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# ADDITIVE EFFECTS OF $CuSO_4$ AND AROMATIC COMPOUNDS ON LACCASE PRODUCTION BY *Pleurotus sajor-caju* PS-2001 USING SUCROSE AS A CARBON SOURCE

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**Abstract -** Laccase enzymes are now commercially available, and a laccase/mediator combination is currently marketed for indigo dye bleaching in textile manufacturing; replacing traditional chemical-based processes with enzymatic technology reduces the need for effluent treatment. However, an inexpensive source of these enzymes will be needed to enable wider application of this technology. In the present work, the main objective was to increase laccase production by the mushroom *Pleurotus sajor-caju* strain PS-2001 grown on sucrose derived from sugar cane, one of most economical carbon sources known, by the addition of compounds that are known to affect laccase production**.** High laccase activities (45–62 U $mL^{-1}$) were obtained with additions of syringaldazine, benzoic acid, gallic acid, and vanillin. When $CuSO_4$ was used in conjunction with these aromatic compounds, the levels of laccase activity were further improved, reaching 58–80 U $mL^{-1}$. These laccase activities indicate the potential of this strain as an enzyme producer, which has also been detected in media containing glucose, but with activity lower than that observed with sucrose.
*Keywords*: *Pleurotus sajor-caju*; Laccase; Submerged culture; $CuSO_4$; Aromatic compounds; Shake-flasks.

## INTRODUCTION

The implementation of laccase-mediated systems holds promise for biotechnological processes of industrial and environmental interest, including cellulose pulp bleaching, textile dye decolorization, polycyclic aromatic hydrocarbon oxidation, effluent detoxification, and phenol removal (Breen and Singleton, 1999; Santos *et al*., 2002; Munari *et al*., 2007; Munari *et al*., 2008; Schmitt *et al*., 2012). Additionally, laccases have found use in the pharmaceutical, chemical, and cosmetic industries, as well as in foods and beverages, e.g., for the clarification and stabilization of fruit juices. Further, laccases are employed in clinical diagnostics, the enzymatic conversion of chemical intermediates, and the upgrading of animal feed (Dhawan *et al*., 2005; Couto and Herrera, 2006). In general, white-rot fungi are able to secrete ligninolytic enzymes, including laccases (Lac - E.C. 1.10.3.2), manganese peroxidases (MnP - E.C. 1.11.1.13), and lignin peroxidases (LiP - E.C. 1.11.1.14) (Tien and Kirk, 1984).

Studies have been conducted to verify the effect of carbon sources on ligninolytic enzyme production by distinct species of basidiomycetes (Galhaup *et al*., 2002; Elisashvili *et al*., 2006; Revankar and Lele, 2006; Bettin *et al*., 2009). Laccases can be produced in submerged processes using glucose as the main carbon source and the enzyme titer can be increased by the addition of ethanol (Lee *et al*., 1999; Rodakiewicz-Nowak 1999), $CuSO_4$ (Giardina *et al*., 1999; Palmieri *et al*., 2000; Baldrian and Gabriel, 2002;

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Hess *et al*., 2002; Levin *et al*., 2002; Chen *et al*., 2003), gallic acid (Galhaup *et al*., 2002; Peralta *et al*., 2004; Gnanamani *et al*., 2006; Revankar and Lele, 2006), tannic acid (Galhaup and Haltrich, 2001), syringaldazine (Koroljova-Skorobogat'ko *et al*., 1998), vanillin (Peralta *et al*., 2004; Xiao *et al*., 2003; Elisashvili *et al*., 2010), and xylidine (Lee *et al*., 1999; Elisashvili *et al*., 2006; Jang *et al*. 2002; Mougin *et al*., 2002; Kollmann *et al*., 2005; Pazarlioglu *et al*., 2005; Jang *et al*., 2006; Murugesan *et al*., 2006).

Among the laccase producers, the genus *Pleurotus* is a cosmopolitan group of ligninolytic fungi, including certain mushrooms with high nutritional value, positive therapeutic properties, and several potential environmental and biotechnological applications (Cohen *et al*., 2002). Previous studies have shown that *Pleurotus sajor-caju* strain PS-2001 is able to grow and produce laccase in liquid medium (Confortin *et al*., 2008; Bettin *et al*., 2009; Bettin *et al*., 2011); furthermore, it presents the capacity to reduce phenolic compounds resulting from paper manufacturing and textile dye decolorization (Munari *et al*., 2007; Munari *et al*., 2008; Schmitt *et al*., 2012). The goal of the present study was to obtain higher levels of laccase activity in submerged cultures of *P. sajor-caju* strain PS-2001 using sucrose from sugar cane, the least expensive source of carbon for bioprocesses, and glucose as carbon source, and several compounds alone or in association, including $CuSO_4$, ethanol, and various aromatics: benzoic acid, gallic acid, tannic acid, phenol, syringaldazine, vanillin, and xylidine.

## MATERIALS AND METHODS

### Strain and Culture Conditions

*Pleurotus sajor-caju* strain PS-2001 was obtained from the culture collection of the Institute of Biotechnology at the University of Caxias do Sul (Brazil). The strain was grown and maintained in a medium containing (per liter): 20 g *Pinus* spp. sawdust, 20 g wheat bran, 2 g $CaCO_3$, and 20 g agar. All media, except the maintenance medium, consisted of a mineral solution containing (per liter): 20 g $KH_2PO_4$, 14 g $(NH_4)_2SO_4$, 3 g $MgSO_4 \cdot 7H_2O$, 3 g urea, 3 g $CaCl_2$, 15.6 g $MnSO_4 \cdot H_2O$, 50 mg $FeSO_4$, 14 mg $ZnSO_4$, and 20 mg $CoCl_2$ (Mandels and Reese, 1957).

*P. sajor-caju* liquid cultivations were conducted in 500-mL Erlenmeyer flasks containing 100 mL of medium, previously autoclaved at 121 ºC for 15 minutes, and maintained under reciprocal agitation of 180 rpm at 28±2 ºC (Bettin *et al*., 2009).

