The Journal of Microbiology, June 2004, p.94-98 Vol. 42, No. 2

Degradation of Phenanthrene by *Trametes versicolor* and Its Laccase

Mun-Jung Han, Hyoung-Tae Choi and Hong-Gyu Song*

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

(Received February 5, 2004 / Accepted April 2, 2004)

Phenanthrene is a three-ring polycyclic aromatic hydrocarbon and commonly found as a pollutant in various environments. Degradation of phenanthrene by white rot fungus *Trametes versicolor* 951022 and its laccase, isolated in Korea, was investigated. After 36 h of incubation, about 46% and 65% of 100 mg/l of phenanthrene added in shaken and static fungal cultures were removed, respectively. Phenanthrene degradation was maximal at pH 6 and the optimal temperature for phenanthrene removal was 30°C. Although the removal percentage of phenanthrene was highest (76.7%) at 10 mg/l of phenanthrene concentration, the transformation rate was maximal (0.82 mg/h) at 100 mg/L of phenanthrene concentration in the fungal culture. When the purified laccase of *T. versicolor* 951022 reacted with phenanthrene, phenanthrene was not transformed. The addition of redox mediator, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) or 1-hydroxybenzotriazole (HBT) to the reaction mixture increased oxidation of phenanthrene by laccase about 40% and 30%, respectively.

Key words: white rot fungi, *Trametes versicolor*, biodegradation, phenanthrene, laccase

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants found in air, soil and aquatic environments. Although they can arise from natural events such as forest fires, the amount released from anthropogenic sources are much larger because they are one of the major components of petroleum (Cerniglia, 1993; Schützendübel *et al.*, 1999). Most aromatic hydrocarbons are toxic to living organisms, and some of them and their metabolites are mutagenic and carcinogenic to humans. There have been many studies on the biodegradation of various aromatic hydrocarbons, but their low water solubility and subsequent low degradation rates hamper the bioremediation of PAH-polluted environments (Levin *et al.*, 2003). Therefore, knowledge of microorganisms having a high PAHs degrading capability is essential for efficient remediation of PAHs contamination. To date, most biodegradation studies have focused on degrading bacteria (Kanaly and Harayama, 2000; Kang, 2002). However, white rot fungi that can degrade lignin and various recalcitrant aromatic compounds have several potential advantages over other degrading microorganisms. Compared to most degrading enzymes of bacteria which have narrow substrate specificity, the lignin-olytic enzymes of these fungi are very nonspecific and extracellular. Therefore, white rot fungi can degrade various insoluble organic pollutants simultaneously (Stahl and Aust, 1995). The lignin degrading system is induced in response to nutrient exhaustion rather than by the presence of pollutants. This allows the fungi to degrade pollutants to essentially non-detectable levels (Stahl and Aust, 1995). Accordingly, the use of white rot fungi has been proposed in the bioremediation of PAHs (Lestan and Lamar, 1996; Kotterman *et al.*, 1998). To date, most attention in the study of white rot fungi has been directed at *Phanerochaete chrysosporium* (Bogan and Lamar, 1996; Bogan *et al.*, 1996), although the possibility exists that some other species may be more effective than *P. chrysosporium* in the removal of PAHs. Several white rot fungi, including *Trametes versicolor* have already shown a higher removal ability of PAHs than *P. chrysosporium* (Sack *et al.*, 1997; Song, 1997). In this study the effects of certain environmental conditions on the degradation of 3-ring PAH, phenanthrene by *T. versicolor*, isolated in Korea, were examined and the transformation of phenanthrene by purified laccase was measured. This work was performed as a preliminary assessment of the potential usefulness of white rot fungus and its enzyme in the bioremediation of PAH pollutants.

Materials and Methods

Chemicals

Phenanthrene was purchased from Aldrich (USA) and all organic solvents were of HPLC grade from Fisher Scientific (USA). DEAE-Sepharose™ Fast Flow, Phenyl Sepharose™ 6 Fast Flow and Standard proteins for SDS-PAGE were purchased from Amersham Pharmacia (Sweden), and Sephadex G-100 was obtained from Sigma (USA).

