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Elimination and detoxification of triclosan by manganese peroxidase from white rot fungus

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ABSTRACT

The antimicrobial and preservative agent triclosan (TCS) is an emerging and persistent pollutant with a ubiquitous presence in the aquatic environment. Thus, TCS was treated with manganese peroxidase (MnP), laccase and the laccase-mediator system with 1-hydroxybenzotriazole. MnP was most effective in eliminating TCS among the three enzymatic treatments, with TCS concentration being reduced by about 94% after 30 min following treatment with 0.5 nkat mL^{-1} MnP and being almost completely eliminated after 60 min. Furthermore, MnP (0.5 nkat mL^{-1}) caused the complete loss of bacterial growth inhibition by TCS after 30 min and reduced the algal growth inhibition of TCS by 75 and 90% after 30 and 60 min, respectively. These results strongly suggest that MnP is effective in removing the ecotoxicity of TCS.

Keywords: Triclosan; Bacterial growth inhibition; Algal growth inhibition; Manganese peroxidase; Ligninolytic enzymes; White rot fungi

1. Introduction

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) (TCS) is a synthetic, non-ionic, broad-spectrum antimicrobial and preservative agent that is widely used in personal care products, such as soaps, shampoos, toothpastes and cosmetics. Many studies have reported the widespread occurrence of TCS with concentrations ranging from 35 to 2700 ng L^{-1} in the effluent from wastewater treatment plants [1-6]. Furthermore, in vitro studies have suggested that TCS may be harmful to aquatic organisms. For example, it has been reported that the green alga *Selenastrum capricornutum* (recently renamed *Pseudokirchneriella subcapitata*) is more sensitive to TCS than bioluminescent bacterium *Vibrio fischeri*, crustacean *Ceriodaphnia dubia* and fishes *Danio rerio* and *Oryzias latipes* [7].

There is currently great interest in lignin-degrading white rot fungi and the ability of their ligninolytic enzymes, such as manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase, to degrade endocrine disruptors and pharmaceuticals [8-17]. To date, several studies on the elimination of TCS using white rot fungus and/or laccase have been carried out. It has been reported that *Trametes versicolor* converts TCS to TCS-xyloside, TCS-glucoside and 2,4-dichlorophenol, and that these two conjugates show lower cytotoxic and microbiocidal activity than TCS [18]. Furthermore, recent researches have demonstrated the elimination of TCS by laccase in the absence and presence of redox mediators [19, 20].

Laccase oxidizes various phenolic compounds by reducing molecular oxygen to water. The use of redox mediators such as 1-hydroxybenzotriazol (HBT) can extend the substrate

range of laccase; HBT radicals generated by laccase oxidation can oxidize nonphenolic compounds [21]. MnP catalyzes the oxidation of Mn(II) to Mn(III) in the presence of H_2O_2, while the generated Mn(III) oxidizes various phenolic compounds. Some reports have shown that the redox potential of Mn(III) is higher than those of HBT radicals and laccase itself [22, 23]. These findings suggest that MnP is more effective in eliminating TCS than laccase alone or the laccase-HBT system. Thus, we applied three enzymatic treatments, MnP, laccase and the laccase-HBT system, to the treatment of TCS and compared the rates of TCS elimination. Furthermore, we examined the removal of TCS toxicity towards two bacterial strains and a freshwater green alga following treatment with MnP.

2. Materials and methods

2.1. Enzyme assay and preparation

Activities of MnP and laccase were determined by monitoring the oxidation of 2,6-dimethoxyphenol (DMP), as described previously [16]. Enzyme activity (katal; kat) was calculated using extinction coefficient of 49.3 mM^{-1} cm^{-1} (at 470 nm) for DMP. *Phanerochaete chrysosporium* and *Trametes versicolor*, which are the best known lignin-degrading white rot fungi, were used for production of ligninolytic enzymes. Partially purified MnP was prepared from cultures of *P. chrysosporium* ME-446 [8], and laccase was prepared from cultures of *T. versicolor* IFO-6482 [24], as described previously.

