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# Textural and Structural Characterizations of Mesoporous Chitosan Beads for Immobilization of Alpha-Amylase: Diffusivity and Sustainability of Biocatalyst

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*ABSTRACT*

In the present study, textural and structural characterizations of chitosan bead for immobilization of alpha amylase in detail by $N_2$ adsorption–desorption, Microspore Analysis (MP), Barrett–Joyner–Halenda (BJH) plots and Field Emission Scanning Electron Microscope (FESEM) observations were investigated. Pore structure observation revealed chemical activation of chitosan bead by glutaraldehyde can change both the isotherm type of adsorption and pores shape. In consistence with textural analysis, the high value of pore volume distribution with range of mesopores region indicated the porosity of activated chitosan bead was uniformly increased. Intra-particle diffusion model designated that 97.6% of amylase was adsorbed inside the mesopores of activated chitosan bead owing to increase in $k_{id}$ (rate constant) and reduce of boundary layer effect on diffusion process. In addition, the stability experiments (pH, storage and thermal stability), enzyme leakage, $Ca^{2+}$ and salt concentration effects were evaluated for immobilized amylase and compared with its free activities. $Ca^{2+}$ concentration of 1 mM shows an excellent impact on relative activity of amylase on its free and immobilized forms. NaCl experiments indicated that 84% of amylase was covalently immobilized on activated chitosan beads. Further, the Michaelis–Menten kinetic coefficients, $K_m$ (~0.4mg/ml) and, $V_{max}$(~227 U/mg Enzyme), point out strong affinity and high activity of immobilized enzym.

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## 1. INTRODUCTION

During the past two decades, enzymes have been used as a biocatalyst in complicated chemical processes. Several developments have been achieved in enzyme technology which assist their usage in many industrial applications [1-3]. The mild reaction conditions have made them to become ideal biological catalyst. Thermo-stable enzymes, like some species of amylase, have numerous commercial applications in baking, brewing, alcohol production, cheese making, pulp, and textile industries because of their inherent stability in wide range of temperature [4-6].

In recent years, there has been universal increase in starch contaminate in processing of sugar cane juice which must be removed. Enzymes of various types are used in these processes. Amylases are enzymes, which hydrolyze starch molecules to give a smaller components composed of glucose units [7, 8]. Also, amylases are widely exploited in various fields ranging from food, fermentation, paper, detergents, pharmaceutical, and agriculture industries [9-12]. Amylases have approximately 25% of the world enzyme market. However, the specificities of enzymes and the abilities to increase reaction rates promise more developments in many industrial applications [9, 13].

Beyond of many profits of enzyme, it is not sufficiently stable in stringent reaction conditions. The major challenge in enzymatic reactions is that even slight conformational changes of enzyme structure can make a significant decline in its activity. Enzymes can be deactivated by making little change in temperature

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or pH value of reaction medium and physical forces [14].

There are several strategies to overcome these challenges. In order to benefit the advantages of enzyme can employ different immobilization methods, chemical modification and even manipulate of the protein structure or its reaction medium. The immobilization of enzymes has been recognized as the simple and an operational method. The immobilization techniques consist of physical adsorption, covalent attachment and entrapment in a suitable prefabricated support.

In chemical immobilization techniques, covalent bond formed between the carrier and the enzyme, while in physical method, enzymes can adsorbed or encapsulated inside a solid carrier or attached on it [1, 5, 15, 16]. Generally, finding a proper method of immobilization is important for increasing of enzyme stability and loading capacity, reusability of enzyme and carrier, less time and labor consumption. As evident from literatures, covalent bonding can attach enzymes to the carriers strongly whereas in physical adsorption, enzymes release from porous materials. Subsequently, it causes to enzyme loss and poor operational stability [2, 4, 17].

The essential factors for selecting the appropriate carrier are porosity, hardness, insoluble in water, capable of enzyme accommodation and stabilization. Most of solid support materials are synthetic polymers which are not safe for consumption in the food processing owing to the possibility of its chemical components leakage. Therefore, solid support materials which are natural, non-toxic and inexpensive have gained extensive attention in recent years [4, 18].

Chitosan which is obtained by the deacetylation of chitin has been used as a solid support material for enzyme immobilization due to highly biocompatible and easily biodegradable, non-toxicity, physiological inertness and high affinity to proteins [19]. Enzyme immobilization on chitosan can be achieved by means of glutaraldehyde crosslinking the amino groups of chitosan and the enzyme molecule to form covalent linkages [3, 14, 16]. These covalent bonds can improve the mechanical characteristics of the enzyme on chitosan. Based on our findings reported in previous researches, as a new approach of cross-linking agent effect, glutaraldehyde was greatly increased chitosan beads (Chit. bead) porosity and its total surface area more than 4 folds [20-22].

In the present study, textural analysis of Chit. bead and effect of glutaraldehyde on structural properties of Chit. beads were studied by $N_2$ adsorption-desorption plot; also, distribution of pores size was evaluated by MP and BJH plots for both carriers. Furthermore, intra-particle diffusion was investigated to confirm the results of pore structure analysis. Characteristic experiments such as $Ca^{2+}$ ion and salt effect, pH stability, thermal stability, leakage and storage stability of immobilized amylase were considered for immobilized enzyme. The obtained results were compared with free enzyme. The Michaelis–Menten kinetic was examined and its constant values were determined. All experiments were conducted three times and the average data were reported.

