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Technical Note

Hydroxylation of bisphenol A by hyper lignin-degrading fungus *Phanerochaete sordida* YK-624 under non-ligninolytic condition

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Key words: *Phanerochaete sordida* YK-624; Bisphenol A; Hydroxylation; Cytochrome P450

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ABSTRACT

Bisphenol A (BPA) is one of the representative compounds of the endocrine disrupting compounds group and the highest volume chemicals produced worldwide. As a result, BPA is often detected in many soil and water environments. In this study, we demonstrated the transformation of BPA from liquid cultures inoculated with hyper lignin-degrading fungus *Phanerochaete sordida* YK-624. Under non-ligninolytic conditions, approximately 80% of BPA was eliminated after 7 d of incubation. High-resolution electrospray ionization mass spectra and nuclear magnetic resonance analyses of a metabolite isolated from the culture supernatant suggested that BPA was metabolized to hydroxy-BPA, 4-(2-(4-hydroxyphenyl)propan-2-yl)benzene-1,2-diol, which has a much lower estrogenic activity than BPA. In addition, we investigated the effect of the cytochrome P450 inhibitor piperonyl butoxide (PB) on the hydroxylation of BPA, markedly lower transformation activity of BPA was observed in cultures containing PB. These results suggest that cytochrome P450 plays an important role in the hydroxylation of BPA by *P. sordida* YK-624 under non-ligninolytic conditions.

1. Introduction

The occurrence of endocrine disrupting compounds (EDCs) in the aquatic environment has generated worldwide interest because these chemicals can cause feminization of fish as well as interfere with the reproduction and development of other aquatic organisms (Purdom et al., 1994; Harries et al., 1996; Larsson et al., 1999). One of the representative compounds of the EDCs group is bisphenol A (2,2-bis(4-hydroxyphenyl)propane; BPA). BPA, a monomer component of polycarbonate plastics, is used in many consumer products, including lacquers applied as food-can linings and dental composite fillings and sealants (Brotons et al., 1995; Olea et al., 1996). Many countries throughout the world have large production capacities for BPA, especially Germany, the Netherlands, the USA and Japan. BPA is one of the highest volume chemicals produced worldwide, with an estimated production of 5 Mt in 2010 (Ballesteros-Gómez et al., 2009). As a result, BPA has been detected often in many soil and water environments (Kitada et al., 2008; Matsumura et al., 2009).

Lignin-degrading white-rot fungi, which have the unique ability to degrade lignin to the level of CO_2 (Kirk and Farrell, 1987), and their ligninolytic enzymes have also attracted interest for the biotransformation of contaminants because of their industrial potential for degrading recalcitrant environmental pollutants, such as polychlorinated dibenzodioxin (Kamei et al., 2005), lindene (Bumpus et al., 1985), chlorophenols (Joshi and Gold, 1993), mycotoxin aflatoxin B_1 (Wang et al., 2011), and acetamiprid as a neonicotinoid insecticide (Wang et al., 2012a).

The degradation of EDCs by ligninolytic enzymes has attracted the attention of many researchers (Hirano et al., 2000; Suzuki et al., 2003; Saito et al., 2004; Tamagawa et al., 2006). The degradation of BPA using fungi is also reported (Chai et al., 2005; Shin et al., 2007) but the detail mechanisms are still unknown. More recently, we have reported that the removal of BPA by lignin peroxidase from hyper lignin-degrading fungus *Phanerochaete sordida* YK-624 (Wang et al., 2012b) and *P. sordida* YK-624 under ligninolytic condition (Wang et al., 2013). In our previous reports, BPA dimers have been formed as metabolites by radical couplings of BPA phenoxy radical generated by ligninolytic enzymes. Unfortunately, these BPA dimers are possible to be decomposed to toxic BPA by various reactions. Therefore, the transformation techniques of BPA without polymerization are necessary. In the present study, we examined the removal of BPA by *P. sordida* YK-624 under non-ligninolytic condition which hardly oxidize BPA to BPA phenoxy radical. We also detected the metabolite from BPA and proposed the metabolic pathway of BPA by *P. sordida* YK-624.

2. Materials and methods

2.1. Fungi and chemicals

P. sordida YK-624 (ATCC 90872), which has been isolated from rotted wood, was used in the present study (Hirai et al., 1994). The fungus was maintained on potato dextrose agar (PDA) slants at 4 °C.

