Characterization of Several Clostridia for Production of Ethanol and Volatile Fatty Acids from Plant-based Substrates

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Citation

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Abstract

The products of four species of Clostridium were characterized in order to explore potential for biofuel and biochemical production in a consolidated bioprocessing (CBP) system. C. phytofermentans, C. cellulolyticum, C. cellulovorans, and C. thermocellum were grown on several plant-based substrates. These six substrates were tested for ethanol and co-product yields: cellobiose, crystalline cellulose, fibrous cellulose, xylan, crystalline cellulose + xylan, fibrous cellulose + xylan. Cellobiose was found to be the highest ethanol yielding substrate, and among the four microorganisms, C. cellulolyticum and C. phytofermentans had higher ethanol yields averaged across all substrates. Between these two bacteria, C. cellulolyticum had higher acetic acid production. Therefore, C. cellulolyticum has the greatest potential for ethanol and co-product production from lignocelluloses biomass. C. cellulovorans had the highest organic acid production on complex substrates. The effect of yeast extract concentration on growth and product formation by C. cellulolyticum and C. phytofermentans were also investigated. Tests within the range of 0.5-2.0 g/L yeast extract revealed that 2.0 g/L was the best concentration to ensure that nutrients were not the growth limiting factor.

INTRODUCTION

Growing awareness of petroleum's finite existence and negative environmental impacts has accentuated the need for clean, renewable energy and chemical sources.Ethanol is one of the popular biofuels at present. There were approximately 11.6 billion gallons of ethanol produced from 4.3 billion bushels of corn in the United States during 2010 (Institute, 2011).However, studies have shown that the energy balance of corn ethanol is not optimal and ethanol from lignocellulosic substrates has a more positive energy balance than corn (Hammerschlag, 2006).Lignocellulosic substrates include crop and forestry residues, grasses, and other plant materials.These complex materials are composed of cellulose, hemicellulose and lignin.Cellulose is made up of glucose molecules joined together by b-1-4 glycosidic bonds into chains. The chains are hydrogen bonded together to form thick fibers. Lignocellulosic ethanol processing is considerably more complex than corn and currently uneconomical.Pretreatment, enzyme production, hydrolysis and fermentation are the main four steps involved in lignocellulosic ethanol production. Consolidated bioprocessing combines the enzyme production, hydrolysis and fermentation steps and has potential to simplify the process and make it more economical (Cardona and Sanchez, 2007).It is well known that some species of *Clostridium* bacteria are capable of producing ethanol directly from lignocellulosic materials and serve as good microbial models for achieving consolidated bioprocessing. These organisms may have lower ethanol yields than yeast (Bai, et al., 2008) but they produce cellulolytic enzymes as well as other products, such as organic acids, which can potentially be converted into valuable co-products, like methane by acetoclastic methanogens.

*C. thermocellum, C. cellulolyticum, C. cellulovorans*and *C. phytofermentans*,have been documented in the literature for their abilities to secrete glycosyl hydrolase enzymes and ferment these hydrolysis products to ethanol and other products.Previous studies have reported the products of *C. thermocellum* (Chinn, et al., 2007, Levin, et al., 2006), *C. cellulolyticum* (Desvaux, 2005),(Ren, et al., 2007), *C. phytofermentans* (Ren, et al., 2007, Warnick, et al., 2002) and *C. cellulovorans* (Sleat, et al., 1984) on a variety of substrates, including cellobiose, crystalline cellulose and mixed cellulose. However, the bacterial growth and reaction conditions were not consistent between different studies,

making accurate comparisons difficult.No literature has been found on the products of these bacteria from hemicellulosic substrates. Cellulosomes are known to be highly adaptable to different substrates; the composition will vary depending on the substrate it encounters (Blouzard, et al., 2010).This study will take this concept a step further by examining the product yields on different substrates as well as similar substrates with different structures.Two different celluloses were tested: crystalline (Avicel) and fibrous cellulose.In addition to pure celluloses, these microbes were also grown on birch xylan to simulate a simplified pure hemicellulose and a mixture of cellulose and xylan to roughly simulate lignocellulosic substrates.

In this study, the effect and concentration of yeast extract as a nutrient source for the bacteria were also investigated.Yeast extract is commonly used in a variety of microbiological media as a source of nitrogen (Ataf, et al., 2005) and growth factors (Selmer-Olsen and Sorhaug, 1998) and consists of the water-soluble contents of autolysed yeast cells.The effect of yeast extract concentration on growth and metabolism has been shown to vary among organisms (Leclerc, et al., 1998).Yeast extract concentrations reported in the literature range from 0.5-6 g/L (Gehin, et al., 1995, Ren, et al., 2007, Sleat, et al., 1984, Warnick, et al., 2002), but there is currently no optimization data in the literature specifically for *C. thermocellum*, *C. cellulovorans, C. cellulolyticum* or *C. phytofermentans*. Yeast extract is a costly item for industrial fermentation so it is important to determine the minimum amount to allow optimum growth for the bacteria.

