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4-Chlorophenol Degradation by Chloroperoxidase from *Caldariomyces fumago*

Formation of Insoluble Products

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Abstract

This study investigated the degradation of 4-chlorophenol (4-CP) by *Caldariomyces fumago* chloroperoxidase (CPO). Enzymatic oxidations were studied in reaction mixtures at pH 3.0, 4.0, and 6.0 in the presence and absence of Cl^- containing 3.5 IU of CPO and 4-CP and hydrogen peroxide concentrations within the range of 0.5–50 and 0.005–50 m*M*, respectively. Distinct patterns of products regarding color, concentration, and solubility were observed. Reaction mixtures at pH 6.0 containing 3.5 IU of CPO and 5.0 m*M* 4-CP and H_2O_2 (1:1 stoichiometry) showed the highest 4-CP removal of 95% and the highest formation of a dark precipitate.

Index Entries: *Caldariomyces fumago*; chloroperoxidase; 4-chlorophenol degradation; polymerization reactions; industrial effluents treatment.

Introduction

The American and Canadian environmental legislations, represented by the Safe Drinking Water Act, the Surface Water Treatment Rule, and the Clean Water Act and controlled by the Environmental Pollution Agency limit the number, kind, and concentration of pollutants in domestic and industrial wastewaters *(1,2)*. This scenario reflects the growing concern related to the pollution of rivers, oceans, and groundwater, which has a direct impact on both the aquatic and terrestrial ecosystems and, by extension, on human health and quality of life. These circumstances are promot-

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ing the development of new technologies for pollution control and the improvement of the traditional processes *(1,3,4)*.

Nontoxic phenols resulting from plant degradation into tannin-like compounds, responsible for the flavor and color of potable water, are produced naturally in the environment *(2)*. However, phenol and phenolic compounds, generated by the petrochemical industry, such as aromatic amines and chlorinated and methoxy-substituted derivatives, are toxic, caustic, and therefore highly polluting *(2,5,6)*. They are present in various materials used in die-casting processes, such as lubes, hydraulic oils, and coolants, and can also be found in waste streams generated by mold release agents and machine wash-down. Petroleum refining; coal conversion; mining; production of resins, plastics, textiles and dyes; and timber, pulp, and paper processing are examples of activities that release phenols into the environment *(1–3,7)*.

Conventional methods for the removal of phenol in industrial wastewater include chemical and photooxidation, adsorption on activated charcoal, solvent extraction, and microbial degradation *(6,8)*. The application of these methods can be impeded by their cost, detoxification efficiency in relation to the phenol characteristics and concentration range, and the formation of hazardous byproducts *(2,5,6,9)*. The transfer of these technologies, often developed in industrialized countries, to developing countries may be hindered by cost and differences in industrial effluent compositions *(1–4)*. Moreover, geographic and climate characteristics also affect the effectiveness of wastewater treatment processes. This is particularly true regarding biologic processes that are more sensitive to the aforementioned factors. Within this context, the need for a worldwide improvement in processes for pollution control is paving the way for the development of new technologies.

Oxidative enzymes, which present a low catalytic selectivity, are able to act on a wide range of substrates. Thus, oxidases such as laccase from *Trametes versicolor (10)* and tyrosinase or polyphenol oxidase from *Agaricus bisporos (16,10–12)* and peroxidases such as horseradish peroxidases (HRPs)*(12–15)*, lignin peroxidase from *Phanerochaete crysosporium (12)* and from *Streptomyces viridosporus (7)*, and chloroperoxidase (CPO) from *Caldariomyces fumago (5,12)* have been reported for the oxidation of several phenolic compounds. Thus, the use of oxidases and peroxidases is emerging as a promising alternative for the treatment of effluents containing mono-, di-, and polysubstituted phenols and aromatic amines, pesticides, and hydrocarbons *(3,7,9–12,16–18)*. Because the majority of these compounds are poisonous to microorganisms, the direct use of biologic treatment is not effective. Therefore, an enzymatic pretreatment would allow the formation of biologically compatible products that could serve as nutrients in the subsequent microbiologic step. Moreover, depending on the bio-catalyst, reaction conditions, and phenolic characteristics, the sole use of enzymes could result in the formation of oligo or polymeric products that could be removed by filtration *(6,7,11)*.

