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Enhancement of laccase production and malachite green decolorization by co-culturing *Ganoderma lucidum* and *Trametes versicolor* in solid-state fermentation

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ABSTRACT

Optimization of laccase production in white-rot fungi has been extensively studied. Metallic and aromatic compounds have been found to enhance enzyme production, but the development of bioremediation as an application field of this enzyme requires clean technologies. In this work, co-cultivation of *Ganoderma lucidum* and *Trametes versicolor* was performed, showing remarkable enhancement of laccase activity. Dual cultures were assayed for malachite green degradation (MG) in solid state fermentation (SSF) using a sawdust-based medium. The time for achieving complete decolorization of MG in co-cultivation was markedly shorter than that observed in monocultures. Dual-species treatment did not differ in wood dry weight loss and lignin, cellulose and hemicellulose degradation, compared to monocultures; but the selectivity index (lignin loss/cellulose loss) of dual cultures was markedly higher than those attained by monocultures. Moreover, a modified isoenzymatic laccase pattern was observed, showing one isoenzyme that was absent in monocultures. Dual cultures were able to decolorize and detoxify the dye more efficiently than the monocultures. The noticeable increase in laccase activity along with the more efficient decolorization and detoxification of MG by co-cultures of *G. lucidum* and *T. versicolor* in SSF makes this system a viable strategy for large scale application of white-rot cultures in bioremediation.

1. Introduction

White-rot fungi (WRF) are the most efficient degraders of recalcitrant biopolymers such as lignin, due to their extracellular non-specific and non-stereoselective enzyme system composed by laccases and high redox peroxidases such as lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), and new representatives like dye-decolorizing peroxidases (DyPs), which function together with H_2O_2-producing oxidases and secondary metabolites (Liers et al., 2013). The same unique nonspecific mechanisms that give these fungi the ability to degrade lignin also allow them to degrade a wide range of pollutants, including: polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), explosives, pesticides and dyes (Singh, 2006). The enzyme laccase belongs to a family of multicopper oxidases, which preferentially oxidize phenolic lignin dimers (Li et al., 1999). Laccase oxidizes model lignin compounds if appropriate primary substrates such as ABTS [2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)], violuric acid (Pogni et al., 2007) or HBT (1-hydroxybenzotriazole) are present (Bourbonnais et al., 1998). Under these conditions laccase can oxidize substrates that are restrictive to LiP, such as veratryl alcohol or PAHs (Johannes et al., 1996). Because of their high non-specific oxidation capacity and the high yields obtained in cultures, laccases are useful biocatalysts for a wide range of biotechnological applications, including dye decolorization, detoxification of a wide range of environmental pollutants, pulp bleaching and black liquor decolorization (Giardina et al., 2010). Laccases secreted by white-rot fungi are generally the result of isoenzymes encoded by complex multi-gene families (Lettera et al., 2010). These laccase isoenzymes usually have slight differences in their catalytic properties along with different regulatory mechanisms and localization. Their synthesis and secretion are strictly influenced by nutrient levels, culture conditions, developmental stage, as well as by the addition of a wide range of

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inducers to culture media. Most of these factors have been shown to act at the level of transcription, producing different responses among different isoenzymes in the same strain and different fungal species as well (Piscitelli et al., 2011). Activation of Cup1 expression protein (ACE1) seems to be responsible for copper regulation of laccase synthesis since in some species such induction occurred even in absence of metal responsive element (MRE) sites in the laccase promoter region (Litvintseva and Henson, 2002). Although copper is the most efficient enhancer of laccase production, the use of this metal is not suitable for application in bioremediation processes due to its toxicity. Interspecific interactions between white-rot fungi and other fungi or bacteria have shown to induce laccase activity (Baldrian, 2004; Zhang et al., 2006; Carabajal et al., 2012). Biotechnological processes such as biopulping have also shown to be improved by co-culturing. Co-cultivation of fungi could imply oxidative stress on both fungal partners, and it may accelerate a fungal metabolic switch to secondary metabolism, thus stimulating wood decay and production of lignin-degrading enzymes (Chi et al., 2007).

