Utilization of Mixed Agricultural Waste for Production of Low (C1 To C3) and High Carbon Content (C4 Plus) Alcohols Under Aerobic Conditions

N.Dlangamandla and SKO. Ntwampe*

***Abstract*— Alcohol production using a variety of microbial species has had challenges due to the toxicity and inhibition of alcohols produced on microbial populations during fermentation, which generally results in low yields. This has resulted in research focusing on a variety of strategies to produce alcohols using suitable biocatalysts and efficient downstream processes for alcohol recovery. Engineered strains also add value on the improvement of the alcohol production process. In the present study, a delignification process was used, which consisted of mild acid (H_2SO_4) pretreatment followed by hot water pretreatment. Subsequently, cellulases were used to hydrolyse the pretreated agrowaste, for which the filtrates from each hydrolysis steps were mixed together. A bacterial strain isolated from crude oil was used to produce low (C1 to C3) and high carbon content (C4+) alcohols under aerobic conditions for the benefit of the biorefinery industry.**

Keywords - Agricultural waste, Aerobic, Biorefinery,

I. Introduction

Recent renewable energy studies have focused on biofuels as an alternative source of transportation fuel [1], with bioethanol and biobutanol being valued higher than most alcohols, particularly for the biofuels market. Alcohols such as bioethanol (C2) and biobutanol (C4) have been used as fuel additives, with bioethanol and biobutanol being preferred. For biobutanol production, Acetone–Butanol–Ethanol (ABE) fermentation has been the primary method used to produce biobutanol [2]. Biobutanol is one of the C4+ alcohols which show potential to revolutionize the renewable biofuels industry and reduce the reliance on fossils fuels.

Manuscript received July 19, 2016; revised August 20, 2016. The authors would like to acknowledge funding from the Cape Peninsula University of Technology (CPUT) and University Research Fund (URF RK 16) for financing this project. The financial assistance of the South Africa National Research Foundation (NRF) towards this research is hereby acknowledged

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Furthermore, biobutanol that is produced from agricultural waste (Agro-waste) materials is currently regarded as a source for clean energy that can easily blend with diesel or gasoline [3, 4]. Since, biobutanol has a high energy content and is water resistant, as compared to bioethanol; it can be distributed through the existing fossil fuel pipeline system and can be used as a fuel, without modifying the engines' system. *Clostridium acetobutylicum* has been used as a major strain for biobutanol production from biomass [3-9]. When the *C. acetobutylicum* is used during alcohol production via fermentation, 10 g/L of biobutanol is normally obtained [4]. However, the major challenge of the biobutanol produced from *C. acetobutylicum* is the lower yield during fermentation, which leads to higher cost associated with biobutanol recovery at the end of the process [10]. *C. beijerinckii* has also been used for biobutanol production[5]. Various engineered strains such as *Escherichia coli (E. coli)* have been reported for the improvement of the biobutanol yield during fermentation [6]. The engineered strains are mostly designed to improve the yield of the production and the efficiency[7] using genetic material from *Clostridium* sp. the highest biobutanol produced from a mutant strain was obtained by Chen and Blaschek (1999) in a study for which 21 g/L of biobutanol was achieved. Most biobutanol production systems are operated under anaerobic conditions. Ng *et al*. [9] have recently reported on biobutanol production under aerobic conditions using different *Bacillus* sp. isolated from soil [5]. Additionally, the production of a variety of alcohols can make the biorefinery processes sustainable, as various alcohols can be used for other uses.

This study focused on the production of low (C1 to C3) and high carbon content (C4+) alcohols under aerobic conditions using agricultural waste as the sole carbon source. The alcohol production system was analyzed under different operational conditions to determine favorable conditions for the fermentation process.

II. Materials and Methods

A. Raw materials used for fermentation

Fermentation feedstock, i.e. Agro-waste (Orange Peel, Apple peel, cobs from corn) was collected from a citrus fruit market (Western Cape, Cape Town, South Africa), dried at 80°C and milled to <100µm. additionally, rotting wood was also used as an alternative feedstock.

ISBN: 978-988-14048-2-4
ISSN: 2078-0958 (Print); ISSN: 2078-0966 (Online)

B. Isolation of an alcohol producing strain and identification

A bacterial strain was isolated from crude oil sludge obtained from a well, off the east coast of Africa. The culture was grown on Potato Dextrose Agar (PDA) and incubated at 30°C for 24 h. Thereafter, single colonies were obtained on PDA plates and further transferred to other PDA plates under sterile conditions to purity the strain for identification.

A staining procedure was performed on the isolated strain for morphological assessment, followed by both biochemical tests and DNA extraction including sequencing for identification.

