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A novel cold-adapted lipase from *Acinetobacter* sp. XMZ-26: gene cloning and characterisation

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Abstract *Acinetobacter* sp. XMZ-26 (ACCC 05422) was isolated from soil samples obtained from glaciers in Xinjiang Province, China. The partial nucleotide sequence of a lipase gene was obtained by touchdown PCR using degenerate primers designed based on the conserved domains of cold-adapted lipases. Subsequently, a complete gene sequence encoding a 317 amino acid polypeptide was identified. Our novel lipase gene, lipA, was overexpressed in *Escherichia coli*. The recombinant protein (LipA) was purified by Ni-affinity chromatography, and then deeply characterised. The LipA resulted to hydrolyse *p*NP esters of fatty acids with acyl chain length from C2 to C16, and the preferred substrate was *p*NP octanoate showing a k_{cat}= 560.52±28.32 s^{-1}, K_m=0.075±0.008 mM, and a k_{cat}/K_m= 7,377.29±118.88 s^{-1} mM^{-1}. Maximal LipA activity was observed at a temperature of 15°C and pH 10.0 using *p*NP decanoate as substrate. That LipA peaked at such a low temperature and remained most activity between 5°C and 35°C indicated that it was a cold-adapted enzyme. Remarkably, this lipase retained much of its activity in the presence of commercial detergents and organic solvents, including Ninol, Triton X-100, methanol, PEG-600, and DMSO. This cold-adapted lipase may find applications in the detergent industry and organic synthesis.

Keywords Cold-adapted lipase · *Acinetobacter* sp. · Detergent additives · Organic synthesis

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Introduction

Lipases (carboxylesterases, EC 3.1.1.3) hydrolyse triacylglycerols to fatty acids, diacylglycerol, monoacylglycerol, and glycerol (Carriere et al. 1994). Owing to their availability and various reactions they catalyse, microbial lipases have been the subject of many important studies (Jaeger et al. 1999). Moreover, due to their substrate specificities, thermostability, and alkaline stability, microbial lipases have been widely utilised in various industries, including detergent, oil processing, cosmetics, medicine, and food industry (Kademi et al. 2000).

Cold-adapted lipases, which display maximal activity at low temperatures, have attracted a great deal of attention in recent years (Joseph et al. 2008). They have evolved a range of structural features that confer a high level of flexibility, particularly around the active site, which translate into a low activation enthalpy, low substrate affinity, and high specific activity at low temperatures. This shift towards low temperatures at which maximal activity occurs is accompanied by a concomitant decrease in thermal stability (Joseph et al. 2008). Therefore, cold-adapted lipases are attractive biocatalysts, which can be used at lower temperatures as additives in the food industry and in laundry detergents to allow washing in cold water (Choo et al. 1998; Park et al. 2009; Suzuki et al. 2001). Additionally, cold-adapted lipases have the potential to be used as catalysts in the organic synthesis of chiral intermediates, allowing relatively unstable compounds to be produced at low temperatures (Ryu et al. 2001). Cold-adapted lipases are normally found in microorganisms that survive at low temperatures (around 5°C) (Joseph et al. 2008). Permanently cold regions such as glaciers and mountain regions provide the typical habitat for such microorganisms.

In the last two decades, a great number of cold-adapted lipase genes have been cloned and much related research has been conducted. Cold-adapted lipases from *Psychrobacter* sp. strain TA 144 (Feller et al. 1991; De Santi et al. 2010), *Pseudomonas fluorescens* strain C9 (Dieckelmann et al. 1998), *Pseudomonas* sp. strain B11-1 (Choo et al. 1998), *Pseudomonas fragi* (Alquati et al. 2002), *Pseudomonas* sp. strain KB700A (Rashid et al. 2001), *Psychrobacter* sp. Ant 300 (Kulakova et al. 2004), *Pseudomonas lipolyticum* M37 (Ryu et al. 2001), and *Moritella* sp. 2-5-10-1 (Yang et al. 2008) have all been cloned and expressed. However, the expression of cold-adapted lipases in mesophilic organisms such as *Escherichia coli* present problems, including the formation of inclusion bodies and protein misfolding (Feller et al. 1991).

In this study, an *Acinetobacter* sp. strain, which we named XMZ-26, was isolated from the soil surrounding glaciers in Xinjiang, China. A novel cold-adapted lipase gene was cloned from this strain and successfully expressed in *E. coli*. Characterisation of the recombinant enzyme revealed that it displayed maximal activity at low temperatures and retained its activity well in the presence of detergents and organic solvents.

Materials and methods

Bacterial strains, plasmids, and chemicals

E. coli strains JM109 (TaKaRa, Shiga, Japan) and BL21 (DE3) (Novagen, Darmstadt, Germany) were maintained at 37°C in Luria-Bertani (LB) broth or on agar plates for recombinant plasmid amplification and protein expression, respectively. The vectors pGEM-T Easy (Promega, Madison, WI, USA) and pET-30a (+) (Novagen, Gibbstown, NJ, USA) were used for gene cloning, and gene expression, respectively. Genome walking kits, DNA purification kits, restriction endonucleases, T4 DNA ligase, and *Taq* DNA polymerase were all purchased from TaKaRa. To validate the accuracy of our gene insertion technique, DNA sequencing and peptide mass fingerprinting were performed at the State Key Laboratory of Crop Genetic Improvement, Chinese Academy of Agricultural Sciences, Beijing, China.

Substrates tributyrin, *p*-nitrophenyl (*p*NP) acetate (C2), butyrate (C4), caprate (C10), myristate (C14), and palmitate (C16) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade and are commercially available.

Isolation of lipase-producing strains

Strains exhibiting lipolytic activity were isolated from the soil of glaciers in Xinjiang, China. The medium used for the selection and enrichment of the soil samples contained 1% olive oil, 0.5% peptone, and 0.5% NaCl. After incubation at 20°C in shake flask (200 rpm) for overnight, the culture was diluted and spread onto agar plates containing a 1% olive oil emulsion. Pure cultures, obtained through repeated streaking, were tested for lipase production by screening on growth plates using 1% tributyrin as the substrate and Victoria blue as an indicator. Based on the level of lipase production of the strains, one strain, designated XMZ-26, was selected for further study. XMZ-26's taxon was identified by comparison of its 16S rDNA sequence with those listed in GenBank. The strain XMZ-26 was deposited in Agricultural Culture Collection Center (ACCC, http://www.accc.org.cn), and the collection number was ACCC 05422.

Cloning of the lipase gene fragment

By comparing the amino acid sequences of cold-adapted lipases, two highly conserved regions (HGGG and GDSAG) were identified. The degenerate primers lipF and lipR (in Table 1) were designed based on the sequences of these highly conserved regions. The partial lipase sequence between these two conserved motifs was amplified from XMZ-26 genomic DNA by touchdown PCR. The reaction conditions were 94°C for 5 min, 20 cycles (94°C for 30 s, annealing temperatures ranging from 65°C to 55°C, with a decrease of 0.5°C from one cycle to the next, 72°C for 45 s), 30 cycles (94°C for 30 s, 55°C for 45 s, 72°C for 45 s), and 72°C for 10 min. The amplified PCR product was cloned into pGEM-T Easy and sequenced.

