Parameters Affecting the Improvement of Properties and Stabilities of Immobilized α-amylase on Chitosan-metal Oxide Composites

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Abstract Chitosan-metal oxide composites, chitosan-Fe₃O₄ (CSM) and chitosan-ZnO (CSZ) were synthesized and intended as suitable supports for immobilization of starch hydrolyzing enzyme, α-amylase. Here α-amylase was chosen as the enzyme to immobilize on these supports due to its wide implementation in industrial applications. The immobilized enzyme, CSZE showed better immobilization yield of 76%, whereas CSME provides high speed and easy separation from the reaction system due to its magnetic property. The quality of the immobilized α-amylases was demonstrated and assessed based on its activity and stability. The immobilized enzymes with higher Ed values provide them more stability towards denaturation compared to free enzyme. At 50°C the thermodynamic parameters such as ∆Hº, ∆Gº and ∆Sº for thermal deactivation of free enzyme were evaluated as 104.29, 12.83 and -0.28 KJ mol⁻¹ respectively and are found to be higher in case of both immobilized enzymes. The Km values for CSME and CSZE are 0.65 and 0.5mg/mL respectively, which are higher than that of free enzyme (0.45mg/mL). The Vmax of free enzyme is decreased from 34.48μmol mg⁻¹ min⁻¹ to 16.39(CSME) and 23.81 (CSZE) μmol mg⁻¹ min⁻¹ as a result of immobilization. The immobilized enzymes have exhibited better storage stability over 6 months and retained more than 50% of their initial activities after 10 cycles of reuses.

Keywords Chitosan-metal Oxide Composite, α-Amylase, Immobilization, Thermal Deactivation, Catalytic Activity, Maltose

1. Introduction

Enzymes are biocatalysts having high catalytic activity and excellent selectivity. The use of enzymes in industrial or medical applications has been limited because most enzymes are relatively unstable and the cost of their isolation and purification is still high. The free enzymes used in solution have inherent susceptibility to protease-catalyzed breakdown [1-2], it is technically very expensive to recover active enzyme from the reaction mixture at the end of a process. Furthermore, in order to purify the product, the enzymes have to be depleted from the reaction solution, whereby they often get inactivated. These drawbacks can be overcome by immobilizing the biocatalysts onto solid supports which enables the easy separation from the reaction system by filtration or centrifugation. In addition, immobilization provide the stability to the enzyme by hindering the opening of the protein molecules and protecting the polypeptide bonds against rupture which leads to the conformational stability in the active sites [3]. The enzyme attained stability due to the formation of numerous hydrogen bonds with amino acid units in its structure [4].

There are several methods for immobilization of enzymes onto various carriers such as adsorption, covalent binding, entrapment or encapsulation. Among this adsorption is the most general, simple to perform and the oldest method. Various materials used as supports for enzyme immobilization have been described in the literatures. Metal oxides such as TiO₂ [5], ZnO [6], SnO₂ [7] and Fe₃O₄ [8] have shown significant attraction towards the field of enzyme immobilization. Among these ZnO gained more attention due to their unique properties like high specific surface area, strong adsorption ability, high catalytic efficiency, high isoelectric point (IEP 9.5), biocompatibility and high electron transfer properties. The high IEP of ZnO is more favorable for immobilizing enzymes with low IEP through electrostatic interactions [9]. Moreover, high chemical stability, nontoxicity, and high electron transfer capability make ZnO a promising material for immobilization of enzymes involved in electron or charge transfer [10]. Magnetite particles are more effective carriers for the immobilization of enzymes, [11-12] as they
allow the easy separation of the catalyst from the reaction system using an external magnetic field. The magnetic particles possess a high surface area, helps to decrease the diffusion barriers in the transport of the substrate and the reaction products, thereby improving the efficiency of the immobilized enzyme [13].

For the immobilization of the bioactive reagent onto metal oxides, the presence of functional groups on their surface is very important. The surface functionalized materials on immobilization of biomolecules are found to be increased loading capacity and stability. Generally, natural and synthetic polymers are used for the functionalization of the metal oxides [14-16]. In our study we have chosen the biopolymer, chitosan for the surface functionalization of Fe$_3$O$_4$ and ZnO particles. Chitosan is a naturally occurring polymer which is obtained by the deacetylation of chitin. It is the second most abundant biopolymer after cellulose and consists of 2-amino-2-deoxy-D-glucose units, is joined by β (1→4) linkages. It is one of the most promising immobilization matrices due to several properties such as, hydrophilicity, biocompatibility, biodegradability and antibacterial properties [17-18]. Because of their relatively low production cost and higher adsorption, the preparation of organic-inorganic composites has attracted great interest.

