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# Removal of Phenols with Encapsulated Horseradish Peroxidase in Calcium Alginate

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**ABSTRACT:** *Horseradish peroxidase was encapsulated in calcium alginate for the purpose of phenol removal. Considering enzyme encapsulation efficiency, retention activity and enzyme leakage of the capsules, the best gelation condition was found to be 1 % w/v of sodium alginate solution and 5.5 % w/v of calcium chloride hexahydrate. Upon immobilization, pH profile of enzyme activity changes as it shows higher value at basic and acidic solution. Besides, for each phenol concentration there would be an enzyme concentration which going further than this value has no significant effect on phenol removal. Investigation into time course of phenol removal for both encapsulated and free enzyme showed that encapsulated enzyme had nearly similar efficiency in comparison with the same concentration of free enzyme; however the capsules were reusable up to four cycles without any changes in their efficiency. The ratio of hydrogen peroxide/phenol at which highest phenol removal obtained, found to be dependent on initial phenol concentration and in the solution of 2 and 8 mM phenol it were 1.15 and 0.94 respectively.*

**KEY WORDS:** *Horseradish Peroxidase, Alginate, Gelation, Encapsulation, Phenol removal.*

## INTRODUCTION

Enzymes as biocatalysts have been used in many biological reactions but they mostly suffer from certain disadvantages. Enzymatic removal of phenolic compounds have been investigated by many researchers and it has been shown that peroxidases are able to react with aqueous phenolic compounds and form non-soluble materials that could be easily removed from the aqueous phase [1-7], however; these processes suffer from enzyme inactivation.

Therefore attentions came into immobilization of peroxidases for the purpose of phenolic compound

removal. Among most abundant peroxidases investigated, horseradish peroxidase (HRP) has been successfully used to remove phenol from waste effluent and it is by far the most researched peroxidase. Using alternative peroxidase was also investigated due to need for cheaper catalyst and it has been shown that soybean peroxidase which is abundant in soybean seed hull can also remove phenolic compound from waste stream with acceptable removal efficiency comparable to HRP [8-10]. media containing immobilized enzyme seems to be more suitable when large amount of wastewater need to be processed.

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*1021-9986/09/2/43*

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Many materials and different methods have been used for HRP immobilization, glass beads, polymers, ion exchange resins, magnetite and aluminum-pillared clay [11-15].

Although immobilization highly improves HRP catalytic efficiency, the inactivation of the enzyme remains a major problem in phenolic wastewater treatment. Some investigators attribute low removal efficiency of biocatalysts to the interaction between the phenoxy radicals and enzyme active site [16]. Besides, the hindrance effect of excess hydrogen peroxidase has been also reported [9]. To surmount this difficulty and minimize the enzyme inactivation some researchers introduced adding compound such as polyethylene glycol (PEG) to form a protective layer by which higher efficiency especially at low enzyme concentration obtained [17]. However, in accordance with the recent publication, a great part of PEG added to the reaction remained in the solution after separation of radicals [18].

In the present work, we attempted to use a new support for immobilization of HRP for the purpose of phenol removal from a synthetic wastewater. We used one step encapsulation method for immobilization of HRP in a semi permeable alginate membrane. The application of immobilized peroxidase for the removal of phenol from aqueous solution was studied at different enzyme, phenol and hydrogen peroxide concentrations. From the results obtained in the present work, the possibility of continuous phenol removal was shown to be promising.

## MATERIALS AND METHODS

### Chemicals

Horseradish peroxidase, HRP (lyophilized powder, 200U mg<sup>-1</sup>), Phenol 99 % and H<sub>2</sub>O<sub>2</sub> 30 % w/v were purchased from Merck also the analytical chemicals 4-aminoantipyrine (AAP) and potassium ferricyanide. Sodium alginate (reach in guluronic acid) from *Lamirania hyperborean* and calcium chloride hexahydrate were obtained from BDH (U.K). Catalase enzyme from aspergillus Niger (EC.1.11.1.6) (lyophilized powder 2993 U mg<sup>-1</sup>) was purchased from SERNA. Other chemical were of analytical grade and were used without further purification.

### Enzyme encapsulation

The immobilization method was carried out according to the following steps:

Sodium alginate was dissolved in reagent water. For dissolving sodium alginate in water a backer equipped with a magnetic stirrer was used. Dissolving process was so slow that it took up to 5 hours for preparing a 2 % w/v of alginate gel.

