

Partial purification, immobilization and preliminary biochemical characterization of lipases from *Rhizomucor pusillus*

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ABSTRACT

Lipases have important applications in biotechnological processes, motivating us to produce, purify, immobilize and perform a biochemical characterization of the lipase from *Rhizomucor pusillus*. The fungus was cultivated by solid state fermentation producing lipolytic activity of about 0.5 U/mL(4U/g). A partial purification by gel filtration chromatography in Sephacryl S-100 allowed obtaining a yield of about 85% and a purification factor of 5.7. Our results revealed that the purified enzyme is very stable with some significant differences in its properties when compared to crude extract. The crude enzyme extract has an optimum pH and temperature of 7.5°C and 40°C, respectively. After purification, a shift of the optimum pH from 7 to 8 was observed, as well as a rise in optimum-temperature to 60°C and an increase in stability. The enzyme was immobilized on CNBr-Agarose and Octyl-Agarose supports, having the highest immobilization yield of 94% in the second resin. The major advantage of immobilization in hydrophobic media such as Octyl is in its hyper activation, which in this case was over 200%, a very interesting finding. Another advantage of this type of immobilization is the possibility of using the derivatives in biotechnological applications, such as in oil enriched with omega-3 as the results obtained in this study display the hy-

drolysis of 40% EPA and 7% DHA from sardine oil, promising results compared to the literature.

Keywords: Enzyme Immobilization; Solid State Fermentation; Purification; n-3 Polyunsaturated Fatty Acids; *Rhizomucor pusillus*

1. INTRODUCTION

Lipases are among the most used enzymes in organic chemistry due to their high versatility [1-3], recognizing a broad range of substrates and being used in a very wide range of reactions—from synthesis of structured oils in food technology with fish oil enrichment of polyunsaturated fatty acids (PUFAs), to fine chemicals resolution via hydrolytic reactions [4-7]. This diversity of reactions makes lipase derivatives be employed in a wide range of reaction media: from fully aqueous media or water/co-solvent systems to anhydrous systems [1-3,5-8]. This makes the adsorbed lipases be exposed to very different medium conditions, and makes it very interesting to compare different reversible immobilization approaches, useful for each specific application. In this paper, we study different strategies for immobilization by hydrophobic and covalent supports as well as characterization of lipase from *Rhizomucor pusillus*.

Enzymes, due to their high selectivity, specificity and activity under mild conditions may be a good option as catalyst for a sustainable chemistry [4,9,10]. However, they are biological catalysts and their features need to be improved for most industrial applications [11-13]. One

of these requirements is the convenience of preparing a heterogeneous catalyst, that is, to have the enzyme in an immobilized form [14-16]. Among the existing immobilization strategies, the physical adsorption of the enzyme on suitable supports is a very simple one, just by mixing the enzyme and the support, the enzymes become rapidly immobilized, usually with low impact on the enzyme activity [17,18]. This produces a reversible immobilization, and makes reuse of the supports possible after enzyme inactivation, thus, reducing the cost of the production of the biocatalysts [17,18]. However, this reversibility may be a problem because the enzyme could desorb from the support during operation, reducing the range of conditions where the immobilized enzymes may be used.

Moreover, based on the particular mechanism of action of lipases, there is one specific immobilization strategy for lipases which is the use of hydrophobic supports. Lipases present two very different structures, changing between a closed form, where the active centre is secluded from the medium by an oligopeptide chain called flap or lid, and an open form where this lid is shifted and the active centre becomes accessible to the substrate [19-21]. When in contact with hydrophobic surfaces, such as oil drops, the open form of lipase is adsorbed, involving the large hydrophobic surface formed by the inner side of the lid and the active centre [22,23]. Thus, in the specific case of lipases, one useful immobilization technique is the interfacial activation of the lipase by adsorption of the open form of the enzyme on hydrophobic supports at low ionic strength [19-29].

This simple strategy allows the preparation of hyper-activated lipase preparations, since the open form of the enzyme is stabilized (at least when acting against hydrophobic substrates).

On the other hand, it has been described that the specificity and selectivity of lipases may be greatly altered by the immobilization protocol, due to the conformational changes that the lipases suffer during catalysis [22,23,28] giving, as final result, an immobilized enzyme with very different catalytic properties [14]. Therefore, it is important to evaluate both the activity and stability of the immobilized enzyme before selecting the optimal biocatalyst.

2. MATERIALS AND METHODS

2.1. Preparation of the Fungus by Solid-State Fermentation (SSF)

R. pusillus was preserved on PDA slants. Inoculum was by growing the fungus for 3 days at 45°C. Solid state fermentation (SSF) was performed in polypropylene bags (21 cm × 12 cm) containing solid substrate (wheat bran), a spore suspension of (5×10^5 colonies forming units) and standard nutrient solution, containing 20 g·L⁻¹

peptone, 2 g·L⁻¹ of K₂HPO₄ and 0.5 g·L⁻¹ of MgSO₄. The crude enzyme extract was obtained by addition of 10 mL of distilled water per gram of fermented material, stirred for 30 min, filtered, and centrifuged at 10,000 g, at 6°C. Finally, the supernatant was used as a crude enzyme solution for the preliminary biochemical characterization and immobilization.

Samples were withdrawn every 24 hours for 5 days and then their enzymatic activities and protein concentration were determined.

2.2. Determination of Lipase Activity

These experiments were carried out by triplicate and the standard error was under 5%.

