Ann Microbiol (2012) 62:1071–1077
DOI 10.1007/s13213-011-0348-9

ORIGINAL ARTICLE

Cloning, characterization and expression of a novel lipase gene from marine psychrotrophic *Yarrowia lipolytica*

Jun Sheng · Fang Wang · HaiYing Wang · Mi Sun

Received: 13 April 2011 / Accepted: 29 August 2011 / Published online: 10 November 2011

Abstract A marine *Yarrowia lipolytica* yeast, named Bohaisea-9145, was found to secrete a large amount of lipase into the medium. A gene coding for lipase was cloned from the genome of this strain and expressed successfully in *Escherichia coli* BL21 (DE3). A maximum activity of 17.6 U/mg was obtained from cellular extract of *E. coli* harboring the lipase gene. The recombinant lipase exhibits one band with a molecular mass of about 44 kDa on SDS-PAGE. The optimal temperature and pH of the purified lipase were 35°C and 8.5, respectively. The K_m and V_{max} values of the lipase for *p*-nitrophenyl laurate were 0.582 μM and 0.124 mmol min^{-1} mg^{-1} under 35°C, respectively. Additionally, the purified lipase showed a high activity and stability over a wide range of temperatures, especially in the low and moderate temperatures, suggesting its potential for industrial applications.

Keywords Recombinant lipase · Expression · Characterization · Psychrotrophic *Yarrowia lipolytica*

Introduction

Lipases (EC 3.1.1.3), one of the hydrolases, can catalyze the hydrolysis of long-chain acyl glycerols (Jaeger et al. 1999). In addition to being used in fat hydrolysis, interfacial activation and enantioselectivity make lipases highly useful as catalysts in synthetic organic chemistry, in detergents and in the food industry (Brzozowski et al. 1991; Schmid and Verger 1998; Verger 1997). In the medical and therapeutic fields, lipases can also be used for the treatment of cystic fibrosis (Jaeger et al. 1999; Vellard 2003). Lipases have emerged as the third most important enzyme in the enzyme market worldwide.

Many microorganisms, including bacteria, yeasts and fungi, are known as potential producers of lipases (Abada 2008; Jaeger et al. 1999; Lopez et al. 2004; Singh and Banerjee 2007). *Yarrowia lipolytica*, a "generally recognized as safe" (GRAS) microorganism with no adverse effect on humans, has attracted much attention. Lipases from *Y. lipolytica* are usually divided into two families, with most being produced by family II (Dujon et al. 2004). These lipases contain a conserved GHSLG motif matching the consensus GXSXG sequence found in the active sites of lipolytic enzymes. A search of the *Y. lipolytica* genome database showed that it possesses 16 paralogs of genes coding for family II. However, little information on these paralogs is yet available, and only three isoenzymes, namely Lip2p, Lip7p and Lip8p, have been partly characterized so far (Fickers et al. 2011).

In previous studies, we screened over 500 yeast strains from different marine environments. We found that psychrotrophic *Y. lipolytica* (Bohaisea-9145) isolated from the sediment of China Bohai sea could secrete a new type of lipase (LipY) into the medium (Shao et al. 2004). This kind of lipase not only showed high catalytic activity at low temperatures but also maintained good stability in several metal ions and detergents. To be used in basic scientific experiments and for commercial exploitation, the single lipase must be purified. However, many enzymes secreted by *Y. lipolytica* have a similar molecular mass and other physical chemistry characteristics, thus hampering purification of a single lipase from this organism. So, the aims of the present work were to clone the lipase gene from psychrotrophic *Y. lipolytica* and to characterize the recombinant lipase expressed in *Escherichia coli*.

J. Sheng · F. Wang · H. Wang · M. Sun (✉)
Laboratory of Enzyme Engineering, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, People's Republic of China
e-mail: sunmi0532@yahoo.com

Materials and methods

Strains and media

Amplification and sequencing of 18S rDNA and ITS from the psychrotrophic *Y. lipolytica* Bohaisea-9145 DNA (GenBank accession number EU 086828) were performed according to the methods described by Chi et al. (2007). Routine identification of the yeast was performed by the methods described by Kurtzman and Fell (1998). *E. coli* DH5α and BL21 (DE3) were used as the host cells to amplify the plasmids carrying the cloned gene and to express the target gene, respectively, in LB medium (10 g/l NaCl, 5 g/l yeast extract, 10 g/l Bacto tryptone).

