



# Characterisation of electron beam irradiation-immobilised laccase for application in wastewater treatment

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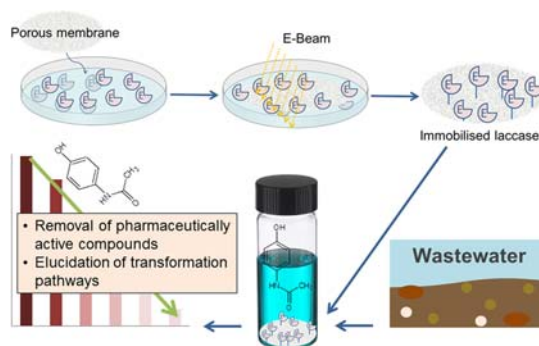
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## HIGHLIGHTS

- Laccase was cross-linked to PVDF membranes by electron beam irradiation.
- Immobilised laccase displayed a remarkable functional stability in real wastewater.
- The batch-wise and continuous enzymatic removal of pharmaceuticals was demonstrated.
- Pathways for acetaminophen and acetaminophen-triclosan conversions are proposed.
- Enzymatic acetaminophen oxidation enhances triclosan removal via cross-coupling.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Laccase from *Phoma* sp. UHH 5-1-03 was cross-linked to polyvinylidene fluoride membranes by electron beam irradiation. Immobilised laccase displayed a higher stability than the non-immobilised enzyme with respect to typical wastewater temperatures, and pH at a range of 5 to 9. Batch tests addressed the removal of pharmaceutically active compounds (PhACs; applied as a mixture of acetaminophen, bezafibrate, indometacin, ketoprofen, mefenamic acid, and naproxen) by both immobilised and non-immobilised laccase in municipal wastewater. High removal rates (>85%) of the most efficiently oxidised PhACs (acetaminophen and mefenamic acid) indicated a high efficiency of the immobilised laccase in wastewater. Continuous elimination of the aforementioned PhACs by the immobilised enzyme in a continuously operated diffusion basket reactor yielded a PhAC removal pattern qualitatively similar to those observed in batch tests. Clearly higher apparent  $V_{max}$  values and catalytic efficiencies (in terms of both  $V_{max}/S_{0.5}$  as well as  $V_{max}/K_m$  values obtained from data fitting according to the Hill and the Michaelis-Menten model, respectively) observed for acetaminophen oxidation by the immobilised compared to the non-immobilised enzyme are in support of a considerably higher functional stability of the immobilised laccase especially in wastewater. The potential influence of acetaminophen on the removal of comparatively less laccase-oxidisable water pollutants such as the antimicrobial triclosan (TCS) was investigated. TCS was increasingly removed upon increasing the initial acetaminophen concentration in immobilised as well as non-immobilised laccase reaction systems until saturation became evident. Acetaminophen was consumed and not recycled during laccase reactions, which was accompanied by the formation of various acetaminophen-TCS cross-coupling products. Nevertheless, the simultaneous presence of acetaminophen (and

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potentially even more pollutant removal-enhancing laccase substrates) and more recalcitrant pollutants in wastewater represents an interesting option for the efficiency enhancement of enzyme-based wastewater treatment approaches.

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## 1. Introduction

A rapid industrial development and growing demands for various chemicals are accompanied by the continuous introduction of persistent and sometimes eco-toxic micro-pollutants (micropollutants) into aquatic systems, mainly from discharges of conventional wastewater treatment plants (WWTPs) (Luo et al., 2014). Due to the incomplete removal of micropollutants in conventional WWTPs, their toxicity potential, and potential long-term detrimental impacts even at the ng/L to the lower µg/L range, current challenges in developed countries relate to the development of advanced water treatment methods (Loos et al., 2013; Verlicchi et al., 2012). However, the application of advanced processes based on chemical oxidation, membrane filtration, or adsorption (e.g. by activated carbon) entails considerable costs (Loh et al., 2000). Whereas filtration and adsorption methods would require further treatment of the generated waste, chemical conversions of micropollutants could lead to undesirable by-products potentially being even more toxic than their parent compounds (Andreozzi et al., 2005; Gasser et al., 2014).

Enzymes as industrial biocatalysts offer promising advanced treatment methods, which potentially may overcome known drawbacks of conventional processes (Cabana et al., 2007a, 2007b; Gasser et al., 2014). In recent years, oxidative enzymes such as laccases (EC 1.10.3.2; benzenediol: oxygen oxidoreductase or phenol oxidase) have attracted considerable interest in this respect because of a relatively low substrate specificity and the usability of available air oxygen as an electron acceptor (Cabana et al., 2011). Laccase is a copper containing enzyme, which is able to oxidise a wide range of micropollutants including endocrine disrupting chemicals (EDCs), and pharmaceutically active compounds (PhACs) (Arca-Ramos et al., 2016; Gasser et al., 2014; Cabana et al., 2007a, 2007b; Marco-Urrea et al., 2010a, 2010b). A remarkable characteristic of laccases relates to the possibility to enhance pollutant oxidation rates and expand the range of oxidisable compounds through laccase redox mediators. These are diffusible low-molecular-mass laccase substrates first being enzymatically oxidised to yield organic radicals, which subsequently oxidise further compounds in an abiotic manner (Jahangiri et al., 2017). Ideally, redox mediators should regenerate during pollutant oxidation thus becoming available for a next catalytic cycle. Such effects have been claimed for laccase oxidation systems involving the lignin-related phenolic syringaldehyde as a natural redox mediator and the pesticide dichlorophen as a target pollutant (Torres-Duarte et al., 2009). Contrary to such reports, presumed laccase redox mediators such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), acetosyringone and syringaldehyde have been found to be consumed instead of being recycled in other studies (Jahangiri et al., 2017; Margot et al., 2015).