The inocula for the study tests were obtained from a 100-mL liquid medium containing (per liter): 5 g sucrose or glucose, 1.5 g pure casein, and 100 mL mineral solution. To start the inoculum cultivation, three mycelial disks (1.5 cm in diameter), each scraped from Petri dishes containing strain PS-2001 grown on the maintenance medium, were added to the flasks. Growth occurred upon agitation for 7 days, under the same conditions described for the experiments. For each treatment, 5 mL of inoculum were used (Bettin *et al*., 2009).

The basic culture medium contained (per liter): 5 g sucrose or glucose, 1.5 g pure casein (Synth®), and 100 mL mineral solution (Bettin *et al*., 2009). Several assays were performed with different media formulations. To the basic culture medium was added $CuSO_4$, absolute ethanol, and/or various aromatic compounds, including benzoic acid (Vetec®, Brazil), gallic acid (Nuclear®, Brazil), tannic acid (Vetec®, Brazil), phenol (Synth®), syringaldazine (Sigma®), vanillin (Sigma®), and xylidine (Aldrich®), at concentrations detailed in the text. All tests were performed in triplicate.

### Sampling Procedure and Determination of Fungal Biomass

For each study, samples were collected, centrifuged at 5,000 rpm (3,000 × *g*) for 30 min at 4 ºC, and the supernatant was used for the analytical procedures and pH determination. The samples were filtered through Whatman № 1 paper and dried at 80 ºC for 24 hours to determine the fungal biomass, in g $L^{-1}$ (Bettin *et al*., 2009).

### Enzyme Assays

Laccase (Lac) activity was determined at 25 °C using 0.45 mM 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS; Sigma®) as a substrate in reaction mixtures containing 90 mM pH 5.0 sodium acetate buffer and an appropriate amount of culture supernatant. The oxidation of ABTS was estimated by measuring the increase in absorbance at 420 nm ($\varepsilon_{420} = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$) for 90 seconds (Wolfenden and Willson, 1982).

### Determination of the Sucrose and Soluble Protein Concentrations

Sucrose was hydrolyzed with 2 M HCl at 65 ºC, and the reaction was neutralized with 1 M NaOH (Falcone and Marques, 1965). The total reducing sugars resulting from the acidic hydrolysis of sucrose

were quantified using the DNS (3,5-dinitrosalicylic acid; Sigma®) method proposed by Miller (1959). Protein levels were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

### Yield Factors, Productivities and Specific Activity

### Enzymatic and Cellular Yields

The substrate yield in terms of laccase activity was calculated by the relationship $Y_{E/S} = (E_f - E_i) / (S_i - S_f)$, where $Y_{E/S}$ is the substrate yield of laccase activity in U $g^{-1}$, $E_f$ is the final enzymatic activity in U $mL^{-1}$, $E_i$ is the initial enzymatic activity in U $mL^{-1}$, $S_i$ is the initial substrate concentration in g $L^{-1}$, and $S_f$ is the final substrate concentration in g $L^{-1}$. The results were expressed in enzymatic units formed per gram of substrate (glucose or sucrose) present in the culture medium (U $g^{-1}$).

The substrate yield in terms of biomass was calculated by the relationship $Y_{X/S} = (X_f - X_i) / (S_i - S_f)$, where $Y_{X/S}$ is substrate yield based on biomass in g $g^{-1}$, $X_f$ is the final biomass concentration in g $L^{-1}$, $X_i$ is the initial biomass concentration in g $L^{-1}$, $S_i$ is the initial substrate concentration in g $L^{-1}$, and $S_f$ is the final substrate concentration in g $L^{-1}$. The results were expressed in grams of biomass formed per gram of substrate (glucose or sucrose) present in the culture medium (g $g^{-1}$).

### Enzymatic and Cellular Productivities

The enzymatic productivity was calculated by the relationship $P_E = (E_f - E_i) / t$, where $P_E$ is the volumetric productivity of laccase activity in U $mL^{-1}$ $d^{-1}$, $E_f$ is the final enzymatic activity in U $mL^{-1}$, $E_i$ is the initial enzymatic activity in U $mL^{-1}$, and $t$ is the cultivation time in days). The results were expressed in enzymatic units formed per mL of sample per day (U $mL^{-1}$ $d^{-1}$).

The cellular productivity was calculated by the relationship $P_X = (X_f - X_i) / t$, where $P_X$ is the volumetric productivity of biomass in g $L^{-1}$ $d^{-1}$, $X_f$ is the final cellular concentration in g $L^{-1}$, $X_i$ is the initial cellular concentration in g $L^{-1}$), and $t$ is the cultivation time in days. The results were expressed in grams of biomass formed per liter of culture medium per day (g $L^{-1}$ $d^{-1}$).

### Laccase Specific Activity

The specific activity of the laccase was calculated by the relationship $SA_{Lac} = U / [TSP]$, where $SA_{Lac}$ is the specific activity in U $mg^{-1}$, $U$ represents laccase enzymatic units in U $mL^{-1}$, and [*TSP*] is the total soluble protein concentration in mg $L^{-1}$. The results were expressed in enzymatic units of laccase produced per mg of total soluble protein (U $mg^{-1}$).

### Statistical Analysis

All statistical tests were performed by analysis of variance (one-way ANOVA) and a post-hoc Tukey test, using a probability level below 5% ($p < 0.05$).

## RESULTS AND DISCUSSION

### Effects of Ethanol and Copper Sulfate on Growth and Laccase Production in the Submerged Cultivation of *Pleurotus sajor-caju* PS-2001 Using Sucrose as a Carbon Source

The data related to the production of laccase in media supplemented with ethanol and $CuSO_4$ are presented in Table 1. The addition of ethanol to the culture medium resulted in laccase activities inferior to the control at the beginning of cultivation (5, 7, and 9 days). However, no significant differences were observed at days 11, 13, and 15. In the presence of 1.56 mg $L^{-1}$ $CuSO_4$, which is the same concentration of $MnSO_4$ used in the mineral solution, no significant variation in laccase activity was observed. In contrast, in the medium containing 100 mg $L^{-1}$ $CuSO_4$, a peak in the laccase activity occurred between the $11^{th}$ and $13^{th}$ days of culture, reaching titers close to 35 U $mL^{-1}$.

The concentration of sucrose (Fig. 1) in the culture medium indicated that initially added sucrose (5 g $L^{-1}$) was not completely consumed over the 15 days of culture. During these 15 days, the pH values varied between 5.9 and 6.7, and they tended to rise at the end of culture in all treatments.