The primary objectives of this study were to characterize and compare the product profiles and yields of the four species of *Clostridium* on pure plant-based substrates and determine which species are the most promising candidates for ethanol and volatile fatty acid production from lignocellulose. The secondary objective was to determine the necessary amounts and effects of yeast extract on the growth and metabolism of these *Clostridium* spp.

MATERIALS AND METHODS

Substrates and Yeast extract

Two types of cellulose were tested: long fibrous and crystalline.The fibrous cellulose was from Sigma (C-6663) and has an aspect ratio of 10-21 as quantified by scanning electron microscopy (Sundar, 2011).The crystalline cellulose was from Fluka (Avicel PH-101) and has an aspect ratio of 1.5-3.0 (ref2). Avicel PH101 is granular with particle sizes ranging from <50 (40%)-140um (Carlson and Hancock, 2006).The xylan used in experiments was birchwood xylan from Sigma.The yeast extract used in these experiments was by Fluka BioChemika (#70161) and contained 38% carbon and 10% nitrogen as measured with a CHN analyzer.

Microorganisms and growth media

Bacteria were purchased from the American Type Culture Collection (ATCC) and include: *C. cellulolyticum* (ATCC# 35319), *C. phytofermentans* (ATCC#700394), *C. thermocellum* (ATCC #35609) and *C. cellulovorans* (ATCC #35296).The media composition for both culture stocks and experiments was as follows (mg/L): 343.5 K_2HPO_4, 450 KH_2PO_4, 367.5 NH_4Cl, 900 NaCl, 157.5 $MgCl_2.6H_2O$, 120 $CaCl_2.2H_2O$, 0.75 $MnCl_2.4H_2O$, 0.75 $CoCl_2.4H_2O$, 5.2 Na_2EDTA, 1.5 $FeCl_2.4H_2O$, 0.07 $ZnCl_2$, 0.1 $MnCl_2.4H_2O$, 0.062 H_3BO_4, 0.192 $CoCl_2.6H_2O$, 0.017 $CuCl_2.6H_2O$, 0.024 $NiCl_2.6H_2O$, 0.036 $Na_2MoO_4.2H_2O$, 0.001 Rezazurin and 2,000 yeast extract (except where noted in experimental design).This mixture turns a light purple color due to the Rezazurin, which is used as a reducing indicator (Tizzard, et al., 2006).After all components were added to one liter of deionized water, the mixture was boiled until the color changed to light pink, indicating the first step of Rezazurin reduction.While continuing to boil, 1,000 mg Cysteine.HCl (reducing agent) was added, further reducing the Rezazurin and causing it to lose color, leaving the media a light yellow color.The media was then dispensed into flasks or tubes containing sodium bicarbonate.For experiments, 10 mL of media was added to 15mL Hungate tubes containing 0.3 grams of sodium bicarbonate.For stocks, 150 mL of media was added 500 mL round bottom flasks containing 5 grams sodium bicarbonate. This transfer caused the media to change back to pink, but all containers were purged with carbon dioxide until color changed back to light yellow before being sealed and autoclaved at 121°C and 15 psi for 30 minutes.Soluble substrates were dissolved in deionized water, filter sterilized and added to the cultures after autoclaving via sterile needle and syringe.Insoluble substrates were added to the flasks with the sodium bicarbonate and autoclaved with the rest of the media.Stock cultures for each bacterium were grown up on cellobiose to an optical density (OD) of 0.7-0.9.Cellobiose was used so that optical density could be easily measured and used as an indicator of approximate cell number.All experimental tubes were inoculated from the stock cultures to a starting OD 0.1

in a 10 mL working volume.

Cultures were incubated at 50°C for *C. thermocellum* and 37°C for *C. cellulolyticum, C. phytofermentans* and *C. cellulovorans*. Cultures were static since agitation has been shown to have negative effects on *C. thermocellum* ethanol yields, most likely due to shear forces that denature cellulase enzyme complexes or interfere with cellulase binding (Zertuche and Zall, 1982). The culture volume was 10 mL with an initial carbon source concentration of 4 g/L.For the purpose of this investigation, carbon sources are defined as glucose, cellobiose, cellulose and xylan.Yeast extract also provides carbon, but will not be included as a carbon source for clarity of discussion.The mixtures of cellulose and xylan contained 2 g/L of each substrate.Liquid samples were taken at 5, 10 and 20 days for product analysis.Gas pressure was measured and released once a day for the first five days.After five days, gas pressure was monitored regularly, but there was no increase in pressure to release.A control was also run with no carbon source (media only) for each organism. Triplicates were run for run for all treatments and controls.

