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# Switchgrass (*Panicum virgatum*) Fermentation by *Clostridium thermocellum* and *Clostridium beijerinckii* Sequential Culture: Effect of Feedstock Particle Size on Gas Production

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## Abstract

**Fermentation of cellulosic biomass can be done in a single step with cellulolytic, solventogenic bacteria, such as *Clostridium thermocellum*. However, the suite of products is limited in consolidated bioprocessing. Fortunately, the thermophilic nature of *C. thermocellum* can be exploited in sequential culture. Experiments were conducted to determine the effect of feedstock particle size on fermentation by sequential cultures and to demonstrate this effect could be shown by gas production. Dual-temperature sequential cultures were conducted by first culturing with *C. thermocellum* (63˚C, 48 h) before culturing with *C. beijerinckii* (35˚C, 24 h). Switchgrass (2, 5 or 15 mm particle size) was the feedstock in submerged substrate (10% w/v) fermentation. The extent of fermentation was evaluated by gas production and compared by analysis of variance with Tukey's test *post hoc*. *C. thermocellum* alone produced 78 kPa cumulative pressure (approx. 680 mL gas) when the particle size was 2 or 5 mm. The *C. thermocellum* cultures with 15 mm feedstock particles had a mean cumulative pressure of 15 kPa after 48 h, which was less than the 2 and 5 mm treatments ($P < 0.05$). When the culture vessels were cooled (to 35˚C) and inoculated with *C. beijerinckii*, and the cumulative pressures were reset to ambient, cumulative pressure values as great as 70 kPa (equivalent to an additional 670 mL gas) were produced in 24 h. Again, the longer (15 mm) particle size produced less gas ($P < 0.05$). When the substrates were inoculated with *C. beijerinckii* without previous fermentation by *C. thermocellum*, the mean cumulative pressures were**

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**approximately 10 kPa. These results indicate that biological pretreatment with *C. thermocellum* increased the availability of switchgrass carbohydrates to *C. beijerinckii*, and that gas production is suitable method to show the effectiveness of a pretreatment.**

## Keywords

**Bioenergy, Cellulosic Butanol, Co-Culture, Consolidated Bioprocessing**

## 1. Introduction

The availability and low cost of cellulosic biomass has led to interest in its use as a raw material for bioenergy [1]. Cellulosic biomass can be used to produce liquid fuels via fermentation. However, most microorganisms do not produce the enzymes to catabolize lignocellulose. The biomass must be pre-treated (e.g. exogenous cellulases, sodium hydroxide and high temperature) before saccharification. After saccharification, solventogenic bacteria or fungi can ferment the sugars.

Saccharification and fermentation can be done in a single step with cellulolytic, solventogenic bacteria, such as *Clostridium thermocellum* [2]. *C. thermocellum* conducts a mixed-acid fermentation, in which $CO_2$, $H_2$, lactate, acetate, formate and ethanol are the primary products [3] [4]. Consolidated bioprocessing with *C. thermocellum* is not sufficient when other products are desired. However, *C. thermocellum* is a thermophile, and the enzyme system retains ~25% activity at mesophilic temperatures [5]. This characteristic enables sequential culture with a non-cellulolytic mesophile. In this way, the complex, highly active cellulosome of *C. thermocellum* [6] can be used to liberate sugars for use by mesophilic organisms, such as *C. acetobutylicum* [7].

The following experiments were initiated to determine if biological pretreatment with *C. thermocellum* would promote the fermentation of switchgrass (*Panicum virgatum*) by *C. beijerinckii*. The hypotheses were: 1) *C. thermocellum* would liberate sugars and increase gas production by *C. beijerinckii*, and 2) the rate and extent of gas production would be surface area-dependent.

## 2. Materials and Methods

### 2.1. Feedstock Preparation

The switchgrass was grown on the University of Kentucky Research Farm. It was harvested in November 2011. The switchgrass was cut at 15 cm and stored in small square bales that were later ground to pass through a 2, 5 or 15 mm sieve (hereafter called particle sizes 2, 5 and 15 mm) using a hammer mill (C.S. Bell, CO. Tiffin, OH, USA. Model No. 10HMBD, Serial No. 375 Bratt 03/05).The switchgrass was analyzed by DairyOne (Ithaca, NY, USA) using the wet chemistry package.

