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Short title: Exploring laccase and mediators for bioethanol production

Abstract

BACKGROUND: Laccases represent a very powerful tool to improve biorefining processes from lignocellulosic feedstocks. These enzymes are being investigated not only for potential use as pretreatment agents in bioethanol production, mainly as a delignifying agent, but also as a biotechnological tool for removal of inhibitors (mainly phenols) of subsequent fermentation processes.

RESULTS: In this work, the treatment of water insoluble solids (WIS) fraction from steam-exploded wheat straw with *Pycnoporus cinnabarinus* laccase and different laccase-mediator (LMS) systems did not decrease the lignin content, resulting in lower glucose recoveries during the subsequent saccharification. In combination with an alkaline extraction, the treatment with laccase/LMS produced no synergistic effect on enhancing the delignification or saccharification of WIS. In contrast, laccase reduced the soluble phenols (95% of the total phenols identified) of the whole slurry from steam-exploded wheat straw, improving the yeast performance during the fermentation and enhancing the ethanol yields.

CONCLUSIONS: The efficiency of *P. cinnabarinus* laccase with or without mediators as delignifying agent on steam-exploded wheat straw for bioethanol production was not observed, whereas its detoxification ability was showed. Thus, new laccases or designing laccases with ability to delignify and detoxify simultaneously needs be explored in order to produce major ethanol global yields.

Keywords: bioethanol; delignification; detoxification; laccase; mediator

INTRODUCTION

Biofuels provide one of the few options for the short-term substitution of fossil fuels in the transportation sector. In this sense, European policies have boosted the use of biofuels during last years. In particular, the Directive 2009/28/EC of the European Parliament establishes a minimum share of 10% of energy from renewable sources, including biofuels in the transportation sector of every Member State by 2020.[1] This target supports the European Union objective to promote the deployment of advanced biofuels. To meet this target, the progress towards the commercialisation of lignocellulosic bioethanol is fundamental. As opposed to starch or sugar-based biomass, lignocellulose is abundant, widely distributed, low-cost, and not used for food or animal feeding.

Lignocellulose is a highly recalcitrant complex matrix built up of cellulose, hemicelluloses and lignin. Lignocellulosic biomass can be converted to ethanol through different processes, being the biochemical route, where carbohydrates are enzymatically hydrolysed to fermentable sugars that are subsequently fermented into ethanol, the most favourable option.[2] This process however, is hindered by recalcitrant lignocellulose structure. Thus, a pretreatment step that precedes the saccharification stage is necessary to increase the digestibility of carbohydrates. A large number of pretreatment approaches, mainly physical and/or chemical, have been developed and optimized for a wide variety of feedstocks.[3] Among them, hydrothermal pretreatments, such as steam explosion,[3] are considered cost-effective methods for lignocellulosic bioethanol production.

Hydrothermal pretreatments promote hemicelluloses solubilisation together with lignin modification and redistribution, which outstandingly increase cellulose accessibility without the necessity of using any catalyst. However, pretreatment

technologies also involve some disadvantages, which need to be overcome. First, pretreated materials contain residual lignin, which limits the enzymatic hydrolysis of carbohydrates, promoting the non-specific adsorption of hydrolytic enzymes to pretreated lignocellulosic fibres.[4] On the other hand, the harsh conditions applied during pretreatment on biomass produces its further degradation. This degradation process generates several byproducts from cellulose and hemicelluloses (furan aldehydes and weak acids) and also from lignin (aromatic acids, alcohols and aldehydes) that inhibit cellulolytic enzymes and fermentative microorganisms.[5] Delignification step has been shown to be effective for reducing the non-specific adsorption of enzymes into lignin and a detoxification step decreases the amount of inhibitory compounds in pretreated materials, boosting saccharification and fermentation steps, respectively. The integration of delignification and detoxification methods is, therefore, beneficial for lignocellulosic bioethanol production.

Different physico/chemical methodologies have been successfully evaluated and applied either for delignification or detoxification of hydrothermally pretreated materials.[3,6] Nevertheless, most of these methods require extra equipment, have high energetic demand and represent new additional steps adding an extra-cost to the overall process. An alternative to physico/chemical methods lies in the use of ligninolytic enzymes such as laccases or laccase-mediator systems. Laccases are multicopper oxidases that catalyze one-electron oxidation of phenols, anilines and aromatic thiols to their corresponding radicals with the concomitant reduction of molecular oxygen to water.[7] The redox potential of laccases is low and it only allows the direct oxidation of phenolic lignin units, a small percentage (20-30%) of the lignin polymer. However, in the presence of low molecular weight compounds that act as redox mediators, laccases can also oxidize non-phenolic lignin units by forming stable radicals.[8]

Laccases and laccase-mediator systems have been extensively studied in paper pulp manufacture for removing the residual lignin responsible for pulp color.[8,9] Moreover, the oxidative capacity of laccases toward soluble phenols makes them an excellent candidate for the detoxification of industrial streams with high phenolic content.[10] Taking advantages from the vast experience gained by the investigation of laccases or laccase-mediator systems in the paper pulp industry, these ligninolytic enzymes can be applied for improving the efficiency of lignocellulosic ethanol production. The use of laccases in the lignocellulosic ethanol industry would provide a better integration of delignification and detoxification processes with less energy requirements (Figure 1).[11]

The present work evaluates the use of *Pycnoporus cinnabarinus* laccase for the delignification and detoxification of steam-exploded wheat straw with the aim of increasing the enzymatic saccharification and ethanol production from the pretreated material. For that purpose, the water insoluble solids (WIS) fraction without the main inhibitory compounds was used for delignification studies. These assays were performed in the presence or absence of different synthetic mediators (1-hydroxybenzotriazole (HBT), violuric acid (VIO) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)) in an attempt to increase the delignification capacity. On the other hand, the whole pretreated slurry, including the inhibitory compounds, was used for detoxification studies.

EXPERIMENTAL

Raw material and steam explosion pretreatment

Wheat straw, supplied by CEDER-CIEMAT (Soria, Spain), was used as raw material. It presented the following composition (% dry weight (DW)): cellulose, 40.5;

hemicelluloses, 26.1 (xylan, 22.7; arabinan 2.1; and galactan, 1.3); lignin, 18.1; ashes, 5.1; and extractives, 14.6.[12]

Prior to steam explosion, wheat straw was milled with a laboratory hammer mill to obtain a particle size between 2 and 10 mm. The milled material (200 g with 5-6% moisture content) was then subjected to steam-explosion pretreatment in a 10 L reactor at two conditions: 210 ºC, 2.5 min for delignification experiments and 220 ºC, 2.5 min for detoxification assays. For analytical purposes, a portion of both recovered slurries was vacuum-filtered to separate the respective liquid and solid fractions. Afterwards, the solid fractions were thoroughly washed with distilled water to obtain the WIS fraction. Chemical composition of raw and pretreated materials (WIS fraction) was determined using the Laboratory Analytical Procedures (LAP) for biomass analysis provided by the National Renewable Energies Laboratory.[13] Sugars and degradation compounds contained in the liquid fraction were also determined by high-performance liquid chromatography (HPLC) as described below in analytical methods. Most of sugars present in the liquid fraction were in oligomeric form; therefore a mild acid hydrolysis (4% (v/v) H_2SO_4, 120 ºC and 30 min) was required to determine monomeric sugars concentration.

The remaining pretreated materials (whole slurry) were handled differently depending on its further use. For delignification studies, the pretreated slurry obtained at 210 ºC, 2.5 min was vacuum-filtered and thoroughly washed to remove the majority of inhibitory compounds. On the other hand, due to its higher inhibitors content, the whole slurry obtained at 220 ºC, 2.5 min was used as substrate for detoxification purposes. Both WIS (210 ºC, 2.5 min) and slurry (220 ºC, 2.5 min) were stored at 4 ºC until use.

