Entrapment of Urease in Poly(1-vinyl imidazole)/Poly(2-acrylamido-2-methyl-1-propanesulfonic acid) Network

Mehmet Şenel,[1] Emre Çevik,[1] M. Fatih Abasıyanık,[2] Ayhan Bozkurt[1]

[1]*Department of Chemistry, Faculty of Arts and Sciences, Fatih University, B.Cekmece, Istanbul 34500, Turkey*
[2]*Department of Genetics and Bioengineering, Faculty of Engineering, Fatih University, B.Cekmece, Istanbul 34500, Turkey*

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ABSTRACT: In this article, urease was immobilized in a conducting network via complexation of poly(1-vinyl imidazole) (PVI) with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPS). The preparation method for the polymer network was adjusted by using Fourier transform infrared (FTIR) spectroscopy. A scanning electron microscope (SEM) study revealed that enzyme immobilization had a strong effect on film morphology. The proton conductivity of the PVI/PAMPS network was measured via impedance spectroscopy, under humidified conditions. The basic characteristics (Michealis-Menten constants, pH_{opt}, $pH_{stability}$, T_{opt}, $T_{stability}$, reusability, and storage stability) of the immobilized urease were determined. The obtained results showed that the PAA/PVI polymer network was suitable for enzyme immobilization. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 119: 1931–1939, 2011

Key words: urease; immobilization; proton conductivity; polymernNetwork

INTRODUCTION

Significant attention has been paid to proton conducting polymer electrolyte membranes (PEMs), due to their potential application in advanced electrochemical devices such as fuel cells, sensors, and electrochromics, since their development in the 1960s.[1,2] To obtain high proton conducting membranes, several attempts have been made, in which one of the promising approaches was the doping of the polymers bearing basic units such as amide, imine, and ether, with strong acids, such as, H_3PO_4 or H_2SO_4.[3,4] Of late, neutral (or basic) proton conducting polymer electrolytes have already been announced as they may probably be more stable in the presence of electrode materials. In this context, heterocycles such as imidazole or benzimidazole have been reported to be promising, under anhydrous and intermediate temperature conditions.[3–6] In these systems, proton transport occurs among hydrogen-bonded neighboring heterocyclic units through the Grotthuss mechanism.[7]

Immobilization of enzymes on the desired biosensor surface and maintaining their activity during a desired application is important factors for the success of enzyme biosensors. Enzymes are expensive, and hence, it is cost-effective to use them more than once. However, it is difficult and expensive to separate them from the reaction mixture. On the other hand, immobilized enzymes have advantages of repeated use, easy separation from the product environment, enhanced stability, and reduction in the cost of operation.[8]

Biological molecules, enzymes, can be immobilized on a biosensor surface using different methods. These methods are: crosslinking, covalent attachments, adsorption, and entrapment/encapsulation of the biological molecules within polymeric gels or carbon paste.[9–11] The urease enzyme is a highly specific enzyme and catalyzes the hydrolysis of urea to ammonium and carbon dioxide.[12] It has been immobilized for various analytical and biomedical purposes. One of the main applications of immobilized urease is the direct removal of urea from the blood, for detoxification,[13] or in the dialysis regeneration systems of artificial kidney machines[14] and for the electrochemical detection of urea.[15] Other applications of immobilized urease will be in a bioreactor, for the conversion of urea, present in fertilizer wastewater effluents, to ammonia and carbon dioxide,[16] or in the food industry for the removal of urea from beverages and foods.[17,18] The urease enzyme has been immobilized/coimmobilized through several methods.[15] It was immobilized in sol-gel films,[19] polyaniline,[20] polypyrrole,[21] and membranes.[22] The

Correspondence to: M. şenel (msenel@fatih.edu.tr).
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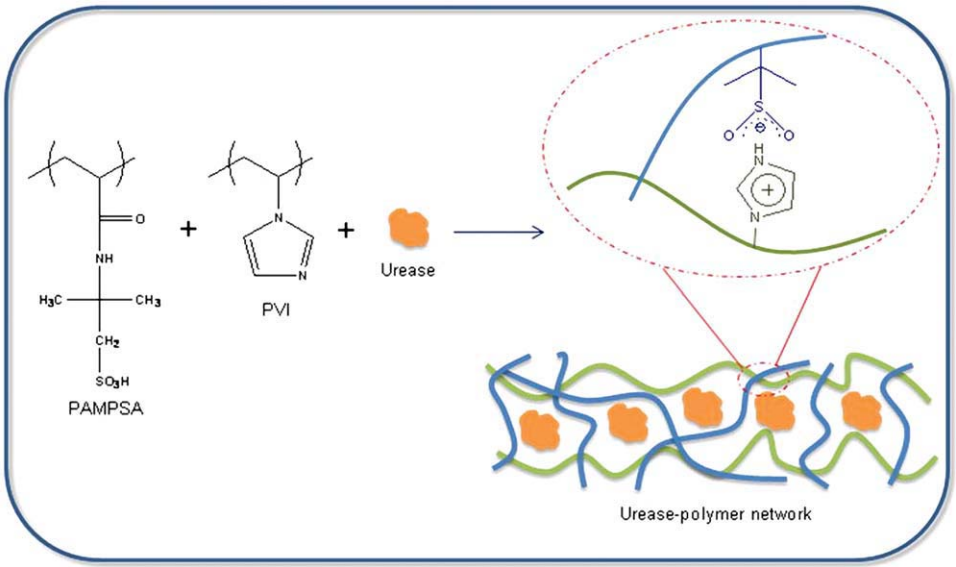