2.2. Treatment of TCS with ligninolytic enzymes

The reaction mixture consisted of 10^{-4} M (28.95 mg L^{-1}) TCS (Wako, Osaka, Japan), partially purified MnP or laccase (0.5 and 2.0 nkat mL^{-1}) and 50 mM malonate buffer (pH 4.5). For MnP treatment, $MnSO_4$ (0.1 mM) and glucose (25 mM) plus glucose oxidase (3.33 nkat mL^{-1}; Wako) for H_2O_2 supply were added to the reaction mixture. For the laccase-mediator system, 0.2 mM HBT was added to the reaction mixture for laccase treatment. Reactions were performed at 30°C with stirring at 150 rpm. Each reaction mixture (500 μL), before and after enzymatic treatment, was mixed with methanol (1000 μL) containing 0.25 M phosphate to stop the enzymatic reaction, and was kept at -20°C for the high-performance liquid chromatography (HPLC) analysis and growth inhibition test using two bacterial strains and a freshwater green alga.

2.3. TCS analysis by HPLC

Residual TCS concentrations in the enzymatic reaction mixtures were determined by HPLC. HPLC analytical conditions were as follows: Wakosil-II 5C18HG column (250 mm × 4.6 mm i.d.; Wako, Osaka, Japan); mobile phase of 20 mM phosphate (X) and methanol (Y); isocratic elution with 75% Y; flow rate of 0.8 mL min^{-1}; and detection at 230 nm.

2.4. Bacterial growth inhibition of TCS treated with MnP

Antibacterial activity of TCS before and after MnP treatment was evaluated by bacterial

growth inhibition test using *Escherichia coli* (NBRC 14249) and *Bacillus subtilis* (NBRC 3134). *E. coli* and *B. subtilis* were precultured at 37 and 30°C, respectively, with shaking (150 rpm) for 24 h in nutrient broth (NB) medium (KYOKUTO, Tokyo, Japan). The culture was diluted with NB medium to give an optical density at 620 nm (OD_{620}) of 0.001. This bacterial suspension (100 μL) was added to a 50-mL Erlenmeyer flask containing NB medium (9.86 mL) and test sample (40 μL). A total of three test flasks were used for controls and each test solution. After 24 h of incubation, bacterial growth inhibition (%) was calculated based on the OD_{620} of controls versus that of each test solution.

2.5. Algal growth inhibition of TCS treated with MnP

The algal growth inhibition test using the freshwater green alga *P. subcapitata* (NIES-35) was performed according to standard ISO methods [25]. A typical test solution (5 mL) containing test sample (75 μL) and algae in culture medium (4.925 mL) was prepared in a 10-mL test tube capped with a thin silicone stopper. Algal cells of *P. subcapitata* were counted by direct microscopic procedure using the Petroff-Hausser counting chamber, and the initial number of algal cells was adjusted to 10^4 cells mL^{-1}. A total of three test tubes were used for controls and each test solution, and the test tubes were then kept at 25°C in an incubator under continuous illumination. After 72 h of incubation, algal growth inhibition (%) was calculated based on the number of cells in controls versus that in each test solution.

3. Results and discussion

3.1. Elimination of TCS by treatment with ligninolytic enzymes

Cabana et al. reported that TCS decreased by less than 20% after 1 h of treatment with laccase and by 65% after either 4 or 8 h of treatment, and that the addition of a redox mediator HBT does not significantly increase the elimination of TCS when compared to laccase in the absence of HBT [19]. More recently, Murugesan et al. reported that the addition of HBT enhanced the elimination of TCS, and that the molar ration between mediator (HBT) and substrate (TCS) is an important factor in the effective elimination of TCS [20].

In the present study, we also confirmed that laccase removed TCS from the reaction mixture and that about 10 and 51% elimination of TCS was achieved after 90 min of treatment with 0.5 and 2.0 nkat mL^{-1} laccase, respectively (Table 1). The laccase-HBT system enhanced the elimination of TCS to some extent when compared to laccase alone; in the presence of 0.2 mM HBT, TCS concentration decreased by about 30% with 0.5 nkat mL^{-1} laccase and by about 66% with 2.0 nkat mL^{-1} laccase after 90 min of treatment, respectively (Table 1). On the other hand, 0.5 nkat mL^{-1} MnP helped eliminate 94% of TCS after 30 min and TCS completely disappeared after 90 min, and no significant differences were seen in TCS elimination between 0.5 and 2.0 nkat mL^{-1} MnP (Table 1). These results indicate that the laccase and the laccase-HBT system are less effective at eliminating TCS when compared to MnP, and that MnP is most effective in eliminating TCS among the three enzymatic treatments.

