Folia Microbiol. **48** (6), 775–779 (2003) **http://www.biomed.cas.cz/mbu/folia/**

Decolorization of Orange G by *Pleurotus ostreatus* Monokaryotic Isolates with Different Laccase Activity

I. EICHLEROVÁ, L. HOMOLKA, F. NERUD

Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Prague, Czechia
e-mail eichler@biomed.cas.cz

Received 20 August 2003
Revised version 4 December 2003

ABSTRACT. The effect of enhanced laccase (Lac) activity (obtained after copper addition to cultivation media) on decolorization of azo dye Orange G in two basidiospore-derived monokaryotic isolates of *Pleurotus ostreatus* was determined. The high Lac-producing isolate efficiently decolorized Orange G. The low-producing isolate showed only poor decolorization ability during cultivation in liquid medium and no decolorization on agar plates containing Orange G after a 25-d growth. A substantial enhancement of Lac activity caused by copper addition into cultivation media was detected in both isolates but, at the same time, the biomass production decreased and decolorization rate was reduced.

During the past decade, white-rot fungi were studied in relation to their ability to degrade recalcitrant organopollutants (Šašek *et al*. 1998; Novotný *et al*. 1999, 2000; Rodrígues *et al*. 1999; Yuxing and Yu 1999; Bhatt *et al*. 2000; Sam and Yeşilada 2001; Couto *et al*. 2002). They use a highly nonspecific, free-radical-mediated process, requiring enzymes able to degrade lignin and structurally related compounds (Chagas and Durrant 2001). Among such compounds there are many synthetic dyes that are very resistant to degradation. Azo dyes characterized by the presence of one or more azo groups (–N=N–) represent the largest group of synthetic organic dyes.

Laccase (EC 1.10.3.2; benzenediol:oxygen oxidoreductase; Lac) is an extracellular lignin-modifying enzyme produced by almost all white-rot fungi (Pérez *et al*. 1996) which plays an important role in degradation processes. It is a copper-containing oxidase that catalyzes oxidation of methoxy-substituted monophenols, *ortho*- and *para*-diphenols, aromatic amines, syringaldazine and nonphenolic compounds such as ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (Thurston 1994; Yaropolov *et al*. 1994). Recently, potential biotechnological application of fungal laccases has been broadly studied, *e.g.*, biodegradation of environmental pollutants (*e.g*. oligomembered ring-fused aromates,'polycyclic' aromatic hydrocarbons) and textile dyes, stain bleaching, bioconversion of lignin, biobleaching and biopulping of wood chips or delignification of agricultural plant residues (Orth *et al*. 1993; Hölker *et al*. 2002). Sufficient production and secretion of these enzymes is, among other things, necessary to ensure the efficiency of the process. Enhancement of Lac activity can be achieved also by the addition of different inducers – copper, manganese, ABTS, 4-methoxyaniline ('*p*-anisidine'), calcium chlorate, ethanol, *etc*. – into the cultivation media (Mai *et al*. 2000; Palmieri *et al*. 2000; Arora and Gill 2001; Crowe and Olsson 2001; Klonowska *et al*. 2001).

The aim of this study was to determine the role of Lac activity in decolorization of the synthetic azo-dye Orange G (OG) using two *Pleurotus ostreatus* basidiospore-derived isolates. We tested the influence of copper addition on Lac activity and on mycelial growth rate, biomass production, and decolorization capacity. The isolates used (monokaryotic isolates, prepared in our laboratory; Eichlerová and Homolka 1999), markedly differing in production of ligninolytic enzymes, especially in Lac, were more stable than almost all dikaryotic ones.

MATERIAL AND METHODS

Organism. Pleurotus ostreatus cv. Florida f6 (Block *et al.* 1959) was obtained from the *Department of Plant Pathology and Microbiology* (*Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem*, Israel); monokaryotic isolates 27 and 29 were prepared by monosporic isolation of basidiospores obtained after fructification of the parental strain *P. ostreatus* Florida f6 (Eichlerová and Homolka 1999). All strains were maintained by serial transfers and kept on wort agar slants at 4 °C. The organisms were repeatedly tested for the ability to keep their basic properties during several last years and are considered to be stable.

