Postprint of: *Biomacromolecules* (Just Accepted Manuscript) (2017)

This document is confidential and is proprietary to the American Chemical Society and its authors. Do not copy or disclose without written permission. If you have received this item in error, notify the sender and delete all copies.

**Lignin films from spruce, eucalyptus and wheat straw
studied with electroacoustic and optical sensors: Effect of
composition and electrostatic screening on enzyme binding**

Journal:	<i>Biomacromolecules</i>
Manuscript ID	bm-2017-00071x.R1
Manuscript Type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Pereira, Antonio; IRNAS-CSIC, Plant Biotechnology Hoeger, Ingrid; North Carolina State University Ferrer Carrera, Ana; North Carolina State University, Forest Biomaterials Rencoret, Jorge; IRNAS-CSIC, Plant Biotechnology Del Rio, Jose; CSIC, Instituto de Recursos Naturales y Agrobiologia de Sevilla Kruus, Kristiina; 3VTT Technical Research Centre of Finland Ltd Rahikainen, Jenni; VTT, Kellock, Miriam; VTT Gutierrez, Ana; IRNAS-CSIC, Rojas, Orlando; Aalto University , Bioproducts and Biosystems, School of Chemical Engineering

SCHOLARONE™
Manuscripts

Lignin films from spruce, eucalyptus and wheat straw studied with electroacoustic and optical sensors: Effect of composition and electrostatic screening on enzyme binding

Antonio Pereira^{1,2}, Ingrid C. Hoeger², Ana Ferrer Carrera², Jorge Rencoret¹, José C del Rio¹, Kristiina Kruus³, Jenni Rahikainen³, Miriam Kellock³, Ana Gutierrez¹, Orlando J. Rojas^{2,4,}*

¹Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS), CSIC, Avenida de la Reina Mercedes, 10, E-41012, Sevilla, Spain.

²Departments of Forest Biomaterials and Chemical and Biomolecular Engineering, North Carolina State University, Raleigh 27695, North Carolina, United States.

³VTT Technical Research Centre of Finland Ltd, P.O. Box 1000, FI-02044 VTT, Espoo, Finland.

⁴Department of Bioproducts and Biosystems, School of Chemical Engineering, Aalto University, FI-00076 Aalto, Espoo, Finland

ABSTRACT

Lignins were isolated from spruce, wheat straw and eucalyptus by using the milled wood lignin (MWL) method. Functional groups and compositional analyses were assessed via 2D NMR and ^{31}P to realize their effect on enzyme binding. Films of the lignins were fabricated and ellipsometry, atomic force microscopy and water contact angle measurements were used for their characterization and to reveal the changes upon enzyme adsorption. Moreover, lignin thin films were deposited on quartz crystal microgravimetry (QCM) and surface plasmon (SPR) resonance sensors and used to gain further insights into the lignin-cellulase interactions. For this purpose, a commercial multicomponent enzyme system and a monocomponent *Trichoderma reesei* exoglucanase (CBH-I) were considered. Strong enzyme adsorption was observed on the various lignins but compared to the multicomponent cellulases, CBH-I displayed lower surface affinity and higher binding reversibility. This resolved prevalent questions related to the affinity of this enzyme with lignin. Remarkably, a strong correlation between enzyme binding and the S/G ratio was found for the lignins, which presented a similar hydroxyl group content (^{31}P NMR): higher protein affinity was determined on isolated spruce lignin (99% G units) while the lowest adsorption occurred on isolated eucalyptus lignin (70% S units). The effect of electrostatic interactions in enzyme adsorption was investigated by SPR, which clearly indicated that the screening of charges allowed more extensive protein adsorption. Overall, this work furthers our understanding of lignin-cellulase interactions relevant to biomass that has been subjected to no or little pre-treatment and highlights the widely contrasting effects of the nature of lignin, which gives guidance to improve lignocellulosic saccharification and related processes.

KEYWORDS. Lignins; cellulases; thin films; hydrophobic interactions; electrostatic interactions; binding; thin films; spruce; eucalyptus; wheat straw; Quartz Crystal Microbalance (QCM); Surface Plasmon Resonance (SPR); NMR.

INTRODUCTION

The replacement of non-renewable resources in the production of chemical building blocks and liquid fuels have intensified research in the bioconversion area. The biorefinery concept aims to utilize lignocellulosics toward bioproducts, bioenergy and high value streams for optimal utilization of nonfood biomass resources, widely available in the form of agricultural and forestry residues or from dedicated energy crops. A significant limitation in related efforts is the recalcitrance of lignocellulosic in bioconversion, which impacts severely the cost-effectiveness of biorefineries.^{1,2} A typical lignocellulose bioconversion process involves multiple steps, including physical, chemical or biological pretreatments, enzymatic hydrolysis of cellulose and/or the residual hemicelluloses into sugars and fermentation.^{3,4} The effectiveness of these processes is critically affected by the type, distribution and chemical nature of the residual lignin.⁵

High value applications of lignin have been limited by the structural complexity of the molecule and its variability; thus, they are mostly utilized in energy cogeneration.⁶ This is related to the fact that the main functions of lignin in nature is to provide microbial resistance and structural strength to the plant.⁷ Lignin is synthesized by chemical polymerization of three main precursors, coniferyl, sinapyl and *p*-coumaryl alcohols. These monolignols produce respectively guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H) lignin units, which are incorporated in the macromolecule. The concentration of each of these unit is highly variable and depends on the botanical origin of the plant. Softwoods, for instance, are made mostly of G units and small amounts of H units while hardwoods are composed of both S and

G units in different relative amounts. Grasses typically comprise the three units.⁸ Some pretreatment methods partially dissolve lignin or rearrange it in different fragments that remain in the biomass while other, less severe methods, may preserve lignin in a form that is close to its native form.

Cellulases, which comprise a set of enzymes, act synergistically, among others, to break down the β -1,4 glycosidic bond that holds together the glucose units in cellulose. These enzymes are used in commercial mixtures to hydrolyze cellulose. They contain cellobiohydrolases (CBH) that hydrolyze the cellulose chains liberating cellobiose from the reducing and the non-reducing ends. The mixtures also contain endo-glucanases (EG), which act on linkages in the amorphous regions of cellulose chains, forming new chain ends and oligomers. They release low molecular weight oligomers and cellobiose that are further hydrolyzed into glucose units by β -glucosidases.⁹ The main enzyme in commercial mixtures of cellulases produced by *Trichoderma reesei* TrCel7A (CBH I, EC 3.2.1.176) is an exo-acting cellulase that hydrolyses the ends of cellulose chain from the reducing end. For complete saccharification of cellulose within a reasonable time, relatively high enzyme loadings are needed, which makes the process costly.¹⁰ Thus, recent efforts have been directed to enhance the enzyme activity in economically-viable processes.^{11,12}

