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α-Amylase immobilization on functionalized glass beads by covalent attachment

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Abstract

In this study, α-amylase was covalently immobilized onto phthaloyl chloride-containing amino group functionalized glass beads. In this procedure, amide bonds were formed between amino groups on the protein and acid chloride groups on the glass surface. The surface modified beads were characterized using Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), energy dispersion spectrum (EDS) and UV–Vis spectroscopy. Immobilization was successfully performed under very mild conditions (15 °C, 4 h). The amount of covalently bound α-amylase was found 25.2 ± 3.1 mg/g glass support. The optimum pH value for the free amylase was at pH 6.5. The optimum pH of the immobilized enzyme was shifted 1.0 pH unit to the acidic region. The immobilized α-amylase exhibited better thermostability than the free one. The free enzyme lost all its activity with in 15 days. Covalently bound amylase was stable up to 5 days and lost only 20% of activity in 25 days. The covalently bound enzyme demonstrated more than 98% activity after 6 runs and 81.4% activity after 25 runs.

Keywords: Covalent immobilization; α-Amylase; Enzyme; Glass beads; EDS–SEM

1. Introduction

Enzymes are catalysts as they are highly effective and specific under ambient conditions; therefore enzymatic processes have great industrial application. General expectations from the commercially used enzymes are efficient use of reactants, maximizing catalytic velocity and enhancement of the operational lifetime (Kadima & Pickard, 1990). To improve their economic feasibility in food, pharmaceutical, medical, industrial and technological processes, soluble enzymes are usually immobilized onto a solid support. Immobilization of the enzymes onto solid supports that are either organic or inorganic is a very effective way to increase enzyme stability and operational lifetime (Géraldine, 2002). Besides that it facilitates the separation of enzymes from reaction media easily, hence the recovery and purification of the final products from enzymes become more reliable simple and efficient (Bajpai & Bhanu, 2003).

There have been many reports about immobilization of α-amylase used for the hydrolysis of starch and production of maltose. Some examples involve glass beads and glutaraldehyde fixation (De Cordt, Hendrickx, Maesmans, & Tobback, 1994), polymeric microspheres (Tümtürk, Aksoy, & Hasırcı, 2000), covalent bonding (Varlan, Sansen, Vanloey, & Hendrickx, 1996), UV-curable polymer (Kahraman, Kayaman-Apohan, Ogan, & Güngör, 2006), functionalized silica (Leng, Douglas, & Gordon, 2003), adsorption on zirconia (Reshmi, Sanjay, & Sugunan, 2007). α-Amylase has been immobilized to collagen (Groom, Meising, & White, 1988), kaolin and sand (Karube, Mitsuda, & Suzuki, 1979). Bacterial amylase has been covalently bound to silica carriers using glutaraldehyde or titanium chloride (Kvesitadze & Dvali, 1982).

The support material can have a critical effect on the stability of the enzyme and the efficiency of enzyme immobilization,

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although it is difficult to predict in advance which support will be the most suitable for a particular enzyme. The support must be insoluble in water, should have a high capacity to bind enzyme, be mechanically stable and must not have deleterious effect on the enzyme action. Various modified supports for covalent immobilization such as polymers, silica and glass, have been widely investigated. Hydrophilic biopolymers based on natural polysaccharides such as dextran and cellulose, synthetic organic polymers such as polyacrylamide and polystyrene can be given of the most widely studied polymeric supports. Silica gel, aluminum oxide, apatite and glass supports are also preferred inorganic support materials. Organic polymeric carriers are the most widely studied materials because of the presence of rich functional groups, which provide essential interactions with the enzymes. However, the organic supports suffer a number of problems such as poor stability towards microbial attacks and organic solvents and disposal issues. In contrast, inorganic materials such as silica, alumina, and layered double hydroxides are known to be thermally and mechanically stable, non-toxic, and highly resistant against microbial attacks and organic solvents (Reshmi et al., 2007).

