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Ethanol induction of laccase depends on nitrogen conditions of *Pycnoporus sanguineus*

Christian A. Hernández [a], Norma Sandoval [a], Julieta Mallerman [b], José Antonio García-Pérez [c], Anne-Marie Farnet [d], Isabelle Perraud-Gaime [e], Enrique Alarcón [a,*]

[a] *Instituto de Biotecnología y Ecología Aplicada (INBIOTECA), Universidad Veracruzana, Xalapa, Veracruz, Mexico*
[b] *Universidad de Buenos Aires (UBA), Buenos Aires, Argentina*
[c] *Facultad de Biología, Universidad Veracruzana, Xalapa, Veracruz, Mexico*
[d] *Equipe Systèmes Microbiens IMBE, UMR CNRS 7263, IRD 237, Faculté des Sciences Techniques de St. Jérôme, Aix Marseille Université, Marseille, France*
[e] *Equipe Ecotechnologies et Bioremediation, IMBE, UMR CNRS 7263, IRD 237, Faculté des Sciences et Techniques de St. Jérôme, Aix Marseille Université, Marseille, France*

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ABSTRACT

Background: Ethanol has been pointed out as a laccase inducer. However, there are controversial reports about its efficiency with some fungi. In this study, we hypothesized that ethanol laccase induced in *Pycnoporus sanguineus* depends on nitrogen nutriment conditions. To prove this, we assessed laccase production in submerged cultures of *P. sanguineus*, with different nitrogen concentrations and with, or without ethanol added in a factorial designed experiment.
Results: In order to analyze the effects of factors on the response variables, a factorial ANOVA, and response-surface models were performed. It was found that the nitrogen source was the main factor that affected laccase production in *P. sanguineus*. The treatments with yeast extract (2 g/L) and ethanol (3 g/L) induced the highest laccase activity (31.01 ± 4.9 U/L), while the treatments with urea reached the lowest activity (less than 1.6 U/L). Ethanol had positive and synergic effects on laccase production, in accordance with the surface response model, as long as simple nitrogen sources (urea) were not available.
Conclusions: We suggest that laccase in *P. sanguineus* is regulated by a catabolic nitrogen repression mechanism; laccase activity is strongly inhibited by urea used as nitrogen source and it decreases when the amount of urea increases; contrarily, a synergic positive effect was observed between yeast extract and ethanol on laccase production.

1. Introduction

It is well known that nitrogen is one of the main nutrients for microbial metabolism because it is an essential component of proteins and nucleic acids, as well as carbon. Its concentration in the medium and type of source (*e.g.* mineral or organic) can affect enzyme production in submerged fungal cultures [1,2,3]. In fungi, the nitrogen catabolite repression, homologous to the economic theory of microbial metabolism for carbon sets that, simple sources of nitrogen (*e.g.* ammonium) are preferred and consumed before complex sources (*e.g.* organic complex sources, such as yeast extract), with the objective of saving resources. For some enzymes linked to secondary metabolism, *e.g.* proteases, L-asparaginase and laccase, it is well known that this mechanism drives their extracellular production [4,5,6,7]; thus, for fungal laccases, for example, some studies pointed that this enzyme is regulated by the nitrogen catabolite repression system [8] and besides, their production, it responds differentially to diverse nitrogen sources in the culture medium. Thus, it is assumed that fungal laccases are activated when carbon and nitrogen are limited [9,10]. Thereby, it has been observed that laccase production in submerged cultures is performed in the presence of nitrogen as a simple mineral source (such as ammonium nitrate and ammonium sulfate [11]), or complex organic supplies (*e.g.* a mixture of amino acids, peptone, and yeast extract, [12,13,14]). In this sense, high laccase yields have been reported, when complex organic nitrogen was added to the medium, in contrast with a simple mineral nitrogen source, for *Agaricus bisporius* [15], *Pleurotus ostreatus* [3], *Trametes hirsuta* [16] and *Trametes pubescens* [17]. Besides, the effect of the nitrogen source could be species and strain specific and varies with culture conditions (*e.g.* carbon source, C/N ratio, micro-nutriments content, presence of inducers). Hence, not only the source, but also the concentration of nitrogen in the medium, influences laccase production [10,18].

