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ORIGINAL PAPER

Production of laccases in submerged process by *Pleurotus sajor-caju* PS-2001 in relation to carbon and organic nitrogen sources, antifoams and Tween 80

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Abstract Some conditions in media composition for laccases production, such as different sources of carbon and organic nitrogen, antifoams and a surfactant, were studied in liquid cultures of *Pleurotus sajor-caju* strain PS-2001. Cultivation with fructose or glucose as carbon sources produced maximum enzyme activities of 37 and 36 U mL^{-1}, respectively. When sucrose was present in the medium, the best results were obtained using 5 g L^{-1} of this carbohydrate, on the 11th day of the process, attaining laccase titres of 13 U mL^{-1}. In a medium without casein, practically no enzyme was produced during the experiments; among the sources of nitrogen studied, pure casein led to the highest titres of laccase activity. Different concentrations of pure casein and sucrose were also tested. As to the different concentrations of casein, the addition of 1.5 g L^{-1} resulted in the highest titres of laccase activity. Negligible levels of manganese peroxidase activity were also detected in the culture medium. In low concentrations, polypropylene glycol or silicon-based antifoams and the surfactant Tween 80 have no significant influence on the formation of laccases by *P. sajor-caju*. However, enhanced concentration of polypropylene glycol negatively affected the production of laccases but favored the titres in total peroxidases, lignin peroxidase and veratryl alcohol oxidase.

Keywords Laccases · *Pleurotus sajor-caju* · Submerged process · Carbon and organic nitrogen sources · Antifoam · Tween 80

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Introduction

In addition to their high gastronomic value, fungi belonging to the genus *Pleurotus* can colonize and degrade a broad variety of lignocellulosic residues and pollutants. These organisms produce different molecules with interesting biological activities, among them some ligninolytic enzymes [1]. These enzymes include phenoloxidases such as manganese peroxidase (MnP; E.C. 1.11.1.13), lignin peroxidase (LiP; E.C. 1.11.1.14) and laccases (Lac; E.C. 1.10.3.2), which are secreted in the growth medium when nutrient sources, particularly carbon and nitrogen, are in low levels [2–4].

Laccases are multi-copper enzymes, which oxidize phenolic compounds reducing oxygen to water by the one-electron abstraction from the aromatic substrate, generating phenoxyl radicals [5–8]. Since these enzymes catalyze the oxidation of a broad range of phenolic and aromatic amines, laccase-mediated processes become a very promising alternative for cellulose pulp bleaching, effluent detoxification, phenol removal, and discoloring textile dyes [9–16]. Among the phenoloxidases, fungal laccases provide an efficient degradation of xenobiotic compounds and can degrade some persistent aromatic pollutants, such as pesticides, organophosphorus insecticides, chlorophenols and polycyclic aromatic hydrocarbons, whose compounds may bioaccumulate in the trophic chains [17–20].

Cultivating basidiomycete fungi in liquid media allows the formation of more biomass in less time, favors fungal dispersion and adaptation and is easier to manipulate, besides producing ligninolytic enzymes [21, 22].

The ligninolytic enzymes are produced during the secondary metabolism under conditions of limited nutrients. The carbon and nitrogen sources in the medium are necessary to the production of ligninolytic enzymes [23, 24].

Laccases and peroxidases production depends on the species of fungi, conditions of cultivation, carbon and nitrogen sources and their concentrations [25].

Studies have been conducted to verify the effect of carbon sources on ligninolytic enzyme production by distinct species of basidiomycetes [23–29]. Some of these studies have shown that both the nature and concentration of nitrogen sources are powerful nutrition factors regulating ligninolytic enzyme production by white rot basidiomycetes [23–25, 27–29]. Additions of aromatic compounds in carbon-nitrogen limited media of different fungi lead to a considerable increase in ligninolytic enzyme titres [30–35].

In the production of enzyme, especially in large-scale processing, antifoam and surfactant are normally used. Surfactants are utilized in the degradation of polyaromatic compounds since they facilitate the solubilization of these compounds in the medium [36]. Tween 80 is a non-ionic surfactant that is helpful in releasing fungi enzymes to the external environment [37]. It presents low toxicity to the cellular membrane, but it can alter the structure and the morphology of fungi and bacteria cell wall, leading to the increase of protein secretion [38].

