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ORIGINAL PAPER

Increasing *Pleurotus ostreatus* laccase production by culture medium optimization and copper/lignin synergistic induction

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Abstract Laccases have great biotechnological potential in diverse industries as they catalyze the oxidation of a broad variety of chemical compounds. Production of laccases by basidiomycetes has been broadly studied as they secrete the enzymes, grow on cheap substrates, and they generally produce more than one isoenzyme (constitutive and/or inducible). Laccase production and isoenzyme profile can be modified through medium composition and the use of inducers. The objective of this work was to increase laccase production by *Pleurotus ostreatus* CP-50 through culture medium optimization and the simultaneous use of copper and lignin as inducers. Increased fungal growth was obtained through the use of a factorial fractional experimental design 2^{6-2} where the influence of the nature and concentration of carbon and nitrogen sources was assessed. Although specific laccase production (U/mg biomass) decreased when malt extract medium was supplemented with carbon and nitrogen sources, fungal growth and laccase volumetric activity increased four and sixfold, respectively. The effect of media supplementation with copper and/or lignin on laccase production by *P. ostreatus* CP-50 was studied. A positive synergistic effect between copper and lignin was observed on laccase production. Overall, the use of an optimized medium and the simultaneous addition of copper and lignin improved growth, laccase volumetric activity, and process productivity by 4-, 60-, and 10-fold, respectively.

Keywords Laccase · *Pleurotus ostreatus* · Media optimization · Inducers

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Introduction

Laccases (p-diphenol:oxygen oxidoreductases, EC 1.10.3.2) are multicopper enzymes that catalyze the oxidation of a broad variety of substrates such as mono-, di-, and polyphenols, aminophenols, methoxyphenols, aromatic amines, and ascorbic acid [19]. They have a great biotechnological potential in diverse fields of industrial application, including effluent detoxification, kraft pulp and dye bleaching, polymer synthesis, bioremediation of contaminated soils, baking, wine and beverage stabilization, the manufacture of anticancer drugs, and recently, in nanobiotechnology as part of biosensors for immunoassays [2, 16]. Production of laccases by basidiomycetes has been broadly studied as they secrete enzymes and grow on cheap substrates. Furthermore, more than one laccase isoenzyme, either constitutive or inducible, has been detected in most white-rot fungi [19]. However, as constitutive extracellular laccases from basidiomycetes are produced only in small amounts, the use of laccases for industrial applications has been limited by low process productivities and, as a consequence, high enzyme costs [12, 22]. Enhancing laccase production through the use of inducers and different nutritional conditions has been reported [6, 11, 25, 28]. Particularly, laccase production is influenced by carbon and

nitrogen concentrations in culture media [3, 6, 20, 21, 26]. The use of enzyme inducers has also been investigated as they not only influence the type of isoenzymes produced but also increase volumetric activity. Much attention has been paid to the chemical induction of laccases by the addition of aromatic or phenolic compounds [12, 18], copper [15, 23], lignin [7], and ethanol [17], among others. However, the use of a combination of inducers for increasing laccase production remains almost unexplored [13].

Despite the large number of reports dealing with factors affecting laccase production by basidiomycetes, the effects of such factors on mycelial growth have been generally neglected. This is particularly important when inducers are added to the cultures, as most of them are toxic to the producing strains. Although the specific production (U/mg biomass) is frequently enhanced, enzyme titers and process productivities remain low as mycelial growth is severely affected [28].

The purpose of this work was to increase the production of laccases by *Pleurotus ostreatus* CP-50, which is a widely distributed cultivar for commercial mushroom production in Mexico. This strain produces two different laccases in a non-induced malt extract medium [9]. Laccases from this strain have been successfully applied for the oxidation of phenolic compounds (unpublished results). However, maximal volumetric activities were low (200 U l^{-1}) as biomass growth was scarce (up to 2.5 g l^{-1}). In order to increase laccase production by *P. ostreatus* CP50, we propose to firstly enhance fungal growth by medium improvement to increase biosynthesis potential of the culture and then evaluate the addition of inducers in order to maximize laccase production. In line with this strategy, the effect of different carbon and nitrogen sources on *Pleurotus ostreatus* growth was assessed, through a fractional factorial experimental design, and a medium composition improving biomass growth was selected. Then the individual and interaction effects of the culture media supplementation with copper and lignin on laccase production were evaluated.

Materials and methods

Fungal strain

P. ostreatus CP-50 was obtained from the Postgraduate College at Puebla (Colegio de Postgraduados, Unidad Puebla, Km 125.5 Carretera México-Puebla La Libertad, Cholula, 72130, Mexico). The microorganism was maintained on 2% (w/v) malt extract-agar slants at 4°C.

Chemicals

ABTS (2,2′-azinobis [3-ethylbenzo-thiazoline-6-sulfonic acid]) was from Sigma-Aldrich (St. Louis, MO). Glucose, buffer salts, and all other chemicals were from J.T. Baker (Phillipsburg, NJ). Food-grade malt extract was from Drogueria Cosmopolita, S.A. de C. V. (Mexico City, Mexico). Bacto™ Agar, Yeast extract, Bacto™ Peptone and Bacto™ Tryptone were from DIFCO (Lawrence, KS). $CuSO_4 \cdot 5H_2O$ was obtained from J.T. Baker (Phillipsburg, NJ) and soluble lignin (alkali, low sulfonate content) was from Sigma-Aldrich (St. Louis, MO).

Inoculum

P. ostreatus was grown on plates containing 2% (w/v) malt extract-agar at 30°C. Pre-inoculum was produced in 500-ml Erlenmeyer flasks containing 100 ml malt extract (2% w/v, pH 6). The flasks were inoculated with 2-cm^2 agar plugs covered by mycelia from a 6-day-old culture previously homogenized with 10 ml of culture medium. After 4 days of incubation at 150 rev min^{-1} and 30°C, the mycelium was collected, washed twice with a salt solution (NaCl, 0.9% w/v), and homogenized for 10 s in a Sorvall Omnimixer (model 17150; Ivan Sorvall Inc., Newtown, CT) with distilled water (1:3 v/v, mycelium:water). One ml of this suspension was used as inoculum for 100-ml liquid cultures in 500-ml Erlenmeyer flasks and incubated on a rotary shaker at 30°C and 150 rev min^{-1}.

