β-Galactosidase Immobilization into Poly(hydroxyethyl methacrylate) Membrane and Performance in a Continuous System

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ABSTRACT: The activity of β-galactosidase immobilized into a poly(2-hydroxyethyl methacrylate) (pHEMA) membrane increased from 1.5 to 10.8 U/g pHEMA upon increase in enzyme loading. The K_m values for the free and the entrapped enzyme were found to be 0.26 and 0.81 m*M,* respectively. The optimum reaction temperatures for the free and the entrapped β-galactosidase were both found to be 50°C. Similarly, the optimum reaction pH was 7.5 for both the free and the entrapped enzyme. The immobilized β-galactosidase was characterized in a continuous system during lactose hydrolysis and the operational inactivation rate constant (k_{iop}) of the entrapped enzyme was found to be 3.1×10^{-5} min^{-1}. © 1999 John Wiley & Sons, Inc. J Appl Polym Sci 72: 1367–1373, 1999

Key words: β-galactosidase; enzyme immobilization; pHEMA; lactose hydrolysis; enzyme-membrane reactor

INTRODUCTION

Polymer membranes play an important role in the immobilization of enzymes, especially in the construction of enzyme-membrane reactors and enzyme electrodes.[1–4] Suitable matrices include hydrogels that are highly compatible for immobilization of enzymes due to their hydrophilic nature. Poly(hydroxyethyl methacrylate) (pHEMA) hydrogels are among the major synthetic polymers approved by federal agencies like the Food and Drug Administration (FDA) in the United States for biomedical, pharmaceutical, and industrial applications. It is a nontoxic, hydrophilic, and biocompatible material which is often employed as an enzyme carrier.[5–9] It has been used in previous enzyme immobilization studies either by adsorption onto its membrane or by covalent binding onto its microspheres.[5,6]

β-Galactosidase, which hydrolyzes the main milk sugar, lactose, into its constituent monosaccharides, glucose, and galactose, has long been attracting attention because of its great potential for use in the dairy industry in the prevention of crystallization of lactose in milk products and in the reduction of lactose content in milk processed for consumers who are intolerant of lactose. This hydrolytic reaction could also be applied in the upgrading of whey, which is a by-product of cheese making and whose disposal currently constitutes a considerable pollution problem.[10–12] For the treatment of large volumes of milk or whey, efficient and inexpensive processing is desired.

Several methods have been developed for the preparation of immobilized β-galactosidase,

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each having its own advantages and disadvantages.[13–17] After immobilization, some changes were observed in the enzymatic activity, optimum pH, temperature, and affinity to substrate and stability. Generally, the changes in the enzymatic activity and in the affinity to substrate were unfavorable,[15,16] whereas the optimum temperature and the stability were improved. The extent of these changes depended on the nature of the enzyme, type of support, and the immobilization conditions.

In the present study, an immobilized β-galactosidase-membrane system was prepared by UV-initiated photopolymerization in the presence of an initiator (α,α′-azobisisobutyronitrile). The immobilized enzyme-membrane system was applied to a enzyme reactor to study the behavior of the enzyme in a continuous flow system.

EXPERIMENTAL

Materials

β-Galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23, Grade IV from *E. coli,* 300 units/mg), was purchased from the Sigma Chemical Co. (St. Louis, MO) and was used without further purification. Glucose oxidase (EC.1.1.3.4, Type II), peroxidase (EC. 1.11.1.7, Type II), *o*-nitrophenyl β-D-galactopyroniside (ONPG), bovine serum albumin (BSA), folin-ciocalteu reagent, lactose, and *o*-dianisidine dihydrochloride were purchased from Sigma and were used as received. 2-Hydroxyethyl methacrylate (HEMA) was obtained from Sigma and distilled under reduced pressure in the presence of hydroquinone and stored at 4°C until use. α-α′-Azobisisobutyronitrile (AIBN) was purchased from Fluka AG (Buchs, Switzerland) and used as received. All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany).

