CHAPTER - 4

IMMOBILIZATION OF PEROXIDASE

CONTENTS

4.1 Properties of horseradish peroxidase

With few exceptions the typical peroxidase (EC 1.11.1.7) belonging to group is haemoproteins. Haemoprotein peroxidase occurs in animals and higher plants, e.g. horseradish, pineapples, potatoes, legumes, corn, root vegetables, tobacco plants, yeast, moulds and in bacteria.[1-4] The widely studied peroxidase is horse radish peroxidase (HRP). In mammals peroxidase occurs in leucocytes, liver, spleen, lung, uterus, thyroid glands,[5-10] etc. They have been found in mammalian cells in the supernatant, microsomal and mitochondrial fractions.

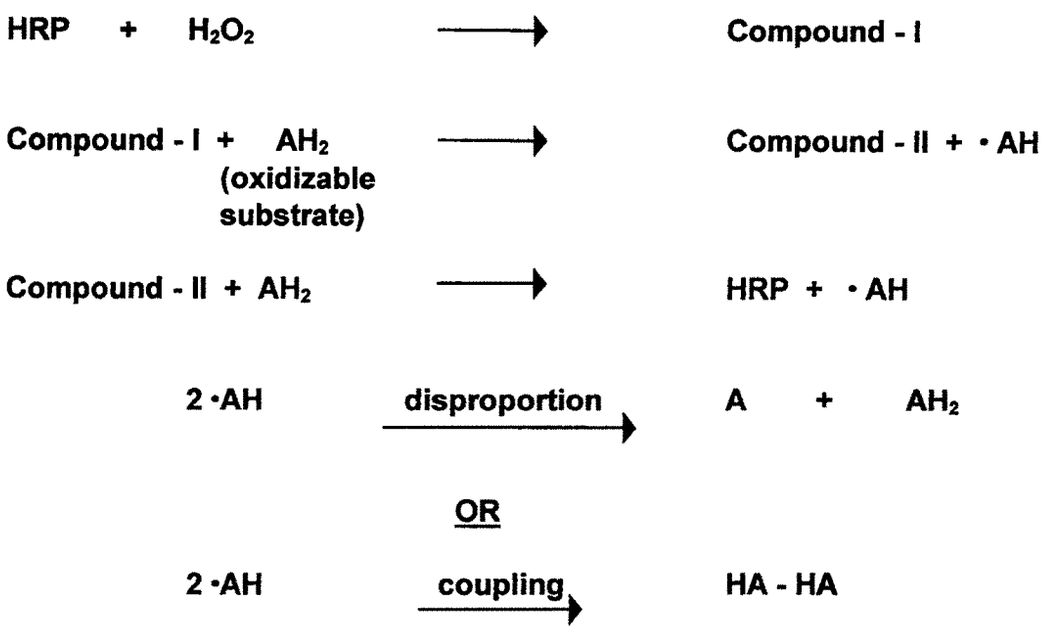
Molecular weight of HRP is 40,000 daltons and its isoelectric point is 7.2. Optimum pH for activity is observed to be 7.0. HRP can be reversibly inhibited by cyanide and sulfide at a concentration of 10^{-5} M. The enzyme exhibits a high specificity towards hydrogen peroxide (H_2O_2). The enzyme is quite stable, and as lyophilized dry powder, it can be stored for several years at low temperature. An aqueous solution of purified peroxidase can maintain its activity practically undiminished for over a year at 5 °C.

Other well known peroxidases that have been characterized are cytochrome C peroxidase, glutathione peroxidase and myeloperoxidase.

Peroxidase catalyses the oxidation of a large number of organic compounds such as phenols and aromatic amines, hydroquinoes and hydroquionid amines, especially benzidine derivatives. Peroxidase can be determined by the decrease of H_2O_2 or the hydrogen donor or the formation of the oxidized compound.[11] Usually the later method is employed for the catalytic activity determination of peroxidase.

HRP catalyzes the oxidation of aromatic compounds by H_2O_2 through an oxidation process that involves a cycle of changes in the oxidation state of an

iron atom located at the catalytic site of the enzyme.[12] The catalytic cycle is shown below :



In the catalytic cycle, the native HRP is oxidized by H_2O_2 and passes through two intermediate oxidation states, designated as compound - I and compound - II before returning to the native form. During this process the intermediate enzyme form reacts with aqueous aromatic oxidizable substrates (AH_2) resulting in the formation of phenoxy radicals (AH). Thus, the oxidation proceeds in one electron step with the final formation of product occurring by disproportion or coupling of the one electron oxidized intermediate.

4.2 Literature survey

The immobilization of enzyme at the interface in a heterogeneous system is currently an active research area in biotechnology. Immobilized enzymes exhibit increased stability and enable a continuous conversion process with good product recovery and minimal loss of enzyme activity. The conventional methods

of enzyme immobilization include physical adsorption, entrapment and covalent binding of enzymes on various supports.

Bartling and coworkers[13-17] immobilized HRP covalently onto various supports for the synthesis of quinone and benzoquinone derivatives. They prepared cellulose acetate hollow fiber of 0.4 mm inner diameter activated using CNBr and poly(4-methacryloxy benzoic acid) support activated using N-ethoxycarboxyl-2-ethoxy-1,2,dihydroquinoline. No significant change in the values of Michaleis constants for free and immobilized enzymes was reported. The polymer-enzyme complex had better thermal and storage stability Immobilization of HRP in methylene chloride using N-N'carboxyldiimedazol cross-linking agent was also carried out by them. The immobilized HRP showed better stability towards denaturation in the aqueous mixtures of methanol, ethanol and 1,4 dioxane.

Schell et. al.[18] immobilized HRP on CNBr activated cellulose a commonly used natural support. They observed that the optimum pH for the immobilized enzyme activity remained unchanged, and enzyme was used several times without loss in activity. Storage of the immobilized HRP at room temperature for two weeks caused the initial activity to decrease by half.

HRP was immobilized onto natural and synthetic polymeric supports by Berezin and coworkers[19-20] using two different techniques. HRP was bound covalently to AH-sepharose 4B through the enzyme carbohydrate residues. They observed that upon immobilization the K_m value did not altered, but the V_{max} value was decreased by 2 fold when o-dianisidine was used as substrate. They also immobilized HRP by entrapping into poly(AAm) using bis-AAm cross-linking agent, and observed decrease in the thermal stability of entrapped enzyme than free enzyme. However, by increasing ionic strength of the solution from 0.01 to 1.0 increase in the thermal stability of immobilized HRP at 64 °C was reported.

