Original Research Article

Isolation, Identification and Production of Biobutanol by Different *Clostridium* species Isolated from Soil Using Waste Paper and Sugar Cane Molasses.

ABSTRACT

Butanol as fuel component has some advantages compared to ethanol. The Study was aimed at isolation of different types of *Clostridium* species from the soil and screening for their capacity for production of biobutanol. Soil samples were collected from three different areas in keffi. Clostridium species were isolated using Reinforce Clostridial media and identified using standard microbiological methods, mutation was induced in the *Clostridium* isolates and 16s rRNA identification techinques was used to identify the isolates. Quantitative screening of the *Clostridium* species isolates capacity in biobutanol was carried out using sugar cane molasses and waste paper substrate. The result using 16s rRNA identification the *clostridium* isolated from old barracks was *C. acetobutylicium* the one isolated from Amgwan kwara was *C. perfringenes* and *C. difficile* was isolated from Amgwan Jaba. Screening for their biobutanol producing capacity it was observed that *C. acetobutylicium* produce the highest before exposing to UV light to induce mutation with 9.61± 0.94 mg/l using sugar cane molasses and 4.89± 0.19mg/l using waste paper follow by *C. perfringenes* 6.24± 0.61 mg/l using molasses and 3.21±0.11mg/l using waste paper. After exposing to UV light to induce mutation *C. acetobutylicium* produced 18.03 ± 0.17 mg/l using molasses and 8.63± 0.06 mg/l using waste paper substrate follow by *C. perfringenes* 14.19 ± 0.11 mg/l and 5.99 ± 0.31 mg/l. From this study it was observed that *C. acetobutylicium* has the capacity to produce biobutanol and also inducing mutation will enhance the capacity of *Clostridium* species in the production of biobutanol.

Key: *Clostridium,* Biobutanol, Molasses, Substrate

1. Introduction

The rate of energy demands combined with little resources of petroleum and environmental condition have raised a new interest in production of renewable energy from biomass, such as agricultural crops residuals, lignocellulosic waste and from industrial waste. Apart from ethanol, butanol is superior in energy content, has lower volatility and is also less corrosive to existing infrastructure [1]. They are three main area remain to be sorted out if biobutanol is to become a major counterpart in the bioenergy market. This includes optimizing feedstock utilization, reaching theoretical maximum yields of butanol and minimizing energy consumption during separation and purification [2].

One important driver for a biobased economy is the exploitation of the biorefinery concept [3] where maximum value can be derived from the biomass through the generation of multiple products and the effective use of process integration. Chemical pulp mills are current examples of biorefineries that can convert lignocellulosic biomass into energy, pulp, cellulose derivatives, tall oil, etc. One strategy for mills to counteract competition from tropical countries using fast-growing raw materials is to further expand the product portfolio into additional value-added products. Cellulose is the primary fraction utilized for chemical pulp production, while lignin, hemicellulose and extractives could be considered as by-products in the process. Currently lignin and a portion of the hemicellulose, and the pulping inorganics end up in the black liquor which is concentrated and burnt in a recovery boiler to

recover the chemicals and to supply energy for a large fraction of the mills process steam requirements. Considering lignin has double the heating value as polysaccharides, the hemicellulose fraction solubilized during alkaline pulping represents an underutilized resource in many mills.

One option to enhance the value of the hemicellulose is to recover this fraction prior pulping by using hot-water extraction [4, 5]. However, despite high yields of xylan, the removal of hemicellulose from birch wood chips prior Kraft cooking has a negative impact of some pulp properties affecting the quality of the paper. If a decrease in pulp strength properties cannot be accepted, another option is to recover the xylan fraction from the black liquor. Acidification of alkaline pulping liquors precipitates lignin as well as any hemicellulose present in the liquor [6]. Technologies for recovery of lignin from Kraft liquors via CO_2 acidification has been the subject of pilot and demonstration-scale processes [7,8] with proposed process of generating fuels, materials, and chemicals from the lignin as well as the opportunity for “de-bottlenecking” capacity-limited recovery-boilers. Hemicelluloses are typically degraded to hydroxy acids during Kraft pulping [9], although oligomeric xylan from hardwoods may be more resistant to alkaline pulping than the glucomannans which are the predominant softwood hemicelluloses due to the protection of glucuronic acid substitutions against end-wise alkaline degradation, and as such, these xylans may offer an opportunity for recovery and utilization as a feedstock for bioconversion. In the present study, the aim was to produce biobutanol using waste paper by two clostridium species isolated in soil of Keffi.