Lignin content in biomass and its detailed composition play critical roles in the saccharification of lignocellulosic materials, usually decreasing the performance and efficiency of enzymatic hydrolysis. Several mechanisms of inhibition have been reported. For example, lignin may act as a physical barrier that restricts the access of the cellulases to cellulose, namely steric hindrance, or may adsorb cellulases, resulting in non-productive binding.^{3,13} Hydrogen bonding, hydrophobic and electrostatic interactions have been hypothesized to participate in non-specific adsorption.^{14,15} However, enzyme inhibition activity and related mechanisms remain untapped for elucidation in order to advance

enzymatic hydrolysis processes.^{13,16,17,18} Quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) techniques combined with model films of isolated lignins have been useful for this purpose.^{13,17,19,20,21} For example, isolated lignin films have been prepared directly by drying from solution or by using the Langmuir-Blodgett technique^{23,24,25} or by spin coating^{6,21,25,26} and several studies have addressed the interactions between lignin and different types of proteins (see for example our previous studies in Refs.^{13,19,27,28}). The control of the thickness and roughness the model films is essential to study lignin-cellulase interactions. In fact, the surface properties of the lignin films depend on the type of lignin, the method used for its extraction and the technique used for film preparation.²⁰ Previous studies of lignin-cellulase adsorption were performed on isolated lignins, including protease-treated lignin (PTL) and cellulolytic enzymatic lignin (CEL),²⁹ enzymatic mild acidolysis lignin (EMAL) and lignin-rich enzymatic hydrolysis residues (EnzHR).¹⁷ These and other studies indicate different adsorption capacity depending on the lignin type.¹⁹

Commonly, pre-treatments are necessary for increasing the accessibility of enzymes to biomass. Such steps modify the lignin structure in various, complex ways. Consequently, differences are expected for the adsorption of cellulases on residual and native lignins. Extensive research, including our own,¹⁷ has attempted to address the first case while the present work focuses on the more native forms of lignins. Indeed, despite the many related seminal efforts, no reports exist in the context of cellulase binding on milled wood (MWL) or Björkman lignins,³⁰ which are considered close versions of the corresponding native structures.^{31,32,33,34} Therefore, in this contribution we elucidate the hydrophobic and electrostatic interactions between isolated MWL and cellulases by using electroacoustic and optical techniques capable of sensing the extent and dynamics of the interactions, namely, the quartz crystal microgravimetry (QCM) and the surface plasmon resonance (SPR), respectively.

MATERIALS AND METHODS

Lignins were extracted from *Eucalyptus globulus* wood (ENCE, Pontevedra, Spain), wheat straw (Spain) and spruce wood (Sweden) according to the traditional Björkman procedure.³⁰ Briefly, ~40 g of extractive-free material were finely ball-milled in a Retsch PM100 planetary ball mill (Restch, Haan, Germany) at 400 rpm using a 500 mL agate vessel with 20 agate ball-bearings (20 mm diameter). The total ball-milling time was 24 h, carried out by using 15 min on and off cycles. The ball-milled materials were then extracted with dioxane-water 96:4 (v/v) and the respective isolated crude MWL was then subsequently purified, as described elsewhere.³⁵ Thereafter, for simplicity, these milled wood lignins are referred to as MWL. All other solvents were of analytical grade.

Studies on enzyme adsorption were carried out with a purified *Trichoderma reesei* cellobiohydrolase TrCel7A (CBH I) as well as a commercial enzyme mixture. The cellobiohydrolase TrCel7A is an exocellulase that was purified according to Suurnäkki et al.³⁶ except for the omission of the last step of hydrophobic interaction chromatography on phenyl sepharose. The commercial cellulase used was under trade name CTec2 (Novozymes A/S, Denmark) and is a multicomponent enzymes based on the *T. reesei*. Naturally, the exact composition of this commercial mixture is not disclosed; such system is used here simply as a reference of industrial relevance. It can be assumed, however, that Ctec2 is an augmented mixture of proteins consisting of xylanases, endoglucanases, beta-glucanases and other proteins.

MWL characterization

Lignin content. Klason lignin was estimated as the residue after sulfuric acid hydrolysis of the purified MWL, according to Tappi Standard T222 om-88.³⁷ The acid-soluble lignin content was determined by spectrophotometry (205 nm, extinction coefficient of 110 L/cm/g)

according to Tappi Standard UM-250. Besides these components, it is worth noting the possible contributions of carbohydrates and minerals. Carbohydrates, for instance, are always present in MWL preparations, in the form of lignin-carbohydrate complexes (LCC).

Two-dimensional nuclear magnetic resonance spectroscopy. For the 2D-NMR analysis, 30 mg of MWL were dissolved in 0.75 mL deuterated dimethylsulfoxide (DMSO- d_6). The heteronuclear single quantum correlation (HSQC) spectra were recorded at 300K on a Bruker AVANCE III 500 MHz spectrometer (Bruker, Karlsruhe, Germany), equipped with a cryogenically cooled 5 mm TCI gradient probe with inverse geometry (proton coils closest to the sample). The 2D ^{13}C - ^1H correlation spectra were obtained using an adiabatic HSQC pulse program (Bruker standard pulse sequence 'hsqcetgpsisp2.2'). The spectral width was from 10 to 0 ppm for the ^1H dimension, with an acquisition time of 145 ms, and a recycle delay (d1) of 1s. For the ^{13}C dimension, the spectral width was from 165 to 0 ppm, collected in 256 increments of 32 scans for a total acquisition time of 2h 40 min. The $^1J_{\text{CH}}$ used was 145 Hz. Processing used typical matched Gaussian apodization in ^1H and a squared cosine bell in ^{13}C . The central solvent peak was used as an internal reference (δ_{C} 39.5; δ_{H} 2.49 ppm).

2D NMR HSQC cross-signals were assigned after comparison with data from literature.^{38,39,40} A semiquantitative analysis of the volume integrals of the HSQC correlation peaks was performed using Bruker's Topspin3.1 processing software, according to previous studies.³⁸ The integration of the cross-signals was performed separately for the different regions of the HSQC spectrum, which contain signals that correspond to chemically analogous carbon-proton pairs. For these signals, the $^1J_{\text{CH}}$ coupling value is similar and integrals can be used semi-quantitatively to estimate the relative abundance of the different species. In the aliphatic oxygenated region, the relative abundances of side-chains involved in inter-unit linkages were estimated from the $\text{C}_\alpha/\text{H}_\alpha$ correlations, except for substructures Aox and I, for which $\text{C}_\beta/\text{H}_\beta$ and $\text{C}_\gamma/\text{H}_\gamma$ correlations were used, respectively. In the aromatic region,

C₂/H₂ and C₆/H₆ correlations from H, G and S lignin units and from *p*-coumarate were used to estimate their relative abundances.

³¹P nuclear magnetic resonance. Methylation and oxypropylation of the MWLs were analyzed by quantitative ³¹P NMR. Around 40–45 mg of dried isolated lignin were dissolved in 500 μL of a mixture of anhydrous pyridine/CDC13 (1.6:1, v/v). A volume of 200 μL of an endo-N-hydroxy-5-norbornene-2,3-dicarboximide solution (9.2 mg/mL) was used as internal standard and 50 μL of a chromium(III) acetylacetonate solution (5.6 mg/mL), used as relaxation reagent, were added. Finally, 100 μL of phosphorylating reagent II (2-chloro-4,4,5,5-tetramethyl-1,2,3-dioxaphospholane) was added and transferred into a 5-mm NMR tube for subsequent NMR acquisition. NMR spectra were acquired using a Bruker 300 MHz spectrometer equipped with a Quad probe dedicated to ³¹P, ¹³C, ¹⁹F, and ¹H NMR acquisition.

