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Research article

Switchgrass (*Panicum virgatum*) fermentation by *Clostridium thermocellum* and *Clostridium saccharoperbutylacetonicum* sequential culture in a continuous flow reactor

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Abstract: The study was conducted to evaluate fermentation by *Clostridium thermocellum* and *C. saccharoperbutylacetonicum* in a continuous-flow, high-solids reactor. Liquid medium was continuously flowed through switchgrass (2 mm particle size) at one of three flow rates: 83.33 mL h^{-1} (2 L d^{-1}), 41.66 mL h^{-1} (1 L d^{-1}), and 20.833 mL h^{-1} (0.5 L d^{-1}). The cellulolytic phase was initiated by culturing *C. thermocellum* (63 °C, 24 h). The temperature was decreased (35) and *C. saccharoperbutylacetonicum* was inoculated. When metabolism decreased (96 h), the temperature was increased (63 °C; 24 h) to permit cellulosome production by *C. thermocellum*. The *C. saccharoperbutylacetonicum* was re-inoculated and the temperature returned to 35 °C. The average gross production over 9 d was 1480 mg total acids (formic, acetic lactic butyric), 207 mg total solvents (acetone, butanol, ethanol), and average dry matter disappearance was 2.8 g from 25 g non-pretreated switchgrass. There was no effect of flow rate on the product formation. These results indicate that *C. thermocellum* can survive and produce cellulases with *C. saccharoperbutylacetonicum*in a continuous-flow, high-solids reactor temperature with temperature cycling.

Keywords: Continuous product removal; co-culture; consolidated bioprocessing; switchgrass; bioenergy

1. Introduction

Lignocellulosic crops are evaluated for biofuel production because of low cost, high biomass yields and availability [1]. Switchgrass (*Panicum virgatum*) is a dedicated energy crop that has been used in several bioenergy conversion processes, including cellulosic ethanol production, biogas and direct combustion for thermal energy production [2]. Clostridia have the potential to convert biomass because they are often cellulolytic as well as solventogenic [3]. Such solventogenic microorganisms carrying out acetone, butanol and ethanol (ABE) fermentation is a promising way to make biofuels and platform chemicals [4].

Clostridium saccharoperbutylacetonicum is a mesophilic, solventogenic anaerobe [5]. This butanol hyperproducing bacterium has been used commercially as inoculant in butanol production [6-9]. Like many solvent-producing bacteria, *C. saccharoperbutylacetonicum*cannot catabolize cellulosic substrates. *Clostridium thermocellum* is a thermophilic anaerobe able to degrade cellulose at high rates and carry out mixed–acid fermentation [10,11]. Also, *C. thermocellum* combines the enzymatic production, hydrolysis and fermentation into one step, also known as consolidated bioprocessing [12]. When ABE products are desired, *C. thermocellum*has been used as a biological treatment making available the sugars to solventogenic bacteria. Sequential culture of *C. thermocellum* ATCC 27405 and *C. beijerinckii* ATCC 51743 has been reported to improve product formation by the solventogenic bacterium, *C. beijerinckii*, when switchgrass was the substrate [13,18].

Although, ABE fermentation is traditionally carried out in liquid batch fermentation, *C. thermocellum* has been reported to grow better on solid substrate cultivation (SSC) [14]. Indeed, *C. thermocellum* grown on solid substrates had greater ethanol yields than liquid media. However, end product inhibition occurred even though sufficient substrate remained [15]. In spite of the advantages of SSC, water availability, unfavorable osmotic conditions, fermentation acid accumulation and the resulting metabolic inhibition are still a problem [15-17]. Solid substrate cultivation with media replacement by periodic flushing (FSSC) maintained favorable growth conditions for *C. thermocellum* with constant amounts of ethanol, acetate and lactate yields [15]. Also, a recent study of *C. thermocellum* and solventogenic bacterium, *C. beijerinckii* growing on high solids lignocellulosic substrate was carried out by flushing periodically new media into the system. The results indicated that the cycling flushing improved the solvent production [18].