Laccase and manganese peroxidase assay. Static cultivation was carried out in 100-mL Erlenmeyer flasks with 20 mL of N-limited Kirk medium (Tien and Kirk 1988) containing OG at a final concentration of 200 mg/L (Kirk-OG) or supplemented with $CuSO_4$ at a final concentration of 300 μmol/L (Kirk-OG-Cu). The flasks were inoculated with two wort agar plugs (diameter 10 mm) cut from the actively growing part of a colony on a Petri dish and incubated at 27 °C. Enzyme activity was measured in filtrates from 4 parallel flasks detained after mycelia removal. The mycelia were used for dry mass determination. Activity of extracellular Lac and manganese peroxidase (MnP) were determined spectrophotometrically by monitoring the absorbance increase at 425 nm (Lac) or 590 nm (MnP) in medium filtrates. Lac activity was determined according to Bourbonnais and Paice (1990) by monitoring the oxidation of ABTS. Determination of MnP activity using MBTH (3-methyl-2-benzothiazolinone hydrazone) and DMAB (3-dimethylaminobenzoic acid) was based on the method of Ngo and Lenhoff (1980) modified according to Daniel *et al*. (1994). All measurements were done in quadruplicate and repeated three times.

Decolorization assays. One hundred-mL Erlenmeyer flasks with 20 mL of Kirk-OG (or with 20 mL of Kirk-OG-Cu) were inoculated in the same way as for enzyme assay. The systems without fungus served as an abiotic control. The dye sorption effect of mycelia during the decolorization process was determined using the biotic control according to Wang and Yu (1998). Decolorization of the liquid medium was measured in the filtrates (4 parallel flasks) after removing the mycelia and absorbance monitored spectrophotometrically at the maximum visible wavelength (478 nm). Decolorization activity was also tested on Petri dishes (diameter 90 mm) with solid Kirk-OG and solid Kirk-OG-Cu. Plates (4 parallels) were inoculated with mycelial plugs (diameter 3 mm) cut from actively growing mycelia. Radial growth and the zone of color change on the agar plates were measured daily. All measurements were repeated three times.

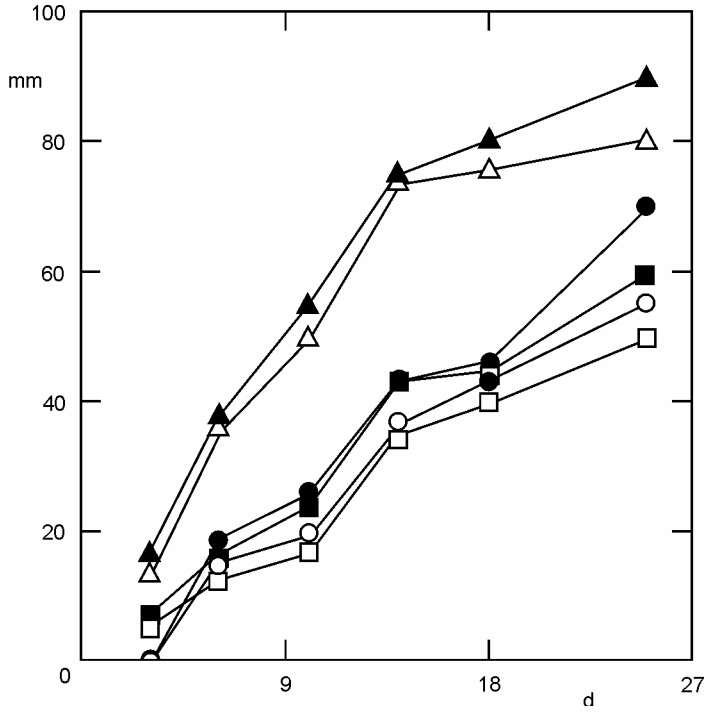

Fig. 1. Decolorization and radial growth rate of *Pleurotus ostreatus* isolates on solid media; *closed symbols* – Kirk-Orange G medium, *empty symbols* – Kirk-Orange G-Cu medium; radial growth rate in isolate 29 (*squares*) and 27 (*triangles*; for both diameter of mycelial colonies in mm), *circles* – decolorization by isolate 29 (diameter of decolorized zone in mm).

RESULTS AND DISCUSSION

In order to characterize in more detail the influence of Lac activity on the decolorization of OG, $CuSO_4$ was used as an efficient inducer of Lac activity in two monokaryotic basidiospore-derived *Pleurotus ostreatus* isolates.

A substantial increase of Lac activity (2-fold and 1.3-fold in isolate 27 and 29, respectively) was detected in liquid Kirk medium with 300 μmol/L $CuSO_4$ (Table I). After the copper treatment, the isolate 27 showed a remarkably lower Lac activity than isolate 29; the MnP activity was not influenced by the presence of copper. Isolate 29 showed slightly higher MnP activity than isolate 27 on both Kirk-OG and Kirk-OG-Cu.

