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**The detection of phenol by using tyrosinase  
immobilized sodium alginate beads**

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**Abstract**

Phenol and its derivatives are common contaminants encountered in the effluents of various industries like petrochemical, coal conversion and phenol producing industries which is very hazardous to human health. It is therefore of utmost importance to detect and monitor the presence of phenol in water. We report the immobilization of the enzyme tyrosinase on sodium alginate to serve as a tool for the detection of phenol. The various operational parameters for immobilization of with respect to pH and loading of enzyme onto the sodium alginate beads have been optimized. The lowest detectable concentration of phenol by tyrosinase immobilized sodium alginate beads was observed to be 3 ppm.

**Keywords:** Tyrosinase, Sodium alginate beads, Immobilization, Phenol detection

**Introduction**

Phenol is highly toxic to humans, aquatic ecosystem and environment even at very low concentrations, degradation is poorly observed. Phenol is utilized in various industries like metals, manufacturing of paper, plastics, pesticides, pharmaceuticals, resin manufacturing, wood products. So, discharging of phenol and its derivatives as a waste product in rivers is highly hazardous. International regulatory bodies such as The US Environmental Protection Agency (EPA) and the National Pollutant Release Inventory (NPRI) of Canada have already declared phenol as a potent pollutant for environment and have already set limits for its safe disposal. Health hazards for humans can both being acute and chronic, so the phenol detection and monitoring is strictly required. Many strategies and methods for removal and detection of phenol have been implemented previously which include solvent extraction, photocatalytic degradation, activated carbon adsorption, catalytic wet air oxidation, photo-oxidation, ozonation and so on, but due to their high cost and harmful byproducts formation their uses are limited. In place of harmful chemical reactions, a simpler practical and beneficial approach can be achieved, i.e., the use of enzymes for monitoring phenol in waste water. It is a cleaner and greener approach. One such enzyme is tyrosinase, which appropriately detect the presence of phenol. Tyrosinase, a Cu containing enzyme is widely distributed in bacteria, fruits, and vegetables and is capable of converting phenolic substance to o-diphenols and then their oxidation to o-quinones. Although Enzymes are being operational over a wide range of pH and temperature sometimes get deactivated and denatured under influence of extreme circumstances. To avoid the same, enzyme immobilization is a process to increase the activity, stability and reusability of an enzyme is administered. Tyrosinase is isolated from unspecified mushroom has been immobilized on a wide variety of supports such as polyacrylamide gel, controlled pore glass, chitosan gel films, nylon-66 etc using different immobilization procedures. In brief, there is report available on an optical biosensor based on the immobilized tyrosinase in a chitosan film (Jafar *et al.*, 2006) [2], MCM-41 (Mangrulkar *et al.*, 2012) [5] is described for the detection of phenol. In the present investigation, we have used this sodium alginate beads to immobilize the enzyme tyrosinase. By our detailed study, experiment and report we have tried to establish a suitable and promising strategy to detect and monitor the presence of phenol in wastewater. The lowest detectable concentration of phenol by tyrosinase immobilized sodium alginate beads was observed to be 3 ppm.

## Material and Methods

All the reagents used for experimental studies were of analytical grade. The purified tyrosinase from mushroom and L-dihydroxyphenylalanine (L-DOPA) was purchased by Sigma (U.S.). Sodium alginate,  $\text{CaCl}_2$ ,  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , sodium potassium tartarate,  $\text{Na}_2\text{CO}_3$ ,  $\text{CuSO}_4$ , Folin reagent, BSA and phenol were purchased from Merck, India Ltd. The stock solutions were prepared in deionized water.

### Synthesis of Sodium Alginate

4g sodium alginate was dissolved in 100ml of distilled water with stirring for an hour. This solution was added through a column of diameter of tapering end about 0.2mm and with constant stirring in  $\text{CaCl}_2$  solution, prepared by dissolving 4g of it in 100ml of distilled water. It was stirred for half an hour and filtered. Formed beads were washed with distilled water and dried at  $60^\circ\text{C}$  for 12 hrs.

