Mokchae Konghak 32(6) : 14~22, 2004

Effect of Cadium Ions on the Activity of Fungal Laccase and Its Decolorization of Dye, RBBR*1

A. Jarosz-Wilkolazka*2, E. Malarczyk*2, A. Leonowicz*2, and Nam-Seok Cho*3†

ABSTRACT

The effect of cadmium ions on ligninolytic and decolourizing activities in cultures of two white-rot fungi, *Cerrena unicolor* and *Trametes versicolor*, were examined. Cadmium was added to the shallow stationary cultures growing on a liquid mineral medium. Both examined strains sorbed Cd ions in the first 24 hr of incubation. An appreciable stimulation of the activity of extracellular laccase (LAC) and inhibition of the extracellular manganese-dependent peroxidase (MnP) were simultaneously observed when 25 mgL^{-1} and 50 mgL^{-1} of cadmium ions were added to the cultures. On the other hand, the addition of cadmium ions also resulted in stimulating the decolorization activity of *C. unicolor* to decolorize Remazol Brilliant Blue R (RBBR) in the cultures, but decreasing it in the culture of *T. versicolor*, which is compared to the inhibition of MnP activity in this fungus. Our data indicate that the presence of Cd(II) ions can affect the ligninolytic activity of white-rot fungi. It was found that *C. unicolor* is a strain resistant to the presence of Cd ions in the liquid culture media, and has a potential to use this strain for bioremediation of sites contaminated with both heavy metals and aromatic pollutants.

Keywords : cadmium, white-rot fungi, laccase; Mn-peroxidase, remazol brilliant blue R (RBBR), decolorization, *Cerrena unicolor*, *Trametes versicolor*

1. INTRODUCTION

White-rot fungi produce an extracellular complex of non-specific, delignifying enzymes that are able to degrade different recalcitrant molecules in the environment, for example, lignin, polycyclic aromatic hydrocarbons, and a number of chlorinated pollutants and synthetic dyes (Arisoy, 1998; Heinfling *et al.*, 1998; Tuomela *et al.*, 1999). Fungal delignifying enzymes have important potential applications in lignocellulose-based industries, as well as in bioremediation of aromatic xenobiotics (Tuor *et al.*, 1995; D'Annible *et al.*, 1998). These fungi produce various combinations of oxidases and peroxidases: lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (LAC) that are involved in the degradation and biotransformation of lignin

*1 Received on April 23, 2004; accepted on May 26, 2004.

*2 Department of Biochemistry, Maria Curie-Sklodowska University, Lublin, Poland
*3 Department of Wood and Paper Science, Chungbuk National University, Cheongju, Korea
† Corresponding author : Nam-Seok Cho (nscho@chungbuk.ac.kr)

in their natural lignocellulosic substrates (Breen and Singleton, 1999). These enzymes are thought to play important roles in removing certain aromatic xenobiotics from natural and industrial waste materials. LAC (EC 1.10.3.2), the multi-copper-containing polyphenol oxidase, is an enzyme oxidizing a number of aromatic hydrogen donors which form free phenoxy radicals and catalyzing reactions of decarboxylation and demethylation of phenolic and methoxyphenolic acids. MnP (EC 1.11.1.13) oxidize substrates in two consecutive one-electron oxidation steps with intermediate cation radical formation and act on phenolic substrates using Mn(II)/Mn(III) as an intermediate redox couple (Tuor *et al.*, 1995). These reactions become an important step in the initial degradation of lignin polymers.

