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# Decolorization of remazol brilliant blue R with laccase from *Lentinus crinitus* grown in agro-industrial by-products

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**ABSTRACT**

*Lentinus crinitus* is a white-rot fungus that produces laccase, an enzyme used for dye decolorization. Enzyme production depends on cultivation conditions, mainly agro-industrial by-products. We aimed to produce laccase from *Lentinus crinitus* with agro-industrial by-products for dye decolorization. Culture medium had coffee husk (CH) or citric pulp pellet (CP) and different nitrogen sources (urea, yeast extract, ammonium sulfate and sodium nitrate) at concentrations of 0, 0.7, 1.4, 2.8, 5.6 and 11.2 g/L. Enzymatic extract was used in the decolorization of remazol brilliant blue R. CH medium promoted greater laccase production than CP in all evaluated conditions. Urea provided the greatest laccase production for CH (37280 U/L) as well as for CP (34107 U/L). In CH medium, laccase activity was suppressed when carbon-to-nitrogen ratio changed from 4.5 to 1.56, but the other nitrogen concentrations did not affect laccase activity. For CP medium, reduction in carbon-to-nitrogen ratio from 6 to 1.76 increased laccase activity in 17%. The peak of laccase activity in CH medium occurred on the 11$^{th}$ day (41246 U/L) and in CP medium on the 12$^{th}$ day (32660 U/L). The maximum decolorization within 24 h was observed with CP enzymatic extract (74%) and with CH extract (76%).

**Key words:** Dye decolorization, ligninolytic enzymes, remazol brilliant blue R, white-rot fungi.

## INTRODUCTION

There is a worldwide trend to develop more efficient productive chains, where the management of industrial residues favors the economic and environmental sustainability of processes. The efficient utilization of agro-industrial by-products and wastes allows the appreciation of this material and represents an alternative to generate highly-aggregated value products with the recovery of chemicals and metabolites through biotechnological processes (Federici et al. 2009). Several processes have been developed for this purpose such as mushroom production, organic acids, biofuels, and enzymes (Rodrigues et al. 2009). The great availability of carbon allows agro-industrial by-products to be used as substrate or support for fermentation processes, and it may reduce costs and environmental impacts on industrial production (Pandey et al. 2000).

In Brazil, by-products from coffee and citric industries are abundant sources of nutrients and

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were chosen for this study. Brazil produced more than 51 million coffee bags in 2016 (CONAB 2017). Coffee husks are obtained when coffee cherries are dried and the coffee beans are separated from the pericarp by hulling (Pandey et al. 2000) representing 12% of the fruit dry mass. Coffee husks have been utilized successfully in different bioprocesses to produce mushroom, citric acid, ethanol, aroma compounds, enzymes, to name but a few (Murthy and Naidu 2012). Citric pulp consists of a mixture of orange peels, seeds and bagasse from the orange juice processing. The yearly volume of this by-product is approximately 2 million tons, and in 2016 the Brazilian industry produced more than 860 thousand tons of concentrated orange juice. Citric pulp is mainly utilized in animal feeding, production of citric acid (Torrado et al. 2011), and pectin (Kaya et al. 2014), besides being an excellent substrate for enzymatic production (Spier et al. 2008).

White-rot basidiomycetes are known for their efficient ligninolytic system that counts with the secretion of extracellular oxidases such as laccase, manganese peroxidase, lignin peroxidase and versatile peroxidases. This enzymatic set cooperates to degrade lignin, and each enzyme could be characterized by a wide substrate range (Leonowicz et al. 1999). Therefore, these enzymes are able to act on different molecules with similar lignin structure such as polycyclic aromatic hydrocarbons, synthetic polymers, and dyes (Bazanella et al. 2013). Laccases (EC 1.10.3.2, *p*-diphenyl: dioxygen oxidoreductases) are stable and require simple conditions for catalysis, making them suitable and attractive for biotechnological applications (Majeau et al. 2010) in the food, paper and cellulose, textile and bioremediation industries (Couto and Toca-Herrera 2006). However, biotechnological applications require a great quantity of enzymes at a low cost. Thus, the search for more efficient processes and the expansion of agro-industrial by-product utilization on ligninolytic enzyme production are stimulated (Kapich et al. 2004, Valle et al. 2014b).

