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REMOVAL OF PHENOL BY ENZYMATIC OXIDATION AND FLOTATION

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Abstract - This work presents a process for phenol removal comprising a reaction step in which phenol is polymerized in the presence of an enzyme followed by a separation step involving dissolved air flotation (DAF). A crude preparation from horseradish roots was used as a low purity source of the enzyme horseradish peroxidase (HRP). The technical feasibility of the process was studied at bench scale using 1 to 10 mM synthetic phenol solutions. Experimental results showed the potential of the proposed technique. A phenol conversion higher than 99 % was observed at the polymerization step and an efficiency higher than 94 % was achieved at the separation stage. Despite the use of a low purity source of HRP, which increases the input of organic matter, the chemical oxygen demand (COD) decreased by 50 %.
Keywords: Phenol removal, horseradish peroxidase, flotation

INTRODUCTION

Aromatic compounds are found in wastewaters of various industries such as petroleum refining, coal conversion, plastics, resins, textiles, iron and steel manufacturing as well as pulp and paper manufacturing. Many of these compounds are toxic and some are known or suspected carcinogens (Nicell *et al.*, 1993).

Conventional processes for removal of phenols and aromatic amines from industrial wastewaters include extraction, adsorption on activated carbon, steam distillation, bacterial and chemical oxidation, electrochemical techniques, irradiation, etc. All of these methods suffer from serious drawbacks as high costs, incompleteness of purification, formation of hazardous by-products, low efficiency and applicability to a limited concentration range (Klibanov *et al.*, 1980). Due to these drawbacks alternative methods may become important in a large scale in the near future. The treatment through enzymatic catalysis seems to have the potential to substitute conventional methods (Karam and Nicell, 1997).

A catalytic process which uses horseradish peroxidase (HRP), an enzyme isolated from the roots of horseradishes, has been shown to be applicable for the treatment of several industrial wastewaters (Alberti and Klibanov, 1980; Klibanov *et al.*, 1983; Cooper and Nicell, 1996). The main advantages of this method, according to some researchers (Klibanov *et al.*, 1981, 1983, 1986) are:

1) broad substrate specificity;

2) effectiveness over a wide range of operating conditions including pH, temperature, salinity and substrate concentrations;

3) ability to remove other organic compounds by co-precipitation.

One-electron oxidation of aromatic substrates (AH_2) catalyzed by peroxidases is depicted by the Chance-George mechanism (Nicell, 1994) as the following:

$$E + H_2O_2 \rightarrow E_i + H_2O \quad (1)$$

$$E_i + AH_2 \rightarrow E_{ii} + AH^{\bullet} \quad (2)$$

$$E_{ii} + AH_2 \rightarrow E + AH^{\bullet} + H_2O \quad (3)$$

$$E_{ii} + H_2O_2 \rightarrow E_{iii} + H_2O \quad (4)$$

The native enzyme (E) is oxidized by peroxide (H_2O_2) to an active intermediate enzymatic form called compound I (E_i). Compound I accepts an aromatic compound (AH_2) into its active site and carries out its oxidation. A free radical ($AH^{\bullet}$) is produced and released into solution leaving the enzyme in the compound II (E_{ii}) state. Compound II oxidizes a second aromatic molecule, releasing another free radical product and returning the enzyme to its native state, thereby completing the cycle. The overall peroxidase reaction consists of the reactions described by Equations (1), (2) and (3). Free radicals formed during the cycle diffuse from the enzyme into the bulk solution where they react to form polyaromatic products. These polymers are water-insoluble and may be removed by solid-liquid operations (Nicell, 1994).

In the presence of excess peroxide, the reaction of Equation (4) becomes important because compound III (E_{iii}) is a reversibly inactivated form of the enzyme. This implies that HRP is inhibited by H_2O_2 in excess. On the other hand, lack of peroxide during the reaction step limits the rate of reaction. The semi-batch addition of H_2O_2 to maintain an optimized ratio between peroxide and HRP concentrations was found to suppress this inhibition (Wu *et al.*, 1994).