Amylases are the enzymes with attractive industrial applications, which belong to the class of hydrolases. They catalyze the hydrolysis of starch into sugars. In starch processing industries, a large number of commercially available microbial amylases are used for hydrolysis. There have been many reports about the immobilization of amylase and much research has focused on developing polymer based supports [19-24]. α-amylases (1,4-α-D-glucan-glucanohydrolase) have more importance in the field of biotechnology with applications ranging from food, fermentation, detergent, paper and textile industry [25]. The use of immobilized α-amylase in these industries will reduce the cost of overall enzyme as it can be reused several times, ultimately affect positively their economy.

Therefore in this study, we aimed to immobilize α-amylase onto chitosan-metal oxide composites, chitosan-Fe$_3$O$_4$ (CSM) and chitosan-ZnO (CSZ). To the best of our knowledge, this is the first report on α-amylase immobilization on these organic-inorganic composites. We have done the enzyme immobilization studies on these two composites. In case of CSZ composite, as a result of centrifugation, undesirable dilution of the sample and the loss of carrier during washing, complicate its usage. Support with magnetic core, CSM composite is chosen to avoid these problems. It can be used favourably for high speed separation and for the elimination of mechanical damage by centrifugation. Various parameters such as pH, temperature, contact time and amount of enzyme required for immobilization were optimized so as to acquire immobilized enzyme with maximum activity and stability. The kinetics of the hydrolysis reaction was studied at various substrate concentrations and the kinetic parameters (Km and Vmax) were calculated from the Lineweaver-Burke plot. Performance of thermal deactivation at different temperatures on free and immobilized enzymes has done and thermodynamic parameters were evaluated by determining the deactivation energy and deactivation constant. Thermal stability, reusability and storage stability of the immobilized enzymes were also studied.

2. Materials and Methods

2.1. Materials

Diastase α-amylase (1,4 α-D-glucanglucanohydrolase, EC 3.2.1.1) was acquired from Himedia Laboratories Pvt Ltd, Mumbai. Soluble Starch (potato) was obtained from S.D. Fine-Chem. Ltd, Mumbai. Chitosan (95% degree of deacylation) was purchased from Meron marine chemicals, Cochin. All other chemicals used were in analytical grade.

2.2. Synthesis of Composites

2.2.1. Chitosan-Fe$_3$O$_4$ (CSM) composite

Chitosan-Fe$_3$O$_4$ (CSM) composite was synthesized by reduction–precipitation process, based on the already reported method with slight modification, in which glutaraldehyde was added as the cross-linking agent instead of epoxy chloropropane [26]. About 70mL FeCl$_3$ solution (0.13 mol L$^{-1}$ in 0.13 mol L$^{-1}$ HCl) was added into 50mL of 2% chitosan solution in 1% HCl under continuous stirring for 1h. Then 30mL 0.1M Na$_2$SO$_3$ solution was added into the colloidal solution and the mixed solution was poured quickly into 40mL of 12% (v/v) ammonia solution under vigorous stirring at room temperature. The black precipitate suspension obtained immediately. After that, 5mL glutaraldehyde (25%) was added to the suspension and stirred for 3hrs in water bath at 60°C in order to stabilize the particles in acid solution. The black precipitate collected with the aid of a magnet and washed several times with distilled water, ethanol and acetone respectively, then dried in vacuum oven at 60°C.

2.2.2. Chitosan-ZnO (CSZ) composite

Firstly zinc oxide particles were prepared by wet chemical method. About 100mL 0.9M aqueous ethanol solution of NaOH was added drop by drop into 100mL 0.5M aqueous ethanol solution of zinc nitrate under high speed constant stirring. Then the solution was allowed to settle for overnight. The obtained precipitate were washed three times with distilled water and ethanol to remove the by-products and then dried in air atmosphere at about 60°C [27]. For the preparation of chitosan-ZnO (CSZ) composites, 1g ZnO powder was dissolved in 100mL 1% acetic acid to produce zinc cations. To this solution 1gm chitosan powder was dissolved under magnetic stirring. Then 1M NaOH
added drop by drop until the solution reached pH 10. The solution was placed in a water bath at 80°C for about 3hrs. The precipitate obtained were filtered, washed with distilled water several times and then dried in an oven at 50°C for 1hr [28].

