α-Amilase *Aspergillus oryzae* Immobilized on Expanded Perlite Modified

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Abstract

The α-amylase from *Aspergillus oryzae* was immobilized covalently onto expanded perlite (EP) modified by treatment with TiO_2 (EP-TiO_2), dye HE3B (EP-HE3B) polyethyleneterephthalate (PET)-hydrazide (EP -PET) and magnetite (PE-magnetite). The modified perlite were characterized using Fourier transform infrared spectroscopy (FT-IR) and scanning electron microscopy (SEM). The supports were functionalized with aminopropyltriethoxysilane (APTES) and glutaraldehyde (GA). The optimum pH for free and immobilized α-amylase was 5.5. Temperature of maximum activity for free enzyme and immobilized enzyme on EP-HE3B was 50°C, whereas for the immobilized enzyme in EP-APTES this value was 55°C. The immobilized α-amylase in EP-APTES and EP-HE3B exhibited better thermostability than free enzyme. The immobilized derivatives showed moderate operational stability by retaining 50% of initial activity after seven successive reuses.

Key words: perlites, zeolites, immobilized enzyme, alpha amylase

Introduction

The enzyme α-amylase (1,4-α-D-glucan glucanohidrolase, EC 3.2.1.1) hydrolyzes starch by breaking of the internal α-1,4-glycosidic bonds. This enzyme is produced by a wide variety of organisms, fungi, bacteria and plants. Amylases are one of the most important industrial enzymes that have a wide variety of applications ranging from conversion of starch to sugar syrups, to the production of cyclodextrins for the pharmaceutical industry (Sivaramakrishnan et al. 2006). For the hydrolysis of the starch on industrial scale the enzyme use is necessary with high thermal stability. The enzyme immobilization frequently produces an increase of the thermal and operational stability of the enzyme (Klibanov 1983). The immobilization process can be carried out fixing the enzyme to some organic and/or inorganic material. The main advantage derived from immobilization is the easy recovery of products and substrates surpluses and the reusability of the catalyst (Buchholz 1992). The ideal support must be of low cost, inert, physically hard and resistant to the microbial attack. The perlite is a volcanic glass of high content in silica and alkalis, that can expand when it is exposed to high temperatures. The perlite combines two characteristics that make it particularly attractive to be used like support for immobilized enzymes, one is its abundance and another is its low cost. α-amylase has been immobilized on several supports such as membranes (Bayramoglu et al. 2004); cellulose-coated magnetite nanoparticles (Namdeo et al. 2009); magnetic beads (Tuzmen et al. 2012; Akkaya et al. 2012); Sepa beads (Nwagu et al. 2012); gelatin (Jaiswal et al. 2012); for their applications in the starch hydrolysis.

In the present study α-amylase of *A. oryzae* was covalently immobilized on expanded perlite modified by treatment with TiO_2 (EP-TiO_2), HE3B dye (EP-HE3B), dacron-hydrazide (EP-PET) and magnetite (EP-magnetite), for application to the hydrolysis of starch. Also study the following properties: retention of activity, optimum pH and temperature, thermal stability and reuse of derivative.

Materials and Methods

Materials

Expanded perlite (Green perlite, Broken of Chiron-Dpto. of the Andes, provided by the Company Perlite Salta). Hydrazine hydrate, α-amylase *A.oryzae,* glutaraldehyde (5%) and Aminopropyltriethoxysilane (APTES) were from SIGMA (St.Louis, Mo, USA). Soluble starch was Mallinckodt, dinitrosalicylic acid (Morton Thiokol Inc.-Alfa Catalog Chemicals), Dacron was produced by Terphane S.A (Cabo, Brazil). All other reagents were of analytical grade.

Enzyme assays

The activity of α-amylase was determined using starch, prepared in 20 mM sodium phosphate buffer, pH 5.5. The progress of the reaction was continued measuring the amount of maltose produced by assay of the DNS (Miller 1959). The unit of catalytic activity katal (kat) was taken as the amount of enzyme that liberates 1 mol of maltose per second.

For the free enzyme was used the following procedure: 0.8 ml of starch 2 mg ml^{-1} in 20 mM sodium phosphate buffer pH 5.5 was added 30 µl enzyme 268 ηkat ml^{-1} (0,81 mg ml^{-1} protein). The mixture was incubated during 10 minutes at 50°C and the reaction was stopped by adding 0.8 ml of DNS, and the mixture was heated to boiling in a water bath for 5 minutes. The absorbance was measured at 540 nm.

