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Polymerization of Phenol using Free and Immobilized Horseradish Peroxidase

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Abstract

Phenol is a toxic compound and need to be treated before discharged. Phenol polymerization has been studied using free and immobilized Horse Radish Peroxidase (HRP). During the study period the ambient room temperature was between 27-32^{o}C. Phenol concentration of 100-500 mg/L was fed to both free and immobilized HRP. Free enzyme studies were carried out in Erlenmayer flasks. Immobilized HRP Enzyme Bed Reactor was fabricated and used for polymerization of phenol. Free HRP polymerized 84% of phenol when fed with 100 mg/L where as Immobilized HRP polymerized 62% with the same phenol concentration. Free enzyme showed a better polymerization effect than immobilized enzyme. This might be attributed to the availability of more active sites in free enzyme when compared to immobilized enzymes. Reduction in phenol polymerization could be noticed with the increase in phenol concentration.

Keywords: Phenol, phenol polymerizing enzymes, horse radish peroxidase, enzyme immobilization, bioremediation.

1. Introduction

Widespread contamination of water by phenol has been recognized as an issue of growing importance in recent years (Idrish and Saed, 2002). Phenols and anilines are toxic contaminants in the wastewater of different industries such as plastics, resins, steels, dyes and organic chemicals (Naghibi *et al.*, 2003). Current methods applied to remove phenolic materials from wastewater include microbial degradation, adsorption on activated carbon, chemical oxidation, incineration, solvent extraction, etc. However, these methods have certain disadvantages, such as low efficiency, high cost or generation of some products that are even more toxic than the original phenols (Hejri and Saboora, 2009). Biological processes are gaining more importance over physicochemical process, as biological systems are more effective and the end products formed are non toxic (Pradeep *et al.*, 2011).

Enzymes are biological catalysts that increase the rate of chemical reactions taking place within living cells. The acceleration achieved by enzymatic catalysis is often tremendous (Bailey and Ollis, 1986). The use of enzyme based techniques to remove organic compounds from aqueous solution was first proposed by Klibanov and colleagues and has been continuously improved since then. The use of enzymatic proteins may represent a good alternative for overcoming most disadvantages related to the use of microorganisms (Rao *et al.*, 2010).

Peroxidases have been isolated from many species of plants, animals, and micro-organisms. Horseradish peroxidase (HRP) has received particular attention because of its catalytic ability under a wide range of conditions of temperature, pH, and contaminant concentrations and because its catalytic function is well-understood (**Ghioureliotis and Nicell, 1999**). Authors have reported the use of purified horseradish peroxidase (HRP) to remove 30 different phenols and aromatics amines (Cooper and Nicell, 1996; Wilberg *et al.*, 2000). Phenol conversion is activated by H_2O_2, the enzyme catalyses the oxidation of aromatic compounds, forming free radicals which undergo spontaneous polymerisation (Wilberg *et al.*, 2002). The

resulting high molecular weight compounds are less soluble in water and can be removed by gravity sedimentation and/or filtration (Zou and Taylor, 1994).

Enzymatic removal of phenolic compounds has been investigated by many researchers and it has been shown that peroxidases are able to react with aqueous phenolic compounds, however; these processes suffer from enzyme inactivation. Therefore more importance has been given to immobilization of peroxidases for the purpose of phenol removal (Iran and Siamak, 2009). For the treatment of large volumes of waste-waters, reactors containing immobilized enzymes are desirable because of the high cost of enzymes.

Most reactions catalyzed by HRP can be expressed by the following equation, in which AH_2 and AH· represent a reducing substrate and its radical product, respectively. Typical reducing substrates include aromatic phenols, phenolic acids, indoles, amines and sulfonates (Veitch, 2004).

$$H_2O_2+2AH_2 \xrightarrow{HRP} 2H_2O+2AH\cdot \quad \text{---- (1)}$$

Enzyme immobilization techniques usually provide, in addition to the desired reuse of the enzyme, unexcelled advantages such as product separation and continuous operation (Loncar *et al.*, 2011).