2.2.1. p-Nitrophenylpalmitate (p-NPP)

A modified method of [30] was used for soluble and partial pure enzyme. The synthetic substrate used was *p*-nitrophenylpalmitate (*p*-NPP), and absorbance was read in a Varian Cary 100 spectrophotometer at 410 nm

For the assay was used 0.05 M sodium phosphate buffer (pH 7,0), incubation proceeded at 45°C for 1 min, and the reaction was stopped with 0.5 mL of a saturated solution of sodium tetraborate (Na₂B₄O₇). The molar extinction coefficient of *p*-NP ($\epsilon = 3.4 \times 10^3 \text{ mol} \cdot \text{L}^{-1}$) was used to calculate the initial rate. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 mmol of *p*-NP (*p*-nitrophenol) per minute. For the partially purified enzymes the assays were performed at the optimum conditions: pH 8.5 and 55°C.

2.2.2. p-Nitrophenyl Butyrate (p-NPB)

The analysis of the activities of the soluble lipases and their immobilized preparations were performed in a quartz cuvette and analyzed spectrophotometrically by measuring the increase in absorbance at 348 nm (extinction molar coefficient = $5150 \text{ M}^{-1} \cdot \text{cm}^{-1}$) using 0.4 mM pNPB in 25 mM sodium phosphate buffer at pH 7,0 and 25°C. Enzyme activity is given as μmol of *p*-NP produced per minute per mg of enzyme (U) under the conditions described earlier.

2.2.3. Biochemical Characterization of the Crude Extract and Partially Pure Enzyme

1) Determination of the Optimum pH

The effect of pH on the enzyme activity was determined at 45°C in suitable buffer and pH ranges of 3 - 10 (sodium citrate 0.05 M at pH 3, sodium acetate 0.05 M at pH 4.0 - 5.0, sodium phosphate 0.05 M at pH 6.0 to 8.0 and glycine-NAOH 0.05 M at pH 9 - 10).

2) Determination of pH Stability

The effect of pH was tested at 45°C, checking the enzyme activity every 30 minutes during 4 hours. The

samples were kept in different buffers from pH 6.5, to 9.5 respectively. The enzyme assays were performed as described earlier.

3) Determination of the Optimum Temperature

The optimum temperature of enzyme activity was determined at temperature ranging from 25°C to 70°C in sodium phosphate 0.05 M pH 7.5.

4) Determination of Thermal Stability of the Partially Purified Lipases

The thermostability was measured by incubating 4 mL of enzyme extract at pH 8.5 and 55°C, the optimum values for the partially purified enzymes. Samples were withdrawn at chosen intervals, cooled and residual activity measured at optimum pH and temperatures.

2.3. Determination of Protein Concentration

Protein concentration was determined as proposed by Bradford [31], using bovine serum albumin as a standard.

2.4. Gel Filtration Chromatography

A column (2.6 × 100 cm) from Pharmacia filled with Sephacryl S-100 HR was attached to an FPLC (fast performance liquid chromatography) Biologic System (Bio-Rad). The column was equilibrated with 0.05 M phosphate buffer pH 7.5 containing 0.3 M NaCl. The elution profile was monitored at 280 nm, collecting fractions of 2 mL.

2.5. SDS-PAGE of the Free (Crude or Partial Pure) and the Immobilized Enzyme

Proteins were analyzed by SDS-PAGE according to Laemmli [32], using a precast 12% gradient gel (model protean 16; Bio-Rad Laboratories, Richmond, Calif.). Gels were silver-stained. To analyze amount of proteins adsorbed on supports, a sample of the support was first boiled in the presence of 1% SDS (w/v) and 2% 2-mercaptoethanol (v/v) to desorb the proteins. Low molecular weight markers from GE Healthcare were used (14 - 205 kDa).

2.6. Zymogram Analysis

The zymogram was used to evaluate the presence of isoenzymes. After the electrophoresis the gel was immersed for 30 minutes in 0.05 M phosphate buffer pH 7.5 containing 2.5% Triton X-100 to remove SDS. The gel was subsequently incubated in a mixture of two solutions: Solution A: 1 mL of acetone was dissolved in 40 mg of α -naphthyl acetate and 30 mg of β -naphthyl acetate. Solution B: 120 mg of RR-fast-blue was dissolved in 10 mL of isopropanol. Both solutions were mixed and diluted with 0.1 M phosphate buffer pH 6.2 to 100 mL.

The gel was soaked in the solution mentioned earlier, and incubated at 50°C until the bands appeared [33].

2.7. Stability of the Crude Extract against Different Storage Temperatures

Samples were kept in different environments: -80°C, -20°C and 4°C for 30 days. Aliquots of these samples were tested for enzymatic activity at 3 days intervals. The experiments were carried out by triplicate and the standard error was under 5%.

2.8. Effect of Ions on the Lipolytic Activity

To determine the effects of ions on the enzyme activity 20 mL of enzyme solution was mixed with different salt solutions: CaCl₂, MgCl₂, NaCl, KCl, MnCl₂ and ZnCl₂ at a final concentration of 2 mM. The enzyme activity assays were performed at the optimal conditions of pH and temperature. The experiments were carried out by triplicate and the standard error was under 5%.

2.9. Effect of the Fatty Acid Length on the Lipolytic Activity

The standard substrate used in this study was *p*-nitrophenylpalmitate. However, tests were performed with the following modifications: solution A consisted of each substrate tested: *p*-nitrophenyl stearate, myristate *p*-nitrophenyl, *p*-nitrophenylcaprylate and *p*-nitrophenyl butyrate. Assays were performed in the optimal conditions of activity. The experiments were carried out by triplicate and the standard error was under 5%.

2.10. Enzyme Kinetics

Enzyme assays were performed with different concentrations (0.1 to 1.0 mM) of *p*-nitrophenylpalmitate, allowing calculating the initial velocity V_0 . The kinetic constants K_m and V_{max} were calculated by nonlinear regression using the Michaelis-Menten equation $V_0 = (V_{max} \cdot [S]) / (K_m + [S])$ as fitting model, using the software QtiPlot version 0.9.8.10 (ProIndepServ SRL, Romania). The experiments were carried out by triplicate and the standard error was under 5%.

