Removal of Phenolic Compounds from Synthetic Wastewaters by Enzymatic Treatments

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Abstract

Many industries generate phenolic pollutants during their manufacturing processes. Most of these compounds are toxic and have been classified as hazardous pollutants. Soybean peroxidase (SBP) catalyzes the oxidation and polymerization of phenolic compounds in the presence of hydrogen peroxide. The polymerized products can easily precipitate and be filtered from the solution. In this study phenolic solutions such as synthetic wastewater containing phenol, *o*-cresol and *m*-cresol, were treated with soybean peroxidase (SBP). The effect of some parameters was investigated on the removal process. The results showed that, an increase in hydrogen peroxide up to the optimum amount leads to an elevated removal of phenolic compounds. Higher concentrations of H_2O_2 inhibited the reaction. Treatment in the presence of PEG (polyethylene glycol) as an additive increased the effect of enzymatic removal. Application of different concentrations of the enzyme in the reaction mixture showed positive regression between enzyme concentration and removal of phenols. Studies to determine the optimum pH for enzyme activity revealed that elimination of phenols was improved in neutral pH. In addition, this study revealed that intact soybean seed-hulls were effective on the elimination of phenolic compounds in synthetic wastewater; treatment time significantly affected the concentration of seed-hulls needed for removal of phenols.

Keywords: Glycine max, Soybean peroxidase, phytoremediation, phenolic compounds, PEG

Introduction

Many industries such as coal conversion, wood preservation, metal casting, the petroleum refining industry and pulp and paper manufacturing, generate phenolic pollutants during their manufacturing processes. Most phenolic compounds are toxic and have been classified as hazardous pollutants (Caza *et al.* 1999, Ikehata *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. (Miland *et al.* 1996). 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. For these reasons, more attention has been paid the development of alternative and/or complementary technologies for removal of toxic organic pollutants in water and soil, such as phytoremediation, or reduction of their concentration (Pletsch *et al.* 1999).

The plant enzymatic systems involved in phytoremediation include nitroreductases, glycosyl and glutathione transferases, oxidases, phosphatases, etc. These enzymes are implicated in the transformation of toxic xenobiotic compounds such as explosives, pesticides and halogenated organic compounds. In addition, plants and many microorganisms contain several oxidases, such as peroxidases, which are involved in the removal of different pollutants (Wolfe & Hoehamer 2003). In particular, peroxidases are constitutive and inducible enzymes that are expressed as soluble, extracellular and membrane bound proteins from some plant tissues, where oxidation of aromatic substrates occur using H_2O_2 as a co-substrate (González *et al.* 2006).

Enzymatic treatment using peroxidase and hydrogen peroxide was proposed in the early 1980s as an alternative treatment, which is highly selective and efficient for removing phenols from their aqueous solutions (Klibanov *et al.* 1980). Enzymatic polymerization offers the advantages of low process energy requirements and the low solubility of the polymerized product (Flock *et al.* 1999). The roles of different classes of peroxidase isoenzymes in the removal process need to be clarified. In this work, we studied the use of partially purified peroxidase from soybean hulls to remove phenolic compounds

from synthetic wastewater. We established the optimum conditions in order to obtain the maximum efficiency in the process. Furthermore, we analyzed the participation of different factors in phenol removal to elucidate which of them were most likely to be involved in this process, based on their removal efficiencies.

Materials and methods

Materials: Soybean seeds (*Glycine max* var. Williams) were supplied from Oil Seed Company (Tehran, Iran). Polyethylene glycol (PEG with average molecular mass of 35000 and 6000), hydrogen peroxide (30% v/v), phenolic compounds (phenol, *m*-cresol and *o*-cresol), 4-aminoantipyrine (4-AAP), potassium ferricyanide, sodium phosphate dibasic, sodium phosphate monobasic, sodium bicarbonate, sodium citrate and guaiacol were purchased from Merck Chemical Company (Germany).