BPA was purchased from Alfa Aesar, A Johnson Matthey (Lancashire, United Kingdom). All other chemicals were analytical pure grade and were used without further purification.

2.2. Transformation of BPA

Potato dextrose broth (PDB) medium which is a non-lignolytic (no production of lignolytic enzymes) condition was used for BPA transformation experiments. The fungus was incubated on a PDA plate at 30 °C for 3 d, and then 10 mm-diameter disks were punched out from the growing edge of mycelium. Two disks were each placed into a 100-mL Erlenmeyer flask containing 10 mL of PDB medium. After statically incubating the flasks at 30 °C for 3 d, 100 μL of 100 mM BPA/dimethyl sulfoxide were added to the cultures (final concentration at 1 mM), and then further incubated for 0-7 d (each in triplicate). The internal standard material was added to the culture, and then the culture was homogenized with 20 mL of acetone by using a Polytron PT1200E (Kinematica, Canada). The homogenate was filtrated and then evaporated to dryness. The residue was analyzed by HPLC for the quantification of BPA under the following conditions: column, Wakosil-II 5C18HG (4.6×150 mm; Wako Pure Chemical Industries, Japan); mobile phase, 10 to 100% MeOH gradient for 60 min; flow rate, 1 mL min^{-1}; and UV wavelength, 277 nm.

2.3. Metabolism experiment of BPA

Inoculated cultures of *P. sordida* YK-624 were prepared under the PDB medium. After static incubation at 30 °C for 3 d, BPA (final concentration, 1 mM) was added to these cultures. These cultures were further incubated for 7 d and then filtrated with membrane filters (pore size; 0.2 μm). The resulting solution was extracted 3 times with equal amount of ethyl acetate (EtOAc). The EtOAc extract was dried over anhydrous sodium sulfate and then evaporated to dryness. The residue was cleaned on a thin-layer chromatography (TLC, glass plates) to obtain 3 fractions. Each fraction was analyzed by TLC (aluminum sheets). Silica gel plates (Merck F_{254}; Merck, Darmstadt, Germany) were used for TLC. The metabolite was further separated by HPLC (column: CAPCELL PAK C18 AQ; 20×250 mm, Shiseido,

78 Japan) using 10 to 100% MeOH gradient. The purified metabolite was analyzed by
79 HR-ESI-MS and NMR, including distorsionless enhancement by polarization transfer,
80 correlated spectroscopy, heteronuclear multi quantum correlation, and heteronuclear multiple
81 bond correlation (HMBC) experiments, respectively. The HR-ESI-MS data were measured
82 using a JMS-T100LC mass spectrometer. ^{1}H-NMR spectra were recorded using a Jeol
83 Lambda-500 spectrometer at 500 MHz, while ^{13}C-NMR spectra were recorded on the same
84 instrument at 125 MHz.

85 *2.4. Cytochrome P450 inhibitor experiment*

86 After preculturing *P. sordida* YK-624 for 3 d, BPA (final concentration at 1 mM) and
87 the cytochrome P450 inhibitor piperonyl butoxide (PB, final concentration, 0, 0.1 and 1 mM)
88 were added to cultures. The cultures were further incubated for 1-4 d, and each culture was
89 then subjected to HPLC for the quantification of BPA, as described above.

90 *2.5. Competition assay using membrane estrogen receptor*

91 Culture of HEK231 cells were stably transfected with membrane estrogen receptor
92 (GPR30) and estrogen receptor competition studies were carried out as described before
93 (Thomas and Dong, 2006). Briefly, human HEK293 cells stably transfected with human
94 GPR30 were cultured in dulbecco's modification of eagle's medium/Ham's F-12 medium with
95 5% fetal bovine serum and 100 μg mL^{-1} of G418 in 150 mm diameter plates. The medium
96 was replaced every 1-2 d and the cells which were reached 80% confluence after 3 d were
97 used in the experiments.

