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Immobilization of *Beauveria bassiana* Lipase on Silica Gel by Physical Adsorption

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ABSTRACT

Extracellular lipase from Beauveria bassiana *strain CG481 was immobilized by using thirteen different immobilization protocols. Silica gel was chosen as the most suitable adsorbent with 94.8% of activity yield. The adsorption on silica gel did not change the optimum pH (8.5) and temperature (45ºC) values of the free lipase (FL) for lipolytic activity, and it showed higher activities in extreme conditions (pH 9.0 to 10.5, 60ºC). The lipase immobilized on silica gel (ILS) showed enhanced stability at pH 7.0 after 120 h incubation (69.0%) when compared to FL (33.3%). The thermal stability was also enhanced by immobilization at 60ºC in aqueous (64.6%) and organic medium (95.1%), while FL showed only 40.6% of residual activity in aqueous medium and exhibited no activity for esterification reaction in n-heptane. The treatment of ILS with 0.8 M NaCl prevented lipase desorption while Triton X-100 (0.1%) resulted the enzyme leakage. The ILS was reused for four times for esterification reaction with 80.8% of initial activity.*

Key words: immobilization, lipase, *Beauveria bassiana*, silica gel

INTRODUCTION

Enzyme immobilization offers numerous advantages as recovery of biocatalyst for reuse and enables its application in continuous process with separation of reaction products without enzyme contamination. Furthermore, immobilized enzymes generally show increased stability under operational condition, heat, organic solvent and extreme pH values (Mateo et al. 2007; Kumari et al. 2008; Nicolic et al. 2009). The support for immobilization must be carefully selected because its characteristics (nature, reactive group and interaction forms with enzymes) can affect the activity of the enzyme (Knezevic et al. 2004). Adsorption on solid supports is a simple and inexpensive method for enzyme immobilization and it is more suitable for use in organic solvents than aqueous solutions (Wu et al. 2007). In this case, porous inorganic materials, such as silica gel, have the advantage of large surface for enzyme retention and protection within the pores (Nicolic et al. 2009).

Lipases (glycerol ester hydrolases, E.C.3.1.1.3) are considered the most promising group of biocatalysts due to their ability to act in different types of reactions as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis (Hasan et al. 2009). They can act on a wide range of substrates and show interesting properties such as regio- and stereo-specificity, and chemoselectivity that allows their several applications, including the production of detergents, wastewater treatment, textile and leather tanning (Castro-Ochoa et al. 2005; Fernandez-Lorente et al. 2008). Lipases on immobilized forms have been successfully applied in organic catalysis production of flavorings,

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pharmaceuticals and agrochemicals (Abbas and Comeau 2003). However, as with other enzymes used in industrial operations, the use of lipases can be limited by the high cost of production and purification, difficulties of enzyme recovery at the end of process and low enzyme stability (Chaubey et al. 2006) that can be solved by immobilization techniques.

Studies involving the enzymes of entomopathogenic fungi have been mostly focused on biological control of insects. However, Cruz et al. (2009) showed good results for the activity and stability of *B. bassiana* lipase, making it interesting for biotechnological applications. The use of entomopathogenic fungus *B. bassiana* has the advantage of being harmless to humans and animals and is easily found in the environment (Health Canada 2010).

The present work evaluated different carriers and techniques for the immobilization of extracellular lipase produced by *B. bassiana* and studied the effect of adsorption on silica gel on enzyme activity and stability.

MATERIAL AND METHODS

Material

Silica gel (pore size 60 Å, 15 µm), celite 545, $CaCO_3$, Al_2O_3, $CaSO_4.2H_2O$, sodium alginate, gelatin, polyvinyl alcohol and bovine serum albumin were purchased from Sigma Chemical (St. Louis, USA). Amberlite MB-1 was purchased from Fisher Scientific Co. (Pittsburgh, USA). Dowex 50x8 was obtained from Dow Co. (Midland, USA). Olive oil (low acidity) was purchased locally. All the other substances were of analytical grade.