Similar results were obtained by Ugarova et. al.[21,22] when they entrapped HRP in poly(AAm) gel. They observed that the value of k_m for immobilized enzyme was very close to free enzyme, while the value of V_{max} decreased by 3 fold at pH 7.0. The thermostability of immobilized HRP was also decreased by 3 fold and 17 fold at 20 and 56 °C respectively. To increase the thermostability of immobilized HRP they first cross-linked HRP with glutaraldehyde and then entrapped into poly(AAm) gel, and observed that thermostability of immobilized enzymes depends on the concentration of cross-linking reagent used. However, they obtained maximum entrapped enzyme activity in 40% poly(AAm) using 10% cross-linking agent bis-AAm, previously cross-linked enzyme with 9% glutaraldehyde.

The preparation and properties of HRP covalently bound to diazotized polyamino styrene beads was reported by Miller et. al.[23,24] Using homovanillic acid as substrate they observed that immobilized HRP lost 90% of its initial activity after storage of 30 days. They used polystyrene beads containing Immobilized enzyme in a packed bed reactor of glass coil for the detection of low concentration of H_2O_2 in the solution having concentration ~ 1 ng L^{-1}.

Coupling of HRP to CNBr activated cellulose was done by Cremonesi et. al.[25,26] They obtained quantitative coupling of HRP within 40 minutes of coupling time at pH 7.0. However, after only two cycles, immobilized HRP lost its activity completely. Properties of HRP and other enzymes covalently immobilized onto diazotized m-diaminobenzene were studied by Gray et. al.[27] They studied the effect of sorbitol on the free and immobilized enzyme and observed that sorbitol has more protective effect for thermostability of immobilized HRP at 60 °C in phosphate buffer of pH 8.0.

A method was developed whereby peroxidase was covalently coupled to polystyrene by Chin and Lanks.[28] The coupling was reported to be effective only

when nitration of polystyrene with HNO_3 - H_2SO_4 was followed by reduction with Na hydrosulfite and simultaneous incubation of HRP.

Peroxidase covalently coupled to aminated porous glass in the presence of $NaBH_4$ was reported to be suitable for automatic serum analysis by Kikutake[29]. They observed maximum loading of 11.7 mg peroxidase per g of support and storage stability was 100% after 4 months at 5 °C.

Covalent coupling of HRP was also reported by Epton et. al.[30,31] using poly(acryloyl-morpholine) gel network and porous spherosil beads. They reported quantitative coupling of 2.9 and 0.8 mg enzyme per g of supports. They also studied the effect of THF in pH 6.0 for free and immobilized enzymes activity and observed that immobilized enzymes retained better activity than equivalent amount of free enzyme.

Adalsteinsson et. al.[32] also covalently bound HRP and other enzymes to poly(AAm) prepared by free radical polymerization using bis-AAm as cross-linking agent in the presence of enzyme protecting reagent such as dithiothreitol. They observed that immobilized enzyme activity depends critically on monomer concentration and cross-linking reagent used and obtained approximately 90%. of enzyme loading by optimum coupling conditions.

Polymethylglutamate (PMG) a synthetic polypeptide was used as a carrier to immobilize peroxidase and other enzymes by Minamoto.[33] The enzymes immobilized through covalent coupling to PMG coated glass beads retained more than 90% enzyme activity. Quantitative coupling of 23 mg g^{-1} of PMG was reported by them. They observed improved thermostability of immobilized peroxidase due to increase in hydrophilicity of the polymer and the multipoint binding of enzyme by covalent and ionic bonding.

D'Angiuro and coworkers[34-36] synthesized graft copolymers of natural polysaccharide such as cellulose, sepharose, sephadex and starch using glycidyl methacrylate monomer for immobilization of HRP either by *insitu* entrapment or by covalent binding. Identical values of K_m and V_{max} for the free and immobilized enzyme indicate that the immobilization process did not altered the active site of enzyme. Nagy and Horkay[37] immobilized peroxidase onto various copolymers of vinyl alcohol with vinyl ether, vinyl ester and vinyl acetal. They reported that in all cases immobilized peroxidase retained 30 - 40% of its initial activity.

Nilsson and coworkers[38,39] immobilized HRP through covalent binding by activating free hydroxyl groups of agarose with p-toluene sulfonylchloride (PTS). The tosylated agarose showed excellent stability and swelling property similar to that of untreated agarose indicating that no cross-linking of the support occurs during tosylation. They optimized various coupling conditions for the maximum loading of HRP and reported loading of 70 mg HRP per g of dry support with only 18% retention of its initial activity. Loading of 6 - 10 μ mol HRP g^{-1} wet gel was obtained in phosphate buffer of pH 8.0 at room temperature for 10 h. Gavrilova et. al.[40] immobilized peroxidase on alginate fibers by adsorption and reported 11.2 mg enzyme per g of support when initial concentration of 89.6 mg was used for loading at pH 4.6.

Immobilization of peroxidase was carried out on two different types of inorganic supports by Thibault et. al.[41] They prepared aliphatic amino silica activated through glutaraldehyde, and aromatic amino silica activated through nitrite. The immobilized derivatives exhibited maximum activity at pH 7.0. However, peroxidase immobilized on glutaraldehyde activated aliphatic amino silica was more resistant to various concentrations of 10 to 20% ethanol than free as well as nitrite activated aromatic amino silica enzyme conjugate.

Kennedy et. al.[42] immobilized peroxidase on hydrous metal oxides of titanium and zirconium. They observed that when ion-exchange resin was used as an internal matrix the loading of enzyme increased to 90% and showed better storage stability than previous system.

Cremonesi and D'Angiuro[43] immobilized HRP onto sepharose by a photochemical initiated graft copolymerization. The polymer was activated using bisacryloylpiperazine and triazine as functional monomers. The values of Michaleis constant for free and immobilized enzymes were similar. They also studied thermostability of free and immobilized HRP in water and in phosphate buffer and observed that after 240 min incubation at 60 °C free-HRP, piperazine-HRP and triazine-HRP retained 30, 50 and 75% respectively of its initial activity

A number of commercially available, activated supports were evaluated and compared for the immobilization of peroxidase and other enzymes by Taylor.[44] He used agarose and sepharose activated with CNBr and separon HEMA activated with epichlorhydrine. The pH required for maximum coupling and retention of activity varied with both protein and support. He observed that the optimum pH for coupling did not necessarily correlate with the optimum pH for retention of activity. Coupling of peroxidase onto HEMA support at pH 7.0 and 9.0 was 7.1 and 5.9 U g^{-1} with 31 and 29% retention of activity, while for sepharose 5.9 and 7.6 U g^{-1} with 59 and 50% retention of activity respectively.

