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Research Article

# A Novel Support for Immobilization of Horseradish Peroxidase: Its Future Aspects in Biosensor Applications

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## Abstract

Several new types of carrier and technology have been implemented in the past to improve traditional enzyme immobilization with the aims to enhance enzyme loading, activity and stability in order to decrease the cost of enzyme in industrial processes and in sensing technology. Thus, the present study aimed to work out a simple and high yield procedure for the immobilization of HRP on a novel chitosan-β-cyclodextrin complex. TGA and molecular modeling were performed to confirm the formation of β-CD-chitosan complex as did not described in our previous study. The molecular modeling study of the complexation of chitosan to β-CD shows the formation was stabilized through hydrogen bonding. A comparative stability of soluble and crosslinked enzyme preparation was investigated against pH, temperature and chaotropic agent like urea, heavy metals and detergent. Crosslinked peroxidase was significantly more stable as compared to soluble enzyme even though the optimum pH and temperature. The reusability of immobilized enzyme preparation was also investigated and it was found that even after the five repetitions use, the chitosan-β-CD bound HRP retained 95% of the original activity.

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## 1. Introduction

Biotechnology is considered as a useful alternative to conventional process technology various industrial, biochemical and analytical fields. Over last few decades, intense research in enzyme technology has provided many approaches that facilitate their practical applications in various industrial, biochemical and analytical fields [1, 2]. Among them, new technological development in the field of immobilized biocatalyst offers the possibility of a wider and more economical exploitation of biocatalyst in industry, medicine, waste treatment and in the development of bioprocess monitoring devices like biosensors [3-5]. The selected procedure of immobilizing enzyme should be able to stabilize the macromolecules to allow for easier diffusion of substrates and products [6, 7]. However, only a few immobilization methods can control the spatial distribution of the catalyst. Therefore, stabilization of enzymes against several physical and chemical denaturants can be accomplished using a multitude of immobilization strategies which include covalent coupling, adsorption, microencapsulation, polymer entrapment and chemical aggregation [8-10]. Currently, affinity immobilization techniques are based on biospecific molecular interactions given much attention to researcher in the field of enzyme technology. Thus, they are extremely powerful tools for the immobilization of valuable biological macromolecules, especially the enzymes and proteins [11]. Lectins are proteins or glycoproteins, which display specificity for certain structural features of oligosaccharides. The ability of lectins to bind glycoconjugates specifically facilitates the immobilized of the target compounds from a complex mixture in a single step.

Cyclodextrins (CDs) are cyclic compounds consisting of six, seven, or eight α-D-glucopyranose units connected by α-(1-4) linkages commonly referred to as α-, β-, and γ-CDs, respectively [12]. CDs possess a characteristic toroidal shape with a well-defined lipophilic cavity and a hydrophilic exterior that is suitable for inclusion and binding of appropriate sized guest compounds. CDs are of interest, in part, because of their ability to form stable inclusion complexes in aqueous solution as well as in solvents [13]. These macromolecules (CDs), which can be spatially represented as a torus with and narrow openings corresponding to secondary and primary hydroxyl

groups respectively, can encapsulate a large variety of compounds due to the hydrophobic character of their internal cavity [14,15].

In this paper, we report the feasibility of a novel support, β-CD-chitosan (β-CD-chitosan) complex for the immobilization of horseradish peroxidase (HRP). Molecular modeling study was also performed to understand the possible binding conformation of chitosan and β-CD complex. A comparative stability study of soluble and surface immobilized peroxidase on chitosan and β-CD complex has been carried out against various physical and chemical denaturants. Immobilized HRP preparations have also been studied for their reusability towards the development of biosensor in future.

## 2. Materials and methods

### *2.1. Materials*

The β-cyclodextrin, chitosan, dimethyl formamide (DMF), horseradish peroxidase (HRP), *o*-dianisidine HCl, cadmium chloride ($CdCl_2$), mercuric chloride ($HgCl_2$), Tween 20, bovine serum albumin, glutaraldehyde and ethanolamine were procured from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents employed were of analytical grade and were used without any further purification.

### *2.2. Preparation of β-CD-chitosan complex*

The β-CD-chitosan complex was prepared according to the method as described in our previous published research article [16]. First, an amount of 0.325 mmol of β-CD was stirred until it dissolved in 20 ml of distilled water. Then, chitosan was added to the mixture with constant stirring to form a chitosan-β-CD complex solution (1:1 ratio). The resulting solution was then centrifuged, freeze-dried [16].

### *2.3. Immobilization of HRP on the surface of complex and crosslinked by glutaraldehyde*

The obtained chitosan-β-CD complex was dissolved in buffer of varying pH at 4 $^oC$ with different units of HRP to achieve best chitosan-β-CD-peroxidase ratio for the immobilization. The buffers used were sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0-8.0) and Tris-HCl (pH 9.0 and 10.0). The mixture was homogenized at 150 rpm for 20 min at 4 $^oC$. The resulting solution was then centrifuged at 3000 rpm for 15 min. The filtrate was freeze-dried for 24 h. This chitosan-β-CD-HRP complex preparation was stored at 4 $^oC$ for further used.

