Use of poly(HEMA-MAH)-Cu^{2+} Microbeads for α-Amylase Immobilization

Poli(HEMA-MAH)-Cu^{2+} Mikrokürelerin α-Amilaz İmmobilizasyonu için Kullanılması

Research Article

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ABSTRACT

In this article poly(2-hydroxyethyl methacrylate-N-methacryloyl-(L)-histidin methylester) [P(HEMA-MAH)] microbeads were prepared by suspension polymerization of 2-hydroxyethyl methacrylate (HEMA) and N-methacryloyl-(L)-histidine methyl ester (MAH). Some properties of the p(HEMA-MAH) microbeads were determined by using scanning electron microscopy (SEM) and swelling tests. Cu^{2+} ions were chelated on the p(HEMA-MAH) microbeads, then these microbeads were used in the immobilization of α-amylase in batch system. The maximum α-amylase adsorption capacity of the p(HEMA-MAH)-Cu^{2+} beads was found to be 14.88 mg/g at pH 6.0. The optimum temperatures for the immobilized and free enzyme were determined to be 30ºC and 25 ºC, respectively. Kinetic parameters (K_m, V_{max}) of the immobilized enzyme were also determined and compared with those of free enzyme. Reuse studies showed that the immobilized enzyme could reuse six times while retaining 76% of its activity.

Key Words

α-amylase, P(HEMA-MAH), microbead, IMAC

ÖZET

Bu çalışmada poli(2-hidroksietil metakrilat-N-metakriloil-(L)-histidin metilester [P(HEMA-MAH)] mikroküreler süspansiyon polimerizasyon yöntemiyle 2-hidroksietil metakrilat (HEMA) ve N-metakriloil-(L)-histidin metil ester (MAH) ile hazırlandı. P(HEMA-MAH) mikrokürelerin bazı özellikleri taramalı elektron mikroskobu (SEM) ve şişme testleriyle belirlendi. P(HEMA-MAH) mikrokürelere Cu^{2+} bağlandıktan sonra bu mikroküreler α-amilaz immobilizasyonu için kullanıldı. P(HEMA-MAH)-Cu^{2+} mikrokürelerin maksimum α-amilaz adsorpsiyon kapasitesi pH 6.0'da 14.88 mg/g olarak bulundu. İmmobilize ve serbest enzim için optimum sıcaklık sırasıyla 30ºC ve 25ºC olarak belirlendi. İmmobilize enzim için kinetik parametreler de (K_m, V_{max}) belirlenerek serbest enzim ile karşılaştırıldı. Tekrarlılık çalışmaları, immobilize enzimin orijinal aktivitesinin %76'sını kaybetmeden altı kez tekrar kulanılabilirliğini gösterdi.

Anahtar Kelimeler
α-amilaz, P(HEMA-MAH), mikroküre, İMAK.

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INTRODUCTION

α-Amylase is an enzyme (1,4-α-D-glucan glucanohydrolase) which hydrolyses starch and related polysaccharides, such as, amylose, amylopectin and glycogen. This enzyme is an endo-acting and generally distributed one hydrolysing the α-1,4-glycosidic bonds, but α-1,6 linkages or other branch points do not hydrolyse by α-amylase [1,2]. In recent years, α-amylase is of great interest with applications ranging from food, fermentation, baking, textile and detergent industries [3-5]. Amylases are usable from various sources, including fungi, yeasts, bacteria and actinomycetes, however, particularly bacterial and fungal origin enzymes have been considerably employed in various industrial sectors, such as, paper manufacture, pharmacology and starch processing [6-8]. The sale of amylolytic enzymes accounts for almost US$25 million worldwide [9].

The enzymatic reactions and instability of the enzyme under repetitive or prolonged use are inhibited at high substrate or product concentrations. Immobilization is an important method for continuous and repeated use of enzymes in industrial application and also rapid separation of the enzyme from reaction medium [10]. Immobilized enzymes are often used in food technology, analytical chemistry and biotechnology. There are several methods of immobilization of an enzyme. Immobilization can occur by different ways, for example, an ionic or covalent bond formation between the enzyme and the support material by adsorption or copolymerization, cross linking, entrapment of the enzyme in or on a solid support [11-17]. Immobilization of α-amylase has been studied with different support materials, such as, poly (hydroxyethyl methacrylate) based nanoparticles [18], poly ethyleneglycol dimethacrylate-n-vinyl imidazole-Cu^{2+} hydrogels [19], chitosan and its amino acid condensation adducts [20].