Enzymes and mediators

149 *P. cinnabarinus* laccase (60 IU mL^{-1} of laccase activity; 7-8 mg mL^{-1} of protein
150 content), from Beldem (Belgium), was used for delignification and detoxification
151 assays. Enzymatic activity was measured by oxidation of 5 mM 2,2′-azino-bis(3-
152 ethylbenzothiazoline-6-sulphonic acid) (ABTS) to its cation radical (ε_{436} = 29 300 M^{-1}
153 cm^{-1}) in 0.1 M sodium acetate (pH 5) at 24 °C. HBT and VIO, both from Sigma-Aldrich
154 (Steinheim, Germany), and ABTS from Roche, were assayed as laccase mediators in
155 delignification experiments.

156 A mixture of NS50013 (60 FPU mL^{-1} of cellulase activity; 140 mg mL^{-1} of protein
157 content) and NS50010 (810 IU mL^{-1} of β-glucosidase activity; 188 mg mL^{-1} of protein
158 content), both provided by Novozymes (Denmark), was employed for lignocellulose
159 saccharification. The overall cellulase activity was determined using filter paper
160 (Whatman No. 1 filter paper strips) and β-glucosidase activity was measured using
161 cellobiose. To detect any direct effect of laccase and/or laccase-mediator systems,
162 cellulase (NS50013) and β-glucosidase (NS50010) activities were determined in the
163 presence of 0.5 IU mL^{-1} of *P. cinnabarinus* laccase and 5.5 mM of the different
164 mediator compounds. The enzymatic activities were followed by the release of reducing
165 sugars.[14] One unit of enzyme activity was defined as the amount of enzyme that
166 transforms 1 μmol of substrate per minute.

167 Total protein content from all enzymatic preparations was analyzed by BCA protein
168 assay kit (Pierce Ref. 23225), using bovine serum albumin as standard.

169 **Microorganism and growth conditions**

170 *Saccharomyces cerevisiae* (Fermentis Ethanol Red, France), an industrial strain used in
171 the fuel alcohol industry, was employed in this study. Active culture for inoculation was
172 obtained in 100-mL shake flasks with 50 mL of growth medium containing 30 g L^{-1}

glucose, 5 g L^{-1} yeast extract, 2 g L^{-1} NH_4Cl, 1 g L^{-1} KH_2PO_4, and 0.3 g L^{-1} $MgSO_4 \cdot 7H_2O$. After 16 h on a rotary shaker at 150 rpm and 35 ºC, the precultures were centrifuged at 9000 rpm for 10 min. Supernatant was discarded and cells were washed once with distilled water and then diluted accordingly to obtain an inoculum level of 1 g L^{-1} (dry weight).

Delignification and saccharification experiments

The WIS fraction obtained after pretreatment at 210 ºC, 2.5 min was subjected to three different laccase delignification and saccharification strategies. Before adding laccase and mediators, 2.5 g DW of WIS was diluted with 50 mM sodium citrate buffer (pH 5) in 100-mL shake flasks to reach a final concentration of 5% DW (w/v).

Strategy 1, *consecutive laccase delignification and saccharification*: the diluted WIS fraction was treated with *P. cinnabarinus* laccase in the presence and absence of HBT, VIO and ABTS. A dosage of 10 IU g^{-1} DW of substrate of laccase was assayed together with 1.5% DW (w/w) of substrate of the corresponding mediator. Laccase and mediators dosages as well as treatment conditions were selected according to Ibarra *et al.*[15] Laccase or laccase-mediator treatments were carried out for 3 h at 50 ºC and 150 rpm in a rotatory shaker. Immediately after laccase treatment, 15 FPU g^{-1} DW of substrate of NS50013 and 15 IU g^{-1} DW of substrate of NS50010 were added to the different assays and trigger the enzymatic hydrolysis (enzyme loading added according to Tomás-Pejó *et al.*[16]). Afterwards, all flasks were further incubated for 72 h at the same conditions than for laccase treatment (50 ºC and 150 rpm).

Strategy 2, *separate laccase delignification and saccharification*: after 3 h of laccase or laccase-mediator treatment, carried out as explained above, all diluted WIS fractions were filtered through a Büchner funnel. Supernatants were discarded and the solid residues were washed with 1 L of water to eliminate laccase and mediators. The

198 remaining solid residue was diluted again with 50 mM sodium citrate buffer (pH 5) in
199 100-mL shake flasks to reach a final concentration of total solids of 5% (w/v). This
200 mixture was supplemented with the corresponding hydrolytic enzymes and a
201 saccharification process was performed as reported before.
202 Strategy 3*, separate laccase delignification and saccharification with an alkaline*
203 *extraction between them*: after 3 h of laccase or laccase-mediator treatment, the solid
204 residues were recovered as explained above and, sequentially, extracted with alkali
205 (1.5% (w/w) NaOH) for 1 h at 60 ºC.[15] After the extraction, the solid residues were
206 collected again by filtration and washed with water. Finally, the alkali extracted residues
207 were diluted with 50 mM sodium citrate buffer (pH 5) in 100-mL shake flasks to reach
208 a final concentration of total solids of 5% (w/v) and subjected to enzymatic hydrolysis
209 as explained above.
210 In all strategies, control assays were performed under the same conditions without
211 addition of laccase and mediators. All the experiments were carried out in triplicate and
212 the average and standard deviation values are shown.
213 The effects of laccase delignification strategies were evaluated according to both
214 chemical composition of the solid residue after treatment and sugar recovery after
215 enzymatic hydrolysis of treated substrates. The chemical composition of the solid
216 residues treated with laccase or laccase-mediator, subjected or not to alkaline extraction,
217 was determined using the Laboratory Analytical Procedures (LAP) for biomass analysis
218 provided by the National Renewable Energies Laboratory.[13] Samples from the
219 enzymatic hydrolysis with and without the alkaline extraction step, were centrifuged
220 and analyzed by HPLC to determine glucose concentration in supernatants. For better
221 comparison between assays, relative glucose recovery (RGR) was calculated as follow:
222

$$RGR(\%) = \frac{\frac{g}{L}glucose_{assay} \times 100}{\frac{g}{L}glucose_{control}}$$

Detoxification and fermentation experiments

The slurry obtained after pretreatment at 220 ºC, 2.5 min was subjected to laccase detoxification procedures. Before adding laccase, 2.5 g DW of the corresponding slurry was diluted with 50 mM sodium citrate buffer (pH 5) in 100-mL shake flasks to reach a final concentration of total solids of 7% (w/v). Then, a laccase dosage of 10 IU g^{-1} DW of substrate was added and flasks were incubated for 8 h at 50 ºC and 150 rpm in a rotatory shaker, according to its optimal conditions.[15] After laccase treatment, temperature was reduce to 35 ºC and slurries were subsequently subjected to a simultaneous saccharification and fermentation (SSF) process for 72 h in a rotatory shaker (150 rpm) with 15 FPU g^{-1} DW of substrate of NS50013, 15 IU g^{-1} DW of substrate of NS50010 and 1 g L^{-1} DW of *S. cerevisiae*. In addition, apart from glucose SSF experiments were supplemented with all the nutrients previously described.

Before SSF process, representative samples were withdrawn, centrifuged and the supernatants analyzed to evaluate the effect of laccase treatment on inhibitory compounds. During the SSF process, representative samples were withdrawn and processed to determine cell viability and glucose and ethanol concentrations.

Control assays were performed under the same conditions without addition of laccase. All the experiments were carried out in triplicate and the average and standard deviation values are shown.

Analytical methods

245 Ethanol was analyzed by gas chromatography, using a 7890A GC System (Agilent,
246 Waldbronn, Germany) equipped with an Agilent 7683B series injector, a flame
247 ionization detector and a column of Carbowax 20 M at 85 ºC. Injector and detector
248 temperature was maintained at 175 ºC.

249 Sugar concentration was quantified by HPLC in a Waters chromatograph equipped
250 with a refractive index detector (Waters, Mildford, MA). A CarboSep CHO-682
251 carbohydrate analysis column (Transgenomic, San Jose, CA) was employed for the
252 separation, operating at 80 ºC with ultrapure water (0.5 mL min^{-1}) as mobile-phase.

