

EXTRACELLULAR LACCASE PRODUCTION IN SUBMERGED CULTURE OF SOME WHITE-ROT FUNGI AND THEIR IMPACT FOR TEXTILE DYES DECOLORISATION

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Abstract

White-rot fungi are a group of organisms capable of biodegrading lignin due to production of a ligninolytic enzyme complex. This enzyme complex is rich in several laccase isoenzymes which found to have the ability to decolorize different classes of industrial dyes. Laccases (EC 1.10.3.2) are phenol oxidases that catalyse one-electron oxidation of many aromatic substrates. This study aimed to evaluate extracellular laccase production in submerged culture of some white-rot fungi and their potential to decolorized three textile dyes like Bemacid red (BR), Bemacid yellow (BY) and Bemacid blue (BB). We found that the best laccase producing fungi were *Pleurotus ostreatus* var. *Florida*, *Ganoderma applanatum*, and *Trametes versicolor*. Enzyme production by selected strains was recorded in six different culture media: malt extract (ME), potato dextrose (PD), mushroom complete medium (MCM), potato-malt-peptone (PMP), glucose - malt extract - yeast (GMY) and yeast - malt extract (YM). *T. versicolor* and *P. ostreatus* showed the highest laccase activities in malt extract medium. Laccase activity and dyes discoloration in submerged cultures were determined spectrophotometrically. All fungal strains were able to discolouring BB dye. These studies underline the need to explore more laccase-producing fungi which could be involved in the textile dyes degradation.

Key words: laccase, submerged culture, white rot fungi, textile dyes, decolorisation.

INTRODUCTION

Synthetic dyes are toxic for environmental and physical or chemical treatments of textile wastewaters are expensive and sometimes unsuccessful. In the last years, research in the dye bioremediation technologies has gained more attention. Studies performed with different microbial species including fungi, bacteria or yeasts suggest that they may discolour azo dyes under certain environmental conditions (Khelifi et al., 2008). Studies on the degradation abilities of synthetic dyes or other pollutants by the white-rot fungi proved to be promising. This biodegradation capacity is supposed to result from the activities of non-specific ligninolytic enzymes secreted by these fungi, including lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases (Lac) (Singh, 2006). These enzymes are responsible for degradation of cellulose, hemicellulose and lignin into low-molecular-weight compounds used in fungal nutrition (Songulashvili et al., 2007). The expression of these enzymes depends on the producing strain.

Thus, some fungi (e.g. *Phanerochaete chrysosporium*) can produce LiP and MnP, but not laccase (Faraco et al., 2009), others produce MnP and laccase but not LiP (e.g. *Ceriporiopsis subvermispora*) (Tanaka et al., 2009), while others produce all these enzymes (e.g. *Irpex lacteus*) (Novotny, 2009). Laccases (benzene diol: oxygen oxidoreductases; EC1.10.3.2) are multi-copper enzymes belonging to the group of blue oxidases widely distributed in nature (Kiiskinen and Saloheimo, 2004; Thurston, 1994). Laccases catalyse the oxidation of a wide spectrum of substrates, including phenolic and nonphenolic compounds as well as some recalcitrant environmental pollutants (Blanquez and Guieysse, 2008; D'Annibale et al., 2005). Due to these properties, laccases are used for many industrial purposes such as paper processing, detoxification of environmental pollutants, oxidation of dye or production of chemicals from lignin (Gianfreda et al., 1999). Laccases are also useful for the decomposition of azo dyes by oxidative methods (Michael et al., 2005). In our previous studies on laccase

producing fungi in submerged conditions and their potential in the decolorisation textile dyes it was found that laccase production is closely related to the synthetic dyes degradation and decolorisation processes (Iordache et al., 2016). In the present study the goals were to investigate the production of extracellular laccase of some white-rot fungi such as: *Flammulina velutipes*, *Laetiporus sulphureus*, *Pleurotus ostreatus* var. 'Florida', *Ganoderma applanatum*, and *Trametes versicolor* and their ability to degrade textile dyes from culture media.

MATERIALS AND METHODS

Fungal material. Five white-rot fungi: *Flammulina velutipes*, *Laetiporus sulphureus*, *Pleurotus ostreatus* var. 'Florida', *Ganoderma applanatum*, and *Trametes versicolor*, provided from the macromycete collection of the Faculty of Biotechnologies - Bucharest were used. The fungi were maintained in collection on malt extract 2% agar slants at 4⁰C. White-rot fungi cultures of 5 days old obtained on malt extract 2% agar were used for the experiments.

