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Keywords (separated by '-')	*A. niger* lipase - Immobilization on magnetic nanoparticles coated with chitosan - Glycidol and glutaraldehyde as coupling agents
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ORIGINAL PAPER

Immobilization of *Aspergillus niger* lipase on chitosan-coated magnetic nanoparticles using two covalent-binding methods

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Keywords *A. niger* lipase · Immobilization on magnetic nanoparticles coated with chitosan · Glycidol and glutaraldehyde as coupling agents

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Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are part of the family of hydrolases, which act on carboxylic ester bonds. Their natural function is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. These enzymes are widely distributed throughout the plant and animal kingdoms, as well as in fungi and bacteria [1]. Lipases are versatile biocatalysts. In addition to their hydrolytic activity on triglycerides, they can catalyze other reactions such as esterification, interesterification, acidolysis, alcoholysis, and aminolysis. Lipases have potential applications in the detergent, food, leather, textile, oil and fat, cosmetic, paper, and pharmaceutical industries [1, 2].

To completely exploit the economic and technical advantages of these enzymes, their application in an immobilized state is recommended. Immobilization facilitates the separation of products, improves lipase properties such as stability and enzymatic activity, and provides more tractability for applications in the reactors with various configurations [3]. Lipases have been immobilized through several methods: adsorption and covalent attachment, cross-linking, adsorption

Journal : Large 449 | Dispatch : 4-3-2015 | Pages : 9
Article No. : 1385 | LE | TYPESET
MS Code : BPBSE-14-0766 | CP | DISK

followed by cross-linking, and physical entrapment using many commercial supports [2, 4]. Covalent binding of an enzyme to a support is probably the most remarkable method of immobilization from an industrial point of view [5]. The enzyme is usually anchored via multiple points and this generally imparts greater thermal, pH, ionic strength, and organic solvent stability onto the enzyme since it is more rigid and less susceptible to denaturation [6–8]. Covalently immobilized enzymes are often more resistant to degradation by proteolysis. Several covalent immobilization methodologies have been described previously [5, 7, 9] but the selection of the method depends on the support and the enzyme nature, as well as process type which will be applied.

Recently, nanostructured magnetic materials have been employed as supports in the immobilization of enzymes [10–12] due to the their many advantages such as: (1) higher specific surface favors the binding efficiency, (2) lower mass transfer resistance and less fouling, (3) the selective separation of immobilized enzymes under a magnetic field and hence lower operation cost, and (4) the application of a continuous biocatalysis system [11].

These magnetic nanoparticles have been prepared with different coatings. Chitosan is one of the most used coatings due to its excellent properties such as non-toxicity, biocompatibility, and chemical reactivity, which allow an easy fixation of enzymes [13].

In the present work, chitosan-coated magnetic nanoparticles (CMNP) were used as support for *Aspergillus niger* lipase immobilization to evaluate the operational properties of derivatives and compare them with those of free enzyme. These magnetic nanoparticles were obtained by simple method of one-step co-precipitation for application in magnetic carrier technology [14–16]. Here we reported their use for lipase immobilization by two covalent-binding methods. The coupling agents used in the immobilization were: glutaraldehyde and glycidol, aimed to form intermolecular bonds between enzyme's amino groups and polymer's chemical groups, to obtain immobilized derivatives with high enzymatic activity, stability, and with a good reusing capacity.

Materials and methods

Materials

Low molecular weight chitosan with 75 % deacetylation degree, ferric chloride ($FeCl_3{\cdot}6H_2O$, 99 %), ferrous chloride ($FeCl_2{\cdot}4H_2O$, 98 %), aqueous ammonia (NH_4OH, 57.6 wt%), glycidol (96 %) and lipase from *Aspergillus niger* ($\sim$200 U g^{-1}) were purchased from Sigma-Aldrich. Glutaraldehyde (25 %) was purchased from J. T. Baker. Deionized, and Milli-Q water were drawn from a Millipore system.

Preparation of chitosan-coated magnetic nanoparticles

Chitosan-coated magnetic nanoparticles (CMNP) were prepared according to published procedures [15]: 50 ml of $FeCl_3{\cdot}6H_2O$ (0.32 M), 50 ml of $FeCl_2{\cdot}4H_2O$ (0.2 M), and 0.125 g of chitosan were mixed in a reactor at 400 rpm. The temperature was set to 50 °C and a dosing (0.67 ml min^{-1}) of 20 ml of aqueous ammonia was started. After dosing, the reaction continued during 20 min with stirring. Finally, CMNP were recovered with a permanent magnet, and were washed 25 times with deionized water.

Immobilization of *Aspergillus niger* lipase on CMNP

CMNP were activated with glutaraldehyde and glycidol. To form the active group with glutaraldehyde, 1 g of CMNP was suspended in 1.68 ml of glutaraldehyde solution (25 %) and 1.12 ml of sodium phosphate buffer (0.2 M, pH 7.0). The mixture was stirred at 120 rpm, at 25 °C during 1 h. Finally, the activated CMNP were recovered with a permanent magnet, and washed with deionized water to remove the unreacted glutaraldehyde.

