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Investigation of Yeast Invertase Immobilization onto Cupric Ion-Chelated, Porous, and Biocompatible Poly(Hydroxyethyl Methacrylate-*n*-Vinyl Imidazole) Microspheres

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Abstract Cupric ion-chelated poly(hydroxyethyl methacrylate-*n*-vinyl imidazole) (poly (HEMA-VIM)) microspheres prepared by suspension polymerization were investigated as a specific adsorbent for immobilization of yeast invertase in a batch system. They were characterized by scanning electron microscopy, surface area, and pore size measurements. They have spherical shape and porous structure. The specific surface area of the p(HEMA-VIM) spheres was found to be 81.2 m^2/g with a size range of 70–120 μm in diameter, and the swelling ratio was 86.9%. Then, Cu(II) ion chelated on the microspheres (546 μmol Cu (II)/g), and they were used in the invertase adsorption. Maximum invertase adsorption was 51.2 mg/g at pH 4.5. Cu(II) chelation increases the tendency from Freundlich-type to Langmuir-type adsorption model. The optimum activity for both free and adsorbed invertase was observed at pH 4.5. The optimum temperature for the poly(HEMA-VIM)/Cu (II)-invertase system was found to be at 55 °C, 10 °C higher than that of the free enzyme at 45 °C. V_{max} values were determined as 342 and 304 U/mg enzyme, for free and adsorbed invertase, respectively. K_m values were found to be same for free and adsorbed invertase (20 mM). Thermal and pH stability and reusability of invertase increased with immobilization.

Keywords Enzyme immobilization · Invertase · Poly(HEMA-VIM) microsphere · Metal chelating · Cu(II) chelation · *n*-Vinyl imidazole

Introduction

Invertase (β-D-fructofuranosidase fructohydrolase, EC 3.2.1.26), a yeast-derived enzyme, is one of the most important enzymes used in industry and can be considered as an ideal enzyme for mainly use in the food (especially confectionery) field. This is used for the manufacture of artificial honey, as plasticizing agent in cosmetics, pharmaceutical, and paper industries as well as enzyme electrodes for the detection of sucrose and one of the

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important applications of invertase lies in the production of noncrystallizable sugar syrup from sucrose. Invertase is also used whenever sucrose containing substrates are subjected to fermentation viz. production of alcoholic beverages, lactic acid, glycerol, etc. Due to the associated inulinase activity, it is also used for the hydrolysis of inulin (polyfructose) to fructose [1]. Invertase finds large-scale application especially in the production of truffles, creams, mints, marshmallow, invert syrup, and other fondants since preference of fructose over sucrose in the food industry. One of the main characteristics of invertase is highly efficient conversion of sucrose to glucose and fructose (invert syrup) [2]. Invertase improves shelf life of confections and has large usability because of the fructose that is widely used as sweeter and crystallizes less readily than sucrose. The hydrolyzed sugar mixture obtained by invertase has the advantage of being colorless in contrast to the colored products obtained by acid hydrolysis, and the applications of hydrolyzed sugar include the brewing industry, in jams since recovery of scrap confectionery [3, 4]. Besides the several advantages, the use of invertase is rather limited because another enzyme, glucose isomerase, can be used to convert glucose to fructose much cheaper. The use of invertase is more preferable for health and taste reasons, and its use in food industry requires that invertase be highly purified.

From the scientific and commercial point of view, purification, separation, and reuse of enzymes have great importance for both scientific treatment and large-scale industrial applications that have been accelerated by rapid developments in biotechnology and genetic engineering [5]. In this regard, the immobilization of enzymes offers several advantages including improvement in enzyme stability and continuous operation in reactors [6, 7]. The enzyme immobilized onto insoluble polymeric supports can be repeatedly used, the products can be easily isolated, and the immobilization onto polymeric matrices is a very effective way to stabilize enzymes against several forms of denaturation. Also, it is cost-effective to use them more than once because of they are not changed during the reactions [8]. Immobilization techniques are becoming increasingly popular especially for these reasons in recent years. A large number of enzymes were successfully immobilized with very high activity yields on appropriate supports, and many methods of enzyme immobilization have been reported in the literature, such as entrapment [9], covalent attachment [10], and adsorption [11, 12]. Among the immobilization methods, adsorption to a solid support material is the most general, easiest to perform, and oldest protocol of physical immobilization methods and may have a higher commercial potential than other methods [13]. Immobilization of enzymes onto polymeric support materials via adsorption has been widely used in biotechnological applications. Hence, invertase should be immobilized for effective use, and the use of immobilized invertase is an attractive option because the re-utilization and the increase in stability of the catalyst can often lead to the cost reduction of the process. Immobilization of invertase on corn grits, gelatin, and various agarose polymers has already been achieved, while its immobilization onto hydrogel polymer has been limited. The latter have good chemical properties and mechanical stability and are not susceptible to microbial attack [14–16].

Note that one of the main criteria for enzyme adsorption is the structure of the polymeric solid support because the specific applications of the supports are closely related to their physical and chemical characteristics such as particle diameter, porosity, toxicity, hydrophilicity, hydrophobicity, and biocompatability. An optimal water-insoluble polymeric support material should provide large surface area/unit volume such as microspheres for effective adsorption of the desired amount of biomolecule, should allow transport with the least diffusional restriction, and also should be easily available and nontoxic [12].

Generally, natural or synthetic polymer-based microspheres are extensively used in biochemical and biotechnological processes in recent years. For example, Soppimath et al. prepared the cellulose-based matrix microspheres and used for encapsulation of antihypertensive drugs viz., nifedipine and verapamil hydrochloride [17]. Encapsulation and controlled release of nifedipine was also achieved by Soppimath and Aminabhavi using biodegradable and tissue-compatible poly(DL-lactide-co-glycolide) microspheres [18]. Similarly, cellulose acetate microspheres were prepared for the release of ibuprofen drug [19]. Moreover, Kulkarni et al. prepared sodium alginate interpenetrating network beads and studied with it for the easy encapsulation and in vitro release of cefadroxil, an antibiotic used in the treatment of bacterial infections [20]. As a synthetic polymer matrix, hydrophilic, nontoxic, and biocompatible poly(hydroxyethyl methacrylate) (poly (HEMA))-based solid supports are among the major synthetic polymers approved by federal agencies such as American Food and Drug Administration for biomedical, pharmaceutical, and industrial applications [21, 22]. Especially, this polymer-based microspheres have adequate mechanical strength for most biotechnological applications, have higher capacity to bind large enzymes with a suitable specific functional group on the surface due to larger surface-to-volume ratios, and they can be considered as attractive enzyme carriers. The properties of these supports, have hydroxyl groups act as attachment sites for bioactive species, can be modified with a wide variety of functional groups including enzymes, coenzymes, cofactors, antibodies, amino acids, proteins, nucleic acids, monomers, organic dyes, and metal ions using copolymerization, incorporation, grafting, chelation, and the other activation methods to gain specificity [23].

