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Affinity Covalent Immobilization of Glucoamylase onto ρ-Benzoquinone Activated Alginate Beads: I. Beads Preparation and Characterization

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Abstract ρ-Benzoquinone-activated alginate beads were presented as a new carrier for affinity covalent immobilization of glucoamylase enzyme. Evidences of alginate modification were extracted from FT-IR and thermal gravimetric analysis and supported by morphological changes recognized through SEM examination. Factors affecting the modification process such as ρ-benzoquinone (PBQ) concentration, reaction time, reaction temperature, reaction pH and finally alginate concentration, have been studied. Its influence on the amount of coupled PBQ was consequently correlated to the changes of the catalytic activity and the retained activity of immobilized enzyme, the main parameters judging the success of the immobilization process. The immobilized glucoamylase was found kept almost 80% of its native activity giving proof of non-significant substrate, starch, diffusion limitation. The proposed affinity covalent immobilizing technique would rank among the potential strategies for efficient immobilization of glucoamylase enzyme.

Keywords Covalent immobilization · p-Benzoquinone · Alginate · Glucoamylase · Affinity

Introduction

The immobilization of enzymes onto insoluble carriers has been an active research topic in enzyme technology, and it is also essential for their application to industrial processes [1, 2]. Carriers which play an important role in the utility of an immobilized enzyme should

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provide a large surface area being suitable for enzymatic reactions, substrate and product transport with the least diffuse restriction [3]. Immobilization leads to heterogeneous systems which enable separation from the reaction Media [4, 5]. Glucoamylase (EC 3.2.1.3) is an important enzyme used in the food industry to hydrolyze α-1, 4- and α-l, 6-glycosidic linkages of starch to produce glucose [6]. Many methods of enzyme immobilization, such as covalent and non-covalent methods, have been employed in the immobilization of glucoamylase [7–11]. Alginate entrapment method has been used to immobilize glucoamylase. However, simple alginate immobilization is not suitable for glucoamylase (MW_82,500) since the enzyme has been found to leak out of alginate beads quickly, and glucoamylase entrapped in calcium alginate beads lost all of its activity within 8 h [12, 13]. Tanriseven et al. [8] observed a similar result when immobilized the enzyme in alginate capsules and fibers. Glucoamylase in capsules lost its half activity after four batch reactions, each reaction taking 20 min and 36% of the retained activity leaked out of alginate fibers during three batch reactions. An additional process requires for the immobilization of enzymes due to the fact that even larger enzymes with molecular weights of 300,000 leak out of alginate beads [8, 13]. Tanriseven et al. [8] immobilized glucoamylase using a newly developed combined method of adsorption of the enzyme to gelatinized corn starch and subsequent alginate fiber entrapment. The immobilization resulted in 22% relative activity. The fibers retained their activities for 21 days. There was also no decrease in glucoamylase activity during 20 batch reactions. Tanaka et al. [13] immobilized glucoamylase (MW_97,000) in alginate coated with poly (ethyleneimine) and partially quaternized poly (ethyleneimine; QPEI) and found that QPEI-coated beads could be used for the immobilization of glucoamylase with 30% yield. In general, the non-covalent method has many advantages such as the simple procedure of immobilization, reusable carrier after desorption of the inactivated enzyme and reduction of the final price and less residues generation [14, 15], but the vital disadvantage of the non-covalent methods is that immobilized enzymes would easily be desquamated. Therefore, the covalent methods have been applied to immobilize enzymes and have gained recognition. Immobilization of enzymes through covalent attachment has been demonstrated to prepare of stable enzyme derivatives [16–25]. The extent of these improvements may depend on other conditions of the system, i.e., the nature of the enzyme, the type of support, and the method of immobilization. Indeed, losing of activity was observed due to incorporation of the active sites through covalent bonds which affects its accessibility to substrate. Protecting active site during the immobilization process using materials with an affinity to the enzyme such as substrate, product, and/or substrate-like materials (inhibitor) was introduced as a solution to overcome this drawback [26, 27]. Many publications reported the affinity of amylases (from various sources) for alginate [28, 29]. This character was used in the separation and purification of amylases [30]. Very few publications have been found addressing the covalent immobilization of enzymes onto alginate [22, 23, 31, 32]. No publications, from those ones, dealing with the covalent immobilization of glucoamylase. The affinity of glucoamylase towards alginate [33] in addition to previous publications of the authors on the covalent immobilization of beta-galactosidase onto ρ-benzoquinone (PBQ)-activated alginate [22, 23] inspired them to combine these advantages together to prepare a new alginate matrix for affinity covalent immobilization of glucoamylase. Due to the affinity of glucoamylase towards alginate, it will work as protecting agent of its active site during the formation of covalent bond and as a matrix simultaneously. This duel function of PBQ-activated alginate beads incorporates in reducing the number of required steps for immobilization and consequently its cost. This goal has been achieved through activation of alginate OH groups using PBQ. The conditions affecting the activation

