Covalent Immobilization of β-Galactosidase onto Amino-Functionalized PVC Microspheres

M. S. Mohy Eldin,[1] M. R. Elaassar,[1]* A. A. Elzatahry,[1]* M. M. B. Al-Sabah,[2] E. A. Hassan[2]

[1]*Polymer Materials Research Department, Advanced Technology and New Materials Research Institute, City for Scientific Research and Technology Applications, New Borg El-Arab City 21934, Alexandria, Egypt*
[2]*Department of Chemistry, Faculty of Science, Al-Azhar University, Cairo, Egypt*

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ABSTRACT: β-Galactosidase enzyme, from *Aspergillus oryzae*, was covalently immobilized onto amino-functionalized PVC microspheres after activation with gultaraldehyde. PVC microspheres were functionalized by introducing terminal amine groups via amination process with ethylene diamine (EDA). Furthermore, verifications of amination process were obtained using TGA, FT-IR, and SEM analysis. Different factors affecting the amination process were investigated and their impact on the activity and the retention of immobilized enzyme's activity was monitored. Concentration of ethylene diamine, amination temperature, and time were found for a determined effect. Reaction with EDA (0.025%) at 30°C for 40 minutes was found for optimum conditions. Variation of PVC : EDA mass ratio over 1 : 1 was found with neglectable effect. Thermal stability of immobilized enzyme was recognized where the immobilized form kept 80% of its original activity compared with 20% for the free form. Denaturation tolerance against pH was observed where immobilized form kept 60% of its original activity after 300 minutes incubation at pH 7.0 in the absence of substrate while the free form kept only 15% of its activity under the same conditions. Moreover, immobilized form show storage stabilities where immobilized form kept 40% of its original activity after four weeks while the free form kept only 25% of its initial activity. Under optimum conditions for enzyme immobilization, 1 kg of immobilized enzyme retains 87% of its native activity and has 11,000 activity units. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 125: 1724–1735, 2012

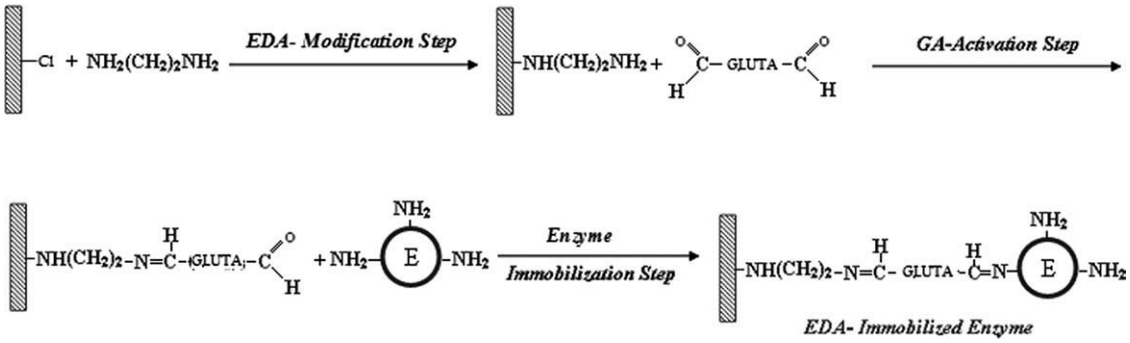
Key words: PVC functionalization; enzyme covalent immobilization; aminoalkylation; stability; characterization

INTRODUCTION

Enzyme immobilization is considered as one of the most popular biotechnological applications of different synthetic polymers including PVC.[1–9] Enzyme immobilization facilitates the purification of the reaction system and the recovery of enzyme and makes it possible to use the enzyme repetitively or continuously. Immobilization is often accompanied by some changes in the enzymatic activity, optimum pH, affinity toward substrate, stability of the enzyme, etc. Such changes depend on the combination of enzyme and carrier and the immobilization conditions.[10–15] β-Galactosidase (3.2.1.23) is a hydrolytic enzyme that catalyzes the breakdown of lactose into glucose and galactose. This enzyme is widely present in microbes, plants, and animals.[16,17] β-Galactosidase from *Aspergillus oryzae* is monomeric, having a molecular weight of 105 kDa and a PI of 4.6. This enzyme showed a pH optima of 4.5 with ONPG and 4.8 with lactose.[18–20] Enzymatic hydrolysis of lactose is a popular technology used to produce lactose-reduced milk and its derived dairy products for consumption by lactose intolerant persons whose metabolism exhibited a decline in the level of β-galactosidase activity.[21–23]

Immobilization of β-galactosidase can be achieved by various methods such as adsorption, covalent attachment, chemical aggregation, entrapment, and micro-encapsulation.[17,22,24–27] Among the various methods available for enzyme immobilization, covalent binding is particularly important because it leads to preparation of stable enzyme derivatives.[28–37] Such kind of stability results from the formation of multipoint or multisubunit attachment which causes changes in the enzyme structure and microenvironments.[28–32] Suitable functional groups are essential to conduct such immobilization techniques, since PVC has no functional groups in its structure, so chemical modification was carried out to introduce proper functional groups. Grafting technique, with different types of polymers possessing different functional groups, was intensively presented as the main

**Present address:* Chair of Petrochemicals, Chemistry Department, Faculty of Science, King Saud University, Riyadh, KSA..
Correspondence to: M. S. Mohy Eldin (mohyeldin mohamed@yahoo.com).
Contract grant sponsor: CSRTA, Alexandria, Egypt.



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

solution using either a chemical or a radiation initiation system.[38–41] On the other hand, another simple technique using the aminoalkylation reaction with diamine has been presented.[42] This technique depends on the reaction between the available chlorine atoms on the PVC surface and the amine groups of diamine. The introduced amine groups were further activated using a symmetric coupling agent, glutaraldehyde (GA), which finally covalently bound with enzyme. The mechanism of PVC modification, activation, and immobilization with the enzyme is presented in Scheme 1. The structure of glutaraldehyde in the scheme may be monomeric, dimeric, or cyclic depending on the carrier activation conditions. Each structure has its influence on the activity and stability of the immobilized enzyme.[28–32] Despite the fact that such a technique has been investigated almost 28 years ago,[9] still the optimization of the modification and the activation process need further investigations.

