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Fermentation of Sweet Sorghum Derived Sugars to Butyric Acid at High Titer and Productivity by a Moderate Thermophile *Clostridium thermobutyricum* at 50°C

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Running Title: Butyric acid production by *C. thermobutyricum*

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Abstract

In this study, a moderate thermophile *Clostridium thermobutyricum* is shown to ferment the sugars in sweet sorghum juice treated with invertase and supplemented with tryptone (10 $g.L^{-1}$) and yeast extract (10 $g.L^{-1}$) at 50°C to 44 $g.L^{-1}$ butyrate at a calculated highest volumetric productivity of 1.45 $g.L^{-1}.h^{-1}$ (molar butyrate yield of 0.85 based on sugars fermented). This volumetric productivity is among the highest reported for batch fermentations. Sugars from acid and enzyme-treated sweet sorghum bagasse were also fermented to butyrate by this organism with a molar yield of 0.81 (based on the amount of cellulose and hemicellulose). By combining the results from juice and bagasse, the calculated yield of butyric acid is approximately 90 kg per tonne of fresh sweet sorghum stalk. This study demonstrates that *C. thermobutyricum* can be an effective microbial biocatalyst for production of bio-based butyrate from renewable feedstocks at 50°C.

Key words: Butyrate; Fermentation; *Clostridium thermobutyricum*; Sweet sorghum juice; Sweet sorghum bagasse

1. Introduction

Butyric acid is a commodity chemical produced at industrial scale by chemical synthesis and by fermentation (Bagby et al., 2003; Dwidar et al., 2012). Butyric acid is used as a feedstock for synthesis of plastics, plasticizers and surfactants (Dwidar et al., 2012). Various derivatives of butyric acid, such as esters, are used in food, perfume, as well as in the chemical and pharmaceutical industries. Recently, butyric acid has been implicated in improving intestinal health in humans as well as in animals (Leonel & Alvarez-Leite, 2012). It is used as an animal feed additive (Fernandez-Rubio et al., 2009) and is expected to become a nutraceutical. Production of butyrate from renewable biological feedstocks rather than from petroleum has the additional benefit of supporting rural economies (Leistritz & Hodur, 2008).

Only anaerobic bacteria (with a rare exception of an aerobic *Thermobifida fusca*) (Merklein et al., 2014) are known to ferment sugars to butyric acid. These anaerobic bacteria are found in various environments and are also natural inhabitants of the human intestine. In these microorganisms, the butyric acid pathway starts from acetyl-CoA generated by glycolysis and pyruvate-ferredoxin oxidoreductase. Conversion of butyryl-CoA to butyrate by the enzymes phosphate butyryltransferase (PTB) and butyrate kinase (BUK) yields an additional ATP per glucose, in addition to two net ATPs from glycolysis, resulting in a total ATP yield of 3 per glucose to support bacterial growth. Butyrate production from glucose by Clostridia is also accompanied by varying amounts of acetate as a co-product to maintain redox balance. To eliminate co-product production during fermentation, the butyrate pathway has been engineered into *Escherichia coli*. However, the reported titer of butyric acid in batch fermentations by these *E. coli* derivatives was $\leq$10 $g.L^{-1}$ compared to *Clostridium* spp. (highest titer of about 45 $g.L^{-1}$ in batch fermentations) (Vandak et al., 1995; Volker et al., 2014) with one study reporting as high

as 62.5 $g.L^{-1}$ using *C. tyrobutyricum* (Song et al., 2010). Although a mutant strain of *C. acetobutylicum* produced very low level of acetate and lactate as co-products during the butyrate production phase, the butyrate titer and yield of 32.5 $g.L^{-1}$ and 0.39 $g.g^{-1}$ (theoretical yield of butyrate from glucose is 0.49 $g.g^{-1}$), respectively (Jang et al., 2014), are lower than the reported values for *C. butyricum* and *C. tyrobutyricum* listed above.

Industrial application of Clostridia is limited in part by the obligate requirement of complex nutrients for high productivity, although *Clostridium acetobutylicum* has been used industrially for butanol production (Jones & Woods, 1986). Thermophilic butyric acid producers (optimum growth temperature of 50-60 °C) could overcome some of the other limitations of mesophilic Clostridia such as potential contamination of large scale fermentations (Abdel-Banat et al., 2010). In addition, the higher fermentation temperature is optimal for cellulase and could lower the amount of enzyme required for effective utilization of non-food carbohydrates from lignocellulosic biomass (Ou et al., 2009). Two butyrate-producing thermotolerant Clostridia, *C. thermobutyricum* and *C. thermopalmarium*, have been isolated and characterized (Soh et al., 1991; Wiegel et al., 1989). Both bacteria grew optimally at 55°C and produced butyrate as the major product and the highest reported butyrate titer of about 15 $g.L^{-1}$ (Canganella et al., 2002) was significantly lower than the highest reported titer of 62.5 $g.L^{-1}$ obtained with mesophilic *Clostridium tyrobutyricum* in batch fermentation (Song et al., 2010). Among the two, *C. thermobutyricum* may have the potential for high butyrate titer since it has been reported to have a volumetric productivity of 2.4 $g.L^{-1}.\ h^{-1}$ in a continuous culture (Canganella et al., 2002; Canganella & Wiegel, 2000).

The dominant source of sugars for fermentation today is corn starch or sucrose from sugarcane or sugar beets, all of which compete with food. The dual use of these feedstocks for

food and fuel/chemicals raises ethical concerns that limit further expansion of fermentation using these sugar sources. Alternative sources of sugars are needed that do not compete with food. Energy crops such as sweet sorghum are under active development. Sweet sorghum has a wider environmental range than sugarcane due to its higher cold and drought tolerance (Saballos, 2008). Sweet sorghum accumulates large amounts of soluble sugars (sucrose, glucose and fructose) in its stem and is considered an attractive energy crop for tropical and subtropical climates (Regassa & Wortmann, 2014). The sugar content of the juice can be as high as 20% (w/v) (Erickson et al., 2011; Kim & Day, 2011). Due to the abundance of glucose and fructose, the sucrose in sweet sorghum juice will not crystallize, limiting use for food applications. Sweet sorghum thus represents an alternate source of non-food sugars that can be fermented to butyrate. In addition, sweet sorghum also produces large amounts of biomass, which remains in the form of bagasse following juice extraction. Sorghum bagasse contains about 70% of structural carbohydrates, which can provide an additional source of fermentable, non-food sugars.

In this present study, we have evaluated *C. thermobutyricum* for production of butyric acid at high titer and yield at 50°C from sugars present in sweet sorghum juice and acid-treated sweet sorghum bagasse. The results presented here show that a butyrate titer of about 44 $g.L^{-1}$ can be achieved at 50°C in less than 40 hours using sweet sorghum juice. The butyrate volumetric productivity of 1.45 $g.L^{-1}.h^{-1}$ using sweet sorghum juice as feedstock is one of the highest values reported for batch fermentation.

2. Materials and methods

2.1. Materials.

Organic and inorganic chemicals were from Fisher Scientific (Pittsburgh, PA) and were analytical grade. Biochemicals were from Sigma-Aldrich (St. Louis, MO). Oxyrase (30 units of enzyme activity .ml^{-1}) was from Oxyrase, Inc. (Mansfield, OH). Cellulase (Cellic CTec2) (145 filter paper units (FPU). ml^{-1}) was a generous gift from Novozymes, Inc. (Franklinton, NC). Invertase (2,400 units.ml^{-1}) was from LorAnn Oils (Lansing, MI).

2.2. Microorganism and culture condition

C. thermobutyricum strain JW171K was obtained from the German Culture Collection as DSM4928. *C. butyricum* strain E.VI.3.2.1 (B-1024) and *C. acetobutylicum* strain 824 (B-527) were from the USDA-ARS culture collection. All cultures were maintained in reinforced clostridium medium (RCM; Oxoid, Hants, UK) under N_2 atmosphere. The RCM medium at pH 6.8 contained per liter, yeast extract (13 g), peptone (10 g), soluble starch (1 g), glucose (5 g), cysteine.HCl (0.5 g), NaCl (5 g), sodium acetate (3 g) and agar (0.5 g). Batch fermentations were conducted in custom-made units (Beall et al., 1991; Ohta et al., 1991) using LB medium (Tryptone, 10 $g.L^{-1}$; yeast extract, 5 $g.L^{-1}$; and NaCl, 5 $g.L^{-1}$; pH 7.0) (Research Products International, Inc., Prospect, IL) supplemented with cysteine (0.1 $g.L^{-1}$) and additional yeast extract in some cases (final concentration, 10 $g.L^{-1}$; LB-YE). Betaine (1 mM) was added to fermentations when the total sugar concentration was ≥100 $g.L^{-1}$. In some experiments LB medium was supplemented with RCM medium at 10% (v/v) final concentration (M-LB). Fermentations of sugars from sweet sorghum juice and pre-treated sweet sorghum bagasse slurry were carried out in LB-YE medium.