DNA manipulation

Genomic DNA and plasmid DNA were isolated using genomic and plasmid DNA isolation kits, respectively (Bioflux, Tokyo, Japan). Total RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's protocol. RNA was reversely transcribed using Universal RiboClone cDNA synthesis system (Promega). Molecular cloning was performed according to standard methods (Sambrook et al. 1989). Restriction enzymes were purchased from Takara Biotechnology (Tokyo, Japan).

Cloning and bioinformatics analysis of the lipase gene

The partial lipase gene sequence was cloned with degenerate primers that were designed based on the conserved amino acid sequences of lipases of different microorganism downloaded from GenBank (http://www.ncbi.nlm.nih.gov/). A full-length lipase gene sequence was prepared using an inverse PCR (IPCR) technique (Triglia et al. 1988). For IPCR, the *Y. lipolytica* Bohaisea-9145 genomic DNA digested by *Eco*RI (37°C, 120 min) was used as a template. The sense and antisense primers involved were designed according to the cloned partial lipase gene. The lipase cDNA was amplified by PCR, and the template was the initial cDNA prepared via reverse transcription of RNA as described above. The PCR product was cloned into a plasmid vector and sequenced. Alignment of multiple sequences was performed with the program DNAMAN (http://www.lynnon.com/).

Expression of the lipase gene in *E. coli*

The forward primer (F_0) 5′-GACGAATTCG CAGGCGTGTCTCAAGG-3′ and the reverse primer (R_0) 5′-AACGCGGCCGCGTTCTCAACTTGTGGGG-3′ were designed on the basis of the lipase gene cloned above. Restriction sites for *Eco*RI and *Not*I were designed at the 5′-terminal of the forward and reverse primers, respectively. The amplified gene fragment was ligated into pET-21a(+). Expressed lipase carries a C-terminal His-tag encoded by the expression vector. The transformants were cultured at 37°C in LB medium supplemented with 100 μg/ml ampicillin until an OD_{600} of 0.4–0.6 was reached. IPTG was added to the medium at a final concentration of 0.5 mM. The cells were further cultured at 16°C for 12 h. Cells transformed with the plasmid pET-21a(+) were used as a control.

Purification of the lipase

The recombinant lipase was purified using a Ni-affinity column (GE; http://www.gelifesciences.com) according to the manufacturer's protocol. SDS-PAGE was performed with a 15% polyacrylamide gel on a two-dimensional electrophoresis system (Bio-Rad, Richmond, CA). The proteins were stained by Coomassie brilliant blue R-250 (0.01%) and destained with a solvent system of methanol-water-acetic acid (9:9:2). The standard proteins used for SDS-PAGE were bovine serum albumin (66.4 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa) and lysozyme (14.3 kDa).

Determination of kinetic parameters and lipase activity

Lipase activity was determined by the methods described by Liu et al (2008). The K_m and V_{max} values of purified lipase were calculated using *p*-nitrophenol laurate as substrate, assuming that the reactions followed a simple Michaelis-Menten kinetics. An aliquot of 0.1 ml recombinant lipase (final lipase concentration was 17 U/ml) was added to 1.5 ml substrate solution and the mixture was incubated for 10 min in a shaking water bath at 35°C. One unit (U) of enzyme activity was defined as the amount of enzyme required for the liberation of 1.0 μmol *p*-nitrophenol per minute under the assay conditions.

