Low carbon/nitrogen ratio increases laccase production from basidiomycetes in solid substrate cultivation

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ABSTRACT: Basidiomycetes are laccase producers used for hydrolysis of lignocellulosic byproducts in fermentative processes and could be used on biofuel production or ruminant feeding. The objective of this study was to evaluate the effect of concentrations of non-protein nitrogen sources on laccase production and mycelial growth of *Pleurotus ostreatus*, *Lentinula edodes* and *Agaricus blazei*. The fungi were grown on soybean hulls to which urea (U), ammonium sulfate (AS) or mixture of AS:U (1:1) were added to achieve carbon/nitrogen (C/N) ratios of 5, 15, 20 or 30. The average longitudinal mycelial growth was measured and laccase activity was determined by the oxidation of 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid. Higher C/N ratios increased mycelial growth and decreased laccase production. The highest activities were obtained with a C/N ratio of 5. *P. ostreatus*, *L. edodes* and *A. blazei* produced more laccase when AS, AS:U and U, respectively, were added. In addition, C/N ratios lower than 30 induced laccase syntheses, inhibited mycelial growth and were a better condition for pre-hydrolysis of plant residues.
Key words: industrial byproduct, lignocellulolytic, enzyme, lignolytic, soybean hull

Reduzida relação carbono/nitrogênio aumenta a produção de lacase por basidiomicetos em cultivo de semissólido

RESUMO: Basidiomicetos são produtores de lacases utilizadas na hidrólise de subprodutos lignocelulósicos em processos fermentativos e pode ser utilizado na produção de biocombustíveis ou na alimentação de ruminantes. Avaliou-se o efeito da adição de fontes e concentrações de nitrogênio não-protéico na produção de lacase e no crescimento micelial de *Pleurotus ostreatus*, *Lentinula edodes* e *Agaricus blazei*. Os fungos foram cultivados em cascas de soja com adição de uréia (U), sulfato de amônio (AS) ou AS:U (1:1) de forma a manter a relação carbono/nitrogênio (C/N) de 5, 15, 20 ou 30. O crescimento micelial longitudinal médio foi medido, e a atividade da lacase foi determinada pela oxidação do ácido 2,2'-azino-bis-3-etilbenzotiazolina-6-sulfônico. O crescimento micelial foi diretamente proporcional à relação C/N, enquanto a atividade de lacase foi inversamente proporcional. Os maiores valores de atividade foram obtidos para a relação C/N de 5. As melhores fontes de N para a produção de lacase por *P. ostreatus*, *L. edodes* e *A. blazei* foram, respectivamente, AS, AS:U e U. Relações C/N menores que 30 induziram a síntese de lacase e inibiram o crescimento micelial, proporcionando condições ideais para a pré-hidrólise de resíduos vegetais.
Palavras-chave: subproduto, lignocelulolítica, enzima, lignolítico, casca de soja

Introduction

In 2008, Brazil produced approximately 60 million tons of soybean (*Glycine max*) (IBGE, 2008) and 1.2 million ton of soybean hull, which contains 66-69% of neutral detergent fiber and 3-8% of lignin (Zambom et al., 2001). Soybean hulls have been used in the feeding of ruminants (Nakamura and Owen, 1989); however, lignin is not well digested by rumen bacteria, reducing digestive efficiency of this material (Van Soest, 1994). Lignin is a recalcitrant compound, covalently linked to hemicellulose, which forms a physical barrier to cellulose degradation (Kerem and Hadar, 1993). Laccase enzyme has been used for previous lignin degradation in order to increase cellulose exposure to fungal cellulases (Anderson et al., 1988).