process and its impact on the characteristics of the immobilized glucoamylase, activity and retained of activity, have been explored.

Experimental

Materials

- Sodium alginate (low viscosity 200 C.P.) obtained from Sigma-Aldrich Chemicals Ltd. (Germany).
- Calcium chloride (anhydrous Fine GRG 90%) was purchased from Fisher Scientific (Fairlawn, NJ, USA).
- Glucoamylase from *Picrophilus torridus* prepared and supplied from Mubarak City for Scientific Research and Technology Applications, GEBRI [34].
- P-Benzoquinone (purity 99+ %) was obtained from Sigma-Aldrich Chemicals Ltd. (Germany).
- Ethyl alcohol absolute was obtained from El-Nasr Pharmaceutical Co. for chemicals (Egypt).
- Starch soluble, pure, Erg.B.6 was obtained from Riedel–de Haen Co. (Germany).
- Glucose kit (enzymatic colorimetric method) was purchased from Diamond Diagnostics Co. for Modern Laboratory Chemicals (Egypt).
- Sodium chloride (purity 99.5%) was obtained from BDH Laboratory Supplies Pool (England).
- Sodium acetate–hydrochloric acid buffer was prepared from two components; hydrochloric acid (purity 30–34%) was obtained from El-Nasr Pharmaceutical Co. for Chemicals (Egypt) and sodium acetate trihydrate (purity 99%, M.wt. 136.08) was obtained from El-Nasr Pharmaceutical Co. for chemicals (Egypt).

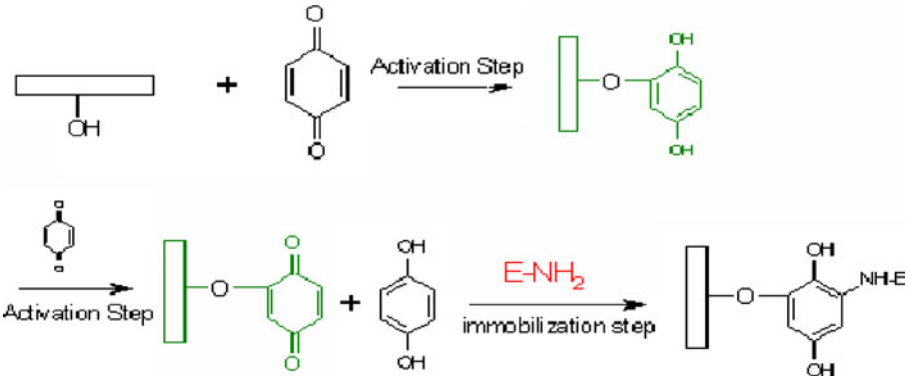
Methods

Preparation of Catalytic Ca-Alginate Gel Beads

The Ca-alginate gel beads were prepared by dissolving sodium alginate (low viscosity) in distilled water with continuous heating the solution until become completely clear to acquire finally 4% (*w*/*v*) concentration. The alginate solution was mixed with equal volume of 0.02 M PBQ solution and kept for continuous stirring at room temperature to have 2% (*w*/*v*) alginate and 0.01 M (PBQ) solution final concentration. The glucoamylase (1 ml of pure enzyme) was added to the mixture and stirring until it becomes completely clear solution (homogenous solution). The mixture was added drop wise, using 10 cm^3 plastic syringes, to calcium chloride solution (3% *w*/*v*) to give a known measurable diameter of beads and left for harden for 30 min at room temperature. Then the beads were kept at 4 °C for 16 h. The mechanism of activation process and enzyme immobilization is presented in Scheme 1.

Determination of Immobilized Enzyme Activity

The activities of both the free and the immobilized glucoamylase preparations were determined by measuring the glucose content in the medium according to a method described previously [34].