Immobilization of peroxidase either through its free amino or carboxyl groups onto keratin and polyamide coated silica gel was reported by Lobarzewski and Wolski[45]. Drastic decrease in enzyme activity through the carboxyl groups of protein was reported. The decrease observed was relatively lower in the case of immobilization through free amino groups. This indicates that the carboxyl group is essential for the activity of peroxidase.

D'Angiuro et. al.[46] covalently coupled HRP to sepharose 4B using two different functional monomers bisacryloylpiperazine and triazine derivatives. They observed coupling efficiency of 22.7% (100 U) and 22.2% (125 U) at enzyme/polymer ratio 0.111 and 0.212 respectively. They also reported that the K_m values for immobilized enzymes (0.6 - 0.8 x 10^{-4} M) were close to that of free HRP (0.57 x 10^{-4} M).

Lobarzewski et. al.[47] prepared copolymer of methyl methacrylate and di(methacryloxymethyl)naphthalene by suspension polymerization for the immobilization of peroxidase. The polymer beads with diameter 0.1 - 0.15 mm were coated with different concentrations of polyamide or keratin. They obtained maximum loading of 8.9 to 10.5 mg protein g^{-1} supports. Husian et. al.[48] immobilized HRP ionically onto sepharose and observed that by addition of 2 mM calcium ion increases stability of immobilized HRP significantly.

Brzyska et. al.[49] immobilized HRP through carboxyl and amino groups of protein. They studied the effect of metal ions such as Hg and Cd and observed that by immobilizing peroxidase through its amino groups one can achieve a considerable decrease in the toxic effect of Hg or Cd. They also observed that activity of immobilized enzyme increases in the presence of metal ions such as Mn, Mg or Ca.

Enzyme electrode was constructed for the detection of H_2O_2 by Liu et. al.[50,51] by immobilizing HRP in the silica gel matrix on carbon paste electrode. The observed value of Michaleis constant K_m for the immobilized HRP was 4.8 mM. They also reported that the immobilized enzyme electrode retained about 60% of its original activity after 35 days of storage in phosphate buffer at 4 °C.

Yotova and Ivanov[52] immobilized HRP onto acetylcellulose beads. The immobilized enzymes manifested no change in their pH and temperature optima but slight increase in the value of K_m compared to free enzyme was reported.

Peroxidase entrapment in different sol-gel matrices of alkoxysilane derivatives was reported by El-Essi et. al.[53] The sol-gel immobilized peroxidase was used for the determination of glucose in serum. They observed that the optimum temperature for maximum enzymatic activity depends on the type of sol-gel matrix. However, entrapped enzymes did not show decrease in activity for two months when stored at room temperature.

Sakuragawa et. al.[54] immobilized HRP onto chitosan beads activated with glutaraldehyde. The immobilized enzyme was further used to detect microamounts of H_2O_2 in environmental samples. The HRP immobilized on chitosan beads was reported to be useful for seven days when stored in solution containing BSA and NaCl at 4 °C.

HRP was immobilized onto commercially available supports such as nitrocellulose, nylon and N^+ nylon by Lukasheva et. al.[55] The immobilization was performed either by adsorption or by covalent binding through aldehyde groups. While Zamora[56] immobilized peroxidase onto the anion exchanger resin Amberlite IRA 400. He obtained maximum enzyme coupling at pH 7.0 and 4 °C within 2 h coupling time. The enzyme immobilized resin was further used to remove phenols from paper and pulp manufacturing effluents.

Horak et. al.[57] prepared nonporous cross-linked poly(HEMA-co-EDMA) microspheres by dispersion polymerization of HEMA and EDMA. They immobilized HRP by covalent coupling onto activated copolymer through hydrazide formation. However, only 7.3 μg of enzyme per g of copolymer was reported without significant loss in enzyme activity. They also observed that the

immobilized peroxidase was stable and retained more than 97% of its initial activity when stored for 23 days at 5 °C.

Rao et. al.[58] immobilized HRP through covalent binding to polystyrene beads activated through water soluble carbodiimide. They observed that the kinetic parameters for free and immobilized HRP were unaltered. They also observed that immobilized HRP retained 81% of its initial activity when stored at 5 °C for 30 days.

Lin et. al.[59] fabricated a novel H_2O_2 biosensor based on immobilzation of HRP on the methylene blue modified graphite electrode by cross-linking with glutaridehyde. This electrode exhibited excellent stability and was used to detect low concentration (3×10^{-6} M) of H_2O_2 in the solution.

Recently a fast, simple and general method for encapsulation of HRP in microporous material was developed by Xu et. al.[60] they studied the effect of fructose, glucose, sucrose and glycerol on the enzymatic activity for free and immobilized enzymes. They also reported that the activity of entrapped HRP had excellent thermal stability in the range of 40 to 60 °C.

There are number of publications in the literature that indicate the advantages of immobilized enzyme. Literature survey reveals that covalent coupling of peroxidase onto various supports increases the stability but decreases the activity of the enzymes while entrapped enzyme retains much of its activity. The present work investigates the maximum entrapment of enzyme during copolymerization of AAm and HEMA and the optimum conditions for covalent coupling of HRP onto chitosan beads. The covalently immobilized HRP was further studied for the biodegradation of phenol in a batch process.

4.3 Experimental

➤ Chemicals used

Peroxidase (from Horseradish of strength 200 U mg^{-1})	:	
Pyrogallol	:	Sigma chemical Co. Ltd. USA
Purpurogallin	:	
Phenol	:	
Hydrogen peroxide (30 % w/w)	:	E.Merk, Mumbai, India
Methanol	:	

All other solvents and reagents used were of A.R grade and double distilled deionized water was used throughout the study.

