Quim. Nova, Vol. 35, No. 6, 1173-1178, 2012

Artigo

BIOCHEMICAL CHARACTERISATION OF LIPASE FROM A NEW STRAIN OF *Bacillus* sp. ITP-001

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Recebido em 26/9/11; aceito em 24/1/12; publicado na web em 15/5/12

Lipases are characterised mainly by catalytic versatility and application in different industrial segments. The aim of this study was to biochemically characterise a lipase from a new strain of *Bacillus* sp. ITP-001. The isoelectric point and molecular mass were 3.12 and 54 kDa, respectively. The optima lipase activity was 276 U g^{-1} at pH 7.0 and a temperature of 80 °C, showing greater stability at pH 5.0 and 37 °C. Enzymatic activity was stimulated by various ions and pyridine, and inhibited by Cu^{+} and ethanol. The values of K_m and v_{max} were 105.26 mmol and 0.116 mmol min^{-1} g^{-1}, respectively determined by the Eadie-Scatchard method.

Keywords: lipase; biochemical characterisation; *Bacillus* sp. ITP 001.

INTRODUCTION

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are enzymes that not only possess the natural function of hydrolysing triacylglycerol to glycerol and free fatty acids, but also catalyse the synthesis of esters and transesterification under microaqueous conditions.[1,2] Lipases are important biocatalysts because they act under mild conditions, are highly stable in organic solvents, exhibit broad substrate specificity, and typically present high region and/or stereoselectivity in catalysis.[3,4] Consequently, lipases have gained importance and been applied widely in various fields such as foods, pharmaceuticals, detergents, biosensors, biodiesel, cosmetics, fine chemicals and agrochemicals.[5]

In view of their wide variety of applications, there has been renewed interest in the development of sources of lipases. They are widely distributed in nature in different organisms such as fungi, bacteria, plants and animals, and have been studied extensively for their properties. Bacterial lipases are most suitable for industrial applications due to their low price, simple production (since they are mostly extracellular), susceptibility to expression in host microorganisms, diverse specificity, wide range of pH and thermal operational optima.[6] A large number of lipase-producing bacteria have been characterised, including those from *Serratia marcescens,*[7] *Staphylococcus aureus*[8] and *Bacillus stearothermophilus* MC 7.[9] Taxonomically close strains may produce lipases of different types, and due to their interesting biological properties, bacteria of the genus *Bacillus* are attractive candidates for a number of industrial applications. Several species of this group are non-pathogenic, simple to cultivate and secrete enzymes with distinctive structure-function properties of high thermostability and optimal activity.[10]

Bacterial lipase is obtained using fermentation processes comprising three main techniques: submerged fermentation, solid-state fermentation, and immobilised-cell fermentation.[11,12] However, the fermentation process generates, besides the desired components, by-products that frequently hinder the use of fermented broth in industrial procedures. After obtaining the fermented broth, purification strategies must be employed, such as reverse micellar systems, membrane processes, chromatography and aqueous two-phase systems (ATPS) with or without ionic liquids.[13-16] ATPS has been shown to be a powerful technique and offers many advantages as it is environmentally biocompatible, requires low interfacial tension, low energy, scales-up easily, and can operate continuously. The purification processes can change the structural and functional properties of the enzymes, rendering enzymatic characterization an important step in determining the process conditions and application potential.[17]

Generally, lipases have alkaline or neutral optimum pH and demonstrate stability over a wide pH range of 4 to 11. The thermal stability of lipases ranges from 30 to 60 °C, and a larger number are stable in organic solvents.[6] Considering that lipases from different sources display variation in properties especially in terms of regioselectivity, fatty acid specificity, thermostability, optimum pH and kinetics in non-aqueous system, the objective of the present study was to biochemically characterise the lipase produced from a new strain of *Bacillus* sp. ITP-001.

