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Cellulases adsorb reversibly on biomass lignin

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Abstract
Adsorption of cellulases onto lignin is considered a major factor in retarding enzymatic cellulose degradation of lignocellulosic biomass. However, the adsorption mechanisms and kinetics are not well understood for individual types of cellulases. This study examines the binding affinity, kinetics of adsorption, and competition of four monocomponent cellulases of Trichoderma reesei during adsorption onto lignin. TrCel7A, TrCel6A, TrCel7B, and TrCel5A were radiolabeled for adsorption experiments on lignin-rich residues (LRRs) isolated from hydrothermally pretreated spruce (L-HPS) and wheat straw (L-HPWS), respectively. On the basis of adsorption isotherms fitted to the Langmuir model, the ranking of binding affinities was TrCel5A > TrCel6A > TrCel7B > TrCel7A on both types of LRRs. The enzymes had a higher affinity to the L-HPS than to the L-HPWS. Adsorption experiments with dilution after 1 and 24 hr and kinetic modeling were performed to quantify any irreversible binding over time. Models with reversible binding parameters fitted well and can explain the results obtained. The adsorption constants obtained from the reversible models agreed with the fitted Langmuir isotherms and suggested that reversible adsorption–desorption existed at equilibrium. Competitive binding experiments showed that individual types of cellulases competed for binding sites on the lignin and the adsorption data fitted the Langmuir adsorption model. Overall, the data strongly indicate that the adsorption of cellulases onto lignin is reversible and the findings have implications for the development of more efficient cellulose degrading enzymes.

KEYWORDS
adsorption, biomass, cellulase, competition, lignin, reversible

1 | INTRODUCTION

Lignin has been considered as one of the major obstructions in biorefinery operations aiming at enzymatically converting cellulose in lignocellulosic biomass into glucose before further downstream processing (Li, Pu, & Ragauskas, 2016). Nonproductive adsorption of cellulases onto lignin is considered an important mechanism behind retardation of enzymatic cellulose degradation in lignocellulose-based processes (Liu, Sun, Leu, & Chen, 2016; Saini, Patel, Adsul, & Singhania, 2016; Sipponen et al., 2017). Studies have reported adsorption of cellulases onto lignin isolated from various biomass feedstocks and have correlated such adsorption with the observed retardation of enzymatic degradation of pure model cellulose in the presence of the isolated lignin (Kellock, Rahikainen, Marjamaa, & Kruus, 2017; Rahikainen et al., 2011; Tu, Pan, & Saddler, 2009). Hydrophobic interaction (Sammond et al., 2014; Tu et al., 2009), electrostatic...
interaction (Lan, Lou, & Zhu, 2013; Yarbrough et al., 2015), and hydrogen bonding (Sewalt, Glasser, & Beauchemin, 1997; Yu et al., 2014) have been regarded as the cause of the nonproductive binding of cellulases to lignin. However, more recently, it has been recognized that several interactions between the different chemical groups in the lignin and in the enzymes may be occurring simultaneously (Liu et al., 2013; Sipponen et al., 2017).

Accordingly, several mitigating efforts by including additives such as bovine serum albumin (BSA) and surfactants in the hydrolysis reaction (Börjesson, Engqvist, Sipos, & Tjerneld, 2007; Yang & Wyman, 2006), engineering the charge of the enzymes (Whitehead, Bandi, Berger, Park, & Chundawat, 2017) or changing the pH of the reaction (Lan et al., 2013) have been used with varying degrees of success. However, the precise mechanism in the enzyme–lignin interaction that leads to reduced recoverable activity or cellulose conversion is not well understood, especially with respect to the individual types of enzymes present in a cellulolytic mixture. Several studies have indicated irreversible binding and/or reduced recovery of activity during adsorption of cellulases to lignin (Kellock et al., 2017; Rahikainen et al., 2011) or during enzymatic hydrolysis of pretreated lignocellulosic biomass (Gao et al., 2014; Várnai, Viikari, Marjamaa, & Siika-Aho, 2011). Yet, there are also studies reporting that isolated lignin neither retarded the enzymatic cellulose degradation (Barsberg, Selig, & Felby, 2013; Weiss, Börjesson, Pedersen, & Meyer, 2013; Djajadi et al., 2018) nor reduced the recoverable cellulase activity after adsorption (Rodrigues, Leitão, Moreira, Felby, & Gama, 2012). These studies suggested that the binding of the enzymes on lignin is reversible by nature. However, such a phenomenon has not been investigated up to date as the loss of enzyme activity because of nonproductive adsorption onto lignin has in general been considered as irreversible (Saini et al., 2016).

Generally, adsorption of protein onto solid surfaces is known as a dynamic process involving partial exchange of adsorbed and desorbed states. During the process, however, the constant conformational rearrangements between the two states can compromise the structural integrity of the protein, leading to irreversible structural change(s) that can affect subsequent adsorption behavior (Norde, 1986). This denaturation because of protein unfolding has been suggested as the cause of reduced enzymatic cellulose degradation in the presence of lignin (Rahikainen et al., 2011; Sammond et al., 2014), especially at elevated temperature (Börjesson et al., 2007; Rahikainen et al., 2011). Consequently, cellulose hydrolysis by thermostable enzymes was affected less by lignin compared with that performed by enzymes with lesser thermostability (Rahikainen, Mollanen et al., 2013). In this study, well-characterized monocomponent cellulases derived from Trichoderma reesei were studied to assess their binding affinity on lignin-rich residues (LRRs) from different biomass feedstocks, to distinguish reversible and irreversible bindings over extended reaction time using kinetic experiments and modeling, as well as to assess their competition with one another during adsorption on lignin.