The chitosan-β-CD-HRP complex at pH 8.0 was crosslinked with 0.5% glutaraldehyde for 1 h at 4 $^oC$. Glutaraldehyde treated adsorbed complex was incubated with 0.01% (v/v) ethanolamine for 90 min at 4 $^oC$ in order to stop crosslinking. Excess ethanolamine was removed by centrifugation and crosslinked chitosan-β-CD-HRP complex was washed thrice [16]. Finally crosslinked chitosan-β-CD-HRP complex was suspended in 100 mM sodium phosphate buffer, pH 8.0 and stored at 4 $^oC$ for further use.

### *2.4. Molecular modeling of chitosan-β-CD complex*

The atomic coordinates of the starting molecules of β-CD and chitosan monomer were retrieved from Cambridge structural database [17,18]. Each of the single molecules was fully optimized using semi-empirical PM3 method available in Gaussion03 software package [19]. Ligand partial charges were assigned based on Kollman charges and a grid map of 40 x 40 x 40 $Å^3$ grid dimension and spacing of 0.375 Å were generated. The molecular docking was performed by employing Lamarckian genetic algorithm with pseudo-Solis and Wets local search method available in Autodock4 [20]. The docked chitosan-β-CD with the best energy score were carefully analyzed and since lower affinity energy indicate stronger binding, the conformation which have the lowest energy were chosen and fully optimized with PM3 using Gaussian03 to obtain the structure with the lowest binding energy.

### *2.5. Conformation of chitosan-β-CD complex*

Thermogravimetric analysis (TGA) was used to examine the freeze-dried mixture using a Nicolet 5700 with TGA/FT-IR interface (Thermo Electron Corporation, UK.). The TGA temperature axis was calibrated using the ferromagnetic material supplied by Perkin Elmer. Samples were heated at 10 °C $min^{-1}$ atmospheric condition.

All enzymatic reactions were stopped in a boiling water bath for 20 min. Insoluble product was removed by centrifugation at 3000 rpm for 15 min. Absorbance were recorded at JASCO V-530 U.V-visible spectrophotometer. The freeze drying was performed by LABCONCO freeze dry, shell freeze system, freezone 12, Germany.

### *2.6. Effect of ion concentration, pH, temperature, urea, metals and detergent*

Crosslinked chitosan-β-CD-HRP complex preparation was incubated with 1.0 M NaCl for varying time interval in 100 mM sodium phosphate buffer, pH 8.0 at 40 $^oC$ to examine desorption of bound enzyme from complex. The remaining percentage of activity on support was calculated by taking the activity of chitosan-β-CD-HRP complex without exposure to NaCl as control (100%).

Appropriate and equal amounts of soluble and crosslinked chitosan-β-CD-HRP complex (2.0 U each) were taken to determine the activity of peroxidase in the buffers of different pH. The buffers used were glycine–

HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0 and 5.0) and Tris–HCl (pH 6.0–10.0). The activity at pH-optimum was considered as control (100%) for the calculation of percent activity at other pH.

The activity of soluble and crosslinked chitosan-β-CD-HRP complex (2.0 U) was determined at various temperatures (30–80 °C) in 100 mM Tris-HCl buffer, pH 8.0. The activity at temperature-optimum was considered as control (100%) for the calculation of percent activity at other temperatures.

In another set of experiment, soluble and crosslinked chitosan-β-CD-HRP complex (2.0 U) preparations were incubated at 60 °C for varying time intervals in 100 mM Tris-HCl buffer, pH 8.0. After each incubation period the enzyme was quickly chilled in crushed ice for 5 min. The enzyme was brought to room temperature and the peroxidase activity was measured. The activity without incubation at 60 °C was taken as control (100%) for the calculation of remaining percentage of activity.

Soluble and crosslinked chitosan-β-CD-HRP complex (2.0 U) were incubated with 4.0 M urea for varying times in 100 mM Tris-HCl buffer, pH 8.0 at 40 °C. Peroxidase activity was determined at intervals. The activity of enzyme without incubation with urea was taken as control (100%) for the calculation of remaining percent activity.

Both preparations were incubated independently with $HgCl_2$ and $CdCl_2$ (0.01–0.1 mM) in 100 mM Tris-HCl buffer, pH 8.0 at 40 °C for 1 h. The activity of enzyme without exposure to heavy metal was taken as control (100%) for the calculation of remaining percent activity.

Soluble and prepared complex in same quantity as described in previously were incubated with Tween 20 (0.5–5.0%, v/v) in 100 mM Tris-HCl buffer, pH 5.0 at 40 °C for 1 h. The activity of enzyme without Tween 20 was taken as control (100%) for the calculation of remaining percent activity.

### *2.7. Reusability of chitosan-β-CD bound HRP*

Chitosan-β-CD bound peroxidase was taken in triplicates for assaying the peroxidase activity. After each assay the immobilized enzyme preparations were taken out, washed and stored in 100 mM Tris-HCl buffer, pH 8.0 overnight at 4 °C. The activity was assayed for 5 successive days. The activity determined for the first time was considered as control (100%) for the calculation of remaining percent activity after each use.

