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Modification and characterization of natural aluminosilicates, expanded perlite, and its application to immobilise α – amylase from *A. oryzae*

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ABSTRACT

The aim of this study was to modify natural silicoaluminates as expanded perlite (EP) through simple and inexpensive treatments in order to get a greater reactivity of surface and immobilise α-amylase from *A. oryzae* by adsorption and covalent binding. The materials obtained (expanded perlite treated with HCl, rehydroxylated expanded perlite, zeolite, and the incorporation of polystyrene in expanded perlite) were analysed by infrared spectroscopy, thermal analysis, zeta potential, adsorption of N_2, and Scanning Electron Microscopy. The analysis allowed us to confirm that the modifications on the materials studied were effective. This is perceived by an increase in bands corresponding to OH groups, which is beneficial when considering functional groups that interact directly with the enzyme. It also allowed us to determine that the materials tested exhibit good thermal stability at the temperature range in which enzymatic studies are conducted. The conditions for immobilisation (pH, concentration of glutaraldehyde, time of contact between the enzyme and the substrate, enzyme concentration) and some properties of the immobilised derivatives (optimum pH, temperature of maximum activity, and reuse) were analysed. The suitable range for the immobilisation of α-amylase from A. *oryzae* by covalent binding was pH between 5 and 6, and for immobilisation by adsorption the suitable pH range was between 5 and 5.5. These values are consistent with the zeta potential values previously determined for the different media studied. The reuse of the immobilised enzyme by covalent binding resulted in a residual activity of 90% up to the sixth reuse. Immobilisation by covalent binding was more effective than immobilisation by adsorption.

1. Introduction

Obsidian is a volcanic igneous rock composed of volcanic glass (mainly SiO_2). It also presents a number of elements with concentrations lower than 0.1%, commonly called trace elements. Its color depends on the composition of impurities. Although obsidian has a water content of about 1% [1], it progressively hydrates when exposed to groundwater. Thus, obsidian becomes a perlitic structure over time, causing the breakdown of the rock into millimetric spherules and fragments due to unevenly contracted cracks. These cracks and tiny bubbles (spherules) give rise to devitrification. First, microscopic crystals of quartz, feldspar, and cristobalite form, and an orderly growth of crystals like radial fibers (spherulites) can be observed. Obsidian is characterized by spherulites called perlite which give it the appearance of a pearl when viewed microscopically [2]. A perlite characterizes and differs from other volcanic glasses in that its original volume can expand four to twenty times when subjected to heating. The crude rock inflates causing countless tiny bubbles, due to the impossibility of diffusion of water generated in the dehydration process, when it is rapidly heated by industrial processes at temperature ranges 900 °C–1000 °C. This occurs by the reaction of the silanol groups:

$$\equiv Si{-}OH + HO{-}Si\equiv - > \equiv Si{-}O{-}Si\equiv + H_2O$$

This product demonstrates a low density and other exceptional physical properties of expanded perlite. The use of expanded perlite has spread to many sectors, including construction and industrial applications [3], being United States, Greece, Japan, Argentina, Turkey, Hungary, Italy, Mexico, Armenia, and Slovakia the main producers. Perlite, together with borates and lithium salts, is one of the main minerals exported. In Argentina, there are perlite deposits in the provinces of Catamarca, Jujuy, Mendoza, Rio Negro, Chubut, and Salta. There are more than fourteen varieties of obsidian in Northwestern Argentina (NWA) [4], with six identified in Cata-

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marca, one on the border between Catamarca and Jujuy provinces, another one on the border among Jujuy, Bolivia and Chile [5,6] and three in Salta (Quirón, Ramada, Alto Tocomar). Salta deposits are characterized by the mineral quality and quantity. The main deposits are located in the Puna, near San Antonio de los Cobres. The oldest sources of perlite are Ramada and Quirón [7,8].

The characteristics of expanded perlite, including its low cost and abundance in the region [9], make it attractive as a support for enzyme immobilisation.

The α-amylase (EC 3.2.1.1) catalyse the hydrolysis of glycosidic bonds in the starch, as well as in other polysaccharides. These enzymes are produced by plants, animals and microorganisms, and are commonly used in various industries such as starch processing, papermaking, and pharmacology [10]. Starch, a polysaccharide found in plants, is very important as a raw material for the production of sweeteners, thickening agents, and adhesives. The use of starch requires degradation to maltodextrins, maltose and glucose, which could be done with amylolytic enzymes of different specificities.

The α-amylase hydrolyses glycosidic α- internal bonds (1,4), the starch produces glucose, maltose or dextrin.

For the industrial application of enzymes, immobilisation seems to be the most satisfactory method to obtain a more stable biocatalyst. The advantages of using immobilised enzymes are well known; mainly, they facilitate the recovery of remaining products and substrates, as well as catalyst recovery and reuse [11,12].

The methods proposed in this paper are immobilisation by adsorption and covalent binding, using a commercial preparation of α-amylase from *A. oryzae,* and some of its properties are also studied.

2. Materials and methods

2.1. Materials

Expanded perlite (Perlita Verde, Quebrada de Quirón – Dpto. de los Andes – provided by Sociedad Perlita de Salta). Glutaraldehyde, α-amylase from A. *Oryzae,* and aminopropyltriethoxysilane (APTES) were obtained from Sigma (St. Louis, Mo, USA). Dacron was produced by Terphane S.A. (Cabo, Brazil); Mallinckrodt soluble starch and dinitrosalicylic acid was obtained from Morton Thiokol Inc. (Productos Químicos Alfa catalog). All other reagents were of analytical grade.

2.2. Preparation of expanded perlite

The expansion of perlite was carried out in a fluidized bed furnace at 1000 °C [13]. A fraction of expanded perlite (EP) was used directly as support, with a 50/70 particle mesh size, while different modification treatments were applied to other fractions.

2.2.1. Modification of expanded perlite

2.2.1.1. Rehydroxylation of EP (EP-RH). Expanded perlite applications are limited due to its low superficial reactivity. However the superficial reactivity can be increased by different treatments. Hydrothermal treatments are adequate for expanded perlite. When expanded perlite is exposed to water vapor [13] a hydrated glass having OH groups on the surface is obtained by the following process:

$$\equiv Si - O^- Na^+ + H_2O \rightarrow \equiv Si - OH + Na^+OH^- \quad (1)$$

A free hydroxyl ion is formed in the hydrolysis reactions and a second important step in the glass corrosion takes place:

$$\equiv Si{-}O{-}Si\equiv + OH^- \rightarrow \equiv Si{-}OH + \equiv Si{-}O^- \quad (2)$$

In reaction (2) the very strong bond (Si-O-Si) is broken and gives rise to another active group, wich reacts with water as:

$$\equiv Si{-}O^- + H_2O \rightarrow \equiv Si{-}OH + OH^- \quad (3)$$

The overall reaction may be written as:

$$\equiv Si{-}O{-}Si\equiv + H_2O \rightarrow 2\equiv Si{-}OH \quad (4)$$

The OH groups on the surface can interact with special chemical groups, like organics.

The rehydroxylation of perlite was performed by treating the EP at 250 °C, 800 psi for 72 h.

2.2.1.2. Zeolitization (Zeolite). Sodium silicate (Fisher; Na_2O 27.7 wt% with a SiO_2/Na_2O ratio of 2:1 approximately) was used as additional Si source and sodium form of a commercial ZSM-5 zeolite (ALSI-Penta SN-55, $SiO_2/Al_2O_3 = 23$) was used as a crystallization seed. EP was used as the only Al source and sodium silicate was employed as a supplementary Si source to obtain the optimum Si/Al molar ratio.

The mixture was subjected to a hydrothermal treatment at 453 K for 24 h. This was conducted at various pH values ranging from 10.1 to 13 [14].

