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Acrylamide–Sepiolite Based Composite Hydrogels for Immobilization of Invertase

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ABSTRACT: Novel composite hydrogels, poly(acrylamide)–sepiolite (PAS), poly(acrylamide/acrylic acid)–sepiolite (PAAS), and poly(acrylamide/itaconic acid)–sepiolite (PAIS) were prepared and used for the immobilization of invertase. The parameters of equilibrium swelling, diffusional exponent, and diffusion coefficient of these hydrogels were calculated from swelling experiments. Invertase was immobilized onto PAS, PAAS, and PAIS and immobilized invertases (PASI, PAASI, and PAISI) were prepared. Optimum pH values for free invertase, PASI, PAASI, and PAISI are found to be 5, 5.5, 4.5, and 6, respectively, and the optimum temperatures were 30, 50, 50, and 35 °C for free invertase PASI, PAASI, and PAISI. It was found that K_m values of free invertase, PASI, PAASI, and PAISI were 11.3, 41.0, 94.5, and 56.0 mM, respectively. V_{max} values were 2 μmol/min for free invertase, 8.10 μmol/min for PASI, 1.30 μmol/min for PAASI, and 0.42 μmol/min for PAISI, respectively. The invertase immobilized hydrogels showed excellent, temperature, storage, and operational stability.

Keywords: acrylamide, composite, immobilization, invertase, sepiolite

Introduction

Invertase (β-D-Fructofuranosidase E.C.3.2.1.26) is a glycoprotein catalyzing the hydrolysis of sucrose to form invert sugar, widely used in the food and drink industry. Enzymatic production of invert sugar is superior to acid catalyzed production due to formation of colored products during hydrolysis of sucrose by acids. Economic production of invert sugar could be carried out by immobilized invertase that has been immobilized on a variety of support materials by different methods for invert sugar production (D'Souza and Godbole 2002; Işık and others 2003; Gürsel and others 2003; Sanjay and Sugunan 2005; H-Dudra and others 2006; Marquez and others 2007).

Superabsorbents are the types of loosely cross-linked hydrophilic polymers that can swell, absorb, and retain a large volume of water or other biological fluids. Due to their potential applications in bioengineering, biomedicine, food industry, communication technology, building industry, chromatography, water purification, separation processes, and agriculture, super absorbent polymeric materials have received considerable attention. Polyacrylamide or modified polyacrylamide hydrogels exhibit superior water absorption capacity, and are permeable to oxygen and poses a good biocompatibility (Karadağ and others 2004).

Composites are engineered materials made from 2 or more constituent materials with significantly different physical or chemical properties and these materials remain separate and distinct on a macroscopic level within the finished structure (Gasser 2000). Hydrogels loaded with dispersed clays are a new class of composite materials that combine elasticity and permeability of the hydrogels with the additional abilities offered by clays; that is, clays have high tendency to adsorb different substances. In previous studies, several kinds of superabsorbent acrylamide composites based on bentonite (Kundakci and others 2008), sepiolite (Ekici and others 2006), attapulgite, kaolinite, mica, vermiculate, Na^+-montmorillonite (Zhang and Wang 2007) and so on have been synthesized and studied for various applications.

Sepiolite is a naturally occurring clay mineral of sedimentary origin. It is a nonswelling, lightweight, porous clay with a large specific surface area. Chemically, sepiolite is a hydrous magnesium silicate with the ideal formula $Si_{12}Mg_8O_{30}(OH)_4(OH_2)_4.8H_2O$. Sepiolite shows an alternation of blocks and tunnels that grow up in the fiber direction. These nanostructured tunnels account in large part for the high specific surface area and excellent sorption properties. Therefore, sepiolite is a reactive mineral exhibiting strong absorbing ability to water and other substances, and it is also a good candidate to prepare organic/inorganic absorbing composites (Brauner and Preisinger 1956; Helmy and de Bussetti 2008; Lazarević and others 2009).

