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Impact of 1-Hydroxybenzotriazole Dosing on Trace Organic Contaminant Degradation by Laccase

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ABSTRACT

This study investigated the removal of five selected non-phenolic trace organic contaminants (TrOC) by extracellular enzyme extract from a white-rot fungus. Except diclofenac, no other investigated non-phenolic TrOC were degraded by the extracellular enzyme extract. Improvement of enzymatic degradation of all TrOC was achieved in the presence of a redox mediator, namely, 1-hydroxybenzotriazole (HBT). However, the enhancement of degradation was mediator concentration-specific. A significant improvement in degradation at higher dosage than 0.1 mM (HBT) was achieved.

Keywords: Trace organic contaminants; crude enzyme extract; laccase; mediator; 1-hydroxybenzotriazole (HBT)

1. INTRODUCTION

White-rot fungi and their lignin modifying enzymes can degrade a wide range of trace organic contaminants (TrOC), which are suspected to cause adverse health effects in humans and other biota (Yang et al., 2013). Recent studies have successfully applied either whole-cell white-rot fungal preparations or their extracellular culture extract to remove TrOC from the aqueous phase. Laccases are copper-containing enzymes which can oxidise various chemicals such as diphenols. Some high redox potential fungal laccases can also oxidise non-phenolics, but in general non-phenolic TrOC are not easily amenable substrates for laccase.

Enzyme-catalysis has been shown to be enhanced by the addition of low molecular weight mediator compounds which act as an 'electron shuttle' between the oxidizing enzyme and target compounds (Kurniawati and Nicell, 2007). The degree of enhancement, however, has been observed to depend predominantly on the type of mediator and the TrOC structure (Baiocco et al., 2003; Tran et al., 2010; Yang et al., 2012). To date these mediators have been studied mainly in conjunction with other groups of resistant pollutants than TrOC. Particularly, the effect of mediator concentration on a set of non-phenolic TrOC having diverse chemical structures has not been studied systematically.

With the aim of addressing the research gaps outlined above, a series of batch tests was conducted in this study to compare the removal of selected non-phenolic TrOC by augmenting

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enzymatic transformation with selected dosage of a redox mediator, namely, 1-hydroxybenzotriazole (HBT).

2. MATERIALS AND METHODS

2.1 Trace organic contaminants and mediator

Three pharmaceuticals and two pesticides were used in this study. Key properties of these compounds are listed in Table 1. These TrOC were selected based on their widespread occurrence in water and wastewater. All the selected TrOC were non-phenolic compounds but contained different functional groups. All compounds were purchased from Sigma-Aldrich (Australia). A mixed stock solution of the TrOC was prepared at a concentration of 1 g/L (each) in pure methanol, stored at -18°C and used within one month.

An N-OH derivative mediator (d'Acunzo et al., 2003), namely 1-hydroxybenzotriazole (HBT) was used in this study. HBT was purchased from Sigma-Aldrich (Australia). The stock solution (50 mM) of HBT was stored at 4°C prior to use.

2.2 Crude enzyme extract

Pure cultures of white-rot fungi (*Trametes versicolor* (ATCC 7731)) were grown in an Erlenmeyer flask, which contained 50 mL of malt extract broth (Merck, Germany) at a concentration of 5 g/L (pH = 4.5). The cultures were incubated in a rotary shaker at 70 rpm and 28°C for a week. The medium was harvested as 'crude enzyme' by decanting the media into sterilized bottle and stored at 4°C.

2.3 Batch test description

The experiments were conducted in 400 mL beakers. The crude enzyme solution (25 mL) having an initial enzymatic activity of 36 ± 3 µM/min was added to the beakers. The TrOC stock solution was added to the crude enzyme at a concentration of 100 µg/L each.

The impact of three different HBT concentrations (0.1, 0.5 and 1 mM) in enhancing the performance of the crude enzyme was tested. All experiments were conducted in triplicate. Control samples comprised TrOC in Milli-Q water. The beakers were covered with aluminum foil and incubated in a rotary shaker at 70 rpm and 25°C for 24 h.

