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Original Research Paper

Direct fermentation of sweet sorghum juice by *Clostridium acetobutylicum* and *Clostridium tetanomorphum* to produce bio-butanol and organic acids

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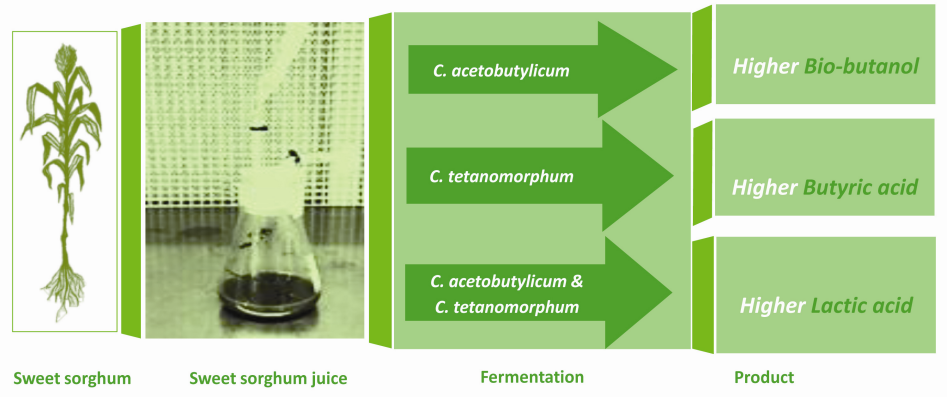
HIGHLIGHTS

- Sweet sorghum juice was fermented with single and mixed cultures of *C. acetobutylicum* and *C. tetanomorphum* at varying inoculum ratios.
- Microbial cell growth as well as ABE and acid concentrations were monitored over 96 h.
- *C. acetobutylicum* produced high concentrations of bio-butanol, whilst co-fermentation showed a significant increase in the yield of organic acids.
- Single and co-culture fermentation of sweet sorghum juice using clostridium species was found a promising method for producing bio-butanol and organic acids.

GRAPHICAL ABSTRACT



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ABSTRACT

Single- and co-culture clostridial fermentation was conducted to obtain organic alcohols and acids from sweet sorghum juice as a low cost feedstock. Different inoculum concentrations of single cultures (3, 5, 10 v/v %) as well as different ratios of *C. acetobutylicum* to *C. tetanomorphum* (3:10, 10:3, 6.5:6.5, 3:3, and 10:10 v/v %, respectively) were utilized for the fermentation. The maximum butanol concentration of 6.49 g/L was obtained after 96 h fermentation with 10 % v/v *C. acetobutylicum* as a single culture. The fermentation with 10% v/v *C. tetanomorphum* resulted in more than 5 g/l butyric acid production. Major organic acid concentration (lactic acid) of 2.7 g/L was produced when an inoculum ratio of 6.5: 6.5 %v/v *C. acetobutylicum* to *C. tetanomorphum* was used.

1. Introduction

Bio-butanol has been considered as a better transportation fuel than bio-ethanol mainly due to its higher number of carbon atoms and consequently higher energy content, its miscibility in diesel, and higher blending capacity. There is a plethora of methods employed by the bio-chemical industries to produce bio-butanol. Over the past decades, direct fermentation of sugars derived from enzymatic conversion of starchy crops or by acid/ enzymatic hydrolysis of lignocellulosic feedstock have dominated (Ranjan et al., 2013; Yang et al., 2014). In the butanol production processes, the production of other byproducts, e.g. acetone, ethanol, and acetic, butyric, and lactic acids, are undesirable; however, these byproducts are considered as main and

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valuable products in a number of processes (Zamani, 2015). Sweet sorghum is one of the common feedstock for bio-butanol production. This is ascribed to the fact that it is grown in diverse temperature climates in both dry and wet areas (Goshadrou and Karimi, 2010). However, the application of this plant as biofuel feedstock has also been the subject of the famous food *vs.* fuel debate.

To avoid such concerns, research has deviated from the starchy biomass to non-edible parts of the plant i.e. the lignocellulosic biomass which could contribute to improving the socio-economic conditions of sweet sorghum as a dedicated energy crop.

