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Isolation and identification of solvent producing clostridia from the soil environment within Jos Metropolis, Nigeria

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Abstract
Indigenous bacteria in the soil environment can produce a wide range of solvents and metabolites more efficiently. This study was carried out to screen *Clostridium* species isolated from the soil environment within Jos metropolis for solvent production. A total of 30 samples were collected from farmlands, nursery gardens, floral gardens and waste disposal sites. The pH, temperature and moisture content at various locations was determined. The mean temperature of the soil ranges from 19.00 °C to 22.4°C, mean percentage moisture content of the soil samples ranged from 9.88 to 13.22 and the mean pH ranged from 6.23 to 7.37. The soil samples were enriched in Reinforced Clostridial Medium (RCM) in an anaerobic medium and isolation of *Clostridia* was carried out in Reinforced Clostridial Medium (RCM) agar using pour plate technique. Fifty-eight (58) isolates were isolated and identified on the basis of morphological characteristics. Ten (10) isolates were suspected as *Clostridium* species and subjected to further biochemical tests. The solvents produced by the isolated bacteria during fermentation was detected using a standard qualitative test for alcohols. The biochemical and physiological test were used to identify the *Clostridium* species by ABIS (Advanced Bacterial Identification Software) online laboratory tool. It was confirmed that two species namely *Clostridium acetobutylicum* and *Clostridium auranbutyricum* are non-pathogenic. The pathogenic *Clostridium* species isolated were *Clostridium colinum, Clostridium perfringens, Clostridium botulinum* and *Clostridium difficile. Clostridium botulinum* had the highest frequency of occurrence. The waste disposal site and nursery gardens had the highest occurrence of *Clostridium* species, while farmlands and floral gardens had the least. The ten (10) isolates produced alcohol during fermentation.
Objective: To isolate and identify Clostridium species from some garden soils, farmlands and waste disposal sites. Determine some physicochemical properties of the soil samples and the solvent-producing potential of the isolates

Keywords: soil, solvents, clostridium, metabolites, jos

Introduction

Microbial production of acetone and butanol was one of the first large-scale industrial fermentation processes of global importance. Production of commercially important solvents (acetone and n-butanol) by clostridia was a major industry during the first half of the 20th century, ranking second in importance only to ethanol fermentation. After a rapid decline after the 1950s, acetone-butanol-ethanol (ABE) fermentation has recently gained renewed interest in the context of biorefinery approaches for the production of fuels and chemicals from renewable resources. The availability of new methods and knowledge opens many new doors for industrial microbiology, and a comprehensive view on this process is worthwhile due to the new interest (Sauer, 2016) [8].
The interest in large-scale production of solvents by fermentation has never ceased because of the benefits of using renewable biomass as the raw material, which is strategically important for petroleum-importing countries. Since then, solvent fermentation has not competed economically with the chemical processes. However, solvent fermentation is considered viable in niche markets, especially when the merits of resource conservation are also considered. In addition, the acid- and solvent-producing pathways of the solvent-producing clostridia serve as a good experimental system for studying the regulation of the enzymes and their genes involved in branched metabolic pathways (Kasap, 2002) [4]. The polysaccharides in plant biomass preserve a huge amount of energy that can be partly made accessible through bioconversion into storable fuels. The ability to ferment a wide range of carbohydrates to C2, C3 and C4 compounds ethanol, acetone, isopropanol, butanol is especially pronounced in the saccharolytic mesophilic bacteria *Clostridium acetobutylicum*, *Clostridium beijerinckii* and related strains (Uppin, 2013) [9]. Currently, the predominant microbial produced biofuel is from starch or sugar-derived ethanol. However, ethanol is not an ideal fuel molecule, and lignocellulosic feedstocks are considerably more abundant than both starch and sugar. Thus, many improvements in both the feedstock and the fuel have been proposed (Uppin, 2013) [9]. Physical maps of the chromosomes of *C. acetobutylicum* ATCC 824, C. beijerinckii NCIMB 8052 and *C. saccharobutylicum* NCP 262 have been constructed (Keis *et al*., 2001a) [5]. Taxonomic studies helped to correct identification of the laboratory strains that were mislabeled as *C. acetobutylicum* (Keis *et al*.,

2001b). Many of the solvent-forming enzymes from either *C. acetobutylicum* or *C. beijerinckii* or both have been purified (Kasap, 2002) [4]. Most recently, the genome sequencing project of *C. acetobutylicum* ATCC 824 has been completed, and the genome sequence is now available for functional analysis (Noelling *et al*., 2001) [7]. The wide range of natural occurrence of solvent producing strains is interesting and reviews concerned to the properties of solvents, microorganisms that produce the solvents, their habitat and their characteristics is being presented here.