The activity of immobilized enzymes was determined by incubating 0.8 ml of starch 2 mg ml^{-1} in 20 mM sodium phosphate buffer pH 5.5 with 30 mg of the water insoluble derivative, under shaking at 50°C. The mixture was incubated during 10 minutes, soon was separated the derivative, added to 0.8 ml of DNS and was heated to boiling in a water bath for 5 minutes. The absorbance was measured at 540 nm.

Support Preparation

The process of expansion of the perlite was conducted in a fluid bed furnace at a temperature of 1000°C, obtaining expanded perlite (EP) was used as a support. We worked with a particle size mesh 50/70.

Modified Support Preparation

Perlite – TiO_2 (EP-TiO_2)

A mixture of 0.5 g of TiO_2 and 18 ml of ethanol was added 1.5 ml of HNO_3 diluted with a pH of 3.5. The suspension was sonicated 5 minutes and was added 1 g of EP, was stirred for 30

minutes. Was filtered and calcined at 450°C for 30 minutes, once cold was washed 2 times with water. The sample was dried at 120°C for 24 hours.

Perlite – dye HE3B (EP-HE3B)

To 0.5 g of EP was treated with 15 ml water and 375 mg of dye HE3B (Red HE-3B), in addition to this mixture was added 7.5 ml of NaCl (2 M) and 3.75 ml of NaOH (1M .) The mixture was stirred for 16 hours at 60°C. Then washed with water, NaCl (1M), NH_4Cl (2M) and water until clear filtrate, then dried at 50°C.

Perlite - polyethyleneterephthalate-hydrazide (EP-PET)

Dacron Films (5.0 g) were cut in strips and incubated with 50 ml of methanol and 12.5 ml of hydrazine hydrate at 40ºC for 17 hours with stirring. Afterwards, the mixture was filtered under vacuum, dried at 50ºC and particles smaller than 250 μm collected by sieving (Soria et al. 2012). Dacron-hydrazide obtained was mixed with PE at a ratio (1:1), and then the mixture was calcined at 250°C for 7 minutes.

Perlite – magnetite (EP-magnetite)

A 2 g of EP was treated with 10% HCl, stirred for 30 min at 60°C. The EP (2 g) was suspended in 100 ml of distilled water and 10 ml of 0.6 M $FeCl_2$ and 1.1 M $FeCl_3$ were added with stirring. The pH value was adjusted to 10 with 28% (w/v) NH_4OH. After a 30 min incubation at 95ºC with stirring, the magnetized particles were washed with deionized water until pH 7 and dried at 50ºC overnight. The treated particles were recovered using magnetic field (6,000 Oe).

Functionalization of the supports with APTES and activation with GA

Were added 2 ml of APTES (10% in acetone) 0.1 g of each of the supports, stirred for 2 hours at 20°C. Were washed with water and 20 mM sodium phosphate buffer pH 5.5 and dried at 50°C for 12 hours. The supports particles (30 mg) were incubated with 1 ml of 5% v/v glutaraldehyde in water under mild stirring for 2 h at 25°C. Afterwards they were washed ten times with buffer.

α-amylase immobilization

APTES-GA activated particles (30 mg), were incubated with 1 ml of α-amylase solutions in 20 mM sodium phosphate pH 5.5, at a concentration of 268 ηkat ml^{-1} (0,81 mg ml^{-1} protein) during 16 h at 4ºC and 20 rpm. The α-amylase derivatives were recovered by centrifugation, the magnetized derivative was recovered by action of a magnetic field (6,000 Oe). The supernatants were collected for protein determination according to Lowry method. The immobilized enzyme

were washed five times with 1 ml of 20 mM sodium phosphate buffer pH 5.5, and stored at 4ºC for their later use.

Characterization of the supports

The supports were characterized by Fourier transform infrared spectroscopy (FT-IR) on a Bruker IFS 88, samples dispersed in KBr and working range of 4000-400 cm^{-1}. SEM (scanning electron microscopy) analysis was done on Scanning Electron Microscope JEOL model JSM 6480 LV.

Effect of pH and temperature on enzymatic activity

Optimal pH and temperature of the free and immobilized α-amylases were established by measuring their activities at several pH values from 4.5 to 8.5, using Na_2HPO_4 citric acid at 55°C and at different temperatures (in 20 mM sodium phosphate buffer pH 5.5) in the range from 30° to 70°C, respectively.

