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

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Abstract A novel affinity covalent immobilization technique of glucoamylase enzyme onto ρ-benzoquinone-activated alginate beads was presented and compared with traditional entrapment one. Factors affecting the immobilization process such as enzyme concentration, alginate concentration, calcium chloride concentration, cross-linking time, and temperature were studied. No shift in the optimum temperature and pH of immobilized enzymes was observed. In addition, K_m values of free and entrapped glucoamylase were found to be almost identical, while the covalently immobilized enzyme shows the lowest affinity for substrate. In accordance, V_m value of covalently immobilized enzyme was found lowest among free and immobilized counter parts. On the other hand, the retained activity of covalently immobilized glucoamylase has been improved and was found higher than that of entrapped one. Finally, the industrial applicability of covalently immobilized glucoamylase has been investigated through monitoring both shelf and operational stability characters. The covalently immobilized enzyme kept its activity over 36 days of shelf storage and after 30 repeated use runs. Drying the catalytic beads greatly reduced its activity in the beginning but recovered its lost part during use. In general, the newly developed affinity covalent immobilization technique of glucoamylase onto ρ-benzoquinone-activated alginate carrier is simple yet effective and could be used for the immobilization of some other enzymes especially amylases.

Keywords Bead formulation conditions · Affinity immobilization · Retention of activity · Shelf stability · Operational stability

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Introduction

Immobilized enzymes are used in food technology, biotechnology, biomedicine, and analytical chemistry. They have various advantages over free enzymes including easy separation of the reactants, products and reaction media, easy recovery of the enzyme, and repeated or continuous reuse. Enzymes can be immobilized to a multitude of different carriers by entrapment, adsorption, ionic binding, and covalent binding. Covalent binding is very effective in retaining the enzyme and can achieve high activity after immobilization, if amino acid residues that are covalently bound with the support material are not involved in the active site or substrate-binding site.

Enzyme immobilization by covalent binding has the following advantages: (1) Enzymes do not leak or detach from the support during utilization because of tight binding; (2) the immobilized enzyme can easily make contact with the substrate because it is localized on the support surface; and (3) an increase in heat stability is often observed because of the strong interaction between the enzyme and support material [1].

Glucoamylase is an industrially important enzyme and is used for large-scale saccharification of malto-oligosaccharides into glucose and various syrups required in the food and beverages industry [2]. Studies on immobilization of glucoamylase are in rapid progress, and many supports have been utilized. These include ceramic membranes [3], polymer microspheres [4], magnetic supports [5, 6], etc.

Alginate entrapment method has been used to immobilize glucoamylase [7, 8], but it requires an additional process for the immobilization of enzymes due to the fact that even larger enzymes with molecular weights of 300,000 Da leak out of alginate beads.

Very few publications have been found addressing the covalent immobilization of enzymes onto alginate [9–12]. There were no publications dealing with the covalent immobilization of glucoamylase. The affinity of glucoamylase towards alginate [13] in addition to previous publications of the authors on the covalent immobilization of beta-galactosidase onto alginate [9, 10] inspired them to use these advantages in preparation of a new alginate matrix for affinity covalent immobilization of glucoamylase. This goal has been achieved through activation of alginate OH groups using ρ-benzoquinone (PBQ). The conditions affecting the immobilization process and its impact on the characteristics of the immobilized glucoamylase have been explored and compared with a simple entrapment process to show the benefits of using covalent technique in combination with entrapment one. Moreover, the applicability of immobilized glucoamylase has been explored through investigation of its storage stability and reuse ability in addition to the effect of bead dryness. The obtained data were compared with those published by the other groups [14–17].

Experimental

Materials

- Sodium alginate (low viscosity 200 cP) 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, Genetic Engineering, Protein Department [18].

- ρ-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).

