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EFFECTS OF CULTURE MEDIA ON LACCASE PRODUCING WHITE-ROT FUNGI

Gabriela POPA[1], Corina BUBUIANU[2], Georgeta FIDLER[3], Calina Petruta CORNEA[1]

[1]University of Agronomical Sciences and Veterinary Medicine, Faculty of Biotechnology, 59 Marasti, district 1, 011464, Bucharest, Romania, Phone: +40 (21) 318 22 66, Fax: +40 (21) 318 28 88, e-mail: popagabiro@yahoo.com
[2]National Research Institute of Chemical-Pharmaceutical Development – ICCF, Calea Vitan, no. 112, district 3, 031299, Bucharest, Romania, Phone: +40 (21) 321 2117; Fax: +40 (21) 322 2917; e-mail: iccf@ncpri.ro
[3]National Institute of Biology, Splaiul Independenței No. 296, Bucharest, Romania

Corresponding author e-mail: popagabiro@yahoo.com

Abstract

White rot basidiomycetes are known as producers of several oxidative and hydrolytic enzymes that act together on the degradation of certain components of the plant cell wall. Among these enzymes, laccases represent a family of copper-containing polyphenol oxidases, and can be involved in various processes such as, morphogenesis, pathogenesis, and lignin degradation. Because of their capability to oxidize a wide range of substrates, fungal laccases are currently studied for their potential agricultural, industrial, and medicinal applications. Hence, the aim of this study was to investigate the efficacy of culture media on laccase production by six white rot fungal species: Ganoderma applanatum, Flammulina velutipes, Hericium coraloides, Laetiporus sulphureus, Pleurotus ostreatus var. Florida, and Trametes versicolor. The white-rot fungi were inoculated in PD broth (potato dextrose) and Hwang et al (2008) medium. Extracellular laccase formation by these fungi was recorded by spectrophotometry using guaiacol as substrate. During 30 days of incubation was found that laccase production reached maximum values in the filtrates of both culture media and declined along incubation period. PD medium was optimum for laccase producing white-rot fungi. The maximum laccase activity was obtained from the culture filtrates of Trametes versicolor and Hericium coraloides. The optimal culture medium for laccase producing fungi was PD broth. Also, pH values of the fungal culture media were changed during incubation period. The minimal pH values were recorded in culture filtrates of Laetiporus sulphureus (2.8 and 2.4). The highest laccase activity was detected at pH values between 5.0 and 6.0 in PD medium. A good biomass yield was recorded by Pleurotus ostreatus var. Florida grown on both media tested. It was found that a high production of laccase did not dependent on high biomass yields.

Key words*: laccase activity, guaiacol, macromycetes.*

INTRODUCTION

Laccases (EC 1.10.2.3; benzenediol: oxygen oxidoreductase) are multicopper-containing enzymes, found widely in plants and fungi, especially in white rot fungi which can degrade natural lignin. Laccases can *c*atalyze the oxidation of many aromatic compounds, particularly phenols. Laccase activity has been demonstrated in many fungal species belonging to ascomycetes and basidiomycetes. Among basidiomycetes that produce laccases can be mentioned *Agaricus bisporus* (Wood, 1980), *Pleurotus ostreatus* (Sannia et al., 1986), *Hericium coralloides* (Zou YJ et al., 2012), *Ganoderma lucidum* (Ko et al., 2001) and *Trametes versicolor* (Rogalski et al., 1991). A major role in enzyme activity acts the culture conditions and medium composition. Laccase production by fungi is strongly affected by many parameters like temperature, time of cultivation, stationary or submerged cultures, medium composition (Palmieri et al., 2000). Temperature plays a major role in growth and laccase production of the fungi. In general the most fungi were cultivated at temperatures between 25°C and 30°C for optimal laccase production (Minussi et al., 2007). It was found that the optimal temperature for fruiting body formation and laccase production is 25°C in the presence of light and 30°C for laccase production when the cultures are incubated in the dark (Thurston, 1994). When the temperatures are higher than 30°C, the activity of laccase decreased significantly (Zadrazil et al., 1999). In white-rot fungi laccases are produced as multiple isoenzymes (Solomon et al., 1996). Extracellular laccases production

can be stimulated by a variety of inducing substances especially phenolic compounds related to lignin or lignin derivatives (Hess et al., 2002). *Ganoderma lucidum* produced higher levels of laccases in medium reached in nitrogen and glucose as carbon sources (D'Souza et al., 1999). The aim of the present study was *to investigate the efficacy of culture media on laccase production by six white rot fungal species: Ganoderma applanatum, Flammulina velutipes, Hericium coraloides, Laetiporus sulphureus, Pleurotus ostreatus var. Florida, and Trametes versicolor in submerged cultures.*