Cloning of the flanking sequence of the lipase gene by genome walking

To obtain the sequences upstream and downstream of our partial lipase gene, we performed genome walking PCR using a genome walking kit according to the manufacturer's instructions. The primers were designed based the partial sequence and listed in Table 1. Sequence assembly was performed using Vector NTI Suite 7.0 software (InforMax, Gaithersburg, MD, USA). Alignments of the DNA and protein sequences were carried out using blastn and blastp, respectively (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignments were performed using ClustalW (Thompson et al. 1994).

Expression of the recombinant LipA in *E. coli*

The following primers were used in the construction of the *lipA* expression plasmid: lipFm (with a *Bam*HI site) and lipRm (with a *Hin*dIII site) (in Table 1). The PCR products were inserted into pET-30a (+) digested with *Bam*HI and

Table 1 Primers used for gene cloning and expression

Name	Function	Sequence (5′→3′)
lipAF	Degenerate primer	GTGGTGTAYTTYCAYGGBGG
lipAR	Degenerate primer	CAGGTTGCCRCCSGCRCTRTC
lipAFsp1	Upstream genome walking	CCATTGCAGGCCATGTTGATAG
lipAFsp2	Upstream genome walking	TGGTGTTAAGCGATAATCCACAC
lipAFsp3	Upstream genome walking	AATCACGGCATAAATAGCGAGTG
lipARsp1	Downstream genome walking	AGTTTATCACTCGCTATTTATGCCG
lipARsp2	Downstream genome walking	TGAGTGTGGATTATCGCTTAACAC
lipARsp3	Downstream genome walking	TCTATCAACATGGCCTGCAATG
lipFm	Expression vector construction	*GGATCC*ATGACACAACAATCGAGC
lipRm	Expression vector construction	*AAGCTT*TCAATTGAACATTGGCTTT

Note: Y identifies C or T; B represents C, G, or T; R represents A or G; S represents C or G; N represents A, C, G, or T. Restriction sites are italicised.

*Hin*dIII. The recombinant plasmid, pET–lipA, was sequenced to confirmation. The recombinant plasmid pET–lipA was transformed into *E. coli* BL21 (DE3) cells. Transformed cells were picked from a single colony and grown in shake flask containing 3 ml LB broth supplemented with 100 μg/ml kanamycin overnight at 37°C. They were then inoculated at a dilution of 1:100 in 100 ml fresh LB medium containing kanamycin and were grown aerobically at 37°C. When the culture density reached to 0.6 at OD_{600}, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the growth medium at a final concentration of 0.5 mM for induction. After incubation overnight at 16°C, the cells were harvested by centrifugation at 8,000×*g* for 10 min at 4°C.

Purification and identification of the recombinant LipA

The bacterial pellets were resuspended in 15 ml lysis buffer (50 mM Tris–Cl pH 8.0) and disrupted by sonication. The suspension was centrifuged at 10,000×*g* for 30 min at 4°C. Then the concentrated supernatant (crude enzyme) was applied to the Ni-NTA affinity chromatography column (GE Healthcare Life Sciences, Piscataway, NJ, USA) that had been previously equilibrated with washing buffer (50 mM Tris–Cl pH 8.0, 500 mM NaCl, and 10% glycerol). The protein was eluted using an imidazole step gradient (0, 20, 40, 80, 100, and 200 mM) in washing buffer. Finally, the imidazoles in the purified protein were removed by dialysis in 50 mM Tris–Cl pH 8.0.

The purified enzyme was evaluated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). A prestained Low Molecular Weight Calibration Kit for SDS Electrophoresis (Fermentas, Glen Burnie, Maryland, USA) was used as a standard. The separated proteins were visualised through staining with Coomassie brilliant blue R-250. The single protein band after purification was confirmed by the peptide mass fingerprinting. Additionally, after SDS-PAGE, the proteins were transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) to be analysed by Western blotting using an anti-His antibody and BCIP/NBT detection kit (Beijing CoWin Biotech, Beijing, China) according to the manufacturer's instructions. Protein concentrations were determined by the Bradford assay (Bradford 1976) using bovine serum albumin as a standard.

Lipase activity assay

Lipase activity was determined by measuring the release of *p*NP using the chromometer method with some modifications (Maia et al. 2001). Unless otherwise described, lipase activity was measured by a standard assay at 20°C with 500 μM *p*NP decanoate (C10 dissolved in ethanol) in 50 mM Tris–HCl buffer (pH 9.0) containing 1% ethanol. The reaction was started by the addition of 100 μl of purified enzyme at a concentration of 0.25 mg/ml. Following incubation at 20°C for 10 min, the reaction was terminated through the addition of 0.5 ml of 10% trichloroacetic acid, and then 0.5 ml of 10% sodium carbonate was added to each reaction mixture to generate the coloured product. Blank reactions were performed with identical composition as the assay mixture without the enzyme. The mixtures were centrifuged, and the absorbance of the resulting supernatants was measured at 405 nm by use of UNIC 7200 spectrophotometer (UNIC, Shanghai, China). One unit (U) of enzyme activity was defined as the amount of enzyme that releases of 1 μmol of *p*NP per minute at 20°C.

Enzyme characterisation of recombinant LipA

The substrate selectivity of LipA for *p*NP esters was assayed using *p*NP acetate (C2), *p*NP butyrate (C4), *p*NP octanoate (C8), *p*NP decanoate (C10), *p*NP myristate (C14), and *p*NP palmitate (C16) as substrates in 50 mM Tris–HCl (pH 9.0) at 20°C. The *p*NP ester substrates except *p*NP palmitate were dissolved in ethanol at a final concentration of 50 mM as the stock solution, and *p*NP palmitate was dissolved in isopropanol. The working

concentration range of the *p*NP esters was 0.025 to 3 mM. Initial velocity versus substrate concentration data were fitted to the Lineweaver–Burk transformation of the Michaelis–Menten equation. Kinetic parameters of LipA for different substrates were determined by the GraphPad Prism software (http://www.graphpad.com).

The *p*NP decanoate was subsequently used to assay the enzyme's other characteristics. Its optimal pH at 20°C was determined using buffers ranging in pH from 3.0 to 12.0. The following buffers were used; 20 mM disodium hydrogen phosphate-citric acid buffer (pH 3.0–8.0), 50 mM Tris–HCl buffer (pH 8.0–10.0), and 20 mM glycine–NaOH (pH 10.0–12.0). To test its pH stability, 100 μl of the lipase (2.5 mg/ml) was pre-incubated without substrate in 900 μl of different buffers with pH values from 3.0 to 12.0 for 1 h at 25°C, and then lipase activity was measured under standard conditions.