In this work, β-galactosidase was immobilized onto amino-functionalized PVC microspheres. Functionalization and activation processes of the PVC microspheres' surface were investigated and the optimum conditions were reached. Moreover, that the changes occurred as a result of the functionalization process was proved using TGA, FT-IR, and SEM analysis. Finally, different forms of induced stabilities, as a result of immobilization, were monitored.

MATERIALS AND METHODS

Materials

Poly(vinyl chloride) (PVC), ethylene diamine (EDA), and β-galactosidase (E.C.3.2.1.23), from *Aspergillus oryzae*, were obtained from Sigma Chem. Co. (St. Louis, USA). Glutaraldehyde (GA) was obtained from Fluka (packed in Switzerland) and lactose 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

PVC microspheres surface modification

PVC microspheres, mean particle size 1600 nm, were aminated by treating with a large excess of an aqueous solution of ethylene diamine. Thus, 1 g of PVC was mixed with 0.025% EDA, 20 ml solution in distilled water and kept in a water bath maintained at 80°C for 1 h. After completion of the reaction, the microspheres were filtered and washed with distilled water to remove unreacted ethylene diamine were and then dried in an air dryer.[43] By studying the optimum conditions for PVC's surface modification, EDA concentration was varied from 0.025% to 1%, the reaction temperature from 30 to 80°C, reaction time from 10 to 60 minutes and PVC amount from 0.25 to 3.0 g.

Determination of nitrogen content by Kjeldahl method

Overview. This method consists in mineralizing the sample with concentrated sulfuric acid and alkalinizing with NaOH. The ammonium liberated is carried by distillation and recovered on boric acid. The subsequent titration with HCl allows the calculation of the amount of ammonium in the sample.

Digestion. To the weight of the sample (0.5 g), 1 g of cupper sulfate catalyst, 10 ml of sulfuric acid at 96% ($d = 1.84$), and some granules of glass were added. The digestion tube was placed with the sample into the Bloc-digest with the fume removal in working condition, and the digestion was done at a temperature between 350 and 420°C.

Distillation. Boric acid (50 ml) was mixed with indicator in an Erlenmeyer flask. The distillation was extended for some time, approximately between 5 and 10 minutes, in order to distill a minimum of 150 ml.

Titration. The distilled solution was titrated with HCl (0.25*N*) until the color changed from green to violet.

The quantity of nitrogen detected was calculated by means of the formula

$$\% \text{ Nitrogen} = 1.4\ (V_1 - V_0) \times N/P$$

where P = weight g of sample, V_1 = HCl consummation on titration (ml), V_0 = HCl consummation on blank (ml), N = normality of HCl.

Enzyme immobilization

Aminated PVC microspheres, 1 g, were activated using 20 ml of glutaraldehyde (1%, pH 8.0) at 40°C for 60 minutes. After completion of the activation process, the activated PVC microspheres were filtered and washed with distilled water to remove unreacted glutaraldehyde. The activated PVC microspheres were then transferred to enzyme phosphate-citrate buffer solution of pH 6.0, 20 ml, containing 0.005 g of β-galactosidase and stirred at room temperature, for 1 h, and then for 16 h at 4°C to complete the immobilization process. For optimization of the activation process, GA concentration was varied from 0.1% to 5%, reaction temperature from 30 to 80°C, reaction time from 10 to 60 minutes, and pH from 2.0 to 10.0.

Determination of immobilized enzyme activity

The catalytic activity of the immobilized enzyme was determined by mixing 1 g of catalytic PVC microspheres with 50 ml of 100 mM lactose in phosphate- citrate solution of pH = 6.0 at 40°C with stirring, 250 rpm, for 30 minutes. Samples (0.1 ml each) were withdrawn every 5 minutes to assess the produced glucose using glucose Kit. The enzymatic activity was determined by the angular coefficient of the liner plot of the glucose production as a function of time.[8]

The retention of activity percentage (RAP) is the ratio of the immobilized enzyme's activity to free enzyme's activity and is given as

retention of activity (%) = (activity of immobilized enzyme × 100)/activity of free enzyme

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

Temperature and pH stability

The thermal stability of β-galactosidase and immobilized β-galactosidase on PVC was tested by incubating the enzyme at 60°C in buffer (pH 4.2) for varying time. Residual activities were calculated as the ratio of the activity of the immobilized enzyme after incubation to the activity at zero incubation time.

The pH stability of β-galactosidase and immobilized β-galactosidase on PVC was studied by incubating the enzyme at 4°C in buffers with pH 4.2 and 7 at varying time and then determining the catalytic activity. Residual activities were calculated as the ratio of the activity of the immobilized enzyme after incubation to the activity at zero incubation time.

Storage stability

The free and immobilized β-galactosidase forms in phosphate/citrate buffer, pH 4.2, were stored at 4°C in the absence of substrate. The storage stability was evaluated by determining the activity of free and immobilized β-galactosidase at regular time intervals up to 42 days.

FT-IR spectroscopic analysis

The structure of the PVC microspheres and modified PVC microspheres was analyzed by FT-IR spectra. Samples were mixed with KBr to make pellets. FT-IR spectra in the absorbance mode were recorded using an FT-IR spectrometer (Shimadzu FTIR- 8400 S, Japan), connected to a PC, and the data was analyzed by IR solution software (version 1.21).

Thermal characterization (TGA)

The thermal degradation behaviors of the PVC were studied using a thermo gravimetric analyzer instrument (Shimadzu TGA–50, Japan) in the temperature range from 20 to 400°C under nitrogen at a flow rate of 20 ml/min and at a heating rate of 10°C/min.

Morphological characterization (SEM)

The surface morphology of PVC and modified PVC was observed with the help of a scanning electron microscope (Joel JSM 6360LA, Japan) at an accelerated voltage of 20 kV. The fracture surfaces were vacuum coated with gold for SEM.

RESULTS AND DISCUSSION

Few publications about the covalent immobilization of enzymes onto the non-grafted, PVC matrix have been found in the literature.[44–46] Their studies were limited only to use readymade functionalized PVC membranes either with amine activated with GA[44,46] or carboxylic groups activated with GA and/or carobdiimide.[45] None of them have studied the optimization process for modification of PVC matrices. Also, a certain activation protocol was used without any optimization process.

