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Cellulose fermentation by *Clostridium thermocellum* and a mixed consortium in an automated repetitive batch reactor

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HIGHLIGHTS

- An automated repetitive batch fermentation system was developed.
- A strong correlation was observed between CO_2 produced and cellulose consumed.
- *C. thermocellum* and a mixed consortium exhibit accelerated substrate utilization.
- Similar numbers of cycles were needed to reach maximum CO_2 production rates.
- Maximum CO_2 production rates and cycle times were very similar for both cultures.

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ABSTRACT

An automated repetitive batch fermentation system was developed to facilitate the study of microbial cellulose utilization. The system was operated with Avicel as the carbon source and either *Clostridium thermocellum* ATCC 27405 or a consortium enriched from compost as inocula. Multiple cycles of growth medium addition, incubation, and medium removal were performed with each inoculum. Removal and addition of media were automatically initiated when CO_2 production fell to 90% of the cycle's peak. A strong correlation was observed between CO_2 production and cellulose consumption, suggesting that the online signal was a good proxy for substrate utilization. Both cultures exhibited accelerated substrate utilization and a decrease in cycle time. About the same number of cycles was required to reach maximum CO_2 production for both cultures. Notably, the magnitudes of the maximum CO_2 production rate and cycle times were very similar for both *C. thermocellum* in pure culture and an environmental consortium.

1. Introduction

Cellulolytic microorganisms play key roles in the global carbon cycle and are of significant interest for the production of renewable fuels and chemicals (Lynd et al., 2002; Himmel et al., 2007). Microbes which rely on non-oxidative processes for provision of metabolically useful energy are of particular interest in the latter context, since a very high fraction of the substrate enthalpy is conserved in products (Lynd et al., 2005).

Pure culture studies play a key role in understanding microbial cellulosic utilization. *Clostridium thermocellum* is a widely-studied cellulolytic, thermophilic anaerobic bacterium, which solubilizes cellulose primarily via a cell surface-associated multi-enzyme complex called the cellulosome (Shoham et al., 1999; Gilbert, 2007). The structure of the cellulosome is believed to foster synergistic interactions between enzyme components, and there is evidence that cellulosomes are several-fold more effective when they act as part of cellulose–enzyme–microbe complexes as compared to when they act without a physical association with cells (Lu et al., 2006). On the other hand, enrichment cultures derived from inocula taken from nature are also useful in the discovery of novel species and cell functions, and in gaining valuable insights into the mechanics of lignocellulose degradation (Haruta et al., 2002; Burrell et al., 2004; Izquierdo et al., 2010; Carver et al., 2011; De Angelis et al., 2012; Ronan et al., 2013). In particular, studies using enrichment cultures allow evaluation of lignocellulose solubilization under conditions that more nearly approximate those present in nature as compared to pure cultures.

Although batch and continuous cultures each have merits for characterizing microbial solubilization of lignocellulose, batch cultures are of interest because cultures grow at their maximum

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growth rate, often achieve a higher fractional substrate utilization (Wisselink et al., 2007), can avoid metabolic responses associated with stationary phase (Huang et al., 2008), and for some purposes are more time-efficient to study. On the other hand, continuous cultures allow for cultures to evolve, and in the case of mixed enrichment adjust their species composition. Automated repeated batch (ARB) culture, in which a fraction of the cell broth is used as an inoculum for successive batch cycles, combines some of the merits of conventional batch and continuous cultures. ARB cultures have been used to enrich and characterize microbes and microbial communities on a variety of substrates (Wisselink et al., 2007; Huang et al., 2008; Tolvanen et al., 2011), although not for cellulosic substrates.

Here, an ARB system was developed for the cultivation of thermophilic, cellulolytic bacteria on a model cellulosic substrate (Avicel), observe the behavior of pure cultures of *C. thermocellum* in this system, and compare the performance of mixed consortia obtained from an environmental inoculum.

2. Methods

2.1. Inocula

C. thermocellum ATCC 27405 was obtained from the American Type Culture Collection (Manassas, Virginia), maintained on MTC medium, and stored at −80 °C until grown in a reactor containing 1 L of low carbon (LC) medium with 5 g/L Avicel PH-105 to 90% of peak CO_2 and stored in 50 mL aliquots in purged serum bottles at −80 °C until needed. Bottles were thawed at room temperature with no agitation and injected directly into the reactor for inoculation.

