Effect of hydrothermal pretreatment severity on lignin inhibition in enzymatic hydrolysis

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Abstract

Hydrothermal pretreatment is commonly used for enhancing enzymatic hydrolysis of lignocellulosics. Spruce and wheat straw were pretreated with increasing severity and lignin characteristics were analysed. The effect of enzymatically isolated lignin on the hydrolysis of Avicel and the adsorption of a cellobiohydrolase onto lignin was measured. Non-pretreated lignins had only a minor effect on Avicel hydrolysis. The structural changes in lignin accompanying hydrothermal pretreatment were associated with increased binding and inactivation of the cellulase on the lignin surface. The inhibitory effect was more pronounced in spruce than in wheat straw lignin. However, similar pretreatment severities caused similar levels of inhibition in Avicel hydrolysis

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for both biomass sources. The combined severity factor of the pretreatment correlated well with the inhibitory effect of lignin.

Keywords

Saccharification, Cellulase, Non-productive binding, Spruce, Wheat straw

1. Introduction

Lignocellulosic biomass is an abundant raw material and a promising alternative for fossil-based energy carriers and chemicals. Lignocellulose is composed of three main polymers, namely cellulose, hemicelluloses, and lignin, as well as small amounts of pectins, extractives, and proteins. Hydrolysis of the polysaccharides to fermentable sugars is commonly carried out using cellulases and hemicellulases.

Pretreatment is a prerequisite for enzymatic hydrolysis in order to reduce the cell wall recalcitrance and enable enzymes to access the structural carbohydrates in the cell wall. Hydrothermal pretreatment has been widely employed, due to its simplicity and low cost. Hydrothermal pretreatments are performed at high temperature (120-230 °C) in a pressurised vessel (reviewed by Pu et al. (2013)). During hydrothermal pretreatment most of the hemicelluloses are hydrolysed and solubilised, whereas lignin is partially modified and redistributed, but it mainly remains in the solid phase with the bulk of the polysaccharides. Acetyl groups in the biomass can be released in the form of acetic acid which catalyses the hydrolysis reactions during pretreatment. Hydrothermal pretreatment is effective on biomass with high acetyl content, such as many agricultural residues. Other biomass sources, such as softwoods, require addition of an external acid catalyst. Typically, a low concentration (<5% w/w) of H_2SO_4 is used (Pu et al., 2013). When an acid catalyst is used, the pretreatment is often referred to as a dilute acid pretreatment. However, both hydrothermal and dilute acid pretreatments are acidic processes and produce similar changes to the biomass (Pu et al., 2013).

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Hydrothermal pretreatment alters the distribution and chemical structure of lignin in biomass. After pretreatment, the majority of lignin remains in the solid phase, although soluble phenolic compounds may also be released. At high temperatures lignin will partially migrate out of the cell wall, condense, and form droplets on the biomass surface (Donohoe et al., 2008). During pretreatment, lignin structure and molecular weight will change due to the cleavage and reformation of internal bonds. The main chemical changes are the cleavage of β-aryl ether (β-O-4) bonds and the formation of new carbon-carbon bonds (Li et al., 2007). In general it appears that, at lower pretreatment severities, the molecular weight of lignin decreases whereas, at higher severities, condensation reactions dominate and the molecular weight increases (Cao et al., 2012; Li et al., 2007). Lignins isolated from pretreated grasses (monocots), have been reported to be less inhibitory to enzymatic hydrolysis than lignin isolated from pretreated softwood (Nakagame et al., 2010; Rahikainen et al., 2013b). Likewise, higher pretreatment temperatures increase the inhibitory effects of lignin in enzymatic hydrolysis (Nakagame et al., 2011; Sun et al., 2016).

Lignin in hydrothermally pretreated lignocellulose restricts enzymatic hydrolysis by physically preventing access of the enzyme to the polysaccharides and by inhibiting the enzymes. The latter is caused by non-productive adsorption of enzymes onto the lignin surface, or by the interaction of soluble phenolic compounds released from lignin during pretreatment with the enzyme. Non-productive adsorption is thought to be the main inhibitory mechanism. Lignin surface hydrophobicity, charge, and functional groups such as carbonyls and phenolic hydroxyls, affect enzyme adsorption onto lignin (Rahikainen et al., 2013a; Sun et al., 2016; Yang and Pan, 2016). In cellulases, enzyme surface hydrophobicity and, especially, the carbohydrate binding module (CBM) which contains a hydrophobic surface, increase cellulase adsorption onto lignin (Kellock et al., 2017; Rahikainen et al., 2013b; Sammond et al., 2014). In addition, the pH-dependent surface charge of the enzyme has been shown to influence enzyme adsorption (Nakagame et al., 2011).

The aim of this work was to study the impact of pretreatment severity on the inhibitory effect of lignins on enzymatic hydrolysis. Lignins from non-pretreated and pretreated spruce and wheat straw were enzymatically isolated and characterised to identify the chemical and physical properties associated with an increased inhibitory effect.

2 Materials and methods

2.1 Biomass sources, and pretreatment conditions

Spruce harvested in 2014 in Eastern Finland was milled through a 5 mm sieve (Wiley mill, Arthur H. Thomas Co., PA, USA). Wheat straw harvested in August 2010, Northern Sealand, Denmark, was cut to 1 cm length. Hydrothermal pretreatment was performed in a small-scale 4-chamber pretreatment equipment using an in-house built pressurised heating system at the University of Copenhagen (Zhang et al., 2014). The biomasses (2 g dry matter (DM)) were pretreated at 180, 200 or 220 °C for 10 min with or without an acid catalyst with moisture adjusted to 30% DM (Table 1). When acid catalyst was used, the biomass was immersed into dilute sulphuric acid (0.4% w/w acid to DM) for 30 min at room temperature and overnight at +4 °C. After pretreatment, the biomass was washed with distilled water, freeze dried, milled through a 0.5 mm sieve (Fritsch Pulverisette 14, Germany) and extracted with acetone for 5 h (Soxhlet). Pretreatment severity factor log R_0 was calculated with the following equation (Overend et al., 1987):

$$R_0 = t * exp\left(\frac{T_r - 100}{14,75}\right) \qquad (1)$$

where T_r is the reaction temperature (°C) and *t* is the reaction time (min).

The combined severity factor (CSF) takes the reaction pH into account and was calculated with the following equation (Chum et al., 1990):

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$$CSF = \log R_0 - pH \qquad (2)$$

where pH is the measured pH after pretreatment.

2.2 Optimisation of milling time for lignin isolation

The extracted biomass samples were milled with a Retsch mixer mill (MM301, Germany) to reduce particle size for isolation of the enzymatic hydrolysis residual (EnzHR) lignins. Milling time was optimised using the most recalcitrant samples: non-pretreated spruce and wheat straw. The milling was performed in intervals of 15 min and the jars were kept on ice for 5 min in between to prevent excess heating of the mixing vessel. The optimal milling time was evaluated by the efficiency of enzymatic hydrolysis: triplicate samples were hydrolysed at a loading of 3% DM, in 50 mM Na-acetate buffer, pH 5, mixing at 45 °C, with Celluclast 1.5L (20 FPU/g substrate DM) supplemented with Novozyme 188 (500 nkat β-glucosidase activity/g substrate DM). The hydrolysis was carried out for 72 h and fresh buffer and enzyme solution was replaced every 24 h. The reducing sugars were analysed using 3,5-dinitrosalisylic acid (DNS) reagent (Miller, 1959) and quantified against glucose standards. A 90 min milling time was found to be optimal for sugar release and used for all pretreated biomasses. The same milling time was also found suitable for gel formation in DMSO-d_6 for NMR analysis.

2.3 Analysis of lignin interunit linkages from biomass by HSQC-NMR

The NMR (2D ^{1}H–^{13}C HSQC) data was collected at 30 °C using a 600 MHz Bruker Avance III NMR spectrometer equipped with a QCI ($^{1}H/^{13}C/^{15}N/^{31}P$) cryoprobe and a SampleJet sample changer. The milled spruce (~90 mg) and wheat straw (~35 mg) samples were transferred into a 5 mm NMR tube and 700 µl of DMSO-d_6 (EurisoTop) was carefully added (Kim et al., 2008). The samples were sonicated with occasional mixing for 50 min (spruce) or 4 h (wheat straw), until a clear gel was formed. The milled biomass material appeared to produce gels for NMR; however, ball-milling equipment capable of producing more finely-divided material (such as a Retsch PM100 or a Fritsch Planetary micro mill Pulverisette 7 ball-mill) is advocated (Mansfield et al., 2012). As will be noted later in section 3.2, some structural features may not have been revealed in the spectra, likely due to the inadequacy of the ball-milling step. The phase-sensitive HSQC data was acquired using Bruker's pulse program hsqcetgpsisp2.2 as recommended (Kim et al., 2008; Mansfield et al., 2012). A matrix of 2048 x 256 datapoints was collected and zero-filled once in F1. Gaussian apodization (LB = -0.5, GB = 0.001) was used in F2 and a $\pi/2$-shifted squared sine-bell (i.e., cosine-bell) apodization was used in F1. Linear prediction was not used. All spectral processing and the integrations were carried out using Topspin 3.5 pl7 software. The various lignin units, characterized by their distinguishing interunit bonds, were calculated as percentage of C9 units ($0.5\ S_{2/6} + G$). Representative spectra are presented Fig. 1 using the conventions that are commonly accepted (Kim et al., 2008; Mansfield et al., 2012).

2.4 Isolation of lignin-rich enzymatic hydrolysis residue (EnzHR lignin)

Enzymatic hydrolysis residues (EnzHR lignins) were isolated from ball-milled biomass using extensive enzymatic hydrolysis of polysaccharides according to (Rahikainen et al., 2011) with minor modifications. Briefly, the samples were hydrolysed using Celluclast 1.5L (20 FPU/g DM) and Novozyme 188 (500 nkat/g DM) at 6% (w/v) DM content, (45 °C, 50 mM Na-acetate, pH 5, with 0.02 % of NaN_3). Fresh enzyme and buffer was replaced every 24 h. After 72 h hydrolysis the biomass was washed three times with pH-adjusted mQ water (pH 2.5). Protease treatment was performed using Bacterial proteinase, type XXIV (Sigma, Japan) 1 mg protease/50 mg lignin (24 h, 0.5 M $NaHCO_3$, pH 9.6, 5% w/v DM, 37 °C with magnetic stirring). The solid residue was washed three times with pH-adjusted water (pH 2.5) and freeze-dried. The lignin-rich residues (EnzHR lignins), were stored in a desiccator at room temperature prior to use.