In the present study, novel composite hydrogels, poly(acrylamide)–sepiolite (PAS), poli(acrylamide/acrylic acid)–sepiolite (PAAS), and poli(acrylamide/itaconic acid)–sepiolite (PAIS) were prepared, characterized and used for immobilization of invertase. The enzymatic performance of these hydrogels at various temperatures and pH values was studied and compared with free enzyme behavior. The kinetic parameters and thermal, storage, and operational stabilities of the immobilized enzymes were also investigated.

Materials and Methods

Materials

Acrylamide (A), acrylic acid (AA), N, N' methylene bisacrylamide (NNMBA), ammonium persulphate (APS), Na_2HPO_4, NaH_2PO_4, CH_3COOH, CH_3COONa were obtained from Merck (Darmstadt, Germany). Sucrose, invertase (*S. cerevisiae*), glucose assay kit (GAGO-20), and itaconic acid (I) N,N,N',N'-tetramethylenediamine (TEMED) were obtained from Sigma (St. Louis. Mo., U.S.A.). Sepiolite was obtained from Yıldız Kimya (Ankara, Turkey). The chemical composition of sepiolite in mass percent is: 58.83% SiO_2, 8.27%

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Al_2O_3, 3.26% Fe_2O_3, 1.23% CaO, 13.55% MgO, 0.93% K_2O, 0.444% TiO_2, 0.038% Mn_2O_3, 0.025% P_2O_5, 0.018% Cr_2O_3, and 0.19% Na_2O.

Preparation of composite hydrogels

For preparation of poly(acrylamide)–sepiolite composite hydrogels, 14×10^{-3} mole of A and 0.17 mmole of NNMBA were dissolved in distilled water in the presence of 50 mg of sepiolite then mixed thoroughly to form a homogeneous solution. Following the addition of 0.03 mmole of APS and 2.5 mmole of TEMED, the mixture was placed in PVC straws of 3 mm dia. A gel formed after 2 h of reaction at ambient temperature. For preparation of PAAS composite hydrogels, 5.55×10^{-4} mol AA was added into the PAS reaction mixture. For preparation of PAIS composite hydrogels, 3.07×10^{-4} mol itaconic acid (I) was added into the PAS reaction mixture. After 24 h, the hydrogel composite rods were cut into 4 to 5 mm in length and washed with distilled water and dried in air and vacuum.

Swelling experiments of composite hydrogels

PAS, PAAS, and PAIS were swelled in distilled water at 25 and 60 °C to determine the parameters of swelling and diffusion. Swollen composites, removed from the water bath at regular time intervals were dried superficially with filter paper, weighed, and placed in the same bath. The reversibility of swelling with pH was also determined. The samples were first swollen in a solution of pH 3 until equilibrium degree, and the swelling degree was measured. Then the hydrogels were transferred to a solution of pH 8 and the swelling degree was measured again. These experiments were carried for 14 d.

Immobilization of invertase onto/into the composite hydrogels

One gram weighed dry PAS, PAAS, and PAIS were immersed in a solution containing invertase (50 mL from 1 mg/mL). Immobilization of invertase was carried out at 22 °C for 3 h by continuous stirring in the reaction medium. After this period, these hydrogels were removed first by washing with distilled water and then with acetate buffer (50 mM, pH 4.8), and prepared PASI, PAASI, and PAISI were stored at 4 °C in fresh buffer until use.

Activity assays of free and immobilized invertases

The activities of both the free invertase (I) and the immobilized invertases PASI, PAASI, and PAISI were determined by measuring the amount of glucose liberated from the invertase-catalyzed hydrolysis of sucrose per unit time. Enzyme activity was assayed in the presence of sucrose as a substrate (300 mM) in 50 mM of acetate buffer pH 5 and 37 °C. Following a preincubation period (5 min at 37 °C), the assay was started by the addition of the enzyme solution (0.1 mL from 1 mg/mL invertase solution). After 15 min of the reaction, the released amount of glucose was measured by glucose oxidase–peroxidase method (Glucose oxidase assay kit from Sigma). The absorbance was measured at 540 nm. One unit of enzyme was defined as a quantity of enzyme that hydrolyzes 1 μmol of sucrose to glucose per minute (Lampen 1971; Bergmeyer and Bernt 1974).

