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Biodegradation of Endocrine-Disrupting Phthalates by *Pleurotus ostreatus*

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Biodegradation of endocrine-disrupting phthalates [diethyl phthalate (DEP), dimethyl phthalate (DMP), butylbenzyl phthalate (BBP)] was investigated with 10 white rot fungi isolated in Korea. When the fungal mycelia were added together with 100 mg/l of phthalate into yeast extract-malt extract-glucose (YMG) medium, *Pleurotus ostreatus*, *Irpex lacteus*, *Polyporus brumalis*, *Merulius tremellosus*, *Trametes versicolor*, and *T. versicolor* MrP1 and MrP13 (transformant of the Mn-repressed peroxidase gene of *T. versicolor*) could remove almost all of the 3 kinds of phthalates within 12 days of incubation. When the phthalates were added to 5-day pregrown fungal cultures, most fungi except *I. lacteus* showed the increased removal of the phthalates compared with those of the non-pregrown cultures. In both culture conditions, *P. ostreatus* showed the highest degradation rates for the 3 phthalates tested. BBP was degraded with the highest rates among the 3 phthalates by all fungal strains. Only 14.9% of 100 mg/l BBP was degraded by the supernatant of *P. ostreatus* culture in YMG medium in 4 days of incubation, but the washed or homogenized mycelium of *P. ostreatus* could remove 100% of BBP within 2 days even in distilled water, indicating that the initial BBP biodegradation by *P. ostreatus* may be attributed to mycelium-associated enzymes rather than extracellular enzymes. The biodegradation rate of BBP by the immobilized cells of *P. ostreatus* was almost same as that in the suspended culture. The estrogenic activity of 100 mg/l DMP decreased during biodegradation by *P. ostreatus*.

Keywords: White rot fungi, *Pleurotus ostreatus*, endocrine-disrupting phthalates, biodegradation, estrogenic activity

Endocrine-disrupting chemicals (EDCs) have been defined by the European Commission as the exogenous materials or compounds that alter the normal hormone regulations and damage the health of intact organisms or their progenies or subpopulations. Recently, it has been reported that depression of reproduction, deformity of reproductive organs, inhibition of growth, induction of cancer, and obstruction of immunity could be caused by EDCs in various organisms [27]. Numerous EDCs are released into the environment through many routes as the result of industrial activities around the world. EDCs as environmental pollutants must be blocked and removed for the protection of the health of all organisms including human beings.

Among the various EDCs, phthalates have been widely used as plasticizers in the industry of plastics, and are also contained in paints, adhesives, cardboard, and fragrances. Phthalates were detected in various environments such as surface waters, transported deposits, biological samples of beef, and sewage sludge at very low concentrations [26]. Endocrine-disrupting chemicals could be biodegraded by some fungi, bacteria, and algae [1, 3, 7, 9, 22, 23]. Among the fungi, most white rot fungi also have the capability to degrade EDCs such as nonylphenol, bisphenol A, and phthalates [3, 7, 18, 22]. Ligninolytic enzymes including lignin peroxidase, manganese-dependent peroxidase, and laccase are related to the biodegradation of endocrine-disrupting chemicals by white rot fungi, and the purified enzymes have been shown to oxidize the EDCs [19, 21]. Most of all, the white rot fungi have various merits for the biodegradation of EDCs, such as mineralizing ability, oxidation of water-insoluble substrates, hyphal extension to distant places, and secretion of extracellular degrading enzymes [8, 17].

In this study, the biodegradation of three phthalates by 10 white rot fungi isolated in Korea was investigated. Estrogenic activity during the phthalate biodegradation by *P. ostreatus* was also measured by a yeast two-hybrid system. The final aim of this study was the selection of fungal strains that have high degradation capability on endocrine-disrupting phthalates.

Materials and Methods

Chemicals and Fungal Strains

Dimethyl phthalate (DMP), diethyl phthalate (DEP), and butylbenzyl phthalate (BBP) were purchased from Sigma Chemical Co. (St.

