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Lignocellulose fermentation and residual solids characterization for senescent switchgrass fermentation by *Clostridium thermocellum* in the presence and absence of continuous *in situ* ball-milling†

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Milling during lignocellulosic fermentation, henceforth referred to as cotreatment, is investigated as an alternative to thermochemical pretreatment as a means of enhancing biological solubilization of lignocellulose. We investigate the impact of milling on soluble substrate fermentation by *Clostridium thermocellum* with comparison to yeast, document solubilization for fermentation of senescent switchgrass with and without ball milling, and characterize residual solids. Soluble substrate fermentation by *C. thermocellum* proceeded readily in the presence of continuous ball milling but was completely arrested for yeast. Total fractional carbohydrate solubilization achieved after fermentation of senescent switchgrass by *C. thermocellum* for 5 days was 0.45 without cotreatment or pretreatment, 0.81 with hydrothermal pretreatment (200 °C, 15 minutes, severity 4.2), and 0.88 with cotreatment. Acetate and ethanol were the main fermentation products, and were produced at similar ratios with and without cotreatment. Analysis of solid residues was undertaken using molecular beam mass spectrometry (PyMBMS) and solid-state nuclear magnetic resonance spectroscopy (NMR) in order to provide insight into changes in plant cell walls during processing *via* various modes. The structure of lignin present in residual solids remaining after fermentation with cotreatment appeared to change little, with substantially greater changes observed for hydrothermal pretreatment – particularly with respect to formation of C–C bonds. The observation of high solubilization with little apparent modification of the residue is consistent with cotreatment enhancing solubilization primarily by increasing the access of saccharolytic enzymes to the feedstock, and *C. thermocellum* being able to attack all the major linkages in cellulosic biomass provided that these linkages are accessible.

Broader context

Cellulosic biofuels are a leading option for decarbonization of a substantial fraction of the transport sector, but cost-competitive production using today's technology is impeded by process features related to overcoming biomass recalcitrance: thermochemical pretreatment and addition of saccharolytic enzymes. Here we investigate a different approach for addressing the recalcitrance barrier for liquid biofuel production. This approach involves fermentation by cellulolytic anaerobic bacteria without added enzymes, consolidated bioprocessing (CBP), augmented by milling during fermentation, cotreatment (CT). We show that *Clostridium thermocellum* – a thermophilic, cellulolytic bacterium – is able to ferment actively in the presence of continuous ball milling whereas yeast, widely used for industrial production of ethanol and other biofuels, is not. Processing *via* CBP-CT using *C. thermocellum* was found to achieve high fractional solubilization of senescent switchgrass without added enzymes and without thermochemical pretreatment other than autoclaving. The more limited modification of lignin-rich residues arising from CBP-CT as compared to hydrothermal pretreatment is potentially advantageous for production of coproducts, and thereby reducing the price of fuels and other products of carbohydrate conversion. These results support the potential for low-cost processing *via* CBP-CT, although much remains to be done to develop and assess this nascent processing paradigm following this initial report.

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Introduction

Biofuels will likely be needed for climate change mitigation at a scale most readily accommodated by cellulosic biomass,[1] are important for rural economic development and economic competitiveness today, and could play a larger role in this context in the future.[2,3] Lignocellulosic plant tissues have evolved to be resistant to deconstruction, and this recalcitrance is the most important barrier impeding low-cost processing of cellulosic feedstocks.[4,5] Thermochemical pretreatment involving heat and/or added chemicals has generally been thought to be necessary in order for biologically-mediated solubilization to occur at high yields.[6] Semi-commercial industrial facilities for cellulosic ethanol production that have come online over the last few years use thermochemical pretreatment and added enzyme preparations based on the cellulase system of aerobic fungi such as *Trichoderma reesei*. The high cost of current cellulosic biofuel production is attributable primarily to costs associated with thermochemical pretreatment and added enzymes.[7] Enzyme addition can in principle be avoided by using cellulolytic microorganisms to produce biofuels, a concept called consolidated bioprocessing,[8] although this approach requires further development to be practical.[9] Recent comparative studies indicate that thermophilic cellulolytic anaerobes, and in particular *Clostridium thermocellum*, are decisively more effective at deconstructing minimally pretreated (*i.e.* autoclaved under standard conditions used for sterilization) cellulosic biomass compared to industry standard preparations based on the cellulase system of *Trichoderma reesei*.[10,11] In particular, total carbohydrate solubilization achieved by *C. thermocellum* cultures was 2 to 4-fold higher than by β-glucosidase-supplemented commercial cellulase (Ctec2/Htec2) over a broad range of conditions, including 5 different feedstocks, >10-fold range of biocatalyst and substrate loading, >10-fold range of particle size, with and without yeast present, and different fungal cellulase incubation temperatures.

Mechanical disruption *via* milling has been considered as a means to increase the accessibility of plant cell walls to biological attack. Ball milling as a stand-alone pretreatment for enzymatic hydrolysis using fungal cellulase increases hydrolysis but is widely thought to be too energy-intensive to be practical.[12–15] Milling of cellulosic feedstocks during enzymatic hydrolysis in the absence of cells has received considerable study,[16–21] mostly during the 1980s and 1990s. Although substantial enhancement of hydrolysis was observed, energy requirements have not to our knowledge been reported and this approach has received little if any study in the last 15 years. More recently, milling has been shown to enhance the effectiveness of thermochemical pretreatment prior to hydrolysis using fungal cellulase preparations at the same time that pretreatment lowers the energy required for milling.[21–27] Paye *et al.*[10] showed that brief (5 min) ball milling of solids remaining after fermentation of unpretreated senescent switchgrass by *C. thermocellum* nearly doubled carbohydrate solubilization upon re-inoculation as compared to a control without ball milling between fermentations. Greater particle size reduction and solubilization were observed for ball milling of partially fermented solids than for unfermented solids.