2.11. Immobilization Process of the Isolipases from *R. pusillus*

2.11.1. Octyl-Agarose Support

A volume of 10 mL of enzyme solution (7 mg total protein/mL determined by Bradford's assay [31]) were mixed with 90 mL of 10 mM sodium phosphate at pH 7.0 and 4°C. Ten grams of octyl-Sepharose previously equilibrated with the immobilization buffer were added. The supernatant and suspension activities were periodi-

cally checked by the method described previously. After the immobilization, the enzyme derivative was recovered by filtration under vacuum. These adsorbed lipases were used as biocatalyst in the resolution reactions.

To perform other studies, 1.5 mg proteins of enzyme solution were mixed with 1 g of octyl-Sepharose, in order to prevent diffusion problems. All the other steps were performed as in the standard preparations.

2.11.2. CNBr-Activated Sepharose Support

The immobilization of enzyme on CNBr-Sepharose was performed for 15 minutes at 4°C to reduce the possibilities of a multipoint covalent attachment between the enzyme and the support. Ten mL of prepared enzyme solution were added to one gram of support and the reaction was maintained for 40 minutes by constant stirring. Periodically, activity of suspensions and supernatants was measured by *p*-nitrophenyl butyrate as substrate. The enzyme-support immobilization was ended by incubating the support with 1 M ethanolamine at pH 8 for 2 h. Finally, the immobilized preparation was washed several times with 5 mM of sodium phosphate buffer pH 7 [28].

2.11.3. Immobilization on Glyoxyl-Agarose

Ten milliliters of enzyme solution were added to 100 mM sodium bicarbonate buffer at pH 10.5 and the pH of the final solution was adjusted to pH 10.1. Then one gram of glyoxyl-agarose (aldehyde activated support) was added and the reaction was maintained for 24 h. Periodically, activity of suspensions and supernatants was measured by using the *p*NPB assay. When the immobilization was finished, 20 mg of NaBH₄ were added during 15 min and then the suspension was filtered and washed abundantly with distilled water (200 mL × 5). The immobilization yield was >95%.

2.11.4. pH Stability of the Immobilized Preparations

Preparations of Octyl and CNBr activated support was carried out with different buffers. Immobilized preparations were incubated in sodium acetate buffer 10 mM pH 5; Sodium phosphate buffer 10 mM pH 7 and Sodium bicarbonate buffer 10 mM pH 10. The activity was measured using the *p*NPB assay.

2.11.5. Thermal Inactivation of Different Lipase Immobilized Preparations

The different lipases preparations (0.5 mg/g) were incubated in 10 mM sodium phosphate at pH 7.0 and 45°C - 55°C. Samples were withdrawn intermittently and their activities were measured using the *p*NPB assay in the interval 1 - 24 hours. The experiments were carried out by triplicate and error was never over 5%.

2.12. Analysis of Polyunsaturated Free Fatty Acids (PUFA) by HPLC–UV

The immobilized lipases were submitted to sardine oil hydrolysis to obtain PUFAs. After a given time, aliquots of 0.1 mL of organic phase were withdrawn and dissolved in 0.8 mL of acetonitrile. The organic phase was easily separated (in less than 5 min) from the aqueous phase when stirring of the biphasic system was stopped. The unsaturated fatty acids produced were analyzed by RP-HPLC [Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450 (Spectra Physics, Santa Clara, CA. USA)] using a Kromasil C8 (15 cm 9 0.4 cm) column. Products were eluted at a flow rate of 1.0 mL/min using acetonitrile-10 mM Tris–OH buffer at pH 3 (70:30, v/v) and UV detection performed at 215 nm. The retention times for the unsaturated fatty acids were: 9.4 min for EPA and 13.5 min for DHA. These enzymatically produced PUFA were compared to their corresponding pure commercial standards.

3. RESULTS

3.1. Lipase Production

Maximum enzyme activity was observed at 72 h, as shown in **Figure 1**, and 0.5 U/ml equivalent to 4.1 U/g enzyme activity was obtained during enzyme production.

Maximum lipolytic activity was reached at the third day, decreasing afterwards. This is likely due to the presence of a protease. Another explanation for the drop in enzyme activity could be an inhibitory effect of other metabolites secreted in the course of the fermentation.

3.2. Partial Purification of Lipases Produced by the Thermophilic Fungus *R. pusillus*

The lipases present in the extract were partially purified through gel filtration chromatography (**Figure 2**), showing at least two enzymes, as deduced from the activity profile. After pooling the fractions with highest activity, we found an 84.9% yield and a 5.7 purification factor (**Table 1**). These results can be considered promising when compared to previous works (results not shown). However, when we tried to increase the degree of purification by ion exchange chromatography (DEAE-Sepharose) or hydrophobic interaction (Octyl-Sepharose) the activity decreased significantly. Thus, the characterization experiments were performed using the isolipases partially pure.

When these data are compared to those reported for the lipase from *Aeromonascaviae* AU04, a mesophilic bacterium by submerged fermentation [34], the authors obtained a total recovery of 28.76% with a purification factor of 3.4 using phenyl sepharose. After purification by ion exchange chromatography with DEAE-Sepharose,

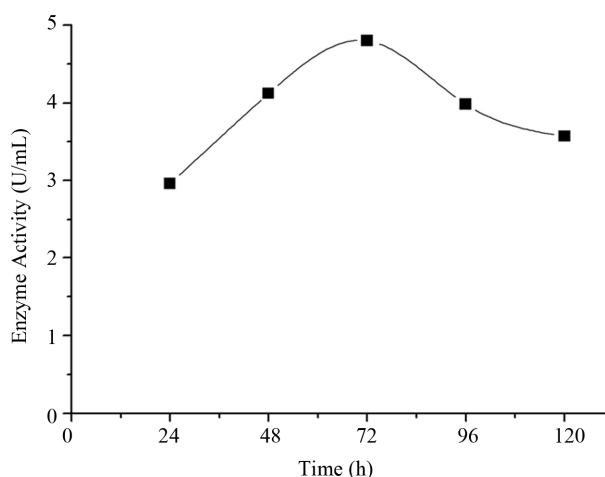


Figure 1. Hydrolytic activity of the crude extract obtained by solid fermentation of the thermophilic fungus *Rhizomucor pusillus*. The experiments were performed in triplicate and the standard error was under 5%.

