Covalent Immobilization of Penicillin G Acylase onto Amine-Functionalized PVC Membranes for 6-APA Production from Penicillin Hydrolysis Process. II. Enzyme Immobilization and Characterization

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ABSTRACT: This article describes the covalent immobilization of penicillin G acylase (PGA) onto glutaraldehyde-activated NH_2-PVC membranes. The immobilized enzyme was used for 6-aminopenicillanic acid production from penicillin hydrolysis. Parameters affecting the immobilization process, which affecting the catalytic activity of the immobilized enzyme, such as enzyme concentration, immobilization's time and temperature were investigated. Enzyme concentration and immobilization's time were found of determine effect. Higher activity was obtained through performing enzyme immobilization at room temperature. Both optimum temperature (35°C) and pH (8.0) of immobilized enzyme have not been altered upon immobilization. However, immobilized enzyme acquires stability against changes in the substrate's pH and temperature values especially in the higher temperature region and lower pH region. The residual relative activities after incubation at 60°C were more than 75% compared to 45% for free enzyme and above 50% compared to 20% for free enzyme after incubation at pH 4.5. The apparent kinetic parameters K_M and V_M were determined. K_M of the immobilized PGA (125.8 m*M*) was higher than that of the free enzyme (5.4 m*M*), indicating a lower substrate affinity of the immobilized PGA. Operational stability for immobilized PGA was monitored over 21 repeated cycles. The catalytic membranes were retained up to 40% of its initial activity after 10.5 working h. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 000: 000–000, 2012

Key words: PVC membranes amination; enzyme covalent immobilization; stability of the immobilized enzyme; 6-APA; penicillin G acylase; TGA

INTRODUCTION

Enzyme immobilization is considered as one of the most biotechnological applications of different synthetic polymers including PVC.[1–9] Among the various methods available for enzyme immobilization, covalent binding is particularly important because it leads to preparation of stable enzyme derivatives.[10–19] Suitable functional groups are essential to conduct such immobilization technique. PVC has no functional groups in its structure, so chemical modification was carried out to introduce the proper functional groups. Grafting technique, with different types of polymers possess different functional groups, was intensively presented as the main solution using either chemical or radiation initiation system.[20–23] On the other hand, another simple technique using the aminoalkylation reaction with diamine has been presented.[24] This technique depends on the reaction between the available chlorine atoms on PVC surface and the amine groups of diamine. The introduced amine groups were further activated using symmetric coupling agent, glutaraldehyde (GA), which finally covalently binding with enzyme. The mechanism of PVC modification, activation, and immobilization with enzyme are presented in Scheme 1. Despite the fact that such technique has been investigated since almost 29 years ago,[9] still no studies concern the optimization of the modification and the activation processes have been published. Recently, the same technique has been used by other authors in immobilization of *Candida rugosa* lipase via GA coupling onto functionalized hydrophobic polypropylene chloride membrane prepared by the amination of chlorinated polypropylene with hexamethylene

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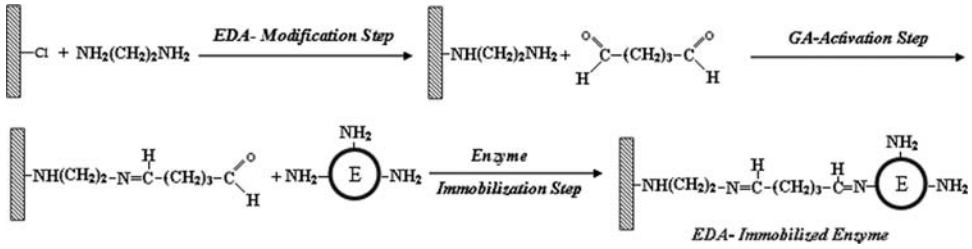

Scheme 1 Mechanism of PVC modification, activation, and enzyme immobilization.

diamine.[25] Other authors immobilized invertase by covalent linking on the inner surface of a polyvinyl chloride (PVC) tube. This is achieved by introducing an active functional group on the surface of an inert PVC tube through 1-fluoro-2-nitro-4-azidobenzene (FNAB), a precursor of highly reactive nitrene, which can insert to any C—H bond. CCl_4 lacking C—H bond is taken as a solvent for loading FNAB solution into the tube. FNAB-loaded tube is then allowed to expose to sunlight for 20 min during which azido group of FNAB generates nitrene and attaches itself to PVC tube through insertion reaction. Invertase is immobilized in the activated PVC tube at 50°C in 45 min. Invertase-embedded PVC tube is used as a flow-through reactor to convert sucrose to invert sugar.[26] Different enzymes, urease and creatinase, were covalently immobilized onto COOH-PVC membranes[27] using carbodiimide or GA for biosensing of urea and creatine or L-ariginine. Glucose oxidase and/or urease were covalently immobilized onto NH_2-PVC for biosensing of glucose and urea.[28] No optimization studies were performed to optimize the immobilization process of any of the above mentioned enzymes. Recently, Zhao et al.[29] covalently immobilized penicillin G acylase (PGA) onto aminopropyl-functionalized microstructured cellular foam through Schiff base reaction. The authors optimized the relation between the amount of immobilized PGA and the degree of matrix functionalization and reached the optimum enzyme distribution density. The porosity and the degree of functionality were found of determined effect. This work is similar to work done here through the immobilization of PGA covalently through Schiff base. Dissimilar, our PVC membrane matrix is nonporous.

