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Degradation of Three Aromatic Dyes by White Rot Fungi and the Production of Ligninolytic Enzymes

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This study was conducted to evaluate the degradation of aromatic dyes and the production of ligninolytic enzymes by 10 white rot fungi. The results of this study revealed that *Pycnoporus cinnabarinus*, *Pleurotus pulmonarius*, *Ganoderma lucidum*, *Trametes suaveolens*, *Stereum ostrea* and *Fomes fomentarius* have the ability to efficiently degrade congo red on solid media. However, malachite green inhibited the mycelial growth of these organisms. Therefore, they did not effectively decolorize malachite green on solid media. However, *P. cinnabarinus* and *P. pulmonarius* were able to effectively decolorize malachite green on solid media. *T. suaveolens* and *F. rosea* decolorized methylene blue more effectively than any of the other fungi evaluated in this study. In liquid culture, *G. lucidum*, *P. cinnabarinus*, *Naematoloma fasciculare* and *Pycnoporus coccineus* were found to have a greater ability to decolorize congo red. In addition, *P. cinnabarinus*, *G. lucidum* and *T. suaveolens* decolorized methylene blue in liquid media more effectively than any of the other organisms evaluated in this study. Only *F. fomentarius* was able to decolorize malachite green in liquid media, and its ability to do so was limited. To investigate the production of ligninolytic enzymes in media containing aromatic compounds, fungi were cultured in naphthalene supplemented liquid media. *P. coccineus*, *Coriolus versicolor* and *P. cinnabarinus* were found to produce a large amount of laccase when grown in medium that contained napthalene.

KEYWORDS : Aromatic dyes, Decolorization, Ligninolytic enzymes, White rot fungi

Soil is a natural resource that can be polluted in many ways. Petroleum hydrocarbon is one of the most common contaminants found in soil. Hydrocarbons are often purposefully released into the environment via the dumping of petroleum waste or wastewater. In addition, petroleum is often introduced to soil as a result of accidents during the production, transport and use of chemicals. Petrochemical industries also generate a series of liquid effluents during the petroleum-refining process and as the refineries are cleaned. The sludges generated by these processes have a high content of petroleum-derived hydrocarbons, primarily alkanes and paraffins with 1~40 carbon atoms, as well as cycloalkanes and aromatic compounds (Overcash and Pal, 1979). Simply dumping these wastes or burning them with no previous treatment can have serious environmental consequence. Therefore, these compounds present a risk to the environment and human health (Baheri and Meysami, 2001).

Bioaugmentation has been found to be a useful type of bioremediation for the treatment of petroleum waste. Recently, the use of fungi to bioremediate petroleum waste has grown (Lestan *et al.*, 1997). The potential for the fungal biodegradation of Tri-Nitro-Toluene (TNT) and other explosives was investigated by Johnston *et al.* (1997). Subsequently, Takada *et al.* (1996) studied the degradation of polychlorinated dibenzofurans and dioxins by fungi. The potential of different fungi to degrade hydrocarbons has also been the subject of studies conducted by McGughan (1995), Ravelet *et al.* (2001) and Chavez-Gomez *et al.* (2003). Although most studies within the field of bioremediation have focused on bacteria, the toxicity of many of the above- named pollutants limits the natural attenuation by bacteria. However, white rot fungi can withstand toxic levels of most organopollutants (Aust *et al.*, 2004).

White rot fungi is a physiological grouping of fungi that can degrade lignin and lignin- like substances. Four main genera of white rot fungi such as *Phanerochaete*, *Trametes*, *Bjerkandera* and *Pleurotus* have been found to have the potential for bioremediation (Hestbjerg *et al.*, 2003). White rot basidiomycetes produce three major classes of enzymes designated lignin peroxidases (LIPs), manganese dependent peroxidases (MNPs) and laccases. These enzymes play an important role in the fungal degradation of lignin (Boominathan and Reddy, 1992; Buswell *et al.*, 1987; Hatakka, 1994; Kirk and Farrell, 1987). Some wood-degrading fungi contain all three classes of lignin-modifying enzymes, while others contain only one