Methods

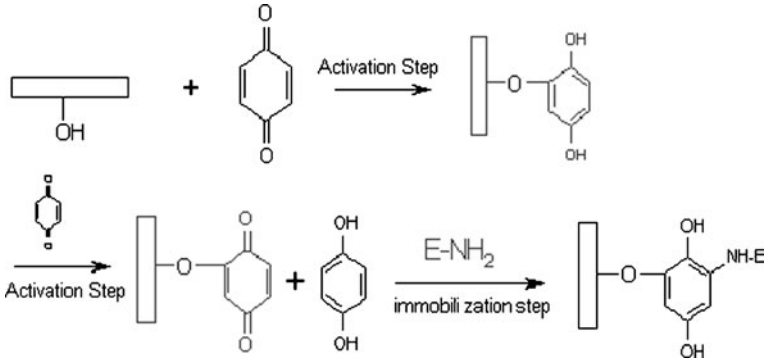
Preparation of Catalytic Ca-Alginate Gel Beads

The Ca-alginate gel beads were prepared by dissolving sodium alginate (low viscosity) in hot distilled water until the solution become completely clear to acquire finally 4% (*w*/*v*) concentration. The alginate solution was mixed with equal volume of 0.02 M PBQ solution at room temperature to have 2% (*w*/*v*) alginate and 0.01 M PBQ. One milliliter of pure glucoamylase enzyme was added to the mixture with stirring to have homogenous solution. The mixture was then dropped wisely using 10-cm^3 plastic syringes into calcium chloride solution (3% *w*/*v*) at room temperature to obtain 3-ml-diameter beads. The formed beads were left for 30 min to complete harden process. To complete the immobilization process, the catalytic 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 free and immobilized glucoamylase preparations were determined by measuring the glucose content in the medium according to a method described previously [19].

The catalytic beads were mixed with 20 ml of 1% starch–sodium acetate buffer solution (pH=2) in shaking water bath (100 rpm) at 80 °C for 30 min. Samples were taken to assess the glucose production using glucose kit. The activity of 1 ml free enzyme estimated under these conditions was found to be 1.8–2.1 μmol/min.



Scheme 1 Mechanism of activation and immobilization process

Determination of Retained Activity of Immobilized Enzyme

The retained activity of enzymes after immobilization is given as,

$$\text{Activity Retention}(\%) = \frac{\text{Activity of immobilized enzyme}}{\text{Activity of free enzyme}}$$

Retained catalytic activity 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 [20].

Results and Discussion

Immobilization Process

Effect of Enzyme Amount

The dependence of the catalytic activity of immobilized enzyme forms, entrapped and covalently bound, on enzyme amount used in the immobilization process is illustrated in Fig. 1. Beads lost its forming ability using enzyme amount over 1.5 ml, so the study was limited to such amount. It is clear from the figure that the activity increases linearly along with the enzyme amount. The catalytic activity of entrapped form was found lower than that of covalently immobilized one. The latter shows a leveling off of activity at an enzyme amount of over 1 ml. On the other hand, the retained activity of immobilized forms is almost identical and shows slight changes with variation of enzyme amount. The covalently bound enzyme has the higher value. This behavior may be referred to as "fixation" of the enzyme molecule's three-dimensional structures in its best form as a result of formation of covalent bonds between the enzyme molecules and the alginate matrix. The interaction between enzyme molecules itself, protein–protein interaction, rather than interaction with alginate matrix might be the cause of getting lower retention of activity for the entrapped enzyme. These results are in accordance with that obtained previously by other authors [21, 22]. Silva et al. [21] found that the activity of covalently immobilized glucoamylase