C. Mild acid hydrolysis of the agrowaste

Dilute sulphuric acid pretreament was performed in a batch system [11]. The experiment was performed using different sulphuric acid concentrations, i.e. 1%, 2%, 3% and 4% v/v, which were applied to determine the suitable acid concentration for delignification at various temperatures, i.e. 80°C, 90°C, 100°C, for 2hrs, with the same process being repeated at 121°C for 15 min [12]; whereby, 2g of the agrowaste and the rotting wood were used for the pretreatment process. The suitable process was determined to be at an acid concentration of 1% v/v and a temperature of 121°C. The filtrate obtained from this process was stored at 4°C until further use.

D. Hot water hydrolysis of the agrowaste

The recovered acid hydrolysed biomass (agrowaste) was further treated with hot water at high temperature (121°C) using an autoclave for 15 min in 250 mL of Schott bottles, where 100 mL of distilled water was added. Most studies have shown hot water treatment to be effective at temperature between 120 to 200°C [12]. The filtrate obtained from this process was stored at 4°C until further use.

E. Enzymatic hydolysis of the agrowaste

After the hot water hydrolysis process, the agrowaste was further treated with cellulases (5 mg enzyme per g agrowaste in 50 mL of sterile distilled water). The mixture was then heated to 55°C for 72 h at pH 4, a pH attained using sodium acetate buffer. Thereafter, the filtrate obtained from this process was stored at 4°C until further use.

F. Medium and inoculum preparation

The filtrate from acid, hot water and enzyme hydrolysis were mixed in equal proportions subsequent to quantifying the total reducing sugars. A supplementation media containing: 0.3 g/L $MgSO_4.7H_2O$; 0.5 g/L KH_2PO_4; 0.01 g/L $FeSO_4.7H_2O$; 3 g/L $CaCO_3$; 3 g/L ammonium acetate; 5 g/L yeast extract; 6 g/L Tryptone; 0.5 g/L Cysteine HCl in sterile distilled water, was added to the mixture of the filtrates, i.e. 50 mL of the filtrates was added to 100 mL of the supplementation media. The medium was adjusted to pH 6.5 using a phosphate buffer.

The inoculum was harvested from agar plates using 5 to 10 mL of sterile distilled water, and transferred directly into each fermentation flask.

G. Fermentation process

The fermentation was conducted at 30°C in 250 mL flasks, containing 100 mL of the fermentation medium. The experiments were run for 7 days in a shaking incubator (120 rpm), while the samples were withdrawn every 24 h for analyses.

H. Analytical methods

The reducing sugars from pretreatment were analyzed using Dinitrosalicylic acid (DNS) method using a spectrophotometer at 575 nm.

Prior to alcohol analysis, the broth was distilled for 30 min, using a bench scale distillation system, with the distillate being used for alcohol analysis.

Preliminary alcohol tests were done using Jones and Lucas method. An additional test for further quantification of the presence of alcohols was done using gas chromatography (GC-FID). Nitrogen was used as a carrier gas (1.5 mL/min). The column used was a packed Q column. The oven temperature was initially held at 100 °C for 1 min, which was increased at 15 °C/min to 250 °C, and held for 3 min. A volume, i.e. 10µL, of the distillate, was used for the GC-FID.

III. Results

In this study, pre-selected agro waste were pre-treated with a three stage pretreatment (i.e. acidic, hot water and enzymatic) recovering the filtrate at each stage. The results for total reducing sugar analysis are illustrated below in Figure 1, where the residual sugars concentration of orange peel, apple peel, corn cob, African potato and rotting wood, are highlighted. The highest achievable TRS was 3.9 g/L, for the filtrate obtained from a combined agro-waste mixture.

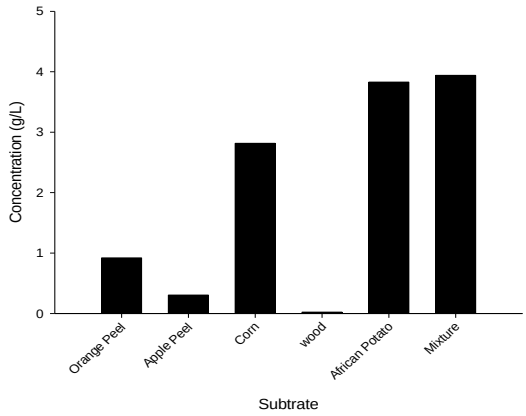


Figure 1: Total reducing sugar (TRS) concentration from agro-waste treatment process

The results obtained at the end of the experiment showed that the fermentation with the mixed agro-waste, performed marginally better , than those with individual feed stock pretreated mixed agro-waste in combination with the isolated strain has an ability to produce different alcohols under aerobic conditions. Different concentrations of alcohols were obtained.

