South Asian Journal of Research in Microbiology

1(3): 1-6, 2018; Article no.SAJRM.42328

Isolation and Screening for Biobutanol Producing *Clostridium* Species from the Soil Environment of Keffi Metropolis

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Authors' contributions

This work was carried out in collaboration between all authors. Author MDM designed the study and performed the statistical analysis, author SOO wrote the protocol and wrote the first draft of the manuscript and managed the analyses of the study. Author GO managed the literature searches and the laboratory work of the Research. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/SAJRM/2018/v1i3805

Editor(s):

(1) Dr. Eliton da Silva Vasconcelos, Department of Physiological Sciences, Federal University of Sao Carlos – UFSCar, Rod. Washington Luiz, Sao Carlos, Brazil.

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Complete Peer review History: http://www.sciencedomain.org/review-history/25351

Original Research Article

Received 12th April 2018
Accepted 25th June 2018
Published 30th June 2018

ABSTRACT

Clostridium species have been reported as one of the most common groups of bacteria that produce biobutanol. The Study was aimed at isolation of Clostridium species from the soil environment in Keffi Metropolis and screening for their ability to produce biobutanol. Ten different location soil samples were collected within keffi Metropolis. Nutrient Blood agar was used for the isolation of the Clostridium species. biobutanol production was carried out using Clostridial isolates and Lucas test was used to detect biobutanol production Clostridium isolates after fermentation. The results show that six (6) species of Clostridial were isolated with a percentage frequency of *Clostridium difficile* (30%), *Clostridium botulinum* (10%), *Clostridium cylindrosporum* (20%), *Clostridium perfringens* (10%), *Clostridium acetobutylicum* (20%) and *Clostridium celerecrescens* (10%). The result further revealed that all the six (6) isolates were found to be able to produce biobutanol. Biobutanol is an industrial organic chemical that can place fossil fuel if harnessed and production of Biobutanol using *Clostridium* in large quantity can be alternative to fossil fuel.

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Keywords: Clostridial; biobutanol; Keffi; metropolis.

1. INTRODUCTION

For centuries wastes has been released into our environment by activities human [1]. The build-up of wastes both in concentrated form as refuse and its haphazard form as litters is of ever-growing concern. Studies on the use of wastes for biofuel production have been reported by many researchers [2,3,4,5].

Biobutanol production by fermentation has been practised commercially for over a century [6]. In addition to other important uses, butanol has been recently recognised as a commercially viable biofuel.

Butanol (butyl alcohol or 1-butanol) or biobutanol if it is produced through the biological process is primary alcohol of four carbon having C_4H_9OH molecular formula. It is a known fact that up to 10 - 12 billion pounds of biobutanol are produced annually [7], which amount 7 - 8.4 dollar market expansion of 3% a year [8].

Biobutanol is reflected as a promising product of biomass fermentations for prospective industrial use as a solvent, feedstock biochemical, and mainly liquid fuel [9]. There is a lot of plant biomass existing as low-value agricultural produce or wastes needed good disposal to shun pollution harms. The ample inexpensive renewable means as feedstock for fermentation have resulted in a renewed interest in the fermentation of n-butanol.

Initial large-scale production of biobutanol was centered on fermentation by *Clostridium acetobutylicum* which break down starch, and produces mostly butanol and acetone [10].

Acetone-Butanol-Ethanol (ABE) is produced at the advanced phase of batch fermentations which some *Clostridium* strains (predominantly saccharolytic butyric acid-producing bacteria) under suitable conditions. Typical examples of such clostridia include *Clostridium saccharoperbutylacetonicum*, *C. acetobutylicum*, *C. beijerinckii*, and *C. aurantibutylicum*. This study was aimed at Isolation and Screening for Biobutanol Producing *Clostridium* Species from the Soil Environment of Keffi.

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 68 km away from the Federal Capital Territory (FCT), Abuja and 128km away from Lafia, the capital town of Nasarawa State. The location Keffi is at longitude 8º5'E along the Greenwich Meridian and at the equator and situated on longitude 850 m above sea level [11].