2 | MATERIALS AND METHODS

2.1 | Biomass pretreatment and lignin isolation

LRRs were obtained from extensive enzymatic hydrolysis of hydrothermally pretreated spruce (HPS) and wheat straw (HPWS) followed by protease treatment optimized from a previous method (Rahikainen et al., 2011). The hydrothermal pretreatment conditions were 195°C for 15 min (log R0 = 3.97) for wheat straw (Djajadi et al., 2017) and 200°C for 10 min (log R0 = 3.94) for spruce. The composition of the LRRs have been determined using the National Renewable Energy Laboratory protocol (Sluiter et al., 2008). The LRRs contained 82.3% and 83.7% total lignin for lignin from HPS (L-HPS) and HPWS (L-HPWS), respectively. The isolation method was shown to remove adsorbed enzymes as indicated by the reduction in nitrogen content of the LRRs (Djajadi et al., 2018; Rahikainen et al., 2011). Even though the isolated LRRs contained residual carbohydrates, the carbohydrates were not accessible to the enzymes and were not traceable to the surface of the LRRs (Djajadi et al., 2018).

2.2 | Enzyme purification and characterization

Monocomponent cellulases, that is, cellulohydrolases (TrCel7A and TrCel6A) and endoglucanases (EGs: TrCel7B and TrCel5A) were produced from Trichoderma reesei (Teleomorph Hypocrea jecorina) at VTT and were purified according to previous work (Suurnäkki et al., 2000). The molecular weights (Mw), isoelectric point (PI), and hydrophobic surface characteristics (patch score) of the enzymes have been determined previously (Kellock et al., 2017; Várnai, Siika-Aho, & Viikari, 2013). The activity of TrCel7A and TrCel6A was assessed by hydrolyzing 0.1% (w/v) regenerated amorphous cellulase as a substrate using 50 mg/g dosage for 2 hr at 45°C and pH 5.0. The activity of TrCel7B and TrCel5A was determined using hydroxyethyl cellulose (Sigma-Aldrich Co., MO) 1% (w/v) as a substrate for 2 hr at 45°C and pH 5.0. The products were quantified as reducing sugars using 3,5-dinitrosalicylic acid (Merck, Darmstadt, Germany). Final protein purity and protein concentrations were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (SDS-PAGE) using the Criterion Imaging System (Bio-Rad Laboratories Inc., CA) and the Detergent Compatible (DC) Protein assay (Bio-Rad Laboratories Inc., CA), respectively. The monocomponent enzymes were pure as indicated by the presence of single bands (Supporting Information Figure S1). The details of the enzymes used in this study are presented in Table 1.

2.3 | Radiolabeling of the enzymes through reductive methylation

The enzymes (TrCel7A, TrCel6A, TrCel7B, and TrCel5A) were radiolabeled with tritium through reductive methylation using tritiated sodium borohydride ([3H]NaBH4) and formaldehyde (CH2O) (Means & Feeney, 1968; Tack, Dean, Ellett, Lorenz, & Schechter, 1980) with modifications according to previous works (Rahikainen, Evans et al., 2013; Wahlström, Rahikainen, Kruus, & Suurnäkki, 2014). For the
reaction, 3 mg enzyme was buffer exchanged in 0.2 M sodium borate buffer pH 8.5 at 4°C and was incubated on ice. Formaldehyde solution (Sigma–Aldrich Co., MO) was added in fivefold molar excess of the molar concentration of free amino groups in the enzyme. \(^{3}H\)NaBH\(_4\) with 100 mCi activity (5–15 Ci/mm, PerkinElmer, MA) was dissolved in 0.01 M NaOH (1 Ci/ml) and added to the reaction. After 60 min, the reaction was stopped by transferring the mixture to Econo-Pac 10 DC gel filtration column (Bio-Rad Laboratories Inc., CA) and eluting it with 0.05 M sodium acetate buffer pH 5.0 to exchange the buffer solution. The protein-rich fractions were pooled and transferred to another gel filtration column. The specific radioactivities as determined by liquid scintillation counting (LSC) and protein concentration assay were 0.5, 0.5, 1.7, and 2.8 Ci/mmol for \(\text{TrCel7A}\), \(\text{TrCel6A}\), \(\text{TrCel7B}\), and \(\text{TrCel5A}\) respectively. Accordingly, in the subsequent adsorption experiments, the \(^{3}H\)-labeled enzymes were mixed in 1:20 (for \(\text{TrCel7A}\) and \(\text{TrCel6A}\)) and 1:50 dilution ratio (for \(\text{TrCel7B}\) and \(\text{TrCel5A}\)) with their nonradiolabeled counterparts to allow accurate detection as done previously (Rahikainen et al., 2013; Wahlström et al., 2014). SDS-PAGE analysis indicated that there was no degradation of the radiolabeled enzymes (Supporting Information Figure S1).

### 2.4 Adsorption experiments and LSC

All of the enzyme adsorption experiments were performed in 0.05 M sodium acetate buffer pH 5.0 at a substrate concentration of 1% DM (dry matter) and at a temperature of 45°C with moderate mixing. The temperature was chosen because of its relevance to large-scale commercial applications that operate at 37–50°C (Larsen, Haven, & Thirup, 2012). After 1 hr incubation, the experiment was terminated by centrifugation and the supernatant was collected for determination of unbound enzymes using LSC. The supernatant was mixed with Ultima Gold\(^{TM}\) XR liquid scintillation cocktail (PerkinElmer, Waltham, MA) and the counts per minute values of the \(^{3}H\)-labeled enzymes were measured using Tri-Carb 2810 TR LSC (PerkinElmer, Waltham, MA) with 15 min counting time. Enzyme blanks were used to determine the fraction of bound enzyme. Adsorption isotherms were established at the initial protein concentration range of 2–16 μM for L-HPS and 1–8 μM for L-HPWS in triplicates for each concentration. The adsorption isotherms data were fitted to the one binding-site Langmuir adsorption model:

\[
B = B_{\text{max}} \frac{K_{\text{ads}} [F]}{1 + K_{\text{ads}} [F]} \tag{1}
\]

where \(B\) is the amount of bound enzyme, \(B_{\text{max}}\) is the maximum adsorption capacity, \(K_{\text{ads}}\) is the Langmuir affinity constant, and \([F]\) is the concentration of unbound enzyme.

