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The transformation of triclosan by laccase: Effect of humic acid on the reaction kinetics, products and pathway

Rong-Ni Dou [a], Jing-Hao Wang [a], Yuan-Cai Chen [a, b, *], Yong-You Hu [a, b]

[a] School of Environment and Energy, South China University of Technology, Guangzhou 510006, China
[b] Ministry of Education Key Laboratory of Pollution Control and Ecological Remediation for Industrial Agglomeration Area, South China University of Technology, Guangzhou 510006, China

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ABSTRACT

This study systematically explored the effect of humic acid (HA) (as model of natural organic matter) on the kinetics, products and transformation pathway of triclosan (TCS) by laccase-catalyzed oxidation. It was found that TCS could be effectively transformed by laccase-catalysis, with the apparent second-order rate constant being 0.056 U^{-1} mL min^{-1}. HA inhibited the removal rate of TCS. HA-induced inhibition was negatively correlated with HA concentration in the range of 0–10 mg L^{-1} and pH-dependent from 3.5 to 9.5. FT-IR and ^{13}C NMR spectra showed a decrease of aromatic hydroxyl (phenolic) groups and an increase of aromatic ether groups, indicating the cross-linking of HA via C-O-C and C-N-C bonds during enzyme-catalyzed oxidation. Ten principle oxidative products, including two quinone-like products (2-chlorohydroquinone, 2-chloro-5-(2,4-dichlodichlorophenoxy)-(1,4)benzoquinone), one chlorinated phenol (2,4-dichlorophenol (2,4-DCP)), three dimers, two trimmers and two tetramers, were detected by gas chromatograghy/mass spectrometry (GC-MS) and high performance liquid chromatography/quadrupole time-of-flight/mass spectrometry (HPLC/Q-TOF/MS). The presence of HA induced significantly lesser generation of self-polymers and enhanced cross-coupling between HA and self-polymers via C-O-C, C-N-C and C-C coupling pathways. A plausible transformation pathway was proposed as follows: TCS was initially oxidized to form reactive phenoxyl radicals, which self-coupled to each other subsequently by C-C and C-O pathway, yielding self-polymers. In addition, the scission of ether bond was also observed. The presence of HA can promote scission of ether bond and further oxidation of phenoxyl radicals, forming hydroxylated or quinone-like TCS. This study shed light on the behavior of TCS in natural environment and engineered processes, as well provided a perspective for the water/wastewater treatment using enzyme-catalyzed oxidation techniques.

1. Introduction

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol, TCS) is a multi-purpose biocide widely used in many household and personal care products, and it has been frequently detected in the various environmental medium, including surface waters, sediments, and groundwater (Zhao et al., 2010). The primary emission source of TCS comes from the effluents discharged from municipal sewage treatment plants (STPs), due to uncomplete removal of TCS by the conventional biological treatment process. Moreover, improper disposal of TCS can lead to the formation of more toxic and persistent products, such as highly toxic polychlorinated dioxins (Buth et al., 2010; Jimmy et al., 2006), more lipophilic methyl TCS (DeLorenzo et al., 2008; Pycke et al., 2014) and more toxic chlorinated TCS derivatives (Fiss et al., 2007). Because of their accumulative and toxic effect on various organisms (Bedoux et al., 2012; DeLorenzo et al., 2008), TCS was banned using as additive in food plastic pachaging by European Commission.

Recent studies demonstrated that enzyme-catalyzed oxidation was one of potential ways to effectively remove TCS. For example, several oxidizing enzymes secreted from the white rot fungi could be used to eliminate TCS (Cabana et al., 2007a, 2007b). Manganese peroxidase has been shown to nearly completely oxidize TCS in 60 min using a dose of 0.5 nkat mL^{-1} (Inoue et al., 2010). Horseradish peroxidase (HRP) and soybean peroxidase had been

* Corresponding author. School of Environment and Energy, South China University of Technology, Guangzhou Higher Education Mega Center, Guangzhou 510006, China.
E-mail address: chenyc@scut.edu.cn (Y.-C. Chen).

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demonstrated to remove TCS (Li et al., 2016; Melo and Dezotti, 2013). Laccase also has been applied to effective oxidation of TCS in the presence or absence of redox mediator (Kim and Nicell, 2006; Murugesan et al., 2010). It is well recognized that enzyme-catalyzed oxidation of substrates occur through oxidative coupling reactions, which involve one-electron oxidation of the substrate into highly reactive radicals by its phenolic or anilinic structures. These reactive radicals subsequently couple to each other to form coupling products (Auriol et al., 2007; Lu et al., 2015, 2009; Murugesan et al., 2010). In such a way, micropollutants can be removed by formation of larger and insoluble coupling products. A number of micropollutants, including bisphenol A (BPA), nonylphenol, natural and synthetic hormones and pharmaceuticals and personal care products have been demonstrated to undergo such coupling degradation mechanisms in enzyme-catalyzed reaction system (Catherine et al., 2016). Therefore, it is hopeful to take advantage of the enzyme-catalyzed oxidative coupling processes to control TCS.

