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Lignin Exhibits Recalcitrance-Associated Features Following the Consolidated Bioprocessing of *Populus trichocarpa* Natural Variants

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Because cellulosic ethanol production remains cost-prohibitive„ advances in consolidated bioprocessing (CBP) have been directed towards lifting this restriction. CBP reduces the need for added enzymes and can potentially slash ethanol production costs through process integration. *Clostridium thermocellum*, a CBP microorganism, organizes its enzymes in a multi-enzyme complex - a stark contrast to fungal enzymes. Nonetheless, recalcitrance may limit the extent of biomass deconstruction. Herein, six *Populus* were treated with *C. thermocellum* (ATCC 27405) and characterized to determine structural changes that resulted from CBP. The 2D HSQC NMR spectra of lignin-enriched residues revealed that higher S/G ratio (2.6) and fewer carbon-carbon interunit linkages (generally 2–5%) were present in the top performing poplar. Furthermore, cellulose degree of polymerization data suggests that *C. thermocellum* likely circumvents long chain cellulose, while cellulose crystallinity and hemicellulose molecular weight data do not provide a direct indication of features connected to recalcitrance. Hence, *C. thermocellum* is similarly impacted by the proposed lignin properties that negatively impact biomass deconstruction using fungal enzymes.

Introduction

Industrially, starch has received much more attention and commercial success than cellulose for ethanol production. Starch is depolymerized into glucose more easily than cellulose, which renders it a more popular substrate. Difficulties in cellulose bioconversion are rooted in several inherent structural features of biomass that restrict fungal enzyme activities. Recalcitrance describes this resistance to enzymatic deconstruction and severely complicates the conversion of cellulose into ethanol. While pretreatments[1] and genetic modifications[2] have been investigated to minimize recalcitrance, consolidated bioprocessing (CBP) is an alternative approach for addressing difficulties in biomass deconstruction that have contributed to the high costs of ethanol production.

The common scheme for ethanol production from lignocellulosic biomass follows a four-step process. Biomass feedstocks such as switchgrass or poplar are pretreated to enhance biomass accessibility for the subsequent enzymatic hydrolysis. Fungal cellulases are traditionally employed to depolymerize cellulose into glucose units, which are often fermented by yeasts to yield ethanol. The final stage is distillation, where ethanol is separated from other fermentation products. Alternatively, CBP uses microorganisms to solubilize and ferment five- and/or six-carbon sugars in lignocellulosic biomass in a single step and without added enzymes. While a number of microorganisms carry out CBP,[3] *Clostridium thermocellum*, a thermophilic anaerobic bacterium, has attracted considerable attention as a robust CBP microorganism.[4] Several other CBP microorganisms derive their attractiveness from their ability to hydrolyze a wide variety of plant polymers and/or utilize the sugars derived from cellulose and/or hemicellulose hydrolysis; however, these properties do not adequately address two important contributors to high ethanol production costs: recalcitrance, which requires costly pretreatments, and low enzyme activities, which are further limited by recalcitrance. In contrast, *C. thermocellum* has the potential to mitigate the high costs associated enzyme production and hydrolysis. *C. thermo-*

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cellum's cellulosome fosters enzyme synergy and has imparted exceptional enzymatic hydrolysis properties.

C. thermocellum contains a cellulosome, a multi-enzyme assembly that is attributed to the efficient solubilization of biomass and saccharification of cellulose. The cellulosome promotes synergistic relationships between enzymes,[5] which in turn are associated with its impressive cellulose hydrolysis rates compared to that of fungal cellulases, particularly in the presence of Avicel,[6] The cellulosome also contains dockerin binding sites for enzymes such as hemicellulases, pectinases, and chitinases that hydrolyze a wide array of biomass components. Furthermore, the enzymes that bind to cellulosome change depending on the substrate composition. Enzyme diversity has been identified as an influential factor for the extent of substrate degradation.[5b] Several of these enzymes disrupt plant cell wall features that restrict cellulase accessibility.