Much work has been conducted so far with the aforementioned enzymes owing to the general agreement as to the importance and usefulness of these biocatalysts for the degradation of xenobiotics. Although the mechanisms involved in the enzymatic oxidations and peroxidations have been studied *(5–7,10,13,14)*, the existing diversity of oxidases and peroxidases from microbial and plant origin, the structural variety of the pollutants, and their degradation products under different reaction conditions call for detailed studies related to the optimization of enzymatic reaction and the identification of the oxidation products. The data obtained will support the development of more effective conditions for effluent treatment and will be valuable for the design of enzyme blends for the treatment of complex mixtures.

In the present study, the degradation of 4-chlorophenol (4-CP) by the enzyme CPO from *C. fumago* was studied. This compound was chosen because of its toxicity, widespread distribution in industrial effluents and the reported formation of insoluble products upon enzymatic oxidation. We focused on the efficiency of phenol removal and the pattern of products formation on the use of reaction mixtures with different phenol and hydrogen peroxide concentrations and stoichiometric relations, phenol:H_2O_2, with the aim of better understanding the reaction conditions that would result in the selective formation of colored, insoluble, or polymeric products.

Materials and Methods

Production of CPO

Shake-flask fermentations of *C. fumago*, strain CMI #89362, were carried out according to Pickard and colleagues *(19,20)*. The enzyme was purified from the culture supernatant by ethanol precipitation *(19,21,22)* and dissolved in 0.1 *M* potassium phosphate buffer, pH 5.5. Enzyme preparations were kept at –20°C.

CPO Halogenase and Peroxidase Activity

Enzyme activity, throughout this work, was measured by the monochlorodimedone (MCDO) method, which measures its halogenasic activity *(22)*. Reaction mixtures contained in 2.1 mL, 100 µL of the CPO preparation, 0.1 m*M* MCDO, 0.5 m*M* H_2O_2, and 20 m*M* KCl, as the source of Cl^-, in 100 m*M* potassium phosphate buffer, pH 2.75. Reactions were started by the addition of the peroxide. One unit of enzyme activity was defined as the activity that effected the halogenation of 1 µmol of MCDO to dichlorodimedone per minute, if under initial rate conditions. The concentrations of the enzyme preparations were found to be within 1500–2000 IU/mL ($Rz_{398/280}$ of about 1.0). The concentration of the enzymatic protein of 27 µ*M* was determined using the CPO molar extinction coefficient ($E_{398\,nm}$) of 86 mM^{-1} cm^{-1} *(22)*.

The CPO peroxidase activity was evaluated using as substrate *ortho*-aminophenol (OAP). This compound was previously reported as substrate for HRP's *(23)*. Reaction mixtures contained, in 2.5 mL, 14 IU of CPO, 1.0 m*M* OAP, and 4.0 m*M* H_2O_2. Reactions occurred at pH 3.0, 4.0, and 6.0 (100 m*M* potassium phosphate buffer) in the presence or absence of 20 m*M* KCl. The 3-min reactions were started by the addition of the enzyme and interrupted by 0.5 mL of 1.0 *N* HCl. One unit of enzyme activity was defined as the activity that effected the oxidation of 1.0 μmol of OAP per minute, if under initial rate conditions. Note that, similar to the oxidation of OAP by HRP, red compounds (quinones) were formed *(23)*. Using OAP as substrate, 1.0 IU of CPO corresponded to 4.46 IU of CPO as measured by the MCDO method.

Study of Reaction Conditions for 4- CP Degradation

Enzyme concentration was optimized at pH 3.0 (100 m*M* potassium phosphate biffer) in 2.5-mL reaction mixtures containing 1 m*M* 4-CP, 4 m*M* H_2O_2, 20 m*M* KCl; and 140, 14, 7.0, and 3.5 IU of CPO. The 3 min reactions, at room temperature, were started by the addition of the enzyme and interrupted by 0.5 mL of 1.0 *N* HCl. Considering the possibility of CPO inactivation by H_2O_2, the enzyme was added in five equivalent portions during the first minute. Mixtures containing 3.5 and 7.0 IU of CPO provided an adequate reaction rate and allowed the use of spectrophotometry (4-CP λ_{max} 280 nm) to measure product formation without major interference from the enzyme, which absorbs in the same region. Subsequently, the effect of pH was studied with reaction mixtures containing 3.5 IU of CPO and were carried out at pH 3.0, 4.0, and 6.0 in the presence and absence of Cl^- in 100 m*M* potassium phosphate buffer according to reported conditions for optimal enzyme activity and phenol removal *(5,19,21,22,24).*