Table I. Activity (pkat/L) of Lac (*first columns*) and MnP (*second columns*) during a 3–14-d cultivation (3, 6, 10 and 14 d) of *Pleurotus ostreatus* in liquid media[a]

Strain	Medium[a]	3		6		10		14	
27	KOG	0.17 ± 0.05	0.18 ± 0.04	0.67 ± 0.08	0.19 ± 0.06	0.41 ± 0.05	0.30 ± 0.05	0.33 ± 0.06	0.29 ± 0.04
	KOGCu	0.50 ± 0.09	0.15 ± 0.01	1.00 ± 0.14	0.20 ± 0.04	1.30 ± 0.12	0.32 ± 0.06	0.50 ± 0.08	0.33 ± 0.02
29	KOG	176 ± 30.3	0.26 ± 0.05	346 ± 56.2	0.30 ± 0.08	441 ± 45.2	0.48 ± 0.08	261 ± 42.5	0.44 ± 0.09
	KOGCu	201 ± 30.7	0.24 ± 0.06	586 ± 90.2	0.30 ± 0.07	580 ± 62.5	0.47 ± 0.10	455 ± 58.8	0.46 ± 0.09

[a]KOG – N-limited Kirk medium (Tien and Kirk 1988) containing Orange G at a final concentration of 200 mg/L, KOGCu – KOG supplemented with $CuSO_4$ at a final concentration of 300 µmol/L.

Table II. Decolorization (% of initial amount; *first columns*) of Orange G and biomass production (g/L; *second columns*) in liquid media[a]

Strain	Medium	3		6		10		14	
27	KOG	6.3 ± 0.95	1.37 ± 0.16	15.3 ± 2.25	1.75 ± 0.41	15.6 ± 2.59	1.58 ± 0.22	37.5 ± 7.22	2.17 ± 0.44
	KOGCu	0	1.23 ± 0.11	10.0 ± 1.54	1.57 ± 0.30	15.0 ± 1.75	1.43 ± 0.16	39.1 ± 7.82	1.78 ± 0.28
29	KOG	15.3 ± 3.06	1.98 ± 0.24	90.5 ± 12.0	3.20 ± 0.55	95.3 ± 11.4	3.48 ± 0.88	96.5 ± 11.0	5.17 ± 0.79
	KOGCu	4.23 ± 0.49	1.78 ± 0.16	82.2 ± 9.92	2.80 ± 0.68	92.0 ± 14.1	2.60 ± 0.65	98.9 ± 14.5	4.73 ± 0.90

[a]For further details *see* Table I.

Isolate 29 rapidly decolorized OG, while isolate 27 decolorized the dye only partly on the medium without $CuSO_4$. Although the Lac activity increased substantially in both isolates after the addition of inducer (Table I) the decolorization was to some extent inhibited and in the first 10 d of cultivation both isolates decolorized the dye more slowly than on the medium without copper (Table II). The decrease of biomass production can be explained by the copper treatment. $CuSO_4$ inhibited the biomass production of both isolates. Nevertheless, isolate 29 showed a higher biomass production than isolate 27.

The physical adsorption of the dye on the mycelia was followed during the cultivation process. The sorption effect was insignificant for 10 d of cultivation. However, after 14 d, 5–10 or 8–15 % of the initial concentration of the dye was removed by mycelial sorption in isolate 27 and 29, respectively. Biomass production decrease on the copper-supplemented media brought about only negligible decrease of the sorption capacity of the mycelia.

The inhibition of radial growth and of the decolorization after addition of copper was apparent also in the experiment on agar plates (Fig. 1). No decolorized zone or decolorized spots were found in isolate 27 either with or without addition of copper. Isolate 29 decolorized OG on agar plates quite easily but, in the presence of $CuSO_4$, with a simultaneous decrease of the decolorization rate. Interestingly, isolate 27 exhibited during all measurements a higher radial growth rate than isolate 29 while the biomass production in liquid medium was higher in isolate 29. Moreover, observation of colony and hypha appearance showed that the density and character of mycelia was quite different in the two isolates (thin long hyphae and sparse mycelium in isolate 27 *vs.* mycelium with very high density of hyphae and with abundant air mycelia in isolate 29), which could explain the above differences in biomass production and radial growth.