### *2.8. Measurement of peroxidase activity*

Peroxidase activity was estimated from the change in optical density ($A_{460}$ nm) at 40 °C by measuring initial rate of oxidation of *o*-dianisidine HCl (18 mM) by $H_2O_2$ (6.0 mM). The assay mixture with immobilized BGP was continuously stirred for the entire duration of assay [21]. One unit (1.0 U) of peroxidase activity was defined as the amount of enzyme protein that catalyzes the oxidation of 1.0 mol of *o*-dianisidine HCl per min at 37 °C.

### *2.9. Statistical analysis*

Each value represents mean of three independent experiments performed in duplicates, with average deviations, < 5%. Data expressed in various studies was plotted using Sigma Plot-10.0 and Microsoft Excel 2003. *P*-values < 0.05 were considered statically significant.

## 3. Results and Discussion

### 3.1. Molecular modeling studies

In order to further understand the molecular recognition that takes place at the atomic level between β-cyclodextrin and chitosan, a molecular modeling study were conducted to provide further insight into the interactions as well as the determine the preferred binding orientations between cyclodextrin and chitosan. The binding energy of the inclusion complexes is defined by Eq 1.

Binding energy,

$$[BE] = E\,[\beta\text{-CD/chitosan}] - E\,[\beta\text{-CD}] - E[\text{chitosan}] \quad (1)$$

Where, E [β-CD/chitosan], E [β-CD] and E [chitosan] represent the total energies of the complex, the free chitosan and the free β-CD, respectively. The more negative value of the binding energy corresponds to the most stable conformation. Our calculations show that β-cyclodextrin is a better host for chitosan than alpha and gamma CDs (**Table 1**). The inclusion took place inside the cavity of the cyclodextrin and the results are consistent when we repeat the calculation using the single monomer and two monomer of chitosan. The driving forces of these types of complexation are due to hydrogen bonding interactions between the hosts (CDs) and the guest molecule. It appears that the recognition is predominantly due its molecular size and shape while maximizing the hydrophobic interaction within the CD cavity. The structure of the lowest energy conformation for the chitosan/β-CD complex and the five O—H···O type hydrogen bondings formed to stabilize the complex is shown in **Fig. 1.**

### 3.2. Confirmation of chitosan-β-CD binding

The TGA thermograms of chitosan, β-CD and the complex are presented in **Fig. 2.** The thermograms of the pure and complexed compound show two decomposition

**Table 1** The energies of the most stable chitosan-CDs complexes

| Host | E (CDs) kcal/mol | E (GlcN) kcal/mol | E complex kcal/mol | BE kcal/mol |
|---|---|---|---|---|
| alpha | -1243.72 | | 1468.61 | -2.53 |
| beta | -1455.58 | -222.36 | -1690.3 | -12.34 |
| gamma | -1668.14 | | -1891.58 | -10.08 |
| | | E (GlcN-GlcN) kcal/mol | | |
| alpha | -1243.72 | | -1636.4 | -4.75 |
| beta | -1455.58 | -388.17 | -1849.18 | -5.43 |
| gamma | -1668.14 | | -2059.01 | -2.73 |