### 2.2. Strain and Media Composition

The *C. thermocellum* ATCC 27405 cell line used in the study came from the culture collection of Herbert J. Strobel, University of Kentucky. *C. thermocellum* cells were grown anaerobically at 63˚C. The basal medium contained (per liter): 1.53 g $Na_2HPO_4$, 1.5 g $KH_2PO_4$, 90 mg $NH_4Cl$, 25 mg $(NH_4)_2SO_4$, 90 mg $MgCl_2 \cdot 6\text{-}H_2O$, 30 mg $CaCl_2$, 2.0 g yeast extract, 10 ml vitamin mixture [8], 5.0 ml modified mineral mixture (Pfennings Metals plus 10 mg $Na_2WO_4 \cdot 2H_2O$ and 1 mg $Na_2SeO_3$ perliter, as described by Strobel [9]) and 1 ml resazurin. The pH was adjusted to 6.7 with NaOH. The medium was autoclaved (121˚C, 104 kPa, 20 min) and cooled under an $O_2$-free $CO_2$ sparge. The buffer, $Na_2CO_3$ (4 $mg \cdot ml^{-1}$), was added before the broth was room temperature. Media for batch cultures were anaerobically dispensed into serum bottles and sealed with butyl rubber stoppers, and autoclaved for sterility.

*C. beijerinckii* ATCC 51743 was obtained from the American Type Culture Collection (Manassas, VA, USA). *C.beijerinckii* cells were grown anaerobically at 35˚C in Reinforced Clostridial Media (Difco Laboratories, Detroit, MI, USA). Reinforced clostridial medium (RCM) contained (per liter) 10.0 g peptone, 10.0 g beef extract, 3.0 g yeast extract, 5.0 g dextrose, 5.0 g NaCl, 1.0 g soluble starch, 0.5 g cysteine, 3.0 g $C_2H_3NaO_2$, and 0.5 g agar. The medium was autoclaved and cooled under $O_2$-free $N_2$. Media for batch cultures were anaerobically

dispensed into serum bottles with butyl rubber stoppers, and autoclaved for sterility.

*C. thermocellum* was routinely transferred in the basal medium with Whatman #1 filter paper (4 $mg \cdot ml^{-1}$). *C. beijerinckii* was routinely transferred in RCM. Growth was monitored by optical density (absorbance 600 nm) using a Biowave II spectrophotometer (Biochrom, Cambridge, UK).

### 2.3. Effect of the Feedstock Particle Size on Gas Production

Gas production was monitored with the Ankom RF Gas Pressure System (Ankom, Macedon, NY, USA). Ground switchgrass (5 g) was added to the fermentation vessels (Pyrex bottle with sidearm port, 1140 mL actual volume). The vessels were sealed and purged of air with $O_2$-free $CO_2$ through the sidearm septum. Basal medium (50 mL) was added through the septa of the sidearm ports and the vessels were warmed in a water bath (63˚C) prior to inoculation. The vessels were inoculated (10% v/v) through the septa with *C. thermocellum* (48 h cultures, approximately $10^7$ viable cells $ml^{-1}$). After 48 h incubation (63˚C), the vessels were removed from the water bath, permitted to cool, and inoculated (10% v/v) with *C. beijerinckii* (24 h cultures, approximately $10^8$ viable cells $ml^{-1}$). Incubation continued at 35˚C for an additional 48 h. Gas pressure was tested in 1 min intervals, and cumulative pressure was recorded every 5 min. The global pressure release was set at 104 kPa. An uninoculated vessel was included as a control in each iteration of the experiment. The pressure values of the uninoculated vessels were subtracted from each treatment to control for the effects of temperature.

To test gas production from *C. beijerinckii* without *C. thermocellum*, control bottles were inoculated with *C. beijerinckii* (10% v/v). Basal medium was added and the vessel was warmed in a water bath (35˚C, 48h). Gas pressure was tested in 1 min intervals, and cumulative pressure was recorded every 5 min for 48 h.

