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THE PROPERTIES OF IMMOBILIZED INVERTASE ONTO A NEW SUPPORT MATERIAL; POLY(METHACRYLAMIDE/ MALEIC ACID) COPOLYMERIC HYDROGEL

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Abstract: Poly (methacrylamide / maleic acid) PM/MA and poly (methacrylamide) PM hydrogels were prepared aiming to be used as a support for invertase. Spectrophotometric, thermal analysis methods, swelling and diffusion experiments were used for the characterization of hydrogels. The swelling of PM/MA was higher than that of PM in water. The diffusion of water within the hydrogel was found to be *non-Fickian*. Invertase was immobilized onto PM and PM/MA (samples named PM-I and PM/MA-I respectively). The optimum pH values were found to be; 6.0, 5.0 and 5.5 for free invertase, PM-I and PM/MA-I respectively. The optimum temperature values were found to be 30 °C, 35 °C and 40 °C for free invertase, PM-I and PM/MA-I respectively. The Michaelis constant (K_m) and maximum velocity of the enzymes (V_{max}) were K_m: 11,75 mM, V_{max}: 1,95 μmol min^{-1} for free invertase, K_m: 67,24 mM, V_{max}: 60,6 μmol min^{-1} for PM-I and K_m: 74,55 mM, V_{max}: 18,12 μmol min^{-1} for PM/MA-I. PM/MA-I showed excellent thermal, operational and storage stability.

***Keywords*:** Invertase, Immobilization, Hydrogel, Methacrylamide, Maleic acid

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Introduction

Enzymes have low stability in aqueous solutions, therefore immobilized enzymes are used different industrial processes. The enzyme immobilization provides greater thermal, operational, storage and pH stability and continuous enzymatic activity. Furthermore, the immobilized enzymes can be easily separated at the end of the processes and reused in new catalytic production.

Invertase (E.C.3.2.1.26), also known as β-D-fructofuranosidase is a glycoprotein catalyzing the hydrolysis of sucrose to form invert sugar. Invert sugar is sweeter and does not crystallize at high concentrations, being widely used in food and drink industry. The 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.[1-5]

Hydrogels having three-dimensional polymer networks are used in enzyme immobilization as support materials. Hydrogels highly swell in water because of the hydrophilic nature of their functional groups. Being superabsorbent, hydrogels can absorb and retain a large volume of water or other biological fluids. Because of this feature, hydrogels are used applications in bioengineering, biomedicine, food industry, chromatography, water purification, separation processes and agriculture.[6-10]

In the present study, we describe the preparation and characterization of a novel copolymeric hydrogel; the poly(methacrylamide/maleic acid) hydrogel; PM/MA, used for immobilization of invertase. For the sake of comparison, poly(methacrylamide) hydrogel PM was prepared. The enzymatic performance of the immobilized enzymes at various temperatures and pH values was studied and compared with these of the free enzyme. The

kinetic parameters and thermal, storage, and operational stabilities of the immobilized enzymes were also investigated.

Results and Discussion

Preparation of PM/MA Hydrogel

Poly(methacrylamide/maleic acid) copolymeric hydrogel (PM/MA) as a novel supporting matrix for the immobilization of invertase was prepared by free-radical crosslinking and copolymerization of methacrylamide (M) and maleic acid (MA), with ethylene glycol dimethacrylate (EGDMA) as crosslinker, in aqueous solution. Ammonium persulphate (APS) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were used as reaction initiator and accelerator, respectively. For the polymerization reaction, the possible initial step is a reaction between M and the crosslinker molecules, by the transfer of an unpaired electron to the monomeric units, so that they become reactive. Another monomer can be attached and activated similarly, resulting in a three dimensional network. The purposed polymerization mechanism of PM/MA is shown in Figure 1. Methacrylamide homopolymer or metachrylamide/maleic acid copolymer can be polymerized and crosslinked by using a chemical initiator, such as ammonium persulfate (APS). APS forms free radicals by decomposing in the presence of tetramethyl ethylene diamine (TEMED), which acts as an accelerator for APS decomposition, due to the possibility to transform in a radical species itself during the reaction (first line from the scheme in Fig. 1).

