Biodegradation Characteristics of Pharmaceutical Substances by Whole Fungal Culture *Trametes versicolor* and its Laccase

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ABSTRACT
The degradation of 10 selected pharmaceutically active compounds (PhACs) by whole fungal culture *Trametes versicolor*, culture filtrates and commercial laccase preparation was conducted. Complete removal of diclofenac (DCF), naproxen (NPX), indomethacin (IDM), ibuprofen (IBP), and fenoprofen (FEP) and partial degradation of other selected PhACs were observed after 48 hours of incubation with the 7-day-old liquid fungal culture both in the presence and absence of ABTS (2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid)) as a laccase-mediator. The catalytic activity of laccase in the degradation of selected PhACs was examined for both crude and commercial extracellular laccase preparations. The results showed that laccase preferentially removed DCF, NPX and IDM among the target PhACs removed by the whole fungal culture. Intracellular enzymes may be involved in the degradation of ketoprofen (KEP), clofibric acid (CA), carbamazepine (CBZ), propyphenazone (PPZ), fenoprofen (FEP) and gemfibrozil (GFZ). The removal of most selected PhACs was increased with the increase in laccase activity. The presence of redox mediators such as ABTS and HBT (1-hyroxybenzotriazole) promoted the degradation of selected PhACs, in which complete removal of DCF, NPX and IDM was observed after 3 hours of incubation with laccase activity (2000 U/L) in the presence of ABTS/HBT. The degradation spectrum by laccase for ionic PhACs with nitrogen-containing structure was quite different from that of the activated sludge process.

Keywords: degradation, white-rot fungal laccase, pharmaceutically active compounds

INTRODUCTION

The frequent detection of organic micropollutants such as pharmaceutically active compounds (PhACs) in the aquatic environment has become an increasing concern because of these pollutants' potential to cause undesirable ecological and human health effects (Daughton and Ternes 1999; Kümmerer 2001). A great variety of PhACs used by humans are discharged into the environment through wastewater treatment plants (WWTPs) and through solid wastes (Metcalfe *et al.,* 2003; Glassmeyer *et al.,* 2005). Due to the hydrophilicity, persistent nature, and the relatively low concentration in wastewater (Heberer 2002; Kolpin *et al.,* 2002), the elimination of these chemicals in conventional sewage treatment plants (STPs) is more difficult than that of other organic pollutants in wastewater.

Most studies to date have focused on the degradation of pharmaceuticals by individual bacteria or consortia (Shi *et al.,* 2004; Batt *et al.,* 2006; Joss *et al.,* 2006; Tran *et al.,* 2009) in which different bacterial oxygenases play key roles in the degradation of pharmaceuticals under aerobically co-metabolic conditions (Shi *et al.,* 2004; Batt *et al.,*

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2006; Joss *et al.,* 2006; Tran *et al.,* 2009).

In recent years, there has been a growing interest in the study of enzyme activities in wastewater treatment, especially those of the oxidoreductase enzymes from white-rot fungi, such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac), due to their recognized potential for oxidizing recalcitrant environmental pollutants, such as dyes (Asgher *et al.,* 2008; Faraco *et al.,* 2009), endocrine disrupting chemicals (Cabana *et al.,* 2007; Cajthaml *et al.,* 2009), herbicides (Maruyama *et al.,* 2006), aromatic hydrocarbons (Canas *et al.,* 2007; Cambria *et al.,* 2008), and antibiotics (Wen *et al.,* 2009). These ligninolytic enzymes are relatively non-specific and use free radical mechanisms to catalyze the degradation of a wide spectrum of organic pollutants (Blánquez *et al.,* 2004; Rodríguez *et al.,* 2004; Cabana *et al.,* 2007; Cajthaml *et al.,* 2009; Wen *et al.,* 2009).

White-rot fungal laccase, a multi-copper oxidase, has been known to catalyze the oxidation of various organic compounds (Solomon *et al.,* 1996; Rodríguez *et al.,* 2004; Morozova *et al.,* 2007; Mizuno *et al.,* 2009). Especially, it has been found that laccase can be used as a novel biocatalyst for removing the organic micropollutants such as natural estrogens (estrone-E1; 17β-estradiol-E2; and estriol-E3) and a synthetic estrogen (17α-ethinylestradiol-EE2) from municipal wastewater (Auriol *et al.,* 2007; Auriol *et al.,* 2008). There has been only a little information reported on the degradation of polar and ionic compounds, such as PhACs at trace level.

Therefore, the objective of this study was to examine the degradation ability of 10 selected PhACs at environmentally relevant concentrations by whole fungal culture *Trametes versicolor* and its laccase.

