# BIO-BUTANOL FERMENTATION USING *CLOSTRIDIUM ACETOBUTYLICUM* AND *CLOSTRIDIUM TETANOMORPHUM* IN MODIFIED BIOREACTOR FLASKS

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ABSTRACT:An alternative new generation biofuel to bio-ethanol has attracted researchers to investigate various approaches for optimization of traditional ABE fermentation to enable bio-butanol to be produced cheaper at an industrial scale. For instance, the use of different or modified *Clostridium* strains to enhance bio-butanol yields has been extensively studied. In this study, single-culture fermentation was investigated usingdifferentinoculum concentrations of *Clostridium acetobutylicum* and*Clostridiumtetanomorphum*(3, 5, 10 % v/v) to ferment sweet sorghum juicewith a Brix index of 14°.Furthermore, co-culture fermentation were conducted using varying ratios of the two *clostridium species*in modified reactor flasks at pH 6-6.5, and incubated at 37ºC while shaking (150rpm) for 96 hrs. Cell growth profileswere monitored using UV spectrophotometry at 600nm during both single and co-culture fermentation. Meanwhile, bio-butanol, residual sugars and by-products were analysed by high-performance liquid chromatography (HPLC).The results demonstrated that butanol and organic acids can be produced from sweet sorghum juice using a single culture, whereas co-culture only produced acids with no organic solvents. The highest butanol concentrationof6.49 g/L was obtained after96hrs using 10 % v/v *C. acetobutylicum*as a single culture. The highest 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* were used and fermented for 96 hrs. Sweet sorghum juicehas potential as a cheaper substrate in producing high bio-butanol yield as a product in the presence of *C.acetobutylicum*. Co-culturescan also be used in fermentation of interest to produce high yields of organic acids.
Keywords: bio-butanol,*Clostridium species*, fermentation, sweet sorghum juice, organic acids

## 1 INTRODUCTION

Bio-butanol is a better transportation fuel than bio-ethanol due to its higher number of carbon atoms, its miscibility in diesel, and higher blending capacity. There are a number of processes that are applied by the bio-chemical industries to produce bio-butanol, but direct fermentation of sugars derived from enzymatic conversion of starchy crops or by acid/ enzymatic hydrolysis of lignocellulosic feedstock [1,2] is preferred.

Sweet sorghum is one in many crops that is characterized by high photosynthetic efficiency and can be grown in diverse temperate climates in both dry and wet areas [3] and this makes it an ideal feedstock for the production of biofuels. The use of non-edible parts ofsweet sorghum as a feedstock can aid in improving the socio-economic conditions of sweet sorghum grain farmers as the production of biofuels from the sugar containing stalks can be used on the farm as fuel or sold into the transportation market for blending.

Fresh sweet sorghum stalks contain monomeric sugars (sucrose, glucose and fructose) that can be extracted from the stalks through pressing. According to [4, 5, 6], sweet sorghum juice contains approximately 15-22°Brix, but the values can vary depending on where the crop was planted. These sugars can be directly fermented using *clostridium species* to produce acetone, butanol and ethanol (ABE).

There are two known stages of ABE fermentation, i.e. an exponential growth stage (acidogenesis) and a stationary stage (solvontogenesis). Acids such as butyric and acetic are formed during acidogenesis and acetone, butanol and ethanol (ABE) are produced during solvontogenesis[7].

A cheaper approach forthe production ofbio-butanol as a fuel is required before it is accessible for widespread commercial utilization.The use of different *clostridium species* has been widely explored for fermentation of sugars [8; 9,10].*Clostridium species* are able to utilize a wide range of monomeric sugar, including carbon 5 and carbon 6 sugars from cellulose and hemicellulose. However, in order to make ABE fermentationviable without competing with food, more efficient and suitable feedstock (lignocellulose, algal biomass) are required and efforts in research for additional substrates should be stepped up. Various studies have reported on the use of organisms for co-culture fermentation. [11] used *C. beijerinckii* and *C. tyrobutyricum*, [12] conducted research on *C. beijerinckii* and *C. cellulovorans*, [13] studied *C. thermocellum* and *C. saccharoperbutylacetonicum* N1-4 and [14] focused on *B. subtilis* in a co-culture with *C. butylicum*. The aforementioned studies only focused on butanol as a product via organic acids as intermediates. Few studies have paid attention to the synergetic effect of inoculum concentrations for the two microorganisms on acid formation during fermentation. To the authors' knowledge, the production of organic acids from sweet sorghum juice through co-culture fermentation has not been reported before.