A cellulolytic consortium was obtained after three transfers from a primary enrichment using thermophilic horse manure compost from the Dartmouth College composting facility, as previously described (Izquierdo et al., 2010), and designated DC3-3. These transfers were grown in Defined Minimal Media (DMM) with 3 g/L Avicel PH-105 in 125 mL serum bottles. The inoculum for fermentation was prepared through an additional transfer in Low Carbon (LC) media with 5 g/L Avicel PH-105 then grown in a 1 L fermentor on LC media with 5 g/L Avicel PH-105. 50 mL aliquots were taken at 90% of peak CO_2 and stored in purged serum bottles at −80 °C until needed. Bottles were thawed at room temperature with no agitation and injected directly into the reactor to inoculate.

2.2. Media formulation

Low carbon (LC) medium, used for repetitive batch experiments, was previously developed (Holwerda et al., 2012) to reduce the amount of carbon other than the primary growth substrate and to increase the accuracy of analytical techniques such as total organic carbon analysis. The medium was prepared as previously described (Holwerda et al., 2012), with the addition of 1 mL SL-10 trace elements per liter (Atlas, 1996). For media volumes greater than 10 L, media were prepared in 2 1 L carboys as described above, and then transferred to a sterile, nitrogen-purged 20 L carboy after all components had been added. Defined mineral medium (DMM), used for the original enrichment of consortium DC3-3, was prepared as described in Izquierdo et al. (2010) with the following modifications: Avicel, PH-105 (FMC CORP., Philadelphia), 5 g/L; $NaHCO_3$, 2 g/L; $MgCl{\cdot}6H_2O$, 0.1 g/L. MTC medium was prepared as described in Holwerda et al. (2012) with the following modifications: resazurin, 2 mg/L; 3-(N-morpholino) propanesulfonic acid (MOPS), 10 g/L; urea, 5 g/L; Avicel PH105, 5 g/L as the sole carbon substrate.

2.3. Automated repetitive batch fermentation system

A system was developed to conduct repetitive batch fermentation experiments with the benefits of online measurements and automatic batch transfers. For each batch transfer, a well-mixed reactor was drained to a specified liquid level of 15% or 150 mL of the original 1 L and refilled to the original volume of 1 L with 850 mL of fresh media. This allowed the cells that remained in the vessel to serve as the inoculum for the next transfer. The movement of liquid from one vessel to another was achieved by pressurization of the donor vessel, while the recipient vessel was kept at slightly higher than atmospheric pressure.

The system studied consisted of a 1 L water-jacket-heated Sartorius Biostat A fermentor (Sartorius Stedim, Gottingen, Germany), a large 10 or 20 L media carboy with a magnetic stir plate, a second 1 L water-jacket-heated vessel used as a media preheater, an automated sampler system, a waste carboy, and an off gas CO_2 analyzer (Fig. 1). A control program was created in LabVIEW (National Instruments, Austin, TX) to control the functions of the automated repetitive batch system and aid the user in programming experiments. The system used an electromagnetic relay board controlled by LabVIEW (Table 1). This relay board controlled 11 solenoid pinch valves, the liquid level sensors, fraction collector, and sample pump, as depicted in Fig. 1 and Table 1. All connections in the system were made with size 16 Norprene tubing.

An electronic liquid level sensor was designed to accurately measure the two volumes of liquid, 150 mL and 850 mL, required for the batch transfer process. The liquid level system (Fig. 1) consisted of a stainless steel probe electrically isolated from the reactor headplate.

The automated sampler module consisted of 3 normally closed pinch valves, a port that reached the bottom of the reactor, a N_2 gas line, a peristaltic pump, and a BioFrac fraction collector (Bio-Rad, Hercules, CA). The fraction collector and the pump were both controlled using LabVIEW by relays in an USB-IIRO 16 relay board. The fraction collector featured a robotic arm and 15 mL conical tubes in a water bath that was cooled to 1 °C by an immersion coil fed by an external chiller using propylene glycol. The sample port and all tubes were purged at the end of each sampling event. 22 mL samples were taken at the beginning and end of each batch cycle as well as every 2 h during the cycle.

2.4. System operation

Each repetitive batch experiment consisted of an initial fermentation and then 14 subsequent transfers for a total of 15 fermentation cycles. Growth media was prepared in one 15 L batch in a 20 L carboy and used for all of the cycles of a given experiment to maintain consistency. The first cycle was inoculated with 50 mL of culture from a serum bottle by syringe. The LabVIEW control program tracked the maximum CO_2 rate in real time during the fermentation and reactor emptying and refilling was initiated when the CO_2 rate fell to 90% of the maximum value. Samples were taken at the beginning and end of each cycle as well as every 2 h during cycles 2, 8, and 15.

