J. Microbiol. Biotechnol. (2000), **10**(3), 344–348

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Biodegradation of Pyrene by the White Rot Fungus, *Irpex lacteus*

HWANG, SOON-SUK AND HONG-GYU SONG*

Division of Biological Sciences, Kangwon National University, Chuncheon 200-701, Korea

Received: January 20, 2000

Abstract The removal percentage (94%) of 100 ppm of pyrene in a shaken culture of white rot fungus, *Irpex lacteus*, was much higher than that in a static culture (37.9%). Over 90% of the pyrene disappeared with *I. lacteus* grown at 15-27°C, yet less than 50% was removed at 37°C. The transformation rates of pyrene (4.5-5.0 μg/ml/day) were not very different among cultures with 5-30% inoculum sizes, and over 90% of the 100 ppm pyrene was removed in every case during 20 days of incubation. The biodegradation of pyrene by *I. lacteus* was confirmed by measuring the CO_2 evolved from the mineralization of the added pyrene. The activity of lignin peroxidase (LiP), which is known to be involved in the biodegradation by white rot fungi, was high between 8 to 12 days of incubation. Although manganese peroxidase activity was demonstrated during the same period as LiP, its activity was quite low, and no laccase activity was detected. Even though the activity patterns of ligninolytic enzymes did not coincide with the pyrene removal, this study shows that *I. lacteus* has a high biodegrading capability and can be a candidate for the bioremediation of polycyclic aromatic hydrocarbon contaminants.

Key words: Biodegradation, pyrene, *Irpex lacteus*, ligninolytic enzymes

White rot fungi can degrade lignin and a variety of other recalcitrant environmental pollutants, including some polycyclic aromatic hydrocarbons (PAHs) [6, 7, 19]. PAHs are common environmental pollutants arising from industrial operations as well as certain natural events such as forest fires [8]. Some of these PAHs and/or their metabolites are toxic, mutagenic, and carcinogenic. There have been many reports on the biodegradation and bioremediation of various PAHs, most of them being focused on the degrading bacteria [8, 9, 19]. White rot fungi have several potential advantages over other degrading microorganisms in the degradation of recalcitrants, including PAHs. Compared to most degrading enzymes of bacteria which have substrate specificities, the ligninolytic enzymes of these fungi involved in degradation of recalcitrants are very nonspecific and extracellular. Therefore, white rot fungi can degrade various insoluble organic pollutants [24]. The lignin degrading system is induced in response to nutrient exhaustion rather than by the presence of pollutants, allowing the fungi to degrade pollutants to essentially nondetectable levels [24]. Accordingly, the use of white rot fungi in the bioremediation of PAHs has been proposed [5, 17]. To-date, most attention on the study of white rot fungi has been directed to *Phanerochaete chrysosporium* [3, 4, 5, 6, 7, 26], although a possibility exists that some other species may be more effective. Indeed, several white rot fungi, including *Irpex lacteus*, have already shown a higher removal activity than *P. chrysosporium* for a 4-ring PAH pyrene [23]. In this study, the effects of certain environmental conditions on the degradation of pyrene by *I. lacteus* were examined and the activities of ligninolytic enzymes during pyrene degradation were measured. The results serve as a preliminary assessment of the potential usefulness of white rot fungi in the bioremediation of PAH pollutants.

Materials and Methods

Effects of Environmental Conditions on Pyrene Biodegradation

The *I. lacteus* was obtained from the Mycology Laboratory in the Department of Biology at Kangnung National University, where this strain had been isolated from soil and identified. The preparation of the fungal inoculum for the biodegradation test has been described elsewhere [23]. To investigate the effect of temperature on pyrene degradation, a YMG medium (yeast extract 4 g, malt extract 10 g, glucose 4 g, distilled water 1 l) including 100 ppm pyrene with 10% (w/v) fungal inoculum was incubated at 15, 20, 27, or 37°C. At every 4 days, duplicate samples were extracted with methylene chloride, and the residual pyrene was analyzed using a gas chromatograph (Hewlett Packard Co., Model 5890) equipped with a flame ionization

*Corresponding author
Phone: 82-361-250-8545; Fax: 82-361-241-4627;
E-mail: hgsong@cc.kangwon.ac.kr

detector. The operating conditions have been described elsewhere [11]. The pyrene removal under different sizes of fungal inoculum was examined. The YMG medium with the addition of 100 ppm of pyrene was inoculated with 5, 10, 20, or 30% (w/v) of the fungal inoculum and the residual pyrene was quantified during incubation at 27°C. The effect of agitation on the degradation of pyrene was also tested. A culture shaken in a rotary shaker (130 rpm) to remove 100 ppm of pyrene was also compared to a static culture.

