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Laccases production by *A.blazei* mushroom grown either in composted or non-composted substrates. Effects of copper and zinc

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ABSTRACT

Agaricus blazei is an edible and medicinal mushroom commonly cultivated on compost. However, non-composted substrates are being particularly studied for specialty mushrooms because their economic and labour advantages. Addition of salt minerals to the substrate or casing materials may stimulate both the synthesis and activity of enzymes involved in the mushroom substrate biotransformation and eventually lead to an increase not only in mushroom productivity but in the fruitbody mineral content too. Remaining substrates from mushroom cultivation are a potential source for the extraction of high valued ligninolytic enzymes like laccases. The main objective of this study was to determine the laccase activity level produced by *A. blazei* on different phases of its growth cycle, using composted and non-composted substrates. It was also studied the *A. blazei* laccase production in substrates, either in the presence or in the absence of Cu^{2+} or Zn^{2+} (100 or 200 ppm). Results showed that laccase activity depends on the substrate used and varies along the different phases of cultivation. It was also demonstrated, that laccase production and its subsequent accumulation in a substrate are not necessarily correlated with the mycelia growth rates and mushroom production yields. The incorporation of Cu^{2+} (100 and 200 ppm) solutions on top of the casing layer (composted substrate) or as part of the formula (non-composted substrates), stimulated the laccase production in the studied substrates. In the case of Zn^{2+}, only the addition of 100 ppm had a positive effect on laccases and mainly in composted substrates.

KEYWORDS

A.brasiliensis;
Almond portobello;
Oligoelements;
Bioremediation;
White rot fungi enzymes.

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductase, EEC 1.10.3.2) have been studied since the end of the 19th century. However, it was only after the studies related to the degradation of ligno-cellulosic materials performed by white rot fungi enzymes that they began to draw more attention[1]. They catalyze the one electron

transfer in the oxidation of several aromatic substrates with the simultaneous reduction of dioxygen to two molecules of water[21].

Laccases can be used in remediation practices (chlorophenols, synthetic dyes, pesticides and polycyclic aromatic hydrocarbons[1]), beverage processing (wine, fruit juice, and beer), ascorbic acid determination, sugar beet pectin gelation, baking, and as part of a biosensor[17]. They can also whiten pulp paper and detoxify agricultural by-products, such as the phenolic waste present in the resulting waters of olives grinding, those of the coffee pulp[1], and even biodegrade the herbicide methyl metsulfuron. Moreover, for substrate oxidation, laccases can also immobilize soil contaminants by chemical coupling to soil humic substances, an analogous process to the humic acid synthesis in soils[3,4].

Laccase activity has been extensively demonstrated in more than 60 fungal mushroom species, among them *Agaricus bisporus*, *Pleurotus ostreatus* and *Trametes versicolor*[18,22], and many laccases have been extracted and purified[20,22]. The typical fungal laccase is a protein of approximately 60-70 kDa with an acidic isoelectric point around pH 4.0[1].

While the majority of the laccases so far purified are extracellular enzymes, the laccases of wood rotting fungi are also usually present in the interior of cells[2]. The location of these is probably associated with their physiological function. It is possible that intracellular laccases of fungi participate in the transformation of low molecular weight phenolic compounds in the cells. Laccases also have been linked to the possible formation of melanin and other protective compounds in the cell wall[8,9]. Other fungi laccase roles include morphogenesis, fungal plant-pathogen/host interaction, stress defence, and lignin degradation[14,25-27].

Laccases activity reflects the course of the organic substances and varies over time. It is also an indicator of mycelium presence[1]. In a particular fungus, laccases are normally produced in several phases of its growth and during this process some are excreted to the lignocellulosic environment; from where they can be easily extracted, as well as from some soils or from spent mushroom substrates[7,15,16]. The possibility of increasing the laccases production through addition of fungal inductors to the mushroom growing media[19] and using a relatively simple purification process is another advantage[23].

Despite many efforts to understand the laccase role in the lignocellulose transformation, it is not completely clear how important this role is in the lignin degradation and in what extension contributes to the formation and decomposition of humic substances in soils. Brum[5] conducted a study on enzyme production, including that of laccases, during the vegetative growing phase of *A. brasiliensis* (syn. *A. blazei*) on composted and non-composted substrates, verifying the adaptability of *A. brasiliensis* to produce mycelial biomass under unfavourable conditions, showing a competitive capacity determined by the production of oxidative enzymes to degrade recalcitrant constituents of the substrates employed and by the access to carbon. However, there is no data available on laccases production during the *A. blazei* reproductive phase as well as on the effect that the addition of either Cu (II) or Zn (II) produces on the mushroom productivity.

The aims of this search were i) to determine the laccase activity level during the cultivation of *A. blazei* (2 flushes), grown on composted and non-composted substrates based on sunflower seed hulls (SSH) and on spent mushroom SSH based substrate, (after two *Pleurotus pulmonarius* flushes), in the absence or presence of supplements. Laccase activity was also investigated on substrates used for running the mycelial linear growth test for this mushroom, when studying the ability to grown in different substrates based on sunflower seed hulls (SSH) and on spent mushroom SSH based substrates (SMS); and ii) to investigate the effect that the addition of Cu^{2+} or Zn^{2+} (100 or 200 ppm) has on laccase production in the *A. blazei* substrate, when their salts are incorporated either during the substrate preparation or at irrigation of casing beds on composted substrates or during the preparation of non-composted substrates.