The sequence of events for initiating a new batch cycle was as follows: The pre-heating vessel was filled at the beginning of the cycle so that it had ample time (1 h) to reach operating temperature. When the control program was about to initiate a transfer (determined from the online data trigger described above), the automated sampler took an endpoint sample. The reactor contents were then drained to a volume of 150 mL and pre-heated medium was transferred into to the reactor.

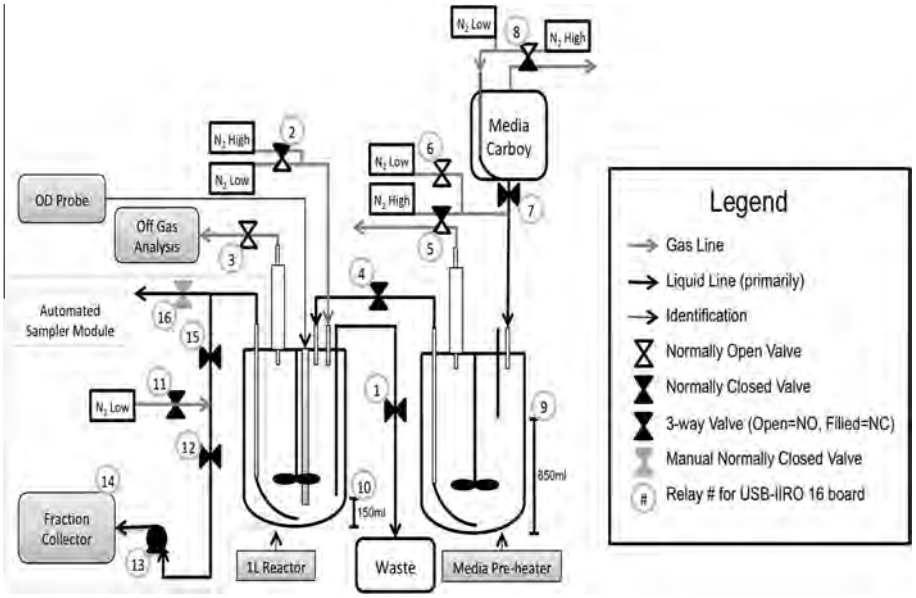

Fig. 1. Detailed diagram of Automated Repetitive Batch (ARB) System. The system allows for automatic sequential batch transfers with automatic sample taking capabilities and online measurement of CO_2 and optical density (OD). The fermentation takes place in a 1 L reactor. Fluid is transferred from vessel to vessel with pressurized N_2 and samples are taken with a pump. Connections between vessels are made with noroprene tubing and pinch valves (1–8, 11, 12, 15, 16). Media is transferred from the media carboy to a separate reactor vessel, the media pre-heater, to heat it to fermentation temperature before batch transfer. Liquid volumes are measured using liquid level sensors (9, 10) During the transfer to the next cycle, 800 mL of fermentation broth is removed from the reactor and refilled with 800 mL of pre-heated media.

Table 1
Detailed description of relay connections, including valve descriptions, liquid level sensors, pump control, and automated sampler fraction collector control.

Relay #	Device description	Relay #	Device description
1	Reactor liquid-level to waste carboy (NC) valve	9	Heater liquid-level on/off
2	Reactor N_2 input high (NC) and low (NO) pressure 3-way valve	10	Reactor liquid-level on/off
3	Reactor off gas (NO) valve	11	Automated sampler line purging gas input valve (NC)
4	Heater to reactor valve (NC)	12	Sample liquid before pump valve (NC)
5	Heater off gas (NO) and high N_2 pressure input (NC) valve	13	Pump control relay (normally off)
6	Heater low pressure N_2 input	14	Fraction collector (starts a new sample while on)
7	Media to heater (NC) valve	15	Sample out port from reactor (NC) valve
8	Media carboy off gas (NO) and high pressure N_2 input (NC) valve.	16	Manual valve for completely draining the reactor (not controlled by relay board)

2.5. Analytical techniques

Approximately 22 mL of fermentation broth were obtained for each sample by the automated sampler and preserved in a 1 °C circulating water bath for no more than 24 h. Once removed from the water bath, the following steps were immediately taken. A total of 3 aliquots of 475 μL of broth supernatant were used for HPLC analysis. From 3 samples of each 1 mL, aliquots of 850 μL of supernatant were used for supernatant Total Organic Carbon analysis and the washed pellets were used for Total Organic Carbon and Nitrogen (TOCN) analysis. The washed pellet sample was diluted 1:10 in Milli-Q DI water and combusted using a TOCN analyzer, Shimadzu Scientific Instruments, to measure pellet nitrogen (PN) and pellet carbon (PC). Solids were mixed before injection.