Malachite green (MG) is a triphenylmethane dye used as a fungicide which also possesses a high toxicity to mammalian cells (Papinutti and Forchiassin, 2004). Decolorization of MG and other compounds in relation to laccase activity was studied in different species of the genus *Trametes* (Levin et al., 2005; Maalej-Kammoun et al., 2009; Yang et al., 2009). MG degradation mediated by laccase of *Ganoderma lucidum* has shown to be induced by phenolic compounds (Murugesan et al., 2009). The goal of this work was to study the effects of the interaction of *Trametes versicolor* and *G. lucidum* on the production of the ligninolytic enzymes laccase and MnP, in solid state fermentation and to evaluate its application to degradation of lignocellulosic residues and the industrial dye MG.

2. Materials and methods

2.1. Fungal strains and culture conditions

G. lucidum strain E47 (University of Guelph, Canada) and a new isolation of *T. versicolor* (BAFC 4272, deposited in the Culture Collection of the University of Buenos Aires) were used in these experiments. Stock cultures were maintained on MEA medium [malt extract (1.2%) agar (2%)] slants at 4 °C with periodic transfer. The organisms were cultivated in solid-state fermentation (SSF) using poplar sawdust as substrate. Humidity in the substrate was adjusted to 75% (w/w) before autoclaving 1 h at 121 °C. After cooling, media were inoculated with 5% (wet weight) of spawn grown on oat seeds. Cylindrical flasks containing inoculated solid substrate were incubated at 28 °C. After 14 days of undisturbed growth, the whole cultures were mechanically disrupted using a domestic food processor. Co-cultures were conducted by mixing and processing equal amounts of SSF media of each strain. Unmixed processed media of both species were used as control monocultures. The co-culture and monocultures were incubated for other 14 days at 28 °C.

2.2. Laccase assay in plates

Qualitative laccase activity assay was performed on mycelia of *G. lucidum* and *T. versicolor* interacting with each other in pair in agar plates containing MEA and PGA (100 g l^{-1} boiled potato extract, agar 20 g l^{-1}, dextrose 10 g l^{-1}) media. The mycelia were flooded with 5 mM of 2,6 dimethoxyphenol (DMP) for 5 min and washed away with distilled water. An orange coloration, which developed instantly at 25 °C, indicated extracellular laccase activity. The intensity of the color was assessed visually and expressed in arbitrary units ranging from 0 to 5 according to Davidson et al. (1938), which gives a qualitative estimation of laccase activity. In order to determine whether enhancement is triggered by proximity or by contact between hyphae of both strains, cultures in the presence of thermally inactivated colonies of the other species were performed.

2.3. Quantitative enzyme assays

Laccase activity (E.C:1.10.3.2) was determined by measuring the increase in A468 ($\varepsilon 468 = 49.6\ mM^{-1}\ cm^{-1}$) due to the oxidation of DMP 5 Mm in 0.1 M Na–acetate buffer (pH 3,6) at 30 °C (Edens et al., 1999). One unit of laccase activity (U) was defined as the amount of enzyme required to oxidize 1 μmol of DMP in 1 min. MnP activity was assayed with 0.1 mM $MnSO_4$ and H_2O_2 and 0.01% Phenol Red as the substrate at 30 °C (Paszczyński et al., 1988). Reactions were halted by adding NaOH 5 N, and increase in A_{610} was measured ($\varepsilon_{610} = 22\ mM^{-1}\ cm^{-1}$). One unit of enzyme activity (U) was defined as the amount of enzyme required to oxidize 1 μmol of Phenol Red in 1 min. LiP activity (E.C:1.11.1.14) was measured by the decrease in absorbance at 651 nm due to the oxidation of Azure B Archibald (1992). To detect activity in solid cultures, aliquots of extracts were used in reaction mixtures. For agarized media, agar plugs with mycelium were added to 2.5 ml of the reaction buffer containing the enzyme substrate (at a ratio of 20 mg plug per ml of reaction buffer). Samples were taken from different Petri dishes for each of the following interaction zones: contact zone, interaction side, non-interaction side and edge. Samples were stored at −20 °C until used. For the extraction of extracellular proteins, 500 mg of solid samples from cultures were stirred 30 min at 20 °C with 2.5 ml of distilled water, followed by centrifugation and filtration. Supernatant was stored at −20 °C until needed for assays.