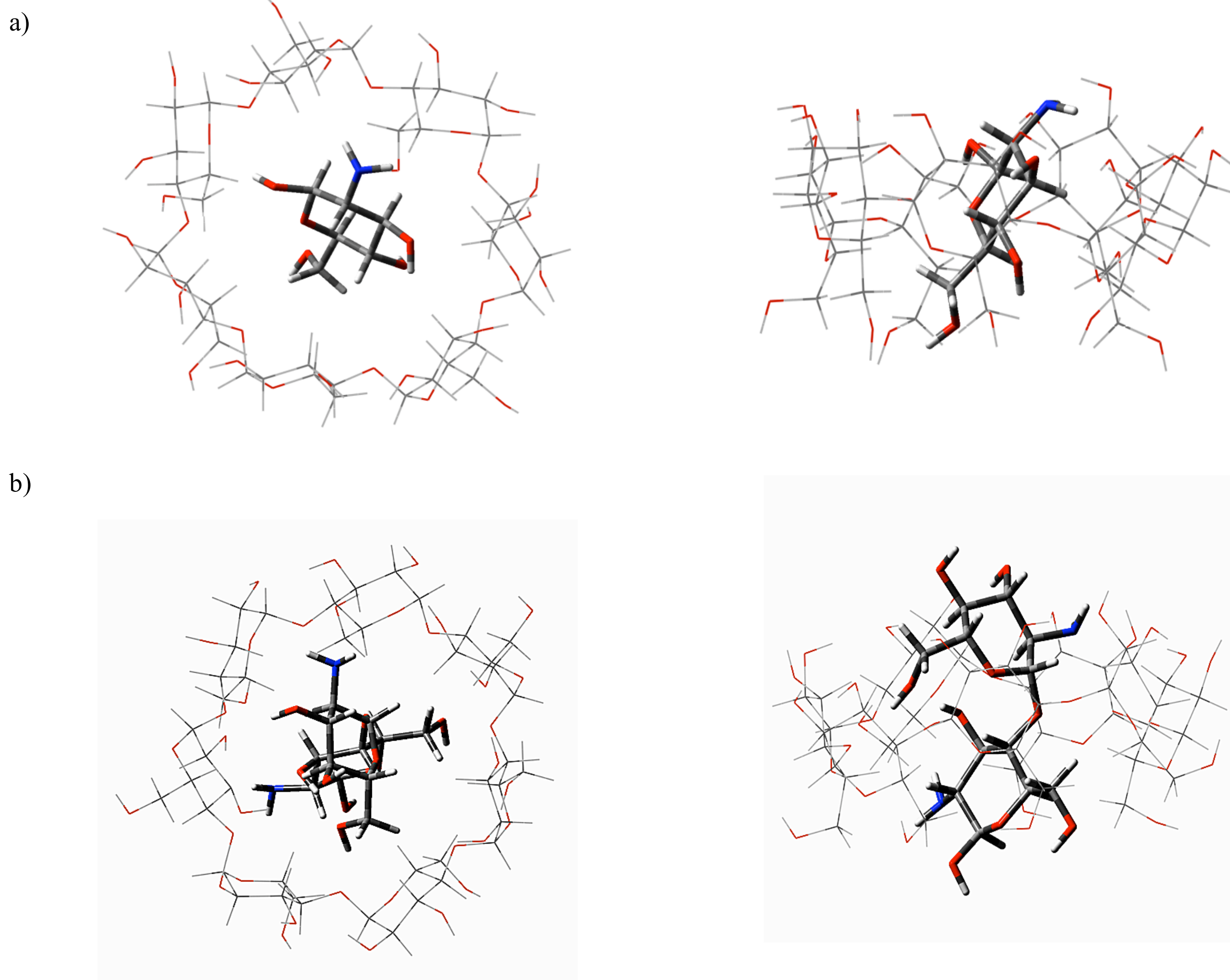


**Figure 1** Energy minimized structures obtained from PM3 calculations seen from the top view and from the side wall of β-CD for the (a) *Glc-N*/β-CD complex (b) (GlcN-GlcN)/β-CD complex.

