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Fermentation of Dilute Acid Pretreated *Populus* by *Clostridium thermocellum*, *Caldicellulosiruptor bescii*, and *Caldicellulosiruptor obsidiansis*

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Abstract Consolidated bioprocessing (CBP), which merges enzyme production, biomass hydrolysis, and fermentation into a single step, has the potential to become an efficient and economic strategy for the bioconversion of lignocellulosic feedstocks to transportation fuels or chemicals. In this study, we evaluated wild-type *Clostridium thermocellum*, *Caldicellulosiruptor bescii*, and *Caldicellulosiruptor obsidiansis*, three thermophilic, cellulolytic, mixed-acid fermenting candidate CBP microorganisms, for their fermentation capabilities using dilute acid pretreated *Populus* as a model biomass feedstock. Under pH-controlled anaerobic fermentation conditions, each candidate successfully digested a minimum of 75 % of the cellulose from dilute acid pretreated *Populus*, as indicated by an increase in planktonic cells and end-product metabolites and a concurrent decrease in glucan content. *C. thermocellum*, which employs a cellulosomal approach to biomass degradation, required approximately 50 h to achieve 75 % cellulose utilization. In contrast, the noncellulosomal, secreted hydrolytic enzyme system of the *Caldicellulosiruptor* sp. required about 100 h after a significant lag phase to achieve similar results. End-point fermentation conversions for *C. thermocellum*, *C. bescii*, and *C. obsidiansis* were determined to be 0.29, 0.34, and 0.38 g of total metabolites per gram of loaded glucan, respectively. These data provide a starting point for future strain engineering efforts that can serve to improve the biomass fermentation capabilities of these three promising candidate CBP platforms.

Keywords *Clostridium thermocellum* · *Caldicellulosiruptor bescii* · *Caldicellulosiruptor obsidiansis* · Consolidated bioprocessing · Dilute acid pretreated *Populus* · Thermophilic fermentation

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Abbreviations

SSF	Simultaneous saccharification and fermentation
CBP	Consolidated bioprocessing
DA	Dilute acid pretreatment
HW	Hot water
HPLC	High-performance liquid chromatography
ATCC	American Type Culture Collection

Introduction

Lignocellulosic biomass is an abundant, low-cost, and renewable source of carbon that has the potential to be converted through enzymatic hydrolysis and fermentation into liquid transportation fuels [1–4]. However, there are technical challenges and economic barriers, several of which are directly related to the inherent recalcitrance of biomass to solubilization. These could potentially be reduced through biomass processing and pretreatment and/or the development of transgenic feedstocks with reduced recalcitrance [5, 6]. The conventional approach for the biological production of cellulosic ethanol has four main process steps: biomass size reduction and pretreatment, production of hydrolytic enzymes, hydrolysis to fermentable sugars, and fermentation of the hexose and pentose sugars to ethanol. Even though the cost of enzyme production has been significantly reduced, the economic burden of producing exogenous hydrolytic enzymes is still formidable [7, 8]. A consolidated bioprocessing (CBP) approach has the potential to lower the cost by merging hydrolytic enzyme production, hydrolysis, and fermentation of sugars into a single step [9–12] and thermophilic lignocellulose deconstruction has been reviewed recently [13].

A candidate organism for CBP is the strictly anaerobic, thermophilic bacterium, *Clostridium thermocellum*, that can rapidly hydrolyze crystalline cellulose [9, 14–17]. However, it lacks the ability to ferment pentose sugars while producing some xylanases [18] and is considered a mixed-acid fermenter, producing acetate, lactate, ethanol, formate, CO_2, and H_2 [18, 19]. The wild-type strain in continuous culture reached 80–90 % glucan conversion yield on pretreated hardwood [20]. More recently, studies of fermentation performance on pretreated biomass substrates have reported that *C. thermocellum* utilizes ammonia fiber expansion (AFEX) pretreated corn stover, dilute acid pretreated transgenic and wild-type switchgrass, and dilute acid pretreated *Populus* without the addition of hydrolytic enzymes digesting approximately 75 % of the cellulose [14, 15, 21]. Moreover, there is a tractable genetic system and an engineered and evolved strain of *C. thermocellum* reached 5.61 g/L of ethanol and in co-culture with *Thermoanaerobacterium saccharolyticum* reached 38 g/L ethanol, both in Avicel fermentations [19]. The mutant strain of *C. thermocellum* achieved a yield of 0.27 g ethanol/g glucan released from dilute acid pretreated transgenic COMT3 switchgrass [22]. These recent developments have made progress but have not yet developed a strain with a phenotype and fermentation performance that is relevant to an industrial process.

The extremely thermophilic *Caldicellulosiruptor* species are also Gram-positive, anaerobic bacteria that hydrolyze hemicellulose and cellulose, utilizing both pentose and hexose sugars [23]. In contrast to *C. thermocellum*, for hydrolysis they use a noncellulosomal approach, but like *C. thermocellum*, they are mixed-acid fermenters producing primarily acetate, lactate, CO_2, H_2, and some strains produce trace amounts of ethanol [24]. The wild-type strains not producing ethanol have been used primarily for hydrogen production from lignocellulosic substrates [25–28]. Recently, there was a *Caldicellulosiruptor* strain isolated that produced 1.12 g/L ethanol from unwashed pretreated *Populus* and in co-culture with *Thermoanaerobacter* reached 1.60 g/L of ethanol [24].

