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Poly(acrylamide/maleic acid)–sepiolite composite hydrogels for immobilization of invertase

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Abstract Poly(acrylamide/maleic acid) and sepiolite (PAMS) composite hydrogel was prepared and used for the immobilization of invertase. In FTIR analysis, the characteristic bands of composite such as –OH, –COOH, Si–OH show the evidence of sepiolite and maleic acid. In TGA analysis, water loss, decomposition of amide side groups and breakdown of main chain regions of the composite were found. The parameters of equilibrium swelling, initial swelling rate, diffusional exponent, and diffusion coefficient were calculated and evaluated from swelling experiments in distilled water. Invertase was immobilized onto PAMS by adsorption and poly(acrylamide/maleic acid)–sepiolite–invertase (PAMSI) was prepared. Optimum pH, optimum temperature values for free invertase and PAMSI were found. It was found that K_m values of free invertase and PAMSI were 11.3 and 34.1 mM, respectively. V_{max} value was found that 2.0 μmol min^{-1} for free invertase and 13.9 μmol min^{-1} for PAMSI, respectively. PAMSI showed excellent temperature, operational and storage stability.

Keywords Acrylamide · Composite · Invertase · Immobilization · Sepiolite

Introduction

Superabsorbent polymers are the types of loosely crosslinked hydrophilic polymers that can swell, absorb and retain a large volume of water or other biological fluids

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[1, 2]. 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 [3].

Hydrogels loaded with dispersed clays are a new class of composite materials which combine elasticity and permeability of the gels with high ability of the clays to adsorb different substances In previous studies, several kinds of superabsorbent acrylamide composites based on bentonite [4], sepiolite [5], attapulgite, kaolinite, mica, vermiculate and Na^+-montmorillonite [6] etc. have been synthesized and studied.

Sepiolite is a naturally occurring clay mineral of sedimentary origin. It is a non swelling, lightweight, porous clay with a large specific surface area. Chemically, sepiolite is a hydrous magnesium silicate with the ideal formula $Si_{12}Mg_8O_{30}(OH)_4(OH_2)_4{\cdot}8H_2O$. 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 of sepiolite. Therefore, sepiolite is a reactive mineral exhibiting strong absorbing ability to water and other substances, and it is also candidate to prepare organic/inorganic absorbing composites [7].

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. Invertase has been immobilized on a variety of support materials, by different methods, for invert sugar production [8–13].

The aim of the present study is preparation and characterization of a novel composite hydrogel, poly(acrylamide/maleic acid)–sepiolite (PAMS), for immobilization of invertase and, investigation of the enzymatic performance of this hydrogel at various temperatures and pH values.

Experimental

Reagents

Acrylamide (A), *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 (I) (*Saccharomyces cerevisiae*), glucose assay kit (GAGO-20), maleic acid (M), *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED) were obtained from Sigma (St. Louis MO, USA). Sepiolite was obtained Yildiz Kimya (Ankara, Turkey). The chemical composition of sepiolite in weight percent is: SiO_2 (58.83%), Al_2O_3 (8.27%), Fe_2O_3 (3.26%), CaO (1.23%), MgO (13.55%),

K_2O (0.93%), TiO_2 (0.444%), Mn_2O_3 (0.038%), P_2O_5 (0.025%), Cr_2O_3 (0.018%), and Na_2O (0.19%).

Preparation of composite hydrogels

For preparation of PAMS, 14×10^{-3} mole of A, 3.44×10^{-4} mole of M, 0.16 mmole of NNMBA dissolved in distilled water in the presence of 50 mg of sepiolite then mixed thoroughly to form a homogeneous solution. Following the addition of 0.027 mmole of APS, and 2.5 mmole of TEMED the mixture was injected through a syringe to PVC straws of 3 mm diameter. A gel formed after 2 h of reaction at ambient temperature. After 24 h, the hydrogel composites rods were cut into 4–5 mm in length and washed with distilled water and dried in air and vacuum.

Characterizations of composite hydrogels

FTIR spectrum of PAMS composite hydrogels were recorded using KBr technique.

Thermal gravimetric analysis (Shimadzu TGA50) studies are carried out in the temperature range 25–600 °C at a heating rate 10 °C min^{-1}.