Acetic acid, cellobiose, ethanol, formic acid, glucose, and lactic acid were measured using High-Performance Liquid Chromatography (HPLC) on a Bio-Rad HPX-87H column (Hercules, CA) operated at 60 °C with 0.01% (v/v) H_2SO_4 mobile phase using the ultra violet (λ = 210 nm) detector.

Cellulose concentration was measured in samples from transfers 1, 7, and 13. Aliquots of 5 mL were added to Corning (Lowell, MA) 15 mL conical tubes containing 250 μL 72% sulfuric acid to stop enzymatic activity and frozen at −4 °C. Cellulose concentration was then assayed by quantitative saccharification, as previously described (Sluiter et al., 2011). The procedure was modified to measure approximately 25 mg of substrate per sample.

A least-squares regression for substrate sugar consumed (mol glucose) vs total CO_2 released (mol) was performed on the data from both fermentations. The regression coefficients were compared as described in Paternoster et al. (1998). Hypothesis tests on the differences in means were performed using the t-distribution. Since these were small sample sizes, the sample populations are assumed to be normal. The population variances are unknown and assumed to be equal.

2.6. 16S rRNA gene PCR

In order to verify the purity of *C. thermocellum* cultures, samples from ARB runs were collected and stored at −80 °C until ready for analyses. DNA was extracted from 1 mL of fermentation broth and PCR was carried out using the universal oligonucleotide primers 8F and 1492R, designed to anneal to conserved regions of bacterial 16S rRNA gene, as previously described (Izquierdo et al., 2010).

3. Results and discussion

An automated repetitive batch (ARB) fermentation system was successfully operated for both a pure culture of *C. thermocellum*

