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Title: The effect of switchgrass plant cell wall properties on its deconstruction by thermochemical pretreatments coupled with fungal enzymatic hydrolysis or *Clostridium thermocellum* consolidated bioprocessing

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Abstract: A combination of thermochemical pretreatment and biological digestion technologies is usually required to overcome lignocellulosic recalcitrance and accomplish effective biomass deconstruction. This study aimed at understanding switchgrass breakdown by hydrothermal, dilute acid, dilute alkali, and co-solvent enhanced lignocellulosic fractionation (CELF) pretreatments followed by application of traditional fungal enzymatic hydrolysis (EH) and *Clostridium thermocellum* consolidated bioprocessing (CBP) to the resulting solids. Unpretreated and pretreated switchgrass and their EH and CBP residues were characterized by a suite of analytical techniques to understand structural changes that occurred during deconstruction. CELF pretreated solids showed the highest accessibility and digestibility by both EH and CBP followed by dilute alkali and then dilute acid / hydrothermal pretreated solids. Lignin removal from biomass had a more positive impact on substrate accessibility and digestibility than did xylan removal, while xyloglucan removal by pretreatment appeared essential for cellulose digestion by fungal enzymes. The extent of CBP digestion of cellulose and non-cellulosic glycans was larger than that by EH. Unlike dilute alkali pretreatment, cellulose crystallinity increased for acid-based pretreatments in the following order: hydrothermal, dilute acid, and CELF. CELF also substantially reduced cellulose degree of polymerization. All thermochemical and biological digestion approaches increased syringyl to guaiacyl lignin (S/G) ratios and reduced β-O-4 lignin interunit linkages and hydroxycinnamates content from levels in unpretreated switchgrass. The substantial increase in S/G ratio after hydrothermal and dilute alkali preatreatments suggested that high temperatures or alkali removed a large portion of G lignin from switchgrass.

Keywords: Bioethanol, *Clostridium thermocellum*, consolidated bioprocessing, pretreatment, switchgrass, enzymatic hydrolysis, fungal enzymes, lignocellulosic biomass

Introduction:

Lignocellulosic biomass cell wall structure is comprised of cellulose, hemicellulose, and lignin making up the lignocellulosic matrix [1, 2]. Cellulose and hemicellulose from the biomass can be broken down to simpler sugars that can then be fermented to ethanol and other useful metabolites. However, the complex cell wall structure in plant biomass is aimed at, among other things, plant survival in the environment against physical, chemical, and biological breakdown [2]. Even though ethanol production from lignocellulosic biomass has been studied extensively, biomass recalcitrance is still a hindrance that must be overcome for effective recovery of simple, fermentable sugars [2-6]. The traditional approach of ethanol production from lignocellulosic biomass thus involves particle size reduction, biomass pretreatment, enzyme production, enzymatic saccharification, hexose fermentation, pentose fermentation, and product recovery [4-6]. A separate enzyme production step, typically using *Trichoderma reesei*, is necessary and can be the most expensive operation in this process [7]. Biomass augmentation by mechanical or thermochemical pretreatments is therefore used to aid fungal enzymes in biomass digestion, thereby reducing enzyme dosage and associated costs required for high yields [5, 6, 8-14]. In contrast, consolidated bioprocessing (CBP) is a simple process that combines enzyme production, enzymatic hydrolysis, and fermentation into one operation [15-22]. *Clostridium thermocellum* is a promising native cellulolytic strategy-based CBP organism that can produce a complex, multi-functional cellulosome to digest lignocellulosic biomass [19, 22-25]. However, biomass augmentation, such as by thermochemical pretreatments, is still essential in achieving high polysaccharides solubilization and metabolite production by *C. thermocellum* [13, 14, 26-30].

A number of biomass structural features including lignin, cellulose, hemicellulose, and other glycan characteristics impact both thermochemical and biological deconstruction of biomass. Different biomass deconstruction protocols in turn affect the biomass itself and can alter its properties uniquely. A number of reports have shown that the extent of biological digestion of lignocellulosics, especially by fungal enzymes, is impacted by cellulose crystallinity, degree of polymerization and other cellulosic properties[31-39]. In our previous work we have shown that, unlike fungal enzymes, *C. thermocellum* is unaffected by cellulose micro-accessibility that is influenced by cellulose properties, such as crystallinity, water retention value, surface area, molecular weight, etc[29]. We have also shown that cellulose macro-accessibility or physical availability, especially as influenced by the presence of bulk lignin, drives *C. thermocellum* digestion[30]. Further, Dumitrache et al. have shown that *C. thermocellum* ATCC 27405 is sensitive to the composition of biomass, particularly the presence of lignin, and that a high syringyl to guaiacyl lignin (S/G) ratio is correlated with increased cellulose accessibility and higher fermentation ethanol yield[40]. High syringyl content was also linked to high molecular weight lignin or longer lignin chain that have a lower interference with enzymatic activity [40]. Other studies have shown that the downregulation of caffeic acid-O-methyl transferase (COMT) gene in the lignin biosynthesis pathway in switchgrass led to increased digestion and ethanol production by *C. thermocellum* while requiring milder pretreatment conditions when compared to the wild type plant biomass [41-43]. Similarly, low recalcitrant poplar natural variants SKWE 24-2 and BESC 876 with mutations in their 5-enolpyruvylshikimate-2-phosphate (EPSP) synthase gene affecting lignin biosynthesis were shown to have loosely held cell wall structures, high water retention value, and high S/G leading to higher glucan solubilization in the pretreated solids compared to the more recalcitrant BESC standard poplar [28].

Further, *C. thermocellum* was also shown to more effectively digest glucan and reduce lignin molecular weight compared to that achieved by fungal enzymes [28]. Biomass accessibility, lignin, and structural features of lignin have been shown to impact and be impacted by *C. thermocellum* fermentations [44, 45]. Sugar release via hydrothermal pretreatment and fungal enzymatic digestion is also affected by *Populus trichocarpa* lignin content and composition [46]. Hydroxycinnamates involved in the lignin carbohydrate complexes have also been shown to increase biomass recalcitrance by increasing the proximity of lignin to polysaccharides [47]. Further, thermochemical pretreatments affect lignin and cellulose characteristics substantially [46, 48-50]. Different lignocellulosic biomass are known to have varying lignin interunit linkages, to an extent depending on lignin composition (S,G, and H lignin), that are broken down during biomass digestion to varying degrees [45]

Thus, a number of factors impact lignocellulosic biomass bulk composition and substrate properties which, in turn, impact biological digestion of lignocellulosics. Biomass type, species, genetic modifications, location, growth conditions, fertilizers, water, soil salinity, harvesting and storage conditions are all expected to impact biomass that is ultimately used for ethanol production [51-59]. In light of the complexity of lignocellulosic biomass, it is important to reduce or eliminate process sensitivity to such variation in biomass structure, composition, and properties. To aid feedstock agnostic process development, a thorough understanding of biomass properties, the impact of different substrates and their characteristics on thermochemical as well as biological digestion, and the overall mechanism of biomass digestion are essential. Specifically, *C. thermocellum* is known to adapt its cellulosomal composition based on the substrate it encounters and is therefore a step toward a feedstock agnostic process [25]. Further, biological

digestion by fungal enzymes and *C. thermocellum* and the impact of various substrate properties on the two biological approaches are expected to be different. Therefore, here, we employed a suite of techniques to characterize unpretreated switchgrass compared to hydrothermal, dilute acid, dilute alkali, and co-solvent enhanced lignocellulose fractionation (CELF) pretreated switchgrass to determine changes in the substrate during pretreatment and understand the impact of these measured properties on the ability of *C. thermocellum* and fungal enzymes to digest these substrates. Further, we also characterized residues left undigested after CBP and fungal enzymatic hydrolysis in order to gain insight into the biological digestion process. First, we determined the extent of glucan digestion of unpretreated and pretreated switchgrass by both fungal enzymatic hydrolysis and *C. thermocellum* CBP. We related cellulose digestion to cellulose accessibility of unpretreated and pretreated switchgrass determined via Simons' staining technique. We further compared Scanning Electron Microscope (SEM) images of all materials including the CBP and EH residues to compare the distinctive physical changes that occurred during thermochemical and biological digestion of switchgrass. Then we looked at changes in cellulose crystallinity and degree of polymerization throughout the digestion process to relate the impact of thermochemical and biological digestion on cellulose in the substrate. We also characterized lignin isolated from all materials to determine relative abundances of syringyl (S), guaiacyl (G), and *p*-hydroxyphenol (H) lignin, lignin interunit linkages (β-O-4, β-β, and β-5), and hydroxycinnamates (ferulate and *p*-coumarate) involved in lignin carbohydrate complexes (LCC). Finally, we also looked at the fate of various glycans in switchgrass, including xyloglucans, xylans, homogalacturonans, rhamnogalacturonan I, mannans, and arabinogalactan, during pretreatment and biological digestion via glycome profiling, a high throughput semi-quantitative immunological assay[60, 61]. Such a comprehensive, unrivaled characterization of a

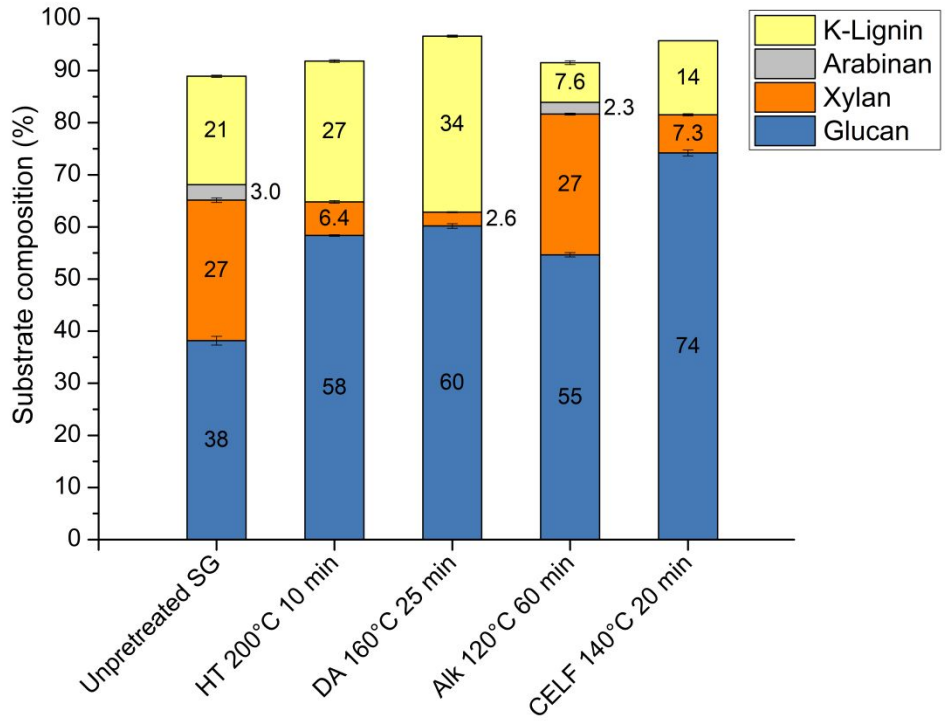
wide variety of materials was performed to reveal biomass structural changes during thermochemical and biological digestion of switchgrass with the goal of understanding the impact of these changes on the extent of digestion.

Results and Discussion:

Impact of pretreatment on the substrate and its biological digestion

Alamo switchgrass was pretreated using four different thermochemical pretreatment technologies: Hydrothermal, dilute acid, dilute alkali, and CELF. These pretreatments are well established in the field of lignocellulosic deconstruction and were chosen because of their ability to produce solids with varying compositional characteristics [62, 63] as shown in our previous work[30]. These pretreatments also represent a diversity in thermochemical pretreatments with the use of distinct catalysts aimed at helping us understand the mechanism of thermochemical digestion of switchgrass. We have previously optimized these pretreatments on switchgrass for maximum total sugar release (glucan + xylan) from pretreatment and *C. thermocellum* CBP combined[30]. We decided it was appropriate to characterize the various pretreatments at experimentally determined optimal conditions reported in our previous work that resulted in the highest digestion for each pretreatment type, instead of testing pretreatments all run at the same conditions[30]. The percent composition of solids produced after hydrothermal, dilute acid, dilute alkali, and CELF pretreatments of switchgrass performed at optimized conditions for maximum sugar release are shown in Figure 1. Hydrothermal and dilute acid pretreatments are acid based pretreatments that focus on hemicellulose removal as evidenced by high xylan and arabinan removal from switchgrass leaving behind solids with very low (<7%) xylan content and no arabinan. Hemicelluloses are amorphous and more branched than cellulose that is mostly

crystalline and are therefore, more prone to acid based hydrolysis[64]. Hydrothermal and dilute acid pretreatments at the conditions chosen for this work achieved 85% and 94% xylan removal, respectively, but removed only 19% and 4% of the lignin, respectively. Higher lignin removal during hydrothermal pretreatment was possibly due to the higher pretreatment temperature / low acidic conditions used during this pretreatment, resulting in less pseudo lignin[64] formation than after dilute acid pretreatment due to dehydration of carbohydrates during pretreatment. Dilute alkali pretreatment on the other hand has been shown to remove substantial amounts of lignin from lignocellulosic biomass and has been extensively used in the paper pulping industry[65]. Alkali based pretreatments may also break bonds between lignin and polysaccharides, especially hemicellulose, leading to some hemicellulose removal[65]. As expected, in this work dilute alkali pretreatment removed 75% of the lignin but removed only 32% of the xylan and therefore produced solids with high xylan content (~27%). CELF pretreatment removed both lignin and xylan in large quantities achieving 67% and 87% lignin and xylan removal respectively. The high digestive ability of CELF compared to other pretreatment techniques can be attributed to tetrahydrofuran (THF) solvent used during CELF pretreatment that has been shown elsewhere to cause lignin expansion and expose interunit linkages that can then be more effectively digested by acid[66]. Thus, CELF-pretreated solids showed the highest glucan content amounting to 74% glucan as opposed to 38%, 58%. 60%, and 55% glucan in unpretreated switchgrass and hydrothermal, dilute acid, and dilute alkali pretreated solids, respectively as reported in Figure 1 and our previous work[30].