2.0 MATERIAL AND METHODS

2.1 Study Area

This study was carried out in Keffi, 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^{o}5'E along the Greenwich Meridian and at the equator and situated on longitude 850m above sea level [10].

2.2 Sample Collection

Three (3) soil samples were randomly collected (at the topsoil) from three different locations of Keffi metropolis such as Angwan Jaba, Old Barracks and Angwan kwara using a clean spoons and stored using disposable screw bottle and transported immediately to the Microbiology Laboratory, Nasarawa State University, Keffi for isolation.

2.2.1 Isolation of *Clostridium* species

The isolation of *Clostridium* species from the three areas in Keffi was carried out using spread plate method. One (1.0g) of the soil sample was suspended in 9.0ml of sterile distilled water containing tween 80 and 7 folds dilutions were made and 0.1ml of the aliquot was spread on petri dishes of fresh prepared Reinforce Clostridial media containing yeast extract 3.0; lab-lemco powder 10.0; peptone 10.0; soluble starch 1.0; glucose 5.0; cysteine hydrochloride 0.5; sodium chloride 5.0, sodium acetate 3.0 and agar agar 1.0. Incubated in anaerobic jar at 37^{o}C for 48 hours.

2.1.3. Identification of *Clostridium* species

The cultural and morphological identifications of *Clostridium* species were carried out in accordance with Bergey's Manual of Determinative Bacteriology [11] such as swarming test, gram staining test, **e**ndospore Staining egg yolk agar test, nagler test, lipase test indole test, catalase test and urease test.

2.1.4 Hank's Buffer/UV light treatment of clostridial isolates

Induction of mutation in *Clostridium* isolates was carried out by the modification of a method described by [12]. Clostridial isolates grown on Reinforce Clostridial medium maintain in Hank's buffer (137mM NaCl, 5.4mM KCl, 4.4mM $KHIO_4$, 0.33mM Na_2HPO_4, 1.3mM $CaCl_2$, 0.81mM $MgSO_4$, 4.2mM $NaHCO_3$, 1g/L glucose, pH 7.4) and were subsequently exposed for 1 hour to ultra violet light rays from 2 white fluorescent tubes (TFC FL-20 SD/18W Day Light). Isolates were exposed at a distance of 15cm. isolates were added to a mixture of 75% methanol and 25% acetic acid for 1 hour. The isolates were subsequently incubated in 70% ethanol containing 0.07N NaOH overnight at 4 ^{o}C. isolates were analysed using 16S rRNA gene sequencing for identifiction.

2.1.5 Extraction of bacterial DNA for 16S rRNA gene sequencing

Eighty (80 µl) of NaOH (0.05M) was added to 20 µl of clostridial isolates were suspended in distilled water and the mixture was incubated at temperature of 60C for 45 minutes, 6 µl of Tris/HCl (pH 7.0) were addition to achieve a final pH of 8.0. The mixture was diluted by ×100 and 5 µl of the diluted extract was used for the polymerase chain reaction (PCR).

2.1.6 PCR, gel electrophoresis, and 16S rRNA gene sequencing

Treated distilled water DNase I and PCR master mix (containing dNTPs, PCR buffer, and Taq polymerase) were used in all PCR reactions by adding 1 U of DNase I (Pharmacia, Uppsala, Sweden) to 40 µl of distilled water or PCR master mix, the mixture was incubated at 25°C for 15 minutes, and later at 95°C for 10 minutes to inactivate the DNase I. The clostridial isolates DNA extracts and control were amplified with 0.5µM primers (27F, 5′-TGGCGAACGGGTGAGTAA-3′ and 1525R, 5′-AGGCCCGGGAACGTATTCAC-3′; Inqaba Biotechnical Industries (Pty) UK). The PCR mixture (50 µl) contained clostridial isolates DNA, PCR buffer (10mM Tris/HCl (pH 8.3), 50mM KCl, 2mM $MgCl_2$, and 0.01% gelatin), 200 µM of each dNTP, and 1.0 U Taq polymerase (Boehringer Mannheim, Mannheim, Germany). The mixtures were amplified by 40 cycles of 94°C for one minute, 55°C for one

