A New Enzymatic Process for the Treatment of Phenolic Pollutants

Mauri Sergio Alves Palma¹, Harald Horn², Mario Zilli³, Gisele Pigatto¹,³ and Attilio Converti³*
¹Departamento de Tecnologia Bioquímico-Farmacêutica; Faculdade de Ciências Farmacêuticas; Universidade de São Paulo; 05508-000; São Paulo - SP - Brasil. ²Institute of Water Quality Control; Technical University of Munich; 85748; Garching - Germany. ³Department of Civil, Chemical and Environmental Engineering; University of Genoa;16145; Genoa - Italy

ABSTRACT

This work aimed to develop a new economic enzymatic process to treat the phenolic pollutants using pure tyrosinase in stirred vessel and adopting temperature (T), pH, rotational speed (N), initial phenol (Cₚₒ) and enzyme (Cₑ) concentrations as independent variables. Experimental data of the residual phenol concentration (Cₚ) were used to calculate the oxidation efficiency (η), initial oxidation rate (-rₒ) and time required to reach the end of reaction (t) that were selected as the responses. Under the optimal conditions (T = 45°C, pH 6.6, N = 400 rpm, Cₚₒ = 100 ppm and Cₑ = 50 U/mL), η was 88.1%, -rₒ = 10.2 mg L⁻¹ min⁻¹, t = 40 min. These results suggested that tyrosinase-rich crude extracts from vegetable byproducts could be quite promising.

Key words: phenols, tyrosinase, wastes, enzymatic treatment

INTRODUCTION

Phenol and other phenolic compounds are common constituents of wastewaters from various activities in different industrial sectors such as electrolytic strip tin coating, chemical (polymeric resins, bisphenol A, alkyl phenols, caprolactams, adipic acid, etc.), petrochemical (oil refining), metallurgical (smelting, iron, steel, and coke), pharmaceutical, textile, plastics, explosive, coffee, ceramic, paint, varnish and pesticide productions (Rosatto et al. 2001). Phenols released into the environment may directly or indirectly cause serious health and odor problems. They can in fact inhibit the growth, or exert lethal effects to aquatic organisms even at relatively low concentrations (5 to 25 mg/l, depending on the temperature and state of maturity of the organism), and impart off flavors in drinking water and food processing wastewater (Theodore et al. 1997). Due to these constraints, the Brazilian legislation is very restrictive on phenols discharge, and the maximum concentration allowed in the treated effluents is 0.5 ppm (Conselho Nacional do Meio Ambiente 2005). Eleven of these compounds are among the 129 major pollutants present in the list of the Environmental Protection Agency (Shibata and Palma 2009). Most of the overall world production of phenol, which was 7.78 x 10⁶ tons in 2001, has been used for the syntheses of bisphenol A (39%), phenolic resins (27%), caprolactam (16%), alkylphenols (5%), 2,6-xylenol (3%), anilines (2%), and others (8%) (Anonymous 2002).

