APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1997, p. 4120–4122 Vol. 63, No. 10
0099-2240/97/$04.00+0

Importance of Laccase in Vegetative Growth of *Pleurotus florida*

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Received 26 November 1996/Accepted 16 July 1997

Mycelial culture of *Pleurotus florida* produced highest extracellular laccase in optimum growth medium. At least two laccases (L_1 and L_2) were shown to be present in the culture filtrate. Low-laccase-yielding mutants with impaired L_2 activity had poor mycelial growth and could not form fruit body, whereas the revertants from the same mutants were similar to the parent in mycelial growth and fruit body formation.

The biological function of laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2), produced by many fungi, is not clear (13, 19). However, the involvement of the enzyme in rapid cell growth, formation of primordia (6), lignin degradation (1, 9), pathogenesis (17, 20), and detoxification of pollutants (2) has been suggested.

A few laccases have been identified, purified, and cloned from *Pleurotus* spp. (3, 10, 15, 18, 22). However, the physiological significance of laccase production by the white rot fungi is not known. We report that mycelial culture of *Pleurotus florida* produces at least two laccases, one of which appears to be linked with the mycelial growth of the fungus.

The mycelial strain of *P. florida* was obtained from the Society for Rural Industrialisation, Ranchi, India. Production of laccase was studied in PD medium, containing (wt/vol) 2% dextrose and 20% potato extract, pH 7.0, with shaking (150 cycles/min) at 30 ± 1°C. The effects on growth and enzyme production of the replacement of dextrose in PD medium with lactose (PL), fructose (PF), or sucrose (PS) and the addition of 0.5% yeast extract (PDY), 0.5% peptone (PDP), or 0.5% glutamic acid (PDG) were studied. Enzyme activity was assayed at 30°C by using 0.3 mM *o*-dianisidine in 0.1 M acetate buffer, pH 5.0, or 10 mM guaiacol in the same buffer containing 10% (vol/vol) acetone (15). The changes in absorbance of the reaction mixtures containing guaiacol or *o*-dianisidine were monitored, respectively, at 470 nm (ε = 6,740 M^{-1} cm^{-1}) and 460 nm (ε = 11,300 M^{-1} cm^{-1}), for 5 min of incubation (11, 14). Enzyme activity was expressed in katals (1 mol of substrate conversion/s). Activity staining of enzyme after native polyacrylamide gel electrophoresis (PAGE) of the concentrated culture filtrate was done with solutions of 0.3 mM *o*-dianisidine or 10 mM guaiacol in acetate buffer, pH 5.0. The laccase activity present in the culture filtrate was partially purified by 80% $(NH_4)_2SO_4$ precipitation, followed by DEAE-Sephadex (A-50) anion exchange chromatography with a 0-to-0.6 M NaCl gradient in 10 mM acetate buffer, pH 5.0. Induced mutations of basidiospores and mycelial protoplasts were carried out by exposing 10^6 cells/ml to 200 μg and 50 μg of *N*-methyl-*N*′-nitrosoguanidine/ml for 20 and 5 min, respectively. Mutants producing high (HLP) and low (LLP) amounts of laccase were selected on PD agar plates containing guaiacol (10 mM), according to the method of Fiegel et al. (8). The method for determining fruit body production was reported previously (5).

Extracellular laccase produced by *P. florida* oxidized *o*-dia-

TABLE 1. Substrate specificity of *P. florida* culture filtrate from PD medium

Substrate (wavelength [nm])[a]	Relative activity[b]
ABTS[c] (420)	303.0
Syringaldazine (530)	252.0
o-Dianisidine (460)	100.0
Diaminobenzidine (420)	65.0
Guaiacol (470)	33.0
o-Phenylenediamine (420)	15.0
Tannic acid (420)	12.0
Ferulic acid (420)	2.0
2-Naphthol (420)	0.6
DOPA[d] (475)	—
Veratrylalcohol (310)	—
Gallic acid (420)	—
Orcinol (420)	—
o-Toluidine (420)	—

[a] The reaction mixtures contained 0.1 mM substrate in 100 mM acetate buffer (pH 5.0). Activity was measured after addition of 1.6 μg of culture filtrate protein. Color development was measured at the wavelengths indicated.
[b] Relative activities were obtained by comparing optical density changes to the optical density with *o*-dianisidine (defined as 100%).
[c] ABTS, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid).
[d] DOPA, 3,4-dihydroxyphenylalanine.

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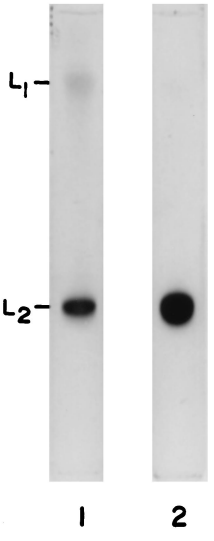

FIG. 1. Activity staining of *P. florida* culture filtrate with *o*-dianisidine (lane 1) and guaiacol (lane 2) after native PAGE.

