B−20 Removal of pharmaceutically active compounds by crude laccase from *Trametes versicolor*-A white rot fungus

(白色腐朽菌 *Trametes versicolor* 由来のラッカーゼによる医薬品の除去)

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1. INTRODUCTION

Environmental pollution by organic micro-pollutants such as pharmaceutically active compounds (PhACs) has increasingly gained attention due to their potential to cause undesirable ecosystem and human health effects (1). This growing environmental problem requires the development of bioprocesses capable of effectively degrading these compounds. Among the bioprocesses developed, white rot fungal cultures and their ligninolytic enzymes have been successfully used for treatment of several persistent organic or environmentally toxic compounds. Thus, the objective of the study was to examine the degradability of the selected PhACs by the fungal culture *Trametes versicolor* and its crude laccase.

2. MATERIALS AND METHODS

(1) Target compounds

The target compounds used in this study were 10 selected PhACs, clofibric acid (CA), gemfibrozil (GFZ), ibuprofen (IBP), fenoprofen (FEP), ketoprofen (KEP), naproxen (NPX), diclofenac (DCF), indomethacin (IDM), propyphenazone (PPZ) and carbamazepine (CBZ).

(2) Fungal culture and preparation of crude laccase

Trametes versicolor (ATCC # 42530) was purchased from American Type Culture Collection (ATCC) and remained by sub-culturing on malt extract medium (20 g/l malt extract, 20 g/l glucose, 1 g/l peptone) with the addition of 2% agar, pH 4.5. For laccase production, *T. versicolor* was induced in basal liquid medium (BLM) consisting of (per liter): glucose 10 g, peptone 5 g, yeast extract 2 g, malt extract 5 g, ammonium tartrate 2g, KH_2PO_4 1g, $MgSO_4 \cdot 7H_2O$ 0.3 g, KCl 0.2 g, $CuSO_4 \cdot 5H_2O$ 0.12 g, and the addition of trace elements. The pH of the BLM was adjusted to 4.5 before autoclaving at 121 °C for 20 min. Laccase production was carried out in 300 ml-Erlenmeyer flask with air permeable stoppers (AS ONE, Japan) containing 100 ml BLM. Flasks were incubated at 30°C in shaken conditions (125 rpm).

(3) Degradation of PhACs by the whole fungal culture

To examine the degradation ability of selected PhACs by whole fungal culture (i.e. mycelium and crude enzymes), a series of batch experiments were conducted with 100 ml the 7-day-old liquid culture of *T. versicolor* and the mixed stock solution of PhACs to achieve an initial PhACs concentration of 10 µg/l. The incubation was continued at 30 °C in shaken condition (125 rpm) for 48 hours.

The effect of redox mediator on degradability of selected PhACs was estimated by adding 1mM ABTS (2, 2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid)), a typical laccase-mediator, into the 7 day-old fungal culture. To serve as abiotic control, a control test with the same amount of a heat-killed fungal culture was performed. All batch experiments were carried out in duplicate.

(4) Degradation of selected PhACs by crude laccase

To examine catalytic activities of culture filtrates obtained from the 7-day-old liquid fungal culture *T. versicolor* in the degradation of selected PhACs, a series of batch tests were performed. The reaction mixture consisted of culture filtrate with laccase activity of 1500 U/L, MnP activity of 30 U/l, and 10 µg/l selected PhACs to give a total volume reaction mixture of 10 ml. The enzymatic reaction was incubated at 30 °C in shaken conditions (125 rpm).

To distinguish the contributive role of MnP containing in culture filtrates in the degradation of selected PhACs, 0.1 mM $MnSO_4$ and 0.4 mM H_2O_2 were added into the reaction mixture. To serve as

abiotic controls, a control test containing the same amount of a heat-denaturated enzyme and conditions was performed. An additional control test without adding enzyme into reaction mixture was also conducted. All experiments were carried out in duplicate.

(5) Enzyme activity assays

Manganese peroxidase: MnP (E.C 1.11.1.13) activity was measured according to the method of Paszczynski et al. (2).

Laccase: Lac (E.C 1.10.3.2) activity was determined by using 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) as the substrate (3).

(6) Analyses

The concentrations of target pharmaceutical substances such as CA, GFZ, IBP, FEP, KEP, NPX, DCF, and IDM were measured by the method using GC/MS after solid phase extraction and pentafluorobenzyl derivatization suggested by Sacher et al.(3). PPZ and CBZ, which require no derivatization, were quantified in the same chromatogram.

3. RESULTS AND DISCUSSION

(1) Production of crude laccase

It has been known that *T. versicolor* is an excellent producer of industrially important laccase (4, 5). To have a sufficient amount of crude laccase for the further investigations in this study laccase was biosynthesized during fermentation in BLM (pH 4.5) under shaken condition at 30 °C. Figure 1 shows the profile of ligninolytic enzymes and fungal mass during fermentation process.

Experimental results showed that no LiP activity was detected in the cultures during the fermentation period. The activities of laccase appeared from the beginning of fermentation and reached a maximum on the 7 th day (1550 U/l), and then slightly decreased until the end of the fermentation process.

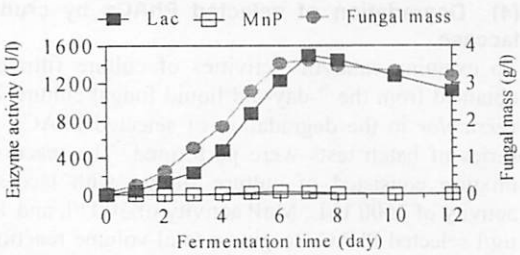

Fig.1 Production of laccase and MnP during agitated fermentation *T. versicolor*

MnP activity was found on the 4 th day and peaked on the 6 th day of fermentation process (30 U/l). It can be seen from Fig.1 that a significantly higher laccase activity was observed by *T. versicolor* compared with MnP in culture filtrate is likely to be due to the characteristics of producer, the compositions of medium and fermentation conditions (e.g. rich nitrogen nutrient, agitated fermentation condition), which are more favorable for laccase production than that for MnP and LiP. The results are consistent with those reported in the previous literatures (4, 6). The fermentation broth should be used as crude laccase preparation for the following experimental purposes.