Adsorption of biomolecules onto metal-chelated polymeric support has become a widespread analytical and preparative separation method for therapeutic proteins, peptides, nucleic acids, hormones, and enzymes [24–26]. This immobilization method introduces a new approach for selectively interacting materials on the basis of their affinities for chelated metal ions. Many first-row transition metals (Zn^{2+}, Ni^{2+}, Cu^{2+}, and Fe^{3+}) can form stable complexes with electron-rich compounds and may coordinate molecules containing O, N, and S by ion–dipole interactions [27]. The separation is based on the interaction of a Lewis acid (electron pair donor), i.e., a chelated metal ion, with an electron acceptor group on the surface of the enzyme [28–30].

In this study, the aim was to investigate the possibility of immobilization of yeast invertase using a new polymeric solid support with a significantly higher capacity and specificity and reach to the higher activity, stability, and reusability for the invertase immobilized on these microspheres. Micron-sized poly(hydroxyethyl methacrylate-*n*-vinyl imidazole) (poly(HEMA-VIM)) microspheres are proposed as a new model polymeric support in order to achieve this aim. The microspheres were produced by suspension polymerization of HEMA and VIM. They were chelated with Cu(II) ions to reach the higher specificity for invertase adsorption, and then, they have been employed in the immobilization experiments. This approach for the production and modification of enzyme carrier has several advantages over conventional immobilization methods. An expensive, time-consuming, and critical step in the preparation of immobilized metal affinity carrier is coupling of a chelating ligand to the adsorption matrix. Comonomer VIM acted as metal-chelating ligand, and it is possible to load metal ions directly on the microspheres without further activation and ligand immobilization steps. Cu(II) chelating was achieved by the adding poly(HEMA-VIM) microspheres to the aqueous solutions of Cu(II) ions. This ion coordinates to the chelating ligand (VIM), and the enzyme binds the microsphere via the chelated metal ion. In the present work, prepared poly(HEMA-VIM) microspheres were

characterized; their enzyme adsorption capacities, storage stabilities, enzymatic activities, and reusabilities were analyzed; effects of external stimuli such as pH and temperature were investigated; and results were compared to that of free counterpart.

Experimental

Materials

HEMA, ethylene glycol dimethacrylate (EGDMA), and *N*,*N*′-azobisisobutyronitrile (AIBN) were purchased from Fluka A.G. (Buchs, Switzerland), and HEMA was distilled under reduced pressure in the presence of hydroquinone and stored at 4 °C until use. VIM was supplied from Aldrich (Steinheim, Germany), and it was diluted under vacuum (74–76 °C, 10 mmHg). Invertase (β-D-fructofuranosidase fructohydrolase, EC 3.2.1.26, grade VII from baker's yeast), was purchased from Sigma (St. Louis, MO, USA) and used as received. Poly(vinyl alcohol) (PVAL; M_w=100,000, 98% hydrolyzed) was supplied from Aldrich Chemicals Company (USA). All other chemicals were of reagent grade and purchased from Merck A.G. (Darmstadt, Germany). All water used in the metal chelation experiments was purified using a Barnstead (Dubuque, IA, USA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed bed system.

Methods

Preparation of Poly(HEMA-VIM) Microspheres and Cu(II) Chelation

In order to produce poly(HEMA-VIM) microspheres, HEMA (base monomer) and VIM (functional monomer) were copolymerized by suspension polymerization in the presence of EGDMA, AIBN, and PVAL as the cross-linker, initiator, and the stabilizer, respectively. Continuous medium was prepared by dissolving PVAL (200 mg) in the purified water (50 ml). Toluene was included in the polymerization recipe as the diluent (as a poreformer). For the preparation of dispersion phase, HEMA (6 ml) and toluene (12 ml) were stirred magnetically at 250 rpm for 15 min at room temperature. Then, VIM (3 ml), EGDMA (6 ml), and AIBN (100 mg) were dissolved in the homogeneous organic phase. The organic phase was dispersed in the aqueous medium by stirring the mixture magnetically (400 rpm) in a sealed pyrex polymerization reactor. The reactor content was heated to polymerization temperature (i.e., 70 °C) within 4 h, and the polymerization was conducted for 2 h with a 600-rpm stirring rate at 90 °C. Final microspheres were extensively washed with ethanol and water to remove any unreacted monomer or diluent and then stored in distilled water at 4 °C.

Chelates of Cu(II) ions with poly(HEMA-VIM) microsphere were prepared as follows: 1.0 g of the microspheres was mixed with 50 ml of aqueous solutions containing 50 ppm Cu(II) ions, at constant pH of 5.0 (adjusted with HCl and NaOH), which was the optimum pH for Cu(II) chelate formation at room temperature. A 1-mg/ml atomic absorption standard solution (containing 10% HNO_3) was used as the source of Cu(II) ions. The flask was stirred magnetically at 100 rpm for 1 h (sufficient to reach equilibrium). The concentration of the Cu(II) ions in the resulting solution was determined with a graphite furnace atomic absorption spectrometer (Analyst 800/Perkin-Elmer, USA). The amount of adsorbed Cu(II) ions was calculated by using the concentrations of the ions in the initial

solution and in the equilibrium. Cu(II) leakage from the poly(HEMA-VIM) microspheres was investigated with media pH (3.0–7.0) and also in a medium containing 1.0 M NaCl. The microsphere suspensions were stirred 24 h at room temperature. Cu(II) ion concentration was then determined in the supernatants using an atomic absorption spectrophotometer. It should be also noted that metal-chelated microspheres were stored at 4 °C in the 10 mM Tris–HCl buffer (pH 7.4).

