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Treatment of tetracycline antibiotics by laccase in the presence of 1-hydroxybenzotriazole

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ABSTRACT

Tetracycline antibiotics are widely used in human and veterinary medicine; however, residual amounts of these antibiotics in the environment are of concern since they could contribute to selection of resistant bacteria. In this study, tetracycline (TC), chlortetracycline (CTC), doxycycline (DC) and oxytetracycline (OTC) were treated with laccase from the white rot fungus *Trametes versicolor* in the presence of the redox mediator 1-hydroxybenzotriazole (HBT). High performance liquid chromatography demonstrated that DC and CTC were completely eliminated after 15 min, while TC and CTC were eliminated after 1 h. This system also resulted in a complete loss of inhibition of growth of *Escherichia coli* and *Bacillus subtilis* and the green alga *Pseudokirchneriella subcapitata* with decreasing tetracycline antibiotic concentration. These results suggest that the laccase-HBT system is effective in eliminating tetracycline antibiotics and removing their ecotoxicity.

Keywords:

Tetracycline antibiotics; Bacterial growth inhibition; Algal growth inhibition; Laccase; 1-Hydroxybenzotriazole

1. Introduction

Tetracycline antibiotics, such as tetracycline (TC), chlortetracycline (CTC), doxycycline (DC) and oxytetracycline (OTC), are broad-spectrum antimicrobial agents that are widely used in human therapy, animal husbandry and aquaculture. Some studies have demonstrated that tetracycline antibiotics are toxic to microalgae and aerobic sludge bacteria (Halling-Sørensen, 2000; Halling-Sørensen et al., 2002), and furthermore, residual antibiotics in the environment can select for resistant bacteria. Several studies have reported the widespread presence of tetracycline antibiotics (Kolpin et al., 2002; Karthikeyan and Meyer, 2006; Gulkowska et al., 2007). For example, it has been demonstrated that TC is present at concentrations ranging from 50 to 850 ng L^{-1} in 80% in the effluents of seven sewage treatment plants in Wisconsin (Karthikeyan and Meyer, 2006).

Recent studies have confirmed the ability of ligninolytic enzymes (manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase) from lignin-degrading white rot fungi to eliminate endocrine disruptors (Tsutsumi et al., 2001; Suzuki et al., 2003; Tamagawa et al., 2005, 2007; Sei et al., 2008), pharmaceuticals and personal care products (Hata et al., 2010; Inoue et al., 2010; Marco-Urrea et al., 2010; Murugesan et al., 2010; Zhang et al., 2010). Wen et al. (2010) reported that MnP is effective in eliminating TC and OTC, although removal of the toxicity of these antibiotics by treatment with MnP was not examined. It is known that MnP and laccase are able to oxidize various phenolic compounds and the substrate range of laccase is extended in the presence of redox mediators such as 1-hydroxybenzotriazole (HBT).

Thus, we applied the laccase-HBT system, laccase and MnP to the treatment of TC, CTC, DC and OTC, and compared the rates of elimination of the antibiotics. Treated samples were tested for growth inhibition of *Escherichia coli*, *Bacillus subtilis*, and the alga *Pseudokirchneriella subcapitata*.

2. Materials and methods

2.1. Enzyme assay and preparation

Laccase and MnP activities were determined by monitoring the oxidation of 2,6-dimethoxyphenol, as reported previously (Mizuno et al., 2009). Partially purified MnP and laccase were prepared from cultures of *Phanerochaete chrysosporium* (ME-446) and *Trametes versicolor* (IFO-6482), respectively, as described previously (Fujisawa et al., 2001; Tsutsumi et al., 2001).

2.2. Treatment of tetracycline antibiotics with ligninolytic enzymes

TC hydrochloride, CTC hydrochloride and OTC hydrochloride were obtained from Wako (Osaka, Japan) and DC hydrochloride from MP Biomedicals LLC (Santa Ana, CA). For treatment with laccase, the reaction mixture consisted of 10^{-4} M test compound, partially purified laccase (10 nkat mL^{-1}) and 50 mM malonate buffer (pH 4.5). For the laccase-HBT system, 0.2 mM HBT was added to the reaction mixture for laccase treatment. For MnP treatment, the reaction mixture consisted of 10^{-4} M test compound, partially purified MnP (10