Microorganisms

Eight *B. bassiana* strains were used: CG71, CG152, CG432, CG481, Unioeste4, Unioeste40, Bbio44 and Bbio61. All of them were isolated from different hosts and geographic regions of Brazil and were granted by Department of Agronomy of UEL and Biological Institute of São Paulo. The frozen spores (-20°C) were subcultured in solid medium containing (%) 1.0 D-glucose, 0.5 yeast extract, 0.16 $NaNO_3$, 0.11 $Na_2HPO_4.7H_2O$, 0.1 KCl, 0.06 $MgSO_4.7H_2O$, 0.036 KH_2PO_4, 2.0 agar (Alves 1998). The plates were incubated at 27 ± 1°C, 12 h photoperiod and 70 ± 5% of humidity for 14 days.

Production and ultrafiltration of lipase

Extracellular lipase was obtained by submerged cultures in 250 mL Erlenmeyer flasks containing 25 mL of Vogel (1956) salts broth, supplemented with 1% olive oil, 1.5% urea, 0.1% $CaCl_2.2H_2O$ and 0.1% Triton X-100. All the cultures were inoculated with 10^6 spores/mL and incubated at 28°C and 200 rpm (orbital shaker) for four days. The crude lipase extracts were obtained by centrifugation of the cultures (8000 g, 4°C, 15 min) and were dialyzed against 5.0 mM phosphate buffer (pH 7.0) at 4°C during 24 h (Cruz 2009).

For immobilization studies, four cultures of *B. bassiana* CG481 with twenty-four repetitions each were performed. The lipase extracts were concentrated three times by ultrafiltration using membrane cut off 100 kDa (Millipore) in Stirred cell (Sigma) at 4°C and denominated as free lipase (FL).

Screening of carriers for lipase immobilization

Thirteen different protocols for lipase immobilization were employed. Except for entrapment technique, 20 mL of FL (containing 54 U/mL lipase activity and protein at 0.35 mg/mL) and 10 mL of 50 mM phosphate buffer (pH 7.0) (immobilization buffer) was added to 1.0 g of each support. The immobilization was carried out into 100 mL glass vessel placed on a magnet stirring plate at 200 rpm and 25°C during 1 h. The immobilized lipase was washed two times using the same immobilization buffer, separated by vacuum filtration, dried in desiccator at 25°C and stocked at -20°C. Lipase immobilization by physical adsorption was carried by five inorganic carriers - Al_2O_3, $CaCO_3$, $CaSO_4.2H_2O$, celite 545 and silica gel, using the same technique described above, but modified by including 15 mL of acetone in the last 30 min of incubation.

The synthetic ion-exchange resins Amberlite MB-1 and Dowex were tested for ionic binding of lipase. Both carriers were equilibrated overnight in 50mM phosphate buffer (pH 7.0) before contact with FL. The same supports were also tested with pretreatment with methanol (2 h, 200 rpm, washed two times with 50 mM phosphate buffer, pH 7.0) before lipase addition. Amberlite MB-1 and Dowex were also used for covalent binding of lipases by addition of 2.5% glutaraldehyde in immobilization buffer (1 h, 200 rpm) prior to lipase contact with the carriers.

Lipase was immobilized by gel entrapment by two techniques. The first one used a mixture of 10 mL

of FL and 40 mL of 2% sodium alginate in 50 mM TRIS-HCl buffer (pH 7.0). The second technique used 10 mL of FL and 90 mL of 5% sodium alginate and 3% gelatin blend in 50 mM TRIS-HCl buffer (pH 7.0), mixed to 3.0 mL 25% glutaraldeyde. In both the cases, after 30 min incubation at 200 rpm on a magnet stirring plate, the mixture was dripped with a pipette into a 2% $CaCl_2$ solution at 4ºC. The gel beads formed were maintained into $CaCl_2$ solution until used.

Immobilization yield (*Y*) was determined by calculating the hydrolytic activity of lipase before and after immobilization using the equation as below:

$$\text{Yield (\%)} = (\text{immobilized lipase activity/ initial lipase activity}).100$$

Protein retention (*PR*) was estimated indirectly by the difference between the amount of protein introduced for immobilization and the amount of protein in the filtrate and washing solution and calculated as follows:

$$\text{Protein retention (\%)} = [1\text{-(total protein on filtrate / total protein added)}].100$$