In the present study, the use of C.acetobutylicumandC. tetanonomorphumfor butanol and organic acid production from sweet sorghum juice is studied. The parameters studied in this study were; the type of microorganisms (*C.acetobutylicum*and*C. tetanomorphum*), inoculum ratios and the synergistic effect of the two micro-organisms to co-ferment the sugar contained insweet sorghum juice.

## 2 MATERIALS AND METHODS

### 2.1 Biomass

Sweet sorghum stalks were harvested in May 2013 at the test farm of ARC-GCI (Agricultural Research Council- Grain Crops Institute of South Africa), Potchefstroom(26°43'43.16"S - 27°04'47.71"E), South Africa. The juice was extracted from the stalks using a mechanical press roller and collected manually using a 10L bucket (See Figure 1). Approximately 4 L of juice was extracted from 26.80kg fresh stems. The juice was transported to the laboratories of the North-West University in buckets and stored at 4ºC until used.

2.2 Long-term storage of the sweet sorghum juice

The prolonged storage of sweet sorghum juice was adopted from [4]. 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 juicewas done for 45 min at 70°Cin a hot plate with continuous stirring. The heating was controlled to allow gradual temperature increases to avoid charring of the sugars. When the juice was cooled down to 40°Cit resulted in clarified sweet sorghum juice of73° Brix index. Thereafter, the cooked juice was stored in PET bottles at 4°C for further use. Figure 1 provides an overview of the sweet sorghum juice processing from harvest to juice concentration.


**Figure 1:**Extraction and processing of sweet sorghum juice

2.3 Strains and medium

C. *acetobutylicum* ATCC 824 and*C. tetanomorphum* ATCC 49273 were purchased from American Type Culture Collection. The stock culture was maintained in the form of a cell suspension in 25% (v/v) sterile glycerol at -80°C. The organisms were grown on a Reinforced ClostridialMedium (RCM)for 24-48 hrs at 37°C before inoculation (see Table I).

**Table I:** Nutrients used for Reinforced Clostridial Medium (Ventura *et al*., 2013)

| Nutrients | Composition (g/L) |
|---|---|
| Tryptose | 10 |
| Beef extract | 10 |
| Yeast extract | 3 |
| Sodium Chloride | 5 |
| Sodium acetate | 3 |
| Cystein hydrochloride | 0.5 |
| Soluble starch | 1 |

The bacteria were then sub-cultured from RCM to Reinforced ClostridialMedium (CGM) for main batch fermentation (see Table II).

**Table II**: Nutrients used for Clostridium Growth Medium[15]

| Nutrients | Composition (g/L) |
|---|---|
| NaCl | 1 |
| $(NH_4)SO_4$ | 2 |
| Yeast extract | 5 |
| $KH_2PO_4$ | 0.75 |
| $K_2HPO_4$ | 0.75 |
| Asparagine | 2 |
| $MgSO_4.7H_2O$ | 0.70 |
| $MnSO_4.H_2O$ | 0.01 |
| $FeSO_4.7H_2O$ | 0.01 |

Clostridialgrowth medium was used as an inoculum to sweet sorghum juice for fermentation. Both media were sterilized at 121°C for 15 min. Short term stock culture was prepared on Clostridium Growth Agar (CGA). Single colonies were revived after every two weeks.