98 Procedures for the preparation of plasma membrane fractions and assay of steroid
99 membrane receptors were followed with few modifications for measurement of [^{3}H] estradiol
100 (E_2) binding to plasma membranes of HEK293 cells transfected with goldfish mPRα
101 (Tokumoto et al., 2007) except that presoaking with Tween 80 was omitted. As a
102 radio-labeled steroid [2, 4, 6, 7-^{3}H]E_2 ([^{3}H]E_2, ~89 Ci mmol^{-1} was purchased from
103 PerkinElmer Life & Analytical Sciences (Waltham, MA). The competitive binding assay
104 tubes contained 4 nM [^{3}H]E_2 and the competitors (concentration range: 1 nM to 10 μM).
105 After a 30 min incubation at 4 °C with the membrane fractions, the reaction was stopped by
106 filtration (Whatman GF/B filters), the filters were washed and bound radioactivity measured
107 by scintillation counting. The displacement of [^{3}H]E_2 binding by the competitors was
108 expressed as a percentage of the maximum specific binding of E_2.

3. Results

3.1. Transformation of BPA by P. sordida YK-624 under non-ligninolytic condition

In the present study, PDB medium was used for the transformation experiment of BPA. When *P. sordida* YK-624 was cultured in PDB medium, BPA was decreased by 51, 71, 83% after 1, 4, and 7 d of incubation, respectively (Fig. 1a). These results suggested that *P. sordida* YK-624 had the ability for the transformation of BPA under the non-ligninolytic conditions such as PDB medium.

3.2. Identification of the metabolite from BPA

To determine the metabolite(s) produced during the degradation of BPA, 7-d cultures of *P. sordida* YK-624 in PDB medium was supplemented with 0.5 mM BPA were subjected to TLC and HPLC. From this analysis, one potential BPA metabolite was detected (data not shown). The purified metabolite was then subjected to high-resolution electrospray ionization mass spectrometry analysis, which yielded a molecular ion at *m/z* 243.1015 $[M-H]^-$ (calculated for $C_{15}H_{15}O_3$, 243.1021), indicating that the molecular formula of this compound was $C_{15}H_{16}O_3$. This formula suggested that the metabolite might be a hydroxylation form of BPA. The structure of the purified metabolite was further characterized by NMR analyses. Table 1 lists the chemical-shift assignment data for the metabolite of BPA. HMBC (Fig. 2) (H-3/C-2′, H-5/C-2′) confirmed that the metabolite was 4-(2-(4-hydroxyphenyl)propan-2-yl)benzene-1,2-diol, which hydroxylated at the *ortho*-position of BPA.

3.3. Effect of cytochrome P450 inhibitor

The effect of the cytochrome P450 inhibitor on the transformation of BPA by *P. sordida* YK-624 was investigated using PB. The transformation rate of BPA after the addition of 0.1 and 1 mM PB into cultures of *P. sordida* YK-624 is shown in Fig. 1b. In contrast to the transformation rate of BPA in cultures without PB, markedly lower transformation activity of BPA was observed in cultures containing PB. In the presence of 0.1 and 1 mM PB, 63 and 38% of BPA were eliminated after 4 d of incubation, respectively, compared to 70% in cultures lacking PB.

3.4. Steroid binding assay of BPA and the metabolite

To evaluate the estrogenic activity of BPA metabolite, we used the steroid binding assay in the present study. BPA showed significant competition with IC_{50} of 1.8 μM. Just a few affinities for binding to the membrane estrogen receptor were detected in hydroxy-BPA. These

results demonstrated that hydroxylation of BPA by *P. sordida* YK-624 causes the 67% loss of estrogenic activity of BPA (Fig. 3). In other words hydroxylation of BPA by *P. sordida* YK-624 is sufficient for diminish possible environmental estrogenic activity of BPA.

4. Discussion

Biotransformation of BPA has also been reported by many researchers. More recently, we reported the removal of BPA by *P. sordida* YK-624 under ligninolytic condition (Wang et al., 2013). BPA dimers are formed by radical couplings at extracellular region without the incorporation into the cell of *P. sordida* YK-624. However, BPA dimers may be returned to BPA by various reactions. BPA glucuronide is the major metabolite of BPA in rat hepatocytes (Nakagawa and Tayama, 2000; Pritchett et al., 2002). In plants, cells of *Eucalyptus perriniana* and *Portulaca oleracea cv.* metabolize BPA to its hydroxyl products (Hamada et al., 2002; Watanabe et al., 2012). In fungi, BPA is metabolized to BPA-*O*-β-D-glucopyranoside by *Aspergillus fumigatus* (Yim et al., 2003). In the present study, we detected a hydroxy-BPA as a metabolite of BPA.