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195 **Fig. 1** Composition of unpretreated switchgrass (SG) and solids produced by hydrothermal (HT),
196 dilute acid (DA), dilute alkali (Alk), and co-solvent enhanced lignocellulosic fraction (CELF)
197 pretreatments of SG performed at optimized conditions for maximum sugar release for each
198 pretreatment technology

199

200 SEM images of hydrothermal and dilute acid pretreated solids showed striations and
201 surface removal of matter when compared to unpretreated switchgrass probably representing
202 xylan removal as shown in Figure 2. Dilute alkali pretreated solids looked less ordered and more
203 crumpled compared to dilute acid and hydrothermal pretreated solids, perhaps representing the
204 effects of lignin removal from switchgrass. CELF pretreated solids showed a striated structure
205 similar to the other acid-based pretreatments along with deeper removal of matter compared to
206 other pretreatments, most likely due to the high removal of both xylan and lignin from these
207 solids.

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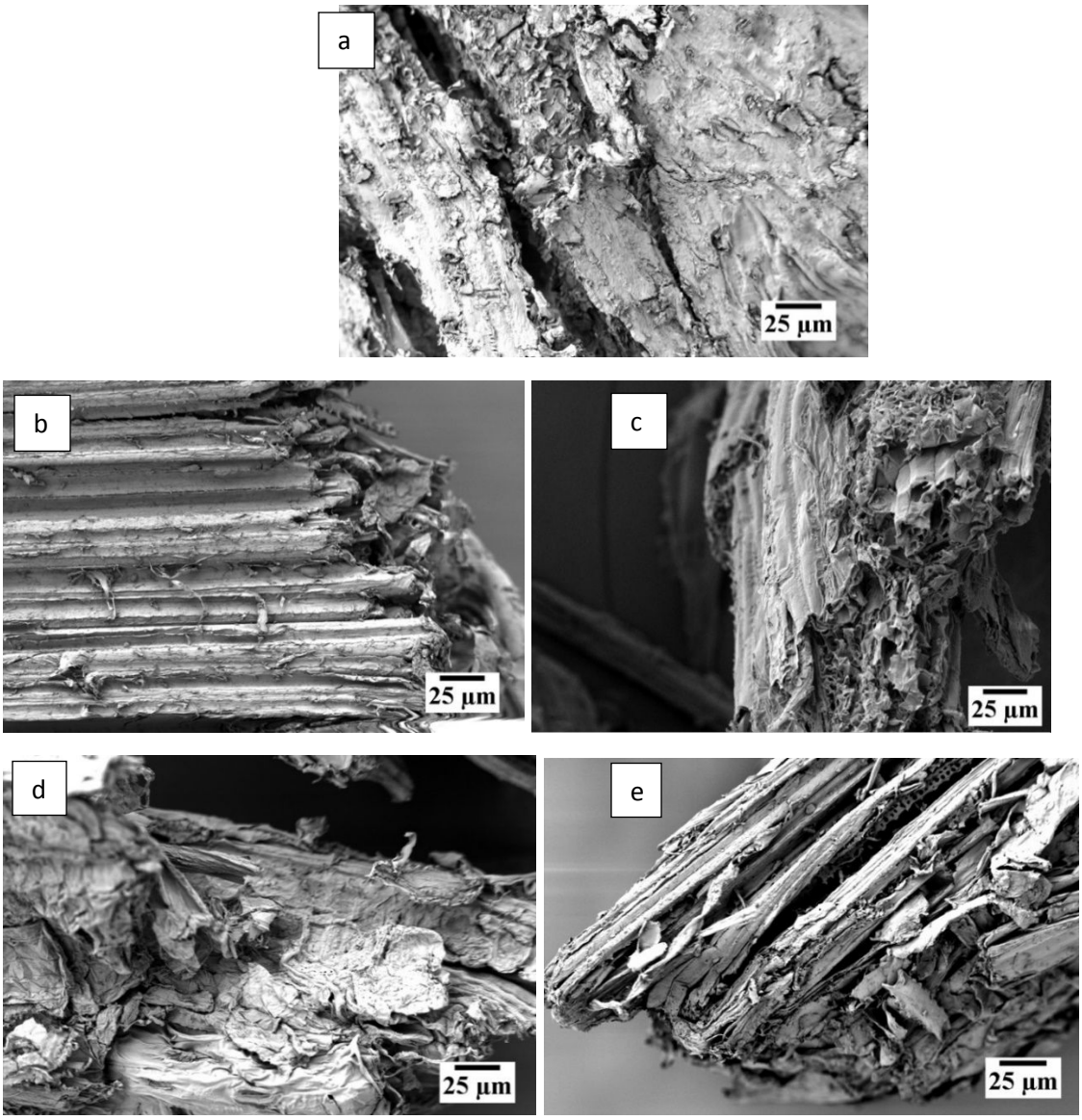


Fig. 2 Scanning Electron Microscope (SEM) images of **(a)** unpretreated switchgrass and **(b)** hydrothermal, **(c)** dilute acid, **(d)** dilute alkali, and **(e)** co-solvent enhanced lignocellulosic fraction (CELF) pretreated switchgrass. All images were taken at 1000x magnification

Cellulose accessibility to biological catalysts for digestion is generally considered to be of two types: macro- and micro-accessibility [1, 29]. Cellulose micro-accessibility is impacted by cellulose structural properties, such as crystallinity and degree of polymerization that may influence cellulose digestion[1, 31, 39, 67, 68]. But, before cellulose digestion by enzymes/microbes can be influenced by cellulose structural properties, the biological entities will need to gain

physical access to cellulose within the lignocellulosic matrix. Thus, macro-accessibility or the physical availability of cellulose is dictated by lignin, hemicellulose, and other physical barriers in the lignocellulosic matrix. By disrupting the complex lignocellulosic matrix, pretreatment of biomass increases the macro-accessibility of cellulose to cellulolytic enzymes/microbes [30]. The cellulose surface area and thus cellulose accessibility, specifically macro-accessibility, can be determined semi-quantitatively by measuring the amount of Direct Orange 15 dye adsorbed by a substrate [40, 44, 55]. The high molecular fraction of Direct Orange 15 has been shown to have a high affinity to cellulose, as opposed to other components of the plant cell wall structure, and is similar to cellulases based on size and structure [69-71]. Cellulose accessibility of unpretreated and pretreated substrates used in this study was found to be in the following order: CELF ≈ dilute alkali > dilute acid > hydrothermal > unpretreated switchgrass as shown in Figure 3.

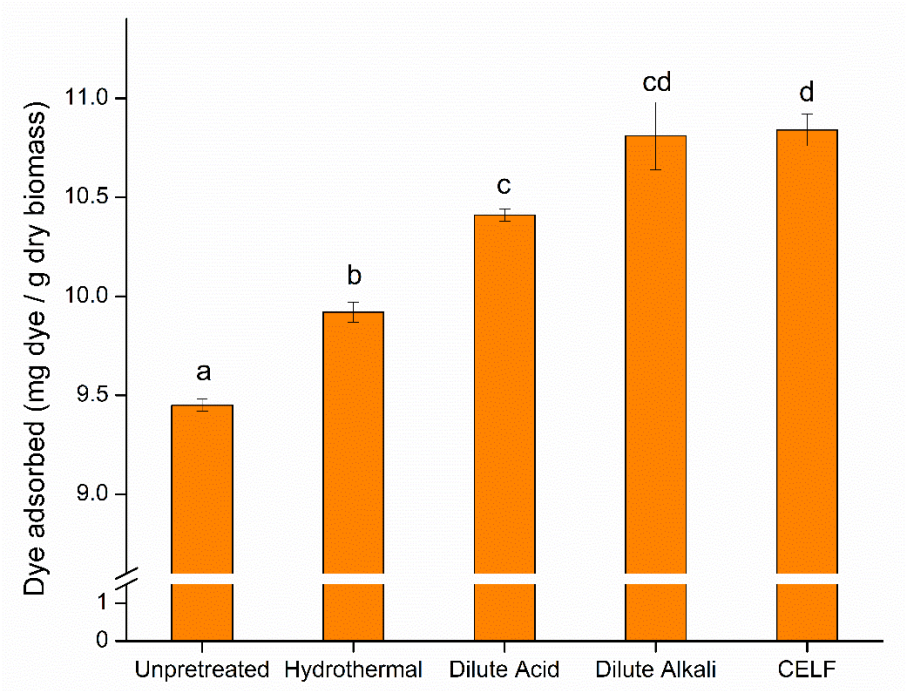
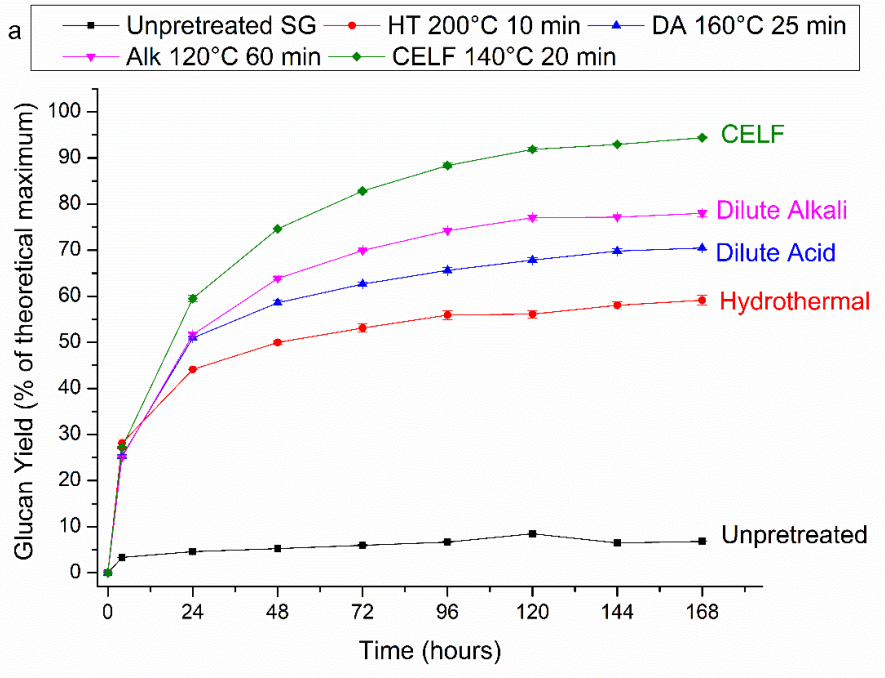


Fig. 3: Effect of hydrothermal, dilute acid, dilute alkali, and co-solvent enhanced lignocellulosic fractionation (CELF) pretreatments on cellulose accessibility of switchgrass measured by dye adsorption via Simons' staining method. Samples were analyzed in triplicate. A one-way ANOVA was performed at 95% significance level post-hoc using Bonferroni method with a p-value of 0.0000777 and F-statistic of 89.26. Same letter indicates no significant difference.



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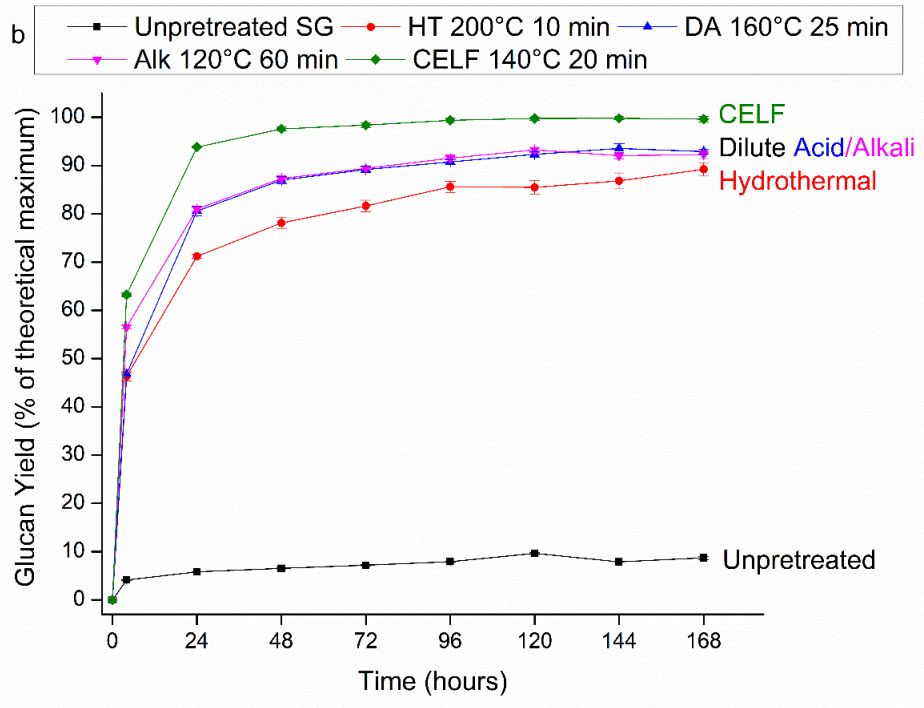

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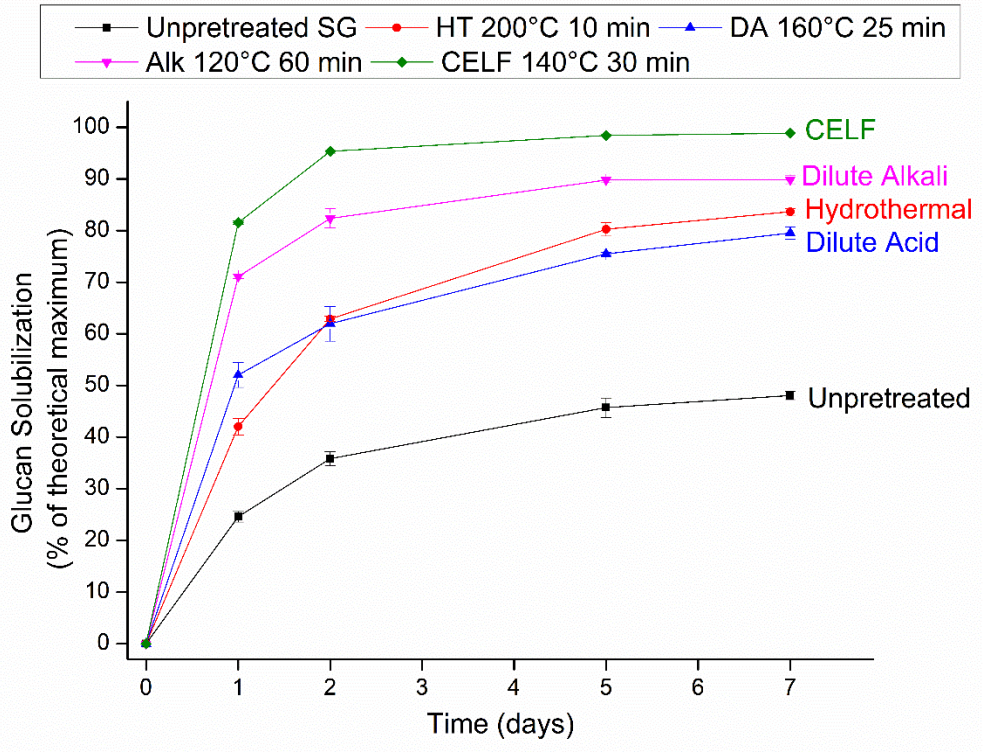
240 **Fig. 4** Fungal enzymatic hydrolysis glucan yield time profiles for unpretreated and hydrothermal
241 (HT), dilute acid (DA), dilute alkali (Alk), and co-solvent enhanced lignocellulosic fractionation
242 (CELF) pretreated switchgrass (SG) with **(a)** 15 mg protein/g-glucan enzyme loading and **(b)** 65
243 mg protein/g-glucan enzyme loading. Data and standard deviation reported are for three
244 biological replicates.

