Microbial lipase obtained from the fermentation of pumpkin seeds: immobilization potential of hydrophobic matrices

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ABSTRACT. Immobilization potential of lipase from *Aspergillus niger* on sol-gel matrix was evaluated by physical adsorption and covalent binding and the biochemical characterization of free and immobilized enzyme was performed. Lipase was produced by solid state fermentation of pumpkin seed flour with 30% moisture, at 30°C for 120h. The enzyme was pre-purified with ammonium sulfate and immobilized in the sol-gel matrix by covalent attachment and physical adsorption. A higher yield of immobilization (81.88%) was obtained in the latter. The free enzyme presented higher hydrolytic activity with pH 4.0, at 37°C; moreover, it was more stable with pH between 6.0 and 7.0, at 35°C. The immobilized lipase showed maximum hydrolytic activity with pH 11.0, at 50°C; it was more stable with pH 11.0, at 37°C. Parameters K_m and V_{max} were best determined by Hanes-Woolf linearization.

Keywords: enzyme, sol-gel, agroindustrial residue.

Lipase microbiana obtida pela fermentação de sementes de abóbora: potencial de imobilização em matrizes hidrofóbicas

RESUMO. O objetivo deste trabalho foi avaliar o potencial de imobilização da lipase de *Aspergillus niger* em sílica por adsorção física e ligação covalente, e realizar a caracterização bioquímica da mesma na forma livre e imobilizada. A lipase foi produzida por fermentação em estado sólido da farinha de sementes de abóbora, contendo 30% de umidade, à temperatura de 30°C em 120h. A seguir, a enzima foi pré-purificada com sulfato de amônio e imobilizada em matriz sol-gel por ligação covalente e adsorção física, obtendo-se nesta última maior rendimento de imobilização (81,88%). A enzima livre apresentou maior atividade hidrolítica em pH 4,0 a 37°C, sendo mais estável em valores de pH entre 6,0 e 7,0 e temperatura de 35°C. A lipase imobilizada por adsorção física apresentou máxima atividade hidrolítica em pH 11,0 e temperatura de 50°C sendo mais estável em pH 11,0 e temperatura de 37°C. Os parâmetros K_m e $V_{máx}$ foram melhor determinados pela linearização de Hanes-Woolf.

Palavras-chave: enzima, sol-gel, resíduo agroindustrial.
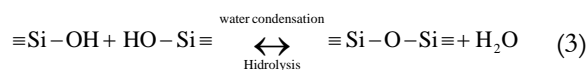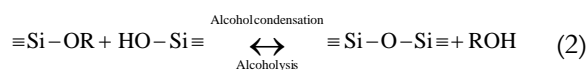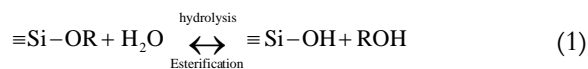
Introduction

Due to their functional properties (activity, selectivity and specificity), lipases (triacylglycerol hydrolase, EC 3.1.1.3) have become rather attractive as catalysts capable of acting on ester bonds of tri-, di- and mono-acylglycerols in mild reaction conditions. In an organic medium, they catalyze a number of useful reactions including esterification, transesterification, acidolysis, alcoholysis, regioselective acylation of glycols and menthols, and synthesis of peptides and other chemicals (SHARMA et al., 2001). Lipases are actually versatile catalysts applicable in a wide range of industrial processes, such as textile, paper, detergents, oil chemicals, food and pharmaceutical industries (SAXENA et al., 2003; YANG et al., 2010). Solid-state fermentation (SSF) is frequently used for lipase production and cultivation media with different residues have been reported, e.g. by-products from the refining process of corn oil or olive oil (DAMASO et al., 2008) or pumpkin seed flour (SANTOS et al., 2012).