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Louis, U.S.A.). Hexane used in the extraction of residual phthalates was of HPLC grade. Ten fungal strains used in phthalates biodegradation were *Irpex lacteus*, *Merullius tremellosus*, *Polyporus brumalis*, *Trametes versicolor*, *Schizophyllum commune*, *Formitella fraxinea*, *Pleurotus ostreatus* species, which were isolated from various forest soils in Korea by Dr. J. Sung in Kangwon National University and Dr. K. Kim in Kangnung National University. Three transformants of *T. versicolor* (MrP1, MrP13, and MnP2-6) were generated through genetic transformation to overexpress the genes for manganese-repressed peroxidase (MrP) and manganese-dependent peroxidase (MnP) of *T. versicolor*, respectively [24].

Biodegradation of Phthalates in Fungal Culture

All white rot fungi were cultured on PDA (potato dextrose agar) plates. Ten pieces of agar plug (diameter: 1 cm) covered with fungal mycelia were added to 100 ml of YMG medium (yeast extract 4 g/l, malt extract 10 g/l, glucose 4 g/l) followed by homogenization for 30 sec with a homogenizer (X120; CAT, Germany), and then incubated (30°C, 200 rpm) for 5 days. After the incubation, fungal cultures were homogenized again for 30 sec with a blender (Model PH91; SMT Co., Japan) followed by centrifugation at 6,140 ×*g* for 30 min. After centrifugation, supernatants and precipitated mycelia were separately collected into the sterile bottles, and 10% fungal inoculum (wet w/v) was prepared by the addition of supernatants to collected mycelia.

Fungal inoculum (0.5 ml) was added with 100 mg/l phthalates into 20 ml of YMG medium, and incubated for 12 days (30°C, 130 rpm). Residual phthalates in the fungal cultures were analyzed every 2 days. Alternatively, 100 mg/l phthalates was added into 5-day pregrown fungal cultures that had been prepared as above, and incubated further for 12 days.

For the determination of residual phthalates in fungal culture, replicate culture was homogenized for 1 min followed by addition of 10 ml of hexane. The mixture was strongly shaken in a vertical extraction shaker (Resipro Shaker RS-1, JeioTech, Korea) at 350 strokes/min for 30 min, and then the solvent layer was separated from the aqueous phase by centrifugation at 6,140 ×*g* for 30 min. The collected hexane was concentrated to 0.5 ml by a vacuum evaporator. The quantification of residual phthalates in the hexane concentrate was determined with a gas chromatograph (GC) (HP 5890; Hewlett Packard Co., U.S.A.) equipped with a flame ionization detector. The operating conditions of GC were as follows: column of HP-1 (25 m×0.32 mm× 0.17 μm film thickness); isocratic oven temperatures of 150, 140, and 230°C for DEP, DMP, and BBP, respectively; injector temperature of 270°C; injection volume of 1 μl; column flow of 1 ml/min (N_2); auxiliary gas (N_2) flow of 30 ml/min; hydrogen flow of 32 ml/min; air flow of 395 ml/min; and split ratio of 30:1.

Biodegradation of Phthalates by Different Parts of Fungal Culture

Five-day pregrown cultures of *P. ostreatus* in 20 ml of YMG medium were centrifuged at 6,140 ×*g* for 30 min and the resulting supernatant and pellet of fungal mycelia were separated. The culture supernatant was passed through filter paper (Whatman No. 3) followed by refiltration with the membrane filter of 0.2 μm pore size (Millipore, U.S.A.). The fungal pellet was washed twice with distilled water and resuspended with distilled water to make a final volume of 20 ml. At the same time, another set of fungal pellet was washed twice with distilled water, resuspended with distilled water (final volume 20 ml), and homogenized. The filtrated supernatant, washed mycelium, and homogenized mycelium in distilled water were added to BBP (final conc. 100 mg/l) and reacted in an incubator (30°C, 130 rpm) for 4 days. Residual BBP was analyzed by GC after 24 and 48 h.