Fungi as *Pleurotus ostreatus*, *Lentinula edodes* and *Agaricus blazei* are edible mushroom and laccase producers (Colauto et al., 1998; Colauto et al., 2010; Colauto and Eira, 1995; Eira et al., 2005; Moda et al., 2005; Regina et al., 2004) with culinary importance (Braga et al., 1998; Escouto et al., 2005) and biological activities such as antitumor (Israilides et al., 2008; Mourão et al., 2009; Sarangi et al., 2006). Fungal enzyme synthesis is strongly influenced by the strain, substrate composition and nitrogen concentration in the cultivation medium (Elisashvili et al., 2008; Elisashvili and Kachlishvili, 2009; Stajić et al., 2006). Among the cultivation parameters, the carbon/nitrogen (C/N) ratio is one of the most important factors to balance biomass and biocomposite productions. The excess or lack of nitrogen content in the substrate may be a limiting factor for fungus

growth (Mantovani et al., 2007). There is a reduction of substratum degradation when nitrogen is excessively added (Rajarathnam and Bano, 1989). Laccase production was related to mycelial growth for *P. ostreatus* when C/N ratios were over 40 (Elisashvili and Kachlishvili, 2009; Stajić et al., 2006). Although *Pleurotus ostreatus, Lentinula edodes* and *Agaricus blazei* are widely cultivated worldwide, little is known about the effect of C/N ratios lower than 40 on laccase production for those fungi. In order to decrease the C/N ratio, non-protein nitrogen can be added. Non-protein nitrogen is a term used to refer to components such as urea and ammonium sulfate, which are not proteins but can be converted into proteins by microorganisms (Fonnesbeck et al., 1975).

Therefore, due to the ability to produce enzymes, basidiomycetes are potential agents on recalcitrant compounds digestion. This condition may allow carbon releases from substrate to contributing for microbial succession processes on biofuel and functional silage productions. Thus, the objective of this study was to evaluate the effect of the addition of sources and concentrations of non-protein nitrogen in soybean hulls on laccase production and mycelial growth of *P. ostreatus*, *L. edodes* and *A. blazei*.

Material and Methods

P. ostreatus (U6/8), *L. edodes* (U6/12) and *A. blazei* (U2/2) strains were maintained at 25ºC. The first two were kept in 3.9% potato dextrose agar (PDA), and the latter in 3.4% malt extract agar (MEA). Nitrogen concentration was determined in the soybean hulls by Kjeldahl method, ashes by burning at 550ºC, and moisture by drying at 105ºC until constant mass. Considering 50% of dry organic mass as carbon mass, the soybean hull C/N ratio was calculated using the results from chemical analysis, according to Mantovani et al. (2007). The soybean hull had 20.2 g kg^{-1} moisture, and, on dry basis, 86.6 g kg^{-1} protein, 29.7 g kg^{-1} ashes, 485.1 g kg^{-1} carbon, and 13.9 g kg^{-1} nitrogen. These results allowed the calculation of nitrogen amounts to be added to soybean hulls in order to obtain C/N ratios presented in Table 1.

From this information, the cultivation medium was prepared with 5 g of soybean hulls mixed with 5.71 mL of water in 15 mL tubes and then autoclaved at 121ºC for 60 min. Each tube received 5.71 mL of concentrations of urea (U), ammonium sulfate (AS) or AS:U (1:1) solutions (previously filtered through 0.22 μm-diameter-pore membrane) in order to achieve C/N ratios of 5, 15, 20 or 30. Each treatment was replicated three times. The culture media were inoculated with a 3 mm-diameter disc containing mycelial grown on PDA or MEA. The material was incubated at 25ºC for ten days. The average longitudinal mycelial growth was determined using a pachymeter with five replications. The colonized cultivation media were kindly homogenized and stored in plastic bags at -20ºC for posterior determination of enzymatic activity and moisture.

