Interactions between Cellulolytic Enzymes with Native, Autohydrolysis, and Technical Lignins and the Effect of a Polysorbate Amphiphile in Reducing Nonproductive Binding

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ABSTRACT: Understanding enzyme–substrate interactions is critical in designing strategies for bioconversion of lignocellulosic biomass. In this study we monitored molecular events, in situ and in real time, including the adsorption and desorption of cellulolytic enzymes on lignins and cellulose, by using quartz crystal microgravimetry and surface plasmon resonance. The effect of a nonionic surface active molecule was also elucidated. Three lignin substrates relevant to the sugar platform in biorefinery efforts were considered, namely, hardwood autohydrolysis cellulolytic (HWAH), hardwood native cellulolytic (MPCEL), and nonwood native cellulolytic (WSCEL) lignin. In addition, Kraft lignins derived from softwoods (SWK) and hardwoods (HWK) were used as references. The results indicated a high affinity between the lignins with both, monocomponent and multicomponent enzymes. More importantly, the addition of nonionic surfactants at concentrations above their critical micelle concentration reduced remarkably (by over 90%) the nonproductive interactions between the cellulolytic enzymes and the lignins. This effect was hypothesized to be a consequence of the balance of hydrophobic and hydrogen bonding interactions. Moreover, the reduction of surface roughness and increased wettability of lignin surfaces upon surfactant treatment contributed to a lower affinity with the enzymes. Conformational changes of cellulases were observed upon their adsorption on lignin carrying preadsorbed surfactant. Weak electrostatic interactions were determined in aqueous media at pH between 4.8 and 5.5 for the native cellulolytic lignins (MPCEL and WSCEL), whereby a ∼20% reduction in the enzyme affinity was observed. This was mainly explained by electrostatic interactions (osmotic pressure effects) between charged lignins and cellulases. Noteworthy, adsorption of nonionic surfactants onto cellulose, in the form cellulose nanofibrils, did not affect its hydrolytic conversion. Overall, our results highlight the benefit of nonionic surfactant pretreatment to reduce nonproductive enzyme binding while maintaining the reactivity of the cellulose substrate.

INTRODUCTION

It has been shown that during the hydrolysis process of lignocellulosic biomass enzymes adsorb on the substrate and also undergo desorption and readsorption cycles.1 The enzyme adsorption is affected by various factors, such as the presence of lignin,2,3 type of pretreatment method used,4–6 as well as lignocellulose surface area and pore volume.1 The kinetics of cellulase adsorption on cellulose is considerably different from that on lignin.3 The lignin-rich components in biomass cause a nonproductive interaction with cellulolytic enzymes,2,4,5,7–11 thereby decreasing the hydrolysis rate.4 The most common types of interactions reported to occur between lignin and enzymes include hydrophobic,5,7 electrostatic,2 and hydrogen bonding.12 They explain some of the fundamental reasons for observed changes in affinity of proteins in aqueous media.13 Specifically, hydrophobic effects are favorable owing to entropic contributions associated with changes in water structuring; as a result, hydrophobic molecules tend to aggregate in order to minimize the surface area exposed to water. The hydrophobic interactions between lignin and enzymes have been discussed by Palonen et al., who found that the carbohydrate binding module (CBM) of Trichoderma reesei enzymes plays a significant role in the nonproductive adsorption on lignin.3 The amino acids exposed on the surface of T. reesei enzymes have hydrophobic character,14 and therefore, the enzyme may bind to the hydrophobic surface of lignin.

The electrostatic interactions were studied by Nakagame et al., who determined that at pH 4.8 some cellulases carry negative charges, whereas others are in fact oppositely charged.7 It was concluded that the positively charged proteins are preferentially adsorbed onto lignin, which display an anionic character at the same pH. In contrast, enzymes and lignin become negatively...
charged at high pH and, thus, electrostatic repulsion reduces their affinity. Moreover, the main acidic groups that exist on the surface of lignocellulosic fibers are associated with the uronic acid in xylans and the carboxylic acid in lignin, both of which display different isoelectric points and thus affect to different degree the interactions, depending on the pH of the medium. The amount of carboxylic groups present in the substrate varies depending on the plant species and the method used in its isolation. In milled wood lignin from aspen and pine these groups are measured to be 0.28 and 0.30 mmol/g of lignin, respectively, whereas from technical hardwood and softwood these increase to 0.41 and 0.47, respectively. hydrogen bonding occurs through hydroxyl groups present in lignins and enzymes. Among these groups, the phenolic hydroxyls are directly responsible for enzyme adsorption, while aliphatic and carboxylic hydroxyl groups have been indicated to contribute to lignin–enzyme ionic interactions. Despite the evidence accumulated, however, the detailed nature of these interactions still needs further examination.

Surfactants are known to reduce the affinity between enzymes and lignin, mainly by affecting the hydrophobic effects and also by bonding (H-bonds) with lignin. Among these groups, the phenolic hydroxyls and nonionic surfactants have been proven to be most effective to enhance enzymatic hydrolysis of lignocellulosic biomass. Proposed mechanisms for the effect of surfactant molecules on bioconversion include their ability to (1) open the structure of the lignocellulose substrate, making cellulose more accessible to enzymes, (2) improve enzyme stability, and (3) change enzyme–substrate interactions by blocking the lignin and reducing its affinity with enzymes. Direct evidence of these mechanisms have been elusive, however. Understanding the molecular role of surfactants and lignin surfaces remain highly relevant not only for enhancing sugar yields during lignocellulosic hydrolysis but also for considering possible enzyme recycling options.

Polyoxyethylene (20) sorbitan monoooleate, a nonionic surfactant, has been recognized to improve enzymatic hydrolysis; however, mechanisms aspects related to its interaction with lignin substrates are only known to a limited extent. In order to demonstrate how nonionic surfactants reduce nonproductive binding between lignin and enzymes, we carried out studies in real time and in situ by using surface-sensitive methods such as quartz crystal microbalance with dissipation monitoring (QCM-D) and surface plasmon resonance (SPR). The role of pH in the binding between lignin and enzymes was also elucidated. Five different lignin substrates were used, including native, enzymatically produced, autohydrolysis and reference (technical) lignins. In addition, a cellulose substrate in the form of cellulose nanofibrils (CNF) was used to evaluate the adsorption of the nonionic surfactant onto such hydrophilic material and its impact on enzymatic digestibility.