The enzyme's optimal temperature was determined by measuring its activity in 50 mM Tris–HCl buffer (pH 9.0) at temperatures ranging from 0°C to 60°C. Its thermostability was subsequently monitored by pre-incubating the enzyme without substrate in Tris–HCl buffer (pH 9.0) for 2, 5, 10, 15, and 20 min at 30, 40, 50, and 60°C. Lipase activity was then measured under standard conditions.

The effects of different metal ions, chemical reagents, detergents, and organic solvents on enzymatic activity were assessed in 50 mM Tris–HCl buffer (pH 9.0) at 20°C. The reactions contained 1, 5, and 10 mM NaCl, KCl, $BaCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $NiCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $CdCl_2$, and EDTA; 0.05% and 0.1% (*v*/*v*) sodium dodecyl sulphate (SDS), alcohol ether carboxylate (AEC), Tween-20, Tween-80, Triton X-100, Ninol, 3-([3-cholanidopropyl] dimethylammonio)-1-propanesulfonate (CHAPS), and cetyltrimethylammonium bromide (CTAB); and 15% and 30% methanol, ethanol, isopropanol, propanediol, propanetriol, polyethylene glycol 600 (PEG-600), and dimethyl sulphoxide (DMSO).

GenBank accession number

The nucleotide sequences of the *Acinetobacter* sp. XMZ-26 16S rDNA and lipase gene (*lipA*) were deposited in the GenBank database under accession numbers GQ227698 and GQ227702, respectively.

Results

Identification of strain XMZ-26

Strain XMZ-26 showed a clear degrading halo when grown on agar supplemented with tributyrin and Victoria blue. It was found to be an aerobic, Gram-negative bacterium able to grow in the presence of 10% glucose (*w*/*v*) and produce lipases. Its 16S rDNA sequence shared 99% identity with 33 different species of the genus *Acinetobacter*. These results indicated that it was a species of *Acinetobacter*.

Gene cloning and sequence analysis

A partial *lipA* gene fragment (240 bp) was amplified by touchdown PCR using the degenerate primers lipAF and lipAR, and then sequenced. Sequence alignment confirmed that the 240-bp nucleotide fragment belonged to a lipase gene. DNA fragments of 737 and 1,019 bp, containing upstream and downstream regions flanking the partial *lipA* gene fragment, respectively, were amplified by genome walking. These two sequences and the 240-bp fragment were assembled using Seqman 6.0 (Lasergene Suite) software, and a 1,915-bp DNA sequence was reconstructed. Sequence analysis identified a 954-bp lipase ORF between nucleotides 171 and 1,124. This putative ORF encoded a 317 amino acid polypeptide with a calculated molecular mass of 35.5 kDa. A putative ribosomal-binding site (RBS) was identified 5-bp upstream of the start codon. The deduced amino acid sequence of the ORF was aligned with available protein sequences held in the GenBank and SWISSPROT databases. It was 69%, 68%, 42%, and 33% identical to lipases from *Acinetobacter baumannii* AB900, *A. baumannii* AYE, *Pseudomonas* sp. B11-1, and *P. fluorescens* Pf-5, respectively. According to these alignments (Fig. 1), LipA contained the consensus lipase catalytic triad, comprised of Ser 165 (in the motif GDSAGG), Asp 264, and His 289. An HGGG motif, which can form an oxyanion hole to stabilise tetrahedral intermediates (Grochulski et al. 1993; Martinez et al. 1994) was also identified. Moreover, tiny amino acids (Ala, Cys, Gly, Ser, and Thr) reached to 28.7% in total amino acids, and many of them distributed around the active site.

Enzyme expression and purification

The transformant harbouring *pET–lipA* was cultivated in LB medium containing kanamycin. After induction with 0.5 mM IPTG at 16°C overnight, recombinant LipA protein with a six-histidine tag at its N terminus was overexpressed in a soluble, catalytically adapted form. It was not expressed in un-induced cultures or induced transformants harbouring empty pET-30a (+) (Fig. 2a).

Recombinant LipA was purified from the supernatants of cell lysates by single-step affinity chromatography on a Ni-NTA column, and mostly eluted in the elution buffers containing 100 and 200 mM imidazol (Fig. 2a). The fractions from affinity chromatography were combined,

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LipA                MTQQSSMHYRLSEEMQSLVYWSSIYSPADDDIDSIRAAYDAMCLHYTLPRDGTVNIEDKTVPQLNHPVNVRLYSPLGEAP 80
A.baumannii AB900   MTTYEQATYALSEEMQSLVYWSSIYSPADADIDSVRAAYDAMCRHYTLPRDDKLDVEDRVIANEEHPVPVRVYLPKTNRP 80
A.baumannii AYE     MTTYEQATYALSEEMQSLVYWSSIYSPADADIDSVRAAYDAMCRHYTLPRDDKLDVEDRVIANEEHPVPVRVYLPKTNRP 80
P.fluorescens Pf-5  -----MNHYPLSAELTAFVQRTLSYTSVDSSFQGLRDSYERMCRAFTPPRPAGLAVQELQLSGVP----LRLYHPERPAA 71
P.fluorescens Pf0-1 -----MGTYPLSPSMAAFVARTESFASDDSSLGGLRKSYDDMCRAFTPERPAGLEVVDFQLSGVA----VRSYRPPVRPS 71
P.aeruginosa PAO1  ----MAAKYPLSPAMWRFVEHSRAFASDSPRLDAQRAAYARMCQAFAPPRPAGLRVLDSCLPAAP-PVRVRRYRPDRPAP 75

LipA                ATGWPCILYLHGGGWMVGNLDSHEFITRYLCRDLNVAVLSVDYRLTPEHHFPAAYEDCETVYHWLYQHGLQWKINPNQIV 160
A.baumannii AB900   ESGWPCVLYLHGGGWMVGGLDSHEFITSYLCKDLNAVVIGVDYRLAPEHRFPAAFEDCLAVYHWLKQHGSAWQIDSENIV 160
A.baumannii AYE     ESGWPCVLYLHGGGWMVGGLDSHEFITSYLCKDLNAVVISVDYRLAPEHRFPAAFEDCLAVYQWLKQHGSVWQIDSENIV 160
P.fluorescens Pf-5  PSGWPWLLYLHGGGWVLGGLDSHEFITARLASELGILVIAVDYRLAPEHPFPAAFDDCLGVWRALEQGLLPLPLDPQRRV 151
P.fluorescens Pf0-1 ASGWPCVLYLHGGGWVVGGLDSHDFICCELAMALGAMVVVDYRLAPEHPFPAGFEDCLSVWRALRSG--PFWFDPGRML 151
P.aeruginosa PAO1  PGGWPALLYLHGGGWMLGGLDSHDFICADLAARLGLLVLAVDYRLAPEHPFPAALQDCLRAWQALSLGELDEALDGRRLL 155