2.3. Characterization of Composites

The composites were characterized by FT-IR spectrometry using JASCO FT/IR-4100. Bruker AXS D8 Advance is used for XRD analysis. The TEM images of composites were taken by JEM 2100 having 0.24nm resolution with an acceleration voltage of 200kV. Thermograms were obtained Perkin Elmer, Diamond TG/DTA. The surface areas of both the composites were measured using a surface area analyzer (TriStar II 3020 V1.04), plotting nitrogen adsorption isotherms at liquid nitrogen temperature.

2.4. Immobilization of α-amylase

The immobilization of α-amylase was performed by stirring 1g of each of the composites with enzyme in buffer at room temperature. The immobilization parameters like pH, time and the amount of enzyme for immobilization were optimized. The pH of the immobilization medium varied from 5 to 9, the incubation time varied in the interval from 30- 150 minutes and the enzyme concentration from 2 to 20mg enzyme g⁻¹ support. The biocatalyst then filtered and washed with the same buffer solution. The supernatant and washings were subjected to protein estimation using Folin Ciocaltaue's reagent by measuring the absorption at 660nm in a Thermo Scientific Evolution 201 UV-Visible spectrophotometer [29]. The prepared immobilized enzymes were kept in a refrigerator at 4 °C for further studies. Immobilization yield (IY) was calculated (Eq. (1)) by measuring the difference in protein concentration of the supernatants after and before immobilization.

Immobilization Yield,

\[ IY\% = \frac{C1 - C2}{C1} \times 100 \] (1)

Where C1 is the concentration of protein introduced for immobilization and C2 is concentration of protein present in the supernatant after immobilization.

The activity yield (Eq. (2)) was determined by measuring the activity of the immobilized enzyme and the activity of the initial enzyme used in the immobilization reaction.

Activity Yield (AY%) = \( \frac{\text{Activity of immobilized enzyme}}{\text{Activity of free enzyme}} \) (2)

Immobilization efficiency was calculated using Eq. (3).

\[ IE\% = \frac{AY}{IY} \times 100 \] (3)

2.5. Determination of Activity of Free and Immobilized α-amylase

The activity of free and immobilized α-amylase were determined by the detection of released reducing sugars from starch using 3, 5-dinitrosalicylic acid (DNS). The enzymatic reaction was carried out by adding 1 mL of free α-amylase (0.5 mg/mL) or 0.05g of immobilized enzyme to 1mL of 1% starch solution in desired buffer and the system was incubated in a water bath with constant shaking at 30°C for exactly 15 min. The reaction was stopped by adding 1 mL of 3, 5-dinitrosalicylic acid reagent. Incubation was performed in a boiling water bath for 5 min and cooled the reaction tubes to room temperature. The amount of reduced sugar (maltose) produced was determined spectrophotometrically at 540nm [30]. An enzyme activity unit (EU) was defined as the amount of enzyme liberating 1μmol maltose per minute under the assay conditions.

2.6. Optimization of Immobilization Parameters

The optimum pH for maximum activity of free and immobilized enzymes (CSME and CSZE) was assayed by incubating the enzyme with starch over a pH range 5- 9 at 30°C. The temperature for maximum activity was also assayed by varying the temperature from 30- 60°C. In order to test the thermal stability of free and immobilized enzymes, they were subjected to various temperatures ranging from 30- 70°C for 1hour in a water bath. After 1hour of pre-incubation both free and immobilized enzymes in buffer were cooled to optimum temperature and enzymatic reaction was performed with the addition of definite amount of 1% starch solution to each reaction medium for a definite time interval.

2.7. Thermal Deactivation Study

2.7.1 Kinetic study and estimation of Deactivation energy

Study on kinetics was conducted by incubating the free and immobilized enzymes at different temperatures in the absence of starch substrate. After definite time intervals, the enzyme activity assay was done and the residual activity expressed in terms of percent of initial activity. The slope of the logarithmic plot of percent residual activity versus time gives the deactivation constant (kd) and the deactivation energy (Ed) was calculated from the slope of Arrhenius plot drawn between ln kd and reciprocal of temperature, 1/T (K) using Eqn. (4),

\[ \text{Slope} = -\frac{Ed}{R} \] (4)

2.7.2. Estimation of thermodynamic parameters

The values for thermodynamic parameters, ΔHº, ΔGº and ΔSº were estimated by using the kd and Ed values.
2.7. Determination of Kinetic Constants

The kinetic parameters were determined by measuring the rates of the reaction at various substrate concentrations ranging from 0.2 to 1.0 mg/mL at optimum temperature and pH. Michaelis constant (Km) and maximum rate (Vmax) were calculated from the Lineweaver - Burk plot. This double-reciprocal plot, 1/V₀ as a function of 1/[S₀], gives the y-intercept representing the inverse of Vmax and the x-intercept of the plot as −1/km.