Lipases may be used in free and in immobilized forms. However, a major disadvantage in the free form is the impossibility of re-utilization, with economical liabilities, due to their high market value. So that the above problem may be overcome, the enzyme may be immobilized by physical adsorption, covalent bonding, ionic interactions or encapsulation (DALLA-VECCHIA et al., 2004; UYANIK et al., 2011). There are several materials that may be used as supports for enzyme

immobilization, or rather, natural materials such as alginate (ELLAIAH et al., 2004), agar (LI et al., 2008) and agarose (RODRIGUES et al., 2008); synthetic ones, such as nylon (PAHUJANI et al., 2008), polyacrylamide (ELLAIAH et al., 2004); inorganic ones, such as silica (CRUZ et al., 2009; YANG et al., 2010) and glass (KAHRAMAN et al., 2007) and others. Among the methodologies used to obtain silica, the supports produced by sol-gel technique are known as hydrophobic matrices (MEUNIER; LEGGE, 2010).

Sol-gel process involves the transition of a sol composition from a liquid sol into a solid gel phase. A sol is first formed by mixing an alkoxide precursor such as tetramethyl-orthosilicate (TMOS) or tetraethyl-orthosilicate (TEOS) with water, a co-solvent and an acid or base catalyst at room temperature. The basic sol–gel reaction starts when the metal alkoxide (Si–OR) is mixed with water (hydrolysis). The following reactions (Equations 1-3) are generally used to describe sol–gel processes (GUPTA; CHAUDHURY, 2007). The progress of hydrolysis and condensation reactions is one of the parameters that influence the intrinsic properties of sol–gel matrix, namely, porosity, surface area, polarity and rigidity. When the pores of the gel are filled with water and alcohol, it is known as aquagel; when the aquagel is dried by evaporation, a xerogel is obtained. After the evaporation of the carbon dioxide, the structure of the aquagel is maintained and a brittle aerogel is obtained. Consequently, hydrophilic aqua, xero- and aero-gels are made. Sol–gels with a hydrophobic surface may be obtained by adding alkyltrialkoxysilanes (methyltrime-thoxysilane, MTMS) to the synthesis mixture (HANEFELD et al., 2009).

$$\equiv Si-OR + H_2O \underset{Esterification}{\overset{hydrolysis}{\longleftrightarrow}} \equiv Si-OH + ROH \qquad (1)$$

$$\equiv Si-OR + HO-Si \equiv \underset{Alcoholysis}{\overset{Alcohol\,condensation}{\longleftrightarrow}} \equiv Si-O-Si \equiv + ROH \qquad (2)$$

$$\equiv Si-OH + HO-Si \equiv \underset{Hidrolysis}{\overset{water\,condensation}{\longleftrightarrow}} \equiv Si-O-Si \equiv + H_2O \qquad (3)$$

Hydrophobic surfaces may have a very positive influence on the reactivity of lipases, since they may induce interfacial activation; i.e. the lipase may be in its active conformation. Compared to other immobilization processes, the advantages of lipases' immobilization in sol-gel matrices comprise higher thermal and chemical stability and higher hydrolytic

activity of the biocatalyst (MEUNIER; LEGGE, 2010). Several researchers have immobilized microbial lipases in silica matrices obtained with sol-gel technique (MEUNIER; LEGGE, 2010; SIMÕES et al., 2011; SOARES et al., 2004b; UYANIK et al., 2011). Current study investigated the immobilization of lipase from *Aspergillus niger* (obtained from SSF pumpkin seed flour) in sol-gel matrix and also the enzyme's biochemical characterization in free and immobilized form.

Material and methods

The *Aspergillus niger* IOC 3677 microorganism was obtained from the culture collection of the Oswaldo Cruz Institute (Rio de Janeiro), preserved in tubes with nutrient agar and stored at 4°C. The pumpkin seeds were purchased from a local market. The silica obtained by sol-gel technique (SOARES et al., 2004b) with particle size 100 µm was kindly donated by the Institute of Technology and Research of Tiradentes University (Aracaju, Sergipe State). All reagents used were of analytical grade.

Solid state fermentation

The production of lipase was performed according to methodology described by Santos et al. (2012). First, the pumpkin seeds were dried in a dryer (Pardal – PE 100, Brazil) at 60°C, for 8h, ground in a Wiley-type mill with an average diameter of 1.06 mm and sterilized in an autoclave at 121°C for 15 min. Fermentations (in triplicate) were conducted in petri dishes containing 10g of pumpkin seed flour (30% moisture content) and 10^5 spores g^{-1} of *Aspergillus niger,* at 30°C. After 120h fermentation, the enzyme extraction was carried out by adding 50 mL of sodium phosphate buffer 0.1 M, pH 7.0, maintained under stirring at 30°C, during 1 5 min. It was then centrifuged during 10 min. at 120 x g, obtaining crude enzyme extract. The enzyme was partially purified through precipitation with ammonium sulfate at 80%, maintained at 4°C, for 30 min.; it was further centrifuged at 120 x g during 10 min. The supernatant obtained was filtered, dialyzed and lyophilized (WOLSKI et al., 2009).

