The Journal of Microbiology (2011) Vol. 49, No. 5, pp. 824-827
DOI 10.1007/s12275-011-1230-y


# Degradation of Endocrine Disrupting Chemicals by Genetic Transformants with Two Lignin Degrading Enzymes in *Phlebia tremellosa*

**Hyunwoo Kum[1], Sungsuk Lee[2], Sunhwa Ryu[2], and Hyoung T. Choi[1]***

[1]*Molecular Microbiology Lab, Department of Biochemistry, Kangwon National University, Chunchon 200-701, Republic of Korea*
[2] *Division of Forest Bioenergy, Korea Forest Research Institute, Seoul 130-712, Republic of Korea*

(Received May 2, 2011 / Accepted October 18, 2011)

**A white rot fungus *Phlebia tremellosa* produced lignin degrading enzymes, which showed degrading activity against various recalcitrant compounds. However, manganese peroxidase (MnP) activity, one of lignin degrading enzymes, was very low in this fungus under various culture conditions. An expression vector that carried both the laccase and MnP genes was constructed using laccase genomic DNA of *P. tremellosa* and MnP cDNA from *Polyporus brumalis*. *P. tremellosa* was genetically transformed using the expression vector to obtain fungal transformants showing increased laccase and MnP activity. Many transformants showed highly increased laccase and MnP activity at the same time in liquid medium, and three of them were used to degrade endocrine disrupting chemicals. The transformant not only degraded bisphenol A and nonylphenol more rapidly but also removed the estrogenic activities of the chemicals faster than the wild type strain.**

***Keywords*:** bisphenol A, genetic transformation, nonylphenol, *Phlebia tremellosa*

Our environment is becoming increasingly polluted by various man-made chemicals including agricultural chemicals, paints and plastics. Many of them have been known to affect human reproductive systems, and the compounds mimicking or interfering with the action of endogenous gonadal steroid hormones have been called endocrine disrupting chemicals (EDCs) (Colborn *et al*., 1996). Various EDCs are used in the plastic synthesis industry, and are also generated during the treatment of plastic wastes. Bisphenol A is widely used as a material for the production of epoxy and phenol resins, and lacquer coatings of food cans (Staples *et al*., 1998). Nonylphenol, a nonionic surfactant is widely used in the generation of emulsifying agents and cleaning product. Even at very low concentrations, EDCs interfere with the animal reproductive system (Schonfelder *et al*., 2002). Since physical and chemical treatments for these EDCs can generate secondary pollutants, biological treatments can be useful because there is almost no need to use other chemicals to remove recalcitrant EDCs, even with a slow removal rate.

White rot fungi have lignin degrading enzymes, such as laccase, lignin peroxidase and manganese peroxidase (MnP). These enzymes are also involved in the degradation of many recalcitrant chemicals such as dyes (Champagne and Ramsay, 2005), EDCs (Cabana *et al*., 2007; Tamagawa *et al*., 2007), explosives (Cheong *et al*., 2006) and pesticides (Rezenda *et al*., 2005; Maruyama *et al*., 2006). A white rot fungus *Phlebia tremellosa* has been used in the degradation of EDCs (Yeo *et al*., 2008a), and its purified laccase was reported to have this degrading activity (Kim *et al*., 2008). We have synthesized a laccase cDNA of this fungus, and constructed an expression vector. A genetic transformant using the laccase expression vector showed about 2-fold increase in laccase activity, and also showed 10-600% faster degradation of 4 different EDCs (Yeo *et al*., 2008b). Another two genetic transformants with the MnP expression vector also showed increased enzyme activity and higher degrading ability to 3 different EDCs (Kum *et al*., 2009). In order to obtain fungal strains with increased degrading abilities, we attempted to construct a recombinant expression vector for two lignin degrading enzymes, laccase and manganese peroxidase. We used laccase genomic DNA from *P. tremellosa* (Kum *et al*., 2009) and MnP cDNA from *Polyporus brumalis* for the construction of the expression vector. We then generated transformants to get fungal strains showing increased degrading ability against two EDCs.

