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Estrogenic Reduction of Styrene Monomer Degraded by *Phanerochaete chrysosporium* KFRI 20742

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The characteristic biodegradation of monomeric styrene by *Phanerochaete chrysosporium* KFRI 20742, *Trametes versicolor* KFRI 20251 and *Daldinia concentrica* KFRI 40-1 was carried out to examine the resistance, its degradation efficiency and metabolites analysis. The estrogenic reduction effect of styrene by the fungi was also evaluated. The mycelium growth of fungi differentiated depending on the concentration levels of styrene. Additionally *P. chrysosporium* KFRI 20742 showed superior mycelium growth at less than 200 mg/l, while *D. concentrica* KFRI 40-1 was more than 200 mg/l. The degradation efficiency reached 99% during one day of incubation for all the fungi. Both manganese-dependent peroxidase and laccase activities in liquid medium were the highest at the initial stage of incubation, whereas the lowest was after the addition of styrene. However, both activities were gradually recovered after. The major metabolites of styrene by *P. chrysosporium* KFRI 20742 were 2-phenyl ethanol, benzoic acid, cyclohexadiene-1,4-dione, butanol and succinic acid. From one to seven days of incubating the fungi, the expression of pS2 mRNA widely known as an estrogen response gene was decreased down to the level of baseline after one day. Also, the estrogenic effect of styrene completely disappeared after treatment with supernatant of *P. chrysosporium* KFRI 20742 from one week of culture down to the levels of vehicle.

***Keywords*:** styrene, *Daldinia concentrica, Phanerochaete chrysosporium,* white rot fungi, biodegradation, ligninase

Today, synthetic chemicals are being used in housewares, agricultural chemicals, medicines, food and industrial materials. However, some of these materials cannot be readily biodegraded. Therefore, they have accumulated extensively in soil, water and etc., and made considerable problems.

Recently, it has been reported that endocrine disruptors act like normal hormones due to their similar chemical structure which disturb or confuse the function of normal hormones in humans or animals. Therefore, these may cause a malformation, decline of reproductive function, change of action, cancer of sexual glands etc. (Sato *et al*., 1991; While *et al*., 1994). These materials can be exposed to living organisms by various routes in our environment. The 67 kinds of chemicals were already designated as endocrine disrupting chemicals by the World Wildlife Fund (WWF) (US EPA, 1997).

Styrene, an important industrial polymer, has been used as a raw material for wrapping and transporting goods. Styrene was eluted from these products and then was polluted in water, air and soil. Exposure to styrene, as in the case of workers in polystyrene producing factories, results in the irritation of the eyes and respiratory system. It has a toxic effect on the liver, kidneys and nervous systems. Also, the transformation product of styrene in the liver, styrene oxide can cause a mutagenic and carcinogenic effect (Braum *et al*., 1997; Lu *et al*., 2001; Mario *et al*., 2001), therefore styrene was sometimes considered as a suspect endocrine disrupting chemical.

One feature of styrene is the natural occurrence of this compound, even though it has undoubtedly been influenced by the evolution of microbial styrene

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catabolic routes. Since the 1970s, styrene degrading microbes including species of *Pseudomonas* (O'Conner *et al*., 1995), *Rhodococcus* (Cripps *et al*., 1978), *Norcardia* (Hartmans *et al*., 1990), and *Xanthobacter* (O'Conner and Dobson, 1996) have been isolated from various soils around the world. Two main pathways for the aerobic degradation of styrene have been described, one that involves an initial oxidation of the vinyl side-chain (Warhurst *et al*., 1994), and the other based on a direct attack of the aromatic nucleus by *Rhodococcus* species (Gbric-Gallic *et al*., 1990). A side chain oxidation pathway involves oxidation of the vinyl side-chain, subsequently oxidized to phenylacetic acid through the action of dehydrogenase. Direct aromatic ring cleavage by a *Rhodococcus* species revealed that styrene metabolism in this organism proceeded via a 3-vinyl-catechol. Under anaerobic conditions, the degradation of styrene was observed in an anaerobic consortium enriched with styrene and ferulic acid, and finally converted to phenylacetic acid (Choi and Ahn, 1998).