The same assay medium was used to determine the activity of the immobilized enzyme. The enzyme reaction was started by the introduction of 0.6 g swelled PASI, PAASI, and PAISI into the assay medium at 37 °C. After 15 min, the reaction was terminated by removal of the composites from the reaction mixture. The produced amount of glucose was determined as described previously.

The activity assays were carried out over the pH range of 3 to 8 and temperature range of 20 to 70 °C to determine the pH and temperature profiles of free and immobilized enzyme. The results of pH and temperature of the medium are presented in a normalized form with the highest value of each set being assigned the value of 100% activity.

The kinetic parameters of Michaelis–Menten, K_m and V_{max} values of the free enzyme were determined by measuring initial rates of the reaction with sucrose (5 to 300 mM) in acetate buffer (50 mM, pH 5) at 30 °C. The kinetic parameters of immobilized invertases were determined in a batch system by varying the concentrations of sucrose (5 to 300 mM) in their optimum pH and optimum temperature values.

Thermal stability of free and immobilized invertases

The thermal stability of free and immobilized invertases was ascertained by measuring the residual activity of enzyme exposed to various temperatures (50 and 70 °C) in 50 mM acetate buffer (pH 4.8) for 160 min.

The storage stability of free and immobilized invertases

The activity of free and immobilized invertases after storage in 50 mM acetate buffer (pH 4.8) at 4 °C was measured in a batch operation mode with the experimental conditions given previously. The activity measurements are carried out for a period of 30 d.

Operational stability of immobilized invertases

The retention of the immobilized enzyme activity was tested as described in activity assays of invertase. After each reaction run, PASI, PAASI, and PAISI were removed and washed with distilled water and 50 mM acetate buffer (pH 4.8) to remove any residual substrate within the composites. They were then reintroduced into fresh reaction medium and enzyme activity was detected.

Results and Discussion

Preparation of composite hydrogels

PAS, PAAS, and PAIS composite hydrogels were prepared by free radical cross-linking and copolymerization of acrylamide with acrylic or itaconic acid and a small amount cross-linker (NNMBA) in aqueous solution in the presence of sepiolite. APS and TEMED were used as the initiator and the accelerator, respectively. At polymerization, the possible step is a reaction among A and anionic comonomers, AA or I and crosslinker molecules by the process of the unpaired electron transfer to the monomeric units, so that they in turn become reactive. Another monomer or comonomers can therefore be attached and activated in the same way resulting in a 3-dimensional network. Sepiolite molecules can be incorporated into chains simultaneously. Sepiolite fibers were dispersed inside in the PVA matrix in the study of Alkan and Benlikaya (2009).

Swelling parameters of composite hydrogels

Dried PAS, PAAS, and PAIS hydrogels are glassy and very hard, but swollen gels are soft. Upon swelling the hydrogels were strong and elastic enough to retain their shape.

The swelling S% of the hydrogels was calculated from the following relationship:

$$S\% = \frac{m_t - m_o}{m_t} \times 100 \quad (1)$$

Here m_t is the mass of swollen hydrogel at time t and m_o is the mass of dry hydrogel at time 0. Swelling curves of the hydrogels in distilled water at 25 and 60 °C are shown in Figure 1. Swelling of the hydrogels increased with time and reached a constant value after a certain point. This value may be named equilibrium or maximum swelling (S_{eq}%). The S_{eq} values are shown in Table 1.