Effects of pH and temperature on lipase activity and stability

The optimal pH for recombinant lipase activity was determined by incubating the purified enzyme between pH 4.0 and 10.0 using standard buffers: 0.1 M acetate buffer (pH 4.0–5.5), phosphate buffer (pH 5.5–8.0) and 0.1 M glycine/NaOH buffer (pH 8.5–10.5). The pH stability was tested by 120 min preincubation of the purified lipase in various buffers (pH 4.0–10.5) at a temperature of 4°C. The lipase activity remaining was measured immediately after this treatment with the standard method described above. The optimal temperature for

recombinant lipase activity was determined at various temperatures (15–70°C) in 0.1 M phosphate buffer at pH 8.5. Temperature stability of the lipase was tested by pre-incubating the lipase for 120 min at different temperatures ranging from 15 to 70°; residual activity was measured immediately as described above. The activity of the preincubated lipase at 4°C was taken as 100%.

Effect of different ions and compounds on lipase activity

To examine the effects of different metal ions on lipase activity, the enzyme was incubated for 120 min at 4°C with various metal ion ($FeCl_2$, NaCl, $BaCl_2$, $Li_2SO_4{\cdot}H_2O$, $CaCl_2$, $MgSO_4{\cdot}7H_2O$, $HgCl_2$, $CuSO_4$, $CoCl_2$, $ZnSO_4{\cdot}7H_2O$, $AgNO_3$, $FeCl_3$, $MnCl_2{\cdot}4H_2O$) at a final concentration of between 1.0 mM and 5.0 mM. The effects of the protein chelating agents ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), reducing agent dithiothreitol (DTT), protein inhibitor phenylmethylsulfonyl fluoride (PMSF) and detergent sodium dodecyl sulfate (SDS) were also measured in the reaction mixture as described above. Residual lipase activity was measured at the optimal pH 8.5 and 35°C. Lipase activity assayed in the absence of metal ions and compounds was defined as the control.

Results

Cloning and bioinformatics analysis of the lipase gene from Bohaisea-9145

Degenerate primers were designed to clone the partial lipase gene based on conserved amino acid sequences of lipases from different microorganisms (Fig. 1) and the codon usage specificity of *Y. lipolytica* (http://www.kazusa.or.jp/codon). A 267-bp gene fragment was amplified. Analysis of the sequence by BLAST indicated that the PCR-generated fragment has the typical conserved catalytic triad (Gly-His-Ser-Leu-Gly) characteristic of lipase and that is essential for enzymatic activity (Fig. 1). For preparation of the full-length lipase gene, the *Y. lipolytica* Bohaisea-9145 genomic DNA was then digested by *Eco*RI. Unimolecular self-ligation was induced to yield a circular DNA. New sense and the antisense primers were designed according to the partial gene sequence cloned. A DNA fragment of 1,425 bp, including a 1,116 bp open reading frame, partial 5′-untranslated regions (5′-UTR) and 3′-UTR, was obtained by IPCR (data not shown). Sequence analysis of the DNA fragment (1,116 bp) indicated that it encoded a protein of 371 amino acids with a predicted molecular mass of 41,290 Da and a G + C content of 52%. This DNA fragment (GenBank accession number HM194605) likely encodes the lipase LipY. Alignment of this cloned sequence with the cDNA indicated that there was no intron in the LipY gene. The protein deduced from the LipY gene sequence showed 46% identity with *Y. lipolytica* lipase (CAD70713.1) and 41% identity with *Y. lipolytica* lipase2 (ABG81956.1). Interestingly, the LipY amino acid sequence is almost identical to the Lip8p (YALI0A20350p) of *Y. lipolytica* with the exception of five amino acid residues, but there is a significant difference in their optimal temperatures (35°C in LipY versus 45°C in Lip8p) and substrate specificity (C12 in LipY versus C10 in Lip8p) (Fickers et al. 2005, 2011). This difference may be caused by long-term evolution of *Y. lipolytica* for surviving in cold marine environment.

Expression of the lipase gene in *E. coli*

The LipY gene, with no signal peptide coding region and native stop codon, was cloned in the expression vector pET-21a(+) containing a 6×His tag at the C-terminus. The recombinant plasmids pET-21a(+)-LipY were transformed into BL21 (DE3) as described in Materials and methods. The recombinant LipY gene was expressed in the transformed cells upon induction with IPTG at 16°C. The specific lipase activity expressed by induced cells of BL21 (DE3)/pET-21a(+)-LipY was 17.6 U/mg while no lipase activity was detected in BL21 (DE3)/pET-21a(+). The amount of the soluble active lipase reached 452 mg L^{-1}, indicating expression of the cloned lipase gene in *E. coli* cells. Cell-free extracts from induced cells harboring pET-21a(+)-LipY were purified by Ni^{2+}-affinity column, and SDS-PAGE showed that recombinant lipase exhibited one specific band with a molecular mass of about 44 kDa (Lane 3 in Fig. 2), which is close to the theoretical value calculated from the deduced amino acid sequences of the LipY gene.

