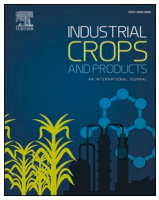
Industrial Crops & Products 233 (2025) 121367



Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop





Assessing process variable effects on laccase-mediated treatment for enhanced saccharification efficiency

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ARTICLE INFO

Keywords:
Enzymatic hydrolysis
Laccase
Phenolic compounds
Inhibition
Eucalyptus bark

ABSTRACT

Lignocellulosic materials (LCM) have gained popularity as a substitute for petroleum, enabling the production of biofuels and various compounds. Eucalyptus bark residue (EBR), generated by pulp and paper mills, is a prime illustration of such materials. The conversion of holocellulose in these materials typically involves the application of enzymes. Nevertheless, these materials may still contain lignin, widely recognized as hindering enzymatic processes. In this work, laccase was evaluated as an accessory enzyme on the hydrolysis of EBR. It underwent autohydrolysis with a severity (S0) of 3.84. The pretreated solid was then hydrolyzed using Cellic CTec2 in conjunction with a laccase-mediated treatment (LMT) employing an in-house produced laccase extract by *Lentinus sajor-caju*. The potential effects of this enzyme were assessed through the glucose released over time and the enzyme adsorption onto the solid substrate. No positive effects were observed when laccase was added simultaneously with cellulases; however, adding laccase 24 h before cellulases increased glucose production by 11 %. Increasing the laccase load notably resulted in a visible decrease in hydrolysis efficiency, suggesting potential toxicity or inhibition effects. Implementing a washing step proved effective in removing phenolics (inhibitors). LMT apparently suffered mass transfer issues and showed differences in enzyme adsorption for different solids, as verified by the free Cel7A levels. However, when an efficient laccase treatment preceded the washing step, with reduced mass transfer limitations, subsequent enzymatic hydrolysis yielded nearly 30 % more glucose. The utilization of laccase in the hydrolysis of LCM can be promising for enhancing the efficiency and cost-effectiveness of related processes, however process design, particularly the timing of enzyme addition and inhibitor removal, plays a critical role in optimizing enzymatic hydrolysis.

1. Introduction

Environmental issues have led to the urgent necessity of research to focus on fossil fuel dependency detachment in the near future. Under the biorefinery concept, enzymatic hydrolysis is a crucial bioprocess for lignocellulosic biomass conversion into biofuels and bioproducts (Zanuso et al., 2021). Even though taking place under milder conditions and without the use of hazardous chemicals compared, for example, with acid hydrolysis, the use of enzymes significantly increases the cost of the process (Gomes et al., 2015; Gomes et al., 2021b). Thus, ensuring high yields in the enzymatic hydrolysis of pretreated lignocellulosic materials (LCM) to fermentable sugars is crucial for economically viable ethanol production (Raj and Krishnan, 2019). However, this production faces challenges due to the recalcitrance of biomass towards enzymatic hydrolysis related to the compact structure of the lignocellulose and the presence of lignin (Rico et al., 2014). Lignin can indiscriminately bind to hydrolytic enzymes, reducing their effectiveness on the enzymatic hydrolysis of pretreated LCM (Moilanen et al., 2011).

Several authors have reported strategies to achieve efficient yields on LCM conversion at low enzyme loads. These strategies include adding the so-called accessory or helper protein enzymes to the cellulase mixture (Oliveira et al., 2018). For example, Sun et al. (2015) showed

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https://doi.org/10.1016/j.indcrop.2025.121367
Received 3 June 2024; Received in revised form 17 May 2025; Accepted 15 June 2025
Available online 21 June 2025
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that the supplementation of cellulase extracts with β-glucosidase, xylanase, and lytic polysaccharide monooxygenases AA9 improved the enzymatic hydrolysis of several lignocellulosic substrates. Hu et al. 2013 reported the synergistic action between cellulase monocomponents and hemicellulases from family 10 and 11 endo-xylanases (GH10 and GH11) and family 5 xyloglucanase (GH5) to improve the hydrolysis of various steam-pretreated LCM. Still, the extent of hydrolysis improvement was highly substrate-specific. Mukasekuru et al. (2018) reported that the synergism between some additives (Tween 80, tea saponin, and BSA) and accessory enzymes (xylanase and AA9) significantly boost the sugar release in high-solids hydrolysis (20 % solid load) of LCM. Maitan-Alfenas et al. (2015) verified that an enzymatic extract from *Chrysoporthe cubensis* provided higher glucose release than other commercial enzymes, and that could be related to the presence of high specific enzyme activities, such as endoglucanase, β-glucosidase, β-xylosidase, pectinase, and laccase.

The importance of these accessory enzymes is evident for efficient cellulose conversion. The current commercial enzymatic cocktails, such as Cellic® CTec3 HS, contain most of these enzymatic activities (cellulase components boosted by enzyme activities such as AA9 molecules, β-glucosidases, and hemicellulases), except laccase. Laccase can be used to modify or partially remove lignin from the biomass (Moilanen et al., 2011). It is an oxidase (EC 1.10.3.2) with low oxidation potentials (<800 mV) that uses molecular oxygen to perform one-electron oxidations of aromatic substrates. Laccases can oxidize the phenolic lignin subunits (less than 20 % of lignin polymer), which have sufficiently low redox potentials, but oxidation of the nonphenolic subunits having higher redox potentials is hindered. Nevertheless, using low Mw mediators such as HBT (1-hydroxybenzotriazole) and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), laccases can extend their oxidation performance to larger molecules as non-phenolic lignin subunits. Thus, laccase oxidizes the mediator, subsequently oxidizing the nonphenolic lignin structure (Hilgers et al., 2020, 2018). That way, using a laccase-mediator can improve saccharification efficiency by cellulase enzymes due to the increase of the porosity of biomass and accessibility to cellulose caused by lignin degradation (Fillat et al., 2017; Moilanen et al., 2011). Moreover, it assists in the detoxification of phenolic inhibitors released in the pretreatment (Raj and Krishnan, 2019).

However, the reports regarding the role of laccase in improving enzymatic hydrolysis are controversial. While some authors have verified a beneficial effect of laccase during the enzymatic hydrolysis of pretreated biomass, others have verified an adverse effect. For example, according to Moilanen et al. (2014), increases of up to 54 % in enzymatic hydrolysis of steam-pretreated spruce (SPS) were verified using laccase-ABTS treatment, which were due to lignin modification caused by laccase-mediator treatments, which reduced the adsorption of endoglucanase Cel5A and cellobiohydrolase Cel7A on lignin. Raj and Krishnan (2018) reported an increase of 35.5 % in the sugar yield during the enzymatic hydrolysis of pretreated sugarcane bagasse using cellulase supplemented with 50 U/g laccase and 2.5 % (w/v) HBT. However, further increase in the laccase level led to a decrease in the sugar yields, in which 22.2 % reduction was verified with 400 U/g laccase compared with the control. Oliva-Taravilla et al. (2015a) have also reported a decrease in glucose yields after laccase treatment, which could be related to an increase in the competition of cellulose binding sites between cellulase and laccase and also with a slight inhibition of β-glucosidase; but other analyses have shown that the lower accessibility of cellulases to cellulose in pretreated biomass could be related to a grafting process of phenoxy radicals onto the lignin fiber.

Various authors have proposed several hypotheses to account for the reduced glucose yields following laccase treatment. These include laccases' potential to enhance the non-specific binding of hydrolytic enzymes to lignocellulosic fibers through catalytic reactions on lignin, its role in strengthening carbohydrate-lignin bonds (Moilanen et al., 2011; Oliva-Taravilla et al., 2015a), and the creation of lignin-derived compounds that may hinder hydrolytic enzymes (Jurado et al., 2009; Michelin et al., 2023, 2016). While inhibition mechanisms are widely acknowledged, there is a lack of information on how to overcome this obstacle. This study seeks to fill that gap by shedding light on laccase's role in improving LCM conversion. It focuses on using autohydrolyzed Eucalyptus bark residue as a model substrate to investigate this aspect, aiming to offer insights about the laccase's role that could enhance the efficiency of the conversion process.

