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# Effect of Fungal Pellet Morphology on Enzyme Activities Involved in Phthalate Degradation

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**Pellet size of white rot fungus, *Pleurotus ostreatus* may affect the secretion of its degradative enzymes and accompanying biodegrading capability, but could be controlled by several physical culture conditions in liquid culture. The pellet size of *P. ostreatus* was affected by the volume of inoculum, flask, and medium, but the agitation speed was the most important control factor. At the lower agitation speed of 100 rpm, the large pellets were formed and the laccase activity was higher than that of small pelleted culture at 150 rpm, which might be due to loose intrapellet structure. However, the biodegradation rates of benzylbutylphthalate and dimethylphthalate were higher in the small pelleted culture, which indicated the involvement of other degradative enzyme rather than laccase. The activity of esterase which catalyzes the nonphenolic compounds before the reaction of ligninolytic enzymes was higher in the small pelleted culture, and coincided with the degradation pattern of phthalates. This study suggests the optimization of pellet morphology and subsequent secretion of degradative enzymes is necessary for the efficient removal of recalcitrants by white rot fungi.**

***Keywords*:** white rot fungus, pellet morphology, laccase, esterase, phthalate biodegradation

Due to the metabolic versatility, white rot fungi have an enormous potential for industrial application, especially bioremediation of a variety of recalcitrant pollutants (Reddy, 1995; Asgher *et al*., 2008). When they grow in submerged culture, they exhibit different growth morphologies from loose mycelial aggregate to dense pelleted growth forms (Grimm *et al*., 2005). Morphological changes are occurred by many factors including inoculum size and culture conditions (Birhanli and Yesilada, 2006; El-Enshasy *et al*., 2006; Park *et al*., 2007), and may affect the fungal metabolism such as phthalate degradation due to the change of secretion pattern of extracellular enzymes involved in degradation (Jiménez-Tobon *et al*., 1997; Hwang *et al*., 2008). Pelleted morphology has some advantages including the decrease of viscosity of culture fluid and facilitation of solid-liquid separation, but it also causes mass transfer limitation finally resulting in autolysis within the inner part of large pellets (El-Enshasy *et al*., 2006). White rot fungi produce many enzymes involved in degradation including ligninolytic enzymes, and their production varies according to the fungal strain, cultivation conditions, and many other factors (Tari *et al*., 2007). Therefore, fungal morphology may affect the enzyme production and the degradation rate of specific target compounds by certain white rot fungus. However, the precise relationship between pellet morphology and enzyme activity in white rot fungi has not been established.

In this study, the pellet size of *Pleurotus ostreatus* that had been reported to have a high degrading capability for phthalates, recalcitrant endocrine-disrupting compounds (Hwang *et al*., 2008) was controlled to enhance the activity of the degradative enzymes and subsequent degradation rate of phthalates.

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## Materials and Methods

### Fungal culture

The fungal strain used in phthalates biodegradation was *Pleurotus ostreatus*, which was isolated from decaying wood by Dr. K. Kim in Kangnung National University. *P. ostreatus* was cultured on potato dextrose agar plate. Ten pieces of agar plug (dia. 1 cm) covered with fungal mycelia were added to a 100 ml YMG medium (yeast extract 4 g, malt extract 10 g, glucose 4 g, DW 1 L) followed by homogenization for 30 sec with a homogenizer (Model ×120, CAT, Germany), and then incubated (30°C, 130 rpm) for 5 days. After 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. Supernatants and precipitated mycelia were separately collected into sterile bottles, and fungal inoculum was prepared by the addition of supernatants to the collected mycelia.

### Control of fungal pellet size and its effect on pellet morphology and enzyme activity

Fungal inoculum was added into YMG medium, and enzyme activity was measured during incubation at 30°C. Pellet size of *P. ostreatus* in the culture was controlled by inoculum size (0.5, 3.0, and 5.5 ml in 100 ml medium), mycelial concentration in the inoculum [3, 5, 10, and 30% (wet w/v)], agitation speed (100 and 150 rpm), flask size (100, 250, and