Where R is the universal gas constant, T is the absolute temperature (K), h is the Planck constant, and kₜ is the Boltzmann constant.

\[ \Delta H^\circ = Ed - RT \]  
\[ \Delta G^\circ = -RT \ln \frac{k_d h}{k_B T} \]  
\[ \Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \]  

2.8. Reusability and Storage Stability

The reusability of the immobilized enzyme was examined by repeated batch experiments maintaining a couple hours in each cycle. The residual activity of immobilized enzymes at its optimum conditions was measured at fixed time intervals. After each run, the immobilized enzyme was removed, washed with buffer solution and mixed with fresh substrate solution. The reaction was carried out continuously for 10 cycles. The storage stability of immobilized enzymes was studied by calculating their activities after being stored at 4°C in buffer solution for 6 months. The measurement was conducted at regular intervals of time. The activity was compared with initial activity and was represented as percentage relative activity.

3. Results and Discussion

3.1. The Spectral Analysis of Prepared Composites

In the infrared spectrum (supplementary data- Appendix A) of chitosan-magnetite (CSM) composite, the absorption band at 3310 cm⁻¹ attributed to the combined peaks of amino (-NH₂) and hydroxyl (-OH) groups stretching vibration and the bands at 1614 cm⁻¹ and 1514 cm⁻¹ were related to the C=O stretching vibration of amide and the bending vibration of -NH₂ groups respectively. The absorption band at 1056 cm⁻¹ represents the C-O stretching vibration of primary alcoholic group in chitosan. In case of chitosan-ZnO (CSZ) composite, the above absorption bands are at 3419, 1610, 1515, 1050 cm⁻¹ respectively. These absorption bands are slightly shifted to lower vibrational frequency when compared to the IR spectrum of chitosan particle in which they are at 3429, 1644, 1540 and 1074 cm⁻¹ respectively. This indicates that the amino and the hydroxyl groups on chitosan were involved in complexation with the metal oxide. IR spectrum of CSM composite exhibits a strong adsorption band at 576 cm⁻¹ corresponding to the Fe-O bond vibration of Fe₂O₃. The electrostatic interaction between positively protonated chitosan and surface negative charged Fe₂O₃ also contribute to this IR change. It means that Fe₂O₃ is coated by chitosan and no chemical bonding between chitosan and Fe₂O₃ was formed. The IR spectrum of CSZ composite shows a new broad absorption band at the range 590-560 cm⁻¹ which is ascribed to the stretching vibration of N-Zn and O-Zn groups.

The TGA curve (supplementary data- Appendix B) for pure chitosan particles (CS nano) exhibited a weight loss of 5% below 100°C and a rapid and main weight loss at 250–500°C, referring to the removal of physically adsorbed water and the thermal degradation of the pure chitosan, respectively. No significant weight change was observed above 500°C. However, the TGA curve of CSM composite was very different from that of the pure chitosan. Three stages of weight loss were observed in the TGA curve of CSM composite. The first stage was about 8% weight loss from room temperature to 200°C, as a result of the evaporation of adsorbed water. The other two stages of main weight loss at 200–400°C and 600–730°C were caused by the decomposition of primary chains of biopolymer and cross-linking chitosan with metal oxide respectively. The conformational changes of chitosan and the additional bridging between Fe₂O₃ and chitosan enhanced the thermal stability of chitosan in CSM composite [26]. Thus, the final temperature of decomposition for CSM composite was higher than that for the pure chitosan. The similar TGA curves have also been reported [31]. In case of CSZ composite, there are three stages of weight loss observed. The initial stage of weight loss from room temperature to 200°C is due to the evaporation of adsorbed water. The other two stages of weight loss at 250°C – 400°C and 600°C -730°C are due to the decomposition of primary chains of biopolymer and cross-linking of metal oxide with the biopolymer respectively. At the end of the heating it was found that the total weight % of the composite with around 55%. From this it is clear that the thermal stability of the composite is higher than that of pure chitosan. Addition of ZnO particles into the chitosan matrix increases the thermal stability due to the strong interaction between ZnO and chitosan. Similar observations have been reported [32].