Textile dyes. Three textile azo-dyes named Bemacid Red N-TF (BR), Bemacid Yellow N-TF (BY) and Bemacid Blue N-TF (BB) (Figure 1), produced by Bezema AG Company, were used for decolorisation tests.

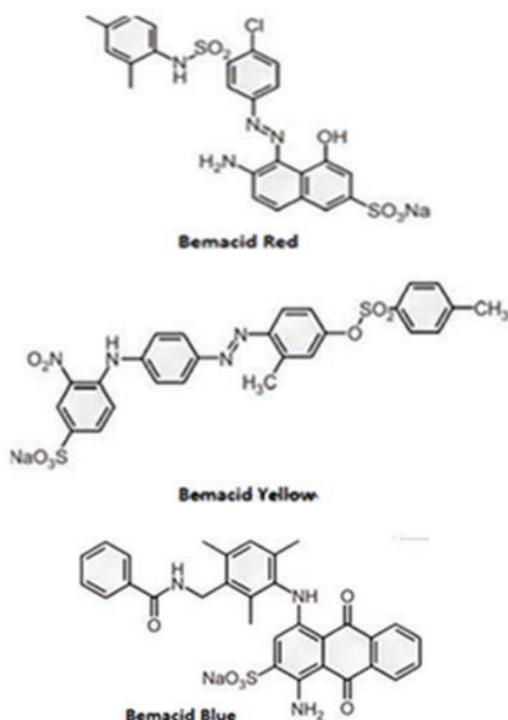


Figure 1. Dyes chemical structure

The industrial dyes were kindly provided from the National Research and Development Institute for Textiles and Leather, Bucharest.

Screening for laccase activity. Determination of potent strains for laccase production was performed under solid state conditions by using malt extract agar 2% supplemented with 0.01% guaiacol. The presence of brown red colour around the mycelium was considered as guaiacol oxidizing laccase secreting organism.

Liquid media for laccase production. Fungal isolates that produced laccase under solid state conditions were cultivated on six different liquid media: malt extract broth (ME), potato dextrose broth (PD), mushroom complete medium broth (MCM), potato-malt-peptone broth (PMP), yeast - malt extract broth (YM) (Kim et al., 2002) and glucose - malt extract - yeast broth (GMY) (Pickard et al., 1999). The media composition and their pH values are shown in Table 1.

Table 1. Media composition used in this study

Composition (g/l)	Culture media					
	ME	PD	PMP	MCM	YM	GMY
Glucose				20	10	10
KH ₂ PO ₄				0.46		2
K ₂ HPO ₄				1		
Malt extract	17		10		3	3.5
MgSO ₄ ·7H ₂ O				0.5		0.5
Peptone	3		1	2	5	
Potato dextrose broth		24	24			
Yeast extract				2	3	2.5
pH	5.4	5.0	4.6	5.9	5.9	5.2

Fungal inocula (5 mm agar plugs) were placed into separate labelled Erlenmeyer flasks (200 mL) with 100 ml of each medium. The flasks were incubated with rotary stirring (120 rpm) at 25⁰C for 7 days, in the dark. After incubation, the fungal cultures were filtered through Whatman No. 1 filter paper (Merck). The filtrates were used for laccase activity determination.

Estimation of laccase activity. Laccase activity was determined spectrophotometrically according to Kalra et al. (2013) 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 (filtrates of fungal culture). The mixture was incubated at 30⁰C for 15 min. The changes in absorbance due the oxidation of guaiacol in the reaction mixture were recorded by spectrophotometer at λ 450 nm. One unit of

enzyme activity is defined as the amount of enzyme that oxidized 1 μ mol of guaiacol per minute.

Dye decolorisation tests

Qualitative assay. For the qualitative assay the fungal inocula were transferred on malt extract agar medium supplemented with 200 mg/l of each dye. All samples were incubated at 25^oC for 7 days. For this treatment was used a control sample with no dye added.

Quantitative assay. For quantitative assay fungal inocula were transferred into 100 ml malt extract broth supplemented with 200mg/l of each dye. The samples were incubated in the dark, with stirring (120 rpm), at 25^oC, for 10 days. After incubation, the fungal cultures were filtered through Whatman No. 1 filter paper (Merck). These filtrates were centrifuged at 8000 rpm for 10 minutes at room temperature (25^oC). The clear supernatants were analysed spectrophotometrically (Eppendorf UV/Vis) at a wavelength corresponding to each dye (λ 340 nm for BR, λ 440 nm for BY and λ 640 nm for BB), in order to determine the decolorisation rate (%). The discoloration percentage of the dyes was calculated using formula: $D (\%) = 100 \times (ODc - ODs) / ODc$, where: ODc = absorbance (nm) of the controls, and ODs = absorbance (nm) of the samples.