2.2.1.3. Treatment with HCl (EP-HCl). When acid aqueous solution acts on sodium silicate glasses, the primary results is the rupture of the O—Na bond. Sodium ions, being very mobile, are readily replace by hydrogen ions present in the solution and the following exchange reaction occurs [15]:

≡Si—O—Na + HCl → ≡Si—O—H + NaCl

EP was treated with HCl 10% v/v at 60 °C, to provide the surface with hydroxyl groups. The treatment was performed at varying times (30, 60, and 90 min).

2.2.1.4. Perlite - polystyrene (EP-MC). Then, 0.3 mL of divinylbenzene, 2.7 mL of styrene, and 0.6 mL of Tween 80 were added to 1.00 g EP and stirred for 10 min. After that, 10.8 mL of $K_2S_2O_8$ 0.025 g mL^{-1} (flow 0.80 mL/min.) was added. The sample was polymerized at 80 °C for 6 h. It was cooled, filtered, and dried at 50° C for 12 h [16].

2.2.2. Functionalization of the supports with APTES

The supports were treated with APTES as follows: in a reflux system, 1.50 g of the sample was suspended in 100 mL of toluene. It was heated to 50 °C, and then 5.5 mL of APTES was added. Heating was continued up to 100 °C, and 5.5 mL of APTES was added again. Reflux was maintained for 6 h. Once the process was over, the sample was allowed to stand for 24 h under a hood to remove the toluene by evaporation. The modified silica was washed with ethyl ether and allowed to dry for 24 h [17].

2.3. Characterization of the supports

2.3.1. Structural and textural properties

2.3.1.1. Infrared spectroscopy (FTIR). FTIR spectra were obtained using a GX Perkin Elmer spectrometer. To obtain the positions of the absorption bands, the spectra were analysed using the Perkin Elmer Spectrum Applications software package and literature data. Tablets for FTIR studies were prepared with dried samples dispersed in KBr.

2.3.1.2. Thermal analysis. The prepared supports were subjected to high temperatures and a controlled heating rate, and the evolution of the structural properties was analysed by differential thermal analysis (DTA) and thermogravimetry (TG). These studies were performed simultaneously on a Rigaku TAS 1100 equipment using a temperature range from ambient conditions up to 1200 °C, with a heating rate of 20 °C/min in static air atmosphere.

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2.3.1.3. N_2 adsorption. The surface area of the supports was calculated using the Brunauer – Emmet – Teller (BET) method, and was obtained by N_2 adsorption in a single-point sorptometer, in a Micromeritics Flow Sorb II equipment. Complete adsorption-desorption isotherms of N_2 were obtained in a Micromeritics ASAP 2020 sorptometer at −195.8 °C.

2.3.2. Surface properties

2.3.2.1. Zeta potential. Zeta potential measurements were made on a Z-Meter 3.0 equipment. Suspensions at a concentration of 5×10^{-3}% (m/v) and an ionic strength of 10^{-2} M of KNO_3 were prepared. Then in order to reach equilibrium, different volumes of HNO_3 or KOH, at 0.1 M concentration in both cases, were added and kept in contact long enough to change the pH.

2.3.2.2. Scanning electron microscopy (SEM). The materials were analysed using a Scanning Electron Microscope (JEOL JSM 6480 LV), with dispersive energy analyser of X-rays (Thermo Electron, NORAM system, SIX NSS-100).

2.4. Free of α-amylase activity assay

The α-amylase activity was determined using starch as substrate; the reaction progress was followed by measuring the amount of maltose produced, according to the method described by Miller et al., [18] (DNS assay).

The following procedure was used: 30 μL of enzyme solution was added to 0.80 mL of substrate (15 mg mL^{-1}) dissolved in sodium phosphate buffer (20 mM pH 5.5). The mixture was incubated at 50 °C for 10 min, an aliquot of 0.5 mL of the reaction mixture was taken, and 0.5 mL of DNS was added; the mixture was heated to boiling point in a water bath for 5 min, cooled, then 5 mL of distilled water was added. The absorbance was measured at 540 nm.

The unit katal for catalytic activity (kat) was defined as the amount of catalyst that liberates 1 mol of maltose per second at pH 5.5 and 50 °C. An enzyme unit activity (U) = 1 μmol min^{-1} and 1 U corresponds to 16.67 nanokatals (ηkat).

2.5. Immobilised α-amylase activity assay

The following procedure was used: 0.8 mL of substrate (15 mg mL^{-1}) dissolved in sodium phosphate buffer (20 mM pH 5.5) was added to 30 mg of the immobilised derivative. The mixture was incubated at 50 °C for 10 min; then the immobilised enzyme was separated from the reaction mixture; 0.5 mL of the sample was taken and 0.5 mL of DNS was added; it was heated to boiling point in a water bath for 5 min, cooled, and 5 mL of distilled water was added. The absorbance was measured at 540 nm.

The unit katal for catalytic activity (kat) was defined as the amount of catalyst that liberates 1 mol of maltose per second at pH 5.5 and 50 °C. An enzyme unit activity (U) = 1 μmol min^{-1} and 1 U corresponds to 16.67 nanokatals (ηkat).

2.6. Immobilisation of α-amylase

2.6.1. Determination of the conditions for the immobilisation of α-amylase

2.6.1.1. The concentration of glutaraldehyde (GA). In determining the optimal concentration of GA for immobilisation of α-amylase by covalent binding, the following procedure was used: 1 mL of various concentrations of GA (2. 5, 5, 10, 15, 20 and 25% v/v in distilled water) were added to different samples of 30 mg of support treated with APTES, and stirred for 3 h at 20 rpm and 20 °C. After this time, the immobilised derivative was separated and washed three times with phosphate buffer pH 5.5. Subsequently, 1 mL of the enzyme solution 5.636 μg of protein mg^{-1}, support in phosphate buffer pH 5, was added. It was stirred at 20 rpm for 8 h at 4 °C. The immobilised derivative was separated and washed five times with phosphate buffer pH 5.5.

2.6.2. Determining the contact time between the enzyme and the support during immobilisation

In determining the contact time between the enzyme and the support during immobilisation by α-amylase adsorption (support without any treatment) and immobilisation by covalent binding of α-amylase (treated with APTES and GA 5% v/v support), the following procedure was performed: 1 mL of the enzyme solution 5.636 μg of protein mg^{-1} support in phosphate buffer pH 5.5 was added to different samples of 30 mg support. It was stirred at 20 rpm, 4 °C for different times (3, 6, 9, 12, 16, 20 and 25 h). The immobilised derivative was separated and washed five times with phosphate buffer pH 5.5.

2.6.3. Determination of pH for immobilisation

The suitable pH for immobilising α-amylase by adsorption and covalent binding was determined by the following procedure: solutions of α-amylase at concentration 11.005 μg of protein mg^{-1} support in buffer Na_2HPO_4/citric acid in a pH range 4.5 – 8.5 were prepared. Then 1 mL of each of the enzyme solutions was added to the different samples of 30 mg support, stirred for 16 h at 20 rpm and 4 °C. The immobilised derivative was separated and washed five times with its own buffer.

2.6.4. Determination of optimal concentration of α-amylase for immobilisation

2.6.4.1. Immobilisation by adsorption. One mL of the enzyme in 20 mM phosphate buffer pH 5.5, incubated for 16 h at 20 rpm and 4 °C, was added to 30 mg of each of the supports, without any pre-treatment. The immobilised derivatives were separated by centrifugation at 6000 rpm and the supernatants were separated for protein determination. The supports containing the immobilised α-amylase were washed five times with 1 mL phosphate buffer 20 mM pH 5.5 and stored at 4° C for later use.

