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Hydrolysis of sucrose by invertase immobilized onto novel magnetic polyvinylalcohol microspheres

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Abstract

The magnetic polyvinylalcohol (PVAL) microspheres were prepared by crosslinking glutaraldehyde. 1,1′-Carbonyldiimidazole (CDI), a carbonylating agent was used for the activation of hydroxyl groups of polyvinylalcohol, and invertase immobilized onto the magnetic PVAL microspheres by covalent bonding through the amino group. The retained activity of the immobilized invertase was 74%. Kinetic parameters were determined for immobilized invertase, as well as for the free enzyme. The K_m values for immobilized invertase (55 mM sucrose) were higher than that of the free enzyme (24 mM sucrose), whereas V_{max} values were smaller for the immobilized invertase. The optimum operational temperature was 5°C higher for immobilized enzyme than that of the free enzyme. The operational inactivation rate constant (k_{opi}) of the immobilized invertase at 35°C with 200 mM sucrose was 5.83×10^{-5} min^{-1}. Thermal and storage stabilities were found to increase with immobilization.

Keywords: Polyvinylalcohol; Magnetite; Microspheres; Covalent bonding; Enzyme immobilization; Invertase

1. Introduction

The immobilization of enzymes onto insoluble supports has been a topic of active research in enzyme technology and is essential for their application to industrial processes. A large number of enzymes were successfully immobilized with very high activity yields on appropriate supports. These immobilized products were intended for use in the construction of artificial organs, biosensors, or bioreactors. Immobilization is advantageous because (1) it extends the stability of the enzyme by protecting the active material from deactivation; (2) it enables repeated use; (3) it provides significant reduction in the operation costs; and (4) it facilitates easy separation and speeds up recovery of the enzyme. The availability of a large number of support materials and methods of immobilization leave virtually no enzyme without a feasible route of immobilization. It is, thus, important that the choice of support materials and immobilization method over the free enzyme should be well justified (Arıca, Hasırcı, & Alaeddinoğlu, 1995; Gordon, 1997; Peterson & Kennedy, 1997; Uhlich, Ulbricht, & Tomaschewski, 1996).

Polyvinylalcohol (PVAL) is a polymer that is frequently used as matrix for the immobilization of various enzymes and cells because of its easy availability, low price, hydrophilic character and hydroxyl groups on the surface capable of chemical reaction. In addition, its chemical structure can cause protein stabilization by attachment to the polymer chain or resemblance to e.g. polysaccharides known as stabilizing agents for proteins due to their impact onto the water structure (Arakawa & Timasheff, 1982; Uhlich et al. 1996).

Magnetic fields have been utilized in the support systems for the study of enzyme immobilization. Rotational and vibrational movements were observed in the alternating magnetic fields by polymeric microspheres exhibiting magnetically anisotropic properties. In a packed bed reactor, these magnetic phenomena could be used for preventing product film formation around the enzyme-magnetic microspheres by an alternating magnetic field. In such a system, the vibrating reaction medium around the support could facilitate substrate transfer through the surface of the enzyme-microspheres and this fact could also provide a key for controlling

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immobilized enzyme activity in a bioreactor. In addition, the magnetic support could be easily separated from the reaction medium and stabilized in a fluidized-bed reactor by applying a magnetic field. The use of magnetic microspheres could also reduce capital and operation costs (Arica, Yavuz, Patır, & Denizli, 2000b; Bahar & Çelebi, 2000; Pietes, Bardeletti, & Coulet, 1991).

Invertase is a highly efficient enzyme that has been described as specific for converting sucrose to glucose and fructose. The hydrolyzed sugar mixture obtained by invertase has the advantage of being colourless in contrast to the coloured products obtained by acid hydrolysis (Arruda & Vıtole, 1999; Monsan & Combes, 1984). Immobilization of invertase on corn grits, gelatine, and various agorose 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 (Arıca et al., 1995; Arıca, Şenel, Alaeddinoğlu, Patır, & Denizli, 2000; Arslan, Tümtürk, Caykara, Şen, & Güven, 2000).

Immobilization of enzymes through covalent attachment has also been demonstrated to induce higher resistance to temperature, denaturants, and organic solvents in several cases. The extent of these improvements may depend on other conditions of the system, i.e. the nature of the enzyme, the type of support, and the method of immobilization (Boy, Dominik, & Voss, 1999; Kotzelski & Staude, 1996; Laska, Wlodarczyk, & Zaborksa, 1999).