The acrylamide hydrogels swell in the water low. Swelling degree of acrylamide hydrogel is increased in addition of sepiolite and hydrophilic monomers (acrylic acid and itaconic acid). This significant increase is related to the presence of many hydrophilic groups such as silanol groups, hydroxyl groups (in sepiolite), and carboxyl groups (in acrylic and itaconic acid). The swelling order of acrylamide-based hydrogels is PAS < PAAS < PAIS at 25 and 60 °C. The swelling of PAIS is higher than the others, because, it has 2 ionizable carboxyl groups while PAAS has 1 carboxyl group and PAS has no carboxyl group. As can be seen from Figure 1, the swelling of hydrogels increased with an increase in the temperature. The swelling degree is increased at 60 °C, this situation could be opening up the pores of sepiolite or increased mobility of water molecules to diffuse in deep into speiolite structure.

Analysis of the mechanisms of water diffusion in swellable polymeric systems has received considerable attention in recent years because of the important applications of swellable polymers in the industrial, biomedical, environmental, and agricultural fields. The following equation was used to determine the nature of diffusion of water and nutrient medium into the hydrogels (Peppas and Ritger 1987):

$$F = kt^n \tag{2}$$

In this equation, F denotes the amount of penetrant fraction at time t; k is a constant incorporating the characteristics of the polymeric network system and the penetrant; n is the diffusional exponent, which is the indicative of the transport mechanism. n and k values were calculated, and are presented in Table 2.

The values of n were found to be between 0.50 and 1 and hence the diffusion of the water into PAS was taken to be of non-Fickian in character. This is generally explained as being a consequence of the slow relaxation rate of the hydrogel. The diffusion of the water into PAAS and PAIS was taken to be of super case. This type of diffusion is diffusion type in which diffusion rate is greater than the speed of the clarification ($n = 1$) (Peppas and Ritger 1987).

The reversibility of swelling with pH was also determined. These experiments were carried out for 14 d. The swelling of PAAS and PAIS increased with pH. The hydrogels have good reswelling ability (Figure 2).

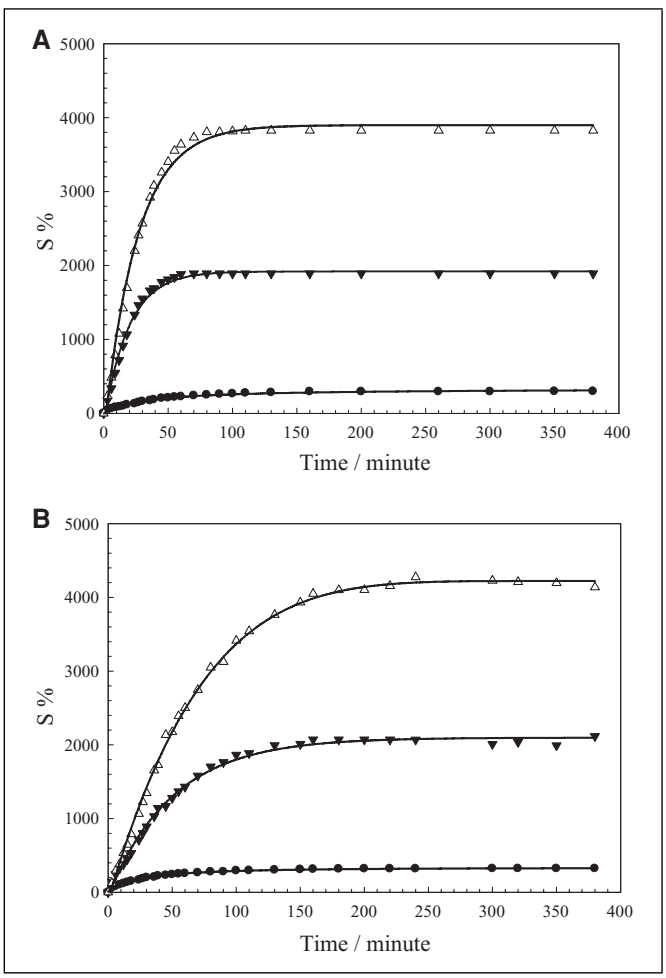

Figure 1—Time depending swelling of the hydrogels in distilled water. (A) at 25 °C, (B) at 60 °C (•, PAS; ▼, PAAS; △, PAIS).