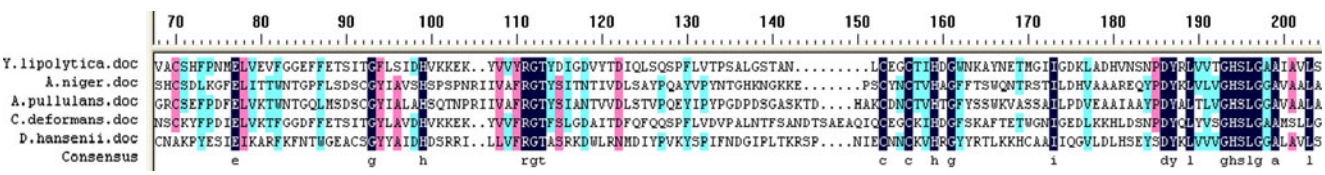

Fig. 1 Multiple alignments of lipase amino acid sequences from *Yarrowia lipolytica* (XP_500777.1) *Aspergillus niger* (ABG37906.1), *Aureobasidium pullulans* (ABV03820.1), *Candida deformans* (CAD21428.1), *Debaryomyces hansenii* (XP_460224.1). Conserved motifs (*shaded dark blue*) were used to design the primers to clone the partial and whole lipase gene

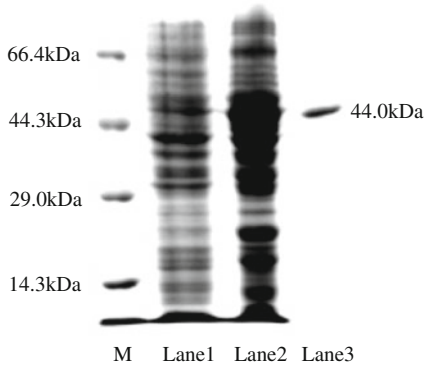

Fig. 2 SDS-PAGE (15%) analysis of lipase expression. Lanes: *M* Marker proteins with relative molecular masses, *1* whole cell extract of control transformants, *2* whole cell extract of induced BL21 (DE3)/pET-21a(+) LipY, *3* purified lipase (44.0 kDa)

Optimum temperature and thermal stability of recombinant lipase

The recombinant lipase exhibited maximal activity at 35°C (Fig. 3a) and retained over 90% of its maximum activity at 30°C. The activity of mesophilic lipases has been reported to be close to zero below 20°C (Joseph et al. 2008). However, the lipase expressed in present study still kept 50% activity at 15°C, which is quite different from other mesophilic lipases. By contrast to the optimal temperatures from *Y. lipolytica* lipases (37°C for Lip2p, 40°C for Lip7p, 45°C for Lip8p), our recombinant lipase showed a higher activity at low and moderate temperatures (Fickers et al. 2011; Song et al. 2006). In addition, it is noteworthy that this recombinant lipase is relatively thermostable, as indicated by the maintenance of at least 70% residual activity after incubation of the enzyme at a wide range of temperatures from 0 to 70°C (Fig.3a).

Optimum pH and pH stability of recombinant lipase

The optimal pH and pH stability of recombinant lipase was measured at various pHs as described in Materials and methods. Figure 3b showed optimal lipase activity at pH 8.5, with significant activity in the range of pH 7.5–10.5. In comparison, the optimal pH of lipases by different yeasts is at a lower range of pH 3–8, such as 5.0 for *Candida rugosa*, 5.5 for *Trichosporon fermentans*, 7 for *Y. lipolytica* LgX64.81, 8 for *Y. lipolytica* Lip2 (Arai et al. 1997; Fickers et al. 2006; Lotti et al. 1993; Yu et al. 2007). Thus, the recombinant lipase expressed here can be characterized as an alkaline lipase.