Trapping of CO_2 Evolved from Pyrene Mineralization

The fungal suspension [10, 20, or 30% (v/v)] was inoculated into pyrene (200 ppm) containing YMG broth in a 250-ml modified Fernbach flask. The fungal cultures were incubated on a rotary shaker (130 rpm) at 15, 20, 27, or 37°C. The CO_2 evolved from the pyrene degradation was periodically trapped with a gas trapping device [18], while providing pure oxygen for flushing and maintaining aerobic conditions in the culture flask. The CO_2 trapped in 0.5 N KOH solution was then titrated with 0.05 M sulfuric acid and quantified.

Activities of Ligninolytic Enzymes During Pyrene Degradation

The activities of lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and laccase were examined during the pyrene degradation. The LiP activity was determined by the conversion rate of veratryl alcohol to veratryl aldehyde [25]. Thus, the culture supernatant (0.5 ml) was mixed with 0.45 ml of solution B (220 mM Na-tartrate, 3.33 mM veratryl alcohol, pH 3.0) in a 1 ml cuvette. Fifty microliters of 10 mM H_2O_2 was added and the optical density (OD) at 310 nm was measured before and after 1 min of enzyme reaction. The increased OD due to the formation of veratryl aldehyde by LiP was then converted to unit/l, and 1 unit of activity indicated that 1 micromole of veratryl alcohol was transformed to 1 micromole of veratryl aldehyde per minute. The MnP activity was expressed as the conversion rate of Mn(II) to Mn(III) [10]. Thus, 0.5 ml of the sample and 0.5 ml of reaction mixture (150 mM sodium lactate, 150 μM $MnSO_4$, pH 4.5) were mixed in a 1-ml cuvette, and the increased OD at 240 nm was measured after the addition of 0.5 ml of 150 μM H_2O_2. The activity was represented by the OD value itself. The laccase activity was examined by the OD at 590 nm after the addition of the *o*-tolidine reagent to the culture supernatant [22].

RESULTS AND DISCUSSION

Effects of Environmental Conditions on Pyrene Biodegradation

The white rot fungal strain, *I. lacteus*, used in the present study has already been shown to have a higher removal activity for pyrene than several other fungal strains including *P. chrysosporium* [23]. When the effect of agitation on pyrene removal was compared with static and shaken cultures (Fig. 1), the removal percentage of 94% in the shaken culture of *I. lacteus* was much higher than that in the static culture (37.9%). It is known that a static culture of *P. chrysosporium* is more efficient in biodegradation and enzyme production [27]. However, cases where a shaken culture exhibits a higher biodegradation capability than a static culture may be due to a better oxygen supply to the aerobic fungi and subsequent better growth [12]. *I. lacteus* also showed a higher decolorizing rate of landfill leachate in a shaken culture [13]. Since the shaken culture showed a higher removal rate of pyrene, the rest of the experiments in this study were carried out using shaken cultures.

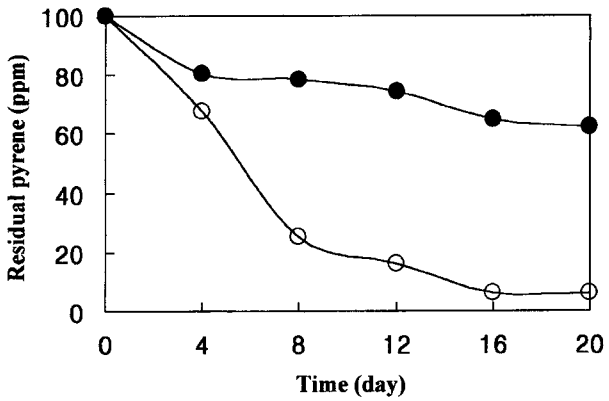

Fig. 1. Effect of agitation on removal of pyrene by *Irpex lacteus*.
Symbols: (●), static culture; (○), shaken culture (130 rpm).

