J. Gen. Appl. Microbiol., **59**, 119–134 (2013)

Full Paper

Molecular cloning and characterization of a thermostable esterase/lipase produced by a novel *Anoxybacillus flavithermus* strain

Laura Chiş,[1] Monica Hriscu,[1] Adriana Bica,[2] Monica Toşa,[1] Gergely Nagy,[3] Gergely Róna,[3] Beata G. Vértessy,[3,4] and Florin Dan Irimie[1,*]

[1] *Department of Biochemistry and Biochemical Engineering, Faculty of Chemistry and Chemical Engineering, Babeş-Bolyai University, Cluj-Napoca, Romania*
[2] *Institute of Biological Research, Cluj-Napoca, Romania*
[3] *Institute of Enzymology, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Budapest, Hungary*
[4] *Depertment of Applied Biotechnology and Food Sciences, Faculty of Chemical and BioEngineering, University of Technology and Economics, Budapest, Hungary*

(Received October 9, 2012; Accepted November 26, 2012)

A thermophilic strain producing an extracellular esterase/lipase was isolated from a hot spring in Tăşnad, Romania, and was identified phenotypically and by 16S rDNA sequencing as *Anoxybacillus flavithermus* (GenBank ID: JQ267733). The gene encoding the putative carboxyl esterase (GenBank ID: JX494348) was cloned by direct PCR amplification from genomic DNA. The protein, consisting of 246 amino acids and having a predicted molecular weight of 28.03 kDa, is encoded by an ORF of 741 bps. Expression was achieved in *Escherichia coli* and a recombinant protein with esterolytic activity and estimated molecular weight of 25 kDa was recovered and purified from the periplasmic fraction by IMAC. The purified enzyme, most active at 60–65°C and in the near-neutral range (pH 6.5–8), displayed a half-life at 60°C of about 5 h. Est/Lip displayed a relative tolerance to methanol, DMSO, acetonitrile, and low detergent concentrations (SDS, Triton) increased its thermostability. Highest activity was attained with *p*-nitrophenyl butyrate, but the enzyme was also able to hydrolyze long chain fatty acid esters, as well as triolein. The primary sequence and predicted tridimensional structure of the enzyme are very similar to those of other *Anoxybacillus* and *Geobacillus* carboxyl esterases in a distinct, recently described lipase family. Est/Lip was highly enantioselective, with preference for the (*S*)-enantiomer of substrates.

Key Words——*Anoxybacillus flavithermus*; biochemical characterization; enantioselectivity; gene cloning and expression; thermostable esterase/lipase; tridimensional structure

Introduction

Carboxyl esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are widely occurring hydrolytic enzymes which catalyze cleavage of ester bonds. These two types of esterases, despite a high degree of sequence similarity, have very different substrate specificities (Gilham and Lehner, 2005). Esterases hydrolyze 'simple' esters and triglycerides with short fatty acid residues, while lipases preferentially address long-chain acyl glycerols and other water-insoluble esters, operating at the water-substrate interface (Arpigny and Jaeger, 1999; Bornscheuer, 2002). Esterases tend to

*Corresponding author: Prof. Florin Dan Irimie, Department of Biochemistry and Biochemical Engineering, Faculty of Chemistry and Chemical Engineering, Babeş-Bolyai University, 11, Arany János St., Cluj-Napoca, RO-400028.
Tel: +40(0)264 591–897 Fax: +40(0)264 190–818
E-mail: irimie@chem.ubbcluj.ro

be regio, stereo and/or enantiospecific enzymes, which renders them interesting as biocatalysts in fine organic synthesis; however, these features are often more pronounced with lipases, which are also associated with a wider substrate acceptance and a better tolerance towards organic solvents. The latter also recommends lipases for use in (trans) esterification reactions. As a whole, carboxyl esterases and lipases have a considerable biotechnological potential in high turnover industrial areas including food (fat, dairy, fragrances), detergents, organic synthesis, drugs and cosmetics, paper and pulp, biofuels, etc. (for reviews see Hasan et al., 2006; Jaeger and Reetz, 1998; Jaeger and Eggert, 2002; Sharma et al., 2011).

Thermophilic strains such as *Bacillus* sp., *Geobacillus* sp., and *Burkholderia* sp. are reputable sources for industrially relevant enzymes displaying an inherent higher stability than their mesophilic counterparts, not only towards temperature, but towards other factors as well. The related genus *Anoxybacillus* has been less studied as yet, although it has been reported in thermal springs from all around the world: Malaysia (Zuridah et al, 2011), Fiji (Narayan et al, 2008), China (Dai et al, 2011), and Spain (Deive et al, 2010). Very few *Anoxybacillus* strains have been investigated for their enzymatic production up to now. *Anoxybacillus flavithermus* was reported to produce a cellulase (Ibrahim and Ahmed, 2007), as well as a xylanase of biotechnological interest (Kambourova et al., 2007). An esterase from *A. gonensis* (Çolak et al., 2005; Faiz et al., 2007) and a thermostable lipase from *A. kamchatkensis*, which retained over 50% of its activity after 30 min at 80˚C (Olusesan et al., 2009), have been also reported. A few other enzymes with esterolytic or lipolytic activity have been described more recently (Pinzón-Martínez et al., 2010; Zuridah et al., 2011; Ay et al., 2011).

In this paper we describe the identification and characterization of a local strain of the thermophile *Anoxybacillus flavithermus*, producing an extracellular hydrolase with esterolytic/lipolytic activity; further, the molecular cloning, purification and detailed characterization of the recombinant *A. flavithermus* esterase/lipase are described here for the first time.

Materials and Methods

Materials. *p*-Nitrophenyl esters (acetate, propionate, butyrate, methyl butyrate, palmitate, and oleate), as well as the chiral substrates tested for kinetic resolution with the enzyme were synthetized in the fine organic synthesis laboratory of our department. LB medium and HPLC grade solvents were from BDH Prolabo (VWR International, Vienna, Austria). IPTG (isopropyl-D-thiogalactopyranoside) and agar-agar were from Merck, Darmstadt, Germany. All other chemicals used were commercially available reagents of high purity grade. All solutions were made with ultrapure deionized water.

Anoxybacillus flavithermus T1: sample collection, isolation and cultivation. The bacterial strain, coded T1, was isolated from a hot spring in Tăşnad, Satu Mare County, located in NW Romania. According to the official data sheet published by local authorities, the water emerges from a depth of 1,354 m and has a total mineral content of 9.84 g/L; the main anions are chloride, bicarbonate and sulphate; the predominant metals are sodium, calcium, and magnesium. The surface temperature of the spring is 72˚C and the pH upon cooling is about 8.

A water sample was collected in a sterile receptacle, brought into the laboratory and inoculated on LB-agar plates as soon as possible after collection. After 24 h, incubation at 60˚C, the plates showed distinct colonies. One isolated colony was used as inoculum for liquid cultures. Purity of the isolate was assessed microscopically.

For both primary and production cultures, NaCl-supplemented LB medium was used, with the following final composition per litre: tryptone 10 g, yeast extract 5 g, and NaCl 10 g, pH 8.2. Primary cultures were grown in wide neck 250 ml Erlenmeyer flasks containing 25−50 ml inoculated medium, incubated at 60˚C with shaking (200 rpm) for 22−24 h. Primary cultures were either used as fresh inocula for larger scale cultures or dispersed over glass beads and deep frozen, serving as inocula whenever necessary. Larger scale cultivation was carried out under the same conditions as for primary cultures, in 2-l Erlenmeyer flasks containing 200−250 ml of inoculated medium.

Identification and characterization of the bacterial strain.

Phenotypic characterization. Colonies on streak plates were assessed for appearance, margin, elevation, colour and transparency. Wet mounts were investigated by light microscopy. Smears were subjected to Gram staining and to specific endospore (Schaeffer-Fulton) staining. To further establish the Gram type of

the isolate, a KOH test was performed (Ryu, 1938).

The strain was characterized biochemically according to the methods described by Logan and Berkeley (1984). The API test (bioMérieux) was incubated at 60˚C, with due care to avoid evaporation, and read after 24 h. Catalase and amylase activity were passessed according to standard protocols (Smibert and Krieg, 1994). NaCl tolerance (0.5−5%) was tested in LB broth, at 60˚C. The physiological temperature and pH ranges of aerobic growth were determined over a 24 h period. The temperature range was assessed between 20˚C and 80˚C, at pH 7.5. The pH range was assessed between 5.0 and 10.0, at 60˚C. The initial pH of the LB broth was adjusted at the respective growth temperature and was checked on a small aliquot after sterilization.

Genetic identification: DNA extraction, PCR amplification and DNA sequencing. The GeneJET Genomic DNA Purification Kit (Fermentas, Burlington, Ontario, Canada) was used to isolate the genomic DNA, according to the manufacturer's instructions. The 16S rRNA gene was amplified using two universal bacterial primers: 16S-F (5′-GAG TTT GAT CCT GGC TCA G-3′) and 16S-R (5′-CGG CTA CCT TGT TAG GAC TT-3′). The PCR mix contained 1.25 units of DreamTaq® DNA Polymerase (Fermentas, Burlington, Ontario, Canada) in the manufacturer's buffer, 1.5 mmol $MgCl_2$, 0.2 mmol dNTP, 0.4 μmol primer in a final volume of 50 μl. The tubes were incubated using a Biometra TGradient thermal cycler with an initial denaturation at 95˚C for 5 min followed by 32 cycles of 95˚C for 45s, 56˚C for 55 s, 72˚C for 2 min and a final incubation at 72˚C for 10 min. Electrophoresis was performed in 1% agarose gel and staining was carried out with 1 $\mu g \cdot ml^{-1}$ ethidium bromide. Photographs were taken with the Bio-Doc-it® Imaging System. Direct sequencing was carried out after PCR product purification with the GeneJET Gel Extraction Kit (Fermentas, Burlington, Ontario, Canada) following the manufacturer's instructions. Each DNA template was sequenced from both ends with the forward and reverse primers used for PCR amplification. The probes were processed with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) in an ABI PRISM 3130 genetic analyzer, according to the provided protocol. The fragments were assembled with ContigExpress (Life Technologies Invitrogen, Carlsbad, California, USA). A 1459 base pair fragment was used to search the public databases for similarities, based on the *blastn 2.2.25* program (Zhang et al., 2000).

