APPLICATION OF LACCASES, PRODUCED BY *GANODERMA* SPECIES, FOR THE DETOXIFICATION OF SOME ANILINE AND PHENOL DERIVATIVES

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ABSTRACT
The objective of this work was to collect isolates of the white rot fungus *Ganoderma* from decaying woods and investigate their laccase producing and detoxification abilities against aniline and phenol compounds. Sporocarps were collected and the tissue culturing technique was used for fungal isolation. In these experiments specific medium, containing benomyl, dichloran and guaiacol was used. Laccase producing of the isolates were tested in liquid media containing different inducers. Crude enzyme extracts were prepared and characterized. The best laccase-producing strains were identified with partial ITS sequence analysis. Their secreted laccases were investigated with SDS-PAGE and the molecular weights of these enzymes were estimated. Degradation of 7 different aniline and phenol derivates (2,4-dichlorophenol, 2-methyl-4-chlorophenol, 3-chloroaniline, 4-chloroaniline, 2,6-dimethylaniline, 3,4-dichloroaniline and 3-chloro-4-methylaniline) were investigated, observing high degradation rates in most cases.

Keywords: biodegradation, *Ganoderma*, laccase, aniline derivatives, phenol derivatives

INTRODUCTION

Harmful xenobiotics are abundant in the environment because of the intensive application of different pesticides in the agriculture. Most of these compounds are harmful with proved or suspected toxic, carcinogenic, mutagenic, teratogenic or endocrine disruptor effect. Some of them enter into the food chain when plants take up them from contaminated water or agricultural soil.

Basidiomycetous white rot fungi could mineralize lignin and other aromatic compounds. The degradation of these compounds depends on their lignin peroxidase, manganese-peroxidase and laccase production (XIAO et al., 2003). Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are polyphenol oxidases, containing four copper atoms, so they usually called multicopper oxidases. Laccases have received much attention from researchers during the past decades, because they have wide substrate range, and shown to be useful for the removal of toxic compounds, like chlorinated aniline and phenol xenobiotics (BOLLAG, 1992). Members of *Ganoderma* genus are well known medicinal mushrooms. This genus has been extensively studied also because they were identified as excellent laccase producers (MURUGESAN et al., 2007). Laccase from *G. lucidum* was able to degrade different types of PAHs (polycyclic aromatic hydrocarbons) and antracene (PUNNAPAYAK et al., 2009; REVANKAR and LELE (2006).

The aim of this study was to isolate good laccase producing *Ganoderma* strains and to characterize their degradation abilities on some aniline and phenol compounds.

MATERIAL AND METHOD

Screening and isolation

Eleven fruiting bodies of *Ganoderma* mushrooms, grown on the barks of various trees, were collected near Szeged and Hódmezővásárhely (Hungary). They were washed with water, cut into small pieces and surface sterilized. These pieces were inoculated onto medium containing KH_2PO_4 (0.05%), $MgSO_4.7H_2O$ (0.02%), NH_4NO_3, (0.01%), KCl (0.01%), $FeSO_4$ (0.002%), $Ca(NO_3)_2.4H_2O$ (0.005%), malt extract (0.2%) and agar (1.5%). After sterilization, benomyl and dichloran were added for selective grow of basidiomycetes and guaiacol for indicating the laccase activity. Strains were maintained on malt extract (MEA) media, containing malt extract (2%), glucose (2%), peptone (1%) and agar (2%). The isolates were deposited into the Szeged Microbial Collection (SzMC).

DNA extraction and identification of the isolates

The fungal DNA extractions were carried out from fresh mycelia with Aqua Genomic Solution Kit, according to the instructions. The identification of the isolates were carried out by partial sequence analysis of the ITS region, using ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'- GGAAGTAAAAGTCGTAACAAGG-3') primers according to WHITE et al. (1990). Sequence analyses were carried using the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST, ALTSCHUL et al., 1990).