2.6.4.2. Immobilisation by covalent binding. Fractions of 30 mg of support treated with APTES were incubated with 1 mL of aqueous solution of glutaraldehyde 5% (v/v) for three hours at 4 °C and 20 rpm. For removal of free glutaraldehyde, the incubated fractions were washed five times with 1 mL of 20 mM phosphate buffer pH 5.5. During washing the activated supports were separated by centrifugation at 6000 rpm. Then 1 mL of the enzyme in 20 mM phosphate buffer pH 5.5 (protein concentrations ranging between 30 and 600 μg mL^{-1}, incubated for 16 h at 4 °C and 20 rpm) was added to the activated supports. The immobilised derivatives were separated by centrifugation at 6000 rpm and the supernatants were separated for protein determination. The supports containing the immobilised α-amylase were washed five times with 1 mL phosphate buffer 20 mM pH 5.5 and stored at 4° C for later use.

The protein concentration immbolised on the support was determined by the difference between the initial amount of protein and the concentration obtained in the supernatants at the end of the incubations. Protein determinations were performed according with the method of Lowry et al. [19].

2.6.4.3. Effect of pH and temperature on enzyme activity. The optimum pH for the free enzyme and the different immobilised derivatives was measured at various pH values from 4.5 to 8.5 using buffer Na_2HPO_4/citric acid (McIlvaine buffer) at 50 °C. The maximum activity temperature for the free enzyme and the various immobilised derivatives was determined by measuring the activ-

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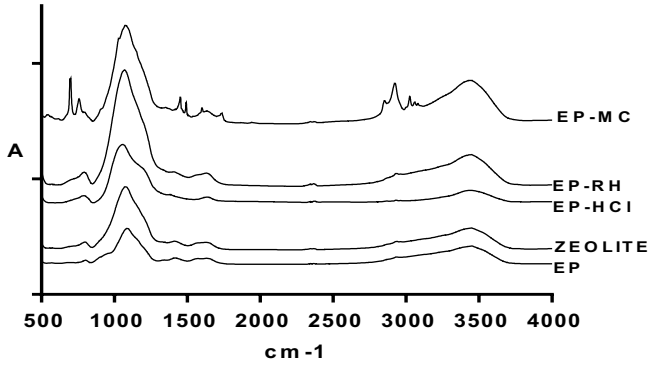

Fig. 1. FTIR spectrum of modified expanded perlite. Expanded perlite (EP), expanded perlite-polystyrene (EP-MC), expanded perlite treated with HCl (EP-HCl), rehydroxylated expanded perlite (EP-RH) and zeolite.

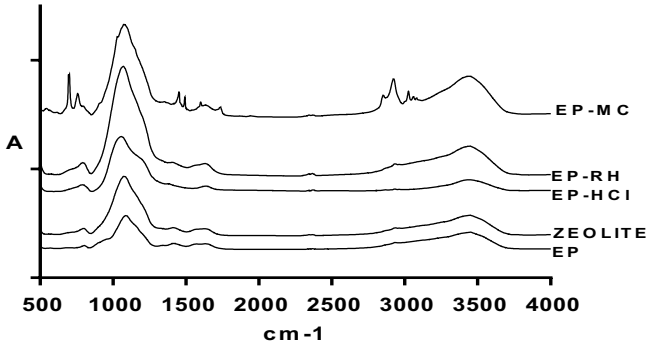

Fig. 2. FTIR spectrum of materials functionalised with APTES. Expanded perlite (EP), expanded perlite-polystyrene (EP-MC), expanded perlite treated with HCl (EP-HCl), rehydroxylated expanded perlite (EP-RH) and zeolite.

ity in buffer Na_2HPO_4/citric acid at different temperatures ranging from 30 °C to 70 °C.

2.6.5. Reuse of immobilised derivatives

The activity of each immobilised derivative was measured ten consecutive times, between each determination, the derivatives were recovered by centrifugation and washed with sodium phosphate buffer (20 mM, pH 5.5).

3. Results and discussion

3.1. Characterization

3.1.1. Infrared spectroscopy (FTIR)

Fig. 1 shows the FTIR spectrum for the expanded perlite (EP) and modified expanded perlite (EP-MC, EP-HCl, EP-RH and Zeolite), prior to functionalization with APTES.

The FTIR of EP without any treatment shows characteristic bands corresponding to groups OH (3450 cm^{-1}). The stretching of the Si—O results in absorbance peaks was observed at 1100 cm^{-1}, while the vibration (deformation) of Si—O at 460 cm^{-1}. Spectra of EP-HCl, EP-RH and Zeolite do not differ significantly from the FTIR of untreated EP.

Fig. 2 presents the FTIR spectra of each of the materials functionalized with APTES.

The FTIR of the materials treated with APTES shows new bands indicating that functionalization with this reagent was effective. A peak at 2927 cm^{-1} and a shift of the band at 1075 cm^{-1} are also shown. These correspond to stretching vibration of CH ethoxy groups and Si-OC, respectively. Small shoulders were observed at 1564 cm^{-1} corresponding to the out-of-plane deformation NH, and 1329 cm^{-1} shows a band due to CN stretching.

Table 1
Surface area and pore distribution for EP and modified EP.

Support	EP	Zeolite	EP-HCl	EP-RH	EP-MC
Surface BET (m^2/g)	2.0524	2.0159	2.2113	6.3703	3.1517

Table 2
Z potential EP, Zeolite, EP-HCl, EP-MC and EP-RH.

Materials	pH (point of zero charge)
EP	5.3
Zeolite	5.5
EP-HCl	6.0
EP-RH	5.5
EP-MC	5.1

3.1.2. Thermal analysis

Expanded perlite (EP) and modified expanded perlite (EP-HCl, EP-RH, zeolite and EP-MC) were analysed by thermogravimetry (TG). Fig. 3 presents the results obtained for the different materials.

The TG analysis for expanded perlite shows a mass loss of 20% corresponding to an associated exothermic event of about 400 °C. Similar behavior is observed in the materials of EP modified with HCl, EP-RH, and zeolite. This is attributed to the removal of water incorporated into the structure during the treatment applied to increase the OH groups.

The TG curve for the EP-MC sample shows a mass loss of 80% in relation to three endothermic events centered at 150 °C, 350 °C, and 500 °C, corresponding to the removal of physisorbed water in the solid and the elimination of structural water. Subsequently, two exothermic events are observed between 500 °C and 800 °C, attributed to the decomposition and removal of organic groups by combustion.

3.1.3. N_2 adsorption

Table 1 shows the values of specific surface area of the solid, measured by nitrogen adsorption using the BET single-point method, degassing temperature at 100 °C and residence time of 30 min.

From the values obtained (Table 1), it can be noted that there is no significant variation in the surface of the different materials except for EP-RH. For a more detailed analysis of the acquired porous structure, the adsorption-desorption isotherms of N_2 at 77 K were obtained (Fig. 4). EP, Zeolite, EP-HCl, and EP-RH present type II isotherms, according to the classification of adsorption isotherms proposed by Brunauer, Emmett and Teller. This is characteristic of adsorption processes in macroporous or non-porous solids. It represents the case of mono-multilayer adsorption, which study is well defined by the BET model. The so called point B is characteristic, indicating the time when the monolayer is complete.

Considering the isotherms according to IUPAC classification (classification of Hysteresis Loops), it can be noted that EP, Zeolite, EP-HCl, EP-RH, and EP-MC present H4 hysteresis loop, characteristic of porous materials having narrow pores shaped like cracks and uniform pore size distribution.

3.1.4. Zeta potential

The nature of the solid surface plays an important role in the adsorption of species. To analyse this property, the zeta potential was determined for each material (Fig. 5) and Table 2 shows the results for the different materials.