Effect of 4-CP and H_2O_2 Concentrations on Degradation of 4-CP

Phenol and H_2O_2 concentrations of 0.5, 5.0, and 50 m*M* and 0.005, 0.05, 0.5, 5.0, and 50 m*M*, respectively (concentration matrix of 3×5), were studied. Reactions were performed according to the previously optimized conditions for enzyme concentration, pH, and presence of Cl^-. The aforementioned 4-CP and H_2O_2 concentrations were defined by taking into account the pattern of products that was observed in preliminary experimentsin in which eight concentrations of 4-CP (0.125, 0.25, 0.5, 1.0, 5.0, 10.0, 50.0, and 100 m*M*) were studied in reaction mixtures containing 4 m*M* H_2O_2. Kinetic experiments were also carried out for 180 min to determine the degradation rates at pH 3.0 and 6.0. Control experiments containing enzyme plus substrate without peroxide, substrate plus peroxide without enzyme, and enzyme plus peroxide without substrate were performed.

Spectrophotometric Analysis

Spectrophotometric analyses were carried out using a CARY 13E spectrophotometer. The λ_{max} for 4-CP and the reaction products were obtained

from the absorption spectra in the range of 190–900 nm using scan rates of 100, 1000, and 2000 nm/min. The adequate choice of the reaction conditions regarding CPO and 4-CP concentrations allowed the use of spectrophotometry to measure product formation without major interference owing to the presence of the enzyme, which absorbs in the same region.

Analysis by Thin-Layer Chromatography

Two elution systems and two methods for band visualization were used. In plaques eluted with methanol:toluene (40:60), 4-CP and phenolic derivatives were revealed by spraying a saturate solution of $AgNO_3$ in acetone *(25)*. The $AgNO_3$ reacts with phenols, some biphenyl compounds, and quinones to form pink to brown nitrated derivatives. In plaques eluted with methanol:toluene:formic acid (20:10:1), product visualization was performed through the addition of 4-aminoantipyrine (4-AAP) directly to the reaction mixtures. 4-AAP exerts an electrophilic attack on para and ortho positions of the oxidized phenols producing red to purple derivatives. The R_f values were determined for the bands observed by direct visualization and under ultraviolet (UV) light. For comparison, the R_f values for 4-CP, phenol, ortho- and parabiphenyl compounds were determined. Reaction intensity was evaluated by direct visual observations ranging from zero (no reaction) up to five (maximum reaction).

Analysis by High Performance Liquid Chromatography

Samples were analyzed by high-performance liquid chromatography (HPLC), in a reverse-phase C_{18} column (250 × 4.6 m*M*, Spherisorb Octadecylsilane [ODS] of 2.5µ). Elution (methanol:water [60:40]) was performed using isocratic analysis with a flow rate of 1.0 mL/min with an ISCO 2350 pump. UV detection was performed at 245 and 280 nm using a UV-VIS detector (LKB-VWM 2141; Pharmacia). These chromatographic conditions were selected to resolve weakly polar and nonpolar products *(12)*. Insoluble products were separated by filtration prior to the HPLC analysis. Solubilized by the addition of nitrile acetate and subsequently analyzed.

Results and Discussion

Effect of pH and Cl^- on CPO Activity Using OAP and on 4-CP Degradation

Higher and equivalent enzyme levels of 37.6 and 34.3 IU were observed at pH 6.0 in either the presence or absence of Cl^-. Enzyme activity at pH 3.0 in the presence of Cl^- (24.21 IU) was higher in comparison to its absence (19.1 U). Very low activity (0.50 IU) was observed at pH 4.0. In previous works, 4-CP degradation by CPO was studied at pH 4.0 *(5,12)*. In accordance with these data, a higher degree of 4-CP removal of about 70% was observed at pH 6.0 in either the presence or absence of Cl^- (Fig. 1).