## 2. MATERIALS AND METHODS

**2. 1. Materials** The alpha amylase from *Bacillus Subtilis* (EC 3.2.1.1), medium viscosity chitosan, and glutaraldehyde 50% were purchased from Sigma Chemical Co. (St. Louis, USA). All other chemicals were analytical grade and purchased from Merck (GmbH, Darmstadt, Germany).

**2. 2. Preparation of Chit. Beads** Chit. beads were prepared based on procedure described by Pallavi Tripathi et al. [23]. Chit. beads were activated with 3% (v/v) glutaraldehyde at 30°C, 150 rpm, for 24 h in incubator shaker (Stuart, S1500 and UK). To remove excess glutaraldehyde, activated beads were washed with distilled water and phosphate buffer. Chit. beads were treated with desired protein concentration and left overnight at 37°C and 150 rpm. After 24h, the solution was decanted and carriers were washed with the same buffer to remove any unbounded enzyme [17].

**2. 3. Protein Assay** Protein concentration was determined by the Bradford protein method [21] using bovine serum albumin as a standard solution.

**2. 4. Amylase Activity Assay** Amylase activity was examined by DNS method [23]. Based on definition, one unit of activity was defined as the amount of enzyme required to produce 1 µmol of reducing sugar/min. Chit. beads incubated in 1 ml starch solution (1%); after 10 min, they were removed from starch solution, and 1 ml DNS was added. Finally, absorbance values were measured at 540 nm in Nano Drop spectrophotometer (Thermo Fisher Scientific Wilmington, Delaware USA).

Also, optimum pH and temperature were obtained for maximum activity; then for calculation of relative activity, the optimized condition was applied (data are not reported).

**2. 5. Immobilization Efficiency** The efficiency of immobilization was evaluated in terms of protein coupling and activity yields as follows:

Immobilization Efficiency (%) = (amount of protein coupled/amount of protein introduced)× 100

Activity (U/ g chitosan) = activity of immobilized amylase on one Chit. bead/ weight of one Chit. Bead

Relative activity = (activity of immobilized amylase / maximum activity of immobilized amylase)× 100

**2. 6. Textural and Structural Characterization** The textural characterization of the Chit. and Chit. glu. beads were measured using an adsorption apparatus (BELSORP 28, BEL Japan Inc.) reported in previous work [21]. In present study, the relations between reported textural properties [21] (surface area, average pore diameter and pore volume) and pores structure were more discussed. The pore structure was examined by employing $N_2$ adsorption–desorption isotherm at 77 K at the relative pressure ($P/P_0$) range of 0.0001–0.99. In order to finding pores size distribution, MP plot and BJH plot were applied for Chit. beads and Chit. glu. beads. Prior to analysis, Chit. beads were separately degassed under vacuum environment at 393 K for 15 h before the nitrogen adsorption measurements. As complementary observation, the structural characterization of chitosan beads were evaluated by a field emission scanning electron microscope (FESEM) (MIRA3, TESCAN) [21] .

**2. 7. Intra-particle Diffusion Study** In order to confirm the results obtained from pore structure analysis, the adsorption kinetic experiments were performed. Weber and Moris had proposed the intra-particle diffusion theory for the rate controlling step of adsorption. In Equation (1), the total amount of adsorption is proportional to the square root of contact time. The mathematical expression of the model is presented as follows:

$$q_t = k_{id} t^{0.5} + C \quad (1)$$

where, $q_t$ is the total amount of adsorption at time $t$, $k_{id}$ (mg/mg.$h^{1/2}$), the intra-particle diffusion rate constant, is slope, and $C$ is the intercept which is proportional to the extent of boundary layer thickness. The slope and intercept can be calculated by extrapolation of data. In fact, the intercept of Equation (1) represents the adsorbate amount on the external surface, $q_{ex.}$. It can be determined by subtracting the adsorbate amount in the pores ($q_{meso}=k_{id}.t^{0.5}$) from the total ($q_t$). In the other word, the Equation (1) can be written as follows:

$$q_t = q_{meso} + q_{ex} \quad (2)$$

In intra-particle diffusion experiments 50 Chit. beads (containing 37.75 mg chitosan dry powder) were added to 50 ml of the enzyme solution and then incubated in an incubator shaker with agitation rate of 150 rpm during 24h [18]. The adsorption capacity, $q_t$, was calculated using the following equation:

$$q_t = \frac{(C_0 - C_t)V}{m} \quad (3)$$

where $q_t$ is the amount of adsorbed enzyme per unit weight of chitosan at time t (mg/mg), $C_0$ is the initial enzyme concentrations (mg/ml) and $C_t$ is enzyme concentration in the solution at time $t$ (mg/ml), V is the volume of the enzyme solution (ml) and $m$ is the dry weight of the chitosan beads (mg)

**2. 8. Stability Tests and Leakage of Immobilized Amylase** pH stability of immobilized amylase was studied by incubating the immobilized enzyme at 70°C in buffer solution while pH varying in the range of 5–9 for duration of 1h and then determined the hydrolytic activity of the enzyme at the optimum pH. Relative activities were calculated based on the ratio of activity of immobilized enzyme after incubation to the activity of enzyme at the optimal condition. In order to examine thermal stability, free and immobilized amylases were stored in buffer solution (50 mM, pH 7.0) at 100°C for 120 min. Samples were periodically withdrawn for activity assay every 30 min. The relative activities were determined as stated above. In addition, immobilized amylase was kept at 25°C to evaluate storage stability. The enzyme activity assays were conducted after each 3 days within 15 days. In addition, the leakage of immobilized enzyme was considered while stored in 4°C within 15 days. The amount of enzyme was eluted in buffer solution, measured by protein assay every 3 days.