Here we extend investigation of mechanical disruption during fermentation, termed cotreatment, in lieu of thermochemical pretreatment to increase the accessibility of lignocellulose to biological attack. In particular, we investigate the impact of continuous ball milling on soluble substrate fermentation by *C. thermocellum* with comparison to yeast, document solubilization during fermentation of senescent switchgrass with and without ball milling, and characterize residual solids pursuant to insights of interest in both fundamental and applied contexts. Whereas prior work has evaluated milling of cellulosic feedstocks prior to or during enzymatic hydrolysis in the absence of cells, this report is the first study that has investigated milling during microbially-mediated fermentation to enhance solubilization of lignocellulose pursuant to biological production of liquid fuels.

Materials and methods

Strain and growth medium

Clostridium thermocellum (*Ruminiclostridium thermocellum*) DSM 1313 (DSMZ, Braunschweig, Germany) was cultured in LC medium.[28] *Saccharomyces cerevisiae* D_5A (ATCC 200062, a gift from the National Renewable Energy Laboratory, Golden, CO) was cultured in a corn steep liquor medium.[29]

Substrates

Switchgrass (*Panicum virgatum*), Alamo variety, was harvested in December after senescence at the University of Tennessee Knoxville. Switchgrass stems were pre-washed as described in Garlock *et al.*,[30] and then milled with a Retsch mill (Haan, Germany) through a 2 mm screen. Substrate loading was 5 g L^{-1} glucan equivalent (~15.4 g L^{-1} total solids, 0.02 gram arabinan per gram total solid, 0.32 g per g glucan 0.21 g per g xylan). Cellobiose and glucose (for fermentations with *S. cerevisiae*) were obtained from Sigma (St. Louis, MO) and loaded at 5 g L^{-1} and 10 g L^{-1} respectively.

For hydrothermally pretreated biomass, the switchgrass was soaked overnight in deionized water at 5–7 wt% solids loading followed by squeezing of solids by hand to remove excess water and to bring the solids moisture content to about 70%. Uncatalyzed (hydrothermal) steam explosion pretreatment was performed in a 4L steam gun directly heated by steam from a steam boiler (FB-075-L, Fulton Companies, Pulaski, NY, USA) with the steam temperature controlled by setting the boiler pressure to the saturated steam pressure corresponding to the target temperature of 200 °C. Pretreatment was performed at 200 °C for 15 min. At the end of the reaction time, the temperature and pressure were suddenly dropped by opening a valve at the bottom of the vessel and pretreated solids were collected in a high temperature round bottom polypropylene drum liner (Cat #9772T48; McMaster, Los Angeles, CA) housed in a drum. The solids were then washed with DI water repeatedly to remove soluble sugars and degradation products that may have been generated during pretreatment.

Reactor configuration and cultivation conditions

Fermentations were conducted in a custom built bioreactor fabricated from a 4″-diameter section of Tri-Clover sanitary piping (Alfa Laval; Lund, Sweden) as shown in Fig. 1. Baffles made from 3/8″ OD stainless steel rod were welded to the inner walls spaced 2″ between rod centers. The agitator was made of the same material with the same spacing offset halfway between baffles. For runs with cotreatment, the reactor was filled with approximately 10 000 3/16″ (~4.8 mm) 316 Stainless Steel ball bearings totaling ~1 L bed volume (McMaster-Carr; Elmhurst, IL). Liquid fermentation volume was 600 mL for runs with ball milling and 1200 mL for runs without ball bearings or milling to maintain a consistent liquid height. For runs with cotreatment, the bed height was 80% of the height of liquid in the fermenter.

The cotreatment reactor was set up by layering beads and feedstock such that all feedstock is exposed to milling in the middle portion of the bed prior to starting ball milling. Once loaded the reactor was autoclaved at 121 °C for 1 hour and then incubated at 30 °C overnight while purging with 30 mL min^{-1} of 20%/80% CO_2/N_2 gas mix (Airgas; White River Junction, VT). Components of LC medium were added *via* syringe as five separate solutions as described in Holwerda *et al.*[28] Ball milling was initiated at 100 rpm immediately prior to inoculation.

Reactors were inoculated with a 5% v/v inoculum. The inoculum was grown in 125 mL sealed serum vials on 5 g L^{-1} Avicel® PH105 (FMC biopolymers, Philadelphia PA) in LC medium with 5 g L^{-1} 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (Sigma; St. Louis, MO), and incubated at 60 °C for 24 hours.

Lignocellulose fermentations were run for 5 days (120 hours) with or without constant milling at 100 rpm. The pH was maintained at 7.0 with 2 N KOH solutions and the temperature was controlled at 60.0 °C by a Sartorius Aplus control tower (Sartorius Stedim, Bohemia NY). 2 mL samples were collected every 24 hours for analysis by HPLC. Total gas production was measured *via* Milligascounter gas tip meters (Ritter, Bochum, Germany) filled with a solution of 0.5 N HCl.