The data in Table 2 quantifies the concentration of *P. sajor-caju* biomass, showing no significant difference between the control and the treatments containing $CuSO_4$. However, the addition of 100 mg $L^{-1}$ of $CuSO_4$ to the medium led to a substantial increase in laccase activity, as mentioned previously (Table 1). The highest final biomass was achieved in the medium containing ethanol, which displayed a cellular productivity that was statistically higher than in the other media over the 15 days of culture (Table 2). However, laccase activity (Table 1) in the treatment containing ethanol was lower than in the other treatments.

**Table 1: Laccase activity in submerged cultivation of *Pleurotus sajor-caju* PS-2001 in media containing ethanol or $CuSO_4$.**

| Time (days) | Laccase activity (U $mL^{-1}$) | | | |
|---|---|---|---|---|
| | Control | Ethanol | $CuSO_4$ | |
| | | | 1.56 mg $L^{-1}$ | 100 mg $L^{-1}$ |
| **5** | $6.60 \pm 0.73^{bc}$ | $2.68 \pm 0.84^{c}$ | $7.57 \pm 1.84^{b}$ | $14.91 \pm 2.77^{a}$ |
| **7** | $12.22 \pm 1.84^{b}$ | $3.91 \pm 0.84^{c}$ | $14.17 \pm 2.24^{b}$ | $29.33 \pm 1.27^{a}$ |
| **9** | $12.46 \pm 1.46^{b}$ | $3.91 \pm 0.84^{c}$ | $16.86 \pm 3.36^{b}$ | $32.26 \pm 5.53^{a}$ |
| **11** | $13.20 \pm 1.94^{b}$ | $17.35 \pm 5.15^{b}$ | $17.84 \pm 4.17^{b}$ | $34.71 \pm 4.88^{a}$ |
| **13** | $16.13 \pm 3.11^{b}$ | $17.11 \pm 4.17^{b}$ | $19.55 \pm 5.60^{b}$ | $35.19 \pm 7.72^{a}$ |
| **15** | $14.30 \pm 4.66^{b}$ | $13.44 \pm 2.77^{b}$ | $17.60 \pm 5.13^{b}$ | $32.02 \pm 6.81^{a}$ |

Medium composition (per liter): 5 g sucrose, 1.5 g pure casein, 100 mL mineral solution, 10 mL absolute ethanol, and 1.56 mg or 100 mg copper sulfate. The control did not contain ethanol or copper sulfate. The values correspond to the average of three replicates, to the standard deviation (SD), and refer to the control and to the treatments to which ethanol or CuSO4 were added. The treatments with the same letter for each day evaluated are not statistically different at a level of 5% ($p < 0.05$).

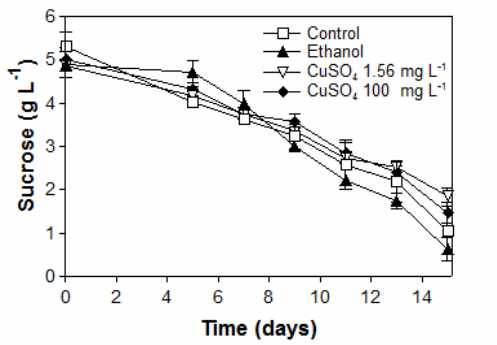


**Figure 1:** Sucrose concentration in submerged culture of *Pleurotus sajor-caju* PS-2001 with the addition of ethanol and different concentrations of $CuSO_4$ to the culture medium. Medium composition (per liter): 5 g sucrose, 1.5 g pure casein, 100 mL mineral solution, 10 mL absolute ethanol, and 1.56 mg or 100 mg copper sulfate. Control did not contain ethanol or copper sulfate. The values correspond to the average of three replicates, and the bars to the standard deviation (SD). The legends refer to the control and to the treatments to which ethanol or $CuSO_4$ were added.

**Table 2: Laccase yield from consumed sucrose ($Y_{E/S}$), enzymatic productivity ($P_E$), biomass yield from consumed sucrose ($Y_{X/S}$), and biomass productivity after 15 days of submerged cultivation of *Pleurotus sajor-caju* PS-2001 in media containing ethanol or $CuSO_4$.**

| Treatment | $Y_{E/S}$ (U $g^{-1}$) | $P_E$ (U $mL^{-1}$ $d^{-1}$) | Biomass (g $L^{-1}$) | $Y_{X/S}$ (g $g^{-1}$) | $P_X$ (g $L^{-1}$ $d^{-1}$) |
|---|---|---|---|---|---|
| **Control** | $3621 \pm 1635^{b}$ | $0.953 \pm 0.013^{b}$ | $1.29 \pm 0.14^{b}$ | $0.307 \pm 0.046^{b}$ | $0.086 \pm 0.009^{b}$ |
| **Ethanol** | $4074 \pm 1793^{b}$ | $0.896 \pm 0.007^{b}$ | $2.30 \pm 0.12^{a}$ | $0.676 \pm 0.174^{a}$ | $0.153 \pm 0.008^{a}$ |
| **$CuSO_4$ 1.56 mg $L^{-1}$** | $4522 \pm 2489^{b}$ | $1.173 \pm 0.014^{b}$ | $1.32 \pm 0.06^{b}$ | $0.326 \pm 0.095^{b}$ | $0.088 \pm 0.004^{b}$ |
| **$CuSO_4$ 100 mg $L^{-1}$** | $10636 \pm 585^{a}$ | $2.134 \pm 0.019^{a}$ | $1.19 \pm 0.15^{b}$ | $0.405 \pm 0.080^{ab}$ | $0.079 \pm 0.010^{b}$ |

Medium composition (per liter): 5 g sucrose, 1.5 g pure casein, 100 mL mineral solution, 10 mL absolute ethanol, 1.56 mg or 100 mg copper sulfate. The control did not contain ethanol or copper sulfate. The values correspond to the average of three replicates, to the standard deviation (SD), and refer to the control and to the treatments to which ethanol or $CuSO_4$ were added. Treatments with the same letter within each column are not statistically different at a level of 5% ($p < 0.05$).