Other factors also affect laccase production, like the type of inducer: metal ions (*e.g.* copper and manganese) [19,20,21]; phenolic

* Corresponding author.
E-mail address: enalarcon@uv.mx (E. Alarcón).
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Table 1
Levels of factors evaluated.

Nitrogen supply	N concentration (g/L)	Ethanol concentration (g/L)	Code
Yeast extract	1	0	Y_1E-
Yeast extract	2	0	Y_2E-
Yeast extract	3	0	Y_3E-
Yeast extract	1	3	Y_1E+
Yeast extract	2	3	Y_2E+
Yeast extract	3	3	Y_3E+
Urea	1	0	U_1E-
Urea	2	0	U_2E-
Urea	3	0	U_3E-
Urea	1	3	U_1E+
Urea	2	3	U_2E+
Urea	3	3	U_3E+

compounds; some organic molecules, like tannic acids [22], and ethanol [23,24,25] in white-rot fungi. It has been reported that these inducers increase the production rate of fungal laccases, both in solid state fermentation and submerged fermentation. Ethanol has been pointed out as cheap and less toxic inducer for laccase production [26]; nevertheless, little is known about its induction mode. Some have speculated that ethanol could cause oxidative stress via membrane disruption, and/or by the segregation of intracellular Ca_2^+, which acts as a secondary messenger and induces the laccase genes [27]. Some authors had recommended the use of ethanol to increase laccase production, meanwhile other studies have not reported positive effects of using this alcohol as an inducer; even negative effects were observed in some cases [23,24]. The effectiveness of ethanol as an inducer of laccase could be related to other relevant culture conditions, *e.g.* the concentration and source of nitrogen. However, little is known in this field about *P. sanguineus*. It has been reported that 2,5-xylidine and cooper sulfate had a synergic and positive effect on laccase production when combined with organic nitrogen sources [16,17], but there are no results with ethanol. With this in mind, we hypothesized that the different results reported so far about the inductive effect of ethanol on fungal laccase could be due to the differences in nitrogen availability. Thus, this study was conducted to evaluate the effectiveness of ethanol as a laccase inducer, in the tropical white-rot fungus *Pycnoporus sanguineus*, using different and contrasting nitrogen nutriment conditions (various sources and concentrations).

2. Materials and methods

2.1. Strain and inoculum

The strain of *P. sanguineus* used was isolated from a wild mushroom found in the sugar cane fields of Jalcomulco, Veracruz, Mexico (19°20′ 00″N, 96°46′00″W) and preserved in a potato-dextrose-agar medium (PDA) at 4°C. The collected fungus was identified by Ramírez-Guillén F., a classical taxonomy expert, and deposited at XAL Herbarium (INECOL A.C., Xalapa, Mexico) as F. Ramírez-Guillén 932. The fungus strain was also identified by sequencing the ITS 1 and ITS 4 regions, compared with sequences in GenBank and the DNA sequence obtained was deposited in GenBank under the accession number KR013138.

The fungus was reactivated in Petri dishes with PDA at 30°C for seven d prior to the experiment. As inoculum during the experimental phase, agar squares of 0.25 cm^2 in size with the reactivated mycelium of *P. sanguineus* were used.

2.2. Experimental design

To determine the response of laccase production in *P. sanguineus* under different culture conditions (Table 1) a factorial design was used, where the independent variables were: i) source of nitrogen with two levels, yeast extract and urea; ii) concentration of nitrogen in the source with three levels, 1, 2 and 3 g/L; and iii) ethanol as laccase inducer with two levels, 0 and 3 g/L.

The dependent variables measured were: i) laccase activity (U/L), ii) extracellular protein (mg/mL), and iii) simple sugars released from the substrate (g/L). Each treatment was replicated four times. Samples for the determination of laccase activity, protein production and simple sugars released were taken each 4 d for a 20 d period.