The hydroxyl groups on the surface of silica and glass provide desired functionality that can react with hydrolysable groups of organosilane and cyanogens bromide coupling agents (Ho & Liao, 1980). Silanisation is a widespread used technique for altering the chemical and physical properties of solid hydrophilic surface properties of silicates and glass (Plueddemann, 1991). The surface modification of glass is carried out using variety silane coupling agents having organofunctional groups with di- or tri-alkoxy structures and the reactions were performed either in gaseous or liquid phase. (Halliwell & Cass, 2001). The silanol groups then condenses with the surface residues to form siloxane linkages. In the case of trialkoxysilanes the presence of three silanol residues in the hydrolysis product can lead to the possibility of multiple surface attachments. Aminosilanes as 3-aminopropyl-triethoxysilane (APTES) are attractive coupling agents for immobilization of enzymes. Amino groups have catalytic activity on formation of siloxane bonds with silanols on the surface of the glass. On the other hand, in literature it has been studied that when APTES is used as a coupling agent, the preferred conformation of the reaction product of monomeric APTES on a glass or metal oxide has two sites of attachment and the third silanols remains free even the number of attachment onto the surface is not limited (Kallury, Krull, & Thompson, 1988). Amino groups can also react with organic molecules, which have functional groups such as aldehyde (Ho & Liao, 1980) and epoxide.

In this study, α-amylase was covalently immobilized on functionalized glass support. For this purpose, inexpensive and renewable glass beads was functionalized with 3-aminopropyl-triethoxysilane then coupled with phthaloyl chloride which can be covalently bind the enzyme; the acid chloride sites can serve as centers of binding through the $-NH_2$ group of enzyme. α-Amylase was successfully covalently attached onto glass support under very mild conditions (15 °C, 4 h). Immobilization efficiency and enzyme activity of α-amylase was examined at various pH (5.0–8.0) and temperature (30–90 °C). The storage stability and reusability of immobilized α-amylase were investigated. Each value reported in this article is an average of three separate experiments.

2. Experimental

2.1. Materials

Glass bead with average diameters of 0.3 mm was provided by Izomas—Turkey. 3-Aminopropyl-triethoxysilane (APTES) was kindly supplied by Wacker. Phthaloyl chloride was purchased from Fluka AG. α-Amylase (1,4-α-D-glucanglucanohydrolase; EC 3.2.1.1, Type VI-B from porcine pancreas, extra pure 35 U/mg), starch, maltose and 3,5-dinitrosalicylic acid (DNSA), bovine serum albumin (BSA) were all obtained from Sigma Chemical Co. Coomassie® Brilliant Blue G-250 was obtained from BIO-RAD. All other chemicals were of analytical grade and were purchased from Merck AG. Freshly double distilled water was used throughout.

2.2. Characterization

FT-IR spectrum was recorded on Shimadzu 8300 FT-IR Spectrometer. Elemental concentrations for carbon, nitrogen, oxygen and silicon, and mapping technique were performed with an energy dispersion spectrum (EDS) OXFORD INSTRUMENTS-INCA, Model No. 7274. SEM (Scanning Electron Microscope) imaging was performed on a JEOL-JSM-5919LV. UV–Visible spectrum was performed on SHIMADZU 240.

2.3. Surface activation of glass beads

Prior to silanization, the glass substrates were pre-cleaned by treatment in 30% v/v NaOH/30% v/v H_2O_2/water (1:1:4), followed by piranha solution, HCl/30% v/v H_2O_2/water (1:1:4) at 85–90 °C for 60 min. The glass beads were removed and rinsed with water subsequently filtered and dried under vacuum at 50 °C. This pre-conditioning method renders surfaces with sufficient amount of silanol groups for silane derivation to form covalent siloxane bonds.

2.4. Synthesis of the organofunctionalized glass beads

Activated glass beads (42 g), 200 mL of dry toluene and 5 mL of 3-aminopropyl-triethoxysilane were added into reaction flask under nitrogen atmosphere. The mixture was stirred under solvent reflux for 18 h. Afterwards, amino functionalized glass beads were removed from solution by filtration and kept in the oven at 110 °C for 2 h to achieve for complete formation of Si–O–Si bonds, via condensation.

In the next step, amino functionalized 40 g of glass beads, triethylamine (3.7 g) and 150 mL dry cyclohexane were

charged into a three-necked 250 ml round-bottom flask, equipped with a nitrogen inlet and dropping funnel. Large excess of phthaloyl chloride (15 g) was then added drop wise to the well-stirred reaction mixture over a period 30 min. The reaction mixture was stirred at 25 °C for 3 h. The product was filtered and washed with dry cyclohexane and dried. A representation of this reaction is shown in Scheme 1.