Caldicellulosiruptor obsidiansis and *Caldicellulosiruptor bescii* have the potential to be CBP candidates, especially with the recent development of a tractable genetic engineering system for *C. bescii* which does not natively produce ethanol [29–32]. A strain of *C. bescii* was genetically engineered and produced 0.59 g/L ethanol on switchgrass with reduced acetate production [33]. Moreover, characterization of microbial growth on different substrates of *C. bescii* and *C. obsidiansis* showed that both microorganisms utilize hexose and pentose sugars, grow on crystalline cellulose; plus, hydrolyze and ferment pretreated switchgrass without the addition of hydrolytic enzymes [16, 34–36]. In the case of *C. bescii*, it has been shown that this microorganism can partially hydrolyze and ferment unpretreated switchgrass without the addition of hydrolytic enzymes [33, 37] and Basen et al. showed that fermentations of unpretreated switchgrass at a solids loading of 50 g/L showed approximately 30 % solubilization of the biomass [38].

There are few comparative studies of these anaerobic thermophilic cellulolytic bacteria on a pretreated biomass substrate. As a result, we report the fermentation performance of *C. thermocellum*, *C. bescii*, and *C. obsidiansis* on a consistent substrate (dilute acid pretreated *Populus*) and media to minimize the effects of varying nutrient levels, substrate accessibility, particle size, and pretreatment-generated compounds on fermentation performance. This allowed for a consistent platform to compare the fermentation profiles of the native strains of the three potential CBP microorganisms.

Materials and Methods

Strains and Fermentation Conditions

All biological duplicate batch fermentations were cultivated on the same pretreated biomass; however, the

washing procedure was performed on smaller subset preparations of biomass per set of fermentations. The same medium composition used for all three strains is provided in Yee et al. [39].

The fermentation conditions for the candidate CBP microorganisms were as follows: *C. thermocellum* (ATCC 27405) temperature of 58 °C, pH 6.8, and agitation 300 rpm, *Caldicellulosiruptor obsidiansis* (ATCC BAA-2073) temperature 75 °C, pH 7.0, and agitation 300 rpm, and *Caldicellulosiruptor bescii* (ATCC BAA-1888) temperature 75 °C, pH 7.0, and agitation 300 rpm. Fermentations were conducted in 2.0-L Applikon jacketed glass fermenters (Applikon Biotechnology, Dover, NJ). Fermenters with water, heat-insensitive media components, and biomass were sterilized at 121 °C for 30 min and then sparged overnight with O_2-free nitrogen, at 300 rpm, while equilibrating to the appropriate growth temperature (58 or 75 °C). The remaining media components, including vitamins and minerals, were added the following day as described previously by Yee et al. [39], and pH was actively controlled with 10 %*w*/*v* $NaHCO_3$. The off gas was bubbled through a water trap, which captured volatile metabolites such as ethanol [14, 21, 36]. The inoculum was grown in 125-mL anaerobic serum bottles containing 50 mL of growth medium with 5.0 g/L Avicel (FMC BioPolymer) as the carbon source. The bottles were incubated at the proper growth temperature for each strain with shaking at 125 rpm for 14 h and then added at 10 %*v*/*v* to the fermentors. The cell density of the fermentations was monitored using planktonic cell counts in a metallized hemacytometer Reichert Bright-Line (Hausser Scientific).

Dilute Acid Pretreated *Populus*

The dilute acid pretreated biomass substrate for the fermentations was a *Populus trichocarpadeltoides* F1 hybrid clone 53–239 male that was chipped, milled, and dilute acid pretreated by the National Renewable Energy Laboratory (NREL) in a SUNDs reactor at 190 °C, 0.050 g sulfuric acid/g dry biomass, 1-min residence time (flow through mode), and 25 % (*w*/*w*) total solids during reaction conditions. The pretreated biomass was stored under acidic conditions at 4 °C. The substrate was washed prior to use with milli-Q water until less than 0.01 g/L of glucose was present in the washate as determined by high-performance liquid chromatography (HPLC) analysis [14, 40]. This substrate was previously used for several *C. thermocellum* experiments [14, 40, 41]. The carbohydrate composition of the biomass solids after dilute acid pretreatment was primarily glucan from cellulose measured by the quantitative saccharification assay. The dilute acid pretreatment solubilized the majority of the hemicellulose and the extensive washing removed soluble compounds and extractives.

Analytical Methods

Fermentation broth samples were analyzed for products (acetic acid, lactic acid, formic acid, and ethanol) and soluble carbohydrates (cellobiose, glucose, xylose, arabinose) using HPLC La chrom elite system (Hitachi High Technologies America, Inc.) equipped with a refractive index detector (model L-2490). The products and carbohydrates were separated using an Aminex HPX-87H column (Bio-Rad Laboratories, Inc.), with a flow rate at 0.5 mL/min of 5.0 mM sulfuric acid and a column temperature 60 °C.

Fermented biomass residues were analyzed for carbohydrate composition using quantitative saccharification (quansacch) assay ASTME 1758–01 (ASTM 2003) and HPLC method NREL/TP 51–42623. Briefly, the technical triplicate samples per biological duplicate fermentation were analyzed using HPLC La chrom elite system (Hitachi High Technologies America, Inc.) equipped with a refractive index (model L-2490) and UV–vis (model L-2420) detectors. The carbohydrates (glucose, xylose, galactose, mannose, and arabinose) and pentose and hexose sugar degradation products (furfural and 5-hydroxy methyl furfural) were separated using an Aminex HPX-87P column (Bio-Rad Laboratories, Inc.), with a 0.6 mL/min flow rate of water and a column temperature of 80 °C.

