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Impacts of redox-mediator type on trace organic contaminants degradation by laccase: Degradation efficiency, laccase stability and effluent toxicity

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ABSTRACT

This study compares the effectiveness of seven redox-mediating compounds namely, 1-hydrozybenzotriazole (HBT), N-hydroxyphthalimide (HPI), 2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO), violuric acid (VA), syringaldehyde (SA), vanillin (VA), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), which follow distinct oxidation pathways, for the degradation of trace organic contaminants (TrOCs). These redox-mediators were investigated for improved degradation of four TrOCs showing resistance to degradation by crude laccase from the white-rot fungus *Pleurotus ostreatus*. ABTS and VA achieved the highest degradation of the phenolic compounds (*i.e.*, oxybenzone and pentachlorophenol), whereas the non-phenolic compounds (*i.e.*, naproxen and atrazine) were best removed using VA or HBT. This implies that the non-phenolic compounds are more effectively removed by the radical species generated by the N–OH type mediators (*i.e.*, VA and HBT), while removal of the phenolic compounds may depend more on the stability and the redox potential of the radicals generated from the mediator, irrespective of the type. Notably, enzyme stability was greatly affected by the N–OH type mediators but it was compensated by their rapid degradation capacity. Overall, VA and HBT (N–OH type) appear to be the best mediators for enhanced degradation of the selected compounds without causing significant toxicity in the effluent.

1. Introduction

Laccase (EC 1.10.3.2) is a type 1 (blue) copper oxidase. It is widely distributed among white rot fungi, which are responsible for the degradation of complex organic polymeric lignins in nature (Yang et al., 2013). Laccase predominantly attacks the phenolic moieties in lignin, catalysing a one-electron oxidation via phenoxyl radicals. Laccase has been used in various industries such as textiles, pulp and paper, food manufacture and in remediation processes especially in the degradation of phenols and anilines (Hai et al., 2013; Witayakran and Ragauskas, 2009). In recent years, the removal of trace organic contaminants (TrOCs) from water and wastewater by laccase has received increasing attention. TrOCs are detected in various water bodies at concentrations as low as a few ng/L. However, many TrOCs are ineffectively removed by the conventional wastewater treatment processes (Hai et al., 2014; Luo et al., 2014) and pose a threat to aquatic ecosystems and to the safety of drinking water resources.

The ability of laccase to degrade TrOCs has been shown for several subgroups such as industrial chemicals (Gros et al., 2014), anti-inflammatory drugs (Tran et al., 2010), antibiotics (Auriol et al., 2007; Weng et al., 2012), personal care products *e.g.*, UV filters (Garcia et al., 2011) and pesticides (Majeau et al., 2010). The oxidative efficiency of laccases depends on the redox potential difference between the reducing substrate and type 1 copper in laccase. Given the range of redox potentials that laccases from different fungi possess (0.17–0.80 V) (Cañas and Camarero, 2010), non-phenolic substrates are often not amenable to direct oxidation

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by laccase. The chemical structure of the substrate is also an important factor influencing its degradation by laccase. Particularly, the distribution of functional groups within the TrOC has a large influence on the efficiency of the oxidation process by laccase (Yang et al., 2013). There are two categories of functional groups: i) electron donating functional groups (EDGs) including hydroxyl and amine that are strong activating groups, meaning that compounds containing these groups are more susceptible to electrophilic attack; and ii) electron withdrawing functional groups (EWGs) such as nitro, halogen, carboxyl and amide groups that are strong deactivating groups making the oxidation of these compounds much slower and more complicated (Yang et al., 2013). TrOCs that may not be directly oxidized by laccase include bulky compounds that cannot access the laccase-active site and those with a high redox potential. This limitation can be overcome by adding a small molecular weight redox-mediating substrate of laccase whereby highly reactive radicals generated due to oxidation of the mediator by laccase can in turn degrade the target compound of interest. This mechanism is analogous to the natural lignocellulose biodegradation by white- and brown-rot fungi, which produce reactive oxygen species (*e.g.*, hydroxyl, peroxyl and hydroperoxyl radicals) to initiate the biodegradation of biopolymers found in wood (Hammel et al., 2002).

Three major mechanisms by which a mediator can oxidize a substrate have been reported in the literature: hydrogen atom transfer, electron transfer and ionic mechanisms (Astolfi et al., 2005). Mediators differ from each other in terms of optimal reaction conditions and in specificity towards a given target compound (Baiocco et al., 2003). Previous studies have focused on performance comparison of different laccase – mediator combinations for degradation of dyes (Khlifi et al., 2010; Mendoza et al., 2011) and TrOCs (Garcia et al., 2011; Jeon et al., 2008). However, critical aspects such as laccase stability and the treated effluent toxicity are often overlooked or not comprehensively covered. A study with such a focus would help identify the type and dose of mediators that improve TrOC removal while minimizing effluent toxicity and laccase inactivation.