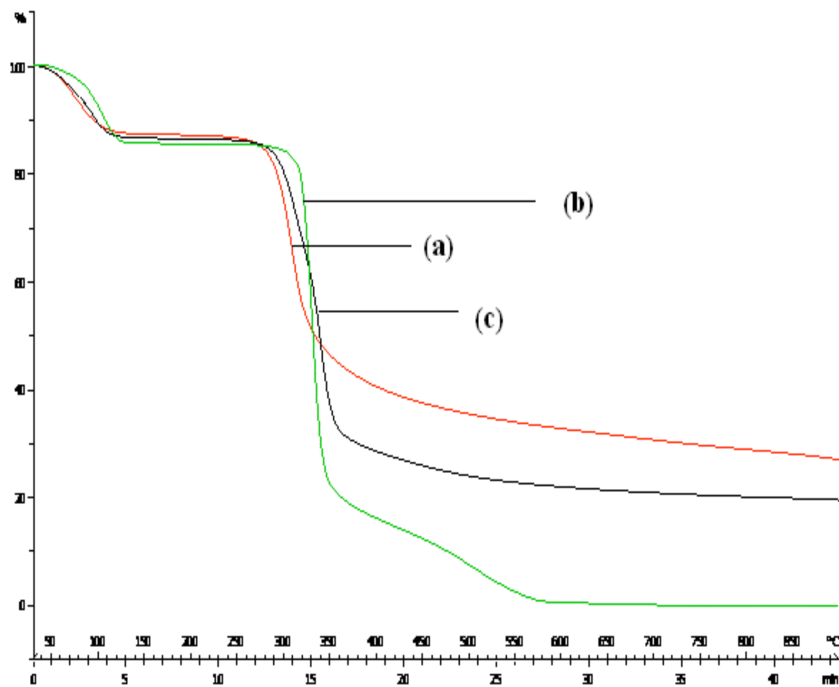


**Figure 2** TGA of (a) chitosan (b) β-CD (c) chitosan- β-CD complex

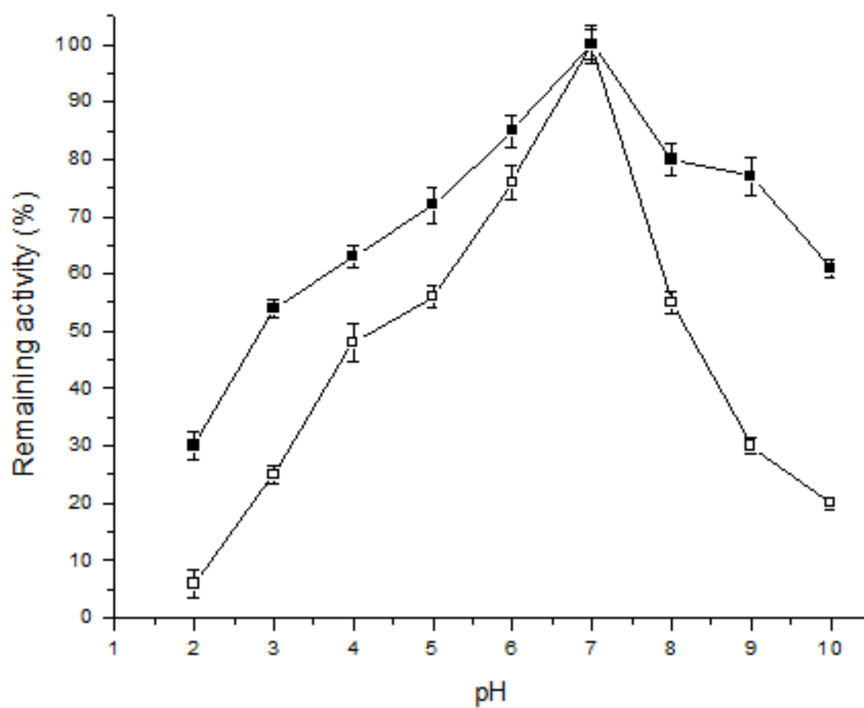


**Figure 3** The pH activity profile of soluble (□) and crosslinked HRP enzyme (■) preparation.

steps where the early decomposition which took place at 30 - 180 °C is due to the evaporation of hydrated water molecules existing in the chitosan polymeric structure and in the case of cyclodextrin, the water present within the cavity of the CD. At higher temperature, between 250 to 800 °C, the weight loss was due to decomposition of chitosan and β-CD. The thermal analysis results also shows that the complex is thermally more stable than the pure chitosan as the decomposition temperature had increased from initially around 300 °C to 340 °C for the complex.

### 3.3. Preparation of chitosan-β-CD-HRP complex

The chitosan-β-CD complex has been exploited for immobilization of peroxidase from horseradish. Binding of HRP on chitosan-β-CD was performed as described in our previous study [22]. In this case, the yield of immobilization was quite superior over other methods used for the immobilization of peroxidases [23-26]. The maximum binding of peroxidase on chitosan-β-CD complex was at pH 8.0. Several earlier workers have also reported the pH-dependent adsorption of other enzymes on various supports [26, 27].

The wastewaters may also contain several types of ions; therefore it was necessary to examine the detachment of HRP from chitosan-β-CD matrix in the presence of various concentrations of ions. The exposure of adsorbed enzyme with increasing concentration of NaCl (0.1–1.0 M) for 1 h exhibited retention of very high enzyme activity even in the presence of 1.0 M NaCl. The incubation of immobilized HRP upto 0.5 M NaCl for 1 h had no detachment of enzyme activity. In order to monitor the effect of long exposure of immobilized enzyme with ions, the adsorbed HRP was incubated with 0.1 M NaCl upto 24 h. The incubation of immobilized enzyme with 0.1 M NaCl for 24 h resulted in a slight loss of only 2% of the initial activity (data not given). These observations suggested that the binding of HRP with chitosan-β-CD was quite strong and such type of immobilized enzyme preparations can be easily exploited for its use in the treatment of wastewater containing aromatic pollutants. Several earlier investigators have also reported the use of various other adsorbents for high yield and stable immobilization of enzymes and proteins [22, 28, 29].

### 3.4. Stability properties of soluble and chitosan-β-CD bound HRP preparations

The stability of soluble and chitosan-β-CD bound HRP preparations was monitored against various physical and chemical parameters because these parameters can affect the activity of the enzymes used for the treatment as well as monitoring of organic pollutants present in the wastewater.

Chitosan-β-CD bound HRP and the native enzyme showed similar optimum pH **(Fig. 3).** Immobilized enzyme retained significantly higher enzyme activity on both sides of the optimum pH in comparison to free enzyme. The optimum pH of immobilized enzyme had slight difference from pH 5.0 to 6.0, although soluble enzyme showed pH optimum at pH 7.0. Recently, Karim and Husain [21] performed a study for the immobilization of bitter gourd peroxidase (BGP) on fly ash. The broadening of pH profile was observed in the case of immobilized peroxidase. In another study, a group of scientist has shown the brinjal polyphenol oxidases (PPO) bound to Celite-545 had higher pH optima as compared to potato PPO [30]. In both studies the immobilized enzymes showed more stability as compare to soluble counterpart. One earlier study reported the entrapment of HRP in K-carrageenan bead, the free enzyme showed the optimum activity at pH 8.0 and the immobilized enzyme showed the maximum activity at pH 7.0 [31]. It could be concluded that every immobilized enzymes have their own optimum pH for their activity. As the enzyme is immobilized through chitosan-β-CD complex, it is not expected to observe any conformational changes. Therefore, no shift in pH towards acidic as well as alkaline sides on immobilization can be attributed due to the nature of β-CD polysaccharide.

Chitosan-β-CD bound HRP preparation had no change in temperature-optima as compared to its soluble counterpart. Both the preparations exhibited temperature optima at 40 °C. However, chitosan-β-CD bound HRP retained significantly greater fractions of catalytic activity at high temperatures **(Fig. 4).** Free enzymes suffer with poor thermal stability. Immobilization of enzymes generally improves thermal stability, which is desirable for the reactions to be carried out at elevated temperature. From the results illustrated in (Fig. 4), it is observed that entrapped enzyme has better thermal stability. This can be attributed to a protective sheath formed due to the chitosan acting as the support and the glutaraldehyde as cross-linker during immobilization, which helps in decreasing the thermal impact and hence deactivation effect. Some earlier workers also reported the same finding in the case of BGP immobilization on fly ash [21].