In this study, invertase was covalently immobilized onto new magnetic microspheres based on PVAL. The effects of the immobilization process on the enzyme activity, the kinetic parameters, operational, thermal and storage stabilities of the enzyme were investigated.

2. Materials and methods

2.1. Materials

Invertase (β-fructofuranosidase, EC 3.2.1.26, Grade VII from baker's yeast), glucose oxidase (GOD, EC 1.1.3.4. Type II from *Aspergillus niger*), peroxidase (POD, EC 1.11.1.7, Type II from horseradish), bovine serum albumin (BSA), *o*-dianisidine dihydrochloride, sucrose, glucose, magnetite (Fe_3O_4; diameter was less than 5 μm), Tween 20 and 1,1′-carbonyldiimidazole (CDI) were all obtained from the Sigma Chemical Company (USA) and used as received. PVAL (MW 50000) was obtained from Aldrich Chemical Co. (USA) and used as received. All other chemicals were of analytical grade and were purchased from Merck AG (Germany).

2.2. Preparation of magnetic PVAL microspheres

The magnetic PVAL microspheres were prepared by chemical cross-linking of PVAL. The dispersion medium was comprised of corn oil (80 ml) containing Tween 20 (20 mg ml^{-1}) and 1-butanol (20 ml). The medium was transferred into a 250-ml round-bottomed two-necked flask fitted with a glass paddle stirrer. The system was placed in a waterbath, heated to 50°C and stirred at 700 rpm. A 40-ml PVAL solution (10% w/v), containing 0.5 g magnetite (Fe_30_4), was introduced into the dispersion medium. After a 10-min equilibrium period, glutaraldehyde solution (1.0 ml, 25% w/v) and concentrated hydrochloric acid solution (0.6 ml, 37.5% w/v) were added to the final mixture. The cross-linking reaction was maintained at 50°C for 1 h. After the reaction, the formed magnetite PVAL microspheres were filtered under suction and were first washed with ethanol (50% v/v), then several times with hot and cold distilled water and dried in a vacuum oven at 25°C.

2.3. Activation of magnetic PVAL microspheres

The activation of hydroxyl groups of magnetic PVAL microspheres for covalent immobilization of enzyme was achieved by reaction with CDI. Magnetic PVAL microspheres (5 g) were immersed in CDI solution (10 ml acetone, 20 mg ml^{-1} CDI), and stirred at 25°C for 24 h. After removal by filtration, the microspheres were washed twice with acetone, dried under reduced pressure at 25°C and kept at 4°C until use.

2.4. Immobilization of invertase onto magnetic PVAL microspheres

The activated magnetic PVAL microspheres were equilibrated in phosphate buffer (50 mM, pH 7.0) and immersed in the same fresh medium containing invertase (2 mg ml^{-1}). The immobilization was carried out at 4°C for 24 h, while continuously stirring the medium. Ionically bound enzyme was removed first by washing with saline solution (10 ml, 0.5 M) and then with acetate buffer (50 mM, pH 5.5), and was stored at 4°C in fresh buffer until use.

2.5. Determination of immobilization efficiency

The amounts of protein in the enzyme solution and in the wash solutions were determined by using Coomassie Brilliant Blue, as described by Bradford (Bradford, 1976) with BSA as a standard. The amount of bound enzyme was calculated as:

$$q = [(C_i - C_f) \cdot V]/W \quad (1)$$

where q is the amount of bound enzyme onto magnetic PVAL microspheres (mg g^{-1}), C_i and C_f are the

concentrations of the enzyme (initial and final) in the reaction medium, respectively (mg ml^{-1}), V is the volume of the reaction medium (ml), W is the weight of the microspheres (g). All data used in this formula are averages of at least duplicated experiments.

2.6. Activity assays

The activities of both the free and the immobilized invertase preparations were determined by measuring the amount of glucose liberated from the invertase-catalyzed hydrolysis of sucrose per 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 5.5), 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^{-1}) and incubation was continued for 5 min. In order to terminate the enzymatic reaction, the reaction medium was then placed in a boiling water bath for 5 min.

The same assay medium was used for the determination of the activity of the immobilized enzyme. The enzymatic reaction was started by the introduction of ten disks into the assay medium (10 ml) and was carried out at 35°C with shaking in a water bath. After 15 min, the reaction was terminated by removal of the magnetic PVAL microspheres from the reaction mixture.

