BUTTERWORTH HEINEMANN

Polymer Vol. 36 No. 21, pp. 4091–4096, 1995

0032-3861/95/$10.00+0.00

Entrapment of urease in glycol-containing polymeric matrices and estimation of effective diffusion coefficient of urea

Hüsniye Demircioğlu*
Middle East Technical University, Faculty of Education, Department of Science Education, 06531 Ankara, Turkey

and Haluk Beyenal and Abdurrahman Tanyolaç
Hacettepe University, Faculty of Engineering, Department of Chemical Engineering, 06532 Ankara, Turkey

and Nesrin Hasırcı
Middle East Technical University, Faculty of Arts and Sciences, Department of Chemistry, 06531 Ankara, Turkey
(Received 15 August 1994; revised 19 December 1994)

Urease enzyme was immobilized in copolymer matrices containing poly(ethylene glycol) (PEG; *MW* 14000 and 1000) or polypropylene glycol (PPG; *MW* 2000), prepared with 2-hydroxyethyl methacrylate (HEMA) and/or *N*-vinyl-2-pyrrolidone (VP). Urease-immobilized copolymer matrices were stored in phosphate buffer solutions of pH 7.0 at 4°C, and the activity of the enzyme was determined periodically for up to 90 days. The highest activity was observed for HEMA and VP containing PEG (*MW* 14000) matrix. The matrices comprising VP and PEG (*MW* 14000) did not have mechanical stability and degraded in 3 weeks, while all the others were stable and demonstrated enzyme activities for up to 3 months. The effective diffusion coefficients of urea in these polymer matrices were calculated with a 'diffusion and reaction model' and the highest effective diffusion coefficient was found in the HEMA and VP containing PEG matrix, possibly due to its highly porous structure.

(Keywords: immobilized urease; copolymer matrix; effective diffusion coefficient)

INTRODUCTION

Enzymes are proteins that catalyse many chemical reactions in living organisms. Unlike most ordinary chemical catalysts, they can operate under very mild conditions of pH, temperature and pressure, and are extremely specific in the types of reactions in which they are involved. To facilitate the separation of an enzyme from the reaction products and to increase its stability, the enzyme can be immobilized. An immobilized enzyme is physically confined on or within an inert support but retains its catalytic activity, allowing it to be used repeatedly and continuously.

There are many different methods for the immobilization of an enzyme. Covalent bonding to an activated support, copolymerization of the enzyme molecules with polymers, crosslinking between enzyme molecules, physical adsorption of the enzyme onto a solid support and entrapment of enzyme molecules in a polymeric structure, are some of the ways of immobilization. In the entrapment method, the enzyme is confined in the pores of a matrix structure, allowing low molecular weight substrate and products to diffuse through the matrix, while retaining the high molecular weight enzyme in the structure.

The enzyme urease catalyses the hydrolysis of urea, with the production of ammonia and carbon dioxide. The substrate urea is one of the toxic substances present in blood plasma and is normally removed by the kidneys. Therefore, a potential application for immobilized urease is in the treatment of kidney failure. In addition, systems utilizing urease can be used in the detection of the urea content of plasma. Therefore there have been many studies on the immobilization of urease in polymeric matrices, and varying degrees of efficiency and specific activity retention were reported[1–5].

In immobilized enzyme systems, mass transfer of the substrate occurs in three different zones: from the bulk fluid to the matrix system, through the pores of the matrix system and finally inwards from the pores to the site of reaction. The first zone is often referred to as the one with external mass transfer resistance and the latter two are considered to show internal mass transfer resistances[6].

One important factor in the design of immobilized enzyme systems is the effective diffusion coefficient D_f of

* To whom correspondence should be addressed

the reacting substrate(s) within the matrix used for immobilization. Measuring the concentration gradient through the matrix in a diffusion cell and coupling it with substrate flux is the direct method of estimating D_f (ref. 7). In the estimation, some assumptions are made for a laminated matrix such that it forms a membrane for the diffusion cell and should contain no enzyme, i.e. no reaction occurs within the membrane. However, in this method the sensitivity of the measurements is very critical and, in addition, a matrix without enzyme may not simulate the real performance of the natural system. The effectiveness factor or 'diffusion and reaction technique' is an indirect method for the estimation of D_f and does not alter the characteristics of the enzyme–support structure. In this technique, numerical methods are used to solve the continuity equation within the matrix for known diffusion coefficient, the value of which is stabilized after successive iterations in the solution[8]. By this method, diffusion coefficients of reactive substrates are reasonably evaluated in their natural environment[9].

