

# Effects of Biomass Accessibility and Klason Lignin Contents during Consolidated Bioprocessing in *Populus trichocarpa*

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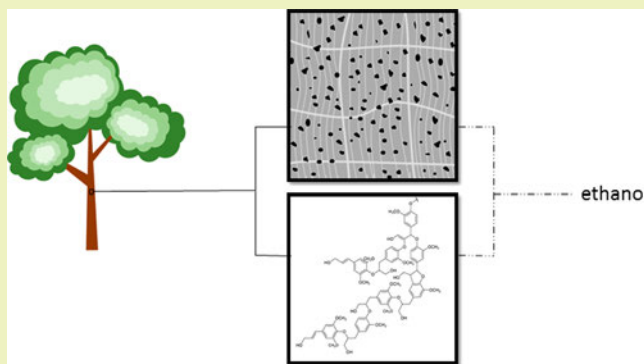
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**ABSTRACT:** The bacterium *Clostridium thermocellum* offers a distinct and integrated approach to ethanol production through consolidated bioprocessing (CBP). The Simons' stain technique, which assays the accessibility of lignocellulosic biomass, has been traditionally applied to fungal cellulase systems; however, its application to CBP has not been fully explored. For this reason, the structural properties of eight *Populus trichocarpa* with either high or low biomass densities were compared to determine bioconversion differences during separate hydrolysis and fermentation (SHF) and CBP with *C. thermocellum*. Simons' staining generally identifies low density poplar as more accessible than high density poplar. Additionally, low density *P. trichocarpa* generally contained less Klason lignin than high density poplar. SHF and CBP treatments consistently identified BESC-7 (high density, low accessibility, low surface roughness) as a low ethanol yielding biomass and GW-9914 (low density, high accessibility, high surface roughness) as a high ethanol yielding biomass. Upon further investigation, BESC-7 also contained a high Klason lignin content (~25%), while GW-9914 had a low lignin content (~20%). Cellulose degree of polymerization (DP) measurements exhibited a weak linear correlation with accessibility ( $r^2 = 0.17$ ). Therefore, the ethanol yields were correlated with accessibility and lignin content extremes but not cellulose DP.

**KEYWORDS:** consolidated bioprocessing, *Clostridium thermocellum*, Simons' stain, Klason lignin, wood density, ethanol



## INTRODUCTION

In biomass, pore sizes are purportedly related to the synergism between fungal enzymes. Depending on their sizes, certain pores may filter enzymes that are too large to pass, thereby separating them from smaller enzymes and reducing the synergy between these enzymes.<sup>1</sup> During biomass deconstruction, synergism has been shown to improve the rate and extent of solubilization<sup>2</sup> with fungal cellulases. Hence, reductions in enzyme synergism have detrimental effects in overcoming biomass recalcitrance and lowering the production costs for cellulosic ethanol. Similarly, *Clostridium thermocellum*, a consolidated bioprocessing (CBP) microorganism, relies heavily on enzyme synergism with a certain organelle to achieve unprecedented hydrolysis rates; however, these synergistic relationships are not clearly defined in terms of accessibility.

The Simons' stain technique assays a fiber's specific surface area (SSA) through semiquantitative measurements. The SSA, which has been linked to enzymatic hydrolysis properties, estimates the fiber's exterior and interior surface areas to assess surface area per mass of cellulose. The exterior surface area depends on the fiber's width and length, while the interior surface area is determined by the amount of pores and crevices in the fiber as well as the size of the lumen.<sup>3</sup> Certain pretreatments remove plant cell wall constituents, thereby altering the cell wall structure and increasing the interior surface area. Accordingly, the Simons' stain is routinely used to determine accessibility following a pretreatment.<sup>4–6</sup>

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The Simons' stain technique depends on two dyes, Direct Blue 1 (DB1) and Direct Orange 15 (DO15), which typically bind to either small (1 nm) or large (5–36 nm) diameter pores, respectively.<sup>7</sup> The dye binding patterns are also related to their individual cellulose affinities; DO15 has an affinity to cellulose that is greater than that of DB1. The ratio of the DO15 and DB1 dyes provides a basis for pore size distribution and accessibility predictions.