4.3.1 Assay of HRP activity

Horse radish peroxidase activity was determined using pyrogallol substrate as described in the standard procedure by Worthington.[61]

Pyrogallol **Purpurogallin**

A 0.2 mL portion of 150 mM H_2O_2 was added under stirring at 30 °C to a mixture 2.4 mL of 100 mM phosphate buffer (pH 6.0), 0.3 mL of 0.4 mM pyrogallol, and 0.1 mL of free HRP (or immobilized HRP) in phosphate buffer. The reaction

mixture was terminated after 1 min by keeping it into 70 °C for 5 min. The quantity of liberated purpurogallin was estimated spectrophotometrically from the change in absorbance at 420 nm for the activity of HRP.

One unit of HRP will form 1 mg of purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20 °C.

4.3.2 Preparation of organic polymeric supports

The polymeric supports previously used for the immobilization of α-chymotrypsin were used for the immobilization of HRP. The details of the preparation of these supports are given in **section 2.3.1**. For the immobilization of HRP copolymer of AAm-HEMA of mesh size 400 - 250 μ and chitosan beads of ~2.0 mm diameter were used.

4.3.3 Immobilization of HRP

(a) *Insitu* entrapment of HRP

To obtain maximum entrapped enzyme activity for HRP, optimization of *insitu* entrapment was done by varying enzyme addition time and cross-linking agent bis-AAm as discussed in **sections 2.3.1** and **2.3.5**.

(b) Covalent coupling of HRP

Various coupling conditions were optimized for the maximum retention of enzyme activity and stability of immobilized HRP using 200 mg of semidry chitosan beads.

Effects of glutaraldehyde concentration (0.0 to 60 mg g^{-1}), cross-linking time (30 to 120 min), coupling medium (100 mM phosphate buffer of range 4 to 10), enzyme to carrier ratio (0.25 to 2.0) and coupling time (1 to 16 h) on the extent

used immobilized tyrosinase onto chitosan films for the removal of phenol. Barton and Russell[68] immobilized polyphenol oxidase onto nylon for the detection of phenol pollutants in water.

The present study investigates the biodegradation of phenol using covalently bound HRP onto activated chitosan beads. The optimum phenol concentration, degradation time and optimum pH for degradation of phenol by free and immobilized HRP in batch scale was studied. Aliquots of 10 mL of various concentrations of phenol were used to study biodegradation of phenol. Residual phenol concentrations were determined by direct spectroscopic measurement of the absorbance of ultraviolet light at 269 nm.

4.4 Results and Discussion

4.4.1 Optimization of coupling conditions

A covalent coupling of HRP was carried out using glutaraldehyde as cross-linking agent. 200 mg of chitosan beads were immersed in 4 mL of 0.15% aqueous solution of glutaraldehyde for 60 min at room temperature. The unreacted glutaraldehyde was removed by washing. This was followed by coupling of HRP at optimum pH 7 0 using enzyme concentration 0.5 mg g^{-1} of support for 12 h coupling time. The results are summarized in **Table 4.1**.

4.4.2 *Insitu* entrapment of HRP

Insitu entrapment of HRP was done in copolymer of AAm and HEMA of ratio 1:1(w/w) using 2% cross-linker bis-AAm at constant temperature 293 ± 1 °K in nitrogen atmosphere. 10 mL of phosphate buffer of pH 7.0 containing 400 U of HRP was added after 5 min of initiation time. Reaction was allowed to complete for 1 h. The copolymer containing entrapped HRP was washed with cold water

Table - 4.1

Optimized conditions for covalently immobilized HRP on chitosan

Parameter	Optimum conditions	Protein coupled (mg g^{-1})	Active enzyme bound (mg g^{-1})	Retention of activity (%)
Glutaraldehyde concentration	30 mg g^{-1}	0.15	0.04	27
Cross-linking time	60 min	0.18	0.052	29
pH of coupling medium	6.0	0.20	0.05	25
Enzyme Concentration	0.5 mg g^{-1}	0.23	0.074	32
Coupling time (h)	14	0.24	0.076	32

followed by buffer solution and meshed to 400-250 μ. Dried copolymer containing entrapped enzyme was stored at 4 °C till further use. Approximately 90% enzyme activity was observed to be retained after entrapment.

4.4.3 Comparative account of free and immobilized HRP

(a) pH activity profile

The pH activity profile of the free and immobilized HRP is given in **Fig. 4.1**. It is observed that the free enzyme exhibited maximum enzyme activity at pH 6.0 whereas CB-HRP and ENT-HRP at pH 7.0, indicating no change in structure-function relation of enzyme protein on immobilization.

(b) Thermal stability

Thermal stability plays an important role in designing the enzymatic applications. Hence, effect of temperatures on the activity of free and immobilized HRP was studied at 40 to 60 °C. From the results obtained the thermodeactivation constant (K_d) was calculated and results are given in **Table 4.2**. It was observed from the results that the free enzyme has higher value of K_d compared to that of immobilized at all temperatures indicating improved thermal stability of enzyme upon immobilization.

(c) Kinetic parameters

The initial reaction rates were determined at different pyrogallol concentrations varying from 0.1 to 1.0 mM. Michaleis constant K_m and maximum reaction velocity V_{max} for the free and immobilized enzymes were calculated from the Lineweaver-Burk plots. The values of K_m and V_{max} for free HRP, CB-HRP and