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or two of these enzymes (De Jong *et al*., 1994; Hatakka, 1994; Peláez, *et al*., 1995). Several previous investigations demonstrated that laccase is produced by many genera of white rot fungi (De Jong *et al*., 1994; Hatakka, 1994; Peláez, *et al*., 1995), and various strains of white-rot fungi capable of degrading aromatic compounds were reported by Barr and Aust (1994). In addition, Reddy (1998) found that lignin degrading white-rot fungi have the unique ability to degrade or mineralize a broad spectrum of structurally diverse toxic environmental pollutants. Similarly, Lang *et al*. (1995) reported that lignin-decomposing white-rot fungi showed extraordinary abilities to transform recalcitrant pollutants, such as polycyclic aromatic hydrocarbons (PAHs). Furthermore, white-rot fungi have also been found to degrade a wide variety of synthetic dyes (Radha *et al*., 2005; Selvam *et al*., 2003). Therefore, this study was conducted to investigate the degradation of aromatic dyes and the production of ligninolytic enzymes by white rot fungi under adverse environmental conditions.

Materials and Methods

Fungal strains. Mycelial cultures of the following 10 species of white rot fungi were obtained from the Culture Collection and DNA Bank of Mushrooms (CCDBM) at the University of Incheon, Korea: *Fomes fomentarius* (IUM 144), *Pycnoporus cinnabarinus* (IUM 253), *Pleurotus pulmonarius* (IUM 795), *Ganoderma lucidum* (IUM 805), *Naematoloma fasciculare* (IUM 810), *Coriolus versicolor* (IUM 863), *Trametes suaveolens* (IUM 1091), *Stereum ostrea* (IUM 1139), *Fomitopsis rosea* (IUM 1191) and *Pycnoporus coccineus* (IUM 1126). The selected fungi were maintained on Potato Dextrose Agar (PDA) at 25°C until used in the experiments described below.

Chemicals. The common names of all dyes have been used for convenience. Malachite Green (MG), Congo Red (CR) and Methylene Blue (MB) were obtained from Merck (Germany). All other chemicals were of analytical grade and obtained from Sigma (U.S.A.).

Solid phase decolorization of aromatic dyes. The dyes used in this study were selected to screen fungi for the ability to degrade 3 classes of aromatic dyes [azo (congo red), heterocyclic (methylene blue), and triphenyl methane (malachite green)]. The ability of the selected fungal strains to decolorize the dyes was evaluated using PDA plates supplemented with 100 mgl^{-1} of the individual dyes. A disc 5 mm in diameter that contained mycelia from each fungi was placed in the center of each plate. In addition, un-inoculated plates containing each of the dyes were used as controls. The plates were then incubated at 25°C for 10 days, after which the mycelia diameter (MD) and decolorization diameter (DD) were determined. The ability of the fungi to decolorize the dye was then expressed as the decolorization index (DI), which was calculated using the following formula DI = DD/MD. Each test was replicated 4 times.

Liquid state decolorization. The decolorization of dyes in the liquid phase was evaluated using PDB (Potato Dextrose Broth) medium that contained 100 mgl^{-1} of aromatic dyes. Flasks containing 100 *ml* of liquid medium were inoculated with five agar plugs of mycelia (5 mm diameter) obtained from the edge of actively growing mycelia on plates that contained the selected fungi. These flasks were then incubated at 25°C in a shaking incubator (140 rpm) for 23 days. Once a day, a 2 *ml* aliquot was taken from the cultures and then centrifuged 2 minutes at 25°C (4000 rpm). The maximum absorbance (Є max **A**) at 617, 644 and 562 nm was then measured using an OPTIZEN 2120UV spectrophotometer to determine the concentrations of MG, MB and CR, respectively. In addition, control samples consisting of media that contained dye but was not inoculated were maintained. The decolorization of the dyes was then calculated using the following formula: decolorization (%) = $(A_0 - A) \times 100/A_0$, where A_0 is the initial absorbance and A is maximum absorbance at the current time period. Each test was replicated 4 times.

Assay of ligninolytic enzymes. Fungal mycelia were grown in 20% potato broth supplemented with 1% glucose and 1% naphthalene. After 10 days, the mycelia were separated from the supernatants by filtering the cultures through Whatman No. 2 filter paper. Following filtration, the supernatants from the cultures were stored at 4°C until used analyzed for the presence of extracellular enzymes.

