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# Degradation of high loads of crystalline cellulose and of unpretreated plant biomass by the thermophilic bacterium *Caldicellulosiruptor bescii*

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## HIGHLIGHTS

- *C. bescii* degrades 60% of 50 g $L^{-1}$ crystalline cellulose.
- *C. bescii* grows on industrially relevant loads of unpretreated biomass (200 g $L^{-1}$).
- Growth is inhibited by low concentrations (20 g $L^{-1}$) of acid-treated switchgrass.
- Carbon balances are closed after degradation of crystalline cellulose and switchgrass.
- Growth on crystalline cellulose was limited by nitrogen and vitamin availability.

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## ABSTRACT

The thermophilic bacterium *Caldicellulosiruptor bescii* grows at 78 °C on high concentrations (200 g $L^{-1}$) of both crystalline cellulose and unpretreated switchgrass, while low concentrations (<20 g $L^{-1}$) of acid-pretreated switchgrass inhibit growth. Degradation of crystalline cellulose, but not that of unpretreated switchgrass, was limited by nitrogen and vitamin (folate) availability. Under optimal conditions, *C. bescii* solubilized approximately 60% of the crystalline cellulose and 30% of the unpretreated switchgrass using initial substrate concentrations of 50 g $L^{-1}$. Further fermentation of crystalline cellulose and of switchgrass was inhibited by organic acid end-products and by a specific inhibitor of *C. bescii* growth that did not affect other thermophilic bacteria, respectively. Soluble mono- and oligosaccharides, organic acids, carbon dioxide, and microbial biomass, quantitatively accounted for the crystalline cellulose and plant biomass carbon utilized. *C. bescii* therefore degrades industrially-relevant concentrations of lignocellulosic biomass that have not undergone pretreatment thereby demonstrating its potential utility in biomass conversion.

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## 1. Introduction

Conversion of plant biomass into liquid biofuels is viewed as a potential sustainable and cost-effective alternative to fossil fuels (Demain, 2009). Plants have evolved numerous mechanisms to counteract microbial and enzymatic attacks. The major obstacle for their efficient and economic conversion to fuels is the recalcitrance of their three major components, the glycan polymers cellulose and hemicellulose and the polyaromatic lignin. Harsh thermochemical and physical pretreatments of the biomass are required to enable the cellulose and hemicellulose to be enzymatically hydrolyzed (Alvira et al., 2010; Yang and Wyman, 2008). It has also been suggested to use microorganisms that produce cellulolytic and hemicellulolytic enzymes and subsequently ferment the sugars to biofuels in a process known as consolidated bioprocessing (CBP) (Lynd et al., 2002). However, to date no microorganism is known that can at the same time ferment polysaccharides from plant biomass and produce a biofuel at the needed rates and efficiencies.

Advances have been made recently in genetically-modifying non-saccharolytic, ethanol-producing microorganisms, particularly *Saccharomyces cerevisiae*, to produce polysaccharide-degrading enzymes (Olson et al., 2012) but to do so in sufficient amounts for efficient biomass conversion is a major challenge. An alternative strategy is to use microorganisms that are naturally cellulolytic and hemicellulolytic and engineer them to produce the biofuel of choice. Among those relatively few microorganisms that have the capability to ferment crystalline cellulose, most studies have focussed on the moderately thermophilic bacterium *Clostridium*

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(*Cm.*) *thermocellum*. This organism grows optimally ($T_{opt}$) at 60 °C and is a potential CBP organism. It degrades crystalline cellulose using a multienzyme complex known as the cellulosome and it also produces ethanol as fermentation product (Lynd et al., 2002). *Cm. thermocellum* does not utilize hemicellulose, consequently it has been grown in co-culture with xylan- or xylose-utilizing, ethanol-producing bacteria such as species of *Thermoanaerobacter* and *Thermoanaerobacterium* in order to increase ethanol yield and titer (Argyros et al., 2011; Demain et al., 2005; Ng et al., 1981). As yet utilization of high loads of pretreated plant biomass (above 10 g $L^{-1}$) has not been reported for such co-cultures.

Some species of the thermophilic bacterial genus *Caldicellulosiruptor* utilize both crystalline cellulose and hemicellulose and metabolize both $C_5$ and $C_6$ sugars (Blumer-Schuette et al., 2012; van de Werken et al., 2008). In addition, they grow optimally at higher temperatures (70–80 °C) than species of *Clostridia* and *Thermoanaerobacter* (typically near 60 °C). Advantages of fermentations at elevated temperatures include higher mixing rates, a lower risk of contamination and facilitated biofuel recovery (Taylor et al., 2009). Moreover, genome sequencing of several *Caldicellulosiruptor* species has revealed that they do not contain cellulosomes. Rather, they represent the second paradigm for degrading crystalline cellulose that involves free enzymes rather than a large multienzyme complex. They contain a variety of glycosyl hydrolases and polysaccharide lyases that include many multidomain cellulases and xylanases (Blumer-Schuette et al., 2012; Van Fossen et al., 2009). With one exception (Svetlitchnyi et al., 2013), the *Caldicellulosiruptor* species produce only trace amounts of ethanol (Hamilton-Brehm et al., 2010). Nevertheless, a genetic system has been recently developed for one species (Chung et al., 2012), so metabolic engineering of this group for biofuel production should be feasible in the near future.

A major development within the *Caldicellulosiruptor* genus was the demonstration that one species, *C. bescii* ($T_{opt}$ 78 °C), degraded switchgrass, a model plant for bioenergy production, that had not been subjected to thermochemical pretreatment (Yang et al., 2009). The organism also degraded switchgrass after it had simply been washed with hot water at the growth temperature of the microorganism (78 °C). This showed that the microbe was not simply utilizing non-recalcitrant soluble sugars present in unpretreated biomass (Kataeva et al., 2013). However, these studies were performed at a relatively low biomass concentration (5 g $L^{-1}$), at least an order of magnitude lower that what would be required for any industrial process. High concentrations of biomass require less water and energy for heating and cooling, therefore reducing processing costs (Kristensen et al., 2009), but the degradation of industrial loads of unpretreated plant biomass (>10 g $L^{-1}$) has not been previously studied. Surprisingly, there are also only a few reports on the microbial degradation of high loads (>10 g $L^{-1}$) of either thermochemically-pretreated lignocellulosic material or on crystalline cellulose (Argyros et al., 2011; Desvaux et al., 2001; Jin et al., 2012; Svetlitchnyi et al., 2013).