Invertase Adsorption Studies

Invertase adsorption of the Cu(II)-chelated poly(HEMA-VIM) microspheres was studied at various pH values, either in acetate buffer (0.1 M, pH 3.0–5.5) or in phosphate buffer (0.1 M, pH 6.0–8.0). Initial invertase concentration was 1.0 mg/ml. The adsorption experiments were conducted for 4 h at 25 °C while stirring continuously. At the end of this period, the enzyme adsorbed microspheres were removed from the enzyme solution, and they were washed with the same buffer three times. The microspheres were stored at 4 °C in fresh buffer until use. Invertase concentrations were determined using absorbance at 280 nm before and after adsorption processes. The amount of adsorbed invertase was calculated using the following equation:

$$Q = [(C_o - C)V]/m \tag{1}$$

Here, Q is the amount of adsorbed invertase onto unit mass of the microspheres (milligrams per gram), C_o and C are the concentrations of invertase in the initial solution and in the aqueous phase after treatment for certain period of time, respectively (milligrams per milliliter), V is the volume of the aqueous phase (milliliters), and m is the mass of the microspheres used (grams). In order to obtain adsorption capacities of cupric ion-chelated microspheres (shown as poly(HEMA-VIM)/Cu(II)), the concentration of invertase in the medium was varied in the range 0.5–3.5 mg/ml.

Activity Assays of Free and Adsorbed Invertase

The kinetic parameters, K_m and V_{max} constants, were determined by measuring initial reaction rates at optimum conditions (pH 5.0, 35 °C) for adsorbed invertase and free invertase with sucrose in acetate buffer. For this purpose, 0.6 mg free or adsorbed invertase was added to 50 ml sucrose solution of different concentrations between 30 and 300 mM, and initial activities were determined as described below. The activities of both the free and the adsorbed invertase preparations were determined by measuring the amount of glucose liberated from the invertase-catalyzed hydrolysis of sucrose/unit time. In the determination of the activity of the free enzyme, the reaction medium consisted of acetate buffer (2.5 ml, 50 mM, pH 5.0) and sucrose (0.1 ml, 300 mM). Following a pre-incubation period (5 min at 35 °C), the assay was started by the addition of the enzyme solution (0.1 ml, 10 mg/ml), and incubation was continued for 5 min. In order to terminate the enzymatic reaction, the medium was then placed in a boiling water bath for 5 min. The same assay medium was used to determine the activity of the adsorbed enzyme. The enzymatic reaction was started by the introduction of 0.5 g of metal-chelated microspheres into the assay medium (10 ml) and was carried out at 35 °C with shaking in a water bath. After 15 min, there action was terminated by removal of the microspheres from the reaction mixture.

Sucrose hydrolysis performances of the free and adsorbed enzyme preparations were determined by measuring the glucose content of the medium. Assay mixture contained

glucose oxidase (25 mg), peroxidase (6 mg), and *o*-dianisidine (13.2 mg) in phosphate buffer (100 ml, 0.1 M, pH 7.0). An aliquot (2.5 ml) of enzymatically hydrolyzed sample was mixed and then incubated in a water bath at 35 °C for 30 min. After addition of sulfuric acid solution (1.5 ml, 30%), absorbance was measured by a UV–vis spectrophotometer (Shimadzu, Model 1601, Tokyo, Japan) at 530 nm. The activity of the adsorbed invertase preparations was presented as relative activity related to the activity of free enzyme within same quantity.

Determination of the Kinetic Constants

The kinetic parameters K_m and V_{max} were determined by measuring initial reaction rates at different conditions given above for adsorbed and free invertase with sucrose. For this purpose, free invertase (0.6 mg) or invertase adsorbed microspheres were added to 50 ml sucrose solution of different concentrations between 25 and 250 mM, and initial activities were determined as described above. K_m and V_{max} values were calculated from Lineweaver–Burk plots.

Incubation Period and Substrate Specificity

The incubation period of free and adsorbed enzyme was determined by measuring the residual activity of the enzyme exposed to three different time (in a range 1–5 min) at the same enzyme assay conditions. The specificity of enzyme activity was investigated by exposing the enzyme to other disaccharides, maltose and lactose. In place of sucrose solution in assay procedure, same amount of disaccharide was used as substrate.

Storage and Thermal Stability

The activity of free and immobilized invertase in acetate buffer (50 mM, pH 5.0) was measured in a batch-operation mode at 4 °C under the experimental conditions given above. Thermal stability studies of the free and the adsorbed invertase were carried out by measuring the residual activity of the enzyme exposed to three different temperatures (50–70 °C) in acetate buffer (50 mM, pH 5.0). After every 10-min time interval, a sample was removed and assayed for enzymatic activity as described above.

Results and Discussion

Characterization of Poly(HEMA-VIM) Microspheres

In this study, it was focused on the preparation of metal chelate affinity sorbents for invertase adsorption from aqueous solutions in a batch system. Poly(HEMA-VIM) microspheres were selected for this purpose and used as solid carrier. HEMA is a hydrophilic monomer, and the purpose of using it as a base material for adsorbent is to increase the hydrophilicity of the porous polymer supports, thus encouraging the immobilization of invertase onto microspheres. Also, HEMA is nontoxic and biocompatible as mentioned above. The simple incorporation of water weakens the secondary bonds with the hydrophilic structure, and this increases the distance between the polymer chains and causes the uptake of water. Comonomer VIM has the ability to form chelate with Cu(II) specifically. The specific interactions of proteins with attached metal ions onto solid

supports are strongly dependent on the changes in protein surface topography and chemical and physical variable parameters. The metal-chelated poly(HEMA-VIM) microspheres were extensively characterized to obtain water content, scanning electron microscopy (SEM), and Fourier transform infrared spectroscopy (FTIR) measurements. Cross-linked poly(HEMA-VIM) microspheres are insoluble in water and have sufficient mechanical and thermal stability. The equilibrium water content of the microspheres was found to be 86.9%. Poly(HEMA-VIM) microspheres are hydrophilic networks capable of imbibing large amount of water penetrate into entanglement polymer chains more easily, yet remain insoluble and protect their three-dimensional shape. This increases the water uptake capacity of polymeric microspheres.