2.3. Culture conditions

The experimental units were glass containers with 150 mL of basal medium [11] with the following composition per liter: 1 g of KH_2PO_4; 0.26 g of NaH_2PO_4; 0.317 g of $(NH_4)_2SO_4$; 0.5 g of $MgSO_4 \times 7H_2O$; 0.5 mg of $CuSO_4$; 74 mg of $CaCl_2$; 6 mg of $ZnSO_4$; 5 mg of $FeSO_4$; 5 mg of $MnSO_4$; and 1 mg of $CoCl_2$ supplemented with sugar cane bagasse as a carbon source (2%). The experimental units were incubated in darkness at 30°C in an environmental chamber (Binder, GmbH), without agitation.

2.4. Statistical analysis

To identify if the independent variables have significant effects on laccase production, a factorial ANOVA was carried out ($\alpha = 0.05$). The combined effect of nitrogen concentration in the source and the ethanol concentration in the medium was analyzed by response surface methodology [28]. The analysis was carried out using Statistica 7 (Statsoft, Inc.) and GraphPad PRISM (GraphPad Software, Inc.) software.

2.5. Analytical methods

Lacasse activity was estimated using syringaldazine as a substrate, according to Leonowicz and Grzywnowicz [29]. 10 μL of a solution of syringaldazine at 5 mM in 0.1 M sodium acetate buffer, pH 4.5, were added to 990 μL of culture sample, according to Criquet [30]. The oxidation kinetics from syringaldazine to quinone was followed at

Table 2
ANOVA effect of all measured parameters on laccase activity, *i.e.* N source (yeast extract/urea), N concentration (1, 2, 3 g/L), ethanol concentration (0, 3 g/L) and their interactions. Significant effects are showed in bold.

Day	Effect	Df	MS	*F* value	*P*
4	N source	**1**	**2.04**	**7.28**	**0.01**
	N concentration	2	0.22	0.80	0.24
	Ethanol concentration	1	0.69	2.46	0.12
	Ethanol concentration * N concentration	2	0.02	0.08	0.91
	Ethanol * N source	1	0.29	1.04	0.31
	N concentration * N source	2	0.33	1.20	0.31
	Ethanol * N concentration * N source	2	0.10	0.35	0.70
8	N source	**1**	**601.98**	**59.08**	**<0.001**
	N concentration	**2**	**56.53**	**5.54**	**0.007**
	Ethanol concentration	1	14.63	1.43	0.23
	Ethanol concentration * N concentration	**2**	**52.95**	**5.19**	**0.01**
	Ethanol * N source	1	14.90	1.46	0.23
	N concentration * N source	**2**	**57.26**	**5.61**	**0.007**
	Ethanol * N concentration * N source	**2**	**53.18**	**5.22**	**0.01**
12	N source	**1**	**1584.39**	**282.12**	**<0.001**
	N concentration	**2**	**404.55**	**72.03**	**<0.001**
	Ethanol concentration	**1**	**240.51**	**42.82**	**<0.001**
	Ethanol concentration * N concentration	**2**	**369.76**	**65.84**	**<0.001**
	Ethanol * N source	**1**	**240.41**	**42.80**	**<0.001**
	N concentration * N source	**2**	**404.59**	**72.04**	**<0.001**
	Ethanol * N concentration * N source	**2**	**369.80**	**65.84**	**<0.001**
16	N source	**1**	**222.70**	**11.15**	**0.001**
	N concentration	2	44.51	2.22	0.12
	Ethanol concentration	1	34.25	1.71	0.19
	Ethanol concentration * N concentration	2	65.02	3.25	0.05
	Ethanol * N source	1	35.19	1.76	0.19
	N concentration * N source	2	45.49	2.27	0.11
	Ethanol * N concentration * N source	2	64.58	3.23	0.05

