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Transformation of the Recalcitrant Pharmaceutical Compound Carbamazepine by *Pleurotus ostreatus*: Role of Cytochrome P450 Monooxygenase and Manganese Peroxidase

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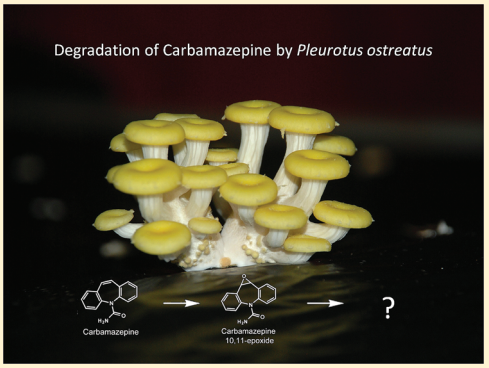
ABSTRACT: Carbamazepine (CBZ) is an environmentally recalcitrant compound highly stable in soil and during wastewater treatment. In this study, we examined the mechanisms by which the white-rot fungus *Pleurotus ostreatus* metabolizes CBZ in liquid culture using a physiological approach. *P. ostreatus* PC9 was grown in media known to support different levels of a multiplicity of enzyme systems such as cytochrome P450 (CYP450) and manganese peroxidase (MnP). When both CYP450 and MnP systems were active, 99% of the added CBZ was eliminated from the solution and transformed to 10,11-epoxycarbamazepine. High removal of CBZ was also obtained when either MnP or CYP450 was active. When both CYP450 and MnP were inactivated, only 10 to 30% of the added CBZ was removed. In this latter system, removal of CBZ might be partially attributed to the activity of versatile peroxidase. *P. ostreatus* was able to eliminate CBZ in liquid culture even when CBZ was added at an environmentally relevant concentration (1 μg L^{-1}). On the basis of our study, we suggest that two families of enzymes are involved in the oxidation of CBZ in liquid culture: MnP in a Mn^{2+}-dependent or independent manner and CYP450. Our study also highlights the potential of using *P. ostreatus* for bioremediation systems.



INTRODUCTION

Environmental concern over the presence of active pharmaceutical compounds in the environment is increasing. Many pharmaceutical compounds are not fully removed during common municipal wastewater treatment, so they are released to the environment with the treated effluents.[1] Pharmaceutical compounds are found in wastewater effluents, surface water and groundwater, and even in drinking water.[2] They can also be introduced into agricultural soils, mainly via irrigation with reclaimed wastewater and biosolids application.[3] In the soil, pharmaceutical compounds can accumulate, affect the soil's microbial community,[4] and can be taken up by crops.[5]

Carbamazepine (CBZ) is an anticonvulsant drug used primarily for the treatment of epilepsy. It exhibits very limited removal efficiency in municipal wastewater-treatment plants [6] and shows high persistence in the environment. This is mainly due to its remarkably high resistance to microbial degradation [7] and high adsorption capabilities.[3] Thus, CBZ has been suggested as an anthropogenic marker in the environment.[8] It has also been found to have an ecotoxicological impact on aquatic organisms.[9]

CBZ removal and transformation by different fungi have been recently reported.[10−13] When CBZ was introduced at an initial level of 10 mg L^{-1} to pure cultures of *Cunninghamella elegans* or *Umbelopsis ramanniana*, its concentration was reduced after 25 d to 5.7 and 7.4 mg L^{-1}, respectively.[10] The white-rot fungi *Trametes versicolor* and *Ganoderma lucidum* were shown to eliminate 57 and 46% of the added CBZ after 7 d of incubation, respectively.[11] Similar removal efficiency of CBZ was observed by *T. versicolor* in a solid-phase bioreactor containing sewage sludge and mycelium.[13] When cytochrome P450 (CYP450) inhibitor was added to the growing medium, CBZ removal was significantly reduced, suggesting its possible involvement in the process.[11] CYP450 monooxygenases have also been reported to metabolize CBZ in the human liver.[14]