Various techniques are available for the treatment of phenolic effluents at different concentrations,
but one should take into consideration that industrial effluents have very complex composition, and a specific treatment would be needed for each particular effluent (Freire et al. 2000). The treatment of phenolic effluents can be subdivided into two main categories, the destruction and the recovery methods (Britto and Rangel 2008). Among the destruction methods, there are biological treatments (Freire et al. 2000), incineration, ozonation in the presence of UV radiation, oxidation with wet air (Britto and Rangel 2008) and electrochemical oxidation (Mojović et al. 2009). The recovery methods include liquid-liquid extraction (Jiang et al. 2003a, b; Palma et al. 2007, 2010), adsorption and electro-adsorption with activated charcoal (Ayranci and Conway 2001; Jain et al. 2002), ion exchange with resins, membrane processes such as a pervaporation, and extraction with membrane, supported liquid membrane and liquid membrane in emulsion (Kujawski et al. 2004). Taking into account these issues, there is the need to develop a new, ecological and auto-sustainable way for the treatment of phenolic effluents. A promising process appears to be the enzymatic oxidation (Durán and Esposito 2000; Faria et al. 2007), particularly by polyphenol oxidase (PPO) that is more commonly referred to as tyrosinase (Martin et al. 2008; Romanovskaya et al. 2009; Yamada et al. 2009). Another enzyme equally efficient in the oxidation of phenols is the polyphenol peroxidase (Campeanu et al. 1999), but it has the disadvantage of requiring hydrogen peroxide instead of atmospheric oxygen as a substrate (Ikehata and Nicell 2000a, b; López-Molina et al. 2003). Tyrosinase, which is present in many fruits, vegetables, seafood and mushrooms, is responsible for the browning of fruits when they have their internal tissue exposed to oxygen. It is a tetrameric enzyme with molecular weight of 120 kDa, two active sites, and the highest activity at pH in the range 5.0-8.0. It is usually obtained from mushroom and used in biosensors for phenols detection (Faria et al. 2007; Ikehata and Nicell 2000a, b). Several fruits and vegetables have been used to obtain PPO, which include artichoke (López-Molina et al. 2003), wheat grain (Fuerst et al. 2006), apple (Eidhin et al. 2006), pawpaw (Fang et al. 2007), loquat fruit (Sellés-Marchart et al. 2006) and banana (Wuyts et al. 2006). The reactions of phenols oxidation catalyzed by tyrosinase can be summarized as follows (Quan et al. 2004).

\[
\text{Phenol} + \frac{1}{2} \text{O}_2 \rightarrow \text{Catechol} \quad (1)
\]

\[
\text{Catechol} + \frac{1}{2} \text{O}_2 \rightarrow \text{o-Quinone} + \text{H}_2\text{O} \quad (2)
\]

But similar mechanisms are followed for other phenols such as \text{o}-cresol and \text{p}-nitrophenol. The use of this enzyme is of particular interest because it oxidizes phenols to oligomers similar to melanins and imparts a brown color to the reaction medium, making it possible to quantify in real time the degradation of phenolic compounds.

The aim of this study was to investigate the kinetics and efficiency of phenols oxidation as well as the time to reach the end of reaction, so as to apply this process for the treatment of polluted wastewaters. However, since the use of pure tyrosinase in the treatment of effluents would be uneconomical, further effort has to be made to obtain crude extracts of this enzyme from the vegetables as cheap – albeit less active – catalysts (Kameda et al. 2006).

**MATERIALS AND METHODS**

**Chemicals**

Chemicals used in this study were tyrosinase T3824-25KU (5370 U/mg) and 4-aminoantipyrine from Sigma-Aldrich (São Paulo, Brazil), and phenol, \text{o}-cresol, \text{p}-nitrophenol, \text{K}_2\text{HPO}_4, \text{KH}_2\text{PO}_4 and \text{H}_3\text{PO}_4 of analytical grade from Labsynth Ltda (Diadema-SP, Brazil). Distilled water was used in the experiments.

**Equipment**

Figure 1 illustrates the experimental set-up used to study the oxidation of phenols in aqueous solution by tyrosinase. It was carried out as described by Quadros and Baptista (2003) using a standard Rushton mixed vessel described in detail in previous work (Palma et al. 2007, 2010; Shibata and Palma 2009). Briefly, the mixed glass reactor (R) with 1.0 L-working volume was provided with an external jacket for temperature regulation by a water bath (B) (0 < \(T < 60\degree C\)) and four holes to host the mixing device (M) (100-1500 rpm), a thermometer (T), a syringe for sampling (S) and a funnel for feeding either phenol or enzyme buffer solution (F). It was also provided with a valve (V) located in the reactor bottom to allow aqueous phase discharge. An air pump (P) provided the oxygen necessary for the enzymatic reaction.
Phenols Oxidation Tests

Batch oxidation tests were performed by adding into the reactor, under mixing at given rotational speed, 0.5 L of 0.05 M phosphate buffer solution having the desired pH and containing the selected phenolic pollutant. After reaching the selected temperature, 3.0 mL of 0.05 M phosphate buffer solution (pH = 6.6) containing 25,000 U tyrosinase were added in the shortest time as possible through a separation funnel, and a chronometer started measuring the reaction time.

