TRANSFORMATION OF TRICLOSAN BY LACCASE FROM THE WHITE ROT FUNGUS *GANODERMA LUCIDUM*

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Abstract

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) (TCS) is used as an antimicrobial agent in wide range of health care products. This compound is widely found in various environmental matrices and is a precursor for unwanted chlorinated organic pollutants. In this study, a thermostable laccase from the white rot fungus *Ganoderma lucidum* was evaluated for its capability to transform triclosan. Purified laccase of this fungus was able to transform TCS. A maximum of 79.2 % TCS was transformed during 12 h incubation by laccase (5 U ml^{-1}). Addition of redox mediator 1-hydroxybenzotriaozle (HBT) enhanced the laccase-mediated TCS transformation. Apart from syringaldehyde all the lignin related phenolic compounds drastically decreased the TCS transformation. HPLC analysis showed that TCS was transformed into more non-polar product by laccase in the absence of redox mediator while more polar products were formed in the presence of redox mediator. GC-MS analysis revealed that laccase plus HBT system degraded the ether bond of TCS and produced 2,4-dichlorophenol. Our study shows that *G. lucidum* laccase was able to destruct the TCS structure and hence could be useful to eliminate TCS from wastewater. This is the first study on degradation of TCS into 2,4-dichlorophenol by laccase-mediated system.

Introduction

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) (TCS) is used as an antimicrobial agent in wide range of health care products and consumer products. Due to general use of heath care products TCS entered into wastewater and TCS has been detected in various environmental matrices such as wastewaters, freshwater, seawater and sediments at various concentrations[1]. Because of toxicity, hydrophobicity nature, and precursor for unwanted chemicals such as chlorinated organic pollutants[2] TCS removal has been received more attention. TCS was shown to inhibit the bacterial fatty acid biosynthetic enzyme, enoyl-[acyl-carrier protein] reductase, in Gram-negative and Gram-positive bacteria, as well as in the Mycobacteria[3]. Due to its inhibitory activity to wide range of bacteria, bacterial degradation of TCS is very scarce. However, few bacteria were able to degrade it[4]. TCS transformation has been reported in white rot fungi *Trametes versicolor* and *Pycnoporus cinnabarinus*[5]. These fungi lowered the cytotoxic and microbicidal activity of TCS by converting it to methylated form. Laccases from the white rot fungi have been known for its detoxification to wide range of chlorinated compounds. Recent studies reported that laccases from *T. versicolor* and *Coriolopsis polyzona* were able to oxidize and detoxify TCS[6,7]. It has been previously observed that polymerization was the mechanism of laccase-mediated TCS removal[7]. The aim of this study was to find out any other degradative mechanisms in laccase-mediated TCS transformation in the presence of redox mediators. For this purpose we evaluated the capability of a thermostable enzyme isolated from the white rot fungus *Ganoderma lucidum*[8] and, different synthetic and natural redox mediators.

Materials and Methods

Triclosan (TCS), redox mediators (as shown in Figure 3) and other chemicals were obtained from Sigma-Aldrich. Laccase preparation from *G. lucidum* KMK2 was carried out by solid state fermentation (SSF) as described earlier[8]. The crude laccase was purified through ammonium sulphate precipitation, Ion-exchange and gel filtration chromatography using FPLC system (BIO-RAD BIOLOGIC). The purified laccase was filter sterilized and stored in refrigerator for further use. Laccase activity was estimated as described in previous report[8] using ABTS as a substrate. TCS stock (0.1 M) was prepared in acetonitrile. Biotransformation of TCS was carried out in glass in vials (2 mL) using citrate-phosphate buffer (50 mM; pH 4.0) and purified laccase. For the effect of TCS concentration, various amounts of TCS from 0.1 to 0.5 mM was tested with 5 U ml^{-1} laccase. For the optimum enzyme concentration, different amount of laccase (1, 2, 5 10 and 15 U ml^{-1}) were tested. The reaction mixture (0.5 or 1.0 mL) was incubated at 30°C under dark. The enzymatic reaction was stopped by decreasing the pH of the reaction mixture using acetic acid. Then 0.5