2. Materials and methods

2.1. Materials

Eucalyptus bark residue (EBR) from *Eucalyptus globulus* was provided by RAIZ (Forest and Paper Research Institute; Portugal). After receiving, EBR samples were air-dried (until humidity levels < 10 %) and milled through a 6 mm screen; the final solid was sieved to remove small particles (< 0.1 mm) and stored in a dark and dry place. RAIZ also provided primary sludge, which was employed in complementary tests. Primary sludge was distributed over small lots, sterilized (121 °C, 20 min), and stored at 4 °C. Before use, a carbonate neutralization was performed with HCl 37 % and then washed with distilled water.

2.2. Laccase production

The laccase extract was produced by cultivating *Lentinus sajor-caju* under solid-state fermentation (SSF) using orange pomace as substrate, according to Freitas et al. 2021. Briefly, cultures of *L. sajor caju* were inoculated in 250 mL Erlenmeyer flasks containing 7 g of orange pomace and 80 % initial humidity using mineral solution (Vogel, 1956) and incubated at 28 °C for 21 days. Crude extracts were obtained by adding 20 mL of water to each flask. The mycelia were separated by gauze filtration. Finally, the supernatant was centrifuged at 9500 × *g* at 4 °C for 10 min and used as a laccase crude extract.

The laccase activity was determined spectrophotometrically by monitoring the oxidation of 2,2′-azino bis (3-ethylbenzthiazoline-6-sulphonic acid; ABTS) in 0.1 M sodium acetate buffer (30 °C, pH 5.0) at 420 nm ($\varepsilon = 36\ mM^{-1}\ cm^{-1}$) (Bourbonnais and Paice, 1990). One enzyme activity unit was defined as the amount of enzyme that oxidized 1 μmol of substrate per min. The crude extract containing 43.2 U/mL was stored in a freezer at −18 °C for further use.

2.3. Hydrothermal pretreatment of Eucalyptus bark residues

EBR was subjected to a hydrothermal pretreatment (autohydrolysis) with a severity of 3.84 following a non-isothermal regime (T_{max} − 211 °C). Briefly, EBR solid was mixed with water under a liquid-to-solid ratio 10 in a pressurized Parr reactor (Model 4848). This solid mixture was heated until a maximum temperature of 211 °C, and once reached, the cooling stage was initiated by removing the heating source (cf. (Gomes et al., 2021a for more details). Once the atmospheric pressure (and a temperature below 80 °C) was reached, the reactor content was removed, and the pretreated solid was separated from the autohydrolysis liquor by vacuum filtration. The pretreated solid was then abundantly washed with water and finally stored until further use. Pretreated EBR presented 55.3 % glucan, 7.83 % xylan, and 28.9 % Klason lignin.

2.4. Preliminary assessment of factors potentially affecting laccase action

A first set of tests was conducted to determine potential factors that can significantly influence the positive action of laccase towards cellulases' action, comprising cellulases load, the presence of a laccase mediator, and the moment of laccase addition. Potential benefits were assessed by comparing the glucose levels produced from the enzymatic hydrolysis of pretreated EBR in the presence of laccase and absence

Table 1
CCC (top) and CCI (bottom) designs matrix presenting the normalized and the real values for each run.

Central Composite Circumscribed Design						
	Normalized value			Real value		
Run	X_1	X_2	X_3	X_1 [% (w/v)]	X_2 [FPU/g_{solid}]	X_3 [IU/g_{solid}]
1	−1	−1	−1	5	5	5
2	+ 1	−1	−1	15	5	5
3	−1	+ 1	−1	5	15	5
4	+ 1	+ 1	−1	15	15	5
5	−1	−1	+ 1	5	5	15
6	+ 1	−1	+ 1	15	5	15
7	−1	+ 1	+ 1	5	15	15
8	+ 1	+ 1	+ 1	15	15	15
9	−1.41	0	0	2.95	10	10
10	+ 1.41	0	0	17.05	10	10
11	0	−1.41	0	10	2.95	10
12	0	+ 1.41	0	10	17.05	10
13	0	0	−1.41	10	10	2.95
14	0	0	+ 1.41	10	10	17.05
15	0	0	0	10	10	10
16	0	0	0	10	10	10
17	0	0	0	10	10	10

Central Composite Inscribed Design						
	Normalized value			Real value		
Run	X_1	X_2	X_3	X_1 [% (w/v)]	X_2 [FPU/g_{solid}]	X_3 [IU/g_{solid}]
1	−0.6	−0.6	−0.6	4	7	2
2	+ 0.6	−0.6	−0.6	10	7	2
3	−0.6	+ 0.6	−0.6	4	13	2
4	+ 0.6	+ 0.6	−0.6	10	13	2
5	−0.6	−0.6	+ 0.6	4	7	8
6	+ 0.6	−0.6	+ 0.6	10	7	8
7	−0.6	+ 0.6	+ 0.6	4	13	8
8	+ 0.6	+ 0.6	+ 0.6	10	13	8
9	−1	0	0	2	10	5
10	+ 1	0	0	12	10	5
11	0	−1	0	7	5	5
12	0	+ 1	0	7	15	5
13	0	0	−1	7	10	0
14	0	0	+ 1	7	10	10
15	0	0	0	7	10	5
16	0	0	0	7	10	5
17	0	0	0	7	10	5

X_1 – Solids load.
X_2 – Cellulase load.
X_3 – Laccase load.

(control). For the enzymatic hydrolysis, the hydrothermally pretreated solids were initially suspended in 0.1 M acetic acid-sodium acetate buffer (pH 4.8) at a solid loading of 2 % (w/v). After sterilization, 10 (or 5) PFU/g_{glucan} of Cellic CTec2 were added to the solids suspension and incubated at 45 °C for 72 h. Potential benefits from a laccase mediator were explored by utilizing 2 mM of ABTS on the medium, one of the most commonly used laccase mediators.

Regarding the moment of laccase addition, two main configurations were tested. One option considered was adding laccase simultaneously with cellulases to the solid suspension, corresponding to a simultaneous laccase-mediated treatment and enzymatic hydrolysis. A distinct approach involved the addition of laccase 24 h before cellulases, without any intermediate steps. This is intended to assess possible benefits from a specific period on which laccase can act without the potential competition for oxygen donors with the LPMOs (lytic polysaccharide monooxygenase) present on Cellic CTec 2, as reported in previous studies (Brenelli et al., 2018).

2.5. Influence of target factors on LMT efficiency to improve cellulose hydrolysis

Two central composite designs were conducted to assess the influence of solids concentration, cellulases, and laccase load on the LMT's potential improvements in enzymatic hydrolysis (Table 1). Firstly, a central composite circumscribed (CCC) design evaluated each variable on five levels, corresponding to the normalized values of −1.41, −1, 0, + 1, and + 1.41. Solids concentration (X_1) was tested in the range of 2.95–17.05 % (w/v), which may allow comparing potential improvements in a scenario with very low viscosity issues, with a highly challenging condition where the concentration of solids (and lignin) is already critical. Similarly, a load of cellulases (X_2) between 2.95 and 17.05 FPU/g_{solid} allows covering a particularly favorable level of cellulases (levels typically reported in the literature are around 10 FPU/g_{solid}) and an enzyme deficit scenario, where LMT effects can be superior. Finally, a load of laccase (X_3) was tested in the range of 2.95–17.05 IU/g_{solid}, which covers the usual interval found in LMT studies (Oliva-Taravilla et al., 2015a) while allowing exploration of slightly superior values.

Following the results of the first composite design, a central composite inscribed (CCI) design was conducted equally addressing five levels of each variable, corresponding to the nominal values of −1, −0.6, 0, + 0.6, and + 1 (Table 1). The range of X_1 was refined to 2–12 % (w/v) to reduce reproducibility issues more frequently for high solid loadings. Additionally, the range of laccase load (X_3) was also readjusted to 0–10 IU/g_{solid}, which accounts for previous indications produced from the first CCD, suggesting a maximum LMT effect for laccase loads below 10 IU/g_{solid}.

Each run of the central composite designs followed the methodology described in Section 2.4, with an initial 24-h period of exclusive laccase action, after which cellulases were added. Periodic samples were collected at intervals of 48–96 h of hydrolysis, which were later analyzed by HPLC to estimate the amount of glucose produced (after accounting for the glucose levels introduced with the enzymes).

2.6. Removal of laccase-produced inhibitors

Following the strong evidence for potential inhibitory effects originated by laccase-produced compounds, an integrated washing step was established to be performed immediately before cellulase addition. Under sterile conditions, the liquid in the solids suspension obtained after laccase treatment was exchanged by fresh buffer over three washing steps; on each step, the solid was pressed against the flask interior, releasing a given amount of liquid (where inhibitors would be present), being then replaced by the same volume of fresh buffer. Throughout these three steps, the total volume of exchanged liquid was up to 100 % of the working volume.