It is likely that the presence of ethanol in the medium disfavored laccase production in the original culture (Fig. 1), as the levels of laccase activity were similar to or lower than the control throughout the incubation period. As a consequence of biomass growth in the medium containing ethanol, the value of $Y_{X/S}$ (Table 2) observed in the treatments containing ethanol was high; however, a high yield was not observed with respect to laccase activity (Table 1).

Based on the laccase activity levels observed in the medium containing ethanol, it was concluded that this compound did not induce the production of this enzyme. These results are contrary to those obtained by Lee *et al*. (1999), who used ethanol to induce laccase activity in *Trametes versicolor*; they observed a twenty-fold increase compared to the control.

Interestingly, Rodakiewicz-Nowak *et al*. (1999) studied the effect of ethanol on the blue laccases of *Coriolus versicolor* and the yellow laccases of *Panus tigrinus* and found that the blue form of these enzymes was inhibited, whereas the yellow form displayed increased activity in the presence of ethanol.

The fungal biomass data in Table 2 indicate that the $CuSO_4$ concentrations employed in this study did not affect fungal growth, as has been observed in cultures of *P. ostreatus* supplemented with 150 μM of $CuSO_4$ (Palmieri *et al*., 2000). In previous studies, it was shown that the addition of up to 1 mM copper had no effect on the growth of *T. trogii,* but stimulated the production of laccases and glyoxal oxidases (Levin *et al*., 2002; Trupkin *et al*., 2003).

The treatments employing $CuSO_4$ displayed activities that were significantly higher than those measured in the control or in any other treatments by the fifth day of culture. While we have not tested other concentrations of $CuSO_4$, our results strongly suggest that the induction of laccases by $CuSO_4$ in *P. sajor-caju* is dependent on salt concentration.

The presence of sucrose in the medium, even on day 15 of culture, indicates a relatively slow metabolism, which may have been the result of insufficient oxygen supply, a characteristic limitation of shake-flask tests (Kumar *et al*., 2004).

The present results are in agreement with those of Baldrian and Gabriel (2002), who reported that the presence of copper in the cultivation medium of *Pleurotus ostreatus* resulted in increased stable laccase production, independent of the time at which copper was added to the culture medium. Furthermore, in liquid cultures of *Grammothele subargentea*, Saparrat (2004) observed that the addition of 0.6 mM $CuSO_4$ promoted a peak production of 1.95 U $mL^{-1}$, whereas higher concentrations of this compound resulted in lower laccase activities. These results are contradictory to those of the present work. Palmieri *et al*. (2000) reported that, in cultures of *P. ostreatus* supplemented with 150 μM $CuSO_4$, laccase gene induction occurred at the transcription level and that the production of three isoenzymes was greatly increased under these conditions.

### Effects of Aromatic Compounds on Growth and Laccase Production in the Submerged Cultivation of *Pleurotus sajor-caju* PS-2001 Using Sucrose and Glucose as a Carbon Source

The data related to the production of laccase in media supplemented with 100 mg $L^{-1}$ of phenol, syringaldazine, vanillin, benzoic acid, gallic acid, or tannic acid are presented in Table 3. The media containing benzoic acid and syringaldazine displayed laccase activities higher than the other treatments from the beginning of culture, and the activities with each compound were not significantly different. The medium containing gallic acid showed peak laccase activity on the 9$^{th}$ day, at levels similar to those of benzoic acid. The treatment with vanillin produced higher laccase activity (45 U $mL^{-1}$) on the 11$^{th}$ day. The treatment containing phenol displayed laccase activities similar to those of the control through the 11$^{th}$ day of cultivation, and subsequently displayed higher levels on days 13 and 15. The medium containing tannic acid displayed enzyme levels similar to or lower than the control during the incubation period. After day 11, no laccase activity was detected in this treatment, providing strong evidence that the addition of tannic acid inhibits the induction of these enzymes. Overall, these results suggest that, among the compounds tested and under the conditions of this assay, phenol, syringaldazine, vanillin, benzoic acid, and gallic acid are inducers of laccase activity. The data for the consumption of sucrose are illustrated in Fig. 2. As mentioned, through day 15 of culture the sucrose was not completely consumed, as previously reported. The same downward trend was observed in all treatments.

Data for the mycelial biomass on the 15$^{th}$ day of culture, shown in Table 4, indicate that the highest final concentrations were obtained in media containing benzoic acid and vanillin (1.6 to 1.7 g $L^{-1}$), with the latter displaying values similar to those of the control. Consequently, the best results using these media related to biomass yield and productivity were obtained. The worst results were achieved in media containing tannic acid, suggesting that this compound is detrimental to both enzyme production and cell growth. Treatments containing phenol, syringaldazine, and gallic acid behaved similarly with respect to biomass formation.

The pH values for all experiments other than that containing phenol (which displayed small variations between 5.7 and 5.9), showed a pH drop from the beginning to the end of culture (data not shown). The treatments containing syringaldazine, vanillin, benzoic acid, and gallic acid showed the same trend as the control, varying from 5.9 to 4.4. The medium containing tannic acid displayed the lowest pH values (between 5.8 and 4.0), which was attributed to the low laccase activity observed in this treatment (Table 3).

The data presented in Table 3 corroborate the results obtained by Peralta *et al*. (2004) in experiments

**Table 3: Laccase activity in submerged cultivation of *Pleurotus sajor-caju* PS-2001 in media containing different aromatic compounds.**