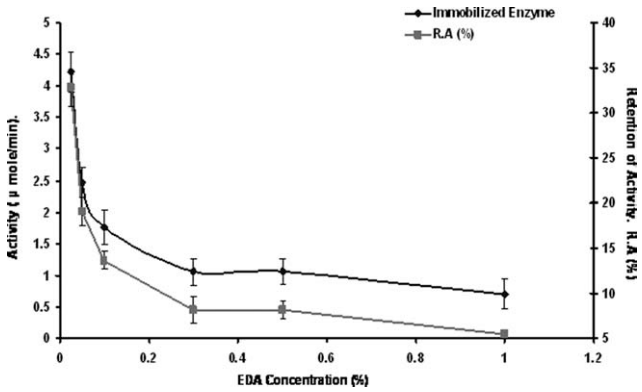


Figure 1 Effect of EDA concentration on the catalytic activity of immobilized enzyme.

In the following, the optimization of the functionalization process of PVC microspheres with amine groups followed by GA activation was studied and the catalytic activity of the immobilized enzyme was correlated to the amine content of modified PVC microspheres for the first time. The amination process with EDA was verified using different characterization techniques such as FT-IR, SEM, and TGA. Different factors affecting the functionalization and activation processes were studied and their effect on the activity of immobilized β-galactosidase was monitored and discussed.

Functionalization (amination) process

Surface functionalization of PVC microspheres with terminal amine groups was carried out using ethylene diamine (EDA). Factors such as ethylene diamine concentration, reaction time, reaction temperature, and PVC : EDA mass ratio were studied, and the obtained results are discussed. Native and treated PVC microspheres, except with the EDA functionalization step, show no catalytic activity, which proves the absence of any adsorption process of the enzyme in the absence of surface amino groups and proves also the non-porous nature of the microspheres.

Effect of EDA concentration

The effect of variation EDA concentration on the catalytic activity of immobilized enzyme was investigated (Fig. 1). A sharp decrease of the activity was observed with a slight increase of EDA concentration from 0.025% to 0.1%. Further increase of the concentration up to 0.3% decreases the activity with slower rate and remains almost constant with increasing EDA concentration up to 0.5%. Further slight activity decrease was observed with increase EDA concentration up to 1%. The determining effect of EDA concentration in the range 0.025% to 0.1% implies the importance of selection the optimum EDA concentration. A similar behavior was noticed by other authors for different enzymes covalently immobilized onto glutaraldehyde activated matrices.[47–49] They explain this behavior based on the presumption that immobilization of the enzyme may occur directly by covalent attachment or first ironically exchange the enzyme molecules and later covalently binding. This may depend on the use of support activation degree. At a very low activation degree, the ionic exchange step will be neglectable and the activation step will be the domain one. At a higher activation degree, a mixture of adsorption followed by covalent binding will be existent. In this way, the orientation of the enzyme molecules will be affected first and consequently the enzyme activity and retained activity will be affected. The behavior of activity decline in Figure 1 could be explained according to the previous mentioned theory. This explanation is reinforced by two findings. The first is the increase of the free terminal amine groups on the microspheres' surface as indicated by nitrogen content (Table I). The second is the fact that a fixed number of enzyme molecules have been immobilized since no trace of enzyme activity was detected in the immobilization medium after completion of the immobilization process. In addition, the formation of Shif's base results from the reaction between GA aldehyde groups and primary amine groups on the surface of PVC microspheres from one side and enzyme molecules from other side inducing ionic changes in the microenvironment around the immobilized enzyme. Possible hydrophobic interactions between the immobilized enzyme molecules and the hydrophobic moieties of PVC structure could also contribute. The individual or accumulated effect of the mentioned factors could give us a reasonable explanation of the presented behavior in Figure 1. The observed low immobilized enzyme's retained activity indicates the importance of optimizing the number of attachment points between the enzyme molecule and the matrix.

TABLE I
Effect of Variation EDA Concentration on the Attached N Content

EDA (%)	Nitrogen (%)
0.025	0.021
0.05	0.0245
0.1	0.0315
0.3	0.098
0.5	0.105
1	0.1015

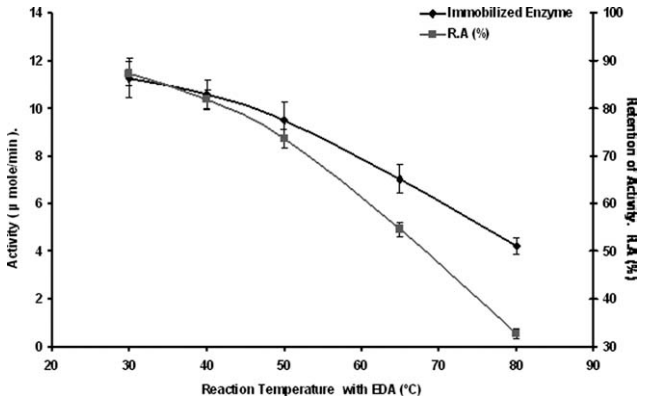


Figure 2 Effect of reaction temperature with EDA on the catalytic activity of immobilized enzyme.

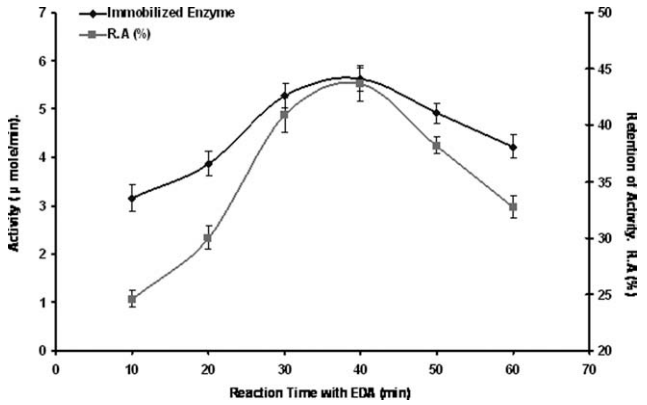


Figure 3 Effect of reaction time with EDA on the catalytic activity of immobilized enzyme.