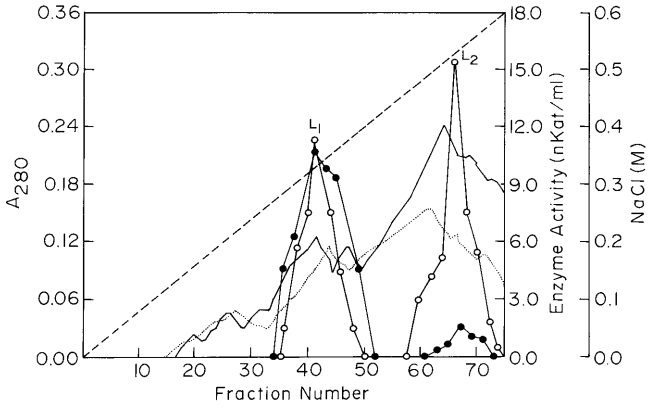

FIG. 2. DEAE-Sephadex A-50 elution profile of laccases secreted by *P. florida* and an LLP mutant (Z_1). ---, NaCl gradient; —, A_{280} of *P. florida*; . . ., A_{280} of Z_1; ○, enzyme elution of *P. florida*; ●, enzyme elution of Z_1.

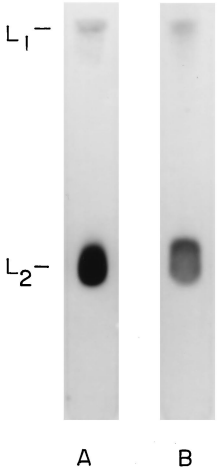

FIG. 4. Activity staining of gel containing culture filtrate of PDY (lane A) and PD (lane B) media after PAGE.

nisidine, guaiacol, and other phenols, phenol analogs, and anilines in the absence of H_2O_2 (Table 1), similar to other laccases (4, 7, 19, 21). Activity staining after native PAGE of the culture filtrate showed the presence of two laccase bands, L_1 and L_2. L_2 had more guaiacol-oxidizing activity than L_1 (Fig. 1). Laccase present in the culture filtrate of *P. florida* was fractionated by anion exchange chromatography into two fractions, also designated L_1 and L_2 (Fig. 2). Native PAGE of the fractions showed that the L_1 and L_2 fractions obtained by chromatography corresponded to the L_1 and L_2 bands in PAGE. The apparent K_m and V_{max} values for L_1 and L_2 were determined (Lineweaver-Burk plot) with guaiacol and *o*-dianisidine as substrates. With guaiacol, L_1 had a K_m value of 28.5 mM (V_{max} = 0.175 μkat/mg) and L_2 had a K_m value of 3.13 mM (V_{max} = 1.353 μkat/mg). On the other hand, with *o*-dianisidine, L_1 had a K_m value of 0.33 mM (V_{max} = 1.47 μkat/mg) while L_2 had a K_m value of 0.29 mM (V_{max} = 2.4 μkat/mg). The V_{max}/K_m of L_1 and L_2 on *o*-dianisidine were not significantly different, but that of L_2 on guaiacol was 70 times higher than that of L_1. Thus, L_2 was catalytically more active on guaiacol than L_1.

It was noted that highest laccase production by *P. florida* occurred in optimum growth medium (Fig. 3). The changes of medium composition were also found to influence both laccase and biomass production simultaneously (Fig. 3). The increase of laccase activity was largely due to higher L_2 production (Fig. 4). The possible relation of laccase production to mycelial growth was also reported previously (12, 16). The linkage between biomass yield and laccase production by *P. florida* was also further supported by the observation that laccase production per milligram of mycelium was significantly lower for different monokaryotic and dikaryotic putative LLP mutants than for the parent strain (Table 2). HLP mutants, however, showed about 20% higher laccase production, but not higher biomass production, than the parent.

