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RESEARCH

Heterologous Expression and Purification of a Heat-Tolerant *Staphylococcus xylosus* Lipase

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Abstract *Staphylococcus xylosus* is a microorganism involved in fermentation of meat products and also a natural producer of extracellular lipases. The aim of the present work was to clone and express in *E. coli* a lipase from *S. xylosus* (AF208229). This lipase gene (1084 bp) was amplified from a *S. xylosus* strain isolated from naturally fermented salami and introduced in pET14b expression vector in order to express the recombinant fusion protein (histidine-tagged lipase) in *E. coli*. Recombinant histidine-tagged *S. xylosus* lipase was purified by affinity chromatography in an HPLC system. The histidine-tagged lipase is a monomer in solution, as determined by size-exclusion chromatography. It presents a high lipase activity at pH 9.0 and 42°C for *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate, among seven different esters assayed ($pNPC_2$, $pNPC_4$, $pNPC_{10}$, $pNPC_{12}$, $pNPC_{14}$, $pNPC_{16}$, $pNPC_{18}$). Moreover, the enzyme presented a quite interesting thermal stability, after an incubation period of 10 min at 95°C, 77% of the initial activity was retained.

Keywords *Staphylococcus xylosus* · Lipase · *E. coli* expression

Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis and synthesis of esters formed from glycerol and fatty acids, being the enzymes with the broadest use in biocatalysis [1]. Despite widely spread in several species, microbial lipases have more industrial potential and are often more useful than enzymes from other sources due to their substrate specificity and ability to remain active in organic solvents, high yields, and ease of genetic manipulation [2]. Moreover, bacterial lipases are preferential enzymes for a wide range of industrial reactions due to its catalytic activity in aqueous and non-aqueous reactions [3]. Lipases act at lipid–water interface and their catalytic activity results from the aminoacid residues triad containing serine, usually occurring in the consensus sequence Gly-X-Ser-X-Gly, Asp/Glu and His. Analyzing the three-dimensional structures of lipases, the presence of a typical α/β-hydrolase fold was observed [4, 5].

Although lipase activity can be defined as the ability to hydrolyze triglycerides, lipases also can catalyze other reactions such as esterification or interesterification. A particular interest relies on lipases capacity of catalyzing such reactions and, consequently, the synthesis of fine compounds used for manufacturing products of high aggregate value, such as trans-free structured lipids [3, 6–8]. Each application requires unique properties with respect to specificity, stability, temperature, and pH-dependence [9].

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In order to use lipases for hydrolysis, esterification, or other applications, it is essential to produce the purified enzyme at high concentrations and to characterize biochemically this enzyme. Only about 2% of world's microorganisms have been tested as enzyme sources until 2006, and lipases from different sources have large variations in enzymatic activity, fatty acid specificity, optimal temperature, and pH [2]. More recently several new lipases have been characterized [10–14].

Staphylococcus xylosus is a coagulase negative *Staphylococcus* species that is commonly isolated from fermented meat products [15–19]. In these products, *Staphylococcus* ssp are responsible for color stabilization, decomposition of peroxides, and aroma formation due to its proteolytic and lipolytic activities [20, 21]. Rosenstein and Götz [6] determined the sequence of nine lipase genes from six *Staphylococcus* species including *S. xylosus* lipase AF208229. All lipases are similarly organized as pre-pro-proteins with pre-regions corresponding to a signal-peptide of 35–38 amino acids, a pro-peptide with hydrophilic characteristics composed of 207–321 amino acids, and a mature peptide containing 383–396 amino acids. Lipases are secreted as pro-proteins, being processed to mature form by specific proteases.

The lipase AF701336 from *S. xylosus* isolated from waste of an oil industry was purified and characterized [22]. Rosenstein and Götz [6] have published the sequence of *gehM* gene corresponding to the lipase AF208229 from *S. xylosus* (DSM 20266 strain); however, this lipase has never been overexpressed or characterized. *S. xylosus* lipase *gehM* expression was studied and it was suggested that an increase in triglycerides content in the growth medium suppresses the expression of this lipase gene [16]. The aim of the present work was to clone, overexpress, purify, and characterize a lipase from *Staphylococcus xylosus* isolated from naturally fermented sausages.

Materials and Methods

Bacterial Strains, Plasmids, and Chemicals

E. coli strain DH5α was used as a host for gene cloning and plasmid propagation, and strain BL21 (DE3) pLysS for protein expression. Plasmid pGEM-T Easy Vector (Promega) was used for sub-cloning and sequencing of the lipase gene, while pET-14b (Novagen) was used as expression vector. Restriction enzymes were purchased from Promega and the HiTrap affinity column from GE Healthcare. The *p*-nitrophenyl esters and isopropyl-L-thio-β-D-galactopyranoside (IPTG) were purchased from Sigma. *S. xylosus* strain was isolated from naturally fermented sausages [23].

