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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

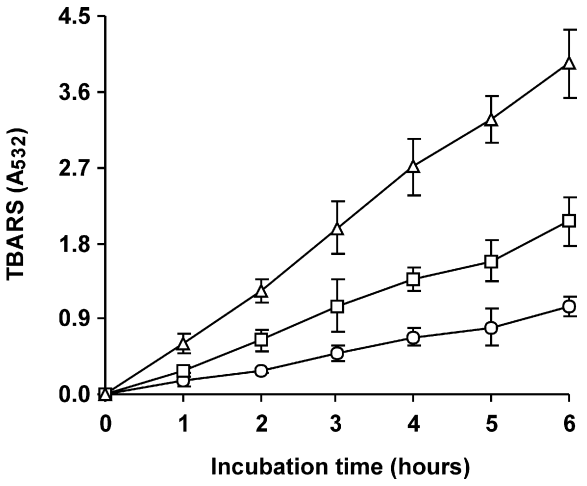

Fig. 1. Production of •OH by *T. versicolor* through DBQ redox cycling as a function of incubation time and amount of mycelium. 30 mg (○), 60 mg (□) and 120 mg (△) (dry weight) 4-day old mycelium were incubated in 40 ml 20 mM phosphate buffer, pH 5, with 500 μM DBQ, 100 μM Fe^{3+}–110 μM EDTA and 2.8 mM 2-deoxyribose. Filtered samples were analysed for TBARS production.

phosphoric acid/acetonitrile (60/40) as the eluent. The diode array detector was set at 201 nm.

2.7. Statistical analysis

All the results included in this work are the mean and standard deviation of three replicates (full biological experiments and technical analyses).

3. Results and discussion

3.1. Production of •OH by T. versicolor via quinone redox cycling

In a previous study, evidence of •OH induction by DBQ and Fe^{3+}–EDTA in several white-rot fungi was obtained using 10-day old mycelium produced in a complex medium containing glucose and peptone [13]. The production of •OH was inferred from the generation of Fenton's reagent and the formation of typical reactions products of •OH attack to 2-deoxyribose and 4-hydroxybenzoic acid (TBARS and 3,4-dihydroxybenzoic acid, respectively). For the present study a culture medium composed exclusively of malt extract was selected. Growing the fungus in this medium and using several amounts of 4-day old pellets in incubations with DBQ, Fe^{3+}–EDTA, and 2-deoxyribose, induction of extracellular •OH production was tested as TBARS formation. Positive results were obtained, showing •OH production on a constant basis during 6 h (Fig. 1). A high correlation between TBARS levels and the amount of mycelium used was also found. Regression analysis of data rendered TBARS production rates of 2.8, 5.6, and 11.0 mU A_{532} min^{-1} in incubations carried out with 30, 60, and 120 mg of mycelium, respectively.

To find out the optimum culture age for the induction of •OH, a time course experiment was carried out. The experiment included the determination of biomass (dry weight), TBARS production rate, and the activities of QR and the ligninolytic enzymes. Fig. 2 shows that *T. versicolor* was able to produce •OH via DBQ redox cycling during both the exponential and stationary phases of growth. Maximum dry weight was reached after 8 days. Differences in the TBARS production rate between most of the samples were not significant, keeping steady around 12 mU A_{532} min^{-1} (average of all samples). However, when referred to the biomass, the efficiency of •OH production was much higher at the beginning of the exponential growth phase. For instance, the specific TBARS production rate in 2–3-day samples was 527 ± 45 mU A_{532} min^{-1} g^{-1}, whereas in the rest of samples was gradually decreased from 198 (4 days) to 120 mU A_{532} min^{-1} g^{-1} (10 days). Enzyme analyses rendered positive results for QR and laccase. Both enzymes presented maximum activity levels during the first 5 days. Based on these results, a 3-day culture time period was chosen for subsequent experiments.

