Cellulose Hydrolysis by Clostridium thermocellum is Agnostic to Substrate Structural Properties in Contrast to Fungal Cellulases

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Title: Cellulose Hydrolysis by *Clostridium thermocellum* is Agnostic to Substrate Structural Properties in Contrast to Fungal Cellulases

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Abstract

The native recalcitrance of lignocellulosic biomass hinders its effective deconstruction for biological conversion to fuel ethanol. However, once cellulose is physically available to enzymes/microbes, i.e., macro-accessible, cellulose micro-accessibility, i.e., the accessibility as influenced by cellulose properties, further affects cellulose conversion. Here, we performed a comparative study of the effect of cellulose micro-accessibility on cellulose conversion by two biological approaches of potential commercial interest: consolidated bioprocessing (CBP) using *Clostridium thermocellum* and cell-free saccharification mediated by fungal enzymes. Commercially available cellulosic substrates, Avicel® PH-101, Sigmacell Cellulose Type 50, cotton linters, Whatman™ 1 milled filter paper, and α-cellulose were employed to constitute different cellulose micro-accessibilities. Physiochemical characterization was performed on these substrates to determine key morphological and chemical differences. Biological conversion of these substrates showed that *C. thermocellum* was unaffected overall by cellulose structural properties, i.e., micro-accessibility, and achieved similar solids solubilization and metabolite production from these structurally different materials. However, fungal enzymes digested these substrates to different extents. Specifically, glucan conversion of these substrates diminished in the following order: milled filter paper > Avicel > Sigmacell and α-cellulose > cotton linters. Here, we propose that *C. thermocellum* digestion of lignocellulosic biomass is primarily controlled by the physical availability of cellulose in the lignocellulosic matrix and largely unaffected by cellulose properties once cellulose is made macro-accessible. In contrast, fungal enzymes require cellulose to be physically accessible, i.e., macro-accessible, as well as have properties amenable to digestion, i.e., micro-accessible.

Introduction

Lignocellulosic biomass is structurally complex with many of the cellulose chains held together by inter chain hydrogen bonds to form crystalline elementary microfibrils, which are in turn hydrogen bonded to a variety of hemicelluloses and pectins. Lignins are covalently bonded to hemicellulose through ferulic acid (and other) ester linkages [1-3]. This complex lignocellulosic architecture contributes to biomass recalcitrance to sugar release, the primary barrier to competitive conversion of this low cost resource to transportation fuels [4]. Furthermore, recalcitrance changes with plant type, which in turn complicates biomass use [5]. Biomass modification is essential through either physical/chemical pretreatment or cotreatment of biomass to achieve high solubilization of polysaccharides by biological systems [6-11]. For ethanol production via a biological route, enzymatic hydrolysis of cellulose is a heterogeneous reaction in which enzymes derived from *Trichoderma reesei* are typically used in solution to breakdown insoluble cellulosic substrates [12-15]. However, the high cost and dosages of enzymes required to achieve industrially relevant sugar yields make them economically challenging [16]. Consolidated bioprocessing, in contrast, is a simple and effective bioprocess that combines enzyme production, enzymatic hydrolysis, and fermentation by organisms such as *C. thermocellum* in one step [4, 11, 17-20]. 24 cellulases have been identified out of the more than 70 known enzyme components on the protein scaffoldin CipA of *C. thermocellum*'s cellulosome, which can form cell-enzyme-substrate complexes [19, 21, 22] as opposed to cell free individual enzyme cocktails produced by *T. reesei* that typically have only one catalytic unit per protein [23]. This complex, multi-enzyme, multi-functional cellulosome produced by *C. thermocellum* enhances biomass solubilization compared to fungal enzymes [24-26]. This augmentation could be attributed to the difference in the mechanism of cellulose hydrolysis by the two biological systems: free fungal

cellulases hydrolyze cellulose through ablation, whereas, *C. thermocellum* cellulosomes separate individual cellulose microfibrils, which enhances hydrolysis [23].

We view the accessibility of cellulose to solubilization mediated by biological catalysts as being comprised of two types based on length scale: macro- and micro-accessibility [27]. Macro-accessibility refers to the availability of cellulose influenced by the presence of lignin, hemicellulose, and other physical barriers in lignocellulosic biomass. Pretreatment of biomass increases the physical access of cellulolytic enzymes/microbes to cellulose by disrupting the complex plant cell wall structure [11]. However, once the enzymes/microbes have gained physical access to cellulose, cellulose structural properties, such as crystallinity and degree of polymerization, control its conversion [13, 27-30]. These properties of cellulose influence the availability of cellulase binding sites on cellulose, thus affecting the micro-accessibility to enzymes/microbes [27]. Even though a number of studies have shown that solubilization by fungal enzymes is affected by cellulose micro-accessibility, the influence of a variety of cellulose properties on fungal enzymatic digestion has not been systematically studied. Furthermore, the effect of cellulose micro-accessibility on substrate solubilization and metabolite production by *C. thermocellum* has not been previously reported.

Cellulose biosynthesis is expected to influence the properties of cellulose that in turn affect cellulose micro-accessibility and therefore, digestibility of the substrate. Cellulose is a polymer of glucose linked via β-(1,4) glycosidic bonds that form at or outside the plasma membrane of plant cells[31]. Cellulose elementary fibrils containing multiple cellulose chains are aligned to form microfibrils, which are eventually decorated with hemicelluloses. Cellulose

microfibrils are also packed together in the cell wall to form larger structures, the cellulose macrofibrils. The cellulose synthase complex in higher plants combines elementary fibrils to form the microfibril, which is thought to comprise between 24 and 36 cellulose chains [32-34]. These cellulose chains are so tightly packed together that even water molecules would be unable to penetrate, resulting in ordered structures that are highly recalcitrant to hydrolysis [12]. Thus, accessible surface area, specific surface area, and pore size of the substrate are expected to affect its hydrolysis [5, 13, 35]. Amorphogenesis, characterized by dispersion or swelling of cellulose to reduce compactness of the cellulose structure and/or cellulose crystallinity, has been proposed to occur in the initial stages of hydrolysis mediated by cell-free enzymes [12] and further increase the available surface area for enzyme adsorption by increasing cellulose micro-accessibility.

Early in evolutionary history, cellulose biosynthesis centered only on polymerization leading to the formation of the more stable cellulose II allomorph, which has a lower degree of polymerization (DP) than what is known today as native cellulose or cellulose I [36]. However, cell elongation and growth would be limited for the lower DP of cellulose II. Therefore, the evolutionary selection process led to the advent of chain ordering and ultimately the formation of microfibrils comprised of high DP cellulose I. These characteristics confer increased functionality of the cell wall and overall enhancement of plant growth [13, 27, 28, 36]. Cellulose DP is known to affect digestion by fungal enzymes, in that lower DP enables greater hydrolysis [37]. Cellulose II and III allomorphs are more susceptible to digestion by fungal enzymes than is cellulose I [29, 38]. Each cellulose microfibril consists of ordered (crystalline) and disordered (amorphous) regions that are thought to coexist in a cross section rather than alternating along the axis of the microfibril [27, 32]. Crystallization during cellulose biosynthesis is thought to occur

when the cellulose elementary fibrils are arranged into a microfibril by proteins in the cellulose synthase complex and is therefore related to hydrogen bond formation [31]. Cellulose hydrolysis by fungal enzymes has been shown to be negatively affected by substrates with high cellulose crystallinity [15, 39, 40]. Furthermore, because moisture uptake by cellulose is expected to increase with a decrease in crystallinity [27], both cellulose water retention value (WRV) and crystallinity are useful indicators of cellulose micro-accessibility [5, 15, 27]. Overall, however, the importance of cellulose crystallinity and DP in achieving high cellulose digestion is still debatable [13, 28, 30].

To better understand how cellulose properties impact biological deconstruction, we report the digestion performance of fungal enzymes compared to *C. thermocellum* on five model cellulosic substrates, Avicel® PH-101 (Avicel), Sigmacell Cellulose Type 50 (Sigmacell), Whatman™ 1 milled filter paper (milled through 40 mesh; henceforth referred to as filter paper), cotton linters, and α-cellulose. These model substrates represent a wide range of commercially available cellulosic substrates [30, 41-47] that were chosen to avoid the negative effect of limited cellulose macro-accessibility on cellulose digestion, which can be observed in lignocellulosic biomass. These substrates, their structural properties, and the impact of their properties on cellulose hydrolysis by fungal enzyme compared to *C. thermocellum* have not been directly considered elsewhere. A sixth substrate was prepared by soaking Avicel in water at 30% solids loading and then drying the solids overnight at 105°C. Understanding differences in deconstruction of cellulose with different characteristics using two distinct biological systems can help us identify critical cellulose properties and catalytic features that influence cellulose digestion.