MATERIALS AND METHODS

Biological material

Agaricus blazei Murrill (PL strain) was obtained from Brasmicel, SP, Brazil.

Preparation and conservation of the mushroom spawn

The mycelial culture was maintained in glass tubes

FULL PAPER

on MYPA medium (20 gl^{-1} malt extract, 2 gl^{-1} yeast extract, 1 gl^{-1} peptone and 20 gl^{-1} agar), covered with sterile liquid Vaseline® at room temperature until use. Another nutrient medium (CDYA) was prepared using the filtrate resulting from boiling 500 g of Phase II sunflower seed hulls-based compost in 1 l water and adding 2 gl^{-1} yeast extract, 20 gl^{-1} dextrose and 20 gl^{-1} agar[10]. This nutrient medium was adjusted to pH 6.5 with HCl and sterilized at 1 atm for 30 min. *A. blazei* mycelium was inoculated on Petri dishes containing CDYA medium and incubated in darkness at 25°C for 10-15 days, at which time the mycelium almost covered the nutrient medium and was ready to be used for spawn preparation.

Wheat (*Triticum durum* L.) grain (250 g) was placed into 1 l glass bottles and 190 ml water with 1.3% $CaCO_3$ were added and allowed to stand overnight at room temperature. Resulting mixture was autoclaved at 1 atm for 1.5 hr. After mycelium inoculation (16 cm^2 of colonized CDYA medium), the spawn was incubated at 25°C in darkness for 30 days, with occasional inspections for detecting contaminations and shakings to facilitate complete grain colonization.

Laccases activity in composted substrate

Two 1 kg bags with phase II compost, based on sunflower seed hulls -*Helianthus annus*- and wheat straw[11], inoculated with 5% of *A. blazei* spawn, were sampled at different phases of the mushroom cultivation process, *i.e.* casing application, fruiting body induction, first and second flush of mushrooms, to determine the laccases activity in both the substrate and casing material. Sample columns were obtained from the bottom to the top of the substrate with help of a 5 cm diameter punch. Both casing and substrate layers were separated and homogenized, and 1 g of each was taken for the laccase activity analysis. Laccase activity was spectrophotometrically measured and average values from three replicates of each extract were analyzed by one way ANOVA and means were compared with Tukey HSD (α=0.01) using the Statistica 6.0 software.

Remaining material was used for dry weight determination by the gravimetric method (50°C for 48 hr).

Laccases activity in non-composted substrates (linear growth test)

Linear growth test on *A. blazei* was performed in glass tubes (20 x 2 cm) (n=10) containing 8.5-10.0 cm tube length of different non-composted substrates (TABLE 1).

TABLE 1 : Non-composted substrates formulations based on sunflower seed hulls (SSH) and spent mushroom substrate either unmilled (SMS) or milled (SMSM), and with different percentages of supplements (vermicompost -verm-, peat or brewery residues -BR-), and 2.0 % $CaSO_4$, 0.5 % $CaCO_3$ and 60.0 % water. Components of the formulations are given in mass units. The total nitrogen content (Kjeldahl) and the carbon/nitrogen ratios are also given.

Substrate	SSH (%)	SMS (%)	SMSM (%)	Verm (%)	Peat (%)	BR (%)	N (%)	C/N
Control 1 (SSH)	37.50	-	-	-	-	-	0.59	71
SSH/SMS (5/5)	18.75	18.75	-	-	-	-	0.63	78
SSH/SMSM (5/5)	-	18.75	18.75	-	-	-	0.63	78
SSH/verm (8/2)	30.00	-	-	7.50	-	-	0.69	55
SSH/verm (6/4)	22.50	-	-	15.00	-	-	0.78	44
SSH/peat (8/2)	30.00	-	-	-	7.50	-	0.78	55
SSH/peat (6/4)	22.50	-	-	-	15.00	-	0.81	54
SSH/BR (8/2)	30.00	-	-	-	-	7.50	0.87	50
SSH/BR (7/3)	26.25	-	-	-	-	11.25	0.99	44
SSH/BR (6/4)	22.50	-	-	-	-	15.00	1.10	40
Control 2 (SMS)	-	37.50	-	-	-	-	0.82	68
SMS/BR (8/2)	-	30.00	-	-	-	7.50	0.90	60
SMS/verm (8/2)	-	30.00	-	-	7.50	-	0.86	58
SMS/verm (6/4)	-	22.50	-	-	15.00	-	0.91	48
SMS/peat (8/2)	-	30.00	-	7.50	-	-	0.88	48
SMS/peat (6/4)	-	22.50	-	15.00	-	-	0.96	45

After 53 days of incubation, three samples per treatment were taken for dry weight measurement. Contents of the seven remaining tubes were emptied and mycelia colonized portions were separated and mixed; then 1 g colonized substrate sample (n=3) was obtained for laccase activity measurement.