The diameter of a representative cellulase is 5.1 nm, but cellulases may range between 4 to 7 nm in size.<sup>5,8</sup> There is also evidence that hemicellulose may influence accessibility measurements, as determined by other Simons' staining studies.<sup>4,9</sup>

During consolidated bioprocessing, the bacterium *C. thermocellum* deconstructs biomass using cell-bound and cell-free cellulosomes and utilizes cellulose-derived degradation products to generate ethanol among other fermentation products. The cell-bound cellulosome is anchored to the bacterial surface via a scaffoldin, whereas the cell-free cellulosome is secreted. The carbohydrate-active enzymes of the cellulosome are anchored to the scaffoldin through hydrophobic mediated cohesin–dockerin interactions. The cellulosome is 18 nm in diameter, much larger than the estimated 5.1 nm cellulase diameter. The pore-dependent filtering effect, and therefore accessibility, may exert much less of an impact during consolidated bioprocessing, as many of the enzymes involved in deconstruction are cellulosomal bound. *C. thermocellum* likely peels cellulose microfibrils from the biomass surface as opposed to fungal enzyme systems, which causes pitting in biomass.<sup>10</sup> This mode of action may also make pore size (and accessibility) irrelevant, as *C. thermocellum* may not require entrance into pores to carry out CBP; however, Dumitrache et al. identified accessibility differences with the Simons' stain before and after CBP, which were possibly linked to structural differences in lignin as contributors to the observed ethanol yields.<sup>11</sup>

The negative correlation between biomass porosity and density<sup>12,13</sup> provides a starting point for the selection and comparison of wood for Simons' staining. Wood density and surface roughness also appear to be interconnected,<sup>14</sup> which is possibly due to their relationship to the number of pores. Therefore, these variables may provide useful information for the proposed surface deconstruction of biomass with *C. thermocellum*. Because density has been linked to surface roughness<sup>14</sup> and porosity,<sup>12,13</sup> either of these parameters may provide indications of accessibility with Simons' staining even if the cellulosome does not need to enter the pores.

This investigation examines Simons' staining's relevance to CBP to assess biomass susceptibility to microbial solubilization. Biomass porosity, Klason lignin content, surface roughness, and cellulose degree of polymerization were all considered as factors that may affect deconstruction during separate hydrolysis and fermentation (SHF) and CBP.

## ■ EXPERIMENTAL SECTION

**Wood Density Characterization.** Wood density analyses were performed on wood cores collected from field grown 4-year-old *Populus trichocarpa* trees in Clatskanie, OR and 3-year old *P. trichocarpa* trees in Corvallis, OR. All *P. trichocarpa* used in this study were natural variants. Field establishment, growth conditions, harvesting, and processing were previously described by Muchero et al.<sup>15</sup> Density was determined using the water displacement method as described by Chave with no modifications.<sup>16</sup> Reproducibility of the density phenotype across the two sites was assessed based on a

common set of 462 genotypes using Spearman rank correlation analysis. Eight genotypes representing the extreme tails of the phenotypic distribution were selected for accessibility measurements.

**Consolidated Bioprocessing and Separate Hydrolysis and Fermentation.** Nonpretreated milled (0.84 mm screen) poplar was used at 50 g/L on a dry-weight basis for the preparation of sterile MTC media for small scale CBP screening. Media for each poplar natural variant were prepared anaerobically at 50 mL culture volume in independent biological duplicates, as previously described.<sup>11</sup> *C. thermocellum* ATCC 27405, grown in freshly prepared cultures, was used at 10% v/v as inoculum of CBP bottles. Aqueous samples were collected from each bottle immediately after inoculation and following 96 h of incubation at 60 °C with constant shaking. Ethanol content was quantified against known standards by HPLC according to standard procedure NREL/TP-510-42623.<sup>17</sup>