### 2.4. Soluble Product Quantification

Soluble product quantification was performed on cultures in serum bottles containing 10% switchgrass (2, 5 or 15 mm). Basal medium was added and the serum bottles were warmed in a water bath (63˚C, 48 h). The experiment was initiated by inoculation with *C. thermocellum* (10% v/v). After 48 h, the temperature was decreased to 35˚, and the bottles were inoculated with *C. beijerinckii* (10% v/v), and sampled daily. Samples (1 ml) were clarified by centrifugation (14800 × g, 2 min), and frozen for later analyses. Acetate, ethanol, butanol, lactate and formate were quantified by HPLC (Dionex, Sunnyvale, CA). The anion exchange column (Aminex 87H; BioRad) was operated at 50˚C, flow rate 0.4 ml $min^{-1}$. Eluting compounds were detected by refractive index (Shodex/Showa).

### 2.5. Statistical Analyses

The experiments were performed in triplicate. The data were analyzed in SAS (version 9.3, SAS Inst. Inc) by MANOVA with Tukey's test *post hoc*. Treatment variables included feedstock particle size, time of measurement and culture type. *P* values less than or equal to 0.05 were considered significant.

## 3. Results

The composition of the switchgrass was (dry matter basis): 54.9% acid detergent fiber, 88.9% neutral detergent fiber, 8.8% lignin, 10.0% calculated non-fibrous carbohydrate, 3.8% crude protein, 1.2% crude fat, 1.2% ash. Gas production caused the pressure in the fermentation vessels to increase when *C. thermocellum* was inoculated into basal media with 5 g switchgrass as the substrate (**Figure 1**). The lag phase was approximately 30 h, after which rapid gas production was observed. When the particle size was 2 or 5 mm, the cumulative pressure after 48 h fermentation was 78 kPa, which is equivalent to the production of approximately 680 mL of gas. The *C. thermocellum* cultures with 15 mm feedstock particles had a mean cumulative pressure of 15 kPa after 48 h, which was significantly less than the 2 and 5 mm treatments ($P < 0.05$).

The culture vessels were cooled (to 35˚C) and inoculated with *C. beijerinckii*, and the cumulative pressures were reset to ambient (**Figure 2**). The cultures continued to produce gas through a 10 h lag phase. An increase in the rate of gas production was observed between 10 and 15 h in the 2 and 5 mm treatments and then production decreased into stationary phase. Cumulative pressure values as great as 70 kPa (equivalent to an additional 670 mL gas) were observed. Again, the longer (15 mm) particle size produced less gas ($P < 0.05$). When the substrates were inoculated with *C. beijerinckii* without previous fermentation by *C. thermocellum*, the mean cumulative pressures were approximately 10 kPa.

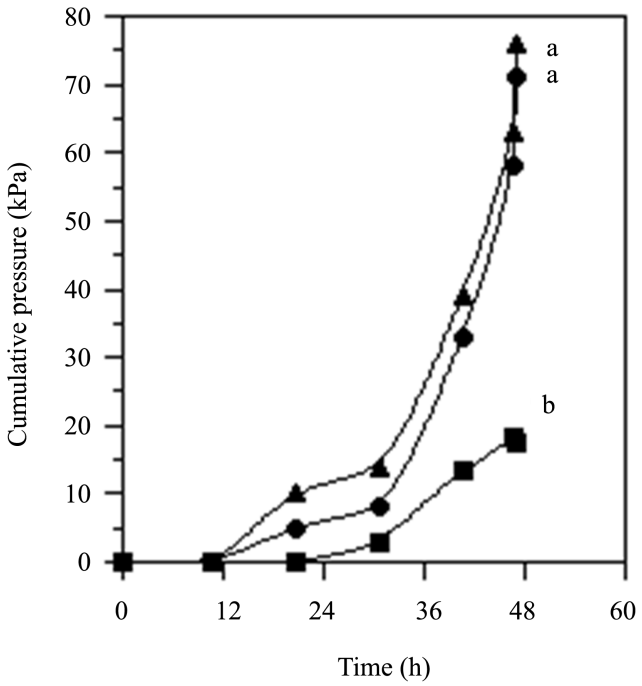


**Figure 1.** Gas production by *Clostridium thermocellum* with 2 (circles), 5 (triangles) or 15 (squares) mm particle size switch grass. The switchgrass (5 g) in basal medium (10% w/v) was inoculated (10% v/v) with *C. thermocellum*, and incubated (65˚C). The markers indicate means of triplicate experiments. Different letters indicate that the means are significantly different ($P < 0.05$).

