Degradation of Bisphenol A by White Rot Fungi, *Stereum hirsutum* and *Heterobasidium insulare*, and Reduction of Its Estrogenic Activity

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Two lignin-degrading basidiomycetes, *Stereum hirsutum* and *Heterobasidium insulare*, were used to degrade bisphenol A (BPA) in culture, and the estrogenic activity of the degradation products was examined using MCF-7 cell proliferation assays (E-screen) and analysis of pS2 mRNA expression in MCF 7 cells. Both *S. hirsutum* and *H. insulare* showed high resistance to BPA 100 ppm, and their mycelial growth was fully completed within 8 d of incubation at 30 °C. It took 7 to 14 d to achieve complete degradation (*ca.* 99%) of BPA by both fungi. MCF-7 cells proliferated actively at a BPA concentration of 10^{-5} M. However, cell line proliferation was significantly inhibited when the cells were incubated in BPA culture media containing *S. hirsutum* and *H. insulare*. Similar results were obtained regarding pS2 mRNA expression. The pS2 mRNA expression levels decreased by 1.5-fold in supernatant from BPA treated with *S. hirsutum* and *H. insulare* compared with those treated with BPA alone.

Key words bisphenol A; biodegradation; *Stereum hirsutum*; *Heterobasidium insulare*; estrogenic activity

Some of the recalcitrant chemicals that accumulate in the natural environment after widespread industrial usage due to resistance to biodegradation are considered to be toxic, mutagenic, carcinogenic, or estrogenic.[1)]

Bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl) propane), which is a major raw material used in the chemical synthesis of industrial polymers (plastics), is thought to be environmentally deleterious and is also suspected to be an endocrine disruptor.[2,3)] To date, the BPA degradation mechanisms have been established using bacteria in laboratory experiments. However, the practical application of such experiments is limited due to a lack of efficacy at high concentrations.[4)] Lignin-degrading basidiomycetes (white rot fungi) are being investigated as new potential tools for the biodegradation of harmful chemicals because they have three nonspecific extracellular enzymes: lignin peroxidase (LiP); manganese-dependent peroxidase (MnP); and laccase. It has been reported that MnP and laccase from *Pleurotus osrteatus* are able to degrade BPA and have the ability to ameliorate or eliminate the estrogenic activity of BPA.[5,6)] However, studies on other lignin-degrading basidiomycetes for the degradation of BPA are few, and the reduction of the estrogenic activity of BPA by those fungi, if any, has not been clearly established.

It has been suggested that *in vitro* assays are ideal for investigating the estrogenic potential of chemicals, and several types of *in vitro* assays have been proposed, including estrogen receptor (ER) binding, MCF-7 cell (or other ER-positive cell) proliferation, and ER-dependent transcription systems in transfected mammalian or yeast cells.[7—11)] Recently, the MCF-7 cell proliferation assay (E-screen) has become common as a rapid and straightforward test for detecting weakly estrogenic compounds. Originally, the MCF-7 human breast cancer cell line was derived from a pleural effusion taken from a woman with metastatic breast carcinoma who had previously been treated with radiation and hormone therapy. The MCF-7 cell line is ER-positive and responds to the presence of estrogens in culture with a proliferation response.[12)] In contrast to binding assays, this method has the advantage of biological response, which can be considered to represent a measure of the direct interaction of agonists with the ER and can hence be equated with estrogenic potential.[8)] The endogenous gene expression assay is an alternative assay that could be based on the quantification of estrogen-induced changes in the expression levels of endogenous genes, either in cultured cells or in selected tissues from exposed animals.[13)] The pS2 gene is expressed in breast cancer cells, but not in normal mammary cells.[14)] pS2 mRNA expression in MCF-7 cells is an ideal model for studying the effects of the estrogenic response. In addition, expression of the pS2 gene has been used as a marker of estrogen responsiveness in ER-containing breast cancer cells, an indicator of disease progression, and a predictor of the success of antiestrogen therapy in breast cancer patients.[15,16)]

In this study, we addressed the possibility of biological degradation of BPA with two selected white rot fungi, *Stereum hirsutum* and *Heterobasidium insulare*. The degree of biodegradation of BPA was determined with high-performance liquid chromatography (HPLC) analysis, and possible degradation products were determined using the gas chromatography-mass spectrometry (GC-MS) technique after TMS derivatization. To evaluate the reduction in BPA toxicity due to fungal treatment, estrogenic activity levels were evaluated in two assays: MCF-7 cell proliferation and pS2 mRNA expression assays.

MATERIALS AND METHODS

Reagents BPA was commercially purchased from Yakuri Pure Chemicals Co. Ltd. Acetone, ethyl acetate, acetonitrile, and hexane for HPLC analysis were used after degassing with helium for 24 h. *N*,*N*-Dimethylformamide (DMF) was obtained from Kanto Chemical Co. All other reagents used for incubation were of reagent grade.