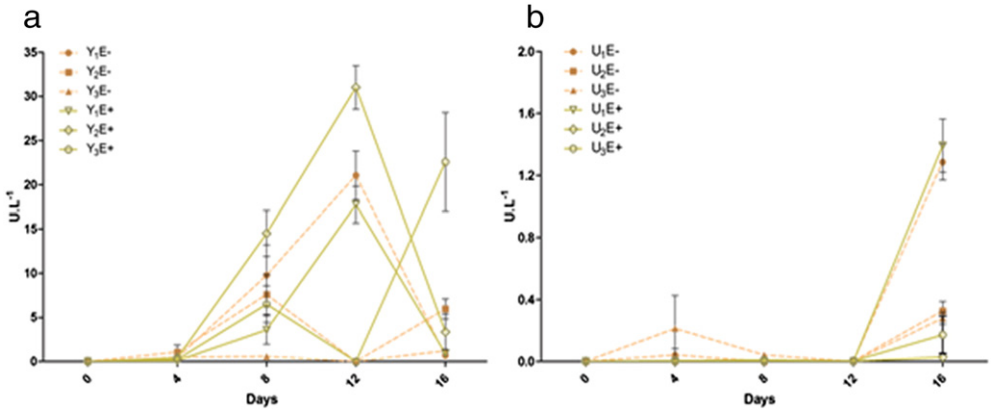

Fig. 1. Laccase activity observed during 16 d of culture with (a) yeast extract, and (b) urea, as nitrogen source; and with 3 g/L (+) or 0 g/L (−) of ethanol. Bars indicate the mean of the data, and whiskers the standard error, $n = 3$. The subscripts numbers indicate concentration in g/L. Note the differences on the ordinate scale (Y axis).

525 nm ($\varepsilon = 65{,}000\ M^{-1}\ cm^{-1}$) for 90 sec. The activity was expressed as μmol of quinone formed from syringaldazine per min (U) per one liter of culture (U/L).

The releasing of simple sugars was determined by the spectrophotometric method of 3,5-dinitrosalicylic acid (DNS), according to Miller [31]. 500 μL of the sample was mixed with 500 μL of DNS reagent and boiled for 5 min; later the reaction mixture was cooled for 10 min in icy water. Subsequently, the reaction mixture was diluted with 5 mL of dH_2O and quantified at 540 nm. The absorbance was transformed into glucose (g/L) through calibration according to a standard curve. The total of extracellular protein was determined according to the Bradford method [32]. 500 μL of the sample were mixed with 500 μL of Bradford reagent (Sigma, USA), the mixture was left standing for 5 min and quantified spectrophotometrically at 595 nm. The absorbance was transformed into protein (mg/mL) using a standard curve of bovine serum albumin (BSA).