For expelling air bubble, occasional mixing was performed with a glass rod. After dissolving sodium alginate, the gel was placed in the room temperature followed by continuous stirring to obtain a homogenous gel. The gel was being stored in 4 °C for further usage. Calcium alginate capsules were prepared by extrusion using a simple one- step process similar to that described by *Nigma et al.* [19]. Pre-determined enzyme was dissolved in 10 mL calcium chloride solution and was dropped through a silicon tube, using a peristaltic pump, into 100 mL of alginate solution.

The sodium alginate solution was maintained under constant stirring (200 rev/min) using a magnetic stirrer situated at the bottom of the backer, in order to avoid the droplets sticking together and minimize the external mass transfer resistance. 10 cm dropping height was chosen to obtain spherical capsule. After 20 min gelation time, the capsule was removed by dilution of alginate solution to 5 times with distilled water followed by filtration of capsules. The average capsule's diameter was 3-4 mm.

### Protein determination

The amount of protein initially offered, in the wash-liquid after encapsulation and also the protein content in capsule after leakage test were obtained by Lowry's procedure as modified by Peterson [20].

### Enzyme encapsulation efficiency

To assess the enzyme encapsulation efficiency, it was necessary to measure HRP concentration both in calcium chloride solution and capsule. To measure the encapsulated enzyme concentration, capsules were cut in half and put in 5 mL phosphate buffer (pH=7.4) solution. The concentration of protein in buffer was measured according to the Lowry's assay after 2 hours in order to obtain encapsulated protein. The percentage of encapsulated enzyme was obtained from the difference between initial protein introduced to the calcium chloride hexahydrate solution and encapsulated protein measured as mentioned above.

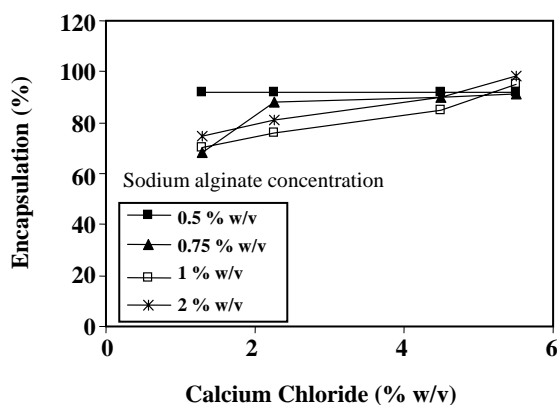


Fig. 1: Encapsulation efficiency, under different gelation condition,  $T= 25\text{ }^{\circ}\text{C}$ , phenol concentration 2 mM.

### Enzyme leakage

Enzyme leakage measurement was carried out by placing capsules in a test tube filled with tris buffer (pH=8.0) for 18 hours. Then the capsules were removed, cut in half and put in phosphate buffer (pH=7.4) solution. The protein concentration was measured according to the Lowry's assay and the leakage percentage was calculated from the differences between encapsulated protein at the beginning of time interval and the value found according to the above procedure.

### Analytical Methods

#### Activity measurement of free and immobilized enzymes

HRP activity was assessed by employing 4-aminoantipyrine method involving colorimetric estimation by using phenol and  $\text{H}_2\text{O}_2$  as substrate and 4-aminoantipyrine (Am-NH<sub>2</sub>) as chromogen [21]. The assay was performed at 25 °C by adding phosphate buffer (pH=7.4) containing  $1.0 \times 10^{-2}$  M phenol,  $2.4 \times 10^{-2}$  M (Am-NH<sub>2</sub>) and  $2.0 \times 10^{-4}$  M  $\text{H}_2\text{O}_2$ . The rate of  $\text{H}_2\text{O}_2$  consumption was estimated by measuring the absorption of the colored products at 510 nm.

#### Phenol assay

Phenol concentration was determined using a colorimetric assay in which the phenolic compounds within a sample react with 2.08 mM AAP in the presence of 8.34 mM potassium ferricyanide reagent. The assay is valid if the phenol concentration does not exceed 0.12 mM in the assay mixture. Following the full development of the color after 9-10 min reaction time, the absorbance

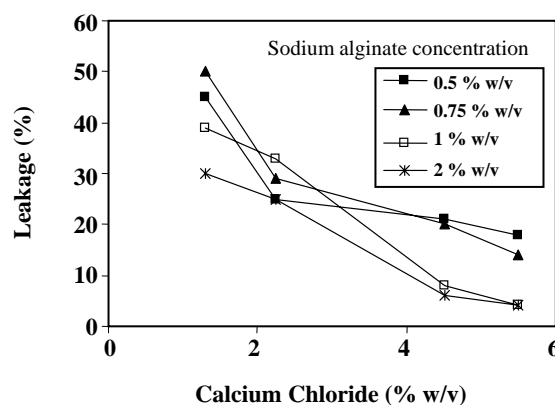


Fig. 2: Leakage percentage under different gelation condition  $T= 25\text{ }^{\circ}\text{C}$ , phenol concentration. 2 mM.

values at 510 nm were transformed to phenol concentration in the samples, obtained from aqueous solutions after immobilized HRP treatment, using a calibration curve.