Results and Discussion:

Substrate characterization

A suite of analytical techniques was applied to determine cellulose crystallinity index (CrI%) and crystallite size, cellulose number average and weight average degree of polymerization (DPn and DPw), and the cellulose surface area of Avicel, Sigmacell, filter paper, cotton linters, and α-cellulose. Scanning electron microscope (SEM) images were also taken to determine structural differences between the cellulosic substrates. Glucan solubilization by both fungal enzymes and *C. thermocellum* was then measured on these substrates to understand the effect of different characteristics of each cellulosic substrate on biological digestion.

The WRV, measured as water retained by a substrate after centrifugation under normal conditions, provides an indication of the amount of water associated with the biomass itself and trapped between biomass particles [48, 49]. WRV could also indicate the surface area of cellulose since water would be expected to form more hydrogen bonds with more accessible hydroxyl groups on cellulose. This ability of the biomass to retain water has been directly correlated to its digestibility by enzymes [49-51]. In this work, cotton linters and filter paper had the highest WRV, as reported in Figure 1, indicating that these materials have higher swell-ability and potentially higher cellulose surface area compared to other materials. Because increased biomass digestibility has been reported with an increase in interaction between biomass and water [49], higher WRV is expected to aid enzyme adsorption and enhance enzymatic digestion. Dried Avicel, which was prepared by rapidly drying a 30 wt% solids suspension of Avicel in 105°C, showed lower WRV compared to that for untreated Avicel. The rapid drying of the material could result in pore collapse and case hardening (hornification), possibly irreversible, that leads

to a lower ability of the material to retain water [52, 53]. However, comparison of digestion performance of Avicel and dried Avicel by fungal enzymes and *C. thermocellum* can indicate the significance of substrate WRV in influencing cellulose digestion [49].

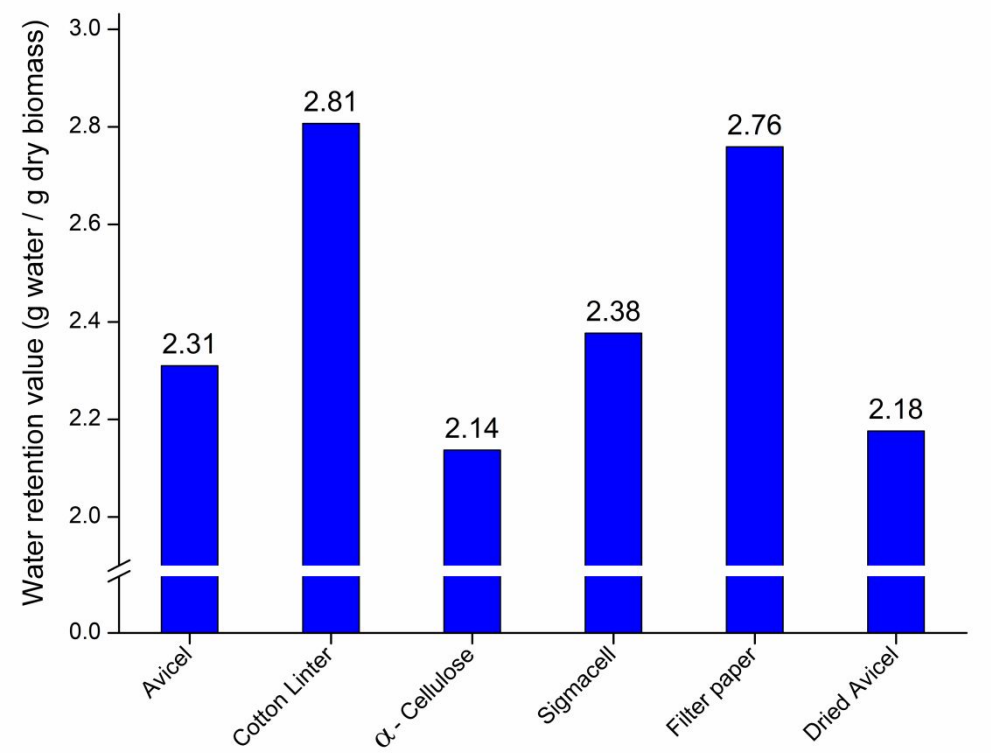


Figure 1. Water retention values (WRV) of untreated Avicel, Sigmacell, cotton linters, filter paper, and α-cellulose and oven dried Avicel.

Modified Simons' staining has been used to estimate substrate specific surface area and indicate cellulose solubilization performance of enzymes acting on different substrates [5, 35, 54]. Dyes used in the Simons' staining method adsorb only to cellulose, as opposed to other polymers in the plant cell wall structure, and have a similar size profile to cellulases [55]. Specifically, the high molecular weight fraction of Direct Orange 15 has a high affinity to cellulose and can access pores of size 5-36 nm [56]. Direct Blue 1, on the other hand, can access smaller pores of about 1 nm, but has lower binding affinity than the orange dye. The ratio of the amount of orange

to blue dye adsorbed by a substrate thus indicates the pore size distribution of the substrate, with higher values indicating larger pores [5, 56]. Pore size distribution has been shown to impact cellulose hydrolysis, with smaller pore sizes reducing the extent of hydrolysis [57, 58]. For reference, fungal cellulases are reported to have an average diameter of 59 Å (5.9 nm) if cellulases are assumed to be spherical[57, 58], whereas, *C. thermocellum* cellulosomes are much larger, about 200 kDa (50-200 nm) as reported in various literature [22, 59-61]. As pointed out earlier, it is important to keep in mind that these two enzymatic systems have different mechanisms of action on cellulose hydrolysis [23] and would therefore be expected to be impacted by pore size distribution differently. Here, measurement of total orange plus blue dye adsorption and orange to blue dye adsorption (O/B) ratio by a modified Simons' staining method was employed to estimate cellulose accessibility and pore size distribution, respectively, for all materials. In particular, the maximum dye adsorption was measured by loading cellulosic substrates with a range of dye concentrations to obtain an adsorption curve for each substrate. As shown in Figure 2, the maximum orange plus blue dye adsorption for the model substrates was found to increase as follows: filter paper > α-cellulose > Avicel > cotton linters > Sigmacell. Even though filter paper had a low O/B ratio, the very high WRV and total dye adsorption for this substrate was indicative of its high cellulose surface area compared to the other materials. Thus, filter paper's higher cellulose accessibility is expected to result in higher enzyme adsorption and, therefore, higher digestibility compared to the other materials. Although α-cellulose also had high total dye adsorption, its lowest O/B ratio may negatively affect biological digestion of this substrate. Interestingly, Avicel had the highest O/B ratio of 1.26 (as compared to 0.9 to 0.95 for the other materials), as can be seen in Figure 2(b), and this result indicates the presence of larger pores in the substrate and therefore, greater cellulose accessibility. Because both cotton linters and

Sigmacell had low total dye adsorption and low O/B ratios, they are expected to have low digestibility.