Materials and Methods

Study Area

Soil sample were collected from waste disposal sites, nursery gardens, floral gardens, and farmlands of Jos Metropolis (Latitude 9.93, Longitude 8.89, elevation 1186 m above sea levels) in Plateau state (elevation 1208m above sea levels), Nigeria, Africa.

Samples Collection

A total of 30 soil samples were collected from different locations within Jos metropolis. The sites were, wastes disposal site, farmlands, nursery gardens and floral gardens. Soil sample was collected with a sterile metal spatula at a depth below 10cm, some plants roots were uprooted from the soil and shaken gently to collect the adhering soil. The soil samples were placed into clean zip-lock plastic bags, labeled according to their source and location and taken to the laboratory for analyses (Abd-Alla *et al*., 2015) [1].

Determination of Percentage Moisture Content and Ph of Soil Samples

Ten grams of freshly collected samples was placed in a crucible and dried in hot air oven at 110^{o}C to constant weight. The moisture content was using the AWPA (1986) American Wood Preserver Association Standard.

$$MC (\%) = (W-w/W) \times 100$$

Where MC is moisture content, W is the original weight and w is the constant weight after oven drying.
pH was determined by Electrometric method with the aid of a Ohaus pH meter starter 3100.

Media Preparation and Sterilization

The media used are Reinforced Clostridial Medium (RCM) and Reinforced Clostridial Agar (RCA). They were prepared according to the manufacturer’s instruction. The media were dissolved in an appropriate volume of sterilized distilled water and warmed for few minutes so as to dissolve completely. The media was sterilized in an autoclave at 121^{o}C for 15 minutes at 15psi.

Pasteurization of the Enriched Media

To counteract the nom-spore formers, pasteurization (high temperature and low time, HTLT) of the enriched samples was done at 80^{o}c for 10 minutes.

Enrichment for Isolation of *Clostridium spp.*

Enrichment of samples was done by suspending 1g of fresh sample in sterile serum bottle containing 10ml RCM anaerobic medium and providing CO2 atmosphere, the bottles were subsequently incubated for 24hours at 37^{o}c under anaerobic conditions (using a gaspac) in an anaerobic jar. Samples having gas production and increased turbidity were selected for isolation.

Isolation of Clostridia from Soil Samples

Based on the turbidity and gas formation enriched samples were selected for isolation of individual colonies. A 10-fold serial dilution to the 4th dilution of the enriched soil samples was carried out. 1ml of the 4th dilution was inoculated on sterilized Reinforced Clostridium Medium (RCM) agar using pour plate technique, this was done in duplicates. The plates were inoculated at 37^o C for 48hours under strict anaerobiosis in an anaerobic jar. The culture was subcultured on RCA to obtain pure cultures.

Morphological, Biochemical and Physiological Characterization of the Isolates

The isolates were characterized morphologically by; gram staining, endospore staining using differential staining technique. Biochemical analyses include; catalase test, indole production test, urease test, hydrolysis of carbon sources (glucose, galactose, sucrose, lactose, cellulose and starch), gelatin liquefaction test. Physiological test was; Rifampicin sensitivity and curd formation test.

Screening for Solvent Production

Inoculum Development and Fermentation for Solvent Production

The isolates were inoculated in Reinforced Clostridial Medium (RCM) as the carbon source at pH 6.2, 37^{o}C for 24 hours under anaerobic conditions. The fermentation for solvent production was done using methods described by Nageeb, El Mubarak and Aidil, (2014) [6] with slight modification. 20ml screw capped bottle containing 15ml of Reinforced Clostridial Medium with initial pH 6.2 and inoculum size of 10% (v/v). The glucose concentration was 40g/L and the culture was incubated at 37ºC for 120 hours under anaerobic conditions. Samples was taken every 24 hours and screened for alcohol production using Lucas test.