Enzyme immobilization in sol-gel matrix

The enzyme immobilization in sol-gel matrix was performed by physical adsorption and covalent bonding according to methodology by Soares et al. (2004a), with modifications. The experiment for immobilization by physical adsorption consisted of mixing 1 mL of enzymatic solution (300 mg mL^{-1}) in sodium acetate buffer 0.2 M pH 4.0, 9 mL of hexane and 1g of support (sol-gel matrix),

maintained under stirring during 3h. After this period, the mixture was left for 24h at 4°C. The immobilized enzyme was recovered by vacuum filtration and successively washed with hexane to remove the lipase which had not been adsorbed in the support.

The enzyme immobilization by covalent attachment initially consisted of the activation of sol-gel matrix with glutaraldehyde 1% (v v^{-1}). Procedure comprised mixing 20 mL glutaraldehyde and 1 g support, kept under stirring at 120 rpm and at 30°C for 1h. The support was afterwards washed with distilled water to remove the excess of glutaraldehyde until pH of washing water reached pH of distilled water. The same procedure described for physical adsorption was employed for enzyme immobilization.

Determination of hydrolytic activity

The enzymatic activity of free and immobilized lipases was determined by the hydrolysis method of olive oil according to procedure by Soares et al. (2004a), with modifications. The substrate was prepared with 25 mL olive oil and 25 mL gum arabic 7% in distilled water. Further, 5 mL of the substrate, 2 mL of sodium phosphate buffer at 0.1 M, pH 7.0, and 1 mL of crude enzyme extract (10 mg mL^{-1}) or 2.5 mL of substrate, 1 mL of sodium phosphate buffer (0.1 M, pH 7.0) and 250 mg of immobilized lipase were added in Erlenmeyer flasks. The mixture was incubated at 37°C for 5 min. and the reaction was stopped with acetone and ethanol (1:1, v v^{-1}). The fatty acids released were titrated with a solution of 0.04 M KOH, using phenolphthalein as indicator. All reactions were carried out in triplicate. One activity unit was defined in terms of amount of enzyme to release 1 μmol of fatty acid per min. of reaction, under the experimental conditions.

Immobilization yield in hydrophobic matrices was calculated according to Equation 4 (SOARES et al., 2004a):

$$\eta(\%) = \frac{U_S}{U_o} x100 \qquad (4)$$

where U_S is the total enzymatic activity present in the support and U_o is the initial activity of enzyme for immobilization.

Effect of pH and temperature in the hydrolytic activity of free and immobilized enzyme

The effect of pH on enzymatic activity of the free lipase and of the immobilized biocatalyst was determined with olive oil hydrolysis reaction by varying the buffers as follows: sodium acetate buffer

0.2 M pH 2.0, 3.0, 4.0 and 5.0; sodium phosphate buffer 0.2 M pH 6.0, 7.0 and 8.0 and Tris-HCL buffer 0.2 M pH 9.0, 10.0 and 11.0.

The buffer with the highest hydrolytic activity was used in the hydrolysis reaction to evaluate the effect of temperatures 30 - 80°C in the enzymatic activity.

Stability of free and immobilized enzyme in different pH values

Stability in different pH rates was determined by incubating 10 mg of free lipase or 250 mg of immobilized biocatalyst at 37°C, in buffers pH 4.0, 5.0, 6.0 and 7.0 and pH 4.0, 6.0 and 11.0, respectively. Periodic samples were retrieved to determine hydrolytic activity in 240 min. incubation.

Thermal stability of free and immobilized enzyme

The thermal stability of free and immobilized lipase was determined as follows: free enzyme (10 mg) was incubated in sodium acetate buffer 0.2 M, pH 4.0 at 30, 37 and 45°C and immobilized biocatalyst (250 mg) was incubated in Tris-HCL buffer 0.2 M pH 11.0 at 37, 50 and 60°C. Periodic samples were retrieved to determine hydrolytic activity in the 240 min. incubation, as described previously.