In this work, various polymeric matrices of poly(ethylene glycol) (PEG; *MW* 14000 and 1000), poly(propylene glycol) (PPG; *MW* 2000), 2-hydroxyethyl methacrylate and *N*-vinyl-2-pyrrolidone with variable compositions were used for the immobilization of urease. After immobilization, the activity, leakage and stability of the enzyme entrapped in these matrices were determined and compared. Then the effective diffusion coefficients of urea in these polymer matrices were calculated with the 'diffusion and reaction model'.

EXPERIMENTAL

Materials

Poly(ethylene glycol)s (*MW* 1000, PEG1 and *MW* 14000, PEG14) were provided by Riedel-de-Haen AG (Germany) and Aldrich Chemical Company (USA) respectively, and used as obtained. 2-Hydroxyethyl methacrylate (HEMA) was obtained from Aldrich Chemical Company (USA) and vacuum distilled to remove the inhibitors. *N*-Vinyl-2-pyrrolidone (VP) and PPG (*MW* 2025) were obtained from BDH Chemicals Ltd (UK). VP was vacuum distilled prior to use. Ethylene glycol dimethyl acrylate (EGDMA) was supplied by Polysciences (USA). Enzyme urease (urea amidohydrolase, EC 3.5.1.5; from sword beans, lyophilized, 5 IU mg^{-1}) was obtained from Merck AG (Germany). A blood urea nitrogen kit (no. 535) was obtained from Sigma Diagnostic (USA).

Preparation of polymeric matrices

Prescribed amounts of the components (*Table 1*) were placed in phosphate buffer (1.5 ml, 0.1 M, pH 7.0) contained in glass ampoules and EGDMA (45 μl) was added to each solution as crosslinker. The ampoules were then degassed three times, heat-sealed, and irradiated with gamma rays from a ^{60}Co Gammacell 220 source for 4 h. The dose rate was 0.43 kGy h^{-1} as determined by conventional Fricke Dosimeter. These matrices were used in spectrophotometric studies and swelling measurements. The matrices in which the enzyme was entrapped, were prepared in the same way. For these preparations, urease (5 mg) was dissolved in phosphate buffer (1.5 ml, 0.1 M, pH 7.0) prior to the addition of the other components and then the same steps were followed.

After the polymerization, the glass ampoules were broken and the gelled matrices were removed. They were cut into smaller discs (6.5 mm radius and 2 mm thickness), immersed in phosphate buffer of pH 7.0 and used for leakage, activity and stability measurements.

Structure of polymeric matrices

The chemical structures of the matrices were analysed using a Nicolet 510 FTIR spectrophotometer from KBr pellets. The surface topography and the physical structure of the matrices were examined using a Cambridge Stereoscan S4-10 scanning electron microscope after coating with gold.

Swelling properties of matrices

The per cent water content (W) of the swollen polymeric matrices was calculated from the weights of the swollen and equilibrated matrices (w_s) and the completely dried matrices (w_d) using the equation $W = [(w_s - w_d)/w_s] \times 100$. For this purpose, the gelly matrices were kept in distilled water about 1 week until maximum swelling was reached. After weighing, they were dried in a vacuum oven until complete dryness, and weighed again.

The per cent volume change (V) values for the matrices were determined by measuring the volumes of the dry (v_d) and the swollen and equilibrated (v_s) matrices, and using the equation $V = [(v_s - v_d)/v_d] \times 100$. W and V values of polymers were utilized directly to estimate the available volume of

Table 1 Composition of polymer matrices and their swelling, diffusion, stability and leakage properties