2.2 Sample Collection

Ten (10) soil samples were randomly collected (at the topsoil) from ten different locations of Keffi metropolis such as Angwan Lambu, Angwan Jaba, Angwan Woje, Dadin Kowa, G. R. A., High Court, Kofar Hausa, Main Campus, Yelwa and Market, using a clean hand trowel and stored using disposable black polythene bags and transported immediately to the Microbiology Laboratory, Nasarawa State University, Keffi for analysis.

2.2.1 Isolation of *Clostridium* species

The isolation of *Clostridium* species from the ten different locations in Keffi metropolis was carried out as described by [12]. Using this method, 1.0 g of the soil sample was suspended in 9.0 ml of sterile distilled water and 10-fold dilutions were made and 0.1 ml of the aliquot was placed on Petri dishes and freshly prepared nutrient agar and blood agar were poured on the aliquot and then incubated in an anaerobic jar at 35ºC for 48 hours.

2.2.2 Identification of *Clostridium* species

The cultural and morphological identifications of *Clostridium* species were carried out in accordance with Bergey's Manual of Determinative Bacteriology [13] such as swarming test, gram staining test, endospore Staining egg yolk agar test, Nagler test, lipase test indole test and urease test.

2.2.3 Preparation of potato starch substrates

Potato tubes were collected cut into smaller sizes, sun-dried and grinded into powder form using clean grinding machine and sieve. Five

hundred gram (500 g) powder form was added into 5 litres of distilled water and sieved to form a homogenous mixture and placed at 4ºC for 24 hours. The starch was allowed to settle and water was discarded. The starch was then dried in an oven at 60ºC, 12 hours. 25 g/l of starch solution was prepared and sterilized using autoclave at 5.0 Ibs (115ºC) for 5 min. To dissolve the starch, alpha-amylase (2.0 μ/ml) was added and animated at 95ºC in a water bath for 15 min. For saccharification, amyloglucosidase (2.0 μ/ml) was added and heated at 55ºC with constant stirring for about 4 hours [14].

2.2.4 Fermentation

The batch fermentation was carried out as described by [14]. The 100 ml from the starter culture was inoculated in 900 ml of fermentation media (potato starch) as described by [15] under strict anaerobic and sterile conditions at 35ºC for 72 hours.

2.2.5 Screening for biobutanol-producing *Clostridium* species

The determination of biobutanol-producing isolates using Lucas' test was carried out as described by [16]. In this test, 5 mL of Lucas' reagent at room temperature was added to 1 mL of the fermentation broth in a test tube. The tube was stoppered, shaken vigorously, and then the mixture was allowed to stand. The solution remained colourless indicating a positive test for biobutanol production.

2.2.6 Extraction and purification of solvents from fermentation broth

Extraction and purification of solvents from the fermentation broth were carried out using a modification of the method described by [17]. In this method, *Clostridium* species were used in the fermentor. The extracting solvent was an alkylate which was substantially free from impurities and had a boiling range of 50º - 150ºC. The tool used in carrying out this process included a normal fermentor receiving fermentation broth including a carbon source and microorganisms at a feed stream which are agitated and preserved at the 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 discharge a light alkylate into the base of the column rising countercurrent movement to the downward flow of the broth. The light alkylates passes from an alkylate source stream, separated in a splitter with the light end boiling in the range of 50º-150ºC.

The extracted broth freed from the butanol was recycled through a stream back to the fermentor where its remaining nutrients and microorganisms were utilized to enrich the feed stream. 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.

2.2.7 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 by [17]. One microliter (1.0 µL) of the acidified sample was injected into “SHIMAZU GC-14, Gas Chromatograph” equipped with the 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 60ºC/min 120ºC, Nitrogen gas (30 mL/minutes) was used as carrier gas. The heats injected and indicator was at 150ºC and 200ºC respectively. The Peaks were documented on “SHIMADZU C-R-4_A, Chromatograph”, and were identified by assessment of the retention times with that of known mixture. The studies were carried out in replicas and the means ± standard deviations of the butanol produced were recorded.