Poly(acrylamide/maleic acid)–sepiolite composite hydrogels were swelled in distilled water and in buffer solutions ranging from pH 3 to 11 at 25 and 60 °C to discover the parameters of swelling and diffusion. KH_2PO_4–K_2HPO_4 and CH_3CCOH–CH_3COONa buffer solutions were used for the desired pH values. Swollen composites, removed from the water bath at regular time intervals were dried superficially with filter paper, weighed, and placed in the same bath.

Immobilization of invertase onto/into the composite hydrogels

Dry PAMS (1 g) was immersed in a distilled water containing invertase (50 mL 1 mg mL^{-1}). Immobilization of invertase was carried out at 22 °C for 3 h, while continuously stirring the reaction medium. After this period, the PAMS composites were removed first by washing with distilled water and then with acetate buffer (50 mM, pH 4.8), and they were stored at 4 °C in fresh buffer until use.

Activity assays of free and immobilized invertase

The activities of both the free invertase (I) and the immobilized invertase poly(acrylamide/maleic acid)–sepiolite–invertase (PAMSI) were determined by measuring the amount of glucose liberated from the invertase-catalyzed hydrolysis of sucrose per unit time. Enzyme activity was assayed in presence of sucrose as a substrate (300 mM) in 50 mM of acetate buffer pH 5.0 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^{-1} invertase solution). After 15 min of reaction released glucose was measured by glucose oxidase–peroxidase method (Glucose GO assay kit). The absorbance was measured at 540 nm. One unit of enzyme was defined as a quantity of enzyme which hydrolysis 1 μmol of sucrose to glucose per minute [14, 15].

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 PAMSI 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 above.

The activity assays were carried out over the pH range 3–8 and temperature range 20–70 °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 (5–300 mM) in acetate buffer (50 mM, pH 5.0) at 30 °C. The kinetic parameters of PAMSI were determined in a batch system by varying the concentrations of sucrose (5–300 mM) in acetate buffer (50 mM, pH 6.0) at 40 °C.

Thermal stability of free and immobilized invertase

The thermal stability of free and immobilized invertase 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 invertase

The activity of free and immobilized invertase 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 above.

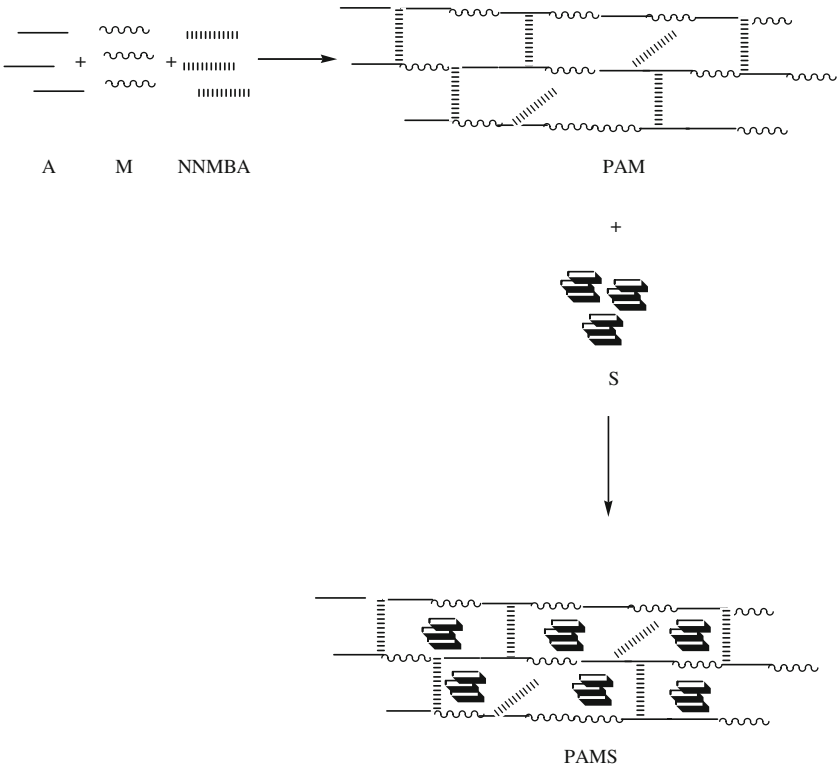
Operational stability of immobilized cells

The retention of the immobilized enzyme activity was tested as described in activity assays of invertase. After each reaction run, PAMSI composites 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 PAMS composite hydrogels