RESULTS AND DISCUSSIONS

Screening for laccase activity

The ability of the *Flammulina velutipes*, *Laetiporus sulphureus*, *Pleurotus ostreatus* var. 'Florida', *Ganodrema applanatum* and *Trametes versicolor* fungal strains to secrete extracellular laccase was carried out using a simple screening method which involves the inoculation of fungi on a solid medium (malt extract agar) containing 0.01% guaiacol as indicator.

Among all isolates tested, *Pleurotus ostreatus* var. 'Florida', *Ganodrema applanatum* and *Trametes versicolor* fungal strains were more efficient in laccase production than *Flammulina velutipes* and *Laetiporus sulphureus*.

After seven days of culture, the extracellular oxidation of laccase by guaiacol led to the formation of characteristic red brown areas around the *Pleurotus ostreatus* var. 'Florida', *Ganodrema applanatum* and *Trametes versicolor* colonies (Figure 2).

Thus, amongst the 5 isolates these strains were found to be more potent for laccase production and, therefore, were used for further studies.



Figure 2. Oxidation of extracellular laccase by guaiacol and reddish brown zone formed around the fungal colonies: 1-*Trametes versicolor*; 2-*Pleurotus ostreatus* var. 'Florida'; 3-*Ganodrema applanatum*

Determination of optimum medium for laccase production. To determine the optimum culture medium for laccase production by selected fungal strains, six different liquid media set at different pH values (ranging from 4.6 to 5.9) were used (see Table 1). Since pH has a significant effect on the growth of fungi and on laccase production, it has been investigated if the initial pH of the culture media undergoes changes during the incubation period. In all culture media it was found that the pH values - recorded at the end of the incubation period - showed significant variations from baseline (Figure 3). Very low

pH value (2.9) was observed in the PD medium of *P. ostreatus* and a high pH value (8.1) in the culture of *P. ostreatus* grown in YM medium. Slight pH changes from baseline were observed in the culture broths of *G. applanatum* and *T. versicolor* (Figure 3).

This variation in pH levels from baseline may be due to the extracellular metabolites elaborated by fungi in the culture media during incubation period. Thus, the level of extracellular metabolites can alter the extracellular pH, acidifying or alkalizing the culture media.

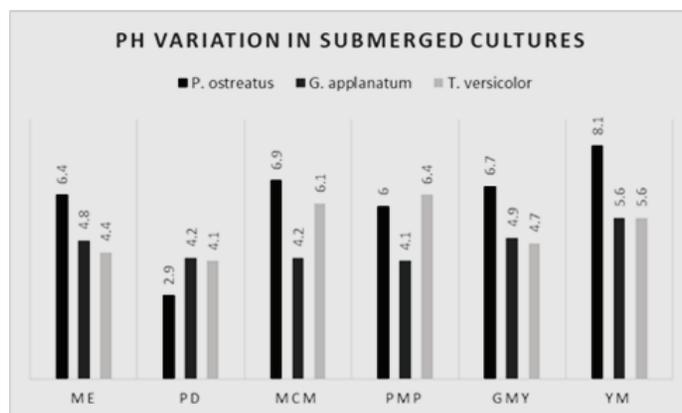


Figure 3. pH variation in culture media of *P. ostreatus* 'Florida', *G. applanatum* and *T. versicolor*

Maximum laccase activity was recorded in the filtrates of all fungal strains grown in malt extract broth, 7 days after. The optimum pH for laccase production was 6.4 for *P. ostreatus* 'Florida' (9.24% U/ml), 4.8 for *G.*

applanatum (1.87 U/ ml) and 4.4 for *T. versicolor* (9.91 U/ml). Among all selected strains, *T. versicolor* and *P. ostreatus* var 'Florida' showed the highest laccase activity (Figures 3 and 4).