Soluble substrate fermentations by *C. thermocellum* and *S. cerevisiae* D_5A were run for 24 hours. 2 mL samples were collected for HPLC analysis at 3 hour intervals.

Controls without inoculation were set up in the same manner as inoculated runs, and incubated for 5 days at 60 °C. Controls with ball milling were termed Milling Controls and controls without ball milling were termed Incubated Controls.

Fermentations with pretreated switchgrass were carried out in 0.5 L Qplus bioreactors (Sartorius Stedim, Bohemia, NY) with 300 mL working volume at 200 rpm and maintained at pH 7.0.

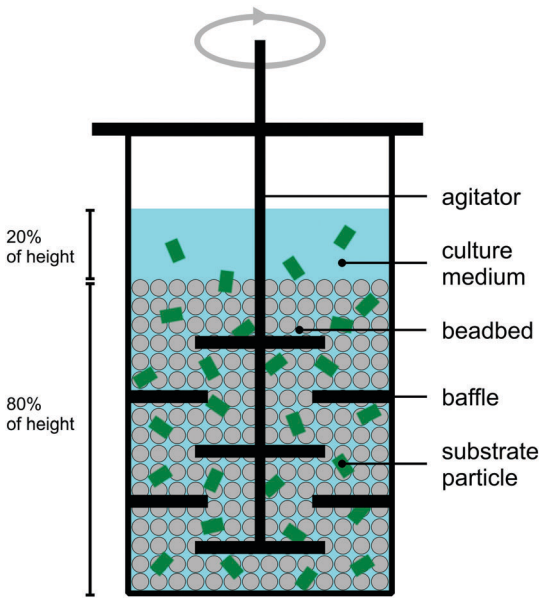

Fig. 1 Schematic of the cotreatment reactor with baffles and bed height shown.

Quantification of carbohydrates and fermentation products

Following fermentation, residual solids were harvested from the reactor by centrifugation at 11 325 × *g* for 10 minutes, washed with deionized water and centrifuged for another 10 minutes. Washed solids were dried for at least 48 hours at 50 °C.

The carbohydrate content of solids present initially and at the end of fermentation was determined by quantitative saccharification (QS) using 72% H_2SO_4 (Fisher; Waltham, MA) as described by Sluiter *et al.*[31] Acid-hydrolyzed sugars (glucose, xylose, and arabinose) were quantified by HPLC (Waters; Milford, MA) with refractive index detection after separation on an Aminex HPX-87H column (Bio-Rad, Hercules, CA) operated at 50 °C with a 5 mM H_2SO_4 eluent. Reported values for xylose and xylan include minor amounts of mannose and galactose which coelute with xylose on the HPX-87H column.

Analysis of liquid-phase components was carried out on the supernatant of centrifuged samples taken at time intervals as noted in the text. Fermentation products (acetate, ethanol, lactate, formate) were analyzed by HPLC as described above. Soluble sugars and oligomers were analyzed by spin filtering, adding 72% H_2SO_4 (0.56 mL sample with 0.02 mL acid), autoclaving for 1 hour, and HPLC analysis.

Calculation of total carbohydrate solubilization and carbon recoveries

The percent total carbohydrate solubilization, S_T, is calculated using eqn (1)

$$S_T = 100\% \cdot \left(1 - \left(\frac{(G_{S,f} + X_{S,f} + A_{S,f})}{(G_{S,i} + X_{S,i} + A_{S,i})}\right)\right) \quad (1)$$

where G denotes glucan or glucose (mmoles), X denotes xylan or xylose (mmoles), A denotes arabinan or arabinose (mmoles), S denotes solid, f denotes final, and i denotes initial.

Initial and final concentrations were determined by multiplying the solids concentration (g per L total solids) by the mass fraction of the sugar (*e.g.* g potential glucose per g total solid) obtained from quantitative saccharification and dividing by molecular weight (*e.g.* 180.16 g per mol for glucose). Recovery of total carbon originally present as carbohydrate, R_T^C, was calculated using eqn (2)

$$R_T^C = \frac{3 \cdot (Ac + E + Lc) + 6 \cdot (G_{S,f} + G_{L,f}) + 5 \cdot (X_{S,f} + X_{L,f}) + 5 \cdot (A_{S,f} + A_{L,f})}{6 \cdot G_{S,i} + 5 \cdot X_{S,i} + 5 \cdot A_{S,i}} \quad (2)$$

where E denotes ethanol (mmoles), Ac denotes acetate (mmoles), and Lc denotes lactate (mmoles), and the subscript L denotes liquid phase concentrations.