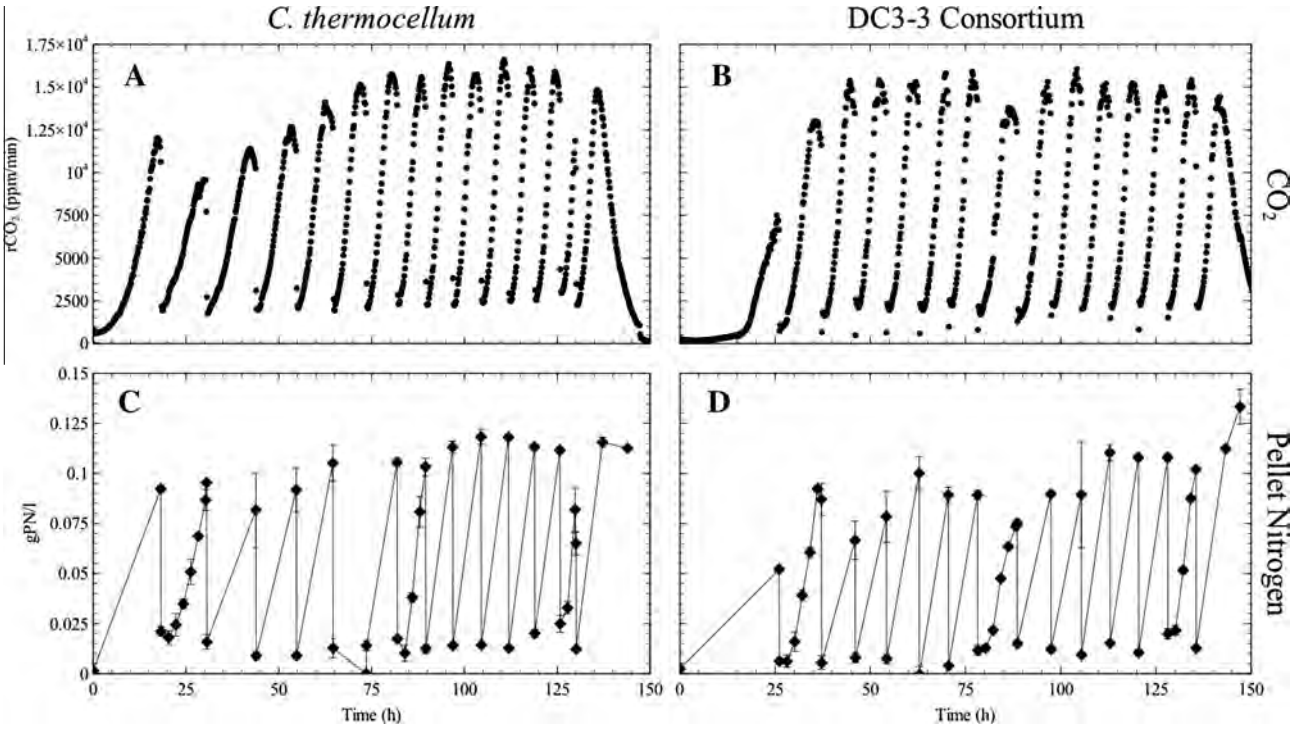

Fig. 2. Rate of production of CO_2 (A and B) and pellet nitrogen (C and D) for *C. thermocellum* (A and C) and DC3-3 (B and D) from 15 cycle repetitive batch experiments. Batch transfer was initiated once the CO_2 production had fallen to 90% of its peak production. Error bars on pellet nitrogen data depict standard deviation calculated from triplicate samples. The endpoint sample for the fifth transfer of the *C. thermocellum* fermentation was not shown, due to a malfunction in the automated sampler.

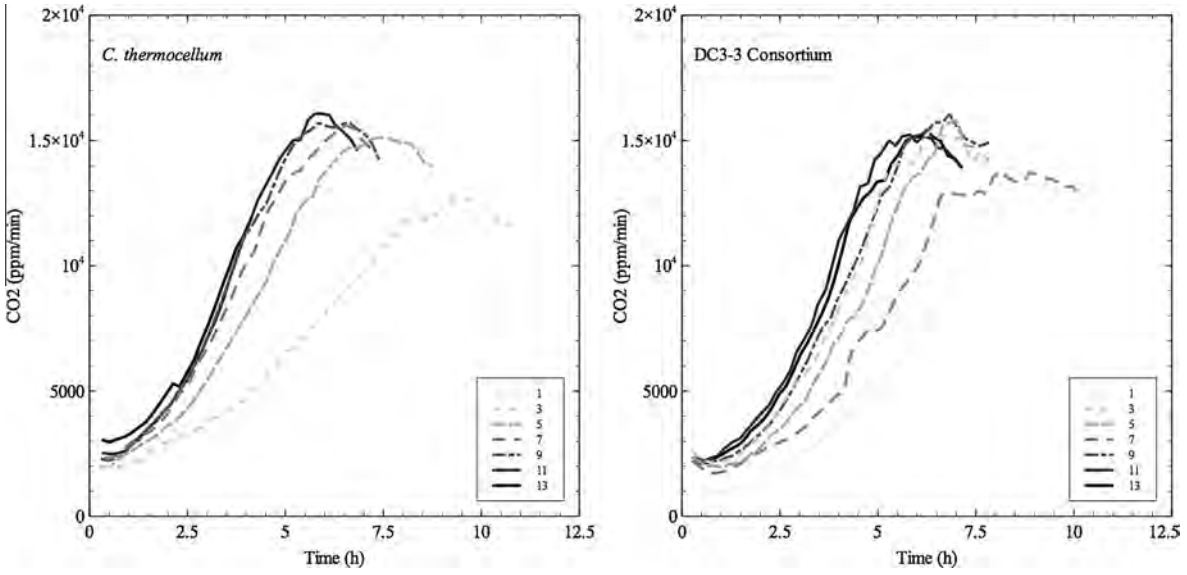

Fig. 3. Rate of CO_2 production by cycle for odd number cycles. *C. thermocellum* (left), DC3-3 (right). Both fermentations exhibited an increase in the maximum rate of CO_2 production as well as a decrease in time to peak CO_2 rate in successive cycles. Data from omitted cycles follow the same trends as data depicted.

and a mixed consortium denoted DC3-3 and obtained from composting horse manure using techniques as described previously (Izquierdo et al., 2010). For each inoculum, the system was operated for 15 growth medium addition cycles. Removal and addition of growth media were initiated when the online CO_2 production measurements fell to 90% of the cycle's peak value. Incubation was maintained at pH 7.0, 60 °C with Avicel as the growth substrate using a medium in which non-substrate organic carbon was a small (1.6%) fraction of substrate carbon (Holwerda et al., 2012).

