



Biobutanol Production from Pineapple Peels Waste using Single and Co-Culture of *Clostridium acetobutylicum* and *Clostridium beijerinckii* Isolated from Keffi Metropolis

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MDM and JOI designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript.

Authors IKE and RHA managed the analyses of the study. Author JO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study investigated the production of biobutanol from pineapple peels using single and co-cultures of locally isolated strains of *Clostridium acetobutylicum* and *Clostridium beijerinckii*.

Methodology: Soil samples were collected from ten different areas in Keffi. *Clostridium* species were isolated using standard methods and identified using standard molecular techniques involving the use of 16S rRNA and compared with type strains.

Results: The yield of biobutanol in this study ranged from 1.2±0.20g/L to 5.6±0.43g/L in single culture fermentation broth and 5.8±0.37g/L to 6.5±0.51g/L in co-culture fermentation broth.

Conclusion: It was observed that in single culture and co-culture, the fermentation broth of *Clostridium acetobutylicum* (SAA1) produced the highest yield of biobutanol in comparison with the yield of other isolates.

Keywords: Biofuel; fermentation; renewable biomass; soil.

1. INTRODUCTION

Butanol is an important industrial and chemical feedstock and it also has potential for use as biofuel. The realization that fossil fuels are a finite resource, the interest about energy security and the cost of crude oil which is on the increase have led to renewed focus on biofuels as a sustainable fuel resource [1]. The use of biofuels is also seen to have advantages in countering further increases in carbon dioxide emissions, these dangerous emissions however could be counterbalanced by using renewable biomass for biofuel production [2]. Biobutanol is already being blended into gasoline and diesel in many countries and these countries have set targets for increasing the biofuel content of transportation fuels [1].

Butanol (butyl alcohol or 1-butanol), sometimes also called biobutanol when produced biologically, is a four-carbon primary alcohol having the molecular formula, C_4H_9OH . It is a colourless, flammable alcohol. Studies by Wladyslaw et al [3] show that the use of butanol as an alternative source of fuel is useful.

Butanol can be obtained using several chemical technologies which include Oxo-synthesis and Aldol condensation. Early industrial production of biobutanol was based on fermentation by the bacterium *Clostridium acetobutylicum* which ferments carbohydrates, and produces mainly butanol, ethanol and acetone [1]. This process occurs under anaerobic conditions, and the butanol produced is called biobutanol [4]. This type of fermentation is called ABE (acetone-butanol-ethanol), as a result of the names of the main products of this process, which are in a typical ratio of 3:6:1. Butanol can therefore be produced from renewable resources by conventional Acetone-Butanol-Ethanol (ABE) fermentation [5]. However, usually less than 2% (w/v) butanol can be obtained in ABE fermentation broth due to the strong cytotoxicity of butanol [6].

Several cellulolytic ABE-producing *Clostridium* species have been isolated, identified, and

characterized, they have been found to produce acetone, butanol, and/or ethanol from agricultural wastes [7]. ABE-producing *Clostridium* species can use many types of carbon sources such as starch, sucrose, glucose, lactose, xylose, xylan, and glycerol, this is due to the fact that they possess a broad substrate utilization ability [8]. There are many reports on ABE fermentation by different strains using various biomass substrates such as agricultural waste, domestic waste, hardwood, corn fibre, palm oil waste, whey, excess sludge [8]. These may help reduce fermentation substrate costs.

Pineapple (*Ananas comosus*) was initially eaten only as a fresh fruit, people obtain the pineapple, remove the peel, consume the inner fleshy part and discard the peels. With the help of the ever-developing technology and due to researches, the fruit is now prepared and consumed in different forms such as pineapple juices, jams, syrups and chunks [9]. There are lots of unused excess parts of the pineapple, specifically the peelings. They are considered as waste and add up to the country's total solid waste count. As a solution to this problem, researchers come up with studies about bioconversion of these waste products where these wastes are used as raw material in the manufacturing of other products including biobutanol.

