JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

EFFECT OF POTENTIAL INDUCTORS ON LACCASE PRODUCTION BY WHITE-ROT FUNGUS *CERIPORIOPSIS SUBVERMISPORA*

Daniela Chmelová[1], Miroslav Ondrejovič[2]*

Address(es): RNDr. Daniela Chmelová;
[1]Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic.
[2]University of Ss. Cyril and Methodius, Faculty of Natural Sciences, Department of Biotechnology, Nám. J. Herdu 2, 917 01 Trnava, Slovak Republic.

*Corresponding author: daniela.chmelova@gmail.com

ARTICLE INFO

Received 17. 10. 2013
Revised 24. 11. 2013
Accepted 16. 12. 2013
Published 1. 2. 2014

Regular article

OPEN ACCESS

ABSTRACT

In this work, the influence of selected inorganic ions (Cu^{2+} and Mn^{2+}) and aromatic amino acids (tryptophan and tyrosine) on laccase production by white-rot fungus *Ceriporiopsis subvermispora* was investigated. This aim was realized by monitoring laccase production during cultivation of *C. subvermispora*. Secondary, we been evaluated glucose concentration in medium and biomass growth after cultivation. Extracellular laccase formation can stimulated by the addition of Cu^{2+} (3.0 mmol/L). The higher laccase activity reached maximum at 7th day (63 U/L), equivalent to 3.7-fold higher than the laccase production without copper (17.2 U/L). Higher concentration of copper ions had a negative effect on laccase production. The addition of copper ions inhibited the biomass growth. Mn^{2+} ions similarly stimulated laccase activity (3.0 and 7.0 mmol/L; 79.6 and 63.8 U/L, respectively) and maximum activities were reached at 6th day. Manganese ions also stimulated fungal biomass of *C. subvermispora*. The addition of aromatic amino acids did not cause an increase laccase production. The highest laccase production was observed in cultivation media without aromatic amino acids (16.0 U/L) at 8th day.

Keywords: *Ceriporiopsis subvermispora*, laccase, inductors

INTRODUCTION

Saprophytic microorganisms (bacteria and fungi) degrade lignin which is component of plant biomass, by the action of ligninolytic enzymes that are secreted from mycelium into the colonized material. Fungi produced ligninolytic enzymes can be classified into three groups: soft-rot, brown-rot and white-rot fungi. White-rot fungi are very selective for lignin degradation, causing high lignin losses with low utilization cellulose **(Blanchette *et al.*, 1992)**. Representative of this group is *Ceriporiopsis subvermispora*, which can preferentially degrade of lignin before cellulose and hemiceluloses. For degradation of lignin, *C. subvermispora* secreted mainly laccase and lesser manganese peroxidase **(Rüttimann *et al.*, 1992)**.
Laccase (EC 1.10.3.2) is extracellular multicopper oxidase which catalyzes the oxidation of several phenolic and non-phenolic compounds using molecular oxygen as electron acceptor **(Pezzella *et al.*, 2009)**. For industrial applications, its low substrate specificity is useful **(Mayer and Staples, 2002)**. In several microorganisms, laccase is constitutively produced in small amounts **(Bollag and Leonowicz, 1984)**, but more often is produced in response to environmental conditions **(Rodriguéz Couto *et al.*, 2006)**. Laccase production can be considerably enhanced by a variety of substances such as inorganic (copper or manganese) **(Palmieri *et al.*, 2000; Dekker *et al.*, 2007; Levin *et al.*, 2008)** and organic compounds (xylidine, aromatic alcohols and amino acids) **(Bollag and Leonowicz, 1984**; **Eggert *et al.*, 1996; Chmelová *et al.*, 2011)**. Copper is essentially microelement for growth of most microorganisms, but its higher concentration is toxic. Copper ions are necessary for laccase synthesis and are considered as laccase inductors **(Galhaup *et al.*, 2001)**. The presence of manganese ions is known to induce the production of MnP in many white-rot fungi, but presence of manganese ions in medium decreases production of lignin peroxidase **(Rothschild *et al.*, 1999)**. Similarly, the addition of aromatic alcohols or amino acids increased the secretion of ligninolytic enzymes by various strains of white-rot fungi **(Collins *et al.*, 1997; Levin *et al.*, 2008)**.
The aim of this study was to evaluate laccase production by white-rot fungus *C. subvermispora* and analyze effect of potential inductors (copper and manganese ions and aromatic amino acids such as tryptophan and tyrosine) on laccase production.