Sucrose hydrolysis performance of the free and immobilized preparation was determined by measuring the glucose content of the medium according to a method described previously (Arıca et al., 2000a). Assay mixture contained GOD (25 mg), POD (6.0 mg) and *o*-dianisidine (13.2 mg) in phosphate buffer (100 ml, 0.1 M, pH 7.0). An aliquot (2.5 ml) of assay mixture and 0.1 ml of enzymatically hydrolyzed sample were mixed and then incubated at 35°C for 30 min in a water bath. After addition of sulphuric acid (1.5 ml, 30%) absorbance was measured in a UV/Vis spectrophotometer (Shimadzu, Model 1601, Tokyo, Japan), at 525 nm.

The activities of the free and the immobilized invertase were expressed in μmol glucose min^{-1} mg^{-1} of enzyme and μmol glucose min^{-1} g^{-1} of enzyme-MPVAL microspheres, respectively.

To determine the pH and temperature profiles for the free and immobilized enzyme, activity assays were carried out over the pH range of 4.0–8.0 and temperature range of 20–60°C. The results of dependence of pH, temperature, storage and repeated use are presented in a normalized form with the highest value of each set being assigned the value of 100% activity.

2.7. Determination of the kinetic constants

K_m and V_{max} values of the free enzyme were determined by measuring initial rates of the reaction with sucrose (30–300 mM) in acetate buffer (50 mM, pH 5.5) at 35°C.

The kinetic parameters of immobilized invertase were determined in a batch system by varying the concentrations of sucrose (30–300 mM) in acetate buffer (50 mM, pH 5.5). The reaction temperature was 35°C. K_m and V_{max} were calculated from the data obtained after 15 min.

2.8. Operational stability of immobilized invertase in packed bed reactor

The reactor (length 8 cm, diameter 1.2 cm, total volume 9 ml), was made from Pyrex glass. The enzyme-magnetic PVAL microspheres were equilibrated in acetate buffer (50 mM, pH 5.5) at 4°C for 1 h. The enzyme microspheres (5 g) were loaded into the reactor yielding a void volume of about 3.5 ml.

To determine operational stability of immobilized invertase, sucrose solution (300 mM) in acetate buffer (50 mM, pH 5.5) was introduced to the reactor at a flow rate of 20 ml h^{-1} with a peristaltic pump (Cole Parmer, Model 7521-00, Miles, IL) through the lower inlet part. It was operated at 35°C for 40 h and the solution leaving the reactor was collected and assayed for invertase activity as described above.

2.9. Storage stability

The activities of free and immobilized invertase after storage in acetate buffer (50 mM, pH 5.5) at 4°C were measured in a batch operation mode with the experimental conditions given above.

2.10. Thermal stability of free and immobilized enzyme

The thermal stabilities of free and immobilized invertase were determined by measuring the residual activity of the enzyme exposed to three different temperatures (50–70°C) in acetate buffer (50 mM, pH 5.5) for 2 h. After every 15 min time interval, 0.1 g magnetic microspheres were removed and assayed for enzymatic activity as described above. The first order inactivation rate constants, k_i were calculated from the equation:

$$\ln A = \ln A_0 - K_i t \quad (2)$$

where A_0 is the initial activity and A is the activity after a time t (min).

2.11. Characterization of magnetic PVAL microspheres

2.11.1. Water content of magnetic PVAL microspheres

The swelling behaviours of dry magnetic PVAL microspheres were determined in distilled water. The apparent volume of the dry magnetic PVAL microspheres (1 g) was measured within a cylindrical glass

tube (10 ml). Distilled water was added into the tube, and the microspheres were allowed to swell at 25°C for 24 h (i.e. the predetermined equilibrium swelling time) with occasional shaking, and then the volume of the swollen microspheres was measured. The water contents of the swollen magnetic PVAL microspheres were calculated by using the following expression:

$$\text{Swelling Ratio } \% = \{(W_s - W_o)/W_o\}100 \quad (3)$$

where W_o and W_s are volume of magnetic PVAL microspheres before and after swelling, respectively.

2.11.2. Scanning electron microscopy

Scanning electron micrographs of the magnetic PVAL microspheres were obtained using a JEOL, JMS-5600 (Japan) after coating with gold under vacuum.

