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Peroxidase Catalyzed Polymerization of Phenol

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ABSTRACT

The effect of horseradish peroxidase (HRP) and H_2O_2 concentrations on the removal efficiency of phenol, defined as the percentage of phenol removed from solution as a function of time, has been investigated. When phenol and H_2O_2 react with an approximately one-to-one stoichiometry, the phenol is almost completely precipitated within 10 min. The reaction is inhibited at higher concentrations of H_2O_2. The removal efficiency increases with an increase in the concentration of HRP, but an increase in the time of treatment cannot be used to offset the reduction in removal efficiency at low concentrations of the enzyme, because of inactivation of the enzyme. One molecule of HRP is needed to remove approximately 1100 molecules of phenol when the reaction is conducted at pH 8.0 and at ambient temperature.

Index Entries: Horseradish peroxidase; phenol; hydrogen peroxide.

Nomenclature: •AH, free radical; AH_2, phenol or aromatic compound; Compound I, intermediate oxidized enzymatic form of horseradish peroxidase by H_2O_2; Compound II, intermediate oxidized enzymatic form of compound I by phenol; HRP, Native enzyme horseradish peroxidase.

INTRODUCTION

A number of investigators (*1,2,3*) have proposed a novel approach to remove phenols from waste water, based on the use of horseradish peroxidase, an oxidoreductase enzyme, with hydrogen peroxide. The treat-

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ment results in polymerization and precipitation of phenols. This approach has been tested with more than 20 different phenols (*4,5*). In most cases, 97–99%, phenols were removed in a wide range of phenol concentrations (0.0005 to 5.0 g/L).

Alberti et al. (*1*) have studied the practicability of the enzymatic approach. However, all the studies reported in the literature have focused on the final removal efficiency of phenols after several h of treatment. Information regarding the effect of horseradish peroxidase and H_2O_2 concentration on phenol removal is vital to the development of an enzymatic approach for wastewater treatment.

The effect of horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) concentration on the removal of phenol (as a function of time) is examined in this article. The aim is to find the stoichiometric relationship between HRP and phenol, and H_2O_2 and phenol. The degree of precipitation of phenol under various conditions is discussed. Studies with immobilized HRP and investigation of reaction products by GC-MS are also presented.

MATERIALS AND METHODS

Materials

Horseradish peroxidase (Type I) from Sigma (St. Louis, MO), with a specific activity of 95 purpuro-gallin units/mg (one unit forms 1.0 mg purpurogallin from pyrogallol in 20 s at pH 6.0 at 20°C), was used in all the experiments. Hydrogen peroxide (30%, specific gravity 1.1) solution was purchased from Fisher Scientific (Pittsburgh, PA). Phenol (purity 99.9%) was purchased from Mallinckrodt Company (St. Louis, MO). Citric acid (No.C-0759), sodium monohydrogen phosphate, glutaraldehyde, acetone and γ-amino-prophyltriethoxy silane were obtained from Sigma.

Immobilization of Enzyme HRP

The enzyme was immobilized on the alumina pellets (from W. R. Grace, Baltimore, MD) by the following procedure. Alumina beads (10 g; average size 3.175 mm in diameter) were silanized by immersing them in 2.5% v/v γ-aminopropyltriethoxy silane in acetone at 45°C for 24 h. The silanized beads were washed with distilled-deonized water thoroughly to remove any superficial γ-aminopropyltriethoxy silane. The beads were then immersed in 2% v/v aqueous glutaraldehyde for 2 h at room temperature. The concentration of glutaraldehyde was maintained uniformly around the beads by constant agitation. The beads were then thoroughly washed with distilled-deionized water and immersed in a known strength of enzyme solution (2000 U/mL) for 5 h at room temperature. After 5 h, the immobilized beads were thoroughly washed with distilled-deionized water and ready for use.

Experimental Procedure: (Soluble HRP)

Treatment of phenol with free horseradish peroxidase and H_2O_2 was routinely carried out in a volume of 100 mL in a 500 mL round-bottom flask at room temperature with stirring. Phenol (50 mg/L and 100 mg/L) in 0.559*M* critic acid-phosphate buffer, pH 8.0, was mixed with H_2O_2 (0.5–3.0 m*M*). The reaction was initiated by addition of horseradish peroxidase (1.0–4.0 units/mL). The solution immediately turned dark. Periodically, 6 mL aliquot was withdrawn and then centrifuged to remove the precipitate formed. The absorbance of the resulting clear supernatant was measured in a Spectronic 1000 spectrophotometer (Rochester, NY) at 271 nm. Neither horseradish peroxidase nor H_2O_2 in the concentrations used interfered with this measurement.