Several parameters effect immobilization conditions. These parameters are surface area, accessibility of the surface for enzymes, number of activated functional groups on the support, binding of functional groups on the protein, distance between the surface of the support and the bound enzyme and the steric position of the active cite [2].

Another important factor is the structure of the support material for enzyme immobilization. Polymeric materials have attracted much attention as support matrices because they can be easily modified. Their particle diameter, porosity, water uptake ability and biocompatibility are related to the specific applications [21]. The preparation of beads has attracted considerable attention because of their physical and chemical properties owing to their small size, large specific surface area and low cost [22]. Among the immobilization techniques, adsorption has a higher commercial potential than the other methods. It is easier, less expensive and a higher catalytic activity may be retained. But adsorption is commonly not very strong and during washing and other operation conditions some of the adsorbed enzyme desorb. Thus, electrostatic interactions occur between enzyme and support material via adsorption [22, 23]. For this aim, pseudo-specific ligands can be suitable in some cases and amino acids can be used as pseudo-specific ligands, and could hold certain advantages as pseudo-affinity ligands for industrial application. Histidine has been used as pseudo-specific ligand because of its carboxyl, amino and imidazole groups interact with several proteins at around their isoelectric points [16].

The low cost of metals and without any loss of metal-chelating properties of adsorbents are the attractive features of immobilized metal affinity techniques. Therefore, over other traditional adsorbents immobilized metal affinity chromatography (IMAC) offer several advantages [24]. IMAC is a sensitive technique for proteins, peptides, nucleic acids and enzymes because it interacts electrodonating aminoacids residues of proteins on their surfaces such as the imidazole group of histidine, thiol group of cysteine and indole group of tryptophan [23-26]. Many transition metals can form stable complexes with electron-rich compounds and may coordinate molecules containing O, N and S by ion dipole interactions. IMAC introduces an interesting approach for selectively interacting biomolecules on the basis of their affinities for metal ions. Co-operation between neighbouring aminoacid side chains and local conformations play important roles in protein binding [27].

In this study, pHEMA based matrix containing MAH was prepared by suspension polymerization of 2-hydroxyethyl methacrylate (HEMA) and N-methacryloyl-(L)-histidine methyl ester (MAH). Then, Cu^{2+} was chelated as a metal ligand because of its affinity to the histidine groups of protein. Then, α-amylase was immobilized onto p(HEMA-MAH)-Cu^{2+} microbeads. Furthermore, the effect of the adsorption conditions (i.e., pH, temperature), kinetic parameters and the reusability of the immobilized enzyme were also studied.

MATERIALS AND METHODS

Materials

α-Amylase (E.C.3.2.1.1, from *Bacillus* sp. Type II-A), L-histidine methylester, methacryloyl chloride and 2-hyroxyethyl methacrylate were obtained from Sigma Chemical Co. (St Louis, MO), ethylene glycol dimethacrylate (EGDMA) was supplied from Fluka A.G. (Buchs, Switzerland). All other chemicals were of reagent grade and purchased from Merck AG (Darmstadt, Germany).

Preparation of MAH

Preparation and characterization of MAH have been described in detail by Prof. Denizli and his co-workers [28] and our previously study [29]:

5.0 g of L-histidine methylester and 0.2 g of hydroquinone were dissolved in 100 mL of dichloromethane. This solution was cooled to 0°C and then 12.7 g of triethylamine was added to the solution. 5.0 mL of methacryloyl chloride was poured slowly into this solution and then this solution was stirred magnetically at room temperature for 2 h. Then, hyroquinone and unreacted methacryloyl chloride were extracted with 10% NaOH. Aqueous phase was evaporated in a rotary evaporator at 30 °C. The residue (i.e., MAH) was crystallized in an ether-cyclohexane mixture (1:1, v/v) and then dissolved in ethyl alcohol.

Preparation of p(HEMA-MAH) microbeads

Firstly, HEMA and MAH monomers were copolymerized to obtain p(HEMA-MAH) microbeads. For this aim poly (vinyl alcohol) (0.2 g; stabilizer) was dissolved in 50 mL deionized water. The dispersion phase was prepared by mixing 4.0 mL of HEMA, 500 mg of MAH, 8.0 mL of EGDMA and 12.0 mL of toluene in a tube. 0.1 g of benzoyl peroxide was dissolved in this solution to be initiator. Polymerization was carried out in a glass-sealed polymerization reactor (250 mL) placed in a water bath equipped with a temperature control system. The polymerization reactor was heated to 65 ºC for 4 h and then 90ºC by stirring the polymerization reactor at 600 rpm. After the polymerization finished, the beads were cooled at room temperature and washed with ethyl alcohol and water several times to remove unreacted monomers.