Table 1—Swelling parameters of PAS, PAAS, and PAIS.

	S_{eq}%		
Temperature/°C	PAS	PAAS	PAIS
25	300	1900	3830
60	324	2200	4300

Immobilization of invertase onto/into the composite hydrogels

Poly(acrylamide)–sepiolite–invertase (PASI), poly(acrylamide/acrylic acid)–sepiolite–invertase (PAASI), and poly(acrylamide/itaconic acid)–sepiolite–invertase (PAISI) were prepared by the immobilization of invertase into PAS, PAAS, and PAIS composite hydrogels.

After immobilization, optimum pH, optimum temperature, kinetic parameters, thermal stability, storage stability, and operational stability of immobilized invertases were investigated. The results are presented in a normalized form with the highest value of each set being assigned the value of 100% activity.

Table 2—Diffusion parameters of PAS, PAAS, and PAIS.

	PAS		PAAS		PAIS	
t/°C	n	$k \times 10^4$	n	$k \times 10^4$	n	$k \times 10^4$
25	0.59	713	1.00	303	1.00	182
60	0.91	271	0.81	261	1.00	103

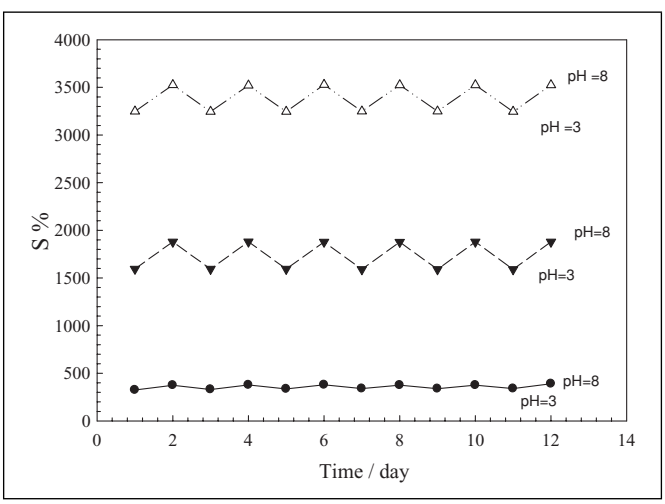

Figure 2—Reversibility of the swelling of the hydrogels (•, PAS; ▼, PAAS; △, PAIS).

Optimum pH, optimum temperature, and kinetic parameters of free and immobilized invertase

The effect of pH on the activity of free and immobilized invertase was studied at various pH values. The results are presented in Table 3. Optimum pH value shifted to alkaline region for PASI and PAISI, and shifted to acidic region for PAASI. This shift is possibly due to the secondary interactions between the enzyme and the polymeric matrix.

The temperature dependence of the activities of the free and immobilized invertases was studied in the temperature range of 20 to 70 °C. Optimum temperatures for the highest activities for the free invertase, PASI, PAASI, and PAISI were determined and are shown in Table 3. The increase in the optimum temperature by immobilization can be the result of changes in the physical and chemical properties of the enzyme.

The kinetic parameters, K_m (Michaelis constant) and V_{max} (maximum reaction rate) for free and immobilized invertases were determined by varying the concentration of sucrose in the reaction medium. These parameters are presented in Table 3. As expected, the K_m value increased with immobilization (Çetinus Akkuş and Öztop 2003; Çetinus Akkuş and others 2007). This result can be attributed to the limited accessibility of sucrose molecules to the active sites of the immobilized invertase as a result of conformational change invertase caused by the immobilization and/or can be the hindrance due to hydrogel network. It was found that the V_{max} value of PASI was bigger than that of the free invertase. Similar results involving change in V_{max} values of enzyme after immobilization have been reported in the literature (Amaya-Delgado and others 2006; Tomotani and Vitolo 2006). Normally, a decrease on V_{max} for an immobilized enzyme would be expected. This result shows that there is no external and internal diffusional resistance for transport of substrate and product in PASI.