### 2.5 Reversibility test and kinetic modeling of adsorption

The reversibility test was conducted at similar conditions as with adsorption isotherms. The experiment was performed using \(\text{TrCel5A}\) and \(\text{TrCel6A}\) on both L-HPS and L-HPWS. The enzymes were incubated with 1% DM LRRs at concentrations of 4, 8, and 16 μM for L-HPS and 2, 4, and 8 μM for L-HPWS. Subsamples were taken at different time points, centrifuged, and measured to determine the amount of enzyme bound. There were two sets of reactions in which twofold buffer dilution was performed at different time points. In the first set of reaction, the “Early Dilution,” the samples were incubated for 1 hr, after which a subsample was taken and dilution was performed. After dilution, the binding of the enzyme was monitored after 1, 5, and 23 hr by taking subsamples. In the second set of reaction, the “Late Dilution,” the samples were incubated for 24 hr during which subsamples were taken after 1, 6, and 24 hr incubation. After 24 hr, buffer dilution was performed and subsamples were taken after 1, 5, and 23 hr to follow the binding of the enzymes. The experiments were done in duplicates and enzyme blanks were used to determine the amount of the enzyme bound.

Kinetic modeling was performed by using MATLAB R2015a (The Mathworks Inc., MA). The differential equations of a kinetic model were solved by numerical integration using ode15s ordinary differential equation solver. The resulting time curves were simultaneously fitted to the combined data from the Early Dilution and Late Dilution experiments of an enzyme–lignin pair by nonlinear regression using lsqcurvefit. The fitting parameters included the rate

### Table 1 Summary of the characteristics of monocomponent cellulases used in this study

| Enzymes | Old name | EC number | Domain architecture | \(M_w\) (kDa)\(^a\) | \(p_l\)\(^b\) | Core | CBM | Total | Activity\(^c\) |
|---------|----------|------------|---------------------|-------------------|--------|------|------|-------|-------------|
| \(\text{TrCel7A}\) | CBHI | 3.2.1.91 | GH7-CBM1 | 56.0 | 3.6–4.3 | 6.7 | 6.6 | 13.3 | 5.7% |
| \(\text{TrCel6A}\) | CBHII | 3.2.1.91 | GH6-CBM1 | 56.7 | 5.4–6.2 | 14.1 | 1.9 | 16.0 | 14.8% |
| \(\text{TrCel7B}\) | EGI | 3.2.1.4 | GH7-CBM1 | 51.9 | 4.5–4.9, 4.7 | 6.2 | 0.8 | 7.0 | 378.2 nkat/mg |
| \(\text{TrCel5A}\) | EGI | 3.2.1.4 | GH5-CBM1 | 48.2 | 5.6 | 2.6 | 7.0 | 9.6 | 568.4 nkat/mg |

Note. CBM: carbohydrate binding module; EC: enzyme commission; \(M_w\): molecular weight; \(p_l\): isoelectric point.

\(^a\)Based on Várnai et al. (2013).

\(^b\)Based on Kellock et al. (2017); major isoform in \(p_l\) measurement is underlined.

\(^c\)Activity of \(\text{TrCel7A}\) and \(\text{TrCel6A}\) is displayed as the degree of regenerated amorphous cellulase hydrolysis, whereas that of \(\text{TrCel7B}\) and \(\text{TrCel5A}\) is displayed as a specific activity.
constants of reversible adsorption $k_{\text{rev}}$, desorption $k_{\text{Rev}}$, and irreversible adsorption $k_v$ and the maximum adsorption capacity of lignin, $B_{\text{max}}$. To find the global maximum for the iterative fitting procedure, the fitting was repeated with a full factorial set of initial value combinations with five different initial values (10, 1, 0.01, 0.0001, and 0) for each rate constant and two initial values for the adsorption capacity $B_{\text{max}}$ including the maximum observed adsorption and its double. For three rate constants and a single $B_{\text{max}}$ this meant 250 repetitions of fitting. The identifiability of the parameters was assessed statistically according to previous work (Pihlajaniemi, Sipponen, Kallioinen, Nyyssölä, & Laakso, 2016), by determining the relative standard deviation (RSD) of each parameter from the set of best fitting parameters, including the sets with the $R^2$ at least 99% of the highest $R^2$.

2.6 | Competitive binding experiment

Competitive binding experiments were performed similarly as with the adsorption isotherms experiments, except that an equimolar amount of another enzyme type was added on top of the other before the experiments to establish adsorption isotherms. TrCel5A and TrCel6A were chosen in this experiment, so that in one experiment a radiolabeled TrCel5A was accompanied with nonradiolabeled TrCel6A and vice versa. The isotherms were established at the ranges of 2–16 $\mu$M for L-HPS and 1–8 $\mu$M for L-HPWS using triplicates for each concentration. Enzyme blanks were used to determine the fraction of bound enzyme.

2.7 | Statistical analysis

One-way analysis of variance was performed using JMP 12 (SAS Institute Inc., NC) with post hoc analysis using the Tukey-Kramer honestly significant difference test at $p \leq 0.05$. Fitting of isotherms data to one binding-site Langmuir adsorption model was performed using OriginPro 2016 (OriginLab Corporation, MA).