Several studies have reported another phenomenon of fungi, namely their ability to accumulate heavy metals from the growing medium (Volesky and Holan, 1995). Fungi and fungal products are used to remove metal cations from industrial effluents, mine water and metal-contaminated soil (Lovley and Coates, 1997; Gray, 1998). Many recent publications account for interactions occurring between microorganisms and metal ions, including (a) extracellular precipitation of metal by a number of microbial polymers and anions, (b) metal binding to the cell surface or adsorption to the cell wall, (c) transport of the metal into the cell, and (d) enzymatic conversion of metal ions to less toxic forms (Krantz-Rülcker *et al.*, 1993; Gadd, 1993). In response to cellular metal stress, fungi utilize (e) intracellular sequestration of metal by cysteine-rich molecules, namely γ-glutamyl peptides, metallothioneins and glutathione (Perego and Howell 1997; Münger and Lerch, 1985; Li *et al.*, 1997). The knowledge of fungal ability to degrade aromatic xenobiotics and accumulate heavy metals, has led to extensive studies on a possibility for bioremediation of natural and industrial waste materials by using the fungal mycelia. This study was carried out to understand the accumulation of cadmium (Cd) ions from a liquid growing media of white-rot fungi, *Cerrena unicolor* and *Trametes versicolor*. In the presence of toxic Cd ions, the decolorizing ability of these fungi also was evaluated.

2. MATERIALS and METHODS

2.1. Fungal Strains and Culture Conditions

Cerrena unicolor (Bull. ex Fr) and *Trametes versicolor* (L. ex Fr.) Pil were obtained from the Culture Collections of the School of Forest Resources, Chungbuk National University, Cheongju, Korea. The fungi were maintained on 2% (w/v) malt agar slants. For inoculation the fungal agar plugs (*ca.* 0.5 cm^2) were cut and grown in a basal medium according to Lindeberg and Holm (1952). The culture medium contained 10 gL^{-1} glucose, 2.5 gL^{-1} *L*-asparagine, 3 gL^{-1} $NaNO_3$, 0.5 gL^{-1} KCl, 0.45 gL^{-1} KH_2PO_4, 0.17 gL^{-1} $Na_2HPO_4 \times 12H_2O$, 0.5 gL^{-1} $MgSO_4 \times 7H_2O$ and trace element's solution. Cultures were grown in static flasks at 25°C until the mycelium colonized the whole surface of the liquid. The mycelial mats were collected and homogenized in a Warning Blender. After inoculation with 4% (v/v) of the homogenate, stationary cultures were incubated at 25°C in 25 mℓ Erlenmeyer flasks. Cadmium chloride ($CdCl_2 \times 2.5H_2O$) was added to the 10-day-old cultures to a final concentration of 50 mgL^{-1}, 25 mgL^{-1} or 10 mgL^{-1}. The extracellular medium was separated from the mycelium by filtration through Miracloth (Calbiochem). All measurements were further recorded using extracellular culture media.

2.2. Enzyme Assay

LAC activity was measured according to

Leonowicz and Grzywnowicz (1983) by monitoring the oxidation of syringaldazine. The reaction mixture contained 0.025 mM syringaldazine, 50 mM citrate-phosphate buffer (pH 5.2) and the enzyme. MnP activity was determined by monitoring the formation of manganese(III) malonate at 270 nm according to Wariishi *et al.* (1992). The enzyme was added to a solution of 0.2 mM $MnSO_4$, 50 mM sodium-malonate buffer (pH 4.5), and the reaction was initiated with 0.1 mM hydrogen peroxide. The initial increase in A_{270} was determined ($=1.159\times10^4$ $M^{-1}cm^{-1}$). The specific activities were expressed in nkat mg^{-1} of protein.

2.3. Dye-decolorization Monitoring

Dye-decolorizing activities were determined *in vitro* with Remazol Brilliant Blue R (RBBR). Decolorization of RBBR was expressed as a decrease in the absorbance ratio A_{585}/A_{500} according to Vyas and Molitoris (1995).

2.4. Determination of Protein

Protein concentrations were determined by using Bradford reagent and bovine serum albumin as the standard (Bradford, 1976).

2.5. Cd Determination

The mycelia of *C. unicolor* and *T. versicolor* were digested by boiling in 70% (v/v) nitric acid for 1 hr. Cd ion was measured by an atomic absorption spectrophotometer (AAS 1N, Carl Zeiss Jena).