In the present study, the aim is to produce biobutanol from pineapple peels using co-cultures of locally isolated strains of *Clostridium acetobutylicum* and *Clostridium beijerinckii*.

2. MATERIALS AND METHODS

2.1 Study Area

This study was carried out in Keffi Local Government Area (LGA), Nasarawa State, Nigeria. Keffi is approximately 68km away from the Federal Capital Territory (FCT), Abuja and 128km away from Lafia, the capital town of Nasarawa State. Keffi is located at longitude 8°5'E along the Greenwich Meridian, latitude 7°5'N along the equator and at 850m above sea level [10].

2.2 Sample Collection

Ten soil samples were randomly collected at a depth of 10-15 cm, from ten different locations within Keffi metropolis such as Nasarawa State University Main Campus, Government Residential Area, High Court, Federal Prison Area, Angwan Kwara, Tudun Amama, Angwan Woje, Dadin Kowa, Nasarawa State University Pyanku Campus and Anto Bridge, using a clean hand trowel into disposable black polythene bags and transported to the Microbiology Laboratory, Nasarawa State University, Keffi for isolation of bacteria.

2.3 Isolation of *Clostridium* species

The isolation of *Clostridium* species from the ten different locations in Keffi metropolis was carried out as described [11]. Using this method, 1.0 g of the soil sample was suspended in 9.0ml of sterile distilled water and 10-fold dilutions were made, then 0.1ml of the aliquot was spread on petri dishes of freshly prepared Reinforce Clostridial media containing yeast extract 3.0 g/L; lab-lemco powder 10.0 g/L; peptone 10.0g/L; soluble starch 1.0 g/L; glucose 5.0 g/L; cysteine hydrochloride 0.5 g/L; sodium chloride 5.0 g/L, sodium acetate 3.0 g/L and agar 1.0 g/L. Incubated in anaerobic jar at 35°C for 48 hours.

2.4 Identification of *Clostridium* species

The cultural, morphological and biochemical identifications of *Clostridium* species were carried out in accordance with Bergey's Manual of Determinative Bacteriology [12]. Such as gram staining test, endospore staining, catalase test, motility test, indole test.

2.4.1. 16S rRNA Amplification

The 16s rRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 25 microliters for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95°C for 5 minutes; denaturation, 95°C for 30 seconds; annealing, 52°C for 30 seconds; extension, 72°C

for 30 seconds for 35 cycles and final extension, 72°C for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 20 minutes and visualized on a blue light transilluminator.

2.5 Screening *Clostridium* Species for Biobutanol Producing Potentials using Lucas Test

The determination of biobutanol-producing ability of the isolates using Lucas' test was carried out as described by Dhingra & Angrish [13]. In this test, 5 mL of Lucas' reagent at room temperature were added to 1ml of the fermentation broth in a test tube. The tube was stoppered, shaken vigorously, and then the mixture was allowed to stand for 1 hour, the solution remained colourless for more than 1 hour and later became cloudy indicating a positive test for biobutanol production.

2.5.1 Preparation of pineapple peels substrate

The pre-treatment of pineapple peels was carried out as described [14]. The pineapple peels were thoroughly washed in distilled water to remove dust and contaminants and dehydrated by air drying [14]. Pineapple peels substrates were mechanically milled with a blender and sieved with 0.45 mm mesh size, then the fraction that passed through the mesh was collected. The pineapple powder was dried in an oven overnight at 110°C. Enzymatic hydrolysis of the pre-treated pineapple peels was carried out as described [15]. One hundred grammes (100 g) pineapple peels were crushed and dissolved in 1000 ml of distilled water. The content was boiled and filtered. The extract was sterilized at 121°C for fifteen minutes. After sterilization, cellulase from *Aspergillus niger* was added at 6 g/kg to the extracts and incubated at 37° C at a pH of 5.0 for 3 hours for enzymatic hydrolysis process.