3 | RESULTS AND DISCUSSION

3.1 | Binding of monocomponent cellulases on LRRs

Adsorption isotherms of TrCel7A, TrCel6A, TrCel7B, and TrCel5A on LRRs isolated from HPS (L-HPS) and wheat straw (L-HPWS) were established to determine their binding affinity in hydrolytic conditions (pH 5.0 and 45°C). The isotherms revealed that TrCel5A had the highest affinity on both L-HPS and L-HPWS (Figure 1). In the adsorption on L-HPS, the binding of TrCel5A was noticeably higher compared with the other enzymes, although less pronounced in the case of binding on L-HPWS. Visually, the order of the enzymes’ affinity was more distinct on L-HPWS compared with L-HPS where the following order of decreasing value can be made: TrCel5A > TrCel6A > TrCel7B > TrCel7A. In general, the enzymes had higher affinity on L-HPS compared with L-HPWS as previously shown in the case of radiolabeled MaCel45A (Cel45 EGs from *Melanocarpus albomyces*) (Rahikainen et al., 2013). The labeling procedure has thus been shown to work consistently despite potential modifications to the surface accessible lysine residues. Change in hydrophobicity because of methylation is minimal due to the low number of total lysine residues in the enzymes (6–13 residues). Furthermore, the procedure is known to not affect the positive charge of lysine residues (Tack et al., 1980), making it unlikely for the pH of the protein to be modified as to affect adsorption.

One binding-site Langmuir adsorption model was fitted to the isotherms data to provide quantitative parameters of the binding. The Langmuir adsorption model has previously been used to model the binding of cellulases to lignin (Börjesson et al., 2007; Rahikainen, Evans et al., 2013; Tu et al., 2009) because of its simplicity and versatility despite the inadequacy and shortcomings to depict the adsorption of proteins on solid surface (Latour, 2015; Rabe, Verdes, & Seeger, 2011). The relative association constant ($a$), in particular, has been shown to reflect the relative affinity during the initial slope of the isotherm (Gilkes et al., 1992; Nidetzky, Steiner, Hayn, &
TABLE 2  Langmuir isotherm parameters from the fitted adsorption data of monocomponent cellulases on isolated lignin-rich residues, L-HPS and L-HPWS, respectively

| Adsorbent | Enzyme  | $10 \times B_{\text{max}}$ (μmol/g) | $10 \times K_{\text{ads}}$ (L/μmol) | $10 \times \alpha$ (L/g) | $R^2$ |
|-----------|---------|-----------------------------------|-----------------------------------|-------------------------|-------|
| L-HPS     | TrCel7A | 3.34 ± 0.28                       | 5.48 ± 0.82                       | 1.83 ± 0.31             | 0.957 |
|           | TrCel6A | 3.66 ± 0.33                       | 8.58 ± 1.86                       | 3.14 ± 0.74             | 0.926 |
|           | TrCel7B | 7.94 ± 1.29                       | 1.42 ± 0.47                       | 1.13 ± 0.42             | 0.972 |
|           | TrCel5A | 9.13 ± 0.61                       | 8.94 ± 1.32                       | 8.16 ± 1.32             | 0.984 |
| L-HPWS    | TrCel7A | 0.84 ± 0.06                       | 6.02 ± 0.96                       | 0.51 ± 0.09             | 0.975 |
|           | TrCel6A | 1.72 ± 0.15                       | 6.57 ± 1.28                       | 1.13 ± 0.24             | 0.974 |
|           | TrCel7B | 4.27 ± 1.64                       | 0.62 ± 0.28                       | 0.26 ± 0.16             | 0.967 |
|           | TrCel5A | 3.07 ± 0.24                       | 4.66 ± 0.61                       | 1.43 ± 0.22             | 0.991 |

Note. $B_{\text{max}}$: maximum adsorption capacity; $K_{\text{ads}}$: Langmuir adsorption constant; L-HPS: lignin-rich residues from hydrothermally pretreated spruce; L-HPWS: lignin-rich residues from hydrothermally pretreated wheat straw; $\alpha$: relative association constant ($B_{\text{max}} \times K_{\text{ads}}$). The reported constants and errors were obtained from the fitting of three experimental replicates using the one binding-site Langmuir adsorption model.

Claeyssens, 1994; Rahikainen, Evans et al., 2013). Accordingly, the order of affinity based on $\alpha$ values (Table 2) fits with the visual observation noticed in the isotherms curve for both L-HPS and L-HPWS (Figure 1) and confirmed the previously mentioned ranking of binding affinity: $\text{TrCel5A} > \text{TrCel6A} > \text{TrCel7B} > \text{TrCel7A}$.

Alternatively, analyzing adsorption at the lower concentration range of an isotherm also provides information on the affinity of the enzyme in nonsaturated conditions. At low initial protein concentration, the ratio of unbound compared to bound enzyme is very low. Therefore the fraction of the bound enzyme reflects the initial affinity toward the substrate without oversaturation of the surface of the adsorbent or excessive interaction among adsorbate molecules. The fraction of bound enzyme at the initial protein concentration of 2 μM after 1 hr showed that TrCel5A had the highest binding with 88% and 55% of enzymes adsorbed on both L-HPS and L-HPWS, respectively (Figure 2). The degree of binding affinity based on the fraction of bound enzyme both on L-HPS and L-HPWS (Figure 2) was: $\text{TrCel5A} > \text{TrCel6A} > \text{TrCel7A} = \text{TrCel7B}$. To a certain extent, this also confirmed the similar previously established order based on visual observation of the isotherms curve (Figure 1) and fitted $\alpha$ values (Table 2).