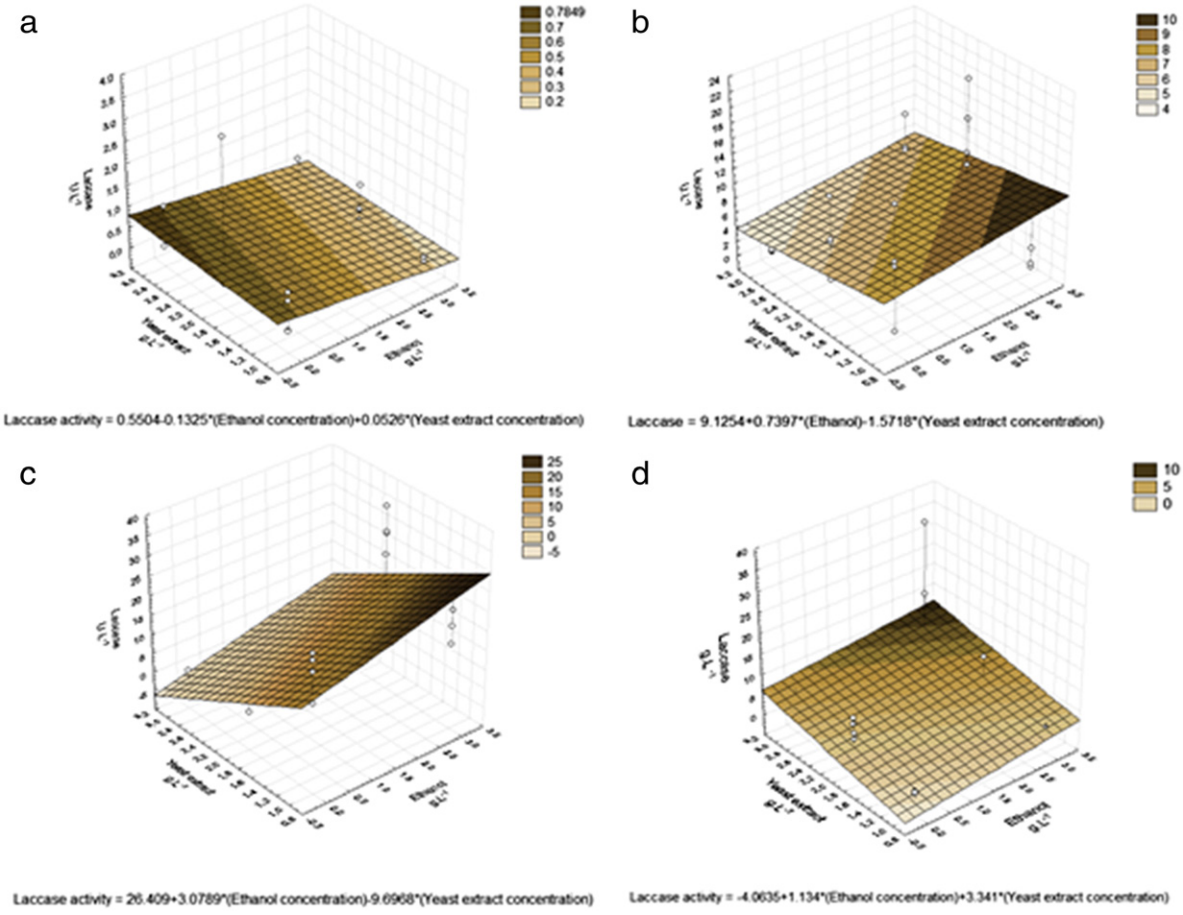

Fig. 2. Influence of yeast extract and ethanol concentrations on laccase produced by *P. sanguineus* after (a) 4 d; (b) 8 d; (c) 12 d, and (d) 16 d. Changes in color patterns indicate changes on the observed laccase activity. The response surface graph follows a linear fit of the data; the proposed model is showed.

3. Results

Laccase production was highly dependent on nitrogen supply conditions ($P < 0.05$, Table 2). In cultures with yeast extract as nitrogen supply (Fig. 1a), the highest activity was observed (31.01 ± 4.9 U/L) after 12 d in a culture treated with 2 g/L of yeast extract as a nitrogen source and 3 g/L of ethanol (Y_2E+). Meanwhile, in all treatments with urea as nitrogen supply (Fig. 1b) laccase activity was lower than 1.6 U/L.

Nitrogen concentration and ethanol had significant effects on laccase production only at certain moments. Nitrogen concentration had significant effects after 8 d ($F = 5.54$; $P = 0.007$) and 12 d ($F = 72.03$; $P < 0.001$) of culture (when laccase expressed high activity), and ethanol concentration had significant effects only at d 12 ($F = 42.82$; $P < 0.001$). Combined effects of ethanol and nitrogen supply/nitrogen concentration were found at d 8 and 12, and as well as between nitrogen supply and nitrogen concentration (Table 2). The surface response analysis showed combined effects of ethanol and nitrogen concentration on laccase production when yeast extract was supplied (Fig. 2); ethanol showed positive effects after 8 ($m = 0.73$; Fig. 2b), 12 ($m = 3.07$ Fig. 2c) and 16 d ($m = 1.13$). At d 4, non-synergic effects were observed between ethanol and nitrogen concentrations (nitrogen concentration did not show effect, Fig. 2a). However, at d 8 and 12 the highest laccase activities were observed in treatments with 3 g/L of ethanol and low nitrogen concentration (Fig. 2b, Fig. 2c).

In cultures with urea, non-effects were observed when ethanol was added (Fig. 3), and urea concentration only showed an effect after 16 d of culture (Fig. 3d). When urea was used, the highest laccase activities were observed at d 16 on treatments with 1 g/L (Fig. 3d).

Extracellular protein production was slightly higher in treatments with yeast extract than those with urea (Fig. 4a and Fig. 4b). However, no effects of ethanol addition or nitrogen concentration were observed. The sugar content in cultures with urea was slightly higher than those with yeast extract (Fig. 5), but no significant effect was observed.

4. Discussion

In accordance with previous reports for other fungi [3] such as *P. ostreatus*, our ANOVA results indicate that the source of nitrogen is the most significant source of variation in laccase activity, and that the yeast extract (an organic complex source) promotes more laccase production in a submerged culture than urea (a mineral simple source).

