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Original Paper

Dimerization of Bisphenol A by hyper lignin-degrading fungus *Phanerochaete sordida* YK-624 under ligninolytic condition

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Running title: Dimerization of BPA

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Dimerization of Bisphenol A by hyper lignin-degrading fungus *Phanerochaete sordida* YK-624 under ligninolytic condition

Bisphenol A (BPA) was treated with hyper lignin-degrading fungus *Phanerochaete sordida* YK-624 under ligninolytic condition. After preculturing *P. sordida* YK-624 for 4 days, BPA (final concentration, 0.1 mM and 1.0 mM) was added to cultures. Both 1 mM and 0.1 mM BPA were effectively decreased within a 24 h treatment and two metabolites were detected. Two metabolites (5,5’-bis-[1-(4-hydroxy-phenyl)1-methyl-ethyl]-biphenyl-2,2’-diol and 4-(2-(4-hydroxy- phenyl) propan-2-yl)-2-(4-(2-(4-hydroxyphenyl) propan-2-yl) phenoxy)phenol) were identified by ESI-MS and NMR analysis. These results indicated that BPA was oxidized to BPA phenoxy radicals by ligninolytic enzymes and then dimerized at extracellular region.

1 **Introduction**

2 Endocrine-disrupting compounds (EDCs) are organic compounds widely used, which are
3 ubiquitous in the environment. Many synthetic chemicals released into the environment, such as
4 plasticizers, pesticides, antifouling paints, raw materials for polymer and hormonal
5 pharmaceuticals, have been identified to be potent EDCs and their environmental occurrence and
6 ecotoxicological effects are being actively studied in recent years [4, 11]. One of the
7 representative compounds of the EDCs group is bisphenol A (2,2-bis(4-hydroxyphenyl)propane;
8 BPA). BPA, a monomer component of polycarbonate plastics, is used in many consumer products,
9 including lacquers applied as food-can linings and dental composite fillings and sealants [3, 14].
10 Many countries throughout the world have large production capacities for BPA, especially
11 Germany, the Netherlands, the USA and Japan. BPA is one of the highest volume chemicals
12 produced worldwide, with an estimated production of 5 million tons in 2010 [1]. As a result, BPA
13 is often detected in many soil and water environments [10, 12].

14 Lignin-degrading white-rot fungi, which have the unique ability to degrade lignin to the
15 level of CO_2 [9], and their ligninolytic enzymes have also attracted interest for the
16 biotransformation of contaminants because of their industrial potential for degrading and
17 detoxifying recalcitrant environmental pollutants such as aflatoxin [24], neonicotinoid insecticides
18 [22], polycyclic aromatic hydrocarbons [2, 6], and dyes [15].

19 In recent years, the degradation of EDCs by ligninolytic enzymes has attracted the attention
20 of many researchers [8, 16, 18, 19]. More recently, we also reported that the removal of BPA by
21 lignin peroxidase from hyper lignin-degrading fungus *P. sordida* YK-624 [23]. However,
22 degradation of BPA using these fungi is also limited [5, 17] and the detail mechanisms are still
23 unknown. In the present study, we examined the removal of BPA by *P. sordida* YK-624 under
24 ligninolytic condition. We also detected the metabolites from BPA and proposed a metabolic
25 pathway for the metabolism of BPA by *P. sordida* YK-624.

26

27 **Materials and methods**

28 **Fungi.**

29 *P. sordida* YK-624 (ATCC 90872), which has been isolated from rotted wood, was used in
30 the present study [7]. The fungus was maintained on potato dextrose agar (PDA) slants at 4°C.

31 **Chemicals**.

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

1 **Elimination experiments.**

2 Nitrogen-limited (NL) medium described by Tien and Kirk [20] was used for BPA
3 elimination experiments. The fungus was incubated on a PDA plate at 30°C for 3 days, and then
4 10 mm-diameter disks were punched out from the growing edge of mycelium. Two disks were
5 each placed into a 100-mL Erlenmeyer flask containing 10 mL of NL medium. After statically
6 incubating the flasks at 30°C for 4 days, 100 µL of 10 or 100 mM BPA/DMSO were added to the
7 cultures (final concentration at 0.1 or 1 mM, respectively), and then further incubated for 24, 48,
8 72 hours (each in triplicate). The culture was filtrated with a 0.2-µm membrane filter, and the
9 filtrate was then subjected to high-performance liquid chromatography (HPLC) for the
10 quantification of BPA under the following conditions: column, Wakosil-II 5C18HG (4.6 x 150
11 mm; Wako Pure Chemical Industries); mobile phase, 60% MeOH in 1% acetic acid aq.; flow rate,
12 1 mL/min; and UV wavelength, 277 nm.