The break of the peroxidic bonding also occurs, so the reaction produces two different radical species. The free radical reacts subsequently with metacrylamide monomer or maleic acid comonomer, breaking the double bond of the monomer or comonomer and generating a new free

radical by preserving an unpaired electron at the bottom of the formed chain (line 2, Fig. 1). This species then interacts with the crosslinker (Ethyleneglycole dimethacrylate, EGDMA), generating species with two radical sites in the molecule, which combine with each other, generating crosslinked metachrylamide/maleic acid copolymers.

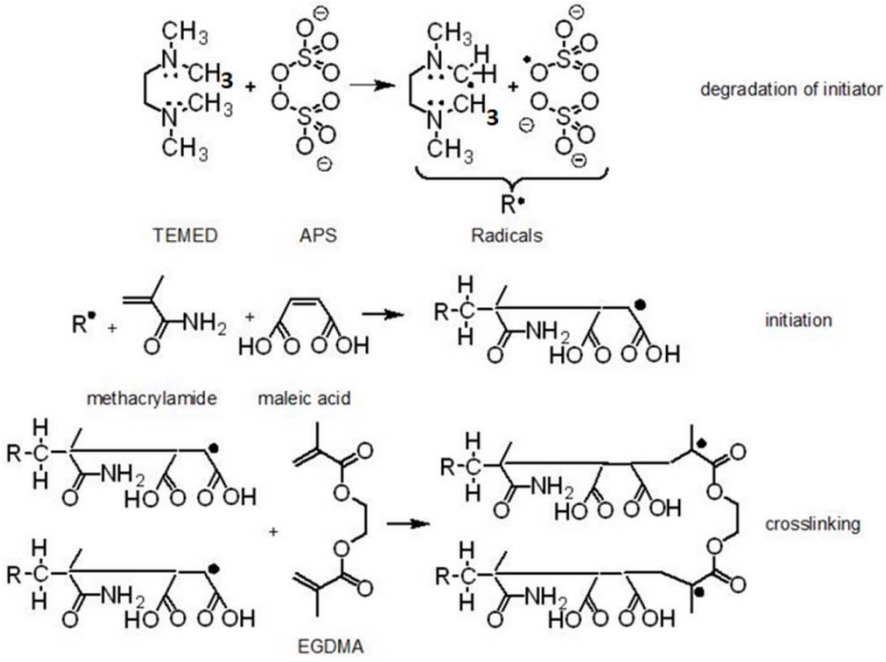


Figure 1. Possible polymerization mechanism of PM/MA.

The poly(methacrylamide) hydrogel (PM) was prepared by free-radical crosslinking in order to allow the comparison with PM/MA in the invertase immobilization.

Thermal Gravimetric Analysis of PM/MA Hydrogel

The thermal analysis of the hydrogels indicates a multistep decomposition (Figure 2). The first significant decomposition occurred at 198^{o}C for PM and 224^{o}C for PM/MA (T_1). This decomposition shows the loss of water adsorbed both on the surface and in the pores of the hydrogel.

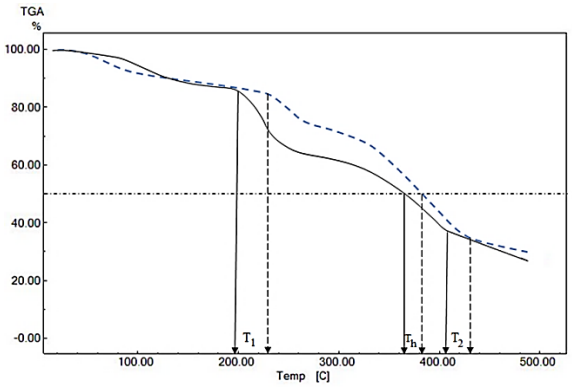


Figure 2. Thermograms of the hydrogels (PM: straight line; PM/MA: dashed line).

As shown in the Figure 2, the weight loss within the temperature ranges of 198−425 ^{o}C for PM and 224−445 ^{o}C for PM/MA can be attributed to the thermal decomposition of the organic network, starting with the amide groups and carboxyl groups from the methacrylamide and the crosslinker on the PM network. Above 425 ^{o}C for PM and 445 ^{o}C for PM/MA (T_2), a substantial mass loss appears, normally attributed to breakdown of the main chain of (co)polymer from the hydrogel.