Electrophoretic mobility. The electrophoretic mobility, evaluated here as the zeta potential, of the MWLs at different buffer concentrations and pH were measured. Prior to the measurements, the lignins were mixed in buffer solutions (50 mL) by using a shaker/incubator at 50 °C and 200 rpm.⁴¹ Acetate buffer solutions of 50, 100, 200 and 500 mM concentration at pH 5.2 were used. Acetate buffers at pH 3.3, and 6.5 at 200 mM were also applied. The lignin concentration in all the buffer solutions was 0.033 % (w/v). The zeta potential was measured on the supernatant of the dispersions by using a Zetasizer Nano series (Nano ZS, Malvern, UK). All measurements were performed in triplicate with three readings each. The size of the lignin colloids was determined at 100 mM concentration in buffer solution at pH 5.2, 25 °C. A refractive index of 1.6 was assumed for the lignins¹⁶ while that for the enzymes was assumed to be 1.45.⁴²

Spin-coated MWL thin films. A 0.5 wt% MWL lignin solution was prepared by dissolving MWL in 1,4-dioxane two days prior to use. The supernatant of the dispersion was used to produce the spin-coated films (spin-coater from Laurell Technologies Corporation

WS-400A-6NPP/LITE, USA.). Prior to spin coating, silica (AFM) or gold-coated quartz sensors (QCM, SPR) were cleaned by rinsing with ethanol and dried with nitrogen. Silicon wafers were also used as substrates (AFM, contact angle). They were cut in 1 x 1 cm² pieces and their surface activated by immersing them in a 1 M sodium hydroxide for 15 seconds.

For spin coating, the speed, time, and lignin concentration were optimized in order to produce films of reproducible and suitable thickness (2000 rpm for 20 s, 1750 rpm^s⁻¹ acceleration). Prior to the lignin deposition, the silica/gold surface was pre-coated with polystyrene (PS) dissolved in toluene (0.5 wt%) using same conditions as those for lignin. The sensors were dried at 80°C for 30 min. For each sample, MWL was spin coated eight times. The films were stored in a desiccator until further used.

Characterization of lignin thin films. AFM imaging was performed to assess the morphology and roughness of the lignin films. The lignin films were mounted on aluminum holders and examined with a Dimension 3000 scanning probe microscope from Veeco Metrology Group. Scanning was performed in air using the tapping mode with silicon cantilevers (NSC15/AIBS) delivered by Olympus AC160TS. The drive frequency of the cantilever was about 275–325 kHz (nominal resonance of 300 kHz). The areas scanned included 200x200 nm² and 3x3 μm² sizes. No image processing except flattening was performed. Images were acquired with a feedback loop to keep the amplitude of oscillation constant and measured the response of the feedback loop. The response of the feedback loop was used to measure how far the scanner was moved in Z in order to keep the amplitude of oscillation constant.

Ellipsometry. A variable angle spectroscopic ellipsometer (J.A. Woollam) with coincident He-Ne laser and capable of spatial mapping of dielectric properties was used to determine the thicknesses of the prepared lignin model surfaces. The variation in optical properties over a large area can the distribution of the ξ (psi/delta) and ψ (wavelength) ellipsometric

parameters was measured over an area with approximately 2 mm sides. The thickness of the lignin film was determined from the measured ξ and ψ parameters as the angle of incidence was varied between 65° and 70°. The model assumed was air/lignin/PS/silica/silicon and all materials were assumed to be optically isotropic. The refractive indices of the materials were assumed to be 3.5 (silicon) and 1.45 (silica). The calculations employed a least squares iterative fitting procedure using both thickness and refractive index of the lignin and polystyrene layers as fitting parameters. The measurements were performed under ambient air and relative humidity conditions. The measurements were repeated several times at different positions on the surface and also with different surfaces that were prepared under the same conditions.

Contact Angle Measurements. The water contact angle on the lignin surfaces were measured using a video-controlled PHX 300 contact angle goniometer (Surface Electro Optics, Phoenix). The contact angles were determined by curve fitting of the drop shape the of ImageJ software.

Enzyme adsorption studied in electroacoustic experiments. Enzyme adsorption on the lignin films was followed with an E4 Quartz crystal microbalance (Q-Sense, Gothenburg, Sweden) operating in a continuous mode. The QCM-D was used to monitor the adsorption of CTec2 and CBH-I on the three different MWL films. CTec2 was used at 5 mg / mL and CBH-I was applied at 1 mg / mL, the enzymes were diluted with freshly prepared 100 mM sodium acetate buffer at pH 5.2 (injection rate of 100 μ L/min and 25 °C). The base signal was obtained while keeping the sensors in buffer for 1 h. Once equilibrated, data acquisition was restarted and the enzyme solution was introduced after 5 min. After a given time, enzyme-free buffer was injected for rinsing and the signals was used to determine the level of reversible and irreversible adsorption. All measurements were recorded at 5 MHz fundamental resonance frequency and its overtones corresponding to 15, 25, 35, 55, and 75

MHz. The third, fifth and seventh overtones were used for data processing. The Johannsmann method was used to calculate the mass of enzyme adsorbed on the surface of the sensor.⁴³

Enzyme adsorption via surface plasmon resonance. The effect of the electrostatic interactions between the enzymes and lignin was investigated with a Surface Plasmon Resonance unit (SPR Navi 200, BioNavis Oy Ltd., Tampere, Finland), operated at fixed angle mode. The experiments were carried out at 25 °C, with a flow rate of 50 µL/min. The concentration of sodium acetate buffer at pH 5.2 was varied between 50 mM and 200 mM. The amount of protein absorbed on the lignin per unit area, Γ , was calculated using equations 1 and 2:

$$d = \frac{l_d}{2} \frac{\Delta\theta}{m(\eta_a - \eta_o)} \tag{1}$$

$$\Gamma = \rho d \tag{2}$$

where d is the thickness of adsorbed layer, $\Delta\theta$ is the angle shift, l_d is a characteristic evanescent electromagnetic field decay, estimated to be ~ 0.37 times the wavelength of the incident light (240 nm),⁴⁴ m is a sensitivity factor for the sensor (109.95°/RIU, RIU: refractive index units) obtained by calculating the slope of a $\Delta\theta$ calibration with solutions of known refractive indices,⁴⁵ η_o is the refractive index of the background solution (buffer, 1.3342) and η_a is the refractive index of the adsorbed species (enzyme), which was assumed to be 1.57. ρ is the bulk density of the enzyme (1370 kg/m³) was determined from specific volume data (0.73 mL/g).