Five-day pregrown fungal cultures in 100 ml of YMG medium were used for the construction of an agar block where mycelia of *P. ostreatus* were immobilized. Intact fungal cultures (100 ml) were quickly mixed with 100 ml of sterilized agar solution (4%, v/v) at 42°C. The mixture was quickly poured into the 96-well plate followed by solidification at 4°C. After the solidified agar block was suspended into 4°C distilled water for 12 h, the distilled water was discarded and this process was repeated twice. Agar blocks (dia. 0.7 cm, height 1 cm) contained 13 mg dry wt/ml of fungal biomass. Ten g of agar block immobilized with fungal mycelia was added into distilled water and the mixture was adjusted to a final volume of 20 ml followed by addition of 100 mg/l DMP. After incubation of this mixture for 5 days (30°C, 130 rpm), the agar blocks were homogenized for 60 sec and residual DMP was extracted with 10 ml of hexane by the vertical extraction shaker at 350 strokes/min for 30 min followed by GC analysis.

Change of Estrogenic Activity During Degradation of Phthalate

Change of estrogenic activity during the degradation of phthalate by white rot fungus was measured by the yeast two-hybrid assay system developed by Nishikawa *et al.* [13]. Supernatants of *P. ostreatus* were collected from the phthalate-added fungal cultures by centrifugation at 6,140 ×*g* for 30 min. Overnight culture of yeast transformant Y 190 (200 μl) in SD medium [minimal SD base (Clontech, U.S.A.) 27.6 g^{-1}, DO Supplement (Clontech, U.S.A.) 0.64 g^{-1}] was added to 800 μl of fresh SD medium with 12-μl supernatants of fungal cultures. After incubation of these mixtures for 4 or 12 h (30°C, 150 rpm), 400 μl of the mixture was centrifuged at 387 ×*g* for 2 min. Collected yeast pellets were resuspended with 800 μl of Z-buffer [0.1 M sodium phosphate (pH 7.0), 10 mM KCl, 1 mM $MgSO_4$, 0.001% sodium dodecyl sulfate], and 16 μl of chloroform was added and the sample was incubated at 30°C for 15 min. After the sample had reacted with 160 μl of 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside for up to 8 h and the color of reaction mixture had changed to a yellow color, 1 ml of 1 M Na_2CO_3 was added to the reaction mixture. Finally, β-galactosidase activity as the estrogenic activity was calculated based on the equation described by Nishikawa *et al.* [13].

Results and Discussion

Biodegradation of Phthalates in Fungal Culture

When the fungal inocula were added together with 100 mg/l phthalates into YMG medium, DMP was completely removed by *P. ostreatus* in 8 days (Table 1, Type A). *I. lacteus* and *T. versicolor* MrP13 could also remove 100% of DMP in 10 days. In the DEP biodegradation, most fungi showed the lower degradation rates compared with those of DMP degradation. However, *P. ostreatus* could completely remove DEP within 8 days as in DMP degradation. BBP was degraded faster than DMP and DEP by most fungi,

Table 1. Biodegradation of 100 mg/l phthalates (DMP: dimethyl phthalate; DEP: diethyl phthalate; BBP: butylbenzyl phthalate) by various white rot fungi.