volume of acetonitrile was added into the reaction mixture and vortexed well. The samples were filtered through 0.45 µM filter and analyzed through RP-HPLC (1100 Series, Agilent, Germany) with ZORBOX SB-C_{18} column using 70% acetonitrile as eluent at a constant flow rate of 1 ml min^{-1}. TCS was monitored at 277 nm by DAD-UV detector. The effect of different synthetic redox mediator compounds, and natural phenolic and non-phenolic compounds (see Fig. 3) were screened in order to check their capabilities to enhance the rate of TCS transformation. To identify the product of TCS transformation and its reaction mechanism, the acidified reaction mixture was extracted thrice with equal volume of ethyl acetate. Then the extract was dehydrated with anhydrous sodium sulphate, dried under N_2 gas and dissolved in acetone. This extract was analyzed through GC-MS using 60 m DB-5 column (Trace GC system – ThermoQuest, Jan Jose, CA and Finnigan Polaris Q, Thermoquest).

Results and discussion

The laccase isolated from *G. lucidum* by SSF was a thermostable enzyme that effectively degraded the synthetic dyes[8]. In this study, our aim was to evaluate *G. lucidum* laccase for its capability to transform TCS in aqueous solution. In our preliminary assay, we observed the tolerance ability of our laccase against TCS at micromolar concentrations. Then we tested different concentration of TCS from 0.1 mM to 0.5 mM. As shown in Fig. 1., at 1 mM concentration 79.2% of TCS was transformed by laccase alone during 12 h incubation. The removal efficiency was slightly decreased when increasing the TCS concentration however, it was significantly decreased at higher concentration of TCS (0.4-0.5 mM). Addition of redox mediator, HBT, enhanced the transformation extent of TCS. In the presence of HBT, maximum of 87.6% TCS was transformed at 0.1 mM concentration. However, the transformation level was not significantly varied up to 0.4 mM TCS concentration. This suggests that at this level the enzymatic activity might have retarded by reaction products. Our results revealed that *G. lucidum* laccase alone effectively removed TCS at lower concentration. For the effect of enzyme concentration, we tested the enzyme from 1-15 U ml^{-1}. As can be seen in Fig 2, the TCS transformation was doubled when increasing the enzyme from 1 to 2 U ml^{-1}. There was no significant increase when adding laccase above 5 U ml^{-1}; suggesting the less amount of enzyme is sufficient to achieve maximum conversion of TCS.

Fig. 3 shows the effect of different synthetic redox mediators and various phenolic compounds on transformation of TCS. Among the synthetic mediators, HBT was found to be effective. Whereas ABTS was more effective for other laccases[6,7]. Among the phenolic compounds tested, 4-hydroxy-3,5-methoxy benzaldehyde (Syringaldehyde) was found to be very effective to enhance the TCS transformation and even more efficient than that of synthetic mediators. Whereas TCS transformation was drastically decreased in the presence of other phenolic compounds (Fig. 3). This might be due to substrate competition between phenolic compounds and TCS because all these phenolic compounds are preferred substrate for laccase. The HPLC analysis of TCS transformation by laccase in the presence or absence of redox mediators showed different profiles. As shown in Fig. 4a, the retention time of TCS was 3.05 min. In the presence of laccase, two new peaks were appeared. Of which one was non-polar compound than TCS (Fig 4b). This could be a dimer product of the triclosan. The other product was a polar compound that could possibly a breakdown product of TCS. On the other hand, in the presence of redox mediators TCS transformation resulted in formation of more polar compounds. In the presence of HBT, accumulation of a new product was observed during TCS transformation (Fig. 4c). Similar result was also found with ABTS (Fig 4d). GC-MS analysis of TCS transformation also revealed the formation of new product from TCS in the presence of HBT (Fig 5 a). GC peak eluted at 22.86 min was identified as TCS by comparison with standard TCS and control sample. The large peak detected at 17.47 was identified as HBT. The peak eluted at 14.74 min was identified as 2,4-dichlorophenol (Fig. 5c). This result indicates that the ether bond of the TCS was degraded to 2,4-dichlorophenol and other products. A previous study on TCS transformation reported that TCS was polymerized by laccase[7]. Our study is the first report on degradation of TCS into 2,4-dichlorophenol by laccase-mediated system.