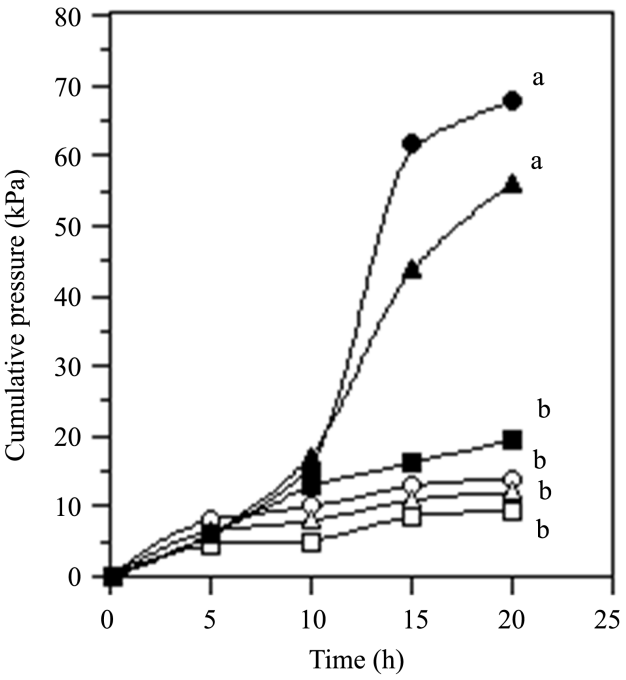


**Figure 2.** Gas production by *Clostridium beijerinckii* with 2 (circles), 5 (triangles) or 15 (squares) mm particle size switchgrass. The switchgrass (5 g) in basal medium (10% w/v) was inoculated (10% v/v). Open symbols indicated culture with *C. beijerinckii* alone (35˚C). Filled symbols indicate switchgrass that was cultured with *C. thermocellum* (63˚C, 48 h) prior to inoculation with *C. beijerinckii*. The markers indicate means of triplicate experiments. Different letters indicate that the means are significantly different ($P < 0.05$).

In sequential cultures on switchgrass, acids (acetate, formate, lactate) and ethanol were produced *by C. thermocellum* (**Table 1**). After inoculation with *C. beijerinckii*, butyric acid and butanol were also produced. Acetone was not detected. Sugars (xylose, glucose) were detected close to or below the limits of quantification early in the fermentations. There was no significant effect of particle size ($P > 0.05$). However, the acetate concentration was numerically higher in the treatment with the smallest particle size.

## 4. Discussion

Physical pretreatment of lignocellulosic biomass, such as milling, increases the surface area and generally makes the feedstock more amenable to chemical or biological conversion [10]. Decreasing particle size has been shown to increase microbial digestion of the feedstock bypure cultures [10] and by natural, poly-microbial fermentations, like the bovine rumen [11].

Co-cultures, either sequential or simultaneous, have been previously used to maximize conversion [12]. Yu and coworkers used a sequential culture of *C. thermocellum* followed by *C. acetobutylicum* to produce ethanol and butanol from an artificial substrate [7]. These results were repeated using corn (*Zea mays*) residuals that were also cultured with a fungus that produces a lignin peroxidase [13]. In the current study, we employed a sequential culture of *C. thermocellum* followed by *C. beijerinckii* to ferment switchgrass. Please note that *C. beijerinckii* and *C. acetobutylicum* are very similar bacteria that are formerly categorized as the same species [14].

In the current experiment, switchgrass was first fermented with the thermophile, *C. thermocellum* prior to fermentation with *C. beijerinckii*. The cellulosome system retained a portion of its fibrolytic activity at mesophilic temperatures, which was originally observed by Ng and co-workers [5] and is consistent with previous results [7] [13]. In this way, *C. thermocellum* could be considered a biological pretreatment for the mesophile *C. beijerinckii*. This type of sequential culture could be considered a biological saccharification to replace enzymatic treatment of lignocellulose. However, it is important to note that much of the cost of acetone-butanol-ethanol production comes from downstream processing of the fermentation broth [1].

