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Laccase induction by synthetic dyes in *Pycnoporus sanguineus* and their possible use for sugar cane bagasse delignification

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Abstract The use of synthetic dyes for laccase induction in vivo has been scarcely explored. We characterized the effect of adding different synthetic dyes to liquid cultures of *Pycnoporus sanguineus* on laccase production. We found that carminic acid (CA) can induce 722 % and alizarin yellow 317 % more laccase than control does, and they promoted better fungal biomass development in liquid cultures. Aniline blue and crystal violet did not show such positive effect. CA and alizarin yellow were degraded up to 95 % during *P. sanguineus* culturing (12 days). With this basis, CA was selected as the best inducer and used to evaluate the induction of laccase on solid-state fermentation (SSF), using sugarcane bagasse (SCB) as substrate, in an attempt to reach selective delignification. We found that laccase induction occurred in SSF, and a slight inhibition of cellulase production was observed when CA was added to the substrate; also, a transformation of SCB under SSF was followed by the ^{13}C cross polarization magic angle spinning (CPMAS) solid-state nuclear magnetic resonance (NMR). Results showed that *P. sanguineus* can selectively delignify SCB, decreasing aromatic C compounds by 32.67 % in 16 days; *O*-alkyl C region (polysaccharides) was degraded less than 2 %; delignification values were not correlated with laccase activities. Cellulose-crystallinity index was increased by 27.24 % in absence of CA and 15.94 % when 0.01 mM of CA was added to SCB; this dye also inhibits the production of fungal biomass in SSF (measured as alkyl C gain). We conclude that CA is a good inducer of laccase in liquid media, and that *P. sanguineus* is a fungus with high potential for biomass delignification.

Keywords Alizarin yellow · Carminic acid · Endoglucanase · ^{13}C CPMAS solid-state NMR · Cellulose crystallinity

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Introduction

Laccases (benzenediol/oxygen oxidoreductase, EC 1.10.3.2) are enzymes with the capacity to degrade lignin and other phenolic compounds, like mono-, di-, and polyphenols, aminophenols, methoxyphenols, aromatic amines, and ascorbic acid (Ding et al. 2014). Recently, laccases have been proposed as biocatalyst for many industrial and bioremediation processes, including biobleaching (Zheng et al. 2012; Monteiro and De Carvalho 1998), azo dyes degradation for wastewater treatment (Chhabra et al. 2015; Kanagaraj et al. 2015; Teerapatsakul et al. 2007, 2008; Junghanns et al. 2008; Vanhulle et al. 2007), herbicide degradation (Pizzul et al.

2009; Jolivalt et al. 1999), petroleum-derivate phenols degradation (Vargas and Ramírez 2002), and biomass delignification (Rico et al. 2015; Ryu et al. 2013).

For industrial applications, vast amounts of protein are extremely important (Songulashvili et al. 2015); thus, many studies have focused in enhancing the production of this enzyme with different organisms. Laccases are produced by bacteria (Dhiman and Shirkot 2015; Galai et al. 2009), filamentous ascomycetes (Cázares-García et al. 2013), and brown-rotting basidiomycetes (An et al. 2015); however, white-rot fungi are the desired producers because of the high yields obtained, and the high catalytic laccases produced (Viswanath et al. 2014; Ramírez-Cavazos et al. 2014). The production of laccase by culturing white-rot fungi can be done in liquid media (Songulashvili et al. 2006, 2015) and in solid-state fermentation (Karp et al. 2012); in both systems, the use of inducers can enhance the production of laccase (Hernández et al. 2015, Khammuang et al. 2013; Meza et al. 2005).

Inducer compounds must be abundant and not expensive to be used at large scale; industrial wastes meet these assumptions. Many metals (e.g., cupper, manganese, and cadmium) phenolic compounds (Mann et al. 2015) and synthetic dyes can induce laccase production in fungi (Kuhar and Papinutti 2014), and much of them are discarded in wastewaters. Phenolic dyes are of special interest due to textile industries that generate a large amount of wastewater (120 m^3/Ton fiber produced) enriched with synthetic dyes (1100–1300 Hazen units; Anjaneyulu et al. 2005), which generate environmental problems, e.g., when the azo dyes are degraded in anaerobic conditions, they can generate carcinogenic aromatic amines (Arslan et al. 2013).

Besides the degradation of many phenolic dyes that has been proven via laccase catalysis (Kanagaraj et al. 2015; Younes et al. 2011; Bayramoglu et al. 2012), there are few studies that evaluate in vivo induction of laccase by synthetic dyes (Table 1). The induction of high laccase production in vivo by synthetic dyes can be very useful for lignocellulose pretreatment, in order to enhance lignin decay and, indirectly, decrease the cellulose crystallinity index (CI). Achieving the reduction of lignin and CI, cellulose and hemicellulose are more easily hydrolyzed by enzymes (Yu et al. 2014; Yoshida et al. 2008), which are very important for bioethanol production.

This work aimed to test the induction of laccase with four synthetic/phenolic dyes: carminic acid, alizarin yellow, aniline blue, and violet crystal at different concentrations in liquid media and, subsequently, utilize the best inducer to enhance the production of laccase in solid-state fermentation (SSF). Carminic acid is an anthraquinone dye which is naturally present in the cochineal insect *Dactylopius coccus* (Eisner et al. 1980) and has been used since pre-Hispanic time as a textile and ceramic dye in Mexico. On the other hand, alizarin yellow is a common azo dye used in textile industry, such as dye cotton, nylon, and silk (Bowes et al. 1991), and as other azo dyes, which are potentially hazardous for the environment because of their toxicity (Bae et al. 2006), mutagenic (Freeman et al. 1996) and carcinogenic (Hildenbrand et al. 1999). The other two tested dyes were aniline blue, a common dye used for wool and cotton staining, and crystal violet, which has uses in paper and ink industries.