We show here that that *C. bescii* can grow on and degrade high concentrations of both unpretreated switchgrass and crystalline cellulose (up to 200 g $L^{-1}$), while it grows poorly on even low loads of thermochemically-pretreated switchgrass (>10 g $L^{-1}$). Unexpectedly, we also demonstrate that different physiological mechanisms are responsible for limiting growth of *C. bescii* on crystalline cellulose and on unpretreated plant biomass.

## 2. Methods

### 2.1. Growth experiments

*C. bescii* DSM6725 was grown in the medium described previously (Yang et al., 2010). The carbon substrates used were cellobiose, crystalline cellulose (Avicel PH-101) (both obtained from Sigma, St. Louis, MO) or switchgrass (sieved 20/80-mesh fraction; provided by Dr. Brian Davison, Oak Ridge National Laboratory, Oak Ridge, TN). Samples of plant biomass were used without chemical or physical pretreatment other than washing for 18 h with water at 78 °C. This material is referred to as unpretreated switchgrass. Switchgrass referred to as acid-pretreated was subjected to thermochemical treatment (0.05 g $H_2SO_4$ per g dry switchgrass, 190 °C, 5 min) and was obtained from Dr. Jonathan Mielenz of Oak Ridge National Laboratory, TN. Before use, the acid-pretreated biomass was frozen, thawed, washed four times with four volumes of water to neutralize and to remove simple oligomeric sugars, and autoclaved (Yee et al., 2012) for 25 min at 121 °C. Vitamins and bicarbonate were added after autoclaving. Growth experiments were performed at 78 °C, either as small scale closed cultures without pH control (50 mL volume, 75 mL headspace of $N_2/CO_2$ 80/20; shaken at 150 rpm), or in pH-controlled, gas flushed or gas sparged fermentors. These were either the DASGIP parallel bioreactor system (0.5 L, DASGIP, Jülich, Germany) or a 20 L custom fermentor. DASGIP bioreactors were equipped with heating jackets with a custom-made feed-back temperature control. The pH was maintained at 6.8–7.2 by the automated addition of $NaHCO_3$. Gas exchange in the fermentors was achieved by continuous flushing of the gas headspace with $N_2/CO_2$ (80/20 v/v) at a flow rate of 2 L per hour, or alternatively by sparging the culture at the same flow rate. To collect the spent media after microbial growth on crystalline cellulose or switchgrass, the DASGIP fermentors were transferred to an anaerobic chamber containing a $N_2/H_2$ (95/5, v/v) atmosphere. The insoluble substrate was allowed to settle and then separated from the medium by decanting. Cells were removed by centrifugation at 6000*g* for 10 min under anaerobic conditions. In the anaerobic chamber, the cell free media was dispensed into 20 mL aliquots in 50 mL bottles, and the atmosphere was replaced by $N_2/CO_2$ (80/20, v/v), before it was used to support growth of new cultures. Cell densities were monitored using a Petroff–Hauser counting chamber. Before counting, a significant fraction of the insoluble switchgrass was allowed to settle and then removed. Total cell protein was determined using a standard Bradford assay.

### 2.2. Quantification of substrates and products

The amount of residual insoluble substrates was determined as described previously (Yang et al., 2009). The products of the fermentations were determined after removal of cells and insoluble substrates by centrifugation. Total sugars in the culture supernatant were determined photometrically at 490 nm after hydrolysis in concentrated sulfuric acid and derivatization of the sugar with 5% phenol (phenol–sulfuric acid method), using glucose as the standard. Acetate, formate, malate, butyrate, uracil, cellobiose, glucose, and xylose were determined by high-performance liquid chromatography (HPLC) on a 2690 separations module (Waters, Milford, MA) equipped with an Aminex HPX-87H column (300 mm by 7.8 mm; Bio-Rad, Hercules, CA), a photodiode array detector (model 996; Waters) and a refractive index detector (model 410; Waters). The system was operated with 5 mM $H_2SO_4$ as the eluent at a flow rate of 0.5 mL $min^{-1}$. Samples for HPLC were acidified with 0.1 M $H_2SO_4$ and centrifuged before analysis to remove particles. Lactate and ethanol concentrations were determined by using assay kits from Megazyme (Wicklow, Ireland). Soluble protein was measured using the Bradford assay. Amino acids and free ammonium were determined by HPLC after phenyl isothiocyanate derivatization (PITC, Edman's reagent). Prior to derivatization, samples were filtered through a 0.2 μm filter. 5 μl of PITC and 10 μl of the filtered sample were added to 85 μl of a mixture of methanol and triethylamine (7/1 v/v), and

incubated for 20 min in the dark. 400 μl of water were added to stop the derivatization, and precipitates were removed by centrifugation. Ammonium and amino acids in the soluble fraction were separated using the HPLC 2690 separations module (Waters, Milford, MA) equipped with a LiChrosorb $C_8$ reversed phase column (Supelco, Bellefonte, PA) in a linear gradient from 100% solvent A (0.05% v/v trifluoroacetic acid) to 100% solvent B (100% acetonitrile). Ammonium was alternatively quantified by the salicylate method using the solutions of a test kit (Mars Fishcare, Chalfont, PA, USA) in a modified plate reader assay.

### 2.3. Carbon mass balances

Carbon mass balances were calculated using the amount of residual substrates and products as described previously (Kataeva et al., 2013). Uronic acids only contribute a minor fraction (<2%) to the total carbon in the cell wall and were therefore not included in the analysis. *C. bescii* does not use the lignin from the cell walls as a carbon source or as an electron donor (Kataeva et al., 2013) and the lignin content was therefore not included in the carbon balance.

For the estimation of the concentration of carbon in total sugars (determined as described above), glucose was used as a standard and total sugars were expressed in glucose units. Carbon dioxide was not experimentally determined as the gas headspace from the microbial cultures was removed by flushing. It was assumed that carbon dioxide equaled the amount of acetate produced (via the pyruvate ferredoxin oxidoreductase, phosphotransacetylase, and acetate kinase reactions). Microbial biomass formation was estimated from the amount of protein formed assuming that 50% of dry weight is protein and 50% of dry weight is carbon (Norland et al., 1995).