Significant enzyme inactivation may also occur during the polymerization step. Free radicals generated in the catalytic cycle adsorb to the enzyme's active site hindering the access of substrates. By employing additives, such as polyethylene glycol (PEG), the apparent enzyme inactivation is alleviated to drastically reduce the amount of enzyme required. PEG combines with the polymers and is separated from the solution as precipitate (Wu *et al.*, 1993).

The enzymatic treatment efficiency was found to be independent of the enzyme purity and therefore, it was possible to utilize a crude enzyme preparation instead of a purified one. This feature leads to a significant reduction in treatment costs (Alberti and Klibanov, 1982; Cooper and Nicell, 1996).

This work aims at developing an enzymatic process to remove toxic organic compounds from industrial wastewaters comprising the following aspects:

1) Use of crude enzyme preparations as a peroxidase source;

2) Meet COD emission values;

3) Provide means for continuous polymerization of phenol in the wastewater;

4) Separation of the polymers formed by flotation;

5) Utilization (use as a raw material) of the polymers generated.

6) Process economics

The present study focuses on the evaluation of parameters leading to phenol polymerization using crude enzyme preparation from horseradish roots over the phenol concentration range of 1 to 10 mM (0.1 to 1 g/L - typical in wastewaters). Reactions were conducted under the action of PEG, an enzyme protector additive and H_2O_2 was added from time to time in a semi-batch manner.

It was also investigated the feasibility of using flotation to separate the polymers produced from the reaction medium. The polymers formed adhere to air bubbles injected in the suspension and are carried to the liquid-air interface. Air bubbles used in the dissolved air flotation (DAF) technique are generated by pressurizing a liquid in a saturation vessel. As the liquid is released to a flotation cell through a flux constraint, bubbles ranging from 0.02 to 0.10 mm diameter are generated, collide and adhere to the polymer fine particles through hydrophobic forces (Rubio, 1998).

MATERIALS AND METHODS

Materials

Purified catalase from bovine liver (EC 1.11.1.6, 1,300,000 U/mL suspension) was purchased from Merck (Germany). Polyethylene glycol (average molecular mass of 1,500) was obtained from Synth (Brazil). Hydrogen peroxide (30 % w/w) and phenol (99 %, loose crystals) were purchased from Nuclear (Brazil). All other chemical used were of analytical grade.

Low purity HRP was obtained by passing washed horseradish roots through a commercial juicer. The extract was centrifuged at 8,000 rpm, 25 ^{o}C for 30 min. Stock enzyme solution was stored at 4 ^{o}C and warmed to room temperature immediately prior to use.

Peroxidase Activity Measurements

Peroxidase activity in the stock enzyme solution was measured before use. The enzyme activity was assayed at 25 ^{o}C using phenol, 4-aminoantipyrine (AAP) and hydrogen peroxide as substrates (Buchanan *et al.*, 1998). The assay mixture contained 1.5 mL of 20 a mM phenol solution, 0.75 mL of a 9.6 AAP solution, 0.3 mL of a 2 mM H_2O_2 solution, 0.15 to 0.45 mL of HRP solution, and up to 0.3 mL phosphate buffer (pH 7.4) (Gomori, 1955). All reagents were prepared using the same buffer. The total volume of the assay mixture was 3 mL and the light path of the cuvette used was 1 cm. The HRP active concentration is proportional to the color development rate measured at 510 nm, during a period of time in which the substrate concentration is not significantly reduced. The color development rate during this period 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 1.0 micromol of hydrogen peroxide per minute at 25 ^{o}C and pH 7.4.

Phenol Concentration Measurements

Phenol concentration was measured using a colorimetric assay (Buchanan *et al.*, 1998). The analytic range covers phenol concentrations from 0.03 to 0.12 mM. In a 16 mm path rounded cell, 2 mL samples comprised of 1.6 mL of 0.25 M sodium bicarbonate and 0.45 mL of 20.8 mM AAP were added. After vigorous mixing 0.45 mL of 83.4 mM potassium ferricyanide were added and mixed again. Samples absorbance was measured at 510 nm, 9 minutes after the ferricyanide addition and converted to concentration

using a calibration curve. The results presented herein are mean values of duplicate measurements.

