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Biocatalytic degradation of carbamazepine with immobilized laccase-mediator membrane hybrid reactor

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ABSTRACT

Carbamazepine (CBZ) is one of the most recalcitrant pharmaceutically active compounds routinely detected in wastewater effluent-impacted environment. Biocatalytic degradation with enzymes such as laccase provides a promising approach for the elimination of CBZ. However, the relatively low redox potential of laccase makes its efficient CBZ degradation difficult. Therefore, an environmentally benign and effective mediator is required. In this study, three natural phenolic compounds, namely p-coumaric acid (PCA), syringaldehyde (SYR), and acetosyringone (ACE), were investigated as redox mediators for the enzymatic removal of CBZ by both free and immobilized laccase. Among the tested mediators, PCA resulted in the optimal CBZ removal performance with 60% removal rate (20 μM initial CBZ) after 96 h incubation with immobilized laccase in a conventional suspension system. The degradation of CBZ was then carried out in a membrane hybrid reactor with the effluent recirculating through suspended biocatalytic TiO_2 nanoparticles. The effect of operation parameters including PCA concentrations, initial enzyme activity and operational flux on CBZ removal were investigated. Further CBZ metabolites study identified 10,11-dihydro-10,11-dihydroxy-CBZ (CBZD), 10,11-dihydro-10,11-epoxy-CBZ (CBZE) and acridone as the major metabolites of CBZ oxidation by laccase. The toxicity tests determined by algal viability using the fluorometric indicator alamarBlue indicated that the CBZ treatment via the hybrid reactor could effectively remove the toxicity of parent CBZ compound.

1. Introduction

Currently, pharmaceutically active compounds (PhACs), a large group of drugs used to prevent, cure and treat disease, are routinely detected in the urban aquatic environment [1–3]. As the PhACs are designed to obtain a biological effect at very low concentrations, the discharge of these compounds imposes a significant ecological impact [4,5]. Carbamazepine (5 H-dibenzazepine-5-carboxamide, CBZ), a widely prescribed antiepileptic drug, is one of the most frequently detected PhACs in the wastewater effluent-impacted surface water and groundwater [4,6]. Approximately one-third of the dosed CBZ is excreted in unaltered form after human consumption. It is highly resistant to biodegradation and also hardly adsorbs onto the solids [6], which results in poor elimination of CBZ in conventional wastewater treatment process [7,8]. The stability of CBZ allows its long-term transportation within the aquatic environments and makes it an ideal indicator of PhACs contamination [9]. Its ecotoxicological impact on aquatic organisms has been reported [10]. Jos et al. [11] found that CBZ had the chronic and synergistic effects with other chemicals. Based on European classification and labeling of chemicals (92/32/EEC), CBZ is "R52/53 harmful to aquatic organisms and may cause long-term adverse effects in aquatic environment" [12]. Therefore, considerable research efforts have been dedicated to developing optimal strategies for degradation of CBZ from wastewater.

However, satisfactory removal of CBZ via the traditional physicochemical water treatment process is difficult to achieve due to the robust chemical stability and hydrophilicity of CBZ [1,13]. On the other hand, other wastewater treatment methods, including advanced oxidation processes, membrane separation and activated carbon adsorption, can achieve high levels of CBZ removal [14–18]. However, there are still challenges involved with these technologies: the formation of undesirable and even more toxic by-products during the advanced oxidation process [19], the disposal of concentrated retentates for membrane separation [20] and the regeneration of absorbents [21]. As an alternative, the biocatalytic methods with the use of enzymes provide an environmentally benign option as such approaches possess lower energy

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requirements and more moderate operational conditions. In addition, the high specificity of the biocatalytic methods helps to minimize the undesirable side reactions during the pollutant degradation. Hence enzymes represent a promising tool for the selective removal of pollutants from waste streams [22].

Among different biocatalytic approaches, the application of the white-rot fungi (WRF) is especially attractive [23]. WRF is proven capable to degrade xenobiotics and recalcitration contaminants through the oxidization catalysed by the WRF harbored enzymes including laccase, lignin peroxidase and manganese peroxidase. Different WRF species such as *Tinea versicolour, Irpex lacteus*, *Pycnoporus cinnabarinus*, *Bjerkandera adusta*, *Pleurotus ostreatus* have been applied for degradation of PhACs including CBZ, and satisfactory removals have been achieved [12,24–28]. However, the difficulty in WRF incubation and susceptibility to attack by other microorganisms significantly constrain its wider application.

On the other hand, the use of purified enzymes for CBZ elimination has also been investigated. Laccase (polyphenoloxidase, E. C.1.10.3.2), a copper-containing enzyme found in many plants, fungi and microorganisms, is one of the most targeted enzymes used for detoxification of contaminants due to its low cost, relatively good stability and broad substrate specificity [29,30]. This enzyme can oxidize both phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants (including dyes, pesticides, endocrine disrupters, and polycyclic aromatic hydrocarbons) to less toxic derivatives, which made it especially attractive for bioremediation of wastewater [30–34]. However, recent studies on laccase catalysed degradation of CBZ revealed its poor activity towards CBZ [35]. This is due to the presence of strong electron withdrawing functional group (amide) in CBZ that generates severe electron deficiency and further renders CBZ less susceptible to laccase catalysed oxidation [36]. Therefore, the use of redox mediators is required. Mediators are normally small-molecular weight compounds, such as 2,2′-azino-bis-(3-ethyl-benzothiazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT), which could enhance the oxidizing capability of laccase to the recalcitrant compounds with high-redox potentials [32,35,37]. The oxidization of the redox mediators by enzyme forms radicals which can further diffuse away from enzyme's active site and reacts with other substrates. With the mediators, it is possible to oxidize the target compounds that in principle are not substrates of laccase due to their special conformation or high redox potential [37]. Hata et al. [35] found that with repeated addition of laccase and a redox mediator HBT, up to 60% of CBZ could be removed after 48 h. However, repeated addition of the HBT implies high additional costs, and also leads to other safety concerns considering the explosiveness and toxicity of HBT, which therefore calls for the application of more benign and natural mediators [37,38].

