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# Lipase from *Aspergillus niger* obtained from mangaba residue fermentation: biochemical characterization of free and immobilized enzymes on a sol-gel matrix

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**ABSTRACT.** In this study, mangaba residue (seeds) was used as a substrate for *Aspergillus niger* lipase production by solid-state fermentation. The partially purified enzyme was efficiently immobilized in a sol-gel matrix by covalent bonding with an immobilization yield of 91.2%. The immobilized biocatalyst and free lipase had an optimum pH of 2.0 and 5.0, respectively. However, greater stability was obtained at pH 4.0 and 7.0, respectively. The biocatalysts showed stability at the optimum temperature of 55°C, where the residual activity was above 87% after 240 min., of incubation. The lower deactivation constant ($k_d$) and higher half-life of the immobilized biocatalyst indicated greater thermal stability than those obtained with the free enzyme. The Michaelis Constant ($K_m$) (77 and 115 mM for free and immobilized lipase, respectively) and maximum reaction rate ($V_{max}$) (1250 and 714 U $mg^{-1}$ for free and immobilized lipase, respectively) indicated that the immobilization process reduced enzyme-substrate affinity. Regarding the operational stability, the biocatalyst showed relative activity above 50% until seven cycles of reuse in olive oil hydrolysis. This novel biocatalyst obtained from a tropical fruit residue showed biochemical characteristics that support its application in future biocatalysis studies.

**Keywords:** fruit residue, sol-gel, biotechnology, enzyme.

## Lipase de *Aspergillus niger* obtida a partir da fermentação do resíduo de mangaba: caracterização bioquímica das enzimas livre e imobilizada em matriz de sol-gel

**RESUMO.** Neste estudo, o resíduo de mangaba (sementes) foi utilizado como substrato para a produção de lipase de *Aspergillus niger* por fermentação em estado sólido. A enzima parcialmente purificada foi eficientemente imobilizada em matriz de sol-gel por ligação covalente visto que o rendimento de imobilização foi de 91,2%. O biocatalisador imobilizado e a lipase livre mostraram pH ótimo de 2,0 e 5,0, respectivamente. Entretanto, a maior estabilidade foi obtida nos pHs 4,0 e 7,0, respectivamente. Os biocatalisadores mostraram estabilidade na temperatura ótima de 55°C em que a atividade residual foi superior a 87% após 240 min., de incubação. A menor constante de desativação ($k_d$) e o maior tempo de meia-vida do biocatalisador imobilizados demonstraram maior estabilidade térmica do que a obtida pela enzima livre. Os parâmetros constantes de Michaelis ($K_m$) (77 e 115 mm, para as enzimas livre e imobilizada, respectivamente) e velocidade máxima de reação ($V_{máx}$) (1250 e 714 U $mg^{-1}$, para as enzimas livre e imobilizada, respectivamente) indicaram que o processo de imobilização reduziu a afinidade enzima-substrato. Em relação à estabilidade operacional, o biocatalisador demonstrou atividade relativa acima de 50% até sete ciclos de reutilização na hidrólise do azeite. Este novo biocatalisador, obtido a partir de um resíduo de fruta tropical, apresentou características bioquímicas que justificam sua aplicação em estudos futuros de biocatálise.

**Palavras-chave:** resíduo de fruta, sol-gel, biotecnologia, enzima.

## Introduction

Agricultural residues represent large potential resources for use in biotechnological processes, mainly due to their low cost, accessibility and nutrient composition, as they contain carbon, nitrogen and minerals. Particularly, by-products of fruits, composed of peels, rinds, seeds, and unused flesh, generated at different steps of the industrial process, normally have no further use and are commonly wasted or discarded (Ajila, Bhat, & Rao, 2007; Salihu, Alam, Karim, & Salleh, 2012).

Several studies have been performed on the use of agroindustrial residues as substrates in solid-state fermentation systems (SSF) for the production of value-added products such as microbial lipases (Graminha et al., 2008; Malilas et al., 2013;

Mussatto, 2009; Pandey, Soccol, & Mitchell, 2000; Salihu et al., 2012; Santos et al., 2014). These enzymes are important due to their versatility in industrial applications since they catalyze a variety of reactions, such as partial or complete hydrolysis of triacylglycerols and the esterification, transesterification and interesterification of lipids (Colla et al., 2010; Salihu et al., 2012).