Medium (250 ml) in 500-ml vessels was sparged with N_2 at a flow rate of 23 ml.min^{-1} for 30 min before inoculation with 12.5 ml of an actively growing culture in RCM. Sparging of the culture with N_2 continued through the experiment. The pH of the culture was maintained by automatic addition of 2N KOH. Medium was additionally buffered with $CaCO_3$ (15-24 g.L^{-1}) that also minimized butyric acid toxicity. Fermentation temperatures were 50°C for *C. thermobutyricum* and 37°C for *C. butyricum* and *C. acetobutylicum*. The inoculum for fermentations was grown in capped serum bottles (Wheaton Scientific) in RCM supplemented with oxyrase (0.3 unit of enzyme activity .ml^{-1} of medium). The gas phase in the bottles was replaced by repeated evacuation and filling with N_2. The medium was pre-incubated for 30 min at 37°C to aid in the removal of residual O_2 by the oxyrase before inoculation. Oxyrase, an *E. coli* membrane preparation enriched for membrane bound dehydrogenases, including lactate dehydrogenases, is inactivated during growth of *C. thermobutyricum* at 50°C.

2.3. Sweet sorghum juice

Sweet sorghum (*Sorghum bicolor* (L.) Moench) cultivar M-81E was grown at the University of Florida Plant Science Research and Education Center near Citra, FL during the summer growing season and harvested when the seed reached soft-dough stage, just prior to full maturity. After removal of leaves and panicles, the stems were pressed using a roller press at about 12 MPa of pressure in a grooved, two-roller electric mill, and the juice from several plants was collected and pooled. Large particles in the juice were removed by passing through two layers of cheese cloth before use and the small particles were removed after centrifugation at 10,000 x g for 20 min. The sugar concentration in the juice was measured using HPLC and contained 92 g.L^{-1} sucrose, 32 g.L^{-1} glucose and 27 g.L^{-1} fructose for a total of 151 g.L^{-1} total sugars. Other sugars, if present in the juice, were below the detection limit of the HPLC (0.5 mM). Before inoculation,

153 invertase (0.4 %, v/v; 9.6 units .ml^{-1}) was added to hydrolyze the sucrose and at this
154 concentration of invertase, the sucrose in the juice was completely hydrolyzed within 15 min.

155 *2.4. Sweet sorghum bagasse*

156 Sweet sorghum bagasse was treated with 0.5% phosphoric acid (w/w of bagasse-dry weight) and
157 steam at 190°C for 5 min as described previously for sugarcane bagasse (Geddes et al., 2011).
158 The dry matter content of this pre-treated bagasse slurry was 25.3% (w/w). The amount of free
159 sugars released during the pre-treatment process was determined using the liquid (moisture
160 content) associated with the acid-treated bagasse obtained after centrifugation (10,000 x g; 20
161 min). The composition of free sugars is expressed as g.kg^{-1} dry weight of solids in the slurry
162 (cellobiose, 29.4; glucose, 30.5; xylose, 129.0; arabinose, 22.6; galactose, 6.9). Pre-treated
163 bagasse that contained both solids and associated liquid (moisture) at 50 g.L^{-1} (based on solids
164 dry weight) was liquefied with cellulases (20 FPU of Cellic CTec2.(g bagasse solids dry
165 weight)$^{-1}$) at 50°C and pH 5.0 in LB-YE medium to lower the viscosity, as described previously
166 for sugar cane bagasse (Geddes et al., 2010). After 6.0 hours of liquefaction at pH 5.0, the pH
167 was raised to 6.5 and the slurry was inoculated with a fresh culture of *C. thermobutyricum* grown
168 in RCM medium, to an initial cell density of 0.1 OD $_{420\,nm}$ unit. The culture pH was maintained
169 at 6.5 for 12.5 hours to support growth of the bacterium by automatic addition of KOH. After
170 12.5 h, the culture pH was lowered to 6.0 to support higher cellulase activity for simultaneous
171 saccharification and co-fermentation (SScF) of remaining glycans.

172 *2.5 Analytical methods*

173 Sugars and fermentation products were determined using HPLC as described before (Ou et al.,
174 2009). A 1-ml sample from the fermentations was centrifuged at 13,000 x g for 5 min and the

supernatant was filtered through 0.2 µm filter. Ten µl of 1M H_2SO_4 was added to the filtrate to acidify the spent medium before analysis by HPLC for sugars and organic acids. The HPLC (Agilent 1100) was equipped with Aminex HPX-87H column (Bio-Rad laboratories, Hercules, CA) and variable wavelength detector and refractive index detector, in series. The mobile phase was 4 mM H_2SO_4 at a flow rate of 0.4 $ml.min^{-1}$ and the column temperature was 45°C. Cell density was determined as optical density at 420 nm (Beckman 640) and a culture at one OD unit contained 0.18 g dry weight of cells per liter. The cellulose, hemicellulose and lignin composition of sorghum bagasse was determined using NREL method (Sluiter et al., 2012). Enzyme activity of Cellic CTec2 cocktail was determined as described previously (Ou et al., 2009) and is expressed as filter paper unit ($FPU.ml^{-1}$). Experiments were repeated at least three times and the mean and standard deviations from three experiments are presented, unless stated otherwise. Productivity values were calculated for different time intervals during the fermentation and either the highest or the average values are presented, as indicated in specific experiments.

3. Results and discussion

3.1. Growth characteristics of C. thermobutyricum

When *C. thermobutyricum* was cultured in M-LB with glucose, growth started after a short lag of 25 min and reached the highest cell density at about 5 hours of incubation (Fig. 1). Since specific productivity provides a measure of the organism's intrinsic metabolic ability to convert sugars to product(s), the specific productivity of butyrate for *C. thermobutyricum* was determined during the growth cycle in a medium that supported the highest growth rate of the bacterium. The highest specific productivity of butyrate was observed during the early exponential growth phase of the culture (1.8 $g.h^{-1}.g$ cell dry $weight^{-1}$) and this rate was maintained through the growth phase although the specific growth rate (μ) continued to decline through the growth phase. Specific productivity of butyrate started to decrease as the culture entered late-exponential phase of growth. At this stage, the average growth rate (0.2 h^{-1}) of the population was less than 30% of the peak value of 0.7 h^{-1}. A specific productivity of 0.4 (g butyrate. h^{-1}. g cell dry $weight^{-1}$) was maintained through the stationary phase. These results suggest that during the active growth period (higher μ), the ATP produced by the butyrate pathway (calculated net of three ATPs per glucose) was rapidly consumed to support a higher sugar flux to butyrate (higher specific productivity). It has been reported previously with *E. coli* and other organisms that lowering the ATP pool by mutations increased the glycolytic flux (Causey et al., 2003; Koebmann et al., 2002) indicating a direct link between the ratios of the concentration of ATP, ADP and AMP in the cytoplasm and glycolytic flux, and associated ATP production. Based on this analogy, it is possible that the decrease in specific productivity observed during the later stages of growth is a result of lower ATP need of a culture growing at a lower specific growth rate compared to an exponentially growing culture (Fig. 1). The volumetric productivity of butyrate for *C.*

thermobutyricum with glucose was 0.8 ± 0.03 $g.L^{-1}.h^{-1}$ and this value compares favorably with 0.89 $g.L^{-1}.h^{-1}$ for *C. acetobutylicum* on glucose (Jang et al., 2014) and 0.9 $g.L^{-1}.h^{-1}$ for *C. butyricum* (Vandak et al., 1995).

3.2. Fermentation characteristics of C. thermobutyricum

Various nutrient levels (RCM, LB and M-LB) were tested for butyrate production by *C. thermobutyricum*. Each medium supported growth of *C. thermobutyricum* to different level (specific growth rates in RCM, LB and M-LB were 1.4, 0.6, and 0.7 $.h^{-1}$, respectively). It is likely that the cost of these media is too high for commercial production but RCM provided near maximal butyrate productivity for this organism (specific productivity, 1.8 ± 0.09 $g.h^{-1}.g$ cell dry $weight^{-1}$; volumetric productivity, 1.3 ± 0.11 $g.L^{-1}.h^{-1}$). M-LB medium did support butyrate specific productivity to the same level as RCM (1.7 ± 0.07 $g.h^{-1}.g$ cell dry $weight^{-1}$) but the volumetric productivity was lower (0.8 ± 0.03 $g.L^{-1}.h^{-1}$) compared to cells grown in RCM medium. The butyrate yield in the three media (RCM, LB, M-LB) were 0.37, 0.46 and 0.45 $g.g^{-1}$ of glucose fermented, respectively. Under similar experimental condition, the butyrate yield from glucose in one of the medium tested (M-LB) for the mesophiles *C. butyricum* and *C. acetobutylicum* was 0.29 and 0.36 $g.g^{-1}$of glucose fermented, respectively. Due to the lower butyrate yield, the specific productivity of butyrate by these two mesophilic Clostridia was also lower compared to *C. thermobutyricum*.