Incorporation of Cu^{2+} ions

Cu^{2+} chelates with p(HEMA-MAH) microbeads were prepared as follows: 50 mL of 50 ppm Cu^{2+} solution at constant pH (pH 5.0; adjusted with 0.1 M HCl and 0.1 M NaOH), which was the optimum pH for Cu^{2+} chelate formation, was added to the 1.0 g of the microbeads. A 1000 ppm atomic absorption standard solution was used as the Cu^{2+} ion source. The flask was stirred at 100 rpm for 24 h at room temperature. The Cu^{2+} ion concentration in the resulting solution was determined by an atomic absorption spectrometer. The amount of adsorbed Cu^{2+} was calculated by using the Cu^{2+} concentration of the initial solution and the equilibrium. Cu^{2+} leakage from the p(HEMA-MAH)-Cu^{2+} microbeads was studied at pH 5.0-8.0 and 0.1 M NaCl. Cu^{2+} concentration of supernatant was determined by using an atomic absorption spectrometer.

Swelling test

Swelling studies of the p(HEMA) and p(HEMA-MAH) microbeads were performed in distilled water. Initially, dry microbeads were weighed and then placed in a 15 mL vial containing distilled water. The vial put into a water bath at 25 ºC for 2 h. The microbead sample was taken out from the water and wiped using a filter paper and weighed again. The weight ratio was determined for dry and wet samples. The water contents of p(HEMA) and p(HEMA-MAH) microbeads were calculated by using the following equation:

$$\text{Water uptake ratio \%} = [(W_s - W_o)/W_o] \times 100$$

where W_o and W_s are the weights of microbeads before and after uptake of water, respectively.

Scanning electron microscopy

The surface morphology of the p(HEMA-MAH) microbeads was examined by scanning electron microscope (SEM, Jeol JSH 5600, Tokyo, Japan). The dried specimens were coated with a gold metal layer to provide proper surface conduction.

Adsorption experiments

α-Amylase adsorption studies were studied at different pHs (0.1 M pH 5.0 acetate; 0.1 M pH 6.0; 6.5 and 8.0 phosphate buffer) and temperatures (15; 25; 35; 37 and 40 ºC). Initial enzyme concentration and microbead amount were selected to be 0.5 mg/mL and 0.5 g, respectively. The adsorption studies were carried out for 2 h at 150 rpm and 25 ºC. The adsorption was followed by monitoring of the decrease in α-amylase concentration by ultraviolet absorbance at 280 nm.

The amount of adsorbed α-amylase on the p(HEMA-MAH)-Cu^{2+} microbeads was determined as follows:

$$Q = (C_o - C)\, V/m$$

where Q is the amount of α-amylase adsorbed onto unit mass of microbeads (mg/g), C_o and C are the initial and in the aqueous phase after treatment of certain period time, respectively (mg/mL), V is the volume of aqueous phase (mL), m is the mass of the microbeads used (g).

Repeated use

To determine the reusability of the immobilized α-amylase, 5 mL of 0.1% starch solution (in 0.1 M phosphate buffer at pH 6.5) was added to the immobilized enzyme and incubating for 30 min at 25 ºC under constant stirring rate for each cycle. Then, immobilized enzyme was taken from the medium and washed with distilled water and then added a substrate solution to start a new cycle.

Activity assays of free and immobilized enzyme

α-Amylase enzyme activity was determined by Bernfeld method [30]. Known amounts of free or immobilized α-amylase were incubated with 5 mL of 0.1% soluble starch solution for 30 min at 25ºC and 120 rpm. 800 μL of 3,5-dinitrosalicylic acid solution was added to the 400 μL of reaction mixture and was incubated in a boiling water bath for 5 min, cooling at room temperature and then 8 mL of deionized water was added. Absorbance of each solution was recorded at 489 nm.

A calibration curve drawn with maltose (100-1000 μg/mL) was used to convert the absorbance readings into the concentration of maltose. One unit of amylase activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol maltose per minute under the specified conditions. The protein concentration was determined by Lowry's method [31] with crystalline BSA as the standard. All experiments were performed in three replicates. For each set of data, standard statistical methods were used to determine the mean values and standard deviations. Confidence intervals of 95% were calculated for each set of samples in order to determine the margin of error.

