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ORIGINAL PAPER

Isolation and characterization of a new alkali-thermostable lipase cloned from a metagenomic library

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Abstract The construction of a cosmid library from the biomass produced in an enriched Sequencing Fed-Batch Reactor allowed the isolation of a new lipase by functional screening. The open reading frame of 928 bp encoded a polypeptide of 308 amino acids with a molecular mass of 32.6 kDa. The amino acid sequence analysis revealed the presence of the conserved pentapeptide **GXSXG** essential for lipase activity. Alignment with known sequences of proteins showed no more than 52% identity with different lipases, confirming the discovery of a novel gene sequence. The lipase was cloned and expressed in *Streptomyces lividans* and further purified by a combination of hydrophobic interaction and size-exclusion chromatography. Spectrophotometric assays with different *p*-nitrophenyl esters demonstrated a preference for long-length acyl chains, especially *p*-nitrophenylmyristate (C14). Moreover, the enzyme presented an optimal activity at 60°C and at alkaline pH of 10.5.

Keywords Alkalithermostable lipase · Metagenomics · Purification · Bioreactors

Introduction

Preservation of the environment has become a major concern in the last decade. Many chemical compounds found in different commercial products are slowly being replaced by microbial enzymes. This biological material has the advantage of being biodegradable and thus has minimum impact on the environment. Many companies spend more money each year in the research and development area in order to find novel interesting enzymes. According to Business Communications Company Inc., the average annual growth rate of this market is thought to increase by about 4% each year. The predictions are that the global market for industrial enzymes will reach close to $2.5 billion by 2009 [24].

As they catalyze many reactions in biotechnology, true lipases (EC 3.1.1.3) represent an important target for the enzyme industry. Their stability in organic solvents, their substrate specificity and their enantioselectivity are only a few of the characteristics making lipolytic enzymes interesting for industries [15]. These enzymes catalyze the hydrolysis of triacylglycerols to release fatty acids and glycerol at the lipid-water interface [1, 9]. Lipases belong to the structural superfamily of α/β hydrolase, defined by the catalytic triad usually formed by serine (S), histidine (H) and aspartate (D) residues [14, 23]. The serine residue is often included in the conserved pentapeptide sequence **GXSXG** [23].

Lipases can be used for the synthesis of compounds via esterification and transesterification reactions [10, 14]. Their use in the enzyme industry is wide, including their presence in detergents, in the pulp and paper industry, in the development of dairy products and in waste treatment [15, 17]. Many industrial processes occur at high temperatures, orienting research to identify new thermostable lipases [30].

The majority of known lipolytic enzymes are from bacterial origin. However, it is well known that only between 0.1 and 1% of microorganisms from an environmental sample can be cultivated using standard techniques, leaving more than 99% of bacterial DNA unexplored [31]. Cultivation-independent techniques became an interesting

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approach to reach all this unculturable resource [2, 16, 32, 34, 36, 38]. Metagenomics, defined as the genomic or functional analysis of a bacterial community without its cultivation, represents an interesting technique to discover genes of industrial interests [26, 28]. In the present study, a metagenome library constructed with the total DNA extracted from the biomass collected from a gelatin-enriched fed-batch reactor was used to identify novel lipase genes. This method has proved its reliability to generate more positive hits than the direct cloning of DNA from soil [4, 8, 26, 35]. This report presents the isolation and characterization of a new thermostable lipase of unknown microbial origin.

Materials and methods

Bacterial strains and plasmids

Escherichia coli LE392MP strain (Epicentre) in combination with the pIAFS2 shuttle cosmid (INRS-Institut Armand-Frappier) was used for the metagenomic library construction. *E. coli* DH11S and the vector pUC18 were used for subcloning and DNA sequencing. Finally, expression vector pIAFD95A (INRS-Institut Armand-Frappier) and gram-positive strain *Streptomyces lividans msiK*$^{-}$ [13] were used for lipase expression.