In the present study HRP was used for phenol polymerization. Studies were carried out using free HRP and an Immobilized HRP Enzyme Bed Reactor was fabricated and used.

2. Materials and Methods

2.1. Enzyme isolation, Activity measurement and Immobilization

Horse Radish roots were used for the extraction of Peroxidase (HRP) enzyme in the laboratory. Buffer of pH 6.8 was used to soak the roots in order to avoid the enzyme inactivation during crushing and extraction. Further, the extract was filtered using whatman filter paper No. 40 and the filtrate was centrifuged at 8,000 rpm at 25^{o}C for 30 min. Stock enzyme solution was stored at 4^{o}C and warmed to room temperature prior to use.

The activity of HRP was assayed as prescribed by Sadasivam and Manickan (2004). The assay was carried out at 25^{o}C using phenol, 4-aminoantipyrine (AAP) and hydrogen peroxide as substrates. The assay mixture contained 1.5 ml of a 20 mM phenol solution, 0.75 ml of a 9.6 mM AAP solution, 0.3 ml of 2 mM H_2O_2 solution, 0.45 ml of enzyme solution, and 0.3 ml phosphate buffer. The active enzyme concentration is proportional to the colour development rate measured at 500 nm (Sadasivam and Manickan 2004).

The colour development rate was converted to activity using an extinction coefficient of 7,100 $M^{-1}cm^{-1}$ based on hydrogen peroxide. One unit of enzymatic activity is defined as the amount of enzyme which transforms 1 micromol of hydrogen peroxide per minute at 25^{o}C.

Sodium alginate (4 grams) was weighed and transferred into a beaker containing 100 ml distilled water, the beaker was transferred on to a hot water bath to dissolve sodium alginate. Sodium alginate solution was cooled and crude enzyme (4%) was added. $CaCl_2$ solution of 0.2 M was placed on a magnetic stirrer and sodium alginate mixture containing enzyme was added drop by drop with the help of a burette. Uniform sized beads were obtained. The diameter of each bead was 8 mm. The beads were stored at 4^{o}C and warmed to room temperature prior to use.

2.2. Fabrication of immobilized enzyme bed reactor (IEBR)

The plastic column of 20.3 cm was selected and was fitted to the iron stand. A sampling port was fixed at the bottom of plastic column as shown in Figure 1. Phenol and hydrogen peroxide were added from the top of the column. The immobilized enzyme beads were filled upto 15.2 cm of the plastic column for the degradation of Phenol. The hydrogen peroxide activates the enzymes for phenol conversion (Wilberg *et al.* 2000).

2.3. Assay using free enzyme

Assay using free HRP were carried out in 100 ml Erlenmeyer flasks at room temperature. Reaction medium was prepared by adding 100 to 500 mg/L with an increment of 100 mg/L phenol, HRP enzyme (4%) and H_2O_2 (1.1 to 5.5 mM) into the phosphate buffer (pH 6.8). The volume of assay mixture was 50 ml. The reaction was initiated by H_2O_2 which was added discretely for every 30 minutes to assure phenol conversion. The conical flasks containing the assay mixture was placed on a rotary shaker to ensure complete mixing.

2.4. Assay using immobilized enzyme bed reactor

Assays were carried out in Immobilized Enzyme Bed Reactor at room temperature. Initially phenol and hydrogen peroxide of known concentrations were added from the top of the reactor. H_2O_2 was added discretely for every 30 minutes to assure phenol conversion.

2.5. Analysis of Sample for Phenol

The sample was drawn for every 30 minutes and phenol concentration was measured. The phenol concentration was varied when there was no considerable difference in two consecutive readings. The phenol concentration was detected using 4-Amino anti pyrine (4-AAP) method (Dannis 1951; Standard Methods for Examination of Water and Wastewaterm, 1985).