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°Brix (16.31g/L)[5].High purity nitrogen (≥95 %) was sparged through the flasks before the inoculum addition to remove unwanted oxygen in the flasks and maintain anaerobic conditions.A single colony from the plates was inoculated in to 10mL CGM and incubated for 12 hrsand $OD_{600}$reading reached 0.798 and 0.810 for *C. tetanomorphum* and *C. acetobutylicum*, respectively. Fermentation was done by transferring an appropriate volume of the starter-culture (3, 5 or 10 mL) into the medium (sweet sorghum juice + nutrients) contained in a modified 250mL Erlenmeyer flasks with a 100mL working volume (see Figure 2).

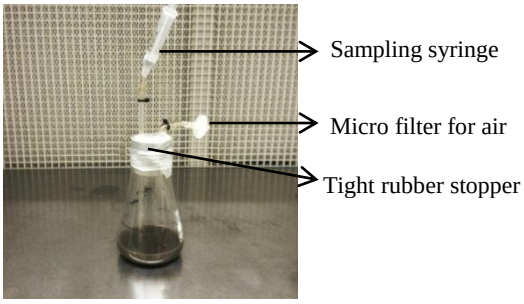


**Figure 2:**Modified reactor flask for fermentation experiments

During fermentation, the temperature was maintained at 37°C, the pH was adjusted to 6.5 by the addition of NaOH or HCl, and the mixture was agitated at 150 rpm. Fermentation was conducted for 92 hrs and samples were taken at set time intervals during the fermentation. Co-culture fermentation of sweet sorghum juice followed after single fermentation. All experiments were conducted in triplicates.

Different inoculum concentration mixtures (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 juiceto investigate the influence of multi-culture inoculavariables on the product yields.

2.5Analytical methods

Cell growth was analysed by measuring $OD_{600}$using a spectrophotometer (UV 7300, Jenway). The samples were then centrifuged at 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 glucose, acids, and solvent concentration were measured using High Performance Liquid Chromatography (HPLC) with an HPX-87H aminex column at 55°C RID and 30°C column temperature. The mobile phase used was 0.005M $H_2SO_4$ at a flow rate of 0.6 $mL.min^{-1}$with aninjection volume of 5µL.

## 3 RESULTS AND DISCUSSION

### 3.1 Characterization of raw material

The initial sugar concentration for all the experiment was 16.31 g/L The concentration and distribution of sugars in the juice is given in Table III.

**Table III** Most prominent sugars concentrationsin sweet sorghum juice used in this study

| Sugars | Concentration (g/L) |
|---|---|
| **Glucose** | 6.25 |
| **Fructose** | 0.31 |
| **Sucrose** | 9.75 |

### 3.2 Changes in pH of fermentation broth during fermentation with *C. tetanomorphum and C. acetobutylicum*

Fermentable sugars (glucose, fructose and sucrose) would normally undergo conversion to a range of intermediates after being consumed by the bacteria. In this study, the pH was monitored to assess the pathway of the fermentation process. During fermentation using *C. tetanomorphum*and*acetobutylicum*, the initial pH of the culture medium dropped sharplyfrom 6.5 to 4.6 and 4.1 for 5% v/v *C. tetanomorphum* and 10%v/v *C.acetobutylicum*, respectively after 96 hrs, which indicates the formation of organic acids. According to [16] rapid pH reduction during clostridial fermentation signals the beginningof product formation and it also gives a clear indication of the metabolic shift in the juice when organic acids are produced.

### 3.3 The effect of *C. tetanomorphum*loading on butanol concentration during fermentation

Single culture fermentation using different inoculum loadings (3, 5, 10%v/v) of *C. tetanomorphum*were investigated for the production of bio-butanol. Figure 3 shows the effect of inoculum concentration of bio-butanol production in 96 hrs of fermentation. The results from acid and solvent analysis showed formation of butanol with an increase in fermentation time. Enhancedcell growth was observed for *C.tetanomorphum*which reached an optical density more than 2 after 24 hrs. Butanol production was accompanied by ethanol production, in the absence of acetone formation which is consistent withfindings from [17]. The ability to produce n-butanol from glucose in large quantities has been found to be an additional characteristic of the strains of this group. Prior to fermentationof sweet sorghum juice, broth with nutrients was inoculated with the organism and incubated for 12 hrs. The initial optical density after 12 hrs of incubation and at 0 hour of juice fermentation was 0.798.