The substrate (sugar cane bagasse) modifications were evaluated by ^{13}C cross-polarization magic angle spinning (CPMAS) nuclear magnetic resonance (NMR), in order to determine if the addition of dyes to lignocellulose promotes a decay in lignin amount and CI values.

Materials and methods

Strain and inoculum

Pycnoporus sanguineus strain was isolated from the sugarcane fields of Jalcomulco, Veracruz, México (19° 20′ 00″ N, 96° 46′ 00″ W), cultured on a potato-dextrose-agar medium

Table 1 Phenolic dyes used for laccase induction or for decoloration test in vivo with fungi

Dye	Type	Fungi	Reference
Reactive black 5	Azo dye	*Leptosphaerulina* sp.	Copete et al. (2015)
Amaranth, reactive black 5, cibacron brilliant yellow	Azo dyes	*Trametes versicolor*	Champagne and Ramsay (2005)
Amido black	Azo dye	*Pleurotus pulmonarius*	Zilly et al. (2002)
Congo red	Azo dye	*Pleurotus pulmonarius*	Zilly et al. (2002)
Bromophenol blue	Triphenylmethane dye	*Pycnoporus sanguineus*	Pointing and Vrijmoed (2000)
Malachite green	Triphenylmethane dye	*Pycnoporus sanguineus*	Pointing and Vrijmoed (2000)
Violet crystal	Triphenylmethane dye	*Trametes maxima*	Hernández-Luna et al. (2008)
Amaranth	Azo dye	*Trametes versicolor*	Swamy and Ramsay (1999)
Textile wastewater	Mixture of colorants	*Pycnoporus* sp. SYBC-L3	Lui et al. (2012)
Remazol brilliant blue reactive	Anthraquinonic dye	*Trametes villosa*	Hernández-Luna et al. (2008)

(PDA), stored at 4 °C, and reactivated in Petri dishes with PDA medium at 30 °C for 7 days prior to the experiment. 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. In addition, this fungal strain was identified by sequencing ITS 1 region, compared with sequences in GenBank, and the DNA sequence obtained was deposited in GenBank under the accession number KR013138.

Experimental design and culture conditions for liquid cultures

In order to determine whether the different aromatic dyes induced laccase production in *P. sanguineus*, a bifactorial experiment was designed. Different synthetic dyes (Fig. S1 in the Supplementary Material) were tested to induce laccase production in liquid cultures of *P. sanguineus*: carminic acid, alizarin yellow, aniline blue, and violet crystal. The dyes were added, at three different concentrations (0, 0.01, 0.02, and 0.03 mM), to 150 ml of culture medium (basal medium; Eggert et al. 1996) 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 $MgSO_4$ $7H_2O$, 0.5 mg of $CuSO_4$, 74 mg of $CaCl_2$, 6 mg of $ZnSO_4$, 5 mg of $FeSO_4$, 5 mg of $MnSO_4$, 1 mg of $CoCl_2$, supplemented with carboxy-methyl cellulose as carbon source (2 %) and yeast extract (1 g L^{-1}) as nutrient source; pH was adjusted to 6.

The cultures were performed in quadruplicate, in plastic containers of 500 ml. Each experimental replicate was inoculated with two plugs of agar-containing mycelium (6 days old) and were incubated in darkness at 30 °C in an environmental chamber (Binder, GmbH; Tuttlingen, Germany). Samplings were performed each 3 days for 12 days of incubation to test laccase activity (U L^{-1}); meanwhile, biomass production (mg of dry mass) and dye degradation (%) were determined at the end of the incubation time.

Analytical methods for liquid cultures

Laccase activity in liquid cultures was estimated according to Criquet et al. (1999). Twenty microliters of a solution of 5 mM syringaldazine was added to 980 μL of the culture sample. The oxidation kinetics from syringaldazine to quinone was followed at 525 nm ($\varepsilon = 65{,}000\ M^{-1}\ cm^{-1}$) at 37 °C for 90 s. The activity was expressed as μmoles of quinone formed from syringaldazine per minute (U) per one liter of culture (U L^{-1}).

Fungal biomass from liquid cultures was calculated at the end of the experiment: cultures were vacuum-filtered and recovered biomass was dried at 60 °C by 48 h. Biomass weight was estimated by the gravimetric method and was reported as milligrams of dry mass (mg.DM^{-1}). Aromatic dye degradation was estimated according to linear models obtained from known concentrations (0 to 0.03 mM) and the absorbance measured at the wavelength of the main peaks (nm) in the visible light spectrum (aniline blue: 595, 610 nm; violet crystal: 588, 592 nm; carminic acid 494 nm; alizarine yellow: 358, 363 nm). Absorbance was quantified with a UV spectrophotometer and transformed into mM according to linear models (Table 2).

Experimental design and culture conditions for solid-state fermentation

The conditions that induced the highest laccase activity were used for the second experimental phase. Because the lowest CA concentration in liquid cultures reach the highest laccase activity, different concentrations of carminic acid were tested in solid-state fermentation (SSF): 0.00, 0.003, 0.006, and 0.01 mM; a volume of basal medium enriched with yeast extract and carminic acid was added to 50 g of pre-dried sugar cane bagasse (SCB) until a final humidity of 80 % *v/w*. The wet SCB was deposited into poly-paper plastic bags and sterilized by autoclaving (120 °C:1.5 atm) for 20 min. After cooling, three-agar squares 0.25 cm^2 in size with reactivated mycelium (7 days of culture) were used as inoculum. The mesocosms were incubated in darkness at 30 °C in an environmental chamber (Binder, GmbH; Tuttlingen, Germany). Each treatment was replicated four times, and samplings to determine laccase and endoglucanase activities were done each for 4 days during 16 days from expendable replicates; consequently, a total of 80 mesocosms (4 replicates × 4 carminic acid concentrations × 5 sampling dates) were set up. SCB samples were analyzed by ^{13}C CPMAS NMR methodology, in order to determine the substrate modification by *P. sanguineus*.