2.7. Laccase-mediated treatment under lower rheological constraints

Given the possibility of mass transfer limitations arising from the standard laccase treatment procedure, mainly when a washing step is included (resulting in early laccase removal), we evaluated an alternative treatment approach. This was conducted with a solids consistency of 3 % (w/v), which significantly minimizes mass transfer issues, for 96 h and under a temperature of 40 °C. In addition, a laccase loading of 10 IU/g_{solid} was employed, which should not present a potential risk of higher inhibitor levels since a more efficient washing is being conducted. Unlike the previous procedure, the laccase pretreated solid was washed by vacuum filtration with 5–7 volumes of distilled water, having as reference the volume of liquid recovered after the laccase treatment.

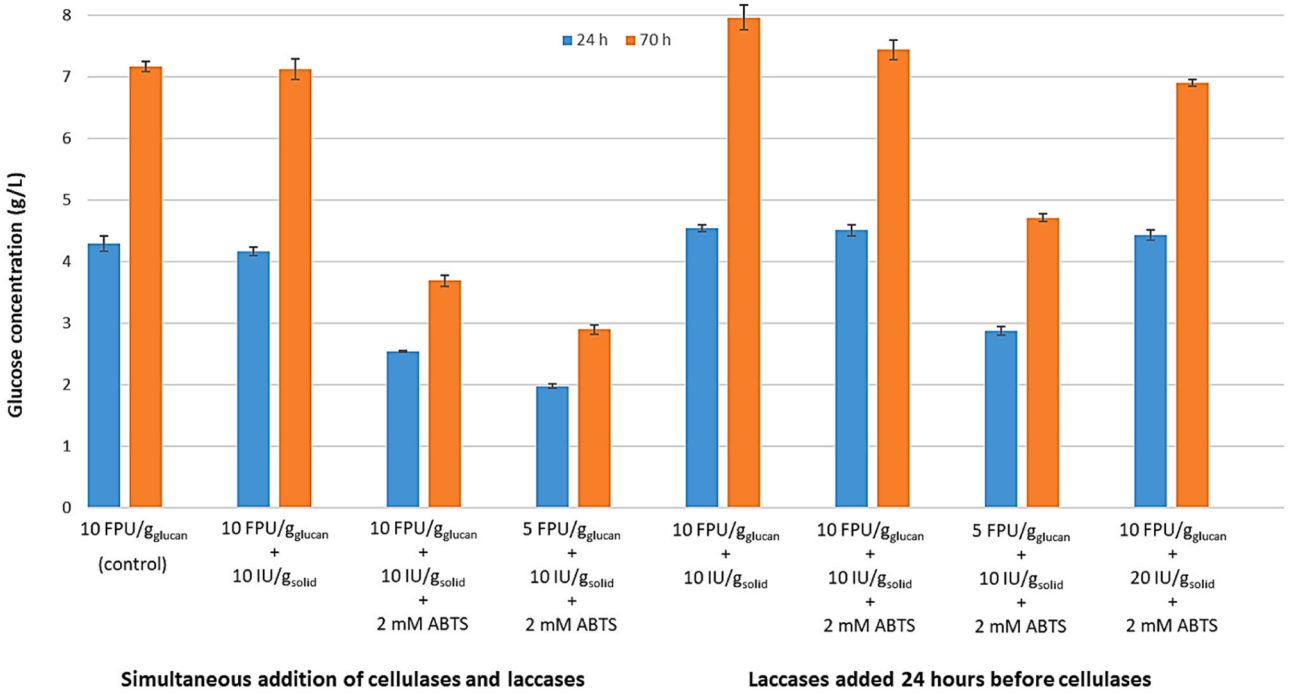


Fig. 1. Glucose production from the enzymatic hydrolysis of 2 % (w/v) EBR after 24 and 70 h under different configurations of a synergetic LMT.

2.8. Analytical procedures

2.8.1. Quantification of the main lignocellulosic components in the solid

The solid composition regarding its main lignocellulosic components was determined by quantitative acid hydrolysis (Sluiter et al., 2011). After drying to a humidity below 10 %, approximately 0.5 g of solid was added to a test tube and mixed with 5 mL of 72 % (w/v) H_2SO_4 for 1 h at 30 °C. The content of the tube was then subjected to dilute acid hydrolysis by raising the reaction volume with water to a total mass of 148.67 g and then autoclaved for 1 h at 121 °C. The solid residue was recovered by filtration (cresol Gooch no.3) and dried (at 105 °C) until constant weight. Sugar monomers released during hydrolysis were quantified by HPLC of the filtrate (*c.f. 2.7.2*).

2.8.2. Quantification of sugar monomers by HPLC

Glucose, xylose, and arabinose were quantified by HPLC (High-Performance Liquid Chromatography). Samples were diluted (when applied), filtered, and then eluted on a Varian MetaCarb 87 H column (300 × 7.8 mm) at 60 °C using 0.5 mL/min of 0.005 M H_2SO_4; a refractive-index detector was used to analyze the compounds.

2.8.3. Quantification of total phenolic compounds

To estimate the extent to which the LMT may have produced cellulase inhibitors, the content of total phenolic compounds was quantified at different points of the process employing the Folin-Ciocalteu method (Singleton and Rossi., 1965). Briefly, 100 µL of the reaction media (filtered and properly diluted) were mixed in a test tube with 2 mL of 7.5 % (w/v) sodium carbonate, 500 µL of Folin reagent (Sigma-Aldrich, 47,641), and completed with distilled water to a total of 10 mL. After well mixed, the test tube was incubated at 50 °C for 5 min and then allowed to cool at room temperature; the absorbance at 700 nm was then measured using a spectrophotometric microplate reader (Bio-TeK Synergy HT). Similarly, a calibration curve was prepared using gallic acid ranging from 0.2 to 2.5 g/L, with total phenolic content being expressed as g of gallic acid equivalent (GAE)/L. All measures employing this method were performed in triplicate.

2.8.4. Estimation of free-adsorbed Cel7A to the solid

A simple way to determine possible modifications introduced by the LMT over lignin, which may ultimately result in superior access to cellulose, refers to potential differences in how cellulases will adsorb-desorb towards the solid. Cel7A represents one of the most abundant cellulolytic domains in cellulase cocktails produced from *Trichoderma reesei*; hence, it is commonly used as an overall cellulase activity indicator. Given its high affinity towards not only cellulose but also lignin (though manifesting distinct desorption behaviors), the distribution of Cel7A among the solid and liquid fractions can provide important indications of potential changes that may have occurred over the solid structure.

Cel7A fractionation was estimated in this work by determining the levels of this component on the liquid fraction (free enzymes) and on the solid fraction (enzymes bound to the solid and within the solid matrix). Cel7A activity was measured following the method, as early described by Bailey and Tähtiharju (2003) with some adjustments. Briefly, 50 µL of the diluted sample was mixed with 400 µL of a freshly prepared solution of 1 mM 4-methylumbelliferyl-β-D-cellobioside (Sigma-Aldrich, M6018) and incubated at 50 °C for 15 min. Afterward, 550 µL of 1 M Na_2CO_3 was added to stop the reaction, which was finally measured on a black bottom 96-well UV fluorescence microplate (Bio-TeK Synergy HT). The reaction of Cel7A over the substrate releases 4-methylumbelliferone, which is measured by fluorescence spectroscopy with excitation and emission wavelengths of 360 and 460 nm, respectively. Applying a calibration curve previously prepared with this compound, it was possible to estimate the amount of substrate converted and consequently determine the level of enzyme activity. For this purpose, one unit of enzyme activity (U) is defined as the amount of enzyme that catalyzes the hydrolysis of one micromole of substrate per minute (cf. details on (Gomes et al., 2016; Rodrigues et al., 2012).