The previous research employed FSSC in each case. The current study was initiated to evaluate if sequential culture of *Clostridium thermocellum* followed by *Clostridium saccharoperbutylacetonicum* could be conducted in a continuous flow, high-solids system, and to determine the effect of mobile phase flow rate on switchgrass catalysis and product formation.

2. Materials and Method

2.1. Strains and culture conditions

2.1.1. Solvent-producer strain

C. saccharoperbutylacetonicum ATCC 27021 N1-4 was obtained from the American Type Culture Collection (Manassas, VA, USA). The bacteria were grown anaerobically at 35 °C in basal medium containing (per L): 1.53 g Na_2HPO_4, 1.5 g KH_2PO_4, 0.5 g NH_4Cl, 0.5 g $(NH_4)_2SO_4$, 90 mg

$MgCl_2 \cdot 6H_2O$, 30 mg $CaCl_2$, 2.0 g yeast extract, 10 mL standard vitamin mixture [19], 5.0 mL modified mineral mixture (Pfennings Metals plus 10 mg $Na_2WO_4 \cdot 2H_2O$ and 1 mg Na_2SeO_3 per liter) and 1 mL resazurin (1%). The pH was adjusted to 6.7 using NaOH. The buffer, Na_2CO_3 (4 mg mL^{-1}), was added after autoclaving (121 °C, 104 kPa, 20 min) once the medium cooled under O_2-free CO_2 gas sparge. Broth for batch liquid cultures was anaerobically dispensed into serum bottles with butyl rubber stoppers, and autoclaved. N1-4 was routinely transferred in the basal medium and the substrate (sterilized glucose, 4 g L^{-1}) was aseptically added to the basal medium prior to inoculation.

2.1.2. Cellulolytic strain

C. thermocellum ATCC 27405 was maintained in our culture collection at the University of Kentucky (chain of custody: ATCC, Herbert Strobel, Michael Flythe). *C. thermocellum* was grown anaerobically at 63 °C in the basal medium described above. Media for batch liquid cultures was anaerobically dispensed into serum bottles with amorphous cellulose (Whatman #1 filter paper; 4.5 mg mL^{-1}) and capped with butyl rubber stoppers, and autoclaved (121 °C, 104 kPa, 20 min). C. *thermocellum* was transferred every 48 hours in the basal medium with the filter paper.

2.2. Substrate preparation

The switchgrass was obtained from small plots at the University of Kentucky's research farm (38.13; −84.50). The switchgrass was collected in November, 2011. Switchgrass was cut at least 15.24 cm high and stored in small square bales that were ground to pass through 2 screen using a hammer mill (C.S. Bell, CO. Tiffin, OH, USA. Model No. 10HMBD, Serial NO. 375 Bratt 03/05). The chemical characterization of the switchgrass was done by Dairy One Forage Testing Laboratory, Ithaca, NY.

2.3. Continuous flow fermentation

Continuous flow fermented switchgrass was conducted in water-jacketed Buchner funnels (hereafter called fermentation vessels; Figure 1). The fermentation vessels (500 mL working volume) contained of switchgrass (25 g, 6.15 % moisture content, ground to pass through a 2 mm screen) were autoclaved (121 °C, 104 kPa, 20 min). The fermentation vessels were sealed with rubber stoppers and continually gassed with O_2 -free CO_2. Basal medium (60 L total, in 10 L batches) was prepared for the continuous flow fermentation and autoclaved (121 °C, 104 kPa, 20 min). The media was pumped to the fermentation vessels using a Watson Marlow Pump (205/CA, Watson Marlow, Inc, Wilmington, Wilmington, MA) through PHARMED BPT NSF-51 tubing.