An important aspect of ligninolytic enzyme production by white-rot basidiomycetes is the nature and concentration of nitrogen. These microorganisms prefer simple sources of nitrogen instead of more complex ones and this seems to affect the enzyme production (Hernández et al. 2015). However, there are reports of laccase production in the presence of non-protein nitrogen sources (Hou et al. 2004, Valle et al. 2014a) as well as in the presence of protein nitrogen sources (Sarnthima and Khammuang 2013, Hernández et al. 2015), and the fungal response depends on the evaluated species and strain. This shows the importance of investigating which is the best nitrogen source in the culture medium when the objective is to produce laccase. Besides the appropriate source, the concentration of available nitrogen plays an important role in the laccase expression. On the other hand, when investigating this cultivation parameter, it is necessary to consider that the microorganism response varies among the species and strains and depends on other cultivation conditions (D'Agostini et al. 2011).

Our research team has sought for fungal strains and cultivation conditions that broaden laccase production for dye degradation. *Lentinus crinitus* is a white-rot fungus found all over Brazil and we verified its high ability to produce laccase compared to other basidiomycetes and also the effects of different nitrogen concentrations in the production of this enzyme (Valle et al. 2014a). Its ability to degrade dyes was also reported (Niebisch et al. 2010). However, its submerged cultivation in media supplemented with agro-industrial by-products has not yet been described. In this study, we followed *L. crinitus* laccase production in submerged cultivation with two agro-industrial by-products as the only carbon sources and different sources and concentrations of nitrogen. The enzymatic extract was used in recalcitrant remazol

brilliant blue R decolorization in order to find alternatives to reduce the environmental impact of dyes.

## MATERIALS AND METHODS

### MICROORGANISM

*Lentinus crinitus* (L.) Fr. (Polyporaceae) strain U9-1 from the culture collection of the Molecular Biology Laboratory of Paranaense University was utilized for laccase production. Mycelium utilized as inoculum was cultivated on malt extract agar medium (MEA) 2% (mass/volume) at 28 ± 1 ºC, in the dark for seven days. Four MEA disks (6 mm diameter) containing mycelia were utilized as inoculum for laccase production.

### LACCASE PRODUCTION BY SUBMERGED CULTIVATION WITH AGRO-INDUSTRIAL BY-PRODUCTS

Laccase production occurred in Erlenmeyer flasks (250 mL) containing 100 mL of culture medium consisting of 1.5 g/L $KH_2PO_4$, 0.5 g/L $MgSO_4$, 0.5 g/L KCl, 0.036 g/L $FeSO_4$, 0.035 g/L $ZnSO_4$ and agro-industrial by-products (50 g/L) such as coffee husk (CH) or citric pulp pellet (CP) as the only carbon source. CH came from a dry-processed coffee industry and CP from an orange juice industry. Both CH and CP were ground in a blender and the utilized granulometry varied from 0.8 mm to 2.0 mm. Solutions of nitrogen compounds were filtered (0.22 μm pore membrane) and added to the previously sterilized culture medium (autoclaved at 121 ºC for 20 min) in sufficient amount to obtain final nitrogen concentration of 2.8 g/L (Valle et al. 2014a). Urea (UR), yeast extract (YE), ammonium sulfate (AS), or sodium nitrate (SN) was used as nitrogen source.

After selecting the best nitrogen source for laccase production, the effect of different concentrations of nitrogen on the enzymatic activity was verified. The nitrogen solution, previously filtered (0.22 μm pore membrane), was added to CH or CP culture medium (previously autoclaved at 121 ºC for 20 min) in sufficient volume to obtain final nitrogen concentration of 0, 0.7, 1.4, 2.8, 5.6 and 11.2 g/L. All assays were kept for 12 days at 28 ± 1 ºC, in the dark without agitation, and at the end of this period, laccase activity was determined.