MATERIALS AND METHODS

Target compounds

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

The selection of these pharmaceuticals for biological wastewater treatment study is preferred on the following basic criteria: high consumption in the world, frequent detection in wastewater, and harmful potential to human health and ecosystem. The chemical structures and physicochemical properties of these compounds are shown in Table 1 (Ternes and Joss, 2006). As seen in the table, ionic pharmaceuticals have very low logD values at neutral pH and their adsorption onto biosolids is considered low.

Table 1 - Physicochemical properties and chemical structure of the target compounds (Ternes and Joss, 2006)

Target compound	Chemical structure	MW	log K_{ow}	pK_a	log D pH 5.5	log D pH 7.4	K_H
CA		241.65	2.57	3.0	0.42	-0.9	2.19×10^{-8}
GFZ		250.34	4.8	n.r.	3.57	1.77	n.r.
IBP		206.29	3.97	4.91	2.6	0.8	1.50×10^{-7}
FEP		242.28	3.9	4.5	2.52	0.75	n.r.
KEP		254.29	3.12	4. 5	1.52	-0.25	2.12×10^{-11}
NPX		230.27	3.2	4.2	2.26	0.47	3.39×10^{-10}
DCF		296.16	4.5	4.15	2.72	0.95	4.73×10^{-12}
IDM		357.80	4.27	4.50	1.56	-0.16	3.13×10^{-14}
PPZ		230.31	1.94	n/a	1.94	1.94	1.84×10^{-9}
CBZ		236.28	2.45	n/a	2.45	2.45	1.08×10^{-10}

n.r.: not reported
n/a: not applicable
MW: molecular weight
K_{ow}: octanol-water partitioning coefficient
K_H: Henry's coefficient
D: octanol-water distribution coefficient, logD reflects the true behavior of an ionizable compound in a solution at a given pH value or range, taking into account all the different ionized species present. For un-ionizable compounds, log K_{ow} = log D at any pH.

Fungal culture and preparation of the crude laccase

Trametes versicolor (ATCC#42530) was purchased from American Type Culture Collection (ATCC). A culture of *T. versicolor* was maintained by sub-culturing on malt extract medium (20 g/L malt extract, 20.0 g/L glucose, 1 g/L peptone) with the addition of 2% agar at pH 4.5. The culture was kept at 4 ºC and sub-culturing was routinely carried out every month. For laccase production, *T. versicolor* was induced in basal liquid medium (BLM) consisting per liter of 10 g glucose, 5 g peptone, 2 g yeast extract, 5 g malt extract, 2 g ammonium tartrate, 1 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.12 g $CuSO_4 \cdot 5H_2O$, and 2 mL of stock trace element solution. The composition of trace elements per liter was 0.05 g $B_4O_7Na_2 \cdot 10H_2O$, 0.03 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 7H_2O$, 0.04 g $ZnSO_4 \cdot 7H_2O$, and 0.01 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. The pH of the basal medium was adjusted to 4.5 before autoclaving at 121 ºC 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 ºC in shaken conditions (125 rpm).

Degradation of selected PhACs by whole fungal culture

To examine the degradation ability of the selected PhACs by whole fungal culture (i.e. mycelium and crude enzymes), a series of batch experiments were conducted with 100 ml of 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 ^{o}C in shaken conditions (125 rpm) for 48 hours. The effect of redox mediator on the degradability of selected PhACs was pre-estimated by adding 1 mM 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 an abiotic control, a control test with the same amount of a heat-killed fungal culture (121 ^{o}C, 30 min) was performed. All batch experiments were carried out in duplicate.

Degradation of selected PhACs by crude laccase

To examine catalytic activities of culture filtrates (crude laccase) 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 of selected PhACs to give a total volume reaction mixture of 10 ml. The enzymatic reaction was incubated at 30 ^{o}C in shaken conditions (125 rpm). To distinguish the contributive role of MnP contained 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 (through oven at 110 ^{o}C, 20 min) was performed under the same condition. An additional control test without adding enzymes into the reaction mixture was also conducted. All experiments were carried out in duplicate.

Degradation of selected PhACs by commercial laccase

To evaluate the degradability of PhACs by laccase, a series of in vitro enzymatic treatment tests were performed using the commercial laccase from *T. versicolor* with specific activity of 23 U/mg proteins (Sigma-Aldrich Co., Ltd, Germany). The reaction mixture consisted of 10 µg/L PhACs, laccase activities ranging from 2000-6000 U/L, and 50 mM sodium acetate (pH 4.5), and in the presence or absence of 1 mM mediators

such as ABTS and HBT (1-hyroxybenzotriazole) to give a total volume of 10 mL in a 30 mL-bottle. A control test containing the same amount of heat-denaturated laccase (through oven at 110 ^{o}C, 20 min) and initial concentration of PhACs in the sodium acetate buffer (pH 4.5) was performed in parallel. All bottles were incubated using the orbital shaker (125 rpm) at 30 ^{o}C and each enzymatic treatment was performed in duplicate.