Optimum pH and temperature

The optimum pH of free and immobilized enzyme was determined by assaying at different pH range (pH: 5.0-8.0). For this aim, 0.1% soluble starch was prepared in respective buffers. The optimum temperature of free and immobilized α-amylase was obtained by assaying the enzyme at temperature from 20 to 50 ºC in a water bath at their optimum pHs. The values of pH and temperature of the medium are presented in a normalized form, with the highest value of each set being assigned the value for % activity. Substrate concentration was studied in the range of 0.075-0.175% soluble starch concentrations for determining kinetic parameters (K_m, V_{max}).

RESULTS AND DISCUSSION

Properties of microbeads

The scanning electron micrograph of p(HEMA-MAH) is shown in Figure 1. For the prepared microbead, particle diameter was found to be 40-100 μm. The microbeads have a spherical form and rough surface due to the pores which formed during the polymerization. P(HEMA) and p(HEMA-MAH) microbeads do not dissolve in aqueous media but swell. Because these microbeads are crosslinked hydrophilic matrices. These properties depend on the degree of cross-

linking and on the hydrophilicity of the matrix. The swelling degrees of p(HEMA) and p(HEMA-MAH) microbeads were found to be 40% and 50%, respectively. Hydrophilic functional groups increase into the polymer chain by adding MAH and reaction of MAH with HEMA could effectively decrease the molecular weight of the resulting polymer and reduce the crystallinity of the structure. In this way, the water molecules diffuse into the p(HEMA-MAH) polymer more easily and polymer water uptake behaviour increases in aqueous medium.

Effect of pH and temperature on α-amylase adsorption

The effect of pH on the adsorption of α-amylase onto [p(HEMA-MAH)-Cu^{2+}] beads i.e., no square brackets, was investigated in the pH range of 5.0-8.0. As shown in Figure 2, the maximum α-amylase adsorption was found to be 14.88 mg/g at pH 6.0. The adsorption capacity of microbeads decreased above and below this pH. The decreased in adsorption capacity at lower and higher pHs might be explained an increase in conformational size and the electrostatic repulsion between the opposite charged groups [32].

The effect of temperature on α-amylase adsorption was studied in the range of 15-40 ºC. The adsorption of enzyme significantly decreased with increasing temperature and adsorption capacity was obtained at 25 ºC (Figure 3). The adsorption capacity decreased for 9.97% from 15 to 40 ºC. The exothermic nature of the adsorption process can be conceivable as a basic possible statement for this behaviour. Another explanation is stated that weak van der Waals interaction can take place with increasing temperature [12].

Effect of pH and temperature on the free and immobilized α-amylase activity

The effect of pH on the free and immobilized α-amylase activity was studied in the pH range of 5.0-8.0 and the results are given in Figure 4. Maximum enzyme activity was detected at pH 6.0 for both immobilized and free enzyme. At all pH values immobilized enzyme showed higher activity than free enzyme. Other researchers

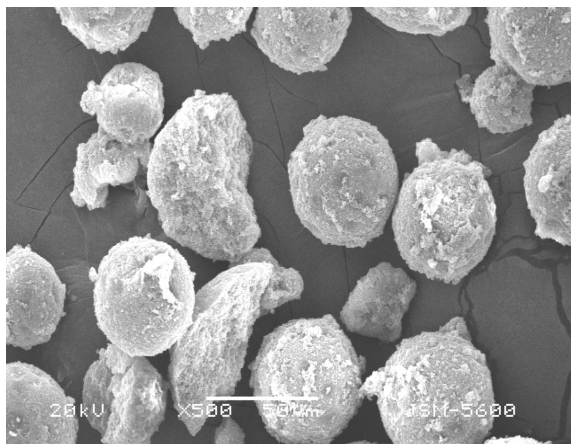

Figure 1. SEM micrograph of poly(HEMA-MAH) microbeads.

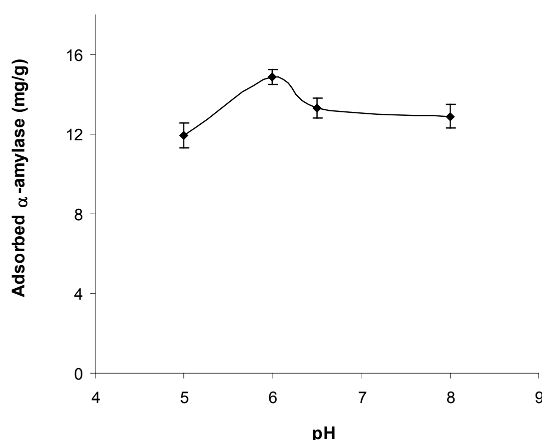

Figure 2. Effect of pH on α-amylase adsorption onto poly(HEMA-MAH)-Cu^{2+} microbeads. Experimental conditions: α-amylase concentration: 0.5 mg/mL; temperature: 25 ºC. Results are expressed as means of three independent experiments, with each experiment run in triplicate.