nkat mL^{-1}), 50 mM malonate buffer (pH 4.5), $MnSO_4$ (0.1 mM) and glucose (25 mM) plus glucose oxidase (3.33 nkat mL^{-1}; Wako, Japan) for H_2O_2 supply. The reaction was performed at 30°C with stirring at 150 rpm. Each reaction mixture (0.5 mL), before and after enzymatic treatment, was mixed with methanol (1.0 mL) containing 0.25 M phosphate in order to stop the enzymatic reaction, and was kept at -20°C until high-performance liquid chromatography (HPLC) analyses and growth inhibition tests using two bacterial strains and freshwater green alga.

2.3. Analyses of tetracycline antibiotics by HPLC

Residual TC, CTC, DC and OTC concentrations in the enzymatic reaction mixtures were determined by HPLC. HPLC analytical conditions were as follows: Cadenza 5CD-C18 column (75 mm × 4.6 mm i.d.; 5 μm; Imtakt, Kyoto, Japan); mobile phase of 0.1% trifluoroacetic acid (X) and methanol (Y); isocratic elution with 20% Y for TC and OTC, 30% Y for CTC and 40% Y for DC; flow rate of 1.0 mL min^{-1}; and detection at 254 nm.

2.4. Test of growth inhibition of *E. coli*, *B. subtilis* and *P. subcapitata*

Growth inhibition tests using gram-negative *E. coli* (NBRC 14249) and gram-positive *B. subtilis* (NBRC 3134) were performed as described previously (Inoue et al., 2010). *E. coli* and *B. subtilis* were cultured at 37 and 30°C, respectively, with shaking (150 rpm) for 24 h in nutrient broth (NB) (KYOKUTO, Tokyo, Japan). The culture was diluted with NB 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 (9.86 mL) and 40 μL of the test sample. 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, and the 24-h EC_{50} values (50% effective concentration) of TC, CTC, DC or OTC against two bacterial strains were determined.

The bacterial growth inhibition of TC, CTC, DC and OTC before and after treatment with the laccase-HBT system was also evaluated. Each test solution consisted of test sample (reaction mixture before and after enzymatic treatment/methanol containing 0.25 M phosphate = 1/2) of 120 μL for *E. coli* assay and 50 μL for *B. subtilis* assay in 10 ml. Consequently, the initial concentration of each tetracycline antibiotic before enzymatic treatment (at time zero) corresponded to 0.40 and 0.17 μM for the test using *E. coli* and *B. subtilis*, respectively, which is higher than each EC_{50} value as shown in Table 1.

The growth inhibition test using the freshwater green alga *P. subcapitata* (NIES-35) was performed according to the standard ISO method (1989) as described previously (Inoue et al., 2010). 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. *P. subcapitata* cells were counted microscopically using a 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. Then, the 72-h EC_{50} values of TC, CTC, DC or OTC against *P. subcapitata* were determined.

The algal growth inhibition of TC, CTC, DC or OTC before and after treatment with the laccase-HBT system was also evaluated. Each test solution (5 mL) consisted of test sample (reaction mixture before and after enzymatic treatment/methanol containing 0.25 M phosphate = 1/2) of 190 μL and algal medium of 4.81 ml. Consequently, the initial concentration of each tetracycline antibiotic before enzymatic treatment corresponded to 1.3 μM in the assay system, which is higher than each EC_{50} value as shown in Table 1.

3. Results and discussion

3.1. Elimination of tetracycline antibiotics by treatment with ligninolytic enzymes

The decrease in the four tetracycline antibiotics observed during enzymatic treatment with MnP, laccase and the laccase-HBT system is shown in Fig. 1. Wen et al. (2010) reported that TC and OTC decreased by 72.5 and 84.3%, respectively, after a 4-h treatment with MnP. In the present study, TC and OTC decreased by approximately 80 and 93%, respectively, after 1 h of treatment with MnP (Fig. 1). DC and CTC completely disappeared after 1 h of treatment with MnP (Fig. 1), but laccase had a reduced ability to eliminate four tetracycline antibiotics when compared with MnP; TC, CTC, DC and OTC decreased by approximately 16, 48, 34 and 14%, respectively, after 4 h of treatment with laccase alone

(Fig. 1). These results suggest that elimination of the four tetracycline antibiotics by laccase itself is possible, but is not sufficiently effective for application to their bioremediation.