* For correspondence. E-mail: htchoi@kangwon.ac.kr; Tel.: + 82-33-250-8511; Fax: +82-33-242-0459

## Materials and Methods

### Construction of an expression vector carrying both laccase and MnP genes

A laccase genomic DNA used previously in the generation of a transformant of *Irpex lacteus* (Kum *et al*., 2009) was used in this study. MnP cDNA was isolated from *P. brumalis* by RACE (rapid amplification of cDNA ends)-PCR. pBARGPE1 vector was double digested with *Sma*I and *Eco*RI, and then ligated with the MnP cDNA which had each enzyme linker at the 5′- and 3′-end respectively. The final both enzyme expression vector was generated by inserting the laccase genomic gene at the *Xho*I site, designated as pMnP-gLac (Fig. 1). Introduction of pMnP-gLac to *P. tremellosa* was carried out by following the REMI method reported previously (Leem *et al*., 1999). Protoplasts ($5\times10^7$/ml) were mixed with 10 μg of pMnP-gLac plus *Not*I (30 U), and transformants were selected using the selectable marker, phosphinothricin resistance. Genomic DNAs from the transformants and the recipient strain were isolated using the CTAB method (Leem *et al*., 1999). PCR with specific primers at the trpC promoter-*bar* gene (forward primer; 5′-GTCGACAGAAGATGATATTG-3′ and re-

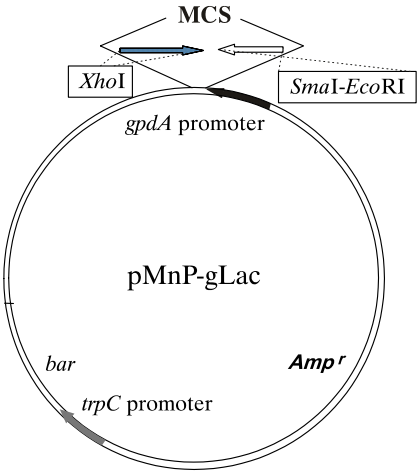


**Fig. 1.** Schematic drawing of the construction of the dual gene expression vector (pMnP-gLac) by inserting MnP cDNA from *Polyporus brumalis* and genomic laccase from *Phlebia tremellosa* to pBARGPE1. *MCS*, multiple cloning site. *Open arrow*, MnP cDNA located at *Sma*I-*Eco*RI site; *grey arrow*, laccase genomic gene at *Xho*I site in MCS.

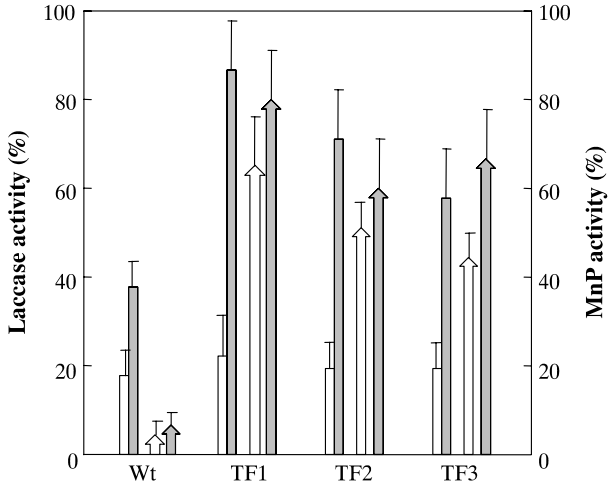


**Fig. 2.** Comparison of extracellular laccase and MnP activities of the recipient and three transformant strains on day 2 and day 5. *White bar*, laccase activity on day 2; *grey bar*, laccase activity on day 5; *white arrow*, MnP activity on day 2; *grey arrow*, MnP activity on day 5.

verse primer; 5-AGTTAGACAACCTGAAGTCT-3′) was run with genomic DNAs as the template to confirm the stable integration of pMnP-gLac in the transformant chromosomes.

**Degradation of EDCs and determination of estrogenic activity**
Four different strains were grown on PDA medium for 5 days to obtain a mycelial lawn. They were grown in the liquid minimal medium (Yeo *et al*., 2008a; 50 ml in 250 ml flask) in a shaking incubator at 30°C by inoculating 20 pieces of fungal mycelia cut with a No. 1 cork borer (diameter 4 mm). Whole fungal cultures were ground in a Waring blender and the homogenates (20 ml) were transferred to fresh liquid minimal medium (100 ml in 500 ml flask). EDCs such as bisphenol A (BPA, 100 mg/L) and nonylphenol (20 mg/L) were added in the minimal medium, and the culture was grown with shaking (160 rpm) at 30°C. The residual concentrations of EDCs were determined as reported previously (Kum *et al*., 2009). The determination of estrogenic activity was performed using yeast two-hybrid system which had been reported by Tsutsumi *et al*. (2001).