PCR Amplification, Cloning, and Sequencing of Lipase Gene

A fragment coding to the mature lipase AF208229 from *S. xylosus* strain was amplified by PCR using primers LIP1 (5′-GCTGCAAAACAAGGACAGTATAA-3′) and LIP2 (5′-TAAGCATCAAATTGCTCGTTACGA-3′). Amplification reactions were performed in a final volume of 25 µl containing 1× PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 mM of each primer, 1 U of Taq DNA polymerase, and 50 ng of template DNA. Amplifications were carried out in a Minicycler™ (MJ Research Inc., Watertown, MA) with the following program: denaturation at 95°C for 5 min; followed by 40 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min; final extension at 72°C for 7 min.

The PCR product was re-amplified using primers LIPET1 (5′-TTCTCGAGCAAGGACAGTATAAAAAC C-3′) and LIPET2 (5′-TTACGGATCCTCAGTAGGATG ATT-3′). Resulting fragment was purified with Concert Rapid PCR kit (Gibco BRL) and inserted into pGEM-T Easy vector (PROMEGA). This plasmid, *pGEM_lip2*, was used for transformation of *E. coli* DH5α.

Assembly and analysis of DNA sequences were performed in a MegaBace1000 (Amersham Biosciences, Piscataway) at the Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Brazil. BLAST was used for database searches. Comparison of the cloned lipase gene with lipase gene AF208229 was carried out using ClustalX (version 2.0.9) software.

Construction of pET14b_lip Expression Vector

DNA fragment obtained from digestion of *pGEM_lip2* vector with *Xho*I/*Sal*I was ligated in frame into pET14b vector previously digested with *Xho*I and dephosphorylated. The insertion of the mature lipase gene was confirmed by restriction analysis with *Eco*RI enzyme. Recombinant plasmid was named *pET14b_lip*. Expressed protein carries N-terminal His_6-tag encoded by the expression vector.

Expression and Purification of Lipase

pET14b_lip was used to transform *E. coli* BL21 (DE3) pLysS strain. Cells harboring the *pET14b_lip* plasmid were inoculated in 10 ml LB broth supplemented with 100 µg ml^{-1} ampicillin and 50 µg ml^{-1} chloramphenicol. Overnight cultures were transferred to 250 ml of the same medium and maintained at 37°C until an OD_{600} of 0.8 was reached. IPTG was added to a final concentration of 1 mM and different culture conditions were tested, two temperatures (30 and 37°C) and two induction periods (5 and 18 h). After induction, cells were harvested by centrifugation

(3000×*g*, 15 min, 4°C) and the pellet was washed once with 50 mM NaH_2PO_4, pH 8.0. Cells were resuspended in Binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and PMSF (40 μg ml^{-1} final concentration) and disrupted by gentle sonication (7 cycles, 20 s) on ice. Cell debris were separated from supernatant by centrifugation (10000×*g*, 20 min, 4°C) in order to obtain the crude extract. The crude extract containing the recombinant lipase was loaded on a HiTrap column (GE Healthcare) previously equilibrated with Binding buffer and connected to an ÄKTA HPLC system (GE Healthcare). Recombinant lipase was eluted with an imidazole gradient ranging from 60 to 500 mM and fractions of 1 ml were collected. Purified lipase was submitted to dialysis (twice) for 24 h against 1 l of 50 mM NaH_2PO_4 buffer, 300 mM NaCl, pH 8.0. Purity and apparent molecular mass were determined by SDS polyacrylamide gel electrophoresis. SDS-PAGE was carried out in gels containing 10% (w/v) polyacrylamide according to standard protocols using Bio-Rad Mini-PROTEAN® system. SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250 and destained with methanol/acetic acid/water (5:1:4 v/v/v). Protein concentration was determined using a Bio-Rad protein assay kit with BSA as standard. Additional experiments of peptide mass fingerprinting by LC/MS of the tryptic digested lipase were performed using UFLC Prominence (Shimadzu Co. Japan) coupled to an HCT Ultra ETD II (Bruker Daltonics, Bremen, Germany) ion trap mass spectrometer.

Determination of Quaternary Structure of the Recombinant Lipase by Gel Filtration

The quaternary structure of recombinant lipase in solution was estimated in triplicate by gel filtration on a Superdex 200 Prep Grade (GE Healthcare) connected to an ÄKTA HPLC System (GE Healthcare). Column was equilibrated with NaH_2PO_4 50 mM buffer, pH 8.0, containing 300 mM NaCl and calibrated with: BSA, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome C, 12.4 kDa; aprotinin, 6.5 kDa. Protein was eluted with the same buffer.

Triglyceride Hydrolysis

The ability to hydrolyze triglycerides was tested in a plate assay. LB medium was supplemented with 2% agar and 2.5% of olive oil and autoclaved. After sterilization, rhodamine B was added to a final concentration of 0.1 mg ml^{-1} and thoroughly blended before pouring into petri dishes. Then, 100 μl of crude extracts from expression tests were applied on plate and incubated at 37°C for 24 h. Triglyceride hydrolysis was identified by fluorescence under UV light at 350 nm.