SEM micrographs, present surface morphology and internal structure of the poly (HEMA-VIM) microspheres, are illustrated in Fig. 1. The microspheres had a spherical geometry and a particle size distribution in the range of 70–120 μm. Figure 1a shows that the microspheres were spherical and had a rough surface due to the pores which formed during the polymerization procedure. Figure 1b was taken with broken spheres so that it could be observed in the internal part. The presence of micropores within the microspheres surface can be clearly seen in this photograph. It can be concluded that the poly(HEMA-VIM) microspheres have a porous interior surrounded by a reasonably rough surface in the dry state. The roughness of the surface and pores having varying dimensions should be considered as a factor providing an increase in the surface area. In addition, these pores reduce diffusional resistance and facilitate mass transfer because of high internal surface area and diffusion to interior of the microsphere increases. Thus, cupric ion and invertase enzyme would be interacted both at the external surface of the microspheres and within the pore space. This factor also provided higher cupric ion chelation and increased the invertase adsorption capacity. Experimental results correspond to these previsions. The amount of chelated Cu(II) ion onto microspheres was observed as 546 μmol Cu(II)/g microsphere. The Cu(II) chelation step is depicted in Fig. 2. Also, average pore diameter of the microspheres was found to be 640 nm using mercury porosimetry, and the size of elliptic shaped invertase was obtained as 9.4×11.3×12.9 nm from the literature [31]. According to these data, it can be considered that poly(HEMA-VIM) microspheres have suitable pore structure for diffusion of invertase.

FTIR spectra were undertaken to determine the structures of poly(HEMA) (a) and poly (HEMA-VIM) (b) microspheres. Figure 3a shows the hydroxyl group at 2,956 cm^{-1},

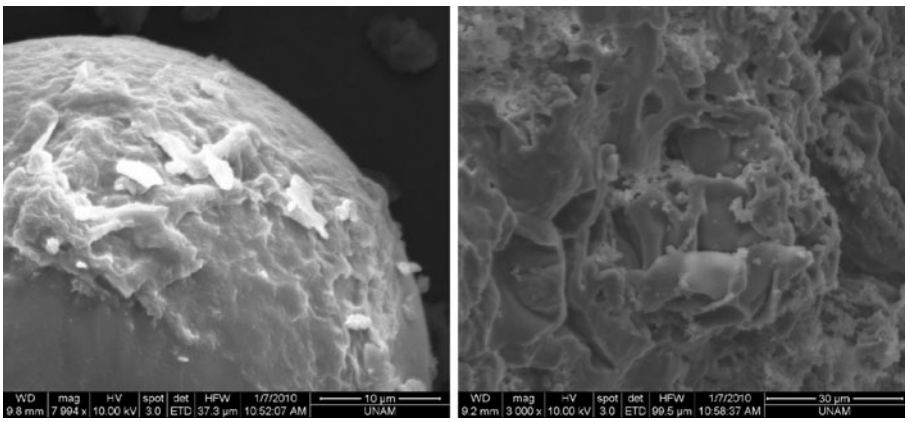

Fig. 1 SEM micrographs of poly(HEMA-VIM) microspheres: **a** surface and **b** cross section

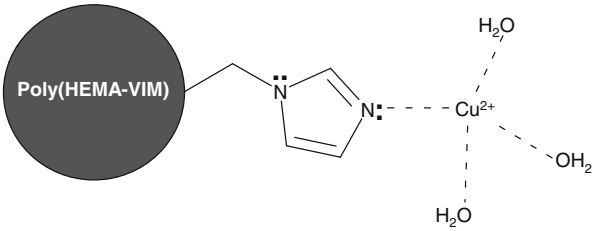

Fig. 2 The chelation of Cu(II) ions through VIM, as functional group of the microsphere

carbonyl at 1,729 cm^{-1}, and ester stretchings (C–O and C–O–C) at 1,263 and 1,162 cm^{-1}, respectively. Poly(HEMA-VIM) microspheres' more bands stemmed from VIM monomer. The bands can be listed as C–H imidazole ring stretching at 3,118 cm^{-1}; C–C and C=N ring stretching at 1,502 cm^{-1}; C–H ring bending (in-plane); and C=N ring stretching at 1,320, 1,110, 1,080, and 918 cm^{-1}, respectively. In addition, according to changes in chemical environment of main polymeric backbone of poly(HEMA), characteristic bands shifted approximately ±10 cm^{-1}, respectively. Under light of the FTIR results, we can say that the synthesis of poly(HEMA) and poly(HEMA-VIM) microspheres were achieved.

Immobilization of Invertase onto Cu(II)-Chelated Poly(HEMA-VIM) Microspheres

Although mechanism of metal chelate affinity with protein has been proposed [32], the macromolecular recognition of protein with attached metal ions obviously remains unclear. In one proposed mechanism, the formation of a coordinated complex between protein and attached metal ion is considered to be the major binding mode. However, more than one type of interaction mechanism is operational [33, 34]. Factors influencing the interactions include the number of functional group on the protein surface, pH value of the interaction, salt type, concentration, temperature, type of metal ion, ligand density, and type of chelating agent [35]. Vinyl imidazole was used as the Cu(II) chelating agent for specific binding of invertase. The unmodified and Cu(II)-chelated poly(HEMA-VIM) microspheres were incubated with the invertase solutions for about 7 h (420 min) at room temperature in the dark. Figure 4 gives the equilibrium adsorption time curves which were obtained by following the decrease of the concentration of invertase within the samples with time. As seen here, the amount of adsorbed invertase onto poly(HEMA) microspheres can be

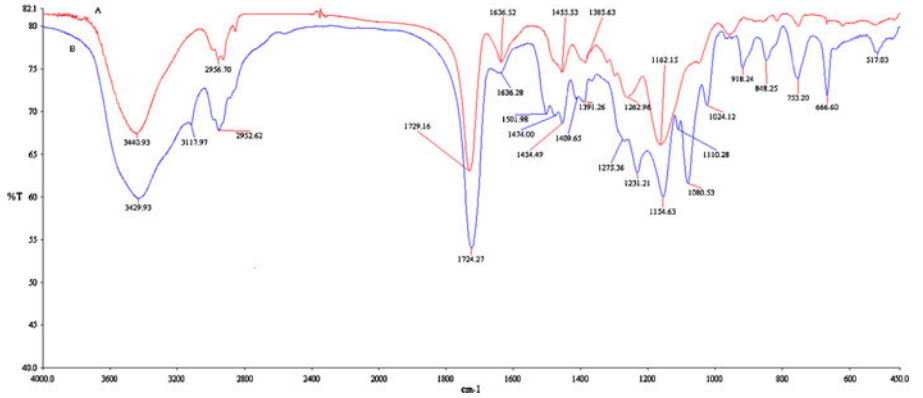

Fig. 3 FTIR spectra of poly(HEMA) (**a**) and poly(HEMA-VIM) (**b**) microspheres

