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Biodegradation of endocrine disruptors in urban wastewater using *Pleurotus ostreatus* bioreactor

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ABSTRACT

The white rot fungus *Pleurotus ostreatus* HK 35, which is also an edible industrial mushroom commonly cultivated in farms, was tested in the degradation of typical representatives of endocrine disrupters (EDCs; bisphenol A, estrone, 17β-estradiol, estriol, 17α-ethinylestradiol, triclosan and 4-*n*-nonylphenol); its degradation efficiency under model laboratory conditions was greater than 90% within 12 days and better than that of another published strain *P. ostreatus* 3004. A spent mushroom substrate from a local farm was tested for its applicability in various batch and trickle-bed reactors in degrading EDCs in model fortified and real communal wastewater. The reactors were tested under various regimes including a pilot-scale trickle-bed reactor, which was finally tested at a wastewater treatment plant. The result revealed that the spent substrate is an efficient biodegradation agent, where the fungus was usually able to remove about 95% of EDCs together with suppression of the estrogenic activity of the sample. The results showed the fungus was able to operate in the presence of bacterial microflora in wastewater without any substantial negative effects on the degradation abilities. Finally, a pilot-scale trickle-bed reactor was installed in a wastewater treatment plant and successfully operated for 10 days, where the bioreactor was able to remove more than 76% of EDCs present in the wastewater.

Introduction

Wastewater treatment plants (WWTPs) are assumed to be one of the main sources of various micropollutants in aquatic environments through their insufficient cleaning processes [1–5]. Most WWTPs are not designed to completely eliminate micropollutants, especially when only conventional processes are employed. The removal efficiency of WWTPs varies depending on the physicochemical characteristics of the pollutants and on the treatment processes involved [1,2]. Secondary treatment (mostly in activated sludge or membrane biological reactors) is the main mechanism of pollutant removal in conventional WWTPs [6,7]. Application of additional treatments, also called tertiary treatment or advanced treatment, in WWTP processes might improve pollutant removal [1,2]. Advanced treatments include natural systems (e.g. constructed wetlands, aquifer recharge and recovery [8]), membrane and advanced chemical/oxidation technologies [9], electro-oxidation [10] and biological treatment [11–13]. The cost of most processes listed above limits their broad full-scale application [14].

Endocrine disrupting compounds (EDCs) belong among the most recently targeted micropollutants detected in WWTP effluents and also in aquatic environments. EDCs are specific in their high biological activities towards various organisms. The most adverse and risky effect lies in their ability to cause reproductive problems in a number of species and probably also in humans. The estrogenic effect of EDCs is often expressed in terms of the estradiol equivalent (EEQ) and it has been documented that a concentration of 1 ng L^{-1} EEQ has a significant negative effect on fish and other aquatic organisms [15,16]. Due to their high estrogenic activity, estrone (E1), 17β-estradiol (E2), estriol (E3) and synthetic 17α-ethinylestradiol (EE2) are considered to be significant contributors to the estrogenic activity of wastewaters. Bisphenol A (BPA) and 4-*n*-nonylphenol (4-NP) have many orders of magnitude lower estrogenic activity, but their elevated concentrations in wastewater also draw attention to these EDCs [17].

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In this context, the search for environmentally friendly and low-cost technologies is of great importance. Bioremediation is a popular alternative to conventional treatment methods and especially white rot fungi (WRF) have been successfully documented for their ability to remove various organic pollutants i.e. PAHs, PCBs, textile dyes, pesticides, as well as EDCs (as described in a review elsewhere [18–21]).

The white rot fungus *Pleurotus* sp., also called oyster, abalone or tree mushroom, is one of the most commonly cultivated edible mushrooms in the world. Mushroom farming is a profitable business and because *Pleurotus* (mainly *Pleurotus ostreatus*) is fairly easy to cultivate and to fructificate, there are many growing farms around the world (mainly in Europe and the U.S.A.), producing fruiting bodies as well as tons of re-usable biowaste. This biowaste usually consists of ligno-cellulosic substrate (straw, wood chips, fruit waste etc.) and *Pleurotus* mycelia. Because of the great bioremediation potential of *Pleurotus ostreatus* [21–23], re-use of this biological waste in organic pollutant degradation represents a promising environmentally friendly technology.

This study was performed to examine the degradation of the main representatives of endocrine disrupting compounds by the commercially available, edible white rot *P. ostreatus* strain HK 35 in augmented bioreactors when our preliminary experiments without fungal inoculation did not show significant removal of EDCs. The experiments using fungi were designed to explore the degradation ability under various regimes and conditions, including different matrices (tap water and wastewater) representing distinct microbial populations as well as different EDC concentration levels in fortified and real wastewater. Initially, the degradation ability of HK 35 strain was compared with the known 3004 strain described in the literature for its degradation efficiency under model laboratory conditions (static cultivation, complex medium) [21]. In the next step, the strain was tested in a laboratory-scale continuous-flow reactor. Then the strain was examined in a scaled-up reactor on a stationary packed bed and for continuous flow trickle-bed regimes. Finally, the function of the trickle-bed arrangement was verified at a WWTP locality. The removal process and its efficiency were assessed using various methods including analytical EDC determination, detection of residual estrogenic activity, ligninolytic enzyme activity and phospholipid fatty acid (PLFA) analysis, in order to monitor the fungal and bacterial biomass.