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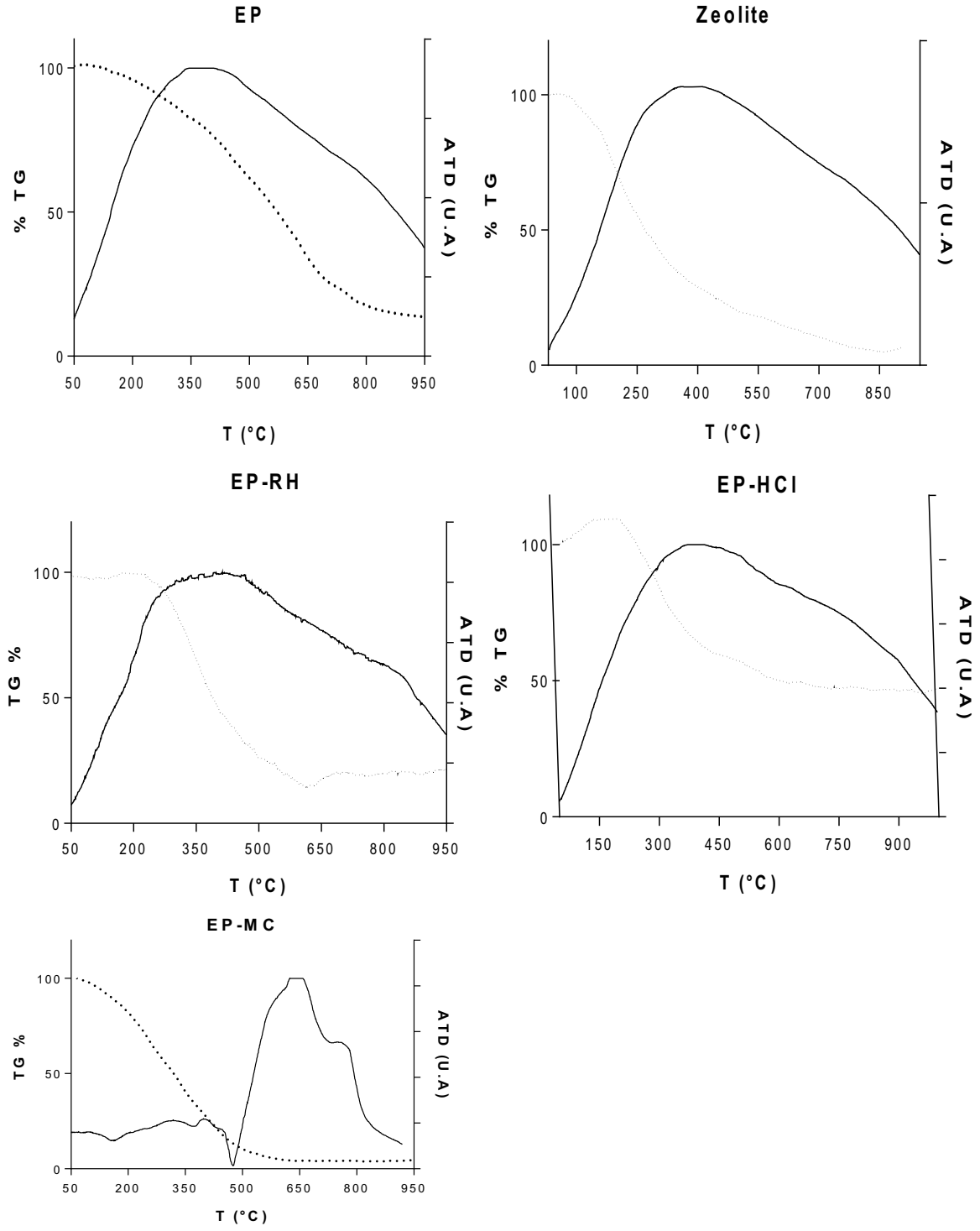

Fig. 3. Thermal Analysis ATD (—) TG (. . . . .). EP, ZEOLITE, EP-RH, EP-HCl and EP-MC.

According to the determined Z potential and the isoelectric point found in current literature for α-amylase enzyme from A. *Oryzae* [20], the possible pH range for the best interaction between the enzymes and the supports studied is between 4 and 6.

3.1.5. Scanning electron microscopy (SEM)

Fig. 6 shows the microscopies of the expanded perlite modified by the different treatments.

The micrographs performed and with reference to the untreated EP (Fig. 6A), show that the EP loses the original globular structure.

3.2. Enzyme immobilisation

3.2.1. Determination of the conditions for the immobilisation of α-amylase

3.2.1.1. The concentration of glutaraldehyde. The α-amylase enzyme from *A. oryzae* was covalently immobilised on the following supports: EP, Zeolite, EP-HCl, EP-RH and EP-MC, after functionalization of the supports with APTES and glutaraldehyde. To study the effect of glutaraldehyde concentration on

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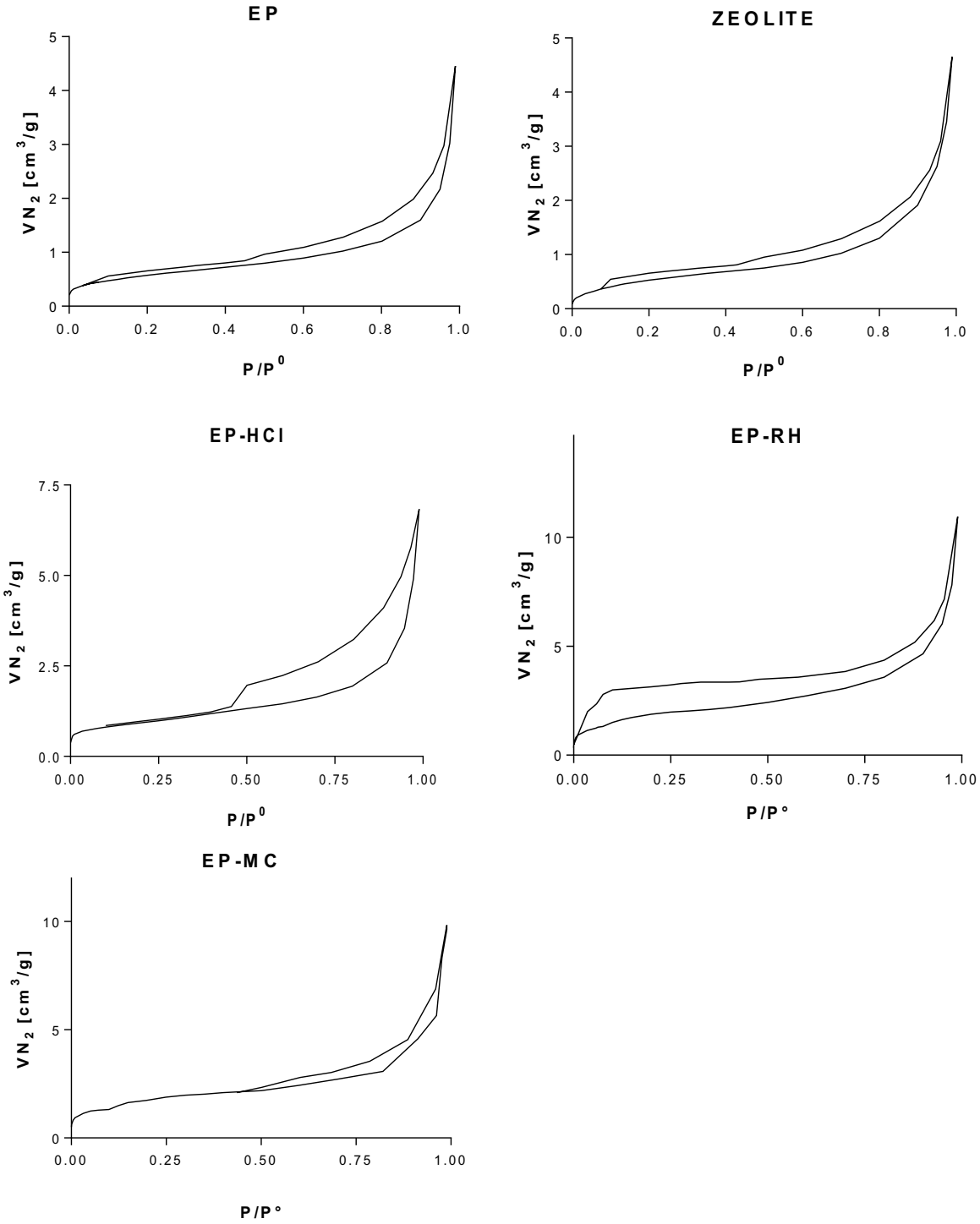

Fig. 4. Adsorption-desorption isotherms of N_2 at 77 K for EP, Zeolite, EP-HCl, EP-RH and EP-MC.

amylase immobilisation, enzyme was immobilised with different concentrations of glutaraldehyde ranging from 5% to 25%.