In this work, for the first time, PGA has been covalently immobilized on the surface of GA-activated NH_2-PVC nonporous membranes for production of 6-APA from penicillin hydrolysis process. Parameters affecting the immobilization process, which affecting the catalytic activity of the immobilized enzyme, such as enzyme concentration, immobilization's time, and temperature were investigated. Various properties including optimum pH, temperature, kinetic parameters, operational and pH stability of immobilized enzyme were explored. Finally, the resultant structural and morphological changes of the activated PVC membranes have been monitored using FT-IR, thermal gravimetric analysis (TGA), and scanning electron microscopy (SEM) analysis.

EXPERIMENTAL

Materials

Poly (vinyl chloride) (PVC; 70 KD) analytical grade, ethylene diamine (EDA) analytical grade, and *E. coli* PGA (E.C.3.5.1.11) were obtained from Sigma Chem. Co. (St. Louis, USA), Glutaraldehyde (GA); analytical grade was obtained from Fluka (packed in Switzerland), and (penicillin G); pharmaceutical grade was obtained from El-Nasr Pharmaceutical Co for Chemicals. (Egypt). All other chemicals used were of analytical reagent grade. Buffer solutions were prepared with distilled water.

Methods

Each experimental point represents the average of three measurements. The experimental errors never exceed 6%.

Preparation of PVC membranes

PVC membranes prepared by dissolving 0.125 g of PVC in 10 mL of tetrahydrofuran (THF). The polymer solution was poured into Petri dish of (diameter: 9 cm). It was rotated on a horizontal flat surface to get an even distribution of polymer over the glass. Then, the membrane was washed many times by distilled water to remove the excess amount of THF solvent.[30]

PVC membranes surface modification

PVC membranes were aminated by treating with a large excess of a 0.020% aqueous solution of ethylene diamine. Thus, small piece of PVC (2×1 cm^2) was added to 20 mL of 0.020% solution of the amine in distilled water in shaking water bath maintained at 80°C for 60 min. After completion of the reaction, the membranes washed with distilled water to remove unreacted ethylene diamine and dried in an air dryer.[31]

Enzyme immobilization

Modified PVC membranes were activated using 20 mL of GA (1%) of pH 8.0 at 40°C for 60 min.

TABLE I
Summary of Immobilization Conditions

Immobilization temperature (°C)	4, 20, 30
Immobilization time (h)	1–24
Enzyme concentration (mL)	0.5–5

After completion of the activation process, the activated PVC membranes were washed with distilled water to remove unreacted GA. The activated PVC membranes were then transferred to 20 mL of enzyme-phosphate buffer solution of pH 7.8 containing 9.36 activity units at 4°C for 16 h to complete the immobilization process. After completion of the immobilization process, the catalytic membranes were washed thoroughly with phosphate buffer solution (pH 7.8) to remove physically adsorbed enzyme. The immobilization conditions are summarized in the following Table I.

Determination of immobilized enzyme activity

The catalytic activity of the immobilized enzyme was measured using 20 mL of 4% (w/v) PGK-phosphate buffer solution (pH 7.8) at 37°C as a substrate. The hydrolysis of PGK by PGA yields 6-APA was determined by means of spectrophotometric method using para-dimethylaminobenzaldehyde as a colorimetric substrate.[32]

The retained activity percentage is the ratio of the immobilized enzyme's activity to free enzyme's activity and given as:

$$\text{Retained Activity } (\%) = \frac{\text{Activity of immobilized enzyme}}{\text{Activity of free enzyme}} \times 100$$

Retained activity percentage provides information on the role of substrate diffusion in the reaction. A value of 100 is obtained under conditions of complete diffusion, i.e., in case of homogenous reaction with the free enzyme.

The optimum temperature and pH were determined under the above mentioned conditions. The substrate temperature and pH were varied from 25 to 60°C and from 4.5 to 10, respectively.