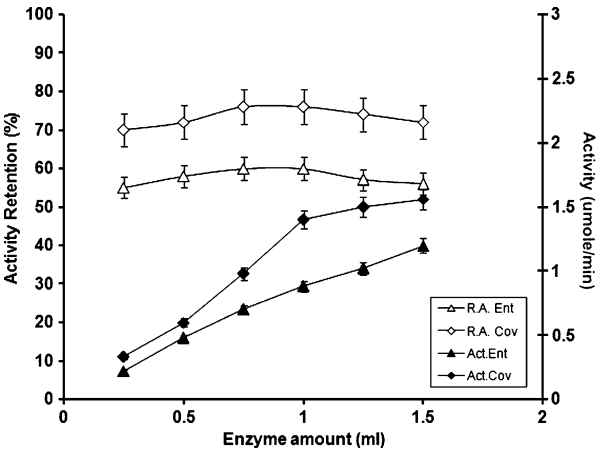


Fig. 1 Effect of enzyme concentration on bead catalytic activity

onto polyaniline polymer leveled off when 0.36 EU was offered per milligram of polymer. The immobilized enzyme retained up to 67% of its native activity. Bai et al. [22] studied the effect of glucoamylase amount added on activity recovery of covalently immobilized enzyme onto hydrophilic bead carriers containing epoxy groups. They found that the maximal activity recovery, retained activity percentage, could reach to 78% when the enzyme-adding amount was 0.5 mg with 50 mg of the carrier.

Effect of Alginate Concentration

It is clear from Fig. 2 that increase the concentration of alginate, in the studied range, has a linear positive effect on the catalytic activity of immobilized enzymes. Indeed, the activity increment was found to be very small, 16% only. The same behavior was observed with the retained activity increment percentage. In comparison, the catalytic activity of covalently immobilized enzyme is 60% higher than its entrapped counterpart. This behavior may be explained by having more covalently bound enzyme, as a result of the increase in the amount of activated OH groups, compared with physically entrapped one supposing that the immobilized enzyme into ρ-benzoquinone-activated alginate beads is divided into two parts. One part is covalently linked, and the other part is entrapped.

Effect of $CaCl_2$ Concentration

As shown in Fig. 3, the activity of the immobilized enzymes has not been significantly affected by increase the concentration of $CaCl_2$ solution. Maximum activity was obtained with beads cross-linked at 3% $CaCl_2$ solution. The retained activity was followed the same manner. Indeed, the activity of covalently immobilized enzyme is almost double of its entrapped counterpart. A balance between the prevention of enzyme leakage out and limitation of starch diffusion into the catalytic beads will be the determining factor in the selection of optimum $CaCl_2$ concentration.

Effect of Cross-linking Time

Figure 4 shows the effect of variation of the cross-linking time on the activity of immobilized enzyme. It is clear that the activity of covalently immobilized enzyme