2.5. Enzyme immobilization

Functionalized glass beads (10 g), 50 mL of dry cyclohexane and 0.8 g of α-amylase were charged into round bottom flask. Immobilization of α-amylase on the glass bead substrates was carried out for 4 h at 15 °C shaking in a water bath under the nitrogen atmosphere. Enzyme bound glass substrate was filtered. Physically bound enzyme was removed by washing the substrates with phosphate buffer. The washing solution was kept for measuring the amount of immobilized enzyme. The enzyme protein concentration within the extract was measured using Coomassie Brillant Blue reagent as described by Bradford (1976). A calibration curve prepared with BSA solution. From the results for the protein recovery, the amount of bound protein per weight of support was calculated. Enzyme immobilization experiments were repeated four times and the average immobilization yield was found as 25.2 ± 3.1 mg/g glass support.

2.6. Activity assays of immobilized and free α-amylase

α-Amylase activity was measured according to the assay suggested by Bernfield (Bernfield, 1951). Briefly 1 wt.% starch solution was prepared by dissolving soluble starch in 100 mL 0.02 M sodium phosphate buffer, pH = 6.9 with 0.006 M NaCl. Then in a test vial, a known amount of glass beads containing alpha-amylase was placed. Subsequently 0.5 mL starch solution was added and the system was incubated in a water bath with constant shaking at 25 °C for exactly 5 min. The reaction was stopped by adding 1 mL of 3,5-dinitrosalicylic acid reagent. Incubation was also performed in a boiling water bath for 5 min. After dilution of the reaction content with distilled water, the amount of reducing sugar (maltose) produced was determined spectrophotometrically at 540 nm. In each set of experiments, a standard curve was prepared with maltose solutions of different concentrations. Each results is an average of three separate experiments. Activity of the enzyme is calculated from the following equation:

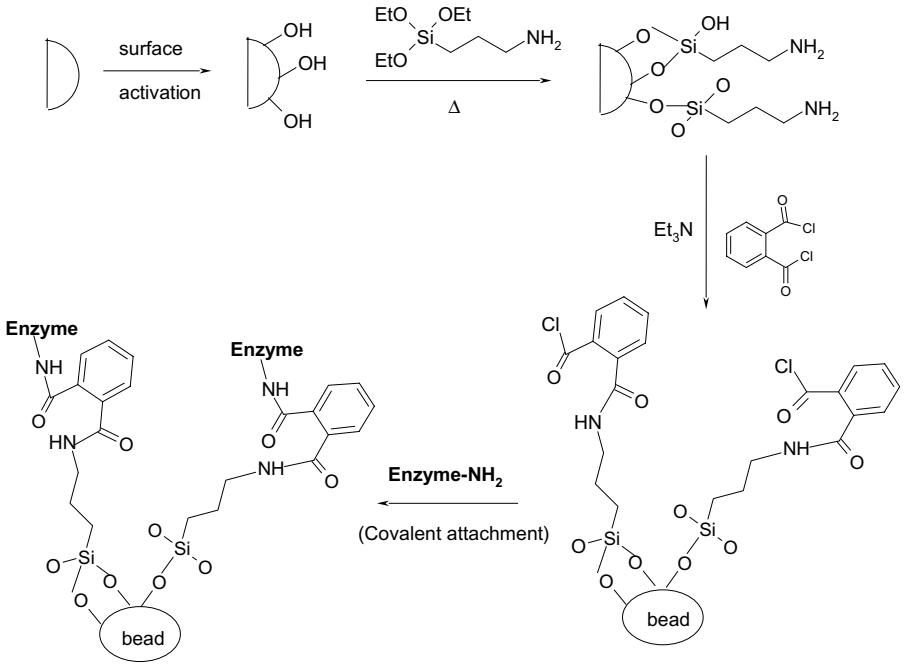
$$\text{Activity } (\text{IUmg}^{-1}) = \frac{\text{released maltose } (\mu\text{mol})}{\text{amount of } \alpha\text{-amylase (mg)} \times 5 \text{ min}}$$

The immobilized enzyme had to be removed by washing with distilled water before re-measuring its activity in successive used cycles. To determine the pH profiles for the free and immobilized α-amylase activity assays were carried out over the pH range of 5–8. The results for dependence on pH, storage, temperature effect and repeated use are presented in a normalized form with the highest value of each set being assigned the value of 100% activity.

3. Results and discussion

3.1. Preparation of support

When choosing a support for enzyme immobilization for industrial application, the most important criteria are



Scheme 1. Representation of the mechanism of enzyme immobilization on functionalized support.

the stability of the carrier and the cost. In this study α-amylase enzyme was covalently immobilized on commercially available inexpensive and renewable glass beads. Covalent immobilization method requires chemical modification of the glass surface, so that functionally inert silanols (Si–OH) of a glass surface are modified to posses either nucleophilic or electrophilic functionalities that react with the enzyme reactive functional groups ($-NH_2$) (Halliwell & Cass, 2001). Its surface modification and enzyme immobilization achieved via a two-step process. The first stage involves surface modification of the glass beads with a silane-coupling agent (3-aminopropyl triethoxysilane). After modification with the organosilane, the surface modified glass beads is reacted with phthaloyl chloride followed by covalent attachment of the α-amylase enzyme to the newly introduced functional group on the surface. Scheme 1 illustrates the general synthetic procedure used for covalently enzyme immobilization on the modified glass bead surface.