The pH stability of PGA and immobilized PGA on PVC were studied by incubating the enzyme at 4°C in buffer solution of different pH ranged from 2.0 to 10 in the varying time and then determining the catalytic activity. Residual activities were calculated as the ratio of the activity of immobilized enzyme after incubation to the activity at zero incubation time.

The operational stability of the immobilized PGA was examined under batch operation mode. After each activity measurement, the immobilized PGA was separated from medium and washed three times with phosphate buffer (20 mL, pH 7.8) and then fresh reaction medium was introduced onto the immobilized enzyme. By this way, the next activity measurement was carried out.

FT-IR spectroscopic analysis

The structure of the PVC membranes and modified PVC membranes were analyzed by FT-IR spectra. FT-IR spectra in the absorbance mode were recorded using FT-IR spectrometer (Shimadzu FTIR- 8400 S, Japan), connected to a PC, and analysis the data by IR Solution software, Version 1.21.

Thermal gravimetric analysis

The thermal degradation behaviors of the PVC membranes were studied using Thermo Gravimetric Analyzer (Shimadzu TGA–50, Japan); instrument in the temperature range from 20 to 600°C under nitrogen at a flow rate of 20 mL/min and at a heating rate of 10°C/min.

Morphological characterization

The surface morphology of PVC and modified PVC were observed by SEM. The dried PVC and modified PVC membranes were coated with gold under reduced pressure, and their scanning electron micrographs were obtained using a JEOL (Model JSM 6360LA; Japan) at an accelerated voltage of 20 kV.

RESULTS AND DISCUSSION

The processes of membrane modification, activation, and enzyme immobilization are illustrated in Scheme 1.

Immobilization process

Effect of enzyme concentration

Figure 1 shows the effect of variation enzyme amount from 2.34 to 23.4 U on the catalytic parameters of immobilized enzyme. The immobilization temperature was kept at 4°C and pH at 7.8 for 16 h. From the Figure, it is clear that enzyme activity increased along with enzyme amount till specific amount, 16.38 U. Moreover, beyond this amount, the activity tends to be leveled off. Immobilization yield percentage has a different behavior where maximum immobilization yield percentage (35%) was observed at 9.39 U. The immobilization yield percentage starts to decrease with further increase of enzyme amount to reach minimum value, 17%, at higher enzyme amount. On the other hand, retained activity percentage was exponentially decreases with increase enzyme amount to level off at the end with enzyme amount starting at 9.36 U. This behavior may be

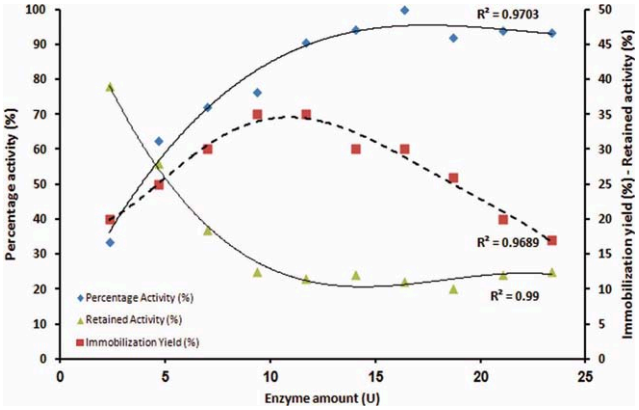

Figure 1 Effect of enzyme concentration on the immobilized enzyme activity. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

explained bearing in mind that 16.38 enzyme units are enough to react with all available aldehyde groups on the surface of PVC membranes, so further increase of enzyme amount has no effect on immobilized amount of enzyme (Fig. 1). The obtained result is in accordance with previous published results by the authors in Ref. 33.

Effect of immobilization's temperature

The effect of variation immobilization's temperature on the catalytic parameters of immobilized enzyme was presented in Table II. The immobilization pH was kept at 7.8 using 9.36 U of enzyme for 16 h. The table shows that the activity increases with increase of immobilization's temperature from 4 to 30°C. This could be referred to the fact that with increasing the immobilization's temperature, the amount of immobilized enzyme increased as the immobilization yield percentage increases to reach maximum value (50%) at 30°C. In addition, at higher temperature, the enzyme changes its conformational structure to the best form. This explanation has been confirmed by the results obtained from temperature profile of immobilized enzyme where maximum activity was obtained around 30°C. This finding considers as an advantage because the immobilization process could be performed without need of cooling or in other words at room temperature. On the other hand, the retained activity percentage of the immobilized enzyme was almost constant where indicates that both the catalytic activity and the immobilized amount of enzyme were increased with equal rate.

