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Advanced oxidation of benzene, toluene, ethylbenzene and xylene isomers (BTEX) by *Trametes versicolor*

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ABSTRACT

Advanced oxidation of benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylene (BTEX) by the extracellular hydroxyl radicals (•OH) generated by the white-rot fungus *Trametes versicolor* is for the first time demonstrated. The production of •OH was induced by incubating the fungus with 2,6-dimethoxy-1,4-benzoquinone (DBQ) and Fe^{3+}–EDTA. Under these conditions, •OH were generated through DBQ redox cycling catalyzed by quinone reductase and laccase. The capability of *T. versicolor* growing in malt extract medium to produce •OH by this mechanism was shown during primary and secondary metabolism, and was quantitatively modulated by the replacement of EDTA by oxalate and Mn^{2+} addition to DBQ incubations. Oxidation of BTEX was observed only under •OH induction conditions. •OH involvement was inferred from the high correlation observed between the rates at which they were produced under different DBQ redox cycling conditions and those of benzene removal, and the production of phenol as a typical hydroxylation product of •OH attack on benzene. All the BTEX compounds (500 μM) were oxidized at a similar rate, reaching an average of 71% degradation in 6 h samples. After this time oxidation stopped due to O_2 depletion in the closed vials used in the incubations.

1. Introduction

Advanced oxidation is a term widely used in the field of pollution remediation to define a form of oxidation where the degradative agents are highly reactive radical species, mainly hydroxyl radicals (•OH). These radicals are produced by different physico-chemical procedures, referred to as advanced oxidation processes (AOP), including Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + •OH$) [1–4]. The most interesting characteristics that •OH present for the degradation of pollutants are high reactivity and low selectivity, in such a way that at sufficient concentration and contact time they are able to mineralize most organic pollutants.

Compared with AOP, biological procedures used for pollutant degradation are much more selective as they are based on the action of enzymes. However, there exists a group of microorganisms, the white-rot fungi, which has been proven to degrade a high number and variety of pollutants [5,6]. This ability is mainly due to the low substrate specificity and high reactivity of the enzymes that they produce to degrade lignin (laccase and peroxidases) [7]. These enzymes catalyze the one-electron oxidation of substrates producing free radicals that undergo a variety of spontaneous degradative reactions [8]. The ligninolytic enzymes can also degrade lignin and environmental pollutants through the oxidation of low molecular weight chemical species acting as enzyme mediators. This mediated oxidation mechanism resembles AOP in the generation of highly reactive radicals. It also expands the range of pollutants susceptible to degradation by the ligninolytic system since substrate specificity stops being a limitation [9]. White-rot fungi also present the ability to produce extracellular •OH [10] and two mechanisms involving the ligninolytic enzymes and Fenton reaction have been proposed to sustain it [11,12]. However, evidence of advanced oxidation of pollutants by white-rot fungi is still in its incipient stages. A recent study has described a simple strategy for the induction of extracellular •OH production in several white-rot fungi, including *Pleurotus eryngii* and *Trametes versicolor* [13]. The strategy consists in the incubation of fungi with a lignin-derived quinone (2,6-dimethoxy-1,4-benzoquinone, DBQ) and chelated ferric ion (Fe^{3+}–EDTA). Under these conditions, •OH is produced through a quinone redox cycling mechanism, which is

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catalyzed in these fungi by an intracellular quinone reductase (QR) and any of the extracellular ligninolytic enzymes. QR converts the quinone into hydroquinone ($DBQH_2$) and the ligninolytic enzymes oxidize $DBQH_2$ to semiquinone radicals ($DBQ^{\bullet -}$) in the extracellular medium. Fenton's reagent is formed by $DBQ^{\bullet -}$ autoxidation catalyzed by Fe^{3+}, in which Fe^{2+} and superoxide anion radical ($O_2^{\bullet -}$) are generated ($DBQ^{\bullet -} + Fe^{3+}$–EDTA → DBQ + Fe^{2+}–EDTA; and Fe^{2+}–EDTA + O_2 ⇌ Fe^{3+}–EDTA + $O_2^{\bullet -}$). This is followed by $O_2^{\bullet -}$ dismutation ($O_2^{\bullet -} + HO_2^{\bullet} + H^+ \rightarrow O_2 + H_2O_2$). This strategy has been used to provide first evidence of advanced oxidation of two pollutants (phenol and the dye reactive black 5) by a white-rot fungus, *P. eryngii* [14].

The aim of the present study was to demonstrate advanced oxidation of benzene, toluene, ethylbenzene and xylene isomers (BTEX) by *T. versicolor* by means of the induction of extracellular •OH production. BTEX are volatile monoaromatic hydrocarbons present in petroleum and gasoline. These compounds are one of the major causes of environmental pollution because of widespread occurrences of leakage from petroleum and fuel storage tanks and spills at petroleum production wells, refineries, pipelines, and distribution terminals. Although aerobic and anaerobic degradation of BTEX by bacteria has been studied for several decades [15,16], fungal studies on this subject are scarce. In white-rot fungi, BTEX degradation has been only described in *Phanerochaete chrysosporium* with the non-involvement of the ligninolytic system [17].