In the case of glycidol, 1 g of CMNP was added to a solution consisting in 0.3 ml of Milli-Q water, 0.5 ml of NaOH (1.7 M) and 0.015 g of sodium borohydride. Later, 0.36 ml of glycidol was added and the mixture was incubated at 4 °C and 100 rpm for 15 h. Then, the mixture was suspended in 6 ml of Milli-Q water and 3 ml of sodium peryodate (0.1 M) to produce glyoxyl groups. The suspension was kept under gentle stirring for 2 h at 25 °C.

The enzymatic solution (100 μg ml^{-1}) was added to 9 ml of phosphate buffer (0.1 M, pH 8.0), and was mixed with 1 g of the activated CMNP under gentle stirring for 12 h at 25 °C. Followed this, CMNP with the immobilized lipase were separated with a permanent magnet and were washed with Tris–HCl buffer (0.2 M, pH 7.0) and Milli-Q water.

Assays of the enzymatic activity

The hydrolytic activity of free and immobilized *Aspergillus niger* lipase was determined spectrophotometrically using *p*-nitrophenyl propionate (*p*-NPP) as substrate in sodium phosphate buffer 25 mM at pH 8.0 and 37 °C. The reaction mixture contained 2.5 ml of buffer, 0.1 ml of free or immobilized enzyme preparation and 0.02 ml of 50 mM *p*-NPP. The reaction was initiated by substrate addition. Absorbance was detected at 348 nm for 30 min each 5 min in the case of free enzyme in Cary-50 spectrophotometer. In the case of immobilized preparation, the reaction was carried out for 30 min and the immobilized derivative was separated using magnet before absorbance measurement. The reaction time was selected based on

Journal : Large 449 | Dispatch : 4-3-2015 | Pages : 9
Article No. : 1385 | ☐ LE | ☐ TYPESET
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preliminary assay, which demonstrated the linear kinetic response for at least 30 min. An activity unit was defined as the amount of enzyme required to hydrolyze 1 μmol of *p*-NPP per minute under described conditions.

Determination of the amount of immobilized enzyme and immobilization yield

Protein content was determined in solution obtained before and after immobilization, according to the Bradford method [17] using bovine serum albumin (BSA) as standard.

The amount of immobilized enzyme (A) and immobilization yield (IY) was calculated by the following equations:

$$A = C_i \times V_i - C_s \times V_s - \Sigma\, C_l \times V_l$$

$$\%\ \mathrm{IY} = (A \times 100)\,(C_i \times V_i)^{-1}$$

where:

A is the amount of immobilized enzyme (mg).

C_i, C_s and C_l are initial, supernatant and washed concentrations of the enzyme (mg ml^{-1}), respectively, and V_i, V_s and V_l are initial, supernatant and washed volumes generated during immobilization (ml), respectively.

Characterization of nanostructured magnetic biocomposites

Fourier transform infrared spectrometry (FT-IR) was used for determining spectra of the pure lipase, the pure CMNP, and immobilized derivatives. Spectra were recorded in a Magna IR 550 from Nicolet with germanium crystal. The size and morphology of CMNP and immobilized derivatives were determined by a high-resolution transmission electron microscope (HRTEM), Titan-300 kV, for which samples were prepared by dispersing the resulting powders in water with ultrasonication, and then depositing the dispersion on a copper grid. Thermogravimetric analysis (TGA) was performed in a TGA Q500 from TA Instruments. Analyzed samples were heated from 30 to 700 °C, at a heating rate of 10 °C min^{-1} under a nitrogen flow of 50 ml min^{-1}.

Assay of thermal and pH stabilities of free and immobilized *A. niger* lipase

The influence of pH on the stability of the free and immobilized enzyme was studied by exposure of lipase at different pH at 37 °C for 4 h, while the change in thermal stability was studied by incubating free and immobilized enzyme at different temperatures and at the optimum pH for 4 h. Residual activity was expressed as a percentage of the initial activity presented by the enzyme.

Determination of kinetic parameters K_m y V_{max}

Michaelis–Menten kinetic parameters (K_m and V_{max}) of the free and immobilized lipase were calculated using different concentrations (5, 10, 12.5, 25, and 50 mM) of *p*-NPP as substrate. The enzymes were incubated with the substrate at optimum pH and temperature. The K_m and V_{max} parameters were calculated by Lineweaver–Burk plots.

Storage stability

Free and immobilized lipase were immersed in phosphate buffer (0.1 M, pH 7.0) and stored at 4 °C. The storage stability was evaluated by determining the enzymatic activity of *p*-NPP hydrolysis, at regular times during 50 days.

Repetitive use of immobilized lipase

Lipase activity was determined by the *p*-NPP hydrolysis. The immobilized lipases were recovered with a permanent magnet, and were washed with sodium phosphate buffer. Then, the enzyme was suspended again in a substrate solution in order to start a new catalytic cycle. For each cycle, the lipase activity was determined spectrophotometrically according to the technique described above.

Statistical analysis

The results are presented as means ± standard deviations. All statistical calculations were performed using Microsoft Excel.