3.2. Characterization of enzyme support

Structural characterization of 3-aminopropyl-triethoxysilane modified glass bead was investigated by analysis of the amount of nitrogen (Kaiser test) (Sarin & Kent, 1981) and energy-dispersion spectrometer of a scanning electron microscope (SEM–EDS) techniques.

The quantitative Kaiser test has here been used to spectrophotometrically determine the number of aminosilanes attached to the surfaces of inorganic matrix. The principle in test is the reaction of ninhydrin with a primary amine with a free proton on the carbon next to the amine to form Ruheman's purple, which is highly conjugated with a strong absorption around 570 nm. The measurements are done by measuring the absorbancy of Ruheman's purple at the wavelength of 570 nm. The test is identical with the test for polypeptide, but is calibrated for 3-aminopropyl triethoxysilane. From Kaiser test analysis the amount of the free amine group was found 0.95 ± 0.10 mmol/g glass beads. Several reviews concerning grafting on silicas have been published (Clark & Macquarrie, 1998; Moller & Bein, 1998). Depending on the organosilane and available number of surface silanol groups, organic loadings of 0.3 to 2 mmol per g solid can be obtained. The covalently attached organic groups can be sufficiently stable for recycling and reuse, and can easily be modified to create a variety of catalytic sites.

SEM image of the dried APTES modified glass beads were obtained using a JEOL-JSM-5919LV scanning electron microscope, after coating gold under reduced pressure. Fig. 1 shows the SEM images of 3-aminopropyl triethoxysilane modified glass beads. It can be seen that the surface of the support presents uniform texture. Fig. 2a and b shows the SEM–EDS mapping images of 3-aminopropyl triethoxysilane modified glass beads. It can be seen from the SEM–EDS mapping image (Fig. 2a and b), glass beads surface was modified with organofunctional group and clearly showed that the organofunctional group homogenically spread out on glass surface. Fig. 3 shows the EDS spectrum energetic lines for carbon, nitrogen, oxygen and silicon. The contents of elemental concentrations are determined with the energy-dispersion spectrometer of a scanning electron microscope (SEM–EDS). Its weight percentages were 21.18, 7.04, 39.77 and 32.01, respectively.

The FT-IR spectrum of immobilized enzyme on glass support is shown in Fig. 4. The FT-IR spectrum clearly marks the presence of amide group at 3550 cm^{-1} (N–H

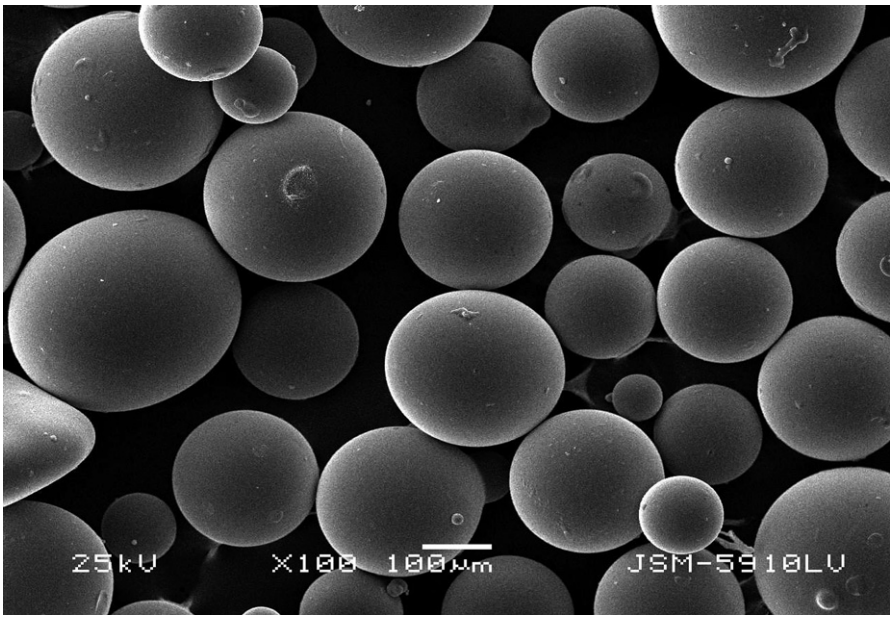


Fig. 1. SEM image of 3-APTES modified glass beads.

