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Immobilization of *Penicillium citrinum* Lipase on Ferromagnetic Azide-Dacron

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Abstract: The immobilization of *Penicillium citrinum* lipase by covalent bond onto ferromagnetic azide-Dacron showed 73% of specific lipase activity retention with 7.3 mg protein/g support. There was no change in either optimal temperature (37°C) or pH (8.0-8.5) after immobilization. The thermal stability of ferromagnetic azide-Dacron-*Penicillium citrinum* lipase derivative was significant, retaining 86.8% of lipolytic activity while the soluble enzyme retained only 34.2% after 1 h at 45°C, this represented an improvement of 154% in the thermal stability. This lipase did not show inhibition by isopropanol. Both soluble and immobilized *Penicillium citrinum* lipases showed a kinetic of Michaellis-Menten to hydrolysis of 4-nitrophenyl palmitate of (4NPP) at 37°C and pH 8.0. The values of K_M were for soluble enzyme of 233 μM and immobilized derivative of 276 μM. This immobilized derivative showed a good operational stability keeping about 75% of initial activity after its reuse for 5 times. Also, it was stable to storage at 4°C in Tris-HCl buffer pH 7.2 with a half-life of 25 days. Immobilized *Penicillium citrinum* lipase was able to catalyse the synthesis of triolein using glycerol and olive oil as substrates free of organic solvent, with 80% of conversion after 20 h at 40°C.

Key words: Lipases, *Penicillium citrinum*, Ferromagnetic azide-Dacron, triacylglycerol acyl-hydrolases

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture and the production of cosmetics. However, the major commercial application for lipases is their use in laundry detergents, which account for about 32% of the total lipases sales. An estimated 1000 tons of lipases are added to approximately 13 billion tons of detergents produced each year. The expectation is that lipases will be as industrially important as the proteases and carbohydrases are currently (Sharma *et al*., 2001).

In addition, lipases are presently considered as choice tools of chemists owing to their ability to catalyse various types of synthetic reaction in non-aqueous environments. Filamentous fungi are preferred sources of lipases because generally they produce extracellular enzymes (Saxena *et al*., 2003).

Enzymes immobilized on magnetic materials can be more easily separated from the from reaction system and stabilized in a fluidized-bed reactor by applying an external magnetic field, reducing operation costs. Many magnetic materials have been developed using various polymers containing magnetic particles, which are easily dispersed to give hydrophobic support with high adsorption capacity and magnetic responsiveness (Guo *et al*., 2003).

Lipase from *Fusarium solani* FS1 has been immobilized by covalent attachment to polyacrylamide beads and onto magnetized Dacron, presenting high activity retention (Knight *et al*. 2000). *Mucor miehei* lipase was immobilized on magnetic polysiloxane-polyvinyl alcohol by covalent binding, employing glutaraldehyde as bifuntional agent (Bruno *et al*., 2005). Lipase from *Pseudomonas fluorescens* (PFL), an enzyme with a great tendency to yield bimolecular aggregates, was immobilized via multipoint covalent attachment on glyoxyl-agarose in the presence of Triton X-100. The enzyme was also able to adsorb other lipases selectively being a very simple purification strategy (Palomo *et al*., 2004).

This study shows a very simple and cheap method of covalent immobilization of *Penicillium citrinum* lipase from broth free of cells on ferromagnetic azide-Dacron (ferromagnetic azide polyethyleneterepthalate), producing an immobilized derivative easily separated using a magnetic field.

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MATERIALS AND METHODS

Materials: *Penicillium citrinum* lipase was obtained according to Pimentel *et al.* (1994, 1996, 1997) and Miranda *et al.* (1999), using MA medium: 1.0% olive oil and 0.75% ammonium sulphate. This preparation was less contaminated than one obtained using yeast extract as nitrogen source as revealed by SDS-PAGE electrophoresis (data not shown).