TABLE II
Effect of Immobilization Temperature on Immobilization Parameters

Immobilization temperature (°C)	Percentage activity (%)	Immobilization yield (%)	Retained activity (%)
4	81 ± 4	35 ± 2	25 ± 1.7
20	88 ± 3.6	41 ± 2.5	23 ± 1.9
30	100 ± 4.9	50 ± 3.2	21.5 ± 1.2

Effect of immobilization's time

Figure 2 shows the effect of variation immobilization's time on the catalytic parameters of immobilized enzyme. The immobilization temperature was kept at 4°C and pH at 7.8 using 9.36 U of enzyme. Catalytic activity at 25 h was considered as 100%. Almost linear increment of the activity has been observed with immobilization's time increase up to 16 h, where 86% of activity was obtained. Prolongation of immobilization's time after 16 h does not significantly affect the obtained activity. The immobilization's time could be shortened in case of performing the immobilization process at high temperature benefits from the results obtained in the study of immobilization's temperature (Table II). The immobilized amount of enzyme increased with higher rate compared to activity increment. Accordingly, the retained activity percentage reaches its maximum value, 30%, after 4 h immobilization time. Prolongation of immobilization time from 16 to 25 h has a slight effect on the retained activity percentage of immobilized enzyme.

Biochemical characterization

Kinetic parameters

Figure 3 presents the effect of the immobilization process on the kinetic parameters of immobilized enzyme. It is evident that the apparent K_m value for the immobilized enzyme is 23 times higher than that of the free one, whereas the V_m of the immobilized form is six times lower than that of free form. These results are in agreement with other published in

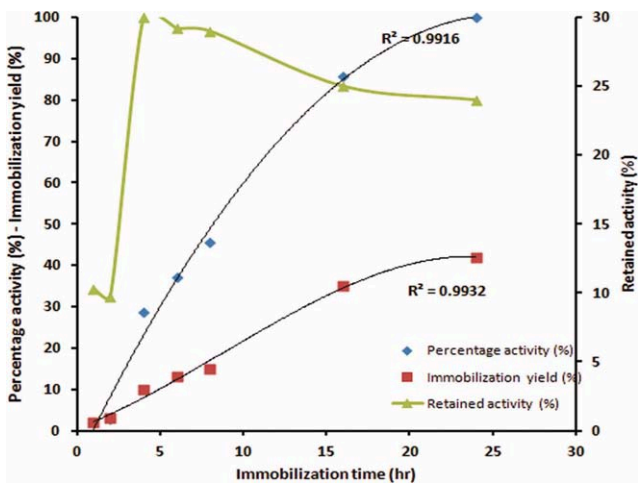

Figure 2 Effect of immobilization's time on the immobilized enzyme activity. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

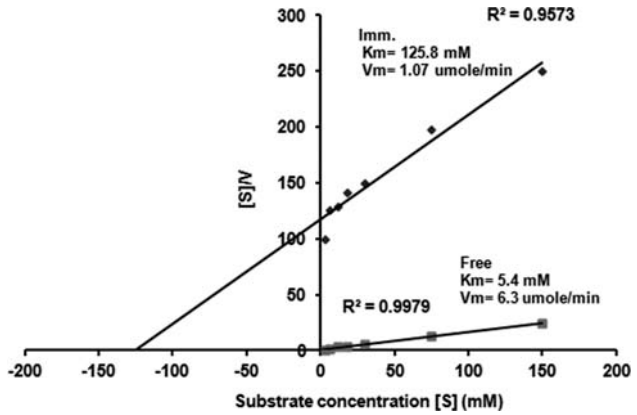

Figure 3 Han's Plot Curve for free and immobilized enzyme.

literature by Žuža et al.[34] where PGA covalently immobilized onto sepabeads EC-EP. They found that the apparent value of K_m for the immobilized enzyme was approximately fivefold higher than that of the free enzyme, suggesting that enzyme immobilization by this method caused a decrease in the enzyme-substrate affinity. Similarly, the value of V_m for the immobilized enzyme appeared to be approximately ninefold lower than that of the free enzyme, indicating that the catalytic property of the enzyme was significantly modified by the immobilization process. Wang et al.[35] reported that K_m value of immobilized penicillin acylase was seven times higher than that of the free enzyme owing to the influence of the support. These changes in kinetic parameters may be a consequence of either structural change in the enzyme occurring upon immobilization[36,37] or lower accessibility of substrate to the active sites of the immobilized enzyme. Depending on the of support's activation degree, immobilization of the enzyme may occur directly by covalent attachment or first ionically exchange the enzyme molecules and later covalently binding. At very low activation degree, the ionic exchange step will be neglect able and the covalent step will be the domain one. At higher activation degree, a mixture of adsorption followed by covalent binding will be existence.[36,37] In this way, the orientation of the enzyme molecules will be affected first and consequently the enzyme activity and retained activity.