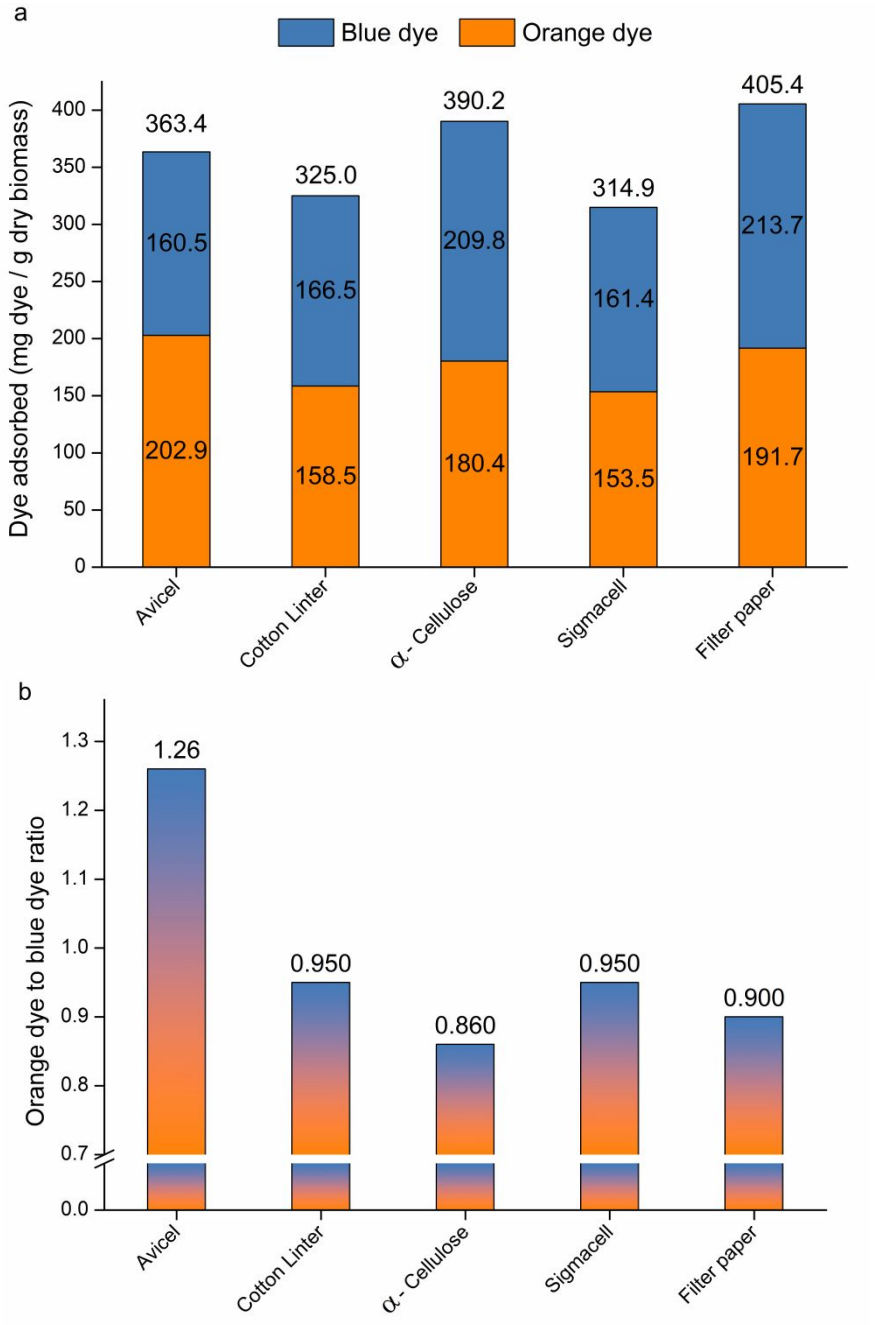


Figure 2. Cellulose accessibility of Avicel, Sigmacell, cotton linter, filter paper, and α-cellulose based on maximum dye adsorption as determined by modified Simons' staining adsorption isotherms: **(a)** total orange dye plus blue dye adsorption and **(b)** orange dye to blue dye adsorption ratio.

Solid state nuclear magnetic resonance (SSNMR) and X-ray diffraction (XRD) techniques were employed to measure the cellulose crystallinity index (CrI%) of Avicel, Sigmacell, filter paper, cotton linters, and α-cellulose reported in Table 1. Both techniques showed the same trend for CrI% of all materials. The relatively high CrI% of cotton linter is expected to impede biological digestion the most. α-cellulose, in contrast, had the lowest CrI% and therefore, would be expected to be highly digestible by both fungal enzymes and *C. thermocellum*. Similar CrI% values have been reported for some of these substrates elsewhere [43].

Material	Crystallinity Index		Crystallite Size (nm)	Molecular Weight		
	CrI (%) SSNMR	CrI (%) XRD		DPw	DPn	PDI
Avicel	59.4	71.3	34.3	309	71	4.35
cotton linters	70.9	76.0	45.1	1578	111	14.26
α-cellulose	45.8	54.0	20.5	4389	393	11.17
Sigmacell	57.8	71.4	33.4	321	68	4.74
filter paper	62.4	68.8	40.5	4266	1008	4.23
dried Avicel	ND	70.7	32.3	ND	ND	ND

Table 1: Cellulose crystallinity measured by solid state nuclear magnetic resonance (SSNMR) and X-ray diffraction (XRD) peak height techniques, crystallite size by XRD, and weight average (DPw) and number average (DPn) degree of polymerization and polydispersity index (PDI) measured by gel permeation chromatography for model cellulosic substrates. Red color indicates the most negative impact expected and green color indicates the most positive impact of the property under consideration on biological digestion of cellulose. ND = Not Determined.

The effect of substrates with different crystallinities on digestion performance has not been studied extensively for *C. thermocellum*, whereas a direct relation between cellulose crystallinity and the rate of cellulose hydrolysis by fungal enzymes has been shown [62, 63]. The latter studies have shown that cellulases preferentially attack amorphous over crystalline regions of cellulose and thereby increase crystallinity in the initial stages of hydrolysis [39]. Furthermore, *T. reseei* Cel7A cellobiohydrolase has been shown to be negatively affected by cellulose crystallinity[40]. In addition, cellulose crystallinity has been shown to impede the effectiveness of

enzymes adsorbed onto the surface of cellulose [15]. However, reports of the effect of cellulose crystallinity on enzymatic digestion have been inconsistent, with some literature showing an insignificant effect of cellulose crystallinity on cellulose digestion [14, 28]. Furthermore, the size of the cellulose crystallite structure for each material can influence its overall crystallinity and surface area. A larger crystal would be expected to reduce the surface to volume ratio and lower water and enzyme adsorption per mass of cellulose [64]. In this study; however, even though both cotton linters and filter paper had higher crystallite sizes, their WRV was greater than that found for the other materials. In addition, the highest crystallite size and crystallinity index values for cotton linters and lowest values for α-cellulose were consistent with prior reports that crystallinity index increased with crystallite size of cellulose for different wood species [65, 66].

As reported in Table 1, cellulose in filter paper and α-cellulose showed high weight average degree of polymerization (DPw). However, because the cellulose in α-cellulose had a much lower number average degree of polymerization (DPn), its polydispersity index (PDI = DPw/DPn) was higher, indicating a wider molecular weight distribution [14, 67]. Cotton linters, comparatively, had a lower cellulose DPn and DPw, which was, however, much higher than those values found for Avicel and Sigmacell. Cotton linters had the highest PDI and therefore showed the greatest molecular weight distribution. Cellulose DP has long been considered an important characteristic that could have a significant impact on cellulose digestion [27]. Higher DP would mean few free chain ends and longer cellulose chains packed together with strong hydrogen bonds between them resulting in lower cellulose accessibility and digestibility [28]. High DP has been reported to have a negative impact on fungal enzymatic digestion of cellulose [68, 69]. However, a levelling off in cellulose DP after a slight drop has been observed despite an

insignificant overall change in DP before and after hydrolysis [13]. There is yet to be a consensus on the extent to which DP impacts cellulose digestion [27].

