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Changes in the Activities of Enzymes Involved in the Degradation of Butylbenzyl Phthalate by *Pleurotus ostreatus*

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Degradation of butylbenzyl phthalate (BBP) by the white rot fungus *Pleurotus ostreatus* and the activities of some degrading enzymes were examined in two different media containing 100 mg/l of the compound. *P. ostreatus* pre-grown for 7 days in complex YMG medium was able to completely degrade BBP within an additional 24 h but degraded only 35 mg/l of BBP in 5 days of incubation in minimal medium. Fungal cell mass in the culture in YMG medium was higher in the presence than in the absence of BBP. The esterase activity of the fungal culture in YMG medium was higher than that in minimal medium and increased with the addition of BBP. On the contrary, laccase activity was higher in minimal medium and it did not increase upon the addition of BBP. General peroxidase activity increased for a few days after the addition of BBP to both media. The degradation of BBP and its metabolites by *P. ostreatus* thus may be attributed mostly to esterase rather than lignin-degrading laccase. In addition, the activities of the enzymes involved in BBP degradation and their changes varied significantly in the different media and culture conditions.

Keywords: Biodegradation, butylbenzyl phthalate, esterase, laccase, *Pleurotus ostreatus*

Endocrine-disrupting chemicals (EDCs) are exogenous substances that interfere with synthesis, secretion, transport, and binding in the human body and disrupt and eliminate natural hormones responsible for the maintenance of homeostasis [24]. EDCs encompass a variety of chemical classes, including pesticides, compounds used in the plastics industry and in consumer products, and other industrial by-products and pollutants. Among these, butylbenzyl phthalate (BBP) is mainly used in vinyl tile and as a plasticizer. Because of its widespread use in many industrial fields, BBP as an environmental pollutant is widely dispersed and is a threat to the health of all organisms including human beings.

Recently, white rot fungi have been found to degrade EDCs such as alkylphenol, bisphenol A, and phthalates [6, 11, 25]. The capability of most white rot fungi to degrade a wide range of EDCs is due to extracellular ligninolytic enzymes such as lignin peroxidase, manganese-dependent peroxidase, and laccase [3, 8, 18, 21]. Although white rot fungi have many advantages for the biodegradation of recalcitrants, their degradation abilities and the enzymes involved may change depending on the types and characteristics of the chemicals and culture conditions. As an example, *Irpex lacteus* showed the highest TNT degrading activity among the white rot fungi tested but exhibited the lowest BBP degradation rate [8, 10]. Tapia and Vicuña [19] reported that lignin mineralization by *Ceriporiopsis subvermispora* and manganese peroxidase and laccase titers changed as a result of pH changes in the culture according to the concentrations of the nitrogen sources.

To date, studies on the degradation of phthalates by white rot fungi have been focused mainly on their degrading capability, enzymatic transformation, and the degradation pathway [8, 13, 22]. Studies on the type and activity of the enzymes involved and the effects of environmental conditions are also important for understanding the mechanisms of phthalate biodegradation by white rot fungi. To this end, the BBP degradation capability of *P. ostreatus* in two different media was examined in this study, and culture parameters affecting BBP degradation were also investigated.

Initially, BBP degradation by, and culture conditions for, *P. ostreatus* isolated from forest soil in Kangwon-Do, Korea were investigated. *P. ostreatus* was grown in YMG broth medium (4 g yeast extract, 10 g malt extract, and 4 g glucose per 1 l distilled water, pH 6.0) on a rotary shaker

