Bioavailability of carbohydrate content in natural and transgenic switchgrasses for the extreme thermophile *Caldicellulosiruptor bescii*

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ABSTRACT

Improving access to the carbohydrate content of lignocellulose is key to reducing recalcitrance for microbial deconstruction and conversion to fuels and chemicals. *Caldicellulosiruptor bescii* completely solubilizes naked microcrystalline cellulose, yet this transformation is impeded within the context of the plant cell wall by a network of lignin and hemicellulose. Here, the bioavailability of carbohydrates to *C. bescii* at 70°C was examined for reduced lignin transgenic switchgrass lines COMT3(+) and MYB Trans, their corresponding parental lines (cultivar Alamo) COMT3(-) and MYB WT, and natural variant cultivar Cave-in-Rock (CR). Transgenic modification improved carbohydrate solubilization by *C. bescii* to 15% (2.3-fold) for MYB, and to 36% (1.5-fold) for COMT, comparable to the levels achieved for the natural variant, CR (36%). Carbohydrate solubilization was nearly doubled after two consecutive microbial fermentations compared to one microbial step, but never exceeded 50% overall. Hydrothermal treatment (180°C) prior to microbial steps improved solubilization 3.7-fold for the most recalcitrant line (MYB WT), and increased carbohydrate recovery to nearly 50% for the least recalcitrant lines (COMT3(+) and CR). Alternating microbial and hydrothermal steps (T→M→T→M) further increased bioavailability, achieving carbohydrate solubilization ranging from 50% for MYB WT to above 70% for COMT3(-), COMT3(+) and CR. Incomplete carbohydrate solubilization suggests that cellulose in the highly lignified residue was inaccessible; indeed, residue from T→M→T→M treatment was primarily glucan and inerts (lignin and ash). While *C. bescii* could significantly solubilize the transgenic switchgrass lines and natural variant tested here, additional or alternative strategies (physical, chemical, enzymatic, and/or genetic) are needed to eliminate recalcitrance.

IMPORTANCE

Key to a microbial process for solubilization of plant biomass is the organism's access to the carbohydrate content of lignocellulose. Economically viable routes will characteristically minimize physical, chemical and biological pretreatment such that microbial steps contribute to the greatest extent possible. Recently, transgenic versions of plants and trees have been developed with the intention of lowering the barrier to lignocellulose conversion, with particular focus on lignin content and composition. Here, the extremely thermophilic bacterium, *Caldicellulosiruptor bescii*, was used to solubilize natural and genetically modified switchgrass lines, with and without the aid of hydrothermal treatment. For lignocellulose conversion, it is clear that the integration of microorganism, plant biomass substrate and processing steps must all be considered simultaneously to achieve optimal results. Whether switchgrass lines engineered for low lignin or natural variants with desirable properties are used, conversion will depend on microbial access to crystalline cellulose in the plant cell wall.

INTRODUCTION

The intrinsic recalcitrance of plant biomass renders its deconstruction a major technical and economic hurdle to the development of conversion processes for fuels and chemicals (1-3). Lignocellulose is composed of three tightly interconnected biopolymers – cellulose, hemicellulose and lignin (4, 5). For microbially-based bioprocesses, the bioavailability of the carbohydrate content of the plant cell wall is a critical factor in achieving high yields and conversion efficiencies. Carbohydrate access to microbial attack varies considerably with plant properties (6), but this issue must be addressed to develop optimal bioprocesses for generating bio-based products from renewable resources.

Extremely thermophilic bacteria in the genus *Caldicellulosiruptor* can metabolize the carbohydrate content of plant cell walls (7, 8). These gram-positive, anaerobic bacteria grow optimally at 70-78°C and co-ferment C5 and C6 sugars to generate acetate, lactate, H_2 and CO_2 as primary fermentation products (9-12). Thus, they offer a distinct advantage over microbes that can ferment only C6 sugars or that are subject to carbon catabolite repression (7). When encountering plant biomass, *Caldicellulosiruptor* responds by up-regulating carbohydrate ABC transporters and unique multidomain extracellular glycoside hydrolases (6, 8), enzymes that are crucial to the degradation capacity of the microbe (13, 14). The model organism in this genus, *Caldicellulosiruptor bescii*, not only utilizes unpretreated plant biomass (8, 15, 16), but has been genetically modified for improved crystalline cellulose degradation (17), xylan degradation (18), and ethanol production (19). These characteristics make *C. bescii* a promising metabolic engineering platform for consolidated bioprocessing (CBP) of lignocellulosic feedstocks. Given that *C. bescii* can utilize pentose sugars (hemicellulose) and crystalline cellulose (15), lignin is the major barrier preventing microbial access to the entire store of plant carbohydrates.

Approaches for improving microbial accessibility to the plant's sugars, thereby making them more susceptible to degradation, has primarily focused on either developing genetically

102 modified plants with reduced lignin composition or exposing the plant biomass to physical,
103 chemical and enzymatic treatments (1, 20, 21). In particular, genetic modification of feedstocks
104 to reduce and/or modify lignin structure can improve fermentation yield by increasing enzymatic
105 digestion and conversion of hemicellulose and cellulose (22-28). Switchgrass (*Panicum*
106 *virgatum* L.), one of the most promising renewable feedstocks (29, 30) in part due to its
107 geographic versatility, high biomass yields, and ability to grow on marginal land (low agronomic
108 input requirements) (29, 31-33), has been the focus of extensive efforts to further optimize its
109 use as a biofuels substrate through genetic engineering. Engineering strategies include
110 modifying lignin (e.g., altering the syringyl:guaiacyl lignin monomer ratio), decreasing cellulose
111 crystallinity, increasing plant polysaccharide content and overall plant biomass, and expressing
112 recombinant cellulases and hemicellulases in the plant (34). Two such efforts of interest here
113 are down-regulation of the caffeic acid 3-O-methyltransferase (COMT3) (23) and
114 overexpression of the R2-R3 MYB transcription factor PvMYB4 (MYB (25). The COMT enzyme
115 converts hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol to sinapaldehyde and sinapyl
116 alcohol (35), respectively; thus its down-regulation reduces S-lignin content (36). With
117 COMT3(+) switchgrass, the syringyl:guaiacyl lignin ratio was reduced and acetyl bromide (AcBr)
118 lignin was reduced 13% (~18.5% to ~16%) relative to the unmodified control line (23).
119 Saccharification efficiency with COMT3(+) switchgrass increased 29-38%, required a 3- to 4-
120 fold lower cellulase dosage for simultaneous saccharification and fermentation (SSF), and led to
121 higher ethanol yield (23). The R2-R3 MYB transcription factors comprise a large family of plant
122 proteins that play a role in a variety of plant processes including development, metabolism, and
123 responses to biotic and abiotic stresses (37). Among other effects, MYB4 specifically represses
124 the expression of the cinnamate 3-hydroxylase (*C4H*) gene (38), which encodes the enzyme
125 responsible for the conversion of cinnamic acid to 4-coumaric acid in the lignin biosynthetic
126 pathway (39). In the overexpressing switchgrass line (MYB4-OE), the ratio of ester linked *p*-

127 coumarate:ferulate (*p*-CA:FA ratio) was reduced ~50% together with a 40% reduction of AcBr
128 lignin (~25% to ~15%) relative to the unmodified control line (25). These changes to lignin in
129 the MYB Trans switchgrass led to a 3-fold increase in saccharification efficiency and ethanol
130 yield (25, 26).

131 Here, we examined the bioavailability of the carbohydrate content of several switchgrass
132 lines to *C. bescii* by assessing the bacterium's capacity to process unmodified Cave-in-Rock
133 switchgrass and two reduced lignin switchgrass lines (and their WT parent strains) derived from
134 the Alamo variety, each with and without hydrothermal treatment (180°C for 25 min). The results
135 of this study show (i) that the combined action of hydrothermal treatment and reduced-lignin
136 (genetically modified) switchgrass increases the bioavailability of plant carbohydrates to the
137 fermenting microbe (*C. bescii*) relative to the unmodified parental switchgrass line, (ii) that
138 hydrothermal treatment releases mostly hemicellulose (xylan and arabinan), which would be
139 preserved and consumed in an industrial bioprocess, and improves *C. bescii*'s access to glucan,
140 and (iii) that certain unmodified natural variants (e.g., Cave-in-Rock used in this study) may
141 inherently have reduced recalcitrance relative to the genetically modified developed lines,
142 enabling improved deconstruction and fermentation by *C. bescii*.
143
144 **MATERIALS AND METHODS**
145
146 **Bacterial strains and growth conditions.** *Caldicellulosiruptor bescii* was obtained as
147 freeze dried culture from the German Collection of Microorganisms and Cell Cultures (DSMZ
148 [http://www.dsmz.de]). *C. bescii* was grown anaerobically in a defined modified version of DSMZ
149 medium 671 (671d), as described previously (8), containing the specified switchgrass type as
150 the sole carbon source. Although the optimal growth temperature (T_{opt}) for *C. bescii* is 75°C (9),
151 Zurawski et al. (8) employed a growth temperature of 70°C for consistency during growth of

three different *Caldicellulosiruptor* species (containing different T_{opt}). Thus, to be comparable to the work in (8), we grew *C. bescii* at 70°C. Switchgrass was prepared as described below. Medium 671d pH was adjusted to 7.2 with 10 M NaOH and filter sterilized through 0.2 µm filters, prior to addition of switchgrass (nominally at 5 g L^{-1}), and prepared anaerobically under N_2/CO_2 (80/20 v/v) headspace. Unless otherwise specified, cultures were grown as 50 ml batch cultures in 125 ml closed serum bottles at 70°C, agitated at 100 RPM. Prior to all experiments *C. bescii* was passaged on the specified switchgrass 2 to 3 times at intervals of 1-2 days to allow for cell acclimation.