All the culture filtrates of LLP strains showed a low guaiacol oxidizing (GX)/*o*-dianisidine oxidizing (ODX) activity ratio, with lowest being 1:20, whereas the ratio was 1:2 both in the parent strain and in HLP mutants. The ODX and GX activities of LLP strains were 29 to 65% and 2 to 16% lower, respectively, than those of the parent strain. Since L_2 was catalytically more active than these of L_1 on guaiacol, as indicated by higher V_{max}/K_m values and by activity staining after PAGE (Fig. 1), it was suggested that low biomass production might be related to low L_2 production by the LLP strains. Activity staining of LLP mutant Z_1 culture filtrate (Fig. 5) and separation of laccase activities of the same strain by ion exchange chromatography

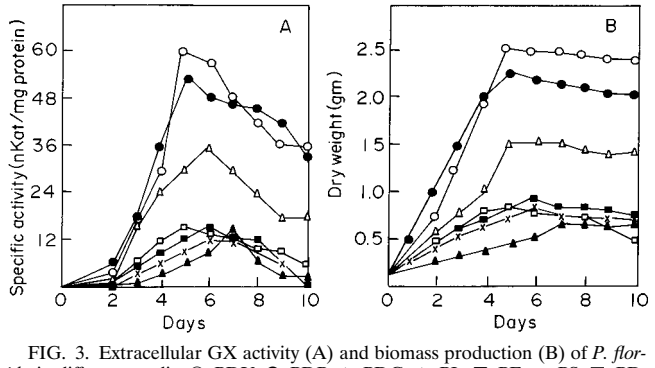

FIG. 3. Extracellular GX activity (A) and biomass production (B) of *P. florida* in different media. ○, PDY; ●, PDP; △, PDG; ▲, PL; ■, PF; ×, PS; □, PD.

TABLE 2. Laccase production and mycelial growth of mutant strains of *P. florida*

Strain	Nature[a]	Source[b]	Dry wt ± SD (g)	Laccase production (nkat/mg of mycelium)[c]		Sp act (nkat/mg of protein)[d]
				ODX activity	GX activity	
P. florida	D	C	0.750 ± 0.008	1.8	0.98	16.20
Z_1	D	P	0.282 ± 0.012	1.17	0.16	2.13
Z_6	M	P	0.385 ± 0.022	1.14	0.15	2.09
Ng	D	P	0.288 ± 0.010	1.05	0.12	1.05
N_2	M	B	0.298 ± 0.016	1.05	0.13	0.42
Z_{16}	M	B	0.225 ± 0.021	1.05	0.12	0.84
Ne_1	M	B	0.223 ± 0.029	1.01	0.10	0.43
N_7	D	P	0.204 ± 0.018	0.77	0.07	0.85
N_5	M	P	0.210 ± 0.006	0.64	0.05	0.64
Z_{10}	D	P	0.219 ± 0.011	0.54	0.03	0.42
Z_5	M	B	0.210 ± 0.013	0.54	0.03	0.43
Ne_7	D	P	0.210 ± 0.038	0.44	0.02	0.35

[a] D, dikaryotic; M, monokaryotic.
[b] C, control parent strain; B, strains isolated from basidiospores; P, strains isolated from protoplasts.
[c] ODX and GX activity was measured with 0.3 mM *o*-dianisidine and 10 mM guaiacol, respectively.
[d] Activity was measured with guaiacol.

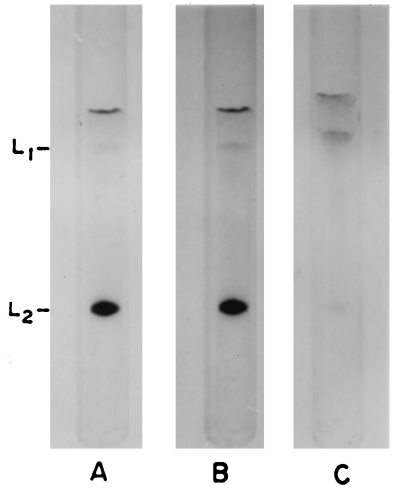

FIG. 5. Activity staining of culture filtrate of LLP and HLP strains. The electrophorized gels were stained with *o*-dianisidine. Lanes: A, control; B, HLP; C, LLP.

(Fig. 2) supported the association of L_2 production with biomass yield. However, no significant changes in band patterns or intensity of bands were noted for HLP mutants (Fig. 5).

Among the 500 colonies of the mutagenized Z_1 population, 35 revertants were screened. Five of the revertants studied were found to regain both laccase-producing capacity (70 to 88% of the parent) and biomass production with an increase in the intensity of L_2 bands (data not shown).

Laccase was reported to determine the fruiting efficiency of *Schizophyllum commune* (6). With *P. florida* it was observed that the dikaryotic LLP strain Z_1 did not produce fruit bodies due to inefficient colonization on the substrate. Normal mycelial growth on the substrate and fruit body production were found to be regained by the revertants of strain Z_1 (data not shown).

All of the evidence tentatively suggests that the L_2 enzyme of *P. florida* is involved in the regulation of mycelial growth of the mushroom.

This work was supported by a grant from the Department of Science and Technology, West Bengal, India.