Effect of reaction temperature with EDA

Figure 2 shows the effect of variation of amination reaction temperature on the catalytic activity of the immobilized enzyme. From the figure it is clear that the temperature increment, within the studied range (30–80°C) negatively affects the catalytic activity in a proportional manner. Taking into consideration the fact that a fixed number of enzyme molecules were immobilized onto all samples, it is expected that an increase in the amination reaction temperature increases the number of attached primary amine groups on the PVC surface and consequently the number of attachment points with each enzyme molecule. This kind of multiattachment points may cause formation of covalent bonds in the enzyme molecule active center, leading to retard the reach of the substrate and consequently reduces the activity. Data obtained from the determined nitrogen content supports this theory in the temperature range 30–50°C where the activity declines in an almost constant rate (Table II). Beyond 50°C, the nitrogen content starts to decrease at a slow rate contrary to the nitrogen content obtained at lower temperature. However, the activity was found to decline at a faster rate. This is may be due to the reaction of some terminal amino groups with other chlorine atoms on the PVC surface as a result of higher reaction rate at elevated temperature. As a sequence, higher concentration of imide groups formed on the microspheres surface, which represent an ionic site, cause changes in the conformational structure of enzyme molecules and may be induced by bad orientation. Following this step, covalent binding of the enzyme molecules with aldehyde terminal groups leads to reduce the catalytic activity in such a dramatic way. As a result, a strong decrease in retained activity with elevated reaction temperature was observed.

Effect of reaction time with EDA

The effect of variation reaction time was investigated (Fig. 3). From the illustrated results it is clear that, the activity increased linearly with the reaction time till reaching a maximum value at 40 minutes. Further increase of the reaction time induced a regular decrease of the activity. This trend is also reflected on the immobilized enzyme's retention of activity where the maximum value was obtained at 40 minutes of amination reaction time. On the other hand, the nitrogen content (Table III) shows a different behavior where it deceases continuously with the reaction time. This behavior may be explained by the erosion of aminated PVC layers with time and consuming of some terminal amino groups while bridging between the two sites of secondary chlorine atoms. The optimum activity and retention of activity was reached with 0.0196% nitrogen

TABLE II
Effect of Reaction Temperature with EDA on Attached N Content

Reaction temperature with EDA (°C)	Nitrogen (%)
30	0.0035
40	0.01225
50	0.02275
65	0.0203
80	0.0189

TABLE III
Effect of Reaction Time with EDA on the Attached N Content

Reaction time with EDA (min)	Nitrogen
10	0.0266
20	0.02485
30	0.0217
40	0.0196
50	0.01925
60	0.01785

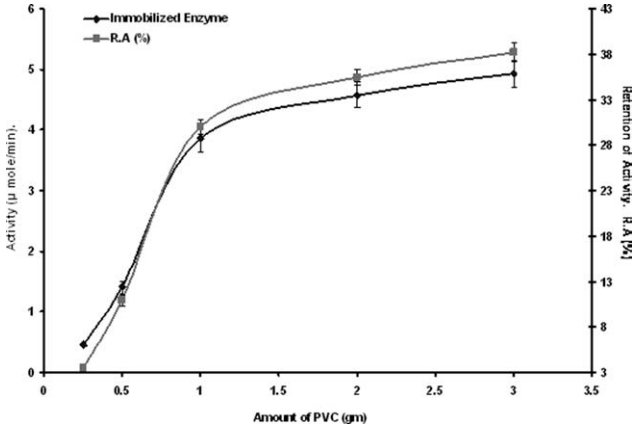


Figure 4 Effect of PVC–EDA ratio on the catalytic activity of immobilized enzyme.

content. Beyond this nitrogen content, the activity and retention of activity started to decline. This is may be due to the formation of larger number of imide groups which are considered as ionic sites with a bad impact on the orientation of the enzyme molecules and the subsequent immobilization step.

Effect of PVC: EDA mass ratio

The last step in exploring the optimum conditions for the amination process was performed by studying the effect of variation of PVC amount under fixed reaction conditions (Fig. 4). Two trends were noticed from inspection of the figure. The first is a linear activity increment with an increase of PVC amount in the range from 0.25 to 1.0 g. The second trend of the curve started from 1 to 3 g in which the activity increment rate was found to be lower and about to level off. This behavior is explained according to the linear correlation between the increase in the number of induced amine groups and the activity. The retained activity has the same trend. The nitrogen content results (Table IV) reinforced our explanation.

Activation process

Functionalized PVC microspheres, with surface primary amine groups, were activated using glutaraldehyde. The factors affecting the activation step, namely, glutaraldehyde concentration, reaction time, reaction temperature, and reaction pH were investigated and the obtained results are discussed. Glutaraldehyde[51] is a very versatile reagent that may be used in different ways: using preactivated supports (and permitting the enzyme ionic exchange as a first immobilization step),[50] or adsorbing the enzyme on aminated supports and further treatment of the adsorbed enzyme with glutaraldehyde to crosslink the enzyme and support.[32] In both cases, the enzyme will be finally attached to the support by Lys residues in the most negatively charged areas because the first step will be an ionic exchange.

TABLE IV
Effect of PVC:EDA Mass Ratio on the Attached N Content

PVC	Nitrogen (%)
0.25	0.0084
0.5	0.0126
1	0.0196
1.5	0.0205
2	0.0216
3	0.0231

Effect glutaraldehyde concentration

Figure 5 shows the effect of variation of gultaraldehyde concentration on the catalytic and retained activity of the immobilized enzyme. From the figure, it is clear that linear increment of the activity was observed with glutaraldehyde concentration increasing up to 1%. Further increase of GA concentration to 5% increased the activity linearly but with lower rate. This behavior could be explained according to the existence of some left ionic charges on unactivated, protonated attached amine groups. These charges may lead to the adsorption of the enzyme molecules in the first stage. However, the enzyme molecules are not in the best conformational form, so with the further binding step, the enzyme molecules lost some of their activity. Increasing the gultaraldehyde concentration leads to two simultaneous things. The first is possible yield of an uncontrolled reaction that generated glutaraldehyde polymerization in solution[50,51] to have dimeric and cyclic gultaraldehyde forms with variable and less activity toward reaction with amine groups. The second possibility is decrease in the left ionic charges on the microspheres surface leading to immobilization of

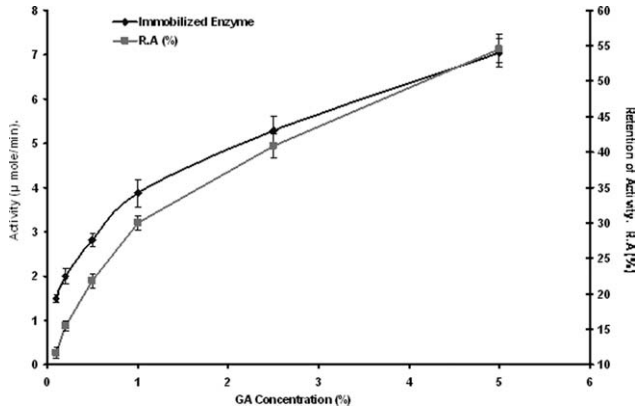


Figure 5 Effect of GA concentration on the catalytic activity of immobilized enzyme.

