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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

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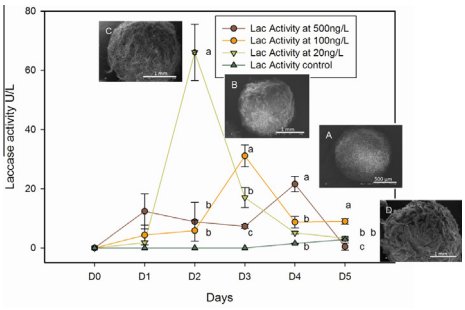
HIGHLIGHTS

- 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

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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

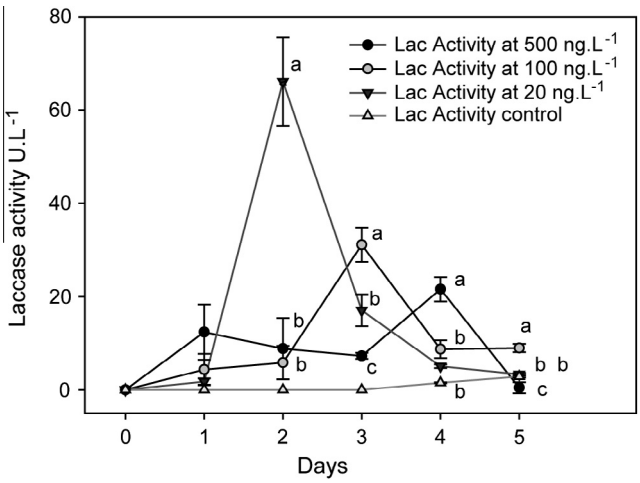

Fig. 1. Lacasse activity over time (5 days) at different PhAC initial concentrations. Values plotted are the means and the error bar represents the standard deviation of triplicate culture. Similar letters indicate no significant difference according to one way analysis of variance (ANOVA), Holm–Sidak,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

PhACs concentrations. The maximum activity of LAC was observed the earliest in the presence of the lowest PhACs concentration.

Results show that *T. hirsuta* is very efficient at removing non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and naproxen (Fig. 2A) which are poor substrates for LAC (Touahar et al., 2014). It is now well documented that many low molecular weight compounds, referred to as mediators, can extend the oxidative capacity of LAC to molecules that are poor substrates to this enzyme (Johannes and Majcherczyk, 2000). Mediators can be efficient at trace concentrations (Malarczyk et al., 2009). LAC mediators are extremely diverse (Cañas and Camarero, 2010) and include various naturally occurring molecules such as alcohol, phenols and aromatic amines. In a recent study, Malarczyk et al. (2009) showed, in *Trametes versicolor* and *Cerrena unicolor*, that the response of LAC activity to mediators concentration was non-linear and that decreasing mediator concentrations improved LAC activity. The efficient degradation of NSAIDs by *T. hirsuta* and the observed stimulation of LAC activity at lower PhACs concentration could reflect the modulation of enzyme activity by LAC mediators present in the tested mixture.

Overall data suggest that PhACs act as inducers and might also act as mediators of LAC activity, with the lowest concentrations achieving the highest and earliest activity. However, it is not clear whether or not the observed induction of LAC activity is a response to specific compounds, or degradation by-products within the mixture, or is a common response to all compounds. The exact identity of the mediators, if any, within the 17 tested compounds, or their by-products, remains to be fully investigated. In wastewater and environmental situations complex mixtures of compounds present at low concentration is the rule (Verlicchi et al., 2012). In this perspective the results are informative on the behavior of *T. hirsuta*. More research must be performed to better evaluate the enzymatic response of *T. hirsuta* to specific classes of compounds that could be relevant to specific applications such as the treatment of hospital wastewater.

3.3. T. hirsuta efficiency to remove multiple PhACs

In all experiments, NSAIDs were totally removed (Fig. 2A). The other PhACs tested (carbamazepine, caffeine, bezafibrate, ifosfamide, cyclophosphamide, atenolol, trimethroprim, ofloxacin) were not or only partially removed; less than 40% of removal for most of these molecules. Some molecules, such as trimethroprim, atenolol and ofloxacin, were poorly removed, especially under low concentrations (<100 ng L^{-1}). Some of these substances (i.e. atenolol and ofloxacin) are known to be quite resistant to biodegradation (Yamamoto et al., 2009). In some cases, the removal efficiency was concentration dependent, but the trends were not always clear.

Interestingly, previous studies on commercially available LAC (Touahar et al., 2014), showed that compounds such as fenofibrate, diazepam, ketoprofen and most NSAI are poorly removed by LAC (~20% of removal). This is in contrast with the present results in which these compounds were very efficiently removed (~80–100%). Experiments were conducted on a mixture of several PhACs under low concentration, while many published studies report experiments conducted at much higher concentrations and often using a single PhAC. This might contribute to explain these contrasted results. However, many studies using simple PhAC solutions under high concentrations also report the higher efficiency of WRF (i.e. *T. versicolor*) to remove compounds usually recalcitrant to LAC (Marco-Urrea et al., 2009). This suggests that mechanisms, other than extracellular LAC, could contribute to the removal of some molecules.

Overall, data show that *T. hirsuta* is able to degrade multiple PhACs under very low and environmentally realistic concentrations. This confirms that WRF bear great biotechnological potential for the removal of PhACs. However, it also appears that many gaps in the understanding of the degradation pathways remain to be filled in order to fully exploit this potential. A particular attention should be given to degradation by-products produced at various concentrations and complexity of PhAC mixtures.