An increase in the amount of immobilised enzyme can be seen when concentration of GA used in the activation of the support is increased (Fig. 7). Probably, the increase of glutaraldehyde concentration increases the amount of free aldehydes groups on the support surface causing a higher amylase loading and activity.

From GA concentration of 5%, the activity of each immobilised derivative does not change significantly.

3.2.1.2. Immobilisation time determination. The time for covalent immobilisation of α-amylase from *A.oryzae*, after which no increased activity was observed, was about 15 h (Fig. 8).

When α-amylase from *A.oryzae* was immobilised by adsorption, a similar behavior was observed. After 12 h no increased activity was observed in the different immobilised derivatives (Fig. 9).

No more enzyme molecules were immobilised on the supports when the immobilisation time was extended.

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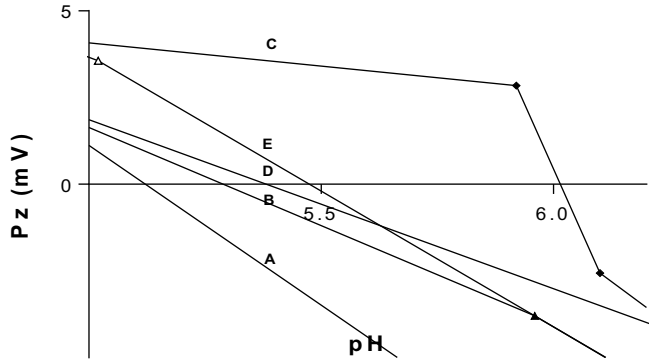

Fig. 5. Electrophoretic mobilities of (A) EP, (B) Zeolite, (C) EP-HCl, (D) EP-RH and (E) EP-MC.

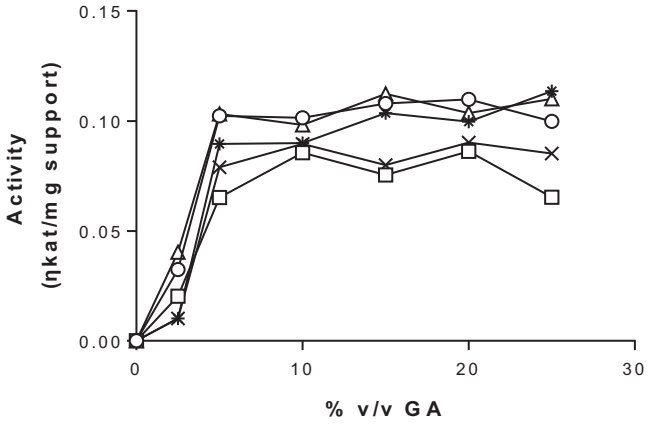

Fig. 7. Effect of concentration of glutaraldehyde on enzyme immobilisation. α-amylase immobilised on (o) EP, (□) zeolite, (Δ) EP-RH, (x) EP-MC and (*) EP-HCl.

3.2.1.3. Determination of pH for immobilisation. The suitable pH range for covalent immobilisation of α-amylase from *A. oryzae* in the different supports was between 5 and 6 (Fig. 10).

When the enzyme was immobilised by adsorption, the most appropriate pH range was between 5 and 5.5 (Fig. 11).

In both cases there was a drastic reduction in activity when the pH value was outside this range, probably due to the enzyme exhibiting a lower stability.

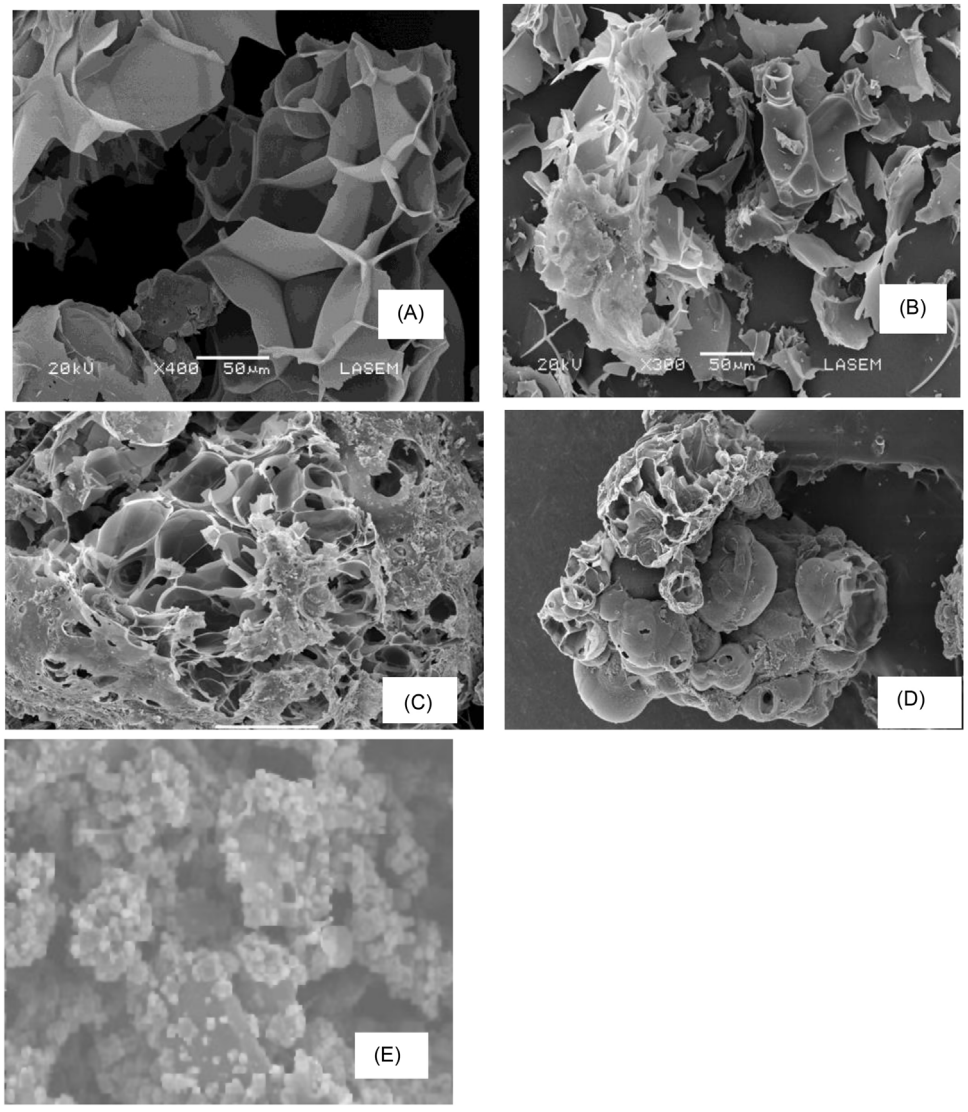

Fig. 6. SEM images. (A) EP (× 400), (B) EP-HCl (X300), (C) EP-MC (X230), (D) EP-RH (X170) and (E) Zeolite (X130).