Lipolytic activity determination

Lipase activity was determined by titrimetric method (Watanabe et al. 1977) with some modifications. Emulsified substrate was prepared by agitation at 200 rpm for 1 min in 125 mL Erlenmeyer flasks; it consisted of 5% olive oil and 2% polivinylic alcohol (PVA) as emulsifier in 50 mM TRIS-HCl buffer (pH 8.5) containing 50 mM NaCl and 5 mM $CaCl_2$ to obtain 20 mL final volume. The hydrolysis reaction was initiated by adding of 1.0 mL of FL or 50 mg of immobilized lipase and incubated at 37ºC at 200 rpm in a orbital shaker during 2 h and was stopped by adding 20 mL acetone-ethanol (1:1). The amount of free fatty acid released during the hydrolysis was measured by titration with 0.01 N NaOH solution using thymolphtalein as indicator. One unit (U) of lipolytic activity was defined as the amount of enzymes that liberated one µmol free fatty acid from olive oil per hour under the assay conditions. All lipolytic assays were running in triplicate and with a parallel control without addition of enzyme under the same conditions.

Esterification reactions

Esterification reaction was carried out to determine the catalytic activity of immobilized lipases in organic medium and was performed in screw-capped flasks: 50 mg of lipase immobilized on silica gel were added to 5.0 mL of a mixture composed by 0.1 M oleic acid and 0.1 M 1-butanol in n-heptane at 37°C and 200 rpm during 24 h (Ghamgui et al. 2007). The reaction was stopped by adding 5.0 mL of acetone:ethanol (1:1) and immobilized enzyme was separated by centrifugation at 8000*g* for 10 min. The supernatant obtained was measured for the residual oleic acid by titrimetric method. The tests were conducted in three replicates and the controls were run without the addition of enzyme under the same conditions. One unit (U) of esterification activity was defined as one µmol oleic acid consumed in the esterification reaction per hour under the assay conditions.

Protein estimation

Protein was estimated according to Bradford (1976) method using bovine serum albumin as standard.

Effect of pH and temperature on lipase activity

Effects of pH and temperature on FL and immobilized lipase on silica gel (ILS) activities were tested using 50 mM buffers at pH range from 3.0 to 10.0: sodium acetate buffer (pH 3.0, 4.0 and 5.0); maleate buffer (pH 6.0); TRIS-HCl buffer (pH 7.0, 8.0, 9.0); glycine-NaOH buffer (pH 10.0), and at 30, 40, 50 and 60°C by lipolytic method.

Effect of pH and temperature on lipase stability

pH stability of the FL and ILS was determined by measuring the residual lipolytic activity after preincubation of samples at pH 6.0, 7.0, 8.0 and 9.0 for 24, 48, 72 and 120 h. The thermal stability was tested at 30, 40, 50 and 60ºC for 1 h of incubation in aqueous medium (TRIS-HCl buffer pH 8.5) for FL and ILS, and organic medium (n-heptane) only for ILS. The residual activity was assayed by lipolytic and esterification methods. For all stability tests, the untreated enzyme was taken as 100% activity (control).

Effect of NaCl and Triton X-100 on ILS desorption

In order to investigate the nature of interaction between lipase and silica gel, 0.1 g ILS was stirred magnetically at 200 rpm in 20 mL 0.1 M phosphate buffer (pH 7.0) containing 0.8 M NaCl or 0.1% (v/v) Triton X-100. After 30 and 60 min, the suspension was centrifuged at 8000*g* for 15

min and the precipitate was assayed for lipolytic activity.

Operational stability

ILS (0.2g) was assayed by successive batches of esterification reaction. After each cycle of 24 h, ILS was washed with n-heptane, separated by centrifugation at 8000 g for 10 min and reused in a fresh medium.