Watanabe et al. (2012) suggested polyphenol oxidases were likely to contribute to BPA hydroxylation by portulaca. However, *P. sordida* YK-624 produces lignin peroxidases and manganese peroxidase as ligninolytic enzymes but not laccase. The functional diversity of cytochrome P450s in white-rot fungi has been studied because cytochrome P450s play an important role on degrading a wide variety of recalcitrant aromatic compounds (Hiratsuka et al. 2001; Xiao et al., 2011; Wang et al., 2012a). Recently, Hata et al. (2010) suggested that hydroxylation catalyzed by cytochrome P450 in *P. sordida* YK-624 might be involved in the elimination and detoxification of diclofenac and mefenamic acid. Our present study showed that the transformation of BPA was efficiently inhibited by the addition of PB (Fig. 1b), which is a common inhibitor of cytochrome P450 and is often used for demonstrating whether a reaction is catalyzed by cytochrome P450 enzymes (Mori and Kondo, 2002; Mori et al., 2003; Kamei et al., 2005). Since hydroxylation of BPA was inhibited by PB, we propose that cytochrome P450s are involved in the hydroxylation of BPA by *P. sordida* YK-624 under non-ligninolytic condition. We have described for the first time the hydroxylation of BPA by a white-rot fungus.

Due to the high production volumes, the toxicity of BPA has been intensively studied since the 1970s. Estrogenic activity of BPA was first reported in 1993 (Krishnan et al., 1993). We used the steroid binding assay to evaluate the estrogenic activity of BPA and BPA metabolite in the present study. BPA showed significant competition with IC_{50} of 1.8 μM. Just

a few affinities for binding to the membrane estrogen receptor were detected in hydroxy-BPA (Fig. 3). Since the metabolite hydroxy-BPA has lower estrogenic toxicity than BPA, diminishing environmental estrogenic activity of BPA is possible using *P. sordida* YK-624.

The findings of the present study confirm that *P. sordida* YK-624 can remove the estrogenic activity of BPA by converting to hydroxy-BPA. In the next study, we will investigate whether hydroxy-BPA could be further metabolized by *P. sordida* YK-624 for complete removal of estrogenic activity.

5. Acknowledgement

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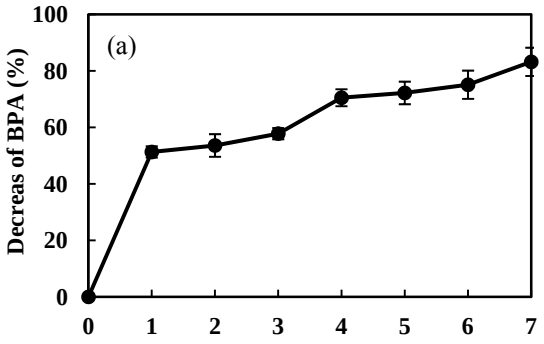
283 Fig. 1 Time course for BPA transformation by P. sordida YK-624 under non-ligninolytic
284 condition (a) and effect of cytochrome P450 inhibitor PB on the transformation of BPA by P.
285 sordida YK-624 (b). ● without PB, ▲ 0.1 mM PB, ■ 1 mM PB. Values are the means±
286 SD of triplicate samples.
287
288 Fig. 2 COSY and HMBC correlations for the identified BPA metabolite.
289
290 Fig. 3 Competition by BPA and hydroxy-BPA for binding to the membrane estrogen
291 receptor. Samples were incubated with 4 nM [3H]E2 and 10 □M competitor. Competition rate
292 (%) is represented as relative value of the displacement of [3H]E2 binding by BPA set as
293 100 %.
294