Production of laccases

Laccase production was carried out in liquid minimal media with inducers. 1 L contained: 20 g glucose, 2.5 g L-Asparagine, 0.15 g D,L-Phenylalanine, 27.5 mg adenine, 50 µl thiamin (from 1mg/ml solution), 1 g KH_2PO_4, 0.1 g Na_2HPO_4, 0.5 g $MgSO_4$, 0.01 g $CaCl_2$, 0.01 g $FeSO_4$, 0.001 g $MnSO_4$, 0.001 g $ZnSO_4$, 0.002 g $CuSO_4$ according to FÅHRAEUS and REINHAMMAR (1967). Inoculated flasks were incubated in rotary shaker at 28 °C for 10 days. Culture supernatants were frozen, thawed and filtered to remove precipitated polysaccharides. They were concentrated according to YANG et al. (2009). Laccase activity was determined by measuring the oxidation of ABTS [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate)] spectrophotometrically after 5 min incubation at 436 nm. The reaction mixture contained 5 mM ABTS in 25 mM succinate buffer (pH 4.5) (KIISKINEN ET AL., 2002 and 2004).

Characterization of *Ganoderma* laccases

Temperature tolerance was investigated at 10, 20, 30, 40, 60 and 70 °C. pH dependence of growth was examined at pH 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7 and 8, in 25 mM succinate buffer. After concentration and pre-purification steps, the molecular weights were determined by SDS-PAGE in a 4-12% gradient gel. After electrophoresis, SDS was removed by incubating the gel in 50 mM sodium acetate with 0.2% Triton X-100 (MURUGESAN et al. 2006). Activity staining was performed with 5 mM ABTS in 25 mM succinate buffer.

Investigation the degradation of different aniline and phenol derivatives

The following xenobiotics were investigated: 2,4-dichlorophenol; 2-methyl-4-chlorophenol; 3-chloroaniline; 4-chloroaniline; 2,6-dimethylaniline; 3,4-dichloroaniline, 3-chloro-4-methylaniline. Reaction mixtures contained 1.5 ml of the ferment broth, which was mixed with equal volume of the distinct pesticides (0.25 mM in 50 mM succinate buffer, pH 4.5). Final concentrations of the xenobiotics were 0.125 mM in 25 mM succinate buffer. If applied, the concentration of the mediator (guaiacol) was 1 mM. The mixtures were incubated at 25 °C overnight. The reactions were terminated by removing the laccase from the system with membrane filtration. The remaining concentrations of the xenobiotics were determined with HPLC-MS.

RESULTS

Isolation and identification of *Ganoderma* strains

Seven *Ganoderma* strains were isolated on selective media with tissue culture technique. After 5 days of incubation the white mycelium, with brownish-reddish surround were subcultured. This color around the colonies indicates laccase production ability. After ITS based identification, these isolates were deposited into the SzMC (*Table 1.*).

Table 1. Identity numbers and source places of *Ganoderma* strains

SzMC Number	Lab Code	Strain	Place of isolation
SzMC 21029	DP1	*Ganoderma resinaceum*	Szeged
SzMC 21030	DP2	*Ganoderma resinaceum*	Szeged
SzMC 21031	DP3	*Ganoderma resinaceum*	Szeged
SzMC 21032	HM3	*Ganoderma adspersum*	Szeged
SzMC 21033	GM1	*Ganoderma resinaceum*	Szeged
SzMC 21034	GM2	*Ganoderma resinaceum*	Szeged
SzMC 21035	GM3	*Ganoderma resinaceum*	Szeged

The two best laccase producers, DP1 (*G. resinacearum*) and HM3 (*G. adspersum*) were selected for further studies. Molecular weights of laccases were determined by SDS-PAGE: according this the molecular weights of these *Ganoderma* laccases were about 90-98 kDa. They find to be thermostable, with pH 3.5-4.5 as an optimum.

Degradation of aniline and phenol derivatives

The degradation of aniline and phenol derivatives was investigated with or without a mediator. Without a mediator, DP3 strain significantly decreased the concentration of 2-methyl-4-chlorophenol and 3,4-dichloroaniline. Similarly, HM3 carried out remarkable degradation of 2,4-dichlorophenol and 2-methyl-4-chlorophenol (*Figure 1.*).
Applying guaiacol as mediator, we observed increased degradation of the investigated xenobiotics almost in all cases (*Figure 2.*).

