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Research article

Pre-treatment of a sugarcane bagasse-based substrate prior to saccharification: Effect of coffee pulp and urea on laccase and cellulase activities of *Pycnoporus sanguineus*

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A B S T R A C T

Production of second-generation bioethanol uses lignocellulose from agricultural by-products such as sugarcane bagasse (SCB). A lignocellulose pre-treatment is required to degrade lignin, ensuring further efficient saccharification. Two experimental designs were set up to define culture conditions of *Pycnoporus sanguineus* in mesocosms to increase laccase activities and thus delignification. The first experimental design tested the effect of phenolic complementation (via coffee pulp) and the use of urea as a simple nitrogen source and the second defined more precisely the percentages of coffee pulp and urea to enhance delignification. The responses measured were: lignocellulolytic activities, laccase isoform profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the chemical transformation of the substrate using solid-state NMR of ¹³C. Adding 10% of coffee pulp increased laccase activities and fungal biomass (32.5% and 16% respectively), enhanced two constitutive isoforms (R_f 0.23 and 0.27), induced a new isoform (R_f 0.19) and led to a decrease in total aromatics. However, higher concentrations of coffee pulp (25%) decreased laccase and cellulase activities but no decrease in aromaticity was observed, potentially due to the toxic effect of phenols from coffee pulp. Moreover, laccase production was still inhibited even for lower concentrations of urea (0–5%). Our findings revealed that an agricultural by-product like coffee pulp can enhance laccase activity -though to a threshold- and that urea limited this process, indicating that other N-sources should be tested for the biological delignification of SCB.

1. Introduction

Because of the increment of global population, the demand for energy has increased about 13-fold during the 20th century (Gupta and Verma, 2015). The use of fossil-fuel-based energy has provoked climate change and concerns about energy supply, which strengthened the interest in alternative renewable sources of energy like biofuels (Koppram et al., 2014). The current industrial production of bioethanol (first generation) mainly relies on starch-containing crops (i.e. sweet-corn, sugarcane and sweet-sorghum). On the other hand, ethanol produced from lignocellulose (second generation) does not compete for arable lands and uses lignocellulosic biomass from wastes (i.e. SCB, wheat straw, corn cobs, etc.) (Koppram et al., 2014). This type of biomass is one of the most abundant on earth, storing about 14% of the total

global energy (Gupta and Verma, 2015).

The process to obtain second-generation ethanol requires a pre-treatment i.e. delignification of the substrate, then hydrolysis of cellulose and hemicellulose (saccharification), sugar fermentation and purification of ethanol to reach fuel specifications (Mosier et al., 2005). However, one of the most challenging steps prior saccharification, is to increase the availability of polysaccharides for enzymes by delignification of plant fibres. Many types of physicochemical, chemical and biological pre-treatments have been developed to achieve this goal (Alvira et al., 2010). Of all these strategies, biological pre-treatments using white-rot fungi involve no chemical hazard unlike other methods but they have never been used for second-generation bioethanol production. White-rot fungi are known to produce phenoloxidases (Baldrian, 2006; Sinsabaugh, 2010): lignin peroxidases (LiP) (Hammel

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et al., 1986), manganese peroxidases (MnP) (Sack et al., 1997) and laccases (Collins et al., 1996). These enzymes can enhance dephenolisation of lignocellulose and thus make polysaccharides more available for subsequent enzymatic hydrolysis during saccharification (Singh et al., 2008; Mood et al., 2013). Laccases (benzenediol/oxygen oxidoreductase, EC 1.10.3.2) are non-specific blue multicopper oxidases, which catalyse one-electron oxidation of various phenolic compounds and use molecular oxygen as the final electron acceptor (D'Agostini et al., 2015). Due to that potential, laccase can be used in biological pre-treatment for delignification (thermostability, low substrate specificity), and promotion of its production has attracted much attention.

Production of laccases is influenced by nutrient levels (Dekker et al., 2007), fermentation parameter conditions such as culture time, shaking, temperature (Vasconcelos et al., 2000), and fungal growth stage (Chen et al., 2003) as well as the addition of a wide range of inducers to culture media (Minussi et al., 2007). Laccase gene transcription is regulated by various aromatic compounds related to lignin or lignin derivatives (Mansur et al., 1998; Janusz et al., 2006; Periasamy and Palvannan, 2010). Laccase production is also regulated by the type and concentration of nitrogen source in the medium (Kachlishvili et al., 2006; D'Agostini et al., 2015; Hernández et al., 2015). Organic nitrogen sources like urea or yeast extract usually lead to high laccase yields and good fungal growth (Arora and Rampal, 2002). However, there is no clear consensus concerning laccase production and nitrogen complementation: various tendencies were observed depending on the white-rot fungi and the type of nitrogen source (Piscitelli et al., 2011). Urea has been used as a nitrogen source for laccase production from *P. sanguineus* in solid-state fermentation (SSF) (Vikineswary et al., 2006). Reddy et al. (2003) reported that urea at 40–50% of total nitrogen in the substrate stimulated fungal growth. This is thus of huge importance to determine the amount of nitrogen added in cultures in order to enhance laccase activity and lignin degradation without reaching inhibition concentrations.