In a previous paper, we demonstrated that oxidation of bisphenol A (BPA) by MnP resulted in oligomeric reaction products through the formation of phenoxy radicals of BPA followed by radical coupling [8]. Moreover, Cabana et al. reported that elimination of TCS, BPA and nonylphenol by laccase is due to oligomerization brought about by laccase oxidation [19, 26]. Thus, the oligomerization mechanism of TCS by MnP oxidation may also occur, resulting in TCS elimination. More recently, Murugesan et al. demonstrated the involvement of two mechanisms of lacacse-mediated TCS elimination: (i) oligomerization in the absence of HBT, and (ii) ether bond cleavage followed by dechlorination and oligomerization in the presence of HBT [20]. Further investigation is needed to verify whether or not the oligomerization of TCS by MnP may proceed via ether bond cleavage and partial dechlorination of TCS.

3.2. Removal of TCS toxicity by treatment with MnP

The greatest concern with regard to the biodegradation of recalcitrant and toxic pollutants in the environment should be on the removal of their toxicities. TCS is widely used as broad-spectrum antimicrobial agent and is suspected to have estrogenic activity. It has been reported that the putative estrogenic activity of TCS could not be determined using the recombinant yeast estrogenic screen assay due to the antimicrobial action of TCS [19]. Thus, in order to confirm the detoxification of TCS by treatment with MnP, the antibacterial activity of TCS before and after treatment was evaluated by bacterial growth inhibition test using

gram-negative *E. coli* and gram-positive *B. subtilis*. The 24-h EC_{50} values (50% effective concentration) of TCS for *E. coli* and *B. subtilis* were 19 μg L^{-1} (6.6×10^{-8} M) and 15 μg L^{-1} (5.2×10^{-8} M), respectively. As shown in Fig. 1, growth of *E. coli* and *B. subtilis* was completely inhibited by the initial concentrations of TCS (38 μg L^{-1}, 1.3×10^{-7} M at time zero) in the assay system, and growth inhibition towards the two bacterial strains was completely lost after 30 min of treatment with 0.5 nkat mL^{-1} MnP.

It has been demonstrated that green algae are more sensitive to TCS toxicity than other aquatic organisms, such as the marine bacteria, crustaceans and fish [7]. Thus, removal of growth inhibition towards the freshwater green alga *P. subcapitata* was also evaluated. In the present study, it was confirmed that the 72-h EC_{50} value of TCS for *P. subcapitata* was 32 μg L^{-1} (1.1×10^{-7} M), although some studies have reported a value of 4.5 μg L^{-1} (1.5×10^{-8} M) [27] or 4.7 μg L^{-1} (1.6×10^{-8} M) [7] at 72 h. The reduction in algal growth inhibition observed during treatment with 0.5 nkat mL^{-1} MnP is shown in Fig. 2. The initial concentration of TCS (145 μg L^{-1}, 5.0×10^{-7} M at time zero) in the assay system showed 83% algal growth inhibition, and treatment with 0.5 nkat mL^{-1} MnP reduced this by about 75 and 90% after 30 and 60 min, respectively.

4. Conclusions

In the present study, we demonstrated that MnP is more effective at eliminating TCS than

laccase and the laccase-HBT system, and the ecotoxicity of TCS towards bacterial and algal growth decreased with time after the start of MnP treatment. To our knowledge, there is no data on the enzymatic removal of TCS toxicity; thus, the present report is the first to showing that the ligninolytic enzyme MnP from white rot fungus is able to effectively remove the ecotoxicity of TCS.

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Figure Legends

Fig. 1. Decrease in bacterial growth inhibition of TCS towards *E. coli* and *B. subtilis* by treatment with 0.5 nkat mL^{-1} MnP. Indicated for each point are the mean and standard deviation of triplicate experiments. (□), *E. coli*; (●), *B. subtilis*.

Fig. 2. Decrease in algal growth inhibition of TCS towards *P. subcapitata* by treatment with 0.5 nkat mL^{-1} MnP. Indicated for each point are the mean and standard deviation of triplicate experiments.