Kinetics studies

The kinetics of thermal inactivation of the free enzyme was determined by incubating 500 μl of the enzyme 268 ηkat ml^{-1} (0.81 mg ml^{-1} protein) in 20 mM sodium phosphate buffer pH 5.5, at temperatures between 40°C and 55°C. Samples of 5 μl were withdrawn at time intervals of fifteen minutes and its activity was measured immediately as described previously.

The kinetics of thermal inactivation of the immobilized derivatives EP-APTES and EP-HE3B was determined incubating 30 mg of support containing 0.090 ηkat/mg and 0.100 ηkat/mg of support respectively, in 20 mM sodium phosphate buffer pH 5.5, at 55°C. Every fifteen minutes a tube of the respective immobilized derivative was separated and its activity was measured immediately as described previously. Once determined the activity of the samples, these were discarded.

The reversibility of the inactivation was tested incubating 500 μl of the free enzyme (268 ηkat ml^{-1}), and 30 mg of immobilized derivatives EP-APTES and EP-HE3B (0.090 ηkat / mg and 0.100 ηkat / mg respectively) in 1 ml of buffer 20 mM sodium phosphate pH 5.5 at 55°C for 45 minutes. The samples were then incubated at 20°C up to no more increase of activity was observed.

Reusability of the immobilized derivatives

The activity of the immobilized derivative was measured seven times consecutively. After each derivative activity determination they were recovered by centrifugation, washed with 20 mM sodium phosphate pH 5.5 buffer and reused.

The graphics, non-linear regressions, and statistic calculations were done using the PRISM software of GraphPad, USA.

Results and Discussion

Characterization of the supports

Figure 1 shows the FTIR spectrum for each of the supports modified before the functionalization with APTES and activation with GA. The FTIR EP without any treatment (Figure 1A) shows characteristic bands corresponding to OH-groups (3450 cm^{-1}), the streching of the Si-O gives rise to peaks of absorbance at 1100 cm^{-1}, while vibration of Si-O bond is observed at 460cm^{-1}. The EP spectra treated with the dye HE3B and TiO_2 (Figure 1B and 1C) are not significantly different to that of untreated EP. Figure 1D shows the FTIR spectra of the EP-magnetite, the characteristic bands of ν_{Fe-O} are 632 cm^{-1} and 548 cm^{-1}. However, the FTIR spectrum of the EP-PET, Figure 1E, shows characteristic bands of PET. Treatment with hydrazine PET replaces the most of the ester bonds by amide bonds. The band at 3324 cm^{-1} can be attributable to the ν_{N-H} amino group. The absorption band at 1628 cm^{-1} can be assigned to ν_{C-O} of an amide carbonyl, 1542 cm^{-1} ν_{C-N} amide, 1338 cm^{-1} δ_{N-H} amide. Bands are also observed at 1490 cm^{-1} and 1608 cm^{-1} corresponding to $\nu_{C=C}$ aromatic ring, and a band at 732 cm^{-1} assigned to an aromatic ring δ_{C-H}. These results indicate that different materials (TiO_2, HE3B, PET and magnetite) were incorporated into the EP, without changing the structure of it.

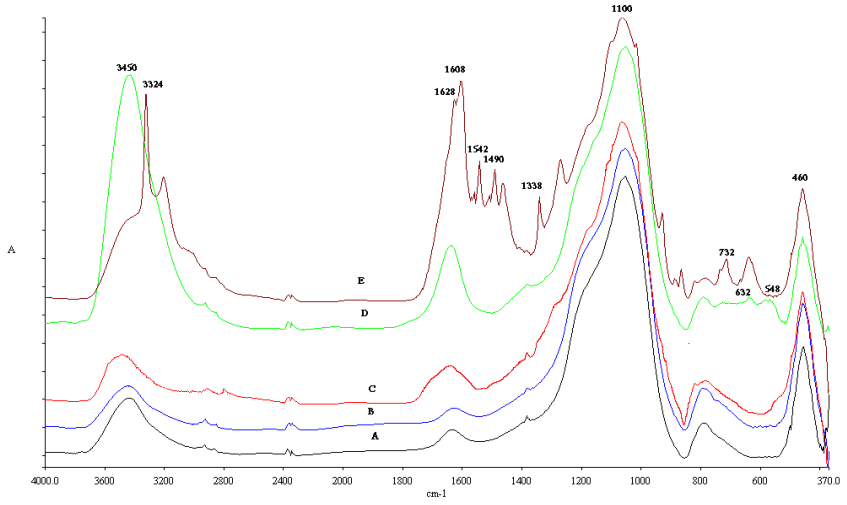


Figure 1. FTIR spectrum. (A) Expanded Perlite no treatment; (B) Expanded Perlite-HE3B; (C) Expanded Perlite-TiO_2; (D) Expanded Perlite-magnetite (E) Expanded Perlite-PET.