In the current study, we focused on the white-rot fungus *Pleurotus ostreatus*. The ligninolytic system of *Pleurotus* species has been found to be mainly composed of the lignin-modifying enzymes laccase, aryl-alcohol oxidase, and two types of peroxidases: Mn^{2+}-dependent peroxidase (MnP) and versatile peroxidase (VP). These enzymes can function separately and/or in cooperation.[15] VP has been suggested to be a MnP−lignin peroxidase hybrid based on its ability to oxidize different substrates in the presence or absence of Mn^{2+}.[16,17] The CYP450 superfamily of *P. ostreatus* consists of 153 genes capable of varied chemical reactions such as hydroxylation, epoxidation, decarboxylation, and aryl-transformation.[18,19] *P. ostreatus* has been shown to successfully metabolize several polycyclic aromatic hydrocarbons

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via CYP450 monooxygenases.[20] *P. ostreatus* is known to degrade natural macromolecules such as lignin, humic substances, and anthropogenic compounds via nonspecific oxidative enzymatic systems. Thus, in this study, we investigated the mechanisms involved in degradation and transformation of CBZ by *P. ostreatus* in liquid culture.

EXPERIMENTAL SECTION

Chemicals. Carbamazepine (CBZ; 5H-dibenzo[b,f]azepine-5-carboxamide, 98%), 10,11-epoxycarbamazepine (10,11EPCBZ; 1a,10b-dihydro-6H-dibenzo(b,f)oxireno[d]azepine-6-carboxamide, ≥98%), and 1-aminobenzotriazole (1-ABT; 98%) were purchased from Sigma-Aldrich (Rehovot, Israel). D_3,^{13}C-labeled carbamazepine (Toronto Research Chemicals, Ontario, Canada) was used as an internal standard for LC-MS analysis.

Fungal Strains and Culture Conditions. The followings strains of *P. ostreatus* were used: Florida N001 (mating type genotype *A1A2B1B2*), PC9 (Spanish Type Culture Collection accession number CECT20311) and Florida F6. Strain PC9 is a monokaryotic derivative of the dikaryon commercial strain Florida N001.[21] Fungal strains were grown and maintained in glucose peptone (GP) or basidiomycetes salt (BSM) media. The GP medium contained 20 g L^{-1} glucose, 5 g L^{-1} peptone, 2 g L^{-1} yeast extract, 1 g L^{-1} K_2HPO_4, and 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$; the BSM contained 5 g L^{-1} glucose, 1 g L^{-1} K_2HPO_4, 0.6 g L^{-1} asparagine, 0.1 g L^{-1} yeast extract, 0.5 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 3 mg L^{-1} $Zn(NO_3)_2 \cdot 6H_2O$, 6 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, and 3 mg L^{-1} $CuSO_4 \cdot 5H_2O$.[22] When specified, Mn^{2+} (27 μM) was added to the media as $MnSO_4$.

Solid cultures were grown in 9 cm diameter Petri dishes containing GP or BSM media solidified with 1.5% (w/v) agar. Liquid cultures were maintained in stationary 250 mL Erlenmeyer flasks containing 50 mL media and covered with paper tops. Cultures were incubated at 28 °C in the dark. The inocula for liquid cultures were two disks (5 mm in diameter) of mycelium collected from a freshly grown colony in solid culture. Liquid cultures of *P. ostreatus* were incubated for 10 d. Then the medium was decanted and replaced with fresh medium containing 10 mg L^{-1} (or 1 μg L^{-1} for environmentally relevant concentration experiment) CBZ. For the treatments containing CYP450 inhibitor, 1-ABT (100 mM) was added to the medium on d 1 of incubation, after the medium replacement and after each sampling (in total, 1-ABT was spiked eight times into each medium). During incubation, the media were sampled twice a week. For each treatment (different media, with or without Mn^{2+}, with or without 1-ABT), four replicates were performed in addition to a set of controls (without CBZ and uninoculated). All treatments and controls were incubated for 32 d.