* To whom correspondence should be addressed.
(Tel) 82-33-250-8545; (Fax) 82-33-241-4627
(E-mail) hgsong@kangwon.ac.kr

Preparation of fungal inoculums

Trametes versicolor 951022 isolated in Kangwon-do, Korea was obtained from the Mycology Laboratory in the Department of Biology at Kangnung National University. The fungus was maintained on YMG medium (yeast extract 4 g, malt extract 10 g, glucose 4 g per 1 l H_2O) solidified with 2% agar. A small portion of a YMG agar plate covered with white-rot fungus was cut out and transferred to YMG broth in an Erlenmeyer flask and was incubated at 30°C on a rotary shaker at 130 rpm for 5 days. Thirty ml of this culture was transferred to a sterile tube and centrifuged (12,000×g, 15 min). The supernatant was decanted into a sterile tube and the fungal pellet was weighed. The pellet was re-suspended in the supernatant and the concentration of fungal biomass was adjusted to 1 g wet wt/10 ml culture supernatant. This suspension was blended for 1 min using an Omni-mixer (Ivan Sorvall Inc., USA) at 16,000 rpm and the blended fungal culture was used as the inoculum for the biodegradation test.

Effects of environmental conditions on phenanthrene degradation by fungus

At first, the effect of agitation on the degradation of phenanthrene was tested. Erlenmeyer flasks of 100 ml containing YMG medium were inoculated with fungal suspension (10%, v/v) and the cultures were incubated on a rotary shaker at 130 rpm for 5 days. Phenanthrene was added to the pre-grown fungal cultures to a final concentration of 100 mg/l, and replicate cultures were separated into shaken cultures on a rotary shaker (130 rpm) and static cultures. All cultures were incubated at 30°C and the residual phenanthrene was quantified during incubation. The effect of phenanthrene concentration on its removal by fungus was examined with 10, 25 and 100 mg/l of phenanthrene in the cultures of *T. versicolor*. To investigate the effect of pH, fungal cultures were adjusted to pH 3, 4, 5, 6, 7, 8, 9 and 10, respectively, and the residual phenanthrene was measured during incubation. The effect of temperature on phenanthrene degradation was examined after incubation of fungal cultures at 10, 20, 30 and 40°C. The concentration of phenanthrene in the experiment for a pH and temperature effect was 100 mg/l.

For analysis of residual phenanthrene at certain time intervals, the whole replicate fungal culture was mixed with an equal volume of HPLC-grade methylene chloride in a 100 ml separatory funnel and vigorously extracted (300 rpm) on an extraction shaker (Jeio Tech model RS-1, Korea) for 10 min. Extraction was repeated 3 times and methylene chloride phases were separated and combined. After removal of water by passage through anhydrous sodium sulfate, methylene chloride was concentrated with a rotary vacuum evaporator (Eyela model NE, Japan), and residual phenanthrene was analyzed by gas chromatograph (GC) equipped with a flame ionization detector (Hewlett Packard model 5890, USA). The current study followed the operating conditions of GC previously described (Hwang and Song, 1999).

Transformation of phenanthrene by purified laccase

Pure laccase was obtained by ion-exchange chromatography, hydrophobic interaction chromatography and size exclusion chromatography. Laccase-mediated oxidation of phenanthrene was performed in a 1.5 ml Eppendorf tube containing 0.8 ml of sodium acetate buffer (0.1 M, pH 5.0), 0.1 ml of phenanthrene solution in methanol (final concn 100 mg/l) and 0.1 ml of enzyme solution (31.88 μg protein/ml). For the investigation of a redox mediator effect on a laccase-mediated reaction, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) or 1-hydroxybenzotriazole (HBT) was added (final conc. 5 mM) into the reaction mixture. Reaction tubes were incubated on a horizontal shaker at 40°C for 2 h. Residual phenanthrene was extracted with 0.5 ml methylene chloride and analyzed by HPLC using a XTerra™ RP_{18} column (5 μm, 4.6×150 mm, Waters, Ireland) with acetonitrile as a carrier solvent at 1.0 ml/min for 20 min.

All experiments were carried out in duplicate or triplicate, and the mean values are presented.

Results and Discussion

Degradation of phenanthrene by Trametes versicolor in different conditions

Degradation of phenanthrene by *T. versicolor* 951022 was examined and the effects of some environmental conditions were investigated. Among PAHs phenanthrene was selected because it is one of the most abundant PAHs in the environment, and it has been used as an indicator for monitoring PAH-contaminated wastes (Sack *et al.*, 1997).