Pyrolysis molecular beam mass spectrometry (PyMBMS)

A molecular beam mass spectrometer (MBMS) designed specifically for biomass analysis (VeraSpec MBx, Extrel CMS) was interfaced to an automated pyrolysis unit (PY-2020iD, Frontier Labs) and was used for pyrolysis vapor analysis.[32] Approximately 4 mg of air dried 20 mesh biomass was introduced into the quartz pyrolysis reactor *via* 80 μL deactivated stainless steel Eco-Cups. Mass spectral data from *m*/*z* 30–450 were acquired on a Merlin Automation data system version 3.0 using 17 eV electron impact ionization. Using this system, both light gases and heavy tars are sampled simultaneously and in real time and the mass spectrum of the pyrolysis vapor provides a rapid, semiquantitative depiction of the molecular fragments derived from the plant cell wall.[33–35]

S/G ratios were determined by summing the syringyl peaks 154, 167, 168, 182, 194, 208, and 210 and dividing by the sum of guaiacyl peaks 124, 137, 138, 150, 164, and 178 (Table 2). Several lignin peaks were omitted in the syringyl or guaiacyl summations due to individual peaks having associations with both S and G precursors.[36]

Solid-state nuclear magnetic resonance (NMR) spectroscopy

Solid-state NMR spectra were collected on whole biomass and cotreatment residues using high-resolution ^{13}C cross-polarization/magic angle spinning (CP/MAS) with a Bruker Avance 200 MHz spectrometer (50.13 MHz).[37,38] The spinning speed was 6900 Hz and a contact time of 2 ms with a 1 dB ramp on the proton spin locking field was applied during cross polarization. The acquisition time was 24.2 ms and the recycle delay was 2 s. 30k scans were acquired for each sample with the exception of the cotreatment residue sample, which required 60k scans due to a limited amount of recovered sample.

The cotreatment sample contained residual metal from the ball milling process that was removed by sonication after suspension in an ethylenediamine tetraacetic acid (EDTA) solution before analysis by solid-state NMR spectroscopy. Biomass isolated from metallic residue during sonication floated to the surface and was recovered by filtration. The biomass residue collected after decantation was dried under vacuum overnight and then passed over a magnet to remove remaining metal particles. The residue was analyzed and compared to the original starting material using PyMBMS to ensure the composition of metal-free residue was not significantly changed during the isolation procedure (Fig. S1, ESI†).

Results

Effect of ball milling on soluble substrate fermentation by *Clostridium thermocellum* and *Saccharomyces cerevisiae*

Having previously shown that *ex situ* ball milling enhances solubilization by *C. thermocellum* cultures upon subsequent inoculation,[10] we sought to determine the impact of *in situ* ball milling on soluble substrate fermentation by *C. thermocellum.* Comparison was made to *S. cerevisiae*, with cellobiose used as the substrate for *C. thermocellum* and glucose for *S. cerevisiae.* As may be seen from Fig. 2, soluble substrate fermentation was slightly slowed down for *C. thermocellum* but prevented entirely for the yeast even after extended incubation for up to four days (data not shown).

Solubilization of senescent switchgrass by *C. thermocellum* with and without cotreatment

To determine the impact of cotreatment on solubilization of a representative lignocellulosic feedstock, we investigated

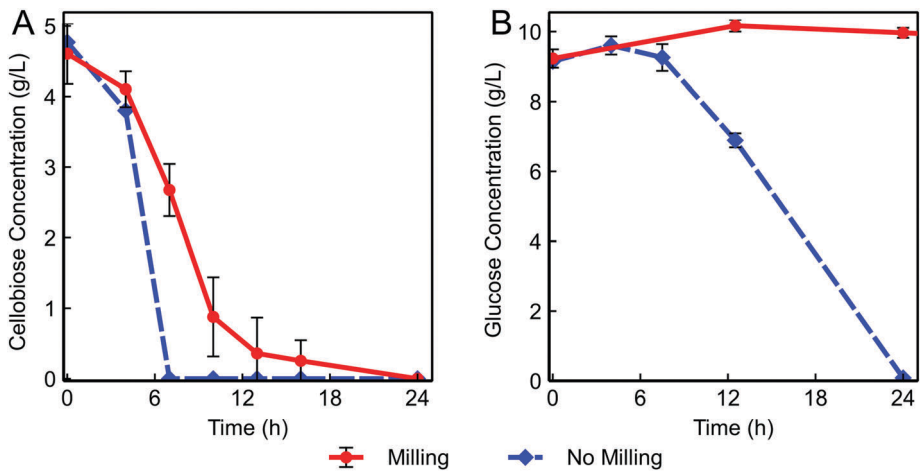

Fig. 2 (A) Cellobiose consumption by *C. thermocellum* and (B) glucose consumption by *S. cerevisiae* D_5A with and without ball milling (duplicate fermentations with error bars representing one standard deviation).

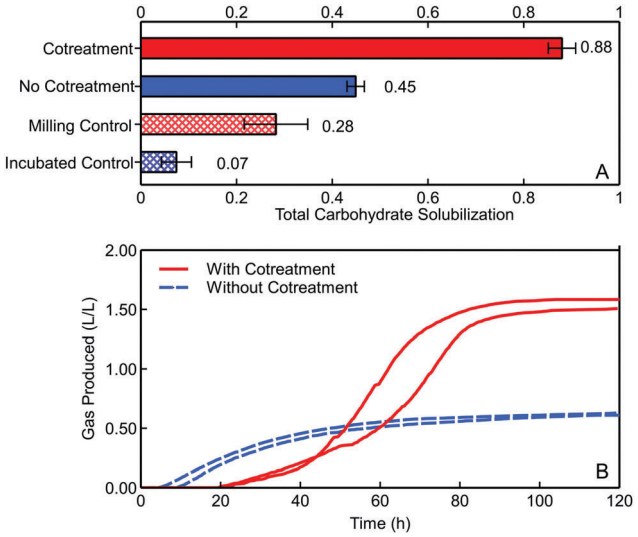

Fig. 3 (A) Final solubilization of senescent switchgrass and (B) gas production throughout fermentation by *C. thermocellum* with and without cotreatment. Solubilization bar plots are at least n = 3 with error bars representing one standard deviation.