Laccase activity was measured using the method described by Bourbounnais *et al*. (1995), which is based on oxidation of the substrate ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) at 5 mM. Briefly, the substrate was dissolved in 2.4 *ml* of sodium acetate buffer (0.1 M, pH 5.0), and 100 μl of the culture supernatant was then added. The mixture was then incubated at 30°C for 2 min, after which the absorbance at 420 nm (ε_{420} = 36000 $M^{-1}cm^{-1}$) was then measured.

Lignin peroxidase (LiP) activity was measured using the method described by Tien and Kirk (1983). In this method, the increase in absorbance at 310 nm due to oxidation of the veratryl alcohol to veratryl aldehyde is measured. Briefly, a reaction mixture containing 2.2 *ml* of sodium tartrate buffer (50 mM, pH 4 at 25°C), 40 μl of veratryl alcohol (2 mM) and 240 μl of the culture supernatant was prepared. Next, the reaction was initiated by the addition of 20 μl of H_2O_2 (0.2 mM) to the reaction mixture. The absorbance was then measured immediately

($\epsilon_{310} = 9333\ M^{-1}cm^{-1}$).

Manganese peroxidase (MnP) activity was measured using the method described by Glenn and Gold (1985). This method is based on the oxidation of Mn(II) to Mn(III), and uses 2.5 *ml* of phenol red (0.01%) and $MnSO_4$ (0.1 mM) in sodium succinate buffer (0.1 M) as the substrate. Briefly, a reaction mixture that contained 2.5 *ml* of substrate and 200 μl of the culture supernatant was prepared. The reaction was then initiated by the addition of H_2O_2 (0.1 mM). After an incubation period of 2 min at 30°C, the reaction was stopped by the addition of NaOH (5 M). The absorbance at 610 nm ($\epsilon_{610} = 22000\ M^{-1}cm^{-1}$) was then measured.

One enzymatic unit of laccase, lignin peroxidase and manganese peroxidase activity was defined as the quantity of enzyme that produced 1 μmol of oxidized product.

Results and Discussion

Solid phase degradation of aromatic dyes. The dyes evaluated in this study contain aromatic compounds that are degraded by white rot fungi during secondary metabolism. The growth and degradation efficiency of the test fungi as determined based on their decolorization ability in solid media are shown in Table 1. Of the 10 fungi cultured on media that contained CR, *C. versicolor*, *F. fomentarius* and *P. coccineus* showed that decolorization activity was higher than that of remaining 7 fungi. When the mycelial growth on media that contained CR was evaluated, *C. versicolor*, *F. fomentarius* and *P. coccineus* were found to have more rapid mycelail growth and decolorization than the other fungal species. However, *P. pulmonarius* was found to have the highest efficiency of decolorization. *G. lucidum*, *S. ostrea*, *F. rosea* and *T. suaveolens* also showed higher decolorization efficiency when compared to the other fungi, whereas the decolorization of *N. fasciculare*, *P. coccineus* and *C. versicolor* was lower than that of the other fungi.

CR belongs to the azo group and white rot fungi are the only microorganisms known to be able to completely mineralize lignocellulosic polymers. Spadaro *et al.* (1992) demonstrated that *Phanerochaete chrysosporium* was capable of mineralizing a variety of aromatic rings present in azo dyes. However, they also found that the mineralization of azo dyes by white rot fungi depended on the nature of the ring substituent.

Malachite green belongs to tryphenyl methane aromatic dye group, and all of the fungi evaluated in this study had a poor ability to decolorize MG. However, *F. fomentarius* showed better mycelial growth and decolorization of MG than the other fungi evaluated here. Decolorization was not observed when *N. fasciculare*, *C. versicolor*, *T. suaveolens* and *S. ostrea* were evaluated, and *C. versicolor* and *T. suaveolens* showed no mycelial growth on media that contained MG. *P. pulmonarius* and *P. cinnabarinus* had the highest DI of the fungi evaluated when grown on media containing MG; however, their growth rate was very low. In addition, although *G. lucidum*, *F. rosea* and *P. coccineus* showed only a slight amount of mycelial growth, they were very effective at decolorizing MG. All of the fungi evaluated in this study were found to grow slowly on media that contained MG. Schnick (1988) reported that MG dye has been widely used as an effective antifungal agent in the fish farming industry, which supports the slow growth rate of the selected white rot fungal species observed in this study.