LipA                LMGDSAGGNLAAALAVQLQHTG-AQACGLAMIYPCLSSGFDTASCQQQADAPLLSLADMHDYLQAYAPNTSDWQDLRLSP 239
A.baumannii AB900   LAGDSAGGNLAAALAVELQHSG-LQAQGLALVYPCLTTAFDTPSAQKHAHAPLLTTEDMHFYLKEYAPNSRDWQDLRLAP 239
A.baumannii AYE     LAGDSAGGNLAAALAVELQHSG-LQAQGLALVYPCLTTAFDTPSAQKHAHAPLLTTEDMHFYLKEYAPDSQDWQDLRLAP 239
P.fluorescens Pf-5  LMGDSAGGNLAAALCLALRDAGEPGPAAQVLVYPGLGGAANLPSRSQCADAPLLSSSDLDCFQALYLPGPQQR-SAYARP 230
P.fluorescens Pf0-1 VAGDSAGGNLAAALCLALRDAGEPLPGAQVLIYPGLGGDAQLPSRSECADAPLLASSDVDCYHALYLRGTAKP-NAYAMP 228
P.aeruginosa PAO1  VAGDSAGGNLAAALCLALRDGGAPSPAAQILLYPLLS-AAPSPSRIDCADAPLLGLGDVQACLDAYLPLAALHRQPLALP 234

LipA                LLAQDFSDMPRSFIAVAEYDPLSDDGRIFADKLKQANIATEFYLGKGLLHGSLRLVRDCPVVQDLYQRMLSSLKPMFN-- 317
A.baumannii AB900   LLATDFSDMPTSFVAVAEYDPLSDDGYLFTQKLEQAGIPNEFHLGKGLLHGSLRLMRDCPEVQQLYQNMLSAIRRMLSSA 319
A.baumannii AYE     LLATDFSDMPTSFVAVAEYDPLSDDGYLFTQKLEQAGIPNEFHLGKGLLHGSLRLMRECPEVQHLYQNMLSAIRRMLSSA 319
P.fluorescens Pf-5  LLAQQFAGLPAAFIALAQFDPLRDDGERYHQHLLAAGGQSQLYPGLGLVHGCLRARGLAPEVDALYSALLAYLEQHLGRP 310
P.fluorescens Pf0-1 LLAGDFSGLPPAWIAVAQFDPLRDDGVCYAERLDAAGVDAALYYGEGLVHGCLRARHQVAEVDRLFENLLGFMADKM--- 305
P.aeruginosa PAO1  LEAADFTGLPPAFVAVAEFDPLRDDGERYGAALRAAGGEAGFYPGSGLVHGCLRG-HGIDEVEALHEALRRAVQGFLAED 313
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Fig. 1 Alignment of the amino acid sequence of LipA with those of five other lipases with high identity. Residues involved in the catalytic triad are identified by *triangles*. Conserved regions are enclosed in *boxes*

dialysed, and concentrated to a final concentration of 1.26 mg/ml, and this purification protocol routinely yielded 12.6 mg of homogeneous LipA from 100 ml bacteria culture (Table 2). By the Western blotting analysis of the various purification steps though using an anti-His antibody, the purified enzyme presented as a single band with a molecular weight of approximately 37 kDa (Fig. 2b). Additionally, peptide mass fingerprinting of the single protein purified from this band also confirmed it to be identical to the predicted lipase encoded by the putative 954-bp ORF (Fig. 3).

Enzyme substrate specificity

To detect substrate specificity, the kinetic parameters on various *p*NP esters with acyl chains of different lengths from 2 to 16 carbon atoms were examined (Table 3). Because all the used esters showed a low solubility in water, we evaluated the effect of the presence of ethanol or

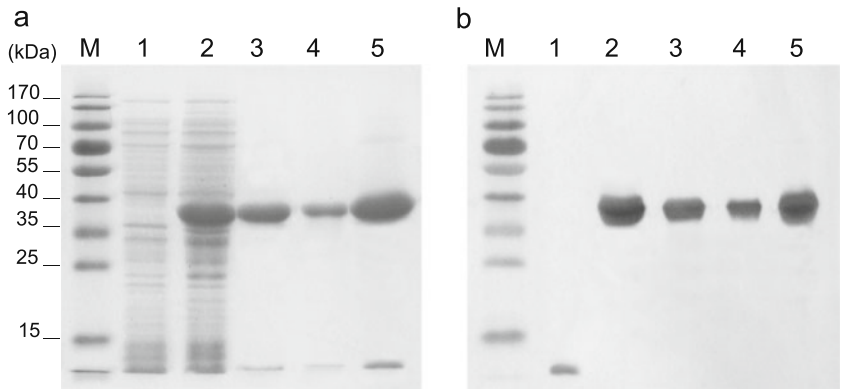

Fig. 2 SDS-PAGE and Western blotting analysis showing the purification of recombinant LipA expressed in *E. coli* BL21 (DE3). **a** Proteins recovered during the various purification steps were analysed by SDS-PAGE. *Lane M*, prestained standard protein molecular weight markers; *Lane 1*, lysate supernatants of induced transformant harbouring empty pET-30a(+); *lane 2*, lysate supernatants (30.40 μg) of induced transformant harbouring pET-LipA; *lane 3*, purified fraction (4.07 μg) from Ni–NTA column eluted by buffers containing 100 mM imidazol; *lane 4*, purified fraction (2.03 μg) from Ni–NTA column eluted by buffers containing 200 mM imidazol; *lane 5*, purified LipA (12.60 μg) after dialysis and concentration; **b** Western blot analysis of purification steps probed with an anti-His antibody. The samples are the same as those described for panel **a**, and the amount of samples is 5% of those described for panel **a**

Table 2 Purification parameters of recombinant LipA

Methods	Concentration (mg/ml)	Volume (ml)	Activity (U/ml)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude enzyme	3.04	15	584.85	192.39	1	100
Affinity chromatography	0.61	20	330.32	541.51	2.81	75.31
Dialysis and concentration	1.26	10	577.07	457.99	2.38	65.77

isopropanol in the assay mixture on LipA activity. No negative effect on LipA catalytic activity was detected when ethanol or isopropanol was added up to 1% (*v*/*v*). Therefore, all the characterisation was carried out at 20°C in the presence of ethanol or isopropanol. In this condition, both the K_m and k_{cat} values of purified LipA decreased with increases in the acyl chain length up to C8. LipA displayed highest specific catalytic efficiency represented by the value of k_{cat}/K_m on *p*NP octanoate (C8). The lowest K_m and highest k_{cat}/K_m values on *p*NP octanoate (C8) indicated it was the best substrate of LipA.

Effects of pH and temperature on lipase activity

Recombinant LipA's optimal pH was 10.0, and it retained at least 80% of its maximum activity between pH 8.0 and 11.0 (Fig. 4a), indicating that it is an alkaline enzyme. LipA was stable over a wide pH range: it remained at least 70% of its maximum activity after incubation in buffers ranging in pH from 6.0 to 12.0 (Fig. 4b).