The agroindustrial residues utilized for lipase production contain lipid components that act as inducers for enzyme production and other components that provide the needed nutrients for the growth of microorganisms. However, some of the nutrients may be available at sub-optimal concentrations or even absent in the substrate. In such cases, exogenous supplementation becomes necessary (Salihu et al., 2012; Pandey, Selvakumar, Soccol, & Nigam, 1999). Residues such as wheat bran, rice bran, soybean bran, barley bran, soy oil cake, olive oil cake, gingelly oil cake, babassu oil cake, coconut oil cake, sugarcane bagasse and pumpkin seeds have been reported to be effective for lipase production (Edwinoliver et al., 2010; Salihu et al., 2012; Zubiolo et al., 2014).

For industrial scale applications, enzymes in the free form are readily inactivated and are difficult to separate for reuse. The process of immobilizing enzymes is considered a very effective method for using these biocatalysts, due to the improved characteristics such as thermal stability of the enzyme, operational ease of separation of the reaction products and reuse of the system for industrial applications (Chaubey, Parshad, Taneja, & Qazi, 2009; Yilmaz, Sezgin, & Yilmaz, 2011). Many microbial lipases have been immobilized by adsorption, covalent bonding or encapsulation on silica supports obtained by sol-gel technology. The synthesis of sol-gels occurs initially with acid-catalyzed hydrolysis of tetraalkoxysilane (e.g. tetramethoxylsilane, TMOS), followed by condensation and formation of the sol, which is formed from a mixture of partially hydrolyzed and partially condensed monomers. By adding alkyltrialkoxysilanes (methyltrimethoxysilane, MTMS) to the synthesis mixture, sol-gels with a hydrophobic surface can be obtained. The mesoporous structure and high pore volume of these gels allow for the diffusion of low to medium molecular weight species and their free interaction with the enzyme (Hanefeld, Gardossi, & Magner, 2009).

In this context, the aim of this work was the production of *Aspergillus niger* lipase from SSF using mangaba fruit residue, enzyme immobilization on a sol-gel matrix and biochemical characterization of the biocatalyst. The fruit *Hancornia speciosa* Gomes (Apocynaceae), popularly called mangaba, is a plant species found in the Cerrado, a region of savannah-like vegetation in Brazil. This fruit is used to make ice cream, cookies, syrup, juice, wine, alcohol and vinegar (Ferreira, Serra, Lemos, Braga, & Cortes, 2007; Endringer, Pezzuto, & Braga, 2009; Lima et al., 2015).

## Material and methods

### Chemicals

γ-(aminopropyl)triethoxy silane (γ-APTS) was purchased from Sigma. Gum Arabic was obtained from Synth (São Paulo State, Brazil). Olive oil was purchased at a local market. All other reagents were analytical grade.

### Microorganism

The microorganism *Aspergillus niger* IOC 3677 was purchased from a collection of cultures from the Oswaldo Cruz Institute (Rio de Janeiro State, Brazil), preserved in tubes with slanted nutrient agar and stored at 4°C.

### Treatment of agroindustrial residue

Mangaba seeds were dried (Pardal-EP 100) at 60°C for 8h and then crushed in an industrial blender until the particles had an average diameter of 1.06 mm. After that, the meal was sterilized by autoclaving at 121°C for 15 min. The product contained about 25% lipids, as determined by the Soxhlet method Instituto Adolfo Lutz (IAL, 2008)

### Enzyme production

The enzyme was obtained by state solid fermentation of mangaba seeds as described by Zubiolo et al. (2014). The fermentations were conducted in Petri dishes containing 10 g of residue (30% moisture content) and a spore suspension of *Aspergillus niger* ($10^6$ spores $g^{-1}$) at a temperature of 30°C. A Petri dish was removed after each 24h of fermentation and the enzyme was extracted using sodium phosphate buffer (0.1 M, pH 7.0) in a 1:5 ratio (mass:volume), which was stirred at 30°C for 15 min. The material was centrifuged (Eppendorf centrifuge 5804R) at 120 x g for 10 min, yielding the crude enzyme extract. The enzyme was precipitated using ammonium sulfate at 80% saturation and centrifuged at 120 x g for 10 min. The supernatant was filtered, dialyzed using a membrane with a cut-off of 10,000-12,000 Da against ultra-pure water for 24h at 4°C and lyophilized (Wolski et al., 2009).