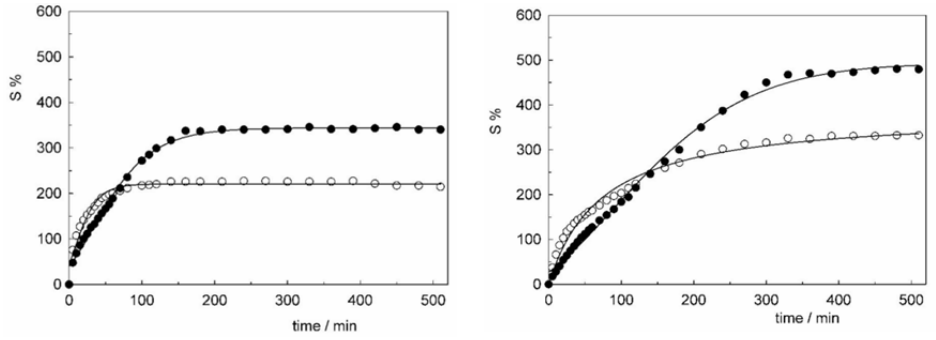
The half-life temperature (T_h) for polymers is defined as the temperature at which the mass loss in TG thermograms is 50 % [11]. From the TG curves, the values of 383 ^{o}C for PM and 404 ^{o}C for PM/MA were obtained. According to these results, the copolymerization reaction, respectively the addition of maleic acid in the preparation recipe of the hydrogel, increased the thermal stability of the product.

Swelling Experiments

The influence of the operating parameters (i.e. pH, temperature) on the hydrogels swelling performance was performed by measuring the percent of water retained in the hydrogel. The swelling capacity; (S%) was calculated using the following relation:

$$S\% = \frac{m_t - m_o}{m_t} x100 \quad (1)$$

where m_t is the mass of swollen hydrogel at time t and m_o is the mass of dry hydrogel at start time. The swelling curves of PM and PM/MA in distilled water are shown in Figure 3.



a) **b)**

Figure 3. Time depending swelling of the hydrogels in distilled water:
a) 25 °C, **b)** 60 °C (○; PM, ●; PM/MA).

The swelling of the hydrogels displays a Langmuir type increase in time, reaching a constant value after a certain value marking equilibrium swelling percent (S_{eq}). The experimental data from Figure 3 show that PM/MA has a higher swelling capacity than PM at both temperature values, 25 °C and 60 °C. The equilibrium swelling values, were, respectively,

220 % for PM and 350 % for PM/MA at 25 °C, 350 % for PM and 500 % for PM/MA at 60 °C. The ionic charge is important for hydrogel swelling capacities. Maleic acid contains many ionic groups that are carboxyl groups. The increase of the swelling capacity is due to the increased number of anionic groups in PM/MA compared to PM. The swelling capacity of the hydrogels increased with the temperature, since this factor favors the expansion of hydrogels.

The following equation was used to define the water model of diffusion in the hydrogels:[12-14]

$$F = k\,t^{n} \qquad (2)$$

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 diffusion exponent, indicating the transport mechanism. The experimental data were fitted to the equation above and the k values were calculated (Table 1). The values of n were between 0.50 and 1.00, so the diffusion of the fluids into PM and PM/MA proved to be *non-Fickian*. This is generally considered a consequence of the slow relaxation rate of the hydrogel.[12-14]

The values of the diffusion constant of the PM/MA are higher than those on PM at both temperature values, 25 ^{o}C and 60 ^{o}C (Table 1), i.e., the diffusion of the water in PM/MA is a little bit slower than in PM, but the swelling capacity is substantially higher on PM/MA.

Table 1. Diffusion parameters of the PM and PM/MA.

T / ^{o}C	PM		PM/MA	
	n	**k**	**n**	**k**
25	0.56	2.91	0.88	4.82
60	0.59	2.57	0.80	3.93

The variation in the swelling degree of the hydrogels with pH at two different temperatures is presented in Figure 4.