**Determination of involvement of laccase and MnP during the degradations of EDCs**
Laccase activity was measured by spectrophotometry using *o*-tolidine as the chromogenic enzyme substrate (Ko *et al*., 2001). Manganese peroxidase activity was determined using 3-amino-9-ethyl-carbazole as the chromogenic substrate (Yeo *et al*., 2007). Total RNAs were isolated from 3 day old fungal strains using Trizol extraction buffer (Invitrogen, USA), and were used as the template in the MnP gene-specific RT-PCR. MnP gene-specific primers were as follows: forward primer 5′-GTCTAGAAGCTCTCTTCCTC-3′ and reverse primer 5′-CACAACCTCACCGCCGA-3′. Both primers were used in the reverse transcriptase PCR (RT-PCR) for the determination of laccase and MnP expression. The actin gene was used as a control in RT-PCR. The amplified products were separated using agarose gel electrophoresis. All experiments for enzyme activity and estrogenic activity were run in triplicates.

## Results and Discussion

The two gene expression vector was used for the genetic transformation of *P. tremellosa* by REMI transformation using the protoplast-$CaCl_2$ procedure. Three transformants that showed higher laccase and MnP activities were selected for further experiments (Fig. 2). These transformants secreted a high amount of MnP under non-inducing conditions (Fig. 2) because the promoter of glyceraldehydes-3-phosphate dehydrogenase gene was used in the expression vector (Fig. 1). However, laccase activity appeared at a later stage, on day 5 (Fig. 2). The stable integration of pMnP-gLac was confirmed by *trpC*/*bar*-specific PCR, and the expected amplified band

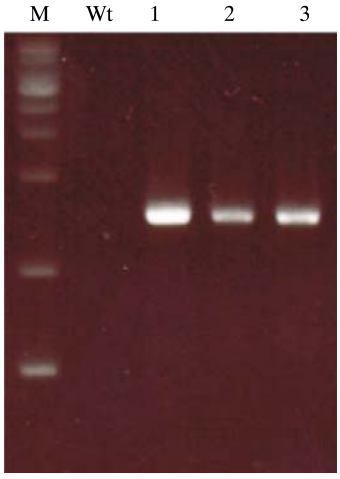


**Fig. 3.** Confirmation of integration of the expression vector (pMnP-gLac) in three transformants by PCR using vector-specific primers.

appeared only in each transformant (TF1 to TF3) (Fig. 3). These transformants were genetically stable since they showed antibiotic resistance after 10 consecutive transfers on non-selective medium.