Polymer matrix	Matrix components: PEG (g)	PPG (g)	HEMA (ml)	VP (ml)	Swelling properties: W (%)	V (%)	Activity of immobilized or leaked enzyme (μmol NH_3 min^{-1}): 1st day	8th day	90th day	Leaked enzyme	Effective diffusion coefficient of urea ($cm^2 min^{-1}$)
PEG14V	1.0	–	0	1.5	95.9	1094	6.88	10.35	degraded	0.00	1.12×10^{-6}
PEG14H	1.0	–	1.5	0	55.4	25	5.64	4.54	0.02	0.74	4.76×10^{-7}
PEG14HV	1.0	–	0.8	0.7	73.5	41	8.53	6.76	0.02	1.99	2.50×10^{-6}
PEG1H	1.0	–	1.5	0	51.6	36	4.92	2.96	0.02	0.00	9.00×10^{-7}
PPG2H	–	1.0	1.5	0	49.5	40	4.95	7.06	0.03	0.92	2.00×10^{-6}
PPG2HV	–	1.0	0.8	0.7	72.5	61	6.46	8.06	0.03	0.00	9.55×10^{-7}

reaction within the polymer matrix and swollen dimensions, respectively.

Activity measurements

The urea concentrations were detected using the diagnostic kit obtained from Sigma, the pink chromogen of unreacted urea being measured spectrophotometrically at 530 nm with an LKB Biochrom Novaspec II colorimeter[10,11]. All experiments were carried out in a thermostated stirred batch reactor in 100 ml phosphate buffer of pH 7.0 at 25°C for an initial urea concentration of 90 mg dl^{-1}. Initial urea decomposition rates were used to calculate urease activity, which was expressed as micromoles of NH_3 produced per min in the presence of 5 mg immobilized or free urease, unless otherwise stated.

From the Lineweaver–Burk[12] plot, the kinetic parameters K_m [the Michaelis–Menten constant[13] (mg l^{-1}] and V_{max} [the maximum reaction rate of the enzyme (mg l^{-1} min^{-1})] for urease were determined. These runs were carried out at 25°C for various urea concentrations (9 to 90 mg dl^{-1}) using a fixed free urease content of 5 mg dl^{-1} in 100 ml phosphate buffer of pH 7.0.

Leakage of enzyme

The discs prepared from the matrices containing immobilized urease were immersed in phosphate buffer (20 ml, pH 7.0) and kept at 4°C for 8 days. Then, the activity of leaked enzyme in the supernatant liquid was measured at 25°C and pH 7.0.

Stability of enzyme in polymeric matrices

The discs prepared from the matrices containing immobilized urease were kept in phosphate buffer (20 ml, pH 7.0) at 4°C. The urease activity of these matrices was measured periodically for up to 90 days.

Calculation of effective diffusion coefficient of urea

For a single cylindrical disc of the enzyme–polymer matrix, a 'diffusion and reaction model'[14] was applied under pseudo steady-state conditions in order to calculate the effective diffusion coefficient of urea. Since most of the substrate flux into the enzyme-entrapped disc is in the z-direction, the substrate continuity equation[15] may be written for the z-direction in a cylindrical pellet with Michaelis–Menten kinetics[13] as:

$$D_f = \frac{d^2S}{dz^2} = \frac{V_{max}S}{K_M + S} \quad (1)$$

The boundary conditions for the equation are

$$S = S_b \quad \text{at } z = +L/2 \text{ and } z = -L/2 \quad (2)$$

$$\frac{dS}{dz} = 0 \quad \text{at } 0 \leq z < +L/2 \text{ and } 0 \geq z > -L/2 \quad (3)$$

$$J_r = 2D_f \frac{DS}{dz} \quad \text{at } z = +L/2 \text{ and } z = -L/2 \quad (4)$$

In equations (1)–(4), D_f is the effective diffusion coefficient of urea in the polymer matrix (cm^2 min^{-1}), S and S_b are respectively the concentrations of urea within the structure and at the particle surface (mg l^{-1}), z is the axial distance along the cylindrical enzyme–polymer matrix (cm), L is the thickness of the polymer disc (cm), and J_r is the total urea flux to the polymer matrix (mg min^{-1} cm^{-2}).

In the application of these equations at pseudo steady-state, the following assumptions were made: (1) the polymer matrix has perfect cylindrical geometry and homogeneous structure (i.e. uniform enzyme distribution in the particle); (2) the substrate urea diffuses in the z-direction only; (3) reaction occurs in the void volume of the particle; (4) the intrinsic immobilized enzyme activity as well as the Michaelis–Menten coefficients[13] are equal to those of the free enzyme; and (5) the surface concentration of urea is equal to the bulk concentration (ignoring liquid film resistance).