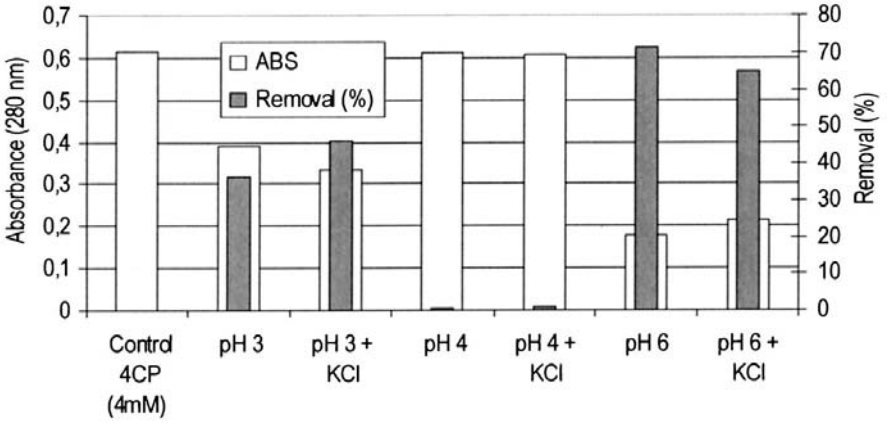

Fig.1. Influence of pH and Cl^- on 4-CP degradation by CPO.

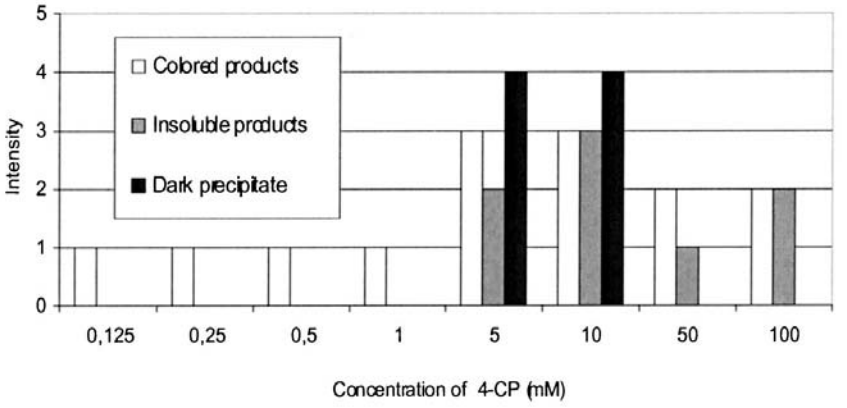

Fig. 2. Pattern of products observed in reaction mixtures containing 3.5 IU of CPO, 0.125–100 m*M* 4-CP, and 4 m*M* H_2O_2 at pH 6.0 in absence of Cl^-.

Degradation of 4-CP and Product Formation

According to visual observation of the test tubes corresponding to the preliminary enzymatic reactions (0.125-100 m*M* 4-CP and 4 m*M* H_2O_2), three categories of products were formed: colored (red solutions), insoluble (cloudy solutions), and a dark precipitate. Depending on the composition of the reaction mixture, a sequential or concomitant formation of the products was observed. For both pH values (3.0 and 6.0), only colored products were observed with concentrations of 4-CP up to 1 m*M*. Reaction mixtures containing 5.0 and 10.0 m*M* 4-CP favored the formation of the three different products, and, interestingly, mixtures containing 4-CP concentrations higher than 10 m*M* did not produce any precipitate. The intensity of the reactions varied and was likewise evaluated by direct visual observation. Reactions were more pronounced at pH 6.0 (Fig. 2).