Films of polyethyleneterepthalate (PET or Dacron) were purchased from Rhodia-PE-Brazil. Triton X-100 and 4-nitrophenylpalmitate (4NPP) were obtained from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Immobilization of *Penicillium citrinum* lipase: Lipase was immobilized on ferromagnetic azide-Dacron as follows:

Hydrazinolysis of dacron: Films of Dacron or PET (4 g) were cut into strips and incubated in methanol (10 mL) containing hydrazine hydrate (10 mL) at 40°C for 48 h with stirring. Afterward, the hydrazine-Dacron (powder) was vacuum washed and filtered twice with methanol (50 mL) and 90% aqueous methanol (50 mL). After this process, hydrazide-Dacron (2 g) was mixed with distilled water (10 mL) in a test tube for eliminating fine particles (Carneiro-Leão *et al.*, 1991).

Magnetisation of hydrazine-dacron: Hydrazine-Dacron (2 g) was stirred in deionised water (100 mL) and an aqueous solution (10 mL) containing $FeCl_3$. $6H_2O$ (300 mg mL^{-1}) and $FeCl_2$. $4H_2O$ (121 mg mL^{-1}) was added drop wise. Under vigorous stirring, the mixture was adjusted to pH 8.3 by addition of (28% v/v) ammonium hydroxide solution and then incubated at 60°C for 30 min. The material obtained was filtered using a vacuum pump and washed exhaustively with distilled water (Carneiro-Leão *et al.*, 1991).

Conversion of groups from hydrazide to azide on magnetised dacron: The ferromagnetic hydrazide-Dacron resulting was converted to ferromagnetic azide-Dacron by incubating the material in 0.6 M HCl (16 mL) containing 5% (w/v) sodium nitrite (2 mL) at 28°C for 25 min with stirring. Then, the ferromagnetic azide-Dacron was isolated from the solution by using a magnet and washed with deionized water (twice), 1 M NaCl (twice) and deionised water (twice) (Carneiro-Leão *et al.*, 1991).

Covalent coupling of lipase to ferromagnetic azide-dacron: Culture broth containing lipase (10 mL) free of cells and containing 0.2 mg mL^{-1} of protein (0.4 U/mg of protein) was incubated with ferromagnetic azide-Dacron (0.1 g) for 3 h under constant low stirring at 4°C. Then, the immobilized derivative (ferromagnetic azide-Dacron-lipase) was isolated from the solution using a magnet and washed according to Knight *et al.* (2000), with 1 M NaCl (50 mL), 0.05 M Tris-HCl buffer pH 7.2 (50 mL), 0.5% (w/v) Triton X-100 (100 mL), 50 mL 0.05 M Tris-HCl buffer pH 7.2 (50 mL) and 1 M NaCl (50 mL). The immobilized enzyme was stored in 0.05 M Tris-HCl buffer pH 7.2 at 4°C.

Lipase activity: The lipolytic activity was measured by the 4-nitrophenylpalmitate (4NPP) method according to Wrinkler and Stuckmann (1979) at 30°C and pH 8.0. Soluble lipase culture broth from *Penicillium citrinum* (0.1 mL) was added to the cuvette containing a uniform emulsion (0.9 mL) of 10-800 μM 4NPP in 50 mM In Tris-HCl buffer pH 8.0 also containing Triton X-100 (4 mg) and gum arabic (1 mg). The amount of 4-nitrophenol liberated was detected at 410 nm (Hitachi spectrophotometer). For immobilized lipase determination, immobilized derivative (0.1 g) was mixed with 4NPP micro-emulsion (10 mL), removing samples (1 mL) of the reaction mixture to read the absorbance at 410 nm and after reading, the sample was returned to the reaction tube to keep initial volume (Pimentel *et al.*, 1994, 1997).

Protein determination: Protein concentration was measured by the classical Lowry (1951) method in both preparations-soluble and immobilized lipase. The amount of protein linked on the immobilized derivative was determined by acid hydrolysis using immobilized lipase suspension (100 mg immobilized lipase powder in 25 mL 50 mM Tris-HCl buffer pH 8.0). This suspension (1.0 mL) was mixed with 6M HCl (0.2 mL), to achieve pH 1.0 and heated in closed tubes at 100°C for 1 h. The hydrolysate was neutralized with 1.0 M sodium hydroxide (to avoid large volume). A sample (100 up to 500 μL depending on protein amount in the sample) was withdrawn for protein measurement using Lowry reagents using BSA as standard. The amount of protein bonded on the support was determined from the spectrophotometric response and the dry weight (to constant weight at 110°C, in triplicate) of the immobilized lipase.