Enzyme activity was normalized with the protein and biomass values. Protein was determined according to Bradford (1976).

Analytical methods for SSF phase

Enzymatic activities were calculated from concentrated protein extracts, obtained according to Criquet et al. (1999): 5 g of SSB (fresh weight) were added to 100 ml of 0.2 M $CaCl_2$

Table 2 Models utilized for phenolic dyes degradation

Model equation	R^2
[Aniline blue, peak 595 nm] = (γ 595 nm + 0.46)/1.93	0.82
[Aniline blue, peak 610 nm] = (γ 610 nm + 0.643)/2.22	0.90
[Violet crystal, peak 588 nm] = (γ 588 nm − 2.813)/1.75	0.85
[Violet crystal, peak 592 nm] = (γ 592 nm − 2.19)/1.98	0.90
[Alizarin yellow, peak 358 nm] = (γ 358 nm − 0.913)/1.24	0.93
[Alizarin yellow, peak 363 nm] = (γ 358 nm − 0.94)/1.105	0.92
[Carminic acid, peak 494 nm] = (γ 494 nm − 0.176)/0.81	0.99

buffer added with Tween 80 0.05 % ,and 6 g of polyvinylpyrrolidone (PVP) stirred per 1 h at ~220 rpm and then filtered with 0.2 μm cellulose filters. Fifty milliliters of the crude extract was dialyzed within cellulose tubular membranes (Sigma-Aldrich; St. Louis, USA) and polyethylene glycol (PEG-8000) overnight at 4 °C; concentrated proteins were recovery in one volume of 0.2 M sodium phosphate buffer with pH 6 and were used for enzymatic determinations.

The reaction mixture of laccase determination was as follows: 100 μl of protein extract, 880 μl of 0.2 M sodium phosphate buffer pH 6, and 20 μl of syringaldazine 5 mM. Endoglucanase activity was estimated according to Ghose (1987), measuring simple sugars released from carboxymethyl cellulose (CMC, 2 %), by the method of dinitrosalicilic acid (DNS). The reaction mixture for endoglucanase estimation was as follows: 250 μl of protein extract was mixed with 250 μl of CMC 2 %; the reaction was incubated in a water bath at 50 °C for 1 h. The reaction was stopped with 500 μl of DNS reagent, boiled for 5 min, and immediately cooled in icy water for 10 min. The reaction mixture was diluted with 5 ml of dH_2O prior spectrophotometer quantification (540 nm). Absorbance was transformed into grams per liter of glucose by comparison with a standard glucose curve ($g\ L^{-1}$). In the case of SSF, both enzymatic activities were reported as μmoles of quinone formed from syringaldazine per minute (U) per g of dry mass ($U\ g\ DM^{-1}$) of SCB.

Substrate modifications

Changes in the chemical composition of SCB were followed via ^{13}C CPMAS NMR in a Bruker Advance DSX 400 MHz spectrometer (Bruker, Madison, WI, USA). One hundred milligrams of dried and ground SCB was placed in a zirconium rotor and spun at the magic angle (54.44°) at 10 KHz. The ^{13}C CPMAS NMR technique was performed with a ramped ^{1}H pulse during a contact time of 3 ms and with ^{1}H decoupling during the acquisition time to improve the resolution. Recording 4 K transients with a recycling delay of 2 s represented standard conditions to obtain a good signal-to-noise ratio.

Deconvolution of NMR spectra was performed using the DmFit software (Massiot et al. 2002). Specifically, regions of NMR spectra were utilized as indicators of chemical shifts in SCB: alkyl C (0–45 ppm) and carboxyl C (160–190 ppm) were related to fungal biomass production since they can be assigned to membrane phospholipids and since only very weak signals were observed in the sugar cane bagasse. Polysaccharides content were estimated measuring the *O*-alkyl C signal (45–110 ppm) and the content in aromatic C compounds measuring the signal between 110 and 160 ppm. Cellulose crystallinity index was calculated according to Park et al. (2010), by separating the C_4 region of the spectrum into crystalline and amorphous peaks, and calculated by dividing the total area of the crystalline peak (87 to 93 ppm) by the total area assigned to the C_4 peak (80–93 ppm). In addition, changes in aromatic C region were evaluated according to Hallac et al. (2009), measuring the decrease of *p*-hydroxyphenyl (162–157 ppm), aromatic quaternary C (160–123 ppm), and aromatic tertiary C (123–103 ppm) signals.

Statistical analysis

Data of laccase activity and biomass production was analyzed by two-way ANOVA ($\alpha = 0.05$), in order to identify if the different dyes added to the medium or their concentrations had significant effects on these variables; when ANOVA was significant, a post hoc Fisher LSD analysis was made. In addition, Pearson product-moment correlation coefficient (r) between laccase activity, biomass production, and percentage of dyes degradation was done. All the analyses were done in the software STATISTICA 7 (StatSoft Inc. 2004).

Results

The effects of aromatic dyes on biomass and laccase production in liquid cultures

Two-way ANOVA showed that the type of dye added to liquid media affects the amount of biomass produced ($F_2 = 6.10$; $P = 0.006$); meanwhile, dye concentration had no significant effects ($F_2 = 1.29$; $P = 0.29$), but the interaction between dye type and dye concentration was significant ($F_4 = 4.31$; $P = 0.007$). Treatments with aniline blue (whatever the concentration), alizarin yellow (0.01 mM), and carminic acid (0.03 mM) were those that produced more fungal biomass (Fig. 1). More precisely, the production of biomass increased with carminic acid concentrations while an inhibiting effect was found for alzarine yellow concentrations of 0.02 and 0.03 mM. Moreover, fungal biomass production was positively correlated with laccase production only in cultures with carminic acid ($r = 0.66$; $P = 0.001$).