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2.5 Analysis of the EnzHR lignins

Carbohydrate and lignin content (Klason + acid soluble) were analysed after a two-step sulphuric acid hydrolysis (Sluiter et al., 2012). The released monosaccharides were analysed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Tenkanen and Siika-Aho, 2000). An anhydro correction of 0.88 for pentoses and 0.9 for hexoses was used for conversion to polysaccharides. Klason lignin was determined gravimetrically. Acid-soluble lignin was quantified by measuring absorbance at 215 and 280 nm (Goldschmid, 1971). Analysis was scaled down to 50 mg DM and run in duplicate. Protein content of the EnzHR lignins was evaluated by analysing nitrogen content using a FLASH 2000 Organic Elemental Analyser (Thermo Fisher Scientific, Bremen, Germany). The molar mass distribution of EnzHR lignins was analysed in duplicate using size-exclusion chromatography (SEC) (Rahikainen et al., 2013b). Briefly, the samples were dissolved in 0.1 M NaOH and run on HPLC with UV detection at 280 nm. The average molar masses ($\overline{M}_n$, $\overline{M}_w$) and the polydispersity ($\overline{M}_n/\overline{M}_w$) were calculated with reference to Na-polystyrene sulphonate standards.

2.6 Microscopy of pretreated biomass and EnzHR lignin

Fluorescence microscopy was used to image the microstructure of pretreated spruce and wheat straw (200 °C) before lignin isolation. The samples were embedded in hydroxyethyl methacrylate resin and cut into sections according to Rahikainen et al. (2011). Aqueous 0.01% (w/v) Calcofluor White (Fluorescent brightener 28, Aldrich, Germany) stain was used to visualise cellulose (blue stain) and aqueous 0.1% (w/v) Acid Fuchsin (BDH Chemicals Ltd., Poole, Dorset UK) in 1.0% acetic acid was used as a counter stain. Imaging was performed using excitation wavelength light, epifluorescence 390-420 nm, fluorescence 450-700 nm, with a Zeiss AxioImager M.2

microscope (Carl Zeiss GmbH, Göttingen, Germany). Micrographs were obtained using a Zeiss Axiocam 506 CCD colour camera (Zeiss) and the Zen imaging software (Zeiss).

2.7 Inhibitory effect of EnzHR lignin in Avicel hydrolysis

Inhibitory effect of EnzHR lignin in the enzymatic hydrolysis of microcrystalline cellulose (Avicel, Serva, Heidelberg, Germany) was studied by adding 1% (w/v) of EnzHR lignin to the hydrolysis of 1% (w/v) Avicel. Enzyme preparations, Celluclast 1.5L and Novozyme 188 (Novozymes, Bagsvaerd, Denmark), were dosed at 10 FPU/g cellulose and 200 nkat β-glucosidase activity/g cellulose, respectively. Control reactions without added lignin or Avicel were used. Triplicate reactions were carried out in 50 mM Na-acetate buffer, pH 5, at 45 °C in 0.5 ml volume in a shaking incubator (Thermo micromixer Mxi4t, Finepcr, Seoul, Korea) for 4, 24 and 48 h. Hydrolysis yields were quantified by measuring reducing sugars using the DNS method (Miller, 1959).

2.8 Enzyme activity and adsorption assays

Trichoderma reesei cellobiohydrolase *Tr*Cel7A was produced and purified in-house according to (Suurnäkki et al., 2000). Adsorption and inactivation of *Tr*Cel7A by EnzHR lignins was analysed as described in Rahikainen et al. (2011) with minor modifications. Briefly, 1.4 µM *Tr*Cel7A with 1% of EnzHR lignin in 50 mM Na-acetate buffer, pH 5, 45 °C in 1.5 ml volume was mixed at 30 rpm, in triplicate. A homogenous aliquot of 250 µl was taken at time points 4 and 24 h and centrifuged to separate the supernatant (free enzyme in solution) and solid fraction (lignin-bound enzyme). Control reactions containing either enzyme without lignin or lignin without enzyme were carried out simultaneously. The lignin fraction was washed with 250 µl of buffer (wash buffer) to remove weakly bound enzymes and resuspended with buffer to the initial volume. Cellobiohydrolase activity was determined with an end-point measurement (45 °C, pH 5, 10 min) from the supernatant, wash buffer and solid fraction using 1 mM 4-methyl umbelliferyl-β-D-lactoside (MULac) as substrate (Bailey and Tähtiharju, 2003). The

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MU released by the enzyme was quantified by measuring absorbance at 370 nm (Varioskan, Thermo electron corporation, Finland) against MU standards. The lignin suspensions were mixed during the activity measurement and the solids were removed by centrifuge prior to measuring absorbance. Distribution of *Tr*Cel7A in soluble and lignin-bound fractions was analysed after 24 h incubation with sodium dodecyl sulphate polyacrylamide gel-electrophoresis (SDS-PAGE) (Laemmli, 1970) using 4-20 % Tris-HCl 1.0 mm Criterion precast gels. Undiluted samples (20 µl) from the supernatant and solid fraction were mixed with 7 µl of SDS-sample buffer and boiled for 5 min. The gel was run at 180 V for 47 min. Results were visualised by a stain-free system using ImageLab 1.0 software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

3. Results and discussion

3.1 Pretreatment severity and post-processing of the biomasses

Spruce and wheat straw were hydrothermally pretreated for 10 min at 180, 200 and 220 °C with or without an acid catalyst (0.4 % w/w H_2SO_4) (Table 1). Pretreatment conditions are usually milder for wheat straw than for spruce (Jensen et al., 2017; Nakagame et al., 2010; Rahikainen et al., 2011). However, in this study, similar conditions were chosen for both biomass types to compare the effect of pretreatment severity on the lignins. Two methods were applied for calculation of the severity factors. The severity factor log R_0 calculated according to Overend et al. (1987) is based on the pretreatment time and temperature and, as such, it did not differentiate between the acid catalysed and non-catalysed pretreatments (Table 1). The combined severity factor (CSF) (Chum et al., 1990) includes the pH after pretreatment. It varied from 0.2 to 2.8 for spruce and -1.4 to 3.2 for wheat straw (Table 1). The CSF was higher for spruce in each condition. The most extreme pretreatment for spruce, 220 °C with acid, resulted in a black charred product and this sample was excluded from further analysis.

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Acetone extraction of non-pretreated and pretreated biomasses was carried out in order to remove soluble compounds that may interfere with the lignin analysis. It was noted that during acetone extraction a significant amount (1-25 w%) of acetone-soluble compounds were removed, especially from biomasses from the higher pretreatment severities (Table 1). The most severe pretreatment resulted in a mass loss of 17% for spruce, and 25% for wheat straw (Table 1). Acetone is a polar solvent, which dissolves phenols, carbohydrates and inorganic salts. Previously, hydrothermal pretreatment has been noted to increase the amount of acetone-soluble compounds in lignocellulosics (Rahikainen et al., 2013b). The acetone-soluble fraction presumably contained extractives as well as degradation products of lignin and hemicelluloses. Harsher pretreatment conditions apparently caused more degradation of lignin and hemicelluloses and, as a result, low molecular weight fractions were lost in the extraction process.

The acetone-extracted biomass was ball-milled for 90 min with a Retsch mixer mill to reduce particle size and make the susceptibility of non-pretreated and pretreated biomass to enzymatic hydrolysis more similar. Milling enhances enzymatic hydrolysis by increasing the accessible surface area and decreasing the cellulose crystallinity (Pew, 1957). Ball-milling also enabled swelling of the sample in DMSO-d_6 for NMR analysis.

3.2 Effect of pretreatment conditions on residual lignin structure, as analysed by HSQC-NMR

HSQC-NMR analysis was performed on finely milled plant cell wall samples using DMSO-d_6 as a swelling agent (Fig. 1). Traditional lignin analytics require isolation of lignin prior to analysis, whereas HSQC can be performed on finely milled biomass (Kim et al., 2008; Mansfield et al., 2012). The use of biomass minimizes the possible modifications made to the lignin during any lignin isolation step. However, milling time and intensity affects the lignin structure by decreasing the amount of β-O-4 bonds, especially in the high molecular weight region (Zinovyev et al., 2018). In order to keep the possible modification caused by milling constant, a fixed milling time was selected for all materials. The optimal milling time for lignin isolation, may have not been optimal for HSQC analyses. This could have resulted in a loss of some information mainly in the carbohydrate fraction of the most recalcitrant samples, non-pretreated spruce and wheat straw. The bonds detected in the HSQC spectra were calculated in relation to the C9 signals (0.5 $S_{2.6}$ + G). The amount of native-type bonds decreased as pretreatment severity increased for both spruce and wheat straw (Fig. 1). For both biomass types, the level of β-O-4 bonds decreased as pretreatment temperature increased and the acid catalyst further decreased the level (Table 1). In general, the β-O-4 bonds account for approximately half of the internal bonds in native lignin and are the first ones to cleave during pretreatment. Reduction in the amount of β-O-4 bonds during pretreatment is well documented for softwoods, hardwoods, and grasses (Jensen et al., 2017; Li et al., 2007; Rahikainen et al., 2013b).

The internal carbon-carbon (β-5 and β-β) bonds in spruce and wheat straw either decreased or relatively increased depending on the pretreatment conditions and the source of the raw material (Fig. 1). The level of β-5 bonds in spruce remained at 6-8% in samples without an acid catalyst, but slightly increased to 9-12% when an acid catalyst was used (Table 1). The rearrangement of β-O-4 bonds to β-5 bonds has been observed after treatment at high temperature in aqueous environments (Heikkinen et al., 2014; Nimz, 1966). In the case of pretreated wheat straw, the level of β-5 bonds varied between 5-9% and the amount of β-β bonds remained constant at 1% (Table 1). Under the most severe pretreatment condition (220 °C with acid), the signals for the C9 units became weak, affecting the interpretation of the data. Previously, the β-5 and β-β bonds have been observed to either remain constant or increase, when comparing non-

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pretreated wheat straw to hydrothermally pretreated wheat straw (Jensen et al., 2017; Rahikainen et al., 2013b).