The *Pleurotus sajor-caju* strain PS-2001, which is commercially used to produce fruiting bodies, has shown ability to degrade dye [15] and to reduce polyphenol content and colour intensity of effluents from pulp and paper plants [16], being both activities associated with the presence of laccase in the medium.

Considering the potential application of *P. sajor-caju* PS-2001 phenoloxidases for solving environmental problems, it is essential to define the best conditions for the production of such enzymes. However, no results with respect to the production of phenoloxidases by *P. sajor-caju* in medium containing a low-cost carbon source, as sucrose, as well as the use of casein as nitrogen source, are available in the literature. Moreover, there are no published studies about the effects of surfactants and antifoams on this process. Thus, the aim of this work was to study the production of phenoloxidases, especially laccases, by *P. sajor-caju* strain PS-2001 in liquid media containing different sources and concentrations of carbon and organic nitrogen, particularly sucrose and casein. Furthermore, the influence of two antifoams, polypropylene glycol and silicon, and the surfactant Tween 80 on this process was assessed.

Materials and methods

Organism and culture conditions

P. sajor-caju strain PS-2001, from the fungus collection of the Institute of Biotechnology, University of Caxias do Sul (Brazil), was used in this work. The strain was maintained in a medium containing (per litre) 20 g *Pinus* spp. sawdust, 20 g wheat bran, 2 g $CaCO_3$ and 20 g agar.

The liquid media used in this work for both inoculum preparation and *P. sajor-caju* cultivation contained 10% (v/v) of the mineral solution described by Mandels and Reese [39] with the following composition (per litre): 20 g KH_2PO_4, 14 g $(NH_4)_2SO_4$, 3 g $MgSO_4{\cdot}7H_2O$, 3 g urea, 4 g $CaCl_2$, 15.6 mg $MnSO_4{\cdot}H_2O$, 50 mg $FeSO_4$, 14 mg $ZnSO_4$ and 20 mg $CoCl_2$.

The media were autoclaved at 121°C and 1 atm for 15 min. Inocula were prepared in 500-mL Erlenmeyer flasks containing 100 mL of liquid media with the following composition (per litre): 5 or 10 g fructose, glucose, lactose, or sucrose (the inocula were prepared with the same carbon source used in the different treatments); 1.0 or 1.5 g pure casein and 100 mL mineral solution. To start the inoculum cultivation, three mycelial disks (with ca. 1.5 cm in diameter), scraped from Petri dishes containing strain PS-2001 grown on maintenance medium, were added to the flasks. Cell growth occurred under reciprocal agitation at 180 rpm and 28 ± 2°C for seven days. In each treatment, 5 mL of inoculum were used.

P. sajor-caju liquid cultures were carried out in triplicate under the same conditions as described for the inoculum preparation. In these assays, different media formulations were evaluated to assess the following: (1) comparison of organic nitrogen sources—pure casein (Synth®), meat peptone (Merck®), beer yeast (Jasmine®, Brazil), powdered soy extract (Olvebra®, Brazil), and isolated soy protein (Samprosoy®, Brazil); (2) effect of pure casein concentration; (3) effect of sucrose concentration; (4) comparison of carbon sources—fructose, glucose, lactose, and sucrose. The effects of polypropylene glycol antifoam (Fluent Cane 114®), silicon antifoam (Vetec®, Brazil), and Tween 80 (Vetec®, Brazil) surfactant on the production of ligninolytic enzymes were also tested. The concentration of each component is mentioned within the text.

Sampling procedures

For each study, samples were collected periodically, centrifuged at 5,000 rpm (3,000*g*) for 30 min at 4°C and the supernatant was used for the analytical procedures and pH measurement. On the last day of cultivation, the samples were filtered through Whatman no. 1 paper and dried at 80°C in order to determined the fungal biomass, in g L^{-1} [33, 40].

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 substrate in reaction mixtures

containing 90 mM pH 5.0 sodium acetate buffer and an appropriate amount of culture supernatant. ABTS oxidation was estimated by measuring increase in absorbance at 420 nm ($\varepsilon_{420} = 3.6 \times 10^4\ cm^{-1}\ M^{-1}$) for 90 s [41]. Total peroxidase activity was estimated in a similar procedure as laccase determination in a reaction medium containing ABTS as substrate and 200 µM of H_2O_2 as described by Heinzkill et al. [42].