| Time (days) | Laccase activity (U mL$^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | Phenol | SYR | Vanillin | Benzoic acid | Gallic acid | Tannic acid |
| 5 | 16.86 ± 4.46$^{b}$ | 3.91 ± 1.27$^{b}$ | 42.04 ± 9.73$^{a}$ | 19.31 ± 5.69$^{b}$ | 35.68 ± 6.15$^{a}$ | 15.88 ± 3.69$^{b}$ | 5.37 ± 1.52$^{b}$ |
| 7 | 21.75 ± 6.15$^{bc}$ | 4.88 ± 0.73$^{cd}$ | 51.57 ± 3.38$^{a}$ | 25.17 ± 5.20$^{b}$ | 49.13 ± 8.92$^{a}$ | 38.50 ± 11.92$^{ab}$ | 2.93 ± 3.88$^{d}$ |
| 9 | 17.84 ± 6.93$^{d}$ | 8.55 ± 1.69$^{de}$ | 62.33 ± 2.07$^{a}$ | 37.15 ± 7.34$^{c}$ | 50.84 ± 4.03$^{ab}$ | 48.39 ± 2.07$^{bc}$ | 0.73 ± 1.27$^{e}$ |
| 11 | 8.80 ± 8.06$^{cd}$ | 29.08 ± 4.77$^{bc}$ | 28.23 ± 9.85$^{bc}$ | 45.22 ± 7.04$^{ab}$ | 50.23 ± 8.81$^{a}$ | 30.43 ± 7.77$^{ab}$ | 0.00 ± 0.00$^{e}$ |
| 13 | 4.15 ± 3.30$^{d}$ | 21.51 ± 2.57$^{bc}$ | 13.56 ± 2.59$^{c}$ | 27.13 ± 6.00$^{b}$ | 38.13 ± 4.14$^{a}$ | 15.03 ± 0.51$^{c}$ | 0.00 ± 0.00$^{d}$ |
| 15 | 1.46 ± 1.27$^{b}$ | 19.55 ± 2.96$^{a}$ | 1.46 ± 0.00$^{b}$ | 22.97 ± 2.57$^{a}$ | 21.26 ± 8.29$^{a}$ | 4.03 ± 3.62$^{b}$ | 0.00 ± 0.00$^{b}$ |

Medium composition (per liter): 5 g sucrose, 1.5 g pure casein, 100 mL mineral solution, and 100 mg aromatic compounds. The control did not contain aromatic compounds. The values correspond to the average of three replicates and refer to the control and to the treatments where phenol, syringaldazine (SYR), vanillin, benzoic acid, gallic acid, or tannic acid were added. The treatments with the same letter for each day evaluated are not statistically different at a level of 5% ($p < 0.05$).

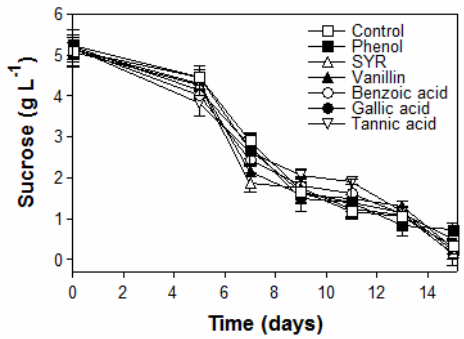


**Figure 2:** Sucrose concentration in submerged culture of *Pleurotus sajor-caju* PS-2001 with the addition of different aromatic compounds to the culture medium. Medium composition (per liter): 5 g sucrose, 1.5 g pure casein, 100 mL mineral solution, and 100 mg aromatic compounds. Control did not contain aromatic compounds. The values correspond to the average of three replicates, and the bars to the standard deviation (SD). The legends refer to the control and to the treatments in which phenol, syringaldazine (SYR), vanillin, benzoic acid, gallic acid, or tannic acid were added.

**Table 4: Biomass concentration, biomass yield from consumed sucrose ($Y_{X/S}$) and biomass productivity ($P_X$) after 15 days of submerged cultivation of *Pleurotus sajor-caju* PS-2001 in media containing different aromatic compounds.**

| Treatment | Biomass (g L$^{-1}$) | $Y_{X/S}$ (g g$^{-1}$) | $P_X$ (g L$^{-1}$ d$^{-1}$) |
|---|---|---|---|
| **Control** | 1.52 ± 0.03$^{b}$ | 0.322 ± 0.040$^{abc}$ | 0.101 ± 0.002$^{b}$ |
| **Phenol** | 1.31 ± 0.04$^{c}$ | 0.291 ± 0.007$^{abc}$ | 0.087 ± 0.003$^{c}$ |
| **Syringaldazine** | 1.40 ± 0.01$^{c}$ | 0.289 ± 0.028$^{abc}$ | 0.093 ± 0.001$^{c}$ |
| **Vanillin** | 1.63 ± 0.07$^{ab}$ | 0.337 ± 0.037$^{ab}$ | 0.108 ± 0.002$^{ab}$ |
| **Benzoic acid** | 1.66 ± 0.03$^{a}$ | 0.365 ± 0.073$^{a}$ | 0.110 ± 0.002$^{a}$ |
| **Gallic acid** | 1.33 ± 0.01$^{c}$ | 0.269 ± 0.014$^{abc}$ | 0.088 ± 0.001$^{c}$ |
| **Tannic acid** | 1.14 ± 0.02$^{d}$ | 0.231 ± 0.013$^{c}$ | 0.076 ± 0.005$^{d}$ |

Medium composition (per liter): 5 g sucrose, 1.5 g pure casein, 100 mL mineral solution, and 100 mg aromatic compounds. The control did not contain aromatic compounds. The values correspond to the average of three replicates, to the standard deviation (SD), and to the treatments where phenol, syringaldazine, vanillin, benzoic acid, gallic acid, or tannic acid were added. Treatments with the same letter within each column are not statistically different at a level of 5% ($p < 0.05$).

performed with *Pleurotus pulmonarius.*

In their experiments, vanillin and several soluble phenolic compounds induced the greatest laccase activity. In the same study, the treatment containing gallic acid also showed increased enzyme levels, and the media supplemented with syringaldazine and tannic acid displayed titers close to those observed in the control. Studies by Galhaup and Haltrich (2001) with the fungus *Trametes pubescens* confirmed that laccase levels were raised by the addition of gallic acid, but not in the presence of tannic acid, as observed in the present work. The higher laccase activity titers obtained with basidiomycetes in the presence of some aromatic compounds may be related to the fact that these compounds have structures that are similar to those derived from lignin.

We also studied an alternate medium composition, in which the sucrose carbon source was replaced by glucose. Higher titers were obtained in media containing phenol, benzoic acid, and xylidine at 60, 52, and 48 U $mL^{-1}$, respectively; all showed peak activities during the 9th day of cultivation (Fig. 3). Interestingly, the media containing $CuSO_4$ and gallic acid displayed the lowest enzyme activities, similar to the control but significantly different than in the sucrose experiments. The pH values again showed the same trend during the evaluation period, varying between 5.4 and 6.2. The data in the studies of the various aromatic compounds with glucose showed that sucrose is a better carbon source than glucose for laccase production.