### Phenol removal studies

Experiments were conducted to assess the HRP catalyzed the removal of phenol from aqueous phase by both free and immobilized enzyme to determine the time required for completion of enzymatic reaction and efficiency of removal. The experiments were carried out at 25 °C in 1 lit beaker equipped with magnetic stirrer. Phenol and buffer solution were introduced to reaction media followed by addition of enzyme and hydrogen peroxide. Different concentration of phenol, hydrogen peroxide and enzyme were used. The samples from the reactor were poured into 1 mL of catalase solution to stop the reaction by breaking down the hydrogen peroxidase.

## RESULTS AND DISCUSSION

### Effect of alginate and calcium chloride concentration on encapsulation

Different concentration of sodium alginate and calcium chloride solution were used to obtain the optimal condition for producing biocatalysts, effective in phenol removal from aqueous phase. In order to find these concentrations two factors were taken into consideration: enzyme leakage, and encapsulation. The results demonstrate the influence of sodium alginate and calcium chloride concentration on the biocatalyst characteristics and are represented in Figs. 1 and 2. Irrespective of the

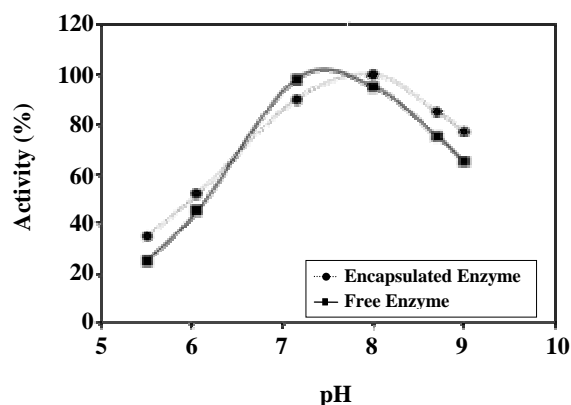


Fig. 3: Effect of pH on the Activity of free and immobilized HRP Free enzyme (■) Encapsulated HRP (●).

alginate concentration, using high concentration of calcium chloride solution results in lower leakage percentage. It has been also shown that the change in enzyme encapsulation relates to calcium chloride concentrations.

On the other hand, changing alginate solution concentration had a significant effect on encapsulation efficiency. The best biocatalytic properties including lower enzyme leakage and higher enzyme encapsulation achieved when the calcium chloride hexa hydrate and sodium alginate solution were 5.5 % w/v and 1 % w/v respectively. According to Figs. 1 and 2, by selecting favorable alginate concentration and calcium chloride solution, leakage decreases to lower than 5 %.

#### Effect of pH

The pH activity profile of free and encapsulated HRP was obtained by incubating both the free and immobilized enzyme at 25 °C for 15 min in 5 mL Tris buffer solution followed by measuring the enzyme activity at 510 nm. Fig. 3 depicts the results of these measurements. This behavior might be the result of interior microenvironment of capsule that is slightly cationic and separated from bulk with a semi-permeable membrane which is anionic in nature.

#### Optimum contact time

Initially experiments were performed in order to assess the optimum contact time required for phenol removal. To a series of beakers each one containing 100 mL of 2 mM phenol, 20  $\mu$ L hydrogen peroxide along with enzyme concentration (0.8 units/mL) were added and reaction

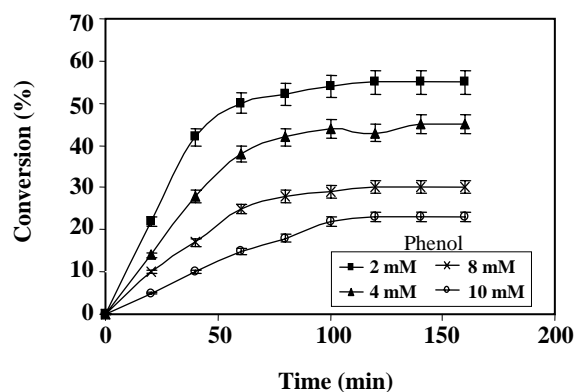


Fig. 4: Time course of phenol conversion with 0.75 units/g of encapsulated HRP for four different initial phenol concentrations. (A) 2 mM, (B) 4 mM, (C) 8 mM, (D) 10 mM.