Figure 1 Entrapment of urease into PAMPS/PVI network. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

use of complex polymer electrolytes in enzyme immobilization is rather interesting and several works have been reported in recent studies.[23–25] Arslan et al. reported a novel conducting polymer electrolyte network system that was used to immobilize invertase. Their results showed that this new method is easy to prepare and improve the stability and the performance of the polymer electrolyte-based enzyme biosensor.[25]

poly(2-acrylamido-2-methyl-1-propanesulfonic acid)/poly(1-vinyl imidazole) (PAMPS/PVI) network has been used for the first time in the immobilization of enzymes. In this network, an enzyme can be entrapped within a polymeric network of porous gel. Compared with other immobilization matrices, in this case, the enzyme molecules become entrapped in a covalent network rather than being chemically bound to an organic matrix, which may inactivate the enzyme. Immobilization of the enzyme into a complex polymer electrolyte network is rather new and easy, where the process is carried out during ionic complexation.

The immobilization of enzymes into complex electrolytes based on the PAMPS/PVI network system, PVI would be interesting where the proton conducting network can affect the activity of the urease enzyme under different conditions. The complexation phenomenon is shown in Figure 1. In this study, we prepared a novel enzyme-based biosensor by immobilizing urease into a polymer network obtained by the complexation of PAMS with PVI. The proton conducting polymer networks were obtained by mixing PAMPS and PVI in several stoichometric ratios, and used for the immobilization of urease. The proton conductivity of the polymer networks was studied by impedance spectroscopy and the thermal properties were investigated by thermogravimetric analysis (TGA) methods. The maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) were investigated for the immobilized urease. The temperature and pH optimization, reusability, and storage stability of the urease-polymer network were also evaluated.

EXPERIMENTAL

Materials

Urease (EC 3.5.1.5 from jack beans) was obtained from Sigma. 2-acrylamido-2-methyl-1-propanesulfonic acid, 1-vinylimidazole and 2,2′-azobis(isobutyronitrile) (AIBN) was obtained from Fluka. All other chemicals were of analytical grade and were used without further purification.

Synthesis of PAMPS

PAMPS used in this study was synthesized in 1,4-dioxane/deionized water cosolvent (2 : 1 by volume), where 1 mol % of potassium persulfate to the number of the moles of the monomer was used as an initiator. The reaction mixture was bubbled with dry nitrogen for 20 min and polymerization was carried out at 65°C for 36 h. To remove unreacted monomer and oligomer, dilute solution of the polymer was dialyzed in deionized water using 2000-molecular weight cut-off membrane.[26]

Synthesis of PVI

PVI was synthesized by solution polymerization using (AIBN) as initiator. 1-Vinylimidazole was

dissolved in absolute ethanol and dry nitrogen was bubbled through the solution with vigorous stirring. AIBN was added to the solution and polymerization was carried out at 70°C for 40 h. PVI was obtained by precipitating the ethanol solution in excess THF/hexane (3 : 8, v/v) solution. The polymer was washed repeatedly with the nonsolvent mixture and dried *in vacuo* at 80°C.[27]

Instrumentation

Activities of the free and immobilized enzymes were determined by using the UV-Visible spectrophotometer (SHIMADZU UV-1700). The polymer networks were studied by Fourier transform infrared (FTIR) spectra and scanned in the range of 4000–400 cm^{-1} on an FTIR Spectrometer (Perkin–Elmer Spectrum BX). A scanning electron microscope was employed to observe the surface of the polymer networks. For SEM, pieces of the membranes were mounted on stubs and then coated with gold using a sputter coater. For SEM, after drying, the pieces of the polymer network were mounted on stub and then coated with gold using a sputter coater. The SEM micrographs of the polymer networks (without enzyme and with enzyme) were taken using a Philips XL30S-FEG scanning electron microscope. The thermal stabilities of the polymer electrolytes were examined by TG analyses with a Mettler-Toledo TG-50. The samples ($\sim$ 10 mg) were heated from room temperature to 700°C under N_2 atmosphere at a scanning rate of 10°C/min. The proton conductivity studies of the samples were performed using a Novocontrol α-N high-resolution, dielectric-impedance analyzer. The films were sandwiched between gold blocking electrodes and the conductivities were measured in the frequency range of 0.1 Hz to 3 MHz, at various temperatures. The temperature was controlled with a Novocontrol cryosystem, which was applicable between −100 and 250°C.