LipA activity peaked at a temperature of 15°C. The enzyme retained over 50% of its maximum activity when assayed at temperatures ranging from 5°C to

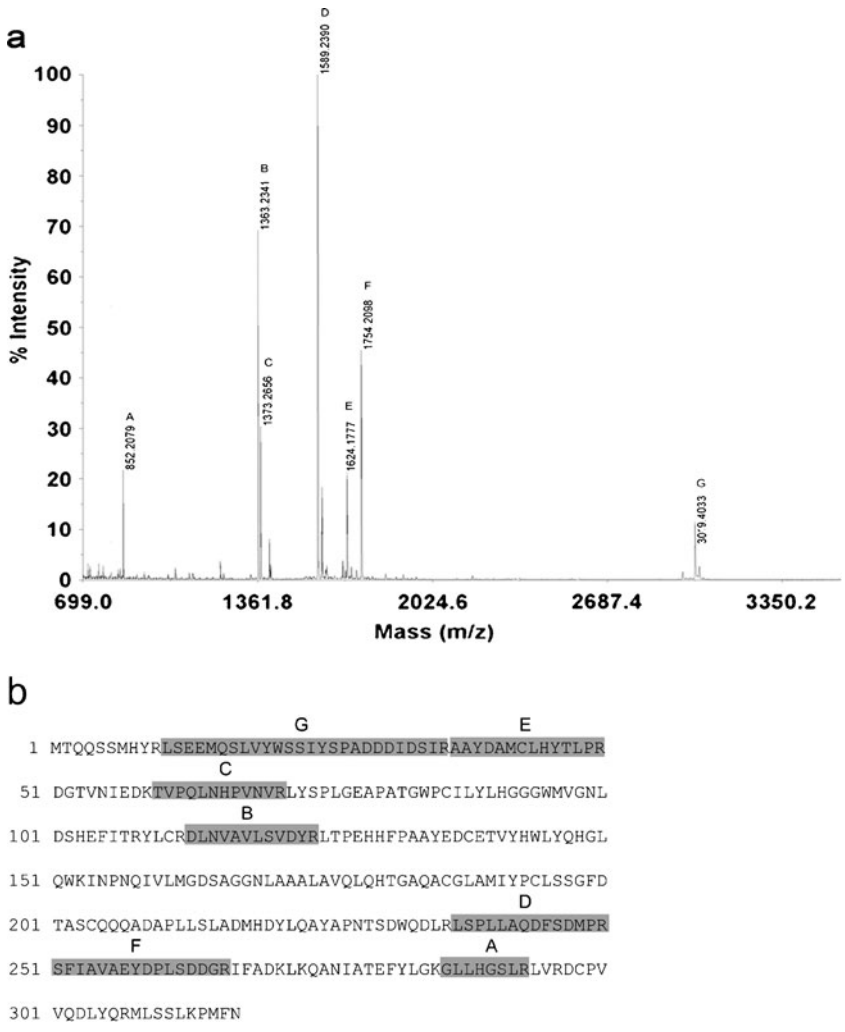

Fig. 3 Identification of LipA from a single band extracted from a polyacrylamide gel by peptide mass fingerprinting following SDS-PAGE. **a** Peptide mass fingerprint generated by MALDI-TOF mass spectrometry of the products produced by trypsinisation of the LipA-containing band (Fig. 2, *lane 5*) (*x*-axis, *m*/*z* ratio; *y*-axis, species abundance in terms of percent signal intensity). Major peaks corresponding to the predicted products of LipA trypsinisation are labelled alphabetically according to their molecular masses (*insets*). Peptide A is the arginine-extended equivalent of A. **b** Predicted trypsinisation products responsible for the highlighted peaks in the peptide mass fingerprint. *Labels* identify the peptide fragments of LipA whose molecular weights correspond to those of the peaks shown in the peptide mass fingerprint

Table 3 Kinetic parameters for recombinant LipA

Substrate (*p*-nitrophenyl ester)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
*p*NP acetate (C2)	1.563±0.051	2,565±127.80	1,641.07±134.79
*p*NP butyrate (C4)	0.867±0.029	2,452±108.10	2,829.76±218.97
*p*NP octanoate (C8)	0.075±0.008	560.52±28.32	7,377.29±118.88
*p*NP decanoate (C10)	0.155±0.006	589.70±17.25	3,809.43±271.46
*p*NP myristate (C14)	0.282±0.023	58.86±1.83	208.50±11.52
*p*NP palmitate (C16)	0.349±0.031	22.18±1.18	63.57±9.08

50°C, and the relative activity still reached to 38.8% even at 0°C (Fig. 5a). The recombinant lipase was, respectively, 71% and 45% active after 30 min of incubation at 30°C and 40°C. However, it was inactivated when incubated at 50°C and 60°C in a pseudo-first-order manner, with $t_{1/2}$ values of 8.5 and 6.2 min, respectively (Fig. 5b).

Effects of metal ions, detergents, and organic solvents on lipase activity

The effects of different metal ions, detergents, and organic solvents on the activity of LipA are shown in Tables 4, 5, and 6, respectively. Lipase activity was fully inhibited by Zn^{2+} and strongly inhibited by Cu^{2+}, Cd^{2+}, and Ni^{2+}, even

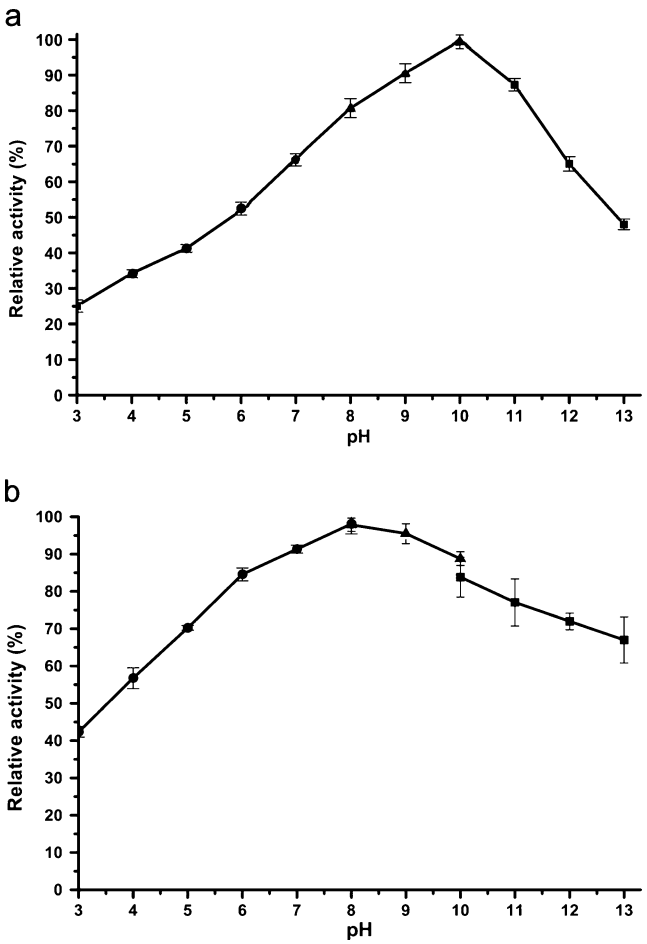

Fig. 4 Effects of pH on the activity and stability of purified LipA. **a** Influence of pH on lipase activity. Assays were performed at 20°C in buffers ranging in pH from 3.0 to 12.0. **b** Maintenance of lipase activity across a range of pH values. After incubation at 25°C for 1 h in buffers ranging in pH from 3.0 to 12.0, the activity of the purified enzyme in 50 mM Tris–HCl buffer (pH 9.0) at 20°C was measured. The following buffers were used; 20 mM disodium hydrogen phosphate-citric acid buffer (pH 3.0–8.0, *filled circle*); 50 mM Tris–HCl buffer (pH 8.0–10.0, *filled triangle*); and 20 mM glycine–NaOH (pH 10.0–12.0, *filled square*)