Laccases activity in non-composted substrates (cultivation trial)

The non-composted substrates supporting the better mycelium development in the linear growth test were used in a cultivation trial. Substrates formulations were prepared in an appropriate container, soaked with water for 15 to 20 hr, then 750 g of each substrate were put in 13 bags (35 x 25 cm) of high density polyethylene (75 μm) and closed with the help of a cotton plug - to allow gas exchange- fitted with a rubber band. Bags

were sterilized at 1 atm for 120 min. After cooling, a substrate sample was obtained from each treatment for moisture and pH measurement, and bags were inoculated with 6.5% (wet weight) of *A. blazei* wheat grain spawn. Treatments consisted of: [*Control (SSH)*]; [*SSH/SMS (5/5)*]; [*SSH/SMSM (5/5)*]; [*SSH/verm (8/2)*]; [*SSH/peat (8/2)*]; [*SSH/BR (8/2)*]; [*SSH/BR (7/3)*]; [*SSH/BR (6/4)*]; and [*SMS/BR (8/2)*] (see TABLE 1). Upon completion of the cultivation trial, three bags were randomly taken from each treatment, the casing material of each bag was removed and a sample representing the entire substrate depth was taken, homogenized and then 1 g of the resulting material was used for measuring laccase activity. Also, to obtain the dry weight, an aliquot of the remaining material was taken and dried in stove. Measures of laccases activity from the different substrate formulations were analyzed by simple ANOVA and the mean values of laccase activities were then compared by Tukey HSD (α=0.01).

Laccases activity in composted substrate. Effect of copper and zinc

High density polyethylene (25 x 35 cm, 75 μm) bags were filled with 350 g phase II compost, inoculated with 5% of *A. blazei* wheat grain spawn, and closed with a cotton plug as mentioned in the previous section. Then they were placed in darkness in an incubation room at 25±3°C and 80% of R.H. Casing bed (140 g, 34% peat, 29% $CaCO_3$, 37% water) was layered on day 17 on top of all bag substrates, and salt treatments were applied at this time. The nine treatments (n = 12) were: C1, control; C2 to C5, incorporation of $CuSO_4.5H_2O$ or $ZnSO_4.7H_2O$ to the casing bed during its preparation (CBP); and C6 to C9 incorporation of the same salts during waterings (W). They were labelled as: *Control*; *100 ppm Cu CBP*; *200 ppm Cu CBP*; *100 ppm Zn CBP*; *200 ppm Zn CBP*; *100 ppm Cu W*; *200 ppm Cu W*; *100 ppm Zn W*; *200 ppm Zn W*.

All bags were opened 27 days after casing and exposed for the fruitbody induction to 300 lx (ca 16 hr photoperiod), 27±3°C, and 85% R.H. with a ventilation rate exchange of 3-4 room air volume per hour. Watering, consisting of five shots with sprayer, was done 5 days after bag opening and continued, usually every two days, until the end of the trial. In the case of watering with salt solutions, different sprayers were used for each treatment, and after use they were stored under refrigeration till the next salt solution application. Seven applications were done every two days after which common watering continued. The assay was ended by harvesting the first flush of mushrooms. From remaining bags, three of them were randomly chosen per treatment, even from those where no fruitbodies were obtained (*100 ppm Cu^{2+} CBP* and *200 ppm Zn^{2+} W*). Also, a sample from each treatment was obtained from the top of the casing material to the bottom of the substrate. The casing layer was separated from the substrate, and each fraction was then homogenized to extract 1 g for laccase activity measurement. Also, the casing layer and substrate were joined again (maintaining their original proportion), mixed uniformly and another 1 g was taken from this mixture. These three samples, *i.e.* from substrate, casing and substrate/casing, were then used for laccase extraction and activity determination. Dry weight of each of the studied fractions was also determined. Results were statistically analysed by using the one way-ANOVA and the Tukey HSD test (α = 0.05).

Laccases activity in non-composted substrate. Effect of copper and zinc

Laccase activity was also analyzed in non-composted substrates with or without addition of those metal salts.

The composition of the non-composted substrate formula was 11.9% SSH, 11.5% poplar (*Populus nigra* L. var. *italica*) sawdust, 6.0% wheat bran, 4.5% soybean meal, 4.0% peat, 2.0% $CaCO_3$, 0.1% $(NH_4)_2SO_4$, and 60.0% water. Copper and zinc salt solutions were applied during substrate preparation in the following concentrations: 100 ppm Cu^{2+}; 200 ppm Cu^{2+}; 100 ppm Zn^{2+}; 200 ppm Zn^{2+}; substrates with no addition of these salts were used as controls. High-density polyethylene bags (25 x 35 cm, 75 μm) were filled with 350 g of the corresponding substrate (n=12) and closed with a cotton neck fitted with an elastic band, to allow appropriate gas exchange. The bags were sterilized at 1 atm for 120 min and this operation was repeated 24 hr later. When the substrate reached room temperature, it was inoculated with *A. blazei* wheat grain spawn at a rate of 6.5%, within a sterile laminar air flow cabinet. Then, bags were passed to an incuba-