EXPERIMENTAL

Microorganism and culture conditions

Bacillus sp. ITP-001, isolated from petroleum-contaminated soil in a field of petroleum exploration from Carmópolis, Sergipe, Brazil, was used in the present work. The bacterium strain was maintained in YPD medium at 4 ºC. For enzyme production, *Bacillus* sp. ITP-001 was cultivated in a medium consisting of (% w/v): 0.5% KH_2PO_4 (0.1), $MgSO_4.7H_2O$ (0.05), $NaNO_3$ (0.3), yeast extract (0.6), peptone (0.13), starch (2.0), 1% Triton-X 100 and 4% coconut oil added after 72 h of reaction and sterilised in the autoclave at 121 °C for 22 min. The fermentation conditions were initial pH of 7.0; incubation temperature of 37 °C and stirring speed of 170 rpm. At the end of fermentation, the cells were harvested by centrifugation and the supernatant was used for enzyme purification.[14]

Purification of lipase

The purification procedure for lipase from *Bacillus* ITP-001 involved a number of steps. First, the cell-free supernatant was precipitated using ammonium sulphate at 80% saturation and centrifuged at 10,000 rpm for 30 min. Subsequently, the aqueous phase was dialysed using membrane dialysise 25 (cut-off 10,000-12,000 Da) against ultra-pure water for 24 h at 4 ºC. Aqueous two-phase systems were prepared in graduated tubes containing 20% polyethylene glycol (PEG) 8,000; 18% phosphate buffer, formed by a mixture of monobasic and bibasic potassium phosphate at a ratio of 1.087

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(w/w), 6% (w/w) NaCl and completed with the solution of dialyzed enzyme. After homogenisation, the pH value was readjusted to 6.0 and the systems were centrifuged at 3,000 rpm for 20 min and kept at 4 °C overnight. After this treatment, the bottom phase was collected carefully using a pipette, dialysed again, lyophilised (on FreeZone Labconco equipment), and kept at 4 °C.[18]

Lipolytic activity assay

Enzyme activity was assayed using the oil emulsion method according to Soares *et al*..[19] The substrate was prepared by mixing 50 mL of olive oil with 50 mL of Arabic gum solution (7% w/v). The assay mixture, consisting of 5 mL of the substrate, 2 mL of 100 mM sodium phosphate buffer (pH 7.0) and 1 mL of lyophilised enzyme solution (0.1 g/mL) was incubated for 5 min at 37 °C under stirring at 80 rpm. The reaction was terminated by the addition of 3 mL of acetone:ethanol:water (1:1:1) in approximately 0.3 g of reaction, and the amount of liberated fatty acids during incubation was titred with 0.04 N KOH in the presence of phenolphthalein as an indicator. One unit of enzyme activity was defined as the number of μmoles of free fatty acids released per mL per min under the assay conditions. All enzymatic activity assays were replicated at least three times.

Determination of protein

The protein concentration was assayed by the method of Bradford *et al*.[20] using BSA as the standard.

Isoelectric point

The zeta potential of purified lipase was determined in a KCl solution (10 mM) with pH values adjusted (2-6) using 0.1 M HCl and 0.1 M KOH. The electrode chamber was filled with the enzyme solution and the charge of each enzyme solution monitored to determine zero charge. Experiments were performed at 25 °C and the isoelectric point corresponded to the pH at which the charge was zero.

Polyacrylamide gel electrophoresis

The molecular mass and purity of lipase were analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[21] These standards (bovine albumin: 66 kDa; ovalbumin: 45 kDa; glyceraldehyde-3-phosphate Dehydrogenase: 36 kDa; carbonic anhydrase: 29 kDa; trypsinogen: 24; trypsin inhibitor: 20.1; αlactalbumin: 14.2) were used as protein markers in the method. Proteins were visualized by staining using the silver stain procedure.

pH and temperature optima and stability

The effect of pH on the activity of the purified lipase was determined by incubating the purified enzyme at pH 2.0-10.5. The buffers used were 0.1 M citric acid-sodium citrate (pH 2.0-5.0), 0.1 M potassium phosphate (pH 6.0-8.0) and 0.1 M bicarbonate-carbonate (pH 8.0-10.5). The pH stability was evaluated over the 5 h incubation period (sampling every 1 h) of the purified enzyme in the appropriate buffers at different pH values (5.0–8.0) at 37°C. The optimal temperature for activity of the enzyme was determined at 25-120°C in the same 0.1 M potassium phosphate buffer (pH 7.0). Thermostability of the purified enzyme was investigated over 5 h (sampling every 1 h) by pre-incubating the enzyme at different temperatures (37, 50, 65 and 80 °C) in buffer of pH 5.0. The remaining activities of lipase were measured immediately after this treatment with the standard method as outlined above.

