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Short communication

Continuous biodegradation of 17β-estradiol and 17α-ethynylestradiol by *Trametes versicolor*

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Abstract

The feasibility of 17β-estradiol (E2) and 17α-ethynylestradiol (EE2) removal by *Trametes versicolor* was demonstrated in batch and continuous cultures. In batch, E2 and EE2 initially supplied at 10 mg l^{-1} were removed by more than 97% in 24 h, which corresponded to volumetric removal rates of 0.43 and 0.44 mg l^{-1} h^{-1}, respectively. A bioreactor inoculated with *T. versicolor* pellets was then continuously operated during 26 days at a hydraulic retention time of 120 h. E2 and EE2 were completely removed at volumetric removal rates of 0.16 and 0.09 mg l^{-1} h^{-1}, respectively, when fed at 18.8 and 7.3 mg l^{-1}, respectively. Evidence was found that removal was caused by laccase. This study demonstrates the technical feasibility of fungal treatment of estrogens using continuous bioreactor with suspended fungal biomass.

Keywords: Bioreactor; Emerging contaminant; Estrogens; Endocrine disrupter; White-rot fungi

1. Introduction

Endocrine disrupting contaminants (EDCs) are ubiquitous environmental pollutants that can cause dramatic environmental effects even when present at trace concentration, such as the feminization of entire fish populations in contaminated streams. Unfortunately, there are today no good methods for their removal [1].

This study was conducted to establish the potential of white-rot fungi (WRF) to biodegrade 17β-estradiol (E2) and 17α-ethynylestradiol (EE2) as model EDCs. WRF are capable to biodegrade a wide range of organic pollutants due to their capacity to release unspecific extracellular enzymes [2]. Their potential for the removal of emerging trace contaminants has not yet been extensively studied although various recent studies have shown WRFs or their enzymes were suitable to degrade various EDCs [3–9]. This study represents, to the best of our knowledge, the first report of successful continuous E2 and EE2 removal by an active WRF.

2. Materials and methods

All chemicals used were reagent grade. E2 and EE2 were purchased from Sigma–Aldrich and dissolved at 2 g l^{-1} in acetone for further use. All aqueous solutions were prepared in deionized water, except for the HPLC mobile phases, which were prepared with ultrapure water. *Trametes versicolor* was obtained from the American Type Culture Collection (ATCC 45230). The fungus was maintained on 2% malt agar slanks at 23 °C by monthly preparing fresh cultures. A mycelia suspension of *T. versicolor* was obtained by inoculating four 1 cm-diameter plugs from the fungi growing zone into 150 ml malt extract medium (2%) in a 500 ml Erlenmeyer flask. The flasks were incubated in an orbital shaker (135 rpm, $r = 25$ mm) at 22 ± 2 °C. After 4–5 days, the thick mycelium formed was grounded with an X10/20homogenizer (Ystral GmbH) and the resulting mycelium suspension was stored in a sterilized 0.85% NaCl solution at 4 °C. This suspension was used to produce pellets by inoculat-

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ing 1 ml of the suspension in 250 ml 2% malt extract medium in a 1 L Erlenmeyer flask. The flasks were incubated in an orbital shaker (135 rpm, $r = 25$ mm) at 22 ± 2 °C for 5 days. The pellets obtained were used as inocula in the subsequent biodegradation tests and stored in sterilized 0.85% NaCl solution at 4 °C for up to 2 months without lost of activity or morphology [10].

2.1. Biodegradation batch assay

E2 was added to 3×100 ml serum flasks closed with rubber septum to reach a final concentration of 10 mg l^{-1}. Once the solvent had evaporated, 9 ml of culture medium (pH 4.5) was added. This contained (per litre of deionised water): glucose, 5 g; NH_4Cl, 1.9 g; 2,2-dimethylsuccinate, 1.46 g and 11 ml of micronutrient medium [11]. The flasks were then inoculated with 2 g of *T. versicolor* pellets to reach a final concentration of 11.1 g l^{-1} (dried weight) according to Blánquez et al. [12,13]. The flasks were finally sealed under sterile conditions and incubated under agitation at 120 rpm at 22 ± 2 °C for 7 days. To serve as abiotic controls, three additional flasks were inoculated with *T. versicolor*, immediately autoclaved and incubated under the same conditions. An additional abiotic control without biomass was also conducted.