Industrial production of butyrate as a chemical feedstock would require a chemically defined medium to minimize the cost of medium and product purification. Although a defined medium was reported for *C. thermobutyricum*, the cell yield of the bacterium in this medium was very low (about 70 $mg.L^{-1}$) (Canganella et al., 2002). Addition of yeast extract in the range of

0.5-20 $g.L^{-1}$ increased the cell yield in this defined medium (0.7 to 1.0 $g.L^{-1}$) indicating an absolute requirement for complex nutrients. Our results confirmed these findings. Due to the lack of a chemically defined medium for *C. thermobutyricum*, we have used LB with the lowest amount of ingredients as the base medium of choice for further studies. In LB medium at pH 7.0, *C. thermobutyricum* only fermented about 17 $g.L^{-1}$ of glucose in 125 h with a butyrate titer of 7 $g.L^{-1}$ and a butyrate yield of 0.41 $g.g^{-1}$ glucose fermented. This butyrate was produced only during the growth period and the decline in cell density during the stationary phase (apparently not due to sporulation) could have a negative effect on butyrate production. This limitation in butyrate production was alleviated by addition of $CaCO_3$ to the medium that in addition to buffering the medium has apparently minimized butyrate toxicity.

With an actively growing culture as the inoculum, *C. thermobutyricum* fermented 50 ± 0.9 $g.L^{-1}$ glucose in LB with $CaCO_3$ (24 $g.L^{-1}$) in 120 hours to 24 ± 0.35 $g.L^{-1}$ butyrate with 2 ± 0.2 $g.L^{-1}$acetate as the co-product (Fig. 2; Table 1). Almost all of the acetate was produced during the first 24 hours and the acetate-to-butyrate ratio was 0.08 ($g.g^{-1}$), a value that is among the lowest reported (Vandak et al., 1995). The average volumetric productivity over the first 48 h was 0.32 g butyrate.L^{-1}.h^{-1}. In this fermentation, 0.49 g butyric acid was produced from each gram of glucose fermented, matching the theoretical yield of 0.49. Apparently, nutrients in the LB also contributed to the butyrate yield, although the concentration of individual sugars in LB was below the detection limit of the HPLC. With this carbon yield, about 78% of the energy in glucose ($C_6H_{12}O_6$; ΔH, -2805 KJ.$mole^{-1}$) was conserved in butyrate ($C_4H_8O_2$; ΔH, -2182.5 KJ.$mole^{-1}$). One of the limiting factors in the LB medium was the low volumetric productivity of *C. thermobutyricum* that increased the length of fermentation.

In earlier studies, Canganella et al. (Canganella et al., 2002) reported that increasing the yeast extract concentration in the medium to as high as 20 $g.L^{-1}$ increased butyrate titer of *C. thermobutyricum* by about four-fold. In an attempt to increase volumetric productivity with the aim of reducing the fermentation time, the yeast extract concentration in LB was increased from 5 $g.L^{-1}$ to 10 $g.L^{-1}$ (LB-YE). In this medium, with an initial glucose concentration of 150 $g.L^{-1}$, the butyrate titer reached to 44.5 $g.L^{-1}$ (Table 1). This fermentation required addition of 1 mM betaine (to minimize osmotic effect of 0.83 M glucose) and was essentially complete in 96 hours. The average volumetric productivity over the first 48 hours when about 60% of the glucose was fermented was 0.62 $g.L^{-1}.h^{-1}$ (Table 1). This high titer of butyrate was comparable to the reported titers with *C. butyricum* (Vandak et al., 1995). However, at this glucose concentration, acetate to butyrate ratio (0.2) was higher than in fermentations at a glucose concentration of 50 $g.L^{-1}$ (0.08). In addition, lactate (3.6 $g.L^{-1}$) was also detected in the medium suggesting that one or more steps in the butyrate pathway is rate limiting and that excess pyruvate from glycolysis is channeled to lactate. It is also possible that the higher acetate-to-butyrate ratio represents the lack of utilization of acetate towards butyrate production, as reported for butyrate-fermenting bacteria including *C. thermobutyricum* (Canganella et al., 2002). The presence of residual glucose in the medium may account for this high acetate to butyrate ratio. These co-products lowered the yield of butyrate from glucose fermented to 0.37 $g.g^{-1}$, compared to 0.49 $g.g^{-1}$ at the lower glucose concentration of 50 $g.L^{-1}$ (Table 1). At an intermediate level of glucose (100 g $.L^{-1}$), the acetate-to-butyrate ratio was lower (0.08), although a significant amount of lactate was detected as a co-product. At this glucose concentration, the yield of butyrate from the amount of glucose fermented was 0.44 $g.g^{-1}$ (Table 1).

It is interesting to note that when the initial glucose concentration was 150 $g.L^{-1}$, the bacterium fermented about 110 $g.L^{-1}$ of the sugar in about 72 hours. Based on this result, glucose supplied at an initial concentration of 100 $g.L^{-1}$ was expected to be completely fermented within that time frame. However, with an initial glucose concentration of 100 $g.L^{-1}$, fermentation started to slow down when the residual glucose concentration reached about 30 $g.L^{-1}$ (about 72 hours), and even after 120 hours of incubation, about 25% of the sugar was left unfermented (22.5 $g.L^{-1}$). These results suggest a metabolic slow down of the bacterium after about 72 hours and improving the productivity during the early periods of fermentation is expected to increase the butyrate titer.

3.3. Fermentation of sweet sorghum juice to butyrate

The glucose and fructose in sweet sorghum juice (without any additional nutrients) were fermented by *C. thermobutyricum* to butyric acid, while the sucrose was left behind. However, this organism only fermented about 30% of the hexoses in the juice to butyric acid, even with added $CaCO_3$ (15 $g.L^{-1}$) and pH control at 7.0. Supplementation of the juice with tryptone (10 $g.L^{-1}$) and yeast extract (10 $g.L^{-1}$) improved the rate of fermentation leading to production of 44 $g.L^{-1}$ butyric acid within 40 hours from 104 $g.L^{-1}$ hexoses, with fructose being the preferred sugar (Fig. 3). In this experiment, invertase was added to hydrolyze sucrose to its constituent sugars glucose and fructose before inoculation, since *C. thermobutyricum*, in contrast to other Clostridia, failed to ferment sucrose. Acetate was the only co-product detected and the acetate-to-butyrate ratio was 0.21. The butyrate yield was 0.42 $g.g^{-1}$ based on the total hexoses fermented. The highest volumetric productivity of butyrate in this sweet sorghum juice fermentation was 1.45 $g.L^{-1}.h^{-1}$. This value is the highest for this bacterium in batch

fermentation. The average volumetric productivity over the 48 hour fermentation of 0.9 $g.L^{-1}.h^{-1}$ is higher than the rate of glucose fermentation in LB-YE medium (0.6 $g.L^{-1}.h^{-1}$; Table 1).

It should be noted that all hexoses, provided at a total concentration of 105 ± 3.8 $g.L^{-1}$, were fermented in less than 48 hours, while fermentation in LB-YE medium with 100 $g.L^{-1}$ glucose resulted in a residual glucose concentration of 22.5 $g.L^{-1}$. Apparently, the sorghum juice contains yet to be identified nutrients that supported the high productivity and these are either absent or present at limiting levels in LB-YE medium. Additionally, the highest butyrate titer of about 44 $g.L^{-1}$ is apparently the upper limit of butyrate concentration that can be reached under this experimental condition, using cells in batch fermentation, most likely due to product toxicity.

3.4. Fermentation of pre-treated sweet sorghum bagasse

Sweet sorghum bagasse obtained after juice extraction contained 45% cellulose and 25% hemicellulose. The remainder includes lignin, protein, waxes, minerals and extractives. Phosphoric acid pre-treated sugarcane bagasse at higher solids loading was previously reported to be difficult to handle, and a short liquefaction step with fungal cellulases greatly decreased the viscosity and mixing of the bagasse suspension during fermentation (liquefaction plus simultaneous saccharification and co-fermentation; L+SScF) (Geddes et al., 2010). In this present study, pre-treated bagasse (190°C; 0.5% acid (w/w); 5 min) at 50 $g.L^{-1}$ (dry wt) was added to LB-YE medium and CTec2 cellulase was added to an initial loading of 20 FPU per g bagasse (dry weight). This mixture was incubated at 50°C, pH 5.0 for 6 hours to liquefy the solids to aid in mixing during fermentation. The liquefaction step increased the glucose concentration from less than 0.2 $g.L^{-1}$ to 13.5 $g.L^{-1}$, and the xylose concentration from 4.5 $g.L^{-1}$

to 8.1 $g.L^{-1}$. After inoculation, both glucose and xylose were rapidly fermented to butyric acid and acetic acid in about 12 hours (Fig. 4). The butyrate titer at the end of 12 hours was 8.5 $g.L^{-1}$ (yield of 0.44 $g.g^{-1}$ of fermented glucose and xylose). These values are comparable to the butyrate titer and yield reported by Liu et al. (Liu et al., 2013) with wheat straw and switch grass hydrolysates as feedstock and *C. tyrobutyricum* as microbial biocatalyst during the first 24 h of batch fermentation (Wheat straw hydrolysate – titer, 9.9 $g.L^{-1}$; yield, 0.44 $g.g^{-1}$ glucose) (switch grass hydrolysate – titer, 7.1 $g.L^{-1}$; yield, 0.42 $g.g^{-1}$ glucose).