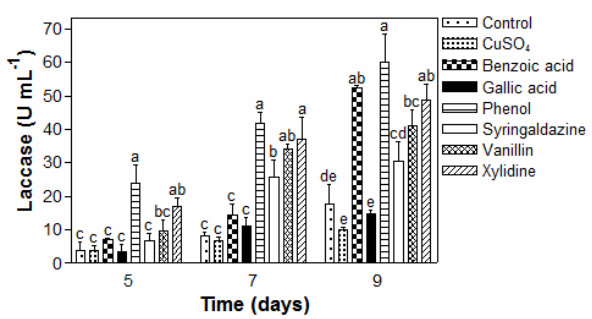


**Figure 3:** Laccase activity in submerged culture of *Pleurotus sajor-caju* PS-2001 with the addition of different aromatic compounds to the culture medium. Medium composition (per liter): 5 g glucose, 1.5 g pure casein, 100 mL mineral solution, and 100 mg inducer compounds. Control did not contain inducer compounds. The values correspond to the average of three replicates, and the bars to the standard deviation (SD). The legends refer to the control and to the treatments in which copper sulfate, benzoic acid, gallic acid, phenol, syringaldazine, vanillin, or xylidine were added. Treatments with the same letter for each day evaluated are not statistically different at a level of 5% ($p < 0.05$).

### Effects of Copper Sulfate in Combination with Different Aromatic Compounds on Growth and Laccase Production in the Submerged Cultivation of *Pleurotus sajor-caju* PS-2001 Using Sucrose and Glucose as a Carbon Source

After observing that $CuSO_4$ and aromatic compounds both showed positive effects on laccase production, we examined the laccase activities in media containing both $CuSO_4$ and various aromatic compounds; the data are presented in Table 5. The treatments with phenol displayed lower laccase activities, while the association of benzoic acid, gallic acid, syringaldazine, and vanillin with $CuSO_4$ resulted in laccase activities of up to 80 U $mL^{-1}$. These values are higher than those obtained in the previous assay in which the compounds were used alone, displaying values near 60 U $mL^{-1}$. A possible explanation for these results is that an additive effect is operative when $CuSO_4$ was associated with benzoic acid, gallic acid, syringaldazine, and vanillin in the culture medium. As observed by Chen *et al*. (2003), enzymatic synthesis in *Volvariella volvacea* is associated with secondary metabolism and is positively regulated by the addition of 200 μM $CuSO_4$ and various aromatic compounds.

In the present assay, the pH (data not shown) did not change during the course of culture, remaining at between 6.1 and 6.5. The sucrose concentration data confirmed the presence of residual carbohydrate levels (data not shown), which were not fully consumed until the last day of culture, as shown in Fig. 1 and Fig. 2. All treatments presented the same tendencies during the culture.

The mycelial biomass data, quantified after 15 days of culture, were similar among the treatments, with values near 1 g $L^{-1}$ (data not shown), suggesting that the variation in media composition directly influenced laccase production (Table 5), but not biomass formation. The data for biomass yield (data not shown) also indicate that this parameter, like the biomass data, did not differ significantly among the treatments, showing values between 0.2 and 0.3 g $g^{-1}$.

The soluble protein profile (Fig. 4a), which represents both media components and the enzyme, was similar across the different treatments during the cultivation. Fig. 4b shows the specific activities that relate laccase activity with the soluble protein in the culture medium. It is evident that variation occurred during the culturing process as a consequence of high or low enzymatic activities. Specific activities up to 360 U $mg^{-1}$ were observed in the treatment with benzoic acid, as consequence of high laccase activities (Table 5). Due to its low laccase activity (Table 5), the medium containing phenol displayed the lowest specific activity, of 40 U $mg^{-1}$. The media containing syringaldazine, vanillin and gallic acid presented higher specific laccase activities (Fig. 4b).

As for sucrose, $CuSO_4$ was added to a medium containing glucose along with each of the different aromatic compounds, including benzoic acid, gallic acid, phenol, syringaldazine, vanillin, and xylidine (Fig. 5). The media containing $CuSO_4$ and xylidine, benzoic acid, and phenol displayed the highest laccase activities, of 40, 38, and 35 U $mL^{-1}$, respectively. Among the treatments that showed high laccase activity, the medium containing $CuSO_4$ associated with benzoic acid had the most stable activity, with high enzymatic activities on all days studied.

**Table 5: Laccase activity in submerged cultivation of *Pleurotus sajor-caju* PS-2001 in media containing copper sulfate and different aromatic compounds.**

| Time (days) | Laccase activity (U mL$^{-1}$) | | | | |
|---|---|---|---|---|---|
| | Phenol | Syringaldazine | Vanillin | Benzoic acid | Gallic acid |
| **5** | $2.68 \pm 2.24^{b}$ | $7.57 \pm 2.24^{b}$ | $29.08 \pm 7.63^{a}$ | $22.24 \pm 3.70^{a}$ | $31.28 \pm 1.69^{a}$ |
| **7** | $5.62 \pm 3.69^{c}$ | $13.20 \pm 2.64^{c}$ | $35.68 \pm 5.70^{b}$ | $30.55 \pm 4.42^{b}$ | $49.86 \pm 4.40^{a}$ |
| **9** | $6.11 \pm 4.48^{b}$ | $19.06 \pm 5.29^{b}$ | $47.91 \pm 5.70^{a}$ | $46.93 \pm 11.6^{a}$ | $63.06 \pm 2.20^{a}$ |
| **11** | $5.86 \pm 3.66^{c}$ | $28.60 \pm 11.1^{b}$ | $54.26 \pm 9.59^{a}$ | $55.48 \pm 7.34^{a}$ | $67.71 \pm 0.42^{a}$ |
| **13** | $4.40 \pm 3.36^{c}$ | $58.91 \pm 5.92^{b}$ | $66.73 \pm 11.5^{ab}$ | $72.11 \pm 9.76^{ab}$ | $80.66 \pm 3.36^{a}$ |
| **15** | $3.91 \pm 2.57^{b}$ | $45.22 \pm 11.6^{a}$ | $59.88 \pm 12.6^{a}$ | $65.75 \pm 6.65^{a}$ | $63.80 \pm 2.93^{a}$ |

Medium composition (per liter): 5 g sucrose, 1.5 g pure casein, 100 mL mineral solution, 100 mg copper sulfate, and 100 mg aromatic compounds. The values correspond to the average of three replicates, to the standard deviation (SD), and refer to the treatments where phenol, syringaldazine, vanillin, benzoic acid, or gallic acid were added. The treatments with the same letter for each day evaluated are not statistically different at a level of 5% ($p < 0.05$).