2.11.3. Analysis of magnetism

The degrees of magnetism of the PVAL microspheres were measured in a magnetic field by using a vibrating-sample magnetometer (Princeton Applied Research Corporation, USA). The presence of magnetite in the cross-linked PVAL polymeric structure was investigated with an ESR spectrophotometer (Model El 9, Varian).

3. Results and discussion

3.1. Properties of magnetic PVAL microspheres

The magnetic PVAL microspheres were prepared by chemical cross-linking of PVAL with glutaraldehyde in an organic dispersion medium. The reaction between hydroxyl groups of polyvinyl alcohol and aldehyde groups of glutaraldehyde is acid catalyzed, leading to acetal linkage (Peterson & Kennedy, 1997). The magnetic PVAL based microspheres which were prepared in this study are rather hydrophilic structures. Note that the equilibrium swelling ratio was about 76% in volume base.

A SEM micrograph of the magnetic PVAL microspheres is presented in Fig. 1. The microspheres had a spherical geometry and a particle size distribution in the range of 5–40 μm. The surface morphology of the magnetic PVAL microspheres shows irregular pores of varying dimensions which may lead to high internal surface area (implying high immobilization capacity). The enzyme would be immobilized both at the external surface of the microspheres and within the pore space, and thus provides a large surface area for the reaction of substrate with the immobilized enzyme molecule.

The magnetic property of the PVAL structure was confirmed by electron spin resonance (ESR). A peak of magnetite (i.e. Fe_3O_4 fine particle) was detected in the ESR spectrum and is presented in Fig. 2. In these spectra, a 600-Gauss magnetic field was found sufficient to excite all of the dipole moments of 1.0 g of a microsphere sample that consists of magnetite. Magnetic field intensity is a very important design parameter for magnetically stabilized reactors with immobilized enzyme systems and for magnetic filtration systems. Bahar and Çelebi (2000) reported that, by applying different magnetic field intensities, the void volume of the fluidized bed reactor could be adjusted, and the pressure drop in the system could be controlled. The value of this magnetic field is a function of the flow velocity, particle size and magnetic susceptibility of solids to be displaced. In the literature, this value changes from 800 to 20 000 Gauss for various applications, thus our magnetic

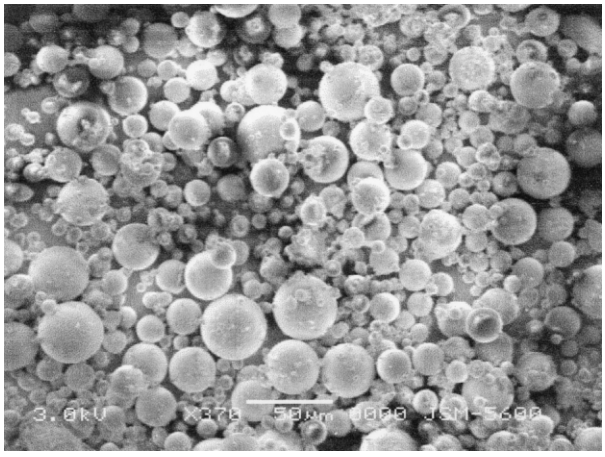

Fig. 1. SEM micrographs of magnetic polyvinylalcohol microspheres (×370 magnification).

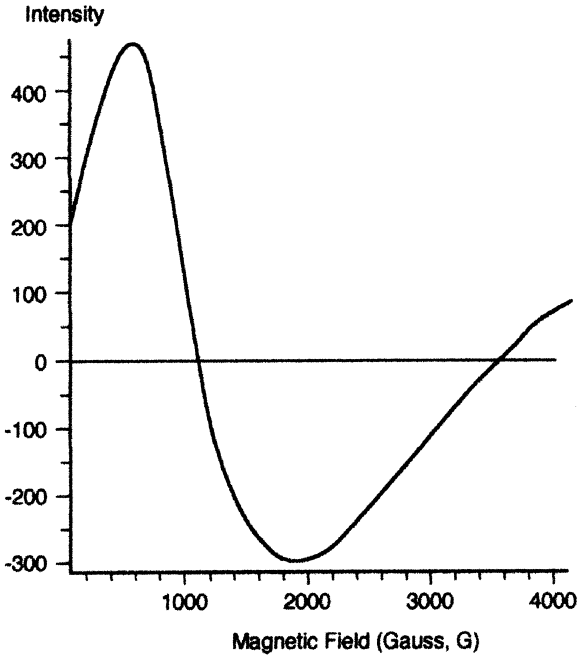

Fig. 2. The electron spin resonance spectra of magnetic polyvinylalcohol microspheres.

microspheres will need less magnetic intensity in a magnetic bed or a magnetic filter.