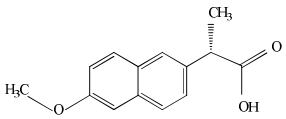
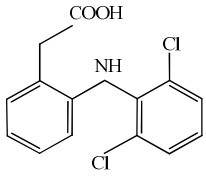
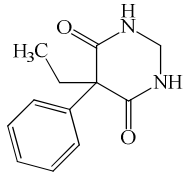
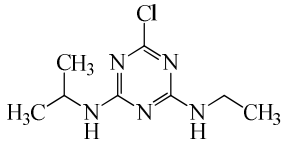
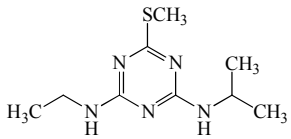
2.4 Analytical methods

The TrOC concentrations in the batch test samples were measured by a previously reported analytical technique involving SPE, derivatisation and quantitative determination by a Shimadzu GC/MS (QP 5000) system (Hai et al., 2011).

The quantitative detection limits of this analytical method were compound specific and in the range from 1 to 10 ng/L (Table 1). At the end of the incubation period (see Section 2.3), the whole test medium was harvested as GC/MS samples. The samples were diluted to 500 mL with Milli-Q water and filtered through 0.45 µm glass fiber filter (Filtech Australia). The pH of the samples was adjusted to 2 by using 4 M H_2SO_4 and the solid-phase extraction (for GC/MS analysis) was carried out on the HLB cartridges (Waters, Australia).

Laccase activity was measured by monitoring the change in absorbance at 468 nm due to the oxidation of 2,6-dimethoxy phenol at room temperature over 2 min using a spectrophotometer (PharmaSpec UV-1700, Shimadzu, Kyoto, Japan) (Hai et al., 2009). Laccase activity was calculated from the molar extinction coefficient ε = 49.6/(mM·cm) and expressed in µM substrate/ min (Hai et al., 2009). Oxidation reduction potential was measured using a WP-80D dual channel waterproof pH-mV-Temperature meter (TPS, Australia) with the accuracy of ± 1 mV.

Table 1 Physicochemical properties and categories of the TrOC studied

Category	Compound (CAS number)	Molecular weight (g /mol)	Log D (pH 5)[a]	Dissociation constant (pKa)[a]	Limit of detection (ng/L)[b]	Chemical structure
Pharmaceuticals	Naproxen ($C_{14}H_{14}O_3$) (22204-53-1)	230.26	2.49	4.84 ± 0.30	1	
	Diclofenac ($C_{14}H_{11}Cl_2NO_2$) (15307-86-5)	296.15	3.66	4.18 ± 0.10 -2.26 ± 0.50	5	
	Primidone ($C_{12}H_{14}N_2O_2$) (125-33-7)	218.25	0.83	12.26 ± 0.40 -1.07 ± 0.40	10	
Pesticides	Atrazine ($C_8H_{14}ClN_5$) (1912-24-9)	215.68	2.63	2.27 ± 0.10	10	
	Ametryn ($C_9H_{17}N_5S$) (843-12-8)	227.33	2.92	3.71 ± 0.41	10	

[a] Source: SciFinder database https://scifinder.cas.org/scifinder/view/scifinder/scifinderExplore.jsf

Log *D* is logarithm of the distribution coefficient which is the ratio of the sum of concentrations of all forms of the compound (ionised and unionised) in octanol and water at a given pH.

na: data not available

[b]Limit of detection (LOD) of the compounds during GC-MS analysis as described in Section 2.4. LOD is defined as the concentration of an analyte giving a signal to noise (S/N) ratio greater than 3. The limit of reporting was determined using an S/N ration of greater than 10

3. RESULTS AND DISCUSSION

3.1 Enzymatic degradation of TrOC

In this study, no or low (< 30%) enzymatic degradation of primidone, atrazine, ametryn and naproxen was obtained (Fig. 1). It is noteworthy that of these resistant TrOC, the fungal removal of only atrazine and naproxen has been studied before. The resistance of the aforementioned TrOC can be attributed to the presence of electron withdrawing groups (EWG) in their structures. The presence of EWG has been reported to render the compounds less susceptible to oxidative catabolism (Tadkaew et al., 2011).