2.5.2 Preparation of inoculums

The seed culture was prepared as described [16]. The cultivated organism was inoculated into 10 ml of Nutrient broth containing lab-lemco powder 1.0g/L; yeast extract 2.0g/L; peptone 5.0g/L; sodium chloride 5.0g/L, and incubated in an anaerobic jar at 35° C for 6 hours. The starter culture was carried out as described [16]. The 10 ml seed culture at 6 hours of inoculum were inoculated into 90 ml of freshly prepared nutrient broth and incubated at 35° C for 18 hours.

2.6 Fermentation

The batch fermentation was carried out as described [16]. The 100 ml from the starter culture was inoculated in 900 ml of fermentation media (enzymatically hydrolysed pineapple peels) and were placed in 1000 ml conical flasks anaerobic jars. The fermentation media were later incubated in a MINI/75 Genlab incubator under strict anaerobic and sterile conditions at 35°C for 24 hours, 48 hours and 72 hours.

2.7 Separation and Quantification of Biobutanol

The separation and quantification of biobutanol produced by *Clostridium* species were carried out by Gas Chromatography and Mass Spectrometry (GC & MS) as described [17] One microliter (1.0 µL) of acidified sample was injected into “SHIMAZU GC-14, Gas Chromatograph” equipped with flame-ionization detector.

The column used for the separation of solvent was PEG (2.1 m x3.0 mm). The temperature programming of the column oven was 60°C/min. One hundred and twenty degrees centigrades (120°C), Nitrogen gas (30 ml/minutes) was used as carrier gas. The temperatures of injector and detector were 150°C and 200°C respectively. The Peaks were recorded on “SHIMADZU C-R-4-A, Chromatograph”, and were identified by comparison of the retention times with that of standard mixture. The experiment was carried out in triplicates and the means ± standard deviations of the yield of butanol were recorded.

The standard calibration curve was used in calculating the concentration of biobutanol produced by the various strains of *Clostridium acetobutylicum* and *Clostridium beijerinckii* isolates.

3. RESULTS AND DISCUSSION

Their cultural, morphological and biochemical characteristics of the isolates are given in Table1.

Fig. 1 shows the agarose gel electrophoresis of the amplified 16s rRNA gene bands of the Different species of *Clostridium* Isolated from the soil of Keffi Metropolis.

A total of 14 isolates of *Clostridium* species were obtained from the soil environment of Keffi metropolis Nigeria as indicated in Table 2.

Three (3) of the isolates were identified as *Clostridium acetobutylicum*, one (1) was identified as *Clostridium beijerinckii*, One (1) was identified as *Clostridium celerecrescens*, Two (2) were identified as *Clostridium cylindrosporium*, Four (4) were identified as *Clostridium difficile* and Three (3) were identified as *Clostridium perfringens* as indicated in Table 2.

Table 3 shows the isolates that had the ability to produce biobutanol qualitatively which is in agreement with study reported by [18]. Table 4 shows the mean yields of biobutanol produced in single culture fermentation of pineapple peels.

Table 5 shows the mean yields of biobutanol produced in co-culture fermentation of pineapple peels.



Fig. 1. Agarose gel electrophoresis showing the amplified 16s rRNA gene bands of the Different Species of *Clostridium* Isolated from the Soil of Keffi Metropolis

KEY: 1= *Clostridium* sp., 2= *Clostridium* sp., 3=*Clostridium* sp., 4=negative amplification, 5= *Clostridium* sp., 6= *Clostridium* sp., 7= *Clostridium* sp., 8= negative amplification(E), 9= *Clostridium* sp., 10= *Clostridium* sp., 11= *Clostridium* sp. 12= *Clostridium* sp., 13= *Clostridium* sp., 14= *Clostridium* sp., 15= *Clostridium* sp., 16= *Clostridium* sp., M= 100bp molecular ladder

Table 1. Cultural, morphological and biochemical characteristics of *Clostridium* species Isolated from the soil samples in Keffi, Nasarawa State, Nigeria