Sugarcane is one of the most produced crops in the world: 42.8 million tons of bagasse were produced from juice extraction of milled sugarcane all around the world in 2010, and this agricultural waste can thus be used for second-generation ethanol production (Gupta and Verma, 2015). Similarly, coffee pulp is a primary by-product obtained during coffee processing (234 000 tons were produced in Mexico in 2016) and is an environmental concern because of the toxicity of caffeine and tannins (Pandey et al., 2000). This by-product has been used to enhance the production of laccase enzymes in white-rot fungi (Bhat, 2000; Niladevi and Prema, 2008). Thus, both by-products can complement each other to produce a suitable substrate which can be used in a process integrating a biological pre-treatment based on white-rot fungus potential of delignification. This study aimed at defining the amounts of coffee pulp and urea added to SCB to enhance and/or induce laccase activities of *P. sanguineus* and therefore degradation of lignin. To our knowledge this is the first report about the use i) of a white-rot fungus in the delignification pre-treatment of lignocellulose for second-generation ethanol production and ii) of coffee pulp as an inducer of laccase activities in such context.

A response surface experimental design was performed to test the influence of two factors (coffee pulp and urea) on the delignification of SCB. As responses, laccase and cellulase activities were measured and transformation of lignocellulose was evaluated by measuring the amount of total phenolics, cellulose, and the degree of cellulose crystallinity by solid-state ^{13}C CP/MAS NMR. The potential induction of laccase isoforms by the addition of coffee pulp was followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

2. Materials and methods

2.1. Fungal strain and preparation of the inoculum

The strain of *P. sanguineus* used was isolated from a wild mushroom

found in the sugarcane 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 fungal strain was also identified by sequencing 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 (Hernández et al., 2016).

The fungal strain was reactivated in plates of malt extract agar 1.5% at 30 °C for seven days. Five round agar pieces of 1 cm, after seven days of growth, were used as inoculum to propagate the strain in sorghum grain plates, they were incubated for 10 day at 30 °C and sorghum grains were pre-rinsed two times and kept in water overnight. 150 mL of water and 0.5 g of gypsum were added for every 100 g of sorghum used, then sterilised by autoclaving. The inoculum for the SSF was 5% of the total substrate mass (Hernández et al., 2017).

2.2. Experimental design

To evaluate delignification of the SCB by *P. sanguineus* two factorial designs were used (Tables 1 and 2), where the independent variables were: the amount of exogenous source of phenolic compounds (coffee pulp) and the amount of nitrogen source (urea). The first experimental design helped to understand how the factors tested (urea and coffee pulp) influenced lignocellulolytic activities of the fungus and thus SCB dephenolisation. For each factor, three concentrations ranging from 0 to 10% were tested. Then, a more specific experimental design was performed, using 5 concentrations for each factor: the amount of urea was lowered from 0 to 5% to avoid catabolic repression without limiting nitrogen (Piscitelli et al., 2011) and coffee pulp concentrations were higher (from 10 to 25%) to test to which extent coffee pulp addition can enhance laccase activities and thus dephenolisation of the substrate. The dependent variables measured were: laccase and cellulase activities, and the chemical characterization of the substrate using solid-state ^{13}C CP/MAS NMR. A $\{p, n\}$ simplex-lattice design for p components consists of points defined by the following coordinate settings: the proportions assumed by each component take the $n + 1$ equally spaced values from 0 to 1,

$$x_i = 0, 1/n, 2/n, \dots, 1 \text{ for } i = 1, 2, \dots, p$$

All possible combinations (mixtures) of the proportions from this equation were used (Tables 1 and 2). Considering a three-component mixture for which the numbers of spaced levels for each component are three and five (first and second experimental design). The centre point was triplicate to obtain a repeatability idea of the data. The following polynomial equation of function X_i was fitted for each factor assessed at each experimental point. The polynomial model Eq. (1) was used:

$$Y = b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (1)$$

Table 1

Factors and levels evaluated in the first experimental design (coffee pulp ranging from 0 to 10% and urea from 0 to 10%).

No.	Coffee Pulp (%)	Urea (%)	Bagasse (%)
1	0	0	100
2	10	10	80
3	5	10	85
4	10	5	85
5	10	0	90
6	0	10	90
7	5	0	95
8	0	5	95
9	5	5	90

Table 2

Factors and levels evaluated in the second design of experiments (coffee pulp ranging from 0 to 25% and urea from 0 to 5%).

No.	Coffee Pulp (%)	Urea (%)	Bagasse (%)
1	13,1	1,3	85,6
2	17,5	0,0	82,5
3	10,0	5,0	85,0
4	10,0	2,5	87,5
5	25,0	0,0	75,0
6	16,3	2,5	81,3
7	13,1	3,8	83,1
8	20,0	5,0	75,0
9	22,5	2,5	75,0
10	10,0	0,0	90,0
11	20,6	1,3	78,1
12	15,0	5,0	80,0

Where Y is the estimated response; b_1 , b_2 , b_3 , b_{12} , b_{13} , and b_{23} are constant coefficients for each linear and interaction term produced for the prediction models of processing components. The significance level was considered at $\alpha = 0.05$ in all calculations. The correlations between the parameters were determined using UNSCRAMBLER X 10.4 (CAMO). To find out whether the independent variables have significant effects on each response, a two-way ANOVA was carried out. Homoscedasticity and normality were analysed previously. The combined effect of urea and coffee pulp concentrations in the medium was analysed by response surface methodology (Box and Wilson, 1992).

2.3. Mesocosm preparation

The SCB and coffee pulp were previously milled and sterilised by autoclave, the SCB was sterilised using plastic bags at 30% of humidity, the coffee pulp was sterilised by autoclaving using flasks of 125 mL and the humidity was adjusted at the end to 80%. The mesocosms were placed in 2.6 L glass jars ($21 \times 16 \times 6 \text{ cm}^3$) containing 70 g of the substrate (dry weight, DW) per mesocosm, covered with pierced lids for oxygen diffusion. For each experiment (Tables 1 and 2), mesocosms were realised in three replicates ($n = 3$), thus, a total of 28 or 36 mesocosms (for the first and second experimental design) were prepared and incubated at 30 °C at 80% of the WHC in the dark for 1 month. Humidity was maintained constant over incubation at 80% of the WHC by adding sterilised water.