Fresh sweet sorghum comprises of sugars, such as; sucrose, glucose and fructose that can be extracted from the stalks. According to Datta Mazumdar et al. (2012), Sobrinho et al. (2011) and Kundiyana et al. (2010), high quality sweet sorghum juice contains approximately 14-22 °Bx, but the values could vary depending on the soil origin of the plant. These sugars can be directly fermented using *Clostridium* spp. to produce acetone, butanol and ethanol (ABE). ABE fermentation involves two stages, namely; i) exponential growth stage (acidogenesis) and, ii) stationary stage (solventogenesis). During the acidogenesis, mainly acetic and butyric acids are produced while during the latter ABE solvents are generated (Borner et al., 2014).

Improving the cost effectiveness aspects of bio-butanol production has been widely investigated. Several studies have strived to use different *Clostridium* spp. for fermentation of sugars into bio-butanol (Ezeji et al., 2004; Kominkiat and Cheirsilp, 2013; Li et al., 2014). In addition, co-culture fermentation has also been explored aiming to enhance ABE production. Examples of such include co-cultivation of *C. beijerinckii* and *C. tyrobutyricum* (Li et al., 2013), *C. beijerinckii* and *C. cellulovorans* (Wen et al., 2014), *C. thermocellum* and *C. saccharoperbutylacetonicum* N1-4 (Nakayama et al., 2011) and *Bacillus subtilis* in a co-culture with *C. butylicum* (Tran et al., 2010).

Nevertheless, the aforementioned studies have only focused on bio-butanol as a product with organic acids as intermediates and few studies have paid attention to the synergetic effect of inoculum concentrations and the ratios at which microorganisms were added, on acid formation during ABE fermentation. To the authors' best of knowledge, the production of organic acids from sweet sorghum juice through co-culture fermentation has not been reported before. Hence, this study was set to investigate bio-butanol and organic acid production from sweet sorghum juice using *C. acetobutylicum* and *C. tetanonomorphum*. Different parameters were taken into account during the course of this study including the type of microorganisms (*C. acetobutylicum* and *C. tetanomorphum*), inoculum ratios and the synergistic effect of the two micro-organisms in co-fermenting the sugars contained in sweet sorghum juice. All final products were analysed and identified by the high performance liquid chromatography (HPLC) technique.

2. Materials and methods

2.1. Biomass

Sweet sorghum stalks were harvested in May 2013 at the test farm of the Agricultural Research Council - Grain Crops Institute of South Africa (ARC-GCI), Potchefstroom (26°43'43.16"S - 27°04'47.71"E). The juice was extracted from the stalks without grains using a mechanical press roller. Approximately 4 L of the juice was extracted from 26.80 kg of fresh stalks and was stored at 4°C until used.

2.2. Long-term storage of the sweet sorghum juice

The prolonged storage protocol of sweet sorghum juice was adopted from Datta Mazumdar et al. (2012). Briefly, the juice was initially filtered using vacuum filtration to remove all the unwanted solid particles. Prior to heating, Brix and pH of the sample were determined using a refractometer and pH meter, respectively. Concentration of the juice was performed at 70 °C for 45 min in a hot plate with continuous stirring. The heating was controlled to allow gradual temperature increases to avoid charring of the sugars. The juice was then cooled down to 40 °C resulting in clarified sweet sorghum juice of 73° Bx. Thereafter, the cooked juice was stored in PET bottles at 4°C till further use. **Figure 1** provides an overview of the sweet sorghum juice processing from harvest to juice concentration.

2.3. Strains and medium

C. *acetobutylicum* ATCC 824 and *C. tetanomorphum* ATCC 49273 were purchased from the American Type Culture Collection (ATCC). The stock cultures were maintained in the form of cell suspensions in 25% (v/v) sterile glycerol at -80°C. The organisms were grown on a Reinforced Clostridial Medium (RCM) for 24-48 h at 37°C before being sub-cultured into Clostridial Growth Medium (CGM) which was used to inoculate sweet sorghum juice for fermentation (**Table 1**). Both media were sterilized at 121°C for 15 min. Short term stock cultures were prepared on Clostridium Growth Agar (CGA) and single colonies were revived every two weeks.

Table 1.
The compositions of the growth media used.