Figure 1. Degradation of xenobiotics by DP3 and HM3 strains without a mediator

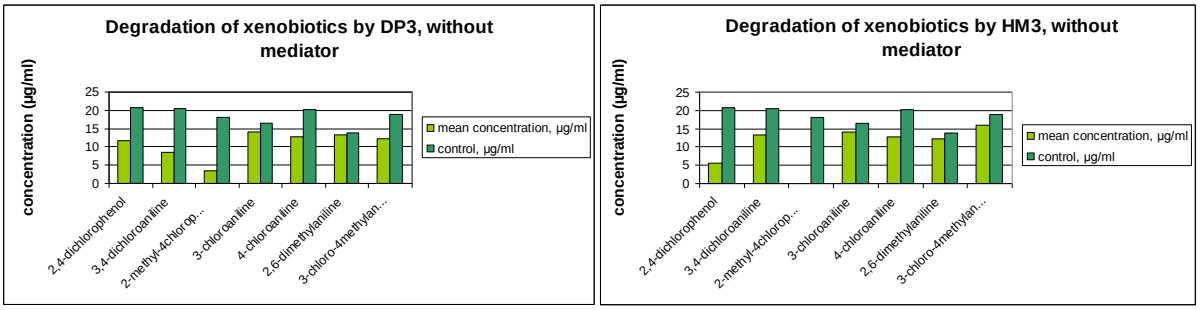
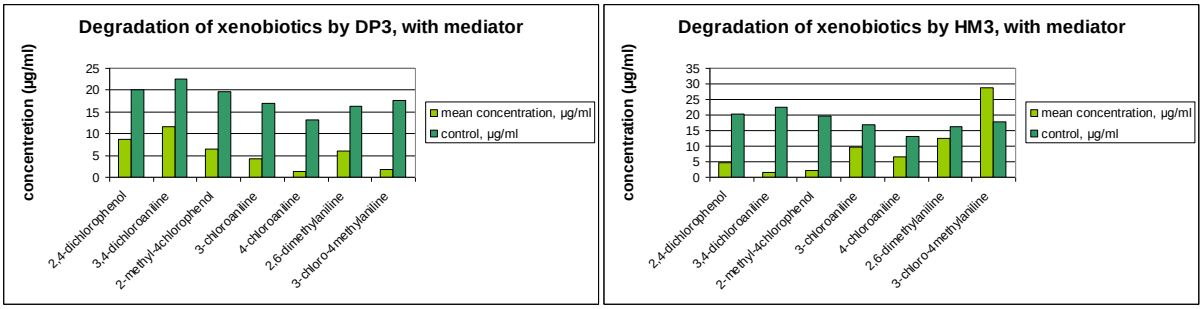


Figure 2. Degradation of xenobiotics by DP3 and HM3 strains after guaiacol addition



Comparison the degradation rates in the two systems (with or without a mediator) clearly shows that the presence of a mediator in most cases significantly increased the detoxification effectiveness of laccases (*Table 2.*).

Table 2. Comparison the remaining percentages of xenobiotics in the samples

Xenobiotics	Fungal isolate	without mediator %	with mediator %
2,4-dichlorophenol	DP3 (*G. resinaceum.*)	56,92	43,21
2,4-dichlorophenol	HM3 (*G. adspersum.*)	27,32	23,16
3,4-dichloroaniline	DP3 (*G. resinaceum.*)	41,78	51,6
3,4-dichloroaniline	HM3 (*G. adspersum.*)	65,26	7,6
2-methyl-4chlorophenol	DP3 (*G. resinaceum.*)	18,94	32,94
2-methyl-4chlorophenol	HM3 (*G. adspersum.*)	0	11,6
3-chloroaniline	DP3 (*G. resinaceum.*)	85,8	24,8
3-chloroaniline	HM3 (*G. adspersum.*)	74,3	57,2
4-chloroaniline	DP3 (*G. resinaceum.*)	63,6	9,7
4-chloroaniline	HM3 (*G. adspersum.*)	68,4	49,5
2,6-dimethylaniline	DP3 (*G. resinaceum.*)	95,5	36,7
2,6-dimethylaniline	HM3 (*G. adspersum.*)	89	75,9
3-chloro-4methylaniline	DP3 (*G. resinaceum.*)	65,5	10
3-chloro-4methylaniline	HM3 (*G. adspersum.*)	85,8	100

