COMPARATIVE CHARACTERIZATION OF SOME FUNGAL LACCASES

E. SAJBEN-NAGY (1), L. MANCZINGER (1), B. ŠKRBIĆ (2), N. ĐURIŠIĆ-MLADENOVIĆ (2), CS. VÁGVÖLGYI (1)

(1) University of Szeged, Faculty of Science and Informatics
Department of Microbiology
Közép fasor 52, H-6726 Szeged, Hungary

(2) University of Novi Sad, Faculty of Technology
Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
sajben@gmail.com

ABSTRACT
Laccase producing fungi were isolated from environmental samples. They were identified on the basis of ITS (Internal Transcribed Spacer) sequence analysis. The laccase production of the isolates were investigated and compared with those of other strains deriving from the Szeged Microbiological Collection and from mushroom producer's spawn. The enzyme production were examined in various (e.g. basic, mineral and inducer containing) liquid media. The pH optimum determinations for laccase activities were carried out in cell free ferment broths, at pH 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7 and 8, applying 25 mM succinate buffer. The investigations were based on ABTS [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate)] substrate measurement method. The results showed that under these conditions, among the ascomycetes strains investigated the best laccase producer was a *Rhizoctonia solani* isolate (the SzMC 6252J strain). Among the basidiomycetes tested, the best laccase producer was a *Ganoderma* isolate (HM3).

Keywords: bioremediation, laccase, pH optimum determination

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are produced in many fungal species belonging to ascomycetes and basidiomycetes groups, accordingly, the enzyme has already been purified from various fungal species. Laccases are typical for the wood-rotting basidiomycetes and a related group of litter-decomposing saprotrophic fungi. They have received much attention from researchers during the past decades because of their capability to oxidize wide range of toxic and environmentally harmful substrates including chlorinated phenolics. Presence of these substances even at very low concentrations in waters and agricultural soils creates substantial danger: they and their degradation products could be mutagenic, carcinogenic and teratogenic. In certain cases, the degradation products may pose greater risks than parent compounds (WEI et al., 2011). Laccases utilize board range of substrates, so they could oxidize various xenobiotics and their degradation products. Moreover, they could react with different redox mediators such as ABTS, methionine, phenol and syringaldehyde. These are low molecular weight compounds which could act as redox mediators after oxidization and could oxidize other molecules expanding the spectrum of laccases (CAMARERO et al., 2004). Up to now, more than 100 laccases have been purified and more or less characterized from fungi. The molecular weight of a typical fungal laccase is about 60-70 kDa, and they have around pH 4.0 isoelectric point. Several laccase isoenzymes have been detected in many fungal species (BALDRIAN, 2005), for example *Pleurotus ostreatus* have at least 10 of them (TÉLLEZ-TÉLLEZ et al., 2005). Like most fungal extracellular enzymes, laccases are glycoproteins. The glycosylation ranges are between 10% and 25%. (SHLEEV et al., 2004). Fungal

laccases have pH optimum in the lower pH range. In the case of ABTS substrate the pH optimum is generally lower than 4.0 (BALDRIAN, 2005).
In this study, we isolated new laccase producer Ascomycota and Basidiomycota fungi from environmental samples and we compared their enzyme producing ability under different culture conditions with other known laccase positive strains.

MATERIAL AND METHOD

Strains and conditions

Fungal strains

DP1 (*Ganoderma sp*.), DP2 (*Ganoderma sp*.), DP3 (*Ganoderma sp*.), HM3 (*Ganoderma sp*.) and Lac22, were isolated from environmental samples; HK35 (*Pleurotus ostreatus*), deriving from a mushroom producer; SzMC 6244J (*Botrytis cinerea*) and SzMC 6252J (*Rhizoctonia solani*) from the Szeged Microbiological Collection. The strains were maintained on malt extract agar (MEA), one liter containing 20 g malt extract, 20 g glucose, 1 g peptone and 20 g agar.

Isolation and identification of laccase producers from environmental samples

For the isolation of laccase producer white-rot fungi one liter of basal medium (BM) contained: 0.5 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NH_4NO_3, 0.1 g KCl, 0.02 g $FeSO_4$, 0.05 g $Ca(NO_3)_2.4H_2O$, 2 g malt extract, 15 g agar. After sterilization 0.4 ml guaiacol was added for indicating the laccase activity. For the isolation of laccase producers from air the isolation medium (LI) was the following for 1 liter: 10 ml glicerol, 1 g arginine, 1 g KH_2PO_4, 1 g K_2HPO_4, 1 g $MgSO_4.7H_2O$, 0.002 g $FeSO_4$, 0.002 $ZnSO_4$ and 0.002 $CuSO_4$. The identification was carried out by sequence analysis of ITS (Internal Transcribed Spacer) region, using ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') universal primers (WHITE et al., 1990). We searched for homologies using NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST, ALTSCHUL et al., 1990).