MATERIALS AND METHODS

Biological material and culture conditions

Six mushroom species, namely *Ganoderma applanatum, Flammulina velutipes, Hericium coraloides, Laetiporus sulphureus, Pleurotus ostreatus var.* Florida and *Trametes versicolor* were used in this study. The biological material, from the indigenous macromycetes species collection of the Faculty of Biotechnology, was maintained on PDA slants at 4^0C until use. For the experiments the fungal inoculums consisted of 5 mm agar plugs of one week old culture grown on PDA at 25^0C were placed on two variants of liquid media: a PD (potato-dextrose), pH 5.2, and a medium described by Hwang et al. (2008): 20g/l glucose, 2g/l peptone, 2g/l yeast extract, 0,46g/l KH_2PO_4, 1g/l K_2HPO_4, 0.5 g/l $MgSO_4$, pH 6.7. Cultures were incubated at 25^0C in 250 ml Erlenmeyer flasks containing 50 ml of culture medium and agitated at 140 rpm. After 15 and 30 days respectively the samples were analysed for the biomass production, pH and laccase activities in the fungal filtrates. The fungal culture was filtered with Whatman No. 1 filter paper and used as enzyme source. Mycelium, collected and dried at 70°C for 48 h, was used for determination of biomass.

Determination of laccase activity

Laccase activity was determined according Savitha et al. (2011) protocol using the guaiacol as substrate. The reaction mixture contained 3 ml sodium acetate buffer (10 mM, pH 5.0), 1ml guaiacol (2 mM) and 1 ml enzyme source. The mixture was incubated at 30^0C for 15 min. The changes in absorbance due the oxidation of guaiacol in the reaction mixture were recorded by spectrophotometer at 450 nm. One unit of enzyme activity is defined as the amount of enzyme that oxidized 1 µmol of guaiacol per minute. The laccase activity (U/ml) is calculated by the formula: U/ml = (A x Vt x dilution factor) / (t x € x Vs) where A = absorbance at 450nm, Vt = total volume of reaction mixture (ml), Vs = enzyme volume (ml), t = incubation time (min) and € = extinction coefficient of guaiacol at 450nm (12,100 $M^{-1}cm^{-1}$) (Savitha et al., 2011).

RESULTS AND DISCUSSIONS

Basidiomycete isolates of *Ganoderma applanatum, Flammulina velutipes, Hericium coraloides, Laetiporus sulphureus, Pleurotus ostreatus var.* Florida and *Trametes versicolor*, were investigated for the maximum biomass achieving and laccase production in submerged conditions. The experiments were performed using two different culture media: PD and Hwang liquid medium. After 30 days of incubation, the highest fungal biomass quantity was recorded by *Pleurotus ostreatus* var. Florida in the Hwang medium (0.50 g), followed by PD medium (0.48 g) (Figure 1).

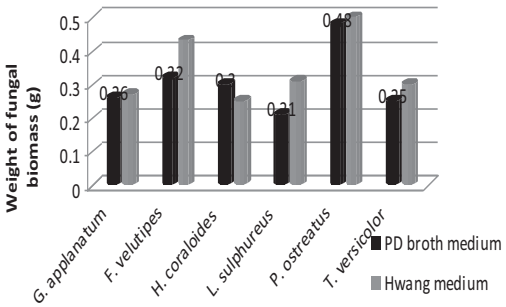

Figure1. Fungal biomass grown on different media after 30 days of incubation

Moreover, Hwang medium offered the optimum chemical composition for mycelium development in all the white-rot fungi tested excepting *Hericium coralloides*: in this case the best biomass production was obtained in PD medium (0.30 g).

During fungal mycelium development in the culture broths (Hwang and PD), pH changes occurred (Figures 2 and 3). Very low pH values were detected in *Laetiporus sulphurous* culture filtrate: pH 2.4 in PD filtrate and pH 2.5 in

Hwang filtrate. These values were closely maintained during the entire period of incubation. Low pH values were also recorded in *Ganoderma applanatum* culture filtrate (pH 4.1 in PD, and pH 3.8 in Hwang medium) and in *Trametes versicolor* culture filtrate (pH 4.2 in Hwang medium) after 15 days of cultivation. Higher pH values were observed in culture filtrated of *F.velutipes* and *P.ostreatus* (Figure 2).