The laccase-HBT system facilitated the elimination of four tetracycline antibiotics when compared to laccase alone; CTC and DC were completely eliminated after 0.25 h, while TC and OTC were eliminated after 1 h (Fig. 1). Furthermore, the elimination of these tetracycline antibiotics with the laccase-HBT system progressed at a faster rate than with MnP (Fig. 1). These results indicate that the laccase-HBT system is more effective in eliminating tetracycline antibiotics than MnP.

3.2. Decrease in growth inhibition of tetracycline antibiotics after treatment with the laccase-HBT system

It has been reported that the 72-h EC_{50} values for TC against cyanobacterium *Microcystis aeruginosa* and green alga *P. subcapitata* (formerly *Selenastrum capricornutum*) were 0.19 and 4.6 μM, respectively, and the values for CTC against *M. aeruginosa* and *P. subcapitata* were 0.10 and 6.0 μM, respectively (Halling-Sørensen, 2000). Moreover, the 48-h EC_{50} values for TC, CTC and OTC against aerobic sludge bacteria were 0.17, 0.06 and 0.16 μM, respectively (Halling-Sørensen et al., 2002). The present study showed that the 72-h EC_{50} values for TC, CTC, DC and OTC against *P. subcapitata* were 0.47, 0.84, 0.14 and 1.2 μM, respectively (Table 1).

As shown in Table 2, growth inhibition of the test bacteria by CTC and DC was

completely lost after 0.25 h of treatment with the laccase-HBT system, and this system completely removed the growth inhibition of TC and OTC after 1 h. Furthermore, the laccase-HBT system caused the complete loss of algal growth inhibition by CTC and DC after 0.25 h and by TC and OTC after 1 h (Table 2).

Laccase catalyzes one-electron oxidation of phenolic compounds by reducing molecular oxygen to water. The elimination of phenolic pollutants, such as bisphenol A (BPA), nonylphenol (NP) and triclosan (TCS), by laccase is due to oligomerization brought about by laccase oxidation (Cabana et al., 2007), and the involvement of oligomerization in the absence of HBT, and ether bond cleavage followed by dechlorination and oligomerization in the presence of HBT have been shown to be mechanisms of laccase-mediated TCS elimination (Murugesan et al., 2010). Thus, elimination of tetracycline antibiotics by the laccase-HBT system may be mainly due to oligomerization of oxidized tetracycline antibiotics via radical-radical coupling, as tetracycline antibiotics have similar phenolic hydroxyl group to BPA, NP and TCS. Further investigation is needed to verify this mechanism.

4. Conclusions

The present study demonstrated that tetracycline antibiotics (TC, CTC, DC and OTC) are effectively eliminated by treatment with the ligninolytic enzyme laccase in the presence of the redox mediator HBT. Furthermore, it was confirmed that the laccase-HBT system removed the growth inhibition of tetracycline antibiotics towards test bacteria and the alga *P.*

subcapitata. Future studies need to be directed towards establishing the nature and toxicity levels of the enzyme-generated degradation products, and towards finding replacements for the costly and potentially toxic HBT redox mediator.

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Figure Legend

Fig. 1. Decrease in concentrations of TC (A), CTC (B), DC (C) and OTC (D) by enzymatic treatment with MnP, laccase or laccase-HBT system. Indicated for each point are the mean and standard deviation of five experiments. (▲), MnP; (♦), laccase; (●), laccase-HBT system.

