thermostable alkaline lipase from a newly isolated thermophilic *Bacillus*, strain A30-1 (ATCC 53841). *J Ferment Bioeng* **79**, 433–8.
43. Laane C, Boeren S, Vos K, Veeger C (1987) Rules for optimization of biocatalysis in organic solvent. *Biotechnol Bioeng* **30**, 81–7.
44. Sugihara A, Tani T, Tominaga Y (1991) Purification and characterization of a novel thermostable lipase from *Bacillus* sp. *J Biochem* **109**, 211–6.
45. Shimada Y, Watanabe Y, Samukawa T (1999) Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase. *J Am Oil Chem Soc* **76**, 789–93.
46. Ogino H, Ishikawa H (2001) Enzymes which are stable in the presence of organic solvent. *J Biosci Bioeng* **91**, 109–16.
47. Brockerhoff H, Jensen RG (1974) *Lipolytic Enzymes*, Academic Press, New York.
48. Patkar SA, Bjorkling F (1994) Lipase inhibitors. In: Woolley P, Petersen SB (eds) *Lipases - their Structure, Biochemistry and Application*, Cambridge Univ Press, Cambridge, pp 207–24.
49. Yan G, Yang G, Xu L, Yan Y (2007) Gene cloning, overexpression and characterization of a novel organic solvent tolerant and thermostable lipase from *Galactomyces geotrichum* Y05. *J Mol Catal B* **49**, 28–35.
50. Uttatree S, Winayanuwattikun P, Charoenpanich J (2010) Isolation and characterization of a novel thermophilic-organic solvent stable lipase from *Acinetobacter baylyi*. *Appl Biochem Biotechnol* **162**, 1362–76.