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Abstract

Laccase enzymes are promising detoxifying agents during lignocellulosic bioethanol production from wheat straw. However, they affect the enzymatic hydrolysis of this material by lowering the glucose recovery yields. This work aimed at explaining the negative effects of laccase on enzymatic hydrolysis.

Relative glucose recovery in presence of laccase (10 IU/g substrate) with model cellulosic substrate (Sigmacell) at 10% (w/v) was almost 10 percentage points lower ($P<0.01$) than in the absence of laccase. This fact could be due to an increase in the competition of cellulose binding sites between the enzymes and a slight inhibition of β-glucosidase activity. However, enzymatic hydrolysis and infrared spectra of laccase-treated and untreated wheat straw filtered pretreated residue (WS-FPR), revealed that a grafting process of phenoxy radicals onto the lignin fiber could be the cause of diminished accessibility of cellulases to cellulose in pretreated wheat straw.

Highlights 3-5 (maximum 85 characters, including spaces, per bullet point)

- The activity of β-glucosidases was slightly reduced by laccases
- Total phenolic content decreased 80 percentage points after laccase treatment
- Laccase treatment increased the Klason lignin in the lignocellulosic substrate
- Laccases modified the infrared adsorption spectra of the lignocellulosic substrate
- Grafting process of lignin units into the wheat straw fibres limiting the accessibility of cellulolytic enzymes to cellulose

Keywords

Lignocellulose, enzymatic hydrolysis, laccase detoxification, grafting, ATR-FTIR.

1. Introduction

Concerns about climate change and uncertainties about fuel supply make renewable biofuels, such as bioethanol and biodiesel, attractive options for oil replacement in the short-term perspective. Presently, international governments have adopted some policies, such as the European Union Directive 2009/28/CE (2009), to gradually replace fossil fuels by biomass-based fuels or biofuels. Moreover, innovative technologies and advanced biorefineries based on lignocellulosic biomass are expected to play an important role in future bio-economy in Europe. Lignocellulosic biorefineries are expected to provide different forms of energy such as bioethanol and create new markets for bio-based products such as food, feed, chemicals and materials.

Lignocellulosic biomass is an abundant, low-cost and widely distributed feedstock. Among different lignocellulosic raw materials, wheat straw is an ideal candidate for bioethanol production worldwide (Tomás-Pejó et al. 2008). The biochemical conversion of lignocellulose into ethanol is performed via enzymatic hydrolysis of the carbohydrates to monomeric sugars, which are subsequently converted into ethanol by a fermentation step. The process is, however, hindered by the complex and recalcitrant structure of the lignocellulosic materials and thus, a pretreatment step is needed to increase biomass digestibility. Hydrothermal methods, such as steam explosion, are cost-effective technologies to increase the accessibility of enzymes to cellulose by solubilization of hemicelluloses and lignin redistribution (Alvira et al. 2010). Nevertheless, harsh conditions applied in these pretreatments also promote sugar and lignin degradation, triggering the formation of weak acids, furan derivatives and phenolic compounds which inhibit enzymes and microorganisms performance (Palmqvist and Hahn-Hägerdal 2000; Ximenes et al. 2010). The removal of the inhibitors from the fermentation broth, also known as detoxification, is an interesting alternative to overcome the negative effect of these compounds. As a detoxification method, *in situ* laccase treatment represents an appropriate option since the process is carried out in the same vessel under mild conditions (Parawira and Tekere 2011; Moreno et al. 2014). Laccases are multicopper-containing phenoloxidases that catalyze the oxidation of substituted phenols, anilines and aromatic thiols at the expense of molecular oxygen. Laccase addition to pretreated lignocellulosic materials

selectively removes the phenolic compounds formed during the hydrothermal pretreatment (Jönsson et al. 1998; Jurado et al. 2009; Moreno et al. 2012, 2013a). This phenol removal by laccases leads to improved fermentation performance of microorganisms, shortening the lag phase and boosting cell viability. In consequence, ethanol volumetric productivities are enhanced and the overall process time is reduced. Nevertheless, in case of steam-exploded wheat straw, laccase-detoxified biomass shows lower glucose hydrolysis yields during the saccharification step, which implies a reduction in the final ethanol concentrations (Jurado et al. 2009; Moreno et al. 2012, 2013a, 2013b).

Several hypotheses have been proposed to explain the lower glucose recovery yields after laccase treatment: 1) laccases increase the non-specific adsorption of hydrolytic enzymes onto the lignocellulosic fibers by catalyzing reactions on lignin (Moilanen et al. 2011); 2) laccases cause strengthening of carbohydrate-lignin bonds (Moilanen et al. 2011) and 3) formation of laccase-derived compounds can inhibit the hydrolytic enzymes (Jurado et al. 2009; Tejirian and Xu 2011). Although all these inhibition mechanisms seem to be accepted, there is no information about the alternatives to overcome this limitation. The present work attempts to establish a better understanding of the effects observed during the enzymatic hydrolysis of laccase-treated steam-exploded wheat straw for the optimum integration of the detoxification step into the current lignocellulosic bioethanol production process.