After 30 days of cultivation the pH values were comparable with those after 15 day for *H. coraloides, T. versicolor, P. ostreatus, L. sulfureus* but only in Hwang mediu. For PD medium, the pH values of culture filtrates were generally higher (Figures 3). The highest pH were detected in *F.velutipes* in both media, and in *T.versicolor* cultivated in PD.

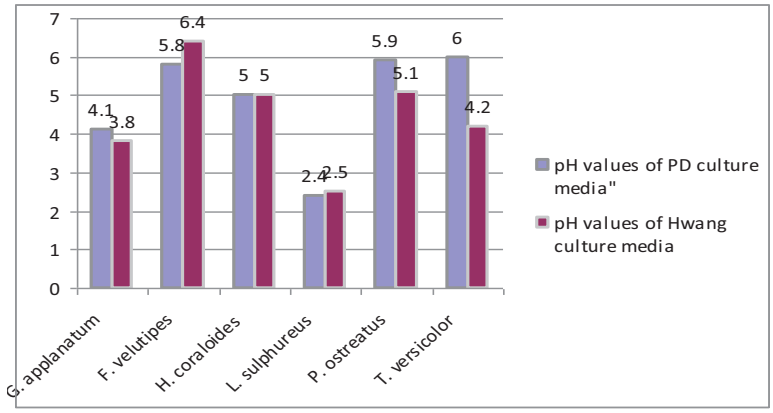

Figure 2. pH changes in the fungal filtrates 15 days after

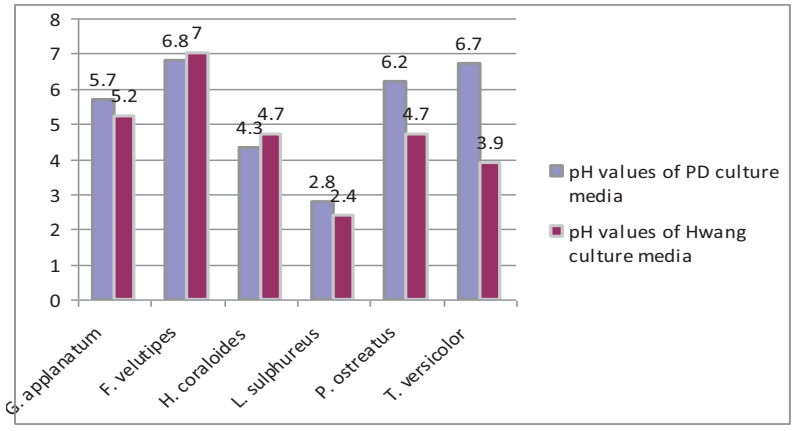

Figure 3. pH changes in the fungal filtrates 30 days after

Regarding the laccase activity, differences among the fungal species cultivated in PD and Hwang liquid media were registered. After 15 days of cultivation, laccase activities were detected in both types of culture filtrates (PD and Hwang medium), with different values depending on fungal strains.

However, PD medium was optimum for laccase producing white-rot fungi. After 15 days of incubation in this medium, *Hericium coraloides* and *Trametes versicolor* presented the highest laccase activities (29.48 U/ml and 17.99 U/ml respectively) (Figures 4).

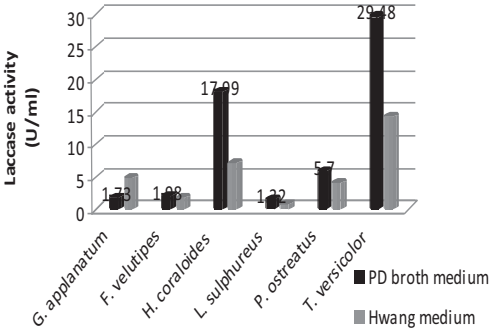

Figure 4. Laccase activity in the fungal filtrates after 15 days of incubation

After 30 days of cultivation the laccase activity decreased, but the best producers remain *H.coraloides* and *T.versicolor*: 12.54 U/ml and 11.07 U/ml, respectively (figure 5). The reduction of the activities was with 27% for *H.coraloides* and more than 60% for *T.versicolor* and could be due to inhibitory compounds released by aged mycelium.