No β-β units were detected in non-pretreated wheat straw, although low levels have been previously detected (Jensen et al., 2017; Rahikainen et al., 2013b). Due to the preponderance of lignin γ-acylation, grasses often have some or all of their β-β units in the form of tetrahydrofuran (THF) structures rather than the traditional resinols, as noted in corn lignin (Lan et al., 2015). However, THF-type structures were not detected either. The β-β resinol or THF structures can only be formed in initial monomer dimerization reactions and can therefore be an initiation point for the lignin chain. Thus, the plausible explanation is that other lignin components, such as tricin, which is particularly high in wheat straw lignin, and ferulates, have been the only initiation points in lignification in wheat straw (Eloy et al., 2017).

In grasses, the hydroxycinnamates, mainly *p*-coumarate (*p*CA) and ferulate (FA), acylate the plant cell wall (Ralph, 2010). *p*CA primarily acylates lignin units, but is also found on arabinoxylans; FA is largely on arabinoxylans but has recently been found acylating lignins (Karlen et al., 2016). FA, in particular, is involved in cross-linking hemicelluloses covalently with each other and to lignin via radical coupling (Ralph, 2010). Both the amount of FA and *p*CA decreased as pretreatment temperature increased and, in pretreatments with the acid catalyst, the amount of FA and *p*CA decreased substantially even at lower temperatures (Table 1). Logically, the ester bond between *p*CA and lignin as well as, perhaps, the ether bonds between FA and lignin were cleaved under the acidic pretreatment conditions. These results are in agreement with previous work in which pretreatment at 200 °C resulted in the levels of *p*CA and FA decreasing compared to those in native wheat straw (Heikkinen et al., 2014).

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The flavonoid tricin has recently been discovered to be incorporated into the lignin structure in monocots (Lan et al., 2016). The amount of detected tricin from the HSQC spectra decreased from 19% in non-pretreated wheat straw to zero in pretreatments at higher severities (Table 1). However, the NMR integration severely overestimates end-groups in lignin, such as tricin (Mansfield et al., 2012), and the level of tricin in wheat straw has been measured at ~3% on a lignin basis (Lan et al., 2016). Previously, the amount of tricin has been seen to decrease during pretreatment (Heikkinen et al., 2014; Jensen et al., 2017). Jensen et al. (2017) suggest that tricin may act as a repolymerisation inhibitor, in a similar way as 2-naphtanol, reducing the amount of condensation reactions during pretreatment. Hydrothermal pretreatment conditions will cause the cleavage of bonds and formation of new carbon-carbon bonds through condensation reactions (Li et al., 2007). Acid-catalysed condensation preferentially occurs between the aromatic C5 or C6 and the α-carbon of a neighbouring unit (Li et al., 2007). Tricin has a particularly electron-rich aromatic ring that could be a preferential reaction site for electrophilic aromatic substitution via the benzylic carbonium ions produced under acidic conditions, leading to reduced condensation reactions between the traditional lignin units.

The S/G ratio of non-pretreated wheat straw was 0.54 (Table 1) and is in accordance with previously reported values (Rahikainen et al., 2013b). The S/G ratio for wheat straw appeared to increase from 0.54 to 0.99 as the pretreatment severity increased (Table 1). However, under higher pretreatment severities, the signals for G and S units become weaker and more disperse (Fig. 1), presumably due to complex conversions, which may affect the calculations. The dissociation energy for β-O-S-units is lower than for analogous G-units (Heikkinen et al., 2014); previously the β-O-4 bonds in S-units have been seen to be cleaved during pretreatment more readily than G-units (Cao et al., 2012; Rahikainen et al., 2013b).

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3.4 Composition of EnzHR lignins

The level of residual hemicelluloses (xylans, mannans) in EnzHR lignins was high in non-pretreated materials whereas, in the pretreated materials, the hemicellulose content decreased as pretreatment severity increased for both spruce and wheat straw (Table 2). Approximately 80% of enzymes on a protein weight basis in the cellulase cocktail Celluclast 1.5L are cellulases (Hu et al., 2013). Limited levels of hemicellulase activity in the enzyme cocktail can explain the relatively high level of residual hemicelluloses in non-pretreated raw materials. A large part of hemicelluloses are typically solubilized during hydrothermal pretreatment. A reduced hemicellulose content in the pretreated biomasses likely contributed to the relatively low hemicellulose content in the EnzHR lignins isolated from pretreated materials. It should be noted that some of the glucose detected in the non-pretreated or mildly pretreated (180 and 200 °C) spruce biomass originated from the hemicelluloses, such as glucomannan and galactoglucomannan.

At higher pretreatment severities, the total carbohydrates were more effectively hydrolysed from pretreated wheat straw compared to pretreated spruce (Table 2). For wheat straw EnzHR lignins, the carbohydrate content decreased from 27 to 4% as pretreatment severity increased, whereas for spruce EnzHR lignins the carbohydrate content did not depend on pretreatment severity and varied between 13 and 23% (Table 2). The total lignin content of the EnzHR lignin samples varied between 70-96% of mass (Table 2). The distribution of cellulose and lignin in the pretreated (200 °C) spruce and wheat straw before and after lignin isolation was studied by fluorescence microscopy imaging of resin-embedded semi-thin sections. Prior to lignin isolation from spruce, the green autofluorescence of lignin was evenly distributed and overlapped with the blue stain of cellulose, whereas in wheat straw, lignin appeared in distinct concentrated areas, separate from cellulose. During pretreatment wheat straw lignin was possibly relocated and separated from cellulose. Cellulose was therefore more easily accessible to enzymes resulting in the lower residual carbohydrate content in the wheat straw EnzHR lignin as compared to spruce lignin (Table 2). The high content of residual carbohydrates in lignin isolated from hydrothermally pretreated spruce without an acid catalyst indicated that the pretreatment was inefficient for this raw material. The residual carbohydrates and proteins of isolated lignins can affect the binding of enzymes as well as lignin solubility in lignin analytics. However, regardless of the raw material and lignin isolation method, isolated lignins always contain some residual carbohydrates. Enzymatically isolated lignins have been reported to contain 6-25% of residual carbohydrates (Nakagame et al., 2011; Rahikainen et al., 2013a).

Nitrogen content was measured from lignin samples to give an indication of the protein content of the EnzHR lignins and estimate the efficiency of the protease treatment in the lignin isolation process. The nitrogen content of EnzHR lignins were higher in the more severe pretreatment conditions than in the non-pretreated or mildly pretreated EnzHR lignins (Table 2). The nitrogen detected in spruce EnzHR lignins is shown to result mainly from the cellulases used in the lignin isolation process (Rahikainen et al., 2011). Native wheat straw has a higher protein content than spruce (Rahikainen et al., 2013b). The higher nitrogen content in lignin from pretreated wheat straw is suggested to result from heat induced denaturation of the endogenous protein, resulting in insoluble protein accumulation into the solid residue (Rahikainen et al., 2013b).

3.5 Molar mass distribution of EnzHR lignins

Molar mass distributions (weight-average molecular weight ($\overline{M}_w$), number-average molecular weight ($\overline{M}_n$) and polydispersity) of the EnzHR lignins were analysed using size-exclusion chromatography (SEC) with 0.1 M NaOH as a solvent. The data represents the alkaline soluble fraction of the EnzHR lignin. The $\overline{M}_w$ of non-pretreated spruce and wheat straw are slightly higher than has been previously reported

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(Rahikainen et al., 2013b). The higher $\overline{M}_w$ reported in the current work is most likely due to the higher amount of residual carbohydrates covalently linked to the lignin in the enzymatically isolated EnzHR lignins and possibly because of the milder ball-milling conditions. The $\overline{M}_w$ and polydispersity of spruce EnzHR lignin decreased rapidly as pretreatment severity increased (Table 2). The use of acid catalyst decreased the $\overline{M}_w$ of spruce even at the lower pretreatment temperatures. The decrease in polydispersity from 9.8 to 2.8 as pretreatment severity increased (Table 2) indicated that even the high molecular weight fractions were degraded at higher pretreatment severities. The decrease in $\overline{M}_w$ and polydispersity are likely to be caused by degradation of lignin, but also through the cleavage of lignin carbohydrate complexes (LCCs). Nakagame et al. (2011a) has observed a decrease in the $\overline{M}_w$ of steam-pretreated softwood as pretreatment temperature increased from 190 to 210 °C. In this work, it was observed that a higher degree of degradation of spruce lignin took place in the presence of the acid catalyst when compared to pretreatment without the acid catalyst.

Contrary to spruce, both increase in pretreatment temperature and the addition of an acid catalyst increased the $\overline{M}_w$ and polydispersity of wheat straw EnzHR lignins indicating that high molecular weight fractions were likely being formed (Table 2). Only at the highest pretreatment temperature of 220 °C did the $\overline{M}_w$ of wheat straw lignin decrease when the acid catalyst was used. When comparing the $\overline{M}_w$ of lignin from non-pretreated wheat straw to pretreated wheat straw, both an increase or decrease of $\overline{M}_w$ have been reported (Jensen et al., 2017; Rahikainen et al., 2013b). Although Jensen et al. (2017) initially observes a decrease in $\overline{M}_w$ as pretreatment severity increases, they note that at the highest pretreatment severity of 190 °C with 1% sulphuric acid, a larger molecular fraction is formed. Possibly the higher pretreatment severities employed in

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the current work explains the increase in $\overline{M}_w$ of wheat straw. Nevertheless, it should be noted that a large amount of acetone-soluble fraction was removed prior to lignin isolation, especially for wheat straw after higher pretreatment severities (Table 1). Small lignin fragments may have also been lost during the lignin isolation and acetone extraction resulting in a higher average $\overline{M}_w$ in the SEC analysis of the lignins.