RESULTS AND DISCUSSION

Removal of Phenol in the Immobilized HRP System

The reaction was initiated by addition of 3.9745 g of immobilized HRP to a 100 mL buffer solution containing 50 mg/L phenol and 0.5 m*M* H_2O_2. The solution remained clear during the reaction, instead of turning dark brown as observed with the soluble enzyme system. However, the alumina beads, on which HRP was immobilized, changed from original dark pink to dark brown.

Figure 1 is a plot of phenol removal efficiency vs reaction time. It is clear that the phenol removal efficiency increases quickly during the initial reaction period, and then remains unchanged after 50 min. It was observed that the polymer precipitate generated during the reaction deposited almost entirely on the beads, thus inactivating the enzyme. This may account for the fact that the alumina beads eventually turned dark brown (because of polymer formation), though the solution containing phenol remained clear.

Removal of Phenol in the Soluble HRP System

Effect of H_2O_2 on Phenol Removal Efficiency and Apparent Reaction Stoichiometry

Figure 2 is a plot of phenol removal efficiency vs time at different initial concentrations of H_2O_2. Figure 2 also demonstrates the dependence of the removal efficiency of phenol (100 mg/L $\simeq$ 1.0 m*M*) on the concentration of H_2O_2. The concentration of HRP was 4.0 U/mL in all the runs. The phenol removal efficiency almost reaches its maximum value within the first 10 min at each corresponding initial H_2O_2 concentration. For instance, 96.90% of the phenol present is removed in the first 10 min at 1.0 m*M* initial H_2O_2 concentration. It is clear from Fig. 2 that, at H_2O_2 concentrations around 1.0 m*M*, the removal of phenol is nearly complete.

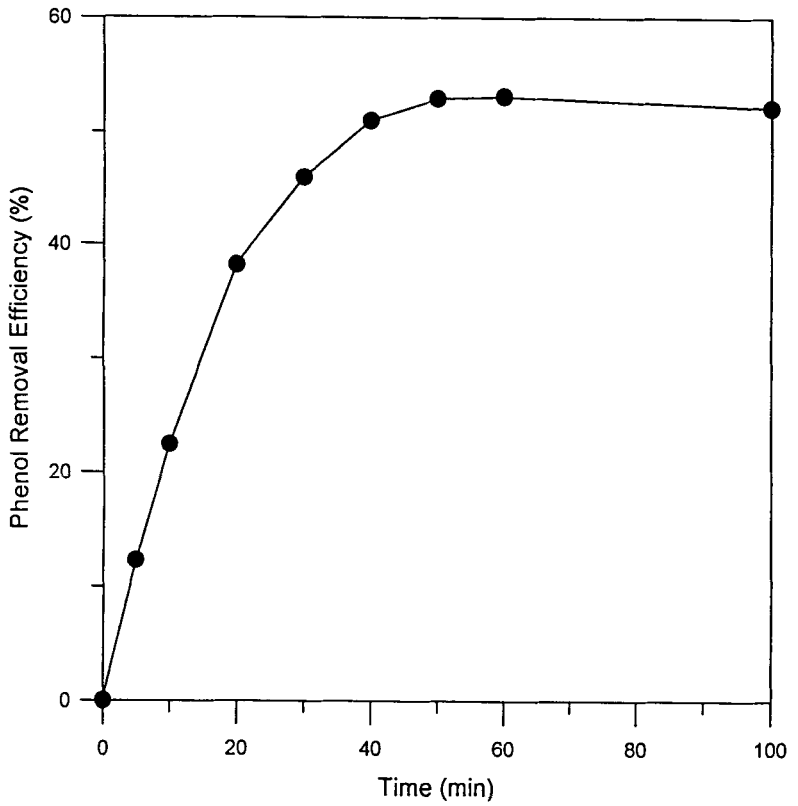

Fig. 1. Phenol removal efficiency in the immobilized HRP system. 3.9745 g HRP/α-$A1_2O_3$, 50 mg/L phenol, 0.5 m*M* H_2O_2, pH 8.0, 20°C.

When the concentration of H_2O_2 is less than 1.0 m*M*, the phenol is not completely removed because the H_2O_2 amount is limiting. However, when the concentration of H_2O_2 is increased beyond 1.0 m*M*, the removal efficiency gradually decreases, probably because of inhibition of the enzyme by H_2O_2.