13 **Metabolism experiment.**

14 Inoculated cultures (1 L of NL medium) of *P. sordida* YK-624 were prepared under the
15 conditions described above. After static incubation at 30 °C for 4 days, BPA (final concentration,
16 1 mM) as added to these cultures. The cultures were further incubated for 24 hours and then
17 homogenized 2 times by Waring blender for 30 seconds with low speed. The cell suspensions
18 were extracted 3 times with equal amount of ethyl acetate (EtOAc). The EtOAc extract was dried
19 over anhydrous sodium sulfate and then evaporated to dryness. The residue was cleaned on a silica
20 gel chromatography (silica gel 60N, φ40 × 600 mm) and eluted with
21 dichloromethane/acetone/MeOH (9/1/0, 8/2/0, 5/5/0, 0/7/3, 0/5/5, and 0/0/10; vol/vol/vol) to
22 obtain 11 fractions. Each fraction was analyzed by thin-layer chromatography (TLC). Silica gel
23 plates (Merck F254; Merck, Darmstadt, Germany) were used for analytical TLC. The metabolite
24 was further separated by HPLC (column: C18; Wakosil-II 5C18HG Prep, 50×250 mm, Wako
25 Pure Chemicals, Japan) using 60% MeOH. The purified metabolite was analyzed by HR-ESI-MS
26 and NMR, including DEPT, COSY, HMQC, and HMBC experiments. The HR-ESI-MS data were
27 measured using a JMS-T100LC mass spectrometer. ^{1}H-NMR spectra were recorded using a Jeol
28 Lambda-500 spectrometer at 500 MHz, while ^{13}C-NMR spectra were recorded on the same
29 instrument at 125 MHz.

30

31 **Results and Discussion**

32 We have previously investigated the removal of BPA by lignin peroxidase from hyper
33 lignin-degrading fungus *P. sordida* YK-624 [22]. In the present study, NL media were used for the
34 elimination experiment of BPA. After preculturing by *P. sordida* YK-624, BPA (final

concentration, 1 mM or 0.1 mM) was added to cultures. After a 24 h treatment, 60% or 100% of BPA were eliminated, respectively (Fig. 1).

The removal of EDCs’ estrogenic activities by ligninolytic enzymes from white-rot fungi has been reported [18, 19, 22]. However, there have been no studies focusing on the metabolic product of these ECDs by fungi. Therefore, we attempted to identify the metabolites of BPA, and two metabolites were detected in the analysis of TLC and HPLC (data not shown). Molecular formula of compound 1 was determined as $C_{30}H_{30}O_4$ by HR-ESI-MS, *m/z* 453.2062$[M-H]^-$(calcd. for $C_{30}H_{29}O_4$, 453.2022). This formula suggested that the metabolite might be a dimer of BPA. The complete assignment of all the protons and carbons was accomplished by DEPT, HMQC, COSY and HMBC experiments. The structure of compound 1 was identified as 5,5’-bis-[1-(4-hydroxy-phenyl)1-methyl-ethyl]-biphenyl-2,2’-diol (Fig. 2a). Compound 1 was the same as the metabolite which has been detected by the treatment of BPA by lignin peroxidase from hyper lignin-degrading fungus *P. sordida* YK-624, and we have demonstrated that the estrogenic activity of the metabolite was lower than that of BPA [22]. Molecular formula of compound 2 was the same to compound 1. The complete assignment of all the protons and carbons was accomplished by DEPT, HMQC, COSY and HMBC experiments. The DEPT experiment indicated the presence of 4 methyls, 9 methylenes, and 11 quaternary carbons (data not shown). Compound 2 also had 1,2,4-trisubstituted phenyl group and 1,4-substituted group. The complete structure was determined by interpretating HMBC correlations (Fig. 2b) (H-1’”, 3’”/phenol C-4’, C-2”, hydroxyphenyl C-1’, H-1”, 3”/phenol C-4, C-2”, hydroxyphenyl C-1), and the connection to phenol C-1 was not determined. The structure of compound 2 was identified as 4-(2-(4-hydroxy- phenyl) propan-2-yl)-2-(4-(2-(4-hydroxyphenyl) propan-2-yl) phenoxy)phenol. This is the first report that compound 2 was chemically identified. We have assayed the formation of compound I and II by HPLC. However, trace amounts of two metabolites have detected. This result suggests that further radical coupling would occur during the fungal treatments. Tsutsumi et al. have reported that BPA tetramers were formed by manganese peroxidase treatment (21). Similarly, it is thought that BPA tetramers are formed as main products by fungal treatment, following the dimerization of BPA.

