Full Length Research Paper

Immobilization of urease on copper chelated EC-Tri beads and reversible adsorption

Emre Çevik[1], Mehmet Şenel[2] and M. Fatih Abasiyanik[1]*

[1]Department of Genetics and Bioengineering, Faculty of Engineering, Fatih University, B.Cekmece, Istanbul 34500, Turkey.
[2]Department of Chemistry, Faculty of Arts and Sciences, Fatih University, B.Cekmece, Istanbul 34500, Turkey.

In the present study, Eupergit C® macroporous beads were functionalized with amino triazole and characterized by FTIR-ATR and SEM. Cu^{2+} ions were chelated on the triazole modified Eupergit C® (EC®), and then the metal chelated beads were used in the adsorption of urease. Maximum reaction rate (Vmax) and Michaelis-Menten constant (km) were determined for the free and immobilized enzymes. Various characteristics of immobilized urease such as the temperature activity curve, thermal stability, operational stability and storage stability were evaluated. The results demonstrated that triazole functionalized Eupergit C® beads can be applied to metal sorption and enzyme immobilization.

Key words: Urease, immobilization, Eupergit C®, triazole, chelating beads.

INTRODUCTION

Enzymes have been utilized in a large number of practical applications, particularly in biomedical and biotechnological fields, through immobilization on a variety of supports. These immobilized products were intended for use in the construction of artificial organ systems, biosensors or bioreactors. Immobilization is advantageous because; (1) it extends the stability of the bioactive species by protecting the active material from deactivation; (2) enables repeated use; (3) it provides significant reduction in the operation costs; (4) facilitates easy separation and speeds up recovery of the bioactive agent. Many methods exist for the immobilization of enzymes but usually one of four methods is used, physical adsorption, entrapment, co-polymerization, and covalent attachment (Arica et al., 1997; Arica et al., 1998; Bailey et al., 1988; Gursel et al., 1997; Gacesa and Hubble 1987). The methods and supports employed for enzyme immobilization are chosen to ensure the highest retention of enzyme activity and its stability and durability (Arica et al., 1995; Busto et al., 1987).

The metallochelate-method for immobilization of enzymes has significant prospects. The process of co-ordination immobilization does not require available

reagents and takes place under soft conditions for a comparatively short time which is very important for the labile enzymes. Usually, carriers with a high specific area like oxides of nickel, aluminium, silicon, etc., are used (Slobodianikova et al., 1979; Iamskov and Budanov, 1979; Kvesitadze and Dvali, 1982). The method is based on the ability of the transition metals oxides to produce complex compounds with the hydroxyl groups of the carrier and the enzyme (Slobodianikova et al., 1979; Iamskov and Budanov, 1979). The enzymes immobilized by this method show high activity and good performance stability (Bailey et al., 1988). Various transition metals have been used; Co(II); Ni(II); Cu(II); Vd(III); Ti(III); Ti(IV) and good results were reported for immobilization (Iamskov and Budanov, 1979; Kvesitadze and Dvali, 1982; Kvesitadze et al., 1977).

Urease hydrolyses urea, yielding ammonia and carbonate. Jack bean urease active as a monomer is found in the form of multimeric aggregates, mainly trimers and hexamers. The enzyme originates mainly from plants and micro-organism and is found both as intra- and extracellular enzyme (El-Sherif et al., 2001; Ciurli et al., 1996). Immobilized urease has been widely used in biosensors for diagnostic purposes, in the determination of urea in biological fluids, in artificial kidney devices for the removal of urea from blood for extracorporeal detoxification, in enzyme reactor for the conversion of urea

---

*Corresponding author. E-mail: msenel@fatih.edu.tr.
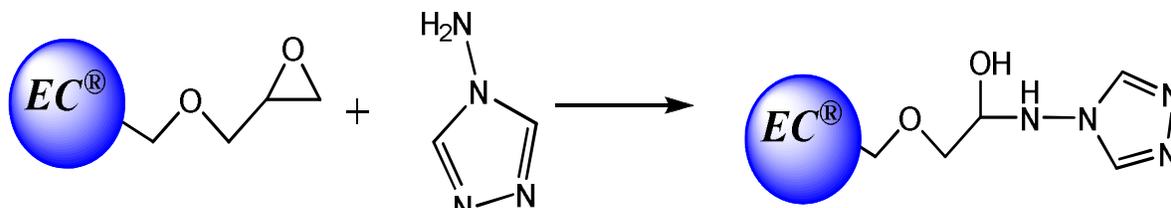
Figure 1. Modification of Eupergit C® with aminotriazole.

present in fertilizer wastewater effluents or in the food industry for removal of urea from beverages and foods (Chen and Chiu, 2000; Hearn and Neufeld, 2000).