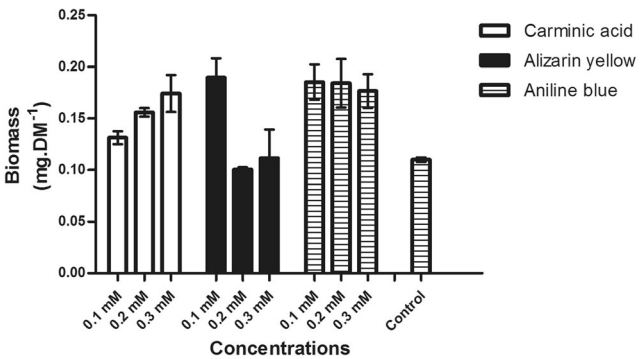


Fig. 1 Biomass production in liquid media enriched with phenolic dyes at different concentrations. *Bars* indicate the mean and *whiskers* the standard error

According to our ANOVA results, the addition of different aromatic dyes to culture medium of *P. sanguineus* affects laccase production (F = 79.07; $P < 0.001$); meanwhile, the concentration was not significant ($F = 2.28$; $P = 0.11$) as well as the interaction between the type of dye and dye concentration ($F = 1.31$; $P = 0.27$). Cultures with carminic acid showed the highest (Fig. 2) total laccase activities (i.e., 0.01 mM = 273.65 ± 90.72 U L^{-1}), followed by alizarin yellow (0.01 mM = 120.85 ± 81.51 U L^{-1}) and control (37.8 ± 12.25 U L^{-1}). Cultures with aniline blue showed lower values (0.01 mM = 34.46 ± 10.16 U L^{-1}). No laccase activity nor fungal development was observed in cultures with violet crystal (Fig. 2).

Because the effect of the dyes on laccase production was compromised by the effect of the dyes on the biomass development (Fig. 1), total laccase activity was normalized with the amount of total protein (specific activity), and with the amount of total biomass. The higher the concentration of CA, the fewer the activity of laccase (U g protein^{-1} g biomass^{-}1; Table 3), with the exception of the control. With this basis, we found that carminic acid at the concentration of 0.01 mM was the best inducer of laccase (Table 3), and we determine to use it in the following tests (SSF).

Dye degradation

Carminic acid and alizarin yellow were degraded in *P. sanguineus* liquid cultures (Table 4). Carminic acid was degraded above of 92 % in all concentrations tested, and alizarin yellow by 97 % in cultures with an initial concentration of 0.01 mM, 48 % in cultures with 0.02 mM, and 60 % in cultures with 0.03 mM. This is consistent with the fact that laccase activity was weaker for alizarin yellow concentrations of 0.02 and 0.03 mM. Dye degradation was positively correlated with laccase activity (carminic acid $r = 0.91$ $P < 0.0001$; alizarin yellow $r = 0.64$, $P = 0.02$).

Enzyme production in SSF

Enzyme activities in SSF showed maximum values after 12 days for endoglucanase (Fig. 3a) and after 8 days for laccase (Fig. 3b). ANOVA results indicate that the amount of carminic acid added to SCB affects the endoglucanase activity (F_3 = 10.96; P = 0.004). According to Fisher LSD post hoc test, cultures without carminic acid had the highest endoglucanase activity (1116.45 ± 127.12 U g DM^{-1}; Fig. 3a); meanwhile, the higher the carminic acid, the lower the endoglucanase activity was observed (Fig. 3a). On the other hand, laccase activity was detected at 4 and 8 days of culture, and laccase increased when carminic acid was added. Treatments with carminic acid showed higher laccase activity, with an average of 1.09 ± 0.40 U g DM^{-1}, whatever the concentration of CA than control (0.75 ± 0.31 U g DM^{-1}). Normalized laccase activity (U biomass^{-1}; Table 5) corroborate that CA induces laccase production in SSF, in a range of 21.05 to 42 % more than control, after 8 days of culture.

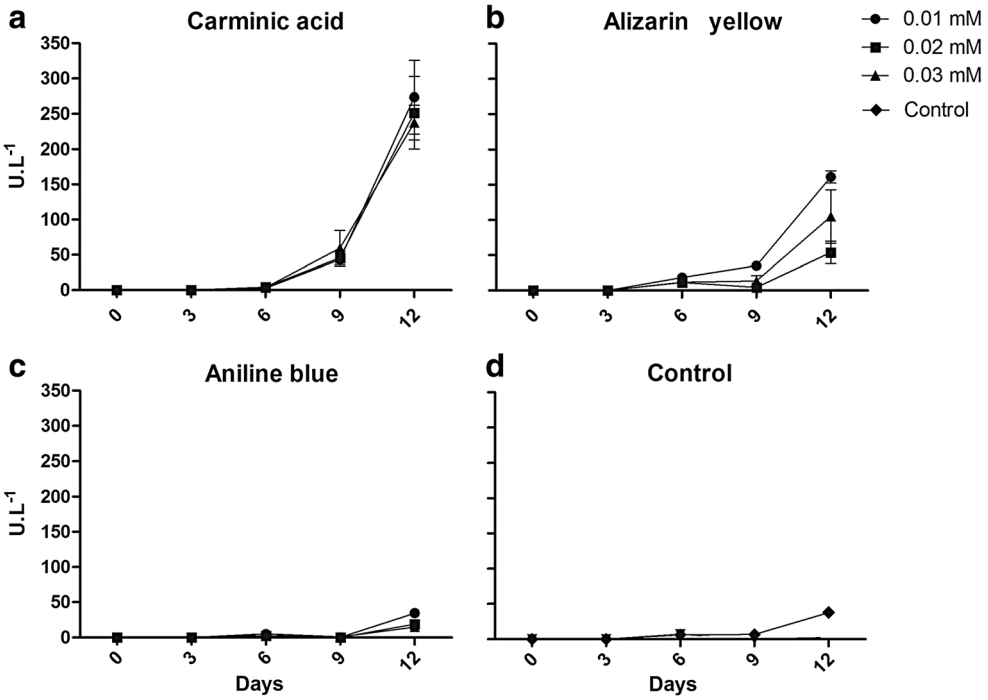


Fig. 2 Laccase activity (U L^{-1}) kinetics of liquid cultures with different dyes addition, in concentration from 0 to 0.01 mM. *Middle points* indicate the mean and *whiskers* the standard error. **a** A red glucosidal hydroxyanthrapurin dye. **b** an azo-dye. **c** a trisulfonate dye. **d** treatment without dye