Effect of different ions and compounds on lipase stability

The effect of various ions on lipase activity was investigated. Fe^{3+}, Na^{+}, Li^{+}, Ca^{2+}, Zn^{2+} (at concentrations of 1 mM) activated the activity of the purified lipase. Among the various metal ions tested, Ba^{2+}, Hg^{2+}, Co^{2+}, Ag^{+} and Mn^{2+} (at concentrations of 5 mM) inhibited lipase activity to 91%, 74%, 92%, 88% and 78% of control values, respectively (Fig. 4). The inhibition by Ag^{+} may indicate the importance of thiol-containing amino acid residues for lipase activity. The greatest stimulation was achieved by Ca^{2+}, and the recombinant lipase activity was enhanced by 122%. We believe that the primary role of Ca^{2+} is to remove fatty acids as insoluble Ca^{2+} salts in certain cases and thus change the interfacial substrate–water relationship to conditions favorable for enzyme action. The lipase from marine *Pseudomonas* sp. 7323 is inhibited by Zn^{2+} and Fe^{2+} by 41% and 48% (Rashid et al. 2001), whereas lipase from marine *Pseudomonas* sp. MSI057 is inhibited by Fe^{2+}, Li^{+}, and Cu^{2+} (Kiran et al. 2008). These results imply that some physical and biochemical properties of the recombinant lipase in this study differ from those of other marine microbial lipases.

The effects of several compounds on lipase activity are depicted in Fig. 5. PMSF had a slight effect on lipase activity at 1 mM but strongly reduced lipase activity to 45.1% at 10 mM. Lipase activity was also inhibited by two known chelating agents, EGTA and EDTA. The calcium-specific chelating agent EGTA reduced lipase activity to 73% at 10 mM, indicating the importance of Ca^{2+} in maintaining lipase structure. In addition, we found that

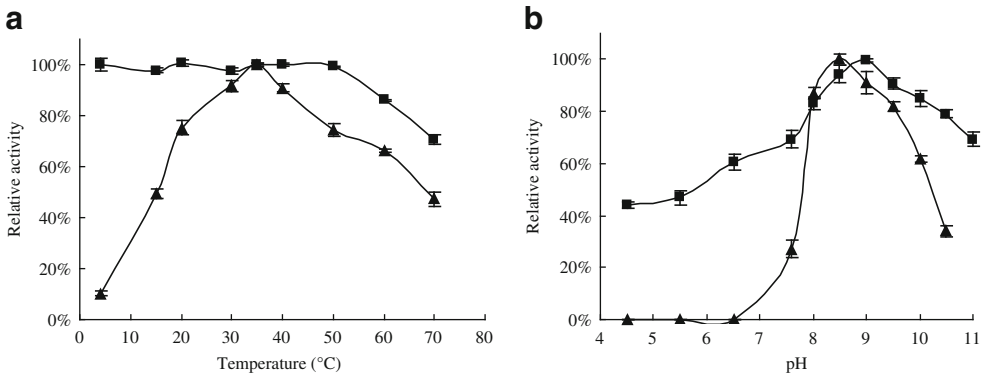

Fig. 3 Effects of temperature and pH on lipase activity (*triangles*) and stability (*squares*). Data are given as means ± SD, $n=3$