253 Furfural, 5-hydroxymethylfurfural (5-HMF), vanillin, syringaldehyde, *p*-coumaric
254 acid and ferulic acid were analyzed and quantified by HPLC in an Agilent
255 chromatograph (Agilent, Waldbronn, Germany) equipped with a 1050 photodiode-array
256 detector and a Coregel 87H3 column (Transgenomic, San Jose, CA), operating at 65 ºC
257 with 89% 5 mM H_2SO_4 and 11% acetonitrile (0.7 mL min^{-1}) as a mobile phase.

258 Formic acid and acetic acid were also quantified by HPLC (Waters, Mildford, MA)
259 using a 2414 refractive index detector (Waters, Mildford, MA) and a Bio-Rad Aminex
260 HPX-87H (Bio-Rad Labs) column maintained at 65 ºC with a mobile phase (5 mmol L^{-1}
261 H_2SO_4) at a flow rate of 0.6 mL min^{-1}.

262 The total phenolic content was analyzed according to a slightly modified version of
263 Folin-Ciocalteau method. 20 µL of sample and the serial standard solution were diluted
264 with 88 µL of water on a 96-well microplate. After the addition of 12 µL Folin-
265 Ciocalteu reagent, the plate was incubated for 5 min at room temperature in dark
266 conditions. The reaction was stopped with 80 µL of 7.5% sodium carbonate solution.
267 Before reading, the plate was incubated for 2 h at room temperature in the dark. The
268 absorbance was measured at 765 nm with a spectrophotometric microplate reader
269 (Anthos Zenyth 200rt, Biochrom, UK).[17]

Cell viability was measured by cell counting using agar plates (30 g L^{-1} glucose, 5 g L^{-1} yeast extract, 2 g L^{-1} NH_4Cl, 1 g L^{-1} KH_2PO_4, and 0.3 g L^{-1} $MgSO_4 \cdot 7H_2O$, 20 g L^{-1} agar) that were incubated at 35 ºC for 24 h.

Statistical analyses were performed using IBM SPSS Statistics v22.0 for MacOs X Software (SPSS, Inc., Chicago, IL, USA). The mean and standard deviation were calculated for descriptive statistics. When appropriate, analysis of variance (ANOVA) with or without Bonferroni's post-test was used for comparisons between assays. The level of significance was set at $P<0.05$, $P<0.01$ or $P<0.001$.

RESULTS AND DISCUSSION

Pretreated wheat straw composition

Steam explosion pretreatments were performed at two temperatures conditions (210 ºC and 220 ºC) to obtain pretreated materials with different compositions (Table 1). Compared to cellulose content of the untreated wheat straw (40.5%), both pretreatments increased cellulose (60.3% and 66.6% at 210 ºC and 220 ºC, respectively) and lignin proportion (30.0% at 210 ºC and 36.7% at 220 ºC), due to an extensive hemicelluloses solubilization and degradation. This hemicelluloses removal was more pronounced at 220 ºC, as reflected by the lower proportion of the remaining hemicelluloses (1.9%) in the WIS fraction compared to that at 210 ºC (6.6%). Moreover, significant biomass degradation was also seen at 220 ºC, obtaining lower sugars concentration in the liquid fraction together with different degradation products. Among them, acetic acid, formic acid, furfural and 5-HMF were predominant in both liquid fractions (Table 1). Acetic acid is formed by the hydrolysis of acetyl groups contained in the hemicelluloses structure. Formic acid derives from furfural and 5-HMF degradation, which in turn

295 results from pentoses (mainly xylose) and hexoses degradation, respectively.[5,18] Both
296 liquid fractions also showed low amounts of some phenols, such as ferulic and *p*-
297 coumaric acids, derived from *p*-hydroxycinnamic acids. These compounds are
298 characteristic of herbaceous plants, acting as linkages between lignin and
299 hemicelluloses.[19] Vanillin, derived from guaiacyl (G) propane lignin units, and
300 syringaldehyde, released from syringyl (S) propane lignin units were also found.

301 **Laccase delignification and saccharification experiments**

302 The residual lignin contained in the pretreated materials represents an important limiting
303 factor for lignocellulosic ethanol production processes. This polymer affects the
304 enzymatic hydrolysis of carbohydrates by decreasing the amount of hydrolytic enzymes
305 catalytically active. As enzyme cost is considered one of the major bottlenecks in the
306 scale-up and commercialization of lignocellulosic ethanol, the non-specific adsorption
307 and inactivation of enzymes are important factors to be controlled.[20] In this context, the
308 oxidative capacity of laccases makes them potential tools for the modification of lignin
309 or its partial removal from the pretreated biomass, thereby improving the
310 saccharification yields.

311 *Effect of laccase on chemical composition of treated samples*

312 In a first attempt to evaluate the different delignification strategies, the chemical
313 composition of the pretreated biomass (WIS) obtained after laccase treatments was
314 determined and compared with their respective controls (Table 2). Independently of the
315 strategy, the lignin content of the solid residues treated with laccase was slightly higher
316 in comparison to their respective controls. In the case of those pretreated materials that
317 were not subjected to an alkaline extraction, no relevant change in the lignin content, or
318 even a slight increment was observed. Lignin content increased from 30.0% DW (w/w)

in control assays to 31.4-32% DW (w/w) in laccase-treated assays (P<0.05). Similar results have previously been reported after laccase (*Cerrena unicolor*) treatment in the absence of mediators of steam-pretreated giant read (*Arundo donax*) and spruce (*Picea abies*), respectively.[21] Likewise, no substantial variation in the lignin content and composition was described in steam-exploded eucalypt treated with *Myceliophtora thermophila* laccase and HBT as mediator.[22,23] By contrast, *Pleurotus sp*. laccase without mediator was used by Mukhopadhyay *et al*.[24] to treat a milled material from a castor oil plant (*Ricinus communis*), obtaining an optimun delignification of 86%. Similar lignin loss (84-89%) was obtained using the same laccase and milled Indian thorny bamboo (*Bambusa bambos*) or Spanish flag.[25,26] These differences reported by the literature about laccase behavior on lignocellulosic materials are likely due to the combination of few factors, being the pretreatment one of the most relevant. When milling is used as pretreatment, the material shows little structural alteration of lignin,[3] and therefore the main action of laccase would be lignin oxidation. It leads to the formation of aromatic lignin radicals that give rise to a variety of reactions, such as ether and C–C bonds degradation, and aromatic ring cleavage (Figure 1), resulting in lignin degradation and finally delignification of lignocellulose. By contrast, during steam explosion pretreatment there is high lignin degradation and consequently high generation of soluble phenolic compounds.[3] Thus, in steam-exploded material, lignin oxidation by laccase would coexist with radical coupling reactions (Figure 1). In this case, soluble phenols from lignin degradation are oxidized by laccase to phenoxy radicals, which are covalently coupled to the aromatic lignin radicals of the fibers, and consequently increasing the lignin content. This effect, known as grafting process, has been widely described in chemical pulps treated with laccase and different lignin-derived phenols as mediators.[27,28] Recently, Oliva-Taravilla *et al*.[29] has reported for the

first time the grafting process of *p*-hydroxycinnamic acids on steam-exploded wheat straw after treatment with *P. cinnabarinus* laccase. In spite of washed WIS fraction without the main phenolic compounds was used in this work for laccase delignification experiments, it was shown that phenols were released when WIS fraction was diluted into the laccase buffer (0.75 g L^{-1} total phenolic compounds). This effect was also reportet by Alvira *et al*.[17] who observed the release of inhibitory compounds trapped within the washed WIS fraction when it was diluted into the saccharification buffer. Thus, these new phenols released to the media could be substrate for laccase enzyme, tiggering the grafting process, and consequently the increment of Klason lignin observed herein.