Optimum pH

The catalytic activity of immobilized enzyme was performed in substrate solutions with varied pH values. Different behavior of the immobilized enzyme was observed in Figure 4, where no change of the optimum pH was noticed upon immobilization. However, the pH profile of the immobilized enzyme became broader, which indicates getting stability over a wide range of pH upon immobilization. The withstanding pH stability at acidic region is well known as effect of Schiff's base formation in accordance with other previous published data.[8] For instance, at pH value 5.5, immobilized enzyme retained 70% of its activity, whereas the free enzyme retained only 40%. Consequently, the high stability of the immobilized enzyme in both acidic and alkaline medium suggests that immobilized enzyme is less sensitive to the alteration induced by pH than the free enzyme. This behavior can be related to the covalent linkage and secondary interactions (ionic and polar stabilization, hydrogen bonding, etc) between the enzyme and the PVC membrane, which enhance the stability of immobilized enzyme structure at various pH values.[38] Abdel-Naby has the same observation with immobilized cyclodextrin glucosyltransferase onto aminated PVC.[24] The author explained this behavior based on the positive charges on the surface of aminated PVC microspheres. Bayramŏglu et al.[25] have the same observation with lipase covalently immobilized onto aminated polypropylene membranes. The optimum pH value of the immobilized lipase was shifted 0.5 U to the basic region (pH 7.5). This shift depended on the method of immobilization and the structure of the matrix. The pH profile of the immobilized enzyme was much broader with respect to the free enzyme. The authors stated that the shift to neutral and basic region of the optimal pH upon immobilized could be expected as a result of the diffusional constraint of the support retaining a higher concentration of enzyme product, fatty acids, on the surface of the membrane that immobilized lipase present. Thus, the microenvironment around the immobilized lipase was more acidic than that of the bulk solution. These results could probably be attributed to the stabilization of immobilized lipase molecules resulting from multipoint

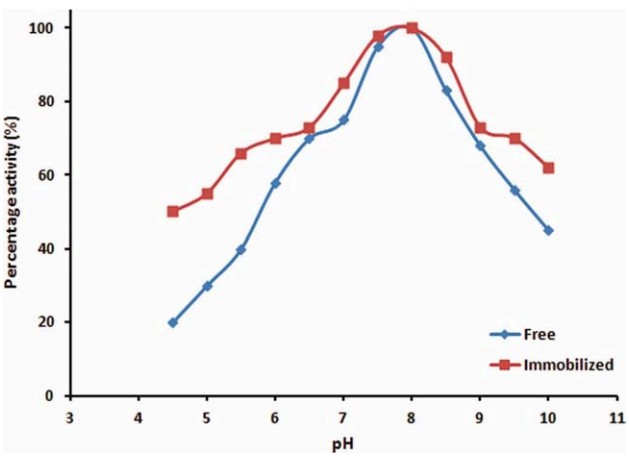

Figure 4 Effect of substrate's pH on the activity of free and immobilized enzyme. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

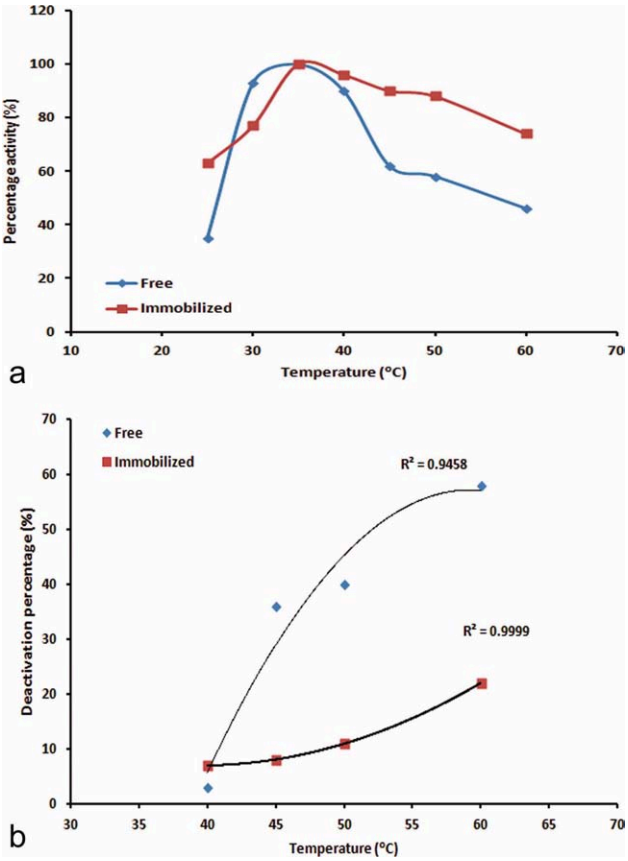

Figure 5 (a) Effect of substrate's temperature on the activity of free and immobilized. (b) Deactivation percentage as a function of substrate's temperature for free and immobilized enzyme. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

attachment on the surface of the APP membrane by covalent bonds, which limited the transition of enzyme conformation against the change of pH.

