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Degradation of tetracycline and oxytetracycline by crude lignin peroxidase prepared from *Phanerochaete chrysosporium* – A white rot fungus

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ABSTRACT

Pharmaceuticals are becoming an emerging environmental issue that attracts increasing attention in recent years. This study chose tetracycline (TC) and oxytetracycline (OTC) as examples of pharmaceuticals. Crude lignin peroxidase (LiP) produced by *Phanerochaete chrysosporium* were used to degrade TC and OTC in vitro. The results illustrated that LiP has a strong degrading ability towards TC and OTC. At 50 mg L^{-1} of TC and OTC and 40 U L^{-1} of the enzyme activity, the degradation of TC and OTC reached to about 95% in 5 min. The degradation of TC and OTC by LiP was dependent on pH and temperature and was largely enhanced by increasing the concentrations of veratryl alcohol (VA) and initial hydrogen peroxide (H_2O_2). The optimized degradation conditions were determined as pH 4.2, 37 °C, 2 mM VA, 0.4 mM H_2O_2.

1. Introduction

Pharmaceuticals had been released to the environment with very little attention till a decade ago. The first overview on the knowledge of the exposures, fates and effects of pharmaceuticals was published by Halling-Sørensen et al. (1998). Only in the last few years has the issue of pharmaceuticals in our environment emerged as an important research topic and attracted increasing attention (Sarmah et al., 2006). In 1999, 24 kinds of pharmaceuticals were reported in aquatic environment in the United States of America (Daughton and Ternes, 1999). Increasing numbers of reports from either developed and developing countries showed that pharmaceuticals and their metabolites exist in a wide range of environmental samples including surface water, groundwater and drinking water (Heberer, 2002; Jones et al., 2002; Batt et al., 2006b; Konishi et al., 2006; Moldovan, 2006; Sarmah et al., 2006; Duong et al., 2008). So far, conventional wastewater treatment plants are not designed and operated to remove very low concentrations of contaminants including pharmaceuticals (Batt et al., 2006a, 2007). Since there are vast arrays of pharmaceutics, their removal efficiencies varied in a wide range based upon the treatment processes, but in all cases results are insufficient to avoid their presences in treated water and subsequently in the environment (Batt et al., 2006a, 2007; Gómez et al., 2007). Generally, conventional biological treatment technologies do not remove many of the pharmaceuticals efficiently (Heberer, 2002; Ternes et al., 2002; Carballa et al., 2004; Seino et al., 2004; Batt et al., 2006a, 2007).

Among various kinds of pharmaceuticals, antibiotics were more frequently detected than others. They are difficult to be removed through common biological treatment methods. Meanwhile, they may adversely affect key biotransformation processes of other pollutants (denitrification, nitrogen fixation, degradation of organic compounds, etc.), including biological sewage treatment processes (Heberer, 2002; Rooklidge, 2004), and promote antibiotic resistance (Batt et al., 2007). According to recent research, variety of antibiotics were detected in various water samples including hospital wastewater, municipal wastewater, effluent of wastewater treatment plant, antibiotics industry wastewater, livestock farm mud and wastewater, surface water, underground water and drinking water (Kümmerer, 2001; Hirsch et al., 1999; Golet et al., 2001, 2002; Sacher et al., 2001; Campagnolo et al., 2002; Heberer, 2002; Löffler and Ternes, 2003; Reverte et al., 2003; Lalumera et al., 2004; Batt et al., 2006b; Sarmah et al., 2006). Soil-aquifer treatment can remove some kinds of antibiotics, such as trimethoprim, clarithromycin, erythromycin and roxithromycin (Ternes et al., 2007). Adding ozone to biologically treated wastewater is sufficient for oxidizing many pharmaceuticals (Carballa et al., 2007). In the present study, tetracycline (TC) and oxytetracycline (OTC) were selected as research objectives because of their wide application, high-solubility in water, high residual toxicity and non-biodegradation characteristics. Limited research has so far been conducted on the photodegradation of TC and OTC using TiO_2, ZnO or other semiconductor materials as catalysts (Delepee et al., 2000; Pena et al., 2000; Halling-Sørensen et al., 2002).

It is well known that white rot fungi produce extracellular lignin-degrading enzymes that are promising in the degradation of xenobiotic organic compounds. Great potential exists in their

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application in environmental pollution control. Compared with the treatment method by using the fungi directly, in vitro treatment by the lignin-degrading enzymes produced through white rot fungi fermentation can exclude several potential limiting factors, such as the difficulties in growth of the fungi on a large scale, the long incubation processes and adsorption effects of the pollutants on the mycelia, and may represent a simpler and more effective method (Karam and Nicell, 1997). For practical applications, exploitation of crude enzymes is nearly a must because of the high cost related to the enzyme purification procedure. Our former research indicated that lignin peroxidase (LiP) showed a stronger degradation capability than manganese peroxidase (MnP) (Yu et al., 2006). In vitro degradation of pollutants by the lignin-degrading enzyme, especially LiP, has been described in some papers (Reddy et al., 1998; Haas et al., 2004; López et al., 2004; Eibes et al., 2005; Tamagawa et al., 2005). In fact, using crude LiP but not pure LiP has been becoming a trend in environmental field. Crude LiP and MnP can be harvested directly from the supernatant during a period of time in liquid culturing white rot fungi. Tamagawa et al. (2006) used crude MnP to remove the estrogenic activity of endocrine-disrupting genistein and natural steroidal hormone estrone. Eibes et al. (2005) used crude MnP to degrade anthrane in organic solvent. Therefore, it is worthy to assess the feasibility of using crude LiP to degrade TC and OTC. In this study, crude LiP produced by *Phanerochaete chrysosporium* was used to degrade TC and OTC in vitro. The objective was (1) to figure out the capability of crude LiP to degrade TC and OTC and, (2) to optimize the reaction parameters of the degradation system.