**2. 9. Effect of Salt and $Ca^{2+}$ Ions** In order to recognize the ratio of covalent immobilization of amylase onto Chit. beads, the effect of NaCl on immobilized enzyme was investigated. To this purpose, relative activity was monitored when immobilized enzyme was placed in 10% (v/v) sodium chloride solution over a time of 24 hours. In similar way, the relative activity of free enzyme was investigated in presence of salt. Based on literatures, presence of this salt can lead to breakage non-covalent bond of enzyme and carrier, by way of ion exchange or washing out the hydrogen bond [24]. As complementary experiment, the leakage of immobilized enzyme in presence of salt was monitored by protein assay. Furthermore, effect of $Ca^{2+}$ ions on the enzyme activity was studied. Free and immobilized enzymes were incubated in Tris-maleate buffer containing calcium ions in concentration of 0.5 to 2.5 mMol for 120 min at 35°C and then the relative activity was measured. Tris-maleate buffer was just used here because of phosphate buffer could sequester divalent cations such as $Ca^{2+}$.

**2. 10. Enzymatic Reaction Kinetics** The Michaelis-Menten constant, $K_m$, and the maximum specific reaction velocity, $V_{max}$, for the immobilized amylase were determined. These factors were measured by the initial velocity of the reaction and varying the substrate (starch) concentrations (0.5-5 mg); while enzyme concentration was kept constant. The initial rate

was determined at 5 min. The kinetic constants ($K_m$ and $V_{max}$) were determined by Lineweaver–Burk plot stated in Equation (4).

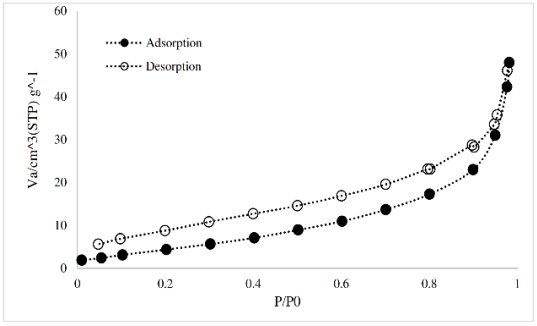
$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad (4)$$

## 3. RESULTS AND DISCUSSION

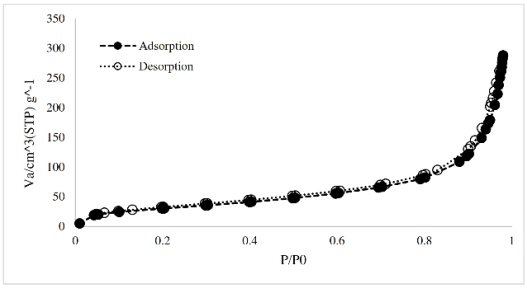
### 3. 1. Textural and Structural Characterization

Figures 1a and 1b show the $N_2$ adsorption–desorption isotherms of the Chit. beads and Chit. glu. beads respectively. According to Figure 1a, adsorption on Chit. beads demonstrates the type III isotherm based on the Brunauer classification which give isotherms with gradual curvature and an indistinct Point B. Although this isotherm is not common, there are systems of this type like nitrogen adsorption on polyethylene at which the adsorbate-adsorbent interactions is important. Hysteresis loops, which are related with capillarity condensation, are characteristic for substances include mesopores. Also, it seems that the loop's shape is categorized as type $H_3$ indicative of the presence of slit-shaped pores [25]. However, after activation of Chit. beads by glutaraldehyde the type of isotherm was changed to type II (see Figure 1b). Based on literatures, type II isotherm indicates unrestricted monolayer-multilayer adsorption. This type of isotherm indicates the condition at which monolayer coverage is complete at point B, start of linear middle section, and then multilayer adsorption going to start [25]. As can be seen from hysteresis loops of Chit. glu. beads, it can be classified as type $H_4$ that could be associated with narrow slit-like pore.

The micropore (MP plot) and mesopore (Barrett–Joyner–Halenda (BJH) plot) size distributions of Chit. and Chit. glu. beads were shown in Figures 2a to 2d, respectively. The pores volume distribution ($dVp/dr_p$) of Chit. beads, increased especially near to the mesopores region indicating the prepared Chit. beads have mainly mesopores with pores sizes in the range of 1.5–40 nm and also have some micropores (see Figures 2a and 2c).