3.2. Immobilization of invertase onto magnetic PVAL microspheres

The activation of alcoholic groups of the magnetic PVAL microspheres was achieved by the reaction with CDI, a carbonylating agent. It has been proven to be a suitable agent for the activation of hydroxyl-containing supports for immobilization technology. The activation of a support containing hydroxyl groups by CDI and the subsequent coupling of an enzyme to the support is presented in Fig. 3. The intermediate activated support reacts with N-nucleophiles, such as free amino groups in protein, to give non-basic uncharged N-alkyl carbonate. Invertase was covalently immobilized via the amino group to the activated magnetic PVAL microspheres by the above-explained reaction mechanism. Analysis of the wash solutions showed that the immobilization process was irreversible. The enzyme loading and retained enzyme activity after immobilization on the magnetic PVAL microspheres were 7.18 mg g^{-1} support, and 74%, respectively.

3.3. Kinetic constants

Kinetic parameters of the enzymatic reaction can be estimated by the direct linear method of the Lineweaver–Burk plot of the initial sucrose hydrolysis rates, from experimental data. The Michaelis constants K_m and V_{max} for free invertase estimated from the Lineweaver–Burk plot were 24 mM and, 120 U mg^{-1} protein for sucrose, respectively. The value of apparent K_m for covalently bound invertase was approximately 2.3-fold higher than that of the free enzyme (Table 1). A comparison with the results obtained by Kotzelski and Staude (1996) for invertase immobilized on the polysulfone membrane, showed that the K_m values of covalently bound enzyme were about 13 times larger than that of the native invertase. The change in the affinity of the enzyme for its substrate is probably caused by structural changes in the enzyme introduced by the immobilization procedure and by lower accessibility of the substrate to the active site of the immobilized enzyme (Arıca et al., 1995). The V_{max} values of immobilized invertase were estimated from the data as 97 U per mg immobilized protein. For sucrose hydrolysis with the immobilized enzyme microspheres, K_m strongly increased while V_{max} was significantly more reduced than that of the free counterpart. However, K_m and V_{max} values of the free and immobilized invertase for sucrose are in the same order of magnitude. This indicates that the catalytic function of invertase was not very much impaired by this immobilization method.

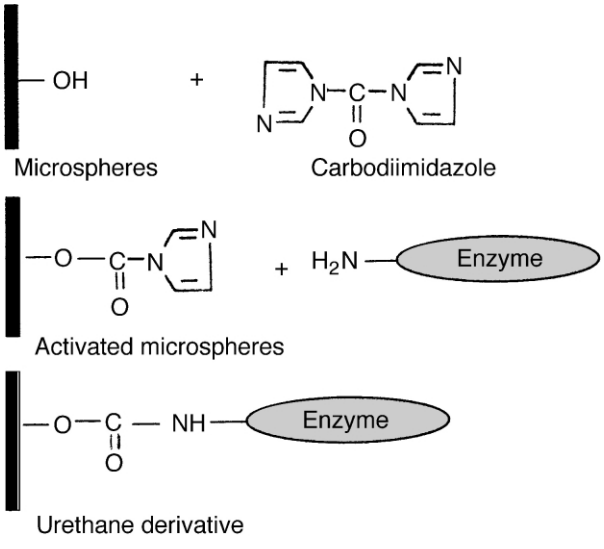

Fig. 3. Activation of the magnetic polyvinylalcohol microspheres with 1,1′-carbonyldiimidazole and covalent immobilization of invertase.