An increase in accessibility has been shown similarly for *Populus* natural variants after hydrothermal pretreatment [55]. Here, however, lignin removal after CELF and dilute alkali pretreatments affected cellulose accessibility more than xylan removal after dilute acid and hydrothermal pretreatments of switchgrass. This correlated well with fungal enzymatic hydrolysis (EH) of these substrates using 15 mg protein/g-glucan enzyme loading during which final glucan yield was found to be in the following order: CELF > dilute alkali > dilute acid > hydrothermal > unpretreated switchgrass as seen in Figure 4 and reported in our previous work[30]. Similarly, even though both dilute alkali pretreated solids and unpretreated switchgrass had similar xylan contents, the low lignin content in dilute alkali pretreated solids resulted in higher cellulose accessibility and therefore, higher fungal enzymatic glucan digestion compared to high lignin content in unpretreated switchgrass. The greater positive impact of lignin removal and thus increased cellulose accessibility on glucan digestion was also found to be true for *C. thermocellum* CBP as shown here in Figure 5 and reported in our previous work[30]. The slightly higher cellulose accessibility and enzymatic glucan digestion for dilute acid pretreated solids compared to hydrothermal pretreated solids might be due to lower xylan content in the former. Hemicelluloses have been previously shown to similarly influence cellulose accessibility measurements via Simon's staining technique [72, 73]. Fungal enzymatic digestion, unlike that for *C. thermocellum*, was negatively affected by the presence of xylan in hydrothermal pretreated solids compared to lower xylan content in dilute acid pretreated solids. Digestion by *C. thermocellum* generally showed the same trends as fungal enzymatic hydrolysis of these substrates with the exception of slightly higher glucan solubilization of hydrothermal pretreated solids compared to dilute acid pretreated solids by *C. thermocellum* shown in Figure 5. This may be attributed to the presence of xylanases in the *C. thermocellum* cellulosomal system, even

268 though the organism is not known to metabolize either xylose or xylo-oligomers, which helped
269 the organism effectively digest hydrothermal pretreated solids with slightly higher xylan content
270 [25].



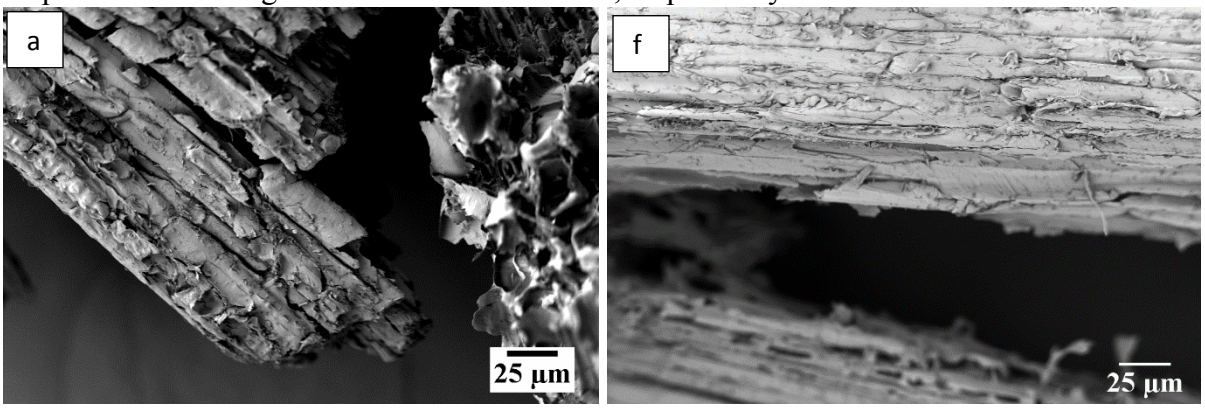
271

272 **Fig. 5** *C. thermocellum* consolidated bioprocessing (CBP) glucan solubilization time profiles for
273 unpretreated and hydrothermal (HT), dilute acid (DA), dilute alkali (Alk), and Co-solvent
274 enhanced lignocellulosic fractionation (CELF) pretreated switchgrass (SG). Data and standard
275 deviation reported are for three biological replicates.
276

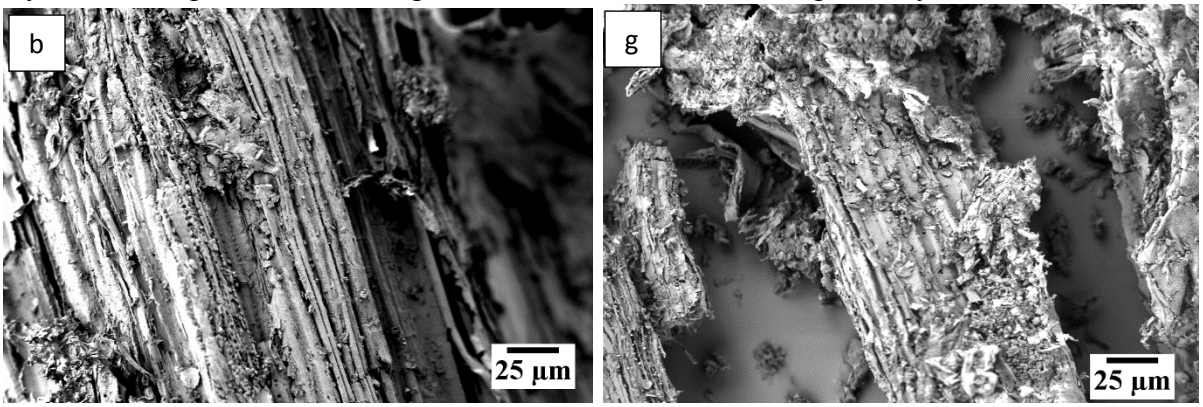
277 The biggest difference in digestion of the different substrates by the two biological
278 approaches, EH (at 15 and 65 mg protein/g-glucan enzyme loads) and *C. thermocellum* CBP (2%
279 by volume inoculum), was in the digestion of unpretreated switchgrass as shown in Figures 4 and
280 5. *C. thermocellum* was able to achieve 48% glucan solubilization from unpretreated switchgrass
281 within 5 days of fermentation as opposed to fungal enzymes that achieved <10% glucan yield
282 within the first day of hydrolysis and then ceased to solubilize the substrate further even at the
283 high enzyme loading of 65 mg protein/g-glucan. This result points to more effective and holistic

lignocellulosic digestion ability of the cellulosome, which has a wider variety of enzyme types, compared to conventional cellulase enzyme cocktails[25, 29, 30, 55]. Fungal enzymes seem unable to digest switchgrass effectively without some form of biomass pretreatment. The SEM image of solid residues obtained after EH (65 mg protein/g-glucan) of unpretreated switchgrass as shown in Figure 6(a) looks similar to that of unpretreated switchgrass itself shown in Figure 2(a) with minimal change. In comparison, CBP residue of unpretreated switchgrass shown in Figure 6(f) looks digested and smoothed out in comparison to unpretreated switchgrass. SEM images showed highly digested residues with smaller size particles left behind after both EH and CBP of CELF and dilute alkali pretreated solids compared to residues obtained after EH and CBP of hydrothermal and dilute acid pretreated solids as shown in Figure 6. This corroborates the overall more positive impact of lignin removal on biological digestion compared to xylan removal from switchgrass. Overall, *C. thermocellum* performed equivalently to 65 mg protein/g-glucan and better than 15 mg protein/g-glucan EH in terms of glucan solubilization. CBP residues of all substrates, overall, look more digested with smaller size particles compared to EH (65 mg protein/g-glucan) residues as seen in SEM images in Figure 6. CELF pretreated solids were the most digestible due to the high removal of both lignin and xylan from switchgrass and residues obtained after both EH and CBP look highly digested in the SEM images.

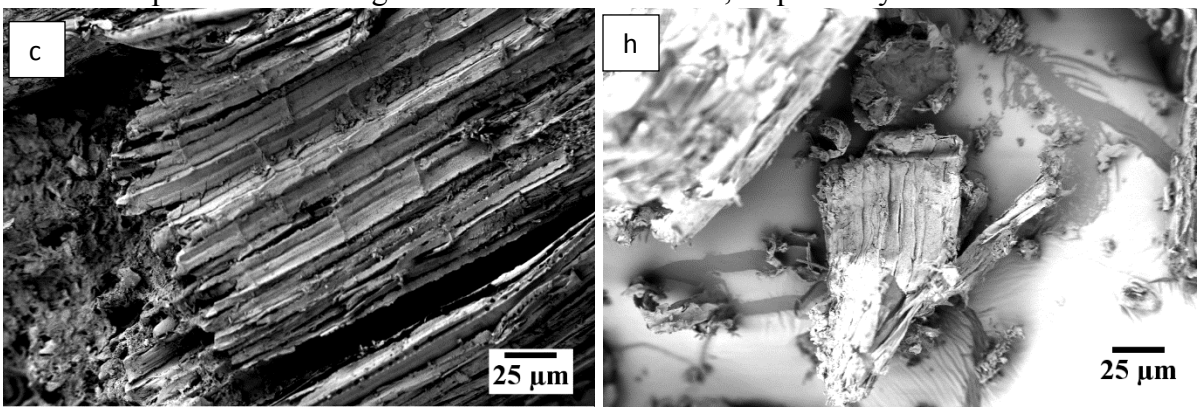
Unpretreated switchgrass EH and CBP residues, respectively:



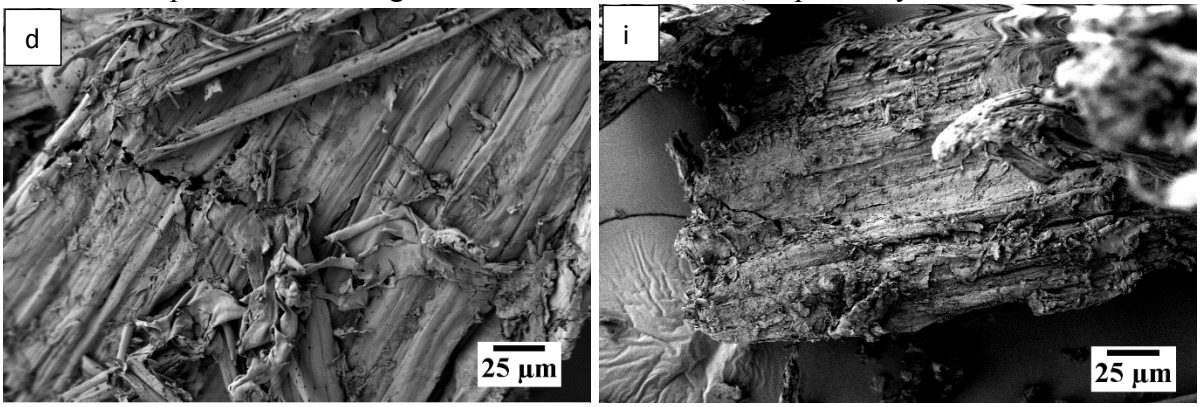
Hydrothermal pretreated switchgrass EH and CBP residues, respectively:



Dilute acid pretreated switchgrass EH and CBP residues, respectively:



Dilute alkali pretreated switchgrass EH and CBP residues, respectively:



CELF pretreated switchgrass EH and CBP residues, respectively:

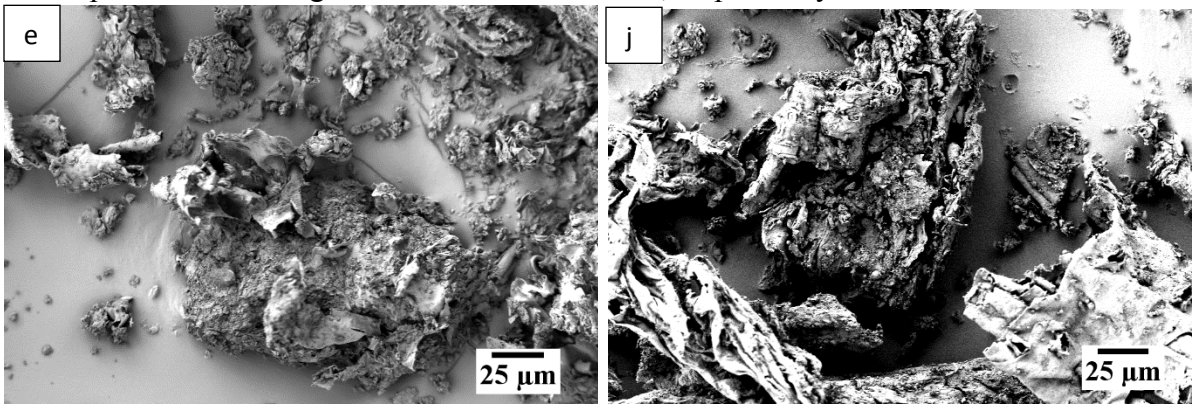


Fig. 6: Scanning Electron Microscopy (SEM) images of residues recovered after fungal enzymatic hydrolysis (EH) (65 mg protein / g glucan enzyme load) of **(a)** unpretreated switchgrass and **(b)** hydrothermal, **(c)** dilute acid, **(d)** dilute alkali, and **(e)** co-solvent enhanced lignocellulosic fraction (CELF) pretreated switchgrass and residues recovered after *C. thermocellum* consolidated bioprocessing (CBP) of **(f)** unpretreated switchgrass and **(g)** hydrothermal, **(h)** dilute acid, **(i)** dilute alkali, and **(j)** CELF pretreated switchgrass. All images were taken at 1000x magnification.