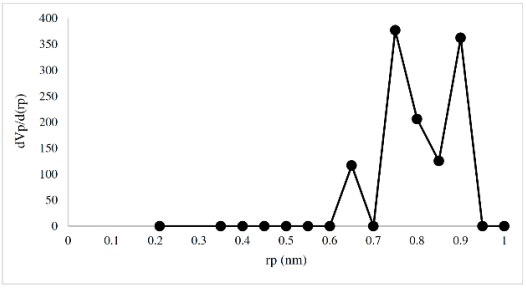



(a)

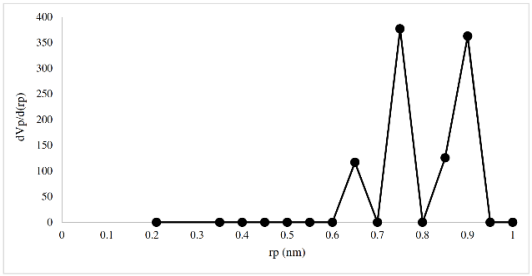



(b)

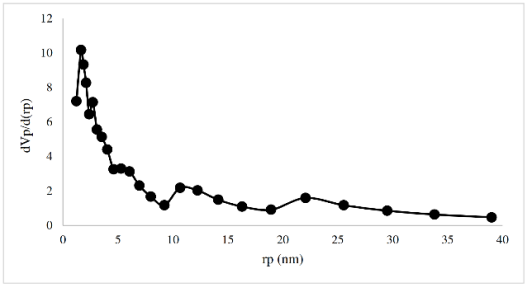
**Figure 1.** Adsorption/desorption isotherms of $N_2$ for (a) Chit. bead and (b) Chit. glu. bead



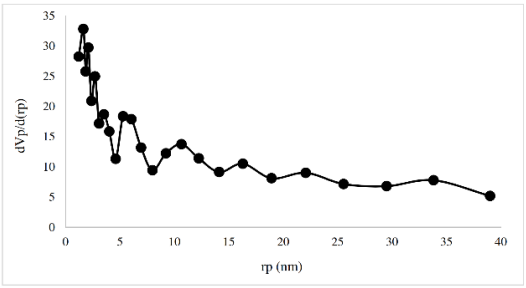

(a) MP - Chit. Bead




(b) MP- Chit. glu. bead,




(c) BJH- Chit. bead

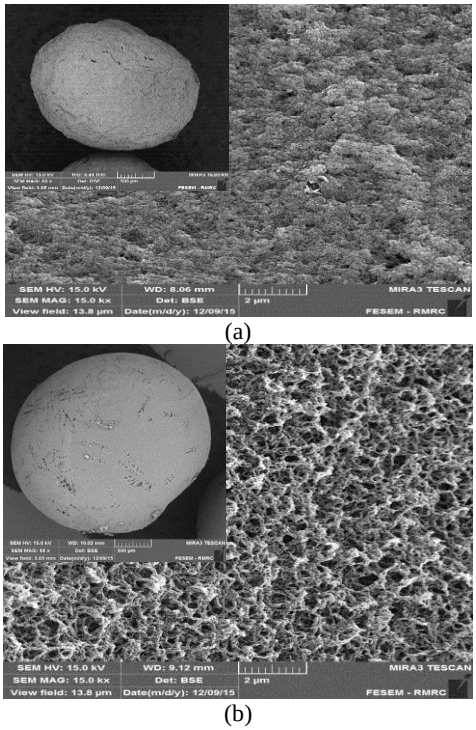



(d) BJH- Chit. glu. bead

**Figure 2.** Micropore and mesopore size distributions plots

However, Chit. glu. beads show high value of pore volume distribution in the range of mesopores region (see Figures 2b and 2d) demonstrating the porosity of Chit. beads were uniformly increased by treating them with glutaraldehyde. These results are confirmed by our previous studies. Based on the results of our previous research on textural properties were obtained by BET analysis; the surface area exposure and pore volume of Chit. beads through activation with glutaraldehyde were increased from 20.1 to 108.3 and 0.0763 to 0.4103, respectively [20-22]. These results were confirmed with FESEM observations. Figure 3 shows that glutaraldehyde has greatly increased the surface porosity of Chit. beads. Lastly, it can be concluded that treating of Chit. beads by glutaraldehyde not only increased the porosity of Chit. beads but also, it can obviously change the isotherm type of adsorption and pores shape of Chit. beads. These promotions on texture and pore structure of Chit. beads may have consequences on immobilization of enzyme or mass transfer of enzymatic reaction. In general, alteration in pore structure of carrier may affect the diffusion of adsorbate into micro/mesopores of adsorbent as a major step of adsorption process. To make this point clear, intra-particle diffusion model was investigated.

**3. 2. Intra-particle Diffusion Study** The adsorption mechanism of adsorbate onto adsorbent follows two general steps: (1) mass diffusion from the boundary film to the external surface of the adsorbent; (2) diffusion of the adsorbate through adsorbent pores; that the slowest step controls the overall rate of the process [18]. In order to make a distinction between adsorption inside pores and external surface of Chit. bead, amylase uptake was evaluated using an intra-particle diffusion model.




(a)

(b)

**Figure 3.** FESEM image of (a) chitosan bead; (b) activated chitosan bead with glutaraldehyde; image magnification of 15kx.