The potential of alkaline extraction to remove lignin from lignocellulosic biomass is well known.[30–32] Likewise, a synergistic effect on lignin removal between laccase treatment, with and without mediators, and alkaline extraction has already been described with successful results.[33] In our study, alkali treatment caused 32% delignification (P<0.001) of steam-exploded wheat straw (Table 2). However, the laccase treatment, in the presence and absence of the different mediators, followed by alkaline extraction did not improve the delignification range produced by the alkaline extraction itself. Actually, a slight increment (P<0.01) of the lignin content was observed (from 20.5% DW (w/w) to 22.3-23.9% DW (w/w)) when comparing control assays with laccase-treated assays (Table 2), as described above for the samples that were not extracted with alkali.

Saccharification of laccase treated samples

The final RGRs after 72 h of saccharification from the different laccase-treated assays without alkali extraction are shown in Figure 2 . In the case of consecutive laccase delignification and saccharification (strategy 1; Figure 2A), RGR of laccase-treated

assay without mediators was decreased by almost 6-7% (P<0.05) compared to control probably due to the slight increase in lignin content or the generation of reactive phenoxy radicals. The effect of laccases on enzymatic hydrolysis has not been elucidated yet and contradictory results have been reported in this matter. Tabka *et al*.[34] and Jurado *et al*.[35] described lower glucose concentration after enzymatic hydrolysis of steam-exploded wheat straw treated with different laccases from *P. cinnabarinus*, *Trametes villosa* and *Coriolopsis rigida*. This phenomenon was attributed to the formation of laccase-derived compounds that inhibit cellulolytic enzymes. Moilanen *et al*.[21] also reported a hydrolysis decrease by laccase (*C. unicolor*) treatment of steam-pretreated giant read. In this case, the lower glucose production was explained by an increment of the non-specific adsorption of hydrolytic enzymes onto the lignocellulosic fibers and a major strengthening of lignin-carbohydrate complexes. Nevertheless, the same study reported that laccase treatment of steam-exploded spruce decreased the non-specific adsorption of cellulases to lignin and consequently the glucose hydrolysis yields were improved. Finally, Oliva-Taravilla *et al*.[29] recently suggested the increase in the competition of cellulose binding sites between cellulases and laccase together with the inhibition of β-glucosidase activity as the main reasons for RGR reduction in enzymatic hydrolysis of model cellulosic substrate (Sigmacell).

The negative effect of laccase on the saccharification was more pronounced when the different mediators were used (Figure 2A), with a 30% reduction (P<0.001) in final RGRs. To evaluate this marked reduction, a possible direct interaction between hydrolytic enzymes and the different mediators was studied. For it, cellulase (NS50013) and β-glucosidase (NS50010) activities were measured in the presence of laccase and mediators. In the case of cellulase activity, a decrease of about 34% was observed in the presence of the different mediators. The enzymatic deactivation was even more

394 remarkable in the case of β-glucosidase activity, showing a reduction of about 50%.
395 Palonen and Viikari previously reported this inhibitory effect on hydrolytic enzymes by
396 oxidized mediators.[36] They found that the mediator N-hydroxy-N-phenylacetamide
397 (NHA) oxidized by *Trametes hirsuta* laccase decreases notably the activity of
398 Celluclast, a cellulase preparation with low β-glucosidase activity.
399 When introducing the filtration and washing steps between laccase delignification
400 and saccharification (strategy 2, Figure 2B), a lower RGR (6%; $P<0.05$) compared to
401 control was also observed. However, the inhibitory effect of the different mediators was
402 markedly prevented, although the RGRs after 72 h of saccharification remained lower
403 (2-11%) than control. Besides the reasons mentioned above, Oliva-Taravilla *et al*.[29] also
404 suggested the grafting process described previously as other possible negative effect on
405 enzymatic hydrolysis. Thus, the incorporation of some phenols onto the fibers by
406 laccase, responsible of the slight increase in lignin content, might limit the accessibility
407 of enzymes to cellulose, either by reducing the number and/or the size of pores or
408 hindering the progress of cellulases. Moreover, the grafting process could also lead to
409 an increase of the lignin surface area, thereby limiting the accessibility of hydrolytic
410 enzymes to cellulose.
411 An alkaline extraction between the sequential laccase delignification and
412 saccharification (strategy 3; Figure 3) did not improve the glucose recovery. In this set
413 of assays, saccharification of samples subjected to alkaline extraction without laccase
414 treatment produced higher RGRs (31%; $P<0.001$) than samples not subjected to alkaline
415 treatment. This enhancement of saccharification by alkaline extraction is a phenomenon
416 widely described.[30–32] The generation of some irregular pores, resulted from the removal
417 of lignin and the breaking of lignocellulose complex during the alkaline treatment
418 contributes to increase the enzyme accessibility of alkaline extracted samples. The

positive effect of laccase (*T. villosa*) treatment, with and without mediators (HBT), and alkaline extraction reported in milled material from Elephant grass (*Pennisetum purpureum*) and eucalypt,[33] was not observed herein. In our case, the RGRs after saccharification of all laccase treated samples with alkaline extraction was slightly lower (4-12%; $P<0.01$) than control.

Laccase detoxification and fermentation experiments

Inhibitors profile of laccase treated samples

The presence of inhibitory compounds released during steam-explosion pretreatment in the whole slurry can negatively affect the sugar conversion into ethanol by fermenting microorganisms, hindering its use for bioethanol production. Usually, the whole slurry is filtered and washed. However, from an economical and environmental point of view, the filtration and washing steps should be avoided because they increase both operational costs and wastewater.[37] In this context, the oxidative capacity of laccases makes them suitable tools as green detoxification agent for the partial removal of phenols from lignin degradation during steam explosion pretreatment, thereby improving the fermentation yields.

In this study, the whole slurry obtained after pretreatment of wheat straw at 220 ºC, 2.5 min was subjected to laccase detoxification followed by a subsequent simultaneous saccharification and fermentation (SSF) process. No synthetic mediators were added during detoxification assays due to their direct interaction with the hydrolytic enzymes.

The identification and quantification of inhibitory compounds of control and laccase-treated samples is shown in Table 3. These compounds can alter the growth of the fermenting microorganisms and inhibit cellulolytic enzymes, decreasing final yields and productivities.[5,38,39] Both formic and acetic acids reduce biomass formation by modifying the intracelular pH that promotes an imbalance in the ATP/ADP ratio.[5] 5-

444 HMF and furfural have a direct inhibition effect on either the glycolytic or fermentative
445 enzymes of the yeast, reducing equally biomass formation and ethanol yields.[5] Finally,
446 phenols also affect biological membranes, thus decreasing growth rates and also
447 inhibiting or deactivating hydrolytic enzmes.[38,39]

448 As shown in table 3, laccase produced a remarkable reduction in the measured
449 phenols (95% of the total phenols identified). This reduction can be produced in several
450 ways. As shown in Figure 1, the phenoxy radicals generated by laccase can polymerise
451 to yield less toxic oligomers or can undergo grafting reactions onto pretreated material
452 (via radical coupling to lignin polymer).[11,27,28] These reaction mechanisms are
453 determined by the structure of the different phenolic compounds. Phenols with two
454 methoxy groups in *ortho*-ring positions, such as syringaldehyde, are more reactive and
455 show the highest tendency to undergo polymerization. While, phenols without any
456 methoxy groups in *ortho*-ring positions (*p*-coumaric acid) are the best to graft onto the
457 fibres. Finally, phenols with one methoxy groups in *ortho*-ring position (vanillin and
458 ferulic acid) show an intermediate behavior.[28] In addition, the structure of the different
459 phenols also establishes their conversion rates by laccase.[40] Thus, syringaldehyde or *p*-
460 hydroxycinnamic acids are quickly converted, whilst vanillin is oxidized with lower
461 rates, showing a remaining content after 8 h of laccase treatment (Table 3).