Replicate samples of nonpretreated milled poplar were also subjected to an SHF bioconversion process according to previously described methodology.<sup>11</sup> In short, poplar samples were first enzymatically hydrolyzed at 50 °C for 5 days with a mixture of Cellic Ctec2 cellulases (Novozymes North America, Franklinton, NC, United States), Htec2 hemicellulases (Novozymes North America, Franklinton, NC, United States), and the Novozymes 188  $\beta$ -glucosidase (Novozymes North America, Franklinton, NC, United States), and the resulting aqueous sugars were then fermented at 35 °C for 3 days with *Saccharomyces cerevisiae* D7A ATCC 200062 (American Type Culture Collection, Manassas, VA, United States). At the end-point, fermentation ethanol yield was determined by HPLC with the same procedure utilized for the CBP process.

**Simons' Stain.** The Simons' stain procedure was based on published methodology, and no modifications were made.<sup>11</sup> The DO15 and DB1 dyes, which were supplied by the Pylam Products, Corp. (Garden City, NY), were prepared in Milli-Q water (10 mg/mL). The DB1 dye solution was used as prepared. The DO15 dye was filtered through a 100 K membrane on an Amicon ultrafiltration apparatus (EMD Millipore Corp., Billerica, MA) under 200 kPa of nitrogen gas to remove low molecular mass components from the dye (<100 kDa). Following filtration, 1 mL of the concentrated DO15 dye was weighed and dried for 3 d in a 105 °C oven. After the 3 d drying period, the mass of DO15 was recorded, and the concentrated solution was diluted to 10 mg/mL. Standard curves were constructed individually for the orange and blue dyes and were measured at 455 and 624 nm. Each tube contained 0.1 mL of phosphate buffered saline solution (aqueous 0.3 M sodium phosphate and 1.4 mM sodium chloride at pH 6). DO15 or DB1 were added to test tubes in their respective volumes for a total of 7 test tubes (0, 0.1, 0.2, 0.3, 0.4, 0.6, and 0.8 mL). The test tubes were diluted to volume (1 mL) with water.

Untreated *P. trichocarpa* (10 mg) was weighed into 7 centrifuge tubes. DO15 and DB1 were added in a 1:1 ratio in increasing concentrations. Phosphate buffered saline solution was added to each test tube (0.1 mL), and water was added to dilute the mixture to volume (1 mL). The mixture incubated at 70 °C for 6 h in a shaking incubator and subsequently centrifuged at 10252 g for 5 min to separate the remaining dye from the biomass. The supernatant was recovered and diluted, and its absorbance was measured on a Lambda 35 UV–vis spectrophotometer at 455 and 624 nm (PerkinElmer, Waltham, MA). The absorbance was transformed into the O:B ratio according to calculations from Chandra et al.<sup>18</sup>

**Klason Lignin Content Determinations.** Klason lignin contents were determined using a modified version of NREL's Determination of Structural Carbohydrates and Lignin in Biomass. Dry, milled (0.84 mm screen) poplar ( $0.175 \pm 0.005$  g) were weighed in flat-bottomed tubes, and 1.5 mL of 72% v/v sulfuric acid (VWR North America, Radnor, PA) was subsequently added. The tubes were placed into the Digiblock digital block heater (Sigma-Aldrich, St. Louis, MO) to maintain the temperature at 30 °C and stirred every 3 to 5 min for 1 h. Each sample was diluted to 42 mL while minimizing Klason lignin losses. The diluted samples were autoclaved for 1 h at 121 °C and cooled to room temperature. Following the secondary acid hydrolysis, the samples were diluted to volume (50 mL). The hydrolysate was

filtered through G8 glass filters to separate sugars from the Klason lignin. The remaining solids (Klason lignin) were heated at 105 °C and weighed 24 h later to determine Klason lignin content. The Klason lignin content was calculated by determining the mass (g) of lignin that remains after 24 h as a percentage of the initial mass of the biomass.