The estrogenic activity of more than 500 chemicals were tested by the yeast two-hybrid system (13), deducing that most of the compounds which have a phenol ring with substitution of a bulky moiety at the *ortho*-position reduces the activity. We propose that the estrogenic activity of two metabolites should be reduced because the radical coupling occurred at the *ortho*-position.

Compound 1 was completely disappeared after 48 h treatment under ligninolytic condition (data not shown). It is possible that compound II is also removed under the condition. Furthermore,

1 a proteomic differential display technique was utilized to study cellular responses of this fungus
2 exposed to BPA, and both BPA and BPA polymers were not incorporated into intracellular region
3 under ligninolytic condition since proteome map of cytosolic fraction of this fungus grown in the
4 presence of BPA is same as that grown in the absence of BPA (data not shown). Therefore, we
5 propose that BPA is oxidized to these phenoxy radicals by ligninolytic enzymes, and BPA dimers
6 are formed by radical couplings at extracellular region without the incorporation into the cell of *P.*
7 *sordida* YK-624. Moreover, BPA dimer is converted to BPA tetramer, and then, the toxicity is
8 decreased by the polymerization of BPA.

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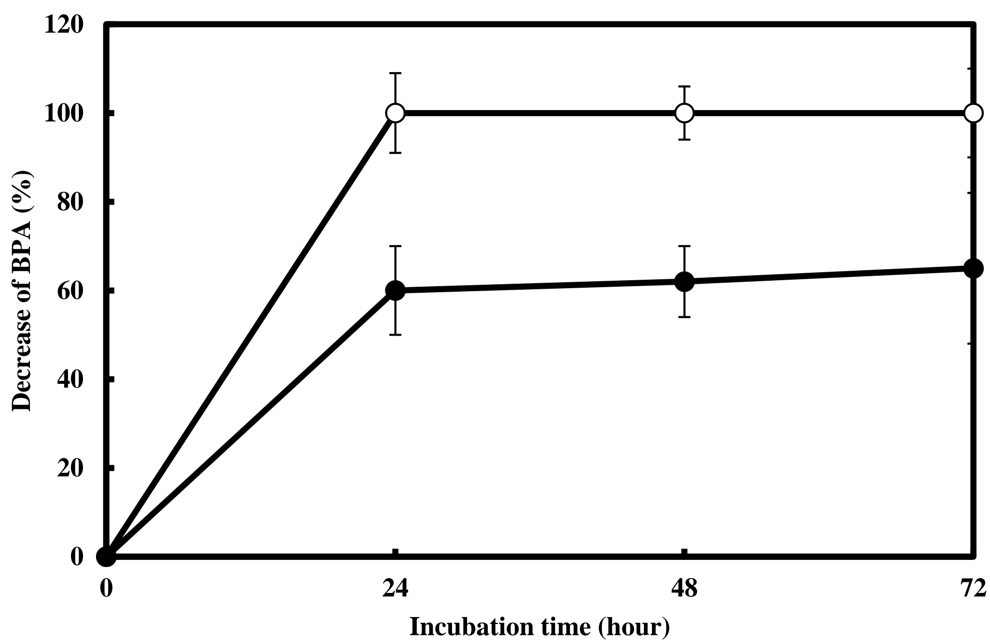

Fig. 1 Time course for BPA elimination by *P. sordida* YK-624. *Closed circles* 1 mM BPA, *open circles* 0.1 mM. Values are the means±SD of triplicate samples