## 3. Results and discussion

### 3.1. Fermentation of high concentrations of crystalline cellulose

The anaerobic thermophilic bacterium *C. bescii* has been shown to degrade crystalline cellulose and switchgrass that had not been chemically-pretreated (Kataeva et al., 2009; Yang et al., 2009) but concentrations high enough to be industrially-relevant have not been previously tested. Here we show that *C. bescii* grows on concentrations of crystalline cellulose as high as 200 g $L^{-1}$ (Fig. 1A and Appendix A; Fig. S1), the highest yet reported for a microbial culture. Concentrations of cellulose above 200 g $L^{-1}$ have been studied using enzymatic degradation, with conversion efficiencies decreasing from ~90% at 20 g $L^{-1}$ to ~30% at 400 g $L^{-1}$ (Kristensen et al., 2009). The highest concentrations using microbial conversion previously studied were 100 g $L^{-1}$ using an unclassified *Caldicellulosiruptor* strain (Svetlitchnyi et al., 2013) and 92 g $L^{-1}$ by a co-culture of a genetically-engineered *Cm. thermocellum* strain and *Thermoanaerobacterium saccharolyticum* (Argyros et al., 2011). Crystalline cellulose has also been used in Solid Substrate/State Cultivation (SSC) where the substrate is soaked with media thereby maximizing substrate and product concentrations. However, the conversion efficiency is very low and efficient mixing and control of pH are significant problems with SSC (Dharmagadda et al., 2010).

In closed bottle cultures with no pH control, *C. bescii* degraded a comparable amount of crystalline cellulose (2.1–2.6 g $L^{-1}$) after 48 h incubation at 78 °C independent of the initial crystalline cellulose concentration over the range 5–50 g $L^{-1}$ (Appendix A; Table S1). Accordingly, the concentrations of the primary end products, lactate and acetate, and the final pH values (4.8 ± 0.1) of the cultures, were similarly independent of the initial crystalline cellulose concentration. These data suggested that the decrease in pH due to organic acid production and the build-up of the hydrogen gas generated by fermentation are likely limiting factors in degrading high loads of crystalline cellulose in closed cultures.

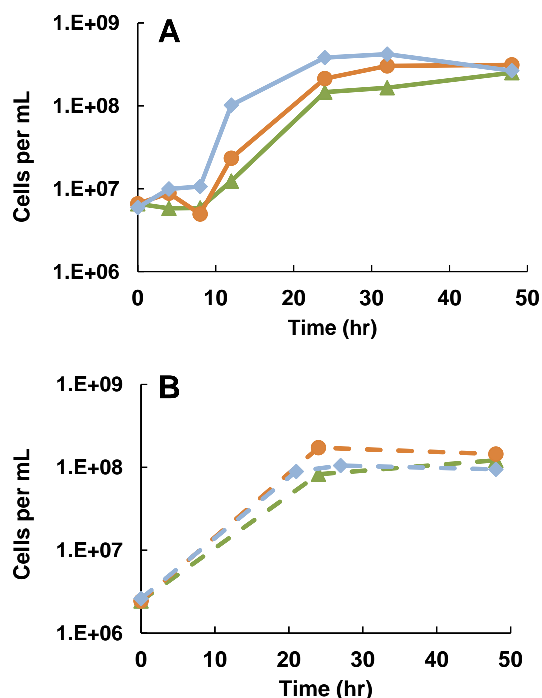


**Fig. 1.** Growth of *C. bescii* on high biomass loads. *C. bescii* was grown in closed cultures on 5 g $L^{-1}$ (green triangles), 50 g $L^{-1}$ (orange circles), or 200 g $L^{-1}$ (blue diamonds) of crystalline cellulose (A) or unpretreated switchgrass (B). Values are averages of three (A) or two (B) biological replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Therefore, *C. bescii* was grown in pH-controlled cultures with continuous flushing of the headspace or sparging of the culture medium with $N_2/CO_2$ (80/20 v/v). Such conditions had a marked effect on the microorganism's ability to degrade crystalline cellulose. With pH control and gas flushing, *C. bescii* completely solubilized crystalline cellulose at substrate loads of both 5 and 10 g $L^{-1}$ and there was approximately 20% conversion using a load of 50 g $L^{-1}$ (see Table 1). The initial crystalline cellulose degradation rate was 96 mg $h^{-1}$ (Appendix A; Fig. S2). When the medium was sparged with $N_2/CO_2$, rather than simply replacing the gas phase by flushing with $N_2/CO_2$, the growth rate also improved (from 0.19 $h^{-1}$ to 0.24 $h^{-1}$), suggesting that efficient removal of hydrogen gas from the medium is not achieved simply by flushing (Appendix A; Fig. S3).

The main carbon end products of crystalline cellulose fermentation by *C. bescii* were acetate, lactate, and $CO_2$ (Fig. 2). Using 50 g $L^{-1}$ and with pH control and gas sparging, approximately 100 mM of these products were generated in the first 35 h (Appendix A; Fig. S4AB). This is similar in terms of soluble carbon yield to what was reported previously with *Cm. thermocellum*, where approximately 40 mM acetate and 35 mM ethanol were formed in 50 h, although the substrate load was lower (10 g $L^{-1}$) (Ellis et al., 2012). For *C. bescii*, the carbon balances (substrates used *versus* products generated) were closed using both low and high crystalline cellulose loads (5 and 50 g $L^{-1}$) showing that the major carbon products and microbial biomass accounted for all of the crystalline cellulose carbon consumed (Fig. 2). Minor products (ethanol, alanine, and pyruvate) accounted for less than 2% of the total carbon. This is in contrast to crystalline cellulose conversion by *Cm. thermocellum*, where a significant number of minor end products were generated that together were equivalent to 11% of the crystalline cellulose utilized (Ellis et al., 2012). Growth and fer-

**Table 1**
Conversion of crystalline cellulose and unpretreated switchgrass by *C. bescii* in pH-controlled (pH 7.2), flushed 10 L fermentors (gas overlay: see Figs. 2 and 5).