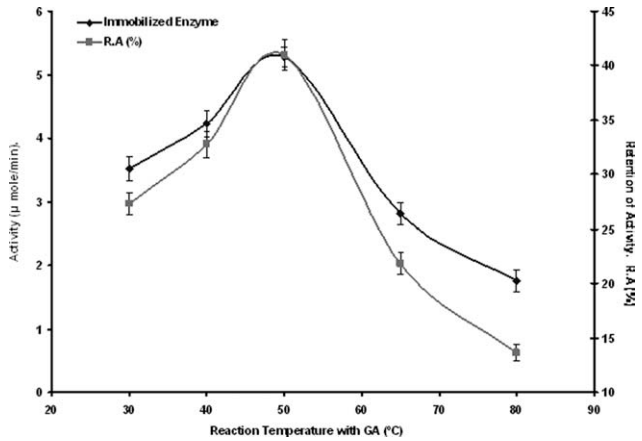


Figure 6 Effect of reaction temperature with GA on the catalytic activity of immobilized enzyme.

the enzyme molecules in a better conformational structure and consequently gaining higher activity. The retained activity of the immobilized enzymes behaves similarly.

Effect of reaction temperature with GA

Figure 6 describes the effect of variation of the reaction temperature with GA on the catalytic activity of immobilization enzyme. From the illustrated data it is clear that the activity linearly increases with temperature to reach its maximum value at 50°C. Further increase of temperature beyond 50°C, the activity linearly decreased to reach the lowest value at 80°C. According to the published results by Roberto Fern'andez-Lafuente et al.[50] and Monsan,[51] proceeding of glutaraldehyde reaction at higher temperature may promote the polymerization and yield dimeric and cyclic forms, which are less reactive toward the amine groups. This may explain the linear decrease of activity beyond 50°C.

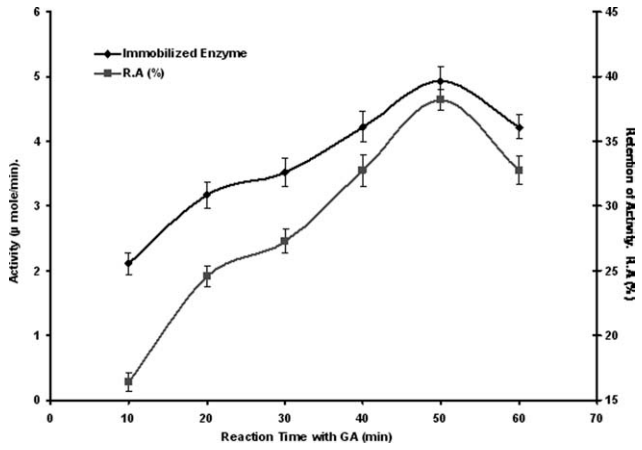


Figure 7 Effect of reaction time with GA on the catalytic activity of immobilized enzyme.

Effect of reaction time with GA

Variation of reaction time with GA clearly affects the catalytic activity of the immobilized enzyme (Fig. 7). Liner increment of activity was observed with increasing reaction time up to 50 minutes in which the maximum activity was obtained. This may be explained as mentioned before; increase in the reaction time decreases the number of protonated unactivated surface amino groups which consequently allow the enzyme molecule to be immobilized in the best conformational structure and hence gain better activity. Prolongation of the reaction time to 60 minutes negatively affected the catalytic activity due to promotion of the formation of dimeric and cyclic forms of glutaraldehyde which are less reactive toward amine groups.[50,51] This will lead consequently to reduce the number of active centers on the PVC microspheres available to bind the enzyme molecules. The behavior of the catalytic activity and retained activity is very similar. They showed approximately the same behavior when the reaction time increased.

Effect of GA solution pH

Figure 8 presents the effect of variation of GA solution pH on the catalytic activity of the immobilized enzyme. The curve has two stages. The first stage is linear increment of activity in the pH range from 2.0 to 6.0. The second stage, in the pH range from 6.0 to 10.0, increment turned to be exponential. This behavior may be referred to the de-protonation effect of alkaline medium on the amine groups which increases its reaction rate with GA and aldehyde groups. This consequently leads to eliminate the negative effect of the first adsorption step with un-activated protonated amino groups and consequently leads to immobilize the enzyme in the best conformational structure and gains better activity.

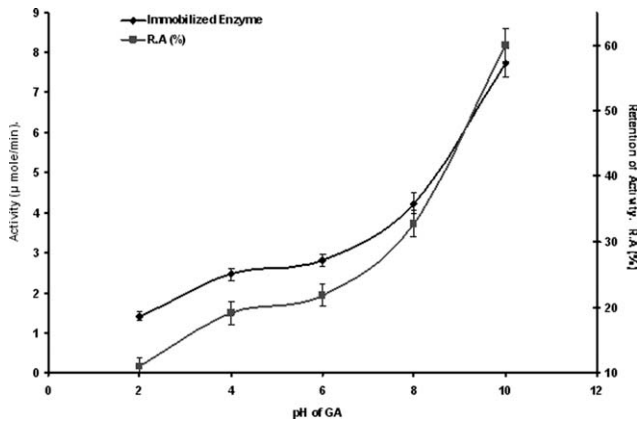


Figure 8 Effect of reaction pH with GA on the catalytic activity of immobilized enzyme.