Optimum temperature

In studying the dependence of enzyme activity on substrate's temperature, a bell-shaped curve, with a maximum value of activity, is obtained. When compared with the activity of the free enzyme, the curve for the immobilized enzyme can be broader, wider, or equal, whereas maximum activity generally presents a shift toward higher temperatures upon immobilization. This means a higher resistance of the enzyme toward thermal deactivation since the structure of the catalytic site is strengthened by the immobilization procedure that created strong bonds between the macromolecule and the carrier.

The effect of the immobilization process on the temperature profile of immobilized enzyme has been presented in Figure 5. From the Figure, it is evident that the immobilized enzyme acquired more thermal stability. Two interesting observations results from a close inspection of the Figure; First one is the existence of wide range of temperature, about 20°C, in which the immobilized enzyme kept from 90 to 100% of its relative activity compared to 10°C only for the free counter part. The second observation is evidenced in Figure 5(b), where the enzyme deactivation is reported as a function of temperature beyond the maximum temperature; 35°C. From the Figure, it is clear that immobilization process has induced thermal stability to the enzyme. For instance, at 60°C, immobilized enzyme lost only 20% of its maximum relative activity, where the free enzyme lost 60%. It is worthy to mention here that the response of the immobilized enzyme to variation of environment temperature is less sensitive than the free one. This behavior can be related to the formation of multicovalent linkage with the enzyme. This leads to "restrict" of enzyme structure conformational changes as a result of environmental changes.

pH stability

Figure 6 show the pH stability for the immobilized enzyme. An optimum pH for the storage was at pH 8 and through days the activity decreased but it still the highest one between other pH ranges. Also, it is clear that after 34 days of storage at pH 8 the

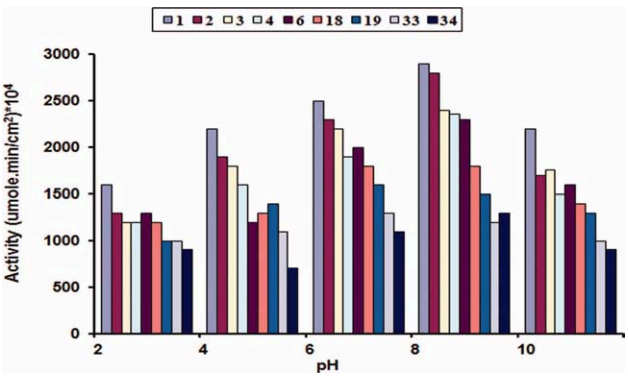

Figure 6 pH stability of the immobilized enzyme. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

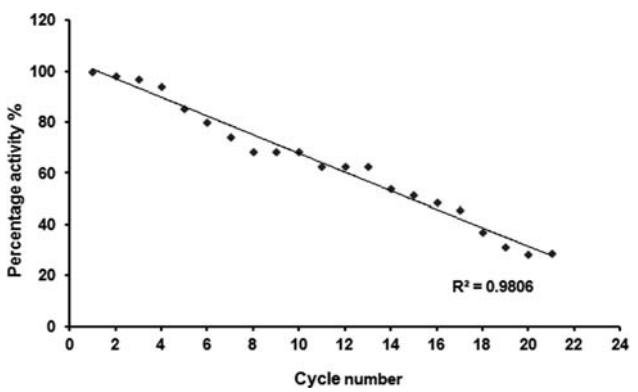

Figure 7 Operational stability of the immobilized enzyme.

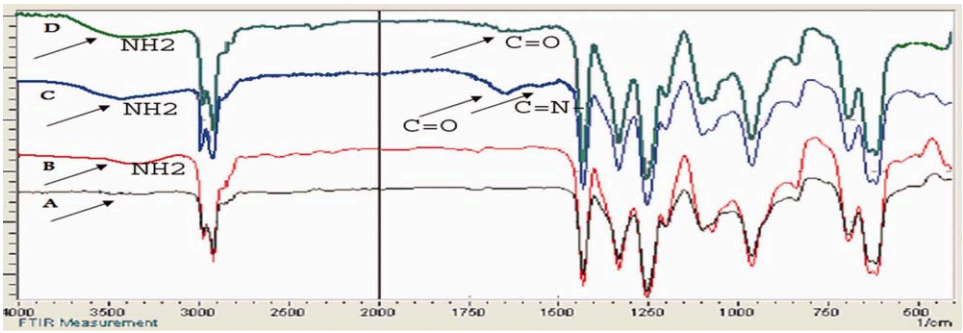

Figure 8 FT-IR spectra of PVC membrane (A), aminated PVC membrane (B), activated PVC membrane (C), and immobilized enzyme (D). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