Gas production is a universal measure of fermentation because carbon dioxide is the most common metabolic product of fermentative organisms [15]. Furthermore, gas production has long been used to evaluate the ability of rumen microorganisms to digest cellulosic feeds [16]. More gas was produced from smaller rather than from larger switchgrass particles, which was consistent with idea that milling increased surface area for catalysis. The effect of particle size on gas production was consistent during both thermophilic, *C. thermocellum* fermentation and mesophilic *C. beijerinckii* fermentation. *C. beijerinckii* alone produced very little gas pressure from the substrate. This latter observation supports the hypothesis that the *C. thermocellum* cellulosome system was deconstructing lignocellulose or other non-soluble carbohydrate into substrates that could then be catabolized by *C. beijerinckii.*

**Table 1.** Product formation by sequential culture of *C. thermocellum* and *C. beijerinckii*. t, trace; -, not detected.

| | Xylose (mM ) | | | Glucose (mM) | | | Lactate (mM) | | | Formate (mM) | | | Acetate (mM) | | | Ethanol (mM) | | | Butyrate (mM) | | | Butanol (mM) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time (d) | 2 (mm) | 5 (mm) | 15 (mm) | 2 (mm) | 5 (mm) | 15 (mm) | 2 (mm) | 5 (mm) | 15 (mm) | 2 (mm) | 5 (mm) | 15 (mm) | 2 (mm) | 5 (mm) | 15 (mm) | 2 (mm) | 5 (mm) | 15 (mm) | 2 (mm) | 5 (mm) | 15 (mm) | 2 (mm) | 5 (mm) | 15 (mm) |
| 0 | t | - | - | t | t | t | - | - | t | - | - | - | 1.7 | 2.0 | 2.0 | 4.0 | 4.0 | 4.3 | - | - | - | - | - | - |
| 1 | t | t | - | - | - | - | t | t | t | 8.0 | - | 3.0 | 8.7 | 9.0 | 8.3 | 8.7 | 8.3 | 6.7 | - | - | - | - | - | - |
| 2 | t | t | - | - | - | - | 1.5 | 1.5 | t | 9.3 | 5.3 | 5.0 | 22.7 | 22.3 | 18.3 | 18.3 | 19.3 | 11.3 | - | - | - | - | - | - |
| 3 | t | t | - | - | - | - | 1.4 | 1.2 | - | 11.0 | 4.0 | 4.3 | 20.7 | 20.0 | 17.0 | - | 5.0 | 6.3 | 3.3 | 1.0 | t | - | - | - |
| 4 | - | - | - | - | - | - | - | - | - | 8.7 | 7.3 | 7.0 | 22.3 | 23.7 | 16.5 | - | - | 6.0 | 10.0 | 11.7 | 6.0 | t | t | t |
| 5 | - | - | - | - | - | - | - | - | - | 10.3 | 7.3 | 6.3 | 24.3 | 23.3 | 22.0 | - | - | 4.0 | 11.7 | 13.0 | 7.3 | t | t | t |
| 6 | - | - | - | - | - | - | - | - | - | 6.3 | 7.7 | 5.3 | 31.7 | 23.7 | 26.3 | - | - | - | 12.0 | 14.0 | 8.0 | 1.0 | t | 1.0 |
| 7 | - | - | - | - | - | - | - | - | - | 5.0 | 4.7 | - | 36.7 | 23.7 | 41.3 | - | - | - | 12.3 | 16.7 | 8.7 | 1.2 | 1.0 | t |
| 8 | - | - | - | - | - | - | - | - | - | 3.7 | 4.7 | - | 42.0 | 14.0 | 44.3 | - | - | - | 13.0 | 12.7 | 8.7 | 1.4 | t | t |
| 10 | - | - | - | - | - | - | - | - | - | 1.0 | 4.7 | t | 51.7 | 13.3 | 47.3 | - | - | - | 13.3 | 14.0 | 9.3 | 1.5 | t | t |

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