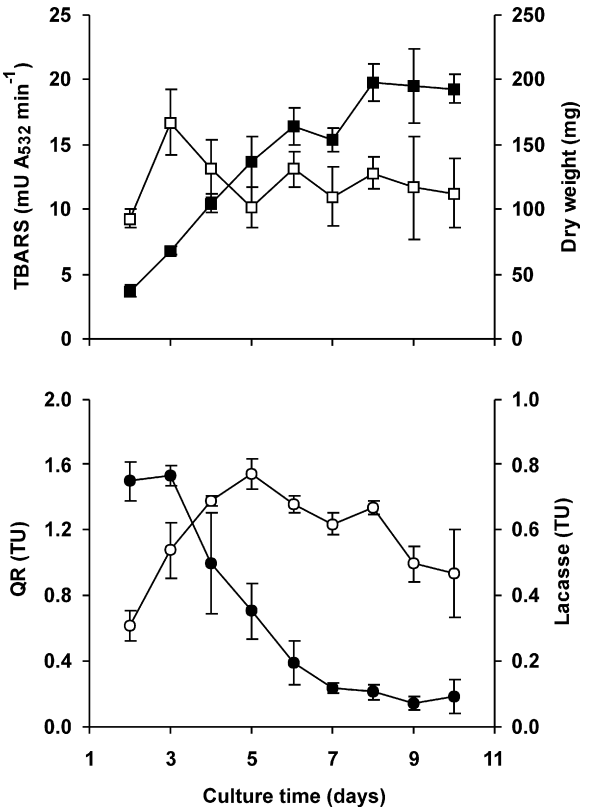

Fig. 2. Capability of *T. versicolor* to produce •OH, estimated as TBARS production from 2-deoxyribose, via DBQ redox cycling as a function of culture time. Besides TBARS (□), time courses of mycelium dry weight (■) and the activity (total units) of the enzymes catalyzing DBQ redox cycle, i.e. QR (○) and laccase (●), are shown.

3.2. Oxidation of benzene by T. versicolor under different •OH induction conditions

Volatility of BTEX compounds addressed the performance of degradative experiments in tightly closed containers that were sacrificed at each time point of analysis. Small capacity vials (8 ml) were used. First experiments were performed with benzene and were aimed to ascribe a role to •OH in the degradative process. Washed mycelium was used to prevent the reactions of •OH with components of the culture medium and/or fungal metabolites, that could lead to the production of other reactive radicals oxidizing the pollutant. Besides, two modifications in the incubation mixture composition that were previously described to quantitatively modulate •OH production in *P. eryngii* were considered: replacement of Fe^{3+}–EDTA by Fe^{3+}–oxalate in the absence and presence of Mn^{2+}, which increased TBARS production rate around 3 and 6 times, respectively [14]. In comparison to Fe^{3+}–EDTA, the complex Fe^{3+}–oxalate is able to oxidize the hydroquinone ($DBQH_2 + Fe^{3+}$–oxalate → $DBQ^{\bullet-} + Fe^{2+}$–oxalate), increasing the rate of DBQ redox cycle. The presence of Mn^{2+} has a double positive effect in •OH generation. First, by reducing $O_2^{\bullet-}$ ($Mn^{2+} + O_2^{\bullet-} \rightarrow Mn^{3+} + H_2O_2$) it doubles the amount of H_2O_2 generated in relation to $O_2^{\bullet-}$ dismutation. Sec-