2.4. Enzymatic activities

Laccases and cellulases were quantified after a 1-month incubation. The reaction mixtures for all the enzyme activities performed with the enzyme extract consisted of 3 g of the substrate with 8 mL of the corresponding buffer 30 °C for laccases and 50 °C for cellulases. Laccase activity was measured by monitoring the oxidation of syringaldazine to its quinone ($\epsilon^M = 65\,000 \text{ M}^{-1} \text{ cm}^{-1}$) at 525 nm in acetate buffer (100 mM, pH 4.5) (Farnet et al., 2004). CM-cellulase (EC 3.2.1.4) activity was assayed using Carboxy Methyl Cellulose (CMC) at 0.1% (w/v) as substrate with sodium acetate buffer (50 mM, pH 5). After a 3 h-incubation, glucose was quantified according to the Somogyi-Nelson method and absorption was read at 870 nm (Farnet et al., 2010). All analytical experiments were performed in triplicate for each mesocosm. One unit (U) of enzyme activity is defined as one μmole of the reaction product formed per h and per g of dry weight (dw).

2.5. Solid-state ^{13}C CP/MAS NMR

Each mesocosm at t_0 and after incubation was chemically characterised by solid-state ^{13}C CP/MAS NMR on a spectrophotometer Bruker DSX 400 MHz operating at 100.7 MHz. Samples (400 mg) were spun at 10 kHz at the magic angle. Contact times of 2 ms were applied

with a pulse width of 2.8 μs and a recycle delay of 3 s. Chemical shift values were referenced to tetra-methyl-silane and calibrated to glycine carbonyl signal set at 176.03 ppm. The relative C distribution in ^{13}C NMR spectra was determined by integrating the signal intensity in different chemical shift regions (Massiot et al., 2002), with an integration routine supplied with Dmfit 2003 software. Seven common chemical shift regions were defined according to Mathers and Xu (2003): Alkyl-C (0–45 ppm), methoxyl-C (46–60 ppm), O-Alkyl-C (61–91 ppm), di-O-Alkyl-C (92–112 ppm), aromatic-C (112–142 ppm), phenolic-C (142–160 ppm) and carboxyl-C (160–185 ppm). Two decomposition indexes have been calculated according to Baldock et al. (1997): (Alkyl-C/O-Alkyl-C) as the humification ratio and an aromaticity ratio by dividing the sum of aromatic-C and phenolic-C to the sum of all regions except methoxyl-C; the crystallinity of cellulose of the substrate after the experiment was also measured using crystalline cellulose (90 ppm)/amorphous plus crystalline cellulose (83 and 90 ppm) (Park et al., 2010).

2.6. Characterization of laccase isoform patterns

SDS-PAGE under semi-denaturalizing conditions were carried out according to Farnet et al. (2004) using 4% stacking gel and 15% separating gel at 220 V with the Mini-Protean II electrophoresis cell (Bio-Rad). Laccase isoforms were revealed using laccase activity staining: gels were immersed for 30 min in acetate buffer, 0.1 M, pH 4.5, with *p*-phenylenediamine 0.1% as laccase substrate and the R_f was calculated for each isoform.

3. Results

3.1. The effect of coffee pulp and urea addition on lignocellulolytic activities and chemical properties of the substrate

Ideally, the addition of urea and coffee pulp has to lead to enhanced laccase activities, while avoiding degradation of polysaccharides within the SCB. For laccase activities, ANOVA results showed a significant effect ($p < 0.001$) of urea in interaction with either SCB or coffee pulp (Table 3). According to the surface plots obtained, the interactions between urea and coffee pulp (Fig. 1a) or urea and SCB (Fig. 1b), showed a negative effect of urea on laccase activities ($p < 0.001$). On the other hand, coffee pulp had a strong significant effect alone ($p < 0.001$) on laccase activities that were enhanced (32.5% more than control when using 10% of coffee pulp) by the addition of these agricultural residues rich in phenols. SDS-PAGE revealed that coffee pulp at 10% enhanced the production of the two constitutive laccase isoforms with $R_f = 0.23$ and 0.27 and of an induced another isoform with $R_f = 0.19$.

ANOVA results showed significant effects ($p < 0.001$) of coffee pulp in interaction with SCB on cellulase activities (Table 3). Analysing the interaction surface plots between coffee pulp and bagasse (data not

Table 3

Three-way ANOVA testing the effect of coffee pulp (CP, 0–10%) and urea (U 0–10%) in SCB (B) on lignocellulolytic activities and chemical properties of the substrate after a one-month incubation.

Response	Coffee Pulp (CP)	Urea (U)	Bagasse (B)	CP*U	CP*B	U*B
Laccase activity	+++	+++	+++	+++	NS	+++
Cellulase activity	NS	NS	NS	NS	+++	NS
Aromatics	NS	+	+++	+++	NS	+
O-Alkyl	NS	+	+++	+++	+	+++
Methoxyl-C	NS	NS	NS	NS	NS	NS
Aromaticity ratio	NS	+	+++	+++	NS	+
Crystallinity	NS	NS	NS	NS	NS	NS
Alkyl-C/COOH	+	NS	NS	NS	+	NS

(NS = Non significant, + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$).