| | Initial ($g L^{-1}$) | Converted ($g L^{-1}$) | Acetate (mM) | Lactate (mM) | Glucose (mM) | Cellobiose (mM) | Total sugars (mM) | Cell protein ($\mu g mL^{-1}$) | Cell density ($mL^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| Cellulose | 5 | 5.0 | 32.9 | 23.1 | 0.0 | 0.2 | *n.d.* | 134 | $3.8 \times 10^8$ |
| | 10 | 9.9[a] | 53.5 | 18.5 | 5.7 | 2.0 | *n.d.* | 269 | $5.5 \times 10^8$ |
| | 50 | 10.5[b] | 35.4 | 23.1 | 32.9 | 2.4 | *n.d.* | 162 | $7.8 \times 10^8$ |
| Switchgrass | 5 | 1.8 | 12.1 | 0.6 | 0.0 | 0.0 | 10.0 | 44 | $3.2 \times 10^8$ |
| | 50 | 10.0 | 58.5 | 3.7 | 1.5 | 0.6 | 12.0 | 118 | $4.8 \times 10^8$ |

*n.d.* not determined.
[a] Rate of crystalline cellulose degradation 96 mg $h^{-1}$ (Appendix A; Fig. S2).
[b] Nitrogen limited (see text and Fig. 2). With sufficient $NH_4Cl$ 30 g $L^{-1}$ were degraded (Fig. 3).

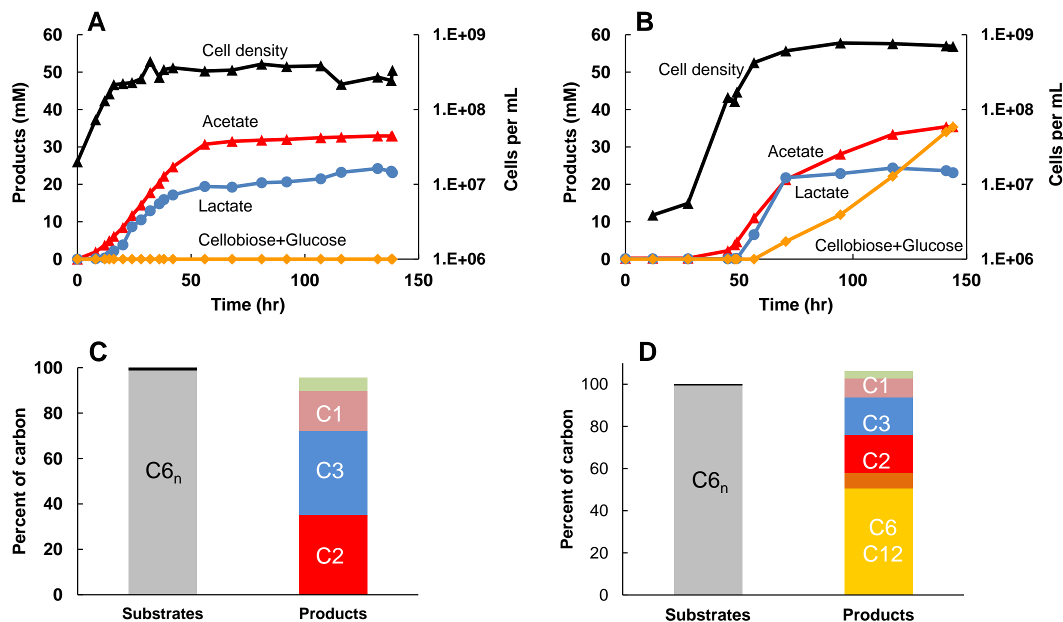


**Fig. 2.** Growth of *C. bescii* on crystalline cellulose with gas-flushing and pH-control. Cell density and fermentation products of *C. bescii* grown on 5 g $L^{-1}$ (A, C) and 50 g $L^{-1}$ (B, D) of crystalline cellulose in a 10 L pH-controlled fermentor with gas flushing. Time courses (A, B) of cell densities (black triangles), and concentrations of acetate (red triangles), lactate (blue circles), and glucose plus cellobiose (orange diamonds). The corresponding carbon balances (C, D) include crystalline cellulose (grey), sugars from yeast extract (black) on the substrate side; and glucose (yellow), cellobiose (orange), acetate (red), lactate (blue), carbon dioxide (pink), and carbon calculated from biomass formation (light green) on the product side. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mentation of crystalline cellulose by *C. bescii* appeared complete after 55 h independent of the initial substrate concentration (Fig. 2). However, in the 50 g $L^{-1}$ culture, glucose, and cellobiose were still being generated after 100 h (Fig. 2) and represented more than 50% of the carbon products. Hence, at high loads, once *C. bescii* reaches a high cell density and ceases to grow, it continues to hydrolyze cellulose to glucose and cellobiose but does not ferment these sugars.

To investigate what limits growth of *C. bescii* on crystalline cellulose, attempts were made to grow the organism on the spent growth medium after cells had been previously grown for 160 h on 50 g $L^{-1}$ crystalline cellulose (Fig. 3). The spent medium contained cellobiose (11 mM) and glucose (19 mM) so addition of carbon substrates was not necessary. However, as shown in Fig. 3A, growth was not observed when the spent medium was inoculated with a fresh culture of *C. bescii.* This was due to nitrogen limitation since the spent medium, which contained only 90 μM ammonium, supported growth to high cell densities ($1.60 \times 10^8$ cells $mL^{-1}$, Fig. 3A) when supplemented with the concentration of ammonium ($NH_4Cl$) that was present in the standard medium (6.2 mM). *C. bescii* was therefore grown on the high crystalline cellulose load (50 g $L^{-1}$) in a pH-controlled, culture sparged with $N_2/CO_2$ (80/20, v/v) in media containing various ammonium concentrations. When the initial ammonium supply was increased from 6.2 to 12.3 mM, the cultures exhausted the nitrogen supply (<100 μM $NH_4^+$) and acetate production ceased (near 80 mM at 35 h). Adding further ammonium (to 12.3 mM) stimulated growth and acetate production resumed, reaching a maximum of 140 mM at 130 h before plateauing (Fig. 3B). Further degradation of the crystalline cellulose was not limited by nitrogen as more than sufficient ammonium (7 mM) remained.