Eupergit C®, that is epoxy-activated bead polymers formed from a hydrophilic acrylamide with ally glycidyl (epoxide) groups as the active components responsible for binding, is usually used as enzyme immobilization through different functional groups (amino, sulfhydryl, hydroxyl, phenolic ones) of enzymes (Kramer et al., 1975).

This present paper describes the new triazole function-nalized Eupergit C® beads used as a carrier in order to co-ordinate Cu²⁺ ions. Cu²⁺ ions coordinate to the triazole groups of the Eupergit C®-Tri beads and the enzyme bind the beads via chelated metal ion. The protein adsorption capacity, coupling efficiency, Michaelis-Menten kinetic constants (K_m and V_{max}), reuse and storage stability of free and immobilized enzymes were evaluated.

MATERIALS AND METHODS

Reagents

Urease (EC 3.5.1.5 from jack beans) was obtained from sigma. Eupergit C® was kindly donated by Röhme GmbH and Co., Degussa (Darmstadt, Germany). 4-amino-1,2,4-triazole was obtained from Fluka. All other chemicals were of analytical grade and were used without further purification.

Instrumentation

Activities of the free and immobilized enzymes were determined by using UV-visible spectrophotometer (SHIMADZU UV-1700). Modification of Eupergit C® with triazole was studied by FTIR-ATR spectra and scanned in the range of 4000 to 400 cm⁻¹ on FTIR spectrometer (Perkin Elmer Spectrum BX).

Modification of Eupergit C® and adsorption of urease

Eupergit C® is a spherical acrylic polymer (particles with diameter of 150 μm) containing epoxide groups as reactive components. These groups function as active components for the covalent binding of ligands containing amino, mercapto or hydroxyl groups. Eupergit C®-Tri (EC®-Tri) beads were prepared by conventional method on Eupergit C® supports which involves direct triazole binding on polymers via oxirane groups as shown in Figure 1. Eupergit C® was incubated with excess amount of triazole in 1.0 M phosphate buffer solution (pH 7.0) at 25°C. After incubation for 4 days, the beads were collected on a sintered-glass filter, washed with 1.0 M NaCl for

three times with same buffer solution. The excess of epoxide groups on the Eupergit C®-Tri beads were blocked by incubation with glycerol for 4 h at 25 °C.

Chelates of Cu²⁺ ions with EC®-Tri beads were prepared by mixing 0.1 g of beads with 5 ml aqueous solutions containing 50 ppm Cu²⁺ ions at constant pH 5.0 (adjusted with HCl and NaOH). The flask was shaked at 25°C for 24 h. The concentration of Cu²⁺ ions in the resulting solution was determined with a graphite furnace atomic adsorption spectrometer (Analyst 800/Perkin Elmer, USA). The amount of adsorbed Cu²⁺ ions was calculated by using the concentrations of the Cu²⁺ ions in the initial solution and in the equilibrium.

Urease was adsorbed onto the Cu²⁺ chelated Eupergit C®-Tri beads at various pH values, either in acetate buffer (0.1 M, pH 3.0-5.5 or in phosphate buffer (0.1 M, pH 6.0-9.0). Initial urease concentration was 1 mg/ml. The adsorption experiments were conducted for 3 h at 25°C while shaking continuously. At the end of the time period, the enzyme adsorbed beads were removed and was washed three times with the same buffer solution. Then, the enzyme adsorbed beads were stored at 4°C in fresh buffer solution until use. The amount of adsorbed urease (Q) was calculated using the following equation:

$$Q = \frac{(C_o - C_e)_x V}{m}$$

Where, Q is the adsorbed urease (mg protein/g Eupergit C®-Tri beads), C_o and C_e are the initial and equilibrium concentrations in the solution (mg/ml), V is the solution volume (ml) and m is the mass of Eupergit C®-Tri beads (g).

Urease desorption studies

Desorption study was carried out for enzyme immobilized Eupergit C®-Tri–Cu²⁺ beads stirring with EDTA solution for 1 h at 20°C. In order to determine the desorption of urease, enzyme assay was applied on Eupergit C®-Tri–Cu²⁺ beads. The beads were washed several times with phosphate buffer (0.1 M, pH 7.5) and then, reused for enzyme immobilization.