Poly(acrylamide/maleic acid)–sepiolite composite hydrogels were prepared by free-radical crosslinking and copolymerization of acrylamide, sepiolite and maleic acid with a small amount crosslinker (NNMBA) in aqueous solution. APS and TEMED were used as the initiator and the accelerator, respectively. At polymerization, the possible step is a reaction amongst AAm and anionic comonomer, M and



Scheme 1 Preparation of poly(acrylamide/maleic acid) hydrogel (PAM), and poly(acrylamide/maleic acid)-sepiolite composite hydrogel (PAMS). (*A* Acrylamide, *M* maleic acid, *NNMBA* *N*,*N*′-methylenebisacryl amide, *S* sepiolite)

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 there dimensional network. Sepiolite molecules can be incorporated into chains simultaneously (Scheme 1).

Characterizations of composite hydrogels

An FTIR spectrum of PAMS composite was taken and is presented in Fig. 1a. The absorption bands in the range of 3,000–3,500 cm^{-1} are attributed to the stretching of –OH groups of sepiolite. The bands appearing between 500 and 1,100 cm^{-1} belong to Si–O asymmetrical stretching vibrations. The absorption peak at 1,670 cm^{-1} is attributed to CO–NH band of acrylamide. The absorption band at 1,710 cm^{-1} is due to asymmetrical stretching for COOH group of maleic acid. And all these bands are in accordance with the corresponding functional groups reported in the literature [5, 16].

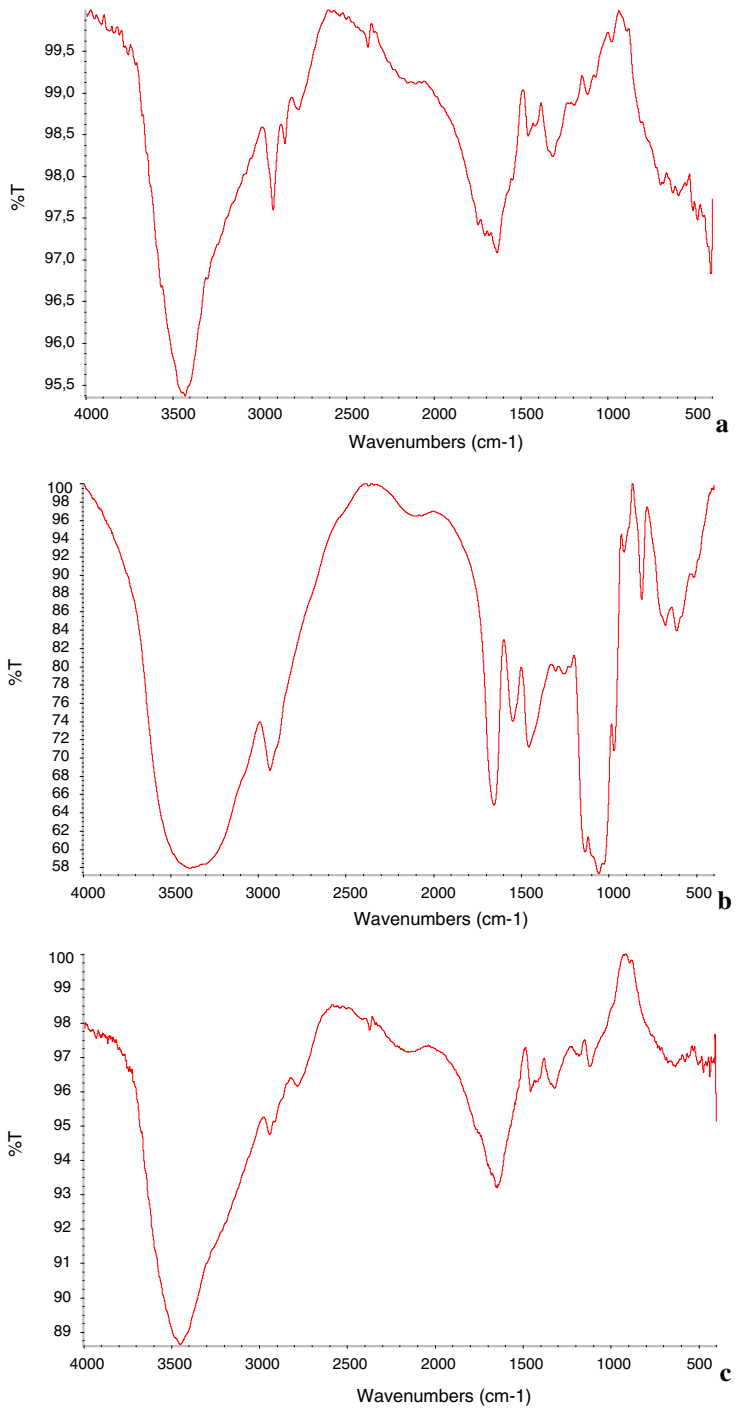


Fig. 1 FTIR spectrum of PAMS (**a**), invertase (**b**) and PAMSI (**c**)