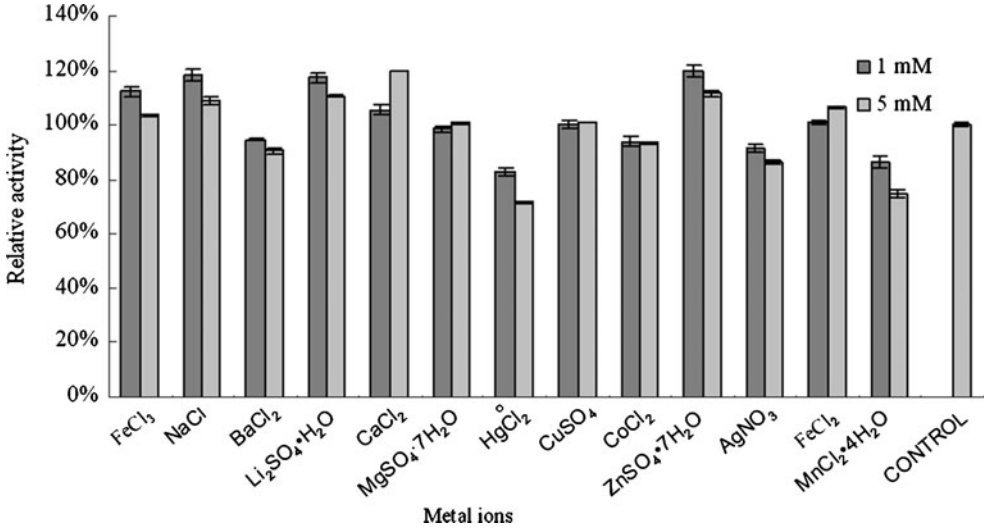

Fig. 4 Effect of different ions on purified lipase activity. Data are given as the means ± SD, $n=3$

DTT could activate the recombinant lipase but SDS had no significant effect on it.

Kinetic parameters

Michaelis-Menten constant and V_{max} were calculated from a Lineweaver-Burk plot. The K_m and V_{max} values of the expressed lipase for the substrate *p*-nitrophenol laurate were 0.582 μM and 0.124 mmol min^{-1} mg^{-1} , respectively, at 35°C (data not shown). The K_m value presented by lipase from *Y. lipolytica* IMUFRJ 50682 was 0.234 mM, whereas the K_m values were 3.3 mM for *Bacillus* lipase, 0.192 mM for *C. antarctica* lipase type B (Brígida et al. 2007; Khurana et al. 2011). These results revealed that the lipase expressed in this study displayed high affinity for *p*-nitrophenol laurate.

Substrate specificity

Substrate specificity of recombinant lipase was assessed by the use of *p*-nitrophenyl (*p*NP) ester with different chain lengths (C2–C16). The recombinant lipase presented the highest activity for *p*NP-laurate (C12), followed by *p*NP-caprylate (C8). The efficiency of recombinant lipase to hydrolyse shorter and longer chain esters decreased rapidly (Fig. 6).

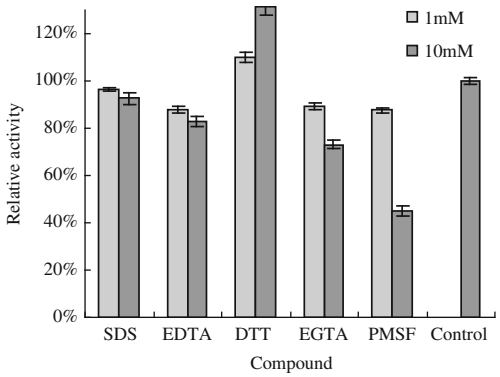

Fig. 5 Effect of various compounds on purified lipase activity. Data are given as the means ± SD, $n=3$

Discussion

Yarrowia lipolytica, formerly known as a good lipase producer, is able to produce extracellular, membrane-bound and intracellular lipases encoded by approximately 16 genes. Although the biochemical and catalytic properties of *Y. lipolytica* lipases have been described extensively in the literature, only three isoenzymes, namely Lip2p, Lip7p and Lip8p, have been partly characterized to date (Fickers et al. 2011; Pignede et al. 2000). Because of huge variation in applications, there is a steadily increasing demand to search for new lipases with different characteristics. In this study, a lipase (LipY) gene was detected and cloned from a psychrotrophic marine *Y. lipolytica*. With the aim of