The results in this study evidently showed that TrCel5A had the highest binding affinity compared with all the tested enzymes, both in L-HPS and L-HPWS (Figures 1,2). In a recent study, where the same set of enzymes were subjected to binding with model surface lignin isolated from HPS and HPWS on quartz crystal microbalance with dissipation monitoring (QCM-D), TrCel7B had the highest binding (Kellock et al., 2017). The finding is in contrast with this study where TrCel7B had the second lowest affinity (Table 2). However, based on maximum adsorption capacity ($B_{\text{max}}$), the values of TrCel7B and TrCel5A were in the same magnitude both in L-HPS and L-HPWS (Table 2), which can explain the discrepancy of the finding in the two works. Nevertheless, a direct comparison between the previous QCM-D work (Kellock et al., 2017) and this current work will be difficult because of different underlying mechanisms in the methods and even properties of the isolated lignin (Rahikainen, Martin-Sampedro et al., 2013). Both current work (Figures 1,2, Table 2) and previous study (Kellock et al., 2017), nevertheless, agreed that TrCel6A had the second highest affinity and TrCel7A had the lowest affinity from the four tested enzymes.

The binding affinity of the enzymes was compared with their intrinsic properties to find the correlation between the two. TrCel5A, which bound the highest, has the lowest molecular weight ($M_W$) of the tested monocomponent cellulases (Table 1). However, the trend is not consistent across the enzymes because TrCel7A, which had the lowest affinity, had the second highest $M_W$. The highest affinity of TrCel5A and TrCel6A correlated to their pI values, which are above the experimental pH value of 5.0. This rendered them to be positively charged and therefore increased the tendency to bind to isolated LRRs from HPS and wheat straw, which were previously found to be negatively charged in the experimental pH (Rahikainen, Evans et al., 2013). However, the trend is not consistent because the pI value of the dominant band was lower in TrCel5A compared with TrCel6A.
(Table 1). Estimated hydrophobic patch score did not provide a clear trend either because both the overall and carbohydrate binding module scores were both second highest in the case of TrCel7A (Table 1), which had the lowest affinity (Figure 1). At this point, correlating the affinity of the enzymes with their properties was not feasible, yet the enzymes displayed similar ranking of affinity in the two substrates. Experiments at longer duration will be needed to assess the nature of the binding.

### 3.2 Reversibility test and kinetic modeling of adsorption

Kinetic modeling was applied for studying the proportions and potential mechanisms of reversible and irreversible adsorption of TrCel6A and TrCel5A on L-HPS and L-HPWS. First, the time course of adsorption and subsequent desorption after dilution of the system by a factor of two were determined. The dilution was performed either after 1 hr (early dilution) or 24 hr of adsorption (late dilution). Three initial enzyme concentrations were used, covering the linear and saturated areas of the adsorption isotherms (Figure 1). The aim was to quantify the proportion of irreversible binding from the difference in desorption after early and late dilution and to provide data for distinguishing the different models. The idea was that the longer incubation before the late dilution would allow irreversible binding to advance further and lead to lower desorption of enzymes compared with the early dilution, which would allow quantification of the proportion and the rate constant of irreversible binding.

Four different kinetic models were studied. Model 1 (Equation (2)) describes reversible adsorption, which may turn into irreversible by a further first-order reaction, resulting in kinetic Equations (3) and (4), where \( E \) stands for free enzymes, \( L \) for free binding sites, and \( EL \) for bound enzymes, and the subscripts Rev and Ir refer to reversible and irreversible binding, respectively, and the corresponding rate constant \( k \). The concentration of free sites is the proportion of unoccupied sites multiplied by lignin concentration, \( [L] = \beta_{max} - (EL_{\text{Rev}} + EL_{\text{Ir}}) \), [lignin].

\[
E + L \xrightarrow{k_{\text{Rev}}} EL_{\text{Rev}}
\]

\[
\frac{dEL_{\text{Rev}}}{dt} = k_{\text{Rev}}[E][L] - (k_{-\text{Rev}} + k_{\text{Ir}})[EL_{\text{Rev}}]
\]

\[
\frac{dEL_{\text{Ir}}}{dt} = k_{\text{Ir}}[EL_{\text{Rev}}]
\]

Model 2 (Equation (5)) describes separate reversible and irreversible binding on the same binding sites, representing a situation where binding may occur differently, depending on, for example, orientation; therefore, following the Langmuir-kinetics of reversible adsorption (Equation (6)) and a second-order reaction of irreversible binding (Equation (7)) in parallel.

\[
E + L \xrightarrow{k_{\text{Rev}}} EL_{\text{Rev}}
\]

\[
k_{\text{Ir}} \leftarrow \frac{1}{EL_{\text{Ir}}}
\]

\[
\frac{dEL_{\text{Rev}}}{dt} = k_{\text{Rev}}[E][L] - k_{-\text{Rev}}[EL_{\text{Rev}}]
\]

\[
\frac{dEL_{\text{Ir}}}{dt} = k_{\text{Ir}}[E][L]
\]

Models 3 and 4 represent completely reversible (Langmuirian) (Equation (8)) and completely irreversible (Equation (9)) adsorption, each follows the kinetics of Equations (6) and (7), respectively.

\[
E + L \xrightarrow{k_{\text{Rev}}} EL_{\text{Rev}}
\]

\[
E + L \xrightarrow{k_{\text{Ir}}} EL_{\text{Ir}}
\]

The models were fitted to the experimental data and compared in terms of \( R^2 \) and parameter identifiability. Identifiability describes whether the parameter value can be determined exclusively, displaying importance (or significance) of the fit, or whether it can adopt an arbitrary value, deeming it irrelevant. The identifiability was described as RSD of each parameter at the optimum fit, determined from the set of repetitions reaching at least 99% of the best fit, according to \( R^2 \).