Continued production of butyrate by the *C. thermobutyricum* culture was from the release of sugars from carbohydrates by the enzymes that are still active during the fermentation process (SScF) at 50°C (Fig. 4). After 55 hours of fermentation, the concentration of butyrate was about 15.5 ± 0.5 $g.L^{-1}$, with 2.7 ± 0.2 $g.L^{-1}$ acetate as a co-product. The molar yield of butyrate from the glucose and xylose that can be released from the cellulose and hemicelluloses (45 and 25%, respectively) in the bagasse was 0.81. Including acetate, 92% of the expected sugars were converted to products in this L+SScF process. This is comparable to the 90% of theoretical yield of cellulosic ethanol produced from phosphoric acid treated sweet sorghum bagasse reported previously (Geddes et al., 2013).

Decreasing the enzyme loading from 20 FPU per g bagasse lowered the amount of glucose released during liquefaction as well as the final butyrate titer and yield (Table 2). Although the enzymes are still present during fermentation at 50°C, the operating pH of 6.0, a value that is higher than the optimal pH of 5.0 for activity of the cellulases, could have diminished the amount of glucose released for fermentation. Increasing the enzyme loading to 25 FPU per g bagasse (dry weight) did not increase the final butyrate titer or yield over that of 20 FPU per g bagasse, although the higher enzyme loading did increase the amount of glucose

released during the liquefaction step. Increasing the time of liquefaction to 24 h at pH 5.0 led to higher glucose and xylose release and the sugars were fermented within 48 h. This longer liquefaction time led to a molar yield of 0.9 for butyrate (calculated theoretical yield of about 380 g butyric acid .kg^{-1} of dry bagasse). These results show that the sugars derived from sweet sorghum bagasse after appropriate pre-treatment can be readily fermented by *C. thermobutyricum* to butyric acid at high yield using the L+SScF process.

3.5 Potential of C. thermobutyricum as a microbial biocatalyst for butyrate production

Fermentation of glucose to butyrate has been reported by several investigators and the butyrate titer, yield and productivity of free cells of these Clostridia in batch fermentations are listed in Table 3 for comparison with *C. thermobutyricum*. Based on these results, the highest butyrate titer was reported with *C. tyrobutyricum* at 62.5 $g.L^{-1}$ (150 $g.L^{-1}$ glucose) in about 80 h and a volumetric productivity of 0.99 $g.L^{-1}.h^{-1}$ at 120 $g.L^{-1}$ glucose (Song et al., 2010). The next highest titer was about 45 $g.L^{-1}$ (Table 3) and this is matched by *C. thermobutyricum* with glucose and the sugars from sweet sorghum juice (Table 1 and Fig. 3). Although a highest volumetric productivity of 1.45 g $butyrate.L^{-1}.h^{-1}$ can be calculated for this bacterium with sweet sorghum juice, the average volumetric productivity of butyrate over a 48 h period with this feedstock was 0.9 $g.L^{-1}.h^{-1}$ (Table 3). This value is comparable to the volumetric productivities reported for other butyrate producers (Table 3). In addition, *C. thermobutyricum* has the advantage of growing and fermenting sugars at 50-60 °C that would minimize contamination in industrial fermentations while also reducing cooling costs. Adaptive metabolic evolution for high cell yield and titer is expected to enhance the potential of *C. thermobutyricum* as a microbial biocatalyst for butyrate production. In addition, process modifications such as immobilization and fed-

batch/continuous fermentation are also projected to increase butyrate titer and volumetric productivity.

4. Conclusion

The results presented above with sweet sorghum juice and bagasse show that *C. thermobutyricum* is an effective biocatalyst for fermentation at 50°C of sugars from sweet sorghum juice and pre-treated bagasse. Based on these results, a tonne of fresh sweet sorghum stalk is expected to yield about 90 kg of butyric acid at a conversion rate of 85% of the total sugars (based on the composition of juice and bagasse listed above). This compares favorably to a reported theoretical yield of 97 kg of ethanol per tonne of sweet sorghum (Kim & Day, 2011).

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References

1. Abdel-Banat, B.M.A., Hoshida, H., Ano, A., Nonklang, S., Akada, R. 2010. High-temperature fermentation: how can processs for ethanol production at high temperatures become superior to the traditional process using mesophilic yeast? Appl. Microbiol. Biotechnol. 85, 861-867.
2. Bagby, M.O., Johnson, R.W., Daniels, R.W., Contrell, R.R., Sauer, E.T., Keenan, M.J., Krevalis, M.A., Staff, a.U.B. 2003. Carboxylic Acids. Kirk-Othmer Encyclopedia of Chemical Technology 5, 27-78.
3. Beall, D.S., Ohta, K., Ingram, L.O. 1991. Parametric studies of ethanol production form xylose and other sugars by recombinant *Escherichia coli*. Biotechnol. Bioeng. 38, 296-303.
4. Canganella, F., Kuk, S.U., Morgan, H., Wiegel, J. 2002. *Clostridium thermobutyricum:* growth studies and stimulation of butyrate formation by acetate supplementation. Microbiol. Res. 157, 149-156.
5. Canganella, F., Wiegel, J. 2000. Continuous cultivation of *Clostridium thermobutyricum* in a rotary fermentor system. J. Ind. Microbiol. Biotechnol. 24, 7-13.
6. Causey, T.B., Zhou, S., Shanmugam, K.T., Ingram, L.O. 2003. Engineering the metabolism of *Escherichia coli* W3110 for the conversion of sugar to redox-neutral and oxidized products: homoacetate production. Proc. Natl. Acad. Sci. U S A 100, 825-832.
7. Dwidar, M., Park, J.Y., Mitchell, R.J., Sang, B.I. 2012. The future of butyric acid in industry. The Scientific World J. 2012, 471417.

8. Erickson, J.E., Helsel, Z.R., Woodard, K.R., Vendramini, J.M.B., Wang, Y., Sollenberger, L.E., Gilbert, R.A. 2011. Planting date affects biomass and brix of sweet sorghum grown for biofuel across Florida. Agron. J. 103, 1827-1833.

9. Fayolle, F., Marchal, R., Ballerini, D. 1990. Effect of controlled substrate feeding on butyric acid production by *Clostridium tyrobutyricum*. J. Ind. Microbiol. 6, 179-183.

10. Fernandez-Rubio, C., Ordonez, C., Abad-Gonzalez, J., Garcia-Gallego, A., Honrubia, M.P., Mallo, J.J., Balaria-Fouce, R. 2009. Butyric acid-based feed additives help protect broiler chickens from *Salmonella enteritidis* infection. Poultry Sci. 88, 943-948.

11. Geddes, C.C., Mullinnix, M.T., Nieves, I.U., Hoffman, R.W., Sagues, W.J., York, S.W., Shanmugam, K.T., Erickson, J.E., Vermerris, W.E., Ingram, L.O. 2013. Seed train development for the fermentation of bagasse from sweet sorghum and sugarcane using a simplified fermentation process. Bioresour. Technol. 128, 716-724.

12. Geddes, C.C., Mullinnix, M.T., Nieves, I.U., Peterson, J.J., Hoffman, R.W., York, S.W., Yomano, L.P., Miller, E.N., Shanmugam, K.T., Ingram, L.O. 2011. Simplified process for ethanol production from sugarcane bagasse using hydrolysate-resistant *Escherichia coli* strain MM160. Bioresour. Technol. 102, 2702-2711.

13. Geddes, C.C., Peterson, J.J., Mullinnix, M.T., Svoronos, S.A., Shanmugam, K.T., Ingram, L.O. 2010. Optimizing cellulase usage for improved mixing and rheological properties of acid-pretreated sugarcane bagasse. Bioresour. Technol. 101, 9128-9136.

14. He, G.Q., Kong, Q., Chen, Q.H., Ruan, H. 2005. Batch and fed-batch production of butyric acid by *Clostridium butyricum* ZJUCB. J. Zhejiang Univ. Sci. B 6, 1076-1080.

15. Jang, Y.S., Im, J.A., Choi, S.Y., Lee, J.I., Lee, S.Y. 2014. Metabolic engineering of *Clostridium acetobutylicum* for butyric acid production with high butyric acid selectivity. Metab. Eng. 23, 165-174.
16. Jones, D.T., Woods, D.R. 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. 50, 484-524.
17. Kim, M., Day, D.F. 2011. Composition of sugar cane, energy cane, and sweet sorghum suitable for ethanol production at Louisiana sugar mills. J. Ind. Microbiol. Biotechnol. 38, 803-807.
18. Koebmann, B.J., Westerhoff, H.V., Snoep, J.L., Solem, C., Pedersen, M.B., Nilsson, D., Michelsen, O., Jensen, P.R. 2002. The extent to which ATP demand controls the glycolytic flux depends strongly on the organism and conditions for growth. Mol. Biol. Rep. 29, 41-45.
19. Leistritz, F.L., Hodur, N.M. 2008. Biofuels: a major rural economic development opportunity. Biofuels Bioprod. Bioref. 2, 501-504.
20. Leonel, A.J., Alvarez-Leite, J.I. 2012. Butyrate: implications for intestinal function. Curr. Opin. Clin. Nutr. Metab. Care 15, 474-479.
21. Liu, S., Bischoff, K.M., Leathers, T.D., Qureshi, N., Rich, J.O., Hughes, S.R. 2013. Butyric acid from anaerobic fermentation of lignocellulosic biomass hydrolysates by *Clostridium tyributyricum* RPT-4213. Bioresour. Technol. 143, 322-329.
22. Liu, X., Zhu, Y., Yang, S.T. 2006. Construction and characterization of *ack* deleted mutant of *Clostridium tyrobutyricum* for enhanced butyric acid and hydrogen production. Biotechnol. Prog. 22, 1265-1275.