Biomass feedstocks. COMT3(+) and MYB Trans transgenic switchgrass and their corresponding unmodified parental genotypes COMT(-) and MYB WT (*Panicum virgatum L.* cv Alamo) were prepared, as described previously (23, 25). These were obtained from collaborators in the BioEnergy Science Center (BESC) and were gathered from senesced material from the second year of field studies at the University of Tennessee (40-42). COMT3(+) and MYB Trans were identified as the two best lines in sugar release studies (23, 25, 26, 43) and thus were chosen for analysis in this work. In these studies, COMT3(+) is often referred to as COMT-KD and MYB Trans as Myb4-OE, respectively. Cultivar Cave-in-Rock (CR) (*Panicum virgatum* L.) was field grown in Monroe County, Iowa, seeded in 2000, harvested in 2010, and obtained from the National Renewable Energy Laboratory (NREL).

Biomass substrate preparation. All varieties were mechanically ground and sieved to 20/80 mesh for closed bottle experiments. Material from several replicate plant clones was combined after milling. Hydrothermal treatment was done by first soaking switchgrass overnight at 4°C in water (9 mL g^{-1}). The switchgrass/water slurry was then centrifuged at 5,000 × g for 20 min, after which the supernatant was discarded and the switchgrass was loaded into stainless

steel tubular reactors (4 or 6 by 0.5 in, McMaster Carr), as described in (44). The reactors were pre-heated in boiling water for 2 minutes and then transferred to a fluidized sand bath (SBS18, Techne) at 180°C for 25 minutes (23, 44, 45). The reactors were then immediately cooled in an ice bath. Hydrothermally-treated and untreated switchgrass were washed with water at 25˚C by centrifugation at 6,000 × g for 10 minutes and the supernatant was discarded. This was repeated (~4 times) until no sugars were present in the wash, as measured by HPLC (Empire 1515 separations module; Waters), using a refractive index detector (model 2414; Waters). Washed switchgrass was oven-dried overnight at 70°C and used as growth substrate in all experiments. All switchgrass types were not autoclaved to eliminate the confounding effect of further treatment prior to use in experiments. At the elevated temperature of *C. bescii* growth, autoclaving to prevent growth by contaminants was not necessary.

Switchgrass solubilization experiments. Batch cultures (50 ml) were prepared in triplicate on 671d medium with specified switchgrass (5 g L^{-1}), inoculated with 1 x 10^6 cells/ml, agitated at 100 rpm, and incubated at 70˚C for 12 days. Run in parallel to solubilization cultures, the growth curve cultures (measure cell density) were carried out for an additional 2 days (14 days total). Solubilization cultures were harvested by centrifugation at 5,000 × g for 15 minutes. Residual substrate was washed with two volumes (100 mL) of 25˚C sterile water and oven-dried at 70˚C, until constant mass was achieved. The extent of solubilization was determined from the mass difference between switchgrass used to prepare cultures and insoluble substrate remaining after harvest. For the sequential microbial and hydrothermal co-treatment experiments, cultures were prepared and harvested, as described above, except with a 7-day incubation period. Residual switchgrass was then harvested, quantified for solubilization and divided; approximately half was hydrothermally-treated and washed, as described above. Solubilization by hydrothermal treatment was determined from the mass difference between

202 switchgrass loaded into the reactor treatment tubes and the switchgrass remaining after
203 washing. A second round of batch cultures using fresh medium was prepared in triplicate, as
204 described above, with either untreated or hydrothermally treated spent switchgrass, incubated
205 for 7 days, and harvested for solubilization determination. All switchgrass loadings for these
206 experiments were 5 g L^{-1}.
207
208 **Determination of switchgrass composition.** Carbohydrate content of switchgrass,
209 before and after incubation with *C. bescii*, was analyzed using a modified version of the NREL
210 Procedure (http://www.nrel.gov/biomass/analytical_procedures.html): Determination of
211 Structural Carbohydrates and Lignin in Biomass (46). Sulfuric acid (600 µl of 72% (w/w)) was
212 added to 40 mg switchgrass, and mixed using a glass stir rod. Samples were incubated in a
213 30°C constant temperature water bath for 70 minutes; samples were mixed with a glass rod
214 every 10 minutes. Sulfuric acid was then diluted to 4% (w/w) with 16.8 ml deionized (DI) water.
215 Tubes were sealed and autoclaved for 1 hour on the liquid cycle. Sugar concentrations were
216 determined using HPLC (Empire 1515 separations module; Waters), equipped with a refractive
217 index (model 2414; Waters) detector. Acetate, cellobiose, glucose, xylose and arabinose were
218 quantified using a Rezex-ROA column (300 mm by 7.8 mm; Phenomenex), operated with a
219 mobile phase of 5 mM H_2SO_4 at 0.6 mL/min, 60°C. The inert components (lignin and ash) were
220 determined as the difference between the mass of total carbohydrate and the total mass.
221
222 **Characterization of switchgrass for cellulose accessibility and crystallinity.**
223 Cellulose accessibility was completed using the Simons' stain procedure, as described in (47).
224 Direct orange 15 and direct blue 1 dyes were obtained from Pylam Products Company, Inc.
225 (Tempe, AZ, USA) and used in working concentrations of 10 mg L^{-1}. For direct orange 15, low
226 molecular weight components were removed by ultrafiltration through 100 K membranes (EMD

Millipore Corp) on an Amicon ultrafiltration apparatus. Following filtration, one mL of direct orange 15 was dried on a Petri dish in a 105°C oven over three days. The recovered solid was weighed and the concentrated solution was diluted to the working concentration (10 mg $L^{-}1$).

Switchgrass (~10 mg) was added to seven 2.5 mL centrifuge tubes. The dye mixture adsorption isotherm was determined by adding a series of 1:1 dye mixtures at increasing concentrations to each tube. Phosphate buffered saline solution (0.3 M Na_3PO_4 and 1.4 mM NaCl at pH 6) was also added (0.1 mL) to each test tube, and the final volume was adjusted to 1.0 mL with deionized water. Samples were incubated with shaking at 70°C for 6 h, then centrifuged at 10,250 x g. Supernatant was recovered and its absorbance was measured using the Lambda 35 UV-Vis Spectrophotometer (PerkinElmer). Calculation of the amount of dye absorbed (Orange (O): Blue (B) ratio) to the switchgrass was adapted from standard curves constructed from and equations located in (48).

For cellulose crystallinity (49), air-dried switchgrass (~1 g) was placed inside of Whatman cellulose extraction thimbles, and Soxhlet-extracted for 24 h in toluene:ethanol (2:1 v/v) under reflux. Following extraction, the biomass was air-dried overnight in the fume hood to evaporate residual solvent. Peracetic acid (5 g peracetic acid/g biomass) was added to 0.6 g of the extractive-free biomass, and delignification proceeded at 25ºC for 24 h. The de-lignified material was then refluxed in 2.5 M HCl at 100ºC for 1.5 h to hydrolyze hemicellulose. The mixture was cooled to room temperature and filtered to recover cellulose. The retentate was washed with water (100 ml) through filtration. The cellulose was analyzed on an attenuated total reflectance Fourier transform infrared spectrometer (Spectrum 100N, Perkin-Elmer, CT, USA), equipped with a ZnSe crystal. The spectral width was 4000 to 600 cm^{-1}, and the spectra were acquired with 32 scans.

Analysis of fermentation products and soluble sugars. Acetate and lactate were analyzed by HPLC using an Rezex-ROA column (300 mm by 7.8 mm; Phenomenex), operated with a mobile phase of 5 mM H_2SO_4 at 0.6 mL/min, 60°C. Samples were acidified to 0.05 wt% H_2SO_4 and detected by refractive index. Total saccharide content was determined by addition of 35 µl 72% (w/w) H_2SO_4 to 1 mL of supernatant. Screw top tubes were sealed and autoclaved for 1 h to hydrolyze soluble oligosaccharides to their respective monosaccharides. Samples were then analyzed by HPLC as described above.

RESULTS

Biosolubilization of untreated transgenic switchgrass. Cave-in-Rock (CR) switchgrass (field-grown, unmodified), two transgenic switchgrass lines, and their respective WT parent were assessed for deconstruction by *C. bescii* following 12 days post-inoculation at 5 g/L loading. CR was included in our analysis as a reference genotype, given that it has been previously studied as a biofuels feedstock (8, 32, 33, 50). In one transgenic switchgrass line, COMT3(+), the caffeic acid 3-O-methyltransferase was down-regulated, generating a transgenic switchgrass line with 13% lower AcBr lignin, altered lignin composition (less S-lignin), and a normal growth phenotype (23, 27, 28). In the other transgenic line, MYB Trans, the R2-R3 MYB transcription factor PvMYB4 was overexpressed resulting in switchgrass with 40% lower AcBr lignin, altered lignin composition (lower *p*-CA:FA ratio), as well as an improved tillering growth phenotype (25, 26). The corresponding parental lines, COMT3(-) and MYB WT, were also examined.

The recalcitrance of each switchgrass line was assessed by tracking *C. bescii* planktonic growth and the corresponding amount of carbohydrate that was solubilized. Growth and solubilization experiments were done at 70°C in small batch fermentations (closed serum bottles with 50 mL working volume) with washed switchgrass at a loading of 5 g/L. Low switchgrass