Majority of adsorption occurred during the first hour, after which only minor changes were observed (Figure 3), indicating that equilibrium was reached within 1 hr of adsorption. TrCel6A and TrCel5A showed similar adsorption patterns, whereas they differed on L-HPS and L-HPWS (Supporting Information Figures S2–S5). After dilution, minor or no release of enzymes occurred from L-HPS, whereas considerable desorption from L-HPWS-lignin was observed. The lack of desorption from L-HPS appears to suggest irreversible binding, but on a closer look this conclusion turns out to be premature. In fact, completely irreversible adsorption fitted poorly to the data (Supporting Information Figure S6) with \( R^2 \) below 0.78 in each case (Table 3). Given the high initial rate of adsorption, the long incubation should have easily allowed completion of irreversible binding, thus leading to either depletion of free enzymes or complete saturation of binding sites. However, such behavior was not observed and instead, equilibrium was reached at each concentration between free and adsorbed enzymes and the endpoints followed a Langmuir isotherm (Supporting Information Figure S6). By definition, both the dynamic equilibrium and Langmuirian behavior indicate reversible adsorption.

Displaying the data from the dilution experiments as binding isotherms revealed that most of the points after dilution either fully or partially returned to the original point before dilution (Supporting Information Figures S7 and S8). In other words, the ascending isotherm (before dilution) overlaps with the descending isotherm (after dilution), displaying no or limited hysteresis in the adsorption. This behavior has also been described as a display of
fully reversible binding during studies on the binding of monocomponent cellulases on cellulose (Palonen, Tenkanen, & Linder, 1999; Pellegrini et al., 2014). The Langmuir constants $K_{ads} = \frac{K_{rev}}{K_{rev}}$ and $B_{max}$ determined from the kinetic modeling (Table 3) and those determined from the adsorption isotherms data (Table 2) are found to be in agreement with one another (Supporting Information Figure S9). These observations further gave a strong indication of reversible binding on lignin. The adsorption constant $K_{ads}$ of $Tr\text{Cel6A}$ and $Tr\text{Cel5A}$ were lower on L-HPWS compared with L-HPS both in the adsorption isotherms fitting (Table 2) and modeling data (Table 3). This indicated lower binding affinity of cellulases on L-HPWS than L-HPS, which is in accordance with the high desorption on L-HPWS after dilution (Figure 3). The difference in affinity can offer an explanation on the previous observations where L-HPS was found to retard the enzymatic hydrolysis of model cellulose more than L-HPWS (Kellock et al., 2017; Rahikainen, Moilanen et al., 2013).

For L-HPS, the Models 1 and 2 showed a similar fit ($R^2$ of 0.896–0.923) and parameter values as that of completely reversible adsorption. In contrast, poor identifiability was observed for the irreversible adsorption rate constant $k_r$ (RSD from 140% to $4.8 \times 10^{7%}$), indicating that reversible adsorption behavior can fully

FIGURE 3 Response surface graphs displaying the fitting of experimental data of $Tr\text{Cel6A}$ adsorption on lignin-rich residues isolated from hydrothermally pretreated spruce (L-HPS) (a,b) and hydrothermally pretreated wheat straw (L-HPWS) (c–f) modeled as reversible adsorption (a–d) and using Model 1 (e,f) with early (a,c,e) and late dilution (b,d,f) [Color figure can be viewed at wileyonlinelibrary.com]
explain the results. No quantifiable irreversible binding was observed and the reason for low desorption was high affinity of L-HPS (Table 3). For L-HPWS, a higher amount of desorption provided a higher resolution for determining irreversible binding. Model 1 showed a slightly better fit for both enzymes ($R^2$ of 0.945 and 0.967) compared with reversible binding (0.936 and 0.965) with a relevant irreversible binding rate ($\text{RSD of } k_{Ir}$ lower than that of $k_{Rev}$ and $k_{Rev}$), whereas Model 2 neither provided improvement in fit nor relevance of $k_{Ir}$ (Table 3). This suggested the possibility of partial irreversible binding on L-HPWS, that is, the enzymes are first bound reversibly, which is then followed by further interactions leading to irreversible binding. This is in line with the idea of protein unfolding taking place

| LRRs | Enzyme | Model | Fit $R^2$ | $k_{Rev}$ | $k_{Rev}$ | $k_{Ir}$ | $B_{max}$ | $K_{ads}$ |
|------|--------|-------|-----------|-----------|-----------|-----------|-----------|-----------|
| HPS  | TrCel6A| Model 1| 0.896     | 0.0157    | 0.0163    | 3.82E-10  | 0.356     | 0.959     |
| HPS  | TrCel6A| Model 2| 0.896     | 0.0120    | 0.0166    | 3.64E-03  | 0.357     | 0.723     |
| HPS  | TrCel6A| Reversible | 0.772 | 0.0157    | 0.0164    | 0.0217    | 0.272     |           |
| HPS  | TrCel5A| Model 1| 0.923     | 0.0165    | 0.0171    | 1.45E-07  | 0.562     | 0.962     |
| HPS  | TrCel5A| Model 2| 0.923     | 0.0154    | 0.0167    | 7.81E-04  | 0.562     | 0.921     |
| HPS  | TrCel5A| Reversible | 0.923 | 0.0160    | 0.0167    |           | 0.562     | 0.958     |
| HPS  | TrCel5A| Irreversible | 0.784 |           |           | 0.0158    | 0.471     |           |
| HPWS | TrCel6A| Model 1| 0.945     | 0.0397    | 0.0855    | 1.18E-03  | 0.223     | 0.464     |
| HPWS | TrCel6A| Model 2| 0.936     | 0.0311    | 0.0515    | 3.01E-04  | 0.217     | 0.603     |
| HPWS | TrCel6A| Reversible | 0.936 | 0.0314    | 0.0518    |           | 0.217     | 0.606     |
| HPWS | TrCel6A| Irreversible | 0.640 |           |           | 0.0743    | 0.124     |           |
| HPWS | TrCel5A| Model 1| 0.967     | 0.0202    | 0.0626    | 4.05E-04  | 0.385     | 0.322     |
| HPWS | TrCel5A| Model 2| 0.965     | 0.0140    | 0.0512    | 4.49E-03  | 0.378     | 0.274     |
| HPWS | TrCel5A| Reversible | 0.965 | 0.0188    | 0.0525    |           | 0.379     | 0.357     |
| HPWS | TrCel5A| Irreversible | 0.570 | 0.0332    | 0.170     |           |           |           |