Screening of Fungi and Optimal Mycelium Growth Conditions *S. hirsutum* and *H. insulare* were selected by screening various white rot fungi stored at the Korea Forest

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Research Institute using the Remazol Brilliant Blue R method and determination of ligninase activity. Both fungi were first grown in potato dextrose agar (PDA) medium at 30 °C for 7 d, then transferred to a complex medium (YMPG), and kept at 4 °C until use. The composition of the YMPG medium (per liter of distilled water) was 10 g of glucose, 10 g of malt extract, 2 g of peptone, 2 g of yeast extract, 1 g of asparagine, 2 g of KH_2PO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 1 mg of thiamine, and 20 g of agar. Optimal growth temperature was determined by measuring the hyphal extension length on 2% malt extract agar medium after optimal pH determination. The 7-mm plugs of inoculum from the YMPG medium slant were transferred to plates containing 2% malt extract agar medium, which were kept at 17, 25, 30, and 34 °C. Three replicates of each fungus at each temperature were prepared. Hyphal extension lengths were measured daily based on the usual colony diameter (unit: cm) in the longest perpendicular direction.

Resistance to Various BPA Concentrations To determine BPA resistance, the inocula of both fungi were cultivated on YMPG medium, which contained BPA concentrations of 50, 100, and 500 ppm. The cultivated medium was incubated at 30 °C for 14 d. Three replicates were prepared of each fungus at each concentration with control. The hyphal extension lengths of fungi were measured daily. Complete growth was assumed when the hyphal length reached 8.5 cm in the Petri dish.

Analysis of Degradation Products Using HPLC and GC-MS Both fungi were incubated in shallow stationary culture (SSC medium) as previously described.[17] The culture medium pH was adjusted to 4.5 using a 2,2-dimethylsuccinic acid buffer (1.46 g/l), and both fungi were incubated in flasks without shaking at 30 °C. After full growth of the mycelium, BPA was added to the culture medium with an inoculated syringe to a final concentration of 100 μM to determine the degrading ability of the fungus. After each indicated interval (1, 3, 7, 14 d), the residual nondegraded BPA was periodically extracted with 40 ml of ethyl acetate and hexane and filtered with a 0.45-μm microfilter. Residual BPA in organic solvent was quantitatively determined using HPLC (HPLC 1100 series). HPLC analysis was performed on a Waters symmetric reverse-phase column (4.6×250 mm) using the linear gradient solvent system in which the mobile phase starts with 50% acetonitrile and 50% water, is held for 10 min, and then increases in a linear fashion to 100% acetonitrile in 20 min. The signals were monitored at 280 nm and the flow rate was 0.6 ml/min. The residual BPA and degraded products were also subjected to metabolic analysis using GC (HP6890)-MS (JMS-600W, JEOL). The organic solvent was evaporated or concentrated by bubbling with N_2 gas to 100 μl, and silylated with *N*,*O*-bis-(trimethylsily) trifluoroacetamide (50 μl) and pyridine (50 μl) at 60 °C for 1 h. The silylated products were separated onto the DB-5 column (60 m×0.25 μm×0.25 μm). The GC conditions were helium as carrier gas, flow rate 1.0 ml/min, injector 270 °C, detection 285 °C, and the oven temperature was initially held at 80 °C for 5 min, and then increased at a rate of 8 °C/min to 160 °C and held for 5 min, again increased at a rate of 5 °C/min, and then finally held for 5 min. The effluent from the GC column underwent MS, the mass fragmentation was obtained in the EI mode, at 70-eV ionization energy, and 50—800 amu scanning for 2 s. The BPA degradation metabolites of both fungi were identified by comparison with MS library information (Wiley Registry of Mass Spectra Data, 6th ed.).

Enzyme Assay To assess the induction effect of ligninase activities such as MnP and laccase due to the addition of BPA, 100-ml flasks were first inoculated with 7-mm plugs of *S. hirsutum* and *H. insulare* and incubated as described above. After 7 d, BPA was added to flasks at a final concentration of 200 ppm. On each incubation day, aliquots of extracellular fluid were collected at 1, 3, 5, 7, and 9 d by separating the supernatant from the fungal mycelia in the culture medium by centrifugation.

Ligninase activities for extracellular fluids were spectrophotometrically determined at 30 °C. MnP and laccase were determined by monitoring the ABTS oxidation (0.08 g/l, $\varepsilon_{414}=36000\ \mathrm{M}^{-1}\,\mathrm{cm}^{-1}$). The reaction mixture for the MnP assay contained ABTS, 0.2 mM of $MnSO_4$ and 0.2 M of sodium lactate buffer (pH 4.5), in a total volume of 3 ml, and the assay was started with the addition of 0.1 mM H_2O_2.

The activities for MnP and laccase were calculated as follows: μkat=(Δabsorbance×total volume×10^6)/(36000×Δtime×sample volume)×100000.