Table 1 shows the experimental conditions under which the tests of phenols oxidation were carried out together with the main results obtained at the end of reaction. The end of reaction was determined by linear fitting of the experimental data and, progressively, omitting the initial points. When the slope of the resulting straight line became lower than 10^{-2}, the time corresponding to the first remaining datum was assumed to be that needed to reach the end of reaction.

Table 1 - Experimental conditions and results at equilibrium of phenols oxidation tests.

<table>
<thead>
<tr>
<th>Run</th>
<th>T (°C)</th>
<th>pH</th>
<th>N (rpm)</th>
<th>C_T (U/mL)</th>
<th>C_P,0 (ppm)</th>
<th>t (min)</th>
<th>η (%)</th>
<th>-r_o (mg L^{-1} min^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>6.6</td>
<td>400</td>
<td>50</td>
<td>100</td>
<td>&gt;500</td>
<td>~77</td>
<td>0.96</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>6.6</td>
<td>400</td>
<td>50</td>
<td>100</td>
<td>100</td>
<td>88.5</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>6.6</td>
<td>400</td>
<td>50</td>
<td>100</td>
<td>80</td>
<td>88.2</td>
<td>3.7</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>6.6</td>
<td>400</td>
<td>50</td>
<td>100</td>
<td>40</td>
<td>88.1</td>
<td>10.2</td>
</tr>
<tr>
<td>5</td>
<td>55</td>
<td>6.6</td>
<td>400</td>
<td>50</td>
<td>100</td>
<td>25</td>
<td>30.9</td>
<td>2.0</td>
</tr>
<tr>
<td>6</td>
<td>45</td>
<td>5.6</td>
<td>400</td>
<td>50</td>
<td>100</td>
<td>120</td>
<td>74.8</td>
<td>9.2</td>
</tr>
<tr>
<td>7</td>
<td>45</td>
<td>7.6</td>
<td>400</td>
<td>50</td>
<td>100</td>
<td>40</td>
<td>89.1</td>
<td>7.3</td>
</tr>
<tr>
<td>8</td>
<td>45</td>
<td>6.6</td>
<td>200</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td>69.3</td>
<td>6.1</td>
</tr>
<tr>
<td>9</td>
<td>45</td>
<td>6.6</td>
<td>300</td>
<td>50</td>
<td>100</td>
<td>40</td>
<td>85.3</td>
<td>10.2</td>
</tr>
<tr>
<td>10</td>
<td>45</td>
<td>6.6</td>
<td>500</td>
<td>50</td>
<td>100</td>
<td>30</td>
<td>83.0</td>
<td>9.5</td>
</tr>
<tr>
<td>11</td>
<td>45</td>
<td>6.6</td>
<td>600</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td>71.8</td>
<td>8.1</td>
</tr>
<tr>
<td>12</td>
<td>45</td>
<td>6.6</td>
<td>800</td>
<td>50</td>
<td>100</td>
<td>&gt;200</td>
<td>~65</td>
<td>7.1</td>
</tr>
<tr>
<td>13</td>
<td>45</td>
<td>6.6</td>
<td>400</td>
<td>25</td>
<td>100</td>
<td>50</td>
<td>78.3</td>
<td>4.6</td>
</tr>
<tr>
<td>14</td>
<td>45</td>
<td>6.6</td>
<td>400</td>
<td>75</td>
<td>100</td>
<td>70</td>
<td>95.3</td>
<td>9.7</td>
</tr>
<tr>
<td>15</td>
<td>45</td>
<td>6.6</td>
<td>400</td>
<td>50</td>
<td>50</td>
<td>30</td>
<td>73.0</td>
<td>2.0</td>
</tr>
<tr>
<td>16</td>
<td>45</td>
<td>6.6</td>
<td>400</td>
<td>50</td>
<td>200</td>
<td>50</td>
<td>61.9</td>
<td>14.4</td>
</tr>
<tr>
<td>17</td>
<td>45</td>
<td>6.6</td>
<td>400</td>
<td>50</td>
<td>400</td>
<td>50</td>
<td>52.0</td>
<td>10.2</td>
</tr>
<tr>
<td>18</td>
<td>45</td>
<td>6.6</td>
<td>400</td>
<td>50</td>
<td>100</td>
<td>40</td>
<td>91.8</td>
<td>7.2</td>
</tr>
<tr>
<td>19</td>
<td>45</td>
<td>6.6</td>
<td>400</td>
<td>50</td>
<td>100</td>
<td>30</td>
<td>85.6</td>
<td>10.4</td>
</tr>
</tbody>
</table>