Effect of isopropanol on the lipase activity: This study was carried out using of (4NPP) in a concentration range of 10 to 800 μM containing isopropanol 0.1 to 10% (v/v), respectively. The amount of others mixture reaction components was kept constant in the micro-emulsion.

Thermal stability: The thermal stability of soluble and immobilized lipase was determined by incubating both enzymes in suspension in 0.05 M Tris-HCl buffer pH 7.2, in the temperature range 30 to 80°C for 1 h. After this treatment, the activities were measured by the 4NPP method.

Storage stability: The immobilized derivative (ferromagnetic azide-Dacron-lipase) was stored at 4°C in 0.05 M Tris-HCl buffer pH 7.2 and its activity was measured periodically using the 4NPP method.

Synthesis of triglycerides: A synthesis reaction mixture was prepared according to Park and Pastore (1989), using a reaction mixture containing oleic acid (0.9 g) and glycerol (8 g) in a closed tube, which was incubated at 40°C under orbital stirring, followed by the addition of immobilized enzyme suspension (0.4 mL). The amount of free fatty acid was measured by a cupric acetate method (Kwon and Rhee, 1986). Samples (0.1 mL) were transferred at defined time intervals to tubes containing isooctane 2.4 mL and mixing for 30 sec. Cupric acetate-pyridine reagent (an aqueous solution of 5% (w/v) cupric acetate, pH adjusted to 6.1 using pyridine) (0.5 mL) was added and mixing continued for 90 sec. The absorbance of the upper layer of isooctane, which is blue due the formation of salt between fatty acid and copper (copper soap), was read at 715 nm. A calibration curve was constructed using oleic acid as standard (0.1 to 10 μmol).

RESULTS AND DISCUSSION

The immobilization of *Penicillium citrinum* lipase by covalent binding to ferromagnetic azide-Dacron showed 73% of specific lipase activity retention with a 7.3 mg protein/g support loading. Considering that the broth culture of *Penicillium citrinum* from culture medium containing ammonium sulphate as nitrogen source was used and which showed less contaminant proteins compared to that obtained using yeast extract by SDS-PAGE electrophoresis (data not shown), this result suggests a selective immobilization, which the proteins with lipolytic activity were preferentially linked to this hydrophobic support, leading to covalent linkage of the enzyme to azide group. It has been reported that lipases are strongly adsorbed to hydrophobic interfaces through several pockets of a large hydrophobic surface which surrounds the catalytic site (Bastida *et al*., 1998).

This result is comparable to that obtained by Knight *et al*. (2000) who, using lipase from *Fusarium solani* FS1, produced an immobilized derivative with magnetized Dacron with 71% of lipolytic activity retention. But, Palomo *et al*. (2004) reported 40% activity retention when preparing the derivative glyoxyl-*Pseudomonas fluorescens* lipase (PFL) by multipoint covalent attachment to glyoxyl-agarose to yield bimolecular aggregates as a simple purification strategy for lipases.

Others lipases have been immobilized, such as *Rhizopus oryzae* lipase covalently attached to alumina by treatment of support with γ-aminopropyltriethoxysilane in acetone followed by reaction with glutaraldehyde solution, but the enzyme retained only 23% of its original activity. Lipase from *H. lanuginosa* immobilized on Amberlite by cross-linking with hexamethylenediamine or glutaraldehyde retained 35.4 and 66.5% of hydrolytic activity, respectively and *Geotrichum candido* lipase immobilized on different supports, azide carboxymethyl Sephadex; azide carboxymethyl cellulose; azopoly-4-amino-styrol and azokieselguhr retained 18, 66, 12 and 22% of hydrolytic activity respectively (Villeneuve *et al*., 2000). Also, porcine pancreatic lipase immobilized on cross linked poly (vinyl alcohol) (CL-PVA) by cross linking with adipoyldichloride showed 63% of activity yield (Kilinç *et al*., 2002). The higher activity retentions were achieved with hydrophobic supports with azide groups.