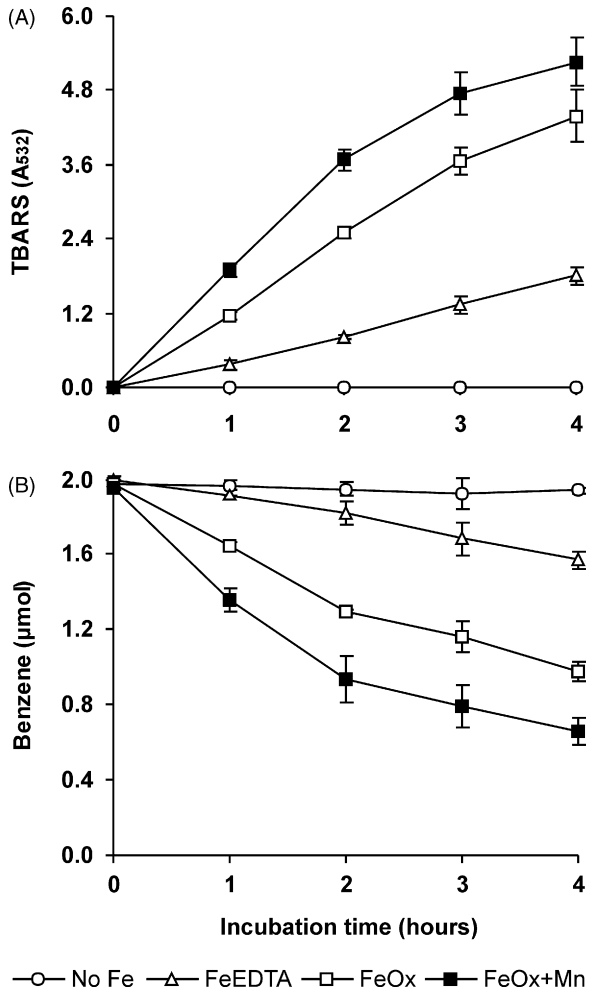

Fig. 3. Effect of oxalate, used as the iron chelating agent, and Mn^{2+} on the production of TBARS (A) and the oxidation of benzene (B) by *T. versicolor*. Incubations were carried out in 8 ml closed vials containing 13.1 ± 0.9 mg (dry weight) 3-day old fungal pellets, 4 ml 20 mM phosphate buffer, pH 5, 500 μM DBQ, and when indicated 100 μM Fe^{3+}–110 μM EDTA (FeEDTA) or 100 μM Fe^{3+}–300 μM oxalate in the absence and presence of 100 μM Mn^{2+} (FeOx and FeOx + Mn, respectively). Incubation blanks contained no iron (No Fe). Vials analysed for TBARS and benzene contained, respectively, 2.8 mM 2-deoxyribose and 2 μmol benzene.

ond, the Mn^{3+} generated propagates hydroquinone oxidation ($DBQH_2 + Mn^{3+} \rightarrow DBQ^{\bullet -} + Mn^{2+}$) [14]. Fig. 3 shows the time courses of TBARS production and benzene oxidation by *T. versicolor* when incubated under DBQ redox cycling conditions including no Fe^{3+}, Fe^{3+}–EDTA, and Fe^{3+}–oxalate with or without Mn^{2+}. Incubations were carried out in 4 ml. Vials were divided in two groups containing either 2-deoxyribose or benzene. Initial TBARS production rates (first 2 h) of 6.6, 20.6, and 30.8 mU A_{532} min^{-1} were obtained in incubations containing Fe^{3+}–EDTA, Fe^{3+}–oxalate, and Fe^{3+}–oxalate plus Mn^{2+}, respectively. Benzene oxidation was observed in the parallel vials, showing initial rates highly correlated with those of TBARS production: 1.5, 5.7, and 8.5 nmol min^{-1} in incubations containing Fe^{3+}–EDTA, Fe^{3+}–oxalate, and Fe^{3+}–oxalate plus Mn^{2+}, respectively. Neither TBARS production nor benzene oxidation was observed in incubations lacking Fe^{3+}.

A longer benzene oxidation experiment (8 h), carried out under optimal •OH production conditions (DBQ redox cycling with Fe^{3+}–oxalate and Mn^{2+}), showed that both TBARS and benzene levels kept steady after 4 h (Fig. 4). The maximum amount of benzene oxidized attained a 78% (8 h sample). Samples from this experiment were also analysed for phenol formation as a primary hydroxylation product of •OH attack to benzene. First, a hydroxycyclohexadienyl