### Glutaraldehyde activation of sol-gel silica

First, the sol-gel silica was subjected to a silanization process using a mixture of 3 mL γ-APTS (γ -(aminopropyl) triethoxysilane) 0.5% (v $v^{-1}$) with 1 g of sol-gel. The solution was maintained under agitation in a route evaporator for 3h at 75°C. After this, the support was washed with heptane and dried at 105°C for 15h. The silanized sol-gel silica was suspended in 10% glutaraldehyde solution at a ratio of 1:35 (silica:solution). The suspension was stirred at room temperature for 24h and vacuum filtered. Then, the solids were washed with distilled water to remove glutaraldehyde and dried under vacuum (Yang, Wu, Xu, & Yang, 2010).

### Enzyme immobilization

The enzyme was immobilized in the support as described by Soares, Santos, Castro, Moraes and Zanin (2004) with some modifications. An enzyme solution (300 mg $mL^{-1}$) in 100 mM of sodium phosphate buffer, pH 7.0 was prepared. For immobilization, 1 mL of this solution, 9 mL of hexane and 1 g of support were mixed and maintained under agitation for 3h at 25°C. This mixture was kept static overnight at 4°C. Then, the immobilized lipase was recovered by vacuum filtration and thoroughly rinsed with hexane.

### Assay of lipase activity

The enzymatic activity of the free and immobilized lipase was determined by the hydrolysis method using olive oil according to the procedure described by Zubiolo et al. (2014). The substrate was prepared using 50 mL of olive oil and 50 mL of gum Arabic solution (7% w $v^{-1}$). The mixture containing emulsion oil (5 mL), 0.1 M sodium phosphate buffer pH 7.0 (2 mL) and free lipase (100 mg) or immobilized lipase (100 mg) was incubated in a thermostated batch reactor for 5 min at 37°C. The reaction was stopped by the addition of 2 mL of acetone-ethanol-water solution (1:1:1). The liberated fatty acids were titrated using potassium hydroxide solution (0.04 M), using phenolphthalein as the indicator. All reactions were carried out in triplicate. One activity unit was defined in terms of the amount of enzyme required to release 1 μmol of fatty acid per min of reaction, under the experimental conditions (37°C, pH 7.0, 150 rpm). The blank sample was replacing enzyme by distilled water. The coupling yield (η,%) was calculated according to Equation 1 (Soares et al., 2004):

$$\eta\,(\%) = U_s/U_0 \;\; x\,100 \qquad (1)$$

where

$U_S$ is the enzyme activity recovered on the dry support and $U_o$ is the total enzyme units offered for immobilization.

### Effect of pH and stability

The effect of pH was determined through the hydrolysis of olive oil ranging the buffers as follows: sodium citrate buffer 0.1 M pH 2.0, 3.0, 4.0 and 5.0; sodium phosphate buffer 0.1 M pH 6.0, 7.0 and 8.0 and sodium carbonate-bicarbonate buffer pH 9.0, 10.0 and 11.0. The stability was performed by measuring the hydrolytic activity during incubation for 5, 15, 30, 60, 90, 120, 150, 180, 210 and 240 min for the free and immobilized lipase.

### Effect of temperature and thermal stability

The effect of temperature on hydrolytic activity was performed at 30, 37, 40, 45, 50, 55, 60, 65 and 70°C using the selected buffer (optimum pH). The data were analyzed by analysis of variance (ANOVA) using Assistat 7.7 beta software, and the difference among means at a significance level of 5% was performed by tukey's range test.