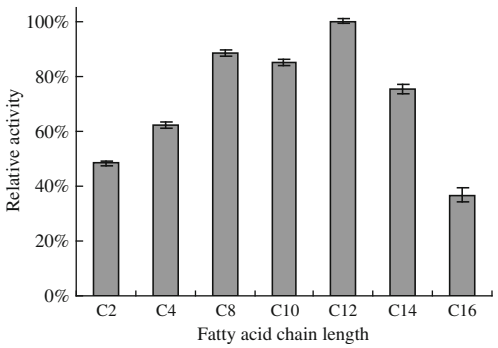

Fig. 6 Hydrolytic activity of recombinant lipase on *p*NPs of varying carbon chain lengths (*p*NP-acetate (C2), *p*NP-butyrate (C4), *p*NP-caprylate (C8), *p*NP-caprate (C10), *p*NP-laurate (C12), *p*NP-myristate (C14) and *p*NP-palmitate (C16) at 35°C and pH 8.5. Data are given as the means ± SD, $n=3$

obtaining a sufficient amount purified lipase and characterizing it, we expressed the LipY gene in *E. coli*.

Our current study indicates that recombinant LipY showed high activity at low and moderate temperatures. It is very active and stable in the presence of several metal ions and detergents under alkaline conditions. Often detergents are potent inhibitors of pancreatic lipase and some microbial lipases (Gargouri et al. 1984). The characteristics of recombinant LipY indicate its potential use as an additive in detergents to enhance effectiveness in cold water washing. The substrate specificity assay indicated that recombinant LipY showed highest activity towards C12 ester. The hydrolysis efficiency of recombinant LipY decreased when the chain length increased from C14 and decreased from C8. By contrast, Lip7p presents an optimal activity for medium chain ester *p*NP-caproate (C6), whereas Lip8p preferred pNP-caprate (C10). It is noteworthy that recombinant LipY retained 75% of maximal activity on *p*NP-myristate (C14), but Lip8p has almost no catalytic activity on this substrate (Fickers et al. 2005). This difference in substrate specificity of lipase isoenzymes was also observed in *C. rugosa*, with lipase A and lipase B having their maximum activity centred on caprylate and on laurate, respectively (Redondo et al. 1995).

In conclusion, we have successfully isolated a lipase gene from *Y. lipolytica*. Despite striking similarities between LipY and LipY8p, LipY exhibited important differences in optimal temperature and substrate specificity. The results obtained here can improve our understanding of lipase features from *Y. lipolytica*. The functional expression of LipY gene is a first step towards making this lipase more broadly accessible to basic studies and industrial applications.

Acknowledgment This research was supported by Hi-Tech Research and Development Program of China (863), grant No. 2007AA091602.

References

Abada EA (2008) Production and characterization of a mesophilic lipase isolated from *Bacillus Stearo thermophilus* AB-1. Pak J Biol Sci 11:1100–1106

Arai T, Yusa S, Kirimura K, Usami S (1997) Cloning and sequencing of the cDNA encoding lipase I from *Trichosporon fermentans* WU-C12. FEMS Microbiol Lett 152:183–188

Brígida AIS, Amaral PF, Gonçalves LR, Coelho MAZ (2007) Characterization of an extracellular lipase from *Yarrowia lipolytica*. Dissertation, Proceedings of European Congress of Chemical Engineering (ECCE-6)

Brzozowski AM, Derewenda U, Derewenda ZS, Dodson GG, Lawson DM (1991) A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. Nature 351:491–499

Chi Z, Ma C, Wang P, Li HF (2007) Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. Bioresour Technol 98:534–538

Dujon B, Sherman D, Fischer G et al (2004) Genome evolution in yeasts. Nature 430:35–44

Fickers P, Fudalej F, Dall MTL, Casaregola S, Gaillardin C, Thonart P, Nicaud JM (2005) Identification and characterization of LIP7 and LIP8 genes encoding two extracellular triacylglycerol lipases in the yeast *Yarrowia lipolytica*. Fungal Genet Biol 42(3):264–274

Fickers P, Ongena M, Destain J, Weekers F, Thonart P (2006) Production and down-stream processing of an extracellular lipase from the yeast *Yarrowia lipolytica*. Enzyme Microb Technol 38:756–759

Fickers P, Marty A, Nicaud JM (2011) The lipases from *Yarrowia lipolytica*: Genetics, production, regulation, biochemical characterization and biotechnological applications. Biotechnol Adv. doi:10.1016/j.biotechadv.2011.04.005