Analysis of the biodiversity

Analysis of 16S ribosomal RNA amplicons of about 100 clones was done to evaluate the microbial diversity. The total DNA from a 1.5-ml sample was extracted by bead beating as described previously [21]. 16S rRNA genes were amplified using pA and pH universal primers [7] and cloned into pGEM-T Easy vector (Promega, Madison, WI) using the manufacturer's protocol. Ninety-eight clones were sequenced using pA primers. Putative chimeras were detected by Bellerophon program [12] and were excluded. The remaining 92 sequences were aligned with ClustalW, and a phylogenetic analysis was conducted using different programs of the Phylip 3.66 package (http://evolution.gs.washington.edu/phylip.html). A distance matrix using the Kimura 2-parameter model was created with all sequences by Dnadist. Based on this matrix, sequences showing distance of 0.015 or less were grouped, and one sequence from each group was picked and used for further analysis. They were compared to those in GenBank using BLASTn, and the closest sequences from known organisms (type strains or strains formally identified in a published paper) were selected for the phylogenetic analysis. Selected sequences from each group and those from the closest reference organisms were aligned and used to build a phylogenetic tree by the Fitch-Margoliash method using a DNA distance matrix, itself based on the Kimura 2-parameter model.

Construction of the metagenomic library and screening for lipolytic activity

Total DNA was extracted from the biomass of a Sequencing Fed-Batch Reactor (SFBR) that had been enriched with gelatin. To enhance the thermoresistant microbial diversity in the SFBR, 30 cycles of 72 h were performed. During those cycles, pH gradually shifted from 7 to 8.5 and dropped again to 7. Simultaneously, the temperature increased from 50 to 70°C. Methods used to extract high-molecular-weight DNA included lysozyme digestion, freeze/thaw cycles and phenol/chloroform extractions. Total DNA was partially digested by *Bam*HI in order to generate approximately 40-Kb fragments that were ligated with pIAFS2 vector digested by *Bgl*II. Lambda phage packaging extracts were added to the ligation, and transduction of *E. coli* LE392MP was performed. Approximately 10,000 transductants were screened for lipolytic activity on 2xTY agar plates supplemented with the antibiotic apramycin (50 μg/ml) and 1% tributyrin. After an overnight incubation at 37°C, colonies showing clearing zones revealed the presence of a lipolytic gene within the cloned DNA insert.

Subcloning and sequence analysis of the lipase gene

From a positive clone that hydrolysed tributyrin, the overnight culture was done at 37°C in 2xTY media supplemented with 50 μg/ml of apramycin. The cosmid was extracted with standard phenol/chloroform method followed by its digestion with the restriction enzyme *Sph*I. The digested cosmid was then ligated on itself in order to randomly delete DNA fragments of the 40-Kb insert. The ligation products were incorporated into *E. coli* DH11S by thermal shock transformation, and screening for lipolytic activity was performed as described in the previous section. This procedure was repeated until small fragments still expressing activity were obtained. Those fragments were then ligated with the ampicillin-resistant pUC18 vector, transformed in *E. coli* DH11S and sequenced at Genome Quebec with universal forward and reverse primers. Comparison of the translated DNA sequence with databases was done with the Blastx program located at NCBI BLAST server. The sequence analysis revealed a *Sph*I restriction site within the lipase gene. Following this step, synthesis of two specific primers to mutate the internal gene *Sph*I site (5′-GCA**C**GCCTCGCCGTTCTATAACC G-3′ and 5′-GATGCGGTTATAGAACGGCGAGGC**G**TG C-3′ the mismatched base is in bold) and two other primers

to amplify the whole gene containing restriction sites *Sph*I and *Sac*I (5′-AAAGCATGCCAATAACCGCCAGAAA-3′ and 5′-AAAGAGCTCAGTACGGGCAGTTACCTCGGT ATT-3′, restriction sites are underlined) allowed overlapping PCR amplification [37]. The corresponding PCR amplicon was ligated in the multi-cloning site vector pIAFD95A in order to increase the production of the lipase and then transformed in *Streptomyces lividans msiK*$^{-}$. The integrity of the PCR insert was confirmed by sequencing.