Molecular cloning and expression of the Est/Lip gene. *Escherichia coli* strains used in this study were XL1 Blue (Stratagene) and BL21(DE3) (Novagen). All *E. coli* strains containing recombinant plasmids were cultured in LB medium supplemented with 100 μg/ml ampicillin (and 50 μg/ml tetracycline for *E.coli* XL1 Blue). Transformed *E.coli* strains were grown at 37˚C, with shaking (200 rpm).

Anoxybacillus flavithermus genomic DNA was extracted using a MasterPure™ DNA Purification Kit (Epicentre). The carboxylesterase gene was amplified using the genomic DNA as a template and two sets of primers, one for periplasmic expression (cloned into the BamHI and XhoI sites of the Novagen pET20b+ vector) and one for intracellular expression (cloned into the NdeI and XhoI sites of pET20b+). Restriction sites (underlined) were incorporated into the forward and reverse primer sequences as follows: FEst/Lip (periplasmic expression, BamH1): 5′-GCGAGGATCCATGATGAAGAT GATTCCCCCAC-3′; FEst/Lip (intracellular expression, NdeI): 5′-GGGAATTCCATATGATGAAGATGATTCCCC CAC-3′; REst/Lip-XhoI: 5′-CATTTATGCATTTTTAGAAT CATTAGATTGGCCGGATCTCGAGCGG-3′. Cloning in the frame with the C-terminal His6-tag in the vector was done in order to allow purification by IMAC.

MyTaq, RedTaq and Phusion polymerases were tested for amplification of the gene. Based on better performance, MyTaq was further used for amplification, using the following conditions: initial denaturation step at 95˚C for 1 min, 35 cycles at 95˚C for 30 s, annealing at 55˚C for 30 s, and extension at 72˚C for 45 s. The final cycle consisted of a 10-min final extension. PCR products were analyzed on 1% agarose gel and subsequently purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel). Cloning was verified by sequencing at Eurofins MWG GmbH.

Plasmids containing the desired insert were also transformed into *E. coli* BL21(DE3) for protein expression. Transformed cells were grown to an optical density of 0.4−0.6; expression of recombinant proteins was induced by 1 mM IPTG and the cells were further grown for 5 h at 37˚C. Cells were harvested by centrifugation at 5,000 rpm for 15 min, at 4˚C. The periplasmic fraction was extracted according to the manufacturer's recommendation (Novagen) and purified on Ni-agarose. Fractions eluted with 250 mM and 500 mM imidazole were tested for esterase activity and electrophoretic homogeneity.

In silico analyses. UniProt was used for alignment of protein sequences (CLUSTAL O 1.1.0) and for building a phylogenetic tree. The deduced amino acid sequence of the protein was analysed with Swiss-Modeller (Arnold et al., 2006; Guex and Peitsch, 1997; Schwede et al., 2003) and PyMol Viewer. The tridimensional structure of Est/Lip was built using as a template the carboxylesterase Est30 from *G. stearothermophilus* (PDB code 1TQH; Liu et al., 2004).

Biochemical characterization of Est/Lip

Temperature—activity and stability. The activity-temperature dependence was determined at pH 8.0, in the temperature range 25–80°C, with both *p*-nitrophenyl acetate (*p*NPA) and *p*-nitrophenyl palmitate (*p*NPP). Thermal stability was studied at 60°C. Samples were taken before and during incubation and residual activity with *p*NPA was determined.

pH—activity and stability. The influence of pH upon Est/Lip activity was assayed in the range pH 3.0–9.0, at 60°C. Determinations at pH values over 9 were hampered by the intense spontaneous hydrolysis of the substrate, which tended to mask enzymatic hydrolysis. Due to the changing equilibrium between *p*-nitrophenol and its anionic form *p*-nitrophenolate, the absorption spectrum of *p*-nitrophenol changes drastically with varying pH; therefore, when studying the pH profile of Est/Lip, the release of *p*-nitrophenol was monitored at 348 nm instead of 410 nm, as suggested by Hotta et al. (2002). At this wavelength, the *p*-nitrophenol/*p*-nitrophenolate spectrum shows an isosbestic point where the absorbance, even though significantly lower, is largely unaffected by pH variations.

Stability at pH values in the range 5.0–9.0 was assessed by preparing double-sized enzyme samples in buffers of the respective pH, of which one aliquot was assessed immediately and the second after a 30-min pre-incubation at room temperature. The used buffers (50 mM, all sodium salts) were: citrate (pH 3.0–6.5), phosphate (pH 7.0–8.5), and bicarbonate (pH 9).

Metals. The effect of cations on enzymatic activity was investigated by measuring residual activity after a 2-h preincubation at room temperature with 1 mM final concentration of the following metal chlorides: Na^+, K^+, Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Zn^{2+}, Hg^{2+}, and Al^{3+}. For every tested cation, a blank sample without enzyme was run in parallel. The samples containing Mn^{2+} and Fe^{3+} were subjected to a brief centrifugation prior to the activity assay, to avoid interfering particulate material.

Solvents. To assess the capacity of the recombinant enzyme to work in the presence of organic solvents, the buffer in the standard reaction mixture was replaced with 10%, 20% or 30% solutions (in Tris-HCl 50 mM, pH 8) of the following water-miscible solvents: acetone, methanol, ethanol, 2-propanol, acetonitrile, and dimethylsulfoxide (DMSO). The reaction with *p*NPA was initiated without extended pre-incubation. Blank samples were run first, to check substrate stability in the respective solvent.

Effectors. In order to assess whether and to what extent detergents and known denaturants interfere with the enzyme's activity and thermostability, activity with *p*NPA was first assayed without extended pre-incubation in the presence of the following reagents: SDS 1, 10, and 100 μM ; β-mercaptoethanol (β-ME) 1, 10, and 100 mM; and Triton X-100 0.01%, 0.05%, 0.1%, and 0.5% (v/v) (all final concentrations). In parallel, identical samples were incubated for 1 h at 60°C and residual activity was then assayed immediately. Results were compared to those of controls with no added effector.

Substrate selectivity and kinetic parameters. Enzymatic assays were carried out with the following esterase and lipase substrates: *p*NP-acetate, *p*NP-propionate, *p*NP-butyrate, *p*NP-methyl butyrate, *p*NP-palmitate, *p*NP-oleate, and triolein. The standard protocol for *p*NPA was used with propionate, butyrate, and methyl butyrate, while the protocol for *p*NPP was used for oleate. Activity against triolein was assessed titrimetrically, as described in the Analytical methods section. Activity vs. substrate concentration plots were built with *p*NPA and *p*NPP, respectively, and analyzed by nonlinear regression, in OriginPro 8.

Biocatalysis with Est/Lip.

The purified recombinant Est/Lip obtained as described was tested as a biocatalyst in hydrolytic reactions with chiral compounds. The reaction mixtures contained 500 μl enzymatic solution (100 μg/ml protein in Tris 50 mM, pH 7.5), 2.5 mg substrate and 2% (v/v) DMSO for improved solubilization of substrates. No DMSO was added for substrates 8–11. The reaction mixtures were shaken for 20 h at 50°C (200 rpm). Extraction was performed with *n*-hexane/isopropanol (IPA) (9 : 1, 500 μl) and samples were dried and filtered. Hydrolysis was monitored by high-performance liquid chromatography (HPLC) with an Agilent 1200 instrument.

The column (4.6 × 250 mm) and eluent used for each substrate are given in Table 1. The absolute configuration of the faster-reacting enantiomer was established by comparison of retention times obtained under similar experimental conditions, as indicated by Brem et al. (2011).

Analytical methods.

Standard enzymatic assay. Esterase activity was determined with *p*-nitrophenyl acetate (*p*NPA) as a substrate, while lipolytic activity was determined with *p*-nitrophenyl palmitate (*p*NPP). Both substrates were solved in isopropanol, at a 5 mM concentration (stock solution). *p*NPP required warming (55–60°C) in order to form a homogenous dispersion.

The released *p*NP was monitored at 410 nm, using a Cary® 50 UV-Vis spectrophotometer (Varian Inc., Australia) equipped with a thermostat. Unless otherwise stated, the reactions were carried out at 60°C, in 50 mM Tris-HCl buffer solution, at pH 8.0. At all times, the pH of the buffer was adjusted at the temperature of the reaction. The reaction mixture contained 900 µl buffer, 90 µl enzyme solution and 10 µl substrate stock solution. The buffer-diluted enzyme was pre-incubated for 3 min at the assay temperature. The reaction was initiated by adding prewarmed substrate solution. With *p*NPA, the initial 0.3 min were used to calculate ΔA/min. With *p*NPP, the reaction was allowed to proceed for 5 min and the absorbance was then read immediately. For each assay, a blank sample was run in parallel and its value subtracted from that of the enzyme sample. All determinations were run at least in duplicate; results are expressed as average ± standard deviation. One unit of activity was defined as the amount of enzyme releasing 1 µmol of *p*NP per minute under assay conditions.