To determine laccase activity, 1 g of colonized medium was homogenized in 4 mL of sodium acetate buffer (10 mmol L^{-1}, pH 4.2). The mixture was kept in ice bath for 1 h with manual agitation every 15 min and then centrifuged at 15300 g at 4ºC for 2 min. The supernatant (400 μL) was mixed with 1400 μL water, 900 μL sodium acetate buffer (0.1 mol L^{-1}, pH 5.0) and 300 μL 2,2-azino-bis-3-ethilbenzotiazoline-6-sulphonic acid (ABTS) 1 mmol L^{-1} (Han et al., 2005). The mixture was kept in water bath at 30ºC for 10 min, and the reaction was interrupted by addition of 100 μL of trichloroacetic acid (5%). The volume was adjusted to 10 mL and the absorbance was measured at 420 nm. A mixture of supernatant (400 μL), water (1,700 μL) and sodium acetate buffer (900 μL) was used as analytical control. One enzymatic activity unit was defined as the amount of enzymes that oxidizes 1 μmol L^{-1} of ABTS per minute under reaction conditions. To calculate enzymatic activity of ABTS, the absorption coefficient of 3.6 10^4 L mol^{-1} cm^{-1} was used. All analyses were replicated three times.

The experimental design was a split plot with nitrogen source (AS, AS:U and U) as the main factor and the C/N ratio (5, 15, 20 or 30) as the split plot factor. All experiments had three repetitions. Data set was tested for homogeneity with Levene's test of equality of error variances and Q-Q plots to test for normality of the data. The presence of outliers was checked with box-plot graphs. Statistical analysis was based on two-way ANOVA using SPSS 15.0.1 for Windows (SPSS Inc. 1989-2006).

Results and Discussion

For each fungus the results obtained for mycelial growth and laccase production were characterized by very low standard deviations among experimental replicates (Figures 1 to 6). Comparing the results of each experimental triplicate, the

Table 1 – Cultivation media composed of soybean hulls added of urea (U) and/or ammonium sulfate (AS) to obtain carbon/nitrogen (C/N) ratios of 5, 15, 20 and 30.

Nitrogen source proportion (AS:U)	C/N ratio	Added nitrogen	Total nitrogen
		mg*	g kg^{-1} on dry basis
1:0	5	83.1641	97.0
1:0	15	18.4809	32.3
1:0	20	10.3955	24.3
1:0	30	2.3101	16.2
1:1	5	83.1641	97.0
1:1	15	18.4809	32.3
1:1	20	10.3955	24.3
1:1	30	2.3101	16.2
0:1	5	83.1641	97.0
0:1	15	18.4809	32.3
0:1	20	10.3955	24.3
0:1	30	2.3101	16.2

*Nitrogen mass added of urea (molar mass of 60) and/or ammonium sulfate (molar mass of 132), both with 28 g of N per mol, and considering that 1 g of soybean hull has 485,124 mg of C and 13,860 mg of N on dry basis.

mycelial growth of *L. edodes* in AS:U culture medium had the highest standard deviation (1,787), which represents a standard error of 13.31%. The variables C/N ratio, fungus and the interaction of C/N ratio x fungus affected ($p \leq 0.05$) both laccase production and mycelial growth.

In general mycelial growth of *P. ostreatus*, *L. edodes* and *A. blazei* increases with increasing of C/N ratios (Figures 1 to 3). However, linear regression coefficients indicate that fungus species respond differently to N sources. Indeed, *P. ostreatus* and *L. edodes* grew more when cultivated with AS whereas *A. blazei* grew more with U. Mycelial growth in all fungus species was hampered when N concentration in the culture media were higher than 16.2 g kg^{-1} or when the C/N ratios were lower than 30 (Figure 1, 2 and 3; Table 1).

Fungus responses also differed due to C/N ratios and N sources when laccase production is considered, but there was a general trend that laccase production reduces when C/N ratios increases. The addition of AS in the culture medium to grow *P. ostreatus* and AS:U for *L. edodes* increased laccase synthesis at C/N ratios lower than 30 (Figures 4 and 5). The highest laccase production by these fungi, in the presence of AS, may be related to the sulfur provided by AS. This compound is a macronutrient used by fungi and is present in the cultivation medium between 0.1 to 0.6 mmol L^{-1}. Sulfur is an essential compound of amino acids as cysteine and methionine as well as thiamine and biotin vitamins and is a structural element of enzymes and antibiotics (Miles and Chang, 1997).