Boundary conditions (2) and (4) refer to experimental conditions prevailing in the reactor. The bulk concentration of urea in equation (2) was determined and the substrate flux J_r in equation (4) was calculated from the total surface area of particles and the overall experimental urea decomposition rate. Boundary condition (3) should be interpreted such that if urea concentration S goes to zero anywhere in the pellet, ($0 \leqslant z < +L/2$ and $0 \geqslant z > -L/2$), the substrate flux (i.e. dS/dz at z) should also converge to zero[16].

There is no analytical solution available for equation (1), so it must be solved numerically. Furthermore, the diffusion coefficient of urea is required for the solution, thus an iterative algorithm was developed. First, an appropriate start-up value for the diffusion coefficient of urea (smaller than that in water) was assigned and boundary condition (4) was calculated from experimental data. Then, using boundary conditions (2) and (4), equation (1) was turned into an initial value problem of a nonlinear differential equation. This equation was solved with a fourth-order Runge–Kutta–Gill algorithm in double precision[17]. During the progress of iterations, equation (3) was checked repeatedly either at $z = 0$ or at any point in the particle where the substrate concentration was found to be zero. If the urea concentration was found to be zero anywhere within the particle, the urea concentration derivative value or flux at this point also had to be zero. If not, a new effective diffusion coefficient was proposed automatically. Finally, the total urea decomposition rate of a single particle, $T_{particle}$ (mg min^{-1}), in equation (5) had to be equal to the observed experimental value. If this condition was not satisfied, again a new effective diffusion coefficient was suggested by the algorithm.

$$T_{particle} = \int_{-L/2}^{+L/2} \left(\frac{V_{max}S}{K_M + S}\right) \pi R^2 dz \quad (5)$$

In equation (5), R is the radius of the cylindrical particle of polymer matrix (cm).

Equation (1) was solved for the substrate urea and the sum of relative errors of predicted urea decomposition rates was found to be $\ll 1\%$. The algorith used was programmed and executed in FORTRAN 77 on a Data General MV 10000 Mainframe computer in double precision. Although Finlayson[18] has recommended the orthogonal collocation method for the solution of 'diffusion and reaction' models, in this study a better and faster technique, the shooting method, was employed[19].

RESULTS AND DISCUSSION

In enzyme reactions, the proper ratio of enzyme to

substrate is crucial for a detectable rate of hydrolysis. To find out that ratio, experiments were carried out with a variable urease concentration at a fixed urea concentration, 15 mM. For sensitive determination, the amounts of urease (either free or immobilized) and urea were chosen as 5 and 90 mg, respectively, in 100 ml buffer solutions for all experiments and every activity measurement was carried out in this reaction medium. For urease, the maximum rate of hydrolysis (V_{max}) and the Michaelis–Menten constant (K_M) were found to be 0.153 mM min^{-1} and 12.432 mM, respectively, for the reaction carried out in phosphate buffer (pH 7.0) at 25°C.

A broad O–H stretching vibration in the range 3550–3420 cm^{-1}), C–O stretching vibrations at 1320–1210 cm^{-1} and C–O–C stretching vibrations in the range 1300–1000 cm^{-1} were observed in the infra-red spectra of all the matrices. Methyl and methylene absorptions were observed in the 1465–1370 cm^{-1} region and C–H stretching absorptions appeared in the 3000–2850 cm^{-1} region. In VP-containing matrices, primary and secondary amides give medium intensity absorption peaks caused by N–H bending vibrations at around 1640 to 1550 cm^{-1} and C–N stretching vibrations at 1400 cm^{-1}. In PPG2HV and PEG14HV matrices, C=O stretching and N–H bending vibrations were observed at 1725 and 1640 cm^{-1}.