3. Results and Discussion

3.1. Free Horse Radish Peroxidase

Horseradish peroxidase (HRP), an enzyme isolated from the roots of horseradish, has been shown to be applicable for the treatment of several industrial wastewaters. HRP catalyzes the oxidation of phenols with hydrogen peroxide to form phenoxy radicals (Cooper and Nicell 1996). The phenol conversion efficiency was 84%, 76%, 62%, 55% and 50% at 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L and 500 mg/L respectively as shown in Figure 2. Wilberg *et al*., (2000) reported 99% phenol conversion efficiency using free HRP enzyme. Iran and Siamak (2009) attained 60% conversion of phenol using free HRP enzyme. As the concentration of phenol was increased the conversion efficiency decreased.

3.2. Immobilized HRP Bed Reactor

HRP enzyme was immobilized using sodium alginate and the so formed beads were used in Immobilized Bed Reactor. The results obtained for various phenol concentrations from 100 to 500 mg/L using HRP IBR are as depicted in Figure 3. The percentage conversion efficiencies of phenol are 62%, 57%, 54%, 50% and 46% for 100, 200, 300, 400 and 500 mg/L respectively. Iran *et al.,* (2009) reported the use of immobilized HRP and obtained 55% phenol conversion at the concentration of 200 mg/L. Lai and Lin (2005) reported the maximum removal efficiency of 25% p-chlorophenol at the concentration of 50 mg/L using immobilized horseradish peroxidase with porous aminopropyl glass beads.

3.3. Comparison of Free and IEBR HRP

Free enzyme converted 84% and immobilized enzyme in IEBR converted 62% at an initial phenol concentration of 100 mg/L. The comparison of free and immobilized HRP enzymes at various phenol concentrations (100-500 mg/L) is as shown in Figure 4. It can be clearly observed that the conversion efficiencies of free HRP enzyme are higher when compared to immobilized HRP enzymes at the concentrations of 100-500 mg/L. This was due to the fact that the free enzyme had more contact with active sites than the immobilized enzyme. Iran *et al.,* (2009) reported 60% conversion and 53% using free and immobilized enzymes respectively at initial concentration of 200 mg/L phenol.

4. Conclusions

The following conclusions are made for phenol polymerization using free and immobilized HRP:

1) Phenol polymerization of 84% was achieved at 100 mg/L using free HRP enzyme.
2) Phenol degradation of 62% was observed using Immobilized HRP Enzyme Bed Reactor.
3) As the concentration of phenol increased, reduction in phenol polymerization efficiency was observed.
4) Free HRP showed better phenol polymerization when compared with immobilized HRP bed reactor.
5) Immobilized enzyme can be recovered and reused.

Enzymatic treatment using peroxidase is a viable option for the polymerization of phenol. The results obtained indicate that HRP can be used for polymerization of phenol from wastewater.

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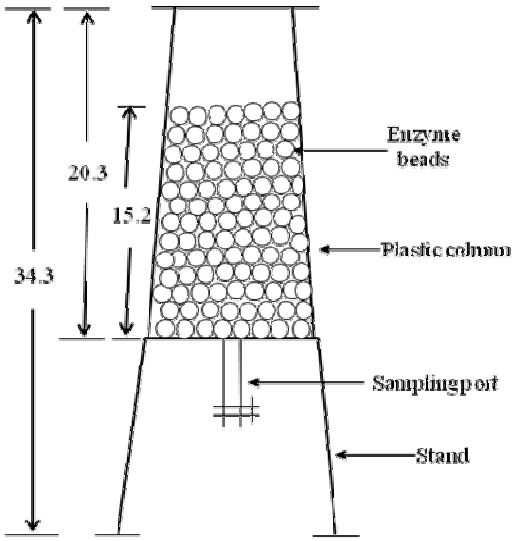

Figure 1. Immobilized Enzyme Bed Reactor. (All dimensions are in cm)