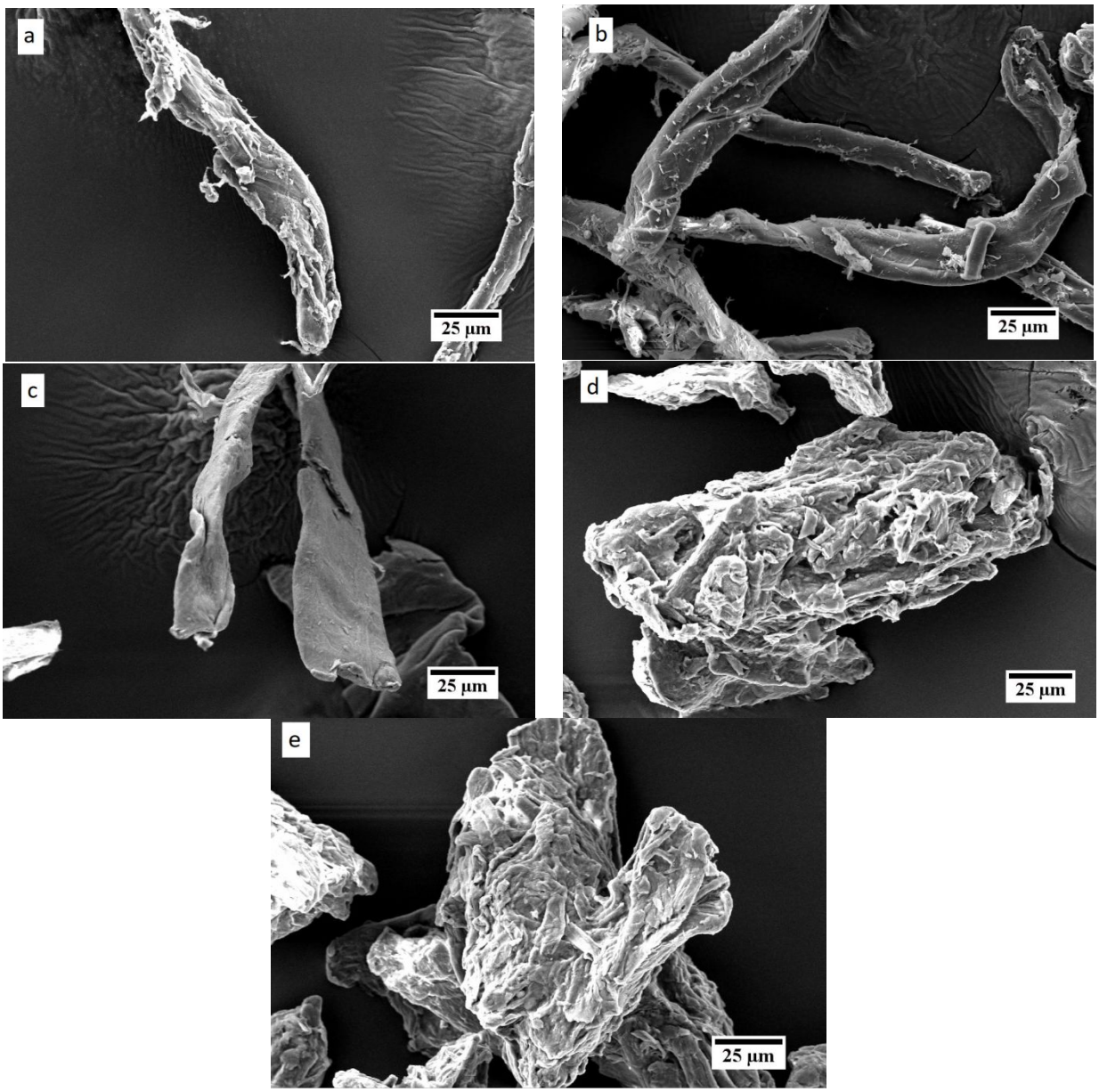


Figure 3. Scanning electron microscope (SEM) images of (a) filter paper, (b) cotton linters, (c) α-cellulose, (d) Avicel, and (e) Sigmacell at a 1.5K times magnification.

The various cellulose substrates were also imaged by SEM, as shown in Figure 3, to reveal possible structural differences between the substrates. Cotton linter and filter paper were elongated and fibrous on the surface and looked structurally similar, as shown in Figure 3. In contrast, even though elongated, α-cellulose was not fibrous on the surface. Avicel and Sigmacell, on the other hand, were not elongated but appeared as small clumped particles. Avicel particles showed more pores in comparison to Sigmacell particles. Overall, even though some structural differences were observed between these substrates, conclusions on the impact of these differences on the extent of cellulose hydrolysis, if any, were not apparent.

Fungal enzymatic hydrolysis of substrates with varying cellulose properties

The cellulose rich substrates with significantly different micro-accessibilities and surface characteristics were hydrolyzed at a 0.5 wt% glucan loading with a cellulase loading of 15 mg protein/g glucan. As shown in Figure 4, fungal enzymes realized substantial differences in the extent of digestion of Avicel, Sigmacell, filter paper, cotton linters, and α-cellulose. The greatest glucan yield of about 90% was achieved on filter paper followed by Avicel, Sigmacell and α-cellulose, whereas the lowest glucan yield was about 30% for cotton linters. Thus, the high WRV (2.8 mg/g dry biomass) and total dye adsorption (405.4 mg/g dry biomass) for filter paper led to higher digestibility by fungal enzymes when compared to Avicel, which demonstrated a WRV of 2.3 mg/g dry biomass and total dye adsorption of 363.4 mg/g dry biomass. This result is consistent with the high overall cellulose surface area being essential for effective fungal enzymatic digestion of cellulose. The higher DP for filter paper compared to Avicel did not seem to affect fungal enzymatic digestion negatively suggesting a lower impact of DP on enzymatic hydrolysis than cellulose surface area. Furthermore, although fungal enzymes digested Avicel

more effectively than Sigmacell, the only difference between the two was regarding orange dye adsorption, whereas blue dye adsorption kinetics was the same for both. As a result, Avicel had a much higher O/B ratio (1.26) compared to 0.95 for Sigmacell, suggesting larger pore sizes for Avicel. Thus, a larger pore size appears essential for effective enzymatic digestion of a substrate.

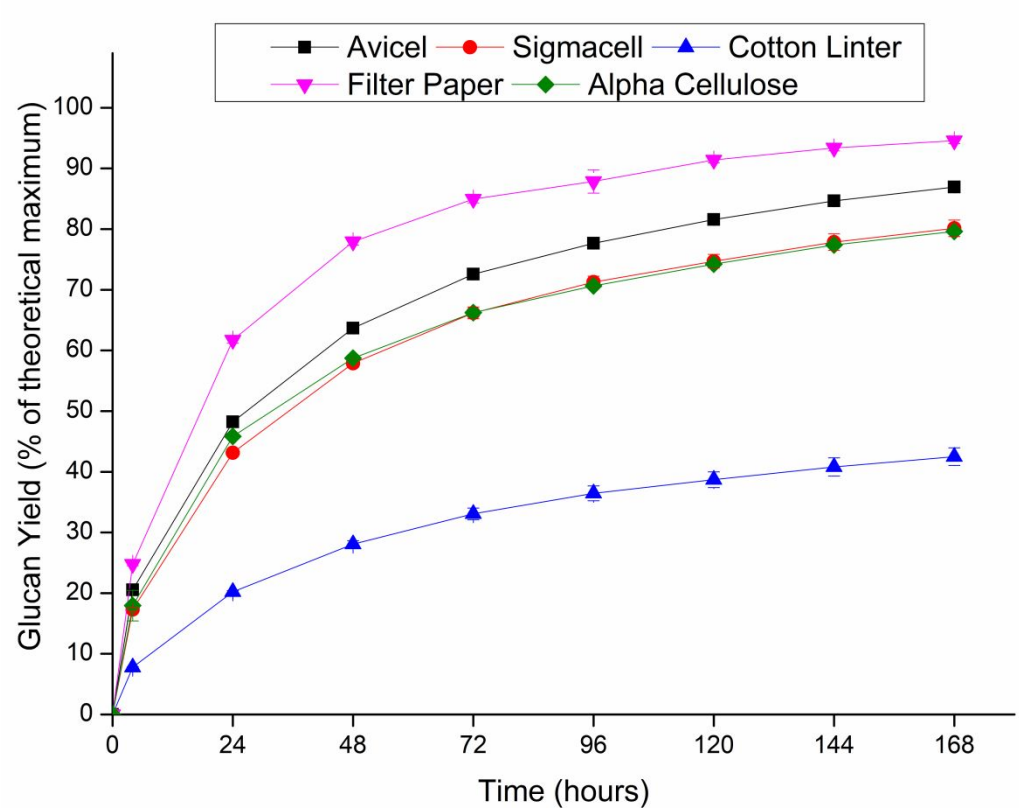


Figure 4. Glucan yields over time for fungal enzymatic hydrolysis of Avicel, Sigmacell, cotton linter, filter paper, and α-cellulose at a 0.5 wt% glucan substrate loading and cellulase loading of 15 mg protein/g glucan. Experiments were performed in triplicate with error bars representing standard deviation.

The high digestibility of α-cellulose by fungal enzymes reported in Figure 4, albeit ~7% lower than that of Avicel, could be attributed to its very low CrI%: 45.8% by SSNMR and 54.0% by XRD Peak Height. Even though α-cellulose had slightly higher total dye adsorption compared to Avicel, the former also showed the lowest O/B ratio (0.86) compared to Avicel (1.26), again

indicating the importance of larger pores in cellulose for effective fungal enzyme digestion. The lowest WRV for α-cellulose was 2.14 mg/g dry biomass, which can be compared to 2.31 mg/g dry biomass for Avicel, which possibly contributed to the lower digestibility of the former. The greater cellulose DP of α-cellulose compared to Avicel may have also negatively impacted its enzymatic digestion. Although Sigmacell had a cellulose DP similar to that for Avicel and higher than that for α-cellulose, Sigmacell achieved the same extent of digestion as did α-cellulose, which was differentiated from the extent of conversion for Avicel. Therefore, cellulose DP could not be credited with substantially influencing enzymatic digestion. Overall, Sigmacell had lower total dye adsorption and higher CrI%, which should have a negative impact on effective cellulose digestion. Sigmacell also had higher WRV, larger pores, and lower cellulose DP which should positively impact cellulose digestion compared to α-cellulose. Therefore, because Sigmacell and α-cellulose showed similar fungal enzymatic digestibility, the parameters identified above are not able to fully explain the digestion performance observed. Cotton linter was least amenable to fungal enzymatic digestion, apparently due to its low cellulose surface area and small pores (Figure 3) and its high crystallinity (Table 1). In fact, cotton linter had the highest CrI% compared to all of the other materials. Even though Sigmacell had a similar surface area and pore size distribution measured via modified Simons' staining compared to cotton linters, the former had a much higher digestibility presumably because of its lower cellulose crystallinity. Despite having a very high WRV that was similar to that of filter paper, the much lower digestibility of cotton linters indicated that low crystallinity is important for effective digestion by fungal enzymes. Typically, the WRV measurement is unable to distinguish between water retained within the biomass and that between biomass particles [49]. WRV of cotton linter was high, but total dye adsorption was low compared to other substrates. The much smaller size of

water molecules compared to the direct dyes indicates that cotton liner possibly has a high number of narrow pores that allow easy penetration of water molecules but a low penetration of the dyes and therefore, cellulases. However, to further test whether a high WRV is important to high cellulose digestion, rapidly dried Avicel with a lower WRV (presumably due to potential pore collapse during rapid drying) was subjected to fungal enzyme hydrolysis. The result was approximately a 7% drop in digestion compared to that for the never dried Avicel, thereby confirming the impact of WRV on cellulose digestibility. However, WRV cannot be used as the sole indicator of substrate digestibility.