277 loadings were chosen to focus on the recalcitrance of the switchgrass by minimizing the
278 accumulation of inhibitory fermentative end products. *C. bescii* growth on all five untreated
279 switchgrass lines was biphasic exponential, with initial rapid growth (phase 1; 11.5 to 18.5 h)
280 followed by a markedly slower growth phase (phase 2; 24.0 to 60.5 h) (**Figure 1A**). Small
281 increases in *C. bescii* growth rate were observed with the transgenic switchgrass lines
282 (COMT3(+) or MYB Trans), relative to their corresponding parental line (COMT3(-) or MYB WT)
283 in both phase 1 and phase 2 (**Figure 1A)**. Larger differences in growth rate were seen between
284 the individual WT switchgrass genotypes (COMT3 vs. MYB vs. CR). For example, *C. bescii*
285 planktonic doubling times during phase 2 (t_{d2}), from fastest to slowest, were CR (16.9 h),
286 COMT3 (25.4 h (+), 26.8 h (-)), and MYB (84.8 h (Trans), 90.3 h (WT)) (**Figure 1A)**. The
287 maximum cell density and the time it took to get there also varied between switchgrass lines.
288 Fermentation of CR resulted in the highest cell density (5.2E+8 cell/mL at 244 h), followed by
289 COMT3(+) (2.8E+8 cells/mL at 244 h), COMT3(-) (2.9E+8 cells/mL at 316 h), MYB Trans
290 (1.9E+8 cells/mL at 316 h), and MYB WT (1.9E+8 cells/mL at 342 h). The *C. bescii* growth rate
291 was slowest on MYB switchgrass (WT and Trans), which corresponded to 10-30% points lower
292 carbohydrate solubilization than the COMT3 lines and the CR line (**Figure 1B, Microbe only**
293 **(M); Table S1**). The slower planktonic growth rate of COMT3(+) compared to CR (reference
294 genotype) did not reflect differences in solubilization, with both switchgrass lines decreasing by
295 ~36% in carbohydrate content (**Figure 1B**). Though minor differences in *C. bescii* growth were
296 observed between the respective transgenic and parental lines, transgenic modification
297 (reduced lignin content) improved carbohydrate solubilization considerably (**Figure 1B, M;**
298 **Table S1**). Solubilization of COMT3(+) (36.3 ± 1.%) was 1.5-fold higher than COMT3(-) (24.4 ±
299 5.4%) and solubilization of MYB Trans (15.0 ± 2.1) was 2.3-fold higher than MYB WT (6.4 ±
300 5.4%) (**Figure 1B, M; Table S1**). Taken together, the growth rate, cell density, and
301 carbohydrate solubilization results indicate large recalcitrance differences between the

individual genotypes (MYB (most recalcitrant) vs. COMT vs. CR (least recalcitrant)), and then among the transgenic vs. parental lines (MYB WT vs. MYB Trans and COMT3(-) vs. COMT3(+)). Interestingly, transgenic COMT3(+) and unmodified CR were considered to have comparable recalcitrance following one microbial pass (~36% carbohydrate solubilization) (**Figure 1B, M; Table S1**).

Glucan, xylan, arabinan solubilization across the less recalcitrant switchgrass lines (COMT and CR) showed that glucan and xylan in CR and all three polysaccharides in COMT3(+) were released in comparable amounts (**Table S1**). For the most recalcitrant genotype MYB (WT and Trans), hemicellulose (xylan and arabinan) accounted for most of the carbohydrate solubilized, indicating that the glucan (cellulose) was highly inaccessible.

Effect of hydrothermal treatment on transgenic switchgrass biosolubilization. Hydrothermal processing has been considered for biomass pre-treatment, since it produces limited amounts of inhibitory degradation compounds (i.e., furfural, hydroxymethylfurfural) (51-54). Hydrothermal (liquid hot water from 170 to 220°C (44)) processing acts to solubilize hemicellulose and causes structural changes in lignin, such that the cellulose becomes more accessible to hydrolytic enzymes and microbial attack (52, 55). To examine the effect of hydrothermal treatment on bioavailability to *C. bescii*, all five switchgrass lines were subjected to elevated temperatures (180°C for 25 min), followed by 12 days of *C. bescii* growth (**Figure 1B**, **T→M; Table S1**). While each specific component (glucan, xylan, arabinan, inert) was solubilized uniformly when only microbial treatments were used (M and M→M) (**Figure S1**), hydrothermal treatment primarily released hemicellulose and lignin, as indicated by an increase in glucan content and a decrease in hemicellulose (xylan and arabinan) and inert material (**Table 1)**. One of the clear advantages of hydrothermal treatment is the release of readily removed saccharides (e.g., hemicellulose (52, 55) and amorphous cellulose (56)) that could

easily be consumed by *C. bescii*. Here, the treated plant material was washed to not obscure the effect of hydrothermal treatment on *C. bescii*'s ability to access plant biomass carbohydrates that were not removed during heat treatment. The planktonic growth rate of *C. bescii* was 2-fold or faster on hydrothermally treated switchgrass relative to growth on untreated material for all switchgrass lines (**Figure 1A**), indicating that hydrothermal treatment improved the accessibility of the plant carbohydrates to *C. bescii* (**see Figure 1B, T→M**) compared to untreated switchgrass. Of particular note, the t_{d2} for MYB WT decreased from 85 h to 16.5 h. Cell growth on all hydrothermally treated switchgrass lines was comparable (t_{d1} ~ 2.1 to 2.6 h) during phase 1, with only slight differences noted during growth phase 2 (t_{d2} ~12.6 to 16.5 h) (**Figure 1A**). The faster growth rates on hydrothermally treated switchgrass corresponded to increased carbohydrate solubilization in all cases (**Figure 1B**, **T→M; Table S1**). The largest increase in carbohydrate solubilization as a result of hydrothermal treatment was for the more recalcitrant MYB WT and MYB Trans. For MYB WT, solubilization increased from 6.4% to 23.5% (3.7-fold) due to hydrothermal treatment. Solubilization of hydrothermally treated and genetically modified MYB switchgrass (MYB Trans) resulted in greater total solubilization than MYB WT, increasing from 15.0% to 33.0% (2.2-fold), but the effect of hydrothermal treatment was smaller (**Figure 1B**, **T→M; Table S1**). In contrast, COMT3(-) and COMT3(+) had the smallest improvements in carbohydrate solubilization following hydrothermal treatment, increasing from 24.4% to 40.9% (1.7-fold) and 36.3% to 47.8% (1.3-fold), respectively (**Figure 1B**, **T→M; Table S1**). Likewise, the reference line CR increased from 36.4% to 49.2% (1.4-fold). Overall, the least recalcitrant lines, COMT3(+) and CR, resulted in the highest levels of carbohydrate solubilization, and transgenic modification led to improvements over the corresponding parental line; however, the impact of hydrothermal treatment on biosolubilization diminished with lower recalcitrance (**Figure 1B**, **T→M; Table S1**).

Organic acid production (which reflects conversion of lignocellulose carbohydrates) correlated with carbohydrate solubilization for both untreated and hydrothermally treated material (**Figure S3**). The absence of any residual sugar in culture supernatants at the end of the fermentation indicated that sugars liberated from insoluble polysaccharides were completely converted to fermentation products (data not shown). Acetate was the primary fermentation product of both untreated and hydrothermally-treated material, with lactate produced in small amounts in cultures grown on hydrothermally-treated switchgrass; in *Caldicellulosiruptor* species, lactate production generally results from H_2 inhibition, diverting carbon flux away from acetate to lactate (57). Organic acid production was variable, with the highest values for hydrothermally-treated COMT3(+) and CR, 329 ± 7 and 326 ± 4 mg per g carbohydrate, respectively, and the lowest for untreated MYB WT at 103 ± 3 mg per g carbohydrate (**Figure S3**).

Sequential hydrothermal and microbial treatments. To determine if carbohydrate solubilization and conversion could be maximized, schemes involving multiple *C. bescii* microbial treatments (7 days each) and hydrothermal treatments of the switchgrass lines were evaluated. After two consecutive microbial treatments (M→M), fermentation of the transgenic switchgrass lines COMT3(+) and MYB Trans resulted in higher carbohydrate solubilization than their parental counterparts. For COMT3(+), carbohydrate solubilization reached 40.3 ± 2.4% vs. 34.6 ± 2.9% for COMT3(-). For MYB Trans, carbohydrate solubilization reached 27.5 ± 2.4% vs. 15.9 ± 3.3% for MYB WT (**Figure 2**; **Table S2**). CR had the highest carbohydrate solubilization at 43.3 ± 2.7%. Yet, after two consecutive *C. bescii* microbial treatments (M→M) of untreated switchgrass, bioavailable carbohydrate still remained (**Figure 2**). Next, a hydrothermal treatment step, either before (T→M→M) (**Figure 2A)** or between (M→T→M) (**Figure 2B)** two rounds of microbial deconstruction was included. Note that hydrothermally treated switchgrass was

376 washed prior to each microbial step and that solubilization resulting from hydrothermal
377 treatment was included in determining total carbohydrate solubilization (not included in **Figure**
378 **1**). Hydrothermal treatment alone, before two rounds of microbial treatment (T→M→M),
379 accounted for about ~30% of the total carbohydrate released for all switchgrass lines, with the
380 exception of MYB WT (22%) (**Figure 2A; Table S2**). On the other hand, the contribution of
381 hydrothermal treatment done between two rounds of microbial treatment (M→T→M) to
382 carbohydrate solubilization ranged from 16.7% for CR to 25.1% for COMT3(+) (**Figure 2B;**
383 **Table S2**). Hydrothermal treatment before two rounds of microbial growth (T→M→M) led to
384 higher carbohydrate solubilization for each switchgrass line than M→T→M. A single
385 hydrothermal treatment prior to sequential microbial treatments (M→M), increased carbohydrate
386 solubilization 1.6-fold (CR) to 2.9-fold (MYB WT) (**Figure 2; Table S2).** Transgenic lines that
387 were hydrothermally treated outperformed their corresponding parental lines by 18.9% points for
388 MYB Trans and 11.3% points for COMT3(+). The transgenic COMT3(+) yielded the highest
389 carbohydrate solubilization of all the lines with 75.1% (T→M→M), with its counterpart MYB
390 Trans yielding 64.5%, which was comparable to COMT3(-) (63.8%) (**Figure 2; Table S2)**.
391 As a final scheme, hydrothermal treatment was carried out both before and between two
392 rounds of microbial deconstruction (T→M→T→M) (**Figure 2C**), resulting in small but meaningful
393 increases in carbohydrate solubilization that ranged from 2.3% points (MYB Trans) to 5.7%
394 points (COMT3(-)) higher than the best single hydrothermal treatment scheme (T→M→M**)**
395 **(Figure 2A)**. Carbohydrate solubilization reached 78.5 ± 1.9% and 70.9 ± 2.4% for COMT3(+)
396 and CR, respectively (**Figure 2C**; **Table S2**); these were the highest levels achieved for any
397 lines with multiple treatment steps. The amount of carbohydrate solubilized from MYB Trans
398 (66.8%) was outperformed by the unmodified COMT3(-) (69.5%).
399