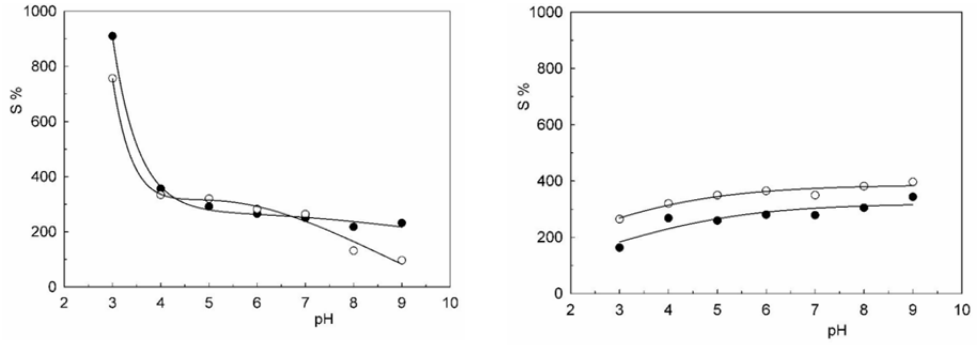


Figure 4. pH responsive swelling curve of the hydrogels:
a) PM; **b)** PM/MA (●; 25°C, ○; 60°C).

The swelling of PM strongly decreased with the increase of pH, especially up to the value of 4; the maximum was found at pH 3. In contrast, the swelling of PM/MA hydrogel the swelling of slightly increased with pH. The pKa value of the carboxyl group of MA is ~ 4.7, meaning that the carboxyl groups of PM/MA are ionized at pH > 4.7. Because of the ionization, the PM/MA swelling increased with pH. The maximum swelling of PM/MA was found at pH 9. At this pH, the carboxyl groups become completely ionized. The electrostatic repulsive force between the charged sites of hydrogel causes an increase in swelling. At acidic pH (pH < 4.7), the carboxyl groups of the hydrogel are protonated, the anion-anion forces are eliminated, so the hydrogel swelling is low at acidic pH.

Preparation and Characterization of Immobilized Invertase

In the immobilization experiments, poly(methacrylamide)-invertase (labeled PM-I) and poly(methacrylamide/maleic acid)-invertase (labeled PM/MA-I) were prepared using PM and PM/MA, respectively, as supports. The immobilization of invertase onto PM/MA is shown in Figure 5 schematically.

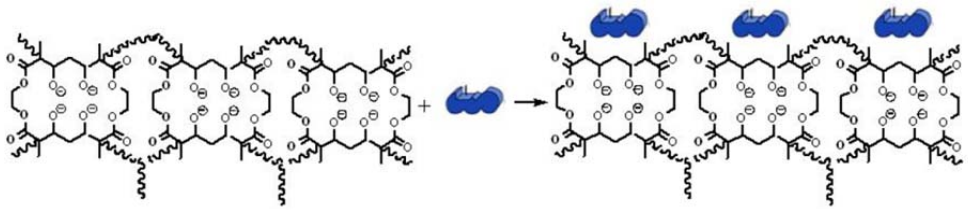


Figure 5. Immobilization of invertase onto PM/MA.

After the immobilization, the optimum pH value, the optimum temperature, the kinetic parameters, thermal stability, storage stability and operational stability of the two samples were investigated.

Optimum pH, Optimum Temperature and Kinetic Parameters of Invertases

Enzymes are amphoteric molecules, because they have many acidic and base groups. The charges on these groups change with the pH of their surroundings. Therefore, the activity dependence on the pH of the free and immobilized invertase was compared while keeping constant the other parameters on the sucrose hydrolysis reaction (300 mM sucrose, 37 ^{o}C). The results are presented in a normalized form with the highest value of each set being assigned as the value of 100 % activity (Figure 6). As seen from the Figure 6, the immobilized enzymes have a of high activity over a broad pH range.

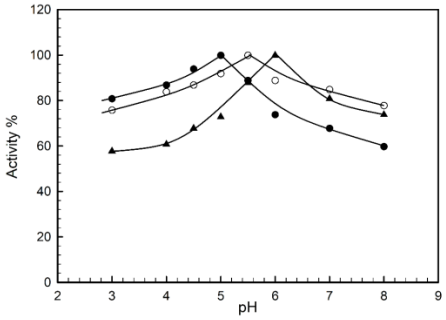


Figure 6. pH profiles of the invertases (▲; free invertase, ●; PM-I, ○; PM/MA-I).

The optimum pH values deduced from the Figure 6 are shown in Table 2.

Table 2. Optimum pH, optimum temperature, K_m and V_{max} parameters of Invertases.