Activity towards triolein. Triolein as a substrate was prepared according to a modified Fluka protocol and consisted of an aqueous solution of 0.15 mM pure triolein, 3 mM NaCl and 0.39% Triton X-100 (w/v). The reaction mixture consisted of 1 ml substrate, 0.5 ml enzyme solution and 0.5 ml of phosphate buffer (50 mM, pH 8). Final concentration of triolein in the reaction mixture was 0.075 mM. The reaction, performed in duplicate, was carried on for 4 h at 60°C with 200 rpm shaking, and was stopped by the addition of 3 ml acetone : ethanol (1 : 1). Liberated fatty acids were titrated against NaOH 50 mM, using phenolphthalein as an indicator. One unit of activity was defined as the amount of enzyme releasing 1 µmol of fatty acids per minute under assay conditions.

Protein content determination. Protein concentration was estimated by means of the Bradford method (Bradford, 1976), using bovine serum albumin for the calibration curve. The concentration was plotted against the 595/450 nm absorbance ratio, which, according to Kruger (2002) and to Ernst and Zor (2010), improves linearity of the curve and increases accuracy and sensitivity of the method by up to 10-fold.

SDS-PAGE. All protein-related electrophoretic procedures were carried out in a discontinuous system, on a vertical mini gel electrophoresis unit (Scie-Plas TV100 YK, Great Britain). Staining was performed with Coo-

Table 1. Substrates, columns and eluents used for HPLC monitorization of hydrolysis reactions with Est/Lip.

Substrate	Name	Column	Eluent *n*-hexane : IPA
(1)	1-phenylethyl acetate	IC[a]	98 : 2
		OJ-H[b]	95 : 5
(2)	ethyl 3-hydroxy-3-phenyl propanoate	IA/IB (tandem)	95 : 5
(3)	ethyl-3-butoxy-4-(4-chlorophenyl) butyrate	IA	96 : 4
(4)–(7)	R-2-(4-isobutylphenyl) propanoate R:	IB[a]	100 : 0[a]
	(4) methyl; (5) ethyl; (6) butyl; (7) octyl	IB-reverse	99 : 0.9
		phase[b]	(: 0.1 acetic acid)[b]
(8)	(ethoxycarbonyl)(4-methoxyphenyl)	IA/AS-H	96 : 4
	methyl decanoate	(tandem)	
(9)	ethyl 3-(furan-2-yl)-3-hydroxypropanoate	IA	95 : 5
(10)	ethyl 3-hydroxy-3-(thiophen-2-yl) propanoate	IB	90 : 10
(11)	1-(benzofuran-3-yl)-3-ethoxy-3-oxopropyl decanoate	IC[a]	90 : 10
		IA[b]	90 : 10
(12)	2-(2-bromo-1-(undecyloxy)ethyl)-7-ethyl benzofuran	Welk	99 : 1

[a] for substrate; [b] for products

massie Brilliant Blue R-250. Molecular weight markers used were beta-galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, beta-lactoglobulin, and lysozyme (Thermo Scientific Pierce Prestained Protein Molecular Weight Markers, 120–20 kDa).

Results and Discussions

Characterization of the bacterial isolate. On agar plates, the strain formed circular, convex, opaque, glossy, yellow colonies with regular margins and diameters between 1 and 3 mm. Microscopic examination of liquid 24-h cultures revealed the presence of moderately motile, singly occurring, straight or slightly bent rods, with lengths varying between 2 and 14 μm. Terminally located, cylindrical or ellipsoidal 0.2/2.0 μm large endospores were detected, in lightly swollen sporangia. The Gram staining reaction was variable (different cells from the same colony stained either positive or negative), but the KOH test defined the strain as Gram-type positive.

The isolate was able to utilize several mono- and disaccharides as a carbon source (production of acid, but no gas), but not glucidic alcohols or citrate. It was positive for gelatinase and amylase and negative for catalase and urease. Indol synthesis, H_2S production and the Voges-Proskauer test were negative. At 60°C, growth was only present with 0.5–3% NaCl added. At pH 8, the strain grew best at 50–60°C; no growth was recorded below 40°C or over 70°C (Fig. 1). At optimal temperature, bacterial growth was approximately constant within the pH range 6.5–8.5. Limit pH values were 5.5–6.0 and 8.5 (Fig. 2). Results of characterization tests of the isolate are summarized in Table 2.

Anoxybacillus flavithermus is described (Pikuta et al., 2000; then, De Clerck et al., 2004) as a facultatively aerobic, endospore forming, moderately thermophilic Gram-positive rod, growing in a broad temperature range (30–72°C; Heinen et al., 1982). Growth temperature, colony and cell morphology, spore-formation, carbon source utilization, of strain T1 match to a large extent the description of the species, although its growth temperature limits appear to be somewhat narrower.

The variability of Gram-staining encountered with this isolate has been described previously in thermophilic bacilli: *A. contaminans* (De Clerck et al., 2004) and *G. stearothermophilus* (Nazina et al., 2001). In all reported cases, the strains were also proven to be chemotypically Gram-positive. A few phenotypic features of our isolate do not match the species description. While *A. flavithermus* has been described as catalase-positive (Pikuta et al., 2000), our isolate was catalase-negative. It also differs from the species description by its ability to hydrolyse both gelatine and starch. Pikuta et al. (2000) reported *A. flavithermus* to utilise starch, but not gelatine, and according to De Clerck et al. (2004) it utilises neither. The above suggest that strain T1 differs to a certain extent in its phenotypic features from previously described strains.

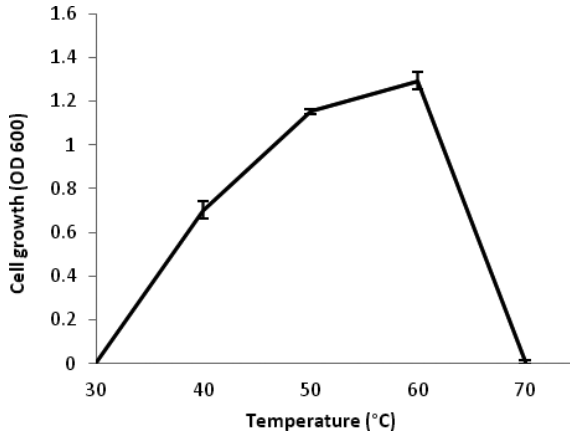

Fig. 1. Bacterial growth at different temperatures, initial pH 8.0.

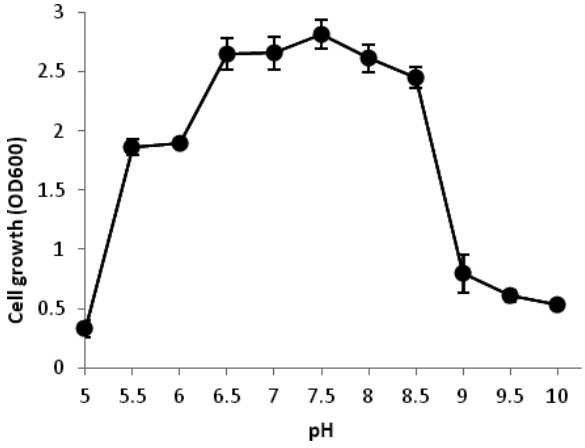

Fig. 2. Bacterial growth at different pH values, at 60°C.

Genetic identification. Sequencing of the 16S rRNA gene identified the isolate as *Anoxybacillus flavithermus* (GenBank ID: JQ267733). Maximum similarity score (2652), query coverage (99%), and maximum identity (99%) were achieved with *A. flavithermus* strain AE3 (FN666242.1). The strain was deposited with the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary, under the accession number NCAIM B 02482.

Table 2. Characterization test results for the isolated strain.

Morphology	
Cell shape	rod
Size (average)	length 4.0–5.0 μm, width >0.5 μm
Endospore	+
Position	terminal
Shape	ellipsoidal/cylindrical
Motility	+/–
Colonies	circular, yellow, convex, Φ1–3 mm
Gram staining	variable
Gram chemotype	positive
Biochemistry	
Growth on:	
NaCl 0.5–3%	+
5%	–
Egg broth	+/–
Blood-agar	–
Meat broth	–
Utilization of:	
D-Glucose	+
Mannitol	–
Inositol	–
D-Sorbitol	–
L-Rhamnose	–
D-Sucrose	+
D-Melibiose	+
Amygdaline	–
L-Arabinose	+
Citrate	–
Hydrolysis of:	
Gelatine	+
Starch	+
Urea	–
Production of catalase	–
Production/formation of:	
H_2S	–
Indol compounds	–
Acetoin	–
Physiology	
Anaerobic growth	+/–
pH range	5.5–8.5
Temperature (°C)	37–70

+, indicates a positive, –, a negative, +/–, a weak result.

Molecular cloning, expression and in silico analysis of Est/Lip. PCR amplification gave best results with the primers designed for periplasmic expression containing the restriction sites BamH1/XhoI, in association with MyTaq. Obtained PCR products were approx. at the 750 bp level, consistent with the length of the expected gene. The putative *A. flavithermus* carboxylesterase, consisting of 247 amino acid residues and having a predicted molecular weight of 28.03 kDa, is encoded by an open reading frame of 741 bps. The protein was successfully expressed in *E. coli* BL21(DE3) and could be recovered in active form from the periplasmic fraction, although a significant amount of activity remained in the cell wall fraction. Elution from Ni-agarose started at imidazole concentrations even lower than 250 mM. Under these conditions, a protein with estimated molecular weight of ~25 kDa and esterolytic ability was the main component of the IMAC-purified fractions (Fig. 3), its ability to operate at 60°C proving its thermophilic origin. The Est/Lip sequence was deposited with GenBank, under accession number JX494348.