Results and discussion

Two covalent-binding methods for the lipase immobilization on CMNP were performed. Table 1 shows the results of the amount of lipase immobilized on the support, the enzymatic activity, and the immobilization yield, as important characteristics of immobilized lipase and immobilization process carried out using glycidol and glutaraldehyde as coupling agents. The higher values of these parameters were detected in the case of glutaraldehyde-treated samples: nearly to 90 % of immobilization yield, the greater activity and amount of immobilized protein, while only 69 % of immobilization yield was achieved using glycidol. However, specific activity of the immobilized enzyme was similar for both assayed systems: 60.7 and 61.7 U mg^{-1} for glutaraldehyde and glycidol treatment, respectively, while for free enzyme was 94.3 U mg^{-1}. Less specific activity could mean a partial inactivation of lipase during covalent immobilization due

Journal : Large 449 | Dispatch : 4-3-2015 | Pages : 9
Article No. : 1385 | LE | TYPESET
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Table 1 Characteristics of immobilized lipase preparations and immobilization process

Coupling agent	Amount of immobilized enzyme [mg (g support)$^{-1}$]	Derivative activity [U(g support)$^{-1}$]	Immobilization yield (%)
Glutaraldehyde	5.10 ± 0.06	309.5 ± 2.0	90.1 ± 1.1
Glycidol	3.86 ± 0.11	238.5 ± 2.4	68.7 ± 2.5

to chemical, diffusional or steric changes after enzyme covalent attachment [4, 7].

These results can be explained since the aldehyde groups of the glutaraldehyde are much more reactive than the glyoxyl groups obtained through the activation with glycidol. Thus, amino groups of chitosan can react directly with glutaraldehyde to generate aldehyde groups, which can form Schiff bases with the enzyme [4, 7]. In addition, the high concentration of amino groups present in chitosan (previously reported [15]) explains the high degree of activation reached. Instead, in the case of glycidol, the chitosan amino groups must be activated first with this agent, in order to be then oxidized with sodium periodate to produce aldehyde groups, which reacts with the lipase. Another reason by which the immobilization yield with glycidol was lower, could be due to the high concentration of formed glyoxyl groups in chitosan (considering that chitosan contains more hydroxyl than amino groups), which provoked them to react between themselves causing aldolic reactions, and therefore could be inactivated for immobilization [3].

Silva et al. [3] reported a yield of 35.8 % and an activity of 27.59 U g^{-1} when immobilized *Candida antarctica* lipase on chitosan using glycidol as binding agent; and a yield of 81 % and an activity of 388.30 U g^{-1}, when used glutaraldehyde as binding agent. Instead, Rodrigues et al. [4] reported a yield of about 40 % for the *Candida Antarctica* lipase immobilization on chitosan spheres activated with glycidol, with activity around 20 U g^{-1}, while using glutaraldehyde a yield of about 90 %. An activity in the range from 300 to 400 U g^{-1} was reported. According to the results obtained in the present study, it can be concluded that the immobilization yields and derivatives activities are very good, since they are higher than those previously reported by other authors.

Moreover, FT-IR analysis has been used to identify the presence of functional groups or chemical bonds in a material due to the presence of modifications on it, because each specific chemical bond often shows a unique energy absorption band [3]. In this study, FT-IR analysis was used to examine the characteristic chemical structures of CMNP (Fig. 1, line a), lipase immobilized on CMNP by means of glycidol (Fig. 1, line b) and glutaraldehyde (Fig. 1, line c), as well as pure lipase (Fig. 1, line d). The spectrum of the pure CMNP shows five characteristic absorption bands:

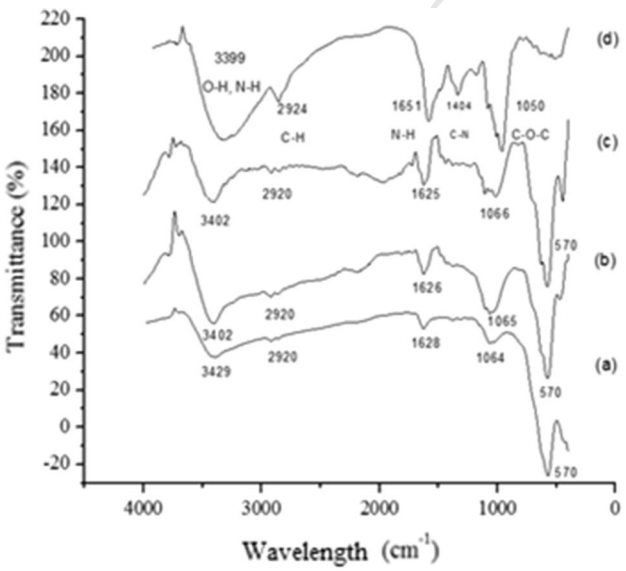

Fig. 1 FT-IR spectra of: *a* CMNP; *b* lipase immobilized on CMNP with glutaraldehyde; *c* lipase immobilized on CMNP activated with glycidol; *d* pure lipase