In this work, the effect of laccase on enzymatic activities of cellulases was examined to discard any direct effect of laccases on those hydrolytic enzymes. Furthermore, the effect of laccase was studied in enzymatic hydrolysis experiments of model cellulosic substrate (Sigmacell) without lignin polymer using different substrate concentration and laccase loadings, which could give information about a potential physical interaction or hindrance between the enzymes. Concurrently, in order to reduce a possible non-specific adsorption of cellulases on to the lignin fiber, steam-exploded wheat straw was enzymatically hydrolyzed in the presence of laccase adding polyethylene glycol (PEG). The chemical composition of the wheat straw filtered pretreated residue (WS-FPR) was studied after 3h incubation with laccase without hydrolytic enzymes. Finally, attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectra were

113 determined for laccase treated or untreated WS-FPR after the enzymatic
114 hydrolysis.

115 2. Material and methods

116 2.1 Nomenclature

ANOVA	Analysis of variance
ATR-FTIR	Attenuated total reflectance - Fourier transform infrared spectroscopy
DW	Dry weight
FPU	Filter paper unit
IU	International unit
PEG	Polyethylene glycol
Raw material	Milled wheat straw (particle size: 2 -10 mm)
RGR	Relative glucose recovery
SF	Severity factor
Sigmacell	Model cellulosic substrate Sigmacell Type 50® (Sigma S5504)
Slurry	Whole steam-exploded wheat straw
WS-FPR	Wheat straw filtered pretreated residue (Vacuum-filtered but not washed)

117

118 2.2 Raw material and pretreatment

119 Wheat straw was supplied by CEDER-CIEMAT (Soria, Spain). This material
120 presented the following composition (% (w/v) dry weight (DW)): cellulose, 40.5;
121 hemicelluloses, 26.1 (xylan, 22.7; arabinan, 2.1; and galactan, 1.3); Klason lignin,
122 18.1; ash, 5.1; and extractives, 14.6 (Alvira et al. 2011).

123 The biomass was milled until a chip size between 2 and 10 mm using a laboratory
124 hammer mill. The milled wheat straw was pretreated by steam explosion in a 2 L
125 reactor without acid impregnation, considering a severity factor (SF) of 3.94 (200
126 ºC for 10 min). The whole steam-exploded wheat straw was vacuum filtered and
127 the wheat straw filtered pretreated residue (WS-FPR) was used as substrate. Since
128 the WS-FPR was unwashed, low amount of phenolic compounds remained soaked

129 in the material. The chemical composition of the WS-FPR was characterized as
130 described in the analytical methods section having the following composition (%
131 DW (w/v)): hemicellulose, 8.4; cellulose, 55.0 and Klason lignin 33.6. The
132 material was kept at 4ºC until use.
133
134 The model cellulosic substrate used was Sigmacell Type 50® (Sigma S5504).
135 This substrate is considered as high purity cellulose according to manufacture
136 information.

137 ## 2.3 Enzymes

138 *Pycnoporus cinnabarinus* laccase (60 IU/mL; 8 g/L), produced by Beldem
139 (Belgium), was used for the evaluation of laccase treatment. Laccase activity was
140 measured by the oxidation of 5 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-
141 sulfonic acid) to its cation radical (ε_{436} = 29300 M^{-1} cm^{-1}) in 0.1 M sodium acetate
142 (pH 5) at 24 ºC.

143 A mixture of the preparations NS50013 (60 FPU/mL cellulase activity; 150 g/L
144 total protein content) and NS50010 (510 IU/mL β-glucosidase activity; 200 g/L
145 total protein content), both produced by Novozymes (Denmark), was used for the
146 enzymatic hydrolysis.

147 Total protein content from all enzymatic preparations was analyzed by BCA
148 protein assay kit (Pierce Ref. 23225), using bovine serum albumin as standard.
149

150 ## 2.4 Enzymatic activities of hydrolytic enzymes

151 Overall cellulase activity of NS50013 was determined using filter paper
152 (Whatman No. 1 filter paper strips), while β-glucosidase activity of NS50010 was
153 measured using cellobiose as substrate (Ghose 1987). One unit of enzyme activity
154 was defined as the amount of enzyme that transforms 1 µmol of substrate per
155 minute.

156 Activities of those hydrolytic enzymes in presence of 0.5, 1 and 2 IU/mL of *P.*
157 *cinnabarinus* laccase were measured in order to detect any direct effect of laccase
158 on hydrolytic enzymes.

2.5 Enzymatic hydrolysis of Sigmacell and wheat straw filtered pretreated residue (WS-FPR)

The model cellulosic substrate Sigmacell and the lignocellulosic substrate (WS-FPR) were hydrolyzed to evaluate the effect of laccase supplementation.

Sigmacell was diluted to 5% or 10% DW (w/v) with citrate buffer 50 mM, pH 5, and subjected to saccharification with optimal dosage of hydrolytic enzymes for a complete enzymatic hydrolysis: 15 FPU/g DW substrate of NS50013 and 15 IU/g DW substrate of NS50010 (Tomás-Pejó et al. 2009). The enzymatic hydrolysis of 5% DW (w/v) Sigmacell was performed in presence of 10 IU laccase/g DW substrate. In case of 10% DW (w/v) Sigmacell, laccase was supplemented at 1, 5, 10 and 15 IU laccase/g DW substrate. Hydrolysis of Sigmacell at 5% and 10% DW (w/v) without laccase were used as control.

