Elucidation of Changes in Cellulose Ultrastructure and Accessibility in Hardwood Fractionation Processes with Carbohydrate Binding Modules

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ABSTRACT: We have recently presented a sequential treatment method, in which steam explosion (STEX) was followed by hydrotropic extraction (HEX), to selectively fractionate cellulose, hemicellulose, and lignin in hardwood into separate process streams. However, above a treatment severity threshold, the structural alterations in the cellulose-enriched fraction appeared to restrict the enzymatic hydrolyzability and delignification efficiency. To better understand the ultrastructural changes in the cellulose, hardwood chips were treated by single (STEX or HEX) and combined treatments (STEX and HEX), and the cellulose accessibility quantified with carbohydrate-binding modules (CBMs) that bind preferentially to crystalline (CBM2a) and paracrystalline cellulose (CBM17). Fluorescent-tagged versions of the CBMs were used to map the spatial distribution of cellulose substructures with confocal laser scanning microscopy. With increasing severities, STEX increased the apparent crystallinity (CBM2a/CBM17-ratio) and overall accessibility (CBM2aH6 + CBM17) of the cellulose, whereas HEX demonstrated the opposite trend. The respective effects could also be discerned in the combined treatments where increasing severities further resulted in higher hemicellulose dissolution and, although initially beneficial, in stagnating accessibility and hydrolyzability. This study suggests that balancing the severities in the two treatments is required to maximize the fractionation and simultaneously achieve a reactive and accessible cellulose that is readily hydrolyzable.

KEYWORDS: cellulose accessibility to enzymes, cellulose ultrastructure, carbohydrate-binding modules, steam pretreatment, hydrotropic extraction, fractionation, hydrolyzability, hardwood

INTRODUCTION

In order to improve the resource utilization and competitiveness of forest biorefineries, all major wood components—lignin, hemicellulose, and cellulose—must be valorized. This often requires the fractionation of woody biomass into separate streams using a process that is efficient and operates with minimal mass losses and with minimal negative effects on key properties essential for further valorization of the wood components. Current fractionation processes, designed for various product portfolios, include the prehydrolysis-Kraft process,1−4 steam explosion (STEX),5−7 and lignin extraction methods using organosolv, alkalai, deep eutectic solvents, ionic liquids, and hydrotropes.8−10 These fractionation methods have been shown to separate one or two of the main wood constituents, but incomplete fractionation and a negative impact on residual components curtail complete biomass valorization. In the prehydrolysis-Kraft process and STEX, the cellulose is enriched in the solid fraction, and the solubilized hemicellulosic sugars can be recovered in the liquid phases. However, the opportunities for valorization of the lignin are limited by covalently bonded sulfur in Kraft-lignin11 and prevalence of highly condensed lignin structures after the high-severity STEX treatments used for recalcitrant biomass.12−14 Consequently, attention has been paid to designing methods, such as hydrotropic extraction (HEX), for delignification of...
woody biomass, where a sulfur-free and reactive lignin can be recovered. However, the recovery of the hemicellulose is compromised by incomplete fractionation in most of these processes.15,16

To address this issue, we recently introduced a two-step process for the fractionation of hardwood, where autocatalyzed STEX was followed by HEX with sodium xylene sulfonate (SXS).17 In the STEX step, the woody biomass was defibrated (i.e., disintegration of wood to free fibers), the lignin was redistributed, and the hemicellulosic sugars were efficiently solubilized and recovered from the liquid phase. Subsequently, the solids were treated by HEX and the extracted lignin precipitated and recovered from the liquid fraction, leaving a cellulose-enriched solid fraction.17 The value proposition of the process is that cellulose, hemicellulose, and a sulfur-free lignin can be isolated in separate product streams in an efficient, sustainable, and scalable process. The cellulose-enriched solids can then be enzymatically hydrolyzed to a sugar platform suitable for further biochemical valorization. However, despite the removal of hemicellulose and lignin with the sequential process, the hydrolyzability of the cellulose-enriched solids was below expectations.17 Scanning electron microscopy of the treated solids showed stark morphological changes in the solid structure.17 Consequently, we hypothesized that the alterations in the cellulose ultrastructure, caused by the treatments, restricted the cellulose accessibility to enzymes and thus the hydrolyzability.18,19