minute, and 72°C for two minutes, with a final extension at 72°C for 10 minutes in an automated 0.5 ml GeneAmp PCR system 9700 (Applied Biosystems, Foster City, California, USA). DNase I treated distilled water was used as the negative control. A 10 µl aliquot of each amplified product was electrophoresed in 1.0% (wt/vol) agarose gel, with a molecular size marker (λ DNA AvaII digest; Boehringer Mannheim) in parallel. Electrophoresis in Tris/borate/EDTA buffer was performed at 100 V for 1.5 hours. The gel was stained with ethidium bromide (0.5 µg/ml) for 15 minutes, rinsed, and photographed under ultraviolet light illumination.

The PCR products were gel purified using the techniques developed by the National Centre for Bioinformatics, Islamabad, Pakistan. Both strands of the PCR products were sequenced twice with an ABI 377 automated sequencer according to the manufacturers' instructions (Inqaba Biotechnical Industries (Pty) Ltd UK) using the PCR primers (27F and 1525R).

2.1.7 The pre-treatment of waste papers

The pre-treatment of waste papers was carried out as decribed by [13]. The waste papers were thoroughly washed in water to remove dust. Waste paper substrates were pre-treated with 3% NaOH for 1 hour. Then, they were washed and neutralized with 0.1N HCl at pH 5. The pre-treated waste papers were dried at 45 ^{o}C in an oven after neutralization.

2.1.7.1 Acid hydrolysis

Acid hydrolysis was done to break down cellulose into glucose units. It was carried out by soaking 10g of paper in different amounts of sulfuric acid 5%: 100, 200 and 300 ml of water for 2 hours. The mixture was autoclave at 121^{o}C. After hydrolysis, the samples were filtered and centrifuged to obtain the hydrolysate product. The pH of the obtained product was adjusted to 5.0 by adding sodium hydroxide solution 5M

2.1.8 Preparation of Molasses

The preparation of molasses from sugarcane was carried out as described by [14, 15]. The sugarcane was stripped off leaves. Its juice was extracted by cutting, crushing and mashing. The juice was boiled over a Bunsen burner to concentrate it, promoting sugar crystallization. The product of this first boiling called first syrup was used as a component of the fermentation medium.

2.1.9 Quantitative Analysis of Reducing Sugar Present in Fermentation medium

The quantitative analysis was carried out using 3, 5–dinitrosalicylic acid. The concentration of the sugar present in the samples was determine by adding 1cm^3 of 3, 5-dinitrosalicylic acid to 1cm^3 of each of the samples and boiled for 5 minutes and 10cm^{3}distille water was added. The absorbance of each of sample was determined at 540nm using JENWAY 6400

spectrophotometer. Thus, the concentration values were extrapolated from the glucose standard curve

2.1.10 Preparation of inoculum

The seed culture was carried out as described by [16]. The cultivated organism two colonies was inoculated into 10 ml of Reinforce Clostridial broth and incubated in an anaerobic jar at 37 ^{o}C for 6 hours.

3.0 Production of Biobutanol

3.1 Fermentation

The batch fermentation was carried out as described by [17]. The 100 ml from the starter culture was inoculated in 900 ml of fermentation media (enzymatically hydrolysed waste papers and molasses) containing 25g of sugar. The fermentation media were incubated under strict anaerobic and sterile conditions at 35^{o}C for 96 hours.

3.1.2 Extraction and purification of biobutanol from fermentation broth

Extraction and purification of solvents from the fermentation broth were carried out using a modification of the method decribed by [13]. In this method, *Clostridium* species were used in the fermentor. The extracting solvent was analkylate which was substantially free from impurities and had a boiling range of 118°C. The apparatus for carrying out the process included a standard fermentor receiving fermentation broth including a carbon source and microorganisms at a feed stream which are agitated and maintained at a desired fermentation temperature. The butanol-containing broth was drained through a first stream discharging into the upper end of an extraction column where it flowed downwardly through the extractor. A second stream released a light alkylate into the bottom of the column for upward countercurrent flow relative to the downward flow of the broth. The light alkylate was fed from an alkylate source stream, separated in a splitter with the light end boiling in the range of 1 °C. The butanol containing alkylate extract was discharged through a stream at the top of the extractor to flow through a dryer (a desiccator), in order to purify it and remove any excess water content. The extraction was carried out at a steady state with a solvent feed ratio ranging from 5–20 ml. The solvent was removed through the column at a rate of 200 – 450 ml per minute.