MnP activity was assayed at 30°C using 50 µg mL^{-1} phenol red (Merck®) as substrate, 50 µM $MnSO_4{\cdot}H_2O$, 50 µM H_2O_2, 12.5 mM sodium lactate, 500 µg mL^{-1} bovine albumin, 20 mM pH 4.5 sodium succinate buffer and 0.5 mL of culture supernatant. The reaction was stopped by adding 40 µL NaOH 2 M to the mixture. The oxidation of phenol red was determined by measuring the increase in absorbance at 610 nm ($\varepsilon_{610} = 4.46 \times 10^4\ M^{-1}\ cm^{-1}$) [43]. One unit of enzyme activity was defined as the amount that catalyzes the production of 1 µmol of coloured product per mL per min.

Veratryl alcohol oxidase (VAO) activity was assayed by measuring the increase in absorbance at 310 nm ($\varepsilon_{310} = 9.3 \times 10^3\ M^{-1}\ cm^{-1}$) in the presence of veratryl alcohol (Sigma®) in sodium tartarate buffer 250 mM pH 5.0 for 5 min [44]. LiP activity was assayed as previously described [2] using veratryl alcohol and H_2O_2 as substrate in sodium tartarate buffer 250 mM pH 3.0 for 5 min ($\varepsilon_{310} = 9.3 \times 10^3\ M^{-1}\ cm^{-1}$).

Determination of soluble proteins concentration

Protein levels were determined by the method of Bradford [45] using bovine serum albumin as standard.

Statistical analysis

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

Results

Effect of organic nitrogen sources on the growth and laccase production by *Pleurotus sajor-caju* PS-2001

Initially, it was verified that amongst the different sources of organic nitrogen, all of which at 1 g L^{-1}, in media with 10 g L^{-1} of sucrose, pure casein led to the highest laccase enzymatic activities on all days evaluated, reaching minimum levels of 1.22 U mL^{-1} and maximum levels of 7.08 U mL^{-1} in up to 13 days of culture. The other treatments containing meat peptone, beer yeast, soy extract, and isolated soy protein only presented laccase titres between 0.24 and 1.22 U mL^{-1}. It should be emphasized that no detectable laccase levels were found in the evaluations performed in treatments without nitrogen source.

MnP activities were detected at levels of up to 4 U mL^{-1} in the treatment containing beer yeast. Similarly as observed for laccase activity, MnP was not found in all treatments, and it did not present a specific relationship with the addition of any of the nitrogen sources tested. The pH of samples remained between 5.6 and 7.1 showing a tendency to increase from the beginning to the end of the culture.

Figure 1 shows biomass concentration after 13 days of culture in media containing different nitrogen sources. It can be observed that the treatments containing beer yeast and soy protein provided the highest biomass concentrations, above 4 g L^{-1}, while for the medium containing pure casein, the concentration achieved was higher than 3 g L^{-1}. These values are statistically similar to the control, which did not contain organic nitrogen and did not show detectable laccase activity.

As shown in Table 1, the medium containing 1.5 g L^{-1} of pure casein, when compared to the other concentration tested for this nitrogen source, significantly increased enzymatic activity from the 7th day of culture onwards, with a peak of 15.4 U mL^{-1} on the 13th day of culture, decreasing on the 15th day.

MnP activity was detected in all treatments only on the 7th day of culture, and in the control (without casein) the highest titres, near to 3.8 U mL^{-1}, were verified. The production of this enzyme was not as regular as that of laccases (Table 1).

During the 15 days of the experiment, pH values varied between 5.5 and 6.5, showing a slight tendency to rise from the beginning to the end of cultivation.

Biomass concentration was measured after 15 days of culture (Fig. 2), and the control (without casein) showed average levels of 3.7 g L^{-1}, without significant difference observed among the different conditions.

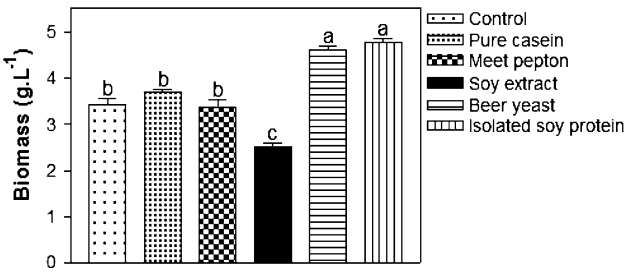

Fig. 1 Biomass concentration after 13 days of submerged culture of *Pleurotus sajor-caju* PS-2001 in media with different sources of organic nitrogen. Medium composition (per litre): 10 g sucrose, 100 mL mineral solution, 1 g organic nitrogen source. Control did not contain organic nitrogen. The values correspond to the average of three replicates. The treatments with the same letter are not statistically different at a level of 5% ($P < 0.05$)