3. RESULTS and DISCUSSION

3.1. Sorption of Cd by Fungi

There are several reports concerning biosorption of Cd by microbial biomass from the viewpoint of environmental purification and metal recovery (Zhou and Kiff, 1991). Most of Cd-sorbing microorganisms belong to algae, fungi, yeasts and bacteria (Volesky and Holan, 1995; Lovley and Coates, 1997). A number of studies have been carried out on heavy metal uptake into hyphae of filamentous fungi, such as *Rhizopus arrhizus, Penicillium spinulum* and *Aspergillus niger* (Zhou and Kiff, 1991; Strasser *et al.*, 1994). This experiment showed that wood rotting fungi are very good Cd biosorbents from aqueous solution. It is evident that the rate of Cd ion adsorption by the mycelia of both strains of fungi, *T. versicolor* and *C. unicolor*, is very fast at the initial 24 hr, then slowed down at each Cd ion concentration level (Fig. 1). Cd adsorption by mycelium of *C. unicolor* was higher than that of *T. versicolor*. At the highest concentration of Cd ions (50 mg L^{-1}) the maximum biosorption capacity was found to be 6~9 μg g^{-1} of fungal cell dry weight over 9 days.

Mechanism which seems to contribute to the accumulation of heavy metals includes adsorption to the fungal cell wall, extracellular precipitation and complexation, and intracellular accumulation, possibly within the vacuole. Fungal cell walls play a major role in biosorption, including carboxyl, hydroxyl, sulphydryl, amino and phosphate groups of their components (Krantz-Rülcker *et al.*, 1993; Gadd, 1993; Galli *et al.*, 1994). Application of fungal biomass to remove heavy metal from industrial wastewater and/or to recover economically valuable metals is attractive to industry (Volesky and Holan, 1995; Lovley and Coates, 1997; Obara *et al.*, 1999).

Food and industrial fermentation processes can provide cheap and constant supply of fungal biomass for removal of heavy metals ions from wastewaters. The dry mycelium of white-rot fungus, *Phanerochaete chrysosporium*, was successfully used by Saglam *et al.* (1999) as

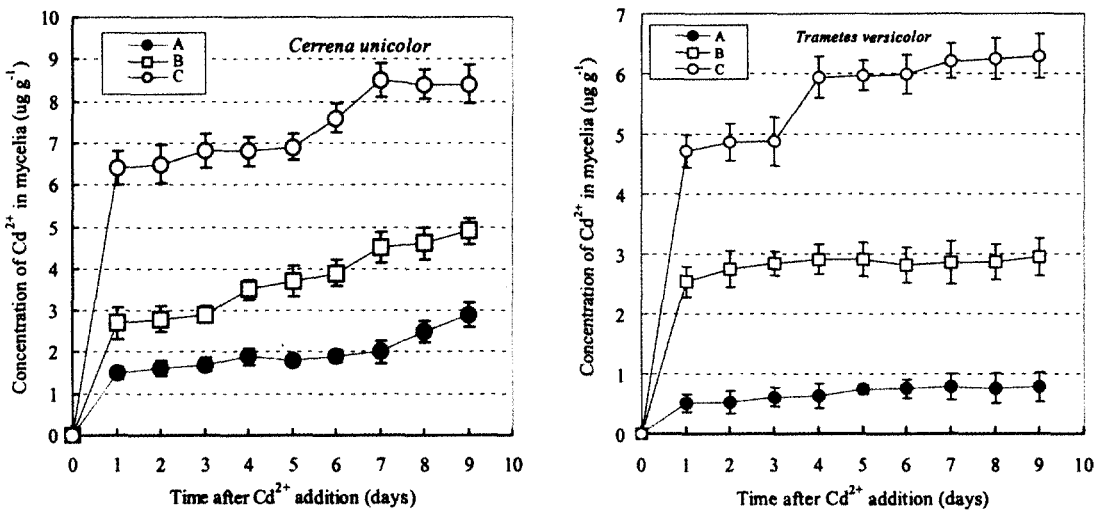

Fig. 1. The biosorption curves of Cd(II) ions at the initial ion concentrations of 10 mg L^{-1} (A), 25 mg L^{-1} (B) and 50 mg L^{-1} (C). Cultures were growing as described under Materials and Methods into liquid, mineral medium. Cadium chloride was added to the 10-day-old cultures. Results are the means SEM of three separate experiments.