Lipase Activity Measurement

Enzymatic activities were determined according to Maia et al. [24] using *p*-nitrophenyl butyrate ($pNPC_4$) as substrate. Production of *p*-nitrophenol was continuously monitored at 410 nm (Microplate spectrophotometer TECAN Infinite Series M200) for 60 min in a final volume of 300 μl. In order to carry out the reaction, a stock solution of 8 mM of $pNPC_4$ was prepared in isopropanol and mixed with 50 mM Tris–HCl, pH 8.0, to a final concentration of 1 mM $pNPC_4$. Then, 270 μl of substrate solution was mixed with 30 μl of enzyme solution. Molar absorptivity of *p*-nitrophenol was experimentally determined as 18015.43 M^{-1} cm^{-1}. One unit of lipase was defined as the amount of enzyme that released 1 nmol of *p*-nitrophenol per minute.

Effect of pH and Temperature on Lipase Activity

Effect of pH was investigated by assaying lipase activity at different pH values (7.0, 8.0, and 9.0). The buffers used for pH tests were PIPES (50 mM, pH 7.0), Tris–HCl (50 mM, pH 8.0), and CHES (50 mM, pH 9.0). Reactions were carried out by mixing the appropriate buffer with a stock solution of 8 mM $pNPC_4$ in order to obtain a substrate final concentration of 1 mM. Reactions were initiated by adding 30 μl of enzyme (25 μg) solution to 270 μl of substrate solution. In order to investigate temperature effect, these reactions were carried out at 25, 37, and 42°C in a Microplate spectrophotometer (TECAN Infinite Series M200) as described above. Molar absorptivity of *p*-nitrophenol was experimentally determined for each pH as 9554.33 M^{-1} cm^{-1} (pH 7.0), 18015.43 M^{-1} cm^{-1} (pH 8.0), and 18669.31 M^{-1} cm^{-1} (pH 9.0).

Substrate Specificity

Substrate specificity was assessed using *p*-nitrophenyl esters ($pNPC_2$, $pNPC_4$, $pNPC_{10}$, $pNPC_{12}$, $pNPC_{14}$, $pNPC_{16}$, $pNPC_{18}$) as substrates. A stock solution (8 mM) of each *p*-nitrophenyl ester was prepared in isopropanol. Substrates were emulsified to a final concentration of 1 mM in 50 mM CHES buffer, pH 9.0, containing gum arabic, 1.1 mg ml^{-1} and Triton X-100, 4.4 mg ml^{-1} [25]. The reaction mixture consisted of 270 μl of emulsified substrate and 30 μl of enzyme solution (25 μg). Reactions were carried out at 42°C in a Microplate spectrophotometer (TECAN Infinite Series M200) as described above.

Kinetic Parameters

Lipase activity was measured as a function of various concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 1, and 2 mM) of

$pNPC_4$. Michaelis–Menten kinetic parameters: affinity constant (K_m), maximal velocity (V_{max}), and the turnover number (k_{cat}) were calculated from the experimental data points using GraphPad Prism 5.01 software. Catalytic efficiency (K_m/k_{cat}) was also determined.

Thermostability Assay

The assay to test the thermostability of recombinant lipase was performed by incubating the enzyme solution at 95°C in a water bath. Aliquots were collected after 10, 20, and 30 min and activity measurements were carried out at 42°C, pH 9.0 using *p*-nitrophenyl butyrate as substrate.

CD Spectroscopy

Protein samples were concentrated to 11 μM in 20 mM NaCl, 20 mM NaH_2PO_4, pH 7.4, and scanned at 20°C using a 5 mm path length cell. CD spectra were recorded on a Jasco J-815 spectrometer equipped with a Peltier temperature control unit. The spectrum represents the average of five scans over the range of 195 to 280 nm with a step size of 0.1 nm, bandwidth 2 nm, and scan speed of 50 nm min^{-1}. The appreciative α-helix content of protein was evaluated using the equation provided by Woody [25]:

$$\%\,\text{alpha helix} = \frac{\left(-[\theta]_{222\,\text{nm}} + 3000\right)}{39000}$$

Results

Genomic DNA extracted from *S. xylosus* strain isolated from naturally fermented sausages [23] was used as the PCR template and the fragment flanked by primers LIP1/LIP2 was amplified in order to obtain the sequence corresponding to mature lipase. This fragment was then re-amplified with primers LIPET1/LIPET2 in order to insert the recognition site for *Xho*I restriction enzyme. The fragment corresponding to the mature region of *S. xylosus* lipase was then cloned into pGEM-T Easy vector and this last recombinant plasmid, named *pGEM_lip2*, was propagated in *E. coli* DH5α and used for sequencing. Sequence analysis revealed a nucleotide sequence of 1084 bp with a homology of 99% when compared with lipase sequence AF208229. Deduced amino acid sequence, which corresponds to the mature protein, presented 361 amino acids, a theoretical molecular weight of 40.47 kDa and following identities with other staphylococcal lipases: 80% for lipase from *S. hominis* SK119 (EEK11887.1), 78% for lipase from *S. warnery* (AB189474.1), and 78% for lipase from *S. epidermidis* (AAA19729).