tion room and maintained at 25±2°C in darkness. Casing bed, prepared as above mentioned, was applied at day 25 after incubation. After casing was colonized during 14 days, bags were open and colonized substrates were exposed to an inductive environment for fruiting (22-30°C, 80% R.H., 300 lx (ca. 16 hr photoperiod), with a ventilation rate exchange of 3-4 room air volume per hour). Upon completion of the first flush of mushrooms, three bags from all treatments, except the one with 200 ppm Cu^{2+}, were randomly sampled. Casing material was retired from the bags and the remaining substrates were homogeneously mixed. Then, two samples of 1 g each were taken at random and extracted for laccase activity determination on two occasions (considered statistically as blocks). From the rest of the material, a sample was obtained to determine dry weight. A bifactorial ANOVA, blocks and treatments, were performed, and as no block effect or treatment interaction was found, the treatment effect was analyzed and means were compared by Tukey HSD ($\alpha = 0.01$).

Determination of laccase activity

Laccase activity was determined following the protocol of Brum[5]. Briefly, 10 ml of distilled water containing 10 µl of Triton-X100 was added to 1 g of fresh substrate or/and casing. The material was macerated, filtered and centrifuged (1500 x g for 15 min at 4°C, Rolco 2036, Rolco S.R.L., Argentina), and the supernatant was immediately used for the enzyme assay based on the oxidation of the syringaldazine (λ525 nm extinction coefficient $\varepsilon = 65000\ M^{-1}\ cm^{-1}$). Syringaldazine [N,N'-bis(3,5-dimethoxy-4-hydroxybenzylidene hydrazine)] is often considered to be a unique laccase substrate[13] as long as hydrogen peroxide is avoided in the reaction, since this compound is also oxidized by peroxidases.

For this, 0.5 ml sodium acetate buffer (pH 5) 0.1 mol/l and 0.5 ml of the supernatant, as explained above, were placed in a glass tube. Four tubes per sample corresponding to three replicates and a blank (without syringaldazine addition) were prepared. The laccase activity was recorded using a spectrophotometer (Metrolab 1600 plus, UV-Vis Metrolab S.A., Bernal, Argentine) at 525 nm. After the blank samples were measured, 25 µl 20 mmol/l syringaldazine (SIGMA) were added to the tubes, placed in a vortex for 10 sec and after a minute of the syringaldazine addition, the absorbance was measured. One unit of enzyme activity was defined as the amount of enzyme which oxidizes 1 µmol of substrate per minute. Laccase activities were expressed as U/g dry weight of the extracted material.

RESULTS

Laccases activity in composted substrate

The evolution of the laccase activity in a composted sunflower seed hull and wheat straw based substrate during *A. blazei* cultivation showed a sigmoid type curve (Figure 1). At casing (day 17) and at fruiting induction (day 27), the laccase average activity were similar and low ca. 0.9 U/g DW, but after the first (day 102) and the second flush (day 117), increased by 8 and 10 times, respectively. The largest increase of laccase activity (576%) was produced during the vegetative phase, *i.e.* from the casing until the first flush; but markedly declined its production rate during the reproductive phase or fruiting, *i.e.* between the first and second flush, when almost reached a *plateau* by increasing 18%.

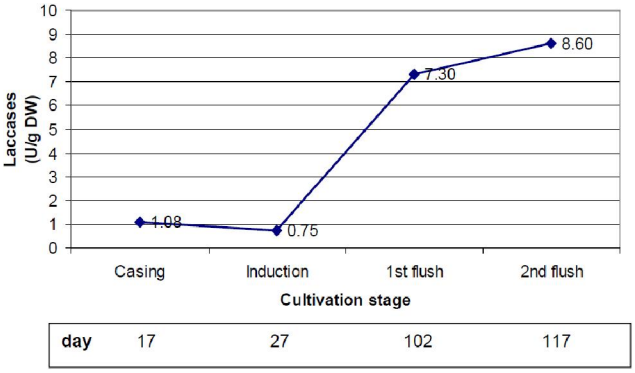

Figure 1 : Mean laccase activity content values (U/g DW substrate) in composted sunflower seed hull based substrate during *A. blazei* cultivation.

The average laccase activity in the casing bed was much lower than that found in the substrate along the crop, *i.e.* 0.2 U/g DW at induction and first flush and 0.4 U/g DW at second flush. This shows that the enzyme is mainly produced and accumulated in the substrate from where the mushroom obtains most of the nutrients for its growth and development.

Laccase activity in non-composted substrates (linear growth test)

Significant differences (p=0.0003) were found be-

tween the laccase activity substrate contents of different non-composted substrates, contained in tubes for linear growth test, colonized by *A. blazei*, after 53 days of incubation, although there were no significant differences with the control (Figure 2). Substrates showing the highest production of *A. blazei* laccases were both the sunflower seed hulls (SSH) and the spent mushroom substrate (SMS) with different proportions of brewery industry residue; an increase in the supplement proportion resulted in an increase of laccases production. The rest of the substrate formulas showed similar laccase production, except for SSH with peat (6/4 ratio) with the lowest content of laccases and the only one that differed significantly with those substrates with highest laccase production (Figure 2).