Fig. 2 presents CO_2 and pellet nitrogen data for fermentation in the ARB system inoculated with either *C. thermocellum* or the DC3-3 consortium. Regular cycles of CO_2 accumulation were rapidly established for both cultures. Pellet nitrogen, increased many-fold during each cycle, indicative of cell synthesis. A few abnormal cycles were observed – e.g. cycle 14 for *C. thermocellum*, cycle 8 for the consortium – indicative of an apparent malfunction or variable input factor. It should be noted that the concentration of reducing agent used in these experiments was exceedingly low (0.1 g/L cysteine), which likely increased the sensitivity of

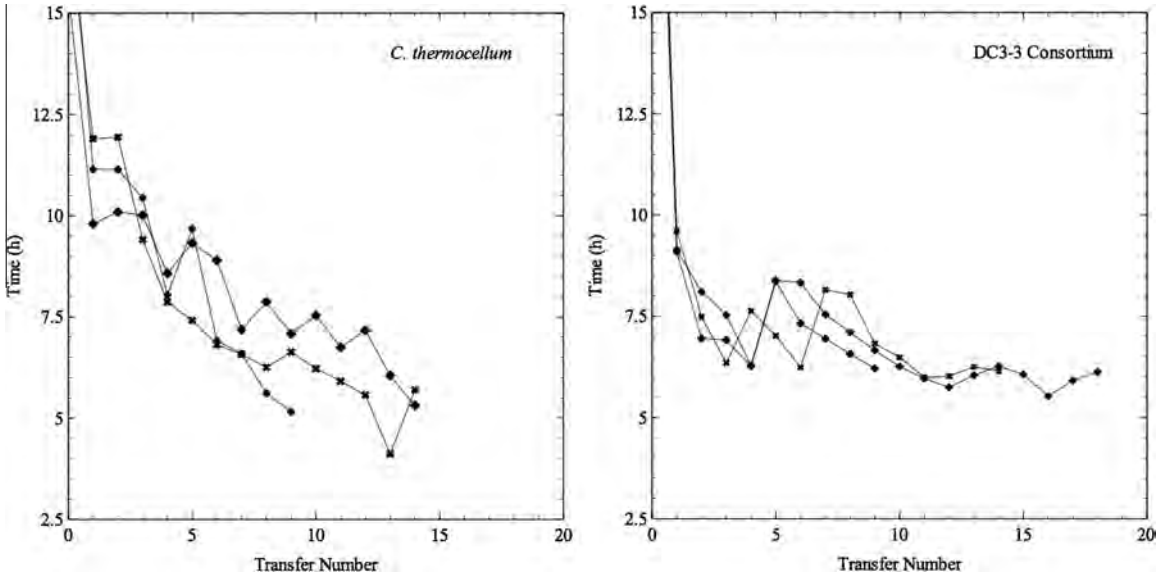

Fig. 4. Reproducibility and acceleration of batch fermentation cycles shown for three replicate runs using ARB system with *C. thermocellum* and DC3-3 enrichment culture as inocula.

the systems to oxygen. 16S rRNA sequencing carried out at the end of the experiments shown in Fig. 2 showed no evidence of contamination for the *C. thermocellum* pure culture and decisively different results for the consortium (data not shown). A pronounced accelerating trend with successive cycles for both cultures can be observed on Fig. 3, which presents the superimposed CO_2 traces from the experiments depicted in Fig. 2.

This acceleration trend can also be observed in Fig. 4, which presents data for the cycle time (t_c) for multiple experiments initiated with either *C. thermocellum* or the DC3-3 culture. Both the rate of decrease in the cycle time (t_c) and the magnitude of t_c eventually realized were similar for both inocula and were also quite reproducible in replicate experiments. For replicate runs exceeding 10 cycles, t_c was initially greater than 9 h for both innocula but decreased to 5.70 ± 0.87 h for *C. thermocellum* and 6.19 ± 0.40 h for the DC3-3 consortium over the final three cycles. The differences between the t_c values for the final three cycles obtained with the two inocula were not statistically significant ($p > 0.05$). Both inocula exhibited substantial increases in the rate of cellulose utilization with successive transfers, as shown by the reduction of time to maximum CO_2 from early transfers to the final transfers by 24% and 46% for *C. thermocellum* and the DC3-3 consortium respectively. More detailed characterization of the phenomena underlying this acceleration would be of considerable interest.

Cumulative CO_2 production is plotted as a function of cellulose consumed in Fig. 5. A proportional relationship is observed with R^2 values of 0.88 and 0.90 for *C. thermocellum* and the consortium respectively. The slopes of the best-fit lines, corresponding to moles of CO_2 produced per mole glucosyl unit consumed, is 2.18 ± 1.27 for *C. thermocellum* and 2.93 ± 1.75 for the consortium. The difference between these slopes, however, is not statistically significant ($p = 0.212$), nor is either slope significantly different from 2.00, which might be anticipated in light of biochemical considerations. A linear correlation between cellulose consumed and CO_2 production suggest that rate of CO_2 production is a reasonable proxy for cellulose consumption under the conditions studied. This is of significant value for studies of cellulose utilization by pure cultures and consortia because of the inherent difficulties associated with calculating rates of growth and substrate consumption on solid substrates (Holwerda et al., 2012).