When the fungi were grown on media that contained MB, the greatest amount of decolorization was exerted by *T. suaveolens* and *F. rosea*, although *N. fasciculare*, *P. pulmonarius* and *G. lucidum* also effectively decolorized MB. However, the mycelia of these two fungal species grew very poorly on media that contained MB. Conversely, although MB did not appear to interfere with the mycelial growth of *F. fomentarius*, *S. ostrea*, *P. coccineus* and *P. cinnabarinus*, these fungi were not able to effectively decolorize MB. In addition, *C. versicolor* showed good

Table 1. Decolorization of aromatic dyes on solid phase by white rot fungi

Scientific names of white rot fungi	Congo Red			Malachite Green			Methylene Blue		
	MD (mm)	DD (mm)	DI	MD (mm)	DD (mm)	DI	MD (mm)	DD (mm)	DI
Fomes fomentarius	74 ± 2.0	68 ± 2.0	0.92	33 ± 1.0	33 ± 1.0	1.00	81 ± 1.5	–	0.00
Pycnoporus cinnabarinus	32 ± 8.3	34 ± 5.1	1.06	3 ± 2.1	8 ± 2.1	2.66	53 ± 1	–	0.00
Pleurotus pulmonarius	18 ±4.1	27 ± 4.4	1.50	8 ± 3.5	16 ± 2.8	2.00	20 ± 3.2	10 ± 4.1	0.50
Ganoderma lucidum	42 ± 5.2	43 ± 1.5	1.02	17 ± 3.2	19 ± 2.4	1.12	44 ± 5.1	18 ± 4.1	0.41
Naematoloma fasciculare	34 ± 2.2	25±3.2	0.74	15 ± 2.0	–	0.00	33 ± 1.2	18 ± 2.2	0.55
Coriolus versicolor	72 ± 3.3	52±4.1	0.72	–	–	0.00	87 ± 5.2	10 ± 3.3	0.11
Trametes suaveolens	26 ± 2.5	33±2.2	1.27	–	–	0.00	23 ± 1.8	28 ± 4.8	1.22
Stereum ostrea	25 ± 3.0	37±6.4	1.48	5 ± 4.2	–	0.00	75 ± 2.2	–	0.00
Fomitopsis rosea	26 ± 2.2	33±3.2	1.27	15 ± 2.2	17 ± 1.5	1.13	10 ± 2.2	14 ± 3.2	1.40
Pycnoporus coccineus	73 ± 2.4	41±4.2	0.56	10 ± 4.1	15 ± 3.0	1.50	72 ± 2.0	–	0.00

MD: Mycelial diameter, DD: Decolorization diameter, DI: Decolorization index = DD/MD. The mycelial diameter and decolorization diameter were measured (mm, n = 4) after 10 days of incubation.