The temperature of the fermentation vessels was set at 63 °C, and 100 mL of sterilized basal media (pH 6.7) was added to increase the water activity of the biomass. The fermentation was initiated by the inoculation of C. thermocellum (100 mL, 108 cells mL^{-1}). This inoculum was prepared in basal media containing amorphous cellulose (Whatman #1 filter paper; 4.5 mg mL^{-1}) and the growth was checked by optical density (absorbance 600 nm). After 24 hours, the vessel temperature was decreased to 35 °C. *C. saccharoperbutylacetonicum* N1-4 (100mL, 10^8 cells mL^{-1}) were added to the vessels. They were previously grown on basal media containing glucose (4 g L^{-1}). After the inoculation with *C. saccharoperbutylacetonicum*, the medium pump was started to initiate

the flow rate indicated: high, medium and low flow rate; 83.33 mL h^{-1} (2 L d^{-1}), 41.66 mL h^{-1} (1 L d^{-1}), and 20.833 mL h^{-1} (0.5 L d^{-1}), respectively.

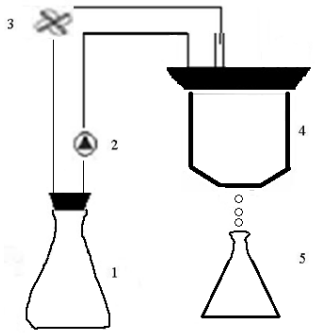

Figure 1. Solid-substrate cultivation system with continuous product removal. 1-medium reservoir, 2-pump, 3-gas administration, 4-fermentation vessel, 5-effluent flask.

Fermentation products were collected in a sterile Erlenmeyer flask (effluent flask). Samples were taken from the effluent flask, which was replaced daily. The samples were frozen for later analysis. The pH and acetate were measured immediately. When the acetate production decreased, the temperature of the fermentation vessels was increased to 65 °C for 24 h to permit cellulosome production by *C. thermocellum*. The temperature was returned to 35 °C, and the fermentation vessels were re-inoculation with *C. saccharoperbutylacetonicum* (100 mL). After 9 d, the fermentation vessels were opened and the residual biomass was dried at 80 °C until the weight was constant. Dry weights were recorded and the residual biomass was subjected to forage analysis as described above.

2.4. Product quantification

The cultures were sampled as indicated. Samples (1 mL) were clarified by centrifugation (14800 g, 2 min), and frozen for later analyses. The pH and acetate were measured daily. Acetate was quantified by a commercial assay that employed acetate kinase (Roche, Nutley, NJ). 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 UV and refractive index (Shodex/Showa) in tandem.

2.5. Mathematical analyses

The experiments were performed in duplicate. The data shown are the means of two replicates unless otherwise indicated. Differences were determined via Kruskal-Wallis Rank Sum Test.

3. Results

The production of acetic acid was used to evaluate establishment of *C. thermocellum* after 24 h. When the temperature was decreased and the vessels were inoculated with *C. saccharoperbutylacetonicum*, daily acetate concentrations revealed that metabolism declined after

4 d at 35 ºC (data not shown). The temperature was increased (63 ºC; 24 h) to permit cellulosome production by *C. thermocellum*, the temperature was returned to 35 ºC and the fermentation vessels were inoculated with *C. saccharoperbutylacetonicum* again.

The chemical characterization of the switchgrass before and after fermentation is shown in Table 1. During the 9 d fermentation, dry matter disappearance was approximately 2.8 g, and there was no statistical difference among treatments (Table 1). Most of the dry matter disappearance was attributable to non-fiber carbohydrate. The protein content of the switchgrass increased as much as 33% during the fermentations ($P < 0.05$).

Table 1. Chemical characterization of the switchgrass before and after fermentation (% total weight).

Treatment	Moisture	Crude Protein	Lignin	NDF	ADF	NFC	DMD
Initial	6.15 ± 0.2	3.8 ± 0	9.45 ± 0.9	87.55 ± 1.9	56.1 ± 1.6	10.6 ± 0.8	
High Rate	6.40 ± 0.3	5.7 ± 0.3	12.15 ± 0.5	86.25 ± 0.4	58.65 ± 1.5	8.85 ± 0.1	11.38
Med. Rate	5.15 ± 0.1	5.4 ± 0.1	11.10 ± 0.4	87.45 ± 0.2	57.95 ± 0.4	7.60 ± 0.1	10.91
Low Rate	6.60 ± 0.1	5.25 ± 0.4	10.75 ± 0.2	85.8 ± 2.4	57.15 ± 1.6	8.75 ± 1.5	14.32

NFC: Non-Fiber Carbohydrates. ADF: Acid Detergent Fiber. NDF: Neutral Detergent Fiber. DMD: Dry Matter Disappearance.