RESULTS AND DISCUSSION

Lipase production

Among eight *B. bassiana* tested, the strain CG481 showed highest lipolytic activity (30.5 U/mL). This strain was selected as the best lipase producer and used for immobilization studies. CG71 and Bbio61 presented intermediary lipolytic activities of 16.8 and 15.5 U/mL, respectively; the other strains, Bbio44, Unioeste40, CG432, CG152 and Unioeste4 exhibited lower lipolytic activities of 7.3, 2.0, 1.5; 1.2; 0.1 U/mL, respectively (Fig. 1). Hegedus and Kachatourians (1988) found an increase of lipase production by *B. bassiana* strain GK2116 in submerged cultivations in yeast-peptone-dextrose culture medium supplied with 5% olive oil. Cruz et al. (2009) reported the optimal condition for *B. bassiana* CG432 lipase production in Vogel salt broth with 0.75% olive oil and without addition of carbon source (D-glucose), evaluated by response surface methodology.
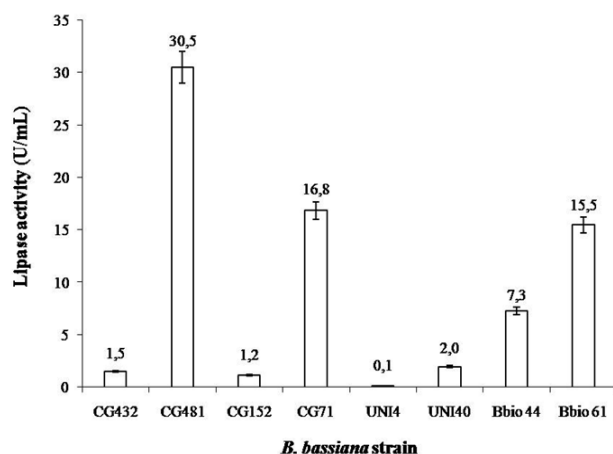

Figure 1 - Production of extracellular lipase from *B. bassiana* by different strains in Vogel salt broth added by 1% olive oil, 1.5% urea, 0.1% CaCl$_2$.2H$_2$O and 0.1% Triton X-100 during 4 days. Lipolytic activity measured by titrimetric method on olive oil substrate.

Screening of supports for immobilization

In order to select a suitable support for lipase immobilization, thirteen immobilization protocols were tested, including adsorption, ionic and covalent binding, and gel entrapment mechanism. Results showed high protein retention (*PR*) among all the carriers used for lipase adsorption, but the lipolytic activity did not correspond to protein loading in the most of cases. Immobilization on silica gel showed the highest immobilization yield (*Y*) of 94.8% while the others carriers presented yield lower than 50% (Table 1). Mustranta et al. (1993) observed similar effects where celite-immobilized lipase exhibited negligible activity despite the high protein retention. Minovska et al. (2005) reported high performance for immobilization of *Candida rugosa* in CaSO$_4$.2H$_2$O (91%) because its fine powder structure with a wide adsorption area. Alloue et al. (2008) found good yield for *Yarrowia lipolytica* lipase adsorption on silica gel (43%) among five carriers tested.

Immobilization of *B. bassiana* lipase by ionic bonds showed very low *Y* (4.7%) and *PR* values (Table 1), but when previously treated with methanol, Amberlite MB-1 showed an increase of 2.6 times of yield (12.4%). Immobilization on Dowex presented no activity retention in both the cases, with or without pretreatment with methanol, but the addition of glutaraldehyde to form covalent bridges between lipases and support favored the immobilization on the Dowex resin, showing 1.2% increase. In this case, immobilization on Amberlite MB-1 exhibited intermediate result of 8.0% or 1.7 times higher than the test without any treatment.The entrapment of lipase on sodium alginate gel showed 100% value of *PR* but without activity detection. One factor that might have influenced it could be the gel pore size that hampered the substrate access to enzyme when the pore was reduced, causing the diffusion effect. The porosity of these gels can be controlled by the concentration of alginate in the gel (Won et al. 2005). However, the addition of gelatin and glutaraldehyde forming alginate-lipase and lipase-lipase bridges (crosslinks) favored the retention of lipase within the gel matrix (*Y*=29.8%). As reported by Fadnavis et al. (2003), the electrostatic interaction between the carboxylic acid groups of alginate and gelatin amino groups allowed the formation of a resistant hydrogel.

Among the thirteen protocols tested, *B. bassiana* lipase adsorption on silica gel presented the best

result of immobilization yield. It is porous, inexpensive and sturdy support (Christensen et al. 2003; Cruz et al. 2009). Hence, silica gel was selected for kinetic and stability experiments.

Table 1 - Immobilization yield and protein retention of *Beauveria bassiana* CG 481 lipase on different types of carriers and interactions.