Effect of different metal ions and organic solvents on lipase

To examine effects of different metal ions on lipase activity, the purified lipase was pre-incubated at 37 °C in citrate buffer (0.1 M, pH 5.0) for 1 h with various metal ions at a final concentration of 0.1, 1.0 or 10 mM. The ions tested included $CoCl_2$, $CuCl_2$, EDTA, $FeCl_3$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$. To study the influence of $CaCl_2$, the purified lipase was pre-incubated for 30 h at concentration of 0.1, 1.0 and 2.0 mM. The effect of some organic solvents on enzyme activity was determined by the addition of 10, 20 and 30% acetone, acetonitrile, ethanol, isopropanol, methanol and pyridine for 3 h at 37 °C in the buffer (pH 5.0). The lipase activity in the presence of metal ions and organic solvents was compared with the control in the absence of metal ions and organic solvents, whose activity was taken as 100%.

Esterification potential

The syntheses of esters were conducted in closed flasks containing 20 mL of heptane and different alcohols (ethanol, methanol, propanol, butanol, iso-amyl and octanol) and carboxylic acids (acetic, lauric, palmitic, and oleic acid) with a fixed molar ratio (1:1.5).[22] The mixtures were incubated with 0.1 g of lipase from *Bacillus* sp. ITP-001. All reactions were performed at 37 °C under constant stirring of 150 rpm for a period of 24 h. Aliquots of 1 mL were removed from the reaction medium at the initial and final timepoints, introduced to 5 mL of acetone:ethanol (1:1) and titrated with NaOH (0.1 N), to determine the concentration of the starting materials consumed. All enzymatic activity determinations were replicated at least three times. The results were calculated by Equation 1.

$$Conversion\ (\%) = \frac{VxNxM}{W} \quad (1)$$

where, M: molecular weight of titrated fatty acid (mol); N: normality of NaOH solution; V: volume of NaOH (mL) used; W: mass of aliquot titrated (g).

Determination of kinetic constants

K_m and v_{max} of the lipase were determined by measuring enzyme activity with various concentrations of olive oil substrate (1-70%, w/v) over 60 min. Kinetic constants were calculated using the linearization methods of Lineweaver-Burk, Hanes-Wolf, Eadie-Scatchard and Wolf-Augustinisson-Hofstee, as well as fitting to a non-linear model. The degree of fit of the models to the experimental data was based on the magnitude of the mean relative error (*P*) and estimated error (SE), calculated by Equations 2 and 3, respectively.

$$P = \frac{100}{n} \sum_{i=1}^{n} \frac{m_{\exp}\ m_{pre}}{m_{\exp}} \quad (2)$$

where m_{exp} and m_{pre} are experimental and predicted units, respectively, and n is the number of observations.

$$SE = \sqrt{\left[\left(\Sigma\ (Y\ \hat{Y})^2\right) / GLR\right]} \quad (3)$$

where Y and $\hat{Y}$ are experimentally observed values and values calculated for the model, respectively, and GLR is the degrees of freedom model.

RESULTS AND DISCUSSION

Lipase production and purification

The purification of lipase from *Bacillus* sp. ITP-001 involved

Table 1. Purification of lipase produced from *Bacillus* sp ITP-001 using several processes

Purification step	Lipolytic activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg)	Purification factor (fold)
Crude culture	6,352	1.20	5,297	1
$(NH_4)_2SO_4$ precipitation	101,413	0.12	826,180	155.98
ATPS (Bottom phase)	48,274	0.04	1,067,486	201

ammonium sulphate precipitation followed by an aqueous two-phase system (ATPS), as summarised in Table 1. After the addition of ammonium sulphate (80% saturation), it was noted that proteins were preferentially precipitated, while lipases remained in the aqueous solution. This phenomenon was possibly due to the molecular weight of lipase or its greater affinity for the aqueous phase. Several authors, including Bacha *et al*.,[23] have reported that enzymes precipitate after the addition of salt.