a sorbing agent for removal of Hg and alkali-Hg species from aqueous solutions. White-rot basidiomycetes are highly specialized groups of microorganisms and seem to be very useful for bioremediation process, since they secrete extracellular enzymes to degrade lignocellulosic and other organic compounds (Tuor *et al.*, 1995; Breen and Singleton, 1999). They could also be used for removal of heavy metals from aqueous solutions by metal adsorption on the mycelium and for biodegradation of various organic xenobiotics. These white-rot strains can gain considerable quantities of Cd(II) ions from the solution by adsorption or related processes, and can still perform its physiological activity.

3.2. LAC Stimulation by Cd Ions

As a result of parallel experiments, we found that addition of Cd ions significantly stimulated the activity of extracellular LAC of these strains as compared with the control cultures (Fig. 2). It is clear that the LAC activity in the cultures of both strains of fungi increases with increasing initial concentration of Cd ions, and attends the maximum at the 6th day of the culture. The higher Cd ions concentration, the more pronounced effect was.

The increase in LAC activity level initiated by high temperature was reported by Fink-Boots *et al.* (1999) in the cultures of selected strains of Basidiomycetes as a consequence of adaptation to changes in environmental temperature conditions. The enhanced LAC activity in the presence of Cd(II) ions plays an important role in fungal adaptation processes, which could be utilized for Cd containing waste materials. LAC catalyses oxidation of many aromatic substrates, such as polyphenols, methoxy substituted monophenols, and aromatic amines, by a single-electron transferring mechanism with concomitant reduction of molecular oxygen to water. In the presence of mediators, this process can be extended to oxidation of substrates that normally do not undergo LAC-catalyzed oxidation (Burbonnaise *et al.*, 1997; Setti *et al.*, 1999).

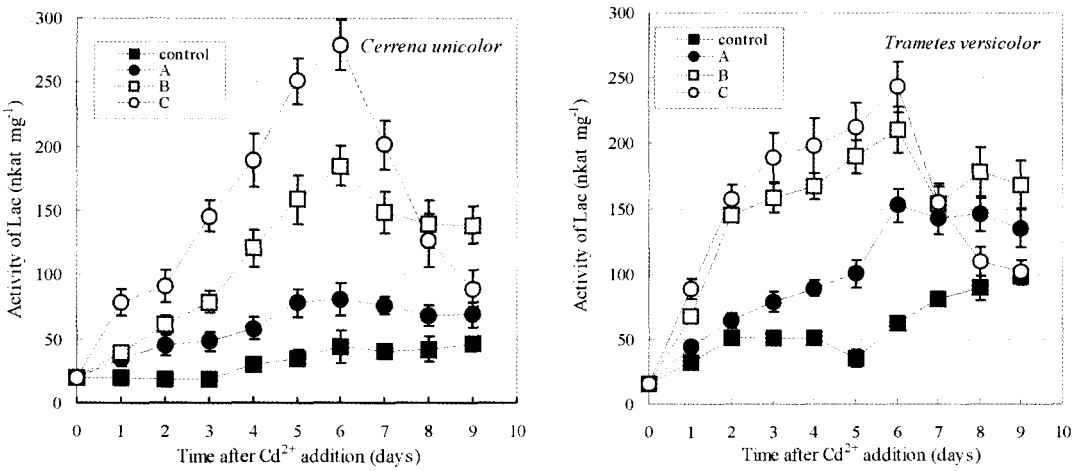

Fig. 2. The effect of Cd ions concentrations on extracellular laccase (LAC) activity in cultures of *C. unicolor* or *T. versicolor*. Cadmium chloride was added to the 10-day-old cultures at the initial ion concentrations of 10 mg L^{-1} (A), 25 mg L^{-1} (B) and 50 mg L^{-1} (C). Results are the means SEM of three separate experiments.