**Experimental Section**

**Materials.** All the chemicals were used as received without purification. Milli-Q water (18 Ω cm) was used in all the experiments. Polyoxyethylene (20) sorbitan monoooleate surfactant (trade name Tween 80), acetic anhydride (certified ACS), polyethylene (Mw 230000), polyethyleneimine (Mw 75000), and toluene were purchased from Sigma-Aldrich. 1,4-Dioxide (certified ACS) was obtained from Acros. Sulfuric acid, hydrogen peroxide (30% v/v), sodium acetate, and acetic acid were purchased from Fisher Scientific. A commercial mixture of enzymes (endoglucanases, cellobiohydrolases, and beta-glucosidases), under the trade name Cellic C7ec2, and a monocomponent enzyme, cellobiohydrolase I (CBH-1) from *Trichoderma reesei*, were kindly provided by Novozymes and the Technical Research Centre of Finland (VTI), respectively. The cellulase activity was 140 FPU/mL, as determined by the filter paper method.

**Lignin and Cellulose Substrates.** A low-ash softwood Kraft lignin (SWK) and a hardwood Kraft lignin (HWK) with high ash content were kindly donated by Domtar Inc. (Plymouth Pulp Mill, NC) and Suzano Pulp Mill (Brazil), respectively. Hardwood autohydrolysis cellulolytic lignin (HWAH) was prepared in our lab after autohydrolysis pretreatment of biomass (180 °C for 1 h). Native cellulolytic lignin from a hardwood (maple; MPEL) and nonwood biomass (wheat straw; SWCS) were also prepared in our lab by sing planetary ball-milling for 10 and 4 h, respectively. These latter lignin samples were extracted with 96% dioxane and isolated by enzymatic hydrolysis by using Ctec2 and Htec (9:1 ratio), also from Novozymes (5% solids content, acetate buffer 50 mM, pH 4.8, 10 FPU/g o.d. fiber, 96 °C, 50 °C). The lignin samples were purified by washing with Milli-Q-water (Millipore) and centrifugation (Beckmann Centrifuge Model J-21C, 3000 rpm) several times. After the purification step, the lignin samples were freeze-dried prior to use. Additionally, cellulose nanofibrils (CNF) were produced by microfluidization of refined, fully bleached birch Kraft fibers that were supplied by International Paper (Riegelwood, North Carolina).

**Lignin Composition and Characterization.** The insoluble and soluble components of the lignins were determined by acid hydrolysis. Briefly, 100 mg of the given lignin source were reacted with 1.5 mL of sulfuric acid (72% v/v H2SO4) at 25 °C for 2 h with stirring every 20 min. The dispersion was then diluted and autoclaved at 1 121 °C for 1.5 h, cooled down overnight, and filtered through a fine crucible for gravimetric determination of acid-insoluble lignin (Klassen lignin). The supernatant was used to determine acid-soluble lignin by UV–vis spectrosopy at 205 nm (Lambda XLS, PerkinElmer, Inc.). After neutralization of this supernatant, sugar analyses were performed in a HPLC unit, as previously reported. Hydroxyl groups content and molecular weight determinations were carried out by 31P NMR and gel permeation chromatography (GPC) following the procedures published elsewhere.

**Lignin Surface Charge and Size of Associated Structures.** The zeta potential of lignins and size of surfactant micelles were determined using a Zetasizer Nano-ZS (Malvern Instrument Ltd.). The zeta potential of the commercial mixture of enzymes and lignin at different pH values were measured in acetate buffer solution (50 mM) and used to evaluate the possible role of electrostatic interactions. Commercial enzymes were diluted to 5 mg/mL in 50 mM acetate buffer and pH of 4.8 and 5.5. Buffer solutions at different pH levels were used with a lignin concentration of 0.03% (w/v). Each lignin solution was mixed during 1 h at 50 °C and 200 rpm using an incubator shaker and allowed to settle for 1 h prior to zeta potential measurement. The change in the size of the micelle upon interaction with lignin was determined in aqueous media. For this, lignin samples were dissolved in 0.1 mg/mL nonionic surfactant solution to a final concentration of 0.03% (w/v) using a sonication bath for 15 min to allow dispersion, then an aliquot was sonicated for 1 h prior to zeta potential measurement. The dispersion was then diluted and autoclaved at 121 °C for 1.5 h, cooled down overnight, and filtered through a fine crucible for gravimetric determination of acid-insoluble lignin (Klassen lignin). The supernatant was used to determine acid-soluble lignin by UV–vis spectrosopy at 205 nm (Lambda XLS, PerkinElmer, Inc.). After neutralization of this supernatant, sugar analyses were performed in a HPLC unit, as previously reported. Hydroxyl groups content and molecular weight determinations were carried out by 31P NMR and gel permeation chromatography (GPC) following the procedures published elsewhere.

**Thin Film Substrate.** Gold-coated QCM-D sensors were cleaned with piranha solution (70% v/v H2SO4 + 30% v/v H2O2 (30%)) for 10 min, rinsed with abundant Milli-Q water, dried with nitrogen, and followed by UV/ozone treatment for 10 min. Silica wafers were also used after treatment with NaOH solution (1 M) during 20 s, followed by Milli-Q water rinsing and drying with nitrogen. Finally, they were subjected to UV/ozone for 5 min. For thin lignin film preparation, each sample was dissolved in 1,4-dioxane overnight (0.5 wt %). The respective lignin solution was allowed to settle, and a given supernatant volume was used for spin coating. Polystyrene (PS) was used as an intermediate layer to hold the lignin film on the gold-coated sensor or silica substrates. PS was dissolved in toluene (0.5 wt %) and spin-coated onto the cleaned surfaces using 2000 rpm and an acceleration of 1785 rpm−1. Then they were dried in an oven at 80 °C for 30 min. After this, four layers of the dissolved lignin were spin-coated on the PS-coated surfaces, using a speed of 2000 rpm and acceleration of 1785 rpm−1. Cellulose thin films were prepared dispersing CNF in Milli-Q water (0.1...
wt % solids content), followed by dispersion for 10 min at 25% amplitude in the tip sonicator and centrifuged at 10000 rpm for 30 min. CNF in the supernatant was spin-coated (3000 rpm for 30 s) on gold sensors carrying preadsorbed layer of polyethylenimine (PEI; 15 min adsorption using 500 ppm solution, washed, and dried with nitrogen gas), and dried in oven at 80 °C for 30 min. These thin films were stored in a desiccator until use.

**Thin Film Characterization.** AFM imaging was performed to assess the morphology, roughness and material distribution of the films. The different dispersions dried on silica surfaces were mounted on aluminum sample holders and examined with a Dimension 3000 scanning probe microscope from Veeco Metrology Group. Scanning was performed in tapping mode in air using 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 scanned areas were imaged. No image processing except flattening was made. Images were taken with a feedback loop to keep the amplitude of oscillation constant and the response of the feedback loop was measured. The response was used to measure how far the scanner was moved in Z in order to keep the amplitude of oscillation constant. Film thickness was measured using a variable angle spectroscopic ellipsometry (VASE; J. A. Woollam, Co., Inc.) with a wide spectral range capability of 190–1100 nm. The thickness was evaluated from the experimentally measured ellipsometric angles Ψ and Δ using the supplied software as the angle of incidence was varied between 65° and 70° between 400 and 800 nm. Water contact angles (WCA) of the thin films were measured by using a contact angle goniometer (SEO Phoenix 300 (Korea) via the sessile drop method with a drop volume of 20 μL at ambient conditions. WCA were calculated using ImageJ software. All measurements were done in triplicate. Silica wafers and PS-coated surfaces exhibited a WCA and roughness of 8 ± 1° and 0.92 nm and 86 ± 3° and 0.38 nm, respectively. The characterization of the different lignin thin films is included in Table 1.