Enzyme and protein assay

Activity of free and immobilized urease was determined using the method of Nessler (Srinivasa et al., 1995). Free (400 μg) or immobilized urease was kept in a test tube and 1 ml of a phosphate buffer (100 mM, pH 7.5) containing 150 mM urea and was added and incubated for 30 min. A volume of 0.3 ml of 0.3% sodium tungstate and 0.3 ml of 0.68 M sulfuric acid was added and the volume was made up to 5 ml. Then, 1 ml of the mentioned solution was treated with 1 ml of the Nessler's reagent and the total volume was made up to 10 ml with distilled water. Absorbance of the solution was measured at λ = 480 nm in a spectrophotometer. The

amount of ammonia released was calculated by comparing the absorbance with a standard curve for ammonium sulfate.

The determination of the amount of enzyme bound to the EC®-Tri-Cu^{2+} beads carriers was carried out by the method of Lowry (Lowry et al., 1951) by using bovine serum albumin as the standard. The quantity of entrapped protein was calculated by subtracting the protein recovered in the combined washings of the urease-EC from the protein used for immobilization.

Properties of free and immobilized enzymes

pH and temperature

In order to determine the effect of pH on activity (incubated for two hour in buffer solution) of free and immobilized urease were assayed in acetate buffer (0.1 M) in the pH range of 4.0 to 6.5 and in phosphate buffer (0.1 M) in the pH range of 7.0 to 9.0 by using the standard activity assay procedure mentioned earlier.

The effect of temperature and the thermal stability on the activity of both free and immobilized urease was studied between 30 and 90°C and assayed under standard assay conditions.

Storage and operational stability

The immobilized urease was repeatedly used to test its reusability under standard assay conditions. After each activity assay, the samples were washed with the buffer and left stored until the next assay in buffer solution. The immobilized urease was stored in phosphate buffer (0.1 M, pH 7.5) at 4 and 25°C for 35 days. Its activity was measured at frequent intervals.

RESULTS AND DISCUSSION

Optimization of immobilization conditions

An ideal membrane support for immobilization of enzyme must have the following requirements; high hydrophilicity, fairly large pore size with a narrow pore size distribution, biological, chemical and mechanical resistance as well as enough reactive functional groups. Polyamide hollow-fibre membranes have several desired properties for enzyme immobilization as a support such as high porosity, large external surface area and high chemical, biological and mechanical stability (Senel et al., 2001).

The bonds formed between the enzyme and the Eupergit C®-Tri-Cu^{2+} beads during the adsorption process depends upon the chemical properties of the matrix and the enzyme. The bonds are formed between Cu^{2+} and with different nucleophiles on the protein as a function of pH.

An adsorption isotherm was constructed to characterize the interaction of the enzyme (urease) with the adsorbent (Eupergit C®-Tri–Cu^{2+} beads). This provides a relationship between the concentration of enzyme in the solution and the amount of enzyme adsorbed on the solid phase, when two phases are at equilibrium. The concentration of urease in the medium was varied between 0.1 and 1.0 mg /ml and the absorption tests were carried out at pH 7.0 at 25°C. The results of the adsorption tests are presented in Figure 2. An increase in urease concentration (from 0.1 to 1.0 mg/ml) led to an increase in adsorption efficiency but this leveled off at an enzyme concentration of 0.8 mg/ml. A maximum adsorption of 40.89 mg/g membrane was reached at 1.0 mg/ml urease solution.

The important parameters including pH and temperature should be controlled during immobilization of enzyme via adsorption to obtain reproducible results, since these parameters influence the stability and conformational structure of proteins. Urease immobilization via adsorption on Eupergit C®-Tri–Cu^{2+} beads was carried out in the pH range of 3.0 to/9.0, at 25°C for 3 h and the initial urease concentration was 1.0 mg/ml in each adsorption medium. Figure 3 shows the amount of enzyme adsorbed onto Eupergit C®-Tri-Cu^{2+} beads at different pH values.

Proteins have no net charge at their isoelectronic points and therefore, the maximum adsorption from aqueous solution is usually observed at this pH. Urease has an isoelectronic point of 4.9 (Marzadori et al., 1998). In this work, the maximum urease adsorption was obtained at pH 6.0; it was shifted to 1.1 units to less acidic pH value. Significantly, a lower urease adsorption was obtained for Eupergit C®-Tri–Cu^{2+} beads in the entire tested pH region. It should be noted that, the affinity between urease molecules and chelated Cu(II) ions on the Eupergit C®-Tri beads should be electron donor/acceptor interactions. Urease contains four surfaces with exposed histidine and several cysteine residues. The presence of these residues affects the immobilization of urease via adsorption and consequently, its activity on the Eupergit C®-Tri–Cu^{2+} beads. The maximum adsorption at pH 6.0 may result from the formation of co-ordination complex between deprotonated amino acid side chain groups (especially imidazole and primary amine groups of histidine and lysine residues, respectively) of urease and incorporated Cu(II) ions onto Eupergit C®-Tri beads.