Interaction	Carrier	Immobilization yield (%)	Protein retention (%)
Adsorption on inorganic carrier	Al_2O_3	0.6	95.1
	$CaCO_3$	1.1	99.6
	$CaSO_4.2H_2O$	27.6	98.5
	Celite	0.0	62.7
	Silica gel	94.8	55.1
Ionic binding	Amberlite MB-1	4.7	54.2
	Dowex	0.0	40.6
	Amberlite treated by methanol	12.4	71.6
	Dowex treated by methanol	0.0	52.0
Covalent binding	Amberlite MB-1+ glutaraldehyde	8.0	79.1
	Dowex + glutaraldehyde	1.2	41.6
Entrapment	Sodium alginate	0.0	100.0
	Sodium alginate/ gelatin + glutaraldehyde	29.8	100.0

Immobilized lipase activity measured by titrimetric method on olive oil substrate. Protein determination by Bradford method.

Effect of pH and temperature on the activity of FL and ILS

Results showed the optimum lipolytic activity at pH 8.5, both for FL and ILS. However, ILS showed higher activities at pH 9.0, 9.5 and 10.0 compared to FL that showed sharp drop in lipolytic activity and no activity was detected at pH 9.5 and 10.0 (Fig. 2). The immobilized and FL showed maximum activity at 45°C. However, with increasing temperature, FL showed a tendency to rapid activity decrease, while immobilized lipase showed slower decrease of activity. At 60°C, FL showed only 59.29% of its optimum activity, while immobilized one remained 79.13% activity (Fig. 3). Similar results were obtained by Minovska et al. (2005) for lipase from *C. rugosa* immobilized on Amberlite IRC50 and Vemuri et al. (1998) for lipase from *Pseudomonas* sp. on alginate gel. These results indicated that the stability was improved at higher pH and temperature by immobilization. Probably, the interaction between lipase and silica gel delayed the enzyme denaturation, showing greater activity in relation to the free enzyme (Cao 2005). Another possibility for the increase in activity of ILS at pH above the optimum value was the accumulation of negative charges on the surface of silica gel, with attraction of H^+ ions near the support, making the apparently more acidic microenvironment (Zanin and Moraes 2004).
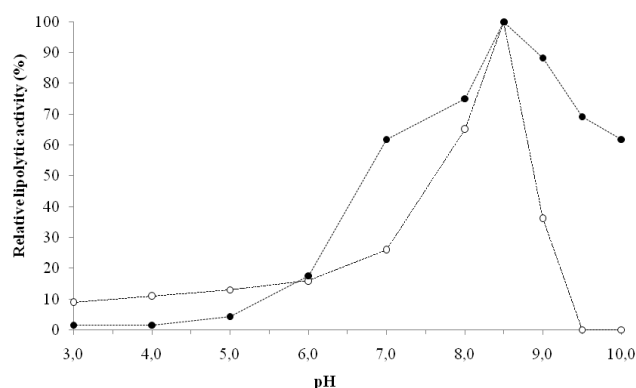

Figure 2 - pH effect on free lipase FL (○) and silica gel immobilized lipase ILS (●) activity. Lipolytic activity measured by titrimetric method on olive oil substrate, at 37ºC.
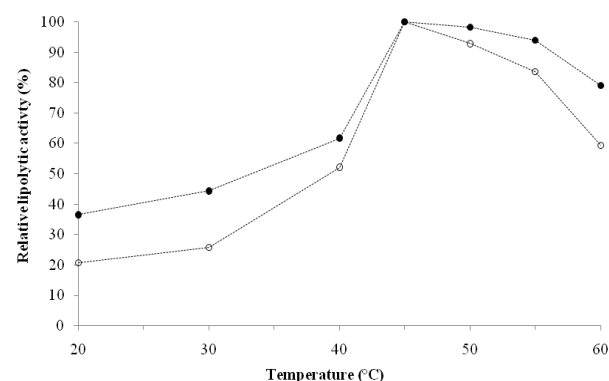

Figure 3 - Temperature effect on FL (○) and ILS (●) activity. Lipolytic activity measured by titrimetric method on olive oil substrate, at pH 8,5.

