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Research Paper

ISOLATION AND CHARACTERIZATION OF NITROGEN-FIXING BACTERIA IN THE SOIL

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Four soil samples were collected from different sources, two of these soil samples were from Thinkers corner land and Emene beans farm land while the remaining two were from maize (*Zea mays*) and legume farm lands, Ugbochime Community all in Enugu State, Nigeria. The nitrogen-fixing media used were *Azotobacter* medium, *Azospirillum* medium and *Clostridium* medium. The pH of these media were adjusted to 7.4, 6.8 and 5.4, respectively. The isolates were subjected to various biochemical tests for differentiation. The bacteria isolated were *Azospirillum*, *Azotobacter* and *Clostridium* species. *Azospirillum* formed colonies were milky, slimy, circular and raised while *Clostridium* spp. produced gas bubbles and rancid odor.

Keywords: *Azospirillum*, *Azotobacter*, *Clostridium*, Isolates, Colonies and Soil samples

INTRODUCTION

Nitrogen-fixing bacteria are widely distributed in nature where they reduce atmospheric nitrogen in soil or in association with plant (Skinner and Banfield, 2005). They have been found in a wide variety of terrestrial and aquatic habitats in both temperate and tropical regions of the world (Yooshinan, 2001). Nitrogen-fixing bacteria are found in symbiotic associations with plants (Klein, 2000).

The only confirmed free living nitrogen-fixing bacteria belonging to the kingdoms *Eubacteria* and *Archaeobacteria* are currently known to fix nitrogen (Perotto and Bonfante, 2010). Many are heterotrophic, needing a supply of reduced

carbon, for example, *Azotobacteria* and *Azospirillum* species. Others are autotrophic, that is, they reduce carbon dioxide (Graham, 3000). For instance *Rhodospirillaceae* use light energy to do this by a process which, unlike photosynthesis in *Cyanobacteria* and higher plants does not evolve oxygen.

Nitrogen fixation generally occurs only under anaerobic or microaerophilic (i.e., low concentration of oxygen) conditions and only few strains of a particular species show activity (Scow, 2007).

The non-autotrophic genera depend indirectly upon light energy for nitrogen fixation. They must be supplied with suitable carbon compounds

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originally derived from photosynthesis, but made available by respiratory pathways. The symbiotic *Rhizobiaceae* and *Streptomycetaceae* obtain these compound from their hosts, but free living forms must obtain them in open competition with other microorganisms (Oliverri and Frank, 2009).

Soil nitrogen is the most difficult nutrient to characterize. It occurs in organic and inorganic forms in solution and as a solvent. Of all the elements that enter into the composition of vegetable and animal substances, nitrogen is the most expensive, evasive, and difficult to replace. The aim of this research was to isolate and characterize nitrogen-fixing bacteria from soil.

MATERIALS

Conical flasks, pipette, petri dishes, bijou bottles, test tubes, hydrogen peroxide, filter paper, cotton wool, durham tubes, aluminium, glass slides, pH meter, wire loop, incubator, autoclave, electronic weighing balance and microscope.

REAGENT USED

Nitrogen free media, normal saline, distilled water, hydrogen peroxide, immersion oil, Kovac reagent, peptone water, gram staining reagents, glucose, galactose, lactose, sucrose and mannitol

MATERIALS AND METHODS

Sterilization of Glass Wares

Properly washed Petri-dishes, test tubes, conical flasks, beakers, pipettes, wire loop knives, spatulas, etc., were sterilized in hot air oven at 180°C for two hours (2 h) and stored at 4 °C.

Sample Collection

The soil samples were collected by the use of a hand glove, spatula and sterile polythene bag. The soil samples were collected from different areas

in Thinkers Corner Emene, Ugbochime environment and farm lands located off the main gate of the area. At each of the collection sites, spatula was used to remove the over laying earth and sample collected from about 3 cm depth.