Acknowledgments

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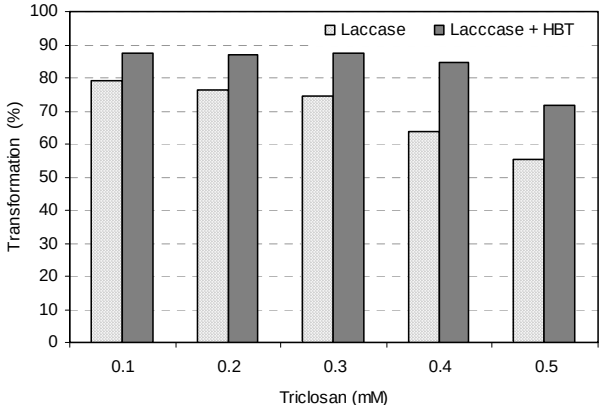


Figure 1. Effect of different initial concentration of triclosan on its transformation by *G. lucidum* laccase (5 U ml^{-1}). Samples were incubated for 12 h in citrate- phosphate buffer (50 mM, pH 4.0)

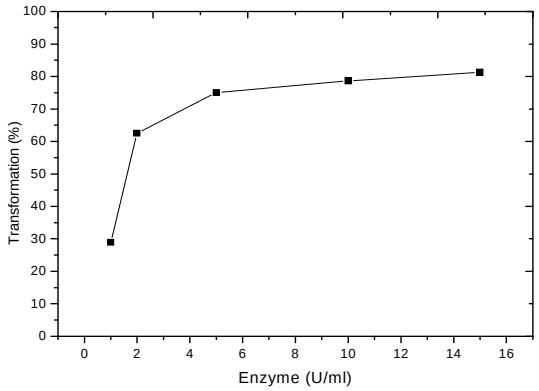


Figure 2. Effect of different amount of laccase on triclosan (0.2 mM) transformation Samples were incubated for 12 h.

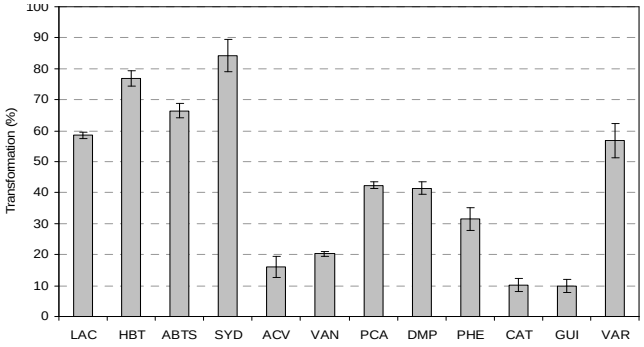


Figure 3. Effect of different redox mediators and phenolic compounds on triclosan (0.2 mM) transformation using 3 U ml^{-1} laccase. Samples were incubated for 12 h. LAC- Laccase only; HBT – 1-hydroxybenzotriazole; ABTS- 2,2-azinobis-(3-ethylbenz-thiazo-line)-6-sulfonic acid); SYD – syringaldehyde; ACV - acetovanillone, VAN - vanillin, PCA - *p*-coumaric acid ; DMP - 2,6-dimethoxy phenol; GUI - guiacol, VA - veratryl alcohol, PHE - phenol and CAT - catechol