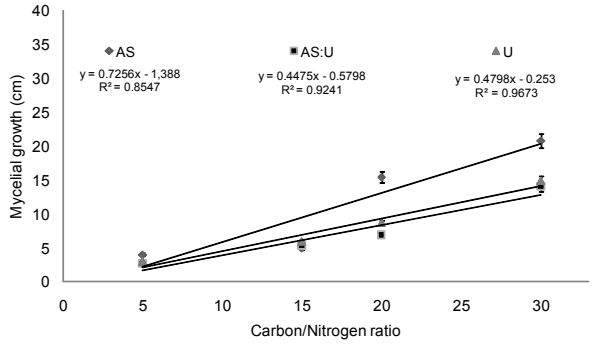

Figure 1 – Relationship between mycelial growth of *Pleurotus ostreatus* and carbon/nitrogen ratio of the cultivation media composed of soybean hulls added with nitrogen sources ammonium sulfate (AS), urea (U) or AS:U (1:1). Vertical bars represent standard deviation.

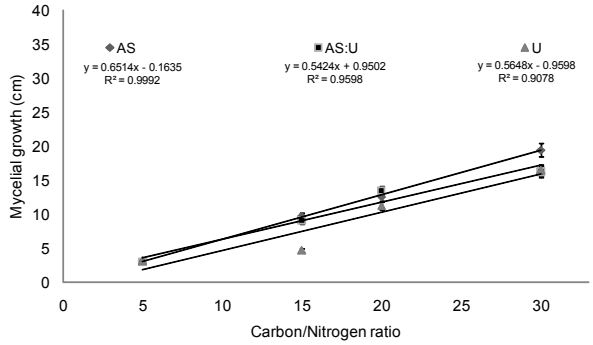

Figure 2 – Relationship between mycelial growth of *Lentinula edodes* and carbon/nitrogen ratio of cultivation media composed of soybean hulls added with nitrogen sources ammonium sulfate (AS), urea (U) or AS:U (1:1). Vertical bars represent standard deviation.

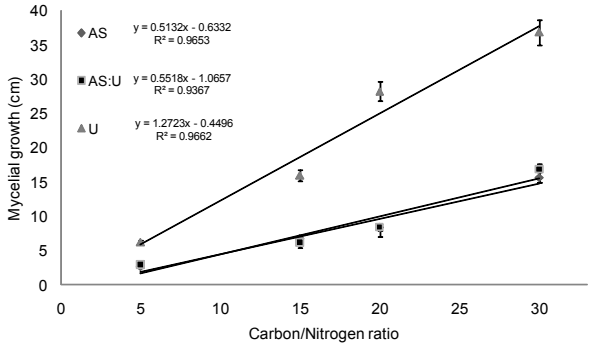

Figure 3 – Relationship between mycelial growth of *Agaricus blazei* and carbon/nitrogen ratio of the cultivation media composed of soybean hulls added with nitrogen sources ammonium sulfate (AS), urea (U) or AS:U (1:1). Vertical bars represent standard deviation.

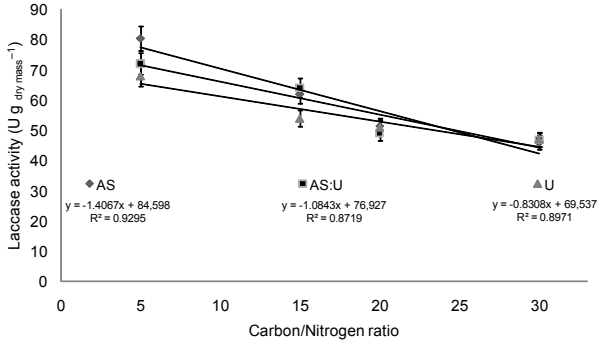

Figure 4 – Relationship between laccase activity of *Pleurotus ostreatus* and carbon/nitrogen ratio of the cultivation media composed of soybean hulls added with nitrogen sources ammonium sulfate (AS), urea (U) or AS:U (1:1). Vertical bars represent standard deviation.