3429 cm^{-1} (O–H and N–H stretching vibrations), 2920 cm^{-1} (C–H stretching vibrations), 1628 cm^{-1} (N–H bending vibrations), 1064 cm^{-1} (C–O-C stretching vibrations), and 570 cm^{-1} (Fe–O stretching vibrations); while pure lipase shows another five peaks: 3399 cm^{-1} (O–H and N–H stretching vibrations), 2924 cm^{-1} (C–H stretching vibrations), 1651 cm^{-1} (N–H bending vibrations), 1404 cm^{-1} (C–N stretching vibrations), and 1050 cm^{-1} (C–O–C stretching vibrations). Spectra corresponding to the lines b and c show some changes: there is a considerable increase on intensity of peaks at 3402, 2920, 1625, and 1065 cm^{-1}, which can indicate the successful immobilization of the lipase in CMNP [14–16]. These changes could be related to the formation of chemical bonds with nitrogen and oxygen, indicating that amine and hydroxyl groups were involved in the reaction with glutaraldehyde and glycidol, respectively. However, glutaraldehyde molecules react with hydroxyl groups and amino groups, while glycidol reacts only with hydroxyl groups [4].The size and morphology of CMNP and immobilized derivatives were evaluated by HRTEM. Measurements of 1000 nanoparticles diameter from different micrographs of each of the samples were carried out by the use of an image

Journal : Large 449 Dispatch : 4-3-2015 Pages : 9
Article No. : 1385 LE TYPESET
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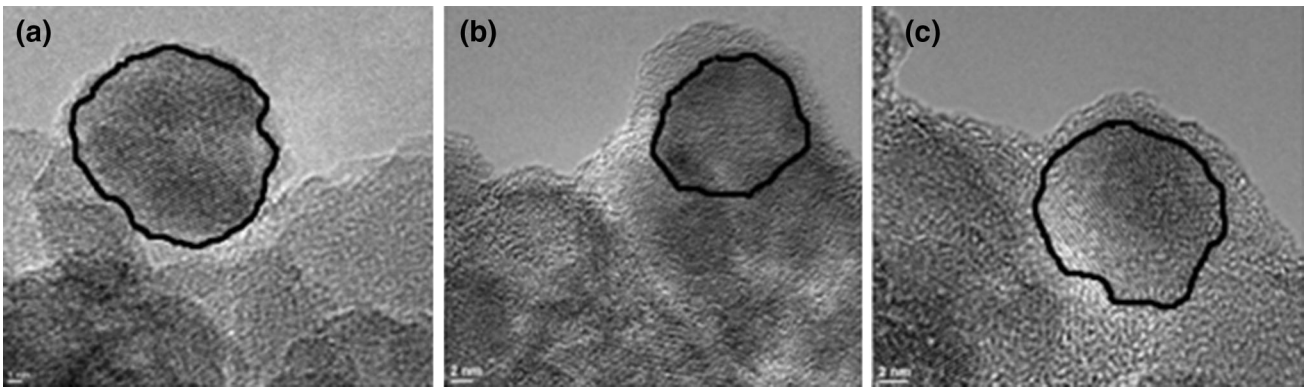

Fig. 2 HRTEM micrographs of CMNP (**a**) and the CMNP with lipase immobilized by means of glutaraldehyde (**b**) and glycidol (**c**). The line delimits the extension of the crystalline region of nanoparticles

analysis program (Image J 1.37C). These results were used to calculate the number-average diameter (D_n) through the following equation [14, 15]: $D_n = \frac{\sum n_i D_i}{\sum n_i}$ where n_i was the number of particles of diameter D_i. D_n values corresponded to CMNP before and after enzyme immobilization with glutaraldehyde and glycidol were 9.9 ± 0.2, 12.5 ± 0.1, and 11.5 ± 0.1 nm, respectively. Xie and Ma [11] reported CMNP with diameters of 11.2 nm approximately and a diameter of 12.9 nm for CMNP with bounded lipase.

Figure 2 shows HRTEM micrographs of CMNP (a), CMNP with lipase immobilized using glutaraldehyde (b) and glycidol (c). The crystal planes of the nanoparticles are identified as parallel lines crossing the particle [14]. However, these lines do not extend to the edges of the particles. The dark line drawn in the image binds the crystal planes of nanoparticles with an area which is not included in these planes, this area corresponds to an amorphous substance, in this case, chitosan [15]. The CMNP micrograph shows a thin chitosan layer (Fig. 2a). The thin layer of chitosan on magnetic nanoparticles is due to the low chitosan content used in the synthesis reaction [15]. In Fig. 2b, c, the area corresponding to the amorphous part is wider, which could be considered as evidence of lipase immobilization onto CMNP [15].

TGA results of CMNP and the immobilized derivatives are shown in Fig. 3. Figure 3 shows TGA measurement of change in the mass of studied sample as a function of temperature in a controlled atmosphere. The materials first suffered a mass loss of about 2–3 % due to moisture and than, as expected, decomposed with temperature increasing to achieve a constant mass corresponding to inorganic part of materials (Fig. 3). An average weight loss of 8.46 ± 0.03 % was calculated for CMNP (a), while immobilized derivatives obtained using glycidol (b) and glutaraldehyde (c) showed weight losses of 10.54 ± 0.74 and 13.59 ± 0.16 %, respectively. Greater weight loss is