Expression and Purification of Recombinant *S. xylosus* Lipase

In order to clone the fragment corresponding to the mature region of the lipase into the pET-14b expression vector, the *pGEM_lip2* plasmid was digested with *Xho*I/*Sal*I enzymes and the resulting fragment was inserted in pET-14b expression vector. Fragment insertion was confirmed by *Eco*RI digestion and sequencing. The fragment was inserted downstream and in-frame with a coding sequence corresponding to the following amino acid residues: MGG SHHHHHHSSGLVPAGSHMLEQGQYKNQD, where Q24 corresponds to Q1 of the mature lipase. The predicted cleavage site of AF208229 is not present in the recombinant lipase. A coding sequence corresponding to two amino acids, Asp and Pro, were added at the 3′ end of the fragment as a cloning artifact. The resulting recombinant plasmid was named *pET14b_lip*.

BL21 (DE3) pLysS *E. coli* strain was transformed with the expression plasmid *pET14b_lip* in order to express the recombinant lipase with a histidine tag. Different induction periods (5 and 18 h) and temperatures (30 and 37°C) were tested for expression of recombinant lipase. It was observed that the best condition was 5 h of induction at 30°C after the addition of 1 mM IPTG in the growth medium, confirmed by an extra band of approximately 42 kDa in SDS-PAGE (Fig. 1). Recombinant lipase was then purified to homogeneity from cell lysates by metal-affinity chromatography in a HiTrap column connected to an ÄKTA system using an imidazole gradient ranging from 60 to 500 mM. Fusion protein bound to resin was eluted in a total volume of 4 ml when a concentration of 150 mM imidazole was achieved (Fig. 1). Once imidazole interferes on lipase activity measurement by spectrophotometry using *p*-nitrophenyl esters as substrates, it was removed by

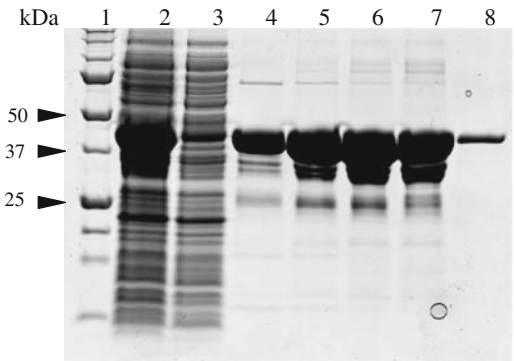

Fig. 1 SDS-PAGE of *S. xylosus* lipase purification in a HiTrap column. *Lane 1* Molecular weight markers, *lane 2* total soluble proteins of *E. coli* BL21(DE3) pLysS expressing *S. xylosus* lipase at 30°C after 5 h of induction, *lane 3* proteins eluted from the column with 60 mM imidazole, *lanes 4–7* eluates containing recombinant lipase, *lane 8* lipase after gel filtration. (15 μl were loaded per lane)

dialysis against phosphate buffer as described in "Material and Methods".

Determination of His-Tagged Lipase Quaternary Structure

In a denaturing SDS-PAGE of the His-tagged lipase, a single band of approximately 42 kDa is observed (Fig. 1). Electrophoretic mobility of His-tagged lipase was consistent with the theoretical molecular mass value (42.9 kDa) predicted for the full-length recombinant protein. The identity of His-tagged lipase was confirmed by mass spectrometry (Fig. 2 and Table 1). Peptide mass fingerprinting of the tryptic digested recombinant lipase yielded at least 16 peptides which represented an overall sequence coverage of 145 residues out of 384 for the intact recombinant molecule in silico digested.

Monomeric state of native His-tagged lipase was determined by comparing its elution profile on a gel filtration column with that of protein size markers (Fig. 3). The recombinant lipase was eluted at 84.16 ml, between the elution volume of carbonic anhydrase (mw 29 kDa) and BSA (mw 66 kDa). From the linear correlation between *elution volume* and *log mw*, the native recombinant lipase can be considered a monomer of approximately 42.6 kDa.

Lipase Activity

Regarding hydrolysis of olive oil by plate assay using rhodamine B, it was observed that crude extracts of *E.coli* harboring recombinant lipase presented ability to cleave this substrate, confirmed by the presence of fluorescence where crude extracts were applied (supplementary data).

Specific activity of recombinant lipase was determined by monitoring the production of *p*-nitrophenol from hydrolysis of *p*-nitrophenyl butyrate ($pNPC_4$) at 410 nm. Purified recombinant lipase presented a specific activity of 1.47 U mg^{-1} at 25°C, pH 8.0.