2.4. Analytical determinations

Samples of SSF wasted media were dried at 50 °C and used for composition analyses. Triplicate independent samples (approximately 500 mg of homogenized substrate) were collected periodically from randomly selected flasks. Hemicellulose was determined according to Cheng et al. (2010). Klason lignin was quantified according to the TAPPI method (TAPPI method: Acid insoluble lignin in wood and pulp T222 om-83 1983). Cellulose was calculated by subtraction [100% − (hemicellulose + Klason lignin)].

2.5. Native polyacrylamide gel electrophoresis (native-PAGE)

Electrophoretic separation was performed in 9% polyacrylamide gels using Pierce Blue Protein Molecular Weight Markers (Thermo Scientific, Rockford, USA). Acrylamide gel was fixed in a mixture of methanol:acetic acid:water (1:1:1) for 5 min, then immersed in a solution of 5 mM DMP in acetate buffer, pH 3.6 and incubated at room temperature (25 °C) until laccase activity bands appeared. Equivalent laccase activities were (approximately) inoculated in each well, and so the intensity of the bands does not represent the total activity in the supernatant.

2.6. Decolorization assay

Decolorization and adsorption of MG was investigated in a batch system. Two g of SSF media were transferred to 100-ml Erlenmeyer flasks containing 30 ml of MG 50 μM in acetate buffer pH 3.6. Dye decolorization was measured spectrophotometrically at 618 nm in the aqueous solution. To quantify the amount of adsorbed MG to the mycelium and solid substrate, samples of solid media were placed in a desorption medium consisting of ethanol:water (1:1) and stirred for 60 min, the concentration of MG in the desorption

medium was spectrophotometrically measured as described above using as baseline the alcoholic desorption of wasted media previously treated with water instead of MG.

2.7. Toxicity against Phanerochaete chrysosporium

In order to verify that the decolorization was not achieved by reducing MG into its equally toxic leucobase, culture media were made using the decolorized solutions (see 2.6.) with the addition of malt extract (12 g l^{-1}) glucose (10 g l^{-1}) and agar (20 g l^{-1}) and inoculated with *P. chrysosporium* BKMF-1767 (ATCC 24725, American Type Culture Collection), according to the method described by Papinutti and Forchiassin (2004).

2.8. Statistical analysis

All data presented are the means of the results of three replicates with a standard deviation of less than 5%. Significant differences between treatments were compared by Fisher's LSD multiple range test at 5% level of probability.

3. Results

3.1. Qualitative estimation of oxidase activity

Incubation of agar cultures in DMP 5 mM buffer phosphate pH 3.6 showed an orange brown halo surrounding the interaction zone that reveals enhanced production of oxidase activity. The magnitude of the oxidation inferred through the color intensity in arbitrary units is shown in Table 1. Control monocultures showed lower color intensity in the entire surface covered by the colony with a slight increase in the marginal zone. Incipient contact between colonies showed no enhanced oxidation. Noticeable differences were achieved after the third day of contact.

In co-cultured plates, oxidation of DMP was higher in the contact zone of mycelia and the intensity of orange color increased further with incubation time (Table 1). In contrast, in control mycelia (monoculture) activity was lower at the front and in contact with the glass. No enhancement of laccase activity was detected in mycelia before contact. Confrontation between one strain and the thermally inactivated mycelium of the other strain, did not produce enhancement, nor did contact between the strains and the lateral glass of the Petri dishes.

3.2. Laccase quantification in plates

Laccase quantification in agar explants taken from confronted agar cultures showed a significantly higher activity in the contact zone of mycelia, as shown in Fig. 1, which was consistent with the pattern observed when treating the culture plates with DMP. This increase in laccase activity was only achieved after physical contact of both growing strains, since no laccase stimulation was observed in pre-contact stages of growth.