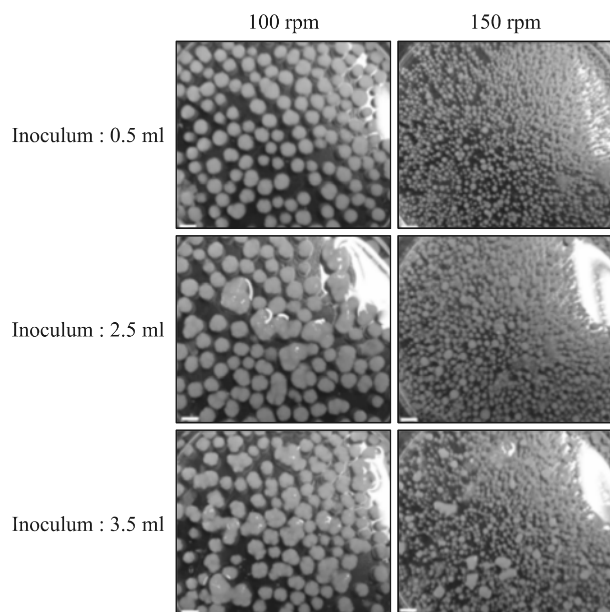


**Fig. 1.** Morphology of the pellets of *P. ostreatus* grown for 8 days in 100 ml YMG medium in 250 ml flask with the different inoculum size and agitation speed.

500 ml) and medium volume (50, 100, and 150 ml in 250 ml flask).

Laccase activity in the culture supernatant was measured by spectrophotometry using 2,6-dimethoxyphenol as an enzyme substrate (Garzillo *et al*., 1998). One unit of laccase activity was defined by oxidation of substrate 1 μmole in 1 min by 1 mg of mycelia ($\varepsilon_{469}$=9800 $M^{-1}$ $cm^{-1}$). Esterase activity in the culture supernatant was also measured by spectrophotometry using *p*-nitrophenylbutyrate as an enzyme substrate (Davies *et al*., 2000; Wang *et al*., 2000). One unit of esterase activity was defined by oxidation of substrate 1 μmole in 30 min by 1 mg of mycelia ($\varepsilon_{405}$=6830 $M^{-1}$ $cm^{-1}$). The surface and inner parts of pellets of *P. ostreatus* grown at the agitation speed of 100 and 150 rpm were observed by scanning electron microscopy. Samples were pretreated by the method described by Lee and Lee (1998). The pellets were sputter-coated with gold-palladium, and observed under a low-vacuum scanning electron microscope (Hitachi Science Systems Ltd., Japan).

### Biodegradation of phthalates in pellet size-controlled fungal culture

*P. ostreatus* was grown for 7 days under the conditions for pellet size control described above, and fungal cultures were separated according to the pellet size of 1~2, 3 and 4~5 mm dia. Separated fungal pellets (30 mg dry wt) were added into 40 ml YMG medium in 100 ml flask with 100 mg/L butylbenzylphthalate (BBP) or dimethylphthalate (DMP) and incubated at 30°C, 150 rpm. For the determination of residual phthalates in the fungal culture, replicate cultures were homogenized for 1 min followed by addition of 10 ml hexane. The mixture was strongly shaken in a vertical extraction shaker (Resipro Shaker RS-1, JeioTech, Korea) at 350 stroke/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 hexane concentrate was determined by gas chromatograph (GC) (HP 5890, Hewlett Packard Co., USA) equipped with 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 140 and 230°C for 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. Change of estrogenic activity during the fungal degradation of phthalates was measured by the yeast two-hybrid assay system developed by Nishikawa *et al*. (1999).

All the experiments were carried out in triplicate, and the mean value with the standard deviations are presented.

## Results and Discussion

### Control of fungal pellet size and its effect on enzyme activity

Pellet size in the culture of *P. ostreatus* was not significantly affected by the size of fungal inoculum, but the agitation

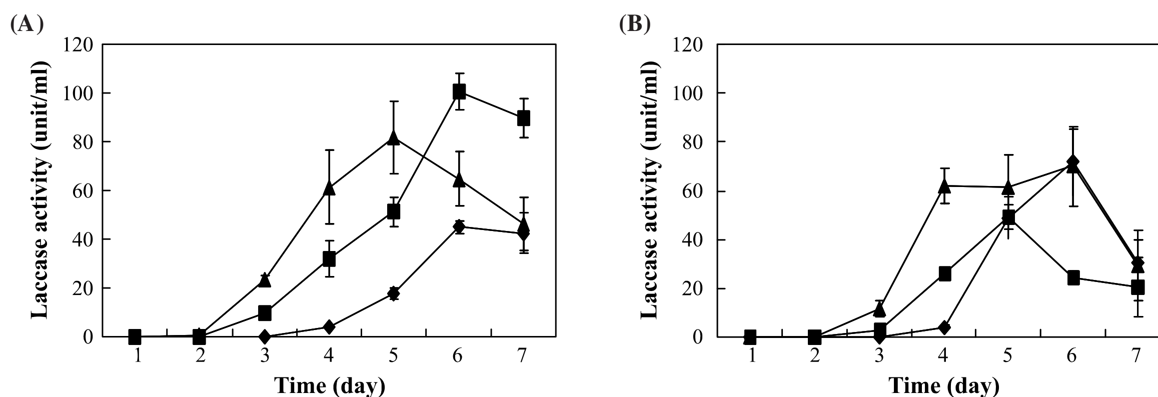


**Fig. 2.** Change of laccase activity in the culture of *P. ostreatus* with different inoculum size (◆, 0.5 ml; ■, 3 ml; ▲, 5.5 ml) and agitation speed (A, 100 rpm; B, 150 rpm) in 100 ml YMG medium.