Preparation of a polymer network and immobilization of urease

To determine the stoichometric ratio for a stable PAMPS/PVI network, 0.10 g PAMPS was mixed with PVI (with respect to the monomer repeating unit) in an aqueous solution. To check the maximum complexation of the polymers, four samples of PAMPS/PVI were prepared at room temperature with x ranging from 0.5 to 3 (where x was the number of moles of imidazole per mole of the $-SO_3H$ group in PAMPS). The FTIR spectra showed that maximum protonation was obtained with $x = 2$. This ratio was used for further urease immobilization studies. In addition, the maximum water absorbing capacity was checked. The maximum capacity of intake was 35% in 2 mL of PAMPS (0.10 g/mL) and PVI (0.10 g/mL).

Optimum enzyme concentration for immobilization studies was determined by using different amounts of urease 1 to 9 mg/mL in 50 m*M* pH 7.5 phosphate buffer. Finally, PAMPS/PVI network urease solutions were stirred to obtain the enzyme entrapped polymer network.

Enzyme and protein assay

The activity of free and immobilized urease was determined using the Nessler's method.[28] Free (400 μg) or immobilized (10 mg) urease was kept in a test tube and 1 mL of phosphate buffer (pH 7.5) containing 150 m*M* urea and 5 m*M* EDTA were added and incubated at 30°C for 30 min. 0.3 mL of 0.3% sodium tungstate and 0.3 mL of 0.68*N* sulfuric acid were added and a total volume of up to 5 mL was made. Then 1 mL of the above solution was treated with 1 mL of Nessler's reagent and the total volume was made up to 10 mL with distilled water. The color produced was read for absorbance against a blank at 480 nm in a spectrophotometer. The amount of ammonia liberated was calculated by comparing the absorbance with a standard curve for ammonium sulfate.

Protein concentrations of polymer networks were determined with the help of the Lowry method,[29] by using bovine serum albumin as the standard. The quantity of entrapped protein was calculated by subtracting the protein recovered in the combined washings of the urease-polymer network from the protein used for immobilization.

Properties of free and immobilized enzymes

Optimum pH

The effect of pH on the activity of free and immobilized urease was assayed in the acetate buffer (0.1*M*) in the pH range 4.0–6.5 and in the phosphate buffer (0.1*M*) in the pH range 7.0–9.0 by using the above-mentioned standard activity assay procedure.

pH stability

The pH stabilities of free and immobilized urease were compared in the acetate and phosphate buffers (0.1*M*) between pH 4.0 and 9.0. Free and immobilized enzymes were incubated at these buffer solutions for one hour. Both forms of urease were assayed using the standard assay conditions.

Optimum temperature

The effect of temperature on the activities of both forms of urease was studied between 30 and 90°C and assayed under standard assay conditions.

Thermal stability

The thermal stability of the free and immobilized urease was evaluated by measuring the residual activity of urease exposed to various temperatures between 30 and 90°C, in phosphate buffer (0.1*M*, pH 7.5), for 15 min. After heating, the samples were rapidly cooled to 30°C and assayed immediately for enzymatic activity using the standard assay conditions. The remaining activities were expressed as relative to the original activities assayed at 25°C.

Storage stability and reusability

The immobilized urease was stored in a phosphate buffer (0.1*M*, pH 7.5) at 4 and 25°C for 35 days. Its activity was measured at frequent intervals. The immobilized urease was repeatedly used for hydrolysis of urea, for reusability evaluation, under standard assay conditions, by using 10, 25, 50, and 100 m*M* of the phosphate buffer. After each activity assay the samples were washed with the buffer and stored until the next assay.

Determination of Michaelis-Menten constants

The kinetic constants, K_m and V_{max}, of the free and the immobilized urease were determined by measuring the initial rates of the reaction with urea (1–150 m*M*) in a phosphate buffer at 30°C. The K_m and V_{max} values were calculated from the Lineweaver-Burk plots.

RESULTS AND DISCUSSION

Preparation of PAMPS/PVI and PAMPS/urease/PVI networks

Throughout this study, we tried to prepare and characterize the PAMPS/PVI interpenetrating polymer network, and then immobilized the urease enzyme in this network. In this context, PAMPS/PVI polymer networks were prepared by mixing the corresponding polymers. The complexation behavior and the structure of the polymer networks were evaluated by FTIR and showed that the maximum protonation of the polymer networks. The swelling behavior of the polymer network increased with an increase in the content of both PVI and PAMPS. Thus $x = 2$ acid-base ratio had the lowest swelling due to complexation. Also, the surface morphology of the PAMPS/PVI and PAMPS/Urease/PVI networks has been discussed in the following section and a significant change was observed after the immobilization of urease in the polymer network.