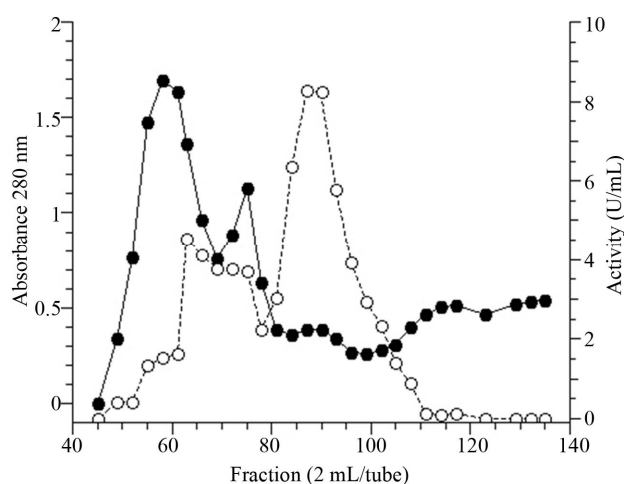


Figure 2. Elution profile of a gel filtration chromatography performed on Sephacryl S-100 support. Conditions: 0.05 M sodium phosphate buffer pH 7.5 containing 0.3 M NaCl. Symbols: (●) protein profile detected at 280 nm; (○) lipolytic activity profile in U/mL.

Table 1. Partial purification of lipases produced by *Rhizomucor pusillus*.

Step	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Crude extract	15	128.3	13.3	9.6	100.0	1.0
Gel filtration	13.5	108.9	2.00	55.0	84.9	5.7

Pastore *et al.* [35] obtained a 0.4% yield and purification factor of 3.9 for lipases from *Rhizopus sp.* In the same study, the authors reported 0.4% of recovery and purifi-

cation factor of 6.9 using the hydrophobic resin phenyl-Sepharose.

Liu *et al.* [36] also purified a microbial lipase; they used gel filtration with Sephadex G-75, obtaining a 12.6% yield and a purification factor of 3. After applying to a DEAE-Sepharose column (ion exchange chromatography) obtained 4.8%, of yield and a purification factor of 4.8 fold.

The analysis of the protein profile by SDS-PAGE shows that there are many contaminants remaining after gel filtration (results not shown). This can be caused by the formation of high molecular weight aggregates, commonly reported in studies of purification of lipases, which can hinder the process [37]. The formation of these aggregates may be due to the presence of lipids or the hydrophobic characteristic of the protein structure.

The zymogram profile (results not shown) showed two spots for lipases that would have molecular weights between 44 and 55 kDa. The lipase displaying higher molecular weight (the first peak at the gel filtration profile, **Figure 2**) displayed higher activity.

3.3. Biochemical Characterization of the Crude Enzymatic Extract and the Partially Purified Lipases

The lipolytic activity profile as a function of pH (**Figure 3(a)**) displayed a peak at pH 7.5 with enzyme activity. The lipolytic activity of the crude extract was the same or higher than 70% in the pH range from 7 to 10, whereas for the partially purified extract the highest activity was recorded at pH 8.5, in agreement with optimum pH values in the literature for lipases, which tend to be alkaline.

This feature is valuable for their potential biotechnological applications, because these lipases could be effective in an alkaline medium, for example in cleaning solutions [38]. It may be noticed that the mixture of these lipases has activity in a wide range of pH values; between 5 and 9 displays activity above 75%, a feature that suggests a potential application of these enzymes in a range of various reaction conditions.

The difference between the optimum pH of the enzyme found for the crude extract (pH 7.5) and the partially purified enzymes could be attributed to contaminants that were removed and would interfere in the activity of these enzymes.

The activity profile as a function of temperature (**Figure 3(b)**) showed that the crude extract had an optimum temperature of 40°C, and that for the partially pure lipase exhibited a higher value: 55°C. It is evident that there was an increase in optimum temperature of the partially pure lipase compared to the crude extract. This could be due to the elimination of proteins and proteases that can inhibit the activity of these lipases or denature at lower