immobilized PGA lost only 55.17% of its activity, whereas at other pH ranges lost 43.75%, 68.18%, 56%, and 59.09% of its activity at pH 2, 4, 6, and 10, respectively. However, at pH 2 lost only 43.755% of its activity (the minimum lose of activity) but we notice that at this pH, the activity value is low comparing with the activity value at pH 8.0. This stability could be further improved through the presence of substrate to fix the conformational structure of enzyme during immobilization.

Operational stability

The operational stability of the immobilized enzyme was investigated (Fig. 7). The immobilized PGA was repeatedly used for 21 cycles, 30 min each, with soaking the membranes in phosphate buffer for washing it after each cycle. The activity was found almost constant for the first four cycles. After that, gradual decrease of the activity was noticed. The immobilized PGA lost nearly 70% of its original activity after 21 cycles.

Membrane characterization

FT-IR analysis

The FTIR spectroscopic analysis of PVC membrane, activated membrane, and immobilized one are carried out from 400 to 4000 cm^{-1} (Fig. 8). The IR spectrum of PVC membrane shows characteristic beaks (curve A). The spectra for aminated PVC show a new broad beak at 3357 cm^{-1}, which is corresponding to NH_2 groups that indicates the presence of amine groups on the surface of the membrane (curve B). GA-activated membranes show new two beaks. The first of them is at 1720 cm^{-1} which refer to the (C=O) groups of a free aldehyde end of GA, and the another beak is at 1670 cm referring to (C=N—) group which is the result of reaction of NH_2 end groups with GA (curve C). Finally the spectra of catalytic membranes give broader beak at 3392 cm^{-1}, indicating to the increase in the concentration of NH_2 groups which found naturally in the enzyme and reduction of the intensity for beaks at 1720 cm^{-1}. From all above, it is can a sure that the process of amination, activation, and immobilization takes place successfully. This result is in agreement with other published results.[38]

TGA analysis

TGA is a simple and accurate method for studying the decomposition pattern and the thermal stability of polymers. As shown in Figure 9, curve A of PVC membrane, it was found that there are two decomposition steps. The first one at 374.4°C results from breaking of C—Cl bond. The second one at 591°C is referring to the decomposition of the ethylene bond. In the second curve B of aminated PVC, a new step appeared at 135°C which results from the evaporation of water molecules result from increase the hydrophilicity after reaction with EDA. The C—Cl decomposition step was shifted to higher temperature, 378.2°C, which indicates some stability of the membrane. In the curve of GA-activated NH_2-PVC membranes [Fig. 9(c)], there is no effect of humidity, and the main decomposition step of C—Cl bond was shifted back to lower temperature, 360°C. In the last curve of catalytic PVC membranes [Fig. 9(d)], the

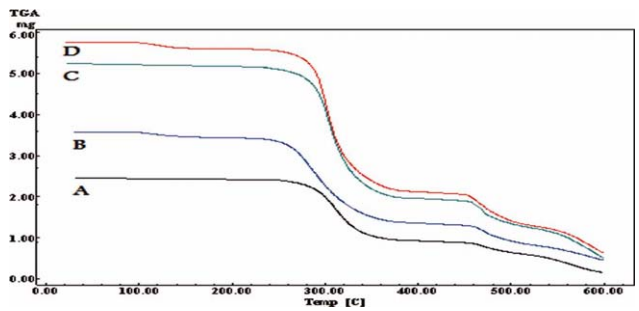

Figure 9 TGA Thermographs of PVC membrane (A), aminated PVC membrane (B), activated PVC membrane (C), and immobilized enzyme (D). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