The activity of an immobilized enzyme depends on the chemical structure and crosslink density of the polymer, as well as on the porosity of the matrix in which reaction occurs. The matrix porosity can be estimated from the maximum adsorbed water content given in *Table 1*. For PEG14H, PEG1H and PPG2H, the per cent water

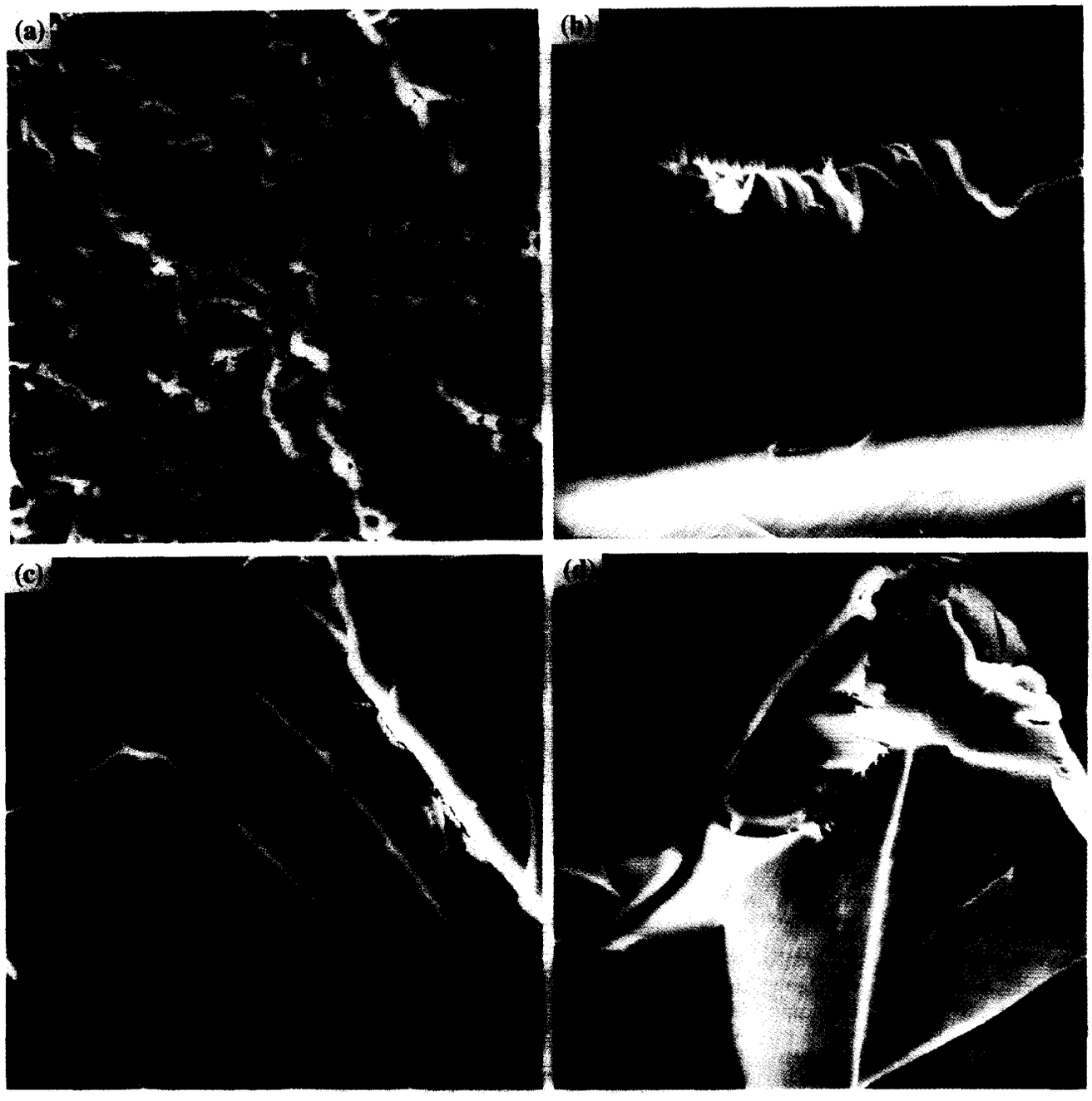

Figure 1 SEM micrographs (×500) of PEG-containing matrices: (a) PEG14V; (b) PEG14H; (c) PEG14HV; (d) PEG1H

content values were found to be 55.4, 51.6 and 49.5% respectively. These close results show that, in these matrices, the amount of adsorbed water was determined mostly by the HEMA present in the structure. The chemistry of glycol was not very effective: the change from PPG to PEG and the increase of the molecular weight caused only a 5% change in the results. A similar trend was observed for the PEG14HV and PPG2HV matrices, which have water content values of 73.5 and 72.5% respectively. On the other hand, the presence of vinyl pyrrolidone in the copolymer increases the water content and the highest value was obtained for the PEG14V matrix as 95.9%.