The best nitrogen source and concentration were utilized in the culture media with CH and CP in order to determine the laccase production throughout 12 cultivation days. The enzymatic extracts were obtained by centrifugation of the cultivation media at 8000 *g* for 10 min at 4 °C. The enzymatic activity was determined every 24 h and in the same interval the extract was utilized to determine pH and reducing sugar contents, using the method of 3,5-dinitrosalicylic (Miller 1959). The assays followed a completely random design with three replications. The results were evaluated using analysis of variance (ANOVA) and significant differences among arithmetic means were determined by Scott-Knott test at 5% of probability.

### COFFEE HUSK AND CITRIC PULP CHARACTERIZATION

The nitrogen concentration of CH and CP was determined by Kjeldahl's method, ashes by burning at 550 ºC, and moisture by drying at 105 ºC until constant mass (Zenebon et al. 2008). Considering that 50% of the dry organic mass corresponds to the by-product carbon mass, the results of the chemical analysis were used to calculate carbon-to-nitrogen (C/N) ratio (Gerrits 1988).

### LACCASE ASSAY

The oxidation of 2,2'-azino-bis3-ethylbenzothiazoline-6-sulfonic acid (ABTS; Sigma; St. Louis, MO, USA) was used to determine laccase activity according to Valle et al. (2014a). The reaction mixture contained 0.2 mL enzymatic extract, 0.7 mL water, 0.45 mL sodium

acetate buffer (0.1 M; pH 5.0), and 0.15 mL ABTS (1 mM). The mixture was incubated at 30 °C for 10 min and the ABTS oxidation was followed by increase in absorbance at 420 nm ($\varepsilon$ = 36000 $M^{-1}$ $cm^{-1}$). A mixture of cultivation medium (0.2 mL), water (0.85 mL) and sodium acetate buffer (0.45 mL), and the mixture of water (0.9 mL), sodium acetate buffer (0.45 mL) and ABTS (0.15 mL) were utilized as analytic controls. Enzymatic activities were expressed in international units (U), which is defined as the amount of enzyme that oxidizes 1 µmol substrate per minute.

### MANGANESE PEROXIDASE ASSAY

The oxidation of $MnSO_4$ was used to determine manganese peroxidase (MnP) activity. The reaction mixture contained 10 mM $MnSO_4$ in 50 mM sodium malonate buffer (pH 4.5) and 0.5 mM hydrogen peroxide (Wariishi et al. 1992). The oxidation was monitored by absorbance increase at 270 nm ($\varepsilon$ = 11590 $M^{-1}$ $cm^{-1}$) caused by the complex formed by $Mn^{3+}$ ion with malonate. The mixture of enzymatic extract and sodium malonate buffer and the mixture of $MnSO_4$ and sodium malonate buffer were used as analytical controls. Enzymatic activities were expressed in international units (U), which is defined as the amount of enzyme that oxidizes 1 µmol substrate per minute.

### RBBR DECOLORIZATION ASSAY

The ability of *L. crinitus* U9-1 enzymatic extract to provoke decolorization of remazol brilliant blue R (RBBR) was evaluated according to Marim et al. (2016) with some modifications. The dye was diluted in sodium acetate buffer (100 mM, pH 5) and utilized in sufficient volume to obtain the final concentration of 1 mg/mL (mass/volume) in all assays. The enzymatic extract consisted of liquid culture medium separated from mycelia and solids by centrifugation (8000 *g*, 4 ºC for 10 min). For the decolorization reaction, 3.2 mL extract, 0.4 mL sodium acetate buffer, and 0.4 mL dye solution were mixed and the mixture was kept at 28 ± 1 ºC for 24 h in the absence of light and decolorization was followed in the maximum absorbance of RBBR (595 nm). The percentage of decolorization within 24 h was calculated in relation to the initial absorbance.

## RESULTS

The composition of agro-industrial by-products of CH and CP was in dry basis, respectively 7.1 and 2.9% ashes; 40.0 and 42.1% carbon and 3.2 and 1.4% nitrogen. Thus, the C/N ratio was 12.5 and 30.1 of CH and CP, respectively. The amounts of nitrogen added to the culture medium to obtain the final concentration of each experimental condition were calculated considering the initial amounts of nitrogen of each by-product.