Increasing the ammonium supply therefore had a dramatic effect on the conversion of crystalline cellulose (Fig. 3C and D). The amount degraded almost doubled to 60% (30 g $L^{-1}$ degraded as determined by weight) compared to 36% conversion (18 g $L^{-1}$ degraded) in the standard medium. This resulted in the production of approximately twice the amount of organic acids (135–165 mM, approx. 90% acetate and 10% lactate) and glucose (50–65 mM) compared to the standard medium (82 mM and 30–35 mM, respectively). In contrast, the amount of cellobiose produced (20–30 mM) was very similar in the different cultures (see Fig. 3C and Appendix A; Fig. S4). The growth rate (0.3 $h^{-1}$) and

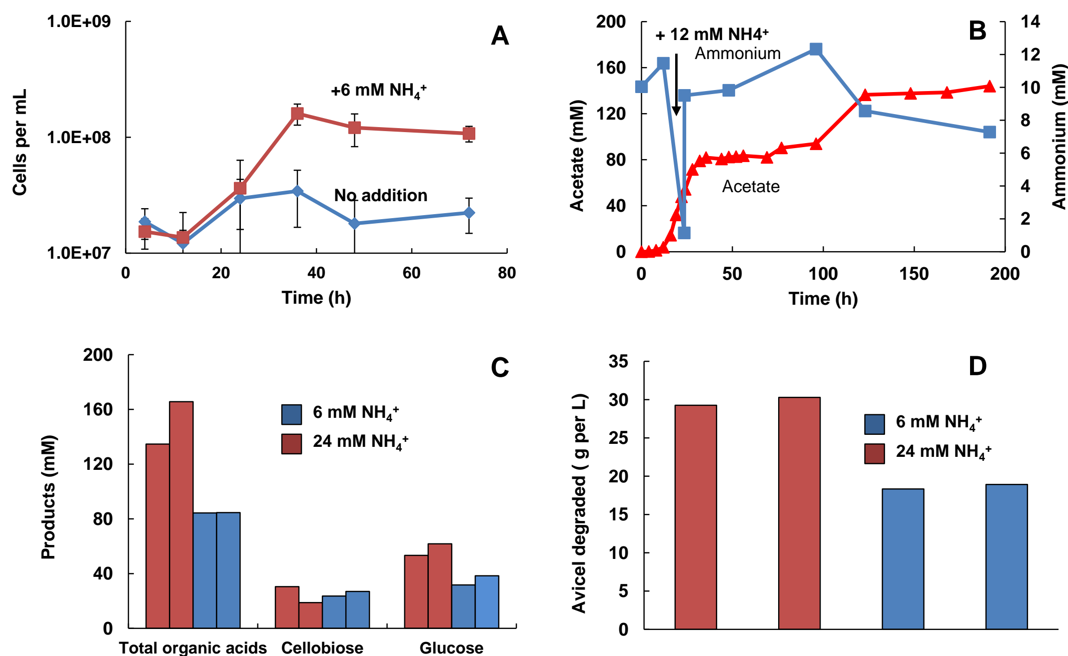


**Fig. 3.** Effect of ammonium addition on conversion of high loads of crystalline cellulose by *C. bescii*. (A) Growth of *C. bescii* on 20 mL spent media in closed cultures without (blue diamonds) and with the addition of 6.2 mM $NH_4Cl$ ($n$ = 3 ± SD). The media was obtained from a pH-controlled *C. bescii* fermentation of 50 g $L^{-1}$ crystalline cellulose after *C. bescii* stopped producing acids. (B) Effect of ammonium addition on acetate production of *C. bescii* in a pH controlled fermentation. (C) Fermentation products of *C.bescii* and (D crystalline cellulose degraded after 192 h by each two replicate pH-controlled, gas-sparged cultures of *C. bescii*. Two cultures were initially supplied with 12.3 mM $NH_4Cl$ concentration (brown bars), twice the concentration that was present in the standard medium (6.2 mM blue bars). After ammonium depletion (Fig. 3B), the culture with the higher initial $NH_4Cl$ concentration was supplied with another 12.3 mM $NH_4Cl$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the rate of product generation were also largely unchanged with the higher concentration of ammonium, indicating that the increased nitrogen supply enables *C. bescii* to continue crystalline cellulose degradation for a longer period (Appendix A; Fig. S4). These results indicate that on high substrate loads, *C. bescii* requires significantly higher amounts of nitrogen, presumably for cell maintenance, to more effectively utilize the substrate and ferment it to end products.

### 3.2. Fermentation of high concentrations of unpretreated lignocellulosic biomass

*C. bescii* also grew to high cell densities on unpretreated lignocellulosic biomass (switchgrass) at high concentrations up to 200 g $L^{-1}$ (Fig. 1 and Appendix A; Fig. S1). The switchgrass samples were first washed with hot water at the growth temperature of the organism (78 °C for 18 h) to remove readily solubilized organic material (sugars, proteins, etc.) that might serve as growth substrates. Hence, the microorganism used the insoluble switchgrass biomass as its carbon source for growth. Previous investigations of microbial biomass degradation used only 5 g $L^{-1}$ (Yang et al., 2009, 2010). The conversion of high loads of thermochemically-treated biomass (50–400 g $L^{-1}$) have been studied using enzymatic hydrolysis (Kristensen et al., 2009) and simultaneous saccharification and fermentation (SSF, 60–115 g $L^{-1}$ (Olofsson et al., 2008)). Pretreated biomass in the form of ammonium-fibre extension (AFEX) corn stover was used at a concentration of 117 g $L^{-1}$ (40 g $L^{-1}$ glucan load) with the mesophilic bacterium *Cm. phytofermentans* (Jin et al., 2012). Hence, the finding that *C. bescii* grew on high loads of biomass that had not undergone thermochemical pretreatment at industrial relevant concentrations of 50–200 g $L^{-1}$ and converted more than 20% to organic acids and sugars in five days bodes well for the concept of CBP in general. We estimate a threshold concentration for industrial fermentations of unpretreated switchgrass between 100 and 150 g $L^{-1}$ because at higher concentrations most of the liquid was absorbed into the substrate (Appendix A; Fig. S1).

Surprisingly, *C. bescii* degraded a constant fraction of the unpretreated switchgrass (27–33%) and this was independent of the initial concentration over the range 1–50 g $L^{-1}$ (Appendix A; Table S2). This is in contrast to the situation with crystalline cellulose, where a constant amount of the substrate was degraded. In pH-controlled fermentor cultures, switchgrass degradation (50 g $L^{-1}$) resulted in the release of soluble sugars (~12 mM as determined by the phenol–sulfuric acid method; Fig. 4B), which accounted for 20% of the product generated (on a carbon basis, Fig. 4D), but less than 10% of these sugars were cellobiose (0.6 mM) and glucose (1.5 mM). The nature of the sugars released from switchgrass remains unknown as the method used measures the degradation products from all soluble carbohydrate. Compared to switchgrass, much more sugar was released from crystalline cellulose (>30 mM, Fig. 2B), accounting for 50% of the carbon products, and in this case all of it was in the form of cellobiose and glucose (Fig. 2D).