As reported previously, mean pore diameter of Chit. bead and Chit. glu. bead were obtained 14 and 16 nm, respectively. High value of pore volume distribution was determined in the range of mesopores region for Chit. glu. bead with narrow slit-like pore in this study. Therefore, it is expected that pore diffusion of amylase will be occurred easily in Chit. glu. bead, on the other hand, a greater part of amylase might be adsorbed on the external surface of Chit. bead. Figure 4 shows the intra-particle diffusion plots for amylase adsorption on Chit. bead and Chit. glu. bead. Good linear correlation is obtained in each case. The intra-particle diffusion rate constants and the intercepts are presented in Table 1. From the slope of plots, it is found that $k_{id}$ of Chit. glu. bead is higher than Chit. bead, indicative of the fast pore diffusion of amylase in mesoporouse Chit. glu. bead. The large intercept represents the greater effect of the boundary layer on molecule diffusion. According to the results, the intercept for Chit. bead was obtained more than 2.5 fold in compared to Chit. glu. bead that indicates the adsorption process on Chit. bead is noticeably of external surface. From the Equation (2), 97.6% of amylase is adsorbed inside the mesopores of Chit. glu. bead whereas 13% stays on the external surface of Chit. bead.

**TABLE 1.** Intra-particle diffusion parameters of amylase immobilization

| Intra-particle Diffusion parameters | $k_{id}$ (mg/mg.(h)$^{1/2}$) | $C_i$ (mg/mg) | $q_{meso}$ (%) | $R^2$ |
|---|---|---|---|---|
| Chit. bead | 0.0112 | 0.0044 | 87 | 0.975 |
| Chit. glu. bead | 0.027 | 0.0017 | 97.6 | 0.988 |

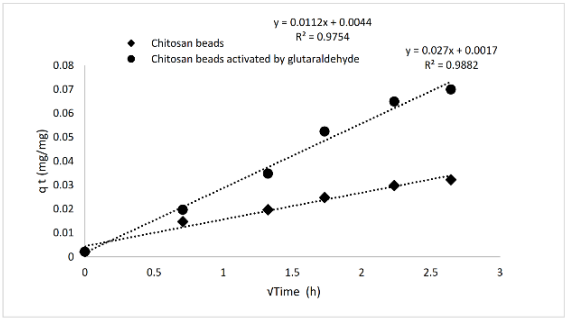



**Figure 4.** Intra-particle diffusion plots for amylase adsorption on Chit. bead and Chit. glu. Bead

**3. 4. Stability Tests and Leakage of Immobilized Amylase** Characteristic analysis including pH stability, thermal stability, leakage and storage stability of immobilized amylase were considered for Chit. beads and Chit. glu. beads.

It is well known that pH of media strictly affect the relative activity of enzyme. In the present research, stability of the immobilized amylase (EC 3.2.1.1) was investigated in various pH. In these experiments, enzyme was placed in solution with defined range of pH (5-9) for an hour and then relative activity was measured in optimum pH. Based on literature, pH stability of immobilized enzyme is principally dependent on the method of immobilization and the interaction of enzyme with carrier [17]. As shown in Figure 5, glutaraldehyde as cross-linker of covalent bond makes enzyme more stable against variations of pH. Enzyme immobilized on Chit. glu. bead retained 85 and 81% of relative activity in pH of 5 and 9, respectively. However, low pH stability (89 and 75% in pH 6 and 9) was achieved by enzyme immobilized on Chit. bead with non-covalent bond. To summarize, the activity of immobilized enzyme was found to be higher than free amylase in whole range of pH.

Furthermore, prepared Chit. glu. beads had high resistance to acidic conditions whereas, Chit. beads were dissolved in pH value below 6. Amine groups of chitosan make it a cationic polyelectrolyte. Therefore, it is soluble in aqueous phase at acidic condition where amine groups are positively charged.

Resistance of enzyme to high temperature during finite time is an important aspect of industrial applications. Thermal stability of alpha amylase was experimented and presented in Figure 6. According to the figure, free enzyme has sharp fall in relative activity about 60% at 100°C within 120 minutes. However, the amylase immobilized on Chit. beads and Chit. glu. beads retained 60 and 80% their initial activities after 120 min of heat treatment, respectively. It must be noted that the immobilization of amylase on Chit. beads make the amylase conformation more rigid and mitigate conformational transitions of the enzyme at high temperatures; therefore the immobilized amylase become more resistant against heat inactivation.