Enzyme inactivation constant (k_d) and half-life ($t_{1/2}$) rates were calculated for immobilized and free lipase at 37°C, respectively by equations 5 and 6 (YANG et al., 2010).

$$A_{in} = A_{in0} \, exp \, (-kd \, x \, t) \qquad (5)$$

$$t_{1/2} = \ln 2 / k_d \qquad (6)$$

where A_{in} is the residual lipase activity after thermal treatment (U) and A_{ino} is the initial lipase activity (U).

Operational stability

The operational stability of the better immobilized system (adsorption or covalent binding) was determined in hydrolysis reactions in consecutive batches with the reuse of immobilized lipase from *Aspergillus niger*. In all batches, 100 mg mass of immobilized bio-catalyst was employed. Batches of 10 min. were carried out for sample immobilized enzyme by adsorption at 37°C and pH 11.0. The immobilized enzyme was then washed with hexane until the complete removal of substrate and used in the next hydrolysis cycle. The procedure was repeated for several cycles.

Determination of kinetic parameters of free and immobilized enzyme

The kinetic parameters of the Michaelis-Menten equation (Equation 7) for free and immobilized enzyme were determined by varying the concentration of olive oil from 1 to 70% in the enzymatic hydrolysis reaction, as described previously. K_m and V_{max} rates were calculated from Lineweaver-Burk, Hanes-Woolf, Scatchard and Eadie-Hofstee plots (CARVALHO et al., 2010), according to equations 8 to 11, respectively.

$$v = \frac{V_{max}[S]}{K_m + [S]} \tag{7}$$

where:

v and V_{max} represented initial and maximum reaction rates, respectively; [S] was the substrate concentration; K_m was the Michaelis constant.

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \tag{8}$$

$$\frac{[S]}{v} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}}[S] \tag{9}$$

$$\frac{v}{[S]} = \frac{V_{max}}{K_m} - \frac{1}{K_m}v \tag{10}$$

$$v = V_{max} - K_m \frac{v}{[S]} \tag{11}$$

Results and discussion

Immobilization of lipase on hydrophobic matrices

In current study, the immobilization of *Aspergillus niger* lipase by physical adsorption presented a higher yield of immobilization (81.88%) than that obtained by covalent attachment (58.18%) (Table 1).

Table 1. Hydrolytic activity of free and immobilized enzyme and immobilization yield.

Method of immobilization	Free lipase activity (U)	Immobilized lipase activity (U)	Immobilization Yield (%)
Physical adsorption	184	150.66	81.88
Covalent attachment	184	107.06	58.18

Soares et al. (2004a) immobilized *Candida rugosa* lipase (LCR) and pancreatic lipase (LPP) in sol-gel matrix and obtained immobilization yields of 12 and 7.49% by physical adsorption and 7.93 and 5.80% by covalent attachment, respectively.

The enzyme was immobilized onto a solid support by low energy bonds, such as the interactions of Van der Waals or hydrophobic, hydrogen and ionic bonds, among others. In the case of covalent bonding, the procedure involved a chemical modification of an amino-acid residue by covalently binding the 'enzyme' to a 'water-insoluble material' and by fixing the enzyme to a matrix by covalent bonding (DALLA-VECCHIA et al., 2004).

In current study, the activity of lipase from *Aspergillus niger* decreased when the glutaraldehyde was employed as a bifunctional immobilization agent by covalent attachment. The above may have been due to the reduction of the amount of water in the system. Moreover, the hypothesis may be raised that the activation process of the support with glutaraldehyde was not efficient to provide active groups for enzyme immobilization, or it may have undergone conformational changes which resulted in the inaccessibility of its bonding groups.

The immobilization yield in sol-gel by physical adsorption achieved in current research (81.88%) was higher than that obtained by Soares et al. (2004b) (32%) and Uyanik et al. (2011) (36%) with *Candida rugosa* lipase immobilized in sol-gel matrix by encapsulation. On the other hand, the immobilization yield of lipase in sol-gel by covalent attachment (58.18%) was lower than that obtained by Yang et al. (2010) (72.1%) and higher than the one obtained by Chaubey et al. (2009) (about 50%) with *Arthrobacter sp* lipase.