Immobilization of β-Galactosidase into pHEMA Membrane

A 5-mL mixture consisting of a 2-mL vacuum-distilled monomer of HEMA, 5 mg of AIBN (as the initiator), and phosphate buffer (3 mL, 0.1*M,* pH 7.5) containing 0.125–3.0 mg of β-galactosidase was placed in a test tube and mixed by vortexing. The mixture was then poured into a round glass mold (diameter: 9 cm) and exposed to long-wave UV (360 nm) for 30 min in a nitrogen atmosphere. Then, the whole enzyme membrane was washed with phosphate buffer (0.1*M,* pH 7.5) and cut with a perforator into small disks (diameter: 1.0 cm, thickness about 0.06 cm) and stored at 4°C until use.

Swelling Behavior of Enzyme Membrane

The swelling behavior of the enzyme membrane was determined in distilled water. Dry membrane pieces (ca. 1 g) were placed in distilled water and kept at a constant temperature: 25 ± 0.5°C. Swollen membranes were periodically removed and weighed by an electronic balance (Shimadzu, EB.280 ± 1.10^{-3} g). The water content of the swollen membrane was calculated using the following expression:

$$\text{Swelling ratio (\%)} = [(W_s - W_0)/W_0] \times 100 \quad (1)$$

where W_0 and W_s are the weights of the membrane before and after swelling, respectively.

Determination of Entrapment Efficiency

The amount of protein in the native enzyme preparation and in the membrane wash solutions were determined according to the method of Lowry et al.[18] and the entrapped enzyme was calculated from the mass balance using a calibration curve prepared with a BSA solution (0.02–0.2 mg/mL).

Activity Assays

The activities of the free and the immobilized β-galactosidase were measured spectrophotometrically according to the procedure of Craven et al.,[19] which essentially measured the increase in the amount of *o*-nitrophenol resulting from the hydrolysis of the artificial substrate ONPG at 405 nm by the use of a UV-visible spectrophotometer (Shimadzu 1601, Japan).

Free Enzyme

The reaction medium contained phosphate buffer (1 mL, 0.1*M,* pH 7.5, containing 3 m*M* $MgCl_2$), ONPG (0.5 mL, 14 m*M*), and distilled water, 1.4 mL. The reaction was started by the addition of the enzyme solution (0.1 mL 6.25×10^{-3} mg/mL) into the assay medium. The absorbance change of the medium was recorded at 25°C.

Immobilized Enzyme

In the determination of entrapped enzyme activity, the same assay medium was used. The enzymatic reaction was started by the addition of four enzyme–membrane disks into the assay medium and the reaction was carried out at 25°C in a shaking water bath for 10 min. The absorbance of the medium was then recorded at 405 nm. One unit of β-galactosidase is defined as the amount of enzyme which hydrolyzes 1.0 μmol of the artificial substrate ONPG per minute at 25°C in the phosphate buffer at pH 7.5. These activity assays were also carried out over the pH range 4.0–9.5, in the temperature range 20–65°C, and in the 0.17–4.67 m*M* ONPG concentration range to characterize the free and the entrapped enzyme.

Lactose Hydrolysis in a Continuous System

The reactor (length 6 cm, diameter 3.0 cm, total volume 42.4 mL) was made from Pyrex® glass. The enzyme-carrying membrane (24 g) was packed into the reactor, yielding a void volume of about 22.2 mL. To determine the effect of the substrate concentration on reactor productivity, a lactose solution (100–400 m*M*) in the phosphate buffer was introduced to the reactor at a flow rate of 40 mL/h using a peristaltic pump (Cole Parmer, Model 7521-00, USA) through the lower inlet part. The solution leaving the reactor was collected in a fraction collector and was assayed for β-galactosidase activity at the end of each hour. Lactose hydrolysis performance of the reactor was determined by measuring the glucose content of the effluent according to a method described previously.[6] The assay mixture contained glucose oxidase (25 mg), peroxidase (6.0 mg), and *o*-dianisidine (13.2 mg) in the phosphate buffer (100 mL, 0.1*M,* pH 7.0). An aliquot of the assay mixture (2.4 mL) and the enzymatically hydrolyzed sample (0.1 mL) were mixed and then incubated at 35°C for 30 min in a water bath. After the addition of sulfuric acid (1.5 mL, 30%), the absorbance was measured at 525 nm.