(2) Degradation of PhACs by the whole fungal culture

The degradability of selected PhACs by whole fungal culture *T. versicolor* was evaluated by incubation of the 7-day-old liquid fungal culture *T. versicolor* with 10 μg/L selected PhACs. After 48 h of incubation with fungal culture, the removal of PhACs is shown in Fig. 2. From Fig. 2 it can be seen that all selected PhACs were removed by the whole fungal culture. Complete removal of IBP, NPX, DCF, IDM, KEP, and FEP and partial removal of other selected PhACs was observed in the cases of both presence and absence of mediator (ABTS).

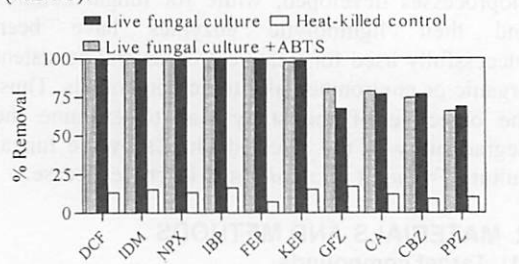

Fig. 2. The removal of PhACs by the whole fungal culture *T. versicolor*

The removal of selected PhACs by whole fungal culture may be caused by the presence of enzymes such as laccase, MnP, and cytochrome P450 oxygenase in the fungal culture. These enzymes are non-specific and use free radical mechanisms to catalyze a broad range of substrates as reported in the published literature (6).

Although the addition of laccase-mediator (ABTS) has been considered to promote degradability, the experimental results showed that a very small difference in the removal of PhACs was obtained in the presence and absence of ABTS for most selected PhACs. No difference in the removal of PhACs was observed in both cases with and without ABTS addition because during the incubation process *T. versicolor* not only produces ligninolytic enzymes such as laccase and MnP but also probably secretes low molecular weight compounds that can act as natural mediators in promoting the oxidation of

pharmaceuticals. Gutierrez et al.(7) and Pointing et al. (8) also reported that white rot fungi secrete low molecular weight mediators that enlarge the spectrum of compounds they are able to oxidize. Another possibility is that the elimination of pharmaceuticals was not only caused by the enzymatic transformations but also probably due to the accumulation, sorption and uptake of the pharmaceuticals onto the fungal mycelia. However, our results on the heat-killed control samples imply that abiotic effects on the elimination were not dominant. The results of the degradation of IBP, CA, and CBZ by whole fungal culture are consistent with those reported in the literature (6).

The comparison of the removal by the fungal culture with those in the activated sludge process shows that the transformed compounds by the whole fungal culture *T. versicolor* are quite different from the activated sludge process (9, 10). Generally speaking, in the case of activated sludge process, IBP can be easily transformed into hydroxyl-ibuprofen (IBP-OH) and carboxyl-ibuprofen (IBP-COOH) (11, 12), while NPX and DCF give a low degradation. On the other hand, the fungal culture *T. versicolor* removed NPX and DCF almost completely. The difference is probably due to wider uptake and wider degradation spectrum of the degrader and its ligninolytic enzymes.

(3) Degradation of selected PhACs by crude laccase

To evaluate biocatalytic activities of culture filtrates of *T. versicolor* (crude laccase) in degradation of selected PhACs, a series of batch enzymatic treatment tests were performed. The reaction mixture contained culture filtrates with laccase activity (1500 U/l), MnP activity of 30 U/l, and 10 µg/l selected PhACs. Figure 3 shows the experimental results of pharmaceutical removal by culture filtrates of *T. versicolor* after 12 h of incubation. From Fig. 3 it can be seen that DCF, NPX and IDM were removed completely within 12 hours of incubation under the in vitro conditions, while some of which are shown to be persistent in the conventional wastewater treatment processes (9).

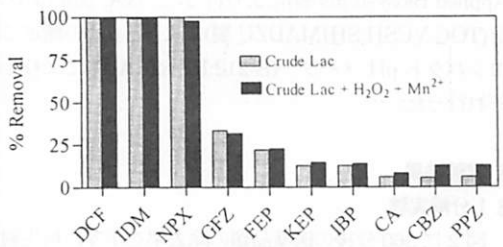

Fig. 3. The removal of PhACs by crude laccase from *T. versicolor*

The complete removal of DCF, NPX, and IDM and a partial elimination of other selected PhACs may be caused by the main contribution of extracellular ligninolytic enzyme activities in culture filtrates of *T. versicolor* in which the laccase activity was dominant compared with other ligninolytic enzyme activities. Laccase has been known as a biocatalyst with a widely broad range of substrates such as natural and synthetic hormones, EDCs, and polycyclic aromatic hydrocarbons (PAHs) as reported in the literature (5, 13-15). Our results also demonstrated that laccase can transform ionic pharmaceuticals. In addition, experimental results show that only a small difference in the removal of selected PhACs was observed in the two cases of with and without addition of $Mn^{2+}$and H_2O_2 into the reaction mixture. The slight difference is likely to be due to the contributive role of MnP activity in degradation. Taken together, the results suggest that the laccase activity from *T. versicolor* can play a considerable role in the degradation of selected PhACs.

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