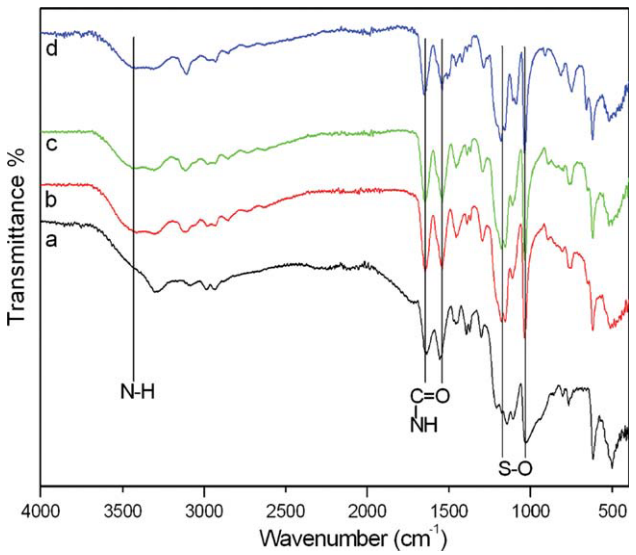

Figure 2 FTIR spectra of polymer network at different x; (a) pure PAMPS, (b) PAMPS/0.5PVI, (c) PAMPS/1.0PVI, and (d) PAMPS/2.0PVI. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FTIR results

FTIR spectra of the polymer networks of PAMPS with various compositions of PVI are compared in Figure 2. The strong absorption bands at 1650 and 1554 cm^{-1} corroborate the amide group of the AMPS unit in the PAMPS. The *N*—H stretching of the same group is observed near 3415 cm^{-1}. In the spectrum, the absorption band in the 1000–1250 cm^{-1} region, in particular, the strong bands at 1036 and 1210 cm^{-1}, evidence an *S*-O stretching typical of the sulfonate groups. The absorptions in the region of 750–800, 1576 and 3135 cm^{-1}, are the evidence of the formation of imidazolium ions. As x varies from 0.5 to 2, these peaks become more intense.

Thermal analysis

The gravimetric thermal analysis curve for PAMPS-2.0 Im is shown in Figure 3. The samples were dried for 2 days under vacuum at 60°C, prior to the measurements. The thermal stability was analyzed under inert atmosphere, with a heating rate of 10°C/min. The PAMPS and PVI homopolymers were reported to be thermally stable up to 150 and 200°C, respectively.[26,27] The initial weight changes were from 220 to 300°C. The reason for this small change was due to the physically bound water in that hygroscopic sample. In addition, the vaporization of the dopant might have occurred above 220°C. The imidazole loss at higher temperatures became clearer for $x = 2$, where excess imidazole was present in the blend. The degradation pathway above 300°C was probably similar to that observed previously, and the evolution of SO_2

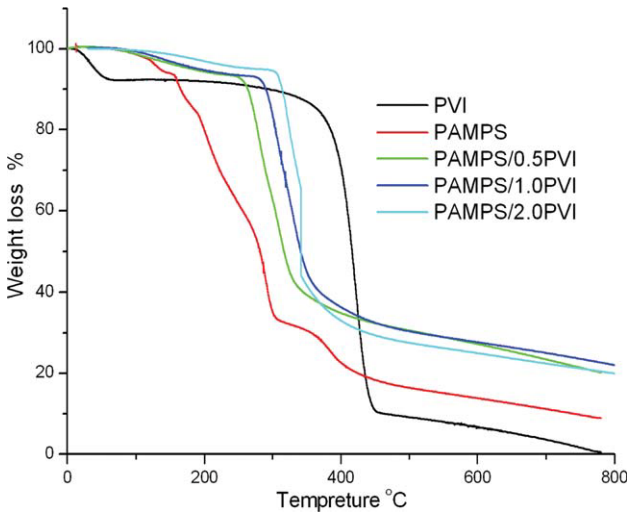

Figure 3 TG thermograms of PAMPS, PVI, PAMPS/0.5PVI, PAMPS/1.0PVI, and PAMPS/2.0PVI recorded at a heating rate of 10°C/min under a nitrogen atmosphere. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

gas and other volatile products, such as, CS_2, COS, and CO might occur.[26]