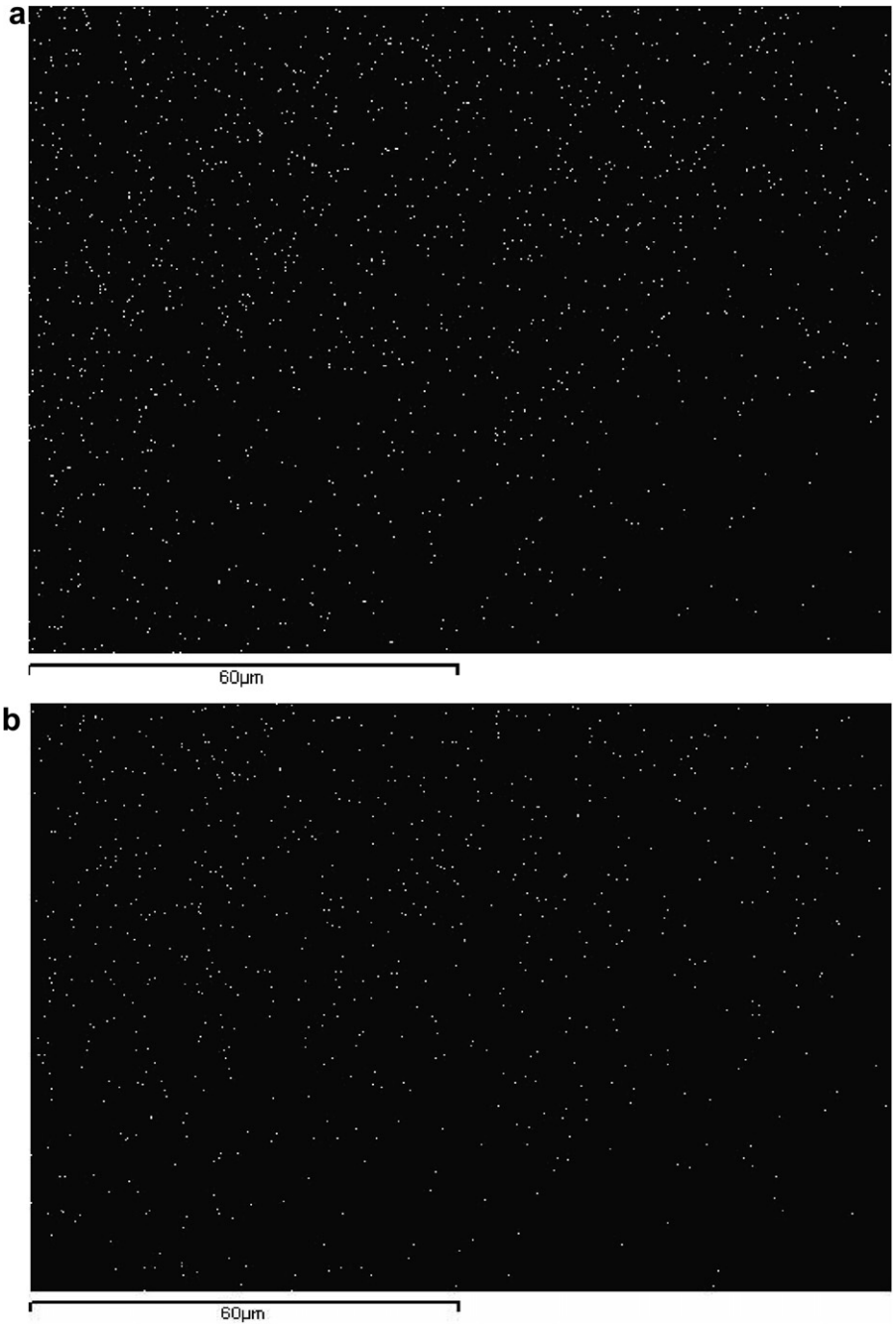


Fig. 2. SEM–EDS map images of 3-APTES modified glass beads: (a) carbon (b) nitrogen.

str.), 1540 cm^{-1} (N–H bending), and at 1660 cm^{-1} C=O stretching (Bajpai & Bhanu, 2003). Enzyme bound glass beads exhibit a Si–O–Si absorption band between 900 and 1170 cm^{-1} which is assigned to the silica network, and a strong band at 460 cm^{-1} due to a Si–O–Si bending vibrational mode.