The accessibility of cellulose to enzymes is a key parameter describing the biomass recalcitrance to enzymatic deconstruction.19,20 Apart from the shielding effects of the ligno-carbohydrate complex, the accessibility is mainly restricted by cellulose ultrastructure (i.e., degree of organization in the micro, macro, and fiber level).19,20 However, supramolecular cellulose structures and their effects on accessibility to enzymes have proven difficult to quantify.21 A promising method that addresses the problem uses the selective adsorption of carbohydrate-binding modules (CBMs) onto the cellulose to assay the structure and accessibility.22,23 CBMs are non-catalytic parts of cellulosytic enzymes,24 which are responsible for substrate targeting and proximity effects in the mature enzymes.25 Currently, more than 80 families of CBMs have been characterized and categorized into three groups according to their substrate affinity, where Type A binds to crystalline cellulose, Type B predominantly binds to single carbohydrate chains (e.g., glucan chains in cellulose regions with a paracrystalline organization), and Type C binds to soluble carbohydrates.25 The diversity and affinity of CBMs toward specific carbohydrate fine structures have been used to qualitatively map the glycoarchitecture of plant cell walls.23,26 CBMs conjugated with fluorescent tags and imaged by confocal laser scanning microscopy (CLSM) have been used to elucidate the spatial distribution of carbohydrate fine structures in processed wood fibers in 2D renditions of the substrate.27–30 Furthermore, because of their properties, CBMs are advantageous to use as probes to analyze and quantify the accessibility of cellulose to different types of enzymes.31–35 Studies have shown that cellulose accessibility to enzymes, quantified by CBM adsorption assays, accurately reflect the hydrolyzability of model cellulose substrates32,35 and “real” lignocellulosic substrates.29

The aim of this study was to derive a more fundamental understanding of the impact of the single (STEX or HEX) and combined (STEX + HEX) treatments on the cellulose ultrastructure in woody biomass and its accessibility to enzymes. The raw material (RM) was treated differentially with STEX at three severity levels, and each STEX-treated solid fraction was further treated by HEX at two severity levels. To elucidate how the structure and accessibility change with varying process conditions, two CBMs, CBM2a (Type A) and CBM17 (Type B), were used to quantify the cellulose accessibility to enzymes by a CBM depletion assay. Additionally, fluorescence-tagged versions of CBM2a and CBM17 were used to map the spatial distribution of supramolecular cellulose structures by CLSM. The study elucidates the effect STEX, HEX, and the combined STEX + HEX treatment has on the cellulose ultrastructure, the accessibility to enzymes, and its hydrolyzability.

### MATERIALS AND METHODS

**Raw Material.** The used mix of hardwood chips, kindly provided by Södra Cell AB (Sweden), consisted of ~80% birch (Betula pendula) and ~20% European beech (Fagus sylvatica). The glucan, xylan, and lignin contents of the RM are depicted in Figure 1, and the complete composition is detailed in the Supporting Information, Table S1. Prior to pretreatment, the wood chips were size-reduced using a knife mill (Retsch GmbH, Germany) fitted with a 20 mm screen, and the 2–10 mm fraction was retrieved by sieving. All treatments were conducted as single experiments. Comparison of the resulting chemical compositions with the parent study proved the reproducibility of the pretreatment setup.
**Pretreatment.** The milled wood was treated with the combined STEX + HEX treatment, as described previously.\(^1\) The HEX conditions used spanned the temperature range investigated previously.\(^1\) The results of the same study further showed that the HEX hold-up time can be shortened to 4 h without any loss in cellulose accessibility to enzymes, and its hydrolyzability. To dissect the impact of the single treatments on these parameters, all treatment conditions were also performed as single treatments on the RM. Table 1 summarizes the single and combined treatment conditions and their denotations. In detail, STEX was performed by aqueous SXS solution (Stepanate SXS-93, Alsiano, Denmark) and washed with an excess of water.\(^1\) HEX was performed in a 2 L vial at the indicated temperature with a solid-to-liquid ratio of 1. The STEX treatment was performed by enzymatic hydrolysis was quenched by bringing the samples to 100 °C for 10 min. Subsequently, the samples were centrifuged (5 min, 13,000 rpm), and the supernatants were filtered through 0.22 μm syringe-driven filters before storage at 4 °C for carbohydrate analysis. Enzymatic hydrolysis reactions were performed in triplicates.

**Analysis of Cellulose Accessibility to Enzymes with CBMs. CBM Production and Fluorescence Tagging.** Two CBMs were used in this study: CBM2a (Type A) and CBM17 (Type B) from *Cellulomonas fimi* and *Clostridium cellulovorans*, respectively.\(^25,\)\(^36\) CBM2a and CBM17, and his-tagged CBM2a (CBM2aH6) were recombinantly produced in *Escherichia coli* and purified as described previously.\(^36\) In brief, CBMs were produced in high cell density fed batch *E. coli* cultivation with a carbon-limited glycerol feed, typically achieving ∼100 g L\(^{-1}\) of cell DM. Harvested cells were lysed chemically with a Bug Buster protein extraction reagent (MilliporeSigma, USA). Subsequently, the CBMs were purified from the cell-free supernatant by affinity chromatography, using cellulose (CBM2a and CBM17) and Ni-NTA beads (CBM2aH6) as the stationary phase.\(^35\) The CBMs were stored at 4 °C in 100 mM potassium phosphate buffer, pH 7 (KPh).

For CLSM studies, CBM2a and CBM17 were fluorescence-tagged with Rhodamine-RedX (RRedX) and Fluorescein (FITC), respectively. The CBMs were purified by size-exclusion chromatography (HiPrep desalting 16/20 column, GE Healthcare Life Sciences, UK), and the correct sizes and purity were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resulting CBM–fluorescent dye conjugates were denoted as CBM17-FITC and CBM2a-RRedX, respectively.