Gargouri Y, Julien R, Sugihara A, Verger R, Sarda L (1984) Inhibition of pancreatic and microbial lipases by proteins. Biochim Biophys Acta 795(2):326–331

Jaeger KE, Dijkstra BW, Reetz MT (1999) Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. Annu Rev Microbiol 53:315–335

Joseph B, Ramteke PW, Thomas G (2008) Cold active microbial lipases: some hot issues and recent developments. Biotechnol Adv 26(5):457–470

Khurana J, Singh R, Kaur J (2011) Engineering of Bacillus lipase by directed evolution for enhanced thermal stability: effect of isoleucine to threonine mutation at protein surface. Mol Biol Rep 38(5):2919–2926

Kiran GS, Shanmughapriya S, Jayalakshmi J, Joseph SR, Gandhimathi S, Sivaramakrishnan M, Arunkumar T, Thangavelu NK (2008) Optimization of extracellular psychro- philic alkaline lipase produced by marine *Pseudomonas* sp. (MSI057). Bioprocess Biosyst Eng 31:483–492

Kurtzman CP, Fell JW (eds) (1998) The yeasts—a taxonomic study, 4rd edn. Elsevier, Amsterdam

Liu Z, Li X, Chi Z, Wang L, Li J, Wang XH (2008) Cloning, characterization and expression of the extracellular lipase gene from *Aureobasidium pullulans* HN2-3 isolated from sea saltern. Antonie van Leeuwenhoek 94:245–255

Lopez N, Pernas MA, Pastrana LM, Sanchez A, Valero F, Rua ML (2004) Reactivity of pure *Candida rugosa* lipase isoenzymes (Lip1, Lip2 and Lip3) in aqueous and organic media, Influence of the isoenzymatic profile on the lipase performance in organic media. Biotechnol Prog 20(1):65–73

Lotti M, Grandosi R, Fusetti F, Longhi S (1993) Cloning and analysis of *Candida cylindracea* lipase sequence. Gene 124(1):45–55

Pignede G, Wang H, Fudalej F, Gaillardin C, Seman M, Nicaud JM (2000) Characterization of an extracellular lipase encoded by LIP2 in *Yarrowia lipolytica*. J Bacteriol 182:2802–2810

Rashid N, Shimada Y, Ezaki S, Atomi H, Imanaka T (2001) Low temperature lipase from psychrotrophic *Pseudomonas* sp. Strain KB700A. Appl Environ Microbiol 67:4064–4069

Redondo O, Herrero A, Bello JF, Roig MG, Calvo MV, Plou FJ, Burguillo FJ (1995) Comparative kinetic study of lipases A and B from *Candida rugosa* in the hydrolysis of lipid *p*-nitrophenyl esters in mixed micelles with Triton X-100. Biochim Biophys Acta 1243(1):15–24

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp 367–370 (Chinese translation)

Schmid RD, Verger R (1998) Lipases: interfacial enzymes with attractive applications. Angew Chem Int Ed 37:1608–1633

Shao TJ, Sun M, Zheng JS (2004) Study on low-temperature alkaline lipase from bohaisea-9145. Wei Sheng Wu Xue Bao 44:789–794

Singh S, Banerjee UC (2007) Purification and characterization of trans-3-(4-methoxyphenyl) glycidic acid methyl ester hydrolyzing lipase from *Pseudomonas aeruginosa*. Process Biochem 42:1063–1068

Song HT, Jiang ZB, Ma LX (2006) Expression and purification of two lipases from *Yarrowia lipolytica* AS 2.1216. Protein Expr Purif 47:393–397

Triglia T, Peterson MG, Kemp DJ (1988) A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acids Res 16(16):8186

Vellard M (2003) The enzyme as drugs: application of enzymes as pharmaceuticals. Curr Opin Biotechnol 14:444–450

Verger R (1997) 'Interfacial activation' of lipases: facts and artifacts. Trends Biotechnol 15(1):32–38

Yu M, Qin S, Tan T (2007) Purification and characterization of the extracellular lipase Lip2 from *Yarrowia lipolytica*. Process Biochem 42(3):384–391