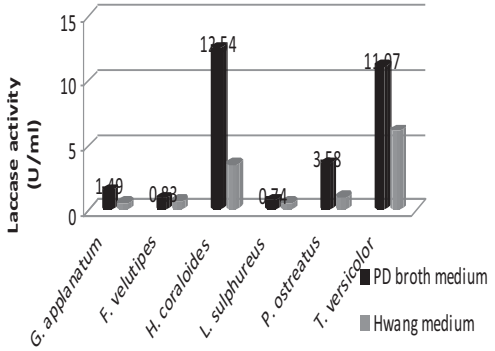

Figure 5. Laccase activity in the fungal filtrates after 30 days of incubation

It was reported that a high production of laccase is not dependent on high biomass yields (Praveen et al., 2011), but the medium formula could influence the enzymatic level (Palmieri et al., 2000; Xavier et al., 2001). Moreover, it was found that laccase production is closely related to the cultivation conditions of the fungi (Praveen et al., 2011; Heinzkill et al., 1998). Among the factors that influence the laccase activity, pH value seem to be very important. Some authors indicated initial pH levels set between 4 and 6 before inoculation, but the pH was not controlled during cultivation period (Vasconcelos et al., 2000; Arora and Gill, 2000). The normal range of pH for typical laccase activity is between 3 and 5, at least for some fungal species (Savitha et al., 2011).

The results obtained in our experiments indicated a correlation between pH value and laccase production: laccase production from the *Trametes versicolor* and *Hericium coraloides* had highest values when the culture filtrate had pH values between 5 and 6 (in PD medium). Similar aspects were observed for *P.ostreatus* cultivated in PD medium. When Hwang medium was used for cultivation, pH had lowest values, and the level of enzymatic activity was also reduced. These results suggested that the medium composition is another factor that influenced the enzymatic activity. However, no correlation between the biomass weight and laccase activity was established.

It is obvious that enhancement of laccase production through optimization of nutritional and physiological conditions during cultivation of white-rot fungi is very important for their utilization at industrial scale (Dhakar and Pandey, 2013). In this order, besides nutritional supplements, some inducers such as organic solvents and metal ions also play important role in production of laccases (Shraddha et al., 2011, Brijwani et al., 2010).

CONCLUSIONS

Indigenous macromycetes isolates of *Ganoderma applanatum, Flammulina velutipes, Hericium coraloides, Laetiporus sulphureus, Pleurotus ostreatus var.* Florida and *Trametes versicolor* were tested for fungal biomass production, pH and laccase production under submerged conditions. The effect of media composition on mycelium growth and laccase activity was also investigated.

During the incubation period was found that laccase production reached maximum values in the filtrates of both culture media after 15 days and significantly decreased after 30 days of incubation. PD medium offered the best nutritional condition for laccase production in almost all the white-rot fungi tested.

Among all mushroom species tested, *Trametes versicolor* and *Hericium coraloides* presented the highest level of laccase activity (29.48 U/ml and 17.99 U/ml).

The influence of pH variation during cultivation on laccase activity was also evaluated. In was shown that the laccase production from the *Trametes versicolor* and *Hericium coraloides* was optimum when the culture media had pH 5-6. No correlation between high production of laccase and high biomass yields was established. Due to the potential biotechnological applications of laccases in various industrial processes and in remediation of soil and contaminated effluents, further studies will be focused on optimization the conditions for enzyme production and recovery.

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REFERENCES

Arora, D. S., Gill, P. K., 2000. Laccase production by some white rot fungi under different nutritional conditions. Bioresource Technology, vol. 73, no. 3, pp. 283–285.

Brijwani, K., Rigdon, A., Vadlani, P. V., 2010. Fungal laccases: production, function, and applications in food processing. Enzyme Research, vol. 2010, pg. 1-10.

Dhakar, K., Pandey, A., 2013. Laccase Production from a Temperature and pH Tolerant Fungal Strain of Trametes hirsuta (MTCC 11397). Enzyme Research, vol. 2013, pg. 1-9.

D'Souza, T. M., Merritt, C. S., Reddy, C. A., 1999. "Lignin-modifying enzymes of the white rot basidiomycete *Ganoderma lucidum*," *Appl. Environ. Microbiol.*, vol. 65, no.12, pp. 5307–5313.