Effect of pH and Temperature on Lipase Activity

Three pH values (7.0, 8.0, and 9.0) and three temperatures (25, 37, and 42°C) were tested with $pNPC_4$ as substrate in order to determine the pH and temperature dependence on the activity of recombinant *S. xylosus* lipase (Fig. 4). The enzyme is active at pH ranging from 7.0 to 9.0 with an optimal activity at pH 9.0 (4.83 U mg^{-1}), the activity being drastically reduced to 0.88 U mg^{-1} at pH 7.0. Regarding the temperature dependence, *S. xylosus* lipase presented the highest activity at 42°C (Fig. 4). Moreover, the enzyme presented an important thermal stability. Recombinant enzyme was incubated at 95°C for 10, 20, and 30 min. After the heat treatment, lipase activity was measured at 42°C and pH 9.0 using *p*-nitrophenyl butyrate as substrate and the enzyme retained 77% of the initial activity (Fig. 5).

Table 1 Detection by LC/MS of peptides derived from recombinant *S. xylosus* lipase after tryptic digestion

Peptide sequence	Position	Mass (Da)
LNIR	60–63	515.33
AVELYYYIK	80–96	1161.62
GGTVDYGAAHAEK	97–109	1275.59
YGHER	110–114	661.30
TYEGVYK	118–124	859.42
DWQPGK	125–130	730.35
VHLVAHSMGGQTVR	132–145	1491.78
QLEELLR	146–152	900.51
NGNQEEIEYQK	153–163	1351.30
QLAFDYAK	208–215	955.49
VDFGFGQWGLK	224–234	1253.63
VQNSGLWK	248–255	931.50
TEDNGFYDLTR	256–266	1330.30
NTSLNPNIVYK	275–285	1262.67
EWR	324–326	490.24
GVWQVTPIK	355–363	1027.59

Substrate Specificity

Substrate specificity was assessed by testing enzymatic activity against several *p*-nitrophenyl esters of different chain lengths (*p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, *p*-nitrophenyl decanoate, *p*-nitrophenyl laurate, *p*-nitrophenyl myristate, *p*-nitrophenyl palmitate, and *p*-nitrophenyl estearate) at pH 9.0 and 42°C (Fig. 6). *S. xylosus* recombinant lipase presented the highest activity for *p*-nitrophenyl acetate (10.15 U mg^{-1}), followed by

Fig. 2 Amino acid sequence of recombinant *S. xylosus* lipase. Peptides identified by LC/MS are showed *underlined* and *gray boxes*. Mass spectrometry was performed in a UFLC Prominence coupled to an HCT Ultra ETD II ion trap

MGGSHHHHHHSSGLVPAGSHMLEQGQYKNQDPIILVHGFNGFTDDINPAVLAH
YWGGDKLNIRQDLESNGYETYEASVGALSSNYDRAVELYYYIKGGTVDYGAA
HAEKYGHERYGKTYEGVYKDWQPGKKVHLVAHSMGGQTVRQLEELLRNGN
QEEIEYQKEHGGEISPLFQGNNDNMVNSITTIGTPHNGTHAADALGNEAIVRQL
AFDYAKFKGNKNSKVDFGFGQWGLKQREGETYAQYVQRVQNSGLWKTEDNG
FYDLTREGAAKLNKNTSLNPNIVYKTYTGESTRPTLFGNQKSDVNLFLPFTVTG
NVIGKAAEKEWRENDGLVSTISSQHPFNQAFIEATDEVKKGVWQVTPIKHGWD
HVDFVGQDSTDSNHPTE

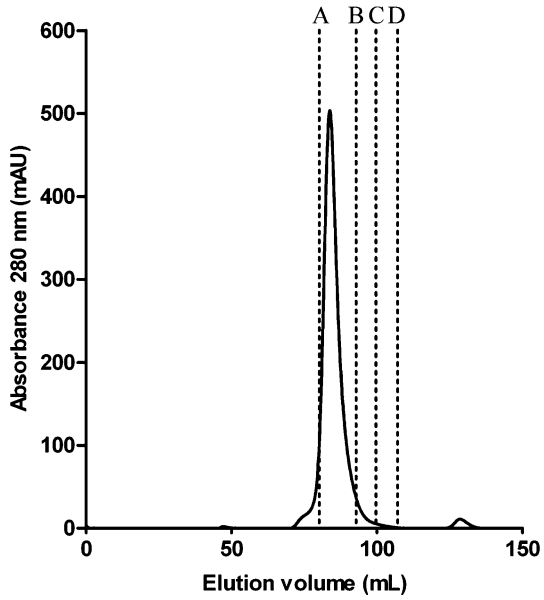

Fig. 3 Gel filtration chromatography of *S. xylosus* lipase on a Superdex 200 Prep Grade column. *Dashed vertical lines* (*A*–*D*) represent molecular markers used for calibrating the column. (*A*) BSA (66 kDa, 80.21 ml), (*B*) carbonic anhydrase (29 kDa, 92.91 ml), (*C*) cytochrome C (12.4 kDa, 99.68 ml), and (*D*) aprotinin (6.5 kDa, 107.09 ml). Elution volume of His-tagged *S. xylosus* lipase was of 84.19 ml