### Characterization

XRD of the materials was obtained by using (PANalytical) X-ray diffractometer, with  $\text{Cu K}\alpha$  radiation ( $\lambda = 1.54060 \text{ \AA}$ ) at 45 kV and 40mA and scanned over the range of diffraction angle  $2\theta = 10\text{--}80$  and the step size for XRD measurement is  $2\theta = 0.0170$ . Scanning electron microscopy (SEM) image of materials and tyrosinase immobilized materials was obtained by using a JEOL scanning electron microscope equipped with an Energy Dispersive X-ray (EDX) analyzer. FTIR spectra of materials (1 wt %) mixed with KBr pellets were recorded by diffused reflectance accessory technique. Spectra of materials were scanned in the range  $400\text{--}4000\text{cm}^{-1}$ . The resolution is  $2 \text{ cm}^{-1}$  and scan number is 16 for FTIR spectra.

### Immobilization of Tyrosinase on Sodium Alginate & Enzyme Assay

The immobilization procedure followed has been reported in our previous study (Prabhu *et al.*, 2009). The enzyme activity of Tyrosinase was estimated spectrophotometrically using L-di-hydroxyphenylalanine (L-DOPA) as a substrate according to the method described by M. Fling *et al.* (1963), with slight modification. The assay system contained 0.2 ml enzyme solution (1 mg/ml) in a 1 cm spectrophotometric cell, containing 1.3 ml of phosphate buffer (0.1 M, pH 6.8) and 1.5 ml of 5 mM L-DOPA. The change in absorbance at 475 nm at  $25^\circ\text{C}$  was recorded over the first 5 min, before and after adding immobilized enzyme. The enzyme activity was calculated by following formula:

$$\text{ENZYME ACTIVITY (U/mL)} = \frac{(\Delta A_{348 \text{ nm}} / \text{minute Test} - \Delta A_{348 \text{ nm}} / \text{minute Blank})}{\text{millimolar extinction coefficient of L-DOPA} \times V \times 1000 \times TV \times DF}$$

Where, 1000 is the conversion to micromoles, TV is the total volume of the reaction mixture, V is the volume of enzyme solution taken, and DF is the dilution factor.

A total of 1 unit of tyrosinase is defined as the amount of enzyme required for liberation of  $1 \mu\text{mol}$  of dopachrome  $\text{min}^{-1} \text{mL}^{-1}$  at  $25^\circ\text{C}$ . All experiments were performed in triplicates.

## Results and Discussion

### Characterization

Figure 1 shows two major peaks at  $2\theta$  of  $10^\circ$  and  $20^\circ$  have been observed in X-ray diffraction patterns of sodium alginate powder and sodium alginate beads indicating that the degree of crystallinity decreases in sodium alginate beads as compared to sodium alginate powder.

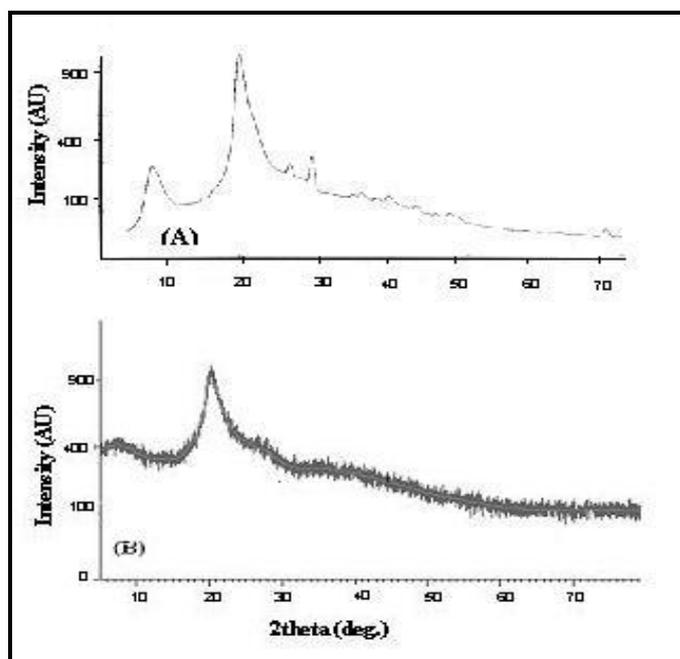


Fig 1:

The functional groups of sodium alginate are amino and hydroxyl groups which are very important for immobilization of enzyme. The FTIR spectra of sodium alginate beads are given in Figure 2. The band at  $3694 \text{ cm}^{-1}$  in sodium alginate beads is attributed to stretching vibration of N–H group. In sodium alginate beads the band at  $1575 \text{ cm}^{-1}$  has a larger intensity due to effective deacetylation. The peaks at  $2888 \text{ cm}^{-1}$  and  $1407 \text{ cm}^{-1}$  in sodium alginate beads are attributed to C–H stretching vibration in polymeric backbone and C–H bending vibration respectively.

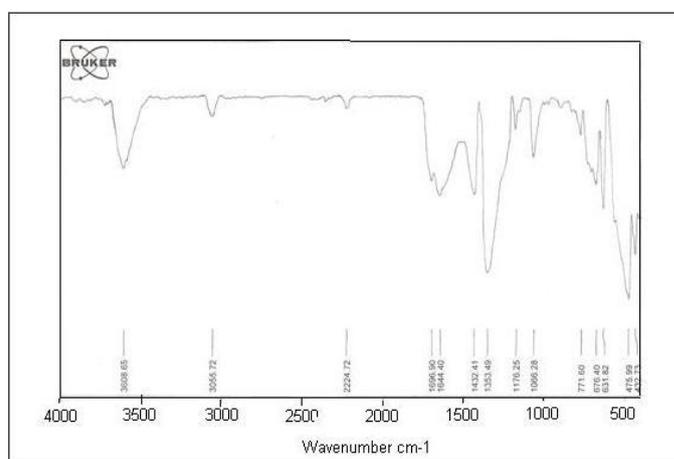


Fig 2:

### Optimization study for Tyrosinase Immobilization

Studies were carried out for optimizing the conditions for immobilization of Tyrosinase. The conditions studied are being discussed in the following sections.

#### Effect of Time Variation on Immobilization of Tyrosinase

The effect of time on immobilization of tyrosinase is shown in Figure 3. The amount of enzyme adsorbed onto the material increased with increasing the contact time up to 6h. Further increase of contact time resulted in decreased activity of immobilized enzyme may be due to leaching of enzyme from the surface of beads.

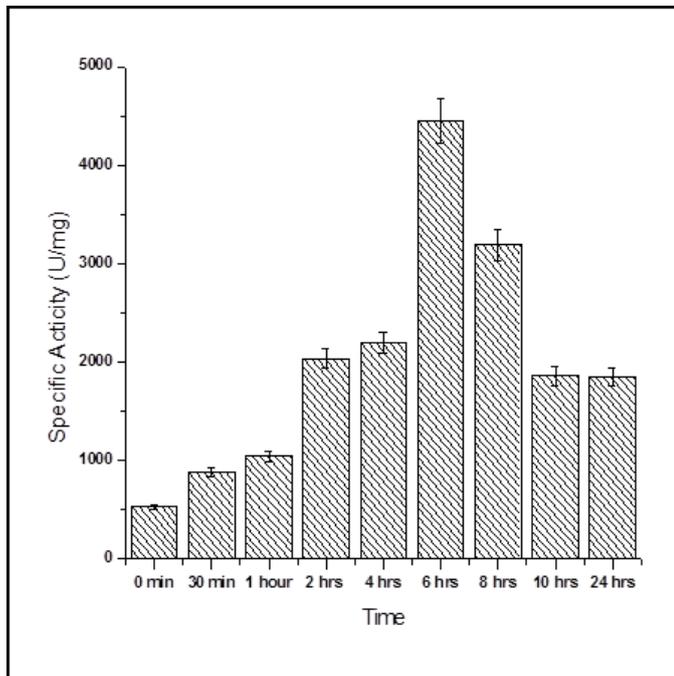


Fig 3

#### Effect of the Material Dose Variation on Immobilization of Tyrosinase

The material dose of sodium alginate beads was varied from 5 and 25 mg/5ml and the optimal dose appear to be 10 mg/5ml for the sodium alginate beads material as shown in Figure 4. Further increase in the dose resulted in decreased enzyme loading probably due to lower concentration of enzyme and higher number of active site on the material due to increased dose of adsorbent.