MCF-7 Cell Culture and Cell Proliferation Assay MCF-7 cells were maintained in 25-cm^2 cell culture flasks in full medium consisting of Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 5% heat-inactivated fetal bovine serum (FBS, Gibco BRL). These were kept in a humidified incubator at 37 °C and 5% CO_2 over a maximum of 12 passages and routinely tested for mycoplasma. The methods used for the cell proliferation assays were a modification of those of Soto *et al.*[8] One 70% confluent 25-cm^2 flask of MCF-7 cells was washed with 5 ml phosphate buffered saline (PBS, Amresco) before the addition of 3 ml of trypsin-EDTA (0.05% trypsin, EDTA 0.53 mM, Gibco BRL). The flask was left for 1 min at room temperature, after which the cells were detached by a firm slap, resuspended in 10 ml of full medium, counted using an improved Neubauer counting chamber, and seeded into well plates at a density of 2×10^4 cells/well in 2 ml of full medium. After 24 h the cells were washed with PBS 5 ml. The medium was changed to 2 ml of estrogen-free medium (phenol red-free DMEM with 5% charcoal dextran-treated human serum, CDHuS) for 48 h. After washing with PBS, one group was treated with original BPA (10^{-5} M) in DMEM for 6 d, and another group was treated with BPA that had been incubated with *S. hirsutum* or *H. insulare* for 1, 3, 7 or 14 d. Cell proliferation was assessed after 6 d in culture using the method of Skehan *et al.*[18]

pS2 mRNA Expression Assay MCF-7 cells (1×10^6) were seeded in 25-cm^2 flasks in 5% FBS-supplemented DMEM. The test substances were treated using the same methods as in the proliferation assay. After 6 d of exposure to BPA, the medium was aspirated, the cells were rinsed with PBS, and total RNA was extracted using Trizol (Sigma Chemical Co.) following the manufacturer's protocol. Five micrograms of total RNA were reverse-transcribed using M-MLV reverse transcriptase (Ambion Inc., Texas, U.S.A.) and random primer (9 mer). The samples contained pS2 primers (pS2-1, 5′-GGCCACCATGGAGAACAAGG-3′ and pS2-2, 5′-CCACGAACGGTGTCGTCGAA-3′) and primers for the 1A gene (1A-1, 5′-GATATGGCGTTTCCCCGCATA-3′ and 1A-2, 5′-GGATTTTGGCGTAGGTTTG G T-3′). To deter-

mine the conditions under which PCR amplification for pS2 and 1A mRNA was in the logarithmic phase, the aliquots (1 μl) were amplified using different numbers of cycles. PCR for the 1A gene was amplified to rule out the possibility of RNA degradation and was used to control the variation in mRNA concentrations in the room-temperature reaction. A linear relationship between PCR products and amplification cycles was observed in both pS2 and 1A mRNA (data not shown). Thirty cycles for pS2 and 25 cycles for 1A gene were employed for quantification. PCR reactions were denatured at 95 °C for 1 min, annealed at 50 °C for 1 min, and extended at 72 °C for 90 s. The products were visualized using agarose gel electrophoresis stained with ethidium bromide, and then the photograph was scanned and analyzed using the molecular analysis program version 1.5 (Gel Doc 1000, Bio-Rad).

RESULTS AND DISCUSSION

Resistance to Various BPA Concentrations The BPA resistance of *S. hirsutum* and *H. insulare* was evaluated by comparing the mycelium growth, growth rate, and required incubation days with the control that did not contain BPA. The fungal mycelium growth of the two fungi at various BPA concentrations is shown in Fig. 1. Before the resistance analysis, the optimal incubation temperature for maximal mycelium growth of *S. hirsutum* and *H. insulare* was 30 °C with an average growth rate of 2 cm/d in the initial stage. Slight inhibition of mycelium growth was shown at 17, 25, and 34 d. The control medium, which contained no BPA, exhibited fast mycelium growth with growth rates of 2.0 and 2.5 cm/d in *S. hirsutum* and *H. insulare*, respectively. However, as the BPA concentration increased, the mycelium growth rate decreased to approximately 1 cm/d. At BPA 50 and 100 ppm, 7 to 8 d were required for full mat coverage by the mycelia of *S. hirsutum* and 8 to 10 d for that of *H. insulare* mycelia. At BPA 500 ppm, the mycelium did not grow. In the resistance analysis, it was found that the two fungi were extremely resistant to low BPA concentration (less than 50—100 ppm), whereas mycelium growth was gradually inhibited at high concentrations (greater than 100 ppm). Significant inhibition was shown at BPA concentrations exceeding 500 ppm.

BPA Fungal Treatment and Estimation of BPA Degradation by HPLC Spivack *et al.* reported that a gram-negative aerobic bacterium could degrade BPA, but degradation of BPA by microorganisms other than bacteria has not yet been attempted.[4] White rot fungi such as *Phanerochaete chrysosporium* have been known to be excellent biodegraders of lignin and resistant pollutants such as pentachlorophe-

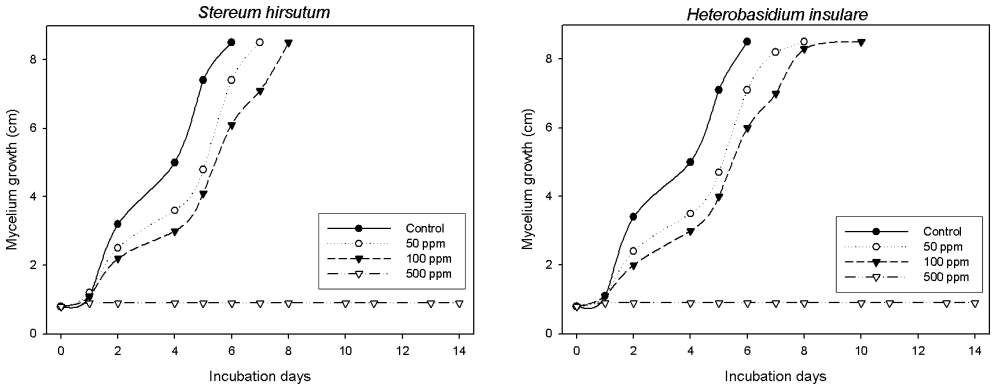


Fig. 1. Resistance of *S. hirsutum* and *H. insulare* to BPA Concentrations of 50, 100, and 500 ppm during 14-d Incubation