Kinetic constants

From the Lineweaver-Burk plot of 1/V versus 1/(S) Michaelis-Menten constants (K_m) and the maximum reaction velocity (V_{max}) of the free and immobilized enzyme were calculated . The V_{max} value free urease (0.01807 µM min^{-1} mg^{-1}enzyme) was found to be lower than that of the immobilized urease (0.08521 µM min^{-1} mg^{-1}enzyme). The calculated K_m values of free and immobilized urease were 4.1 and 32.3 mM, respectively. The increase in K_m after immobilization clearly indicates an apparent low affinity of the enzyme to its substrate compared with the free enzyme; this may be attributed to the tendency of enzyme to leave substrate within short time without giving a product. An increase in K_m was also reported when urease was immobilized onto collagen-poly (GMA) copolymer (Raghunath et al., 1984) and
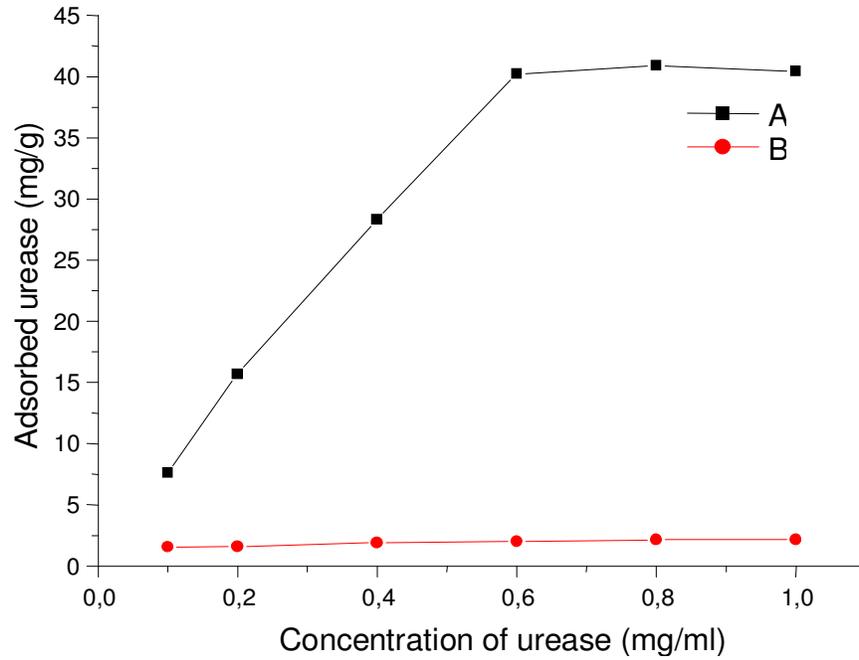
Figure 2. Effect of urease concentration on the adsorption efficiency of (A) Eupergit C$^{®}$-Tri–Cu^{2+} and (B) Eupergit C$^{®}$-Tri beads: pH 7.5 phosphate buffer; adsorption time, 3 h; T, 20°C.
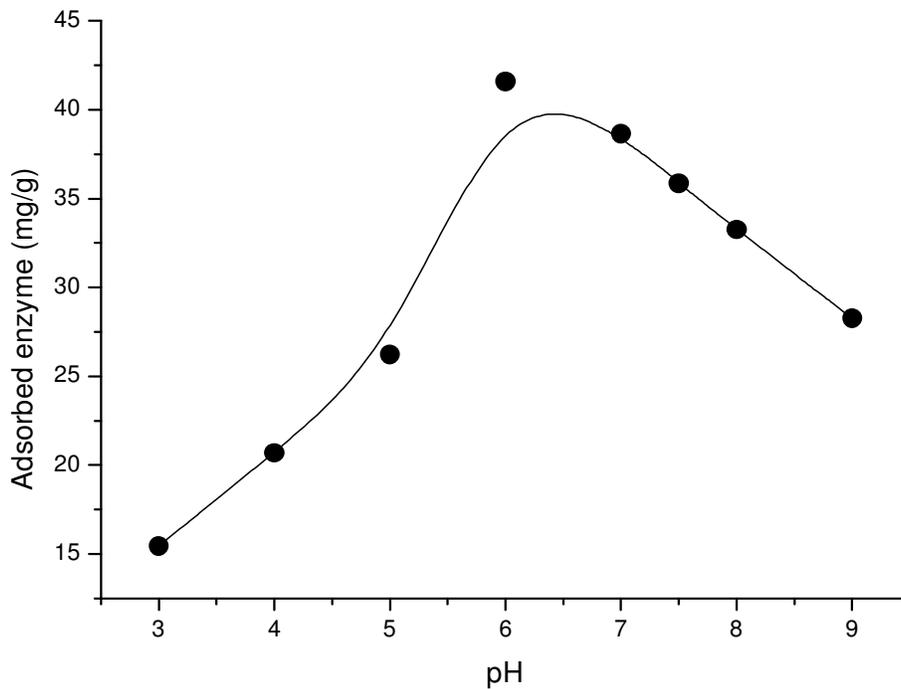
Figure 3. pH effect on the adsorption efficiency of Eupergit C$^{®}$-Tri-Cu^{2+} beads: adsorption time, 3 h; T, 20°C.