Effect of pH and temperature on the stability of free and immobilized B. *Bassiana* lipase on silica gel

A similar kinetic behavior was observed here for the stability of FL at different pH values tested (6.0 to 9.0), presenting decrease of 50% or more of initial activity after 120 h (Fig. 4). ILS showed increased stability when incubated at pH 7.0 after 72 h (92.9%) up to 120 h (69.0%) while FL showed only 66.7 and 33.3% of activity in the same period. At pH 6.0 and 8.0, FL and ILS stabilities were very similar. However, at pH 9.0, a significant decrease was observed in the stability of ILS.
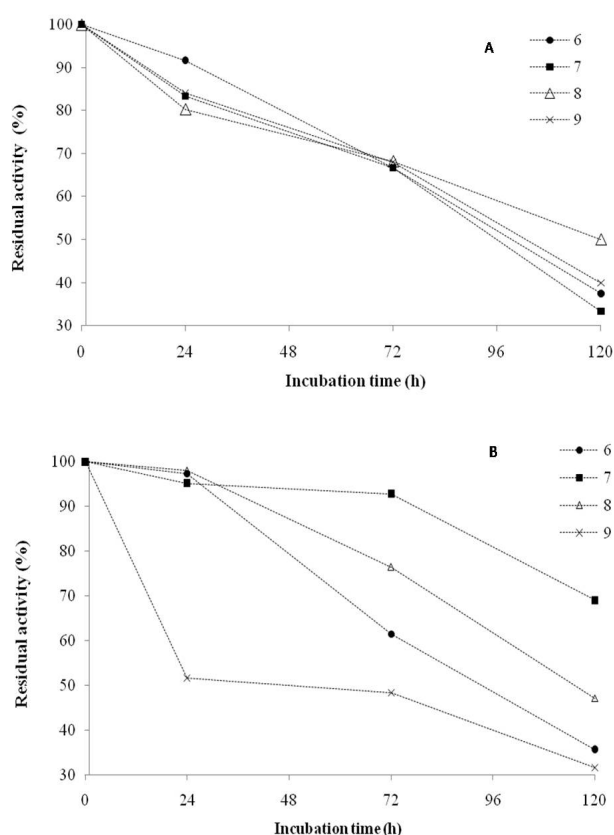

only 40.6% of activity after 1 h, while ILS retained 64.7% in aqueous medium and 95.1% in n-heptane. At 30°C incubation in n-heptane, ILS showed an increase of esterification activity to 173.2%. Lyophilized FL did not show esterification activity. These results suggested that adsorption interactions between lipase and support promoted the thermal stability of the enzyme, preventing denaturation and changes in enzyme conformation in both aqueous and organic medium. The use of organic solvents as reaction media provides many attractive advantages for industrial process compared to traditional aqueous medium systems, such as increased solubility of hydrophobic substrates, often enhanced thermostability and elimination of microbial contamination (Doukyu and Ogino 2010).

Silica gel presents a pore size generally between 6 and 15 nm favoring the localization of lipase inside the silica gel micropores. It has been described with size of 6.88 nm x 6.85 nm x 5.2 nm (Ge et al. 2007), which probably protected the enzyme against microenviroment alterations. Similar results was obtained by Nicolic et al. (2009) after incubation of *C. rugosa* lipase at 50°C during 1 h, where the FL enzyme showed only 21% of initial lipase activity while the lipase immobilized on silica retained 84% in aqueous medium.
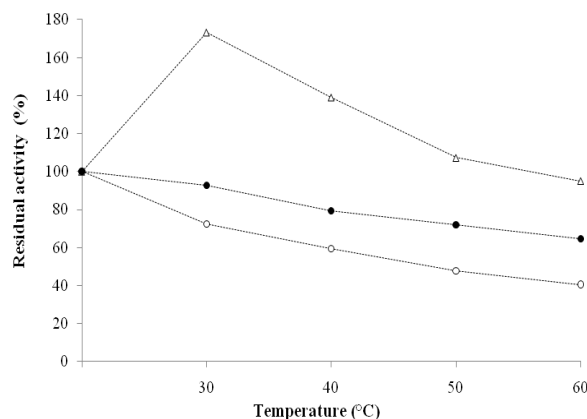

Figure 4 - pH effect on stability of FL (A) and ILS (B) at different incubation time at 25°C. Lipolytic activity measured by titrimetric method on olive oil substrate.