Table 3 Laccase total activity (U L^{-1}), specific activity (U g $protein^{-1}$), and normalized activity (U g $protein^{-1}$ g $biomass^{-1}$). Obtained with the addition of carminic acid and alizarin yellow in liquid cultures. Values are mean and SD; $n = 4$

	U L^{-1}	Protein, g L^{-1}	U g^{-1}	Biomass, g DM^{-1}	U g $protein^{-1}$ g $biomass^{-1}$
Control	37.8	0.33 ± 0.02	114.54 ± 44.9	0.11 ± 0.004	1041.32 ± 368
Carminic acid 0.01	273.65 ± 90.7	0.35 ± 0.006	781.85 ± 197.0	0.13 ± 0.01	6014.29 ± 2242
Carminic acid 0.02	251.43 ± 102.3	0.34 ± 0.020	739.50 ± 297.8	0.15 ± 0.008	4930.00 ± 1930
Carminic acid 0.03	237.69 ± 49.0	0.33 ± 0.03	720.27 ± 241.3	0.17 ± 0.03	4236.90 ± 2340
Alizarin yellow 0.01	161.14 ± 15.1	0.36 ± 0.02	447.61 ± 70.19	0.18 ± 0.03	2486.73 ± 452
Alizarin yellow 0.02	53.9 ± 31.7	0.46 ± 0.01	117.17 ± 71.8	0.1 ± 0.004	1171.74 ± 696
Alizarin yellow 0.03	104.85 ± 75.8	0.46 ± 0.01	227.93 ± 158.5	0.11 ± 0.05	2072.13 ± 3521

NMR chemical shifts in SSF

^{13}C CPMAS NMR showed variations in the chemical composition of sugarcane bagasse under biological pretreatment with *P. sanguineus* and at different concentrations of carminic acid over time (after 16 days under SSF; Fig. 4). Cellulose crystallinity index (CI) increased in all the treatments; SCB had an initial CI ratio of 0.34 and increased 27.34 % in the control. When carminic acid was added, the increment in CI was lower (Table 6).

Changes in the chemical composition of SCB were mainly observed for the aromatic C region, which indicates changes in SCB lignin fraction. However, the reduction of these values was higher in control and when 0.003 mM of carminic acid was added, reaching a reduction in aromatic C signals of 32.67 and 30.65 %, respectively. Treatments with 0.006 mM of carminic acid showed a decrease of 16.33 %; meanwhile, when 0.01 mM were added, the aromatic region only decreased by 1.95 %. However, aromatic quaternary C diminish its signal by 17.60 %. ^{13}C CPMAS NMR analysis also showed that SCB's lignin had very little *p*-hydroxyphenyl (162–157 ppm) radicals, and that the peaks obtained for aromatic C region corresponded mainly to aromatic quaternary C (160–123 ppm) and aromatic tertiary C (123–103 ppm; Table 6).

Carboxylic C and alkyl C regions of the spectra were related to fungal biomass development (membrane phospholipid production); the increase in those regions was lower when

Table 4 Degradation of the dyes used as inducers in liquid media (first experimental phase)

Dye	Wavelength measured, nm	Initial concentration, mM	Final concentration, mM	Degradation percentage
Carminic acid	494	0.01	0.0004	95.97 ± 3.78
Carminic acid	494	0.02	0.001	92.57 ± 1.27
Carminic acid	494	0.03	0.002	92.48 ± 0.79
Alizarin yellow	358	0.01	0.0004	95.88 ± 14.15
Alizarin yellow	363	0.01	0	100 ± 0.5
Alizarin yellow	358	0.02	0.010	49.15 ± 0.57
Alizarin yellow	363	0.02	0.010	46.85 ± 0.82
Alizarin yellow	358	0.03	0.011	61 ± 2.59
Alizarin yellow	363	0.03	0.012	58 ± 4.18
Aniline blue	595	0.01	0.01	0
Aniline blue	610	0.01	0.01	0
Aniline blue	595	0.02	0.03	0
Aniline blue	610	0.02	0.02	0
Aniline blue	595	0.03	0.031	0
Aniline blue	610	0.03	0.029	0
Violet crystal	588	0.01	0.01	0
Violet crystal	592	0.01	0.01	0
Violet crystal	588	0.02	0.015	0
Violet crystal	592	0.02	0.016	0
Violet crystal	588	0.03	0.03	0
Violet crystal	592	0.03	0.03	0

