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Enhancement of trace organic contaminant degradation by crude enzyme extract from *Trametes versicolor* culture: Effect of mediator type and concentration

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ABSTRACT

The performance of two redox mediating compounds, namely 1-hydroxybenzotriazole (HBT) and syringaldehyde (SA), was compared in terms of enhancement of enzymatic degradation of a diverse set of 14 phenolic and 16 non-phenolic trace organic contaminants (TrOCs) and the toxicity of the treated media. Extracellular enzyme extract (predominantly containing laccase) from *Trametes versicolor* culture achieved efficient degradation (70–95%) of nine phenolic and one non-phenolic TrOCs. Mediator dosing extended the spectrum of efficiently degraded TrOCs to 13 phenolic and three non-phenolic compounds, with moderate improvements in removal of a few other non-phenolic compounds. TrOC removal efficiency improved significantly as the HBT dose was increased from 0.1 to 0.5 mM, while SA achieved similar removal over dosage range of 0.1–1 mM. A particular concern was the toxicity of the treated media (1200–2200 times that of the control) for all SA dosages applied. Overall, HBT at a concentration of 0.5 mM achieved the best removal without raising concern regarding toxicity of the treated media. The results are discussed in the light of the redox potential of the enzyme-mediator cocktail, the balance between the stability and reactivity of the radicals generated and their cytotoxic effects.

1. Introduction

The occurrence of trace organic contaminants (TrOCs) such as pharmaceuticals and personal care products, pesticides, steroid hormones and industrial chemicals in water and wastewater is of increasing concern. Many TrOCs have been implicated in endocrine disrupting effects on aquatic organisms and even humans, while others have been linked to ecological perturbations through acute and chronic toxicity on aquatic flora and fauna [1]. Due to the incomplete TrOC degradation in wastewater treatment plants (WWTP), WWTP effluent is a major point source of TrOC pollution. The resistance of certain TrOCs to degradation by conventional wastewater treatment has prompted research on TrOC degradation by white-rot fungi [2].

White-rot fungi can efficiently degrade a wide range of organic compounds, including many that are resistant to bacterial degradation, *via* one or more extracellular enzymes including lignin peroxidases, manganese-dependent peroxidases and laccase. In addition to whole-cell preparations, removal of TrOCs has been investigated either by employing crude culture extract ('crude enzyme') or by purified enzymes [2,3]. Laccases are multi-copper containing enzymes that catalyze the oxidation of a wide range of phenolic substrates using oxygen as an electron acceptor [2]. The oxidation of a substrate typically involves the formation of a free radical after the transfer of a single electron to laccase. 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), non-phenolic substrates are often not amenable to direct oxidation by laccase [4,5]. To overcome this limitation, the use of 'redox mediators' has been proposed.

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Mediators are small molecular weight compounds that are easily oxidized by laccase. The presence of mediators can expand the catalytic activity of laccase by increasing the redox potential of the enzyme solution. Mediators also act as an "electron shuttle," facilitating the oxidation of complex substrates that do not enter the active sites of the enzyme due to steric hindrances. Laccase oxidizes the mediators, generating highly reactive radicals, which then oxidize the target substrates [6,7]. Three major mechanisms by which a mediator can oxidize a substrate have been reported in the literature, namely by hydrogen atom transfer (HAT), electron transfer and ionic mechanisms [6,8]. Commonly used mediators following the HAT pathway include small molecular weight phenolic compounds and compounds containing the structural group =N–OH. The oxidation of these mediators by laccase generates highly reactive phenoxyl ($C_6H_5O^*$) and aminoxyl (=N–O^*) radicals, owing to the enzymatic removal of an electron followed by release of a proton [9]. These radicals then extract a hydrogen atom from the substrate. With a few exceptions (*e.g.* [3,10–12]), studies on the efficacy of these mediators to remove compounds resistant to conventional treatment has to date focused mostly on compounds other than TrOCs. Furthermore, a few studies have raised concern about the toxicity of the media following treatment by laccase-mediator systems [13–15]. However, there appears to be no study which has compared the performance of redox mediators in terms of enhancement of enzymatic degradation of a diverse set of TrOCs and the resulting toxicity of the treated solution. Such a study would help identify the type and dose of mediators that improve TrOC removal while minimizing toxicity of the treated media.

The aim of this study was to investigate the performance of an extracellular enzyme extract from *Trametes versicolor* (ATCC 7731) culture on the removal of a set of 30 TrOCs representing diverse chemical structures (*e.g.*, phenolic and non-phenolic moieties and electron donating/withdrawing functional groups). A special focus was given to the effect of augmenting enzymatic transformation with different dosages of two redox mediators, namely 1-hydroxybenzotriazole (HBT) and syringaldehyde (SA) on the removal performance and ultimate media toxicity. The study provides unique insights in light of the redox potential of the enzyme-mediator cocktail, the balance between the stability and reactivity of the radicals generated and their cytotoxic effects.