Identifiability (RSD at optimum fit)

| LRRs | Enzyme | Model | Fit $R^2$ | $k_{Rev}$ | $k_{Rev}$ | $k_{Ir}$ | $B_{max}$ |
|------|--------|-------|-----------|-----------|-----------|-----------|-----------|
| HPS  | TrCel6A| Model 1| 0.896     | 9%        | 9%        | 4.86E+07% | 3%        |
| HPS  | TrCel6A| Model 2| 0.896     | 46%       | 10%       | 140%      | 3%        |
| HPS  | TrCel6A| Reversible | 0.772 | 4%        | 10%       |           | 3%        |
| HPS  | TrCel5A| Model 1| 0.923     | 19%       | 23%       | 2.35E+05% | 4%        |
| HPS  | TrCel5A| Model 2| 0.923     | 28%       | 11%       | 513%      | 4%        |
| HPS  | TrCel5A| Reversible | 0.923 | 10%       | 10%       |           | 5%        |
| HPS  | TrCel5A| Irreversible | 0.784 |           | 0%        |           | 0%        |
| HPWS | TrCel6A| Model 1| 0.945     | 71%       | 65%       | 9%        | 4%        |
| HPWS | TrCel6A| Model 2| 0.936     | 30%       | 8%        | 3.03E+03% | 3%        |
| HPWS | TrCel6A| Reversible | 0.936 | 3%        | 10%       |           | 4%        |
| HPWS | TrCel6A| Irreversible | 0.640 |           |           | 31%       | 3%        |
| HPWS | TrCel5A| Model 1| 0.967     | 78%       | 71%       | 49%       | 10%       |
| HPWS | TrCel5A| Model 2| 0.965     | 55%       | 10%       | 159%      | 13%       |
| HPWS | TrCel5A| Reversible | 0.965 | 25%       | 8%        |           | 13%       |
| HPWS | TrCel5A| Irreversible | 0.570 |           |           | 0%        | 0%        |

Note. HPS: hydrothermally pretreated spruce; HPWS: hydrothermally pretreated wheat straw; RSD: relative standard deviation. $^*$ $k_{Ir}$ is a first-order rate constant with the unit L/(g·hr)$^{-1}$ in Model 1 and a second-order rate constant with the unit L$^2$·(μmol·g·hr)$^{-1}$ in other models.
after binding on lignin (Rahikainen et al., 2011; Rahikainen, Moilanen et al., 2013; Sammond et al., 2014).

The overall good fitting ($R^2 \approx 0.9$) of the Models 1–3 nevertheless pointed out that the adsorption of monocomponent cellulases on lignin is reversible by nature, instead of being fully irreversible. The good identifiability of reversible adsorption constants, especially in Model 3 where they were better than that in Models 1 and 2, implied that the completely reversible adsorption model alone can explain the findings. Although in some ways the statement might seem contradictory to the previous understanding, this finding illustrates the need for a redefinition of the term irreversibility and highlights that reversibility of adsorption should not be confused with binding affinity. Distinguishing between the two can be complicated, therefore, for practical purposes the activity of the enzyme during binding onto lignin should also be investigated. Loss of activity can correlate to irreversible binding, even though that does not necessarily denote a direct causal relationship. Hence this points to the need to understand the precise mechanism leading to the loss of enzyme activity. Good fitting of Model 1 in this work confirmed and expanded the nuances of the explanation of previous findings (Rahikainen et al., 2011). Initially, the enzymes constantly change structural conformation as they adsorb and desorb reversibly. Incubation at elevated temperature increases the rate of the process and thus the binding affinity. As the process continues, eventually the protein structure unfolds and renders the enzymes to be bound irreversibly at a certain extent, losing activity. In future work, it would be relevant to assess whether the loss of enzyme activity is aggravated at high substrate concentration (10%–30% DM) because of an increased rate of adsorption, and/or whether the binding kinetics may be affected. It remains to be seen by future work whether the loss of enzyme activity and the change to irreversible binding on lignin occur sequentially, separately or simultaneously. Finally, it is important to stress that while the binding is reversible, the loss of activity because of denaturation is irreversible.