Our results confirm that Lac plays a significant role in the decolorization of OG by isolates derived from *P. ostreatus*; nevertheless the process is influenced also by many other factors, *e.g.*, different secondary metabolites, radicals, superoxides

and other oxidizing agents present (Kotterman *et al*. 1996; Kapich *et al*. 1999; Tanaka *et al*. 1999). Biodegradative ability of white-rot fungi is generally assumed to be associated with the production of extracellular ligninolytic enzymes but in our previous work we did not find any positive correlation between higher production of ligninolytic enzyme and more efficient degradation of dyes or other pollutants (Homolka *et al*. 1997; Eichlerová *et al*. 2000). Our present work showed that insufficient secretion of Lac in the lower Lac-producing isolate correlates with the low decolorization ability while the higher Lac-producing isolate decolorizes the dye more efficiently. However, a further enhancement of the enzyme activity did not result in a higher decolorization ability. The decolorization efficiency was strongly influenced by the total yield of biomass produced by individual strains which corresponds to our previous findings (Eichlerová *et al*. 2002) that the poor decolorization ability of strains with low secretion of ligninolytic enzymes was substantially improved by the enhancement of their biomass production. The inhibition of biomass production in liquid media was accompanied by a decrease of decolorization ability even in the Lac higher-producing isolate which, on the other hand, confirms the positive correlation between the biomass production and the decolorization capacity.

This work was supported by grant no. 206/02/D119 from the *Grant Agency of the Czech Republic* and by *Institutional Research Concept* no. AV 0Z 502 0903.

REFERENCES

ARORA D.S., GILL P.K.: Effect of various media and supplements on laccase production by some white rot fungi. *Biores.Technol.* **77**, 89–91 (2001).

BALDRIAN P., GABRIEL J.: Variability of laccase activity in the white-rot basidiomycete *Pleurotus ostreatus. Folia Microbiol.* **47**, 385–390 (2002).

BHATT M., PATEL M., RAWAL B., NOVOTNÝ Č., MOLITORIS H.P., ŠAŠEK V.: Biological decolorization of the synthetic dye RBBR in contaminated soil. *World J.Microbiol.Biotechnol.* **16**, 195-198 (2000).

BLOCK S.S, TSAO G., HAN L.: Experiments in the cultivation of *Pleurotus ostreatus*. *Mushroom Sci*. **4**, 309–325 (1959).

BOURBONNAIS R., PAICE M.G.: Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. *FEBS Lett.* **267**, 99–102 (1990).

CHAGAS E.P., DURRANT L.R.: Decolorization of azo dyes by *Phanerochaete chrysosporium* and *Pleurotus sajorcaju*. *Enzyme Microbiol.Technol.* **29**, 473–477 (2001).

COUTO S.R., DOMÍNGUES A., SANROMÁN Á.: Production of manganese-dependent peroxidase in a new solid-state bioreactor by *Phanerochaete chrysosporium* grown on wood shavings. Application to the decolorization of synthetic dyes. *Folia Microbiol.* **47**, 417–421 (2002).

CROWE J.D., OLSON S.: Induction of laccase activity in *Rhizoctonia solani* by antagonistic *Pseudomonas fluorescens* strains and a range of chemical treatments. *Appl.Environ.Microbiol.* **67**, 2088–2094 (2001).

DANIEL G., VOLC J., KUBÁTOVÁ E.: Pyranose oxidase, a major source of H_2O_2 during wood degradation by *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Oudemansiella mucida*. *Appl.Environ.Microbiol*. **60**, 2524–2532 (1994).

EICHLEROVÁ I., HOMOLKA L.: Preparation and crossing of basidiospore-derived monokaryons – a useful tool for obtaining laccase and other ligninolytic enzyme higher-producing dikaryotic strains of *Pleurotus ostreatus*. *Antonie van Leeuwenhoek* **75**, 321–327 (1999).

EICHLEROVÁ I., HOMOLKA L., NERUD F., ZADRAZIL F., BALDRIAN P., GABRIEL J.: Screening of *Pleurotus ostreatus* isolates for their ligninolytic properties during cultivation on natural substrates. *Biodegradation* **11**, 279–287 (2000).

EICHLEROVÁ I., HOMOLKA L., NERUD F.: Decolorization of synthetic dyes by *Pleurotus ostreatus* isolates differing in ligninolytic properties. *Folia Microbiol.* **47**, 691–695 (2002).

HOMOLKA L., PALTIEL J., VOLÁKOVÁ I., NERUD F., HADAR Y.: The effect of growth conditions and genetic background of laccase production by the fungus *Pleurotus ostreatus*. *Folia Microbiol.* **42**, 527–529 (1997).