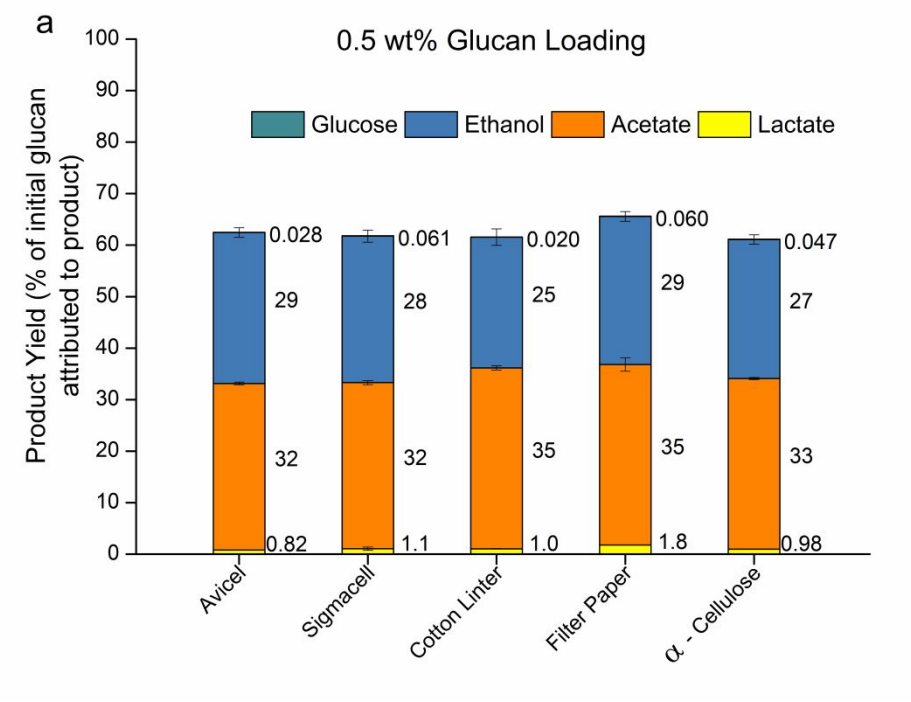
Overall, cellulose surface area and pore size as measured by Simons' staining had the greatest impact on predicting substrate digestibility by fungal enzymes followed by cellulose crystallinity and WRV. DP could not be attributed with substantially influencing fungal enzyme digestion. The minor effect of DP on overall digestion by fungal enzymes complements results reported elsewhere with minimal reduction in molecular weight distribution followed by a leveling off in DP during hydrolysis suggesting that DP may be a limiting factor in cellulose digestion only above a certain molecular weight limit [13]. Furthermore, cellulose digestion appears to be controlled by initial cellulase adsorption onto the substrate, likely followed by a surface peeling-type effect[14]. Cellulose surface area and pore size distribution along with cellulose crystallinity substantially influenced enzyme accessibility and therefore enzyme adsorption. However, once enzymes have adsorbed onto the cellulose surface, they would likely have to attack the outermost layers of cellulose that are readily hydrolyzed and peeled off irrespective of cellulose DP. This mechanism would explain the low importance of DP as opposed to a substantial impact of other cellulose parameters measured in this study on cellulose

digestion. The minor effect of cellulose DP on cellulose digestion after the enzymes adsorb on the substrate is also consistent with the synergistic mechanism of endo- and exoglucanases [14, 30]. Endo- and exoglucanases work together to peel off cellulose fibers completely and synergistically. Thus, effective enzyme adsorption on cellulose could control enzymatic digestion of cellulose. Further, enhancement of cellulose digestion with increasing WRV supports the amorphogenesis mechanism of cellulase action [12, 14]. High WRV of the material should aid in the initial swelling of the substrate to increase its surface area and make amorphous regions more available for effective cellulose digestion.

C. thermocellum CBP of substrates with varying cellulose properties

In addition to the enzymatic hydrolysis results summarized above, a 2% v/v inoculum of *C. thermocellum* was employed to deconstruct all materials in a 50 g working mass with a 0.5 wt% glucan loading of each material. The metabolites and glucose yields reported in Figure 5(a) were measured for *C. thermocellum* CBP as a percentage of initial glucan, with the mass of each product adjusted for the stoichiometry of glucan hydrolysis to glucose and its fermentation to metabolites. Ethanol, acetic acid, and lactic acid were the major metabolites produced by the organism, and about 65% of the initial glucan in the 0.5 wt% glucan loading could be attributed to metabolites production from each substrate with negligible glucose accumulation. As seen in Figure 5(a), the distribution of each metabolite produced by *C. thermocellum* did not vary significantly with substrate type. Furthermore, unlike fungal enzymes, *C. thermocellum* achieved virtually the same solids solubilization and product formation on Avicel and dried Avicel, as reported in Figure 6, with no apparent impact of substrate WRV on *C. thermocellum*

fermentations. Thus, we conclude that the substrate type and cellulose micro-accessibility did not affect cellulose digestion by *C. thermocellum*.



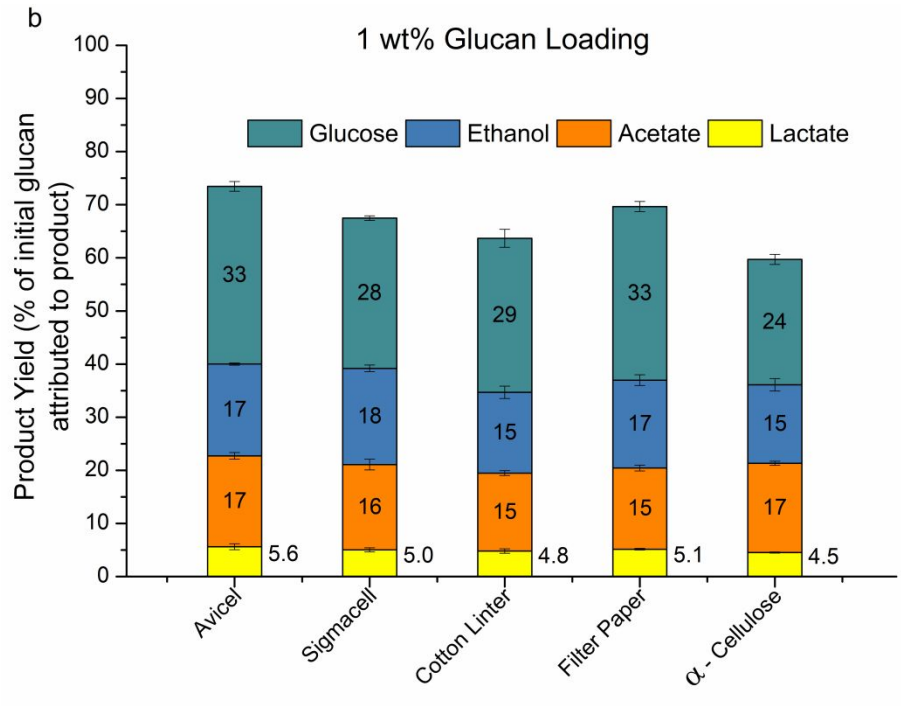


Figure 5. Metabolites and glucose accumulation by *C. thermocellum* from Avicel, Sigmacell, cotton linters, filter paper, and α-cellulose after 7 days at glucan loadings of **(a)** 0.5 wt% and **(b)** 1 wt%.