Impact of thermochemical and biological digestion on cellulose properties of switchgrass

We determined crystallinity, degree of polymerization (DP) and polydispersity index (PDI) of cellulose isolated from unpretreated and pretreated switchgrass (henceforth, collectively referred to as substrates) and residues recovered after CBP and EH (65 mg protein/g-glucan) of the corresponding substrates (henceforth, referred to as either CBP or EH residues) using solid state nuclear magnetic resonance (SSNMR) and gel permeation chromatography (GPC). All

acid-based pretreatments, dilute acid, hydrothermal, and CELF, significantly altered the overall crystallinity index (CrI) of cellulose as shown in Figure 7. In other words, acid-based pretreatments led to digestion of some of the amorphous cellulose in switchgrass leading to an overall increase in crystallinity of cellulose in pretreated solids consistent with other reports [49, 50]. In contrast to acid-based pretreatments, there was negligible change in the CrI of cellulose from dilute alkali pretreated solids compared to CrI of cellulose from unpretreated switchgrass. While the main impact of dilute alkali pretreatment on lignocellulosic biomass is delignification, any change in cellulose is minimal as observed here, especially since dilute alkali pretreatment was performed at comparatively lower temperatures than other pretreatments[65]. Hydrothermal and dilute acid pretreated solids showed higher cellulose crystallinity, which hindered effective hydrolysis, especially by fungal enzymes. On the other hand, CELF and dilute alkali pretreated solids had lower cellulose crystallinity and higher cellulose accessibility leading to better EH performance compared to that for dilute acid and hydrothermal pretreatments. Even though dilute alkali pretreated solids showed lower CrI compared to CELF pretreated solids, the presence of high amounts of xylan in dilute alkali solids may have led to lower EH yields compared to that from CELF pretreated solids. While cellulose from unpretreated switchgrass had relatively low CrI of ~42%, fungal enzymes were only able to digest <10% cellulose from this substrate. In contrast, fungal enzymes effectively digest Avicel, which is a more crystalline model cellulosic substrate [35]. This shows that physical availability of cellulose or cellulose macro-accessibility has a greater impact on digestion by fungal enzymes than cellulose properties or cellulose micro-accessibility[29].

In our previous work with commercially available model cellulosic substrates (comprised mostly of cellulose compositionally), we observed that *C. thermocellum* performance is not impacted substantially by cellulose properties and the organism equally digested model cellulosic substrates with varying cellulose crystallinity and other properties[29]. In contrast, fungal enzymes were negatively impacted by high cellulose crystallinity of these model cellulosic substrates as also reported elsewhere [33, 35-37, 74]. Further, fungal Cel7A cellulase has been reported to be extensively impacted by high cellulose crystallinity compared to CelA from *Caldicellulosiruptor bescii*, a thermophilic, anaerobic bacteria similar to *C. thermocellum*. [74]. Overall, both *C. thermocellum* and fungal enzymes were not able to substantially alter overall cellulose crystallinity during hydrolysis for most substrates as seen in Figure 7. This has also been reported for *C. thermocellum* fermentations of two *Populus* natural variants [40].

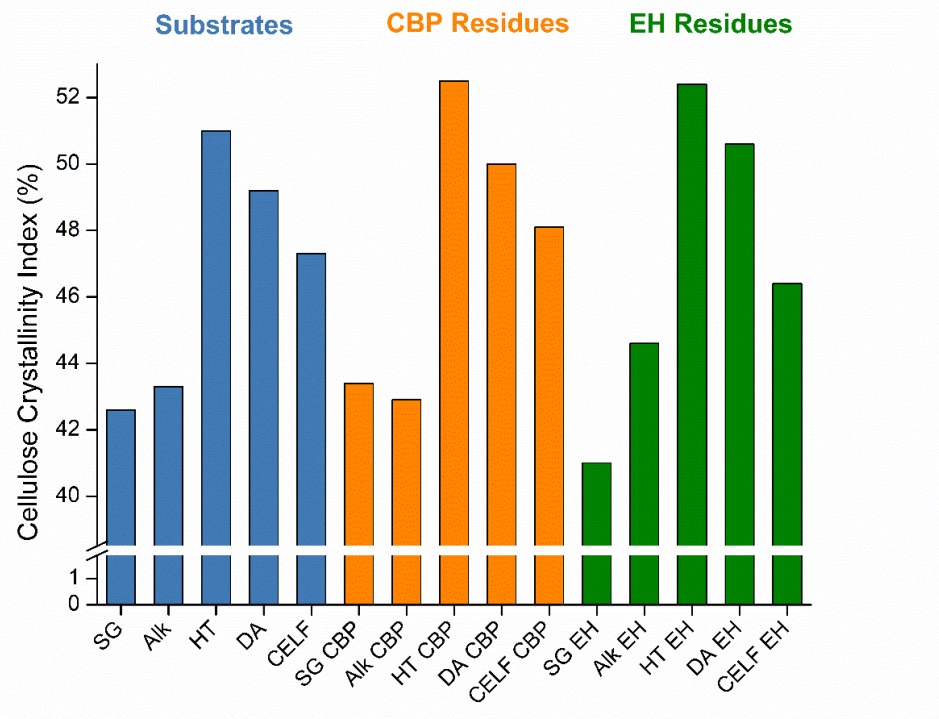
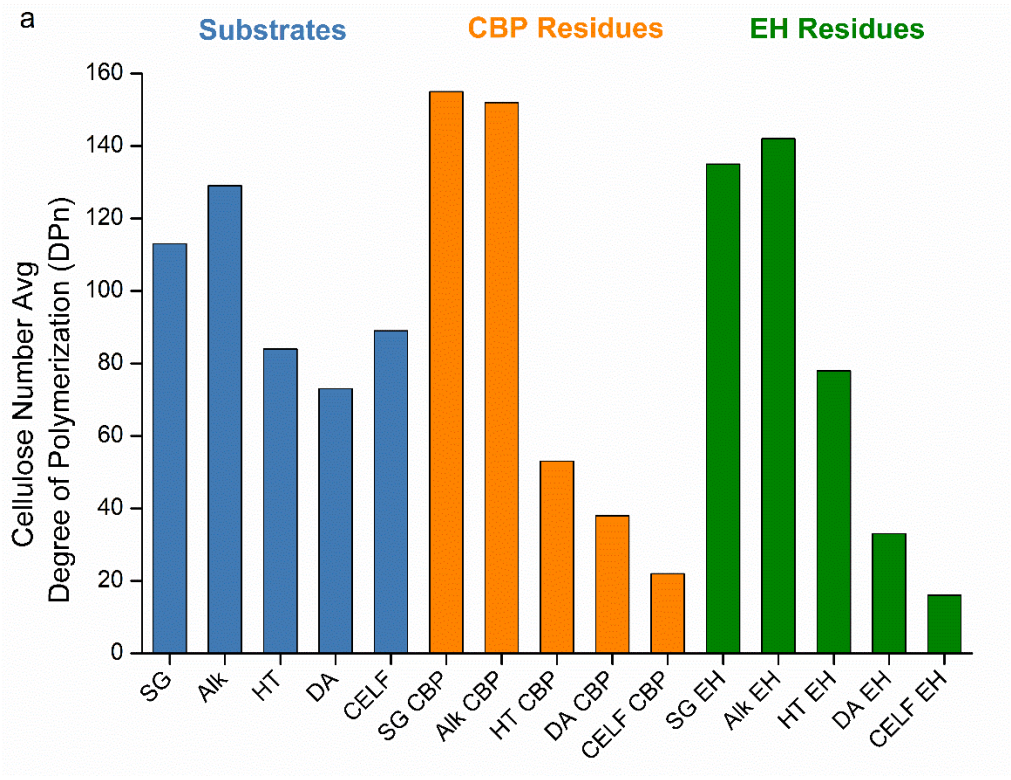
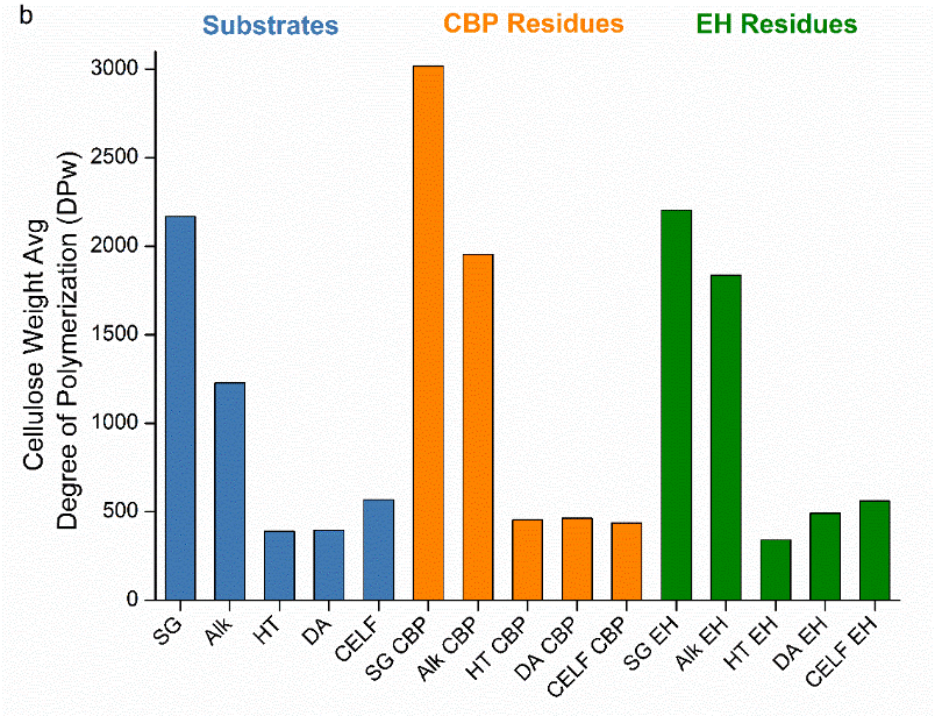


Fig. 7 Crystallinity indices (CrI) of cellulose isolated from unpretreated and hydrothermal (HT), dilute acid (DA), dilute alkali (Alk), and co-solvent enhanced lignocellulosic fractionation (CELF) pretreated switchgrass (SG) and their corresponding *C. thermocellum* consolidated bioprocessing (CBP) and fungal enzymatic hydrolysis (EH) residues.

Higher cellulose DP may reduce cellulose accessibility that could negatively impact biological digestion [1, 31, 75, 76]. Similar to the minor impact of dilute alkali pretreatment on cellulose crystallinity, this pretreatment also had a low to negligible impact on cellulose degree of polymerization compared to other pretreatment technologies as shown in Figure 8. All acid-based pretreatment technologies substantially decreased both the number average and weight average degree of polymerization, DPn and DPw respectively, which were further reduced during hydrolysis by both *C. thermocellum* and fungal enzymes. This decrease in cellulose DP after acid based pretreatments is attributed to preferential removal of amorphous cellulose at acidic conditions, which is confirmed via the increased cellulose crystallinity of solids resulting from after acid-based pretreatments, as shown in Figure 7[64]. Generally, it is understood that the decrease in cellulose DP during acid-based pretreatments occurs early during pretreatment, as amorphous cellulose is preferentially solubilized, followed by a levelling-off of cellulose DP[64]. CELF pretreated CBP and EH residues showed the lowest cellulose DPn suggesting that CELF pretreated solids were more amenable to cellulose DP reduction than the other materials by both biological approaches. Unpretreated switchgrass and dilute alkali pretreated residues left behind after biological digestion by both biocatalysts showed high DP, suggesting that acid-based pretreatments that reduce DP can further aid reduction in DP by both biological approaches. An increase in cellulose DP has also been reported for *C. thermocellum* digestion of two unpretreated *Populus* natural variants [40]. Similar to a decrease in DPn shown here for hydrothermal pretreated switchgrass and further decrease in DPn in their EH and CBP residues, a decrease in DPn has also been reported for three hydrothermal pretreated *Populus* natural variants, BESC standard, SKWE 24-2, and BESC 876 and their EH and CBP residues compared to unpretreated materials [55]. Further, there was an increase in the polydispersity indices after

biological digestion suggesting a larger decrease in DPw than DPn as shown in Figure 8(c). This was in contrast to a negligible change in PDI observed on unpretreated *Populus* natural variants with varying S/G ratios with different PDIs, which did not change after *C. thermocellum* fermentation [40]. However, there was no significant relationship between DP and extent of overall biological digestion, thus cellulose DP does not seem to be a driving factor for digestion by either biological approaches in this study. Overall, acid-based pretreatments impacted both cellulose crystallinity and cellulose degree of polymerization, whereas, dilute alkali pretreatment did not. Further, cellulose crystallinity may affect biological digestion but cellulose DP did not seem to have an influence on the extent of biological digestion. However, cellulose properties may only impact biological digestion of substrates that have high cellulose macro-accessibility.





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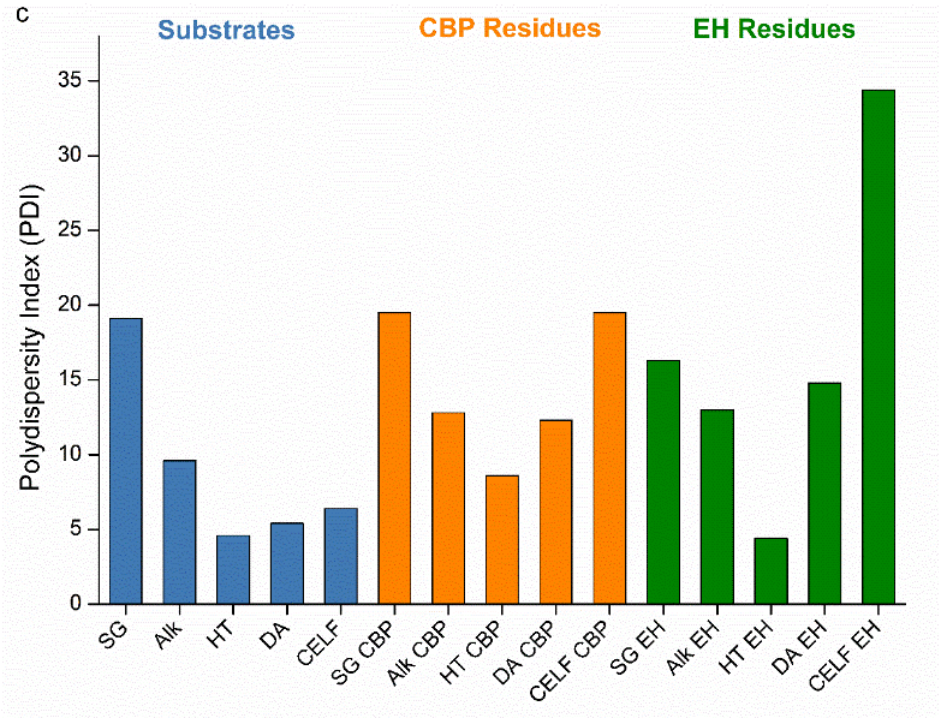


413

414 **Fig. 8 (a)** Number (DPn) and **(b)** weight (DPw) average degree of polymerization and **(c)**
415 polydispersity indices (PDI) of cellulose is unpretreated and hydrothermal (HT), dilute acid
416 (DA), dilute alkali (Alk), and co-solvent enhanced lignocellulosic fractionation (CELF)
417 pretreated switchgrass (SG) and their corresponding *C. thermocellum* consolidated bioprocessing
418 (CBP) and fungal enzymatic hydrolysis (EH) residues.