However, in the present study it is important to observe that all the lipase was covalently linked to ferromagnetic azide-Dacron because Triton X-100 was used for washing the derivative, successfully washing out the adsorbed non-linked lipase and leaving the covalently bound enzyme on the support. This procedure avoided loss of activity during successive re-use of immobilized derivative. Bruno *et al*. (2005) observed loss of activity because they did not wash their derivative with Triton X-100. Palomo *et al*. (2004) prepared a covalent derivative glyoxyl-PFL, using *P. fluorescens* lipase for purification of lipases by bimolecular aggregates. The purified lipase was separated from the aggregates by washing using 0.6% Triton X-100. This could be a suggestion for application of ferromagnetic azide-Dacron *P. citrinum* lipase derivative in a magnetic bioreactor. Because this immobilized derivative could be used to form bimolecular aggregates with crude lipase preparations, as described by Palomo *et al*. (2004), then in turn to produce purer lipase preparations. This is because the *P. citrinum* lipase will be linked onto the Dacron by azide groups on bioreactor wall due the magnet jacket. Hence this would lead to a continuous purification process for lipases due their hydrophobic character i.e., the Triton X-100 will separate the aggregates.

Effect of isopropanol on the lipase activity: There was no effect of isopropanol on lipolytic activity of ferromagnetic azide-Dacron-*Penicillium citrinum* lipase (Table 1), as

Table 1: Effect of isopropanol on soluble and immobilized *Penicillium citrinum* lipase activities

Isopropanol concentration (% v/v)	4NPP concentration (μM)	Relative lipolytic activity (%) Soluble	Immobilized
0.1	10.0	3.15	1.00
1.0	100.0	25.26	28.40
2.0	200.0	44.21	45.00
4.0	300.0	78.94	60.00
6.0	400.0	100.00	69.47
8.0	600.0	94.73	75.79
10.0	800.0	72.63	100.00

some researchers have found for another lipase-García-alles and Gotor (1998) found for *Candida antarctica* lipase that the alcohol inhibition has been shown to be dependent on desolation properties of the alcohol (1-butanol). The inhibition of lipase by alcohols is explained by Zaidi *et al.* (2002) whose studies showed that the alcohol acts on the enzyme reactivity, in the esterification reactions of fatty acids using nylon-immobilized lipase from *Candida rugosa*. There is an immediate reaction between the alcohol and the enzyme, blocking the nucleophilic site of the enzyme that, normally, is engaged in the acylation process. Lipases have a serine group in the active site that it is responsible for the linkage of acyl group in esterification reactions. This may be responsible for the enzyme inhibition by the alcohol. It is well known that this polarity decreases with increasing numbers of C-atoms, due to the size of the alcohol moiety (steric effect), in analogy to the decreasing alcohol acidity. Consequently, when the number of C-atom increases, the alcohol inhibition decreases which is reflected by the increasing inhibition constant values.

Lešcic *et al.* (2001) found a relative stability of *Streptomyces rumosus* lipase in organic solvents and suggested that the cause for much lower hydrolytic activity observed in the presence of 50% ethanol, acetone or dioxane was the absence of enzyme-substrate interface and not instability of the enzyme. This is showing that the alcohol inhibition of lipases, in some cases, depends on amount of alcohol. In this study, it was used a concentration 5 fold lesser than that used by Lešcic *et al.* (2001).

Physical-chemistry properties and thermal stability: There was no improvement in either the optimal temperature (37°C) or pH (8.0-8.5) after immobilization. However, the thermal stability of ferromagnetic azide-Dacron-*Penicillium citrinum* lipase derivative was higher, retaining 86.8% of lipolytic activity after 1 h at 45°C while the soluble enzyme retained only 34.2%; this represented an improvement of 154% in the thermal stability (Fig. 1).

This compares with *Mucor meihei* lipase immobilized on POS-PVA hybrid composite (Bruno *et al.*, 2005) in which there was no change in optimum temperature (45°C), but optimum pH changed from 7.0 to 8.0 for soluble and immobilized preparations respectively. In addition, Kilinç *et al.* (2002) reported that after immobilization of porcine pancreatic lipase on CL-PVA by cross linking with adipoyldichloride the optimum pH was shifted from 8.5 to 9.0 and the optimum temperature from 30 to 37°C.