The culture media with CH promoted greater laccase production than the medium containing CP in all the conditions of evaluated nitrogen sources (Figure 1). On the other hand, other ligninases such as MnP and LiP were not detected in any of the cultivation conditions. Laccase production in CH culture medium without additional nitrogen was 33335 U/L. This value was 15% greater than the one with CP (29056 U/L).

Urea was the nitrogen source that provided greater laccase production in CH medium (37280 U/L) as well as in CP medium (34107 U/L) (Figure 1). Only urea (UR) – a non-protein nitrogen source – favored laccase production and promoted an increase in activity of 12 and 17% in relation to the culture media only with CH and CP, respectively. The activity of the culture medium supplemented with UR was greater even when compared to the culture medium with yeast extract (YE) - a protein nitrogen source. For the culture medium added with YE, the activities were 32% and 48.5% lower for CH and CP media, respectively. The effects of the nitrogen source on laccase production were more

evident for CP medium. CP medium containing sodium nitrate, for example, presented a 69% lower production than the culture medium with urea. Thus, CH promoted greater enzymatic activity as well as robust laccase production by *L. crinitus*.

In the culture medium with CH the laccase activity was totally suppressed when C/N ratio was of 1.56 (11.2 g/L of nitrogen). However, other nitrogen concentrations (C/N ratios from 2.8 to 8.7) did not affect the laccase production when CH was used as carbon source. On the other hand, in culture medium with CP the reduction of C/N ratio from 6 to 1.76 with urea, increased laccase activity in 17% reaching 38067 U/L ($p \leq 0.05$), the greater value obtained in culture medium with CP. These findings suggest that the laccase production by the fungus - in function of nitrogen concentration - depends on the carbon substrate found in the culture medium. The greater laccase activities of the culture media of CH and CP were obtained with 0.7 g/L and 11.2 g/L nitrogen. Thus, these concentrations were chosen for the time follow-up of laccase production in CH and CP media.

Laccase production was initially detected on the fourth cultivation day in CH medium and on the fifth day in CP medium (Figure 3). Between the 7th and 8th days of cultivation, there was an increase of 63% in the laccase activity in CH medium, overlapping with the reduction of sugar concentration ($p \leq 0.05$). By the eighth day of cultivation, the laccase productivity of the CH medium had already reached 192 U $L^{-1}$ $h^{-1}$ although the peak of the activity occurred on the 11th day of cultivation (41246 U/L and 156 U $L^{-1}$ $h^{-1}$). The laccase productivity in CH medium was 390% higher than CP medium (39 U $L^{-1}$ $h^{-1}$) on the 8th day. In the CP medium, there was an expressive increase of 123% of laccase activity between the 9th and 10th day, but there was no change in the sugar concentration in this period. The peak of the activity occurred on the 12th day of the cultivation (32660 U/L). The laccase productivity of CH medium (156 U $L^{-1}$ $h^{-1}$) at the activity peak was 38% greater than in the CP medium (113 U $L^{-1}$ $h^{-1}$); also the CH medium anticipated the enzymatic peak reaching a production of more than 20000 U/L after the 6th day. On the other hand, in CP medium the same production level was only reached on the tenth day. The pH varied little, remaining close to 4.0 in CH medium and close to 6.0 in CP medium.

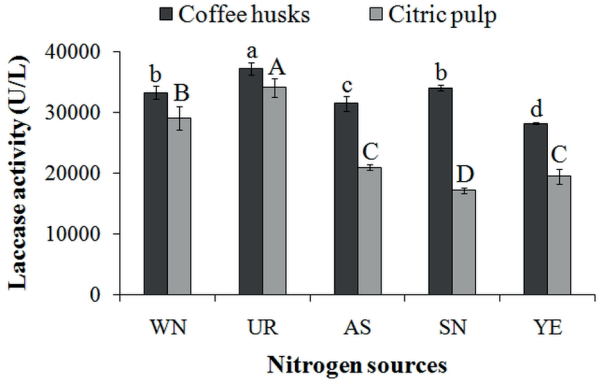


**Figure 1 -** Laccase activity (average ± standard deviation) of *Lentinus crinitus* grown in culture medium added of coffee husk (CH) or citric pulp (CP) (50 g/L) without additional nitrogen (WN) or with different nitrogen sources (2.8 g/L nitrogen) such as urea (UR), ammonium sulfate (AS), sodium nitrate (SN), and yeast extract (YE), after 12 days of cultivation. Averages indicated by the same letter for the same carbon source (CH or CP) do not differ statistically by Scott-Knott's test ($p \leq 0.05$).