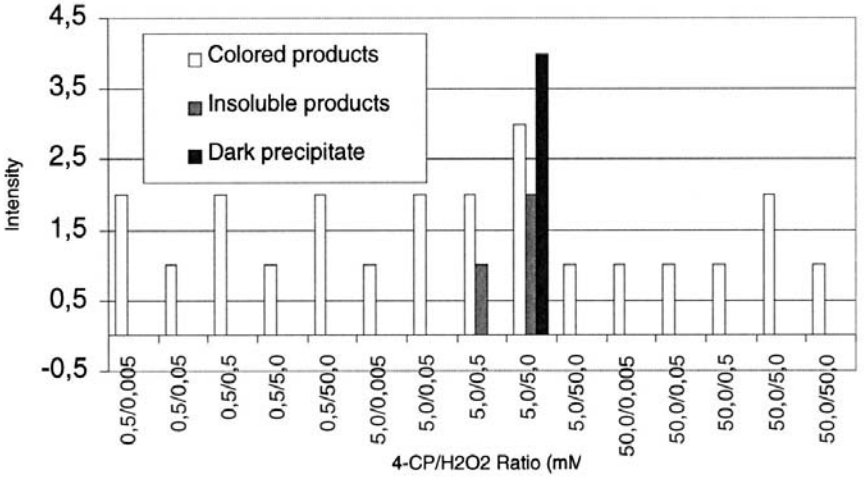

Fig. 3. Pattern and intensity of products, according to direct visual observation in reaction mixtures containing 3.5 IU of CPO; 0.5, 5.0, and 50.0 m*M* 4-CP; and 0.005–50.0 m*M* H_2O_2 at pH 6.0.

Thin layer chromatography (TLC) data confirmed these visual observations. As such, only one product band was observed in reaction mixtures containing 4-CP up to 1.0 m*M*, three bands for 5.0 and 10.0 m*M*, and only two bands for 50 and 100 m*M*. The bands varied in intensity. Fluorescent products were not observed on the reaction with 4-AA, as reported previously *(7)*. Residual 4-CP (R_f 0.5) revealed by the $AgNO_3$ reagent was only observed in samples from reaction mixtures containing initial 4-CP concentrations higher than 5.0 m*M*.

Figure 3 presents the pattern of products obtained at pH 6.0 in the absence of Cl^- from reaction mixtures planned according to the concentration matrix (3 × 5). Although 4-CP degradation occurred in all cases, as seen from the formation of colored products, the highest degree of degradation and the concomitant presence of the three groups of products occurred only with equimolar concentrations of 4-CP and H_2O_2 of 5.0 m*M*. Experiments using TLC analysis reproduced the same pattern of products already observed.

Figure 4 presents the quantification of 4-CP removal as measured by HPLC analysis of 4-CP residual concentrations in the supernatant of reaction mixtures containing 0.5, 5.0, and 50 m*M* 4-CP and 0.005–50 m*M* H_2O_2, respectively, at pH 3.0 and 6.0. For reactions at pH 6.0, no phenol was detected in reaction mixtures containing 0.5 and 5.0 m*M* 4-CP and 0.005 m*M* H_2O_2. As such, the reaction mixtures represented initially 1:100 and 1:1000 molar ratios of H_2O_2/4-CP. When the 4-CP concentration increased to 50 m*M*, total phenol removal occurred in the presence of 0.5 m*M* H_2O_2, and therefore the reaction represented a 1:100 molar ratio. The highest removal at pH 3.0 for the three 4-CP concentrations occurred at a 1:10 molar ratio. The absence of a defined stoichiometric relationship

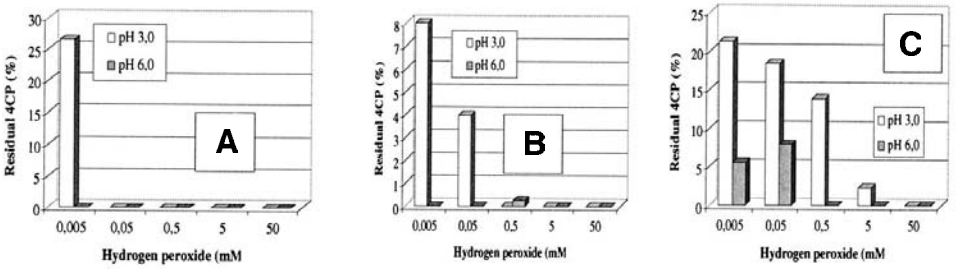

Fig. 4. HPLC quantification of residual 4-CP (percentage) in reaction mixtures containing phenol and H_2O_2 concentrations of (**A**) 0.5,(**B**)5.0 and(**C**)50 m*M* and 0.005–50 m*M* H_2O_2, respectively, at pH 3.0 in presence of Cl^- and pH 6.0 in absence of Cl^-.