Substrate experimental design and statistical analysis

Six substrate combinations were used: cellobiose, crystalline cellulose (Avicel), fibrous cellulose, xylan, crystalline cellulose mixed with xylan, and fibrous cellulose mixed with xylan. A control was run also for each bacterium with no carbon source.The products from the control were subtracted out from each corresponding treatment to quantify the product yields from the substrate only.Yield data was calculated by dividing the product concentration (g/L) by the initial substrate concentration (g/L). The 5-day yield data are presented in this paper for the cellobiose, since values remained constant after this point, and the 20-day data is presented for all other substrates. Total product yields were calculated as the sum of all individual product yields. Analysis of variance (ANOVA) and Tukey tests were conducted using R software (CRAN) on the final ethanol yields using the 5-day data for cellobiose and 20-day data for all other substrates.ANOVA was conducted using the statistical model in equation (1), where Ethanol is yield in g EtOH/g substrate:

Ethanol = Substrate + Bacteria(1)

The interaction term was not included because the model did not pass the Shapiro test for normality or the Levene test for homogeneity of variance with this term included. Tukey tests were also conducted to compare individual bacteria and substrates using a p-value of 0.05.

Yeast extract experimental design

The effects of yeast extract concentration were measured with and without an additional carbon source.*C. cellulolyticum* and *C. phytofermentans* were found to be the most promising of the four bacteria tested so they were used for yeast extract optimization.The first set of experiments measured the growth of *C. cellulolyticum* and *C. phytofermentans* on either a carbon source (cellobiose or glucose) or varying levels of yeast extract. Growing the bacteria on cellobiose and glucose without yeast extract determined the necessity of yeast extract in carbon metabolism.Culturing with yeast extract only showed the bacteria's ability to metabolize yeast extract without additional carbon source and determined whether this type of control is needed in future experiments. The OD was measured over time for 48 hours.

C. cellulolyticum and *C. phytofermentans* were cultured on cellobiose and yeast extract together in the second set of experiments.The effect of yeast extract level on growth and product yields for *C. cellulolyticum* and *C. phytofermentans* growing on cellobiose was measured. The OD and gas production were monitored over time. Final liquid samples were taken for analysis of ethanol and volatile fatty acids.

For both sets of yeast extract experiments, statistical analysis on the net growth between 0 and 48 hours was conducted based on the model in equation (2), where growth is approximated using OD and yeast extract has units of g/L.

Growth = Bacteria + Yeast Extract + Bacteria*Yeast Extract(2)

ANOVA and Tukey tests were conducted using R statistical software (CRAN). Carbon sources were not included in the statistical analysis since yeast extract was the focus of this investigation.

Analytical methods

Cell concentration for stock cultures growing on cellobiose was estimated using OD measured at 660 nm and a 1 cm light path. Product concentrations including ethanol, lactic acid, acetic acid, formic acid, propionic acid, butyric acid and valeric acid were measured using high performance liquid chromatography (HPLC).Glucose and cellobiose were also measured using HPLC.The analysis was run on a

Shimadzu HPLC system with Biorad HPX-87H ion exclusion column, Microguard cation H guard column and 15 mmol sulfuric acid mobile phase at a 0.6 mL/min flow rate.The separation was run at 85°C using a refractive index detector for ethanol and sugars, and photo diode array (205 nm) detector for volatile fatty acids.A 4-point calibration curve was run with each batch of samples to ensure accuracy.

RESULTS

Product yields from plant-based substrates

Ethanol and acetic acid were the main two products for C. cellulolyticum, C. phytofermentans and C. thermocellum, while C. cellulovorans produced mainly formic acid and butyric acid.All organisms produced ethanol with C. cellulolyticum having the highest yield and C. cellulovorans having the lowest.Cellobiose yielded the highest ethanol production of the four substrates for all organisms except C. cellulovorans, which had negligible ethanol production on cellobiose.These results are summarized in Table 1.

Table 1

Clostridium product yields (g/g substrate) on plant based substrates(a)