SEM results show the effect of different treatments on PE, **Figure 2**. Can see that the EP treated with PET presents the greater change, Figure 2e.

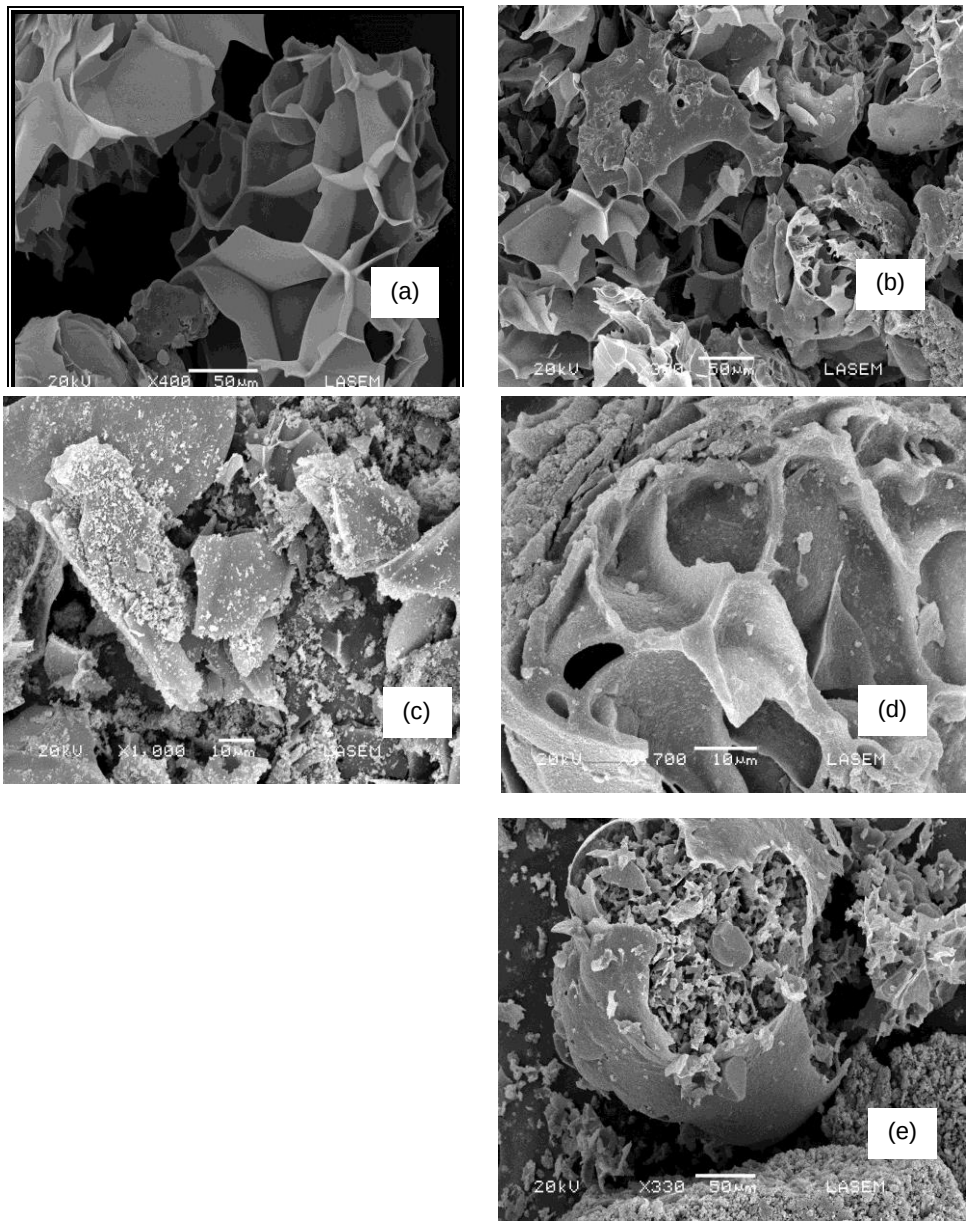


Figure 2. SEM pictures. (a) Expanded Perlite no treatment (x 400); (b) Expanded Perlite-HE3B (x300); (c) Expanded Perlite-TiO_2 (x1000); (d) Expanded Perlite-magnetite (x700); (e) Expanded Perlite-PET (x330).