MATERIAL AND METHODS

Chemicals

K_2HPO_4. 12 H_2O p.a., KH_2PO_4 p.a., NaCl p.a., $CaCl_2$. 2 H_2O p.a., $ZnCl_2$ p.a., glucose p. a., $CuSO_4$. 5 H_2O p.a., $FeSO_4$. 7 H_2O p.a. and $MgSO_4$. 7 H_2O p.a. were obtained from Mikrochem (SK). $MnSO_4$. 4 H_2O p.a. was obtained from Slavus (SK). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) 98 %, casein hydrolysate and 3,5-dinitrosalicylic acid 98 % were obtained from Sigma Aldrich (G). Malt agar was obtained from Biomark (IN).

Microorganism

Culture of *Ceriporiopsis subvermispora* ATTC 90467 was provided from the Centraalburea voor Schimmelcultures (Netherlands). The culture was maintained on malt agar at 4 °C. In all cases, the suspension of fungal mycelium was prepared by scraping of plaque (1 cm^2) of the growth culture from agar plate using microbiological loop and mixing in sterile deionized water (10 mL).

Medium composition

The composition of basic mineral medium is shown in Table 1. 50 mL of liquid medium containing glucose as carbon source (10 g/L) and casein hydrolysate as nitrogen source (5 g/L) was inoculated with 5 mL fungal mycelium suspension. This medium was used as a propagation medium and it was cultivated during 7 days at 30 °C and pH 5.0. After 7 days, medium was decanted and replaced now medium with glucose as carbon source (10 g/L), casein hydrolysate as nitrogen source (5 g/L) and basic mineral medium with addition of inductors such as copper and manganese ions in sulphate form ($CuSO_4$. 5 H_2O and $MnSO_4$. 4 H_2O) in concentrations 1.0, 3.0 and 7.0 mmol/L or aromatic amino acids (tryptophan and tyrosine) in concentrations 2.5, 5.0 and 7.5 mmol/L. In these media, *C. subvermispora* was cultivated for 13 days at 30 °C and pH 5.0 with shaking (min. 200 RPM). In media were determined glucose concentration and laccase activity during different cultivation time (for media with Cu^{2+} and Mn^{2+}: 0;1;2;3;6;7; and 13th day and for media with tryptophan and tyrosine: 0;1;2;3;4;5;7;8;10 and 13th day of cultivation, respectively). Cultivation was ended after 13 days in all media

and it was determined dry fungal biomass. All values are the mean of at three replicates.

Table 1 The composition of basic mineral medium **(Aguiar *et al.*, 2006)**

Components of medium	Concentration
$MgSO_4$. 7 H_2O	0.5 g/L
NaCl	0.1 g/L
$CaCl_2$. 2 H_2O	0.1 g/L
$CuSO_4$. 5 H_2O	0.1 mg/L
$FeSO_4$. 7 H_2O	0.2 mg/L
$MnSO_4$. 4 H_2O	0.02 mg/L
$ZnCl_2$	0.15 mg/L

Glucose concentration

The supernatant from cultivation medium was used for the analysis of residual glucose in a medium by 3,5-dinitrosalicylic acid (DNS) method **(Miller, 1959)**. 0.8 mL of DNS reagent was added to 0.1 mL of supernatant of cultivation medium. Reaction mixture was incubated in boiling water batch for 5 minutes. The mixture was cooled down to room temperature and 8 mL of distilled water was added. The absorbance of the reaction mixture (200 µL) was measured at 540 nm using a microplate reader (BioTek EL 800, Fisher, GE).

Laccase activity

Laccase activity was determined by oxidation of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). The assay mixture contained 150 µL of 50 mmol/L phosphate buffer (pH 5.0) with 1 mmol/L ABTS and 50 µL of enzyme extracts. Oxidation of ABTS was monitored by measuring of absorbance at 405 nm **(Shin *et al.*, 1987)**. Laccase activity was expressed in unit (U) as the amount of enzymes able to oxidation of 1 mg of ABTS per minute.