Purification in the aqueous two-phase systems (ATPS) partitioned lipase to the bottom phase with a purification factor of 201 fold and the highest specific activity (48,274 U/mL).[18] Souza *et al*.[14] used this same system to purify commercial porcine pancreatic lipase and verified a 3.89-fold purification factor with specificity activity of 47.09 U/mL. The bottom phase was dialysed against ultra-pure water, lyophilised at a temperature of -50 °C and pressure of 1.2 x 10^{-2} mbar for 48 h. The purified lipase showed activity of 175 U/g, p*I* of 3.12 and produced a single band on SDS-PAGE electrophoresis with an apparent molecular mass of approximately 54 kDa (Figure 1S, supplementary material). According to Nthangeni *et al*.,[24] lipases from generous *Bacillus* normally have a p*I* of between 6.3 and 9.4.

Effect of pH and temperature on activity and stability

Maximum lipase activity was obtained at pH 7.0 and 80 ºC, while this enzyme activity decreased at strongly alkaline pH and temperatures above 90 ºC (Figure 1). Kim *et al*.[25] studied the influence of pH range (6.0 to 10.0) on lipase activity from *Bacillus pumilus* B26, with results similar to the present work. Moreover, Gupta *et al*.[26] reported that this pH range is optimal for lipases from other microorganisms such as *Acinetobacter calcoaceticus*, *Pseudomonas* sp., *P. cepacia* and *Staphylococcus aureus*. There are also some reports on the optimal pH (10.0) for lipases from generous *Bacillus*.[27]

With regard to the effect of temperature on lipase activity, for most cases, optimal activity has been found between 30 and 65 ºC[23,28] or in some cases, at higher temperatures of 75 ºC.[9] Thermal tolerant lipases are very promising for industrial applications, since processes performed at high temperatures increase the reaction rate, for example in detergent formulations and other biotransformations.[3]

The pH and temperature stability were measured at 5 h of pre-incubation of the purified lipase, with sampling every 1 h, in appropriate buffers at different pH (5.0 to 8.0) and temperature (37 to 80 ºC) values. *Bacillus* sp. ITP-001 was most stable at pH 5.0 and at 37 °C, as shown in Figure 2. This relative activity profile was similar to results obtained by Pastore *et al*.[29] using the lipase produced by a new strain of *Rhizopus* sp., in which greater stability was found at 40 ºC, in contrast to the results reported by Sharma *et al*.[30] and Horchani *et al*.,[8] in which lipase was more stable at alkaline pH (9.5 and 9.0, respectively). Guncheva and Zhiryakova[31] reported that most *Bacillus* lipases are stable in neutral to moderately alkaline media (pH = 7.5-9.0), while a number of enzymes have a significantly higher pH optimum: *B. subtilis* FH5, *B. licheniformis* strains H1 and RSP-09, *B. alcalophilus* B-M20, *B. subtilis* lipA and lipB.

These results failed to show a rapid loss of activity and therefore the half-lives ($t_{½}$) of lipases from *Bacillus* sp. ITP-001 were incubated for 24 h at 80 ºC, 32 days at 65 ºC and 71 days at 50 ºC. Interestingly,

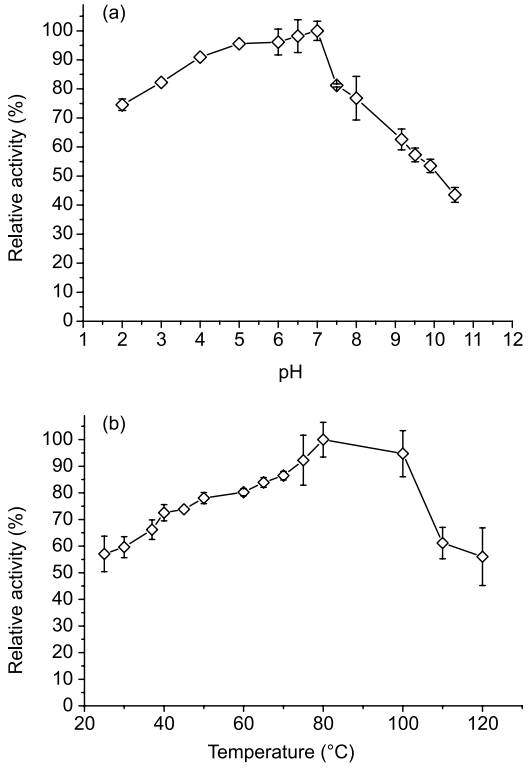

Figure 1. *Effect of different pH values at 37 ºC (a) and temperatures at pH 7.0 (b) on the activity of lipase from Bacillus sp. ITP-001*