462 Formic acid, acetic acid, 5-HMF and furfural were not much alterated by laccase
463 treatment (Table 3). The absence of laccase action on weak acids and furan derivatives
464 has been already reported in previous studies .[41–44] This substrate-specific reaction of
465 laccases towards phenols offers some advantages over chemical and physical
466 detoxification methods, such as mild reactions conditions, fewer toxic sub-products and
467 low energy requirements.[45]

468 *Fermentation of laccase treated samples*

469 Ethanol production with *S. cerevisiae* was followed on the slurry subjected to laccase
470 treatment. As can be seen in Figures 4 and 5, the lag phase of *S. cerevisiae* was
471 extended up to 48 h in the control assay. This long lag phase was due to adaptation of
472 the yeast to fermentation conditions, which depends of the inhibitory compounds type,
473 their concentrations, and the synergistic effects between them.[5] The assimilation of the
474 different inhibitory compounds by yeast, mainly the conversion of 5-HMF, furfural and
475 aromatic aldehydes (vanillin and syringaldehyde) to their less inhibitory forms,
476 determines to a great extent the lag phase time.[5] After the lag phase, *S. cerevisiae*
477 showed a gradual increment in cell viability between 48 h and 72 h of SSF (Figure 4). It
478 was accompanied with complete glucose consumption at 72 h of SSF (Figure 5),
479 attaining an ethanol concentration of 11.6 g L^{-1} (Table 4).
480 The specific phenols removal by laccase improved the cell growth and ethanol
481 production of *S. cerevisiae*. Compared to control assay, laccase treatment shortened the
482 yeast lag phase from 48 h to 6 h (Figures 4 and 5). Cell viability was significantly
483 improved, showing the highest increment of the number of colony forming units
484 between 6 h and 24 h of SSF (Figures 4). In addition, very low glucose accumulation in
485 the early stages of SSF was observed, resulting in faster ethanol production rates with
486 an ethanol concentration of 13.2 g L^{-1} at 32 h of SSF (Figure 5). Ethanol volumetric
487 productivity incremented 4-fold after laccase treatment from 0.11 g L^{-1} h to 0.42 g L^{-1} h
488 (P<0.001) (Table 4). Laccase also enhanced about 20% the ethanol yield from 0.32 g g^{-1}
489 to 0.38 g g^{-1} (P<0.05) (Table 4). Larsson *et al*.[42] reported higher yeast growth together
490 with higher glucose consumption rate, ethanol productivity and ethanol yield when
491 liquid fraction from steam-exploded spruce was subjected to *Trametes versicolor*
492 laccase and fermented with *S. cerevisiae*. In the same way, Moreno *et al*.[46] described
493 higher cell viability and shorter lag phases when steam-exploded wheat straw was

494 treated with *P. cinnabarinus* laccase and fermented with the xylose-consuming *S.*
495 *cerevisiae* F12. Finally, Jurado *et al*.[35] described higher influence on ethanol
496 concentration than yeast growth when enzymatic hydrolyzates from both acid and non
497 acid steam-exploded wheat straw were subjected to laccases from *T. versicolor* or *C.*
498 *rigida* and fermented with *S. cerevisiae*.
499 An important aspect for ethanol production is the substrate loading. By increasing
500 substrate loading during ethanol production process higher final product concentrations
501 will be obtained. This approach could reduce operational cost for hydrolysis and
502 fermentation process and minimize energy consumption during subsequent distillation
503 and evaporation stages, making lignocellulosic ethanol production economically
504 feasible. Then, higher substrate loadings than those assayed in this study for
505 delignification (5% DW (w/v)) and detoxification (7% DW (w/v)) experiments are
506 being studied.
507
508 **CONCLUSIONS**
509 On the basis of the observations from this study, the use of *P. cinnabarinus* laccase with
510 or without mediators on steam-exploded wheat straw leads to polymerization and
511 grafting reactions instead of lignin degradation and delignification. Lignin-derived
512 phenols resulting from steam explosion of wheat straw are oxidized by laccase to
513 phenoxy radicals, which polymerise between them or are grafted to the aromatic lignin
514 radicals, the latter effect explaining the lignin content increment observed and the lower
515 glucose recoveries during the saccharification. In contrast, this effect results in a
516 reduction of soluble phenols content, and consequently the detoxification of steam-
517 exploded material, improving the yeast performance during the fermentation and
518 enhancing the ethanol yields. Nevertheless, major ethanol global yields will be obtained

519 if the laccase effect is also extending to lignin degradation and delignification. These
520 major final ethanol concentrations will reduce the distillation and evaporation costs,
521 which could offset the relative cost of using laccases. In addition, filtration and washing
522 steps of steam-exploded materials could be avoided, reducing operational costs and
523 wastewater.

524

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528

529 **REFERENCES**

530 1 Directive 2009/28/EC, Directive of the European parliament and the council of 23 April 2009
531 on the promotion of the use of energy from renewable sources, in *Official Journal of*
532 *European Union* (2009).

533 2 Ballesteros M, Enzymatic hydrolysis of lignocellulosic biomass, in *Bioalcohol production.*
534 *Biochemical conversion of lignocellulosic biomass*, ed. by Waldron K. Woodhead
535 Publishing, UK, 159−177 (2010).

536 3 Alvira P, Tomás-Pejó E, Ballesteros M and Negro MJ, Pretreatment technologies for an
537 efficient bioethanol production process based on enzymatic hydrolysis: a review.
538 *Bioresource Technol* **101**:4851−4861 (2010).

539 4 Berlin A, Balakshin M, Gilkes N, Kadla J, Maximenko V and Kubo S, Inhibition of cellulase,
540 xylanase and β-glucosidase activities by sofwood lignin preparations. *J Biotechnol*
541 **125**:198−209 (2006).

542 5 Palmqvist E and Hahn-Hägerdal B, Fermentation of lignocellulosic hydrolysates. II: inhibitors
543 and mechanism of inhibition. *Bioresource Technol* **74**:25–33 (2000a).

544 6 Palmqvist E and Hahn-Hägerdal B, Fermentation of lignocellulosic hydrolysates. I: inhibition
545 and detoxification. *Bioresource Technol* **74**:17–24 (2000b).

546 7 Thurston CF, The structure and function of fungal laccases. *Microbiol* **140**:19–26 (1994).

547 8 Paice MG, Bourbonnais R, Reid ID, Archibald FS and Jurasek L, Oxidative bleaching
548 enzymes: a review. *J Pulp Paper Sci* **21**:280–284 (1995).

549 9 Bajpai P, Biological bleaching of chemical pulps. *Crit Rev Biotechnol* **24**:1−58 (2004).

550 10 Milstein O, Haars A, Majcherczyk A, Tautz D, Zanker H and Hüttermann A, Removal of
551 chlorophenols and chlorolignins from bleaching effluents by combined chemical and
552 biological treatment. *Water Sci Technol* **20**:161−170 (1988).

553 11 Kudanga T and Le Roes Hill M, Laccase applications in biofuels production: current status
554 and future prospects. *Appl Microbiol Biotechnol* **98**:6525–6542 (2014).

555 12 Alvira P, Negro MJ and Ballesteros M, Effect of endoxylanase and α-L-arabinofuranosidase
556 supplementation on the enzymatic hydrolysis of steam exploded wheat straw. *Bioresource*
557 *Technol* **102**:4552–4558 (2011).

558 13 NREL, Chemical Analysis and Testing Laboratory Analytical Procedures. National
559 Renewable Energy Laboratory, Golden, CO (2007).
560 www.1.ere.energy.gov/biomass/analytical_procedures.html.

561 14 Ghose TK, Measurement of cellulase activity. *Pure Appl Chem* **59**:257–68 (1987).

562 15 Ibarra D, Romero J, Martínez MJ, Martínez AT and Camarero S, Exploring the enzymatic
563 parameters for optimal delignification of eucalypt pulp by laccase-mediator. *Enzyme*
564 *Microbiol Technol* **39**:1319−1327 (2006).

16 Tomás-Pejó E, García-Aparicio M, Negro MJ, Oliva JM and Ballesteros M, Effect of different cellulase dosages on cell viability and ethanol production Kluyveromyces marxianus in SSF processes. *Bioresource Technol* **100**:890–895 (2009).