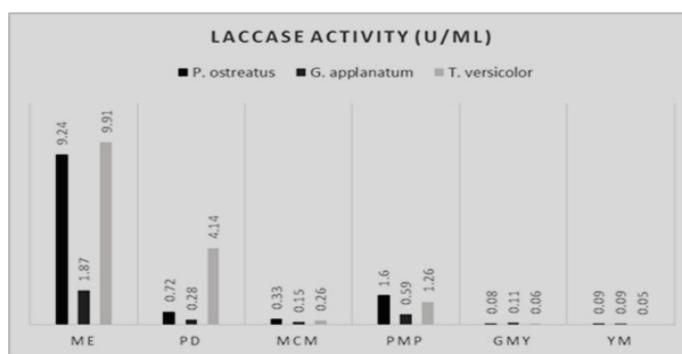


Figure 4. Laccase activity in the filtrates of *P. ostreatus*, *G. applanatum* and *T. versicolor*

According to Singh and Abraham (2013) in general, the growth of fungi is ideal at low pH and the maximum laccase activity of the fungus was found to be at an optimum pH 4 with maltose and peptone as ideal carbon and nitrogen sources. Our data showed a maximum laccase activity in the filtrates of all fungal strains grown in malt extract broth, with malt extract 2% and peptone as carbon and nitrogen sources and with an optimal pH value ranging between 4.4-6.4 (see Figure 3). The influence of pH on the growth, production and activity of ligninolytic enzymes, and the degradation of aromatic compounds such as lignin and environmental pollutants by basidiomycetes have been the subject of several studies (Lechner and Pappinutti, 2006; Zouari-Mechichi et al., 2006). Laccase production by mushrooms has been previously demonstrated to depend significantly on the composition of

the growing medium. The carbon source, nitrogen content and phenolic inducer compounds have been reported to have significant effects on laccase production (El-Batal et al., 2015).

Dye decolourisation tests

Laccase producing fungal isolates were subjected to dye decolourisation experiments. Three azo dyes Bemacid Red N-TF (BR), Bemacid Yellow N-TF (BY) and Bemacid Blue N-TF (BB) use in textile industry were chosen for this study.

Screening for textile dyes decolourisation

was performed on malt extract agar plates supplemented with each dye. The growing of the fungal isolates on solid media showed mycelium extension, but clear area of dyes degradation were observed after 7 days of cultivation (Figure 5).



Figure 5. Removal of dyes from the culture media by *T. versicolor*, *P.ostreatus* 'Florida' and *G. applanatum*:
 Line A: Bemacid Red dye degradation from the culture media of *T. versicolor*, *P. ostreatus* 'Florida' and *G. applanatum*
 Line B: Bemacid Yellow dye degradation from the culture media of *T. versicolor*, *P. ostreatus* 'Florida' and *G. applanatum*;
 Line C: Bemacid Blue dye degradation from the culture media of *T. versicolor*, *P. ostreatus* 'Florida' and *G. applanatum*

Quantitative assay. Fungal isolates grown in 100 ml malt extract broth (pH 5.4) supplemented with 200 mg/l of each dye were analysed to determine their decolourisation potential. The fungal samples showed variation

in dyes decolourisation degree in submerged culture 10 days after incubation (Figure 6). No significant variation in pH level from baseline has been recorded.



Figure 6. Dyes decolourisation degree of fungal submerged cultures:
 Line A: BR-Bemacid Red-control; *T. versicolor*, *P. ostreatus*'Florida', *G. applanatum* cultures supplemented with BR
 Line B: BY-Bemacid Yellow-control; *T. versicolor*, *P. ostreatus*, *G. applanatum* cultures supplemented with BY;
 Line C: BB-Bemacid Blue-control; *T. versicolor*, *P. ostreatus*, *G. applanatum* cultures supplemented with BB

To determine the decolourisation rate (%) the fungal filtrates were analysed spectrophotometrically at a wavelength corresponding to each dye: λ 340 nm for BR, λ 440 nm for BY and λ 640 nm for BB. Dyes decolourisation recorded by spectrophotometer showed that the best results were obtained against BB. The values recorded at the end of the incubation

period (on the 10th day) showed that 45.16% of BB dye was decolorized by *T. versicolor*, 34.12% by *G. applanatum* and 25.65% by *P. ostreatus*. For the other dyes the decolorizing rate was lower. Significant results were obtained with *G. applanatum* 27.71% for BR and 14.62% for BY (Figure 7).