RESULTS AND DISCUSSION

Milled Wood Lignins (MWL). The concentration of acid soluble lignin, ASL, was 2.3, 1.2 and 0.5% for spruce, wheat straw and eucalyptus, respectively. The total lignin concentration (expressed as Klason plus ASL) was quite similar for the three types of MWL,

between 85% and 87%. The S/G ratio of the samples increased with the ASL but the main differences are further shown with respect to phenolic hydroxyl, carboxylic acid and aliphatic hydroxyls groups. Moreover, 2D-NMR was used to access the detailed composition of the MWL samples and their inter-unit linkages. The main lignin cross-signals assigned in the 2D HSQC spectra of the MWL isolated from eucalyptus, wheat straw and spruce (Figure 1) are listed in Table S1 of the Supporting Information. The spectra indicated major differences between the isolated MWL, in terms of composition and linkages: see **Table 1** for the relative abundances of the main inter-unit linkages and end-groups, as well as the percentage of γ -acylation, the molar abundances of the different lignin units (H, G and S), *p*-coumarates, and the molar S/G ratios of the MWL extracted, which were estimated from volume integration of contours in the HSQC spectra.

The MWL from spruce, as a coniferous specie, almost exclusively comprised guaiacyl lignin units (99%), with a very low amount of *p*-hydroxyphenyl units (1%). In contrast, eucalyptus MWL was mainly composed of syringyl lignin units (69%), with lower amount of guaiacyl lignin (31%) and a S/G ratio of 2.1. The MWL extracted from wheat straw was rich in guaiacyl (G) and had a S/G ratio of 0.5 (H:G:S = 3:61:36). The relative abundance of the different interunit linkages reflected the compositional differences noted for the three extracted MWLs. Although, β -O-4' linkages were most abundant in all of these lignins, important amounts of β -5' (phenylcoumaran) and 5-5' (dibenzodioxocin), which are related to the presence of G lignin units, were also found in the MWL extracted from spruce. Interestingly, MWL extracted from wheat straw was acylated at the gamma position of the side chains of *p*-coumarate and presented important amounts of tricinn, as previously reported.³⁸

Figure 1. Heteronuclear single quantum correlation nuclear magnetic resonance spectra of (a) spruce, (b) wheat straw and (c) eucalyptus isolated as milled wood lignins (MWL) with main structures identified. See Table S1 for quantification of the structures.

Table 1. Structural characteristic lignin interunit linkages, end-groups, γ -acylation, aromatic units, S/G ratio and cinnamate content obtained from integration of ^{13}C – ^1H correlation peaks in the HSQC spectra of the MWL isolated from eucalyptus, wheat straw and spruce.

	Eucalyptus	Wheat straw	Spruce
Lignin inter-unit linkages (%)			
β –O–4' aryl ethers (A/A')	80	78	62
α -oxidized β –O–4' aryl ethers (Aox)	3	2	0
Phenylcoumarans (B)	4	9	25
Resinols (C)	9	10	5
Dibenzodioxocins (D)	0	1	8
Spirodienones (F)	4	0	0

Lignin end-groups			
Cinnamyl alcohol end-groups (I)*	6	5	4
Cinnamaldehyde end-groups (J)*	0	3	4
Lignin side-chain γ -acylation (%)			
	0	12	0
Lignin units (%) and S/G ratio			
S	67	36	0
G	33	61	99
H	0	3	1
S/G ratio	2.0	0.5	0
<i>p</i> -coumarates (%)**	0	4	0

*Expressed as a fraction of the total lignin interunit linkage types A-F.

***p*-coumarate molar content reported as percentage of lignin content (S + G + H).

The hydroxyl group content of the different MWL was determined by ^{31}P NMR spectroscopy (see **Figure 2** for the ^{31}P NMR spectra of the MWL). The results obtained from ^{31}P NMR are included in **Table 2** and indicates that the amount of total hydroxyl groups was similar for all the MWL studied. The MWL extracted from wheat straw and eucalyptus presented a relatively higher phenolic hydroxyl group content, whereas the MWL from spruce was enriched in aliphatic hydroxyl groups. Since the total hydroxyl group content was not too different in the isolated lignins, our studies about the adsorption of cellulases on lignins were mainly concerned with the effect of lignin composition, for example, in terms of the syringyl/guaiacyl (S/G) ratio.

Figure 2. ^{31}P NMR spectra of milled wood lignins isolated from wheat straw, eucalyptus and spruce, as indicated.

Table 2. Compositional analysis from ^{31}P NMR spectra of MWL extracted from the lignocellulosic sources studied, as indicated. Data expressed as mmol/g.

Sample	Aliphatic OH	Phenolic OH	S+C	G	H	COOH	Total OH
Spruce	5.40	1.51	0.26	1.18	0.07	0.03	6.95
Wheat Straw	4.72	2.06	0.54	0.92	0.63	0.08	6.89
Eucalyptus	4.91	2.09	1.12	0.63	0.27	0.02	7.02

Zeta potential of lignins and cellulases. In addition to the effects of molecular composition, the interactions between lignins and cellulase enzymes depend on electrostatic interactions in aqueous media, which in turn depend on the charge balance (zeta potential) of the dispersed MWLs (**Figure 3a**) and enzymes (**Figure 3b**). All the MWLs were negatively charged at pH 5.2, as expected; in all the cases, also as expected, the charge was reduced with the increase in ionic strength, due to electrostatic screening effects. **Table S2** of supporting information provides additional data. As noted in **Figure 3a**, in the range between 50 and 100

mM the zeta potential was between -23 and -17 mV while it was reduced to -12 and -6 mV for ionic strength of 200 mM and 500 mM, respectively.

Figure 3. Surface charge reported as zeta potential and measured in aqueous dispersions of given ionic strength and pH = 5.2 for (a) MWL samples: spruce (blue), wheat straw (red) and eucalyptus (green). (b) Also plotted is the zeta potential for the enzymes (CBH-I and CTec2).

The molecular mass (MALDI-ToF) and isoelectric point (pI) of the CBH-I were 56 kDa and 3.82, respectively, in agreement with other reports.^{46,47} Compared with the lignins, the enzymes displayed a lower negative charge at pH=5.2 (**Figure 3b**).

Ultrathin films of the MWLs. AFM height images of thin films obtained by spin-coating on polystyrene of MWL extracted from spruce, wheat straw, and eucalyptus are shown in **Figure 4**. The respective root-mean-square (RMS) AFM roughness measured for at least four images were 5.41 ± 0.16 , 4.15 ± 0.03 and 2.14 ± 0.04 nm (note the roughness measured for the PS support was 0.3 nm). Spherical features, with sizes in the range of 10-20 nm, were observed in the AFM images. The morphology of the surfaces was characteristic of other lignin films prepared by the spin-coating technique.⁶ Images acquired at lower magnification indicated that the films were continuous over large areas (images not shown).

Figure 4. AFM topography ($3 \times 3 \mu\text{m}^2$ images) of MWL films spin coated on polystyrene. (a) Spruce, (b) wheat straw and (c) eucalyptus. The insets include images at higher magnification $200 \times 200 \text{ nm}^2$.

The RMS roughness of MWL films varied considerably depending on the method used to isolate the lignin.⁴⁸ The possibility to control the thickness and roughness of lignin films is critical to study the interactions between lignin and cellulases. Indeed, smooth films are preferred when techniques such as QCM are used.³⁴ From the images and RMS roughness values, it is concluded that the lignin films fully covered the surface and were relatively smooth. However, it is interesting to note that the spruce and wheat straw films were slightly rougher compared to the films obtained from eucalyptus MWL. It is possible that the more significant presence of syringyl units in the latter resulted in smoother lignin films. This is because the positions 3 and 5 of the aromatic rings were blocked by methoxyl groups, which translates into more linear lignin structures.