	Xylan	Cellobiose	Avicel + Xylan	Avicel	F. cellulose + Xylan	F. cellulose
C. phytofermentans						
Ethanol	0.18 (0.14)	0.27 (0.13)	0.05 (0.04)	0 (0)	0 (0)	0 (0)
Lactic Acid	0 (0)	0.04 (0.05)	0 (0)	0 (0)	0 (0)	0 (0)
Formic Acid	0.02 (0)	0.06 (0.03)	0.06 (0.05)	0.01 (0.01)	0 (0.01)	0.01 (0)
Acetic Acid	0.05 (0.02)	0.07 (0.07)	0.08 (0.07)	0.01 (0.02)	0.02 (0.02)	0.01 (0.01)
Propionic Acid	0 (0)	0.05 (0.05)	0 (0)	0 (0)	0 (0)	0 (0)
Isobutryic Acid	0 (0)	0 (0)	0 (0)	0 (0.01)	0 (0)	0 (0)
Butyric Acid	0 (0)	0.01 (0.01)	0.01 (0.01)	0.01 (0.01)	0 (0.01)	0 (0)
Valeric Acid	0.01 (0)	0 (0)	0 (0)	0 (0)	0.01 (0)	0 (0)
C. cellulovorans						
Ethanol	0.07 (0)	0 (0)	0.07 (0)	0.06 (0.03)	0.08 (0.01)	0 (0)
Lactic Acid	0 (0)	0.13 (0.01)	0 (0)	0 (0)	0 (0)	0 (0)
Formic Acid	0.14 (0.02)	0.19 (0.01)	0.22 (0.01)	0.16 (0)	0.11 (0.02)	0.01 (0.01)
Acetic Acid	0.01 (0.02)	0.04 (0.01)	0.07 (0.03)	0.05 (0.01)	0.01 (0.01)	0 (0)
Propionic Acid	0 (0)	0.1 (0.02)	0 (0)	0 (0)	0 (0)	0 (0)
Isobutryic Acid	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Butyric Acid	0.07 (0.01)	0.28 (0.01)	0.16 (0.02)	0.28 (0.02)	0.05 (0.01)	0.01 (0.01)
Valeric Acid	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
C. thermocellum						
Ethanol	0.01 (0)	0.12 (0.02)	0.02 (0.02)	0.01 (0)	0 (0.01)	0.01 (0)
Lactic Acid	0 (0)	0.4 (0.03)	0 (0)	0 (0)	0 (0)	0 (0)
Formic Acid	0.04 (0)	0.28 (0.04)	0.04 (0.04)	0.01 (0)	0.02 (0.01)	0.03 (0.01)
Acetic Acid	0 (0)	0.1 (0.03)	0.01 (0.02)	0 (0)	0.02 (0.03)	0 (0)
Propionic Acid	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Isobutryic Acid	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Butyric Acid	0 (0)	0.01 (0.01)	0 (0)	0 (0)	0.01 (0.02)	0 (0)
Valeric Acid	0 (0)	0.01 (0)	0 (0)	0 (0)	0 (0)	0 (0)
C. cellulolyticum						
Ethanol	0.14 (0.02)	0.38 (0.04)	0.08 (0.06)	0.01 (0.01)	0.08 (0.05)	0.01 (0.01)
Lactic Acid	0.03 (0.02)	0.07 (0.08)	0 (0)	0 (0)	0 (0)	0 (0)
Formic Acid	0.02 (0.02)	0.08 (0.04)	0.01 (0.01)	0 (0.01)	0 (0)	0 (0)
Acetic Acid	0.19 (0.07)	0.11 (0.08)	0.05 (0.04)	0 (0.01)	0.03 (0.01)	0 (0)
Propionic Acid	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Isobutryic Acid	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Butyric Acid	0.02 (0.03)	0.01 (0.01)	0 (0)	0 (0)	0 (0)	0 (0)
Valeric Acid	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] Standard deviation of triplicates in parenthesis

C. cellulolyticum

The major products by C. cellulolyticum were ethanol and acetic acid.The highest ethanol yield was obtained on cellobiose of 0.38 g ethanol/g cellobiose. Ethanol yields from crystalline cellulose, fibrous cellulose, and xylan were 0.01, 0.01 and 0.14 g product/g substrate, respectively. Product yields for fibrous and crystalline celluloses were very similar.The acetic acid yield was 0.16 g acetic acid/g cellobiose on cellobiose, and 0.19 g acetic acid/g xylan on xylan.The total product yield on cellobiose was 0.65 g product/g cellobiose indicating that 65% of the cellobiose was converted to products.There was no residual cellobiose in the cultures, so 35% of the initial cellobiose was left for cell growth and maintenance, and gas production. Other products by C. cellulolyticum were lactic acid, formic acid and butyric acid.

C. phytofermentans

The main products from C. phytofermentans were ethanol, acetic acid and formic acid.The highest ethanol yield was 0.27 g ethanol/g substrate and was obtained on cellobiose. Ethanol yield from crystalline cellulose was negligible and from xylan was 0.18 g ethanol/g cellulose. The acetic acid yield on cellobiose was 0.07 g acetic acid/g cellobiose and 0.05 g ethanol/g xylan from xylan. The total product yield on cellobiose was 0.51 g products/g cellobiose or 51% of initial substrate conversion.There was negligible (0.002 g/L) cellobiose remaining in the culture, allowing 49% initial substrate for cell growth and maintenance, and gas production. Other products by C. phytofermentans were lactic acid, propionic acid, with highest production of both from cellobiose.

C. thermocellum

The main products for C. thermocellum were ethanol and lactic acid.Similar to the previous two strains, the highest ethanol yield was obtained on cellobiose.This study obtained an ethanol yield of 0.12 g ethanol/g cellobiose from C. thermocellum on cellobiose.The lactic acid and total product yields on cellobiose were 0.4 g lactic acid/g cellobiose and 0.9 g products/g cellobiose, respectively. Only 0.01 g/L of cellobiose, or 0.3% of initial substrate remained at the end of the cellobiose fermentation, giving a total of 90.3% initial substrate that was accounted for and leaving around 10% for cell growth and maintenance, and gas production.C. thermocellum had limited products from xylan and crystalline cellulose.Other products by C. thermocellum were formic acid, and small amounts of butyric acid.