The above results showed that it was possible to hold biochemical characterization and determination of kinetic parameters for the immobilized lipase in sol-gel by physical adsorption, known as immobilized biocatalyst (IB).

Effect of pH and temperature on the hydrolytic activity of free and immobilized enzyme

The effect of pH on the relative activity of free and immobilized lipase was determined at 37°C for pH range between 2.0 and 11.0 (Figure 1). Free lipase presented maximum relative activity at acidic pH 4.0. Since the lowest relative activity (around 20%) was at pH 2.0, the enzyme tended towards inactivation at high acid pH rates, which may be caused by excess H^+ ions. Result was similar to that obtained by Namboordiri and Chattopadhyaya (2000), Mahadik et al. (2002) and Mhetras et al. (2009) who produced *Aspergillus niger* lipase with optimum pH between 5.0 and 6.0 (by submerged fermentation of olive oil), between 2.5 and 3.0 (using wheat bran) and 2.5 (by submerged fermentation of a synthetic medium), respectively.
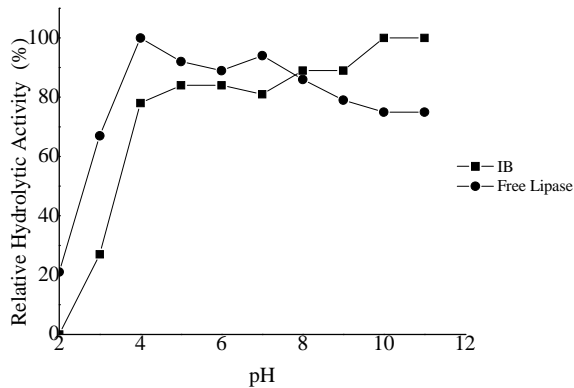
Figure 1. Effect of pH on the relative activity of free lipase and immobilized biocatalyst.

The immobilization process provided maximum (100%) relative activity for lipase at a pH range between 10.0 and 11.0 (Figure 1). At pH 2.0 the immobilized enzyme was completely inactivated (relative activity = 0%) which might have occurred due to the excess of H^+ ions. Optimum pH of IB (between 10.0 and 11.0) was higher than the rates obtained by Salis et al. (2011) and Kim et al. (2006) for *Mucor javanicus* lipase immobilized on silica (optimum pH between 6.0 and 9.0, respectively).

The difference between optimum pH of free and immobilized enzyme was probably due to the immobilization process which may have resulted in conformational changes in the three-dimensional structure of the enzyme or alteration of concentration among the charged species, substrate, product, hydrogen ions, hydroxyl ions, both in the micro-environment of the immobilized enzyme and in the reaction medium (SIMÕES et al., 2011). As a rule, the immobilized enzymes have either the same pH range or a broader high activity than free enzyme (KHARRAT et al., 2011).

Temperature influence on the hydrolytic activity was determined at pH 4.0 for the free lipase and at pH 11.0 for the immobilized biocatalyst. Free enzyme reached maximum relative activity (100%) at 37°C, decreasing the activity at subsequent temperatures (Figure 2). Kamini et al. (1998), Namboordiri and Chattopadhyaya (2000) and Saxena et al. (2003), obtained an optimal temperature of 37°C, between 35 and 55°C and between 28 and 30°C respectively for *Aspergillus niger*, *Aspergillus carneus* and *Aspergillus oryzae* lipase.

The immobilized biocatalyst had maximum relative activity at 50°C, with approximately 80% at other temperatures. The immobilization process provided better enzyme performance at high temperatures when compared with enzyme in the free form. Sol-gel matrix may be protecting the

enzyme against thermal denaturation. The above results were better than those obtained by Soares et al. (2006), with lipase from *Candida Rugosa*, immobilized on silica sol-gel, which provided relative activity between 80 and 100% only at temperatures between 55 and 65°C.
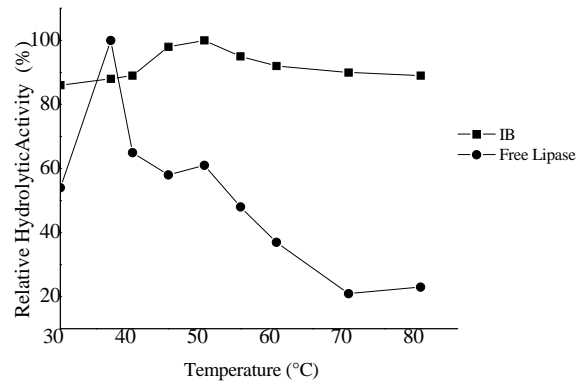

Figure 2. Effect of temperature on the activity of lipase's free and immobilized biocatalyst.