Atrazine and ametryn are two triazine compounds. Atrazine additionally is a chlorinated compound (Table 1). Despite

having the weak electron donating groups (EDG) methyl and secondary amine, atrazine and ametryn were poorly removed. This is consistent with previous reports that triazine pesticides are resistant to fungal treatment (Mougin et al., 1997). In particular, varying removal of atrazine depending on the fungal strain has been reported in the literature. A different strain of *T. versicolor* than that used in this study showed significantly better removal of atrazine (over 60%) (Bending et al., 2002) compared to that (< 40%) by other species (*D. squalens* and *P. velutina*) (Bending et al., 2002; Torres-Duarte et al., 2009).

Primidone, possessing methyl (weak EDG) and amide (strong EWG) groups, was poorly degraded by the crude enzyme extract (Fig. 1). The presence of methyl groups means that the degradation could initiate from conversion of the methyl group to alcohol, by-passing the recalcitrant amide conversion. However, methyl and other aliphatic groups have very weak electron donating capacity, and thus in presence of a strong electron withdrawing group they may have limited activating effect (Tadkaew et al., 2011).

Naproxen, which contains an ether group (EDG) and a carboxyl group (EWG), has been found to be degraded by whole-cell preparations of several fungal species including *T. versicolor*, *B. adusta* and *P. chrysosporium* (Marco-Urrea et al., 2010a; Rodarte-Morales et al., 2011; Tran et al., 2010). However, our observation regarding low enzymatic degradation of naproxen is in agreement with a few other studies. For example, degradation of naproxen was less than 10% with a purified laccase solution (Lloret et al., 2010; Marco-Urrea et al., 2010a).

Unlike the other investigated TrOC, a significant enzymatic degradation of diclofenac was achieved in this study (Fig. 1). Diclofenac contains amine (EDG), carboxylic (EWG) and chlorine (EWG) groups in its structure. It has been reported to be efficiently degraded by white-rot fungal treatment over a concentration range of 0.01 to 5 mg/L (Lloret et al., 2010; Marco-Urrea et al., 2010b; Rodarte-Morales et al., 2011; Tran et al., 2010; Yang et al., 2012; Zhang and Geißen, 2010). The high removal of diclofenac is also in good agreement with a TrOC classification proposed by Yang et al. (2013) based on TrOC degradation by white-rot fungi.

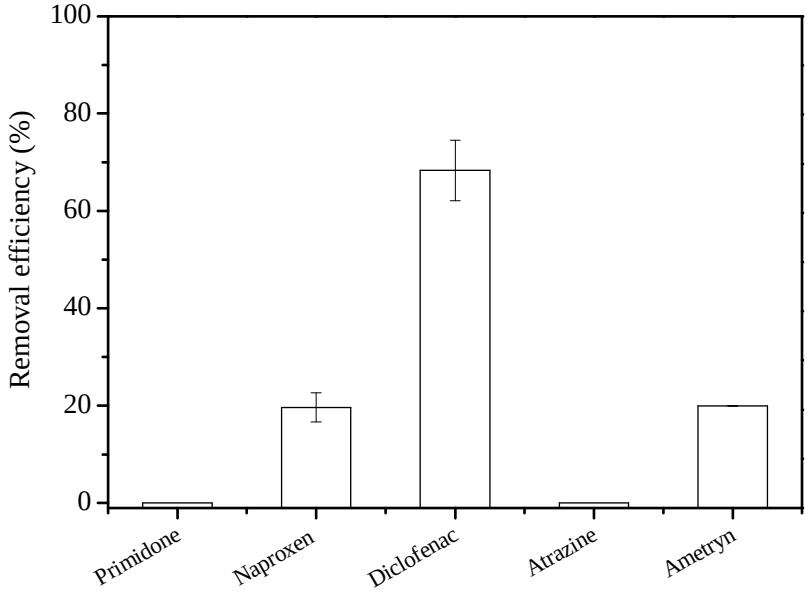


Figure 1 Removal of the selected non-phenolic TrOC by crude enzyme extract. The error bars represent the standard deviation of three replicates