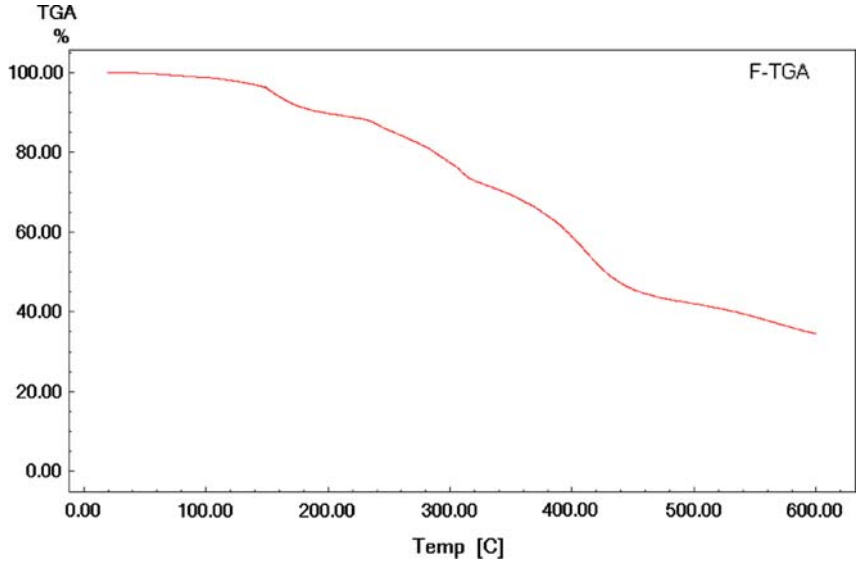


Fig. 2 TGA thermogram of PAMS

In the experiments of thermal analysis, three types of the decomposition region of the composite were found (Fig. 2). The first region is in the range of 20–200 °C due to water loss, which is adsorbed both on the surface and in the pores of the composite. Following the first region, the weight loss within the temperature of 200–400 °C can be attributed to the thermal decomposition of amide side groups of acrylamide and crosslinker on the network. When sepiolite is heated above 350 °C, coordinated water is lost and a phase transformation takes place, folding the structure of the sepiolite. The third region (400 °C) represents substantial mass loss and is normally attributed to main chain of composite breakdown.

Dried PAMS 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 PAMS was calculated from the following relationship:

$$\mathrm{S}\% = \frac{m_t - m_0}{m_t} \times 100 \tag{1}$$

Here m_t is the mass of swollen composite at time t and m_o is the mass of dry composite at time 0.

The swelling degree of the PAMS increased with time and reached a constant value after a certain point. This value may be named as an equilibrium or a maximum swelling ($S_{eq}\%$). The $S_{eq}\%$ values are shown in Table 1. As can be seen from Table 1, the swelling of PAMS at 60 °C was higher than that of PAMS at 25 °C.

For extensive swelling of composites, following second-order kinetics, the following equation can be used [17]:

Table 1 Swelling and diffusion parameters of PAMS

25 °C	Swelling	S_{eq} %	672
		r_i/g water (g composite min)$^{-1}$	1.59
		S_{max}/g water (g composite)$^{-1}$	6.72
		k_s/g composite min (g water)$^{-1}$	3.5×10^{-2}
	Diffusion	n	0.75
		$k \times 10^4$	728
60 °C	Swelling	S_{eq}%	838
		r_i/g water (g composite min)$^{-1}$	0,29
		S_{max}/g water (g composite)$^{-1}$	8,38
		k_s/g composite min (g water)$^{-1}$	4.1×10^{-3}
	Diffusion	n	0.57
		$k \times 10^4$	889

$$(t/S) = A + Bt \tag{2}$$

In which

$$B = 1/S_{eq}$$

is the inverse of the maximum or equilibrium swelling;

$$A = 1/k_s S_{eq}^2$$

is the reciprocal of the initial swelling rate of the composite; and k_s is the swelling rate constant.