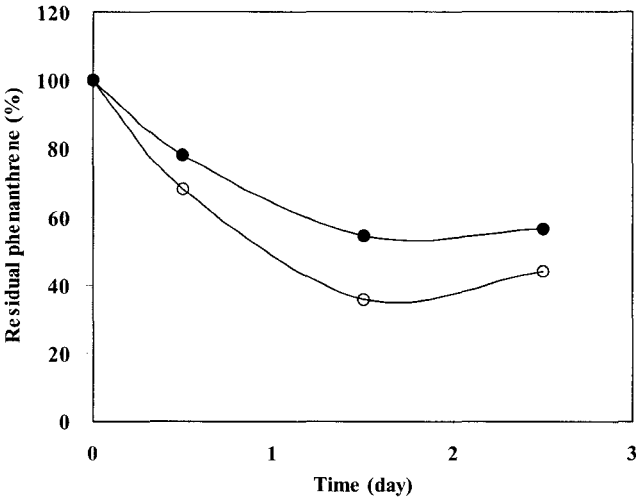

Fig. 1. Degradation of phenanthrene by *Trametes versicolor* 951022. 100 mg/l of phenanthrene was added to 5 day pre-grown mycelial culture. ●, shaken culture; ○, static culture

When the effect of agitation on phenanthrene removal was compared with static and shaken cultures, static culture removal (65%) was higher than shaken culture removal (46%) (Fig. 1). It has been well known that a static culture of white rot fungi is more efficient in biodegradation of aromatic compounds and production of ligninolytic enzymes (Venkatadri and Irvine, 1990), although a shaken culture can exhibit a higher growth and biodegradation capability in some cases (Jäger *et al.*, 1985; Hwang and Song, 2000). Since the static culture showed a higher removal rate of phenanthrene, all other experiments in this study were carried out using static cultures. Further degradation did not occur after 36 h of incubation, and it seemed to be the result of substrate exhaustion because the fungal cultures were already in a 5-day pre-grown state. Addition of fresh growth substrate may induce a further degradation of phenanthrene (Kim and Song, 2003).

The time course and extent of phenanthrene removal at different incubating temperatures are shown in Fig. 2. Degradation rates of phenanthrene by *T. versicolor* were higher in the mesophilic range (20~30°C) than those in lower or higher temperatures. Since the optimum growth temperature range for *T. versicolor* is 25~30°C, its growth decreased at lowered or elevated temperatures (data not shown), and accordingly phenanthrene degradation at 10 and 40°C decreased. When the effect of initial culture pH on phenanthrene degradation was examined, the highest degradation occurred at pH 6 (Fig. 3). Neutral pH conditions were favorable for phenanthrene degradation by *T. versicolor* in this study. The removal percentage of phenanthrene by *T. versicolor* was higher at lower concentration of phenanthrene (Fig. 4), and it is likely due to the toxicity of aromatic hydrocarbons (Song, 1997). Although the removal percentage was highest at 10 mg/l of phenanthrene (76.7%), the transformation rate of phenanthrene in the fungal culture was highest at 100 mg/l (0.82 mg/h) (Table 1). This removal rate was much higher than 30% removal of 5 mg/l phenanthrene by *Bjerkandera adusta* during 3 days (Schützendübel *et al.*, 1999) and 48% removal of 15 mg/l phenanthrene by *Phanerochaete chrysosporium* during 5 days (Kim *et al.*, 1998). This result indicates that *T. versicolor* 951022 may be used for biodegradation of PAHs contamination.