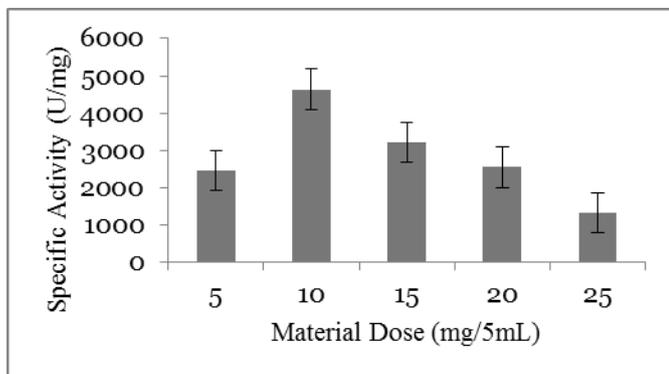


Fig 4

#### Effect of the Variation of the Tyrosinase Concentration on Immobilization

The enzyme concentration was varied from 50  $\mu\text{g}/5\text{ml}$  to 1mg/5ml, and the optimal enzyme concentration appeared to be 250  $\mu\text{g}/5\text{ml}$ , as shown in Figure 5. Further increase in the enzyme loading above 250  $\mu\text{g}/5\text{ml}$  for sodium alginate beads resulted in the gradual decrease in the enzyme activity onto the material. This is probably due to optimal adsorption of the enzyme on the matrix surface at 250  $\mu\text{g}/5\text{ml}$  for sodium alginate beads. Mansour *et al.* (2003) [3] suggested that the gradual decrease in the enzyme activity is probably due to hindrance between the adsorbed enzyme molecules on the matrix, at higher concentrations of enzyme.

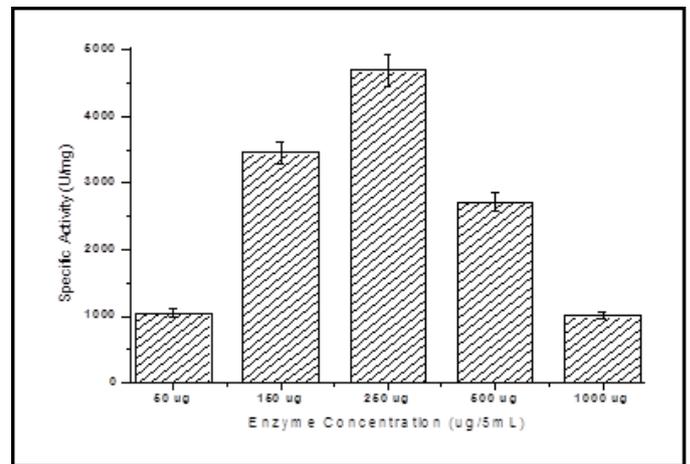


Fig 5

#### Effect of Variation of Shaking Speed on Immobilization of Tyrosinase

The effect of shaking speed on immobilization of enzyme on sodium alginate beads is shown in Figure 6 wherein shaking speed was varied from 60 to 160 rpm. The optimal shaking speed is 120 rpm for the materials. Further increase in shaking speed ultimately lowered the activity of immobilized enzyme probably due to the weakening of the interaction between tyrosinase and sodium alginate beads at high speed. Thus, summarizing from the above studies the optimal conditions for enzyme immobilization on sodium alginate beads are as follows: a) shaking time : 6h, b) material dose: 10 mg/5ml, c) enzyme conc.: 250  $\mu\text{g}/5\text{ml}$  and d) shaking speed : 120 rpm.