Results

Cell Growth

The duplicate batch fermentations of dilute acid pretreated *Populus* were monitored with metabolite time course profiles and planktonic cell counts (using a microscope). *C. thermocellum* grew to a maximum planktonic cell density greater than approximately 5×10^8 cells/mL and entered stationary phase at 70 h. In addition, the metabolite production stopped at 70 h. The cell densities for *C. bescii* and *C. obsidiansis* reached approximately 2×10^9 and 5×10^9 cells/mL at 133 and 120 h, respectively. There was not an apparent lag in planktonic cell growth for the *Caldicellulosiruptor* sp. However, there was a significant lag before metabolite production began. However, for both *Caldicellulosiruptor* species the metabolite production continued after the cell density reached a maximum (Fig. 1a–c). The cell densities were tracked with planktonic cell counts, because the biomass substrate interfered with other methods that are used to monitor cell growth on insoluble substrates such as total nitrogen and total protein [14, 15, 35, 36]. The biological duplicate fermentations for each microbe showed an increase in planktonic cell count qualitatively indicating when the cells were growing and reached stationary phase; however, these measurements are not quantitative.

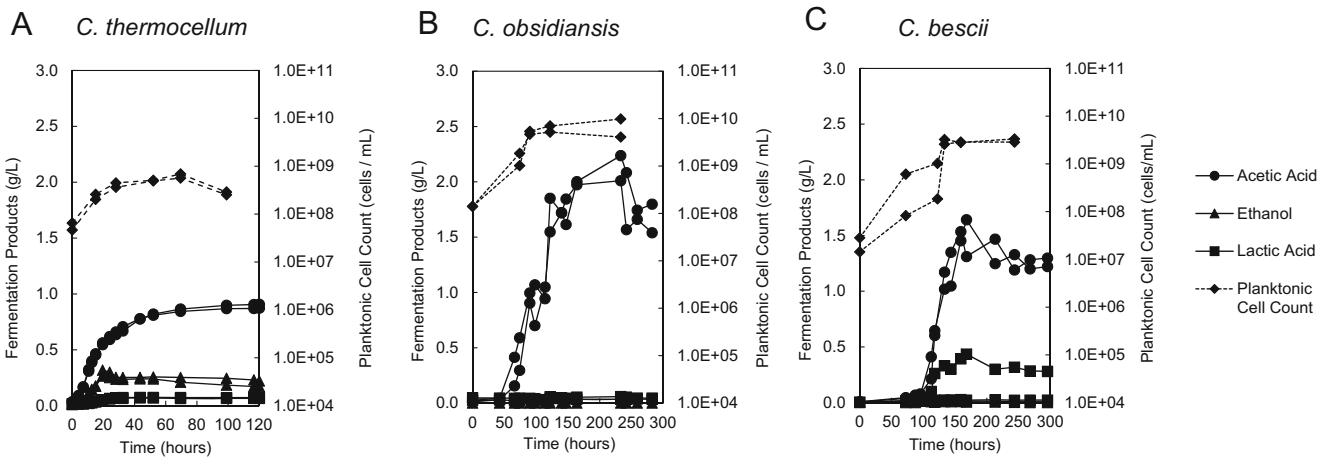

Fig. 1 Time course fermentation profiles for *C. thermocellum* (**a**), *C. obsidiansis* (**b**), and *C. bescii* (**c**) on dilute acid pretreated *Populus* (5 g/L dry biomass) comparing planktonic cell count and metabolite production in biological duplicate. Note the time axis are different

Fermentation Metabolite and Biomass Carbohydrate Composition Profiles

The fermentations of dilute acid pretreated *Populus* with the CBP candidate microbes were monitored over time using HPLC for measuring fermentation metabolites (acetic acid, lactic acid, formic acid, and ethanol) and simple carbohydrates released from the biomass (cellobiose, glucose, xylose, and arabinose). In duplicate fermentations, *C. thermocellum* showed the production of acetic acid and ethanol with minimal lactic acid (Fig. 1a) and an average endpoint ethanol concentration of 0.35 g/L in the water trap. The water trap ethanol was accounted for in the endpoint conversion and yield calculations. The production of ethanol and acetic acid started at approximately 4 h and reached a plateau at 70 h with a ratio of 2.9:1 acetic acid to ethanol. This ratio was slightly higher than what is typically reported 2:1 acetic acid to ethanol [14, 17, 41], and the ethanol concentration appears to decrease in the fermentation broth. Both phenomena are likely due to evaporative ethanol losses which were collected in the water trap, because these fermentations were equipped with a condenser but had continual low flow rate nitrogen sparge into a water trap to maintain anaerobic conditions. The endpoint concentrations of acetic acid and ethanol in the fermentation broth where 0.90 and 0.20 g/L, respectively. In contrast, *C. bescii* and *C. obsidiansis* produced primarily acetic acid, minimal lactic acid, and no detectable amounts of ethanol (Fig. 1b, c). The acetic acid concentration for *C. bescii* and *C. obsidiansis* started to increase at approximately 70 and 40 h, respectively and the metabolite production reached a maximum at approximately 200 h with final acetic acid concentrations on average of 1.2 and 1.7 g/L, respectively. Of the biological duplicate fermentations for *C. bescii*, one of them produced minimal but still measurable amounts of lactic acid at a concentration less than 0.5 g/L.