fermentation of Switchgrass (Alamo, December harvest, fully senescent) by *C. thermocellum* with and without continuous ball milling. Uninoculated controls were also run with and without ball milling. Total carbohydrate solubilization, S_T, after 5 days is presented in Fig. 3A, and gas production is presented in Fig. 3B. Mean fractional S_T observed in replicated experiments increased from 0.45 without cotreatment to 0.88 with cotreatment. S_T observed for uninoculated controls was 0.28 with ball milling and 0.07 without milling. Final gas production correlated well with end-point S_T results (Fig. 3B). Based on the temporal profiles of gas production, it appears that *C. thermocellum* metabolism under the conditions tested is initially slowed by cotreatment with ball milling, but subsequently proceeds more rapidly with milling than without it.

Fig. 4 shows the production of fermentation products over time for the experiment depicted in Fig. 3. Acetate and ethanol

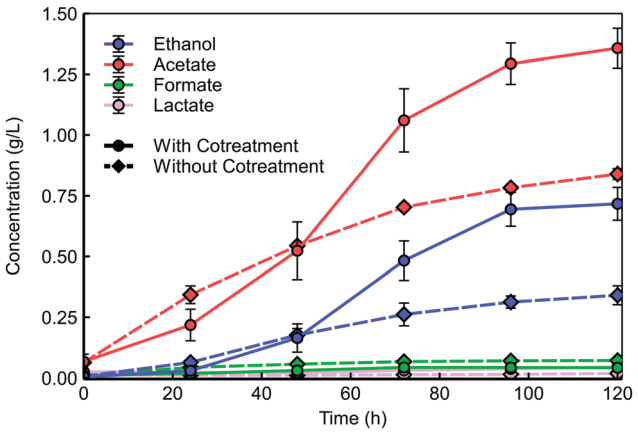

Fig. 4 Major fermentation products by *C. thermocellum* when grown on senescent switchgrass with and without cotreatment, data are at least n = 4 and error bars represent 1 standard deviation.

are the main fermentation products, and produced at similar ratios, with and without cotreatment, with smaller amounts of lactate and formate also observed. Consistent with dynamic gas production data (Fig. 3), the main fermentation products (acetate and ethanol) were initially produced more slowly with cotreatment, but production rates were equal with and without cotreatment at 48 hours, and higher with cotreatment thereafter. At the conclusion of fermentation with cotreatment, intact *C. thermocellum* cells were readily observed in the fermentation broth *via* light microscopy.

Results from the experiments depicted in Fig. 3 and 4 are summarized in Table 1 with respect to initial and final carbohydrate concentrations and final concentrations of fermentation products, sugars present in the liquid (as monomers and oligomers), and sugar polymers present in residual solids. Data are also included in Table 1 for fermentation of the same switchgrass feedstock by *C. thermocellum* following hydrothermal pretreatment. Hydrothermal pretreatment conditions, 200 °C for 15 minutes (severity factor 4.12), were chosen based on the optimal conditions for switchgrass identified by Kim *et al.*[39] and the experience of the Wyman lab (UC Riverside). S_T for fermentation with cotreatment (88 ± 2.9%) is similar but somewhat higher than for fermentation following hydrothermal pretreatment (81% ± 4.5%). Recovery of carbon, R_T^C, initially present as carbohydrate is between 81% and 99% for all cases. Carbon recovery values are likely inflated somewhat by release of acetic acid initially present in the biomass, but do not include conversion of carbohydrate carbon into cells, and into amino acids as known to occur in *C. thermocellum*.[40] Gas production profiles for fermentation of hydrothermally-pretreated switchgrass (Fig. S2, ESI†) confirmed that fermentation was most rapid during the second day of incubation and had largely ceased by day five.

Residual solids characterization

Analysis of solid residues was undertaken using molecular beam mass spectrometry (PyMBMS) and solid-state nuclear magnetic resonance spectroscopy (NMR) in order to provide insight into changes in plant cell walls during processing *via* various modes. Samples tested included autoclaved but otherwise not pretreated switchgrass before and after fermentation by *C. thermocellum* and with and without ball milling. To compare the effects of cotreatment and a representative thermochemical pretreatment, hydrothermally pretreated switchgrass was also characterized before and after fermentation by *C. thermocellum.*

Pyrolysis molecular beam mass spectrometry

The mass spectrum of the untreated switchgrass material is shown in Fig. 5(A) and the assignments of the major peaks resulting from the pyrolysis of hemicellulose, cellulose and lignin are presented in Table 2. Peaks assigned to C5 sugars predominately arising from xylan are indicated by blue arrows in Fig. 5(A). Peaks assigned to C6 sugars, indicated by green arrows in Fig. 5(B), predominately result from the pyrolysis of cellulose although there are contributions from galactose and

Table 1 Concentrations of sugars and products for initial feedstock and tested conditions. Recovery and solubilization calculated as described in the Materials and methods section. Results are $N = 2$ to $N = 6$

Initial carbohydrate (mmoles monomer per L)		
	Unpretreated feedstock	Hydrothermally pretreated feedstock
Glucan	27.7	27.8
Xylan	21.4	5.9
Arabinan	2.21	0.7