Using microorganism for styrene degradation has been extensively studied. However, using lignolytic fungi for styrene degradation, in spite of well-known decomposers of lignin or phenolic compounds, has not been studied extensively.

Lignocellulosics of woody plants are compact and composed of cellulose and lignin in part crystalline complex region. The hyphae of wood-rotting basidiomycetes penetrate into woody plant tissues in order to contact the easily assimilable carbohydrates or lignin constituents of the lignocellulosic complex. Among the enzymes secreted by white rot fungi during degradation of lignin, laccase (EC 1.10.3.2), lignin peroxidase (LiP; EC 1.11.1.14) and manganese-dependent peroxidase (MnP; 1.11.1.13) were acknowledged to be especially significant in the degradation of lignin. This lignin degradation system of white rot fungi was adapted for pollutants degradation of bioremediation field (Han *et al*., 2004; Shin, 2004), e.g. chlorinated phenols like pentachlorophenol, 4,5,6,-trichoroguaiacol (Choi, 1999; Schützendübel *et al*., 1999), polycyclic aromatic hydrocarbons (Levin *et al*., 2003), organic pollutants (Heinfling *et al*., 1997), and dyes (Johannes *et al*., 1998). The oxidation of acenaphthene and acenaphthylene under a laccase mediator system was also studied (Stahl *et al*., 2000).

In this work, monomeric styrene was biodegraded by white rot fungi, *Phanerochaete chrysosporium* strain KFRI 20742, *Trametes versicolor* strain KFRI 20251 and *Daldinia concentrica* strain KFRI 40-1 and its characteristic biodegradation and estrogenic reduction effects were evaluated.

Materials and Methods

Reagents

Styrene monomer (purity 99.0%) was purchased from Showa Chemical Co. (Showa, Japan). Reagents for culture media and extractions were of reagent grade.

Fungi and cultivation

The fungi, *Phanerochaete chrysosporium* strain KFRI 20742, *Trametes versicolor* strain KFRI 20251 and *Daldinia concentrica* strain KFRI 40-1, were obtained from the Microbiology Chemistry Lab. at the Korea Forest Research Institute. The fungi were first cultivated in Potato Dextrose Agar (PDA) medium and recultivated in the YMPG medium (10 g glucose, 10 g malt extract, 2 g peptone, 2 g yeast extract, 1 g asparagine, 1 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 1 mg thiamine, 20 g agar in 1 L distilled water).

Resistance test by fungi against styrene

The fungi in the YMPG medium were recultivated in 2% malt extract agar (MEA) medium which was a nitrogen-limited condition. The stock solution was prepared by dissolving styrene in dimethyl formamide (DMF). The concentrations were 0, 10, 50, 100, 200, and 500 mg/l in medium culture containing 2% malt extract agar. Resistance was determined every other day by measuring the hyphal length growth of each fungus at each concentration.

Enzyme activity and pH

When the mycelium of *P. chrysosporium* KFRI 20742 was completely grown in 100-ml Erlenmeyer flask, the stock solution of styrene was added in the culture medium. Shallow stationary culture (SSC) medium is used to estimate enzyme activity (Kirk *et al*., 1986). The styrene concentration in the culture medium was adjusted to 100 μM by using 200 mM of the stock solution. Extracellular fluids were collected every day, and their pH and both activities, manganese-dependent peroxidase (MnP) and laccase, were assayed. Both activities were spectrophotometrically determined at room temperature. MnP and laccase were determined by monitoring the oxidation of ABTS (2,2'-azino-bis (3-ethylbenzthiazolin-6-sulfonic acid)). The reaction mixture for MnP assay contained ABTS (0.8 g/L), 0.2 mM $MnSO_4$, 0.1 mM H_2O_2 and 0.2 M sodium lactate buffer in a total volume of 3 ml (Wariishi *et al*., 1992). Laccase activity assay contained ABTS (0.8 g/L) and 0.1 M sodium lactate buffer in a total volume of 3 ml. The activities for MnP or laccase were calculated as follows; unit/ml = (Δabsorbance × total volume × 10^6) / (36,000 × Δtime × sample volume).