400 **Crystalline cellulose solubilization and glucan bioavailability in switchgrass.** After
401 7 days at 70°C, *C. bescii* is able to solubilize microcrystalline cellulose (Avicel) to near
402 completion in a single microbial step at 1-2 g/L loading in a closed bottle (**Figure S2**). When
403 plant biomass was tested at 5 g/L (~1.7-1.8 g/L glucan equivalency), *C. bescii* was only able to
404 solubilize up to 36% of the glucan (**Figure 1B, Table S1**). Not surprisingly, elements of the
405 complex lignocellulosic matrix make access to glucan difficult in the plant cell wall versus when
406 pure microcrystalline cellulose is available to *C. bescii*. However, improving microbial access to
407 glucan (34-37% of switchgrass mass) will be essential for high conversion of plant biomass to
408 product fuels and chemicals.
409 Hydrothermal treatment primarily released hemicellulose (xylan and arabinan) and led to
410 higher total carbohydrate solubilization than microbial treatment alone (**Figure 3**). It also
411 improved microbial accessibility to glucan but to varying degrees across the switchgrass lines
412 tested and depended on the placement of the hydrothermal treatment within the treatment
413 scheme (**Figure 3**). A hydrothermal treatment before two microbial treatments (T→M→M) led
414 to higher microbial glucan solubilization than in-between treatment (M→T→M) for only the MYB
415 lines (**Figure 3**), with MYB Trans resulting in higher microbial glucan solubilization than MYB
416 WT for both treatment schemes (**Figure 3 C,D**). A hydrothermal treatment before two microbe
417 passes (T→M→M) led to microbial glucan solubilization of 27% (MYB WT) and 35% (MYB
418 Trans); in contrast, a hydrothermal treatment in-between two microbial passes (M→T→M) for
419 MYB WT and MYB Trans resulted in microbial glucan solubilization of 19% and 31%,
420 respectively (**Figure 3**). A hydrothermal treatment in-between two microbial treatments
421 (M→T→M) led to higher glucan solubilization for the COMT3 lines and CR, with the transgenic
422 COMT3(+) resulting in the highest microbial glucan solubilization of all switchgrass genotypes.
423 For COMT3(-), COMT3(+), and CR, microbial glucan solubilization for M→T→M was 43%,
424 54%, and 47% and for T→M→M was 32%, 49%, and 45%, respectively (**Figure 3 A,B,D**).

When compared to successive microbial (M→M) treatments, the addition of a hydrothermal treatment (at the most effective placement) resulted in microbial glucan solubilization increases of 35% to 43% (COMT3(-)), 44% to 54% (COMT3(+)), 11% to 27% (MYB WT), 25% to 35% (MYB Trans), and 42% to 47% (CR) (**Figure 3**). Transgenic COMT3(+) switchgrass resulted in the largest percentage of glucan solubilized by *C. bescii*, while the largest fold-increase on microbial glucan solubilization from hydrothermal treatment came for the most recalcitrant line MYB WT (**Figure 3**). Hydrothermal treatment had the smallest effect on microbial glucan solubilization for CR (**Figure 3**).

In the final process scheme, the effect on glucan accessibility following alternating hydrothermal and microbial treatments (T→M→T→M) was examined in comparison to M→M (**Figure 3**). The second hydrothermal treatment had a minimal or negative impact on total microbial glucan solubilization for the less recalcitrant lines COMT3(+) (0%), CR (-9%), and COMT3(-) (-23%). Microbial glucan solubilization increased, relative to M→M, for MYB WT (+139%) and MYB Trans (+28%). Although T→M→T→M yielded the highest total carbohydrate solubilization among all the switchgrass lines tested (**Figure 2C**), the second hydrothermal treatment in comparison to the most effective single hydrothermal treatment (M→T→M or T→M→M) had a positive impact only on microbial glucan solubilization for MYB WT (increased 3%) and reduced microbial glucan solubilization for COMT3(-) (-37%), CR (-20%), COMT3(+) (-19%), and MYB Trans (-10%).

To examine recalcitrance more closely, switchgrass lines were tested for cellulose crystallinity and accessibility, before and after microbial deconstruction and hydrothermal treatments (T→M→T→M) (**Figure 4**). Mean cellulose crystallinity was reduced in all lines after exposure to extensive treatment (T→M→T→M), with the largest decrease in the lateral order index (LOI) seen with MYB WT (**Figure 4A**). Furthermore, the cellulose accessibility (O:B ratio) increased for all switchgrass lines following successive treatments (T→M→T→M) (**Figure 4B**),

450 presumably a result of hemicellulose removal. These increases in accessibility ranged from 3.8-
451 fold for COMT3(-) to 12.7-fold for MYB Trans. Compositional analysis of this material found
452 hemicellulose (xylan and arabinan) content to decrease for each switchgrass line following
453 treatments (**Table 2**). Arabinan composition was reduced to below detectable levels for all lines
454 except COMT3(-), which was reduced from 3.9 ± 0.3% to 0.8 ± 0.0% (**Table 2**). Xylan
455 composition was reduced the most in CR, with levels dropping from 25 ± 0.8% to 8.6 ± 0.2%.
456 Thus, the composition of the remaining switchgrass after T→M→T→M was primarily glucan and
457 inert material, with glucan concentrations that ranged from 45% (COMT3(+) to 60% (MYB WT)
458 and inert concentrations that ranged from 27% to 44% (**Table 2**).

459 Cellulose crystallinity and enzyme accessibility were also measured at every process
460 step from untreated material through two microbial and hydrothermal treatments in the
461 transgenic COMT3(+) (T→M→T→M) (**Figure 5**); the untreated results corresponded to previous
462 reports (49). Cellulose crystallinity was found to increase after one hydrothermal treatment (T),
463 likely indicating the removal of amorphous cellulose (**Figure 5A**). Correspondingly, cellulose
464 accessibility only slightly increased after one hydrothermal treatment (**Figure 5B, T**).
465 Subsequent microbial treatments then lowered cellulose crystallinity from 0.99 (T) to 0.95
466 (T→M→T→M) (**Figure 5A**). Cellulose accessibility increased 3-fold after the first microbial
467 treatment (**Figure 5B, T→M**). Compositional analysis of COMT3(+) found that xylan content
468 was reduced the most at this step, decreasing from 25% to 18% (**Table 3, T→M**). Further
469 hydrothermal and microbial treatments had only minor effects on cellulose accessibility. For
470 example, although the residual material after T→M→T→M had decreased cellulose crystallinity,
471 it also had decreased accessibility (**Figure 5**), thus was more recalcitrant than the untreated
472 switchgrass and still contained 45% glucan (**Table 3**).
473
474

475 **DISCUSSION**

476

477 A major challenge to converting lignocellulose into fuels and chemicals is the lack of
478 microbial access to the complex polysaccharides that comprise the plant cell wall. *C. bescii* is
479 unique in its ability to degrade and co-utilize sugars from both hemicellulose (C5) and cellulose
480 (C6). Thus, for this extremely thermophilic bacterium, lignin is the major barrier to complete
481 plant biomass conversion.

482 Transgenic switchgrass lines MYB Trans and COMT3(+), their respective parental lines
483 (cv Alamo), and a naturally-occurring, low-recalcitrant switchgrass (cv CR) were all solubilized
484 by *C. bescii* but to different extents. Significant variations in solubilization were seen between
485 the different parental lines (WT); this validates the observations previously reported (43) using
486 CBP with *Clostridium thermocellum.* While the *C. bescii* growth rate and carbohydrate
487 solubilization were higher on the transgenic lines relative to their parental line, both MYB lines
488 were especially resistant to microbial degradation. The low recalcitrance nature (higher
489 carbohydrate bioaccessibility) of Cave-in-Rock (upland cultivar) when fermented by *C. bescii*
490 compared to the Alamo lines (lowland cultivar) was unexpected given that lowland varieties
491 typically have higher cellulose content, higher biomass yields, lower ash content, and lower fiber
492 concentrations (58, 59). Agronomic factors such as precipitation, temperature, location, and
493 harvest date may influence the plant in ways that impact carbohydrate bioavailability (60, 61).
494 While the reason for differences in recalcitrance between the lines and resistance to *C. bescii*
495 degradation is currently unknown, lignin structure, monolignol ratios, surface accessibility (62)
496 and inhibitory compounds present in the plant are also likely to play a role (28, 63, 64).

497 The impact of hydrothermal treatment on carbohydrate solubilization was greatest for the
498 most naturally recalcitrant switchgrass line, MYB (WT and Trans). Primarily hemicellulose, and
499 very little glucan was solubilized by *C. bescii* in both the untreated MYB WT and MYB Trans.
500 However, following hydrothermal treatment, cellulose (glucan) solubilization dramatically

501 increased for both (**Figure 1B**). This suggests that hydrothermal treatment improved
502 accessibility to cellulose (see **Figure 4** and **6**). This is consistent with reports that hydrothermal
503 treatment removes/disrupts hemicellulose and lignin in the plant biomass matrix with minimal
504 effect on cellulose (52, 65). We would also expect that the greatest effect of pretreatment would
505 be on the most recalcitrant line (thus benefiting most from the removal of some xylan and the
506 lignin aggregation (66, 67). Lignin was solubilized concomitantly with carbohydrate by *C. bescii*
507 in all switchgrass lines and under all conditions examined here (**Figure S1**), consistent with
508 previous studies that showed uniform degradation ('onion peeling' mechanism) of all cell wall
509 components by *C. bescii* (16). The details of how this occurs are not yet clear, but possible
510 contributions from carbohydrate esterases in *C. bescii* acting in concert with other carbohydrate
511 active enzymes (cellulases and hemicellulases) to liberate lignin and carbohydrate cannot be
512 ruled out (68, 69).

513 Inhibition of microbial growth and fermentation processes is a concern when using
514 biomass from transgenic plants that have been subjected to treatment technologies, given the
515 release of a wide range of lignin-derived compounds (70). Previous studies with COMT3(+)
516 switchgrass found that down-regulation of caffeic acid O-methyltransferase resulted in the
517 generation of a novel monolignol (71). The COMT3(+) line also showed no significant change in
518 lignin molecular weight (72). The MYB Trans showed decreased levels of potential phenolic
519 inhibitors (26). Yee et al. (28) found that acid pretreated COMT3(+) switchgrass had an
520 inhibitory effect on fermentation by *C. bescii* and two other thermophilic bacteria. The results
521 presented here, however, show that *C. bescii* growth was apparently not inhibited by transgenic
522 modification or by hydrothermal treatment given that the growth rate increased on the
523 transgenics and on hydrothermally treated switchgrass (see **Figure 1A**). This indicates that the
524 fitness of *C. bescii* is not affected by modification of the lignin biosynthetic pathway in COMT3

525 and MYB and, furthermore, that hydrothermal treatment did not generate lignin by-products
526 inhibitory to *C. bescii* at least at these low biomass solid loadings.