Mycelium was considered to have attained full growth on the day when it fully covered the mat, and reached 8.5 cm in perpendicular length on the Petri dish. Each data point represents the mean of three separate experiments.

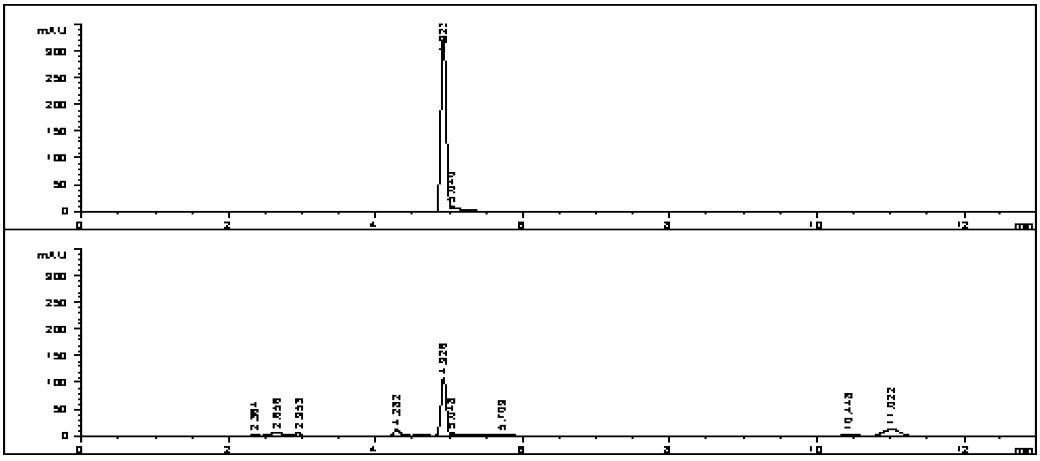


Fig. 2. HPLC Chromatogram of BPA Metabolites after 3-d Incubation

BPA 200 ppm was incubated with *S. hirsutum* and *H. insulare* in liquid SSC culture. The retention time of BPA in the chromatogram was 4.924 min. The upper panel indicates the chromatogram of 0-h treatment, and the lower after 24-h treatment.

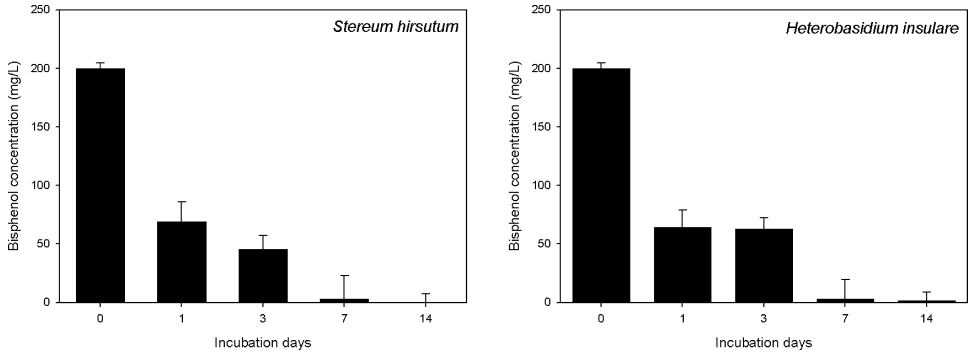


Fig. 3. Time Course of BPA Degradation in Culture Media of *S. hirsutum* and *H. insulare*

Each data point represents the mean of three separate experiments±S.D.

nol, 2,7-dichlorodibenzodioxin, and trichlorophenol.[19—22] Therefore we employed the extracellular white rot fungi *S. hirsutum* and *H. insulare* to degrade BPA and to ameliorate its estrogenic activity.

BPA 200 ppm was added to each culture medium, which was then incubated for 14 d. HPLC chromatograms of metabolites derived from the biodegradation of BPA in liquid SSC culture are shown in Fig. 2. When BPA 200 ppm was added at the beginning of culture, the residual BPA was 64.1 and 62.8 ppm after 1- and 3-d incubation in liquid SSC medium of *H. insulare*, and 63.1 and 45.6 ppm in that of *S. hirsutum*, respectively. These figures suggest that both fungi removed 68 to 77% of BPA after 3-d incubation. Moreover, after 7 d of incubation, only trace amounts of BPA remained in the liquid culture medium, and no residual BPA was determined at 14 d of incubation (Fig. 3). Although there was a difference in the biodegradation of *S. hirsutum* and *H. insulare*, both fungi exhibited impressive BPA conversion. Tsutsumi *et al.* completely removed BPA within 1 h with using laccase and MnP.[6] Compared our results, the ligninolytic enzyme treatments appear to be more efficient in the removal of BPA.