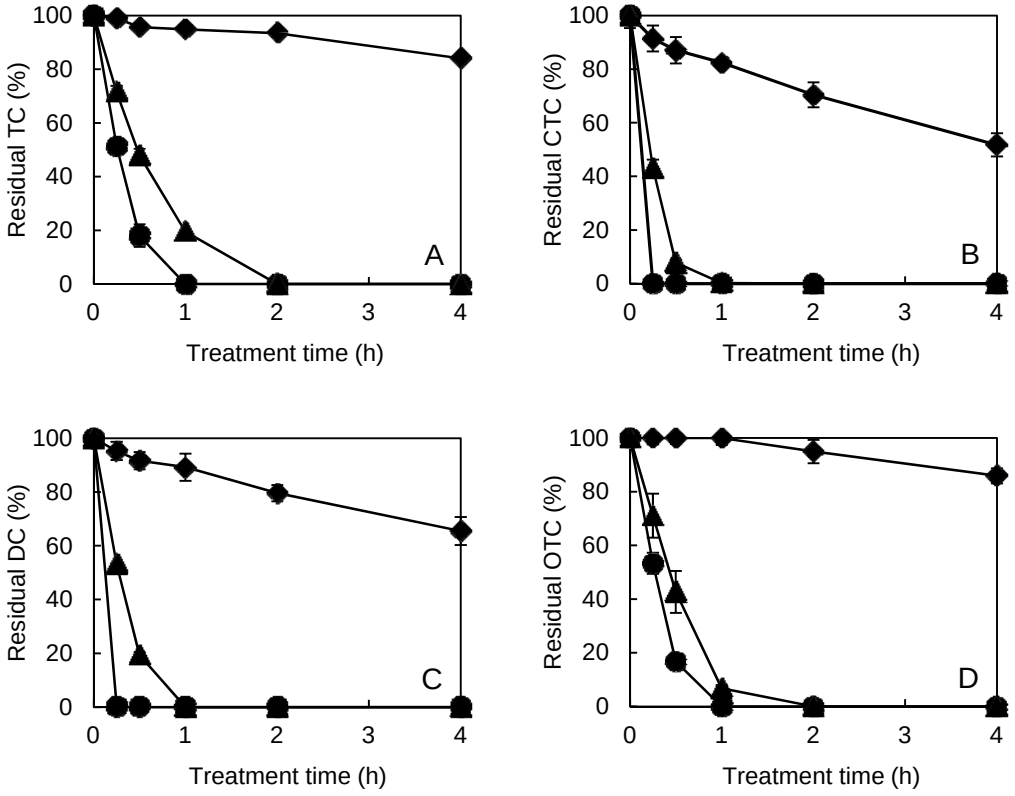
Table 1
EC_{50} values of tetracycline antibiotics for algae and bacteria.

	EC_{50} (μM)				Ref.
	TC [a, b]	CTC [c]	DC [d]	OTC [e]	
P. subcapitata	0.47 [a]	0.84	0.14	1.2	
E. coli	0.30 [a]	0.33	0.13	0.31	
B. subtilis	0.08 [a]	0.03	0.02	0.13	
M. aeruginosa	0.19 [b]	0.10			Halling-Sørensen (2000)
P. subcapitata	4.6 [b]	6.0			Halling-Sørensen (2000)
Aerobic sludge bacteria	0.17 [b]	0.06		0.16	Halling-Sørensen etal.(2000)

[a] TC hydrochloride
[b] TC
[c] CTC hydrochloride
[d] DC hydrochloride
[e] OTC hydrochloride

Table 2
Decrease in algal and bacterial growth inhibition of tetracycline antibiotics by treatment with the laccase-HBT system.

	Treatment Time (h)	Growth inhibition (%)		
		P. subcapitata	*E. coli*	*B. subtilis*
TC	0	87.1 ± 3.7	94.5 ± 0.9	100.0 ± 0.0
	0.25	55.6 ± 9.4	35.6 ± 4.3	44.1 ± 7.2
	0.5	6.5 ± 7.1	7.1 ± 1.1	6.8 ± 5.5
	1.0	0	0	0
	2.0	0	0	0
	4.0	0	0	0
CTC	0	80.1 ± 8.7	100.0 ± 0.0	100.0 ± 0.0
	0.25	0	0	0
	0.5	0	0	0
	1.0	0	0	0
	2.0	0	0	0
	4.0	0	0	0
DC	0	93.7 ± 5.8	100.0 ± 0.0	100.0 ± 0.0
	0.25	0	0	0
	0.5	0	0	0
	1.0	0	0	0
	2.0	0	0	0
	4.0	0	0	0
OTC	0	73.0 ± 6.7	89.5 ± 2.8	100.0 ± 0.0
	0.25	40.8 ± 3.4	12.4 ± 1.6	20.0 ± 4.8
	0.5	12.4 ± 4.5	6.2 ± 1.1	11.0 ± 4.7
	1.0	0	0	0
	2.0	0	0	0
	4.0	0	0	0



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