This study aims to compare the effectiveness of seven selected redox-mediators, representing three different oxidative mechanisms, for enhancing the oxidation of four resistant TrOCs by laccase. The performance of laccase with different mediators was systematically compared particularly focusing on laccase stability, TrOC removal efficiency, and effluent toxicity to pinpoint the best mediator. A series of batch tests with crude laccase preparation from white-rot fungi *Pleurotus ostreatus* were used to evaluate the impact of mediator concentrations, types and reaction times.

2. Materials and methods

2.1. Crude laccase preparation

Erlenmeyer flasks (250 mL) containing 50 mL of malt extract at a concentration of 5 g L^{-1} were inoculated with the white-rot fungus *P. ostreatus* (ATCC 34675). The pH of the solution was adjusted to 4.5 and the culture incubated on a rotary shaker at 70 rpm and 28 °C for one week. The fungus secreted extracellular laccase into the media and this crude enzyme extract was separated from the biomass before storing in sterilized bottles at 4 °C.

2.2. Trace organic contaminants and mediators

Four TrOCs namely oxybenzone, pentachlorophenol, atrazine and naproxen were selected based on their widespread occurrence in water and wastewater and their resistance to degradation by laccase in previous studies (Nguyen et al., 2014b; Yang et al., 2013). These compounds were selected to facilitate a systematic investigation of the effect of mediator addition. The physicochemical properties of these compounds are summarized in Supplementary Data Table S1.

A range of mediators, including 1-hydroxybenzotriazole (HBT), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), syringaldehyde (SA), (2,2,6,6-tetramethylpiperidin-1-yl) oxy (TEMPO), violuric acid (VA), vanillin (VAN) and N-hydroxyphthalimide (HPI) were used to compare the relative efficiency of mediators at aiding the removal of target contaminants. As shown in Table 1, these mediators follow three different mechanisms: hydrogen atom transfer (SA, HBT, VA, VAN and HPI), electron transfer (ABTS) and ionic mechanism (TEMPO).

2.3. Experimental protocol

For the initial screening of the mediators, crude laccase extract (5 mL) with an initial activity of 52 ± 2 ($n = 2$) $\mu M_{(DMP)}$ min^{-1} was added to 10 mL test tubes. Working solution, comprising the four TrOCs, was added to the test tubes containing crude enzyme to yield a final concentration of 500 μg L^{-1} of each compound. Selected mediators were added to the solutions separately to produce final mediator concentrations of 1 mM. Controls included TrOC solution in Milli-Q water and TrOC solution and enzyme solution without mediator. Tests were conducted in duplicate. Test tubes were sealed and incubated on a rotary shaker at 70 rpm and 25 °C for 24 h.

The three mediators that exhibited the greatest TrOC removal in the mediator screening experiments (*i.e.*, ABTS, VA and HBT) were selected for assessment of impact of incubation time (2, 4, 8 and 24 h) and mediator concentration (0.05, 0.1, 0.25, 0.5 and 1 mM). These tests were conducted in the same way as described above.

2.4. Analytical methods

Laccase activity was assayed by recording the change in absorbance (468 nm) due to oxidation of 2,6-dimethoxyl phenol (DMP) in the presence of sodium citrate (pH 4.5). Enzymatic activity was calculated using a molar extinction coefficient of 49.6 $(mM\ cm)^{-1}$ and expressed in $\mu M_{(DMP)}$ min^{-1}. The redox potential of the laccase solution before and after mediator addition was measured utilizing an oxidation-reduction potential meter (WP-80D dual pH-mV meter, Thermo Fisher Scientific, Australia). Toxicity of untreated and treated media was analysed in duplicate by measuring inhibition of luminescence of naturally luminescent bacterium *Photobacterium leiognathi*, and expressed as relative Toxic Unit (rTU), the reciprocal of the IC_{20} value of the inhibition of luminescence *vs.* concentration curve (van de Merwe and Leusch, 2015). The concentration of the four TrOCs utilized in this study (*i.e.*, pentachlorophenol, oxybenzone, naproxen and atrazine) was measured by an HPLC–UV–vis detector system (Shimadzu, Japan) following a previously reported method (Nguyen et al., 2014a).

3. Results and discussion

3.1. Removal of TrOCs by laccase

Substrate degradation by laccase is limited by two factors, principally, the availability of strong EDG within its structure, and the redox potential of that particular type of laccase. Also, steric shielding from EWG may prevent electron abstraction from occurring. Hence, this section explains enzymatic degradation of the selected TrOCs based on their molecular structures and the presence of EDGs and EWGs (Yang et al., 2013). Results show low laccase-catalyzed degradation (15–23%) of the TrOCs tested (Fig. 1).