**BSA Blocking of Lignin.** Prior to CBM binding in the depletion assay, all materials were incubated with BSA (Sigma-Aldrich, USA) to block the lignin and prevent unspecific CBM adsorption. For this, 10 g of wet material was soaked in 100 mL of BSA solution (5 g L\(^{-1}\) in KPh) and incubated at 4 °C overnight. Unbound BSA was removed by thorough washing. For this, excess BSA solution was removed by vacuum filtration (grade 5 filter paper, Munktell, Sweden), and the material was resuspended in 200 mL of KPh. After 30 min of incubation at room temperature under constant agitation, the solids were filtered off and rinsed with an additional 200 mL of KPh over the vacuum filter. The BSA-blocked materials were stored at 4 °C.

**Quantitative CBM Binding Analysis.** The CBM binding on the BSA-blocked materials was analyzed by a depletion assay, as described previously.\(^35\) In brief, the CBMs were added in three different concentrations (0.5–15 μmol L\(^{-1}\) to 10 mg of DM material in KPh in a total volume of 1 mL, resulting in triplicate experiments. The samples were incubated for 30 min at room temperature, the supernatant separated from the solids by centrifugation, and the concentration of CBMs in the supernatant quantified spectrophotometrically at 280 nm. The absence of BSA and lignin interference in the spectrophotometric measurements, due to desorption, was verified by resuspending 10 mg of material in 1 mL of KPh. After 30 min of incubation, the supernatant was measured at 280 nm, where all signals were below 0.0050 absorption units. For CBM quantification, the molar mass and molar extinction coefficient used were 19.7 kDa and 31 010 M\(^{-1}\) cm\(^{-1}\) for CBM12 and 12.4 kDa and 27 625 M\(^{-1}\) cm\(^{-1}\) for CBM2aH6, respectively.

**Analysis of the Supramolecular Structure with Fluorescent-Tagged CBMs and CLSM.** Sample preparation for CLSM imaging was performed as follows: CBM17-FITC and CBM2a-RRedX were mixed with the material and KPh, resulting in ∼5 μg g\(^{-1}\) of each CBM, based on dry weight of the treated wood materials. The samples were incubated for 15 min at room temperature, after which a small amount of the samples was mounted on a microscope slide, covered with a coverslip, and sealed with paraﬃn wax. Of each pretreatment

| denotations | STEX conditions | HEX conditions |
|-------------|----------------|---------------|
| Single Treatments | | |
| STEX180/5 | 180 °C, 5 min, auto-cat.* | 150 °C, 4 h |
| STEX210/5 | 210 °C, 5 min, auto-cat.* | 190 °C, 4 h |
| STEX210/10HAc | 210 °C, 10 min, HAc-cat. | 190 °C, 4 h |
| HEX150/4 | −150 °C, 4 h | |
| HEX190/4 | −190 °C, 4 h | |
| Combined Treatments | | |
| STEX180/5 + HEX150/4 | 180 °C, 5 min, auto-cat.* | 150 °C, 4 h |
| STEX180/5 + HEX190/4 | 180 °C, 5 min, auto-cat.* | 190 °C, 4 h |
| STEX210/5 + HEX150/4 | 210 °C, 10 min, HAc-cat. | 190 °C, 4 h |
| STEX210/5 + HEX190/4 | 210 °C, 10 min, HAc-cat. | 190 °C, 4 h |

*auto-cat.: autocatalyzed, HAc-cat.: acetic acid-catalyzed.*
Renewable Energy Laboratory standard methods. The acid-lignin in the solids were measured in triplicates with National the materials and the composition of structural carbohydrates and RedX (red) and CBM17-FITC (red) projection. Colors were assigned arbitrarily to the imaged CBM2a-RRedX (crystalline cellulose) binding is displayed in green and red, respectively. Micrographs were acquired with 10X (a–e) and 25X (a′–e′) objectives. Scales present 200 μm (a–e) and 20 μm (a′–e′).

Figure 2. Cellulose ultrastructure of solids treated with STEX and HEX alone, visualized by fluorescent-tagged CBM binding and CLSM. Solids were treated with STEX190/5 (a,a′), STEX210/5 (b,b′), and STEX210/190HAc (c,c′) and HEX150/4 (d,d′) and HEX190/4 (e,e′). CBM17-FITC (paracrystalline cellulose) and CBM2a-RRedX (crystalline cellulose) binding is displayed in green and red, respectively. Micrographs were acquired with 10X (a–e) and 25X (a′–e′) objectives. Scales present 200 μm (a–e) and 20 μm (a′–e′).

condition, one specimen was prepared. All steps were conducted in the dark to prevent fluorescence quenching. CLSM imaging was performed with a Leica SP8 DLS (Leica, Germany), using a 10X/0.3 dry and a 25X/0.95 water objective. CBM17-FITC was excited at 552 nm and emissions detected at 590–650 nm, and CBM2a-RRedX was excited at 488 nm and detected at 610–650 nm. The micrographs were acquired in 4.285 μm (10X) and 0.568 μm (25X) thick optical sections. The micrographs presented herein were selected after screening through the entire specimen and then capturing a z-stack image of a representative image frame. All micrographs presented herein are two-dimensional renditions of three-dimensional z-stacks consisting of 13–21 (10X) and 23–52 (25X) optical slices. The instrument settings (i.e., laser power) were kept constant for all images, where the imaging settings (i.e., gain and offset) were optimized for each image, trying to maximize the dynamic range of the detector. Image processing in LAS X (Leica, Germany) included the flattening of the z-stacks by maximum projection. Colors were assigned arbitrarily to the imaged CBM2a-RRedX (red) and CBM17-FITC fluorescence (green).