media (25 °C, pH=8.0) was agitated for a period of 4 hours. Every 20 minutes, a 1 mL sample was taken from solution and was analyzed for the residual phenol concentration. It was shown that 100 min is required to reach acceptable removal efficiency. Subsequent experiments were performed at a beaker containing 100 ml phenol with definite concentration, 4, 8, 10 mM and lasted for 100 min. Further reactions with different phenol concentrations have shown that phenol removal follows the same trends (Fig. 4). Increasing initial phenol concentration results decrease in % conversion due to the inhibitory effect of the substrate for the catalyst. The highest conversion belongs to phenol concentration of 2 mM.

The phenol conversion against time was also studied for both encapsulated and free enzyme. Fig. 5 shows the comparison between free and encapsulated enzyme for phenol removal efficiency versus time which are near. Reaction profile of p-chlorophenol removal with immobilized HRP on the other carrier APG, aminopropyl glass reached the optimal nearly after 100 min, but the percent removal was about 20 % [11].

#### Influence of enzyme concentration

Since the biocatalyst has a finite lifetime and also the conversion is found to be dependent on the contact time, normally removal of phenol is dependent on the amount of catalyst added. To study the effect of enzyme concentration on phenol removal, five different enzyme concentrations were used to compare the efficiency of encapsulated enzyme. The phenol and hydrogen peroxide

Table 1: The effect of  $H_2O_2$ /Phenol on %conversion of phenol for different initial phenol concentration.

$H_2O_2$ /Phenol	0.4	0.7	0.92	1	1.2	1.3	1.5	1.7
Phenol (2Mm)	15	25	43	44	45	44	43	42
Phenol (5Mm)	20	27	44	45	44	44	43	42
Phenol (8Mm)	12	28	40	39	38	35	33	32

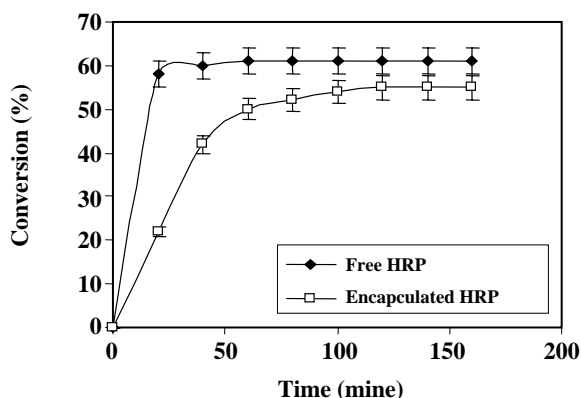


Fig. 5: Comparing time course of phenol conversion of immobilized and free enzyme, Phenol concentration 2 mM. Free enzyme (●) Encapsulated HRP (■)

concentration along with the physical condition of reaction remained unchanged (phenol concentration 2mM, pH=8.0). Fig. 6 depicts the effect of enzyme concentration on phenol concentration. It is found that for a 2 mM phenol solution, increasing enzyme concentration from 0.15 units/mL to 0.8 units/g alginate, results in gradual increase in phenol removal. Further increases in enzyme concentration have no significant effect on phenol removal. The enzyme concentration of 0.8 Units/g was found to be the optimal dose for the experiment condition.

#### Influence of hydrogen peroxide concentration

Increasing phenol removal percentage could be obtained by choosing an appropriate hydrogen peroxide concentration; therefore some authors introduced an optimal molar ratio of hydrogen peroxide to phenol resulting in higher removal efficiency [9, 22]. It has been also described [23] that the optimum peroxide concentration is totally depends on initial phenol concentration and differ from case to case.

Several experiments were carried out by using three different phenol concentration (2, 5 and 8..and hydrogen peroxide varying from 200 to 950 mM In every assay,

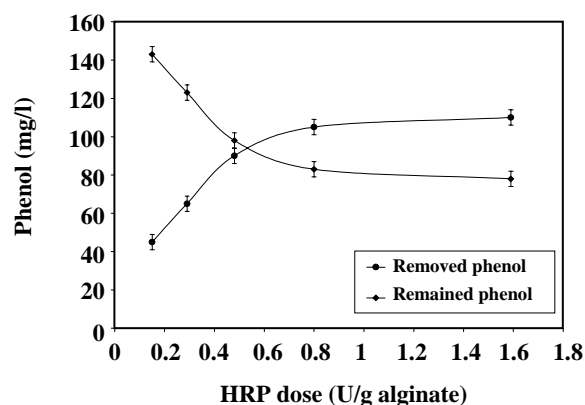


Fig. 6: Effect of encapsulated HRP dose on phenol concentration: (●) removed, (■) remained.