<table>
<thead>
<tr>
<th>Lignin</th>
<th>Roughness (nm)</th>
<th>Thickness (nm)</th>
<th>WCA (deg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWK</td>
<td>10</td>
<td>24.6 ± 5.5</td>
<td>63 ± 1</td>
</tr>
<tr>
<td>HWK</td>
<td>2.4</td>
<td>14.3 ± 2.4</td>
<td>67 ± 3</td>
</tr>
<tr>
<td>HWAH</td>
<td>3.4</td>
<td>8.9 ± 3</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>MPCEL</td>
<td>0.53</td>
<td>21.4 ± 0.02</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>WSCEL</td>
<td>0.55</td>
<td>23.5 ± 0.03</td>
<td>70 ± 3</td>
</tr>
</tbody>
</table>

**Adsorption Experiments.** Adsorption and desorption phenomena were followed by using a quartz crystal microbalance (QCM) with dissipation monitoring (Model E4Q-E; Sense AB, Göteborg, Sweden) operated in continuous mode at 25 °C (40 °C in the case of cellulose model surfaces) and constant flow rate of 100 μL/min. Commercial cellulases (Cellic CTec2 from Novozymes) were diluted at 5 mg/mL in the acetate buffer at 50 mM, whereas the monocomponent CBH-I was used at 1 mg/mL at pH 5.5. Concentrations below and above the critical micelle concentration (CMC) of polyoxyethylene (20) sorbitan monolaurate were used. The CMC was 0.012 mM, as quoted by the supplier and concentrations used are reported as multiple units of the CMC: 0, 0.1 CMC, 0.013 mg/mL, 3 x CMC, 0.005 mg/mL, 6 x CMC, 0.01 mg/mL, and 19 x CMC. The lignin films were stabilized in buffer prior to the adsorption experiments; then, the different surfactant solutions were added until equilibrium. The surfactant excess was rinsed with buffer solution until the QCM signal reached a plateau. In binding experiments, once the thin films were primed with the adsorbed nonionic surfactant, the enzymes were injected and the shift in frequency was monitored. After the enzymes reached the maximum binding capacity, acetate buffer was used to remove the unbound enzymes. The adsorbed mass before and after rinsing was calculated from the shift in QCM frequency according to the Johannsmann model using eq 1:

$$m_a = \frac{\sqrt{n \mu A_f}}{2d_{lo}}$$  

All measurements were recorded at a fundamental resonance frequency of 5 MHz and its overtones corresponding to 15, 25, 35, 55, and 75 MHz. The third, fifth, and seventh were used for data processing. The experiments were run at least for triplicated and an analysis of variance (ANOVA) was performed to test the differences for the irreversible adsorbed mass of cellulases onto lignin surfaces under the given surfactant concentrations. Representative QCM-D curves for the adsorption of enzymes onto lignin pretreated with surfactant can be found as Supporting Information (Figures S1 and S3).

Adsorption was also monitored by using surface plasmon resonance (SPR, Navi 200, Oy BioNavis Ltd., Tampere, Finland); a description of this technique, as it applies to lignin films, can be found in our earlier publication.33 The experimental conditions were similar to those used for QCM-D adsorption experiments, except for the flow rate (15 μL/min). The thickness of the adsorbed surfactant layer was calculated using eq 2.

$$d = \frac{l_0 \Delta \theta}{2m (\eta_s - \eta)}$$  

where $d$ is the thickness, $l_0$ is the evanescent electromagnetic field decay (240 nm), $m$ is the sensitivity factor (109.95°/RIU), $\eta_s$ and $\eta$ are the refractive index of the surfactant and buffer solutions (1.472 and 1.334), respectively.

The extent of binding and hydrolysis were determined by fitting the QCM data to exponential decay (eq 3) and Boltzmann-sigmoidal (eq 4) equations.39 The QCM $\Delta f$ (Hz) shift of the third overtone was used.

$$\Delta f = M_{\text{max}} (1 - e^{-t/T})$$  

$$\Delta f = A + \frac{B - A}{1 + e^{(V_{\text{to}} - V)/C}}$$  

The adsorption parameters $M_{\text{max}}$ and $1/T$ represent the maximum adsorption capacity (determined from the minimum QCM frequency shift in Hz) and the adsorption rate (min⁻¹), respectively. Hydrolytic parameters $A$, $B$, $V_{\text{to}}$ and $1/C$ represent the frequency (Hz) at which hydrolysis starts and ceases, the time for maximum conversion, and the hydrolysis rate, respectively. All these parameters, except $A$, were obtained by minimizing the sum of the squared error between experimental and computed values from QCM data before rinsing. It was found that for CNF the enzyme binding highly correlated to the Boltzmann-sigmoidal eq 5:

$$\Delta f = A + \frac{M_{\text{max}} - A}{1 + e^{(V_{\text{to}} - V)/C}}$$  

The nonionic surfactant adsorption isotherms were fit to empirical functions such as the Langmuir or the one-step models,41 which assume that surfactant molecules interact with the solid substrate forming at equilibrium a solidd or hemimicelle:

**surface site + monomer ⇌ hemimicelle**

where the equilibrium constant is $k = a_{s}a_{r}/a_{w}a_{m}$ with $a_{s}$, $a_{r}$, and $a_{w}$ are the activities of adsorbed hemimicelle, surface site, and surfactant monomer in solution, respectively. At low concentrations, $a$ is equal to the surfactant concentration, $C$. Thus, for $C < \text{CMC}$, $a_{s}$ and $a_{m}$ are the concentration of adsorbed hemimicelle and unoccupied surface areas, respectively. Through mass action law, the activities can be converted to adsorption density ($\Gamma$) by using eq 6:

$$k^c = \frac{\Gamma}{\Gamma_{\text{to}} - \Gamma}$$  

where $\Gamma_{\text{to}}$ is the maximum adsorption density at high solution concentrations and $n$ is the surfactant aggregation number. Furthermore, the thermodynamic surface free energy of micellization ($\Delta G_{\text{mic}}$) and aggregation ($\Delta G_{\text{ag}}$) were calculated according to eqs 7 and 8, respectively.
Δ = −GR T c m c ln m_0 (7)
Δ = −GR T k_0 (ln s/a)^n (8)

where \( R \) and \( T \) are the gas constant and the absolute temperature, respectively. The fitting parameters \( \Gamma_\infty, k_0, \) and \( n \) were obtained by minimizing the sum of the squared error between experimental and computed data.