Biodegradation efficiency of styrene

The inoculated medium was incubated in a stationary culture condition at 30°C. After completely growing mycelium in a 100-ml Erlenmeyer flask, 100 µl of styrene from 200 mM stock solution was added to the culture medium to determine biodegradation efficiency and metabolic pathways. After each indicated interval, the mycelium growth was stopped with the addition of 1 M HCl. The remaining styrene was first extracted with 20 ml *n*-hexane, and the remaining water fraction was re-extracted with the mixture of 10 ml *n*-hexane and 10 ml ethylacetate. The extracted fraction was combined together and filtered with a microfilter (0.22 µm diameter).

The quantitative analysis of styrene in biodegradation efficiency was determined by a Hewlett Packard 1100 series HPLC equipped with a Waters symmetric column. The conditions were as follows; 0.6 ml/min flow rate in mixed eluent of CH_3CN and H_2O (80:20, v/v), 20 min isocratic run, and detection at 240 and 280 nm. Styrene was identified at 8.85 min of retention time. The calibration curve was prepared by using standard solutions of 1, 5, 10, 20, and 30 mg/l.

Metabolites of styrene

The filtrate was completely evaporated by bubbling with nitrogen gas, derivatized by N,O-bis (trimethylsilyl) trifluoroacetamide at 60°C for 1 h, then analyzed by Gas Chromatography (HP 6890)-Mass Spectrometry (JMS-600W, JEOL). The GC/MS conditions were as follows; Shimadzu HiCap-CBP1-M25-O25 (25 m, 0.25 mm) column, and helium carrier gas with a 1.0 ml/min flow rate.

Cell culture for biological evaluation

MCF-7 cells were maintained in 25-cm^2 cell culture flasks in Dulbecco's modified Eagle's medium (DME) supplemented with 5% heat inactivated fetal calf serum. These were kept in a humidified incubator at 37°C, with 5% CO_2 over a maximum of 12 passages, and routinely tested for mycoplasma.

Cell proliferation assay

The methods used for the cell proliferation assays were a modification of those of Soto et al. 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, 0.53 mM EDTA, Gibco BRL). The flask was left for 1 min at room temperature. These cells were detached by a firm slap, re-suspended in 10 ml of full medium, counted using an improved Neubauer counting chamber and seeded to 6 well plates at a density of 2×10^4 cells/well in 2 ml DME medium. After 24 h, the cells were washed with 5 ml PBS. The DME medium was changed to 2 ml estrogen-free medium (phenol red-free DMEM with 5% Charcoal dextran-treated fetal calf serum, FBS) for 48 h. After washing with PBS, one group was treated with original styrene (10^{-5} M) in DMEM only for 6 days, and another group was treated with styrene that have been incubated with *P. chrysosporium* KFRI 20742 for 1, 3, 6 and 12 days. Cell proliferation was assessed after 6 days in culture using the method of Skehan *et al.* (1990).

The pS2 mRNA expression assay

MCF-7 cells were seeded in 25-cm^2 flasks in 5% FBS-supplemented DMEM. Twenty-four hours later, the medium that contained the chemicals to be tested was changed to 5% FBS. Control flasks were treated with a vehicle. The substances to be tested were treated with the same methods as the proliferation assay. After 72 h of exposure to the test chemicals, the medium was aspirated, the cells were rinsed with PBS, and total RNA was extracted using Trizol (Sigma, USA) following the manufacturer's protocol. Five micrograms of total RNA were reverse transcribed using M-MLV reverse transcriptase (Ambion, U.S.A.) and random primer (9 mer). The samples contained pS2 primers (pS2-1; 5'-GGCCACCATGGA GAACAAGG-3' and pS2-2; 5'-CCACGAACGGTGTC GTCGAA-3') and primers for 1A gene (1A-1; 5'-GATATGGCGTTTCCCCGCATA-3' and 1A-2; 5'-GG ATTTTGGCGTAGGTTTGGT-3'). To determine the conditions under which PCR amplification for pS2 and 1A mRNA were in the logarithmic phase, the aliquots (1 µl) were amplified using different numbers of cycles. PCR for 1A gene was amplified to rule out the possibility of RNA degradation and was used to control the variation in mRNA concentrations in the RT reaction. A linear relationship between PCR products and amplification cycles was observed in both the 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 1 min 30 sec. The products were visualized by agarose gel electrophoresis stained with ethidium bromide, and the photograph was scanned and analyzed using a molecular analysis program version 1.5 (Gel Doc 1000, Bio-Rad).