Test for Alcohol production

The test for the presence of alcohol was done using Lucas test. An aliquot of the fermentation broth was centrifuged at 7000rpm for 10 minutes, with the aid of a sterile pipette, a portion (0.2ml) of the supernatant was transferred to attest tube and 2ml Lucas reagent at room temperature was added to it. The test tube was properly covered with a stopper, shaken vigorously and the mixture was allowed to stand. The time required for the fermentation was noted. Appearance of cloudy second layer or emulsion within 10 minutes indicated the presence of alcohol.

Result and Discussion

A total of 30 soil samples were collected from different locations of Jos metropolis from waste disposal sites, gardens, farmlands, sugarcane field, rice paddy field, forest, floral gardens and from compost soil. The soil pH, temperature and moisture content of samples is shown in Table 1. A total of fifty-eight mesophilic bacteria were isolated on the RCM agar. The isolated bacteria formed circular to slightly irregular, flat to raised, milky colonies on RCM agar surface at 37ºC. A total of fifty-six straight, gram-positive rods were isolated. Ten (10) of these were gram positive rods and formed endospores. The 10 isolates were subjected to further biochemical characterization (Table 2). All the 10 isolates developed yellow colour which was indicated as negative due to lack of indole production (Table 2). The results of the urease test showed that 3 isolates developed a pink colour which indicated positive, 7 isolates had no change in the original colour of the medium which indicated negative for urease test (Table 2). The 10 isolates were found to be positive for gelatin liquefaction. The curd formation test showed that 3 isolates were able to coagulate the milk medium and 7 isolates were found not able to coagulate the milk (Table 2). All 10 isolates were susceptible to rifampicin. All the suspected *Clostriduim* isolates were found to utilize glucose, 9 isolates were able to utilize galactose, 5 isolates utilized sucrose, 4 isolates utilized lactose, 5 isolates utilized starch and 4 isolates were able to utilze cellulose (Table 2). Based on the physiological characterization and utilization of different carbon source, between 24- 120 hours' incubation period during the fermentation, the ten *Clostridium* isolates were observed to produce alcohol (Table 3).

Table 1: Description of Site and Physicochemical Properties of Soil Samples

Location	Description Of Site	Crop Grown	Mean Temp (ºC)	Mean Soil pH	Moisture Content (%)
Naraguta	Farm lands, waste sites, gardens	Sweet potatoes, cocoyam, carrot, cabbage	18.2	6.93	12.79
Village	Farm lands, waste sites, gardens	Cocoyam, irish potatoes, maize, okro	19.7	6.94	15.61
Bauchi road	waste sites, garden, apiary, plant nursery	Pawpaw, Eucalyptus camadulensis, guinea corn, carrot	20.5	7.70	7.42
Central bank road	Floral garden	Flowers	20.7	6.63	13.04
Lamingo/kyan	Farmlands	Rice, sweet potatoes,sugar cane, pepper, tomatoes, cocoyam	23.1	5.83	20.98

Table 2: Biochemical Reactions and Sugar Utilization Test

Isolate	Indole	Urease	Curd formation	Rifampicin Sensitivity	Gelatin hydrolysis	Glucose	Galactose	Sucrose	Lactose	Starch	cellulose
4B	-	-	+	+	+	+	+	+	+	+	+
16A	-	+	-	+	+	+	+	+	-	+	+
16Aa	-	+	+	+	+	+	+	+	+	+	+
16B	-	-	-	+	+	+	+	+	+	-	-
16Bb	-	+	+	+	+	+	+	+	+	+	+
17Aa	-	-	-	+	+	+	+	-	-	-	-
18A	-	-	-	+	+	+	+	-	-	-	-
18Aa	-	-	-	+	+	+	+	-	-	-	-
18B	-	-	-	+	+	+	-	-	-	-	-
18Bb	-	-	-	+	+	+	+	-	-	-	-