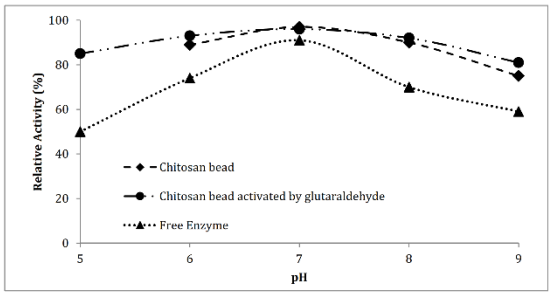



**Figure 5.** pH stability of free and immobilized amylase

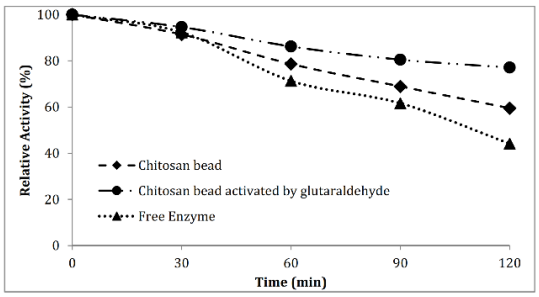



**Figure 6.** Thermal stability of free and immobilized amylase in 100°C within 120 min

Demir et al. [26] were immobilized α-amylase on glutaraldehyde activated silanized calcium carbonate nanoparticles by using covalent binding method. The immobilized enzyme had higher activity at elevated temperature (50–90 °C) than the free one. At 90 °C, the relative activity loss was calculated as approximately 26.2% for immobilized enzyme and 96.8% for the free one.

The storage stability of alpha amylase was considered within 15 days at 25 °C. Based on the obtained results depicted in Figure 7 immobilization can cause enzyme stable during time. Amylase immobilized on Chit. glu. beads was obviously found to be more stable than Amylase immobilized on Chit. beads. The amylase immobilized on Chit. beads had lost its relative activity close to 40% after 15 days. However, the decrease in relative activity of amylase immobilized on Chit. glu. beads was slowly fallen near to 14% after 15 days. Thus, covalent immobilization improves storage stability of enzyme. In fact, covalent bond makes modification in three dimensional structure of the enzyme, which leads to conformation change of the active center [27-29].

Similar results were previously reported by Hasirci et al., who showed that amylase immobilized via covalent bonding on modified glass beads retained almost 80% of its initial activity after 25 days [30] .

Demir et al. [26] were stored the free and immobilized amylase at 4 °C. Free enzyme lost its activity completely within 15 days. However, the immobilized enzyme retained 71% of its activity after 40 days. Klapiszewski et al. [1] were immobilized Amylase on a synthesized titania/lignin support. The immobilized enzyme stored at 5 and 25 °C was stable over 30 days, retaining about 70 and 80%, respectively of its initial activity, while the native biocatalyst lost over 80% of its initial activity [1]. Tumturk et al. [31] were covalently immobilized amylase onto poly(methyl methacrylate– 2-hydroxyethyl methacrylate) microspheres, which were activated by using either epichlorohydrin (ECH) or cyanuric chloride ($C_3N_3Cl_3$). Enzyme activities were found to be 32.7 % for ECH and 41.1 % for $C_3N_3Cl_3$ activated matrices after storage for one month at 4 °C .

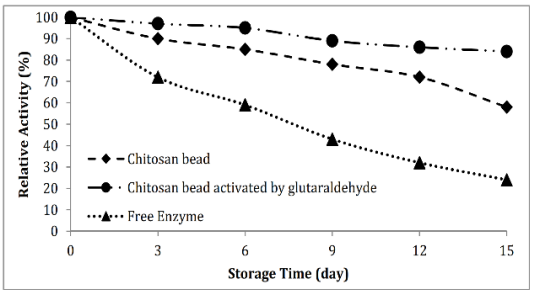



**Figure 7.** The storage stability of free and immobilized amylase within 15 days

In addition, leakage of immobilized enzyme was monitored within 15 days (see Figure 8). Enzyme bind non-covalent with Chit. beads had 20% leakage. On the other hand, covalent immobilization had lower leakage about to 4% of immobilized enzyme. In fact, approximately all immobilized enzyme linked strongly to the carrier by covalent bonds. Therefore, it can be concluded that using glutaraldehyde as cross-linking immobilization agent of amylase on Chit. bead can obviously reduce enzyme leakage.

**3. 5. Effect of Salt and $Ca^{2+}$ Ions** As previously mentioned, amylase is bound to the functional group of the Chit. glu. beads mainly by strong covalent bonds. To verify this, the sodium chloride test was conducted. Presence of sodium chloride may affect the activity of the native enzyme, but may also elute the immobilized enzyme from carrier, as stated in literature [24]. NaCl can cause to desorption of the immobilized enzyme, by way of ion exchange or washed out the hydrogen bonds [24]. The effect of NaCl over a time of 24 h on the relative activity of free and immobilized amylase was examined and presented in Figure 9. The activity of the free enzyme remained almost constant over time. In fact, the sodium chloride has shown no remarkable effect on the activity of the free enzyme, so it was stable under these conditions. On the other hand, the relative activity of enzyme immobilized on Chit. glu. bead reduced to 79% during 24 h that 85% of reduction was occurred after 12 h.