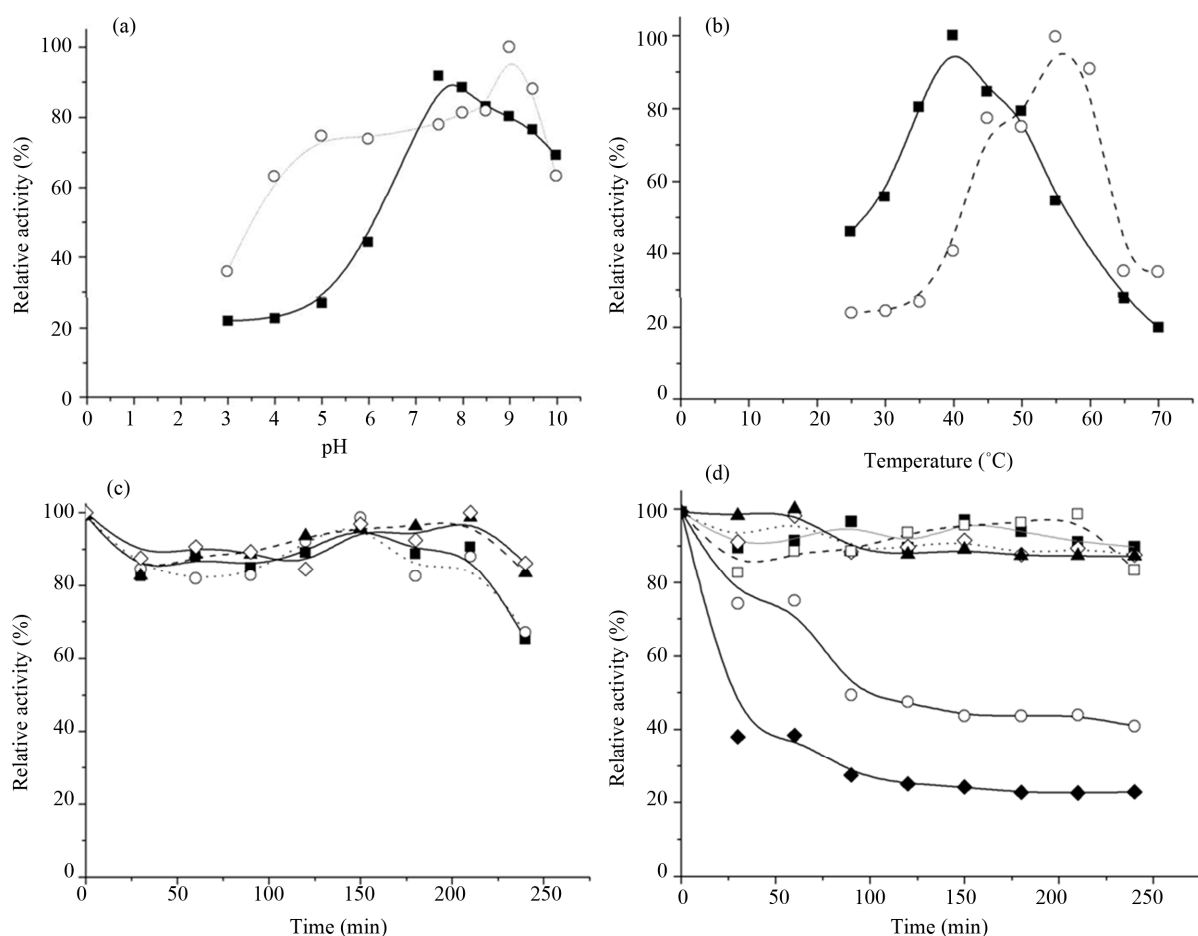


Figure 3. (a) Effect of pH on the lipolytic activity; (b) Effect of the temperature on the lipolytic activity: Symbols: (■) The crude enzymatic extract; (○) partially purified lipases. (c) pH Stability of the partially purified lipase maintained at 45°C for 4 hours. Conditions: pH 8.5 and 55°C. Symbols: (■) pH 9.5; (○) pH 8.5; (▲) pH 7.5; (◇) pH 6.5. (d) Thermal stability of the partially pure lipases from *R. pusillus* maintained at different temperatures at pH 8.5 for up to 4 hours. Conditions: pH 8.5 and 55°C. Symbols: (■) 4°C; (◇) 25°C; (▲) 35°C; (□) 45°C; (○) 55°C; (◆) 65°C.

temperatures promoting aggregation with the enzymes.

At temperatures below 40°C, their activity was low, probably due to the high intrinsic rigidity of thermophilic proteins. These proteins demand high temperature of activity (above 40°C) to promote the thermal motion and increased flexibility essential for catalytic activity [39].

Studies with intracellular lipases from athermophilic *Rhizomucor miehei* [40], obtained an optimum pH and temperature of 7.0 and 40°C respectively. Another lipase which has similar characteristics produced by a mesophilic *Rhizopus oryzae* [41] showed optimal conditions of activity at pH 7.5 and 35°C. Liu *et al.* [36] also obtained similar results for the lipase from a mesophilic *Aureobasidium pullulans* HN2.3, which exhibited optimum temperature at 35°C and pH 8.5.

Velu *et al.* [34] studied lipases of *Aeromonas caviae* and obtained as optimum pH 7.0 and 60°C. Studying a lipase from a thermophilic *Bacillus sp.* PTI-001, Nawani, Kaur. [42] found pH 8 and temperature 60°C as optimum

conditions.

Figure 3(c) shows that the lipases partially purified of *R. pusillus* incubated at different pH were less stable at more alkaline pH values (8.5 and 9.5) after 150 minutes, with steeper decline than for the samples kept at pH 6.5 and 7.5. Most fungal lipases present maximal activity in a temperature range of 30°C to 65°C. Generally, they have optimal activity at pH of (4.5 - 6.0). Furthermore, the enzyme activity is also affected by pH, which affects the enzyme charge distribution and, consequently, the substrate binding and its catalysis.

The thermal stability of these enzymes (**Figure 3(d)**) is greater when kept at 4°C, but the enzymatic activity of these lipases remains constant and close to 100% when kept at temperatures up to 45°C.

The lipase maintained at 55°C showed more than 80% of its activity within 60 minutes of incubation. After 240 minutes, the enzyme still showed approximately 40% of its residual activity.

However, when kept at 65°C, the lipase activity dropped to 40% by 60 minutes, which was soon lost, leaving only about 25% activity at the end of 240 minutes.

3.4. Stability of the Lipases from the Crude Extract at Different Storage Conditions

In order to determine the best storage condition of these crude enzymes, we analyzed the effect of time on enzyme activity for samples stored at different temperatures. When kept frozen at -80°C or -20°C, the enzyme displayed a slight loss of activity, but if stored at 4°C, it lost about more than 30% of its initial activity after 30 days.

