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Alpha-amylase Immobilization on Modified Polyimide Material

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In this study, α-amylase was covalently immobilized on modified polyimide materials. Polyimide (PI) was prepared with pyromellitic dianhydride (PMDA) and 4,4'-oxydianline (4,4'-ODA) in the solution of *N*,*N*-dimethylformamide (DMF). Free amine groups on the surface of the polyimide membranes were generated by the amination reaction of polyimides with hexamethylenediamine (HMDA). Surface-aminated membranes were then subjected to enzyme immobilization. The morphology of the polymeric support was characterized by scanning electron microscopy (SEM). Chemical structure of PMDA-ODA PI membranes was characterized by FTIR. SEM and FTIR results showed that the enzyme was successfully covalently attached to the polymeric support. Immobilization efficiency and enzyme activity of α-amylase was examined at various pH values (3.0 – 8.0) and temperatures (15 – 80 °C). Immobilization yield was found to be 285.45 mg per gram for the modified polyimide films. Enzyme assays demonstrated that the immobilized enzyme exhibited better thermo-stability than the free one.

1. Introduction

Alpha-amylase is one of the most important enzymes used in industry and biotechnology, being of high relevance in food, fermentation, detergent, paper and textile industries (Gupta et al., 2003; Kirk et al., 2002; Polaina and MacCabe, 2007; van der Maarel et al., 2002).

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 and 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 (Gèraldine, 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 and Bhanu, 2003).

Alpha-amylase has been immobilized onto a wide variety of organic and inorganic supports, as for example silica gel (Saville et al., 2004), chitosan (El-Ghaffar and Hashem, 2009), and glass (Kahraman et al., 2007). Although there are only a few studies that assess polyimides for enzyme immobilization, there is no doubt that polyimide membranes are good candidates as immobilization substrates. For instance, Paşahan et al. (2011), prepared naphthalene based polyimide materials and the enzyme immobilization on polyimide was done by physical entrapment. Moreover, polyimide materials were also modified with different bioactive agents such as albumin, RGD rather than enzymes, in order to increase their biocompatibility (Van Vlierberghe et al., 2010; Massia et al., 2004; Kawakami et al., 2001).

In this work, the immobilization of α-amylase on aminated polyimide materials is being reported for the first time. Immobilization efficiency and enzyme activity of α-amylase was examined at various pH values (3.0 – 8.0) and temperatures (15 – 80 °C).

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2. Experimental

2.1 Materials

4,4′-Oxydianiline (ODA) and pyrromellitic dianhydride (PMDA) were purchased from Sigma Aldrich and were dried under vacuum overnight at 120 °C. Hexamethylenediamine (HMDA), methyl alcohol, N,N-dimethylformamide (DMF), 3,5-dinitrosalicylic acid (DNSA), bovine serum albumin (BSA) and α -amylase (1,4-α -D-Glucan-glucanohydrolase; EC 3.2.1.1, Type VI-B from porcine pancreas, extra pure 35 U/mg) were purchased from Sigma Aldrich and used as received. Glutaraldehyde (GA) (25 %) was obtained from Merck AG.

2.2 Preparation of PMDA-ODA polyimide membranes

PMDA-ODA polyimide films were prepared via thermal imidization from their corresponding polyamic acid solutions. To synthesize poly(amic acid) (PAA) solutions, ODA (2.0024 g, 0.01 mol) was first put into a flame dried three-neck flask containing 16 mL of DMF under a nitrogen purge. After ODA was completely dissolved in DMF, PMDA (0.01 mol) was added to the flask batch by batch. The mixture was then stirred at room temperature for 24 h to obtain a viscous PAA solution. The solid concentration was afforded as ~20 (wt/wt) %. Polyimide membranes were prepared by casting viscous PAA solutions on clean dust-free glass plates. Then thermal imidization was performed stepwise at 80, 100, 150, 200, and 300 ˚C for 1 h at each temperature. Polyimide membranes were removed from glass plates by immersing in distilled water at 80˚C.

2.3 Surface modification of polyimide membranes

Polyimide membranes were modified similar to reported procedures (Shao et al., 2008; Aziz and İsmail, 2010). 10 % (w/v) of HMDA solutions in methanol were prepared. The amination of polyimide membranes was performed by immersing the membrane films in the solution for 10 min. Modified films were then washed with methanol immediately after removal from the HMDA/methanol solution in order to wash away all residual HMDA. Then membranes were dried at 40 °C for about 1 day for the complete removal of methanol.