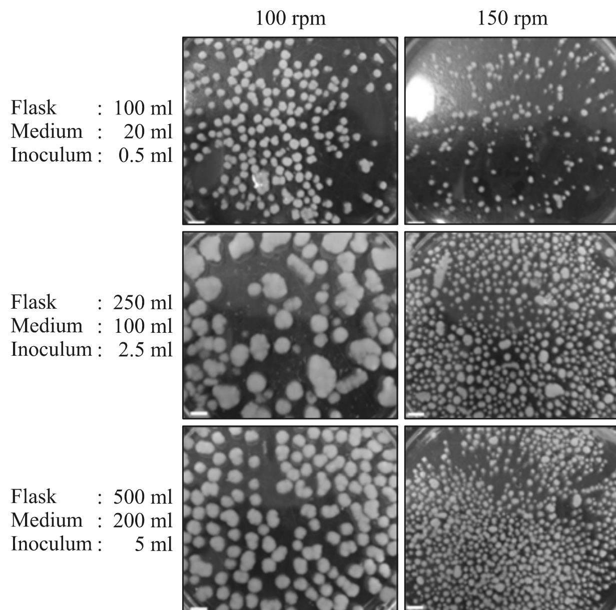


**Fig. 3.** Morphology of the pellets of *P. ostreatus* grown at different agitation speed in different volume of flask, inoculum, and YMG medium for 7 days.

speed of 150 rpm could make smaller pellets than 100 rpm due to the higher shear stress (Fig. 1). Jiménez-Tobon *et al.* (1997) also reported the formation of smaller pellets of white rot fungus *Phanerochaete chrysosporium* at higher agitation rate, but the larger pellets were formed by the lower initial concentration of spore which was different inoculum from the mycelial homogenate in this experiment. When the activities of ligninolytic enzymes were examined in the culture of *P. ostreatus*, laccase seemed to be the major ligninolytic enzyme. The activity of lignin peroxidase was negligible and the Mn peroxidase could not be detected in the culture of *P. ostreatus*. Although the inoculum size did not affect the size of fungal pellet formed, the fungal culture with the large amount of inoculum showed the faster increase and higher levels of laccase activity (Fig. 2). The secretion of ligninolytic enzymes such as laccase usually occurs under the stationary phase of cultivation (Reddy, 1995), which could be achieved earlier by large inoculum size. Lower agitation speed (100 rpm) could also increase the laccase activity faster and higher than that at 150 rpm (Fig. 2). Feng *et al*. (2004) also reported the formation of larger pellet and higher yield of secondary metabolite, destruxin B by fungus *Metarhizium anisopliae*. However, if the fungal pellets become too large, it may reduce the enzyme production due to limitation of oxygen transfer (Jiménez-Tobon *et al*., 1997). The pH 5.0~6.0 in the culture supernatant grown at 100 rpm was lower than pH 7.1~7.5 grown at 150 rpm. Many fungal laccases were induced and showed the higher activity at acidic conditions (Kim *et al*., 2001), and this result also followed the similar pattern.

The higher mycelial concentration in the inoculum seemed to increase the laccase activity faster than the lower mycelial concentration at both 100 and 150 rpm of shaking conditions, but the differences were not significant (data not shown). Mycelial concentrations ranged 3~30% also did not affect the fungal biomass grown measured by dry weight and pH in the culture supernatants.

When the size of flask, medium volume and inoculum were examined for the control of pellet size and laccase activity, they could affect the pellet size, but the agitation speed was more important factor on the determination of pellet size (Fig. 3). Regardless of size of flask, medium, and inoculum, the higher agitation speed, 150 rpm always made the smaller pellets than at 100 rpm. The laccase activities increased faster and generally showed the higher values at 100 rpm than those at 150 rpm as like in Fig. 2 (Fig. 4). It has been known that agitation could suppress ligninolytic enzymes (Venkatadri and Irvine, 1990). The depth of fluid media may be an important factor for growth and degradation rate in fungal cultures. White rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor* exhibited the enhanced growth in a shallow (10 ml) culture than a deep (50 ml) culture, and it might be due to the difference in oxygen transfer (Logan *et al*., 1994). In this study, the dry cell weight of *P. ostreatus* after 8 days incubation was higher in the culture grown at 150 rpm (464±35 mg/L) with better oxygen transfer than that at 100 rpm (353±35 mg/L), but the laccase activity did not coincide with the fungal biomass and the medium volume in the culture (Fig. 4).

