Original Research Article

ISOLATION AND IDENTIFICATION OF MICROORGANISMS ASSOCIATED WITH BIOREMEDIATION OF OIL SPILLED SITE IN BODO RIVERS STATE, NIGERIA.

ABSTRACT

The samples of spilled water collected from Bodo West in Gokana Local Government of Rivers State were isolated to identify microorganisms associated with bioremediation and the isolate was use for biodegradation of hydrocarbons. The microorganisms isolated may have the potential of biodegradation of hydrocarbon which may show evidences of bioremediation. Carbon source from petrol, diesel and kerosene were used. The evidence of degradation was based on the increase in the number of cells from day 1 to day 4. The colony forming unit was used to count the increase in the number of cells. The results of the analysis show that notable number of microorganism of which seven bacteria and seven fungi were isolated and identified. The bacteria are Micrococcus Luteus, Streptococcus Lactic, Streptococcus Epidemidis, Streptococcus Faecalis, Clostridium Sprogenes, Aerococcus Viridems, and Bacillus Anthracis. The fungi are Articulosspara inflate, Dendospora Erecta, Aspergillus Niger, Liodioderium Species, Geotichrum Albdum, Aspergillus Funigatus and Sreptothric Atrax. On the strength of the result, it is inferred that microorganisms are associated with bioremediation and can be used for environmental and petroleum cleanup exercise in an oil spilled site.

Keywords: microorganisms, biodegradation, bioremediation, hydrocarbons, oilspilled, isolation, identification.

1. INTRODUCTION

Petroleum exploration is a lucrative business especially in Nigeria [7,12,14,15,34]. Nigeria since the discovery of oil has survived on the proceeds from oil production, as capital projects and paying of workers' salaries is dependent on income generated from the oil business [7,12,14,15,36]. Though, there had been calls from different quarters for diversification of the economy from the solo means of petroleum exploration into other sectors like agriculture, commerce and manufacturing [14]; however the current gains for petroleum resources has overshadowed government interest in other areas of the economy [13,32].

Petroleum exploration involves a complex process; from drilling, refining to the distribution of the products to the different marketers and end users [13,36]. The processes has its own associated environmental problems as oil spills on a large scale on the land, sea or river and massive air pollution has been reported [1,8,9,11,12,16,17,18,21,29,30]. Government had in the past carried out environmental programmes to educate the people on the consequences of pollution [22,32,35]; but the people has always these rejected government programmes due to their non-participation in the decision making process [31]. Such agitations by the people in the local communities have always resulted in to violent conflicts [10,19,23,24,25].