Materials and methods

Materials and substrate preparation

The spent straw substrate containing the fungal biomass that was utilized in the experiments was obtained from a commercial oyster mushroom farm (Farma Volek, CZ). Wheat straw pellets (8 mm Ø), used as a bulking agent and a fresh nutrition source, were purchased from Atea Praha (CZ). The straw pellets were moisturized with distilled water (1:3 *w/w*), twice sterilized in an autoclave (121 °C; 25 min) and stored at room temperature (max 24 h). For the degradation experiments, the spent straw substrate and sterile straw pellets were mixed in a ratio of 2:1 (*w/w*) and left for 3 days at 28 °C. All the experiments were performed in the darkness. In order to remove color pigments originating from straw, the fungal straw substrate was washed with tap water (1:2; *w/w*), before the experiments. This material was used as the filling for all the reactor experiments.

Wastewater was obtained from a WWTP located in Central Bohemia. WWTP effluent after secondary treatment, which is normally discharged into a recipient, was stored in a stainless tank at 4 °C and used as a matrix in laboratory experiments.

Bisphenol A (BPA); estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethinylestradiol (EE2), triclosan (TRC) and 4-*n*-nonylphenol (4-NP) were used as analytical standards and as substrates for the degradation experiments. All the compounds were purchased from Sigma Aldrich (Steinheim, Germany) with purity of 99.0% or higher. Standard solutions containing 2 mg mL^{-1} of each of the six selected EDCs were prepared in ethyl acetate (EtOAc) and stored in a refrigerator. The stock standard solutions with the respective concentrations were prepared by mixing all 7 EDCs and diluting the stock solution with EtOAc or dimethylsulfoxide (DMSO) or *N*,*N*-dimethylformamide (DMF).

All the analytical grade organic solvents used in present study were purchased from Chromservis (CZ). The analytical standards of fatty acid methyl esters were obtained from Sigma-Aldrich (Prague, Czech Republic) and Matreya LLC (USA). BSTFA:TMCS (99:1); glucose, agar, malt extract broth, DMSO, DMF and all the other chemical used in the present study were purchased from Sigma (Germany).

Fungal culture

The spent straw substrate obtained from the mushroom farm was colonized by industrial *P. ostreatus* HK 35 and stored at 4 °C. The strain was aseptically isolated on Petri dishes with MEG agar medium (malt extract 20 g L^{-1}, glucose 20 g L^{-1}, agar 25 g L^{-1}, pH adjusted to 6.0 with NaOH prior to autoclaving) and stored at 4 °C. After one week, the strain was reinoculated on Petri dishes with fresh MEG medium; the colonized Petri dishes were transferred to 4 °C and used as stock culture. Every two months, the fungus was re-inoculated on fresh medium.

P. ostreatus 3004 was obtained from the Culture Collection of Basidiomycetes of the Czech Academy of Sciences (*P. ostreatus* 3004 CCBAS 278).

The fungal inocula for biodegradation experiments were prepared in 250 mL Erlenmeyer flasks containing 20 mL of malt extract-glucose medium (MEG; 5 g malt extract, 10 g glucose, pH 4.5) starting from 2 mycelial plugs (7 mm Ø). The cultures were grown for 7 days at 28 °C, homogenized by Ultraturrax-T25 (IKA-Labortechnik, Germany) and 5% aliquots of the mycelial suspension were used to inoculate sterile MEG medium.

In vivo biodegradation under sterile conditions

The static MEG medium liquid cultures of *P. ostreatus* 3004 and HK 35 were incubated in 250 mL Erlenmeyer flasks at 28 °C in five parallel samples. The samples for biodegradation experiments were spiked with 100 μL of DMF (final concentration 0.5%, v/v; Sigma Germany) containing a mixture of EDCs (final concentration 2 mg L^{-1} of each representative). Biotic controls were spiked with only 100 μL of DMF. The heat killed controls (HKCs) were performed with mycelia grown for one week and killed in an autoclave. The biodegradation samples were harvested after 3, 12 and 20 d, extracted according to [24] and analyzed using the GC/MS method (see below). All the samples were prepared in triplicate.

Laboratory-scale continuous flow trickle bed bioreactor under non-sterile conditions

A laboratory-scale glass tubular, vertical reactor with total volume of 1 L (working volume 0.8 L) was filled with the mixture of straw substrates (total amount 200 g of wet substrate, 75% humidity), washed with tap water (2 L; pH 6.5) and aerated using a small air pump (3 L min^{-1}; Hailea Aco-6601, Hailea, CZ). These experiments were carried out with fortified WWTP effluent (original EDC concentration sum 356 ng L^{-1}; fortification level 500 μg L^{-1} of individual EDCs; total volume of wastewater 400 mL)