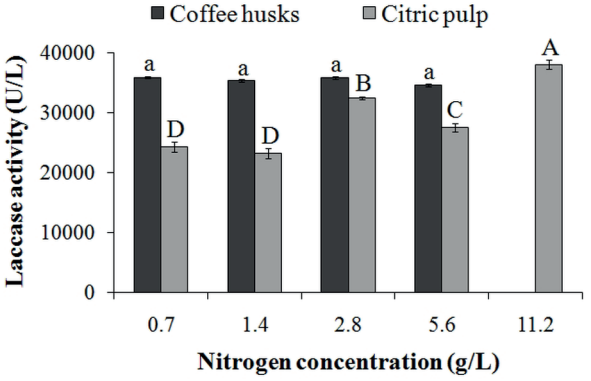


**Figure 2 -** Laccase activity (average ± standard deviation) of *Lentinus crinitus* grown in culture medium added of coffee husk (CH) or citric pulp (CP) (50 g/L) with different nitrogen (urea) concentrations, after 12 days of cultivation. Averages indicated by the same letter for the same carbon source (CH or CP) do not differ statistically by Scott-Knott's test ($p \leq 0.05$).

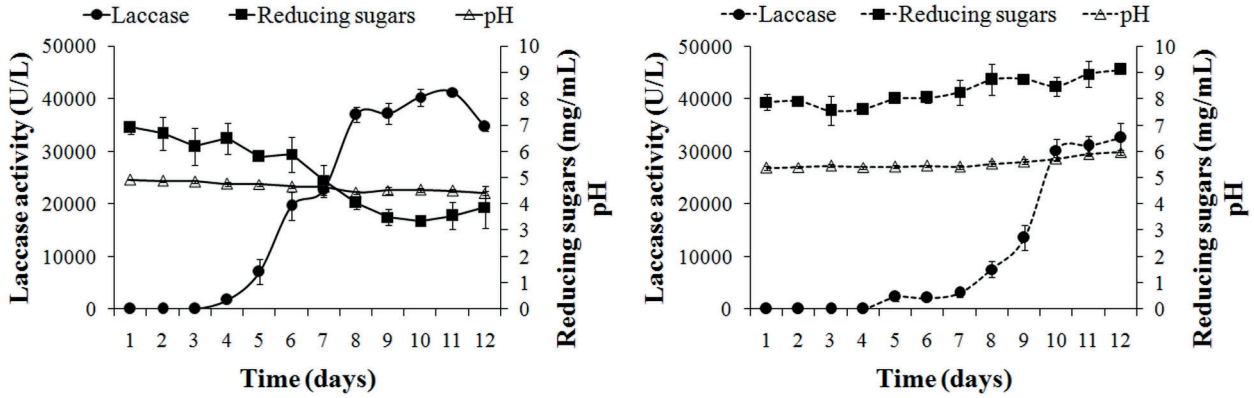


**Figure 3 -** Laccase activity (average ± standard deviation), reducing sugars and pH of culture medium added of coffee husk (left) or citric pulp (right) (50 g/L) and urea in 12 days of cultivation.