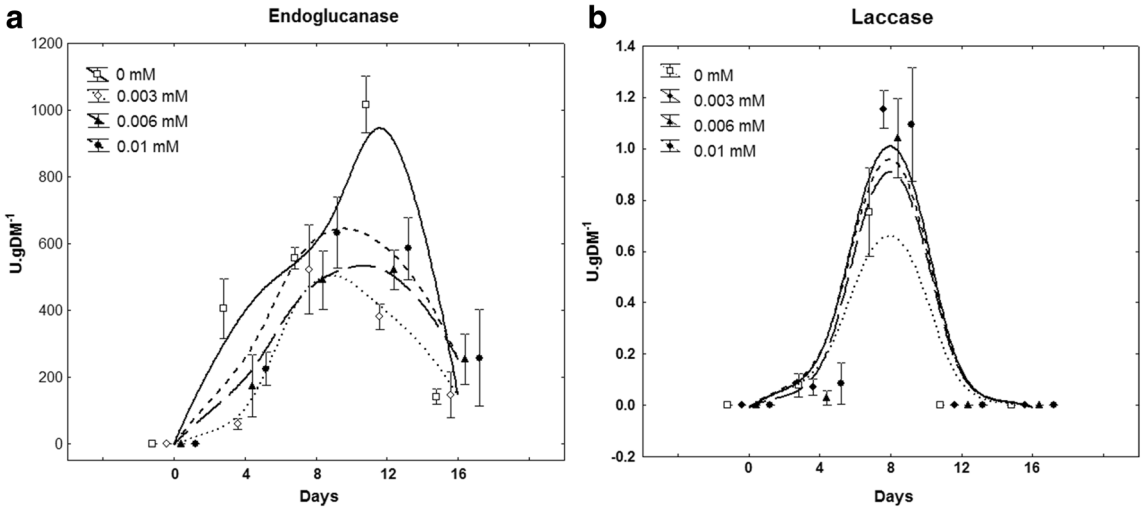


Fig. 3 Enzymatic activities observed in SSF phase. **a** Endoglucanase activity, highest activity was registered in treatments without carminic acid at day 12. **b** Laccase activity was only registered after 4 and 8 days. *Middle points* indicate the mean and *whiskers* the standard error

carminic acid was added to SCB compared to the control (Table 6), which indicated negative effects of carminic acid in biomass production, in SSF. On the other hand, the polysaccharide content (*O*-alkyl C) was almost unchanged in all treatments (decreased less than 2 %) and no differential effects were observed in treatments with and without carminic acid.

Discussion

Liquid cultures

We observed that carminic acid and alizarin yellow can enhance total laccase by 722 and 317 % more than the control, respectively, in liquid cultures with *P. sanguineus*. In an early report, Korniłłowicz-Kowalska and Rybczyńska (2014) indicated that carminic acid can induce the production of other phenoloxidases in *Bjerkandera adusta* CCBAS 930 like Mn peroxidase and horseradish peroxidase, and that the production of these enzymes its accompanied with a degradation of carminic acid and Poly R-478.

Low concentrations of alizarin yellow also increase the laccase activity but in less proportion that CA does. This study contributes to the knowledge on this field, proving that alizarin yellow can induce laccase production and that azo dyes and anthraquinonic dyes can be degraded in liquid cultures of *P. sanguineus*.

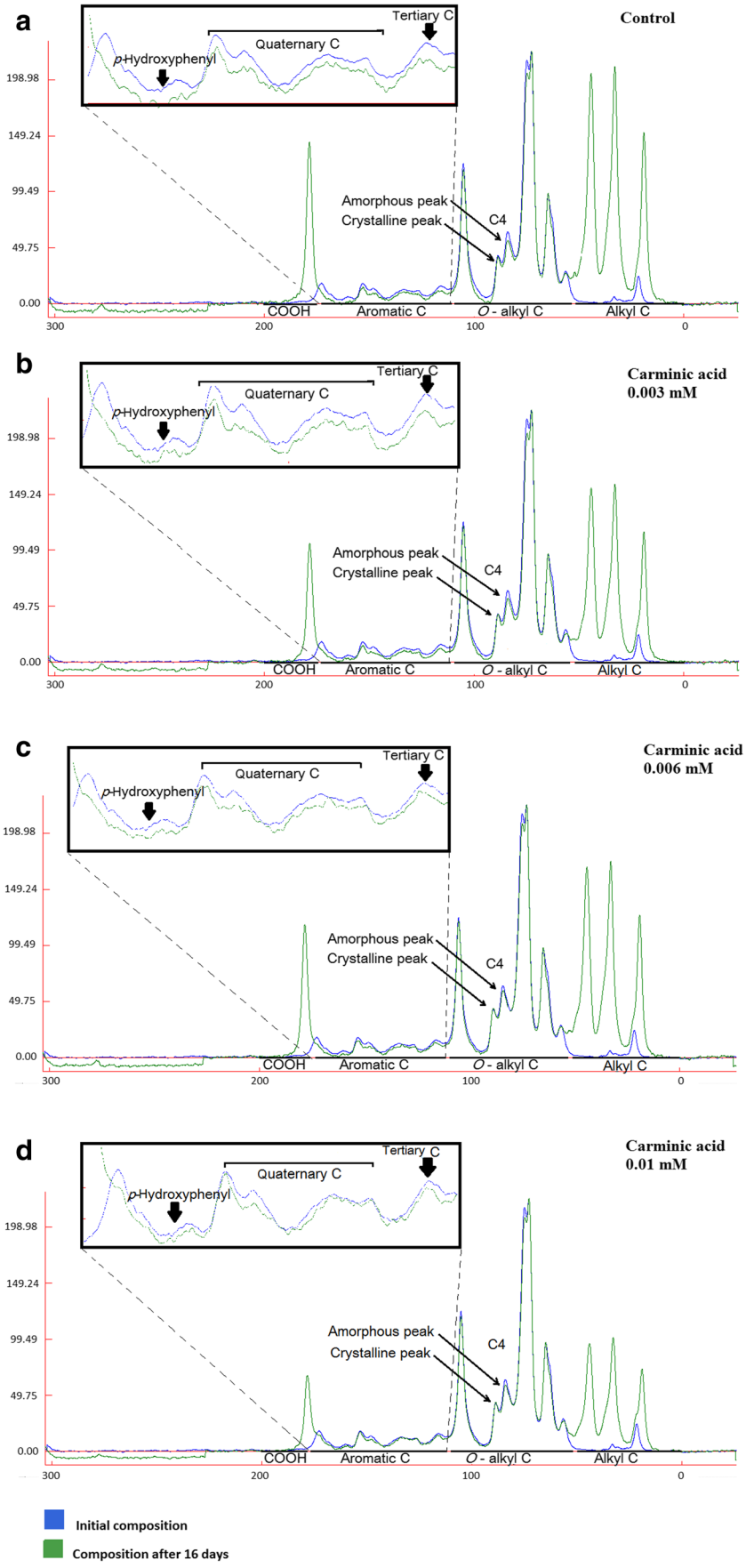
Biomass production was also modified by the addition of dyes into culture medium: aniline blue (all concentrations), carminic acid (0.03 mM), and alizarin yellow (0.01 mM) enhanced the production of biomass in liquid cultures of *P. sanguineus*. The increased biomass production in cultures with these dyes could indicate that *P. sanguineus* can use them as nutrient sources, i.e., alizarin yellow and aniline blue can function as nitrogen and sulfur sources (Dave et al. 2015); however, the nutrimental role of carminic acid is still unclear. Biomass production was positive correlated with alizarin yellow and carminic acid degradation, and with laccase production.