3.1.3 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 by [13]. 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 (2.1m x3.0mm). The temperature programming of the column oven was 60 ^{o}C/min. One hundred and twenty degrees centigrades (120 ^{o}C), Nitrogen gas (30 mL/minutes) was used as carrier gas. The

temperatures of injector and detector were 150 ^{o}C and 200 ^{o}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 duplicates and the means ± standard deviations of the yield of butanol were recorded. The standard calibration curve presented was used in calculating the concentration of biobutanol produced by the various Clostridal isolates.

3. Results

The species of *clostridium* that were isolated from soil of Keffi and were identified using cultural, morphology and different biochemical test as given in table 1. The three species of *clostridium* isolated from different location in keffi were able to produce biobutanol after screening for biobutanol production at pH 5.5, temperature of 37^{o}C for 96hours were *Clostridium* specie isolated from Old barracks produced the highest butanol 9.61± 0.94 mg/l using sugar cane molasses and sugar utilization of 9.61±0.71 g/l and 4.8±0. 19mg/l using waste paper substrate followed by *Clostridium* species isolated from Angwan Jaba 6.21± 0.61 mg/l, 9.05±1.11 g/l of sugar utilization using molasses and 3.21 ± 0.11 mg/l using waste paper substrate *Clostridium* isolated from Angwan kwara 5.48±0.99 mg/l and 10.11± 0.99 g/l of sugar utilization sugar cane molasses and 1.97 ± 0.33 mg/l using waste paper substrate. Mutation was induced in the clostridium isolates and were identify using 16s rRNA . biobutanol produced pH 5.5, temperature of 37^{o}C for 72hours after the induce of mutation as given in table 2 using waste paper as fermentation substrate shows that clostridium species isolated from old barracks was *C. acetobutylicium* aslo produce the highest butanol after mutation with 8.63±0.06 mg/l sugar utilization of 6.21±0.11 g/l followed by *C. perfringenes* isolated from Amgwan kwara 5.99 ± 0.31 mg/l sugar utilization of 4.89±0.19 g/l and *C. difficile* isolated from Angwan Jaba 3.01± 0.54 mg/l and sugar utilization of 3.97±0.33 g/l respectively. Table 3 shows biobutanol produce using sugar cane molasses by the *Clostridium* isolates were *C. acetobutylicium* produced highest 16.63±0.06 mg/l and sugar utilization of 16.98±0.51 mg/l followed by *C. perfringenes* 14.19 ± 0.11mg/l sugar utilization of 12.99±0.06 mg/l and the lowest was produced by *C. difficile* 10.01± 0.01mg/l and 9.89±0.19 mg/l utilization of sugar respectively.

Figure 1, 2 and 3 shows the phylogenetic tree of the different species of the *clostridium* isolated from different location.

Table1. Cultural, Morphology and Biochemical Characteristics Test

Cultural	Morphology characteristics							Inference
	Gram stain	Endo spore	Egg york test					
			lipase IND UV	NR Sg/UV	LE	cat		
Glossy, grey	-	+	-	+	+	-	+	*C. difficile*
Large smooth	-	+	Nil	nil	-	-	-	*C. perfringenes*
Raised	-	+	+		-	-	-	*C. acetobutylicium*

Key: UV fluorescence , NR- No reaction, SW- swarming growth, LE- Lecithinnase. Cat-catalase, IND- indole.