The XRD spectra of composites were shown in the supplementary data (Appendix C). The typical peaks of chitosan appeared at 5.5° and 20°, but these peaks become weak in the case of CSM and CSZ composite. The other strong and sharp peaks for CSM composite were observed at 2θ = 29.9°,35.6°, 43.1°, 53.5°, 57.2°, and 62.7°;
corresponding to (220), (311), (400), (422), (511), and (440) crystal planes of face centred cubic \( \text{Fe}_3\text{O}_4 \) respectively (JCPDS File No: 75-0033). These revealed that the crystal structure of \( \text{Fe}_3\text{O}_4 \) in CSM composite prepared with the reduction–precipitation method was a cubic spinel structure [33]. Diffraction peaks in XRD pattern of CSZ composite are sharper and stronger at 31.7°, 34.36°, 36.2°, 56.59°, 62.7°, and 67.90° and were assigned to the (1 0 0), (0 0 2), (1 1 0), (1 0 3), and (1 1 2) planes of hexagonal zinc oxide can be indexed to the wurtzite \( \text{ZnO} \) with high crystallinity. All the diffraction peaks are in good agreement with those of hexagonal wurtzite structure of \( \text{ZnO} \) (JCPDS card 36-1451). The particle sizes can be quantitatively evaluated from the XRD data using the Debye–Scherrer equation which gives a relationship between peak broadening in XRD and particle size:

\[
 d = \frac{k\lambda}{\beta \cos \theta}
\]

Where \( d \) is the particles size, \( k \) is the Debye–Scherrer constant (0.89), \( \lambda \) is the X-ray wavelength (0.15406nm) and \( \beta \) is the full width at half maximum, \( \theta \) is the Bragg angle. According to the Debye-Scherrer equation, the particle size of the chitosan coated \( \text{Fe}_3\text{O}_4 \) particles is 30nm and that of the chitosan coated \( \text{ZnO} \) particles is 25.8nm.

The transmission electron microscope photographs of composites were shown in the supplementary material (Appendix D). The images are not agglomerated since polymer is incorporated with the metal oxides. The average size was 20 and 30nm respectively for CSZ and CSM which were close agreement with the values obtained from XRD characterization.

The surface areas of composites were 1.99 and 2.4 m\(^2\)/g for CSM and CSZ respectively.

### 3.2. Immobilization Conditions

To find out the optimum immobilization condition for the enzyme, we have investigated the effect of pH of the immobilization medium, contact time of support and amount of enzyme taken for immobilization. The retained activity of the immobilized enzymes was recorded and the results were designated in terms of relative activity. The effect of solution pH during immobilization process was given in Figure 1a. Both of the immobilized enzymes, CSME and CSZE have shown maximum activity at pH 6. The isoelectric point of \( \alpha \)-amylase is around 4.6 and the amino group in chitosan has a pKa value of about 6.5. At pH 6, the amino groups of chitosan have net positive charge and these amino groups attract the enzyme molecule which has negative charge. This is due to the significant electrostatic interaction between the supports and the enzyme. When the immobilization is carried out at higher pH, the enzyme activity found to be decreased. This is because at higher pH both the enzyme and the chitosan having net negative charge which results in the electrostatic repulsion.

The effect of contact time on the activity of immobilized enzyme was given in the Figure 1b. The figure illustrates that the activity of the immobilized enzyme increased with contact time of enzyme and support. The highest activity is obtained at 120 and 90 min for CSME and CSZE respectively. The decrease in the immobilized enzyme activity can be due to the formation of a disordered multilayer or formation of multiple bonds which deformed the active site of the enzyme [34].
Figure 1. (a) Effect of pH of immobilization medium on the relative activity of immobilized α-amylases, buffers used are acetate buffer (5-5.5), phosphate buffer (6-8) and glycine buffer (8.5-9) (b) Effect of contact time on immobilized enzymes activity. (c) Effect of reaction pH on the activity of free and immobilized α-amylases. (d) Effect of temperature on activity of free and immobilized enzymes.

Table 1. Immobilization yield, activity yield and immobilization efficiency of α-amylase loaded on CSM and CSZ composites. The enzyme was immobilized on different supports at various initial concentrations at optimum experimental conditions.

| Support | Initial protein (mg) | Immobilized protein mg/g support | IY (%) | Initial activity (EU) | Immobilized enzyme activity (EU) | AY (%) | IE (%) |
|---------|----------------------|----------------------------------|--------|-----------------------|----------------------------------|--------|-------|
| CSM     | 12.6                 | 7.2                              | 57.14  | 23.49                 | 10.28                            | 43.76  | 76.58 |
| CSZ     | 14.2                 | 10.8                             | 76.05  | 26.06                 | 13.7                              | 52.57  | 69.12 |

The effect of protein loaded on the activity of immobilized enzymes were investigated and shown in the figure (supplementary material-Appendix D1). As the enzyme concentration increases, the amount of enzyme immobilized on the support increased and then reached a saturation point indicating a saturated monolayer formation. Thereafter both the enzyme loading and activity decreases denoting the multilayer adsorption. The decrease in the activity after the saturation point is due to the changes in the conformations of the enzyme or the steric hindrance caused by the support at the active site [35]. The results are depicted in the figure (supplementary material-Appendix D2). The immobilization yield, activity yield and immobilization efficiency were evaluated and the results are given in the table1.