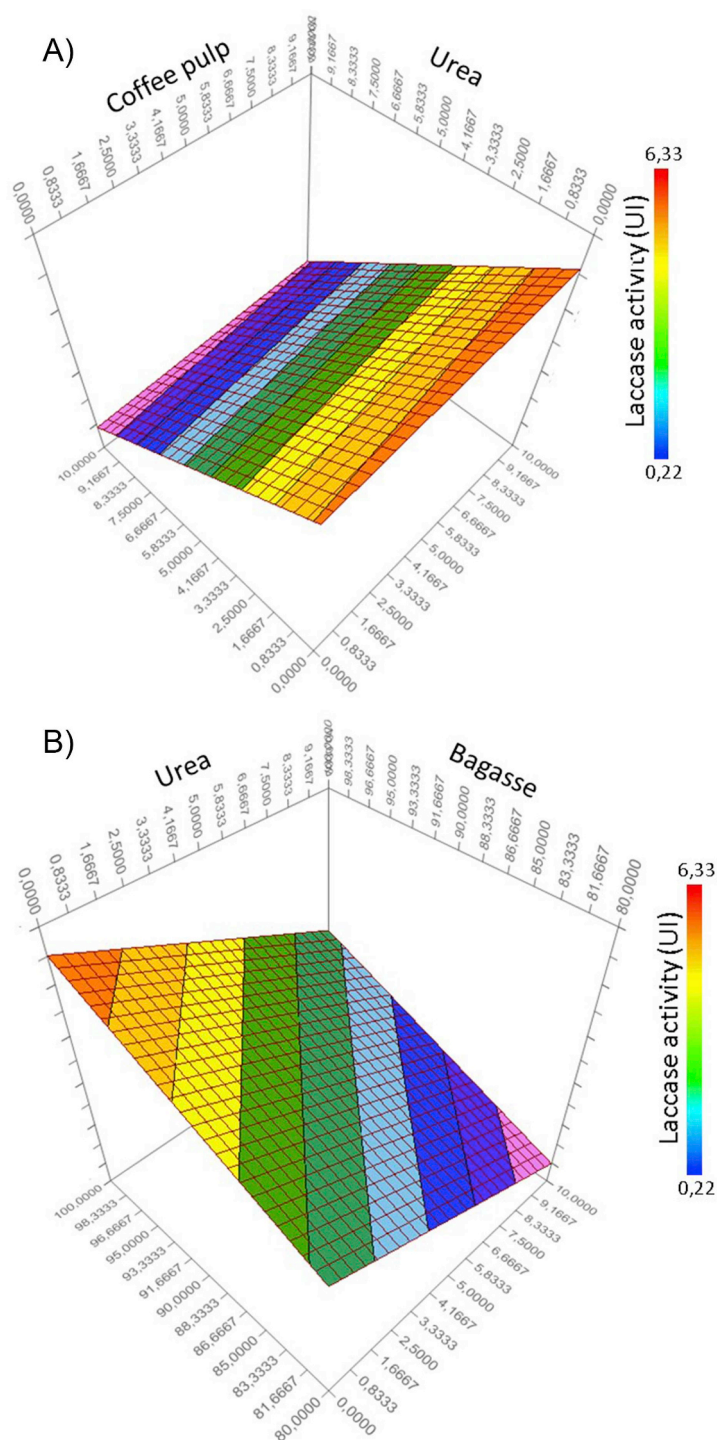


Fig. 1. Laccase activity surface plots showing interactions between Urea and coffee pulp (A) and Bagasse and urea (B) during the first experimental design (Coffee pulp 0–10%, Urea 0–10%).

shown), we observed that these activities increased when both factors were at the maximum level i.e. 10% and 100% respectively, proving that mixing coffee pulp and bagasse can enhance such activities.

At t_0 , no differences in aromaticity ratio or methoxyl-C signals (linked to lignin) were found between all the substrates (data not shown). In order to determine the degree of substrate delignification, the aromaticity ratio was calculated at the end of incubation and ANOVA showed a significant effect of interactions between urea and coffee pulp ($p < 0.01$) on aromaticity ratio: it increased with urea, while it decreased with coffee pulp (Fig. 2a). Interestingly, the

crystallinity of cellulose (crystalline cellulose/amorphous cellulose) was never significantly affected no matter the factor considered (Table 3). This result indicates that crystalline cellulose was barely hydrolysed during a one-month incubation.

For AlkylC/COOH ratio, ANOVA (Table 3) showed the effect of the interaction between coffee pulp and SCB on this ratio: Alkyl-C/COOH ratio increased with coffee pulp (Fig. 2b). When analysing the coffee pulp effect alone, we observed an increase in 16% of Alkyl-C/COOH using the highest concentration of coffee pulp without urea ($p < 0.05$) compared to control.