Expression and purification of the lipase LipIAF5.2

Strains were first incubated at 34°C for a 48-h period in TSB medium with 1% glycerol in a shaker. The antibiotic thiostrepton was added to a final concentration of 5 μg/ml. Lipase expression was done in 1 l of M14 minimal medium (0.14% of $(NH_4)_2SO_4$, 0.5% of K_2HPO_4, 0.1% of KH_2PO_4, 0.2% of Tween 80 and 0.1% of Mandels salts solution) supplemented with 0.03% of $MgSO_4$, 0.15% of $CaCl_2$, 1% of xylose and 5 μg/ml of thiostrepton. After 72 h of incubation at 34°C with 240 rpm of agitation, the supernatant was filtered under vacuum with a Whatman 3 MM filter in order to remove the mycelium. A second filtration was performed with Whatman 0.2 μm nylon membrane to remove small particles. Supernatant was then equilibrated with phosphate buffer and ammonium sulfate to a final volume of 1 l buffered at 50 mM pH 7 and 1 M of salt. This mixture was adsorbed overnight to a hydrophobic column HiTrap Phenyl FF (GE Healthcare) at 4°C. The lipase was eluted at a flow rate of 1 ml/min from 1 M of ammonium sulfate to 0 M by ten steps of 2 ml each, followed by five steps from 10 to 50% of ethylene glycol in order to remove all highly hydrophobic proteins bounded on the column. The active fractions analyzed on tributyrin agar plates were dialyzed against water, pooled and concentrated with nanosep 3 K column (VWR). About 500 μg of the protein mixture was concentrated to a final volume of 100 μl and filtered with 0.45 μm Acrodisc Syringe Filter with Versapor 4 mm, before it was submitted to size-exclusion chromatography. Bovine serum albumin (66 kDa), carboxylase A (29 kDa) and chimotrypsinogene-A (20.2 kDa) were used as standards (Sigma). The fractions were collected with 0.2 M sodium phosphate buffer (pH 7) and analyzed for lipase activity as described previously. The purified lipase was dialyzed against water and stored at −20°C in aliquots. Enzyme purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis and zymographic analysis

Protein analysis was done using standard SDS-PAGE. However, the protein sample buffer did not contain any denaturing agents to allow further analysis. To detect lipase activity, the gel was washed 30 min in a Tris–HCl 50 mM pH 7 buffer supplemented with 2.5% of Triton X-100. The lipolytic activity was then visualized under UV detector (Alpha Imager) after 5 min of incubation in the same Tris buffer containing 100 μM of 4-methylumbelliferyl-butyrate as substrate [6]. Gels were then stained with Coomassie Blue R250 or silver nitrate.

Lipase characterization

Enzymatic assays were done by measuring the hydrolysis rate of with *p*-nitrophenylmyristate (*p*-NPM) or other *p*-nitrophenyl esters. All reactions were done in 1-ml cuvettes that contained a final concentration of 0.3 mM of the appropriate substrate dissolved in isopropanol and 75 ng of the purified lipase. All buffers had a molarity of 50 mM supplemented with 0.05% of $CaCl_2$. Reactions were done in duplicate over a period of time of 5 min and monitored at a wavelength of 405 nm with a Cary 300 Win/UV spectrophotometer. The protein concentration was determined with the Bradford method, using γ-globuline as the standard. One international unit (IU) was defined as the amount of enzyme needed to liberate 1 μmol of *p*-nitrophenol per minute using *p*-nitrophenylmyristate as substrate.

Substrate specificity was determined by using *p*-nitrophenylbutyrate (C4), *p*-nitrophenylvalerate (C5), *p*-nitrophenylcaprate (C10), *p*-nitrophenyllaurate (C12), *p*-nitrophenylmyristate (C14), *p*-nitrophenylpalmitate (C16) and *p*-nitrophenylstearate (C18). The optimal temperature and pH established for the lipase were used in these assays.

Optimal temperature was studied from 40 to 75°C. Reactions were carried out with glycine–NaOH buffer pH 10.5 using *p*-NPM as a substrate. Thermostability of the lipase was performed at 90°C in water. At 1-h intervals, an aliquot of the incubation mix was removed and kept on ice before the enzymatic reaction in the optimal conditions was done. Optimal pH was determined at 60°C using *p*-NPM as a substrate. The 50-mM buffers used for the pH ranges of 7.0–9.0, 8.0–10.0 and 9.0–11.0 were, respectively, Tris–HCl, H_3BO_3–KCl–NaOH and glycine–NaOH; the pH was adjusted at room temperature.

Effect of different compounds on lipase stability

The purified lipase was incubated for 1 h at 37°C with 1 and 10 mM of metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, K^{+}), oxidizing agent (ammonium sulfate), chelating agents (EDTA, EGTA), inhibitor (PMSF) and 0.1 and 1% of detergents (SDS, Triton X-100, Tween 20 and Tween 80). Residual activity compared to that of a standard reaction was measured at pH, temperature and with the optimal substrate.

Nucleotide accession number

The nucleotide sequence of the LipIAF5.2 gene and the flanking region is available in the Genbank database under accession number EU660533.