3. Results and discussion

3.1. Assessment of the laccase extract as an LCM hydrolysis booster

A first evaluation was conducted to determine the potential benefits of laccase on the enzymatic hydrolysis of EBR. This first approach

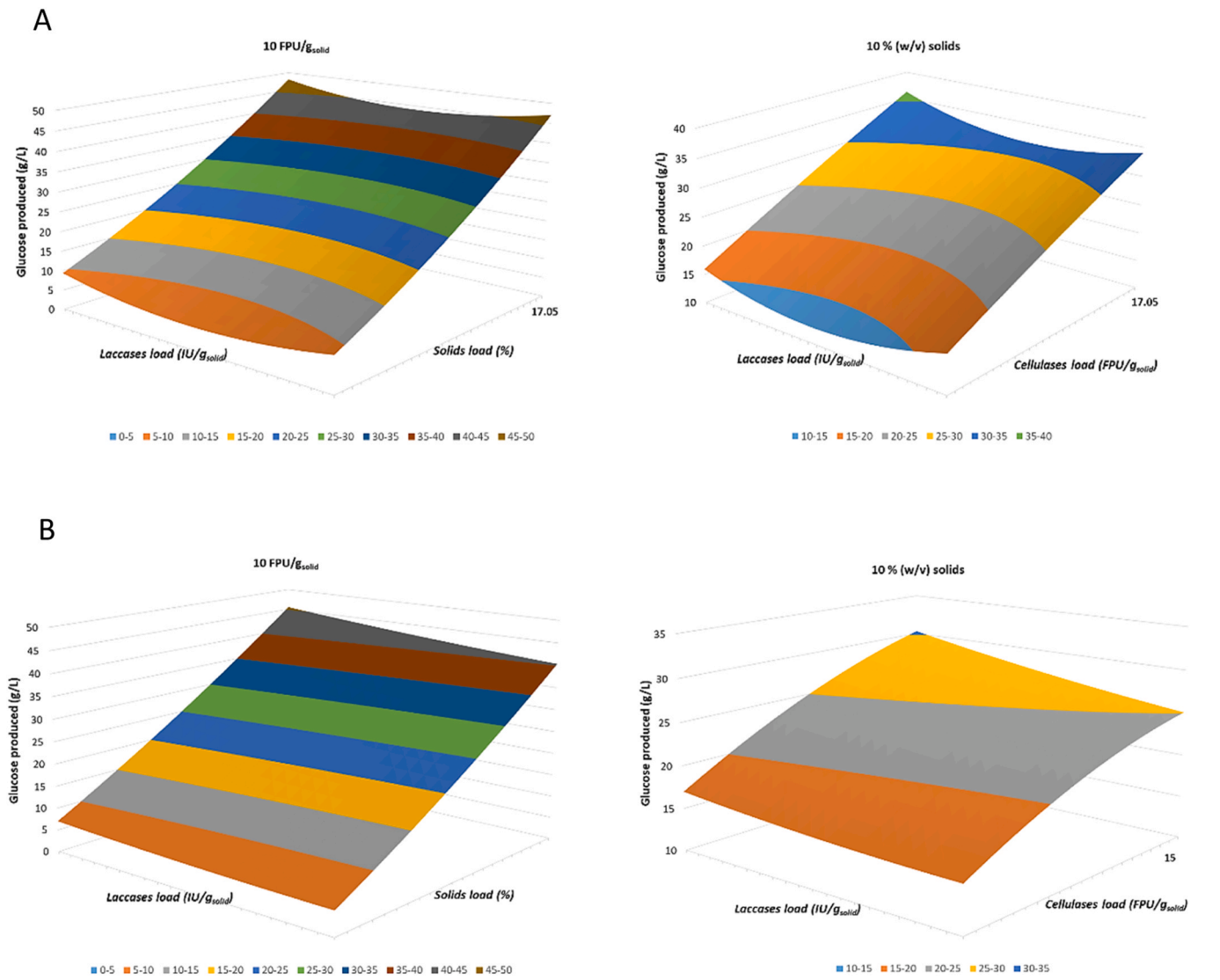


Fig. 2. Response surfaces obtained on the CCC (A) and CCI (B) designs, referring to the glucose produced after 72 h hydrolysis as a function of laccases and solids loadings (left), and laccases and cellulases loadings (right).

employed a low solids loading to avoid mass transfer issues. Concerning the laccase load (10 IU/g_{solid}), it met the typical range found in similar works (Oliva-Taravilla et al., 2015a, 2015b; Rico et al., 2014).

Fig. 1 presents the glucose levels produced from the enzymatic hydrolysis of hydrothermally pretreated EBR combined with a laccase pretreatment conducted under distinct conditions. First, glucose production showed no improvement when laccase pretreatment was performed with the simultaneous addition of laccase and cellulases. Additionally, adding 2 mM ABTS, a laccase mediator, resulted in a noticeable reduction of glucose production, around 48 % (70 h of hydrolysis).

When performed under a different configuration, referring to cellulases added 24 h after laccase, the LMT produced a much different result. Conducted under standard conditions (10 IU/g_{solid}; no mediator), the LMT allowed an increase of 11 % in glucose production after 70 h of hydrolysis compared to the control; when 2 mM ABTS was used, this improvement was lower (4 %). This noticeable influence of the time of cellulase addition meets the previous findings from Brenelli et al. (2018) where the authors reported a competition of laccase and LPMOs towards oxygen, reducing enzymatic hydrolysis. Oliva-Taravilla et al. (2016) reported a competition of cellulose-binding sites between cellulases and laccases as a decrease in the glucose recovery was observed in the presence of laccase (10 IU/$g_{substrate}$) using a (lignin-free) model cellulosic substrate (Sigmacell).

On the other hand, considering that the ABTS concentration used meets the lower values of this compound typically found in similar works, the negative effects here observed could suggest that ABTS possibly potentiated the action of laccase towards undesirable mechanisms, namely the conversion of lignin structures into cellulase inhibitors. Additionally, this claim is supported by the observation that doubling the laccase load to 20 IU/g_{solid} did not improve cellulase performance. On the contrary, it resulted in a 4 % decrease in glucose production compared to the control. These results seem to indicate that the potential benefits of a reduction/modification of lignin structures could be outweighed by possible toxicity effects resulting from the presence of lignin-derived inhibitors. This becomes even more relevant given the fact that the current LMT approach does not predict a step to remove such potential inhibitors. On the other hand, this may also suggest that laccase effects may already be saturated and that the optimum load of these enzymes should correspond to a value inferior to 10 IU/g_{solid}. This is supported by the work of Qiu and Chen (2012), who reported an increase in the hydrolysis yields of steam-exploded wheat straw with low laccase load (0.55 IU/g substrate) while a further increase in laccase dosage decreased the cellulose conversion rate.

Table 2
General indicators and analysis of variance (ANOVA) for the different regression models.

		CCC Design		CCI Design		
		72 h	120 h	48 h	72 h	120 h
p-value	X_1	6.26E−9	5.04E−10	7.66E−8	3.11E−8	3.70E−9
	X_2	8.57E−7	1.21E−7	0.00020	7.98E−5	2.61E−5
	X_3	0.53501	0.29692	0.05789	0.04540	0.05779
	X_1^2	0.06711	0.00643	0.81270	0.36250	0.04271
	X_2^2	0.73654	0.75904	0.20404	0.32692	0.25792
	X_3^2	0.01108	0.09667	0.97373	0.79463	0.73367
	X_1X_2	0.00169	0.00025	0.42717	0.46040	0.39979
	X_2X_3	0.50407	0.97629	0.77275	0.56993	0.81159
	X_1X_3	0.91815	0.48457	0.50483	0.57682	0.53839
R^2		0.99496	0.99746	0.98815	0.99084	0.99491
R^2_{adj}		0.98847	0.99420	0.97292	0.97907	0.98836
Lack of Fit (*p*)		0.98730	0.71710	0.00348	0.00343	0.05789

Another important aspect to consider is the load of solids on the enzymatic hydrolysis, which in this case represented a very modest value. When operating under higher solids loadings, which is typically pursued for LCMs-based processes (Zanuso et al., 2022), the concentration of lignin will equally reach superior levels; while a significant increase of non-productive binding may not necessarily occur since the amount of enzymes added will also increase (enzyme addition is based on the amount of solid), lignin effect as a physical barrier blocking cellulose access is very likely to increase, and so the potential benefits of conducting an LMT.