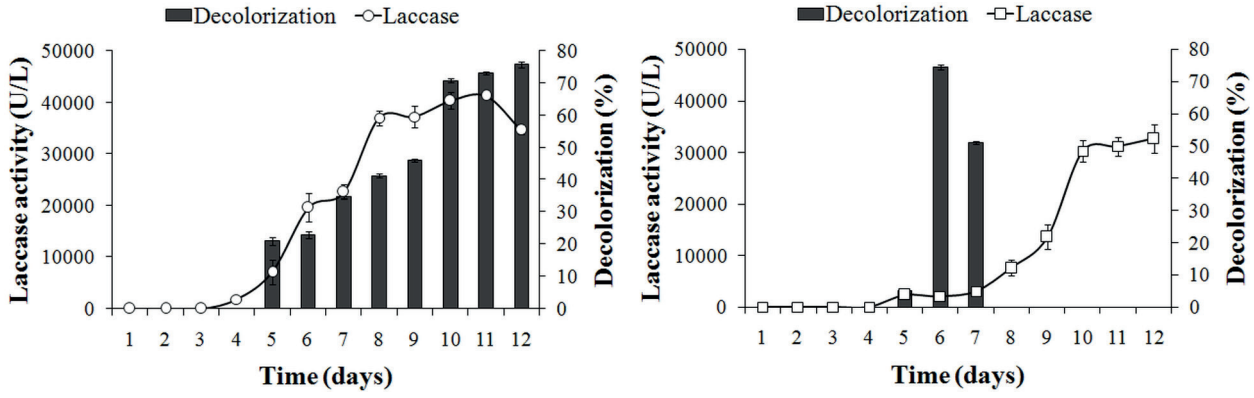


**Figure 4 -** Laccase activity and decolorization of remazol brilliant blue R (RBBR) obtained with the enzymatic extract of *Lentinus crinitus* grown on culture medium added of coffee husk (left) or citric pulp (right) (50 g/L) and urea in 12 days of cultivation.

The enzymatic extract obtained with CP on the 6$^{th}$ and 7$^{th}$ day of cultivation reduced the color of remazol brilliant blue R (RBBR) in 74% and 51%, respectively (Figure 4). On the other days of cultivation, the extract did not cause decolorization of RBBR solution. The extract obtained in CH medium started decolorizing the dye on the 5$^{th}$ day (20%). The higher discoloration value was 76% reached on the 12$^{th}$ day of cultivation. The maximum decolorization, however, did not coincide with the maximum laccase production for CH or CP culture medium. The maximum decolorization observed with the CP extract occurred when the laccase productivity was only 14.5 U $L^{-1}$ $h^{-1}$ while with the CH extract the maximum decolorization occurred when the laccase productivity was 120 U $L^{-1}$ $h^{-1}$.

## DISCUSSION

Our study showed that *L. crinitus* did not produce MnP in the submerged cultivation using media added with two different agro industrial by-products. This is in agreement with Niebisch et al. (2010) that cultivated the same species but with synthetic media supplemented with different nitrogen sources and concentrations and reported production of laccase but not MnP. On the other hand, Conceição et al. (2017) that cultivated *L. crinitus* on solid medium with barley and cassava residues (1:1) reported production of MnP but not laccase. Michniewicz et al. (2006) also demonstrated that *Cerrena unicolor* did not produce MnP in synthetic media with glucose or in complex

culture medium with tomato juice. However, Kachlishvili et al. (2014) observed that *C. unicolor* grown on medium with different carbon sources or lignocellulosic material, produced a great amount of both ligninases. The results suggest that the production of MnP is related to the fungus species and also the cultivation media.

Our study is the first report of submerged cultivation of this basidiomycete in culture medium added with agro-industrial residues as the only carbon source. CH and CP showed to be appropriate for laccase production for this species. However, CH promoted greater activity and robust laccase production. CH contains caffeine (1%), polyphenols (0.8%) and tannins (5%) (Murthy and Naidu 2012). CP contains carbohydrates like pectin and polyphenols (0.08%), mainly flavonoids (0.02%) (Santos et al. 2014). Phenolic compounds – soluble in water – can induce the production of ligninolytic enzymes (Elisashvili and Kachlishvili 2009, Kachlishvili et al. 2014), and these compounds can have induced the laccase production by *L. crinitus*. Still, the greater polyphenol concentrations and tannins described for CH can explain the greater enzymatic activities and productivity obtained with this by-product.