Humic acid (HA) is an important form of humic substances and ubiquitously present in the nature, water treatment plan and so on, therefore inevitably influences water or wastewater treatment processes. It has been demonstrated that HA is able to affect the processes enzyme-catalyzed oxidation, because of highly reactive properties of the functional groups (Feng et al., 2013; Lu et al., 2017, 2009; Lu and Huang, 2009; Sun et al., 2016). Many studies have reported that the presence of HA can decrease the removal kinetics of contaminants by competing for enzymes. For example, Feng et al. (2013) reported that HA could participate in the oxidation reaction and inhibited the oxidation of tetrabromobisphenol A (TBrBPA) by laccase, especially in environmentally relevant concentrations. Sun et al. (2016) found that the removal of 17 β-estradiol decreased with the concentration of HA increased. Recently, Lu et al. (2015, 2017) suggested that HA could inhibit the enzyme-catalyzed oxidation rate through quenching the radical intermediates and reversion the radical intermediates back to the original form. However, other studies have found contrary results. For example, Lu and Huang (2009) observed that the presence of humic matter could enhance the removal of acetaminophen by laccase, and they ascribed the promoting effect of humic matter to the formation of humic matter radicals, which can react with substrate by the hydrogen exchange. Li et al. (2013) found that the presence of HA promoted the removal of BPA, due to the chain cross-coupling oxidation between HA and contaminant self-polymerization, in which contaminant self-polymers could incorporate into HA, thus enhancing the removal of substrates. These results provide insights into the role of humic substances in enzyme-catalyzed oxidation process. However, the interaction mechanism involving humic substances and substrates transformation remains unclear and deserves further study.

The purpose of this study is to systematically investigate the effect of HA on the transformation kinetics of TCS by enzyme-catalyzed oxidation using laccase, which is a representative naturally occurring extracellular enzyme. The effect of HA on the transformation pathway was particularly addressed by characterization of the products on gas chromatograghy/mass spectrometry (GC-MS) and high performance liquid chromatography/quadrupole time-of-flight/mass spectrometry (HPLC/Q-TOF/MS). Finally, the tentative reaction pathways of TCS transformation in laccase-catalyzed oxidation process in the presence of HA was proposed.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade or higher grade. Triclosan (TCS, 99.5%, CAS: 3380-34-5) was purchased from Sigma-Aldrich (St. Louis, MO). A stock solution of TCS was prepared in HPLC-grade acetonitrile at a concentration of 1000 mg L^{-1} and stored at 4 °C. Working solutions of TCS were prepared by dilution in phosphate buffer solution (PBS) and contained less than 0.5% acetonitrile, which has no impact on the enzyme. Laccase from *Trametes versicolor* was supplied from Sigma-Aldrich, and enzyme solutions were freshly prepared in PBS before used. Diammonium 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS, 98%, CAS: 30931-67-0) was purchased from Aladdin (Shanghai, China). Humic acid (HA) in the form of a sodium salt (Sigma-Aldrich, Shanghai), which consists of 18.37% water (air dry basis), 20.84% ash (dry basis), 59.97% HA (dry basis), 3.7% N, 6.6% Na, 0.1% K, 2.3% Fe, 0.5% Mg, was dissolved in ultra-water. After 24 h stirring, the solution was filtered through a 0.45 μm GF/C filter to remove the insoluble and suspended solid and the total organic carbon (TOC) content was measured with a TOC analyzer (TOC-V model, Shimazdu, Kyoto, Japan). HA concentration used in the experiments was expressed as milligrams of organic carbon per liter water.

2.2. Enzyme activity assay

Laccase activity was determined using a colorimetric assay by oxidation of 0.5 mM ABTS in 100 mM sodium acetate buffer (pH 4.5). The absorbance at 420 nm (ε_{420} = 36 000 M^{-1} cm^{-1}) was continuously recorded every 30 s in 3 min at 25 °C. One unit (U) of laccase activity is defined as the amount of enzyme required to oxidize 1 μmol of ABTS per min under defined conditions.

2.3. The batch enzyme-catalyzed reactions

The reaction kinetics were conducted in 10 mL brown glass tubes, which containing 2 mL TCS working solutions (5 mg L^{-1}, pH 7.0). All tubes were incubated in a shaker (150 rpm) at 25 °C, the reactions were initiated after adding different amounts of laccase (1–6 U mL^{-1}). Samples were collected at specified intervals and terminated by adding 2 mL acetonitrile immediately, then filtered with a 0.22 μm glass fibre membrane (Shanghai ANPEL, China) and analyzed by a HPLC. The tubes without adding laccase were used as control.

To investigate the impact of HA on TCS oxidation rate, 5 mg L^{-1} TCS working solutions were prepared by dilution in buffer solution (pH 7.0) containing HA with different concentrations between 0 and 20 mg L^{-1}. The reactions were initiated after adding 5 U mL^{-1} laccase. Samples were collected and analyzed as the treatment without HA. For adjusting different pH values, citric acid/di-sodium hydrogen phosphate buffer 0.1 mM was used as buffer for pH < 7.0, while sodium borate of 0.1 mM was employed for pH > 7.0. To assess the impact of HA (5 mg L^{-1}) on Michaelis-Menten reaction kinetics, experiments were conducted to estimate the initial reaction rates of TCS at seven different concentrations (2.0, 2.5, 3.3, 5.0, 6.7, 8.0 and 9.2 mg L^{-1}, respectively) in the absence or presence of HA, the data were used to construct Lineweaver-Buck plot and carried out kinetic analysis. Reactions were initiated by addition of 5 U mL^{-1} laccase and terminated by adding 2 mL acetonitrile after vigorously stirring in the vortex shaker for 40 s. The initial reaction rate (v) was determined by the formulation $v = ([S]_0\text{-}[S]_t)/\Delta t$, where $[S]_0$ and $[S]_t$ represent TCS concentrations at time 0 and specific time t, respectively. The Lineweaver-Buck plot is expressed as following.

$$\frac{1}{v} = \frac{k_m}{v_{max}[S]} + \frac{1}{v_{max}} \quad (1)$$

For intermediate products identification, 100 mL TCS (5 mg L^{-1}) solution with or without HA (5 mg L^{-1}) was treated with laccase (5 U mL^{-1}) at pH 7.0 at 25 °C. After 2 h incubation, reactions were