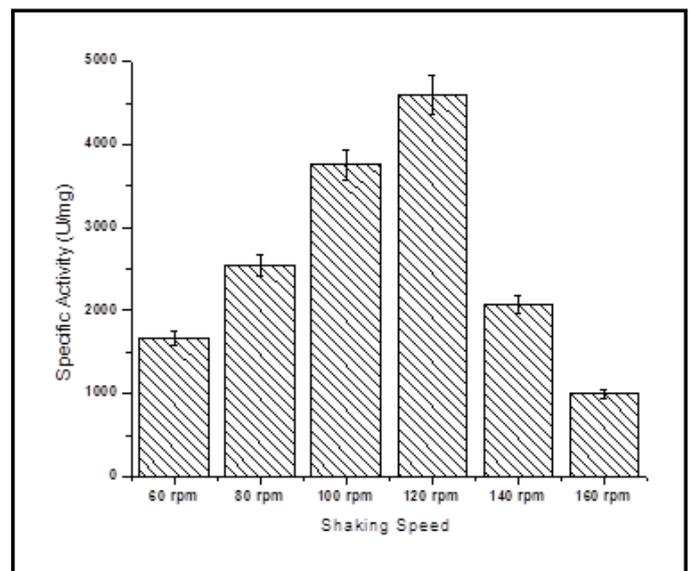


Fig 6

#### Detection of Phenol by Tyrosinase-immobilized Sodium Alginate Beads

Tyrosinase immobilized sodium alginate beads were taken in the culture tube to it added 3 ml of Phosphate Buffer and 7 ml of phenol solution (2mM). It was allowed to stand and colour was observed. The variation of phenol concentration on detection was studied in detail. Blank experiments were also carried out in the absence of enzyme.

#### Effect of Variation of Phenol Concentration

The phenol concentration was varied from 1ppm to 50ppm. Light brown colour was observed for phenol at 5 ppm while the colour was relatively dark with the increase in

concentration and almost became black at 50 ppm. At lower concentration of phenol (3 ppm), the colour change was not very prominent. However the colour change was distinct at 5 ppm. The decrease in concentration of phenol with time is shown in Figure 7. The lowest limit of visual detection of phenol was 3 ppm.

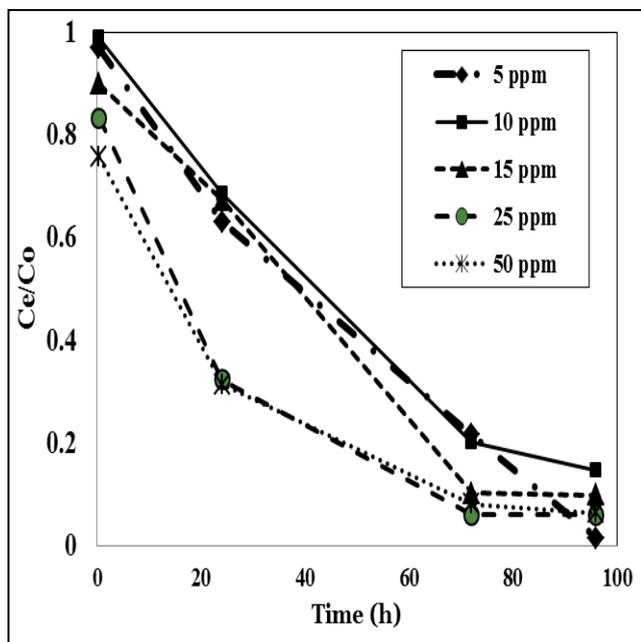


Fig 7

### Conclusions

In this research paper we have made the efforts for the development of sensor material for the detection of phenol by the immobilization of the tyrosinase on sodium alginate beads. It was observed that the immobilized enzyme was quite stable on repeated use for phenol detection. The lower detection concentration of phenol was observed to be  $3\text{ mg l}^{-1}$ . The research findings reported herein have relevance in exploring this biosensor in selective pre-concentration and visual detection of phenol. Moreover, this biosensor helps in enzymatic conversion of phenol to quinones which consequently leads to the formation of humus (humification).

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