Some studies have reported better laccase production with mineral sources of nitrogen than with organic ones [21], for several species of *Pleurotus*. However, it is accepted that inorganic sources lead to lower laccase yields than organic sources, although both of them promote good fungal (biomass) development [33]. However, the source of nitrogen added to the medium and its consequent effect on laccase activity is species and strain specific, as determined by Mikiashvili et al. [3]. In this study, we observed that *P. sanguineus* preferred a

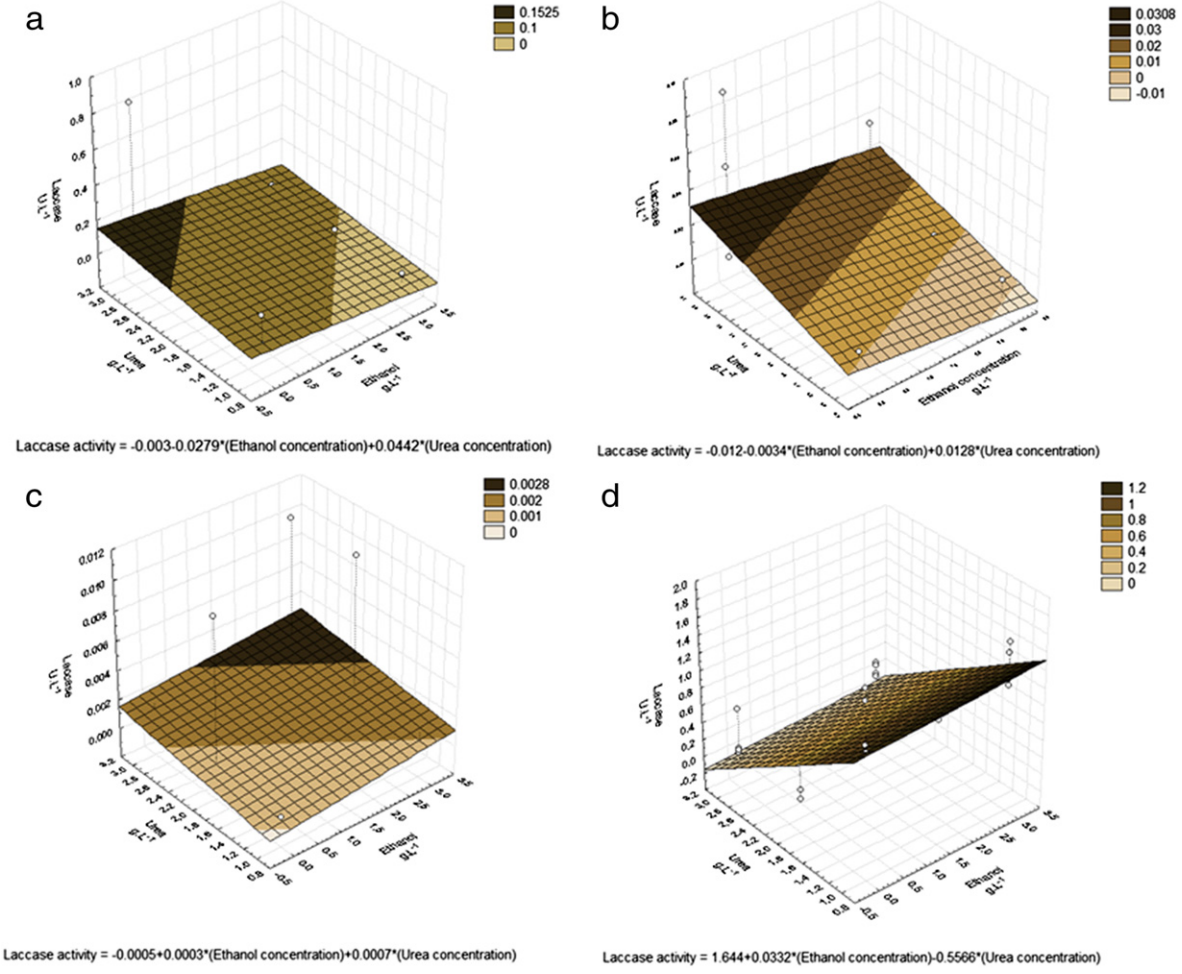

Fig. 3. Influence of urea and ethanol concentrations on laccase produced by *P. sanguineus* (a) after 4 d; (b) 8 d; (c) 12 d, and (d) 16 d. Changes in color patterns indicate changes on the observed laccase activity. The response surface graph follows a linear fit of the data; the proposed model is showed.