Chemical Oxygen Demand (COD) Measurements

COD was measured using a Merck reagent kit to the analytic range from 100 to 1,500 mg/L. Samples were digested using a Merck Thermoreactor TR 300. Colorimetric determinations were performed at 585 nm. The results presented herein are mean values of duplicate measurements.

Total Suspended Solids Measurements

Flotation efficiency was measured as residual total suspended solids using a variation of the method 2540 D (APHA *et al.*, 1995). 100 mL samples were filtered using Gooch crucibles (porosity 5) and washed with 100 mL distilled water. Crucibles were dried until constant weight at 60 ^{o}C to avoid polymer degradation by temperature.

Experimental Procedure

Experiments were carried out in 600 mL beakers at room temperature. Reaction medium was prepared by adding individually certain amounts of phenol, low purity HRP, PEG and H_2O_2 into the phosphate buffer (pH 7.4). The operating conditions based on the optimizations performed by Wu *et al*. (1993, 1994) who used purified HRP are listed in Table 1. All reagent concentrations were 10 % greater than the optimized values to assure reaction efficiency. PEG was available with a molecular mass of 1,500 instead of 3,350 as used by Wu *et al*. (1993, 1994). This was taken into account by calculating the PEG concentration as the optimized concentration plus 10 % multiplied by 3,350 and divided by 1,500.

Table 1:Experimental conditions.

Test	C_{ph} initial		Low purity HRP	PEG	H_2O_2	
	(mM)	(mg/L)	(U/mL)	(g/L)	(mM)*	(Aliquots)**
1	1.0	94	0.06	0.08	1.1	1
2	2.0	188	0.07	0.14	2.2	2
3	4.0	376	0.12	0.27	4.4	3
4	6.0	565	0.20	0.39	6.6	2
5	8.0	753	0.31	0.52	8.8	2
6	10.0	941	0.44	0.64	11.0	1

* Total H_2O_2 added. ** H_2O_2 aliquots added in a semi-batch manner at 30 minutes intervals.

The phenol concentration in the synthetic wastewater varied from 1 to 10 mM. A mechanic stirrer at 15 rpm agitated the reacting mixtures. The reaction was initiated by H_2O_2, which was added in a semi-batch manner. H_2O_2 was added discretely in one, two or three aliquots at 30 minutes intervals. The reaction time was fixed in 4 hours and 30 minutes to assure total phenol conversion (Wu *et al*., 1993, 1994).

Phenol polymerization efficiency was evaluated after reaction completion. For the phenol concentration and COD measurements, samples were first filtered using fritted glass filters (porosity 5) to remove phenolic polymers. The reaction kinetics was evaluated in samples collected at 45 minute intervals. For instantaneous phenol concentration analysis, 2 mL samples drawn from the reaction mixtures were first mixed with 2 mL of 0.4 mL/L catalase to stop the reaction (Nicell, 1994) and then filtered.

Polymers were removed from solution using a bench scale DAF unit having a 2 L saturation vessel and a 1 L flotation cell as shown in Figure 1. Distilled water was pressurized at 4 atm and released to the cell as 20 % of the initial suspension volume (Féris, 1998). For the residual suspended solids determination, clarified samples were drawn after 3 minutes flotation at 5 cm from the bottom of the cell.