Calculation of the Performance of the Immobilized Enzyme in the Bed Reactor

The performance of immobilized β-galactosidase in the bed reactor can be described under steady-state conditions using the integrated form of the Michaelis–Menten equation[20]:

$$-d[R]/dt = V_{max}[R]/(K_m + [R]) = V_{max}/\{(K_m/[R]) + 1\} \quad (2)$$

where $[R]$ is the reactant concentration (M) in the reactor; V_{max}, the maximum rate of the reaction ($M\ s^{-1}$); K_m, the Michaelis constant (M); and t, the time (s).

For a bed reactor, an integrated equation can be written by replacing the reaction time with the residence time, V_{tot}/Q [where V_{tot} is the reactor volume (dm^3) and Q is the volumetric flow rate ($dm^3\ s^{-1}$), the time which each fluid element spends in the reactor]. The voidage of the reactor can be expressed as $\varepsilon = V_1/V_{tot}$, where V_1 is the volume of the enzyme membrane in the system (dm^3) and eq. (2) can be rearranged as

$$V_{max}V_{tot}\varepsilon/Q = K_m \ln([R_0]/[R]) + ([R_0] - [R]) \quad (3)$$

where $[R_0]$ is the reactant concentration in the feed (M). The performance equation for the enzyme reactor [eq. (3)] can be rearranged for the calculation of the kinetic constants from the experimental data:

$$[R_0]\,X = K_m \ln(1 - X) + V_{max}V_{tot}\varepsilon/Q \quad (4)$$

where X is defined as $([R_0] - [R])/[R_0]$. Thus, plotting the experimentally obtained values of $[R_0]X$ versus $\ln(1 - X)$ will give a graph having a slope of K_m and an intercept of $V_{max}V_{tot}\varepsilon/Q$.

Effect of Flow Rate

The effect of the flow rate on the reactor performance was studied by varying the flow rate in the range of 20–60 mL/h at 25°C for 6 h, while keeping constant the concentration of lactose 200 m*M* in the phosphate buffer (0.1*M,* pH 7.5).

Operational Stability of Entrapped β-Galactosidase

To test the operational stability of the immobilized enzyme, the reactor was loaded with immobilized β-galactosidase and operated at 25°C for 48 h. The feed solution contained lactose (200 m*M*) in the phosphate buffer (0.1*M,* pH 7.5) and was introduced at a flow rate of 40 mL/h. The samples (0.1 mL) were removed 1-h time intervals from the reaction effluent and the hydrolysis rate of the lactose was determined as described above.

RESULTS AND DISCUSSION

Immobilization and Kinetic Constants

In this study, the pHEMA membrane was selected as the carrier matrix because of its high

mechanical strength, high stability, nontoxicity, and high water content, which provides a microenvironment similar to that *in vivo* for the enzymes.[21] The variation in the swelling degree of the pHEMA enzyme membrane with time is presented in Figure 1. As seen here, swelling occurs rapidly and the equilibrium swelling is reached in about 20 min. The equilibrium swelling value of the enzyme membrane was about 62% (w/w). The SEM micrographs given in Figure 2(A,B) show the cross-sectional and surface structures of the enzyme membrane, respectively. As seen in the figures, the enzyme membrane exhibits a rough external surface and a highly open pore structure, which may lead to a high surface area for diffusion of both substrate and product.