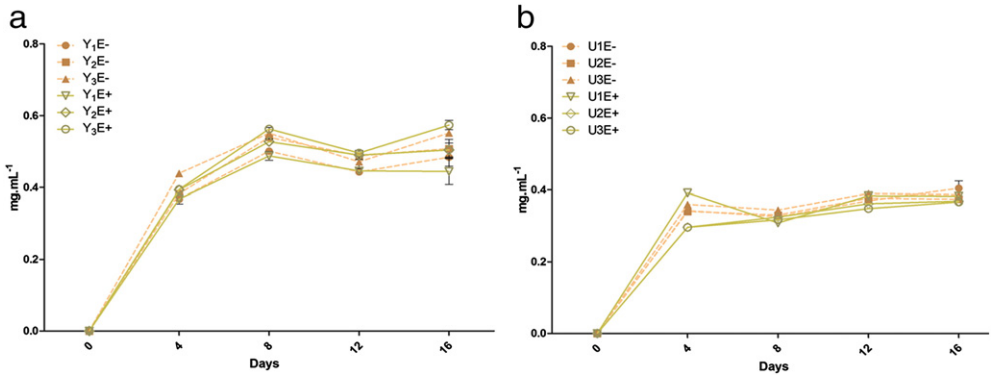

Fig. 4. Extracellular protein kinetics produced during 16 d of culture with (a) yeast extract, and (b) urea, as nitrogen source; and with 3 g/L (+) or 0 g/L (−) of ethanol. Bars indicate the mean of the data, and whiskers the standard error, $n = 3$. The subscripts numbers indicate concentration in g/L.

medium with an organic nitrogen source for the production of laccase, as reported previously. Eugenio et al. [23] stated that asparagine is the best nitrogen source (even better than yeast extract) for laccase production using *P. sanguineus*.

According to our results, the nitrogen source concentration used showed significant effects. When yeast extract (at d 8 and 12) and urea (at d 16) concentrations were significant, the highest laccase activities were registered. It suggests that nitrogen concentration has a strong effect on laccase production during the late stages of development of fungi, when secondary metabolism is active [34]. Negative correlations between concentrations of an inorganic nitrogen source (ammonium tartrate) and laccase production were reported for *P. sanguineus* by Pointing et al. [35]. That study also reported that the highest laccase production was reached when low carbon (glucose) and low nitrogen (ammonium tartrate) sources were added to the medium. Laccase repression occurred at high nutriment (carbon/inorganic nitrogen) conditions. Our surface response results tend to the same conclusion of Pointing et al. [35]: the amount of inorganic nitrogen source – urea, in our case – affects the production of laccase negatively in *P. sanguineus*.

In our study, the highest laccase activity was observed in the treatments with the lowest urea concentrations (1 g/L). The preference of an organic nitrogen source and the negative correlation between urea concentration and laccase activity may suggest that laccase production in *P. sanguineus* could be subjected to a nitrogen catabolic repression; a mechanism for saving resources that was reported for laccase production in other basidiomycetes, like *Cryptococcus neoformans* [8]. It has also been suggested that *Phanerochaete chrysosporium* (wild-type) has a regulatory system for laccase that inhibits its production under sufficient carbon and nitrogen conditions; this can be deregulated in mutant strains [36]. The catabolic nitrogen repression states that in the presence of simple nitrogen sources the expression of genes for more complex nitrogen sources (like genes of protease, γ-glutamyl transpeptidase, L-asparaginase, and permease) is repressed [37]. In this study we suggest that this regulatory mechanism operates and regulates laccase production in *P. sanguineus*; however, more research must be done to prove that. Nevertheless, the effect of the nitrogen source added to medium on laccase activity is evident.