Reaction medium´s temperature increase usually speeds up reactions catalyzed by lipases, since it increases the number of collisions between the reagents' molecules. However, this effect also causes the increased speed of heat deactivation, resulting in the breakdown of bonds on the enzymatic structure (hydrogen bonds, disulfide bonds and hydrophobic interactions) and in the reduction of the enzyme´s hydrolytic activity. Since the immobilization process provided more rigid conformation to the lipase, it was less significant for the enzyme's three-dimensional structure than the effect of high temperatures (BALCÃO et al., 1996; SIMÕES et al., 2011).

Stability of free and immobilized enzyme in different pH and temperature rates

Free lipase showed higher stability with pH rates closer to neutrality (pH rates between 6.0 and 7.0) in which the relative activity ranged between 90 and 100%. The relative activity of the enzyme at pH rates 4.0 and 5.0 varied from 60 to 85% in 240 min. incubation (Figure 3A). The results agreed with those in the literature, since researchers have obtained *Aspergillus niger* lipase with stability at pH rates between 4.0 and 10.0 at 30°C during 30 min. (KAMINI et al., 1998); between 5.0 and 7.5 at 37°C during 1h (NAMBOORDIRI; CHATTOPADHYAYA, 2000); between 2.5 and 9.0 at 25°C during 24 h (MAHADIK et al., 2002) and between 8.0 and 11.0 at 30°C during 24h (MHETRAS et al., 2009). The immobilized biocatalyst was more stable at pH 11.0, keeping

100% of activity during 150 min. incubation and reaching about 95% of activity in 240 min. At pH 6.0, the immobilized enzyme kept 100% relative activity during 30 min., reaching approximately 90% in 240 min. incubation. At pH 4.0, relative activity remained close to 96% until 125 min., reaching 88% in 240 min. incubation (Figure 3B).
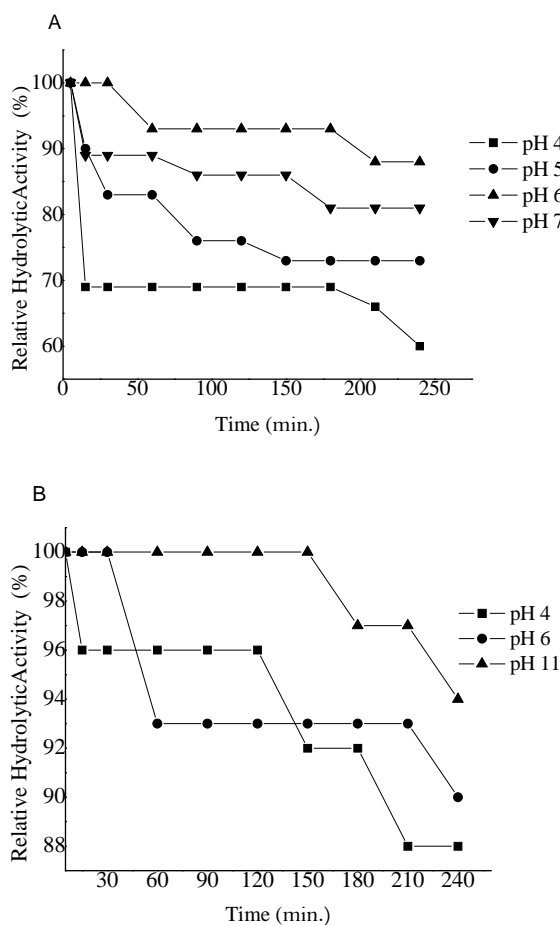

Figure 3. Stability of free lipase (A) and immobilized biocatalyst (B) at different pH rates.