Table 1 Laccase activity in submerged cultures of *Pleurotus sajor-caju* PS-2001 in media with different concentrations of pure casein as a source of organic nitrogen

Time (days)	Laccase activity (U mL^{-1})				
	Control	0.5 g L^{-1}	1.0 g L^{-1}	1.5 g L^{-1}	2.0 g L^{-1}
5	0.00 ± 0.00^{d}	2.68 ± 0.42^{bcd}	3.42 ± 1.12^{abc}	5.62 ± 1.52^{a}	3.91 ± 1.12^{ab}
7	0.00 ± 0.00^{c}	3.42 ± 0.42^{b}	4.88 ± 1.12^{b}	8.06 ± 1.46^{a}	4.88 ± 1.12^{b}
9	0.00 ± 0.00^{c}	5.62 ± 1.12^{b}	6.60 ± 1.46^{b}	12.71 ± 1.84^{a}	6.84 ± 1.12^{b}
11	0.00 ± 0.00^{d}	4.40 ± 0.73^{c}	4.64 ± 2.24^{c}	14.91 ± 0.84^{a}	8.79 ± 1.94^{b}
13	0.00 ± 0.00^{d}	3.17 ± 0.42^{c}	4.39 ± 0.73^{c}	15.40 ± 0.73^{a}	12.22 ± 1.12^{b}
15	0.00 ± 0.00^{d}	2.44 ± 0.42^{c}	3.42 ± 0.42^{c}	11.24 ± 1.12^{a}	7.08 ± 1.12^{b}

Medium composition (per litre): 10 g sucrose; 100 mL mineral solution; 0.5, 1.0, 1.5 or 2.0 g pure casein. The control did not contain casein. The values correspond to the average of three replicates and refer to the control and to the experiments with media containing different concentration of pure casein. The treatments with the same letter for each day evaluated are not statistically different at a level of 5% ($P < 0.05$)

The variation of total soluble protein concentration with the incubation time (Fig. 3) shows that there was a decrease in concentration from the beginning to the end of the culture, indicating casein consumption by the fungus during the assays. When analyzing the data in Table 1 and Fig. 3, it can be observed that the increased laccase production from the 5th day onwards and the peak of activity on the 13th day of culture coincide with the reduction of protein concentration in the medium.

Effect of carbon sources on the growth and laccase production by *Pleurotus sajor-caju* PS-2001

The growth and laccase production by *P. sajor-caju* PS-2001 were evaluated in relation to different sucrose concentrations (0, 5, 7.5, 10 and 15 g L^{-1}) in medium with 1.5 g.L^{-1} of pure casein and the mineral solution. Data on laccase activity (Table 2) showed significantly higher values in the medium containing 5 g L^{-1} of sucrose on the 11th day of culture. Throughout the assay, a trend towards a decrease in laccase activity was found from the 13th day onwards for all treatments. Whereas laccase activity was detected on all days of cultivation, MnP activity was observed only on the 9th and 13th day of culture, in any treatment, reaching approximately 1.2 U mL^{-1}. The pH measured over the 15 days of culture showed small variations (between 5.9 and 6.2) and the same tendency to increase in all treatments.

After 15 days of cultivation, in the tests with 10 and 15 g L^{-1} of sucrose, biomass concentration achieved significantly higher values—4.5 and 4.6 g L^{-1}, respectively—than those found in media containing 5.0 and 7.5 g L^{-1} of sucrose. This corresponds to 2.3 and 3.8 g L^{-1} of fungal biomass, respectively (Fig. 4). The determination of total soluble proteins in an assay of sucrose concentration (Fig. 5) showed a decrease in concentration from the beginning of the culture for all conditions.