In this study, we evaluated submerged cultures of *P. sanguineus*, but other studies have reported that in solid-state fermentation, an increment of urea concentration accompanied an increment of laccase production for *P. ostreatus*, *Lentinula edodes*, and *Agaricus blazei* [10]. This shows how the response of laccase to nitrogen supplies and culture conditions could change accordingly for fungi species.

According to our results, ethanol had a significant effect on laccase activity at d 12 and showed combined effects with nitrogen conditions at d 8 and 12. As we previously hypothesized, the effect of ethanol as an inducer of laccase is linked to other factors. We observed that ethanol induces laccase production when simple nitrogen sources (urea) are not available. In previous reports, Eugenio et al. [23] and Barreto et al. [24] have established that the effect of adding ethanol to the medium was null or negative on laccase production. Eugenio et al. [23] used ammonium tartrate (Kirk's medium) to produce laccase with *P. sanguineus*. Barreto et al. [24] used glutamine (*Trametes* defined medium), a simple organic source of nitrogen to produce laccase with *T. versicolor*. In both cases, the nitrogen source of the medium corresponded to simple sources, one inorganic and one organic. When more complex mediums were used, ethanol addition had a positive effect as a laccase inductor. Valeriano et al. [26] used malt extract medium for the production of laccase, Alves et al. [25] used a mixture of yeast extract and ammonium sulfate as nitrogen

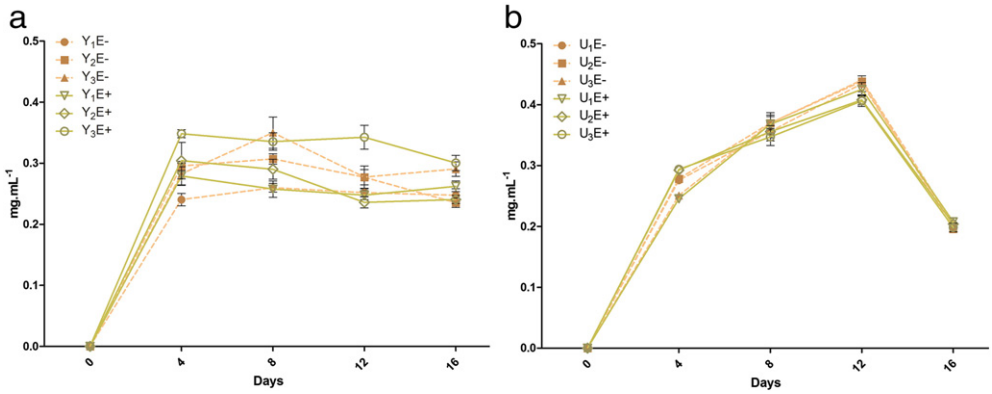

Fig. 5. Sugar content kinetics in the culture medium during 16 d of culture with (a) yeast extract, and (b) urea, as nitrogen source; and with 3 g/L (+) or 0 g/L (−) of ethanol. Bars indicate the mean of the data, and whiskers the standard error, $n = 3$. The subscript numbers indicate concentration in g/L.

sources, and Lomascolo et al. [27] used yeast extract; all those studies suggested that the ethanol is a good laccase-inducer. Following this line of knowledge, and supported by our results, we conclude that ethanol can act as a weak inducer of laccase only in the presence of complex nitrogen sources. Moreover, we hypothesize that this works only when nitrogen catabolite repression is not operating.

Our study contributes to a better understanding of the use of ethanol as an inducer of laccase in *P. sanguineus*, and our results showed that its functioning as an inducer is dependent on nitrogen nutriment conditions.

5. Conclusion

In accordance with our results, we conclude that: i) laccase production in *P. sanguineus* is strongly dependent on the type of nitrogen source added to medium in submerged cultures; urea inhibits its production (simple mineral source) and yeast extract (organic complex source) promotes it. We hypothesize that these results suggest the presence of a mechanism of nitrogen catabolite repression for *P. sanguineus*; however, genomic studies are required to assert it. ii) Ethanol only functions as a laccase inducer when simple nitrogen sources are not available in the culture medium; in the presence of complex nitrogen sources, such as yeast extract, ethanol showed positive synergic effects on laccase production.

Conflict of interest statement

The authors declare that there are no conflict of interest.

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