C. cellulovorans

The main products of C. cellulovorans were formic and butyric acid.Ethanol yields were low (<0.1 g ethanol/g substrate) on all substrates. The acetic acid yields were also low (<0.1 g acetic acid/g substrate) on all substrates.The formic acid and butyric acid yields on cellobiose were 0.19 and 0.28 g product/g cellobiose, respectively. On crystalline cellulose, C. cellulovorans produced 0.05 g acetic acid/g cellulose, 0.16 g formic acid/g cellulose and 0.28 g butyric acid/g cellulose. The C. cellulovorans total product yield on crystalline cellulose is the highest of the four bacteria, which suggests that this organism has a high enzymatic activity on crystalline cellulose.

Statistical analysis

ANOVA tests on the ethanol yields revealed that bacteria and substrate types were significant (p£0.05) in the model, indicating that both factors contributed to determining final ethanol yield. The results of the Tukey tests for the bacteria are shown in Table 2.Averages with the same letter are not significantly difference (p£0.05).The average ethanol yield from C. cellulolyticum over all substrates was 0.111 g ethanol/g substrate and was significantly (p£0.05) higher than C. cellulovorans and C. thermocellum.All other bacteria comparisons were not significantly different.Tukey comparisons for the substrates are shown in Table 3.

Table 2

Tukey tests on the effect of substrate

Substrate	Average Ethanol Yield over all Bacteria[a] (g/g)		
Cellobiose	0.213	a	
Xylan	0.103	b	
Avicel + Xylan	0.057	b	c
F. cellulose + Xylan	0.042	b	c
Avicel	0.021	b	c
F. cellulose	0.005		c

[a]Values with the same letter are not significantly different

The average ethanol yield from cellobiose over all bacteria was 0.213 g/g and was significantly higher (p£0.05) than all other substrates. Xylan was also significantly higher than fibrous cellulose, but no other substrates were significantly different (p£0.05) from each other. Statistical analysis shows that both bacteria and substrate are important for ethanol yield determination and that C. cellulolyticum and cellobiose produce significantly higher ethanol yields than other bacteria and substrates, respectively.

Table 3

Tukey tests on the effect of bacterium type

Bacterium type	Average Ethanol Yield over all Substrates[a] (g/g)		
C. cellulolyticum	0.111	A	
C. phytofermentans	0.096	A	B
C. cellulovorans	0.047		B
C. thermocellum	0.037		B

[a]Values with the same letter are not significantly different

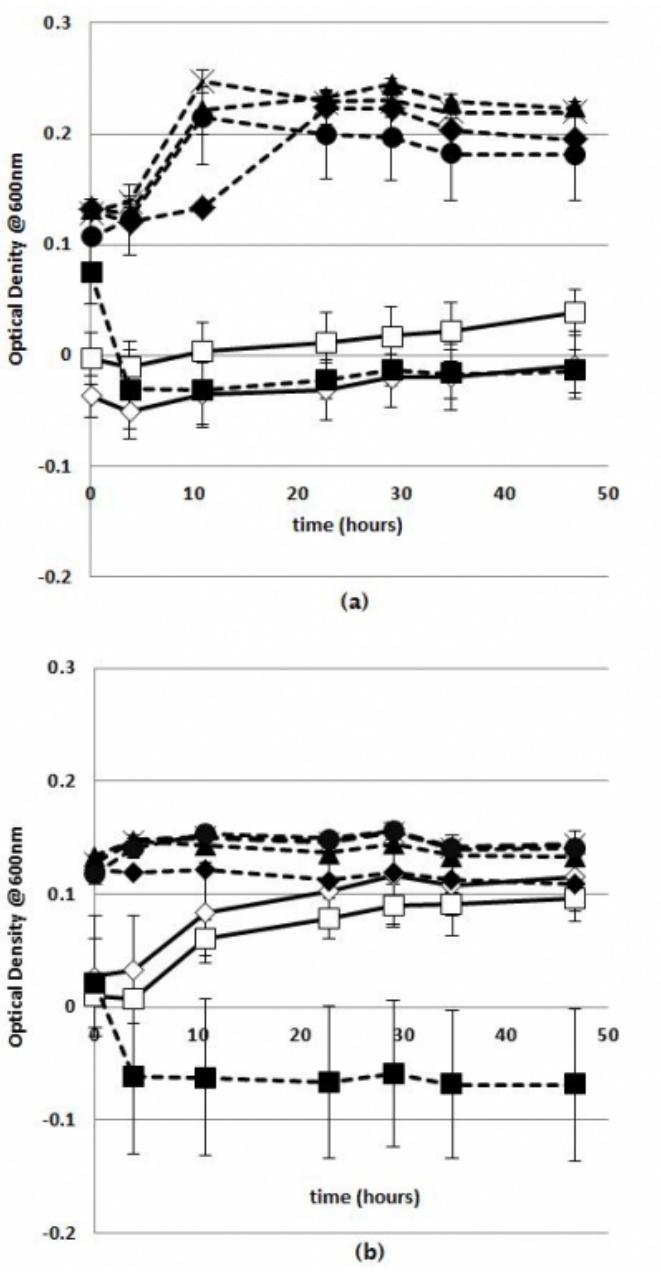
Growth on simple carbon source or yeast extract