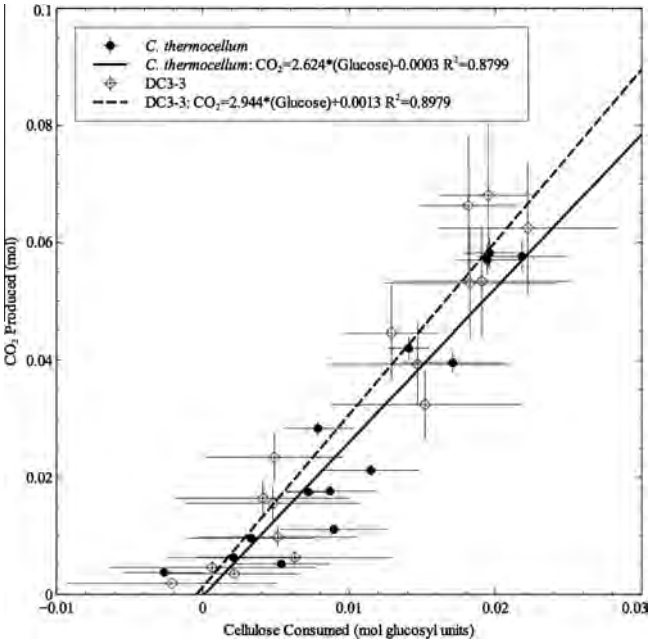

Fig. 5. Totalized CO_2 (mol) produced vs Glucose consumed (mol, as measured by quantitative saccharification) for *C. thermocellum* (filled circles) and DC3-3 (open diamonds). Linear regression lines and equations are depicted for *C. thermocellum* (solid) and DC3-3 (dashed).

Fermentation products are presented in Fig. 6 for *C. thermocellum* and the consortium respectively. The distribution of fermentation products is quite similar for both cultures, with acetate, ethanol and formate as the major organic products observed. Small amounts of lactate consistently appear in DC3-3 cultures, but not in the *C. thermocellum* cultures. For both fermentations, the proportions of products changed little throughout 15 cycles, although *C. thermocellum* produced a higher proportion of formate during early cycles, whereas formate only appears in the DC3-3 fermentation after transfer 8.

In general, stable, reproducible results were obtained for both cultures, albeit with occasional abnormal cycles. The choice of growth medium used ensured that the behaviors observed were