Table 2. Chemical Characteristics of the Lignins Used in This Study

<table>
<thead>
<tr>
<th>Lignin</th>
<th>ALL, %wt</th>
<th>ASL, %wt</th>
<th>Carbohydrates, %wt</th>
<th>M_w</th>
<th>M_n</th>
<th>Aliphatic OH</th>
<th>Condensed OH</th>
<th>Noncondensed OH</th>
<th>Total OH</th>
<th>COOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWK</td>
<td>94</td>
<td>5.4</td>
<td>&lt;1</td>
<td>4473</td>
<td>1062</td>
<td>2.7</td>
<td>2.6</td>
<td>2.8</td>
<td>8.1</td>
<td>0.7</td>
</tr>
<tr>
<td>HWK</td>
<td>85</td>
<td>6</td>
<td>1.2</td>
<td>3236</td>
<td>1002</td>
<td>1.8</td>
<td>3.7</td>
<td>1.4</td>
<td>6.9</td>
<td>0.5</td>
</tr>
<tr>
<td>HWAH</td>
<td>76</td>
<td>2.4</td>
<td>1.2</td>
<td>5696</td>
<td>1383</td>
<td>2.6</td>
<td>1.4</td>
<td>0.6</td>
<td>4.6</td>
<td>0.1</td>
</tr>
<tr>
<td>MPCEL</td>
<td>91</td>
<td>2.2</td>
<td>8.3</td>
<td>17892</td>
<td>3762</td>
<td>1.9</td>
<td>4.7</td>
<td>1.3</td>
<td>7.9</td>
<td>0.5</td>
</tr>
<tr>
<td>WSCEL</td>
<td>87</td>
<td>8</td>
<td>12</td>
<td>9383</td>
<td>4041</td>
<td>8.2</td>
<td>0</td>
<td>1.2</td>
<td>9.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Figure 1. AFM tapping mode images of the lignin thin films used in this study (columns from left to right correspond to scan sizes of 5 × 5 μm, 1 × 1 μm, and 500 × 500 nm). The samples included the following lignins (from top to bottom): SWK, HWK, HWAH, MPCEL, and WSCEL. The height scale corresponds to \( Z \) values between 0 and 90 nm, as indicated by the bar.
RESULTS AND DISCUSSION

Lignins and Thin Films. The chemical characteristics of the lignins used for the preparation of thin films are shown in Table 2, which includes the acid-insoluble (A1, equivalent to Klason lignin) and acid-soluble (ASL) fraction content, carbohydrates, and hydroxyl group concentration (31P NMR) as well as molecular mass.

Based on the nature of the lignin samples, they are regarded as suitable precursor of models to study the interaction with enzymes and nonionic surfactants. Lignins, as those considered here are of great importance not only to study interactions with enzymes but also with different molecules relevant to fiber processing, mainly in biorefinery platforms. Kraft pulping generates phenolic units on fibers due to the cleavage of aryl ether linkages of lignin. In contrast, autohydrolysis pretreatment mainly removes hemicelluloses, leaving the other components less affected, mainly lignin and cellulose. In the case of lignin, the severity of this type of pretreatment caused its recondensation and relocation on the solid substrate during the process. β-O-4 linkages and ester bonds were acid-catalyzed, which resulted in a more condensed lignin with a high molecular weight and more phenolic hydroxyl groups. Samuel et al. pretreated poplar at 200 °C for 30 min and determined a reduction of 43% in aliphatic hydroxyl groups and an increase of 130 and 96% in the total phenolic and carboxylic groups, respectively. Therefore, the autohydrolysis pretreatment can cause a significant effect on lignin. However, we note that the conditions used in this work were comparatively milder (180 °C for 1 h). On the other hand, the cellulolytic lignin, which undergoes minor structural changes, has been considered as representative of the whole lignin present in lignocellulosic biomass.

The AFM images of the different thin lignin films (Figure 1) indicate complete and uniform surface coverage. High magnification images (second and third columns in Figure 1) reveal the arrangement of lignin after spin-coating on the solid support. As the solvent evaporated, lignin self-associated to create distinctive features (spherical, nanosized particles), likely stabilized by hydrogen bonding and van der Waals forces. Section analyses of these images (Supporting Information, Table S1) reveal a dense structure for SWK. Characteristic properties of these lignin films are presented in Table 1. The root-mean-square (RMS) roughness was determined from the images in Figure 1 (5 × 5 μm). SWK thin lignin film showed the highest roughness, 10 nm, whereas those for HWK and HWAH were 2.4 and 3.4 nm, respectively. The native cellulolytic lignins exhibited the lowest values, 0.53 and 0.55 nm for MPCEL and WSCEL, respectively. The thickness of these thin lignin films was determined by ellipsometry and noted to be in the range between 9 and 25 nm. Based on the ellipsometric model used and the fitted data, the refractive indices of the lignins was determined to be 1.60 at 630 nm, which indicate compact and dense films. The wettability of the films was also determined by WCA values included in Table 1. All the lignin exhibited similar values, between 60° and 79°; the native cellulolytic lignins showed the highest WCA.

The stability of the lignin films supported on silica was tested by immersion in deionized water and 1 M NaOH solution for 3 h. It was noted that immersion in water did not affect the wettability of these lignin films, indicating strong attachment to the solid support. However, a slight decrease in roughness was observed, possibly due to hydration or desorption of loosely attached lignin molecules. Immersion in the alkali solution caused partial lignin removal from the solid support.

Effect of pH on Lignin Surface Charge. Electrostatic interactions affect nonproductive binding between lignin and enzymes; therefore, the surface charge, assessed by zeta potential measurements, was determined for the lignins dispersed in aqueous media at given pH. The zeta potential became more negative as the pH increased (Figure 2) due to the deprotonation of carboxyl groups. Similar observations applied to lignin residues after pretreatment with dilute acid and sulfite to overcome recalcitrance of lignocellulose (SPORL). A linear regression indicated that pH has a strong effect on the surface charge of HWAH lignin; however, it showed the lowest zeta potential values, probably because of the reduction in the number density of –COOH groups after autohydrolysis pretreatment. Native lignins exhibited intermediate values, whereas the kraft lignins (SWK and HWK) presented a high surface charge due to the large number of carboxylic groups generated during the kraft pulping process, which can more easily ionize in water.

Enzymatic hydrolysis of biomass with Trichoderma reesei cellulases similar to those used in this work indicated an optimal pH range for hydrolysis of 5.5 to 6.2, while pH = 4.8 has been used for pure cellulotic substrates. In this study, two pH levels, 4.8 and 5.5, were selected to investigate the affinity of enzymes with lignin, as discussed in the next section.