3.5. Effect of Cations on the Lipolytic Activity

As is shown in **Table 2**, most of the tested cations increased activity; the addition of Mg²⁺ to the enzyme solution, 62.1% of its activity was increased on the lipase partially purified from *Rhizomucor pusillus*, which is slightly higher than the activity obtained in the presence of Na⁺. Ca²⁺ and Mn²⁺ also significantly activated the activity of these lipases, while K⁺ did not practically affect its activity. However, when Zn²⁺ was added, there was a significant inhibition of enzyme activity.

Sidhu *et al.* [43] observed that the lipase from *Bacillus* sp. with optimum temperature of 50°C and pH 8 was also activated in the presence of Ca²⁺ and Na⁺. Chartrain *et al.* [44] observed that extracellular lipases of *P. aeruginosa* were activated by Ca²⁺ (1.26-fold) and strongly inhibited by Zn²⁺ (94%). Sharon *et al.* [45] showed that the activity of lipases from *P. aeruginosa* KKA-5 also increased in the presence of Ca²⁺ and Mg²⁺, but were inhibited by Mn²⁺.

Table 2. Effect of ions (2 mM final concentration) in the enzymatic activity of lipases partially purified from *R. pusillus*. Assays were performed in triplicate and analyzed in the optimal conditions of the enzyme (pH 8.5 and 55°C).

Cation	Relative activity % (arithmetic mean ± standard deviation)
CONTROL	100.0 ± 0.11
CaCl ₂	154.6 ± 0.35
MgCl ₂	162.1 ± 0.26
NaCl	159.9 ± 0.16
KCl	106.2 ± 0.27
ZnCl ₂	31.9 ± 0.6
MnCl ₂	132.8 ± 0.21

Many of known enzymes require the presence of metal ions for catalytic activity. These lipases appear to belong to this group (metalloenzymes), behaving as metal-activated enzymes, which they associate weakly to metal ions from solution [46]. In this case, the ions play a structural function, rather than catalytic, since even in the absence of metals, these lipases exhibit high enzyme activity.

3.6. Effect of the Fatty Acid Chain Length on the Activity of the Partially Purified Lipase

Tests with different substrates (**Table 3**) showed that the lipases from *Rhizomucor pusillus* have more activity with the substrate p-nitrophenylmyristate (C14:0), and decreasing activity with palmitate (C16:0) > caprylate (C8:0) > stearate (C18:0). That is, for the fatty acids tested (all saturated) showed a preference for carbon chains longer than eight carbons, since for p-nitrophenyl butyrate (C4:0) the activity was around 25% compared with p-nitrophenyl-myristate.

3.7. Kinetics Parameters of the Enzyme

From the values of enzyme activity (initial velocity) with different substrate concentrations of p-nitrophenyl-palmitate (**Figure 4**), we estimated the kinetic constants, V_{max} and K_m (Michaelis-Menten constant). The values obtained by nonlinear regression were V_{max} = 12.6 ± 0.5 mmol/min and K_m = 0.2 ± 0.0 mM. It is important to stress that the calculated values for the lipases from *R. pusillus* are apparent, since they are a result of the action of two enzymes.

Comparing these values to other kinetic analyses using the same substrate, Nawani, Kaur [42] analyzed lipases from thermophilic *Bacillus* sp, finding a V_{max} = 0.032 μmol/mL and K_m = 0.19 mM. Brabcová *et al.* [47] found a higher K_m for the same substrate for an extracellular lipase from the mesophilic fungus *Geotrichum candidum* 4013: 0.406 mM.

Table 3. Affinity of the partially pure lipase in relation to different synthetic substrates at 0.8 mM final concentration. Assays were performed in optimal conditions of the enzyme for p-NPP (palmitate), pH 8.5 and 55°C.

Substrate (p-Nitrophenyl-fatty acid)	Relative activity (%)
Myristate	100.0 ± 0.79
Palmitate	91.4 ± 0.54
Caprate	81.5 ± 0.86
Stearate	78.2 ± 1.34
Butyrate	24.7 ± 1.00

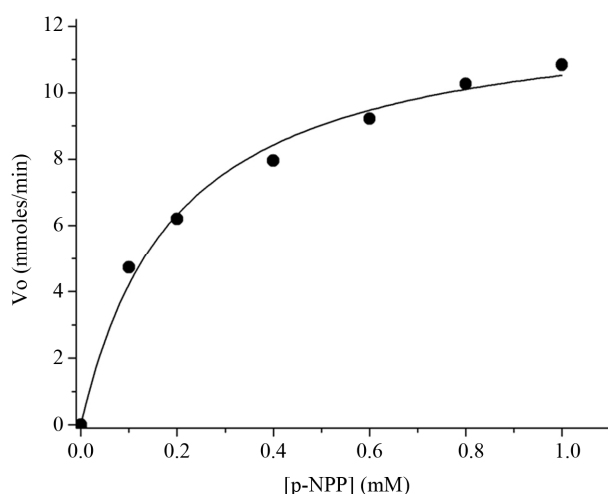


Figure 4. Enzyme Kinetics performed with different concentrations of p-nitrophenyl-palmitate in the optimal conditions of enzymatic activity: pH 8.5 and 55°C.

3.8. Immobilization of Crude Lipases on Octyl-Agarose and CNBr Activated Supports

Table 4 shows the immobilization course of the crude enzyme octyl-Sepharose and CnBr supports. In all cases, immobilization was very rapid (full immobilization was achieved after only 15 minutes on octyl-Sepharose).

However, the effect on enzyme activity was very different. Immobilization on the CNBr did not produce any positive or negative effect on the enzyme activity, while immobilization on octyl-Sepharose increased the enzyme activity by a 10 fold factor. This enhancement in the lipase activity by immobilization on hydrophobic supports has been described previously for many lipases, and it is correlated to its interfacial activation [28,29].

On octyl-Sepharose was achieved almost complete enzyme immobilization and 100% of the initial enzyme activity was expressed on the support, leaving only 25% - 30% of the proteins. In contrast, using the CNBr support only 30% of activity was immobilized. In fact, the SDS-PAGE analysis of the proteins adsorbed on the supports showed that all proteins have been immobilized in a similar way, while octyl-Sepharose selectively adsorbed about only 50% of the proteins (Data not shown).