Agro-industrial by-products have been utilized for submerged cultivation of fungi in an attempt to stimulate the production of ligninolytic enzymes. Besides the reduced cost of agro-industrial by-products, these substrates can have high content of non-soluble carbohydrates (cellulose and hemicellulose), lignin and, laccase inducers (Gassara et al. 2010, Postemsky et al. 2017). In our study, the maximum laccase production was 38000 U/L. These activity values are greater than the ones reported by Valle et al. (2014b) utilizing *L. crinitus* in synthetic culture medium (10 g/L glucose, 2.8 g/L nitrogen from urea with ethanol and guaiacol as inducers). The activity values for the synthetic culture medium were 122% lower than the one obtained with CH and 94% lower than in the medium with CP. The results support that the induction of laccase production occurs with lignocellulosic substrates, mainly CH.

The nitrogen source and concentration in the culture medium can affect the laccase production of fungi (Piscitelli et al. 2011). There are reports on greater laccase production with protein nitrogen sources than with non-protein for other species of basidiomycetes (Hernández et al. 2015). In our study, only urea – in detriment to protein sources and other non-protein sources – induced laccase production by *L. crinitus*. Our results are in agreement with the one observed by Valle et al. (2014a) that reported an increase in laccase production by this species in the presence of urea (2.8 g/L nitrogen) in synthetic culture medium. Cambri et al. (2016) cultivated *L. crinitus* in culture media with different carbon and nitrogen sources and concentrations and verified that the combination of maltose and urea was the one that induced the expression of more complex protein profiles. For these authors, the analysis of *L. crinitus* secretome produced with the maltose-urea combination allowed the identification of 10 multicopper oxidases, besides 3 MnP, 2 lignin peroxidases, other oxidases and several carbohydrate active enzymes. Fungi are known for their capacity to utilize alternative sources of nitrogen as urea when preferential sources like ammonium and glutamine are absent, which depends on the gene expression related to the utilization of these sources (Marzluf 1997). It is known that regulatory proteins NIT2 and their orthologues are expressed in the presence of secondary nitrogen sources and act promoting derepression of several enzymes of nitrogen catabolism (Tudzynski 2014). However, it is known that in basidiomycetes the promoter region of laccase codifying genes contains consensus sequences of NIT2-like proteins binding sites, indicating also a possible regulating role of this kind of protein on laccase expression (Soden and Dobson 2003). There is still no available information on the

promoter region of *L. crinitus* laccase genes, but it is possible to suppose that mechanisms that are similar to the described ones are related to the effect of urea on the laccase expression of this species.

In our study, the nitrogen concentration also affected *L. crinitus* laccase production. We observed that the combination between the carbon source (CH or CP) and the nitrogen concentration strongly affects laccase production. Until now there have been no reports on the physiological behavior of *L. crinitus* in submerged cultivation added with agro-industrial by-products, but this species produced laccase when cultivated in synthetic culture medium with glucose and urea and C/N ratio of 14 (Valle et al. 2014a) or C/N ratio of 17 (Niebisch et al. 2010). However, when the authors substituted glucose for fructose, keeping the same C/N ratio, the laccase activity was expressively lower (Niebisch et al. 2010). Our results are in agreement with the ones observed by D'Agostini et al. (2011) who evaluated the laccase production by *Agaricus subrufescens* (*Agaricus blazei*)*, Lentinula edodes* and *Pleurotus ostreatus* in solid culture medium of soybean husks supplemented with different non-protein nitrogen sources. For these authors, the enzymatic activity increased with the reduction of C/N ratio from 30 to 5. Elisashvili et al. (2006) observed that laccase production by *Pleurotus dryinus* grown on submerged cultivation with mandarin peels and yeast extract (0.5 g/L) was not affected by additional nitrogen sources (potassium nitrate, ammonium sulfate, ammonium nitrate or peptone) or concentration (10 mM). Hernández et al. (2015), however, verified that *Pycnoporus sanguineus* grown on submerged culture medium with sugarcane bagasse presented significant reduction of laccase activity when the amount of urea (1 to 3 g/L) is increased to the culture medium.