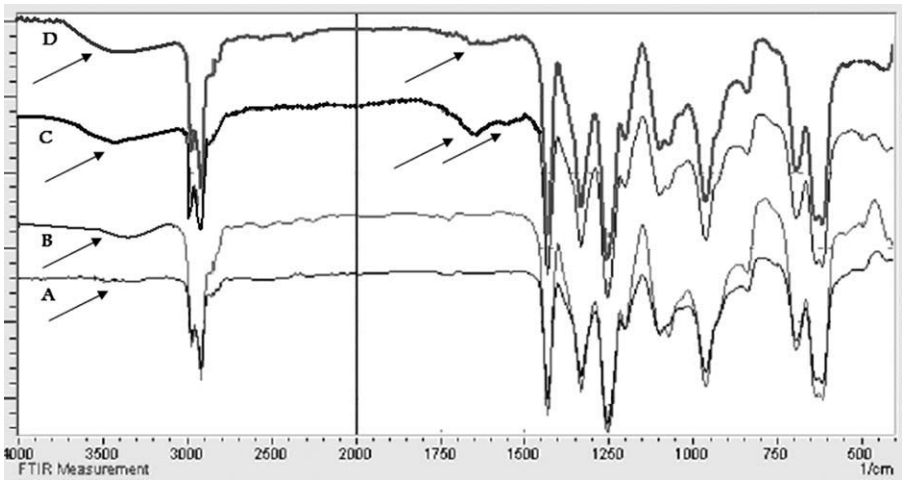


Figure 9 FT-IR analysis: PVC (A), aminated PVC (NH_2-PVC) with 0.025% EDA (B), glutaraldehyde activated NH_2-PVC (C), and glutaraldehyde activated NH_2-PVC-immobilized enzyme (D).

As a result, the retained activity of immobilized β-galactosidase gained the same trend. The same trend has been observed by Mohy Eldin et al.[6] with immobilization of PGA onto amino functionalized nylon particles. At high reaction pH, the possibility of glutaraldehyde polymerization is raised. According to the published results by Roberto Fern'andez-Lafuente et al,[50] using lower concentrations or shorter reaction times, an average of more than one glutaraldehyde molecule and less than two glutaraldehyde molecules were incorporated. The use of more drastic conditions (e.g., pH over 8, higher glutaraldehyde concentrations) yielded an uncontrolled reaction that generated the polymerization of glutaraldehyde in solution. These results are in agreement with those previously reported by Monsan.[51] Polymerized glutaraldehyde at higher pH (10) plays double functions. The first is the regular binding one. The other one is "spacer" which puts the enzyme molecules away from the support surface and makes them more available for the substrates. This normally leads to have more activity and higher retained activity for the immobilized enzyme.

Matrix characterization

The verification of surface functionalization process was obtained through performing FT-IR and TGA analysis of the modified PVC microspheres. In addition, morphology changes resulting from surface modification has been monitored through SEM micrographs. The obtained results are discussed.

FT-IR analysis

The FT-IR spectroscopic analysis of PVC microspheres, activated microspheres, and catalytic one is carried out from 400 to 4000 cm^{-1} (Fig. 9). The IR spectrum of PVC microspheres shows characteristic beaks (curve A). The spectra for aminated PVC shows a new broad beak at 3357 cm^{-1} which is corresponding to the NH_2 groups that indicates the presence of amine groups on the surface of the microspheres (curve B). Glutaraldehyde activated microspheres show new two peaks. The first of them at 1720 cm^{-1} refers to the carbonyl groups (C=O) of the free aldehyde end of glutaraldehyde, and the other peak at 1670 cm^{-1} refers to (C=N—) bonds which result from reaction of NH_2 end groups with glutaraldehyde (curve C). Finally, the spectra of catalytic microspheres gives a broader peak at 3392 cm^{-1}, indicating the increase in the concentration of NH_2 groups which are found naturally in the enzyme and reduction of the intensity for peaks at 1720 cm^{-1} as a result of the reaction with enzyme surface amine groups. From all of the above, it is sure that the process of amination, activation, and immobilization takes place successfully. This result is in agreement with other published results.[52]

TGA analysis

Functionalization of the PVC surface with amine groups is expected to modify the hydrophilicity of modified PVC microspheres. Figure 10 confirms our expectations. The modified PVC with 0.025 and 0.05% EDA shows weight loss at 30–100°C range as a result of water loss ranging from 29.5 to 33.26%. This thermo-gram is absent in native PVC. The second weight loss stage appeared at the 250–400°C range. The weight loss was reduced from 62% to 42% for PVC and modified PVC with 0.025 and 0.05% EDA [Fig 10(B,C)], respectively. This behavior confirmed the substitution of surface secondary chlorine atoms with primary amine groups and

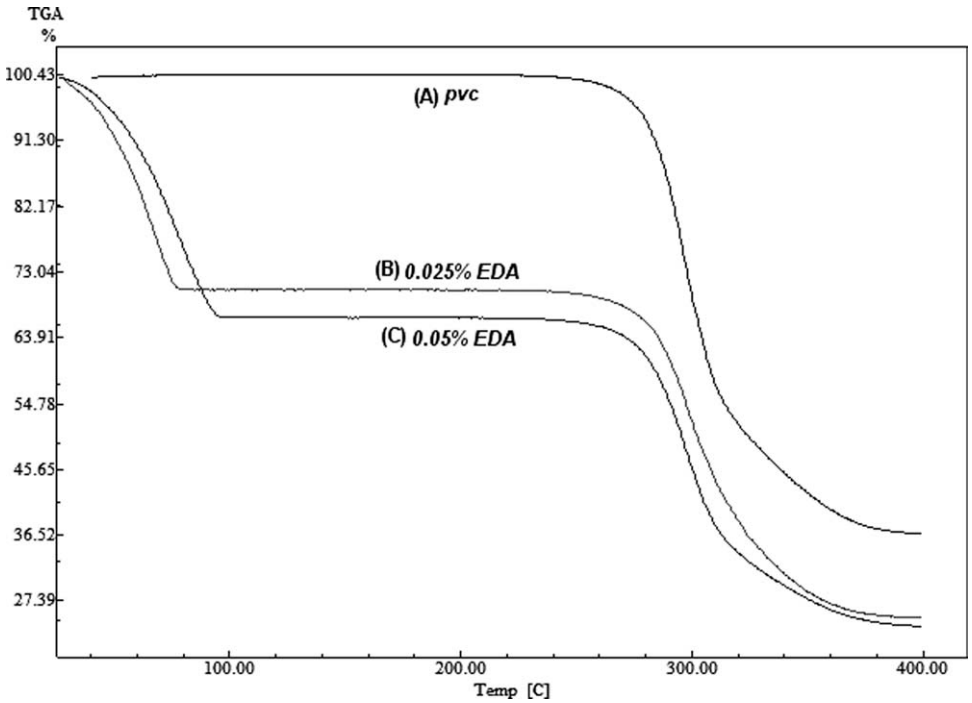


Figure 10 TGA analysis: PVC (A), aminated PVC (NH_2-PVC) with 0.025% EDA (B), and aminated PVC (NH_2-PVC) with 0.05% EDA (C).