Effect of carbon and nitrogen sources on growth and laccase production by *Pleurotus ostreatus* CP50

Three carbon sources (xylose, glycerol, and glucose; 0 and 10 g l^{-1}) and three nitrogen sources (peptone, yeast extract, and tryptone; 5 and 10 g l^{-1}) were evaluated (Table 1). All media contained 2% malt extract. Cultures were conducted by triplicate using 500-ml Erlenmeyer flasks with 100 ml of liquid medium and were harvested after 96 h of incubation on a rotary shaker at 30°C and 150 rev min^{-1}. The effect of the medium composition on mycelial growth and laccase production was assessed using a fractional factorial design 2^{6-2}. This factorial design is known as a IV resolution design, which allows independent estimation of individual and two-factor interactions effects. As it is often true that high-order interactions (triple or above) tend to become negligible, it is possible to assess significant effects keeping reasonable the number of experiments (16 vs. 64 for a full factorial) [4]. The main effects for each of the factors evaluated on the response, were:

Table 1 Fractional factorial 2^{6-2} experimental design used to evaluate the influence of the nature and concentration of carbon and nitrogen sources on growth and laccases production by *P. ostreatus* CP-50

Run	Variables in coded levels						Responses			
	Peptone	Yeast extract	Tryptone	Xylose	Glycerol	Glucose	Biomass[a] (mg ml^{-1})	Biomass[b] (mg ml^{-1})	Laccase[a] (U ml^{-1})	Laccase[b] (U ml^{-1})
1	−1	−1	−1	−1	−1	−1	6.8 ± 0.60	7.1	0.6 ± 0.028	0.6
2	+1	−1	−1	−1	+1	−1	10.0 ± 0.89	9.8	0.6 ± 0.029	0.6
3	−1	+1	−1	−1	+1	+1	9.4 ± 1.54	9.0	0.6 ± 0.061	0.6
4	+1	+1	−1	−1	−1	+1	9.8 ± 1.15	10.6	1.2 ± 0.115	1.1
5	−1	−1	+1	−1	+1	+1	8.2 ± 0.88	7.6	0.7 ± 0.061	0.7
6	+1	−1	+1	−1	−1	+1	9.7 ± 1.57	9.2	0.7 ± 0.105	0.7
7	−1	+1	+1	−1	−1	−1	5.6 ± 1.37	5.6	0.7 ± 0.047	0.7
8	+1	+1	+1	−1	+1	−1	6.9 ± 0.61	7.3	1.1 ± 0.102	1.2
9	−1	−1	−1	+1	−1	+1	7.0 ± 0.22	7.2	0.7 ± 0.104	0.8
10	+1	−1	−1	+1	+1	+1	7.0 ± 0.27	7.0	1.0 ± 0.070	1.0
11	−1	+1	−1	+1	+1	−1	7.5 ± 0.18	7.3	1.0 ± 0.068	1.0
12	+1	+1	−1	+1	−1	−1	7.0 ± 0.13	7.1	1.2 ± 0.105	1.3
13	−1	−1	+1	+1	+1	−1	7.2 ± 0.38	7.3	1.0 ± 0.092	0.9
14	+1	−1	+1	+1	−1	−1	7.0 ± 0.21	7.1	1.1 ± 0.098	1.0
15	-1	+1	+1	+1	−1	+1	7.1 ± 0.27	7.1	1.0 ± 0.068	1.1
16	+1	+1	+1	+1	+1	+1	7.1 ± 0.11	6.9	1.4 ± 0.152	1.4

[a] Experimental data

[b] Estimated data

$$\beta_i = \left(y_i^+\right) - \left(y_i^-\right) \qquad (1)$$

where β_i is the effect of the ith factor on the response, and y_i^+ and y_i^- are the mean responses for the upper (+) and the lower (−) levels of the ith factor. Interactions of two factors were also calculated by this equation. The general equation is a first-degree polynomial:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_{ij} + \varepsilon \qquad (2)$$

where Y is the estimated response, β_0 is the general mean, $\Sigma\ \beta_i\ X_i$ is the sum of main effects of the factors, $\Sigma\ \beta_{ij}\ X_{ij}$ is the sum of two-factor interaction effects and ε is the lack of fit of the model (error).

Effect of copper and lignin on growth and laccase production by *Pleurotus ostreatus*

Medium 4 (Table 1) was selected as high biomass concentration was obtained and laccase production was significantly improved. Lignin (0.5 g l^{-1}) and $CuSO_4$ (0.5 mM) were added individually or combined at the middle of the growth phase (60 h).

Analytical techniques

Mycelial biomass was measured as reported before [9]. ABTS (2,2′-azinobis [3-ethylbenzothia-zoline-6-sulfonic acid]) was used as laccase substrate (1 mM in acetate buffer pH 3.6). The enzymatic reaction was monitored in a Beckman DU 650 spectrophotometer at 436 nm (ε_{436} = 29,300 M^{-1} cm^{-1}). One enzyme unit (U) was defined as the amount of enzyme catalyzing the production of 1 μmol of oxidized product per minute.

Statistical analysis

All the experiments were independently performed by triplicate and differences among treatments were evaluated by ANOVA analysis ($p = 0.05$) using DESIGN-EXPERT, version 5.0.7 (Stat-Ease Inc., Minneapolis, MN).

Results and discussion

The effect of medium composition (carbon and nitrogen sources) on maximal *P. ostreatus* growth and laccase volumetric activity was evaluated through a fractional factorial 2^{6-2} and the results are summarized in Table 1.