In further experiments, different carbon sources (fructose, glucose, lactose, and sucrose) were compared at concentrations

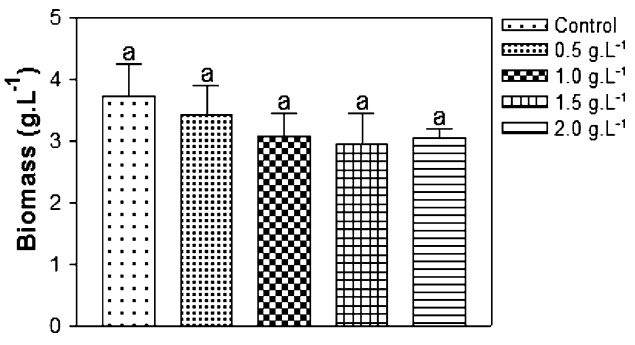

Fig. 2 Biomass concentration after 15 days of submerged cultures of *Pleurotus sajor-caju* PS-2001 in media with different pure casein concentrations as a source of nitrogen. Medium composition (per litre): 10 g sucrose; 100 mL mineral solution; 0.5, 1.0, 1.5 or 2.0 g pure casein. Control did not contain casein. The values correspond to the average of three replicates. The treatments with the same letter are not statistically different at a level of 5% ($P < 0.05$)

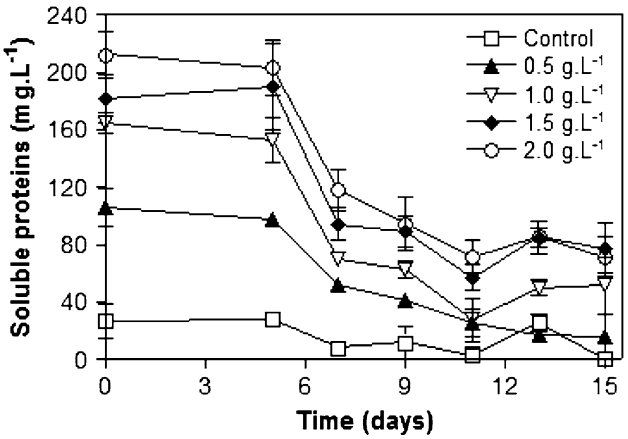

Fig. 3 Time course of total soluble protein in submerged cultures of *Pleurotus sajor-caju* PS-2001 in media with different concentrations of pure casein. Medium composition (per litre): 10 g sucrose; 100 mL mineral solution; 0.5, 1.0, 1.5 or 2.0 g pure casein. Control did not contain casein. The values correspond to the average of three replicates

Table 2 Laccase activity in submerged cultures of *Pleurotus sajor-caju* PS-2001 in media with different sucrose concentrations

Time (days)	Laccase activity (U mL^{-1})				
	Control	5.0 g L^{-1}	7.5 g L^{-1}	10 g L^{-1}	15 g L^{-1}
5	0.48 ± 0.00^{a}	0.97 ± 0.42^{a}	0.97 ± 0.42^{a}	0.73 ± 0.00^{a}	0.73 ± 0.00^{a}
7	0.24 ± 0.00^{b}	3.17 ± 1.12^{a}	2.44 ± 0.42^{a}	2.93 ± 0.73^{a}	2.44 ± 0.42^{a}
9	1.71 ± 0.42^{d}	6.84 ± 1.12^{a}	4.40 ± 0.73^{bc}	5.37 ± 1.12^{ab}	2.68 ± 0.84^{cd}
11	3.66 ± 1.27^{b}	12.46 ± 0.73^{a}	6.11 ± 1.12^{b}	5.13 ± 0.73^{b}	5.37 ± 1.12^{b}
13	5.37 ± 1.12^{abc}	7.33 ± 0.73^{a}	6.35 ± 1.12^{ab}	3.66 ± 0.73^{cd}	2.93 ± 0.73^{d}
15	4.88 ± 0.84^{ab}	6.35 ± 0.42^{a}	4.15 ± 0.42^{b}	1.46 ± 0.73^{c}	1.46 ± 0.73^{c}

Medium composition (per litre): 1.5 g pure casein; 100 mL mineral solution; 5.0, 7.5, 10 or 15 g sucrose. The control did not contain sucrose. The values correspond to the average of three replicates and refer to the control and to the experiments with media containing different concentration of sucrose. The treatments with the same letter for each day evaluated are not statistically different at a level of 5% ($P < 0.05$)