527 By successive hydrothermal and microbial steps (T→M→T→M), over 70% of the
528 carbohydrate content of transgenic COMT3(+) (79%) and natural variant Cave-in-Rock (71%%)
529 could be achieved, with most attributed to the first hydrothermal and microbial treatment (T→M)
530 (69% and 61%, respectively) (**Figure 2D**). Additional hydrothermal and microbial treatments led
531 to diminished benefits, but still improved carbohydrate solubilization, modified the carbohydrate
532 composition, and altered structural characteristics. Given that hemicellulose was easily removed
533 following hydrothermal treatment, the final switchgrass material following T→M→T→M was
534 primarily glucan and inert material (lignin and ash). The initial hydrothermal treatment was
535 responsible for solubilizing the largest quantity of inert material (ranging from 31.6% (CR) to
536 55.5% (COMT3(+)), but left an inert component that was highly resistant to additional
537 hydrothermal and microbial solubilization attempts (**Figure 3**). Thus, the increase of inert
538 content in the final material after all treatments, suggests that lignin content (type) and structure,
539 both in the transgenic and parental switchgrass lines, likely become a limiting factor of microbial
540 accessibility to the 30% (COMT3(+)) to 70% (MYB WT) glucan still remaining (47, 73) (**Figure**
541 **3**). The inability of multiple microbial and hydrothermal treatment steps to overcome this
542 recalcitrance barrier and achieve near complete carbohydrate conversion suggests that further
543 treatments and/or genetic modifications to either plant or microbe may be necessary to achieve
544 80-90% solubilization, typically the goal of industrial processes. Other physical, non-chemical
545 attempts to improve switchgrass degradation achieved 68% carbohydrate solubilization (glucan,
546 xylan, and arabinan) when switchgrass was ball milled between two fermentation stages with *C.*
547 *thermocellum* (50). While *C. thermocellum* is unable to naturally metabolize the available
548 pentose sugars (hemicellulose) (74), physical treatments (hydrothermal and other) should be

549 considered in improving plant biomass degradation for eventual conversion into fuels and
550 chemicals.

551 This study establishes a baseline for evaluating *Caldicellulosiruptor* species for
552 conversion of transgenic and natural variant switchgrass feedstocks. Strategic combination of
553 hydrothermal treatment, biomass with reduced recalcitrance, and extremely thermophilic
554 bacteria is promising, especially if process heat integration can be optimized to minimize energy
555 costs associated with cooling between treatment and fermentation. The work described here is
556 consistent with hydrothermal treatment of other plant feedstocks, such as corn stover (75),
557 prairie cord grass (76), and poplar (77), which effectively removed hemicellulose and lignin,
558 improved sugar yields, and reduced recalcitrance. To date, hydrothermal treatment has been
559 primarily used to improve SSF with yeast (27, 28, 78, 79) and conversion with thermophilic
560 microorganisms (27, 80). Most recently, Yee *et al*. (28) in 2012 reported on the generation of
561 fermentation products by *C. thermocellum, C. bescii,* and *C. obsidiansis* grown on
562 hydrothermally treated wild-type and transgenic switchgrass (COMT3). When transgenic
563 switchgrass was used as the feedstock, there was a 10% and 4% increase in product
564 generation for *C. thermocellum* and *C. obsidiansis,* respectively (28). Yet with *C. bescii*, there
565 was no increase and the fermentation product concentration was low (~50 mg/g carbohydrate)
566 (28). However, the results presented here with *C. bescii* showed 164 to 326 mg organic acids
567 generated per g carbohydrates and an increase in organic acid production of 25% (COMT3(+))
568 and 43% (MYB Trans) when grown on hydrothermally treated transgenic switchgrass, relative to
569 their hydrothermally treated parental line (**Figure S3**). Given the recent improvements in *C.*
570 *bescii* genetics tools (81), there is an opportunity to improve the capacity and use of this
571 extremely thermophilic bacterium for lignocellulose deconstruction and conversion, as has been
572 recently demonstrated for enhancing xylan utilization (18). In this work, the transgenic
573 COMT3(+) led to the highest carbohydrate and highest microbial glucan solubilization, yet the

574 results of unmodified CR highlight the need to identify and target natural variant genotypes with
575 inherently low recalcitrance in future genetic modification efforts. Examination of the solid
576 residues may also provide indications of the remaining polymers and linkages that require
577 specific targeting *in planta*, microbially or via pretreatment. If plant and microbial metabolic
578 engineering can lead to processes that require at most only a single hydrothermal treatment
579 step, the prospects for an economically viable route to bio-based fuels and chemicals is
580 promising.
581

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583

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596

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861 **FIGURE CAPTIONS**

862

863 **Figure 1.** (**A**) Planktonic growth of *C. bescii* on microbe only treated (M) (filled circles),
864 hydrothermally-treated (T→M) (open squares), control (solid lines), and transgenic (dashed
865 lines) switchgrass lines. Phase 1 doubling times (t_{d1}) were calculated between 11.5 to 18.5 h
866 and phase 2 doubling times (t_{d2}) were calculated between 24.0 and 60.5 h. **(B)** Carbohydrate
867 solubilized by *C. bescii* (percent arabinan, xylan, and glucan of total carbohydrate) after 12
868 days. CR, Cave-in-Rock.

869

870 **Figure 2.** Carbohydrate solubilization after one hydrothermal treatment (hydrothermal) either
871 **(A)** before (T→M→M) two rounds of microbial deconstruction, **(B)** in-between (M→T→M) two
872 rounds of microbial deconstruction, or **(C)** a combination of two hydrothermal treatments and
873 two rounds of microbial deconstruction (T→M→T→M). Each fermentation with *C. bescii* was
874 carried out for 7 days. The indicated fold change is relative to two consecutive rounds of
875 microbial deconstructions (M→M) without hydrothermal treatment.

876

877 **Figure 3.** Percent glucan, xylan, arabinan, and inert material solubilized by *C. bescii* following
878 various microbial and hydrothermal treatment schemes for **(A)** COMT3(-) **(B)** COMT3(+) **(C)**
879 MYB WT **(D)** MYB Trans and **(E)** Cave-in-Rock switchgrass.

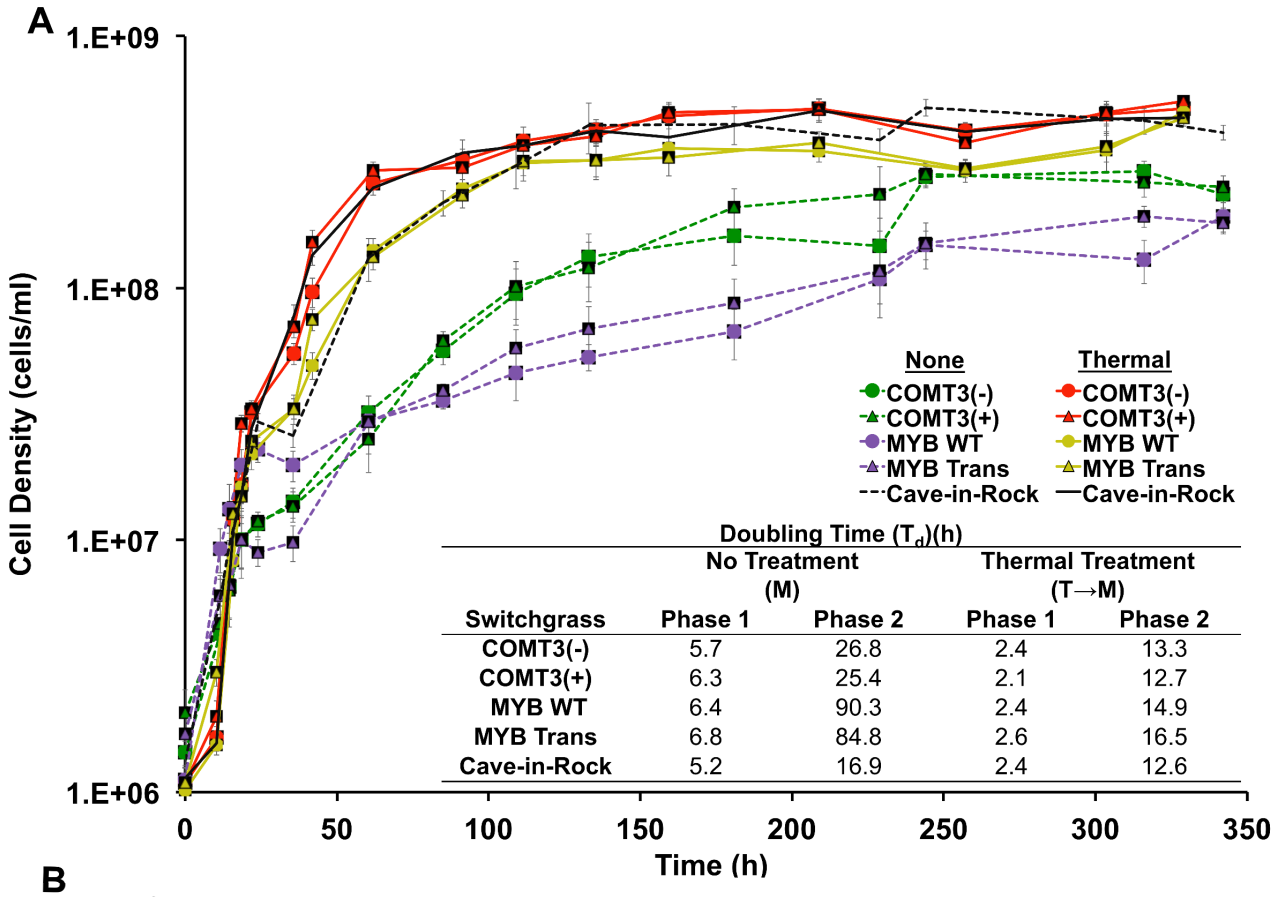
880

881 **Figure 4.** (**A**) Cellulose crystallinity and (**B**) enzyme accessibility of untreated switchgrass and
882 switchgrass lines after two hydrothermal and microbial deconstructions. CR, Cave-in-Rock