In addition to tracking fermentation metabolites, HPLC analysis was used to monitor the cellobiose, glucose, xylose, and arabinose levels that were liberated and potentially consumed from the biomass. The fermentation broth of *C. thermocellum* revealed minimal concentrations of cellobiose (0.03 g/L), xylose (0.01 g/L), and no detectable glucose or arabinose. *C. bescii* and *C. obsidiansis* fermentations had minimal cellobiose and glucose concentrations, but both fermentations had residual but minimal xylose concentrations of 0.08 and 0.09 g/L, respectively (data not shown).

The carbohydrate composition of the dilute acid pretreated *Populus* was monitored throughout the fermentations to evaluate hydrolysis and extent of sugar consumption. The biomass was subjected to a severe dilute acid pretreatment and extensive washing. The carbohydrate content of the pretreated biomass was primarily glucan (~600 mg/g dry biomass) with minimal xylan at approximately 16–17 mg/g dry biomass and with negligible amounts of arabinose, galactose, and mannose in the starting biomass solids prior to fermentation. The three CBP candidate microorganisms' hydrolytic systems significantly reduced the glucan content of the biomass leaving 25, 10, and 16 % of the initial content for *C. thermocellum*, *C. obsidiansis*, and *C. bescii*, respectively (Table 1). Overall, the three CBP microorganisms did not fully hydrolyze the biomass leaving residual glucan and xylan at the end of the fermentation (Figs. 2, S1, S2, and S3). In the *C. thermocellum* fermentations, the biomass glucan content was reduced from 593 to 155 (mg/g dry biomass). In the *C. bescii* and *C. obsidiansis* fermentations, the biomass had a starting glucan content of 670 (mg/g dry biomass) and it was reduced to 70 and 102 (mg/g dry biomass), respectively (Figs. S1, S2, and S3). While *C. thermocellum* does not ferment xylose, it can still solubilize some xylan. The *Caldicellulosiruptor* sp. can both solubilize and ferment xylan, and more xylan was solublized. However, in all cases, the amount of xylan

Table 1 Summary of fermentation capabilities of *C. thermocellum*, *C. bescii*, and *C. obsidiansis* with dilute acid pretreated poplar

CBP microorganism	Active fermentation time (h)	Lag (h)	% of initial cellulose remaining in solids[a]	Endpoint yield (g/g liberated glucan)[a,b]	Endpoint conversion(g total products/g glucan loaded)[a,b]	(h) to hydrolyze 75 % of the cellulose	Max rate of cellulose hydrolysis (mg/g/h)
C.thermocellum	120.0	0	25.6±0.8	0.39±0.01	0.29±0.01	~45	~18
C. bescii	~200	~50	10.7±0.3	0.35±0.02	0.34±0.04	~140	~7
C. obsidiansis	~220	~50	16.7±2.3	0.45±0.03	0.38±0.02	~140	~5

[a] Average of biological duplicate fermentations and standard deviation of technical triplicates analyses; endpoint analyses were after last time point (120, 295, and 285 h, respectively)

[b] Total products=acetic acid+lactic acid+ethanol

available and released was small compared to the glucan activity. The *Caldicellulosiruptor* species had a greater total extent of hydrolysis than *C. thermocellum* as seen in Fig. 2, but a slower rate. *C. thermocellum* had an overall glucose release of approximately 9 mg glucose/g dry biomass/h in comparison to the *Caldicellulosiruptor* species which had a rate of approximately 3 mg glucose/g dry biomass/h. The maximum rate of glucose release which occurred during the solublization of the first 50 % of total solubilized glucan was 18 mg/g/hr, 7 mg/g/hr, and 5 mg/g/hr for *C. thermocellum*, *C. bescii*, and *C. obsidiansis*, respectively. The decrease in glucan content of the residual pretreated *Populus* for *C. thermocellum* fermentations halted at approximately 45 h while the glucan decrease from *Caldicellulosiruptor* species halted at approximately 175 h after the lag phase.

C. thermocellum fermentations did not exhibit a lag phase as measured by planktonic cell count, metabolite production, and hydrolysis but *C. bescii* and *C. obsidiansis* both exhibited lag phases of approximately 70 and 40 h. Despite the differences in lag phase between the three microorganisms, *C. thermocellum*, *C. obsidiansis*, and *C. bescii* liberated and consumed fermentable carbohydrates from the pretreated *Populus* corresponding to yields of 0.39, 0.42, and 0.35 g of total metabolites per gram glucan liberated, respectively (Table 1). In addition, the amount of carbon going to cellular biomass has been shown to be minimal <5 % for the *C. bescii* and ≤10 % for *C. thermocellum* [42–44]. These fermentations showed substantial glucan liberated from pretreated *Populus* and assuming minimal carbon going to cellular biomass with the metabolites measured the carbon balance remains not fully closed.