**Fig. 5** demonstrates the thermal denaturation of soluble and immobilized HRP at 60 °C for 5 h. Soluble HRP incubated at 60 °C for 5 h retained 25% of its initial enzyme activity while the immobilized enzyme incubated under similar conditions was significantly more stable to heat inactivation. The immobilized HRP exhibited 75% of the original activity after 5 h of heat treatment. These observations show that resistance of enzyme to high temperatures was greatly increased by immobilization. Chitosan-β-CD-peroxidase complex retained its structure and remarkably high activity at elevated temperatures. Therefore, such enzyme preparation could be exploited at relatively high temperatures. Improvement in thermal stability of this complex preparation may come from multipoint

attachment of peroxidases with chitosan or β-CD. This enhancement in thermal stability is due to formation of several linkages between enzyme and support [32, 33].

Chitosan-β-CD bound HRP was more resistant to inactivation induced by 4.0 M urea compared to its soluble counterpart. Exposure of soluble enzyme with 4.0 M urea for 2 h resulted in the loss of 70% activity whereas the immobilized enzyme retained more than 80% of the initial enzyme activity **(Fig. 6).** Urea (4.0 M) is a strong denaturant of some proteins and it irreversibly denatures enzymes [34, 35]. However, action mechanism of urea on protein structure has not yet been completely understood, several earlier findings have suggested that protein could be unfolded by direct interaction of urea molecule with a peptide backbone via hydrogen bonding and/or hydrophobic interaction, which contributes to preservation of protein conformation [36]. Complexing of glycoenzymes with lectin also resulted in an enhancement of their resistance to denaturation mediated by urea [37]. Thus, these observations indicate that chitosan-β-CD-peroxidase complex from urea has induced inactivation.

Unused detergents are normally present in the wastewater coming out of municipal waste. Somewhere such wastewater can mix with the effluents released by the industries. In order to make immobilized enzyme preparation more efficient for wastewater treatment and the monitoring of aromatic pollutants in wastewater, we have investigated the effect of Tween 20 on the activity of soluble and immobilized HRP. Soluble HRP was more sensitive to Tween 20 exposure and lost nearly 38% enzyme activity after 1 h incubation with 1.0% (w/v) detergent. Moreover, the immobilized HRP was markedly more resistant to inactivation induced by Tween 20 and retained over 97% of its initial activity **(Table 2).** Furthermore, (Table 2) demonstrates the effect of increasing concentration of (1.0–3.5%) Tween 20 on the activity of soluble and immobilized HRP. The soluble enzyme retained a marginal activity of 35% after 1 h exposure to 3.5 % Tween 20. However, the immobilized HRP exhibited 78% of the original activity under similar exposure conditions. Moreover, the immobilized HRP preparation was more resistant to denaturation induced by Tween 20 as compared to its soluble counterpart. In a study entrapped Con A-BGP could work quite efficiently in presence of contaminants like soaps and detergents. Immobilized peroxidases are reported to be significantly stabilized against denaturation induced by some house hold detergents containing Tween 20 [24, 37].

The immobilization of HRP on the surface of chitosan-β-CD bound HRP has presented a strategy to overcome the problem of diffusion limitation of the substrate/product and to increase the surface area of contact between enzyme and substrate so that such preparation could be exploited for monitoring of pollutants present in industrial effluent as well as for the treatment of large volume of industrial waste. The chemical contamination of water from a wide range of toxic compounds, in particular heavy metals, is a serious environmental problem owing to their potential human toxicity. In view of their presence in wastewater, it is important to examine the effect of some heavy metals on the activity of soluble HRP and immobilized HRP. Soluble HRP and immobilized HRP retained 57% and 78% activity in the presence of 0.1 mM $HgCl_2$, respectively whereas both enzyme preparations retains 61% and 87% activity, respectively with exposure of 0.1 mM $CdCl_2$ (Table 3).

Table 3 further demonstrates the loss of activity with increasing concentration of heavy metals. Metals induce conformational changes in enzymes; however peroxidases remain active even in the presence of a number of metal ions, as a part of their detoxifying role. Immobilized HRP has exhibited more resistant to inactivation induced by heavy metals. Some recent reports have indicated that HRP was remarkably inhibited by heavy metal ions [38, 39]. However, in this study the strength of inhibition of immobilized HRP by heavy metal ions was quite low as compared to the free enzyme **(Table 3)**. The stability of immobilized HRP against these heavy metals showed that such preparations could be exploited to treat aromatic pollutants even in the presence of heavy metals.

### 3.5. Reusability of immobilized HRP

Reusability of immobilized preparations of HRP has been shown in **Fig. 7**. After 5 cycles of repeated use, the chitosan-β-CD bound HRP retained 95% of the original activity. Enzyme reusability provides a number of cost effective advantages that are often an essential prerequisite for establishing an economically viable enzyme-catalyzed process. The activity loss during repeated use might be due to the inhibition of enzyme by product or by leaching of enzyme from the gel bead or due to damage of the beads [34, 40].

## Conclusion

The results presented in the present work showed that chitosan-β-CD-peroxidase complex was more stable as compared to soluble HRP against various types of denaturants. This preparation retained more activity in its immobilized form and this preparation has no problem of substrate and product diffusion. Thus, such immobilized enzyme preparation would be useful for the preparation of biosensors for the detection and monitoring of aromatic pollutants present in wastewater. These immobilized complex used in a reactor might be not affect the flow rate of the column. In view of these advantages offered by surface immobilized HRP preparation, we can suggest that this immobilized preparation would be most suitable for the treatment of huge volume of effluents/aromatic pollutants.