The deduced amino acid sequence of *A. flavithermus* T1 Est/Lip showed highest similarity with carboxyl-esterases from *A. flavithermus* WK1 (B7GLX5) and *Anoxybacillus* sp. PDF1 (G9C5W9)—97% and 96%, respectively. The following most similar sequences, with identity scores between 86% and 84%, all belong to various *Geobacillus* strains, among which are *G. thermoglucosidans* TNO (I0UCF0), *G. kaustophilus* HTA426 (Q5KVF6), *G. thermoleovorans* -EstA (Q2V6P5) and *G. stearothermophilus* Est30 (Q06174), the last being used as a template for 3D modeling of the *A. flavithermus* T1 Est/Lip. Figure 4 shows the sequence alignment of these

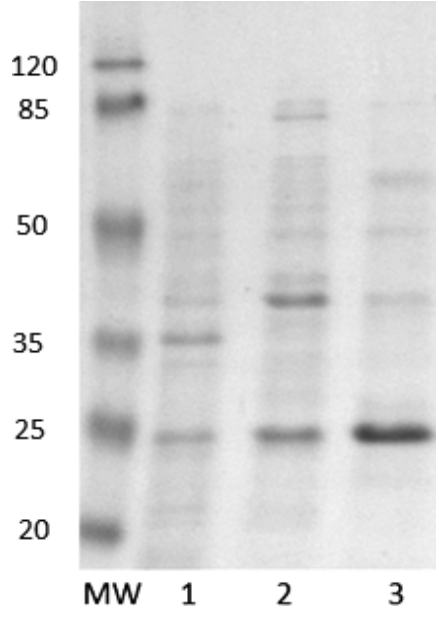

Fig. 3. Denaturing SDS-PAGE gel showing purification steps of the cloned Est/Lip.

MW, molecular weight markers; lane 1, crude extract of *E. coli* BL21(DE3) cells, 5 h after IPTG induction; lane 2, periplasmic fraction; lane 3, purified Est/Lip after Ni^{2+}-affinity chromatography.

```
A.flavithermus_T1       MMKMIPPQPFTFEAGERAVLLLHGFTGNSADVRMLGRFLQSKGYTCHAPIYKGHGVPPEE 60
A.flavithermus_WK1      MMKMIPPQPFTFEAGERAVLLLHGFTGNSADVRMLGRFLQSKGYTCHAPIYKGHGVPPEE 60
Anoxybacillus_sp._PDF1 MVKMIPPQPFTFEAGERAVLLLHGFTGNSADVRMLGRFLQAKGYTCHAPIYKGHGVPPEE 60
G.stearothermophilus    -MKIVPPKPFFFEAGERAVLLLHGFTGNSADVRMLGRFLESKGYTCHAPIYKGHGVPPEE 59
G.kaustophilus_HTA426   -MKIVPPKPFFFEAGERAVLLLHGFTGNSADVRMLGRFLESKGYTCHAPIYKGHGVPPEE 59
G.thermoglucosidans     -MKVVPPKPFTFEAGERAVLLLHGFTGNSSDVRMLGRFLEAKGYTCHAPIYKGHGVPPEE 59
G.thermoleovorans       MMKIVPPKPFFFEAGERAVLLLHGFTGNSADVRMLGRFLESKGYTCHAPIYKGHGVPPEE 60
                         :*::**:** ******************:*********::******************

A.flavithermus_T1       LVHTGPEDWWQDVINAYEHLKQK-HEKIAVVGLSLGGVFSLKLGYTVPVVGIVPMCAPMY 119
A.flavithermus_WK1      LVHTGPEDWWQDVINAYEYLKQT-HEKIAVVGLSLGGVFSLKLGYTVPVVGIVPMCAPMY 119
Anoxybacillus_sp._PDF1 LVHTGPEDWWQDVMNAYEHLKQT-HEKIAVVGLSLGGVFSLKLGYTVPVVGIVPMCAPMY 119
G.stearothermophilus    LVHTGPDDWWQDVMNGYEFLKNKGYEKIAVAGLSLGGVFSLKLGYTVPIEGIVTMCAPMY 119
G.kaustophilus_HTA426   LVHTGPDDWWQDVMNGYQFLKNKGYEKIAVAGLSLGGVFSLKLGYTVPIQGIVTMCAPMY 119
G.thermoglucosidans     LVHTGPDDWWQDVINAYEFLKNKGYEKIAVAGLSLGGVFSLKLGYTVPVVGIIPMCAPMY 119
G.thermoleovorans       LVHTGPDDWWQDVMNGYQFLKNKGYEKIAVAGLSLGGVFSLKLGYTVPTQGIVTMCAPMY 120
                        ******:******:*.*:.**:. :*****.*****************  **: ******

A.flavithermus_T1       IKSEQTMYEGVLAYAREYKKREGKSEDQIEREMVEFAKTPMKTLKALQQLIAEVRDHLDF 179
A.flavithermus_WK1      IKSEQTMYEGVLAYAREYKKREGKSEEQIEREMAEFAKTPMKTLKALQQLIADVRDHLDF 179
Anoxybacillus_sp._PDF1 IKSEQTMYEGVLAYAREYKKREGKDEEQIEREMMEFAKTPMKTLKALQQLIAEVRDHLDF 179
G.stearothermophilus    IKSEETMYEGVLEYAREYKKREGKSEEQIEQEMEKFKQTPMKTLKALQELIADVRDHLDL 179
G.kaustophilus_HTA426   IKSEETMYEGVLEYAREYKKREGKSEEQIEQEMERFKQTPMKTLKALQELIADVRAHLDL 179
G.thermoglucosidans     IKSEETMYEGVLKYAREYKKREGKTPEQIEKEMEAFQKTPMKTLKALQQLIADVRERIDL 179
G.thermoleovorans       IKSEETMYEGVLEYAREYKKREGKSEEQIEQEMERFKQTPMKTLKALQELIADVRAHLDL 180
                        ****:******* **********  :***:**  * :**********:***:** ::*:

A.flavithermus_T1       IYAPVFVVQARHDDMINPDSANIIYNGVESPVKQMKWYEESGHVITLDKEKEQLHEDIYA 239
A.flavithermus_WK1      IYAPVFVVQARHDDMINPDSANIIYNGVESPVKQIKWYEESGHVITLDKEKEQLHEDIYA 239
Anoxybacillus_sp._PDF1 IYAPIFVVQARHDDMINPDSANIIYNGVESPVKQMKWYEESGHVITLDKEKEQLHEDIYT 239
G.stearothermophilus    IYAPTFVVQARHDEMINPDSANIIYNEIESPVKQIKWYEQSGHVITLDQEKDQLHEDIYA 239
G.kaustophilus_HTA426   VYAPTFVVQARHDEMINPDSANIIYNEIESPVKQIKWYEQSGHVITLDQEKDQLHEDIYA 239
G.thermoglucosidans     IYAPTFVVQARHDEMINPDSANIIYNGIESPVKKIKWYEESGHVITLDKEKEQLHEDIYE 239
G.thermoleovorans       VYAPTFVVQARHDEMINPDSANIIYNEIESPVKQIKWYEQSGHVITLDQEKDQLHEDIYA 240
                        :*** ********:************ :*****::****:********:**:*******

A.flavithermus_T1       FLESLDW 246
A.flavithermus_WK1      FLESLDW 246
Anoxybacillus_sp._PDF1 FLESLDW 246
G.stearothermophilus    FLESLDW 246
G.kaustophilus_HTA426   FLESLDW 246
G.thermoglucosidans     FLESLDW 246
G.thermoleovorans       FLESLDW 247
                        *******
```

Fig. 4. Multiple sequence alignment for *A. flavithermus* T1 Est/Lip and closely related carboxyl esterases, showing the highly conserved GXSXG sequence.

Black triangles indicate the proposed catalytic residues. Protein sequences were aligned with CLUSTAL O (1.1.0) under UniProt.

related carboxylesterases.

Sequence analysis of the plasmid harboring the desired insert proved the presence of the highly conserved domain GXSXG specific for α/β hydrolases including esterases and lipases, which contains the catalytic serine residue (Jaeger and Reetz, 1998; Ollis et al., 1992). The amino acid residues composing the signature motif are Gly91-Leu92-Ser93-Leu94-Gly95 and, by comparison with the tridimensional model of the template enzyme Est30 from *G. stearothermophilus* (Liu et al., 2004), we proposed a catalytic triad consisting of Ser93, Asp192 and His222. In this case, the catalytic serine would be located on the 'nucleophilic elbow' situated after β-sheet 5 and before α-helix 4, and all three catalytic residues would be located at the top of the C-terminal β-sheet (Liu et al., 2004). The relative position, as well as the distances between the catalytic residues in *A. flavithermus* Est/Lip and in the template enzyme are almost identical, bringing a further argument for the similarity of the two enzymes (Fig. 5).

Analysis of the modeled tridimensional structure of *A. flavithermus* T1 Est/Lip showed that the central β-sheet is composed of seven strands, with the short N-terminal strand antiparallel to the rest, while the lid, apparently a helical region distinct from the core domain, consists of two α-helices and one 3_{10}-helix (Fig. 6).

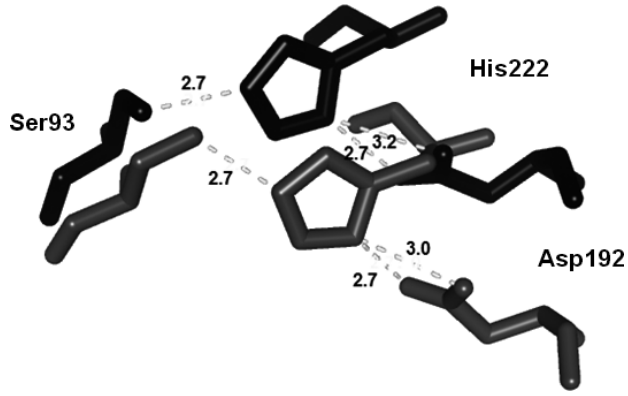

Fig. 5. Position and relative distances of the catalytic amino acid residues in the active sites of *A. flavithermus* T1 Est/Lip (in grey) and *G. stearothermophilus* Est30 (in black), respectively.