Immobilization of α-amylase

The immobilization of enzymes adds additional cost and is only justified if economic or the use of derivative represents is an advantage to the process regarding the use of free enzymes (Chaplin 1990). The nature of the support could have a significant effect on enzyme activity expressed by the enzyme and the apparent kinetic. The natural silicoaluminates can be modified by means of hydrothermal treatments, in order to obtain a higher reactivity of the surface, so as to transform the amorphous silicate zeolitic structures. The immobilization by covalent bonding has the advantage of practically lack of loss of enzyme from the support by washing during use. Covalent bond methodology most commonly used is based on a two step process firstly binds to support a reagent such as aminopropyltriethoxysilane (APTES), which then reacts with a bifunctional reagent such as glutaraldehyde, so that this finally react with the proteins via formation of Schiff's base.

A.oryzae α-amylase was covalently bound via APTES-GA on EP, EP-TiO_2, EP-HE3B, EP-PET and EP-magnetite. The retained an activity on activated supports, EP-APTES, EP-TiO_2, EP-HE3B, EP-PET y EP-magnetite, were 0.0902; 0.0486; 0.100; 0.113 and 0.137 ηkat mg^{-1} of support respectively. Although the amount of enzyme bound to activated supports EP-APTES, EP-HE3B, EP-PET y EP-magnetite were similar, the α-amylase immobilized on EP-APTES and EP-HE3B have the highest percentages of retention of activity after the third reuse. The latter result suggests that modifications of the EP with APTES and HE3B promote more effective interactions with the enzyme. The supports EP-APTES and EP-HE3B were selected as the best performance for subsequent immobilization studies α -amylase.

The dependence of the amount of protein fixed to the supports EP-APTES and EP-HE3B and that offered in the immobilization process is shown in **Figure 3a**. An increase of the fixed protein is observed for both materials in the tested range (0 to 20 μg of protein per milligram of support). The amount of fixed protein for EP-APTES and EP-HE3B was 2.6 and 2.1 μg mg^{-1} of support respectively at the highest amount of offered protein. In the literature, there are many different loading values for different supports, for example fixed protein were reported as 4.0 μg mg^{-1} on PMMA beads support (Aksoy et al. 1998); 18.2 μg mg^{-1} on cellulose-coated magnetite nanoparticles support (Namdeo et al. 2009) and 25.5 μg mg^{-1} glass support (Kahraman et al. 2007). Figure 3b and c displays the retained and specific activities for both derivatives, respectively, the maximum values of specific activity in EP-APTES and EP-HE3B were 0.22 and

0.32 ηkat / mg of support respectively. These values decrease by increasing the amount of protein offered, this decline can be attributed to enzyme overloading.