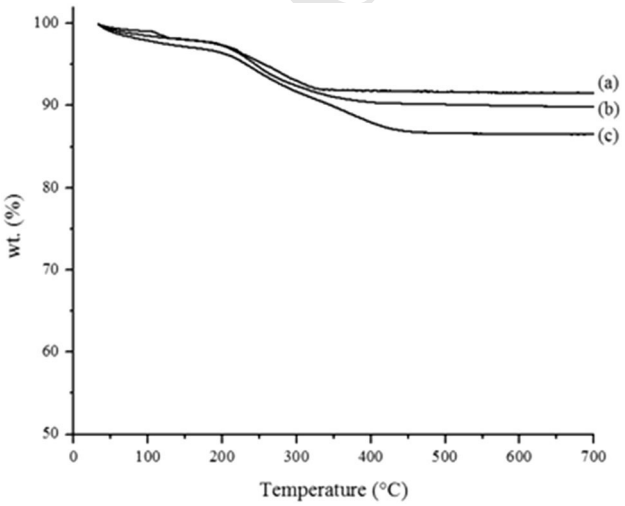

Fig. 3 Thermal degradation curves of: *a* CMNP, *b* CMNP with lipase immobilized using glycidol (*b*) and glutaraldehyde (*c*) as coupling agents

interpreted as evidence of increased organic matter content [16]. These results mean that the attachment using glutaraldehyde could be more effective than when using glycidol, because the amount of lipase on CMNP treated with glutaraldehyde is greater than in those treated with glycidol.

The stability of the immobilized enzyme was studied at different pH and temperatures. The variation in the residual activity at different pH is shown in Fig. 4. The immobilized lipase was more stable as free enzyme. After enzyme incubation at pH 7, 8, and 9 for 4 h prior to enzymatic activity detection, it lost no more than 10 % of activity in the case of derivative obtained with glutaraldehyde and no more than 20 % in the case of the other derivative obtained with glycidol. Instead, the free lipase reduced its activity by 40 %. These results indicate that immobilization improves the stability of the lipase in the neutral and alkaline region.

Journal : Large 449 Dispatch : 4-3-2015 Pages : 9
Article No. : 1385 LE TYPESET
MS Code : BPBSE-14-0766 CP DISK

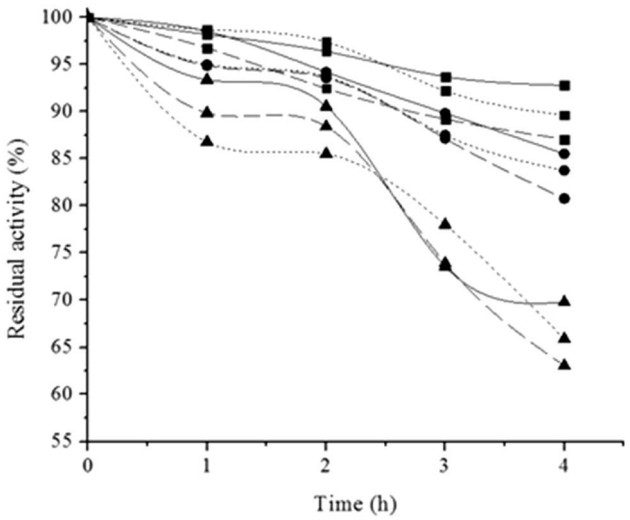

Fig. 4 Residual activity of free lipase (*closed triangle*) and immobilized derivatives obtained using glutaraldehyde (*closed square*) and glycidol (*closed circle*) after their incubation at pH 7 (*solid line*), 8 (*dotted line*) and 9 (*dashed line*)

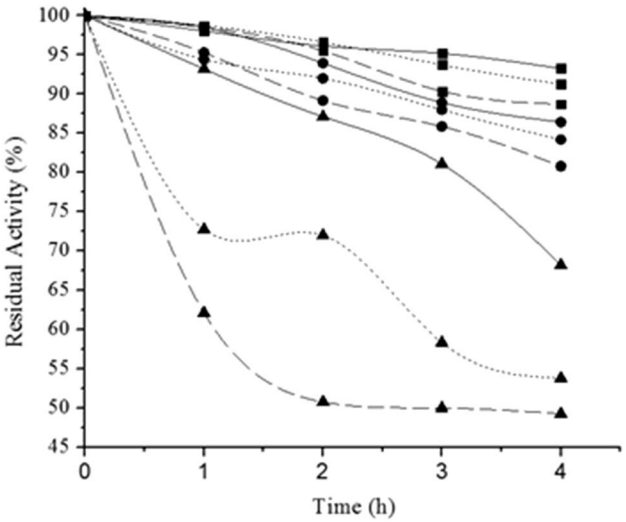

Fig. 5 Thermal deactivation profiles at 30 °C (*solid line*), 40 °C (*dotted line*), and 50 °C (*dashed line*) for: free lipase (*closed triangle*), for immobilized derivative obtained using glutaraldehyde (*closed square*), and lipase immobilized by means of glycidol (*closed circle*)

Similar results were obtained in the study of *C. Rugosa* immobilized in chitosan gels [18].

Results of thermal stability of the immobilized enzyme at three different temperatures (30, 40, and 50 °C) were compared with the stability of the free enzyme at the same temperatures (Fig. 5). The thermal stability of immobilized enzyme was higher than the stability of the free enzyme. For example, after enzyme pre-incubation at 50 °C for 4 h, the enzyme immobilized on CMNP with glutaraldehyde lost 10 % of activity, with glycidol by no more than 20 % of activity, while the free enzyme lost approximately 50 % of activity (Fig. 5).

These results are consistent with the concept that lipase immobilization on a solid support leads to enzyme protection that prevents its rapid denaturation, due to the linkages to the support. High temperatures normally cause breakdown of the enzyme catalytic structure, which is characterized by some flexibility, which means that its active site undergoes conformational changes that are often irreversible, causing inactivity [19, 20]. Immobilization decreases the enzyme's flexibility, helps to maintain the structure of the active site, which is important for its activity.