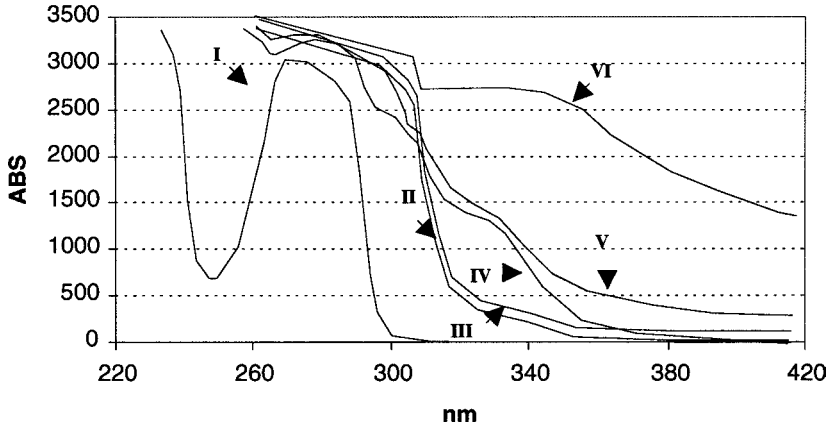

Fig. 5. Absorbance spectra for 5.0 m*M* 4-CP (I) and reaction mixtures containing: 5.0 m*M* 4-CP and 0.005 (II), 0.05 (III), 0.5 (IV), 5.0 (V), and 50.0 m*M*(VI) H_2O_2 at pH 6.0 in absence of Cl^-.

between 4-CP and H_2O_2 for phenol removal indicates that removal of 4-CP was not solely related to CPO-mediated reaction. The occurrence of nonenzymatic reactions after the primary CPO-catalyzed reaction has been previously reported *(13,14,26)*.

Spectrophotometric analysis of reaction mixtures containing 5.0 m*M* 4-CP and 0.005–50.0 m*M* H_2O_2, pH 6.0, are shown in Fig. 5, in comparison to the spectrum of 5.0 m*M* 4-CP. Different degradation profiles were observed for each case, indicating heterogeneity of the composition of the mixture. Similar profiles, however, were observed. As such, mixtures containing H_2O_2 concentrations of 0.005 and 0.05 m*M* showed an absorbance increase at 300 nm, which, in accordance to the visual observation, corresponded to the sole formation of colored products. The use of H_2O_2 concentrations of 0.5 and 5.0 m*M* resulted in a decrease in absorbance at 300 nm and the presence of a shoulder at 330 nm, suggesting the disappearance of colored products and formation of insoluble compounds. Finally, 50.0 m*M* H_2O_2 originated an increase in absorbance in the range of 300–500 nm,