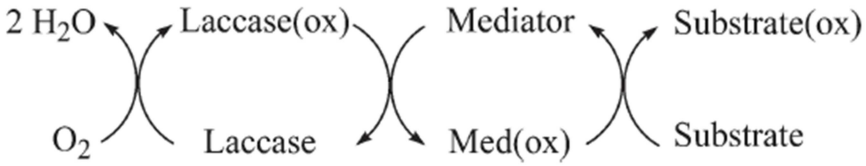


Figure 2 Schematic diagram illustrating the working principle of laccase-mediator systems (d'Acunzo et al., 2003)

Table 2 Oxidation reduction potential (mV) and enzyme inactivation (%) due to the addition of different concentrations of HBT

Parameters	Mediator concentration (mM)			
	0	**0.1**	**0.5**	**1**
Oxidation reduction potential (mV)[a]	207	363	420	441
Enzyme inactivation (%)[b]	8	14	28	36

[a]Values represent the average of duplicate samples with standard deviation of less than ± 5 mV
[b]Enzyme inactivation (%) = (Initial - Final enzymatic activity) / Initial enzymatic activity

3.2 Effect of mediator addition on enzymatic stability

As noted earlier, in the laccase-mediators systems, mediators act as "electron shuttle," facilitating the oxidation of complex substrates that do not enter the active sites of the enzyme due to steric hindrances. Laccase oxidises the mediators, generating highly reactive radicals, which then oxidise the target substrates (d'Acunzo et al., 2003).

The laccase-mediator systems can achieve better removal of non-phenolic compounds or compounds with high electrochemical potential as the radicals generated in laccase-mediator systems have redox potentials higher than that of laccase only (Klonowska et al., 2002). Table 2 confirms that, in our study, the oxidation reduction potential of the crude enzyme extract increased significantly due to the addition of the mediators. The oxidation reduction potential of the crude enzyme extract amended with HBT (1 mM) was 441 mV, which was significantly higher than that of the crude enzyme extract (207 mV) (Table 2). Our results are consistent with that of Weng et al. (2012) who reported that the oxidation reduction potential of a range of mediators was higher than that of laccase or the mediator separately.

The radicals (generated from laccase-mediator systems) that can improve pollutant degradation may on the other hand inactivate laccase (Khlifi-Slama et al., 2012). In this study, when no mediators were added, the loss of laccase activity during incubation was 8%, possibly due to incubation under agitation (Hai et al., 2012). By contrast, depending on the mediator concentration, the loss of laccase activity ranged from 14 to 36% (Table 2). This may have implications on the performance with different dosages of mediators, which has been discussed in the following section.

3.3 Effect of mediator addition on TrOC degradation

The addition of HBT improved the removal of the TrOC which showed resistance to degradation by the crude enzyme extract (Fig. 3). For an HBT dose of 1 mM, the removal of naproxen, ametryn, atrazine and primidone was improved by 78, 16, 95, and 40%, respectively. HBT addition also improved the

removal of diclofenac by 25%, achieving above 90% removal. The role of HBT in improving the enzymatic degradation of these TrOC is in good agreement with previous studies (Lloret et al., 2010; Nguyen et al., 2013). For example, naproxen (Marco-Urrea et al., 2010a; Rodríguez-Rodríguez et al., 2010) was removed only by 10% by a laccase preparation, however, the addition of HBT improved the removal to 95% (Marco-Urrea et al., 2010a). Lloret et al. (2010) observed improvement in the removal efficiency of diclofenac (from ~ 65% to 95%) in the presence of HBT.