2.4 Immobilization of α-amylase

Functionalized polyimide films were immersed in 250 mL glutaraldehyde (25 %) solution. The solution was magnetically stirred at room temperature for 1 h. Then films were removed from the glutaraldehyde solution and washed extensively with distilled water to remove unreacted GA. Glutaraldehyde activated polyimide films were dried at 40 °C in vacuum. Glutaraldehyde modified polyimide membranes were added to 2 g (70, 000 U) α-amylase in 200 mL distilled water. The covalently immobilization process was performed over 12 h at 25 °C with shaking at 110 rpm shaking frequency. The protein concentration within the supernatant was measured by Bradford assay (Bradford, 1976). A calibration curve prepared with BSA solution of known concentration was used in the calculation of protein in the enzyme and wash solution. From the results of protein recovery, the amount of immobilized enzyme per weight of material was calculated.

2.5 Effects on enzyme activity

In this work, the activity of immobilized α-amylase was determined through the detection of released reducing sugars from starch using 3,5-dinitrosalicyclic acid (DNS) (Bernfield, 1951). Briefly 1 wt % starch solution was prepared by dissolving soluble starch in 100 mL 10 mM phosphate buffer (pH 6.9). In a test vial, a known amount of polyimide material containing α-amylase was placed. Then 1 mL starch solution was added and the system was incubated in a water bath with constant shaking at 30 °C for exactly 5 min. The reaction was stopped by adding 1 mL of 3,5-dinitrosalicylic acid reagent. Incubation was performed in a boiling water bath for 5 min and cooling the reaction tubes to room temperature. The amount of reduced 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. An enzyme activity unit (U) was defined as the amount of enzyme liberating 1 μmol maltose per minute under the assay conditions. Each determination was carried out in triplicate.

$$\text{Activity } (\text{Umg}^{-1}) = \frac{\text{release maltose } (\mu\text{mol})}{\text{Amount of } \alpha\text{-amylase (mg)x min}} \quad (1)$$

2.6 Kinetics of free and immobilized α-amylase

K_m and V_{max} were determined from the initial rates of the reaction of the enzyme with starch solution using different concentration of the substrate (0.25 – 2.5 mg/mL).

2.7 Characterization

FT-IR spectrum was recorded on Perkin Elmer Spectrum 100 ATR-FTIR spectrophotometer. The conditions of analysis were as follows; resolution 2 cm^{-1} and a frequency range of 400 – 4,000 cm^{-1}. Scanning Electron Microscope (SEM) image of the polyimide membranes were acquired at acceleration voltage of 10 kV by using Philips XL30SEM FEG

3. Result and Discussion

To our best knowledge there is no published work on the immobilization of alpha-amylase on aminated polyimides. In this study, α-amylase enzyme was covalently immobilized on surface modified polyimide films. First amine groups were generated on polyimide's surface via the amination reaction with HMDA. Then for the enzyme bonding, amine groups on the surface of polyimide were activated by glutaraldehyde, followed by covalent attachment of the α-amylase enzyme to the activated polyimide surface.

3.1 Characterization of the polymeric support materials

The FT-IR spectra of polyimide and amylase immobilized polyimide can be seen in Figure 1. PMDA-ODA polyimide films shows the characteristic –NHCO- peak at 1,546 cm^{-1}. Polyimide showed characteristic imide absorption bands at 1,780-1,785 cm^{-1} and 1,727-1,735 cm^{-1}. The peak at 1,780-1,785 cm^{-1} was attributable to the asymmetrical carbonyl stretching vibrations, and that at 1,727-1,735 cm^{-1} to the symmetrical carbonyl stretching vibrations. Also the peak at around 1,375cm^{-1} is attributed to C-N bond stretchings and the peak at 724 cm^{-1} is due to -C=O bending. Furthermore Figure 1 shows the FTIR spectrum of α-amylase immobilized polyimide membranes. In Figure 1b), the peak at 1669 cm^{-1} was attributed to the newly formed amide bond which confirms that the amination reaction was successfully achieved. After GA treatment a new peak was formed at 1,658 cm^{-1}. This peak is due to imine Schiff-base groups that result from the reaction between free amine groups and GA. These spectra prove that the enzyme was successfully covalently attached to the polymeric support material rather than being physically entrapped in the polymer matrix.