Results

Phylogenetic analysis

The biodiversity of the enriched biomass was relatively wide, with 20 phylotypes (defined as 16S rDNA sequences having a distance of no more than 0.015) found in 92 cloned 16S rDNA sequences. However, the community was dominated by two groups: gr01 with 38 sequences (41%) and gr04 with 27 sequences (29%). Based on the phylogenetic analysis, they belong to *Geobacillus toebii* and *Symbiobacterium thermophilum*, respectively (Fig. 1). However, there are many other sequences that are relatively distant from known organisms and cannot be classified into a defined species or even genus.

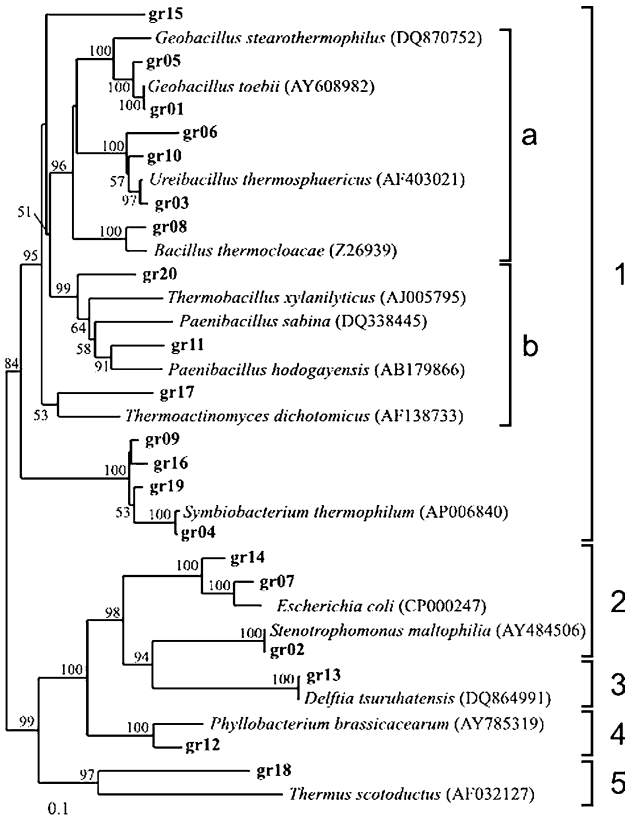

Fig. 1 Biodiversity in the SFBR. The analysis was conducted on partial 16S rDNA sequences (*E. coli* position 74 to 715). Sequences obtained in the present study are in bold face. They are representative of groups of sequences that show a distance of 0.015 or less to each others. *Cryptanaerobacter phenolicus* (AY327251) was used as the outgroup organism (not shown). One thousand bootstrap trees were generated, and bootstrap confidence levels (shown as percentages close to nodes) were determined. Numbers on the right identify the following bacterial classes: 1 *Bacilli*, 2 *γ-Proteobacteria*, 3 *β-Proteobacteria*, 4 *α-Proteobacteria*, 5 *Deinococci*. Two families are also identified: **a** *Bacillaceae*, **b** *Paenibacillaceae*. *Bar* 0.1 nucleotide substitutions per position

Construction and screening of the metagenomic library

The enrichment of the biomass of a SFBR with gelatin was done as described in Materials and Methods. A cosmid DNA library was constructed into pIAFS2 vector followed by activity screening on 2xTY plates supplemented with tributyrin. From approximately 10,000 colonies screened, 10 positive clones were selected, including the LipIAF5.2 clone.

Sequence analysis of a novel lipase and the flanking DNA

The vector that contained LipIAF5.2 was extracted, and through digestion and subcloning, the 40-Kb vector was reduced in two *Sph*I fragments of 1,400 and 2,800 bp each (Fig. 2). The individual cloning of these fragments did not show activity, suggesting a *Sph*I site within the lipolytic gene. The sequencing of the gene presented one open reading frame (ORF) of 928 bp, encoding a polypeptide of 308 amino acids with a deduced molecular mass of 32,584 Da. The sequence analysis showed a G + C of 65.2%, which supported the use of the *Streptomyces* genus as the producer of LipIAF5.2. Also, a putative ribosomal binding site (gaggag) located 8 bp upstream the ATG start codon was identified (data not shown).

The sequencing of the flanking regions (Fig. 2) revealed an ABC transporter (89% homology with *Marinobacter algicola*), an AraC transcriptional regulator (62% homology with *Ralstonia eutropha* and finally a transcriptional regulator (72% homology with *Mesorhizobium loti*). Based on these results, we could not affiliate the lipase IAF5.2 with any already known lipases produced by a culturable bacteria.