Most researchers have considered that the major lignin-degrading extracellular systems under secondary metabolic conditions were good prospects for the degradation of recalcitrants.[23—25] As shown in Fig. 4, however, the extracellular enzyme systems of both fungi were not strongly induced by the addition of BPA, while the activities of MnP and laccase were slightly increased in the *H. insulare* and *S. hirsutum* culture media. However, laccase activity was always higher than that of MnP and remained stable throughout the incubation. Therefore laccase might play a key role in BPA biodegradation.[6,26]

Analysis of BPA Metabolites Using GC-MS The change in the amount of metabolites depending on the incubation period could be used to determine the degradation pathway. According to the above results, the two fungi appeared to degrade BPA. The following data explain the metabolites or intermediates produced.

The metabolites and intermediates obtained were analyzed using GC-MS during BPA degradation (Table 1). There was little difference in metabolites and intermediates in terms of the incubation length and fungus, implying that the degradation abilities of the two fungi rely on similar metabolization mechanisms and reactions.

The most abundant product among analyzed compounds

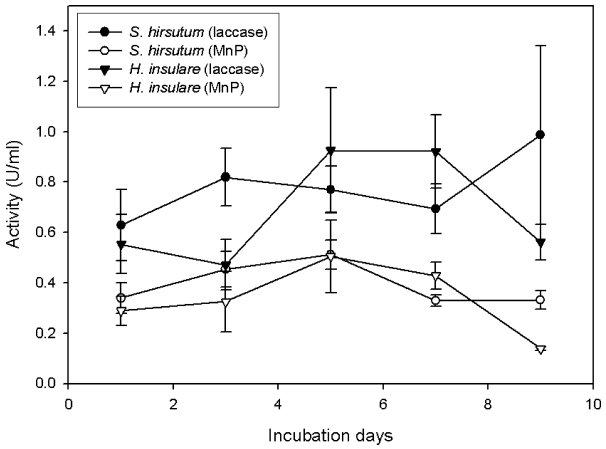


Fig. 4. Change in Ligninase Activities Induced by the Addition of BPA to Culture Media of *S. hirsutum* and *H. insulare* Depending on Incubation Time

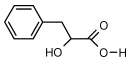
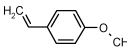
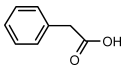
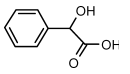
One unit is defined as that forming the oxidation product of ABTS 1 mmol for 1 min. Each data point represents the mean of three separate experiments±S.D.

was 2-hydroxy-3-phenyl propanoic acid, followed by 1-ethenyl-4-methoxybenzene, and then by phenylacetic acid and its hydroxylated compound at C2. These compounds were assumed to originate from the phenol of BPA through dehydroxylation, carboxylation, and hydroxylation on the side chain, in that order. In the case of 1-ethenyl-4-methoxybenzene, methylation was assumed to occur on the phenyl ring.

These results illustrate that the hydroxylation of degradation compounds by oxidoreductases in the medium occurs more easily on alkyl side chains than on aromatic rings. This finding requires more study in the future. Hirano *et al.*[5] reported that the major metabolites of BPA treated with MnP from *P. ostreatus* were phenol, 4-isophenyl phenol, 4-isopropyl phenol, and hexestrol. Some phenolic compounds were also detected in this study, but the compounds were slightly different (data not shown). We could not identify some ring cleaved compounds that occurred on a pathway involving peroxidase- or laccase-catalyzed reactions. Instead, various fatty acids were observed during fungal metabolization of BPA, such as tetradecanoic, hexadecanoic, and octadecen-1-enoic acids, which ranged from C8 to C18. However, it was not clear what role the fatty acids might play in BPA biodegradation.

Proliferative Responses in MCF-7 Cells To determine the responsiveness of BPA as an endocrine disruptor, we car-

Table 1. Degradation Products of Bisphenol A by *S. hirsutum* and *H. insulare*

Name	Chemical formula	Structure	Major peaks in mass spectrum (bp, base peak; mp, molecular peak)
2-Hydroxy-3-phenyl propanoic acid	$C_9H_{10}O_3$		73, 147, 193 (bp), 220, 295, 310 (mp)
4-Methoxyphenyl ethene	$C_9H_{10}O$		65, 91, 119, 134 (bp, mp)
2-Phenyl acetic acid	$C_8H_8O_2$		73 (bp), 91, 164, 193, 208 (mp)
2-Hydroxy-2-phenyl acetic acid	$C_8H_8O_3$		73, 147, 179 (bp), 253, 296 (mp)