Although frequently being considered for wastewater treatment, laccase freely suspended in real wastewater would undergo rapid denaturation and not provide long-term operational stability (Cabana et al., 2009b; Gasser et al., 2014). The use of enzymes in immobilised form is advancing in industrial and environmental applications, and has the potential to overcome shortcomings related to the use of free enzymes (Ba et al., 2014; Gasser et al., 2014; Touahar et al., 2014). Covalent enzyme binding to solid support materials has been reported to be the preferred laccase immobilisation method for wastewater treatment applications, and the corresponding biocatalysts are thought to be more stable than those obtained with other immobilisation techniques especially under the harsh conditions of real wastewaters (Gasser et al., 2014). However, "classical" covalent enzyme immobilisation methods are usually quite

time-consuming and commonly involve coupling steps ranging from several hours up to about one day (Arca-Ramos et al., 2016; Cabana et al., 2009a; Hommes et al., 2012; Kumar et al., 2014; Zimmermann et al., 2011). They are additionally complicated due to the need for appropriate cross-linkers, and often involve numerous further time-consuming processing (e.g. surface modification, drying, washing) steps (Arca-Ramos et al., 2016; Cabana et al., 2009a; Hommes et al., 2012; Kumar et al., 2014; Zimmermann et al., 2011). Using commercial *Trametes versicolor* laccase (TvL), we have recently firstly demonstrated the general applicability of a very rapid, simple, and inexpensive one-step immobilisation procedure based on the electron beam (E-Beam) irradiation-induced covalent linking of the enzyme to commercial porous polyvinylidene fluoride (PVDF) membranes as the support material (Jahangiri et al., 2014). The laccase-containing membranes thus achieved are advantageous with respect to long-term mechanical stability and easy separation from a reaction medium, and potentially hold promise for wastewater treatment approaches.

The present study aimed to establish and partly optimise a particularly rapid one-pot procedure for the immobilisation of laccase from the aquatic ascomycete *Phoma* sp. UHH 5-1-03 (Junghanns et al., 2008; Junghanns et al., 2009), based on E-Beam irradiation-induced cross-linking onto PVDF membranes as introduced above. Laccase from *Phoma* sp. was chosen because of its ability to oxidise substrates still at neutral to slightly alkaline pH values (Junghanns et al., 2009) relevant for wastewaters. However, the *Phoma* laccase was found to be quite sensitive towards glutaraldehyde used as a cross-linking agent in a previous study (Hommes et al., 2012). The E-Beam procedure allows enzyme immobilisation within the range of minutes (see [Subsection 2.3](#) of the materials and methods section) instead of the several hours to days needed for conventional chemical enzyme coupling as described before. We further aimed to assess important characteristics of the immobilised laccase such as pH and thermal stability, reusability, and apparent kinetic parameters for micropollutant oxidation in comparison with the non-immobilised enzyme. For this, we have investigated the performance of laccase in either form in various aqueous matrices also including real wastewater. Enzymatic micropollutant removal was demonstrated using a cocktail of phenolic and non-phenolic PhACs in glass vial-based batch experiments, and a lab-scale perfusion basket reactor (BR) (Langford and Thomas, 2009). Among these PhACs, the phenolic acetaminophen was chosen as a model compound for comparing apparent kinetic parameters of PhAC oxidation by the immobilised and the free laccase in real wastewater and buffer. The formation of free acetaminophen radicals as primary products of acetaminophen oxidation by laccase (Lu et al., 2009) qualifies the compound as a potential laccase redox mediator, possibly enhancing the laccase-catalysed transformation of other MPs being more resistant towards laccase attack (Arca-Ramos et al., 2016; Touahar et al., 2014). We have therefore studied potential redox-mediating effects of acetaminophen in laccase reaction systems (Hachi et al., 2017) in more detail. In this regard, the influence of acetaminophen on both target pollutant removal and the formation of transformation products was assessed. Triclosan (TCS), a persistent environmental contaminant with antimicrobial activity being comparatively slowly oxidised by the laccase from *Phoma* sp. (Hofmann and Schlosser, 2016; Jahangiri et al., 2017), was considered to be a suitable model target pollutant for these investigations. TCS concomitantly also offers previously established knowledge with respect to the mode of action and related transformation pathways of other compounds enhancing its laccase oxidation such as syringaldehyde (Jahangiri et al., 2017). Based on such data the present

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