**Analytical Methods and Data Processing.** The DM content in the materials and the composition of structural carbohydrates and lignin in the solids were measured in triplicates with National Renewable Energy Laboratory standard methods. The acid-soluble lignin content was measured spectrophotometrically at 240 nm and quantified using an extinction coefficient of 25 L g⁻¹ cm⁻¹. Quantification of soluble carbohydrates was performed by isocratic high-performance anion-exchange chromatography with pulsed amperometric detection (ICS-3000, Dionex, USA), using a Carbo Pac PA1 analytical column (Dionex). Measurements were performed at 30 °C with deionized water as the mobile phase at a flow rate of 1 mL min⁻¹. The conversion of cellulose to glucose was performed as described previously. The initial uptake rates represent the slope of the linear regression of the glucose consumption [g L⁻¹] versus time [h] plot, generated from data points taken in the first 4 h of reaction time.

**RESULTS**

**Impact of the Treatments on the Chemical Composition of the Solids.** As summarized in Table 1, STEX was performed under three different conditions (180 °C, 5 min, autocatalyzed; 210 °C, 5 min, autocatalyzed; and 210 °C, 10 min, acetic acid-catalyzed) and HEX under two conditions (150 °C, 4 h; and 190 °C, 4 h). All combinations of conditions in the STEX and following HEX treatments were performed for the combined treatment. Figure 1 shows the impact of the respective treatments on the chemical composition of the resulting solid material. The complete characterization is detailed in the Supporting Information, Table S1.

For the single treatments, clear trends in the effects of the increasingly severe STEX and HEX conditions on glucan, xylan, and lignin content were observed (Figure 1a). With increasing severity of the STEX treatment (STEX180/5 → STEX210/5 → STEX210/190HAc), the glucan content in the solids was enriched progressively from 40% in the RM to 62%. The enrichment was mainly a result of dissolution of xylan in the hemicellulose, which dropped from 22 to 4%. Only incremental changes in the lignin content could be observed, from 29% in the RM to 31% in STEX210/190HAc (Figure 1a), indicating that the STEX treatment solubilizes hemicellulose with good selectivity. The mild HEX150/4 only demonstrated a modest effect on the composition. The glucan content in the solids was enriched from 40% in the RM to 54%, mainly as a result of dissolution of lignin (from 29 to 22%) and xylan (from 22 to 18%, Figure 1a). The more severe HEX190/4 showed significant delignification (reduced to 12%) and xylan decreased to 4%, which resulted in the glucan content being enriched to 81% (Figure 1a). Because the solubilized xylose cannot easily be separated and recovered from the HEX liquid phase, this result highlights that a prehydrolysis step is required for extensive fractionation and recovery of all wood components.

Despite the clear differences in the composition of the solids after single treatments, the differences in the resulting composition after the combined treatments were less pronounced. The most significant differences in chemical composition were obtained for the mildest conditions (STEX180/5 + HEX150/4 and STEX190/5 + HEX190/4). Here, the glucan enrichments were improved to 65 and 80%, respectively. The lignin content was decreased from 18 and 13%, respectively, and xylan content decreased to 12 and 5%, respectively (Figure 1b). Under other conditions, the glucan, lignin, and xylan contents were in the ranges 81–85, 13–16, and 3–7%, respectively. Increased hemicellulose dissolution was obtained progressively with increased severity in STEX and HEX, while the delignification efficiency stagnated. The perceived increase and variation in lignin content is largely an effect of increased dissolution of hemicellulose with higher severity in STEX and HEX and, to a certain extent, cellulose dissolution at the highest STEX and HEX severities. As both
STEX and HEX severities have a profound effect on xylan content but only a limited impact on lignin content, it is advantageous to increase the severity in the STEX step to enhance fractionation on the components between different process streams.