In a second set of experiments, WS-FPR was diluted to 10 % DW (w/v) with citrate buffer 50 mM, pH 5. In order to enable a better detection of any difference on enzymatic hydrolysis yields, low enzyme dosages were used. More specifically, enzyme dosage was 5 FPU NS50013/g DW of substrate and 5 IU NS50010/g of DW substrate. PEG was added at different concentrations: 0.5, 1, 2.5 and 5% DW (w/w) of substrate with or without laccase (10 IU /g DW of substrate). WS-FPR with hydrolytic enzymes and without PEG or laccase was used as control. Hydrolytic enzymes, PEG and laccase were added simultaneously when applied.

After the hydrolysis step, laccase and non-laccase supplemented WS-FPR without PEG were centrifuged at 5000 rpm for 10 min to collect the solid fraction. Solids were dried in an oven at 40ºC during 3 days and analyzed by ATR-FTIR.

All assays were run in triplicate in 100 mL-flasks at 50ºC and 150 rpm for 72 h. Representative samples were withdrawn periodically, centrifuged and supernatants were analyzed for glucose concentration. For better comparison between assays, relative glucose recovery (RGR) was calculated as follow:

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

2.6 Effect of laccase treatment on WS-FPR without hydrolytic enzymes

To evaluate the changes in the chemical composition of the WS-FPR after 3h of laccase treatment, 10% DW (w/v) WS-FPR was supplemented with 10 IU/g DW of substrate of *P. cinnabarinus* laccase without hydrolytic enzymes. This assay was run in triplicate in 100 mL-flasks at 50ºC and 150 rpm for 3 h in a rotary shaker. Laccase-treated and non-treated WS-FPR were filtered and washed and the chemical composition of the solid residues was analyzed as explained in analytical methods section.

2.7 Analytical methods

The chemical composition of non-treated and laccase-treated (3 h) WS-FPR was analyzed using the National Renewable Energy Laboratory (NREL) standard methods for determination of structural carbohydrates and lignin in biomass (LAP-002, LAP- 003, and LAP-019) (Sluiter et al 2010). Dry weight of WS-FPR was determined by drying a representative sample at 105ºC for 24 h (LAP-001). Total phenolic content of the supernatants was determined according to a modified version of the Folin-Ciocalteau method (Alvira et al. 2013).

Glucose concentration was quantified by high-performance liquid chromatography (HPLC) equipped with a refractive index detector. An Aminex HPX-87H column (Bio-Rad Labs, Hercules, CA), operated at 50ºC with 5 mM H_2SO_4 as a mobile-phase (0.4 mL/min), was employed for separation.

The dried solid residues obtained after the enzymatic hydrolysis of laccase-treated or non-treated WS-FPR without PEG were analyzed by ATR-FTIR in order to determine chemical changes in the fibers. Sample preparation was carried out with KBr. 5% (w/w) of dried biomass was analyzed in a FTIR spectrometer (Thermo Scientific Nicolet 6700 spectrometer) using an attenuated total reflectance (ATR) accessory and deuterated triglycine sulphate detector. Spectra were collected at room temperature in the 4000–400 cm^{-1} range with a 1.928 cm^{-1} resolution and being an average of 64 scans.

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 Bonferroni's post-test was used for comparisons between assays. The level of significance was set at $P<0.05$, $P<0.01$ or $P<0.001$. In addition, the effects of laccase dosage during the enzymatic hydrolysis of Sigmacell at 10% DW (w/v) were evaluated by regression analysis.

3. Results and discussion

3.1 Effect of laccase on cellulase/β-glucosidase activities

The effect of laccase on cellulolytic enzymes was evaluated in absence of soluble phenolic compounds, lignin and cellulose. Cellulase and β-glucosidase activities were measured in the presence of 0.5, 1 and 2 IU/mL of *P. cinnabarinus* laccase, using filter paper and cellobiose as substrates, respectively, to evaluate the direct interaction between hydrolytic and laccase enzymes (Table 1). In the case of overall cellulase activity (NS50013), ANOVA analysis showed no significant differences at any of the laccase loadings tested. By contrast, β-glucosidase activity (NS50010) showed a significant slight decrease of about 7 percentage points ($P<0.001$) in the presence of 2 IU/mL of laccase. These results suggest that, among the enzymatic activities present in a cellulolytic cocktail, laccase has more marked effect on the activity of β-glucosidase enzymes.

3.2. Effect of laccase supplementation on enzymatic hydrolysis of model cellulosic substrate (Sigmacell)

Two different consistencies (5% and 10% DW (w/v)) of model cellulosic substrate Sigmacell were subjected to enzymatic hydrolysis in the presence of laccase (Table 2 and Fig. 1). It is worth to mention that laccase and cellulase preparations were added considering the DW of Sigmacell, so that at 10% (w/v) substrate, the total protein concentration was the double than at 5% (w/v). The addition of 10 IU/g substrate (0.5 IU/mL) of laccase during the enzymatic hydrolysis of Sigmacell at 5% DW (w/v) did not show any significant difference in terms of final RGR. When the substrate concentration increased from 5% to 10% DW (w/v), the addition of 10 IU/g (1 IU/mL) of laccase decreased significantly the final RGR by almost 10 percentage points ($P<0.01$) compared to those assays performed without laccase. As there were no potential laccase targets

due to the absence of lignin and phenols in the medium, this result might be explained by an increase in the competition of cellulose adsorption sites between cellulases and laccases.