P. sanguineus does not produce either Mn peroxidase (MnP) or lignin peroxidase (LiP) (Pointing and Vrijmoed 2000; Eggert et al. 1997), but it is considered as a good laccase producer (Pointing et al. 2000); thus, the decoloration capacity is probably related to the laccase production observed. Laccase-mediated dye degradation has been previously reported for carminic acid in agar plates and with purified enzymes from *Pleutorus ostreatus*, *Kuehneromyces mutabilis*, *Trametes versicolor*, and *Cerrena unicolor* (Cho et al. 1999, 2007).

In addition, alizarin yellow degradation in cultures of *P. sanguineus* reached values up to 95 % (with an initial concentration of 0.01 mM), similar to other technological methods, e.g., laser-induced photocatalytic degradation (Hayat et al. 2010) or hybrid processes like iron–carbon

Table 5 Fungal biomass indicators (COOH, alkyl-C) in SSF after 8 days of culture. Their relationship with laccase production (U ΣCOOH alkyl-C^{-1})

	COOH	Alkyl-C	U gDM^{-1}	Biomass (ΣCOO, alkyl-C)	U $biomass^{-1}$
Control	7.92	32.14	0.75206154	40.06	0.019
Carminic acid 0.003	8.54	34.06	1.15373077	42.60	0.027
Carminic acid 0.006	8.91	35.98	1.04263077	44.89	0.023
Carminic acid 0.01	9.04	35.40	1.09390769	44.44	0.025


a
Control
Tertiary C
Quaternary C
p-Hydroxyphenyl
198.98
149.24
99.49
49.75
0.00
Amorphous peak
Crystalline peak
C4
COOH
Aromatic C
O - alkyl C
Alkyl C
300
200
100
0
b
Carminic acid
0.003 mM
c
Carminic acid
0.006 mM
d
Carminic acid
0.01 mM
Initial composition
Composition after 16 days

◀ **Fig. 4** Specters obtained from ^{13}C CPMAS NMR analysis. **a** Cultures without carminic acid. **c** Cultures with 0.003 mM of carminic acid. **b** Cultures with 0.006 mM of carminic acid. **d** Cultures with 0.01 mM of carminic acid. Signals of different carbon functional groups are indicated in the basis of the specters, and the *square* maximizes the aromatic C region. Note that C4 has two signal peaks, which corresponds to crystalline and amorphous forms

microelectrolysis combined to aerobic bio-contact oxidation (Liang et al. 2012). This azo dye has been degraded in vitro by chloroperoxidases of *Caldariomyces fumago* (Li et al. 2013), but we did not find previous reports of laccase-mediated decolorization of alizarin yellow. However, in this study, we found a high correlation with alizarin yellow decolorization and laccase production in vivo. Laccase has been utilized in many studies for azo dyes degradation in vitro (Abadulla et al. 2000; Pointing and Vrijmoed 2000; Rodriguez et al. 1999). The other tested dyes (aniline blue and crystal violet) were not degraded in liquid cultures, and no laccase production was registered.

Thus, carminic acid was the stronger laccase inducer tested here in liquid cultures of *P. sanguineus*: It promoted higher laccase and biomass production than the other dyes and was degraded more than 95 % at the end of incubation time (12 days); even if the induction mechanism is not clear, some anthraquinone-derived compounds could be involved in the induction of phenol-oxidases. For these reasons, this anthraquinone dye was selected for being utilized in solid-state fermentation, in an attempt to induce laccase activity and consequently depolymerize the lignin faction of sugar cane bagasse (SCB).

Solid-state fermentation

Many studies reported the induction of laccase in SSF using white-rot fungi, and most of them tested the effect of different carbon sources, nitrogen sources, the use of surfactants (e.g., Tween) and metal or organic inducers, like cooper and xylidine (El-Batal et al. 2015; Karp et al. 2015; Boran and Yeşilada 2011). However, only few have evaluated the effect of dye for ligninolytic enzymes induction. We found that, as well as in liquid cultures, carminic acid was able to enhance laccase production in SSF, compared to control treatments, even though induction was lower. In spite of that, laccases showed to have none or negative effect on the delignification with respect to control.

Usha et al. (2014) reported that bioabsorption of synthetic dyes such as remazol brilliant blue and remazol brilliant violet 5R, onto lignocellulose (wheat bran and rice bran), and its further use for laccase and lignin peroxidase production was possible, using *Stereum ostrea*. Other studies reported that malachite green absorbed onto wheat bran could be used as solid substrate to produce lignin peroxidase from *Fomes sclerodermeus* (Papinutti et al. 2006). Remazol brilliant blue and remazol brilliant violet 5R are anthraquinone dyes, as well as carminic acid; thus, this work along with these previous reports showed evidence that anthraquinone dyes can be added onto lignocellulosic material, and this substrate can enhance phenol-oxidases production.