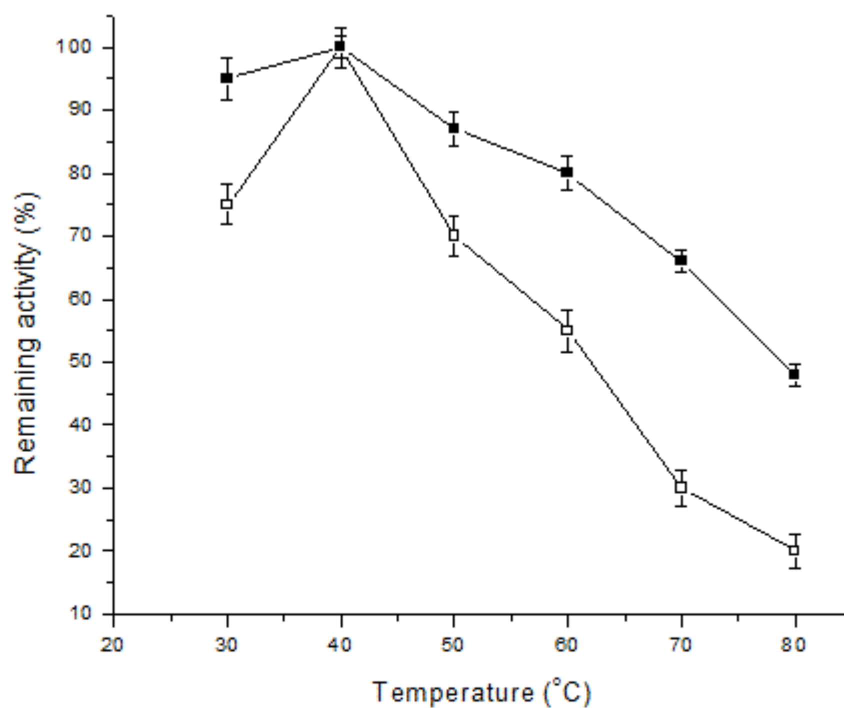


**Figure 4** Effect of temperature on soluble (□) and crosslinked enzyme (■) preparation.

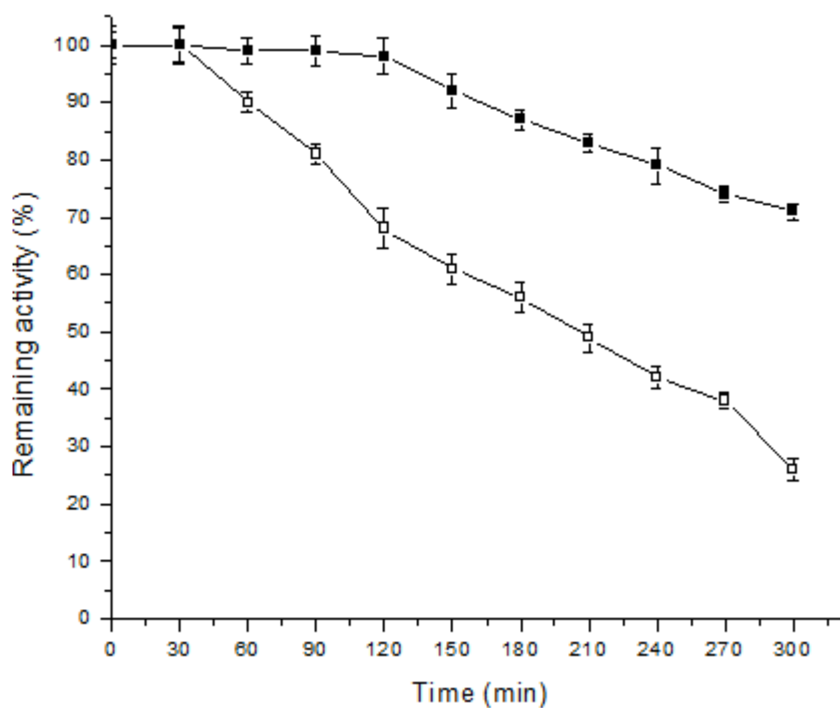


**Figure 5** Thermal denaturation profile of soluble (□) and crosslinked enzyme (■) preparations.

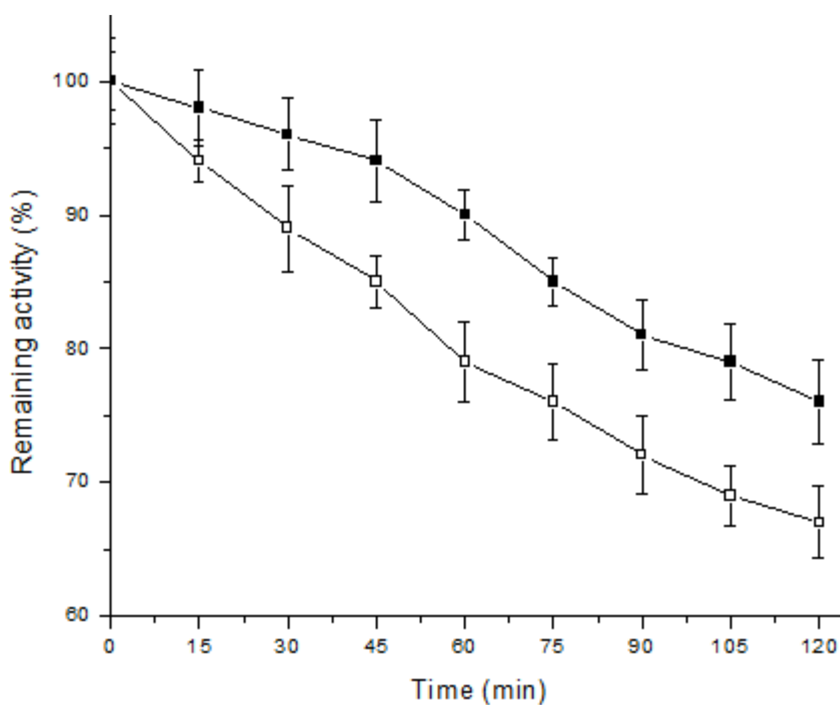


**Figure 6** Effect of 4 M urea on the soluble (□) and crosslinked enzyme (■) preparation.

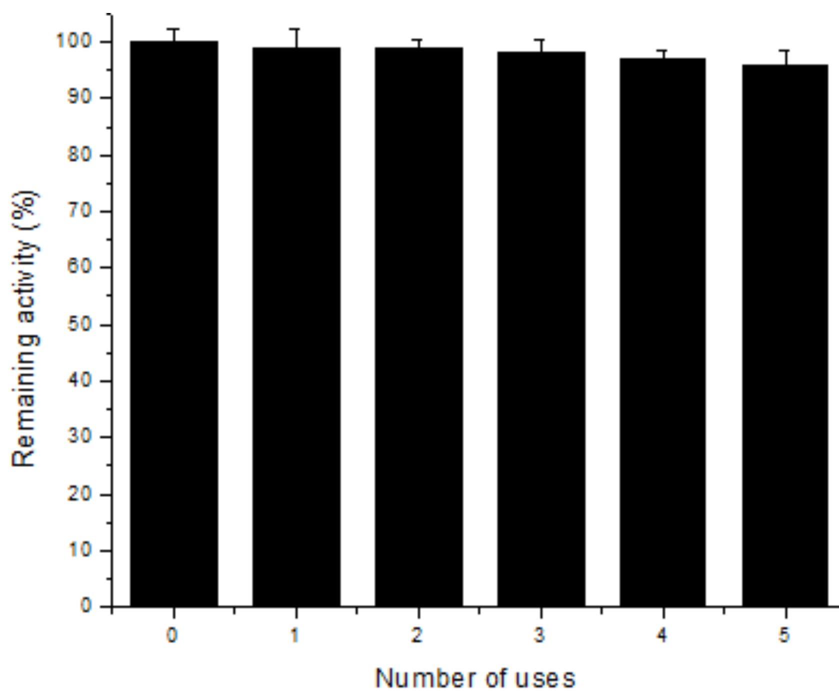


**Fig. 7:** Reusability of immobilized enzyme preparation.

**Table 2** Effect of detergent on the stability of soluble and immobilized HRP

| Tween 20 (%, v/v) | Remaining activity (%) | |
|---|---|---|
| | Soluble HRP | immobilized HRP |
| 1.0 | 62±2.7 | 97±3.1 |
| 1.5 | 54±2.8 | 94±2.9 |
| 2.0 | 49±1.6 | 92±2.4 |
| 2.5 | 44± 1.9 | 88±2.6 |
| 3.0 | 38±1.7 | 83±2.1 |
| 3.5 | 35±3.2 | 78±1.9 |