This set of experiments looked at carbon source and yeast extract separately; all yeast extract levels have no cellobiose or glucose, and the cellobiose and glucose cultures have no yeast extract. Growth curves for C. cellulolyticum and C. phytofermentans for all treatments are shown in Figures 1a and 1b, respectively.C. cellulolyticum had minimal growth on cellobiose and glucose with no yeast extract.This indicates that at least some amount of yeast extract is required for utilization of cellobiose and glucose by C. cellulolyticum. Cultures also had minimal growth with 0 g/L yeast extract and no carbon source, while cultures grew up to an optical density between 0.2 – 0.25 for the other yeast extract levels with no glucose or cellobiose.

Like C. cellulolyticum, C. phytofermentans had no growth on 0 g/L yeast extract without glucose or cellobiose, indicating that the base media is not sufficient for growth without yeast extract or a carbon source.However, C. phytofermentans did have limited growth on cellobiose and glucose without yeast extract, suggesting that C. phytofermentans is not as dependent on the yeast extract nutrients as C. cellulolyticum.There was also very limited net growth in all the yeast extract only cultures, suggesting that C. phytofermentans does not utilize the yeast extract as readily as C. cellulolyticum.

Figure 1

Growth curves of C. cellulolyticum and C. phytofemermentans cultured with 4 g/L glucose (white squares) and 4 g/L cellobiose (white diamonds) without yeast extract and 0 (black squares), 0.5 (black diamonds), 1.0 (black triangles), 1.5 (black crosses), 2.0 g/L (black circles) yeast extract



Tukey tests revealed that the average net growth over all yeast extract levels for C. cellulolyticum was significantly higher (p£ 0.05) than that of C. phytofermentans.This supports the hypothesis that C. cellulolyticum utilizes yeast extract more easily than C. phytofermentans.Tukey tests on the net growth at each yeast extract level with no cellobiose or glucose, averaged over both bacteria, showed that the 0 g/L level was significantly different than all other levels, with no other significant differences between levels.

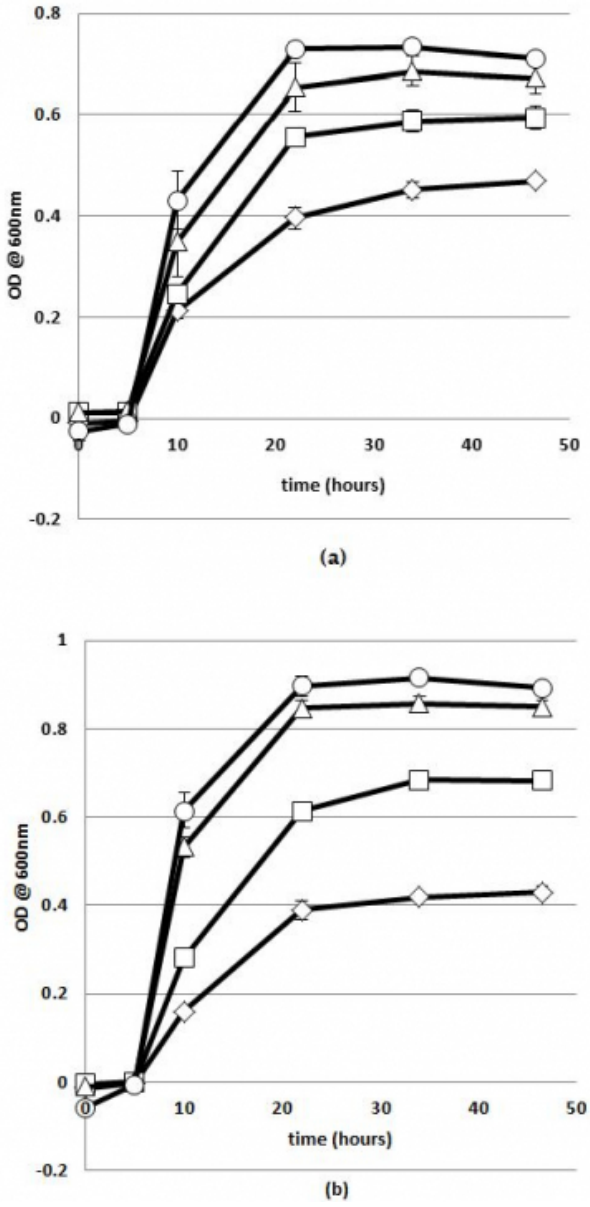
Tukey comparisons for the interaction term showed that C. cellulolyticum had significantly higher (p£0.05) growth at all yeast extract levels, except 0 and 2 g/L.Both bacteria had limited growth without yeast extract (0 g/L).Larger standard deviations on the 2 g/L C. cellulolyticum data prevented significant differences at this level. These differences support the hypothesis that the two organisms metabolize yeast extract differently. For each bacterium, growth on 0 g/L yeast extract was significantly lower than all other yeast extract levels. There were no other significant differences between yeast extract levels within each bacterium group.