3.4. Effect of temperature on the catalytic activity

Fig. 4 shows the effect of temperature on the activity of the free and covalently linked invertase. The activities obtained in a temperature range of 20–60°C were expressed as percentage of the maximum activity. In the resultant bell-shaped curve, the maximum activity for the free enzyme was at 45°C. For the immobilized invertase it was at 50°C and the temperature profile was slightly broader than that of the free enzyme. Arrhenius plots in the temperature range from 20°C to optimum appear linear and activation energies were found to be 1.48 kcal mol^{-1} and 1.99 kcal mol^{-1} for free and immobilized invertase, respectively. The increases in optimum temperature and activation energy were caused by the changing physical and chemical properties of the enzyme. The covalent bond formation via amino groups

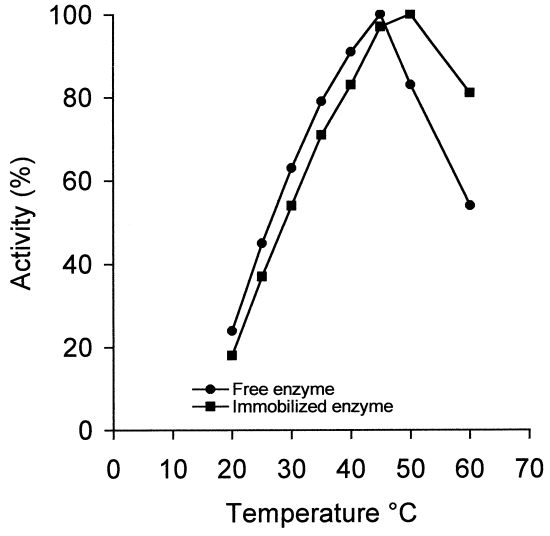

Fig. 4. Temperature profiles of free and immobilized invertase.

of the immobilized invertase might also reduce the conformational flexibility and may result in a higher activation energy for the molecule to reorganize the proper conformation for the binding to substrate. One of the main reasons for enzyme immobilization is the anticipated increase in its stability to various deactivating forces, due to restricted conformational mobility of the molecules following immobilization (Arıca et al., 1995; Tien & Chiang, 1999).

3.5. Effect of pH on the activity

The pH effect on the activity of the free and immobilized invertase preparations for sucrose hydrolysis was studied at various pHs at 45°C. The reactions were carried out in acetate and phosphate buffers and the results are presented in Fig. 5. The pH for maximum sucrose hydrolysis was determined as 5.0 for free invertase. Upon immobilization on magnetic PVAL microspheres, the optimum pH for sucrose hydrolysis was slightly shifted toward a less acidic region (5.5). This shift is possibly due to the secondary interactions (e.g. ionic and polar interactions, hydrogen bonding) between the enzyme and polymeric matrix. Similar observations, upon immobilization of invertase and other enzymes have been reported by other researchers (Gordon, 1997; Peterson, & Kennedy, 1997; Tien & Chiang, 1999; Uhlich et al., 1996).

3.6. Thermal stability

Thermal stability experiments were carried out with free and immobilized enzymes, which were incubated in the absence of substrate at various temperatures. Figs. 6 and 7 show the heat inactivation curves from 50 to 70°C for the free and immobilized enzymes, respectively. The immobilized invertase preserved its activity at 50°C and free enzyme retained its initial activity (about 92%) during a 100-min incubation period. At 60°C the immobilized and free enzymes retained their activities at levels of 93 and 45%, respectively. The immobilized form was inactivated at a much slower rate than the native form. Both the free and immobilized enzymes lost their initial activity at 70°C after 30 and 60 min treatments, respectively. The half-life values and thermal inactivation rate constants for the free and immobilized enzyme were determined from the percent residual activity vs. time at three different temperatures, and are presented in Table 2. These results suggest that the thermostability of immobilized invertase increased considerably as a result of covalent immobilization onto magnetic PVAL microspheres. The activity of the immobilized enzyme, especially in a covalently bound system, is more resistant than that of the soluble form against heat and denaturing agents. If the thermal stability of an enzyme were enhanced by immobilization, the potential utilization of such enzymes would be

Table 1
Properties of the free and immobilized invertase on magnetic polyvinylalcohol microspheres

Form of enzyme	K_m (mM)	V_{max} (U mg^{-1} enzyme)	Enzyme loading mg enzyme g^{-1} support)	Recovered activity (%)
Free invertase	24	120	–	100
Immobilized invertase	55	97	7.18	74