The sugars were diluted accordingly and analyzed on a high-performance anion exchange chromatography with pulsed amperometric detection (Dionex ICS-3000, Dionex Corp., United States). The instrument was equipped with a PC10 pneumatic controller, CarboPac PA1 guard column (2 × 50 mm, Dionex, Dionex Corp., United States), a CarboPac PA1 column (2 × 250 mm, Dionex), a conductivity detector, and an AS40 automated sampler. The eluent (0.2 and 0.4 M NaOH) flow rate was 0.4 mL/min, and the analysis duration was 70 min per sample. The sugar contents were quantified with five standard curves constructed from known glucose, mannose, galactose, xylose, and arabinose concentrations. Fucose (1 mg/mL) was used as an internal standard.

**Surface Roughness Measurements.** The logs for the surface roughness measurements were cut to form slices that were further cut to yield 100 mm-thick slices. The surfaces of the logs were sanded with 109 μm sandpaper<sup>19</sup> to mitigate irregularities on the log surface from cutting with the band saw. The logs were held at 65% relative humidity until the moisture content of the wood was approximately 12%.<sup>20</sup> The surface roughness measurements were carried out at room temperature with a stylus type profilometer (Alpha Step D-600 Stylus Profiler, KLA Tencor, Milpitas, CA, United States) with a 5 μm diameter stylus tip and a 90° pin angle. The force of stylus tip was 5 mN, and the tracing length spanned 2 mm at a scanning 50 μm/s. The data were acquired in 2D.

**Cellulose Degree of Polymerization.** Cellulose degrees of polymerization (DP) were obtained following delignification of *P. trichocarpa* with peracetic acid (3.5 g/g biomass).<sup>21</sup> Cellulose and hemicellulose were subsequently separated from the resulting holocellulose using two successive sodium hydroxide treatments. During the first treatment, 17.5% v/v sodium hydroxide was added to the holocellulose and stirred for 2 h. The NaOH was then diluted to 8.75% v/v and stirred for an additional 2 h. Cellulose, which was insoluble in solution, was separated from hemicellulose through filtration through a 0.45 μm polyamide filter. The cellulose was washed via filtration with Milli-Q water, frozen, and freeze-dried. The dry cellulose was vacuum-dried overnight and derivatized with phenyl isocyanate (0.5 mL) in pyridine (4 mL). The derivatization took place over 3 d at 55 °C, and the derivatized cellulose was recovered through precipitation in 7:3 methanol:water. The derivatized cellulose was separated from the solution with centrifugation at 8228 g for 10 min, washed with 7:3 methanol:water once, and rinsed two additional times with Milli-Q water. The solid was air-dried and vacuum-dried prior to dissolution (1–2 mg/mL) in the mobile phase (THF).

**Statistical Analysis.** Outlier detection was assessed using the modified z-score.<sup>11</sup> Any datum possessing a modified z-score of ±1.5 was considered an outlier and removed from the data set. Statistical analyses for the Simon's stain were conducted on the orange and blue dyes individually for the calculation of the O:B ratio. One-way ANOVA and Tukey's tests were used at 95% confidence to determine significant differences between means.

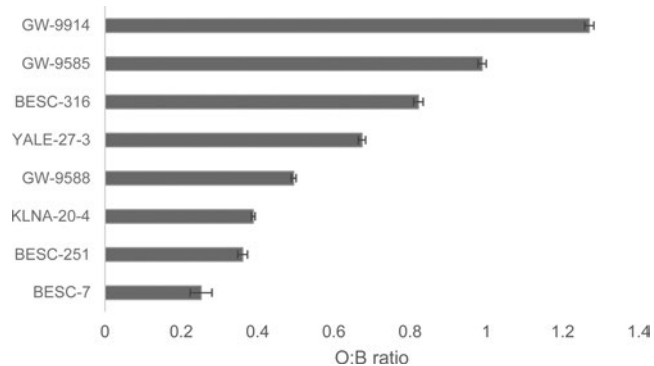
## RESULTS AND DISCUSSION

**Wood Density.** Significant phenotypic variation was observed for the 462 *P. trichocarpa* genotypes at both field sites. At Clatskanie, wood densities ranged from 0.22 to 0.72 g/cm<sup>3</sup>, while ranges of 0.29 to 0.55 g/cm<sup>3</sup> were observed at the Corvallis field site. Furthermore, the wood density phenotypes exhibited significant levels of reproducibility across the two field sites ( $r^2 = 0.35$ ,  $p = 0.0$ ). On the basis of these data, eight genotypes that exhibited consistently high and low density at both sites were selected for accessibility assessment (Table 1).