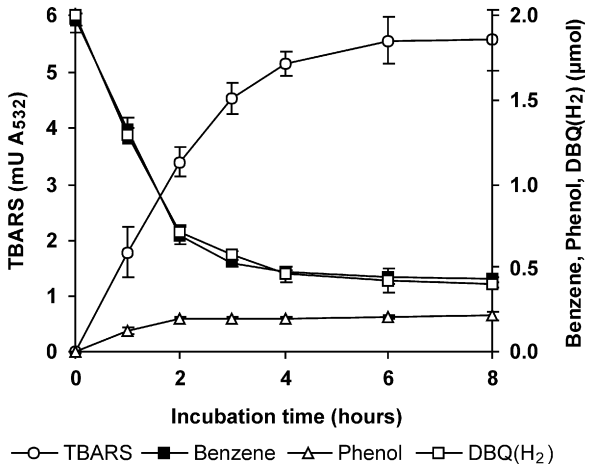

Fig. 4. Time courses of TBARS formation, benzene and $DBQ(H_2)$ removal, and phenol production during the incubation of *T. versicolor* with DBQ under optimal •OH production conditions. Incubations were carried out in 8 ml closed vials containing 17.6 ± 0.7 mg (dry weight) 3-day old fungal pellets, 4 ml 20 mM phosphate buffer, pH 5, 500 μM DBQ, 100 μM Fe^{3+}–300 μM oxalate, 100 μM Mn^{2+}, and either 2.8 mM 2-deoxyribose (for TBARS analysis) or 2 μmol benzene.

radical intermediate is formed [20,21]. In the presence of an oxidant like Fe^{3+}, this intermediate produces phenol and returns the iron catalyst to its divalent state, ready for •OH production by reducing H_2O_2. The oxidation of hydroxycyclohexadienyl radicals by Fe^{3+} has been shown to be catalyzed by quinones [22], such as the one used in the present study to induce •OH production. Phenol was detected in all samples (Fig. 4), showing a progressive increase for 2 h and then keeping constant around 0.2 μmol. Conversion of phenol into 1,4- and 1,2-benzohydroquinone (catechol) by •OH has been previously described in incubations of *P. eryngii* with DBQ and Fe^{3+}–oxalate [14]. Oxidation studies of benzene and phenol based on Fenton and UV/H_2O_2 AOPs have shown the generation of these dihydroxybenzene derivatives, along with quinones and ring-opened products (mainly carboxylic acids), as common degradation intermediates [23,24]. Therefore, it is likely that the plateau observed in phenol levels after 2 h (Fig. 4), while benzene being still oxidized, was due to phenol oxidation by •OH. In this respect, it is interesting to mention that some of the above phenol hydroxylation intermediates, such as 1,4-benzohydroquinone, can support •OH radical production via quinone redox cycling [14].

3.3. Factors limiting advanced oxidation of benzene by T. versicolor

Among the likely factors that could be limiting •OH production and benzene oxidation in Fig. 4 experiment, the following were evaluated: (i) consumption of the reducing equivalents required for DBQ reduction; (ii) degradation of DBQ; (iii) depletion of O_2 due to its consumption for •OH production and fungal respiration; and (iv) some mycelium damage. First, •OH production was estimated under the same quinone redox cycling conditions used in Fig. 4 experiment but in the presence of 2.8 mM glucose. No significant differences in the time course of TBARS production to that shown in Fig. 4 were observed (data not shown). This indicates that the fungus had enough reducing equivalents for QR activity. To discard TBARS formation from glucose, incubation blanks in this experiment were performed in the absence of 2-deoxyribose, rendering negative results. Second, the degradation of DBQ was studied in the same samples used for the determination of benzene levels in Fig. 4 experiment. This analysis included the determination of DBQ and its reduction counterpart ($DBQH_2$) levels, and is shown in Fig. 4 as the sum of both: $DBQ(H_2)$. An extremely sim-