Enzyme activity was analyzed as the function of substrate concentration. Michaelis–Menten behavior was verified. Lineweaver–Burk plots were obtained (Fig. 6). The Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) for free and immobilized derivatives were calculated. Results are shown in Table 2.

Michaelis constant (K_m) is related to the affinity between enzyme and its substrate. Lower values of K_m mean higher affinity between enzyme and substrate, while higher values of K_m mean less affinity. According to Table 2, K_m value quantified for enzyme immobilized by means of glutaraldehyde was 1.7 times lower than for the free lipase. It probably means that the affinity of the immobilized enzyme for its substrate is higher than for the free enzyme. This result is in agreement with previous reports, which suggest that this could be due to more efficient conformation of immobilized lipase, or owing to enzyme expanding on the surface of the nanoscale particles with a better orientation leading to higher affinity to substrate and more available active sites [21].

Moreover, V_{max} values corresponding to both derivatives with immobilized enzyme were slightly less than for free enzyme (Table 2). It may be related to lower amount of enzyme bound to support or partial enzyme inactivation. V_{max} is the theoretically quantified maximum rate of the reaction. However, this condition can be achieved only with high substrate concentration, since V_{max} would require that all enzyme molecules have bound substrate. Higher V_{max} value means greater rates of reaction under optimum condition. From results in Table 2, V_{max} values of immobilized enzymes using glycidol were slightly higher than enzyme immobilized with glutaraldehyde, as well as specific activity was slightly higher in this case. It means that the reaction of *p*-NPP hydrolysis catalyzed by this immobilized enzyme is more efficient than for other immobilized derivate. The less V_{max} of the immobilized enzymes generally could be related to partial enzyme inactivation mentioned above, as well as diffusional limitations, steric effects, or structural changes in

Journal : Large 449 Dispatch : 4-3-2015 Pages : 9
Article No. : 1385 LE TYPESET
MS Code : BPBSE-14-0766 CP DISK

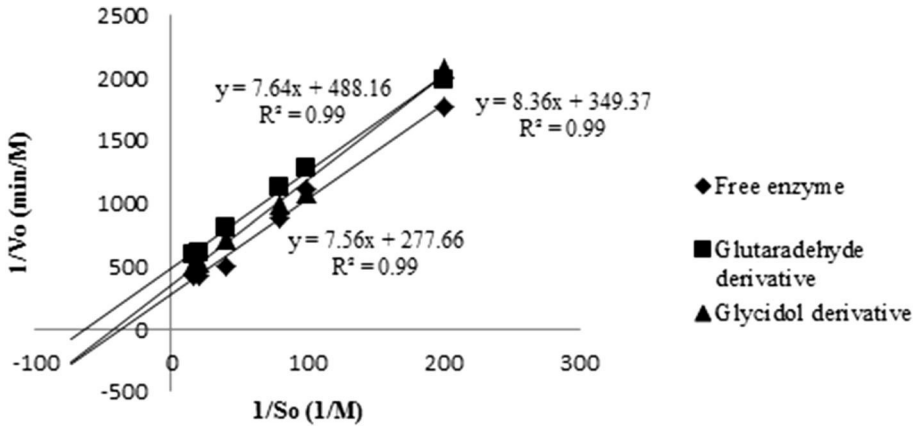

Fig. 6 Lineweaver–Burk plots obtained for *p*-NPP hydrolysis catalyzed by free (*closed diamond*) and lipase immobilized on CMNP activated with glutaraldehyde (*closed square*) and glycidol (*closed triangle*)

Table 2 K_m and V_{max} values for free lipase and enzyme immobilized on CMNP

Enzymatic preparation	K_m (mM)	V_{max} (mM min^{-1})
Free lipase	27.2	3.6
Immobilized derivative activated with glutaraldehyde	15.7	2.1
Immobilized derivative activated with glycidol	23.9	2.9

the enzyme occurring upon covalent coupling or lower accessibility of substrate to the active sites of the immobilized enzyme [7].

From these results, it can be concluded that the enzyme kinetic properties slightly change after the immobilization process. However, the difference cannot be considered statistically significant according to variability of kinetic data.

Li et al. [22] reported values of 87.7 mM and 0.40 mM min^{-1} for K_m and V_{max}, respectively, when the *B. cepacia* lipase was immobilized on polystyrene beads, while Handayani et al. [23] obtained K_m values in the 130–170 mM range and V_{max} within a range of 1.3–4.7 mmol s^{-1} for the *C. antarctica* lipase immobilized on six beads macroporous copolymers (poly(GMA-*co*-EGDMA). The immobilized enzyme was characterized by higher V_{max} in comparison to free enzyme [23].

The storage stability of immobilized lipases was determined during 50 days. Figure 7 shows the stability profiles of the free and immobilized lipase. The free lipase maintained activity at 75 % after 50 days of storage at 4 °C, while lipase immobilized using glycidol and glutaraldehyde as coupling agent, maintained 85 and 95 % of activity, respectively. These results confirm that CMNP are a suitable matrix to immobilize *A. niger* lipase due to their ability to increase its storage stability.