No enzyme leakage was observed during washing of the freshly prepared enzyme membrane or during activity assays in the batch mode and continuous operation. This indicated that the entrapment process was irreversible. As shown in Figure 3, the activity of the immobilized β-galactosidase into the pHEMA membrane increased from 1.5 to 10.8 U/g pHEMA upon increase in enzyme loading. The highest activity retention (9.5%) was obtained with a loading of 27 μg enzyme/g pHEMA membrane, and the lowest activity retention (2.0%), with a loading of 650 μg enzyme/g pHEMA membrane. These recovered activities are low, in general, but similar low-activity retention was also reported by other researchers.[12–17] Carrara and Rubiolo reported that the activity retention of covalently linked β-galactosidase on chitosan was 10.7%.[14] Bodalo et al.[16] reported that the activity retention of entrapped β-galactosidase was between 2.0 and 5.2% in alginate and *k*-carrageenan, respectively. The overall activity retention of the entrapped β-galactosidase was decreased as the enzyme loading increased. This was also observed in many previous studies.[5–8] Several explanations could be offered for these results, but probably the overall activity is adversely influenced by the oversaturation of the enzyme within the pore space of the matrix, and as a result, substrate diffusion limitations occur.[21]

The kinetic parameters of free and entrapped enzyme activities were determined using the artificial substrate ONPG in a concentration range

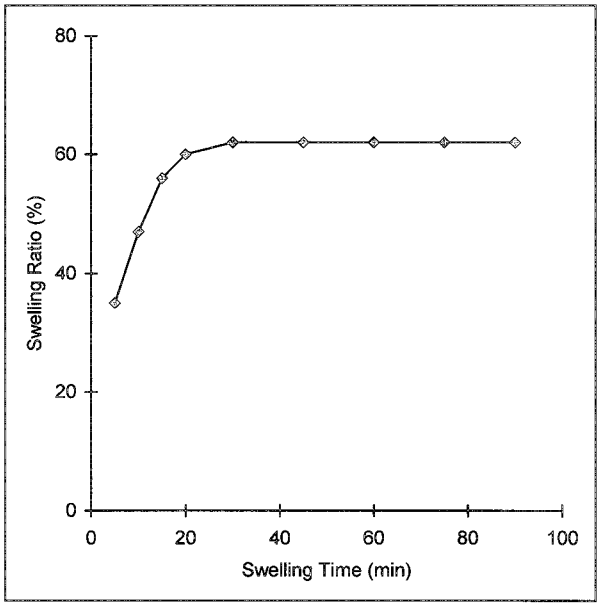

Figure 1 Swelling of enzyme membrane as a function of time.