Enzyme Adsorption on Lignin Thin Films. The nonproductive adsorption of cellulases on lignin was investigated. The isolated lignins used in this study are expected to differ from unextracted or native lignin present in the biomass. In fact, any fractionation method would unavoidably affect the characteristics and properties of the isolated lignin. Keeping this in mind, electrostatic forces are expected to influence nonproductive interactions between enzymes and lignin. QCM adsorption profiles for cellulases adsorbing onto lignin substrates at two pH levels, 4.8 and 5.5, are shown in Figure 3. The binding parameters were determined and included as Supporting Information (see Table S2). At pH 4.8 the maximum binding was higher for SWK and HWK (46 Hz or 8.2 mg/m²) and 51 Hz or 8.6 mg/m², respectively.
respectively); however, the adsorption rate was similar for the five lignin types. After rinsing with background aqueous solution and upon reaching equilibrium, the amount of irreversibly adsorbed enzymes was calculated: the native cellulolytic lignins, MPCEL and WSCEL, adsorbed enzymes to a lesser extent compared to the other samples: 4.4 ± 0.2 and 4.7 ± 0.1 mg/m², respectively, whereas for SWK, HWK, and HWAH, the corresponding values were 6.5 ± 0.2, 6.8 ± 0.1, and 5.7 ± 0.1 mg/m². The same pattern was observed at pH 5.5. However, as the pH increased, the maximum amount of adsorbed enzymes was lower. The most notorious effect was observed for the adsorption rate, which decreased significantly by changing the pH of the medium from 4.8 to 5.5 (Table S2). This reduction in the rate of adsorption is due to the increased negative charge of both lignin (Figure 2) and enzymes. In fact, the zeta potential of the enzyme mixture diluted in acetate buffer exhibited values of −6.4 and −9.6 mV at pH 4.8 and 5.5, respectively. The higher negative values for both lignin and enzymes explain increased electrostatic repulsion. The enzyme irreversibly adsorbed at pH 5.5 was calculated to be 6.3 ± 1.6, 6.5 ± 0.5, 5.5 ± 0.1, 3.3 ± 0.1, and 3.8 ± 0.1 mg/m² for SWK, HWK, HWAH, MPCEL, and WSCEL lignins, respectively. Although electrostatic repulsion by the increased osmotic pressure is likely to have a major role, the difference in the amount of enzymes adsorbed irreversibly on SWH, HWK, and HWAH substrates at pH 4.8 and 5.5 was not significant (<5%). In contrast, the reduction of irreversibly adsorbed enzymes onto native cellulolytic lignins was ∼20%, suggesting that a weak electrostatic interaction existed between these lignin and the cellulases. The similar adsorbed amount measured here for the commercial cellulases suggests that hydrophobic interactions are a driving factor that control the affinity to lignin. Correlations among the different hydroxyl groups in lignin and the maximum frequency upon enzyme adsorption are plotted in Figure S2 in the Supporting Information. Phenolic hydroxyl groups have been related to increased enzyme affinity for lignin.12,19 In this work, MPCEL, SWK, and HWK lignins contained more phenolic hydroxyl groups than HWAH and WSCEL (Table 2). This fact partially explains the higher amount of bound enzyme at the two pH levels tested for SWK, HWK, HWAH, and WSCEL lignins, with the exception of the MPCEL sample (Figure S2b, Supporting Information). In contrast, a reduction in the amount of absorbed
enzymes was observed as the carboxylic group content increased (Figure S2c of Supporting Information), which can favor a reduction in nonproductive interactions,\textsuperscript{54} for example, by increasing the negative charge of lignin.\textsuperscript{51,52,54} Lignin not only acts as a physical barrier, but due to its nature and pretreatment method used for isolation, it is also a chemically heterogeneous macromolecule that takes part in nonspecific interactions with the enzymes.\textsuperscript{12}

Mechanism of Surfactant Adsorption onto Lignin. Hydrophobic interactions between the alkyl chains and the hydrophobic sites of the surface have been ascribed as the main driving force for surfactant adsorption.\textsuperscript{35} Figure 4 shows the adsorption isotherms for the polyeorbate surfactant onto the thin lignin films before (total adsorption, Figure 4a) and after (irreversible adsorption, Figure 4b) rinsing. As expected, the surfactant adsorbed mass increased as the concentration increased. Upon rinsing, the adsorbed mass decreased by \( \sim 50\% \) and a plateau was reached for surfactant concentrations above the CMC. A sharp increase in adsorption at concentration close to the CMC is due to the lateral interactions between surfactant monomers which start to associate.\textsuperscript{56} Using Gibbs adsorption isotherms, the maximum surface excess (\( \Gamma_{\text{sa}, \text{air/liquid}} \)) for the surfactant at the air–liquid interface was found to be \( 29.3 \times 10^{-7} \) mol/m\(^2\), similar to values reported in previous work.\textsuperscript{57} Significantly lower adsorbed amounts were determined after rinsing, indicating the removal of loosely bound molecules and yielding an irreversibly adsorbed surfactant layer. These findings suggest the complexity of surfactant adsorption onto lignin surfaces, mainly due to their heterogeneous structural features.

The QCM frequency data obtained upon surfactant adsorption before rinsing was fitted to the kinetic models introduced before for the maximum adsorption capacity (eq 3) and the one-step model (eq 6). The results for the first model are presented in Table S3 whereas those for the one-step model are shown in Table 3. Both models described satisfactorily the maximum adsorption of surfactant onto lignin (high correlation values except for SWK lignin). As the surfactant concentration increased, the binding rate (1/\( \tau \)) calculated by the exponential decay model increased. The highest maximum binding (\( M_{\text{max}} \)) was found for HWK, while the highest binding rate corresponded to MPCEL lignin. Remarkably, a high total adsorption generally correlated with a high total hydroxyl content (Table 2), which is expected to contribute to hydrogen bonding with the surfactant polar groups. In the one-step model the surfactant adsorption is described as reactions between unoccupied sites and surfactant molecules to form hemimicelles.\textsuperscript{41} Furthermore, the standard free energy for surface micellization (eq 7) was found to be 28 kJ/mol, whereas the standard free energy of aggregates (eq 8) adsorbed onto the different lignin substrates yielded lower values (Table 3), indicating that surface aggregation was energetically favored. Maximum adsorption density, \( \Gamma_{\infty} \) (\( 10^{-7} \) mol/m\(^2\)), values were similar for all the lignins (Table 3). A value of average aggregation number parameter \( n > 1 \) suggests surface micellization, whereas a value <1 suggests that the each adsorbed molecule occupies more than one site. Interestingly, in the case of the HWK lignin \( n > 1 \), indicating surface micellization. This would explain partial removal of lignin molecules upon surfactant adsorption and rinsing (note Figure S1b–d, where the surfactant adsorption is followed by a sharp increase in frequency due to the presence of surfactant). A total lignin removal of 19 ± 2% was calculated by QCM measurements in air for the sensor with HWK lignin before and after surfactant adsorption. Surfactants can solubilize lignin by hydrophobic interactions.\textsuperscript{36} The micelle size of polyoxyethylene (20) sorbitan monooleate in water is 10.3 ± 0.4 nm at 25 °C, as determined by dynamic light scattering. It was found that in the presence of HWK lignin the surfactant tended to form aggregates with lignin, with sizes up to 128 ± 4 nm and lignin solubility increased up to 25% in the aqueous medium.