Thermal stability of free and immobilized invertases

Thermal stability studies of free and immobilized invertases were carried out at 50 and 70 °C (Figure 3A and 3B). The activity of free enzyme decreased with an increase in temperature. While free invertase lost about 50% of its initial activity within first 10 min, PAASI and PAISI retained about 85% of their initial activities during 150 min incubation period at 50 °C. After 150 min, PASI has 70% activities at 50 °C (Figure 3A). It is shown in Figure 3B that the immobilized invertases preserved 75% to 80% of initial activities at 70 °C, whereas the free enzyme lost all its activity after an 80 min incubation period.

According to these results, we can say that immobilization of invertase in PAS, PAAS, and PAIS composite hydrogels preserves tertiary structure of the enzyme and it protects the enzyme from conformational changes caused from environmental effect. It is often observed that immobilized enzyme has a higher thermal stability than the corresponding free enzyme because of the reduction of conformational flexibility in the immobilized enzyme.

Table 3—The various parameters of free invertase, PASI, PAASI, and PAISI.

Enzyme	Optimum pH	Optimum temperature/°C	K_m/ mM	V_{max}/ μmol/min
I	5	30	11.3	2
PASI	5.5	50	41	8.10
PAASI	4.5	50	94.5	1.30
PAISI	6	35	56	0.42

Storage stability of free and immobilized invertases

Storage stability of immobilized enzymes is important for their practical application. Free and immobilized invertases were stored in 50 mM acetate buffer (pH 4.8) at 4 °C and the activity measurements are carried out for a period of 30 d (Figure 4). Free invertase lost about 90% of its initial activity within 30 d, whereas

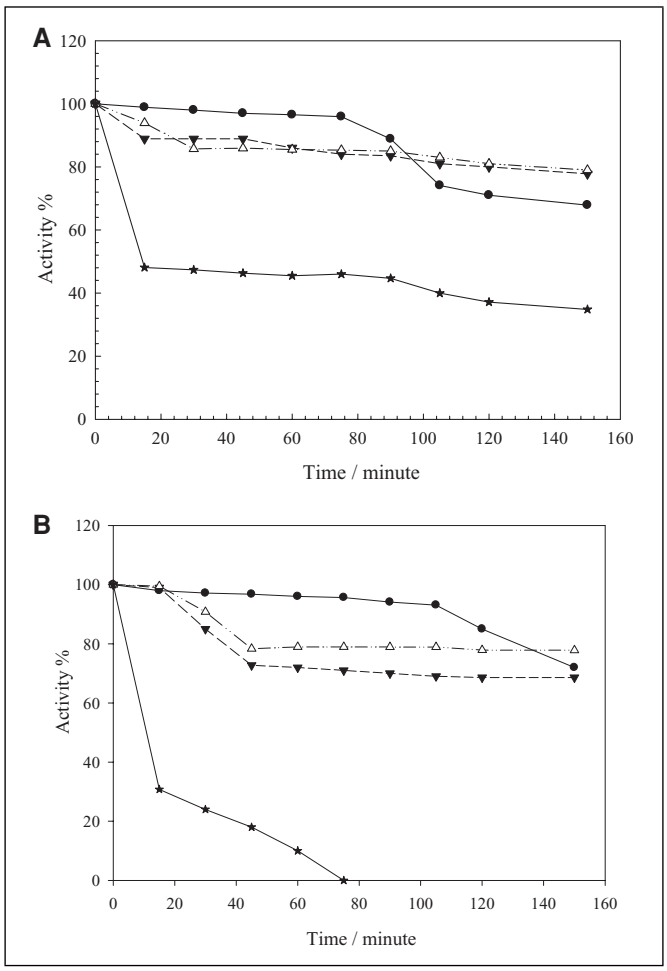

Figure 3—(A) Thermal stability of invertase at 50 °C. (B) Thermal stability of invertase at 70 °C. (•, PASI; ▼, PAASI; △, PAISI; ∗, free invertase).