Effects of carbon and nitrogen sources on *P. ostreatus* growth

P. ostreatus growth was significantly influenced by the nature and concentration of carbon and nitrogen sources.

ANOVA showed that the effect of the factors studied was significantly different from zero (F test, $p < 0.001$) while lack of fit of the model was not (F test, $p = 0.09$). Analysis of the most significant effects demonstrated that Eq. (2) can be reduced to:

$$\begin{aligned}\text{Biomass}(gl^{-1}) = {} & 7.64 + 0.35\text{Pep} - 0.36\text{Tryp} \\ & - 0.51\text{Xyl} + 0.47\text{Glu} \\ & - 0.45\text{PepXyl} - 0.52\text{PepGly} \\ & + 0.34\text{YexGlu} + \varepsilon\end{aligned}$$

In order to understand the main factor effects it is necessary to deduce the nature of the interactions. Figure 1a–c shows the interaction graphs for the three significant two-factor effects. Interaction graphs show the mean value (and the standard deviation) of the response (biomass) at the upper (+) and lower (−) level of the factors involved. Figure 1a shows that biomass concentration was practically unaffected by peptone concentration if xylose concentration is 10 g l^{-1} (level +). However, when xylose is absent (level −), biomass concentration was significantly increased when 10 g l^{-1} of peptone (level +) was used. As the main effect of xylose is negative (−0.51), it can be concluded that xylose negatively affected *P. ostreatus* growth. Figure 1b shows a peptone–glycerol interaction graph. Biomass concentration was not affected by peptone concentration when glycerol (10 g l^{-1}) was present in the culture medium. However, in the absence of glycerol, peptone concentration had a positive effect on biomass concentration. The main effect of glycerol was not significant and therefore should not be used, whereas peptone has to be at the highest level (10 g l^{-1}) to increase biomass concentration. Figure 1c shows the yeast extract–glucose interaction graph. For instance, when yeast extract is at the upper level (10 g/l), a significant increase of biomass concentration occurs when glucose is added to the medium. This is not the case when yeast extract was at 5 g/l (low level). In this case, a higher biomass concentration can be obtained when both yeast extract and glucose are at the upper level (10 g l^{-1}).

Figure 2a, b shows the surface response of maximal biomass concentration as a function of peptone, glucose, and xylose (most significant factors), in a medium containing yeast extract 10 g l^{-1} and tryptone 5 g l^{-1}. According to the model, medium 4 (peptone 10 g l^{-1}; yeast extract 10 g l^{-1}; tryptone 5 g l^{-1}, and glucose 10 g l^{-1}) is (within the experimental domain) the best choice in order to maximize biomass concentration with an estimated value of 10.6 g l^{-1}, which is not significantly different to the experimental value of 9.8 g l^{-1}. The influence of the type and concentration of carbon and nitrogen sources on fungal growth and laccase production by *P. ostreatus* was initially assessed in order to enhance fungal growth before the medium was supplemented with copper and lignin.

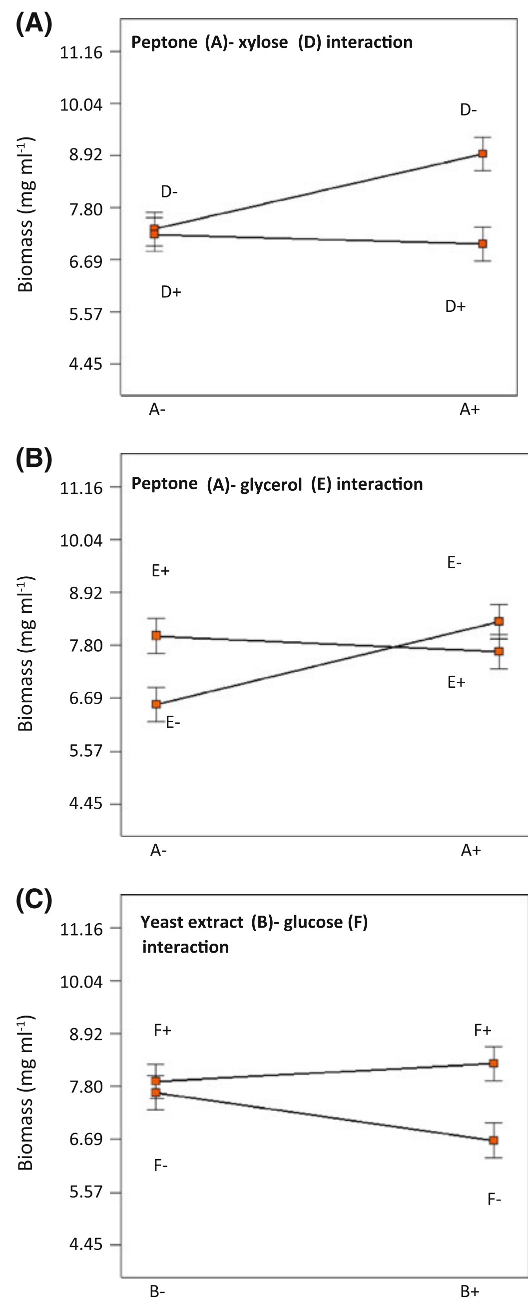

Fig. 1 Relevant factor interactions ($p < 0.05$) affecting growth of *P. ostreatus* CP-50. **a** Peptone–xylose interaction. **b** Peptone–glycerol interaction. **c** Yeast extract–glucose interaction

Effects of carbon and nitrogen sources on laccase production by *P. ostreatus*

As it was the case for fungal growth, laccase production was also significantly influenced by carbon and nitrogen sources. ANOVA showed that the effect of the factors