3.3. Efficiency of covalent immobilization

In the literature, there are many different loading values for different structures with various levels of activity retention for α-amylase immobilization. For example, coupling capacities were reported as 3–29 mg/g, respectively, on polystyrene and silica based supports (Kvesitadze & Dvali, 1982; Ulbrich, Schellenberger, & Damerau, 1986) and 25–90 mg/g for cellulosic supports (Kennedy & Paterson, 1993). In our previous study on α-amylase immobilization capacity on UV curable polymeric support material (methacrylated/fumaric acid modified cycloaliphatic epoxide) (Kahraman, Bayramoğlu, Kayaman-Apohan, & Güngör, 2007) was found as 94 mg/g support. In this study the amount of covalently bound α-amylase enzyme was found as 25.2 ± 3.1 mg/g glass support. In the present study, covalently bonded enzyme was achieved under very mild exper-

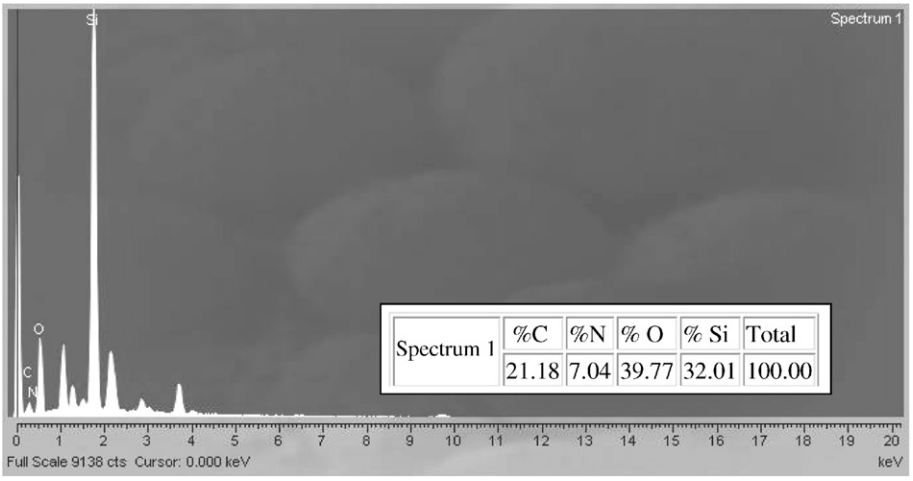


Fig. 3. EDS pattern of the 3-APTES modified surface.

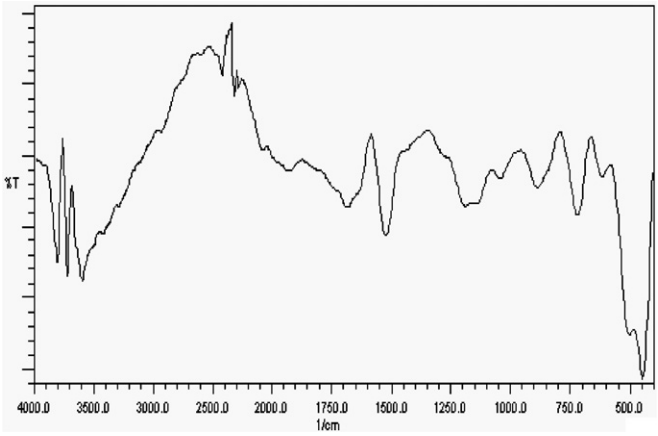


Fig. 4. FT-IR spectrum of covalently immobilized α-amylase.

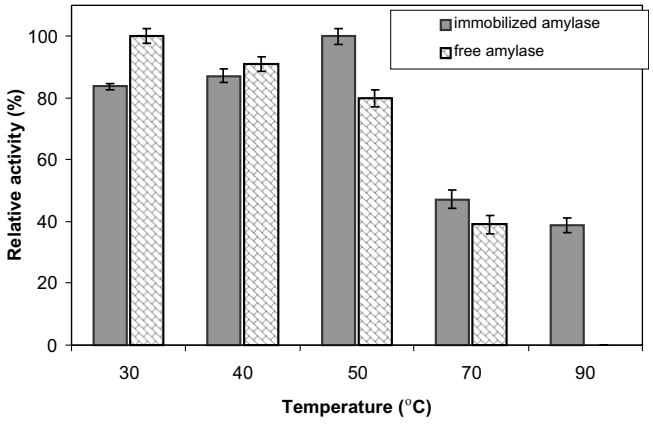


Fig. 5. Effect of temperature on enzyme activity (average of three separate experiments).

imental conditions (15 °C, 4 h) permits higher enzyme stability; the preserved activity was 87% for the immobilized enzyme after 4 h coupling time. The maximum preserved activity was reported as 75–100% on multifunctional epoxy support, which is capable of absorbing proteins via cationic, anionic and metal chelate groups located on the support surface (Mateo, Fernandez-Lorente, Abian, Fernandez-LaFuente, & Guisan, 2000).