Structural changes in lignin after thermochemical and biological digestion of switchgrass

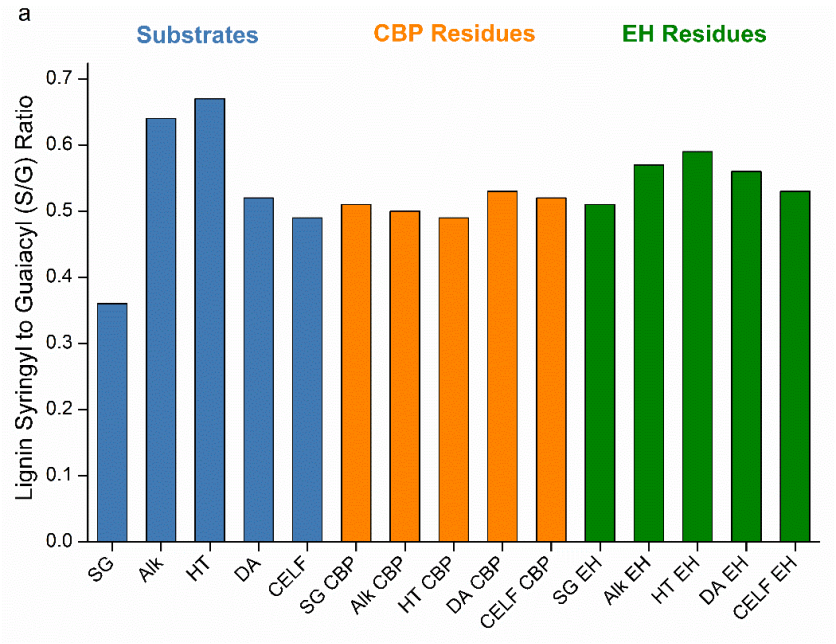
Syringyl (S), guaiacyl (G), *p*-hydroxyphenyl (H) are the predominant types of lignin found in lignocellulosics that are polymerized from sinapyl alcohol (4-(3-hydroxyprop-1-enyl)-2,6-dimethoxyphenol), coniferyl alcohol 4-(3-hydroxy-1-propenyl)-2-methoxyphenol), and p-coumaryl alcohol (4-(3-hydroxy-1-propenyl)phenol), respectively. Lignin composition in terms of proportions of monolignol subunits has been proposed to affect thermochemical and biological degradation of lignocelluloses [47]. Here, we determined the relative abundance of monolignol subunits in unpretreated and pretreated substrates as well as in residues obtained after CBP and EH via 2D Heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) as reported in Figure 9.

Even though the composition of lignin is expected to influence the digestion of lignocellulosics, there are conflicting reports on the specific impact and fate of S/G ratio during thermochemical pretreatments. Generally, S lignin has linear chains with less cross-linking compared to G lignin because C-5 position in the S unit is methoxylated and therefore blocked [77]. This potentially leads to a higher occurrence of β-β (resinol) bonds leading to less cross-linking and a lower occurrence of the more stable β-5 (phenylcoumaran) and 5-5 bonds (C-5 position is blocked) [77-79]. S-rich lignin has been reported to have a lower molecular weight than G-rich lignin due to its high abundance of β-β bonds[46]. S lignin is also reported to have a high proportion of β-O-4 (β-aryl-ether) bonds that are highly reactive leading to an increased susceptibility to lignin removal during pretreatment and an overall increase in biomass susceptibility to enzymatic hydrolysis [80]. S/G ratio has been shown to drop with hydrothermal,

dilute acid, and alkaline pretreatments suggesting more susceptibility of S lignin to breakdown with higher pretreatment severities impacting S/G ratio more [46]. A faster cleavage of β-O-4 has been shown to occur during pretreatments under alkaline conditions [81]. Further, significant S lignin fragmentation has been shown after steam explosion pretreatment of Miscanthus (unpretreated S/G = 1.34) and wheat straw (unpretreated S/G = 1.12) [82].

However, there are opposing reports of the impact of S/G ratio on overall digestibility of lignocellulosics. Specifically, a study showed that higher S/G ratio in *Populus* natural variants possibly led to longer linear lignin chain lengths with an overall high lignin molecular weight [40]. It has also been reported that the relative amount of the labile β-O-4 bonds does not depend on S/G ratio and remains constant as long as some syringyl units are present [46]. Further, higher proportion of lignin with high β-O-4 bonds has also been shown to negatively correlate with extent of cell wall degradation [80]. Similarly, through modeling, the long chain β-O-4 containing lignin has been shown to be able to linearly orient parallel to the cellulose surface with increased interaction with cellulose as opposed to β-5 and 5-5 bonds that stiffen lignin overall causing a flat adsorption onto cellulose [83]. The same study mentioned above that showed significant S lignin fragmentation in Miscanthus after steam explosion pretreatment also showed that pretreatment of poplar led to more removal of G lignin (unpretreated S/G = 1.29) [82]. Similarly, another study showed an increase in S/G ratio after hydrothermal pretreatment of three *Populus* natural variants [84]. Further, a similar increase in S/G ratio after alkaline hydrogen peroxide pretreatments of switchgrass was also reported with a simultaneous substantial break down of β-O-4 bonds [48]. Overall, there is limited consensus on the significance of the impact of S/G ratio and lignin interunit linkages on biological cellulose digestibility.

In the current study, hydrothermal, dilute acid, dilute alkali, and CELF pretreatments were all responsible for an increase in S/G ratio in switchgrass as shown in Figure 9(a). This could be because of the presence of very low S lignin in unpretreated switchgrass (S/G ratio = 0.36) and the sheer presence of high amounts of G lignin possibly leading to more degradation of the latter during pretreatment causing an increase in S/G ratio in pretreated solids. S/G ratio was still less than 1 even in the pretreated solids. Greater G lignin breakdown and a concomitant increase in S/G ratio has been reported for dilute acid hydrolysis of wood samples from a second generation *Populus* cross (S/G ratio of unpretreated samples varied from 1.8 to 2.3) [85]. Interestingly, forage maize with lower S lignin has also been shown to lead to higher milk and meat production [86]. It was hypothesized that higher G content produced more crosslinked but thinner cell walls and is therefore easier to break down. Further, it was also speculated that cell walls with lower S lignin were less mature and less lignified leading to better chemical and enzymatic digestion [87]. Here, hydrothermal and dilute alkali pretreated solids showed higher S/G ratios of 0.67 and 0.64, presumably due to significant removal of G lignin, in comparison to other substrates as shown in Figure 9(a). It is important to remember that hydrothermal pretreatment achieved only 20% lignin removal, whereas dilute alkali pretreatment achieved 75% lignin removal. However out of the respective amounts of lignin removed by both pretreatments, both pretreatments achieved G lignin removal more than S lignin removal leading to a 78% and 86% increase in S/G ratio after hydrothermal and dilute alkali pretreatments, respectively. Here, alkali seems to have catalyzed substantial G lignin removal compared to acid catalysts, whereas, the high temperature (200 ^{0}C) used in hydrothermal pretreatment may have contributed to high G lignin removal compared to either dilute acid or CELF pretreatments.



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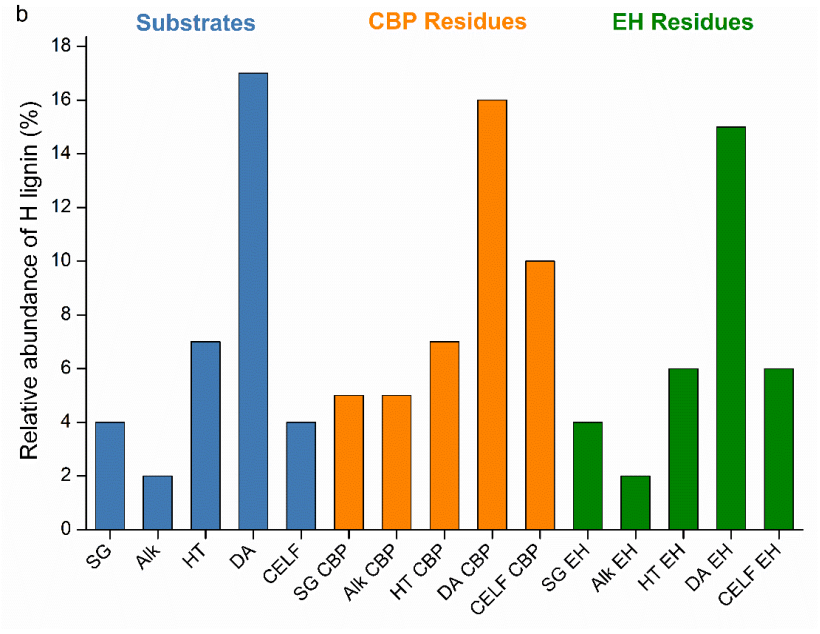


488

489 **Fig. 9** Relative abundance of (a) Syringyl to guaiacyl monolignol subunit ratio (S/G) and (b) *p*-
490 hydroxyphenol (H) monolignol subunit content in lignin isolated from unpretreated and
491 hydrothermal (HT), dilute acid (DA), dilute alkali (Alk), and co-solvent enhanced lignocellulosic
492 fractionation (CELF) pretreated switchgrass (SG) and their corresponding *C. thermocellum*
493 consolidated bioprocessing (CBP) and fungal enzymatic hydrolysis (EH) residues
494

495

496

A number of reports have speculated that better biological digestibility correlated with the presence of higher amounts of S lignin and therefore higher S/G ratios. A majority of studies report a positive impact of S/G ratio on hydrolysis by fungal enzymes as well as *C. thermocellum* [40, 46, 85]. Further, the influence of bulk lignin content on enzymatic hydrolysis has been shown to diminish at higher S/G ratios (≥ 2) suggesting the positive effect of the presence of S lignin [46]. In contrast, multiple reports have also shown minimal effect of S/G ratio on enzymatic digestion of lignocelluloses [80, 88, 89]. Others showed negative impact of high S/G on enzymatic digestion of alkaline and acid pretreated miscanthus [84, 90]. In the present work, no direct impact of S/G ratio on glucan solubilization by *C. thermocellum* or fungal enzymes can be determined, especially since each of the materials have varying amounts of lignin and the overall lignin content itself had a stronger impact on solubilization. Interestingly, as in the case for thermochemical digestion of switchgrass, *C. thermocellum* increased the S/G ratio of unpretreated switchgrass during hydrolysis consistent with multiple reports on *C. thermocellum* [40, 45].This has been attributed to removal of G lignin that has a less sterically hindered phenolic group than S lignin (2 methoxy groups in S lignin units compared to 1 in G lignin units presumably induces more steric hindrance) [45]. However, since G lignin removal during biological digestion of unpretreated switchgrass was observed by both *C. thermocellum* and fungal enzymes, the removed G lignin is possibly carbohydrate associated. Further, higher G lignin removal than S lignin from unpretreated switchgrass by CBP and EH could just be due to the sheer presence of high amount of G lignin in unpretreated switchgrass. This could also explain the decrease in S/G ratio for dilute alkali and hydrothermal pretreated solids after both CBP and EH. Higher presence of S lignin in these substrates could be why S lignin breakdown observed was higher compared to G lignin breakdown, especially if this is only a manifestation of carbohydrate associated lignin

breakdown. S/G ratio of all CBP residues was around 0.5, whereas, those of all EH residues varied from 0.5-0.6. S/G ratio of EH residues of all substrates followed the same trend as the substrates themselves (hydrothermal > dilute alkali > dilute acid > CELF > unpretreated switchgrass), whereas, the substrates lost this trend after *C. thermocellum* fermentation with residues showing equal S/G ratios.

H-lignin, which has not been studied as extensively as the other monolignol subunits of lignin, is negatively correlated to biomass recalcitrance and therefore, positively to enzymatic hydrolysis yields [47]. H lignin is reported to negatively impact cellulose crystallinity improving cellulose digestion in wheat and rice samples [91]. Further, H lignin is shown to be more reactive than S lignin using density functional theory [92]. High sugar yields were obtained from an H-rich Arabidopsis mutant as opposed to G- or S-rich mutants [92]. Here, Figure 9(b) shows that dilute alkali and CELF pretreatments that substantially reduce lignin content also reduced H lignin content specifically. In contrast, dilute acid and hydrothermal pretreatments showed a relative increase in H lignin content possibly due to higher removal of G lignin instead as shown by an increase in S/G ratio after pretreatment. Substrates with high lignin content, unpretreated switchgrass and hydrothermal and dilute acid pretreated solids, showed an increase in solubilization by both *C. thermocellum* and fungal enzymatic hydrolysis with an increase in H lignin content in the substrates. Thus, for substrates with high lignin content, the presence of higher H lignin could possibly lead to an improvement in the extent of biological digestion.

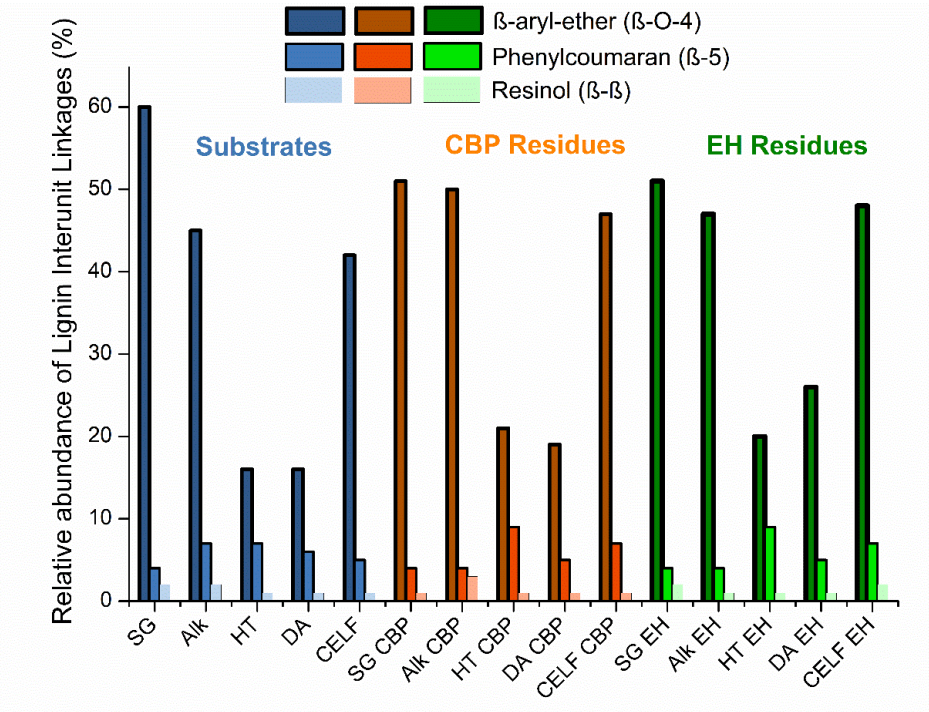


Fig. 10 Relative abundance of interunit linkages in lignin isolated from unpretreated and hydrothermal (HT), dilute acid (DA), dilute alkali (Alk), and co-solvent enhanced lignocellulosic fractionation (CELF) pretreated switchgrass (SG) and their corresponding *C. thermocellum* consolidated bioprocessing (CBP) and fungal enzymatic hydrolysis (EH) residues.