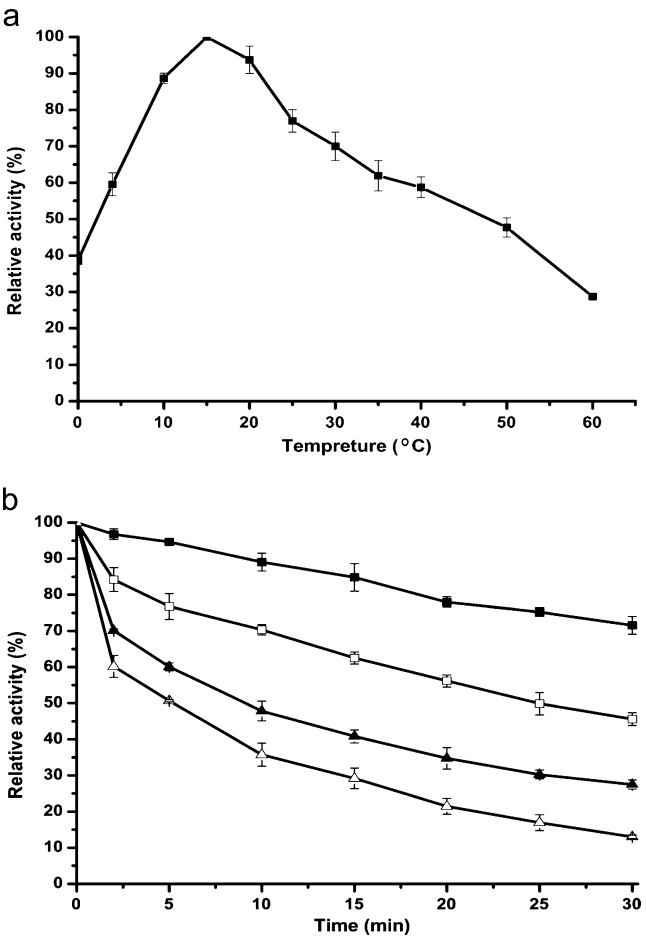

Fig. 5 Effects of temperature on the activity and stability of purified LipA. **a** Influence of temperature on lipase activity. Activity of LipA (using *p*NP decanoate as substrate) was assayed at pH 9.0 and a range of different temperatures. **b** Thermostability of purified LipA. LipA was incubated in 50 mM Tris–HCl buffer (pH 9.0) at 30°C (*filled square*), 40°C (*empty square*), 50°C (*filled triangle*), and 60°C (*empty triangle*) for the indicated periods of time, and enzymatic activity was measured (using *p*NP decanoate as substrate)

Table 4 Effects of various metal ions on LipA activity

Metal ion	Relative activity (%) at a metal ion concentration of:		
	1 mM	5 mM	10 mM
Na^+	105.22±3.81	113.35±0.30	115.54±7.80
K^+	116.05±8.97	123.31±8.03	125.14±9.77
Ca^{2+}	162.85±4.19	236.20±2.07	250.00±0.96
Mg^{2+}	166.98±0.69	197.17±0.96	206.68±2.95
Ba^{2+}	200.88±2.75	218.38±2.74	234.28±4.28
Zn^{2+}	6.85±1.66	3.47±1.35	1.03±0.03
Mn^{2+}	84.42±0.53	55.67±0.27	45.38±0.69
Ni^{2+}	14.91±0.68	9.23±0.27	7.77±1.76
Cu^{2+}	9.87±0.31	7.31±0.54	4.66±0.31
EDTA	85.68±0.62	79.85±4.41	61.41±7.06

at the lowest tested concentration (1 mM) and enhanced by Ca^{2+}, Mg^{2+}, and Ba^{2+}. Ca^{2+}, Mg^{2+}, and Ba^{2+} at a concentration of 10 mM increased the activity of LipA approximately 2.5-, 2.34-, and 2.07-fold, respectively. Na^+ and K^+ had little or no effect on lipase activity. EDTA had little influence on the activity of the lipase at the lower tested concentrations, but inhibited lipase activity by 39.39% at a concentration of 10 mM.

Similar to other *Acinetobacter* lipases, including those of *Acinetobacter* sp. SY-01 (Han et al. 2003), RAG-1 (Snellman et al. 2002), O16 (Breuil and Kushner 1975), *Acinetobacter calcoaceticus* BD413 (Kok et al. 1995), and *A. baumannii* BD5 (Park et al. 2009), the activity of LipA was increased 2.5-fold in the presence of Ca^{2+}. As in *Acinetobacter radioresistens* CMC-1 (Hong and Chang 1988), certain heavy metal ions, such as Zn^{2+} Ni^{2+}, and Cu^{2+}, also caused LipA inactivation.

Apart from SDS, the tested detergents did not inhibit the activity of LipA at a concentration of 0.05%. In fact, enzymatic activity was increased by 126.11% using 0.05% Ninol, by 47.71% using 0.05% Tween-80, by 44.04% using 0.05% Triton X-100, and by 30.65% using 0.05% AEC. LipA activity was reduced by 35.63%, 21.84%, 19.92%, 37.11%, 34.16%, and 79.64% using 0.1% Tween-20, Triton X-100, CHAPS, CTAB, AEC, and SDS, respectively. In contrast, 0.1% Ninol increased the enzymatic activity by approximately 44%. In addition, the result of detergents' resistance showed that activity of LipA remained over 80% after incubating in 0.1% above-mentioned detergents except Triton X-100 and SDS at 25°C for 30 min (data not shown).