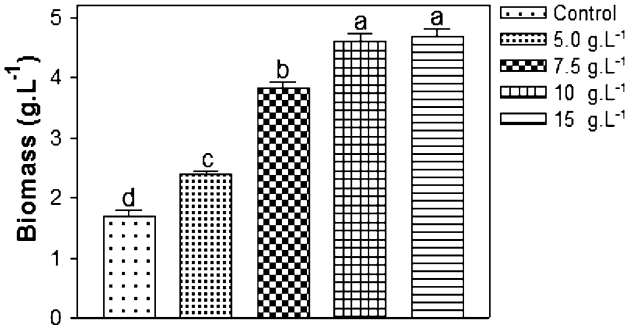

Fig. 4 Biomass concentration after fifteen days of submerged cultures of *Pleurotus sajor-caju* PS-2001 in media with different concentrations of sucrose. Medium composition (per litre): 1.5 g pure casein; 100 mL mineral solution; 5.0, 7.5, 10 or 15 g sucrose. Control did not contain sucrose. The values correspond to the average of three replicates. The treatments with the same letter are not statistically different at a level of 5% ($P < 0.05$)

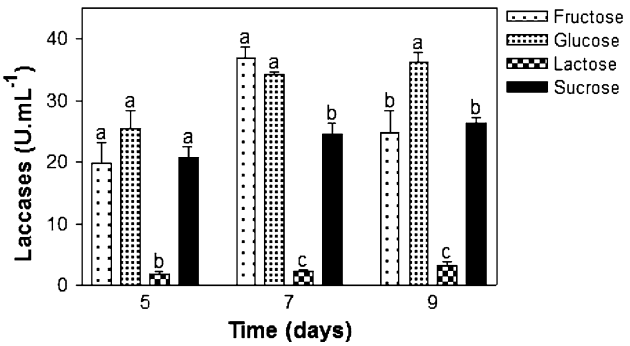

Fig. 6 Laccase activity in submerged cultures of *Pleurotus sajor-caju* PS-2001 in media with different carbon sources. Medium composition (per litre): 1.5 g pure casein; 100 mL mineral solution; 5.0 g fructose, glucose, lactose or sucrose. The values correspond to the average of three replicates. The treatments with the same letter are not statistically different at a level of 5% ($P < 0.05$)

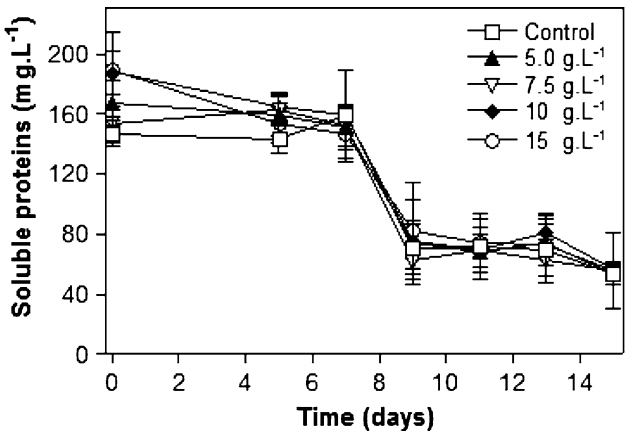

Fig. 5 Time course of total soluble protein in submerged cultures of *Pleurotus sajor-caju* PS-2001 in media with different concentrations of sucrose. Medium composition (per litre): 1.5 g pure casein; 100 mL mineral solution; 5.0, 7.5, 10 or 15 g sucrose. Control did not contain sucrose. The values correspond to the average of three replicates

of 5 g L^{-1} with respect to laccase production (Fig. 6). The results obtained with fructose and glucose (37 and 36 U mL^{-1}, respectively) were higher than those obtained with sucrose (26 U mL^{-1}). With lactose, a negligible value of laccase activity (3 U mL^{-1}) was found. The higher enzymatic activity presented in Fig. 6 in comparison to those of Tables 1 and 2, for media with sucrose, could be associated with variations of mycelia concentration during inoculum production.

Effect of antifoam and Tween 80 on the ligninolytic enzymes production by *Pleurotus sajor-caju* PS-2001

Figure 7 shows the data of phenoloxidase production in media containing 2 mL L^{-1} of polypropylene glycol antifoam, in medium containing 5 g L^{-1} of glucose, 1.5 g L^{-1} of pure casein and mineral solution. On the 7th day of cultivation of *P. sajor-caju*, it can be seen that the addition of polypropylene glycol to the medium had a negative effect on the production of laccases (Fig. 7a). Nevertheless, with regard to the production of total peroxidases, LiP and VAO, favorable effects of this antifoam were observed (Fig. 7b–d, respectively). MnP was not detected at any time in this group of experiments.