Results and Discussion

Resistance test by fungi against styrene

The resistance of three fungi, *Daldinia concentrica* KFRI 40-1, *Trametes versicolor* KFRI 20251, and *Phanerochaete chrysosporium* KFRI 20742 against

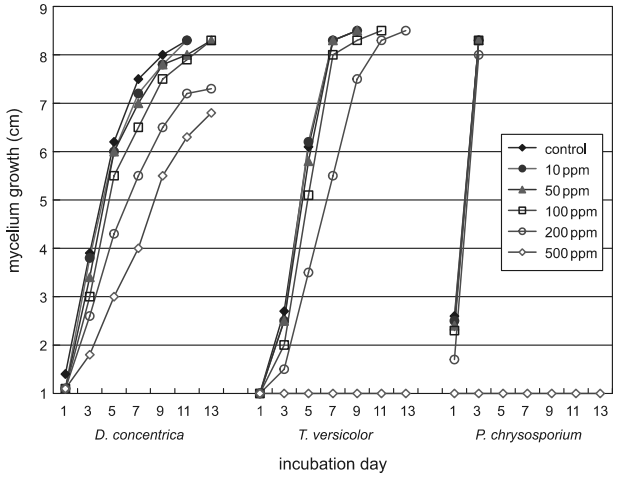

Fig. 1. Resistance of *D. concentrica* KFRI 40-1, *T. versicolor* KFRI 20251 and *P. chysosporium* KFRI 20742 to various styrene concentrations depending on incubation days.

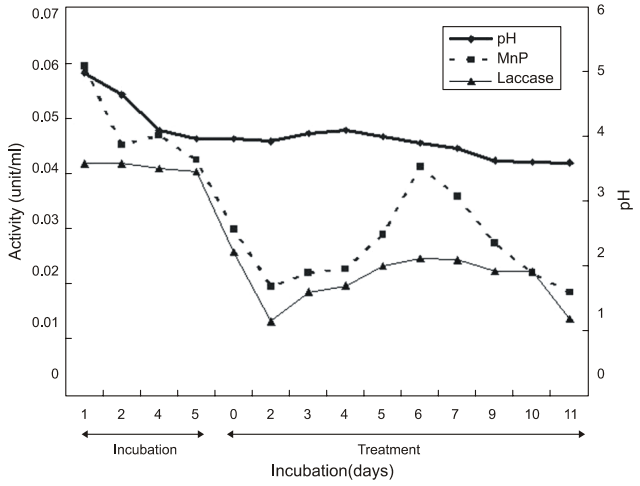

Fig. 2. Changes in extracellular ligninase activities of *P. chrysosporium* KFRI 20742, MnP and laccase during incubation.

monomeric styrene was determined by comparing the mycelium growth and required incubation days with the control, which did not contain styrene. The mycelium growth of fungi according to various concentrations was shown in Fig. 1.

The mycelium growth rate was approximately 2 to 3 cm/day with a little inhibition until the styrene concentration reached to 200 mg/l. It was found that the three fungi were quietly resistant in low concentrations of styrene, such as 10, 50, 100 and 200 mg/l, but in high styrene concentrations the mycelium growth was gradually inhibited. Significant inhibition appeared with the addition of 500 mg/l, and *T. versicolor* KFRI 20251 and *P. chrysosporium* KFRI 20742 did not even grow. However, the mycelium growth of *D. concentrica* KFRI 40-1 was continuously preceded in the concentration of 500 mg/l without any lag time at the initial stage of incubation, even though the growth rate was less than 1 cm/day. According to those results, the highest concentration for the growth of *T. versicolor* KFRI 20251 and *P. chrysosporium* KFRI 20742 was approximately less than 500 mg/l, while that of *D. concentrica* KFRI 40-1 was more than 500 mg/l. Finally, *T. versicolor* KFRI 20251 and *P. chrysosporium* KFRI 20742 were resistant to low concentrations of styrene, and *D. concentrica* KFRI 40-1 was very quickly adjusted to the new hazardous environment, even in high concentrations of styrene.