a Temperature; b Rotational speed; c Enzyme concentration; d Initial pollutant concentration; e Time to reach the end of reaction; f Oxidation efficiency; g Initial reaction rate; h Best conditions determined in this study; i Test performed using o-cresol; j Test performed using p-nitrophenol.
The phenolics (phenol, o-cresol and p-nitrophenol) and the temperature, pH and initial phenols concentration were selected from those usually reported for the treatment of phenol emissions in Brazil (15 ≤ T ≤ 55°C; 5.6 ≤ pH ≤ 7.6; 50 ≤ C_{P,o} ≤ 400 ppm) (Britto and Rangel 2008; Palma et al. 2007), being T = 45°C and pH 6.6 the optimum values for tyrosinase activity (Worthington Enzyme Manual 2013), while the range of enzyme concentration studied was that (25 ≤ C_{T} ≤ 75 U/mL) reported by Kameda et al. (2006). In addition, the range of rotational speed (200 ≤ N ≤ 800 rpm) was selected according to Jiang et al. (2003a, b), who demonstrated the importance of this variable as well as the reaction time (1 ≤ t ≤ 30 min) to reduce the operating costs of the treatment. Since at the start of the work, a reaction time of 30 min was shown to be insufficient to reach the end of reaction, it was prolonged particularly at T < 45°C, at which the reaction was very slow. The experimental data accuracy was checked by carrying out in quadruplicate the run (n. 4) that provided the best combination of results and supposing the same reproducibility (average standard deviation of 3 ppm) for all the other conditions. The remaining tests were done in duplicate and the results were expressed as average values.

Analytical Techniques
The aqueous samples were analyzed for phenol concentration using a UV-VIS spectrophotometer, model DU 640 (Beckman Coulter, Brea-CA, USA), having the following features: 10 mm path length cuvettes; wavelength range 190-1100 nm; -4.5 to +4.5 Abs reads out to eight places and displays out four places; UV and/or VIS lamps turned off independently. At selected time intervals, 1.0 mL-liquid samples of phenol, or p-nitrophenol solutions were collected from the reacting vessel by means of a syringe and filtered through cellulose acetate membranes with 0.45 μm-pore diameter (Sartorius, Göttingen, Germany). The filtered samples (300 μL) were transferred to the capped flasks containing 600 μL of distilled water and 60 μL of 8.5% (w/v) phosphoric acid solution to inactivate the enzyme. For o-cresol analyses, sample and reactants volumes were twice those of the other two pollutants. Phenol concentration was determined spectrophotometrically according to Eaton et al. (2005), as follows. 960 μL of each, borate (pH = 9.0), 0.1% (w/v) 4-aminoantipyrine and 5% (w/v) potassium ferricyanide solutions, were added to the samples prepared as above and the absorbance of the resulting solution was recorded at λ = 546 nm exactly after 10 min.

p-Nitrophenol concentration was determined similarly to phenol after the addition of 2.0 mL of 0.1 M NaOH solution to the samples (Toral et al. 2002). Since the p-nitrophenol produced yellow color in alkaline medium, the absorbance of the resulting solution was recorded at λ = 405 nm, and, unlike phenol, its absorbance did not vary with time.

o-Cresol concentration was determined according to Neufeld and Paladino (1985). For this, the samples were supplemented with 900 μL of each, 5% (w/v) ammonium chloride, 2% (w/v) 4-aminoantipyrine and 29% (w/w) ammonium hydroxide solutions, the last in order to set the pH at 10.0. After vigorous shaking of the mixture, 900 μL of 8% (w/v) potassium ferricyanide were added and the absorbance of the resulting solution was recorded at λ = 510 nm exactly after 15 min. The concentrations of phenol, o-cresol and p-nitrophenol were determined by using blank solutions without phenols as a reference and comparing the sample absorbance with those of calibration curves previously obtained using standard specimens with known concentrations. The detection limits were 3, 0.05 and 1.25 ppm for phenol, p-nitrophenol and o-cresol, respectively.