It is worth considering whether CBP microorganisms are negatively influenced by recalcitrance as fungal cellulases are. While *C. thermocellum* has been heavily investigated from a diverse group of perspectives (e.g. genetic engineering to improve ethanol yields, cellulosome assembly to understand synergy, etc.),[3–4] the structural features of biomass that facilitate CBP remain obscure. Previous studies provide few clues, suggesting that short DP (or high crystallinity) cellulose[6a,7] and high S/G ratio lignin{Dumitrache, 2016 #3} positively affect CBP. Additional studies are required to identify and/or clarify the structural features of biomass that are problematic during CBP. Upon understanding recalcitrance during CBP, pretreatments and/or genetic modifications will be selected more efficiently to attain substrates that undergo facile deconstruction. Hence, this investigation aims to characterize cellulose, hemicellulose, and lignin structure before and after CBP in nonpretreated *Populus trichocarpa* to associate structural changes to difficulties during CBP.

Results

Carbohydrate compositions and fermentation yields

Carbohydrate compositions for six *Populus trichocarpa* were obtained prior to microbial treatment. Glucose contents ranged between 455.2 mg/g dry biomass in BESC-316 to 498.9 mg/g dry biomass in GW-9947. Xylose, galactose, arabinose, and mannose contents exhibited minor variations between samples. Xylose, specifically, demonstrated the greatest difference in content between samples, attaining a maximum difference of 13.9 mg/g dry biomass. Acetate was the dominant fermentation product followed by ethanol and lactate (Table 1). Ethanol yields ranged from 7.6 mg/g in BESC-316 to 32.2 mg/g glucan in GW-9947 and are similar to values previously reported for *C. thermocellum*.[8] Accordingly, GW-9947 produced the highest concentrations of lactate (2.4 mg/g glucan) and acetate (123 mg/g glucan), while BESC-316 produced the lowest concentrations (0.3 mg/g and 68.4 mg/g glucan, respectively).

Table 1. Fermentation product yields from the six natural variants of *P. trichocarpa*

Variant	mg/g cellulose Ethanol (SD [a])	Lactate	Acetate	Ethanol Yield (%)[b]
BESC-876	18.0 (0.3)	0.67 (0.3)	94.4 (0.8)	3.2
GW-9920	29.3 (0.8)	1.4 (0.1)	114.4 (1.4)	5.2
GW-9947	32.2 (0.7)	2.4 (0.3)	123.3 (0.1)	5.7
BESC-292	24.5 (0.4)	1.1 (0.07)	104.6 (0.9)	4.3
BESC-316	7.6 (0.3)	0.34 (0.09)	68.4 (1.9)	1.3
GW-9762	22.6 (1.2)	1.1 (0.02)	99.3 (1.7)	4.0

[a] SD; standard deviation of three biological replicates [b] Based on 100% theoretical mg ethanol/g glucan being 568.255 g.

Carbohydrate structure

Cellulose DP and crystallinity

Cellulose weight-average degrees of polymerization, or DP_w, from untreated poplar (TAPPI test method T203 cm-09) ranged between roughly 2000 and 3000 units in length and did not demonstrate statistically significant differences ($p > 0.05$). Following CBP, cellulose DPws increased in all samples (Figure 1),

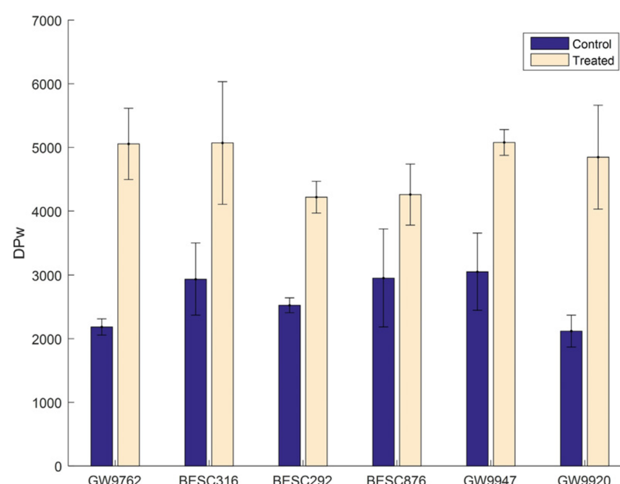

Figure 1. Cellulose DPw from the control and *C. thermocellum* treated *P. trichocarpa*.