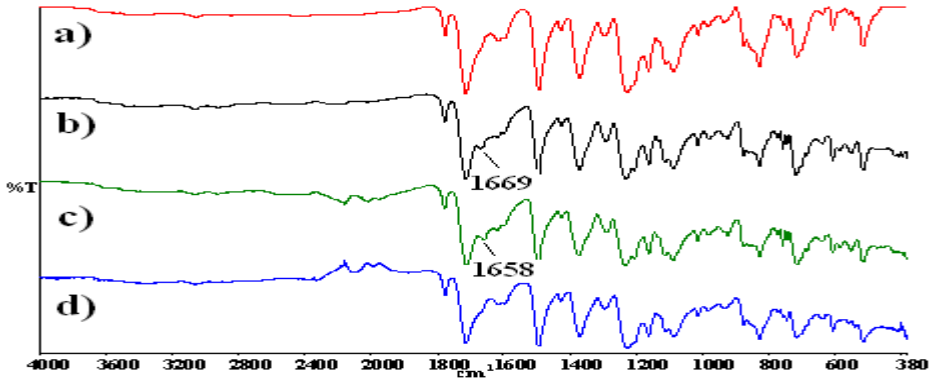

Figure 1: ATR-FTIR spectra of a) PMDA-ODA polyimide film b)Aminated PMDA-ODA polyimide membrane c) Gulataraldehyde modified membrane d)Enzyme immobilized PMDA-ODA polyimide membrane

Figure 2 shows the SEM micrographs of the surface morphology of polymeric support before and after enzyme immobilization. It can be seen from these micrographs that the polymeric film has a uniform, homogeneous and crack-free surface before enzyme immobilization. On the other hand it's clearly seen that the surface morphology of the PMDA-ODA polyimide films changed after immobilization. Also these SEM micrographs show the covalent attachment of the enzyme on the surface of the polymer films.

3.2 Immobilization efficiency

The amount of covalently bound α-amylase enzyme was found as 285.45 mg per gram of modified polyimide films. There are several studies including α-amylase immobilization in which the binding capacity of the support materials is labile due to the characteristic properties of the prepared materials. Demir et al., (2012) studied α-amylase immobilization on nano $CaCO_3$ particles and found its capacity as 199.43 mg/g. Hasirci et al. (2006) studied poly(dimer acid-co-alkyl polyamine) particles that were activated by CDI, EDA, and HMDA, respectively. The amount of bound enzyme was found as 7.6; 6.5 and 39.3 mg/g of each particle orderly.

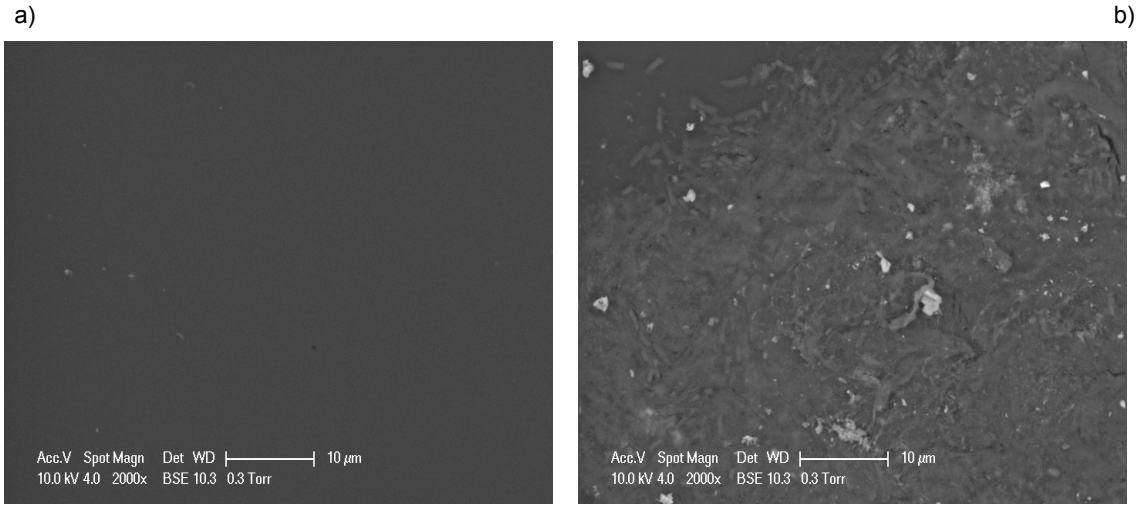

Figure 2: SEM micrographs of enzyme free PI surface (a) and enzyme immobilized PI film (b)