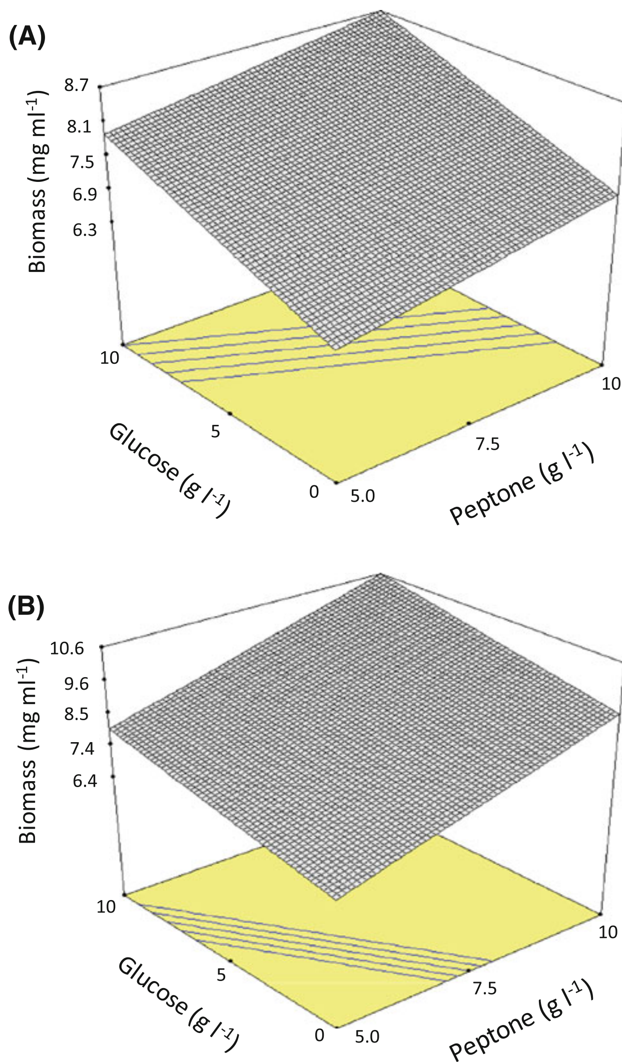

Fig. 2 Response surface representing maximal biomass concentration versus glucose and peptone concentration. Media composition (g l^{-1}): **a** tryptone 5, yeast extract 10, glycerol 0, xylose 10; **b** tryptone 5, yeast extract 10, glycerol 0, xylose 0

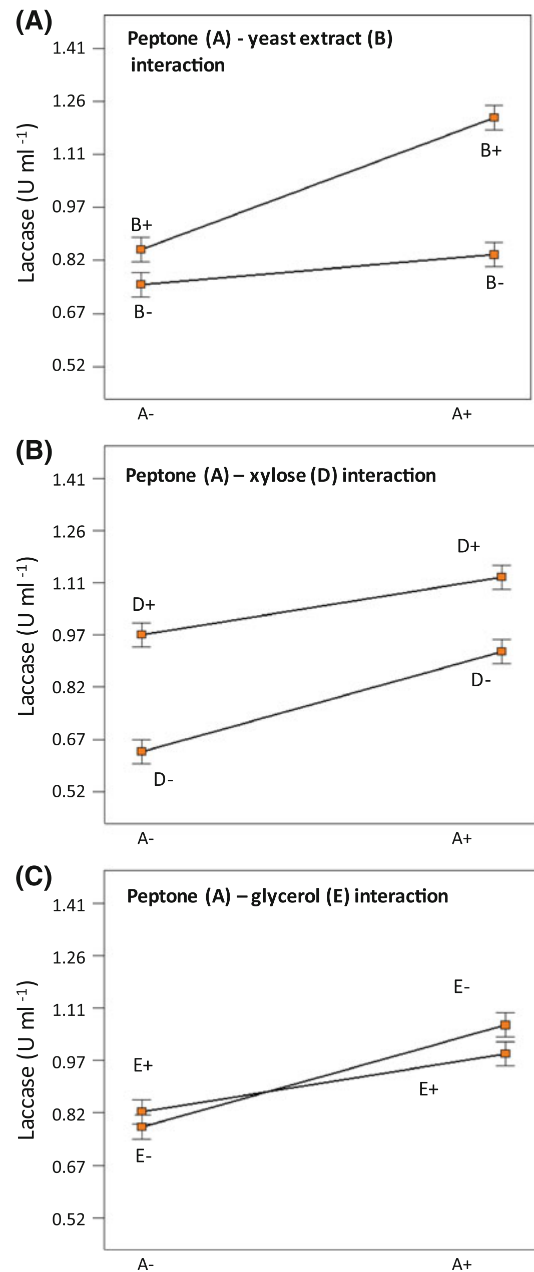

Fig. 3 Relevant factor interactions ($p < 0.05$) affecting laccase production by *P. ostreatus* CP-50. **a** Peptone–yeast extract interaction. **b** Peptone–xylose interaction. **c** Peptone–glycerol interaction

studied was significantly different to zero (*F* test, $p < 0.001$), while the lack of fit of the model was not (*F* test, $p = 0.14$). Analysis of the most significant effects demonstrated that Eq. (2) can be reduced to:

$$\begin{aligned}\text{Laccase}(\text{U ml}^{-1}) = 0.91 &+ 0.11\text{Pep} + 0.12\text{Yex}\\ &+ 0.04\text{Try} + 0.14\text{Xyl}\\ &+ 0.07\text{PepYex} - 0.03\text{PepXyl}\\ &- 0.03\text{PepGly} + \varepsilon\end{aligned}$$

Analysis of two-factor interaction is shown in Fig. 3a–c. Interaction between peptone and yeast extract is shown in Fig. 3a. When yeast extract is at the lower level (5 g l^{-1}) the peptone effect on laccase production is insignificant. However, high titers of laccase activity can be achieved if both peptone and yeast extract are at the upper level (10 g l^{-1}). Increasing peptone and xylose concentration has a significant positive effect on laccase production (Fig. 3b). Regarding the peptone–glycerol interaction (Fig. 3c), the best conditions for increasing laccase production titers were when peptone was at the upper level (10 g l^{-1}) regardless of the glycerol concentration.