3.3. Activity of Enzymes

3.3.1. Effect of pH and Temperature on the Activity of Free and Immobilized α-amylases

The pH of the immobilization medium will affect the distribution of the ionic groups on the enzyme surface and the net charge on the enzyme, also to the reactivity of the catalytically active groups. The change in pH may cause the denaturation of the protein structure and the loss of activity. We have studied the effect of pH on the activity of free and immobilized enzyme and are shown in the Fig. 1c. The free enzyme showed its maximum activity for starch hydrolysis in the range of pH 5-5.5. From the figure it is clear that for immobilized enzyme, CSME the maximum activity at pH 7 and for CSZE the optimum activity at pH 6. The optimum pH of the immobilized enzymes shifted to the alkaline region which is due to the unequal partitioning of the $H^+$ and $OH^-$ ions between the microenvironment of the immobilized enzyme and the bulk phase due to the electrostatic interactions with the matrix [36]. The effect of pH on the enzyme activity is expressed on the basis of external solution pH, which in these cases increases, the pH optimum shifts to the alkaline region. The results showed improved stability of the immobilized enzymes at higher pH values than the free enzyme. Similar observations, for immobilization of α-amylase on glutaraldehyde-agarose support have been reported [37].

The effect of temperature on the activity of free and immobilized α-amylase was studied by changing the temperature in the range of 30-60°C. The results are depicted in the figure 1d. The temperature optimum for starch hydrolysis was 50°C for free enzyme. As a result of immobilization, it gets shifted to 35°C for both CSME and CSZE. The decrease in the optimum temperature might be due to change in conformational integrity of the enzyme structure by immobilization which favoured amylase activity below 50°C. The attachment of the support might have affected the three dimensional protein structure which either caused the denaturation of the protein structure or affected the conformation of protein which resulted in an alteration of enzyme substrate affinity and hence showed a decrease in the immobilized enzyme catalytic activity at
higher temperature. The decrease in activity of $\alpha$-amylase at high temperature was reported when immobilized on chitosan-clay composite [38].

Arrhenius plot was drawn between log relative activity (%) and $1/T$ in order to estimate activation energy, $E_a$ (figure 2a). The values were found to be 11.75, 28.23 and 23.77 KJmol$^{-1}$ for free $\alpha$-amylase, CSME and CSZE respectively. The decline in the activation energy of enzyme might be due to its reduced conformational flexibility as a result of immobilization. Similar observation was reported when $\alpha$-amylase immobilized on coconut fiber support [39].

3.4. Thermal Stability

Thermal stability is one of the most important properties of enzymes for different industrial applications. We have studied this property over the range of 30–70$^\circ$C by pre-incubating the free and immobilized enzymes in buffer for 60 min followed by enzyme reaction at optimum temperature. The results are depicted in figure 2b, which shows that the immobilized enzymes exhibited enhanced heat resistance than free enzyme. This emphasizes the rate of deactivation is lowered as a result of immobilization. The thermal denaturation might be as a result of unfolding of protein structure that is hindered due to immobilization, since the immobilization brings about a restriction in the free movement of enzyme. After 1 hr pre-incubation at 50$^\circ$C, only less than 50% of the free enzyme remained active whereas 80–85% of the immobilized enzymes retained their activity. At 70$^\circ$C the free enzyme lost almost 90% of its activity, whereas immobilized enzymes lost 25-30%. From the figure it is clear that stability at higher temperature is more for the immobilized enzyme, CSZE. The gentle decline in the enzyme activity of CSME, may be due to the influence of iron core on the biological activity of the enzyme [40].