23. Merklein, K., Fong, S.S., Deng, Y.Z. 2014. Production of butyric acid by a cellulolytic actinobacterium *Thermobifida fusca* on cellulose. Biochem. Eng. J. 90, 239-244.

24. Michel-Savin, D., Marchal, R., Vandecasteele, J.P. 1990. Control of the selectivity of butyric acid production and improvement of fermentative performance with *Clostridium tyrobutyricum*. Appl. Microbiol. Biotechnol. 32, 387-392.

25. Ohta, K., Beall, D.S., Mejia, J.P., Shanmugam, K.T., Ingram, L.O. 1991. Genetic improvement of *Escherichia coli* for ethanol production: chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl. Environ. Microbiol. 57, 893-900.

26. Ou, M.S., Mohammed, N., Ingram, L.O., Shanmugam, K.T. 2009. Thermophilic *Bacillus coagulans* requires less cellulases for simultaneous saccharification and fermentation of cellulose to products than mesophilic microbial biocatalysts. Appl. Biochem. Biotechnol. 155, 379-385.

27. Regassa, T.H., Wortmann, C.S. 2014. Sweet sorghum as a bioenergy crop: literature review. Biomass and Bioenergy 64, 348-355.

28. Saballos, A. 2008. Development and utilization of sorghum as a bioenergy crop. in: *Genetic Improvement of Bioenergy Crops*, (Ed.) W. Vermerris, Springer. New York, N.Y., pp. 211-248.

29. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. 2012. Determination of structural carbohydrates and lignin in biomass: laboratory analytical procedure (LAP). NREL/TP-510-42618 http://www.nrel.gov/biomass/analytical_procedures.html.

30. Soh, A.L.A., Ralambotiana, H., Ollivier, B., Prensier, G., Tine, E., Garcia, J.L. 1991. *Clostridium thermopalmarium* Sp. Nov., a moderately thermophilic butyrate-producing bacterium isolated from palm wine in Senegal. Systematic and Applied Microbiology 14, 135-139.

31. Song, H., Eom, M.-H., Lee, S., Lee, J., Cho, J.-H., Seung, D. 2010. Modeling of batch experimental kinetics and application to fed-batch fermentation of *Clostridium tyrobutyricum* for enhanced butyric acid production. Biochem. Eng. J. 53, 71-76.

32. Vandak, D., Telgarsky, M., Sturdik, E. 1995. Influence of growth factor supplements on butyric acid production from sucrose by *Clostridium butyricum*. Folia Microbiol. 40, 669-672.

33. Volker, A.R., Gogerty, D.S., Bartholomay, C., Hennen-Bierwagen, T., Zhu, H., Bobik, T.A. 2014. Fermentative production of short-chain fatty acids in *Escherichia coli*. Microbiology 160, 1513-1522.

34. Wiegel, J., Kuk, S.U., Kohring, G.W. 1989. *Clostridium thermobutyricum* sp. nov., a moderate thermophile isolated from a cellulolytic culture, that produces butyrate as the major product. Int. J. Syst. Bacteriol. 39, 199-204.

35. Zhang, C.H., Ma, Y.J., Yang, F.X., Liu, W., Zhang, Y.D. 2009. Optimization of medium composition for butyric acid production by *Clostridium thermobutyricum* using response surface methodology. Bioresour. Technol. 100, 4284-4288.

Figure Legend

Fig. 1. Specific productivity of butyrate as a function of growth rate of *C. thermobutyricum*.

C. thermobutyricum was cultured in M-LB + glucose (30 $g.L^{-1}$) at 50°C. Solid bars represent the highest calculated specific productivity of butyrate (g butyrate. h^{-1}. g dry cell $weight^{-1}$) and the bars with dotted lines indicate the specific growth rate (h^{-1}).

Fig. 2. Fermentation of glucose by *C. thermobutyricum* at 50°C, pH 7.0.

Glucose (50 $g.L^{-1}$) was fermented in LB medium with $CaCO_3$ (24 $g.L^{-1}$) at 50°C and pH 7.0. See Methods section for other details.

Fig. 3. Fermentation of sweet sorghum juice by *C. thermobutyricum*.

Sweet sorghum juice at 60% (v/v) was fermented at 50°C, pH 7.0. Invertase (9.6 $units.ml^{-1}$) was added before the start of fermentation to hydrolyze sucrose. See text for other details.

Fig. 4. Fermentation of pre-treated sweet sorghum bagasse by *C. thermobutyricum* by L+SScF process.

Sweet sorghum solids (50 g dry $weight.L^{-1}$) in LB-YE medium was liquefied with 20 FPU.(g dry $weight\ of\ bagasse)^{-1}$ of CTec2 cellulase preparation for 6 hours at pH 5.0 and 50°C. The pH of the bagasse slurry was adjusted to 6.5 with KOH and inoculated with an active culture. After 12.5 hours, the pH was lowered to 6.0 to support cellulase activity and maintained by automatic addition of KOH.

Table 1. Effect of increasing glucose concentration on fermentation characteristics of *C. thermobutyricum*

Initial [glucose] ($g.L^{-1}$)	Glucose consumed ($g.L^{-1}$)	[Product] ($g.L^{-1}$)			Ratio ($g.g^{-1}$)		Butyrate yield[a] ($g.g^{-1}$ glucose)	Average volumetric productivity[b]	C-recovery[c]	
		Butyrate	Acetate	Lactate	Acetate / Butyrate	Co-products / Butyrate			Butyrate	Total
50	49.5	24.2	2.0	0.1	0.08	0.09	0.49	0.32	1.00	1.06
100	76.5	33.9	2.6	2.3	0.08	0.14	0.44	0.28	0.91	0.96
150	120.6	44.5	9.0	3.6	0.20	0.28	0.37	0.62	0.75	0.90

Fermentation data with an initial glucose concentration of 50 $g.L^{-1}$ are from Fig. 2 and included for comparison. Other fermentations were in LB-YE medium with betaine (1 mM) and $CaCO_3$ (24 $g.L^{-1}$). All the data are from the end of fermentations (120-128 hours) and the results from a typical experiment are presented.

[a]Theoretical butyrate yield from glucose is 0.49.

[b]Average volumetric productivity of butyrate was calculated during the first 48 hours of fermentation when 60% of the sugar was fermented and is presented as $g.L^{-1}\ h^{-1}$.

[c]C-recovery represents the fraction of fermented glucose in either butyrate or in total fermentation products.

Table 2. Effect of cellulase concentration and liquefaction time on the amount of sugar released from pre-treated sweet sorghum bagasse and SScF to butyric acid by *C. thermobutyricum*

	[Glucose][a] ($g.L^{-1}$)	Glucose yield (%)[b]	[Xylose][a] ($g.L^{-1}$)	[Butyrate][c] ($g.L^{-1}$)	Butyrate yield[d] ($g.g^{-1}$ sugars)
Cellulase concentration (FPU. g^{-1} bagasse dry wt)					
10	9.5	38	6.9	11.7	0.29
15	11.9	48	7.2	15.0	0.37
20	13.7	55	8.0	16.3	0.41
25	16.4	66	8.6	16.3	0.41
Liquefaction time at 20 FPU.g^{-1} bagasse dry wt					
3 h	11.5	47	5.3	9.7	0.24
6 h	13.5	54	8.1	15.4	0.38
24 h	20.5	83	10.2	17.6	0.44

[a]The amount of sugars released after liquefaction from 50 $g.L^{-1}$ (dry weight) of acid-treated sweet sorghum bagasse. For the experiments with different cellulase concentration, the liquefaction time was 6 hours. Results from a typical experiment are presented.

[b]Glucose yield represents the amount of glucose released from the cellulose in bagasse.

[c]The amount of butyrate in the broth after 72 hours of fermentation at 50°C.

[d]Butyrate yield was calculated as g butyrate produced per g of total calculated amount of sugars from the carbohydrates (cellulose + hemicellulose) in sweet sorghum bagasse. Xylose was used as the sugar in the hemicellulose for this calculation.