3.3. Laccase activity and MG decolorization using lignocellulosic solid media

Wasted solid culture media based on poplar sawdust as substrate and oat seeds as inoculum, from mono- and co-cultures significantly decolorized MG 50 μM after different incubation periods (Fig. 2). *T. versicolor* wasted media reached the 80% of the absorbance of the original solution in 13 h, and *G. lucidum* in 16 h, while the co-culture wasted media achieved the same level of decolorization in 3 h. Data of decrease in absorbance were fitted to one phase decay equation for calculating half-lives of MG

Table 1
Color reaction of agar cultured at different distances from the contact line after 5, 10 and 15 min scored as (0) = no brown decolorization of the agar under or about the mat, (1) = diffusion zone light brown (2) = diffusion zone dark brown visible from the underside only, (3) = diffusion zone dark brown visible from the upper side, (4) = diffusion zone very dark brown and opaque (5) = diffusion zone intensely dark brown, opaque, with crystals of oxidized substrate. Positive values of distance to the contact zone (Dist.) represent the direction to the inoculum of *T. versicolor* and negative to the inoculum of *G. lucidum*. Color reaction scores are shown as co-culture/control. No further increase in intensity was observed.

Dist. (cm)	5′	10′	15′
−3	1/0	1/0	1/0
−2	0/0	1/0	2/0
−1	0/0	1/0	1/1
0	0/–	4/0	5/–
1	0/0	1/1	3/1
2	1/0	1/0	2/0
3	0/0	0/0	1/0

decolorization as the time needed for the solid medium to reduce the absorbance of the solution to one half of the original value. The half-life calculated for co-culture was much lower than that of the respective monocultures, showing the potential of the co-culture in bioremediation applications. The absorbance of the alcoholic

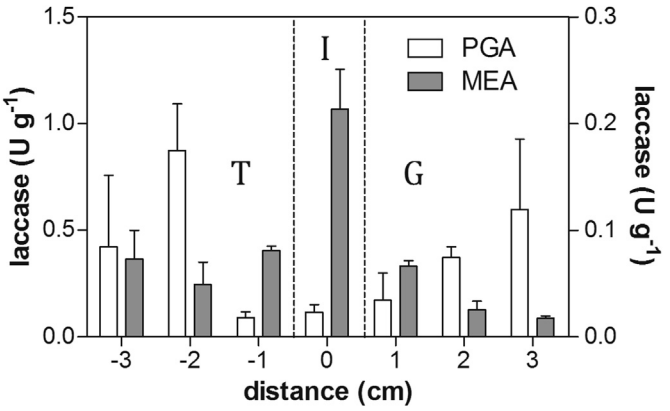

Fig. 1. Laccase activity (U g^{-1}) of co-cultures at different distances of the contact zone in MEA (left axis) and PGA (right axis). Bars represent standard deviation.

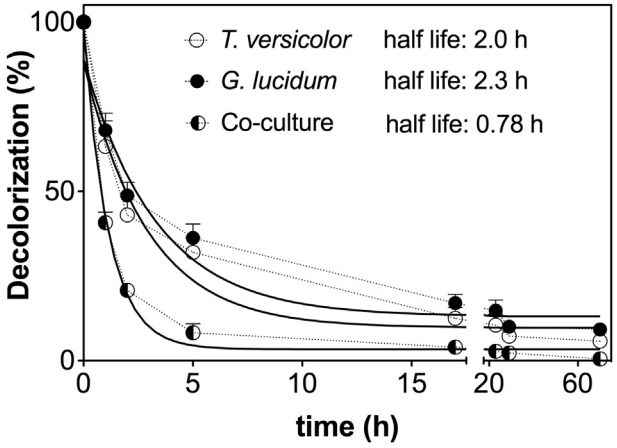

Fig. 2. Decolorization of an MG 50 μM solution by bio-treated sawdust-based solid media of single cultures of *G. lucidum* and *T. versicolor* and of the co-culture. Bars represent standard deviation and solid lines correspond to the fitted monophasic decay equations. Half-life was calculated as the time that it takes to reduce the absorbance to 1/2 of the original value.

elution of the remaining solid media was 3% (co-culture) of the MG solution, 7% (*T. versicolor*) and 9% (*G. lucidum*) above the desorption solution of the wasted medium. Similarly to that observed in plates, laccase activity was also enhanced in co-cultures cultivated in sawdust (Table 2). After 14 d of spawn mixing, laccase registered in co-culture was up to 9 times higher than those measured in the respective monocultures. MnP activity was only detectable in *T. versicolor* monocultures and no LiP activity was detected.