The changes in morphology and wettability of lignin films after adsorption of polyoxyethylene (20) sorbitan monooleate (6 xCMC) are shown in Figure 5. Compared to the values of the original films of SWK, HWK, and HWAH, the surface roughness was reduced upon surfactant adsorption (Table 1). In contrast, the roughness increased for MPCEL and WSCSEL, suggesting the possibility of steric hindrance effects, which correlates with lower enzyme accessibility. As revealed by AFM images, the morphology of the lignin surface changed after surfactant adsorption, which can be associated with the formation of surfactant aggregates since polyoxyethylene (20) sorbitan monooleate forms half-micelles or hemimicelles on hydrophobic surfaces.\textsuperscript{59,60} Imaging of the micelles was challenging due to the high roughness of the surfaces. The mechanism of micellar break up leading to the formation of hemimicelles on hydrophobic surfaces may occur by the contribution of various effects: hydrophobic lateral interactions and hydrophobic interactions between the hydrocarbon chains and the surface,\textsuperscript{64} as well as hydrogen bonding.\textsuperscript{61} Surfactant adsorption at 6 xCMC was monitored by SPR, which allows to determine the thickness of the adsorbed layer by using eq 2. The results indicated a layer thickness between 2.3 and 4.3 nm at maximum adsorption (before rinsing) and 0.9–3 nm for the molecules irreversibly bounded (measured after rinsing) onto the lignin surfaces (Table S4). At close-packed coverage, the surfactant molecules would be arranged in an end-to-end configuration with a molecule distance \( \sim 5 \) nm.\textsuperscript{62} Surfactant layer of 6.7 nm has been reported on hydrophobic surfaces (WCA 110°) by using the surface force apparatus,\textsuperscript{65} whereas on surfaces with a WCA \( \sim 71^\circ \), the thickness was found to be \( \sim 1.2 \) nm for polyoxyethylene (20) sorbitan monooleate by using a dual slab waveguide interferometer.\textsuperscript{65} A rough estimation of the apparent layer thickness on the solid can be determined by multiplying the maximum surfactant adsorption density at the solid/liquid interface (\( \Gamma_{\infty, \text{solid/liquid}} \)) by the maximum adsorption density at the air/liquid interface (\( \Gamma_{\infty, \text{air/liquid}} \)), which gives values close to 1 (before rinsing) and <1 (after rinsing) surfactant adsorption. Moreover, the number of molecules per nm\(^2\) and the aggregation number \( n \) were both <1 at irreversible adsorption (Figure 5). Therefore, the results in this study suggest the presence of a monolayer or a disorganized

Table 3. Fitting Parameters for the One-Step Model upon Adsorption of Polyoxyethylene (20) Sorbitan Monooleate onto Different Lignins before Rinsing

<table>
<thead>
<tr>
<th>lignin</th>
<th>( \Gamma_{\infty} ) (10(^{-7}) mol/m(^2))</th>
<th>( k )</th>
<th>( n )</th>
<th>( R^2 )</th>
<th>( -\Delta G_{\infty}^a ) (kJ/mol)</th>
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<tr>
<td>SWK</td>
<td>50.7</td>
<td>3.7</td>
<td>0.14</td>
<td>0.71</td>
<td>23.6</td>
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<tr>
<td>HWK</td>
<td>37.2</td>
<td>4.0 ( \times 10^7 )</td>
<td>1.7</td>
<td>0.99</td>
<td>25.5</td>
</tr>
<tr>
<td>HWAH</td>
<td>27.7</td>
<td>3.6 ( \times 10^6 )</td>
<td>0.9</td>
<td>0.98</td>
<td>29.9</td>
</tr>
<tr>
<td>MPCEL</td>
<td>23.9</td>
<td>7.0 ( \times 10^6 )</td>
<td>1.0</td>
<td>0.98</td>
<td>33.4</td>
</tr>
<tr>
<td>WSCSEL</td>
<td>30.9</td>
<td>3.2 ( \times 10^7 )</td>
<td>0.7</td>
<td>0.99</td>
<td>30.5</td>
</tr>
</tbody>
</table>

\( \Gamma_{\infty} \) (10\(^{-7}\) mol/m\(^2\)) corresponds to the maximum adsorption density; \( k \) is the equilibrium constant; \( n \) is the aggregation number; \( R^2 \) corresponds to the coefficient of determination for the model; and \( -\Delta G_{\infty}^a \) (kJ/mol) is the standard free energy of surface aggregation.
or patchy bilayer structure since the thickness values obtained in this study after rinsing are comparable to those obtained with similar WCA values. The low thickness values compare to the end-to-end conformation can be related to the chemical structure of the surfactant used, which possess a double-carbon bond in the aliphatic chain that would force the molecule to stand at an angle of the surfactant used, which possess a double-carbon bond in the end-to-end conformation can be related to the chemical structure of the surfactant used, which possess a double-carbon bond in the alkyl chain of the surfactant that would force the molecule to stand at an angle.<ref> Moreover, the WCA values decreased after surfactant adsorption, indicating that the surfactant head groups form the outmost layer.