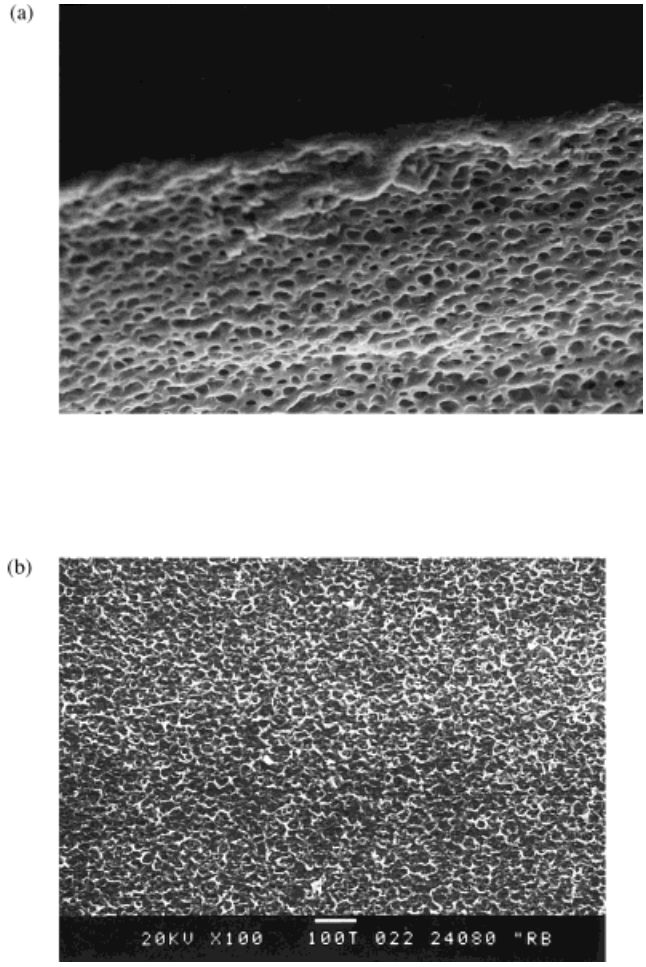

Figure 2 SEM micrographs of enzyme membrane: (A) cross section; (B) surface.

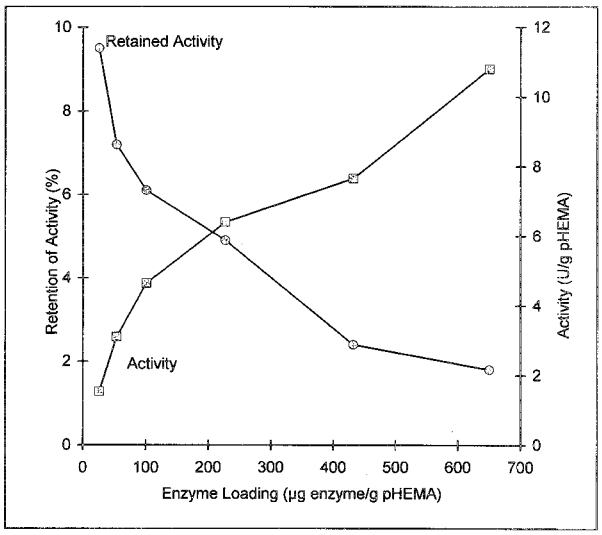

Figure 3 Effect of loading on the activity retention and activity of the enzyme membrane.

of 0.17–4.67 m*M*. A Lineweaver–Burk plot was used for the calculation of the apparent Michaelis constant, K_m, and the maximum velocity, V_{max}, of the free and entrapped β-galactosidase. K_m and V_{max} for the free enzyme were found to be 0.26 m*M* and 266 U/mg, respectively. As expected, the K_m and V_{max} values were significantly changed after entrapment in the pHEMA membranes. The K_m value (0.81 m*M*) was threefold higher and V_{max} decreased ninefold (30 U/mg) upon immobilization. Similar significant changes in the K_m value were also reported in the literature for immobilized β-galactosidase.[5–7,22] Park and Hoffman,[21] and Bodalo et al.[16] reported that the apparent K_m value of β-galactosidase upon entrapment in *N*-isopropylacrylamide and in alginate gel were increased about two- and threefold with respect to the free enzyme, respectively. The change in K_m reflects a change in the affinity of the enzyme to its substrate and this probably was caused by the restriction in the conformational change of the enzyme upon entrapment in a matrix consisting of polymer chains.

Effect of Temperature and of pH on the Catalytic Activity

The temperature dependence of the activities of the soluble and immobilized β-galactosidase were studied in the phosphate buffer (0.1*M,* pH 7.5) in the temperature range 25–65°C (Fig. 4). The data revealed bell-shaped curves with optimum activity at 50°C for free and entrapped enzymes, but the one for the immobilized enzyme was significantly broader. The activity of both free and immobilized β-galactosidase increased with increasing temperature up to 50°C and then declined with further increase in the temperature. A plateau region is seen for immobilized enzyme at temperature between 45 and 55°C that was not apparent for the free enzyme. Arrhenius plots in the temperature range from 25 to 50°C appeared linear and activation energies from the plots were found to be 1.18 and 1.26 kcal mol^{-1} for the free and immobilized β-galactosidase, respectively. The activation energy of β-galactosidase was not increased significantly upon immobilization. This result suggests that the enzyme structure was not altered during the immobilization processes.