the lipase retained 69% of its original activity at 37 °C for up to 100 days. The half-life of lipase from *Bacillus* sp. ITP-001 was longer than that reported for other lipases, e.g. by Kambourova *et al*.[9] ($t_{½}$ = 3 h at 70 ºC) and by Demir and Tükel[2] ($t_{½}$ = 30 days at 25 ºC).

Table 2 shows a comparison of optimal temperatures and pH values of several lipases from different microorganisms. Most lipases have the same features, particularly with regard to the optimum pH for activity and thermal stability. Thermophilic lipases often show higher resistance to chemical denaturation, making them ideal tools in industrial and chemical processes where relatively high reaction temperatures and/or organic solvents are used. The elevated reaction temperature provides a higher conversion rate, minimal risk of microbial contamination, higher solubility of the substrates and lower viscosity of the reaction medium, favouring mass transfer.[6]

Effect of Ca^{2+} on activity and stability

The influence of Ca^{+2} ions on lipase activity was studied, as shown in Figure 3. At low concentrations of calcium ions, there was a relative activity profile similar to the control (without Ca^{+2} ions) with decreased relative activity. However, the relative activity of lipase was enhanced by approximately 10% (4 h) and 23% (12 h), when the concentration of Ca^{+2} ions increased to 1.0 and 2.0 mM. After this time, the relative activity was negatively influenced by the presence of Ca^{+2} ions. According to Gupta *et al*.,[26] calcium also

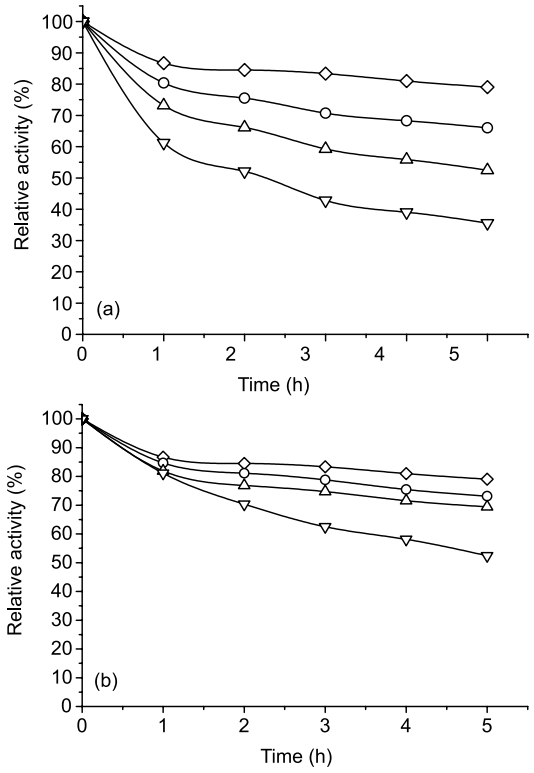

Figure 2. *Effect of pH [Diamond (5.0), Circle (6.0), Up triangle (7.0), Down triangle (8.0)] at 37 °C (a) and temperature [Diamond (37 °C), Circle (50 °C), Up triangle (65 °C), Down triangle (80 °C)] at pH 5.0 (b) on the stability of lipase from Bacillus sp. ITP-001*

stimulated the activity of lipases from different microorganisms (*B. subtilis* 168, *B. thermoleovorans* ID-1, *P. aeruginosa* EF2, *S. aureus* 226, *Candida viscosum* and *Acinectobacter* sp. RAG-1). The increase in lipase activity due to calcium induction can be attributed to the vital role played by increased Ca^{2+} in building a stable catalytic enzyme structure as a result of calcium ions binding to the internal structure of the enzyme, thereby changing the solubility and behaviour of the ionised fatty acids at interfaces.[32]

The positive influence of calcium has been observed by many researchers, e.g. Ghanem *et al*.,[27] who reported an increase of 150% in lipase activity in the presence of 50 mM Ca^{2+}, and Yu *et al*.,[33] who reported a 4-fold increase in lipase activity using 2 μmol L^{-1} Ca^{2+}. However, in both these cases the enzyme's thermostability was not good. Conversely, Côté and Shareck[34] did not achieve significant effects with 10 mM of Ca^{2+} using two lipases from *Streptomyces coelicolor* A3[2].