Enzyme reuse was evaluated because it is of great importance in its use for batch and continuous processes.

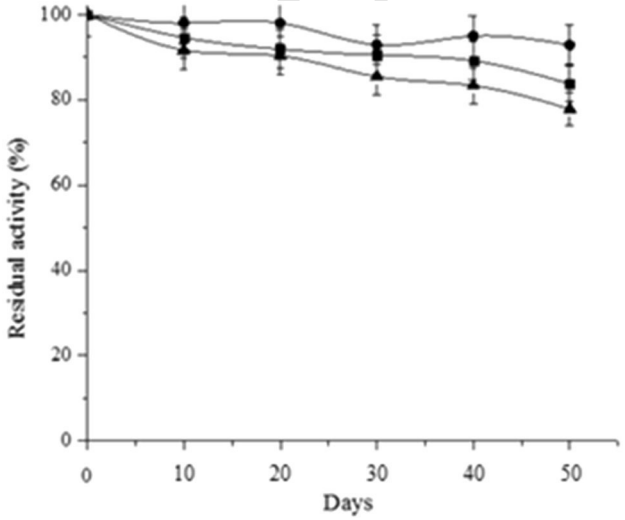

Fig. 7 Storage stability at 4 °C of free and immobilized on CMNP *A. niger* lipase: free lipase (*closed triangle*), lipase immobilized using glutaraldehyde (*closed circle*), and glycidol (*closed square*) as coupling agents

Tests were performed by means of *p*-NPP enzymatic hydrolysis. Figure 8 shows the decrease of lipase activity after 15 reaction cycles. The immobilized lipase (with glutaraldehyde, and glycidol as coupling agents) retained its activity at more than 80 %. The gradual but slight decrease in enzyme activity could be due to denaturation and/or release of lipase from the chitosan-coated magnetic nanoparticles. Figure 8 also demonstrates advantage of glutaraldehyde as coupling agent due to less decrease in enzymatic activity. These results are in agreement with previously reported data [24], where authors observed a slight decrease of enzyme activity (<15 %) after 18 cycles of reuse. With this excellent reusability and easy retrieval (by simple magnetic separation), lipase immobilized on chitosan-coated magnetic nanoparticles prepared by one-step method would be useful for industrial applications.

Journal : Large 449 Dispatch : 4-3-2015 Pages : 9
Article No. : 1385 LE TYPESET
MS Code : BPBSE-14-0766 CP DISK

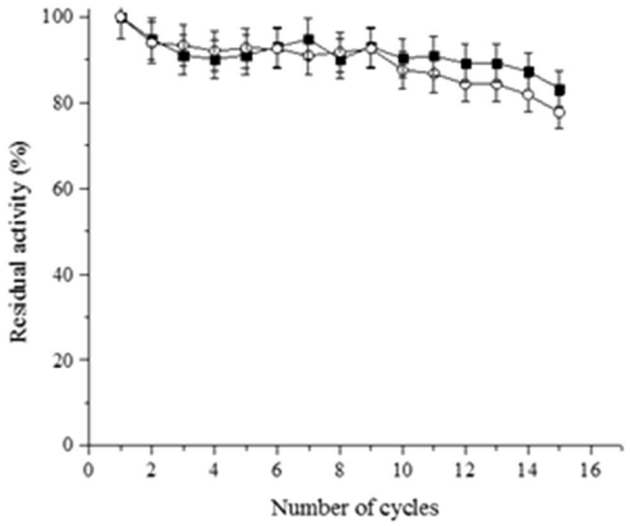

Fig. 8 Reusability of lipase immobilized on CMNP using glutaraldehyde (*closed square*) and glycidol (*open circle*) as couplings agents, in the *p*-NPP hydrolysis reaction

Conclusions

Chitosan-coated magnetic nanoparticles obtained by one-step co-precipitation method were successfully applied for *A. niger* lipase immobilization using glutaraldehyde and glycidol as coupling agents. The greater enzymatic activity value was 309.5 ± 2.0 U (g support)$^{-1}$, using glutaraldehyde as an activation agent. FT-IR, HRTEM and TG analysis confirmed the lipase immobilization. Immobilization by both methods led to increase of pH, thermal and storage stability of lipase. Enzyme kinetic properties slightly changed after the immobilization process. However, the difference cannot be considered statistically significant according to variability of kinetic data. After 15 cycles of *p*-NPP hydrolysis, they lost less than 20 % of activity. It means that the covalent-binding immobilization is very effective. Derivatives of lipase immobilized on CMNP have a great potential for commercial use due to simplicity of their preparation and use by magnetic separation. Some advantages of glutaraldehyde in comparison with glycidol as coupling agent were demonstrated.