Scheme 1 Mechanism of activation and immobilization process

The catalytic beads were mixed with 20 ml substrate (1% starch) solution. This substrate solution was prepared by dissolving soluble starch (0.5 gm) in 50 ml Sodium acetate buffer solution (PH=2). The run is carried out using shaker water bath at temperature 80 °C for 30 min and at 100 rpm. Samples were taken to assess the glucose production using glucose kit.

The activity of free enzyme estimated under these conditions was found 1.8–2.1 μmol/min.

Determination of Effectiveness Factor of Immobilized Enzyme

The effectiveness factor η is the ratio of rates of the free and immobilized enzymes given as,

$$\eta = \frac{\text{Rate(activity) of immobilized enzyme}}{\text{Rate(activity) of free enzyme}}$$

Effectiveness factor provides information on the role of substrate diffusion in the reaction. A value of η=1 is obtained under conditions of complete diffusion, i.e., in case of homogenous reaction with the free enzyme [35].

Determination of Starch Concentration

A starch calibration curve was created to relate the absorbance reading (O.D. at 620 nm) to starch concentration (*w*/*v*, %) in the medium (Fig. 1). Five starch concentrations (2 mL) ranging from 1% to 6% were placed in 10-mL test tubes. A 200 μl sample of each starch concentration was added to a 10-mL test tube containing 1 mL 0.5 N hydrochloric acid. From this solution, a 200-μl sample was taken and added to 5 mL of iodine solution. The absorbance of each sample was measured after 15 min. The absorbance (O.D. at 620 nm) is plotted as a function of starch concentration.

Determination of ρ-Benzoquinone Concentration

Amount of attached PBQ was determined according to procedure published by deOlive Ira et al. [36]. A calibration curve was constructed using different concentrations PBQ. In brief, 1.0 mL of PBQ–alginate solution was taken, it was added to a test tube and the volume was

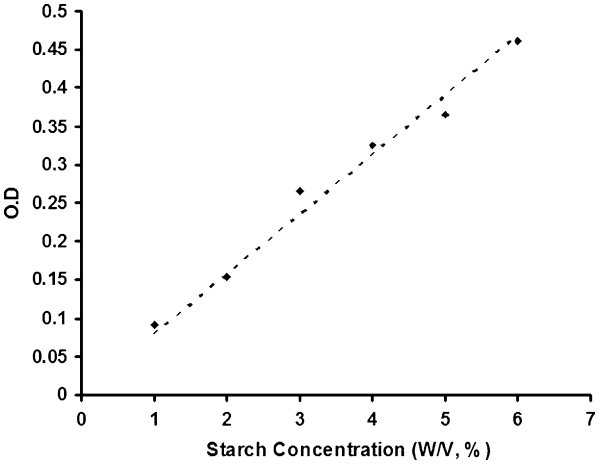


Fig. 1 Effect of starch concentration on the substrate diffusion ability in PBQ-modified alginate beads. (Activation conditions: 2% alginate, 30 °C, 0.01 M PBQ, 1 h and 1% BSA)

adjusted to 2.0 mL with 0.2 m acetic acid/acetate of sodium pH 3.0. To each tube, 200 mL of 0.1 m thiourea was added; the tubes were shaken, incubated at 37 °C for 10 min and cooled to room temperature. After 20 min at room temperature, the absorbencies at 410 nm were read against the blank.

FT-IR Spectroscopic Analysis

The structure of the alginate and alginate–PBQ-modified beads were analyzed by Fourier transform infrared spectroscopy (FT-IR) spectra. Samples were mixed with KBr to make pellets. 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 alginate and alginate-PBQ modified beads were studied using Thermo Gravimetric Analyzer (Shimadzu Thermal Gravimetric Analysis (TGA)–50, Japan); instrument in the temperature range from 20 °C 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 alginate and alginate-PBQ modified beads were observed with the help of a scanning electron microscopy (Joel Jsm 6360LA, Japan) at an accelerated voltage of 20 kV. The fracture surfaces were vacuum coated with gold for scanning electron microscope (SEM).