which is consistent with other observations.[7a,9] Cellulose DPws in the solid residuals were generally two or more times greater than the initial DPw. Cellulose crystallinity indexes (CrIs) isolated from the untreated biomass ranged between 54.1 and 61.4%, and only GW-9920 was significantly different from the others ($p < 0.05$). Following CBP, the CrIs of the solid residuals ranged between 54.7 and 57.7%, and the differences were negligible ($p > 0.05$). In this study, a significant association of cellulose DPw or CrI with fermentation yields was not observed.

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Hemicellulose molecular weights

Hemicellulose molecular weights were not significantly different from one another neither in the controls nor in the treated materials ($p > 0.05$). Accordingly, the molecular weights were relatively similar between the controls (between 3.6×10^4 and 5.6×10^4 g/mol) and between the treated (4.0×10^4 and 5.2×10^4 g/mol) samples. Lastly, the variability in molecular weights tended to be smaller in the treated samples than the controls.

Characteristics of lignin

Lignin contents

Lignin contents varied by less than five percent in the untreated samples. GW-9947 had the lowest total lignin content (22.8%), while BESC-316 had the highest (27.2%). Although the differences appeared minor, lignin contents potentially influence fermentation according to the previous studies.[10] Specifically, natural variants with lower lignin contents tended to generate higher lactate, ethanol, and acetate concentrations (Figure 2).

Lignin S/G ratio and interunit linkages

The 2D HSQC NMR spectra of a top (GW-9947), moderate (BESC-292), and poor (BESC-316) ethanol yielding poplar were obtained to understand structural differences in lignin that potentially influence the extent of CBP (Figure 3). The primary structural differences in lignin-enriched residues between untreated BESC-292, BESC-316, and GW-9947 are listed in Table 2. Following integration, the NMR spectra revealed distinct structural features in lignins that were isolated from GW-9947, BESC-292, and BESC-316. Firstly, the relative proportion of S lignin in GW-9947 was higher than the remaining two lignins, and its S/G ratio was approximately 1.5 times greater than that of BESC-316 and BESC-292. Secondly, the *p*-hydroxybenzoate (PB) content of GW-9947 was between 2 and 3% lower than in the remaining lignin-enriched residues. Additionally, the contents of carbon-carbon linkages (β-β and β-5 linkages) were approximately 2% lower in GW-9947 than in BESC-316. The data revealed that GW-9947 and BESC-316, which varied greatly in their CBP performances, exhibited very different lignin structural features.

Discussion

Fermentation yields differ between natural variants

The ethanol concentrations and yields (<6%) for these fermentations are low but are in line with previous reports for the wild-type strain where 17–18% of the glucan is consumed by *C. thermocellum* on poplar.[8] Acetate is released from poplar during incubation and if this is considered, acetate to ethanol ratios are in the typical range for a wild-type strain. Further strain development and process optimizations are required to meet more applied outcomes with this bacterium utilizing