2. Materials and methods

2.1. Preparation of the crude enzyme

P. chrysosporium strain BKM-F-1767 was maintained at 37 °C on potato dextrose agar (PDA) (200 g L^{-1} potato extract, 20 g L^{-1} glucose and 20 g L^{-1} agar) plates. *P. chrysosporium* was cultured in an immersed liquid culture system. The culture medium was prepared as described by Tien and Kirk (1988) but containing 20 mM acetate buffer (pH 4.4) instead of dimethyl succinate buffer. In addition, 1.5 mM vertryl alcohol (VA), 0.2 g L^{-1} yeast extract powder, and 1 g L^{-1} Tween 80 were introduced. The final spore concentration of 1×10^5 spores mL^{-1} was introduced into a 250 mL Erlenmeyer flask containing 100 mL medium under an immersed state. Then the flasks were incubated in 37 °C in a rotary shaker with agitation of 160 rpm and 2.5 cm diameter throw. The cultures were harvested at the time when the maximum activities of LiP was detected at approximately day 6 and centrifuged at 16200*g* for 30 min at 4 °C. The resultant supernatant was used directly as crude ligninolytic enzymes in the degradation experiments.

2.2. Measurement of the activity of LiP

The activity of LiP was measured as described by Tien and Kirk (1988), with 1 U defined as 1 μmol of veratryl alcohol (VA) oxidized to veratraldehyde per minute by using a Shimadzu UV-2401 spectrophotometer. The general procedure was as follows: at 25 °C, 0.2 mL tartaric buffer (1 M, pH 2.5) was first mixed in a cuvette with aliquots of VA stock solution (10 mM) and LiP solution, then an aliquot of hydrogen peroxide H_2O_2 stock solution (4 mM) was added to initiate the LiP catalyzed oxidation of VA. After quick mixing, a plot of absorbance (A) at 310 nm versus the reaction time (t) was recorded against the corresponding reference (all chemicals without LiP). The molar extinction coefficient of veratraldehyde was 9300 M^{-1} cm^{-1}. From the slope of the linear portion of the A–t curve we could calculate the quantity of veratraldehyde produced per minute.

2.3. Enzymatic degradation

The reaction mixture of enzymatic decolorization consisted of crude LiP, veratryl alcohol, H_2O_2, TC and OTC in 50 mM tartrate buffer. The amount of each component in the mixture as well as the buffer pH was arranged as indicated. The reaction was carried out in a total volume of 8 mL liquid in 20 mL tube at 37 °C on 120 rpm rotary shaker (except special conditions given). The reaction was initiated by the addition of H_2O_2, and final concentration of the antibiotics was measured at about 30 min after the commencement. Our first experiment showed that TC and OTC concentration did not change much after 5 min reaction and was stable after 30 min. Control tests were conducted with the crude enzyme replaced by heat-inactivated crude enzyme at the same time. The control tests by using H_2O_2 (alone) to degrade TC and OTC were also conducted. These experiments were performed in triplicate and results are expressed as the mean values and one standard deviation.

2.4. Chemicals

Veratryl alcohol, and nitrilotriacetate (used in medium) were purchased from Fluka (Buchs, Switzerland). TC and OTC were obtained from Sino-American Biotec, Beijing. All other chemicals used were of analytical grade.

2.5. Analyses of TC and OTC

The TC and OTC and its metabolite was monitored using HPLC according to the procedure described in the Chinese pharmacopoeia (2005). A Shimadzu HPLC system (Japan) consisting of the following components was used: LC-10AT pump, CTO-10A column oven and SPD-10A UV-VIS detector. The system was equipped with a HYPERSIL BDS C18 (5 μm, 4.6 × 250 mm, No. 1217290). The oven was set to 35 °C. Separations were run using 68% (v/v) 0.1 M oxalic acid ammonium, 27% (v/v) N,N-dimethylformamide, 5% (v/v) 0.2 M diammonium phosphate in high purity water with a constant flow rate of 1.0 mL min^{-1} for TC, and 75% (v/v) 0.05 M oxalic acid ammonium, 20% (v/v) N,N-dimethylformamide, 5% (v/v) 0.2 M diammonium phosphate in high purity water with a constant flow rate of 0.8 mL min^{-1} for OTC. Eluted substances were detected at 353 nm. Degradation of TC and OTC was expressed as a concentration percentage which was calculated based upon the peak area of known standards.

3. Results and discussion

3.1. Production of LiP

The production profiles of LiP and MnP in immersed liquid culture (C/N ratio 56/8.8 mM) of *P. chrysosporium*, respectively, were depicted in Fig. 1. Activities of LiP appeared from day 4, peaked on day 6, and then decreased gradually till the end of the culture. The peak activity of LiP (1350 U L^{-1}) attained was far higher than that of MnP (136 U L^{-1}), indicating an LiP-dominated composition in the ligninolytic extracellular products.

3.2. The degradation effect of TC and OTC by LiP

TC and OTC were removed rapidly by LiP in the reaction system (Fig. 2). About 95% of the initial TC and OTC were converted within the first 5 min of incubation. After 30 min, only traces of TC and