Reinforced Clostridial Medium (RCM)		**Clostridial Growth Medium (CGM)**	
Nutrients	*Composition (g/L)*	*Nutrients*	*Composition (g/L)*
Tryptose	10	NaCl	1
Beef extract	10	$(NH_4)SO_4$	2
Yeast extract	3	Yeast extract	5
Sodium Chloride	5	KH_2PO_4	0.75
Sodium acetate	3	K_2HPO_4	0.75
Cystein hydrochloride	0.5	Asparagine	2
Soluble starch	1	$MgSO_4.7H_2O$	0.70
		$MnSO_4.H_2O$	0.01
		$FeSO_4.7H_2O$	0.01

2.4. Fermentation of sweet sorghum juice

Prior to fermentation, the concentrated sweet sorghum juice was diluted with distilled water to revert to the original Brix index of 14°Bx (16.3 g/L) (Sobrinho et al., 2011). High purity nitrogen (99.9 %) was sparged through the modified flasks before the inoculum addition to remove oxygen from the flasks and to maintain anaerobic conditions. The flask was modified using a tight rubber stopper for sealing the opening, a micro filter outlet, and sampling syringe to avoid closing and opening of the flask. A single colony from the plates was inoculated into 10 mL CGM and was incubated for 12 h. After the incubation the OD_{600} reached 0.798 and 0.810 for *C. tetanomorphum* and *C. acetobutylicum*, respectively. Fermentation was done by transferring certain volumes of the starter-culture (3, 5 or 10 % v/v) into the medium (sweet sorghum juice + nutrients) contained in the modified 250mL Erlenmeyer flasks with a 100 mL working volume.

During fermentation, the temperature was maintained at 37°C, the pH was adjusted between 6-6.5 by addition of NaOH or HCl, and the mixture was agitated at 150 rpm. Fermentation was conducted for 92 h and samples were taken at set time intervals during the experiment. In addition to the single species fermentation, co-culture fermentation of sweet sorghum juice was also conducted. Different inoculum concentration ratios (3:10, 10:3, 6.5:6.5, 3:3, and 10:10% v/v of *C. acetobutylicum* to *C. tetanomorphum*) were used to ferment sweet sorghum juice to investigate the influence of co-culture inocula variables on the product yields. All experiments were conducted in triplicates.

2.5. Analytical techniques used

Cell growth was analysed by measuring OD_{600} using a spectrophotometer (UV 7300, Jenway). The samples were then centrifuged for 5 min and the supernatants were used to determine the concentrations of glucose, fructose and solvent after filtration with a 0.22-0.45 µm syringe filter. The reducing sugars, acids, and solvent concentrations were measured using HPLC with an HPX-87H aminex column at 55°C RID and 30°C column temperature. The

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mobile phase used was 0.005M H_2SO_4 at a flow rate of 0.6 mL.min^{-1} with an injection volume of 5 µL.

3. Results and discussion

Sweet sorghum juice was subjected to fermentation with different cultures using *C. tetanomorphum* and *C. acetobutylicum*. The effect of different parameters on the fermentation of the juice containing 16.31 g/l sugars (6.25 g/l glucose, 0.31 g/l fructose and 9.75 g/l sucrose), was evaluated.

3.1. Fermentation with C. tetanomorphum

Fermentable sugars, i.e., glucose, fructose and sucrose, would normally undergo conversion to a range of intermediates after being consumed by the bacteria. It was observed that after fermentation with *C. tetanomorphum*, the pH of the culture medium decreased from 6.5 to 4.6 as a result of organic acids formation.

Single culture fermentation using different inoculum loadings (3, 5, 10 %v/v) of *C. tetanomorphum* was investigated for the production of bio-butanol. **Figure 1** shows the effect of inoculum concentration on bio-butanol production within 96 h fermentation. Even with 3 % inoculum, the optical density of the culture increased to more than 2 in the first 24 h of fermentation. Butanol production was accompanied by ethanol production, in the absence of acetone formation which is consistent with the findings of Gottwald et al. (1984). Prior to the fermentation of sweet sorghum juice, broth medium containing nutrients was inoculated with the organism and was incubated for 12 h. The initial optical density after 12 h of incubation and at 0 h after the initiation of the iuice fermentation was at 0.798.