Culture	Fungal strains	Biodegradation (%)					
		DMP		DEP		BBP	
		6 day	12 day	6 day	12 day	6 day	12 day
Type A	*Irpex lacteus*	93.6±0.5	100^{10}±0.0	83.6±0.4	98.9±0.4	82.4±0.2	100^{10}±0.0
	Merullius tremellosus	70.5±0.7	100±0.0	69.8±2.3	93.8±0.2	56±0.6	100^{10}±0.0
	Polyporus brumalis	41.3±0.7	100±0.0	47.1±0.8	97.2±3.5	100±0.0	-
	Trametes versicolor	52.3±0.2	93.8±0.1	43.9±9.6	96.1±0.8	100±0.0	-
	Schizophyllum commune	8.2±0.5	27.1±2.3	16.4±0.2	55.5±1.5	74.6±0.9	80.3±0.3
	Fomitella fraxinea	1±0.1	34.6±0.3	36.1±0.3	84.9±3.5	98±1.5	100^{8}±0.0
	Pleurotus ostreatus	67.2±5.9	100^{8}±0.0	48.6±8.2	100^{8}±0.0	88.7±0.6	100^{8}±0.0
	T. versicolor MrP1	20.8±1.2	98±0.5	48±1.2	86.2±2.0	100±0.0	-
	T. versicolor MrP13	97.1±2.3	100^{10}±0.0	44.8±0.5	95.0±6.1	45.7±7.4	95±2.7
	T. versicolor MnP2-6	6.4±0.0	69.4±0.8	19±0.7	50.1±2.5	100±0.0	-
Type B	*Irpex lacteus*	77.8±1.5	97.7±1.2	93.7±0.1	98.6±0.7	83.8±0.7	92.7±2.8
	Merullius tremellosus	16.7±1.4	37.4±0.7	63±4.6	66.7±4.3	100^{4}±0.0	-
	Polyporus brumalis	39±1.9	81.3±1.7	94.4±3.6	97.6±1.6	100^{2}±0.0	-
	Trametes versicolor	84.9±2.1	100^{8}±0.0	87±0.1	98.8±0.4	100^{4}±0.0	-
	Schizophyllum commune	27±2.0	46.6±4.0	24.7±3.1	43.3±4.5	97.1±4.2	91±6.3
	Fomitella fraxinea	17.2±3.0	26±1.4	66.9±1.3	87.1±0.3	97.6±0.7	100^{8}±0.0
	Pleurotus ostreatus	100±0.0	-	100^{4}±0.0	-	100^{2}±0.0	-
	T. versicolor MrP1	93.4±0.0	97.9±0.9	92.1±0.3	100^{10}±0.0	100^{2}±0.0	-
	T. versicolor MrP13	94.9±1.48	100±0.0	68.5±0.1	92.1±2.9	100^{2}±0.0	-
	T. versicolor MnP2-6	66.9±7.7	96.4±2.4	75±0.5	86.1±0.2	100^{4}±0.0	-

Phthalates were added to the fresh YMG media together with the fungal inoculum (Type A) or to 5-day preincubated fungal cultures in YMG media (Type B). The number in superscript means the day of 100% removal of the phthalate.

and several strains could completely remove BBP within 6 days. When 100 mg/l of phthalates was added to 5-day pregrown fungal cultures in YMG medium, the biodegradation rates of DMP and DEP were varied among fungal strains compared with those of non-preincubated cultures (Table 1, Type B). In a previous study for 2,4,6-trinitrotoluene (TNT) biodegradation, the 5-day preincubated fungal cultures showed higher removal rates than those of non-preincubated cultures, and it might be due to the large amounts of extracellular enzymes produced in the 5-day preincubated cultures and inhibition of growth by chemical toxicity in the non-preincubated cultures [5]. The degradation mechanisms and toxicity of DMP and DEP on the growth of most fungi tested in this study may be somewhat different from those of TNT degradation. However, *P. ostreatus* showed the highest degradation rates for the 3 phthalates among fungi tested with or without a preincubation. The BBP degradation rates in the preincubated cultures were also higher than those of non-preincubated cultures.

The degradation rates of DMP by most fungal strains were higher than that of green alga *Closterium lunular*, which could remove less than 40% of 100 mg/l of DMP until 6 days [23]. Five-day pregrown *P. ostreatus* showed faster degradation of 100 mg/l DEP and BBP than that in a bioreactor study with sludge, in which 100% removals of DEP and BBP were achieved in 5 and 4 days, respectively [2]. Generally, the biodegradation rates of phthalates decreased with the increase of alkyl chain length and alkyl branch chains [28]. However, BBP degradation by the white rot fungi in this study was faster than DMP and DEP degradation. The biodegradation rates of the 3 phthalates were in the order of BBP>DMP>DEP, and this order was similar to that in a bioreactor study with sludge [2]. BBP degradations by 5-day pregrown white rot fungi such as *P. ostreatus*, *P. brumalis*, and *T. versicolor* MrP1 were also much faster than that of a *Gordonia* sp. that could remove 100% of 25 mg/l BBP in 4 days [3]. It was reported that 28 mg/l dibutyl phthalate (DBP), of which structure is similar to BBP, could be completely removed in 6 days by the white rot fungus *Daldinia concentrica* [7]. When this DBP transformation rate of 0.2 mg/h was compared with the BBP transformation rate of 3.94 mg/h by *P. ostreatus* in this study, the transformation rate of *P. ostreatus* was much higher than that of *D. concentrica*, although the phthalates used were slightly different. Even the degradation rate of DBP was reported to be higher than BBP in sludge [2]. Not all the fungi tested had a high biodegrading capability for all phthalates, but some of them, especially *P. ostreatus*, seemed to be very efficient for degradation of various phthalates.