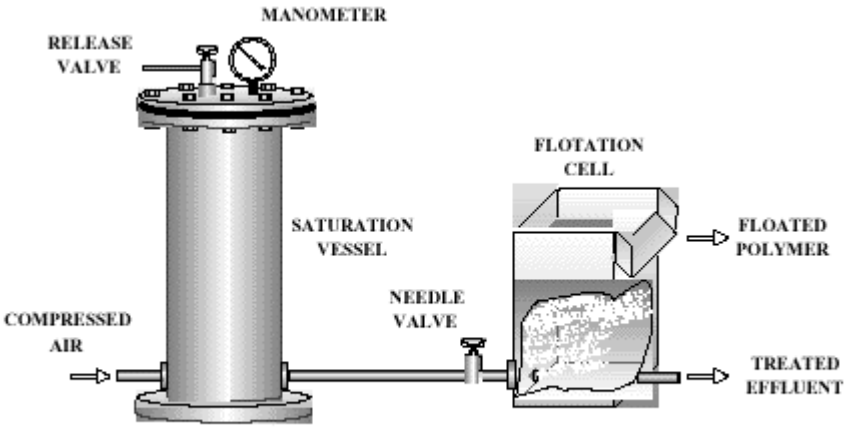

Figure 1: Bench scale dissolved air flotation (DAF) unit.

RESULTS AND DISCUSSION

The peroxidase activity measured for horseradish roots was approximately 21 U/g. This value is in accordance with published results of 33 U/g using guaiacol assay (Alberti and Klibanov, 1981) and 21 ± 2 U/g using a 3,5-dichloro-2-hydroxy-benzenesulphonic acid assay (Cooper and Nicell, 1996).

Experiments were designed to achieve a conversion of at least 95 % of the phenol initially present in the synthetic wastewater. The results using a low purity HRP and synthetic wastewaters are summarized in Table 2. No phenol polymerization was observed when either HRP or hydrogen peroxide were added to the wastewater alone.

Table 2: Phenol conversion.

Test	C_{ph} initial		C_{ph} final		Phenol conversion
	(mM)	(mg/L)	(mM)	(mg/L)	(%)
1	1.03	97	0.011	1.0	99
2	1.98	186	0.003	0.3	100
3	3.96	373	0.005	0.5	100
4	5.99	564	0.002	0.2	100
5	8.34	785	0.012	1.1	100
6	9.93	934	0.009	0.9	100

Phenol conversion in all experimented conditions was greater than 99 %. The high efficiency observed is in accordance with conditions optimized to guarantee 95 % polymerization using purified HRP (Wu *et al.*, 1993, 1994). Cooper and Nicell (1996) also suggested that crude enzyme preparation is protected from inactivation due to the significant quantity of proteinaceous matter present. This feature added to the greater quantity of reagents used can explain the improved results obtained.

The reactor residence time is one of the main parameters that determine the economics of an enzymatic process. A low enzyme concentration will decrease materials costs but will increase the reactor residence time needed to obtain the same level of phenol separation. This would lead to the use of larger reactors to treat the same wastewater flow rate. Clearly, there is a compromise between the reduction in variable costs by the use of less enzyme and the increase in capital investment at the time of building the treatment facility. Phenol conversion is depicted on Figure 2 for a period of 4.5 hours.

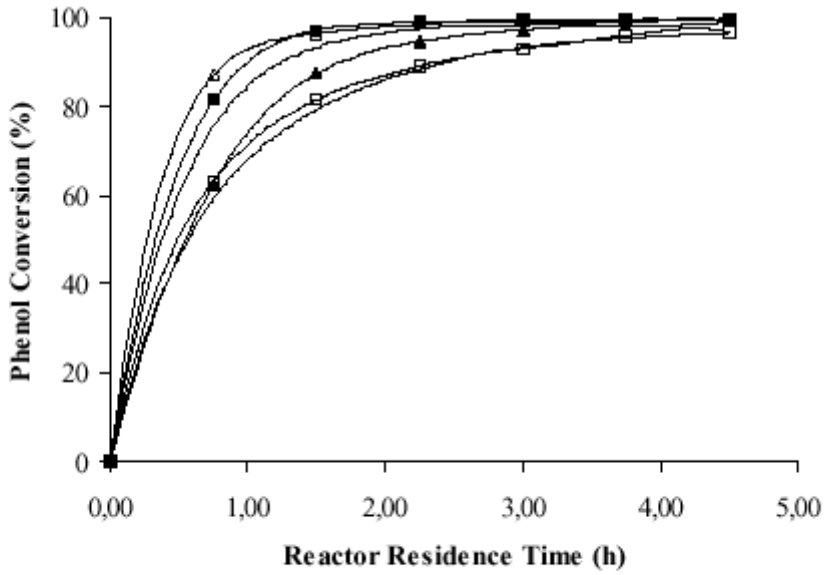

Figure 2: Phenol conversion for a period of 4.5 h. Experimental conditions of Table 1. Initial phenol concentration: ● 1mM 2 mM ▲ 4 mM ○6 mM ■ 8 mM Δ 10 mM

Optimized experimental conditions assure that residence time must be no longer than 4.5 hours for all phenol concentrations studied. It should be also noted that more concentrated wastewaters need less time for reaction completion.