3.2. Effect of enzymes and solid loadings on the LMT efficiency

Considering the beneficial effects of LMT and the preliminary observations presented in Section 3.1, the impact of process variables on LMT efficiency was studied in greater detail, with the outline of empirical models focusing a full coverage of variable effects and their interactions. The enzymatic hydrolysis of EBR following a laccase treatment was evaluated under different conditions: solids concentration, cellulases load, and laccase load. In the first stage, a central composite circumscribed design was conducted, quantifying the levels of glucose produced at 72 and 120 h of hydrolysis. The levels of glucose production were fitted to second-order polynomial models as a function of the normalized values of solid concentration (X_1), cellulases loading (X_2), and laccase loading (X_3), from which it was possible to construct the correspondent response surfaces (Fig. 2A). Also, an ANOVA analysis was conducted over the different models to determine the significance of the distinct effects possibly associated with each independent variable (Table 2).

On a first note, and as would be expected, Fig. 2A shows that both solids concentration and cellulase loading had a significant effect on glucose production, further confirmed by a positive linear coefficient (*data not shown*) that was statistically significant on a confidence level of 99 % (Table 2). Differently, increasing laccase (the central element of LMT) loadings resulted in more complex effects on the levels of glucose production. In this sense, while a (negative) linear effect was not detected with statistical significance, a quadratic effect was observed with a significance of 95 % (for 72 h of hydrolysis). This result is somehow unexpected given the indications from the previous results (c. f. 3.1), which suggest either a negative linear or quadratic effect. Instead, response surfaces seem to indicate that an increase of laccase loadings may result in a decrease of glucose production only until some point, where there is then an inflection of laccase effects. Furthermore, there was no evidence of a relevant effect of either solids or cellulase loadings in the potential effects from laccase; increasingly challenging conditions on the enzymatic hydrolysis would theoretically favor the effect of laccase, which did not happen. We hypothesize that, under this LMT configuration, there was a specific level of laccase loading above which the possible negative effects associated with these enzymes (either inhibitors production or a negative modification of solid components) were outweighed by the potential benefits from their action.

Table 3
Experimental values for a selected set of runs of the CCI design.

			Glucose produced (g/L)		
% Solids	FPU/g_{solid}	IU/g_{solid}	48 h	72 h	120 h
4	7	2	9.15	10.32	12.13
		8	7.74	8.70	9.05
10	7	2	29.41	33.06	39.65
		8	26.34	29.88	36.83
7	10	0	20.85	22.97	26.20
		5	20.81	23.06	27.63
		10	19.24	21.96	26.40

Moilanen et al. (2011), in a study with pretreated spruce and giant reed, in which the substrates were first treated with laccase and then hydrolyzed with commercial cellulases, verified an improvement of 12 % in the hydrolysis yield of spruce and an adverse effect on a giant reed, with a reduction of 17 % in the hydrolysis yield. It is important to highlight that no washing step was performed in this work for inhibitor removal, which could hamper the enzymatic hydrolysis. This statement is supported by the findings in which the authors verified that for giant reed, laccase treatment led to a decrease in the lignin content, implying a removal of inhibitory compounds from the lignin. On the other hand, this was not verified for spruce, suggesting a polymerization reaction of soluble aromatic substrates instead of a lignin degradation.

So, while the previous results have given some indication for the general effects that we may expect from an LMT on enzymatic hydrolysis, it can be especially relevant to investigate laccase effects specifically in a range of lower laccase loadings, which naturally would be desirable for economic reasons, especially with the inclusion of a laccase-free level (0 IU/g_{solid}) that will allow us to better determine the real effects of these enzymes under very low loadings, which did not occur on the 1st CCD. In this sense, the first central composite design was followed by a second (central composite) inscribed design with some adjustments on each variable range, especially a reduction of laccase loadings, varying between 0 and 10 IU/g_{solid}.

As can be observed from Fig. 2B, the first consequence of reducing the levels of laccase loadings is that the quadratic effect previously observed for this variable (with an inflection after a glucose production minimum) was no longer visible, also confirmed by the ANOVA analysis. On the other hand, laccase loading had a linear negative effect on glucose production, which is statistically significant on a confidence level of 95 % (for 72 h of hydrolysis). Although under this full range of laccase loadings (0–10 IU/g_{solid}), their action was overall translated by a linear negative effect on hydrolysis efficiency, it is important to note that for low laccase levels, there was evidence of an improving effect, possibly reflecting very attenuated toxicity effects from laccase. Analyzing the detailed results from the CCI design (Table 3), it was possible to observe that while glucose production generally decreased when laccase levels increased, in the particular case of the tests performed with 7 % (w/v) solids and 10 FPU/g_{solid}, glucose production at 120 h of hydrolysis increased 5.5 % when 5 IU/g_{solid} were used. Still, it is unclear whether a maximum effect of laccase would be expected for 5 IU/g_{solid} or an inferior enzyme loading. Understanding how laccase affects enzymatic hydrolysis rather than improving it will help design an optimum LMT with direct benefits for enzymatic hydrolysis.

3.3. Potential effects of a washing step to remove intermediate inhibitors

As results from the previous CCDs (central composite designs) have suggested possible inhibitory effects originating from laccase action, which apparently occurred for a laccase loading around 5 IU/g_{solid}, and considering other studies where a solid washing step was conducted

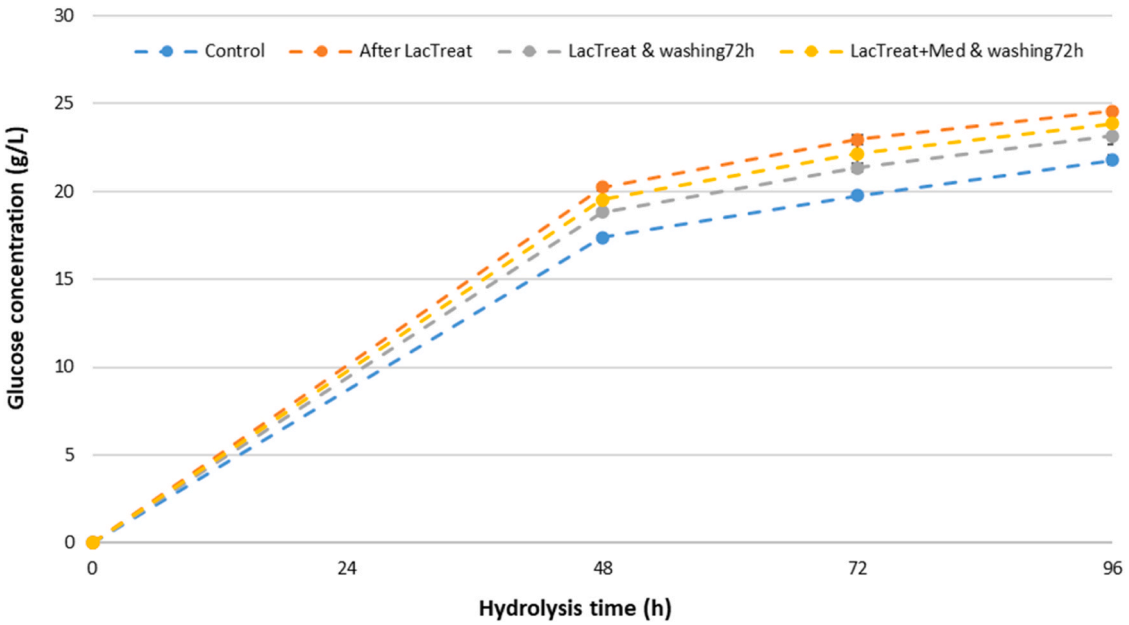


Fig. 3. Glucose production from the enzymatic hydrolysis of EBR (7 % (w/v) solids, 10 FPU/g_{solid}), combined with a previous laccase-mediated treatment (3 IU/g_{solid}; 72 h) and a facultative inhibitors-washing step. Control corresponds to the treatment with only cellulases (10 FPU/g_{solid}).

after laccase treatment (aiming to remove toxic compounds formed) (Rico et al., 2014), the next step involved assessing the possible effects of such a procedure on our process. For that, a washing step was conducted after 72 h of laccase treatment, which was followed by cellulase addition for cellulose hydrolysis (Fig. 3). Under aseptic conditions (to enable a direct application of the laccase-pretreated solid in a saccharification process), the washing step was conducted through buffer exchange (up to a maximum of 1 working volume), this was also replicated with the utilization of ABTS to investigate potential benefits from the presence of a mediator. For this test, only 3 IU/g_{solid} of laccase were employed to facilitate the observation of any hydrolysis improvements, even in the absence of the washing step.