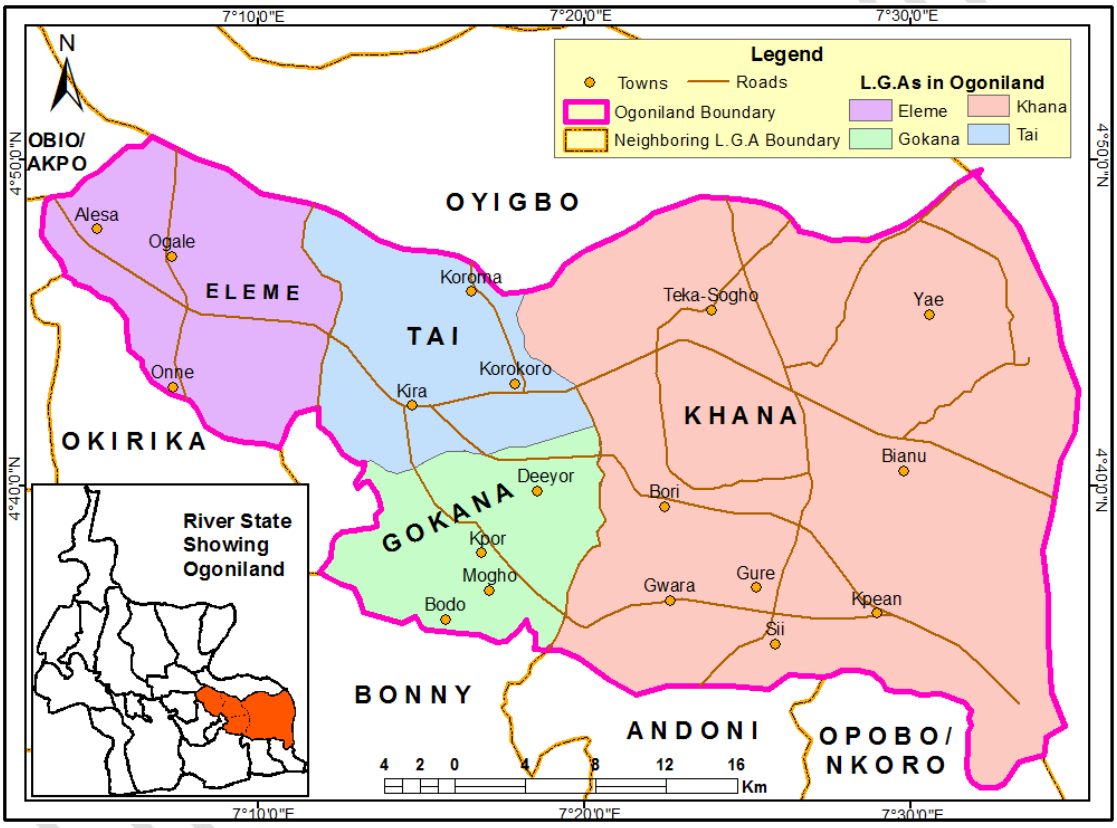
Hydrocarbon contamination of the environment has not only destroyed the ecosystem, but has also resulted in several health challenges and deaths [33]. Thus, there had been calls for remediation of polluted land in the Niger Delta [31]. Mechanical and chemical methods are generally used to remove hydrocarbons from contaminated sites [26-28]. These methods have limited effectiveness and can be expensive; so bioremediation is a promising technology for the treatment of these contaminated sites,

since it is cost effective and will lead to complete mineralisation [26-28]. The process of bioremediation is simply the used of microorganisms to remove pollutants from the polluted environment through the establishment and maintenance of a condition that favours oil biodegradation rates in the contaminated environment [26-28,2-5].

Bioremediation become a process of interest in the petroleum industry due to the success in the cleanup of the oil tanker Exxon Valdez of oil spill of 1989 [6,26-28). Bioremediation is an attractive technology that has gained popularity in the global conservation and sustainability strategies [26-28].

2. THE STUDY AREA

Bodo West is a village settlement in Gokana Local Government Area in Ogoni. Ogoni which is a superset of Bodo West lies between latitude $4^{0}05^{1}$ and $4^{0}20^{1}$ North and longitude between $7^{0}10^{1}$ and $7^{0}30^{1}$ East [36] as shown on figure 1. The site is in Rivers Basin. It is accessible by roads and footpath and some parts that are covered by thick vegetation were inaccessible.



Map of Ogoni indicating the position of Bodo West [36]

3. MATERIALS AND METHODS

Source of Samples

Samples were collected from contaminated oil spilled sites at Bodo West, Gokana Local Government Area of Rivers State and analysed in Jossy laboratory, Port Harcourt.

Sterilization Techniques

Throughout the case of this project, a sterile condition was maintained. The material used was sterilized in different form according to the nature of the materials.

Method of Sample Collection

Samples were collected using aluminium foil or glass wares. PVC materials, plastics or polythene materials were avoided because they contain hydrocarbon. This was done to ensure quality control.

Culture Media

The sterilization of the media was carried out by heating under pressure autoclave. This was carried out at 121^{o}C for 15 minutes.

Inoculating Chambers

Since air always contained dust particles that generally have a population of micro organism, it was carried out in inoculating chamber. The chamber was sterilizer with ethanol by swabbing.

Inoculatin Loops

Aseptic transfer of culture from one tube of another was accomplished with an inoculating loop. This was sterilized by incinerating the loop in a flame while forceps was sterilized by dipping it in alcohol and flaming.

Glass Ware

This glass ware was washed, rinsed with tap water and finally with distilled water. The glass ware was sterilized first in autoclave at 121^{o}C for 15 minutes to get rid of all the microorganisms before normal washing. After washing, they were heated at a mode rate temperature with the use of hot air oven. The sterilization period was 160^{o}C for 1 hour. In the process precaution was taken to pack them so tightly to impede the circulation of trapped air.