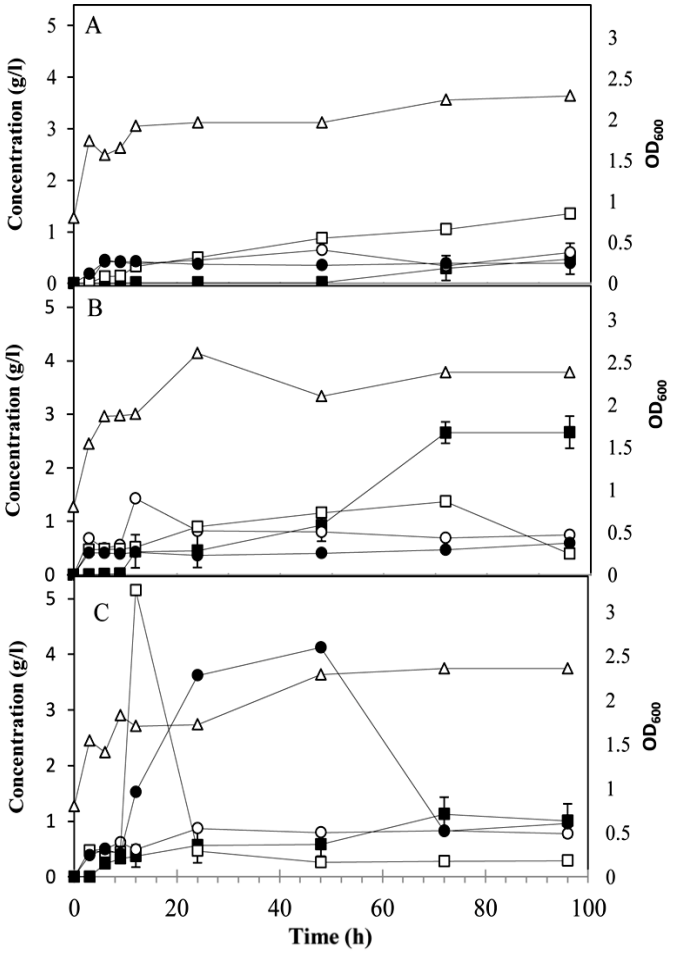

Fig.1. Concentration of butanol (■), ethanol (●), acetic acid (○), and butyric acid (□) as well as optical density (Δ) using 3% (A), 5% (B), and 10% *C. tetanomorphum* inoculum concentrations.

Through the fermentation with *C. tetanomorphum*, organic acids i.e. acetic and butyric acids were initially generated followed by the production of alcohols, i.e., ethanol and butanol. The maximum acetic acid concentrations of 0.64, 1.43, and 0.87 g/l, were obtained in the fermentation with 3, 5, and 10% *C. tetanomorphum* after 48, 12, and 24 h, respectively. In addition, butyric acid concentration increased to 1.35, 1.37, and 5.15 g/L after 96, 72, and 12 h fermentation with 3, 5, and 10% inoculum loadings, respectively.

With 3% inoculation, no detectable level of butanol was produced within the first 48 h of fermentation. The butanol concentration of less than 0.3 g/l and ethanol concentration of 0.42 g/l were obtained after 72 h fermentation, which were produced mostly during the stationery phase. Organic acids showed a rapid increase after 12 h. Inoculating with 5 %v/v inoculum resulted in obtaining higher butanol and ethanol concentrations. During 96 h fermentation, more than 2.5 g/L butanol and 0.5 g/L ethanol were produced. With increasing the initial inoculation volume to 10% of the medium, the final butanol and ethanol concentration increased to 1.14 and 0.96 g/L, respectively. Using different inoculation regimes, no acetone was produced during the fermentation with *C. tetanomorphum*. Butanol was the main alcohol produced in the fermentation with 5 % inoculation, whereas ethanol production was prevailed when fermentation was conducted with 10 % inoculum.

Optical density of the medium increased within the initial 12 h of the fermentation. Moreover, as a result of developing high concentrations of acids in the fermentation broth, the cell growth gradually decreased (Wu et al., 2013).