The thickness of the prepared lignin thin films was determined by ellipsometry: 14, 9 and 9 nm for spruce, wheat straw, and eucalyptus MWL, respectively. Interestingly, the film roughness remained relatively low independent of the film thickness. Based on fitted data from more than 50 ellipsometry measurements, the refractive index was determined to be $\mu=1.60 \pm 0.03$ for the MWLs studied, similar to the values reported by Norgren et al.,⁶ for kraft lignins ($\mu=1.61 \pm 0.04$). However, they were larger than those for lignins extracted, via

1
2
3 acetosolv or organosolv methods, from sugar cane bagasse and from *Pinus caribaea* var.
4 *hondurensis* ($\mu=1.04\text{-}1.30$ for 350-800 nm wavelength).²³ The porosity of these latter lignin
5 films was lower, yielding denser films. Interestingly, the roughness remained relatively low
6 independent of the film thickness.
7
8
9

10
11 **Cellulase binding on MWL films.** Images of sessile water droplets in contact with the
12 MWL films, taken before and after CBH-1 adsorption, revealed a clear reduction in contact
13 angle, from 66-69 degrees (depending on lignin source) to 30-40 degrees (**Figure S1** and
14 **Table S3**). This was a consequence of enzyme adsorption on the surface, as will be explained
15 in more detail with QCM experiments, whereby hydrophobic amino acids of the cellulases
16 interacted with the lignin, leaving exposed hydrophilic residues of the enzymes.
17
18
19

20
21 Two different concentrations of CTec2 were used in experiments to reveal the binding
22 and dynamics of enzyme adsorption on the lignin-coated QCM sensors (**Figure 5a** and **b** for
23 1 and 5 mg/ml CTec2). An increase in the negative value of the frequency shift, $-Af_3$,
24 indicated mass uptake or adsorption onto the film. After the baseline acquired in the
25 background electrolyte and upon introduction of the enzyme (in the same electrolyte solution,
26 left arrow to indicate approximate time of injection), an increase in the adsorbed mass
27 (increase in $-Af_3$) was noted for all the lignin films. The rate at which the enzyme was
28 adsorbed can be taken as indicative of the early stages of the adsorption process. Only small
29 differences were observed if one compares the adsorption profiles on the different MWL
30 films, **Figure 5a,b**. The enzymes adsorbed to a larger extent onto spruce MWL but the
31 opposite was observed for eucalyptus MWL. The apparent mass of enzyme (CTec2 and
32 CBH-I) adsorbed on the different MWL films, calculated from QCM sensograms, is reported
33 in **Table 3**. Enzyme adsorption increased with equilibrium concentration (isotherms acquired
34 at other concentrations, not shown) but only to a limited extend, indicating the possibility that
35 surface saturation was nearly reached at 5 mg/mL solution concentration.
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

As far as the interactions between lignin and Ctec2, we would like to point out that given the complex mixture of proteins that exist in this commercial system, caution must be exercised in any effort to correlate the binding signature with that of purified CBH1. For this purpose, other enzyme cocktails known to comprise enzyme preparations from *T. reesei* and which may not be augmented, may be more relevant. However, Ctec2 is simply taken here as an example of a commercial system that is widely reported and used for total hydrolysis. Here, we attempted to identify any significant differences in the lignin-binding behavior, which as indicated by the data, seems to be sensitive to the exact composition of the reference enzymes.

Figure 5. Quartz crystal microgravimetry (QCM) sensograms upon injection of Ctec2 on MWL thin films. Two different enzyme concentrations were used, (a) 1 and (b) 5 mg/mL. (c) Included is also the sensogram for CBH-I added at 1 mg/mL concentration. In each figure, the arrow on the left indicates the approximated time at which enzyme was injected after film equilibration in background electrolyte and the “drop” symbol represents the time at which the enzyme solution was replaced with background electrolyte solution (rinsing step). The source of the MWL used in film preparation is indicated (spruce, wheat straw and eucalyptus).

Rinsing with background buffer was carried out to determine the extent at which lignin was removed and thus to gain some understanding on the irreversibility of the adsorption process. The adsorbed mass was calculated by using the Johannsmann method⁵⁰ from QCM frequency values. In the calculation, the density of the AT-cut quartz crystals, ρ_q , was

assumed to be 2648 Kg/m³. The shear modulus, μ_q , was assumed to be 2.95x10¹⁰ Kg/m². The third, fifth and seventh overtones of the resonance frequencies were used to calculate the mass m_i at each frequency ($i = 3, 5$ or 7) using Equation 3

$$m_i = \frac{\sqrt{\rho_q \mu_q} \Delta f}{2f_i} \quad (3)$$

m_i was plotted as a function of the square of the resonance frequency and the mass of the adsorbed layer was determined by extrapolation at a resonance frequency zero. The frequencies of the QCM sensor were measured in buffer before the deposition of polystyrene and lignin. The obtained values of adsorbed mass, at 40 min and after rinsing at 80 min, for the reversible and irreversible adsorption, respectively, are included in **Table 3** together. The percentage of enzyme that irreversibly adsorbed onto lignin was noted to be similar for both of the enzyme doses investigated. Please note that as any other approach to calculate the adsorbed mass, the Johannsmann model is subject to assumptions and can be only taken on a relative basis.

Table 3. Apparent QCM mass of enzyme (CTec2 or CBH-I) adsorbed on thin MWL films as measured by the respective sensograms (see Figure 5 with examples in the case of CTec2 introduced at two concentrations). The data is reported in units of mass per unit area, mg/m². The reversible adsorbed mass calculated upon enzyme injection is determined after the frequency signals reaches equilibrium (about 40 min after injection). The mass determined from frequency values obtained after rinsing with background electrolyte is taken as the enzyme that is irreversibly adsorbed on the substrate.

MWL	CTec2 (1 mg/mL)		CTec2 (5 mg/mL)		CBH-I (1 mg/mL)	
	Reversible	Irreversible	Reversible	Irreversible	Reversible	Irreversible
Spruce	5.4 ± 0.4	4.3±0.7 (80%)	7.9 ± 0.9	6.2±0.9 (79%)	2.5 ± 0.3	1.3±0.2 (52%)
Wheat	4.9 ± 0.3	3.7±0.1 (76%)	7.5 ± 0.4	6.0±0.3 (80%)	2.4 ± 0.1	1.1±0.3 (46%)
Eucalyptus	4.3 ± 0.5	3.3±0.3 (77%)	7.2 ± 0.4	5.7±0.3 (79%)	2.2 ± 0.4	1.0±0.1 (45%)

Enzyme adsorption has been indicated to scale with both the S/G ratio and the amount of phenolic and aliphatic hydroxyl groups.^{17,49,51} For the MWLs studied here, the concentration

of total hydroxyl groups was very similar (^{31}P analysis) while there was significant difference in the S/G ratio (NMR data). Thus, considering the extent of enzyme adsorption, there was clear indication that it increased with the guaiacyl (G) content of the lignin.

