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Evaluation of the efficiency of *Trametes hirsuta* for the removal of multiple pharmaceutical compounds under low concentrations relevant to the environment

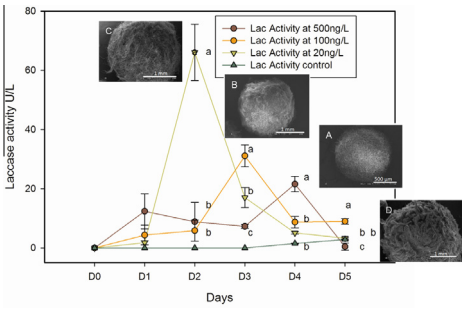
Lounès Haroune [a], Sabrina Saibi [b], Jean-Philippe Bellenger [a], Hubert Cabana [b,*]

[a] Department of Chemistry, Université de Sherbrooke, 2500 Boul. de l'Université, Sherbrooke (Qc) J1K 2R1, Canada
[b] Department of Civil Engineering, Université de Sherbrooke, 2500 Boul. de l'Université, Sherbrooke (Qc) J1K 2R1, Canada

HIGHLIGHTS

- The removal of 17 PhACs compounds by *Trametes hirsuta* was evaluated.
- The activity of extracellular enzymes was monitored.
- *Trametes hirsuta* is able to remove multi-class PhACs at low concentration.
- The PhACs concentration plays a major role on removal efficiency.
- Biosorption plays a key role in removal of several PhACs.

GRAPHICAL ABSTRACT



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ABSTRACT

An evaluation of the efficiency of the White-rot fungi (WRF) *Trametes hirsuta* to remove multi-classes pharmaceutical active compounds (17 PhACs) at low and environmentally realistic concentrations (20–500 ng L^{-1}) was performed. The importance of biosorption over enzymatic activity on PhACs removal was also evaluated. Results highlight the importance to consider environmentally relevant PhACs concentrations while evaluating the removal capacities of WRF in wastewaters treatment processes, as PhACs concentration strongly influence both the enzymatic activity profile and the removal efficiency. Results also show that under tested experimental conditions, laccase was the only active extracellular lignin modifying enzyme and that biosorption and possibly intracellular enzymes also contribute to the removal of some PhACs.

1. Introduction

Pharmaceutical active compounds (PhACs) have been reported in all compartments of the environment (Verlicchi et al., 2012), highlighting the relatively poor efficiency of wastewater treatment plants (WWTP) to remove these chemicals. To overcome this inefficiency, new processes of elimination are being explored. Biological treatments are receiving increasing interest as they offer the right balance between versatility and renewability (see Sup. Info. Table S1). One of the most promising biological processes for PhACs removal is the use of white-rot fungi (WRF) (Lebkowska and Załęska-Radziwill, 2014). These lignivore fungi produce a variety of extra cellular lignin modifying enzymes (LME) [including; Lignin peroxidase (LiP; EC 1.11.1.14), manganese-dependent peroxidase (MnP; EC 1.11.1.13), and laccase (LAC; EC 1.10.3.2)] that not only decompose wood materials but also a wide array of organic compounds, including emerging recalcitrant contaminants (Touahar et al., 2014; Jelic et al., 2012). However, most studies using WRF have been carried out using

* Corresponding author. Tel.: +1 (819)821 8000 (65457); fax: +1 (819)821 7974.
E-mail address: Hubert.Cabana@USherbrooke.ca (H. Cabana).

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single PhAC under concentrations that are not representative of wastewaters or the environment (100–1000 times higher). This strongly limits the evaluation of the real potential of WRF to remove PhACs in a comprehensive manner (Marco-Urrea et al., 2009).

In this study, the efficiency of the WRF *Trametes hirsuta* to remove a mixture of 17 PhACs, representing various chemical and therapeutic classes, under low PhACs concentrations (20, 100 and 500 ng L^{-1}) was evaluated. The degradation kinetics (5 days) were measured by UPLC/MS–MS. The activities of three major extracellular enzymes (LAC, and MnP, LiP) were monitored by UV–Vis spectrophotometry. The potential contribution of biosorption and intracellular enzymes to the removal of PhACs were also evaluated. This is one of the first studies that considers the effect of low PhAC concentrations on WRF efficiency to remove multiple PhACs.

2. Methods

2.1. Chemicals

All chemicals were analytical grade. Formic acid, methanol and acetonitrile (Optima® grade for LC/MS) were purchased from Fisher Scientific (Ottawa, ON, Canada). PhACs, malt extract, yeast extract and D-glucose from Sigma–Aldrich (Saint-Louis, MO, USA).