Cultural (RCMA)	Morphological Characteristics	Biochemical Characteristics			M	I	G	Inference
		C	O					
	Gram							
	Endospore							
	Stain stain							
Circular, grayish, glossy, flat	+, rods +	-	-		+	-	+	<i>Clostridium</i> sp.
Irregular, grayish, shiny, raised	+, rods +	-	-		+	-	+	<i>Clostridium</i> sp.
Circular, grayish, shiny, flat	+, rods +	-	-		+	-	+	<i>Clostridium</i> sp.
Circular, grayish, glossy, flat	+, rods +	-	-		+	-	+	<i>Clostridium</i> sp.
Circular, grayish, glossy, raised	+, rods +	-	-		+	-	+	<i>Clostridium</i> sp.
Irregular, grayish, glossy, raised	+, rods +	-	-		+	-	+	<i>Clostridium</i> sp.

KEY: - = Negative; + = positive, RCMA = Reinforced Clostridium Medium Agar C=catalase, O=oxidase, M=motility, I=Indole, G=glucose

Table 2. *Clostridium* species Isolated from Soil of Keffi

Isolates	Soil sample location									
	A	B	C	D	E	F	G	H	I	J
<i>C. acetobutylicum</i>	+	-	-	-	-	+	-	+	-	-
<i>C. beijerinckii</i>	-	-	-	-	+	-	-	-	-	-
<i>C. celerecrescens</i>	+	-	-	-	-	-	-	-	-	-
<i>C. cylindrosporum</i>	-	+	-	-	-	-	-	-	+	-
<i>C. difficile</i>	-	-	+	-	+	-	+	-	-	+
<i>C. perfringens</i>	-	-	+	+	-	-	-	+	-	-

KEY: A = Nasarawa State University Main Campus; B = Government Residential Area; C = High Court; D = Federal Prison Area; E = Angwan Kwara; F = Tudun Amama; G = Angwan Woje; H = Dadin Kowa; I = Nasarawa State University Pyanku Campus; J = Anto Bridge; - = Absent; + = Present

This study focused on the isolation of *Clostridium acetobutylicum* and *Clostridium beijerinckii* from the soil environment of Keffi and the production of biobutanol using these isolates in co-culture fermentation of pineapple peel wastes.

All the sampled areas yielded growth of *Clostridium* isolates which is in agreement with the work of [19]. Three (3) isolates of *Clostridium acetobutylicum* and one (1) isolate of *Clostridium beijerinckii* were successfully identified using the 16S rRNA sequencing, this report agrees with independent studies carried out by Laurentiu et al., [20].

During the research, other species of *Clostridium* such as *Clostridium celerecrescens*, *Clostridium*

cylindrosporum, *Clostridium difficile* and *Clostridium perfringens*, were isolated in addition to the species of *Clostridium acetobutylicum* and *Clostridium beijerinckii* which were isolated from the soil environment in the study location. This however, is in agreement with other studies earlier reported by Makut et al., [18]. It also agrees with studies reported by Logan & Vos [21] that *Clostridium* species are common bacteria isolated from the soil environment. Yang & Lu [22] reported a high frequency of occurrence of *Clostridium* in the United Kingdom, the occurrence of *Clostridium* species from the soil environment of Keffi metropolis has been quite high probably due to optimum temperature of 30–45°C and ability to ferment glucose [22].

Table 3. Biobutanol production of Clostridium species using Lucas test

Sample	Clostridial Isolate	Biobutanol Production
SAA1	<i>Clostridium acetobutylicum</i>	+
SAA2	<i>Clostridium celerecrescens</i>	-
SAB	<i>Clostridium cylindrosporum</i>	-
SAC1	<i>Clostridium difficile</i>	-
SAC2	<i>Clostridium perfringens</i>	-
SAD	<i>Clostridium perfringens</i>	-
SAE1	<i>Clostridium beijerinckii</i>	+
SAE2	<i>Clostridium difficile</i>	-
SAF	<i>Clostridium acetobutylicum</i>	+
SAG	<i>Clostridium difficile</i>	-
SAH1	<i>Clostridium acetobutylicum</i>	+
SAH2	<i>Clostridium perfringens</i>	-
SAI	<i>Clostridium cylindrosporum</i>	-
SAJ	<i>Clostridium difficile</i>	-