Then, the stability in optimum temperature was evaluated by incubation of free and immobilized lipase for 05, 15, 30, 60, 90, 120, 150, 180, 210 and 240 min. The residual activities were determined through the hydrolysis of olive oil at 37°C. The enzyme inactivation rate constant ($k_d$) and half-life ($t_{1/2}$) were calculated using Equations 2 and 3, respectively (Yang et al., 2010).

$$A_{in} = A_{in0}\, x\, exp\,(-k_d x\, t\,) \qquad (2)$$

$$t_{1/2} = ln2/k_d \qquad (3)$$

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 immobilized lipase was assayed by using immobilized lipase (100 mg) in successive batches carried out under hydrolysis reaction conditions at 37°C and the optimum pH. Batch tests were carried out for 10 min, using the same mass of immobilized enzyme. Then, the biocatalyst was removed from the reaction medium and rinsed with hexane, in order to extract any substrate or product eventually retained in the support. This procedure was repeated for several cycles.

### Determination of kinetic parameters

The kinetic parameters of the Michaelis-Menten equation (Equation 4) were determined by varying the olive oil concentration from 1 to 70% in the enzymatic hydrolysis reaction. The $K_m$ and $V_{max}$ values were calculated from Lineweaver-Burk plot (Yang et al., 2010) for free and immobilized lipase according to equation 5.

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \quad (4)$$

where

ν and $V_{max}$ are the initial and maximum reaction rates, respectively, [S] is the substrate concentration and $K_m$ is the Michaelis constant.

$$\frac{1}{v} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad (5)$$

## Results and discussion

### Lipase immobilization

The lipase yield was determined every 24h during the fermentation process; the maximum production was 62.5 U $g^{-1}$ obtained at 120h, as shown in Figure 1. It is likely that microbial growth was limited due to the lack of key nutrients such as carbon, nitrogen and phosphate sources after 120h of fermentation, causing a decrease in lipase production (Barrios-González, 2012). This result was better than that obtained by Colla et al. (2010) and Contesini et al. (2009), who showed maximum *Aspergillus niger* lipase production of 25.2 and 33.0 U $g^{-1}$ from soy bran and a mixture of rice bran and wheat bran fermentation, respectively.

The enzyme was efficiently immobilized on the sol-gel support by covalent bonding, with an immobilization yield of 91.6%. Covalent bonding of an enzyme to a carrier has the advantage that the enzyme is tightly fixed, minimizing leaching into the aqueous medium. As a consequence, this type of immobilization should be preferred when working in aqueous solution and when denaturing factors exist. Shorter spacers used for lipase immobilization, such as glutaraldehyde used in this work, can confer greater thermal stability since they restrict enzyme mobility and prevent unfolding. Furthermore, the fact that lipase was immobilized on a sol-gel matrix can have a very positive influence on the catalytic reactivity, since the enzyme might remain its active conformation (Hanefeld et al., 2009).

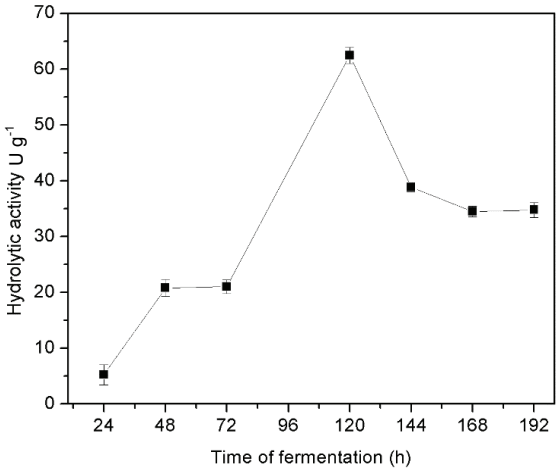


**Figure 1.** Lipase production during solid-state fermentation of "mangaba" residue.

### Effect of pH on hydrolytic activity and stability

The pH is an important factor that influences the hydrolytic activity of both free and immobilized enzymes, since it can change the performance of the support, the enzyme conformation and the enzymatic reaction. Figure 2 shows the relative activity of biocatalysts at different pH levels of the reaction medium, ranging from 2.0 to 11.0. The maximum relative activity (100%) of the free lipase was at pH 5.0, around 85% at pH 6.0 and 7.0 and from 40 to 50% at pH 8.0 to 11.0. According to Figure 3, the free enzyme showed good stability at pH 5.0, 6.0 and 7.0, with relative activity values above 80% for up for 240 min, of incubation. These results are in accordance with the literature, since lipases from *Aspergillus* sp. have been shown to have an optimum pH between 2.5 and 6.0 and greater stability between pH 7.0 and 11.0 (Contesini, Lopes, Macedo, Nascimento, & Carvalho, 2010; Mahadik, Puntambekar, Bastawde, Khire, & Gokhale, 2002; Mhetras, Bastawde, & Gokhale, 2009; Zubiolo et al., 2014).