Sigmacell at 10% DW (w/v) was enzymatically hydrolyzed with increasing laccase loadings to evaluate if higher laccase dosages promote its reversible adsorption to cellulose, lowering the adsorption sites of hydrolytic enzymes towards this polymer. As listed in Table 2, although the total protein concentration did not change markedly after laccase addition, an increase from 0.14% to 2.06% in the ratio between laccase and hydrolytic enzymes (L/H values) was enough to reduce the RGR about 5 percentage points. Linear and quadratic effects were found when evaluating the decrease of RGR at different laccase dosage (Fig. 2). Although both equations were statistically significant ($P_{linear}<0.01$; $P_{quadratic}<0.05$), a better trend fit was obtained with the quadratic model ($R^2 = 0.999$). Supporting these results, Qiu and Chen (2012) described an increase in the hydrolysis yields of steam-exploded wheat straw when laccase was added at 0.55 IU/g substrate, however, further increase of laccase dosage decreased the cellulose conversion rate.

The time course for enzymatic hydrolysis of Sigmacell at 5 and 10% DW (w/v) in presence or absence of 10 IU/g substrate of laccase is shown in Fig. 1. As it was showed previously, at 5% DW (w/v) Sigmacell, the addition of laccase did not show any difference in terms of hydrolysis rates and about 40 g/L of glucose were released in both cases. By contrast at 10% DW (w/v), the addition of laccase decreased the final glucose concentration from 59.7 g/L to 55.2 g/L. Moreover, a significant marked decrease in the hydrolysis rate ($P<0.01$) was observed after 24h. As it is well known, endo- and exo-glucanase enzymes are acting on cellulosic polymer at the first stage of the hydrolysis and β-glucosidase is acting sequentially after. Thus, the decrease in hydrolysis rate after 24h could be explained by the slight inhibition on β-glucosidase activity observed previously in the presence of laccase (Table 1).

3.3 Effect of PEG supplementation on enzymatic hydrolysis of WS-FPR in presence of laccase

Since it has been already shown that the addition of non-ionic surfactants such as Berol or Tween 80 and polymers such as polyethylene glycol (PEG) reduces the non-specific adsorption of cellulases (Kristensen et al. 2007), 10% (w/v) of WS-FPR substrate was enzymatically hydrolyzed in the presence or absence of PEG and laccase. PEG concentrations were 0.5, 1, 2.5 and 5% (w/w) of substrate. As expected, when WS-FPR was supplemented with 5% (w/w) PEG without laccases, the RGR increased 1.4 fold (P<0.001) compared to control assays without PEG (Fig. 3a). PEG has previously demonstrated to be efficient for enhancing the enzymatic hydrolysis of steam-exploded wheat straw by decreasing the non-specific adsorption of cellulases (Kristensen et al. 2007; Cannella and Jørgensen. 2014). However, regardless the PEG concentrations, RGRs were significantly (P<0.05) lower than the control in the presence of laccase (Fig. 3b). These results suggest that the increase in the non-specific adsorption of hydrolytic enzymes in the presence of laccases is not the reason for the reduction in final RGR when using WS-FPR. As it was explained before in the case of Sigmacell, when no lignin neither phenols were in the media, an increase in the competition of cellulose adsorption sites was supposed to be the main reason for reducing the RGR. However, this effect seemed to be negligible when lignin and phenols are in the media.

After 24 h of enzymatic hydrolysis, total phenol concentration was measured to verify the laccase detoxification ability in presence of 5% (w/w) of PEG. The total phenolic concentration decreased about 80 percentage points (P<0.001) after laccase treatment, regardless the presence or absence of PEG (no significant differences were found in terms of total phenolic content between assays supplemented with laccase or laccase+PEG) (Fig. 4). Therefore, PEG does not affect laccase activity in terms of phenol removal.

As ligninolytic enzymes, in addition to soluble phenols removal, laccases have the ability to interact with phenolic units present in lignin polymer. In this context, laccase can alter the hydrophobicity of lignin by increasing or decreasing the non-specific adsorption of hydrolytic enzymes onto this polymer (Palonen and Viikari 2004; Moilanen et al., 2011). The high variability of lignin composition among different lignocellulosic materials could explain the contradictory effects regarding laccase interaction with lignin phenolic units. Palonen and Viikari (2004) showed that laccase treatment of steam-exploded spruce increased

carboxyl groups of lignin, reducing the hydrophobicity and increasing surface charge. Subsequently, the non-specific adsorption of cellulases to lignin decreased and the glucose hydrolysis yields were improved. Moilanen et al. (2011) obtained similar results when using steam-exploded spruce treated with laccase. Nevertheless, these authors also reported an increase in the non-specific adsorption of hydrolytic enzymes and lower glucose yields, when laccase treatment was performed on steam-exploded giant reed (*Arundo donax*).