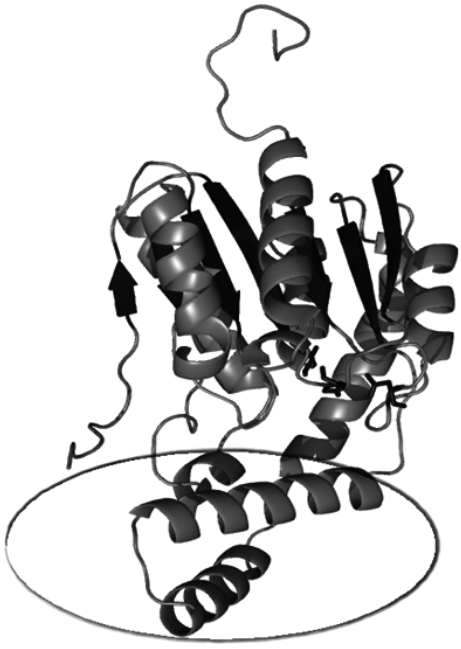

Fig. 6. Three-dimensional model of *A. flavithermus* T1 Est/Lip, showing α-helices (in grey) and β-strands (as black arrows).

The three helices constituting the lid are marked at the top of the model. Catalytic amino-acid residues are represented as black stick models.

These structural features, together with the high degree of homology with the aforementioned *Geobacillus* esterases, suggest that *A. flavithermus* T1 Est/Lip belongs to the new class of microbial carboxylesterases proposed by Montoro-García et al. in 2009, distinct from any of the eight classic lipase families described by Arpigny and Jaeger (1999). Esterases in this class, which as for now comprises only a limited number of *Geobacillus* and *Anoxybacillus* enzymes, lack the first β strand of the eight present in the typical α/β hydrolase core of esterases in family IV. Another difference consists in the structure and position of the lid, which is made up of only three helices instead of four or five, as in esterases of family IV, and does not surround the catalytic triad, instead being positioned closer to the N-terminal end of the central sheet. Esterases in this family have a similar molecular weight and core structure to enzymes in family VI, differing however from the latter in their lid, which in family VI esterases consists of four small β-strands covering the catalytic site and limiting the ability of these enzymes to hydrolyse long chain fatty acid esters. A phylogenetic tree illustrates the relative position of several carboxylesterases related to *A. flavithermus* T1 Est/Lip, based on the same gene sequences used for multiple sequence alignment (Fig. 7).

Biochemical characterization of Est/Lip.

The purified recombinant *A. flavithermus* Est/Lip was fully stable at 4°C for at least 15 days at a concentration of 11.28 μg/ml in Tris/HCl 50 mM pH 8. A sample of this enzyme stock, freeze-dried, kept at −20°C for 15 days and reconstituted in the same buffer, retained 65.5% activity compared with the native enzyme (data not shown).

Temperature—activity and stability. Activity with *p*NPA was already present at room temperature, at a rate amounting to 64% of the highest value, and kept increasing up to 60°C. Above 65°C, the enzyme started to dena-

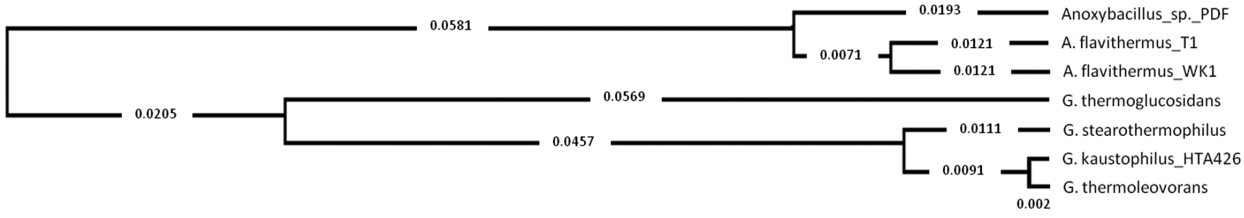

Fig. 7. Phylogenetic tree showing the degree of relatedness between several carboxyl esterases highly homologous with *A. flavithermus* T1 Est/Lip.

Numbers denote phylogenetic distances.

ture quickly; nevertheless, it still retained 50% residual activity at 70°C (Fig. 8). With *p*NPP as a substrate, the enzyme required a minimal temperature of 50°C in order to perform catalytically, but activity values peaked at a higher temperature (65°C) than with *p*NPA. We noted that at high temperatures (80°C) chemical hydrolysis in the blank tended to be higher than enzymatic release of *p*-nitrophenyl, which led to negative activity values. It appears that the mere presence of the enzyme exerts a certain protective effect upon the substrate, probably by allowing it to attach to the active site even after having lost catalytic activity.

At 60°C, the pure enzyme had a half-life of at least 5 h and retained over 75% of its activity for over 2 h. The decay rate was linear over the first ~8 h (Fig. 9).

pH—activity and stability. Est/Lip exhibited highest activity at pH 6.5; nevertheless, this peak was rather small and overall high activity was encountered over a broader pH ranging from slightly acidic to slightly alkaline (6.0–8.5), within which the average activity level was approx. 85% of the maximum (Fig. 10). Unlike lipases, which are usually optimally active at slightly alkaline pH (Fojan et al., 2000; or see Guncheva and Zhiryakova, 2011 for a comprehensive review on *Bacillus* lipases), esterases appear to display a higher degree of heterogeneity in terms of pH preference. A brief literature review shows that thermophilic microbial esterases have been reported to be optimally active at pHs ranging from 5.5 (Faiz et al., 2007—*A. gonensis*) to 9.5 (Ewis et al., 2004—*G. stearothermophilus*). However, many esterases seem to be characterized by a plateau-like rather than a narrow-peaked pH profile, with a relatively broad pH range of submaximal activities and/or high stability (e.g., Ay et al., 2011—*A. flavithermus*; Montoro-García et al., 2009—*G. kaustophilus*; Soliman et al., 2007—*G. thermoleovorans*; Tekedar and Şanli-Mohamed, 2011—*Geobacillus* sp.).

The enzyme was stable over the whole assessed pH range (5.0–9.0). Moreover, at pH values over 7, it was activated by the 30 min pre-incubation, even though this was carried out at room temperature (Fig. 10). It appears that this longer and milder prewarming allows the enzyme to assume a more favourable conformation, more so than an abrupt change from 4°C (temperature of the enzyme stock solution) to 60°C (temperature of the reaction mixture). Given the satisfactory level of activity of Est/Lip at pH 8 (over 80% of the maximum), all other characterization experiments were

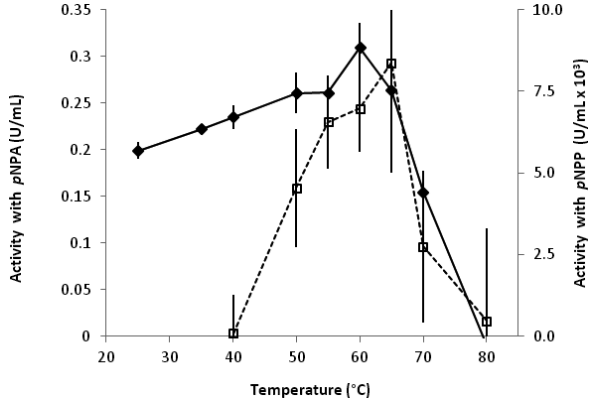

Fig. 8. Temperature profile of the recombinant Est/Lip, determined at pH 8.0, with *p*NPA (solid line, closed symbols) and *p*NPP (dashed line, open symbols), respectively.

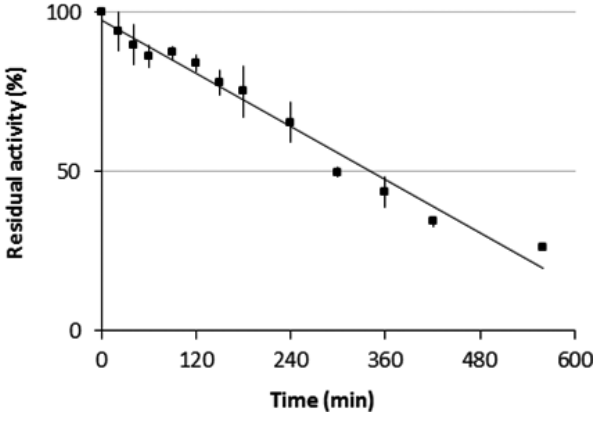

Fig. 9. Stability of Est/Lip at 60°C.

Residual activity at different experimental time points was expressed as percentage of the activity before incubation.

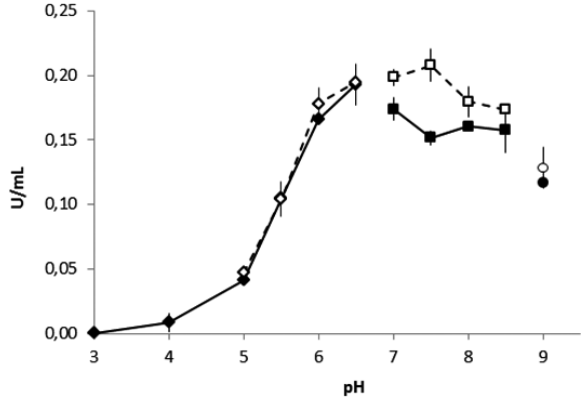

Fig. 10. pH profile of the recombinant Est/Lip, determined at 60°C (solid line, closed symbols).