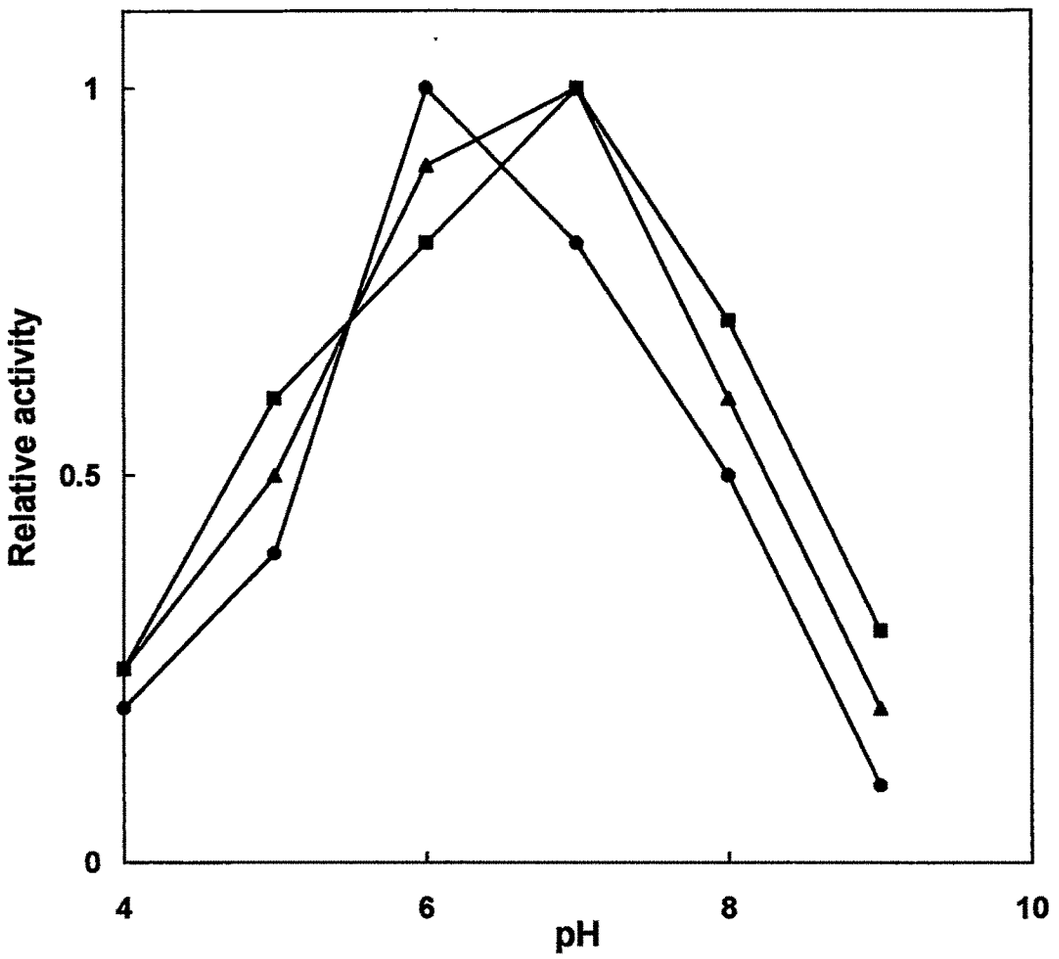


Fig. 4.1 pH activity profile at 30 °C using pyrogallol as substrate

Free HRP (●), CB-HRP (▲) and ENT-HRP (■)

Table - 4.2

Kinetics and thermodeactivation parameters for free and immobilized HRP

Sample	Michaleis constant (K_m) (mM)	Maximum reaction velocity (V_{max}) (mM min^{-1})	Deactivation rate constant ($K_d \times 10^{-3}$)		
			40 °C	50 °C	60 °C
Free HRP	6.67	1.11	0.12	3.7	5.0
CB-HRP	4.17	0.80	0.08	1.3	2.0
ENT-HRP	4.65	1.10	0.09	1.1	0.92

ENT-HRP are given in the **Table 4.2.** The decrease in the K_m value for immobilized enzymes indicates the limitation of diffusion resistance to reaction or partially kinetically controlled reactions. The decrease in the K_m and V_{max} values on immobilization of enzymes on polymeric materials is known to be frequently observed phenomena.

(d) Storage stability

Storage stability of free and immobilized HRP was determined by storing them at room temperature (30 °C). Free HRP and CB-HRP was stored in phosphate buffer of pH 6.0 and 7.0 respectively, while ENT-HRP was stored without buffer solution. The results are given in **Fig. 4.2** From the figure it was observed that after 6 days free HRP loses its activity completely, whereas CB-HRP and ENT-HRP retained respectively 40 and 70% of its activity after storage of 30 days.

(e) Reusability of immobilized HRP

The reusability of covalently bound and entrapped peroxidase was evaluated by repeating incubation cycles with 100 mM phosphate buffer of pH 6. After each cycle, the reaction mixture was removed and enzyme activity was determined. The immobilized peroxidase samples were washed with deionized water and incubated with fresh reaction mixture. **Fig. 4.3** shows the activity of the immobilized enzymes at different incubation cycles. The activity of peroxidase immobilized through both the techniques showed an almost linear decrease from cycle to cycle. After eight incubation cycles ENT-HRP loses it activity completely, while CB-HRP retains 70% of the original activity. Rapid decrease in enzyme activity of ENT-HRP may be due to the leakage of immobilized peroxidase during each incubation and wash.