Fermentation acids (acetic, lactic, formic and butyric acid) and solvents (butanol and ethanol) were measured each day. There was no effect of flow rate on the yields (mass product mass substrate^{-1}), and acetic and butyric acids were the primary products (Table 2). Cumulative total production is shown in Figure 2. Approximately, 45 mg of total acids/g dried substrate and 6 mg of total solvents/g dried substrate were collected at the low flow rate. The medium and high flow rates yielded more products numerically, but again, the differences were not significant.

Table 2. Cumulative Yield (mg product per g switchgrass dry weight) after 9 days of fermentation.

Total product (mg g^{-1})	Acetate	Butyrate	Lactate	Formate	Ethanol	Butanol
Low Rate	22.20	21.98	1.12	-	-	6.07
Medium Rate	34.19	31.33	4.60	5.65	2.16	8.61
High Rate	26.33	40.90	0.57	0.38	2.61	7.07

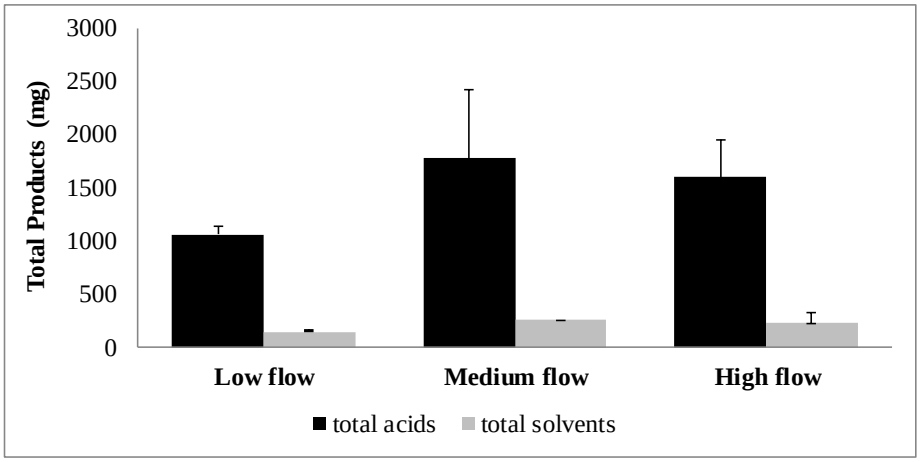

Figure 2. **Total quantity of acids (acetic, lactic, butyric and formic acid) and solvents (ethanol and butanol) collected.** Switchgrass (25 g, 6.15 % moisture content) was inoculated with a sequential culture of *C. thermocellum* and *C. saccharoperbutylacetonicum* and basal medium was continuously flowed through the system to remove the products. Differences between flow rates were not statistically significant.

4. Discussion

Switchgrass is a lignocellulosic substrate that contains 29−45% cellulose, 30−37% hemicellulose and 12−19% lignin [20]. The natural resistance of lignocellulose to degradation by microorganisms requires a prior step of removing or modifying lignin structure as well as liberating the sugars from the cellulose and hemicellulose [3]. In order to avoid high cost artificial enzymatic treatments, studies using sequential cultures or co-cultures demonstrated that *C. thermocellum* could be used as biological pretreatment to liberate sugars from lignocellulose to be fermented for others bacteria [21,22]. Sequential culture of *C. thermocellum* with the solventogenic bacterium *C. acetobutylicum* has been already studied and the total fermentation products increased approximately 1.7−2.6 fold [23]. Sequential cultures in gas production experiments indicated *C. thermocellum* could be used to saccharify switchgrass, liberating sugars for *C. beijerinckii* to produce CO_2 [13]. *C. saccharoperbutylacetonicum*is a close relative of *C. beijerinckii*and *C. acetobutylicum*, which has been shown to product butanol from various substrates [8,14].