emphasizes the possibility of the occurring crosslinking reaction.[53]

SEM morphology examination

Since the modification process occurs on the surface, it is essential to monitor the surface morphology changes resulting from the modification (Fig. 11). From the figure, the nonporous nature of the original PVC microspheres can be seen. The changes in the surface morphology of modified matrix (11B, 11C) compared with un-modified one (11A) show that the surface smoothness increased with increase in the concentration of EDA.

Immobilized enzyme characterization

For evaluation of the studied immobilization protocol, stability characters of the immobilized enzyme were studied. The obtained results are discussed in the following sections.

Thermal stability

The resultant thermal stability for the immobilized enzyme compared to the free form is presented in Figure 12. The half life time for the free form was found to be 240 minutes while the immobilized form onto modified matrix using 0.025% EDA (Immob. 1) increases to be 300 minutes. The immobilized form onto modified matrix using 0.5% EDA (Immob. 2) shows higher stability which lost only 20% of its activity after 300 minutes. Such acquired stability may be referred to the formation of multiattachment points which "fix" the structure of the enzyme molecules. This explanation is reinforced by the obtained nitrogen contents, 0.021% and 0.105%, for immobilized forms 1 and 2 (Table I), respectively. The immobilized enzyme maintained a greater rigidity and was more resistant to unfolding at higher temperatures than its free form.[54–56] Therefore, the immobilized enzyme could work in harsh environmental conditions with less activity loss compared to its free counterpart.[57–59]

pH stability

Figure 13 shows the pH stability of free and immobilized enzyme in acidic medium, pH 4.2, and in neutral medium; pH 7.0. Inspecting the figure shows that the stability of immobilized form is higher in general. Almost linear decrement of the immobilized enzyme relative activity has been noticed at pH 4.2 where the immobilized form kept around 70% of its activity after five hours incubation compared to about 55% of free form. Real advantage of immobilization is clear at pH 7.0 where the free form keeps around 15% of its original activity while the immobilized form keeps 60%. The presence of covalent bonds between the enzyme molecules and the microspheres surface contributes in inducing

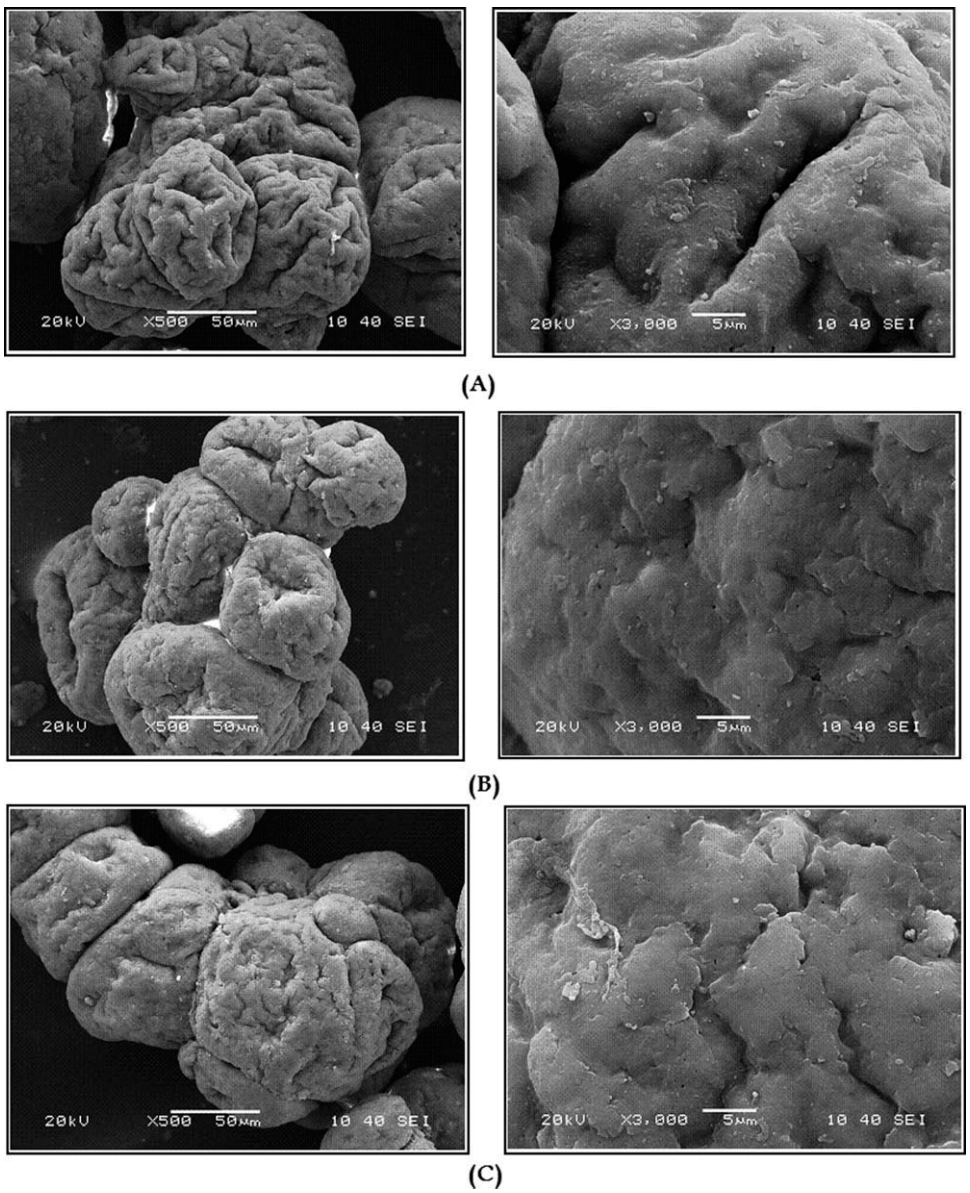


Figure 11 SEM morphology analysis: PVC (A), aminated PVC (NH_2-PVC) with 0.025% EDA (B) and aminated PVC (NH_2-PVC) with 0.05% EDA (C).

structural stability which restricts the conformational changes leading to the inactivation effect.[60]

Storage stability

High replacement costs of enzymes used for industrial purposes and the time necessary for their immobilization have led to increasing interest in the storage stability of these enzymes for longer periods. After six weeks of storage at 4°C, the immobilized β-galactosidase shows high stability where it retained 40% of its activity while the free enzyme retained only 25% of its activity (Fig. 14).

