IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 7, Issue 5 (Sep. – Oct. 2021), PP 14-18
www.iosrjournals.org

Isolation and Characterization of *Azotobactr* Species from Three Soil Samples in Enugu State Nigeria

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Abstract
As an alternative to chemical fertilizers, biofertilizersare becoming increasingly used in agriculture to enhance the growth of crops. Azotobacter species is among the microorganisms exploited in this technology. This study aimed at isolation and characterization of Azotobacter species used in biofertilizer. The organism was isolated from composite soil samples and cultured using Ashby's agar. Biochemical characterization and Gram staining technique were carried out on the isolates. Analysis of the soil samples were further carried out to determine the nitrogen contents, electrical conductivity and pH. Biochemical characteristics indicated that the Azotobacter was a Gram negative organism and positive for indole, starch hydrolysis, catalase and methyl red tests. The results indicated the possibility of isolation of Azotobacter species in soil samples could be further used in biofertilizer.

Key words: *Biofertilizer, Azotobacter, Microorganism, Chemical fertilizer, Analysis, Isolation and Biochemical test*

Date of Submission: 09-10-2021 Date of Acceptance: 23-10-2021

I. Introduction

Biofertilizers are preparations that are made up of living cells of efficient strains of microbes that help crop plants take up of nutrients by their interaction in the rhizosphere when applied through seed or to the soil (Nur, 2018). Biofertilizers are classified based on the type of micro-organism: algal biofertilizers such as blue green algae (BGA), fungal biofertilizers which includes mycorrhiza and bacterial biofertilizers, such as Azospirilum, Azotobacter, phosphobacteria and Rhizobium (Fernandes and Satish, 2015). Biofertilizer from nitrogen fixing bacteria are in three forms: liquid, solid and lyophilized. For liquid and lyophilized ones, only solution medium is used but for solid form, carriers such as peat, activated charcoal and chicken dung are needed (Gomare *et al*, 2013).The use of chemical nitrogen and phosphorus fertilizers at high level has an adverse effect upon accumulation of NH_4^+, $N0_3^-$, NO_2^- and PO_4^- in vegetable tissues and other crop plants (Bhattracharyga *et al*, 2010). Thus the need for an alternative like biofertilizers which are organic, produce high yields with the best commodity quality without contamination and less accumulation with heavy metal (Rao *et al*, 2014). The application of biofertilizers in preference to chemical fertilizers offers economic and ecological benefits by improving soil's health and fertility (Abbasniazare *et al*, 2012). The intensive use of biofertilizer naturally activate the micro-organisms found in the soil, restoring the soil's natural fertility and protecting it against drought and soil diseases and stimulating plants' growth (Abbasniazare *et al*, 2012). Azotobacter are known to produce different types of secondary metabolites such as vitamins, amino acids, plant growth hormones, antifungal compounds and siderophore (Myresiotis *et al*, 2012). These growth-promoting substances such indole-3-acetic acids, nicotinic acid,auxin, lumichrome and mimics starch have direct influence on plant growth and development of several agricultural crops (Chennappa *et al*, 2014).

II. Methods

Collection of soil samples

Soil samples were collected from Esut, Amuri and Agbani farmlands of Enugu State Nigeria using a clean sterile polyethene bags. The samples were collected from 5-10 cm depth, pebbles and plant residues were removed from the samples and were labelled

Determination of nitrogen content of the soil

A quantity, 10 g of sodium sulfate anhydrous (Na_2SO_4) was weighed into three different petri dishes and 1 g of NaOH was added into the same petri dishes followed by addition of 1.5 g of soil samples. The above samples were poured into separate Khajadal flasks and 20 ml of tetraoxosulphate (vi) acid (H_2SO_4) was added

into each flask. They were heated until a light greenish colour was observed. The samples were poured into three different beakers and allowed to solidify. During the distillation process, 10 ml of the digested samples were pipetted into Khajadal flasks. A volume, 10 ml of 40% Sodium hydroxide (NaOH) was added and a bluish colour was observed. Then a little portion of zinc powder was added as an antiboiling agent. A volume, 5ml of boric acid was pipetted into three different test tubes and 2 drops of methyl red were added which turned into pink colour after which the distillation process started. They were allowed to start boiling before timing for 15 mins. The distillates were allowed to cool and titrated with 0.1 HCl, a pink colour indicates positive result for nitrogen.