ABE production was improved by cycling through cellulolytic and solventogenic phases, intermittent flushing of new media in high solids fermentation [18]. Solid substrate cultivation with media replacement by periodic flushing (FSSC), maintained favorable growth conditions for *C. thermocellum* with constant amounts of ethanol, acetate and lactate yields [15]. In this study, continuous media replacement was investigated in order to improve lignocellulose conversion. Media flow was tested at three different values (0.5 L d^{-1}, 1.0 L d^{-1} and 2.0 L d^{-1}), and no effects on catalysis or product formation were observed.

Based on the switchgrass composition on a dry matter basis (46.6% cellulose, 31.5% hemicellulose, and 11% lignin), theoretical yield of monosaccharides, glucose and xylose from cellulose and hemicellulose (assuming just xylose), respectively; and assuming 50% of hydrolysis by the cellulolytic strain is calculated as follows:

$$23.46\ g\ switchgrass\ \times \frac{0.47 g\ cellulose}{g\ switchgrass} \times \frac{1.11\ g\ glucose}{g\ cellulose}\ \times\ 0.5 = 6.06\ g\ glucose$$

$$23.46\ g\ switchgrass\ \times \frac{0.32 g\ hemicellulose}{g\ switchgrass} \times \frac{1.11\ g\ pentose}{g\ hemicellulose}\ \times\ 0.5 = 4.10\ g\ pentose$$

The approximate stoichiometric equations of an ABE fermentation process using standard strains and conditions [24] are (M):

$$12C_6H_{12}O \rightarrow 6C_4H_{10}O + 2C2H_6O + 18H_2 + 28CO_2 + 2H_2O$$

Based on this equation, the theoretical yield of butanol is:

$$10.16\ g\ monosaccharide\ \times \frac{0.2\ g\ butanol}{g\ monosaccharide} = 2.03\ g\ butanol$$

The best case (highest numerical values) observed in the current study was obtained from the medium flow rate. In that case, 2.03 g of total products were accounted for. The dry matter disappearance was 2.56 g. The difference (0.53 g) is easily attributable to CO_2 production.

Unlike other solventogenic species, in which the transition from acid to solvent fermentation is related to acetic acid and butyric acid levels, solventogenesis in *C. saccharoperbutylacetonicum* is constitutive [25,26]. In this case, the switchgrass was not pretreated for lignin modification or removal; thus, cellulose availability limited fermentation. It is known that *C. thermocellum* released more glucose when biomass was pretreated with a lignin-degrading fungus [27]. Prior studies indicated that the initial inoculum of *C. thermocellum* was viable even after sequentially culturing a mesophile and the cellulosome system was able to hydrolyze cellulose when the temperature was increased to 65 °C [18]. However, during that stage of 24 h at 65 °C (to reactivate the cellulolytic phase) the new media flushing was stopped. Then, water activity was lessened and it is known that lower water activity decreased the adsorption of cellulases at solid substrates with a concomitant decreased in cellulose conversion [28]. The advantages of continuous flow are to maintain moisture and continuously remove products. A disadvantage to our bench-scale system is that it requires continuous CO_2, which affects product ratios. Accumulation of H_2 has been shown to promote ethanol production in *C. thermocellum* via hydrogenase end product inhibition [29].

5. Conclusion

These results indicate that *C. thermocellum* cellulases retain activity with *C. saccharoperbutylacetonicum* in a continuous-flow, high-solids reactor with temperature cycling. It is unclear if the additional cost of continuous flushing will be justified by the product yield. However, it is promising that the lower flushing rates were just as effective as the higher rate. Clearly, the process requires optimization, including pretreatment for lignin removal or selection of less lignified substrates. The study should be considered a proof-of-concept rather than a feasible, optimized process.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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