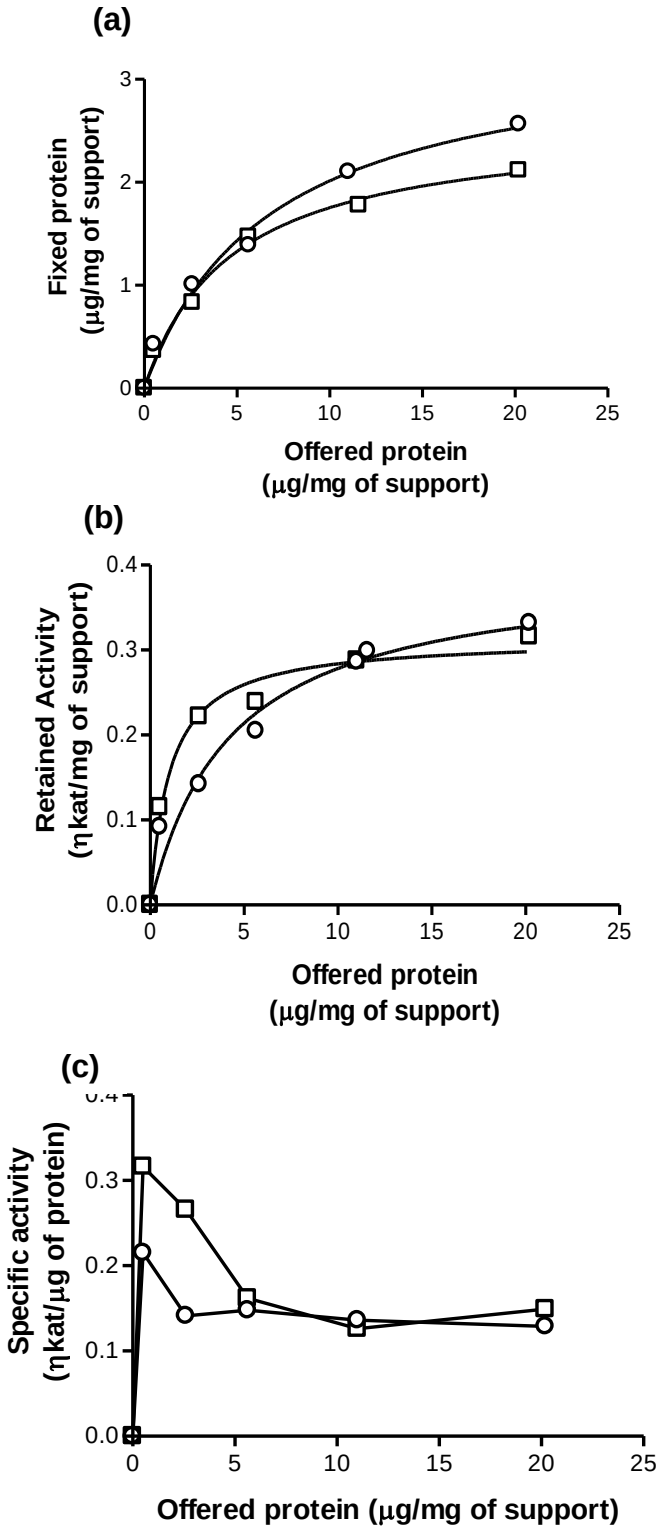


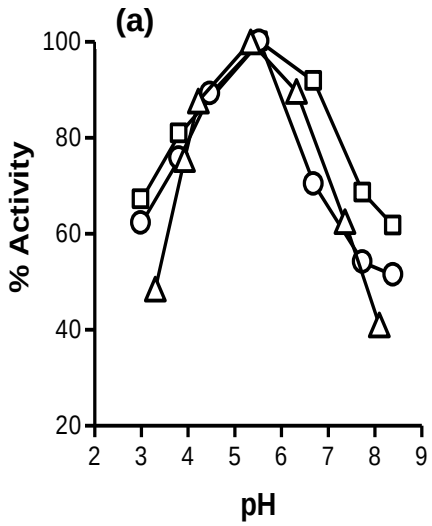
Figure 3. Relationship between the fixed α-amylase (a) retained (b) and specific activities (c) on EP-APTES (white circle) and EP-HE3B (white square) and the amount of offered enzyme. Supports particles (30 mg) were incubated with 1 ml of α-amylase solutions prepared in the

buffer for 16 h at 4°C. (○) α-amylase immobilized on expanded perlite (EP)-APTES; (□) α-amylase immobilized on expanded perlite (EP)-HE3B.

Effect of pH and the temperature on the enzymatic activity

The pH dependence of the enzyme activity free and immobilized on the EP-APTES y EP-HE3B is shown in **Figure 4a**. The optimum pH of free enzyme resulted 5.5, this value does not differ from determined for immobilized derivatives EP-APTES and EP-HE3B. This pH optimum determined are not discrepant from those immobilized α-amylases reported: pH 6.0 (Nwagu et al. 2012; Saikumar et al. 2006; Singh et al. 2012) and pH 5.5 (Kahraman et al. 2007).

The activity of the free and immobilized enzyme as a function of temperature is shown in Figure 4b. The maximum temperature for free and immobilized enzyme on EP-HE3B was 50°C, but for the immobilized enzyme in EP-APTES this value was 55°C, indicating the existence of a thermal stabilization. These temperatures of maximum activity are similar to those reported for other α-amylases: 60°C (Saikumar et al. 2006); 50°C (Nwagu et al. 2012; Singh et al. 2012) and 57°C (Raviyan et al. 2003).