Similarly, if the initial concentration of phenol is reduced by half (50 mg/L $\cong$ 0.5 m*M*), only 0.5 m*M* H_2O_2 is required to completely remove phenol (*see* Fig. 3, which is a plot of phenol removal efficiency vs time at different initial concentrations of H_2O_2). Again, most of the phenol present is removed within the first 10 min. It is also clear from Fig. 3 that when the concentration of H_2O_2 is greater than 0.5 m*M*, the removal efficiency of phenol gradually deceases with increasing concentration of H_2O_2.

It therefore appears that the optimum ratio of H_2O_2 to phenol is 1:1. Excess of H_2O_2 results in enzyme inhibition, thereby decreasing the removal efficiency of phenol. This differs favorably from the conventional treatment with an iron catalyst, in which at least a 14-fold molar excess of H_2O_2 is employed (*6*).

The one-electron oxidation of phenols (AH_2) catalyzed by horseradish peroxidase is usually depicted by the following mechanism (*7*):

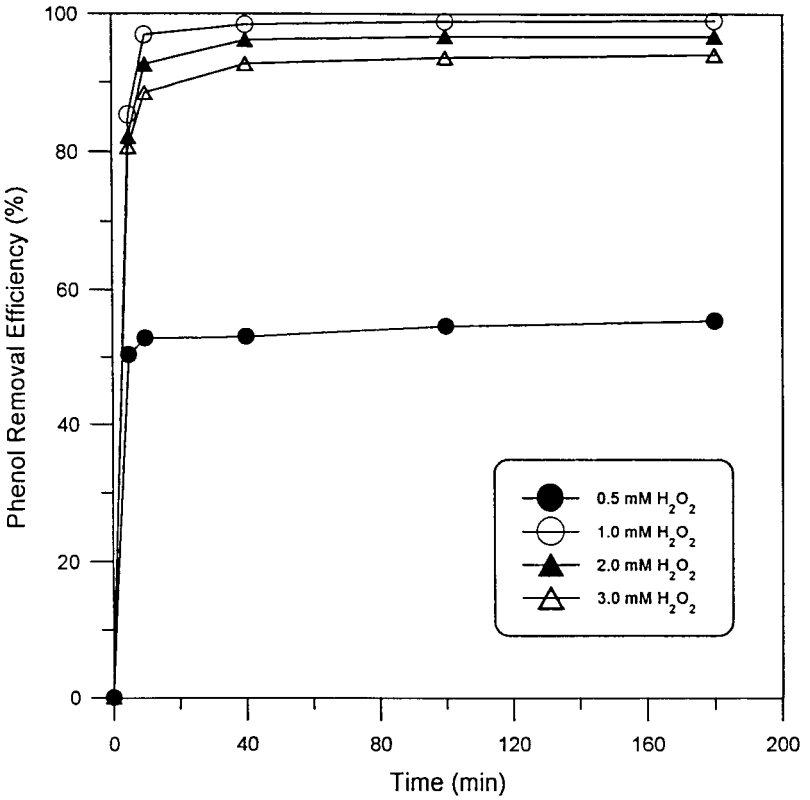

Fig. 2. Removal efficiency of phenol by HRP and H_2O_2. 4.0 U/mL HRP, 100 mg/L phenol, pH 8.0, 20°C.

$$\text{HRP} + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (1)$$

$$\text{Compound I} + AH_2 \rightarrow \text{Compound II} + \bullet AH \quad (2)$$

$$\text{Compound II} + AH_2 \rightarrow \text{HRP} + \bullet AH \quad (3)$$

In the above mechanism, hydrogen peroxide oxidizes the native HRP to an active intermediate form, Compound I. Compound I then reacts with a phenol molecule, to produce a free radical (•AH), and the enzyme complex, Compound II. Compound II then oxidizes a second phenol molecule, producing another free radical and the native HRP. The free radicals formed are released into the solution, where they can combine to form polyphenolic products. These polymers are less soluble in water and can precipitate from solution. If precipitation does not occur, the larger polyphenolic compound can react with HRP (Compound I), resulting in the formation of a still larger polymer which will eventually precipitate out. The significant feature of these final polymers is that, in contrast to their monomeric precursors, these polymers are practically insoluble in water. Therefore, peroxidase-catalyzed oxidation transforms water-soluble phenols into water-insoluble state.