Yeast extract with carbon source growth

This set of experiments examined the effect of yeast extract concentration on the utilization of cellobiose by C. cellulolyticum and C. phytofermentans. Growth curves for the two bacteria, shown in Figures 2a and 2b, show that the bacteria are able to grow to higher optical densities with higher concentrations of yeast extract.The control (yeast extract with no carbon source) values were subtracted from the treatment data.The curves are very different between each step until the 1.5 to 2.0 g/L step.The difference begins to level off, indicating that the yeast extract is no longer the growth-limiting factor.

Figure 2

Growth curves of C. cellulolyticum (a) and C. phytofermentans (b) on 4 g/L cellobiose and 0.5 (diamonds), 1.0 (squares), 1.5 (triangles), 2.0 g/L (circles) yeast extract



ANOVA tests in R revealed that all terms were significant (p£0.05).Tukey tests for the bacteria term showed that the average growth over all yeast extract levels for C. cellulolyticum was significantly (p£0.05) higher than that of C. phytofermentans. Tukey tests for yeast extract levels averaged over bacteria showed that all levels were different, except for 1.5 and 2.0 g/L. However, Tukey tests on the interaction showed that when the yeast extract levels are compared for each bacterium separately, all levels are significantly different from each other. When comparing bacteria at each yeast extract level, all levels were significantly different except 0.5 g/L. For both bacteria, the ethanol yields were comparable for all levels of yeast extract.

DISCUSSION

Product yields

Ethanol yield by *C. thermocellum* from cellobiose (0.12 g/g) is comparable to that reported in literature of 0.10 g/g (Levin, et al., 2006).The ethanol yields from cellobiose by *C. cellulolyticum* (0.38 g/g) and *C. phytofermentans* (0.27g/g) were higher than values of 0.07g/g and 0.12, respectively, reported by Ren et. al (2007).Differing hydrogen partial pressures could explain these differences.Previous studies suggest that changing the hydrogen partial pressure can affect ethanol production (Collet, et al., 2003, Mistry and Cooney, 1989).*Clostridium thermolacticum* cultures with hydrogen partial pressures greater than 0.03 atm resulted in ethanol concentration of 23 mmol/L while cultures with partial pressures of less than 0.01 atm only had 10mmol/L ethanol production (Collet, et al., 2003).Hydrogen partial pressures were not measured for this study or Ren et. al (Ren, et al., 2007). However, the headspace for this study was 33% of the total volume, while in Ren et. al (Ren, et al., 2007) 83% of the total volume was the headspace.Assuming similar hydrogen volume production for both studies, the partial pressure of this study would be higher, which could explain the higher ethanol production as well.The differences hydrogen partial pressure did not appear to affect the ethanol production from crystalline cellulose, most likely because the overall gas production was much lower for crystalline cellulose than cellobiose.

This discrepancy could also be due the fact that the stock cultures in the current experiments were maintained on cellobiose while previous studies were maintained on cellulose.Cellobiose was chosen for stock cultures in the current study so that the cell biomass could be approximated using OD allowing for comparable inoculation for each bacterium.However, it is possible that the cells were not completely adapted to more complex substrates, leading to lower ethanol yields on cellulose and xylan. The current study reached 0.01 g ethanol/g substrate each for crystalline cellulose, fibrous cellulose and xylan by *C. thermocellum* while literature shows ethanol yields on cellulose ranging from 0.09-0.20 g ethanol/g cellulose (Levin, et al., 2006, Shin, et al., 2002, Zertuche and Zall, 1982). Ren et. al (2007) reports ethanol yields from crystalline cellulose of 0.04 and 0.07 g ethanol/g cellulose by *C. cellulolyticum* and *C.*

phytofermentans, respectively. The current study only reached 0.01 and 0.0 g ethanol/g cellulose by *C. cellulolyticum* and *C. phytofermentans,* respectively.There is no previous literature for *C. cellulolyticum* and *C. phytofermentans* growing on xylan, but current yields (0.18 and 0.14 g ethanol/g xylan, respectively) were higher than those reported in literature for cellulose, suggesting that xylan utilization may not be as sensitive to adaptation time as cellulose for these two organisms.On the other hand, *C. thermocellum* seems to be more sensitive to the lack of adaptation time, especially for xylan, with a yield of only 0.01 g ethanol/g xylan.