Table2: Biobutanol produced by different clostridium isolates before mutation at pH5.5, 37^{o}C after 96hours

Isolates	Initial sugar concretion (g/l)	Sugar utilization (g/l)	Biobutanol produced using waste paper (mg/l)	Biobutanol produced using sugar cane molasses (mg/l)
Clostridium (AJ)	20	9.05±1.11	3.21±0.11	6.21±0.61

Clostridium (AK)	20	10.11± 0.04	1.97±0.33	5.48±0.99
Clostridium (OB)	20	9.61±0.71	4.89±0.19	9.61± 0.94

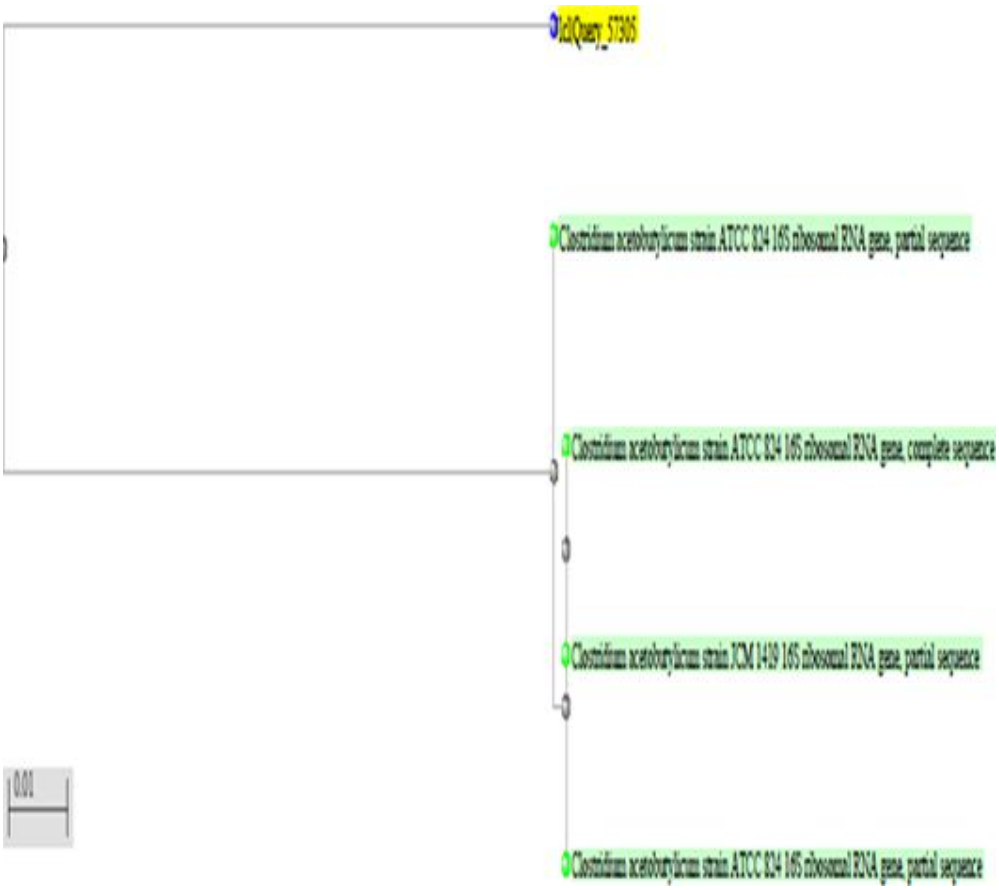
Key: AJ= Angwan Jaba, AK= Amgwan kwara, OB= Old barracks

Table3: Biobutanol produced by different clostridium isolates after mutation at pH5.5, 37°C after 96 hours

Isolates	Initial sugar (g/l)	Sugar utilization (g/l)	Biobutanol produced using waste paper (mg/l)
C. acetobutylicium	20.1±0.50	6.21±0.11	8.63±0.06
C. difficile	20.1±0.50	3.97±0.33	3.01± 0.54
C. perfringenes	20.1±0.50	4.89±0.19	5.99 ± 0.31

Table 4: Biobutanol produced by different clostridium isolates after mutation at pH5.5, 37°C after 96 hours