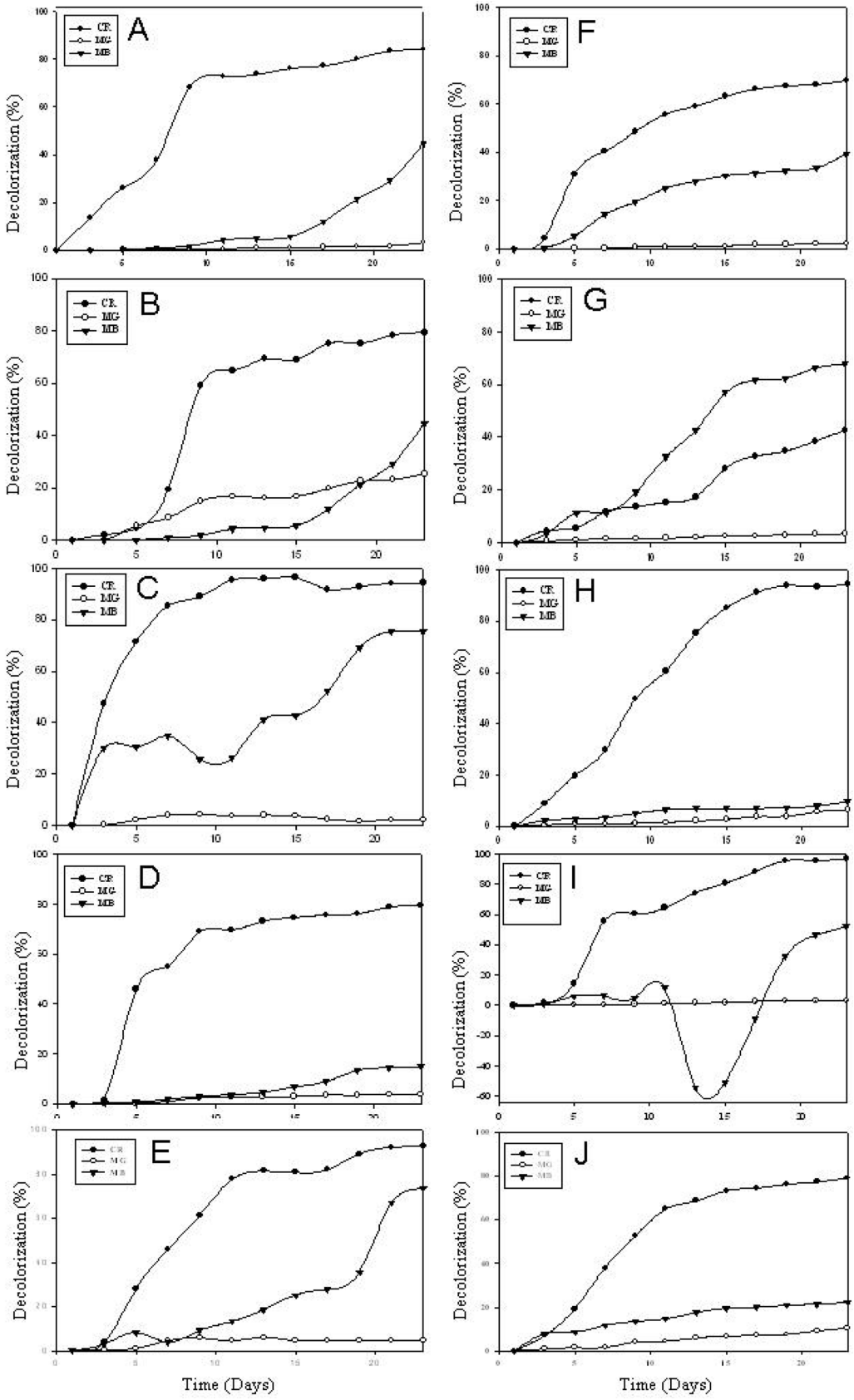

Fig. 1. Fungal decolorization of aromatic dyes in the liquid phase, CR: Congo Red, MG: Malachite Green, MB: Methylene Blue, A: *C.* versicolor, B: *F. fomentarius*, C: *P. cinnabarinus*, D: *P. pulmonarius*, E: *G. lucidum*, F: *T. suaveolens*, G: *S. ostrea*, H: *N. fasciculare*, I: *P. coccineus*, J: *F. rosea.*

mycelial growth on media that contained MB, but its efficiency of decolorization was very low. The value of decolorization of MB on solid media by the selected fungi was not considerably higher. Novotný *et al.* (2004) reported that when the white rot fungus, *Irpex lacteus*, was grown on different media containing MB it did not show any considerable decolorization. In addition, the results of a study conducted by Kling and Neto (1991) also showed that *P. chrysosporium* did not play an important role in the oxidation of MB. However, Boer *et al.* (2003) found that *Lentinula edodes* decolorized media that contained 200ppm MB by 60%.

Liquid state decolorization of aromatic dyes. A study of the decolorization capacity of selected fungi cultured in the PDB containing 100 mg l^{-1} dye revealed that fungi such as *P. cinnabarinus*, *N. fasciculare*, *G. lucidum* and *P. coccineus* decolorized more than 90% of the CR within 20 days, but that they did not decolorize MG or MB (Fig. 1). Some studies have shown that *Pycnoporus* species (Schliephake and Lonergan, 1996), *Ganoderma* species (Levin *at el.*, 2004) and *Naematoloma* species (Nozaki *et al.*, 2008) decolorized CR efficiently in liquid media. Among the species evaluated in this study, *C. versicolor*, *F. fomentarius*, *P. pulmonarius* and *F. rosea* decolorized approximately 80% of the CR, and other species such as *T. suaveolens* and *S. ostrea* decolorized approximately 60% of the CR. Nozaki *et al.* (2008) observed that *C. versicolor*, *Pleurotus eryngii*, *Pleurotus ostreatus* and *Pleurotus salmoneostramineus* decolorized CR successfully in liquid media. In addition, Champagne and Ramsay (2005) observed similar results when the degradation of CR by *Trametes versicolor* was evaluated.