Table 3. Butyric acid production characteristics of select organisms in batch fermentation

Organism	Butyrate titer ($g.L^{-1}$)	Productivity[a] ($g.L^{-1}.h^{-1}$)	Yield[b] ($g.g^{-1}$)	Acetate/butyrate ($g.g^{-1}$)	Reference
C. acetobutylicum ATCC824 (*ctf pta, buk,adhE1)*	32.5	0.89	0.39	0.032	(Jang et al., 2014)
C. butyricum S-21	45.0	0.90	0.45	0.10	(Vandak et al., 1995)
C. butyricum ZJUCB	12.3	0.38	NA	0.42	(He et al., 2005)
C. tyrobutyricum CIP I-776	45.0	0.32	0.34	0.33	(Fayolle et al., 1990)
C. tyrobutyricum CNRZ 596	44.0	0.59	0.38	0.19	(Michel-Savin et al., 1990)
C. tyrobutyricum ATCC25755	20.0	NA	0.34	0.22	(Liu et al., 2006)
C. tyrobutyricum ATCC25755 *(ack)*	41.7	NA	0.42	0.18	(Liu et al., 2006)
C. tyrobutyricum ATCC25755 (glucose, 150 $g.L^{-1}$)	62.5	0.78	0.47	NA	(Song et al., 2010)
C .thermobutyricum (Glucose + acetate) DSM4928	15.3	NA	NA	NA	(Canganella et al., 2002)
C .thermobutyricum (Glucose + acetate) DSM4928	12.1	NA	NA	NA	(Zhang et al., 2009)
Thermobifida fusca (cellulose) muS	2.0	NA	NA	NA	(Merklein et al., 2014)
C. thermobutyricum DSM4928 (glucose, 100 g/L)[c]	34.0	0.32	0.44	0.11	This study
C. thermobutyricum DSM4928 (glucose, 150 g/L)[c]	44.5	0.62	0.39	0.39	This study
C. thermobutyricum DSM4928 (sorghum juice)[c]	44.0	1.45 (0.9)[d]	0.42	0.21	This study

[a]Productivity is gram butyrate produced $.L^{-1}.h^{-1}$.

[b]Yield is the amount of butyrate produced per g of sugar fermented.

[c] *C. thermobutyricum* fermentations were at 50°C, compared to other reported fermentations at 37°C. See Table 1, Fig. 2 and Fig. 3 for details on *C. thermobutyricum* fermentation.

[d]The highest calculated volumetric productivity for this feedstock and in parenthesis, the average volumetric productivity over a 48 hour period are listed.

NA-data not available.

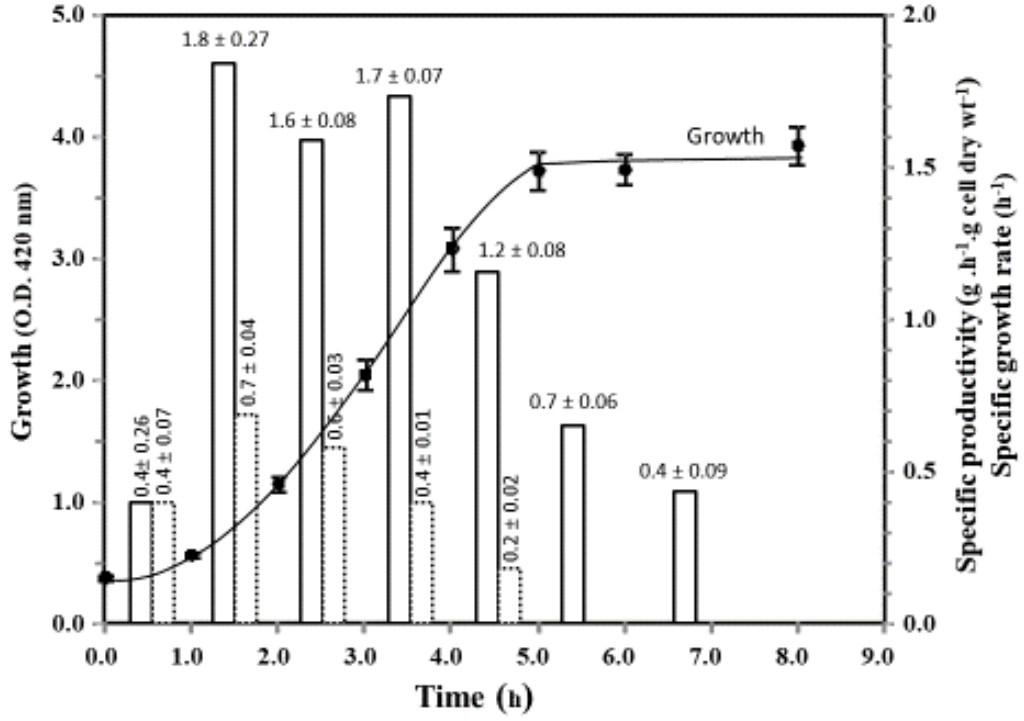
Growth (O.D. 420 nm)
Specific productivity (g .h-1.g cell dry wt-1)
Specific growth rate (h-1)
Time (h)
Growth
1.8 ± 0.27
1.7 ± 0.07
1.6 ± 0.08
1.2 ± 0.08
0.7 ± 0.06
0.4 ± 0.09
0.4± 0.26
0.4 ± 0.07
0.7 ± 0.04
0.6 ± 0.03
0.4 ± 0.01
0.2 ± 0.02

Fig. 1

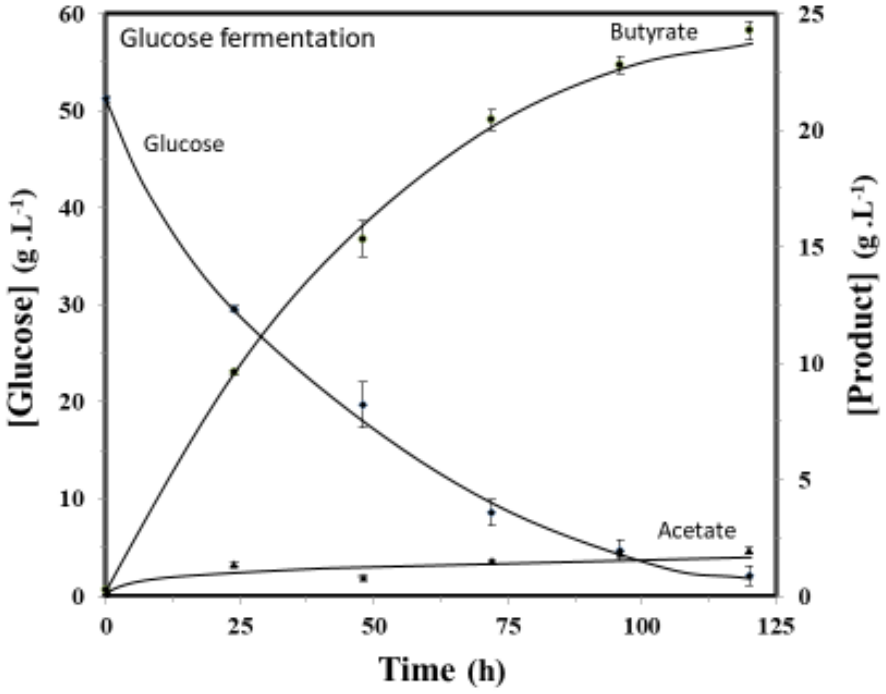
Glucose fermentation
Butyrate
Glucose
Acetate
[Glucose] (g.L-1)
[Product] (g.L-1)
Time (h)
0
10
20
30
40
50
60
0
5
10
15
20
25
0
25
50
75
100
125

Fig. 2

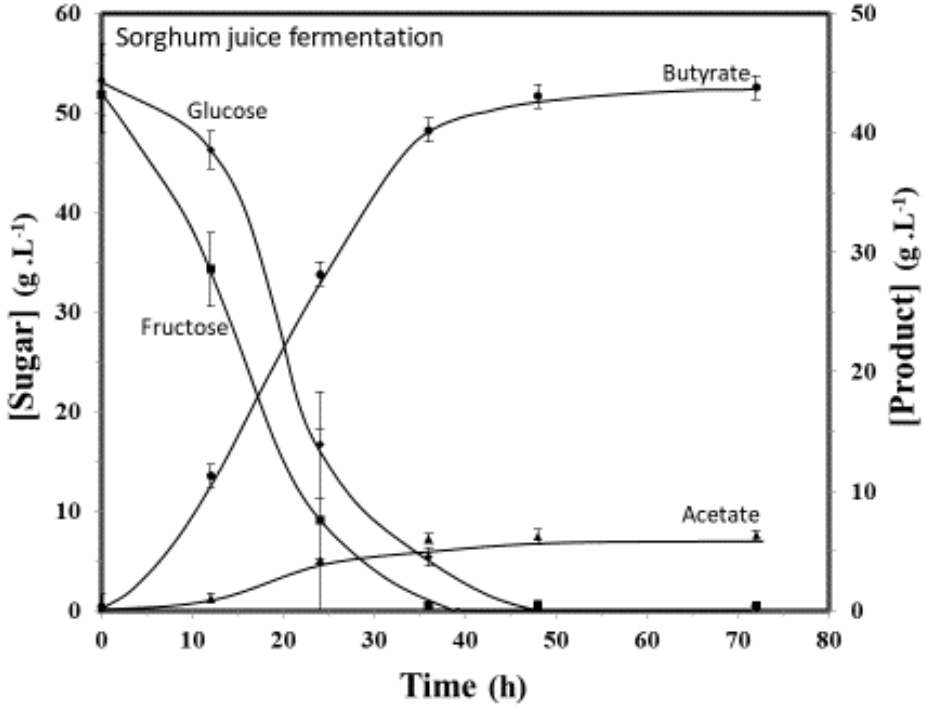
Sorghum juice fermentation
Glucose
Fructose
Butyrate
Acetate
[Sugar] (g.L-1)
[Product] (g.L-1)
Time (h)
0
10
20
30
40
50
60
70
80