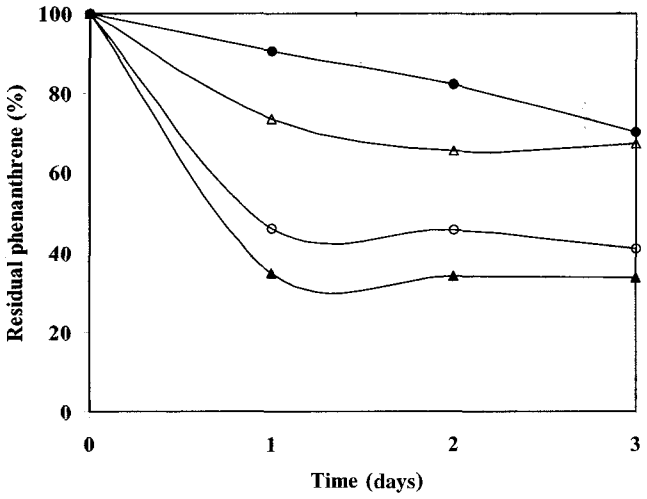

Fig. 2. Degradation of phenanthrene by *T. versicolor* 951022 at different temperatures. ●, 10°C; ○, 20°C; ▲, 30°C; △, 40°C

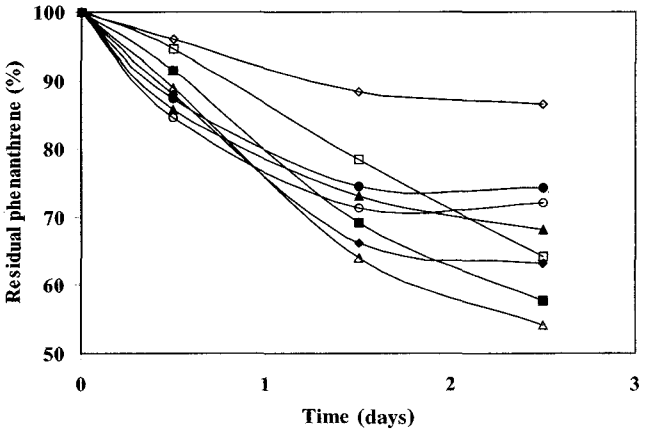

Fig. 3. Degradation of phenanthrene by *T. versicolor* 951022 at different pH levels. ●, pH 3; ○, pH 4; ▲, pH 5; △, pH 6; ■, pH 7; □, pH 8; ◆, pH 9; ◇, pH 10

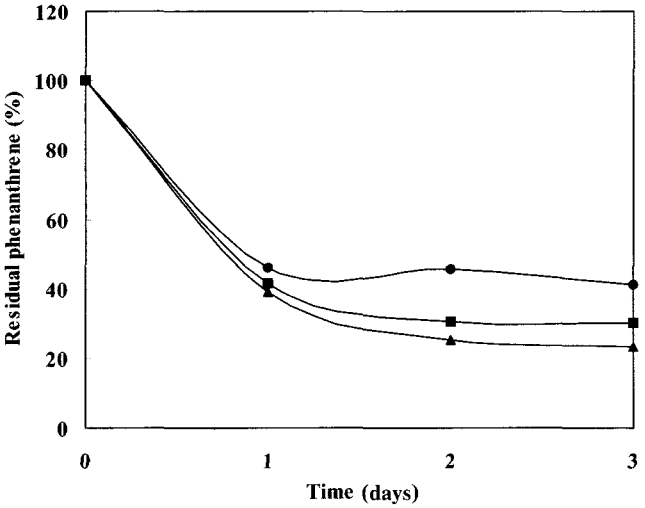

Fig. 4. Degradation of phenanthrene by *T. versicolor* 951022 at different concentrations of phenanthrene. ●, 100 mg/l; ■, 50 mg/l; ▲, 10 mg/l

Table 1. Transformation rates and removal percentages of phenanthrene in the culture of *Trametes versicolor* 951022 at different concentrations of phenanthrene

Concentration of phenanthrene (mg/l)	Phenanthrene removal (%)	Transformation rate (mg phenanthrene/h)
100	59.1	0.82
25	69.9	0.24
10	76.7	0.11