With the exception of *F. fomentarius*, none of the selected fungi decolorized a considerable amount of MG (Fig. 1B). Specifically, *F. fomentarius* decolorized less than 25% of the MG, and all of the other organisms evaluated in this study decolorized less than 10% of the MG present in the liquid broth. Levin *et al.* (2004) reported that Argentinean white rot fungi was not able to successively degrade MG in liquid media. Furthermore, Eichlerová *et al.* (2005, 2006) also found that *Dichomitus squalens* and *Ischnoderma resinosum* could not degrade MG in liquid culture.

When the ability of the selected fungi to degrade MB in liquid culture was evaluated, *P. cinnabarinus* and *G. lucidum* were found to have the ability to degrade approximately 80% of the MB present in broth within 20 days and *C. versicolor*, *F. fomentarius*, *T. suaveolens*, *S. ostrea* and *P. coccineus* were able to degrade approximately 40% of the MB within 20 days (Fig. 1). However, *P. pulmonarius* and *F. rosea* degraded less than 20% of the MB in liquid media within 20 days. Methylene blue belongs to the heterocyclic aromatic group. Cripps *et al.* (1990) evaluated the decolorization of azo and heterocyclic dyes by liquid culture extract of *Phanerochaete chrysosporium* and found that *P. chrysosporium* extract from nitrogen-limited media was capable of degrading 100% of heterocyclic dyes within 5 days, but that culture extract from nitrogen-sufficient media was only capable of degrading 60% of the heterocylic dyes within 5 days. In addition, Svobodova *et al.* (2006) reported that *P. chrysosporium* and *I. lacteus* could degrade 22% and 12% of the MB in nitrogen limited media, respectively. In this experiment, both *Pycnoporous* effectively degraded MB (Fig. 1C and 1I). It should be noted that after 10 days of incubation, the medium containing MB gradually become darker and greener. This drastic color change was observed in media that was inoculated with *P. coccineus* and resulted in the UV-VIS absorption value at 664 nm increasing Due to this exceptional color change, the percentage of decolorization was negative during this period. This extraordinary absorption value may have been due to a reaction of the MB with enzymes secreted by the fungal mycelia.

Production of ligninolytic enzymes. Fungal mycelia were grown in 20% potato broth supplemented with 1% glucose and 1% naphthalene and the laccase production was then determined based on an ABTS assay (Fig. 2). The largest amount of laccase (> 0.5 Uml^{-1}) was secreted by *P. cinnabarinus*, *C. versicolor* and *P. coccineus* in media that contained naphthalene, whereas *F. rosea* and *T. suaveolens* only produced around 0.1 Uml^{-1} of laccase under the same conditions. All other species produced less than 0.1 Uml^{-1} of laccase in media that contained naphthalene. *P. pulmonarius* and *N. fasciculare* were found to produce more LiP in media that contained naphthalene (around 0.16 Uml^{-1}) than the other species. However, *F.*

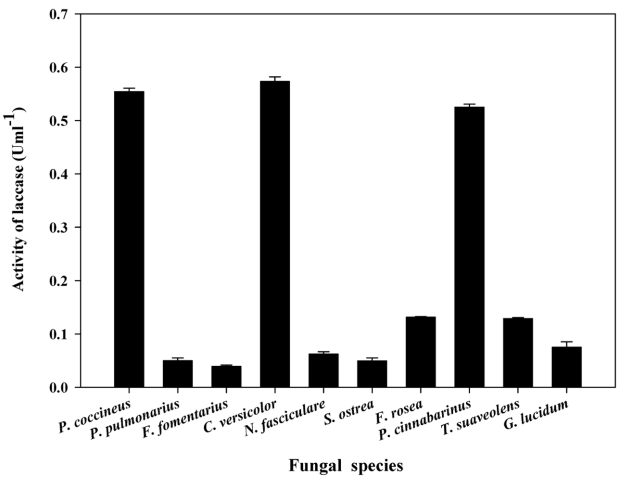

Fig. 2. Production of laccase by white rot fungi in potato broth containing 1% of naphthalene and glucose. Results shown are the means of five replications. Vertical bars represent the standard deviation.