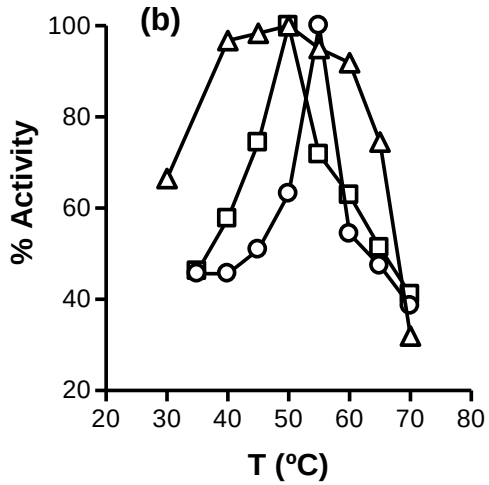


Figure 4. Effect of pH (a) and temperature (b) on the enzymatic activity of free and immobilized α-amylase. Values of pH were obtained by using Na_2HPO_4 citric acid and activities determined at 50°C. The temperature was analyzed between 30° and 70°C at pH 5.5 in 20 mM phosphate buffer. (Δ) free enzyme; (○) α-amylase immobilized on expanded perlite (EP)-APTES; (□) α-amylase immobilized on expanded perlite (EP)-HE3B.

Kinetics studies

In order to obtain useful information for the study of enzymatic hydrolysis of starch, the kinetics of thermal inactivation of free α-amylase was studied between 40°C and 55°C. Residual activity values obtained by measurements carried out immediately after taking samples of the treated solution were plotted as a function of time**, Figure 5.** The results were then compared to 55°C with the immobilized enzyme EP-APTES and EP-HE3B, **Figure 6**. The immobilized derivatives have lower inactivation against the heat treatment, the curves were obtained by nonlinear regression using the equation of first order decay. The immobilization of the enzyme increases the residual activity between 40 and 50% relative to the free enzyme during incubation at 55°C. The immobilization as a strategy to increase the thermal stability of the enzymes is widely used to stabilize the native structure of proteins against unfolding (Klibanov 1983). It was observed, however, that if the inactivated samples, free and immobilized enzymes, were incubated at 20°C, the activity lost during the inactivation at 55°C for 45 minutes was partially recovered in a 20 hours, **Table 1**. The activity recovered for derivatives immobilized EP-APTES and EP-HE3B was 90% and 70% respectively. The free enzyme has a lower activity recovery 35% compared with the immobilized enzyme. These results suggest that the immobilization process the enzyme

stabilizes against unfolding reversible, assuming that the inactivation process should take place according to the classical model of inactivation in two steps (Lumry et al. 1954).

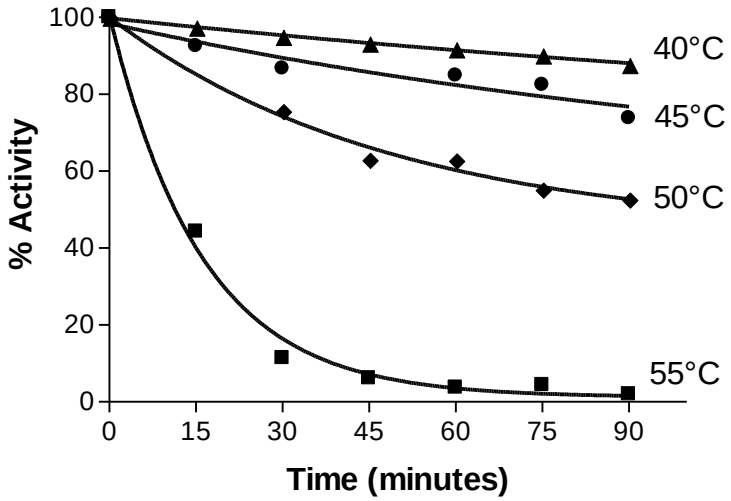


Figure 5. Thermal inactivation of α-amylase free *A.oryzae* at different temperatures.

Solid lines: residual activity immediately after the inactivation treatment. Initial activity 268 kat /ml^{-1} (0.81 mg ml^{-1} protein) in buffer 20 mM sodium phosphate pH 5.5. The solid lines were obtained by nonlinear regression using the equation of first order decay, R^2 0.9085, 0.9922, 0.9933, 0.9598 respectively and P value of test runs 0.700, 0.543, 0.543, and 0.543 respectively.