When observing the *A. blazei* mycelial growth behaviour on these same substrates, we found that in those where a significant increase of laccases activity content was measured, the mycelium growth rate was similar to their respective controls (data not shown), which suggests that the laccase activity, at least in this system of evaluation of growth and growing conditions, is not a good indicator of mycelium growth. However it should be correlated when the mycelial mass were the parameter to be measured for mycelial growth evaluation.

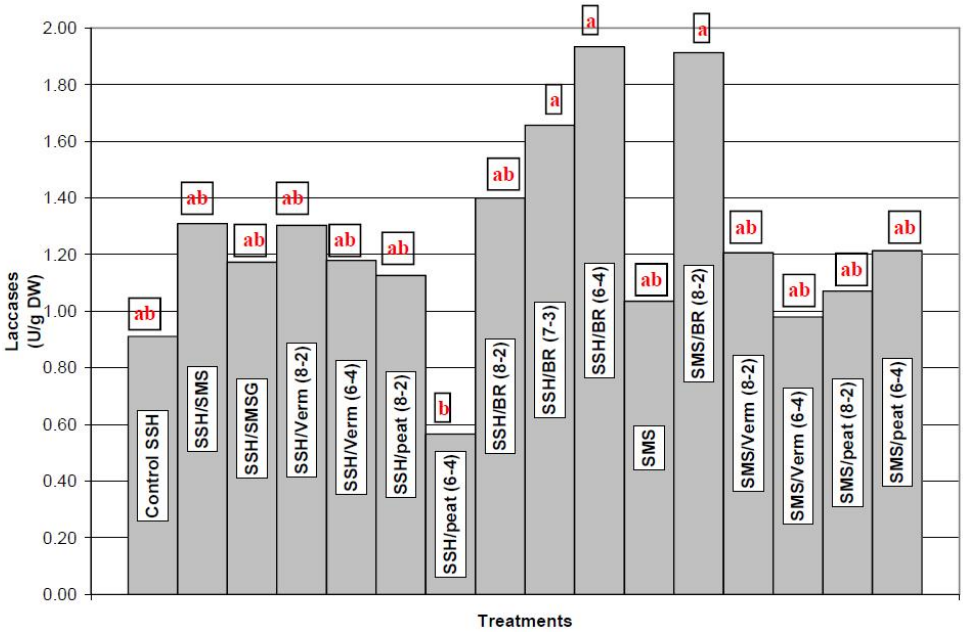

Figure 2 : Mean laccase activity content values (U/g DW substrate) produced by *A. blazei* in different non-composted substrates contained in test tubes for analysis of mycelial growth after 53 days of incubation. For treatment abbreviations see TABLE 1. Different letters between columns represent significant differences (Tukey HSD, α=0.01).

In almost all substrate formulations, the average laccase activity content values, with the exception of SSH/peat (6/4), were in the range of 0.9-1.5 U/g DW, showing no statistical differences.

Laccase activity in non-composted substrates (cultivation trial)

Highly significant differences (p=0.004) were found in the laccase production by *A. blazei* on the residual non-composted substrates after two mushrooms flushes. The highest laccase content was obtained in the control (SSH) and in the SSH/peat (8/2) substrates. These were significantly superior to the laccase content in SMS/BR substrate (8/2) but not to the rest of the substrates (Figure 3). These laccase content values were similar to those found in composted substrate, also after two flushes of *A. blazei*.

Laccases activity in composted substrate. Effect of copper and zinc

No significant differences in the laccase content coming from the casing material, after the first *A. blazei* flush, from different treatments were found. However, laccase content increased around 243% with the addition of 100 ppm of the salts of either element during the preparation of the casing bed compared to the control.

FULL PAPER

When the mineral nutrient dose was increased, only the Cu^{2+} salt solution had a positive effect on the laccase content (181%) with regard to control. Otherwise, when 100 ppm Cu^{2+} was applied by irrigation, it was observed a laccase content lower than the control; however, an increase of 137% was obtained with 200 ppm Cu^{2+} irrigation. When the Zn^{2+} solution was applied during irrigation, laccase content values were similar to the one obtained with the control (Figure 4). This enzyme content in the control casing bed (0.16 U/g DW) is consistent with that measured after a first *A. blazei* flush cultivated on composted substrate (0.20 U/g DW).