It is well known that pH plays an important role on enzyme activity. The effect of pH on the activity of the free and immobilized β-galactosidase was studied at various pH's at 25°C. The reactions were carried out in acetate (pH 4.0–5.0), phosphate (pH 6.0–8.0), and tris-acetate (pH 8.2–9.0) buffers and the results are presented in Figure 5. The optimum activities for both the free and the immobilized enzymes were observed at the same pH (7.5). Generally, the pH profile is displaced toward more alkaline or acid pH values for negatively or positively charged matrices. In our case, pHEMA is a neutral hydrogel; for this reason, the pH profile is coincident for free and immobilized enzymes.

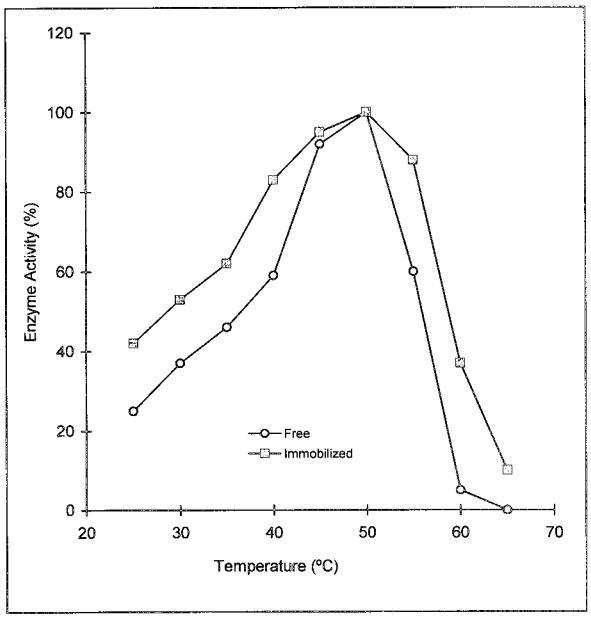

Figure 4 Temperature profiles of free and immobilized β-galactosidase.

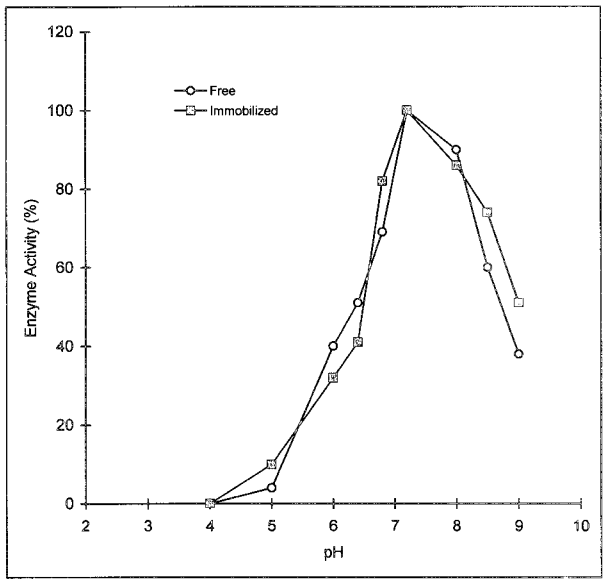

Figure 5 pH profiles of free and immobilized β-galactosidase.