The presence of surfactants and preservatives in commercial enzyme cocktails influence the interactions between lignin and cellulase.⁵² However, in the present case no attempt was made to remove (via solvent exchange, etc.) surfactants or preservatives for two reasons, first the effects were expected to be small given the levels of dilution and, secondly, the system was tested in conditions relevant to actual applications.

Data from the purified enzyme is discussed now in light of the adsorption experiments. The monocomponent cellulase from *T. reesei* Cel7A (CBH-I) was a cellobiohydrolase that represents about 50-60% of total enzyme cocktails produced by *T. reesei*. The function of this enzyme is to break down cellulose by an exo mechanism from the reducing ends. The extent of adsorption of the single component enzyme on the lignin films (**Figure 5c**) was distinctively less than that measured for the commercial enzyme mixture (**Figure 5a**). Compared to the case of the enzyme mixture, CBH-I followed the same adsorption trend on the three MWLs. The amount of CBH-I irreversibly adsorbed on MWL accounted for about half of the total mass adsorbed at equilibrium after injection. An interesting observation was that compared to the two other substrates, adsorption of CBH-I on spruce MWL was faster (data for short adsorption times, not included). One noticeable fact is that the relative amount of enzyme irreversibly adsorbed on spruce MWL was ca. 80% for CTec2, while that for CBH-I was 52%. These figures were 45 and 46% in the case of eucalyptus and wheat straw MWL, respectively. Thus, the monocomponent enzyme adsorption on lignin was more limited.

Electrostatic and rinsing effects revealed by SPR. Electrostatic effects are known to affect the interactions between lignin and enzymes.⁵³ Here, we discuss experiments carried

1
2
3 out at pH 5.2 and different ionic strengths by the optical technique, surface plasmon
4
5 resonance (SPR). For this purpose, wheat straw and spruce lignin were compared since they
6
7 present the largest differences in lignin composition. In the experiments, enzyme was first
8
9 adsorbed for 4 min and then the surface was rinsed with background buffer. After 5 min, data
10
11 was acquired to study the irreversibility of enzyme binding to lignin (SPR sensograms in
12
13 **Figure 6**).

14
15
16
17 Enzyme adsorption experiments were carried out in buffer solutions of 50 and 200 mM
18
19 salt concentration since, as shown in **Figure 3**, an important difference in lignin zeta potential
20
21 was determined in these conditions (see also **Table S2** with the zeta potential of MWLs at
22
23 different ionic strengths). Compared to the case of low (50 mM) background buffer
24
25 concentration, at 200 mM (when lignin is less negative) adsorption occurred to a larger extent,
26
27 **Figure 6a,b**. At the higher ionic strength, the salts screened the electrostatic repulsion
28
29 between the negatively charged enzymes allowing for better binding. Results for adsorption of
30
31 the enzyme mixture, CTec2, is shown in **Table 4**. It is apparent that under similar conditions
32
33 SPR adsorbed mass (**Table 4**) was smaller than that calculated from QCM sensograms
34
35 (**Table 3**). This is explained by the fact that QCM is sensitive to hydration or coupled water.
36
37 Also, SPR revealed, as was the case of QCM data, that adsorption on wheat straw lignin was
38
39 higher for the enzyme cocktail compared to the monocomponent CBH I. More important to
40
41 the present discussion is that the irreversible adsorption of enzyme was significantly affected
42
43 by the ionic strength: for spruce MWL, the relative fraction of enzyme that was irreversibly
44
45 adsorbed was 38% at 50 mM and it increased to 50% at 200 mM. The same applied to wheat
46
47 straw MWL (33 and 50%, respectively). Thus, adsorption was favored under conditions of
48
49 reduced electrostatic repulsion.
50
51
52
53

54
55 Experiments were conducted with enzymes in 500 mM background electrolyte
56
57 concentration followed by rinsing with electrolyte solutions of given concentrations (50, 200
58
59
60

1
2
3 and 500 mM), **Figure 6a',b'**. The initial mass adsorbed was equivalent to $\sim 3.3 \text{ mg/m}^2$ on
4 spruce MWL and 2.7 mg/m^2 on wheat straw MWL. Upon rinsing with 500 mM buffer, the %
5 fraction of the enzyme that was irreversibly adsorbed on spruce and wheat straw MWL were
6
7
8
9
10 41 and 54 %, respectively. Results after rinsing with 200 and 50 mM electrolyte solutions are
11 shown also in **Figure 6a',b'**. The total mass on spruce and wheat straw MWL after rinsing
12 with these solutions were less than the initial mass, which indicated partial removal of the
13 lignin, possibly in the form of lignin-enzyme complexes. Indeed, AFM images obtained from
14 the sensors for the experiments with 50 mM electrolyte concentration indicated partial
15 removal of MWL, though some fragments still remained on the sensor, **Figure S2**. The
16 roughness of the MWL substrate increased to 7 nm (spruce) and to 5.9 nm (wheat straw).
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 6. SPR sensograms upon adsorption of CTec2 (5mg/ml) dissolved in aqueous solution of 50 and 500 mM electrolyte concentration on (a) spruce MWL and (b) wheat straw MWL. In these cases, rinsing was performed with enzyme-free, electrolyte solution of the same concentration. Additional experiments were conducted by introducing the enzyme in 500 mM background electrolyte concentration followed by rinsing with electrolyte solutions of given concentrations (50, 200 and 500 mM): (a') on spruce MWL and (b') on wheat straw MWL.

Experiments similar to (a) and (b) are shown in (c) and (d) for CBH-I adsorption on wheat straw MWL. In each figure, the arrow symbol represents the approximated time at which enzyme was injected after film equilibration in background electrolyte and the “drop” symbol represents the time at which the enzyme solution was replaced with background electrolyte solution of given concentration, as indicated (rinsing).

Table 4. CTec2 adsorbed mass (mg/m²) determined by SPR on spruce and wheat straw MWL upon adsorption at (a) 50 and 200 mM electrolyte concentration (see Figure 6a,b). (b) Adsorption level obtained from 500 mM electrolyte solution after rinsing with enzyme-free, electrolyte concentrations of 50, 200 and 500 mM, as indicated. (c) Mass adsorption (mg/m²) for CBH-I is also included for conditions similar than those in (a).

		(a) CTec2 SPR adsorbed mass at given background electrolyte conc.		(b) CTec2 SPR mass adsorbed from 500 mM after rinsing with given electrolyte conc.			(c) CBH-I SPR adsorbed mass at given background electrolyte conc.	
		50 mM	200 mM	50 mM	200 mM	500 mM	50 mM	200 mM
Spruce	Rev.	1.6	4.2	-	-	3.4	-	-
	Irrev.	0.6	2.1	3.3	3.1	1.4	-	-
Wheat Straw	Rev.	0.9	3.2	-	-	2.8	1.4	1.6
	Irrev.	0.3	1.6	2.6	2.8	1.5	~ 0	0.2

Proteins or enzymes undergo different adsorption mechanisms, which are influenced mainly by hydrophobic and electrostatic interactions between the proteins and the surface. Adsorption is possible to occur in the form of patches or uniformly distributed mono- or multi-layers. The respective surface coverage of the enzymes on the surface films can be estimated from the adsorption data obtained via SPR. Three different scenarios were considered for this purpose, as shown in **Figure S3**. Accordingly, enzyme coverage on the MWL films were calculated for the different experiments and **Table S4** shows the corresponding coverage values. There is an indication that patches are the primary enzyme arrangement on the surface, which was influenced by the structural conformations and charge of the enzymes. Compared to data obtained at high ionic strength, the electrostatic repulsions

that existed in conditions of lower ionic strength was clearly shown as a decrease in enzyme coverage on the surface, by more than 25%.