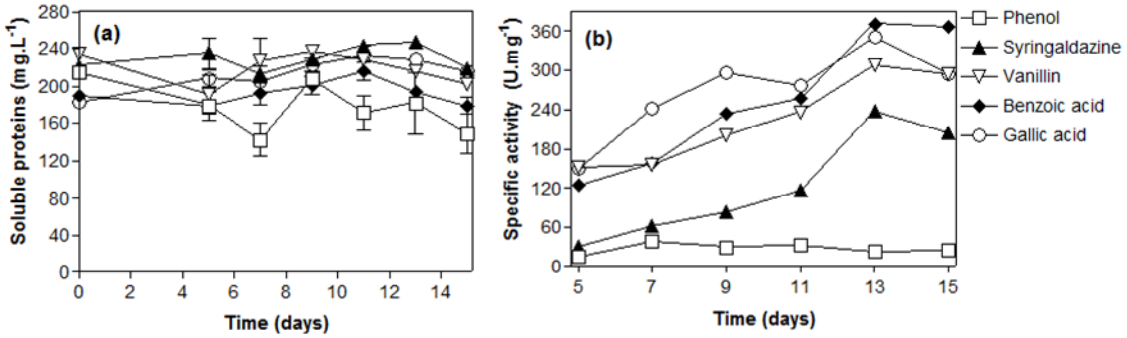


**Figure 4:** Total soluble proteins (a) and laccase specific activity (b) in submerged culture of *Pleurotus sajor-caju* PS-2001 with the addition of copper sulfate and different aromatic compounds to the culture medium. Medium composition (per liter): 5 g sucrose, 1.5 g pure casein, 100 mL mineral solution, 100 mg copper sulfate, and 100 mg aromatic compounds. The values correspond to the average of three replicates, and the bars to the standard deviation (SD). The legends refer to the treatments in which phenol, syringaldazine, vanillin, benzoic acid, or gallic acid were added.

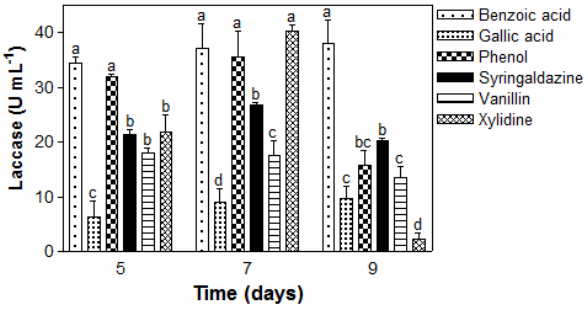


**Figure 5:** Laccase activity in submerged culture of *Pleurotus sajor-caju* PS-2001 with the addition of copper sulfate and different aromatic compounds to the culture medium. Medium composition (per liter): 5 g glucose, 1.5 g pure casein, 100 mL mineral solution, 100 mg copper sulfate, and 100 mg aromatic compounds. The values correspond to the average of three replicates, and the bars to the standard deviation (SD). The legends refer to the treatments in which benzoic acid, gallic acid, phenol, syringaldazine, vanillin, or xylidine were added. Treatments with the same letter for each day evaluated are not statistically different at a level of 5% ($p < 0.05$).

The treatment containing phenol showed the highest concentration of biomass (Fig. 6a), of approximately 2.6 g L$^{-1}$ after 7 days of cultivation. The treatment containing xylidine also displayed a high biomass yield (2.2 g L$^{-1}$ at the 9$^{th}$ day); these treatments were higher than all the others. Fig. 6b shows the high level of residual glucose (up to 1 g L$^{-1}$) remaining after 9 days of growth. As in the sucrose experiments, the fungus did not fully consume the glucose, likely due to an insufficient oxygen supply in the system, as suggested by Kumar *et al*. (2004). In some treatments, the biomass decreased from the 7$^{th}$ to the 9$^{th}$ day of cultivation, especially in the medium containing phenol. We hypothesize that this was related to

mycelial autolysis.

We obtained data for overall yields and productivities (data not shown); due to the similarities in substrate consumption (Fig. 6b) observed across the different treatments, the profiles followed very closely the results for the overall enzymatic activity (Fig. 5) and biomass (Fig. 6a), with peaks similar to laccase activity and cellular concentration. A similar trend was observed with the data obtained for enzymatic and cellular productivity (data not shown), both of which showed peaks similar to laccase activity and biomass concentration (Fig. 5 and Fig. 6a, respectively). Overall, the laccase levels obtained with the glucose medium were lower than those obtained when sucrose was used, suggesting that, under the conditions tested, sucrose favors enzymatic production.

The measurements of laccase activity presented in the current work are similar to or higher than those found in the literature (Table 6). The new data pertaining to *P. sajor-caju*, obtained herein lend support to the belief that this microorganism is of potential industrial utility as a laccase producer. Furthermore, we demonstrated that it is possible to increase its laccase activity when sucrose is used as a carbon source in a medium containing $CuSO_4$ in combination with several aromatic compounds. High laccase activities of *P. sajor-caju* PS-2001 have also been obtained with similar culture medium in a stirred-tank bioreactor (Bettin *et al*., 2011).