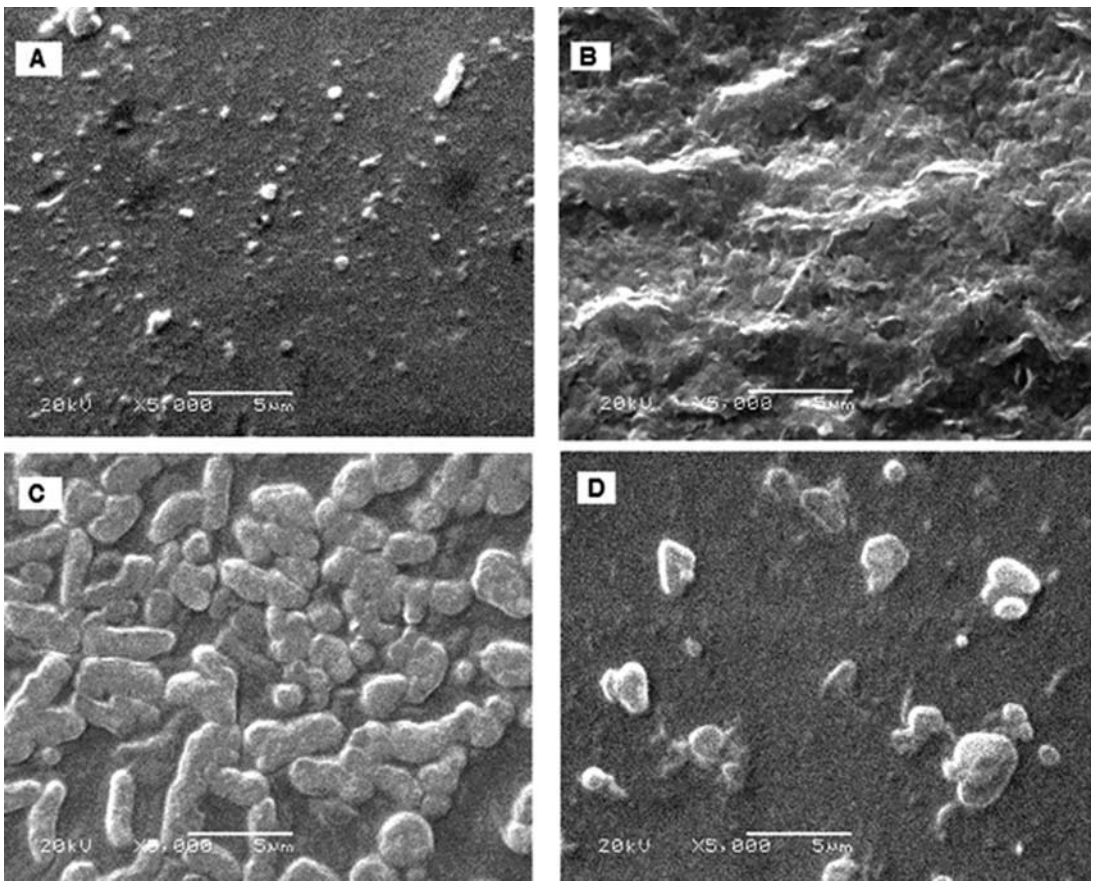

Figure 10 SEM of PVC membrane (A), aminated PVC membrane (B), activated PVC membrane (C), and immobilized enzyme (D).

step of loss humidity is appeared again because of the NH_2 groups present in enzyme. On the other hand, the mean step of C—Cl bond decomposition was shifted to 386.6°C, which refers to more stability for the membrane immobilized with enzyme.

SEM morphology examination

Figure 10 displayed SEM for PVC membrane, aminated PVC, activated PVC, and immobilized one. From these curves (A, B, C, and D), it can be noticed the changes which happened in each step which confirmed that there is change in the surface after each step. With amination step [Fig. 10(B)], new functional groups are introduced, amino and imide groups, which added some kind of charges on the surface causing increase of roughness. With activation of the terminal amino groups with GA, some kind of aggregates have been observed which results from possible crosslinking reaction between two terminal amino groups on the PVC membrane surface. These kind of aggregates have been reduced in number after immobilization of enzyme molecules, which may be referred to formation of monolayer of biomolecules on the surface of the PVC membrane [Fig. 10(D)].

CONCLUSIONS

PGA enzyme was covalently immobilized onto the GA-activated surface of aminated PVC membranes. The conditions of immobilization process such as enzyme concentration, immobilization's temperature, and time were optimized. The immobilized enzyme has a broader temperature profile where kept its optimum temperature unaltered. No change of the optimum pH was obtained upon immobilization. However, the pH profile for the immobilized enzyme became broader, which indicates getting stability over a wide range of pH upon immobilization. The apparent kinetic parameters K_M and V_M were determined. K_M of the immobilized PGA was found 23 times higher than that of the free enzyme indicating a lower substrate affinity of the immobilized PGA, whereas V_M was found six times lower than that of the free enzyme. Operational stability for immobilized PGA was recognized over 21 repeated use cycles where retained up to 40% of its original activity.

In conclusion, this technique has proven to be effective in immobilization of PGA enzyme on PVC membranes with high catalytic activity reaches to 4000 μmole min m^2 and 45% retained activity.

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