Enzyme activity and pH

The changes in MnP and laccase activities by the addition of styrene to the culture medium of *P. chrysosporium* KFRI 20742 were shown in Fig. 2. Both MnP and laccase activities were very minimal. Those values were highest after one day of incubation without the addition of styrene. After the styrene addition, the activities were decreased to the lowest activity at day 2. Then, it was gradually increased to the highest activity at day 6, but decreased at the end of incubation. This result indicated that both activities might affect the degradation and radical polymerization of styrene. The pH variation ranged from 3.5 to 4.9, and the pH gradually decreased as incubation day increased. The decrease of pH might result from the production of fatty acids during the secondary metabolism of white rot fungi (Stahl *et al*., 1996). During identification of styrene metabolites in GC/MS analysis, various C16 and C18 fatty acids were found (data not shown).

Degradation efficiency of styrene

A time course of the degradation of styrene by three fungi in liquid culture was carried out. When the 100 μM (28 mg/l) of styrene was added to culture medium, the degradation efficiencies by three fungi were approximately 99% after one day incubation. After three days, styrene was not detected in the culture medium, suggesting that three fungi completely degraded styrene. But one of interesting points was found that small, broad peaks appeared at the end of the HPLC chromatogram (Fig. 3). It seemed that styrene monomers were polymerized to the styrene dimer or trimer instead of the degraded metabolites. The result also confirmed that the decolorized zone was formed in a petri dish during resistance test by *D. concentrica* KFRI 40-1 in the concentration of 500 mg/l. It has been reported that the decolorized zone could be due to the occurrence of chromorphores which were conjugated bonds formed by phenolic compounds after a radical polymerization by laccase (Li *et al*., 1999).

Metabolites of styrene

According to the above results, three fungi seemed to biodegrade monomeric styrene, therefore, the following discussions focused on whether three fungi could so quickly degrade styrene as they did in the resistance test. We also studied what kinds of metabolites or intermediates occurred. Metabolites and intermediates analyzed by GC/MS during styrene degradation by *P. chrysosporium* KFRI 20742 were shown on Table 1. The major metabolites produced during styrene degradation were 2-phenyl ethanol, benzoic acid, cyclohexadiene-1,4-dione, butanol and succinic acid. Some metabolites, such as 2-phenyl ethanol, benzoic acid, and cyclohexadiene-1,4-dione might be oxidized and hydroxylated, and still had an aromatic ring. However, butanol and succinic acid might be considered as aromatic ring cleaved metabolites. It is necessary to do more detailed investigation of intermediates depending on incubation time in order to evaluate the biodegradation mechanism.

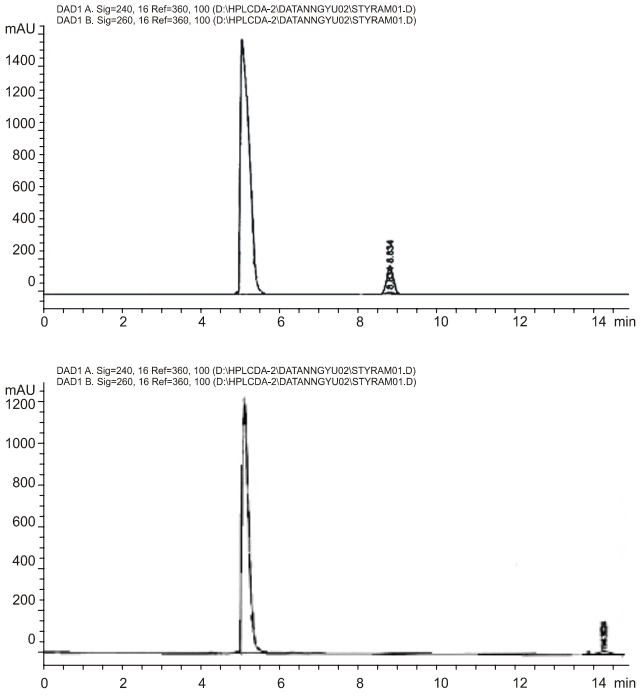

Fig. 3. HPLC chromatogram of styrene standard (upper) and intermediates after 1 day incubation by *P. chrysosporium* (the peak at RT 8.85 min was styrene, and the big one at RT 4.9 min was solvent).