The BLAST search against protein databases (blastx) of the nucleotide sequence of LipIAF5.2 showed similarity to the triacylglycerol lipase of *Thermobifida fusca* YX (YP_288944), the lipase of *Streptomyces coelicolor* A3(2) (AAD09315) and the depolymerase of *Acidovorax delafieldii* (BAB86909) that feature identities of 49, 47 and 52%, respectively. Searching for a potential signal peptide, a SignalP 3.0 Server analysis established a cleavage site probability of 99.7% between positions 27 and 28 that gave a mature polypeptide of 281 amino acids with a deduced molecular weight of 29,898 Da (http://www.cbs.dtu.dk/services/SignalP/). The analysis of the primary sequence showed a putative oxyanion hole HG (positions 119–120)

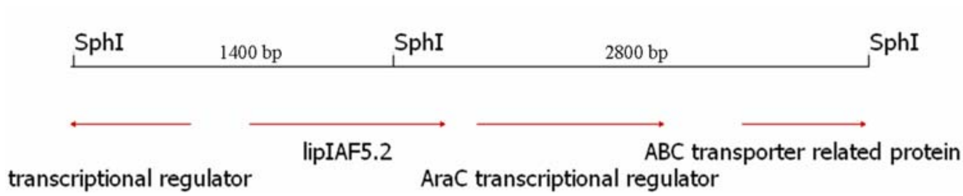

Fig. 2 Sequence analysis of the novel lipase IAF5.2 and the flanking DNA

that is involved in the stabilization of intermediates formed during the ester hydrolysis [11, 14]. This hole is positioned 52 amino acids ahead of the active serine residue, which is included in the conserved pentapeptide sequence **GWSMG**. The amino acid alignment performed with ClustalW program (Fig. 3) identified putative aspartic and histidine residues by homology with other protein sequences. All these characteristics indicated a lipase enzyme with considerably novel sequence that has been designated as LipIAF5.2.

Expression and purification of LipIAF5.2

The high level expression provided by pIAFD95A vector in *S. lividans* showed the secretion of the lipase into the supernatant, with 11.3 mg of pure active lipase per liter. The first purification step using a hydrophobic phenyl sepharose column allowed the elimination of the majority of the supernatant proteins. Most of LipIAF5.2 was eluted with 15% ethylene glycol fraction (from 10 to 20%) with two other proteins. The contaminating proteins were separated by size-exclusion chromatography, and the active purified fraction showed homogeneity on SDS-PAGE (Fig. 4b, lane 3). The protein migration without denaturation (Fig. 4, lane 1) demonstrated one major active protein between 30 and 45 kDa and a minor protein at 30 kDa. With partial denaturation (Fig. 4, lane 2), an active protein at 30 kDa was detected. However, the total denaturation had not showed activity and demonstrated a single protein at 30 kDa that suggested the lipase as a monomer protein. The mass spectrometer analysis had confirmed the identity as LipIAF5.2 (data not shown).

Biochemical characterization of LipIAF5.2

To analyze the effect of fatty acid chain length on the specificity of the lipase, seven *p*-nitrophenyl esters were tested. Under the given conditions, the activity was maximal with *p*-NPM (C14) and about 70% with *p*-nitrophenyl-palmitate (C16) and *p*-nitrophenylstearate (C18), indicating that the enzyme was a lipase (Fig. 5). Furthermore, an aliquot of the purified lipase on an emulsified 2xTY