Discussion

In this study, we provide comparative fermentation profiles of the wild-type strains for *C. thermocellum*, *C. obsidiansis*, and *C. bescii* grown on dilute acid pretreated *Populus* to investigate hydrolysis, rate, and metabolite titer. The substrate used was dilute acid pretreated *Populus*, which was extensively washed to remove any inhibitors and soluble carbohydrates [39, 41]. As described previously, the carbohydrate

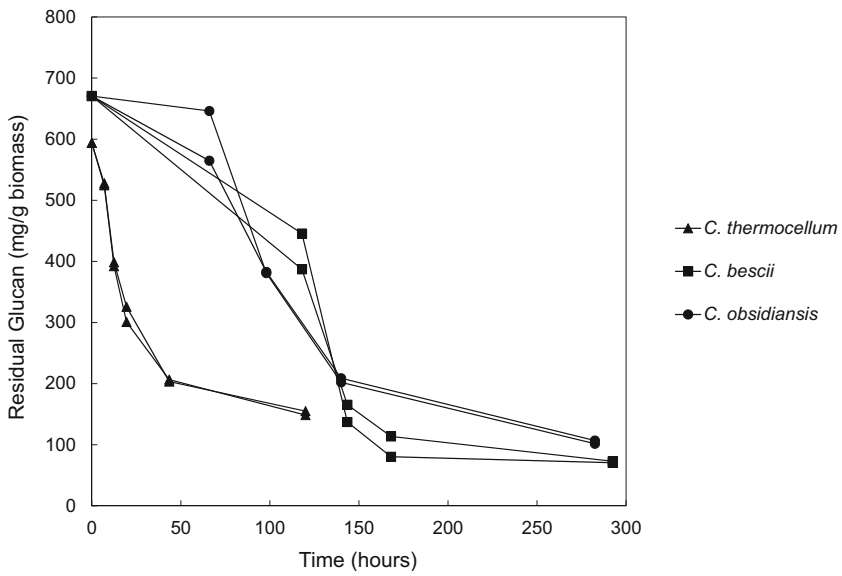

Fig. 2 Time course profile of the biomass residual cellulose composition for *C. thermocellum*, *C. obsidiansis*, and *C. bescii* on dilute acid pretreated *Populus* (5 g/L dry biomass)

composition of the starting substrate was primarily glucan from cellulose with minimal xylose and negligible amounts of mannose, galactose, and arabinose. The fermentations did not require the addition of hydrolytic enzymes for digestion and there was a significant increase of mixed metabolites (acetate, lactate, and ethanol) and planktonic cells throughout the fermentations indicating active metabolism and cell growth at solids loading of 5 g/L on a dry biomass basis. The final extent of growth and metabolism where most of the glucan is consumed in these dilute conditions supports a conclusion that product and substrate inhibition was not very important in these fermentations. Other inhibitors may have slowed the rate but not the extent.

There were apparent slower initial planktonic growth for both *C. bescii* and *C. obsidiansis* that were not observed with *C. thermocellum*. This was paralleled by a significant lag phase in metabolite production. This extensive lag phase has been observed before, and Svetlitchnyi et al. reported approximately a 40-h lag phase of a *Caldicellulosiruptor* species on pretreated *Populus* [24]. In contrast, Hamilton-Brehm et al. did not observe a lag phase for *C. obsidiansis* fermenting dilute acid pretreated switchgrass at a 1 %*w*/*v* loading [36]. Moreover, Basen et al. did not observe as significant of a lag phase for *C. bescii* fermentations of unpretreated switchgrass at 5 g/L loading as was observed during these fermentations [38]. One possible explanation for the lag phases is that the inocula were cultivated on crystalline cellulose, which is a significantly different substrate than dilute acid pretreated *Populus*. The *Caldicellulosiruptor* species may have needed to express different and/or new enzymes to hydrolyze the biomass leading to an adaption time. However, all three species are known to form biofilms on the cellulosic biomass which carry out most of the degradation and metabolite production. Either the initial amounts of planktonic cells results in undetectable metabolites or the *Caldicellulosiruptor* sp. may have a decoupled growth and end-product metabolism. There was no buildup of soluble but unfermented monomeric carbohydrates in the broths for the three fermentations at any time point sampled. This indicates that they are utilizing the liberated carbohydrates at equal or higher rates than they become available. In this case, due to the severe pretreatment, primarily glucan from cellulose was being liberated from the biomass by the hydrolytic enzyme systems. This was confirmed by the significant reduction in glucan content in the residual biomass.

Considering the lag phase, the entry to stationary phase and the maximum production of metabolites, we can estimate an active fermentation time of about 120 h for *C. thermocellum* and about 150 h for the *Caldicellulosiruptor* sp. (Fig. 1). The hydrolysis rate was approximately twofold greater for *C. thermocellum* in comparison to both *Caldicellulosiruptor* species (Fig. 2). The planktonic growth of *C. thermocellum* was less than half of *C. bescii* or *C. obsidiansis*; this may be due to a higher proportion of attached or biofilm growth. In addition, the planktonic numbers of the *C. bescii* and *C. obsidiansis* continued to apparently increase after the production of measured metabolites decreased. However, the measured metabolite production rate during the most active phase was actually greater for the *Caldicellulosiruptor* sp.

The dilute acid pretreatment of the *Populus* increased the accessibility of the biomass to enzymatic hydrolysis, removed the hemicellulose, and likely formed lignin aggregates on the solid surface as previously reported [45, 46]. We noted that the pretreated *Populus* composition during the time course fermentation showed significant reduction in glucose content. This appears to contrast with the previous report by Kataeva et al., who showed that the glucose/xylose/lignin ratio was unchanged for the digestion of unpretreated switchgrass by *C. bescii* [42]. Indeed, the greatly reduced hemicellulose content in our samples after dilute acid fermentation may have left insufficient pentose and other biomass sugars to demonstrate the proportional consumption of minor sugars. Still, Raman et al. and Lochner et al. respectively showed that *C. thermocellum* and *C. bescii* each produced an augmented or altered suite of hydrolytic enzymes depending on the substrate [14, 35]. As a result, it is not unreasonable to have differences in hydrolysis patterns and therefore substrate composition for fermentation of two dissimilar substrates.