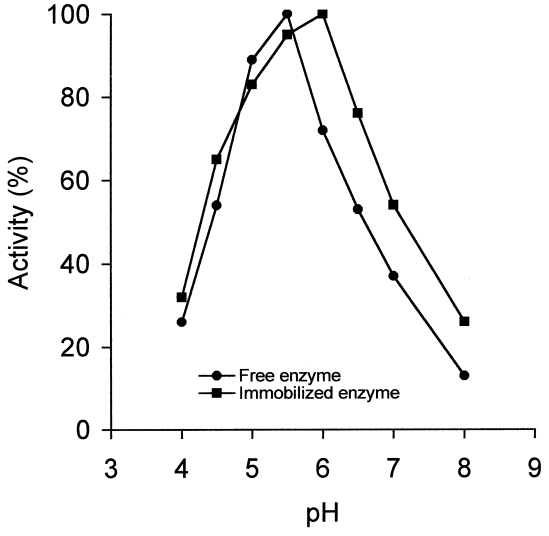

Fig. 5. pH profiles of free and immobilized invertase.

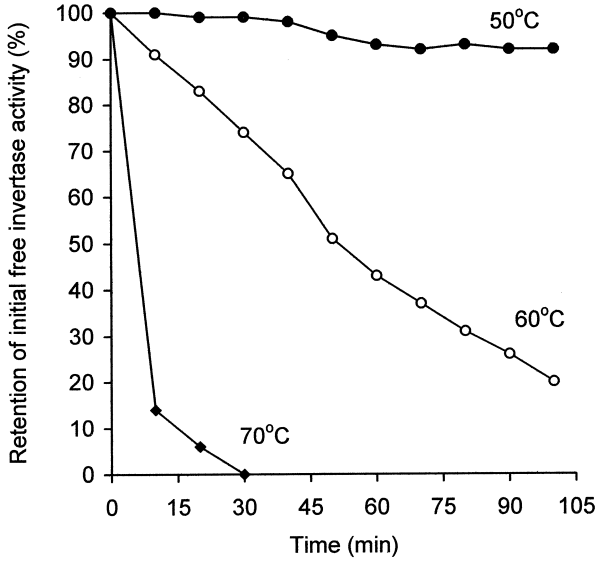

Fig. 6. Influence of temperature on the stability of free invertase.

extensive. In principle, the thermal stability of an immobilized enzyme can be enhanced, diminished, or unchanged relative to free counterparts, and several examples of each kind have been previously reported (Arıca, 2000; Godbole, Kubal, & D'Souza, 1990; Tien & Chiang, 1999).

3.7. Operational stability in a packed bed reactor

It is important, for economical use of an enzyme, as a means for the mass production of the desired product, that the enzyme reaction is continuous. One of the problems in continuous enzyme reactions is the operational stability of the enzyme immobilized on the support.

The operational stability of covalently linked invertase in the packed bed reactor was monitored for 40 h. The operation temperature was set at 30°C because it was observed that the activity is higher at higher temperature but the stability is decreased with increasing temperature. The results are presented in Fig. 8. The data show that, during the initial 12 h, the magnetic PVAL enzyme system preserved all of its initial activity. After this time, a steady decrease in enzyme activity is observed with time. After 40 h, the immobilized enzyme lost about 17% of its initial activity, possibly resulting from the inactivation of invertase upon use.

The operational inactivation rate constant of the immobilized invertase at 35°C with 200 mM sucrose in acetate buffer (50 mM, pH 5.5) was calculated from the above data as $k_{opi} = 5.83 \times 0^{-5}$ min^{-1}.

3.8. Storage stability

In general, if an enzyme is in solution, it is not stable during storage, and the activity is gradually reduced. Fig. 9 shows the stability of free and immobilized invertase stored in acetate buffer (50 mM, pH 5.5) at 4°C. As can be seen, the immobilization increase the storage stability of the enzyme, while the free enzyme

Table 2
Half-lives ($t_{1/2}$) and inactivation rate constant (k_i) of the free and the immobilized invertase at three different temperatures

Temperature (°C)	Free enzyme		Immobilized enzyme	
	$t_{1/2}$ (min)	k_i (min)	$t_{1/2}$ (min)	k_i (10^3 min)
50	625	8.3×10^{-4}	1666	3.0×10^{-4}
60	63	1.6×10^{-2}	75	4.0×10^{-3}
70	11	1.4×10^{-1}	16	2.2×10^{-2}