Enzyme extraction and purification: Soybean peroxidase was extracted from the seed-hulls by the following procedure. One hundred grams of the soybean seed-hulls were mixed with sodium phosphate buffer solution (0.02 M, pH 7.0) containing 2% polyvinylpolypyrrolidon (PVPP) in a cold room. The crude extract was centrifuged at 15000 g in 4ºC for 45 min and then the supernatant was filtered through whatman N. 1. In the first step, ammonium sulfate was added to gain 30% saturation in the supernatant. After 6 hrs, the resulting solution was centrifuged at 15000 g (4ºC) for 45 min. In the second step, ammonium sulfate was again added to 80% saturation in the supernatant and then the solution was kept for 12 hrs. The resulting solution was centrifuged at 15000 g (4ºC) for 45 min. The pellet was re-dissolved in phosphate buffer solution (0.02 M, pH 7.0) and then solution was dialyzed against the same buffer. The dialyzed solution was installed in an ion exchange chromatography column (DEAE Sephadex-A50). The column was equilibrated with an eluent consisting of 0.02 M phosphate buffer (pH 6.8) followed by a gradient of NaCl solution to a final concentration of 1.5 M in the buffer. Each 3 ml of the solution as a fraction was collected from the outlet of the column. Peroxidase activity was assayed as described in below in each step of the purification and for all collected fractions from the column. Fractions with peroxidase activity were mixed and used as enzyme extract.

Enzyme activity assay: Protein content was measured according to Bradford method (1976) using bovine serum albumin (BSA) as standard protein. Enzyme activity of the soybean peroxidase (SBP) was determined according to Liu *et al.* (1999) by measuring the oxidation of guaiacol to tetraguaiacol. Assay solution (3 ml) was contained 0.95 ml sodium citrate buffer (0.1 M, pH 4.6), 1 ml of 15 mM guaiacol, 1 ml of 1.6 mM H_2O_2 and 50 µl of enzyme extract. The initial change in absorbance per min at 470 nm was recorded by a PHILIPS 8620 UV/VIS/NIR spectrophotometer (PHILIPS, England). One unit of peroxidase activity represents the amount of enzyme that catalyzes the oxidation of one micromole of guaiacol in one minute.

Experimental procedures: The enzymatic reaction mixture (10 ml) was consisted of 1 mM H_2O_2, 1 U ml^{-1} SBP and 1 mM from one of the phenolic compounds in sodium phosphate buffer (0.1 M, pH 7.0). Reactions were initiated by adding H_2O_2. The solutions were shaken slowly for 2 hrs. One ml of each reaction mixture was then centrifuged and the supernatant was assayed for remaining phenolic compounds. Treatment condition was modified by changes in pH value and addition of PEG into reaction mixture for increase of enzymatic phenol removal. In other experiments which efficiency of the method was studied in related to the changes of H_2O_2 and SBP concentrations, the amounts of each two component was varied in the reaction mixture while total volume of the reaction mixture was kept constant. All experiments were repeated at least 2 times in each series, concentration of the phenolic compound was detected at the end of experiments in a three replicate.

Total phenol assay: The concentration of phenolic compounds was colorimetrically determined using 4-AAP and potassium ferricyanide. Phenolic compounds were reacted with 4-AAP under alkaline conditions to yield an intermediate species which then was oxidized in the presence of potassium ferricyanide reagent. The resulting compound is a quinonetype dye which absorbs light at 510 nm (Ghioureliotis and Nicell 1999). One ml of the assay cocktail was consisted of 100 µl of 20.8 mM 4-AAP, 100 µl of 83.4 mM potassium ferricyanid, 750 µl of 0.25 M sodium bicarbonate and 50 µl of the phenolic solution. After 10 min, the absorbance was measured at 510 nm. The generated color was directly proportional to the concentration of the

phenolic compound.

Results

Some factors were changed in the reaction mixture to determine optimum conditions. Also, this was made to identify relationship between percentages of total phenol removal and process variables.

In this research the effect of H_2O_2, SBP and PEG concentrations, pH value and also, amount of intact soybean seed-hulls were studied on the removal of phenolic compounds from synthetic wastewaters. Chemical structures of those compounds are shown in Figure 1. Immediately after addition of peroxidase and H_2O_2 to the reaction mixture, the synthetic wastewater (phenolic solutions) was become colored. Color of the solutions was varied between milky white, yellow and brown depending on the phenolic substrate; *m*-cresol, *o*-cresol and phenol, respectively. Subsequently a precipitate was formed.