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References

1. Sharmaa R, Chisti Y, Banerjeea UC (2001) Production, purification, characterization, and application of lipases. Biotech Adv 19:627–662
2. Sakai S, Liu Y, Yamaguchi T, Watanabe R, Kawabe M, Kawakami K (2010) Immobilization of *Pseudomonas cepacia* lipase onto electrospun polyacrylonitrile fibers through physical adsorption and application to transesterification in nonaqueous solvent. Biotechnol Lett 32:1059–1062
3. Silva JA, Macedo GP, Rodrigues DS, Giordano RLC, Gonçalves LRB (2012) Immobilization of *Candida antarctica* lipase B by covalent attachment on chitosan-based hydrogels using different support activation strategies. Biochem Eng J 60:16–24
4. Rodrigues DS, Mendes AA, Adriano WS, Gonçalves LRB, Giordano RLC (2008) Multipoint covalent immobilization of microbial lipase on chitosan and agarose activated by different methods. J Mol Catal B Enzym 51:100–109
5. Manrich A, Komesu A, Adriano WS, Tardioli PW, Giordano RL (2010) Immobilization and stabilization of xylanase by multipoint covalent attachment on agarose and on chitosan supports. Appl Biochem Biotech 161:455–467
6. Tan T, Lu J, Nie K, Deng L, Wang F (2010) Biodiesel production with immobilized lipase: A review. Biotech Adv 28:628–634
7. Prlainović NŽ, Knežević-Jugović ZD, Dušan MŽ, Dejan BI (2011) Immobilization of lipase from *Candida rugosa* on Sepabeads®: the effect of lipase oxidation by periodates. Bioproc Biosyst Eng 34:803–810
8. Huang J, Zhao R, Wang H, Zhao W, Ding L (2010) Immobilization of glucose oxidase on Fe_3O_4/SiO_2 magnetic nanoparticles. Biotechnol Lett 32:817–821
9. Kuo C-H, Liu Y-C, Chang C-MJ, Chen J-H, Chang C, Shieh C-J (2012) Optimum conditions for lipase immobilization on chitosan-coated Fe_3O_4 nanoparticles. Carbohyd Polym 87(4):2538–2545
10. Wu Y, Wang Y, Luo G, Dai Y (2009) *In situ* preparation of magnetic Fe_3O_4-chitosan nanoparticles for lipase immobilization by cross-linking and oxidation in aqueous solution. Bioresour Technol 100:3459–3464
11. Xie W, Ma N (2009) Immobilized lipase on Fe_3O_4 nanoparticles as biocatalyst for biodiesel production. Energ Fuel 23:1347–1353
12. Xie W, Wang J (2012) Immobilized lipase on magnetic chitosan microspheres for transesterification of soybean oil. Biomass Bioenerg 36:373–380
13. Tang Z-X, Qian J-Q, Shi L-E (2007) Preparation of chitosan nanoparticles as carrier for immobilized enzyme. Appl Biochem Biotech 136:77–96
14. Gregorio-Jauregui KM, Pineda MG, Rivera-Salinas JE, Hurtado G, Saade H, Martínez JL, Iliná A, López RG (2012) One-step method for preparation of magnetic nanoparticles coated with chitosan. J Nanomater. doi:10.1155/2012/813958
15. Osuna Y, Gregorio-Jauregui KM, Gaona-Lozano JG, de la Garza-Rodríguez IM, Ilyna A, Barriga-Castro ED, Saade H, López RG (2012) Chitosan-coated magnetic nanoparticles with low chitosan content prepared in one-step. J Nanomater. doi:10.1155/2012/327562
16. Gregorio-Jauregui KM, Carrizalez-Alvarez SA, Rivera-Salinas JE, Saade H, Martinez JL, López RG, Segura EP, Ilyina A (2014) Extraction and immobilization of SA-α-2,6-Gal receptors on magnetic nanoparticles to study receptor stability and interaction with *Sambucus nigra* lectin. Appl Biochem Biotechnol 172:3721–3735
17. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
18. Hung TC, Giridhar R, Chiou SH, Wu WT (2003) Binary immobilization of *Candida rugosa* lipase on chitosan. J Mol Catal B - Enzym 26:69–78
19. Shaw JF, Chang RC, Wang FF, Wang YJ (1990) Lipolytic activities of a lipase immobilized on six selected supporting materials. Biotechnol Bioeng 35:132–137
20. Dove R, Madamwar D (2006) Esterification in organic solvents by lipase immobilized in polymer of PVA-alginate-boric acid. Process Biochem 41:951–955

Journal : Large 449 | Dispatch : 4-3-2015 | Pages : 9
Article No. : 1385 | ☐ LE | ☐ TYPESET
MS Code : BPBSE-14-0766 | ☑ CP | ☑ DISK

21. Ranjbakhsh E, Bordbar AK, Abbasi M, Khosropour AR, Shams E (2012) Enhancement of stability and catalytic activity of immobilized lipase on silica-coated modified magnetite nanoparticles. Chem Eng J 179:272–276
22. Li Y, Gao F, Wei W, Qu J-B, Ma G-H, Zhou W-Q (2010) Pore size of macroporous polystyrene microspheres affects lipase immobilization. J Mol Catal B Enzym 66:182–189
23. Handayani N, Miletic N, Loos K, Achmad A, Wahyuningrum D (2011) Properties of immobilized *Candida antarctica* Lipase B on highly macroporous copolymer. Sains Malays 40:965–972
24. Wang S-G, Zhang W-D, Li Z, Ren Z-Q, Liu H-X (2010) Lipase immobilized on the hydrophobic polytetrafluoroethene membrane with nonwoven fabric and its application in intensifying synthesis of butyl oleate. Appl Biochem Biotech 162:2015–2026

Journal : Large 449 | Dispatch : 4-3-2015 | Pages : 9
Article No. : 1385 | ☐ LE | ☐ TYPESET
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