chitosan (Krajewska et al., 1990).

The reaction of urea hydrolysis by the free and adsorbed urease as a function of pH is presented in

Figure 4. Upon immobilization via adsorption, the optimal pH for the activity of urease shifted down from 7.0 to 8.0 and the pH profile of the adsorbed urease became
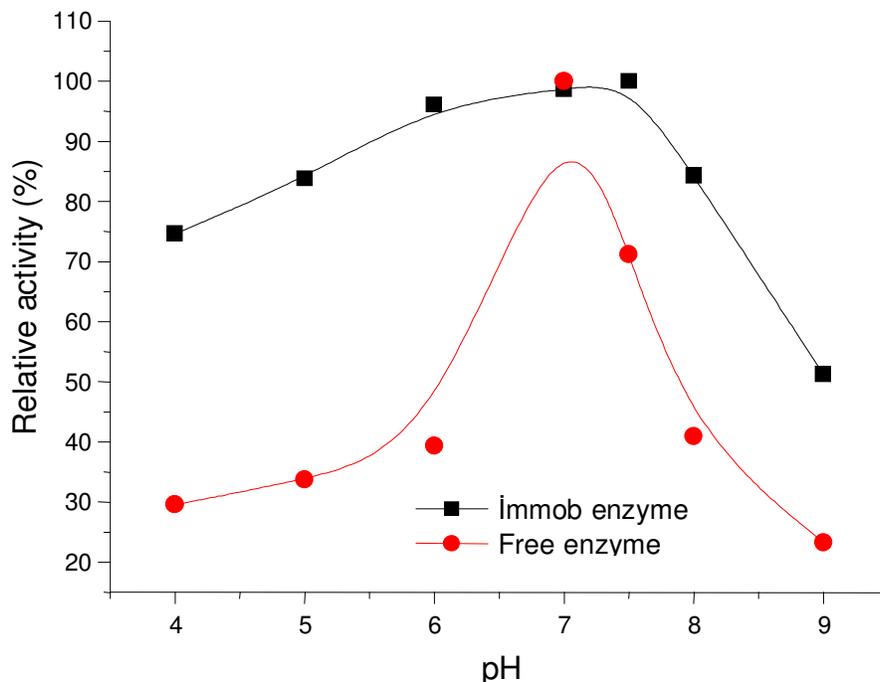
Figure 4. pH profiles of free and adsorbed urease.

broadened particularly at acidic pH values. The shift to more basic optimal pH upon immobilized could be expected as a result of the diffiusional constraint of the support retaining a higher concentration of enzyme product, ammonia, in the vicinity of the pore space of the support that adsorbed enzyme present. Thus, the micro-environment around the enzyme was more basic than that of the bulk solution. Other researchers have reported similar observations upon the immobilization of urease and other enzymes (Arica et al., 2001; Bulmus et al., 1997).

Effect of pH and temperature on the activity

The effect of temperature on the activity of the free and immobilized urease is shown in Figure 5. The activity of the free urease is strongly dependent on temperature and a sharp optimum was obtained at 55°C, whereas the optimum temperature for the immobilized enzyme was 60°C. An increase in optimum temperature was caused by the changing enzyme structure upon immobilization, largely affecting the formation of multi-chelation between enzyme and Eupergit C®-Tri-Cu²⁺ beads.

Adsorption-desorption cycle

The adsorption of urease was studied in the packed reactor continuously stirring at room temperature for 3 h. Desorption studies of enzyme immobilized Eupergit

C®-Tri–Cu²⁺ beads was performed in desorption medium which contain 25 mM EDTA (at room temperature for 2 h) just after Eupergit C®-Tri–Cu²⁺ beads were used in adsorption studies immediately. The Figure 6 shows urease adsorption capacity of the Eupergit C®-Tri-Cu²⁺ beads during ten successive adsorption-desorption cycles. During these adsorption-desorption studies, enzyme activiyies was not significantly changed. These results showed that, Cu²⁺-chelated Eupergit C®-Tri beads can be repeatedly used in enzyme immobilization without detectable losses in their initial adsorption capacities and activities.