Transformants of *T. versicolor* had been made to overexpress the genes for ligninolytic enzymes such as Mn-dependent peroxidase or Mn-repressed peroxidase, which were expected to increase the degradation rates of recalcitrants as in bacteria [25]. Some transformants showed the higher degradation rate for certain phthalates, and the changing patterns of degradation rates of the 3 transformants compared with the wild-type strain were not consistent, and they may be due to the slightly lower growth rates of the transformants (data not shown).

P. ostreatus treated with phthalates DMP, DEP, and BBP showed the highest biodegradation rate among the 10 white rot fungi (Table 1). It was observed that *P. ostreatus* could make more dense spherical pellets compared with the other strains tested, and this observation may be due to hydrophobin. Hydrophobins, proteins of low molecular weight, are produced by filamentous fungi including *P. ostreatus* [16], and they can render fungal structure hydrophobic [10]. Therefore, they may make denser fungal pellets in aqueous culture media and adsorb hydrophobic phthalates more easily, which may be helpful for the degradation of phthalates by *P. ostreatus*. The production of hydrophobins by the *P. ostreatus* strain in this study has to be examined precisely. In conclusion, *P. ostreatus* has the potential feasibility for application to the treatment of phthalates-containing wastes and bioremediation of phthalates-contaminated sites.

Biodegradation of BBP by Different Parts of *P. ostreatus* Culture

Biodegradation of 100 mg/l BBP by the supernatant of *P. ostreatus* culture in YMG medium was 14.9% in 4 days of incubation (Fig. 1). The washed intact mycelium and the homogenized mycelium of *P. ostreatus* could remove 100% of BBP within 2 days of incubation, even in distilled water. The biodegradation, at least the initial metabolism of BBP, by *P. ostreatus*, may not be attributed to the extracellular enzymes contained in the culture supernatant but mainly attributed to the mycelium-associated enzymes like laccase of *I. lacteus* [20] or intracellular enzymes including esterase [12]. When the mycelial pellets collected by centrifugation of a 5-day culture of *P. ostreatus* was reacted with *o*-tolidine (a substrate of laccase) the mycelium-associated laccase activity was higher than those of other white rot fungi tested in this study (data not shown). Intracellular enzymes, such as catechol dioxygenase, which is involved in degradation of aromatic compounds, may be responsible for this kind of result. In fact, the activity of catechol 1,2-dioxygenase was related to degradation of dibutyl phthalate [9], and therefore, it may be necessary to examine the activity of catechol dioxygenase in the culture of this fungus. In a previous study on fungal TNT degradation, the culture supernatant of *Irpex lacteus* could not remove any TNT, and intracellular enzymes including aromatic nitroreductase were involved in the initial metabolism of TNT [6]. According to the result of this study, fungal mycelium was indispensable for the BBP biodegradation by *P. ostreatus*, and this phenomenon should be examined for degradation of other phthalates.

When the intact mycelia of *P. ostreatus* were immobilized in agar block, they could remove 58% of 100 mg/l DMP in 5 days, which was almost the same removal rate as in the suspended culture (Table 1). It has been reported that a more efficient degradation of phthalate was achieved by immobilizing *Bacillus* sp. in alginate and polyurethane foam because immobilized cells can be used for repeated cycles and can be operated for the degradation of phthalate in a packed-bed reactor with much higher dilution rates [15]. More commonly used immobilizing materials such as alginate or polyurethane may increase the removal rate of phthalates by immobilized cells of *P. ostreatus*.