A. Enhancement of alcohol production

To improve alcohol production, the supplementation medium was added to the pretreated mixed agro-waste. Therefore, the addition of the supplementation medium to the fermentation culminated increased alcohol concentration yield. This observation was obtained by comparing fermentations that contained pretreated mixed agro-waste only, with fermentations which contained the supplementation media. Further analyses for this study are currently underway to validate other parameters that can improve the process.

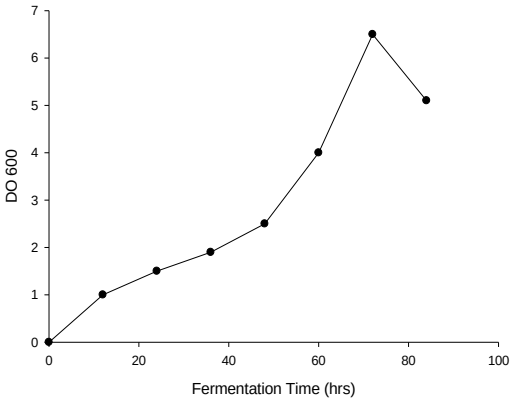
ISBN: 978-988-14048-2-4
ISSN: 2078-0958 (Print); ISSN: 2078-0966 (Online)

TABLE I
PREMININARY ALCOHOL PRODUCTION USING ISOLATE FROM CRUDE OIL

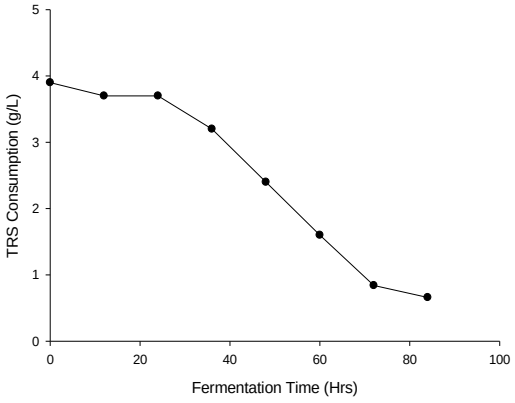
Substrate	C1 to C3	C4 +
Orange Peel	+	+
Apple Peel	+	+
Corn Cob	+	+
Wood	+	+
African Potato	+	+
Mixture of Agro-waste	+	+

Positive results obtained after fermentation
+ Presence of alcohol
– Absence of alcohol

After the fermentation process has been verifying for alcohol production the cell density was measured. Where, the DO_{600} of 6.5+ was obtained in 72 h as shown in fig 2a. Fig.2b shows the TRS consumption during fermentation and was obtained after 84 h at a TRS of 0.66 g/L. Similar activity of this bacterial under aerobic conditions, was observed into other strains that are operating under anaerobic conditions. This strain has ability to utilize sugars during fermentation.



a)



b)

Fig.2 a) Illustration of bacterial growth, while b) show the TRS consumption during fermentation using mixed agro-waste

Table 2, below show the alcohols produce during production. The C_1, C_3 and C+ alcohols that were produce were acetone, ethanol, methanol biobutanol, pentanol and the other group of alcohols that we unidentified by GC standard that was prepare for analysis. Large amount of bioethanol was produced compare to *Clostridia* species. Low amount of the other alcohols were obtained. The results obtained in this study show that the alcohols can be produced under optimized aerobic conditions. Proper optimize is required for further studies under this conditions.

TABLE II
CONCENTRATION OF THE ALCOHOLS THAT WERE PRODUCED

Alcohols	Concentration (g/L)
Acetone	0.13
Ethanol	3.69
Propanol	0.24
Butanol	1.18.
Pentanol	0.34
+	-

+ stand for the alcohol that were unidentified

IV. Conclusion

In this study, a crude oil isolate has been used to ferment agro-waste with medium supplementation under aerobic conditions to produce C1 to C3 and C4+ alcohols. The TRS consumed nearly zero after 84 hrs. High concentration of ethanol was obtained. While different alcohols were produced during the process. The study show that the strain has a potential to produces different type of alcohols. Currently, further studies are still in progress, which include the optimization of the process being developed.

Acknowledgement

The authors would like to acknowledge the support of the Bioresource Engineering Research Group (BioERG) team for their help in the project. The assistance of Honeil Meyo and Clement Utomi is hereby acknowledged.

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ISBN: 978-988-14048-2-4
ISSN: 2078-0958 (Print); ISSN: 2078-0966 (Online)