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Degradation of the Recalcitrant Pharmaceuticals Carbamazepine and 17α-Ethinylestradiol by Ligninolytic Fungi

Ivan J. S. Santos[a], Matthew J. Grossman[a], Adilson Sartoratto[b], Alexandre N. Ponezi[b], Lucia R. Durrant[a*]

[a]Department of Food Science - FEA, Food Eng. Faculty (FEA), University of Campinas (UNICAMP), Rua Monteiro Lobato, 80 CEP: 13083-862 Campinas, São Paulo, Brazil
[b]Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA) - University of Campinas CEP: 13081-970 Campinas, São Paulo, Brazil
durrant@fea.unicamp.br

17α-ethinylestradiol (EE2, synthetic estrogen), carbamazepine (CBZ, anti-epileptic and mood-stabilizer) are pharmaceutical substances widely used worldwide and have been frequently detected in wastewater treatment plant (WTP), effluents and natural waters in several countries, including Brazil. The major concern of residues of these drugs in drinking water and aquatic environments are the potential adverse effects on human and animal health. This study evaluated the potential of ligninolytic fungi (two strains of *Pleurotus* and an as yet unidentified basidiomycete designated as strain BNI), to degrade EE2 and CBZ individually. All three strains were capable of degrading EE2 completely after 7 days of incubation, both in the absence or presence of another carbon source (glucose). While EE2 was extensively degraded by all strains, only strain BNI was able to degrade CBZ in the presence of glucose (47%). *Pleurotus* sp. P1 had the best growth in the presence of EE2 and produced significant enzyme activities for laccase and manganese peroxidase but not lignin peroxidase while degrading EE2. Laccase, manganese peroxidase and lignin peroxidase was observed for basidiomycete strain BNI strain during cometabolic degradation of CBZ.

1. Introduction

Synthetic and semi synthetic pharmaceuticals are chronic pollutants that are continuously released to sewage systems or directly into the aquatic environment largely after ingestion and excretion by humans and animals (Daughton, C., 2003a; Daughton, C. 2003b). Up to 60% of the administered dose of a pharmaceutical is excreted unmetabolized in urine or stool and discharged into domestic wastewater (Zuccato et al., 2000; Calamari et al., 2002; Bound and Voulvoulis, 2004; Wu and Janssen, 2011). About 26 metric tons of pharmaceutical waste is disposed annually down the drain in the US alone (S.M. Gualtero, 2005). Studies in Austria, Brazil, Canada, Croatia, Germany, Greece, Italy, Spain, Switzerland, The Netherlands, the UK and the US have found more than 80 pharmaceutical compound including antibiotics, painkillers, hormones, tranquilizers, anti-inflammatory, chemotherapeutic, antiepileptic and hypolipidemic drugs in waterways at ng L^{-1} to µg L^{-1} levels, which is within the biologically active range for many of these drugs (Castiglioni et al., 2006; Heberer, 2002).

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Analysis of water samples along the Atibaia River, São Paulo State (Brazil) found endocrine disruptor pharmaceuticals in 92% of the samples including 17β-estradiol, 17β-ethynylestradiol, progesterone and levonorgestrel (Montagner and Jardim, 2011).

Pharmaceutical compounds reach surface waters (rivers, lakes, sea, etc.) and groundwater primarily as a result of incomplete removal in wastewater treatment plants, and subsequently drinking waters, creating significant concerns for their potential effect on humans due to continuous low level exposure (Petrovic et al. 2009; Jelic et al., 2011; Reungoat et al., 2011). Among the most abundant pharmaceuticals found in wastewater treatment plants, removal rates vary from 0% (Carbamazepine, clarithromycin, erythromycin, estrone, lincomycin, and spiramycin) to 20%–70% (atenolol, bezafibrate, ciprofloxacin, diclofenac, enalapril, hydrochlorothiazide, ibuprofen, ofloxacin, ranitidine, sulfamethoxazole) with effluent concentrations within the pharmacologically active range of these compounds (Carballa et al., 2004; Castiglioni et al., 2006; Fent et al., 2006).