The biodegradation kinetics of E2 and EE2 (10 mg l^{-1} each) by *T. versicolor* was then studied in serum flasks as described above. Twenty-two flasks were prepared for each fungi, 12 being autoclaved after inoculation. Four flasks (two tests and two controls) were then periodically sacrificed for chemical analysis.

2.2. Continuous biodegradation

A custom-made glass air fluidized bioreactor was filled with 100 ml of the culture medium described above further amended with 3 mg E2 l^{-1} and inoculated at 3.2 g l^{-1} with *T. versicolor* pellets. Fluidized conditions were maintained by injecting 3 l filtered air h^{-1} into the bioreactor through a porous metal diffuser. The reactor has been described elsewhere [14]: it was made of a 130 ml cylindrical glass vessel ($\emptyset = 3.4$ cm, length = 15.5 cm) equipped with two sampling ports located at 8.5 and 13.5 cm from the top of the reactor. Following 1 d of bath operation, the bioreactor was switched to a continuous mode at 120 h hydraulic retention time (HRT) by injecting fresh culture medium successively supplied with 3 mg E2 l^{-1} for 6 d, 18.8 mg E2 l^{-1} for 11 d, and finally a mixture of 10.4 mg E2 l^{-1} and 7.3 mg EE2 l^{-1} for 9 d. The biomass was fully retained into the bioreactor until day 19, when partial biomass renovation was carried out: one-third of the bioreactor broth volume was removed and the biomass was removed using a metal strainer. It was then replaced by an equivalent amount of fresh biomass and the suspension was reintroduced into the bioreactor. All experiments were conducted at room temperature. Ten ml samples were periodically taken from the reactor influent and effluent and saved in 25 ml closed glass tubes at −18 °C prior to analysis. Samples of 2 ml were taken for laccase analysis and immediately analyzed.

2.3. Analysis

Ten ml of acetone was added to each cultivation flasks which were then manually mixed for 5 min. The entire content of each flask was transferred to 25 ml glass tubes and was ultrasonicated for 15 min at 30 °C. The tubes when then centrifuged for 5 min at $2200 \times g$ and portions of the supernatants were collected in HPLC vials for analysis. A similar procedure was followed for samples from the bioreactor samples with the difference that the acetone was directly added to the 25 ml tubes filled with the samples and manually mixed for 5 min thereafter. The extraction recovery from biomass-free medium in flasks was higher than 99% at 10 mg l^{-1} for both estrogens.

The concentrations of E2 and EE2 in the aqueous–acetone mixtures were measured with an HPLC (Waters 2690) equipped with a fluorescence detector (Waters 474) and a Ascentis C18 column (Supelco). Elution was performed with a mixture of HPLC grade acetonitrile (40%) and 25 mM K_2HPO_4 aqueous solution at pH 7 (60%). External standards were used to enable quantitative determination and the limit of detection for both compounds was 0.1 mg l^{-1}.

Laccase and MnP activities were measured using modified versions of the Paszczynski et al. [15] MnP determination method [16] where 2,6-dimethoxyphenol (DMP) is oxidized by laccase even in the absence of a cofactor whereas oxidation by MnP requires the presence of H_2O_2 and catalytically active Mn^{2+}. One activity unit (AU) was defined as the number of micromoles of DMP oxidized per minute. The DMP extinction coefficient was 10,000/Mcm. Two cuvettes were used for the enzymatic activity determination. In the first one, the reaction mixture consisted of 200 ml of 250 mM sodium malonate at pH 4.5; 50 ml of 20 mM DMP and 600 ml of sample. DMP oxidation was recorded during 2 min at 468 nm and converted as laccase activity (run 1). Then 50 ml of $MnSO_4$ 20 mM were added and phenoloxidase activity was recorded during 2 more min (run 2). Hundred ml of 4 mM H_2O_2 were then added to the mixture and penoloxidase activity was measured again during 3 min (run 3). In the second cuvette, the reaction mixture consisted of 200 ml of 250 mM sodium malonate at pH 4.5; 50 ml of 20 mM DMP, 100 ml of EDTA and 550 ml of sample. DMP oxidation was recorded during 2 min at 468 nm (run 4). Then 100 ml of 4 mM H_2O_2 were added and the Mn^{2+}-independent phenoloxidase activity was measured during 2 min (run 5). MnP activity was calculated as (run 3 − run 2) − (run 5 − run 4).