After analyzing Fig. 3, the first result to emphasize is that the improvements originated by the laccase treatment were clearly superior to the ones observed so far, especially when compared to the initial results with 2 % (w/v) solids and 10 IU/g_{solid} (Fig. 1). Such fact can be explained by two main reasons: the utilization of a higher solids concentration (7 % (w/v) solids); and the application of inferior laccase loading (3 IU/g_{solid}). The first one refers to the fact that when increase the solids loading and, consequently, lignin concentration, the potential benefits from laccase are expected to increase (as long as toxicity originated from laccase action is neutralized). On the other hand, considering the previous indications of potential inhibition effects from laccase-generated compounds, an inferior level of laccase may, in fact, constitute an advantage since the level of inhibitors would be lower; under the current circumstances, the main contributor to this result cannot, however, be accurately established.

When analyzing the hydrolysis efficiency for the cases when an intermediate step of inhibitors washing was conducted, the results were somehow unexpected. Although glucose production under this scenario

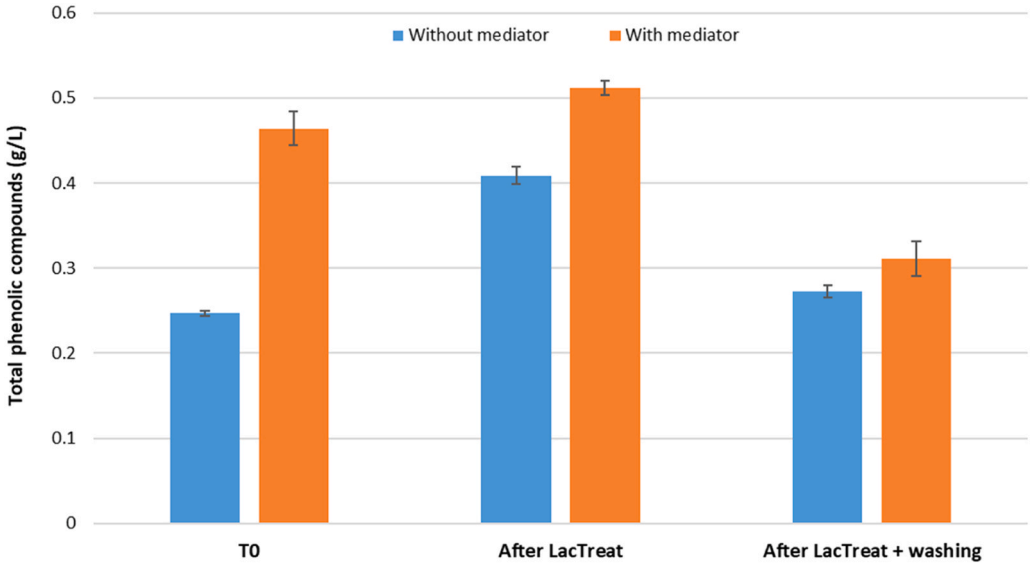


Fig. 4. Variation of total phenolic compounds during an LMT (3 IU/g_{solid}; 72 h) over 7 % (w/v) EBR and after the application of a washing step (buffer exchange up to 1 vol). T0 corresponds to the time immediately after adding the laccases to the solid.

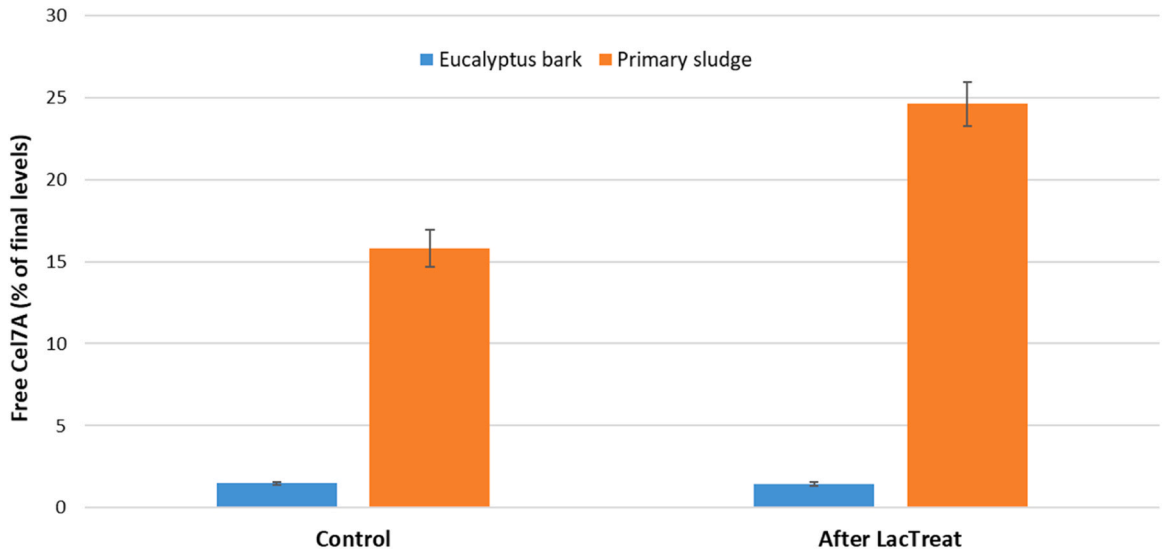


Fig. 5. Variation of free Cel7A after 96 h of enzymatic hydrolysis (7 % solids; 10 FPU/g_{solid}) of solids without and with the previous laccase treatment (7 % solids; 3 IU/g_{solid}; 72 h). Control corresponds the treatment with only cellulases (without previous laccase treatment).

was still superior (9 – 10 %) compared to the control (no laccase treatment), glucose production was lower than in the process where inhibitors were not washed, hence contradicting the initial purpose of this procedure. This could be related to the low load of laccase used and, consequently, a low release of inhibitors from lignin, which meant that the washing step had a low impact on improving the saccharification process.

To better understand this outcome, we aimed to quantify the total phenolics released during laccase treatment and those that persisted after the washing step. Since lignin molecules are known for their phenolic nature, we considered this parameter a reliable indicator for assessing potential inhibitors. As Fig. 4 demonstrates, there was indeed a noticeable increase in total phenolics content after laccase treatment, which in the case of the treatment without mediator was in the range of 66 %, reaching approximately 0.4 g (GAE)/L.

Some studies have shown that the inhibitory effect of phenolic compounds on cellulase activity is mainly related to the type and their concentration (Qin et al., 2016). These phenolics can be released through the laccase treatment (Oliva-Taravilla et al., 2016, 2015b), as well as biomass pretreatment (Chen et al., 2020; Michelin et al., 2023). Zhao et al. (Zhao and Chen, 2014) identified the phenolics generated in steam-exploded corn stover and evaluated their effects on cellulase activity. They verified that phenolic aldehyde significantly inhibited cellulase activity from *Trichoderma reesei* at 0.05 −8 g/L. However, phenolic acids might have a concentration-dependent inhibition/-stimulation effect on cellulase activity.

Independently of the expected effects from these levels of phenolics, it is important to note that after the washing step, their levels were reduced by nearly 33 %, significantly approaching the initial values. This result suggests that any change caused by the laccase treatment in the phenolic content was possibly neutralized by carrying out a simple washing step. Therefore, perhaps the benefit caused by the lignin removal was higher than any inhibition caused by the inhibitors (concentration) generated at a low laccase load. Although the washing step was effective in removing these inhibitors, apparently, this benefit would only be detectable in treatments with higher enzyme load, in which the neutralization of the negative effect of a higher concentration of inhibitors would be easily detectable with the washing step. Another hypothesis could be related to a reduced action of laccase before being "washed", which does not occur when a washing step is not conducted. Cellulolytic enzymes usually face mass transfer limitations and mobility issues when operating under high solid loadings, and the same will likely occur for laccase when exposed under equivalent conditions. While 7 % (w/v) solid loading may not be exceptionally high (Shafiei-Alavijeh et al., 2024), it does pose some mass transfer limitations for this specific solid (pretreated Eucalyptus bark residue). This is evident because, during the initial 48 h of laccase treatment, the solid suspension did not undergo liquefaction.