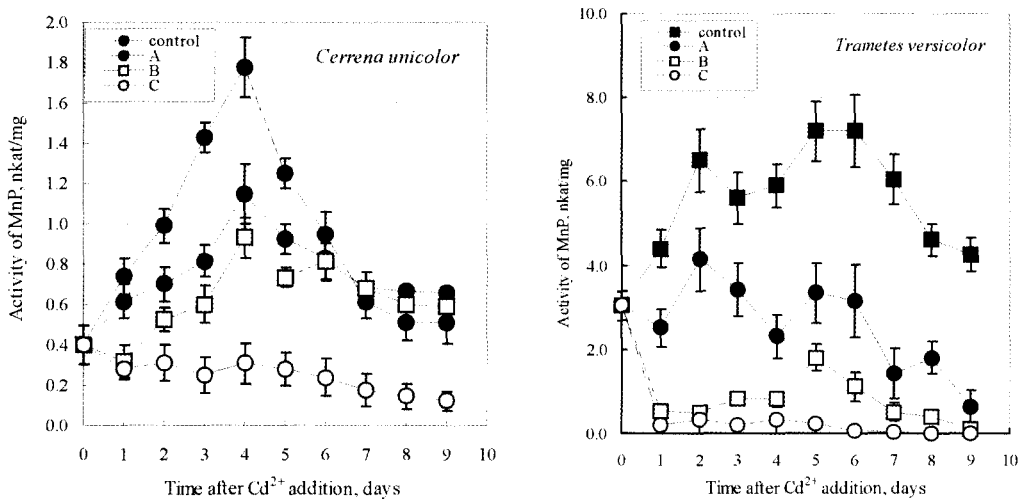

Fig. 3. The effect of Cd ions concentrations on extracellular manganese peroxidase (MnP) activity in cultures of *C. unicolor* or *T. versicolor*. Cadmium chloride was added to the 10-day-old cultures at the initial ion concentrations of 10 mg L^{-1} (A), 25 mg L^{-1} (B) and 50 mg L^{-1} (C). Results are the means SEM of three separate experiments.

3.3. MnP Activity Behavior

The activity of the other examined extracellular enzyme, MnP, increased mainly in the culture of *C. unicolor* or with addition of 10 mg L^{-1} Cd(II) ions, which attained the maximum at the 4th day of the culture (Fig. 3). The addition of Cd(II) ions at the concentrations of 25 mg L^{-1} and 50 mg L^{-1} resulted in the inhibition of this enzyme in the Cd-amended cultures of