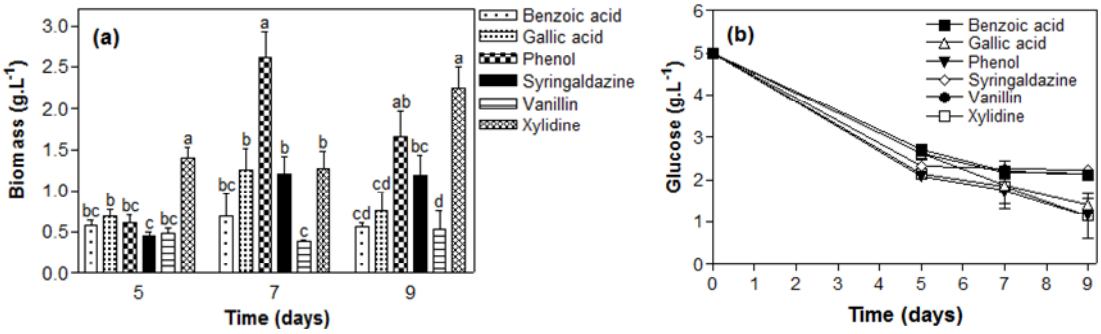


**Figure 6:** Biomass (a) and glucose concentration (b) in submerged culture of *Pleurotus sajor-caju* PS-2001 with the addition of copper sulfate and different aromatic compounds to the culture medium. Medium composition (per liter): 5 g glucose, 1.5 g pure casein, 100 mL mineral solution, 100 mg copper sulfate, and 100 mg aromatic compounds. The values correspond to the average of three replicates, and the bars to the standard deviation (SD). The legends refer to the treatments in which benzoic acid, gallic acid, phenol, syringaldazine, vanillin, or xylidine were added. Treatments with the same letter for each day evaluated are not statistically different at a level of 5% ($p < 0.05$).

**Table 6: Laccase activities produced by different white rot fungi in shake-flasks, quantified with ABTS as substrate.**

| Organism | Peak of laccase activity (U $mL^{-1}$) | Day | Inducer | Reference |
|---|---|---|---|---|
| *Cerrena unicolor* | 170<br>103 | - | Vanillin<br>Xylidine | Elisashvili *et al*. (2010) |
| *Ganoderma lucidum* | 69 | - | Xylidine | Elisashvili *et al*. (2010) |
| *Grammothele subargentea* | 1.95 | 20 | $CuSO_4$ | Saparrat (2004) |
| Unidentified | 692<br>410 | 6 | Gallic acid<br>$CuSO_4$ | Revankar and Lele (2006) |
| *Phanerochaete chrysosporium* | 0.01<br>0.02 | 5<br>5 | Gallic acid<br>$CuSO_4$ | Gnanamani *et al*. (2006) |
| *Pleurotus dryinus* | 7.77<br>8.90 | 6<br>7 | $CuSO_4$<br>Xylidine | Elisashvili *et al*. (2006) |
| *Pleurotus eryngii* | 0.08 | 6 | 2,5-xylidine | Munõz *et al*. (1997) |
| *Pleurotus ostreatus* | 30.0<br>400 | -<br>19 | $CuSO_4$<br>$CuSO_4$ | Giardina *et al*. (1999)<br>Baldrian and Gabriel (2002) |
| *Pleurotus sajor-caju* | 14.0 | 8 | Xylidine | Jang *et al*. (2006) |
| ***Pleurotus sajor-caju* PS-2001** | **72.1**<br>**80.7**<br>**66.7**<br>**58.9** | **13**<br>**13**<br>**13**<br>**13** | **$CuSO_4$ and benzoic acid**<br>**$CuSO_4$ and gallic acid**<br>**$CuSO_4$ and vanillin**<br>**$CuSO_4$ and syringaldazine** | *** This work.** |

*Continuation Table 6*

*Continuation Table 6*

**Table 6: Laccase activities produced by different white rot fungi in shake-flasks, quantified with ABTS as substrate.**

| Organism | Peak of laccase activity (U $mL^{-1}$) | Day | Inducer | Reference |
|---|---|---|---|---|
| *Pycnoporus cinnabarinus* | 9.60 | 8 | 2,5-xylidine | Hess *et al*. (2022) |
| *Trametes multicolor* | 18.0 | - | $CuSO_4$ | Moldes *et al*. (2003) |
| *Trametes pubescens* | 4.40 | 13 | Gallic acid | Galhaup and Haltrich (2001) |
| | 2.50 | 13 | Tannic acid | Galhaup and Haltrich (2001) |
| | 8.00 | 13 | 2,5-xylidine | Galhaup and Haltrich (2001) |
| | 68.5 | 4 | $CuSO_4$ | Galhaup and Haltrich (2001) |
| | 360 | 16 | Gallic acid | Galhaup *et al*. (2002) |
| | 320 | 16 | $CuSO_4$ | Galhaup *et al*. (2002) |
| | 270 | 16 | 2,5-xylidine | Galhaup *et al*. (2002) |
| *Trametes trogii* | 90.3 | 27 | $CuSO_4$ | Levin *et al*. (2002) |
| | 17.2 | 26 | $CuSO_4$ | Trupkin *et al*. (2003) |
| *Trametes versicolor* | 0.90 | 7 | Gallic acid | Lee *et al*. (1999) |
| | 1.10 | 7 | 2,5-xylidine | Lee *et al*. (1999) |
| | 5.69 | 3 | Xylidine | Mougin *et al*. (2002) |
| | 6.71 | 3 | 2,5-xylidine | Kollmann *et al*. (2005) |
| | 3.00 | 3 | Phenol | Pazarlioglu *et al*. (2005) |
| | 14.5 | - | Xylidine | Elisashvili *et al*. (2010) |
| *Trametes* sp. | 12.5 | 2 | 2,5-xylidine | Jang *et al*. (2002) |
| | 15.8 | 2 | 2,5-xylidine and ABTS | Jang *et al*. (2002) |
| | 12.5 | 7 | 2,5-xylidine | Jang *et al*. (2006) |
| | 15.8 | 7 | 2,5-xylidine and ABTS | Jang *et al*. (2006) |
| | 15.0 | 7 | 2,5-xylidine and guaiacol | Jang *et al*. (2006) |
| | 13.8 | 7 | 2,5-xylidine and Syringaldazine | Jang *et al*. (2006) |
| *Trichophyton rubrum* | 0.46 | 7 | $CuSO_4$ | Bermek *et al*. (2004) |

* Culture media containing sucrose as a carbon source.

## CONCLUSIONS

In addition to the information found in the literature on the induction of fungal laccase, the data about the little-studied *P. sajor-caju* obtained in the present work strengthen the case that this micro-organism can potentially be used as an industrial producer of laccase, as it is possible to increase its laccase activity when sucrose or glucose is used as a carbon source in a medium containing $CuSO_4$ associated with aromatic compounds, i.e., benzoic acid, gallic acid, phenol, syringaldazine, vanillin, and xylidine. In comparison to previous reports, relatively high laccase activities were found, indicating the potential of this strain as an enzyme producer.

## ACKNOWLEDGEMENTS

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