The time course and extent of the pyrene removal with different temperatures are shown in Fig. 2. The removal rates of pyrene, 4.6–4.8 μg/ml/day, by *I. lacteus* in the mesophilic range (15–27°C) were higher than those by

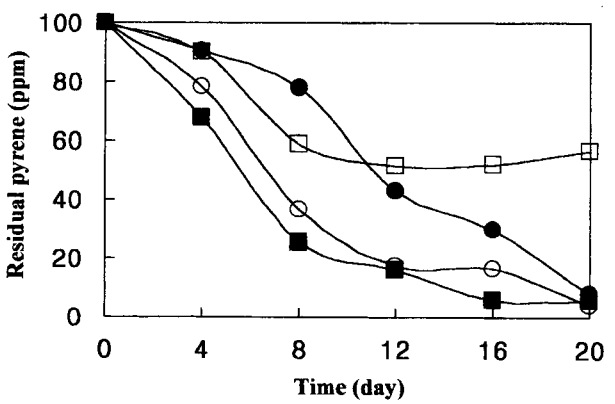

Fig. 2. Effect of temperature on removal of pyrene by *Irpex lacteus*.
Symbols: (●), 15°C; (○), 20°C; (■), 27°C; (□), 37°C.

other fungi [16, 21] and *Mycobacterium* [9]. Although *P. chrysosporium* has an optimal growth temperature of 39°C and most biodegradation studies with *P. chrysosporium* have been carried out at 37-39°C [4, 5, 7, 14], the removal of pyrene by *I. lacteus* was more efficient between 20-27°C than at 37°C. Yadav and Reddy [28] also showed that the level of degradation of monoaromatics by *P. chrysosporium* was relatively higher at 25°C than at 37°C. During 20 days of incubation, *I. lacteus* removed 95.9 and 94% of the pyrene at 20 and 27°C, respectively, and only about 40% at 37°C. Since the optimum growth temperature for *I. lacteus* is 25-27°C, its growth decreased at an elevated temperature (data not shown), therefore, the degradation at 37°C was lower than that at lower temperatures. The mycelium of *I. lacteus* was aggregated during growth at 37°C, and a similar phenomenon also occurred with higher concentrations of pyrene (500-1,000 ppm). This result indicated that *I. lacteus* was more sensitive to the toxicity of pyrene at a higher temperature.

The degradation rates of 4.5-5.0 μg/ml/day were not significantly different among the fungal cultures of 5-30% inoculum sizes (Fig. 3), and over 90% of the pyrene was removed in every case during 20 days of incubation. When the inoculum size was increased, the pyrene degradation was only enhanced in the initial phase. This was due to the fact that the fungal biomass in the cultures with larger inocula aggregated after 4 days of incubation, therefore, as the contact with the insoluble pyrene became limited the degradation rates became similar to those with the smaller inoculum sizes. Based on this result, an inoculum size of 10% was applied in the following experiments. Pickard *et al.* [21] also used 5-10% inocula for the fungal degradation of PAHs and enzyme production.

Disappearance of original compounds does not necessarily mean an absolute removal of those compounds. Some recalcitrants can be transformed rapidly in the initial phases, but the following steps may slow down and even

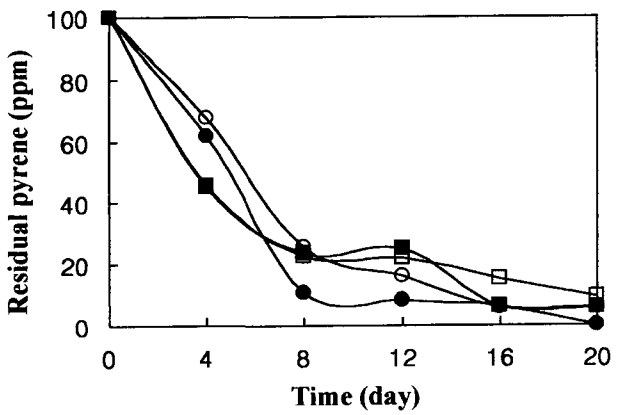

Fig. 3. Effect of inoculum size on removal of pyrene by *Irpex lacteus*.
Symbols: (●), 5%; (○), 10%; (■), 20%; (□), 30% (g wet wt/ml).