3.7 Lignin inhibition in enzymatic hydrolysis of cellulose

An inhibitory effect of EnzHR lignins (1% w/v) on the hydrolysis of microcrystalline cellulose (1% w/v) was studied using a commercial cellulase mixture (Celluclast 1.5L, 10 FPU/g cellulose) and β-glucosidase (Novozyme 188, 200 nkat/g cellulose) at 45 °C. Recently, compositions of hydrothermal or dilute acid pretreated raw materials have been reported to be 35-70% polysaccharides and 15-50% total lignin (Jensen et al., 2017; Nakagame et al., 2010; Rahikainen et al., 2013b). In the hydrolysis experiment, the cellulose (50% of DM) and EnzHR lignin (50% of DM) concentrations were kept constant in all experiments in order to compare the effect of EnzHR lignin on the hydrolysis of Avicel. Enzymatic hydrolysis of Avicel was not remarkably inhibited by the EnzHR lignin isolated from non-pretreated or mildly (at 180 °C) pretreated spruce as the hydrolysis yield at 48 h was 88-92% compared to the Avicel control (Fig. 2a). Similarly, hydrolysis of Avicel was only slightly inhibited by EnzHR lignins from non-pretreated wheat straw, or wheat straw pretreated at 180 or 200 °C, the hydrolysis yields being 89-91% as compared to the Avicel control (Fig. 2c). Previously, Rahikainen et al. (2013b) observed that non-pretreated spruce and wheat straw lignins cause less inhibition to Avicel hydrolysis than the corresponding lignins from hydrothermally pretreated biomasses. The inhibitory effect of spruce EnzHR lignins gradually strengthened to 44-48% compared to Avicel control after 48 h hydrolysis as pretreatment severity increased, either by higher temperatures (220 °C, Fig. 2a) or by an addition of an acid catalyst (Fig. 2b). In the case of wheat straw, the lowest hydrolysis yields (51-57%) were obtained with lignins from pretreatments at 220 °C as well as 200 and 220 °C with acid catalyst (Fig. 2c and d). Minimal inhibition by lignin from non-pretreated and mildly pretreated spruce and wheat straw indicated that the inhibition was mainly caused by the effects of pretreatment and was not an inherent characteristic of the lignins. The pretreatment temperature had a greater impact on lignin inhibition by spruce lignin than by wheat straw lignin (Fig. 2a and b), whereas the acid catalyst had a more similar impact on the inhibitory effect of both lignins (Fig. 2c and d). In previous studies, the increase in pretreatment temperature of softwood from 190 to 210 °C and hardwood from 150 to 190 °C has been shown to increase lignin inhibition in enzymatic hydrolysis experiments (Nakagame et al., 2011; Sun et al., 2016).

3.8 Inactivation and adsorption of cellobiohydrolase *Tr*Cel7A by EnzHR lignins

Non-productive adsorption and inactivation of enzymes on isolated lignins was studied using purified cellobiohydrolase *Tr*Cel7A (CBH I), the major component in cellulase mixtures produced by *Trichoderma reesei*. The cellulase (1.4 μM) was added at a 1% level (w/w) to EnzHR lignin and incubated at hydrolysis temperature (45 °C) for up to 24 h. Inactivation of *Tr*Cel7A was studied from the solid fraction (lignin-bound enzyme), washing buffer (weakly-bound enzyme) and supernatant (unbound enzyme) by activity measurements using MULac as substrate. Adsorption of *Tr*Cel7A onto lignin was followed by an SDS-PAGE analysis from the solid fraction and the supernatant.

EnzHR lignin isolated from non-pretreated and mildly pretreated spruce and wheat straw did not inhibit the activity of *Tr*Cel7A; however, the lignins from more severe pretreatment conditions, especially with the acid catalyst, were clearly inhibitory (Fig. 3). For spruce, EnzHR lignin from pretreatments at 200 °C and 200 °C with acid decreased total residual enzyme activity to 73% and 35% compared to control, respectively, after 24 h incubation time (Fig. 3a). In the case of wheat straw lignin, only

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the most severe pretreatment at 200 °C with acid reduced the residual activity to 72% (Fig. 3b). The inhibitory effect was more pronounced with spruce lignin than with wheat straw lignin and is in agreement with the lignin-derived inhibition observed in Avicel hydrolysis (section 3.7).

The activity of *Tr*Cel7A was also measured from the solid fraction and washing buffer, but only low enzyme activity was detected in these fractions (Fig. 3), indicating that the lignin-bound enzyme was tightly bound and not easily released by washing from the lignin surface. Rahikainen et al. (2011) studied the non-productive adsorption of enzymes and enzyme activities of a cellulase cocktail in the presence of lignin isolated from pretreated spruce. Enzymes incubated with lignin at hydrolysis temperature (45 °C) were observed to bind strongly to the lignin fractions (Rahikainen et al., 2011).

The SDS-PAGE analysis gives a semi-quantitative estimation of the location of enzymes between the lignin-bound and unbound fractions. For both spruce and wheat straw lignins, as pretreatment severity increased a clear shift from the unbound fraction to the lignin-bound fraction was observed. In the SDS-PAGE, no lignin-bound enzyme was detected on mildly pretreated EnzHR lignins, but as pretreatment severity increased, the lignin-bound enzyme fraction increased to 30-47% for spruce and to 29% for wheat straw. The activity analyses indicated that the enzyme retained its activity in the supernatant, whereas the activity was mostly lost in the lignin bound fractions, especially when the enzyme was bound onto lignins from the harsh pretreatment conditions. Previously it has been observed that non-productive binding of enzymes on isolated softwood lignins increased with the increase in pretreatment temperature (Nakagame et al., 2011). Hydrophobic and ionic interactions are proposed to be involved in non-productive enzyme binding on the lignin surface but the binding mechanism is enzyme-specific (Kellock et al., 2017; Nakagame et al., 2011; Rahikainen et al., 2013b; Sammond et al., 2014). Rahikainen et al. (2011) proposes that, lignin-derived inhibition of enzymes is at least partially caused by denaturation of enzymes on lignin-rich surfaces.

3.9 Relationship between lignin inhibition in Avicel hydrolysis to pretreatment parameters and lignin characteristics

The relationship between lignin inhibition in Avicel hydrolysis and lignin characteristics as well as the severity factor was assessed. For both spruce and wheat straw, the increase in the combined severity factor (CSF) correlated well with increased inhibition in Avicel hydrolysis ($R^2 = 0.78$, Fig. 4a). No correlation was observed between the severity factor (log R_0) and lignin inhibition in Avicel hydrolysis ($R^2 = 0.10$, data not shown). The results from lignin-derived inhibition in Avicel hydrolysis with isolated EnzHR lignins strongly suggest that the CSF, which takes the pretreatment pH into account, is better suited for comparing pretreatment conditions than the conventional severity factor (log R_0). Severity factors have been used to compare pretreatment conditions on the carbohydrate conversion of lignocellulosic materials. However, based on a meta-analysis of pretreatment conditions and relation to severity factors it was concluded that no severity factor was able to universally predict total carbohydrate conversion, whereas the pretreatment pH showed some predictability (Pedersen and Meyer, 2010).

A strong correlation ($R^2 = 0.84$, Fig. 4b) between the decrease of β-O-4 bonds and lignin inhibition in Avicel hydrolysis was observed. No clear correlation was seen in relation to other lignin characteristics (data not shown). Free phenolic hydroxyls are formed as β-O-4 bonds are cleaved. The increase in phenolic hydroxyls in lignin is reported to strongly correlate with the inhibition by pretreated lignocellulose in the enzymatic hydrolysis (Sun et al., 2016; Yang and Pan, 2016). According to Sun et al. (2016), the increase in phenolic hydroxyls and hydrophobicity via condensed aromatic rings

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increase hydrogen bonding as well as hydrophobic interactions between enzymes and lignin. Yang and Pan (2016) decreased the level of phenolic hydroxyls on the lignin surface by blocking the phenolic hydroxyl groups with propylene oxide in lignins from organosolv-pretreated pine and poplar. Although the hydrolysis yields are improved, the decrease in phenolic hydroxyls do not significantly affect the adsorption of the enzymes. As discussed previously in section 3.8, hydrophobic interactions between lignin and the enzyme core are proposed to be involved in enzyme inactivation on the lignin surface (Rahikainen et al., 2011). The observation by Yang and Pan (2016), that enzymes adsorb onto the lignin surface but remain active when phenolic hydroxyls are protected, could indicate that phenolic hydroxyls are at least partially involved in inactivation of enzymes on the lignin surface.

Lignin from pretreated softwood is commonly considered more inhibitory to enzymatic hydrolysis than lignin from pretreated grasses (Nakagame et al., 2010; Rahikainen et al., 2013b). The pretreatment temperatures for grasses are generally lower, typically <200 °C, than for softwood, typically >200 °C. Although the pretreatment conditions in the current work were initially set to be the same for both spruce and wheat straw, the severities were greater for spruce than for wheat straw, as indicated by the pH measured after pretreatment and observed in the difference of CSF (Table 1). Under similar pretreatment severities, wheat straw lignin was as inhibitory to Avicel hydrolysis as spruce lignin (Fig. 4a). This indicates that the lower pretreatment severity needed for wheat straw compared to spruce is the main the cause for the observed lower inhibition of wheat straw lignin, rather than differences in lignin chemistry between the raw materials. Wheat straw lignin has hydroxycinnamates incorporated into the lignin structure as well as an initially lower lignin content compared to spruce. This may contribute to the better response of wheat straw to pretreatment, thus leading to lower degree of detrimental alterations in lignin structure at optimal pretreatment severity.

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4 Conclusions

Lignins isolated from non-pretreated or mildly pretreated spruce and wheat straw exhibited minor inhibition on Avicel hydrolysis, whereas lignins from higher pretreatment severities caused greater inhibition. Lignin inhibition was therefore mainly a pretreatment-induced effect. Applying an acid catalyst during pretreatment, even at lower temperatures, increased the inhibition. At similar pretreatment severities, wheat straw lignin was as inhibitory as spruce lignin. The inhibition resulted from non-productive binding and inactivation of enzymes on the lignin surface. The decrease in β-O-4 bonds in lignin, an indication of how severely lignin was modified from its native form, correlated to lignin inhibition in Avicel hydrolysis.

E-supplementary data of this work can be found in e-version of the paper online.

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References

1. Bailey, M.J., Tähtiharju, J., 2003. Efficient cellulase production by *Trichoderma reesei* in continuous cultivation on lactose medium with a computer-controlled feeding strategy. Appl. Microbiol. Biotechnol. 62, 156–162.
2. Cao, S., Pu, Y., Studer, M., Wyman, C., Ragauskas, A.J., 2012. Chemical transformations of *Populus trichocarpa* during dilute acid pretreatment. RSC Adv. 2, 10925–10936.
3. Chum, H.L., Johnson, D.K., Black, S.K., Overend, R.P., 1990. Pretreatment-catalyst

doi: 10.1016/j.biortech.2019.02.051

effects and the combined severity parameter. Appl. Biochem. Biotechnol. 24–25, 1–14.

4. Donohoe, B.S., Decker, S.R., Tucker, M.P., Himmel, M.E., Vinzant, T.B., 2008. Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment. Biotechnol. Bioeng. 101, 913–925.