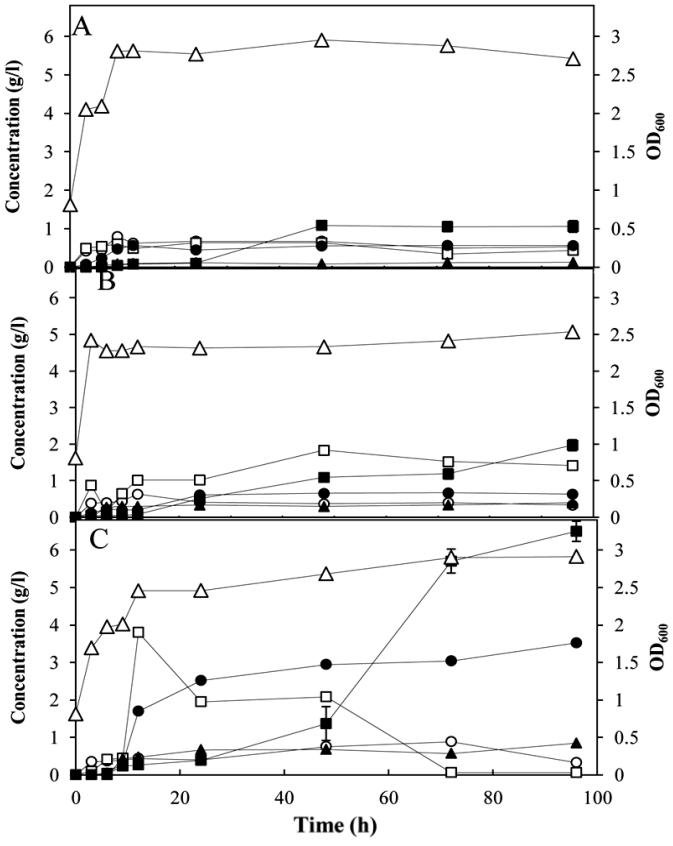

Fig.2. Concentration of acetone (▲), butanol (■), ethanol (●), acetic acid (○), and butyric acid (□) as well as optical density (Δ) using 3% (A), 5% (B), and 10% *C. acetobutylicum* inoculum concentrations.

3.2. Fermentation with C. acetobutylicum

The sweet sorghum juice was subjected to the fermentation with *C. acetobutylicum* with different inoculation ratios of 3, 5, and 10 % v/v for 96 h. The profiles of acids and alcohols as well as their respective concentrations and also the optical density of the medium are shown in **Figure 2** Through the

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first 9 h of fermentation, along with the growth phase of the clostridia, the sugars were utilized and fermented to acetic and butyric acids. After the organic acids concentration increased during the growth phase, alcohols formation was initiated by the cells during their stationary phase. The maximum acetic acid concentration observed for 3, 5 and 10%v/v inoculation were 0.79, 0.63 and 0.89 g/L, respectively. Butyric acid with maximum concentrations of 0.63, 1.83, and 3.30 g/L were produced by using 3, 5 and 10%v/v inoculum, respectively. The pH of the medium decreased from 6.5 to 4.1 through the fermentation by *C. acetobutylicum.* These results were in line with those of Jiang et al. (2014), who reported rapid pH reduction during clostridial fermentation indicating the formation of products.

A gradual increase in the concentrations of both alcohols was generally observed. By increasing the volume of the initial inoculum, the production of acids and alcohols was improved. Using 3 % inoculum of *C. acetobutylicum*, maximum ethanol concentration of 0.56 g/L was obtained within 24 h. In addition, the maximum butanol and acetone concentrations of 1.09 and 0.12 g/L were obtained after 48 h. By increasing the inoculum loading to 5 and 10%, ethanol concentration increased to more than 0.6 and 3.5 g/L, respectively. Moreover, by increasing the inoculum volume to 5%, the final butanol and acetone concentrations were increased to more than 1.9 and 0.3 g/l, respectively. Further increase in the inoculation volume resulted in higher butanol and acetone concentrations of 6.5 and 0.8 g/l, respectively.

Sugar consumption through the fermentation was highly dependent on the culture and its initial concentration. The profiles of sugar concentration during the fermentation with different cultures are presented in **Figure 3**. The sugars contained in the sweet sorghum juice (i.e. 6.25 g/l glucose, 0.31 g/l fructose, and 9.75 g/l sucrose) were consumed by the cultures resulting in 0.3 to 2.5 g/l residual sugar. Through the fermentation with 10% v/v *C. acetobutylicum* more than 98 % of the sugars contained in the juice was consumed.

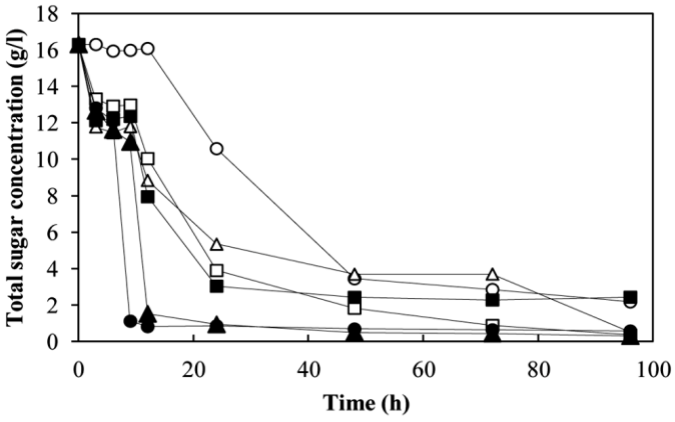

Fig.3. Total concentration of sugars (g/L) during the fermentation using different inoculum volumes 3 % (○), 5% (□), and 10% (Δ) *C. tetanomorphum* and 3% (■), 5 % (●), and 10% (▲) *C. acetobutylicum*.