Preparation Of Soil Samples

The soil samples were first ground with sterile mortar and pestle to liberate the adhering microorganism before their suspension was prepared. 1 g of the soil sample was weighted out and dissolved in 9 ml of distilled water in a beaker and homogenized. Also nine fold serial dilution of the homogenized sample was made.

Media Preparation

Nitrogen free media was used for the isolation of nitrogen fixing bacteria. However, specific types of such organisms and specific components are usually needed for differential and enrich media purposes.

Azospirillum Medium

Preparation of solid agar medium (Hurst *et al.*, 2000).

The components of this medium were as follows:

Distilled water	100 ml
K ₂ HPO ₄	0.5 g
MgSO ₄ .7H ₂ O	0.2 g
NaCl	0.1 g
KOH	4 g
Yeast extract	0.05 g
Agar agar	5 g
PH	6.5 g

Procedure

Each of the components was measured with weighing balance and put into conical flask containing 100 ml distilled water and then covered

with aluminium foil. The pH of the medium 8.2 was adjusted to 6.8 using pH meter after this process. The medium was autoclaved at temperature of 121 °C for 15 min and allowed to cool and was poured into Petri dishes.

Azotobacter Medium

Preparation of solid (agar) medium and the components of the medium are as follows

Distilled water	100 ml
Glucose	2 g
CaCO ₃	2 g
K ₂ HPO ₄	0.1 g
MgSO ₄ .7H ₂ O	0.05 g
pH	7.4
Agar agar	1.5 g

Procedure

Each of the components was measured with the weighing balance and put into a conical flask containing 100 ml distilled water. The pH was adjusted to 7.4 using pH meter and batches of the medium were autoclaved at 121°C for 15 min. After autoclaving, a visible precipitate was present, which disappeared after several days at room temperature. Later, the medium was poured into Petri dishes and conical flask for solid agar medium.

Clostridium Medium

Components of the solid agar medium

Distilled water	100 ml
Sucrose	0.001 g
KH ₂ PO ₄	0.05 g
MgSO ₄ .7H ₂ O	0.05 g
NaCl	0.00015 g

FeSO ₄	0.001 g
Agar agar	1.5 g
pH	5.4

Procedure

This medium was prepared as described in the Reinforced Clostridial Medium. This is a solidified version of Reinforced Clostridial Medium and can be used for the enumeration of anaerobes by pour plate, shake tube or membrane filtration methods. When solidified in tubes or bottles with minimal head space it can be used for anaerobic culture without the need for anaerobic atmosphere.

Bacteria Isolation and Purification

Individual nitrogen-fixing microorganisms were isolated by spread-plate on the solid medium. 0.5 ml portion of each sample was pipetted and plated out on the solid medium. A glass spreader, sterilized with alcohol and flame was used to spread the inoculums evenly on the plates. The plates were incubated at room temperature (30±2 °C).

Purity was achieved by sub-culturing consecutively on nutrient agar plates which were prepared by dissolving 2.8 g of nutrient agar in 100 ml of distilled water and autoclaved at 121°C for 15 min.

Inoculating the Slant Culture Medium

Nutrient agar was homogenized and then poured into well washed bijou bottles and tightly closed. They were sterilized in the autoclave at 121°C for 15 min. After autoclaving they were slanted and allowed to solidify. Slant culture medium was inoculated with purified bacteria culture obtained by isolation and purification processes.

Characterization Of Isolates

Macroscopy was done by observing morphology

and cultural characteristics of the isolates on the nutrient agar plates.

Gram Reaction

This was used to differentiate bacteria into two main groups, gram positive and gram negative.

Procedure

Using a clean slide and sterile wire loop, a loopful of normal saline was dropped at the center of the slide and loop sterilized again, Specimen was collected with the wire loop. A smear was made in a circular manner. After making the smear, it was heat-fixed on the slide by passing it gently over the Bunsen flame. Crystal violet were put on the smear for 30 s, and poured off. Lugol's iodine's (Mordant) was poured on and left for 1 min. It was then washed off with water. The decolonization was done with acetone/alcohol very briefly. The acetone/alcohol was poured off and water poured immediately. Counter staining was done with water within a minute. When the surface was dry immersion oil was added and then viewed with x 100 oil immersion objective.