The enzyme entrapped in polymeric matrices may leak out, especially if the matrix has a very porous structure or the enzyme is loosely bound. Therefore, the matrices were kept in buffer solution at 4°C and the activities of the leaked enzymes were measured in the supernatant solutions after 8 days. Leakage of enzyme was detected for PEG14H, PEG14HV and PPG2H matrices, as shown in *Table 1*. The highest value, obtained with PEG14HV, might be the result of the very high molecular weight of PEG and low crosslink density. Also VP, being a very hydrophilic polymer, can cause breakage of the covalent bonds in the matrix structure.

The stability of immobilized enzyme in buffer solutions at 4°C was tested by measuring the activity on the 1st, 8th and 90th days of storage and the results are presented in *Table 1*. The enzyme activity in PEG14V matrix achieved a 50% increase on the 8th day due to the largest swelling, and then the matrix degraded. Activity on the 8th day decreased for all other PEG-containing matrices and increased for PPG-containing matrices. Activities on the 90th day were found to be $<0.03\ \mu$mol $NH_3\ min^{-1}$ for all matrices.

The effective diffusion coefficients of urea through the polymeric matrices were calculated and are presented in *Table 1*. The lowest effective diffusion coefficient for urea was found for the PEG14H matrix at $4.76 \times 10^{-7}\ cm^2\ min^{-1}$ while the highest value was found for the PEG14HV matrix at $2.50 \times 10^{-6}\ cm^2\ min^{-1}$, based on the 8th day activity values. Thus the diffusion coefficients of urea in all polymeric matrices were less than that in water[20–22] ($8.22 \times 10^{-4}\ cm^2\ min^{-1}$). Diffusion of urea through polymeric matrices and microcapsules has been studied experimentally with some theoretical approximations by several researchers[23–26], and the diffusion coefficient is reported to be of the order of $10^{-6}\ cm^2\ min^{-1}$.

The effective diffusion coefficient of urea through PEG14V was found to be $1.12 \times 10^{-6}\ cm^2\ min^{-1}$. This high value results from the highly porous and swollen structure of the matrix (*Figure 1a*), which degraded in 3 months in aqueous medium. For PEG14H matrix (*Figure 1b*), the replacement of VP with HEMA caused a decrease of $\sim 55\%$ in the effective diffusion coefficient. The highest diffusion coefficient was calculated for the PEG14HV matrix (*Figure 1c*) as $2.50 \times 10^{-6}\ cm^2\ min^{-1}$. HEMA gave the stability to the matrix while VP caused the hydrophilicity. Although PEG14HV was swollen less than PEG14V, the higher diffusion coefficient of PEG14HV may be attributed to laminated and neat infrastructure (*Figures 1a* and *c*).

For the matrices which involve HEMA in their structures, namely PEG14H (*Figure 1b*) and PEG1H (*Figure 1d*), the effective diffusion coefficients were found to be 4.76×10^{-7} and $9.00 \times 10^{-7}\ cm^2\ min^{-1}$ respectively. Decreasing the molecular weight of PEG from 14000 to 1000 increased the diffusion coefficient by almost 50%, most probably as a result of increased packing due to long glycol chains. Addition of VP into PEG-containing matrices caused a large increase in the effective diffusion coefficients: $1.12 \times 10^{-6}\ cm^2\ min^{-1}$ for PEG14V and $2.50 \times 10^{-6}\ cm^2\ min^{-1}$ for PEG14HV matrices. On the contrary, for PPG matrices the addition of VP caused a 50% decrease in the effective diffusion coefficient, from $2.00 \times 10^{-6}\ cm^2\ min^{-1}$ for PPG2H to $9.55 \times 10^{-7}\ cm^2\ min^{-1}$ for PPG2HV, possibly due to a decrease in porosity of macropores within the structure (*Figures 2a* and *b*).