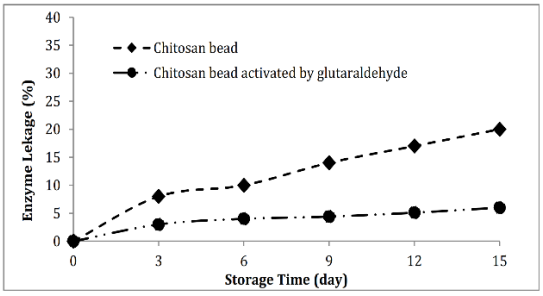



**Figure 8.** The immobilized amylase leakage within 15 days

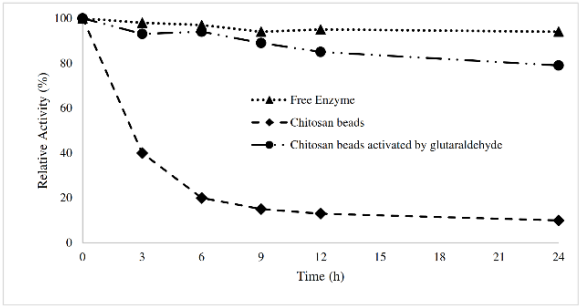



**Figure 9.** The effect of NaCl on the relative activity of free and immobilized amylase within 24h

These results can be verified by measurement of the protein value leaked in the salt solution within 24 h (see Figure 10). According to this experiment Chit. glu. beads had 16% leakage of enzyme that confirmed what was achieved by activity assay. Consequently, amylase was bound to the Chit. glu. beads mainly by relatively stable covalent bonds. This finding is in consistence with what is suggested for other carriers [32]. [1, 33].

In addition, evaluation of enzyme immobilized on Chit. beads in presence of sodium chloride revealed that relative activity sharply decreased to about 20% within 6 hours and finally descended to 10% after 24h (Figure 9). Protein assay shows that activity reduction must be owe to release of adsorbed enzyme in salt solution. Figure 10 shows that 91% of immobilized enzyme was washed by NaCl after 24h.

It should be noted that the beads may contain entrapped released enzyme, due to salt interaction, that can cause inaccuracy in results. Therefore, all of the beads must be perfectly washed with buffer solution before activity analysis. Remaining the released enzyme in chitosan scaffold was verified by protein assay on washing solution. The reported enzyme activities in Figure 9 obtained after perfectly washing the beads.

Alpha amylase is one of the metalloenzymes that can be reactivated by calcium ion and in some cases caused to increase their activity in present of calcium ion [14].

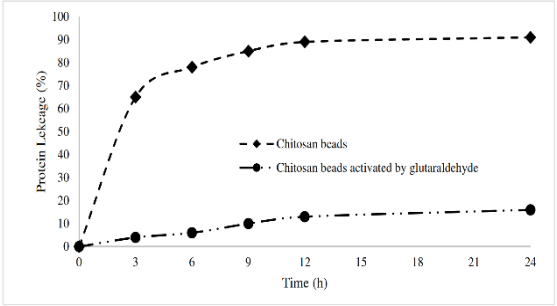



**Figure 10.** The effect of NaCl on the leakage of immobilized amylase within 24h

In the present research, the effects of various concentrations of calcium ion in the activity of alpha amylase were studied. The obtained results were illustrated in Figure 11. The activities of the free and immobilized enzyme initially increased with the concentration of $Ca^{2+}$ ions (0.5–1 mM). The maximum activity of these enzymes were observed in 1 mM concentration of calcium ion. Based on literatures, calcium ion can act as chaperons to help the necessary refolding of denatured enzyme molecules or they may be contributed in the enzyme reactivation reactions. In presence of calcium ion, primary or secondary binding site can take place in alpha amylase structure. The primary calcium binding site can create and stabilize the active site. The reduction in relative activity was observed in high concentration of calcium ions (in 2.5 mM). This is perhaps due to this fact that the primary calcium binding sites have been occupied with calcium ions; whereas, increased concentration of calcium ions will make creation of secondary binding. The secondary binding can prevent the formation of the E–S complex which is necessary before product formation in enzymatic reaction [14].

**3. 6. Enzymatic Reaction Kinetics** Table 2 lists the kinetics parameters $V_m$ and $K_m$ which are measured for free and immobilized enzymes. Figure 12 presents double reciprocal plot also known as Lineweaver–Burk plot for Michaelis-Menten rate equation. From the table, free enzyme had high value of maximum velocity compared to immobilized enzyme. It perhaps for this reason that immobilization can decrease enzyme activity because of enzyme restriction by binding it to the carrier. The Michaelis–Menten constant, $K_m$, represents the affinity of enzymes to substrate. The lower $K_m$ value indicates stronger affinity of the enzyme to substrate. The value of $K_m$ for physically adsorbed enzyme on Chit. beads is higher than free one . It must be due to high diffusion constrain of substrate in mesoporous matrix leading to low accessibility of substrate to confined enzyme. From Table 2, the high value of $K_m$ was obtained for covalently immobilized enzyme relative to other.

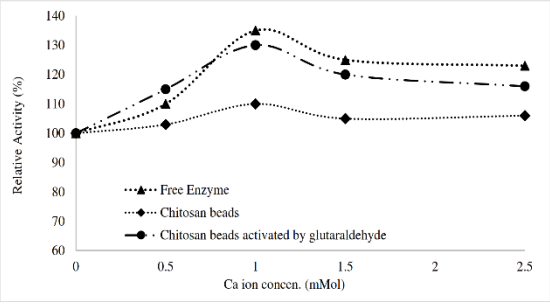



**Figure 11.** The effect of $Ca^{2+}$ concentration on the relative activity of free and immobilized amylase

**TABLE 2.** Kinetic parameters of free and immobilized amylase

| Michaelis-Menten parameters | $K_m$(mg/ml) | $V_{max}$ (U/mg Enzyme) | $R^2$ |
|---|---|---|---|
| Free Enzyme | 0.208 | 416.67 | 0.961 |
| Chit. bead | 0.296 | 370.37 | 0.983 |
| Chit. glu. bead | 0.431 | 227.27 | 0.979 |