A rapid organic acid production (acetic and butyric acid) was observed before alcohols (ethanol and butanol) were formed. Acetic acid formation reached maximum concentrations of 0.64 (48 hrs), 1.43 (12 hrs), and 0.87 g/L (24 hrs) for 3, 5, and 10%v/v of *C. tetanomorphum*, respectively. Butyric acid concentrations of 1.35 (96 hrs), 1.37 (72 hrs), and 5.15 g/L (12 hrs) were reached 3,5, and 10% v/v *C. tetanomorphum*, respectively. For 3% v/v, butanol concentration only started to increase after 72 hrs to approximately 0.29 g/L, and ethanol reached 0.42 g/L during the stationery phase of the cells. Organic acids showed a rapid increase from 12 hrs. The 5%v/v inoculum produced the highest butanol concentration of 2.66 g/L and 0.59 g/L butanol and ethanol, respectively

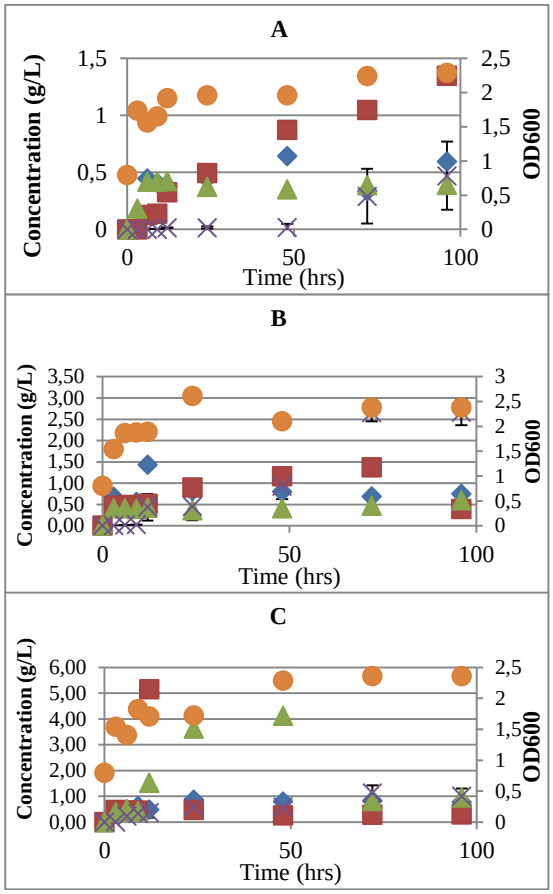


**Figure 3:**Effect of inoculum loading on acid and alcohol concentrations (■-Butyric acid, ♦-Acetic acid, ×-Butanol, ▲-Ethanol, ●-Cell density) for *C.tetanomorphum* inoculum concentrations of 3%v/v **(A),** 5%v/v **(B),** and 10%v/v **(C)**

in 96 hrs of fermentation. Organic acids started increasing after 12 hrs, but dropped sharply after 72 hrs. Optical density increased within 12 hrs and decreased after 24 hrs. This is cumulative accumulation of acids and solvents leading to a decline cell growth [18]. The batch with a loading of 10%v/v inoculum,yielded 1.14 and 0.96 g/L butanol and ethanol, respectively. After 72 hrs, butanol decreased, while optical density showed that the fermentation was inthe stationery phase. It has been shown that the loss of solventogenesis is due to defects in chromosomal regions which are embedded in the solvent genes [19]. There was no acetone produced in any of the different inoculum concentrations.Butanol was the most prominent alcohol produced for an inoculum loading of 5%v/v while ethanol was the most prominent alcohol formed for an inoculum of 10% v/v.