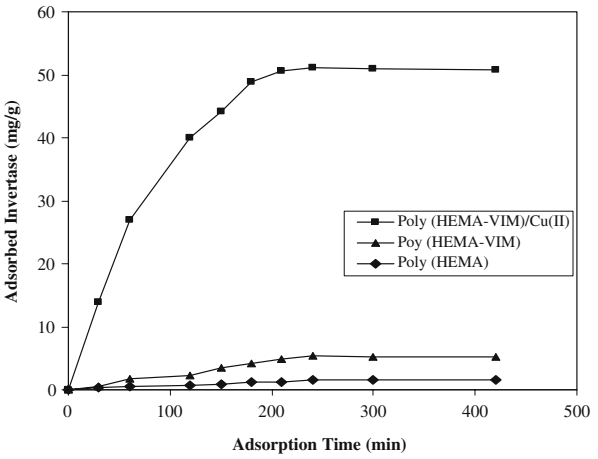

Fig. 4 Equilibrium adsorption times of invertase. Invertase concentration, 2.5 mg/ml; *T*, 25 °C; pH 4.5

negligible. Notice that there was a very low nonspecific invertase adsorption onto the unmodified poly(HEMA-VIM) microspheres (maximum capacity is 5.4 mg invertase/g microsphere). The adsorption may be due to diffusion of invertase to the pores and weak interactions between invertase molecules and the imidazole groups of microspheres. On the other hand, much higher adsorption capacities (up to 51.2 mg invertase/g microsphere) were obtained when the poly(HEMA-VIM)/Cu(II) microspheres were used. There were relatively faster adsorption rates observed at the beginning, and then adsorption equilibria were achieved gradually in about 4 h (240 min). This may be due to a high driving force, which is the invertase concentration difference between the liquid (i.e., the aqueous solution) and the solid (i.e., the microspheres) phases, in the case of high invertase concentration. Also, specific binding of invertase with Cu(II) ion increased the adsorption capacity.

In order to determine the effect of initial invertase concentration on the invertase adsorption of the adsorbent, the initial invertase concentrations were changed in range of 0.0–3.5 mg/ml (Fig. 5). The initial concentration of invertase was increased up to 3.5 mg/ml in order to reach the plateau values which represent saturation of the active sites on the adsorbent, in other terms to obtain the maximum adsorption capacity. The increases in

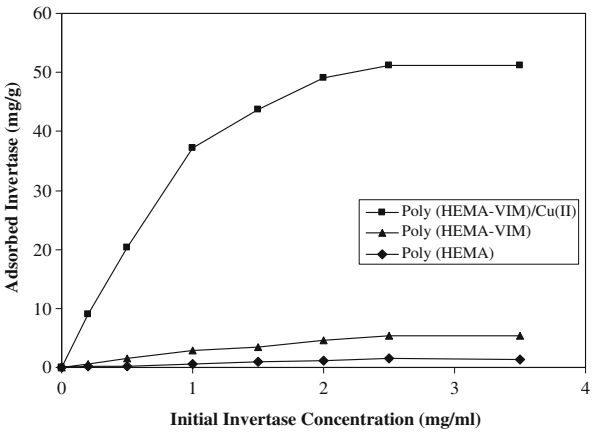

Fig. 5 Effect of initial concentration on invertase adsorption. *T*, 25 °C; pH 4.5

invertase concentration led to a significant increase in adsorption efficiency, but this became constant at an enzyme concentration after 2.5 mg/ml and then approached saturation. There was, thus, a level in the maximum adsorption capacity for the adsorbents. Cu(II) chelation significantly increased the adsorption capacity of the microspheres. It is clear that this increase is due to specific interactions between Cu(II) ions and invertase molecules. The step slope of the initial part of the adsorption isotherms represents a high affinity between invertase and metal-chelated microspheres. This could be considered as a typical example of the occupation of all binding sites on the adsorbent surface which are available for the invertase adsorption. The high invertase concentration may also contribute to this high adsorption rate due to the high driving force between the solution and poly(HEMA-VIM) solid phases. Thus, invertase adsorption is favored at a higher initial concentration. Invertase concentration of 1 mg/ml is approximately in the middle of the initial part of the adsorption curve where initial concentration–adsorption relationship can be easily observed. Hence, the solutions having this concentration were used in the next experiments.

The effect of pH on the adsorption of invertase onto poly(HEMA-VIM)/Cu(II) microspheres was studied in the pH range of 3.0–8.0 (Fig. 6). The maximum adsorption was obtained at pH 4.5. This may be due to specific interactions resulted from both Cu(II)-chelated vinyl imidazole groups on adsorbent and amino acid side chains of the invertase and from the conformational state of invertase at this pH. At pH values lower and higher than pH 4.5, the adsorbed amount of invertase drastically decreased. Obtaining lower adsorption in more acidic and more alkaline pH regions could have been due to the ionization state of invertase. The decrease in the invertase adsorption capacity in these regions can also be attributed to electrostatic repulsion effects between the opposite charged groups. An increase in the conformational size and lateral electrostatic repulsions between adjacent adsorbed invertase molecules may also have caused a decrease in the adsorption efficiency. Proteins have no net charge at their isoelectric points, and therefore, the maximum adsorption from aqueous solutions is usually observed at about their isoelectric points. The isoelectric pH of invertase is 4.7. In the present study, the maximum adsorption was not at this pH and had slightly shifted toward more acidic pH values. This could be due to preferential interactions between invertase molecules and Cu(II)-chelated microspheres at acidic pH.

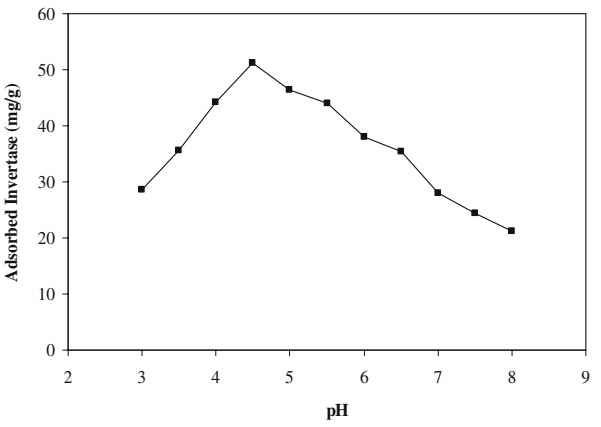

Fig. 6 Effect of pH on invertase adsorption onto poly(HEMA-VIM)/Cu(II) microspheres. Invertase concentration, 1 mg/ml; *T*, 25 °C

Kinetic Constants

Analysis of equilibrium data is important for developing an equation that can be used to design a process. The Langmuir and Freundlich isotherms are represented by Eqs. 2 and 3, respectively.

$$C_e/Q_e = (1/Q_{max}) + [1/(Q_{max}b)](1/C_e) \quad (2)$$

$$\ln Q_e = 1/n(\ln C_e) + \ln K_F \quad (3)$$

where C_e is the equilibrium concentration of invertase molecules (milligrams per milliliter), Q_e is the equilibrium adsorption capacity of the microspheres (milligrams per gram), b is the Langmuir isotherm constant (milliliters per milligram), K_F is the Freundlich constant, and n is the Freundlich exponent. $1/n$ is a measure of the surface heterogeneity ranging between 0 and 1, becoming more heterogeneous as its value gets closer to zero.