Enzyme activity assays

Manganese peroxidase: MnP (E.C 1.11.1.13) activity was measured according to the method of (Paszczynski *et al.,* 1988). A suitable volume of culture filtrate was added to the reaction mixture containing 100 mM sodium lactate buffer (pH 4.5), 0.1 mM $MnSO_4$ and 0.1 mM H_2O_2. Reactions were initiated by the addition of H_2O_2. Oxidation of Mn^{2+} to Mn^{3+} was measured at 30 ^{o}C by the increase in absorbance at a wavelength of 240 nm (ε_{240} = 6500 M^{-1} cm^{-1}) where the transiently stable complex formed between Mn^{3+} and lactic acid was monitored. One unit (U) of MnP activity is defined as the amount of enzyme required to oxidize 1 μmol Mn^{2+} to Mn^{3+} per minute under specified conditions, and activities were expressed as U/L.

Lignin peroxidase: LiP (E.C 1.11.1.14) activity was measured by using veratryl alcohol as the substrate (Tien *et al.,* 1988). A suitable volume of culture filtrate was added to the reaction mixture consisting of 2 mM veratryl alcohol, 50 mM sodium tartrate buffer (pH 2.5) and 0.4 mM H_2O_2. The reaction was started by adding H_2O_2 and maintained at 30 ^{o}C. Oxidation of veratryl alcohol was determined by the increase in absorbance at a wavelength of 310 nm (ε_{310} = 9300 M^{-1} cm^{-1}). The increase in A_{310} was monitored for 5 min at 30 °C using a UV Mini-1240 UV-VIS spectrophotometer (Shimadzu, Inc., Japan) in determining the enzyme activity. One unit (U) of LiP activity is defined as the amount of enzyme required to oxidize 1 μmol of veratryl alcohol per 1 min under specified conditions, and activities were expressed as U/L.

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 (Kissi *et al.,* 2001). The reaction mixture contained 2mM ABTS, 100 mM sodium acetate buffer (pH 4.5) and a suitable amount of properly diluted crude enzyme. Oxidation of ABTS was monitored at 30 ^{o}C by measuring the increase in absorbance at a wavelength of 420 nm (ε_{420}=36 000 M^{-1} cm^{-1}) using a UV Mini-1240 UV-VIS spectrophotometer (Shimadzu, Inc., Japan) for measuring the enzyme activity. One unit (U) of laccase activity is defined as the amount of enzyme required to oxidize 1 μmol of ABTS per 1 min under specified conditions, and activities were expressed as U/L.

Analysis of pharmaceutical residues

Enzymatic reactions in water sample were first stopped by heating at 110 ^{o}C for 20 min to avoid degrading the surrogate standards and internal standards in the analytical process, then the samples were acidified to approximately pH 2 by adding hydrochloric acid and fortified with the target compounds where appropriate. The sample was then mixed with the internal standard solutions, 2,3-dichlorophenoxyacetic acid (2,3-DPAA) and chrysene-d_{12}. A solid-phase extraction disk (C_{18}-Octadecyl, 3M Empore^{TM}) was conditioned with 10 mL acetone and 10 mL methanol. The sample was then passed through the extraction disk at a flow rate of 10 mL min^{-1} using a gentle vacuum. Upon

completion of the extraction, the extraction disk was dried for 30 min under vacuum. The extraction disk was finally eluted with 5 mL of methanol. This fraction contained the acid and neutral PhACs. Then, 2,4-dichlobenzoic acid (2,4-DBA) was added to check the recovery in the derivatization step, and the sample was evaporated to dryness under a stream of nitrogen at 60 ^{o}C to remove a portion of the methanol present. The solution of pentaflourobenzyl bromide (PFBBr) and triethylamine was added as reagent for the derivatization reaction at 110 ^{o}C for 1h suggested by (Koutsouba *et al.,* 2003; Tran *et al.,* 2009). A gas chromatography-mass spectrometry (Shimadzu, Inc., Japan, GC2010/MS PARVUM 2) was used for the quantification of the derivatized materials in toluene solution. PPZ and CBZ, which require no derivatization, were quantified in the same chromatogram.

RESULTS AND DISCUSSION

Production of extracellular ligninolytic enzymes

It has been known that *T. versicolor* is an excellent producer of industrially important laccase (Jang *et al.,* 2006; Auriol *et al.,* 2008). To have a sufficient amount of crude laccase for further investigations in this study, laccase was biosynthesized during fermentation in BLM (pH 4.5) under shaken condition at 30 ^{o}C. Fig. 1 shows the profile of typical ligninolytic enzymes and fungal mass during the 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, reached the maximum on the 7th day (1550 U/L), and then decreased slightly until the end of the fermentation process.