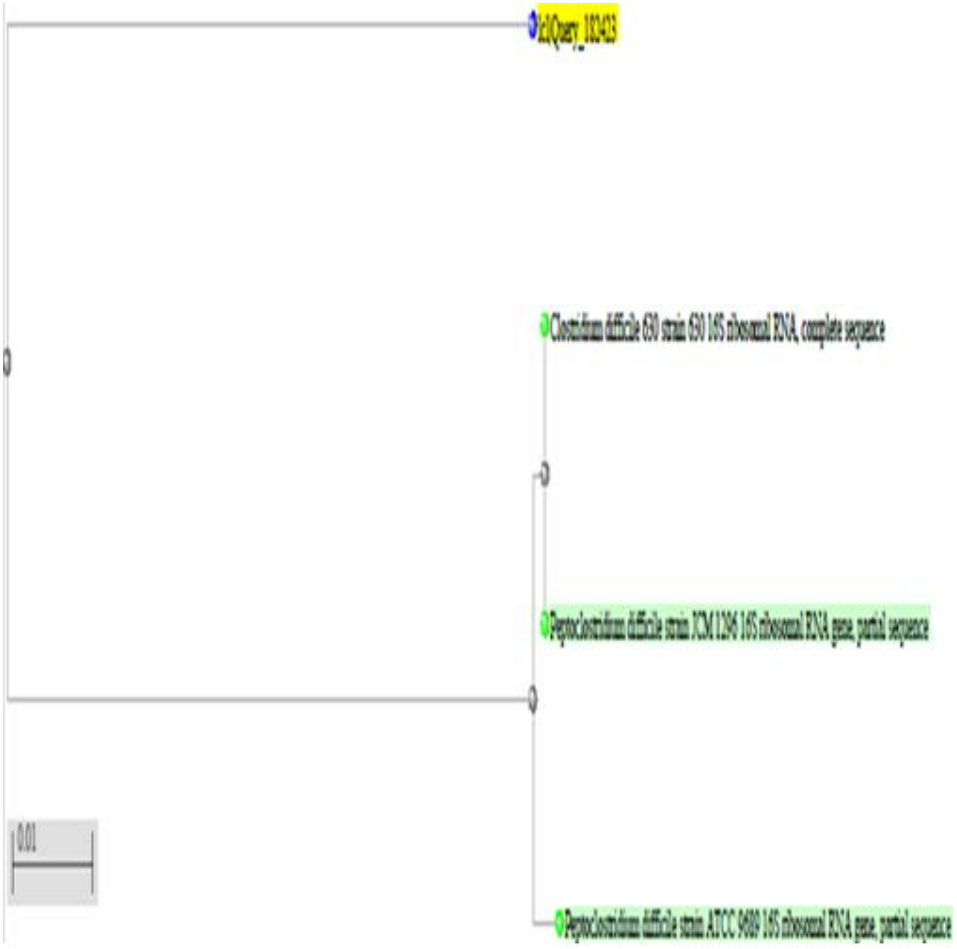
Isolates	Initial sugar (g/l)	Sugar utilization (g/l)	Biobutanol produced using sugar cane molasses (mg/l)
C. acetobutylicium	25.01±0.50	16.98±0.51	18.03±0.17
C. difficile	25.01±0.50	9.57±0.93	10.01± 0.01
C. perfringenes	25.01±0.50	12.99±0.06	14.19 ± 0.11



232

233 **Phylogenetic Tree of the Molecular Characterisation of *Clostridium acetobutylicum***