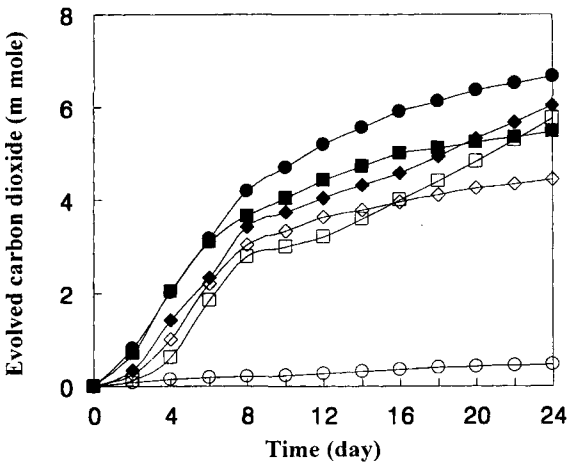

Fig. 4. Evolution of CO_2 during degradation of 200 ppm of pyrene by *Irpex lacteus* under different temperatures in YMG medium.
Symbols: (■), control without pyrene at 27°C; (○), control without inoculum at 27°C; (□), 15°C; (◆), 20°C; (●), 27°C; (◇), 37°C.

be rate-limiting steps. If a dead-end metabolite is produced, it can not be utilized further. Therefore, mineralization of original substrates into carbon dioxide must be confirmed in a biodegradation study. The effects of temperature and inoculum size on pyrene degradation were tested again in a mineralization experiment. The highest mineralization rate of pyrene was shown at 27°C, and CO_2 evolution at 37°C was even lower than the control without pyrene (Fig. 4). The culture with an inoculum size of 10% also mineralized more pyrene than the cultures with 20 and 30% inoculum (Fig. 5). Although there were some differences between the pyrene removal in the previous experiment and the CO_2 evolved in the mineralization experiment, which might have

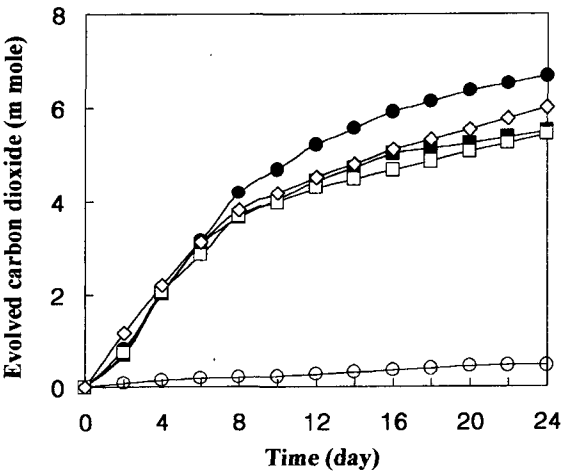

Fig. 5. Evolution of CO_2 during degradation of 200 ppm of pyrene by *Irpex lacteus* with different inoculum sizes in YMG medium at 27°C.
Symbols: (■), control without pyrene; (○), control without inoculum; (●), 10%; (□), 20%; (◇), 30% (g wet wt/ml).

been due to the difference in the cultivation methods, this mineralization experiment nevertheless confirmed the earlier results on the effect of temperature and inoculum size on pyrene degradation by *I. lacteus* (Figs. 2 and 3).

Activities of Ligninolytic Enzymes During Pyrene Degradation

The white rot fungi use the same enzyme system to degrade organic pollutants as they use to degrade lignin. Because of the nonspecific nature of the lignin-degrading enzymes, such as LiP, MnP, and laccase, white rot fungi may be excellent candidates for the bioremediation of contaminated sites [24]. However, each fungal strain may have a different production pattern of ligninolytic enzymes. The time course of LiP and MnP production by *I. lacteus* during pyrene degradation were similar to those of *Pleurotus* sp. [15], however, no laccase activity was detected in this study. The activities of LiP and MnP were the highest between 8 to 12 days of incubation, and the pyrene-containing culture showed much higher activities of both enzymes than the control without pyrene (Fig. 6). This result indicates that the pyrene or pyrene metabolites induced LiP and MnP in the shaken culture. However, their activities in the static culture were much lower than those in the shaken culture (data not shown), which was similar to the pyrene removal results (Fig. 1).