3. Results and discussion

E2 was efficiently removed by *T. versicolor* as inoculation of serum flaks with the active fungi was followed by a disappearance of 93 ± 6% of the pollutant after 7 days of incubation. By comparison, 38 ± 5% of E2 was removed in serum flaks supplied with autoclaved fungal biomass and incubated under the same conditions. Because of the high E2 recovery in the absence of biomass, E2 disappearance in the controls was most likely caused by adsorption to dead biomass (no laccase activity was recorded after sterilization).

These results were confirmed by the kinetic assays as *T. versicolor* supported E2 and EE2 volumetric removal rates of 0.43 and 0.44 mg l^{-1} h^{-1} (1.56 and 1.49 μM h^{-1}), respectively, during the first 24 h of incubation when most removal (>97%) occurred (Fig. 1). Approximately 40% of the E2 initially supplied was adsorbed the dead fungi biomass, compared to 30% for EE2, which is consistent with the fact that E2 (Kow 4.01) exhibit a higher affinity for organic matter than EE2 (Kow 3.67).

E2 removal efficiency (RE) remained above 99% during the entire period of operation. E2 concentration in the effluent remained undetected (<0.1 mg l^{-1}) during the first 7 d of continuous operation (inlet concentration of 3 mg l^{-1}) and remained below 0.21 mg l^{-1} after the E2 inlet concentration was increased to 18.8 mg l^{-1}. Laccase activity increased from day 6 to day 8 when it reached a maximum of 156 AU l^{-1}. Thereafter, the enzyme activity decreased and fluctuated between 37 and 65 AU l^{-1} during 10 d (Fig. 2). Partial biomass renovation was followed by an increase in laccase activity from 57 AU l^{-1} to 89–152 AU l^{-1}. Neither manganese peroxidise (MnP) nor lipase activity was detected. After day 18, EE2 concentration in the reactor remained below 0.20 mg l^{-1} (RE > 97%), expect from a small surge of EE2 concentration observed at day 23 (0.33 mg l^{-1}). The maximum E2 and EE2 volumetric removal rate achieved were 0.16 and 0.06 mg l^{-1} h^{-1}, respectively. Little data is available to compare the rates achieved in this study. However, these are in the same order of magnitude than those achieved with fungal enzymes, bacterial communities or bacterial isolates (Table 1). E2 and EE2 degradation by pure or partially purified laccases from *T. versicolor* has also been reported [3,7].

Following the study of *T. versicolor*, a rapid screen was conducted to test the capacity of *Phanerochaete chrysosporium* ME-446 ATCC 34541, *Irpex lacteus* (AX1), *Ganoderma lucidum* FP-58537-Sp, *Bjerkandera* sp. BOL 13 and *Penicillium ochrochloron* CBS 115580 to biodegrade E2 at 10 mg l^{-1} in serum flasks. Only *P. chrysosporium* (77 ± 7% removal in

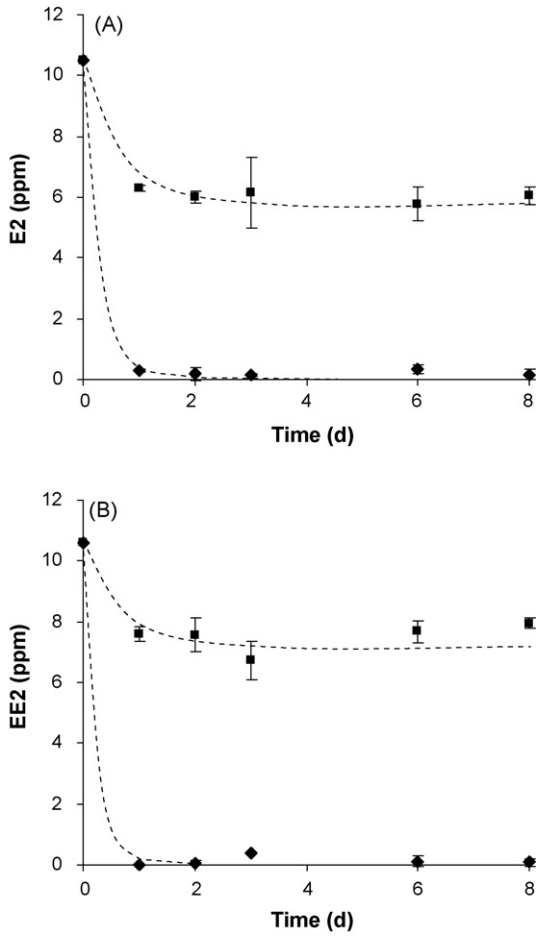

Fig. 1. Changes in E2 (A) and EE2 (B) concentrations in 100 ml flasks inoculated with *T. versicolor* (diamonds) or in control flasks inoculated and autoclaved (squares). The data shown represent average on duplicates ± standard deviation.