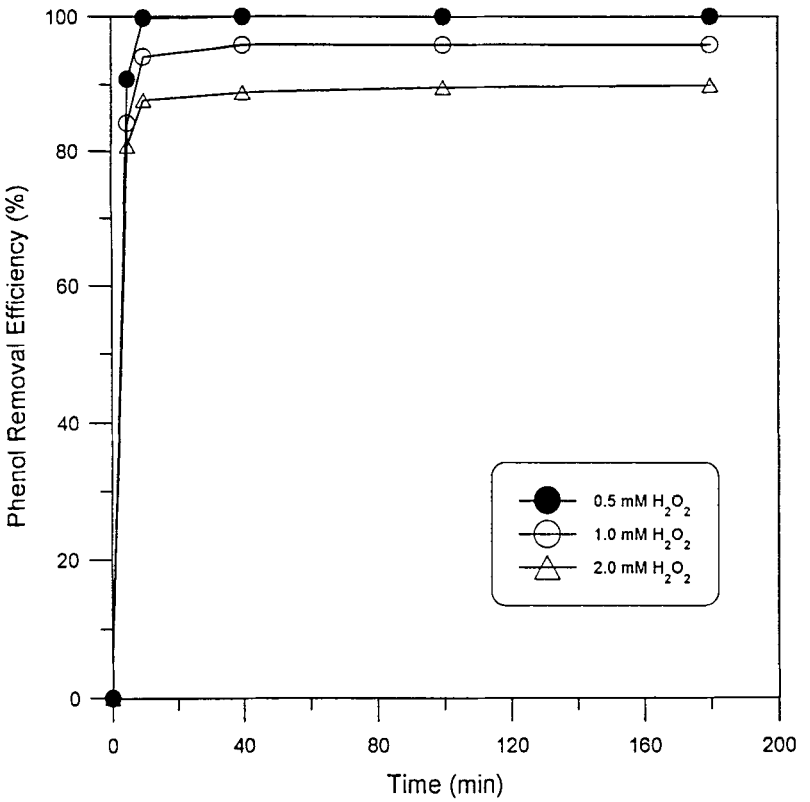

Fig. 3. Removal efficiency of phenol by HRP and H_2O_2. 4.0 U/mL HRP, 50 mg/L phenol, pH 8.0, 20°C.

The inhibition of HRP at higher concentrations of H_2O_2 may be attributed to the formation of an oxidation state of HRP designated as Compound III. HRP in Compound II state can be oxidized by excess H_2O_2 to Compound III, according to the reaction scheme presented by Nakajima and Yamazaki (*8*):

$$\text{Compound II} + H_2O_2 \rightarrow \text{Compound III} + H_2O \quad (4)$$

Compound III is not catalytically active, but its formation does not represent a terminal inactivation of HRP, since Compound III decomposes to native peroxidase according to the reaction (*9*)

$$\text{Compound III} \rightarrow \text{HRP} + O_2^{\cdot} \quad (5)$$

However, the return to native HRP is rather slow, so that, once in Compound III form, the enzyme is restricted in carrying out the catalytic oxidation of phenols. In the presence of excess peroxide, Compound III formation would increase, resulting in further inhibition of the enzyme.

The overall reaction for the enzyme-catalyzed oxidation of phenol by hydrogen peroxide may therefore be represented as (*7*):

$$H_2O_2 + 2AH_2 \rightarrow 2 \bullet AH + 2H_2O \quad (6)$$

It is clear that two free radicals are generated for every molecule of peroxide consumed. Since the free radicals polymerize spontaneously, the stoichiometric ratio of peroxide to phenol should be 0.5:1.0. As suggested by Nicell et al. (*7*), this assumes that the dimers are completely insoluble in water. However, the dimers which remain soluble can react again with HRP to form more free radicals. These radicals can combine further to form larger polymers which then precipitate. In short, as long as the polymers remain soluble, the enzyme can catalyze the oxidation of phenol and its polymers. The consumption of peroxide to phenol therefore approaches unity. This assumes that the formation of large polymer molecules does not take place via a propagation step, but only via the formation of free radicals (requiring HRP). The results from the effect of HRP concentration on phenol removal (discussed below), corroborate this assumption.

In the immobilized enzyme runs, it was observed that the solution remained clear as polymerization and precipitation took place entirely on the immobilized beads. It is theoretically possible for the free radicals to diffuse into the solution, and then combine to form dimers. These soluble dimers can then react with immobilized HRP to form more free radicals. The fact that the precipitation took place on the beads and rendered the enzyme inactive (as seen in Fig. 1, the removal efficiency reached a constant value of 54%), suggests that the polymer growth rate was much faster than the diffusion rate.