Oxidation of phenanthrene by purified laccase

Degradation of aromatic compounds including lignin and PAHs by white rot fungi depends on the production and secretion of ligninolytic enzymes such as lignin peroxidase, manganese peroxidase, and laccase (Kim *et al.*, 1998). In this study phenanthrene was not degraded by purified laccase of *T. versicolor* 951022, but was transformed when ABTS or HBT was added into the reaction mixture (Fig. 5). In the presence of 5 mM of ABTS or HBT, 5 U/ml of laccase was able to oxidize 40% and 30% of 20 mg/l of phenanthrene in 2 h, respectively. Laccase (benzenediol:oxygen oxidoreductase; EC. 1.10.3.2) is a polyphenol oxidase, and the laccase action is commonly limited to aromatic compounds containing a phenolic group. Furthermore, it is not active with nonphenolic compounds (Johannes *et al.*, 1996; Majcherczyk *et al.*, 1998). Kersten *et al.* (1990) reported oxidation results of some non-phenolic substrates by laccase from *T. versicolor*, but laccase was limited to compounds with a very low half-wave redox potential. Phenanthrene has an ionization potential of 8.19 eV and cannot be a substrate of lignin peroxidase and laccase. Mineralization of phenanthrene by manganese peroxidase of *Nematoloma frowardii* was also lower than those of other PAHs (Sack *et al.*, 1997). It has been recently shown, however, that in the presence of appropriate low-molecular-mass compounds, called mediators, laccase is able to oxidize various aromatic compounds (Bourbonnais *et al.*, 1995; Majcherczyk *et al.*, 1996; 1998). In contrast to other PAHs such as anthracene, fluorene, benzo[a]pyrene and perylene which could be almost completely removed from the reaction mixture of laccase of *T. versicolor* after the addition of HBT, phenanthrene was not significantly influenced by the addition of HBT (Majcherczyk *et al.*, 1998). Compared to less than 10% enhancement of phenanthrene transformation in previous studies (Majcherczyk *et al.*, 1998; Cho *et al.*, 2002), the laccase of *T. versicolor* 951022 in this study could transform phenanthrene efficiently with HBT (30% enhancement), and addition of ABTS showed an even higher transformation rate of phenanthrene during 2 h of reaction. The transformation rate may have further increased with a prolonged reaction time.

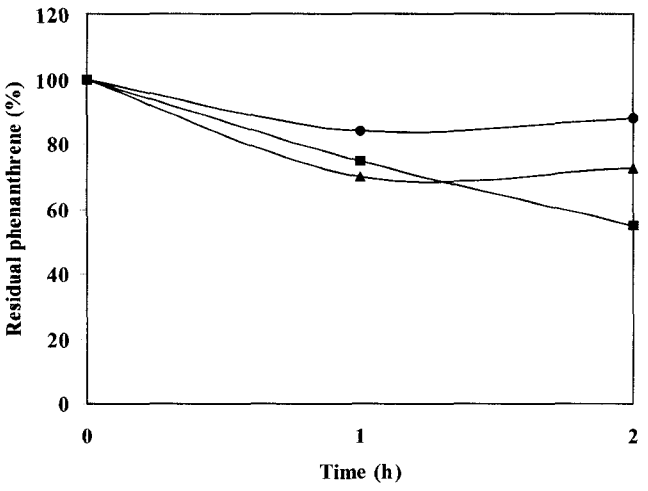

Fig. 5. Effect of mediators on the degradation of phenathrene by purified laccase from *T. versicolor* 951022. ●, Control (without mediator); ■, ABTS; ▲, HBT

Laccase activity could be implicated in the degradation of PAHs by white rot fungi. A high and relatively stable activity of laccase was observed during degradation of anthracene by *Trametes trogii* (Levin *et al.*, 2003). Although laccase production by *T. versicolor* 951022 was not enhanced by an addition of phenanthrene in this study (data not shown), it was increased by many xenobiotic compounds in the cultures of other strains of *T. versicolor* (Mougin *et al.*, 2002). Further study on the induction and enhancement of enzyme activities is necessary for the application of *T. versicolor* to remove PAH pollutants in the environments.

Acknowledgments

This work was supported by Grant No. R01-2002-000-00170-0(2002) from the Basic Research Program of the Korea Science & Engineering Foundation. The authors are thankful to Dr. K. Kim in Kangnung National University for providing the fungal strain.

References

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.

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.

Bourbonnais, R., M.G. Paice, I.D. Reid, P. Lanthier, and M. Yaguchi. 1995. Lignin oxidation by laccase isozymes from *Trametes versicolor* and role of the mediator 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) in kraft lignin depolymerization. *Appl. Environ. Microbiol.* 61, 1876-1880.

Cerniglia, C.E. 1993. Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* 3, 351-368.