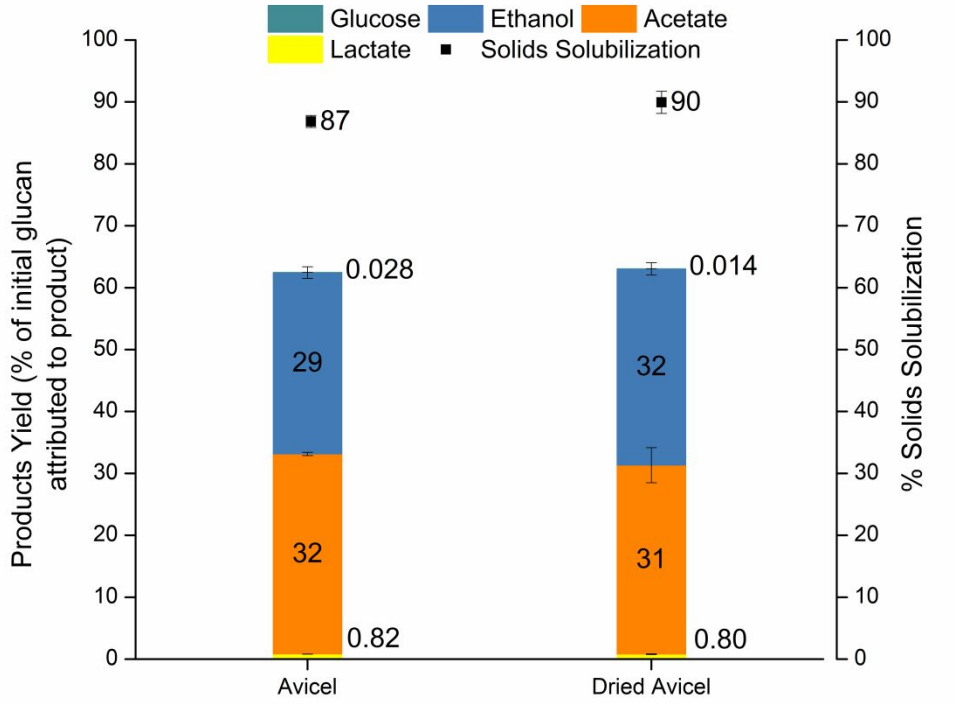
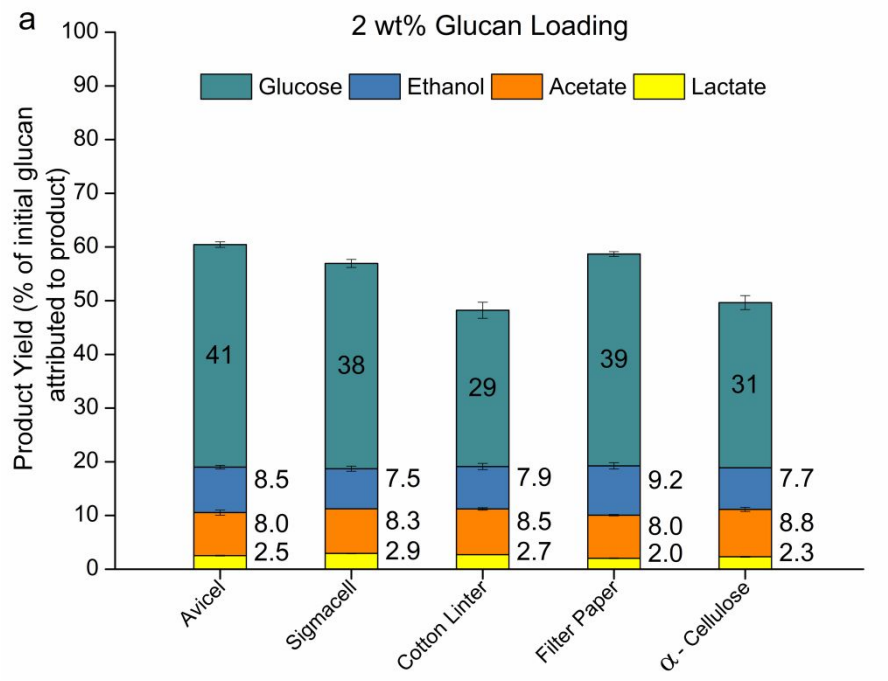


Figure 6. *Clostridium thermocellum* solids solubilization and product yields on Avicel and dried Avicel at a 0.5 wt% substrate glucan loading.

Fermentations were also run at higher glucan loadings to determine if product/substrate accumulation affected digestion of each material by the organism. Any inhibition of *C. thermocellum* by its products should ideally affect digestion of all materials equally, with differences in deconstruction of different substrates attributable to just substrate specific properties. For a 1 wt% glucan loading, metabolite production dropped while glucose accumulation increased, suggesting *C. thermocellum* metabolite accumulation inhibited sugar fermentation but not cellulolytic activity, as shown in Figure 5(b). However, the amounts of each metabolite were similar for all substrates. Although fermentations of 1 wt% glucan of all

substrates did not reveal any significant differences in digestion of the different materials, some differences became evident at substrate loadings of 2 and 5 wt% glucan, as shown in Figures 7(a) and 7(b). Cotton linter and α-cellulose were limited to lower metabolites production and glucose accumulation combined compared to the other materials. This result was consistent with lower solids solubilization, measured as disappearance of mass of solids, of cotton linter and α-cellulose compared to the other materials by *C. thermocellum*, as shown in Figure 8.



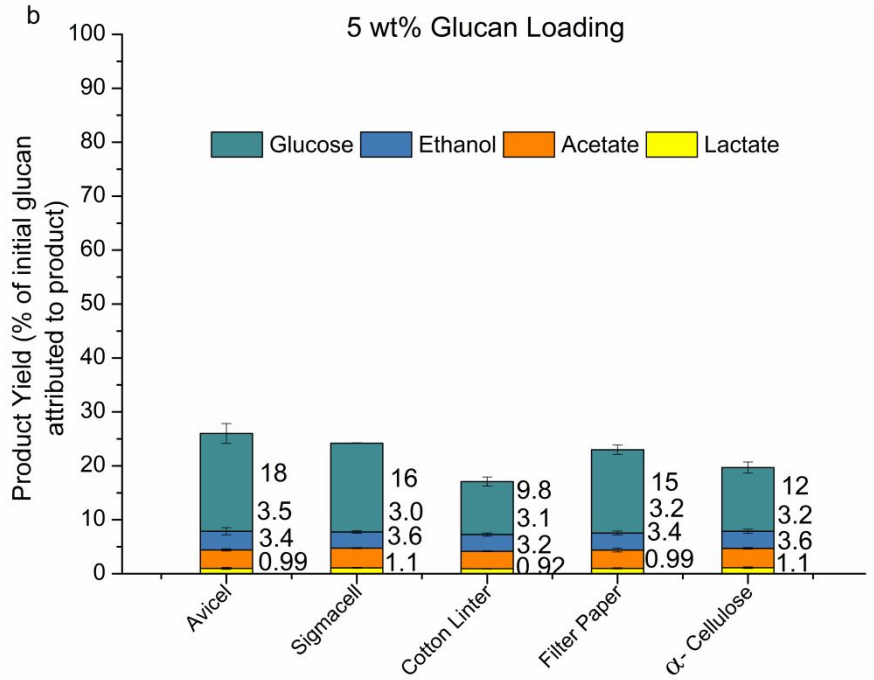


Figure 7. Metabolites and glucose production by *Clostrodium thermocellum* from Avicel, Sigmacell, cotton linters, filter paper, and α-cellulose after 7 days at solids loadings of **(a)** 2 wt% and **(b)** 5 wt% glucan.

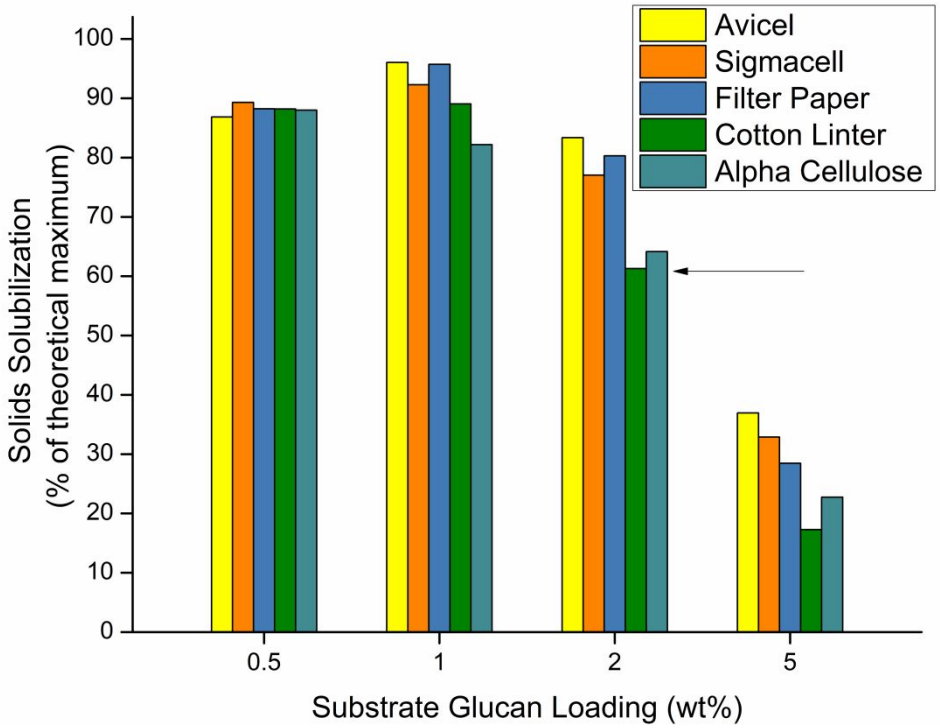


Figure 8. Solids solubilization of Avicel, Sigmacell, cotton linter, filter paper, and α-cellulose at glucan loadings of 0.5 wt%, 1 wt%, 2 wt%, and 5 wt% by *Clostridium thermocellum* after 7 days. The arrow indicates lower solubilization by *C. thermocellum* of cotton linter and α-cellulose at high substrate loadings.