From the analysis of the individual and interaction effects, it is clear that peptone and yeast extract have a highly significant positive effect and can be used at 10 g l^{-1}. Tryptone has a lower positive effect and can be used at either

concentration (5 or 10 g l^{-1}). Regarding the effect of carbon sources, xylose has a high positive effect on laccase production and should be used at 10 g l^{-1}, while glycerol and glucose did not significantly influence laccase production. Figure 4a, b shows the surface response for laccase volumetric activity as a function of peptone, xylose, and glucose concentration (most significant factors) in media containing yeast extract, 10 g l^{-1} and tryptone, 10 g l^{-1}. According to the model, medium 16 (peptone 10 g l^{-1}; yeast extract 10 g l^{-1}; tryptone 10 g l^{-1}; xylose 10 g l^{-1}; glycerol 10 g l^{-1}; glucose 10 g l^{-1}) is the best choice in order to maximize laccase concentration with an estimated value of 1.5 U ml^{-1}, which is not significantly different to the experimental value of 1.4 U ml^{-1}.

The relevant literature shows that the best carbon sources for laccase production by *Trametes pubescens* are cellobiose, glucose, and glycerol, while lactose and cellulose are poor carbon sources for laccase production [11]. Prasad et al. [25] showed that increasing glucose concentration (from 10 to 30 g l^{-1}) resulted in an enhanced laccase production (50% increase) by *P. ostreatus* 1804. Mikiashvili et al. [20, 21] reported that polysaccharides (Avicel and CMC) are poor substrates for laccase production by *Trametes versicolor*, while easily metabolizable sugars (such as glucose and cellobiose) supported high biomass and laccase activities. Our results showed that the use of xylose as the only carbon source yields an increased specific laccase production (U/mg biomass) but in detriment of fungal growth. On the other hand, the use of glucose instead has the opposite effect.

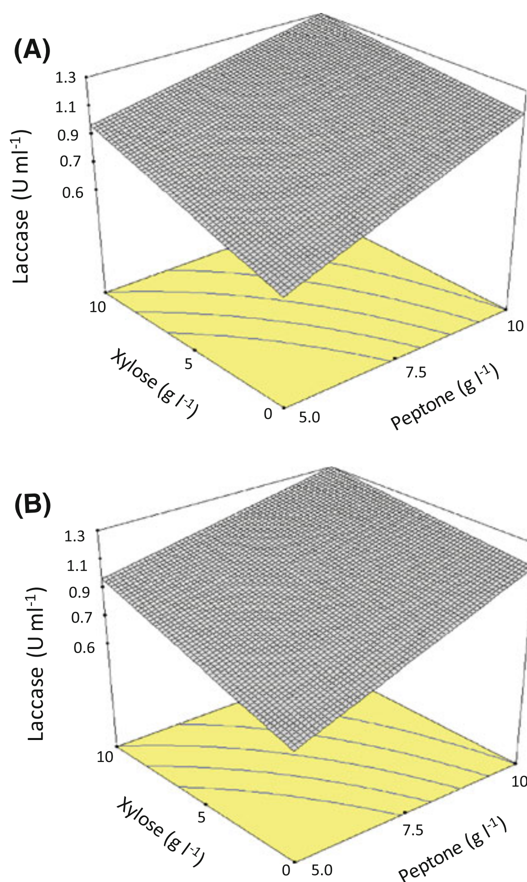

Fig. 4 Response surface representing maximal laccase volumetric activity versus xylose and peptone concentration. Media composition (g l^{-1}): **a** tryptone 5, yeast extract 10, glycerol 0, glucose 0; **b** tryptone 5, yeast extract 10, glycerol 0, glucose 10

Although the type and concentration of nitrogen sources strongly affects fungal growth and metabolite production, it is generally accepted that organic nitrogen sources enhance growth yields of macrofungi; however, the effect of organic nitrogen source on laccase production depends on the microorganism [8, 14]. Laccase activities of both *P. ostreatus* and *Lentinus edodes* were greatly stimulated in a peptone nitrogen-based medium [14]. Galhaup et al. [11] showed that the use of peptone, tryptone, and yeast extract increases laccase production by *Trametes pubescens* on a simple glucose-based medium with 2 mM of copper, although growth of the microorganism was not affected. In the case of *P. ostreatus* 1804, yeast extract has shown a positive effect on laccase production when its concentration does not exceed 15 g l^{-1} [25]. Peptone, followed by casein hydrolysate, was the best nitrogen source for biomass growth and laccases production by two strains of *P. ostreatus* [21]. Recently, it has been reported that casein is the best nitrogen source for laccase production by *Pleurotus sajor-caju* when compared to peptone, beer yeast, and soy extract [3].

In the above-mentioned literature, most of the studies only evaluate the carbon/nitrogen sources effect on laccase production with practically no discussion about the effects on fungal growth. Indeed, to our knowledge, no studies about the individual effects, and their interactions, among different carbon and nitrogen sources on growth and laccases production by any microorganism, have been previously reported.

As can be expected, the medium composition, which allowed to significantly increase the specific laccase production (U/mg biomass), is not the best medium for fungal growth. Our results showed that medium 16 allowed the maximal laccase volumetric activity of 1.4 U ml^{-1} and 7 g l^{-1} of biomass, which is 30% lower than the maximal biomass achieved with other media compositions (i.e., 9.8 g l^{-1} with medium 4). It is worth noting that none of the media suppressed laccase production. However, the best compromise to significantly ($p = 0.05$) increase biomass growth, without a significant ($p = 0.05$) difference in laccase production, was medium 4, in which 9.8 g l^{-1} and 1.2 U ml^{-1} were obtained, respectively. The study of fungal growth and laccase production by *P. ostreatus* in the

control medium (malt extract 2%) and medium 4 was then undertaken.