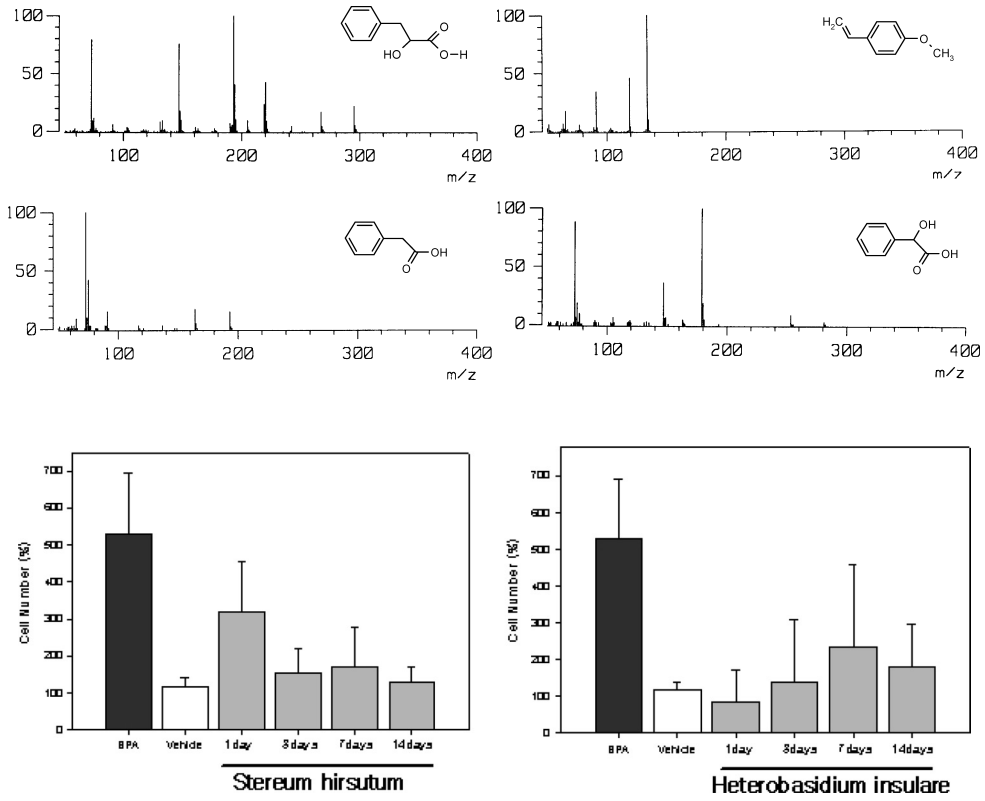


Fig. 5. Reduction of BPA Estrogenic Activity upon Treatment with *S. hirsutum* (A) and *F. insulare* (B)

MCF-7 cells were treated with equal molarity BPA (10^{-5} M) or supernatants preincubated with *S. hirsutum* and *H. insulare*. The rate of proliferation is expressed as the fold increase in cell number above the hormone-free control (vehicle). The results are expressed as mean±S.D.

ried out comprehensive dose–response analyses with the MCF-7 cell line (data not shown). Cells were treated with a range of concentrations of the chemical according to a protocol originally devised by Soto *et al.*[8] There was a significant effect at a BPA concentration of 10^{-5} M. BPA was initially cultured with *S. hirsutum* and *H. insulare* at a concentration of BPA 200 ppm. After 1, 3, 7, and 14 d of culture, the supernatant was harvested and used for proliferation and pS2 expression assays by addition to MCF-7 cells for 6 d. Figure 5 shows the BPA biodegradation effects of both *S. hirsutum* and *H. insulare*. BPA increased the rate of proliferation of MCF-7 cells compared with the vehicle by a factor of 4.5, and these levels were reduced to 40% supernatant treated with *S. hirsutum* after culturing for 1 d. After incubation for 3 d, BPA treated with *S. hirsutum* had no proliferative effects when compared with the vehicle. Unlike *S. hirsutum*, *H. insulare* clearly blocked the proliferation effects of BPA after 1-d culture.

In this study, we estimated the estrogenic activity of BPA using MCF-7 cells and the degradation effects of *S. hirsutum* and *H. insulare* for estrogenic compounds. Although there was insufficient evidence to determine the mechanism of the proliferation, it is likely that estrogenic responses were caused *via* not only the ER but also other factors.[13] Compared with the negative control, BPA clearly increased the rate of proliferation, and these results are consistent with other data, both *in vitro* and *in vivo*.[2,3,27—29] BPA 10^{-5} M significantly increased the rate of proliferation in MCF-7 cells, and the increased rate was blocked when treated with supernatants from *S. hirsutum* and *H. insulare* in BPA.

pS2 mRNA Expression Assay The expression of pS2 is widely considered to be due to an estrogen response gene, in response not only to BPA treatment but also as a result of the BPA degradation effects of *S. hirsutum* and *H. insulare*. The