### 3.4 The effect of *C. acetobutylicum*loading on butanol concentration during fermentation

Figure 4 shows the product concentration profiles for organic acids, butanol and ethanol in culture medium containing inoculum ratios (3, 5, and 10% v/v) fermented for 96 hrs. The cells started producing acetic acid by 9 hrs in all cases and a steady increase in the organic acid concentration could be observed. Moreover, this observation of increase in organic acid concentration correlates with the increase in cell growth. After organic acid formation, alcohols concentrations started to increase. The maximum acetic acid concentrations

observed for 3,5 and 10%v/v were 0.79,0.63 and 0.89 g/L, respectively. The highest concentrations of butyric acid were 0.63, 1.83, and 3.30 g/L for 3, 5and 10%v/v, respectively.

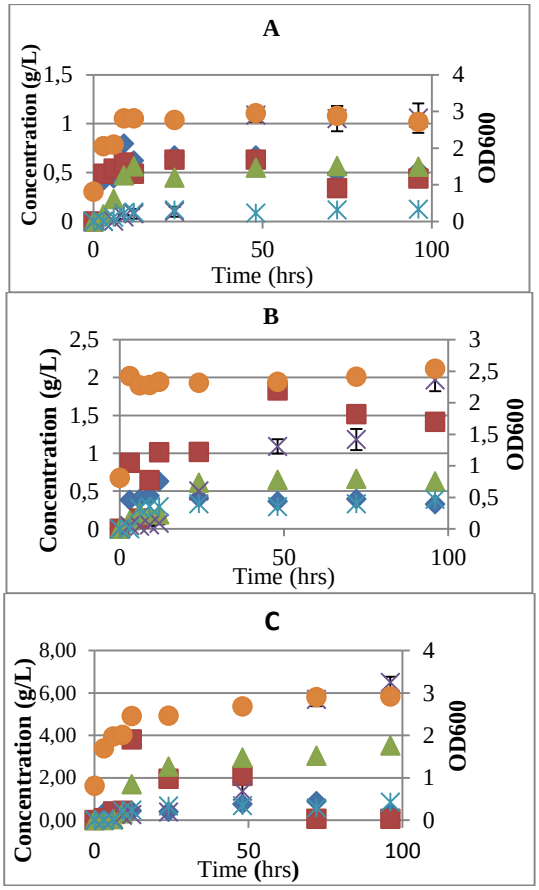


**Figure 4:**Effect of inoculum loading on acid and alcohol concentrations (■-Butyric acid, ♦-Acetic acid, ×-Butanol, ▲-Ethanol, ж-Acetone, and ●-Cell density)for *C.acetobutylicum* inoculum concentrations of 3%v/v **(A),** 5%v/v **(B),** and 10%v/v **(C)**

A steady increase for both alcohols was observed for all inoculum loadings. Ethanol and butanol concentration increased with increase in fermentation time. At an inoculum loading of 3% v/v *C. acetobutylicum* production of ethanol was observed within the first 9 hrs, and a maximum of 0.56g/L ethanol was produced within 24 hrs. An initial increase in butanol and acetone concentration between 20 and 48 hrs to approximately 1.09 and 0.12 g/L respectively were followed by a decrease after 48 hrs. For inoculum loadings of 5 and 10% v/v *C.acetobutlicum*, ethanol concentration increased to approximately 0.66 and 3.52 g/L, respectively. A consistent increase of butanol (1.97 and 6.49 g/L) and acetone (0.39 and 0.85 g/L) with increase in fermentation time and inoculum concentration was observed when using inoculum loadings of 5 and 10% v/v *C. acetobutylicum*, respectively.

It can be concluded that the highest butanol produced was found when 10% v/v *C. acetobutylicum* inoculation was used on sweet sorghum juice. The highest acid concentration formed was butyric acid when using 10% v/v *C. tetanomorphum*. According to the present study, the results show that *C. acetobutylicum*produced the highest butanol concentration, but *C. tetanomorphum* produced the most acids. After single culture fermentation, co-culture experiments were conducted using a combination of *C.acetobutylicum* and *tetanomorphum* to observe the formation of acids comparable to single culture acid production.