As the initial objective of this work was to enhance the growth of *P. ostreatus* without a repression of laccase production, carbon (glucose, xylose, and glycerol) and nitrogen sources (peptone, yeast extract, and tryptone) were selected on the basis of the literature survey (presented earlier in this section) of the effect on medium composition on laccase production by basidiomycetes. The basal medium contains only malt extract (2%), and maximal fungal growth (2.5 g l^{-1}) and laccase volumetric activity (0.5 U ml^{-1}) were very low. In such conditions it is likely that fungal growth was principally limited by nitrogen availability. To overcome this fungal growth limitation, the experimental design focused on increasing nitrogen content (all the nitrogen sources are present in all the tested media) in relation to carbon content. The effect of carbon and nitrogen source on fungal growth and laccase production was assessed through a fractional factorial experimental design 2^{6-2}.

The experimental design allowed to obtain a significant increase of fungal growth as well as laccase production. Under our conditions, all the media tested supported more than 5 g l^{-1} of biomass production after 96 h of incubation, representing an increase of 100% of the biomass concentration in comparison with the basal medium. Furthermore, medium 4 supported a maximal biomass concentration of 9.8 g l^{-1} together with 1.2 U ml^{-1} of laccase volumetric activity after 96 h of cultivation, yielding 370 and 250% for fungal growth and laccase production, respectively, compared to the basal medium. This is an important issue as maximal laccase production by basidiomycetes usually takes much longer cultivation periods on the order of hundreds of hours [27] and yielding low process productivities.

Kinetic characterization of growth and laccase production by *P. ostreatus* CP50

Growth of *P. ostreatus* in basal medium and medium 4 is shown in Fig. 5a. While the *P. ostreatus* growth phase in basal medium lasted only 60 h, reaching 2.5 g l^{-1} of biomass, medium 4 allowed a continuous biomass increase for 108 h when 9.4 g l^{-1} was obtained. However, no significant differences were found between culture media in terms of specific growth rate (0.1 h^{-1}). Laccase production in basal medium started after 12 h of cultivation and maximal volumetric activity (0.8 U ml^{-1}) was obtained at 60 h (Fig. 5b). On the other hand, the onset of laccase production in medium 4 was at 36 h reaching a maximal volumetric activity of 1.2 U ml^{-1} at 84 h. It is interesting to point out that in both media, laccase activity reached its maximal value before *P. ostreatus* growth ended.

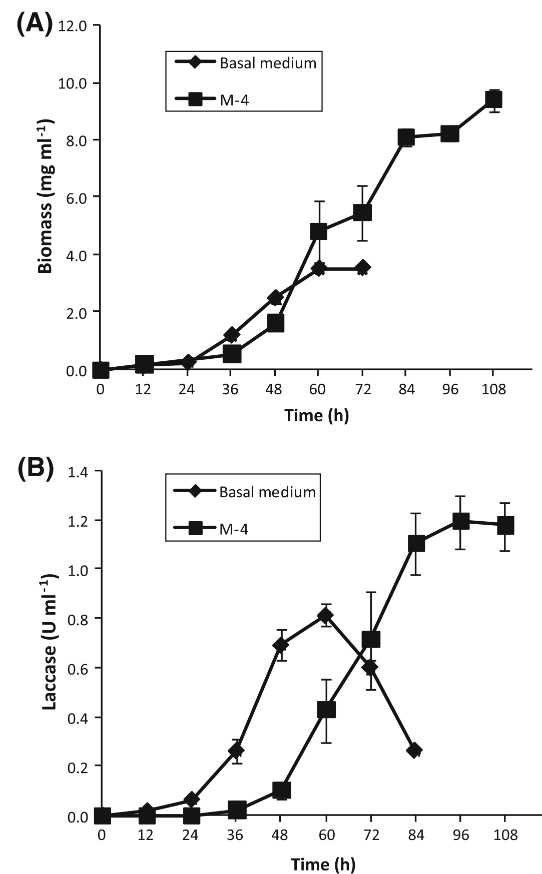

Fig. 5 Kinetics of growth and laccase volumetric activity in *P. ostreatus* CP-50 cultures as a function of the medium composition

Regarding laccase specific productivity, significant differences were found between the two media. Although in medium 4 the highest volumetric activity was found, the average laccase specific productivity in such conditions (13 ± 3 U g biomass^{-1} h^{-1}) was only a third of that obtained in the control medium (41 ± 13 U g biomass^{-1} h^{-1}). Based on growth evolution of *P. ostreatus* on medium 4, the effect of the addition of copper and/or lignin to the culture was evaluated after 60 h of cultivation (middle of growth phase).

Based on our results, medium 4 was selected in order to study the influence of the addition of copper and/or lignin on the laccase production by *P. ostreatus*. Laccase production by *P. ostreatus* CP-50 is growth-associated when cultured in either medium 4 and basal medium and no differences were found in their specific growth rates (0.1 h^{-1}). Comparing our results with previous reports, it has been shown that laccase production by *P. ostreatus* ATCC 32783 is growth-associated, with a specific growth rate of 0.02 h^{-1}, in glucose-yeast extract based medium supplemented with copper [27]. Differences in the specific growth rate could be due to the timing of the addition of copper. In the case of *P. ostreatus* ATCC 32783, 1.5 mM

copper was added at the beginning of the culture and it is well known that copper can be toxic for microorganisms [1]. Nevertheless, maximal growth (8 g l^{-1}) and laccase volumetric activity (1.2 U ml^{-1}) were almost identical to that obtained in this work; however, an incubation time of 500 h was required.

Our results showed that maximal laccase volumetric activity was reached before fungal growth stopped. However, while no significant decrease on laccase activity was found in cultures carried out in medium 4, this was not the case for the basal medium, where 50% of the activity was lost. Production of proteases by *P. ostreatus* has been reported at the later stages of fungal growth [24]. Differences in the stability of laccase activity in both cultures media could be explained by the high protein content of medium 4, which can compete for proteolysis with laccase.