Thermal stability is a very important feature for the industrial application of the enzymes and the immobilization promotes, in most cases, a significant increase in this parameter (Cao 2005). At all the temperatures examined, the adsorption increased the thermal stability of ILS in aqueous and organic medium (Fig. 5). At 60°C, FL retained

Figure 5 - Thermal stability of FL (○) and ILS (●) in aqueous medium (TRIS-HCl buffer pH 8,5), and ILS (Δ) in organic medium (n-heptane). Activity measured by titrimetric method on olive oil substrate (aqueous medium) and esterification method (n-heptane).

The addition of acetone during the immobilization might have promoted the thermal stability of the

enzyme, probably acting in the withdrawal of water around the enzyme molecule as reported by Wu et al. (2007) for *Mucor javanicus* lipase immobilization on Amberlite XAD-7, resulting in increased enzyme stiffness resistant to thermo-deactivation.

Effect of NaCl and Triton X-100 on ILS desorption
After 30 min of incubation in phosphate buffer, ILS showed 12.1% of lipolytic activity decrease (Table 2). However, ILS treated with 0.8 M NaCl hampered lipase desorption with decrease of only 2.2% and 3.8% of lipolytic activity after 30 and 60 min, respectively. The presence of 0.1% Triton X-100 facilitated the desorption of lipase with decrease of 33.7 and 37.0% of activity after 30 and 60 min, respectively. These results agree with Nikolic et al. (2009), who obtained similar results with *C. rugosa* lipase adsorbed on silica gel. The desorption prevention by NaCl could be explained by the fact that sodium ions helped to reduce the negative charges of silica gel (isoelectric point 2,5), resulting in less repulsion between the molecule and the lipase carrier (Tohver et al. 2001). Triton X-100 acts as the opposite, improving desorption because of the competition with lipases for hydrophobic regions of silica gel (Palomo et al. 2004). These results suggested that the nature of adsorption of *B. bassiana* lipase on silica gel was hydrophobic probably by interfacial activation (Fernandez-Lorente et al. 2008).

Table 2 - Effect of the environment on desorption of *B. bassiana* CG481 lipase immobilized on silica gel.

Time of incubation (min)	Desorption of lipase (%)		
	0.1 M phosphate buffer pH 7.0	0.8M NaCl	0.1% (v/v) Triton X-100
30	12.1	2.2	33.7
60	13.8	3.8	37.0

Operational stability
Adsorbed lipase shows a great reuse capacity in organic solvents because of the less leakage of enzyme from support (Kumari et al. 2008; Doukyu and Ogino 2010). The operational stability can make the process feasible despite the high costs of production, purification and immobilization of enzymes (Mateo et al. 2007). The ILS could be reused up to four cycles with 80.8% of initial activity (Fig. 6). However, after five cycles of esterification reaction, the activity decreased to

53.8%. This reduction of activity probably occurred due to lipase desorption from silica gel during repeated use. Kumari et al. (2008) reported 90% of activity retention after four cycles of isoamyl acetate synthesis by *Enterobacter aerogenes* lipase immobilized on silica gel by cross-link method.
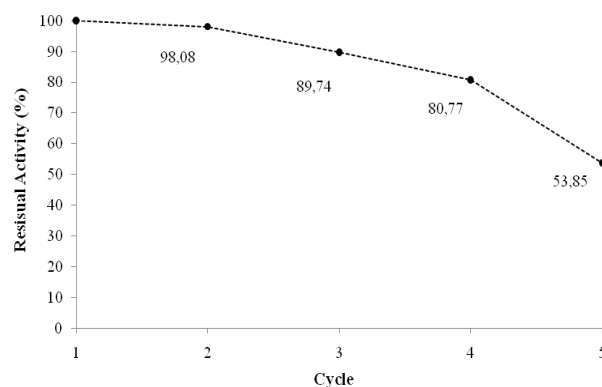

Figure 6 - Operational stability of ILS. Activity measured by esterification method with 24 h incubation each cycle.

CONCLUSIONS

The adsorption of *B. bassiana* CG481 lipase on silica gel enhanced the lipolytic activity at higher pH and temperature conditions and showed improvement of stability in organic solvents. This is the first study involving the immobilization of lipase produced by *B. bassiana,* showing the advantage of being safe for human health and opens new perspectives for the use of *B. bassiana* lipase in industrial catalysis and further studies with other immobilization carriers.

ACKNOWLEDGEMENTS

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