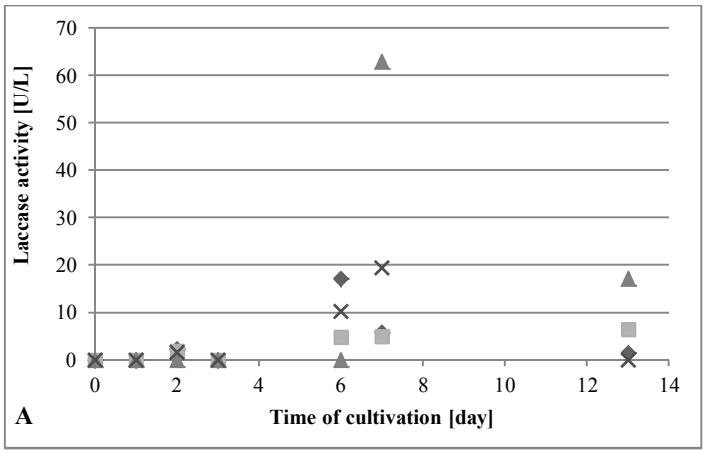
Determination of dry fungal biomass

After filtration and washing of fungal mycelium with distilled water, dry biomass was determined by a moister analyzer IR-35 (Denver Instrument, USA).Dry fungal biomass was expressed in g/L of cultivation medium.

RESULTS AND DISCUSSION

Laccase production in media with addition of inorganic ions

The choice of culture conditions had a significant impact on the final amount of enzymes produced **(Levin *et al.*, 2008)**. Extracellular ligninolytic enzymes are regulated by heavy metal ions during their catalytic action **(Baldrian, 2003)**. Two heavy metal ions are directly involved in the reactions catalyzed by ligninolytic enzymes, specifically copper and manganese. Copper is the cofactor of laccase. The positive effect of copper addition on laccase production was observed in many white-rot fungi **(Galhaup *et al.*, 2002; Fonseca *et al.*, 2010)**. Manganese is involved in the catalytic cycle of manganese peroxidase and also plays significant role in expression of peroxidases and laccase **(Périe and Gold, 1991; Perez and Jeffries, 1992)**. Both copper and manganese ions were added into the cultivation medium of *C. subvermispora* in three different concentrations (1.0; 3.0 and 7.0 mmol/L, respectively) and kinetic of laccase activity in cultivation medium during 13 days of white-rot fungus cultivation was measured (Figure 1).



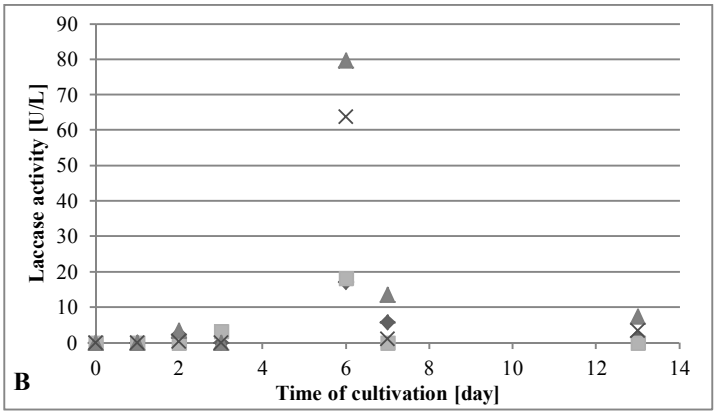
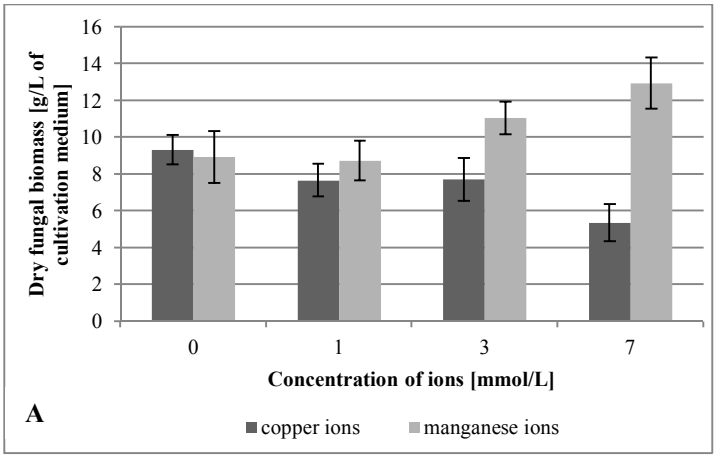