In our hands, under these conditions, we noted a significant reduction in glucan content of the entire fermented biomass residue. However, there were varying amounts of residual fermentable carbohydrates remaining in the solid residues depending on the fermentation microbe and this has been noted by others [14, 17, 21]. These observed differences could be a result of the use of varying hydrolytic enzymatic approaches. *C. thermocellum* produces a cellulosome which is a multienzyme complex while the *Caldicellulosiruptor* species rely on noncellulosomal secreted cellulolytic and hemicellulolytic enzymes that are multifunctional [16, 23, 34, 35]. In addition, Lochner et al. showed that there was variation in the secreted cellulolytic systems between *C. obsidiansis* and *C. bescii* [35]. This could partially explain the difference in glucan content in the endpoint residues between the *Caldicellulosiruptor* species. There was significant glucan reduction but incomplete hydrolysis of the biomass substrate. It is possible that as the hydrolysis progressed, the remaining recalcitrance increased mainly due to increasing cellulose crystallinity, lignin content, and possible lignin-carbohydrate complexes within the remaining solid substrate.

Conclusions

The wild-type cellulolytic organisms *C. thermocellum* and the *Caldicellulosiruptor* spp. are thermophilic anaerobic bacteria that successfully hydrolyze and ferment dilute acid pretreated

Populus without the addition of exogenous hydrolytic enzymes. This study directly compared the fermentation performance of these organisms on acid pretreated *Populus* under parallel conditions (with the exception of growth temperature), which so far, has not been reported in the literature. There are differences in metabolite production and carbohydrate content of the biomass residues for the fermentation profiles. *C. thermocellum* had minimal to no lag phase and a faster rate of hydrolysis than the *Caldicellulosiruptor* sp. However, the *Caldicellulosiruptor* species eventually removed a slightly larger percentage of the glucan and *C. obsidiansis* had the greatest endpoint metabolite yield. Now that genetic engineering is beginning to demonstrate product yield and titer improvements [19, 30, 32], this data can be taken into consideration for guiding future strain engineering of the three candidate CBP platforms to improve performance on real biomass substrates.

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Conflict of Interest The authors declare that they have no competing interests.

Authors' Contributions KLY planned the work, conducted the experiments, and wrote the manuscript. MR Jr helped conduct experiments, assisted in data acquisition/analysis, and edited the manuscript. CYH helped conduct experiments. SDHB helped with enumeration of planktonic cells and edit the manuscript. OAT edited the manuscript. JRM helped plan the experiments and edited the manuscript. BHD and JGE helped plan and edit the manuscript. All authors have read and approved the final manuscript.

References

1. Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T (2006) The path forward for biofuels and biomaterials. Science 311:484–489. doi:10.1126/science.1114736
2. Farrell AE, Plevin RJ, Turner BT, Jones AD, O'Hare M, Kammen DM (2006) Ethanol can contribute to energy and environmental goals. Science 311:506–508. doi:10.1126/science.1121416
3. Wyman CE (2003) Potential synergies and challenges in refining cellulosic biomass to fuels, chemicals, and power. Biotechnol Prog 19:254–262. doi:10.1021/bp025654l
4. Lin Y, Tanaka S (2006) Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 69:627–642. doi:10.1007/s00253-005-0229-x
5. Chen F, Dixon RA (2007) Lignin modification improves fermentable sugar yields for biofuel production. Nat Biotechnol 25:759–761. doi:10.1038/nbt1316
6. Hisano H, Nandakumar R, Wang ZY (2009) Genetic modification of lignin biosynthesis for improved biofuel production. In Vitro Cell Dev Biol Plant 45:306–313. doi:10.1007/s11627-009-9219-5
7. Zhang YHP, Himmel ME, Mielenz JR (2006) Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 24:452–481. doi:10.1016/j.biotechadv.2006.03.003
8. Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch HW (2012) The challenge of enzyme cost in the production of lignocellulosic biofuels. Biotechnol Bioeng 109:1083–1087. doi:10.1002/bit.24370
9. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66:506–577. doi:10.1128/mmbr.66.4.739.2002
10. Lynd LR, van Zyl WH, McBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 16:577–583. doi:10.1016/j.copbio.2005.08.009
11. Wooley R, Ruth M, Glassner D, Sheehan J (1999) Process design and costing of bioethanol technology: a tool for determining the status and direction of research and development. Biotechnol Prog 15:794–803. doi:10.1021/bp990107u
12. Olson DG, McBride JE, Shaw AJ, Lynd LR (2012) Recent progress in consolidated bioprocessing. Curr Opin Biotechnol 23:396–405. doi:10.1016/j.copbio.2011.11.026
13. Blumer-Schuette SE, Brown SD, Sander KB, Bayer EA, Kataeva I, Zurawski JV, Conway JM, Adams MW, Kelly RM (2014) Thermophilic lignocellulose deconstruction. FEMS Microbiol Rev 38(3):393–448. doi:10.1111/1574-6976.12044
14. Raman B, Pan C, Hurst GB, Rodriguez M, McKeown CK, Lankford PK, Samatova NF, Mielenz JR (2009) Impact of pretreated switchgrass and biomass carbohydrates on *Clostridium thermocellum* ATCC27405 cellulosome composition: a quantitative proteomic analysis. PLoS One 4, e5271. doi:10.1371/journal.pone.0005271
15. Shao XJ, Jin MJ, Guseva A, Liu CG, Balan V, Hogsett D, Dale BE, Lynd L (2011) Conversion for Avicel and AFEX pretreated corn stover by *Clostridium thermocellum* and simultaneous saccharification and fermentation: insights into microbial conversion of pretreated cellulosic biomass. Bioresour Technol 102:8040–8045. doi:10.1016/j.biortech.2011.05.021
16. Blumer-Schuette SE, Kataeva I, Westpheling J, Adams MWW, Kelly RM (2008) Extremely thermophilic microorganisms for biomass conversion: status and prospects. Curr Opin Biotechnol 19:210–217. doi:10.1016/j.copbio.2008.04.007
17. Wilson CM, Yang S, Rodriguez M Jr, Ma Q, Johnson CM, Dice L, Xu Y, Brown SD (2013) *Clostridium thermocellum* transcriptomic profiles after exposure to furfural or heat stress. Biotechnol Biofuels 6(1):131. doi:10.1186/1754-6834-6-131
18. Demain AL, Newcomb M, Wu JHD (2005) Cellulase, clostridia, and ethanol. Microbiol Mol Biol Rev 69:124. doi:10.1128/mmbr.69.1.124-154.2005
19. Argyros DA, Tripathi SA, Barrett TF, Rogers SR, Feinberg LF, Olson DG, Foden JM, Miller BB, Lynd LR, Hogsett DA, Caiazza NC (2011) High ethanol titers from cellulose by using metabolically engineered thermophilic, anaerobic microbes. Appl Environ Microbiol 77:8288–8294. doi:10.1128/aem.00646-11
20. Lynd LR, Grethlein HE, Wolkin RH (1989) Fermentation of cellulosic substrates in batch and continuous culture by *Clostridium thermocellum*. Appl Environ Microbiol 55:3131–3139
21. Fu CX, Mielenz JR, Xiao XR, Ge YX, Hamilton CY, Rodriguez M, Chen F, Foston M, Ragauskas A, Bouton J, Dixon RA, Wang ZY (2011) Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. Proc Natl Acad Sci U S A A108:3803–3808. doi:10.1073/pnas.1100310108
22. Yee KL, Rodriguez M, Thompson OA, Fu CX, Wang ZY, Davison BH, Mielenz JR (2014) Consolidated bioprocessing of transgenic switchgrass by an engineered and evolved *Clostridium*