3.4 Characterization of WS-FPR before and after the enzymatic hydrolysis in presence/absence of laccase.

Although the increment in the non-specific bindings of hydrolytic enzymes to the lignocellulosic fibers did not seem to be the main reason for the lower RGR registered after laccase treatment, there are other factors that could explain this outcome. Since the reaction mechanism of laccase involves an oxidative polymerization process via formation of unstable radicals, the formed phenolic polymers could exert a greater inhibitory effect on hydrolytic enzymes. As a matter of fact, Tejirian and Xu (2011) observed that oligomeric phenols formed after the pretreatment of lignocellulosic materials could inhibit with a greater extent the enzymatic hydrolysis than single soluble phenols**.**

On the other hand, radicals formed by laccase action can also interact with WS-FPR fibers via grafting (Aracri et al. 2010; Barneto et al. 2012). This reaction mechanism of laccases might incorporate some of the phenols that were solubilized during the steam explosion pretreatment into the pretreated fibers, limiting the enzymatic hydrolysis.

Considering the previous effects, the chemical composition of WS-FPR treated 3h with laccase was analyzed in the absence of hydrolytic enzymes. Interestingly, in accordance to Aracri et al. (2011), an increase of Klason lignin of 2-3 percentage points was observed. This increase in the lignin content suggests a reintegration of some soluble phenols to the fibers. It has been described that the reaction mechanism of soluble phenols after laccase treatment (oxidative polymerization or grafting) is determined by the nature of the phenolic compound itself. Among different phenols, *p*-hydroxycinnamic acids, such as *p*-coumaric acid or ferulic acid, can be covalently coupled to the lignin component of the fibers after laccase treatment, while others such as syringaldehyde lead to the oxidative

352 polymerization (Aracri et al. 2010; Barneto et al. 2012). As an herbaceous crop,
353 wheat straw contains considerable amount of *p*-coumaric acid (5% DW w/w) and
354 ferulic acid (5% DW w/w) on its composition that can be released during the
355 pretreatment (Buranov and Mazza 2008; Moreno et al. 2012). In this sense,
356 laccase would incorporate these compounds onto the fiber, justifying the slight
357 increase in Klason lignin content and, therefore, limiting the accessibility of
358 cellulose.

359 For a better understanding of the grafting process, solid residues that remained
360 after the enzymatic hydrolysis of WS-FPR in the presence or absence of laccase
361 were analyzed by ATR-FTIR (Fig. 5). The absorbance in the mid-infrared region
362 (2000-800 cm^{-1}) corresponds to lignin and carbohydrate bonds (Sun et al. 2005).
363 Both spectra were clearly dominated by carbohydrate bands, including a band at
364 900 cm^{-1} characteristic of ß-glycosidic linkages; a region at 1160-1035 cm^{-1}
365 dominated by ring vibrations overlapped with stretching vibration of (C-OH) side
366 groups; and a band at 1375 cm^{-1} due to symmetric bending of aliphatic C–H.
367 Lignin bands were also observed, including the characteristic triplet at 1600, 1512
368 and the 1429 cm^{-1} band ascribed to aromatic ring vibrations, together with the
369 bands at 1657 cm^{-1} (stretching of C=O conjugated to aromatic rings) and at 1726-
370 1710 cm^{-1} (stretching of C=O unconjugated to aromatic rings) (Sun et al. 2005;
371 Ibarra et al. 2004). In general, no significant differences were found between both
372 spectra, except the increase and reduction of intensities observed at 1160 and
373 1512 cm^{-1}, respectively (Fig. 5 arrows). The 1160 cm^{-1} band, previously assigned
374 to carbohydrates, is also attributed to antisymmetric C-O stretching vibration,
375 indicating the ester-linked hydroxycinnamic acids such as esterified *p*-coumaric
376 acid and ferulic acid (Sun et al. 2002). On the other hand, variations at 1512 cm^{-1}
377 are indicative of changes in surface lignin and its lower intensity proves the action
378 of laccase towards phenolic lignin units present in the surface of the fibers
379 (Mattinen et al. 2005; Kahar 2013). This outcome, together with the increase in
380 Klason lignin reported above, supports the grafting hypothesis where *p*-
381 hydroxycinnamic acids are incorporated into the WS-FPR fibers. This hypothesis
382 was also recently supported by Rencoret et al. (2014) that showed the grafting
383 process of ferulic acid on high lignin sisal pulp after laccase treatment.

384 In addition to the differences observed at 1512 and 1160 cm^{-1}, the ATR-FTIR
385 spectrum of laccase-treated WS-FPR exhibited higher intensities values in the

carbohydrate region (1035-1109 cm^{-1}) (Fig. 5). In agreement with this result, Molainen et al. (2011) also reported higher residual glucan content after the enzymatic hydrolysis of laccase-treated giant reed (*Arundo donax*). Assuming that the grafting process is taking place, the new-formed bonds might affect the accessibility of enzymes to cellulose, probably by reducing the number and/or the size of pores or hindering the progress of cellulases. The grafting process could also result in an increment of the lignin surface area by incorporating new lignin units therefore limiting the accessibility of cellulolytic enzymes to cellulose.