Indole Test

The tryptophan broth was inoculated with the test sample and incubated at 37°C for 28 h then 0.5 ml of the Kovac's reagent was added and gently agitated and examined after 1 min. The upper layer of the liquid in the test tube turned red, indicating a positive result.

Motility Test

This test was done using the hanging drop method. A drop of the test organisms in a saline suspension was placed on a cover slip. The cover slip was inverted and placed on a cavity slide, this was viewed under the microscope; a sharp darting movement in different directions across the field of view of the microscope indicated a

positive result motility and showed that the organism had locomotive apparatus like *flagella* on that they can move.

Carbohydrate Fermentation

This is also called sugar fermentation test. These tests were used in distinguishing fastidious and non fastidious organisms. The sugars used were Glucose, Sucrose, Lactose, Mannitol and Galactose. 1.5 g of peptone water was added to 100 ml of distilled water plus 1 g of each sugar in Erlenmeyer flasks. A sterile pipette was used to transfer 0.5 ml of methyl red indicator into the flask. It was dispensed into test tubes and inverted Durham tubes introduced into the test tubes. They were autoclaved at 100 °C for 10 min, allowed to cool and inoculated. It was incubated for 24 h. Acid production occurred when there was a breakdown of sugar by the bacteria.

Catalase Test

The isolates were transferred with sterile wire loop into hydrogen peroxide (H₂O₂) on a slide. Effervescence showed a positive result.

Spore Staining

This is used to test the presence, shape, and position of spores in bacterial isolates. Heat-fixed smear of the test isolates were heated to boiling with a mixture of 5% aqueous solution of malachite green and 0.5% safranin. Heat fixed smear of the sample was made on a clean slides. The slide was flooded with malachite green and heated from below with the bunsen flame. Until when the flame was removed, steam was seen rising from the stain.

The dye was allowed to act with the steam rising from it for a minute. The stain was washed off thoroughly with distilled water and the slides drained. The slides were also flooded with

safranin and left to act for 30 s. The slides were washed off with distilled water, drained and blotted dry with absorbent paper. When completely dry a drop of oil immersion was added and observed under the microscope which showed the pale green colour of the spores.

Methyl Red-voges Proskauer Test

In methyl red test, glucose 0.5 g, peptone water 1.5 g and di-potassium hydrogen phosphate 0.5 g were added into 100 ml of distilled water. Sterilization lasted for 15 min at 121 °C. After cooling it was inoculated and incubated for three days. Five drops of methyl red was added into the bottles. Red color showed a positive result for methyl red while negative result was positive for voges proskauer.

Coagulase Test

Here slide coagulase test was used. Distilled water was used to emulsify the culture on a slide. A drop of human plasma was placed on the culture and mixed with the aid of a wire loop. After the mixing, the slide was examined for coagulation after 2 min. Presence of clumping indicated a positive result. The wire loop was sterilized after each test by passing it over the flame.

RESULTS AND DISCUSSION

According to the results, only a few bacteria genera were isolated and they were *Azospirillum*, *Azotobacter* and *Clostridium* species. This could be as a result of few media that was used (nitrogen free-media). Isolation of these organisms from soil samples were examined clearly and confirmed their ubiquity as noted by Skinner and Banfield (2005). *Azospirillum* and *Azotobacter* were grown under aerobic conditions while *Clostridium* was under anaerobic condition.

Table 1 shows the morphological Characteristics of bacteria isolated from soil samples gotten from roots of maize and Beans plants, and the presumptive organisms were *Azotobacter* sp, *Azospirillum* sp, and *Clostridium* sp.