Further, as expected, the β-O-4 interunit linkage was the most common in unpretreated switchgrass and all pretreatments were able breakdown β-O-4 and β-β bonds as shown in Figure 10. While all pretreatments reduced the relative abundance of β-O-4 bonds in switchgrass, dilute acid and hydrothermal pretreatments caused a substantial reduction (~73%) of this type of interunit linkage. Both hydrothermal and dilute acid pretreatments do not remove a lot of lignin from switchgrass as seen in this work but they are still able to cause a significant change in the lignin structure, possibly due to the use of high temperatures. Most of the lignin is still left behind in dilute acid and hydrothermal pretreated solids with a simultaneous reduction in β-O-4 bonds, which suggest condensation and possible redeposition of lignin in the substrate [13]. Thus lignin, once isolated, from hydrothermal and dilute acid pretreated solids would not keep a native

structure impacting downstream lignin valorization and utilization. While dilute alkali and CELF pretreatments remove substantial amounts of lignin from the biomass, lignin left in the solids shows similar abundance of interunit linkages as unpretreated switchgrass. Even though CELF pretreatment utilizes a 0.5 wt% sulfuric acid solution, similar to dilute acid pretreatment, THF used in the former has been shown to prevent aggregation of lignin. Without the presence of THF, lignin aggregation is expected in aqueous environments leading to recondensation of lignin as in the case of dilute acid pretreatment [93]. Further, as expected, the strong β-5 bonds were not broken down during any pretreatment and the relative abundance of this type of interunit linkage increased possibly due to reduction in the relative abundance of other types of linkages. Both *C. thermocellum* and fungal enzymes caused a 15% reduction in the relative abundance of β-O-4 bonds during hydrolysis of unpretreated switchgrass with no substantial change in abundance of β-β and β-5 bonds in unpretreated switchgrass.

Fate of Lignin-carbohydrate linkages during digestion of switchgrass

Hydroxycinnamates, namely ferulates (FA) and *p*-coumarates (*p*CA), are common in grasses and are part of lignin-carbohydrate complexes (LCCs) [47, 94]. Bifunctional *p*-coumaric and ferulic acids form ester linkage from their carboxyl group or an ether linkage from their phenolic groups [95]. Ferulic acid can cross link with lignin and hemicellulose by esterification of their carboxylate groups to arabinose in arabinoglucuronoxylan and etherification of the hydroxyl group to phenyl hydroxyls in lignin. Ferulic bridges of this kind are common in grasses, in contrast to wood LCCs, and are sometimes referred to as "lignin/phenolic carbohydrate complexes" [96].The carbohydrate part of LCCs in grasses are composed predominantly of arabino-4-O-methylglucuronoxylan [95]. LCCs draw lignin closer to polysaccharides and thus

increase overall biomass recalcitrance [47]. Alkali treatments break the ester linkages freeing carbohydrates from lignin leaving behind hydroxycinnamic acids and their residues [96]. A number of alkaline pretreatment technologies have been reported to cleave and/or modify FA and *p*CA to increase biomass digestibility [48, 97-99]. The ether linkages can be broken down through acid catalyzed reactions while the ester linkage may remain intact [96, 100, 101]. Here, dilute alkali pretreatment was able to break the ester bonds of both FA and *p*CA leading to a sharp decrease in their relative abundance as shown in Figure 11, consistent with other reports.

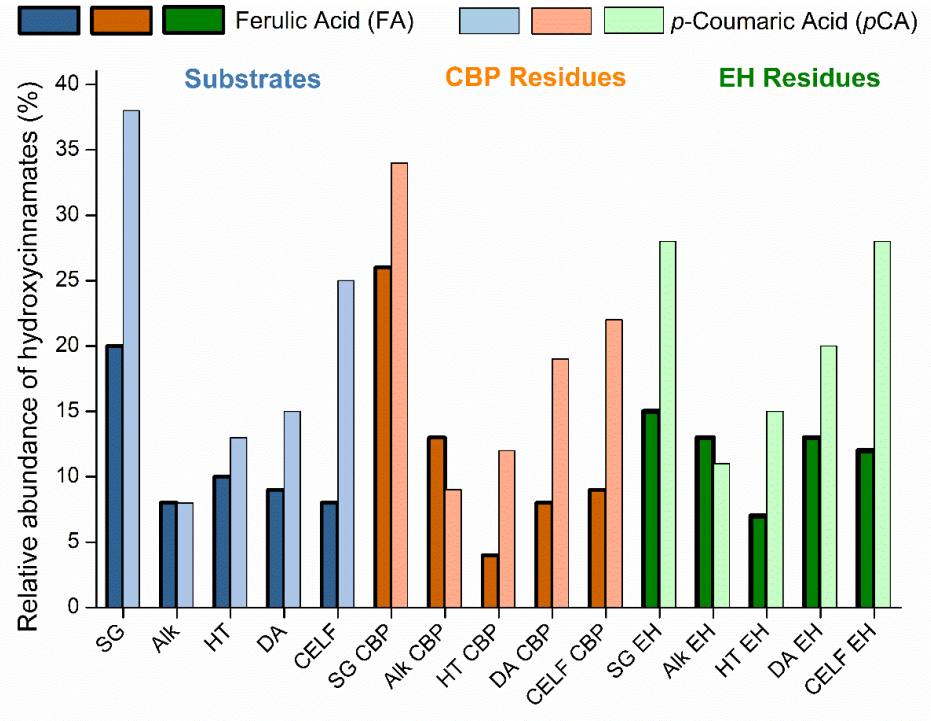


Fig. 11 Relative abundance of hydroxycinnamates in unpretreated and hydrothermal (HT), dilute acid (DA), dilute alkali (Alk), and co-solvent enhanced lignocellulosic fractionation (CELF) pretreated switchgrass (SG) and their corresponding *C. thermocellum* consolidated bioprocessing (CBP) and fungal enzymatic hydrolysis (EH) residues.

Even though both FA and *p*CA were reduced substantially by acid based pretreatments, FA was removed in larger quantities than *p*CA. CELF pretreatment, especially, showed low

595 removal of *p*CA (34%) compared to dilute acid pretreatment, which yielded a 60% reduction in
596 relative *p*CA abundance. Overall, all pretreatments reduce the amounts of hydroxycinnamates
597 present in switchgrass, while also reducing recalcitrance. Even though *C. thermocellum* has been
598 shown to have ferulic acid esterases and to produce *p*CA in the fermentation broth, the organism
599 in the absence of pretreatments was the least effective in hydroxycinnamates removal compared
600 to all other thermochemical and biological digestion techniques [102]. Hydrothermal pretreated
601 solids were the most amenable to reduction in FA after both CBP and EH.
602
603 **Fate of glycans after thermochemical and biological digestion of switchgrass as observed by**
604 **glycome profiling:**
605 Unpretreated and pretreated switchgrass and the corresponding CBP and EH solid
606 residues were subjected to sequential extraction with a set of increasingly harsh reagents and the
607 resulting wall extracts were screened using a diverse library of glycan-directed monoclonal
608 antibodies (mAbs)[103] in a high throughput, semi-quantitative assay called glycome profiling[55, 60,
609 61, 104-106]. This sequential extraction protocol leads to the solubilization of non-cellulosic cell wall
610 components into glycan rich extracts depending on the relative tightness with which the glycans
611 are integrated into the cell walls. Epitope specific mAbs then reveal the contents of the extracts
612 allowing us to determine what glycans are present in the walls/residues and the relative strength
613 with which these glycans are bonded to the cell wall. Thus, we can gauge the impact of
614 thermochemical and biological digestion on cell wall structure through examination of the
615 antibody binding intensity heat map shown in Figure 12. The reagents used in the sequential
616 extractions are oxalate, carbonate, 1M KOH, 4M KOH, Chlorite, 4M KOH post chlorite. It can
617 be concluded that glycan epitopes that appear in the later, harsher extracts were more tightly

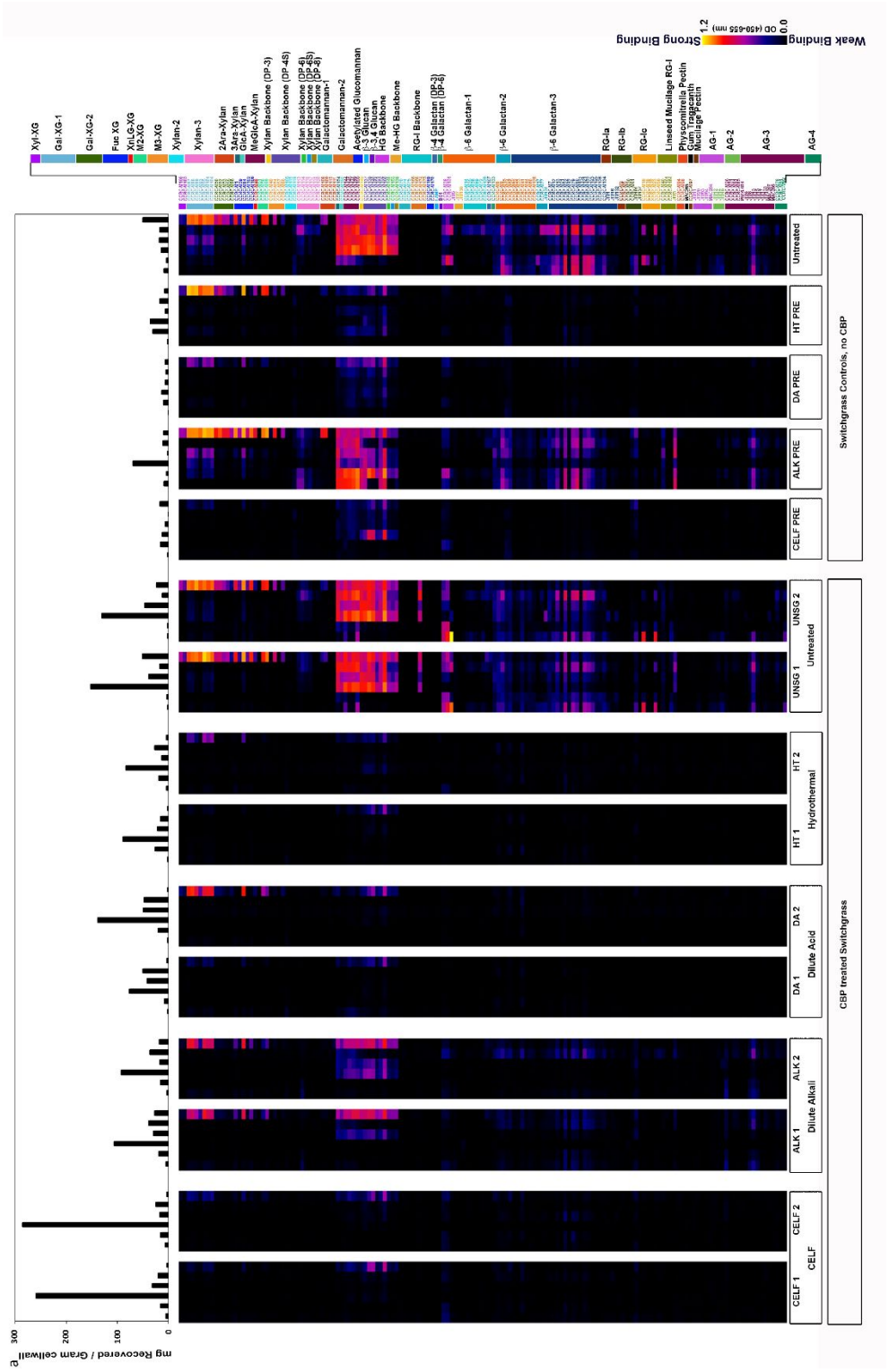
bound to the plant cell wall than the ones that appear in the earlier milder extracts. Further, the level of monoclonal antibody binding is depicted using a range of colors; lighter yellow-orange-red colors represent high levels of binding and darker purple-blue-black colors represent lower levels of binding. Thus, the more yellows the color intensity on the plot, the higher the quantity of the corresponding epitope present in that extract. Glycome profiling has been used to determine the sequence of changes, namely, breakdown of lignin-polysaccharide interaction along with removal of pectins and arabinogalactans followed xylans and xyloglucans that occurred during hydrothermal pretreatment of Populus biomass[104]. A similar study revealed the impact of Ammonia Fiber Extraction or AFEX™ pretreatment that led to loosening of xylan, pectin, and xyloglucan from eight distinct biomass types[105]. Further, glycome profiling on Populus natural variants revealed that removal of xyloglucan during hydrothermal pretreatment was essential to higher digestion by both *C. thermocellum* and fungal enzymes based biological digestion[55].