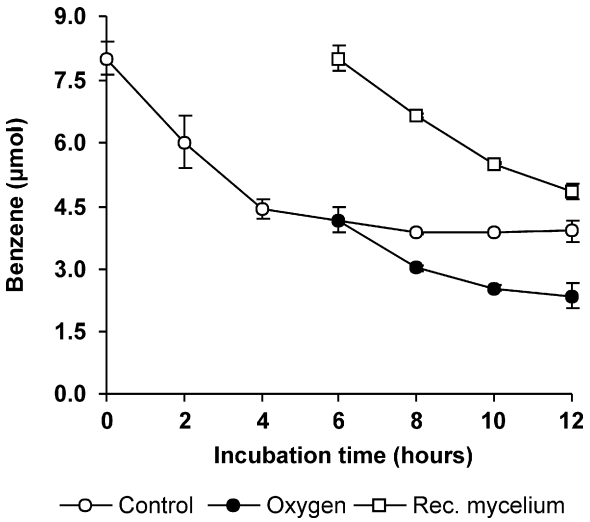

Fig. 5. Effect of oxygen and mycelium recycling on advanced oxidation of benzene by *T. versicolor*. Incubations contained 14.2 ± 0.9 mg (dry weight) 3-day old fungal pellets, 4 ml 20 mM phosphate buffer, pH 5, 1 mM DBQ, 100 μM Fe^{3+}–300 μM oxalate, 100 μM Mn^{2+}, and 8 μmol benzene. Control vials were kept during 12 h under these conditions. Oxygen vials were supplemented with 1 ml pure O_2 after 6 h. Recycled mycelium vials contained pellets previously incubated under the control conditions during 6 h.

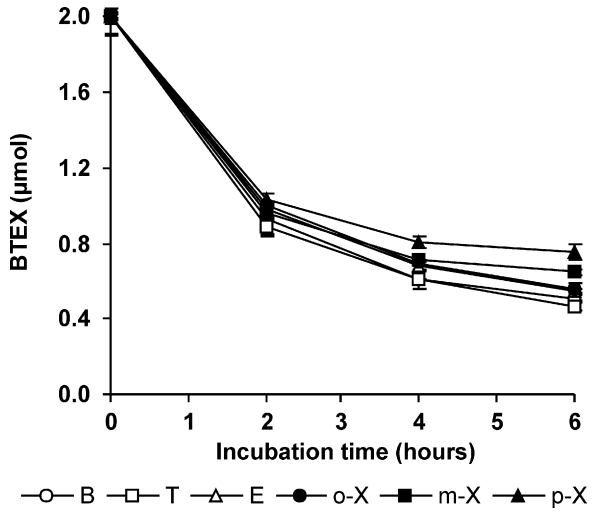

Fig. 6. Effect of inducing the production of •OH in *T. versicolor* on the oxidation of BTEX compounds. Incubations were carried with 15.9 ± 0.7 mg (dry weight) 3-day old mycelium in 4 ml 20 mM phosphate buffer containing 500 μM DBQ, 100 μM Fe^{3+}–300 μM oxalate, 100 μM Mn^{2+}, and 2 μmol of either benzene (B), toluene (T), ethylbenzene (E), *o*-xylene (*o*-X), *m*-xylene (*m*-X), or *p*-xylene (*p*-X). No oxidation was observed in incubation blanks without Fe^{3+}–oxalate.

ilar time course for DBQ(H_2) disappearance to that of benzene was observed, suggesting DBQ(H_2) consumption by •OH attack. However, the remaining DBQ(H_2) concentration present in samples after 4 h (around 100 μM) could still sustain •OH production, as tested by TBARS production in a DBQ redox cycling experiment carried out with an initial quinone concentration of 125 μM (data not shown). And third, a new benzene oxidation experiment evidenced that between O_2 depletion and some mycelium damage, the first one was the main factor limiting TBARS production and benzene oxidation. Incubations of *T. versicolor* with benzene were performed during 12 h. After 6 h, the vials were divided in three groups: (i) the control group which was kept under invariable incubation conditions; (ii) the oxygen group which was supplemented after 6 h with 1 ml of pure O_2 by injection through the vial septa; and (iii) the recycled mycelium group in which the mycelium was separated from the incubation solution by filtration, washed 3 times with MilliQ water, and put again under the initial benzene oxidation conditions. To better evaluate the effects of these treatments, the amount of benzene and DBQ was increased to 8 and 4 μmol, respectively. The oxidation of benzene in control incubations was observed during the first 4 h (Fig. 5), keeping steady at around 51% afterwards (average of 6–12 h samples). The injection of O_2 restarted benzene oxidation reaching 71% after 12 h. The recycled mycelium oxidized benzene at a rate slightly lower to that observed during the first degradation cycle, showing a high resistance against the possible toxic effect of benzene and its degradation intermediates, as well as to •OH attack.