The laccase-HBT system can achieve better removal of non-phenolic compounds as the aminoxyl radicals generated (d'Acunzo et al., 2006) have redox potentials greater than that of laccase only (Klonowska et al., 2002). For instance, Bernini et al. (2011) reported that the laccase-mediator system can facilitate abstraction of hydrogen from benzylic carbons of non-phenolic compounds. This can explain the improvement in naproxen, ametryn, atrazine, and primidone removal in our study in the presence of HBT. Notably, despite very similar chemical structures (Table 1) of atrazine and ametryn, better removal of the former was achieved in this study. Probably the thio-group attached to the s-triazine ring (of ametryn), imparts greater resistance to degradation.

Except for primidone, the removals improved significantly as the HBT dose was increased from 0.1 to 0.5 mM but did not improve beyond that (Fig. 3). This is consistent with the dosage-specific profile of oxidation reduction potential of the mediator-amended enzyme extract (Fig. 2). Our results are in line with the general trend observed in the literature that pollutant removal may not increase beyond a certain mediator concentration. For example, Lloret et al. (2010) observed diclofenac removal to range from 40% to 80% for the SGD concentrations ranging from 0.1 to 0.5 mM, while a complete removal was achieved at 1 mM.

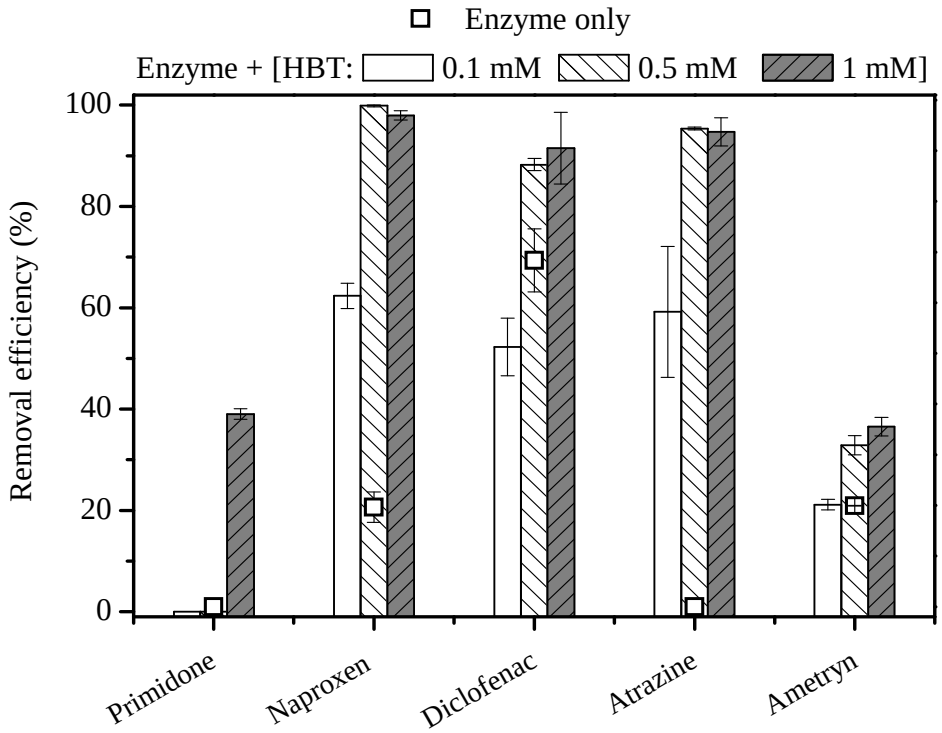


Figure 3 Removal of the selected non-phenolic TrOC by crude enzyme extract in the presence of different concentrations of HBT. The error bars represent the standard deviation of three replicates

CONCLUSIONS

Addition of the mediator 1-hydroxy-benzotriazole to the crude enzyme extract harvested from the culture of *T. versicolor* led to different levels of improvement of the investigated non-phenolic TrOC degradation. This was attributed to the increase in redox potential of the enzyme solution following mediator addition. A significant impact of the mediator concentration on the redox potential of the mediator-amended enzyme extract and inactivation of enzyme, and, consequently, on enzymatic degradation was observed.

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