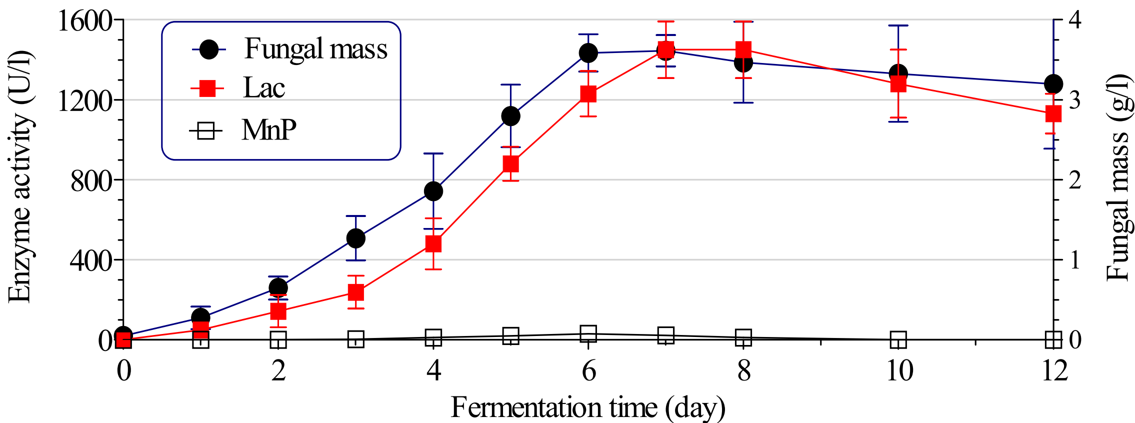

Fig. 1 - Production of laccase and MnP during agitated fermentation of *T. versicolor*. The error bars represent standard deviation of two replicates.

MnP activity was found on the 4th day and peaked on the 6th day of fermentation process (30 U/L). It can be seen from Fig. 1 that laccase activity showed laccase at the highest activity produced by *T. versicolor* compared with MnP in culture filtrate, which is likely to be due to the characteristics of the producer, the composition of media, and the fermentation conditions (e.g. rich nitrogen nutrient and agitated fermentation conditions), which are more favorable for laccase production than that for other ligninolytic enzymes, such as MnP and LiP. Though manganese-independent peroxidase

(MIP) activity was not measured in this study, the production of MIP by *T. versicolor* was not reported in previous studies (Lee *et al.,* 1999; Blánquez *et al.,* 2004; Jang *et al.,* 2006). The fermentation broth should be used as crude laccase in preparation for the following experimental purposes.

Degradation of selected PhACs by whole fungal culture

The degradability of selected PhACs by whole fungal culture *T. versicolor* was evaluated by the incubation of the 7-day-old liquid fungal culture *T. versicolor* with 10 μg/L of selected PhACs and the pH was adjusted to 4.5. After 48 h of incubation with fungal culture, the degradation of PhACs was observed. Fig. 2 shows that all selected PhACs were degraded by the whole fungal culture *T. versicolor*. Complete removal of IBP, NPX, DCF, IDM, KEP, and FEP and partial removal of other selected PhACs was observed both in the presence and absence of mediator (ABTS). The degradation 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 published literature (Canas *et al.,* 2007; Mizuno *et al.,* 2009).

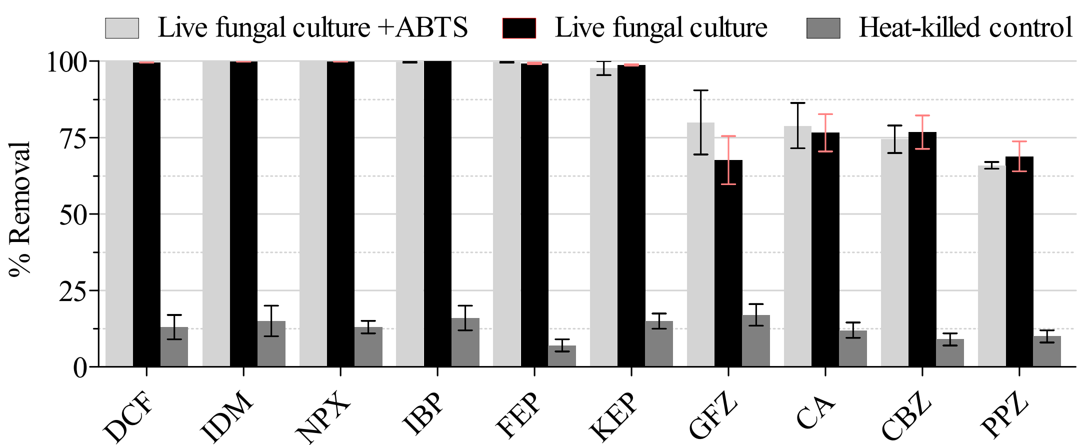

Fig. 2 - Removal of selected PhACs by the whole fungal culture *T. versicolor*. The error bars represent standard deviation of two replicates.