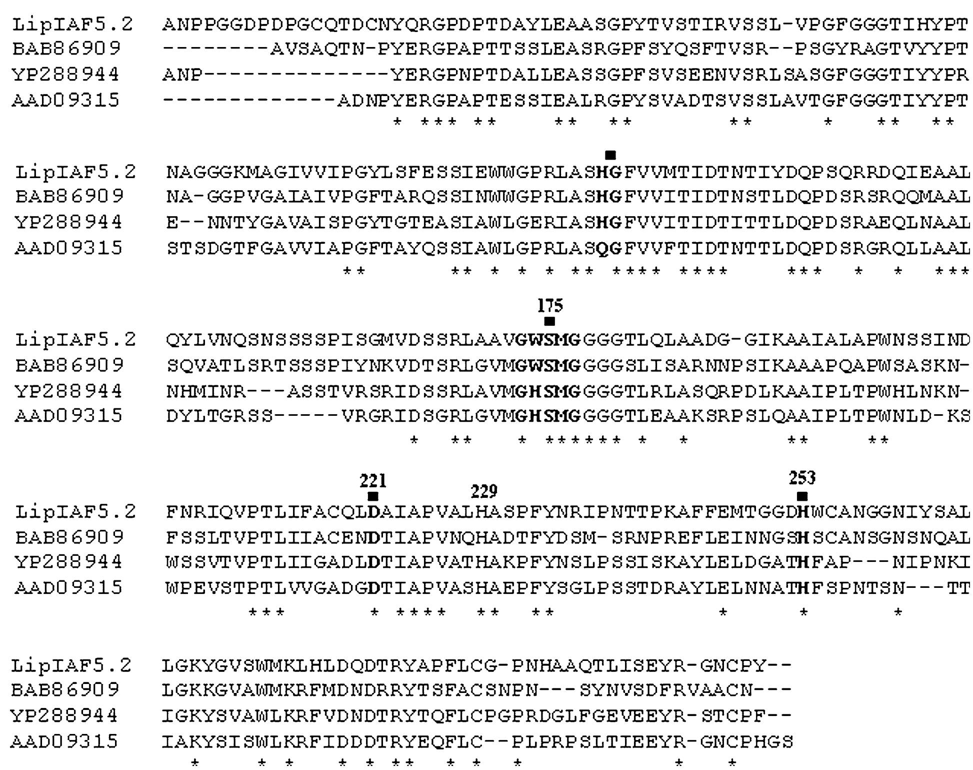

Fig. 3 The amino acid sequence alignment of LipIAF5.2 was compared with *Thermobifida fusca* YX triacylglycerol (YP_288944), lipase from *Streptomyces coelicolor* A3(2) (AAD09315) and depolymerase from *Acidovorax delafieldii* (BAB86909) using the ClustalW program. *Asterisks* indicate amino acids conserved; *filled square* indicates putative oxyanion hole (HG), as well as serine, aspartic acid and histidine as putative residues of the active site

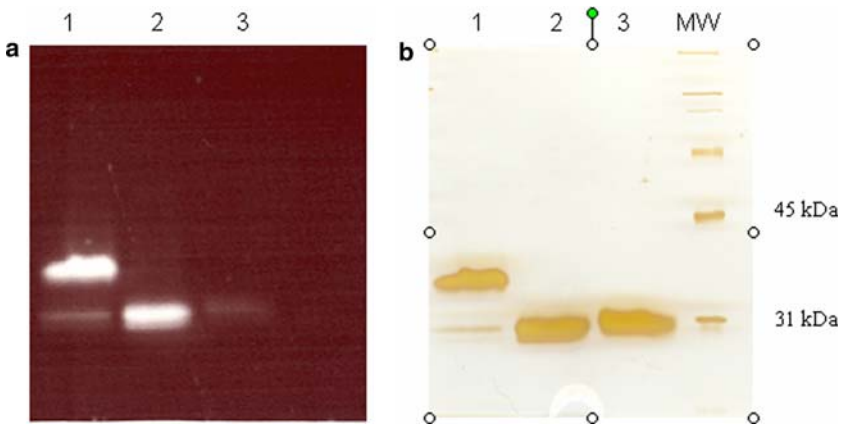

Fig. 4 SDS-PAGE of purified LipIAF5.2. **a** A 4-methylumbelliferyl-butyrate zymogram was done after the protein migration. *Lane 1* crude extract (nonheated sample); *lane 2* crude extract (heated sample); *lane 3* protein extract treated with iodoacetamide and DTT (heated sample). **b** Protein silver staining. *Lanes 1–3* were the same as in **a**

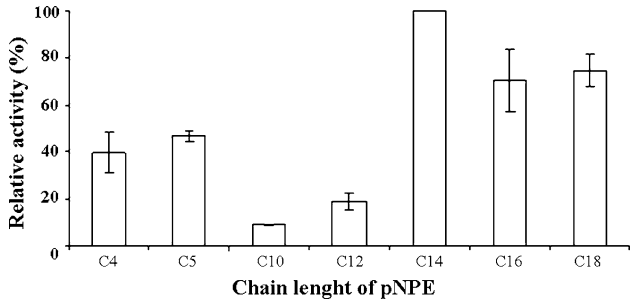

Fig. 5 Effect of chain length on specificity of LipIAF5.2. The activity was assayed toward various *p*-NPEs at 60°C in 50 mM glycine–NaOH pH 10.5