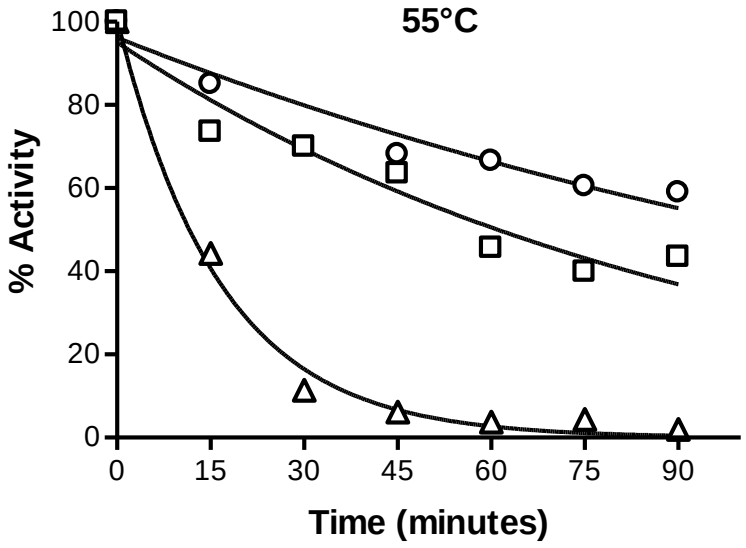


Figure 6. Thermal inactivation of α-amylase free and immobilized at 55°C.

Solid lines: residual activity immediately after the inactivation treatment. Initial activity of the immobilized enzyme: EP-APTES 0.090 ηkat mg^{-1} and EP-HE3B 0.100 ηkat mg^{-1} support in

buffer 20 mM sodium phosphate pH 5.5. The solid lines were obtained by nonlinear regression using the equation of first order decay, R2 0.9545, 0.9341, 0.9933 respectively and P value of test runs 0.900, 0.800, 0.543 respectively. (Δ) free enzyme; (○) α-amylase immobilized on expanded perlite (EP)-APTES; (□) α-amylase immobilized on expanded perlite (EP)-HE3B.

Table 1. Thermal Inactivation at 55°C of α-amylase free and immobilized.

α-Amylase preparations	Treatment	Residual Activity (%) Time (h)		
		3	7	20
Free enzyme	Inactivation + Recovery[a]	30±1	35±1	35±0
Immobilyzed enzyme EP-HE3B	Inactivation + Recovery	53±1	55±0	59±1
Immobilyzed enzyme EP-APTES	Inactivation + Recovery	59±1	65±2	76±1

Values are then mean of two experiments ± standard deviation.
[a]Inactivation follow by incubation at 20°C, 20 hours.

Reuse of immobilized derivative

An important feature arising from the immobilization of an enzyme, is the ability to reuse, which justifies immobilize evaluate when considering costs with respect to the use of a soluble enzyme. **Figure 7** shows that the derivative PE-APTES maintained a 45% of its initial activity after seven consecutive reuses. Whereas the derivative PE-HE3B maintained a 58% of its initial activity under the same test conditions.

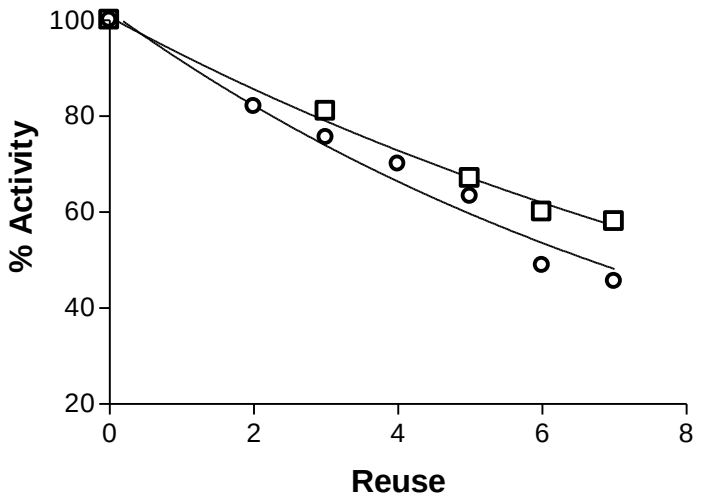


Figure 7. Stability of the derivative immobilized for reuse at 55°C.

Free enzyme initial activity: 268 ηkat ml $^{-1}$ in buffer 20 mM sodium phosphate pH 5.5. Initial activity of immobilized enzyme: EP-APTES 0.090 ηkat mg^{-1} and EP-HE3B 0.100 ηkat mg^{-1} support in buffer 20 mM sodium phosphate pH 5.5. The solid lines were obtained by nonlinear regression using the equation of first order decay. (○) α-amylase immobilized on expanded perlite (EP)-APTES; (□) α-amylase immobilized on expanded perlite (EP)-HE3B.

Conclusions

It can be concluded that *Aspergillus oryzae* α-amylase has a low thermal stability, this limits their use in the hydrolysis of starch solutions on a large scale. Immobilization of α-amylase in expanded perlite functionalized by covalent attachment increases its thermal stability allowing its application in several biotechnological areas.

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