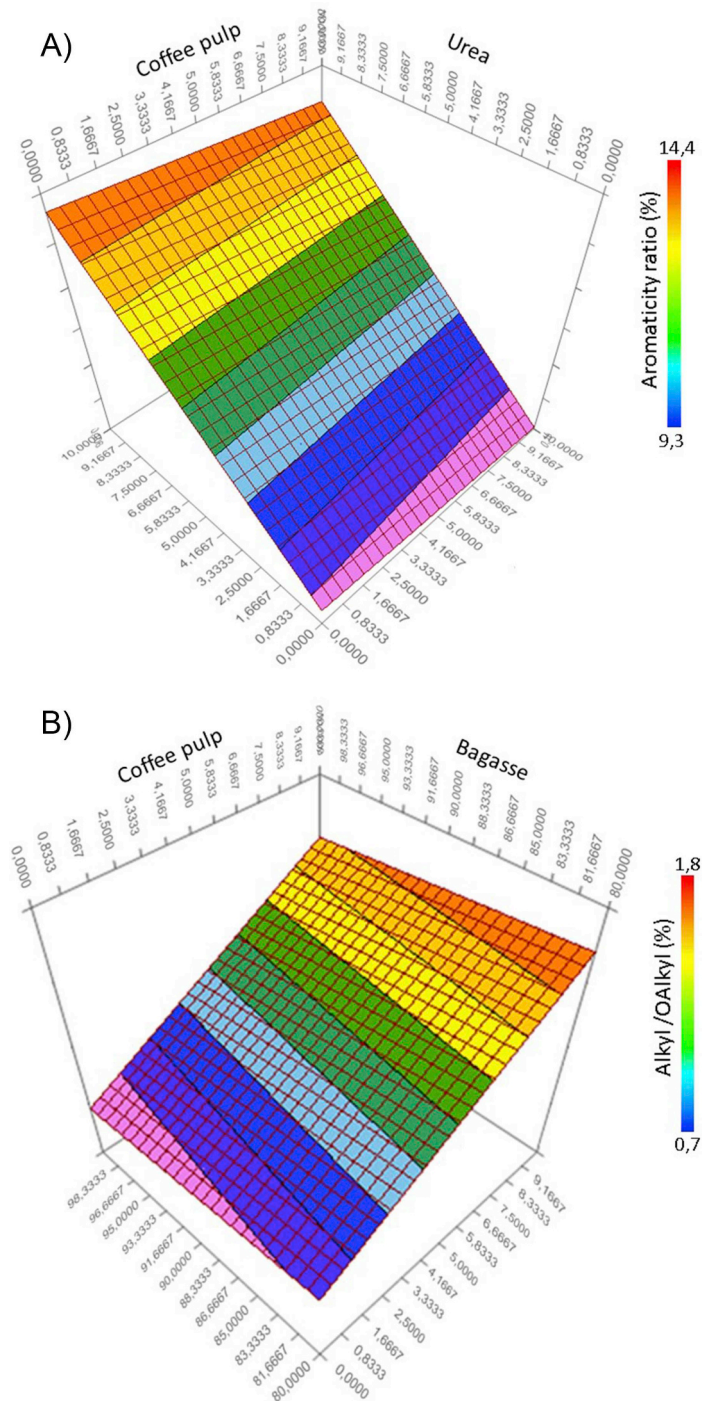


Fig. 2. Aromaticity ratio showing surface plots interactions between coffee pulp and urea (A) and Alkyl-C/O-Alkyl-C ratio surface plots showing interactions between coffee pulp and bagasse (B) during the first experimental design (Coffee pulp 0–10%, Urea 0–10%).

3.2. Defining to what extent coffee pulp and urea can modify laccase activities

For the second experimental design (Table 2), lower concentrations of urea were assayed to determine to what extent urea can affect laccase activities. ANOVA revealed a significant effect ($p < 0.05$) of urea addition on laccase activities (Table 4). Analysing the surface plot interaction between urea and SCB, we observed an inhibitory effect on laccase activities with the addition of urea but to a lesser extent compared to that found in the first experimental design (data not shown). ANOVA also showed the effect of higher amounts of coffee pulp on

laccase activities, which differed from the results of the first experimental design. The addition of a higher quantity of coffee pulp indeed led to a decrease in laccase activities (Fig. 3) and this suggests that coffee pulp can enhance laccase activities of *P. sanguineus* up to a threshold. SDS-PAGE revealed the same results as those obtained when adding 10% of coffee pulp i.e. an enhanced production of the two constitutive laccase isoforms and another induced isoform produced.

Interestingly, for cellulases, we found a significant effect of interaction between urea and coffee pulp ($p < 0.001$). An increase in urea (even for lower concentrations i.e. 0–5%) together with a decrease in coffee pulp concentrations enhanced cellulase activities (Fig. 4). An

Table 4

Three-way ANOVA testing the effect of coffee pulp (CP, 0–25%) and urea (U, 0–5%) in SCB (B) on lignocellulolytic activities and chemical properties of the substrate after a one-month incubation.

Response	Coffee Pulp (CP)	Urea (U)	Bagasse (B)	CP*U	CP*B	U*B
Laccase activity	++	NS	+++	NS	+++	NS
Cellulase activity	NS	+	+++	+++	+	+
Aromatics	NS	++	NS	NS	NS	NS
O-Alkyl	NS	NS	NS	NS	NS	NS
Methoxyl-C	+	+++	+++	+++	NS	+++
Aromaticity ratio	NS	+	NS	NS	NS	NS
Crystallinity	NS	NS	?	NS	NS	NS
Alkyl-C/COOH	+	+	NS	+	+	+

(NS = Non significant, + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$).

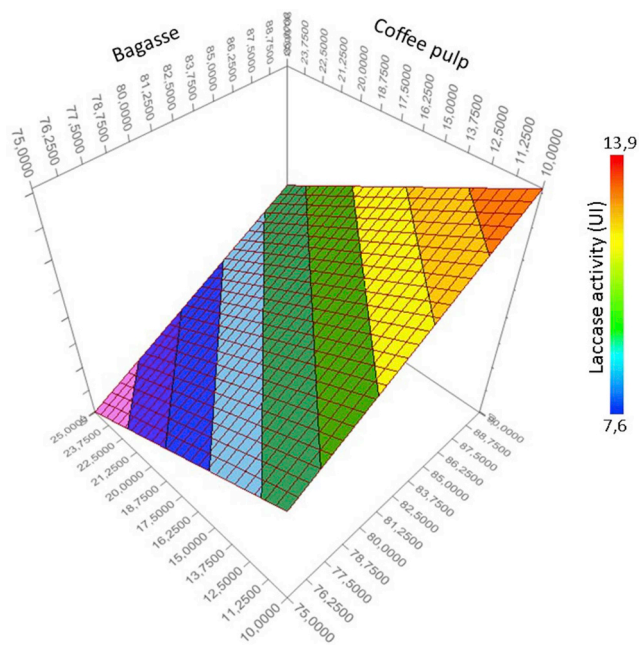


Fig. 3. Laccase activity surface plot of showing the interaction between bagasse and coffee pulp for the second experimental design (Coffee pulp 10–25%, Urea 0–5%).

opposite result was found for Alkyl-C/COOH marker: the surface plot interaction between coffee pulp and urea (Fig. 5) showed higher Alkyl-C/COOH at higher concentrations of coffee pulp and lower concentrations of urea.