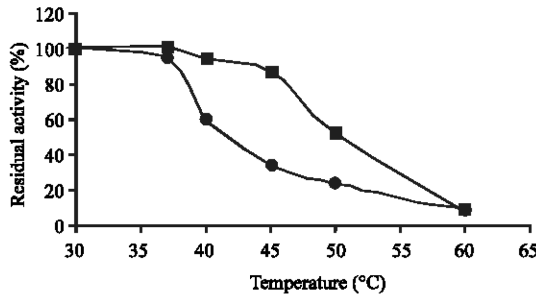

Fig. 1: Thermal stabilities of soluble (●) and immobilized (■) *Penicillium citrinum* lipases

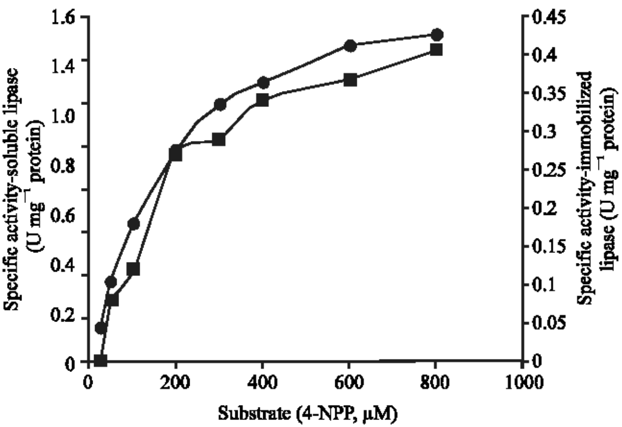

Fig. 2: Effect of substrate concentration on the soluble(●) and immobilized (■) activities of *Penicillium citrinum* lipase

Kinetic studies: Both soluble and immobilized *Penicillium citrinum* lipases showed Michaellis-Menten kinetics for hydrolysis of 4NPP at 37°C and pH 8.0. Figure 2 yields the values of K_M for soluble enzyme (233 μM) and immobilized derivative (276 μM). There was no inhibition of either of the enzyme preparations by variation of 4NPP concentration. The values in Table 1 show only the alcohol effect on the lipolytic activity, because the enzyme is not inhibited by high concentrations of 4NPP.

Candida cylindracea lipase immobilized by adsorption to magnetic poly(VAC-DVB) microspheres showed a K_M 4-nitrophenylacetate of 3.6 mM at 20°C while for the native enzyme the K_M was 2.2 mM (Guo *et al.*, 2003).

According to Bruno *et al.* (2005) the kinetic parameters changed after covalent immobilization of *Mucor meihei* lipase on POS-PVA hybrid composite from K_M of 390.4 μM for free enzyme to 228.3 μM for immobilized derivative, using 4NPP as substrate. Montero *et al.* (1993) found only a small difference between the K_M values (2700 and 2600 μM for free and immobilized enzyme from *Candida rugosa*). And Knight *et al.* (2000) showed that *Fusarium solani* FS1 lipase immobilized on magnetized Dacron was inhibited by substrate 4NPP excess.

This shows that the K_M values can change or not depending on the form of immobilization, which sometimes can involve a change on the special structure of the enzyme, as in Bruno *et al.* (2005) improving the affinity of enzyme and substrate. In our case, there was neither improvement nor inhibition by substrate excess.

Operational and storage stabilities: The Ferromagnetic azide-Dacron-*Penicillium citrinum* lipase derivative was stable, keeping about 75% of initial activity after its reuse for 5 times (Fig. 3). Also, it was stable to storage at 4°C in Tris-HCl buffer pH 7.2 with a half-life of 25 days (Fig. 4). These results were better than that reported by Kilinç *et al.* (2002) in which porcine pancreatic lipase on CL-PVA by cross linking with adipoyldichloride lost about 50% of its activity within 8 h of continuous process and a half life of 8 h. Also the results showed when *Candida rugosa* lipase was immobilized to chitosan beads utilizing both its amino and hydroxyl groups, called binary immobilization, 74% of residual activity after 10 hydrolysis cycles was retained and 67% after 7 days of storage (Hung *et al.*, 2003) and the same lipase when immobilized by adsorption to magnetic poly (VAC-DVB) microspheres retained about 74% activity after 6 hydrolytic cycles (Guo *et al.*, 2003).