The production of laccases

The production of laccases was investigated in different liquid media. ME contained 20 g malt extract, 20 g glucose, 1 g peptone per liter. MEM liquid media: one liter of ME base media supplemented with 80 µl of minerals (1.0 g $CaCl_2 \cdot 2H_2O$, 1.0 g $FeSO_4 \cdot 7H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, and 1.0 g Na_2EDTA per liter). MEMX media contained in addition to MEM, 1% xylan as indicator. Fifty milliliters of liquid media were inoculated with agar pieces cut from well-grown mycelium. After 6 days of incubation at 28 °C (180 rpm), the samples were centrifuged at 8000g for 10 minutes. The tenfold dilution of the cell free ferment broths served as a basis for the activity assays.

The optimum pH values

The optimum pH values of the secreted laccases were measured at pH 3.5, 4, 4.5, 5, 5.5, 6, 6.5,7 and 8, applying 25mM succinate buffer from cell free tenfold diluted supernatants. In the experiments we used 5mM ABTS [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate)] as substrate, and we measured the OD at 436 nm wavelength after 5 minutes of incubation (KIISKINEN ET AL., 2002 and 2004).

RESULTS

Isolation and identification of laccase producers

We isolated 4 promising laccase positive Basidiomycota strains, DP1, DP2, DP3 and HM3 on BM media, all of them were identified as *Ganoderma sp*. on the basis of their ITS sequence analysis. On LI media only one applicable laccase producer, Lac22 Ascomycota fungus was isolated. Both strains could grow under laboratory conditions, at 25 °C.

Effect of liquid media to the laccase production

Both fungi could grow in liquid media, ME, MEM and MEMX. The ME basic media was the best for laccase production in the most cases at pH 4.5. DP3 (*Ganoderma sp*.), HK35 (*P. ostreatus*), SzMC 6252J (*R. solani*) and SzMC 6244J (*B. cinerea*) showed the highest activity under this condition. The MEM, (ME supplemented with minerals) inhibited the production in several times, except at DP1 (*Ganoderma sp*.), HM3 (*Ganoderma sp*.) and Lac22, when increased activities were shown. The addition of xylan as inducer increased the activity in only one case, only at DP2 (*Ganoderma sp*.). Outstandingly promising producers were DP1, HM3 and HK35 among the basidiomycetes and SzMC 6252J from the ascomycetes (*Figure 1*).

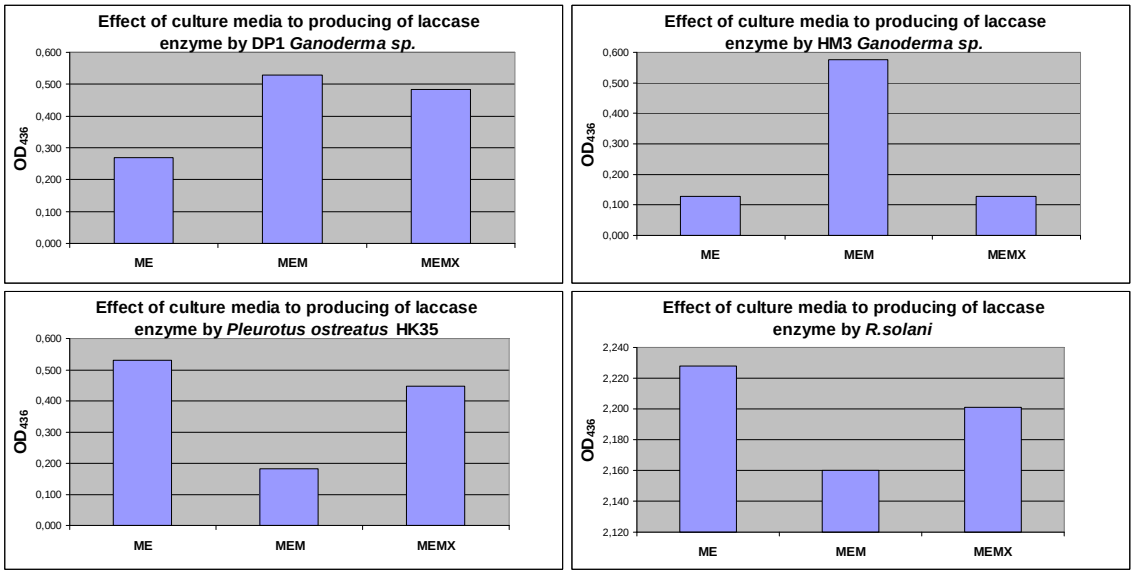

Figure 1: Enzyme activities of the best laccase producer fungi using different liquid media.