3.4. Detoxification of MG

A bioassay was used to verify the loss of toxicity of the decolorized solution. The sensitive strain *P. chrysosporium* was able to grow at 37 °C in the agarized media detoxified by the co-culture, reaching 1.4 ± 0.22 diam. after 5 days, but in those treated with wasted monocultures media the colony consisted of short hyphae emerging from the inoculum (*G. lucidum*: 0.3 ± 0.09 cm, *T. versicolor*: 0.2 ± 0.04 cm). No growth was observed in the controls treated with boiled wasted media of the three treatments, although a slight decolorization due to adsorption was verified.

3.5. Effect of co-cultivation on laccase extracellular protein profile

To study the effect of co-cultivations on laccase protein secretion, native-PAGE profiles were obtained for all cultivations (Fig. 3). Native-PAGE separation of proteins eluted from wasted co-cultured solid media revealed a band with laccase activity after incubation in DMP (140 kDa), which was absent in elution supernatants of wasted media of the strains in monocultures. This band showed lower electrophoretic mobility than those of *G. lucidum* and *T. versicolor* growing separately.

3.6. Spectrum analysis

Absorption spectra of an MG solution decolorized by wasted solid media of monocultures of both species showed a notable decrease in the absorbance, but the two characteristic peaks (445 nm and 621 nm) were still present (Fig. 4). After treatment of the same original solution with the wasted solid media from the co-culture, the decolorization was complete and no characteristic peak of MG remained. However, as already remarked in 3.4, the solid residue retained a small amount of the original absorbance.

3.7. Wasted media composition analysis

Compositional analysis of media consisting in poplar sawdust and wasted culture media of both species and co-culture showed that weight losses are not related to one fraction but to the whole wood matter. Fig. 5 shows that neither delignification nor weight loss was influenced by the improved laccase production attained in co-cultivation. Monocultures of *T. versicolor* caused 42% of dry weight loss (the highest observed value), while no significant differences were detected between monocultures of *G. lucidum* and the co-culture. A similar trend was also observed in dry weight loss of Klason lignin. Although the higher production of laccase in co-cultures did not cause higher lignin dry weight loss, selectivity index (lignin loss/cellulose loss) of dual cultures was markedly higher than those attained by both monocultures.

Table 2
Laccase and MnP activities (U g^{-1} ± SD) of wasted solid media after 14 days of undisturbed growth plus 14 days after processing and mixing. No LiP activity was detected.

Treatment	Lacasse	MnP
G. lucidum	2.24 ± 0.28	0.00
T. versicolor	0.86 ± 0.12	0.13 ± 0.05
Co-culture	7.93 ± 0.65	0.00

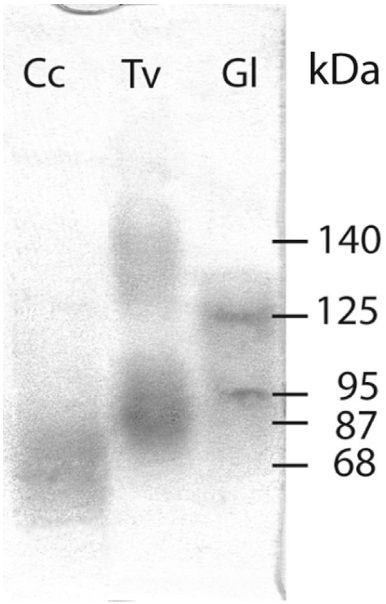

Fig. 3. Native-PAGE separation of laccase isoenzymes eluted from bio-treated sawdust with co-culture (Cc) and single strain cultures of *G. lucidum* (Gl) and *T. versicolor* (Tv). Molecular weight markers are detailed in Materials and methods.

