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Laccase- and electrochemically mediated conversion of triclosan: Metabolite formation and influence on antibacterial activity

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HIGHLIGHTS

- Laccase oxidation of triclosan (TCS) led to oligomerization and ether cleavage.
- Ether cleavage was more prominent during electrochemical TCS oxidation.
- Syringaldehyde (SYD) promoted TCS ether cleavage by laccase.
- SYD was consumed thus not acting as a recyclable "true" laccase redox mediator.
- Dimethoxybenzoquinone formed from SYD contributes to SYD-laccase systems' toxicity.

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ABSTRACT

Metabolite formation from radical-based oxidation of the environmental pollutant triclosan (TCS) was compared using an ascomycete (*Phoma* sp. UHH 5-1-03) and a basidiomycete (*Trametes versicolor*) laccase, laccase-redox mediator systems, and electrochemical oxidation (EC). Laccase oxidation predominantly yielded TCS di- and trimers, but notably also caused TCS ether bond cleavage. The latter was more prominent during EC-catalysed TCS oxidation, which generally resulted in a broader and more divergent product spectrum. By contrast, only quantitative but not qualitative differences in TCS metabolite formation were observed for the two laccases. Application of the presumable natural laccase redox mediator syringaldehyde (SYD) shifted the TCS-transforming reactions of laccase systems from oligomerization more towards ether bond cleavage. However, the observed rapid removal of SYD from reaction systems caused by predominant adduct formation from SYD and TCS, and concomitant conversion of SYD into 2,6-dimethoxy-1,4-benzoquinone (DMBQ) clearly demonstrates that SYD does not function as a "true" laccase redox mediator in the sense of being recycled during TCS oxidation. Laccase treatment of TCS without SYD decreased the anti-bacterial TCS activity more than treatment employing SYD in addition, indicating that SYD and/or its transformation products contribute to bacterial toxicity. DMBQ was found to be about 80% more active in a bacterial growth inhibition test than its parent compound SYD in terms of IC_{20} values. These observations establish DMBQ as a potential cause of toxicity effects of SYD-laccase systems. They further illustrate that a natural origin of a redox mediator does not automatically qualify its use as environmentally benign or non-hazardous.

1. Introduction

Triclosan (TCS; 2,4,4′-trichloro-2′-hydroxydiphenyl ether or 5-chloro-2-[2,4-dichloro-phenoxy]-phenol) is a biocide with a long history of use in health care and household products, cosmetics and plastic materials. The widespread use of TCS and its long-term persistence led to the ubiquitous occurrence of this compound in ecosystems including surface and sea water, living organisms such as fish, algae, and plants (Kolpin et al., 2002; Singer et al., 2002; Balmer et al., 2004; Chau et al., 2008; Coogan and La Point, 2008), human blood, hair (Martin et al., 2015), and breast milk (Allmyr et al., 2006; Calafat et al., 2008). Exposure to TCS and its accumulation can increase antimicrobial resistance through gene mutations, and may result in cross-resistance to antibiotics (Drury

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et al., 2013). TCS is further a suspected cause of endocrine disruption (Witorsch, 2014). Recently, the U.S. Food & Drug Administration (FDA) has decided to ban TCS from over-the-counter consumer antiseptic wash products like hand and bar soaps, body washes, and others (http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm378393.htm).

Various water treatment processes, e.g. water chlorination, UV irradiation and ozonation, have been considered for TCS degradation. However, some of them are costly and may lead to toxic and carcinogenic by-products including dioxins (Suarez et al., 2007; Buth et al., 2010; Murugesan et al., 2010; Ding et al., 2015). Despite considerable challenges related to the practical feasibility of wastewater treatment methods, bio-oxidation of TCS by oxidative enzymes such as laccases (EC 1.10.3.2) is a potentially attractive option. Laccases are widely distributed multi-copper enzymes found in many kinds of environmentally frequent organisms including fungi, bacteria, plants and insects. These enzymes can effectively catalyse the one-electron oxidation of phenols and aromatic amines, anilines and thiols using atmospheric oxygen as the terminal oxidant (Cañas and Camarero, 2010). In recent years, laccases have been employed in several studies targeting TCS degradation (Cabana et al., 2007; Murugesan et al., 2010; Melo et al., 2016; Sun et al., 2016). An enhanced TCS transformation is commonly observed in the additional presence of laccase redox mediators (Cabana et al., 2007; Murugesan et al., 2010), i.e. diffusible low-molecular-mass laccase substrates first being oxidized by laccases and subsequently oxidizing further compounds in a non-enzymatic manner. Due to high costs and possible toxic effects of synthetic redox mediators, natural redox mediators from lignocellulose have been considered as an eco-friendly and easily available alternative (Cañas and Camarero, 2010). Furthermore, ideal redox mediators should regenerate during their catalytic cycle to the greatest possible extent. The natural lignin-related phenolic syringaldehyde (SYD) was reported to fulfil the aforementioned criteria and become essentially fully recycled by re-reduction of its enzymatically formed primary radical oxidation product while in turn abiotically oxidizing the pesticide dichlorophen (Torres-Duarte et al., 2009, 2011).