Keys: + = Positive, - = Negative

Table 3: Preliminary Solvent Production Test

Isolates	0 (hr)	24 (hrs)	48(hrs)	120(hrs)
4B	_	1^o	1^o	1^o
16A	_	1^o	1^o	1^o
16Aa	_	2^o	2^o	2^o
16B	_	3^o	3^o	1^o
16Bb	_	3^o	1^o	1^o
17Aa	_	3^o	3^o	3^o
18A	_	3^o	3^o	3^o
18Aa	_	3^o	2^o	2^o
18B	_	3^o	3^o	3^o
18Bb	_	3^o	3^o	2^o

Keys: 1^0= Primary 2^0= Secondary Alcohols 3^0= Tertiary Alcohols

Table 4: Percentage Frequency of Occurrence of Isolates in the Various Locations within Jos Metropolis

Location	Frequency of occurrence	Total Number of isolates Studied	Clostridium species isolated	Percentage Frequency (%)
Naraguta	1	24	*Clostridium acetobutylicum*	4.17
Village	0	24	——	0.00
Bauchi Road (Forestry /Unijos)	9	24	*C. colinum,C.perfringens, C. acetobutylicum, C.botulinum, C. difficile*	37.50
Central Bank Road	0	24	____	0.00
Lamingo/Kwan	0	24	_____	0.00

Table 5: Clostidium Species Isolated From Soil Environment within Jos Metropolis and their Frequencies of Occurrence

Clostridium species	Frequency of Occurrence	Percentage Frequency
Clostridium acetobutylicum	2	20
Clostridium perfringens	2	20
Clostridium colinum	1	10
Clostridium botilinum	4	40
Clostridium difficile	1	10

Conclusion

This study illustrates the solvent producing potential of *Clostridium* species from the soil environment. Two species namely *Clostridium acetobutylicum* and *Clostridium auranbutyricum* are non-pathogenic. The pathogenic *Clostridium* species isolated were *Clostridium colinum, Clostridium perfringens, Clostridium botulinum* and *Clostridium difficile.* These isolates produced alcohol during fermentation. Wastes disposal sites and nursery gardens had the highest frequency of occurrence of isolates of the genus *Clostridium.* The data from the study proof that the soil environment is colonized by strains which are industrially useful. Advance techniques such as molecular analysis of the 16S rRNA of the Clostridium isolates can be used, so as to ascertain the identity of the isolates. The isolates could be screen for solvent production using HPLC (High performance Liquid Chromatography) and other more advanced methods. Quantification of the solvents produced by the solventogenic Clostridia during the fermentation can be employed.

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References

1. Abd-Alla *et al*. Acetone-butanol-ethanol production from substandard and surplus dates by Egyptian native Clostridium strains.Anaerobe,2015:32:77-86.
2. Advanced Bacterial Identification Software (ABIS) https://www.tgw1916.net/bacteria_logare_desktop.html
3. AWPA. American Wood Preserver Association Standard, 1986.
4. Kasap M. Nitrogen-fixation genes and nitrogenase activity in Clostridium acetobutylicum and Clostridium beijerinckii. Journal of Industrial Microbiology and Biotechnology,2002:26:1-6.
5. Keis S, Sullivan JT, Jones DT. Physical and genetic map of the Clostridium saccharobutylicum (formerly Clostridium acetobutylicum) NCP 262 chromosome. Microbiology,2001a:147(7):1909-1922.
6. Nageeb K, El Mubarak TS, Aidil AH. Bio butanol-production by a New Local Isolate of Clostridium acetobutylicum YML. AIP Conference Proceedings,2014:1614:523.
7. Noelling J, Breton G, Omelchenko MV, Makarova KS, Zeng Q, Gibson R, *et al*. Genome Sequence and Comparative analysis of the solventproducing bacterium Clostridium acetobutylicum J Bacteriol,2001:183(16):4823-4838.
8. Sauer M. Industrial production of acetone and butanol by fermentation—100 years later.Biotechnology & amp; Synthetic Biology, 2016. doi: 10.1093/femsle/fnw134
9. Uppin RM. Isolation and Characterization of Butanol producing Clostridium isolates from the Diverse sources. Department of Agricultural Microbiology College of Agriculture, Dharwad University of Agricultural Sciences, Dharwad, 2013.