Cho, S.-J., S. Park, J.-S. Lim, Y. Rhee, and K.-S. Shin. 2002. Oxidation of polycyclic aromatic hydrocarbons by laccase of *Coriolus hirsutus*. *Biotechnol. Lett.* 24, 1337-1340.

Hwang, S.-S. and H.-G. Song. 1999. Biodegradation of pyrene in marine environment. *Kor. J. Microbiol.* 35, 53-60.

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.

Johannes, C., A. Majcherczyk, and A. Hûttermann. 1996. Degradation of anthracene by laccase of *Trametes versicolor* in the presence of different mediator compounds. *Appl. Microbiol.*

Biotechnol. 46, 313-317.

Kanaly, R. and S. Harayama. 2000. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. *J. Bacteriol.* 182, 2059-2067.

Kang, H.-Y. 2002. Cellular responses of *Pseudomonas* sp. KK1 to two-ring polycyclic aromatic hydrocarbon, naphthalene. *J. Microbiol.* 40, 38-42.

Kersten, P.J., B. Kalyanaraman, K.E. Hammel, and B. Reinhammar. 1990. Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes. *Biochem. J.* 268, 475-480.

Kim, M.S., E.J. Huh, H.K. Kim, and K.W. Moon. 1998. Degradation of polycyclic aromatic hydrocarbons by selected white-rot fungi and the influence of lignin peroxidase. *J. Microbiol. Biotechnol.* 8, 129-133.

Kim, H.-Y. and H.-G. Song. 2003. Transformation and mineralization of 2,4,6-trinitrotoluene by the white rot fungus *Irpex lacteus*. *Appl. Microbiol. Biotechnol.* 61, 150-156.

Kotterman, M.J.J., E.H. Vis, and J.A. Field. 1998. Successive mineralization and detoxification of benzo[a]pyrene by the white rot fungus *Bjerkandera* sp. strain BOS55 and indigenous microflora. *Appl. Environ. Microbiol.* 64, 2853-2858.

Lestan, D. and R.T. Lamar. 1996. Development of fungal inocula for bioaugmentation of contaminated soils. *Appl. Environ. Microbiol.* 62, 2045-2052.

Levin, L., A. Viale, and A. Forchiassin. 2003. Degradation of organic pollutants by the white rot basidiomycete *Trametes trogii*. *Int. Biodet. Biodeg.* 52, 1-5.

Majcherczyk, A., C. Johannes, and A. Hüttermann. 1996. Degradation of anthracene by laccase of *Trametes versicolor* in the presence of different mediator compounds. *Appl. Microbiol. Biotechnol.* 46, 313-317.

Majcherczyk, A., C. Johannes, and A. Hüttermann. 1998. Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of *Trametes versicolor*. *Enzyme Microb. Biotechnol.* 22, 335-341.

Mougin, C., A. Kollmann, and C. Jolivalt. 2002. Enhanced production of laccase in the fungus *Trametes versicolor* by the addition of xenobiotics. *Biotechnol. Lett.* 24, 139-142.

Sack, U., T.M. Heinze, J. Deck, C.E. Cerniglia, R. Martens, F. Zadrazil, and W. Fritsche. 1997. Comparison of phenanthrene and pyrene degradation by different wood-decaying fungi. *Appl. Environ. Microbiol.* 63, 3919-3925.

Schützendübel, A., A. Majcherczyk, C. Johannes, and A. Hüttermann. 1999. Degradation of fluorene, anthracene, phenanthrene, fluoranthene, and pyrene lacks connection to the production of extracellular enzymes by *Pleurotus ostreatus* and *Bjerkandera adusta*. *Int. Biodet. Biodeg.* 43, 93-100.

Song, H.-G. 1997. Biodegradation of aromatic hydrocarbons by several white-rot fungi. *J. Microbiol.* 35, 66-71.

Stahl, J.D. and S.D. Aust. 1995. Biodegradation of 2,4,6-trinitrotoluene by the white rot fungus *Phanerochaete chrysosporium*, p. 117-133. *In* J. Spain (ed.), Biodegradation of Nitroaromatic Compounds, Plenum Press, NY.

Venkatadri, R. and R. Irvine. 1990. Effect of agitation on ligninase activity and ligninase production by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 56, 2684-2291.