The follow-up of laccase production for 12 days showed laccase increase after the 6$^{th}$ cultivation day with CH and after the 10$^{th}$ day in CP medium. Ligninolytic enzymes from white-rot fungi are usually expressed during the secondary metabolism (Singh and Chen 2008) and the profile of *L. crinitus* laccase production observed in our study suggests that this also happens in this species. Our results also showed that CH medium anticipated laccase production at least four days compared to the CP medium resulting in significantly higher productivity. Soluble phenolic compounds found in CH medium could act as laccase inducers, altering the standard of produced isoenzymes as well as anticipating the production (Piscitelli et al. 2011). Our results are in agreement with previous findings reporting that phenolic/aromatic compounds derived from lignocellulosic by-products are required for the production of a specific set and greater amounts of ligninases (Kapich et al. 2004). The replacement of glycerol by milled mandarin peels in cultivation media without any other inducer promoted 3- to 45-fold increase in laccase activity of *C. unicolor, Fomes fomentarius, Funalia trogii, Pycnoporus coccineus*, and *Trametes versicolor* (Kachlishvili et al. 2016). Similar results were observed for *C. unicolor, T. versicolor* and *Ganoderma lucidum* cultivated with different inducers (pyrogallol and 2,4,6-trinitrotoluene) that anticipated laccase production peak (Elisashvili et al. 2010). The same was observed with vanillin and veratryl alcohol that reduced the time to reach the laccase production peak from 20 days to 6 and 15 days, respectively (Valle et al. 2015).

The decolorization caused by crude extract obtained in the cultivation with CP was more restrict than with CH. With CP, decolorization was observed only in the beginning of the laccase production whereas the capacity to decolorize RBBR from crude extract of CH medium extended until the end of the cultivation. On the other hand, decolorization produced by CP extract was more efficient, once the greater percentage of decolorization was obtained with a much lower amount of enzyme (14.5 U $L^{-1}$ $h^{-1}$). It is speculated that the observed decolorization results were the outcome of distinct

laccase isoenzyme expression in CH and CP media with different affinities by the substrate and/or different catalytic properties. It is known that isoenzyme production of laccase codified by genes organized in gene families is common in fungi (Piscitelli et al. 2010) and the expression of at least 10 multicopper oxidases in *L. crinitus* has been demonstrated (Cambri et al. 2016). Moreover, the expression of laccase isoenzymes is differentially regulated and depends on the cultivation conditions as source of carbon, nitrogen and phenolic and/or aromatic compounds (Giardina et al. 2010, Pezzella et al. 2013). The affinity of laccase isoenzymes by the same substrates vary greatly. Palmieri et al. (1997) characterized the catalytic properties of two isoenzymes (POXA1 and POXA2) of *Pleurotus ostreatus* in relation to typical laccase substrates among them 2,6-dimetoxyphenol (DMP). Isoenzymes presented different $K_m$ and while POXA2 presented $K_m$ of 74 μM when DMP was utilized as electron donor, POXA1 presented $K_m$ of 2100 μM. Molecular docking analysis revealed that purified laccase of *Lentinus tigrinus* (LCC3) can interact distinctly with structurally different dyes and this would explain different decolorization rates (Hsu et al. 2012). The authors observed that the enzyme forms five hydrogen bonds with acid blue 80 dye (97% decolorization) and four hydrogen bonds with RBBR (29% decolorization), suggesting that H-bond interactions may explain the decolorization efficiency.

RBBR is a recalcitrant anthraquinone dye with a fusion of aromatic rings in its structure, but its decolorization by *L. crinitus* and some other *Lentinus* spp. was demonstrated. The decolorization caused by extracts obtained in cultivation with CH and CP (76% and 74.4%, respectively) in our study was greater than decolorization described by Machado et al. (2005) and Sarnthima et al. (2009) who studied strains of *L. crinitus* and *L. polychrous*, respectively. The crude extract of two *L. crinitus* strains reduced RBBR between 25% and 49% (Machado et al. 2005). On the other hand, the enzymatic extract of *L. polychrous* cultivated in rice bran culture medium supplemented with rice husk decolorized RBBR approximately 58% in one hour. Therefore, we can state that the cultivation of *L. crinitus* in CH and CP can be an alternative to produce enzymatic extract to decolorize RBBR.

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