Here, the three acid based pretreatment methods, hydrothermal (HT), dilute acid (DA), and CELF pretreatments showed significant removal of most non-cellulosic glycans as evidenced by the absence of binding of most antibodies included in the screens, with CELF being the most effective and HT being the least effective. Some xyloglucans remained in the DA and HT pre-treated biomass, as indicated by the residual binding of xyloglucan-directed mAbs. Because the extent of cellulose digestion by fungal enzymes was also highest on CELF followed by dilute acid and then hydrothermal pretreated solids, it may be concluded that removal of xyloglucans was essential for cellulose digestion by fungal enzymes. Fungal cellulolytic enzyme digestion of HT solids was poorer than that of DA pretreated solids at the 15 mg protein / g

641 glucan loading, suggesting an impact of the amount of xyloglucan present in the two materials.
642 *C. thermocellum* also showed higher cellulose digestion on CELF pretreated solids compared to
643 dilute acid and hydrothermal pretreated solids. However, CELF pretreatments also removed a
644 significant amount of lignin, which may have had a larger impact on digestion by both biological
645 approaches. *C. thermocellum* on the other hand digested both hydrothermal and dilute acid
646 pretreated solids equally and therefore the amount/presence of xyloglucan may not have had an
647 impact on *C. thermocellum* cellulose digestion.
648
649 Dilute alkali preatreated solids showed a significant abundance of xylan epitopes
650 (especially xylans) in the glycome profile which is consistent with the overall compositional data
651 presented in Figure 1. In addition, dilute alkali pretreated solids also retained residual
652 xyloglucan, homogalacturonan and 6-linked galactan epitopes, as indicated by the binding of
653 antibodies to these epitopes in one or more extracts from the pre-treated biomass. Digestion of
654 alkali pretreated solids by fungal enzymes did not lead to significant changes in the glycome
655 profiles of the digested, alkali-pre-treated biomass (Figure 12(b)). In contrast, *C. thermocellum*
656 CBP was able to significantly reduce the residual amounts of non-cellulosic epitopes remaining
657 in alkali-pretreated solids, with the exception of tightly bound xyloglucans and xylans, as
658 observed in the 4M KOHPC extracts (Figure 12(a)). Specifically, *C. thermocellum* digestion of
659 dilute alkali pretreated solids removed essentially all galactan and homogalacturonan epitopes
660 that are present in large quantities in these solids. Furthermore, *C. thermocellum* also removed
661 most of the more loosely bound xylan epitopes, but appeared to leave some tightly bound
662 galactosylated xyloglucan (Gal-XG) epitopes. These results show that *C. thermocellum* can
663 more effectively break down non-cellulosic components of the cell wall than fungal enzymes

664 which in turn helps the former more substantially breakdown and utilize the cellulose in the

665 biomass.



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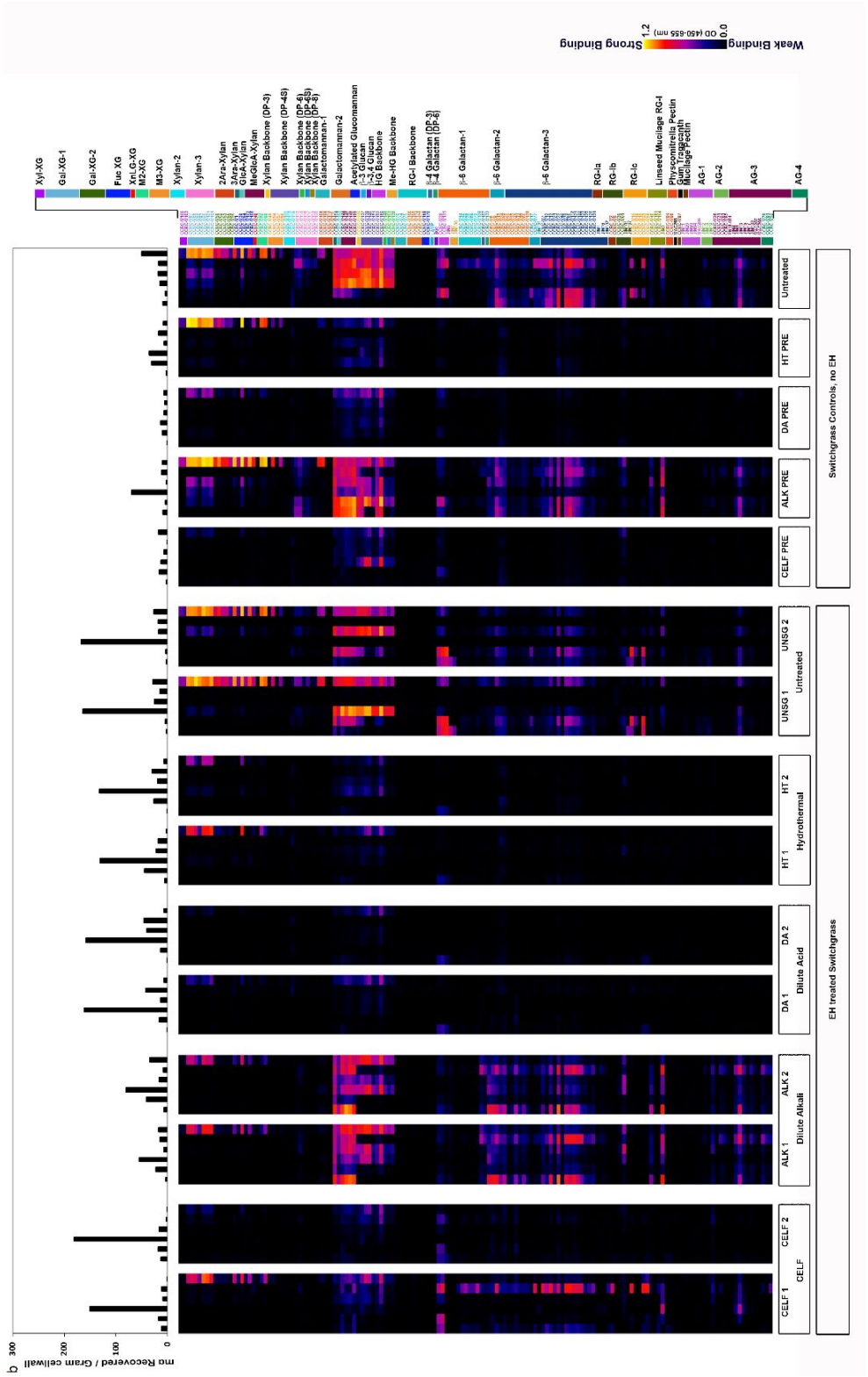

Fig. 12: Glycome profiling of unpretreated and hydrothermal (HT), dilute acid (DA), dilute alkali (Alk), and co-solvent enhanced lignocellulosic fractionation (CELF) pretreated switchgrass (SG) as controls and their corresponding **(a)** *C. thermocellum* consolidated

bioprocessing (CBP) and **(b)** fungal enzymatic hydrolysis (EH) residues. Samples were created by sequential extractions (oxalate, carbonate, 1M KOH, 4M KOH, Chlorite, 4M KOH post chlorite) and epitopes appearing in later extracts (to the right in a column) were more tightly bound to the cell wall than the ones that appear earlier (to the left in a column). The strength of monoclonal antibody binding is represented by light to dark colors as shown in the legend on the right. The legend on the right also shows the glycan epitopes. The bars at the top represent the amount of material recovered in each extraction step. Xyl-XG = xylosylated xyloglucan; Gal-XG = galactosylated XG; Fuc XG = fucosylated xyloglucan, HG = homogalacturonan, RG = rhamnogalacturonan, AG = arabinogalactan. The DP-4S and DP-6S xylan-backbone-directed antibodies tolerate side-chain substitutions on the backbone.

Conclusions:

This study is a comprehensive work on understanding the mechanism of lignocellulosic biomass deconstruction using four different thermochemical pretreatment technologies and two different biological digestion approaches. Each of these deconstruction technologies utilize unique chemical or biological catalytic systems that affect the biomass in different ways. Overall, we tried to elucidate the process of thermochemical and biological breakdown of switchgrass, the structural changes that occur in the biomass during digestion, and the impact of the structural changes on the overall digestibility of the substrate. The major conclusions of this study are summarized in Table 1. Specifically, we showed that CELF pretreatment produced the most accessible substrate, measured via Simons' staining, and was also the most digestible substrate by both CBP and EH. CELF and dilute alkali pretreatments that removed more lignin from switchgrass produced solids with higher accessibility and digestibility compared to solids produced from dilute acid and hydrothermal pretreatments that removed more xylan from switchgrass. Glycome profiling showed that removal of xyloglycan from the cell wall may be important to further biological digestion, especially that by fungal enzymes. *C. thermocellum* was overall able to digest all substrates more effectively compared to fungal enzymes solubilizing more glucan and as also corroborated by smaller particle sizes of material observed

in SEM images of CBP residues compared to EH residues. This was also validated by glycome profiling which showed very low amounts of non-cellulosic glycans present in the material after *C. thermocellum* digestion of all unpretreated and pretreated solids compared to the EH residues. Acid based pretreatments affected cellulose properties more than dilute alkali pretreatment, CBP, or EH. A sharp increase in cellulose CrI was observed after all acid based pretreatments due to the deconstruction of amorphous cellulose more than crystalline cellulose in switchgrass. Amongst the pretreated solids, hydrothermal and dilute acid pretreated solids had the highest crystallinity and the lowest accessibility measured via Simons' staining and were therefore the least digested. Even though dilute alkali pretreated solids had much lower CrI than CELF pretreated solids, the higher amount of xylan in the former led to lower digestibility. Acid based pretreatments caused a decrease in cellulose DP, which was further reduced after CBP and EH. In contrast, dilute alkali pretreatments did not reduce cellulose DP and there was negligible change in DP of cellulose in dilute alkali pretreated solids after CBP and EH. An increase in DP after biological digestion of unpretreated switchgrass was observed and has been shown before.

Both thermochemical pretreatments and biological digestion led to an increase in S/G ratio of lignin from unpretreated switchgrass that can be attributed to greater removal of G lignin than S lignin during biomass deconstruction. This shift provides evidence to a certain degree to support the hypothesis that G lignin potentially leads to the formation of more cross linked lignin with lower molecular weight and thinner cell walls. Lignin with more G and less S monolignol units in grasses is speculated to be less lignified making the biomass overall more susceptible to digestion. Further, G lignin removal was higher with the use of alkali as a catalyst or higher pretreatment temperatures. Lignin reduction was observed during both CBP and EH which was

723 assumed to be carbohydrate associated. G lignin removal from unpretreated switchgrass that had
724 low S/G ratio, whereas, S lignin removal from dilute alkali and hydrothermal pretreated solids
725 that had high S/G ratio was observed. H lignin proportion in substrates with overall higher lignin
726 content was shown to impact digestibility of the substrates. All thermochemical and biological
727 digestion techniques used in this work led to a decrease in β-O-4 lignin interunit linkage.
728 Hydrothermal and dilute acid pretreatments reduced the β-O-4 bonds more than other digestion
729 techniques. However, since both hydrothermal and dilute acid pretreatments do not remove a lot
730 of lignin and simultaneously reduced the β-O-4 linkage substantially, the lignin is thought to
731 have condensed and redeposited. All thermochemical and biological digestion techniques
732 reduced hydroxycinnamates content from unpretreated switchgrass substantially, *C.*
733 *thermocellum* being the least effective. However, overall, hydroxycinnamates content does not
734 seem to impact biological digestion substantially.

Pretreatment → **Analytical method↓**	**Sample Type**	**Hydrothermal**	**Dilute Acid**	**Dilute Alkali**	**CELF**
Compositional analysis	**P**	85% xylan removal 19% K-lignin removal	94% Xylan removal 4% K-lignin removal (possible pseudo lignin formation)	32% xylan removal 75% K-lignin removal	87% xylan removal 67% K-lignin removal
	B	• Higher physical removal of xylan and lignin led to higher biological digestion • Lignin removal has a higher positive impact on EH and CBP than xylan removal • Negative impact of xylan more on EH than CBP since *C. thermocellum* in CBP has xylanases			
SEM	**P**	Striations / surface removal of matter	Striations / surface removal of matter	Crumpled	Striations / deeper removal of matter
	B	• In general, CBP residues appeared more digested than EH residues • Both EH and CBP residues of CELF pretreated appeared most digested			
Cellulose Accessibility (measured via Simons' staining)	**P**	5% higher than unpretreated SG	10% higher than unpretreated SG	14% higher than unpretreated SG	15% higher than unpretreated SG
		• Higher removal of lignin and xylan led to higher cellulose accessibility • Lignin removal had a more positive impact on cellulose accessibility than xylan removal			
	B	• Cellulose accessibility generally correlated positively with cellulose digestion by EH and CBP			
Cellulose crystallinity	**P**	20% higher than unpretreated SG	15% higher than unpretreated SG	2% higher than unpretreated SG	11% higher than unpretreated SG

		• Greater cellulose crystallinity of solids from acid based pretreatments (HT, DA, and CELF) was possibly due to higher amorphous cellulose removal • Dilute alkali pretreatment that focused mainly on delignification did not impact cellulose crystallinity			
	B	• Greater cellulose crystallinity may be correlated to lower biological digestion • Cellulose physical accessibility, influenced by presence of lignin and xylan, has more impact on cellulose digestion than cellulose crystallinity • Biological digestion by EH and CBP did not significantly impact cellulose crystallinity			
Cellulose degree of polymerization (DP)	**P**	• Acid based pretreatments led to a substantial drop in cellulose DP, presumably because amorphous cellulose was preferentially digested as supported by cellulose crystallinity data			
	B	• The initial drop in cellulose DP by acid based pretreatments was essential for a further decrease in cellulose DP by both EH and CBP • CBP and EH were unable to break down high DP cellulose as seen by an increase in the fraction of high cellulose DP in dilute alkali and unpretreated SG CBP and EH residues • No relationship was observed between cellulose DP and extent of cellulose digestion by EH or CBP			
Lignin S/G ratio	**P**	0.67 (86% higher than unpretreated SG)	0.52 (44% higher than unpretreated SG)	0.64 (78% higher than unpretreated SG)	0.49 (36% higher than unpretreated SG)
		• All pretreatments removed more G than S lignin presumably because G lignin is more crosslinked and therefore, weaker as well as due to generally high G lignin availability in SG			
	B	• No correlation of S/G ratio to biological digestion of pretreated substrates was observed • However, CBP and EH both removed G lignin, possibly carbohydrate associated, from unpretreated SG, which still had higher G lignin compared to pretreated substrates			
H Lignin	**P**	75% higher than unpretreated SG	325% higher than unpretreated SG	50% lower than unpretreated SG	No change compared to unpretreated SG