KEY: SAA = Nasarawa State University Main Campus; SAB = Government Residential Area; SAC = High Court; SAD = Federal Prison Area; SAE = Angwan Kwara; SAF = Tudun Amama; SAG = Angwan Woje; SAH = Dadin Kowa; SAI = Nasarawa State University Pyanku Campus; SAJ = Anto Bridge - = Absent; + = Present

Table 4. Mean yields of biobutanol produced by isolates in single culture

Sample	Isolates	Yield (g/L)
SAA1	<i>Clostridium acetobutylicum</i>	5.6±0.43
SAE1	<i>Clostridium beijerinckii</i>	1.2±0.20
SAF	<i>Clostridium acetobutylicum</i>	4.8±0.45
SAH1	<i>Clostridium acetobutylicum</i>	4.9±0.33

KEY: SAA1 = Nasarawa State University Main Campus; SAE1 = Angwan Kwara; SAF = Tudun Amama; SAH1 = Dadin Kowa

Table 5. Mean yields of biobutanol produced by isolates in co-culture

Isolates in Co-culture	Yield (g/L)
<i>Clostridium acetobutylicum</i> (SAA1), <i>Clostridium beijerinckii</i> (SAE1)	6.5±0.51
<i>Clostridium acetobutylicum</i> (SAF), <i>Clostridium beijerinckii</i> (SAE1)	5.8±0.49
<i>Clostridium acetobutylicum</i> (SAH1), <i>Clostridium beijerinckii</i> (SAE1)	5.8±0.37

KEY: SAA1 = Nasarawa State University Main Campus; SAE1 = Angwan Kwara; SAF = Tudun Amama; SAH1 = Dadin Kowa

In this study, it was observed that the isolated strains of *Clostridium acetobutylicum* and *Clostridium beijerinckii* had the ability to produce biobutanol this study is in agreement with other studies earlier described by Tashiro and Sonomoto [8], Abdehagh et al. [23] that *Clostridium acetobutylicum* and *Clostridium beijerinckii* are the common bacteria involved in the production of biobutanol. All other species of *Clostridium* isolated in this study did not produce biobutanol, and this is in agreement with studies earlier reported by Amira et al., [24].

Analysis of the fermentation broth using Gas Chromatography & Mass Spectrometry (GC - MS) of the isolated strains of *Clostridium acetobutylicum* and *Clostridium beijerinckii*

showed the production of biobutanol which is in agreement with studies of Abdehagh et al. [23]. The yield of biobutanol in this study ranged from 1.2±0.20g/L to 5.6±0.43g/L in single culture fermentation broth and 5.8±0.37g/L to 6.5±0.51 g/L in co-culture fermentation broth.

It was observed that in single culture and co-culture, the fermentation broth of *Clostridium acetobutylicum* (SAA1) produced the highest yield of biobutanol in comparison with the yield of other isolates, this is in agreement with earlier studies of Makut et al. [20], Tashiro and Sonomoto [8], by Amira et al. [24] and which agrees that *Clostridium acetobutylicum* are the highest producers of biobutanol.

4. CONCLUSION

Clostridium species isolated in this study had the ability to produce biobutanol hence can be used in the production of biobutanol. The use of pineapple peels waste will be a good source of carbon or media in the production of bio-butanol. The strains of *Clostridium acetobutylicum* and *Clostridium beijerinckii* studied are promising in the fermentation of pineapple residues with potential for the production of biobutanol. The production of biobutanol using single and co-cultures of *clostridium acetobutylicum* and *clostridium beijerinckii* using different sources of environmental biomass (waste) in Keffi metropolis should be carried out in future research.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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