Conductivity measurements

Frequency-dependent proton conductivity of the samples was measured using the impedance method, as a function of temperature. Alternating current (AC) conductivity, σ_{ac}, versus frequency curves was plotted and the direct current (DC) conductivity, σ_{dc}, of the samples was derived from the σ_{ac} data, as described earlier.[26–30] The DC conductivities of the humidified (50%) samples 1 and 2, at several temperatures, are compiled in Table I. Clearly the conductivity of the samples depended on the temperature. In the dry state the sample showed no significant conductivity, however, in a humidified state the conductivity increase could be attributed to the mobilization of ions, that is, H_3O^+. Also, protonation of the imidazole groups resulted in the formation of polysalts, and proton conductivity might also occur over the protonated and unprotonated units in the complex matrix.[26]

TABLE I
Results of Proton Conductivity Measurements

Temperature (K)	PAMPS/ 1.0PVI (S/cm)	PAMPS/ 2.0PVI (S/cm)
293	4.5×10^{-7}	1.2×10^{-6}
313	2×10^{-6}	3.8×10^{-6}
333	6.9×10^{-6}	9.7×10^{-6}
353	1.5×10^{-5}	1.9×10^{-5}
373	6.9×10^{-5}	3.1×10^{-5}

Surface morphologies of polymer networks

A SEM study of the surface of the PAMPS/PVI network, before and after immobilization, was carried out. It was observed that the surface of the PAMPS/PVI network before immobilization had few ridges and roughness and was smoother than the surface of the PAMPS/PVI/Urease network (as shown in Fig. 4). It was observed that enzyme immobilization had a strong effect on film morphology. The smooth structure was damaged as an expected phenomenon according to previous study, by enzyme entrapment, because the entrapped molecule had a big structure.[25]

Effect of enzyme concentration on immobilization

When the concentration of urease was increased during immobilization, relative activity of immobilized urease was found to increase (Fig. 5).[31,32] Urease concentration of 5 mg was found to give the optimum level of activity. Above these urease concentrations, there was no change in the relative activity.

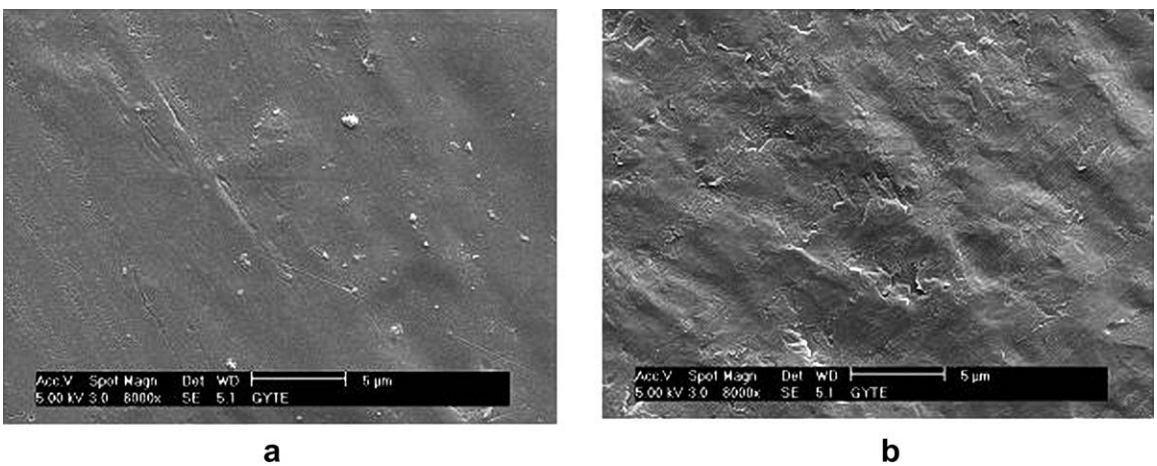

Figure 4 SEM micrographs of polymer network; (a) PAMPS/PVI and (b) PAMPS/PVI/Urease.

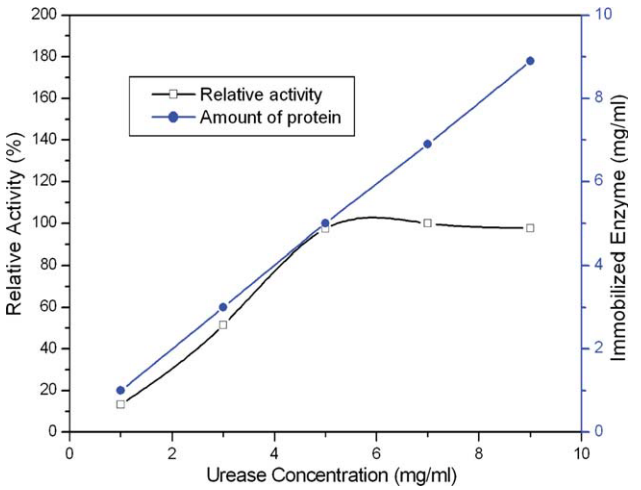

Figure 5 Effect of enzyme concentration on immobilization. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