		• Dilute alkali and CELF pretreatments that remove significant amounts of lignin also remove H lignin • Dilute acid and hydrothermal pretreatments, which don't remove much K-lignin to begin with, removed less H lignin relative to other lignin types			
	B	• H lignin amount was positively correlated to biological digestion by CBP and EH for substrates with high overall K-lignin content			
Lignin interunit linkages	**P**	73% lower β-O-4 relative to unpretreated SG	73% lower β-O-4 relative to unpretreated SG	25% lower β-O-4 relative to unpretreated SG	42% lower β-O-4 relative to unpretreated SG
		• Dilute acid and hydrothermal pretreatments caused a significant change in lignin structure with a sharp decrease in β-O-4 relative abundance that is expected to negatively impact downstream lignin usage • Even though less lignin was left behind in dilute alkali and CELF pretreated solids, the solids had a similar relative abundance of interunit linkages to native lignin from SG			
	B	• The relative abundance of lignin interunit linkages did not have a significant impact on the extent of biological glucan digestion or vice versa			
Hydroxycinnamates: ferulate (FA) and p-coumarate (*p*CA)	**P**	50 % and 66% reduction in FA and *p*CA respectively	55 % and 61% reduction in FA and *p*CA respectively	60 % and 79% reduction in FA and *p*CA respectively	60 % and 34% reduction in FA and *p*CA respectively
		• While all pretreatments reduced relative abundance of hydroxycinnamates in pretreated SG solids compared to unpretreated SG, dilute alkali pretreatment was the most successful in reducing biomass recalcitrance			
	B	• The relative abundance of hydroxycinnamates did not have a significant impact on the extent of biological glucan digestion or vice versa			
Glycome Profiling	**P**	• Acid based pretreatments removed significant amounts of non-cellulosic glycans from SG, with CELF being the most successful followed by dilute acid and hydrothermal pretreatments • Dilute alkali was unable to remove non-cellulosic glycans as also evidenced by compositional analysis			
	B	• Removal of xyloglucans via pretreatment may be essential for successful downstream fungal EH, unlike CBP • *C. thermocellum* CBP broke down non-cellulosic components of the cell wall more effectively than fungal enzymes, thereby making cellulose more macro-accessible			

735 **Table 1: Summary of major conclusions of this study. Sample type P = pretreated solids, sample type B = biologically digested**
736 **residues, SG = switchgrass, EH = fungal enzymatic hydrolysis, CBP = *C. thermocellum* consolidated bioprocessing, CELF = co-**
737 **solvent enhanced lignocellulosic fractionation.**
738

Materials and methods:

Lignocellulosic Biomass

Chopped Alamo switchgrass (~3/4 inch) obtained from Genera Energy Inc. was harvested in January 2014 and was five year old fully mature biomass. This biomass was completely mixed and sorted into multiple gallon sized bags and stored in a freezer. Thomas Wiley® mill (Model 4, Thomas Scientific, Swedesboro NJ) (knife mill) was used to mill the entire contents of each bag and passed through a 1 mm sieve. The milled biomass was mixed thoroughly before each use. The composition of the biomass was determined to be 38.18 (±0.8) % glucan, 26.96 (±0.4) % xylan, 2.97 (±0.05) % arabinan, and 20.8 (±0.2) % Klason-lignin (K-Lignin, acid insoluble lignin).

Thermochemical pretreatments

Pretreatment conditions previously determined best for maximum sugar release (glucan + xylan) from pretreatment and *C. thermocellum* CBP combined were used in this study and are listed as follows: hydrothermal pretreatment at 200°C for 10 minutes, dilute acid pretreatment at 160°C for 25 minutes, dilute alkali pretreatment at 120°C for 60 minutes, and Co-solvent enhanced lignocellulosic fractionation (CELF) pretreatment at 140°C for 20 minutes. Pretreatments were performed as described previously[30]. Briefly, all pretreatments were performed at a 10 wt% solids loading with a total reaction mass of 800 g in a 1 L Hastelloy Parr reactor (236HC series, Parr Instruments Co., Maoline, IL). A 0.5 wt% sulfuric acid solution was used in dilute acid and CELF pretreatments. While dilute acid pretreatment was performed in an aqueous solution, CELF pretreatment utilized tetrahydrofuran (THF) as co-solvent with water at a 1:1 volume ratio. Dilute alkali pretreatment was done with a 1 wt% sodium hydroxide solution. A 10 wt%

762 solids loading was fed to all pretreatments based on a total of 800 g reaction mass. The Parr
763 reactor was equipped with a double stacked pitch blade impeller that was set at 200 rpm. A 4 kW
764 fluidized sand bath (Model SBL-2D, Techne, Princeton, NJ) was used to maintain the
765 pretreatment temperature within ± 2°C which was measured using a K-type thermocouple probe
766 (CAIN-18G-18, Omega Engineering Co., Stamford, CT, USA). The temperature ramp up to
767 target temperature for all pretreatments was around one minute. At the completion of the target
768 pretreatment time, the reactor was lowered into a room temperature water bath to cool its
769 contents which were then vacuum filtered at room temperature using a glass fiber filter paper.
770 The temperature ramp down to below 80°C took about two minutes in all cases. The solids were
771 thoroughly rinsed with room temperature deionized water to remove any soluble sugars,
772 degradation products, acid/alkali, and solvents.
773
774 ***Clostridium thermocellum* consolidated bioprocessing**
775 *Clostridium thermocellum* DSM 1313 was provided by Prof. Lee Lynd at Dartmouth College,
776 Hanover NH. Stock culture was prepared and growth curve using pellet nitrogen content was
777 determined previously[30]. Seed cultures were grown on 5 g/L glucan loading of Avicel® PH101
778 (Sigma Aldrich, St. Louis, MO) in 50 mL volume for 8-9 hours in Media for Thermophilic
779 Clostridia (MTC) without trace minerals (Table 1) with a 2% by volume inoculum. The media
780 composition was described previously[30]. The vitamins solution was sterilized by passing it
781 through 28 mm diameter polyethersulfone (PES) syringe filters with 0.2 μm pores (Corning®
782 Life Sciences, Tewksbury MA), whereas, the other media solution were autoclaved.
783 Fermentations were performed in 125 mL bottles (Wheaton, Millville NJ) with a 0.5 wt% glucan
784 loading of substrates and a working mass of 50 g. Bottles containing biomass and water were

purged with nitrogen to maintain anaerobic conditions and then autoclaved for sterilization. A repeated 45 seconds application of vacuum and 14 psi nitrogen over a total of 27-30 min was used to purge the bottles. Fermentations were performed at 60°C at a shaking speed of 180 rpm in a Multitron Orbital Shaker (Infors HT, Laurel MD) with a 2% by volume inoculum. Insoluble solids left after CBP were recovered and rinsed thoroughly. Compositional analysis was performed on the residues to determine glucan solubilization. Data averages and standard deviations reported are for three biological replicates. Residues recovered from six to twelve flask runs, depending on sample type to ensure enough material availability, were provided to UTK/ORNL and UGA laboratory groups for further characterization.

Enzymatic hydrolysis

Accellerase® 1500 cellulase (DuPont Industrial Biosciences, Palo Alto CA) was used at 15 and 65 mg protein / g glucan loadings for enzymatic hydrolysis. These loadings were based on the amount of glucan in unpretreated switchgrass to not penalize a pretreatment for releasing more sugars before enzymatic hydrolysis as described elsewhere [107, 108]. The BCA protein content of Accellerase® 1500 was 82 mg/mL as reported elsewhere [109]. Hydrolysis was performed following the NREL Laboratory Analytical Procedure "Enzymatic Saccharification of Lignocellulosic Biomass" [110]. Briefly, a 0.5 wt% glucan loading and a working mass of 50 g in 125 mL Erlenmeyer flasks, which were incubated at 50°C and 150 rpm in a Multitron Orbital Shaker (Infors HT, Laurel MD). Data averages and standard deviations reported are for three biological replicates. Flasks were allowed to equilibrate before adding required enzyme solution. Representative samples were collected from each flask after 4 hours, 24 hours, and every 24 hour period thereafter to determine glucan yield. The samples were centrifuged and the supernatant

808 was analyzed by HPLC. Insoluble residues from six to twelve flask runs, depending on sample
809 type, to ensure enough material availability, were recovered, washed, and provided to Prof.
810 Arthur Ragauskas' and Prof. Michael Hahn's laboratory groups for further characterization.
811
812 **Compositional analysis of solids**
813 NREL Laboratory Analytical Procedure "Determination of Structural Carbohydrates and Lignin
814 in Lignocellulosic Biomass" [111] was followed to determine the composition of unpretreated, and
815 pretreated switchgrass and their CBP and enzymatic hydrolysis residues. Solids were dried to
816 moisture content < 10% in either a 40^{0}C and 60^{0}C oven prior to analysis. The amounts of
817 ingredients required for analysis were modified proportionately if the amount of material being
818 analyzed was insufficient to meet the NREL specified amount. Percent composition of glucan,
819 xylan, arabinan, and K-lignin were determined for each material
820
821 **Sugar analysis**
822 A Waters Alliance e2695 HPLC system (Waters Co., Milford MA) was used for analysis of all
823 liquid samples. Bio-Rad Aminex HPX-87H column and a Waters 2414 refractive index detector
824 were used. Mobile phase was a 5 mM sulfuric acid solution eluted at 0.6 mL/min. Integration of
825 the chromatograms was by the Empower™ 2 software package.
826
827 **2D Heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR)**
828 For the lignin characterization, whole cell wall NMR analysis was conducted as described
829 previously [112]. Briefly, the samples were ball-milled using Retsch PM 100 at 600 rpm for 2 hours.
830 About 50 mg of the ball-milled sample was loaded in a 5 mm NMR tube with 0.4 mL of DMSO-

d_6/HMPA-d_{18} (4:1, *v/v*) and sonicated for 2 hours. Two-dimensional ^{1}H-^{13}C HSQC NMR experiment was conducted at 300 K using a Bruker Avance-III 500 MHz spectrometer with a 5 mm cryogenically cooled probe and a Bruker pulse sequence (hsqcetgpspsi2.2). The spectra were measured with spectral width of 12 ppm in F2 (^{1}H) dimension with 1024 time of domain and 166 ppm in F1 (^{13}C) dimension with 256 time of domain, a 1.0-s delay, a $^{1}J_{\mathrm{C-H}}$ of 145 Hz, and 128 scans. Relative abundance of lignin subunits, hydroxycinnamates, and interunit linkages were estimated by volume integration of contours in HSQC spectra.

Solid-state NMR

All the residues were filtrated through 417 Filter paper (VWR Inc.) and the residue detained were freeze-dried. One portion of the dried residue was used to isolate cellulose. The cellulose isolation and cellulose crystallinity measurement was conducted according to literature [113, 114]. In detail, the isolated cellulose samples were stored in a sealed container to prevent moisture loss. The NMR samples were prepared by packing the moisturized cellulose into 4-mm cylindrical Zirconia MAS rotors. Cross polarization magic angle spinning (CP/MAS) NMR analysis of cellulose was carried out on a Bruker Advance-400 spectrometer operating at frequencies of 100.59 MHz for ^{13}C in a Bruker double-resonance MAS probe head at spinning speeds of 10 kHz. CP/MAS experiments utilized a 5 μs (90°) proton pulse, 1.5 ms contact pulse, 4 s recycle delay, and 4000 scans. The cellulose crystallinity index (CrI) was determined from the areas of the crystalline and amorphous C_4 signals using the following formula:

$$\mathrm{CrI} = \frac{A^{86\ -\ 92\ \mathrm{ppm}}}{A^{86\ -\ 92\ \mathrm{ppm}} + A^{79\ -\ 86\ \mathrm{ppm}}}$$

Gel permeation chromatography (GPC)

The weight-average molecular weight (M_w) and number-average molecular weight (M_n) of cellulose were measured by GPC after tricarbanilation. Briefly, the isolated cellulose in previous solid-state NMR measurement was collected and dried under vacuum at 45°C overnight. The dried cellulose samples were then derivatized with phenyl isocyanate in an anhydrous pyridine system prior to GPC analysis. Size-exclusion separation was performed on an Agilent 1200 HPLC system (Agilent Technologies, Inc, Santa Clara, CA) equipped with Waters Styragel columns (HR1, HR4, and HR6; Waters Corporation, Milford, MA). Number-average degree of polymerization (DP_n) and weight-average degree of polymerization (DP_w) of cellulose were obtained by dividing M_n and M_w, respectively, by 519 g/mol, the molecular weight of the tricarbanilated cellulose repeating unit.

Scanning Electron Microscopy (SEM)

Samples for SEM were placed on carbon tape on aluminum stubs and sputter-coated with gold. Zeiss Auriga FIB-SEM at an accelerating voltage of 10 kV with back scatter detector at 100 to 5000 times magnification was used to take SEM images. Raw images were adjusted for brightness and contrast in ImageJ software [115]. Images were merged using Adobe Photoshop CC v. 2017.

Simons' staining

Simons' staining was performed as described previously using the high molecular weight fraction (≥30,000 kDa) of Direct Orange 15 dye (CAS: 1325-35-5) [55, 70]. ANOVA was via OriginPro 2018 software

Glycome profiling

Plant cell wall glycan directed mAbs against epitopes on most major non-cellulosic plant cell wall glycans were procured as hybridoma cell culture supernatants from stocks at the Complex Carbohydrate Research Center (CCRC). Antibodies used in this study are available from CarboSource (http://www.carbosource.net). LAMP and BG-1 antibodies are available from Bio-supplies (Parkville, Victoria, Australia; http://www.biosupplies.com.au). Glycome profiling analyses of unpretreated and hydrothermal (HT), dilute acid (DA), dilute alkali (Alk), and co-solvent enhanced lignocellulosic fractionation (CELF) pretreated switchgrass (SG) as controls and their corresponding *C. thermocellum* consolidated bioprocessing (CBP) and fungal enzymatic hydrolysis (EH) residues were performed as described previously[60]. First, Alcohol Insoluble Residue (AIR) cell wall materials were prepared from various biomass residues and were subjected to sequential extractions with ammonium oxalate (50 mM), sodium carbonate (50 mM), KOH (1 and 4 M) and acidic chlorite. Sodium borohydride (0.5% W/V for carbonate and 1% W/V for all KOH reagents) was added to all alkaline extracting reagents to ensure structural integrity of extracted glycans. The extracts were extensively dialyzed (48 hours against deionized water with 4 changes) and dialyzed extracts were freeze-dried and subsequently were screened with ELISAs against a suite of cell wall glycan-directed mAbs on an equal carbohydrate basis and the results reported as heat maps (Pattathil et al., 2012). The actual amounts of cell wall carbohydrate materials recovered during each extraction steps are depicted as bar graphs above the respective heat map panels.

Conflicts of Interest:

CEW is founding Editor in Chief of the Journal *Biotechnology for Biofuels*. The other authors declare that they have no competing interests

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Authors' Contributions:

NK, RK, and CEW designed the study. NK carried out *C. thermocellum* fermentation and fungal enzymatic hydrolysis experiments and prepared samples for further characterization. SB performed SEM and Simons' staining. YP, CGY, and ML performed SSNMR, 2D HSQC NMR, and GPC for cellulose and lignin characterization. SV performed glycome profiling. NK, SB, YP, MGH, RK, and CEW analyzed the data. NK wrote the first draft of the manuscript. NK, SB, YP, CGY, ML, SV, SP, RK, CMC, MGH, AJR, and CEW edited and approved the final draft of the manuscript.

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