Determination of soil pH

A quantity, 10 g of the soil sample was weighed into a beaker and 40 ml of distilled water was added to it. The suspension was stirred with a glass rod and allowed to stand for 30 mins. The pH meter was calibrated with two buffer solutions (4.0 and 7.0).The sample was stirred again before measuring the pH. The electrode was carefully positioned in the solution just above the sand layer and measurement was repeated three times to ensure accurate results. The pH was recorded to the nearest 0.1 unit and electrode was triple rinsed with distilled water before another sample was tested (AOAC, 2018).

Determination of electric conductivity of the samples

A quantity, 10 g of the air-dried soil sample was weighed into a beaker, 50 ml of distilled water was added to it and the suspension was shaken for one hour to dissolve soluble salts. The conductivity meter was calibrated according to the manufacturer's instructions using KCL reference solution to obtain the cell constant. The cell was rinsed thoroughly and the electrical conductivity of the 0.01 M KCl was measured at the same temperature as the soil suspension. The conductivity cell was refilled without disturbing the settled soil. The value indicated on the conductivity meter was recorded and cell was rinsed with distilled water between samples. The EC calculated using the formula below

$$EC_{25}\ (dS/m) = \frac{S \times 1.413}{K}$$

Isolation of *Azotobacter* from the soil samples

A quantity, 10 g of the composite soil samples was weighed into a conical flask and 95 ml of distilled water was added. The suspension was properly mixed labeled as A and serial dilution carried out. Sterile pipette was used to transfer 1 ml of the suspension into 9 ml of distilled water in a test tube and was labelled B. The dilution steps were repeated three times, each time with 1ml of the previous suspension into 9 ml of distilled water. Tubes C, D and E were labeled sequentially and this result in the serial dilution of 10^{-1} to 10^{-5}. A loopful of 10^{-5} of the serial dilution was streaked onto already prepared Ashby's mannitol agar medium. The inoculated plates were incubated for 3-5days at 37^{o}C. The developed colonies were subcultured to obtain pure cultures. The developed colonies were maintained in Ashby's slant at 4^{o}C in a refrigerator for further identification.

IDENTIFICATION OF THE ISOLATES

Gram staining

A 24 hour old culture was smeared on a clean microscopic slide, air dried and heat fixed. The slide was placed on a staining rack, flooded with crystal violet and allowed for 60 seconds. The slide was then washed with tap water, the smear flooded with iodine solution and allowed for 60 seconds. The slide was washed again, decolourized with acetone by tilting the slide and was washed with distilled water. Care was taken to avoid over decolourization. The smear was flooded with safranin, allowed to stand for 30seconds and then washed with water again, air dried and observed under oil immersion objective lens (x100).

BIOCHEMICAL ANALYSIS OF THE ISOLATES

Starch hydrolysis

A 24 hour old isolate was inoculated on Ashby's agar plate containing 10% starch and it was incubated at 37^{o}C for 24 hours. After incubation period, the plate was flooded with iodine solution. A clear zone indicates starch hydrolysis was observed.

Catalase test

A 24 hour old culture colony was picked and emulsified in a few drops of hydrogen peroxide on a clean slide. Air bubble is a positive test for catalase.

Indole test

A volume, 5 ml of tryptone water was dispensed into test tubes and was sterilized by autoclaving at 121^{o}C 15 psi for 15 mins. A loopful was inoculated into test tubes. It was incubated for 3 days at 37^{o}C. After incubation, 3

drops of Kovac's reagent was added into the test tubes. A pinkish colour which indicates positive test was observed

Methyl red

A volume 5ml of tryptone water was dispensed into the test tubes and was sterilized by autoclaving at 121^{o}C 15 psi for 15 mins. A loopful of the isolates was inoculated into the test tubes and was incubated for 3 days. Thereafter, 3 drops of methyl red reagent was added into the tubes. A pink red colour which indicated positive test was observed.