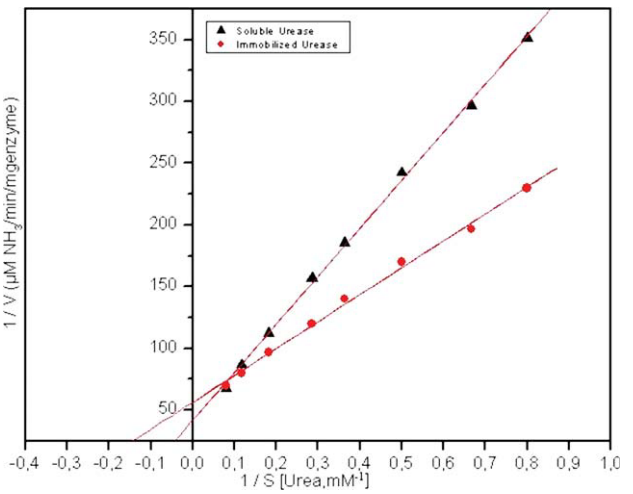

Figure 6 Lineweaver-Burk plots for free and immobilized ureases. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

To optimize the enzyme immobilization procedure it is important to determine the capacity of the support for the highest retention of enzyme activity.

An increase in urease concentration (from 1 to 9 mg/mL) led to an increase in immobilization efficiency but this leveled off at an enzyme concentration of 5 mg/mL. Although the enzyme amount bound to the membrane increased after 5 mg/mL enzyme concentration, the activity did not increase accordingly. The linearity between the enzyme concentration and the enzyme activity was broken, indicating the presence of a constant number of active sites in the polymer network.

Kinetic parameters

From the Lineweaver-Burk plot of 1/V versus 1/[S] Michaelis-Menten constants (K_m) and the maximum reaction velocity (V_{max}), the free and immobilized enzymes have been calculated and the results are shown in Figure 6. The calculated K_m values of the free and immobilized urease were 4.1 and 16.6 mM (about 4.4 times higher than that of the free urease), respectively. The increase in K_m after immobilization clearly indicated an apparent low affinity of the enzyme to its substrate compared to the free enzyme; this could be attributed to the tendency of the enzyme to leave the substrate within a short time without giving a product. In general, K_m of an immobilized urease was different from that of the free urease due to the diffusional limitations, steric effects, and ionic strength. The change in the affinity of the enzyme to the substrate was also caused by structural changes in the enzyme, introduced by the immobilization procedure and by lower accessibility of the substrate to the active site of the immobilized enzyme. An increase in K_m was also reported when urease was immobilized onto the collagen-poly (GMA) copolymer[33] and chitosan.[34] The V_{max} value free urease (0.01807 μM/min/mg enzyme) was found to be lower than that of the immobilized urease (0.03497 μM/min/mg enzyme). Diffusion of the product was easier than that reported in the earlier works. This result could be attributed to the electrostatic interactions between the product and the polymeric matrix. The leaving of the reaction product from the catalytic site became easier due to the structure of the polymers for immobilized urease.

Optimum temperature and thermal stability

The optimum temperatures for the free and immobilized urease were investigated by changing the enzyme activity assay temperature from 30 to 90°C

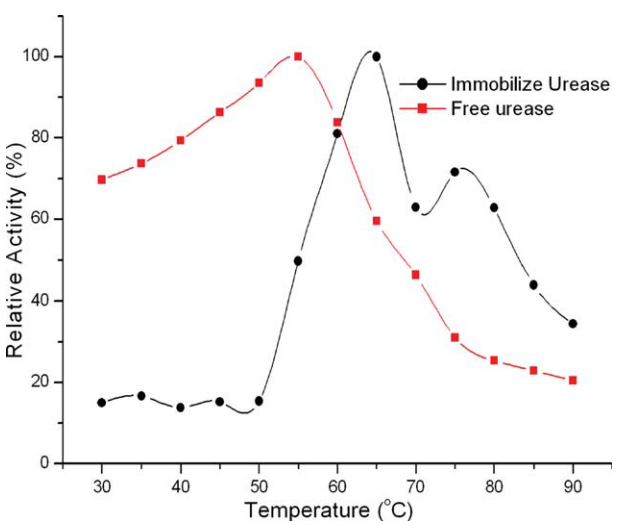

Figure 7 Optimum temperature of the free and immobilized urease. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