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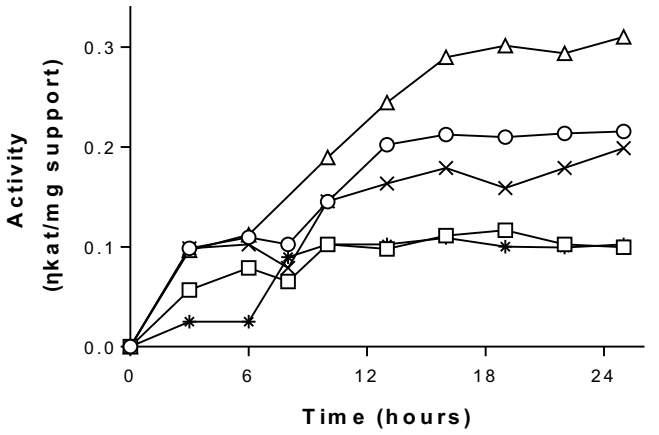

Fig. 8. Effect of time on immobilisation of enzyme. α-amylase covalently immobilised on (o) EP, (□) zeolite, (Δ) EP-RH, (x) EP-MC and (*) EP-HCl.

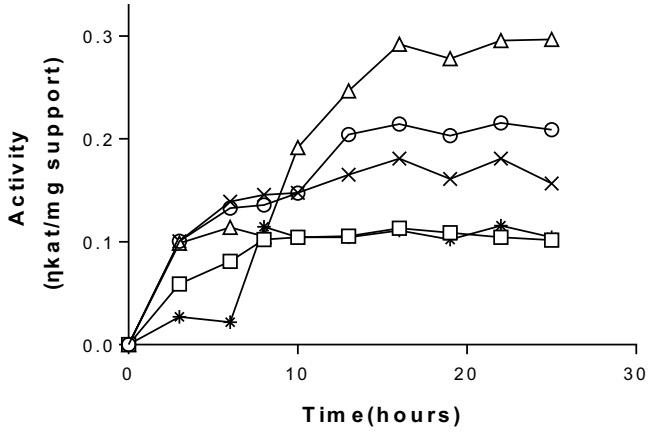

Fig. 9. Effect of time on immobilisation of enzyme.α-amylase immobilised by adsorption on (o) EP, (□) zeolite, (Δ) EP-RH, (x) EP-MC and (*) EP-HCl.

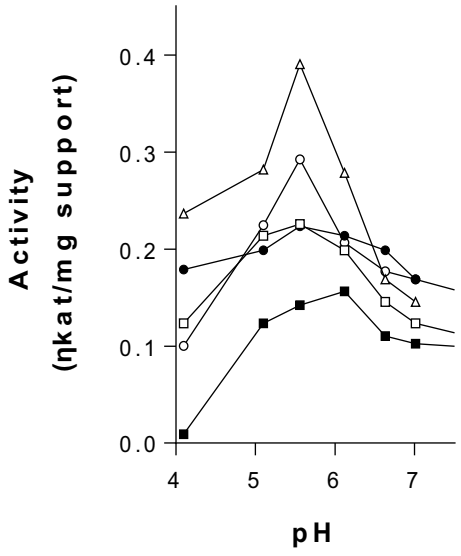

Fig. 10. Effect of pH on immobilisation of enzyme.α-amylase covalently immobilised on (o) EP, (□) EP-HCl, (Δ) EP-RH, () EP-MC and () zeolite.

3.2.1.4. Determination of optimal concentration of α-amylase for immobilisation. Table 3 shows the retained activity in different immobilised derivatives.

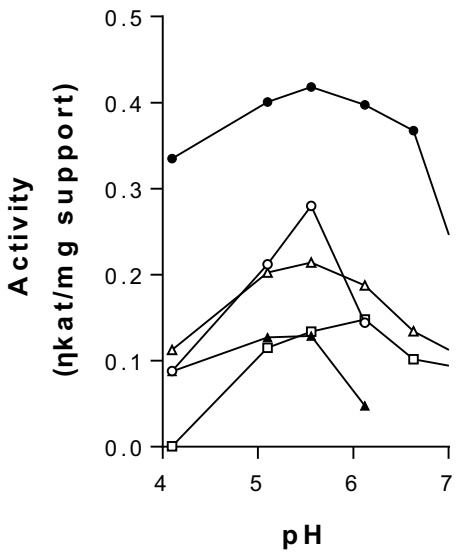

Fig. 11. Effect of pH on immobilisation of enzyme.α-amylase immobilised by adsorption on (o) EP, (□) zeolite, (Δ) EP-HCl, (●) EP-RH and (▲) EP-MC.

Table 3
Retained activity of α-amylase from *A. oryzae* immobilised. Immobilisation by covalent binding: GA 5% (v/v), stirring time 12 h, 20 rpm and 4 °C.

Immobilised derivatives	Retained Activity (ηkat mg^{-1} of support)	
	Immobilisation by adsorption	Immobilisation by covalent binding
EP	0.089	0.144
Zeolite	0.038	0.042
EP-HCl	0.089	0.107
EP-RH	0.085	0.096
EP-MC	0.101	0.113

Immobilisation by adsorption: GA 5% (v/v), stirring time 16 h, 20 rpm and 4 °C. Enzyme concentration offered 0.2027 mg mL^{-1} in 20 mM phosphate buffer pH 5.5 containing activity 1.71 ηkat mL^{-1}.

Dragomirescu et al. [21] immobilised α-amylase from *Bacillus amyloliquefaciens* in ceramic supports, obtaining 5.97 U g^{-1} support. This activity value is similar to that obtained when α-amylase from *A. oryzae* was immobilised by adsorption on EP; EP-HCl and EP-RH, 5.33 U g^{-1} of support (0.089 ηkat mg^{-1} of support), 5.33 U g^{-1} of support (0.089 ηkat mg^{-1} of support) and 5.10 U g^{-1} of support (0.085 ηkat mg^{-1} of support) respectively (Table 3). When Singh et al. [22] immobilised α-amylase from *Bacillus acidocaldarius* in various supports such as ceramics, glass wool, and polystyrene, they obtained 16.70; 6.60; 28.60 U g^{-1} support, respectively. These authors used Apar and Ozbek method to determine the activity of α-amylase. When the enzyme α-amylase from *A. oryzae* was immobilised by covalent binding on the different supports, an increase in protein concentration offered to the support resulted in the increased of the amount of immobilised protein. Fig. 12 shows the relationship between the amount of immobilised protein and the initial amount of protein.

This increase was observed up to a maximum of 20 micrograms of protein per mg of support offered. The highest amount of immobilised protein was for EP and the lowest for zeolite. Fig. 13 shows the relationship between enzyme activity and the initial amount of protein.

EP support presented the highest activity with a maximum amount of 20 μg of protein offered per mg of support. This result corresponds to the amount of immobilised protein. The immo-

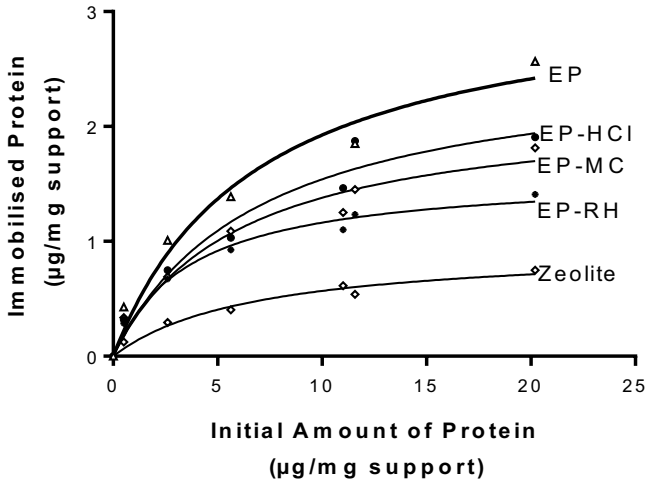

Fig. 12. Relationship between the amounts of immobilised protein and the initial amount of protein. Immobilisation by covalent binding. The supports (30 mg) were incubated with 1 mL of solutions in 20 mM phosphate buffer pH 5.5 for 16 h at 4 °C and 20 rpm.