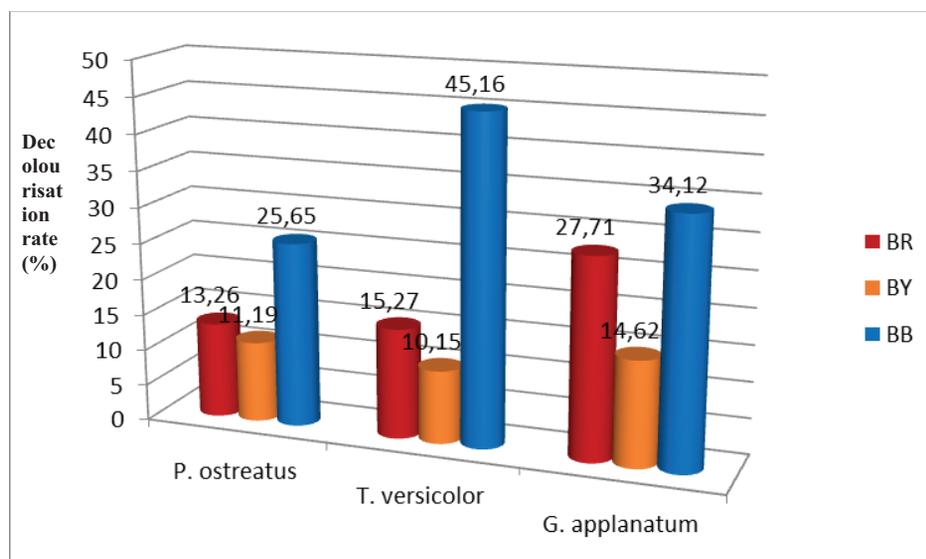


Figure 7. Decolourisation rate (%) of textile dyes by white - rot fungi at the end of the incubation period

The dye decolourisation effect observed and recorded in broth culture and in fungal filtrates is due to the extracellular enzymes produced by fungi. It is well known that most of the white-rot fungi produce at least two of the three nonspecific enzymes like lignin peroxidase, manganese peroxidase and laccases (Pavko, 2011) which can play a synergistic role in the process of dyes degradation or other xenobiotic pollutants. The genus *Trametes*, which belongs to the white-rot fungi group, is assumed to be one of the main producers of laccases. *T. versicolor* produces both laccase and lignin peroxidase as major ligninolytic enzymes (Pazarlioglu et al., 2010); however, the role of these enzymes in decolorisation of azo dyes is not yet clear (Stoilova et al., 2010). According to the literature data, laccase activity in culture filtrate of *T. versicolor* was not able to decolorize azo dyes, thus indicating a role of other enzymes or cell-bound components in azo dye degradation (Swamy and Ramsay, 1999). In our studies *T. versicolor* was the best laccase producing fungal species of all three selected isolates for degradation of azo dyes. *Pleurotus* spp. is one of most extensively studied white-

rot fungi for its ligninolytic properties (Li and Shah, 2016; DaLuz et al., 2015). *Pleurotus ostreatus* produces as major ligninolytic enzymes laccase and lignin peroxidase (Pazarlioglu et al., 2010). *Ganoderma applanatum* is a mushroom used in Chinese traditional medicine and reported to have various medicinal activities, such as antitumor, antiviral, immunomodulated etc. (Lee et al., 2007). Contrary to its medicinal properties, which were studied extensively, its ligninolytic enzyme system has not been studied very well. Many studies reported that the level of ligninolytic enzymes is playing a major role in degradation of dye, which is dependent on time of incubation and species of fungi (Kunjadia et al., 2016; Koyani et al., 2013; Dos Santos et al., 2004). Different factors (dye concentration, pH and chemical media content) influencing the ability of mushroom species to degrade dyes are also documented (Singh and Singh, 2017).

CONCLUSIONS

In the present work, amongst five fungal isolates, three fungal strains: *P. ostreatus* var.

'Florida', *T. versicolor* and *G. applanatum* showed laccase activity in the presence of guaiacol as inducer. The potent strains for laccase production were grown in six different broth media each with a well-defined pH value. It was found that, in all culture media, the pH levels recorded at the end of the incubation period showed significant variations from baseline. Maximum laccase activity in the filtrates of all selected fungal strains was recorded in malt extract broth. Among all strains, *T. versicolor* and *P. ostreatus* var. 'Florida' showed the highest laccase activity at a different pH values. Many studies have been carried out on the efficacy of fungi to produce laccase and its various applications in degradation and decolourisation of synthetic dye. Investigating the dyes decolourisation efficacy of extracellular laccase produced by the three selected white rot fungi we found that these were able to discolouring BB dye. Only *G. applanatum* was able to discolouring both BR and BY dyes. These studies underline the need to explore more laccase-producing fungi which could be involved in the textile dyes degradation.

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