Results and Discussion

The aim of our study is creating active sites on alginate structure for further covalent binding of enzymes taking the advantage of amylase enzymes affinity in general towards

alginate. This goal has been achieved through reaction of hydroxyl groups with PBQ. The selection of hydroxyl groups to create active site on rather than carboxylic ones is preferred since the carboxylic groups are engaged mainly in the process of beads formulation through cross-linking with calcium ions.

Main concern was raised regarding the bulky nature of PBQ which could affect the diffusion of bulk substrate-like starch. This issue was clarified through following the diffused amount of starch into PBQ–alginate-modified beads immobilized with BSA as a model of glucoamylase enzyme. Different starch concentration solution, 1–6 (*w*/*v*, %), were used in this study. The beads were soaked in starch solution under the same experimental conditions for estimation of the catalytic activity, then the beads were dissolved using phosphate buffer solution and the amount of diffused starch was estimated (Fig. 1). From the figure, it is clear that the amount of diffused starch was increased linearly with the concentration of starch solution. This result eliminates the accusation of significant substrate diffusion restrictions results from the bulky nature of attached PBQ and emphasis on the fact that produced catalytic activity is referred to enzyme molecules immobilized both in bulk and on the surface of the beads.

The conditions affecting the activation process such as PBQ concentration, activation time, temperature, and pH and alginate concentration were investigated. The impact of variation conditions on the amount of attached PBQ molecules and consequently on the activity and retention of activity for the immobilized glucoamylase was followed. The obtained results are discussed in the following.

Effect of ρ-Benzoquinone Concentration

Figure 2 shows the effect of variation PBQ concentration on the attached amount of PBQ, catalytic activity, and retention of activity of immobilized glucoamylase. From the figure, it is clear that increasing the PBQ concentration, increase all the above mentioned parameters but with different rates. The attached amount of PBQ increased exponentially up to about 10 mg/g with 0.04 M PBQ solution then slightly decreased. On the other hand, both catalytic activity and retention of activity of immobilized enzyme were found increased almost linearly but with very low rate compared with the attached PBQ amount. Increasing the activity with PBQ concentration can be explained in the light of increasing the number

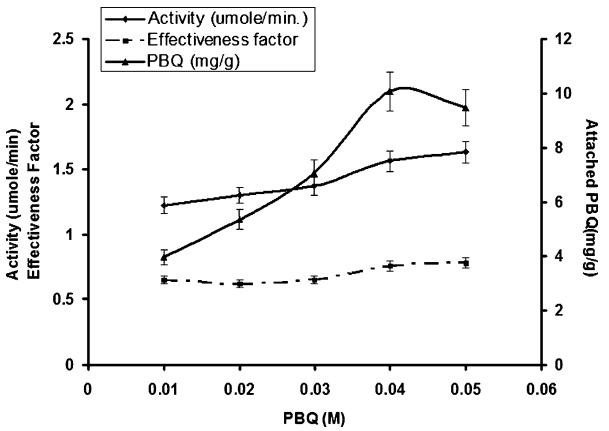


Fig. 2 Effect of PBQ concentrations on immobilized enzyme catalytic activity, retention of activity and attached amount of PBQ. (Activation conditions: 2% alginate, 30 °C, 1 h, and pH 10.0)

of activated OH groups and so the amount of covalently immobilized enzymes which thought to be in the best conformational structure due to the affinity interaction with alginate matrix.

The same behavior was found by Kalman et al. [37] which the amount of immobilized protein onto ρ-benzoquinone-activated polyacrylamide increased linearly with PBQ up to 20 mM and then leveled off. Similar finding was observed with immobilization of β-galactosidase enzyme onto ρ-benzoquinone-activated cellulose beads but the activity increment was leveling off with higher concentration of PBQ; 40 mM, in accordance with our results [38]. Formation of multipoint covalent attachment may explain the increment of effectiveness factor with PBQ concentration to reach up to 0.78 (78% retained activity). Much less retained activity was obtained by Tanriseven et al. [8]. They immobilized glucoamylase using a newly developed combined method of adsorption of the enzyme to gelatinized corn starch and subsequent alginate fiber entrapment. The immobilization resulted in 22% relative activity. Rebroˇs et al. immobilized glucoamylase from *Aspergillus niger* into a poly(vinylalcohol) hydrogel lens-shaped capsules LentiKats [39] with reatained 35% of its activity in free form. The difference in rate of increment for the above mentioned factors, attached amount of PBQ, catalytic activity, and effectiveness factor, may be explained by the limitation effect of enzyme amount used under the experimental conditions.