5. Eloy, N.B., Voorend, W., Lan, W., Saleme, M. de L.S., Cesarino, I., Vanholme, R., Smith, R.A., Goeminne, G., Pallidis, A., Morreel, K., Nicomedes, J., Ralph, J., Boerjan, W., 2017. Silencing *CHALCONE SYNTHASE* in maize impedes the incorporation of tricin into lignin and increases lignin content. Plant Physiol. 173, 998–1016.

6. Goldschmid, O., 1971. Ultraviolet spectra, in: Sakranen, K. V., Ludwig, C.H. (Eds.), Lignins: Occurrence, Formation, Structure and Reactions. John Wiley & Sons, New York, pp. 241–266.

7. Heikkinen, H., Elder, T., Maaheimo, H., Rovio, S., Rahikainen, J., Kruus, K., Tamminen, T., 2014. Impact of steam explosion on the wheat straw lignin structure studied by solution-state nuclear magnetic resonance and density functional methods. J. Agric. Food Chem. 62, 10437–10444.

8. Hu, J., Arantes, V., Pribowo, A., Saddler, J.N., 2013. The synergistic action of accessory enzymes enhances the hydrolytic potential of a “cellulase mixture” but is highly substrate specific. Biotechnol. Biofuels 6, 1–12.

9. Jensen, A., Cabrera, Y., Hsieh, C.W., Nielsen, J., Ralph, J., Felby, C., 2017. 2D NMR characterization of wheat straw residual lignin after dilute acid pretreatment with different severities. Holzforschung 71, 461–469.

10. Karlen, S.D., Zhang, C., Peck, M.L., Smith, R.A., Padmakshan, D., Helmich, K.E., Free, H.C.A., Lee, S., Smith, B.G., Lu, F., Sedbrook, J.C., Sibout, R., Grabber, J.H., Runge, T.M., Mysore, K.S., Harris, P.J., Bartley, L.E., Ralph, J., 2016.

Monolignol ferulate conjugates are naturally incorporated into plant lignins. Sci. Adv. 2.

11. Kellock, M., Rahikainen, J., Marjamaa, K., Kruus, K., 2017. Lignin-derived inhibition of monocomponent cellulases and a xylanase in the hydrolysis of lignocellulosics. Bioresour. Technol. 232, 183–191.
12. Kim, H., Ralph, J., Akiyama, T., 2008. Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d_6. BioEnergy Res. 1, 56–66.
13. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
14. Lan, W., Lu, F., Regner, M., Zhu, Y., Rencoret, J., Ralph, S.A., Zakai, U.I., Morreel, K., Boerjan, W., Ralph, J., 2015. Tricin, a flavonoid monomer in monocot lignification. Plant Physiol. 167, 1284–1295.
15. Lan, W., Rencoret, J., Lu, F., Karlen, S.D., Smith, B.G., Harris, P.J., del Río, J.C., Ralph, J., 2016. Tricin-lignins: occurrence and quantitation of tricin in relation to phylogeny. Plant J. 88, 1046–1057.
16. Li, J., Henriksson, G., Gellerstedt, G., 2007. Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion. Bioresour. Technol. 98, 3061–3068.
17. Mansfield, S.D., Kim, H., Lu, F., Ralph, J., 2012. Whole plant cell wall characterization using solution-state 2D NMR. Nat. Protoc. 7, 1579–1589.
18. Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.
19. Nakagame, S., Chandra, R.P., Kadla, J.F., Saddler, J.N., 2011. The isolation, characterization and effect of lignin isolated from steam pretreated Douglas-fir on the enzymatic hydrolysis of cellulose. Bioresour. Technol. 102, 4507–4517.
20. Nakagame, S., Chandra, R.P., Saddler, J.N., 2010. The effect of isolated lignins,

doi: 10.1016/j.biortech.2019.02.051

obtained from a range of pretreated lignocellulosic substrates, on enzymatic hydrolysis. Biotechnol. Bioeng. 105, 871–879.

21. Nimz, H., 1966. A new type of rearrangement in the lignin field. Angew. Chemie Int. Ed. English 5, 843.

22. Overend, R.P., Chornet, E., Gascoigne, J.A., 1987. Fractionation of lignocellulosics by steam-aqueous pretreatments [and discussion]. Philos. Trans. R. Soc. A Math. Phys. Eng. Sci. 321, 523–536.

23. Pedersen, M., Meyer, A.S., 2010. Lignocellulose pretreatment severity – relating pH to biomatrix opening. N. Biotechnol. 27, 739–750.

24. Pew, J.C., 1957. Properties of powered wood and isolation of lignin by cellulytic enzymes. Tappi 40, 553–557.

25. Pu, Y., Hu, F., Huang, F., Davison, B.H., Ragauskas, A.J., 2013. Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments. Biotechnol. Biofuels 6, 15.

26. Rahikainen, J., Evans, J.D., Mikander, S., Kalliola, A., Puranen, T., Tamminen, T., Marjamaa, K., Kruus, K., 2013a. Cellulase-lignin interactions—the role of carbohydrate-binding module and pH in non-productive binding. Enzyme Microb. Technol. 53, 315–21.

27. Rahikainen, J., Martin-Sampedro, R., Heikkinen, H., Rovio, S., Marjamaa, K., Tamminen, T., Rojas, O.J., Kruus, K., 2013b. Inhibitory effect of lignin during cellulose bioconversion: the effect of lignin chemistry on non-productive enzyme adsorption. Bioresour. Technol. 133, 270–8.

28. Rahikainen, J., Mikander, S., Marjamaa, K., Tamminen, T., Lappas, A., Viikari, L., Kruus, K., 2011. Inhibition of enzymatic hydrolysis by residual lignins from softwood - study of enzyme binding and inactivation on lignin-rich surface. Biotechnol. Bioeng. 108, 2823–2834.

29. Ralph, J., 2010. Hydroxycinnamates in lignification. Phytochem. Rev. 9, 65–83.

30. Sammond, D.W., Yarbrough, J.M., Mansfield, E., Bomble, Y.J., Hobdey, S.E., Decker, S.R., Taylor, L.E., Resch, M.G., Bozell, J.J., Himmel, M.E., Vinzant, T.B., Crowley, M.F., 2014. Predicting enzyme adsorption to lignin films by calculating enzyme surface hydrophobicity. J. Biol. Chem. 289, 20960–9.

31. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2012. NREL, Determination of structural carbohydrates and lignin in Biomass, Laboratory analytical procedure (LAP) Issue date:4/25/2008 17.

32. Sun, S., Huang, Y., Sun, R., Tu, M., 2016. The strong association of condensed phenolic moieties in isolated lignins with their inhibition of enzymatic hydrolysis. Green Chem. 18, 4276–4286.

33. Suurnäkki, A., Tenkanen, M., Siika-Aho, M., Niku-Paavola, M.-L., Viikari, L., Buchert, J., 2000. *Trichoderma reesei* cellulases and their core domains in the hydrolysis and modification of chemical pulp. Cellulose 7, 189–209.

34. Tenkanen, M., Siika-Aho, M., 2000. An α-glucuronidase of *Schizophyllum commune* acting on polymeric xylan. J. Biotechnol. 78, 149–161.

35. Yang, Q., Pan, X., 2016. Correlation between lignin physicochemical properties and inhibition to enzymatic hydrolysis of cellulose. Biotechnol. Bioeng. 113, 1213–1224.

36. Zhang, H., Fangel, J.U., Willats, W.G.T., Selig, M.J., Lindedam, J., Jørgensen, H., Felby, C., 2014. Assessment of leaf/stem ratio in wheat straw feedstock and impact on enzymatic conversion. GCB Bioenergy 6, 90–96.

37. Zinovyev, G., Sumerskii, I., Rosenau, T., Balakshin, M., Potthast, A., 2018. Ball milling's effect on pine milled wood lignin's structure and molar mass. Molecules 23, 2223.

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Figure captions

Table 1. Pretreatment conditions, severity factors, and acetone-soluble compounds for the non-pretreated and pretreated materials. All pretreatments were performed for 10 min at the selected temperature with or without an acid catalyst. Acetone-soluble compounds were extracted after pretreatment from dry biomass. The levels of units (characterized by their interunit linkages) and S/G ratio derived from volume-integrals in whole-cell-wall HSQC-NMR spectra are expressed as % per C_9 units ($0.5S_{2/6}+G_2$) detected in spruce and wheat straw.

Table 2. Chemical composition and characteristics of enzymatically isolated lignin-rich hydrolysis residues (EnzHR). Results shown are % (w/w) of dry mass. Total lignin includes Klason lignin and acid-soluble lignin. Hemicelluloses are the sum of xylose, mannose, galactose, rhamnose and arabinose after anhydro correction. Cellulose is calculated as glucose after anhydro correction. $\overline{M}_w$ = weight-average molecular weight, $\overline{M}_n$ = number-average molecular weight, FA = ferulate, *p*CA = *p*-coumarate.

Fig. 1. ^{1}H–^{13}C HSQC NMR partial spectra of spruce (top row) and wheat straw (bottom row) biomass, each non-pretreated, pretreated at 200 °C, or pretreated at 200 °C with acid.

Fig. 2. Inhibitory effect of lignin-rich enzymatic hydrolysis residue (EnzHR) in Avicel hydrolysis. Avicel 1% (w/v) was hydrolysed for 4, 24 and 48 h in the presence of 1% (w/v) (a, b) spruce EnzHR lignin and (c, d) wheat straw EnzHR lignin. Avicel without added lignin was used as a reference. The enzyme mixture contained 10 FPU/g cellulase Celluclast 1.5L supplemented with 200 nkat/g cellulase Novozyme 188 and the hydrolysis was performed in 50 mM Na-acetate buffer, pH 5 at 45 °C.

Fig 3. Effect of pretreatment severity on the activity of *Tr*Cel7A (1.4 μM) in the presence of lignin-rich enzymatic hydrolysis residue (EnzHR lignin) (1% w/v) at 45 °C,

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pH 5. Aliquots were taken at 4 and 24 h and the solid fraction was separated from the supernatant by centrifugation. The solid fraction was washed once with fresh buffer (washing buffer). Samples of the supernatant (free enzyme) and solid fraction (lignin-bound enzyme) were analysed for enzyme activity using MULac as substrate. Enzyme control without lignin and lignin control without enzyme were used.