2. Materials and methods

2.1. TrOC and mediators

A set of 30 TrOCs, including 11 pharmaceuticals, six pesticides, five steroid hormones, three industrial chemicals, two phytoestrogens, and three UV filters was used in this study. Key properties of these compounds are listed in Supplementary Data Table S1. These TrOCs were selected in view of their widespread occurrence in wastewater and wastewater-impacted water bodies and represent different molecular properties such as phenolic *vs.* non-phenolic moieties and electron donating *vs.* withdrawing functional groups. All compounds were purchased from Sigma–Aldrich (Australia). A stock solution of the TrOCs was prepared at a concentration of 1 g/L (each) in pure methanol, stored at −18 °C and used within one month.

Two redox mediators, namely 1-hydroxybenzotriazole (HBT) and syringaldehyde (SA), were used in this study. HBT is a =N–OH type mediator, while SA is a phenolic mediator (Supplementary Data Table S2). The oxidation of these mediators by laccase generates highly reactive aminoxyl (=N–O^*) and phenoxyl ($C_6H_5O^*$) radicals, respectively, both of which follow the HAT pathway during substrate (TrOC) degradation [9]. These mediators were also purchased from Sigma–Aldrich (Australia). The mediators were prepared as a stock solution at a concentration of 50 mM and stored at 4 °C.

2.2. Extracellular enzyme extract

Stock pure cultures of *T. versicolor* (ATCC 7731) were inoculated into Erlenmeyer flasks, each containing 50 mL of malt extract broth (Merck, Germany) at a concentration of 5 g/L. The pH of the medium was adjusted to 4.5. The cultures were incubated on a rotary shaker at 70 rpm and 28 °C for a week. The culture broth was harvested as 'crude enzyme' by decanting into sterilized bottles, which were stored at 4 °C. Under the culture conditions, the *T. versicolor* strain used in this study secreted laccase as the main extracellular enzyme. Laccase activity was measured according to the method described in Section 2.4.2.

2.3. Batch test description

Crude enzyme solution (25 mL) was added to 400 mL beakers. The initial enzymatic activity was 36 ± 3 μM/min (see Section 2.4.2). The TrOC stock solution was added to the crude enzyme solution at a concentration of 100 μg/L (each). To investigate the effect of mediator addition, each of the mediators was added separately to obtain solutions with final mediator concentrations of 0.1, 0.5 and 1 mM. All experiments were conducted in triplicate. Controls comprised TrOCs in deionized (Milli-Q) water. The beakers were covered with aluminium foil and incubated on a rotary shaker at 70 rpm and 25 °C for 24 h. At the end of the incubation period, the whole test medium was harvested. The samples were diluted to 500 mL with deionized (Milli-Q) water and filtered through 0.45 μm glass fibre filters (Filtech, Australia). The pH of the sample was adjusted to 2 with 4 M H_2SO_4 before refrigerating until solid-phase extraction (SPE) and GC/MS analysis (Section 2.4.1) within 2 d. For toxicity assay, undiluted samples were kept at 4 °C until analyzed.

2.4. Analytical methods

2.4.1. TrOC analysis

TrOC concentration was measured by a previously reported analytical technique involving SPE, derivatisation and quantitative determination by a Shimadzu GC/MS (QP5000) system [16]. The GC/MS system was equipped with a Shimadzu AOC 20i autosampler and a Phenomenex Zebron ZB-5 (5% diphenyl–95% dimethyl polysiloxane) capillary column (30 m × 0.25 mm ID, d_f = 0.25 μm). The quantitative limits of detection of this analytical method were compound specific and in the range from 1 to 20 ng/L (Supplementary Data Table S1). The aqueous phase TrOC removal efficiency was calculated by comparing initial and final concentrations.

2.4.2. Enzymatic activity, redox potential and toxicity assay

Under the experimental conditions of this study, the fungus predominantly secreted the extracellular enzyme laccase. Laccase activity was determined by monitoring the oxidation of 2,6-dimethoxy phenol (DMP) in sodium citrate buffer (pH 4.5) over 2 min at room temperature. The reaction mixture contained 10 mM of DMP, 100 mM sodium citrate and the sample. The reaction was started by the addition of DMP to the mixture of the sample and buffer. The measurement was based on monitoring the change in absorbance at 468 nm by a spectrophotometer (Shimadzu, Japan). Laccase activity was then calculated from the molar extinction coefficient ε = 49.6/(mM cm) and expressed in μM substrate/min [17]. The oxidation reduction potential (ORP) of the enzyme solution with and without mediator dosing was measured by an ORP meter (Orion Star, Thermo Fisher Scientific, Australia).