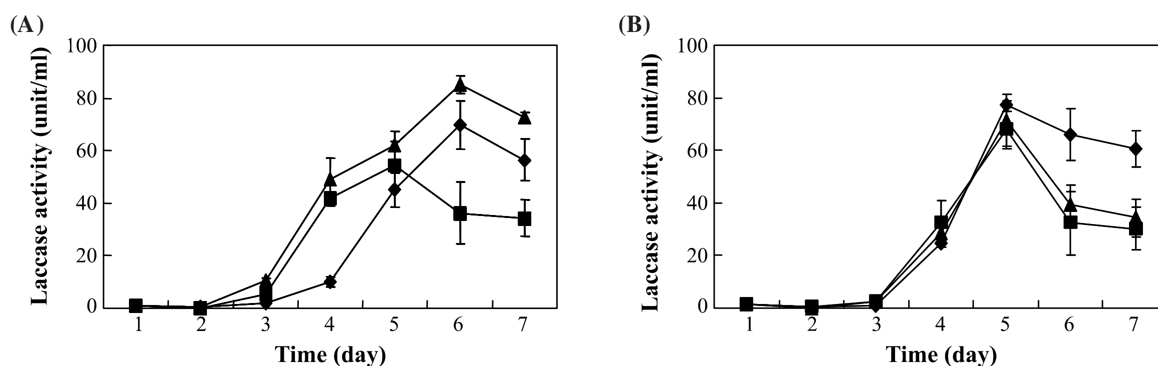


**Fig. 4.** Change of laccase activity in the culture of *P. ostreatus* in different volume of YMG medium (◆, 40 ml; ■, 80 ml; ▲, 120 ml) in 250 ml flask and agitation speed (A, 100 rpm; B, 150 rpm).

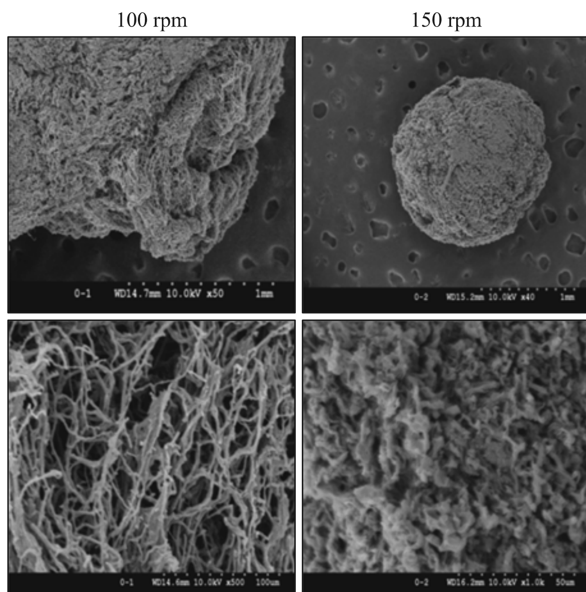


**Fig. 5.** Scanning electron microscopic observation of the pellets of *P. ostreatus* grown at different agitation speed.

When the inner part of the fungal pellets were observed by scanning electron microscopy, the pellets grown at 100 rpm showed more loose structure than the pellets grown at 150 rpm (Fig. 5). The pellets at 100 rpm exhibited the lower compaction due to the weaker shear force, and subsequently became larger than the pellets at 150 rpm. This loose intrapellet structure is likely one of reasons for the higher laccase activity in the large pelleted culture than the small but dense pelleted culture in which intrapellet gradients of nutrients or pH may be formed (Jiménez-Tobon *et al*., 1997). There may be many factors which can control the fungal morphology and activity (Grimm *et al*., 2005). In this experiment, the pellet size of *P. ostreastus* could be controlled by several culture conditions, especially agitation speed. El-Enshasy *et al*. (2006) also reported the agitation effects on growth morphology and enzyme production.

## Biodegradation of phthalates in pellet size-controlled fungal culture

The fungal cultures composed of small or large pellets were examined for the biodegradation of phthalates. The cultures with the small (1~2 mm dia.) and large (3~4 mm dia.) pellets degraded 54 and 27% of 100 mg/L of benzylbutylphthalate (BBP) in 12 h, respectively, however, the laccase activities of the large pelleted cultures were higher (73~75 U/ml) than those of the small pelleted cultures (25~33 U/ml) at both agitation speeds. The degradation of dimethylphthalate (DMP) by *P. ostreastus* showed the similar patterns. The cultures with the smallest pellets (1~2 mm) showed the highest removal rate of DMP than those of 3 and 4~5 mm pellets (Fig. 6A), but also had the lowest growth rate (Fig. 6B).