Enzyme	Optimum pH	Optimum Temperature/ ^oC	K_m mM	V_{max} µmol min^{-1}
Free I	6.0	30	11.75	1.95
PM−I	5.0	35	67.24	60.6
PM/MA−I	5.5	40	74.55	18.12

The optimum pH of the immobilized enzyme has shifted to the acidic region. This shift is possibly due to the secondary interactions between the enzyme and the polymeric matrix, showing that the immobilized invertase is more stable than the free invertase at acidic pH. The immobilization may change the enzyme structure; therefore, this process can modify the optimum pH value. Similar results were found in invertase immobilization studies,[3,15,16] as well as in our studies (the optimum pH values as 5.5, 4.5, 6 and 6 for poly(acrylamide)-sepiolite−invertase, poly(acrylamide/acrylic acid)-sepiolite−invertase, poly(acrylamide/itaconic acid)-sepiolite−invertase, and poly(acrylamide/maleic acid)-sepiolite−invertase respectively).[4,5]

In order to investigate the effects of temperature, the initial activities of free invertase, PM-I and PM/MA-I were determined at temperature ranging between 20-70 ^oC, while keeping constant the other parameters (300 mM sucrose, pH 5). The results are presented in a normalized form with the highest value of each set being assigned as 100% activity (Figure 7).

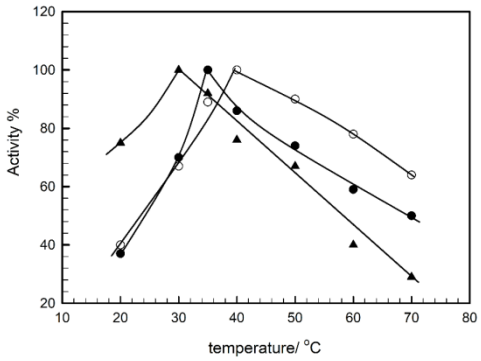


Figure 7. Temperature profiles of the invertases (▲; free invertase, ●; PM-I, ○; PM/MA-I).

The optimum temperature values found from Figure 7 are shown in the Table 2. The optimum temperature of invertase activity has increased with the immobilization. The increase in the optimum temperature of the invertase-mediated reaction by immobilization can be a result of changes in the physical and chemical properties of the enzyme. We have found similar results for the optimum temperature in our other invertase immobilization studies.[4,5] The optimum temperature of free invertase was 30 ^{o}C, while the optimum temperature of PM/MA-I was 40 ^{o}C (Table 2). These results show that PM/MA-I is more stable than free invertase at high temperature and this fact is suitable for the industrial use of invertase.

The initial rates of sucrose hydrolysis were obtained for various concentrations of the sucrose solutions for free and immobilized invertase. These data were plotted according to the Lineweaver–Burk method (Figure 8). The kinetic parameters, K_m and V_{max} deduced from the data in Figure 8 are shown in the Table 2.