(a)
Decreas of BPA (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7

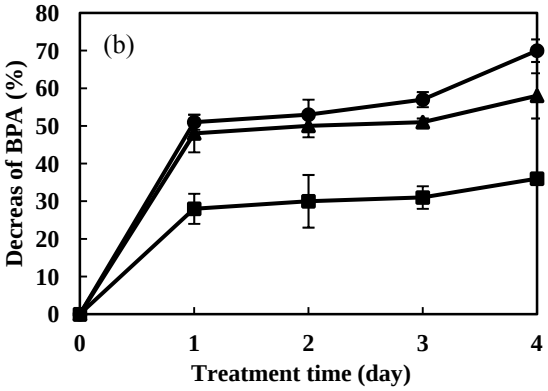
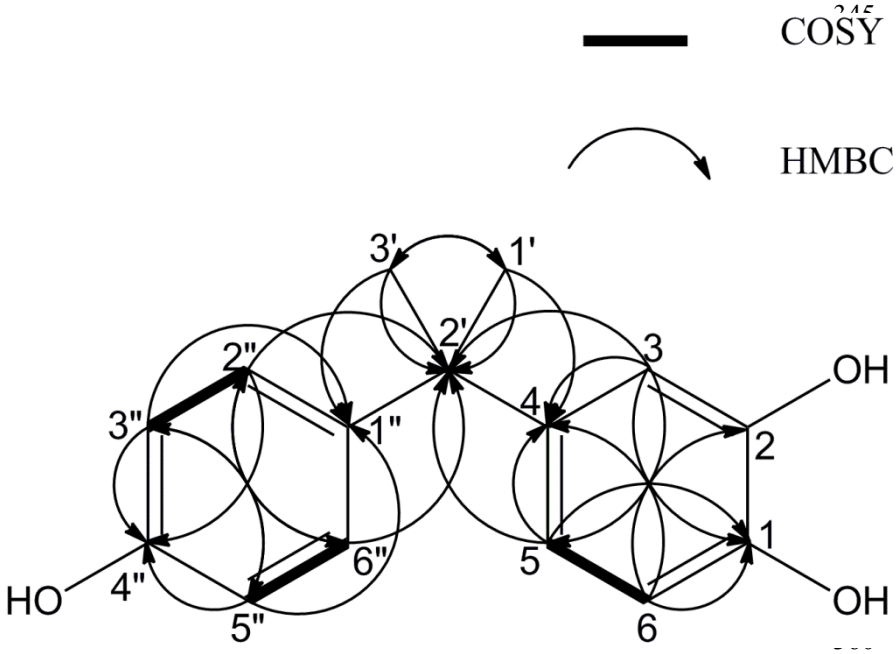

(b)
Decreas of BPA (%)
80
70
60
50
40
30
20
10
0
0
1
2
3
4
Treatment time (day)

Fig. 1



4-(2-(4-hydroxyphenyl)propan-2-yl)benzene-1,2-diol

Fig. 2

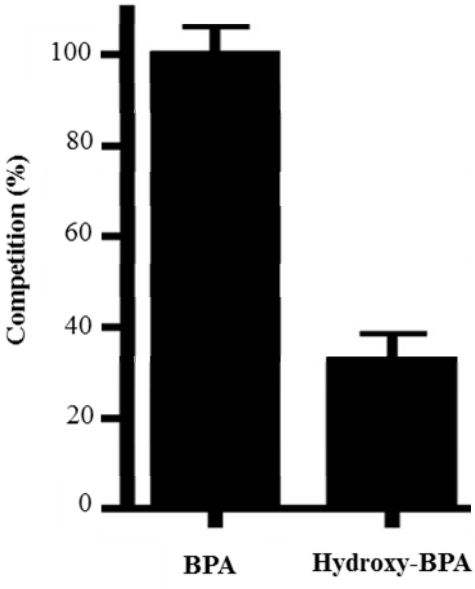


Fig. 3

Table 1 ^{1}H- and^{13}C-NMR data for BPA metabolite (in CD_3OD)

Position	^{1}H	^{13}C
	δ_H (mult, *J* in Hz)	d
1	-	142.3
2	-	142.9
3	6.61 (d, 2.2)	114.1
4	-	141.9
5	6.56 (dd, 7.9, 2.2)	117.3
6	6.64 (d, 7.9)	113.9
1″	-	143.9
2″	7.02 (d, 9.0)	127.2
3″	6.65 (d, 8.5)	113.9
4″	-	154.3
5″	6.65 (d, 8.5)	113.9
6″	7.02 (d, 9.0)	127.2
1′-CH_3	1.54 (s)	30.1
2′	-	41.0
3′-CH_3	1.54 (s)	30.0