So far, only a few studies have provided limited data concerning structures of products and transformation mechanisms related to TCS oxidation by laccases (Cabana et al., 2007; Murugesan et al., 2010; Hofmann and Schlosser, 2016; Sun et al., 2016). The oligomerization of TCS as a result of the oxidation of the compound by laccase has been reported by Cabana et al. (2007). Later on, Murugesan et al. (2010) and Sun et al. (2016) confirmed TCS oligomerization upon laccase oxidation. Furthermore, Murugesan et al. (2010) also described TCS ether bond cleavage followed by partial dechlorination, provided that a laccase redox mediator was additionally present. With the exception of just one report from our own research group demonstrating TCS polymerisation by an ascomycete laccase (Hofmann and Schlosser, 2016), basidiomycete laccases were always investigated in the aforementioned studies.

Our picture of biocatalytic processes potentially diminishing TCS concentrations in wastewater as well as in the environment, and related environmental implications is far from being complete. Knowing TCS transformation products can shed light on (i) enzymatically or abiotically induced reactions and related pathways of TCS breakdown, (ii) the influence of redox mediators on such processes, and (iii) possible consequences for the remaining biological (antibacterial) activity. We have addressed these points by studying TCS transformation using two different fungal laccases, which were chosen in order to cover biotechnologicaly and environmentally relevant biocatalysts sharing the general principle of substrate oxidation but differing in important properties, and concomitantly representing two major fungal groups involved in the catabolism of environmental contaminants (Harms et al., 2011). Laccase from *Trametes versicolor* (TvL) is a typical basidiomycete laccase known to oxidize substrates preferentially in the acidic pH range, and ranges among the high redox potential laccases typically found in basidiomycetes (Baldrian, 2006; Rodgers et al., 2010). The laccase from the aquatic isolate *Phoma* sp. UHH 5-1-03 oxidizes laccase substrates still at neutral to slightly alkaline pH values and originates from an ascomycete, hereby exemplifying a group of fungi apparently mostly producing low to middle rather than high redox potential laccases (Junghanns et al., 2009; Rodgers et al., 2010). In the context of the laccase-catalysed TCS breakdown we have also addressed the question whether the lignin-related phenolic compound SYD acts as a "true" laccase redox mediator in the sense of being significantly recycled during TCS oxidation. The formation of transformation products was investigated using an ultra-performance liquid chromatography (UPLC) system coupled to a quadrupole time-of-flight mass spectrometer to identify the metabolites.

The aforementioned enzymatic investigations were accompanied by the elucidation of metabolites resulting from electrochemical (EC) TCS oxidation, using a Boron-doped diamond (BDD) electrode-equipped EC cell. EC oxidation reactions are known to involve e.g. hydroxylation of activated aromatics, benzylic hydroxylation, N-dealkylation of amines, dealkylation of ethers and thioethers, S- and P-oxidation, and oxidation of alcohols to aldehydes (Jurva et al., 2003). Such reactions may potentially proceed via direct electron transfer from a reductant to the BDD anode surface, or via generation of hydroxyl radicals in solution that may subsequently oxidize organic compounds (Panizza and Cerisola, 2005). As EC oxidation is less site-specific than enzymatic oxidation, a broader range of TCS transformation products could be expected for EC oxidation compared to laccase-catalysed oxidation.

To the best of our knowledge, this study represents the so far most comprehensive elucidation of TCS transformation metabolites and related pathways of radical-based TCS breakdown by different enzymatic and non-enzymatic causes, wherein laccases, laccase-redox mediator systems, and EC oxidation processes were comparatively assessed.

2. Materials and methods

2.1. Chemicals and other materials

Laccase from *Trametes versicolor* (TvL; specific activity $\geq$ 10 U/mg), syringaldehyde (SYD; purity 98%), and 2,6-dimethoxy-1,4-benzoquinone (DMBQ; purity 97%) were purchased from Sigma-Aldrich (Munich, Germany). 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; purity $\geq$ 98%) and triclosan (TCS; purity $\geq$ 97%) were obtained from AppliChem (Darmstadt, Germany) and Merck Millipore (Darmstadt, Germany), respectively. Deuterated triclosan-D3 (TCS-D3) was provided by TRC (Toronto, Canada). All chemicals were of analytical grade and used without further purification. Acetonitrile, ammonium acetate and acetic acid (all UPLC-MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands).

2.2. Production and purification of laccase from Phoma sp.

The isolation, identification and maintenance of *Phoma* sp. strain UHH 5-1-03 (available as *Phoma* sp. DSM 22425 from the German Culture Collection of Microorganisms and Cultures, Braunschweig, Germany) has previously been described (Junghanns et al., 2008).

Phoma sp. was cultivated in flasks (1 L) containing 300 mL medium composed of 2% (w/v) malt extract (pH 5.7), 50 μM $CuSO_4$, and 1 mM vanillic acid (Junghanns et al., 2009). Each flask was