Other important issues of the enzymatic degradation include the long-term operational stability and reusability of the enzymes, and the efficient biocatalytic reactor design. One of the most effective solutions to improve the enzyme's stability is to use enzymes in their immobilized forms. However, so far most studies applying immobilized enzymes focused on phenolic compounds and dyes removal, and their application in CBZ elimination is scarce in the literature. Our group has developed a series of TiO_2 based laccase immobilization techniques and two types of different membrane reactors for bisphenol-A degradation: i.e. the hybrid biocatalytic reactor where biocatalytic TiO_2 nanoparticles are suspended within the membrane feed solution, as well as the biocatalytic membrane reactor where laccase is directly immobilized onto TiO_2 coated membrane surface [39,40]. The unique properties of TiO_2 nanoparticles, including high mechanical strength, low price, physical and chemical stability, low toxicity, coordination ability with amine and carboxyl groups, as well as good biocompatibility, make it an ideal candidate for enzyme immobilization [41]. Furthermore, TiO_2 nanoparticles can be integrated with membrane material to prepare the anti-fouling and biocatalytic membrane, facilitating its high efficiency application in wastewater treatment [33]. However, whether the TiO_2 based biocatalytic reactor is effective in CBZ removal especially under long-term operation is still unknown, and the CBZ degradation metabolites and their toxicity are worth investigation.

In this study, three lignin-related natural phenols, namely a lignin precursor (p-coumaric acid) and two lignin degradation products (syringaldehyde and acetosyringone), were investigated as alternatives to synthetic mediators for promoting the oxidation of CBZ by *Trametes versicolor* laccase. For better stability and reusability, laccase was covalently immobilized on TiO_2 nanoparticles, and a biocatalytic TiO_2 particle suspension membrane hybrid reactor was systematically tested for the CBZ removal. For comparison, we also directly immobilized laccase onto the TiO_2 coated membrane, and applied the resultant biocatalytic membrane for CBZ removal. Furthermore, optimization of CBZ degradation by the hybrid reactor was conducted by varying degradation parameters, and the reusability of the biocatalytic nanoparticles for CBZ degradation was investigated. Finally, the CBZ biodegradation products were identified and the acute toxicity bioassay was carried out to understand the effect of the biocatalytic treatment on the CBZ toxicity, and the membrane fouling during the degradation process was also studied.

2. Materials and methods

2.1. Chemicals and materials

Laccase from *T. versicolor* (EC 1.19.3.2, 33 U/mg), 2,2′-Azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS), carbamazepine (CBZ), syringaldehyde (SYR), acetosyringone (ACE), p-coumaric acid (PCA), amino-propyltriethoxysilane (APTES), glutaraldehyde (GLU) and 2,4-pentanedione were purchased from Sigma-Aldrich. Titanium isopropoxide (TTIP) ($\geq$ 97%, Sigam-Aldrich) was used to prepare TiO_2 sol solution. 0.1 μm hydrophilic polyvinylidene fluoride (PVDF) membranes were supplied by Millipore Pty. Ltd. AlamarBlue (AB) (Sigma-Aldrich) was used for toxicity assay. The marine raphidophyte *Chattonella marina* (CMPL01) and the freshwater algea *Microcystis aeruginosa* (PCC 7806) were generously provided by Professor T. David Waite (School of Civil and Environmental Engineering, the University of New South Wales).

2.2. Laccase immobilization

Laccase was covalently immobilized on TiO_2 nanoparticles [40]. Briefly, TiO_2 nanoparticles were firstly modified by APTES in pure ethanol for 24 h and then dispersed in 4% (v:v) GLU in pH 7 Sørensen's phosphate buffer for 12 h. Then the functionalized TiO_2 nanoparticles were suspended in 5 mL of laccase solution (100 μg/mL) for 48 h under 4 °C. Finally, the biocatalytic nanoparticles were centrifuged and re-dispensed 3 times with Milli-Q water. In addition, TiO_2 nanoparticles coated PVDF membranes were prepared for laccase immobilization. TiO_2 sol solution was prepared by mixing TTIP (5.95 ml), 2,4-pentanedione (0.974 ml), $HClO_4$ (0.99 ml), H_2O (5.5 ml) and ethanol (46.96 ml) in room temperature with well stirring for 1 h. Coating of TiO_2 sol–gel on PVDF membranes (hydrophilic 0.1 μm) was carried out via dip-coating method.as described in our previous publication [33]. Subsequently, the coated membranes were dried in the oven at 120 °C for 16 h, followed by 90 °C water bath for 24 h. Finally, the membranes were rinsed with Milli-Q water and dried at room temperature. In this work, 3 coating cycles were applied based on