Comparison of the pH optimums: On the basis of the experiments carried out with 5 mM ABTS in 25 mM succinate buffer we could conclude that the optimal pH for all of the produced laccases is under pH 4.0. We measured high activities at pH 3.5. The activities dramatically decreased if we increased the pH. Over pH 6.0 much reduced activities occurred, except SzMC 6252J which is a *R. solani* strain, where we could measure activity at pH 7.0 also. It should be mentioned that this strain cannot be really compared to the others, because of its outstandingly high laccase activity (*Figure 2*).

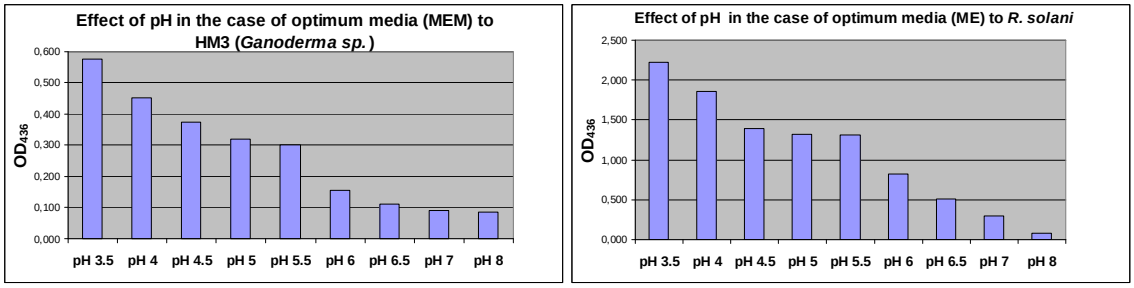

Figure 2: pH dependence of the laccase activities in the ferment broths of HM3 *Ganoderma sp.* and SzMC 6252J *Rhizoctonia solani* strains.

CONCLUSIONS

The results of the investigations on culture media dependence of laccase production showed that the applied inducers mostly decreased the laccase production, but there are some exceptions. For example the addition of minerals increased the activity in case of DP1, HM3 and Lac 22 isolates. The xylan reduced the productivity in all cases. The comparison of the pH dependence curves showed in all cases that the increasing pH decreased the activity, as was expected on the basis of the former work of BALDRIAN, 2006. On the basis of our results we could conclude that the best laccase producer among the investigated strains was the *R. solani* SzMC 6252J. The genus is already known about the good laccase producing ability (BOLLAG et al. 1988; CROWE and OLSSON, 2001). For bioremediation aims, this seems to be the best laccase, because it retains significant activity at higher pH values, which is essential for *in situ* soil treatments.

ACKNOWLEDGEMENTS

The project is co-financed by the European Union through the Hungary-Serbia IPA Cross-border Co-operation Programme (LACREMED, **HUSRB/1002/214/147**).

REFERENCES

ALTSCHUL S.F., GISH W., MILLER W., MYERS E.W., LIPMAN D.J. (1990): Basic local alignment search tool. Journal of Molecular Biology **215,** 403-10.

BALDRIAN P. (2006): Fungal laccases – occurrence and properties. FEMS Microbiolological Review **30,** 215-242.

BOLLAG J.-M., SHUTTLEWORTH K. L., ANDERSON D. H. (1988): Laccase-mediated detoxification of phenolic compound. Applied and Environmental Microbiology 3086-3091.

CAMARERO S., IBARRA D., MARTÍNEZ M.J., MARTÍNEZ A.T. (2005): Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. Applied and Environmental Microbiology 1775-1784.

CROWE J.D, OLSSON S. (2001): Induction of laccase activity in *Rhizoctonia solani* by antagonistic *Pseudomonas fluorescens* strains and a range of chemical treatments. Applied and Environmental Microbiology 2088-2094

KIISKINEN L.-L., RÄTTÖ M., KRUUS K. (2004): Screening for novel laccase-producing microbes. Journal of Applied Microbiology **97,** 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 **59,** 198-204.
SHLEEV S.V., MOROZOVA O., NIKITINA O., GORSHINA E.S., RUSINOVA T., SEREZHENKOV V.A., BURBAEV D.S., GAZARYAN I.G., YAROPOLOV A.I. (2004): Comparison of physico-chemical characteristics of four laccases from different basidiomycetes. Biochimie **86,** 693-703.
TÉLLEZ-TÉLLEZ M., SÁNCHEZ C., LOERA O., DÍAZ-GODÍNEZ G. (2005): Differential patterns of constitutive intracellular laccases of the vegetative phase of *Pleurotus* species. Biotechnology Letters **27,** 1391-1394.
WEI H-R, RHOADES M.G., SHEA P.J. (2011): Formation, adsorption, and stability of N-Nitrosoatrazine in water and soil. American Chemical Society. In: It's all in the water: studies of materials and conditions in fresh and salt water bodies; BENVENUTO M., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2011.
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.