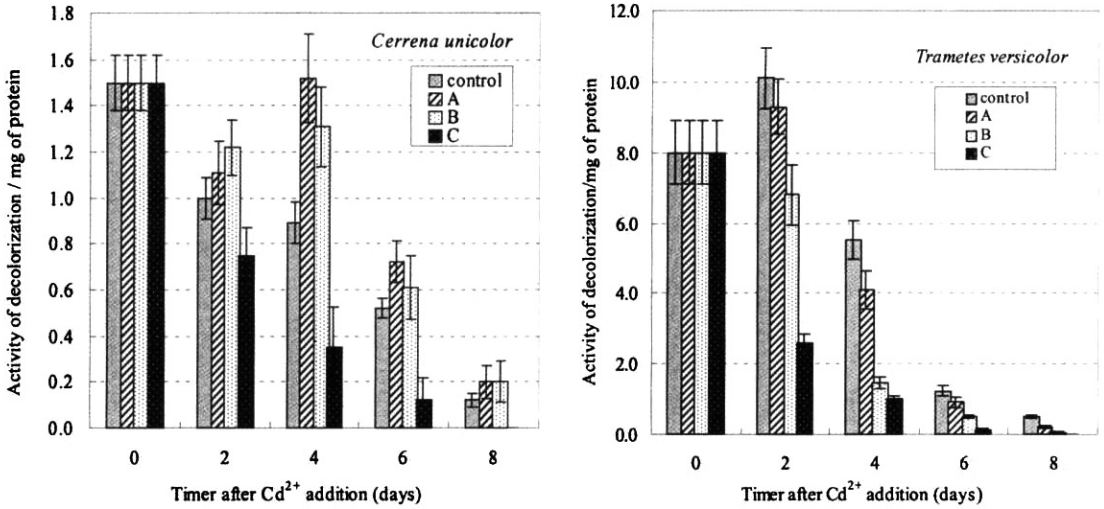

Fig. 4. The effect of Cd ions concentrations added to cultures of *C. unicolor* or *T. versicolor* on the RBBR-decolorizing activity measured in vitro. Cadmium chloride was added to the 10-day-old cultures at the initial ion concentrations of 10 mg L^{-1} (A), 25 mg L^{-1} (B) and 50 mg L^{-1} (C). Results are the means SEM of three separate experiments.

the both fungi. Heavy metals are known as inhibitors of many enzymes belonging to both primary and secondary metabolic pathways. Baldrian *et al.* (1996) reported that MnP activity in Cd-treated cultures of *Stereum hirsutum* and *Phanerochaete chrysosporium* were inhibited, while that of LAC was reduced of about 50%. Cd salt was added to these strains before sterilization, except that in our experiments Cd ions were added to 10-day-old cultures with a mycelium of approximately high ability of biosorption. Youngs *et al.* (2000) studied inhibition of MnP by Cd(II) ions under steady- and transient-states kinetic conditions. Kinetic analysis of MnP suggests that Cd(II) binds at the Mn(II)-binding site of MnP, preventing oxidation of Mn(II) ions, but does not impair oxidation of substrates, because such phenolic substrates do not bind at the Mn(II)-binding site.

3.4. RBBR Decolorization

Ligninolytic cultures of several white-rot fungi have been reported to degrade and decolorize various dyes, and the involvement of MnP has been demonstrated in the degradation pathway of some dyes (Goszczynski *et al.*, 1994; Spadaro *et al.*, 1992). However, white-rot basidiomycetes are expected to differ in their ability and capacity to degrade dyestuffs on the basis of qualitative and quantitative differences in production of that enzyme. In this study, addition of Cd ions stimulated the RBBR-decolorization activity in *C. unicolor* cultures, but decreased it in *T. versicolor* ones (Fig. 4), which can be compared to the inhibition of MnP activity in this fungus. Correlation between dyes decolorization and the MnP activity was obtained by Baldrian *et al.* (1996) in Cd-amended cultures of *Stereum hirsutum* and *Phanerochaete chrysosporium*, but Vyas and Molitoris (1995) determined that MnP did not decolorized RBBR in the culture of *Pleurotus ostreatus.*

Rates of biodegradation by white-rot fungi are dependent on various factors, such as chemicals, soils, quantity of nutrient and presence of

various low molecular-weight compounds, for example metals, and organic acids (Swamy and Ramsay, 1999; Reddy, 1995; Pasty-Grigsby *et al.*, 1992). This experiment indicates that the presence of Cd(II) ions can affect the outcome of ligninolytic activity of white-rot fungi. In addition it was disclosed that *C. unicolor* is a resistant strain to the presence of Cd ions in the liquid culture media, and has a potential application of this strain for bioremediation of sites contaminated with both heavy metals and aromatic pollutants.

4. CONCLUSIONS

This experiment was performed to investigate the effect of cadmium (Cd) ions on the ligninolytic activities of white-rot fungi, *Cerrena unicolor* and *Trametes versicolor*. In the presence of toxic Cd ions, the decolorizing ability of these fungi was also evaluated. Cadmium was added to the shallow stationary cultures growing on a liquid mineral medium. Both examined strains sorbed cadmium ions in the first 24 hr of incubation. An appreciable stimulation of the activity of extracellular laccase (LAC) and inhibition of the extracellular manganese-dependent peroxidase (MnP) were simultaneously observed when 25 mg L^{-1} and 50 mg L^{-1} of cadmium ions were added to the cultures. On the other hand, the addition of Cd ions also resulted in stimulating the decolourization activity of *C. unicolor* to decolourize Remazol Brilliant Blue R (RBBR) in the cultures, but decreasing it in the culture of *T. versicolor*, which is compared to the inhibition of MnP activity of this fungus. Our data indicate that the presence of Cd(II) ions can affect the ligninolytic activity of white-rot fungi. It was found that *C. unicolor* is a strain resistant to the presence of Cd ions in the liquid culture media, and has a potential to use this strain for bioremediation of sites contaminated with both heavy metals and aromatic pollutants.