Figure 2 SEM micrographs (×50) of PPG-containing matrices: (a) PPG2HV; (b) PPG2H

CONCLUSIONS

In the preparation of an immobilized enzyme, the major criteria are the biocompatibility of the support material for a higher apparent activity, the stability of the matrix and immobilized enzyme, and diffusional limitations. In this study glycols were used as the main component of the support structures. HEMA was selected for its high biocompatibility and structural stability, and VP was used to increase surface porosity and void volume fraction in order to enhance diffusion of urea through the polymeric matrices. The presence of VP within the copolymer matrix increased the void volume and surface porosity and, in turn, observed enzymatic activity was maximized for PEG14V matrix; however, poor mechanical stability was the major disadvantage for this matrix. The presence of HEMA together with VP increased mechanical stability as well as favouring a laminated and neat infrastructure for the PEG14HV matrix, and the highest effective diffusion coefficient was realized along with high enzymatic activity. However, an adverse trend was observed for PPG matrices when VP participated in the structure.

REFERENCES

1 Buresh, R. J., De Datta, S. K., Padilla, J. L. and Samson, M. I. *Soil Sci. Soc.* 1988, **52,** 856
2 Sugii, A., Ogava, N. and Matsumoto, M. *J. Appl. Polym. Sci.* 1986, **32,** 4931
3 Ristau, O., Pommering, K., Jung, C., Rein, H. and Scheler, W. *Biochem. Biophys. Acta* 1985, **44,** 1105
4 Raghunath, K., Rao, K. and Joseph, T. K. *Biotech. Bioeng.* 1984, **26,** 104
5 Ramachandran, K. B. and Perlmutter, D. D. *Biotech. Bioeng.* 1976, **18,** 685
6 Dueck, C. L., Neufeld, R. J. and Chang, T. M. S. *Can. J. Chem. Eng.* 1986, **64,** 540
7 Yu, C. Y. and Smith, P. L. 'Fixed-Film Biological Process for Wastewater Treatment', Noyes Data Corporation, New Jersey, 1983, p. 85
8 Matson, J. V. and Charklis, W. G. *Water Res.* 1976, **10,** 877
9 Onuma, M., Onuma, T., Umita, T. and LaMotta, E. J. *Biotech. Bioeng.* 1985, **27,** 1533
10 Natelson, S. 'Techniques of Clinical Chemistry', 3rd Edn, Charles C. Thomas, IL, 1971, p. 728
11 Danielsson, B., Gadd, K., Mattiason, B. and Mosbach, K. *Anal. Lett.* 1976, **9,** 987
12 Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* 1934, **56,** 568
13 Michaelis, L., and Menten, M. L. *Die kinetic der Invertinwirkung Biochemische Zeitschrift* 1913, **49,** 333
14 Hossain M. M. and Bailey, J. E. *AIChE J.* 1986, **32** (7), 1088
15 Bird, R. B., Steward, W. E. and Lightfoot, E. D. 'Transport Phenomena', John Wiley & Sons, New York, 1960, p. 559
16 Andrews, G. F. and Thien, C. *AIChE J.* 1981, **27** (3), 396
17 'Diffeq, Hewlett–Packard contributed program series: Math and Numerical Analysis (300),' H&P Software Department, Santa Clara, CA, USA
18 Finlayson, B. A. 'Nonlinear Analysis in Chemical Engineering', McGraw-Hill, New York, 1980, p. 60
19 Vos, H. J. P., Heedrik, J. and Luyben, M. *Bioprocess Eng.* 1990, **28,** 314
20 Barbara, M., Martins, F., Eugenia, M. and Cruz, M. *J. Chem. Tech.* 1987, **39,** 201
21 Karube, I., Nakomoto, Y. and Suzuki, S. *Biochim. Biophys. Acta* 1976, **445,** 774
22 Timmermans, J. 'The Physicochemical Constants of Binary Systems in Concentrated Solutions', IV Interscience, New York, 1960, p. 76
23 Miyama, H., Kobayashi, T. and Nosaka, Y. *Biotech. Bioeng.* 1982, **24,** 2757
24 Karajewska, B., Lezsko, M. and Zaborska, W. *J. Chem. Tech.* 1990, **48,** 337
25 Sundaram, P. V. *Biochim. Biophys. Acta* 1973, **321,** 319
26 Singh, R. and Nye, P. H. *J. Soil Sci* 1984, **35,** 529