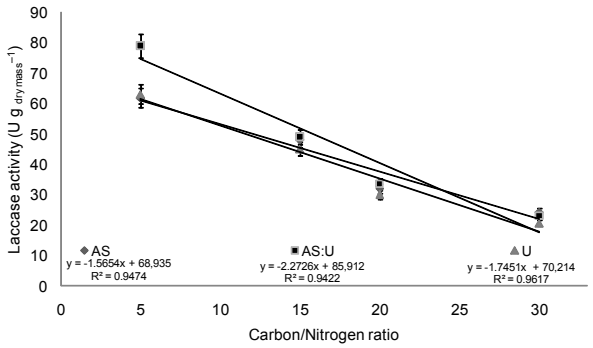

Figure 5 – Relationship between laccase activity of *Lentinula edodes* and carbon/nitrogen ratio of the cultivation media composed of soybean hulls added with nitrogen sources ammonium sulfate (AS), urea (U) or AS:U (1:1). Vertical bars represent standard deviation.

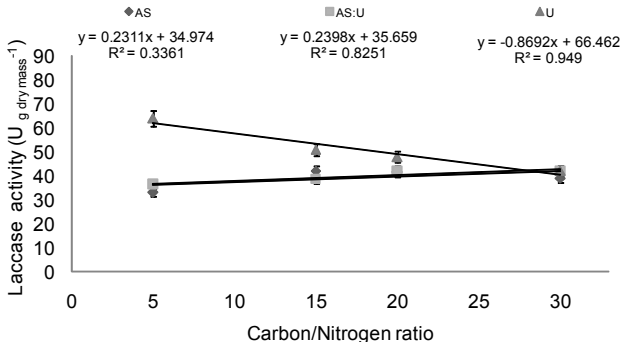

Figure 6 – Relationship between laccase activity of *Agaricus blazei* and carbon/nitrogen ratio of the cultivation media composed of soybean hulls added with nitrogen sources ammonium sulfate (AS), urea (U) or AS:U (1:1). Vertical bars represent standard deviation.

The addition of increasing rates of U to the culture medium of *A. blazei* decreased laccase production (Figure 6) but improved *A. blazei* growth (Figure 3). It corroborates Mantovani et al. (2007) that reported best mycelial growth of *A. brasiliensis* (*A. blazei*) in substrate added with U instead of AS. Other fungi like *P. ostreatus* and *L. edodes* demand for more sulphur, and thus, are able to grow better under highly concentrated sulfur culture media.

Laccase production is positively correlated to mycelial growth for *P. ostreatus* with addition of non-protein nitrogen sources as well as to AS (Elisashvili and Kachlishvili, 2009) or NH_4NO_3 (Elisashvili and Kachlishvili, 2009; Stajić et al., 2006). However, these authors worked with C/N ratios over 40, according to Miles and Chang (1997) who recommend C/N ratios higher than 30 for mushroom cultivation. Thus, these results can not be compared to the ones reported in this study where C/N ratio values were lower than 30. Considering *P. ostreatus*, it is very likely that C/N ratios over 40 stimulate laccase production by increasing of mycelial growth whereas C/N ratios lower than 30 stimulate laccase production due to the activation of enzyme synthesis.

Considering *L. edodes*, laccase activity increased and mycelial growth reduced at lower C/N ratios (Figures 2 and 5). Kachlishvili et al. (2005) reported that for *L. edodes*, the addition of protein and non-protein sources of nitrogen increased laccase production by two-to-four times, although there were no changes in the synthesis of cellulases and xylanases. Leatham and Kirk (1983) and Hatakka (1994) observed increases in the ligninolytic enzyme activity with the supplementation of nitrogen to *L. edodes* culture medium. Furthermore, despite reductions in the hydrolytic enzyme synthesis, Kachlishvili et al. (2005) and Mikiashvili et al. (2005) reported that the addition of nitrogen leads to repression of hydrolytic enzyme synthesis and induces laccase production.