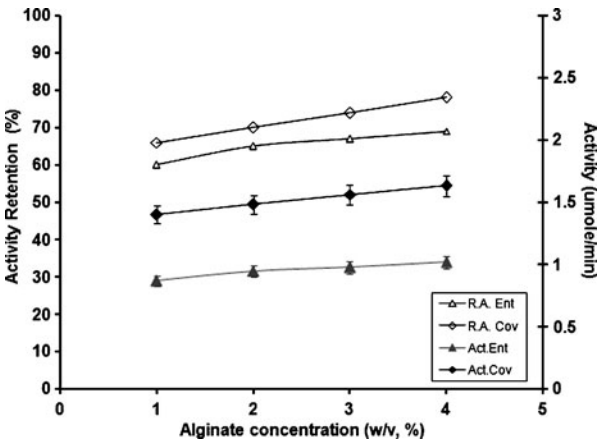


Fig. 2 Effect of alginate concentration on bead catalytic activity

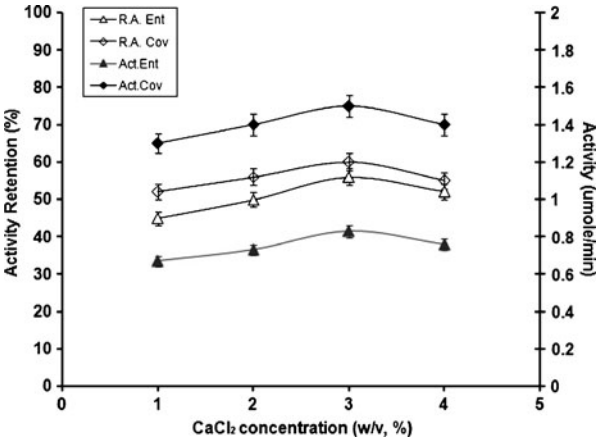


Fig. 3 Effect of $CaCl_2$ concentration on bead catalytic activity

increases by about 30% with time increase from 30 to 180 min, while its entrapped counterpart does not respond effectively under the same conditions. Accordingly, the percentage of activity increment of covalently immobilized enzyme to entrapped one ranged from 58% to 98% at 30 to 180 min cross-linking time. The retained activity of immobilized forms was found to be almost equal with a slight advantage of covalently immobilized one. The behavior of immobilized enzyme onto activated alginate beads could be interpreted according to the allowance of more enzyme molecules to bind covalently in comparison with the entrapped one.

Effect of Cross-linking Temperature

The temperature of cross-linking process in $CaCl_2$ solution was found to be of determined effect on the catalytic activity of immobilized enzymes especially the covalently immobilized one. The activity of immobilized enzymes increases by 26% and 122%, through increase of the cross-linking temperature from 30 to 80 °C, for the entrapped and the covalently immobilized enzymes, respectively. Such results may be explained based on

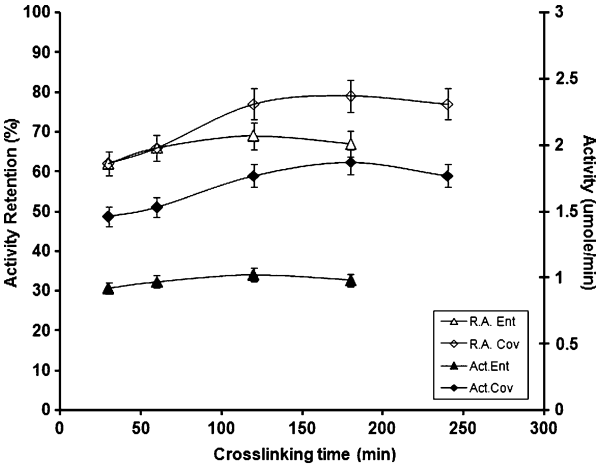


Fig. 4 Effect of cross-linking time on bead catalytic activity

the fact that the conformational integrity of the covalently immobilized enzyme structure changes to the best form at high temperature. Two things happened simultaneously in parallel with that. The first is increase of activated OH groups, which consequently increased the covalently bound enzyme molecules, which leads to reduction of the porosity of the beads. The second is the increase of cross-linking density of the formulated beads. The combination of both effects prevented the physically entrapped part of the enzyme from leaking out of the beads. Maximum retention of activity was obtained at 80 °C where the activity of immobilized enzyme is three times that of its entrapped counterpart (Fig. 5).

Operational Stability

The reusability or operational stability of immobilized enzymes is an important aspect from an industrial application point of view. Figure 6 shows the changes of activity of the immobilized glucoamylase after multiple reuses. The beads were repeatedly used for 30 cycles, every cycle 30 min. After each cycle, the beads were washed with a 1% $CaCl_2$–buffer solution. It was noticed that the covalently immobilized enzyme kept its activity almost constant over 10 h of processing. Twenty percent loss of activity was observed after 21 reuse cycles. This loss was recovered within the next nine cycles. Rebros et al. [15] reported that entrapped glucoamylase in LentiKats® maintained 80% of its initial activity after 100 repeated batch processes. Our obtained results are better than the results published by other authors [16] in which glucoamylase was adsorbed first onto surface of silica gel and/or DEAE-cellulose then entrapped into alginate. The immobilized enzyme kept 92.3% and 88.9% of their initial activity after 2 h of reaction. Tanriseven and Olcer [17] immobilized glucoamylase covalently onto poly-glutaraldehyde-activated gelatin, which retained its full activity during 30 consecutive batch reactions that lasted for 5 h at 48 °C. Czichocki et al. [23] were successful in forming a complex of glucoamylase entrapped with polyelectrolyte. Even after five repeated batches, the enzyme was fully active.