The relative activity of free and immobilized enzymes decreased with increasing temperature of the reaction medium (Figure 4A and B). At 30, 37 and 45°C, the free lipase maintained the relative activity around 85, 67 and 60% in 240 min. incubation, respectively. At 37, 50 and 60°C, immobilized lipase had respectively close to 95, 80 and 72% relative activity in 240 min. incubation.

The immobilization process provided the enzyme with greater heat stability, probably by protecting the enzymatic three-dimensional structure of the negative effect of high temperatures (CARVALHO et al., 2010; SIMÕES et al., 2011). A hypothesis could be raised with regard to the lipase location inside the support micropores which

offered a good protection against alterations. *Aspergillus niger*-derived lipases reported in literature have shown stability at temperatures between 4 and 50°C for 30 min. (KAMINI et al., 1998); between 20 and 50°C for 1h (NAMBOORDIRI; CHATTOPADHYAYA, 2000); between 50 and 60°C for 5h (MAHADIK et al., 2002) and at 40°C for 3h (MHETRAS et al., 2009).

Researchers have as a rule established that immobilized enzyme has a higher thermal stability than free enzyme due to restriction of its conformational flexibility attributed to its multiple attachment point of enzyme on the support which limits the conformational alterations and movements under various temperatures (KHARRAT et al., 2011).
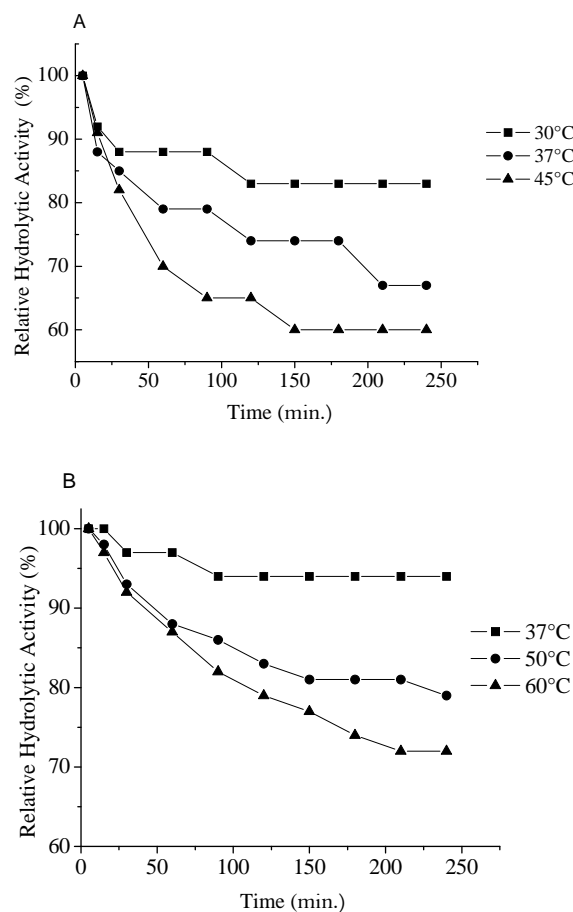

Figure 4. Thermal stability of free lipase (A) and immobilized biocatalyst (B).

The thermal stability of the biocatalysts was also evaluated by calculating the thermal deactivation constant (k_d) and half-life $(t_{1/2})$ of free and immobilized lipases at 37°C, as shown in Table 2.

The k_d rate of immobilized lipase was approximately 7 fold lower and the $t_{1/2}$ value was

7 fold higher than that obtained for free enzyme. The shorter the k_d value, the greater the half-life better thermal stability of the biocatalyst.

Table 2. Thermal deactivation of free and immobilized lipases at 37°C for 240 min. incubation.

Free lipase		Immobilized lipase	
k_d	$t_{1/2}$	k_d	$t_{1/2}$
0.1	6.93	0.014	48.51

These results suggest that the thermal stability of the immobilized enzyme is greater than that of free enzyme, demonstrating a successful immobilization method.