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. This suggests that the degradation of pharmaceuticals by the whole fungal culture probably did not only involve the extracellular ligninolytic activities (laccase and MnP) but also may be caused by intracellular oxidizing activities (e.g., cytochrome P450 oxygenases) or by the synergistic action of ligninolytic enzymes (MnP and laccase) with other components in the cell-free extract. This idea is relatively consistent with those previously reported in the literature (Marco-Urrea *et al.,* 2009; Marco-Urrea *et al.,* 2010b), where researchers found that white rot fungi were able to degrade ibuprofen, carbamazepine, naproxen, diclofenac, atenolol, and propanolol.

Furthermore, in a recent study by Hata *et al.,* (2010) it was found that degradation of diclofenac and mefenamic acid by white rot fungal culture may be involved in the

cytochrome P450, MnP and laccase activities. The possible occurrence of natural mediators in the whole fungal culture can be explained for the non-difference between the results of fungal culture experiments with and without the addition of ABTS. Thus, the natural mediators may play a role similar to that of ABTS in promoting the oxidation of organic compounds as reported by Gutierrez *et al.* (1994) and Cambria *et al.* (2008). That is why in the fungal culture tests ABTS showed less effectivity than that in commercial laccase experiment. Another possibility is that the elimination of pharmaceuticals did not only involve 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 comparison of the degradation 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 (Joss *et al.,* 2006; Tran *et al.,* 2009). In the case of activated sludge process, IBP can be easily transformed into hydroxyl-ibuprofen (IBP-OH) and carboxyl-ibuprofen (IBP-COOH) (Weigel *et al.,* 2004; Clara *et al.,* 2005), while NPX and DCF have a low degradation. On the other hand, in the fungal culture *T. versicolor*, NPX and DCF were completely removed. The difference is probably due to wider uptake and wider degradation spectrum of the degrader and its ligninolytic enzymes. The roles of ligninolytic enzymes from *T. versicolor* in the degradation should be clarified in the following experiments.

In acidic condition, acidic pharmaceuticals were easily removed biologically (Urase and Kikuta 2005). The lower pH condition in the case of fungal reaction may be one of the reasons of higher removal compared with the case of activated sludge.

Degradation of selected PhACs by crude laccase

To evaluate biocatalytic activities of culture filtrates of *T. versicolor* (crude laccase) in the degradation of selected PhACs, a series of batch enzymatic treatment tests was performed. The reaction mixture contained culture filtrates with laccase activity of 1500 U/L, MnP activity of 30 U/L, and initial concentration of 10 μg/L for all selected PhACs. The pH of the reaction mixture was adjusted to 4.5. Fig. 3 shows the pharmaceutical degradation by crude laccase 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 are shown to be persistent in the conventional wastewater treatment processes (Joss *et al.,* 2006). The complete removal of DCF, NPX, and IDM and a partial degradation of other selected PhACs may be caused by extracellular ligninolytic enzymes activities in culture filtrates of *T. versicolor*. In the cell-free extract (crude enzyme), laccase activity was dominant compared with other ligninolytic enzymes. Thus, it may be suggested that the complete removal of DCF, NPX and IDM may involve laccase activity alone or the combination of laccase and natural mediators contained in cell-free extract. In addition, experimental results showed that only small differences between the results of experiments with and without the addition of Mn^{2+} and H_2O_2 into the reaction mixture can be interpreted as being due to MnP activity.

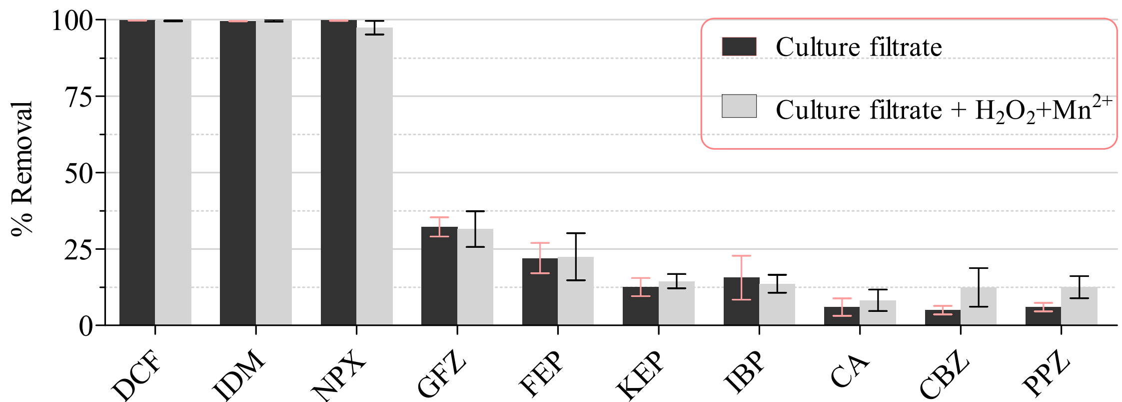

Fig. 3 - Removal of selected PhACs by culture filtrates from *T. versicolor*. The error bars represent standard deviation of two replicates.