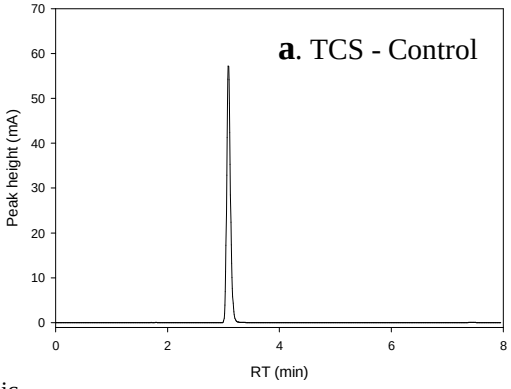
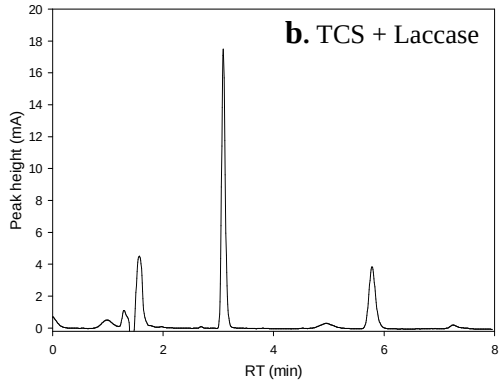
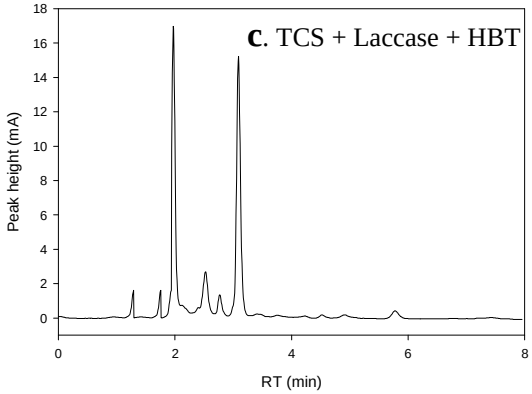
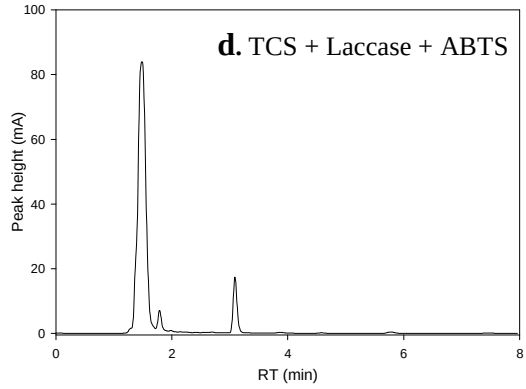
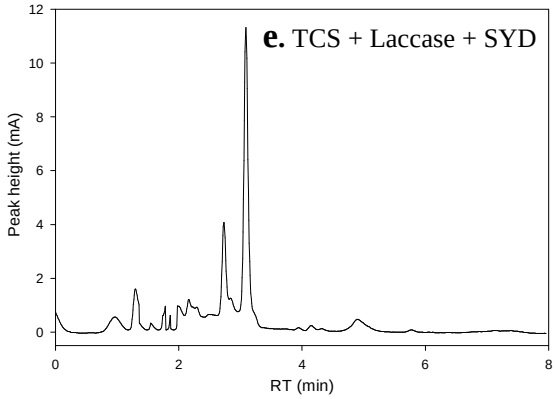


Figure.4a-e. HPLC profile of triclosan transformation by *G. lucidum* laccase with and without redox mediators.









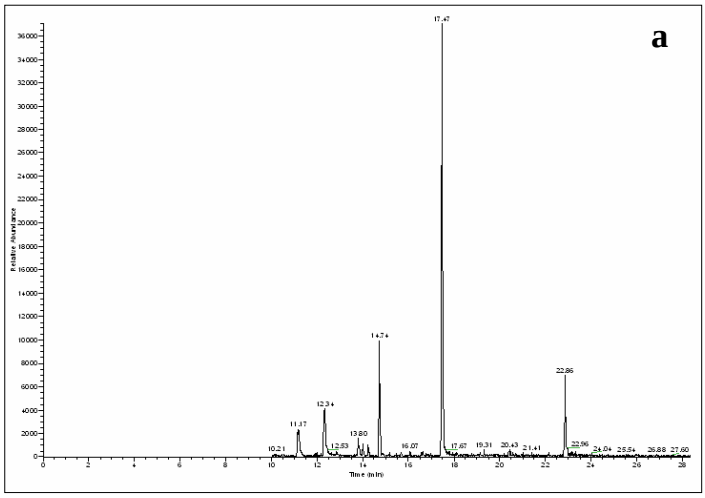


Figure 5a-c. GC-MS analysis of triclosan transformation by *G. lucidum* laccase with HBT as redox mediator.