In further contrast to the situation with crystalline cellulose, the same products were generated from both low and high loads of unpretreated switchgrass (Fig. 4). We had previously shown that at the low load (5 g $L^{-1}$) the carbon balance between substrates utilized and products generated was approximately 100%, based on the $C_6$ and $C_5$ contents of switchgrass (0.45 and 0.31 g $g^{-1}$, respectively) (Kataeva et al., 2013). For unknown reasons, a lower amount (86%) of the substrates was recovered as products for the high load case (50 g $L^{-1}$: Fig. 4C and D). However, in both cases, 3–4% of the substrate carbon was converted to lactate and 4–6% of it was found in the microbial biomass. The major difference between these two balances is in the amount of acetate formed, which represents 45% and 33% of the carbon product for 5 g $L^{-1}$ and 50 g $L^{-1}$, respectively. The abiotic release of acetate from hemi-

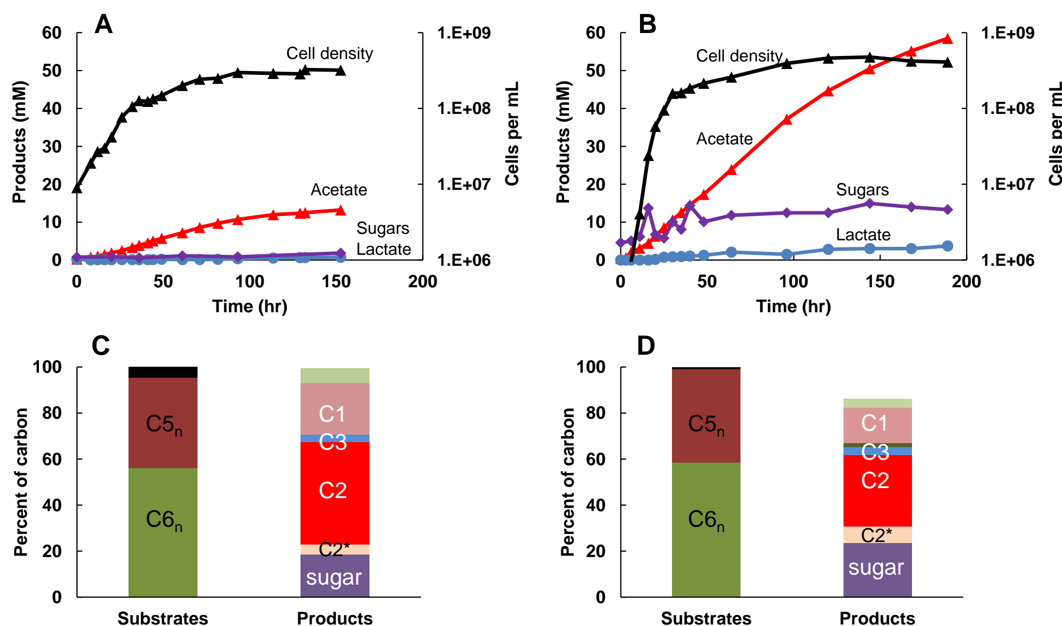


**Fig. 4.** Growth of *C. bescii* on unpretreated switchgrass in a 10 L fermentor and fermentation products. Cell density and fermentation products of *C. bescii* grown on 5 g $L^{-1}$ (A, C) and 50 g $L^{-1}$ (B, D) of unpretreated switchgrass. Time courses (A, B) of cell densities (black triangles), and concentrations of acetate (red triangles), lactate (blue circles), and total sugars (purple diamonds). The corresponding carbon balances (C, D) include C6 sugars (dark green) and C5 sugars (brown) calculated from switchgrass composition and consumption (as determined by weight) and sugars from yeast extract (black) on the substrate side. On the product side totals sugars (determined after acid hydrolysis; pink), acetate (red), acetate derived from hemicellulose acetylation (light pink), acetate (red), lactate (blue), ethanol (dark green), carbon dioxide (pink), and carbon calculated from biomass formation (light green) were quantified. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cellulose acetylation at 78 °C was determined to be 11 mM from 50 g $L^{-1}$ switchgrass. Therefore, up to ~20% of the acetate produced originated from switchgrass deacetylation (Fig. 4C and D). A low concentration of ethanol (2.5 mM) was also produced during degradation of the high switchgrass load representing close to 2% of carbon utilized (Fig. 4D). While there are no previous reports on the degradation of unpretreated biomass by other microorganisms, *C. obsidiansis* (Hamilton-Brehm et al., 2010) and an unclassified *Caldicellulosiruptor* species (Svetlitchnyi et al., 2013) fermented thermoacid-pretreated switchgrass (5 and 10 g $L^{-1}$, respectively) with the products accounting for 74% and up to 97%, respectively, of the carbon consumed. Herein we report the first carbon balance for a high load of unpretreated switchgrass (50 g $L^{-1}$) but in this case the recovery was only 86% and the nature of the 'missing' carbon is not known (although it is not ethanol, alanine, or pyruvate). On the other hand, given the complex composition of plant material such as switchgrass, and the fact that more than 20% of the high load (50 g $L^{-1}$) was solubilized and fermented by *C. bescii*, a recovery of close to 90% of biomass carbon is not unreasonable.

These data therefore demonstrate that *C. bescii* grows to high cell densities using high substrate loads (200 g $L^{-1}$) of unpretreated switchgrass without inhibition of growth with increasing substrate load. However, this was not the case with switchgrass that had undergone thermal acid-pretreatment (with sulfuric acid at 190 °C for 5 min). As shown in Fig. 5, growth of *C. bescii*, as measured by the cell density obtained after 72 h incubation in closed cultures, dramatically decreased with increasing concentrations of acid-treated switchgrass from 10 to 50 g $L^{-1}$, such that there was virtually no growth at the highest biomass concentration tested. The pH at the end of the experiment (72 h) in the cultures with acid-pretreated switchgrass was 6.2 for loads of 10 and 20 g $L^{-1}$and 7.3 for 50 g $L^{-1}$, so growth of *C. bescii* was not inhibited by low pH. In contrast, under the same conditions, high cell densities (at least $2.3 \times 10^8$ cells $mL^{-1}$) were obtained for unpretreated switchgrass using the same biomass concentrations. Moreover, the addition of 50 g $L^{-1}$ acid-pretreated switchgrass inhibited growth of *C. bescii* on 10 g $L^{-1}$ unpretreated switchgrass (Fig. 5).