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(130 rpm) for 5 days at 30°C. After incubation, the fungal culture was blended with a homogenizer (Model X120; CAT, Germany) for 30 s. The blended fungal culture (0.5 ml) was inoculated into 20 ml of fresh YMG or a minimal medium (10 g glucose, 1 g KH_2PO_4, 1 g K_2HPO_4, 3 g $(NH_4)_2PO_4$, 0.3 g $MgSO_4$, and 0.1 g $CaCl_2$ per 1 l distilled water) and incubated for 7 days on a shaking incubator at 30°C. Subsequently, BBP purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA) was added to a final concentration of 100 mg/l, and the fungal cultures were further incubated for an additional 5 days. BBP was added after 7 days of preincubation to allow for the production of sufficient amounts of fungal mycelia and the extracellular enzymes necessary for BBP degradation. During the entire incubation period, the pH of control cultures with and without BBP was determined daily. BBP degradation by the fungi was also determined daily by gas chromatography (HP 5890; Hewlett Packard Co., USA) with a flame ionization detector, the analytical procedure for which was described in a previous report [8]. The concentration of residual glucose in the media was analyzed by high-performance liquid chromatography (Waters Breeze Model; Waters Co., USA). Analytical conditions were as follows: OP NH_2 column (250×46 mm; RS Tech, Korea), 40°C column temperature, 75:25 acetonitrile/water mobile phase, 1.2 ml/min flow rate, and RI detector. For the measurement of fungal mass, the fungal culture was filtered through filter paper (Whatman No. 3; Whatman, USA) and dried at 100°C for 24 h. The dried fungal mass was weighed on an analytical balance.

Since the ligninolytic enzymes involved in most aromatic compound degradation pathways in white rot fungi, which are usually produced during secondary metabolism, are brought about by a deficiency such as in C or N, most fungal degradation studies have used a minimal medium [16, 19, 20, 29]. However, because the nutrient-rich YMG medium may be more effective for the degradation of some aromatics than minimal medium [10], we compared BBP degradation in both media. In YMG medium, *P. ostreatus* pre-grown for 7 days was able to degrade completely 100 mg/l of BBP within 24 h of additional incubation. In contrast, pre-grown *P. ostreatus* in minimal medium could not completely remove BBP, and only 35% of BBP was removed during an additional 5 days of incubation (Fig. 1). Similarly, low-level BBP degradation (30%) by another white rot fungus, *Phlebia tremellosa*, was also reported in a different minimal medium [28]. Although the culture conditions were different, BBP degradation in YMG medium was faster than that by *Arthrobacter* sp., which could degrade 100% of 25 mg/l BBP in 4 days [6], and a sludge sample, which showed a half-life of 0.9–1.2 days for 100 mg BBP/kg [5].

Determining the effects of various culture parameters on BBP degradation may be important for understanding the mechanisms of phthalate biodegradation, because a sufficient amount of fungal mass may be required for the efficient initial degradation of xenobiotics by white rot fungi [8, 10]. In addition, the pH of a fungal culture has a significant effect on fungal growth and the activity of BBP-degrading enzymes [1, 18, 21]. In this study, changes in medium pH and fungal mass were monitored during the degradation of

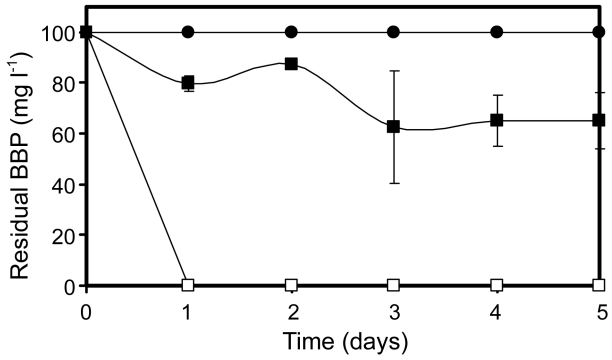

Fig. 1. Degradation of 100 mg/l BBP by *P. ostreatus* precincubated for 7 days before addition of BBP. Symbols: uninoculated control (●), inoculated in minimal medium (■), inoculated in YMG medium (□).