Fig. 1. Biodegradation of 100 mg/l butylbenzyl phthalate (BBP) in 2 days (closed bar) and 4 days (open bar) by different parts of a 5-day preincubated culture of *Pleurotus ostreatus*.
Symbols: culture supernatants (**A**), washed intact mycelia in distilled water (**B**), homogenized mycelia in distilled water (**C**).

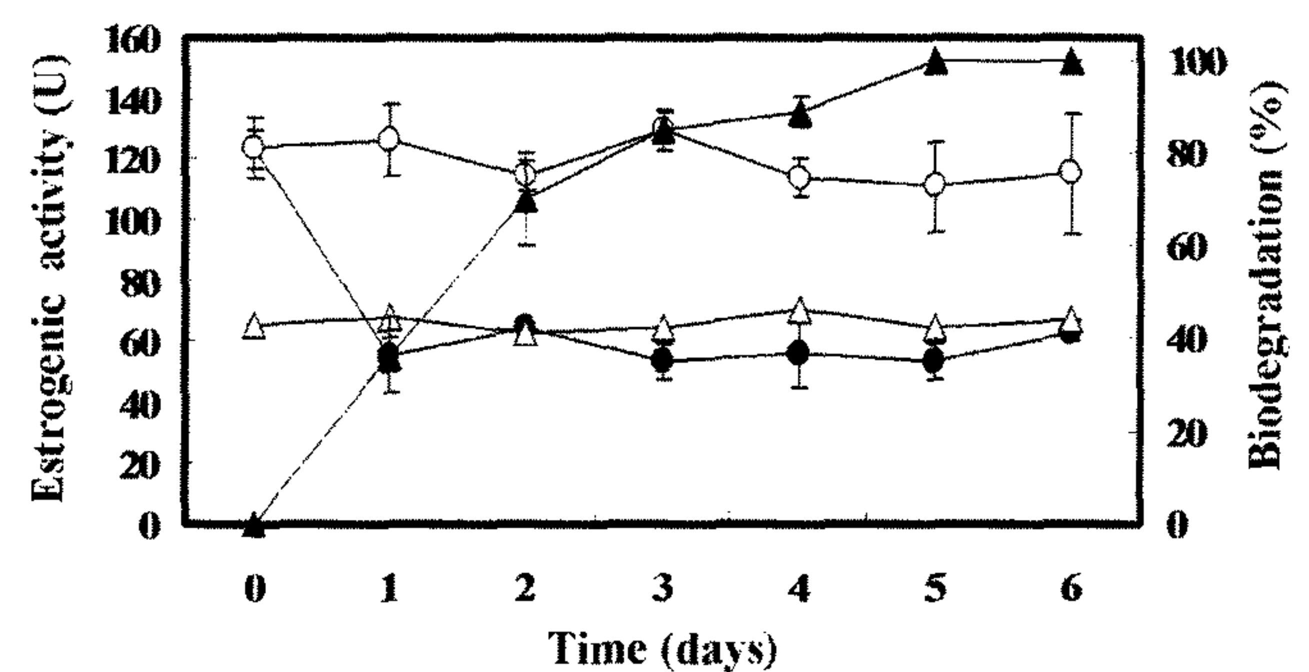

Fig. 2. Change of estrogenic activities during the degradation of 100 mg/l dimethyl phthalate (DMP) by *Pleurotus ostreatus* for 6 days. Estrogenic activity was expressed as Miller unit (U) of β-galactosidase activity.
Symbols: supernatant of fungal culture treated with DMP (●), biodegradation of DMP by *P. ostreatus* (▲), uninoculated control (○), supernatant of fungal culture without DMP addition (△).