Furthermore, when *P. ostreatus* was cultured in medium 4, only a third of the specific productivity of that obtained in the basal medium was achieved and no significant influence of the medium composition on the specific growth rate (0.1 h^{-1}) was found. However, the onset of laccase production on medium 4 showed a delay of 24 h, as compared with that observed in the basal medium. As both media contain malt extract, it is likely that protein supplementation (as sole nitrogen sources) of basal media induces higher proteolytic activities. Therefore, measured laccase activities should reflect the difference between synthesis and degradation rates. However, proteolytic activity was not assessed during cultures.

Effect of copper and lignin on laccase production by *P. ostreatus* CP50

The individual and interaction effects of copper and lignin on laccase production by *P. ostreatus*, cultivated in medium 4, were assessed and the results are shown in Fig. 6. Both compounds had a strong positive influence over laccase production by *P. ostreatus*. For instance, laccase volumetric activity at 96 h in induced cultures was almost threefold higher than in the control culture. After 96 h of cultivation, lignin-added cultures did not show a further increase of laccase activity while cultures induced with copper showed a significant increase of enzymatic activity, reaching 8 U ml^{-1} (sixfold higher than the maximal activity of the control culture) after 132 h of cultivation. More interesting are the results obtained when lignin and copper were added simultaneously: 12 U ml^{-1} were obtained after 108 h of cultivation, a volumetric activity that is higher than the sum of the activities obtained (at the same culture time) with individual inductions. Therefore, there exists a synergistic effect of lignin and copper on laccase production by *P. ostreatus*. As no significant differences were observed

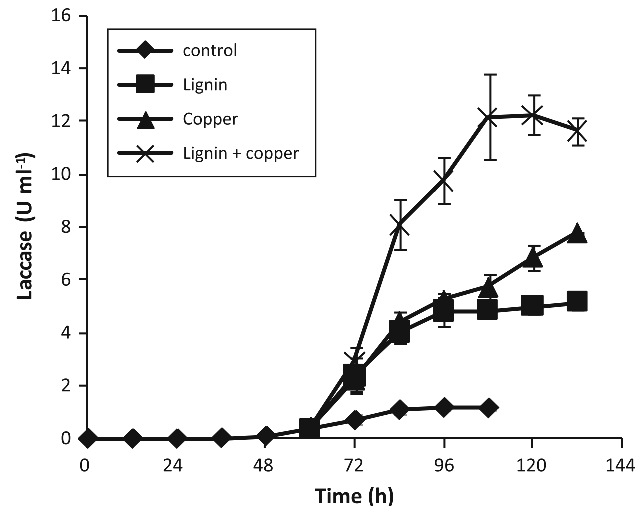

Fig. 6 Laccase production during the cultures of *P. ostreatus* CP-50 where copper and/or lignin were added as inducers at the middle of the growth phase (60 h)

in the biomass concentration for all the treatments (results not shown), it can be assumed that under the present conditions, specific productivity of *P. ostreatus* was effectively enhanced by the use of both inducers without apparent toxic effects on *P. ostreatus*.

Overall, laccase volumetric activity was significantly enhanced by the simultaneous use of copper and lignin in an optimized culture media for *P. ostreatus* CP-50 growth. For instance, in malt extract medium, maximal biomass growth and laccase activity were 2.5 g l^{-1} and 0.2 U ml^{-1} after 72 h of cultivation, respectively; while for the induced system, the values were 10 g l^{-1} and 12 U ml^{-1} after 108 h, respectively. An overall productivity of 1.1 U g $biomass^{-1}$ h^{-1} was obtained in malt extract cultures, while a tenfold increase (11.1 U g $biomass^{-1}$ h^{-1}) was obtained in the induced cultures.

Based on the fungal growth kinetics on medium 4, the influence of copper and/or lignin supplementation on laccase production by *P. ostreatus* CP-50 was evaluated. Copper induction of laccases enzymes has been previously shown in *Trametes versicolor* [5], *Pleurotus ostreatus* [23], *Trametes pubescens* [10], and *Botryosphaeria rhodina* [6] cultures. Such studies showed that regulation of the synthesis of different laccases isoenzymes by copper was at the level of gene transcription. The use of lignin and lignin sulphonate for increased laccase production by *Trametes versicolor* has been recently reported [29]. However, very little has been done on the simultaneous use of two inducers on laccase production. Indeed, to the authors' knowledge, only Minussi et al. [22] have reported that significant enhancement of laccase production by *Trametes versicolor* can be obtained with the simultaneous addition of Cu and 2,5-xylidine.

Our results showed that maximal laccase volumetric activity was reached before fungal growth stopped. Thus, under our conditions, laccase production was growth-associated. In light of this, media design to improve fungal growth and further addition of inducer to increase laccase production was adequate. Previous results showed that the addition of copper at the beginning of culture drastically reduced growth and laccase production by *P. ostreatus* CP-50 (results not shown). However, laccase production by *Pleurotus ostreatus* CP-50 was significantly enhanced when lignin (threefold) or Cu (sevenfold) were added at the middle of the exponential growth phase. Furthermore, laccase volumetric activity was influenced by the type of the inducer. For instance, laccase production in lignin-added cultures was strongly stimulated during the first 36 h after induction, and no significant differences in volumetric activity were found if compared with Cu-induced cultures. After that, no further increase on enzyme activity (4.5 U ml^{-1}) was observed. However, in Cu-induced cultures, laccase production was stimulated until 132 h of cultivation (62 h after Cu addition) when 7.0 U ml^{-1} were obtained.

When Cu and lignin were added simultaneously, laccase volumetric activity was strongly enhanced reaching its maximum (12.2 U ml^{-1}) only 48 h after induction (108 h of cultivation). It is worth noting that by analyzing the laccase increase relative to the sum of laccase volumetric activities of individually induced cultures (at 108 h of cultivation), the simultaneous addition of Cu and lignin exhibited a synergistic effect, resulting in a significant increase (30%) of laccase productivity by *P. ostreatus* CP-50. This was obtained without a formal optimization of the inducers concentration and/or the time of addition.