883

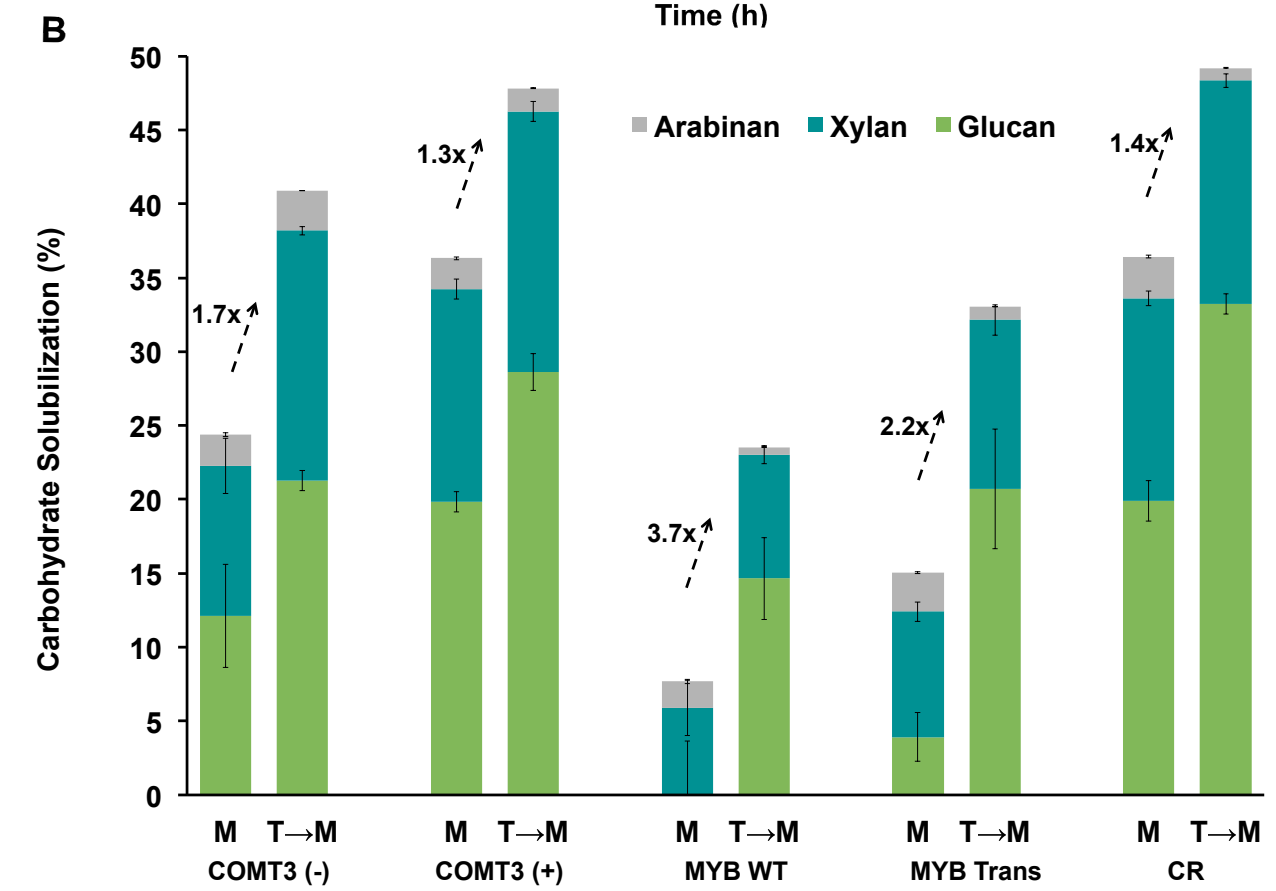
884 **Figure 5.** (**A**) Cellulose crystallinity and (**B**) enzyme accessibility of untreated COMT3(+)
885 switchgrass, after rounds of hydrothermal-treatment (T) and microbial deconstruction (M).