2.3 Statistical Analyses of Data

Statistical studies of data were carried out. The study was conducted in duplicates. Unless otherwise indicated, for each experiment undertaken, the mean level of variables and the standard deviations (SD) was calculated. Comparisons of variables were made with the One–Way Analysis of Variance (ANOVA). A value of $p < 0.05$ was considered statistically significant. Statistical analyses were conducted

using the software programme, Statistical Package for Social Sciences (SPSS 21.0; IBM, USA).

3. RESULTS

3.1 Percentage Frequency of *Clostridial* Isolates from Different Locations in Keffi Metropolis, Nigeria

The frequency (percentage) of occurrence of *clostridial* isolates from different locations of Keffi Metropolis is as given in Table 1. The frequency (percentage) of occurrence of *clostridial* isolates from different locations of Keffi metropolis were within the range of 10.0% and 30.0%. The highest percentage occurrence was recorded for *Clostridium difficile* (30.0%), followed by *Clostridium cylindrosporum* and *Clostridium acetobutylicum* each having 20.0%. However, *Clostridium botulinum*, *Clostridium perfringens* and *Clostridium celerecrescens* had the frequency (percentage) of occurrence of 10.0%.

Cultural, morphology and biochemical test of the clostridium species isolated in Keffi soil is as given in Table 2.

3.2 Screening for Biobutanol-Producing Clostridial Isolates Soil Environment in Keffi

Table 2 shows the screening for biobutanol-producing clostridial isolates from the soil environment of Keffi metropolis, Nigeria. All the *Clostridium* species isolated has the ability to produce biobutanol and the highest butanol produced was obtain from *Clostridium acetobutylicum* isolated from Kofar Hausa and Yelwa which produced 16.87±2.34 mg/l and 15.24±1.88 mg/l of biobutanol with sugar utilization of 15.56±2.59 g/l and 16.14±1.44g/l followed by *Clostridium cylindrosporum* isolated from Angwan Woje and Keffi Market which produced 12.22±1.61 mg/l of biobutanol, sugar utilization of 11.21±2.3 g/l and 11.66±1.11 mg/l of biobutanol and sugar utilization of 14.58±2.55 g/l, *Clostridium perfringens* isolated from G. R. A produced 10.12±0.69 mg/l of biobutanol and sugar utilization of 13.30±1.01 g/l, *Clostridium celerecrescens* isolated from main campus produced 9.50±0.12 mg/l of biobutanol with sugar utilization of 12.41±0.17 g/l, *Clostridium difficile* isolated from Angwan Lambu and Dadin Kowa produced 8.10±1.06 mg/l of biobutanol with sugar utilization of 10.05±1.11 g/l and 7.54±1.51 mg/l of biobutanol with sugar utilization of 9.41±2.11 g/l and the least *Clostridium botulinum* isolated from Angwan Jaba produced 5.41±0.15 mg/l of biobutanol with sugar utilization of 8.12±0.12g/l respectively. Biobutanol produced irrespective of species of *Clostridium* were statistically significant at $P<0.05$ and 95% confidence interval.

4. DISCUSSION

Clostridium species such as *Clostridium difficile* (Cd_1), *Clostridium botulinum* (Cb_1), *Clostridium cylindrosporum* (Cc_1), *Clostridium difficile* (Cd_2), *Clostridium perfringens* (Cp_1), *Clostridium difficile* (Cd_3), *Clostridium acetobutylicum* (Ca_1), *Clostridium acetobutylicum* (Ca_2), *Clostridium celerecrescens* (Cce_1) and *Clostridium cylindrosporum* (Cc_2) were isolated from the soil environment in the study location. This however, is in agreement with other studies earlier reported by [18] that *Clostridium* species are common bacteria isolated from the soil environment.

Also, in this study, the occurrence of *Clostridium difficile* was higher than other species of *Clostridium* such as *Clostridium botulinum*, *Clostridium cylindrosporum*, *Clostridium perfringens* and *Clostridium acetobutylicum*. This appears new in the study area to the best of our knowledge. Although, [19] reported a high frequency of occurrence of *Clostridium* in the United Kingdom, the occurrence of *Clostridium* species from the soil.