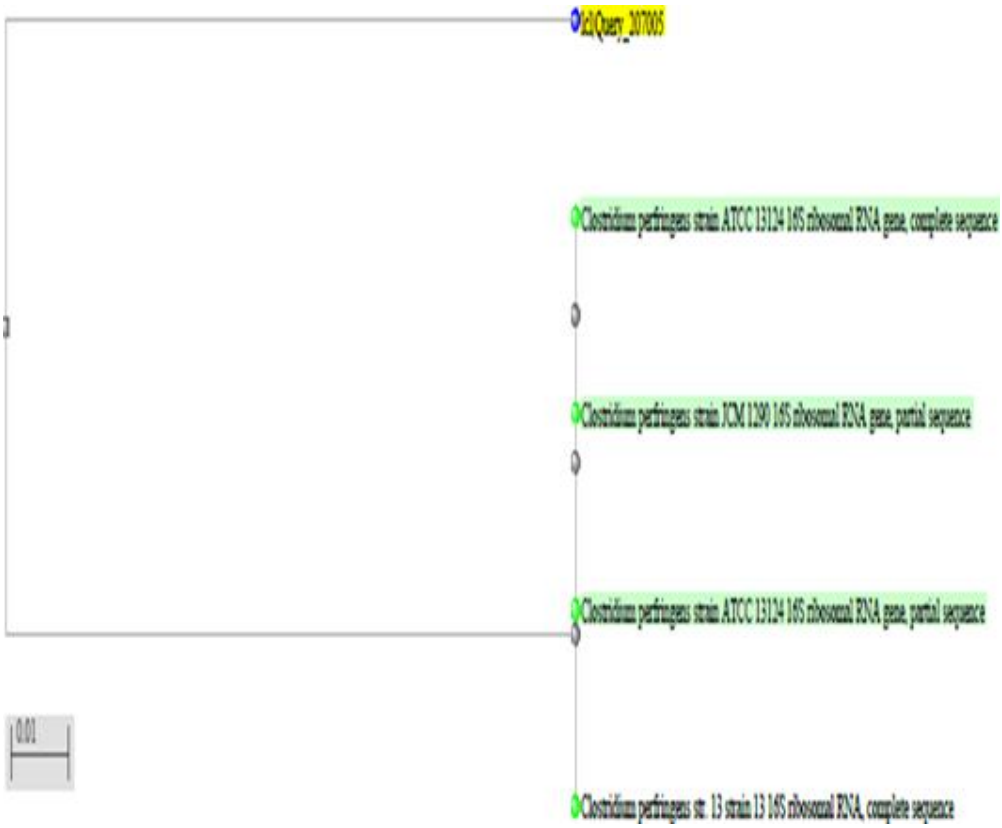
234 **isolated from old Barracks Keffi.**



235

236 **Phylogenetic Tree of the Molecular Characterisation of *Clostridium difficile* isolated**

237 **from** Angwan Jaba in Keffi.



Phylogenetic Tree of the Molecular Characterisation of *Clostridium perfringens* isolated from Angwan kwara in Keffi

4. Discussion

Butanol as fuel or blending component has some advantages compared to ethanol. For example a lower vapour pressure and higher energy density. The production of butanol in a microbial fermentation was first reported by Pasteur in 1861. Three different species of clostridium were isolated and identified using 16s rRNA from different location from soil in keffi, were C. *perfringens, C. difficile* and *C. acetobutylicium.* This however, is not in contrary with studies earlier reported by [18] that *Clostridium* species are one of most common bacteria isolated from the soil environment. Biobutanol production by different species of *Clostridium* isolated in this study is in agreement with other studies earlier described by [19, 20, 21] that *Clostridium* species are some bacteria that has the ability in production of biobutanol. In the study it was observed that *Clostridium* species isolated from old bricks has the highest accumulation of biobutanol at 37^{O}C, pH5.5 after incubation of 96 hours. Fermentation parameters and type of fermentation substrate play an important role in production of biobutanol. It was observed that were was high production of biobutanol using

sugar cane molasses and glucose which contain noncomplex sugar than using waste paper which is made up lignin which need to be detoxified to enhance the used of waste paper in the production bio-butanol to remove the inhibitor that will affect the growth of the *Clostridium* isolates as earlier reported by [22]. In this study it was observed the sugar cane molasses has higher yield of biobutanol that when used in a media containing the waste paper, also base on the isolates that yield has the highest accumulation of the biobutanol it observed that *C. acetobutylicium* has the accumulation of 8.63±0.06 mg/l in use of waste paper as substrate and 18.03±0.17 mg/l on sugar cane molasses as substrate which is agreement with work earlier reported by[18]. Also it was observed that there was increase in the production of biobutanol after the isolate was expose to UV light for mutation which suggested that to economical biobutanol production using *Clostridium* species inducing mutation will help in industrial scale production of biobutanol.

5. Conclusion

Clostridium species isolated from soil environment showed high capacity of production of biobutanol. It was observed that the use of both molasses and waste paper will be a good sources of carbon or media in the production of biobutanol. Inducing mutation in *Clostridium* species will help to overcome the challenges of using clostridium in industrial scale production of biobutanol.

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