Impact of Treatment Conditions on the Ultrastructure of the Solids. To understand how the conditions in the single and the combined treatments affected the ultrastructure of the solids, we analyzed the binding pattern of the fluorescent-tagged CBMs (i.e., distribution of CBM2a-RRedX- and CBM17-FITC-induced fluorescence on the fiber surface) using CLSM. The resulting micrographs from the single and the combined treatments are depicted in Figures 2 and 3, respectively. When comparing the differentially STEX-treated solids, the effects on fiber morphology and the CBM binding pattern were discerned. Thus, with increasing treatment severity, the materials changed their overall structure and increasing defibration occurred. The biomass changed from being bulky with fibers that were held together in a complex matrix (STEX180/5, Figure 2a) to a structure where long separated fibers could be discerned (STEX210/5 and STEX210/10HAc; Figure 2b,c, respectively). Further, the binding pattern of the two CBMs changed. At the lowest severities, seemingly more CBM2a-RRedX (crystalline cellulose binding, indicated in red) was bound to the substrate surface compared to CBM17-FITC (paracrystalline binding indicated in green). This changed with increased STEX severity, where the STEX210/5 and STEX210/10HAc micrographs show higher green (CBM17-FITC) and then red (CBM2a-RRedX) fluorescence intensity (Figure 2b,c). Because of the binding specificities of the two CBMs, this indicates that STEX created more binding sites for the paracrystalline-prefering CBM17-FITC at the fiber surface, which imply decreasing surface crystallinity with increasing treatment severities. The higher magnification micrographs (Figure 2a’–c’) substantiate this observation. Furthermore, they show that with increasing STEX severities, the fiber surface became smoother, creating pulp-like fibers. Interestingly, the fibers developed by the highest severity, STEX210/10HAc (Figure 2c,c’), show discrete zones of high green fluorescence intensity. These zones resemble supramolecular cellulose structures in fiber defects, often referred to as kinks or dislocation zones. HEX had a similar impact on the structure of the RM (Figure 2d,e, HEX150/4 and HEX190/4, respectively) as STEX, where increasing severity resulted in defibration, fiber development, and increased CBM17-FITC binding.

The effects of the combined treatments on the ultrastructure of the solids are shown in Figure 3. At the low STEX severities (STEX180/5 + HEX150/4 and STEX180/5 + HEX190/4; Figure 3a,b, respectively), the different HEX condition did not affect the cellulose ultrastructure significantly compared to STEX180/5 (Figure 2a). The solids remained in their bulky structure and little, or no, additional defibration occurred. The STEX210/5 + HEX150/4-treated solids (Figure 3c) strongly resemble the structure of STEX210/5 (Figure 2b), indicating that mild HEX did not significantly affect the cellulose ultrastructure of the STEX-treated material. Like the solids treated with the lower STEX severity (STEX180/5 + HEX150/4 and STEX180/5 + HEX190/4; Figure 3a,b), the biomass showed a bulky organization and no single fiber structure. However, with increased HEX severity (STEX210/5 + HEX190/4; Figure 3d), a pronounced change was observed. The fibers were shortened and fragmented, with many smaller particles being visible. The latter seemingly has a high surface crystallinity, as indicated by the predominant binding of CBM2a-RRedX (indicated as red). This morphology is shared by STEX210/10HAc + HEX190/4 (Figure 3f), but not by STEX210/10HAc + HEX150/4 (Figure 3e), indicating that this structural alteration was caused by the higher HEX severity.

Please note that the micrographs reflect the CBM binding at the fiber surface, representing the optical section of the CLSM. Thus, the images depicted in Figures 2 and 3 give information on the apparent supramolecular surface structure and accessibility. However, basing conclusions solely on the micrographs has the following disadvantages: (i) they do not reflect the accessibility of the overall fiber to the CBMs, (ii) the information is not quantitative, and (iii) only show a small sample size, here n = 2 (two independent image frames from one specimen), can be displayed. To address these issues, we additionally performed a quantitative depletion assay, which is discussed hereinafter. Together, these two methods give us an understanding of the fiber morphology and the spatial distribution of cellulose substrates on the fiber surface (CLSM, Figures 2 and 3) as well as the overall accessibility and the degree of organization (i.e., crystalline vs paracrystalline) of the CBM-accessible cellulose (depletion assay, Figure 4).

Impact of Treatment Conditions on Cellulose Accessibility to Enzymes. To quantify the cellulose
accessibility to enzymes of the treated substrates, sequential binding of CBM17 and CBM2aH6 was performed. Figure 4 shows the amount of CBM17 and CBM2aH6 adsorbed to the cellulose (Figure 4a1, b1), as well as the sum (CBM2aH6 + CBM17) and the ratio (CBM2aH6/CBM17, Figure 4a2, b2) of bound CBMs for the single (Figure 4a1, a2) and combined treatments (Figure 4b1, b2). The sum of CBM binding is used as an indicator of the overall accessibility of cellulose to enzymes. The binding ratio (CBM2aH6/CBM17) gives information on the degree of order (i.e., crystalline vs paracrystalline) of the cellulose that is accessible to the CBMs. In a recently published study on cellulose model substrates, we showed that changes in the degree of order determined by CBM2a and CBM17 binding corresponded well to parallel 13C NMR measurements,30 a method commonly used to determine cellulose crystallinity.21 CBM2aH6/CBM17 was therefore used as a proxy for the cellulose crystallinity in this study.