The initial swelling rate (r_i), the swelling rate constant (k_s) and the theoretical equilibrium swelling (maximum swelling; S_{max}) of composite are calculated and are presented in Table 1. As can be seen from Table 1, the values of maximum swelling of the composites are in accordance with the swelling behaviors. The results observed that the swelling process of PAMS composites at 60 °C is faster than the swelling of PAMS composites at 25 °C. It was also observed that, the swelling of PAMS composite increased with increase in the temperature.

The following equation was used to determine the nature of diffusion of water and nutrient medium into the hydrogels [18]:

$$F = k t^n \tag{3}$$

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 1.

The values of n were found to be between 0.50 and 1.00 (Table 1), and hence the diffusion of the fluids into PAMS 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 [18].

The variation in swelling degree of composites with pH is presented in Fig. 3 at two different temperatures. As seen from this figure, the swellings increased with pH and

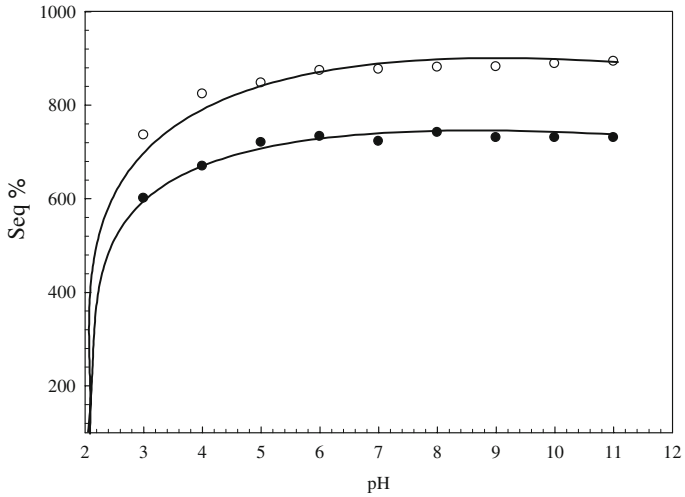


Fig. 3 pH responsive swelling curves of PAMS (*filled circle* 25 °C, *open circle* 60 °C)

reached a constant value up to pH 6. In these composites the maximum swellings were reached at pH 6 as a result of complete dissociation of the acidic groups of maleic acid units at this pH. The first and second dissociation constants of the maleic acid are $pK_{a1} = 1.85$ and $pK_{a2} = 6.06$, respectively. Charged groups attached to the polymeric network structure played an essential role in swelling properties.

The reversibility of swelling was also determined. The sample was first swollen in a solution of pH 3 until equilibrium degree, and the swelling degree was measured. Then the composite was transferred to a solution of pH 8 and the swelling degree was measured again. These experiments were carried on during 14 days. It can be seen in Fig. 4, PAMS have good reswelling ability.

Immobilization of invertase onto/into the composite hydrogel

Poly(acrylamide/maleic acid)–sepiolite–invertase was prepared by the immobilization of invertase into PAMS composite. FTIR spectra of I and PAMSI are shown in Fig. 1b, c. It is seen in these spectra that the absorption bands at 1,710 cm^{-1} ascribed to COOH band of maleic acid (Fig. 1a), and the band at 1,071–1,078 ascribed as Si–OH band for sepiolite (Fig. 1a) and the peak at 1,553 cm^{-1} belongs to amid units in invertase (Fig. 1b) disappeared in the spectrum of PAMSI (Fig 1c). This indicates that the carboxyl groups in maleic acid and hydroxyl groups of sepiolite may interacted with amino groups in invertase.

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 2. The optimum pH of the immobilized enzyme was shifted 1.0 pH unit to the alkaline region.