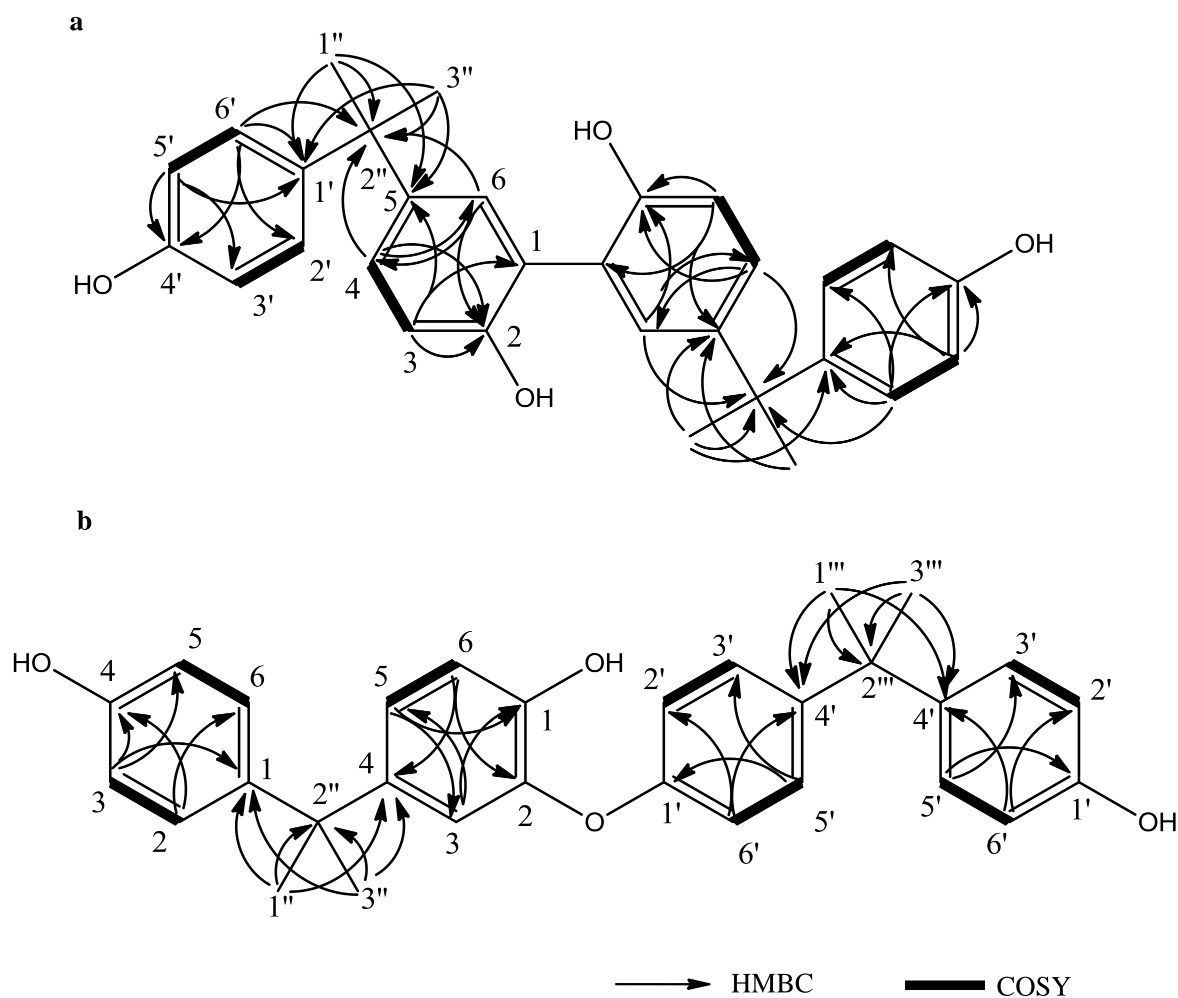

Fig. 2 COSY and HMBC correlations for the identified BPA metabolites

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

	Position	^{1}H δ_H (mult, J in Hz)	^{13}C δ_C
Compound 1	1	-	127.3
	2	-	152.7
	3	6.78(d, 8.2)	116.9
	4	7.05(dd, 8.2, 2.4)	127.8
	5		144.7
	6	6.89(d, 2.4)	131.5
	1′	-	143.2
	4′	-	156.2
	2′, 6′	7.05(d, 8.6)	125.8
	3′, 5′	6.68(d, 8.6)	115.6
	2″	-	42.6
	1″, 3″	1.57(s)	31.5
Compound 2	[phenol]		
	1	-	147.7
	2	-	144.8
	3	6.69(d, 2.0)	120.8
	4	-	144.3
	5	6.86(dd, 8.5, 2.0)	124.0
	6	6.80(d, 8.5)	141.4
	1′	-	156.1
	2′, 6′	6.64(d, 8.5)	115.6
	3′, 5′	7.01(d, 8.5)	128.8
	4′	-	143.1
	[hydroxyphenyl]		
	1	-	143.0
	2,6	6.99(d, 8.5)	128.7
	3,5	6.66(d, 8.5)	115.6
	4	-	156.1
	1′	-	146.5
	2′, 6′	7.10(d, 8.5)	128.9
	3′, 5′	6.72(d, 8.5)	117.3
	4′	-	157.2
	1″, 3″	1.53(s)	31.5
	2″	-	42.9
	1‴, 3‴	1.59(s)	31.6
	2‴	-	42.8