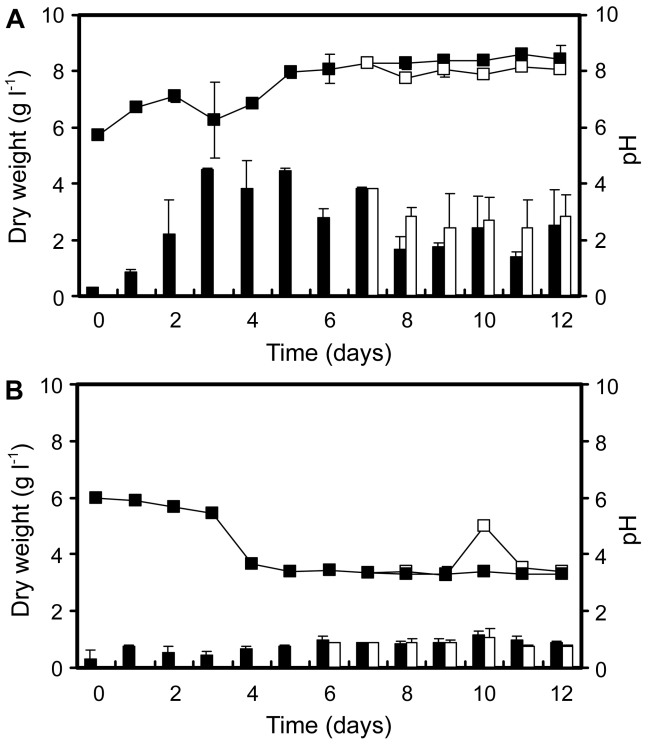

Fig. 2. Changes in pH and fungal mass (g dry weight/l) during degradation of 100 mg/l BBP by *P. ostreatus* in YMG medium (**A**) and minimal medium (**B**).
BBP was added to fungal cultures after 7 days of incubation. Symbols: pH (■) and fungal mass (closed bar) in the culture without BBP; pH (□) and fungal mass (open bar) with BBP.

BBP by *P. ostreatus* (Fig. 2). The dry weight of fungal biomass and pH were higher in YMG medium than in the minimal medium and these differences seemed to be the main reasons for the higher rate of BBP degradation in the former. Culture pH in the YMG medium increased from 5.8 to 8 after 7 days of preincubation, and fungal mass rapidly increased during the initial 3 days of preincubation and then decreased slightly. Xu *et al.* [27] also reported the highest BBP removal rate between pH 7.0 and 7.5 in a culture of *Pseudomonas fluorescens*; this rate was 6 times higher than that at pH 5.0. A similar pH increase was observed during the degradation of synthetic lignin by *Ceriporiopsis subvermispora* at high nitrogen concentrations [19], but the explanation for this phenomenon is unclear. After the addition of 100 mg/l BBP to the fungal culture herein, the pH decreased slightly and was maintained between 7.6 and 8.0 during the additional 5-day incubation. The pH decrease in the YMG medium after the addition of BBP may be due to the subsequent formation of metabolic intermediates from BBP degradation. In the biodegradation of BBP, phthalic acid is produced as a main metabolite and it may change the culture pH [5, 6, 26]. Fungal mass in the YMG medium began to decrease at the end of preincubation owing to the depletion of substrate. However, the culture containing BBP contained more biomass than that without BBP, suggesting that *P. ostreatus* utilized BBP as a carbon source. The concentration of glucose rapidly decreased during the initial 4 days of preincubation and was below 0.5 mg/l at the end of preincubation, perhaps inducing BBP degradation. On the contrary, the pH in the minimal medium dropped from an initial 6.0 to below 4 after 4 days of incubation and was maintained at a similar level until 12 days; this response might be due to the production of various organic acids as metabolites and the weak buffering capacity of the medium. Similar pH decreases during fungal incubation in minimal media were reported in other studies in which they were explained to be the result of the production of organic acids such as oxalate and malonate and the metabolism of buffer by the fungus [15, 19]. This low pH seemed to be the main reason for the lower biomass and degree of BBP degradation compared with those in YMG medium. Medium pH and fungal mass were not affected significantly by the addition of BBP.

It is known that a variety of enzymes are involved in the degradation of recalcitrant aromatics by white rot fungi and that those participating in the initial metabolism differ depending on the compound [14]. For the degradation of phenanthrene by *P. ostreatus*, membrane-bound enzymes such as cytochrome P-450 monooxygenase and epoxide hydrolase were involved in the initial degradation rather than laccase and manganese peroxidase [1]. Unlike the fungal degradation of phenolic EDCs such as bisphenol A and alkylphenol, which can proceed by the action of laccase and peroxidases [4, 14], the initial transformation of nonphenolic compounds including phthalates cannot be carried out by laccase unless some mediators are also present [14]. However, Yeo *et al.* [28] reported an increase in laccase activity and transcript level in a culture of *Phlebia tremellosa* containing BBP.