Stability of Est/Lip at different pH values (dashed line, open symbols) was investigated by incubating enzyme samples at 60°C for 30 min in the buffer of the respective pH prior to assessing hydrolytic activity. Buffers (50 mM): sodium citrate (pH 3.0–6.5), sodium phosphate (pH 7.0–8.5), sodium bicarbonate (pH 9.0).

further performed at this pH, which allowed monitorization at 410 nm, and thus better sensitivity and minimal sample processing.

Metals. All metal ions tested inhibited Est/Lip activity. Metals of groups I and II had a limited effect, except for magnesium, which depressed activity by about 70%. Of the transitional metals, Co^{2+} exerted the strongest inhibitory effect, inactivating the enzyme completely. Est/Lip retained a residual activity close to 10% in the presence of Fe^{3+}, Cu^{2+}, and, surprisingly, Hg^{2+}. The results (Table 3) suggest that the enzyme is not a metalloprotein; this conclusion is also supported by the observation that 1 mM EDTA does not inhibit its activity (data not shown).

Solvents. The enzyme retained a high amount of activity (over 60%) in the presence of 10% methanol and 10% DMSO, although increasing concentrations of methanol inactivated it (Fig. 11). DMSO was especially compatible with Est/Lip, allowing significant levels of activity at a concentration as high as 30%. Acetonitrile also appeared to be relatively well tolerated. Other esterases have been likewise reported to be stable in DMSO (e.g., Kang et al., 2001), including the esterase of *G. kaustophilus* HTA 426, closely related to *A. flavithermus* Est/Lip (Montoro-García et al., 2009). Even more so, an active-site re-arrangement was suggested for an *Aspergillus niger* feruloyl esterase following prolonged incubation in DMSO-water (Faulds et al., 2011).

Table 3. Influence of metal cations on Est/Lip activity (2 h preincubation in presence of the metal, at room temperature; standard enzymatic assay at 60°C and pH 8).

Cation (1 mM)	Residual activity	Cation (1 mM)	Residual activity
Control	100.0	Mn^{2+}	4.5
Na^{+}	81.1	Fe^{3+}	13.7
K^{+}	66.6	Co^{2+}	0.0
Ca^{2+}	61.9	Cu^{2+}	7.3
Mg^{2+}	28.6	Zn^{2+}	3.0
Ba^{2+}	50.8	Hg^{2+}	7.3
		Al^{3+}	0.4

Effectors. SDS in concentrations up to 100 μM and of Triton X-100 in concentrations of 0.01−0.5% exerted a slight inhibitory effect upon Est/Lip activity in unincubated samples (Fig. 12). However, the presence of a low concentration of both detergents (1 μM SDS, respectively 0.01−0.05% Triton X-100), rendered the enzyme significantly more thermostable and concurrently facilitated a temperature-induced activation effect, which has been previously reported for thermostable esterases (Montoro-García et al., 2009). By adding negative charges to the protein surface, SDS in low concentrations could act as a lubricant, increasing electrostatic repulsion both within the protein coil and between adjacent molecules, rendering each molecule more flexible, limiting aggregation and thus increasing activity at high temperatures (Córdova et al., 2008; Santos et al., 2003). Helistö and Korpela (1998) suggest

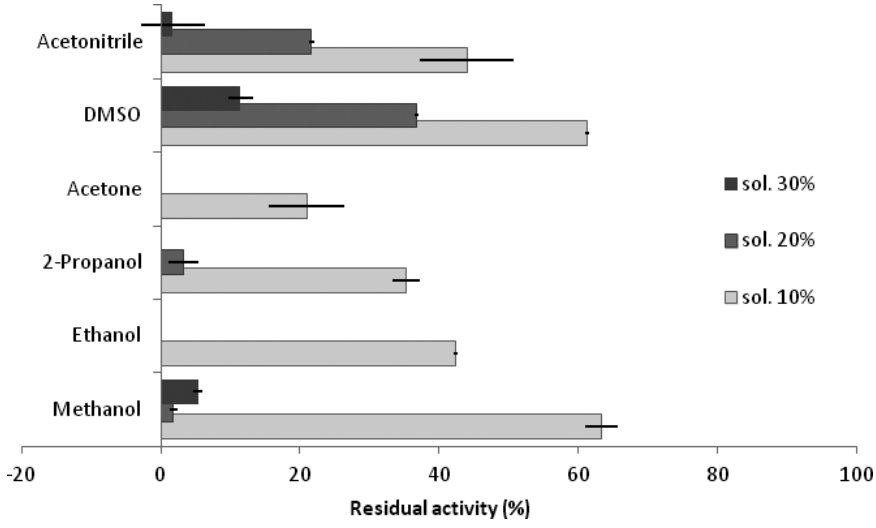

Fig. 11. Activity of the cloned Est/Lip in organic solvents.

Standard enzymatic assay was performed in buffer solution containing 10%, 20% and 30% (v/v) solvent. The activity was expressed as percentage of the activity of control samples (0% solvent).

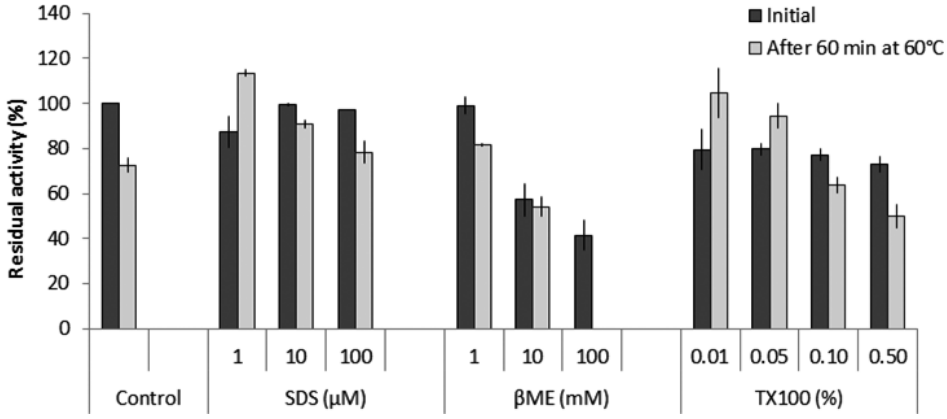

Fig. 12. Influence of effectors on the activity and thermostability of Est/Lip.

Standard enzymatic assay was performed in reaction mixtures containing effectors at the indicated final concentrations, before and after a 30 min pre-incubation at 60°C. The activity of a control sample (no added effector) before incubation was considered as 100%; all other results were expressed as percentage of this value.

that the protective effect of SDS against thermally-induced denaturation may also be due to its amphiphilic nature, which helps preserve the structure by bridging non-polar and charged residues located on different loops. Thirty minutes of incubation at room temperature in the presence of 1 mM SDS completely abolished enzymatic activity (data not shown).

At 1 mM, β-ME did not affect the activity and slightly enhanced the thermostability of the enzyme, possibly by protecting methionine residues in the vicinity of the active site against oxidation (Kim et al., 2001). It is known that sulfoxide formation may modulate enzyme activity by changes in hydrophobicity and by introducing steric limitations (Park et al., 2001). When associated with prolonged incubation at 60°C, 100 mM β-ME led to complete inactivation of the enzyme. Given the low cysteine content of the protein, a non-specific, solvent-like inhibition mechanism is a possible explanation. However, Zappa et al. (2001) also reported a low cysteine content enzyme (a thermostable alkaline phosphatase with only one cysteine residue per monomer and no interchain disulfide bonds) severely affected by the non-alcohol-like reducing agent dithiothreitol (1 mM). It has been shown that β-ME can form adducts with cysteine residues (Begg and Speicher, 1999). In this case, Cys115 is very close to the catalytic core and has its sulfhydryl group oriented directly towards its centre; it is thus reasonable to assume that a bulky Cys-β-ME adduct in this place will interfere decisively with the catalytic act.

Substrate selectivity and kinetic parameters. Est/Lip had highest activity with *p*NP-butyrate and with the slightly larger chiral substrate *p*NP-methyl butyrate. The activity decreased towards both shorter and longer fatty acid chains (Table 4). Despite this definite preference for short chain fatty acid esters, Est/Lip displayed a no-

Table 4. Substrate specificity of the cloned Est/Lip for *p*-nitrophenyl acyl esters and triolein

Substrate	Specific activity (U/mg)	Residual activity (%)
*p*NP-acetate (C2:0)	61.3±1.5	36.7
*p*NP-propionate (C3:0)	84.1±3.4	50.3
*p*NP- butyrate (C4:0)	167.1±2.7	100.0
*p*NP- methyl butyrate (C*5:0)	149.1±32.6	89.2
*p*NP- caprate (C10:0)	73.6±2.9	44.1
*p*NP- palmitate (C16:0)	1.4±0.3	0.8
*p*NP- oleate (C18:1 cis-9)	3.2±0.4	1.9
Triolein	14.3±2.7	8.6

Conditions, 60°C, pH 8, see Materials and Methods for details. Asterisk denotes presence of chiral carbon.

ticeable activity with unsaturated lipase-specific substrates (triolein) and with long chain *p*N esters, if allowed longer reaction times (data not shown).

With both *p*NPA and *p*NPP, the lowest substrate concentration point for which activity could be defined with precision was 10 μM. Table 5 presents the kinetic parameters for the two substrates. The results show that, while the reaction velocity with *p*NPA is almost 60-fold higher than with *p*NPP, the enzyme's affinity is slightly lower for the typical esterase substrate than for the much longer *p*NPP. For both substrates, the best fitting curve for the rate vs. concentration plot was obtained with a Hill equation instead of the expected Michaelis-Menten relation, which also precluded a good linearization. In both cases a substrate inhibition occurred, at concentrations over 350 μM for *p*NPA and much lower (125 μM) for *p*NPP (data not shown). The *N* value close to 2 suggests that the active enzyme would be a dimer.