Different Clostridium species isolated from the study area shows high capability of biobutanol production were *Clostridium acetobutylicum* produced the highest biobutanol after 72 hours of fermentation at temperature of 35^oC which was in agreement with studies reported by [20],[21] that *Clostridium acetobutylicum* produced higher yield of biobutanol than other species of *Clostridium*. Ultimately, the yields of butanol such as 16.87±2.34 mg/l and 15.24±1.88 mg/l varied between the two species of *C. acetobutylicum* (CA_1) and *C. acetobutylicum* (CA_2) observed in this study. This may be due to the fact that the two species of *Clostridium acetobutylicum* are of different strains.

Table 1. Percentage frequency of clostridial isolates from different locations in Keffi

Clostridial isolate	Soil location										Frequency (%)
	A	B	C	D	E	F	G	H	I	J	
C. difficile	+	-	-	+	-	+	-	-	-	-	30.0
C. botulinum	-	+	-	-	-	-	-	-	-	-	10.0
C. cylindrosporum	-	-	+	-	-	-	-	-	-	+	20.0
C. perfringens	-	-	-	-	+	-	-	-	-	-	10.0
C. acetobutylicum	-	-	-	-	-	-	+	-	+	-	20.0
C. celerecrescens	-	-	-	-	-	-	-	+	-	-	10.0

Key: - = Absent; + = Present; A = Angwan Lambu; B = Angwan Jaba; C = Angwan Woje; D = Dadin Kowa; E = G. R. A.; F = High Court; G = Kofar Hausa; H = Main Campus, I = Yelwa; J = Market

Table 2. Screening for biobutanol-producing clostridial isolates from soil environment of Keffi Metropolis, Nigeria

Sample	Clostridial isolate	Initial sugar (g/l)	Sugar utilization (g/l)	Biobutanol produced (mg/l)
A_1	*Clostridium difficile*	25 g/l	10.05±1.11	8.10±1.06
A_2	*Clostridium botulinum*	25 g/l	8.12±0.12	5.41±0.15
A_3	*Clostridium cylindrosporum*	25 g/l	11.21±2.3	12.22±1.61
A_4	*Clostridium difficile*	25 g/l	9.41±2.11	7.54±1.51
A_5	*Clostridium perfringens*	25 g/l	13.30±1.01	10.12±0.69
A_6	*Clostridium difficile*	25 g/l	7.80±1.20	5.72±032
A_7	*Clostridium acetobutylicum*	25 g/l	15.56±2.59	16.87±2.34
A_8	*Clostridium celerecrescens*	25 g/l	12.41±0.17	9.50±0.12
A_9	*Clostridium acetobutylicum*	25 g/l	16.10±1.44	15.24±1.88
A_{10}	*Clostridium cylindrosporum*	25 g/l	14.58±2.55	11.66±1.11

F-value = 0.9997; p-value = 0.4227

Key: A_1 = Angwan Lambu; A_2 = Angwan Jaba; A_3 = Angwan Woje; A_4 = Dadin Kowa; A_5 = G. R. A; A_6 = High Court; A_7 = Kofar Hausa; A_8 = Main Campus; A_9 = Yelwa; A_{10} = Market; + = Positive

5. CONCLUSION

The findings revealed that *Clostridium* species such as *Clostridium difficile*, *Clostridium botulinum*, *Clostridium cylindrosporum*, *Clostridium perfringens*, *Clostridium celerecrescens* and *Clostridium acetobutylicum* were the common species of *Clostridium* isolated from the soil environment of Keffi Metropolis, Nigeria and *Clostridium acetobutylicum* isolated from Kofar Hausa and Yelwa produced the highest biobutanol.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Cabezas R, Plaza A, Merlet G, Romero J. Effect of fluid dynamic conditions on the recovery of ABE fermentation products by membrane-based dense gas extraction. Chemical Engineering and Processing: Process Intensification. 2015;95:80-89. DOI: 10.1016/j.cep.2015.04.003
2. Errico M, Sanchez-ramirez E, Quiroz-ramirez JJ, Segovia-hernandez JG, Rong R. Synthesis and design of new hybrid configurations for biobutanol purification. Computers and Chemical Engineering. 2016;84:482-492.
3. Kurkijarvi AJ, Melin K, Lehtonen J. Comparison of reactive distillation and dual extraction processes for the separation of acetone, butanol, and ethanol from fermentation broth. Industrial Engineering Chemistry Research. 2016;55:1952-1964.
4. Outram V, Lalander CA, Lee JGM, Davis ET, Harvey AP. A comparison of the energy use of *in situ* product recovery techniques for the acetone butanol ethanol fermentation. Bioresource Technology. 2016;220:590-600.
5. Serdof H, Fricke WF, Veith B, Bruhhermann H, Lieseganag H, Strittmatter A, Miethke M, Buckel W, Hinderberger J, Li F, Hagemeier C, Thauer