17 Alvira P, Moreno Antonio D, Ibarra D, Saéz F and Ballesteros M, Improving the fermentation performance of Saccharomyces cerevisiae by laccase during ethanol production from steam-exploded wheat straw at high-substrate loadings. *Biotechnol Prog* **29**:74–82 (2013).

18 Oliva JM, Saez F, Ballesteros I, González A, Negro MJ, Manzanares P and Ballesteros M, Effect of lignocellulosic degradation compounds from steam explosion pretreatment on ethanol fermentation by thermotolerant yeast *Kluyveromyces marxianus*. *Appl Biochem Biotechnol* **105**:141−154 (2003).

19 Buranov AU and Mazza G, Lignin in straw of herbaceous crops. *Ind Crops Prod* **28**:237–259 (2008).

20 Alvira P, Ballesteros M and Negro MJ, Progress on enzymatic saccharification technologies for biofuels production, in *Biofuels Technologies*, ed. by Gupta VK and Tuohy MG. Springer-Verlag Berlin Heiderberg, pp 145−169 (2013).

21 Moilanen U, Kellock M, Galkin S and Viikari L, The laccase-catalyzed modification of lignin for enzymatic hydrolysis. *Enzyme Microbiol Technol* **49**:492−498 (2011).

22 Martín-Sampedro R, Capanema EA, Hoeger I, Villar JC and Rojas OJ, Lignin changes after steam explosion and laccase-mediator treatment of eucalytpus wood chips. *J Agric Food Chem* **59**:8761–8769 (2011).

23 Martín-Sampedro R, Eugenio ME, García JC, López F, Villar JC and Díaz MJ, Steam explosion and enzymatic pre-treatments as an approach to improve the enzymatic hydrolysis of *Eucalytpus globulus*. *Biomass Bioen* **42**:97–106 (2012).

589 24 Mukhopadhyay M, Kuila A, Tuli DK and Banerjee R, Enzymatic depolymerization of
590 *Ricinus communis*, a potential lignocellulosic for improved saccharification. *Biomass*
591 *Bioen* **35**:3584−3591 (2011).

592 25 Kuila A, Mukhopadhyay M, Tuli DK and Banerjee R, Accessibility of enzymatically
593 delignified Bambusa bambos for efficient hydrolysis at minimun cellulase loading: an
594 optimization study. *Enzyme Res* DOI:10.4061/2011/805795 (2011).

595 26 Kuila A, Mukhopadhyay M, Tuli DK and Banerjee R, Production of ethanol from
596 lignocellulosics: an enzymatic venture. *EXCLI J* **10**:85−96 (2011).

597 27 Aracri E, Fillat A, Colom JF, Gutiérrez A, del Río JC, Martínez AT and Vidal T, Enzymatic
598 grafting of simple phenols on flax and sisal pulp fibres using laccases. *Bioresource*
599 *Technol* **101**:8211−8216 (2010).

600 28 Barneto AG, Aracri E, Andreu G and Vidal T, Investigating the structure-effect relationships
601 of various natural phenols used as laccase mediators in the biobleaching of kenaf and sisal
602 pulps. *Bioresource Technol* **112**:327−335 (2012).

603 29 Oliva-Taravilla A, Moreno AD, Demuez M, Ibarra D, Tomás-Pejó E, González-Fernández C
604 and Ballesteros M, Unraveling the effects of laccase treatment on enzymatic hydrolysis of
605 steam-exploded wheat straw. *Bioresource Technol* **175**:209–215 (2015).

606 30 Yang P, Jiang S, Zheng Z, Shuizhong L, Luo S and Pan L, Effect of alkali and laccase
607 pretreatment of Brassica campestris straw: architecture, crystallisation, and
608 saccharification. *Polymers Renew Resour* **2**:21−34 (2011).

609 31 Li J, Sun F, Li X, Yan Z, Yuan Y and Liu XF, Enhanced saccharification of corn straw
610 pretreated by alkali combining crude ligninolytic enzymes. *J Chem Technol Biotecnhol*
611 **87**:1687−1693 (2012).

612 32 Asgher M, Ahmad Z and Iqbal HMN, Alkali and enzymatic delignification of sugarcane
613 bagasse to expose cellulose polymers for saccharification and bioethanol production. *Ind*
614 *Crops Prod* **44**:488−495 (2013).

615 33 Gutiérrez A, Rencoret J, Cadena EM, Rico A, Barth D, del Río JC and Martínez AT,
616 Demostration of laccase-based removal of lignin from wood and non-wood plant
617 feedstocks. *Bioresource Technol* **119**:114−122 (2012).

618 34 Tabka MG, Herpoel-Gimbert I, Monod F, Asther M and Sigoillot JC, Enzymatic
619 saccharification of wheat straw for bioethanol production by a combined cellulase,
620 xylanase and feruloyl esterase treatment. *Enzyme Microbiol Technol* **39**:897−902 (2006).

621 35 Jurado M, Prieto A, Martínez-Alcalá A, Martínez AT and Martínez MJ, Laccase
622 detoxification of steam-exploded wheat straw for second generation bioethanol.
623 *Bioresource Technol* **100**:6378−6384 (2009).

624 36 Palonen H and Viikari L, Role of oxidative enzymatic treatments on enzymatic hydrolysis of
625 softwood. *Biotechnol Bioeng* **86**:550–557 (2004).

626 37 García-Aparicio MP, Ballesteros I, González A, Oliva JM, Ballesteros M and Negro MJ,
627 Effect of inhibitors released during steam-explosion pretreatment of barley straw on
628 enzymatic hydrolysis. *Appl Biochem Biotechnol* **129**:278−288 (2006).

629 38 Ximenes E, Kim Y, Mosier N, Dien B and Ladisch M, Inhibition of cellulases by phenols.
630 *Enzyme Microbiol Technol* **46**:170−176 (2010).

631 39 Ximenes E, Kim Y, Mosier N, Dien B and Ladisch M, Deactivation of cellulases by phenols.
632 *Enzyme Microbiol Technol* **48**:54−60 (2011).

633 40 Kolb M, Sieber V, Amann M, Faulstich M and Schieder M, Removal of monomer
634 delignification products by laccase from *Trametes versicolor*. *Bioresource Technol*
635 **104**:298−304 (2012).

636 41 Chandel AK, Kapoor RK, Singh A and Kuhad RC, Detoxification of sugarcane bagasse
637 hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. *Bioresource*
638 *Technol* **98**:1947−1950 (2007).

639 42 Larsson S, Reimann A, Nilvebrant N.-O and Jönsson JL, Comparison of different methods
640 for the detoxification of lignocellulose hydrolyzates of spruce. *Appl Microbiol Biotechnol*
641 **77−79**:91−103 (1999).

642 43 Martín C, Galbe M, Wahlbom CF, Hahn-Hägerdal B and Jönsson JL, Ethanol production
643 from enzymatic hydrolysates of sugarcane bagasse using recombinant xylose-utilising
644 Saccharomyces cerevisae. *Enzyme Microbiol Technol* **31**:274−282 (2002).

645 44 Moreno AD, Ibarra D, Fernández JL and Ballesteros M, Different laccase detoxification
646 strategies for ethanol production from lignocellulosic biomass by the thermotolerant yeast
647 Kluyveromyces marxianus CECT 10875. *Bioresource Technol* **106**:101−109 (2012).

648 45 Parawira W and Tekere M, Biotechnological strategies to overcome inhibitors in
649 lignocellulose hydrolysates for ethanol production: review. *Critical Reviews Biotechnol*
650 **31**:20–31 (2011).

651 46 Moreno AD, Tomás-Pejó E, Ibarra D, Ballesteros M and Olsson L, In situ laccase treatment
652 enhances the fermentability of steam-exploded wheat straw in SSCF processes at high dry
653 matter consistencias. *Bioresource Technol* **143**:337−343 (2013).

654 47 Zhang J and Bao J, A modified method for calculating practical ethanol yield at high
655 lignocellulosic solids content and high ethanol titer. *Bioresource Technol* **116**:74–79
656 (2012).
657

Table captions

Table 1. Composition of steam-exploded wheat straw at 210 ºC, 2.5 min and 220 ºC, 2.5 min.