Estrogenic activity by the E-SCREEN test

We examined the induction of cell proliferation using the original styrene (10^{-5} M) compared to styrene that had been incubated with *P. chrysosporium* KFRI 20742 for 1, 3, 6 and 12 days. As shown in Fig. 4, proliferation of MCF-7 cells significantly decreased at 1 day in styrene that had been incubated with *P. chrysosporium* KFRI 20742 (p<0.05). When styrene

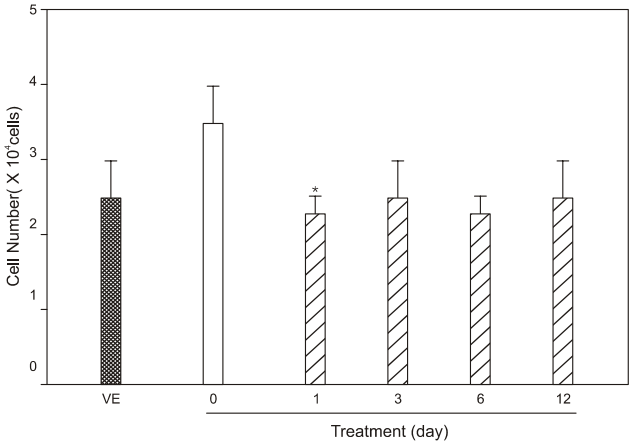

Fig. 4. Cell proliferation assay of styrene and its metabolites degraded by *P. chrysosporium* KFRI 20742.

Table 1. TMS-silylated metabolites during the biodegradation of styrene by white rot fungi, *Phanerochaete chrysosporium* KFRI 20742 (bp : base peak, mp : molecular ion peak)

Metabolites	Formula	Major mass peaks
1-methyl butanol	$C_5H_{12}O$	73(bp), 117, 131, 145, 160(mp)
5-methyl-2-(1-methylethyl)phenol	$C_{10}H_{14}O$	73, 191, 207(bp), 222(mp)
2-phenyl ethanol	$C_8H_{10}O$	73(bp), 103, 161, 179, 184(mp)
benzoic acid	$C_6H_6O_2$	77, 105, 135, 179(bp), 194(mp)
2-methyl-1,3-propanediol	$C_4H_{10}O_2$	73, 129, 147(bp), 177, 191, 219, 234(mp)
succinic acid	$C_4H_6O_4$	73, 129, 147(bp), 172, 247, 262(mp)
4-hydroxy-3-pentene-2-one	$C_5H_8O_2$	73, 157(bp), 172(mp)
2,5-bis(1,1-dimethylpropyl)-2,5-cyclohexadiene-1,4-dione	$C_{16}H_{24}O_2$	37, 247(bp, mp), 249
2-benzyl-3,4,5-triphenylcyclopenta-2,4-dienone	$C_{30}H_{22}O$	370(bp), 398(mp)

was incubated with *P. chrysosporium* KFRI 20742, the other days also decreased proliferation of MCF-7 cells.

The proliferation of MCF-7 cells is basically dependent on estrogen and has been used for the detection of estrogenic activity in the form of E-SCREEN (Soto *et al*., 1995). Styrene dimers and styrene trimers are known as substances eluted in microscopic amounts from food containers made of polystyrene (PS) (Yamada *et al*., 2000).

Styrene dimers and styrene trimers in high concentrations showed estrogenic effects in the ER binding assay and E-SCREEN pointing out the fear that they may disrupt the endocrine system (Ohyma *et al*., 2001). In the MCF-7 cell proliferation assay, styrene trimers had the highest proliferative activities of the compounds tested. Styrene dimers also significantly increased the cell yields. The proliferative activities of styrene dimers were weaker than those of styrene trimers. Estrogenic activities of styrene trimers differed depending on their chemical structures (Ohyma *et al*., 2001). The growth of MCF-7 cell was monitored a period of 6 days. In this assay, the styrene monomer incubated with *P. chrysosporium* KFRI 20742 did not induce proliferation of MCF-7 cells, and also gradually decreased styrene concentrations to reach vehicle level. Therefore, estrogenic activity of styrene completely disappeared after being treated with supernatant of *P. chrysosporium* KFRI 20742 in MCF-7 cell from one week.