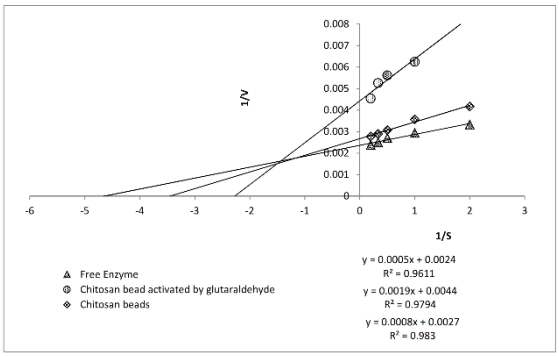



**Figure 12.** Lineweaver–Burk plot of free and immobilized amylase

It has been reported that covalently crosslinked immobilized enzyme might have different conformational flexibility to adsorbed and its free form. The presence of glutaraldehyde as crosslinking agent can alter molecular flexibility and the mobility of enzyme. Rigidification of the enzyme molecule, relative to the free and adsorbed enzyme, is the main reason for lower accessibility and then affinity of substrate to enzyme [34].

## 4. CONCLUSION

In this study, alpha amylase was immobilized on Chit. beads by covalent and non-covalent immobilization. The effect of glutaraldehyde on textural and structural properties of Chit. beads were considered. It was observed that glutaraldehyde had significant effect on Chit. beads porosity, pore shape and isotherm type of adsorption. Intra-particle diffusion was investigated to find out the rate controlling step of adsorption. Based on the results, $k_{id}$ of Chit. glu. beads was obtained higher than Chit. beads. It can be reason for fast pore diffusion of amylase in mesoporouse Chit. glu. beads. Also, 97.6% of amylase is adsorbed inside the mesopores of Chit. glu. beads whereas 13% stays on the external surface of Chit. beads because of higher boundary layer effect in Chit. beads. Thermal, pH and storage stability along with leakage were examined for free and immobilized enzymes. The use of glutaraldehyde can enhance immobilization efficiency and stability of

enzyme. The activity of alpha amylase as a metalloenzyme can be influenced by presence of calcium ions. According to the results, $Ca^{2+}$ concentration of 1 mM had great effect on relative activity to reach maximum activity in both free and immobilized form. Salt solution demonstrated amylase was bound to the Chit. glu. beads mainly (84%) by relatively stable covalent bonds. However, covalent immobilization process had slightly undesirable effect on the affinity of the enzyme and substrate resulted by kinetic consideration.

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# Textural and Structural Characterizations of Mesoporous Chitosan Beads for Immobilization of Alpha-Amylase: Diffusivity and Sustainability of Biocatalyst

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**چکیده**

در این تحقیق خواص بافتی و ساختاری دانه های کیتوزان به منظور تثبیت آنزیم آلفا آمیلاز به وسیله ی جذب و واجذب نیتروژن،نمودارهای انالیز میکروحفرات، نمودارهای بارت–جوینر–هالندا و مشاهدات میکروسکوپ الکترونی روبشی نشر میدانی مورد مطالعه قرار گرفت. مشاهدات ساختاری حفرات نشان داد که فعال سازی شیمیایی دانه کیتوزان توسط گلوتارآلدئید می تواند نوع ایزوترم جذب و همچنین شکل حفرات را تغییر دهد. در تایید نتایج تحلیل بافت، توزیع حجمی حفرات به مقدار زیادی در محدوده مزوحفره ها می باشد که همچنین نشان می دهد، تخلخل دانه کیتوزان فعال شده، به طور یکنواخت افزایش یافته است. مدل نفوذ درون ذره ای نشان می دهد که به دلیل افزایش $k_{id}$ (ثابت سرعت) و کاهش اثر لایه مرزی بر پدیده نفوذ، ۹۷/۶٪ آمیلاز در داخل حفرات دانه فعال شده کیتوزان جذب شده است. همچنین آزمایش های پایداری ( pH ، ذخیره سازی و پایداری حرارتی)، نشت آنزیم، اثرات غلظت نمک و یون کلسیم برای آمیلاز تثبیت شده بررسی و با آنزیم آزاد مقایسه گردید. غلظت ۱ میلی مولار یون کلسیم تاثیر بسیار خوبی بر فعالیت نسبی آمیلاز در شکل آزاد و تثبیت شده نشان داده است. آزمایشات تاثیر نمک کلرید سدیم نشان می دهد که ۸٤٪ از آمیلاز به صورت کوالانسی بر روی دانه های فعال کیتوزان تثبیت شده است. علاوه بر این ثابت معادله میکائیلیس- منتن ( ~ 0.4mg / ml) و بیشترین سرعت در معادله میکائیلیس- منتن و( ~ 227 U /mg Enzyme) به دست امده است که نشان دهنده تمایل قوی و فعالیت بالای آنزیم تثبیت شده می باشد.

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