Storage stability

Storage stability is an important advantage of immobilized enzymes over free enzymes, because free enzymes can lose their activities quickly. In general, if an enzyme is in aqueous solution, it is not stable during storage and the activity gradually reduces. Free- and adsorbed urease was stored in a phosphate buffer (100 mM, pH 7.5) at 4°C and the activity measurements were recorded over a 35 days period. The free enzyme lost 80% of its activity within 35 days. Enzyme immobilized Eupergit C®-Tri-Cu²⁺ beads lost 20% of their activity in the same period (Figure 7). This fall in enzyme activity was explained as a time-dependent natural loss in enzyme activity, which was prevented to a significant degree by adsorption. This observation distinctly indicates that the immobilized urease exhibits greater
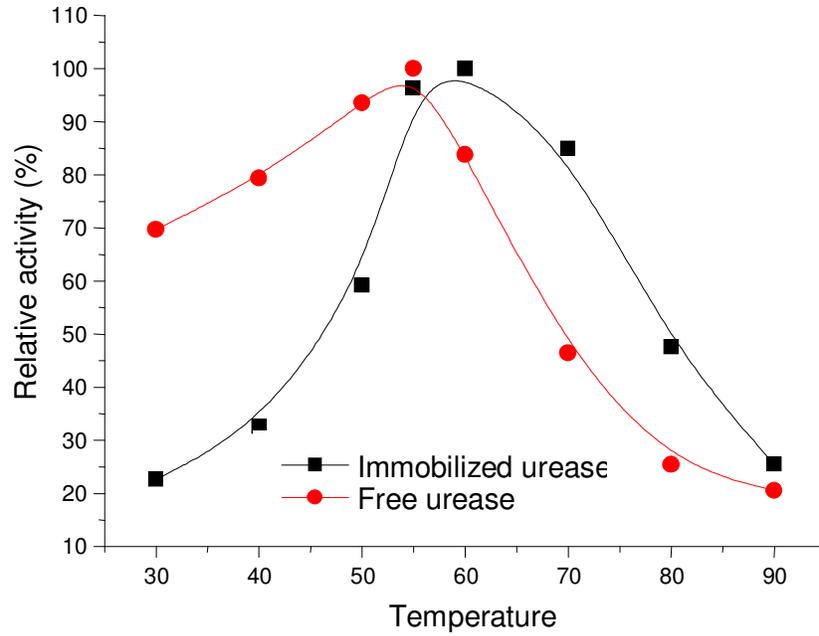
Figure 5. Optimum temperature of the free and immobilized urease.
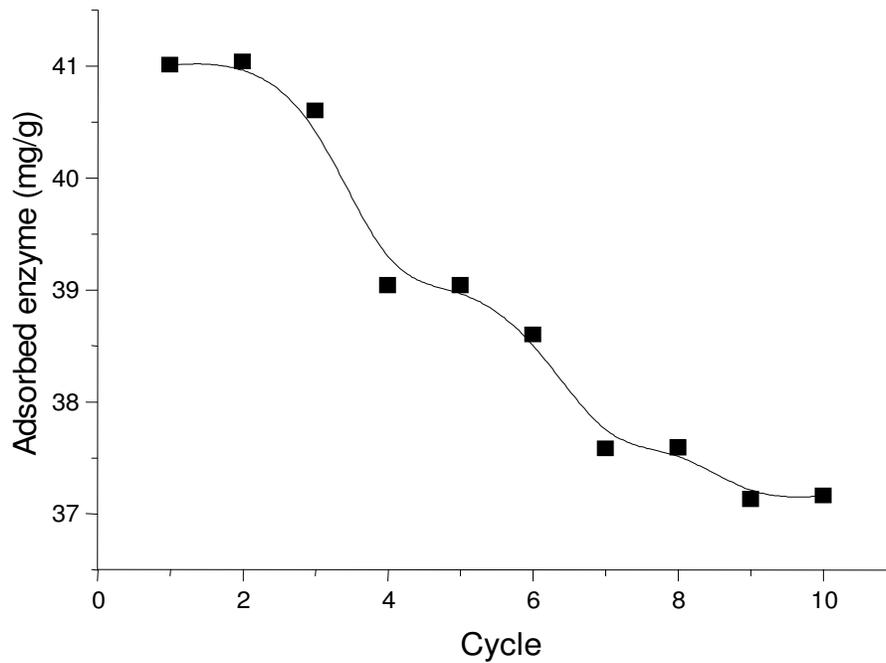
Figure 6. Repeated use of adsorbed invertase; invertase concentration 1.0 mg/ml; pH 7.5; time, 3 h; T, 20°C.

stability than the free enzyme.