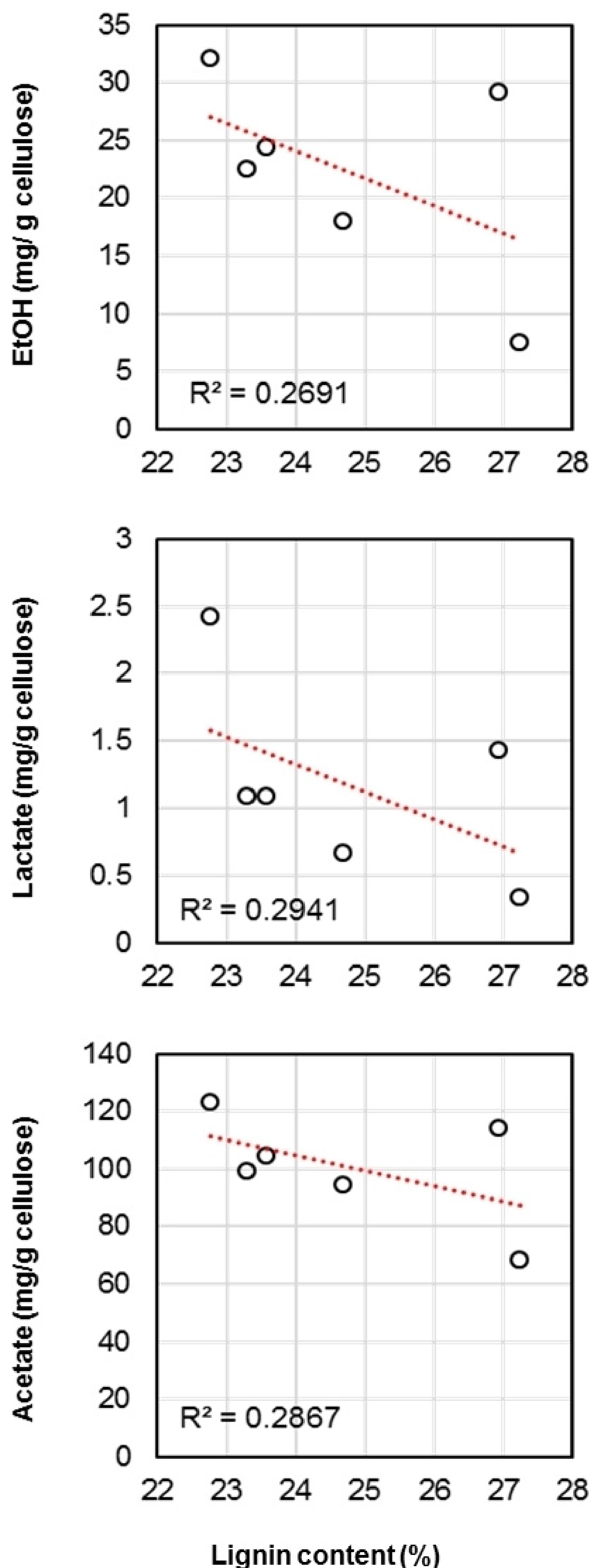

Figure 2. Lignin contents (%) of the six natural variants compared to the fermentation product yields.