When carminic acid was added to sugarcane bagasse, cellulase activity decreased, which is positive as an attempt to reach selective delignification, and it aimed to reduce the lignin fraction and the cellulose crystallinity index, while preserving the carbohydrate fraction (Ramos 2003). Generally, when fungi are used, these characteristics can be achieved with high phenol-oxidase activities (e.g., laccase) and low cellulase activities, in order to reach a selective delignification (Dorado et al. 2001) useful to biomass pretreatments for bioethanol production. Some authors considered that *P. sanguineus* is capable of selective delignification (Gupta et al. 2011; Lomascolo et al. 2011), and it has been reported that this fungus can produce its own redox mediator which enhances lignin decay in the presence of laccase, i.e., 3-hydroxyanthranilic acid (3-HAA; Eggert et al. 1996). This fungal metabolite can mediate the degradation of non-phenolic lignin structures by laccase (Eggert et al. 1996).

The enzymatic responses in SSF with or without carminic acid had to be considered together with chemical shifts in SCB composition measured by ^{13}C CPMAS NMR. We found that the main structural shifts in SSF were taking place in the aromatic C region. Other studies that used ^{13}C CPMAS NMR to evaluate lignocellulosic transformation (sorghum

Table 6 Chemical shifts in SCB after 16 days of culturing. Data are given in percentage of increment or decrement

Treatment	Cellulose crystallinity ratio	COOH (organic acids)	Alkyl C (lipids)	O-alkyl C (polysaccharides)	Aromatic C	Aromatic quaternary C	Aromatic tertiary C
Control	+27.24 %	+572.79 %	+2035.98 %	NC	−32.67 %	−25.89 %	−7.05 %
Carminic acid 0.003 mM	+18.74 %	+400.89 %	+1489.33 %	−1.94 %	−30.65 %	−34.82 %	−7.05 %
Carminic acid 0.006 mM	+15.94 %	+470.64 %	+1696.49 %	−0.94 %	−16.33 %	−27.00 %	−5.18 %
Carminic acid 0.01 mM	+15.94 %	+214.04 %	+936.99 %	−1.35 %	−1.95 %	−17.60 %	−5.57 %

NC no changes

and wheat wastes), in solid-state cultures of white-rot fungi, concluded that the growth of such type of fungi mainly led to a decrease in aromatic C amount (Akin et al. 1996), which is similar to our observations.

The delignification yields obtained here are interesting when comparing with other studies, which used white-rot fungus in SSF. Delignification yields of 33 % have been reported in SSF using *Pleurotus ostreatus* and a combination of ferulic acid and cooper sulfate as laccase inducers (Karp et al. 2015). Also, our results are similar to those obtained by Masayuki et al. (2005) using rice straw inoculated with either *P. ostreatus* (41 %), *Phanerochaete chrysosporium* (21 %), or *Trametes versicolor* (37 %). However, it should be noted that the incubation time in these experiments was longer (about 60 days) than that used here (16 days). Even if carminic acid induced more laccase production in SSF, we did not find higher delignification rates; indeed, surprisingly, when carminic acid was added, less of the aromatic C region was degraded. Dyes could be adsorbed by the solid substrate (Sen et al. 2016), reducing their availability to the fungi. This could reduce the laccase induction effect that we observed in liquid cultures, and thus the effect on lignin degradation was not evident in SSF.

We observed that the *O*-alkyl C region, which corresponds to polysaccharides, was slightly affected over time and decreased less than 2 % in all treatments. This result is in accordance to Gupta et al. (2011) and Lomascolo et al. (2011), which suggested that *P. sanguineus* is a fungus with a selective action on lignin. Selectively delignification of SCB by *P. sanguineus* could be an efficient way of pretreatment, in order to enhance enzymatic hydrolysis in second-generation bioethanol production. This mainly because can diminish 32.67 % of lignin, with less than 2 % of carbohydrate losses.

Crystallinity index (CI) is another important issue to account in biomass pretreatments. Here, we observed that solid-state fermentation with *P. sanguineus* increases 27.24 % the CI after 16 days, mainly when carminic acid was absence. The CI value indicates the relation between the amount of crystalline cellulose and amorphous cellulose (Park et al. 2010); when the aim is to hydrolyze cellulose with cellulases, it is desired to have low CI values because amorphous cellulose is more easily degraded by cellulases (Yu et al. 2014; Yoshida et al. 2008). When fungi degrade lignocellulose, generally the CI value increases (Howell et al. 2009), as we observed with *P. sanguineus*; however, when carminic acid was added, the increment of CI was lower than the controls. According to our results, the addition of carminic acid at low concentrations (0.003 mM) in combination with *P. sanguineus* offers several advantages for SCB pretreatment, i.e., it induces the production of laccase and diminishes by 30.65 % of aromatic compounds; the increment of CI was lower in this treatments (18.79 %) than the control (27.24 %) and partially avoids the biomass production.

In the basis of the results obtained, we can recommended the use of *P. sanguineus* for SCB selective delignification, even though it increased its CI value; also, carminic acid is a good inducer of laccase both in liquid cultures and in SSF. However, its use to enhance delignification could be limited because it affects biomass production. Further experiments could be design to avoid this limiting.

This study showed that the use of carminic acid and alizarin yellow, two important textile and industrial dyes, enhance the production of laccase better in liquid cultures of *P. sanguineus* than in SSF (carminic acid). Also, the use of *P. sanguineus* for SCB delignification could bring many advantages, as a rapid and efficient lignin decay with very few polysaccharides losses, even in the absence of inducers. When carminic acid was added to sugarcane bagasse, cellulase activity decreased, preserving the carbohydrate fraction and increasing the cellulose crystallinity index. Finally, we found that higher laccase amount does not necessary entails to higher delignification yields, biomass and redox mediators could have important roles in this process.

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Compliance with ethical standards

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Human and animal rights and informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

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