2. Materials and methods

2.1. Chemicals

Benzene, toluene, ethylbenzene, *o*-, *m*-, and *p*-xylene, phenol, 2,6-dimethoxyphenol (DMP), 3,4-dimethoxybenzyl (veratryl) alcohol, 1,4-benzoquinone (BQ), 1,4-benzohydroquinone, and DBQ were purchased from Aldrich. $DBQH_2$ was prepared from DBQ by reduction with sodium borohydride [13]. 2-Deoxyribose, and 2-thiobarbituric acid (TBA) were from Sigma. All other chemicals used were of analytical grade.

2.2. Organism and culture conditions

T. versicolor (ATCC 42530) was maintained at 4 °C on 2% malt extract agar. For mycelial pellets production the fungus was grown at 28 °C in shaken (150 rpm) 1 l conical flasks with 400 ml of a medium containing 2% malt extract. In time course studies 100 ml conical flasks with 40 ml of medium were used. Inoculum (0.5 mg dry weight per ml of medium) was prepared by homogenizing 5-day old mycelium.

2.3. Enzyme activities

All the enzymatic assays were performed at room temperature (22 °C). International units of enzyme activity (μmol min^{-1}) were used. Laccase and MnP activities were assayed using DMP and Mn^{2+} as substrates, respectively [18]. LiP activity was estimated as the oxidation of veratryl alcohol [19]. Washed mycelium was used for the determination of QR activity with BQ as substrate [13] and analyzing the production of BQH_2 by HPLC. Samples (20 μl) were injected into a Shimadzu system (model LC-9A, No. 272260LP) equipped with a Mediterranea Sea18 column (5 μm, 15 × 0.46 mm, Teknokroma, Madrid, Spain), and a diode array detector. The eluent was 10 mM phosphoric acid/methanol (80/20) at a flow rate of 1 ml min^{-1}. The detector operated at 280 nm and BQH_2 levels were estimated using a standard calibration curve.

2.4. Quinone redox cycling conditions

In time course experiments, •OH radical induction in *T. versicolor* was performed as follows. Mycelial pellets were collected by filtration, washed three times with MilliQ water, and divided in two equal parts (wet weight), one for the determination of QR activity and the other one for •OH induction. In the latter case, pellets were incubated with 500 μM DBQ and 100 μM Fe^{3+}–110 μM EDTA in 20 ml 20 mM phosphate buffer, pH 5. Iron salt ($FeCl_3$) solutions were made up fresh immediately before use. Incubations also contained 2.8 mM 2-deoxyribose, which was the probe used to detect •OH radicals as the production of TBA reactive substances (TBARS) (see below), and were carried out in 100 ml conical flasks at 28 °C and 150 rpm. Samples (1 ml) were taken every 30 min during 2 h, filtered, lowered their pH to 2 with phosphoric acid, and analysed for TBARS production.

In BTEX degradation experiments, •OH induction was performed in 8 ml screw top vials, sealed with polytetrafluoroethylene (PTFE) lined silicone septa (Teknokroma, Madrid, Spain). Incubations were carried out in 4 ml and, besides Fe^{3+}–EDTA, 100 μM Fe^{3+}–300 μM oxalate in the absence and presence of 100 μM Mn^{2+} was used to induce •OH production. TBARS were analysed in vials not containing BTEX compounds. Vials were incubated horizontally at 28 °C and 150 rpm. Samples (3 vials at each point of analysis) were treated as described above for time course experiments. Incubation blanks contained no iron complexes.

2.5. BTEX degradation experiments

Appropriate amounts of 4-day old pellets were incubated in 4 ml 20 mM phosphate buffer, pH 5, with BTEX under conditions inducing or not inducing •OH production, as described above for quinone redox cycling in BTEX degradation experiments (in the absence of 2-deoxyribose). Acetonitrile was used in the preparation of BTEX stock solutions (100–400 mM in 24 ml) due to the low water solubility of these compounds. Screw top vials (24 ml capacity) sealed with PTFE lined silicone septa (Teknokroma, Madrid, Spain) were used. Unless otherwise stated, 2 μmol of each BTEX compound (500 μM initial concentration in the incubation solution) were added to the 8 ml vials, using a 20 μl Hamilton syringe. Samples (3 vials at each point of analysis) were treated as follows. To release any BTEX compound that could have been adsorbed to the mycelium, 4 ml acetonitrile were added to the vials. Samples were kept under the incubation conditions for a further 30 min period. Then, after pellet decantation, 700 μl were transferred by means of a 1 ml Hamilton syringe to 700 μl screw top vials sealed with PTFE lined silicone septa (Teknokroma, Madrid, Spain). These vials contained 2.5 μl phosphoric acid in order to low the pH of samples to 1.5–2.0 and inactivate any enzyme that could have been released by the fungus. Vials were kept at 4 °C and centrifuged at 12,000 rpm for 5 min before BTEX analyses by HPLC (see below). Uninoculated vials, incubated for 1 h under the same conditions and processed in the same way, were used to correct BTEX levels from those released to the gas phase.

2.6. Analytical techniques

TBARS production from 2-deoxyribose was used to estimate •OH production [13]. The concentration of DBQ, $DBQH_2$, phenol, and BTEX compounds was determined by HPLC using standard calibration curves of each compound. DBQ, $DBQH_2$, and phenol were analysed under the same chromatographic conditions described above for QR activity. BTEX analyses were performed at room temperature with a Mediterranea Sea18 column (3 μm, 3 × 0.46 cm, Teknokroma, Madrid, Spain), a flow rate of 2 ml min^{-1} and 10 mM