**Surfactant Blocking Mechanism for Enzymes Exposed to Lignins.** From previous results (Figure 3), a slight decrease in the amount of enzymes adsorbed on lignin occurred with increased pH, explained by electrostatic repulsion. Therefore, a pH of 5.5 was selected for the aqueous medium to study the effect of surfactant on the reduction of enzyme affinity with lignin, especially at surfactant concentrations above the CMC. The maximum binding was determined according to eq 3 for both surfactant and enzymes (Table S3). As the surfactant concentration increased, the surfactant total adsorbed mass and adsorption rate increased, whereas the opposite behavior was observed for the enzymes. The total enzyme adsorption decreased significantly and the presence of the surfactant layer slowed down enzyme adsorption on the lignin surfaces, especially at concentrations above the CMC. The relative mass of irreversibly adsorbed surfactant and commercial enzyme after rinsing is presented in Table S5 (in the case of HWK lignin, the enzymes were added without rinsing since the surfactant caused partial lignin removal). There was a small amount of adsorbed surfactant at 0.1 x CMC (free surfactant in solution) on the lignin surface (data not shown), and the molecules were removed easily by rinsing; thus, it was not possible to calculate the amount of surfactant irreversibly adsorbed on SWK, HWK, and HWAH films. In the case of SWK and HWK films, and for surfactant concentration close to the CMC (but still at submicellar concentrations), the reduction of enzyme affinity was less than 20%. Remarkably, for the autohydrolysis and native cellulolytic lignins, the surfactant prevented enzyme adsorption by more than 80%. At submicellar concentrations, the surfactant molecules attach to the hydrophobic surfaces, possibly with the hydrocarbon chains aligned parallel to the surface. In fact, the unsaturated carbon—carbon double bond in the alkyl chain of polynoxyethylene (20) sorbitan monooate favors the molecule to adopt a parallel configuration at the surface, whereas lateral interactions between monomers take place at concentrations close to the CMC. Finally, at concentrations above the CMC, the molecules become decreasingly tilted with respect to the surface, and have a greater tendency to form aggregates. It is found that the affinity of polynoxyethylene (20) sorbitan monooate with lignin increases with concentration, for example, increasing concentration from 0.85 to 19 x CMC (Figure 4). As the irreversibly surfactant adsorbed mass increased, the affinity of cellulases with lignin decreased. Using the ANOVA statistical analysis (α-level 5%) a significant difference in the amount of enzymes irreversibly adsorbed was determined (for surfactant concentration below and above the CMC) for all the lignins except for MPCEL. A surfactant concentration close to the CMC was effective in reducing the enzyme affinity. Concentrations above the CMC showed no significant difference in the amount of enzymes irreversible adsorbed. Moreover, the effect of surfactant (6 x CMC) on the reduction of lignin affinity with monocomponent CBH-I enzyme was investigated (QCM) and the results for binding parameters are shown in Table S6. This monocomponent enzyme exhibited higher affinity for the technical and pretreated lignins (SWK, HWK, and HWAH) compared to the native cellulolytic lignins. Interestingly, the surfactant prevented the monocomponent adsorption for all the lignins: except for the case of SWK substrates, negligible or no adsorption was determined on lignins primed by the surfactant. This is explained by the blocking of the hydrophobic sites by the adsorbed surfactant, thus, preventing nonproductive interaction with the CBH-I that contains a carbohydrate binding domain as primary site for hydrophobic interactions with lignin.

The deactivation or inhibitory effect of lignin toward cellulases was studied by enzymatic hydrolysis and SDS-PAGE analyses (data not shown) and by using the fluid containing residual free enzymes that were extracted from the QCM module during the respective experiment. A low glucan conversion was observed upon incubation of microcrystalline cellulose and cellobiose with the fluid recovered, which is expected to be due to the large amount of cellulases adsorbed onto the lignin substrates (Tables S2 and S6). SDS-PAGE gels revealed bands associated with free
enzymes contained in the fluid recovered after adsorption on lignin but they displayed much lower intensity compared to that from fresh enzyme solutions. This indicates the negative effect of enzyme affinity with lignin. In contrast, the reduction of enzymes adsorption upon surface treatment with the polysorbate surfactant, yielded a microcrystalline cellulose glucan conversion similar to that of fresh enzymes. Moreover, the bands in the SDS-PAGE gels displayed similar intensity to those from the fresh enzymes. Monocomponent enzymes, such as cellobiohydrolases (CBH), have a catalytic domain (CD) connected by a linker with a carbohydrate-binding module (CBM).\(^{64}\) CBM facilitates the nonproductive adsorption onto lignin\(^1\) through hydrophobic interactions.\(^{23}\) \(\beta\)-Glucosidase exhibits larger hydrophobic path regions compared to endoglucanases and cellobiohydrolases,\(^65\) which have been found to be responsible for a high binding on lignin, mainly because of hydrophobic interactions.\(^{65,66}\) Phenolic compounds have been reported to inactivate \(\beta\)-glucosidase to a large extent.\(^{67,68}\) Therefore, there is evidence to support that the hydrophobic interactions with the enzymes are greatly reduced by blocking lignin sites with surfactant. By using the residual free enzymes extracted from the QCM module, it was observed that once lignin was pretreated with a nonionic surfactant, the glucan conversion from cellulbiose increased. Although this conversion did not reach the same level as that observed for fresh enzymes, this negative effect was not notorious when the substrate was microcrystalline cellulose, as explained above, indicating that free cellulases were still catalytically active.

**Effects of Polysorbate Surfactant on Cellulose Bioconversion.** Cellulose nanofibrils (CNF) were used to test the effect on enzymatic hydrolysis of surfactant treatment. Polyoxyethylene (20) sorbitan monooleate (6 xCMC) was adsorbed onto CNF films before enzyme addition (Figure S3). Interestingly, the surfactant adsorbed on CNF to a lower extent compared to that on lignin (Figure 4b), 5.1 ± 0.9 x 10\(^{-7}\) mol/m\(^2\) (=0.7 ± 0.1 mg/m\(^2\)), but more irreversibly (rinsing was not effective for surfactant removal). Enzyme affinity of CBH-I was higher for cellulose (Table S7) than for lignin (Table S6), but a different behavior was found for commercial cellulases, where a higher maximum adsorption was found on SWK and HWK lignins compared to that on pure cellulose (Tables S2 and S7). The combination of electrostatic (Table S2) and hydrophobic interactions, mainly caused by the presence of endoglucanases,\(^3,22\) and \(\beta\)-glucosidases\(^{65,66}\) in the commercial cellulases mixture, may contribute to the nonproductive affinity of cellulases with lignin. By fitting the enzyme binding data (eqs 3 and 5) for substrates consisting of neat cellulose film and that after surfactant treatment (Table S7), a decrease in the total cellulase adsorption (\(M_{\text{max}}\)) and adsorption rate (\(1/r\)) was observed: from 36 to 29 (Hz) and from 2.7 to 2.1 (min\(^{-1}\)), respectively, in the case of cellulase mixtures. For CBH-I the maximum adsorption decreased from 43 to 40 (Hz) but the adsorption rate was similar. However, the hydrolytic parameters (eq 4) were similar (Table 4) without and with preadsorbed surfactant. Therefore, there is an indication that the polyelectrolyte amphiphile does not affect negatively the biocatalytic process. The affinity of mononic surfactants with cellulose has been found to be higher than for other hydrophilic surfaces.\(^{29}\) Based on the QCM dissipation data (not reported), the presence of the nonionic surfactant during the application of enzyme increased the swelling of the cellulose film by ~43%. Probably, this swelling prevents enzyme deactivation by facilitating desorption.\(^{21}\) Both the ethylene oxide headgroup and the aliphatic tails contribute cooperatively to the adsorption on cellulose at low concen-