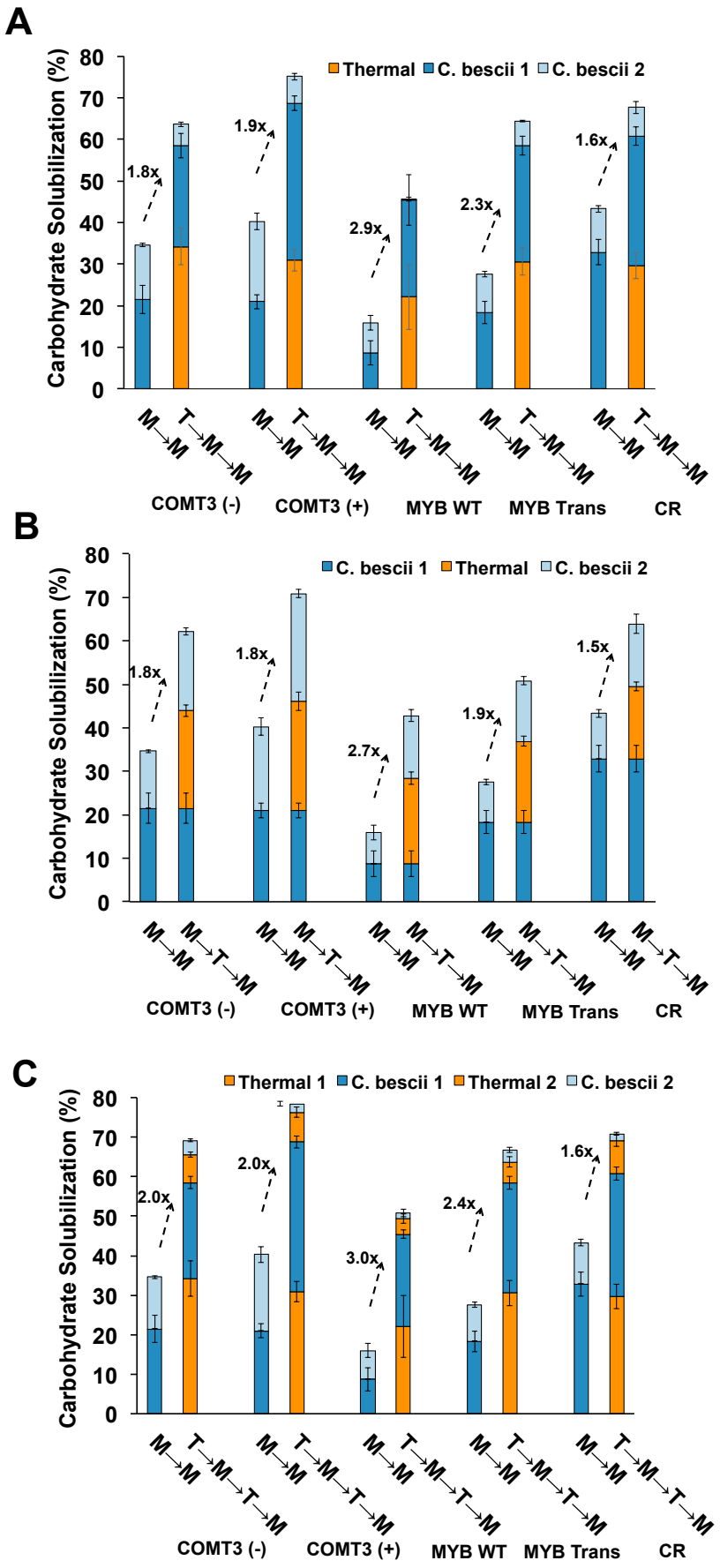
886

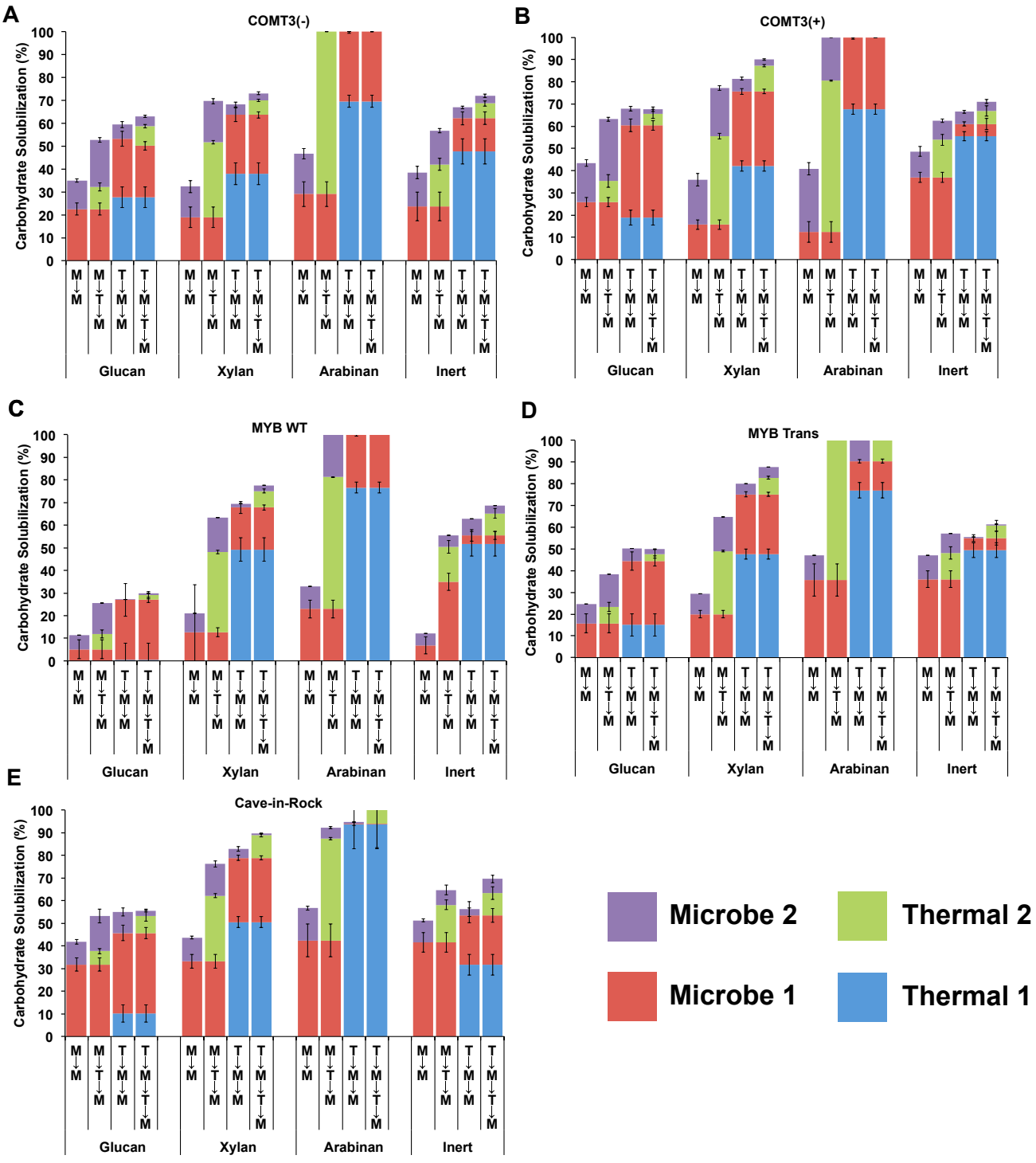


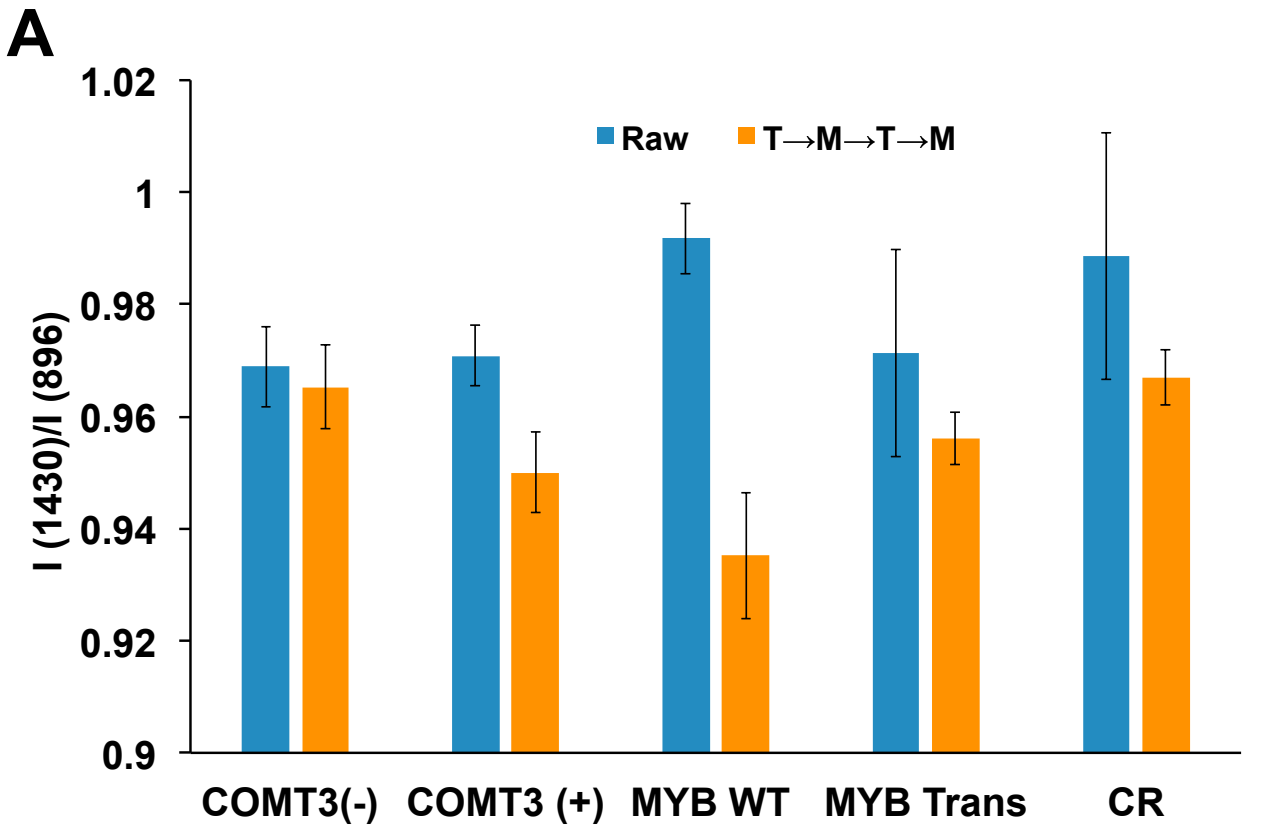
Doubling Time (T_d)(h)

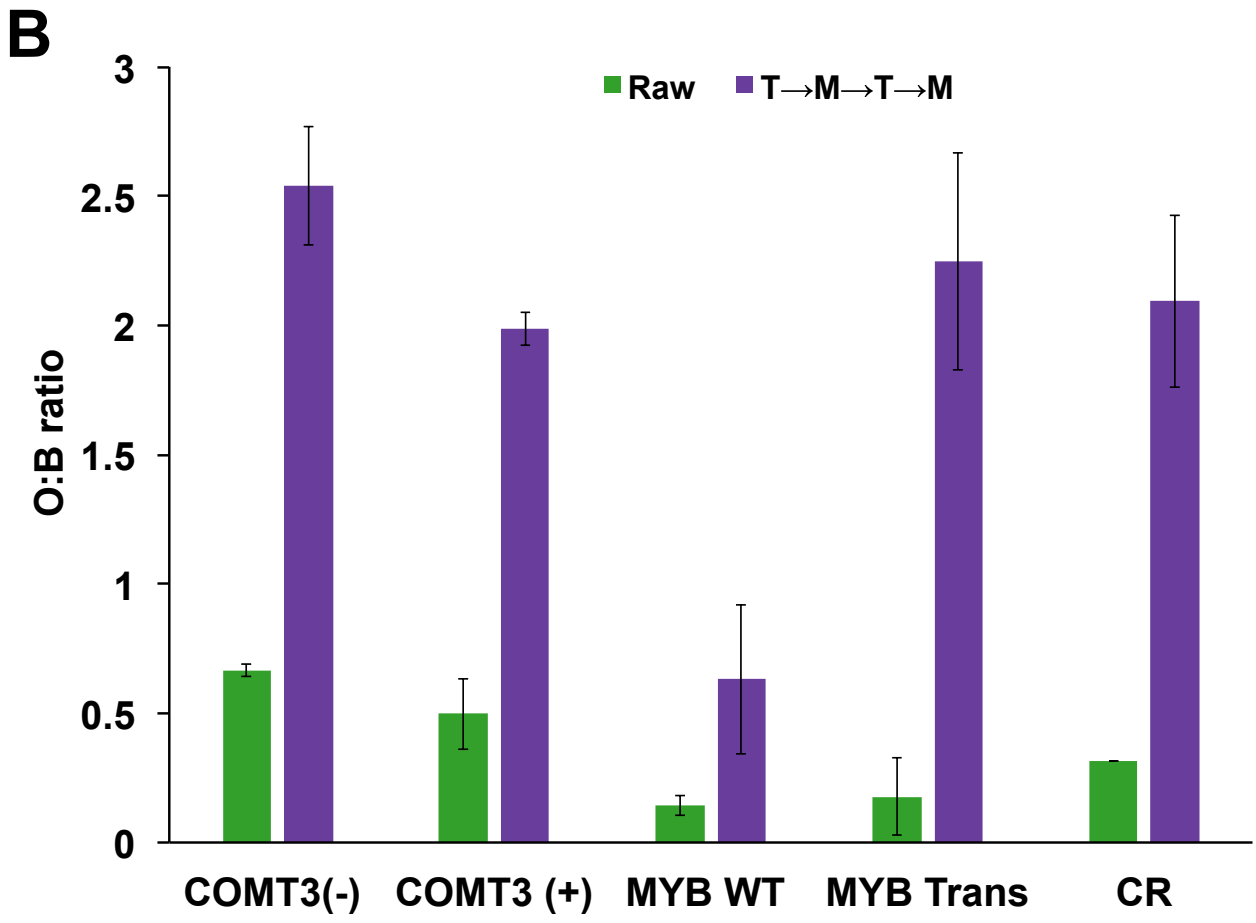
Switchgrass	No Treatment (M) Phase 1	No Treatment (M) Phase 2	Thermal Treatment (T→M) Phase 1	Thermal Treatment (T→M) Phase 2
COMT3(-)	5.7	26.8	2.4	13.3
COMT3(+)	6.3	25.4	2.1	12.7
MYB WT	6.4	90.3	2.4	14.9
MYB Trans	6.8	84.8	2.6	16.5
Cave-in-Rock	5.2	16.9	2.4	12.6