Table 1 shows the isotherm parameters calculated from the results. In the presence of Cu (II) chelation, the correlation coefficient (R^2) is higher (0.9741 for Langmuir and 0.9268 for Freundlich) for Langmuir adsorption model, and the calculated Q_e value is close to the theoretical Q_{max} value. Equilibrium data also fitted very well to the Langmuir model which indicates that the invertase molecules adsorbed onto poly(HEMA-VIM)/Cu(II) microspheres via almost monolayer adsorption process. But the correlation coefficient (R^2) is higher (0.9737 for Freundlich and 0.9408 for Langmuir) for Freundlich adsorption model without metal chelation onto microspheres. This value shows multilayer adsorption, lateral interaction, and nonspecific binding for invertase. Hence, it can be concluded that Cu(II) chelation onto microspheres increased the adsorption tendency from Freundlich to Langmuir type. Also, this result contributed to the suggestion implies the specific binding of invertase with microspheres only via Cu(II) ions.

Kinetic parameters, the Michaelis constant (K_m), and the maximal initial rate of the reaction (V_{max}) for free and adsorbed invertase preparations were determined using sucrose as a substrate. For both free and adsorbed enzymes, K_m was found to be 25 mM, whereas V_{max} was calculated as 342 U/mg enzyme for free enzyme and 304 U/mg for adsorbed enzyme, respectively. The same K_m values of both enzyme preparations may be explained by no diffusional limitations imposed on the substrate. This also shows no decreasing in enzyme affinity and higher accessibility of substrate molecules to the active site of the adsorbed enzyme.

Influence of pH and Temperature on the Catalytic Activity

The effect of pH on the activity of both free and immobilized invertase onto poly(HEMA-VIM)/Cu(II) microspheres was determined in the pH values varied between 3.0 and 8.0

Table 1 Langmuir and Freundlich adsorption isotherm parameters

Microsphere	Langmuir					Freundlich	
	Q_e (mg/g)	Q_{max} (mg/g)	b (ml/mg)	R^2	K_F	$1/n$	R^2
Poly(HEMA-VIM)/Cu(II)	51.2	68.03	1.157	0.9741	32.34	0.5999	0.9268
Poly(HEMA-VIM)	5.4	10.26	0.356	0.9408	2.47	0.7730	0.9737

(Fig. 7). It is well-known that the pH plays a relevant role on enzyme activity. This role is more clearly evidenced when the activities of free and immobilized enzymes are compared. The optimum pH of immobilized enzyme may be different from that of the free one because of the nonuniform distribution of the hydrogen ions between the microenvironment of enzyme and the bulk solution [36]. This effect mostly occurs when the carrier contains ionizable groups [37, 38]. The adsorption process may also affect pH–activity profile of the immobilized enzyme. The maximum activity was found to be at pH 4.5 for free and immobilized forms. This optimum pH value was not changed with immobilization. This shows that invertase does not bind to Cu(II)-chelated microspheres from its active sites because of the optimum pH value and maximum activity of enzymes that are generally changed by binding of enzymes via their active sites onto microspheres. Furthermore, immobilized invertase showed a broader range in the optimum pH activity which may be due to charge effects of the microspheres. This result shows that the possible situations regarding pH effect were not significantly in this case and pH–activity curve was wider than that of free enzyme. This result was attributed to the fact that pH stability of immobilized invertase was better than that of free one.

Activity profile related to temperature of invertase was constructed by measuring activity at various temperatures altered between 20 °C and 70 °C (Fig. 8). The relative activities of both the free and the immobilized invertase as a function of temperature are reported in the figure, together with that of the free enzyme for comparison. From the experimental results, it can be considered that the enzyme activity was found to be dependent upon the temperature. The optimum temperature was found to be 45 °C and 55 °C for the free and immobilized enzyme, respectively. An increase in the relative activity was observed with the increasing temperature. The activity of free enzyme decreased at temperatures higher than 45 °C, probably due to thermal deactivation. But adsorbed invertase keeps its activity until 55 °C. This shift toward higher temperatures with adsorbed invertase could be explained by multipoint chelate interactions, which consequently leads to an increase in the activation energy of the enzyme to reorganize an optimum conformation for binding to its substrate. Thermal stability of immobilized enzyme found to be higher than that observed in free counterpart. Thus, immobilization of invertase onto poly(HEMA-VIM)/Cu(II) microspheres also enhances a significant improvement in the thermal stability and enables broader working temperature range for invertase.