3.4. Extent of laccase action under challenging rheological conditions

Understanding the real extent to which laccase may act under high-solid conditions should be equally important as it is for cellulases since this will help design efficient laccase treatments with the expected lignin modifications over the lignocellulosic solid. Thus, the real action of laccase during 72 h before being partially removed by the washing step was investigated. Upon extensive action towards lignin, laccase would be expected to cause significant changes over its structure and consequently over mechanisms related to it, as is the case of enzyme binding and non-productive cellulase binding. To investigate that, the free Cel7A levels (not bound to the solid) were analyzed over an enzymatic hydrolysis process (96 h) where the initial solid was initially subjected (or not) to a laccase treatment (Fig. 5). Cel7A is one of the most abundant cellulolytic domains on enzyme cocktails from *Trichoderma reesei*, hence typically representing a good indicator of cellulase activity. As a comparison base in this study, a similar experiment, including the laccase treatment, was also performed with another solid corresponding to primary sludge (PS) obtained from a paper mill.

Analyzing Fig. 5, the first result to emphasize is that there was a notorious difference in the ranges of free Cel7A obtained for the different solids, independently of the conduction of the laccase treatment. For EBR, these were always inferior to 3 %, ranging between 15 % and 25 % for PS. Even though this difference is not entirely unexpected (differences in enzyme adsorption are quite common for different solids), it gains special relevance in this context since it suggests that the potential benefits from a laccase treatment will be highly dependent on the employed solid and cellulase interactions that it typically presents. Other authors have already reported differences in enzyme adsorption for different solids (Djajadi et al., 2018; Hao et al., 2019; Moilanen et al., 2011). Moilanen et al. (2011) reported an improvement in the hydrolysis yield of spruce by 12 % but an adverse effect on giant reed (17 % lower hydrolysis yield) after LMT, concluding that the structural differences in lignin affected cellulases binding and consequently the hydrolysis yields by cellulases.

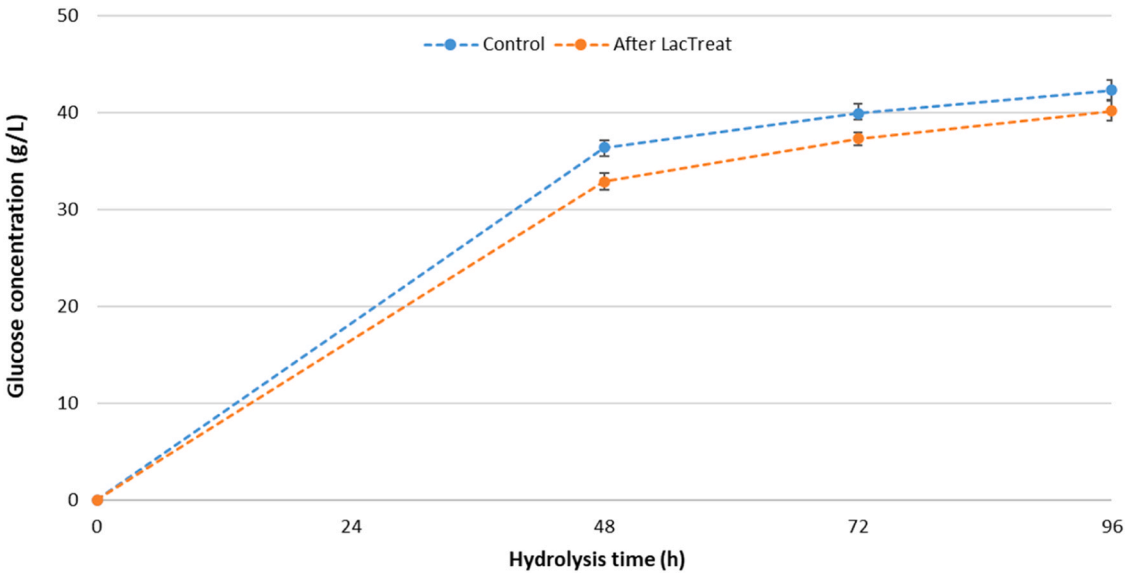


Fig. 6. Glucose production from the enzymatic hydrolysis of PS (7 % solids; 10 FPU/g_{solid}) with or without the combination of a previous laccase treatment (7 % solids; 3 IU/gsolid; 72 h).

However, the most important result was that no visible changes were observed in the levels of final free Cel7A for EBR due to the laccase treatment, remaining at very low values as for the control case. To some extent, this result is a little unexpected since, from the action of laccase, some level of lignin degradation/removal is anticipated, which, in theory, would enable an increase of free Cel7A (that typically presents a high affinity towards lignin). The fact that such improvement was not observed in this case can indicate that the extent to which laccase could act towards lignin efficiently was limited by some factor, with rheological and/or mass transfer limitations being the most likely one. This limited action over lignin structures would explain the poor action of laccase at 3 IU/g_{solid} until they are removed on the washing step after 72 h of action, hence barely producing any improvements on the solid structure. On the other hand, it should also be considered that laccase action does not fully occur through deep lignin degradation but by slight modifications on these structures. Indeed, the variation in total phenolics (Fig. 4) seemed to indicate some lignin hydrolysis, but this does not automatically imply a significant change in the solid structure, which could also be related to the low laccase load (3 IU/g_{solid}) and rheological and/or mass transfer issues.

Arslan et al. (2016) reported that the overall adhesion forces of lignocellulosic substrates to the carbohydrate-binding module (CBM) of Cel7A were related to the surface lignin coverage, suggesting that changing the surface hydrophobicity and surface energy of lignin is a prerequisite to avoid the non-productive adsorption of cellulase onto lignin. However, laccase treatment appears to have had little effect on altering lignin structure, such as in reducing hydrophobic groups in lignin that can make the interaction force between lignin and cellulase weaker and thus improve the efficiency of cellulase hydrolysis (Siqueira et al., 2017).

In clear contrast with EBR, laccase treatment produced a much more visible change in PS, leading to a final free Cel7A increase from 15 % to 25 % when this treatment was performed. PS also presents some rheological issues under these solid loadings, which constitutes a significant difference in the apparent action that the laccase made over the solid. One possible reason for that could be an inferior lignin content on PS (14 %) or the fact that this solid was already highly processed; hence, laccase action can be more easily conducted. Another evidence of this superior action is the effects observed on hydrolysis efficiency, where laccase-pretreated PS had a 9.5 % lower glucose production at 48 h of hydrolysis and 6.5 % at 72 h (Fig. 6). Since no washing step was conducted, an extensive action towards lignin and the consequent production of relevant levels of inhibitors could explain a lower cellulase performance, as verified in other works (Michelin et al., 2023, 2016; Qin et al., 2016).

Still, an important point to stress out is that in this work, the same laccase treatment produced very distinct effects for the two solids: laccase treatment before enzymatic hydrolysis led to a 16 % increase in the glucose produced after 72 h for the case of EBR (Fig. 3), while a 6.5 % decrease in the case of PS was verified (Fig. 6). As mentioned before, this seems to suggest very distinct laccase mechanisms towards the solids, either by acting on different solid components and/or producing distinct inhibitors.

While some early reports on this subject presented laccase as an important component that may improve cellulases' action, our results indicated that their effects are highly specific to process design, with inhibitory effects being a critical aspect that should be addressed. Whereas an integrated approach to remove inhibitors would be ideal from a process perspective, namely by keeping high solid loadings, results above showed that laccase may be affected by similar mass transfer issues as cellulases, so particular adjustments should be performed to get the maximum benefits from these enzymes.