### 3.3 Competitive binding of cellulases

Competitive binding study was performed to find if there is competition between selected monocomponent cellulases TrCel6A and TrCel5A, which had the highest binding affinity based on the adsorption isotherms (Figures 1 and 2, Table 2). In this experimental

### TABLE 4 Langmuir isotherm parameters from the fitted adsorption data of competitive binding of TrCel6A and TrCel5A on isolated lignin-rich residues

| Adsorbent | Enzyme                | $10 \times B_{\text{max}}$ (μmol/g) | $10 \times K_{\text{ads}}$ (L/μmol) | $10 \times \alpha$ (L/g) | $R^2$   |
|-----------|-----------------------|-------------------------------------|-------------------------------------|-------------------------|--------|
| L-HPS     | TrCel6A−[3H]          | 3.66 ± 0.33                         | 8.58 ± 1.86                         | 3.14 ± 0.74             | 0.926  |
|           | TrCel6A−[3H] + TrCel5A| 2.11 ± 0.05                         | 16.3 ± 2.87                         | 3.43 ± 0.61             | 0.948  |
|           | TrCel5A−[3H]          | 9.13 ± 0.61                         | 8.94 ± 1.32                         | 8.16 ± 1.32             | 0.984  |
|           | TrCel5A−[3H] + TrCel6A| 3.64 ± 0.12                         | 5.66 ± 0.45                         | 2.06 ± 0.18             | 0.992  |
| L-HPWS    | TrCel6A−[3H]          | 1.72 ± 0.15                         | 6.57 ± 1.28                         | 1.13 ± 0.24             | 0.974  |
|           | TrCel6A−[3H] + TrCel5A| 1.17 ± 0.11                         | 15.4 ± 3.17                         | 1.80 ± 0.41             | 0.898  |
|           | TrCel5A−[3H]          | 3.07 ± 0.24                         | 4.66 ± 0.61                         | 1.43 ± 0.22             | 0.991  |
|           | TrCel5A−[3H] + TrCel6A| 1.38 ± 0.09                         | 7.79 ± 1.29                         | 1.08 ± 0.19             | 0.979  |

Note. $B_{\text{max}}$: maximum adsorption capacity; $K_{\text{ads}}$: Langmuir adsorption constant; L-HPS: lignin-rich residues from hydrothermally pretreated spruce; L-HPWS: lignin-rich residues from hydrothermally pretreated wheat straw; $\alpha$: relative association constant ($B_{\text{max}} \times K_{\text{ads}}$). The reported constants and errors were obtained from fitting of three experimental replicates using one binding-site Langmuir adsorption model.
setup, only the binding of the radiolabeled enzyme was recorded. In the equimolar presence of one another, the enzymes showed competitive binding in the isotherms (Figure 4). The presence of TrCel6A reduced the binding of labeled TrCel5A significantly, whereas the presence of TrCel5A had a less pronounced effect on the binding of labeled TrCel6A. The reduction of the binding was clearly visible in both L-HPS (Figure 4a) and L-HPWS (Figure 4b).

Fitting of one binding-site Langmuir adsorption model to the competitive adsorption isotherms still showed good fit in general (Table 4). The maximum adsorption capacity ($B_{\text{max}}$) of TrCel6A was less affected by TrCel5A, whereas the $B_{\text{max}}$ of TrCel5A was reduced more significantly by TrCel6A both in L-HPS and L-HPWS (Table 4). The $B_{\text{max}}$ values of the mixture constituted by the two enzymes were nevertheless almost similar in magnitude (Table 4). This indicated that both enzymes competed for similar binding sites and TrCel6A predominated the competitive binding albeit lower $B_{\text{max}}$ value. Previously it was suggested that the Vroman effect was present in a cellulytic enzyme mixture where enzymes of greater affinity displaced others of lesser affinity (Yarbrough et al., 2015). In this study, affinity did not seem to be the factor because TrCel5A had higher, if not similar, affinity to TrCel6A based on both $\alpha$ and $K_{\text{ads}}$ (Table 4). However, in the original study that coined the Vroman effect, it was shown that proteins with larger size ($M_w$) displaced the smaller ones (Vroman & Adams, 1969). Accordingly, TrCel6A is indeed larger than TrCel5A (Table 1), therefore suggesting size as a plausible factor that governs competitive binding.

The presence of competitive binding between two enzymes showed that monitoring the adsorption of a multicomponent system, such as cellulases, can be difficult to perform. Nevertheless, the presence of competition and good fitting to the Langmuir model also suggest that the binding of cellulases on lignin is exchangeable and thus reversible by nature. The finding thus supports the previous observations in this work and points that the binding of cellulases on lignin is both reversible and competitive as in the case of the binding of cellulases on cellulose (Kyriacou, Neufeld, & MacKenzie, 1989; Pellegrini et al., 2014).

4 | CONCLUSIONS

The present work indicates that despite differences in the binding affinity of individual monocomponent cellulases, the binding is reversible by nature. Modeling of kinetic experiments suggests the possibility of previously reversible binding turning to irreversible which can explain the previous observations on retardation of enzymatic cellulose conversion in the presence of lignin. Because of the reversible nature of binding, the negative effect of lignin can plausibly be alleviated by including additives in the reaction. Given the indication that the binding turns irreversible hence losing activity because of structural unfolding over time at elevated temperature, engineering or finding novel enzymes with improved thermostability can be an avenue to pursue. Future studies should be directed into deciphering the underlying mechanism and factors that govern the deactivation of the enzyme by lignin, especially at high substrate concentration. The competition among cellulases in the adsorption on lignin highlights the necessity to develop methods able to distinguish the binding and activity of individual enzymes in a mixture to identify and selectively improve the necessary enzyme component.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

NOMENCLATURE

$B_{\text{max}}$ maximum adsorption constant (μmol/g)
$E$ free enzymes (μmol/L)
$EL$ bound enzymes (μmol/g)
$L$ free binding sites in lignin-rich residues (g/L)
$K_{\text{ads}}$ Langmuir adsorption constant (L/μmol)
$k_{\text{f}}$ irreversible adsorption constant (L·(g·hr)$^{-1}$ in Model 1; otherwise L$^2$·(μmol·g·hr)$^{-1}$ in other models)
$k_{\text{Rev}}$ reversible adsorption constant (L$^2$·(μmol·g·hr)$^{-1}$)
$k_{\text{Rev}}$ reversible desorption constant (L·(g·hr)$^{-1}$)
$\alpha$ relative association constant (L/g)

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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