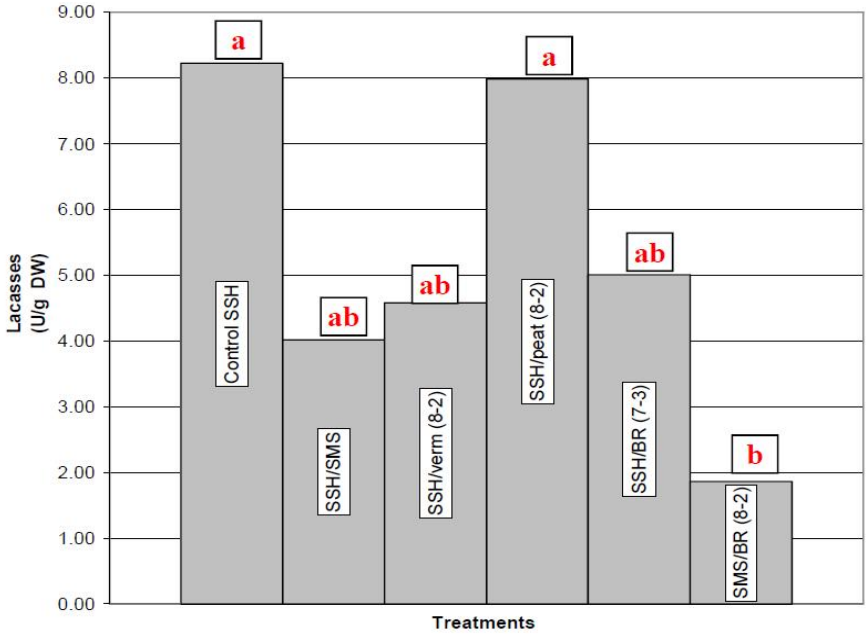

Figure 3 : Mean laccase activity content values (U/g DW substrate) in different spent substrates corresponding to non-composted substrates: sunflower seed hull (control, SSH) or spent mushroom substrate (SMS) and SSH supplemented with different materials (vermicompost -verm-, peat or brewery residues -BR-), at the end of a two flushes cycle of *A. blazei* cultivation, 210 days after spawning. Different letters between columns represent significant differences (Tukey HSD, α=0.01).

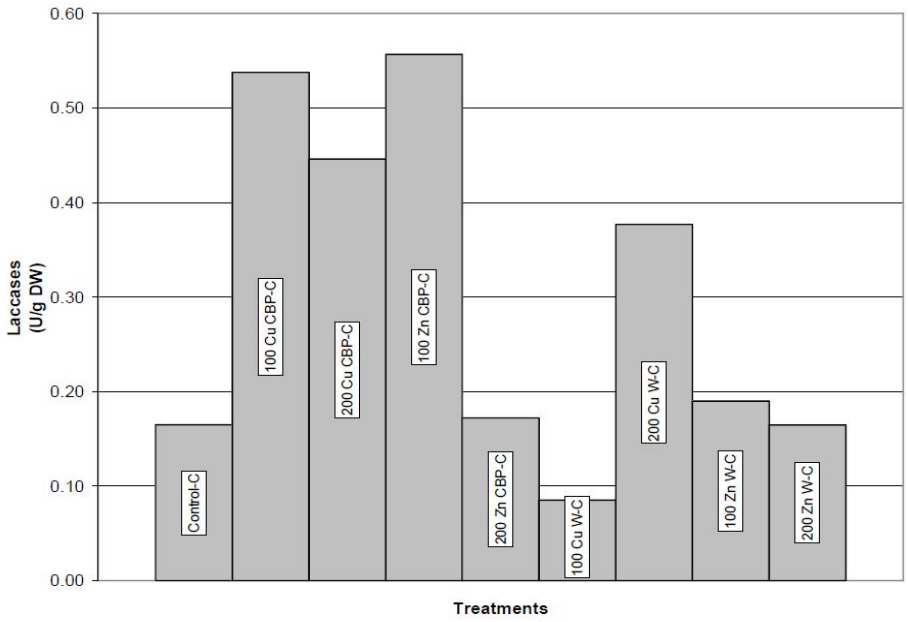

Figure 4 : Mean laccase activity content values (U/g DW) (n=3) in peat casing (C) of composted substrate with addition or not of Cu^{2+} or Zn^{2+} (100 or 200 ppm) during watering (W) or casing bed preparation (CBP), after the first *A. blazei* flush.

Laccase activity content analysis in the substrates of the different treatments showed no significant differences, being all values between 9.83 and 11.97 U/g DW (Figure 5). These values were on average 50 % higher than those measured in composted substrate without addition of minerals after a first flush of *A. blazei*.

In the case of laccase activity in samples of the remaining whole growing medium, *i.e.* substrate and casing layer, it is possible to observe that the differences in the activity values obtained on the casing layer alone between different treatments, disappeared and decreased a little with respect to the values obtained from substrates alone (Figure 6).