4. Discussion

Although enhanced laccase activities as a result of interaction between white-rot fungi have been previously reported (Li et al., 1999; Litvintseva and Henson, 2002; Baldrian, 2004; Chi et al., 2007), there are no reports focusing on their application to dye decolorization and detoxification. The system proposed in this paper was able to accomplish the complete removal of the industrial dye MG. In addition, noticeable toxicity reduction of the treated solutions was also successfully detected. The nature of this enhancement is not yet fully understood; the induction of different laccase isoenzymes has already been reported in other fungal systems such as *Phlebia radiata*/*Dichomitus squalens* (Dong et al., 2012), other works (*e.g.* He et al., 2010) have found an equivalent

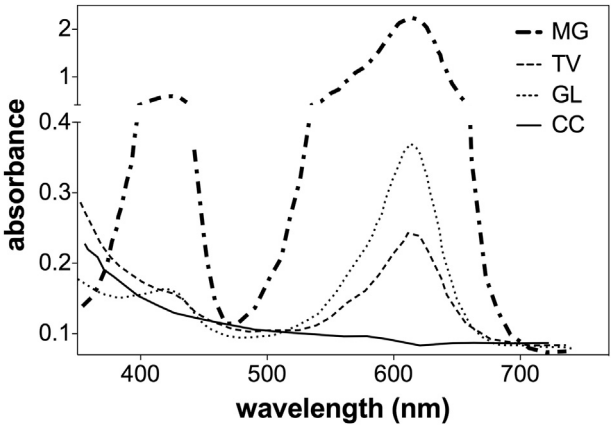

Fig. 4. Absorption spectra of an MG 50 μm solution and of the same solution decolorized by wasted solid media of single strain cultures of *G. lucidum* (GL) and *T. versicolor* (TV), and of co-culture (CC).

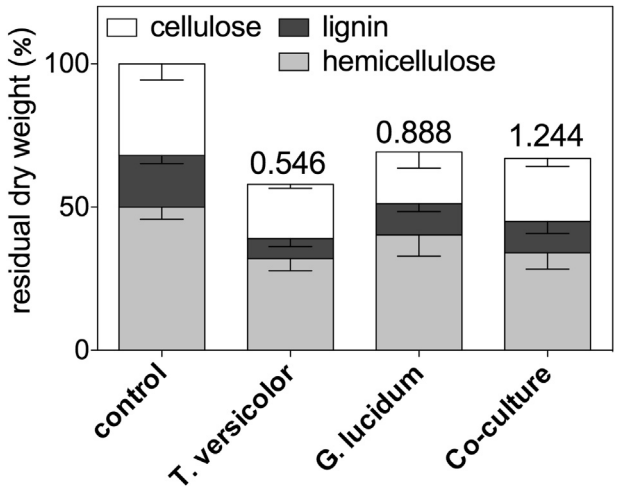

Fig. 5. Chemical composition of control (non-inoculated) and bio-treated sawdust. Hemicellulose, lignin and cellulose fractions are shown proportionally to the total dry weight loss, represented by the high of each entire column. Numbers on the columns are the selectivity indexes (calculated as lignin loss/cellulose loss ratio). Bars represent standard deviation of the mean calculated from three independent replicates.