Final concentration (mmoles L^{-1})	Fermentation with cotreatment	Fermentation without cotreatment	Uninoculated with milling	Uninoculated without milling	Fermentation with pretreatment
Fermentation products (mmoles L^{-1})					
Acetate	23.3	13.7	5.9	3.4	12.6
Ethanol	16.0	7.0	0	0	12.1
Formate	0.9	1.2	0	0	1.1
Lactate	0.5	0.2	0	0	0.0
Stoich CO_2	38.4	19.4			23.6
Residual solid (mmoles L^{-1})					
Glucan	3.1	16.6	21.1	25.6	4.7
Xylan	2.7	10.4	14.4	20.0	1.8
Arabinan	0.4	0.9	1.0	1.9	0.0
Soluble sugars (mmoles L^{-1})					
Glucose/glucan	1.5	1.0	0.8	0.8	0.26
Xylose/xylan	13.3	7.6	3.5	0.6	2.4
Arabinose/arabinan	2.5	1.7	1.1	0.1	0.24
Solubilization and carbon recovery (%)					
S_T[a]	88	45	29	7	81
R_T^{Cb}	84.7	95.8	87.8	99.2	81

[a] Total carbohydrate solubilization. [b] Recovery of carbohydrate carbon.

mannose contained in hemicelluloses. Lignin peaks, highlighted with red arrows in Fig. 5(A), are monomers resulting from the cleavage of C–O–C linkages present within the lignin polymers. Less intense peaks above ~m/z 250 (*e.g.*, 272, 418) have been assigned to dimers containing C–C linkages between the lignin monomeric units not cleaved during the pyrolysis process.[36]

The mass spectra of the residues from different conversion processes are shown in Fig. 5(B–D). The spectrum of the

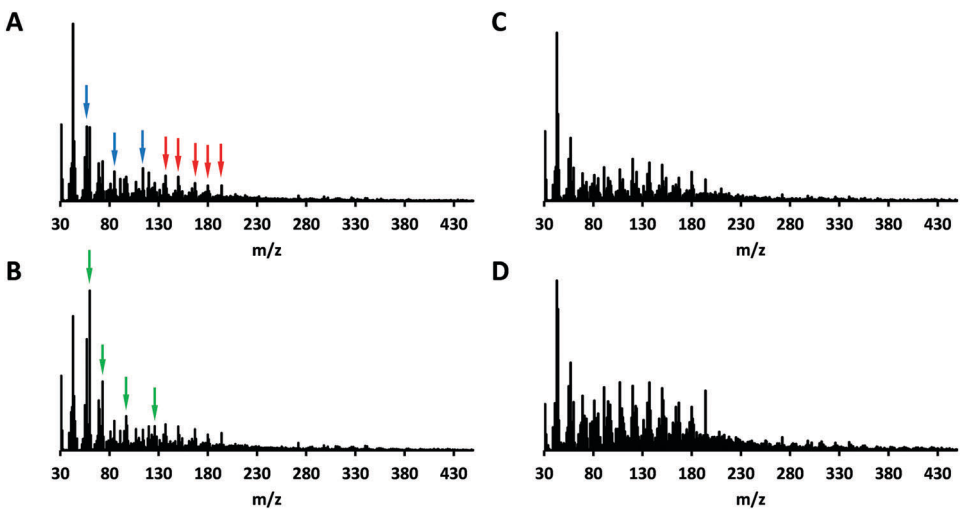

Fig. 5 PyMBMS spectra of switchgrass feedstock before and after conversion. (A) Switchgrass feedstock before conversion. Blue arrows highlight monomers released during pyrolysis derived from xylan. Red arrows highlight monomers released during pyrolysis derived from lignin. (B) Residue after hydrothermal pretreatment. Green arrows highlight monomers released during pyrolysis derived from C6 sugars, predominately cellulose. (C) Residue remaining after *C. thermocellum* fermentation without cotreatment. (D) Residue remaining after *C. thermocellum* fermentation with cotreatment.

Table 2 Assignments of major peaks observed in the mass spectra from the pyrolysis of biomass materials.[19] S, G, and H identify peaks assigned to fragments from syringyl, guaiacyl and *p*-coumaryl lignin moieties, respectively, present in the lignin polymer

m/*z*	Assignment	Type of lignin precursor
57, 73, 85, 96, 114	C5 sugars	
57, 60, 73, 98, 126, 144	C6 sugars	
94	Phenol	H, S, G
120	Vinylphenol	H, S, G
124	Guaiacol	G
137[a]	Ethylguaiacol, homovanillin, coniferyl alcohol	G
138	Methylguaiacol	G
150	Vinylguaiacol, coumaryl alcohol	G
152	4-Ethylguaiacol, vanillin	G
154	Syringol	S
164	Allyl- + propenyl guaiacol	G
167[a]	Ethylsyringol, syringylacetone, propiosyringone	S
168	4-Methyl-2,6-dimethoxy phenol	S
178	Coniferyl aldehyde	G
180	Coniferyl alcohol, vinylsyringol, α-D-glucose	G, S
182	Syringaldehyde	S
194	4-Propenylsyringol	S
208	Sinapylaldehyde	S
210	Sinapylalcohol	S

[a] Fragment ion.