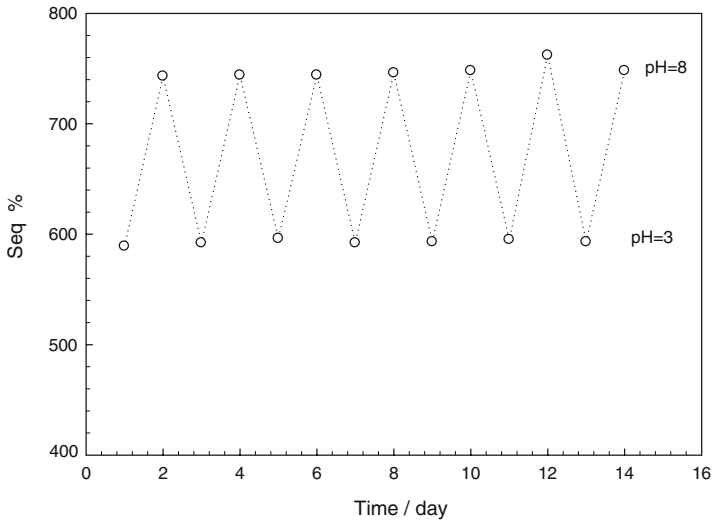


Fig. 4 Reversibility of the swelling PAMS

Table 2 The various parameters of I and PAMSI

Enzyme	Optimum pH	Optimum temperature/ °C	K_m/mM	V_{max}/μmol min^{-1}
I	5.0	30	11.3	2.00
PAMSI	6.0	40	34.1	13.9

The temperature dependence of the activities of the free and immobilized invertase were studied in temperature range 20–70 °C. Optimum temperatures for the highest activities for the free invertase and PAMSI were determined and are shown in Table 2. The increase in the optimum temperature by immobilization can be 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 invertase were determined by varying the concentration of sucrose in the reaction medium. These parameters are presented in Table 2. As expected, the K_m value increased with immobilization [19, 20]. 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. It was found that the V_{max} value of PAMSI was higher than that of the free invertase. Similar results involving change in V_{max} × values of enzyme after immobilization have been reported in literature [21, 22]. Normally a decreasing on V_{max} for an immobilized enzyme would be expected. This result shows that there is a not external and internal diffusional resistance for transport of substrate and product in PAMSI.

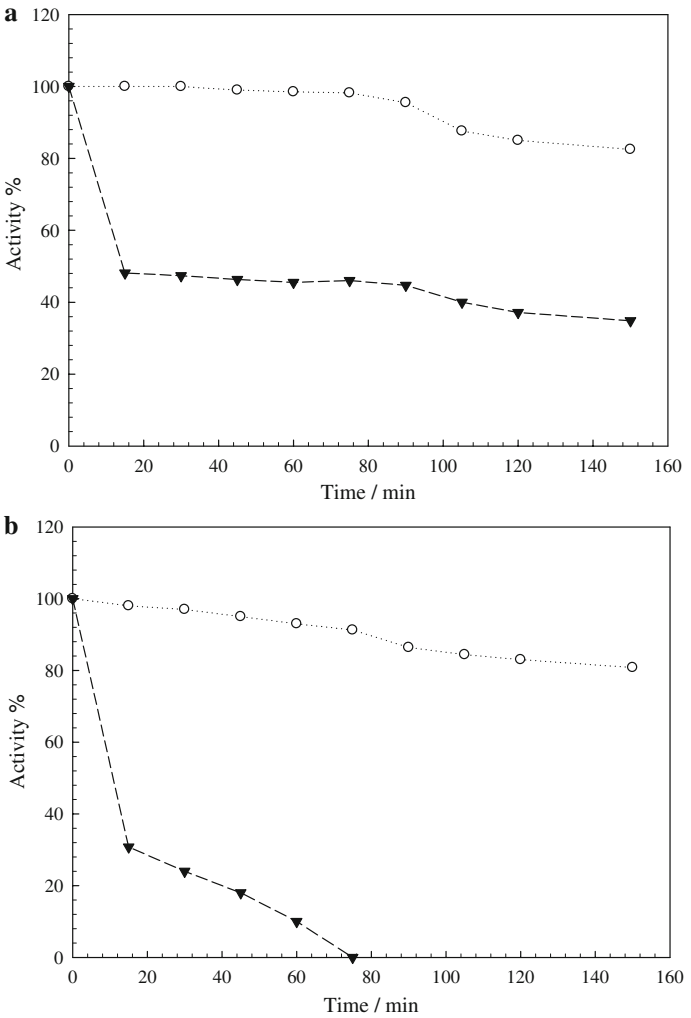


Fig. 5 **a** Thermal stability of invertase at 50 °C. (*filled inverted triangle* I, *open circle* PAMSI). **b** Thermal stability of invertase at 70 °C. (*filled inverted triangle* I,*open circle* PAMSI)