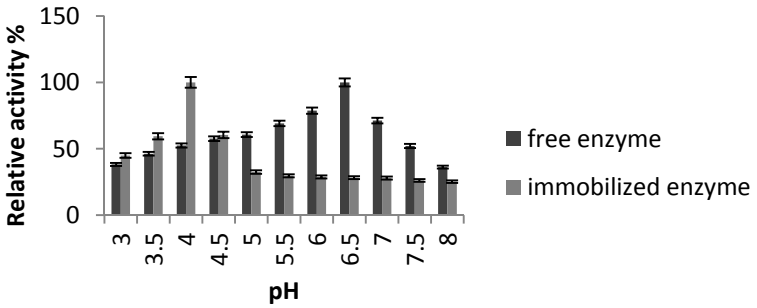

Figure 3: Effect of pH on activity free and immobilized α-amylase.

3.3 Effect of temperature on activity

The activity of the free and immobilized α-amylase was assayed at various temperatures (15 – 80 ˚C). As it can be seen in Figure 4, the maximum catalytic activity was obtained at 30 ˚C for free α-amylase and 50 ˚C for immobilized α-amylase enzymes. However, as the temperature increases, the stability of free enzyme reduces rapidly compared to immobilized form. The immobilized enzyme exhibited better thermal stability than the free one. α-amylase was covalently immobilized onto phthaloyl chloride containing amino group functionalized glass beads. The immobilized α-amylase exhibited better thermostability than the free one (Kahraman et al., 2007). This is probably owing to the covalent immobilization that makes the α-

amylase conformation more rigid and therefore the immobilized α-amylase became more resistant heat inactivation.

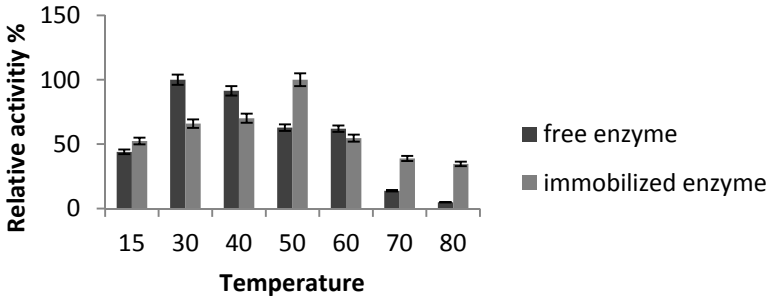

Figure 4: Relative enzyme activity as a function of temperature for free and immobilized α- amylase

3.4 Kinetics

Catalytic properties of the free and immobilized enzymes were evaluated by using soluble starch as a substrate. The Michaelis- Menten constant K_m and V_{max} of the free and immobilized enzymes were estimated at pH 6.5 and 30 ˚C. K_m values were found as 0.550 and 0.488 mg/ mL for free and immobilized enzymes, orderly. The Km value is known as the affinity of the enzymes to substrates and the lower values of K_m emphasize the higher affinity between enzymes and substrates (Cosulich et al., 2000; Park et al., 2005). The results have shown that the affinity of the α-amylase to its substrate was increased by immobilization. V_{max} values for the free and immobilized enzymes were calculated as 10,000 and 0.1146 mg/mL min^{-1}, respectively.

4. Conclusions

In this work aminated PMDA-ODA polyimide membranes were successfully prepared and the immobilization of amylase onto these membranes was obtained by covalently bonding and characterized by FTIR and SEM techniques. Enzyme assays demonstrated that, the immobilized enzyme exhibited better thermo-stability than the free one. The optimum pH of the immobilized enzyme was shifted 2.5 pH unit to the acidic region. These results confirm that α-amylase was successfully immobilized and gained more stable character compared to free one.

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