Effect of Activation Temperature

Effect of variation activation temperature on the attached amount of PBQ, catalytic activity, and retention of activity of immobilized glucoamylase is shown in Fig. 3. Regular increase of all parameters has been noticed with increasing the temperature of the activation process. This behavior could be explained by increase of the rate of the activation reaction due to decreasing of the reaction medium viscosity, so the number of activated OH groups is increased and in turn the amount of covalently immobilized enzyme. With using fixed amount of enzyme molecules, the probability of formation multi-attached covalent bonds with enzyme molecules leads to improvement of the retention of activity measured as effectiveness factor. Maximum retention of activity 78% (effectiveness factor, 0.78) was obtained at 45 °C which is still higher than values obtained with covalent immobilized

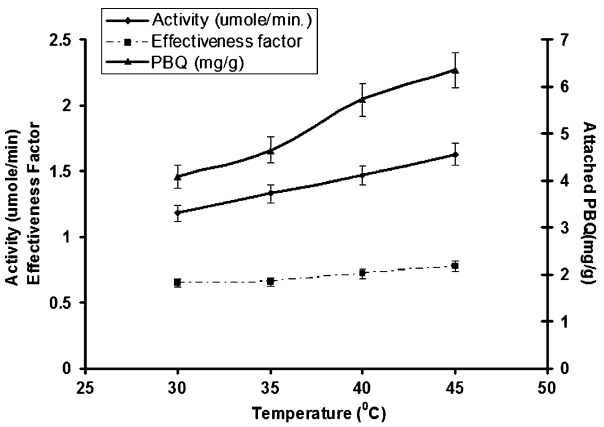


Fig. 3 Effect of temperature of activation process on immobilized enzyme catalytic activity, retention of activity and attached amount of PBQ. (Activation conditions: 2% alginate, 0.01 M PBQ, 1 h, and pH 10.0)

glucoamylase onto glutraldehyde-activated chitosan beads retained 50% of its activity by Dhar et al. [40]. However, this value is less in compared with our results.

Effect of Activation Time

From Fig. 4, it has been observed that variation of the activation time has an almost identical effect on the attached amount of PBQ, catalytic activity, and retention of activity of immobilized glucoamylase. Prolongation of activation time from 120 to 240 min has no effect on the catalytic activity of the beads. This explained by the fact that all the available OH groups in the glucose ring of alginate at were activated after 120 min, so further increase of the time has no effect. Kalman et al. [37] found the same trend with immobilizing BSA onto ρ-benzoquinone-activated polyacrylamide gel but after longer activation time, 20 h. These results are in accordance with that obtained by Chun et al. [38] where activity of immobilized β-galactosidase was leveling off after 5 h. Mateescu et al. [41] studied the effect of variation the activation time with PBQ onto different polymer supports namely; PVA, chitosan, and agarose. The leveling off of attached amount of PBQ was observed after 2, 4, and 6 h, respectively. The shortest time was found in the case of the most compact structure (when mostly surface reactions are supposed), while the longest time was required for the largest network. In our case, the reaction was carried out in homogenous condition which shortened the time needed for activation in contrary to the work of Mateescu et al. where the reaction was a heterogeneous one.

On the other hand, with increasing activation time from 15 to 120 min, the number of covalently immobilized glucoamylase molecules increases relative to the total immobilized molecules. This leads directly to increase the retained activity of immobilized enzyme.

Effect of Activation pH

The effect of variation pH value of PBQ activating solution on the attached amount of PBQ, catalytic activity, and retention of activity of immobilized glucoamylase is presented in Fig. 5. Linear increment of the activated OH groups was obtained as a result of increase the attached amount of PBQ. In general, we can say that the influence of such increment of attached PBQ amount was not observed over both catalytic activity and retention of activity