In concluding this discussion, it is important to point out a few items that are pertinent: residual lignins that result from biomass pre-treatment (saccharification process, etc.) are most relevant as far as their interactions with enzymes. Such subject has been discussed, for example, in Refs.^{17,18} and many others, where the results for exo- and endoglucanases indicated different binding degrees. For example, exo- and endoglucanases showed very little affinity toward the lignin extracted from the pretreated corn stover, which is in contrast with the results presented in this work for the various milled wood lignins tested. In these latter cases, enzyme binding is a scientifically important aspect that, surprisingly, has received little attention. More importantly, the results clearly indicated that lignin-enzyme interactions depended on the nature of the substrates. While this was tested for different biomass sources, the same applies to other factors such as the level of pretreatment severity.

We should point out that the type of surfaces and the surface sensitive methods used here revealed fundamental aspects about the interactions that, nevertheless, are only approximations to actual process conditions, where mechanistic studies are not possible or very challenging. Indeed, the affinity of enzymes with lignin is affected by many physical properties of individual enzymes or enzyme types that influence adsorption rates and mechanisms. Moreover, such factors may not be displayed in mixed population of proteins, where the competitive binding shown by multiple enzymes for the same substrate may affect their interactions.

CONCLUSIONS

Multicomponent and a monocomponent *Trichoderma reesei* exoglucanase (CBH-I) enzymes adsorbed extensively on films of lignins isolated from spruce, wheat straw and

eucalyptus. Compared to the multicomponent cellulases, CBH-I displayed lower affinity with lignin and higher adsorption reversibility. These results challenge the standing assumption that this enzyme has a high affinity toward lignin and further highlights the relevance of different sources of lignins. QCM and SPR indicate that charge screening allows more extensive protein adsorption, revealing the importance of electrostatic interactions in the mechanism of enzyme action in the presence of residual lignins. Finally, a correlation between the extent of adsorption and the S/G ratio of the lignins was found.

SUPPORTING INFORMATION

The following data are available online as complementary information: Assignments of lignin $^{13}\text{C}/^1\text{H}$ correlation signals in the 2D HSQC spectra of the MWL isolated from eucalyptus, wheat straw and spruce; size and zeta potential of the MWL measured in aqueous dispersions at different background electrolyte concentrations; water contact angles before and after CTec2 and CBH-I adsorption; AFM images of wheat straw films corresponding to SPR sensograms after rinsing with 50 mM background electrolyte; schematics of the different scenarios for enzymes adsorption on the surfaces for the calculation of surface coverage.

ACKNOWLEDGEMENTS

This study was partially funded by the INDOX EU-project (KBBE-2013-7-613549); the LIGNOCELL, LIGNIN, NOESIS, and BIORENZYMER Spanish MICINN (co-financed by FEDER funds) projects (AGL2011-25379, CTQ2014-60764-JIN, BIO2014-56388 R and AGL2014-53730-R) and the CSIC Project 201440E097. A.P. is grateful to the Spanish MINECO for a FPI fellowship. O.J.R. would like to thank the Academy of Finland for funding through its Centers of Excellence Program (2014–2019), under Project 132723612

HYBER.

Corresponding Author

*Email: orlando.rojas@aalto.fi, Tel.: +358-(0)50 512 4227

Author Contributions. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

REFERENCES

1. Chundawat, S. P.; Beckham, G. T.; Himmel, M. E.; Dale, B. E. *Annu. Rev. Chem. Biomol. Eng.* **2011**, 2, 121-145.
2. Kumar, R.; Wyman, C. E. *Biotechnol. Bioeng.* **2014**, 111(7), 1341-1353.
3. Chandra, R. P.; Bura, R.; Mabee, W. E.; Berlin, A.; Pan, X.; Saddler, J. N. *Adv. Biochem. Eng./Biotechnol.* **2007**, 108, 67-93.
4. Mosier, N.; Wyman, C.; Dale, B.; Elander, R.; Lee, Y. Y.; Holtzapple, M.; Ladisch, M.. *Bioresour. Technol.* **2005**, 96(6), 673-686.
5. Guo, F.; Shi, W.; Sun, W.; Li, X.; Wang, F.; Zhao, J.; Qu, Y. *Biotechnol. Biofuels* **2014**, 7(1), 38. doi: 10.1186/1754-6834-7-38
6. Norgren, M.; Notley, S. M.; Majtnerova, A.; Gellerstedt, G. *Langmuir* **2006**, 22(3), 1209-1214. doi: 10.1021/la052284c
7. Sarkanen, K. V.; Ludwig, C. H.. *Lignins: Occurrence, Formation, Structure and Reactions*. Wiley-Interscience, New York (1971).
8. Boerjan, W.; Ralph, J.; Baucher, M. *Annu. Rev. Plant. Biol.* **2003**, 54, 519-546.
9. Kumagai, A.; Lee, S.-H.; Endo, T. *Biomacromolecules* **2013**, 14(7), 2420-2426.
10. Bubner, P.; Plank, H.; Nidetzky, B. *Biotechnol. Bioeng.* **2013**, 110(6), 1529-1549.
11. Percival Zhang, Y.-H.; Himmel, M. E.; Mielenz, J. R. *Biotechnol. Adv.* **2006**, 24(5), 452-481.
12. Zhang, Z.; Donaldson, A. A.; Ma, X. *Biotechnol. Adv.* **2012**, 30(4), 913-919.