For both *C. cellulolyticum* and *C. phytofermentans,* the ethanol yield from 2 g/L crystalline cellulose + 2 g/L xylan were approximately the average of the respective individual pure substrate (4 g/L) fermentation yields, which would be expected if there was no interaction effect caused by mixing substrates. Since the substrate particles are separate entities, each cell only interacts with one type of substrate.*C. cellulolyticum* has been shown to adapt its cellulosome according to its substrate (Blouzard, et al., 2010) so each cell would only make one set of enzymes, specific for either cellulose or xylan.On a cell-by-cell basis, mixed substrates look the same as the separate substrates and it is therefore expected that the mixture product yield would simply be the average of those from the separate substrates. However, this conclusion may not transfer to lignocellulosic biomass due to the interconnected nature of the cellulose and hemicellulose fibers.Each cell could interact with multiple polysaccharides, requiring more enzyme variety (and energy) per cell and leading to lower product yields.

The total product yield for *C. thermocellum* was 0.92 g product/g cellobiose, which leaves only 8% of substrate for cell growth and maintenance, and gas production.This high total conversion could be explained by the fact that complex media was used.The yeast extract in the media supplied cell building blocks allowing most of the cellobiose to be used for energy metabolism (Guedon, et al., 1999). All products but butyric acid were found in previous literature (Chinn, et al., 2007).

Low ethanol yields by *C. cellulovorans* agree with those reported previously(Sleat, et al., 1984). Sleat et al. (Sleat, et al., 1984) reported *C. cellulovorans* product yields on 10 g/L avicel of 0.06 g/g acetic acid and 0.39 g/g butyric acid, which are comparable to the results of this study.Formic and butyric acids, like other organic acids, can be used as building blocks for a variety of chemical syntheses (Weissermel and Arpe, 2003) or biologically converted to methane for additional energy production.

Overall, cellobiose was the best substrate for ethanol and volatile fatty acid production. This result is not surprising since cellobiose is the simplest of the substrates tested and the *Clostridium* are capable of using cellodextrins, including cellobiose, directly with no enzyme secretion (Schwarz, 2001).Without the need to produce cellulase enzymes, the bacteria can devote more energy to fermentative metabolism, potentially resulting in higher product yields. Among the four bacteria and four substrates tested in this study, *C. cellulolyticum* achieved the highest ethanol yield of 0.38 g ethanol/g cellobiose on cellobiose with co-product yields of 0.1 g acetic acid/g cellobiose, 0.08 g formic acid/g cellobiose, 0.07 g lactic/g cellobiose, and 0.01 g butyric acid/g cellobiose.Growing on xylan, *C. cellulolyticum* had yields of 0.15 g ethanol/g xylan 0.19 g acetic acid/g xylan. *C. phytofermentans* produced 0.27 g ethanol/g cellobiose from cellobiose and 0.15 g ethanol/g xylan and 0.05 g acetic acid/g xylan from xylan.

Yeast extract effects

The data showed that yeast extract is an important factor in the growth and metabolism of Clostridia and confirmed the hypothesis that at least some Clostridia are capable of metabolizing yeast extract for growth and product formation. Products were not directly measured on yeast extract alone, but it is inferred that fermentation products would be a byproduct of growth since the organism uses a fermentation pathway to produce energy in the form of ATP. Even though growth of both bacteria was limited on yeast extract only, the data suggests that controls without carbon source should be run in future experiments and subtracted out of the substrate treatments.This will prevent the products metabolized from yeast extract from being attributed to the substrate yield and will allow for more accurate mass balance.

For the yeast extract with carbon source experiments, Tukey tests on the yeast extract – bacteria interaction term suggest that 2 g/L yeast extract may be necessary to ensure that yeast extract is not the growth limiting factor.These results are similar to those with *Clostridium* strain F5a15.Growth for this strain changed little between 1 and 2 g/L of yeast extract and decreased for lower concentrations (Leclerc, et al., 1998).

CONCLUSION

Based on its product yields, *C. cellulolyticum* appears to have the highest potential for both ethanol and other co-product production from plant-based substrates. However, further testing on real lignocellulosic substrates is needed to confirm its suitability for industrial use.*C. cellulovorans* had the highest total product yield on a complex substrate: 0.55 g products/g cellulose on crystalline cellulose with individual yields of 0.28 g butyric acid/g cellulose, 0.16 g formic acid/g cellulose, 0.06 g ethanol/g and 0.05 g acetic acid/g cellulose. Therefore, *C. cellulovorans* has the most potential for organic acid production from plant-based substrates. A yeast extract concentration of 2 g/L is acceptable for lab scale research and fermentation, but still high for industrial fermentations. Future research should investigate alternative nitrogen and nutrient sources.

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