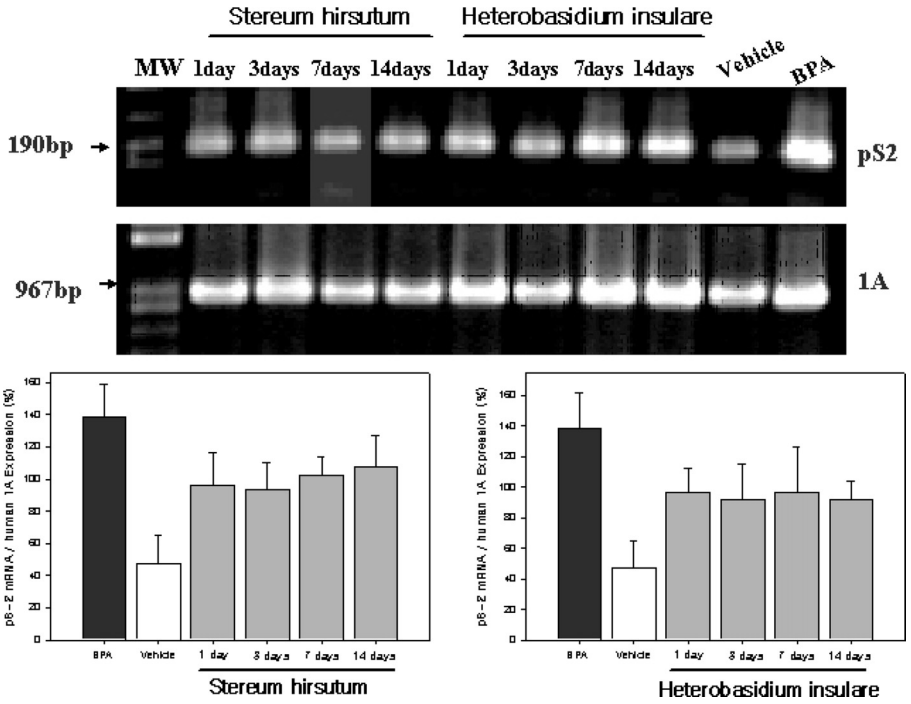


Fig. 6. Effects of BPA and Supernatants from *S. hirsutum* and *H. insulare* Treated with BPA on pS2 mRNA Expression

MCF-7 cells were treated with BPA (10^{-5} M) or supernatants preincubated with *S. hirsutum* and *H. insulare*. RT-PCR products were used for pS2 gene analysis. The pS2 mRNA signal was determined in densitometric analysis and was normalized by the human 1A mRNA signal from each lane. The results are expressed as mean±S.D.

same fungi, *S. hirsutum* and *H. insulare*, were used to degrade BPA as a proliferation assay. The levels of pS2 mRNA were measured using RT-PCR techniques, following exposure of BPA to the fungi, and expression levels were normalized by human 1A. Increased pS2 mRNA levels were detected in cells (10^{-5} M) treated with BPA alone (3-fold *vs.* vehicle) as a positive control, as shown in Fig. 6. Although it was not clear in the proliferation assay, the effects of BPA on the expression of pS2 transcripts were abolished by preincubation with *S. hirsutum* and *H. insulare* (1.5-fold *vs.* BPA). There were no fungal species differences between *S. hirsutum* and *H. insulare* (1.4-fold *vs.* BPA) in the level of the induced pS2 gene expression. The BPA degradation times for both *S. hirsutum* and *H. insulare* were unrelated to pS2 gene expression. Tsutsumi *et al.* also reported that the estrogenic activity of BPA, as determined in the two-hybrid yeast system, could be completely eliminated by MnP and laccase treatments, suggesting that the ligninolytic enzymes are effective in the removal of BPA estrogenic activity.[6)]

The pS2 mRNA expression was increased at a BPA concentration of 10^{-5} M, and the results were also consistent with the data of Jørgensen *et al.*[30)] The BPA decompositional effects of *S. hirsutum* and *H. insulare* were clear, although not significant, and were similar to those of the proliferation assay. The probable reason is that the regulating factors of proliferation and pS2 mRNA expression are different. It is unclear how estrogen regulates pS2 expression and whether or not the ER is involved in the process. The estrogen inducibility of pS2 has been shown to be a primary transcriptional event, resulting from an estrogen responsive element in the 5′ flanking sequence of the pS2 gene. However, in the ER-positive breast cancer cell line T47D and ER-HeLa cells, pS2 cannot be induced by estradiol, which suggests that estradiol may not regulate gene expression *via* the ER.[31)] pS2 gene expression is also found in carcinomas of the gastrointestinal tract, stomach, biliary tract, and pancreas, organs that do not have the ER, indicating that the expression of pS2 is not ER dependent.[32)] BPA exhibited similar estrogenic activity in the proliferation and pS2 mRNA expression assays. In conclusion, in terms of estrogenic activity, both *S. hirsutum* and *H. insulare* had a reducing effect on BPA, with similar tendencies in both these assays.

CONCLUSIONS

The white rot fungi *S. hirsutum* and *H. insulare* are able to resist BPA in culture, even under harsh conditions. Furthermore, they were shown to have excellent potential to degrade BPA in *in vitro* experiments. According to our experimental data, laccase may play a key role in BPA biodegradation mechanisms. However, synergistic effects with other enzymes may also be operating in the biodegradation system. In proportion to the period of fungal treatment of BPA with the two basidiomycetes, a gradual reduction in estrogenic activity was observed in proliferation assays in MCF-7 cell lines, as well as in pS2 gene expression assays, compared with the control experiment. These results strongly suggest that industrial toxic pollutants can be effectively disposed by lignin-degrading fungi without causing environmental pollution and that their toxicity can be eliminated by these biological treatments.