Storage Stability

Immobilized glucoamylase preparations were stored in sodium acetate buffer of pH 2 at 4 °C, and the activity measurements were carried out during the 35 days' period

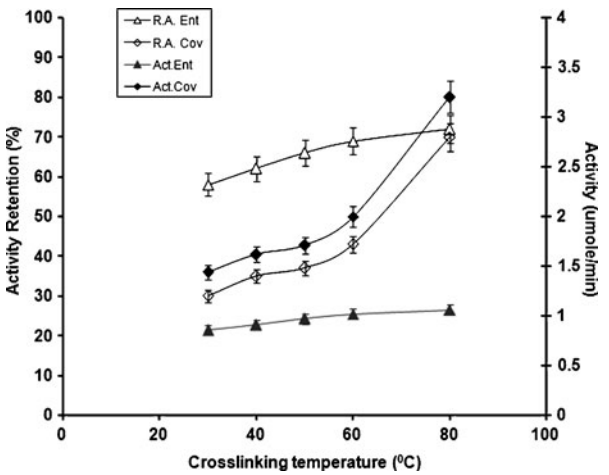


Fig. 5 Effect of cross-linking temperature on bead catalytic activity

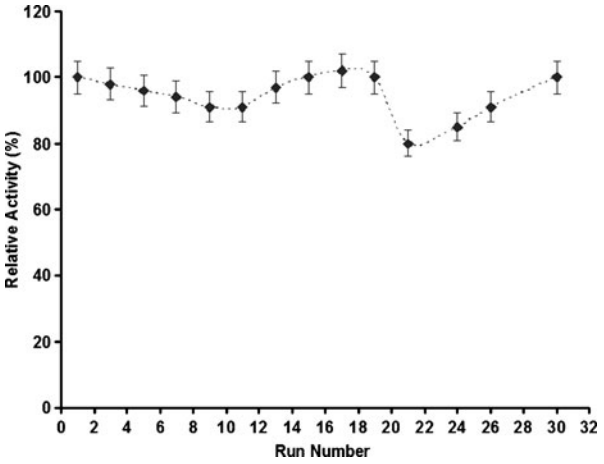


Fig. 6 Effect of operational stability on the catalytic activity of covalently immobilized enzyme

(Fig. 7). The storage stability, among many other factors, presents an important feature or character to give applicability for the immobilized enzyme on the industrial scale. From the figure, it is clear that the activity of immobilized form is oscillating during the studied period and surprisingly increased by about 50% of its native activity at the end of storage time. The oscillation behavior of activity may be referred to swelling–deswelling cycles of the beads along with storage time. Such behavior depends on the "elasticity" of formed ionic network hydrogel structure. The beads swell to a maximum depending on its elasticity then start to "squeeze" or deswell. The authors have no explanation for activity increase with storage time except that the beads swell along with time and lost its "squeezing" power, which as a result kept the hydrogel network structure open, and at the end facilitated the diffusion of substrate in and products out and eliminates the "protein–protein" interaction which was thought to be one of the causes of losing catalytic activity over entrapment immobilization. Tanriseven and Olcer [17] immobilized glucoamylase covalently onto poly-glutaraldehyde-activated gelatin, which retained its full activity for 30 days in storage at 4 °C and retained 90% and 75% of its initial activity in 60 and 90 days, respectively. Dudra et al. [24] reported 100% retained activity of covalently immobilized glucoamylase on thermo-sensitive carriers over 30 days at 4 °C in acetate