Table 2. Composition of WIS samples treated with laccase in the presence and abscence of mediators and with or without a subsequent alkaline extraction.

Table 3. Inhibitory compounds concentration (mg mL^{-1}) of slurry samples resulting from control and laccase treatments.

Table 4. Summary of simultaneous saccharification and fermentation (SSF) assay of slurry samples resulting from control and laccase treatments.

Figure captions

Figure 1. Role of laccase and mediators in bioethanol production from steam-exploded lignocellulose. Different detoxification (1 and 2) and delignification (3, 4 and 5) reactions are indicated. C, cellulose; H, hemicelluloses; L, lignin (adapted from Kudanga and Le Roes Hill[11]).

Figure 2. Relative glucose recovery (RGR) at 72 h of saccharification. Samples resulting from the different laccase delignification and saccharification strategies. A) strategy 1, consecutive laccase delignification and saccharification; B) strategy 2, separate laccase delignification and saccharification. Discontinuous line represents RGR values (100%) in control assays (without laccase). L, laccase samples; L-HBT, laccase-hydroxybenzotriazole system samples; L-VIO, laccase-violuric system samples; L-ABTS, laccase-(2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) system samples. Glucose concentration values after 72 h of saccharification of control samples were 29.8 g L^{-1} and 30 g L^{-1} for strategies 1 and 2, respectively. Mean values and standard deviations were calculated from the triplicates. Analysis of variance (ANOVA) with Bonferroni's post-test was performed to identify differences between control and laccase or laccase-mediator systems assays. The mean different is significant at the (*) 0.05 or (***) 0.001 level.

Figure 3. Relative glucose recovery (RGR) at 72 h of saccharification. Samples resulting from strategy 3, separate laccase delignification and saccharification with an alkaline extraction between them. Discontinuous line represents RGR values (100%) in control assay (without laccase and alkaline treatment). Alk, alkaline extraction; L, laccase samples; L-HBT, laccase-hydroxybenzotriazole system samples; L-VIO,

695 laccase-violuric system samples; L-ABTS, laccase-(2,2-azinobis(3-
696 ethylbenzothiazoline-6-sulfonic acid)) system samples. Glucose concentration value
697 after 72 h of saccharification of control sample was 29.9 g L^{-1} . Mean values and
698 standard deviations were calculated from the triplicates. Analysis of variance (ANOVA)
699 with Bonferroni's post-test was performed to identify differences between control and
700 laccase or laccase-mediator systems assays. The mean different is significant at the (**)
701 0.01 or (***) 0.001 level.
702
703 **Figure 4**. Viable cells during SSF assay of slurry. Samples resulting from control and
704 laccase treatments. Symbols used: control (■) and laccase (▲) samples. Mean values
705 and standard deviations were calculated from the triplicates. A two-way analysis of
706 variance (ANOVA) with Bonferroni's post-test was performed to identify differences
707 between non-supplemented and laccase-supplemented assays at different time points.
708 The mean difference is significant at the (*) 0.05, (**) 0.01 or (***) 0.001 level.
709
710 **Figure 5**. Time course for ethanol (filled symbols) and glucose (open symbols) during
711 SSF assay of slurry. Symbols used: control (■, □) and laccase (▲, △) samples.
712 Mean values and standard deviations were calculated from the triplicates. A two-way
713 analysis of variance (ANOVA) with Bonferroni's post-test was performed to identify
714 differences between non-supplemented and laccase-supplemented assays at different
715 time points. The mean difference is significant at the (**) 0.01 level within 6-12 h and
716 (***) 0.001 level within 12-48 h.
717

718 **Table 1.**

WIS composition (% dry weight, w/w)	**210 ºC 2.5 min**	**220 ºC 2.5 min**
Cellulose	60.3 ± 0.5	66.6 ± 0.8
Hemicellulose	6.6 ± 0.0	1.9 ± 0.2
Lignin	30.0 ± 0.3	36.7 ± 0.5
Prehydrolysate composition (g L^{-1})	**210 ºC 2.5 min**	**220 ºC 2.5 min**
Monosaccharides		
Glucose	4.7 ± 0.3	4.5 ± 0.4
Xylose	26.2 ± 0.6	12.0 ± 0.5
Arabinose	2.1 ± 0.1	0.5 ± 0.1
Galactose	1.9 ± 0.1	0.5 ± 0.0
Mannose	nq	0.5 ± 0.0
Degradation Products		
Formic acid	7.8 ± 0.2	9.3 ± 0.1
Acetic acid	5.6 ± 0.1	11.9 ± 0.2
5-HMF	0.3 ± 0.1	1.1 ± 0.0
Furfural	0.7 ± 0.0	3.5 ± 0.1
Vanillin	0.04 ± 0.00	0.05 ± 0.00
Syringaldehyde	0.02 ± 0.00	0.03 ± 0.00
p-Coumaric acid	0.02 ± 0.01	0.02 ± 0.01
Ferulic acid	nq	0.03 ± 0.01

[nq] not quantified

719

720

721 **Table 2.**

	Composition (% dry weight, w/w)[a]	C	L	L-HBT	L-VIO	L-ABTS
NA	Cellulose	60.3 ± 0.5	59.6 ± 0.9	59.4 ± 0.3	56.6 ± 0.8**	57.6 ± 0.2*
	Hemicellulose	6.6 ± 0.0	6.7 ± 0.1	6.5 ± 0.0	6.5 ± 0.0	5.9 ± 0.0***
	Lignin	30.0 ± 0.3	31.4 ± 0.3*	31.7 ± 0.5**	31.4 ± 0.4*	32.0 ± 0.0**
A	Cellulose	68.7 ± 0.7	66.6 ± 0.0*	66.2 ± 0.5*	68.2 ± 0.4	65.6 ± 1.0**
	Hemicellulose	7.8 ± 0.0	7.3 ± 0.2	7.3 ± 0.0	7.6 ± 0.0	8.1 ± 0.0
	Lignin	20.5 ± 0.5	22.3± 0.1**	22.2 ± 0.2**	22.3 ± 0.2**	23.9 ± 0.1***

[a] The remaining percent (of the whole 100%) for biomass composition is represented by other components, including ashes and acid soluble lignin. NA, no alkaline extraction; A, alkaline extraction; C, control samples; L, laccase samples; L-HBT, laccase-hydroxybenzotriazole system samples; L-VIO, laccase-violuric system samples; L-ABTS, laccase-(2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) system samples. Analysis of variance (ANOVA) with Bonferroni's post-test was performed to identify differences between control and laccase or laccase-mediator systems assays. The mean different is significant at the (*) 0.05, (**) 0.01 or (***) 0.001 level.

722

723

724

725 **Table 3.**

Inhibitor (mg L^{-1})	Slurry 7% (w/v)	
	C	L
Formic acid	2571.7 ± 18.3	nq
Acetic acid	3386.3 ± 51.6	3154.0 ± 25.1
5-HMF	236.0 ± 5.5	202.0 ± 9.6
Furfural	659.0 ± 19.9	647.0 ± 7.8
Vanillin	28.0 ± 0.0	3.0 ± 0.0
Syringaldehyde	6.3 ± 0.6	0.0 ± 0.0
p-Coumaric acid	10.0 ± 0.0	0.0 ± 0.0
Ferulic acid	20.6 ± 1.1	0.0 ± 0.0

C, control samples; L, laccase samples; nq, not quantified

726

727

728 **Table 4.**

Sample	$EtOH_M$ (g L^{-1})	$Y_{E/G}$ (g g^{-1})	$Y_{E/ET}$ (%)	Q_E (g L^{-1} h)
C	11.6 ± 0.3	0.32 ± 0.01	63.4 ± 1.5	0.1 ± 0.0
L	13.9 ± 1.2*	0.39 ± 0.03*	75.8 ± 6.3*	0.4 ± 0.0***

C, control samples; L, laccase samples; $EtOH_M$, maximum ethanol concentration during 72 h of SSF; $Y_{E/G}$, ethanol yield based on total glucose content present in the slurry. The ethanol yield is calculated considering that the liquid volume of the SSF system is constant;[47] $Y_{E/ET}$, theorical ethanol yield assuming ethanol yields on glucose by *S. cerevisiae* 0.51 g g^{-1}; Q_E, volumetric ethanol productivity at 32 h of SSF. Analysis of variance (ANOVA) was performed to identify differences between control and laccase-supplemented assays. The mean different is significant at the (*) 0.05 or (***) 0.001 level.