Effect of HRP on Phenol Removal Efficiency

As might be expected, the removal efficiency of phenol increases with an increase in the HRP concentration. Figure 4 is a plot of phenol removal efficiency vs time at different concentrations of HRP. At a HRP concentration of 1.2 U/mL, the phenol removal efficiency (after 3 h) was around 46%, increasing to about 74% at 2.0 U/mL, and approx 80% at 3.0 U/mL. When [HRP] = 4.0 U/mL, the phenol is almost completely removed (greater than 99% of 100 mg/L phenol). In all these cases, the initial concentration of phenol and hydrogen peroxide were 100 mg/L and 1.0 m*M*, respectively. The temperature and pH were 20°C and 8.0, respectively. The incomplete removal of phenol at low concentrations of HRP may be attributed to the inactivation of the enzyme, probably as a result of the interaction of the phenoxy radicals with the enzyme's active center, and also because of inhibition by hydrogen peroxide. The increase in removal efficiency of phenol with an increase in HRP concentration indicates that the conversion of phenol to polyphenolic compounds occurs via the formation of free radicals, requiring HRP (*see* reaction mechanism, Eqs. 1–3).

It is clear from Fig. 4 that the removal efficiency increases rapidly during the first 10 min, and then remains practically unchanged during the next 110 min. An increase in the time of treatment, therefore, cannot be used to offset the reduction in removal efficiencies at low concentrations of the enzyme. This differs from the removal of carcinogenic aromatic amines with HRP, in which an increase in the time improves the removal even at lower enzyme concentrations (*5*).

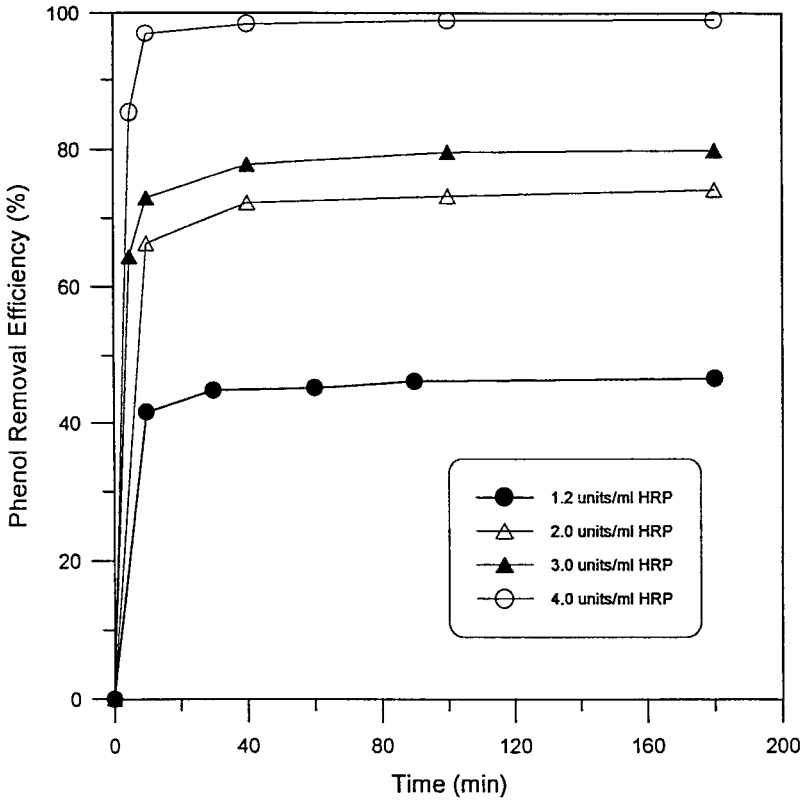

Fig. 4. Phenol removal efficiency by HRP and H_2O_2. 100 mg/L phenol 1.0 m*M* H_2O_2, pH 8.0, 20°C.

Figure 5 is a plot of phenol removal efficiency vs time under two different conditions, but having the same mol ratios of HRP over phenol and H_2O_2 over phenol. From Fig. 5, we can see that by halving the initial concentration of phenol ([phenol] = 50 mg/L), only 2.0 U/mL of HRP are needed to obtain complete removal of phenol. Therefore, peroxidase-catalyzed polymerization for complete removal of phenol is equally effective at different conditions, provided that the [HRP]/[phenol] ≃ 1:1100 (mol) and [H_2O_2]/[phenol] ≃ 1:1 (mol). Nicell et al. (*7*) also observed that by extrapolation of the phenol curve (a plot of phenol concentration vs initial H_2O_2 concentration), the ratio of [H_2O_2]/[phenol] approached unity.