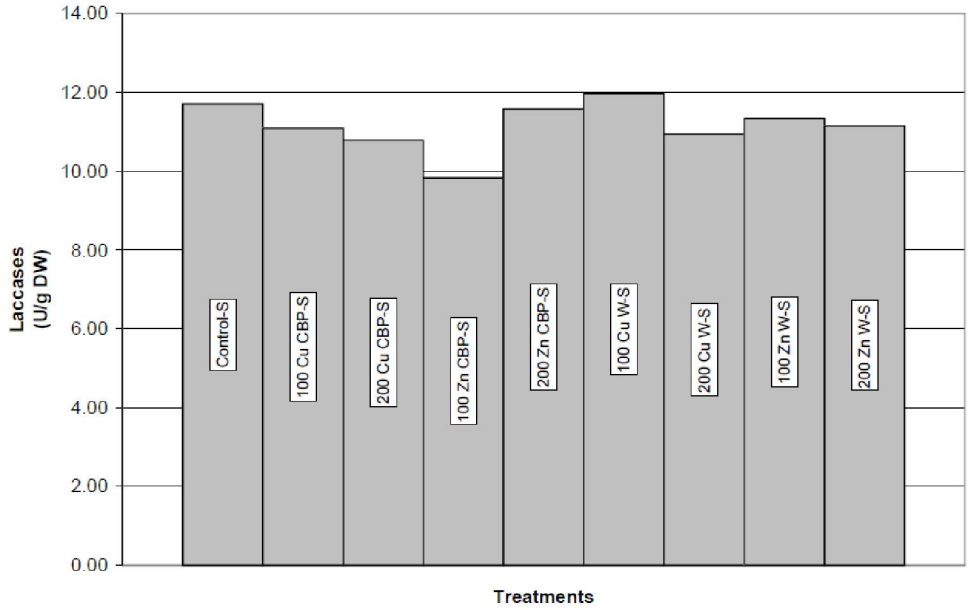

Figure 5 : Mean laccase activity content values (U/g DW) (n=3) in composted substrate (S) with or without Cu^{2+} and Zn^{2+} (100 or 200 ppm) applied during watering (W) or casing bed preparation (CBP), after the first flush of *A. blazei* ushrooms.

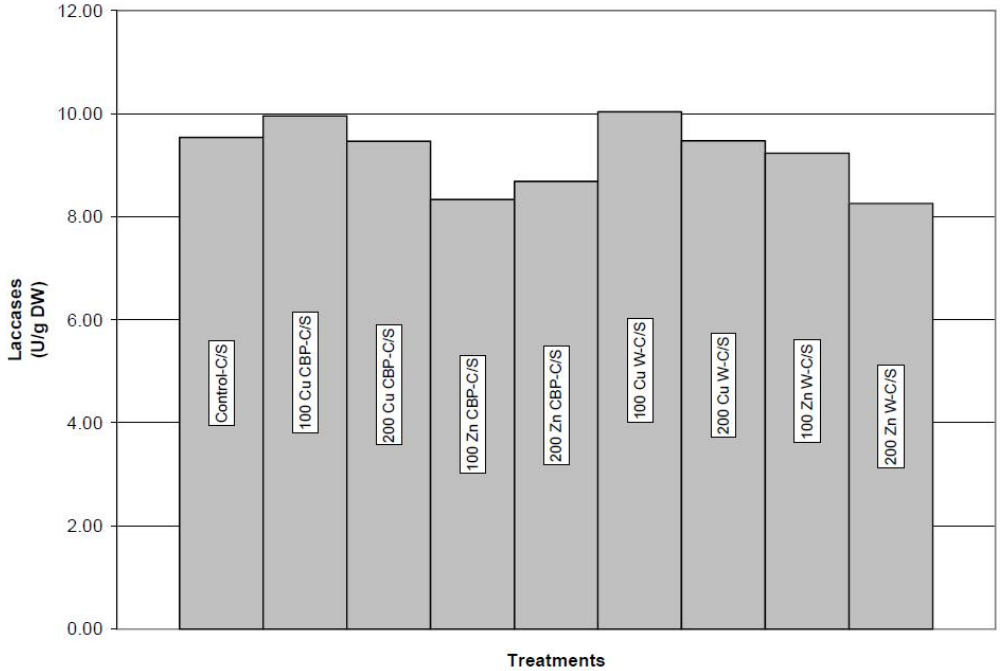

Figure 6 : Mean laccase activity content values (U/g DW) in spent mushroom compost (casing + substrate, C/S) with or without Cu^{2+} and Zn^{2+} (100 or 200 ppm) salt solutions applied during watering (W) or casing bed preparation (CBP), after the first flush of *A. blazei*.