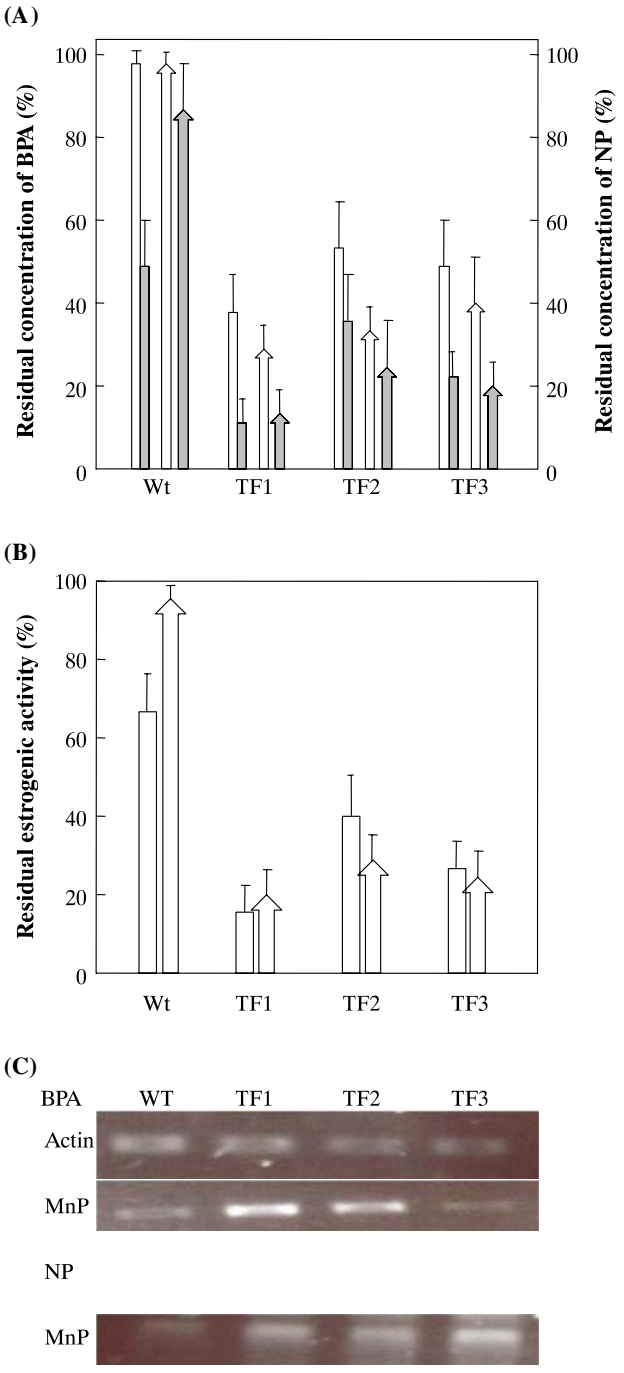


**Fig. 4.** Determination of residual EDCs from the cultures of wild type and three transformant strains on day 2 (*white*) and day 5 (*grey*). *Bar*, BPA; *arrow*, NP. (B) Determination of estrogenic activity on the culture supernatant of the wild type and transformants on day 5. *Bar*, cultures with BBP; *arrow*, cultures with NP. (C) Determination of MnP expression on day 2 cultures by RT-PCR using the MnP-specific primers.

One transformant strain (TF1) was able to remove more than 60% of BPA (100 mg/L) during 48 h incubation when analyzed with HPLC, and two other transformant strains (TF2 and TF3) removed the original compound better than the recipient strain (Fig. 4a). The removal rate by TF1 was better than that of *Irpex lacteus* transformant strains (T7 and T8) which showed 40-50% removal during 3 day incubation (Kum *et al*., 2009). When TF1 was compared with the laccase cDNA transformant of *P. tremellosa* (Yeo *et al*., 2008b) in BPA degradation, it had nearly the same removal rate. However, T1 had a better removal rate (ca. 70%) for NP, while *P. tremellosa* laccase cDNA transformant had only a 20% removal rate during 3 day incubation (Fig. 4a). The three transformants removed the estrogenic activity approximately two-times faster than the recipient strain on day 2 (Fig. 4b). It has been reported that the removal rate of estrogenic activities generated by EDCs in previous reports was slower than that of the original compound (Yeo *et al*., 2008b; Kum *et al*., 2009). The removal rate of the original EDCs by each strain was nearly the same as the removal rate of estrogenic activity in all transformant strains. This result implied that catabolic intermediates of EDCs that showed estrogenic activities were rapidly degraded, and both laccase and MnP enzymes positively enhanced the degradation of these intermediates in the transformants. The white rot fungus, *Coriolopsis polyzona* removed 5 mg/L of BPA completely after 4 h treatment (Cabana *et al*., 2007). It took 7 to 14 days to achieve 99% degradation of BPA (100 mg/L) by two different white rot fungi, *Stereum hirsutum* and *Heterobasidium insulare* (Lee *et al*., 2005).

When EDCs were added in the liquid medium, the increase of MnP expression was more than three times that observed in each transformant (Fig. 4c). Since the introduced MnP gene was regulated by a promoter of a constitutively expressed enzyme (glyceraldehydes-3-phosphate dehydrogenase), MnP was expressed during the early growth phase. Laccase gene expression also increased in all fungal strains (data not shown), because this gene was induced by the addition of many EDCs (Yeo *et al*., 2008a; Kum *et al*., 2009).

There are many reports that lignin degrading enzymes are involved in the degradations of various recalcitrant compounds. Phthalates, which are also components of EDCs, have induced laccase expression in *P. tremellosa* (Yeo *et al*., 2008a). *Trametes versicolor* showed enhanced laccase activity and its expression during the degradation of explosives (Cheong *et al*., 2006), and the addition of nonylphenol or aniline greatly enhanced the enzyme activity in the same fungus (Mougin *et al*., 2002). Manganese peroxidases were also reported to be involved in aflatoxin B degradation (Wang *et al*., 2011) and olive mill waste decolorization (Goudopoulou *et al*., 2010). In conclusion, genetic transformants with dual gene expression vectors showed enhanced laccase and MnP activities, which more efficient for the degradation of EDCs and gave better removal rates of estrogenic activity.

## Acknowledgements

This work was partly supported by a research grant from the Korea Forest Research Institute.

## References


Cabana, H., J.-L.H. Jiwan, R. Rosenberg, V. Elisashvili, M. Penninckx, S.N. Agathos, and J.P. Jones. 2007. Elimination of endocrine disrupting chemicals nonylphenol and bisphenol A and personal care product ingredient triclosan using enzyme preparation from the white rot fungus *Coriolopsis polyzona*. *Chemosphere* 67, 770-778.

Champagne, P.-P. and J.A. Ramsay. 2005. Contribution of manganese peroxidase and laccase to dye decoloration by *Trametes versicolor*. *Appl. Microbiol. Biotechnol.* 69, 276-285.