Thus, immobilization on octyl-Sepharose permitted the one step immobilization, purification and hyperactivation of enzymes preparation, while CNBr is also an efficient method of enzyme immobilization, but neither purify nor hyper activate the lipase.

3.8.1. Stability of the Immobilized Preparations

The stability of the derivatives and the soluble enzyme was tested at room temperature and pH 5. In **Figure 5(a)**, we see that both the soluble enzyme and the derivative

Table 4. Immobilization yield and expressed activity of *R. pusillus* lipase on two different supports.

Immobilization supports	Immobilization yield (%)	Expressed activity (%)
CNBr-activated support	30	80
Octyl-agarose support	95	237

prepared on octyl have the same stability profile. Both have some hyperactivation, while CNBr have a completely different behavior.

The question remains in the behavior of CNBr derivative, since within the first 5 hours, the activity falls to almost 50% at pH 5, when the derivative had a higher enzyme expression.

Regarding pH 7 (**Figure 5(b)**), both the soluble enzyme and derivatives, have a completely different behavior. Here, the two derivatives (CNBr and Octyl) have a similar behavior, while the soluble enzyme activity remained almost unchanged, after 24 hours its activity drops by 25%.

At pH 10, the derivative activity in CNBr, drops sharply and the curious case is presented with the soluble enzyme. As they are not completely pure, so that the protein-protein interaction confers some stability and hyperactivation, at some point, unlike the derivative octylagarose, **Figure 5(c)**.

3.8.2. Thermal Stability of the Immobilized Preparations

Another interesting feature of an immobilization is the study of the effect of temperature on immobilized enzyme. **Figures 6(a)** and **(b)** show the inactivation course of the lipase immobilized on CnBr and octyl-agarose, stability at 45°C and 50°C was compared. CNBr-Sepharose has a mild covalent attachment—having quite similar stability to the soluble enzyme without any intermolecular interactions (e.g., lipase-lipase interactions). Hence, it is intriguing that the CNBr derivative activity and hyper activity were intact and maintained about 180% of its initial activity when analyzed at 45°C.

While the octyl-agarose derivative, which was expected to exhibit this behaviour did not, but rather the hydrophobic and expressed enzyme activity was much higher at the beginning, as shown in **Figures 6(a)** and **(b)**.

3.9. Hydrolysis of Sardine Oil Using Immobilized Lipase on Octyl and BrCN-Agarose

Using octyl-Sepharose derivatives of lipases the hydrolysis of sardine oil under standard reaction conditions was studied, in addition to the HPLC-UV analysis to follow the rate of PUFA release and the EPA/ DHA se-