729

730

731 **Figure 1.**

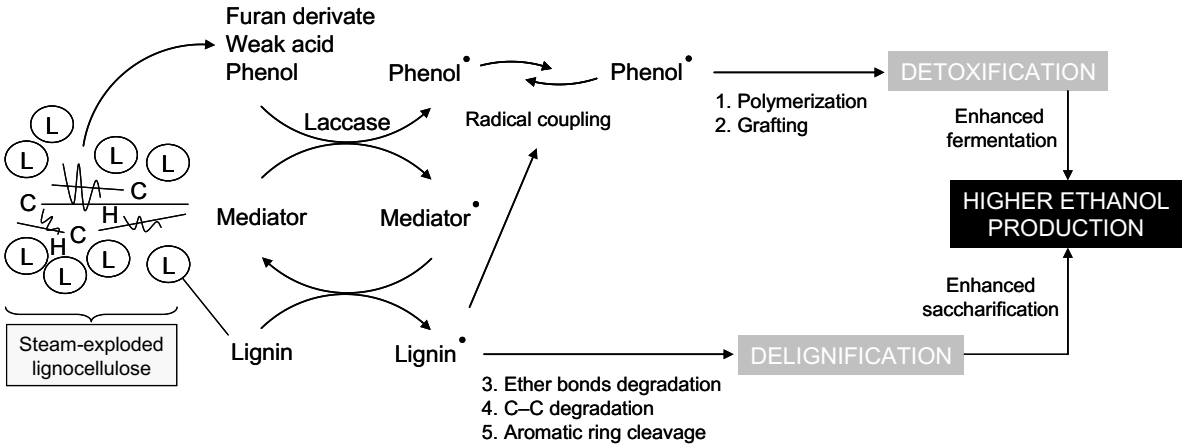

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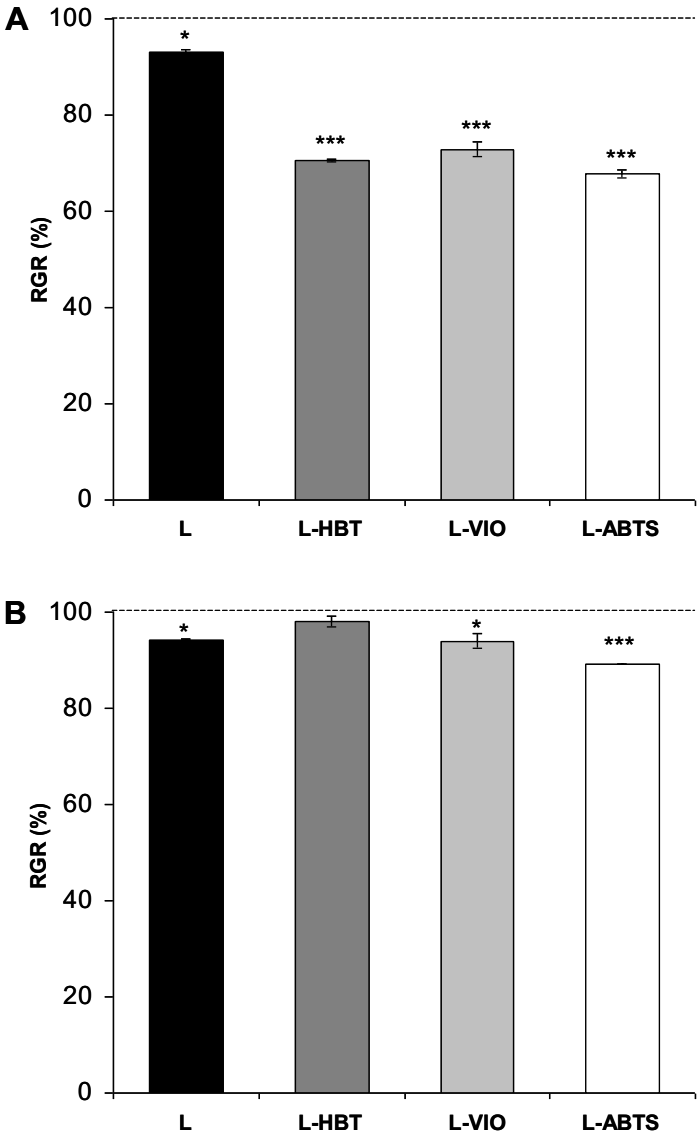
Figure 2.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L-HBT
L-VIO
L-ABTS

748 **Figure 3.**

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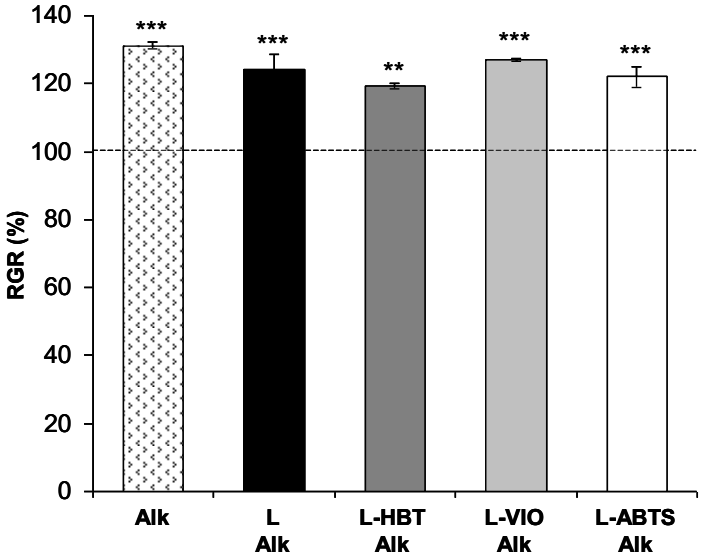

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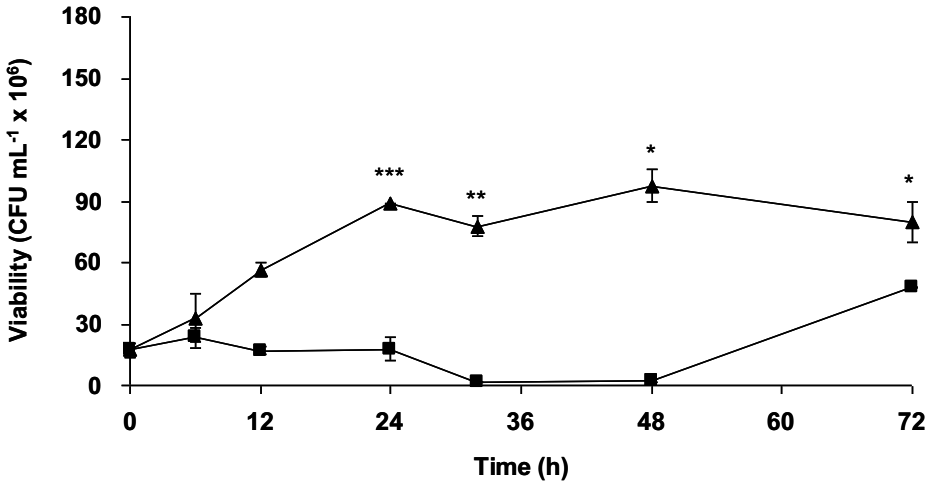
Figure 4.


180
150
120
90
60
30
0
Viability (CFU mL-1 x 10^6)
***
**
*
*
0
12
24
36
48
60
72
Time (h)

759 **Figure 5.**

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