The relatively low *C. thermocellum* digestion of cotton linters was similar to that observed for its breakdown by fungal enzymes. However, the lower digestion of cotton linters by *C. thermocellum* was only observed at 2 wt% and higher glucan loadings, with product formation only ~17% lower compared to other materials at the same substrate loading. On the other hand, the negative impact of cotton linters on fungal enzymes was observed at a low 0.5 wt% glucan loading and the resulting ~57 to 66% lower product formation compared to other materials suggested a much greater impact of cellulose micro-accessibility on fungal enzymes compared to *C. thermocellum*. The low digestion of α-cellulose by *C. thermocellum* at high substrate loadings may be due to the presence of high amounts of xylan in the substrate compared to other materials that are known to be mostly pure cellulose [41, 44-47]. Although *C. thermocellum* cellulosomes are known to contain xylanases that can breakdown xylan, xylose and xylo-oligomers, the breakdown products of xylan, are known to be inhibitory to *C. thermocellum* [70]. In particular, the IC_{50} concentration of xylose or xylobiose has been determined to be 15 g/L for the M1570 strain of *C. thermocellum* with lower concentrations of both the mono- and disaccharide also inhibiting the organism [71]. By comparison, the 2 to 5 wt% glucan loadings of α-cellulose used here would result in 0.5 to 1.3 wt% (~5 to 13 g/L) xylan loadings, in the range for substantial inhibition.

Conclusions

Here, we showed how cellulose micro-accessibility impacts cellulose deconstruction by fungal enzymes and *C. thermocellum* once cellulose from lignocellulosic biomass is made physically accessible to the biological entity through biomass modification. Although prior studies have shown that cellulose crystallinity, DP, WRV, surface area, and other structural features influenced cellulose micro-accessibility, in this work we related the effect of these properties to the extent of fungal enzyme digestion of cellulose compared to that by *C.*

thermocellum. These properties substantially impacted the ability of fungal enzymes to digest cellulose compared to *C. thermocellum*. Surface area and pore size distribution of the substrates had the greatest impact on fungal enzymatic digestion, followed by cellulose crystallinity index (CrI%), water retention value (WRV), and degree of polymerization (DP). The low digestibility of cotton linters compared to Avicel, Sigmacell, filter paper, α-cellulose, and dried Avicel could be related to its low surface area, smaller pore size distribution, and high crystallinity and DP. On the other hand, the high fungal enzyme digestibility of filter paper compared to Avicel was consistent with its greater surface area measured via Simons' staining and higher swelling ability measured by WRV. The better fungal enzyme digestibility of Avicel compared to Sigmacell and α-cellulose could be related to its larger pores as measured via SEM and high O/B dye ratios. The lower cellulose crystallinity of Sigmacell appeared to enhance its digestion by fungal enzymes compared to cotton linters.

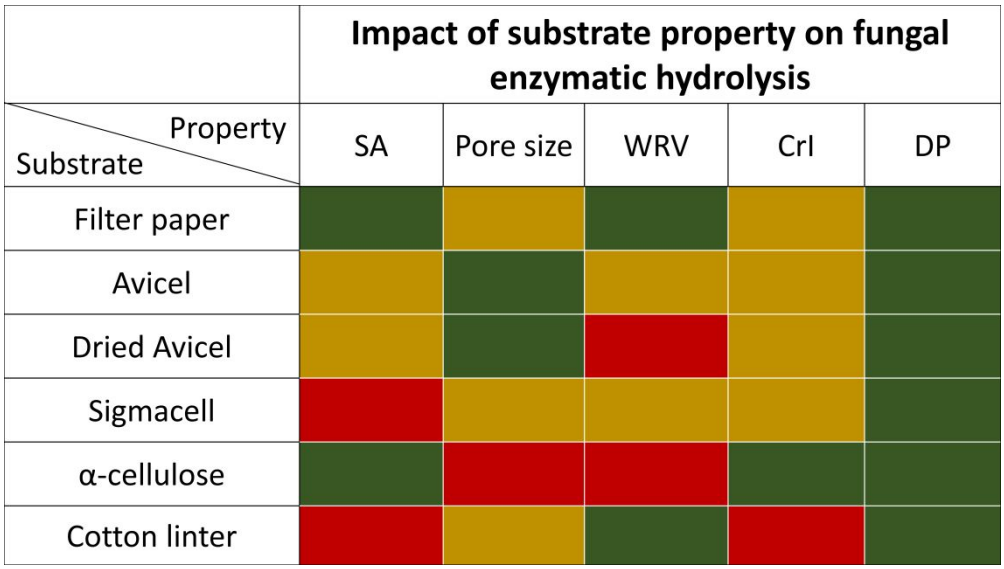


Figure 9: A color matrix to illustrate the relative impact of properties, such as, surface area (SA), pore size, water retention value (WRV), crystallinity index (CrI), and degree of polymerization (DP) of different model cellulosic substrates on the extent of their digestion by fungal enzymes (red refers to a substantial negative impact, yellow refers to a somewhat negative impact, and green refers to an overall positive impact of the respective property on the extent of digestion of the substrate by fungal enzymes).

Although alike in all properties other than WRV, the less effective digestion of dried Avicel compared to regular Avicel by fungal enzymes showed the impact of WRV on fungal enzyme digestion. The fact that filter paper with a higher cellulose DP compared to Avicel was still more digestible by fungal enzymes suggests that DP has a limited impact on enzymatic deconstruction of cellulose. Overall, fungal enzymatic digestion could be related to cellulose surface area, pore size, and crystallinity of the substrate that are expected to influence effective enzyme adsorption. Figure 9 illustrates the relative impact of various substrate properties on the extent of digestion of the substrate by fungal enzymes as a color matrix to aid visualization. It is important to note that the impact of various cellulose properties studied here on the extent of cellulose hydrolysis is multi-factorial, with this matrix type effect clearly evident in Figure 9.

In contrast, *C. thermocellum* showed no differences in cellulose deconstruction of the various substrates at a 0.5 wt% glucan loading. However, at 2 and 5 wt% glucan loadings, *C. thermocellum* digestion of cotton linters and α-cellulose was marginally lower than on the other substrates. The lower digestion of α-cellulose at high loadings may result from inhibition of *C. thermocellum* by accumulation of high amounts of xylan and its breakdown products in solution and that of cotton linter due to high cellulose crystallinity of the substrate. Overall, however, *C. thermocellum* was unaffected by cellulose micro-accessibility as measured by cellulose surface area, pore size, crystallinity, and degree of polymerization. The effective digestion of varied substrates by *C. thermocellum* can be attributed to its complex cellulosome with multiple enzymes working synergistically. As pointed out earlier, 24 out of the over 70 enzymes identified on *C. thermocellum*'s cellulosome are cellulases, along with xylanases, pectinases, and

others comprising the rest [21, 24]. Model substrates were used in this work to differentiate the impact of cellulose micro-accessibility from that of cellulose macro-accessibility on biological digestion. A similar analysis of deconstruction of more complex lignocellulosic biomass will be valuable to fully understand how cellulose micro-accessibility impacts digestion by fungal enzymes compared to *C. thermocellum*.

Experimental:

Materials

Avicel® PH-101 (Cat No. 11365, Lot No. BCBN7864V), Sigmacell Cellulose Type 50 (S5504, Lot No. SLBB7781V), cotton linters (Lot No. 090M0144V), and α-cellulose (C8002, Lot No. 066K0076) were purchased from Sigma-Aldrich (St. Louis, MO). Whatman™ 1 filter paper (Cat. No. 1001-110) was milled using a Thomas Wiley® mill (Model 3383-L20, Thomas Scientific, Swedesboro NJ) and passed through a size 40 mesh. Since all these substrates, except α-cellulose, are known to be of high purity [41, 42, 44-47], they were assumed to contain 100% glucan for calculations. As reported elsewhere and since the same lot of the material as in the reported literature was used, α-cellulose was assumed to be composed of 82.7 wt% glucan with the rest being xylan [42]. Ethanol (E1028), acetic acid (A38-212), and lactic acid (L6661) used as standards for HPLC analysis were obtained from Spectrum® Chemical Mfg. Corp. (Gardena, CA), Fisher Scientific™ (Fair Lawn, NJ), and Sigma-Aldrich® (St. Louis, MO), respectively. Glucose (G8270), also used as an HPLC standard, was obtained from Sigma-Aldrich® (St. Louis, MO). The Accellerase® 1500 cellulase enzyme cocktail was kindly provided by DuPont Industrial Biosciences (Palo Alto, CA). The BCA protein content of Accellerase® 1500, as reported elsewhere [72], was 82 mg/mL. *C. thermocellum* DSM 1313 wild type was kindly provided by

Prof. Lee Lynd at Dartmouth College, Hanover NH. A stock culture was grown in a 500 mL anaerobic media bottle (Chemglass Life Sciences, Vineland NJ) and stored in 5 mL serum vials at -80°C.