*P. ostreastus* already showed the high removal rates of phthalates, and laccase was one of the major degradative enzymes in the culture of *P. ostreastus* (Hwang *et al*., 2008). However, the inverse relationship between the size of fungal pellet and accompanying laccase activity and the phthalate degradation rates suggested that some other degradative enzymes may involve in the initial metabolism of phthalate degradation. Since phthalates do not have the hydroxyl group needed for laccase reaction, esterase is necessary for phthalates degradation before laccase attack (Tanaka *et al*., 2000). When the esterase activity was measured in the different pellet sized culture, the small pelleted (1~2 mm) culture had the higher esterase activity than the large pelleted (3~4 mm) culture (Fig. 7), and this result coincided with the pattern of phthalates degradation. On the contrary, the degradation of phenolic compounds such as bisphenol A and alkylphenols is likely more efficient in the culture with the large pellets which have the higher laccase activity.

Ligninolytic enzymes are important for the degradation of a variety of recalcitrant compounds by white rot fungi. However, the initial metabolisms of phenolic and nonphenolic compounds and the degradative enzymes involved may be quite different. This study showed that the secretion of the

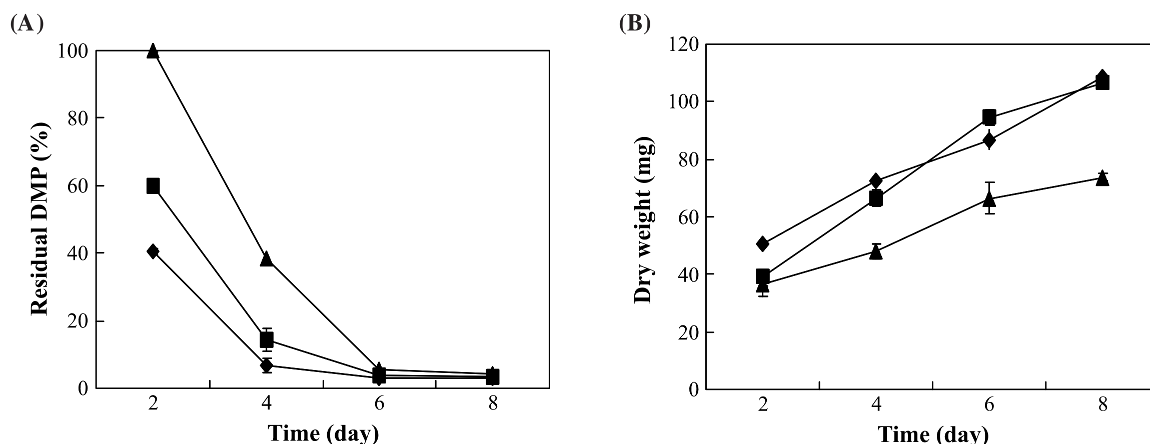


**Fig. 6.** Removal of 100 mg/L dimethylphthalate (A) and fungal dry weight (B) in the culture with different pellet size of *P. ostreatus* (◆, 1~2 mm; ■, 3 mm; ▲, 4~5 mm dia.).

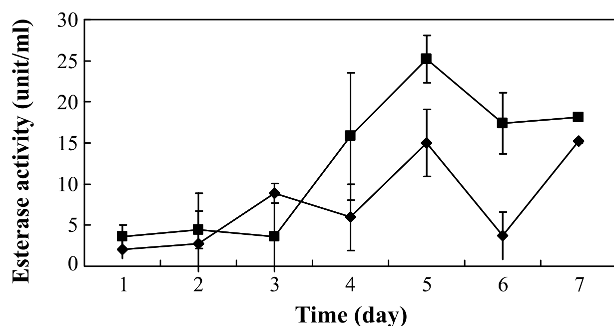


**Fig. 7.** Change of esterase activity in the culture of different pellet size of *P. ostreatus* in YMG medium (◆, 1~2 mm; ■, 3~4 mm dia.).

degradative enzymes in *P. ostreastus* can be changed by the fungal pellet morphology, and the optimization of pellet size and accompanying enzyme activity is necessary to apply the biodegrading capability of white rot fungi to bioremediation. Not only the size but also the internal structure and compaction of pellet should be investigated to examine the precise relationship between the pellet morphology and degradation in white rot fungi.

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