Table 5 Effects of various detergents on LipA activity

Detergent	Relative activity (%) at a detergent concentration of:	
	0.05%	0.1%
Tween-20	115.96±1.79	64.37±2.28
Tween-80	147.71±2.63	96.83±5.74
Triton X-100	144.04±5.03	78.16±3.64
Ninol	226.11±5.60	144.08±0.35
AEC	130.65±2.06	65.84±0.79
SDS	51.18±5.48	20.36±3.11
CTAB	110.35±2.40	62.89±7.33
CHAPS	121.15±2.38	80.08±0.71

Table 6 Effects of various organic solvents on LipA activity

Organic solvent	Relative activity (%) at an organic solvent concentration of:	
	15%	30%
Methanol	141.13±0.29	121.46±3.24
Ethanol	89.31±1.68	66.16±2.72
Isopropanol	51.33±0.41	23.64±1.90
Propanediol	129.52±0.24	138.52±2.08
Propanetriol	103.65±4.22	109.64±1.36
PEG-600	146.04±0.35	176.77±0.79
DMSO	126.10±0.59	125.29±0.84

Activity in organic solvents is an important property of protein catalysts used in organic synthesis reactions. To assess the potential of LipA this context, its activity in selected water-miscible solvents was examined. Although its activity was decreased by 10.69% and 48.67% using 15% (*v*/*v*) ethanol and isopropanol, respectively, similar concentrations of other organic solvents increased its activity. Enzymatic activity was increased by 41.13% using methanol, by 29.52% using propanediol, by 46.04% using PEG-600, and by 26.10% using DMSO. When the solvent concentrations were increased to 30%, enzymatic activity was strongly inhibited by ethanol and isopropanol but enhanced by other organic solvents: 21.46% by methanol, 38.52% by propanediol, 76.77% by PEG-600, and 25.29% by DMSO.

Discussion

Permanently cold regions such as glaciers and mountain regions provide the typical habitat for psychrotrophic microorganisms. Our stain *Acinetobacter* sp. XMZ-26 was also isolated from glaciers soil samples. It showed a clear degrading halo when grown on agar supplemented with tributyrin and Victoria blue, which indicated that it has secreted lipase or esterase to hydrolyse olive oil and tributyrin contained in plates. After calculated by the software SignalP3.0, LipA don't possess a secretion signal

peptide and is a cytoplasmic protein. However, in the research, we also have cloned four other lipase/esterase genes from the strain XMZ-26 and have ascertained that there are secretion signal peptides in two lipases. Therefore, it indicates that LipA is not responsible for hydrolysing olive oil in agar plates observed by this strain. Besides, the related articles of other lipases will be published in the future.

Many lipase/esterase genes have been cloned from *Acinetobacter* sp. However, the expression of the proteins they encoded in *E. coli* has been problematic, and many recombinant proteins, including LipBD5 from *A. baumannii* BD5 (Park et al. 2009) and Lip1, LipA2, and LipA3 from *Acinetobacter* sp. DYL129 (Kim et al. 2008), form inclusion bodies as a result of protein misfolding or excessively rapid production and accumulation (Sabate et al. 2010). In this study, when *lipA* of *Acinetobacter* sp. XMZ-26 was introduced to *E. coli* BL21 (DE3) induced at low temperatures, LipA was overexpressed in a soluble, catalytically active conform. This result indicates that the cold-adapted lipase gene can be expressed in mesophilic organisms such as *E. coli* following low-temperature induction.

Many bacterial IV family lipases display striking amino acid sequence similarities to mammalian HSL family lipases (Hemilä et al. 1994). They contain two highly conserved motifs: GDSAG, which includes the active-site serine residue, and HGGG, which may constitute an oxyanion hole (Grochulski et al. 1993; Martinez et al. 1994). LipA contains both of these motifs. Moreover, there are many tiny amidic acids surrounding the active site of LipA, which allow the structure of LipA more flexible in order to reduce the energetic cost of the conformational changes required to interact with the substrate (Jahandideh et al. 2007). Elsewhere, we showed that the activity of LipA was highest at 15°C, similar to cold-adapted lipase 2 of *Psychrobacter* TA144 (Feller et al. 1991; De Santi et al. 2010) and the lipases of *Psychrobacter* sp. 2-7 (Loreto et al. 2008). This optimal temperature is lower than those reported for lipases/esterases produced by other *Acinetobacter* sp., including *Acinetobacter* sp. No 6 (Suzuki et al. 2001), *Acinetobacter* sp. SY-01 (Han et al. 2003), *Acinetobacter* sp. RAG-1 (Snellman et al. 2002), and *A. baumannii* BD5 (Park et al. 2009), which have been shown to be optimally active at temperatures ranging from 35°C to 55°C. The cold adaptation of LipA, combined with the results of our sequence analysis, indicate that LipA is a cold-adapted enzyme.

Many lipases, such as the lipases from *Psychrobacter* sp. 2-7 (Loreto et al. 2008) and *Pseudomonas* sp. B11-1 (Choo et al. 1998), are capable of hydrolysing water-soluble esters with short chain acyl group. The values for kinetic parameters reveal that medium chain acyl group *p*NP esters (C8 and C10) are better substrates for LipA, which elucidated that LipA is a lipase. Moreover, the catalytic constants of k_{cat}/K_m towards short chain acyl group *p*NP-esters (C2 and C4) are also very high, so it is obvious that our LipA also has high catalytic efficiency to hydrolyse esterase substrates.

The most commercially important field for the application of hydrolytic lipases is in household and industrial laundry detergents and in household dishwashers (Joseph et al. 2008). Some lipases, including *Candida antarctica* lipase, have been produced as recombinant enzymes for use in detergent formulations (Uppenberg et al. 1994). The lipase component increases detergency and prevents scaling (Ito et al. 1988). LipA was found to be active on an unusually wide range of substrates. Moreover, it was able to retain a high level of activity in the presence of commercially available detergents. Indeed, its activity was actually increased by Ninol, Tween-80, Triton X-100, and AEC. These results indicate that this cold-adapted lipolytic enzyme may be useful in detergents designed to be used at cold temperatures.

Stability in the presence of organic solvents is a requisite property of enzymes used in non-aqueous systems (Ogino 2009). Although inhibited by ethanol and isopropanol, the activity of LipA was increased by other organic solvents, notably PEG-600, which increased its activity by 176.77% at a concentration of 30%. These solvents may decrease enzyme aggregation, modify the substrate–water interface or cause beneficial conformational changes (Snellman and Colwell 2004). That LipA demonstrated increased activity following exposure to such solvents suggests that it might be a useful catalyst in organic solvent systems.

In this study, the lipase LipA produced by *Acinetobacter* sp. XMZ-26 exhibited maximal activity at low temperatures, indicating that it is a cold-adapted enzyme. Moreover, LipA retained strong lipolytic activity in various detergents and organic solvents. These results pave the way for further research of this cold-adapted lipase for application in the detergent industry or organic synthesis.