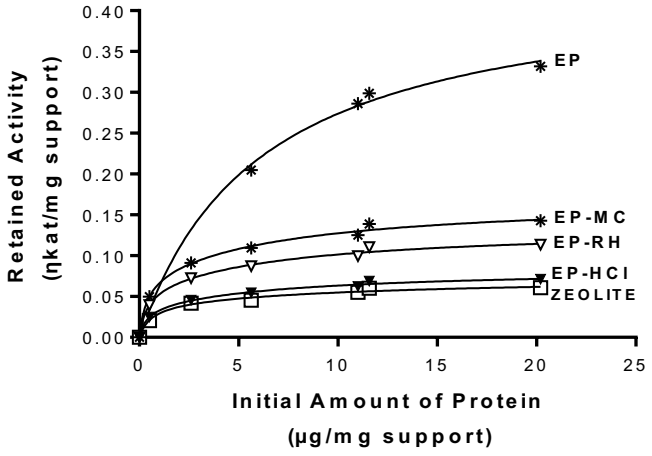

Fig. 13. Relationship between enzyme activity and the initial amount of protein. Immobilisation by covalent binding. The supports (30 mg) were incubated with 1 mL of solutions of α-amylase in 20 mM phosphate buffer pH 5.5 for 16 h at 4 °C and 20 rpm.

bilised derivatives EP-HCl, EP-RH, EP-MC, and zeolite exhibited activities lower than 0.150 ηkat mg^{-1}.

Fig. 14 presents the relationship between specific activity and the amount of initial amount of protein.The maximum specific activity was observed for the EP support, between 0.15 and 0.20 ηkat mg^{-1} protein. Immobilised derivatives EP-HCl, EP-RH, EP-MC, and zeolite exhibited specific activities below 0.10 ηkat mg^{-1} protein. The specific activity in the various immobilised derivatives decreased as the initial amount of protein increased. This behavior may be attributed to the saturation of the enzyme on the support. When the amount of bound enzyme on the support increased, the access of substrate molecules to the active site of enzymes becomes difficult, and therefore, the catalytic capacity decreased [23].

Apparently, the activity of immobilised enzyme depend, primarily, on the properties of the supports and the immobilisation conditions, according to the results observed for α-amylase immobilisation. The high amount of protein load could cause high relative activity, however, the increases in the amount of binding enzyme indicated that diffusion limitation, change of enzyme conformation, and its denaturation under more acidic and alkali conditions also affected the ultimate activity in immobilisation.

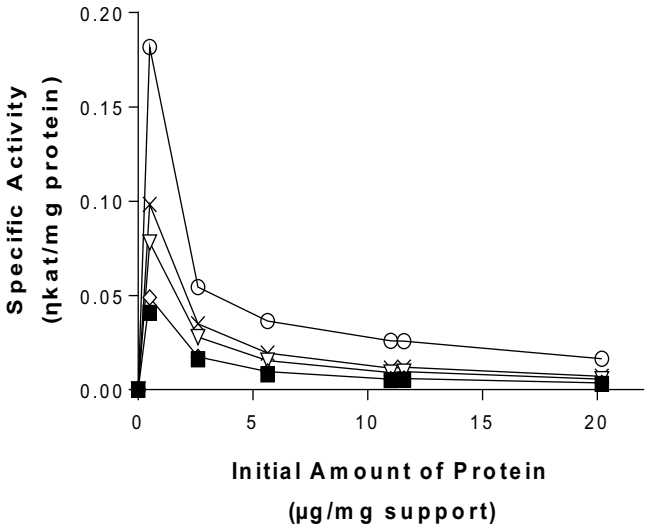

Fig. 14. Relationship between specific activity and the initial amount of protein. α-amylase immobilised on (o) EP, () zeolite, (◊) EP-HCl, (▽) EP-RH and (x) EP-MC.

Table 4
Optimum pH and temperature of maximum activity for free and immobilised α-amylase. pH values were obtained by using citric acid/Na_2HPO_4 at 50 °C. Temperature ranged between 30 °C and 70 °C (McIlvaine Buffer) was analysed.

α-amylase preparation	Optimum pH	Máximum T (°C)
Free	5.5	50
EP	5.5	50
Zeolite	5.0	50
EP-HCl	5.0	50
EP-RH	5.5	50
EP-MC	6.0	50

3.2.2. *Effect of pH and temperature on enzyme activity*

Fig. 15 (A) and (B) shows the dependence of the immobilised enzyme activity, on the different supports with pH.

The optimum pH of α-amylase immobilised on different supports, in McIlvaine buffer and starch substrate ranged between 5.0 and 6.6 (Table 4). These values are comparable to that determined for free enzyme with an optimum pH of 5.5. The α-amylase activity immobilised on different supports was also assayed at different temperatures among 30 °C and 70 °C. Fig. 16 (A) y (B) show the dependence of the activity of the immobilised enzyme on temperature.

The maximum temperature for α-amylase immobilised on different supports was comprised in a range between 50 °C and 60 °C (Table 4). These values are comparable to that determined for the free enzyme with a maximum temperature of 50 °C. The temperatures of maximum specific activity are comparable to those reported for other α-amylase: 60 °C [24], 50 °C [22,25] and 57 °C [26].

The optimum pH determined on the different supports does not differ significantly from that determined for α-amylase immobilised on other supports: pH 6.0 [24–22] and pH 5.5 [27].

3.2.3. *Reuse of immobilised derivative*

An important characteristic derived from the immobilisation of an enzyme is the possibility of reusing it. The stability of immobilised derivatives by adsorption for reuse is shown in Fig. 17. It is noted that the immobilised enzyme loses practically all activity by the tenth reuse.

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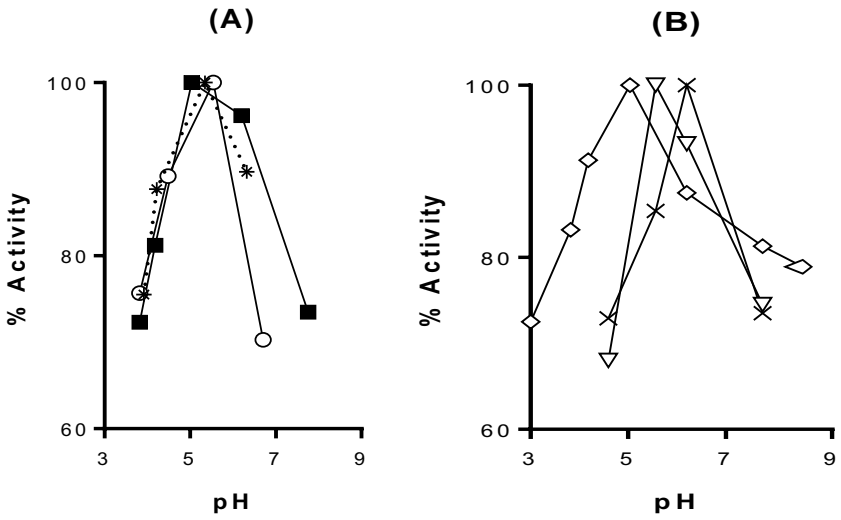

Fig. 15. (A) and Fig. 15 (B). Effect of pH on the enzymatic activity. Citric acid/Na_2HPO_4 (MacIlvaine buffer), 50 °C. α-amylase immobilised on (o) EP, () zeolite, (◊) EP-HCl, (■) EP-RH, (x) EP-MC and (*) α-amylase free.