In comparison with the *C. tetanomorphum* fermentation, the fermentation with *C. acetobutylicum* resulted in higher butanol production. Increasing the inoculum volume in the clostridial fermentation improved the butanol production. The highest butanol concentration obtained was with 10% v/v *C. acetobutylicum* inoculation. On the other hand, through fermentation with 10% v/v *C. tetanomorphum*, the highest concentration of butyric acid was generated. After single culture fermentation, co-culture experiments were conducted using a combination of *C. acetobutylicum* and *C. tetanomorphum*.

3.3. Co-culture fermentation for acid production

C. *acetobutylicum* and *C. tetanomorphum* were inoculated simultaneously into the juice medium under anaerobic conditions. The two strictly anaerobic *C. acetobutylicum* ATCC 824 and *C. tetanomorphum* ATCC 49273 were used in these experiments to investigate their synergistic effect on the product yields. The concentrations of organic acids, i.e., acetic acid, lactic acid, and succinic acid, formed after 96 h fermentation using the co-culture are shown in **Figure 4**. As shown, different amounts of acids were formed depending on the initial inoculation regime of the medium.

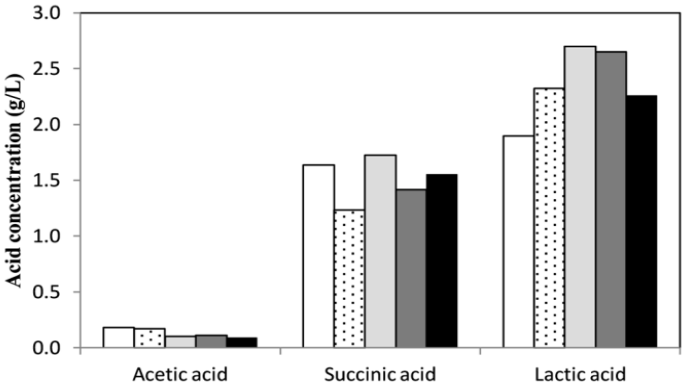

Fig.4. Acid concentrations (g/L) obtained for inoculation with 3:3 (white), 3:10 (dotted withe, 6.5:6.5 (light gray), 10;3 (dark gray), 10:10 %v/v (black) of C.acetobulycum to C. tetanomorphum in 96 hrs.

Fermentation with the co-culture resulted in significantly higher acid formation from the sweet sorghum juice compared to to the pure cultures of *C. acetobutylicum* and *tetanomorphum*. Lactic acid was the most prominent acid produced by all co-cultures. The highest lactic acid concentration of 2.7 g/l was produced after 96 h fermentation with a co-culture of 6.5:6.5 %v/v *C. acetobutylicum* to *C. tetanomorphum*. However, the acetic acid concentration using this treatment was low at 0.1 g/l. The concentrations of the remaining sugars after fermentation with different co-cultures are listed in **Table 2**. Less than 0.2 g/l sugars remained unconverted after 96 h fermentation with the 6.5:6.5 and 10:10 % v/v co-cultures.

Table 2.
Remaining Reducing sugar concentration after co-culture fermentation in 96 h.

***C. acetobutylicum* to *C. tetanomorphum* (% v/v)**	**Residual sugar (g/L)**
3:3	1.44
3:10	1.41
10:3	1.21
6.5: 6.5	0.18
10:10	0.18

Relatively high amounts of organic acids consisting of acetic acid, succinic acid, and lactic acid were obtained through fermentation of sweet sorghum juice with the co-culture of *C. acetobutylicum* and *C. tetanomorphum*. Therefore, co-culture of these strains is recommended for the production of organic acids.

4. Conclusions

The fermentation with single- and co-culture of *C. acetobutylicum and C. tetanomorphum* were evaluated for organic alcohols and acids production from sweet sorghum juice. The results obtained showed that an inoculum loading of 10% v/v *C. acetobutylicum* led to the maximum concentration of 6.49 g/L for butanol. An inoculum loading of 10% v/v *C. tetanomorphum* was found to produce a high acid (butyric acid) concentration of 5.15 g/L. In addition, fermentation using a co-culture approach resulted in the production of lactic acid with a concentration of 2.7 g/L, higher than that obtained by single culture fermentations.

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