Cheong, S., S. Yeo, H.-G. Song, and H.T. Choi. 2006. Determination of laccase gene expression during degradation of 2,4,6-trinitrotoluene and its catabolic intermediates in *Trametes versicolor*. *Microbiol. Res.* 161, 316-320.

Colborn, T., D. Dumanoski, and J. Meyers. 1996. Our Stolen Future. Dutton, New York, USA.

Goudopoulou, A., A. Krimitzas, and M.A. Typas. 2010. Differential gene expression of lignolytic enzymes in *Pleurotus ostreatus* grown on olive oil mill wastewater. *Appl. Microbiol. Biotechnol.* 88, 541-551.

Kim, Y., S. Yeo, M.K. Kim, and H.T. Choi. 2008. Removal of estrogenic activity from endocrine disrupting chemicals by purified laccase of *Phlebia tremellosa*. *FEMS Microbiol. Lett.* 284, 172-175.

Ko, E., Y. Leem, and H.T. Choi. 2001. Purification and characterization of laccase isozymes from the white-rot basidiomycete *Ganoderma lucidum*. *Appl. Microbiol. Biotechnol.* 57, 98-102.

Kum, H., M.K. Kim, and H.T. Choi. 2009. Degradation of endocrine disrupting chemicals by genetic transformants in *Irpex lacterus* with an inducible laccase gene of *Phlebia tremellosa*. *Biodegradation* 20, 673-678.

Lee, S.M., B.W. Koo, J.W. Choi, D.H. Choi, B.S. An, E.B. Jeung, and I.G. Choi. 2005. Degradation of bisphenol A by white rot fungi, *Stereum hirsutum* and *Heterobasidium insulare*, and reduction of its estrogenic activity. *Biol. Pharm. Bull.* 28, 201-207.

Leem, Y., S. Kim, I.K. Ross, and H.T. Choi. 1999. Transformation and laccase mutant isolation in *Coprinus congregatus* by restriction enzyme-mediated integration. *FEMS Microbiol. Lett.* 172, 35-40.

Maruyama, T., C. Komatsu, J. Michizoe, H. Ichinose, and M. Goto. 2006. Laccase-mediated oxidative degradation of the herbicide dymron. *Biotechnol. Prog.* 22, 426-430.

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.

Rezende, M.I., A.M. Barbosa, A.F. Vasconcelos, R. Haddad, and R.F. Decker. 2005. Growth and production of laccase by the ligninolytic fungi, *Pleurotus ostreatus* and *Botryospaeria rhodina*, cultured on basal medium containing the herbicide, Scepter (imazaquin). *J. Basic. Microbiol.* 45, 460-469.

Schonfelder, G., B. Flick, E. Mayr, C. Talsness, M. Paul, and I. Chahoud. 2002. *In utero* exposure to low doses of bisphenol A lead to long-term deleterious effects in the vagina. *Neoplasia* 4, 98-102.

Staples, C., P. Dorn, G. Klecka, S. O'Block, and L. Harris. 1998. A review of the environmental fate, effects, and exposures of bisphenol A. *Chemosphere* 36, 2149-2173.

Tamagawa, Y., H. Hirai, S. Kawai, and T. Nishida. 2007. Removal of estrogenic activity of 4-tert-octylphenol by ligninolytic enzymes from white rot fungi. *Environ. Toxicol.* 22, 281-286.

Tsutsumi, Y., T. Haneda, and T. Nishida. 2001. Removal of estrogenic activities of bisphenol A and nonylphenol by oxidative enzymes from lignin-degrading basidiomycetes. *Chemosphere* 42, 271-276.

Wang, J., M. Ogata, H. Hirai, and H. Kawagishi. 2011. Detoxification of aflatoxin B1 by manganese peroxidase from the white-rot fungus *Phanerochaete sordid* YK-624. *FEMS Microbiol. Lett.* 314, 164-169.

Yeo, S., M.K. Kim, and H.T. Choi. 2008a. Increased expression of laccase by the addition of phthalates in *Phlebia tremellosa*. *FEMS Microbiol. Lett.* 278, 72-77.

Yeo, S., M.K. Kim, and H.T. Choi. 2008b. Degradation of endocrine disrupting chemicals by laccase transformants of *Phlebia tremellosa*. *Korean J. Microbiol.* 44, 10-13.

Yeo, S., N. Park, H.-G. Song, and H.T. Choi. 2007. Generation of a transformant showing higher manganese peroxidase (MnP) activity by overexpression of MnP gene in *Trametes versicolor*. *J. Microbiol.* 45, 213-218.