4. Conclusions

An increase in the competition of cellulose binding sites between cellulases and laccase together with the inhibition on β-glucosidase activity seemed to be the main reasons for RGR reduction on model cellulosic substrate enzymatic hydrolysis. According to experiments with PEG addition, non-specific adsorption of cellulases was negligible on WS-FPR. It could be thus suggested that the presence of phenols and lignin promoted different effects. The increase in Klason lignin together with the changes observed in the ATR-FTIR spectra for laccase-treated or non-treated WS-FPR supported the grafting process that would limit the accessibility of cellulolytic enzymes to cellulose.

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Table captions

Table 1 Cellulase (NS50013) and β–glucosidase (NS50010) activities in the presence of different concentrations of *P. cinnabarinus* laccase.

Table 2 Laccase loading, total protein concentration, laccase/hydrolytic enzymes ratio and relative glucose recovery (RGR) after 72 h of enzymatic hydrolysis of Sigmacell.

Figure captions

Figure 1 Time course for glucose release during enzymatic hydrolysis of Sigmacell at 5% DW (w/v) (**a**) and 10% DW (w/v) (**b**) in the presence (▲) or absence (●) of *P. cinnabarinus* laccase. Mean values and standard deviations were calculated from triplicates to present the results. A two-way analysis of variance (ANOVA) with Bonferroni's post-test was performed to identify differences between non-supplemented and laccase-supplemented assays at different time points. The mean difference is significant at the (**) 0.01 level.

Figure 2 Influence of laccase dosage on relative glucose recovery (RGR) after the enzymatic hydrolysis of 10% DW (w/v) Sigmacell. Linear (·····) and quadratic (–—) trends were calculated by regression analysis.

Figure 3 Relative glucose recovery (RGR) in enzymatic hydrolysis of 10% (w/v) WS-FPR supplemented with (a) PEG (0.5-5% w/v) and (b) PEG (0.5-5% w/w) + laccase (10 UI/g DW of substrate). Black lines represent RGR values (100%) in control assays (a) without PEG or (b) without PEG and laccase. Mean values and standard deviations were calculated from triplicates to present the results. Analysis of variance (ANOVA) with Bonferroni's post-test was performed to identify differences between (a) non-supplemented and PEG-supplemented assays or (b) non-supplemented and laccase-supplemented (with or without PEG) assays. The mean difference is significant at the (*) 0.05, (**) 0.01 or (***) 0.001 level.

Figure 4 Total soluble phenols measured at 24 h of enzymatic hydrolysis of 10% (w/v) WS-FPR. Assays were supplemented with 5% (w/w) PEG and/or 10 UI/g DW of *P. cinnabarinus* laccase. Mean values and standard deviations were

548 calculated from triplicates to present the results. Analysis of variance (ANOVA)
549 with Bonferroni's post-test was performed to identify differences between assays.
550 The mean difference is significant at the (***) 0.001 level
551
552 **Figure 5** Infrared absorption spectra (cm^{-1}) of the solid residue obtained after the
553 enzymatic hydrolysis. Assays were supplemented (Laccase) or not (Control) with
554 *P. cinnabarinus* laccase. Differences (Subtraction) were obtained by subtraction
555 of control spectrum on laccase-supplemented spectrum.
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557

558 # Table 1

Laccase (IU/mL)[a]	Relative activity of NS50013 (% FPU)[b]	Relative activity of NS50010 (% IU)[b]
0.0	100.0 ± 0.8	100.0 ± 0.1
0.5	99.9 ± 0.4	99.9 ± 0.0
1.0	98.3 ± 3.0	95.4 ± 0.1***
2.0	99.5 ± 1.5	92.9 ± 0.1***

Relative cellulase and β–glucosidase activities were calculated as the percentage of the activity obtained in the presence of laccase divided by the activity in the absence of laccase. Mean values and standard deviations were calculated from the triplicates to present the results

[a] Laccase activities in terms of total volume assay.

[b] Analysis of variance (ANOVA) with Bonferroni's post-test was performed to identify differences between non-supplemented and laccase-supplemented assays. The mean different is significant at the (***) 0.001 level.

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562 **Table 2**

Sigmacell (% (w/v))	Laccase (IU/g)[a]	$Protein_{Total}$ (g/L)	L/H[b] (%)	RGR(%)[c]
5	-	4.80	-	100.0 ± 2.0
	10 (0.5)	4.86	1.37	101.1 ± 0.8
10	-	9.59	-	100.0 ± 1.7
	1 (0.1)	9.60	0.14	95.7 ± 1.3
	5 (0.5)	9.66	0.69	93.9 ± 1.9**
	10 (1.0)	9.72	1.37	92.4 ± 0.7**
	15 (1.5)	9.79	2.06	91.4 ± 0.6**

[a] Laccase activities in terms of total volume assay (IU/mL) are indicated in parentheses
[b] Percentage considering the ratio between Laccase (L) and Celluclast + Novo 188 (H)
[c] RGR values were calculated as the percentage of the glucose concentration in laccase-supplemented assays divided by the glucose concentration in non-supplemented assays. Mean values and standard deviations were calculated from the triplicates to present the results. Analysis of variance (ANOVA) with Bonferroni's post-test was performed to identify differences between non-supplemented and laccase-supplemented assays. The mean different is significant at the (**) 0.01 level.