Fig. 3

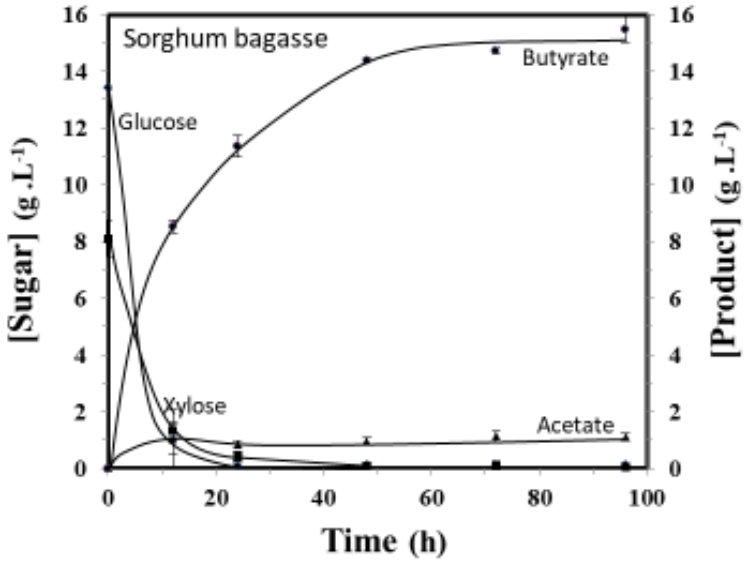
Sorghum bagasse
Butyrate
Glucose
Xylose
Acetate
[Sugar] (g.L-1)
[Product] (g.L-1)
Time (h)
0
2
4
6
8
10
12
14
16
0
20
40
60
80
100

Fig. 4

Supplementary Information

Supplementary Table 1. Growth and butyric acid productivity of *C. thermobutyricum* as compared with *C. butyricum* and *C. acetobutylicum*

Organism	Growth rate (h^{-1})	Cell yield ($g.L^{-1}$)	Specific productivity[a] ($g.\ h^{-1}.gDW^{-1}$)	Volumetric productivity[b] ($g.L^{-1}.h^{-1}$)	Butyrate titer ($g.L^{-1}$)	Total product ($g.L^{-1}$)	Butyrate fraction[c]	C-recovery[d] Butyrate	C-recovery[d] Total products
C. thermobutyricum	0.44 ± 0.02	0.7 ± 0.04	1.8 ± 0.30	0.8 ± 0.03	3.6 ± 0.2	4.4 ± 0.2	0.89 ± 0.01	0.92 ± 0.05	1.05 ± 0.07
C. butyricum	0.80 ± 0.02	5.5 ± 0.80	0.5 ± 0.10	1.4 ± 0.30	6.4 ± 0.5	14.9 ± 0.1	0.60 ± 0.03	0.43 ± 0.03	0.79 ± 0.01
C. acetobutylicum	0.77 ± 0.09	4.7 ± 0.70	0.7 ± 0.20	1.5 ± 0.14	11.4 ± 0.6	19.7 ± 0.9	0.73 ± 0.01	0.65 ± 0.02	0.84 ± 0.03

LB medium supplemented with RCM (10%, v/v) and glucose (32.4 $g.L^{-1}$) was used in this experiment to support growth of all three strains. *C. thermobutyricum* was cultured at 50°C. *C. butyricum* and *C. acetobutylicum* were grown at 37°C. pH of the cultures during fermentation was maintained at 7.0 by addition of 2N KOH. Specific and volumetric productivity values are the calculated highest values.

[a]Specific productivity: gram butyrate $.h^{-1}$. g dry cell $weight^{-1}$

[b]Volumetric productivity: gram butyrate. L^{-1}. h^{-1}

[c] Fraction of fermented glucose in butyrate compared to the total products either at the end of experiment (96 h for *C. thermobutyricum*) or when all the glucose was fermented (*C. butyricum* and *C. acetobutylicum*, 15 and 22 hours, respectively). The major co-products were acetate and lactate for *C. acetobutylicum* (4.4 ± 0.1 $g.L^{-1}$, 3,9 ± 0.3 $g.L^{-1}$, respectively), and acetate (3.6 ± 0.1 $g.L^{-1}$), lactate (4.9 ± 0.4 $g.L^{-1}$) and formate (7.4 ± 0.4 $g.L^{-1}$) for *C. butyricum*. *C. thermobutyricum* produced less than 1g. L^{-1} each of acetate and lactate.

[d]C-recovery represents the fraction of fermented glucose recovered as butyrate or as total products.

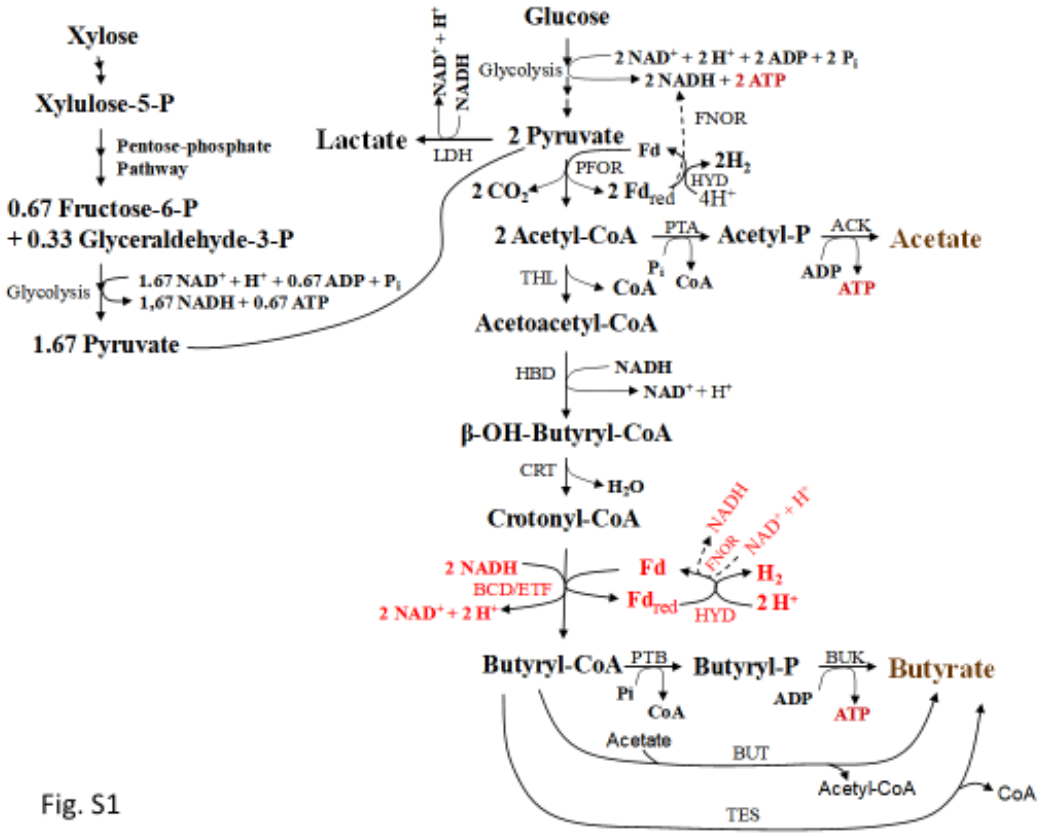

Fig. S1

Fig. S1. Fermentation pathway of *Clostridium* spp. to butyric acid.

ACK, acetate kinase; BCD/ETF, butyryl-CoA dehydrogenase/electron transfer complex; BUK, butyrate kinase; BUT, butyryl-CoA acetate CoA transferase; CRT, crotonase; FNOR, ferredoxin NAD^+ oxidoreductase; HBD, hydroxybutyryl-CoA dehydrogenase; HYD, hydrogenase; LDH, lactate dehydrogenase; PFOR, pyruvate ferredoxin oxidoreductase; PTA, phosphotransacetylase; PTB, phosphate butyryltransferase; TES, thioesterase; THL, thiolase. Pi, phosphate; CoA, coenzyme A; Fd and Fd_{red}, represent oxidized and reduced forms of ferredoxin, respectively. An unknown fraction of reduced ferredoxin is used to produce NADH by FNOR and this ambiguity is represented by dashed line arrows. The reduction of crotonyl-CoA to butyryl-CoA, a two electron (and $2H^+$) step, uses two NADHs due to coupling of crotonyl-CoA reduction by the

BCD/ETF with reduction of ferredoxin (Li et al., 2008). Some of the reductant in the reduced ferredoxin may be recovered as NADH using FNOR while the remainder is released as H_2. The reported fermentation balance of 2.5 H_2 per glucose fermented is in agreement with these observations (Jones & Woods, 1986).