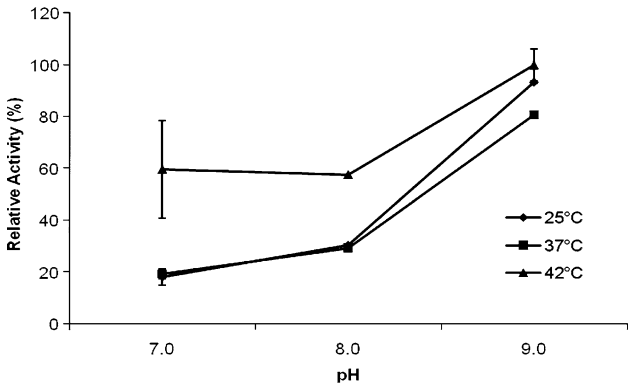

Fig. 4 Effect of pH and temperature on the activity of recombinant *S. xylosus* lipase. Activity assay was carried out with 1 mM *p*-nitrophenyl butyrate as substrate in 50 mM of appropriate buffer at different pH values and temperatures, 25°C (*circles*), 37°C (*squares*), and 42°C (*triangles*). Lipase activity was measured in a microplate spectrophotometer. Results are expressed as percentage of the lipase activity measured at pH 9 and 42°C

p-nitrophenyl butyrate (4.83 U mg^{-1}). The lowest activity resulted when *p*-nitrophenyl estearate was assayed as substrate (0.313 U mg^{-1}).

Kinetic Parameters

Kinetic parameters for recombinant *S. xylosus* lipase were determined by measuring rates of hydrolysis of different

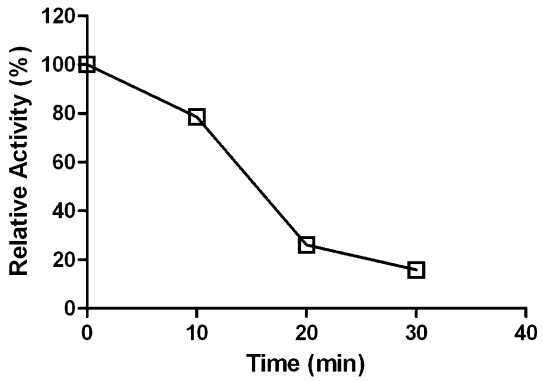

Fig. 5 Thermostability of recombinant *S. xylosus* at 95°C. His-tagged enzyme was incubated at 95°C during 10, 20, and 30 min. After the heat treatment, lipase activity was measured at 42°C and pH 9.0 using *p*-nitrophenyl butyrate as substrate. Results are expressed as percentage of the lipase activity

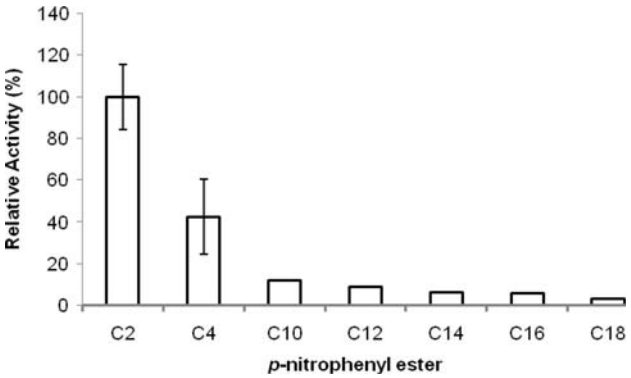

Fig. 6 Relative activity of recombinant *S. xylosus* on *p*-nitrophenyl esters with different chain length at pH 9 and 42°C. Hydrolysis was measured for *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl decanoate (C10), *p*-nitrophenyl laurate (C12), *p*-nitrophenyl myristate (C14), *p*-nitrophenyl palmitate (C16), and *p*-nitrophenyl estearate (C18). Results are expressed as percentage of the lipase activity assayed with *p*-nitrophenyl acetate as substrate

concentrations of $pNPC_4$. Michaelis–Menten data was plotted (Fig. 7) and maximal velocity (V_{max}), affinity constant (K_m), turnover number (k_{cat}), and catalytic efficiency (K_m/k_{cat}) were determined (Table 2).

CD Spectroscopy

S. xylosus recombinant lipase has a typical α-helix profile, the far-UV CD spectrum displayed minimal peaks at 208 nm and 222 nm (Fig. 8). Analysis of the spectrum [26] suggests that the amount of α-helical secondary structures represent a total of 49.65%, which is quite expected for a member of the α/β-hydrolases family, as previously described [5, 27].