Conclusions

Our experiments have shown that, the urease immo-
bilized on Eupergit C® exhibits an improved resistance against thermal and pH denaturation and has a promising operational stability for the immobilized enzyme and its operations. The Eupergit C®-Tri-Cu^{2+} system could be useful not only for urease but also for other enzymes (Teresa et al., 2003).
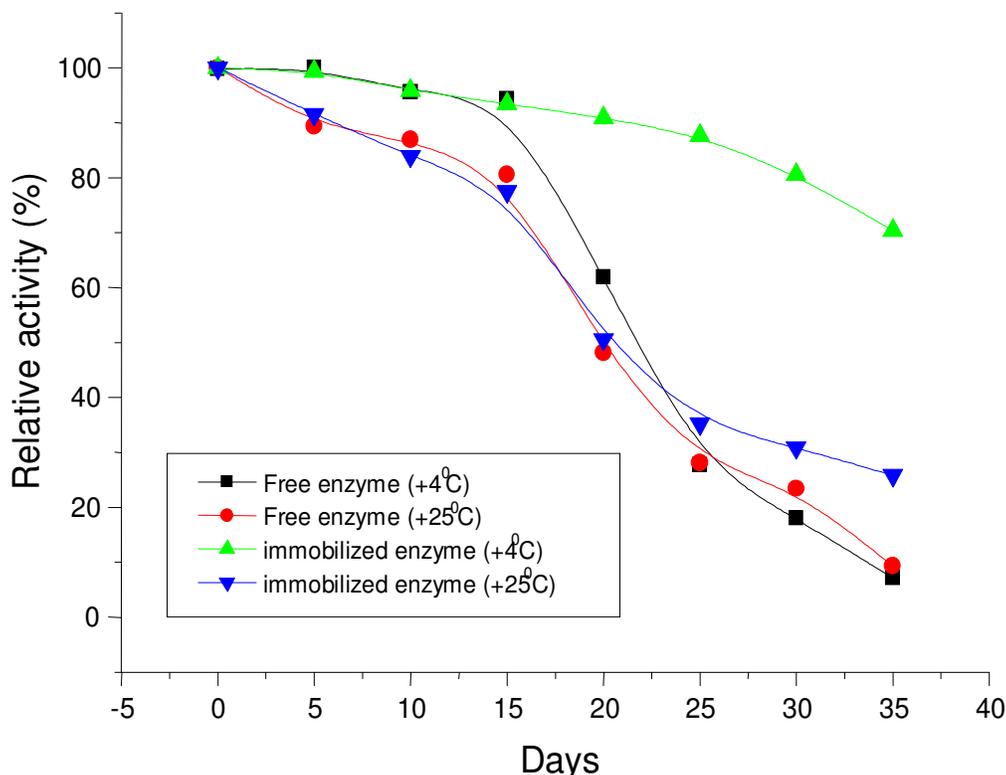
Figure 7. Storage stability of the free and immobilized urease at 4 and 25°C.

The Michaelis-Menten kinetic constants K_m and V_{max} of the free and immobilized urease were determined and it revealed that the affinity of urease to urea decreased after immobilization. The properties of the free and the immobilized urease were compared and the results indicated that, the stability of the immobilized urease towards temperature, pH reusability and storage was enhanced by immobilization. In conclusion, the amino-triazole functionalized EC beads revealed good properties as adsorptive beads and therefore, proved to be useful for applications in biotechnology.

REFERENCES

Arica MY, Kacar Y, Ergene A, Denizli A (2001). Reversible Immobilization of Lipase on Phenylalanine Containing Hydrogel Membranes. Process Biochem., 36: 847-854.
Arıca MY, Denizli A, Baran T, Hasırcı V (1998). Dye Derived and Metal Incorporated Poly(Hydroxyethylmethacrylate) Membranes for Use in Enzyme Immobilization. Polym Int. 46: 345-52.
Arıca MY, Denizli A, Salih B, Piskin E, Hasırcı V (1997). Urease Adsorption on to Cibacron Blue F3ga And Fe(Iii) Derivatized Phema Membranes and Application yo A Continuous System. J. Membr. Sci., 129: 65-76.
Arıca MY, Hasırcı V, Alaeddinoglu NG (1995). Covalent Immobilization of a-Amylase onto Phema Microspheres: Preparation and Application to Fixed Bed Reactor. Biomaterials, 16: 761-781.
Bailey JE (1988). Immobilization of Glucoamylase And Glucose Oxidase in Activated Carbon: Effect of Particle Size and Immobilizationconditions on Enzyme Activity and Effectiveness. Biotechnol. Bioeng., 25: 1923-1935.