Figure 1 Kinetic of laccase production [U/L] in cultivation media with the addition of different concentration Cu^{2+} (A) of Mn^{2+} (B).
♦ - 0 mmol/L; ■ – 1.0 mmol/L; ▲ – 3.0 mmol/L and x – 7.0 mmol/L of copper or manganese ions

The maximum laccase activity was determined in medium with concentration of copper ions 3.0 mmol/L at 7th day (63 U/L) (Figure 1A). Lower (1.0 mmol/L) and higher (7.0 mmol/L) concentration of copper in cultivation medium was not sufficient for laccase production. In both media, laccase activity was compared with activity in medium without copper ions. Our results are comparable to those described by other authors, who have reported increase of laccase production with copper ions **(Galhaup *et al.*, 2002; Fonseca *et al.*, 2010)**. **Mäkälä *et al.* (2013)** observed that laccase activity producing *Phlebia radiata* was increased in media with 1.5 mmol/L of Cu^{2+} while **Levin *et al.* (2008)** found that optimal concentration of copper ions for laccase production by *Trametes trogii* is 11 mmol/L.
The laccase activity in media with manganese ions was increased by increasing the ion concentration. The maximum laccase activity was measured in medium with manganese concentration 3.0 mmol/L (79.6 U/L) and 7.0 mmol/L (63.8 U/L) at 6th day (Figure 1B). In media with lower concentrations of manganese, laccase activity was lower. **Manubens *et al.* (2007)** found that the highest laccase activities were attained at manganese concentration between 80 and 160 µmol/L, but our experiments show that production of laccase increases also at higher concentrations of manganese.
Secondary, we monitored glucose concentration in cultivation media and biomass growth after cultivation. The results are shown in Figure 2.



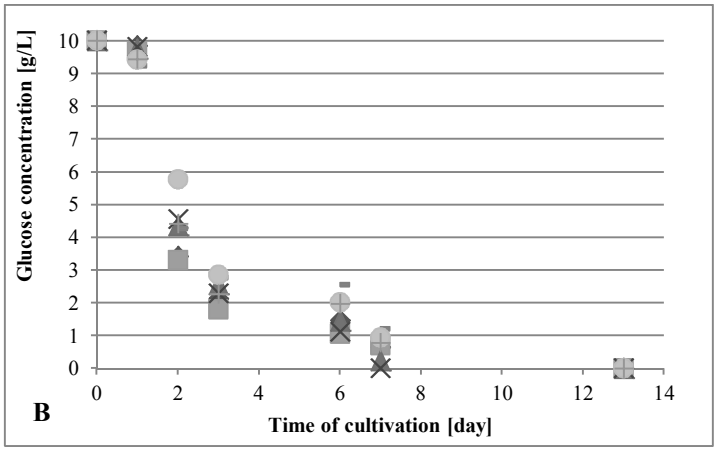
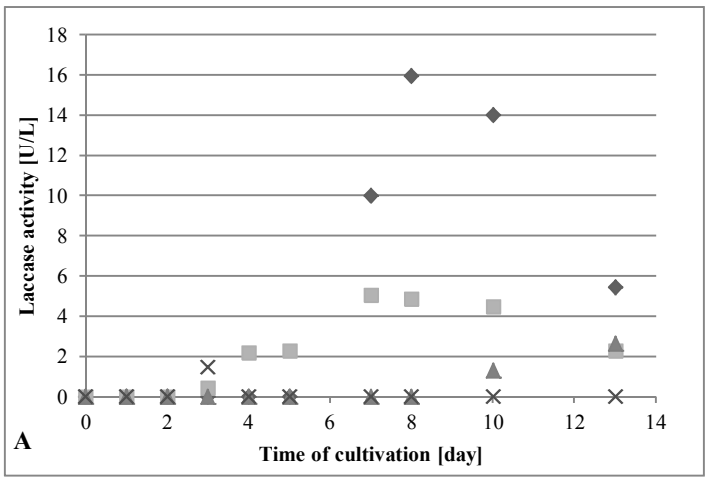