3.4. Advanced oxidation of BTEX by T. versicolor

The effect of •OH production by *T. versicolor* on the oxidation of the rest of BTEX compounds was tested in separated incubations for each compound (benzene was included for comparison). Toluene, ethylbenzene, and xylene isomers were only oxidized under •OH producing conditions (Fig. 6). No significant differences in the oxidation rate of these compounds and benzene were observed. An average of 71% BTEX degradation was calculated from the 6 h sample data. It should be noted that the degradation of all six BTEX compounds has been estimated in relation to their disappearance, this being the result of •OH attack. The most common mechanism for oxidation of aromatic compounds by •OH is electrophilic addition to the rings [21]. Fig. 4 shows the conversion of benzene into phenol, which in turn is transformed in dihydroxybenzene derivatives [14]. Oxidation of toluene by •OH produce a mixture of cresol isomers [25], which have been shown to be converted into quinones, hydroquinones and, finally, aliphatic carboxylic acids and CO_2 [26]. The metabolism of some of these hydroxylated intermediates could involve not only •OH radicals but also the ligninolytic enzymes, and probably intracellular oxygenases [27]. The results shown in Fig. 6 are in agreement with the similar constant rates for reaction of •OH with the six BTEX compounds [28,29]. An advanced oxidation study of BTEX using gamma radiolysis to generate •OH has also shown that the degradation of these compounds takes place at similar rates [30]. This is a distinctive characteristic of the results shown in Fig. 6 when compared with other fungal studies showing enzymatic oxidation of BTEX at different rates. The degradation of the six BTEX compounds has been studied with *P. chrysosporium* [17] and, among non-ligninolytic fungi, with two species belonging to the *Cladophialophora* and *Cladosporium* genera [31,32], and *Paecilomyces variottii* [33]. Among them, only *P. chrysosporium* and *P. variottii* degraded all the BTEX compounds at different rates, and *Cladophialophora* sp. and *Cladosporium* sp. did not degrade benzene. Pollutant degradation by fungi generally implies intracellular oxygenases [27]. In white-rot fungi the ligninolytic system is also implicated. However, little is known about the enzymatic systems involved in the oxidation of BTEX. A microsomal aromatic ring-hydroxylating monooxygenase showing activity on BTEX compounds except benzene has been purified and characterized from *Cladosporium sphaerospermum* [34]. *P. chrysosporium* was shown to degrade BTEX under conditions producing no ligninolytic enzymes [17]. This fungus has an aromatic ring cleavage dioxygenase acting on di- and tri-hydroxylated benzene derivatives [35]. In this regard, the present study provides the first evidence on the involvement of the ligninolytic system in BTEX oxidation through the generation of •OH.

In summary, the results of this study have shown that •OH radical induction in *T. versicolor* is a feasible strategy to the degradation of recalcitrant pollutants, such as BTEX. Further work is needed to identify BTEX intermediates, and to determine the role of •OH, ligninolytic enzymes and intracellular oxygenases in the metabolism

of these intermediates. Mineralization of BTEX is another concern in which we are working at the moment. We have started with toluene, incubated with *P. eryngii* under the same conditions as in the present work, and we have observed that this compound is in part converted to CO_2 in a few hours (data not shown). We are conscious of the fact that a vast amount of work is required in order to evaluate the industrial feasibility of this strategy: optimization of the process, scale in up, evaluation of economic viability, etc. The work described in this paper is a proof of concept.

3.5. Conclusion

The oxidation of BTEX by *T. versicolor* mediated by $^{\bullet}OH$ proves that advanced oxidation of pollutants can be extended to biological processes.

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