However, it cannot be clearly concluded that the complete removal of DCF, NPX and IDM by the cell-free extract is caused by laccase activity or the combination of laccase and natural mediators only, and not by other ligninolytic enzymes. A possible interpretation for the removal of pharmaceuticals may involve the synergic action of ligninolytic enzymes (MnP and laccase) with other components contained in cell-free extract. This explanation is consistent with that in the literature reported by Arantes and Milagres (2007) wherein they found that the synergic action of MnP and laccase with Fe^{3+} reducing activity might be an oxidative pathway for the degradation of Azure B. Marco-Urrea *et al.* (2010b) also found that the oxidation of clofibric acid, carbamazepine, atenolol and propranolol by biological Fenton-like system was mediated by the combination of ligninolytic enzymes (laccase and peroxidases) with 2,6-dimethoxy-1,4-benzoquinone (DBQ) and Fe^{3+}-oxalate. In a recent study, Hata *et al.* (2010) also suggested that the degradation of diclofenac and mefenamic acid might be caused by the combination of laccase and MnP with other enzymes. Further investigation is needed to clarify whether or not the contribution of laccase activity/the synergic action of ligninolytic enzymes (laccase and peroxidases) with other components in cell-free extract may be involved in the degradation of selected pharmaceuticals.

Degradation of selected PhACs by commercial laccase

Laccase catalyzes the oxidation of a wide spectrum of organic compounds such as *o*- and *p*-diphenols, aminophenols, polyphenols, polyamines, heterocyclic compounds, and some inorganic ions coupled with the reduction of molecular dioxygen to water (Solomon *et al.,* 1996; Rodríguez *et al.,* 2004; Morozova *et al.,* 2007; Mizuno *et al.,* 2009). To examine the degradation ability of ionic pharmaceuticals by laccase, a series of batch experiments were performed using commercial laccase with laccase activity of 6000 U/L, initial concentration of 10 µg/L for all selected PhACs, and 50 mM sodium acetate buffer (pH 4.5). The laccase used in the experiments was commercial laccase from *T. versicolor* with specific activity of 23 U/mg proteins.

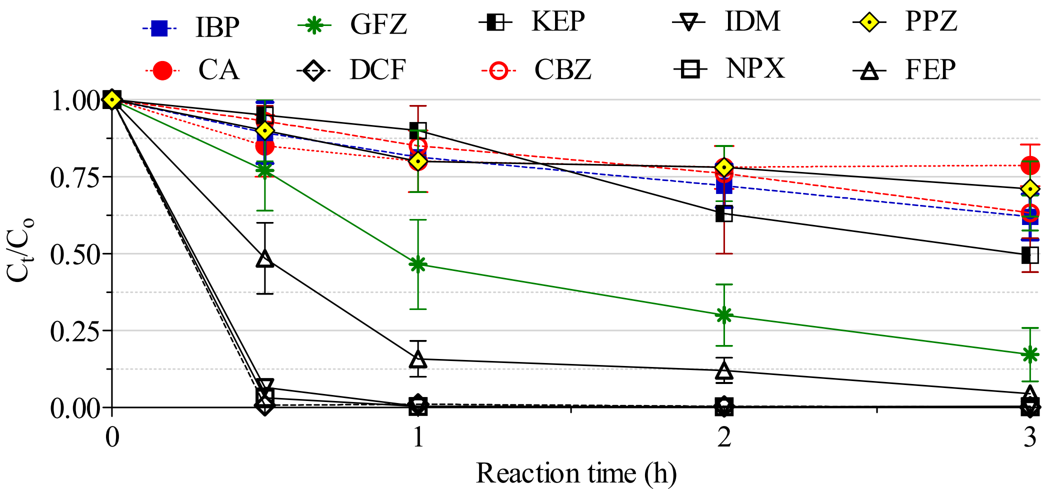

Fig. 4 - Changes in normalized concentrations of selected PhACs during degradation. The error bars represent standard deviation of two replicates.

Fig. 4 shows the representative results obtained for the oxidation of selected PhACs by commercial laccase (6000 U/L) in batch experiments at an initial concentration of 10 μg/L for all selected PhACs. From Fig. 4, it can be seen that DCF, IDM and NPX were rapidly transformed by laccase alone. The degradation characteristics of pharmaceuticals by commercial laccase are similar to those by cell-free extract.