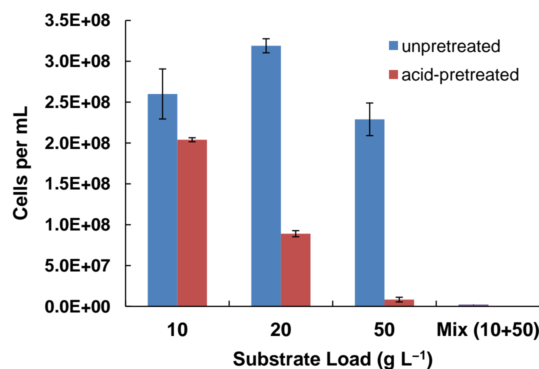


**Fig. 5.** Growth of *C. bescii* on acid-pretreated and unpretreated switchgrass. Cell densities in *C. bescii* cultures after 3 d of incubation at 78 °C (stationary phase) in closed cultures on 10, 20, and 50 g $L^{-1}$ of unpretreated switchgrass (blue bars) and acid-pretreated switchgrass (brown bars); and 10 g $L^{-1}$ unpretreated plus 50 g $L^{-1}$ acid-pretreated switchgrass (purple bars). The experiment was performed in three biological replicates ($n = 3 \pm$ SD). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Clearly, these results show that acid pretreatment of switchgrass releases inhibitors of bacterial growth and characterization of biomass degradation at high substrates loads is only possible with unpretreated switchgrass.

Thermochemical treatment of plant biomass is typically used to greatly reduce the lignin-associated recalcitrance of plant cell walls, rendering the sugar polymers (cellulose and hemicellulose) more accessible to the cellulolytic enzymes. On the other hand, it is one of the most expensive steps in converting plant biomass to biofuels (Yang and Wyman, 2008). In addition, by-products of pretreatment like furan aldehydes, phenols, and certain aliphatic acids can act as inhibitors of microbial fermentations or enzymatic hydrolysis (Jönsson et al., 2013). Pretreatment of biomass with dilute sulfuric acid at high temperature (near 150 °C) is the most commonly used method, and acid-pretreated switchgrass has been

shown to support growth of *Cm. thermocellum* (Yee et al., 2012) and several *Caldicellulosiruptor* species (Blumer-Schuette et al., 2010; Hamilton-Brehm et al., 2010; Yee et al., 2012), However, the highest concentration previously reported was only 10 g (dry weight) $L^{-1}$ (Yee et al., 2012). The results presented herein with *C. bescii* indicate that such a concentration may be close to the limit. Presumably, the higher concentrations of inhibitors that are released from higher concentrations of acid-pretreated switchgrass (above 10 g (dry weight) $L^{-1}$) affect growth and/or enzymatic hydrolysis of the biomass, an effect not observed with unpretreated switchgrass. Since *C. bescii* degrades unpretreated switchgrass (Yang et al., 2009), even at 200 g $L^{-1}$, conventional thermochemical pretreatment of the biomass is clearly unnecessary. This result gives a new perspective on the cost and efficiency of biofuel production using the CBP approach.

### *3.3. Factors limiting further crystalline cellulose and switchgrass fermentation*

Degradation of high loads of crystalline cellulose by *C. bescii* under nitrogen-sufficient conditions resulted in the production of high concentrations of organic acids (~150 mM). Similar product concentrations (up to 150 mM) were achieved with an uncharacterized *Caldicellulosiruptor* species (Svetlitchnyi et al., 2013). As shown in Fig. S5 (see Appendix A), growth of *C. bescii* on the disaccharide cellobiose was affected by much lower organic acid concentrations. The addition of 75 mM sodium acetate to the standard growth medium resulted in a lag phase of about 6.5 h and a decreased growth rate (0.24 $h^{-1}$ compared to a value of 0.40 $h^{-1}$ for the control culture). The effect was even more pronounced at concentrations comparable to those seen during crystalline cellulose degradation. The presence of 150 mM resulted in a lag phase of over 24 h and a resulting growth rate of 0.13 $h^{-1}$. Nevertheless, the cell density eventually reached that seen in the control culture ($\sim 4 \times 10^8$ cells $mL^{-1}$) albeit after 72 h rather than after 20 h. No growth was observed if 200 mM sodium acetate was added to fresh medium (data not shown).

To determine if the production of organic acids was the limiting factor for crystalline cellulose degradation in an *N*-sufficient medium, attempts were made to cultivate a fresh inoculum of *C. bescii* in the spent cell-free medium once cell growth on crystalline cellulose had ceased. The spent medium contained sufficient amounts of sugar for growth (50 mM glucose and 30 mM cellobiose) but also contained 135 mM of organic acids. *C. bescii* grew to a reasonably high cell density ($1 \times 10^8$ cells $mL^{-1}$) after 48 h, but only when a mixture of vitamins and trace elements were added (at concentrations added to a fresh medium: Appendix A, Fig. S8). *C. bescii* requires only the thermally-labile vitamin folate for growth (Kridelbaugh et al., 2013), which is in accordance with its lack of the folic acid biosynthetic pathway, and addition of only folate (rather than a vitamin mixture) stimulated growth of *C. bescii* on the spent medium. However, there was also a significant lag phase for growth of about 50 h (Appendix A; Fig. S8), which might be explained by the energetic burden to synthesize the other vitamins. Continuous addition of folate alone or the vitamin mixture and trace elements throughout the growth phase to fermentations of 50 g $L^{-1}$ crystalline cellulose with sufficient ammonium supply (24 mM) did not lead to a higher concentration of end products (data not shown). Moreover, no growth was observed in the spent medium from a fermentor containing 165 mM of organic acids (acetate plus lactate; data not shown).