Although the pattern of enzyme activities was in agreement with that of Bezalel *et al.* [1], in which the activities of laccase and Mn-inhibited peroxidase of *Pleurotus ostreatus* were increased by the addition of catechol, phenanthrene, and pyrene, the activities of LiP and MnP of *I. lacteus* did not remain continuously in this experiment. It is known that ligninolytic enzymes such as LiP and MnP are involved in the degradation of PAHs by white rot fungi. However, the time courses of LiP and MnP did not coincide with the pyrene removal patterns (Figs. 2 and 3) and mineralization (Figs. 4 and 5) which showed a rapid biodegradation in the initial phase. This difference may be due to the involvement in the degradation of various enzymes by white rot fungus. Each fungal strain may use one or more ligninolytic enzymes among LiP [14, 24, 26], MnP [3, 4], and laccase [1, 21] for the degradation of recalcitrants. Moreover, some other enzymes such as cytochrome P-450 monooxygenase and epoxide hydrolase [2, 19] could be involved in the PAH degradation. Kim *et al.* [14] also reported that the rates of LiP production were not consistent with the PAH hydrolysis rate. Even LiP and MnP were not produced during the degradation of monoaromatics by *P. chrysosporium* [28]. Since some other enzymes in addition to LiP, MnP, and laccase may also participate in ligninolysis and pyrene degradation [15], and these lignin-degrading peroxidases may be uniquely expressed in different species of fungi [20], the precise enzymatic mechanisms involved in PAH degradation by each fungal strain need to be elucidated in order to apply white rot fungi to the bioremediation of contaminated sites.

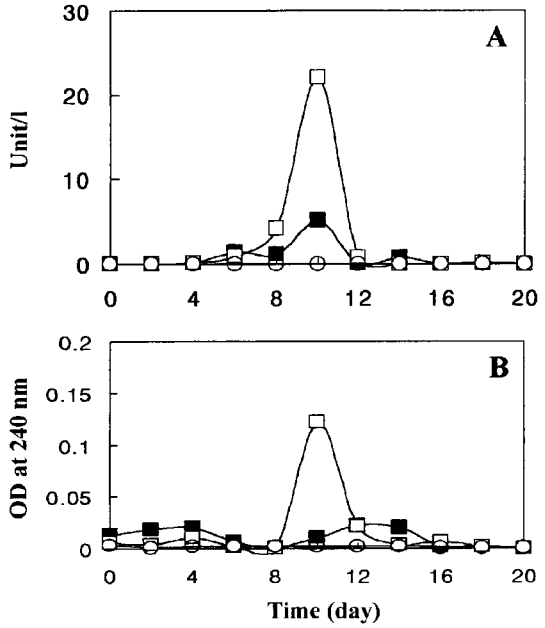

Fig. 6. Activities of lignin peroxidase (A) and manganese-dependent peroxidase (B) during pyrene degradation in shaken cultures of *Irpex lacteus*.
Symbols: (■), control without pyrene; (○), control without inoculum; (□), pyrene 200 ppm.

Acknowledgments

This work was supported by a grant from Korea Science and Engineering Foundation (KOSEF Grant 981-0513-062-2). The authors would like to thank Dr. Kyu-Jung Kim for providing the fungal strain.

REFERENCES

1. Bezalel, L., Y. Hadar, and C. Cerniglia. 1996. Mineralization of polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* **62:** 292-295.
2. Bezalel, L., Y. Hadar, and C. Cerniglia. 1997. Enzymatic mechanisms involved in phenanthrene degradation by the white rot fungus *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* **63:** 2495-2501.
3. Bogan, B. W. and R. T. Lamar. 1996. Polycyclic aromatic hydrocarbon-degrading capabilities of *Phanerochaets laevis* HHb-1625 and its extracelluar ligninolytic enzymes. *Appl. Environ. Microbiol.* **62:** 1597-1603.
4. Bogan, B. W., R. T. Lamar, and K. E. Hammel. 1996. Fluorene oxidation *in vivo* by *Phanerochaete chrysosporium*