Table 2 shows the biochemical test on the organisms isolated. The present report confirms the occurrence of nitrogen-fixing bacteria in the *rhizosphere* of legume and *Zea mays*. In plant roots a large variety of materials are released to their surrounding soil, including various sugars, amino and organic acids, alcohol and vitamins. However, it was observed that these species were able to grow on glucose, galatose, mannitol, lactose and

Table 1: Morphological Characterization of Isolates

Samples	Medium Used	Morphological Characteristics	Gram Stain	Implicatedorganism
1.	<i>Azospirillum</i> Medium	Brownish Smooth and flat	-	<i>Azospirillum</i> Sp
2.	<i>Azospirillum</i> Medium	Brownish Smooth and flat	-	<i>Azospirillum</i> Sp
3.	<i>Azospirillum</i> Medium	Brownish Smooth and flat	-	<i>Azospirillum</i> Sp
4.	<i>Azospirillum</i> Medium	Brownish Smooth and flat	-	<i>Azospirillum</i> Sp
5.	<i>Azotobacter</i> Medium	Milky, Slimy, Circular and raised	-	<i>Azotobacter</i> Sp
6.	<i>Azotobacter</i> Medium	Milky, Slimy, Circular and raised	-	<i>Azotobacter</i> Sp
7.	<i>Azospirillum</i> Medium	Brownish Smooth and flat	-	<i>Azospirillum</i> Sp
8.	<i>Clostridium</i> Medium	Grey white, Irregular and raised	+	<i>Clostridium</i> Sp
9.	<i>Clostridium</i> Medium	Grey white, Irregular and raised	+	<i>Clostridium</i> Sp

Key: - = Negative; + = Positive.

Table 2: Biochemical Characteristics of Isolates

Samples	Motility	Catalase	Hydrogen Sulphide	Coagulase	Indole	Methyl Red	Voges	Glucose	Sucrose	Lactose	Mannitol	Galactose	Implication Organism
1	+	+	+	-	-	+	-	AG	AG	A	A	AG	<i>Azospirillum Sp</i>
2	+	+	+	-	-	+	-	AG	AG	A	A	AG	<i>Azospirillum Sp</i>
3	+	+	+	-	-	+	-	AG	AG	A	A	AG	<i>Azospirillum Sp</i>
4	+	+	+	-	-	+	-	AG	AG	A	A	AG	<i>Azospirillum Sp</i>
5	-	+	-	-	+	-	+	AG	AG	AG	AG	AG	<i>Azotobacter Sp</i>
6	-	+	-	-	+	-	+	AG	AG	AG	AG	AG	<i>Azotobacter Sp</i>
7	+	+	+	-	-	+	-	AG	AG	A	A	AG	<i>Azospirillum Sp</i>
8	+	-	+	+	-	+	-	AG	A	AG	AG	AG	<i>Clostridium Sp</i>
9	+	-	+	+	-	+	-	AG	A	AG	AG	AG	<i>Clostridium SP</i>

Key: - = Negative; A = Acid production; + = Positive; AG = Acid and Gas production.

sucrose. The above observation was in agreement with that of Hurst *et al.*, (2000) who earlier reported the ability of members of these species to use these sugars as sole carbon source in a medium. However, from this research it was found that *Azospirillum* sp dominated almost half of the members of the other two genera *Azotobacter* sp and *Clostridium* sp.

CONCLUSION

Nitrogen-fixing bacteria of the genera *Azospirillum*, *Azotobacter* and *Clostridium* are present in the soil. These organisms were isolated from the soil samples using nitrogen-free media, such as *Azospirillum* medium, *Azotobacter* medium and *Clostridium* medium. Species of these genera were isolated and characterized in this work based on their morphological and biochemical properties. These organisms used various carbohydrates such as galactose, mannitol,

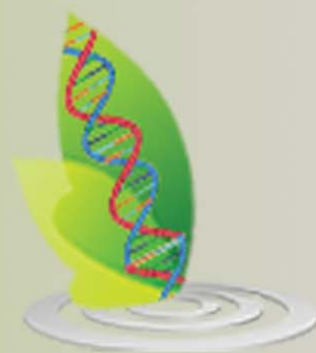
glucose, sucrose and lactose as sources of carbon and energy for growth.

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