Detection of Reaction Soluble Byproducts

In most situations where enzymatic treatment processes might be considered for use in the field, the treated effluent would either be discharged directly to a receiving water (effluent polishing), or to a subsequent on-site or off-site wastewater treatment system. Thus, it is important to detect if there are any soluble byproducts of the enzymatic reaction, and to characterize these byproducts if they are indeed present.

GC-MS analysis was carried out before and after removal of phenol (greater than 99%), respectively. The reaction conditions were 4.0 U/mL

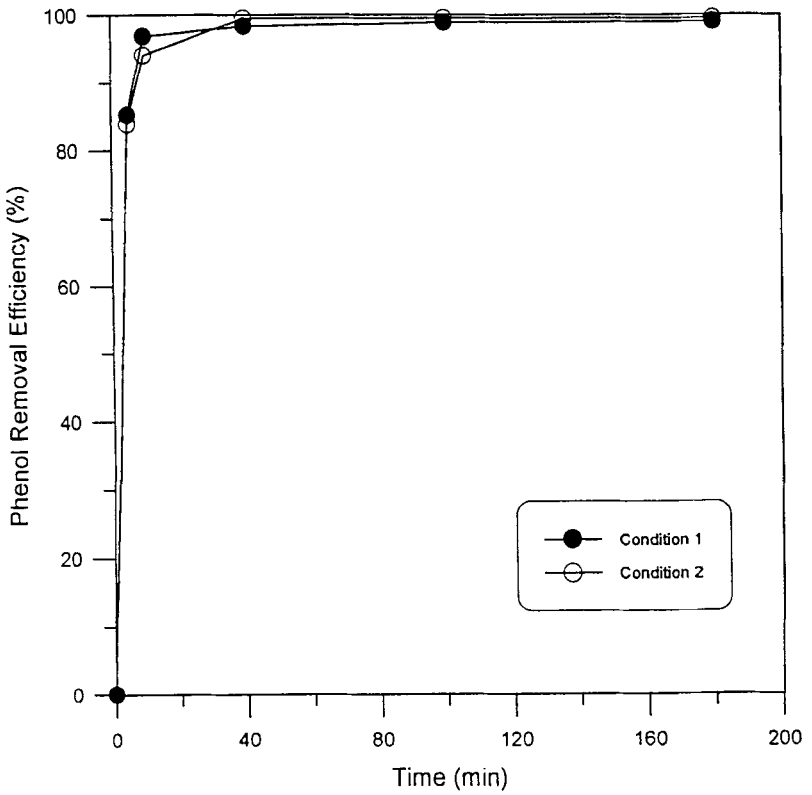

Fig. 5. Comparision of phenol removal under two conditions with the same mol ratios of HRP over phenol and H_2O_2 over phenol: Condition 1 (●)-4.0 U/mL HRP, 1.0 m*M* H_2O_2, 100 mg/L phenol; Condition 2 (○)-2.0 U/mL HRP, 0.5 m*M* H_2O_2, 50 mg/L phenol.

HRP, 100 mg/L (≅ 1.0 m*M*) phenol in 0.559*M* citric acid-phosphate buffer (pH 8.0), and 1.0 m*M* H_2O_2 at room temperature with agitation for 3 h. The analysis revealed that the phenol peak completely disappeared, and that no additional peaks (except the buffer—sodium mono hydrogen phosphate) were present after 3 h treatment. A gravimetric analysis of the reaction was also carried out. Before reaction, the total mass of horseradish peroxidase and phenol was 0.0145 g. After reaction, the precipitate formed was 0.0137 g. Therefore, about 94.5% transformation is achieved, which could be considered as nearly complete precipitation of phenol, indirectly confirming the existence of very little soluble byproducts after 3 h treatment.

CONCLUSIONS

Horseradish peroxidase (HRP) catalyzes the precipitation of phenol from aqueous solution by H_2O_2. The apparent reaction stoichiometric ratio of phenol to H_2O_2 is about 1:1. The reaction is inhibited in the presence

of higher concentrations of H_2O_2. One molecule of HRP is required to remove approximately 1100 molecules of phenol at pH 8.0 and at room temperature. Under optimum reaction conditions, most of the phenol is removed within 10 min. GC-MS studies reveal that no soluble products are present after 3 h treatment. Immobilized enzyme studies indicate that the insoluble products are completely precipitated on the support itself, leaving a clear solution behind.

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