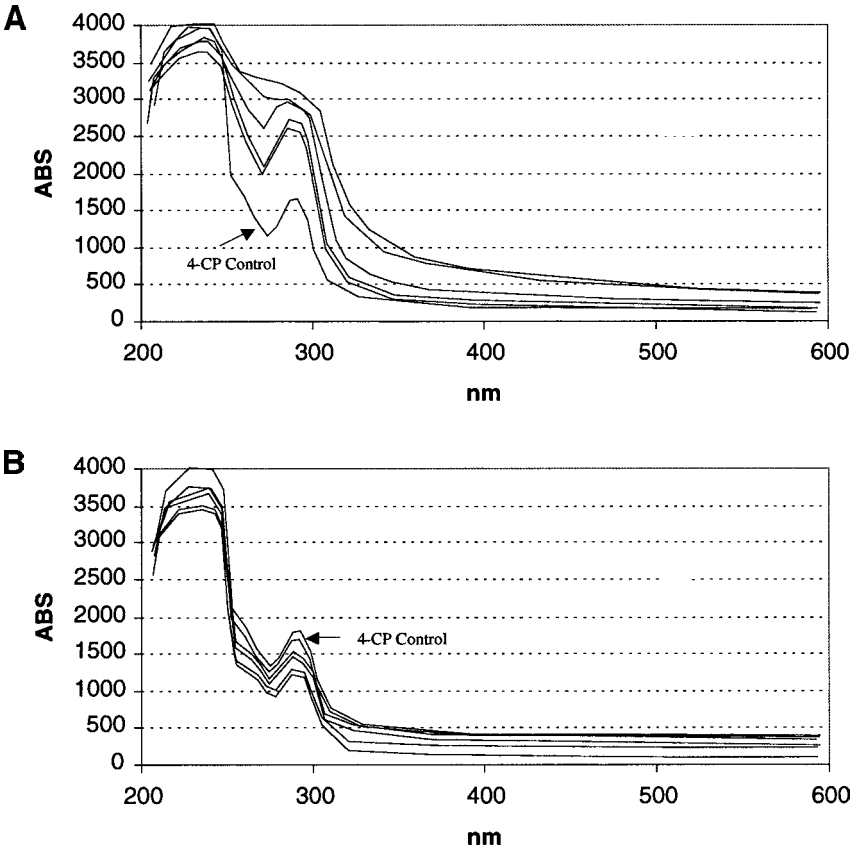

Fig. 6. Degradation kinetics spectra (0–180 minutes) for 4-CP at (**A**) pH 3.0 and (**B**) pH 6.0. Reactions contained 3.5 IU of CPO, 5.0 mM 4-CP, and 5.0 m*M* H_2O_2.

which corresponded to the presence of different colored products and the absence of dark precipitate. Therefore, we could envisage that through the design of the reaction conditions, it would be possible to influence the nature of the reaction products.

Figure 6 presents the spectra for the 4-CP degradation kinetics in reaction mixtures containing equimolar (5.0 m*M*) concentrations of 4-CP and H_2O_2, at pH 3.0 and 6.0. At pH 6.0, a continuous decrease in absorbance at 280 nm was observed. Considering that the substrate and products cause absorbance to overlap to a certain degree, the observed decrease in absorbance could not be directly related to the degradation level (about 70% of the initial 4-CP concentration). At pH 3.0, this overlap was more notorious and transformation of 4-CP resulted in the appearance of new peaks and regions with higher absorbance between 300 and 400 nm. The expected decrease at 280 nm was not observed. This fact suggests that pH directs the formation of different products.

Soluble products formed within 3 and 40 min in reaction mixtures containing 5.0 m*M* equimolar 4-CP and H_2O_2 concentrations at pH 6.0 were

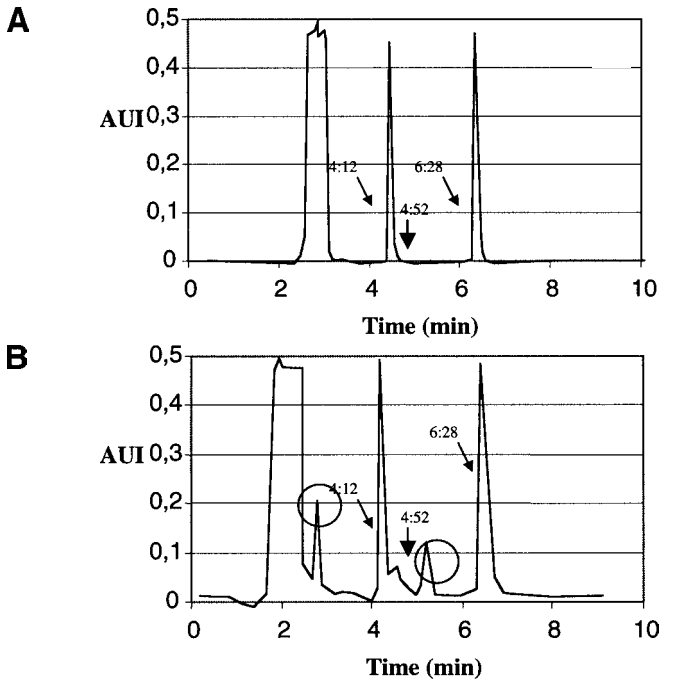

Fig. 7. HPLC analysis of supernatant of reaction mixtures containing equimolar (5.0 m*M*) concentrations of 4-CP and H_2O_2 at pH 6.0 incubated for (**A**) 3 and (**B**) 40 min. The arrows indicate residual 4-CP (R_t 4:25); a more polar compound (R_t 4:12 min), possibly a chloroquinone; and a more hydrophobic compound (R_t 6:28 min), possibly a chlorobiphenyl. The circle peaks (R_t 2:30 and 5:12) correspond to products formed after 30 min. The first peak corresponds to tricholoroacetic acid (TCA) added prior to HPLC analysis. AUI, absorbance units.

analyzed by HPLC (Fig. 7). According to the chromatogram in Fig. 7A, 4-CP was completely degraded. Two peaks corresponding to a more polar compound with a retention time (R_t) of 4:12 min, possibly a chloroquinone, and a more hydrophobic compound (R_t 6:28 min), possibly a chlorobiphenyl or other dimeric compounds (quinone + quinone or quinone + phenol) were also observed. The presence of these products is in accordance with previous reports concerning product retention times and octanol-water partition coefficients *(12,27,28)*.