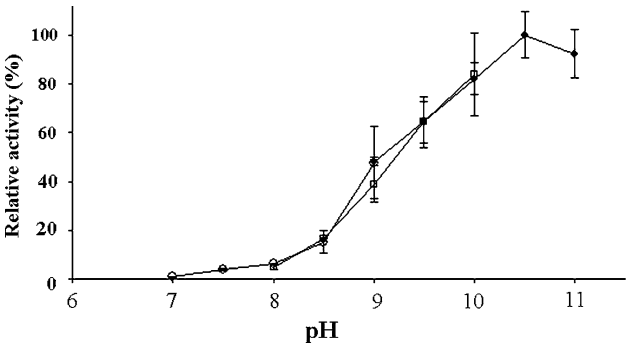

Fig. 6 Effect of pH on LipIAF5.2. Lipase activity was assayed toward *p*-NPM (C14) at 50°C with Tris–HCl (pH 7–9, *open circle*), KCl–H_3BO_3–NaOH (pH 8–10, *open square*) and glycine–NaOH (pH 9–11, *closed circle*) buffers

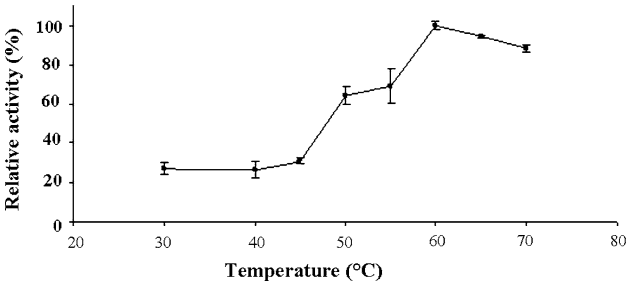

Fig. 7 Effect of temperature on LipIAF5.2. Lipase activity was assayed toward *p*-NPM (C14) in 50 mM glycine–NaOH pH 10.5

supplemented with 1% of pure olive oil and 0.001% of rhodamine B revealed fluorescence under UV detection (result not shown). The LipIAF5.2 exhibited a range of activity at alkaline pH from 9 to 11 with a maximum at 10.5 (Fig. 6). The effect of temperature on enzyme activity was tested using *p*-NPM as substrate. The enzyme showed more than 50% of the highest activity from 50 to 70°C with a maximum at 60°C (Fig. 7). For thermostability of LipIAF5.2, even after 4 h of incubation at 90°C, the enzyme activity remained the same as the control. The specific activity towards *p*-NPM at pH 10.5 and 60°C was 160 IU/mg. Thereafter, the effect of some effectors (metal ions, chelating and oxidizing agents, inhibitors and detergents) on the lipase stability was tested (Table 1).

Calcium and manganese divalent cations were found to enhance the enzyme activity (115 and 134%, respectively, at 10 mM of concentration); magnesium ions and the active site serine specific inhibitor PMSF hardly affected the lipase activity. Cobalt and zinc ions affected activity only at 10 mM, while potassium ion, ammonium sulfate and chelating agent EDTA were found to reduce enzymatic activity at both 1 and 10 mM. With calcium specific chelating agent EGTA, LipIAF5.2 had completely lost its activity. With Triton X-100 and SDS detergents, the enzyme did not show activity. However, Tween 20 did reduce activity only at 10 mM of concentration, while incubation with 1 mM of Tween 80 gave a loss of 25% of activity and absolutely no activity at 10 mM.

Discussion

A novel thermostable lipase was identified from a metagenomic library expressed in the heterologous host *E. coli* and in *S. lividans*. Functional screening of the metagenomic bank on tributyrin plates rapidly identified

Table 1 Effect of various effector molecules on LipIAF5.2 activity

Effector molecule	Residual activity (% of control)	
	1 mM (1%)[a]	10 mM (10%)[a]
Calcium chloride	99 ± 5	115 ± 2
Magnesium chloride	103 ± 1	105 ± 1
Manganese chloride	100 ± 1	134 ± 3
Zinc chloride	99	51 ± 6
Cobaltous chloride	102 ± 4	62 ± 4
Potassium chloride	44 ± 8	34
Ammonium sulfate	70 ± 14	72 ± 1
Ethylene diamine tetraacetic acid	72 ± 3	62 ± 2
Ethylene glycol tetraacetic acid	0	0
Phenylmethanesulphonylfluoride	93 ± 4	99 ± 3
Triton X-100	0	0
Tween 20	101	52 ± 2
Tween 80	76 ± 6	0
Sodium dodecyl sulfate	0	0

[a] For Triton X-100, Tweens and SDS

clones with potential industrial interest. As the screening revealed 0.1% of positive clone compared with less 0.01% without culture enrichment [3], the use of enriched cultures, pH and temperatures controlled showed that the desired gene encoding the targeted reaction can be recovered [18]. Furthermore, LipIAF5.2 presented the physical characteristic chosen for enrichment, such as enzymatic activity at high temperature and at alkaline pH.