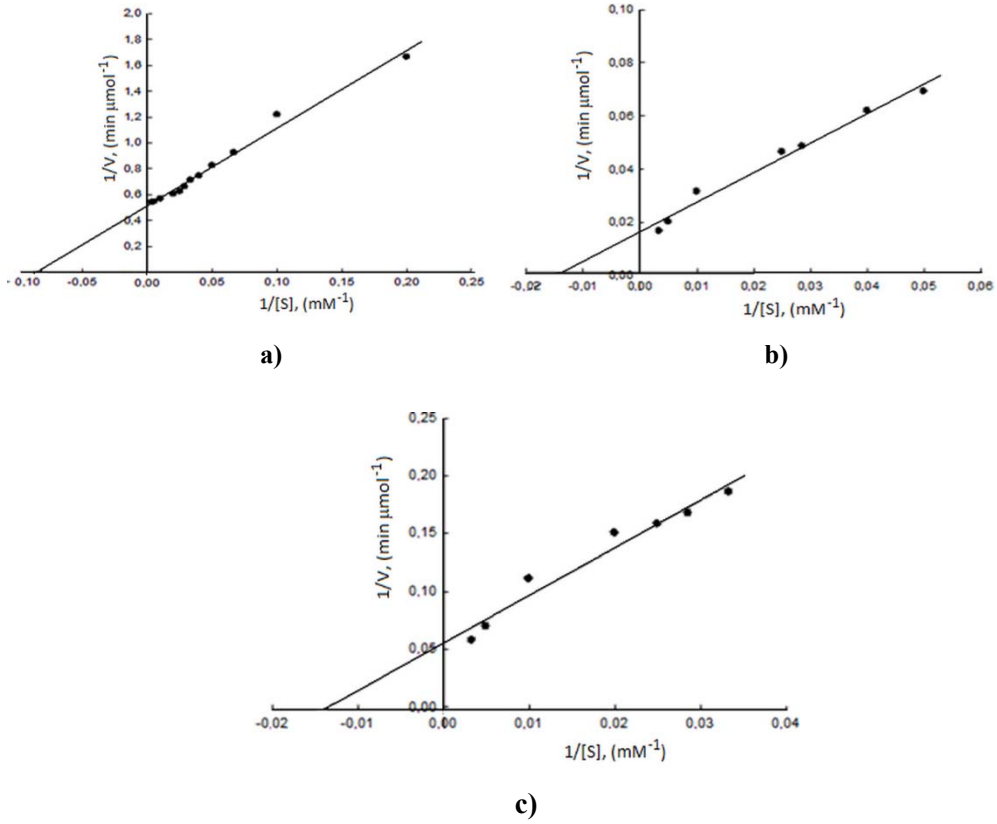
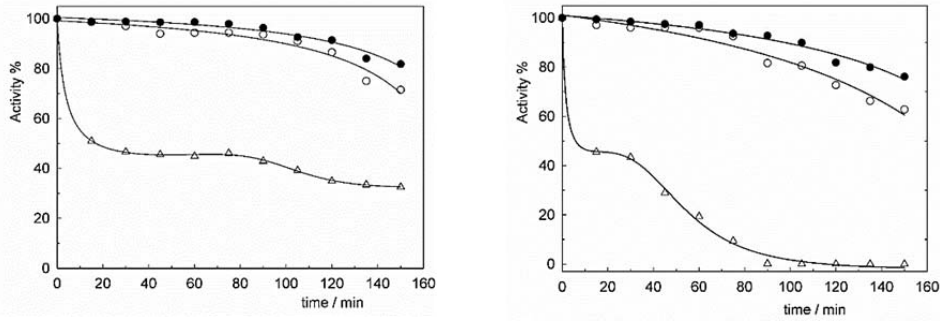


Figure 8. Lineweaver–Burk plots for: **a)** free catalase, **b)** PM-I, and **c)** PM/MA-I.

As expected, the K_m value increased with immobilization (Table 2).[17,18] This result can be attributed to the limited accessibility of the sucrose molecules to the active sites of the immobilized invertase, as a result of the conformational change that invertase suffer by the immobilization or to the hindrance due to the hydrogel network. It was found that the V_{max} value of PM-I and PM/MA-I were higher than those of the free invertase (Table 2). Similar results involving changes in V_{max} values of enzyme after immobilization have been reported in literature.[5,19,20] Normally a decrease of V_{max} for an immobilized enzyme would be expected. This result shows that there is no external and internal diffusional resistance for the transport of substrate and product in and out the PM-I and PM/MA-I supports.

Thermal Stability of Free and Immobilized Invertase

The thermal stability studies of free and immobilized invertase were carried out at 50 ˚C and 70 ˚C (Figure 9).



a) **b)**

Figure 9. Thermal stability of free invertase, PM-I and PM/MA-I; **a)** 50 ^{o}C; **b)** 70 ^{o}C (Δ; Free invertase, ○; PM-I, ●; PM/MA-I).

The activity of the free enzyme decreased quickly in time, especially at the higher temperature values. While free invertase lost about 50 % of its initial activity within the first 10 min, PM-I preserved about 70 % and PM/MA-I 85 % of its initial activity after a duration of 150 min at 50 ^{o}C (Figure 9a). The activity of the free enzyme was completely lost at 70 ^{o}C after 90 minutes, while the immobilized enzymes lost much slighter their activity; PM/MA-I kept about 75% of its activity and PM-I about 60 % after 150 minutes (Figure 9b).

Clearly, PM/MA-I was more stable than both free invertase and PM-I at high temperatures (it has better thermal stability). According to these results, we can say that the immobilization of invertase in PM/MA hydrogel preserved the tertiary structure of the enzyme and protects it from conformational changes caused by the environmental effects.