Degradation spectrum by laccase

Previous studies have reported that the mechanism of substrate oxidation by laccase includes one electron oxidation of the substrate molecule with the oxidized enzyme form. Thus, the substrates are required to have electron donor properties in the laccase reaction (Shleev *et al.,* 2003; Kulys and Vidziunaite 2005; Morozova *et al.,* 2007). Most of the target pharmaceuticals used in this study satisfy the essential criteria for a laccase substrate such as (i) aromatic or heterocyclic ring(s), (ii) coupled bonds or easily oxidized substitutions (e.g., hydroxyl groups), and (iii) electron donor substituent (e.g., phenyl, alkyl) (Shleev *et al.,* 2003; Morozova *et al.,* 2007). Our experimental results can suggest that laccase prefers to catalyze the oxidation of DCF, IDM and NPX rather than those of other pharmaceuticals in the reaction mixture. As a result, more than 90% of initial DCF, IDM, and NPX were transformed within 30 min of incubation (as shown in Fig.4). Interestingly, it can be easily found that in the chemical structure of IDM and DCF and well-known laccase mediators (typical laccase substrates) such as HBT, ABTS, N-hydroxy phthalimide (HPI) and violuric acid (VA), they share the same characteristics that their molecules have at least a nitrogen atom, and they are ionic compounds with negative charge group(s) (Table 2).

These characteristics can explain the reason why DCF and IDM were easily transformed than other pharmaceuticals in the reaction mixture. The results taken together can suggest that pharmaceuticals with nitrogen-containing structure and negative charge(s) may be preferably degraded by laccase. This agrees well with the recent results as reported by Hata *et al.* (2010) and Marco-Urrea *et al.* (2010a) wherein they found that laccase can efficiently degrade diclofenac and mefenamic acid.

Table 2 - Typical substrates as redox mediators for laccase (Morozova *et al.,* 2007)

Substrates	Chemical structure	logD (pH 5.5)	logD (pH 7.4)
ABTS		-2.51	-2.51
HBT		0.68	0.33
HPI		0.32	-0.9
VA		-4.5	-5.59

Effect of laccase activity on the degradation of PhACs

Fig. 5 shows the effect of laccase activity on the degradation of pharmaceuticals in the case of commercial laccase. Experimental results showed that the degradation of most selected PhACs increased with the increase of laccase activity. For example, at a low laccase activity (2000 U/L) most pharmaceuticals were only degraded partially (except DCF), while complete removal of IDM, DCF, and NPX was observed at a laccase activity of more than 4000 U/L. However, it cannot be clearly concluded whether the degradation of the selected pharmaceuticals such as IBP, GFZ, CA, CBZ, PPZ and KEP occurred through laccase activity directly or indirectly via the assistance of redox mediators, because laccase used in this study was commercially prepared. Thus, it is possible that it contains some redox mediators and/or other enzymes.

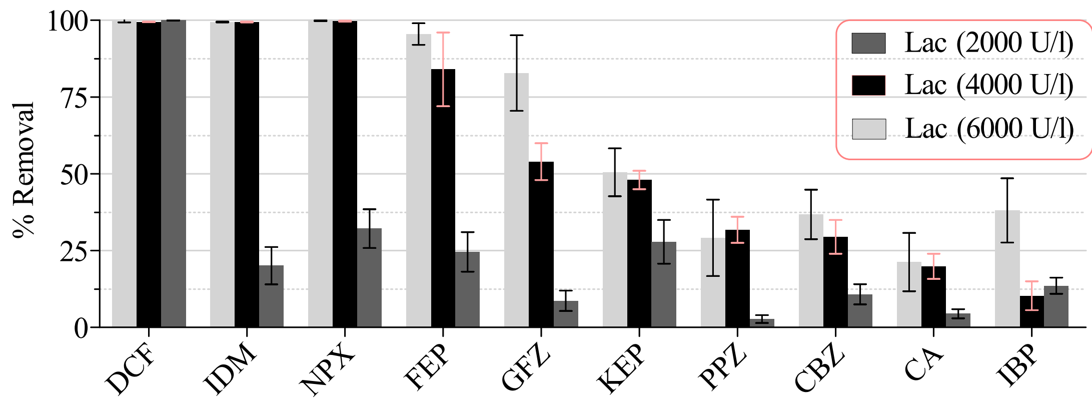

Fig. 5 - Effect of laccase activity on the removal of selected PhACs. The error bars represent standard deviation of two replicates.

Degradation of PhACs in the presence of mediators

The presence of appropriate mediator compounds extends the substrate spectrum of laccase to various organic compounds through the use of a free radical mechanism for catalysis (Fabbrini *et al.,* 2002; Morozova *et al.,* 2007). To reinforce the role of redox mediators in the transformation of selected PhACs using laccase at a relatively low activity (2000 U/L), two typical laccase-mediators (ABTS and HBT) were used in this experiment. The removal of selected PhACs by laccase after 3 hours of enzymatic treatment with and without redox mediators is shown in Fig. 6.