Bench Tops

Bench tops and cabinet was sterilized using 40% formalin absolute alcohol dilated to a concentration of 70%.

Incubator

The incubator was used to create an enabling environment for microorganism to grow on any material. The pleasant and conditioning of an already inoculated culture medium is the specific environment that favours the growth of microorganisms in the laboratory. This was carried out by using incubator with the door and the entire air closed for 48 hours.

Isolation of Organism

Millilitre of sample of water liquor was withdrawn and transferred into test tubes containing sterile distilled water in preparation for sterile distilled water in preparation for serial dilution to 10^{-8} dilution which 10^{-1}, 10^{-4}, 10^{-8} and stock was used. It was carried out with the aid of sterile syringes. Nutrient Agar (2.8g was dispersed into conical flask containing 100m/s distilled water and autoclaved at 121^{o}C at 15 minutes.

About 0.5mls of selected four dilution factors source was dispensed into sterile Petri dishes. Potato dextrose Agar (3.6g) into 100mls distilled water in a conical flask was used for fungi by pour plate method, the Petric dishes already inoculated with diluted isolate and incubated at 37^{o}c for 48 hours for

bacteria and normal room temperature for fungi for 48-72 hours. After incubation, growth was observed and discrete calories developed on the surface of the plate. It was sub-cultured to get the pure culture isolated. Sample was appropriately labelled.

Pour Plate

This involves the preparation of the dilation of inoculums. The inoculums dilution is added to sterile petric dish mixed with molten but cold media and allowed to solidify after incubation; some calories develop embelled and in addition to others on the surface of the plate but the plate were incubated in closed container to avoid oxygen.

Streak Plate

The streak plate method appeared to be the most widely and routinely used method of plating in laboratory. It involves the sterilization of wire loop by passing it over blue flume until it turns reddish.

The sterile, loop was used to pick a loopful of the organism and immediately used to make series of parallel, non overlapping streak on the surface of the already solidify medium. The final streak will not allow touching the source of the inoculums on the plates. The initial streak result to confluent growth well isolate discrete colonies developed along the streaks on the plate. The discrete colonies on the plate are clearly pure culture and further sub-culture was made from them for microbiological investigation.

Total Viable Counts

This test was performed by pour plate techniques using nutrient Agar and fuel Agar (i.e. Kerosene, diesel, and petrol).

The medium was prepared and sterilized; the sterile medium was allowed to cool. Duplicate of pour plate of each sample were prepared by pipeting aseptically 0.1mls of sample into each sterile Petri dish used for each sample and was discarded to avoid cross contamination.

The sterile and cool medium were poured aseptically and used to overlap the sample in the Petric dish. The plate was rocked gently in single circular motion to allow proper mixing of medium and sample. It was allowed to solidify and incubated in an upside position at 28C-37^{o}C for 24 hour in a gallon Kamp incubator. The plates were examine for microbial growth after 24 hour and colonies were counted and recorded as colony forming units per millilitre.

Gram Staining

This test was performed by pour plate techniques using nutrient Agar and fuel Agar i.e (Kerosene, Diesel, and petrol).

The medium was prepared and sterilized; the sterile medium was allowed to cool.

Duplicates of pour plate of each sample was prepared by pipeting aseptically oilms of sample into each sterile Petric dish used from each sample and was discarded to avoid cross contamination. The sterile and cooled mediums were poured aseptically and use to overlap the sample in the Petric dish. The plates were rocked gently in single circular motion to allow proper mixing of medium and sample. It was allowed to solidified and incubated in an upside position at 28^{o}C – 37^{o}C for 24 hour in a gallon

Kamp incubator. The plates were examined for microbial growth after 24 hour and calories were counted and recorded as colouring forming units per millilitre.

Gram Staining

The gram-staining was carried out as follows:- A loopful of water was dropped on a slide by sterile inoculating loop. Inoculums from the young culture mixed with water in the film over the slide. The smear was allowed to dry heated and fixed gently by passing it over the bursen flame. The smear on the slide flooded with crystal violet solution of for 60 second, and rinsed with water. All the cells turned purple, the smear will again flooded with fugal iodine for 1 minute and rinse 95% alcohol was poured on the slide until the crystal violet was completely washed off. It will then counter strained with safaranin and wash with water. At this point gram positive cells remained purple while gram negative remain pink. This was viewed by means of oil immersion objective of the light microscope.