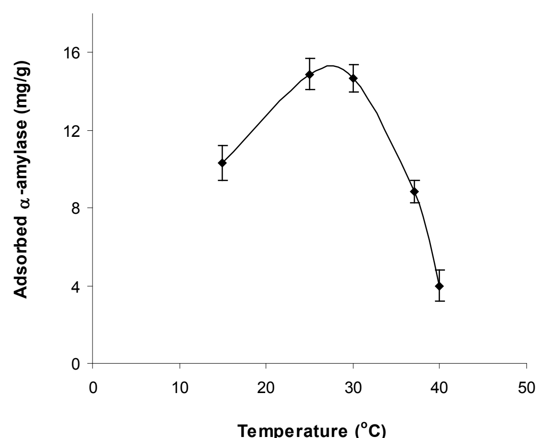

Figure 3. Effect of temperature on α-amylase adsorption onto poly(HEMA-MAH)-Cu^{2+} microbeads. Experimental conditions: α-amylase concentration: 0.5 mg/mL; pH: 6.0. Results are expressed as means of three independent experiments, with each experiment run in triplicate.

have stated similar results upon immobilization of α-amylase [14, 19, 33, 34].

The effect of temperature on enzyme's activity is shown in Figure 5. The optimum temperatures of free and immobilized α-amylase were found to be 25 ºC and 30 ºC, respectively. The increase in optimum temperature of the immobilized enzyme may be caused by the changing physical and chemical properties of the enzyme [14, 35].

Kinetic parameters

The K_m value of enzyme's gives an idea about the affinity of an enzyme for its substrate and the lower K_m value shows the higher affinity between enzyme and substrate. However, when the enzyme active site is saturated by its subtrate the V_{max} value gives the maximum rate of enzymatic reaction [36]. Figs. 6 and 7 show the Lineweaver-Burk plots of free and immobilized α-amylase, respectively. The K_m and V_{max} values of free enzyme were calculated to be 4.977×10^{-3} M and 2.890 U/mg, respectively. For the immobilized enzyme, the K_m and V_{max} values were found to be 5.59×10^{-3} M and 4.90 U/mg, respectively. The K_m value of immobilized α-amylase was slightly higher than that of free α-amylase. The negligible change of K_m could be the conformational changes in tertiary structure of α-amylase and steric effects resulting from limitation of the accessibility of substrate to the active site are affected on immobilization [15, 37].

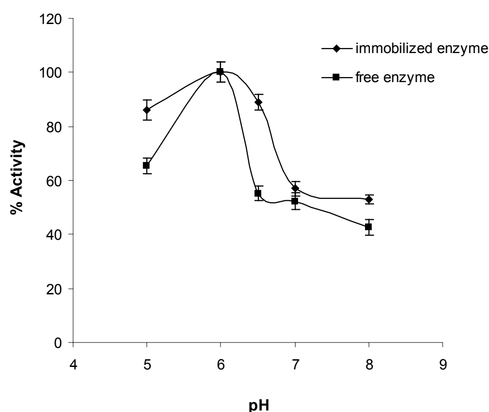

Figure 4. Effect of pH on the free and adsorbed α-amylase activity. Experimental conditions: α-amylase concentration: 0.5 mg/mL; T: 25 ºC. Results are expressed as means of three independent experiments, with each experiment run in triplicate.

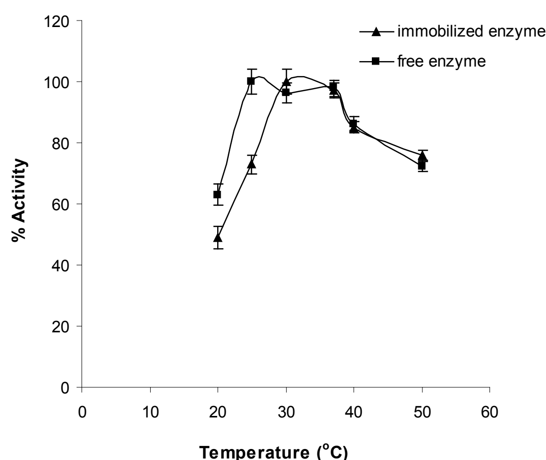

Figure 5. Effect of temperature on the immobilized α-amylase activity. Experimental conditions: α-amylase concentration: 0.5 mg/mL; pH: 6.0. Results are expressed as means of three independent experiments, with each experiment run in triplicate.