The effect of the single treatments on CBM17 binding was limited (Figure 4a1). An incremental rise in CBM17 binding was obtained with increasing STEX severity (5.7 ± 0.0 to 6.3 ± 0.3 μmol g⁻¹ of cellulose). Mild HEX treatment resulted in slightly higher CBM17 binding than STEX (6.9 ± 0.6 μmol g⁻¹ of cellulose), and an increase in HEX severity resulted in decreased CBM17 binding (5.1 ± 0.1 μmol g⁻¹ of cellulose). In contrast, stronger effects on CBM2aH6 binding were observed. With rising STEX severities, CBM2aH6 binding increased 1.9-fold (7.2 ± 0.0 to 13.5 ± 0.9 μmol g⁻¹ of cellulose). The opposite effect on CBM2aH6 binding was observed in the HEX treatments, which showed a decrease from HEX150/4 (10.7 ± 1.0 μmol g⁻¹ of cellulose) to HEX190/4 (7.3 ± 1.1 μmol g⁻¹ of cellulose). Overall, STEX treatment increased the total accessibility (CBM2aH6 + CBM17) as well as the CBM2aH6/CBM17 binding ratio. Increased severity in the HEX resulted in decreased total CBM binding and marginal changes in the CBM2aH6/CBM17 binding ratio.

When performing CBM adsorption on the materials treated with the combined method, several trends could be discerned. Like the single treatments, an increase in STEX severity in the combined treatments resulted in an increase in CBM2aH6 binding (2.4-fold for the lower and 2.8-fold for the higher HEX severities) and more moderate changes in CBM17 binding. CBM17 binding increased ~2-fold from STEX180/5 + HEX150/4 to STEX210/10HAc + HEX150/4 and then slightly decreased again at the highest severity conditions (STEX210/10HAc + HEX150/4). Furthermore, independent of STEX conditions, materials treated with the higher HEX severity (i.e., STEX180/5 + HEX190/4, STEX210/5 + HEX190/4, and STEX210/10HAc + HEX190/4) showed significant decreases in CBM2aH6 and total CBM adsorption (CBM2aH6+CBM17) compared to their lower HEX severity counterparts (Figure 4b1, b2). The CBM2aH6/CBM17 binding ratio showed a similar trend except at the highest STEX severity, where it was higher for STEX210/10HAc + HEX190/4 than for STEX210/10HAc + HEX150/4 (Figure 4b2). The results imply that in the combined treatments, the effects of the respective treatments, that is, an increase in CBM2aH6 and total CBM binding with increasing STEX severities and a decrease in CBM binding with the higher HEX severity, were maintained.

Impact of Treatment Conditions on Hydrolyzability.

The solids treated with the single and combined treatments were hydrolyzed at 3% solid loading and an enzyme loading of 10 FPU g⁻¹ DM. The initial rates and cellulose conversion yields after 24 h of reaction are summarized in Table 2, and the
First, during STEX, the lignin undergoes several cycles of demethoxylation, degradation, and repolymerization reactions, during which the lignin is extruded out of the biomass and deposited onto the surface. This lignin is highly accessible for lignin removal during HEX, even at the lower severity conditions. This is supported by the decline of residual lignin in the biomass with the initial increase in STEX severity (from STEX_{180/5} to STEX_{210/5}) in the combined treatments, Figure 1). However, when further increasing the STEX severity (STEX_{210/10HAc} in the combined treatments, Figure 1), the lignin transforms into more condensed structures with increased carbon–carbon bond formation. This could make the lignin more difficult to remove during HEX and explain the small increase in residual lignin content in the highest severity treated materials (Figure 1). Further, when comparing the CLSM micrographs, a pronounced structural change is occurring during the treatments, which could have affected the penetration of SXS into the material and the transport of SXS–lignin complexes out of the material (Figure 3). At the low STEX severities (STEX_{180/5} + HEX_{150/4} and STEX_{180/5} + HEX_{190/4} Figure 3a,b), the HEX condition did not affect the cellulose ultrastructure significantly. The solids remain in their bulky structure and little defibration occurred. This is interesting, considering that increasing the severity in HEX-alone treatments (Figure 2d,e) resulted in significant defibration. In fact, the lignin removal efficiencies increased less when going from STEX_{180/5} + HEX_{150/4} to STEX_{180/5} + HEX_{190/4} than from HEX_{150/4} to HEX_{190/4} (Figure 1, Table S1). This may indicate that the STEX treatment prevented further defibration, which in turn could negatively affect the mass transfer of the SXS and SXS–lignin complexes. When increasing the STEX severity in the combined treatment to STEX_{210/5} and STEX_{210/10HAc}, the severity of HEX had a significant impact on the solids structure. Thus, under the lower-severity HEX_{150/4} conditions, the solids are in their dispersed structure. Under the higher-severity HEX_{190/4} conditions, however, the fibers are severely fragmented. To verify that this was an effect of HEX, hardwood fibers from Kraft pulping were treated with HEX_{150/4} and HEX_{190/4} and the fragmentation was characterized with a fiber-quality analyzer (information on substrate and methodologies is provided in the Supporting Information). A clear and progressive fiber fragmentation pattern could be observed with increased severity (Supporting Information, Figure S2). Apart from the hemicellulose dissolution caused by the HEX, additional peeling reactions and removal of the easily accessible cellulose may have occurred, resulting in the observed fiber fragmentation. Because of this structural collapse, SXS penetration may have been restricted, lowering the lignin removal efficiencies (Figures 1 and 3).