isoenzymatic pattern. In the present work, co-cultures of *G. lucidum/T. versicolor* produced one new isoenzyme, thus the isoenzymatic pattern was completely different from those observed in monocultures. Interaction by means of diffusible factors can be ruled out by the fact that no enhancement of laccase was detected before physical contact of both strains. This fact also dismisses the enhancement by succession of carbon sources described by Chi et al. (2007). Moreover, contact with a thermally inactivated mycelium did not increase laccase activity, showing that living microorganisms are required for the increase in activity. Similar results were previously found by Baldrian (2004) when studying the effects of interspecific interactions between white-rot fungi and other microorganisms on laccase activity. The band with lower electrophoretic mobility detected in dual cultures, seems to correspond to the aromatic-induced laccase band, found by Kuhar and Papinutti (2014) in the same strain of *G. lucidum*, and this would suggest a similitude between the induction by co-cultivation and aromatic compounds, but further analyses have to be undertaken in order to establish the equivalence of these bands. None of the co-culturing conditions assayed in this work enhanced the activity of the peroxidases assayed (Lip and MnP) (evaluated after 28 days of incubation), nevertheless ligninolytic activities may vary along fermentation. On the contrary, previous reports have shown that MnP produced by co-cultures of *Trametes* sp. with *Chaetomium* sp. was significantly promoted (Chi et al., 2007). Therefore different fungal combinations and culture media should be considered according to the particular requirements of enzyme activities. Laccase enhancement is not only dependent on fungal species but also on the culture medium, which can either increase or decrease laccase production. For example, laccase production did not show any increase from interaction in PGA medium. This significant contrast, with respect to MEA medium, emphasizes the complexity of the physiological processes involved in the enhancement of laccase production as the observable response. The results from the experiments on plates highlight the importance of the extent of contact zones between growing mycelia for achieving increased laccase activities. Co-inoculation of two *Pleurotus* species without further active spawn mixing after colonization showed no enhancement of laccase activities (Papinutti and Forchiassin, 2004). Although ABTS showed to be a more sensitive substrate for many laccase isoenzymes (*e.g.* Eichlerová et al., 2012), might not be adequate for revealing a slow oxidation processes, as in the plate test described in the present work, due to its instability and the diffusion of the colored products. Since the products of the laccase activity on DMP crystallize after a few minutes, a higher definition of differential activity in agarized cultures can be achieved, especially after long incubation times. This substrate was used in the subsequent experiments in order to measure and visualize the same process. For an accurate comparison of laccase yields against other processes, corresponding substrates should be used.

Opposite to the expected, enhanced laccase activity did not cause higher lignin loss from wood. Probably, incubation time should be longer for detecting a differential pattern of lignin degradation in correlation with this enzyme activity. Similar uncorrelated results were observed in co-cultures of other white-rot fungi where results obtained from ligninolytic enzymes could not be directly associated with lignin degradation (Chi et al., 2007; Levin et al., 2007). However, with regard to the comparison of the selectivity index, the co-culture attained up to a 2-fold higher index value compared to monocultures. As revealed in the Native-PAGE, the pattern of isoenzyme production was entirely different in co-culture compared to respective monocultures. Since laccases are involved in different physiological processes, such as conidial pigmentation in *Aspergillus nidulans* (Kurtz and Champe, 1982), phytopathogenic processes in *Gaeumannomyces graminis* var. *tritici* (Edens et al., 1999) and fructification in *Lentinus tigrinus* (Lechner and Papinutti, 2006), it is likely that laccase isoenzymes produced as result of fungal interspecific interaction were not able to cause higher lignin dry weight loss. However, stimulation of laccase in co-cultures was able to more efficiently decolorize MG. Decolorization of MG was accelerated up to 3 times compared to the respective monocultures. Since MG can easily be transformed into its uncolored form, leucomalachite green, without decreasing its toxicity (Srivastava et al., 2004), it is necessary to verify that the decolorization is followed by detoxification. The toxicity of the colorless reaction products was assessed, as in previous reports, in *P. chrysosporium* (Papinutti and Forchiassin, 2004; Maalej-Kammoun et al., 2009). Dual cultures were able to decolorize and detoxify the dye more efficiently than the monocultures.

Optimization of ligninolytic enzyme production using copper, manganese and other metallic ions has been extensively studied on white-rot fungi (Papinutti and Forchiassin, 2006; Janusz et al., 2013; Kuhar and Papinutti, 2014). Despite the high efficiency in enzyme promotion, the use of metals and xenobiotic compounds as inducers would represent an inconsistency for bioremediation processes due to the contamination of the wasted media generated by the addition of those heavy metals and xenobiotic compounds. Thus, induction through co-culture could be positioned as a cleaner and also economically convenient alternative. The noticeable increase in laccase activity along with the more efficient decolorization and detoxification of MG by co-cultures of *G. lucidum* and *T. versicolor* in SSF make this system a viable strategy for large scale application of white-rot cultures to bioremediation and laccase production for industrial uses.

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