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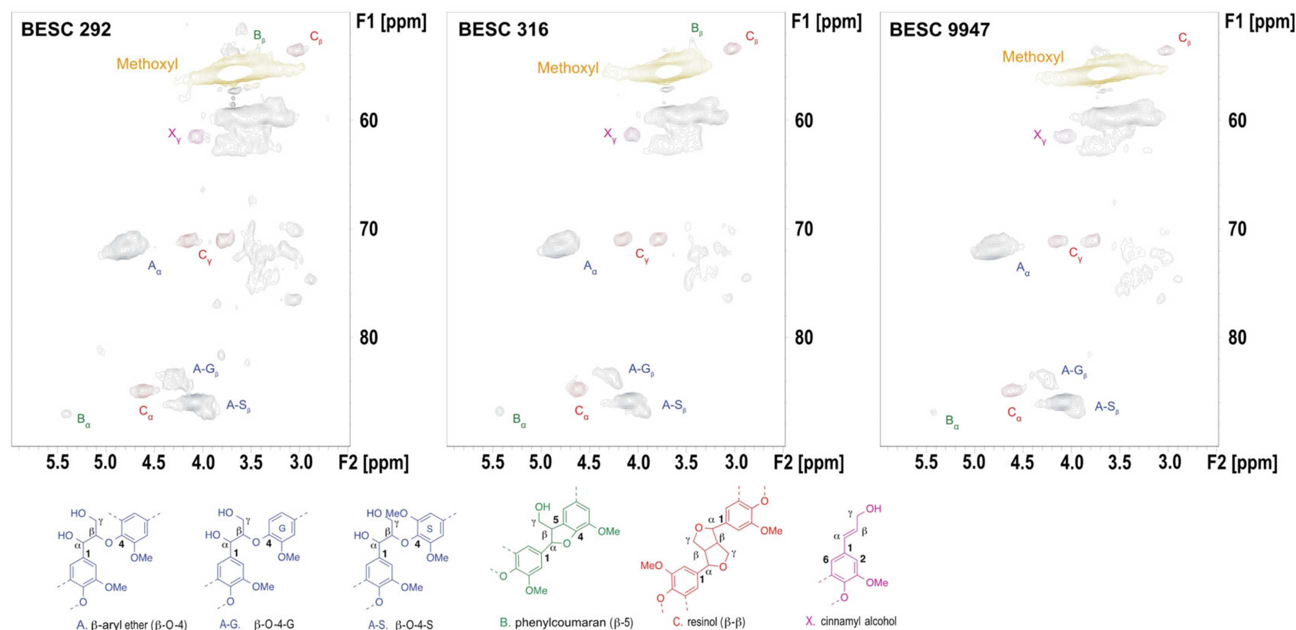

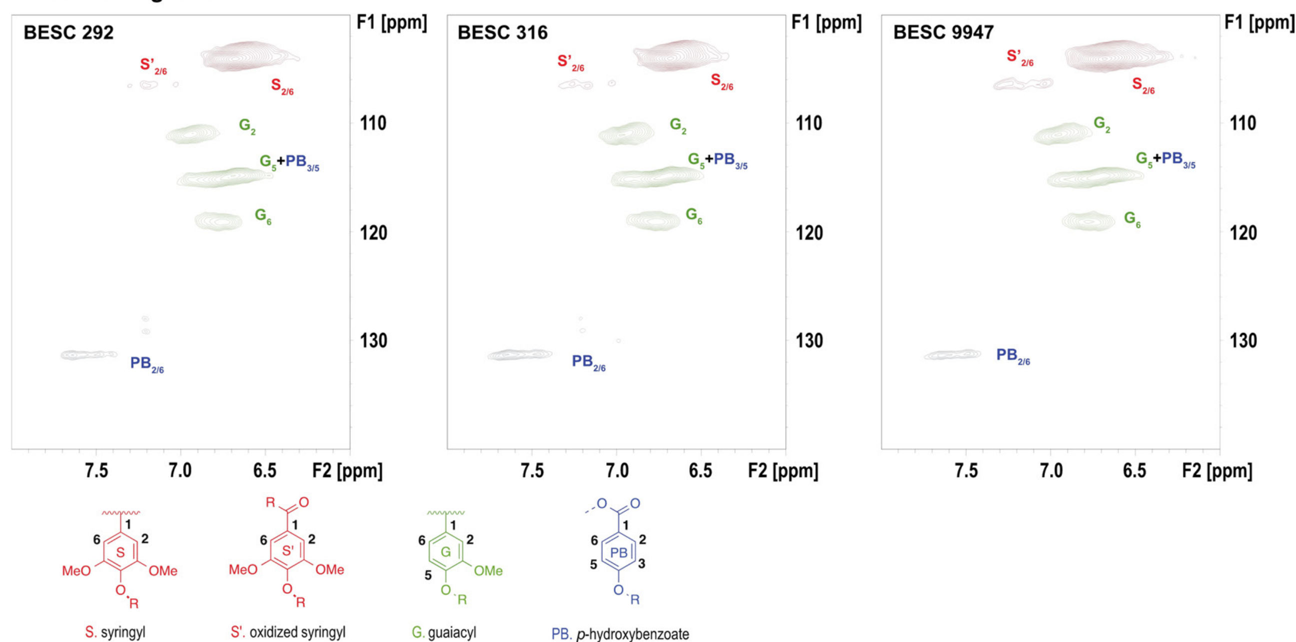

Figure 3. Illustration of the 2D ^{1}H-^{13}C HSQC NMR spectra of the aliphatic and aromatic regions of BESC-292, BESC-316, GW-9947 in lignin isolated from control *P. trichocarpa.*