Heinzkill, M., Bech, L., Halkier, T., Schneider, P., Anke, T., 1998. Characterization of laccases and peroxidases from woodrotting fungi (family *Coprinaceae*). *Appl. Environ. Microbiol.*, vol. 64, no. 5, pp. 1601–1606

Hess, J., Leitner, C., Galhaup, C., et al., 2002. Enhanced Formation of Extracellular Laccase Activity by the White Rot Fungus *Trametes multicolour*. *Appl. Biochem. Biotechnol*, 98-100, pp 229 – 241.

Hwang, H.S., Lee, S.H., Baek, Y.M., Kim, S.W., Jeong, Y.K., Yun, J.W., 2008. Production of extracellular polysaccharides by submerged mycelial culture of *L. sulphureus* var. miniatus and their insulinotropic properties. Appl. Microbiol. Biotech.78: 419-429.

Ko, E.-M., Leem, Y.-E., Choi, H. T., 2001. Purification and characterization of laccase isozymes fromthe white-rot basidiomycete *Ganoderma lucidum*. *Appl. Microbiol. Biotechnol.*, vol. 57, no. 1-2, pp. 98–102.

Minussi, R. C., Miranda, M. A., Silva, J. A. et al., 2007. Purification, characterization and application of laccase from Trametes versicolor for colour and phenolic removal of olive mill wastewater in the presence of 1-hydroxybenzotriazole. African J. Biotechnology, vol. 6, no. 10, pp. 1248–1254.

Palmieri, G., Giardina, P., Bianco, C., et al., 2000. Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol., vol. 66, no. 3, pp. 920–924.

Praveen, K., Viswanath, B., Usha, K. Y. et al., 2011. Lignolytic enzymes of a mushroom *Stereum ostrea* isolated from wood logs. *Enzyme Research*, vol. 2011, Article ID 749518.

Rogalski, J., Lundell, T., Leonowicz, A., Hatakka, A., 1991.Production of laccase, lignin peroxidase and manganese-dependent peroxidase by various strains of *Trametes versicolor* depending on culture conditions. *Acta Microbiologica Polonica*, vol. 40, pp. 221–234.

Sannia, G., P. Giardina, M. Luna, 1986. Laccase from *Pleurotus ostreatus*. *Biotechnology Letters*, vol. 8, no. 11, pp. 797–800.

Savitha S. D., Gururaj B. T., Nityanand, A.C. et al., 2011. Isolation of laccase producing fungi and partial characterization of laccase. Biotechnol. Bioinf. Bioeng., 1(4):543-549.

Shraddha, R. Shekher, S. Sehgal, M. Kamthania, Kumar, A., 2011. Laccase: microbial sources, production, purification, and potential biotechnological applications, Enzyme Research, vol. 2011, pg. 1-11.

Solomon, E.I., Sundaram, U.M., Machonkin, T.E., 1996. Multicopper Oxidases and Oxygenases. Chem Rev. Nov 7; 96 (7), 2563-2606.

Thurston, C. F., 1994.The structure and function of fungal laccases. Microbiology, 140 (1), pp. 19–26.

Vasconcelos, A.F.D., Barbosa, A. M. R., Dekker, F. H., Scarminio,I. S.,Rezende, M. I., 2000. Optimization of laccase production by Botryosphaeria sp. in the presence of veratryl alcohol by the response-surface method. Process Biochemistry, 35 (10), pp. 1131–1138.

Wood, D. A., 1980.Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *J. Gen. Microbiol.*, vol. 117, pp. 327–338, 1980.

Xavier, A. M. R. B., Evtuguin, D. V., Ferreira, R. M. P., Amado, F. L., 2001. Laccase production for lignin oxidative activity, in *Proceedings of the 8th International Conference on Biotechnology in the Pulp and Paper Industry*, pp. 4–8, Helsinki, Finland, June 2001.

Zadrazil, F., Gonser, A., Lang, E., 1999. Influence of incubation temperature on the secretion of extracellular ligninolytic enzymes of *Pleurotus* sp. And *Dichomitus* squalens into soil, in Proceedings of the Conference on Enzymes in the Environment: Activity, Ecology and Applications, pp. 12–16, Granada, Spain, July1999.

Zou, Y.J., Wang, H.X, Ng TB, Huang, C.Y., Zhang, J.X., 2012. Purification and characterization of a novel laccase from the edible mushroom *Hericium coralloides*. J Microbiol.,50 (1):72-8.