2.2. Medium, fungal strain and culture conditions

This work was carried out under sterile conditions using *T. hirsuta* strain IBB 450 under pelleted mycelium forms (see Sup. Info. for details). Biodegradation studies were performed in 250 mL Erlenmeyers, containing 50 mL of medium spiked with 0.5 mL of a mycelium solution (~300 mg dry weight). After two days of fungal growth (26 °C, 135 rpm) homogenous pellets appeared. Fungal cultures (2.06 ± 0.12 g dry weight per flask) were used without further treatment (LF) or were inactivated before addition of PhACs to the medium (20, 100 and 500 ng L^{-1}). Two inactivation treatments were used; (i) a heat-kill treatment (HKF, autoclaved 45 min at 121 °C and 19 psi) and (ii) a biocide treatment (BF, 10 mM of sodium azide). Every day, one triplicate was sacrificed and the supernatants were collected for PhACs quantification and enzyme activity measurements. The pellets were dried (120 °C, 3 h) and weighted in order to estimate fungal biomass. The comparison of the PhACs removal by living fungi and inactivated fungi allows deciphering the contribution of external enzyme activity and biosorption to the observed removal.

2.3. Pharmaceuticals quantification

Analyses of PhACs were performed using a positive electrospray ionization (ESI+) source in Multi-Reaction-Monitoring mode on an Acquity UPLC XEVO TQ mass spectrometer (Waters Corporation, Milford, MA) equipped with an Acquity UPLC HSS-T3 column (100 mm × 2.1 mm, 1.8 µm). No significant matrix effect was observed and the determined LOQ were above 2 ng L^{-1} (See Sup. Info. for details).

2.4. Extracellular enzymatic activity assays

The activities of LAC, MnP, and LiP were monitored by UV–Vis spectrophotometry according to Touahar et al. (2014) (see Sup. Info. for details). One enzymatic activity unit (U) was defined as the amount of enzyme that transforms 1 µmol of substrate per min. Results report the mean and standard deviation of triplicates.

2.5. Statistics

The statistics treatment was performed by a variance analysis (ANOVA), using a Holm–Sidak test. The levels of significance are expressed as a P value <0.05.

3. Results and discussion

3.1. Extracellular enzymes activity

The enzymatic production by WRF is known to be highly dependent on species (Saparrat et al., 2002) and growth conditions (Gao et al., 2010). Laccase was the only significantly active LME in the culture medium of *T. hirsuta* exposed to PhACs under the three conditions. Thus, we only report activity for LAC (Fig. 1). The absence of peroxidase activities is in accordance with previous studies reporting that peroxidase and other enzymes are more often expressed when the WRF are grown on solid medium or immobilized (Songulashvili et al., 2007). It is also known that the LMEs, and particularly LiP and MnP enzymes, are mostly expressed under nitrogen and carbon limiting conditions (Songulashvili et al., 2007; Saparrat et al., 2002). Thus, the relatively nutrient rich medium used in this work might contribute to explain the limited production of peroxidases.

The monitoring of peroxidases activity, based on the oxidation of veratryl alcohol to veratraldehyde, has been subjected to critics as it might induce bias (Arora and Gill, 2001). An alternative assay proposed by Arora and Gill (2001) using dye azure B was tested, but no significant activity for LiP nor MnP was detected.

3.2. Laccase activity as a function of PhACs concentration

Laccase activity was detected solely in cultures containing PhACs and its activity increased with decreasing PhACs concentrations (maximum activity from ~20 U L^{-1} at 500 ng L^{-1} after 96 h to ~70 U L^{-1} at 20 ng L^{-1} after 48 h) suggesting that the presence of PhACs induced LAC secretion (Fig. 1). All experiments were performed using the same initial fungal biomass and fungal biomass did not significantly changed over the 5-day period of PhAC exposure (data not shown). This rule out the potential effect of PhACs on fungal biomass production that could impact LAC activity. It is also worth noting that the pattern of LAC activity was affected by

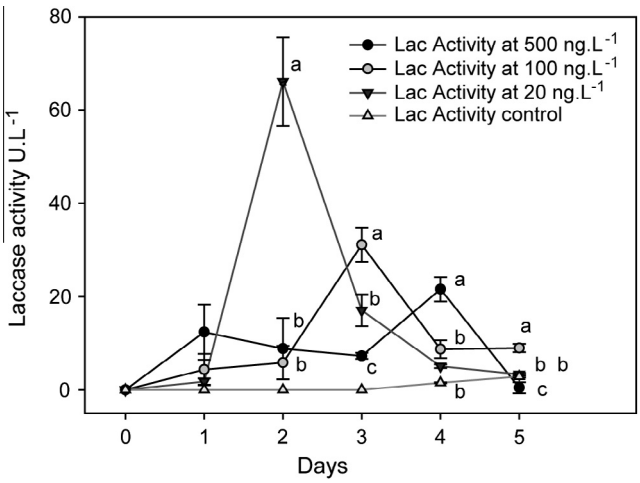

Fig. 1. Lacasse activity over time (5 days) at different PhAC initial concentrations. Values plotted are the means and the error bar represents the standard deviation of triplicate culture. Similar letters indicate no significant difference according to one way analysis of variance (ANOVA), Holm–Sidak, $P < 0.05$.