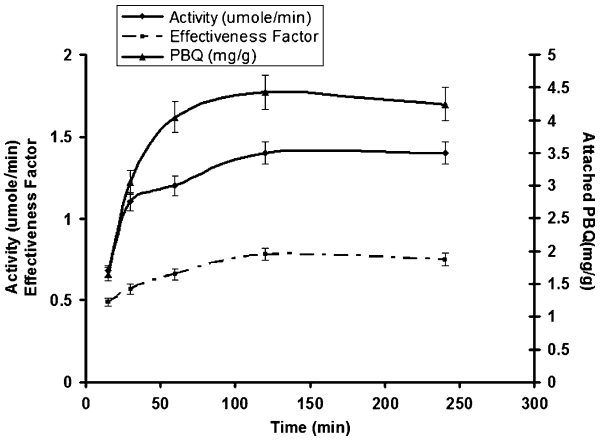


Fig. 4 Effect of activation time on immobilized enzyme catalytic activity, retention of activity and attached amount of PBQ. (Activation conditions: 2% alginate, 30 °C, 0.01 M PBQ, and pH 10.0)

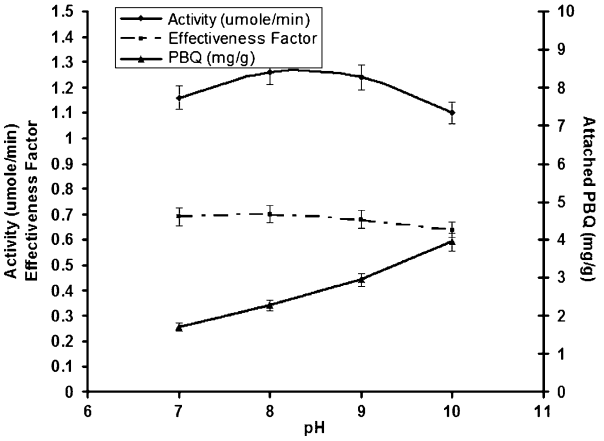


Fig. 5 Effect of activation pH on immobilized enzyme catalytic activity, retention of activity and attached amount of PBQ. (Activation conditions: 2% alginate, 30 °C, 0.01 M PBQ, and 1 h)

of immobilized enzyme. These results are in contrary with previous obtained results, which were explained by increase the rate of reaction between OH groups and PBQ and consequently the amount of immobilized enzyme [22, 37, 38].

In our case, the enzyme immobilized into vacations available in the alginate network, so increasing the number of activated OH groups in alkaline medium will result in formation of multicovalent bonds with the same amount of enzyme molecules. This could be resulted in increase of the thermal stability but not the amount of immobilized enzyme as in the published data in which the enzyme immobilized on the surface of the beads [22]. Also, partial deactivation of enzyme molecules as a result of the alkaline pH is a possible cause for loss of part of the immobilized enzyme activity since the optimum pH of free enzyme is 2.0.

Effect of Alginate Concentration

It is clear from Fig. 6 that increasing the concentration of alginate has a positive effect on the attached amount of PBQ, catalytic activity, and retention of activity of immobilized

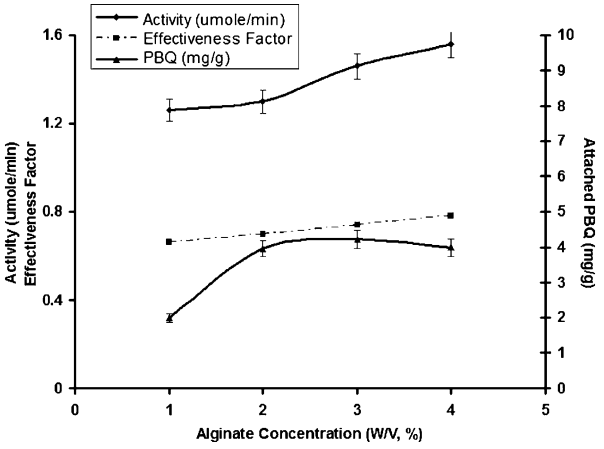


Fig. 6 Effect of alginate concentration on immobilized enzyme catalytic activity, retention of activity and attached amount of PBQ. (Activation conditions: 2% alginate, 30 °C, 0.01 M PBQ, pH 10.0, and 1 h)