Staining of Fungi

Heat fixed smear was obtained with cotton blue lacto phenol for 1 minute, washed with distilled water and dried. The slide was viewed under the microscope using objective x 100.

Biological Test

a. Catalase Test

Most of the aerobic microorganisms are capable of producing the enzymes catalane. A loopful of 48 hour culture of each isolate was placed on clean slide of which a drop of hydrogen peroxide had been added. A formation of oxygen bubbles of effervescent caused by liberation of oxygen gas indicate the presence of catalane.

b. Sugar Fermentation

Sugar fermentation was used to test for the ability of the organism to different sugar by breaking it down to alcohol. Sugar such as sucrose, maltose, lactose, arabinos and mentol was used. 0.5% carbon source was taken and emptied into McCartney bottle, containing peptone water and phenol red as an indicator. The bottle was autoclaved for 121^{o}C for 10 minutes. The autoclaving with tested organisms was incubated with the inoculums. It was later used to compare the colour changes in tubes. Changing in colour turned to yellow which indicates complete fermentation.

c. Coaculate Test

A small quantity of 24 hour old culture was taken with sterile inoculating loop; it then mixed with 1ms of 10% V/V dilution of plasma on clean slide. Clothing of the mixture indicate positive test.

d. Motility Test

A 24 hour broth culture was taken with sterile inculpating loop into a cover slide, hanging drop technique was used. It was viewed with the help of oil emission of X100 objective of light microscope. Movement indicates motility positive while non-movement indicates motility negative.

Biodegradation Test

Kerosene agar was used after serial dilution to know the total number of organism that utilizes such organics solvent (i.e Kerosene), petrol and diesel. This was known through the increase in the number of cell per day.

4. RESULT

Organism isolated and identified were seven fungi and seven bacteria. The bacteria isolate are clostridium sparogerms. Aerococus viridians, streptococcus Lactic, Micrococcus Lutes, Staphylococcus Lactic, Staphylococcus Epidermis, Streptococcus epidermis, Streptococcus Faecalis, Bacillus anthraces. The seven fungi isolated and identified are: *Articulospara Infalta, Dendospora erecta, Aspergillums Niger, Loidioderium Species, Geotichrum Albidum, Aspergillums funigatus and Streptothrix Atrax*. The result is shown on the table below.

Table 1: Biochemical/Morphological and microscopic identification of bacterial isolate.

Catalase	-	-	-	-	-	-	-	Z+
Motility	-	-	-	-	-	-	-	-
Hydrolysis	+	+	+	+	+	+	+	+
Glucose	A	A	A	A	A	A	A	A
Lactose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
maltose	+	+	+	+	+	+	+	+
Arabinos	+	+	+	+	+	+	+	+
Coagulase	-	-	-	-	-	-	-	-
Shape	Circular	Sphere	sphere	Sphere	Round	Round	Dombel	Round
Edge	Dented	Enteric	Dented	Dented	Dented	Dented	Dented	Enteric
Elevation	Raised	Raised	Raised	Raised	Raised	Raised	Raised	Raised
Surface colour	Smooth	Smooth	Smooth	Smooth	Smooth	Rough	Smooth	Smooth
Pigmentation	Creamy	Creamy	Creamy	Creamy	Creamy	Pinkish	Pinkish	Creamy
G-stain	+ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve
Probable organism	1 clostridium sprogenes	2 Aerococcs Viridams	3 Streptococcs lactic epidemis	4 Micrococcs luteus	5 Staphylococucs luteus	6 Streptococcs lactic faecalis	7 Streptococcs lactic faecalis	