Multiple laccase genes are common in fungi, and the production of several laccase isoforms arranged in gene families with differential expression has been reported (Pezzella et al., 2009). Some of them are constitutively expressed and others are induced by physiological and growth conditions (Mansur et al., 1998, Soden and Dobson, 2003; Xiao et al., 2006). The amount of N in the culture medium determines the laccase gene expression for *Trametes versicolor* (Collins and Dobson, 1997) and laccase production for *Pycnoporus cinnabarinus* (Eggert et al., 1996). Soden and Dobson (2001) reported that *Pleurotus sajor-caju* produces five laccases whose expression is induced by C, N, Cu, Mn, and aromatic compounds. Likewise, Pezzella et al. (2009), analyzing the promoter region of seven laccase genes of *P. ostreatus*, reported that the presence of nitrogen in the cultivation media associated with certain genetic sequences may be related to the regulation of laccase expression. The enzymatic synthesis activation can also be explained by the balance between the presence of easily metabolized carbon as glucose for the mycelial growth and the presence of inducers like cellulose and lignin. Thus, it is possible that in cultivation media, with N surplus, the fungus is induced to produce laccase in order to release C that would facilitate restoring the balance between carbon and the free nitrogen.

The slope values of the equations obtained for laccase production (Figures 4, 5 and 6) of the three fungi shown that *L. edodes* had the highest values followed by *P. ostreatus* and *A. blazei*. It suggests that laccase synthesis by *L. edodes* is affected by nitrogen concentrations. In fact, *L. edodes* grows naturally in wood logs that are rich in lignin and poor in nitrogen. It makes this fungus well-adapted in terms of laccase production which does not allow developing strategies to grow in rich N substrate (Elisashvili et al., 2008). These aspects can explain the higher effect of C/N ratio on the laccase production by *L. edodes*. On the other hand, *P. ostreatus* had greater capacity to adapt its enzymatic production in function of N sources and concentrations. This behavior is probably associated to the high adaptation capacity of this fungus that grows in different cultivation media with variability of nitrogen and C/N ratio. The low effect of C/N ratio on laccase synthesis of *A. blazei* may be related to a low laccase synthesis capacity; given that *A. blazei* grows well in culture media based on composted materials, with previous hydrolysis of lignocellulosic materials (Braga et al., 1998), without needing high laccase activity. *A. blazei* use high amounts of nitrogen for its growth *in vitro* corroborating that this fungus has laccase production less affected by low C/N ratios (Mantovani et al., 2007).

When comparing enzymatic activity, *P. ostreatus* and *L. edodes* were better laccase producers than *A. blazei* (Figures 4, 5 and 6). Indeed, Kachlishvili et al. (2005) reported that *L. edodes* is a better laccase producer than *Pleurotus dryinus* and *Pleurotus tuberregium*. But Songulashvili et al. (2007) reported that in 18 basidiomycete fungi the laccase production capacity was higher in *Pleurotus* spp, and that the best producer was *Pleurotus robustus*, although *L. edodes* was not evaluated. Moreover, *P. ostreatus* or *L. edodes* are natural decomposers of decaying wood which presents C/N ratio from 300 to 500 (Alonso et al., 2007) and there is a strong induction of cellulase and laccase synthesis due to high concentration of cellulose and lignin. However, *A.*

blazei grows naturally in decomposing material or on soil with high concentration of degraded biological material, generally straw and leaves, demanding lower cellulose and laccase synthesis.

The higher laccase production and the reduced mycelial growth of *P. ostreatus* and *L. edodes* in cultivation medium with C/N ratio of 5 suggest that these fungi are very useful for pre-hydrolysis of plant residues with higher lignin concentration, helping access carbon from cellulose without necessarily using it to growth on cultivation medium. Furthermore, laccase production may increase carbon bioavailability in the culture medium. It would be useful for animal feed or microbial succession processes for enzyme or biofuel production from lignocellulolytic byproducts.

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