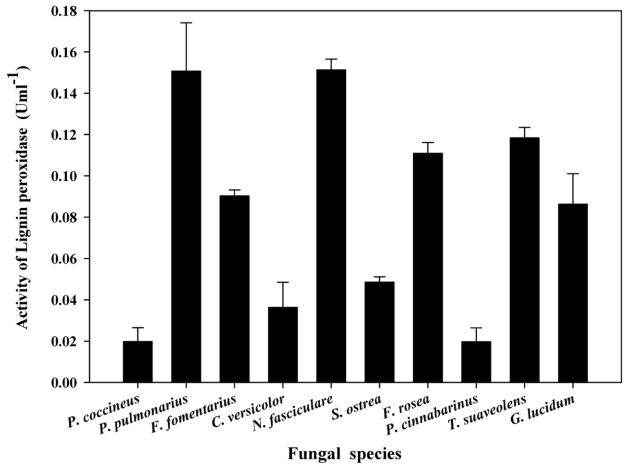

Fig. 3. Production of lignin peroxidase by white rot fungi in potato broth containing 1% naphthalene and glucose. Results shown are the means of five replications. Vertical bars represent the standard deviation.

rosea and *T. suaveolens* produced around 0.11 Uml^{-1} LiP and *G. lucidum* and *F. fomentarius* produced around 0.09 Uml^{-1} LiP under the same conditions. All other species evaluated in this study produced less than 0.05 Uml^{-1} LiP in media that contained naphthalene (Fig. 3). *C. versicolor* produced the highest amount (0.27 Uml^{-1}) of MnP in media that contained naphthalene, followed by *F. fomentarius* (0.16 Uml^{-1}). The remainder of the species evaluated in this study produced less than 0.1 Uml^{-1} of MnP when they were cultured in media that contained naphthalene (Fig. 4). The secretion of laccase by *P. cinnabarinus*, *C. versicolor* and *P. coccineus* was greatly enhanced when they were cultured in media supplemented with naphthalene. Similarly, the production of LiP by *P. pulmonarius*, *N. fasciculare*, *F. rosea* and *T. suaveolens* was also enhanced in response to the addition of naphthalene the media. Furthermore, the production of MnP by *C. versicolor* was improved by the addition of naphthalene to the media. Production of ligninolytic enzymes is affected by many factors during fermentation, including the medium composition, carbon to nitrogen ratio, pH, temperature, and aeration rate. Moreover, many aromatic compounds have been widely used to stimulate the production of ligninolytic enzymes during fermentation (Arora and Gill, 2001; Mansur *et al.*, 1997). Therefore, many studies have been conducted in an attempt to improve the production of ligninolytic enzymes by white rot fungi (Yesilada *et al.*, 1991; Ardon *et al.*, 1996; Kaluskar *et al.*, 1999). The results of this study suggest that supplementation of the medium with a small amount of aromatic hydrocarbons can greatly enhance ligninolytic enzyme production and that fungi have the capability to degrade aromatic compounds efficiently.

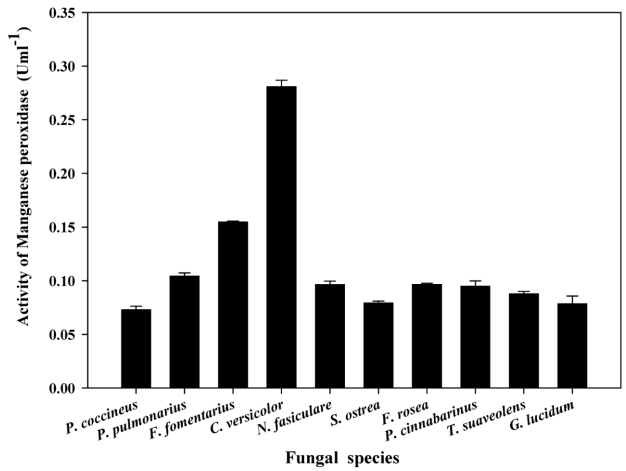

Fig. 4. Production of manganese peroxidase by white rot fungi in potato broth containing 1% naphthalene and glucose. Results shown are the means of five replications. Vertical bars represent the standard deviation of the mean.

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