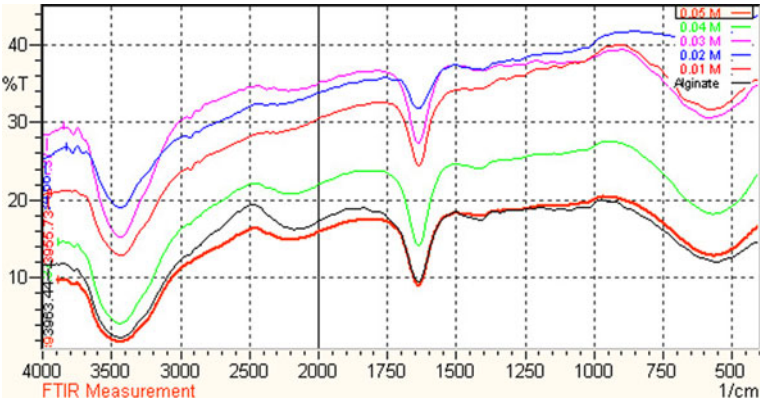


Fig. 7 FT-IR Spectra of alginate and modified alginate–PBQ matrices

glucoamylase. Indeed, the attached PBQ amount increased noticeably with increasing alginate concentration up to 2%, this increment was not observed with increasing alginate concentration from 2% to 4%. This means that used PBQ concentration is the limiting factor in this study. On the other hand, increment of both catalytic activity and retention of activity is very low. This behavior could be explained based on that the immobilized enzyme in alginate beads is divided into two parts, one part is covalently linked and the other part is entrapped. In that case, the main cause could be the increase of the part of enzyme covalently linked with the alginate matrix, as a result of increase the amount of activated OH groups, compared with the other part physically entrapped, since the alginate beads prepared under the studied conditions have the capacity to immobilize all the amount of enzyme. This conclusion has been abstracted from the fact that no traces of enzyme have been found in the calcium chloride cross-linking solution.

Enzyme immobilized onto activated beads prepared from 4% alginate solution has optimum effectiveness factor 0.78 (retained activity; 78%). Sanjay and Sugunan [42] successes in immobilization of glucoamylase covalently onto montmorillonite clay (K-10) with retaining of 100% of free enzyme activity. Covalently bound glucoamylase demonstrates a sharp decrease in surface area and pore volume that suggests binding of the enzyme at the pore entrance. Park et al. [43] immobilized glucoamylase on surface-modified carriers using a co-immobilized as well as a single system. Hydrophilic silica gel

Table 1 Weight loss percentage, as a function of different temperatures, of alginate and treated alginate matrices with different concentrations of PBQ

Polymer	100 °C	200 °C	300 °C	400 °C	500 °C
Alginate	25	40	52	57	65
Alginate/PBQ (0.01 M)	25	40	51	56	65
Alginate/PBQ (0.02 M)	10	26	43	52	59
Alginate/PBQ (0.03 M)	10	26	43	52	57
Alginate/PBQ (0.04 M)	7	22	39	49	52
Alginate/PBQ (0.05 M)	7	18	36	45	48