In this study, the activities of several enzymes expected to be involved in BBP degradation were examined in a culture of *P. ostreatus* in two different media, to elucidate the correlation between the degree of degradation and these activities. To measure the activity of laccase, a typical ligninolytic enzyme, 0.1 ml of fungal culture filtrate was added to 0.4 ml of distilled water followed by the addition of 0.5 ml of 2 mM 2,6-dimethoxyphenol. Laccase activity was determined by measuring the rate of production of 3,3',5,5'-tetramethoxy-diphenoquinone in a spectrophotometer at 469 nm [23]. The activity of general peroxidase, another degrading enzyme of white rot fungi, was measured using carbazole (3-amino-9-ethyl-carbazole) as a substrate. Culture filtrate (0.12 ml) was added to 0.8 ml of a solution containing 0.05% carbazole and 0.3% (v/v) H_2O_2 in 50 mM sodium acetate buffer and then incubated at 30°C for 40 min. After incubation, the OD_{405} of the reaction mixture was measured in a spectrophotometer, and 0.01 of OD_{405} was expressed as 1 U/ml of enzyme activity [17]. In addition, esterase activity was determined by measuring the concentration of *p*-nitrophenol produced by the hydrolysis of *p*-nitrophenyl butyrate (*p*NPB). The fungal cultures were filtered with filter paper (Whatman No. 3) and then centrifuged (16,600 ×*g*, 20 min). Culture filtrate (0.1 ml) was added to 0.9 ml of a solution (10 µl *p*NPB stock solution [1.76% (v/v) *p*NPB in acetonitrile], 790 µl of 50 mM phosphate buffer [pH 7.0], and 100 µl of 0.04% Triton X-100), and the final concentration of *p*NPB was 1.001 mM. After incubation for 5 min at 37°C, the absorbance was measured at 405 nm in a spectrophotometer (UV-1700; Shimadzu Co., Japan). The outcome was expressed as U/ml, which means that one unit of esterase activity converts one micromole of *p*NPB to *p*-nitrophenol per minute [7].

Fig. 3 shows the activities of laccase, esterase, and general peroxidase in the fungal cultures during the 12-day incubation. After the addition of BBP, esterase and general peroxidase titers in the YMG medium increased rapidly and were maintained at higher levels than those in the control without BBP. Esterase activity decreased during the later incubation period in the YMG medium but was higher in the culture containing BBP than in the control. General peroxidase activity in the YMG medium decreased after 3 days of BBP degradation and then became lower than that in the control. Laccase activity in the YMG medium was somewhat low, and BBP-containing medium had lower activity than the control, supporting the observation that the initial transformation of phthalates cannot be carried out by laccase [14]. Laccase activity in the minimal medium was higher than that in the YMG medium, but esterase