The humification rate (Alkyl-C/O-Alkyl-C) was influenced by coffee pulp in interaction with SCB. The surface plot interaction between SCB and coffee pulp showed that for higher concentrations of coffee pulp, this rate increased, which had to be linked with Alkyl-C variations which strongly increased with coffee pulp. Interestingly, the cellulose crystallinity ratio was not affected by any factors under these conditions.

4. Discussion

The addition of coffee pulp (10%) in SCB substrate increased laccase activities, (from 1.6 to 2.1 $\mu\text{mol/g}$ dry weight/h), enhancing delignification. This is supported by SDS-PAGE, which showed that the production of the two constitutive isoforms was enhanced. There are numerous reports about the enhancement of laccase production during SSF using different phenolic compounds which are commonly found in coffee pulp such as ferulic, caffeic, coumaric and chlorogenic acids

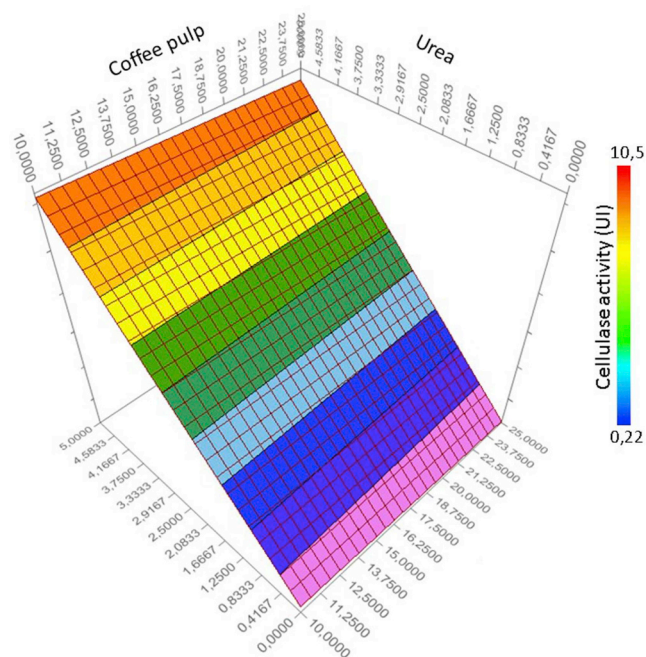


Fig. 4. Cellulase activities surface plot of showing interactions between coffee pulp and urea for the second experimental design (Coffee pulp 10–25%, Urea 0–5%).

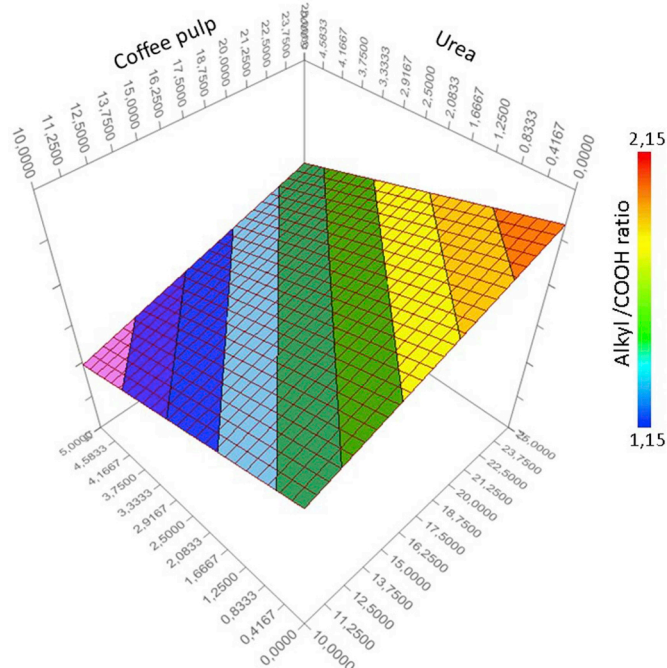


Fig. 5. Alkyl-C/COOH ratio surface plot of showing interactions between coffee pulp and urea for the second experimental design (Coffee pulp 10–25%, Urea 0–5%).

(Torres-Mancera et al., 2011). Wang et al. (2017) showed an increase in laccase activities of 150% when adding a solution of gallic acid in a culture of *Pleurotus ostreatus* during SSF as well as Yang et al. (2013), who also found higher laccase activities in *Trametes velutina* using such phenolic compound as an inducer. Zhuo et al. (2017) found that the transcription levels of most laccase genes increased in the presence of ferulic acid, coumaric acid, and cinnamic acid in a culture of *Pleurotus*

ostreatus. Laccase production was also qualitatively modified since SDS-PAGE revealed that, when adding 10 and 25% of coffee pulp, a new laccase isoform was produced. These enzymes are known to be induced by various aromatic compounds (Qasemian et al., 2011), as a response developed by microorganisms when substrates are present in their surrounding environment. Collins et al. (1996) observed induction of different isoforms of phenoloxidases by lignin-related phenols. Farnet et al. (1999, 2011) found that different phenolic compounds like ferulic acid, gallic acid, vanillic acid or *p*-OH benzoic acid increased the number of isoforms produced by the white-rot fungus *Marasmius quercophilus* compared with control. Laccases from this white-rot fungus were also found to be induced by different aromatic pollutants like anthracene, and this resulted in a better degradation of the toxic compound (Qasemian et al., 2011).