Figure 2 A – glucose concentration in cultivation media with and without addition of Cu^{2+} and Mn^{2+} ions. ♦ - 0 mmol/L; ■ – 1.0 mmol/L Cu^{2+}; ▲ – 3.0 mmol/L Cu^{2+}; × – 7.0 mmol/L Cu^{2+}; - - 1.0 mmol/L Mn^{2+}; ● – 3.0 mmol/L Mn^{2+} and + - 7.0 mmol/L Mn^{2+}. B – dry fungal biomass [g/L of cultivation medium] of *C. subvermispora* after the cultivation

Glucose concentrations in media with and without copper and manganese ions had similar profile (Figure 2A) which means that the tested concentrations of selected inorganic ions not affected fungal growth. Although fungal biomass produced in medium with copper ions decreased with increasing of ion concentration from 9.3 to 5.4 g/L (Figure 2B). The higher concentrations of copper ions are toxic for white-rot fungi **(Baldrian, 2003)**. **Fonseca *et al.* (2010)** found that 0.5 and 1.0 mmol/L of copper ions did not affect biomass production of *Ganoderma applanatum*, *Peniophora* sp. and *Coriolus versicolor* f. antarcticus, but *Pycnoporus sanguineus* showed a marked delay in growth with 0.5 mmol/L Cu^{2+} ions and dramatic growth inhibition with the addition of 1.0 mmol/L $CuSO_4$. In the case of medium with manganese ions, the dry fungal biomass increased with increasing of ion concentration from 9.3 to 12.9 g/L. As previously described above, manganese ions were stimulated biomass growth of white-rot fungus *Ceriporiopsis subvermispora* **(Manubens *et al.* 2007)**.

Laccase production in media with addition of aromatic amino acids

Laccase is enzyme which cans oxidase aromatic compounds **(Levin *et al.*, 2008)**. Present of these compounds in medium may be suppressed its production. Moreover, more authors **(Galhaup *et al.*, 2002; Thiruchelvam and Ramsay, 2007; Levin *et al.*, 2008)** describe that organic source of nitrogen supports the laccase production, but **Levin *et al.* (2008)** found that growth of white-rot fungus *T. trogii*, *T. villosa* and *C. versicolor* f. antarcticus was considerably inhibited in media with tryptophan as only nitrogen source in comparison with complex nitrogen sources as peptone or yeast extract. Therefore, the addition of aromatic amino acids in cultivation medium on laccase production was studied. Tryptophan and tyrosine were added into cultivation medium of *C. subvermispora* in three different concentrations (2.5; 5 and 7.5 mmol/L, respectively). In Figure 3 is shown laccase activities measured during 13 days of cultivation of white-rot fungus *C. subvermispora*.



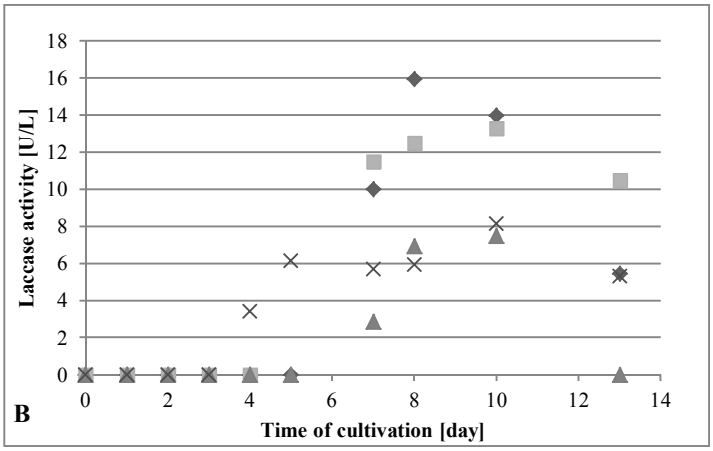


Figure 3 Laccase activities in cultivation media with the addition of different concentration of tryptophan (A) and tyrosine (B).
♦ - 0 mmol/L; ■ – 2.5 mmol/L; ▲ – 5.0 mmol/L and x – 7.5 mmol/L