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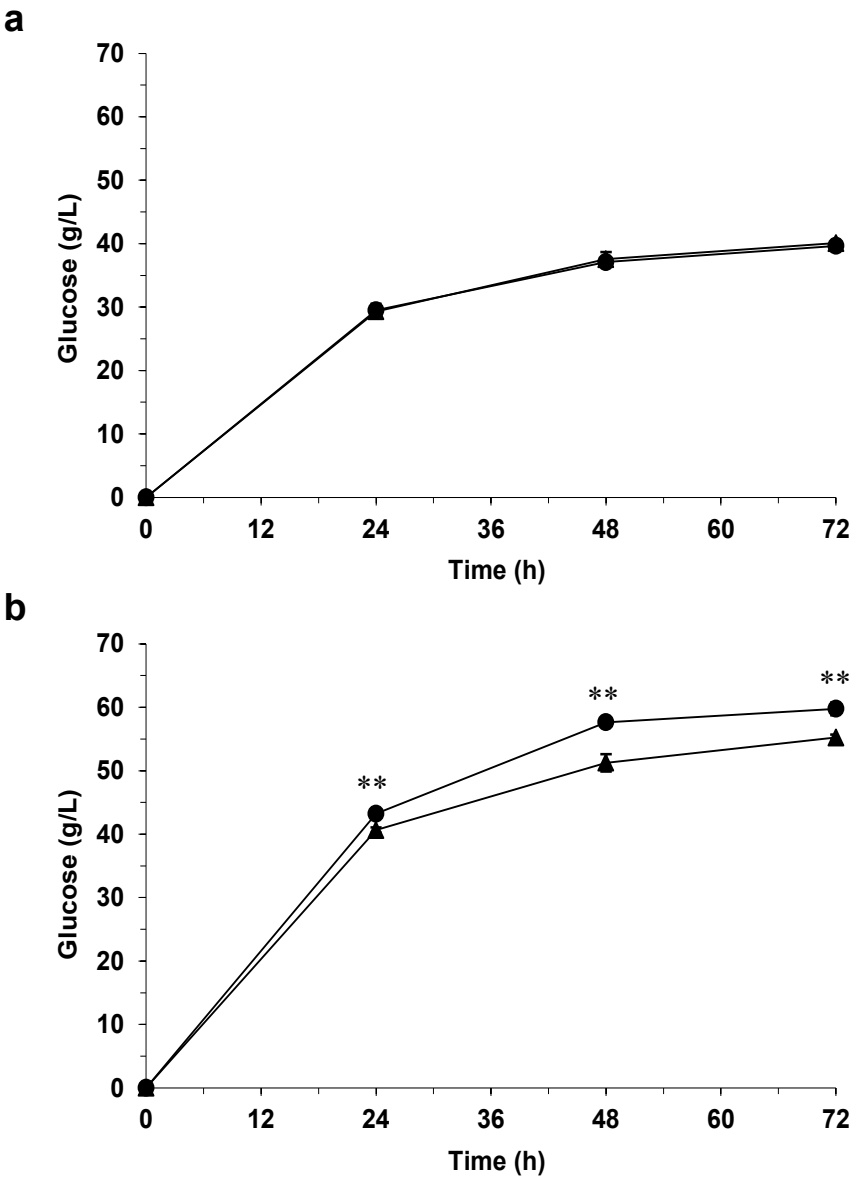
565 **Figure 1**



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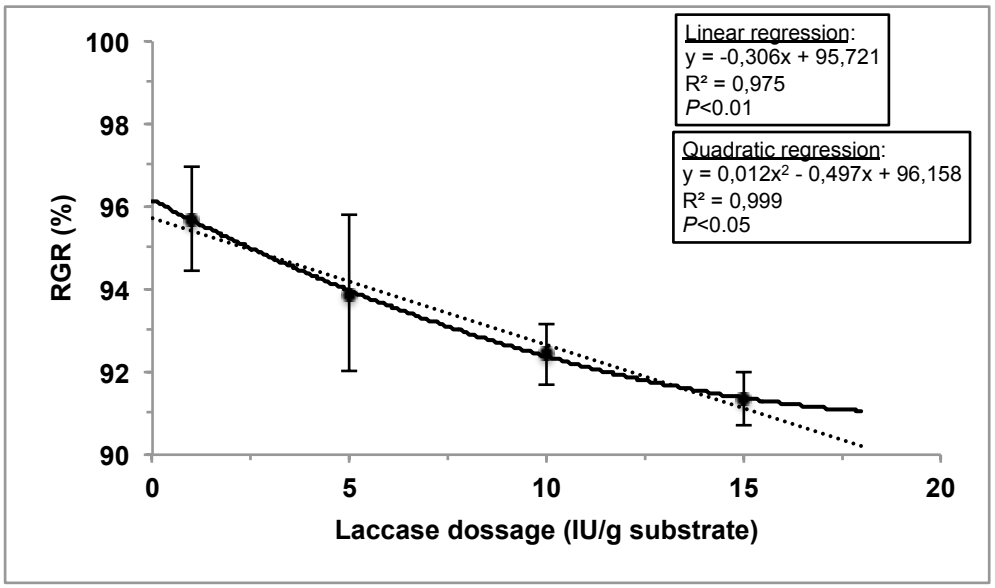
568 **Figure 2**