**Table 1. Densities (g/cm<sup>3</sup>) of the Eight Biomasses Determined by the Water Displacement Values**

	low density biomass	
	Clatskanie (g/cm <sup>3</sup> )	Corvallis (g/cm <sup>3</sup> )
GW-9585	0.32	0.33
BESC-316	0.33	0.35
GW-9914	0.34	0.35
GW-9588	0.34	0.35
	high density biomass	
	Clatskanie (g/cm <sup>3</sup> )	Corvallis (g/cm <sup>3</sup> )
KLNA-20-4	0.50	0.53
YALE-27-3	0.50	0.50
BESC-251	0.47	0.53
BESC-7	0.50	0.50

**Simons' Stain.** During Simons' staining, accessibility differences are quantified with the ratio of the orange and blue dyes bound to the biomass, where higher O:B ratios are associated with higher accessibilities. Figure 1 describes the



**Figure 1.** Association between Simons' stain O:B ratios and *P. trichocarpa* (harvested in Clatskanie, OR) densities (g/cm<sup>3</sup>).

natural variants and their associated O:B ratios. Although the O:B ratios are not optimally grouped into four high and four low extremes, the data illustrate that higher O:B ratios and therefore higher accessibilities ( $r^2 = 0.53$ ) were generally associated with lower density poplar. Theoretically, lower density wood has more void space as density and porosity are inversely correlated.<sup>22</sup> Although the Simons' stain is commonly applied to pretreated substrates,<sup>9,18,23–25</sup> this study demonstrates that accessibility differences can be observed in nonpretreated biomass with Simons' staining.

**Cellulose DP.** Scanning electron microscopy imaging data has suggested that cellulose structural changes (i.e., fragmentation and deformation) result from pretreatments.<sup>26</sup> Accordingly, these changes may influence the cellulose fiber's interior and exterior surface areas, which are assessed during Simons' staining. Though the *P. trichocarpa* in this study were not pretreated, we assessed cellulose DP in the natural variants to establish its relationship to accessibility on this basis. Although the cellulose DPs varied by as much as 4000 units between natural variants (Table 2), it appears to be unrelated to O:B ratio ( $r^2 = 0.17$ ). Furthermore, it appears that cellulose DP are not grouped into high and low extremes as densities are, which further supports the lack of relationship.

**Klason Lignin.** The Klason lignin contents of the 8 natural variants, which varied between 20.3 and 25.1%, are displayed in Figure 2. Klason lignin contents may influence the progression



**Table 2. Cellulose DP and Polydispersity Indices (PDI) from the Low and High Density *P. trichocarpa***

		cellulose DP	SD <sup>a</sup>	PDI	SD <sup>a</sup>
high density	YALE-27-3	2939	56	4.18	0.20
	BESC-251	5452	28	4.11	0.13
	KLNA-20-4	5966	20	6.65	0.03
	BESC-7	3207	114	3.11	0.49
low density	GW-9914	7250	68	3.59	0.11
	GW-9585	5436	64	4.46	0.10
	GW-9588	5870	418	3.97	0.30
	BESC-316	3932	4	3.07	0.0

<sup>a</sup>SD: standard deviation.

of CBP, as *C. thermocellum*'s cellulases have been found to adsorb to lignin.<sup>27</sup> Significant differences in Klason lignin contents existed between several poplars, including GW-9914 and BESC-7, GW-9914 and BESC-316, and GW-9914 and YALE 27-3 ( $P < 0.05$ ). Interestingly, Klason lignin contents exhibited a weak linear relationship with *O:B* ratio ( $r^2 = 0.33$ ). A linear plot between Klason lignin contents and *O:B* ratio revealed two relatively well-defined groupings of high Klason lignin contents and low accessibilities and vice versa (Figure 3). Hemicellulose contents did not demonstrate significant differences between BESC-251, GW-9914, and BESC-7 ( $P > 0.05$ ).