Storage Stability of Free and Immobilized Invertase

The storage stability of the immobilized enzymes is important for their potential practical application. Free and immobilized invertases were stored in 50 mM acetate buffer (pH 4.8) at 4 ^{o}C and the activity measurements were carried out at regular intervals for 30 days (Figure 10). Free invertase lost about 95 % of its initial activity within 30 days, whereas PM-I and PM/MA-I preserved about 78 %, respectively 85 % of their initial activity during this incubation period. The decrease in activity can be considered a time-dependent natural process, which is avoided to a significant extent by immobilization.

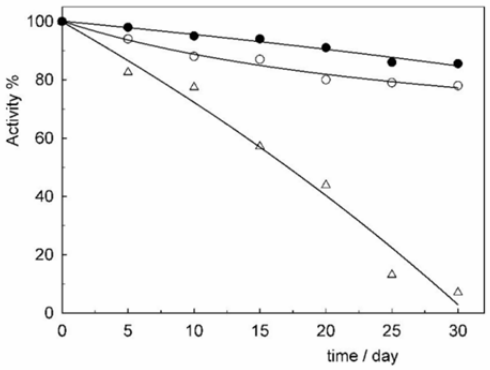


Figure 10. Storage stability of invertases (Δ; Free invertase, ○; PM-I, ●; PM/MA-I).

Operational Stability of Immobilized Invertase

The economical application of enzymes is very important. For the mass production of a desired product by enzymatic catalysis, the continuous process is more efficient than the discontinuous (batch) version. The operating stability of the two immobilized enzymes is shown in Figure 11; their performance was determined for 25 successive batch reactions. At the end, PM-I and PM/MA-I still kept about 45 % and 55 % of their activities,

respectively. PM/MA-I had a higher operational stability than the free invertase and PM-I.

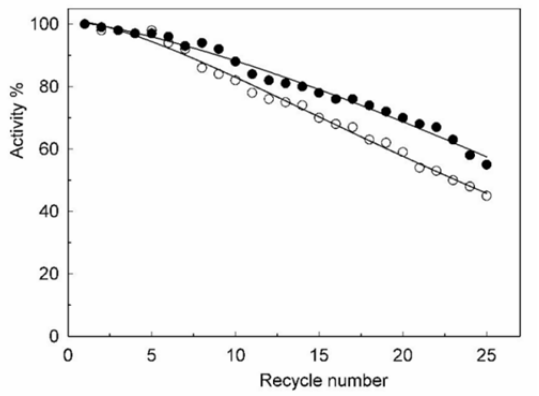


Figure 11. Operational stability of PM-I and PM/MA-I (○; PM-I, ●; PM/MA-I).

Experimental

Materials

Methacrylamide (M), ethylene glycol dimethacrylate (EGDMA), ammonium persulphate (APS), Na_2HPO_4, NaH_2PO_4, CH_3COOH, CH_3COONa were obtained from Merck (Darmstadt, Germany). Sucrose, invertase (*S. cerevisiae*), maleic acid (MA), *N,N,N',N'*-tetramethylethylene diamine (TEMED), glucose assay kit (GAGO-20) were obtained from Sigma (St. Louis. MO, USA).

Preparation of the Hydrogels

For the preparation of poly(methacrylamide) hydrogel (PM), $5x10^{-2}$ mole of M and $2.5x10^{-3}$ mole of EGDMA as crosslinker were dissolved in distilled water then mixed thoroughly. After the addition of $2.5x10^{-3}$ mole APS as initiator and $2.5x10^{-3}$ mole of TEMED as accelerator, the mixture was injected through a syringe to PVC straws of 3 mm diameter. For the preparation of poly(methacrylamide/maleic acid) copolymeric hydrogel

(PM/MA), $5x10^{-3}$ mol MA is added to the fore-mentioned mixture. A gel formed at ambient temperature.

The hydrogel composites rods were cut into pieces of 4–5 mm in length and washed with distilled water and 50 mM (pH 4.8) acetic acid-sodium acetate buffer solution in order to remove the unreacted monomer and initiator, dried in air under vacuum.

Thermal Gravimetric Analysis of the Hydrogels

For the thermal characterization of PM and PM/MA, the TG experiments were carried out in the temperature range 25−500 ^{o}C under nitrogen gas flow rate of 25 mL min^{-1}, with a heating rate of 10 ^{o}C min^{-1}, by using the thermogravimetric analyzer Shimadzu TGA50.