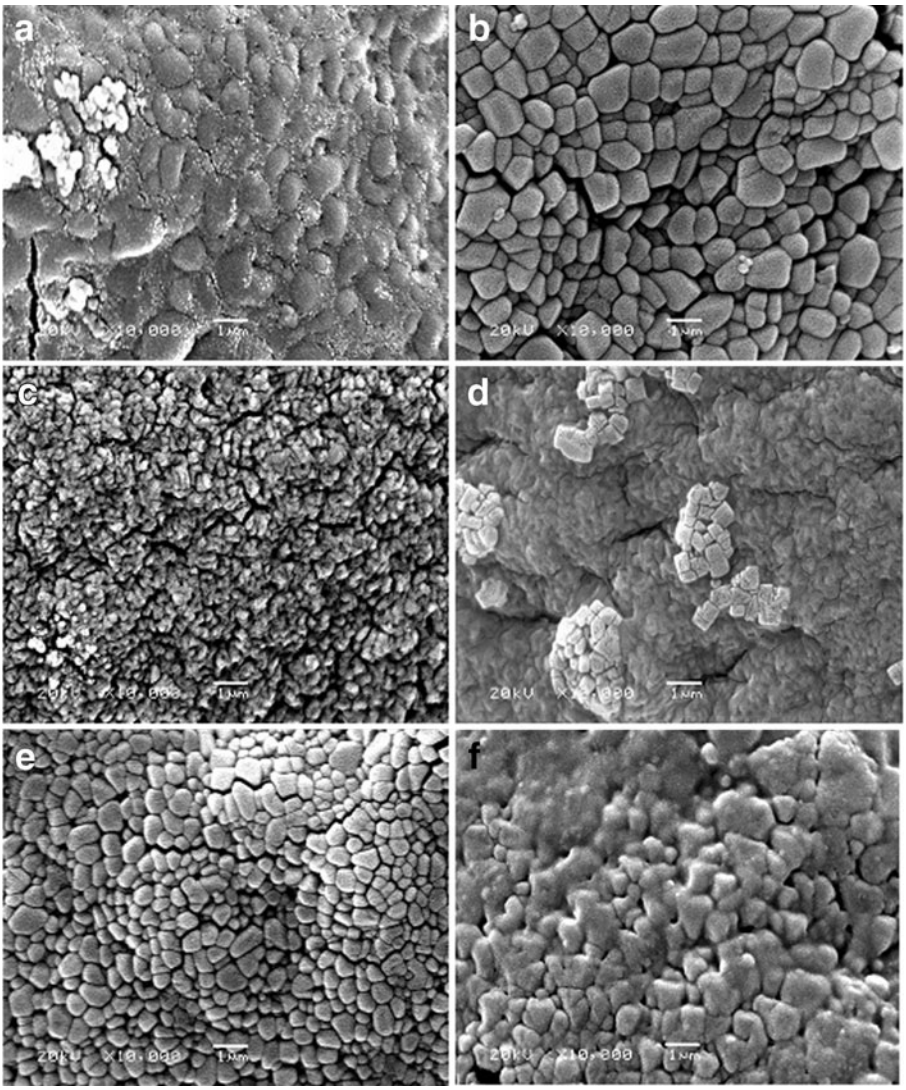


Fig. 8 SEM photographs of **a** alginate, **b** alginate–PBQ (0.01 M), **c** alginate–PBQ (0.02 M), **d** alginate–PBQ (0.03 M), **e** alginate–PBQ (0.04 M), **f** alginate–PBQ (0.05 M)

and DEAE cellulose entrapped in alginate beads were selected as the most effective carriers with 78–80% and 77% retention of activity, respectively.

Beads Characterization

The IR spectra of alginate and PBQ activated alginate beads are shown in Fig. 7. From the IR spectra it was evident that absorption band intensity at 1,560, 1,649 cm^{-1} corresponding to carbonyl bands has been increased in curves of PBQ–alginate via introduce further carbonyl groups of PBQ as illustrated in schema1. On the other hand, intensity of OH

groups at 3,400 cm^{-1} going to be reduce along with increase reacting amount of PBQ due to substitution [44].

Table 1 represents the thermal degradation of alginate and PBQ-activated alginate beads. It is clear that attaching of PBQ molecules onto the polysaccharide backbone enhances the thermal stability of the polysaccharides. This was reflected on the shift of half weight loss temperature (T_{50}) of alginate from 300 °C to 400–500 °C of PBQ-activated alginate beads. Also in the weight loss, percent at temperature ranged from 100 °C to 500 °C. Such behavior confirms the formation of new chemical structure different from the native alginate structure. Possible cross-linking is also expected.

Changes of chemical structure of polymer always reflect on its morphological characters. The scanning electron microscope examination of alginate and PBQ-activated alginate beads was shown in Fig. 8. It is clear that the morphological structure of alginate differ from PBQ-activated alginate beads. The surface morphology of alginate before modification with PBQ shows a less fragmented structure, which has been changed to fragmented form after modification. The fragmentation degree increases with increase of PBQ used in the modification step.

Conclusion

ρ-Benzoquinone-activated alginate beads for glucoamylase affinity covalent immobilization for the first time were prepared. The affinity of the carrier successes in retained of almost 80% of enzyme activity upon immobilization. In addition to the affinity of alginate, covalent binding of glucoamylase was induced under broad range of pH. Activation time was optimized and optimum activity was obtained at 120 min. Higher temperature has a positive effect on the attached amount of PBQ, catalytic activity, and retention of activity. Beads prepared from 4% alginate solution have the highest activity among studied alginate concentrations. FT-IR, TGA, and SEM analysis of the modified beads show changes in the physicochemical characters which prove the occurrence of the modification process. Starch diffusion experiment gives indications about the absence of significant substrate diffusion limitation and emphasis on the contribution of immobilized enzyme molecules both in the bulk and on the surface in the catalytic process. In conclusion, the new carrier has show very high profile for affinity covalent immobilization of glucoamylase.

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