Operational stability

The reuse of immobilized biocatalyst is important to evaluate support potential in industrial applications. Actually immobilized lipase may be reused up to two times while keeping 50% of its initial activity (Figure 5). This result was close to that by Souza et al. (2012), where the lipase from *Bacillus* sp. immobilized by adsorption on sol-gel kept 50% of its initial activity up to 3 cycles of reuse. The detachment of the enzyme from the support during reuse may have occurred. Enzyme immobilization by adsorption may have the highest commercial potential when compared to other techniques due to its relatively low cost, simplicity and retention of high catalytic activity. However, a disadvantage of this technique is the weak interaction between enzyme and support which causes biocatalyst desorption during washing and other steps (KHARRAT et al., 2011).
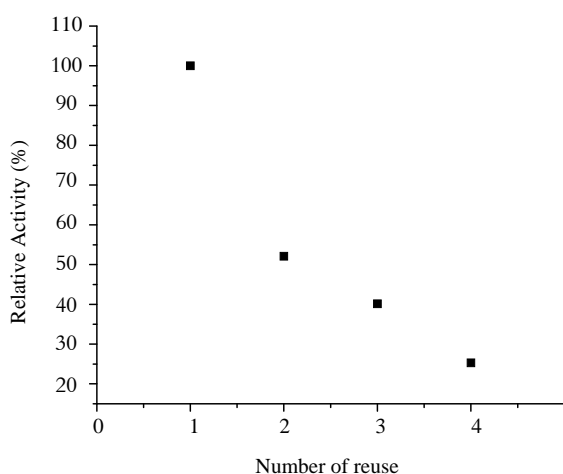
Figure 5. The relative activity of lipase from *Aspergillus niger* immobilized by physical adsorption as a function of reuse numbers.

Kinetic parameters

The Michaelis constant (K_m) and maximum rate of reaction (V_{max}) of free and immobilized lipase were calculated from Lineweaver-Burk (LB), Hanes-Woolf (HW), Scatchard (Scat) and Eadie-Hofstee (EH) plots. The linearization of Hanes-Woolf presented the highest correlation coefficient ($R^2 = 0.926$ for the free enzyme and $R^2 = 0.953$ for the IB). In fact, it was the most indicated for the determination of parameters K_m and V_{max} for the free enzyme as well as for that immobilized in sol-gel (Table 3).

In the case of Michaelis constant (K_m), high rates mean low affinity of the substrate, or rather, higher concentrations of the substrate are required to reach half of the maximum activity (GUPTA et al., 2008). The 'free lipase' had higher affinity to the substrate (K_m of 117 mM) than the immobilized enzyme in sol-gel ($K_m = 170$ mM). On the other hand, the maximum rate of reaction for free (V_{max} of 0.0276 mM min.$^{-1}$) and immobilized enzyme ($V_{max} = 0.0216$ mM min.$^{-1}$) had similar values.

Table 3. Kinetic parameters of the Michaelis-Menten equation obtained through different methods of linearization.

	Free enzyme			Immobilized enzyme		
	R^2	K_m (mM)	V_{max} (mM min^{-1})	R^2	K_m (mM)	V_{max} (mM min^{-1})
LB	0.430	14.53	0.0237	0.126	6.73	0.0171
HW	0.926	117	0.0276	0.953	170	0.0216
Scat	0.330	49	0.0273	0.498	200	0.0220
EH	0.330	16	0.0243	0.116	7.81	0.0175

Lineweaver-Burk (LB), Hanes-Woolf (HW), Scatchard (Scat) and Eadie-Hofstee (EH).

Conclusion

Aspergillus niger lipase obtained from the fermentation of pumpkin seed flour showed greater yield of immobilization in sol-gel matrix by physical adsorption (81.88%) than by covalent attachment (58.18%). The free enzyme presented maximum hydrolytic activity at pH 4.0 and 37°C, and was characterized as acidic lipase. The immobilization process by adsorption in sol-gel matrix has provided changes in the enzyme´s biochemical characteristics, with maximum activity obtained with pH 11.0 at 50°C. The thermal stability of the immobilized biocatalyst was confirmed by the rates of thermal deactivation constant (k_d) and half-life ($t_{1/2}$), which were 7 fold higher than those obtained by the enzyme in the free form. The kinetic parameters of free and immobilized enzyme were better represented by the Hanes-Woolf linearization and the differences in K_m rates showed changes in the affinity of the enzyme to the substrate when immobilized in sol-gel matrix.

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