13. Martin-Sampedro, R.; Rahikainen, J. L.; Johansson, L. S.; Marjamaa, K.; Laine, J.; Kruus, K.; Rojas, O. J. *Biomacromolecules* **2013**, *14*(4), 1231-1239.
14. Berlin, A.; Balakshin, M.; Gilkes, N.; Kadla, J.; Maximenko, V.; Kubo, S.; Saddler, J. J. *Biotechnol.* **2006**, *125*(2), 198-209.
15. Sewalt, V. J. H.; Glasser, W. G.; Beauchemin, K. A. *J. Agric. Food Chem.* **1997**, *45*(5), 1823-1828.
16. Nakagame, S.; Chandra, R. P.; Kadla, J. F.; Saddler, J. N. *Bioresour. Technol.* **2011**, *102*(6), 4507-4517.
17. Rahikainen, J. L.; Martin-Sampedro, R.; Heikkinen, H.; Rovio, S.; Marjamaa, K.; Tamminen, T.; Rojas, O.J.; Kruus, K. *Bioresour. Technol.* **2013**, *133*, 270-278.
18. 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. *J. Biol. Chem.* **2014**, *289*(30), 20960-20969.
19. Fritz, C.; Ferrer, A.; Salas, C.; Jameel, H.; Rojas, O. J. *Biomacromolecules* **2015**, *16*(12), 3878-3888.
20. Hoeger, I. C.; Filpponen, I.; Martin-Sampedro, R.; Johansson, L.-S.; Österberg, M.; Laine, J.; Kelley, S.; Rojas, O. J. *Biomacromolecules* **2012**, *13*(10), 3228-3240.
21. Tammelin, T.; Johnsen, I. A.; Österberg, M.; Stenius, P.; Laine, J. *Nord. Pulp Pap. Res. J.* **2007**, *22*(1), 93-101.
22. Constantino, C.; Dhanabalan, A.; Cotta, M.; Pereira-da-Silva, M.; Curvelo, A.; Oliveira, O. *Holzforschung* **2000**, *54*(1), 55-60.
23. Constantino, C. J. L.; Juliani, L. P.; Botaro, V. R.; Balogh, D. T.; Pereira, M. R.; Ticianelli, E. A.; Curvelo, A.A.S.; Oliveira, O. N. *Thin Solid Films* **1996**, *284-285*(0), 191-194.
24. Micic, M.; Benitez, I.; Ruano, M.; Mavers, M.; Jeremic, M.; Radotic, K.; Moy, V. Leblanc, R. M. *Chem. Phys. Lett.* **2001**, *347*(1), 41-45.
25. Notley, S. M.; Norgren, M. *Biomacromolecules* **2008**, *9*(7), 2081-2086.
26. Tammelin, T.; Österberg, M.; Johansson, L. S.; Laine, J. *Nord. Pulp Pap. Res. J.* **2006**, *21*(4), 444-450.

27. Morales, L. O.; Iakovlev, M.; Martin-Sampedro, R.; Rahikainen, J. L.; Laine, J.; van Heiningen, A.; Rojas, O. J. *Bioresour. Technol.* **2014**, *161*, 55-62.
28. Salas, C.; Rojas, O. J.; Lucia, L. A.; Hubbe, M. A.; Genzer, J. *ACS Appl. Mater. Interfaces* **2013**, *5*(1), 199-206. doi: 10.1021/am3024788
29. Nakagame, S.; Chandra, R. P.; Saddler, J. N. *Biotechnol. Bioeng.* **2010**, *105*(5), 871-879.
30. Björkman, A. *Sven. Papperstidn.* **1956**, *59*(13), 477-485.
31. Capanema, E.; Balakshin, M.; Katahira, R.; Chang, H. M.; Jameel, H. J. *Wood Chem. Technol.* **2015**, *35*(1), 17-26.
32. Crestini, C.; Melone, F.; Sette, M.; Saladino, R. *Biomacromolecules* **2011**, *12*(11), 3928-3935.
33. Martin-Sampedro, R.; Capanema, E. A.; Hoeger, I.; Villar, J. C.; Rojas, O. J. *J. Agric. Food Chem.* **2011**, *59*(16), 8761-8769.
34. Notley, S. M.; Norgren, M. *Langmuir* **2010**, *26*(8), 5484-5490.
35. Rencoret, J.; Marques, G.; Gutierrez, A.; Nieto, L.; Jimenez-Barbero, J.; Martinez, A. T.; del Río, J. C. *Ind. Crops Prod.* **2009**, *30*(1), 137-143.
36. Suurnäkki, A.; Tenkanen, M.; Siika-aho, M.; Niku-Paavola, M.-L.; Viikari, L.; Buchert, J. *Cellulose* **2000**, *7*(2), 189-209.
37. *Tappi Test Methods 2004–2005*, Tappi Press, Norcross, GA 30092 USA.
38. del Rio, J. C.; Rencoret, J.; Prinsen, P.; Martinez, A. T.; Ralph, J.; Gutierrez, A. *J. Agric. Food Chem.* **2012**, *60*(23), 5922-5935.
39. NMR Database of Lignin and Cell Wall Model Compounds. Sally A. Ralph, John Ralph and Larry L. Landucci. 2009. Available at URL www.glbrc.org/databases_and_software/nmrdatabase/.
40. Rencoret, J.; Gutiérrez, A.; Nieto, L.; Jiménez-Barbero, J.; Faulds, C.B.; Kim, H.; Ralph, J.; Martínez, Á.T.; del Río, J. C. *Plant Physiol.* **2010**, 110.167254.
41. Lou, H.; Zhu, J. Y.; Lan, T. Q.; Lai, H.; Qiu, X. *ChemSusChem* **2013**, *6*(5), 919-927.
42. Vörös, J. *Biophys. J.* **2004**, *87*(1), 553-561.
43. Johannsmann, D.; Mathauer, K.; Wegner, G.; Knoll, W. *Phys. Rev. B* **1992**, *46*(12), 7808.

44. Jung, L. S.; Campbell, C. T.; Chinowsky, T. M.; Mar, M. N.; Yee, S. S. *Langmuir* **1998**, *14*(19), 5636-5648.
45. Shen, F. *Affinity Interaction between Hexamer Peptide Ligand HWRGWV and Immunoglobulin G Studied by Quartz Crystal Microbalance and Surface Plasmon Resonance*, PhD. Thesis, North Carolina State University, Raleigh, NC, USA (2010).
46. Hu, J. P.; Lanthier, P.; White, T. C.; McHugh, S. G.; Yaguchi, M.; Roy, R.; Thibault, P. *J. Chromatogr. B: Biomed. Sci. Appl.* **2001**, *752*(2), 349-368.
47. Várnai, A.; Siika-aho, M.; Viikari, L. *Biotechnol. Biofuels* **2013**, *6*(1), 30.
48. Pasquini, D.; Balogh, D.; Antunes, P.; Constantino, C.; Curvelo, A.; Aroca, R.; Oliveira, O. *Langmuir* **2002**, *18*(17), 6593-6596.
49. Pu, Y.; Cao, S.; Ragauskas, A. J. *Energy Environ. Sci.*, *4*(9), 3154-3166.
50. Johannsamnn, D.; Mathauer, K.; Wegner, G.; Knoll, W. *Phys. Rev. B: Condens. Matter Mater. Phys.* **1992**, *46*, 7808-7815
51. Yu, Z.; Gwak, K. S.; Treasure, T.; Jameel, H.; Chang, H. M.; Park, S. *ChemSusChem* **2014**, *7*(7), 1942-1950.
52. Suchy, M.; Linder, M. B.; Tammelin, T.; Campbell, J. M.; Vuorinen, T.; Kontturi, E. *Langmuir* **2011**, *27*(14), 8819-8828.
53. Rahikainen, J.L.; Evans, J.D.; Mikander, S.; Kalliola, A.; Puranen, T.; Tamminen, T.; Marjamaa, K.; Kruus, K. *Enzyme Microb. Technol.* **2013**, *53*(5), 315-321.

FOR TABLE OF CONTENTS USE ONLY

Lignin films from spruce, eucalyptus and wheat straw studied with electroacoustic and optical sensors: Effect of composition and electrostatic screening on enzyme binding.

Antonio Pereira, Ingrid C. Hoeger, Ana Ferrer Carrera, Jorge Rencoret, José C del Rio, Kristiina Kruus, Jenni Rahikainen, Miriam Kellock, Ana Gutierrez, Orlando J. Rojas