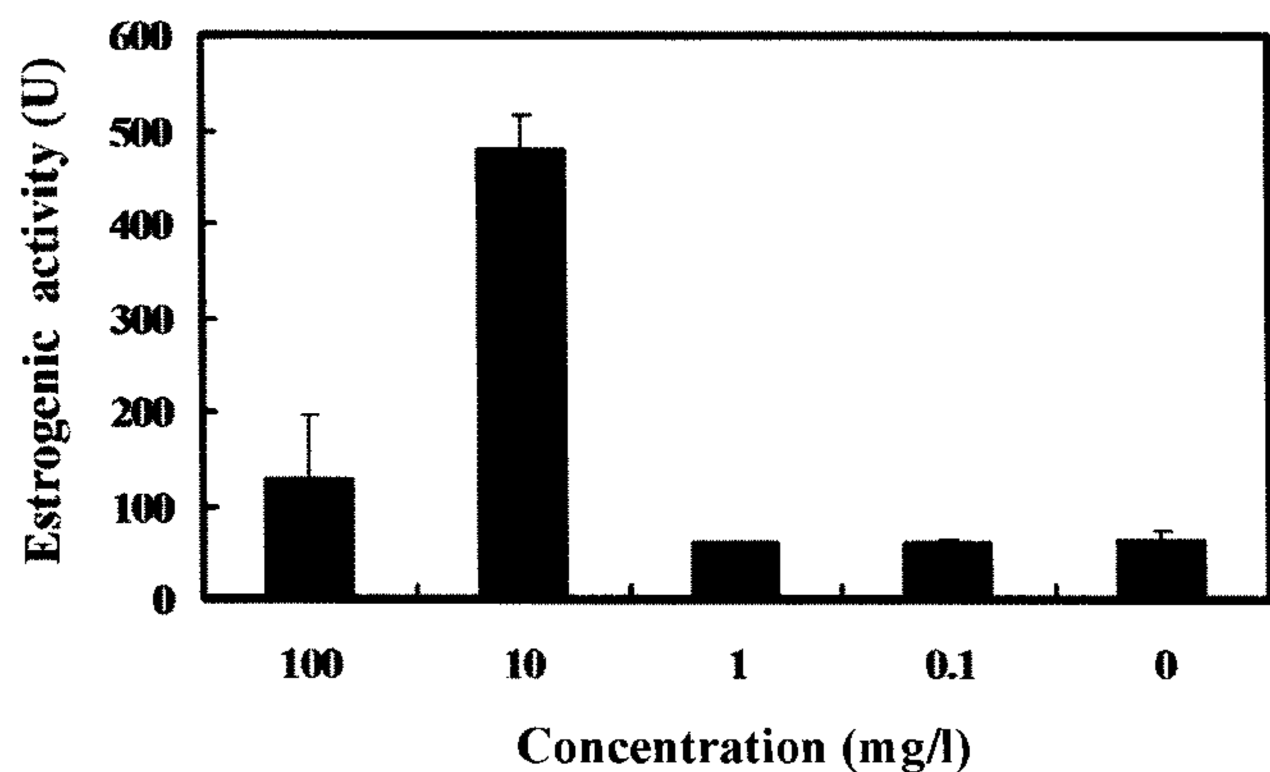

Fig. 3. The estrogenic activity of dimethyl phthalate (DMP) at different concentrations in YMG medium.

Estrogenic Activities During the Biodegradation of Phthalate

During the DMP degradation by 5-day pregrown culture of *P. ostreatus*, the change of estrogenic activity in the fungal culture was estimated for 6 days by measurement of β-galactosidase activity using a yeast transformant of Y190. Estrogenic activity of 100 mg/l DMP, 128 U (Miller units), decreased according to the degradation by *P. ostreatus*, and showed the range of 53–64 U for 6 days, which was the level of the control without DMP addition (Fig. 2). However, the estrogenic activities in YMG medium containing the serially diluted DMP between 100 and 0.1 mg/l were not proportional to the concentration of DMP, and the highest estrogenic activity was shown at 10 mg/l DMP (Fig. 3). As reported by Nishikawa *et al.* [13], this *in vitro* estrogenic assay system might be affected by the time of chemical exposure, difference of chemical permeability, and toxicity of tested chemical to yeast cells. The toxicity of DMP at 100 mg/l might be higher than that of lower concentrations. A similar phenomenon of decrease in the estrogenic activity expressed as β-galactosidase activity at higher chemical concentrations appeared with nonylphenol and bisphenol A using the same yeast transformant [19]. Since estrogenic products may be formed from degradation of phthalates [14] and toxic metabolites can be produced during biodegradation of several phthalates [4, 11], the precise changing pattern of estrogenic activity during phthalate metabolism by white rot fungi should be examined.

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