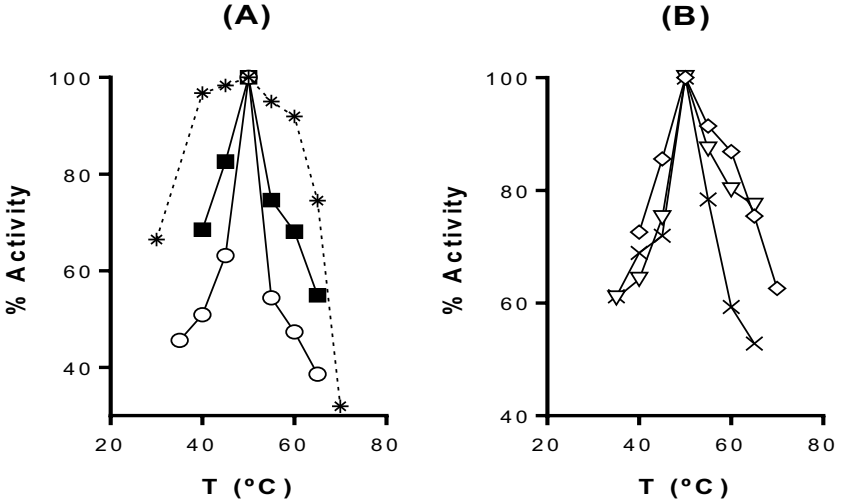

Fig. 16. (A) and Fig. 16 (B). Effect of temperature on enzymatic activity. Citric acid/Na_2HPO_4 (MacIlvaine buffer), 50 °C. α-amylase immobilised on (o) EP, () Zeolite, (◊) EP-HCl, (▽) EP-RH, (x) EP-MC and (*) free α-amylase.

Table 5
Amount of released protein by washing. Immobilisation by adsorption of α-amylase from *A. oryzae*.

N° washes	1	3	6	10	% Total released proteins by washing
Immobilised derivatives	% Released proteins by washing				
EP	10	6.5	4.2	5.1	60.2
Zeolite	14.3	9.7	5.9	4.8	71.4
EP-HCl	12.2	9.8	7.3	2.8	73.9
EP-RH	9.8	6.6	6.3	3.9	58.2
EP-MC	10.5	9.5	4.6	3.6	67.5

To determine whether the loss of activity is due to a process of inactivation or the loss of support-bound enzyme between reuses, the following assay was performed: each of the immobilised derivatives was washed ten times with 20 mM phosphate buffer pH 5.5, after the adsorptive immobilisation process. For each of the washing liquids, proteins were determined (Table 5). From the results, it is concluded that the loss of activity between reuses is due to protein loss. It is noted that the most weakly bound enzyme occurred on the supports EP, zeolite, EP- MC and EP-HCl, with a total loss of protein ranging from 60 and 74%. The support that had the lowest protein loss was EP-RH, with a total protein loss range between 50 and 58%. The enzyme is weakly bound to the support; therefore, immobilisation by adsorption is not effective.

Stability for reuse of the immobilised derivatives obtained when the enzyme was immobilised in supports treated with APTES and GA is shown in Fig. 18.

In this case, the immobilised enzyme retained a high percentage of activity (93%) up to the sixth reuse. This indicates that the interaction between the enzyme and the support is effective and resistant. From the seventh reuse, the percentage of activity declined to reach

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Table 6
Amount of released protein by washing. Immobilisation by covalent binding of α-amylase from *A. oryzae*.

N° washes	1	3	6	10	% Total released proteins by washing
Immobilised derivatives	% Released proteins by washing				
EP	4.5	2.6	0.0	0.0	9.4
Zeolite	8.8	7.5	0.0	0.0	31.7
EP-HCl	7.6	6.9	0.0	0.0	27.5
EP-RH	4.4	3.6	0.0	0.0	12.8
EP-MC	6.5	4.5	0.0	0.0	19.2

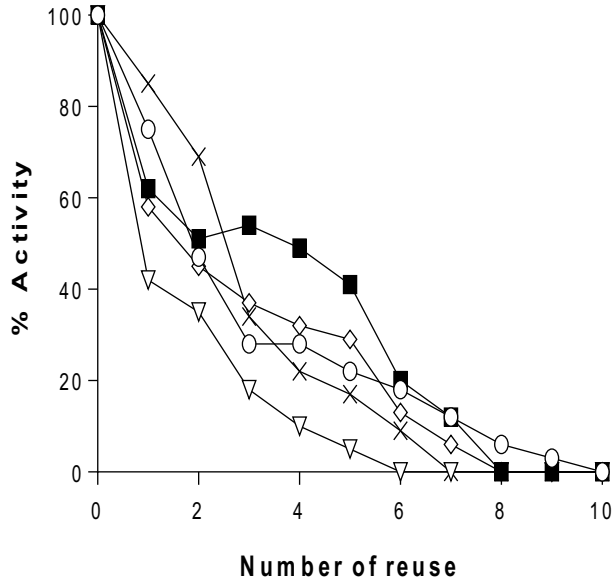

Fig. 17. Percentage of retained activity versus number of reuses of immobilised derivative. Immobilisation by adsorption. α-amylase immobilised on (o) EP, () Zeolite, (◊) EP-HCl, (■) EP-RH and (x) EP-MC.

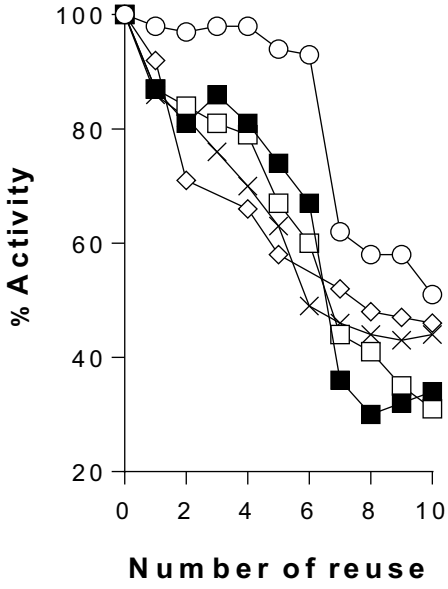

Fig. 18. Percentage of retained activity versus number of reuses of immobilised derivative. Immobilisation by covalent binding. α-amylase immobilised on (o) EP, () Zeolite, (◊) EP-HCl, (▽) EP-RH and (x) EP-MC.

a value close to 60%. This is attributed to thermal inactivation of α-amylase from *A. oryzae* [27,28]. For the rest of the immobilised derivatives, activity decayed more quickly between reuses; however, the tenth reuse presented a considerable activity percentage.

Besides, protein loss was analysed during the washes. Each of the immobilised derivatives was washed ten times with 20 mM phosphate buffer pH 5.5, after the process of immobilisation. Proteins were determined for each of the washings, (Table 6).

Protein loss was observed, during the washes, only until the third wash, with a total protein loss of between 8 and 13% for the EP and EP-RH supports. EP-MC has a total protein loss, about 20% and, zeolite and EP-HCl were the supports with the highest protein loss, about 30%.

Therefore, we conclude that the interaction between the enzyme and the support has the features of covalent binding and the loss of activity after each reuse was probably due to the thermal inactivation process (Table 6 and Fig. 18).

4. Conclusions

FTIR analysis allows us to confirm that the changes in the studied materials were effective. This is perceived by an increase in the bands corresponding to OH groups, which is beneficial when considering functional groups that directly interact with the enzyme (adsorptive binding) or with APTES (covalent binding). The DTA-TG analysis determined that the analysed materials have good thermal stability at the temperature range in which enzymatic studies were performed. The adsorption analysis determined that the materials studied are nonporous solids. SEM microscopy allowed the authors to establish perlite loss of the original globular structure.

The suitable pH range for the immobilisation of α-amylase from *A. oryzae* by covalent binding was between 5 and 6; and the pH range for immobilisation by adsorption was between 5 and 5.5. These values are consistent with the zeta potential values previously determined for the different supports studied.

Immobilisation by covalent binding was more effective than immobilisation by adsorption.

The reuse of the immobilised enzyme by covalent binding resulted in a residual activity of 90% up to the sixth reuse. The result indicated that the α-amylase immobilised, on different support, has a good reusability.

Acknowledgments

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