Chen *et al.* [38] also reported an increase K_m

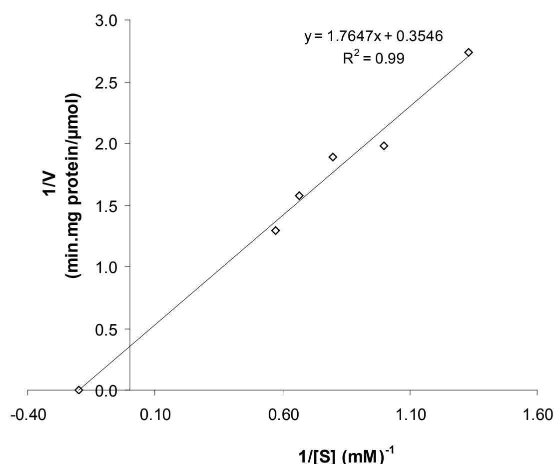

Figure 6. Lineweaver-Burk plot of free enzyme.

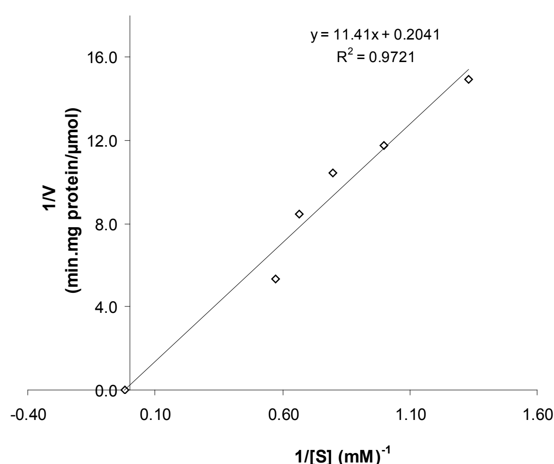

Figure 7. Lineweaver-Burk plot of immobilized enzyme.

indicates an apparent low affinity of the enzyme towards its substrate compared to the enzyme. Recently, Kumari *et al.* [15] reported an apparent K_m values of α-amylase were 4 mg/mL and 2.5 mg/mL for chitosan and amberlite, respectively. Ashly *et al.* also reported [39] the K_m values of α-amylase increased for enzyme which immobilized onto different polyanilines compared to the free enzyme.

Reusability of the immobilized enzyme

The ability of the immobilized enzyme to remain activity was examined by recording the changes in activity after repeated washes. For each repetition of the activity found was compared with the initial activity assuming it possess 100% activity. Six enzyme reaction cycles were studied and changes in activity were given in Figure 8. At the end of the 6th cycle the residual activity retained was approximately 76%.

CONCLUSIONS

Another advantage of enzyme immobilization is recovery and reusability of enzyme. In the present study p(HEMA-MAH)-Cu^{2+} microbeads were prepared to be adsorbent for the immobilization of α-amylase. Thus, there was no need to activate the matrix and this adsorbent was used as a support material for enzyme immobilization. The optimum pH and temperature of the immobilized α-amylase were not drastically changed compared to free one. However, p(HEMA-MAH)-Cu^{2+} beads displayed good adsorption capacities and could be useful in the enzyme adsorption technology and biotechnological processes.

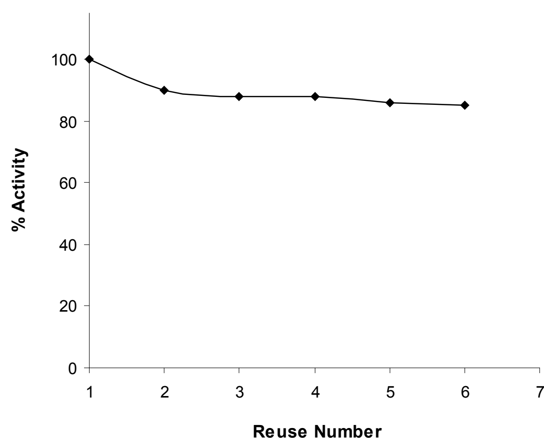

Figure 8. Effect of reusability on immobilized enzyme activity Experimental conditions: α-amylase concentration: 0.5 mg/mL; pH:6.0; T: 25 ºC.