REFERENCES

1. **Ander, P., and K. E. Eriksson.** 1976. The importance of phenol oxidase activity in lignin degradation by the white rot fungus *Sporotrichum pulverulentum*. Arch. Microbiol. **109:**1–8.
2. **Bollag, J.-M., K. L. Shuttleworth, and D. H. Anderson.** 1988. Laccase-mediated detoxification of phenolic compounds. Appl. Environ. Microbiol. **54:**3086–3091.
3. **Bourbonnais, R., and M. G. Paice.** 1988. Veratryl alcohol oxidases from the lignin-degrading basidiomycete *Pleurotus sajor-caju*. Biochem. J. **255:**445–450.
4. **Cai, W., R. Martin, B. Lomaure, J. L. Leuba, and V. Petiard.** 1993. Hydroxyindoles: a new class of laccase substrates. Plant Physiol. Biochem. **31:**441–445.
5. **Das, N., and M. Mukherjee.** 1996. Preparation and regeneration of mycelial protoplasts of *Pleurotus florida* and *Pleurotus ostreatus*. Folia Microbiol. **41:** 208–210.
6. **De Vries, O. M. H., W. H. C. F. Kooistra, and J. G. H. Wessel.** 1986. Formation of an extracellular laccase by a *Schizophyllum commune* dikaryon. J. Gen. Microbiol. **132:**2817–2826.
7. **Faure, D., M.-L. Bouillant, and R. Bally.** 1995. Comparative study of substrates and inhibitors of *Azospirillum lipoferum* and *Pyricularia oryzae* laccases. Appl. Environ. Microbiol. **61:**1144–1146.
8. **Fiegel, T. W., V. Meevootisom, and S. Kiatpapan.** 1982. Indication of ligninolysis by *Trichoderma* strains isolated from soil during simultaneous screening for fungi with cellulase and laccase activity. J. Ferment. Technol. **60:**473–475.
9. **Galliano, H., G. Gas, J. L. Sevis, and A. M. Boudet.** 1991. Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two enzymes: Mn peroxidase and laccase. Enzyme Microb. Technol. **13:**478–482.
10. **Giardina, P., R. Cannio, L. Martirani, L. Marzullo, G. Palmieri, and G. Sannia.** 1995. Cloning and sequencing of a laccase gene from the lignin-degrading basidiomycete *Pleurotus ostreatus*. Appl. Environ. Microbiol. **61:** 2408–2413.
11. **Hosoya, T.** 1960. Turnip peroxidase. IV. The effect of pH and temperature upon the rate of reaction. J. Biochem. (Tokyo) **48:**178–189.
12. **Matcham, S. E., B. R. Jordon, and D. A. Wood.** 1985. Estimation of fungal biomass in a solid substrate by three independent methods. Appl. Microbiol. Biotechnol. **21:**108–112.
13. **Mayer, A. M.** 1987. Polyphenol oxidases in plants—recent progress. Phytochemistry **26:**11–20.
14. **Mliki, A., and W. Zimmermann.** 1992. Purification and characterization of an intracellular peroxidase from *Streptomyces cyaneus*. Appl. Environ. Microbiol. **58:**916–919.
15. **Palmieri, G., P. Giardina, L. Marzullo, B. Desiderio, B. Nitti, R. Cannio, and G. Sannia.** 1993. Stability and activity of phenol oxidase from ligninolytic fungus *Pleurotus ostreatus*. Appl. Microbiol. Biotechnol. **39:**632–636.
16. **Prillinger, H., and K. Esser.** 1977. The phenoloxidases of the ascomycete *Podospora anserina*. XII. Action and interaction of gene controlling the formation of laccase. Mol. Gen. Genet. **156:**333–346.
17. **Rigling, D., and N. K. VanAlfen.** 1991. Regulation of laccase biosynthesis in the plant-pathogenic fungus *Cryphonectria parasitica* by double-stranded RNA. J. Bacteriol. **173:**8000–8003.
18. **Sannia, G., G. Giardina, P. Luna, M. Rossi, and V. Buonocore.** 1986. Laccases from *Pleurotus ostreatus*. Biotechnol. Lett. **8:**797–800.
19. **Thurston, C. F.** 1994. The structure and function of fungal laccases. Microbiology **140:**19–26.
20. **Vaiterbo, A., B. Yagen, and A. M. Mayer.** 1993. Cucurbitacins, attack enzymes and laccase in *Botrytis cinerea*. Phytochemistry **32:**61–65.
21. **Xu, F.** 1996. Oxidation of phenols, anilines and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. Biochemistry **35:**7608–7614.
22. **Youn, H. D., K. J. Kim, J. S. Maeng, Y. H. Han, I. B. Jeong, G. Jeong, S. O. Kang, and Y. C. Hah.** 1995. Single electron transfer by an extracellular laccase from the white rot fungus *Pleurotus ostreatus*. Microbiology **141:**393–398.