RESULTS AND DISCUSSION

Figure 2 shows the results of phenol concentration (C_{P}) versus time at different temperature and pH values, where those of run 4 (carried out at T = 45°C and pH 6.6), which ensured the best performance, were taken as a reference. The residual phenol concentration (C_{P}) and the efficiency of phenol oxidation (η), defined as the percentage of phenol removed at the end of reaction with respect to its initial level, were not significantly influenced by the temperature in the range 25-45°C (Fig. 2A and Table 1). On the other hand, as expected, at T = 15°C C_{P} reached the end of reaction only after a very long time (t > 500 min) and the initial reaction rate (-r_{o}), i.e., the slope of C_{P} vs. time for t = 0, was minimal (0.96 mg L^{-1} min^{-1}). It progressively increased with the temperature, reached a maximum at T = 45°C (10.2 mg L^{-1} min^{-1}) and then decreased markedly.
over this threshold, likely due to thermo-inactivation of the enzyme. Such a bell-shaped behavior of enzyme activity vs. temperature was already observed for most enzymatic systems, the position of the optimum activity being directly related to the temperature of maximum stability of the enzyme tertiary structure in water. For example, similar trends were observed for mycelium-bound carboxylesterases (Converti et al. 2002), lipases (Pastorino et al. 2004), dehydrogenases (Hasmann et al. 2007), proteases (Viana et al. 2010) and oxidases (Porto et al. 2006).

![Figure 2 - Influence of (A) temperature (T) and (B) pH on phenol oxidation by tyrosinase. Conditions: A) N = 400 rpm, pH = 6.6, C_T = 50 U/mL, C_P,o = 100 ppm. Temperature (°C): (●) 15, (X) 25, (△) 35, (□–□) 45, (○) 55. B) N = 400 rpm, T = 45°C, C_T = 50 U/mL, C_P,o = 100 ppm. pH: (◇) 5.6, (□–□) 6.6, (X) 7.6. The line (□–□) represents the best conditions determined in this study (T = 45°C, C_T = 50 U/mL, C_P,o = 100 ppm, N = 400 rpm, pH = 6.6). The average standard deviation was about 3 ppm for all the experimental results.](image)

The effect of temperature on the tyrosinase activity was confirmed by the behavior of the oxidation efficiency that, as expected, exhibited a minimum value (78.6%) at T = 15°C, increased with temperature up to a maximum (88.1-88.5%) in the range 25 ≤ T ≤ 45°C and then decreased to only 30.9% at 55°C. The time to reach the end of reaction progressively decreased from more than 500 to only 25 min with increasing temperature from 15 to 55°C, not only because of the above effects of temperature on enzyme activity and oxidation efficiency, but also due to better mixing and medium homogeneity owing to the resulting decrease in medium viscosity (Converti et al. 1999). Thus, the minimum t value at the highest temperature was the likely result of the...
simultaneous occurrence of enzyme activity reduction, thermo-inactivation and enhanced fluid dynamics.

The pH influenced significantly the values of all the three responses, namely $t$, $\eta$ and $-r_o$ (Fig. 2B and Table 1). In particular, $-r_o$ achieved its maximum (10.2 mg L$^{-1}$ min$^{-1}$) at intermediate pH (6.6), whereas the minimum value of $t$ (40 min) and the maximum of $\eta$ (89.9%) were obtained at pH $\geq$ 6.6 and 7.6, respectively. These results suggested that the enzyme might have been partially inactivated in the acidic environment and displayed high activity under neutral and alkaline conditions.