3.4. Optimum temperature and thermal stability

The inability to enhance the thermal stability of a native enzyme is one of the most important limitations for their application in continuous reactors. The activity of free and immobilized α-amylase was assayed at various temperatures (30–90 °C). The results in Fig. 5 showed that the maximum catalytic activity was obtained at 30 °C for free α-amylase and 50 °C for immobilized α-amylase; however, as the temperature increases, the stability of free enzyme reduces rapidly compared to immobilized form. The immobilized α-amylase exhibited better thermostability than the free one. Sakhukhan et al. reported a similar increase in temperature optima for immobilized α-amylase (Sakhukhan, Roy, & Chakrabarty, 1987). Increased thermal stability has been reported for a number of immobilized enzymes, and the support material is supposed to preserve the tertiary structure of the enzyme. The authors demonstrated that the thermal stability of enzymes might be drastically increased if they are attached to a complementary surface of a relatively rigid support in a multipoint (Martinek, Kilbanov, Goldmacher, & Berezin, 1977).

3.5. Effect of pH on enzyme activity

The behavior of an enzyme molecule may be modified by its immediate microenvironment. An enzyme in solution can have a different optimal pH from the same enzyme immobilized on a solid matrix depending on the surface and residual charges on the solid matrix and the nature of the enzyme-bound pH value in the immediate vicinity of the enzyme environment. A change in the optimum pH normally results in insolubilization of enzymes, depending upon the polymer used as support.

The effect of pH on the activity of free and immobilized α-amylase for the starch hydrolysis was determined from pH 5 to 8 at 25 °C. The enzyme activities obtained are presented in Fig. 6. The maximum activity was observed at pH 6.5 for free α-amylase. However, the maximum pH of the immobilized enzyme was shifted 1.0 pH unit to the acidic region. This may be due to the challenges inflicted on the enzyme on account of the strong covalent bond formed between the support and the enzyme. Strong interactions between enzyme and support will affect the intra-molecular forces responsible for maintaining the conformation of the enzyme that would lead to a change activity. The shift depends on the enzyme reaction as well as on the structure and the charge of the matrix. For amylase immobilization, shift towards the acidic or basic directions have been observed previously (Bayramoğlu, Yılmaz, & Arıca, 2004). In This case the shift to acidic region could be result of some secondary interactions between the enzyme and the polymeric matrix.

3.6. Storage stability

One of the most important parameters to be considered in enzyme immobilization is storage stability. In general, if an enzyme is in solution, it is not stable during storage, and the activity is gradually reduced. The effect of storage conditions on the activity of the immobilized enzyme is an important aspect to ensure that a long shelf life is possible. The decline of enzyme activity is essentially identical for all storage condition tested (Leng et al., 2003). In Fig. 7 the storage stabilities of free and immobilized α-amylase can be seen. The free and the immobilized α-amylase were stored in phosphate buffer solution (pH = 6.9) at +4 °C and the activity measurements were carried out for a period of 25 days. The free enzyme lost all its activity with in 15 days. Covalently bound amylase was stable up to 5 days and lost only 20% of activity in 25 days. It appears that the immobilization process developed here has improved the stability of α-amylase and helps maintain the activity longer. Leng et al. reported that α-amylase immobilized on silanized silica particles lost its activity at a rate of

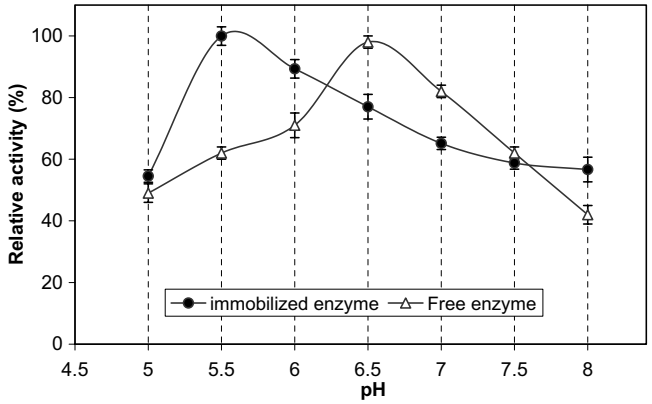


Fig. 6. Effect of pH on enzyme activity (average of three separate experiments).