Fig. 4. Correlation between lignin inhibition by enzymatic hydrolysis residues (EnzHR lignins) in Avicel hydrolysis and (a) combined severity factor (CSF), (b) level of β-O-4 units in EnzHR lignins. Open squares are for spruce EnzHR lignins and black squares for wheat straw EnzHR lignins.

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Table 1.

Raw material	Pretreatment temperature (°C)	Acid catalyst H_2SO_4 (% w/w dm)	pH after pretreatment	Severity factor (log R_0)[a]	Combined severity factor (CSF)[b]	Acetone-soluble compounds (% w/w of dm)	β-O-4	β-5	β-β	S/G	FA	*p*CA	Tricin
Spruce	non-pretreated					0.8	40	6	nq	-	-	-	-
	180	-	3.2	3.4	0.2	1.8	37	8	nq	-	-	-	-
	200	-	3.1	3.9	0.8	3.9	20	6	nq	-	-	-	-
	220	-	2.8	4.5	1.7	9.0	3	8	-	-	-	-	-
	180	0.4	1.1	3.4	2.2	3.7	14	9	-	-	-	-	-
	200	0.4	1.1	3.9	2.8	16.8	2	12	-	-	-	-	-
Wheat straw	non-pretreated					2.1	58	-	-	0.54	7	7	19
	180	-	4.7	3.4	-1.4	2.4	37	5	1	0.61	8	5	1
	200	-	4.1	3.9	-0.2	6.2	28	7	1	0.84	9	5	nq
	220	-	3.5	4.5	1.0	16.0	15	4	1	0.59	-	1	nq
	180	0.4	1.3	3.4	2.1	13.1	16	9	1	0.65	4	5	nq
	200	0.4	1.3	3.9	2.7	17.8	13	5	1	0.99	5	3	nq
	220	0.4	1.3	4.5	3.2	25.1	*	*	*	*	*	*	*

[a] calculated based on Overend et al. (1987)

[b] calculated based on Chum et al. (1990)

dm = dry matter

nq = not quantifiable

*G_2 signal missing, $S_{2/6}$ signal weak

Table 2.

Sample	Pretreatment condition	Total lignin (%)	Total carbohydrates (%)	Cellulose (%)	Hemicelluloses (%)	Nitrogen (%)	$\overline{M}_w$ (Da)	$\overline{M}_n$ (Da)	Polydispersity ($\overline{M}_w/\overline{M}_n$)
Spruce EnzHR lignin	Non-pretreated	74	18	6.5[a]	11.8	0.3	18900	2300	8.2
	180 °C	88	13	5.8[a]	6.7	0.4	20900	2100	9.8
	200 °C	82	19	15.8[a]	2.5	0.6	11600	1800	6.5
	220 °C	82	22	20.9	0.4	0.6	8100	2000	4.1
	180 °C + acid	74	23	22.3	0.7	0.6	4300	1400	3.1
	200 °C + acid	85	13	12.8	0.2	0.6	4700	1700	2.8
Wheat straw EnzHR lignin	Non-pretreated	70	27	11.3	15.1	0.4	7700	2000	3.9
	180 °C	79	17	7.2	9.7	0.6	12300	2500	4.9
	200 °C	91	7	2.3	4.3	1.1	15500	2800	5.5
	220 °C	96	4	3.2	0.3	1.6	20200	3200	6.2
	180 °C + acid	94	5	4.5	0.5	1.4	26600	3000	8.9
	200 °C + acid	92	6	5.7	0.3	1.3	20200	2700	7.4
	220 °C + acid	95	4	4.1	0.1	1.0	9300	2200	4.3

[a] Cellulose may be overestimated as some of the glucose may originate from hemicelluloses such as galactoglucomannan and glucomannan.

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Fig 1. COLOUR FIGURE IN PRINTED VERSION AND ONLINE VERSION

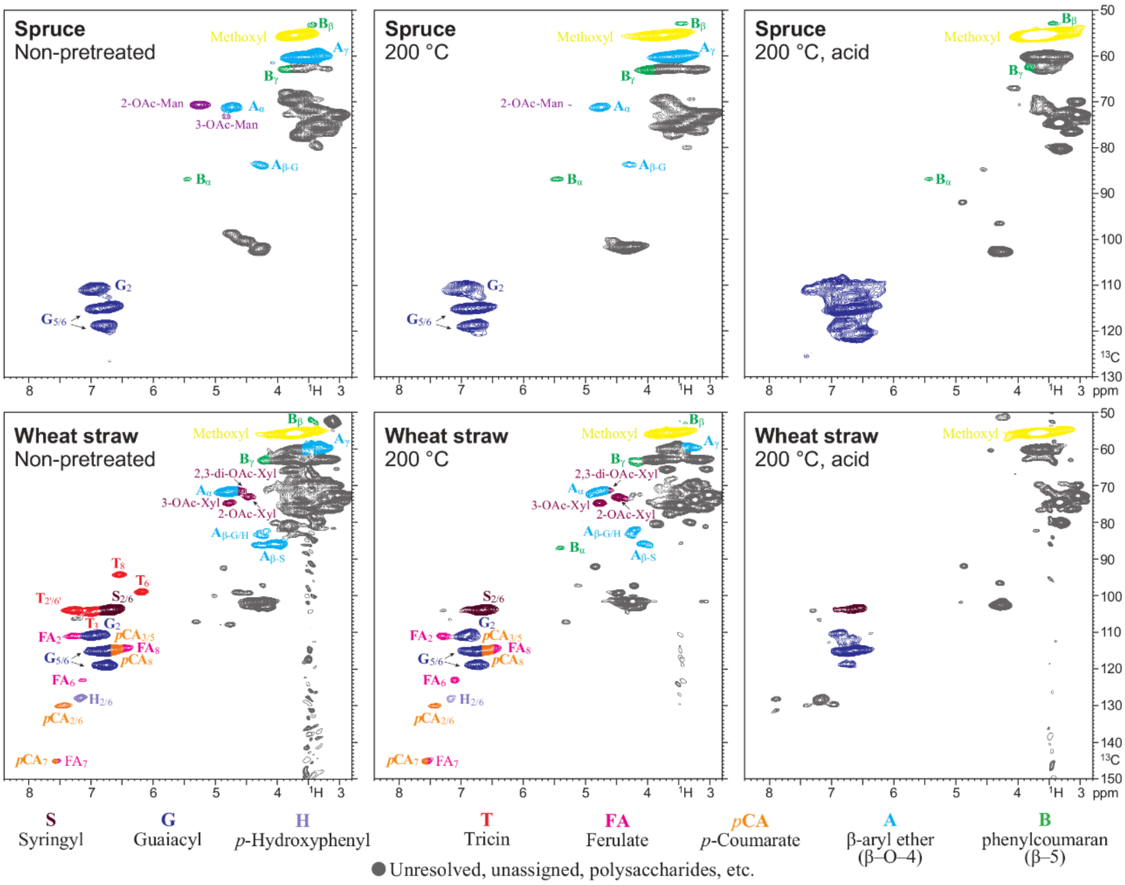
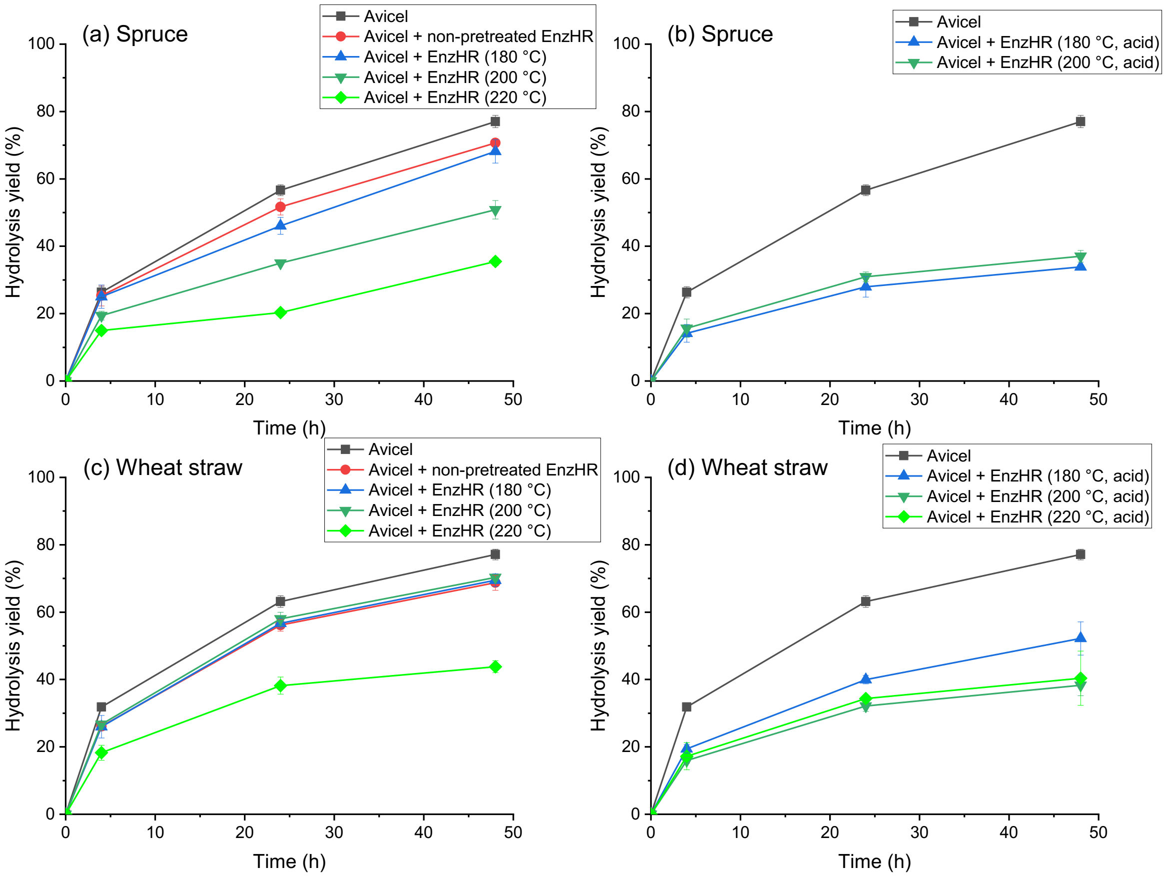