thermocellum strain. Biotechnol Biofuels 7:75. doi:10.1186/1754-6834-7-75

23. Blumer-Schuette SE, Giannone RJ, Zurawski JV, Ozdemir I, Ma Q, Yin YB, Xu Y, Kataeva I, Poole FL, Adams MWW, Hamilton-Brehm SD, Elkins JG, Larimer FW, Land ML, Hauser LJ, Cottingham RW, Hettich RL, Kelly RM (2012) *Caldicellulosiruptor* core and pangenomes reveal determinants for noncellulosomal thermophilic deconstruction of plant biomass. J Bacteriol 194:4015–4028. doi:10.1128/jb.00266-12
24. Svetlitchnyi VA, Kensch O, Falkenhan DA, Korseska SG, Lippert N, Prinz M, Sassi J, Schickor A, Curvers S (2013) Single-step ethanol production from lignocellulose using novel extremely thermophilic bacteria. Biotechnol Biofuels 6(1):31. doi:10.1186/1754-6834-6-31
25. Ivanova G, Rakhely G, Kovacs KL (2009) Thermophilic biohydrogen production from energy plants by *Caldicellulosiruptor saccharolyticus* and comparison with related studies. Int J Hydrog Energy 34:3659–3670. doi:10.1016/j.ijhydene.2009.02.082
26. Ntaikou I, Antonopoulou G, Lyberatos G (2010) Biohydrogen production from biomass and wastes via dark fermentation: a review. Waste Biomass Valoriz 1:21–39. doi:10.1007/s12649-009-9001-2
27. de Vrije T, Bakker RR, Budde MAW, Lai MH, Mars AE, Claassen PAM (2009) Efficient hydrogen production from the lignocellulosic energy crop *Miscanthus* by the extreme thermophilic bacteria *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana*. Biotechnol Biofuels 2:12. doi:10.1186/1754-6834-2-12
28. Zeidan AA, van Niel EWJ (2010) A quantitative analysis of hydrogen production efficiency of the extreme thermophile *Caldicellulosiruptor owensensis* OLT. Int J Hydrog Energy 35:1128–1137. doi:10.1016/j.ijhydene.2009.11.082
29. Chung D, Farkas J, Huddleston JR, Olivar E, Westpheling J (2012) Methylation by a unique alpha-class N4-cytosine methyltransferase is required for DNA transformation of *Caldicellulosiruptor bescii* DSM6725. PLoS One 7(8):e43844. doi:10.1371/journal.pone.0043844
30. Chung D, Cha M, Farkas J, Westpheling J (2013) Construction of a stable replicating shuttle vector for *Caldicellulosiruptor* species: use for extending genetic methodologies to other members of this genus. PLoS One 8(5):e62881. doi:10.1371/journal.pone.0062881
31. Chung DW, Farkas J, Westpheling J (2013) Overcoming restriction as a barrier to DNA transformation in *Caldicellulosiruptor* species results in efficient marker replacement. Biotechnol Biofuels 6(1):82. doi:10.1186/1754-6834-6-82
32. Cha M, Chung DW, Elkins JG, Guss AM, Westpheling J (2013) Metabolic engineering of *Caldicellulosiruptor bescii* yields increased hydrogen production from lignocellulosic biomass. Biotechnol Biofuels 6(1):85. doi:10.1186/1754-6834-6-85
33. Chung D, Cha M, Guss AM, Westpheling J (2014) Direct conversion of plant biomass to ethanol by engineered *Caldicellulosiruptor bescii*. Proc Natl Acad Sci U S A A111:8931–8936. doi:10.1073/pnas.1402210111
34. Lochner A, Giannone RJ, Keller M, Antranikian G, Graham DE, Hettich RL (2011) Label-free quantitative proteomics for the extremely thermophilic bacterium *Caldicellulosiruptor obsidiansis* reveal distinct abundance patterns upon growth on cellobiose, crystalline cellulose, and switchgrass. J Proteome Res 10:5302–5314. doi:10.1021/pr200536j
35. Lochner A, Giannone RJ, Rodriguez M, Shah MB, Mielenz JR, Keller M, Antranikian G, Graham DE, Hettich RL (2011) Use of label-free quantitative proteomics to distinguish the secreted cellulolytic systems of *Caldicellulosiruptor bescii* and *Caldicellulosiruptor obsidiansis*. Appl Environ Microbiol 77:4042–4054. doi:10.1128/aem.02811-10