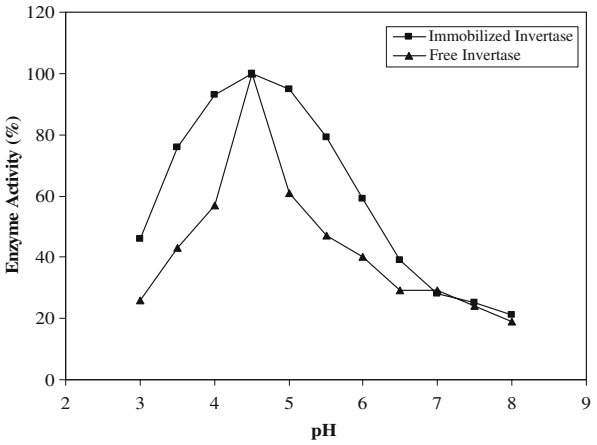

Fig. 7 Effect of pH on the catalytic activity. *T*, 25 °C

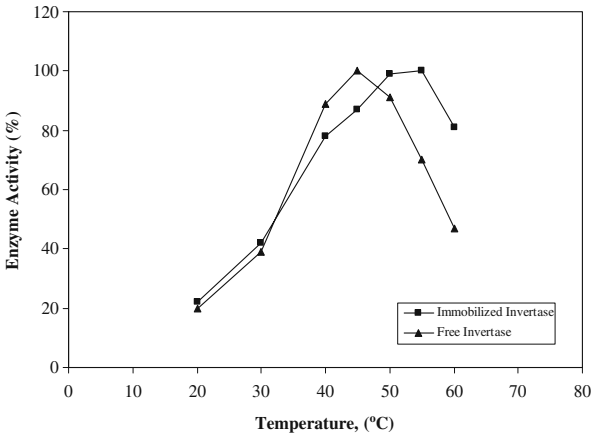

Fig. 8 Effect of temperature on the catalytic activity. pH 4.5

Substrate Specificity

One of the main parameters that affect the productivity of the enzyme is the specificity. To determine the specificity of the enzyme, the enzyme activity toward several saccharides as comparative substrates was investigated (Fig. 9). The activity on sucrose was accepted as 100% and compared to the activity of other substrates. As seen in figure, both enzyme preparations, free and adsorbed invertase, more specifically hydrolyzed sucrose molecules than maltose and lactose. Invertase showed high hydrolyzing activity on sucrose and very low activity lactose and maltose. Lactose and maltose were not split by the invertase. Also, hydrolyzing activities of adsorbed invertase on maltose and lactose were slightly higher than that of free counterpart. Enzymes have a long amino acid chain consist of several recognition sides for the substrates molecules. During the adsorption process, some conformational changes on invertase can be occurred [16]. Thus, the substrate specificity of the adsorbed enzyme was slightly changed.

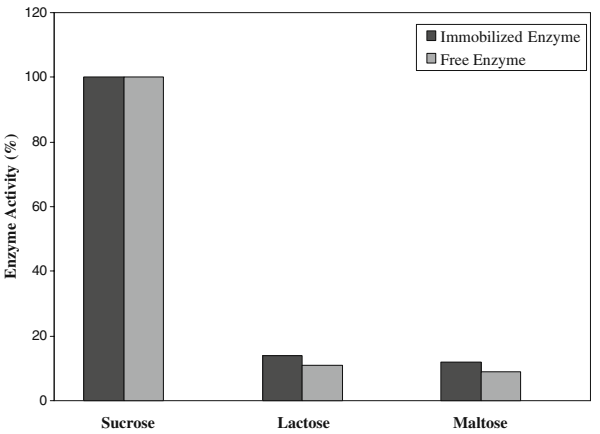

Fig. 9 Substrate specificity of free and immobilized invertase. *T*, 25 °C; pH 4.5

Storage Stability

Generally, enzymes are not stable during storage if they are in solution and their activities decrease with time. Especially, free enzymes can lose their activities so quickly. But immobilization also provides additional advantage for their practical application in biochemical studies as they prevent to gradually reducing the activity of enzymes in solutions. Figure 10 shows that storage period clearly increased with increasing stability of invertase with immobilization. Free and immobilized invertase preparations were stored in an acetate buffer (50 mM, pH 4.5) at 4 °C, and activity measurements were carried out for a period of 28 days for free enzyme and 60 days for immobilized enzyme. No enzyme release was observed. The free enzyme lost its all activity within 28 days. Invertase immobilized poly(HEMA-VIM)/Cu(II) microspheres retained 74% of its activity during the same period. This decrease in enzyme activity was explained as a time-dependent natural loss in enzyme activity, and this was prevented to a significant degree upon adsorption. The result readily indicates that the immobilized invertase exhibits an improved stability over that of the free enzyme. Of the immobilization methods, fixation of enzyme molecules on a surface often gives rise to the highest stabilization effect on enzyme activities because the active conformation of the immobilized enzyme is stabilized by multipoint bond formation between the substrate and the enzyme molecules [39].

Thermal Stability

Heat activity experiments related to temperature were carried out for determining thermal stability and heat inactivity of free and immobilized invertase. These experiments depend on the incubating of invertase in the absence of substrate at the various temperatures altered from 50 °C and 70 °C. Heat inactivation curves between this temperature range for free and immobilized invertase were presented in Fig. 11a, b respectively. At 50 °C, the adsorbed and free invertase retained their activity about to a level 96% and 72%, respectively. At 70 °C, the adsorbed enzyme retained their activity to a level 46% at the 35th min of incubation period whereas free enzyme loss all activity at the same time. The immobilized invertase showed remarkable stability to the free invertase. These results suggest that thermostability of immobilized invertase becomes significantly higher than that of the free counterpart at high temperatures. This is due to adsorbed invertase being protected from conformational changes