3.5 Co-Culture fermentation for acid production

C. *acetobutylicum* and *C.tetanomorphum* were loadedsimultaneouslyinto juice medium contained in the modified flasks and purged with $N_2$ to maintain anaerobic conditions for acid production. 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. Subsequently, acid production was compared to those obtained with single culture fermentation of *C. acetobutylicum*and *C.tetanomorphum*. Figure 5 shows the different acids produced in 96 hrs of fermentation for all the different co-culture loadings investigated in this study.

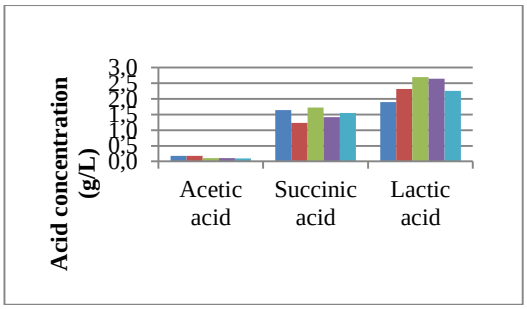


**Figure 5:** Acid concentrations (g/L) obtained for different inoculum combinations (■-3:3, ■-3:10, ■-6.5:6.5, ■-10;3, ■-10:10 %v/v) of *C.acetobulycum* to *C. tetanomorphum*

The results from Figure 5 shows that a combination of the two bacteria produces three (3) acids, i.e. acetic, succinic, and lactic acid. A mixed culture of *C. acetobutylicum* and *tetanomorphum* significantly increased acid production from sweet sorghum juice as compared with a pure culture of *C. acetobutylicum* and *tetanomorphum*.

As shown in Figure 5, lactic acid was produced in the highest concentration (2.56 g/L) after 96 hrs of fermentation using a mixture of 6.5:6.5 %v/v *C.acetobutylicum* to *tetanomorphum*. The acid with the lowest concentration at 6.5:6.5 %v/v *C.acetobutylicum* to *C. tetanomorphum* was acetic acid (0.10 g/L).Lactic acidwas the most prominent acid produced preferentially for all inoculum combinations with small amounts of acetic acid formed. The microorganisms functionedthe best at equalinoculum ratios (6.5:6:5 %v/v) in the juice medium.

There were several beneficial findings observed in the current study, such as high productivity using *C. acetobutylicum* as a single culture and high productivity of lactic acid when co-culture fermentation was used.The acid production performance in co-culture of *C. acetobutylicum*with *C. tetanomorphum*is better than thatseen under single culture fermentation. Therefore, when a desired organic acid has to be produced a co-culture fermentation involving a combination of the two Clostridia species can be used.

## 4 CONCLUSION

Many research studies have focused on the production of bio-butanol using first generation feedstock that is directly in competition with food security. More sustainable feedstock will need to be utilised to achieve a sustainable and viable process that meets the demand for renewable energy. These could include cheap crops residues such as sweet sorghum stem,sugar cane bagasse, and wheat straws. In this study, sugar rich syrup pressed from sweet sorghum stems were used to produced bio-butanol and other useful organic acids through fermentation *using C. acetobutylicum* and *C. tetanomorphum*single and co-cultures. It was demonstrated that an inoculum loading of 10% v/v *C. acetobutylicum*producedthe highest concentration of 6.49 g/L for butanol. An inoculum loading of 10% v/v *C. tetanomorphum*was found toproduce the highest acid concentration (butyric acid) of 5.15 g/L.Co-culturesof the two strainsproduced a highlactic acid concentration(2.7 g/L) which was not generally obtainable using single culture fermentation.The results suggest that when co-culture fermentation involving *C. acetobutylicum* and *C. tetanomorphum*are carried out, some significant synergistic effects were detected that led to a wide range of organic acids being formed, while*C. acetobutylicum*in a single culture is yielded the highest butanol concentration. Additionally, these results provide a basis for hemicellulosic material derived sugars as feedstocks for butanol and organic acid production which would open up several opportunities in biofuels research.

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## 7 LOGO SPACE