Even though the rotational speed had a significant impact on $\eta$ and $-r_o$, its influence on $t$ was small (Fig. 3A and Table 1). In particular, $-r_o$ exhibited a minimum value (6.1 mg L$^{-1}$ min$^{-1}$) at the lowest rotational speed ($N = 200$ rpm), reached a maximum (9.5-10.2 mg L$^{-1}$ min$^{-1}$) at 300-500 rpm and then decreased beyond this threshold. On the other hand, the maximum of $\eta$ (88.1%) and minimum of $t$ (30-40 min) were observed at 400 and 300-500 rpm, respectively, i.e., at intermediate rotational speeds. These results confirmed that an increase in $N$ actually improved the oxidation because of improved mixing and homogeneity, but an excess agitation led to mechanical denaturation of the enzyme (Colombié et al. 2001). It was possible that the low efficiency (69.3%) and initial oxidation rate (6.1 mg L$^{-1}$ min$^{-1}$) detected at $N = 200$ rpm were also the result of increased enzyme thermo-inactivation due to local superheating, but it was impossible to discriminate this effect from that of insufficient mixing.

Figure 3 - Influence of (A) the rotational speed ($N$) and (B) the enzyme concentration ($C_T$) on phenol oxidation by tyrosinase. Conditions: A) pH = 6.6, $T = 45^\circ$C, $C_T = 50$ U/mL, $C_{P,o} = 100$ ppm. $N$ (rpm): (◊) 200, (△) 300, (-□--) 400, (×) 500, (○) 600, (+) 800. B) $N = 400$ rpm, $T = 45^\circ$C, pH = 6.6, $C_{P,o} = 100$ ppm, $C_T$ (U/mL): (◊) 25, (-□--) 50, (×) 75. The line (-□--) represents the best conditions determined in this study ($T = 45^\circ$C, $C_T = 50$ U/mL, $C_{P,o} = 100$ ppm, $N = 400$ rpm, pH = 6.6). The average standard deviation was about 3 ppm for all the experimental results.
Also, the enzyme concentration ($C_T$) significantly influenced the values of $t$, $\eta$ and $-r_o$ (Fig. 3B and Table 1). In particular, $-r_o$ reached a maximum value (10.2 mg L$^{-1}$ min$^{-1}$) at $C_T = 50$ U/mL and a minimum one (4.6 mg L$^{-1}$ min$^{-1}$) at $C_T = 25$ U/mL, whereas $t$ exhibited a minimum value at $C_T = 50$ U/mL, and $\eta$ progressively increased with $C_T$. These results suggested that, at $C_T = 25$ U/mL the reaction was limited by the enzyme level, while the lowest efficiency was the likely consequence of enzyme inactivation caused by the relatively high level of $o$-quinones in relation to that of the enzyme. The highest oxidation efficiency was observed at $C_T = 75$ U/mL, but, surprisingly, $-r_o$ was lower than at $C_T = 50$ U/mL, likely due to self-aggregation of the enzyme molecules at the highest level, forming clusters (dimers, trimers, oligomers) that could have made them unavailable for the catalysis. This phenomenon could have been also the reason of the surprising behavior of $t$ that showed a minimum at $C_T = 50$ U/mL.

The results reported in the panel A of Figure 4 and Table 1 demonstrated that $C_{P,o}$ significantly impacted on the oxidation efficiency and the initial oxidation rate. The values of $t$ did in fact vary only from 30 to 50 min with increasing $C_{P,o}$ from 50 to 400 ppm, while the initial oxidation rate exhibited a bell-shaped behavior with a maximum ($-r_o = 14.4$ mg L$^{-1}$ min$^{-1}$) at $C_{P,o} = 200$ ppm and decreased over this threshold, which was typical of the well-known excess substrate inhibition observed in about 20% of the enzymes (Chaplin and Bucke 1990).