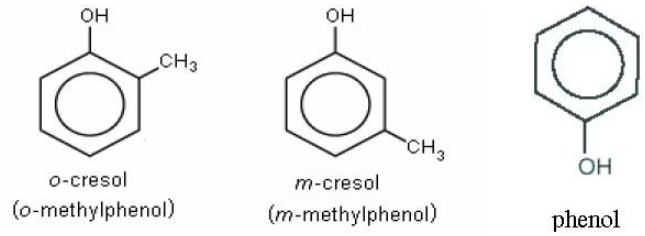

Fig. 1: Chemical structures of phenol, m-cresol and o-cresol.

The effect of pH value: The first parameter that was optimized was pH value. All experiments for determination of the optimum pH were performed in 0.1 M citrate- phosphate buffer with pH 3.0 to pH 7.0. Figure 2a illustrates that the removal efficiency of the phenols improved by increasing the pH value. The percentage of phenol removal was 11% in pH 3 and it was elevated to 47% in pH 7. Experiments with other phenolic compounds showed a similar pattern. Percentages of *m*-cresol and *o*-cresol removal were 20% and 11% in pH 3 and these were elevated to 49% and 57% in pH 7, respectively.

The effect of H_2O_2 concentration: The second parameter which was optimized was H_2O_2 concentration in the reaction mixture. The results for the treatment of 1.0 mM phenolic compounds with 1U ml^{-1} SBP and variable amounts of H_2O_2 are shown in Fig. 2b. The amount of remaining phenolic compounds was decreased with increasing concentrations of initial hydrogen peroxide up to an optimum value. The results indicated a definite optimum point. The highest percentage of the phenols removal was achieved 51% for *o*-cresol, 56% for *m*-cresol and about 50% for phenol by 0.5, 0.75 and 1 mM H_2O_2, respectively. As the amount of H_2O_2 was increased beyond the optimum point, the rate of phenolic conversion was decreased. It seems that high concentrations of H_2O_2 inhibit enzyme activity.

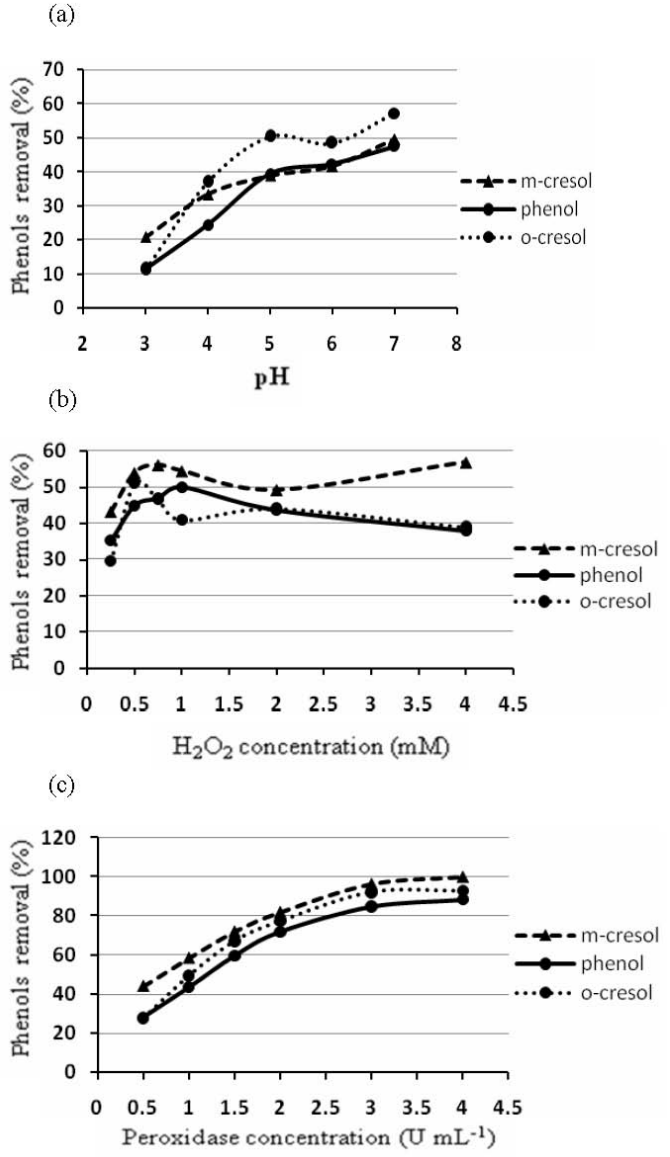

Fig. 2: The effect of pH value, H_2O_2 and SBP concentration on removal of some phenols.