Biocatalysis with Est/Lip.

Structures of the chiral substrates tested with Est/Lip are shown in Fig. 13. The enzyme was hydrolytically active with several compounds. Table 6 lists the substrates transformed by Est/Lip, along with data on enantiomeric excesses, conversion and enantiomeric ratio. Est/Lip showed high selectivity, with a preference for the (*S*)-enantiomer. For substrates (2), (9) and (10), even though the reaction products could not be visualised on the chromatogram, analysis of the substrates proved the same (*S*)-selectivity, with low (<30) ee values (data not shown). Substrates (4–7), (8) and (12)

Table 5. Kinetic parameters for *A. flavithermus* T1 Est/Lip, with *p*NPA, and *p*NPP.

Substrate	V_{max} (μmol min^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	*N*
*p*NPA	82.89±1.56	34.14±1.37	7,400	216.75	1.96±0.16
*p*NPP	1.44±0.08	23.28±2.32	128.5	5.52	1.85±0.32

Reactions were carried out at 60°C and pH 8.

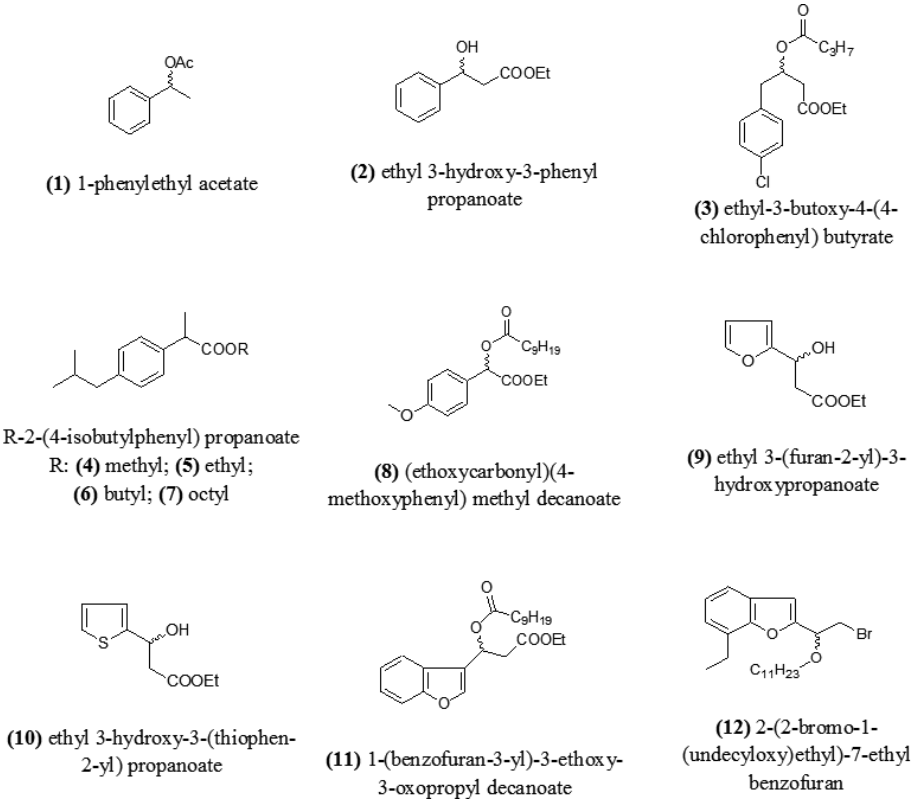

Fig. 13. Structures of chiral substrates tested with Est/Lip.

Table 6. Enantioselectivity data on hydrolysis reactions with Est/Lip.

Substrate	ee_s (%)	ee_p (%)	c (%)	E	t_R[a] (min)	Config.[b]
(1)	40.9	99.9	29.03	>200	13.3	(*S*)
(3)	4.87	99.9	4.65	>200	18.4	(*S*)
(11)	14.9	99.9	13.10	>200	11.8	(*S*)

[a] Retention time of the product; [b] configuration of the faster reacting enantiomer, as determined by comparison of retention times with data in the literature (Brem et al., 2011).

were not hydrolysed under the experimental conditions employed.

In conclusion, we have identified a local *A. flavithermus* strain producing an extracellular esterase/lipase which was cloned and overexpressed in *E. coli*. Use of the primer set designed for periplasmic expression, combined with the use of a His6-tag, allowed a facile recovery of the enzyme in an active form. The purified recombinant enzyme is very similar to several other thermostable *Anoxybacillus* and *Geobacillus* carboxyl esterases, in terms of amino acid sequence, structural features and biochemical characteristics. As proposed before by several authors (Liu et al., 2004; Montoro-García et al., 2009), these enzymes are part of a distinct family, different from other classes comprising thermostable carboxyl esterases of bacterial origin. A brief comparison of related esterases in this family, including *A. flavithermus* T1 Est/Lip characterized herein, shows that they share a sequence of 245–249 amino acid residues, a molecular weight of approx. 25–30 kDa and an optimal working temperature of about 60–65°C. The most favourable pH may go from slightly acidic (as in Est/Lip) to slightly alkaline, with a remarkable stability over a relatively broad pH range. The preferred substrate is frequently C4, but some esterases in this class, including Est/Lip, can hydrolyze, albeit to a low rate, substrates as long as C18. Enzymes in this family are less well characterized as yet with respect to other properties such as regio or enantioselectivity. Our results prove that Est/Lip is active and enantioselective upon several substrates under mild conditions, which makes it an interesting candidate for improvement by directed evolution (Reetz, 2004).

Acknowledgments

LC and MH acknowledge the financial support received through the Sectoral Operational Programme for Human Resources Development 2007–2013, co-financed by the European Social Fund, under the project numbers POSDRU/88/1.5/S/60185 "Innovative doctoral studies in a knowledge-based society" and POSDRU 89/1.5/S/60189 "Postdoctoral Programs for Sustainable Development in a Knowledge Based Society", respectively. MT acknowledges the financial support received through PN-II-PT-PCCA-2011-3.1-1268. BGV acknowledges financial support from grants OTKA NK-84008 and NN 103242.

The authors thank Dr. Laura Ispravă-Pop for NMR analyses of synthesized *p*-NP esters, Dr. Iulia Lupan for kindly providing transformed *E. coli* cells, and colleagues in the fine organic synthesis group for assistance with the biocatalysis experiments.

References

Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2006) The SWISS-MODEL Workspace: A web-based environment for protein structure homology modeling. *Bioinformatics*, **22**, 195–201.

Arpigny, J.L. and Jaeger, K.E. (1999) Bacterial lipolytic enzymes: Classification and properties. *Biochem. J.*, **343**, 177–183.

Ay, F., Karaoglu, H., Inan, K., Çanakçi, S., and Beldüz, A.O. (2011) Cloning, purification and characterization of a thermostable carboxylesterase from *Anoxybacillus* sp. PDF1. *Protein Express. Purif.*, **80**, 74–79.

Begg, G.E. and Speicher, D.W. (1999) Mass spectrometry detection and reduction of disulfide adducts between reducing agents and recombinant proteins with highly reactive cysteines. *J. Biomol. Tech.*, **10**, 17–20.

Bornscheuer, U.T. (2002) Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS Microbiol. Rev.*, **26**, 73–81.

Bradford, M.M. (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

Brem, J., Naghi, M., Toşa, M.I., Boros, Z., Poppe, L., Irimie, F.D., and Paizs, C. (2011) Lipase mediated sequential resolution of aromatic β-hydroxy esters using fatty acid derivatives. *Tetrahedron: Asymmetry*, **22**, 1672–1679.

Çolak, A., Sisik, D., Saglam, N., Guner, S., Çanakçi, S., and Beldüz, A.O. (2005) Characterization of a thermoalkalophilic esterase from a novel thermophilic bacterium, *Anoxybacillus gonensis* G2. *Bioresour. Technol.*, **96**, 625–631.

Córdova, J., Ryan, J.D., Boonyaratanakornkit, B.B., and Clark, D.S. (2008) Esterase activity of bovine serum albumin up to 160° C: A new benchmark for biocatalysis. *Enzyme Microb. Technol.*, **42**, 278–283.

Dai, J., Liu, Y., Lei, Y., Gao, Y., Han, F., Xiao, Y.Z., and Peng, H. (2011) A new subspecies of *Anoxybacillus flavithermus* ssp. *yunnanensis* ssp. nov. with very high ethanol tolerance. *FEMS Microbiol. Lett.*, **320**, 72–78.

De Clerck, E., Rodríguez-Díaz, M., Vanhoutte, T., Heyrman, J., Logan, N.A., and De Vos, P. (2004) *Anoxybacillus contami-*

nans sp. nov. and *Bacillus gelatini* sp. nov., isolated from contaminated gelatin batches. *Int. J. Syst. Evol. Microbiol.*, **54**, 941–946.

Deive, F.J., Dominguez, A., Barrio, T., Moscoso, F.M., Moran, P., Longo, M.A., and Sanroman, M.A. (2010) Decolorization of dye Reactive Black 5 by newly isolated thermophilic microorganisms from geothermal sites in Galicia (Spain). *J. Hazard. Mater.*, **182**, 735–742.

Ernst, O. and Zor, T. (2010) Linearization of the Bradford protein assay. JoVE. 38. http://www.jove.com/details.php?id=1918, doi: 10.3791/1918.