Manganese Peroxidase (MnP) and Versatile Peroxidase (VP) Activities. The activity assays [22] were performed twice a week starting from day 3 of incubation. Reaction mixture (1 mL) contained 0.01% (w/v) phenol red, 25 mM lactate, 0.1% (w/v) bovine serum albumin, 0.1 mM $MnSO_4$, 0.1 mM H_2O_2, and 100 μL sample in 20 mM Na-succinate buffer (pH 4.5). The assays were performed at 32 °C for 10 min and terminated with 50 μL of 2 M NaOH. Absorbance was recorded at 610 nm. Boiled samples were used as blanks. VP activity was determined with phenol red (as described for MnP) in a reaction mixture that did not contain Mn^{2+}. One unit of MnP activity was defined as μmol product in 60 s for 1 mL sample. The activity in the control that did not contain H_2O_2 was negligible.[22] MnP activity was defined as the activity in the samples containing Mn^{2+} in both the growth medium and the reaction mixture. VP activity was defined as the activity in the samples that did not contain Mn^{2+} in the growth medium.

Analytical Procedures. Culture samples were filtered through 0.45 μm Teflon filters (National Scientific, Rockwood, TN, USA). NaN_3 (100 mg L^{-1}) was added to the vial to prevent biotic activity until sample analysis. CBZ concentration was quantified using an L-7100 LaChrom HPLC (Merck-Hitachi, Darmstadt, Germany) equipped with a LiChrospherRP-18 column (25 cm × 4 mm, particle size 5 μm). CBZ was eluted at a flow rate of 1 mL min^{-1} using isocratic conditions of 60/40 acetonitrile/water acidified with 0.1% (v/v) formic acid, and was detected with a photodiode array (PDA) detector at an absorbance of 286 nm.

Quantification of CBZ at lower concentrations (i.e., below 100 μg L^{-1}) and identification and quantification of its metabolites were performed using LC-MS. The samples (100 μL) containing 10 mg L^{-1} CBZ as the initial concentration were diluted with 800 μL of water and spiked with 100 μL of the labeled CBZ (2 mg L^{-1}). The samples taken from the 1 μg L^{-1} CBZ experiment were only spiked with 10 μL of the labeled CBZ prior analysis. Chromatographic analysis was performed on an Accela High Speed LC system (Thermo Fisher Scientific Inc.), which consists of Accela Pump, Accela Autosampler, and Accela PDA detector. HPLC separation was performed with Agilent Zorbax Eclipse XDB-C18 column (2.1 × 100 mm, particle size 1.8 μm) or with Phenomenex Gemini C18 column (2 × 150 mm, particle size 3.5 μm). CBZ was eluted at a flow rate of 200 μL min^{-1} using isocratic conditions of 30:70 water/methanol (both acidified with 0.1% formic acid). The Accela LC system was coupled with the LTQ Orbitrap Discovery hybrid FT mass spectrometer (Thermo Fisher Scientific Inc.) equipped with an electrospray ionization ion source. The mass spectrometer was operated in both negative and positive ionization modes. Ion source parameters were as follows: spray voltage 3.5 kV, capillary temperature 250 °C, sheath gas rate arb 30, and auxiliary gas rate (arb) 10. Mass spectra were acquired in the m/z 150−2000 Da range. The ion transfer parameters were optimized using automatic tune option for m/z 237 (protonated CBZ). The LC-MS system was controlled and data analyzed by *Xcalibur* software (Thermo Fisher Scientific Inc.).

The metabolites were identified based on standards (in the case of 10,11EPCBZ), exact mass (1−2 ppm), and MS or MS/MS spectra. For both CBZ and 10,11EPCBZ, the LOQ value was 0.1 ng mL^{-1}. Statistical analysis (All Pairs, Tukey Kramer, p = 0.05) was performed by *JMPIN* software, version 7.0.2 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Strain Comparison. The ability of three *P. ostreatus* strains F6, N001 (dikaryons) and PC9 (monokaryon) to degrade the environmentally recalcitrant compound CBZ was investigated. The studied strains exhibited different levels of CBZ degradation, ranging from 48 to 99% (Figure 1). It is important to note that with strain PC9, CBZ concentration was reduced from 10 mg L^{-1} to 20 μg L^{-1} within 17 d of incubation. This shows that *P. ostreatus* is more efficient at removing CBZ than other fungi studied to date. In previous reports, *T. versicolor*, *G. lucidum*, *C. elegans*, and *U. ramanniana* only removed 25 to 60% of added