3.5. Kinetics of Thermal Deactivation and Estimation of Deactivation Energy

The thermal deactivation study of free and immobilized enzymes was performed at various pre-incubation times and the corresponding relative activity was determined. The plot of log relative activity (%) versus time describes the first order thermodynamics[41] and depicted in supplementary material (Appendix E). The deactivation energy ($E_d$) of free and immobilized enzymes were determined from the slopes of the linear curve plotted between $\ln(k_d)$ and $1/T$ (figure 3a). The $E_d$ for free $\alpha$-amylase, CSME and CSZE are found to be 15.52, 18.71 and 23.49 KJ mol$^{-1}$ respectively. Higher the $E_d$ values for immobilized enzymes described that more energy was required for thermal deactivation and provide them higher stability towards denaturation compared to free enzyme. Since the thermal stability of the enzyme is the important parameter for industrial applications, the higher $E_d$ values as a result of immobilization made them as a potential candidate [42]. The increased $E_d$ value of $\alpha$-amylase was observed when modified with bovine serum albumin [43].
3.6. Estimation of Thermodynamic Parameters

The enthalpy change (ΔHº) at 50 ºC for free enzyme was found to be 12.83 KJmol⁻¹ and for immobilized enzymes, CSME and CSZE was 16.02 and 20.81 KJmol⁻¹ respectively (table 2). The high ΔHº values of immobilized enzymes showed that more energy is required for the thermal denaturation of enzyme as a result of immobilization [44]. It is clear that in both cases the values were decreased with the rise of temperatures indicated that lesser amount of energy is needed to denature the enzyme at high temperatures, but the decrease is more for immobilized enzymes. There is considerable conformational change caused by the enzyme at higher temperatures which leads to the decrease of enthalpy change [45].

The Gibbs free energy change (ΔGº) for thermal deactivation at 50ºC of free enzyme, CSME and CSZE are 104.59, 107.05 and 109.20 KJmol⁻¹ respectively and are given in the table 2. The high ΔGº value of immobilized enzymes confirmed that the thermal unfolding at high temperatures is controlled by the immobilization process and resistance towards the thermal denaturation measures the increase of their stability [46]. The ΔGº values describe the spontaneity of the thermal deactivation processes and the positive values indicate the non-spontaneous nature of the processes.

Table 2. Thermodynamic parameters for thermal deactivation study of free and immobilized enzymes at different temperatures.

| Temperature | Enzyme | ΔGº KJmol⁻¹ | ΔHº KJmol⁻¹ | ΔSº KJmol⁻¹ |
|-------------|--------|-------------|-------------|-------------|
| 50ºC        | FE     | 104.59      | 12.83       | -0.2839     |
|             | CSME   | 107.05      | 16.02       | -0.2817     |
|             | CSZE   | 109.20      | 20.81       | -0.2735     |
| 60ºC        | FE     | 107.41      | 12.75       | -0.2841     |
|             | CSME   | 109.83      | 15.94       | -0.2818     |
|             | CSZE   | 112.11      | 20.72       | -0.2743     |
| 70ºC        | FE     | 110.28      | 12.67       | -0.2844     |
|             | CSME   | 112.69      | 15.85       | -0.2822     |
|             | CSZE   | 114.67      | 20.64       | -0.2740     |

The entropy of deactivation is related to the thermal unfolding of the enzyme structure. The ΔSº values for both free and immobilized enzymes were found to be negative indicated the processes of their aggregation and more ordered state (table 2). But the magnitude of the value for immobilized enzyme is found to be lower as compared to the free from and the less negative values indicated their ordered state which might be raised as a result of immobilization. Due to the improvement in the ordered state, the stability of the enzyme is enhanced as it provides stronger intermolecular forces [47].

Table 3. Kinetic constants Kᵥ and Vmax of free and immobilized α-amylases, calculated from Lineweaver- Burke plots. The enzymatic reaction was carried out at optimum pH and temperature with varying starch concentration from 0.1 to 1 mg/mL.

| Starch (mg/mL) | Free enzyme | CSM | CSZ |
|---------------|-------------|-----|-----|
| Kᵥ (mg/mL)    | 0.45±0.02   | 0.65±0.03 | 0.5±0.04 |
| Vmax (μmol mg⁻¹ min⁻¹) | 34.48±0.05 | 16.39±0.04 | 23.81±0.02 |