Swelling Experiments of the Hydrogels

Dynamic swelling experiments were done at 25 ^{o}C and 60 ^{o}C. Approximately 6-9 mg dry PM and PM/MA were put in 30 mL of distilled water. The swollen hydrogels were removed from the water bath at regular time intervals, wiped with filter paper, weighed and placed back in the same bath. Swelling experiments were performed till the hydrogels reached a stable weight (6 h). The equilibrium swelling experiments on PM and PM/MA were done in the buffer solutions with pH values ranging from 3 to 9, at 25 ^{o}C and 60 ^{o}C, for the investigation of pH sensitive swelling. 50 mM CH_3COOH− CH_3COONa and 50 mM Na_2HPO_4−NaH_2PO_4 buffer solutions were used.

Immobilization of Invertase onto the Hydrogels

50 mg weighed dry PM and PM/MA were immersed in 50 mL solution containing 1 mg mL^{-1} invertase. The immobilization of invertase

was carried out at 22 °C for 3 h by continuous stirring (150 rpm) in the reaction medium. The resulted product was removed, washed firstly with distilled water, then with acetate buffer (50 mM, pH 4.8) in order to remove the remained invertase. The samples were stored at 4 °C in fresh buffer until use.

Activity Assays of Free and Immobilized Invertase

The activities of both free invertase (I) and immobilized invertases, PM-I and PM/MA-I, were determined by measuring the amount of glucose released from the invertase-catalyzed hydrolysis of sucrose per unit time. The enzyme activity was assayed in the presence of sucrose as 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 (0.1 mL of 1 mg mL^{-1} invertase solution). After 15 min of reaction, the released amount of 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 the amount of enzyme which hydrolyzes 1 μmol of sucrose to glucose per minute.[21,22] The same assay medium was used to determine the activity of the immobilized enzyme. The enzyme reaction started by the introduction of 1.0 g PM-I and PM/MA-I into the assay medium at 37 °C. After 15 min, the reaction was terminated by the removal of the hydrogels from the reaction mixture and the produced amount of glucose was determined.

The activity assays were carried out over the pH range of 3−8 and temperature range of 20–70 °C to determine the pH and temperature profiles of free and immobilized enzymes. 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 (Michaelis constant) and V_{max} (maximum reaction rate) values of the free enzyme were determined by measuring the initial rates of the reaction with sucrose in acetate buffer (50 mM, pH 5.0) at 30 °C. The kinetic parameters of the immobilized invertases were determined in a batch system by varying the concentrations of sucrose (5–300 mM) in their optimum pH and optimum temperature values, determined previously.

Thermal Stability of Free and Immobilized Invertases

The thermal stability of free and immobilized invertases was ascertained by measuring the residual activity of the enzyme exposed to various temperatures (50 °C and 70 °C) in 50 mM acetate buffer (pH 4.8) for 150 min.

The Storage Stability of Free and Immobilized Invertases

In order to investigate the effect of storage time on invertase stability, the activity of free and immobilized invertases after storage in 50 mM acetate buffer (pH 4.8) at 4 °C was measured in batch operation mode with the experimental conditions mentioned above.

Operational Stability of Immobilized Invertases

The preservation of the immobilized enzyme activity was tested as described in activity assays of invertase. After each reaction run, PM-I and PM/MA-I were removed and washed with distilled water and 50 mM acetate buffer (pH 4.8) to remove any residual substrate from the hydrogels. They were reintroduced into fresh reaction medium and enzyme activity was detected.

Conclusion

The poly (methacrylamide/maleic acid) copolymeric hydrogel (PM/MA) prepared to serve as a new support material for the immobilization of invertase showed improved behavior, higher thermal stability and better swelling properties compared with the poly(methacrylamide) hydrogel (PM). The hydrophilicity of PM/MA is higher than that of PM, because the addition of maleic acid, therefore PM/MA swelled better than PM. The PM/MA swelling is sensitive to pH and temperature.

Immobilized invertase onto PM/MA was prepared (PM/MA-I). It showed an improved behavior compared with the free enzyme and invertase immobilized onto PM (PM-I). PM/MA-I provided significantly improved stability over the free form. PM/MA-I showed excellent thermal, operational and storage stability. The main advantage of enzyme immobilization is its easy separation and reusability, together with the better stability to pH differences and high temperature values used in the operating processes.

The new immobilized invertase, PM/MA-I, could be successfully used at industrial scale in a continuous system for the production of glucose and fructose from sucrose.

Acknowledgements

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