Table 1
E2 and EE2 removal rates achieved with fungi, fungal enzyme and bacteria

Catalyst	Estrogen	Initial concentration (mg l^{-1})	Removal rate (mg l^{-1} h^{-1})	Specific removal rates (mg g^{-1} h^{-1})	Reference
T. versicolor					
Batch	E2	10	0.43	0.04	
	EE2	10	0.44	0.04	Present study
Continuous	E2	3–18.8	0.16	0.05	
	EE2	7.3	0.06	0.02	
MnP and laccase[a]	E2	2.7	0.34[b]	–	[7]
	EE2	2.7	0.34[b]	–	
Pure *T. versicolor* laccase	E2	0.11	0.10[c]	–	[3]
	EE2	0.12	0.11[c]	–	
Activated sludge (batch)	E2	0.01–0.05	0.007–0.03[d]	0.004–0.02[d]	[17]
Activated sludge isolates (batch)	E2	3	0.13[e]	0.15–0.31[e]	[18]

The values presented here are given for rough comparison as none of the cited studies, including the present work, specifically aiming to optimized removal rates.
[a] Partially purified *phanaerochaete chrysosporium* MnP and *T. versicolor* laccase.
[b] Based on 100% removal in 8 h.
[c] Based on 90% removal in 1 h.
[d] Based on 99% removal in 1.5 h and a mixed volatile liquor suspended solid (MLVSS) concentration of 1.75 g l^{-1}.
[e] Based on 100% removal in 24 h and a MLVSS concentration of 0.4–0.85 g l^{-1}.

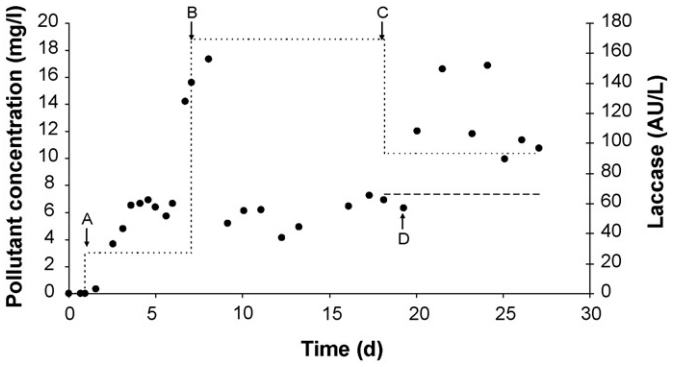

Fig. 2. Changes in laccase activity (●), influent E2 concentration (- - -), and influent EE2 (- -) in a bioreactor inoculated with *T. versicolor*. The vertical arrows represent times when operating conditions were changed: (A) continuous E2 feeding at 3 mg l^{-1} and 120 h HRT started (day 1); (B) E2 feeding concentration increased to 18.8 mg l^{-1} (day 7); (C) E2 feeding concentration decreased to 10.35 mg l^{-1}, EE2 feeding started at 7.3 mg l^{-1} (day 18); and (D) biomass renovation (day 19).

7 d) and *G. lucidum* (>99% removal in 7 d) were capable the remove this compound. The kinetics of E2 and EE2 degradation by *G. lucidum* were also studied and found similar to that of *T. versicolor*. Laccase production by the fungi was also evidenced. These results suggest that the ability of fungi, and especially laccase-producing fungi, might be quite common and could be exploited for remediation purposes.

4. Conclusions

This study demonstrates the feasibility of continuous estrogenic compounds biodegradation by white-rot fungi. Although in the ppm range, the pollutant concentration tested remain above typical environmental EDCs concentrations (ppb–ppt). However, the feasibility of removing estrogens at 100 ng l^{-1} with pure laccase from *T. versicolor* was recently demonstrated [3]. Hence, continuous treatment with fungal pellets should be feasible but it should be optimized and tested at lower influent pollutant concentrations and shorter HRT, at this could offer a more cost-effective alternative than using pure enzymes. Further work should also determine the products of biodegradation and their potential toxicity.

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