On the other hand, when the enzyme was immobilized on the sol-gel matrix by covalent bonding, the maximum relative activity (100%) was obtained at pH 3.0, and remained above 50% until pH 7.0 (Figure 2). The immobilized lipase also showed excellent stability at pH 2.0, 3.0 and 4.0 with relative activities above 70% after 240 min, of incubation (Figure 4). This means that the biocatalyst may be used under more acidic reaction conditions than those supported by the enzyme in its free form. This may be due to the fact that the lipase molecules were covalently bonded to the silica surface, preventing the denaturation of the enzyme under extreme pH conditions (Pandya, Jasra,

Newalkar, & Bhatt, 2005). A similar result was obtained by Zubiolo et al. (2014), who showed that lipase from *Aspergillus niger* (obtained from pumpkin residue SSF) encapsulated in a sol-gel matrix also showed maximum relative activity at pH 3.0.

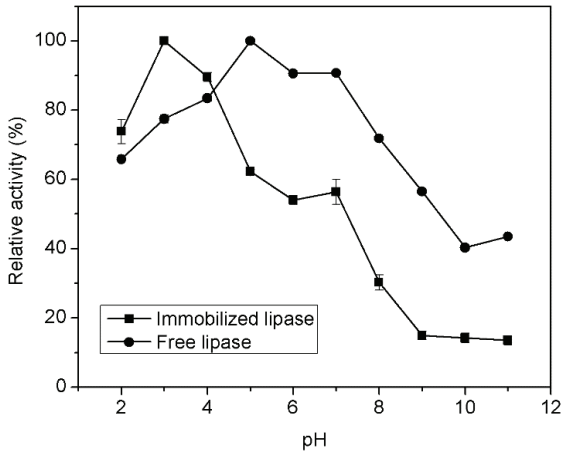


**Figure 2.** Effect of pH on the relative activity of the free and immobilized lipase.

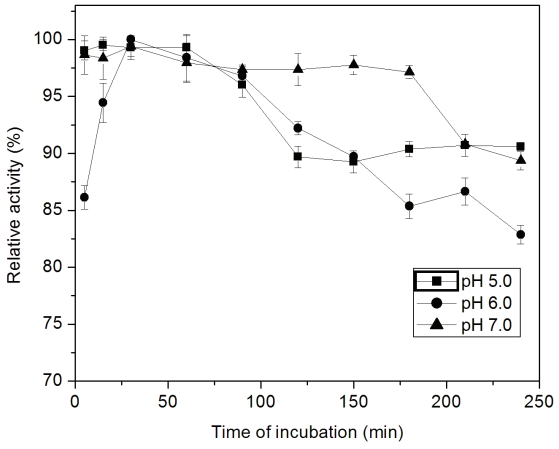


**Figure 3.** pH stability of free lipase.

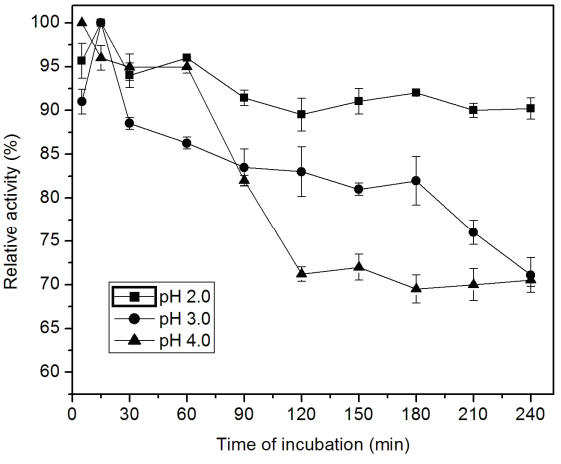


**Figure 4.** pH stability of immobilized lipase.

## Effect of temperature on hydrolytic activity and thermal stability

The influence of temperature was determined at the optimum pH 5.0 and 3.0 for the free and immobilized lipase, respectively. The maximum relative activity (100%) was obtained at a temperature of 55°C and remained above 50% until 70°C for both biocatalysts. Also, the statistical analysis showed that the relative activities of biocatalysts did not differ statistically ($p > 0.05$) until 55°C (Figure 5). This result was better than that obtained by Zubiolo et al. (2014), who showed that the lipase of *Aspergillus niger* from pumpkin seed SSF had an optimum temperature of 37 and 45°C for the free and sol-gel encapsulated enzyme, respectively.

