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The white-rot fungus *Trametes versicolor* reduces the estrogenic activity of a mixture of emerging contaminants in wastewater treatment plant effluent

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ABSTRACT

This study investigated the removal of common emerging contaminants (ECs) and related estrogenic activity from wastewater effluent by a strain of the white-rot fungus *Trametes versicolor* with previously unreported bioremediation potential. *T. versicolor* NRRL 66313 was grown in carbon-amended sterile secondary wastewater treatment plant (WWTP) effluent. Aerated batch reactors containing sterile, glucose-amended (5 g l^{-1}) wastewater were inoculated, incubated for eight days, and then spiked with either 5 mg l^{-1} of 17β-estradiol (E2), or a mixture of E2, atrazine (ATZ), bisphenol A (BPA), carbamazepine (CBZ), N,N-diethyl-3-methylbenzamide (DEET), estrone (E1), 17α-ethynylestradiol (EE2), oxybenzone (OBZ), and triclosan (TCS) to a final concentration of 350 μg l^{-1} each. Abiotic and heat-killed fungus controls were also prepared. EC and metabolite concentrations were measured over time using HPLC and Gas Chromatography coupled with Time-Of-Flight Mass Spectrometry (GC-TOFMS). Estrogenic activity was measured on the same samples using the Yeast Estrogen Screen. In less than 5 h, active fungi decreased the concentration of E2 from 5 mg l^{-1} to below detection, producing E1 as a metabolite and subsequently removing it from solution. Acting on the mixture of ECs, fungi removed BPA, E1, E2, and EE2 to a significant degree (62–100%) vs. controls (0–28%) in only 3.5 h, reducing the estrogenic activity of the mixture by 77% (vs. 4–8% for controls). After 12 h, the total reduction of estrogenic activity was greater than 98% (vs. 24–42% for controls). These results show that *T. versicolor* NRRL 66313 can reduce the estrogenic activity of mixtures of estrogens and BPA, which are typically the most significant contributors to the hormone disrupting activity of domestic WWTP effluent.

1. Introduction

Emerging contaminants (ECs) often pass through domestic wastewater treatment plants (WWTPs) and are discharged into surface waters, where they can threaten aquatic ecosystems and human health by mimicking natural hormones (e.g. estrogen) and disrupting the endocrine systems of exposed individuals. The potential for an EC, or a mixture of ECs, to mimic estrogen can be quantified in terms of its estrogenic activity. Among the ECs detected in WWTP effluent, estrogen compounds (e.g. 17β-estradiol, 17α-ethinylestradiol, and estrone) have been found to contribute the majority of hormone disrupting activity to receiving waters (Desbrow et al., 1998; Aerni et al., 2004; Metcalfe et al., 2013). In addition, risk-based prioritization schemes which consider occurrence, exposure, and hazard effects have been used to narrow the field of potentially harmful ECs and focus research efforts (Murray et al., 2010; Kumar and Xagoraraki, 2010). On the basis of these prior works, nine chemicals were chosen as model ECs for use in this study: atrazine (ATZ), bisphenol A (BPA), carbamazepine (CBZ), N,N-diethyl-3-methylbenzamide (DEET), estrone (E1), 17β-estradiol (E2), 17α-ethynylestradiol (EE2), oxybenzone (OBZ), and triclosan (TCS).

While various physical/chemical technologies (e.g. membrane separation, advanced oxidation, and carbon adsorption) show some

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promise for removing ECs during wastewater treatment, drawbacks include added costs associated with plant modification, energy/chemical consumption, and disposal of concentrated waste streams. In contrast, advanced biocatalysis results in the degradation, or detoxification, of ECs and the potential to enhance existing biological WWTP unit operations through bioaugmentation. White-rot fungi (WRF) have received a great deal of attention in recent years for their ability to transform a variety of recalcitrant ECs in bench-scale experiments via their very powerful and non-specific extracellular oxidative enzymes (Cabana et al., 2007; Yang et al., 2013). In some cases, extracellular fungal enzymes have been isolated and immobilized for use in specialized reactors (Gasser et al., 2014). However, using whole-cell cultures as biocatalysts allows for the potential to exploit intracellular fungal enzymes and potentially degrade a broader range of ECs (Yang et al., 2013; Nguyen et al., 2014). In addition, whole-cell cultures of fungi could be used to enhance microbial populations in existing wastewater treatment processes such as activated sludge, or as a tertiary treatment step to further treat the effluent from existing WWTP operations.

To date, nearly all of the bench-scale experiments involving EC degradation by whole-cell fungal cultures have been conducted in nutrient media or synthetic wastewater (Yang et al., 2013). The use of whole-cell cultures of WRF to treat *actual domestic* wastewater for EC removal has been reported in only a limited number of cases (Zhang and Geißen, 2012; Cruz-Morató et al., 2013). The WRF used in these studies were grown in nutrient media prior to being introduced into wastewater. In order to use WRF as a tertiary treatment step in existing domestic WWTPs, their ability to grow, produce, enzymes, and remove ECs in a wastewater matrix with minimal nutrient supplementation is of interest. With any process involving biological removal of ECs, it is also critical that the overall effect of fungal metabolism on the hormone disrupting potential of mixtures of ECs be investigated. Prior studies using whole-cell cultures of WRF to treat domestic wastewater and hospital wastewater, assess overall toxicity using the Microtox assay, but do not assess the hormone disrupting potential of the treated effluent (Cruz-Morató et al., 2014, 2013). In the present study, these gaps in the literature are addressed by comparing the bioremediation potential of whole-cell cultures of a WRF cultured in nutrient media and domestic wastewater and examining the fungal degradation of a mixture of ECs in actual domestic WWTP effluent in terms of the removal of individual chemicals *and* the overall estrogenic activity of the mixture.