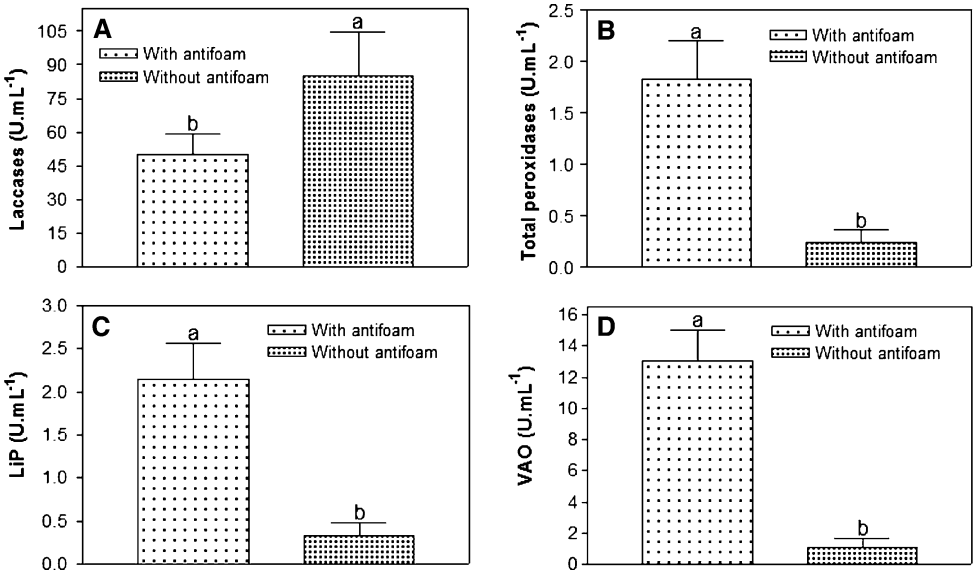

Fig. 7 Laccase (**a**), total peroxidase (**b**), lignin peroxidase (**c**) and veratryl alcohol oxidase (**d**) activity in submerged cultures of *Pleurotus sajor-caju* PS-2001 in media with and without polypropylene glycol antifoam after 7 days of culture. Medium composition (per litre): 5 g glucose, 1.5 g pure casein, 100 mL mineral solution, 2 mL polypropylene glycol antifoam. Control did not contain antifoam. The values correspond to the average of three replicates. The treatments with the same letter are not statistically different at a level of 5% ($P < 0.05$)

In the sequence, the addition of polypropylene glycol to the medium in a lower concentration (1 mL L^{-1}) was evaluated with respect to the production of laccases in comparison to silicon-based antifoam at the same concentration. Furthermore, the presence of the surfactant Tween 80 in the medium (2 mL L^{-1}) was also tested (Fig. 8). In the days of cultivation evaluated, the statistic difference occurred only on the 5th day, for polypropylene glycol, confirming the negative effect of this substance on enzyme production, although the media containing the antifoam showed similarity with the control on the 7th and 9th days. Likewise, the silicon-based antifoam data were not different from the control.

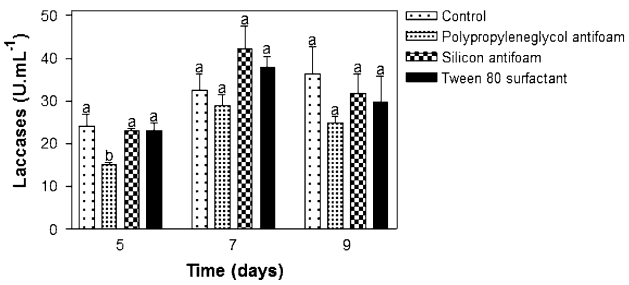

Fig. 8 Laccase activity in submerged cultures of *Pleurotus sajor-caju* PS-2001 in media with different additives. Medium composition (per litre): 5 g glucose, 1.5 g pure casein, 100 mL mineral solution, 1 mL polypropylene glycol or silicon antifoam, 2 mL Tween 80 surfactant. Control did not contain additives. The values correspond to the average of three replicates. The treatments with the same letter are not statistically different at a level of 5% ($P < 0.05$)

Discussion

The results of Fig. 1 indicate that the organic nitrogen source affects fungal growth and enzyme production in different ways, and no association was found between laccase production and biomass formation. The statistical similarity of the data showed in Fig. 2 may be due to the inorganic nitrogen source used besides the presence of sucrose in the culture medium. Furthermore, the analysis performed only after 15 days of culture does not consider a possible autolysis of hyphae that may have occurred previously, which may have influenced the quantification of the fungal biomass.