Fig. 2 COLOUR FIGURE IN ONLINE VERSION ONLY



711 **Fig. 3**

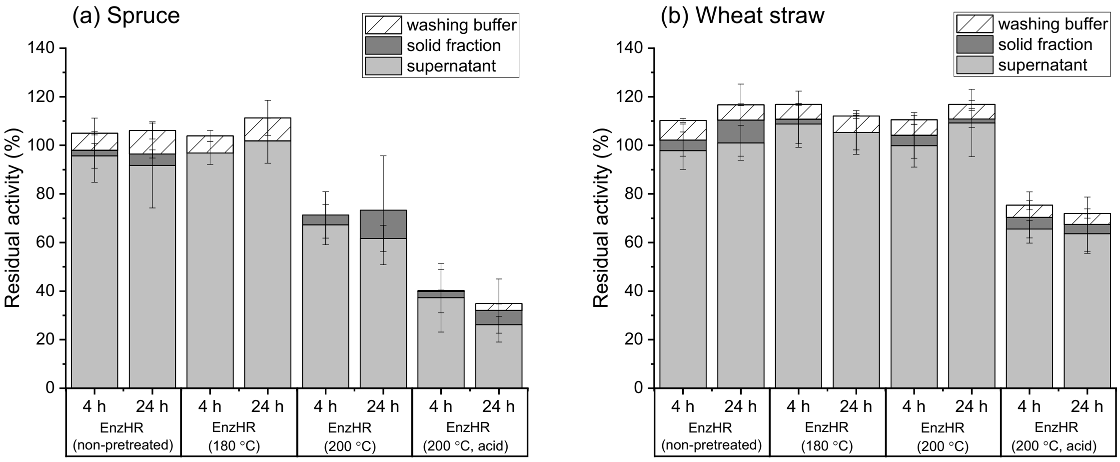


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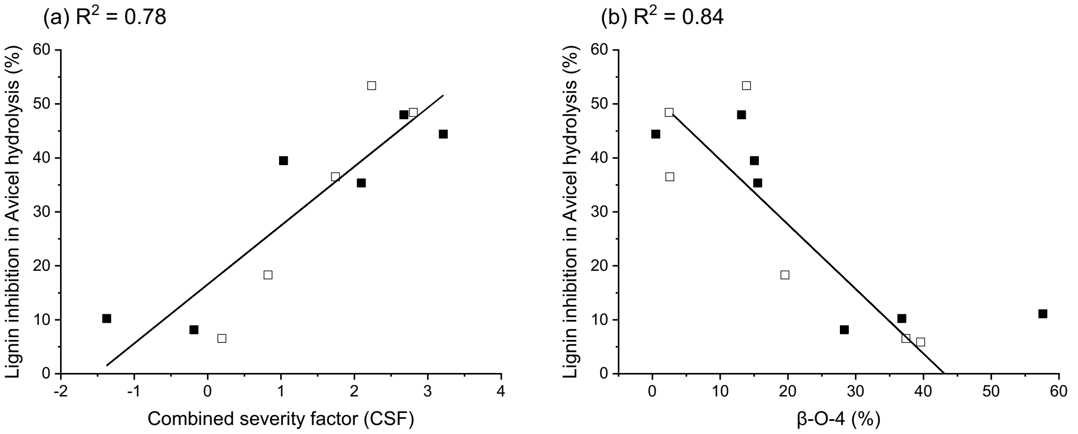
Fig. 4



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Supplementary Material for:

Effect of hydrothermal pretreatment severity on lignin inhibition in enzymatic hydrolysis

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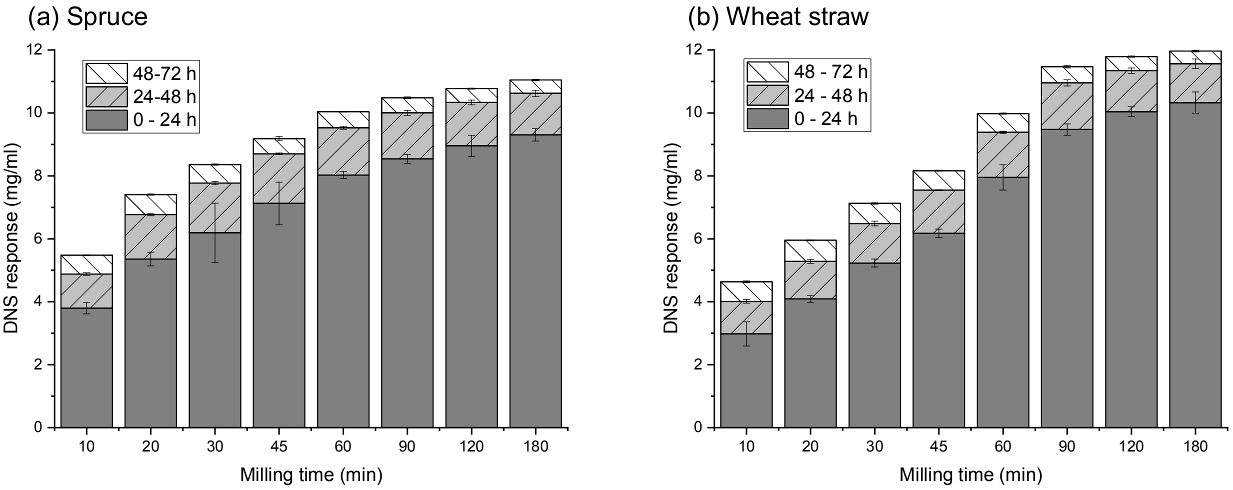

Fig. S1. Effect of milling time in a mixer mill on the enzymatic hydrolysis of (a) non-pretreated spruce and (b) non-pretreated wheat straw. Milling was performed on washed, freeze dried, and acetone-extracted raw material. Milling time was optimised with non-pretreated spruce and wheat straw in order to minimise milling time and potential modification made to lignin, but also maximise enzymatic removal of carbohydrates under hydrolysis conditions. The released sugars were estimated by measuring the DNS response of the hydrolysate. The DNS response includes reducing sugars as well as the background from the biomass.

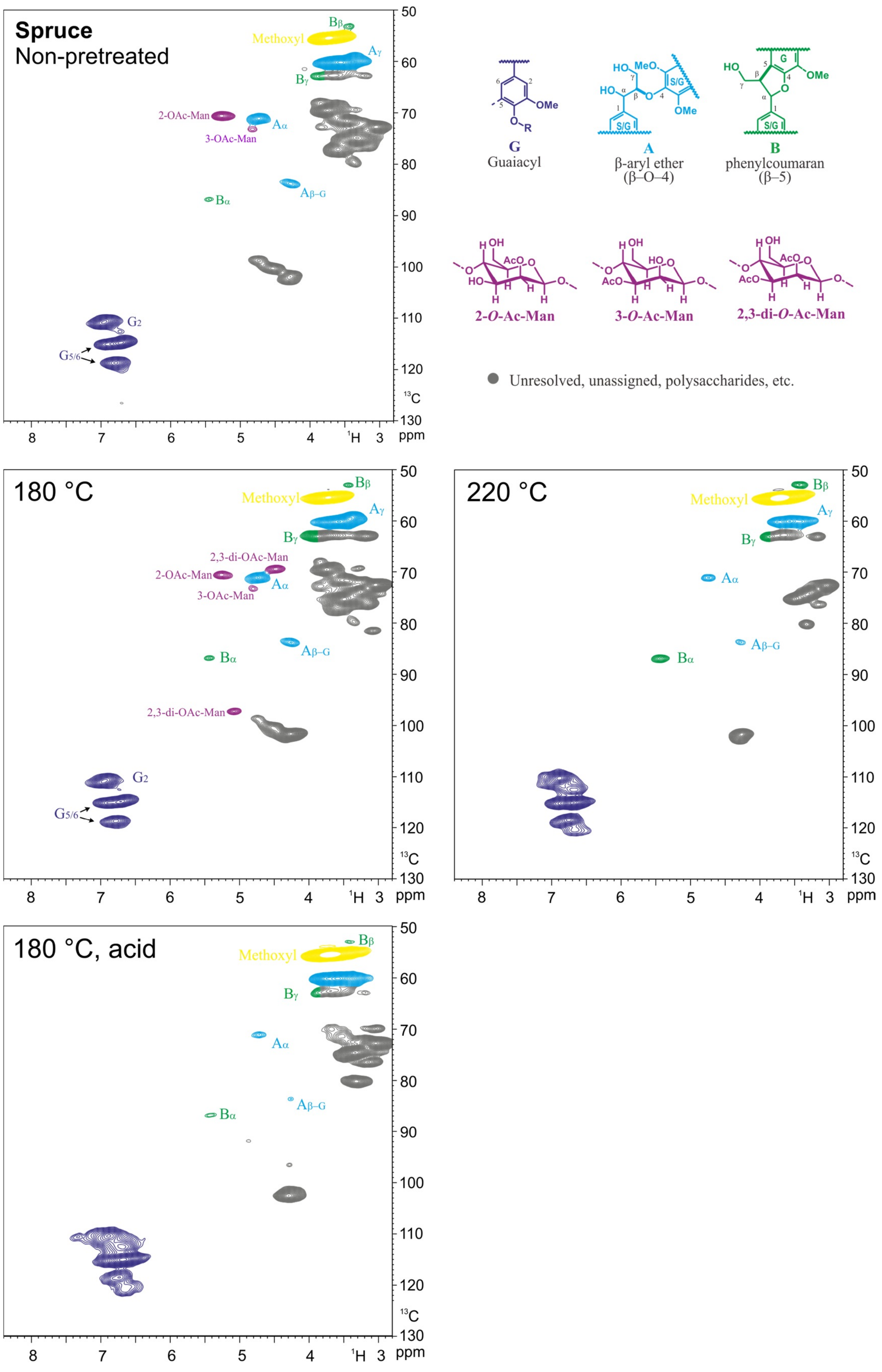

Fig. S2. ^{1}H–^{13}C HSQC NMR partial spectra of non-pretreated and pretreated spruce biomass. Pretreatment was performed for 10 min at 180, 200 or 220 °C with or without an acid catalyst. Spectra of pretreatments at 200 °C with or without an acid catalyst are presented in Fig 1.