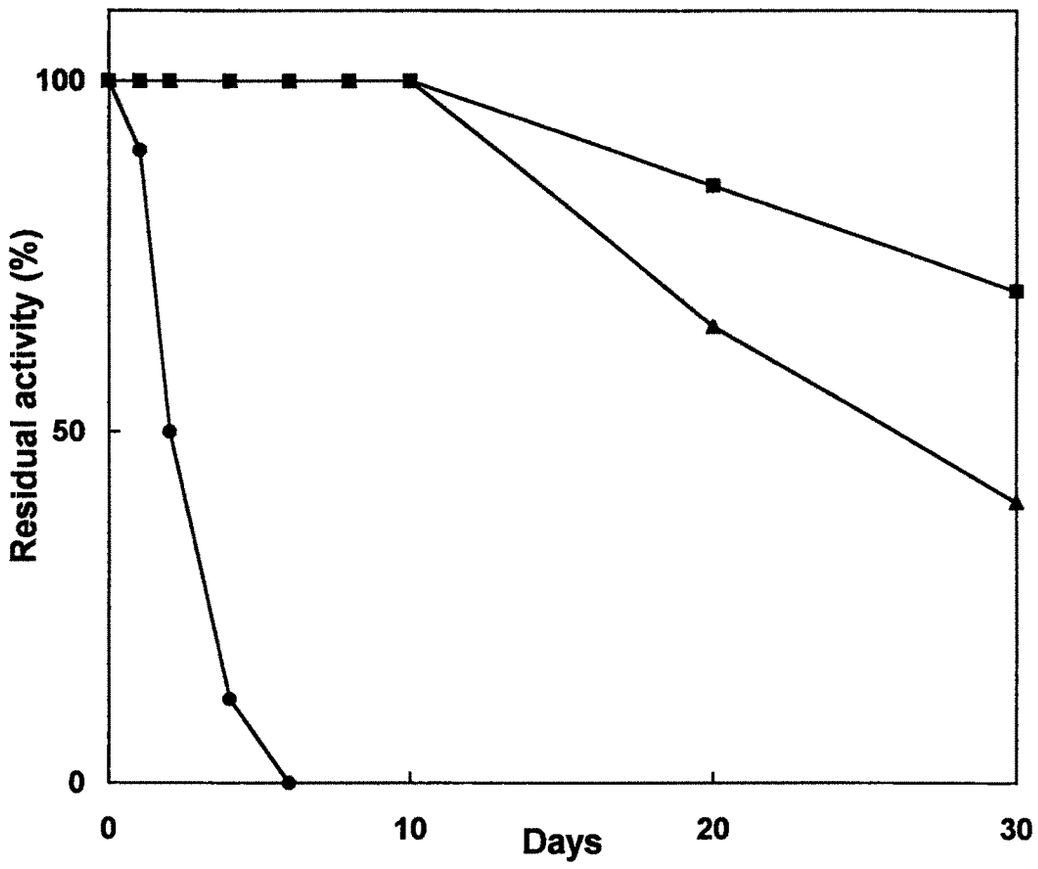


Fig. 4.2 Storage stability of enzymes at room temperature (30 °C)

Free HRP in buffer pH 6 (●), CB-HRP in buffer pH 7 (▲) and ENT-HRP without buffer (■)

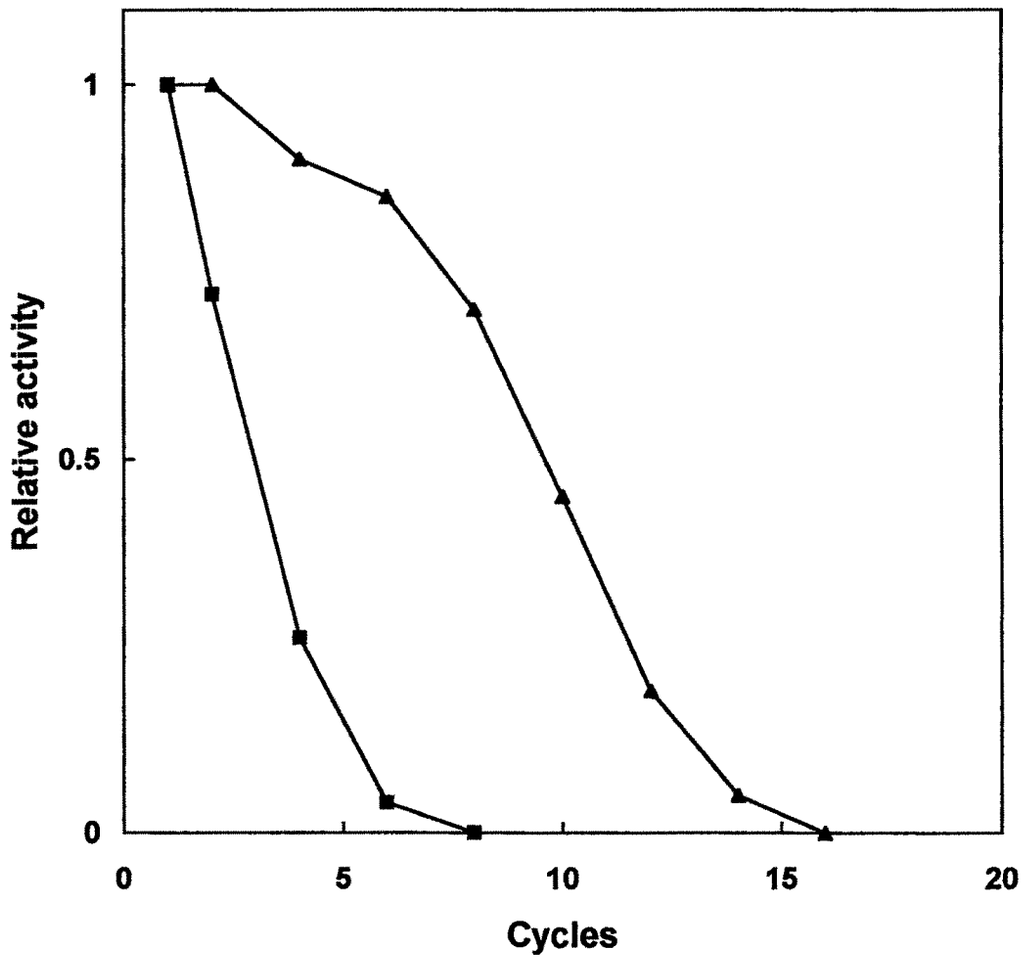


Fig. 4.3 Reusability of immobilized enzymes at 30 °C and pH 6.0

CB-HRP (▲) and ENT-HRP (■)

(f) Solvent stability

The practical importance of solvent stability studies for the immobilized enzyme is described in the previous chapter. Accordingly we have studied the oxidation of pyrogallol by incubating enzymes in 0 to 30% methanol. The results obtained are given in **Table. 4.3**. It was observed from the results that free and immobilized enzymes have similar trend towards water miscible solvent methanol, however the immobilized enzymes had more stability than free enzyme.

4.4.4 Biodegradation of phenol

There are various examples such as petroleum refining, chemical production, plastic, resins, textiles etc., in which phenols appear as contaminant in process stream and their selective removal is required for waste minimization. We have attempted few experiments for the degradation of phenol by covalently immobilized HRP onto activated chitosan beads.

Batch experiments for phenol biodegradation were carried out for free and covalently immobilized HRP. The phenol degradation ability of 10 mL of 1 mM phenol concentration using 1 g of chitosan beads containing immobilized HRP was compared with the equivalent amount of free HRP (76 μg). The results obtained are given in **Fig. 4.4**. From the results it is observed that initial rate for degradation of phenol by free enzyme was more, however the time for complete degradation of 1 mM phenol by immobilized HRP was 3 h, while free enzyme degrades within 4 h.