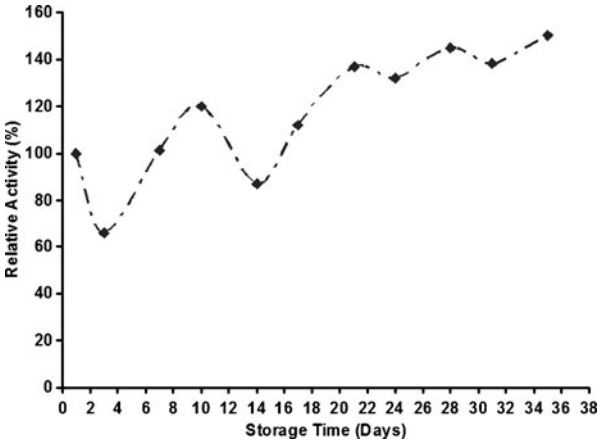


Fig. 7 The effect of storage on the catalytic activity of covalently immobilized enzyme

buffer of pH 4.5. Stability may be explained as a result of a decrease of the conformational space available for protein through multi-point attachment. Bryjak [25] immobilized glucoamylase covalently onto acrylic carriers with different functional groups and got a wide variant of stability depending on the chemical structure of the carrier that ranged from 50% to 100% after storage at 4 °C for 30 days.

Wet and Dry Beads

Figure 8 shows the activity of enzyme immobilized onto dry and wet alginate beads. The dry catalytic beads were prepared by drying the wet catalytic beads in an oven at temperature of 30 °C. Both wet and dry catalytic beads were used for consequent 18 cycles, every cycle 30 min, without washing the beads between cycles. The catalytic activity for the wet beads was around two times or higher than the dry one. This behavior is explained by the increase of the time needed to swell the dry beads and as a result the limitation of substrate diffusion. It has been noticed that the dry catalytic beads never regain its wet status. This indicates that some of the immobilized enzyme, especially in the core of the beads, is not available into contact with the substrate at any time. This was considered as the main reason for the reduction of the activity. Reduction of the bead volume to increase of the working volume of the bio-reaction is considered as an advantage of the dried catalytic beads in the biotechnological process. Indeed, reduction of bead volume should be accompanied by keeping its catalytic activity to have the benefits from the increase of the working volume. In our case, both the bead volume and catalytic activity were reduced in the same percentage, so drying of the beads is not beneficial. The activity increment of both dry and wet catalytic beads in such manner might be referred to as inclusion of product inside its bulk. After a certain number of cycles, the concentration of the product inside the beads became higher than its counterpart in the substrate surrounding solution, so the product starts to migrate outside the beads and consequently increases the product concentration in such obtained manner.

Effect of the Reaction Medium Temperature (Temperature Profile)

Figure 9 illustrates the activity–temperature dependence of free and immobilized enzymes. The highest activity for both free and immobilized enzymes was observed at 80 °C. At a