The 40-min reaction chromatogram (Fig. 7B) showed additional peaks with an R_t of 2:30 and 5:12 min corresponding to lower molecular weight (more polar compound) and higher molecular weight products (less polar compounds), respectively, in comparison to 4-CP. The presence of a lower molecular weight product (R_t 2:30) indicates a further degradation from the main reaction products by CPO. Since this chromatogram was obtained using a higher attenuation, it was possible to observe the presence of residual amounts of 4-CP (R_t 4:52). The formation of higher molecular weight compounds could be related to the enzymatic formation of unstable compounds prompted to polymerize via free-radical reactions, as previously reported

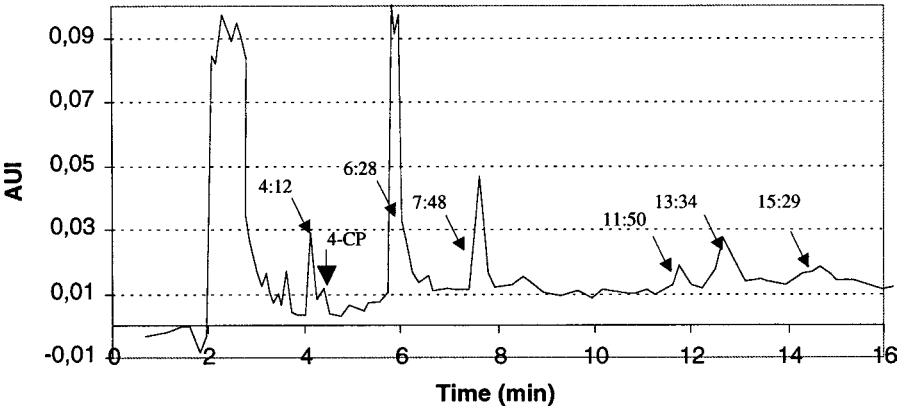

Fig. 8. Chromatogram of insoluble products. Peaks corresponded to residual 4-CP (R_t 4:52 min); a quinone (R_t 4:12 min); and a more hydrophobic compound (R_t 6:28 min), possibly a biphenyl. Four peaks with higher retention times (R_t 7:48, 11:50, 13:34, and 15:29 min) indicate the presence of higher molecular weight products. The first peak corresponds to TCA added prior to HPLC analysis. AUI, absorbance units.

(7,11,13,14,26). In general, the CPO-mediated reactions produce oxidized compounds (chloroquinones) that undergo dimerization spontaneously to form chlorobiphenyl compounds or other dimeric products.

Figure 8 presents the HPLC analysis of insoluble products of a 3-min reaction containing equimolar 5.0 m*M* concentrations of 4-CP and H_2O_2 at pH 6.0. Peaks correspond to residual 4-CP (R_t 4:52), quinones (R_t 4:12), and biphenyl compounds (R_t 6:28). The presence of four additional compounds with higher retention times and higher molecular weight (R_t 7:48, 11:50, 13:34, and 15:29 min) was observed, confirming the occurrence of polymerization reactions.

Conclusions

The enzymatic degradation of 4-CP by CPO from *C. fumago* was evaluated at pH 3.0 and 6.0 in a reaction containing constant CPO concentrations of 3.5 IU (0.5 m*M*) and a stepwise variation in the substrate and H_2O_2, with the aim of identifying selective reaction conditions in terms of yield and product diversity.

Distinct patterns of products regarding color, concentration, and solubility were observed in response to changes in pH, substrate concentration, and stoichiometry. Greater product variety and intensity were observed at pH 6.0, in the absence of chloride ions, and at an equimolar concentration of 5.0 m*M* 4-CP and H_2O_2. Under these conditions up to 95% of phenol removal with formation of a mixture of unidentified quinones and biphenyl-like compound as well as a dark precipitate was observed. HPLC analysis confirmed the presence of other derivatives from the reaction mixtures.

Our current work includes the study of reaction conditions containing equimolar CPO/H_2O_2 concentrations and multiple additions of H_2O_2 and CPO. The characterization of the soluble and insoluble products is also being evaluated with the aim of understanding better how nonenzymatic reactions occur, after a reaction catalyzed by CPO.

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