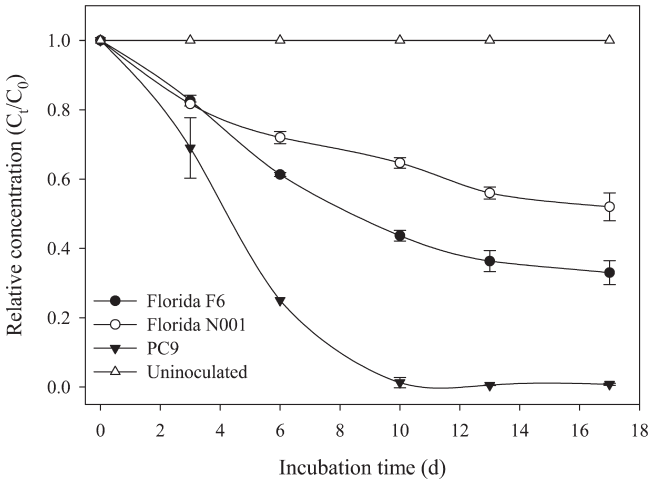

Figure 1. Relative level of carbamazepine during incubation with *P. ostreatus* strains F6, PC9, and N001. Initial carbamazepine concentration was 10 mg L^{-1}; plotted values are means ± standard deviations of four replicates.

CBZ under similar conditions.[10,11,13] Better removal of CBZ (about 80%) was only observed when a lignin-derived quinone was added to the growth medium of *T. versicolor* together with ferrous oxalate to form a Fenton-like reaction.[12] This reaction facilitates the formation of hydroxyl radicals, which oxidize the CBZ molecule faster than the natural enzymatic mechanisms. Because strain PC9 was most efficient at removing CBZ in our study, it was selected for further experiments designed to reveal the involved enzymatic mechanisms.

CBZ Removal in Different Media. At least two enzymatic mechanisms have been suggested to be involved in the oxidation of CBZ: the ligninolytic system of the white-rot fungi and the CYP450 monooxygenase system.[11,23] To dissect the possible role of each, we used a physiological approach, that is, incubation of the fungus in different media known to support the different enzymatic systems. In GP medium with Mn^{2+}, the fungus expresses MnP genes but a lower level of VP; in GP medium in the absence of Mn^{2+}, the fungus expresses the VP genes but not MnP.[22,24] To reduce CYP450 activity, we added the inhibitor 1-ABT to the media.[25] Additional experiments were conducted in BSM, in which neither MnP nor VP are produced at significant levels.[22]

The highest removal rate of CBZ was observed in the presence of Mn^{2+} and absence of 1-ABT (Figure 2A). Under these conditions, the CBZ concentration was reduced from 10 mg L^{-1} to 22.6 μg L^{-1} after 32 d of incubation (99.7% removal). Addition of 1-ABT to this medium (inactivating CYP450) or removal of Mn^{2+} (eliminating MnP system) also resulted in CBZ removal, but at a slower rate. CBZ removal rate was statistically similar between these two treatments but significantly different from its removal rate when both MnP and CYP450 were active. When *P. ostreatus* was grown in GP in the presence of 1-ABT and in the absence of Mn^{2+}, CBZ removal was lowest, only 30% of the initial CBZ was removed from the solution during the 32 d of incubation. In this treatment, both CYP450 and MnP enzymatic systems were inactive. This treatment was significantly different from the other treatments in which at least one system was active. Based on this observation we suggest that both MnP and CYP450 systems are involved in CBZ oxidation.