1. Jones, D.T., Woods, D.R. 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. 50, 484-524.

2. Li, F., Hinderberger, J., Seedorf, H., Zhang, J., Buckel, W., Thauer, R.K. 2008. Coupled ferredoxin and crotonyl coenzyme A (CoA) reduction with NADH catalyzed by the butyryl-CoA dehydrogenase/Etf complex from *Clostridium kluyveri*. J. Bacteriol. 190, 843-850.

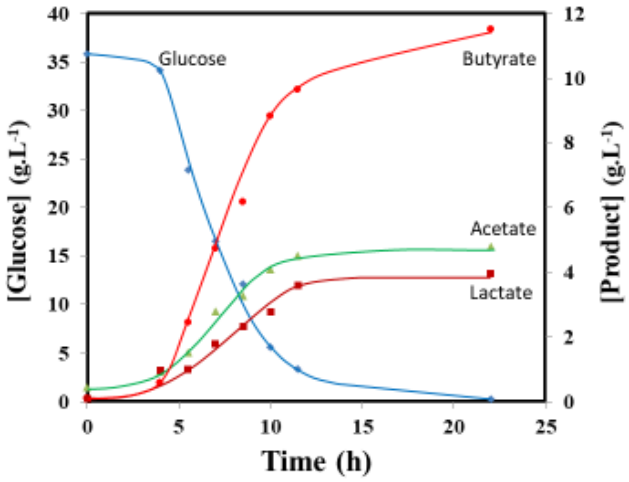

Fig. S2A. Fermentation profile of *C. acetobutylicum* at pH 7.0.

C. acetobutylicum was cultured in M-LB medium with 35 g.L^{-1} of glucose at 37°C (pH 7.0). See text for other details. Average values from three experiments are presented

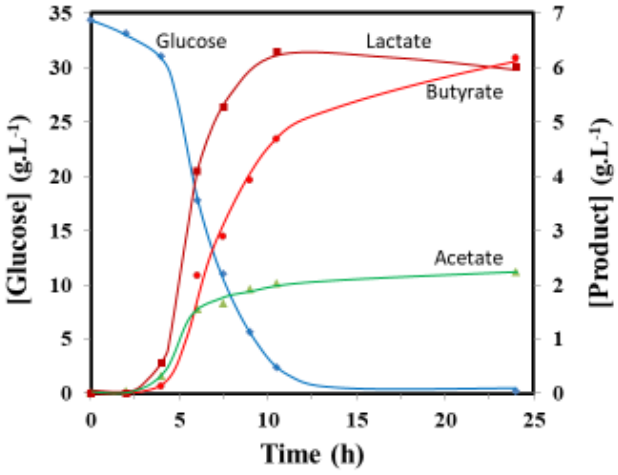

Fig. S2B. Fermentation profile of *C. butyricum* at pH 7.0.

C. butyricum was cultured in M-LB medium with 35 g.L^{-1} of glucose at 37°C (pH 7.0). See text for other details. Average values from three experiments are presented

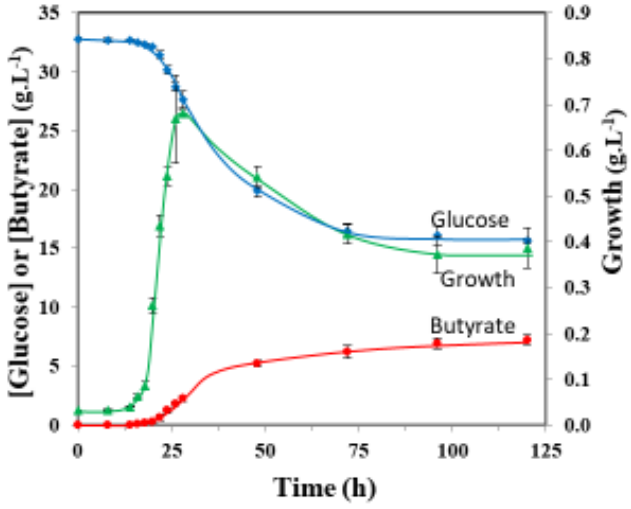

Fig. S3. Fermentation profile of *C. thermobutyricum* at 50°C, in LB medium.

LB medium was supplemented with 32 g.L-1 of glucose.
Culture pH was maintained at 7.0 by automatic addition of 2N KOH. See text for other details.

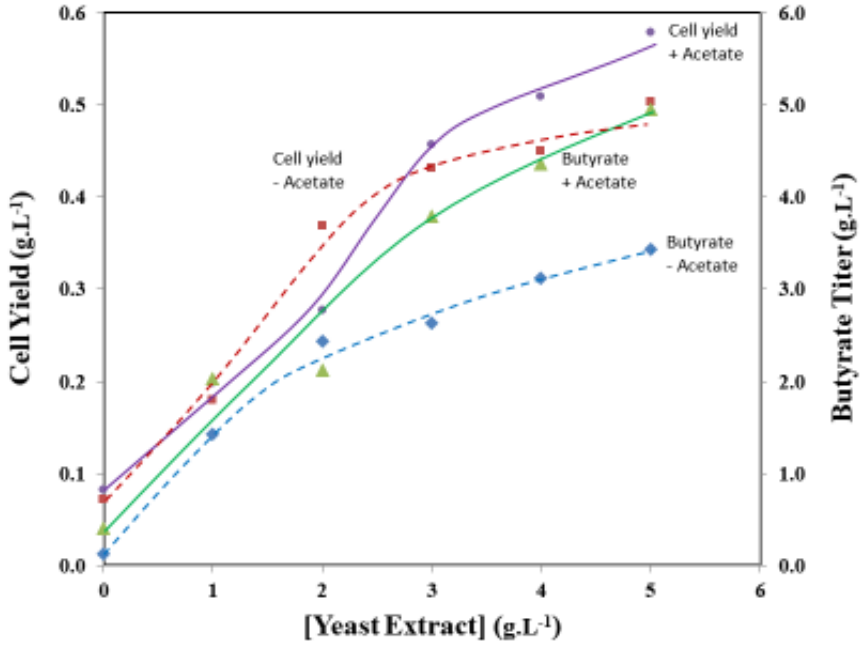

Fig. S4. Cell yield and butyrate titer of *C. thermobutyricum* grown in mineral salts medium With various amounts of yeast extract

The minimal medium contained in $g.L^{-1}$, $(NH_4)_2SO_4$ (2), NaCl (1), KH_2PO_4 (0.75), K_2HPO_4 (0.75), Cysteine (0.5), $MgSO_4 .7H_20$ (0.2), $MnSO_4.H_20$ (0.01), $FeSO_4.7H_20$ (0.01), sodium acetate (2) and Glucose (30) (Sivagnanam et al. 2011 Proteome Science, 9:66). Indicated amount of Yeast extract was added to the medium. Fermentations were at pH 7.0 at 50°C.

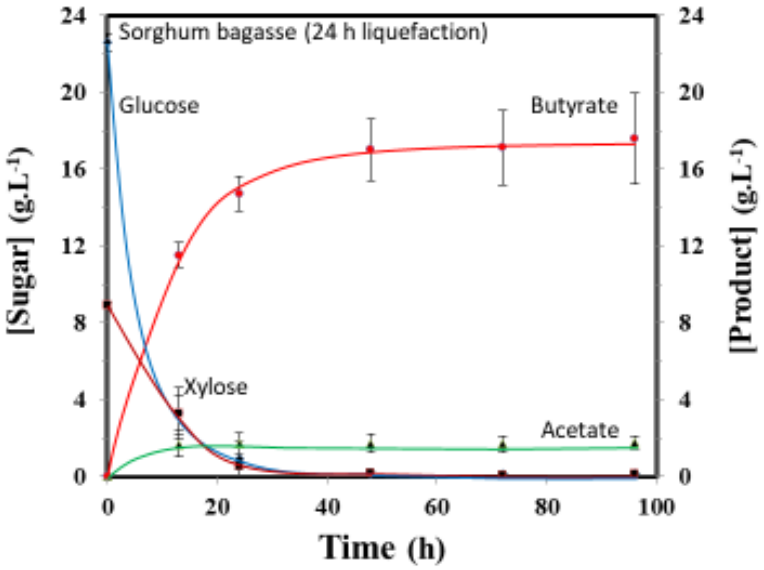

Fig. S5. Fermentation profile of *C. thermobutyricum* on sweet sorghum bagasse after Pretreatment and liquefaction with fungal cellulases for 24 hours.

Sweet sorghum solids (50 g dry weight.L^{-1}) in LB-YE medium was liquefied with 20 FPU.(g dry weight of bagasse)$^{-1}$ of CTec2 cellulase preparation for 24 h at pH 5.0 and 50°C. The pH of the bagasse slurry was adjusted to 6.5 with KOH and inoculated with an active culture. After 12.5 hours, the pH was lowered to 6.0 to support cellulase activity and maintained by automatic addition of KOH.