Table 1

Decrease in TCS concentration by enzymatic treatment with MnP, laccase or laccase-HBT system.

Enzymatic treatment	Treatment time (min)	TCS ($mg\ L^{-1}$)	Residual TCS (%)
0.5 nkat/mL Laccase	0	28.95 ± 0.96	100.0 ± 3.3
	30	27.42 ± 0.98	94.7 ± 3.4
	60	27.42 ± 1.19	94.7 ± 4.1
	90	26.00 ± 0.38	89.8 ± 1.3
0.5 nkat/mL Laccase and 0.2 mM HBT	0	28.95 ± 0.35	100.0 ± 1.2
	30	20.96 ± 0.78	72.4 ± 2.7
	60	20.59 ± 0.20	71.1 ± 0.7
	90	20.35 ± 0.46	70.3 ± 1.6
0.5 nkat/mL MnP	0	28.95 ± 1.16	100.0 ± 4.0
	30	1.73 ± 0.26	6.0 ± 0.9
	60	0.78 ± 0.17	2.7 ± 0.6
	90	0.18 ± 0.03	0.6 ± 0.1
2.0 nkat/mL Laccase	0	28.95 ± 0.90	100.0 ± 3.1
	30	19.34 ± 0.78	66.8 ± 2.7
	60	17.46 ± 0.61	60.3 ± 2.1
	90	14.10 ± 1.45	48.7 ± 5.0
2.0 nkat/mL Laccase and 0.2 mM HBT	0	28.95 ± 0.75	100.0 ± 2.6
	30	17.78 ± 0.84	61.4 ± 2.9
	60	13.23 ± 1.04	45.7 ± 3.6
	90	9.81 ± 1.16	33.9 ± 4.0
2.0 nkat/mL MnP	0	28.95 ± 1.30	100.0 ± 4.5
	30	1.12 ± 0.26	3.9 ± 0.9
	60	0.25 ± 0.17	0.9 ± 0.6
	90	0	0

Data are the mean ± standard deviation of five experiments.

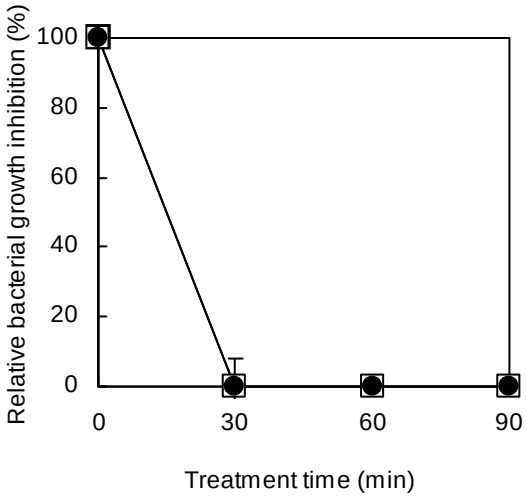

Fig. 1. Decrease in bacterial growth inhibition of TCS towards *E. coli* and *B. subtilis* by treatment with 0.5 nkat mL^{-1} MnP. Indicated for each point are the mean and standard deviation of triplicate experiments. (□), *E. coli*; (●), *B. subtilis*.

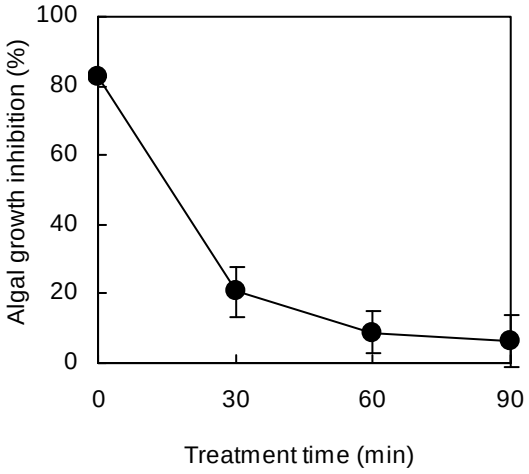

Fig. 2. Decrease in algal growth inhibition of TCS towards *P. subcapitata* by treatment with 0.5 nkat mL^{-1} MnP. Indicated for each point are the mean and standard deviation of triplicate experiments.