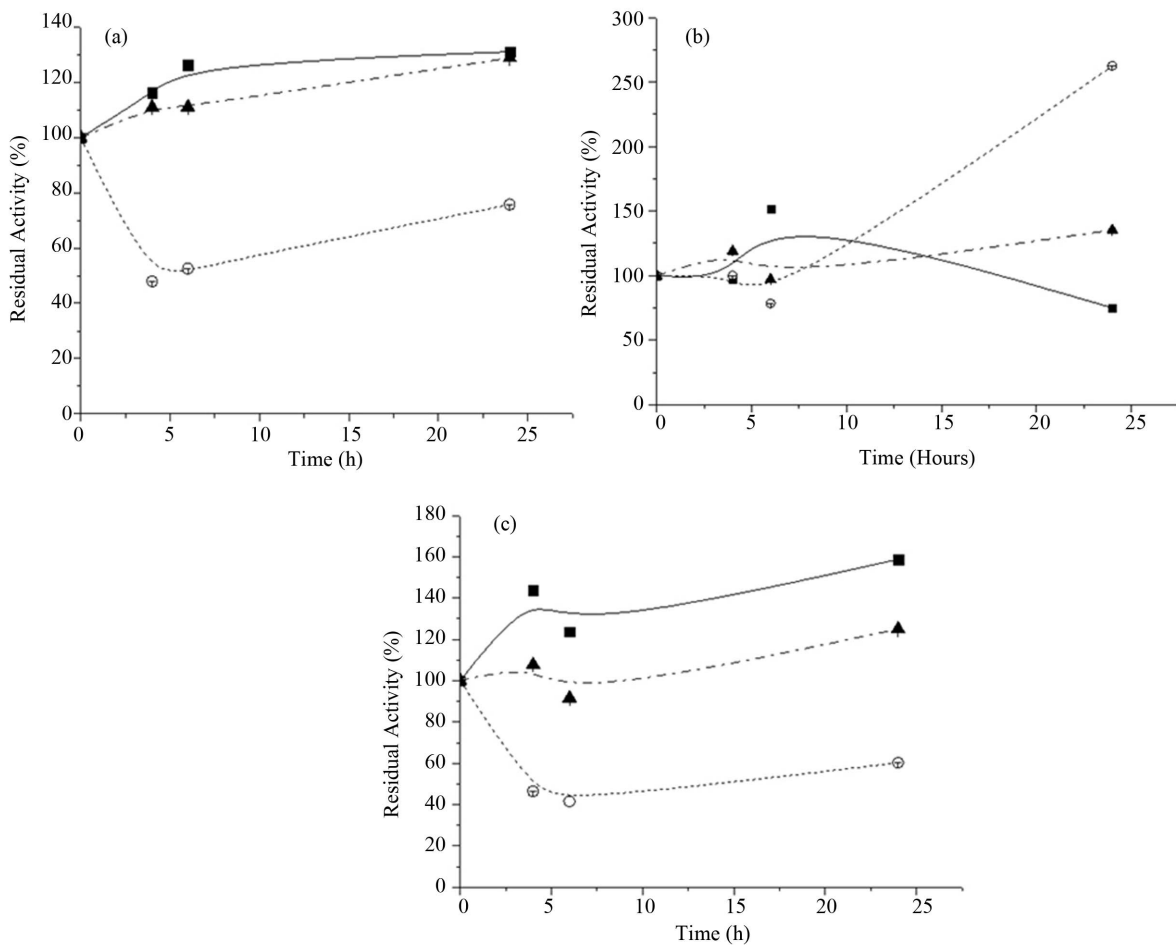


Figure 5. (a) Stability of crude extract and immobilized lipase in pH 5; (b) Stability of lipase in the crude extract and immobilized lipase at pH 7; (c) Stability of lipase in the crude extract and immobilized lipase at pH 10. Symbols: (■) Crude extract of soluble lipase; (○) Lipase adsorbed on CNBr-activated; (▲) Lipase adsorbed on octyl-agarose.

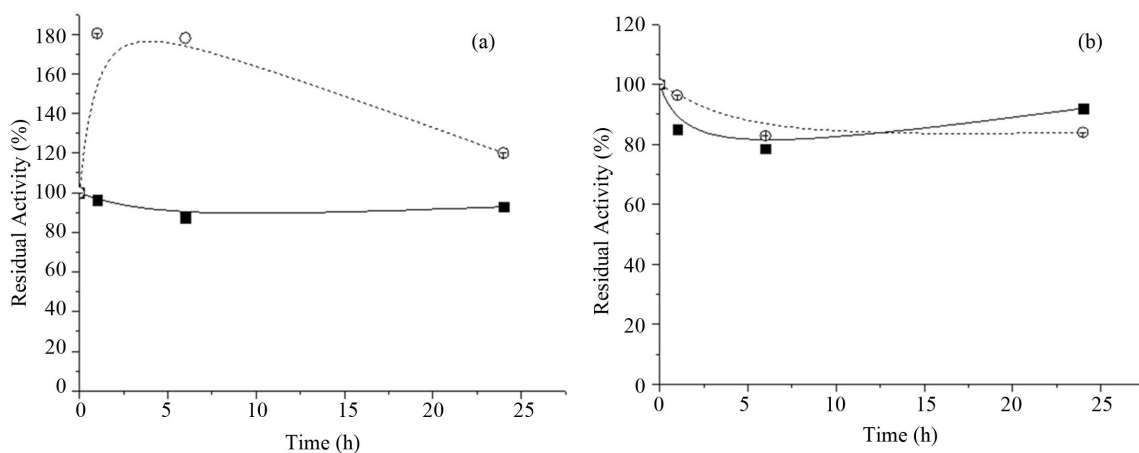


Figure 6. (a) Thermostability of CNBr derivative at pH 7. The activity was measured as was described in materials and methods. (b) Thermostability of the Octyl derivative at pH 7; Symbols: (■) 45°C; (○) 50°C.

lectivity. HPLC-light scattering analysis was also carried out to determine lipase selectivity towards polyunsaturated fatty acids: eicosapentaenoic acid (EPA) (20-5, n-3;

polyunsaturated fatty acid d) and docosahexanoic acid (DHA) (22-6, n-3; polyunsaturated fatty acid). EPA and DHA are widely available in fishoil and are recognized

to have beneficial health effects. In this way, the validity of the release of PUFA for the accurate measurement of lipolytic activity of lipases could be determined.

As a routine our group has developed new methodologies for the analysis of both PUFAs (EPA and DHA). Enabling quick analysis of the hydrolysis of these compound with the derivatives, just to see, how they were or not selective in such reactions. Based on this we analyzed only at pH 7 and different temperatures. As shown in **Table 5**, the octyl derivative achieved the highest yields of EPA and DHA at 37°C, with selectivity close to 6.

Morrissey and Okada [48] reported the hydrolysis of 33% of EPA and 29% of DHA from sardine oil after 9 h of reaction using the commercial lipase of *Candida rugosa* (not immobilized) at a concentration of 500 U. It is worth mentioning that there is no significant selectivity difference between these two fatty acids, differently from what we observed in this study, with a lipase more selective for EPA. The reaction reported by those authors was faster; however, it took 500 U for hydrolysis, whereas in this work only 130 U were able to carry out the hydrolysis of 40% and 7% of EPA and DHA respectively. These promising results have allowed us to work on improving the production of these lipases and search for new and better methods of mobilization for future work.

4. CONCLUSIONS

The enzyme was produced with a very good activity level, subsequently with simple purification processes, even without the separation of the isolipases, and yield close to 90% was obtained.

The expression of the enzyme immobilization mapping is excellent, due to immobilization on octyl-agarose, which reached hyperactivation levels in the order of 200%, which is excellent for studies of enantioselectivity against filing details.

Furthermore, the derivatives stability versus temperature and pH were excellently allowing use in industry where conditions may be somewhat drastic. The method used of enzyme immobilization applied was very simple

Table 5. Yield of EPA and DHA in 72 hours reaction of hydrolysis of fish oil and selectivity for these fatty acids. Conditions: Fish oil in Tris 10 mM pH 7 buffer and cyclohexane-under magnetic agitation.

Derivative	EPA (20:5 n-3)(%)	DHA (22:6 n-3)(%)	Selectivity
Octyl-agarose pH 7, 25°C	11.2	4.2	2.7
Octyl-agarose pH 7, 45°C	10.0	2.5	4.0
Octyl-agarose pH 7, 37°C	40.0	6.8	5.9

and very easy. All amount of the soluble enzyme offered before on each support was completely and quickly adsorbed in all cases with excellent yields on immobilization.

The lipases from *R. pusillus* showed important characteristics with potential for industrial applications; in particular, they displayed promising results in the enrichment of fish oil with PUFA omega 3.

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