References

Alquati C, Gioia L, Santarossa G, Alberghina L, Fantucci P, Lotti M (2002) The cold-adapted lipase of *Pseudomonas fragi*: heterologous expression, biochemical characterization and molecular modeling. Eur J Biochem 269:3321–3328

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 72:248–254

Breuil C, Kushner DJ (1975) Partial purification and characterization of the lipase of a facultatively psychrophilic bacterium (*Acinetobacter* O16). Can J Microbiol 21:434–441

Carriere F, Thirstrup K, Hjorth S, Boel E (1994) Cloning of the classical guinea pig pancreatic lipase and comparison with the lipase related protein. FEBS Lett 338:63–68

Choo DW, Kurihara T, Suzuki T, Soda K, Esaki N (1998) A cold-adapted lipase on an Alaskan psychrotroph, *Pseudomonas* sp. strain B11-1: gene cloning and enzyme purification and characterization. Appl Environ Microbiol 64:486–491

De Santi C, Tutino ML, Mandrich L, Giuliani M, Parrilli E, Del Vecchio P, de Pascale D (2010) The hormone-sensitive lipase from *Psychrobacter* sp. TA144: new insight in the structural/functional characterization. Biochimie 92:949–957

Dieckelmann M, Johnson LA, Beacham IR (1998) The diversity of lipases from psychrotrophic strains of *Pseudomonas*: a novel lipase from a highly lipolytic strain of *Pseudomonas fluorescens*. J Appl Microbiol 85:527–536

Feller G, Thiry M, Arpigny JL, Gerday C (1991) Cloning and expression in *Escherichia coli* of three lipase-coding genes from psychrotrophic Antarctic strain *Moraxella* TA144. Gene 102:111–115

Grochulski P, Li Y, Schrag JD, Bouthillier F, Smith P, Harrison D, Rubin B, Cygler M (1993) Insights into interfacial activation from an open structure of *Candida rugosa* lipase. J Biol Chem 268:12843–12847

Han SJ, Bach JH, Yoon MY, Shin PK, Cheong CS, Sung MH, Hong SP, Chung IY, Han YS (2003) Expression and characterization of a novel enantioselective lipase from *Acinetobacter species* SY-01. Biochimie 85:501–510

Hemilä H, Koivula TT, Palva I (1994) Hormone-sensitive lipase is closely related to several bacterial proteins, and distantly related to acetylcholine esterase and lipoprotein lipase: identification of a superfamily of esterases and lipases. Biochim Biophys Acta 1210:249–253

Hong M, Chang M (1988) Purification and characterization of an alkaline lipase from a newly isolated *Acinetobacter radioresistens* CMC-1. Biotechnol Lett 20:1027–1029

Ito S, Kobayashi T, Ara K, Ozaki K, Kawai S, Hatada Y (1988) Alkaline detergent enzymes from alkalophiles: enzymatic properties, genetics and structures. Extremophiles 2:185–190

Jaeger KE, Dijkstra BW, Reetz MT (1999) Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. Annu Rev Microbiol 53:315–351

Jahandideh S, Abdolmaleki P, Jahandideh M, Barzegari Asadabadi E (2007) Sequence and structural parameters enhancing adaptation of proteins to low temperatures. J Theor Biol 246:159–166

Joseph B, Ramteke PW, Thomas G (2008) Cold adapted microbial lipases: some hot issues and recent developments. Biotechnol Adv 26:457–470

Kademi A, Fakhreddine L, Baratti J (2000) Purification and characterization of a thermostable esterase from the moderate thermophile *Bacillus circulans*. Appl Microbiol Biotechnol 54:173–179

Kim SH, Park IH, Lee SC, Lee YS, Zhou-Yi KCM, Ahn SC, Choi YL (2008) Discovery of three novel lipase (lipA1, lipA2, and lipA3) and lipase-specific chaperone (lipB) genes present in *Acinetobacter* sp. DYL129. Appl Microbiol Biotechnol 77:1041–1051

Kok RG, Thor JJ, Nugteren-Roodzant IM, Brouwer MB, Egmond MR, Nudel CB, Vosman B, Hellingwerf KJ (1995) Characterization of the extracellular lipase, LipA, of *Acinetobacter calcoaceticus* BD413 and sequence analysis of the cloned structural gene. Mol Microbiol 15:803–818

Kulakova L, Galkin A, Nakayama T, Nishino T, Esaki N (2004) Cold-adapted esterase from *Psychrobacter* sp. Ant300: gene cloning, characterization, and the effects of Gly–>Pro substitution near the active site on its catalytic activity and stability. Biochim Biophys Acta 1696:59–65

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Loreto PP, Fernando R, Juan P, Oriana S, Barbara AA, Juan AA (2008) Cloning and fusion expression of a cold-adapted lipase from marine Antarctic origin. Enzyme Microb Technol 42:371–377

Maia MM, Heasley A, Camargo de Morais MM, Melo EH, Morais MA Jr, Ledingham WM, Lima Filho JL (2001) Effect of culture conditions on lipase production by *Fusarium solani* in batch fermentation. Bioresour Technol 76:23–27

Martinez C, Nicolas A, van Tilbeurgh H, Egloff MP, Cudrey C, Verger R, Cambillau C (1994) Cutinase, a lipolytic enzyme with a preformed oxyanion hole. Biochemistry 33:83–89

Ogino H (2009) Organic solvent-tolerant enzymes. Seikagaku 81:1109–1118

Park IH, Kim SH, Lee YS, Lee SC, Zhou Y, Kim CM, Ahn SC, Choi YL (2009) Gene cloning, purification, and characterization of a cold-adapted lipase produced by *Acinetobacter baumannii* BD5. J Microbiol Biotechnol 19:128–135

Rashid N, Yuji S, Satoshi E, Haruyuki A, Tadayuki I (2001) Low-temperature lipase from psychrotrophic *Pseudomonas* sp. strain KB700A. Appl Environ Microbiol 67:4064–4069

Ryu HS, Kim HK, Choi WC, Kim MH, Park SY, Han NS, Oh TK, Lee JK (2001) New cold-adapted lipase from *Photobacterium lipolyticum* sp. nov. that is closely related to filamentous fungal lipases. Appl Microbiol Biotechnol 70:321–326

Sabate R, de Groot NS, Ventura S (2010) Protein folding and aggregation in bacteria. Cell Mol Life Sci (in press)

Snellman EA, Colwell RR (2004) *Acinetobacter* lipases: molecular biology, biochemical properties and biotechnological potential. J Ind Microbiol Biotechnol 31:391–400

Snellman EA, Sullivan ER, Colwell RR (2002) Purification and properties of the extracellular lipase, LipA of *Acinetobacter* sp. RAG-1. J Ind Microbiol Biotechnol 269:5771–5779

Suzuki T, Nakayama T, Kurihara T, Nishino T, Esaki N (2001) Cold-adapted lipolytic activity of psychrotrophic *Acinetobacter* sp. strain No. 6. J Biosci Bioeng 92:144–148

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Uppenberg J, Patkar S, Bergfors T, Jones TA (1994) Cold-adapted lipolytic activity of psychrotrophic *Acinetobacter* sp. strain No. 6. J Mol Biol 235:790–792

Yang X, Lin X, Fan T, Bian J, Huang X (2008) Cloning and expression of lipP, A Gene encoding a cold-adapted lipase from *Moritella* sp. 2-5-10-1. Curr Microbiol 56:194–198