36. Hamilton-Brehm SD, Mosher JJ, Vishnivetskaya T, Podar M, Carroll S, Allman S, Phelps TJ, Keller M, Elkins JG (2010) *Caldicellulosiruptor obsidiansis* sp. nov., an anaerobic, extremely thermophilic, cellulolytic bacterium isolated from Obsidian Pool, Yellowstone National Park. Appl Environ Microbiol 76:1014–1020. doi:10.1128/aem.01903-09
37. Yang SJ, Kataeva I, Hamilton-Brehm SD, Engle NL, Tschaplinski TJ, Doeppke C, Davis M, Westpheling J, Adams MWW (2009) Efficient degradation of lignocellulosic plant biomass, without pretreatment, by the thermophilic anaerobe *Anaerocellum thermophilum* DSM 6725. Appl Environ Microbiol 75:4762–4769
38. Basen M, Rhaesa AM, Kataeva I, Prybol CJ, Scott IM, Poole FL, Adams MWW (2014) Degradation of high loads of crystalline cellulose and of unpretreated plant biomass by the thermophilic bacterium *Caldicellulosiruptor bescii*. Bioresour Technol 152:384–392. doi:10.1016/j.biortech.2013.11.024
39. Yee KL, Rodriguez M, Tschaplinski TJ, Engle NL, Martin MZ, Fu CX, Wang ZY, Hamilton-Brehm SD, Mielenz JR (2012) Evaluation of the bioconversion of genetically modified switchgrass using simultaneous saccharification and fermentation and a consolidated bioprocessing approach. Biotechnol Biofuels 5(1):81. doi:10.1186/1754-6834-5-81
40. Wilson CM, Rodriguez M, Johnson CM, Martin SL, Chu TM, Wolfinger RD, Hauser LJ, Land ML, Klingeman DM, Syed MH, Ragauskas AJ, Tschaplinski TJ, Mielenz JR, Brown SD (2013) Global transcriptome analysis of *Clostridium thermocellum* ATCC 27405 during growth on dilute acid pretreated *Populus* and switchgrass. Biotechnol Biofuels 6(1):179. doi:10.1186/1754-6834-6-179
41. Linville JL, Rodriguez M Jr, Mielenz JR, Cox CD (2013) Kinetic modeling of batch fermentation for *Populus* hydrolysate tolerant mutant and wild type strains of *Clostridium thermocellum*. Bioresour Technol 147:605–613. doi:10.1016/j.biortech.2013.08.086
42. Kataeva I, Foston MB, Yang SJ, Pattathil S, Biswal AK, Poole FL, Basen M, Rhaesa AM, Thomas TP, Azadi P, Olman V, Saffold TD, Mohler KE, Lewis DL, Doeppke C, Zeng YN, Tschaplinski TJ, York WS, Davis M, Mohnen D, Xu Y, Ragauskas AJ, Ding SY, Kelly RM, Hahn MG, Adams MWW (2013) Carbohydrate and lignin are simultaneously solubilized from unpretreated switchgrass by microbial action at high temperature. Energy Environ Sci 6:2186–2195. doi:10.1039/c3ee40932e
43. Holwerda EK, Thorne PG, Olson DG, Amador-Noguez D, Engle NL, Tschaplinski TJ, P van Diijken J, Lynd LR (2014) The exometabolome of *Clostridium thermocellum* reveals overflow metabolism at high cellulose loading. Biotechnol Biofuels 7:155. doi:10.1186/s13068-014-0155-1
44. Ellis LD, Holwerda EK, Hogsett D, Rogers S, Shao X, Tschaplinski T, Thorne P, Lynd LR (2012) Closing the carbon balance for fermentation by *Clostridium thermocellum* (ATCC 27405). Bioresour Technol 103:293–299. doi:10.1016/j.biortech.2011.09.128
45. Selig MJ, Viamajala S, Decker SR, Tucker MP, Himmel ME, Vinzant TB (2007) Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retards enzymatic hydrolysis of cellulose. Biotechnol Prog 23:1333–1339. doi:10.1021/bp0702018
46. Kumar R, Wyman CE (2009) Access of cellulase to cellulose and lignin for poplar solids produced by leading pretreatment technologies. Biotechnol Prog 25:807–819. doi:10.1002/btpr.153