When *P. ostreatus* PC9 was grown in BSM (part B of Figure 2), only limited removal of CBZ occurred in all treatments (with or

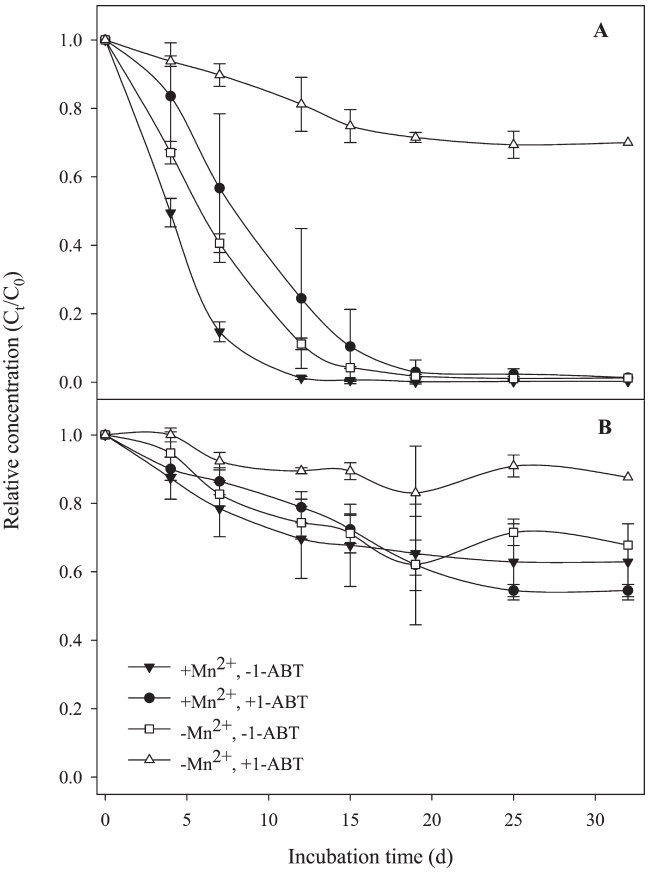

Figure 2. Relative concentrations of carbamazepine (initial concentration was 10 mg L^{-1}) during incubation with *P. ostreatus* PC9 in GP (A) and BSM (B) media. Mean values ± standard deviations of four replicates are presented (1-ABT, cytochrome P450 inhibitor; Mn^{2+}, 27 μM).

without addition of 1-ABT or Mn^{2+}). In the absence of CYP450 inhibitor, CBZ removal was similar, regardless of the presence of Mn^{2+} in the medium. In these treatments, CBZ levels have been reduced to only 63 ± 1.1% and 72 ± 0.4% of the initial CBZ concentration after 32 d of incubation (with and without Mn^{2+}, respectively). Hence, we conclude that in BSM CYP450 plays a major role in CBZ degradation. CYP450 monooxygenases have been shown to be the key enzymes in phenanthrene metabolism by this fungus in this medium: when CYP450 was turned off (in the presence of 1-ABT), phenanthrene degradation was inhibited by more than 80%.[25] Similarly, the presence of 1-ABT has been shown to reduce CBZ degradation rate by *T. versicolor* by more than 50%.[11] In the presence of 1-ABT and absence of Mn^{2+}, CBZ removal was minimal, reaching only 9.1 ± 0.3% of the initial CBZ concentration during the 32 d of incubation. CBZ removal in this treatment was lower than that observed in the same treatment in GP (30%). On the basis of previous studies,[22,24] and our data presented in Table 1, both MnP and VP show negligible activities in BSM relative to GP liquid cultures. Thus, the 30% removal of CBZ in GP medium without Mn^{2+} and with 1-ABT cannot be explained solely by MnP activities, but might be also due to sorption of the CBZ to the fungal biomass (10−15% according to Marco-Urrea et al., ref 11) and biodegradation by VP or other oxidizing systems.