However, *Mucor meihei* lipase immobilized on POS-PVA hybrid composite lost 90% of initial activity after 5 cycles (Bruno *et al.*, 2005). What did not show

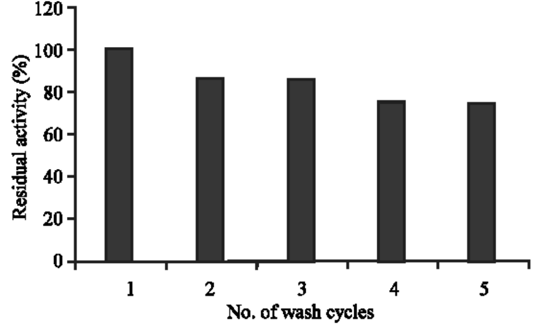

Fig. 3: Operational stability of ferromagnetic azide-Dacron-*Penicillium citrinum* lipase derivative

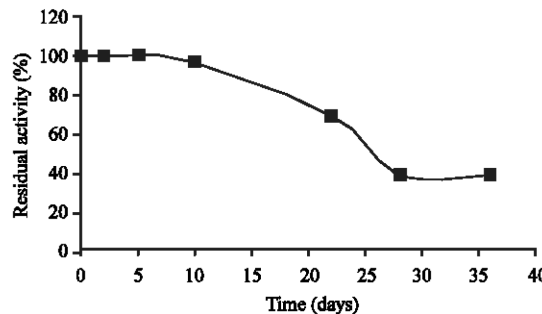

Fig. 4: Storage stability of ferromagnetic azide-Dacron-*Penicillium citrinum* lipase derivative

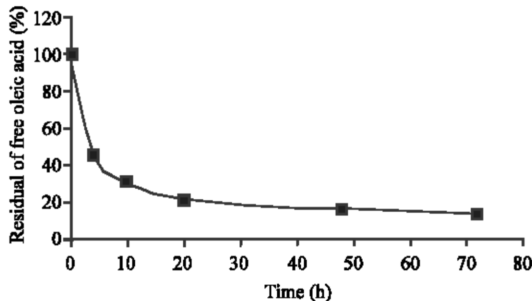

Fig. 5: Synthesis of triolein catalysed by ferromagnetic azide-Dacron-*Penicillium citrinum* lipase derivative

with ferromagnetic azide-Dacron-*Penicillium citrinum* lipase derivative due to the washing using Triton X-100 after immobilization releasing adsorbed lipase, was any significant loss-this enzyme derivative being more stable.

Synthesis of triolein: Immobilized *Penicillium citrinum* lipase was able to catalyse the synthesis of esters using glycerol and oleic acid as substrates, free from organic solvent, with 80% of conversion after 20 h at 40°C (Fig. 5). The mono, di and triglycerides production was confirmed by TLC (data not shown).

CONCLUSION

Penicillium citrinum lipase from the cell free broth culture covalently immobilized on ferromagnetic azide-Dacron retained high specific activity (73%) This can be considered a selective immobilization. This immobilized derivative showed an improvement of 154% in the thermal stability and kept 75% of initial activity after 5 use cycles and was stable to storage at 4°C for 25 days. Also, the results showed that this immobilized derivative was able to catalyse esters biosynthesis from glycerol and oleic acid.

The synthesis reaction was able to be carried out with a large amount of glycerol compared to oleic acid (27:1) -the opposite proportions to that which is generally used-glycerol: Oleic acid (1:3), following the method described by Park and Pastore (1989) without observing the stoichiometric concentration

These results, compared with the literature references reports, show that the immobilized derivative presents biotechnological potential for several applications, such as, extraction and purification of extracellular lipases, hydrolysis and esterification reactions.

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