Ewis, H.E., Abdelal, A.T., and Lu, C.D. **(**2004) Molecular cloning and characterization of two thermostable carboxyl esterases from *Geobacillus stearothermophilus*. *Gene*, **329**, 187–195.

Faiz, Ö., Çolak, A., Saglam, N., Çanakçi, S., and Beldüz, A.O. (2007) Determination and characterization of thermostable esterolytic activity from a novel thermophilic bacterium *Anoxybacillus gonensis* A4. *J. Biochem. Mol. Biol.*, **40**, 588–594.

Faulds, C.B., Pérez-Boada, M., and Martínez, A.T. (2011) Influence of organic co-solvents on the activity and substrate specificity of feruloyl esterases. *Bioresour. Technol.*, **102**, 4962–4967.

Fojan, P., Jonson, P.H., Petersen, M.T., and Petersen, S.B. (2000) What distinguishes an esterase from a lipase: A novel structural approach. *Biochimie*, **82**,1033–1041.

Gilham, D. and Lehner, R. (2005) Techniques to measure lipase and esterase activity in vitro. *Methods*, **36**, 139–147.

Guex, N. and Peitsch, M.C. (1997) SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modelling. *Electrophoresis*, **18**, 2714–2723.

Guncheva, M. and Zhiryakova, D. (2011) Catalytic properties and potential applications of *Bacillus* lipases. *J. Mol. Cat. B: Enzym.*, **68**, 1–21.

Hasan, F., Shah, A.A., and Hameed, A. (2006) Industrial applications of microbial lipases. *Enzyme Microb. Technol.*, **39**, 235–251.

Heinen, W., Lauwers, A.M., and Mulders, J.W.M. (1982) *Bacillus flavothermus*, a newly isolated facultative thermophile. *Antonie Leeuwenhoek*, **48**, 265–272.

Helistö, P. and Korpela, T. (1998) Effects of detergents on activity of microbial lipases as measured by the nitrophenyl alkanoate esters method. *Enzyme Microb. Technol.*, **23**, 113–117.

Hotta, Y., Ezaki, S., Atomi, H., and Imanaka, T. (2002) Extremely stable and versatile carboxylesterase from a hyperthermophilic archaeon. *Appl. Environ. Microbiol.*, **68**, 3925–3931.

Ibrahim, A.S.S. and Ahmed, I.E. (2007) Isolation and identification of new cellulases producing thermophilic bacteria from an Egyptian hot spring and some properties of the crude enzyme. *Aust. J. Basic. Appl. Sci.*, **1**, 473–478.

Jaeger, K.E. and Eggert, T. (2002) Lipases for biotechnology. *Curr. Opin. Biotechnol.*, **13**, 390–397.

Jaeger, K.E. and Reetz, M.T. (1998) Microbial lipases form versatile tools for biotechnology. *Trends Biotechnol.*, **16**, 396–403.

Kambourova, M., Mandeva, R., Fiume, I., Maurelli, L., Rossi, M., and Morana, A. (2007) Hydrolysis of xylan at high temperature by co-action of the xylanase from *Anoxybacillus flavithermus* BC and the β-xylosidase/α-arabinosidase from *Sulfolobus solfataricus* Oα. *J. Appl. Microbiol.*, **102**, 1586–1593.

Kang, Ch. H., Oh, K.H., Lee, M.H., Oh, T.K., Kim, B.H., Yoon, J.H. (2011) A novel family VII esterase with industrial potential from compost metagenomic library. *Microb. Cell Fact*., **10**, 41–48.

Kim, Y.H., Berry, A.H., Spencer, D.S., and Stites, W.E. (2001) Comparing the effect on protein stability of methionine oxidation versus mutagenesis: steps toward engineering oxidative resistance in proteins. *Protein Eng.*, **14**, 343–347.

Kruger, N.J. (2002) The Bradford method for protein quantitation. *In* The Protein Protocols Handbook, 2nd. ed., ed. by Walker, J.M., Humana Press Inc., Totowa, New Jersey, pp. 15–21.

Liu, P., Wang, Y.F., Ewis, H.E., Abdelal, A.T., Lu, C.D., Harrison, R.W., and Weber, I.T. (2004) Covalent reaction intermediate revealed in crystal structure of the *Geobacillus stearothermophilus* carboxylesterase Est30. *J. Mol. Biol.*, **342**, 551–561.

Logan, N.A. and Berkeley, R.C.W. (1984) Identification of *Bacillus* strains using the API system. *J. Gen. Microbiol.*, **130**, 1871–1882.

Montoro-García, S., Martínez-Martínez, I., García-Carmona, F., Navarro-Fernández, J., Takami, H., and Sánchez-Ferrer, A. (2009) Characterization of a novel thermostable carboxylesterase from *Geobacillus kaustophilus* HTA426 shows the existence of a new carboxylesterase family. *J. Bacteriol.*, **19**, 3076–3085.

Narayan, V.V., Hatha, M.A., Morgan, H.W., and Rao, D. (2008) Isolation and characterization of aerobic thermophilic bacteria from the Savusavu Hot Springs in Fiji. *Microbes Environ.*, **23**, 350–352.

Nazina, T.N., Tourova, T.P., Poltaraus, A.B., Novikova, E.V., Grigoryan, A.A., Ivanova, A.E., Lysenko, A.M., Petrunyaka, V.V., Osipov, G.A., Belyaev, S.S., and Ivanov M.V. (2001) Taxonomic study of aerobic thermophilic bacilli: Descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius* and *G. thermodenitrificans*. *Int. J. Syst. Evol. Microbiol.*, **51**, 433–446.

Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S., Harel, M., Remington, S.J., Silman, I., Schrag, J., Sussman, J.L., Verschueren, K.H.G., and Goldman, A. (1992) The α/β hydrolase fold. *Protein Eng.*, **5**, 197–211.

Olusesan, A.T., Kamaruzaman, A.L., Abubakar, F., Hamid, N.S.A., Radu, S., and Saari, N. (2009) Phenotypic and molecular identification of a novel thermophilic *Anoxybacillus* species: A lipase-producing bacterium isolated from a Malaysian hotspring. *World J. Microbiol. Biotechnol.*, **25**,1981−1988.

Park, S.Y., Shin, H.J., and Kim, G.J. (2011) Screening and identification of a novel esterase EstPE from a metagenomic DNA library. *J. Microbiol.*, **49**, 7−14.

Pikuta, E., Lysenko, A., Chuvilskaya, N., Mendrock, U., Hippe, H., Suzina, N., Nikitin, D., Osipov, G., and Laurinavichius, K. (2000) *Anoxybacillus pushchinensis* gen. nov., sp. nov., a novel anaerobic, alkaliphilic, moderately thermophilic bacterium from manure, and description of *Anoxybacillus flavithermus* comb. nov. *Int. J. Syst. Evol. Microbiol.*, **50**, 2109−2117.

Pinzón-Martínez, D.L., Rodríguez-Gómez, C., Miñana-Galbis, D., Carrillo-Chávez, J.A., Valerio-Alfaro, G., and Oliart-Ros, R. (2010) Thermophilic bacteria from Mexican thermal environments: isolation and potential applications. *Environ. Technol.*, **31**, 957−966.

Reetz, M.T. (2004) Controlling the enantioselectivity of enzymes by directed evolution: Practical and theoretical ramifications. *Proc. Natl. Acad. Sci. USA*, **101**, 5716−5722.

Ryu, E. (1938) On the Gram-differentiation of bacteria by the simplest method. *J. Jpn. Soc. Vet. Sci.*, **17**, 31.

Santos, S.F., Zanette, D., Fischer, H., and Itri, R. (2003) A systematic study of bovine serum albumin (BSA) and sodium dodecyl sulfate (SDS) interactions by surface tension and small angle X-ray scattering. *J. Colloid Interface Sci.*, **262**, 400−408.

Schwede, T., Kopp, J., Guex, N., and Peitsch, M.C. (2003) SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Res.*, **31**, 3381−3385.

Sharma, D., Sharma, B., and Shukla, A.K. (2011) Biotechnological approach of microbial lipase: a review. *Biotechnology*, **10**, 23−40.

Smibert, R.M. and Krieg, N.R. (1994) Phenotypic characterization. *In* Methods for General and Molecular Bacteriology, ed. by Gerhardt, P., Murray, R.G.E., Wood, W.A., and Krieg, N.R., American Society for Microbiology, Washington, DC, pp. 607−655.

Soliman, N.A., Knoll, M., Abdel-Fattah, Y.R., Schmid, R.D., and Lange, S. (2007) Molecular cloning and characterization of thermostable esterase and lipase from *Geobacillus thermoleovorans* YN isolated from desert soil in Egypt. *Proc. Biochem.*, **42**, 1090−1100.

Tekedar, H.C. and Şanli-Mohamed, G. (2011) Molecular cloning, over expression and characterization of thermoalkalophilic esterases isolated from *Geobacillus* sp. *Extremophiles*, **15**, 203−211.

Zappa, S., Rolland, J.L., Flament, D., Gueguen, Y., Boudrant, J., and Dietrich, J. (2001) Characterization of a highly thermostable alkaline phosphatase from the euryarchaeon *Pyrococcus abyssi. Appl. Environ. Microbiol.*, **67**, 4504−4511.

Zhang, Z., Schwartz, S., Wagner, L., and Miller, W. (2000) A greedy algorithm for aligning DNA sequences. *J. Comput. Biol.*, **7**, 203−214.

Zuridah, H., Norazwin, N., Siti Aisyah, M., Fakhruzzaman, M.N.A., and Zeenathul, N.A. (2011) Identification of lipase producing thermophilic bacteria from Malaysian hot springs. *Afr. J. Microbiol. Res.* **5**, 3569−3573.