Thermal stability of free and immobilized invertase

Thermal stability studies of free and immobilized invertase were carried out at 50 and 70°C (Fig. 5a, b). The activity of free enzyme decreased with increase in temperature. While free invertase lost about 50% of its initial activity within first 10 min, immobilized invertase retained about 85% of its initial activity during 150 min incubation period at 50 °C (Fig 5a). It is shown in Fig 5b that the immobilized invertase preserved all its initial activity at 70 °C, whereas the free enzyme lost all its activity after 80 min incubation period. Immobilization of

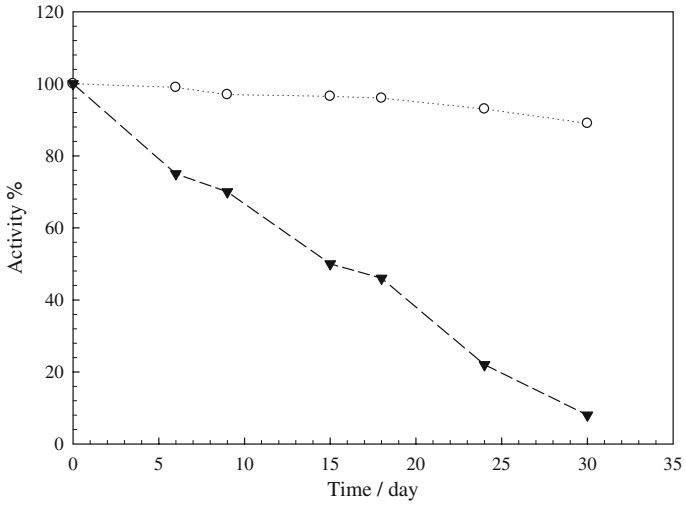


Fig. 6 Storage stability of invertase (*filled inverted triangle* I, *open circle* PAMSI)

invertase in PAMS composite preserve 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.

Storage stability of free and immobilized invertase

Storage stability of immobilized enzymes is important for their practical application. Free and immobilized invertase was stored in 50 mM acetate buffer (pH 4.8) at 4 °C and the activity measurements are carried out for a period of 30 days (Fig. 6). Free invertase lost about 90% of its initial activity within 30 days, whereas immobilized invertase retained about 90% of its initial activity 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 free and immobilized invertase

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 Fig. 7. Operational stability of the immobilized invertase was determined for 26 successive batch reactions at 40 °C. At the end of this period PAMSI retained about 50% of its activity. It was found that PAMSI has higher operational stability.

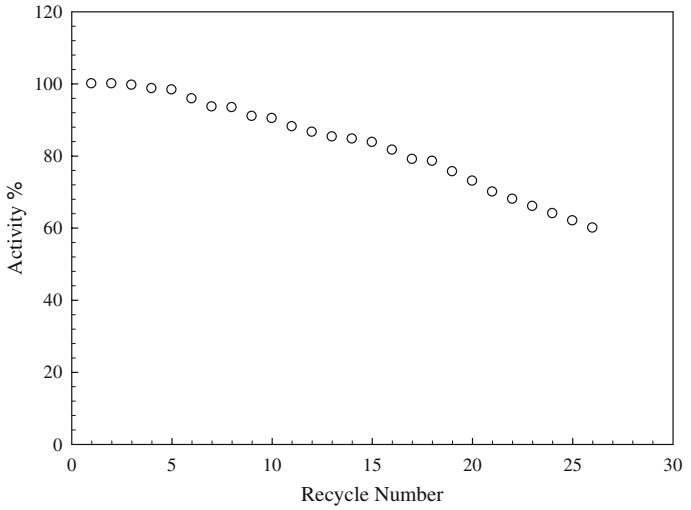


Fig. 7 Operational stability of PAMSI

Conclusions

A novel hydrogel–clay composite hydrogel; PAMS were prepared, characterized and used as support for the immobilization of invertase. Sepiolite was incorporated to poly(acrylamide/maleic acid) crosslinked with NNMBA during polymerization. Some swelling properties of these materials were investigated. Swelling degree of acrylamide hydrogel was increased with addition of maleic acid and sepiolite.

Invertase can be of great value in food and drink industries for hydrolysis of sucrose to form invert sugar. PAMS composite were used for invertase immobilization. The immobilized invertase displays significantly improved stability over the free form. The immobilized invertase was stable a wide range of pH's and temperatures. It showed better thermal and storage stabilities. It was observed that this immobilized enzyme has a high operational stability.

It can be concluded that PAMS is an appropriate matrix for invertase and PAMSI could be successfully used in a continuous system for the production of glucose and fructose from sucrose.

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