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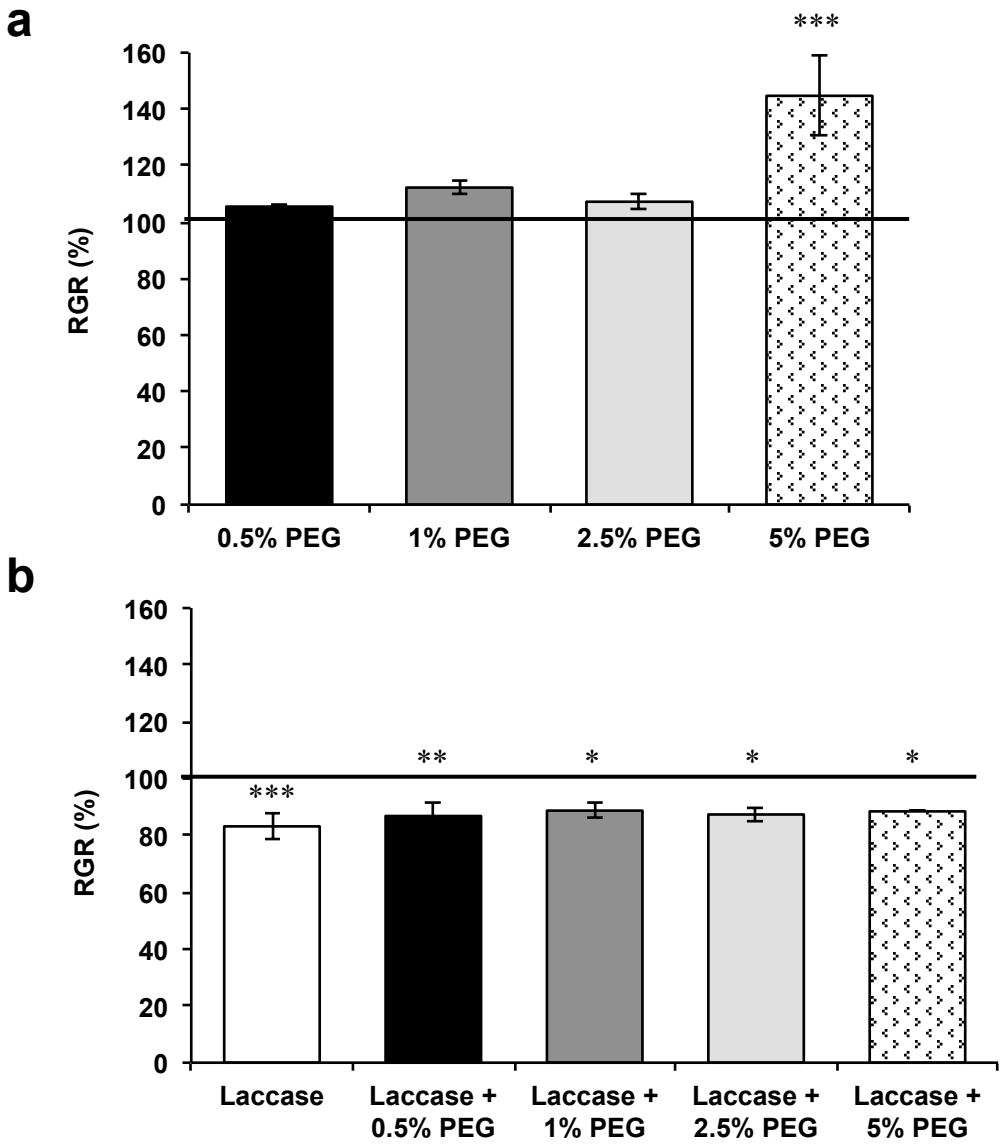
572 **Figure 3**



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580 **Figure 4**

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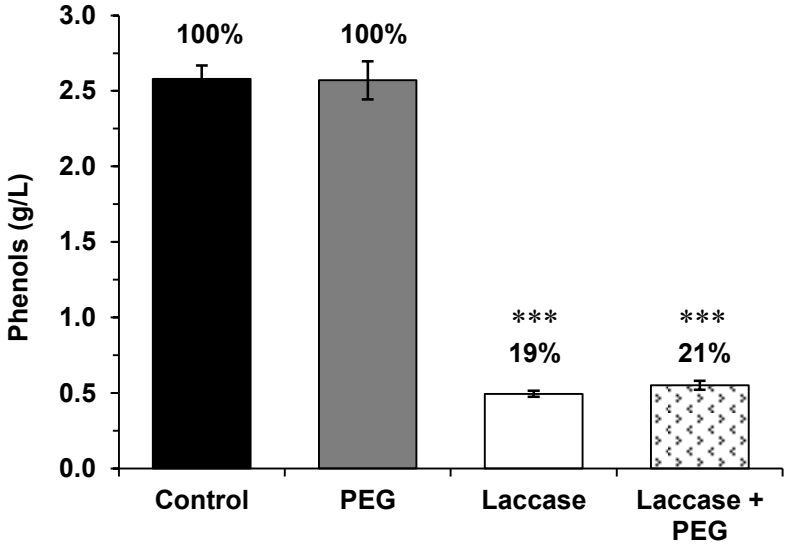

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