Figure 4 - A) Influence of the initial phenol concentration ($C_{P,o}$) on phenol oxidation by tyrosinase and B) oxidation of different phenolics by tyrosinase. Conditions: A) $N = 400$ rpm, $T = 45^\circ$C, $C_T = 50$ U/mL, pH = 6.6, $C_{P,o}$ (ppm): (◇) 50, (□) 100, (△) 200, (●) 400. B) $N = 400$ rpm, pH = 6.6, $C_T = 50$ U/mL, $C_{P,o} = 100$ ppm, $T = 45^\circ$C, (◇) $p$-nitrophenol, (●) $o$- cresol, (□) $o$- cresol, (◇) $o$- cresol. The line (□) represents the best conditions determined in this study ($T = 45^\circ$C, $C_T = 50$ U/mL, $C_{P,o} = 100$ ppm, $N = 400$ rpm, pH = 6.6). The average standard deviation was about 3 ppm for all the experimental results.
On the other hand, the bell-shaped curve of the oxidation efficiency suggested that this parameter could have been modulated by the inhibiting action of o-quinones produced by phenol oxidation. As suggested by the minimum value of this parameter \( (\eta = 73.0\%) \) at \( C_{P,o} = 50 \text{ ppm} \), the lower the phenol level, the stronger was such an inhibition. An increase in phenol concentration to 100 ppm might have accelerated the diffusion of phenol molecules towards the active site, and then led to more effective release of o-quinones molecules from it, thus exerting a sort of active site protection \( (\eta = 88.1\%) \). However, an excess increase in \( C_{P,o} (400 \text{ ppm}) \) led to a sharp decrease in \( \eta \) (52.0\%), as the likely result of limitation of this transfer to the bulk.

The panel B of Figure 4 and Table 1 show the results of oxidation of o-cresol and p-nitrofenol (PNP) compared to phenol. It was evident that the type of substrate had little effect on both \( t \) and \(-r_o\), but strongly influenced \( \eta \). The minimum and maximum values of \( \eta \) were obtained with PNP and o-cresol, respectively, which pointed out higher affinity of tyrosinase for the hydrophilic compounds. The values of \(-r_o\) were similar for o-cresol and phenol and about 30\% lower for PNP, which suggested that the increase in the electrophilicity of the aromatic ring or, more likely, in the steric hindrance, both associated to the presence of the nitro group in PNP, might have significantly reduced its reactivity.

CONCLUSIONS

This work investigated the influence of temperature \((T)\), pH, rotational speed \((N)\), enzyme concentration \((C_r)\) and initial concentration of phenol \((C_{P,o})\) on phenol oxidation by tyrosinase in homogeneous aqueous solutions and compared these results with those obtained using o-cresol and p-nitrophenol as substrates. The process proved to be reproducible, and the results allowed properly assessing the influence of the selected variables. The optimum operating conditions were \( N = 400 \text{ rpm}, T = 45^\circ\text{C}, C_T = 50 \text{ U/mL}, \text{pH} = 6.6-7.6 \) and \( C_{P,o} = 100 \text{ ppm} \). Although under these conditions, the final concentration of phenol in the treated solution was only 11.9 ppm, corresponding to an oxidation efficiency of 88.1\%, this treatment did not allow meeting the limit of 0.5 ppm imposed for phenols by the Brazilian legislation. Therefore, additional study would be required to increase the process efficiency. Moreover, to face the high cost of pure tyrosinase, which would make an industrial process for the treatment of wastewater contaminated by these compounds unfeasible, efforts should be made to obtain and test cheaper tyrosinase extracts of vegetable origin.

ACKNOWLEDGEMENTS

The financial support from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) are gratefully appreciated.

REFERENCES

Enzymatic Treatment of Phenolic Pollutants


Kameda E, Langone MAP, Coelho MAZ. Tyrosinase extract from Agaricus bisporus mushroom and its in natura tissue for specific phenol removal. Environ Technol. 2006; 27: 1209-1215.


Rosatto SR, Freire RS, Durán N, Kubota LT. Amperometric biosensors for phenolic compounds determination in the environmental interest samples (in Portuguese). Quim Nova. 2001; 24: 77-86.