The addition of aromatic amino acids (tryptophan and tyrosine) did not cause the higher laccase production. The maximum laccase activity was reached in cultivation medium without aromatic amino acids (16.0 U/L) at 8th day. This result disagrees with the findings of other authors. In work **Collins *et al.* (1997)** reported a large increase in LiP production when adding tryptophan to the culture of *Trametes versicolor*, *Phanerochaete chrysosporium* and *Chrysosporium lignorum*. In work **Arora and Gill (2001)** used malt extract rich in aromatic amino acids as nitrogen source and they described increasing of the laccase production by *P. radiata* and *Phlebia fascicularia*. However, our results show that the presence of aromatic amino acids in cultivation medium of *C. subvermispora* not increase the production of laccase.

CONCLUSION

In the present study, stimulation of laccase production by *Ceriporiopsis subvermispora* was investigated. Inorganic ions (Cu^{2+} and Mn^{2+}) and aromatic amino acids (tryptophan and tyrosine) were selected as potential inductors of laccase activities. Productivity of laccase reached a maximum of 63 U/L in medium with 3.0 mmol/L of Cu^{2+} and *C. subvermispora* grown in medium with 3.0 mmol/L of Mn^{2+} produced 79.6 U/L of laccase. Media with Cu^{2+} and Mn^{2+} stimulated laccase production than media without these ions (17.2 U/L). Biomass growth was inhibited the addition of high concentration of copper ions, but mycelium biomass was stimulated the addition of manganese ions. The addition of aromatic amino acids did not cause an increase laccase production.

Acknowledgments: This work was supported by KEGA 024SPU-4/2013.

REFERENCES

AQUIAR, A., SOUZA-CRUZ, P.B., FERRAZ, A. 2006. Oxalic acid, Fe^{3+} - reduction activity and oxidative enzymes detected in culture extracts recovered from *Pinus taeda* wood chips biotreated by *Ceriporiopsis subvermispora*. *Enzyme and Microbial Technology*, 38(7), 873-878.

ARORA, D.S., GILL, P.K. 2001. Effects of various media and supplements on laccase production by some white rot fungi. *Bioresource Technology*, 77(1), 89-91.

BALDRIAN, P. 2003. Interactions of heavy metals with white-rot fungi. *Enzyme and Microbial Technology*, 32(1), 78-91.

BLANCHETTE, R.A., BURNES, T.A., EERDMANS, M.M., AKHTAR, M. 1992. Evaluating isolates of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora* for use in biological pulping processes. *Holzforschung*, 46(2), 109-115.

BOLLAG, J.M., LEONOWICZ, A. 1984. Comparative studies of extracellular fungal laccases. *Applied and Environmental Microbiology*, 48(4), 849-854.

CHMELOVÁ, D., ONDREJOVIČ, M., ONDÁŠ, V., ŠTURDÍK, E. 2011. Influence of cultivation conditions on production of lignocellulolytic enzymes by *Ceriporiopsis subvermispora*. *Biologia*, 66(5), 748-754.

COLLINS, P.J., FIELD, J.A., TEUNISSEN, P., DOBSON, A.D. 1997. Stabilization of lignin peroxidases in white rot fungi by tryptophan. *Applied and Environmental Microbiology*, 63(7), 2543-2548.

DEKKER, R.F., BARBOSA, A.M., GIESE, E.C., GODOY, S.D., COVIZZI, L.G. 2007. Influence of nutrients on enhancing laccase production by *Botryosphaeria rhodina* MAMB-05. *International Microbiology*, 10(3), 177-185.

EGGERT, C., TEMP, U., ERIKSSON, K.E. 1996. The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Applied and Environmental Microbiology*, 62(4), 1151-1158.