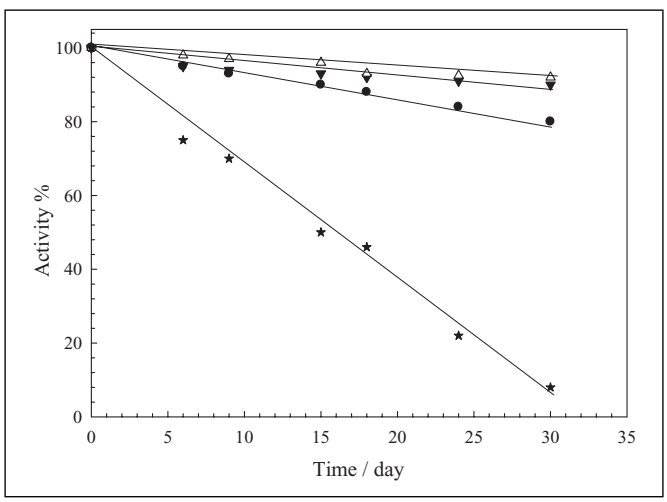

Figure 4—Storage stability of invertase (•, PASI; ▼, PAASI; △, PAISI; ∗, free invertase).

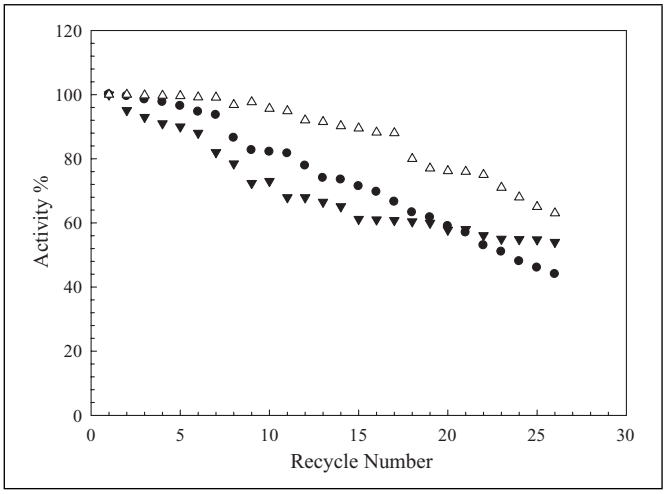

Figure 5—Operational stability of invertase (•, PASI; ▼, PAASI; △, PAISI).

immobilized invertases retained about 85% to 90% of their initial activities during this incubation period. The decrease in activity was explained as a time-dependent natural loss in enzyme activity and this was prevented to a significant degree by immobilization.

Operational stability of immobilized invertases

The economical use for an enzyme is very important, as a means for the mass production of the desired product, that the enzyme catalysis is continuous. Operational stability curve was shown in Figure 5. Operational stability of the immobilized invertases was determined for 26 successive batch reactions at 40 °C. At the end of this period, PASI, PAASI, and PAISI retained about 50% of their activities. It was found that the hydrogels have higher operational stability.

Conclusions

Novel hydrogel–clay composites (PAS, PAAS, and PAIS composite hydrogels) were prepared, characterized, and used as support for the immobilization of invertase. Some swelling properties of these materials were investigated. Swelling degree of acrylamide hydrogel was increased with addition of sepiolite and acrylic and itaconic acid monomers. Invertase can be of great value in food and drink industries for hydrolysis of sucrose to form invert sugar. The hydrogels were used for invertase immobilization and immobilized invertases (PASI, PAASI, and PAISI) were prepared. The immobilized invertases provided significantly improved stability over the free form. They showed better thermal and storage stabilities. It was also found that these immobilized enzymes have a high operational stability. It can be concluded that PAS, PAAS, and PAIS are an appropriate matrix for invertase and at the same time they could be successfully used for the production of glucose and fructose from sucrose.

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