Bulmus V, Ayhan H, Piskin E (1997). Modified Microbeads for Glucose Oxidase Immobilization. Chem. Eng. J., 65: 71-76.
Busto MD, Ortega N, Perez-Mateos M (1987). Effect Of Immobilization on The Stability of Bacterial and Fungal b-D-Glucosidase. Process Biochem., 32: 441-449.
Chen J-Pç, Chiu S-H (2000). A Poly(N-isopropylacrylamide-co-N-acryloxyethyl methacrylate) Composite Hydrogel Membrane for Urease Immobilization to Enhance Urea Hydrolysis Rate by Temperature Swing. Enzyme Microb. Technol. 26: 359-367.
Ciurli S, Marzadori C, Benini S, Deiana S, Gessa Cn (1996). Urease from the Soil Bacterium Bacillus Pasteurii: Immobilization on Capolygalacturonate. Soil Biol. Biochem., 28: 811-817.
El-Sherif H, Martelli PL, Casadio R, Portaccio M, Bencivenga U, Mita DG (2001). Urease immobilisation on chemically grafted nylon membranes part 1: isothermal characterisation. J. Mol. Catal. B: Enzymatic, 14: 15-19.
Gacesa P, Hubble J (1987). Enzyme technology. Oxford: Alden Press, 45-54.
Gursel I, Arıca MY, Hasırcı V (1997). Immobization of Glucose Oxidase and Urease in Hydrogel Matrices. Trends J. Chem., 21: 387-93.
Hearn E, Neufeld RJ (2000). Poly(methylene-co-guanidine) Coated Alginate as an Encapsulation Matrix for Urease. Process Biochem., 35: 1253-1260.
Iamskov IA, Budanov M (1979). Coordinate Immobilization of Enzymes, Bonding of Penicillinamidohydrolases by Cupric Complexes. Bioorg. Chem., 5: 757-765.
Kvesitadze GI, Dvali MSh (1982). Immobilization of Mold and Bacterial Amylases on Silica Carriers. Biotechnol. Bioeng., 24: 1765-1772.
Kvesitadze GI, Gvalia TSh, Svinidze RS, Toxadze ZV, Nichubidze NN (1977). Immobilization of Glucoamylase Aspergillus onto Anorganic Carriers. Bioorg. Chem., 3: 836-841.
Kramer DM, Lehmann K, Pennewiss M, Plainer H (1975). Photo-Beads and Oxirane Beads as Solid Supports for Catalysis and Biospecific Adsorption. In: Peeters H, Editor, Protides of Biological Fluids 23th Colloquium, Pergamon Press, Oxford, pp. 505-511.

Lowry O, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein Measurement with the Folin Phenol Reagent, J. Biol. Chem., 193: 263-276.

Marzadori C, Miletti S, Gessa C, Ciurli S (1998). Immobilization of Jack Bean Urease on Hydroxyapatite: Urease Immobilization in Alkaline Soils. Soil Biol. Biochem., 30: 1485-1490.

Raghunath K, Panduranga RK, Thomas JK (1984). Preparation and characterization of urease immobilized onto collagen-*GMA* graft copolymer. Biotechnol. Bioeng. 26: 104-111.

Srinivasa RM, Chellapandian M, Krishnan MRV (1995). Immobilization of urease on gelatin poly (HEMA) copolymer preparation and characterization Biopro. Bioeng.13: 211-214.

Senel S, Say R, Arica MY, Denizli A (2001). Cibacron Blue F3GA and Cu(II) Derived Poly(2-hydroxyethylmetacylate) Membranes for Lysozyme Adsorption. Colloids Surf. A: Physicochem. Eng. Aspects., 182: 161-173.

Slobodianikova LS, Latov VK, Belikov VM (1979). Immobilization of Protease Bacillus Subtilis onto Copolymere Stirene and Maleic Anhydriteusing Covalent and Coordinate Bonds. J. Appl. Biochem. Microbiol., 15: 262-270.

Teresa Martin M, Francisco JP, Miguel Alcalde AB (2003). Immobilization on Eupergit C of Cyclodextrin Glucosyltransferase (CGTase) and Properties of the Immobilized Biocatalyst. Journal of Molecular Catalysis B: Enzymatic, 21: 299-308.