**Surface Roughness.** Surface roughness measurements were expected to identify whether the porosity extremes are manifested at the biomass surface where *C. thermocellum* is believed to carry out CBP. Surface roughness measurements were designed to determine if these irregularities influence *C. thermocellum*'s interactions with the wood surface and promote the hydrolytic activity of a CBP microorganism. In Figure 4, the low density biomasses generally possessed higher average roughness ( $R_a$ ), reduced valley depth ( $R_v$ ), and reduced peak depth ( $R_p$ ) values compared to those of the high density biomasses. The surface roughness measurements, similar to the density data, were grouped into two extremes (Figure 4), which verify that the porosity differences are manifested at the surface of the wood.

**Ethanol Yields from SHF and CBP.** Although SHF generated ethanol yields better than those of CBP, BESC-7 consistently produced low ethanol yields, whereas GW-9914 consistently produced one of the highest ethanol yields (Table 3). While the differences in ethanol production were not significantly different between BESC-251 and GW-9914 during CBP ( $P > 0.05$ ), the two means were found to be different in SHF ( $P < 0.05$ ). During SHF, the higher porosities may have facilitated cellulase entry during hydrolysis. The higher concentrations of glucose may account for the  $\sim 20$  mg/g discrepancy in ethanol yields between GW-9914 and BESC-

251, assuming the threshold for sugar inhibition was not reached. *S. cerevisiae* possesses impressive ethanol tolerance<sup>28</sup> and achieves high ethanol productivities,<sup>29</sup> which may also account for the higher ethanol yields during SHF than during CBP.

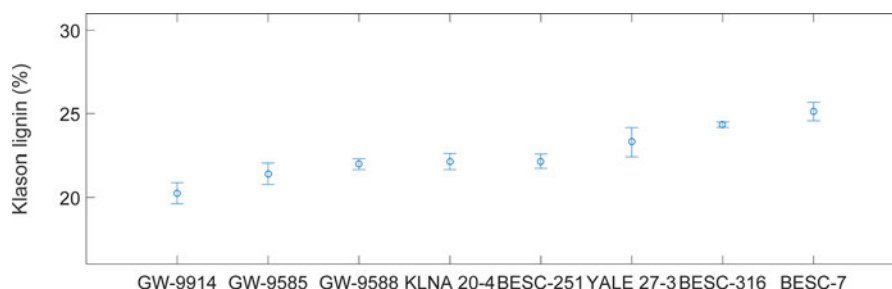
Surface irregularities, which are likely associated with porosity in this study, appear to be relevant to CBP with *C. thermocellum*. Surface roughness values identified GW-9914 and BESC-7 with the roughest and smoothest surfaces, respectively. Hence, Simons' staining porosity measurements can provide useful information about the biomass surface.

Klason lignin content may hold importance in the observed CBP trends. Total lignin content alone, when above 20% (w/w), has not been strongly correlated with improved enzymatic hydrolysis of poplar.<sup>30</sup> In this study, however, Klason lignin content and accessibility may be indirectly related to hydrolysis by fermentation yields. The best and poorest ethanol yielding biomass demonstrated noticeable differences in Klason lignin content and accessibility. GW-9914 possessed the lowest Klason lignin content, whereas BESC-7 possessed the highest. Furthermore, GW-9914 was predicted to be most accessible, whereas BESC-7 was predicted to be least accessible. Hence, the Klason lignin contents and accessibility appear to be related to the extent of fermentation.