Effect of metal ions and solvents on lipase

The effects of other ions and solvents on the purified lipase were

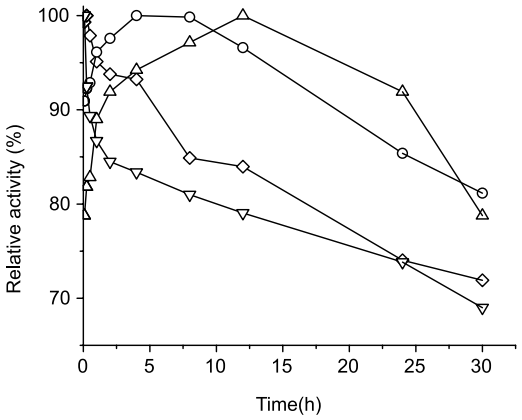

Figure 3. *Effect of different concentrations of Ca^{2+} [Diamond (0.1 mM), Circle (1.0 mM), Up triangle (2.0 mM), Down triangle (Standard at 37 °C and pH 5.0)] on the lipase of Bacillus sp. ITP-001*

evaluated as shown in Tables 3 and 4, respectively, where values are averages of three assays and have coefficients of variation less than 5%. The lipase was stimulated by Co^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+} and Zn^{2+} ions and inhibited by Cu^{2+}. Gaur *et al*.[28] used lipase from *P. aeruginosa* that demonstrated a decrease in activity of 30 and 49% in the presence of 1 mM of Cu^{2+} and Zn^{2+} for 30 min, respectively. The chelating agent EDTA did not affect the lipase. Kumar *et al*.[35] reported that lipase from *Bacillus coagulans* BTS-3 was inactive in the presence 1 mM of Co^{2+}, Mn^{2+} and Zn^{2+}, but was enhanced by K^{+}, Fe^{3+}, Hg^{2+} and Mg^{2+}. Two mechanisms of ion action have been described. One is direct action at the catalytic site, as with many other enzymes whereas another is specific action resulting in the formation of complexes between the metal ions and ionised fatty acids in a manner that changes their solubility and behaviour at interfaces.[5]

The effect of solvents on lipase activity is shown in Table 4, where all values were determined in triplicate with a coefficient of variation of less than 5%. Except for pyridine, all solvents acted as inhibitors, especially ethanol, isopropanol and acetone, which in high concentrations reduced activity by approximately 30, 20 and 12%, respectively. Interestingly, none of the solvents inactivated the lipase completely, possibly due to low penetration of the solvent in the enzyme structure compared with hydrated solvents. Results reported by Wang *et al*.[1] using lipase from *Burkholderia cepacia* ATCC 25416 in the presence of 30% (v/v) acetone, chloroform, n-hexane, cyclohexane and n-heptane in the reaction mixture for 2 h at 37 °C showed activity increases of 1.7, 1.8, 1.7, 1.7 and 2.0-fold, respectively.

One valuable feature of the lipase from *Bacillus* sp. ITP-001 is its incomplete inactivation in the presence of solvents and increased enzyme activity in the presence of metallic ions, which enhances the potential for industrial employment of this enzyme. Table 5 shows the effect of ions on the activity of lipases from different sources.