Soluble and immobilized HRP (2.0 U) were incubated with Tween 20 (1.0–3.5%. v/v) in 100 mM Tris-HCl buffer, pH 5.0 at 40 °C for 1 h. The activity of HRP without exposure to $HgCl_2$/$CdCl_2$ was taken as control (100%) for the calculation of remaining percent activity.

**Table 3** Effect of $HgCl_2$ and $CdCl_2$ on the soluble and immobilized HRP

| $HgCl_2$/$CdCl_2$ (mM) | Remaining activity (%) | | | |
|---|---|---|---|---|
| | $HgCl_2$ | | $CdCl_2$ | |
| | S-HRP | I-HRP | S-HRP | I-HRP |
| 0.01 | 82±2.2 | 97±3.1 | 85±3.1 | 98±2.1 |
| 0.02 | 77±1.7 | 95±2.4 | 79±2.6 | 98±1.3 |
| 0.04 | 73±2.3 | 93±2.8 | 76± 2.1 | 97±1.5 |
| 0.06 | 68±3.2 | 87±1.5 | 72±2.8 | 96±1.5 |
| 0.08 | 63±1.5 | 83±.4 | 65±2.9 | 92±1.8 |
| 0.10 | 57±3.2 | 78±1.9 | 61±2.5 | 87±1.9 |

Soluble (S-) and immobilized (I-) HRP (2.0 U) were incubated with $HgCl_2$ and $CdCl_2$ (0.01–1.0 mM each) in 100 mM Tris-HCl buffer, pH 5.0 at 40 °C for 1 h. The activity of HRP without exposure to $HgCl_2$ and $CdCl_2$ was taken as control (100%) for the calculation of remaining percent activity.

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