The MnP family of *P. ostreatus* consists of nine members, three of which are predominantly expressed. Among these, two

Table 1. Manganese Peroxidase (MnP) and Versatile Peroxidase (VP) Activities (U mL^{-1}) in Glucose Peptone (GP) Medium[a]

	Mn^{2+}amended growth medium		Mn^{2+}nonamended growth medium	
	MnP activity[b]	VP activity[c]	VP activity[c]	VP activity[c]
Day 4	7.7×10^{-3}	4×10^{-4}	$6.5 \times 10^{-1} \pm 1 \times 10^{-1}$	$2.5 \times 10^{-2} \pm 1 \times 10^{-2}$
Day 7	$4.3 \times 10^{-2} \pm 2 \times 10^{-2}$	$1.0 \times 10^{-3} \pm 1 \times 10^{-4}$	$1.1 \times 10^{-2} \pm 7 \times 10^{-3}$	$8.0 \times 10^{-2} \pm 1 \times 10^{-2}$
Day 11	$3.8 \times 10^{-2} \pm 1 \times 10^{-2}$	n.d.	$6.6 \times 10^{-1} \pm 3.0 \times 10^{-1}$	$7.9 \times 10^{-2} \pm 2 \times 10^{-2}$

[a] Values shown are means ± standard deviations for three replicates. n.d., not detected. [b] Mn^{2+} was added to reaction mixture. [c] Mn^{2+} was not added to reaction mixture.

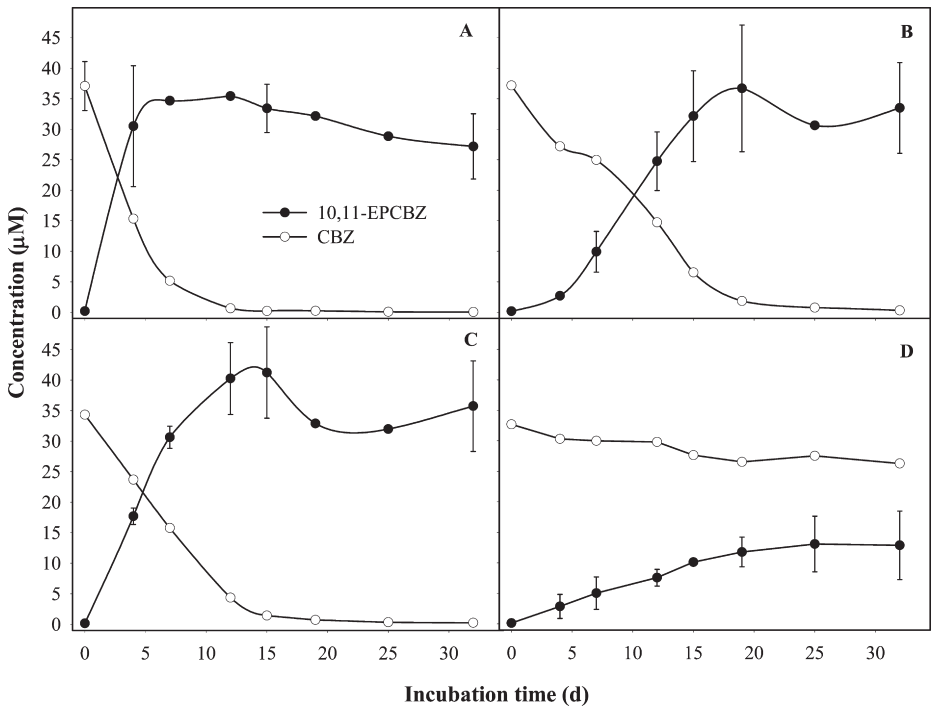

Figure 3. Concentrations of carbamazepine (CBZ) and 10,11-epoxycarbamazepine (10,11-EPCBZ) during incubation with *P. ostreatus* PC9 grown in GP medium: (A) with Mn^{2+} and without 1-ABT (cytochrome P450 inhibitor); (B) with Mn^{2+} and with 1-ABT; (C) without Mn^{2+} and without 1-ABT; and (D) without Mn^{2+} and with 1-ABT. Mean values ± standard deviations of four replicates are plotted.