Table 2. Optimal temperature and pH of some lipases from different microorganisms

Microorganism	Optimum temperature (ºC)	Optimum pH	pH stability	Thermo stability (ºC)
P. aeruginosa LX1[37]	40	7.0	11.0	50
P. gessardii[32]	30	6.0	5.0	30
Nomuraea rileyi MJ[5]	35	8.0	7.0	4
Spirulina platensis[2]	45	6.5	-	25
Bacillus coagulans BTS-3[35]	55	8.5	8.5	70
Bacillus sp. ITP-001	80	7.0	5.0	37

Table 3. Effects of additives on the activity of the purified enzyme

Additives	Relative activity (%) at concentration (mM)		
	0.1	1	10
Co^{2+}	103.4	106.8	120.4
Cu^{2+}	101.8	93.7	82.4
EDTA*	99.8	100.0	104.9
Fe^{3+}	121.5	121.8	129.9
Mg^{2+}	131.3	152.1	187.4
Mn^{2+}	116.7	132.1	139.0
Zn^{2+}	131.3	142.4	153.9

*chelating agent

Table 4. Effects of different concentrations of solvents on lipase activity

Solvents	Relative activity (%) at concentration (%, v/v)		
	10	20	30
Isopropanol	98.3	83.4	79.5
Ethanol	96.5	79.0	69.8
Methanol	98.3	97.1	96.9
Acetone	94.9	91.7	88.0
Acetonitrile	94.5	93.5	93.5
Piridine	98.3	103.6	110.5

Table 5. Metallic ion and EDTA sensitivity of microbial lipases

Microorganism	Activator	Inhibitor
Burkholderia cepacia[1]	Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}	EDTA
Metarhisium anisopliae[36]	Ca^{2+}, Mg^{2+}, Co^{2+}	Mn^{2+}
Spirulina platensis[2]	Ca^{+2}, Mg^{+2}, Zn^{+2}	Li^{+1}, Fe^{+2}, Mn^{+2}, EDTA
Pseudomonas gessardii[32]	Ca^{2+}, Mg^{2+}	K^{+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, EDTA
Bacillus sp. ITP-001	Ca^{2+}, Co^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Zn^{2+}	Cu^{2+},

EDTA (Ethylenediamine tetraacetic acid)

Kinetic parameters

Table 6 shows values of K_m and v_{max} for different methods of kinetic parameters determined using olive oil as a substrate. No significant difference in the v_{max} was noted but K_m values were strongly influenced. Based on the P and SE values (mean P lower than 10%), the model of Eadie-Scatchard proved the best fit overall for K_m and v_{max}, at 105.26 mmol and 0.116 mmol $min^{-1} g^{-1}$, respectively.

Figure 2S, supplementary material, depict the experimental data of purified lipase from *Bacillus* sp. ITP-001 compared with adjusted non-linear and Eadie-Scatchard data from Silva *et al*.[36] on the kinetic parameters of lipase from *Metarhizium anisopliae* for σ-nitrophenyl palmitate and ρ-nitrophenyl laurate substrates, and the K_m and v_{max} values of 0.474 mmol and 1.093 mmol $min^{-1} mg^{-1}$ and 0.712 mmol and 5.969 mmol $min^{-1} mg^{-1}$, respectively. Horchani *et al*.[8] determined the kinetic parameters of lipase from *Staphylococcus aureus* in different substrates (tributyrin, triolein and trioctanoin) and obtained K_m and v_{max}: 4.93 mmol and 7.774 mol $min^{-1} mg^{-1}$, 7.87 mmol and 2.803 mol $min^{-1} mg^{-1}$ and 14.53 mmol and 1.485 mol $min^{-1} mg^{-1}$, respectively.

Table 6. The values of mean relative error (*P*), estimated (SE) and kinetics parameters (K_m and v_{max}) using olive oil as a substrate

Methods	K_m (mmol)	v_{max} (mmol $min^{-1} g^{-1}$)	P	SE
Lineweaver-Burk	91.76	0.118	9.70	0.0103
Hanes-Wolf	29.68	0.101	17.49	0.0124
Eadie-Scatcharel	105.26	0.116	7.98	0.0113
Wolf-Augustinisson-Hofstee	76.85	0.110	8.87	0.009
Non-linear	57.76	0.107	10.87	0.008