3.4. Effect of PhACs exposure on T. hirsuta pellets morphology

All cultures were cultivated under the exact same conditions and initial fungal biomass. While fungal biomass was not significantly affected, the size of the pellets decreased with increasing PhACs concentrations (Sup. Info. Fig. S1). The surface of the pellets was also more homogenous at higher PhACs concentrations. Previous studies have reported that pellets size is directly linked to enzymatic secretion; increasing pellets size increases enzymatic production and secondary metabolites (Kim and Song, 2009). This is in accordance with present observations, where high PhACs concentrations result in smaller and more homogenous pellets and lower LAC activity (Fig. 1 and Sup. Info. Fig. S1). Smaller and more

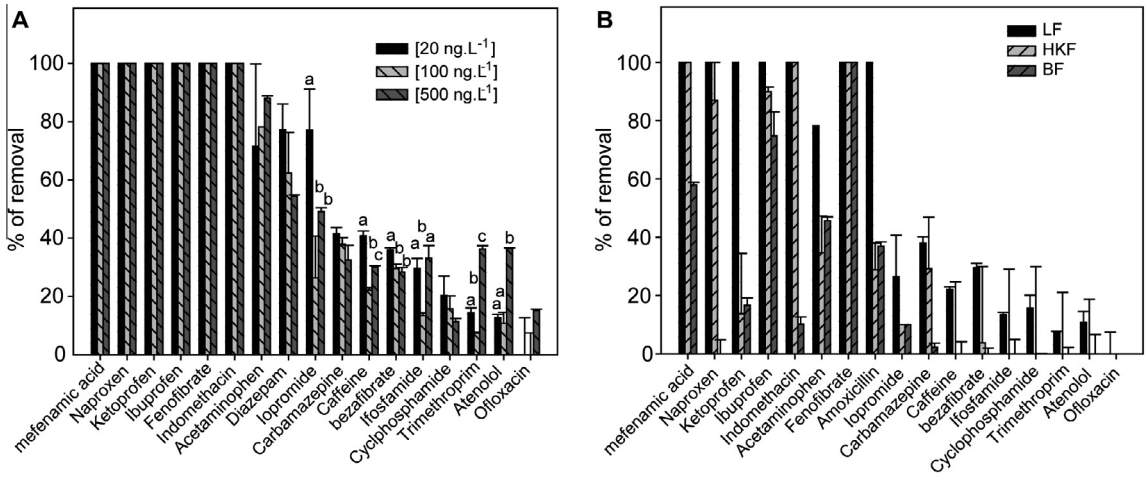

Fig. 2. Percentage of removal of 17 PhACs by the WRF *T. hirsuta*. (A) At three PhAC concentrations in the aqueous media (B) By living fungi (LF), heat-kill treated fungi (HKF) and biocide treated fungi (BF) at 100 ng L^{-1}. (Values plotted = means, error bar = standard deviation, $n = 3$.) Similar letters indicate no significant difference according to one way analysis of variance (ANOVA), Holm–Sidak, $P < 0.05$.

homogenous pellets also offer a higher specific surface for sorption. This change in morphology might also affect the hydrophobicity of the pellet further influencing PhAC sorption. It has been hypothesized that biosorption on fungus surface contributes to remove recalcitrant compounds including PhACs (Nguyen et al., 2014).

3.5. Contribution of biosorption to PhACs removal by T. hirsuta

PhACs poorly removed by LF (bezafibrate, ifosfamide, cyclophosphamide, trimethroprim, atenolol, caffeine and ofloxacin) were as well poorly removed by inactivated fungi (HKF and BF) (Fig. 2B). The other tested PhACs achieved contrasted responses to inactivated fungi. Fenofibrate was completely removed by LF, HKF and BF. This strongly suggests that biosorption is the primary process responsible for the observed removal and that extracellular enzyme (i.e. LAC) activity has a negligible effect. The removal of ketoprofen, acetaminophen, amoxicilline and iopromide was significantly higher in the presence of LF than both HKF and BF. This suggests that both biosorption and degradation by extracellular LAC contributed to the observed removal of these compounds. Finally, the removal of mefenamic acid, ibuprofen, indomethacin, naproxen and carbamazepine was very efficient and comparable with LF and HKF but was significantly lower with BF. This suggests that while biosorption can contributed to the observed removal of these compounds, other processes might be at play. The absence of significant differences between LF and HKF suggests that the extracellular enzyme LAC has a limited or no effect on the removal. The higher removal observed with HKF than BF could reflect a higher biosorption due to improved membrane surface after heat treatment (broken membrane). The heat treatment could also strongly disturb the structure of fungal pellets further improving biosorption compared to BF. The BF and HKF might also have contrasted effects on enzymes contributed to PhACs removal but not accounted for in this study.

Indeed, recent studies suggested the implication of intracellular enzymes (i.e. cytochrome P450s) in the degradation of PhACs by WRF (Marco-Urrea et al., 2009). Differences between HKF and BF could reflect the role of internal cellular content (i.e. enzymes) and its subsequent release into the solution during the heat-kill treatment, on PhACs removal. Further research is required to decipher the real contribution of extracellular and intracellular enzyme activity and biosorption on the removal of multiple PhACs under environmentally realistic concentrations. This variety of potential mechanisms involved in PhACs removal by WRF make processes using whole-fungi likely more versatile in terms of applications and target molecules, than processes based on pure enzymes.

4. Conclusion

This is one of the first studies reporting the efficiency of WRF (*T. hirsuta*) to remove multiple PhACs from contrasted chemical classes under environmentally relevant concentrations (20–500 ng L^{-1}). Data also suggest that some PhACs can act as LAC inducers and possibly mediators. Results also indicate that PhACs concentration significantly affects the morphology of *T. hirsuta* pellets. Finally, results suggest that LAC activity might not be the only process at play; biosorption and intracellular enzyme activity might play a significant role in multiple PhACs removal that remains to be fully investigated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.08.036.

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