FONSECA, M.I., SHIMIZU, E., ZAPATA, P.D., VILLALBA, L.L. 2010. Copper inducing effect on laccase production of white-rot fungi native from Misiones (Argentina). *Enzyme and Microbial Technology*, 46(6), 534-539.
GALHAUP, C., WAGNER, H., HINTERSTOISSER, B., HALTRICH, D. 2002. Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. *Enzyme and Microbial Technology*, 30(4), 529-536.
GALHAUP, C., WAGNER, H., KULBE, K.D., HALTRICH, D. 2001. Efficient production of laccase activity by the white-rot fungus *Trametes pubescens*. *8th international conference on biotechnology in the pulp and paper industry* (Proceeding of the work of the International Conference) Helsinki: Finland, 4-8.
LEVIN, L., HERMANN, C., PAPINUTTI, V.L. 2008. Optimization of lignocellulolytic enzyme production by the white-rot fungus *Trametes trogii* in solid-state fermentation using response surface methodology. *Biochemical Engineering Journal*, 39(1), 207-214.
LEVIN, L., JORDAN, A., FORCHIASSIN, F., VIALE, A. 2011. Degradation of anthraquinone blue by *Trametes trogii*. *Revista Argentina de Microbiologia*, 33(4), 223-228.
MÄKALÄ, M.R., LUNDELL, T., HATAKKA, A., HILDÉN, K. 2013. Effect of copper, nutrient nitrogen, and wood-supplement on the production of lignin-modifying enzymes by the white-rot fungus *Phlebia radiata*. *Fungal Biology*, 117(1), 62-70.
MANUBENS, A., CANESSA, P., FOLCH, C., AVILA, M., SALAS, L., VICUÑA, R. 2007. Manganese affects the production of laccase in the basidiomycete *Ceriporiopsis subvermispora*. *Research Letter*, 275(1), 139-145.

MAYER, A.M., STAPLES, R.C. 2002. Laccase: new functions for an old enzyme. *Phytochemistry*, 60(6), 551-565.
MILLER, G.L. 1959. Use of dinitrosalicylic reagent for the determination of reducing sugar. *Analytical Chemistry*, 31(3), 426-428.
PALMIERI, G., GIARDINA, P., BIANCO, C., FONTANELLA, B., SANNIA, G. 2000. Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. *Applied and Environmental Microbiology*, 66(3), 920-924.
PEREZ, J., JEFFRIES, T.W. 1992. Roles of mangnasese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology*, 58(8), 2402-2409.
PÉRIÉ, F.H., GOLD, M.H. 1991. Manganese regulation of manganese peroxidase expression and lignin degradation by the white-rot fungus *Dichomitus squalens*. *Applied and Environmental Microbiology*, 57(8), 2240-2245.
PEZZELLA, C., AUTORE, F., GIARDINA, P., PISCITELLI, A., SANNIA, G., FARACO, V. 2009. The *Pleurotus ostreatus* laccase multi-gene family: isolation and heterologous expression of new family members. *Current Genetics*, 55(1), 45-57.
RODRÍGUEZ COUTO, S., RODRÍGUEZ, A., PATERSON, R.R., LIMA, N., TEIXEIRA, J.A. 2006. Laccase activity from the fungus *Trametes hirsuta* using an air-lift bioreactor. *Letters in Applied Microbiology*, 42(6), 612-616.
ROTHSCHILD, N., LEVKOWITZ, A., HADAR, Y., DOSORETZ, C.G. 1999. Manganese deficiency can replace high oxygen levels needed for lignin peroxidase formation by *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology*, 65(2), 483-488.
RÜTTIMANN, C., SCHWEMBER, E., SALAS, L., CULLEN, D., VICUÑA. R. 1992. Ligninolytic enzymes of the white rot basidiomycetes *Phlebia brevispora* and *Ceriporiopsis subvermispora*. *Biotechnology and Applied Biochemistry*, 16(1), 64-76.
SHIN, T., MURAO, S., MATSUMURA, E. 1987. A chromogenic oxidative coupling reaction of laccase: application for laccase and angiotensin I converting enzyme assay. *Analytical Biochemistry*, 166(2), 380-388.
THIRUCHELVAM, A.T., RAMSAY, J.A. 2007. Growth and laccase production kinetics of *Trametes versicolor* in a stirred tank reactor. *Applied and Environmental Microbiology*, 74(3), 547-554.