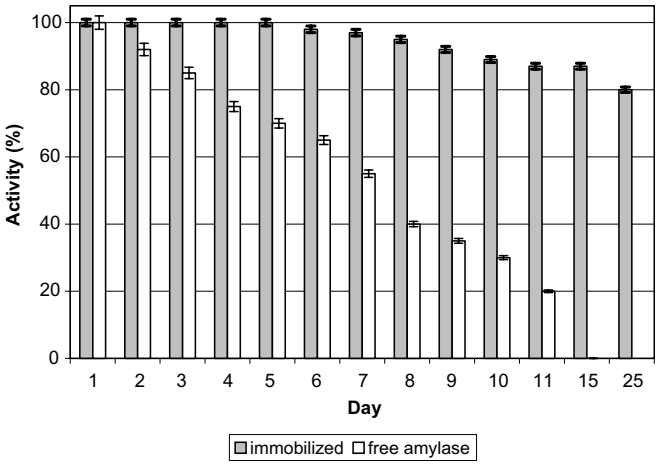


Fig. 7. Storage stability of free and covalently immobilized α-amylase (average of three separate experiments).

10% every 12 days of storage (Leng et al., 2003). Retained activities of α-amylase immobilized on various polymeric supports were previously reported as 75–90% after 25 days of storage (Kahraman et al., 2006; Tümtürk et al., 2000).

3.7. Multiple use of the covalently immobilized enzyme

Enzymes are still quite expensive products. Their recycling and multiple uses could be ensured using the existing enzyme immobilization techniques. The most important advantage of immobilization is repeated use of enzymes. Reusabilities of the immobilized α-amylase samples were examined by using the same conditions repeatedly 25 times within 6 h and the measured activities are shown in Fig. 8. It was observed that the covalently bound enzyme demonstrated more than 98% activity after 6 runs and 81.4% activity after 25 runs. In our previous study, it was found that, the reuse capabilities of α-amylase was in the range of 74–88% when immobilized on UV curable support (Kahraman et al., 2006). In another study, reuse capabilities

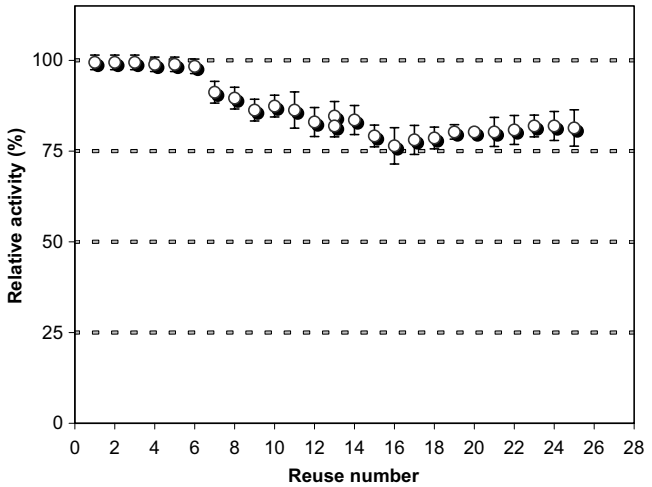


Fig. 8. Reuse of covalently immobilized α-amylase (average of three separate experiments).

of CDI-bound α-amylase demonstrated more than 99% activity after 20 runs and 91.3% activity after 40 runs (Hasirci, Aksoy, & Tumturk, 2006).

4. Conclusions

Glass support reacted with 3-aminopropyltriethoxysilane to introduce amino groups on the surface and follow treated with phthaloyl chloride, and used as solid support materials in the covalent immobilization of α-amylase. Immobilization was successfully performed under very mild conditions (15 °C). Enzyme carrier is prepared by using inexpensive and renewable glass bead material and also it causes less damage to the catalytic activity of α-amylase enzyme. The immobilized enzyme exhibited better thermostability than the free one. The optimum pH of the immobilized enzyme was shifted 1.0 pH unit to the acidic region. pH stability was also improved in region pH 5–6. The storage stability and re-usability improved by the immobilization on this enzyme support. This support is very robust and suitable for use continuous industrial process.

Acknowledgement

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