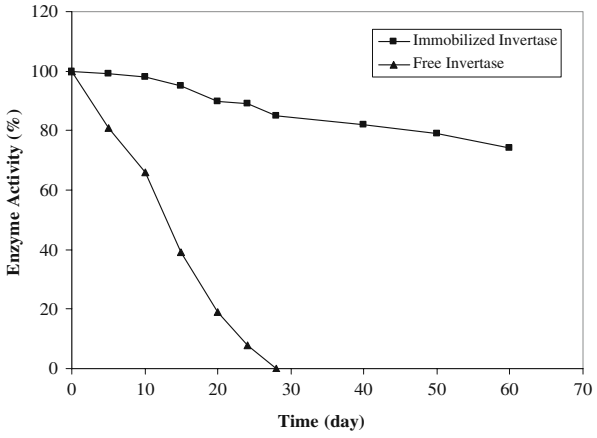

Fig. 10 Storage stability of free and immobilized invertase

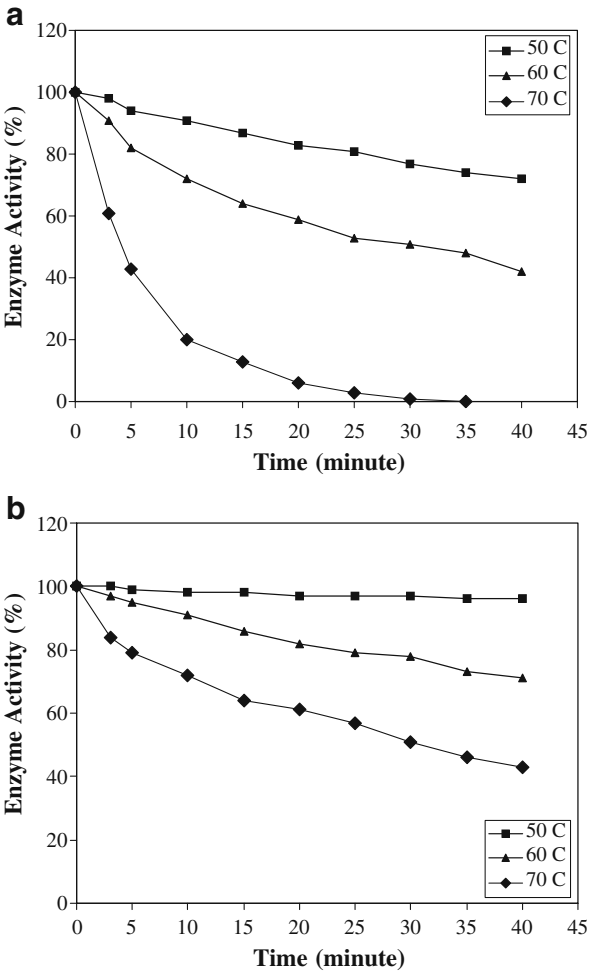

Fig. 11 Comparison of activity of free (**a**) and immobilized (**b**) invertase related to temperature

causing effect of the environment.

Repeated Use

It can be reported that the repeated usability gaining a unique property and immobilization enables this important advantage to enzymes. The reuse of adsorbents without any detectable loss of metal-chelating properties is the attractive features of metal affinity separation [27]. Desorption of invertase from Cu(II)-chelated poly(HEMA-VIM) microspheres was studied in a batch system, and these invertase adsorbed microspheres were placed within the desorption medium containing 25 ml 1.0 M NaCl at room temperature for 3 h. In order to show reusability of poly(HEMA-VIM)/Cu(II) microspheres, adsorption–desorption cycles was repeated ten times using the same microsphere sample to determine the change in invertase adsorption capacity, and results were showed in Fig. 12. As is seen from the figure, it was observed that both the invertase adsorption capacity of microspheres and the activity of immobilized invertase were not significantly changed during the first

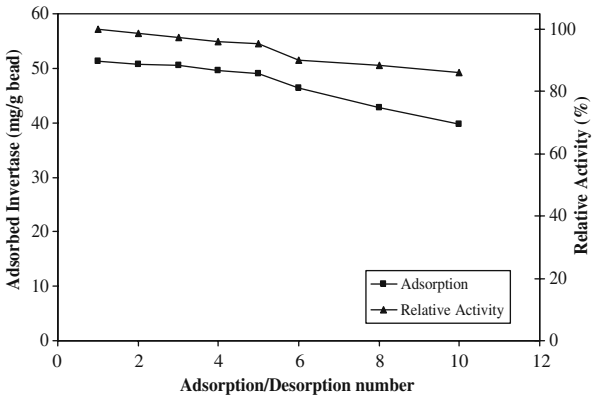

Fig. 12 Desorption and repeated use of immobilized invertase

five successive cycles. Then, both of them showed slightly decreasing. Immobilized invertase retained its activity of 95.3% and 86.0% at the end of the 5th and 10th cycle, respectively. These results showed that poly(HEMA-VIM)/Cu(II) microspheres can be repeatedly used at least ten times (quite satisfactory especially for the first five times) in enzyme immobilization, without detectable losses in their initial adsorption capacity and activities.

Conclusion

This study focused to investigate the potential and effective usability and to determine specific affinity of cupric ion-chelated poly(HEMA-VIM) microspheres for yeast invertase immobilization. It can be reported that reversible immobilization of invertase onto poly (HEMA-VIM)/Cu(II) microspheres was achieved specifically, and the obtained experimental results proved the existence of good adsorptive properties of these microspheres. They showed excellent thermal, storage, and operational stability, hydrophilicity, and reusability. Also, poly(HEMA-VIM)/Cu(II) microspheres has cost-effectiveness, high mechanical strength, and high biological and chemical stability, and they can be easily separated from reaction medium. It should be especially pointed out that one of the main objectives of this work was preparation and used of the highly porous microspheres. Besides the good chemical resistance, nontoxicity, and biocompatibility of hydroxyl ethyl methacrylate [21, 22], porosity is the important property, and this is the main reason for preference of hydroxyethyl methacrylate in this study since the process of biocatalyst immobilization in poly(hydroxyethyl methacrylate)-based materials generally allows good controlled porosity to ensure sufficient mass transfer of reactants and products [40]. Because surface area, directly proportional to the porosity of the microspheres, is the determinative characteristic for enzyme immobilization and increases the number of active binding site for both chelating metal ion and enzyme. The experimental results and data given in the previous literature correspond to this prevision. For example, Bahar and Tuncel studied the immobilization of invertase using nonporous poly(*p*-chloromethylstyrene) beads (surface area 14.1 m^2/g), and maximum immobilization capacity was obtained as 19.0 mg invertase/g bead [36]. Osman et al. achieved invertase immobilization with ethylene glycol dimethacrylate-based microbeads (surface area 59.8 m^2/g), and maximum invertase immobilization capacity was found to be 35.2 mg invertase/g bead [15]. On the other

hand, in this study, using hydroxyl ethyl methacrylate increased the surface area up to 81.2 m^2/g bead, and maximum immobilization capacity was found to be 51.2 mg/g. It should be especially added that although immobilization capacity can be comparable using surface area, the capacity is not only a function of wideness of the surface area, and it is resulted from the cumulative effects of both surface area and other parameters mentioned in the text. Also, *n*-vinyl imidazole monomer shows effective cupric ion chelating capacity, and this ion specifically interacts with active sites of invertase. Monolayer adsorption characteristic of invertase onto microspheres was very well fitted to Langmuir-type model. pH studies showed that the optimum pH value and maximum activity at this pH did not changed after immobilization, and invertase also remained significantly active over a broader pH range. This shows a suitable working opportunity at wider pH interval. It means that the immobilization was occurred far away from active site of invertase. The immobilized invertase kept its stability for longer time period and also at higher temperatures compared to the free invertase in solution. After ten operational cycles, it was observed that the enzyme retained lots of its initial activity of 86.0%. The immobilization studies of invertase reported here offers mild conditions used for the efficient biosensors and enzymatic reactors. Thus, in the light of the results, it can be concluded that Cu(II)-chelated poly(HEMA-VIM) microspheres can be used as a specific affinity sorbents for reversible invertase immobilization for biotechnological and industrial applications.

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