(*mnp3* and *mnp9*) are expressed and active only in the presence of Mn^{2+}, while *mnp4* (i.e., VP) is expressed in the absence of Mn^{2+}.[24] As CBZ transformation was also affected by the presence of Mn^{2+}, MnP activity was monitored under the current experimental conditions. MnP activity was dominant in the samples containing Mn^{2+} both in the growth medium and the reaction mixture (Table 1). VP activity was dominant in samples that did not contain Mn^{2+} in the growth medium. As already mentioned, VP can oxidize different substrates both in the presence or absence of Mn^{2+}.[16,17] In GP medium, VP activity was lower when Mn^{2+} was absent in the reaction mixture (Table 1). However, its activity level without Mn^{2+} was similar to that of MnP. These results support the hypothesis that VP could be involved in CBZ degradation in GP without Mn^{2+}.

Metabolites. The following CBZ metabolites were detected when *P. ostreatus* PC9 was grown in GP medium: 10,11-epoxycarbamazepine (10,11EPCBZ), 10,11-dihydroxycarbamazepine, and 2- or 3-hydroxycarbamazepine. 10,11EPCBZ was the major metabolite detected in all treatments. In the experiment in which the initial concentration of CBZ was 10 mg L^{-1} (Figure 2), the concentration of 10,11EPCBZ increased with time exhibiting an opposite trend to that of CBZ (Figures 3 and 4). On the basis of mass balance calculations, almost all of the added CBZ has been transformed to 10,11EPCBZ, which was stable and accumulated in the medium. In GP medium, when CBZ removal was maximal (without 1-ABT and in the presence of Mn^{2+}), 10,11EPCBZ concentration reached a maximum of 35.4 μM (part A of Figure 3) .When CBZ removal was the lowest (with 1-ABT and in the absence of Mn^{2+}), 10,11EPCBZ reached a maximal concentration of only 12.9 μM (part D of Figure 3). Similar trends were obtained with BSM (Figure 4). These results are consistent with a report showing that *C. elegans* and *U. ramanniana* transformed CBZ (initial concentration was 1 M) mainly to 10,11EPCBZ.[10] In that study,[10] the other metabolites identified were 2-hydroxycarbamazepine and 3-hydroxycarbamazepine, all of them known as CYP450 products of the human liver.[21]

Epoxides that are CYP450 oxidation products of polycyclic aromatic hydrocarbons (such as phenanthrene and pyrene) are unstable and rapidly transformed to hydroxy derivatives or vicinal dihydrodiols.[26,27] However, in our experimental system 10,11EPCBZ was stable. Indeed, 10,11EPCBZ was reported as a