Table 2. Lignin S/G ratio, PB (%) content, and interunit linkages in natural variants of untreated *P. trichocarpa*

Populus trichocarpa	S/G ratio	PB (%)[a], [b]	β-O-4 (%)[c]	β-5 (%)[c]	β-β (%)[c]
BESC-292	1.9	7.7	82.2	2.9	14.9
BESC-316	1.6	8.9	79.4	3.9	16.7
GW-9947	2.6	5.8	83.3	2.3	14.4

[a] PB means para-hydroxybenzoate [b] Content (%) expressed as a fraction of S+G [c] Content (%) expressed as a fraction of β-O-4 + β-5 + β-β

lignocellulosic feedstocks. Recent studies have explored altering *C. thermocellum*'s lactate production,[11] hydrogen production[12], and other metabolic pathways[13] to achieve ethanol increases.

In *C. thermocellum*, enzyme activities are related to fermentation yields.[14] While enzymatic hydrolysis was not measured in this study, recalcitrance restricts enzymatic hydrolysis. The variability in ethanol yields suggest that certain natural variants are more or less recalcitrant than others.

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Low DP cellulose is utilized before high DP cellulose

Cellulose DP data indicate that longer cellulose chains remain after CBP. This observation suggests that *C. thermocellum* utilizes short chain cellulose and leaves behind longer chain cellulose. Similar studies suggest that *C. thermocellum* takes cellulose size into account during enzymatic hydrolysis. For example, cellulosomes that were isolated from *C. thermocellum* have been used to hydrolyze Avicel (PH-101) and Whatman filter paper no. 1 under the same conditions; however, the conversion of shorter chain Avicel proceeded more rapidly and to a greater extent than on the Whatman filter paper.[6a]

When fungal cellulases carry out hydrolysis, lower cellulose DPs are believed to facilitate enzymatic hydrolysis due to fewer intracellular hydrogen bonds, which result in larger numbers of hydroxyl groups that are available to participate in enzymatic hydrolysis;[15] however, studies that explore this relationship lack consensus.[16] Regardless, the data support the observation that cellulose chain length plays a role during substrate selection for enzymatic hydrolysis; however, cellulose DPw's influence on recalcitrance and ethanol yields appears to be nonexistent.

Cellulose crystallinity and hemicellulose molecular weight are likely minor contributors to recalcitrance during CBP

Although several reports have argued it is a critical factor to enzymatic hydrolysis,[17] cellulose crystallinity is likely a minor contributor to deconstruction difficulties in this study. The CrI (%) from GW-9920 differed significantly from the remaining controls ($p < 0.05$); however, the CrIs (%) were not significantly different from one another ($p > 0.05$) in the treated samples. GW-9920 had the highest CrI of all the samples but did not exhibit poorer fermentation properties compared to its counterparts. It is unlikely that cellulose CrI is a dominant factor that is responsible for the CBP differences observed.

Hemicellulose molecular weight has been found to influence enzyme inhibition[18] and increase in size following enzymatic hydrolysis with fungal cellulases.[19] Hemicellulose molecular weights neither increased nor decreased consistently in all of the samples and were not significantly different amongst the controls or treated samples ($p > 0.05$). The changes in the molecular weights indicate that hydrolysis occurs to hemicellulose, but the exact way in which hemicellulose size influences recalcitrance is unclear. Furthermore, hemicellulose molecular weights interestingly approach 4.5×10^4 g/mol, which suggests that *C. thermocellum* focuses primarily on hemicelluloses removal rather than its complete degradation for utilization.