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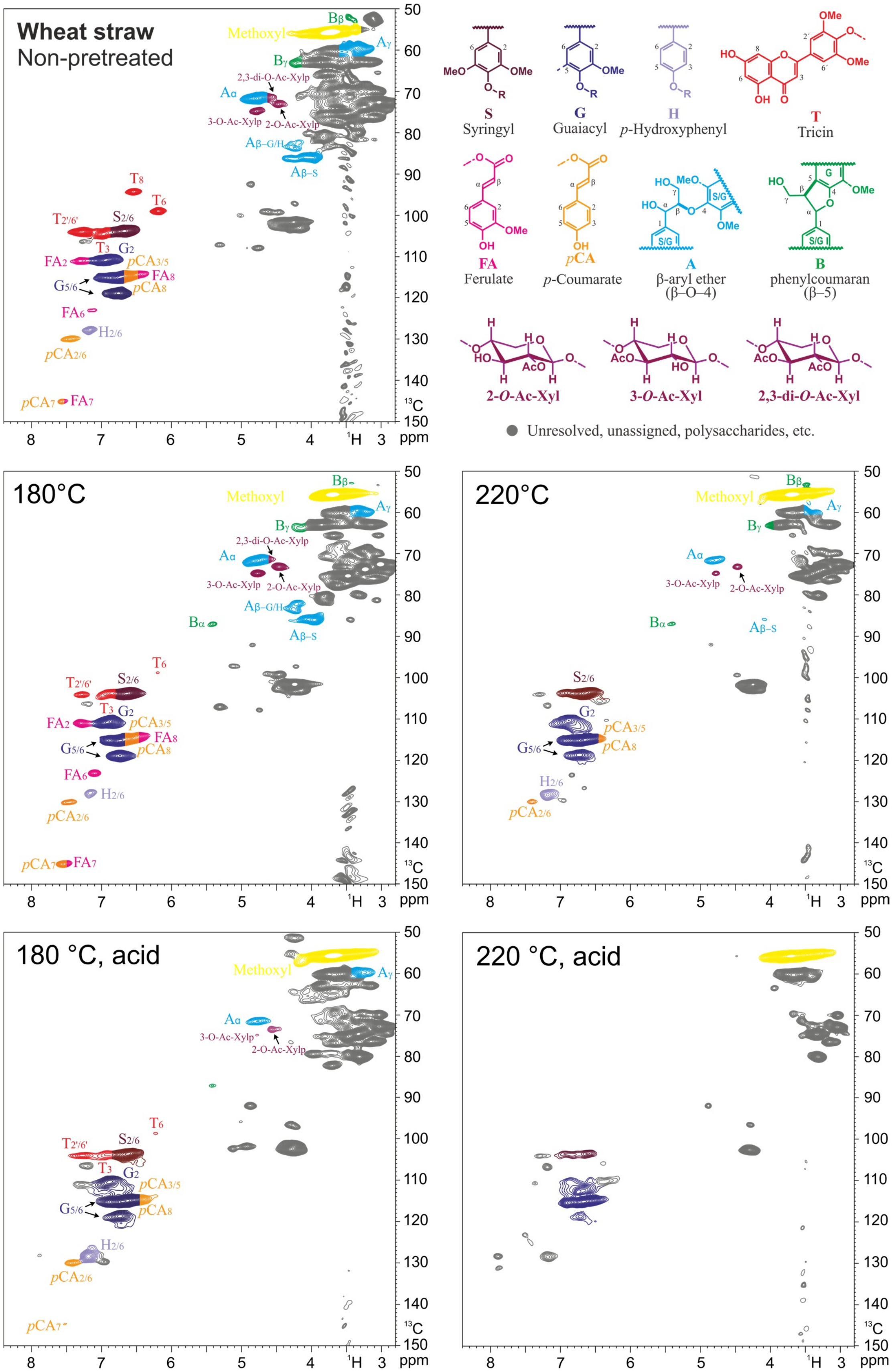

Fig. S3. ^{1}H–^{13}C HSQC NMR partial spectra of non-pretreated and pretreated wheat straw. Pretreatment was performed for 10 min at 180, 200 or 220 °C with or without an acid catalyst. Spectra of pretreatments at 200 °C with or without an acid catalyst are presented in Fig 1.

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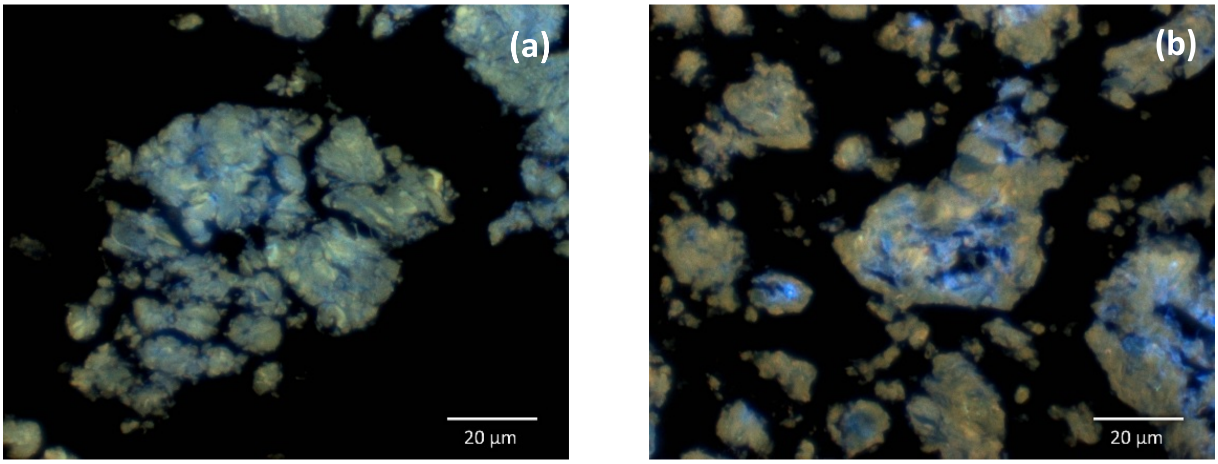
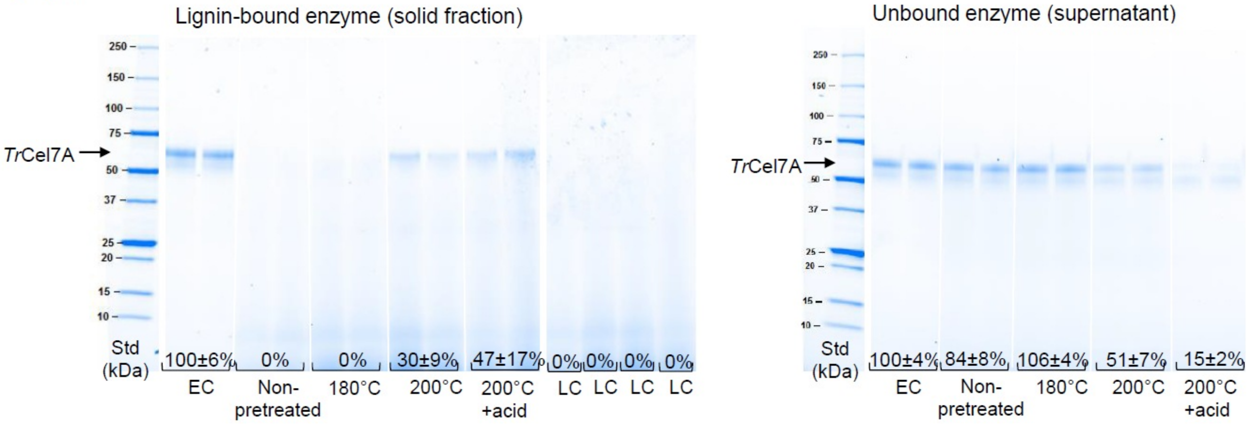


Fig. S4. Fluorescence microscopic images of pretreated (200 ºC) and ball-milled (a) spruce and (b) wheat straw. Cellulose appears blue by staining with Calcofluor White stain and other colours visible arise from the autofluorescence of lignin in the sample.

(a) Spruce EnzHR lignin



(b) Wheat straw EnzHR lignin

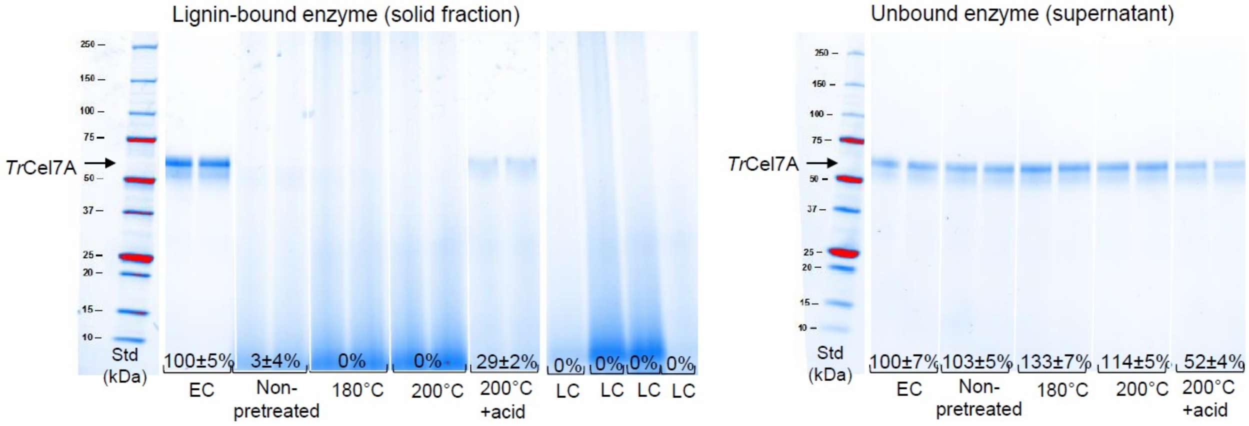


Fig. S5. Adsorption of the major cellulase of *Trichoderma reesei Tr*Cel7A onto (a) lignin-rich enzymatic hydrolysis residues (EnzHR lignin) of spruce and (b) EnzHR lignin of wheat straw studied by SDS-PAGE. The adsorption of 1.4 µM *Tr*Cel7A onto EnzHR lignins was performed at 1% lignin concentration, 50 mM Na-acetate buffer, pH 5, at 45 °C. After mixing for 24 h, an aliquot was taken and the solid fraction was separated from the supernatant by centrifugation. The solid fraction was washed once with fresh buffer. Samples of the supernatant (unbound enzyme) and solid fraction (lignin-bound enzyme) were run on SDS-PAGE. Enzyme control without lignin (EC) and lignin control without enzyme (LC) were used. Average band intensity compared to EC (%) is presented below the bands.

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