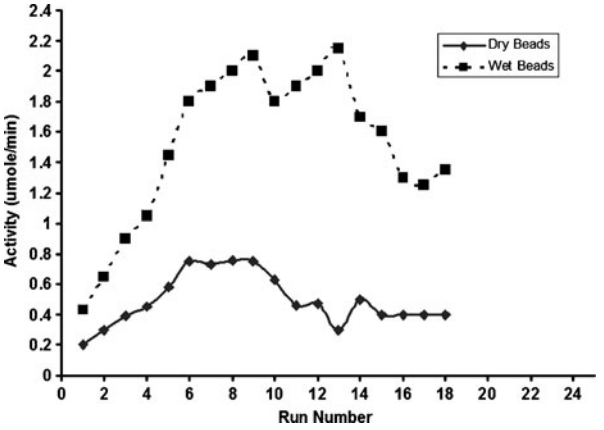


Fig. 8 The activity of the wet and dry catalytic beads

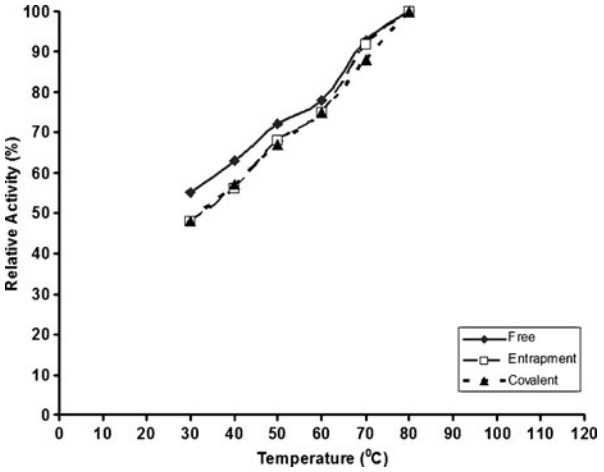


Fig. 9 Effect of the reaction medium temperature on the catalytic activity of immobilized and free enzyme

temperature higher than 80 °C, the beads start to disintegrate, so the study was limited to 80 °C. On achieving an unaltered highest activity position, within the studied temperature range, we can conclude that the structure of the active site and the microenvironment in which the enzyme is operating are probably the same in the free and immobilized forms.

In addition, the temperature profile of immobilized enzymes is almost identical with the free counterpart. In other words, the immobilization process has not changed the temperature profile of the immobilized enzymes. An unchanged temperature profile is a result of the substrate affinity effect of alginate matrix. Uhlich et al. [26] did not observe any change of the temperature dependence on the enzyme's activity upon entrapment of glucoamylase in photo-chemically cross-linked polyvinyl alcohol. Covalently immobilized glucoamylase onto spacer-arm attached magnetic poly-(methylmethacrylate) microspheres shows a shift of its optimum temperature from 50 to 55 °C [27]. The increase in optimum temperature was caused by the changing physical and chemical properties of the enzyme. D'Souza and Kubal [28] immobilized glucoamylase covalently onto polyethyleneimine-coated cloth strips. No change in the temperature activity profile was seen on immobilization. Free and immobilized forms have a maximum activity at 55 °C. Tanriseven et al. [8] immobilized glucoamylase using a novel method combined with adsorption of the enzyme to gelatinized corn starch and subsequent alginate fibers entrapment. No change of the optimum temperature was observed where optimum free and immobilized enzyme activities were observed at 60 °C.

Effect of the Reaction Medium pH (pH Profile)

The effect of substrate pH on the activity of the free and immobilized glucoamylase has been explored, and the results are presented in Fig. 10. From the figure, it is clear that the optimum activity was obtained at pH 2 for all forms of enzyme, and no shift was recognized. A substrate solution with a pH value beyond 2 leads to a quite fast decrease of the free enzyme activity, while the activity of immobilized forms decreases gradually. Change in the pH profile of the immobilized enzyme forms could be attributed to the effect of the presence free COO^- groups inside the matrix. Ida et al. [3] studied the effect of covalent immobilization of glucoamylase on ceramic membrane surfaces modified with a new method of treatment utilizing SPCP-CVD. The obtained results, in accordance with our

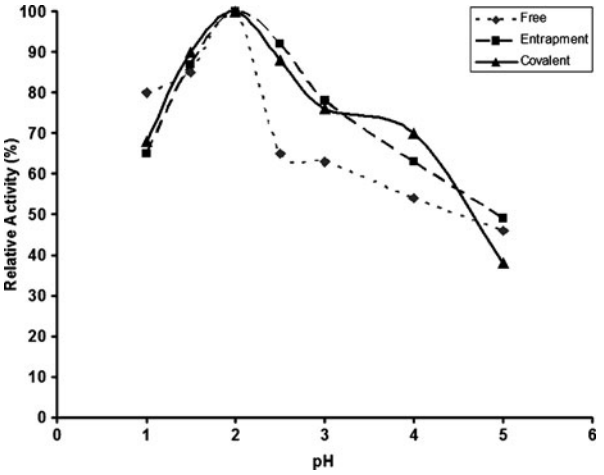


Fig. 10 Effect of the reaction medium pH on the catalytic activity of immobilized and free enzyme

results, show that after immobilization, the optimal pH did not change while the curve profile became much broader. Milosavic et al. [29] immobilized glucoamyalse covalently via its carbohydrate moiety on macroporous poly (GMA-co-EGDMA) polymer. Maximal activities were obtained at pH 4.5 for both the immobilized and the free enzyme. The similarity of pH profiles for both preparations may indicate that no large conformational changes occurred upon immobilization.