Reduction of endogenous estrogen responsive genes

To evaluate the reduced estrogenic potency of styrene that has been incubated with *P. chrysosporium* KFRI 20742, we examined the pS2 mRNA induction in MCF-7 cells. As an internal control, constitutively expressed human 1A mRNA was used. From one to seven days incubating with the fungus, the level of pS2 mRNA was decreased down to the level of baseline at one day. Also, estrogenic activity of styrene completely disappeared after being treated with supernatant of *P. chrysosporium* KFRI 20742 from one week of culture down to the levels of vehicle (Fig. 5).

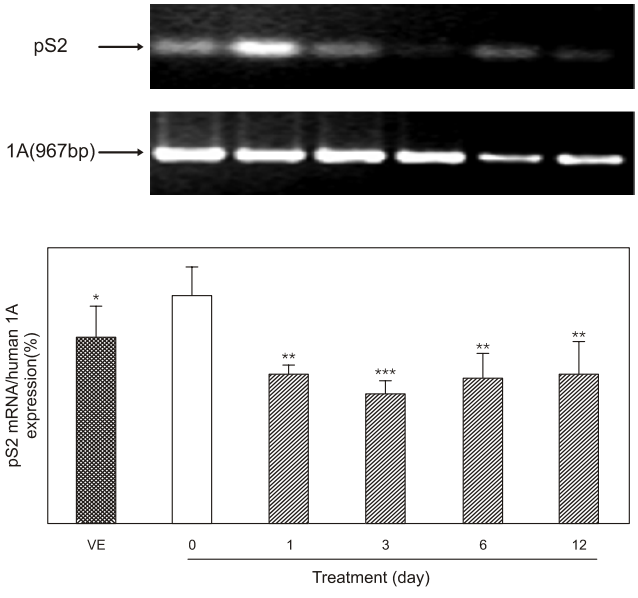

Fig. 5. pS2 mRNA expression of styrene and its metabolites degraded by *P. chrysosporium* KFRI 20742.

Estrogen and estrogenic chemicals induce the expression of pS2, whose gene product has been first identified in the MCF-7 cell (Masiakowski *et al*., 1982). pS2 mRNA can be rapidly induced by estradiol in certain breast cancer biopsies, but neither in normal breast tissue nor in any other cultured human cell lines (Jakowlew *et al*., 1984). Therefore, pS2 mRNA expression in MCF-7 cells is an ideal model to study the effects of estrogenic chemicals. From the styrene incubated with *P. chrysosporium* KFRI 20742, the level of pS2 mRNA was decreased down to the baseline level after one day. With the others of styrene incubated with *P. chrysosporium* KFRI 20742, MCF-7 cell demonstrates antiproliferative effects in vitro.

Conclusion

Three fungi, *P. chrysosporium* KFRI 20742, *T. versicolor* KFRI 20251 and *D. concentrica* KFRI 40-1 showed high resistance to styrene with a little inhibition depending on concentration levels. In the concentration of less than 200 mg/l, *P. chrysosporium* KFRI 20742 was very resistant, while in more than 200 mg/l, *D. concentrica* KFRI 40-1 was superior to other fungi. The resistance of *D. concentrica* KFRI 40-1 was highest at the concentration of 500 mg/l, and its degradation showed 99% even after one day of incubation. After three days of incubation, three fungi completely degraded styrene. The changing patterns of enzyme activity of manganese dependent peroxidase and laccase were very similar. The major metabolites were 2-phenyl ethanol, benzoic acid, cyclohexadiene-1,4-dione, butanol and succinic acid. The presence of butanol and succinic acid, which might be considered as a ring cleaved product confirmed that recalcitrant monomeric styrene could possibly be biodegraded by white rot fungi. However, the detailed pathway of biodegradation mechanism of styrene by *P. chrysosporium* KFRI 20742 should be further studied, and the role of ligninase has to be elucidated for understanding styrene biodegradation by white rot fungi. The estrogenic reduction effect of styrene monomer was shown in its degradation

metabolites by using MCF-7 cell proliferation and pS2 mRNA expression assays.

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