Table 3 S/G ratio of original, pretreated and fermented switchgrass samples determined by pyrolysis MBMS

Sample identification	S/G ratio	Std. dev.
Starting switchgrass	0.64	0.01
Hydrothermal pretreatment	0.83	0.01
Without cotreatment	0.70	0.04
With cotreatment	0.80	0.02

hydrothermal pretreatment residue shows the characteristic decrease in the peak intensity of the C5 sugars relative to the C6 sugars due to the removal of xylan and arabinan.

The mass spectra of the residues following *C. thermocellum* fermentation and cotreatment, Fig. 5(C and D) show a decrease in the carbohydrate components relative to lignin consistent with other chemical analyses performed on the residues. The intensities of carbohydrate peaks *m*/*z* 57, 60, 73, 85, 114, and 126 are greater in the residue of the *C. thermocellum* fermentation, indicating enhanced carbohydrate removal during fermentation by *C. thermocellum* in the presence of cotreatment (Fig. 5(D)). There is little evidence of a change in the lignin structure due to formation of C–C bonds from condensation reactions occurring in the *C. thermocellum* fermentation and cotreatment residues. A comparison of the fermentation and cotreatment residues indicates a slight increase in lignin S/G ratio following fermentation and cotreatment (0.80) *versus* the starting switchgrass feedstock (0.64) (Table 3).

Solid-state nuclear magnetic resonance spectroscopy

The aromatic region of the ^{13}C CP/MAS spectra from the starting switchgrass feedstock, hydrothermal residue, and the *C. thermocellum* fermentation and cotreatment residuals are shown in Fig. 6(A–D). Percent lignin contents estimated from integrations of the NMR spectra (Table S1, ESI†) indicate that some carbohydrates were removed during hydrothermal

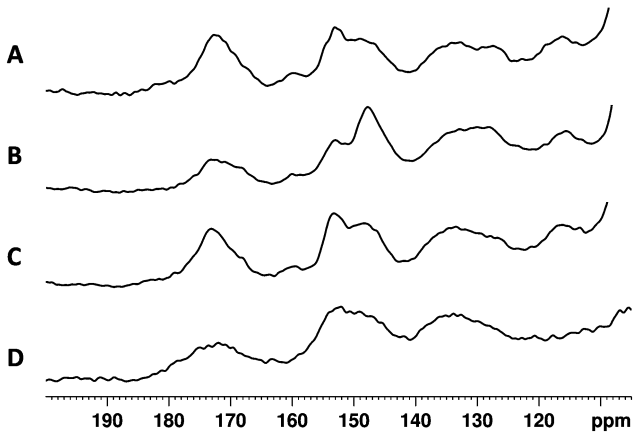

Fig. 6 Aromatic region of solid-state ^{13}C cross polarization magic angle spinning (CP/MAS) spectra of switchgrass feedstock before and after conversion. (A) Switchgrass feedstock before conversion. (B) Residue after hydrothermal pretreatment. (C) Residue remaining after *C. thermocellum* fermentation. (D) Residue remaining after *C. thermocellum* cotreatment following removal of metal particles by sonication after suspension in an EDTA solution.

pretreatment but lignin did not appear to be solubilized in contrast to PyMBMS results[41,42] (Fig. 5D). A change in the lignin structure is observed for the hydrothermally pretreated sample (Fig. 6(B)) consistent with previous studies, most noticeably a decrease in the peak at ~153 ppm relative to the peak at ~148 ppm indicative of the cleavage of β-O-4 linkages between lignin aromatic moieties.[43,44] In addition, liquid-state 2-dimensional HSCQ experiments (Fig. S3 and Table S2, ESI†) verified a significant disruption of lignin C–O–C linkages. Broadening of the NMR resonances is observed in the NMR spectrum of the residue remaining after *C. thermocellum* cotreatment (Fig. 6(D)) presumably due to a small number of residual metal particles.

Table 4 ^{13}C Chemical-shift assignments of the most relevant peaks for syringyl (S), guaiacyl (G) and *p*-coumaryl (H) lignin subunits[43,45,46]

δ ^{13}C (ppm)	Assignment
168–174	Carbonyl or carboxyl of lignin or hemicellulose
160	H4
153	Ether-linked S3, S5, and G3
149	S4 and G4-etherified
146–148	Non-ether-linked S3, S5, and G3
144	C_α in cinnamate
143	G4 with C4OH
133–138	S1
128–132	G1
121	G6
115	G5, C_β in cinnamate

There is a noticeable loss of intensity observed at ~115 ppm, assigned to either the C5 carbon in guaiacyl units or C_β in cinnamate moieties (Table 4) in the cotreatment residue (6D). S/G ratios determined by pyMBMS showed there was no apparent decrease in guaiacyl moieties so the most likely explanation is a loss of cinnamate moieties during cotreatment. Liquid-state 2-dimensional HSCQ experiments (Fig. S3 and Table S2, ESI†) confirmed the presence of cinnamate moieties in the untreated switchgrass feedstock, the hydrothermal pretreated residue, and *C. thermocellum* fermentation residue. HSQC NMR of the cotreatment residue did not show resonances that could be assigned to cinnamate moieties although low signal to noise due to line broadening from the residue metal particles did not allow for a definitive conclusion. A decrease in the intensity of the *m*/*z* 120 peak, assigned to vinylphenol derived from the fragmentation of cinnamate groups (Table 2), relative to the other lignin peaks was also observed in the pyMBMS spectra of the cotreatment residues consistent with a loss of cinnamate groups during cotreatment.