Fig. 6 shows that the removal efficiency by laccase treatment in the absence of redox mediators ranged from 5-99%, in which range only DCF was removed completely. The removal efficiency of most selected PhACs was enhanced when ABTS and HBT were added to the reaction mixture. Especially, Fig. 6 also shows that the nearly complete removal of DCF, NPX, and IDM was obtained in the presence of ABTS or HBT. The presence of an appropriate redox mediator (ABTS or HBT) probably allows laccase to overcome a kinetic barrier and promote the degradation (Bourbonnais *et al.,* 1998). As a result, higher removal efficiency was observed for most selected PhACs in the presence of redox mediators (ABTS or HBT) compared with those in the absence of mediators.

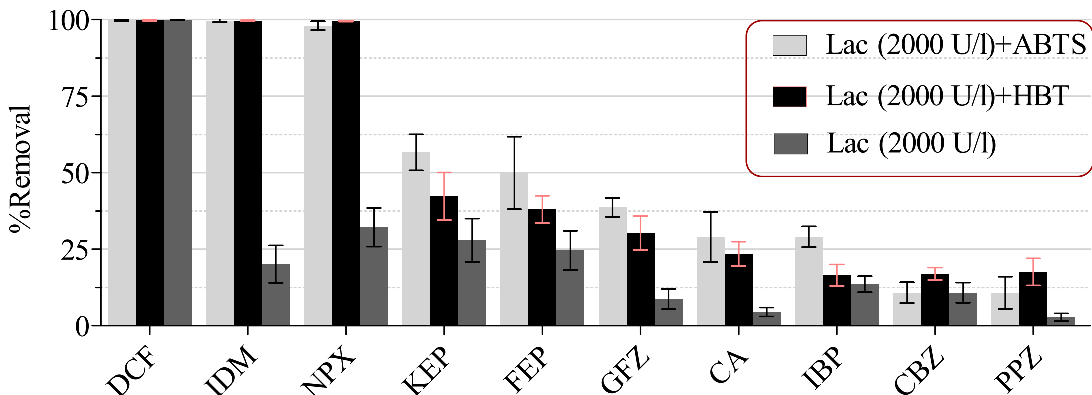

Fig. 6 - Degradation of selected PhACs by laccase with and without redox mediators. The error bars represent standard deviation of two replicates.

The degradation tendency observed in this study is relatively consistent with those reported in the literature (Rodríguez *et al.,* 2004; Tamagawa *et al.,* 2005; Maruyama *et al.,* 2006; Auriol *et al.,* 2007; Canas *et al.,* 2007; Casas *et al.,* 2007), wherein they found that fungal laccase can oxidize a variety of organic compounds in the presence of a redox mediator, such as HBT or ABTS. However, the degradation spectrum in the presence of redox mediators was still mainly dependent on the characteristics of target compounds. Further investigations such as identification of metabolites and degradation mechanism(s) for the experiments (whole fungal culture, cell-free extract and purified laccase with and without redox mediators) are needed to clarify the role of laccase or the synergic action of laccase with other ligninolytic enzymes on the degradation of the selected pharmaceuticals.

CONCLUSION

The characteristics of degradation of ten selected PhACs by the whole fungal culture *T. versicolor,* cell-free extract (crude laccase), and commercial laccase were examined. Experimental results showed DCF, IDM and NPX could be efficiently degraded by the cell-free extract (crude laccase) and the commercial laccase, although the others showed poor degradation. On the other hand, the whole fungal culture showed considerably higher degradation for all the tested PhACs compared with the cell-free extract, for example, IBP, FEP and KEP could be completely removed. This result can suggest that the degradation of selected pharmaceuticals in the whole fungal culture may involve the intracellular enzymes (e.g., cytochrome P450 oxygenases), while the degradation of pharmaceuticals such as DCF, IDM and NPX by the whole fungal culture and the cell-free extract may involve the laccase activity only or the synergic action of laccase with natural mediators and other enzymes in the cell-free extract. The degradation of most selected pharmaceuticals was increased with the increase of laccase activity or increased in the presence of redox mediators. The degradation spectrum of the tested PhACs by the fungal culture is quite different from that by activated sludge processes. The pharmaceuticals with nitrogen-containing structure and negative charge(s) were easily removed by laccase activity. This research provides the preliminary data about the degradation of ten commonly used pharmaceuticals at environmentally relevant concentrations by white rot fungal culture, its linginolytic enzymes, which would be useful for future development of the fungal culture-based bioreactors or ligninolytic enzymes-based reactors for the treatment of pharmaceuticals.

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