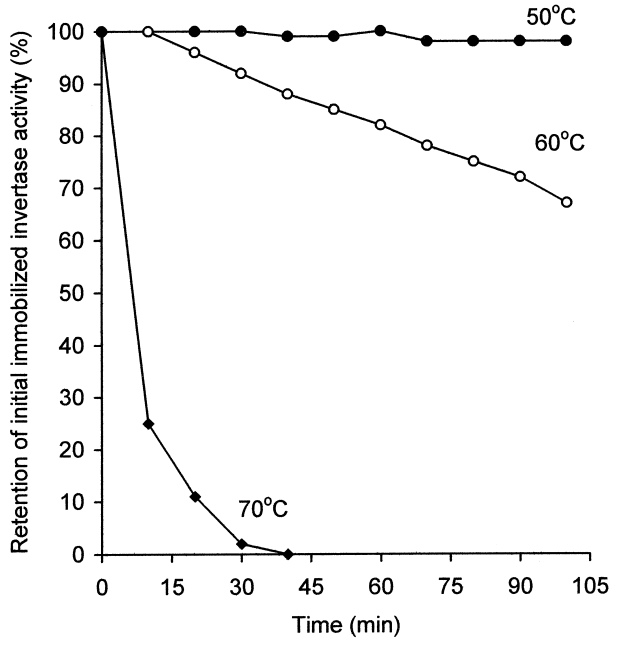

Fig. 7. Influence of temperature on the stability of immobilized invertase.

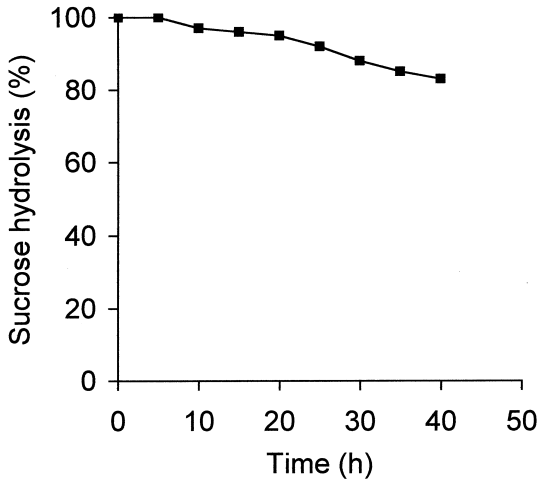

Fig. 8. Operational stability of immobilized invertase in a packed bed reactor.

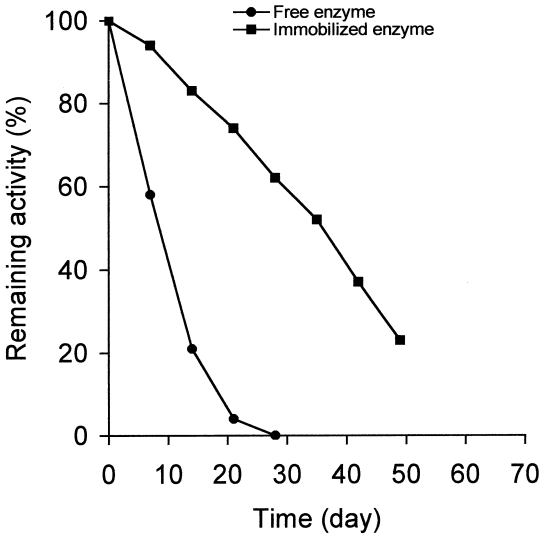

Fig. 9. Storage stability of the free and the immobilized invertase.

lost all its activity within 28 days. Immobilized enzyme retained 62% of its initial during the same storage period. This decrease in activity was explained as the time-dependent natural loss in enzyme activity.

4. Conclusion

In this study, the porous magnetic PVAL microspheres (diameter between 5 and 40 μm) were prepared and used as support for the immobilization invertase. Generally, small particles are used for enzyme immobilization because of their high immobilization area, but packed-bed reactors with small particles show high pressure drops and insufficient flow rates for large scale applications. These disadvantages, arising from small size, could be eliminated by using magnetic particles. As previously mentioned, the optimum pH and temperature profile of the immobilized enzyme are not drastically modified, and the thermal stability of the invertase was increased upon immobilization. The enzyme magnetic microspheres were used continuously for hydrolysis of sucrose with a 17% initial activity loss in a packed bed reactor. The magnetic PVAL microspheres elicit the desired properties, and could be used for covalently bound bioactive macromolecule immobilization. A high operational stability, obtained with the preparation, indicates that the immobilized invertase could be successfully used in a continuous system for the production of glucose and fructose from sucrose.

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