However, higher concentrations of coffee pulp (25%) led to a decrease in both laccase and cellulase activities. In our study, laccase activities were measured after a one-month incubation time. Addition of a high amount of coffee pulp (25% of the substrate) may have accelerated the dynamic of laccase production during the early period of culture. For instance, Téllez-Téllez et al. (2008) found that laccase production of *Pleurotus ostreatus*, reached a peak of production between 8 and 14 days during SSF. However, in our study, no decrease in aromaticity was observed after adding coffee pulp at 25% of the substrate, conversely to what we found with 10% of coffee pulp. Previous studies reported that fungal culture substrate could be complemented with coffee pulp using percentages ranging from 50% to 95% with no toxic effect for the development of the fungi (Salmones et al., 2005; González et al., 2013). This can be explained by the fact that phenols are polymerised within the coffee pulp and are gradually degraded by laccases and other ligninolytic enzymes, limiting their toxicity. In this study, sterilization of coffee pulp in water probably led to the extraction of monophenolic compounds with low-molecular-weight. These compounds can cause loss of biological membrane integrity, affecting their role of selective barriers. Moreover, they can inhibit enzyme activities directly *via* binding on the active site or indirectly *via* protein precipitation (Klinke et al., 2004; Piscitelli et al., 2011). Phenolic compounds like tannins can react with sulfhydryl groups of the enzymes and form a covalent bond, leading to a strong decrease in enzyme activities (Scalbert, 1991). Concerning enzyme inhibition by phenols, several studies have reported the negative effect of these compounds on bacterial (González-Bautista et al., 2017) and fungal cellulases (Kumar et al., 2008).

The addition of 25% of coffee pulp in the substrate led to an increase in the amount of biomass produced as revealed by Alkyl-C/COOH ratio, linked to the aliphatic part of phospholipids from the fungal cell membrane (Farnet et al., 2013; Lundberg et al., 2001). The addition of this by-product rich in nitrogen (approximately 10.5% of dry weight, Pandey et al., 2000) could enhance the production of fungal biomass. Delfín-Alcalá and Durán-de-Bazúa (2003) used a mixture (1:1) of coffee pulp with different urban wastes (cellulosic part of soiled diapers and garden grass residues) in order to improve lignocellulose degradation by *Pleurotus ostreatus*. Pandey et al. (2000) reported that the use of coffee pulp/husk combined with other vegetal debris, has been shown to be an excellent substrate for production of fungal biomass especially fruiting bodies of *Pleurotus ostreatus* and *Volvariella volvacea* in SSF.

Regarding nitrogen sources, this study revealed that urea, even at low concentrations, inhibited laccase production (a range of C/N ratio from 10 to 50 was tested here) and hampered delignification, while coffee pulp, *via* laccase induction, enhanced phenol removal. Laccase expression is regulated by several different biotic and abiotic factors (xenobiotics, metal ions, heat-shock response or microbial antagonisms) and one of the most important is C/N ratio (Janusz et al., 2013). White-rot fungi are more capable of degrading lignocellulosic biomass with C/N ratio around 30:1 (D'Agostini et al., 2015), but the effect of C/N ratio on production of ligninolytic enzymes can vary with the fungal species. For *Pleurotus ostreatus*, *Lentinus edodes* and *Agaricus blazei*,

laccase production was stimulated by low carbon to nitrogen ratio (C/N = 5), (D'Agostini et al., 2015). On the other hand, Tychanowicz (2004) reported an increase in 60% of laccase activities from *Pleurotus pulmonarius* cultured on corn cob with a C/N ratio around 30:1. The nature of the nitrogen source can also affect the expression of laccases in white-rot fungi. Simple organic sources of nitrogen like urea, are consumed by white-rot fungi like *Pycnoporus cinnabarinus*, or *Phlebia radiata*, with the objective of saving cell resources and energy and this usually leads to a low level of laccase activities (Mikiashvili et al., 2006). On the other hand, complex organic nitrogen sources such as mixtures of amino acids, peptones or yeast extract can increase the expression of different enzymes like proteases, L-asparaginase or laccase in order to degrade the different compounds of complex nitrogen sources (Kalisz et al., 1986). Yeast extract can also be considered as an enrichment of the medium, including growth factors, which favour the whole metabolism of the fungus. Low concentrations of nitrogen can unleash the expression of proteases and laccases that are required to improve nitrogen assimilation. This is corroborated by our results since laccase activities of *P. sanguineus* were higher in mesocosms 'control' i.e. without urea and just complemented with yeast extract.

In our study, the addition of urea limited laccase activities but favoured cellulases though neither the crystallinity ratio nor O-Alkyl-C signal varied. Hernández et al., in 2017, reported that, when *P. sanguineus* selectively delignified SCB, by 32.67% in 16 days, less than 2% of polysaccharides were degraded. This means that the polysaccharide fraction remained almost unchanged during SSF. This is of importance since preserving polysaccharides for the further steps of bioethanol production (i.e. saccharification and fermentation) meets the objectives of this study. The weak transformation of labile C-source can be linked to the fact that urea addition did not favour biomass production. Consequently, although urea can be considered as a cheap source of nitrogen for fungal growth in SSF (Reddy et al., 2003) and submerged liquid culture (Hernández et al. 2015; Zimbardi et al., 2016; Marim et al., 2016), in our study, it was not favourable for both induction of laccase activities and fungal growth.