The composition of the wastewater leaving the enzymatic treatment unit must also be considered. The residual organic content of the effluent is of concern, especially since the process involves the addition of crude enzyme preparation and PEG, organic bearing compounds with high carbon contents. These compounds plus the organic reagents in excess, residual phenol and soluble byproducts of the reaction, can adversely impact the aquatic environment due to their high oxygen demand. Yet, some reagents may be employed in the flotation stage resulting in lower COD values.

The residual organic content in the process effluent was evaluated and the results are shown in Table 3. The organic content of the original wastewater, before reagent addition, is given as theoretical oxygen demand (TOD) based on the phenol content. The COD of the treated wastewater and corresponding changes in COD with respect to the original wastewater are presented.

Table 3: Residual organic content in the process effluent.

Test	TOD related to C_{ph} initial (mg O_2/L)	Final COD (mg O_2/L)	COD change (%)
1	231	136	41
2	443	170	62
3	888	428	52
4	1,343	657	51
5	1,867	882	53
6	2,224	1,111	50

The initial COD of the wastewater was lowered by almost 50 % in all experiments. Wu *et al.* (1998) showed that PEG used at the minimum dose is completely removed with the phenolic compound. Since 99 % conversion was accomplished, residual COD is due to the HRP crude preparation, which remains dissolved in the effluent, and an excess of PEG.

In spite of the residual organic content due to PEG and HRP crude preparation, which is easily biodegradable (Wu *et al.*, 1997), further COD reduction can be attained using other flotation reagents or a partially purified enzyme source.

An important point that has not received much attention in the literature is the solid-liquid separation stage. Polymer coagulation using alum and sedimentation is frequently mentioned. However, this technique is time consuming and incomplete, yielding sludge with high water content. Conversely, flotation is a reliable high capacity technique which produces a thicker sludge (Rubio, 1998, Féris, 1998). DAF efficiency in removing polymers coagulated only by the salt content due to the buffered solutions is presented in Table 4.

Table 4 - Flotation efficiency.

Test	Flotation efficiency* (%)
1	99
2	95
3	98
4	99
5	99
6	100

* Using DAF with addition of 20 % v/v of pressurized water at 4 atm.

DAF presented a high level of separation in all tested conditions. Thus, flotation can be suggested as an alternative method to remove the polymers produced by this enzymatic process. Other studies using synthetic wastewaters prepared with tap water instead of buffer resulted in stable colloidal suspensions which need some coagulant addition prior to flotation.

Since the polymers produced present no acute toxicity (Aitken *et al.*, 1994) and feature thermal and photolytic stability, as well as rigidity and conductivity properties they might be considered for a wide range of applications (Johnson *et al.*, 1992). This constitutes the theme of forthcoming research studies.

CONCLUSIONS

Peroxidase enzymes from low purity sources such as horseradish roots crude preparation showed a good potential for phenol polymerization. Experimental results indicated that phenol conversion in synthetic wastewaters is almost complete. The phenolic polymers produced were efficiently removed using a flotation technique. At the reaction step conversions higher than 99% were achieved yielding polymers which were separated by dissolved air flotation with a reduction of at least 95 % of suspended solids. Thus, the proposed process can be considered for the removal of phenol from industrial wastewater.

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NOMENCLATURE

AAP	4 - aminoantipyrine
COD	Chemical oxygen demand (mg O_2/L)
C_{ph}	Phenol concentration
DAF	Dissolved air flotation
HRP	Horseradish peroxidase
PEG	Polyethylene glycol
TOD	Theoretical oxygen demand (mg O_2/L)

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