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References

1. H. Tümtürk, S. Aksoy, N. Hasırcı, Covalent immobilization of α-amylase onto poly(2-hydroxyethyl methacrylate) and poly(styrene -2-hydroxyethyl methacrylate) microspheres and the effect of Ca^{2+} ions on the enzyme activity, Food Chem., 68 (2000) 259.
2. A. Konieczna-Molenda, A. Kochanowski, A. Walaszek, E. Bortel, P. Tomasik, Immobilization of α-amylase on poly(vinylamine) and poly(vinylformamide) supports and its performance, Chem. Eng. J., 146 (2009) 515.
3. S. Sivaramakrishnan, D. Gangadharan, K.M. Nampoothiri, A. Pandey, α-Amylase from microbial sources– an overview on recent developments, Food Technol. Biotechnol., 44 (2006) 173.
4. R. Gupta, P. Gigras, H. Mohapatra, V. Kumar Goswami, B. Chauhan, Microbial α-amylases: a biotechnological perspective, Process Biochem., 38 (2003) 1599.
5. D. Gangadharan, K. Madhavan Nampoothiri, S. Sivaramakrishnan, A. Pandey, Immobilized bacterial α-amylase for effective hydrolysis of raw and soluble starch, Food Research. Int., 42 (2009) 436.
6. A. Pandey, P. Nigam, C.R. Soccol, V.T. Soccol, D. Singh, R. Mohan, Advances in microbial amylases, Biotechnol. Appl. Biochem., 31 (2000) 135.
7. J. Bryjak, Glucoamylase, α-amylase and β-amylase immobilisation on acrylic carriers, Biochem. Eng. J., 16 (2003) 347.
8. A. Kadziola, J. Abe, B. Svensson, R. Haser, Crystal and molecular structure of barley α-amylase, J. Mol. Biol., 239 (1994) 104.
9. P. Tripathi, A. Kumari, P. Rath, A.M. Kayastha, Immobilization of α-amylase from mung beans (*Vigna radiata*) on Amberlite MB 150 and chitosan beads: A comparative study, J. Mol. Catal. B., 49 (2007) 69.
10. M. Safarikova, I. Roy, M.N. Gupta, I. Safarik, Magnetic alginate microparticles for purification of α-amylases, J. Biotechnol., 105 (2003) 255.
11. G. Rajagopalan, C. Krishnan, Immobilization of malto-oligosaccharide forming α-amylase from *Bacillus subtilis* KCC103: properties and application in starch hydrolysis, J. Chem. Technol. Biotechnol., 83 (2008) 1511.