The objective of this study is to evaluate ligninolytic fungi able to degrade the pharmaceuticals carbamazepine (CBZ), and 17α-ethinylestradiol (EE2). Ligninolytic fungi are a group of microorganisms capable of aerobically depolymerizing and mineralizing the highly resistant polyphenolic natural polymer lignin. These fungi produce intracellular enzymes (e.g. cytochrome P450) and extracellular enzymes (lignin and manganese peroxidases and laccase), which typically have low specificity for the substrate and as a result are also capable of degrading a wide variety of xenobiotic compounds (Cabana et al., 2007; Zhang & Geissen, 2010), such as PAHs, azo dyes and many others.

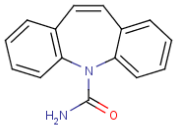
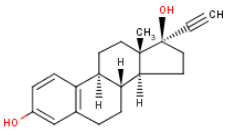
CBZ is an anticonvulsant and mood-stabilizing drug used primarily in the treatment of epilepsy and bipolar disorder, as well as trigeminal neuralgia and it is also used off-label for a variety of other indications (Ceron-Litvoc et al., 2009). CBZ has very low biodegradability and tends to persist even after wastewater treatment and in the environment where it has been shown to exhibit toxicity towards algae (Andreozzi et al., 2002). EE2, also known as ethynylestradiol, is a derivative of the natural estrogen estradiol. EE2 is an orally bio-active estrogen used in almost all modern formulations of combined oral contraceptive pills. EE2 has been found in treated wastewater at significant levels (Nasu et al., 2001). Exposure to environmental concentrations of the pharmaceutical ethynylestradiol has been shown to cause reproductive failure in fish (Nash et al., 2004; Schäfers et al., 2007).

2. Materials and methods

2.1. Pharmaceuticals

CBZ and EE2 were obtained in pure form (HPLC grade) and relevant information about them is presented in Table 1. A stock solution of 2 g L CBZ^{-1} was prepared in ethanol while the EE2 1 gL^{-1} was prepared in acetone.

Table 1: Properties of pharmaceuticals studied

Properties	Carbamazepine	17α-ethinylestradiol
CAS number	298-46-4	57-63-6
Molecular formula	$C_{15}H_{12}N_2O$	$C_{20}H_{24}O_2$
Melting point (°C)	190.2	183
Water solubility ($mg.L^{-1}$)	17.7	11.3
Chemical structure		

2.2 Fungi and maintenance of cultures

The ligninolytic fungi *Pleurotus* sp. P1, *Pleurotus ostreatus* BS and a still unidentified basidiomycete, designated as basidiomycete strain BNI, were the strains used in this work. These microorganisms were obtained from the Collection of Microorganisms and Systematics Laboratory of Microbial Physiology (FEA/UNICAMP). The fungal strains were maintained on potato dextrose agar (PDA) of the following composition: potato extract 4 g L^{-1}, dextrose 20 g L^{-1}, agar 15 g L^{-1}. Periodical transfers to fresh media were performed for the maintenance of active cultures.

2.3 Evaluation of the degradation potential ligninolytic fungi

Fungi were evaluated for their ability to degrade EE2 and CBZ at 10 and 15 mg L^{-1}, respectively, as the sole carbon source (catabolism), and at 7 and 10 mg L^{-1}, respectively, with the addition of 5 g L^{-1} of glucose (cometabolism).

The mineral medium used in these experiments had the following composition per litre: 0.5 g Na_2HPO_4, 0.8 g KH_2PO_4, 0.3 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 1 mL of vitamin solution and 10 mL of mineral solution. The vitamin solution was composed of 2 mg L^{-1} biotin, folic acid 2 mg L^{-1}, thiamine HCl 10 mg L^{-1}, 5 mg L^{-1} riboflavin, pyridoxine HCl 10 mg L^{-1}; cyanocobalamin 0.10 mg L^{-1}, nicotinic acid 5 mg L^{-1}, DL-calcium pantothenate 5 mg L^{-1}; thiotic acid (alpha-lipoic acid) 5 mg L^{-1}. The mineral solution was composed of $MnSO_4 \bullet H_20$ 5 g L^{-1}, $CaCl_2 \cdot 2H_2O$ 60 g L^{-1}, $ZnSO_4 \bullet 7H_2O$ 4 g L^{-1}, $FeSO_4 \bullet 7H_2O$ 5 g L^{-1}.