Various concentrations of phenol were examined using fixed amount of immobilized HRP in order to determine minimum amount of immobilized HRP required for degradation of phenol. Experiment was carried out at various

Table - 4.3

Effect of methanol on HRP activity

Methanol concentration (%)	Retention of activity (%)		
	Free HRP	CB-HRP	ENT-HRP
0	100	100	100
10	79	90	80
20	39	61	73
30	10	44	31

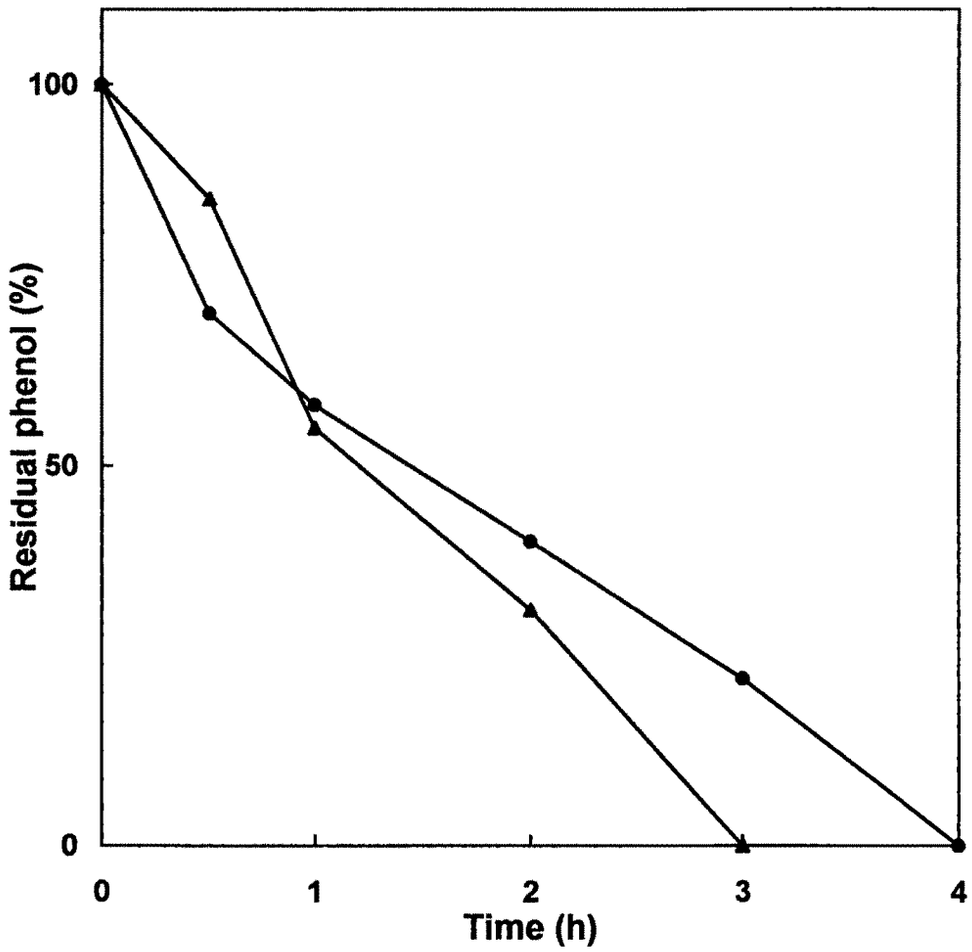


Fig. 4.4 Comparison of 1 mM phenol degradation at 30 °C, for 1 h

Free HRP in pH 6 (●) and CB-HRP in pH 7 (▲)

concentrations of phenol ranging from 0.5 to 2.0 mM per g chitosan containing immobilized HRP. The results obtained are given in **Fig. 4.5**. It is inferred from the results that as the phenol concentrations increase, time to degrade phenol by immobilized enzyme also increases. The time for complete degradation of 1.5 mM phenol was ~ 5 h. However, at higher concentration of 2 mM complete degradation occurs after 8 h. *Pseudomonas putida* immobilized on activated carbon was reported to degrade 1500 mg L^{-1} of phenol by Zhu et. al.[69] However, the time required for complete degradation was 35 h.

Biodegradation of phenol was carried out by covalently coupled and free HRP in different pH range of 4 to 8 for 1 h. The results obtained are given in **Fig. 4.6**. It is observed from the figure that maximum degradation of phenol was obtained at pH 6.0 while same was obtained for immobilized HRP at pH 7.0. At pH 4.0 phenol degradation did not occur in both systems. While at pH 5.0 and above pH 7.0 the degradation was very less. Maximum phenol degradation by immobilized HRP at neutral pH is promising result for the application in wastewater treatment containing low concentration of phenol.

4.5 Summary and Conclusions

1. The successful immobilization of horseradish peroxidase was carried out onto natural polysaccharide chitosan beads activated through glutaraldehyde. HRP was also entrapped into mechanically stable support poly(AAm-co-HEMA) synthesized at low temperature.

2. By varying the optimum coupling conditions for immobilization of HRP onto activated chitosan beads, maximum loading of 0.24 mg of HRP per g of support was obtained within 12 h coupling time at pH 7.0. However, the retention of enzyme activity was obtained only 32%. While a simple and reproducible method for the *insitu* entrapment of HRP was developed