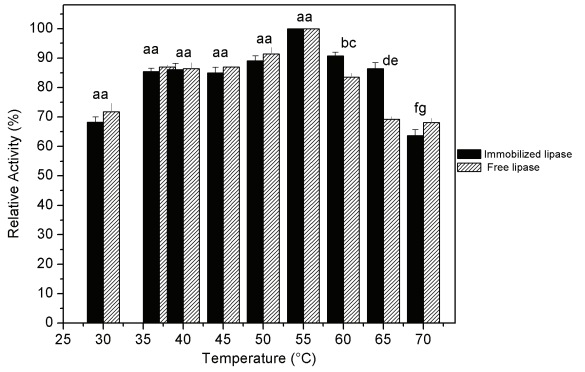


**Figure 5.** Effect of temperature on the relative activity of free an immobilized lipase. a-g for each temperature, different letters indicate significant differences ($p < 0.05$) between the mean values according to tukey's test.

The biocatalysts showed excellent stability at 55°C, since the relative activities were above 87% and 92% for the free and immobilized lipases, respectively, after 240 min, of incubation (Figure 6). This result agrees with studies on lipases from *Aspergillus* sp., which have shown optimum temperatures between 30 and 60°C (Contesini et al., 2010; Mhetras et al., 2009; Zubiolo et al., 2014). The $k_d$ and $t_{1/2}$ values of $0.01h^{-1}$ and 63h, respectively, (correlation coefficient ($R^2$) = 0.90) for the immobilized lipase also suggest higher thermal stability than the free lipase ($k_d$ and $t_{1/2}$ values of $0.02h^{-1}$ and 35h, respectively, $R^2$ = 0.93). These results suggest that immobilization protected the tertiary structure of the enzyme by preventing denaturation at elevated temperatures (Balcão, Paiva, & Malcata, 1996; Yang et al., 2010; Uyanik, Sen, & Yilmaz, 2011). Nevertheless, the lipase covalently immobilized on the sol-gel showed a much longer half-life than that of the lipase of *Aspergillus niger* (obtained from pumpkin seed SSF) encapsulated on

a sol-gel ($t_{1/2}$ = 13.6h at 45°C) (Zubiolo et al., 2014) and the lipase of *Candida antarctica* immobilized on a sol-gel by covalent bonding ($t_{1/2}$ =1.9h at 40°C) (Yang et al., 2010).