**Table 2. Initial Rates and Cellulose Conversion Yields after 24 h of Enzymatic Hydrolysis**

| Treatments | Initial Rates [g L^{-1} h^{-1}] | Yields [%] |
|------------|---------------------------------|-----------|
| Single Treatments | | |
| STEX_{180/5} | 0.23 ± 0.02 | 21 ± 2 |
| STEX_{180/5} + HEX_{150/4} | 0.87 ± 0.04 | 36 ± 2 |
| STEX_{180/5} + HEX_{190/4} | 3.28 ± 0.11 | 89 ± 3 |
| STEX_{180/5} + HEX_{190/4} + HAc | 3.11 ± 0.01 | 82 ± 3 |
| STEX_{180/5} + HEX_{190/4} + HAc | 3.18 ± 0.06 | 91 ± 1 |
| Combined Treatments | | |
| STEX_{180/5} + HEX_{150/4} | 2.64 ± 0.06 | 71 ± 2 |
| STEX_{180/5} + HEX_{150/4} + HAc | 2.85 ± 0.09 | 85 ± 4 |
| STEX_{180/5} + HEX_{190/4} + HAc | 3.02 ± 0.11 | 89 ± 3 |
| STEX_{180/5} + HEX_{190/4} + HAc | 3.11 ± 0.01 | 82 ± 3 |
| STEX_{180/5} + HEX_{190/4} + HAc | 3.18 ± 0.06 | 91 ± 1 |

*Data represent mean values and standard deviations from independent triplicate experiments. Data acquisition and processing are described in the Materials and Methods section. Glucose release rate determined for the first 4 h of reaction. Cellulose conversion yield after 24 h of reaction.*

An increase in severity for the single treatments had a pronounced impact on the hydrolyzability of the solids. Increasing the temperature during STEX from 180 to 210 °C increased the initial rates 12-fold and the yields 3.7-fold, respectively. A similar effect was observed for solids treated with HEX, where the initial rates and the yields where improved 9.9 and 4.5-fold, respectively, when the temperature was increased from 150 to 190 °C. In the combined treatments, the initial rates and yields increased with higher severity in either treatment from STEX_{180/5} + HEX_{150/4} (0.87 g L^{-1} h^{-1} and 36%) to STEX_{210/5} + HEX_{190/4} (3.28 g L^{-1} h^{-1} and 89%). Here, the STEX severity had a stronger positive impact on the hydrolyzability than the HEX severity. After the first increases, the improvement plateaued and the initial rates and yields of the three most severely pretreated substrates (STEX_{210/5} + HEX_{190/4}, STEX_{210/10HAc} + HEX_{150/4} and STEX_{210/10HAc} + HEX_{190/4}) were similar, with STEX_{210/10HAc} + HEX_{150/4} showing a slightly lower hydrolyzability.

**DISCUSSION**

**Effect of Treatment Conditions on the Chemical Composition and Overall Biomass Structure of the Solids.** The total lignin content in the HEX_{150/4} and HEX_{190/4} treated materials decreased from 29% in the RM to 22 and 13% DM, respectively, showing a significantly higher delignification efficiency at higher severity (Figure 1). In the combined treatment, however, the final lignin content only decreased from 18% (STEX_{180/5} + HEX_{150/4}) to ~13% (STEX_{180/5} + HEX_{190/4}, STEX_{210/5} + HEX_{150/4} and STEX_{210/5} + HEX_{190/4}) and increased again with higher STEX severities to ~16% (STEX_{180/10HAc} + HEX_{150/4} and STEX_{210/10HAc} + HEX_{190/4}). Thus, similar to the previously published study on the combined STEX + HEX treatment, the delignification efficiency stagnated, and despite the broad range of severities investigated (Table 1), it was not possible to remove the residual ~13% lignin. There might be several reasons that explain this phenomenon.
the more reactive paracrystalline cellulose. Because enzymatic degradation of crystalline cellulose is thermodynamically less favored and, thus, slower than degradation of paracrystalline cellulose, this cannot explain the observed increase in hydrolyzability with increasing STEX severity. It is more likely that the observed increase in CBM2aH6 binding implies an improved accessibility for the CBM2aH6 to its binding sites, otherwise shielded by the complex biomass ultrastructure. This is supported by the CLSM micrographs, which show the already discussed fibrillation that may have increased the overall accessibility. In addition, an increase in apparent green fluorescence was observed for STEX180/5 (Figure 2b,b') and STEX210/10HAc (Figure 2c,c'), implying an increase in accessible paracrystalline surface structures for CBM17-FITC. It is noteworthy that the CLSM micrographs show the spatial distribution of the CBM binding pattern on the cellulose surface (according to the optical section), whereas the CBM adsorption reflects the overall accessibility. Collectively, this change in the accessible cellulose fine structures was likely the reason for the pronounced increase in hydrolyzability.