RK, Gottschalk G. The genome of *Clostridium kluyveri*, a strict anaerobe with unique metabolic features. Proceedings of National Academy of Sciences of the United States of America. 2008;105:2128-2133.
6. Donaldson GK, Huang LL, Maggio-Hall LA, Nagarajan V, Nakamura CE, Suh W. Fermentative production of four carbon alcohols. International Patent WO20077/041269; 2007.
7. Durre P. Fermentative butanol production of bulk chemical and biofuel. AnnualNew York Academic Science. 2008;1125:353-362.
8. Kirschner M. N-butanol. Chemical Market Reporter January 30th February 5th, ABI/ INFORM Global. 2006;42.
9. Jones DT, Woods DR. Acetone-butanol fermentation revisited. Microbiology Reviews. 2011;50(4):481-524.
10. Atsumi S, Liao JC. Metabolic engineering for advanced biofuel production. Current Opinion in Biotechnology. 2008;19:414-419.
11. Akwa VL, Binbol NL, Marcus ND. Geographical perspective of Nasarawa State. Onaivi Printing and Publishing Company Ltd., Keffi, Nigeria. 2007;3.
12. Reddy CRK, Jha B, Fujita Y, Ohno M. Seaweed micro-propagation techniques and their potentials: An overview. Journal of Applied Phycology. 2010;20:609-617.
13. Breed RS, Murray EGD, Smith NR. Bergey's manual of determinative bacteriology. The Williams and Wilkins Company, USA. 2011;1-1094.
14. Ekeleme IK, Makut MD, Adoga MP, Tsaku PA, Nkene IH, Oti VB. Production of citric acid by *Trichoderma viride* isolated from soil in Keffi, Nigeria using glucose enhanced substrates. South Asian Journal of Research in Microbiology. 2018;1(1):1-6.
15. Vidya R, Jasmine I, Pradeep V. Process for the production and quantification of high yield of biobutanol. United States Reliance Life Sciences Pvt. Ltd., Navi Mumbai. 2010;1-16.
16. Dhingra S, Angrish C. Qualitative organic analysis: An efficient, safer, and economical approach to preliminary tests and functional group analysis. Journal of Chemical Education. 2011;88(5):649–651.
17. Logan NA, Vos PD. Genus I. *Clostridium prazmowski* 1880. In: (Eds.) Vos PD, Garrity G, Jones D, Krieg NR, Luwig W, Rainey FA, Scleifer KH, Whitman WB. Bergey's Manual of Systematic Bacterio-logy: Firmicutes, Springer. 2009;3:738–828.
18. Yang ST, Lu CZ. Extraction-fermentation hybrid (extractive fermentation) in separation and purification technologies in biorefineries. (eds. Ramaswamy S, Huang HJ, Ramarao BV.), John Wiley and Sons, Ltd., Chichester, UK; 2013.
19. Amira H, Karimi K. Organosolvent pretreatment of rice straw for efficient acetone, butanol, and ethanol production. Bioresource Technology. 2014;152:450-456.
20. Amira H, Karimi K, Bankar S, Granstrom T. Biobutanol from lignocellulosic wastes. lignocellulose-based bioproduction. In: Karimi K. Springer International Publishing, Switzerland. 2015;2289-324.
21. Becerra M, Cerdan ME, Gonzatez-Siso MI. Biobutanol from cheese whey. Micro-biological Cell Fact. 2015;14:27.

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Peer-review history:
The peer review history for this paper can be accessed here:
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