Esterification potential

The ability of lipase from *Bacillus* sp. ITP-001 to catalyse an esterification reaction using various fatty acids and alcohols is shown in Figure 4. After 24 h of reaction, the best conversions were obtained by using acetic acid and methanol. This indicates that the lipase from *Bacillus* sp. ITP-001 has greater affinity for small carbon chains. Chen *et al*.[37] isolated seven strains of the genus *Bacillus* (three strains of *B. stearotheromophilus* and *B. licheniformis*, and one of *B. subtilis*) and assessed the potential reaction of different carbon chain lengths. The substrates used were p-nitrophenol butyrate (C_4), p-nitrophenol caproate (C_6), p-nitrophenol caprylate (C_8), p-nitrophenol caprate (C_{10}), p-nitrophenol laurate (C_{12}), p-nitrophenol myristate (C_{14}), p-nitrophenol palmitate (C_{16}) and p-nitrophenol stearate (C_{18}). With the exception of one strain of *B. licheniformis*, which showed greater activity for medium carbon chains (C_{10}), the lipolytic activity of lipases was higher in short chain esters in the hydrolytic reaction. In the results reported by Gomes *et al.,*[22] the maximum conversion

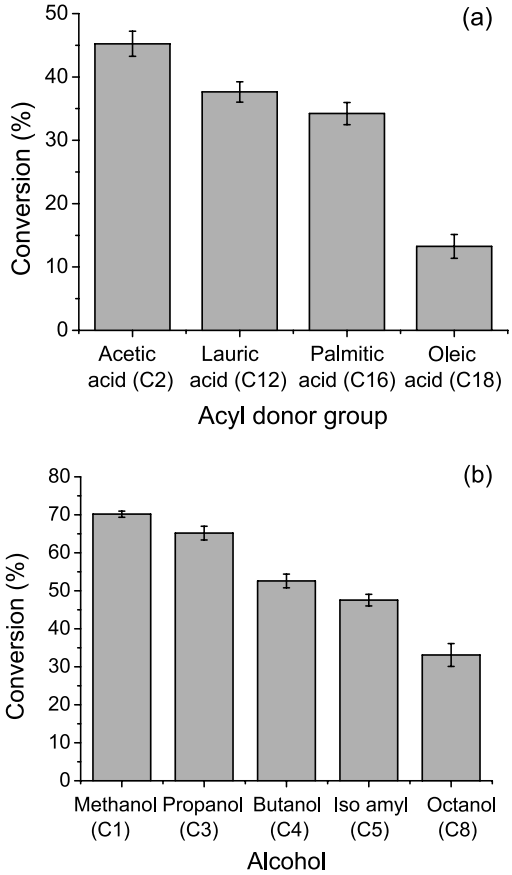

Figure 4. *Influence of alkyl chains of fatty acids (a) on the degree of esterification of ethanol (0.30 M), and of alkyl chains of alcohols (b) on the degree of esterification of acetic acid (0.45 M)*

using lipase from immobilized *Candida* was achieved for the system butanol and octanoic acid (73.9%) and the reduction of the conversion observed for the system butanol and lauric acid (12 carbons), also suggesting the negative effect of carbon chain size, which restricts the access of reagents to the active site of the enzyme. An application of the esters produced from short-chain fatty acids is as flavoring agents in the food industry.[6]

CONCLUSIONS

In this study, the lipase from *Bacillus* sp. ITP-001 was purified around 201 fold using ATPS and showed activity of 276 U g^{-1} at pH 7 and a temperature of 80 ºC. The p*I* and molecular mass of the purified lipase were 3.12 and 54 kDa, respectively. The lipase showed high stability and activity at pH 5.0 and a temperature of 37 ºC. Values of K_m and v_{max} were 105.26 mmol and 0.116 mmol min^{-1} g^{-1}, respectively. The high tolerance against organic solvent along with the positive effect of the majority of the metal ions investigated makes the lipase from *Bacillus* sp. ITP-001 a very attractive enzyme for potential application in industry.

SUPPLEMENTARY MATERIAL

Available at http://quimicanova.sbq.org.br, in the form of a PDF file, with free access. The electrophoresis of the purification process of the enzyme described in this work is presented (Figure 1S), in addition to comparisons between the calculated and experimental values for the reaction velocity at different substrate concentration using the Eadie-Scatchard model (Figure 2S).

ACKNOWLEDGEMENTS

The authors acknowledge financial assistance from CNPq, CAPES and UNIT.

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