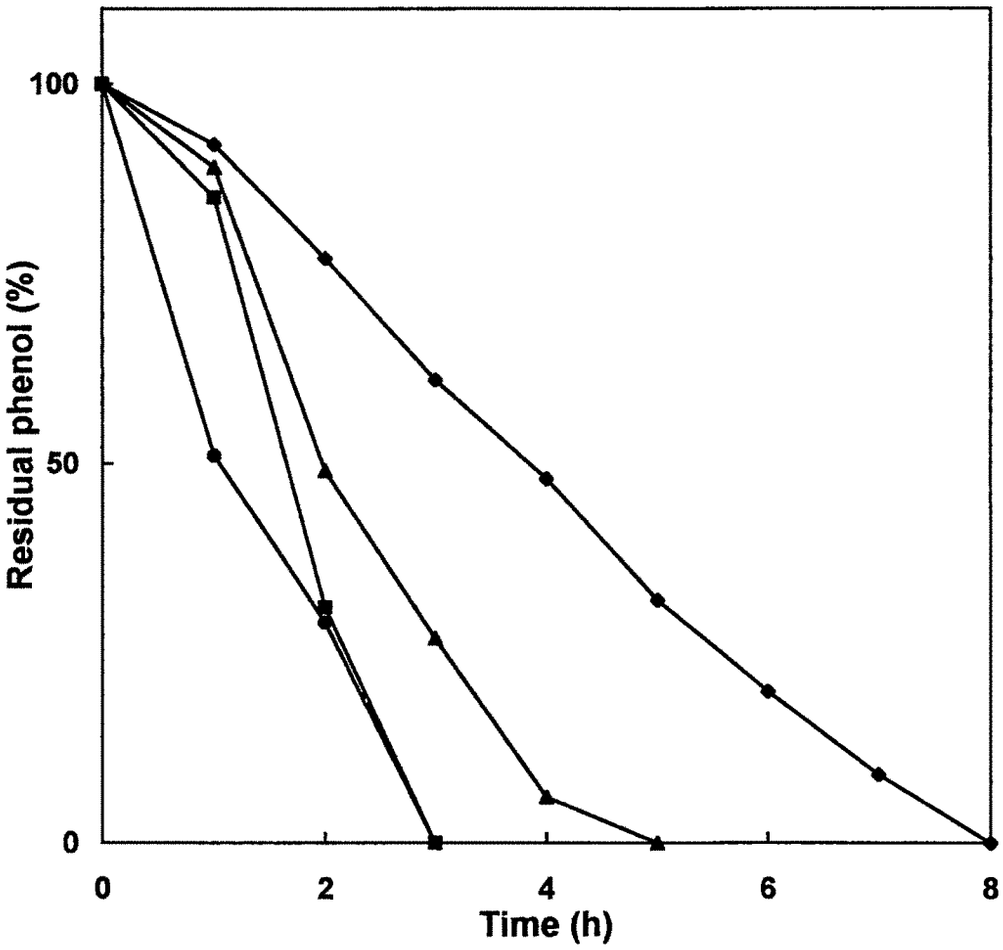


Fig. 4.5 Degradation of phenol using 1 g of CB-HRP at 30 °C, pH 7.0 for 8 h

0.5 mM (●), 1.0 mM (■), 1.5 mM (▲), 2.0 mM (◆)

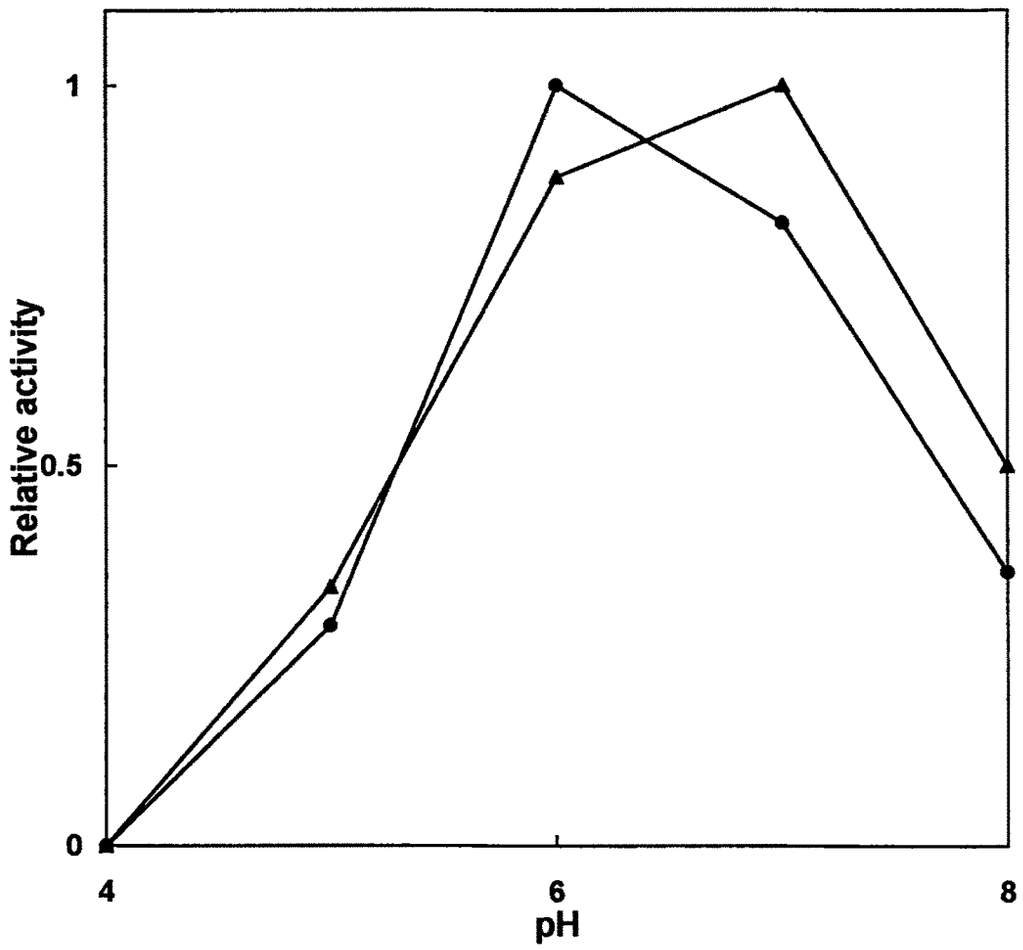


Fig. 4.6 Effect of pH on 1 mM phenol degradation at 30 °C, pH 7.0, for 1 h

Free HRP (●) and CB-HRP (▲)

could retained more than 90% of enzyme activity after entrapment. This method can be applied to any enzyme for the entrapment technique.

3. Properties of the immobilized enzymes were compared with free enzyme. The pH optimum of immobilized enzymes was shifted by 1.0 unit toward the alkaline side when compared to that of free enzyme. It was also observed that the thermal stability, storage stability and solvent stability were better than that of free enzyme. However, the kinetic parameters for both the techniques were observed to be decrease marginally

4. Covalently coupled HRP onto chitosan beads was further used in batch scale for the phenol degradation study. The results obtained indicates as the phenol concentration increases the time required to degrade by CB-HRP also increases. It is possible to achieve the complete degradation of 2 mM phenol within 8 h per g of chitosan beads containing immobilized HRP. The results obtained clearly show the possibility for their application for the purification of waste water containing phenol. The performance of immobilized enzymes with free enzyme was compared and the properties are summarized in **Table 4.4**.

Table - 4.4

Comparison of properties of free and immobilized HRP

Properties	Free enzyme	Covalent binding	*Insitu* entrapment
Optimum pH	6.0	7.0	7.0
Optimum temperature (°C)	20	30	30
Storage stability at RT (days)	2	30	30
Durability (cycles)	-	15	6
Thermodeactivation constant (K_d) at 60 °C	5.0×10^{-3}	2.0×10^{-3}	0.92×10^{-3}
Michaeli's constant, K_m (mM)	6.67	4.17	4.65
Maximum velocity, V_{max} (mM min^{-1})	1.11	0.8	1.10
Stability in 20% methanol (%)	39	61	73

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