CONCLUSION

PVC microspheres were successfully modified with EDA and as a result their surfaces have been functionalized with the amine groups. EDA concentration (0.025%) and 30°C reaction temperature for 40 minutes are the best modification conditions to induce the highest immobilized enzyme catalytic activity. Surface aminated PVC microspheres were activated using glutaraldehyde as a coupling agent. The conditions of the activation process were investigated such as glutaraldehyde concentration, pH, reaction time, and reaction temperature. The optimum conditions within the studied range are GA 5%, pH = 10.0, 50 minutes reaction time, and 50°C

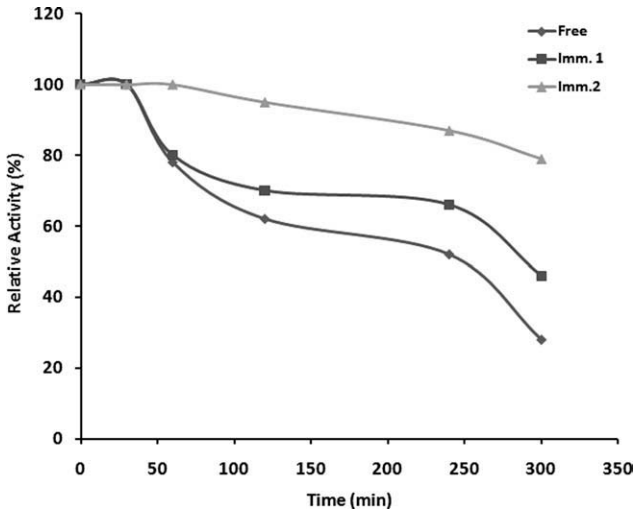


Figure 12 Thermal stability of free and immobilized enzymes onto PVC microspheres; immobilization 1 (0.025% EDA) and immobilization 2 (0.5% EDA).

reaction temperature. The modified PVC microspheres were characterized using FT-IR and TGA analysis to prove the surface functionalization with amine groups. Characteristic band, at 3444 cm^{-1}, for amine groups appeared and their intensity increased with EDA concentration. A new thermogram stage at 25–100°C appeared in the TGA analysis of aminated PVC microspheres in which 30–35% weight loss was detected. This range is referred to loss of water which confirmed the hydrophilicity improvement of the surface due to grafting of amine groups. The second weight loss stage at the range of 250–400°C, which is characteristic for PVC, was reduced from 62% to about 40% with aminated PVC. This thermal stability proves the occurrence of the amination reaction. The monitoring of surface morphology changes was performed using SEM micrographs. A smoother surface was observed as a result of the amination process. The induced stability results from the immobilization process via possible multi-

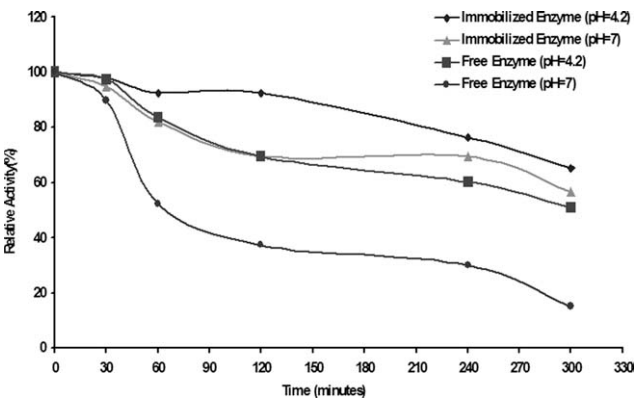


Figure 13 pH stability of free and immobilized enzyme onto PVC microspheres.

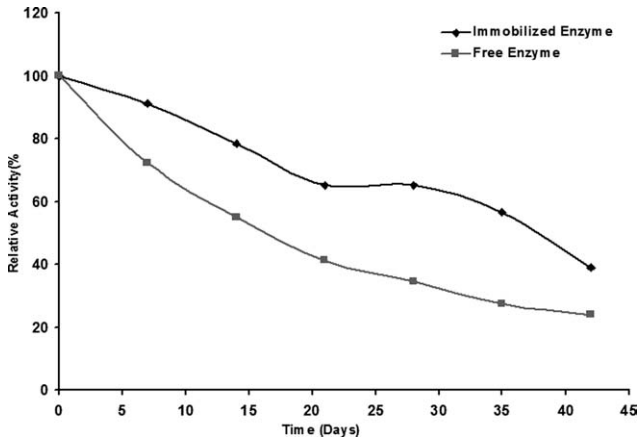


Figure 14 Storage stability of free and immobilized enzyme onto PVC microspheres.

attachment points. Thermal stability for the immobilized enzyme was recognized where the immobilized form kept 80% of its original activity compared with 20% for the free form under the same conditions. pH denaturation tolerance was observed upon immobilization where immobilized form kept 60% of its original activity after 300 minutes incubation at pH 7.0 in the absence of substrate compared to 15% for the form under the same conditions. The storage stability of the immobilized form after four weeks was measured where the immobilized form kept 40% of its original activity compared to 25% of the free form.

In conclusion, this immobilization protocol proves to be effective in the immobilization of β-galactosidase enzyme on PVC microspheres with high catalytic activity reaching to 11,000 units/kg and promising stability. Further work concerning the protection of the active sites during the immobilization step may be of great interest and will eliminate the negative impact of the ionic nature of the modified PVC microspheres surface and induce further stability.

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