Kinetic Studies

The goal of our study was to find the optimum conditions for glucoamylase immobilization onto alginate. To avoid or overcome the diffusion limitation drawback of the substrate, it was essential to study the effect of immobilization process on the kinetic parameters especially K_m, which is considered as the reflection of the presence or absence of diffusion limitation problem. Kinetic constants of the free and immobilized enzyme forms, and K_m and V_{max} values were determined by using soluble starch as substrate. The variation of free

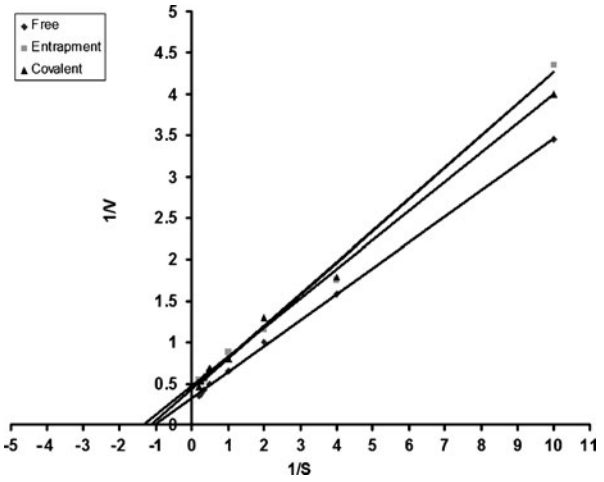


Fig. 11 Lineweaver–Burk plots of free and immobilized enzymes

and immobilized glucoamylase activity with various substrate concentrations was plotted in the form of Lineweaver–Burk curves (Fig. 11). K_m and V_{max} values were calculated from the intercepts on *x*- and *y*-axes, respectively. The obtained results illustrate how close the values of K_m for free (1.03 g/L) and immobilized enzyme forms and for entrapped (1.097 g/L) and covalently immobilized ones (1.31 g/L), respectively. This is strong evidence on the success of covalent immobilization in avoiding substrate diffusion limitation. On the other hand, V_{max} has been reduced upon immobilization in both forms: free 3.1, entrapped 2.37, and covalently 2.16. Similar results were obtained by Tanriseven et al. [8] upon testing immobilized glucoamylase by a novel method onto alginate fibers with maltodextrin substrate. They claimed the substrate diffusion limitation. Entrapped glucoamylase into photo-chemically cross-linked polyvinyl alcohol shows the same behavior [26], but the kinetic parameters in this case were affected dramatically in comparison with our obtained results. Even with changing the immobilization technique and using the covalent one, Arica et al. [27] found higher K_m values and lower V_{max} values for glucoamylase immobilized covalently onto spacer-arm attached magnetic poly (methylmethacrylate) microspheres.

Conclusions

The conditions affecting the affinity covalent immobilization process of glucoamylase enzyme onto modified alginate beads have been studied. The impact of conditions like enzyme concentration, alginate concentration, calcium chloride concentration, bead cross-linking time and temperature on the retention of activity of immobilized glucoamylase, and the reflection of the efficiency of immobilization process has been investigated. Bead cross-linking temperature was found as a determining factor, which increased the immobilized enzyme retained activity from 43% at 60 °C to 70% at 80 °C. Both temperature and pH optima have not changed upon immobilization of glucoamylase. No signs of substrate diffusion limitation were recognized.

In conclusion, the immobilized enzyme shows high shelf and operational stability, which reflects the high profile of the immobilization conditions.

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