HÖLKER U., DOSHE J., HÖFER M.: Extracellular laccases in ascomycetes *Trichoderma atroviride* and *Trichoderma harzianum*. *Folia Microbiol.* **47**, 423–427 (2002).

KAPICH A.N., JENSEN K.A., HAMMEL K.E.: Peroxyl radicals are potential agents of lignin biodegradation. *FEBS Lett.* **461**, 115–119 (1999).

KLONOWSKA A., LE-PETIT J., TRON T.: Enhancement of minor laccases production in the basidiomycete *Marasmius quercophilus* C30. *FEMS Microbiol.Lett.* **200**, 25–30 (2001).

KOTTERMAN M.J.J., WASSEVELD R.A., FIELD J.A.: Hydrogen peroxide production as a limiting factor in xenobiotic coumpons oxidation by nitrogen-sufficient cultures of *Bjerkandera* sp. strain BOS55 overproducing peroxidases. *Appl.Environ.Microbiol*. **62**, 880–885 (1996).

MAI C., SCHORMANN W., MILSTEIN O., HUTTERMANN A.: Enhanced stability of laccase in the presence of phenolic compounds. *Appl. Microbiol.Biotechnol.* **54**, 510–514 (2000).

NGO T.T., LENHOFF H.M.: A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. *Anal. Biochem*. **105**, 389–397 (1980).

NOVOTNÝ Č., ERBANOVÁ P., ŠAŠEK V., KUBÁTOVÁ A., CAJTHAML T., LANG E., KRAHL J., ZADRAZIL F.: Extracellular oxidative enzyme production and PAH removal in soil by exploratory mycelium of white rot fungi. *Biodegradation* **10**, 159–168 (1999).

NOVOTNÝ Č., ERBANOVÁ P., CAJTHAML T., ROTHSCHILD N., DOSORETZ C., ŠAŠEK V.: *Irpex lacteus*, a white rot fungus applicable to water and soil bioremediation. *Appl.Microbiol.Biotechnol*. **54**, 850–853 (2000).

ORTH A.B., ROYSE D.J., TIEN M.: Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. *Appl.Environ. Microbiol.* **59**, 4017–4023 (1993).

PALMIERI G., GIARDINA P., BIANCO C., FONTANELLA B., SANNIA G.: Copper induction of laccase in the ligninolytic fungus *Pleurotus ostreatus*. *Appl.Environ.Microbiol.* **66**, 920–924 (2000).

PÉREZ J., MARTÍNEZ J., DE LA RUBIA T.: Purification and parcial characterization of a laccase from the white rot fungus *Phanerochaete flavido-alba. Appl.Environ.Microbiol.* **62**, 4263–4267 (1996).

RODRÍGUES E., PICKARD A., VAZQUEZ-DUHALT R.: Industrial dye decolorization by laccases from ligninolytic fungi. *Curr.Microbiol.* **38**, 27–32 (1999).

SAM M., YEŞILADA O.: Decolorization of Orange II dye by white-rot fungi. *Folia Microbiol.* **46**, 143–145 (2001).

ŠAŠEK V., NOVOTNÝ Č., VAMPOLA P.: Screening for efficient organopollutant fungal degraders by decolorization. *Czech Mycol.* **50**, 303–311(1998).

TANAKA H., ITAKURA S., ENOKI A.: Hydroxyl radical generation by an extracellular low-molecular-weight substance and phenol oxidase activity during wood degradation by the white-rot basidiomycete *Phanerochaete chrysosporium*. *Holzforschung* **53**, 21–28 (1999).

THURSTON F.: The structure and function of fungal laccases. *Microbiology* **140**, 19–26 (1994).

TIEN M., KIRK T.K.: Lignin peroxidase of *Phanerochaete chrysosporium*. *Meth.Enzymol.* **161B**, 238–248 (1988).

WANG Y., YU J.: Adsorption and degradation of synthetic dyes on the mycelium of *Trametes versicolor*. *Water Sci.Technol*. **38**, 233–238 (1998).

YAROPOLOV A.I., SKOROBOGATKO O.V., VARTANOV S.S., VARFOLOMEYEV S.D.: Laccase: properties, catalytic mechanism and applicability. *Appl.Biochem.Biotechnol.* **49**, 257–279 (1994).

YUXING W., YU J.: Laccase-catalyzed decolorization of synthetic dyes. *Water Res.* **33**, 3512–3520 (1999).