12. M.E. Çorman, N. Öztürk, N. Bereli, S.Akgöl, A. Denizli, Preparation of nanoparticles which contains histidine for immobilization of *Trametes versicolor* laccase, J. Mol. Catal. B: Enzymatic., 63 (2010) 102.
13. M.V. Kahraman, G. Bayramoğlu, N. Kayaman-Apohan, A. Güngör, α-Amylase immobilization on functionalized glass beads by covalent attachment, Food Chem., 104 (2007) 1385.
14. G. Bayramoğlu, M. Yılmaz, M.Y. Arıca, Immobilization of a thermostable α-amylase onto reactive membranes: kinetics characterization and application to continuous starch hydrolysis, Food Chem., 84 (2004) 591.
15. A. Kumari, A.M. Kayastha, Immobilization of soybean (*Glycine max*) α-amylase onto chitosan and amberlite MB-150 beads: optimization and characterization, J. Mol. Catal. B: Enzymatic., 69 (2011) 8.
16. M.M. Sarı, C. Armutçu, N. Bereli, L. Uzun, A. Denizli, Monosize microbeads for pseudo-affinity adsorption of human insulin, Colloid. Surface. B., 84 (2011) 140.
17. E.B. Altıntas, L. Uzun, A. Denizli, Synthesis and characterization of monosize magnetic poly(glycidyl methacrylate) beads, China Particuol., 5 (2007) 174.
18. D. Aktaş Uygun, N. Öztürk, S. Akgöl, A. Denizli, Novel magnetic nanoparticles for the hydrolysis of starch with *Bacillus licheniformis* α-amylase, J. Appl. Polymer Sci., 123 (2012) 2574.
19. A. Kara, B. Osman, H. Yavuz, N. Beşirli, A. Denizli, Immobilization of α-amylase on Cu^{2+} chelated poly(ethylene glycol dimethacrylate-*n*-vinyl imidazole) matrix via adsorption, React. Function. Polym., 62 (2005) 61.
20. M.A. Abd El-Ghaffar, M.S. Hashem, Immobilization of α-amylase onto chitosan and its amino acid condensation adducts, J. Appl. Polymer Sci., 112 (2009) 805.
21. S. Li, J. Hu, B. Liu, Use of chemically modified PMMA microspheres for enzyme immobilization, Biosystems., 77 (2004) 25.
22. G. Bayramoğlu, E. Yalçın, M.Y. Arıca, Immobilization of urease via adsorption onto L-histidine-Ni(II) complexed poly(HEMA-MAH) microspheres: preparation and characterization, Process Biochem., 40 (2005) 3505.
23. S. Akgöl, N. Öztürk, A. Denizli, New generation polymeric nanospheres for catalase immobilization, J. Appl. Polymer Sci., 114 (2009) 962.
24. A. Derazshamshir, B. Ergün, G. Peşint, M. Odabaşı, Preparation of Zn^{2+}-chelated poly(HEMA-MAH) cryogel for affinity purification of chicken egg lysozyme, J. Appl. Polymer Sci., 109 (2008) 2905.
25. N. Ünlü, Ş. Ceylan, M. Erzengin, M. Odabaşı, Investigation of protein adsorption performance of Ni^{2+}-attached diatomite particles embedded in composite monolithic cryogels, J. Sep. Sci., 34 (2011) 2173.
26. M. Karataş, S. Akgöl, H. Yavuz, R. Say, A. Denizli, Immunoglobulin G depletion from human serum with metal-chelated beads under magnetic field, Int. J. Biol. Macromol., 40 (2007) 254.
27. D. Çimen, A. Denizli, Immobilized metal affinity monolithic cryogels for cytochrome c purification, Colloid. Surface. B., 93 (2012) 29.
28. B. Garipcan, A. Denizli, A novel affinity support material for the separation of immunoglobulin G from human plasma, Macromolecular Biosci., 2 (2002) 135.
29. M. Yavuz, Z. Baysal, Preparation and use of poly(hydroxyethyl methacrylate) cryogels containing L-histidine for β-casein adsorption, J. Food Sci., 78 (2013) E238.
30. P. Bernfeld: α-and -amylases. In: *Methods in Enzymology*, S.P. Colowick, N. Kaplan (Eds.), Academic Press, New York, USA (1955) pp. 149-154.
31. O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem., 193 (1951) 265.
32. N. Tüzmen, T. Kalburcu, A. Denizli, α-Amylase immobilization onto dye attached magnetic beads: Optimization and characterization, J. Mol. Catal. B: Enzymatic., 78 (2012) 16.
33. M.E. Sedaghat, M. Ghiaci, H. Aghaei, S. Soleimanian-Zad, Enzyme immobilization. Part 3: immobilization of α-amylase on Na-bentonite and modified bentonite, Appl. Clay Sci., 46 (2009) 125.
34. J.I. Ida, T. Matsuyama, H. Yamamoto, Immobilization of glucoamylase on ceramic membrane surfaces modified with a new method of treatment utilizing SPCP–CVD, Biochem. Eng. J., 5 (2000) 179.
35. I. Gancarz, J. Bryjak, M. Bryjak, G. Poniak, W. Tylus, Plasma modified polymers as a support for enzyme immobilization 1.: allyl alcohol plasma, Eur. Polym. J., 39 (2003) 1615.
36. V. Singh, P. Kumar, Carboxymethyl tamarind gum–silica nanohybrids for effective immobilization of amylase, J. Mol. Catal. B: Enzymatic., 70 (2011) 67.
37. M.Y. Arica, V. Hasırci, N.G. Alaeddinoğlu, Covalent immobilization of α-amylase onto pHEMA microspheres: preparation and application to fixed bed reactor, Biomaterials., 16 (1995) 761.
38. J.P. Chen, Y.M. Sun, D.H. Chu, Immobilization of α-amylase to a composite temperature-sensitive membrane for starch hydrolysis, Biotechnol. Progg., 14 (1998) 473.
39. P.C. Ashly, M.J. Joseph, P.V. Mohanan, Activity of diastase α-amylase immobilized on polyanilines (PANIs), Food Chem., 127 (2011) 1808.