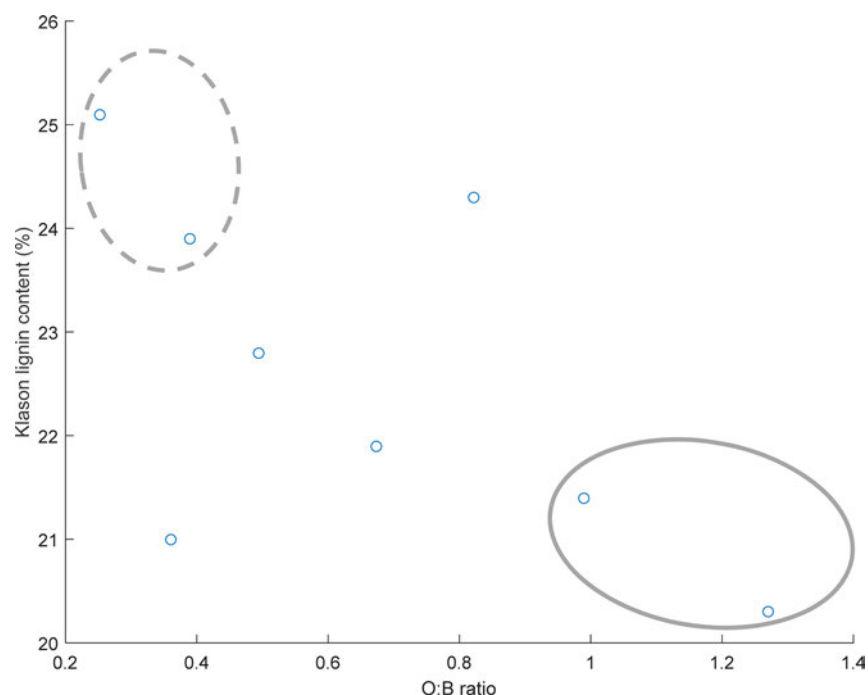
## DISCUSSION

Few studies have suggested that lignin content and/or structure play a role in the efficiency of CBP;<sup>31–33</sup> however, there is only one known study that establishes a relationship between the Simons' stain and CBP.<sup>11</sup> Neither the SHF nor the CBP ethanol yields clearly pointed toward a dominating effect of either variable; however, the two may be related, as higher Klason lignin contents may limit enzyme or cellulosomal accessibilities. Regardless, studies suggest that lignin content contributions among other factors impact ethanol production.<sup>34,35</sup> For example, Grabber et al. reported that lignin content, in addition to lignin–ferulate cross-linking, was more influential than lignin composition during fermentation. In Grabber's study, elevated Klason lignin contents (128 mg/g biomass) were linked to 12-fold increases in hydrolysis lag times compared to the nonlignified counterparts (5 mg/g biomass) when fungal cellulases were employed.<sup>34</sup> In this study, we observed much lower ethanol yields during CBP compared to those of SHF, which may be attributed to a more potent or confounded effect of lignin on *C. thermocellum*'s cellulases and cellulosome.

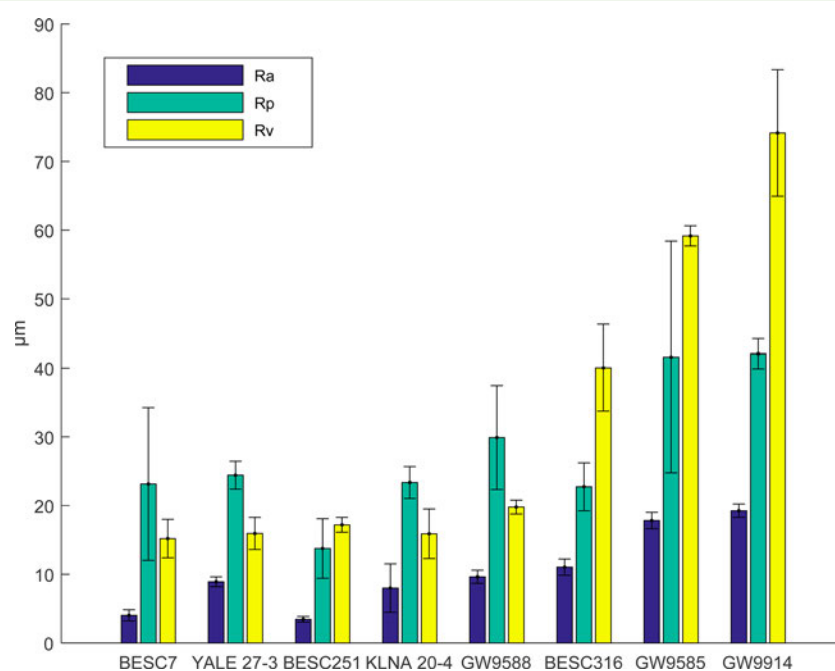
Although accessibility and density were related in this investigation, their relationship with ethanol yields, lignin content, and cellulose DP are less well-defined. There was no significant correlation between wood density and lignin content