Structural variations in lignin structure are apparent between natural variants

Although recalcitrance is not completely understood during CBP, lignin appears to influence the action of *C. thermocellum*.[9,20] Recently, the hydrolysis of poplar was shown to cease prematurely as lignin increased at the surface of the biomass.[21] The lignin content relationships illustrated in Figure 2 indicate that fermentation yields are generally lower in the presence of higher lignin contents. Hence, high lignin concentrations appear to interfere with CBP. Higher lignin contents could result in lowered hydrolysis efficiencies and reduced yields of fermentable sugars. Furthermore, lignin-derived inhibitors can accumulate during CBP and negatively impact intracellular metabolism and fermentation yields. Both high lignin content[10b,22] and inhibitor generation[23] have been linked to decreased end-product yields in *C. thermocellum*. Lignin structural analysis revealed that GW-9947, which demonstrated better fermentation yields during CBP than BESC-316 and BESC-292, was rich in syringyl lignin. Similar to free cellulases,[24] higher S/G ratio facilitates CBP.[8]

Additionally, PB (%) content also influences recalcitrance. The inclusion of hydroxybenzaldehyde and hydroxybenzoate derivatives in transgenic *Arabidopsis* has been associated with lower lignin DPs and improved sugar release properties.[25] GW-9947 had the lowest PB (%) content, while BESC-316 had the highest among the three lignin-enriched residues. Interestingly, BESC-292 was a moderately performing poplar despite its low S/G ratio, which may also be attributed to its high PB (%) content, relative to the other poplar.[26] It is possible that the high PB (%) content promoted a similar lignin DP reducing effect and/or increased terminal hydroxyl groups in lignin,[27] which reduced lignin's surface coverage and improved cellulose accessibility. Lastly, the high proportion of carbon-carbon linkages, β-β and β-5 (%) in BESC-316 compared to the remaining two poplars may have also impeded the progression of CBP.

Conclusions

In this study, obvious associations of hemicellulose molecular weight, cellulose crystallinity, and cellulose DP with the biomass recalcitrance regarding *C. thermocellum*'s mode of deconstruction were not observed. Lignin structural analysis indicated that lignin structural features, either individually or collectively, influenced the extent of CBP with *C. thermocellum* (Table 3). Lignin structure and content contribute to the observed differences in CBP, meaning that biomass structure still presents difficulties for large-scale ethanol production.

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Conflict of Interest

The authors declare no conflict of interest.

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Table 3. Summary of select cellulose, hemicellulose, and lignin properties in comparison with ethanol yields

	Ethanol Yield	Cellulose DPw		Hemicellulose molecular weight (g/mol)		Cellulose Crystallinity CrI (%)		Lignin	Lignin structure			
Phenotype	(mg/g cellulose)	Before	After	Before	After	Before	After	Content (%)	S/G	β-O-4 (%)	β-β (%)	β-5 (%)
GW-9947	32.2	3,050	5,077	37,570	48,590	54.7	57.7	22.8	2.6	83.3	14.4	2.3
BESC-316	7.6	2,934	5,069	34,175	57,167	55.0	54.7	27.5	1.6	79.4	16.7	3.9
BESC-292	24.5	2,525	5,070	41,500	43,200	51.1	56.8	23.6	1.9	83.3	2.3	14.4
BESC-876	18.0	2,952	4,259	55,800	44,700	55.2	54.9	24.7	–	–	–	–
GW-9762	22.6	2,184	5,054	42,300	38,400	54.8	56.7	23.3	–	–	–	–
GW-9920	29.3	2,119	4,846	42,300	29,100	49.7	54.6	26.9	–	–	–	–

Keywords: cellulosic ethanol · *Clostridium thermocellum* · lignin · *Populus trichocarpa* · recalcitrance

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