and *in vitro* during manganese peroxidase-dependent lipid peroxidation. *Appl. Environ. Microbiol.* **62:** 1788-1792.
5. Brodkorb, T. S. and R. L. Legge. 1992. Enhanced biodegradation of phenanthrene in oil tar-contaminated soils supplemented with *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **58:** 3117-3121.
6. Bumpus, J. A. 1989. Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **55:** 154-158.
7. Bumpus, J. A., M. Tien, D. Wright, and S. D. Aust. 1985. Oxidation of persistent environmental pollutants by a white rot fungus. *Science* **228:** 1434-1436.
8. Cerniglia, C. E. 1993. Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* **3:** 351-368.
9. Dean-Ross, D. and C. Cerniglia. 1996. Degradation of pyrene by *Mycobacterium flavescens*. *Appl. Microbiol. Biotechnol.* **46:** 307-312.
10. Gold, M. and J. Glen. 1988. Manganese peroxidase from *Phanerochaete chrysosporium*. pp. 258-264. *In* Wood, W. and S. Kellogg (eds.), *Methods in Enzymology*, vol. 16, Academic Press, NY, U.S.A.
11. Hwang, S.-S. and H.-G. Song. 1999. Biodegradation of pyrene in marine environment. *Kor. J. Microbiol.* **35:** 53-60.
12. Jäger, A., S. Croan, and T. Kirk. 1985. Production of ligninases and degradation of lignin in agitated submerged cultures of *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **50:** 1274-1278.
13. Kim, H.-Y. and H.-G. Song. 1997. Decolorization of landfill leachate by white-rot fungi. *Kor. J. Microbiol.* **33:** 43-48.
14. Kim, M., E. Huh, H. Kim, and K. Moon. 1998. Degradation of polycyclic aromatic hydrocarbons by selected white-rot fungi and the influence of lignin peroxidase. *J. Microbiol Biotechnol.* **8:** 129-133.
15. Lang, E., F. Nerud, E. Novotna, E. Zadrazil, and R. Martens. 1996. Production of ligninolytic exoenzymes and ^{14}C-pyrene mineralization by *Pleurotus* sp. in lignocellulose substrate. *Folia Microbiol.* **41:** 489-493.
16. Lange, B., S. Kremer, O. Sterner, and H. Anke. 1994. Pyrene metabolism in *Crinipellis stipitaria*: Identification of trans-4,5-dihydro-4,5-dihydroxypyrene and 1-pyrenylsulfate in strain JK364. *Appl. Environ. Microbiol.* **60:** 3602-3607.
17. Lestan, D. and R. T. Lamar. 1996. Development of fungal inocula for bioaugmentation of contaminated soils. *Appl. Environ. Microbiol.* **62:** 2045-2052.
18. Marinucci, A. C. and R. Bartha. 1979. Apparatus for monitoring the mineralization of volatile ^{14}C-labeled compounds. *Appl. Environ. Microbiol.* **38:** 1020-1022.
19. Muncnerova. D. and J. Augustin. 1994. Fungal metabolism and detoxification of polyaromatic hydrocarbons: A review. *Bioresource Technol.* **48:** 97-106.
20. Orth, A., D. Royse, and M. Tien. 1993. Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. *Appl. Environ. Microbiol.* **59:** 4017-4023.
21. Pickard, M., R. Roman, R. Tinoco, and R. Vazquez-Duhalt. 1999. Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. *Appl. Environ. Microbiol.* **65:** 3805-3809.
22. Ross, I. 1982. The role of laccase in carpophore initiation in *Coprinus congregatus*. *J. Gen. Microbiol.* **128:** 2763-2770.
23. Song, H. 1999. Comparison of pyrene biodegradation by white rot fungi. *World J. Microbiol. Biotechnol.* **15:** 669-672.
24. Stahl, J. D. and S. D. Aust. 1995. Biodegradation of 2,4,6-trinitrotoluene by the white rot fungus *Phanerochaete chrysosporium*. pp. 117-133. *In* Spain, J. (ed.), *Biodegradation of Nitroaromatic Compounds*, Plenum Press, NY, U.S.A.
25. Tien, M. and T. Kirk. 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. *In* pp. 238-249. Wood, W. and S. Kellogg (eds.), *Methods in Enzymology*, vol. **161**, Academic Press. NY, U.S.A.
26. Vazquez-Duhalt, R., D. W. S. Westlake, and P. M. Fedorak. 1994. Lignin peroxidase oxidation of aromatic compounds in systems containing organic solvent. *Appl. Environ. Microbiol.* **60:** 459-466.
27. Venkatadri, R. and R. Irvine. 1990. Effect of agitation on ligninase activity and ligninase production by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **56:** 2684-2691.
28. Yadav, J. S. and C. A. Reddy. 1993. Degradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **59:** 756-762.