The tests were conducted in duplicate 125 mL Erlenmeyer flasks containing 35 mL of the indicated culture medium with shaking at 90 rpm at 30°C. The flasks were inoculated with 2 pieces of 1 cm^2 of agar-mycelium (fungus grown on PDA medium). Non-inoculated controls and controls where the microorganism was inactivated by autoclaving after growth for 7 days in the absence of the drug, followed by addition of the drug were also performed. The first control was performed to monitor the abiotic degradation of the compounds, while the second control was carried to evaluate the possible adsorption of the drug in cell biomass. All bottles were weighed before incubation and after the incubation period and water loss was corrected by the addition of distilled water. The medium was filtered using glass wool to remove the mycelium and the filtrate was used for quantification of drugs.

The quantification of drug degradation was performed by high performance liquid chromatography (HPLC). For each drug, standard curves were made using the pure compound. Quantification of CBZ was performed using a LC-DAD chromatographic system (Waters Alliance), consisting of a Waters 2695 pump, 2996 detector, Waters Empower software, and an L-10 Phenomenex Luna CN column (250 x 4.6 mm x 5mm). The mobile phase used was a mixture of 80% (v/v) water/methanol/tetrahydrofuran (85:12:3) containing 0.22 ml of formic acid and 0.5 ml of TEA (triethylamine) and 20% (v/v) methanol. The elution of samples was performed with a flow rate of 1 mL/min with UV detection at 280 nm. EE2 was quantified using the same chromatographic system but with a Phenomenex Luna C-18 column (150 mm x 9.3 mm x 5 mm). The mobile phase used was a mixture of water and acetonitrile (50:50), and the elution of samples was performed with a flow rate of 0.6 mL/min with UV detection at 280 nm.

2.4 Enzyme assays

Enzyme assays for lignin-peroxidase (LiP, EC1.11.1.14), manganese-peroxidase (MnP, EC1.11.1.13) and laccase (Lac, EC1.10.3.2) were performed as described by Silva et al. (2009). All the activities are expressed in mmol of substrate oxidized per litre per minute (U L^{-1} min^{-1}).

3. Results

3.1 Biodegradation cultures

Biodegradation of CBZ was examined over a period of 28 days. Significant biodegradation of CBZ occurred only with strain BNI with glucose in the medium (cometabolism). Significant values of abiotic degradation and adsorption to the mycelia biomass were not detected for this compound (Table 2).

A maximum of 47% total removal of CBZ was observed after 28 days of incubation, with 42% attributable to biodegradation. The conversion rate was highest for the first 7 says. The rate was somewhat lower in the second 7 days, however, under the conditions used maximum conversion was almost reached by day 14 and the reduction in rate may reflect kinetic limitations as a result of reduced

CBZ level. CBZ has been shown to be very recalcitrant to biodegradation. In addition to biodegradation studies, studies of chemical and photochemical processes have been evaluated, but none of these techniques have shown significant degradation of this drug. Moreover, in advanced oxidation processes (AOP's), the formation of acridine has been demonstrated, resulting in a higher associated environmental toxicity of this drug.

Table 2: Carbamazepine degradation by basidiomycete strain BNI

	% Reduction of CBZ			
Time (days)	Abiotic control	Autoclaved control	Biotic culture	Biodegraded*
7	0	2	27	25
14	0	9	45	36
21	0	12	50	38
28	0	5	47	42

*Percent biodegraded was calculated as the difference between the percent reduction in CBZ obtained in the biotic cultures and the combined percent reduction in CBZ in the abiotic controls and autoclaved.