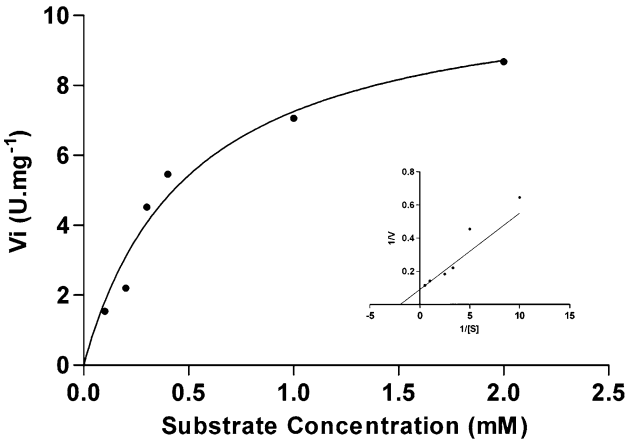

Fig. 7 Michaelis–Menten plot of recombinant lipase from *S. xylosus* determined by measuring release of *p*-nitrophenyl from several $pNPC_4$ concentrations. Inset graph shows the Lineweaver–Burk plot of the transformed data. Assays were performed in 50 mM CHES buffer pH 9.0 at 42°C

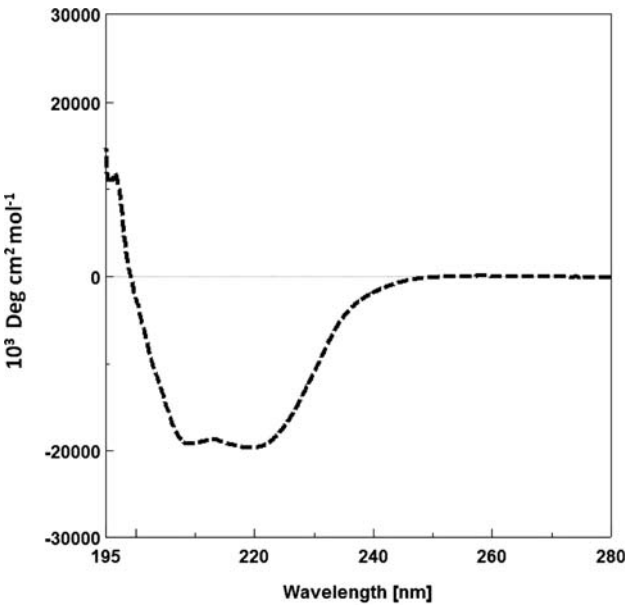

Fig. 8 Circular dichroism spectra of recombinant *S. xylosus* lipase at 20°C. Minima at 208 nm and 222 nm indicate a typical α-helix profile

Table 2 Kinetic parameters of recombinant *S. xylosus* lipase for $pNPC_4$

Specific activity (U/mg)	V_{max} (nmol min^{-1} mg^{-1})	K_M (mM)	k_{cat} (s^{-1})	$kcat/K_M$ (s^{-1} mM^{-1})
7.06	10.93	0.507	0.0078	0.0154

Discussion

In the present work, the fragment corresponding to the mature region of lipase gene from *Staphylococcus xylosus* strain isolated from naturally fermented sausages was cloned, sequenced, and successfully expressed in *E. coli* BL21 (DE3) pLysS. It presented a homology of 99% when compared to the lipase sequence AF208229 cloned from *S. xylosus* DSM 20266 strain [6], this late enzyme was not previously characterized. Activity of lipases is dependent upon a charge substitution system involving an active Ser-Asp/Glu-His triad, called catalytic triad [28]. Besides, the active serine residue of catalytic triad is commonly found in the highly conserved motif Gly-X-Ser-X-Gly [28]. Mosbah et al. [22] cloned and expressed the lipase AF701336 from a *S. xylosus* strain isolated from oil industry waste that presents the complete consensus sequence Gly-X-Ser-X-Gly. Amino acid sequence of *S. xylosus* lipase AF208229 presents 53% identity with the lipase AF701336 [22]. The purified recombinant lipase of *S. xylosus* strain isolated from fermented sausages is a monomer, as determined by size-exclusion chromatography. The lipase AF701336 from *S. xylosus* is also a monomeric protein [22]. Unlike other previously described staphylococcal lipases that presented properties of forming aggregates under native conditions [22], these two *S. xylosus* lipases remain as monomeric proteins in solution.