Most studies reported the combined use of chemical attacks (delignification) and enzymes (xylanases, cellulases...) for lignocellulosic pre-treatment prior to fermentation. Here, a biological delignification was realised by inducing the phenoloxidase enzymatic system of a white-rot fungus cultivated on SCB. Moreover, many previous studies on laccase induction of white-rot fungi showed that various expensive components (monophenols, yeast extract) were used (Table 5). Here we found that coffee pulp, a cheap agricultural by-product rich in phenolic compounds, containing also minerals and nitrogen sources, could efficiently induce laccase activity up to a threshold. Eventually, the culture conditions defined here led to the selective oxidation of lignin by the white-rot fungus without extensive consumption of polysaccharides (Blanchette, 1995).

5. Conclusions

This study revealed that the complementation of SCB with a recalcitrant by-product like coffee pulp, can increase laccase activities and also induce new isoforms of laccases, leading to a decrease in the total aromatics of SCB. However, these promising results were obtained up to a threshold of coffee pulp (10%), which seems to be toxic for the fungus at higher concentrations. Very interestingly, fungal biomass increased with coffee pulp addition but no strong hydrolysis of polysaccharides was observed, saving these polymers for the next step of second-generation ethanol production. On the other hand, we observed that simple organic sources of nitrogen like urea, limited laccase activities and also affected fungal development of *P. sanguineus* during SSF. Thus here, coffee pulp complementation of the substrate seems to be a promising strategy for pre-treatment of lignocellulose, and other nitrogen sources should be further tested to limit laccase inhibition.

Table 5

Lignocellulosic materials used as a substrate for laccase production and different enrichment techniques used to enhance laccase activity during SSF.

Lignocellulosic material	White-rot fungal strain	Culture complementation to induce laccase	Laccase activity	Reference
Apple pomace	<i>Phanerochaete chrysosporium</i>	Veratryl alcohol (3 mmol/kg)	789 U/gdw	Gassara et al. (2011)
Beech tree leaves	<i>Pleurotus dryinus</i> IBB 903	Peptone (40 mM)	15.6U/gdw	Kachlishvili et al. (2006)
Barley bran	<i>Trametes versicolor</i>	Xylidine (1 mM)	1700 U/L	Couto et al. (2002)
Brewery waste	<i>Phanerochaete chrysosporium</i>	Veratryl alcohol (1.5 mmol/kg)	841 U/gdw	Gassara et al. (2011)
Coffee husk	<i>Trametes pubescens</i>	CuSO ₄ (5 mM)	110 U/L	Gonzalez et al. (2013)
Coffee pulp	<i>Ganoderma sp</i>		142U/gdw	Revankar et al. (2007)
Flower Stems	<i>Pycnoporus sanguineus</i> SYBC-L1	Gallic acid (40 µM)	7.26 U/gdw	Wang et al. (2017)
Horticultural waste	<i>Trametes versicolor</i>	Veratryl alcohol (1 mM)	8.6 U/gdw	Xin et al. (2011)
Peanut shell	<i>Pycnoporus spp</i> SYBC-L3		5 U/gdw	Liu et al. (2018)
Rice husks and straw	<i>Ganoderma lucidum</i> E47		10.9 U/gdw	Postemsky et al. (2017)
Rice straw	<i>Polyporus sanguineus</i>	Malt extract (0.5%)	4.5 10 ⁻² U/mL	Arora and Gill (2001)
Sawdust-based medium	<i>Ganoderma lucidum</i>	Malt extract (12 g/L)	1.1 U/gdw	Kuhar et al. (2015)
Sugarcane bagasse	<i>Polyporus sanguineus</i>	Malt extract (0.5%)	8.99 10 ⁻² U/mL	Arora and Gill (2001)
	<i>Pycnoporus sanguineus</i>	Ethanol (3 g/L) and yeast extract (2 g/L)	31.01 U/L	Hernández et al. (2015)
	<i>Pycnoporus cinnabarinus</i> ss3	Ethanol (10 g/m ³)	90U/gdw	Meza et al. (2005)
	<i>Pleurotus ostreatus</i> PO45		35 U/mL	Dong et al. (2013)
	<i>Pleurotus ostreatus</i>		112 U/gdw	Verma et al. (2002)
	<i>Pycnoporus sanguineus</i> U13-4	CuSO ₄ (200 µM)	35 U/mL	Marim et al. (2016)
Sugarcane molasses	<i>Agaricus blazei</i>	Ethanol (1.2 mM)	9.7 U/mL	Valle et al. (2014)
Wheat Bran	<i>Ganoderma sp</i>	CuSO ₄ (1 mg/g)	10,05 U/gdw	Revankar et al. (2007)
	<i>Pleurotus ostreatus</i> IE 137		145 U/gdw	Mata (2005)
	<i>Fomes fomentarius</i> MUCL 35117	CuSO ₄ (5.5 mg/g)	40U/gdw	Neifar et al. (2011)
Wheat bran and corncob	<i>Pycnoporus sanguineus</i> RP15	CuSO ₄ (50 mM)	138.6 U/gdw	Zimbardi et al. (2016)
Wheat straw	<i>Lentinus edodes</i> IBB 363	(NH ₄)NO ₃ (10 mM)	16.5 U/gdw	Kachlishvili et al. (2006)
	<i>Polyporus sanguineus</i>	Malt extract (0.5%)	8.3 10 ⁻² U/mL	Arora and Gill (2001)
	<i>Lentinus edodes</i> IBB 123	Yeast extract (4 g/l)	228 U/L	Elisashvili et al. (2008)

Conflicts of interest

All authors declare that they have no conflict of interest in this study.

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