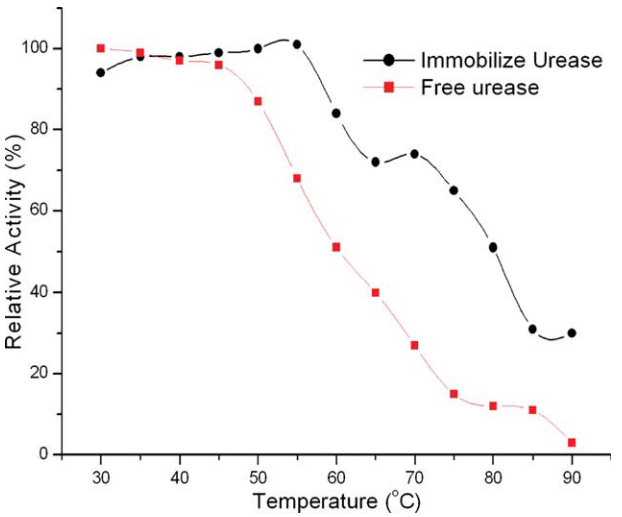

Figure 8 Thermal stability of the free and immobilized urease. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

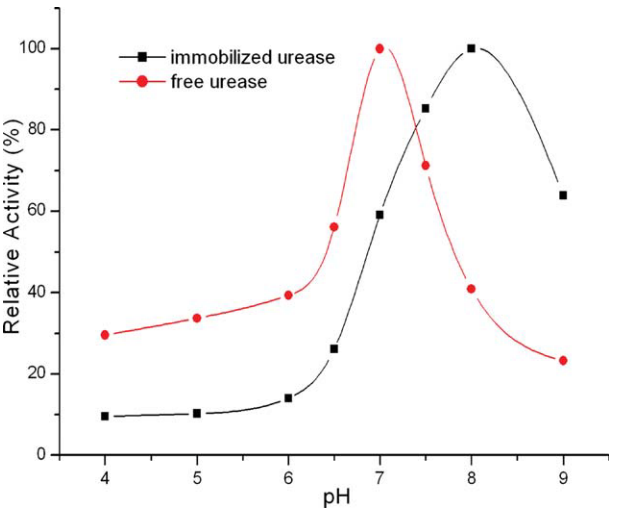

Figure 9 Optimum pH of the free and immobilized urease. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Fig. 7). Free urease had an optimum temperature of approximately 55°C, whereas, the temperature optimum of immobilized urease shifted to 65°C. Further increases in temperature decreased the activity, but it remained higher than the free enzyme. Similar results were reported earlier, when urease was immobilized on a modified polysulfone membrane.[35]

The thermal stability of enzymes after immobilization can be enhanced, decreased, or increased. Figure 8 shows that immobilized urease showed an increase in thermal stability when compared to the free enzyme. The increase in thermal stability is mainly due to minimized denaturation through the protection by the PAMPSA/PVI network. However, there is a sudden decrease in the activity after 70°C, due to the denaturation of urease.

Optimum pH and pH stability

The effect of the pH on the activity of free and immobilized urease was examined in the pH range 4.0–9.0 by changing the pH of the enzyme activity assay and the results are presented in Figure 9. The pH value for optimum activity for free urease was found to be 7.0, which was similar to that reported earlier.[36] On the other hand, the optimum pH for immobilized urease was found to have shifted to pH 8.0. The observed displacement toward the basic region for immobilized urease was caused by the increase in the diffusion of the substrate and the product. The enzyme was immobilized in the PVA/PVI network via complexation of the corresponding polymers, in an aqueous media. Therefore, the strength of the network depended on the pH changes. According to the experimental data [Fig. 8], the diffusion was the highest at pH ~ 8, showing that the pore size of the network was suitable for the diffusion of the substrate and the product.

The pH stability of the free and immobilized urease was determined by incubating the free and immobilized enzyme in different buffers (pH 4.0–9.0) for 2 h (Fig. 10). The activity was then assayed under standard conditions. Immobilized urease showed improved stability, retaining a considerable amount of activity at higher pH values, when compared to the free enzyme. Decrease in activity at pH 7.0 was due to the isoelectric point of the enzyme. At this point the molecule carried no net charge, hence, the activity decreased rapidly due to difficulty in substrate binding. At low pH values, below pH 6.0, the enzyme was irreversibly denaturated.

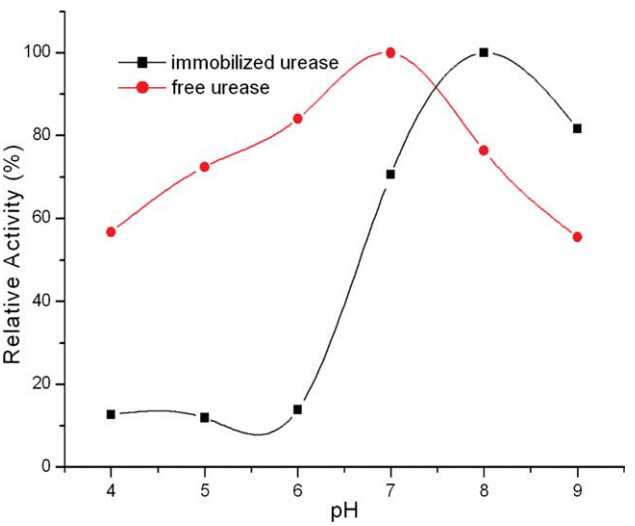

Figure 10 pH stability of the free and immobilized urease. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Stability and reusability