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REFERENCES

1) Bumpus J. A., Tien M., Wright D., Aust S. D., *Science*, **228**, 1434—1436 (1985).
2) Laws S. C., Carey S. A., Ferrell J. M., Bodman G. J., Cooper R. L., *J. Toxicol. Sci.*, **54**, 154—167 (2000).
3) Perez P., Pulgar R., Olea-Serrano F., Villalobos M., Rivas A., Metzler M., Pedraza V., Olea N., *Environ. Health Perspect.*, **106**, 167—174 (1998).
4) Spivack J., Leib T., Lobos J., *J. Biol. Chem.*, **269**, 7323—7329 (1994).
5) Hirano T., Honda Y., Watanabe T., Kuwahara M., *Biosci. Biotechnol. Biochem.*, **64**, 1958—1962 (2000).
6) Tsutsumi Y., Haneda T., Nishida T., *Chemosphere*, **42**, 271—276 (2001).
7) Jobling S., Reynolds T., White R., Parker M. G., Sumpter J. P., *Environ. Health Perspect.*, **103**, 582—587 (1995).
8) Soto A. M., Sonnenschein C., Chung K. L., Fernandez M. F., Olea N., Serrano F. O., *Environ. Health Perspect.*, **1103** (Suppl. 7), 113—122 (1995).
9) Welshons W. V., Rottinghaus G. E., Nonneman D. J., Dolan-Timpe M., Ross P. F., *J. Vet. Diagn. Invest.*, **2**, 268—273 (1990).
10) Arnold S. F., Robinson M. K., Notides A. C., Guillette L. J., McLachlan J. A., *Environ. Health Perspect.*, **104**, 544—548 (1996).
11) Klotz D. M., Beckman B. S., Hill S. M., McLachlan J. A., Walters M. R., Arnold S. F., *Environ. Health Perspect.*, **104**, 1084—1089 (1996).
12) Brooks S. C., Locke E. R., Soule H. D., *J. Biol. Chem.*, **248**, 6251—6253 (1973).
13) Petit F., Le Goff P., Cravedi J. P., Valotaire Y., Pakdel F., *J. Mol. Endocrinol.*, **19**, 321—335 (1997).
14) Jakowlew S. B., Breathnach R., Jeltsch J. M., Masiakowski P., Chambon P., *Nucleic Acids Res.*, **12**, 2861—2878 (1984).
15) Schwartz L. H., Koerner F. C., Edgerton S. M., Sawicka J. M., Rio M. C., Bellocq J. P., Chambon P., Thor A. D., *Cancer Res.*, **51**, 624—628 (1991).
16) Foekens J. A., Rio M. C., Seguin P., van Putten W. L., Fauque J., Nap M., Klijn J. G., Chambon P., *Cancer Res.*, **50**, 3832—3837 (1990).
17) Tien M., Kirk T. K., *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 2280—2284 (1984).
18) Skehan P., Storeng R., Scudiero D., Monks A., McMahon J., Vistica D., Warren J. T., Bokesch H., Kenney B. S., *J. Natl. Cancer Inst.*, **82**, 1107—1112 (1990).
19) Lamar R. T., Dietrich D. M., *Appl. Environ. Microbiol.*, **56**, 3093—3100 (1990).
20) Chung N., Aust S. D., *Arch. Biochem. Biophys.*, **322**, 143—148 (1995).
21) Valli K. V., Wariishi H., Gold M. H., *J. Bacteriol.*, **174**, 2131—2137 (1992).
22) Joshi D. K., Gold M. H., *Appl. Environ. Microbiol.*, **59**, 1779—1785 (1993).
23) De Long E., Field J. A., de Bont J. A. M., *FEBS Lett.*, **299**, 107—110 (1992).
24) Mileski G. J., Bumpus J. A., Jurek M. A., Aust S. D., *Appl. Environ. Microbiol.*, **54**, 2885—2889 (1988).
25) Ruttimann-Johnson C., Lamar R. T., *Appl. Environ. Microbiol.*, **62**, 3890—3893 (1996).
26) Okazaki S., Michizoe J., Goto M., Furusaki S., Wariish H., Tanaka H., *Enzyme Microbial Technol.*, **31**, 227—232 (2002).
27) Jones P. A., Baker V. A., Irwin A. J. E., Earl L. K., *Toxicol. Vitro*, **12**, 373—382 (1998).
28) Andersen H. R., Andersson A. M., Arnold S. F., Autrup H., Barfoed M., Beresford N. A., Bjerregaard P., Christiansen L. B., Hummel G. B., *Environ. Health Perspect.*, **107**, 89—108 (1999).
29) Diel P., Schulz T., Smolnikar K., Strunck E., Vollmer G., Michna H., *J. Steroid Biochem. Mol. Biol.*, **73**, 1—10 (2000).
30) Jørgensen M., Vendelbo B., Skakkebaek N. E., Leffers H., *Environ. Health Perspect.*, **108**, 403—412 (2000).
31) Maminta M. L., Molteni A., Rosen S. T., *Mol. Cell. Endocrinol.*, **78**, 61—69 (1991).
32) Welter C., Theisinger B., Rio M. C., Seitz G., Schuder G., Blin N., *Int. J. Cancer*, **56**, 52—55 (1994).