ACKNOWLEDGEMENTS

Some part of this work was supported by RTD5 EC grant No ICA2-CT-2000-10050 and by KBN grant No. 139/E-SPUB-M-5 PR-UE/DZ 280/2000, and KOFST and KOSEF Brain Pool Program (032S-4-6).

REFERENCES

1. Arisoy M. 1998. Biodegradation of chlorinated organic compounds by white-rot fungi. Bull. Environ. Contam. Toxicol. 60: 872~876.
2. Baldrian P., J. Gabriel, and F. Nerud. 1996. Effect of cadmium on the ligninolytic activity of *Stereum hirsutum* and *Phanerochaete chrysosporium*. *Folia. Microbiol.* 41(4): 363~367.
3. Bradford M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248~254.
4. Breen A. and F. L. Singleton. 1999. Fungi in lignocellulose breakdown and biopulping. Curr. Opin. *Biotechnol.* 10: 252~255.
5. Burbonnaise R., M. G. Paice, B. Freiermuth, E. Bodie, and S. Borneman. 1997. Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. *Appl. Environ. Microbiol.* 63: 4627~4632.
6. D'Annible A., C. Crestini, V. Vinciguerra, and G.G. Sermanni. 1998. The biodegradation of recalcitrant effluents from an olive mill by white-rot fungus. *J. Biotechnol.* 61: 209~218.
7. Fink-Boots M., E. Malarczyk, and A. Leonowicz. 1999. Increased enzymatic activities and levels of superoxide anion and phenolic compounds in cultures of Basidiomycetes after temperature stress. *Acta. Biotechnol.* 19(4): 319~330.
8. Gadd G. M. 1993. Interactions of fungi with toxic metals. *New Phytol.* 124: 25~60.
9. Galli U., H. Schuepp, and C. H. Brunold. 1994. Heavy metal binding by mycorrhizal fungi.

Physiol. Plant 92: 364~368.