CONCLUSIONS

Two of the seven isolated *Ganoderma* isolates have outstandingly high laccase producing ability (DP3 and HM3). On the basis of partial ITS sequencing, DP3 and HM3 were identified as *G. resinaceum* and *G. adspersum*, respectively. They produce thermostable laccases with molecular weight of 90-98 kDa.

Degradations of 2,4-dichlorophenol; 2-methyl-4-chlorophenol; 3-chloroaniline; 4-chloroaniline; 2,6-dimethylaniline; 3,4-dichloroaniline, 3-chloro-4-methylaniline were very efficiently catalyzed by these laccases. These processes were further improved by the presence of guaiacol as mediator molecule. These results suggest the potential applicability of these laccase and laccase-mediator systems for bioremediation purposes.

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REFERENCES

ALTSCHUL, S.F., GISH, W., MILLER, W., MYERS, E.W., LIPMAN D.J. (1990): Basic local alignment search tool. Journal of Molecular Biology, Volume 215. pp. 403-410.

BOLLAG, JM. (1992): Decontaminating soil with enzymes: An *in situ* method using phenolic and anilinic compounds. Environmental Science Technology, Volume 26. pp. 1876-1881.

FÅHRAEUS, G., and REINHAMMAR, B. (1967): Large scale production and purification of laccase from cultures of the fungus *Polyporus versicolor* and some properties of laccase A. Acta Chemica Scandinavica, Volume 21. pp. 2367-2378.

KIISKINEN L.-L., RÄTTÖ M., KRUUS K. (2004): Screening for novel laccase-producing microbes. Journal of Applied Microbiology, Volume 97. pp. 640-646.

KIISKINEN L.-L., VIIKARI L., KRUUS K. (2002): Purification and characterisation of a novel laccase from the ascomycete *Melanocarpus albomyces.* Applied Microbiolology and Biotechnology, Volume 59. pp. 198-204.

MURUGESAN, K., NAM, I.H., KIM, Y.M., CHANG, Y.S. (2007): Decolorization of reactive dyes by a thermostable laccase produced by *Ganoderma lucidum* in solid state culture. Enzyme Microbiology and Technology, Volume 40. pp. 1662-1672.

PUNNAPAYAK, H., PRASONGSUK, S., MESSNER, K., DANMEK, K., LOTRAKUL, P. (2009): Polycyclic aromatic hydrocarbons (PAHs) degradation by laccase from a tropical white rot fungus *Ganoderma lucidum*. African Journal of Biotechnology, Volume 8. Number 21. pp. 5897-5900.

REVANKAR, M. S., LELE, S. S. (2006): Enhanced production of laccase using a new isolate of white rot fungus WR-1. Process Biochemistry, Volume 41. pp. 581-588.

WHITE, T.M., BRUNS, T., LEE, S., TAYLOR, J. (1990): Amplification and direct sequencing of fungal ribosomal RNA for phylogenetics. In: INNIS, M.A., GELFAND, D.H., SNINSKY, J.J., WHITE, T.J. (Eds.). PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA, pp. 315-321.

YANG, X.Q, ZHAO, X.X., LIU C. Y., ZHENG, Y., QIAN S. J. (2009): Decolorization of azo, triphenylmethane and anthraquinone dyes by a newly isolated *Trametes sp*. SQ01 and its laccase. Process Biochemistry, Volume 44. pp. 1185-1189.

XIAO, Y. Z., TU, X. M., WANG, J., ZHANG, M. CHENG, Q.· ZENG, W. Y.·SHI, Y. Y. (2003): Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete *Trametes sp*. strain AH28-2. Applied Microbiology and Biotechnology, Volume 60. pp. 700-707.