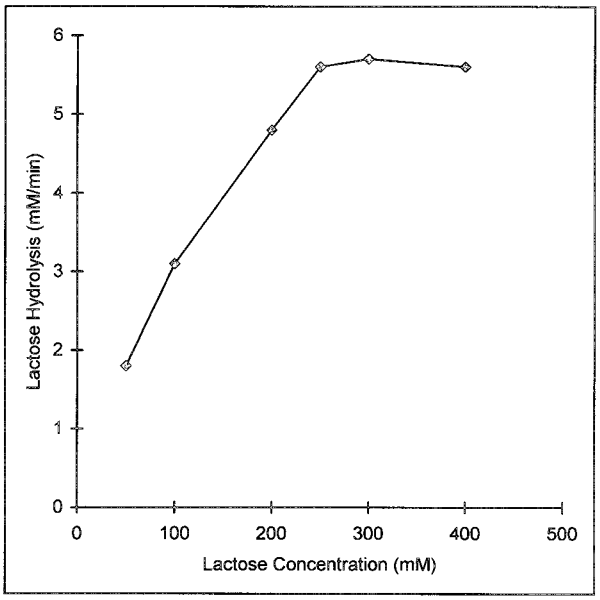

Figure 6 Effect of substrate concentration on the reactor performance. Operation conditions: loading 250 U/24 g enzyme membrane; lactose concentration: 50–400 m*M,* pH 7.5, flow rate 40 mL/h, temperature 25°C.

Reactor Performance

Effect of Substrate Concentration

The effect of the substrate concentration and residence time on reactor performance was determined by using various lactose concentrations and the flow rate. Hydrolysis results were calculated from the amount of glucose produced after enzymatic degradation of the lactose.

An increase in the hydrolysis rate was observed up to lactose concentration of 200 m*M* (Fig. 6). With higher concentrations, a steady level in the hydrolysis rate was observed. This steady level in the hydrolysis rate was due to insufficient contact time between the substrate and the enzyme. Complete hydrolysis was expected even with a 300 m*M* lactose solution at a 40 mL/h flow rate because the reactor was sufficiently loaded with about 250 U/24 g of the enzyme membrane.

Effect of Flow Rate

Figure 7 shows the effect of residence time on the hydrolysis of the lactose by β-galactosidase. The results were converted to relative activities (percentage of the maximum hydrolysis obtained in this series). As the residence time is increased, the extent of hydrolysis is also increased (to almost complete hydrolysis). Eventually, at a contact duration of about 45 min, a plateau is reached (obtained with a flow rate of 30 mL/h and 200 m*M* substrate). Thus, a 200-m*M* lactose concentration and a flow rate of 40 mL/h yield the optimum activity when the reactor is loaded with 250 U/24 g pHEMA enzyme.

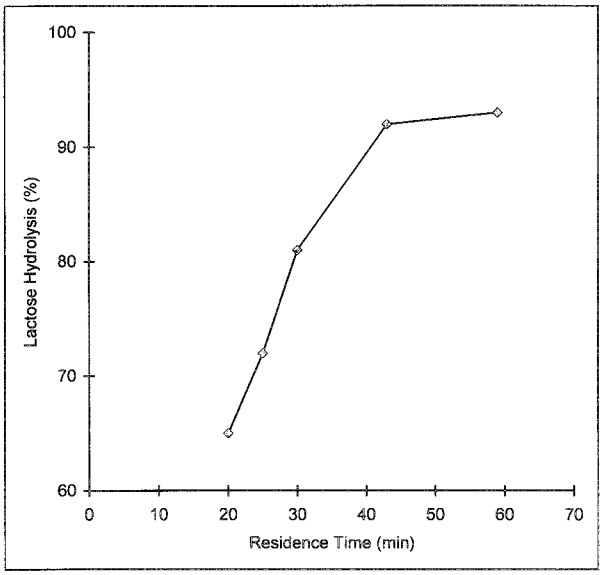

Figure 7 Effect of residence time on the reactor performance. Operation conditions: loading 250 U/24 g enzyme membrane; lactose concentration: 200 m*M,* flow rate 20–60 mL/h, temperature 25°C.

Operational Stability

The operational stability of the immobilized β-galactosidase is also very important in applications because it is subjected to continuous or discontinuous hydrolysis reactions. The operational stability of the entrapped β-galactosidase was studied in the packed bed reactor for 48 h. It was observed that immobilized β-galactosidase lost only about 9% of its activity after 30 h of the continuous operation. The operational inactivation rate constant (k_{iop}) of the immobilized enzyme at 25°C with 200 m*M* lactose in phosphate buffer (0.1*M,* pH 7.5) was calculated to be 3.1×10^{-5} min^{-1}. The operational stability obtained with the entrapped β-galactosidase in the pHEMA membrane indicates that this immobilized enzyme can be used for the hydrolysis of lactose in the milk.