3.7. Kinetic Constants

Kinetic constants of the Michaelis-Menton equation, Km and Vmax for free and immobilized α-amylases were determined by varying the concentration of starch (0.2 to 1mg/mL) in the enzymatic reaction and the results are presented in Table 3. Lineweaver-Burk plot for evaluation
of Km and Vmax for free and immobilized enzymes were shown in the figure 3b. Vmax indicates the intrinsic characteristics of the enzyme and Km is a measure of the substrate’s affinity for the enzyme. In our study, the Km values were increased for all immobilized forms compared to the free enzyme. The Km values were calculated for the immobilized enzymes, CSME and CSZE are 0.65 and 0.5mg/mL respectively, which are higher than that of free enzyme (0.45mg/mL). This can be interpreted that increase in Km value is due to the conformational changes of the immobilized enzymes, leads to the lower affinity for substrate compared to the free enzyme. Similar results were reported when α-amylase immobilized onto DEAE-cellulose [48]. The decrease in affinity towards substrate is caused by the structural changes introduced in the enzyme by immobilization procedure and hence resulted in the lower the accessibility of the substrate to the active sites of the immobilized enzyme[49]. On the other hand, Vmax measures the potential of the catalytic activity follows the opposite trend. As seen from the table 3, the Vmax for the free enzyme is calculated as 34.48 μmol mg\(^{-1}\) min\(^{-1}\) and for CSME and CSZE as 16.39 and 23.81μmol mg\(^{-1}\) min\(^{-1}\) respectively. As a result of immobilization Vmax decreases, the loss of activity may be due to the conformational changes of the enzyme on immobilization or steric hindrance of the enzyme molecule caused by diffusional limitation of the support [50].

3.8. Reusability and Storage Stability

Reusability is the most important advantage of an immobilized enzyme compared to the free enzyme, since it reduce the cost of enzymes for an enzymatic reaction. We investigated the reusability of the two immobilized enzymes maintaining a couple hours in each cycle. The relative activities of the immobilized enzymes for ten cycles of use at optimum conditions were shown in the fig.4a. The figure showed the decreasing tendency of the activity of immobilized enzyme with the reuses. The decreasing activity is caused by the denaturation tendency of the of the immobilized enzyme molecule upon use. The immobilized enzyme, CSZE maintained 90% activity after 5 runs and was about 60% of activity even after 10 cycles of uses and further washing. But CSME retain only 50% of activity after 10 runs. It is very easy to handle the immobilized enzyme, CSME in reuse experiments as it can be recovered by magnetic separation in each cycle because of its magnetic property.

The industrial application for enzymes requires that they could be maintained for long periods. In order to compare the efficiency in storage, the free and immobilized enzymes were stored in buffer at 4\(^°\)C. The free enzyme lost all of its activity within 2 days, while the immobilized enzymes retained activity more than that of free enzyme [9]. The results are depicted in the figure 4b. After storage of 6 months CSZE retained about 60% of its initial activity, while that of CSME was 50%. The loss of activity of the immobilized enzyme, CSME may be caused by the toxic influence of iron ions [40].

![Figure 4](image-url)
4. Conclusions

Chitosan-metal oxide composites, CSM and CSZ were synthesized, characterized and subjected to α-amylase immobilization. In our study the enzyme loading ability of CSZ composite found to be more than that of CSM composite. This is attributed to the higher surface area of CSZ compared to the CSM composite. Because of the magnetic property, CSM composite provides us with the best support for high speed and gentle separation. Both of the immobilized enzymes exhibited good thermal stability compared to the free enzyme. The thermal deactivation study followed first order kinetics and the deactivation energy, Ed was found to be higher for immobilized enzymes. Thermodynamic parameters $\Delta H^\circ$, $\Delta G^\circ$ and $\Delta S^\circ$ were evaluated and revealed that the enhanced stability at high temperatures is due to the immobilization process. The kinetic study showed that the lower substrate affinity of the immobilized enzymes in enzymatic reaction compared to the free enzyme attributed to the conformational change of native enzyme upon immobilization. Reuse assay and studies on pH, temperature and storage stabilities confirmed that the immobilized enzymes are suitable for many industrial applications.

Acknowledgements

The author is thankful to UGC for financial support.

Supplementary Data

Appendix A

FT-IR Spectrum of chitosan, CSM and CSZ

Appendix B

Thermograms of chitosan, CSM and CSZ

Appendix C

1. XRD Spectrum of CSM composite

2. XRD Spectrum of CSZ composite
54 Parameters Affecting the Improvement of Properties and Stabilities of Immobilized \(\alpha\)-amylase on Chitosan-metal Oxide Composites

### Appendix D

1. TEM image of CSM composite

![TEM image of CSM composite](image)

2. TEM image of CSZ composite

![TEM image of CSZ composite](image)

### Appendix E

1. Effect of initial protein amount on protein loading.

![Graph showing protein loading vs initial protein amount](image)

2. Effect of initial protein concentration on immobilized enzyme activity.

![Graph showing enzyme activity vs initial protein amount](image)

### Appendix F

1. First order of thermal deactivation of free enzyme.

![Graph showing log of relative activity vs time](image)
2. First order of thermal deactivation of CSME

![Graph showing first order of thermal deactivation of CSME](image)

3. First order of thermal deactivation of CSZE

![Graph showing first order of thermal deactivation of CSZE](image)

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