KEY

+ = Positive
- = Negative
A = Acid Production

TABLE 2: Culture and Microscope Characterization of Fungi Isolate

Cultural characteristics	Microscopic	Identification
White mycelia growth on PDA after 24 hours.	Cordidiophore hyaline slender upper part sparingly branch conidia.	Articlospara inflate
Submerge aquatic with branched septate mycelium, simple cordidiophore slender hyaline.	Whitish cotton like mycelia which turns red on PDA plate.	Dendropspora erecta
Black mycelia on culture media after 48 hours.	Chain of conididial bonne on phial ides with black glucose head supported by septet were observed.	Aspergillus Niger
Whitish mycelia which later turns grey on APDA plate.	Mycelia external conidiophores upright simple upper portion which increase in length as conidia formed.	Oidioderium species
White septate mycelia on PDA plate	Conidia arthrospore hyaline J. celled shut cylindrical with truncate end.	Geotrichum albidum
Gray mycelia on PDA Plate which were dusty.	Conidiophores upright simple terminating in a globule or elevate swelling bearing phralites at apex.	Aspergillus fumigates
Dark mycelia on PDA plate.	Loosely tall mycelia tall conidiophores branch spirally coiled.	Streptothric Atra

TABLE 3: TOTAL COLONY COUNTS IN (KEROSENE FUEL, DIESEL) AGA R MEDIA.

Day 1

	DIESEL ($X10^{-4}$)	KEROSENE ($X10^{-4}$)	PETROL ($X10^{-4}$)
Clostridium Sprogenes	2	2	2
Aerococcus Viridams	2	3	4
Streptococcus lactic	-	1	2
Micrococcus luteus	1	3	2
Streptococcus epidemidis	-	2	4
Streptococcus faecalis	2	1	3
Bacillus anthracis	-	1	4

Day 2

	DIESEL ($X10^{-4}$)	KEROSENE ($X10^{-4}$)	PETROL ($X10^{-4}$)
Clostridium Sprogenes	3	2	3
Aerococcus Viridams	5	1	5
Streptococcus lactic	2	2	4
Micrococcus luteus	4	2	3
Streptococcus epidemidis	6	3	5
Streptococcus faecalis	5	3	7
Bacillus anthracis	7	2	6

Day 3

	DIESEL ($X10^{-4}$)	KEROSENE ($X10^{-4}$)	PETROL ($X10^{-4}$)
Clostridium Sprogenes	7	5	11
Aerococcus Viridams	9	10	16
Streptococcus lactic	4	8	14
Micrococcus luteus	6	4	11
Streptococcus epidemidis	8	9	4
Streptococcus faecalis	9	6	8
Bacillus anthracis	15	10	12

Day 4

	DIESEL ($X10^{-4}$)	KEROSENE ($X10^{-4}$)	PETROL ($X10^{-4}$)
Clostridium Sprogenes	9	8	20
Aerococcus Viridams	15	17	30
Streptococcus lactic	8	7	22
Micrococcus luteus	11	14	7
Streptococcus epidemidis	15	9	16
Streptococcus faecalis	10	13	22
Bacillus anthracis	70	11	17

5. DISCUSSION AND CONCLUSION

The results obtained from this study revealed that arrays of microorganisms are associated with body of water. A total of seven fungi and seven bacteria were isolated as shown on table 1 and 2. Most of these colonies isolated are predominately microorganism that resides in water. The moisturazation of the water body and its creaks create a conductive environment for the growth of microorganism and relationship exist between the enzymatic activity of fungi and amount of water in colonize substrate. The maximum relative humidity permitting the growth of fungi varies from 75% - 95% for different species of microorganisms.

The presence of microorganism in water which act to degrade the hydrocarbon components of contaminant, get the resident flora of certain contaminated water and play an important role in maintaining and bringing back the contaminated water to its uncontaminated state.

This study focused on bioremediation and treatment of high volume hydrocarbon wastes. Evidence also provided that in land farming practices as in conventional bioremediation systems, a large fraction of the volatile hydrocarbons is not biodegraded but is rather transferred to the atmosphere through volatilization. An increasing focus on regulation and control of volatile organic carbon emissions calls for hydrocarbon remediation and waste treatment system which contain or destroy the volatile organic fraction.

The interest for exploiting petroleum degrading organism for environmental cleanup has become central to petroleum geoscientist as this study has clearly shown that bacteria and fungi can be use to degrade or detoxify pollutants or contaminant in a given environment.

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