All three strains of fungi were capable of degrading EE2 (data not shown). After 6 days of incubation, both in the presence and absence of glucose, 100% degradation was observed. Significant levels of abiotic degradation or adsorption were not found in any of the three strains used. *Pleurotus sp.* P1 was selected for enzyme activity evaluation in the presence of EE2 because it presented better growth in the presence of this compound (data not shown). Table 3 shows the degradation pattern for *Pleurotus sp.* P1. Significant adsorption to biomass was observed after 4 days; however, by day 6, adsorption was no longer detected.

Table 3: Degradation of EE2 by Pleurotus sp. P1 under catabolic conditions

	% Reduction of EE2			
Time (days)	Abiotic control	Autoclaved control	Biotic culture	Biodegraded
2	0	0	48	48
4	1	19	89	69
6	6	0	100	94

*Percent biodegraded was calculated as the difference between the values obtained from the biotic cultures and the combined decrease in EE2 in the abiotic controls and autoclaved.

3.2 Enzyme Activity

Enzyme activity for Lac, LiP and MnP were evaluated for basidiomycete strain BNI every 7 days for a period of 28 days (Table 4). BNI was grown in the presence of CBZ under cometabolic conditions as previously described for the biodegradation tests. Both Lac and LiP activity was observed on day 7 suggesting their involvement in CBZ degradation, which occurred primarily in the first 14 days. Moreover, there is a correspondence between Lac and Lip activities and the period of highest CBZ reduction (days 0-14). The enzyme activity of Lac was higher on day 7, which corresponds to the period the highest rate of CBZ reduction, than day 14, and the lignin peroxidise activity reached a maximum on day 14 (663 U L^{-1}). MnP activity was not detected until day 21 and reached a maximum of 628 U L^{-1} after 28 days.

Table 4: Enzyme activities produced by strain BNI during cometabolic growth in the presence of CBZ

Time (days)	Lac (U L^{-1})	LiP (U L^{-1})	MnP (U L^{-1})
7	282	6	0
14	132	663	0
21	1740	296	239
28	1512	0	628

Lac, LiP and MnP enzyme activities for *Pleurotus sp.* P1 grown in the presence of EE2 were evaluated every 2 days over a period of 6 days (Table 5). P1 was grown under conditions previously described for catabolism in the presence of EE2. The maximum activity of MnP of 5122 UL-1 was found after 6 days of incubation. A maximum Lac activity of 308 UL-1 was observed after 4 days of incubation but was not detectable on day 6. There was no detection of LiP enzyme activity. The results of enzyme activities of Lac and MnP for *Pleurotus* sp. P1, during catabolic growth in the presence of EE2, coincide with EE2 removal rates; with maximum Lac levels on day 4 (corresponding to the midpoint of biodegradation) and high levels of manganese peroxidase detected on days 4 and 6.

Table 5: Enzyme activities produced by Pleurotus sp. P1 during catabolic growth in the presence of EE2

Time (days)	Lac (U L^{-1})	LiP (U L^{-1})	MnP (U L^{-1})
2	97	0	0
4	308	0	3363
6	0	0	5112

4. Conclusions

This work demonstrates that ligninolytic fungi are capable of degrading EE2 and CBZ, making them potentially useful in the treatment of wastewater containing recalcitrant pharmaceuticals. EE2 was found to be complete removed in the presence of all three strains evaluated under both catabolic and cometabolic conditions. The evaluation of enzyme activities for Lac, LiP and MnP in cultures of *Pleurotus* sp. P1 in the presence of EE2 revealed significant activities for only Lac and MnP. In contrast, significant removal of CBZ was only observed for basidiomycete strain BNI under cometabolic conditions, and in this case a maximum of 47% reduction occurred after 28 days of incubation. Significant enzyme activity for Lac, LiP and MnP was also observed for this strain during cometabolic degradation of CBZ. Further studies are needed to optimize the biodegradation process, as well as to evaluate the mechanism of degradation (enzymes involved in the process) and toxicity of metabolites generated.

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