The data showing that the addition of pure casein to the culture medium leads to higher enzyme titres are according to previous studies performed with strains of *Pleurotus eryngii*, *Pleurotus ostreatus,* and *Pleurotus pulmonarius*. These studies showed that among several organic and inorganic sources of nitrogen tested, satisfactory laccase activity was obtained in a medium supplemented with casein and peptone, though for *P. pulmonarius* the highest titres were observed in a medium supplemented only with casein [25]. Mikiashvili et al. [23] also obtained elevated titres of laccases for *P. ostreatus*, when hydrolyzed casein, corn steep liquor, and peptone were used. In this work, corn steep liquor and peptone, among other protein sources, were also those that promoted the largest biomass concentration. The higher laccase titres obtained in experiments containing casein might be related to its amino acid composition.

The analysis of total soluble protein (Fig. 3) and of enzyme activity corroborate the data reported in the literature, namely that the enzymatic activity occurs with a

limitation of nutrients, especially sources of carbon and nitrogen [2, 3]. Studies on the supplementation of protein sources in the culture of different fungi have already been performed [22, 28, 33, 46–48]. However, most of these studies only evaluated inorganic sources of nitrogen. The difference here consists in studying organic nitrogen sources, as in the case of pure casein; and it can be concluded that, among those tested, the concentration of 1.5 g L^{-1} (Table 1) was the most appropriate for laccase production in a submerged culture of *P. sajor-caju* PS-2001.

With regard to the data in Table 2, it as observed that the presence of laccases in the control—which contained pure casein but not sucrose—suggests that adding protein to the culture medium is more important for enzyme production than adding a carbon source, since in previously presented assays the control (without casein) did not show enzymatic activity on any of the days evaluated. As expected, the media without sucrose (control) presented the lowest biomass concentration (Fig. 4), and the cell growth observed may be due to the organic nitrogen source added to the culture medium. As already discussed [48, 49], the peak of laccase activity occurred after 11 days of culture (Table 2), probably when the fungus' metabolism showed a decrease in total soluble proteins concentration (Fig. 5).

This work further showed that sucrose at a concentration of 5 g L^{-1} could also be used as a carbon source for laccase production (Table 2, Fig. 6). Many studies have already been conducted concerning the supplementation of medium with carbon sources, especially glucose, for the cultivation of different fungi [22, 23, 25, 28, 47, 48, 50]. It is important to remark that, in Brazil, sugar cane sucrose is a significantly cheaper carbon source for biotechnological processing in comparison to glucose and fructose.

The presence of LiP during the assays, as presented in Fig. 7, is unusual since reports [30, 51] mention the absence of this enzyme during the liquid culture of *Pleurotus* sp. The results herein indicate that choosing antifoam for laccase production must be carefully evaluated because, depending on the antifoam concentration, the enzymatic activity may be affected (Fig. 7, 8). With respect to the use of Tween 80 (Fig. 8), no significant influence on the process was observed, corroborating the report of Giese et al. [38] on the production of constitutive laccases by *Botryosphaeria* sp.

The results of this work disclose the potential of *P. sajor-caju* strain PS-2001 to produce laccases in a liquid medium. In summary, it is possible to conclude that, among the different sources of organic nitrogen tested, pure casein is the most appropriate for laccase production, especially at a concentration of 1.5 g L^{-1} in the culture medium, under the conditions of the present assays. The addition of beer yeast and soy protein to the medium increases the fungal biomass of *P. sajor-caju* PS-2001, when compared with other protein sources, but this concentration was not followed by laccase activity in the enzyme broth. In addition, it was observed that sucrose, a relatively cheap carbohydrate, is an adequate carbon source for the growth and production of laccases, particularly when added to the medium at a concentration of 5 g L^{-1}. The presence of polypropylene glycol antifoam in the medium can negatively alter laccase production, but apparently, its presence positively influences other enzymes as total peroxidases, LiP and VAO. Silicon-based antifoam and Tween 80, at least at the concentrations tested, do not present any effect on the process.

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