<table>
<thead>
<tr>
<th>enzyme</th>
<th>surfactant (aCMC)</th>
<th>(B)</th>
<th>(V_{50})</th>
<th>1/C</th>
<th>(R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctec2</td>
<td>0</td>
<td>78.4 ± 5.8</td>
<td>1.3 ± 0.2</td>
<td>1.6 ± 0.3</td>
<td>0.9904</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>75 ± 25</td>
<td>1.3 ± 0.2</td>
<td>1.5 ± 0.4</td>
<td>0.9885</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>78 ± 8.3</td>
<td>1.2 ± 0.2</td>
<td>1.5 ± 0.3</td>
<td>0.9883</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>68 ± 7.4</td>
<td>1.4 ± 0.4</td>
<td>1.4 ± 0.3</td>
<td>0.9896</td>
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<tr>
<td>CBH-I</td>
<td>0</td>
<td>49.22 ± 2.2</td>
<td>78.1 ± 0</td>
<td>0.02 ± 0</td>
<td>0.9527</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>49.2 ± 6.1</td>
<td>82 ± 3</td>
<td>0.02 ± 0</td>
<td>0.9860</td>
</tr>
</tbody>
</table>

\(^{44}\) B corresponds to the frequency at which hydrolysis ends, \(V_{50}\) is the time for maximum conversion, and 1/C is the hydrolysis rate.

trations; at higher concentration, lateral attraction becomes dominant.\(^{69}\) Surfactant adsorption data onto CNF at three concentrations (above the CMC) was fitted to the one-step model (eq 6, Table S8); the results indicated an aggregation number \(n > 1\), which suggests micellization.\(^{70}\) Moreover, the number of molecules per area was found to be <0.4 molecules/\(\text{nm}^2\) and the apparent number of layers <1. Also, low dissipation values (Figure 6b, inset) were measured and the layer thickness was found to be <0.5 nm, as determined by SPR (Table S4). Hydrogen bonding between the surfactant polar groups and the hydroxyl groups of the surface may contribute to such results.

**Conformation of the Adsorbed Layers.** The changes in the viscoelastic properties of the adsorbed layers were followed in the QCM experiments. The dissipation factor or total energy dissipation (\(\Delta D\)) is the ratio of the energy dissipated to the energy stored at the interface. Large dissipation values are obtained when the adsorbed molecules are loosely adsorbed or a soft, low density layer accumulates on the surface. By plotting the changes in total energy dissipation (\(\Delta D\)) versus the changes in frequency (\(\Delta f\)), it was possible to describe qualitatively the macromolecule conformation upon layer build up (see Figure 6). A small slope in the \(\Delta D - \Delta f\) profiles indicates that the adsorbed layer does not greatly undergo conformational changes. This was observed in Figure 6a for the adsorption of cellulases on lignin films (the profiles exhibited a straight and small slope, suggesting a flat conformation of the molecule on the surface). In contrast, a steeper slope with a slight curvature was observed for cellulases adsorbed on lignin films pretreated with the polysorbate amphiphile (Figure 6b), suggesting conformational changes of the proteins on the surface upon adsorption. It is thus plausible that the nonionic surfactant prevents the adsorption of enzymes through their hydrophobic domains,\(^9\) therefore, the enzymes tend to adopt an end-to-end, instead of a flat conformation. Similar response was obtained onto CNF substrates; however, here the enzymes needed to overcome the preadsorbed surfactant layer by changing more significantly their conformation, as revealed by a sharp slope in the respective profiles. For all the substrates, the surfactant layer exhibited a small slope and dissipation values upon adsorption (less than 1 unit; see inset in Figure 6b), which indicate that a rigid layer covered the surface.

Although the main purpose of this work was to study the binding mechanism of enzymes on lignin surfaces in the presence of a preadsorbed nonionic surfactant, the results are also useful in elucidating the potential for enzyme recycling. The surfactants can make the substrate more accessible for enzymatic
The interactions between lignin, a nonionic surfactant, and a commercial multicomponent cellulolytic enzyme mixture were studied. Electrostatic interactions were found to have a minor effect on the affinity of enzymes with the lignins studied, with the exception of native lignins, which exhibited a ∼20% reduction in enzyme affinity by inducing lignin charging at pH 5.5. A dominant effect of hydrophobic interactions was found by application of the nonionic surfactant that reduced the nonproductive adsorption of cellulases onto lignin by up to 63, 92, 93, 100, and 100% for SWK, HWK, HWAH, MPCEL, and WSCEL, respectively. The kinetics of surfactant adsorption followed a one-step model and surface aggregation onto lignin was found to be an energetically favorable and reversible process. The low adsorbed thickness values suggest surfactant adsorption as a monolayer or patchy layers. The extent of adsorption of the nonionic surfactant onto the lignins increased with surfactant concentration above the critical micelle concentration (CMC), leading to better protection toward nonproductive binding. Compared to the case of lignin substrates, the polysorbate amphiphile adsorbed to a lower degree but irreversibly on cellulose; however, cellulase digestibility in surfactant-treated cellulose was not affected negatively.

**CONCLUSIONS**

The Interactions between Lignin, a Nonionic Surfactant, and a Commercial Multicomponent Cellulolytic Enzyme Mixture were Studied. Electrostatic Interactions Were Found to Have a Minor Effect on the Affinity of Enzymes with the Lignins Studied, with the Exception of Native Lignins, Which Exhibited a ∼20% Reduction in Enzyme Affinity by Inducing Lignin Charging at pH 5.5. A Dominant Effect of Hydrophobic Interactions Was Found by Application of the Nonionic Surfactant That Reduced the Nonproductive Adsorption of Cellulases Onto Lignin by Up to 63, 92, 93, 100, and 100% for SWK, HWK, HWAH, MPCEL, and WSCEL, Respectively. The Kinetics of Surfactant Adsorption Followed a One-Step Model and Surface Aggregation Onto Lignin Was Found to Be an Energetically Favorable and Reversible Process. The Low Adsorbed Thickness Values Suggest Surfactant Adsorption as a Monolayer or Patchy Layers. The Extent of Adsorption of the Nonionic Surfactant Onto the Lignins Increased With Surfactant Concentration Above the Critical Micelle Concentration (CMC), Leading to Better Protection Toward Nonproductive Binding. Compared to the Case of Lignin Substrates, the Polysorbate Amphiphile Adsorbed to a Lower Degree But Irreversibly on Cellulose; However, Cellulase Digestibility in Surfactant-Treated Cellulose Was Not Affected Negatively.

**ASSOCIATED CONTENT**

Th**e Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biомac.5b01203.

Section analysis for AFM images (Table S1), binding parameters (Tables S2, S3, S6, S7, and S8), thickness of the adsorbed surfactant layer (Table S4), irreversible adsorbed mass (Table S5), representative QCM curves (Figures S1 and S3), and correlations (Figure S2) (PDF).

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**Notes**

The authors declare no competing financial interest.

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