FULL PAPER

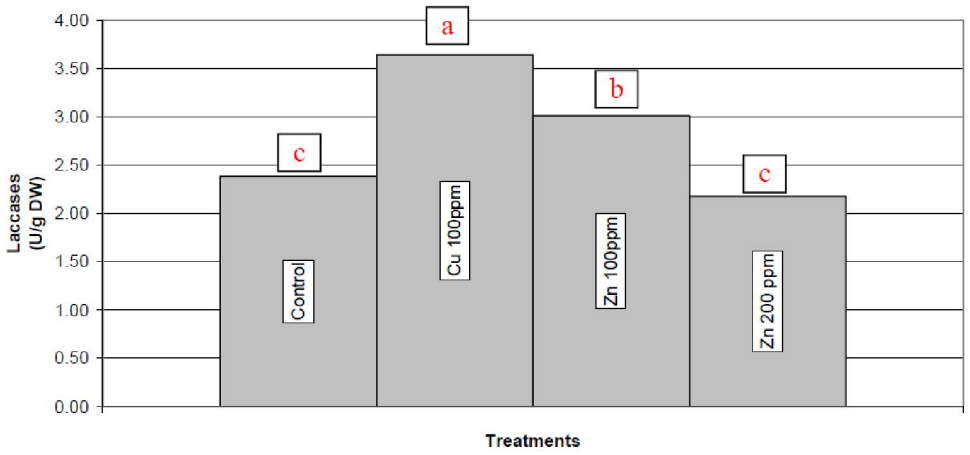

Figure 7 : Mean laccase activity content values (U/g DW) after the first flush of *A. blazei* mushrooms cultivated in non-composted substrate with or without Cu^{2+} (100 ppm) or Zn^{2+} (100 or 200 ppm) addition. Different letters between columns represent significant differences (Tukey HSD, α=0.01).

Laccases activity in non composted substrate. Effect of copper and zinc

We found no interaction between factors (p=0.317) or effect by the block (different samplings) (p=0.031); however, there was a highly significant treatment effect (p<0.010). The addition of 100 ppm of Cu^{2+} to the substrate increased laccase activity in a highly significant way with regard to the control. As for Zn^{2+}addition, the lower dose also increased in a highly significant way the laccase content in the substrate, but was not the case with the higher dose. At the same time, the laccase activity content in the substrate containing 100 ppm of Cu^{2+} was highly significantly superior to that found with 100 ppm of Zn^{2+} (Figure 7).

DISCUSSION

Variation in the laccase activity content along the cultivation time was observed with composted and non-composted substrates. It is likely that during tropophase and before the first flush of mushrooms, mostly available nutrients released during composting were used until its depletion. This new situation which generates nutrient insufficiency would be responsible for the activation of enzyme genes involved to complete the idiophase, including those for laccases, whose activities would allow the access to less available carbon sources within the lignocellulosic matrix.

Different strains cultivated in various substrate materials can result in different laccase activities[6]. In our research, different contents of laccase activities were measured, showing that both the formulation and the initial substrate degradation status can be a determinant of the laccase production and a particular biodegradation strategy settled by this mushroom when adapting to growth in a given substrate.

Comparing the laccase concentration of the non-composted substrates studied with the mushroom yields obtained from these substrates during the cultivation stage (data not shown), it was possible to note that the highest laccase concentration after two flushes of *A. blazei*, about 8 U/g DW, was found on the substrates with lowest yields, *i.e.* control and SSH/peat (8/2). Probably, the shortage of easily available nutrients and the limited access to carbon sources induced laccase production to higher levels. In the more nutrient-rich substrates, with SMS or those containing different supplements, *i.e.* brewery residues (BR) or vermicompost, the laccase production was lower. On the other hand, the lowest laccase concentration (1.9 U/g DW) was observed in the SMS/BR (8/2) substrate which was the best in productive performance. It was also demonstrated that *A. blazei* laccases production and its subsequent accumulation in the substrate is not correlated with the mycelial growth rate and mushroom production yields, *i.e.* SMS/BR (8/2) showed the highest laccase activity content, after 53 days of incubation on the linear growth test.

In the case of non-composted SSH based substrate, laccase content values were lower than those reported by Brum[5] for a non-composted substrate (50% real palm leaf -*Archontophoenix alexandrae* Mart.-, 25%

Eucalyptus sp. sawdust, 12% wheat bran, 8% $CaCO_3$, and 5% $CaSO_4$, with a C/N ratio of 25/1) which were 13.9 and 5.2 U/g DW after 14 and 35 days of incubation of *A. brasiliensis*, respectively.

Since laccases are oxidoreductases enzymes containing Cu^{2+}, in *A. blazei* cultivation it would be expected an increase in its activity content following the addition of non-mycotoxic concentrations of Cu^{2+} in the substrate or casing bed. In fact, it is known that this enzyme production and activity can be stimulated in the presence of adequate amounts of Cu^{2+}[9,28]. In this study, addition of 100 to 200 ppm of Cu^{2+} in the casing bed of a composted substrate does not alter the laccase activity content in the substrate; however, it may have an inductive effect on laccases activity in the casing material, especially when the mineral solution is incorporated during its preparation. In the case of non-composted substrates, 100 ppm of Cu^{2+} as part of the substrate formula stimulated the production of laccases.

Instead, only the addition of Zn^{2+} (100 ppm) had a positive effect on laccases, similar to that found with the addition of Cu^{2+} (100 ppm) in the case of composted substrate, but it was less extensive in the case of the non-composted substrate. The average values of laccase activity contents found in the non-composted substrate with the addition of Cu^{2+} and Zn^{2+} solutions were about 1/3 of those found in composted substrate with the addition of the same salts in the casing layer.

CONCLUSION

Laccase activity increase did not result in a productivity improvement at the first flush of mushrooms. However, should this finding do support the use of this oligoelements when looking for adding economical value to this crop through laccase production, since it would not be expensive or cumbersome to add the copper or zinc salt in the substrate formula.

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