CONCLUSIONS

In the present study, immobilization of β-galactosidase within the hydrogel membrane was successfully achieved via UV-initiated photopolymerization. As previously mentioned, the optimum pH and temperature profile of the immobilized enzyme are not significantly modified, indicating that the environment of the immobilized enzyme is quite like that of the free enzyme. The enzyme–membrane was used continuously for lactose hydrolysis without any considerable losses of activity.

It can be concluded that by using this easy immobilization process the preparation of a stable immobilized enzyme system is possible. This would, therefore, enable researchers to construct durable membrane reactors and biosensors which can be applied in pharmaceutical, chemical, and food industries.

REFERENCES

1. White, C. A.; Kenedy, J. F. Enzyme Microbiol Technol 1980, 2, 82–89.
2. Hayashi, T.; Hyon, S. H.; Cha, W. I.; Ikade, Y. Polym J 1993, 25, 489–495.
3. Clark, D. S. TIBTECH 1994, 12, 439–446.
4. Mohy Elden, M. S.; Bencivenga, U.; Portaccio, M.; Stellato, S.; Rossi, S.; Santucci, M.; Canciglia, P.; Castagnolo, D.; Gaeta, F. S.; Mita, D. C. J Appl Polym Sci 1998, 68, 613–623.
5. Arıca, M. Y.; Denizli, A.; Salih, B.; Pişkin, E.; Hasırcı, V. J Membr Sci 1997, 129, 65–76.
6. Arıca, M. Y.; Alaedinoğlu, N. G.; Hasırcı, V. Enzyme Microbiol Technol 1998, 22, 152–157.
7. Arıca, M. Y.; Testereci, H. N.; Denizli, A. J Chromatogr A 1998, 799, 83–91.
8. Arıca, M. Y.; Hasırcı, V.; Alaedinoğlu, N. G. Biomaterials 1995, 16, 761–768.
9. Piedade, A. P.; Gill, M. H.; Cavaco, M. C.; Andrade, M. E. Polym Int 1995, 38, 269–275.
10. Siso, M. I. G.; Ramil, A. F.; Belmonte, E. R.; Torres, A. R.; Cerdan, E. Process Biochem 1994, 29, 7–15.
11. Gekas, V.; Lopes-Leiva, M. Process Biochem 1985, 22, 2–12.
12. Batsalova, K.; Kunchev, K.; Popova, Y.; Kozhukarova, A.; Kirova, N. Appl Microbiol Biotechnol 1987, 26, 227–230.
13. Saito, T.; Yoshida, Y.; Kawashima, K.; Lin, K. H.; Maeda, S.; Kobayashi, T. Appl Microbiol Biotechnol 1994, 40, 618–621.
14. Carrara, C. R.; Rubiolo, C. A. Biotechnol Prog 1994, 10, 220–224.
15. Park, T. G.; Hoffman, A. S. J Biomed Mater Res 1990, 24, 21–38.
16. Bodalo, A.; Gomez, E.; Gomez, J. L.; Bastida, J.; Maxima, M. F.; Diaz, F. Process Biochem 1991, 26, 349–355.
17. Rejikumar, S.; Devi, S. J Appl Polym Sci 1995, 55, 871–878.
18. Lowry, O. H.; Rosebrough, N.; Farr, A.; Randall, R. J Biol Chem 1951, 193, 263–275.
19. Craven, G. R.; Steers, E.; Anfinsen, C. J. J Biol Chem 1965, 240, 2468–2472.
20. Gacesa, P.; Hubble, J. Enzyme Technology; Alden: Oxford, 1987; pp 45–64.
21. Park, T. G.; Hoffman, A. S. Enzyme Microbiol Technol 1993, 15, 476–485.
22. Khare, S. K.; Gupta, M. G. Biotechnol Bioeng 1988, 31, 829–833.