Lipases are versatile enzymes that express activity of synthesis and hydrolysis besides presenting other esterase type of activities and exhibiting large substrate specificities [29]. Generally, staphylococcal lipases present their highest activities at alkaline pH, with some exceptions such as SAL-1 from *S. aureus* and SEL-3 from *S. epidermidis* [6]. SAL-3 from *S. aureus*, recently characterized showed the highest activity at pH 9.5 and 55°C [30]. Besides, *S. xylosus* lipase AF701336 presented highest activity at pH 8.2 and 45°C [22]. The recombinant *S. xylosus* lipase presented in this work seems to have similar preferences for high pH and high temperature for maximum activity, pH 9.0, and 42°C being the preferred conditions. Besides, it presented quite impressive heat-tolerance when exposed to high temperatures. After 10, 20, and 30 min at 95°C, the enzyme retained 77, 24, and 14% from the initial activity, respectively. Information about the thermostability of staphylococcal lipases is scarce at present, the only examples being lipase from of *S. xylosus* that maintains 50% of its activity after 15 min at 60°C [22] and lipase from *S. saprophyticus*, which maintains 80% of its activity after 15 min at 70°C and 30% after 15 min of boiling [31]. Also, a lipase from *S. simulans* is inactivated after a few minutes at 60°C [32]. In fact, thermostable enzymes have found several applications due to its inherent stability [33, 34] although applications for staphylococcal thermostable lipases have not been reported. An important advantage of conducting biotechnological processes at elevated temperatures is to reduce the risk of contamination by common mesophiles [35]. Besides, elevated temperatures allow also higher reaction rates due to a decrease in viscosity and an

increase in diffusion coefficient of substrates and higher process yield due to increased solubility of substrates and products [34]. Thus, ability of lipases to remain stable at high temperatures is an important and valuable feature for biotechnological applications.

Some staphylococcal lipases, such as SEL3 from *S. epidermidis*, SHaL from *S. haemolyticus*, SAL1 from *S. aureus*, SWL2 from *S. warneri* preferably hydrolyze water-soluble short-chain substrates [6, 31, 36], while SHyL from *S. hyicus* and SXL lipase (AF701336) from *S. xylosus* [22] present no specificity for chain length when hydrolyzing triacylglycerols. Lipase from *S. saprophyticus* presented the same profile of the recombinant enzyme purified in the present work for chain length specificity, with a preference for $pNPC_2$ followed by $pNPC_4$ [31] besides presenting different optimal temperature (30°C) and pH (6.0).

Regarding the kinetic parameters, the low value of K_m (0.5 mM) observed for recombinant lipase of *S. xylosus* shows that this enzyme presents high affinity for the substrate with four carbons in the lateral chain. A lipase from *S. saprophytics* presented a K_m value of 1.47 mM for the same substrate [31] and a recombinant lipase from *S. epidermidis* presented 0.9 mM [37]. The k_{cat} value presented by the recombinant lipase from *S. xylosus* (0.0078 s^{-1}) was lower than recombinant lipase of *S. epidermidis* (25.1 s^{-1}) [37] as well as catalytic efficiency (0.0154 and 28.2 s^{-1} mM^{-1}, respectively).

The α/β-hydrolase fold is characterized by five to eight strands connected by α-helices to form an $\alpha/\beta/\alpha$ sandwich. In most of the family members the β-strands are parallel [38]. The S. *xylosus* recombinant lipase from this work presents a $\sim$47% alpha helix content, compatible with the secondary structure content of the 3D structure determined for *S. hyicus* lipase [5], which presents 3 parallel β-strands and 12 α-helix. The members of this superfamily diverged from a common ancestor into a number of hydrolytic enzymes with a wide range of substrate specificities, together with other proteins with no recognized catalytic activity [38]. Comparing with *S. hyicus* lipase structure, the catalytic triad of *S. xylosus* recombinant lipase is present in loops, one of them, the nucleophilic elbow, is the most conserved feature of the fold, which occurs in the majority of this family members [38].

Esters of short chain fatty acids are known as flavor compounds being widely used in food, beverage, cosmetic, and pharmaceutical industries [39]. There is an increasing preference for "natural" products rather than for chemically synthesized flavors [40] mainly due to the fact that chemical catalysis uses polluting liquid acids as catalysts [41] and leads to dark-colored products and undesired byproducts [42]. Besides, flavor esters produced by enzymatic synthesis may be labeled as "natural" products. Several reports about the production of such interesting compounds by lipases are available [39–41, 43, 44], including *S. warneri*, *S. xylosus* [45], and *S. epidermidis* [42] lipases. We thus propose the recombinant lipase from *S. xylosus* presented in this work to be useful in producing esters of short-chain fatty acids.

Conclusion

A gene coding for *S. xylosus* lipase was successfully cloned and expressed. The amplified sequence obtained in this work presented a 99% similarity with the lipase (AF208229) from *S. xylosus* DSM 20266 strain. This is the first report to show the *S. xylosus* lipase (AF208229) purification to homogeneity, biochemical, and biophysical characterization. The purified histidine-tagged lipase presented high specific activity at pH 9.0 and 42°C for *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate. Also, the recombinant lipase presented an impressive heat-tolerance when submitted to 95°C for 10 min. Further structural and biochemical characterization is necessary to achieve a full understanding of this enzyme and its reaction mechanism, and this will be the subject of future communications.

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