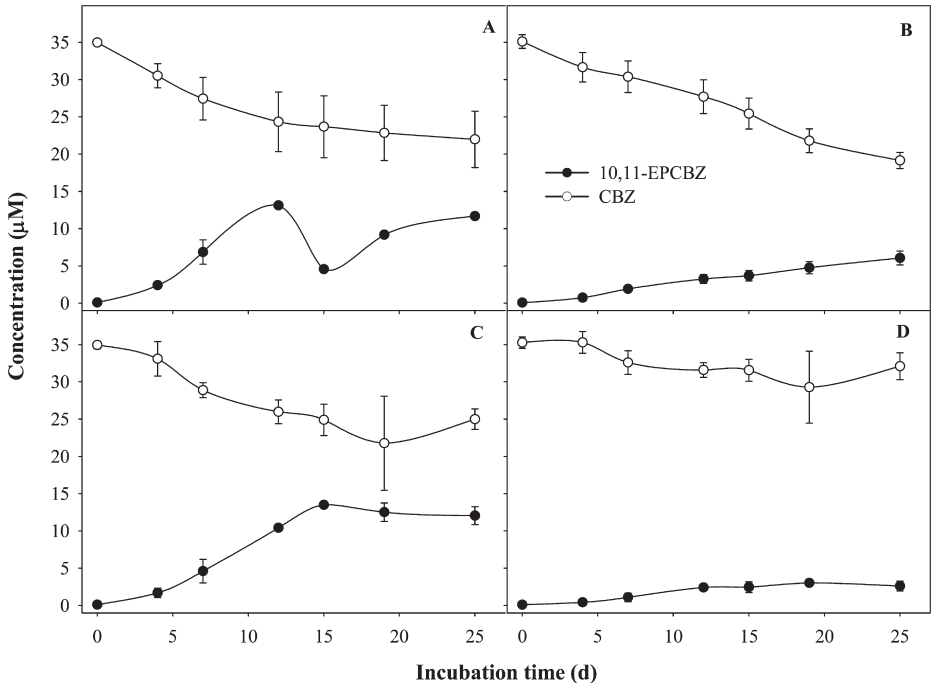

Figure 4. Concentrations of carbamazepine (CBZ) and 10,11-epoxycarbamazepine (10,11-EPCBZ) during incubation with *P. ostreatus* PC9 grown in BSM: (A) with Mn^{2+} and without 1-ABT (cytochrome P450 inhibitor); (B) with Mn^{2+} and with 1-ABT; (C) without Mn^{2+} and without 1-ABT; and (D) without Mn^{2+} and with 1-ABT. Mean values ± standard deviations of four replicates are plotted.

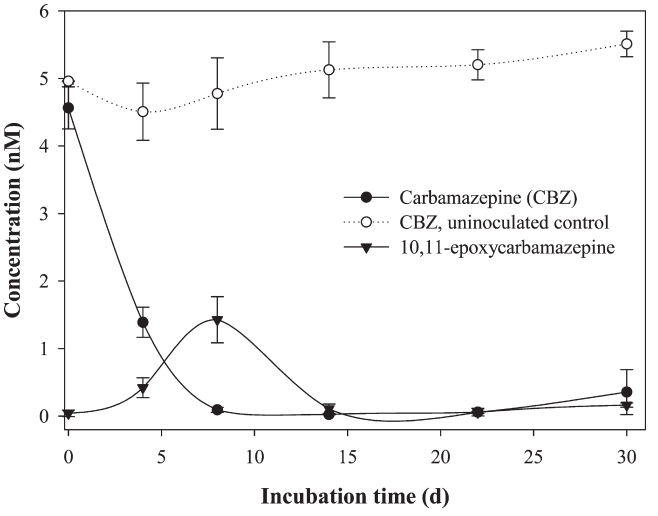

Figure 5. Degradation of carbamazepine (CBZ) at an environmentally relevant concentration (1 $\mu g\ L^{-1}$, 4.5 nM) during incubation with *P. ostreatus* PC9 in GP medium with Mn^{2+}. Mean values ± standard deviations of three replicates are plotted.

very stable compound in vitro and in vivo because it is formed on the pseudoaromatic ring of CBZ.[28]

Environmental Implications. In our initial experimental setup, we used high CBZ concentration (10 mg L^{-1}) similar to other studies [11−13] to study the enzymatic mechanisms involved in its degradation by *P. ostreatus*. Under these conditions, *P. ostreatus* exhibited the highest removal ability of this recalcitrant compound as compared to other tested fungi [10−13] and was transformed to 10,11EPCBZ, which was stable and accumulated in the media. It is important to note that 10,11EPCBZ has pharmacological activity similar to that of its parent compound,[29] and was also detected in wastewater effluents.[30]

To evaluate the potential use of *P. ostreatus* to remediate contaminated water, we studied CBZ removal from its environmental relevance concentration (∼1 $\mu g\ L^{-1}$, 4.6 nM) (ref 5 and references therein). When optimal conditions for enzymes activity were obtained (both CYP450 and MnP enzymatic systems were activated), CBZ concentration decreased by 98% to 0.093 nM (22 ng L^{-1}) within 8 d (Figure 5). Unlike the accumulation of 10,11EPCBZ observed when CBZ was introduced at high concentration (Figure 3), in this experiment 10,11EPCBZ disappeared gradually. It reached a maximum (1.43 nM) on day 8 and decreased from that point on reaching a minimal concentration of 0.1 nM on day 14. These results suggest that at environmentally relevant concentrations *P. ostreatus* can not only transform CBZ to 10,11EPCBZ but may also continue its metabolism probably to 10,11 trans-diol.[14] Such metabolites could be bioavailable to other microorganisms. The current study highlights the potential of *P. ostreatus* as a bioremediation agent together with the need for further investigation concerning the fate of the metabolites.

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