The sequencing of the flanking region revealed protein affiliation with three different microorganisms, combined with no more than 52% of identity, and 66% of homology of the lipase sequence with other identified proteins confirmed that LipIAF5.2 belongs to an unknown organism. However, the phylogenetic analysis of the bacteria in the SFBR from where the DNA was extracted showed the potential of *Geobacillus* and *Symbiobacterium*, two thermophilic genera. The identification of a predicted signal peptide, the HG sequence that formed an oxyanion hole in the three-dimensional protein structure and the catalytic triad composed of Ser, His and Asp residues where the serine is usually located into **GXSXG** pentapeptide are characteristics of known lipases [20]. These characteristics, identified in the LipIAF5.2 amino acid sequence, with the specificity for long-length acyl chain *p*-nitrophenyl esters, allowed the classification of this new enzyme as a true lipase.

Purification of the lipase was performed in two steps, and the final product showed a single protein on SDS-PAGE with an apparent molecular weight of 31 kDa. Despite the fact that the 3D structure of the lipase remained unknown, rough conclusions can be drawn. Furthermore, the activity observed after 10 min of incubation at 100°C (i.e., heated sample for SDS-PAGE) indicated a high thermostability of the protein, confirmed with no lost of activity after 4 h of incubation at 90°C.

The enzymatic characterization of the lipase revealed that it was active over a wide range of pH values from 9 to 11 and at temperatures from 50 to 70°C with maximum activity at pH 10.5 and at 60°C. These results allowed the classification of the lipase as an alkaline and thermophilic enzyme. Most of thermophilic and alkaline lipases come from thermophilic bacterial strains, but few have been isolated by a metagenomic approach. Both calcium and manganese ions enhanced LipIAF5.2 activity, as previously reported for an alkaline lipase of *Burkholderia cepacia* [25] and from a lipase of a new family isolated from a metagenomic library of tidal flat sediments [22]. Calcium ions often acted as a ligand between residues in the active site, and manganese increased enzyme activity and stability at elevated temperature [29]. Also, electrostatic interactions between calcium and fatty acids produced by the hydrolysis of the substrate led to the clearing of the active site and allowed another substrate molecule to access the site [35]. In preincubation with EDTA, the enzymatic activity was inhibited to 62% (10 mM), and with EGTA, a calcium and manganese specific chelating agent [5], total inhibition occurred, suggesting that both calcium and manganese are essential for the catalytic activity of LipIAF5.2. The activity was not significantly affected by magnesium ions. Zinc and cobalt ions did reduce the activity, but at 10 mM concentration.

A main characteristic of lipases proposed by Sarda and Desnuelle in 1958 is their interfacial activation. It is defined by the low activity of lipases on monomeric substrate, but enhanced once substrate molecules formed an emulsion [27]. Many lipase active sites are covered by a lid structure that is composed of an amphiphilic peptidic loop that moves apart when the enzyme is in the presence of a lipid-water interface that is formed by substrate emulsion [14, 19]. In order to determine if LipIAF5.2 could have a lid structure, we pre-incubated the enzyme with PMSF. This agent is a specific serine inhibitor and will link the active site serine residue if it can access it. We observed no loss of activity when PMSF was added to the pre-incubation mix. This means that this agent cannot access the active site, and this could suggest the presence of a lid structure. However, this can also mean that the catalytic residue is not a serine. To resolve this, we added PMSF directly to a reaction mix (i.e., the reaction mix contains substrate in an emulsion form), and we recorded the activity of the lipase. When PMSF is added directly in the reaction mix, there is completed loss of activity compared to the control. This result suggested that the active serine site is covered by a lid structure that moved only at the oil-water interface that is provided by the emulsion of the substrate in the reaction mix [14, 19]. This lipase's

property definitively classifies LipIAF5.2 in the family of true lipases [33].

The metagenomic approach used here allowed the identification of a new enzyme of microbial origin that has not been cultivated yet by standard methods. Furthermore, the enrichment of bioreactors yielded more positive hits than the direct cloning of DNA from soil, and one of them led to the identification of LipIAF5.2, an alkaline and thermostable lipase that can further be used for industrial purposes.

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