0.8 unites/mL of enzyme, introduced to reaction medium. Table 1 shows the results obtained in these series of experiments.

The behavior of phenol removal efficiency was similar for all phenol concentrations. First, the amount of phenol removed was sharply increased with an increase in hydrogen peroxide up to an optimal point. It shows that hydrogen peroxide is a limiting factor in this range. Second, after phenol conversion reached its optimum point adding hydrogen peroxide significantly reduced the conversion. This reduction is sooner for higher initial phenol concentration. For initial 8 mM phenol concentration optimum reaches at 0.92 hydrogen peroxide/phenol, and 1 and 1.2 for the other initial phenol concentration respectively. A reason for this phenomenon would be that an excess amount of hydrogen peroxide results in higher concentrations of intermediate products which inhibit the activity of enzyme, and/or that enzyme is inactivated by an excess of hydrogen peroxide. The deviation of the curve also might be the result of polymer produced in the catalytic process larger than dimmer [24].

#### Reusability

The immobilized enzyme could be easily removed

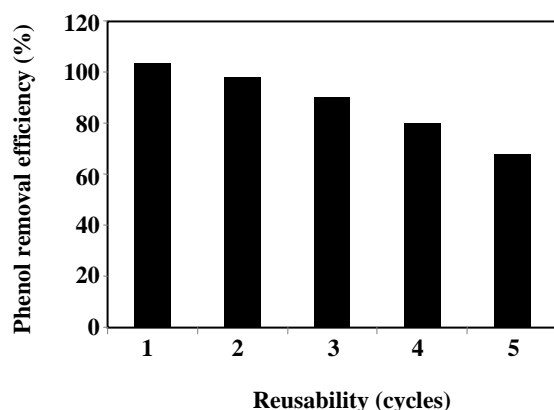


Fig. 7: Reusability of capsules(cycles), Phenol concentration 2 mM and enzyme content 7.5 units/g.

and assessed for its remained catalytic activity. To demonstrate the reusability of encapsulated enzyme, capsules were separated after 100 min of reaction time and then rinsed thoroughly with distilled water. The capsules used for subsequent batches. After 5 times of the repeated test, the phenol removal efficiency was reduced to higher than 75 % of its initial value (Fig. 7). This phenomena might be the result of plugging of the membrane pore and accumulating of radicals and dimmer in the interior environment of each capsule which entrapped the active site of enzyme or even enzyme molecules resulting in enzyme inactivation. Other investigators for immobilized HRP on the other carrier observed that 50% of the initial activity was lost after five cycles [11].

## CONCLUSIONS

The preparation and application of immobilized Horseradish peroxidase in ca-alginate beads for phenol removal from aqueous solution was investigated. The experimental results obtained in the present work revealed the effectiveness of the encapsulated peroxidase in phenol removal. The performance of phenol removal was found to be highly dependent on phenol concentration, enzyme dose, hydrogen peroxidase and aqueous pH. The enzyme concentration of 0.8 Units/g was found to be the optimal dose for the experiment condition. Effect of hydrogen peroxide/phenol for various initial phenol concentration (2, 5 and 8 mM) showed that for initial 8 mM phenol optimum conversion reaches 0.92 hydrogen peroxide/phenol, and 1 and 1.2 for the other initial phenol concentration respectively, which present the inhibitory

effect of substrate at high concentration. The encapsulated enzyme activity shows higher relative activity in acidic and basic solutions which are the most common conditions appeared in waste stream. Encapsulation and leakage percentage of enzymes are influenced by gel preparation condition such as calcium chloride and alginate concentrations which finding a proper value for above quantities totally depends on alginate species used. The best biocatalytic properties including lower enzyme leakage and higher enzyme encapsulation achieved when the calcium chloride hexa hydrate and sodium alginate solution were 5.5 % w/v and 1 % w/v respectively. The reusability experiment showed that these biocatalysts can be used up to five cycles without serious deficiency in their catalytic performance.

Received : 23<sup>rd</sup> October 2007 ; Accepted : 16<sup>th</sup> September 2008

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