The effect of SBP concentration: The effect of the presence of SBP units was examined on the removal of phenols. One mM H_2O_2 and variable concentrations of SBP (based on guaiacol assay) were added to the reaction solutions. The results

showed that by increasing SBP concentration, transformation of the phenols was increased (Fig. 2c). For *o*-cresol and *m*-cresol, the removal percentage was achieved to more than 90% by application of 3 U ml^{-1} SBP. Extra concentration of SBP in the reaction mixture, above the optimum concentration, did not significantly affect the improvement of the phenols removal. For example, the percentage of *o*-cresol removal was 92% by application of 3 U ml^{-1} SBP and 92.7% by application of 4 U ml^{-1} SBP.

The effect of Concentration and molecular weight of PEG: Experiments were conducted to evaluate the effect of PEG concentration and its molecular weight on the catalytic life of SBP during the chemical reaction. The results of the application of PEG-6000 and PEG-35000 are illustrated in Fig. 3a and 3b. More than 94% of *o*-cresol was removed by application of 25 mg/L PEG-6000. The optimum amounts of PEG-6000 for increasing of its effectiveness in removal process were obtained by addition of 25, 50 and 150 mg/L PEG-6000 for *o*-cresol, *m*-cresol and phenol, respectively (Fig. 3a). The higher removal percentage (more than 90%) was achieved by application of 25 mg/L PEG-35000 for *o*-cresol and phenol. Therefore the optimum amounts of PEG-35000 were 25 mg/L for *o*-cresol and phenol but 50 mg/L PEG-6000 was more suitable for remove of *m*-cresol (Fig. 3b).

Application of the intact soybean seed-hulls: In order to protect the enzyme from immediate inactivation, intact seed-hulls were used in the reaction solution. As the amount of seed-hulls was increased, conversion of phenol and *o*-cresol to polymeric products was increased during 2 hrs. Figure 3c shows that maximum conversion for *m*-cresol was achieved with 60 g/L of seed-hulls after 2 hrs. After 24 hrs, the difference between the amounts of the seed-hulls did not affect phenol and *m*-cresol removal (Fig. 3d). In the case of *o*-cresol, 24 hrs after application of 10, 20, 40 and 60 g/L of intact seed-hulls in the reaction mixture, the rate of the conversion did not show any considerable difference. But the presence of 80g/L intact seed-hulls was more effective and caused a greater conversion of phenolic compounds (about 90%).

Discussion

This research showed that, soybean peroxidase is a brilliant molecule to effectively treat a variety of phenolic compounds. Since, the soybean hulls have been shown to be a rich source of peroxidase (Gillikin & Graham 1991, Parsiavash 2007) and the seed coat of the soybean is a waste product of the food industry; therefore soybean shells could provide a cheap and abundant source of peroxidase. The broad pH and temperature stability of SBP and simplicity of its extraction from the seed-hulls make it an excellent candidate for the bioremediation of phenols.

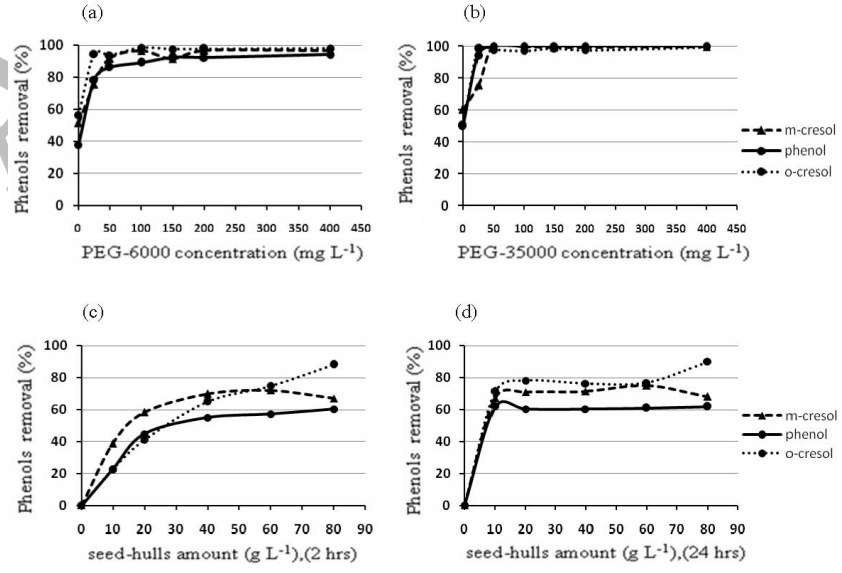

Fig. 3: The effect of addition of PEG (a, b) and seed hulls (c, d) to reaction mixture on removal of some phenols.