In the CLSM micrographs of the HEX-only treated materials, a similar change in the biomass structure and CBM binding pattern could be observed (Figure 2d,d',e,e'). Here, no change in the CBM binding ratio (CBM2aH6/CBM17) and decreased total CBM binding (CBM2aH6 + CBM17, Figure 4a1,a2) was measured. The observed increase in hydrolyzability was therefore likely a result of the decreased lignin content. As shown previously, HEX reduces surface lignin, which in turn enhances the hydrolyzability. In the CBM adsorption studies (both for the CLSM imaging and the quantification), but not in the hydrolysis reactions, the lignin had been blocked with BSA, to prevent unspecific CBM–lignin interactions and lignin autofluorescence. Thus, the CBM adsorption study cannot account for the effects of unproductive binding of enzymes to lignin during enzymatic hydrolysis.

A significant decrease in CBM2aH6 binding was observed when comparing the solids treated with the higher HEX severity with the lower HEX severity, both in the single treatments and in the combined treatment pairs (Figure 4). Because this observation is consistent across all the conditions analyzed, it seems that this structural change is induced in the solids by HEX90/4 independent of the initial treatment. As shown in the Supporting Information, Figure S2, and as discussed above, HEX190/4 but not HEX150/4 results in fiber fragmentation, suggesting significant changes in the ultrastructure by the higher severity HEX. However, the underlying reason for the reduced accessibility is unclear so far. One reason may be that the loss in hemicellulose and the redistribution and removal of the lignin during HEX190/4 result in a collapse of the porous architecture of the cellulose. This could reduce the cellulose accessibility to enzymes, as has been described for the pore collapse introduced by drying.

In the combined treatment, the hydrolyzability increased rapidly from STEX180/5 + HEX190/4 to STEX180/5 + HEX90/4 and then gradually to STEX210/5 + HEX90/4 (Table 2, Figure 4). The initial boost in hydrolyzability was likely caused by the removal of xylan and lignin (Figure 1), similar to the comparison of HEX150/4 with HEX90/4. Further, an interesting effect can be observed. When comparing the accessibility of the solids treated with the low HEX severity (STEX180/5 + HEX150/4) and the high HEX severity (STEX210/5 + HEX190/4), an increase in overall accessibility (CBM2aH6 + CBM17) can be observed, likely explaining the continued increase in hydrolyzability. In contrast, the high HEX severity treatments (STEX180/5 + HEX90/4 and STEX210/5 + HEX90/4) have a lower total accessibility, but also a lower CBM2aH6/CBM17 binding ratio. The latter might imply a more reactive (i.e., less crystalline) cellulose, offsetting the loss in the overall accessibility.

When comparing the CLSM micrographs, discrete zones of high-intensity green are developing on the fiber surfaces with increasing STEX severities (STEX210/5 and STEX210/10HAc), which seemed to be shielded from predominantly “red” cellulose in STEX180/5. These green zones of pronounced CBM17 binding indicate an increase in accessible paracrystalline regions at the fiber surface. They further have been shown to play an essential part in facilitating rapid cellulose hydrolysis of fibers, resulting in fiber fragmentation.

At the highest severity conditions, the improvements in hydrolyzability stagnated. Reasons for this could be unproductive binding of enzymes to an increasingly hydrophobic lignin that captures the enzymes and thus depletion of enzyme activity in the reaction. To test this, we repeated hydrolysis reactions of STEX150/5 + HEX150/4 and STEX210/10HAc + HEX190/4 with 2.5 FPU per gram dry mass and compared it to the hydrolysis reactions where the lignin has been blocked prior to enzyme loading. The results are shown in the Supporting Information, Figure S3. Here, it can be seen that lignin blocking indeed improved the hydrolysis yields substantially. The improvements, however, are similar for the two investigated substrates, suggesting that the extent of the hydrophobic lignin–enzyme interactions was comparable. It is more likely that at the highest severity, most of the reactive and easily accessible cellulose has been removed from the treatment, leaving a more crystalline cellulosic substrate. This is supported by the constant increase in CBM2aH6/CBM17 ratio from STEX150/5 + HEX190/4 to STEX210/10HAc + HEX150/4 and STEX210/10HAc + HEX190/4.

**CONCLUSIONS**

By using Type A and Type B CBMs and performing quantitative CBM binding, this study shows that both STEX and HEX treatments have pronounced but opposing effects on the cellulose ultrastructure and the cellulose accessibility to enzymes. STEX increased the CBM2aH6/CBM17 binding ratio and the overall accessibility (CBM2aH6 + CBM17), whereas HEX decreased the binding ratio and the overall accessibility. The effects of the single treatments were maintained in the combined treatments, which resulted in stagnating improvements in hydrolyzability with increasing severities. This suggests that an optimum exists where the treatment conditions in the respective steps maximizes both cellulose reactivity and accessibility and, thus, results in efficient enzymatic hydrolyzability. This study therefore shows that selection of severity of the respective treatment steps must be performed with regard to the overall fractionation efficiency as well as the downstream processing of the respective biomass components.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.9b07589.
Compositional analysis data, hydrolysis time courses, methods, and fiber morphology data (PDF)

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V.N. and F.N. contributed equally. All authors contributed to the design of the study. V.N., F.N., J.O., and K.A. planned and conducted the experiments. V.N., F.N., J.O., and M.G. analyzed the data and wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes
The authors declare no competing financial interest.

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