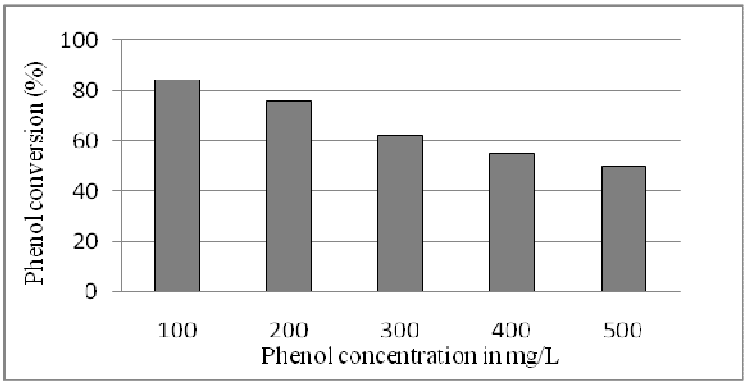

Figure 2. Percentage conversion of phenol using free HRP.

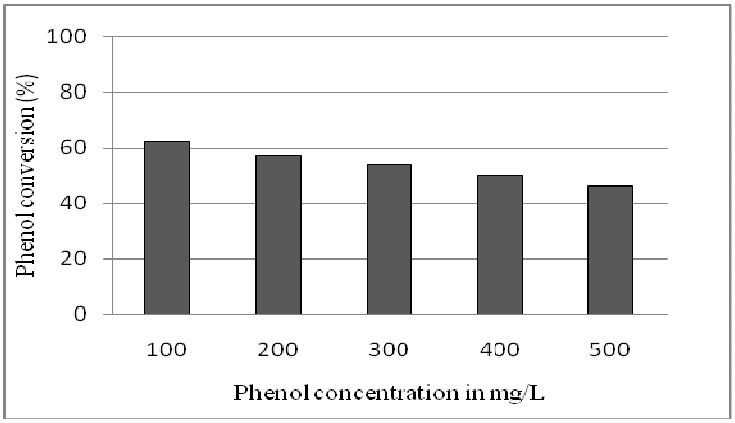

Figure 3. Percentage conversion of phenol using HRP in IEBR.

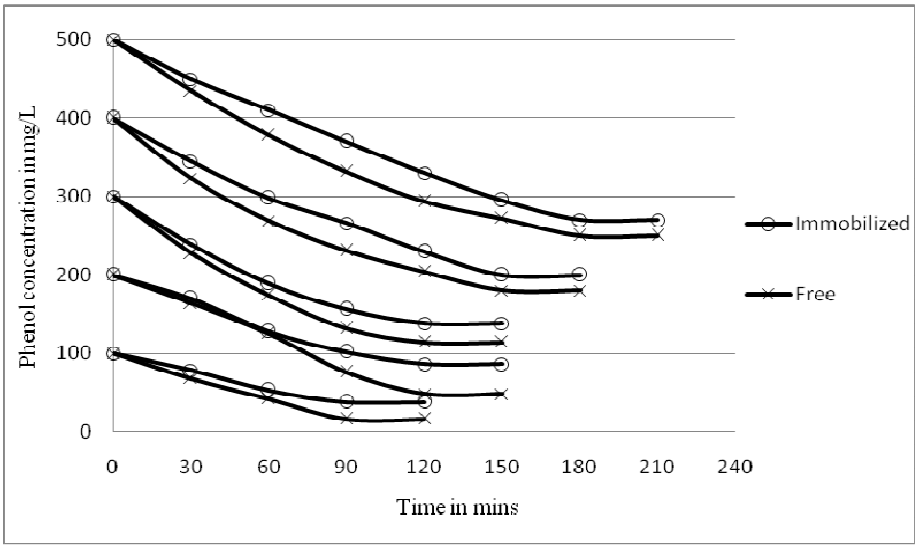

Figure 4. Comparison of free and IEBR (HRP).