Discussion

Soluble substrate fermentation in the presence of ball milling was found to proceed almost as fast as unmilled controls for *C. thermocellum*, but was completely arrested for yeast (Fig. 2). In comparative studies using a bead mill, Schutte *et al.*[47] found bacteria to be more resistant to mechanical disruption than yeast, and Gram-positive bacteria to be more resistant than Gram-negative bacteria. The roughly 10-fold smaller size of bacteria compared to yeast and the thick cell wall of Gram-positive bacteria are believed to be important factors underlying these differences.[47,48]

Having demonstrated the ability of *C. thermocellum* to carry out fermentation in the presence of ball milling, we proceeded to evaluate fermentation of senescent switchgrass, a widely-studied herbaceous energy crop,[49] in the presence of continuous ball milling. Gas production profiles (Fig. 3B) indicated that switchgrass is rapidly fermented by *C. thermocellum* with milling. Ball milling delayed the onset of gas production by about 12 hours compared to unmilled controls. Further study would be of value to investigate the mechanism responsible for this delay and remedial strategies such as strain adaptation. Maximum rates and extents of switchgrass solubilization were substantially greater with cotreatment than without it. Total carbohydrate solubilization in the presence of ball milling, 88%, was nearly twice that in the absence of ball milling, 45% (Fig. 3A and Table 1). The total carbohydrate solubilization observed here for fermentation with cotreatment is generally comparable to values achieved here and elsewhere using thermochemical pretreatment,[6] and is compatible with favorable process economics.[50] In the studies reported here, we washed the feedstock prior to fermentation to remove soluble components, including sugars and some phenolic extractives, in order to focus on conversion of the recalcitrant fraction of lignocellulose. We anticipate somewhat higher sugar yields in the absence of washing but this outcome must still be confirmed.

The properties of lignin residues remaining after fermentation with cotreatment have not been examined previously. Hydrothermal pretreatment altered the switchgrass lignin structure to a greater extent compared to cotreatment. The intensity of the peaks in the pyMBMS spectra (Fig. 5B) assigned to lignin appeared to drop relative to peaks assigned to C6 sugars indicating that lignin was either being solubilized during the hydrothermal pretreatment process or was not pyrolyzed due to a change in the lignin structure, mainly the formation of C–C linkages. We observed lignin structural changes in the hydrothermal pretreated residues consistent with previous NMR studies showing thermochemical pretreatments increased the C–C bonding between aromatic moieties due to aromatic condensation reactions that occur at lower pH and higher temperatures.[43,44] The solid-state NMR results also indicated that the lignin was not solubilized during hydrothermal pretreatment indicating that the most likely explanation for the pyrolysis behavior observed in Fig. 5B was that lignin condensation reactions during hydrothermal pretreatment altered the lignin structure and thus inhibited lignin depolymerization. The cotreatment lignin residue appeared to generate a higher percentage of monomers during analytical pyrolysis compared to the hydrothermal pretreated switchgrass indicating that the cotreatment lignin residue may be more suitable for downstream conversion by processes that require lower molecular weight starting materials such as pyrolysis, catalyzed depolymerization, and biological funneling.[51–54] The value created from coproducts could produce additional revenue that would reduce the final cost of the fuel or chemical products produced during the fermentation.

The observation of high solubilization with little apparent residue modification is consistent with cotreatment enhancing solubilization primarily by increasing the access of saccharolytic enzymes to the feedstock, and *C. thermocellum* being able to attack all the major linkages in cellulosic biomass provided that these linkages are accessible. Mechanically-induced reactions in organic solids have been described[55] and cannot be ruled out for the work presented here.

Key results reported here for the first time support the potential of CBP with cotreatment (CBP-CT) as an alternative to the extensively studied but still expensive thermochemical pretreatment/fungal cellulase processing paradigm:

• The capacity of a cellulolytic anaerobic bacterium to carry out fermentation in the presence of milling at an intensity sufficient to render plant cell walls highly accessible to biological attack;

• Achievement of high carbohydrate solubilization while avoiding key factors responsible for the high cost of current technology for producing cellulosic biofuels: thermochemical pretreatment and added saccharolytic enzymes;

• Production of lignin-rich residues that exhibit less C–C condensation compared to hydrothermal pretreatments and thus may have greater potential for conversion to value-added coproducts compared to residues resulting from thermochemical pretreatment.

Although these observations are notable and promising, CBP-CT is a nascent concept that will require considerable further work before it can be assessed or implemented. The milling configuration investigated here, a fermentor consisting of a radially-agitated ball mill with low fractional void volume, was chosen to document the effects of mechanical disruption on fermentation, solubilization, and the properties of residual solids. We think it unlikely that this particular configuration will be industrially practical. Further development and evaluation of CBP-CT requires investigation of alternative milling modalities, biotechnologically-driven improvements in product formation and robustness of cellulolytic anaerobes, and integrated studies of milling and fermentation including mechanistic aspects and evaluation of new configurations. We hypothesize that the energy required for milling as a cotreatment can be lower than as a pretreatment in light of the changes in the physical character of biomass slurries during the early stages of biological solubilization.[56,57] Definitive testing of this hypothesis is an important topic for future work. It will also be interesting to investigate coproduct opportunities associated with minimally-modified lignin residuals arising from CBP-CT.

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