3.5. Laccase pretreatment through a dedicated step for improved efficiencies

Assuming that laccase action over a lignocellulosic solid will generate some inhibitors, using these enzymes to improve biomass hydrolysis will rely on developing efficient strategies to eliminate these compounds or reduce their effects. Provided that laccase treatment should be efficiently separated from cellulose hydrolysis, and as seen before, laccase can also face similar mass transfer limitations when operating under high solid loadings, increasing potential benefits from this class of enzymes may rely on operating this initial treatment under lower solid loadings. In this sense, a new laccase treatment was next assessed, consisting of two main differences: laccase loading was 10 IU/g_{solid}, and initial solids consistency during laccase pretreatment was only 3 % (w/v). Despite the evidence of inhibition effects for laccase levels above 5 IU/g_{solid}, given that a more efficient washing step will be employed, we did not foresee possible inhibition. On the other hand, operating the laccase treatment with a solids consistency as low as 3 % will drastically attenuate mass transfer problems, which most likely

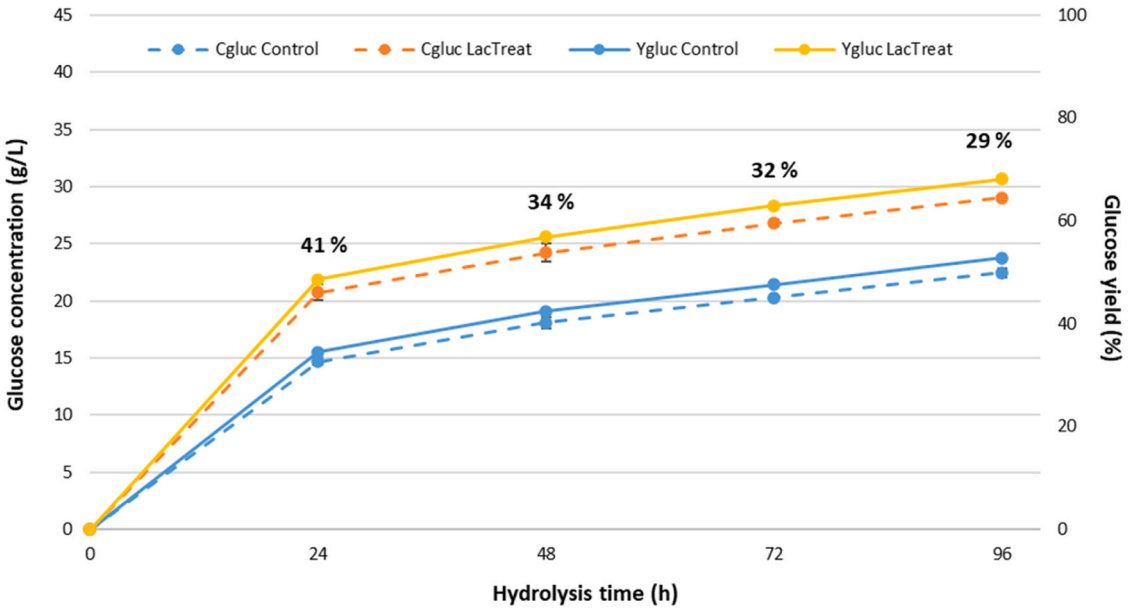


Fig. 7. Glucose production from the enzymatic hydrolysis of EBR (7 % (w/v) solids; 10 FPU/g_{solid}) with or without the combination of an initial laccases-mediated treatment (3 % solids; 10 IU/g_{solid}; 96 h) and a subsequent washing step.

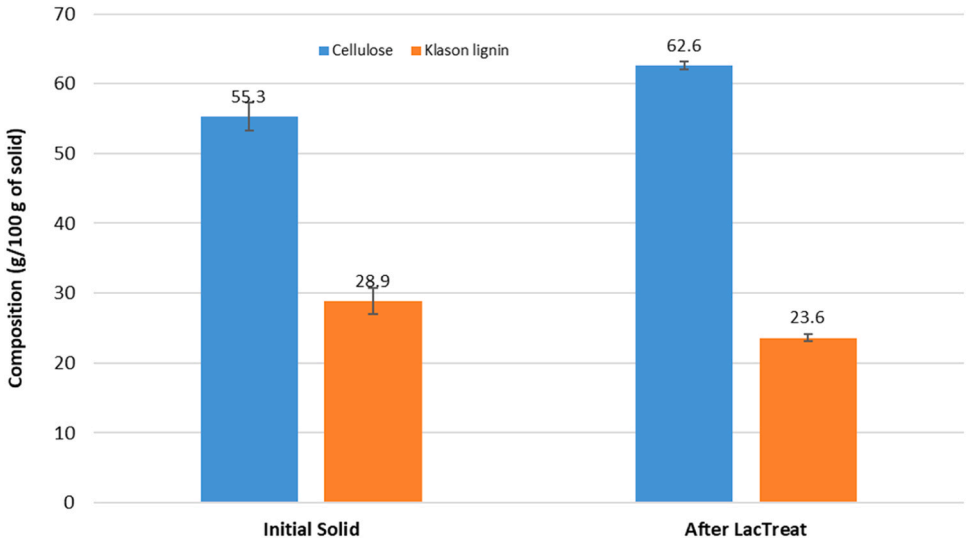


Fig. 8. Variation of cellulose and (Klason) lignin content on EBR after a laccases-mediated treatment (3 % solids; 10 IU/g_{solid}; 96 h).

were affecting laccase action for superior solid loadings. This new treatment was conducted for 96 h, after which the solid was washed by vacuum filtration (with approx. 5–7 volumes). While this washing method is undoubtedly more efficient than the previous one (buffer exchange of up to 1 working volume, under sterile conditions), its contribution should not be leveled to that of the new laccase's treatment. Hydrolysis efficiency and potential changes over the Cel7A adsorption profiles were assessed using the new laccase-pretreated solid.

From the analysis of Fig. 7, it is possible to observe that this new laccase treatment led to a much more visible improvement in the hydrolysis efficiency of EBR. Compared to the previous layout of this treatment, which showed hydrolysis improvements of 16 and 13 % for a hydrolysis time of 72 and 96 h, respectively (Fig. 3), in this case, enhancements of 32 and 29 % were observed, achieving almost 70 % of glucose yield; for 24 h of hydrolysis, this improvement inclusively reached 41 %. Another result that supports this superior action of laccase is that the fraction of free Cel7A after (96 h) hydrolysis increased to 11 % (*data not shown*), opposing to unaltered levels in the previous treatment.

Moilanen et al. reported that the laccase treatment of steam pretreated spruce increased the hydrolysis yield in 12 % (Moilanen et al., 2011) and 19 % (Moilanen et al., 2014) compared with the reference. Also, it was verified that 20 % more sugar yield was obtained with simultaneous or sequential treatment of laccase and cellulase in ammonia-pretreated sugarcane bagasse (Raj and Krishnan, 2018).

One final result also potentially confirming this improvement is the visible changes in the solid composition during laccase treatment alone (Fig. 8). Though detected to a rather moderate extent, it was possible to observe a visible reduction of (Klason) lignin content, which possibly contributed to a similarly moderate increase in cellulose content (i.e., concentration effect). This is corroborated by other works where LMT was used to remove lignin and improve enzymatic hydrolysis (Gutiérrez et al., 2012; Rico et al., 2014). In a work reported by Rico et al. (2014), the treatment with 10 U/g laccase and 1 % methylsyringate mediator

enhanced 39 % glucose yield. In this case, a washing step was performed after the laccase treatment to reduce any potential cellulase inhibition caused by lignin-degradation products.

4. Conclusions

This work showed that the positive impact of laccase-mediator treatment (LMT) on saccharification is influenced by multiple process-related factors, including enzyme addition timing, solid composition, and the removal of inhibitory compounds. When laccase was added simultaneously with cellulases, there was no improvement in the cellulose-to-glucose conversion. However, when laccase was added 24 h before cellulases, a beneficial effect was observed, likely due to reduced competition between laccase and LPMOs. The effectiveness of LMT varied depending on the type of solids, as indicated by the levels of free Cel7A. A potential limitation of LMT could be related to mass transfer issues at high solid load and limited changes in solid structure despite the laccase action, although the release of phenolics was observed. An essential step in the process was the removal of inhibitors through washing, which significantly improved hydrolysis efficiency (by 30 %), evidenced by increased levels of free cel7A and reduced Klason lignin content post-LMT. Thus, our findings suggest that the efficacy of LMT is highly dependent on process design, addressing inhibitory effects crucial for maximizing enzyme benefits.

CRediT authorship contribution statement

Michele Michelin: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Funding acquisition, Conceptualization. **José A. Teixeira:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Lucília Domingues:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Maria de Lourdes T.M. Polizeli:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Eduardo Coelho:** Writing – review & editing, Validation, Formal analysis. **José Carlos S. Salgado:** Methodology, Investigation. **Robson C. Alnoch:** Methodology, Investigation. **Emanuelle N. Freitas:** Methodology, Investigation. **Gomes Daniel:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the EcoTech project (POCI-01-0145-FEDER-032206), the strategic funding of UIDB/04469/2020 unit, and by LABBELS – Associate Laboratory in Biotechnology, Bioengineering and Microelectromechanical Systems, LA/P/0029/2020, and São Paulo Research Foundation (FAPESP), grants No: 2018/07522-6.

Data availability

Data will be made available on request.

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