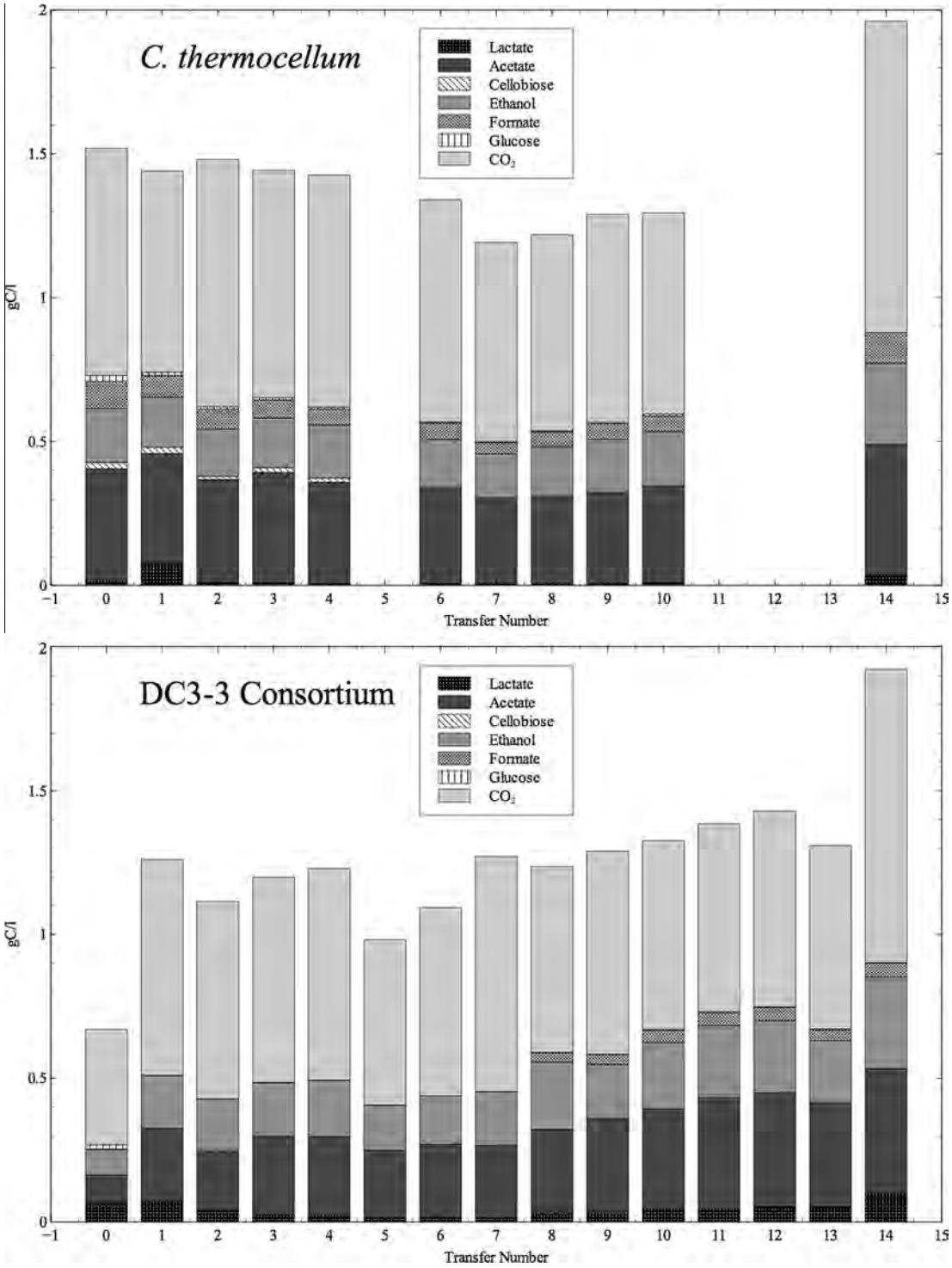

Fig. 6. Fermentation product profile for *C. thermocellum* and DC3-3 consortium at the end of 15 cycle repetitive batch fermentations.

dominated by microbial utilization of cellulose rather than organic compounds added as reducing agents, chelating agents, or growth supplements. Substantially higher cycle-to-cycle consistency was observed for the ARB system compared to manual batch transfer in previous studies using environmental enrichments (Izquierdo et al., 2010). It is possible to speculate that this may be due to the avoidance of metabolic events occurring once substrate was exhausted.

It was expected that higher rates of cellulose utilization will be present in the consortium compared to the *C. thermocellum* monoculture, in light of the greater diversity of enzymatic activities and potential for positive interspecies interactions. However, this was not observed. Indeed, once the acceleration period was complete, the performance of the *C. thermocellum* monoculture was remarkably similar with respect to the time to achieve maximum rates of CO_2 evolution and the magnitude of the maximum CO_2 evolution rate (Figs. 2 and 3), both indicative of the rate of cellulose utilization, as well as end products formed (Fig. 6).

It is well documented that the growth rate of microbial enrichment cultures and pure cultures benefits after repetitive batch fermentations regardless of the substrate used for selection (Yokoyama et al., 2007; Wisselink et al., 2009). However, this has

previously been reported on monomeric sugars or gaseous substrates, and not on a model cellulosic substrate such as Avicel. In other applications, repetitive batch fermentation has been successfully used in increasing growth rates in genetically-modified yeasts (Wisselink et al., 2007) and product formation in acetogens (Sakai et al., 2005). In the case of the former, increases in CO_2 formation were concomitant with accelerated growth rates and substrate utilization, in very similar fashion to the type of evolution observed for both *C. thermocellum* and the DC3-3 consortium throughout ARB fermentations using cellulose.

4. Conclusion

This paper represents a significant step toward establishing automated repetitive batch (ARB) fermentation as a tool to study microbial cellulose utilization. Using a model cellulosic substrate, a marked acceleration was observed with successive medium replacement cycles as well as a proportional relationship between CO_2 production and cellulose consumed. Notably, results were similar for a pure culture of *C. thermocellum* and an environmental consortium. Promising directions for future research include examination of changes during the acceleration period, characterization of the phylogenetic composition of mixed consortia, use of more complex lignocellulosic substrates such as switchgrass, and rate comparisons under different growth conditions.

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