The involvement of peroxidases in the oxidative polymerization of the phenols was previously demonstrated by several authors (Klibanov *et al.* 1980, Buchanan and Nicell 1997, Wright and Nicell 1999). Peroxidase enzymes catalyze the oxidation of a wide variety of aromatic compound by hydrogen peroxide. Some peroxidases from different sources such as horseradish (HRP), *Coprinus cinereus* (CiP), *Coprinus macrorhizus* (CMP), *Arthromyces ramosus* (ARP) have been studied (Klibanov *et al.* 1980, Klibanov *et al.* 1981; Al-Kassim *et al.* 1993, 1994; McEldoon *et al.* 1995).

Our research described the removal of several phenolic compounds from synthetic wastewater via treatment with SBP and H_2O_2. Determination of the optimum pH for enzyme activity revealed that elimination of phenols was improved in neutral pH. The optimum pH was 7 for all three phenolic compounds. Thus based on these results, phosphate buffer with pH 7.0 was used for these experiments. The results showed that, an increase in hydrogen peroxide concentration up to the optimum amount leads to an elevated removal of phenolic compounds. At higher concentrations, H_2O_2 displayed an inhibitory effect. Application of different concentrations of SBP (0.5-4 U ml^{-1} based on guaiacol assay) in the reaction mixture showed positive correlation between enzyme concentration and removal of phenols. But, higher enzyme concentrations in the reaction mixture had no significant effect on the removal efficiency. This finding is similar to the results obtained by Caza *et al.* (1999).

In order to protect the enzyme from immediate inactivation, PEG was used because it was inexpensive and non-toxic. Phenols removal was dramatically increased when PEG was added. The presence of a few amounts of PEG improved the removal of phenolic compounds. Thus, PEG could be an effective additive for protecting SBP during the enzyme-catalyzed polymerization and precipitation of phenols and could reduce enzyme requirements. In the presence of PEG, the removal efficiency for *o*-cresol was higher than other phenolic compounds. There was no considerable difference between the effect of PEG-6000 and PEG-35000 on phenols removal. These results are consistent with the results obtained by Tonegawa *et al.* (2003). The addition of PEG improved the removal efficiency up to an effective minimum concentration. Beyond that, excess amounts of PEG neither increased nor decreased the removal efficiency.

Many researchers have studied the interaction of PEG with water molecules. They have concluded that PEG binds water molecules and creates a relatively large hydrated volume (Osterberg *et al.* 1995, Harris 1992, Donato *et al.* 1995). When PEG molecule attached to a surface of reaction products; it can reject proteins (Gombotz *et al.* 1992). So, too much energy is required for peroxidase to penetrate into a hydrated layer. Thus, peroxidase molecules don't bind to the surface of reaction products and remain active.

Since, SBP has been found to be readily extracted by soaking the soybean seed-hulls in water or buffer solutions (Flock *et al.* 1999); it could be possible to use the seed-hulls directly in the enzymatic treatment. Application of the intact seed-hulls in synthetic wastewaters had a greater effect on the removal efficiency of *o*-cresol in compare with phenol or *m*-cresol. Application of intact soybean seed-hulls instead of the purified enzyme could reduce the cost of remediation in polluted environments.

The seed-hulls would allow the slow leaching of the enzyme and also act to absorb the polymerized products (Flock *et al.* 1999). This hypothesis was investigated using various quantities of seed-hulls directly in the reaction. The efficiency of phenols removal in the presence of 10 g L^{-1} seed-hulls during 24 hrs was equal to the application of 60-80 g L^{-1} seed-hulls in 2 hrs. Thus, in order to avoid environmental pollution, it is recommended that the wastewater is treated by a lower amount of seed-hulls in the long term instead of application of high amounts in a short time.

The obtained results and optimum conditions attained in this research can be used for phytoremediation and increasing the efficiency of removing phenolic compounds in the effluent.

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