10. Goszczynski S., A. Paszczynski, M. B. Pasty-Grigsby, R. L. Crawford, and D. L. Crawford. 1994. New pathway for degradation of sulfonated azo dyes by microbial peroxidases of *Phanerochaete chrysosporium* and *Streptomyces chromofuscus*. *J. Bacteriol.* 176: 1339~1347.
11. Gray S. N. 1998. Fungi as potential bioremediation agents in soil contaminated with heavy or radioactive metals. *Biochem. Society Transactions* 26: 666~670.
12. Heinfling A., M. J. Martinez, A. T. Martinez, M. Bergbauer, and U. Szewczyk. 1998. Transformation of industrial dyes by manganese peroxidases from *Bjerkandera adusta* and *Pleurotus eryngii* in a manganese-independent reaction. *Appl. Environ. Microbiol.* 64(8): 2788~2793.
13. Krantz-Rülcker B., B. Allard, and J. Schnurer. 1993. Interactions between a soil fungus, *Trichoderma harizanum* and IIb metals-adsorption to mycelium and production of complexing metabolites. *Biometals* 6: 223~230.
14. Leonowicz A. and K. Grzywnowicz. 1983. Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substarte. Enzyme Microb. *Technol.* 3: 55~58.
15. Li Z.-S., Y.-P. Lu, R.-G. Zhen, M. Szczypka, D. J. Thiele and P. A. Rea. 1997. A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis(glutathionato)cadmium. Proc. Nat'l. *Acad. Sci.* 94: 42~44.
16. Lindeberg G. and G. Holm. 1952. Occurrence of tyrosinase and laccase in fruit bodies of mycelia of some hymenomycetes. *Physiol. Plant* 5: 100~114.
17. Lovley D. R. and J. Coates. 1997. Bioremediation of metal contamination. *Curr. Opin. Biotechnol.* 8: 285~289.
18. Münger K. and K. Lerch., 1985. Copper metallothionein from the fungus *Agaricus bisporus*: chemical and spectroscopic properties. *Biochemistry* 24: 6751~6756.
19. Obara T., T. Sawaya, K. Hokari, Y. Umehara, M. Mizukami, and F. Tomita. 1999. Removal of cadmium from scallop hepatopancreas by microbial processes. *Biosci. Biotechnol. Biochem.* 63: 500~505.
20. Pasty-Grigsby M. B., A. Paszczynski, S. Goszczynski, D. L. Crawford, and R. L. Crawford. 1992. Influence of aromatic substitution patterns on azo dye degradability by *Streptomyces* spp. and *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 38: 3605~3613.
21. Perego P. and S. B. Howell. 1997. Molecular mechanisms controlling sensitivity to toxic metal ions in yeast. Toxicol. *Appl. Pharmacol.* 147: 312~318.
22. Reddy A. 1995. The potential for white-rot fungi in the treatment of pollutants. *Curr. Opin. Biotechnol.* 6: 320~328.
23. Saglam N., R. Say, A. Denizli, S. Patri, and M. Y. Arica. 1999. Biosorption of inorganic mercury and alkyl mercury species on to *Phanerochaete chrysosporium* mycelium. *Proc. Biochem.* 34: 725~730.
24. Setti L., S. Giuliani, G. Spinozzi, and P. G. Pifferi. 1999. Laccase catalyzed-oxidative coupling of 3-methyl-2-benzothiazoline hydrazone and methoxypxenols. Enzyme Microb. *Technol.* 25: 285~289.
25. Spadaro J. T., M. H. Gold, and V. Renganathan. 1992. Degradation of azo dyes by the lignin-degrading fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 58: 2397~2401.
26. Strasser H., W. Burgstaller, and F. Schinner. 1994. High yield production of oxalic acid for metal leaching purposes by *Aspergillus niger*. FEMS Microbial Lett. 119: 365~370.
28. Swamy J. and J. A. Ramsay. 1999. Effects of glucose and NH_4^+ concentration on sequential dye decoloration by *Trametes versicolor*. Enzyme Microb. *Technol.* 25: 278~284.
29. Tuomela M., M. Lyytikainen, P. Oivanen, and A. Hatakka. 1999. Mineralization and conversion of pentachlorophenol (PCP) in soil inoculated with the white-rot fungus *Trametes versicolor*. Soil *Biol. Biochem.* 31: 65~74.
30. Tuor U., K. Winterhalter, and A. Fechter. 1995. Enzymes of white-rot fungi involved in lignin degradation and ecological determinants for wood decay. *J. Biotechnol.* 41: 1~17.
31. Volesky B. and Z. R. Holan. 1995. Biosorption of heavy metals. *Biotechnol. Prog.* 11(3): 235~250.

32. Vyas B. R. M. and H. P. Molitoris. 1995. Involvement of an extracellular H_2O_2-dependent ligninolytic activity of the white rot fungus *Pleurotus ostreatus* in the decolorization of Remazol Brilliant Blue R. *Appl. Environ. Microbiol.* 61(11): 3919~3927.
33. Wariishi H., K. Valli, and M. H. Gold. 1992. Manganese (II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. *J. Biol. Chem.* 267: 23688~23695.
34. Youngs H. L., M. Sundaramoorthy, and M.H. Gold. 2000. Effect of cadmium on manganese peroxidase Competitive inhibition of Mn-II oxidation and thermal stabilization of the enzyme. Eur. *J. Biochem.* 267(6): 1761~1769.
35. Zhou J. L. and R. J. Kiff. 1991. The uptake of copper from aqueous solution by immobilized fungal biomass. *J. Chem. Tech. Biotechnol.* 52: 317~330.