These results suggest that the supply of the thermolabile vitamin folate may be a limiting factor under prolonged fermentation conditions but that this is overridden by growth inhibition by the high concentrations of organic acids that accumulate. Complete inhibition of growth of *C. bescii* was reported with an osmolarity of 250 mOsmol (50 mM NaCl; (Farkas et al., 2013)) and growth of its close relative, *C. saccharolyticus,* was affected by an osmolarity of 200–300 mOsmol (from sugar, organic acids, and NaCl) (van Niel et al., 2003). However, we found that inhibition of fermentation and crystalline cellulose degradation is not due to osmotic pressure alone, since growth was observed even with 160 mM organic acids where the osmolarity of the medium is 520 mOsmol (440 mM ions). Additionally, we found that *C. bescii* was able to grow on medium containing 90 g $L^{-1}$ glucose (initial osmolarity of >550 mOsmol), albeit with a long lag phase of >50 h (data not shown).

Organic acids such as acetic acid can also act to uncouple membrane function by dissipating ion gradients (Baronofsky et al., 1984). Consequently, *C. bescii* did not grow in the presence of 200 mM acetate and exhibited a lag phase in the presence of concentrations of acetate comparable to those produced during fermentation (150 mM), although the cells reached cell densities comparable to those obtained in the absence of acetate. A similar concentration of acetate (175 mM) completely inhibited growth of *C. saccharolyticus* (van Niel et al., 2003). It has also been reported that concentrations as low 3 mM cellobiose led to a significant decrease in the purified extracellular cellulase of *Cm. thermocellum* (Johnson et al., 1982). With *C. bescii*, however, cellobiose and glucose production continued despite cellobiose accumulating to concentrations up to 30 mM during crystalline cellulose fermentation (Appendix A; Fig. S4C). Hence, crystalline cellulose degradation by *C. bescii* might be much less sensitive to hydrolysis products than is *Cm. thermocellum.* Of course, the generation by *C. bescii* of ethanol or other alcohols instead of organic acids by metabolic engineering would alleviate issues of product inhibition, a scenario that now seems possible given the recent development of a transformation system for this organism (Chung et al., 2012).

In contrast to the situation with crystalline cellulose, the nitrogen supply did not limit growth of *C. bescii* on unpretreated switchgrass, presumably because additional nitrogen is available from the plant biomass. Similarly, the addition every 24 h of folate or a vitamin mixture (equivalent to that in the original medium) to a *C. bescii* culture growing on 50 g $L^{-1}$ unpretreated switchgrass did not affect the fraction of biomass that was solubilized (data not shown). When a fresh culture of *C. bescii* was added to the cell-free spent medium resulting from the fermentation of 50 g $L^{-1}$ switchgrass, some growth was observed when the medium was supplemented with a carbon source (cellobiose, 5 g $L^{-1}$), but both the growth rate (0.02 $h^{-1}$ *versus* 0.28 $h^{-1}$) and the final cell density ($5 \times 10^7$ *versus* $3 \times 10^8$ cells $mL^{-1}$) were about an order of magnitude less that those measured with *C. bescii* in a fresh medium (Appendix A; Fig. S6). In contrast to the situation with crystalline cellulose, the addition of folate did not stimulate growth in the spent medium (data not shown).

These data suggest that deconstruction of plant biomass over prolonged periods by *C. bescii* eventually generates an inhibitor of microbial growth. It has been suggested that fermentation of both unpretreated (Kataeva et al., 2013) and acid-pretreated (Yee et al., 2012) switchgrass result in the release of lignin-derived phenolic compounds that potentially inhibit microbial growth (Kataeva et al., 2013). Either these phenolic compounds or furan aldehydes created by the acid-pretreatment could be responsible for inhibiting growth of *C. bescii* on the thermochemically-treated material at biomass concentrations above 10 g $L^{-1}$. However, this was not true with unpretreated biomass since *C. bescii* degraded more rather than less biomass with increasing loads (Appendix A; Table S2). Nevertheless, *C. bescii* did not grow well on the spent medium from a pH-controlled fermentation of unpretreated switchgrass (50 g $L^{-1}$) (Appendix A; Fig. S6), even though organic acids were at non-inhibitory concentrations (Appendix A; Table S2). Surprisingly, the spent medium supplemented with cel-

lobiose did support vigorous growth of the anaerobic fermentative bacterium *Thermoanaerobacter mathranii* (which grows optimally at 70 °C), to the same extent as a fresh culture medium. (Appendix A; Fig. S6). Hence if a growth inhibitor is involved, it appears to be specific for *C. bescii* and is presumably derived from the plant biomass as it is not generated when *C. bescii* grows on crystalline cellulose and generates similar fermentation products. Further studies of the growth of *C. bescii* on unpretreated switchgrass are needed to identify the nature of this specific inhibitor.

At present there is no clear benchmark for the concentrations of unpretreated plant biomass that are relevant for industrial conversion processes. That is not the case for thermochemically-treated biomass since the results presented herein suggest that the limit is about 10 g $L^{-1}$, at least for *C. bescii.* One requirement for industrial biomass conversion seems to be the formation of at least 4% (w/v) ethanol (40 g $L^{-1}$ or 868 mM) (Jin et al., 2012). Given the $C_6$ and $C_5$ content of the unpretreated switchgrass used in this study (Kataeva et al., 2013) and the maximum theoretical ethanol yields from the two sugar types (Jin and Jeffries, 2004), an initial concentration of 108 $L^{-1}$ of unpretreated biomass would be sufficient to generate 4% (w/v) ethanol. The high load used herein with *C. bescii* (50 g $L^{-1}$) would theoretically generate close to 2% (w/v) ethanol assuming optimal conversion efficiencies. Of course, as discussed above, at present this organism generates organic acids rather than alcohol, but clearly, in principle, a genetically-modified strain of *C. bescii* that generates the appropriate product could be used in industrial fermentations of extremely high concentrations of unpretreated plant biomass.

## 4. Conclusion

*C. bescii* does not contain cellulosomes but is able to degrade crystalline cellulose as efficiently as cellulosome-containing microbes, such as *Cm. thermocellum* and therefore represents the second paradigm for cellulose degradation. More importantly, *C. bescii* degrades unpretreated switchgrass almost as efficiently as it does crystalline cellulose and does so at substrate loads relevant to industrial conversion (up to 200 g $L^{-1}$). In contrast, growth of *C. bescii* was inhibited by much lower concentrations of thermochemically-pretreated switchgrass. Biomass degradation at high substrates loads necessary for industrial processing, at least by this microorganism, is therefore only possible with unpretreated biomass.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.11.024.

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