**Figure 2.** Klason lignin contents of the eight natural variants of *P. trichocarpa*.



**Figure 3.** Association between *O:B* ratio and Klason lignin content (%). The dotted circle represents a high Klason lignin content and low *O:B* ratio, and the solid circle represents a low Klason lignin content and high *O:B* ratio.



**Figure 4.** Average roughness (Ra), reduced peak height (Rp), and reduced peak depth (Rv) of the high and low density biomasses.

in the 462 genotypes evaluated ( $r^2 = 0.004$ ,  $p = 0.92$ ); however, this observation suggests that the two phenotypes may have synergist but independent effects on cell wall deconstruction. During SHF and CBP, low and high density biomasses regularly produced ethanol yields that were not significantly different from one another ( $P > 0.05$ ). The observed trends in BESC-7 and GW-9914 suggest that Klason lignin contents play a role in *O:B* Simons' stain measurements. Meng et al. proposed that lignin exerts a minimal impact on cellulose accessibility; however, lignin likely limits xylan accessibility, which negatively influences cellulase accessibility to cellulose.<sup>4</sup>

Shao et al. formed similar conclusions, arguing that hemicellulose influences accessibility during CBP.<sup>36</sup> Higher Klason lignin contents may indicate a similar trend in lignin on hemicellulose accessibility and the binding of orange and blue dyes to cellulose.

The predicted least and most accessible poplar generated some of the worst and best ethanol yields, respectively. Klason lignin contents and accessibilities of the aforementioned poplar may work together in restricting ethanol yields. To further understand this occurrence, future studies can assess the CBP ethanol yields when lignin contents are not significantly

**Table 3. Ethanol Yields from SHF with *S. cerevisiae* and CBP with *C. thermocellum***

SHF			CBP		
	ethanol yield (mg/g biomass)	SD <sup>a</sup>		ethanol yield (mg/g biomass)	SD <sup>a</sup>
BESC-7	24.24	0.24	BESC-7	0.17	0.04
KLNA-20-4	30.44	1.12	BESC-316	0.34	0.03
GW-9585	33.41	0.57	GW-9588	0.35	0.01
BESC-316	35.96	0.36	GW-9585	0.50	0.08
GW-9588	37.34	1.29	YALE-27-3	1.69	0.16
YALE-27-3	39.23	0.68	KLNA-20-4	1.81	0.33
BESC-251	39.52	0.11	GW-9914	2.68	0.59
GW-9914	61.83	2.61	BESC-251	2.77	0.89

<sup>a</sup>SD: standard deviation.

different from one another and accessibility varies or when accessibilities remain constant while lignin contents vary. In this study, Simons' staining successfully identifies accessibility differences that are present in top- and poor-performing poplar, but Klason lignin content appears to be an important factor in ethanol yields. Accessibility alone, as predicted by Simons' staining, does not appear to be a sole predictor of biomass susceptibility to CBP.

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The manuscript was written by H.A., A.D. and W.M. All authors have given approval to the final version of the manuscript. J.N. and S.S.J. contributed equally.

### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

*C. thermocellum*: *Clostridium thermocellum*

CBP: consolidated bioprocessing

DP: degree of polymerization

O:B: direct orange to direct blue ratio

SHF: separate hydrolysis and fermentation

SSA: specific surface area

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