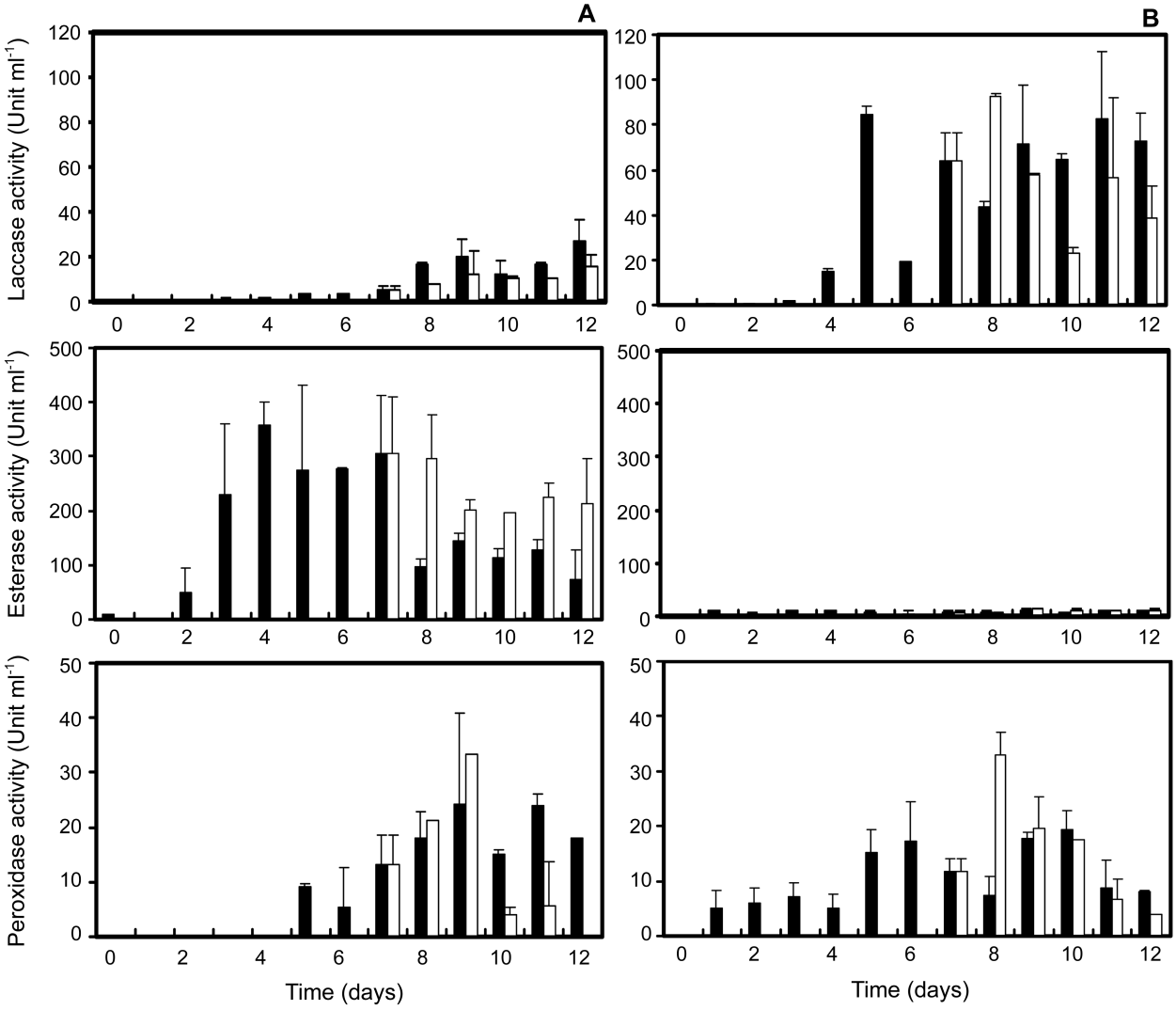

Fig. 3. Activities of laccase, esterase, and peroxidase during degradation of 100 mg/l BBP by *P. ostreatus* in YMG medium (**A**) and minimal medium (**B**).
BBP was added to fungal cultures after 7 days of incubation. Symbols: without BBP (closed bar); with BBP (open bar).

activity was negligible. Although laccase and general peroxidase activities increased with the addition of BBP, they decreased after 3 days of BBP degradation and then became lower than the control. General peroxidase may be involved in the degradation of metabolites produced from the initial degradation of BBP. In both YMG and minimal media, lignin peroxidase and manganese-dependent peroxidase activities were negligible (data not shown).

The inverse pattern of the esterase and laccase activities of *P. ostreatus* in the two different media might be due to the changes in pH of the fungal culture, although the pH optima of the enzymes are highly dependent on the substrate. It was reported that the optimal pH of esterase produced by fungi is between 7.0 and 9.0. The optimal pH of xylan esterase produced by the anaerobic fungus *Orpinomyces* sp. strain PC-2 is 9.0 and that of cholesterol esterase from *Trichoderma* sp. AS59 is 7.0 [2, 12]. In turn, the optimal pH of laccase produced by *Trametes modesta* is in the range of 3.0–5.0 [9]. Since the increased expression of laccase upon addition of phthalates in another white rot fungus, *Phlebia tremellosa*, has been reported [28], the degradation of BBP and its metabolites by the laccase of *P. ostreatus* under optimal pH conditions should be investigated. The biochemical characteristics of the esterase and laccase produced by *P. ostreatus* should also be examined in a further study.

BBP was completely degraded within 24 h in a culture of *P. ostreatus* pre-grown for 7 days in complex YMG medium. BBP degradation could be attributed to esterase rather than lignin-degrading laccase, which may not play a major role in the initial attack of nonphenolic substrates. These results indicate that the production of degrading

enzymes and the subsequent degradation of recalcitrant compounds by white rot fungi are dependent on the culture conditions and the type of chemicals involved.

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