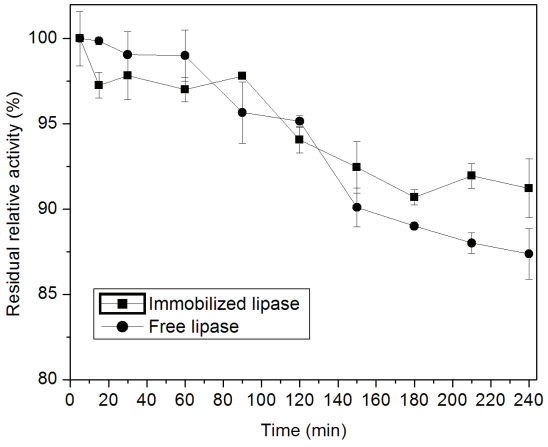


**Figure 6.** Thermal stability of the free and immobilized lipase at 55°C.

### Kinetic parameters

The initial reaction rates of the hydrolysis of olive oil were evaluated for the free and immobilized lipases using different substrate concentrations (1-70%). The Michaelis-Menten constant ($K_m$) and maximum rate of reaction ($V_{max}$) of the free and immobilized lipases were calculated from the Lineweaver-Burk plot (Table 1). The $K_m$ of the immobilized lipase was 1.5 times higher and the $V_{max}$ was 1.8 times lower than the values for the free enzyme, indicating a lower affinity for the substrate. It is likely that immobilization by covalent bonding caused conformational changes in the three-dimensional structure of the enzyme, which hindered substrate access to the immobilized enzyme active site, consequently reducing the maximum reaction speed when compared with the free lipase (Hiol et al., 2000; Yang et al., 2010). On the other hand, both biocatalysts showed higher affinity for the substrate than the free ($K_m$ = 45.38 mM and $V_{max}$ = 526.31 U $mg^{-1}$) and encapsulated sol-gel ($K_m$ = 57.05 mM and $V_{max}$ =1680.67 U $mg^{-1}$) lipase from *Aspergillus niger* (obtained from pumpkin seed SSF) (Zubiolo et al., 2014).

**Table 1.** Kinetic parameters for the free and immobilized lipases.

| | Parameters | |
|---|---|---|
| | $K_m$ (mM) | $V_{max}$ (U $mg^{-1}$) |
| Free lipase[1] | 77 ± 2 | 1250 ± 65 |
| Immobilized lipase[2] | 115 ± 4 | 714 ± 16 |

[1]Correlation coefficient ($R^2$) = 0.90; [2]Correlation coefficient ($R^2$) = 0.88.

### Operational stability

For large-scale applications of an enzyme, the reuse of the biocatalyst is of great significance in terms of process economy (Kharrat, Ali, Marzouk, Gargouri, & Karra-Chaabouni, 2011). The immobilized lipase was reused repeatedly for olive oil hydrolysis using sodium citrate buffer (pH 3.0) and a reaction temperature of 37°C. The relative activity was maintained above 90% in the first four cycles and above 50% until seven cycles of reuse (Figure 7). The biocatalyst showed better operational stability than that of lipases from *Bacillus* sp. ITP-001 (50% relative activity until two cycles of reuse) (Souza et al., 2012) and *Aspergillus niger* (50% relative activity until six cycles of reuse) encapsulated in a sol-gel matrix (Zubiolo et al., 2014).

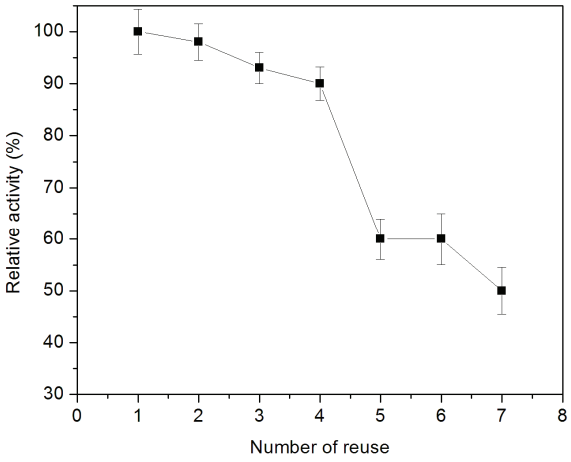


**Figure 7.** The relative activity of immobilized lipase as a function of reuse numbers.

## Conclusion

The lipase of *Aspergillus niger* obtained from mangaba residue solid-state fermentation was efficiently immobilized on a sol-gel support by covalent bonding (immobilization yield of 91.6%). The immobilization process provided the enzyme with higher stability in acidic pH in contrast to the free lipase, with better performance at pH levels close to neutrality. Both biocatalysts showed good stability at the optimum temperature of 55°C (after 240 min, of incubation). However, the greater thermal stability of the immobilized biocatalyst was demonstrated by its lower thermal deactivation constant ($k_d$) and longer half-life than the free enzyme. The kinetic parameters indicate that, probably due to conformational changes, the immobilized lipase had lower affinity for the substrate than the free lipase. On the other hand, the

biocatalyst showed good operational stability in the hydrolysis of olive oil, since the relative activity remained above 50% until seven cycles of reuse. Using SSF, the production of a biocatalyst from mangaba fruit residue, with similar biochemical characteristics to conventional catalysts, could add value to this residue and reduce the final production cost of the enzyme.

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