The investigation consisted of two phases: First, a preliminary experiment compared small batch cultures (150 ml) of the WRF *Trametes versicolor* grown in carbon-amended wastewater (with no additional nutrient supplementation) to those grown in a defined nutrient medium with a focus on quantifying biomass growth and enzyme production. The strain of *T. versicolor* used in this study (NRRL 66313) had not previously been examined with regard to its bioremediation potential. During this phase, E2 (10 mg l^{-1}) was added to assess whether the viability of *T. versicolor* would be negatively affected in the subsequent experimental phase, where high concentrations of E2 were used to evaluate metabolite production. In the second phase, carbon-amended wastewater cultures of *T. versicolor* were grown in larger (1 l) reactors allowing for more efficient collection of time-series EC and estrogenic activity removal data. Two groups of these larger reactors were prepared: the 'estrogen group' was spiked with an elevated concentration of E2 (5 mg l^{-1}) in order to ease the detection of metabolites, while the 'mixed group' was spiked with the aforementioned mixture of nine common ECs at a lower concentration (350 μg l^{-1} each).

2. Materials and methods

2.1. Model emerging contaminants and stock solutions

Bisphenol A (BPA), carbamazepine (CBZ), N,N-diethyl-3-methylbenzamide (DEET), estrone (E1), 17β-estradiol (E2), 17α-ethynylestradiol (EE2), oxybenzone (OBZ), and triclosan (TCS) were purchased from SigmaAldrich (St. Louis, MO, USA) at a purity of 97%, or greater. Atrazine (ATZ) was purchased from Cayman Chemical (Ann Arbor, MI, USA) at a purity of 98%. All model ECs were dissolved in N,N-dimethylformamide (DMF; EMD Millipore, Billerica, MA, USA) prior to spiking into bioreactors.

2.2. Carbon-amended wastewater and nutrient media

Nutrient media used in batch reactors was prepared after Blánquez and Guieysse (2008), where the micronutrient medium was that of Tien and Kirk (1988). Wastewater (secondary effluent, pre-chlorination) was collected from the overflow weir of the secondary clarifier at The Pennsylvania State University WWTP (University Park, PA, USA). The wastewater was prepared for use in batch reactors by chemically adjusting 2 l aliquots as follows: alkalinity was removed by adding 1 M hydrochloric acid (EMD, USA) to a pH of 3.5, followed by the addition of 1 M sodium hydroxide (NaOH; J.T. Baker, Phillipsburg, NJ, USA) to a pH of 4.5 over a period of 3 h, while vigorously stirring. Glucose (EMD, USA) was added to a final concentration of 5 g l^{-1} in both nutrient media and wastewater.

2.3. Organism, maintenance, and cultivation conditions

T. versicolor (WC 861) was obtained from the Mushroom Spawn Laboratory at The Pennsylvania State University, and was deposited in the United States Department of Agriculture Agricultural Research Service (USDA ARS) Culture Collection as strain number NRRL 66313. A partial 18s rRNA gene sequence was obtained via DNA extraction from the pure culture using a PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) followed by PCR amplification using the primer set nu-SSU-0817-5′/nu-SSU-1536-3′ (Borneman and Hartin, 2000) and subsequent Sanger sequencing. The sequence was deposited in GenBank at the National Center for Biotechnology Information (NCBI) under accession number KR869151 (http://www.ncbi.nlm.nih.gov/GenBank/index.html). Solid-phase, stock cultures were maintained on YMPG agar media (Tien and Kirk, 1988). Colonized YMPG plates were stored at 4 °C and *T. versicolor* was transferred to fresh media every 1–2 months.

2.3.1. Preparation of fungal inoculum

Agar plugs were aseptically harvested from a *T. versicolor* populated plate and incubated in YMPG media for 7 days at 30 °C and 120 rpm (MaxQ 4000; Thermo Scientific, Waltham, MA, USA), yielding spherical pellets of *T. versicolor* mycelia with a wide size distribution. These pellets were rinsed of spent growth medium using sterile distilled deionized (DDI) water and blended (WSB33 Quik Stik; Waring, USA) with additional sterile DDI water in an approximate volumetric ratio of 1:3 (DDI:mycelia) for 3 min in order to prepare a homogenous suspension of mycelia. The homogenate was then added to fresh YMPG media in a ratio of 0.8–1.4% (v/v) and incubated again under the above conditions. The second growth phase yielded pellets of uniform size which were rinsed with, and stored in, 0.85% NaCl at 4 °C for up to two months (Romero et al., 2006). As needed, the stored pellets were recovered from the storage solution, rinsed, and blended in equal parts (v/v) with additional sterile DDI water. The resulting inoculum was