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References

1. Baldrian P (2003) Interactions of heavy metals with white-rot fungi. Enzyme Microb Tech 32:78–91
2. Baldrian P (2006) Fungal laccases—occurrence and properties. FEMS Microbiol Rev 30:215–242
3. Bettin F, Montanari Q, Calloni R, Gaio TA, Silveira MM, Dillon AJP (2009) Production of laccases in submerged process by *Pleurotus sajor-caju* PS-2001 in relation to carbon and organic nitrogen sources, antifoams and Tween 80. J Ind Microbiol Biot 36:1–9
4. Box G, Hunter W, Hunter S (1978) Statistics for experimenters. An introduction to design data analysis and model building. Wiley, New York, 653 pp
5. Collins PJ, Field JA, Teunissen P, Dobson ADW (1997) Stabilization of lignin peroxidases in white rot fungi by tryptophan. Appl Environ Microb 63:2543–2548
6. Dekker R, Barbosa A, Giese E, Godoy S, Covizzi L (2007) Influence of nutrients on enhancing laccase production by *Botryosphaeria rhodina* MAMB-05. Int Microbiol 10:177–185
7. Dong J, Zhang Y, Zhang R, Huang W, Zhang Y (2005) Influence of culture conditions on laccase production and isozyme patterns in the white-rot fungus *Trametes gallica*. J Basic Microbiol 45:190–198
8. Fazenda ML, Seviour R, McNeil B, Harvey LM (2008) Submerged culture fermentation of “Higher fungi”: the macrofungi. Adv Appl Microbiol 63:33–103
9. Flores C, Vidal C, Trejo-Hernández M, Galindo E, Serrano-Carreón L (2009) Selection of *Trichoderma* strains capable of increasing laccase production by *Pleurotus ostreatus* and *Agaricus bisporus* in dual cultures. J Appl Microbiol 106:249–257
10. Galhaup C, Haltrich D (2001) Enhanced formation of laccase activity by the white-rot fungus *Trametes pubescens* in the presence of copper. Appl Microbiol Biotechnol 56:225–232
11. Galhaup C, Wagner H, Hinterstoisser B, Haltrich D (2002) Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. Enzyme Microb Technol 30: 529–536
12. Hou H, Zhou J, Wang J, Du C, Yan B (2004) Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. Process Biochem 39:1415–1419
13. Junghans C, Moeder M, Krauss G, Martin C, Schlosser D (2005) Degradation of the xenoestrogen nonylphenol by aquatic fungi and their laccases. Microbiology 151:45–57
14. Kaal EE, Field JA, Joyce TW (1995) Increasing ligninolytic enzyme activities in several white-rot basidiomycetes by nitrogen-sufficient media. Bioresour Technol 53:133–139
15. Klonowska A, Le Petit J, Tron T (2001) Enhancement of minor laccases production in the basidiomycete *Marasmius quercophilus* C30. FEMS Microbiol Lett 200:25–30
16. Kunamneni A, Camarero S, García-Burgos C, Plou F, Ballesteros A, Alcalde M (2008) Engineering and applications of fungal laccases for organic synthesis. Microb Cell Fact 7:1–17
17. Lomascolo A, Record E, Herpoël-Gimbert I, Delattre M, Robert JL, Georis J, Dauvrin T, Sigoillot JC, Ashter M (2003) Overproduction of laccase by a monokaryotic strain of *Pycnoporus cinnabarinus* using ethanol as inducer. J Appl Microbiol 94:618–624
18. Marques de Souza CG, Tychanowicz GK, Farani de Souza D, Peralta RM (2004) Production of laccase isoforms by *Pleurotus pulmonarius* in response to presence of phenolic and aromatic compounds. J Basic Microbiol 44:129–136
19. Mayer AM, Staples RC (2002) Laccase: new functions for an old enzyme. Phytochemistry 60:551–565
20. Mikiashvili N, Elisashvili V, Wasser S, Nevo E (2005) Carbon and nitrogen sources influence the ligninolytic enzyme activity of *Trametes versicolor*. Biotechnol Lett 27:955–959
21. Mikiashvili N, Wasser S, Nevo E, Elisashvili V (2006) Effects of carbon and nitrogen sources on *Pleurotus ostreatus* ligninolytic enzyme activity. World J Microbiol Biotechnol 22:999–1002
22. Minussi R, Pastore G, Durán N (2007) Laccase induction in fungi and laccase/N-OH mediator systems applied in paper mill effluent. Bioresour Technol 98:158–164
23. Palmieri G, Giardina P, Bianco C, Fontanella B, Sannia G (2000) Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. Appl Environ Microbiol 66:920–924
24. Palmieri G, Bianco C, Cennamo G, Giardina P, Marino G, Monti M, Sannia G (2001) Purification, characterization, and functional role of a novel extracellular protease from *Pleurotus ostreatus*. Appl Environ Microbiol 67:2754–2759
25. Prasad K, Mohan V, Rao S, Pati R, Sarma P (2005) Laccase production by *Pleurotus ostreatus* 1804: optimization of

submerged culture conditions by Taguchi DOE methodology. Biochem Eng J 24:17–26

26. Stajic M, Persky L, Friesem D, Hadar Y, Wasser S, Nevo E, Vukojevic J (2006) Effect of different carbon and nitrogen sources on laccase and peroxidases production by selected *Pleurotus* species. Enzyme Microb Technol 38:65–73
27. Tlecuitl-Beristain S, Sánchez C, Loera O, Robson G, Díaz-Godínez G (2008) Laccases of *Pleurotus ostreatus* observed at different phases of its growth in submerged fermentation: production of a novel laccase isoform. Mycol Res 112: 1080–1084
28. Vasconcelos AFD, Barbosa AM, Dekker RFH, Scarminio IS, Rezende MI (2000) Optimization of laccase production by *Botriosphaeria* sp. in the presence of veratryl alcohol by the response-surface method. Process Biochem 35:1131–1138
29. Xavier A, Mora A, Ferreira R, Amado F (2007) *Trametes versicolor* growth and laccase induction with by-products of pulp and paper industry. Electron J Biotechnol 10:444–451