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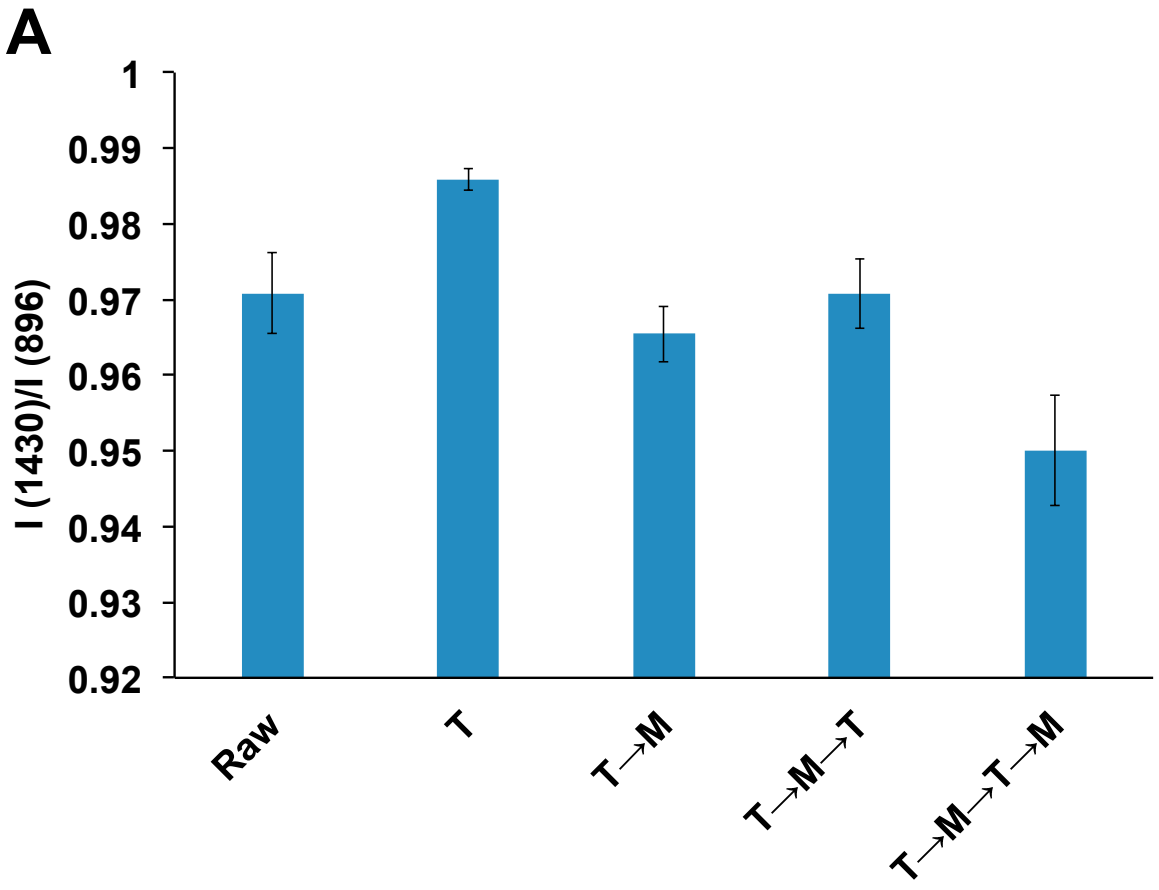

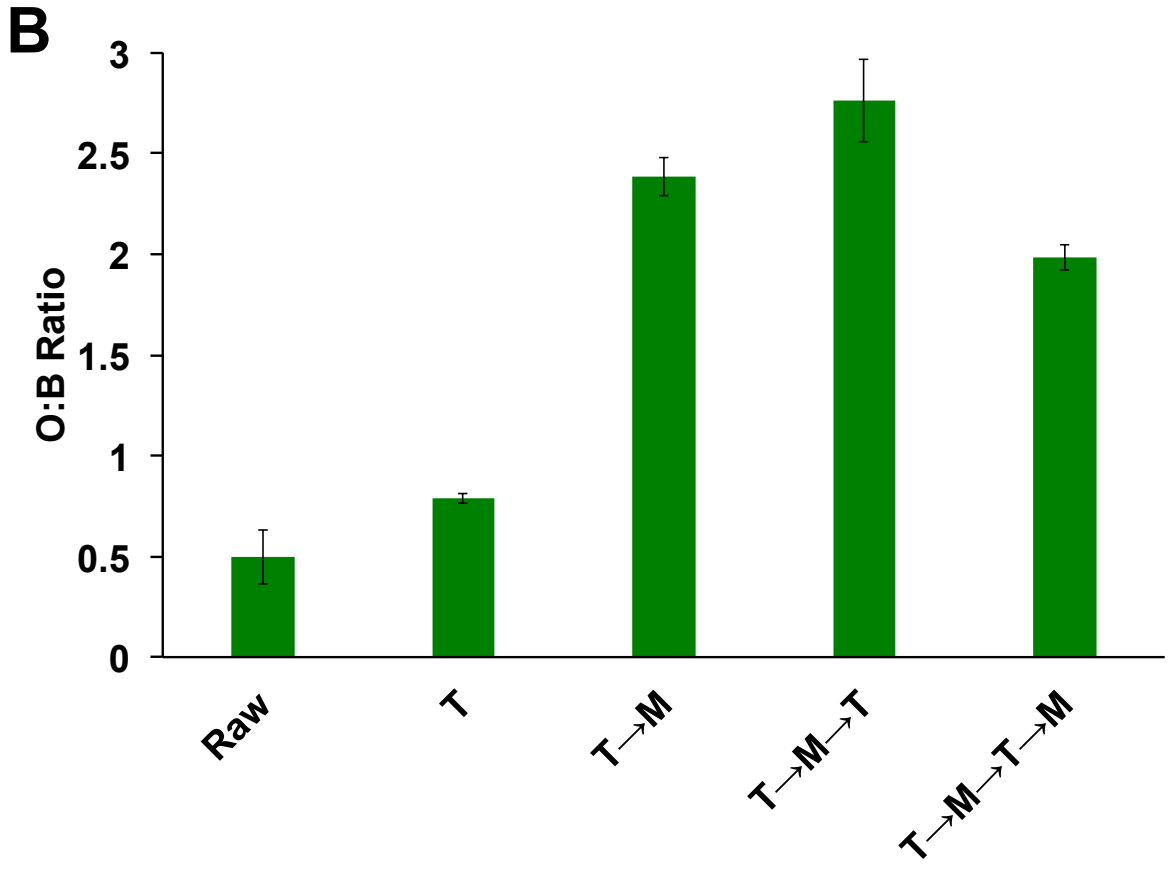

Table 1. Switchgrass composition before (Raw) and after hydrothermal treatment (T).

Switchgrass	Treatment	Carbohydrate (%)	Glucan (%)	Xylan (%)	Arabinan (%)	Inert (%)
COMT3(-)	Raw	66 ± 2.4%	36 ± 0.8%	26 ± 1.3%	3.9 ± 0.3%	34 ± 2.4%
	T	71 ± 1.8%	43 ± 1.1%	27 ± 0.7%	1.9 ± 0.0%	29 ± 1.8%
COMT3(+)	Raw	63 ± 0.9%	34 ± 0.7%	26 ± 0.3%	3.5 ± 0.1%	37 ± 0.9%
	T	73 ± 0.6%	46 ± 0.6%	25 ± 0.4%	1.9 ± 0.1%	28 ± 0.6%
MYB WT	Raw	63 ± 1.6%	37 ± 1.4%	24 ± 0.3%	2.6 ± 0.1%	37 ± 1.6%
	T	73 ± 1.0%	54 ± 0.5%	18 ± 0.5%	0.9 ± 0.0%	27 ± 1.0%
MYB Trans	Raw	65 ± 1.5%	37 ± 1.6%	25 ± 0.3%	3.3 ± 0.4%	35 ± 1.5%
	T	72 ± 1.0%	50 ± 0.7%	21 ± 0.4%	1.2 ± 0.1%	28 ± 1.0%
Cave-in-Rock	Raw	65 ± 2.1%	37 ± 1.1%	25 ± 0.8%	3.2 ± 0.3%	35 ± 2.1%
	T	65 ± 0.5%	47 ± 0.3%	18 ± 0.4%	0.3 ± 0.5%	35 ± 0.5%

Table 2. Switchgrass composition (%) for raw, non-treated (NT) material and material exposed to two microbial (M) and hydrothermal-treatments (T). Switchgrass was washed following each treatment step, which then served as the material for the next planned treatment. The average mass compositional values were calculated based off a 5 g/L initial loading. ND: None detected.

Switchgrass	Treatment	Glucan		Xylan		Arabinan		Inert	
		Conc. (%)	Mass (g)	Conc. (%)	Mass (g)	Conc. (%)	Mass (g)	Conc. (%)	Mass (g)
COMT3(-)	Raw	36 ± 0.8%	1.8	26 ± 1.3%	1.3	3.9 ± 0.3%	0.2	34 ± 2.4%	1.7
	T→M→T→M	46 ± 1.3%	0.7	17 ± 0.8%	0.4	0.8% ± 0.0%	0.0	37 ± 2.1%	0.5
COMT3(+)	Raw	34 ± 7.0%	1.7	26 ± 0.3%	1.3	3.5 ± 0.1%	0.2	37 ± 0.9%	1.9
	T→M→T→M	45 ± 1.2%	0.6	11 ± 0.2%	0.1	ND	0.0	44 ± 1.4%	0.5
MYB WT	Raw	37 ± 1.4%	1.8	24 ± 0.3%	1.1	2.6 ± 0.1%	0.1	37 ± 1.6%	1.9
	T→M→T→M	60 ± 0.9%	1.3	12 ± 0.4%	0.3	ND	0.0	27 ± 1.3%	0.6
MYB Trans	Raw	37 ± 1.6%	1.9	25 ± 0.3%	1.3	3.3 ± 0.4%	0.2	35 ± 1.5%	1.7
	T→M→T→M	53 ± 1.1%	0.9	8.8 ± 0.4%	0.2	ND	0.0	38 ± 1.3%	0.7
Cave-in-Rock	Raw	37 ± 1.1%	1.9	25 ± 0.8%	1.2	3.2 ± 0.3%	0.2	35 ± 2.1%	1.8
	T→M→T→M	55 ± 1.4%	0.9	8.6 ± 0.2%	0.1	ND	0.0	36 ± 1.5%	0.5

Table 3. COMT3(+) composition for no treatment (NT) and following microbial deconstruction (M) and hydrothermal-treatments (T). Switchgrass samples were washed following each treatment step, which then served as the material for the next planned treatment. ND: Not detected

Treatment	Glucan (%)	Xylan (%)	Arabinan (%)	Inert (%)
Raw	34 ± 0.7%	26 ± 0.3%	3.5 ± 0.1%	37 ± 0.9%
T	46 ± 0.6%	25 ± 0.4%	19 ± 1	28 ± 0.6%
T→M	39 ± 2.0%	18 ± 0.6%	ND	42 ± 2.5%
T→M→T	43 ± 14%	12 ± 0.4%	ND	45 ± 1.9%
T→M→T→M	45 ± 1.2%	11 ± 0.2%	ND	44 ± 1.4%