The storage of the immobilized urease in the PAMPS/PVI network at 4 and 25°C was also investigated (see Fig. 11). It was found that the immobilized urease retained 98% of its initial activity after 16 days when stored at 4°C, and 82% of its initial activity after 16 days when stored at 25°C. The higher stability at lower temperature could be ascribed to the protection of denaturation as a result of the attachment of urease to the PAMPS/PVI network.

A reusability study was carried out by measuring the activity of the immobilized urease successive times at different buffer (10, 25, 50, and 100 m*M*) concentrations, with several washes with a phosphate buffer, after each assay. In Figure 12, the maximum activity in the range of 100% was obtained at the beginning of the reusability experiments. An activity loss of 20% was observed after the fourth use. At the end of the tenth use, a total activity loss of 50% was observed. As a consequence, the immobilized enzyme provided multiple uses, whereas, the free enzyme could only be used once unless it was recovered from the reaction mixture without any denaturation, and its maximum activity was highest at 100 m*M* buffer concentration, because the polymer network was more stable at high buffer concentrations.

CONCLUSIONS

In this study, the PAMPS/PVI polymer network was used successfully for the entrapment of urease. This polymer network was characterized by FTIR spectroscopy, and it was found that the maximum protonation occurred with $x = 2$. Furthermore, the surface morphologies of the PAMPS/PVI and PAMPS/PVI/Urease networks showed a significant change after enzyme immobilization in the polymer network. The PAMPS/PVI network revealed high temperature resistance as determined by TGA. The Michaelis-Menten kinetic constants K_m and V_{max} of the free and immobilized urease were also determined and revealed that the affinity of urease to urea decreased after immobilization. The properties of free and immobilized urease were compared and the results indicated that the stability of the immobilized urease toward temperature, pH, reusability, and storage was enhanced by entrapment. The entrapped urease exhibited high stability over a broad pH and temperature range when compared to free urease. The immobilized enzyme was stable and active in operational conditions, with considerable urea degradation. However, immobilized enzymes activity decreased during repeated use due to the leakage of the enzyme from the polymer network.

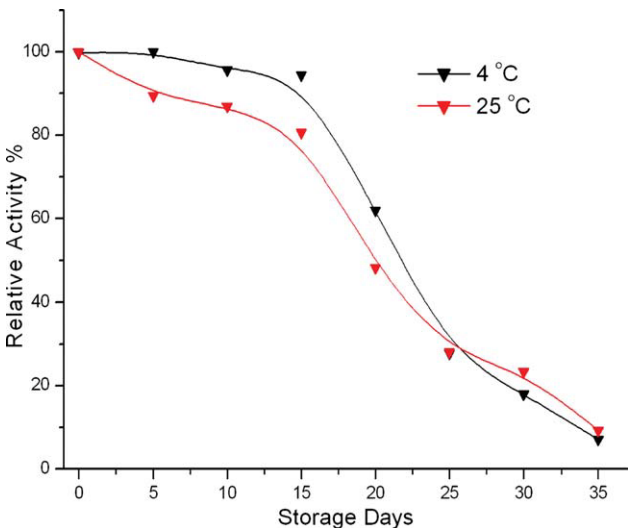

Figure 11 Storage stability of the immobilized urease at 4 and 25°C. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

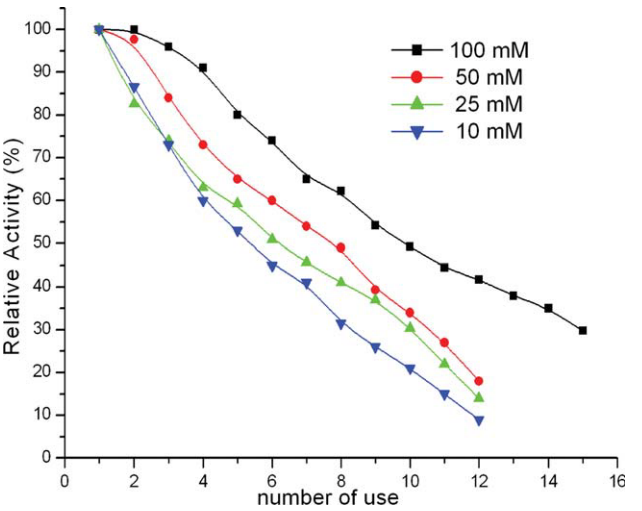

Figure 12 Reusability of urease-polymer network at different buffer concentrations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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