Clostridium thermocellum fermentations

C. thermocellum fermentations were performed as reported elsewhere [11]. Briefly seed cultures were grown with a 5 g/L glucan loading of Avicel® PH-101 (Sigma Aldrich, St. Louis, MO) in a 50 mL working volume for 8-9 h (approximately the start of exponential phase based on pellet nitrogen analysis reported in our earlier work [11]) in Media for Thermophilic Clostridia (MTC) without trace minerals using a 2% by volume inoculum. Fermentations were performed in 125 mL bottles (Wheaton, Millville NJ) with 0.5-1 wt% glucan loadings of cellulosic substrates in triplicates at a working mass of 50 g. Bottles containing substrate and water were purged with nitrogen. An alternating 45 s application of vacuum and 14 psi nitrogen over a total of 27 to 30 min was used for purging. The bottles were then sterilized by autoclaving at 121°C for 35 min. All sterile media solutions and inoculum were injected aseptically into the bottles. Fermentations were run at 60°C with a shaking speed of 180 rpm in a Multitron Orbital Shaker (Infors HT, Laurel MD). Bottles were opened after 7 days (to aid direct comparison to digestion by fungal enzyme hydrolysis of the same substrates that had to be run for 7 days to ensure maximum digestion) of fermentation and liquid samples were taken to measure metabolites and simple sugars content. The liquor samples were centrifuged in 500 µL micro-centrifuge vials (Ultrafree™-MC, EMD Millipore, MA USA) at 15,000 rpm for 10 min. The filtered liquid solution was then analyzed by HPLC. Insoluble solids were also recovered after fermentation

and rinsed thoroughly to determine solids solubilization on aluminum pans in 105°C oven for 24 to 48 h.

Fungal enzymatic hydrolysis

Enzymatic hydrolysis was performed in triplicates in accordance with National Renewable Energy Laboratory (NREL, Colorado) [73] protocol at 0.5 wt% glucan loading and an Accellerase® 1500 cellulase loading of 15 mg protein / g glucan enzyme with a working mass of 50 g in 125 mL Erlenmeyer flasks as reported in our earlier work [11]. The enzyme activity of Accellerase 1500 was ~0.5 filter paper units (FPU)/mg protein[74]. Enzymatic hydrolysis was run at 50°C and 150 rpm in a Multitron Orbital Shaker (Infors HT, Laurel MD). Flasks were allowed to equilibrate at temperature before adding the enzyme solution. One mL representative homogenous samples containing the insoluble substrate and liquor were collected in 1.5 mL Simport® microcentrifuge tubes (Spectrum® Chemical Manufacturing Corporation, New Brunswick, NJ) after 4 h, 24 h, and every 24 h period thereafter for a total of 7 days to ensure maximum digestion of substrates. The samples were centrifuged at 15,000 rpm for 10 min and the supernatant was analyzed by HPLC.

Analytical procedures

Waters Alliance e2695 HPLC system (Waters Co., Milford MA) equipped with a Bio-Rad Aminex HPX-87H column and a Waters 2414 refractive index detector was used for analysis. 5 mM sulfuric acid mobile phase was eluted at a flow rate of 0.6 mL/min. Empower™ 2 software package was used for the integration of chromatograms.

Yield calculations

All experiments were performed in triplicates, unless otherwise specified. Error bars represent the standard deviation in the replicates. Metabolite yield was calculated as the glucan required to produce each metabolite through stoichiometry of balanced glucose to metabolite reactions. Glucose was further converted to glucan using the anhydrous correction factor (= 0.9). Solids solubilization was measured as the percentage loss of solids after 7 days of fermentations compared to solids at time zero. All yields were based on glucan loaded initially.

Solid state nuclear magnetic resonance

The cellulose crystallinity of samples was measured using solid-state cross polarization magic angle spinning (CP/MAS) nuclear magnetic resonance (SSNMR). The samples were moisturized and packed into 4-mm cylindrical Zirconia MAS rotors. All the SSNMR experiments were carried out on a Bruker Avance III HD 500-MHz spectrometer operating at frequencies of 125.77 MHz for ^{13}C in a Bruker double-resonance MAS probe at room temperature. The acquisition conditions for CP/MAS experiments were as follows: a 5 μs (90°) proton pulse, 3.0 ms contact pulse, 3 s recycle delay and 4096 scans. The rotor spin rate was 8000 Hz. The cellulose crystallinity index (CrI%) was determined from the areas of the crystalline and amorphous C_4 signals using the following formula:

$$\mathrm{CrI\%} = \frac{A^{86\text{ - }92\text{ ppm}}}{A^{86\text{ - }92\text{ ppm}} + A^{79\text{ - }86\text{ ppm}}} \text{ x } 100$$

X-ray diffraction

The crystallinity indices (CrI%) of cellulose samples were also measured by X-ray diffraction (XRD) using a Rigaku (Tokyo, Japan) Ultima IV diffractometer with CuKα radiation having a

wavelength λ(Kα1) = 0.15406 nm generated at 40 kV and 44 mA. The diffraction intensities of freeze-dried samples placed on a quartz substrate were measured in the range of 8 to 42° 2θ using a step size of 0.02° at a rate of 2°/min. The CrI% of the cellulose samples was calculated according to the method described by Segal *et al*. [75] by using eq. (1) presented below:

$$CrI\% = \frac{I_{200} - I_{Am}}{I_{200}} x\ 100 \quad (1)$$

where I_{200} and I_{Am} are the diffraction intensity at approximately 2θ = 22.4 - 22.5° and 2θ = 18.0 - 19.0°, respectively.

Scherrer's equation [76, 77] was used for estimating crystallite size:

$$\beta = \frac{k\lambda}{\tau cos\theta} \quad (2)$$

where λ is the wavelength of the incident X-ray (1.5418 Å), θ is the Bragg angle corresponding to (2 0 0) plane, β is the full-width at half maximum (FWHM) of the X-ray peak corresponding to the (2 0 0) plane, τ is the X-ray crystallite size, and k is a constant with a value of 0.89 [78, 79].

Gel permeation chromatographic (GPC) analysis.

GPC after tricarbanilation was used to measure the weight-average molecular weight (M_w) and number-average molecular weight (M_n) of cellulose. Briefly, cellulose substrates were dried overnight under vacuum at 45°C. The dried cellulose samples were then derivatized with phenyl isocyanate in an anhydrous pyridine system. An Agilent 1200 HPLC system (Agilent Technologies, Inc, Santa Clara, CA) equipped with Waters Styragel columns (HR1, HR4, and HR6; Waters Corporation, Milford, MA) was used to perform size-exclusion separation. 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. SEM was carried out on Zeiss Auriga FIB-SEM at an accelerating voltage of 10 kV with back scatter detector at 100 to 5000 times magnification. Raw images were adjusted for brightness and contrast in ImageJ software [80]. Images were merged using Adobe Photoshop CC v. 2017.

Water retention value (WRV)

WRV was measured as described previously [5]. Briefly, 15 ml ultracentrifuge tubes were loaded with 90 mg sample (on a dry basis) in triplicate and equilibrated with excess water at RT overnight. Then, the tubes were spun at 900g for 30 min at RT followed by weight measurements. WRV is defined as the ratio of the mass of water retained in the sample after centrifugation to the mass of dry sample after centrifugation.

Modified Simons' staining

Modified Simons' staining was performed as described previously by Chandra et al. [5, 54] Direct Orange 15 dye (CAS: 1325-35-5) and Direct Blue 1 (CAS: 2610-05-1) were obtained from Pylam Products Company, Inc. (Tempe, AZ)

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Competing interests:

CEW and MEH are Editors-in-Chief of the Journal *Biotechnology for Biofuels*. The other authors declare that they have no competing financial interests.

Author contributions:

NK, SB, RK, and CEW designed the study and analyzed data. NK planned and managed the overall study. NK and MZ performed *C. thermocellum* fermentations and fungal enzymatic hydrolysis. SB performed Simons' staining, water retention value, scanning electron microscopy, and gel permeation chromatography analytical techniques. CGY assisted in running samples for molecular weight. YP performed the solid state nuclear magnetic resonance analysis of all materials. AM performed X-ray diffraction analytical method on all materials. NK wrote the first draft of the manuscript. NK, SB, RK, CEW, MZ, YP, AM, CGY, MEH, and AJR read, edited, and finalized the manuscript.

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Unlike fungal enzymes, *C. thermocellum* digestion of lignocellulosic biomass is largely unaffected by cellulose properties once cellulose is made macro-accessible