III. Results

Growth of *Azotobater* on Ashby's agar plate after primary culture

Plate 1: Shows the growth of *Azotobacter* on the Ashby's agar plate which indicated that only Azotobacter specie which has rod-shaped, cream light chains, capsular slime and formed cysts, was isolated from three soil samples: Amuri, Agbani and Esut farmlands.

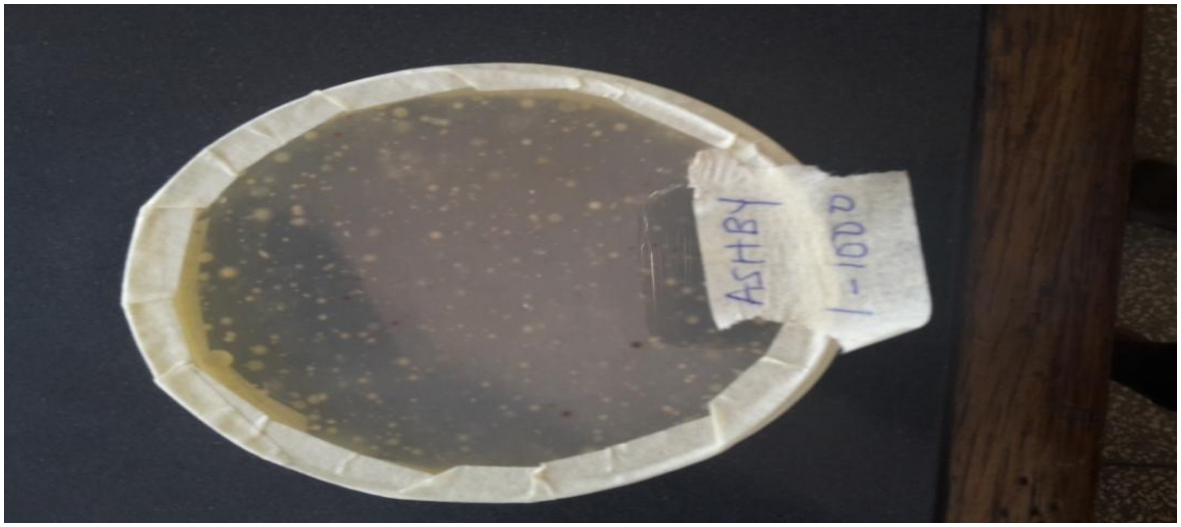

Plate1: Growth of *Azotobacter* on Ashby's agar plate after the primary culture .

Plate 2: Also shows the growth of *Azotobacter* species on Ashby's agar plate after secondary culture. The growth of Azotobacter only was as a result of differential and selective media that was used which inhibited the growth of other organisms.

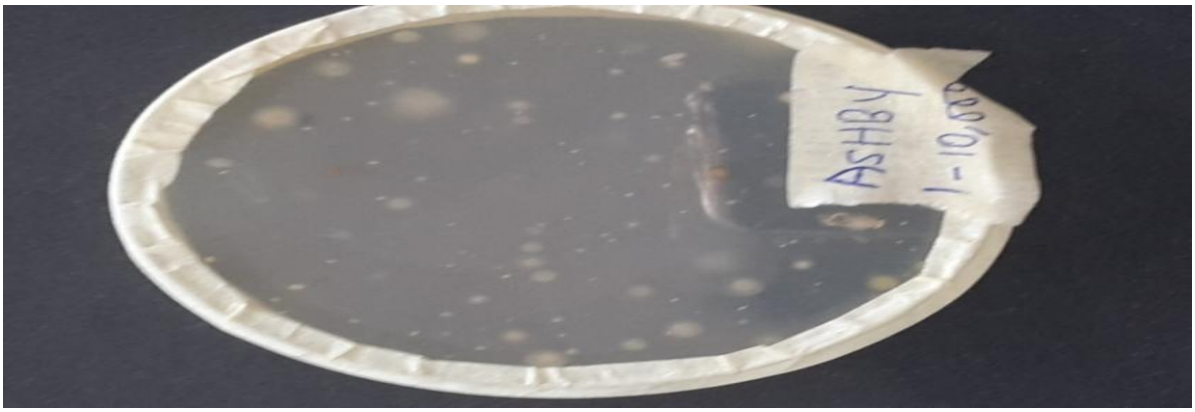

Plate 2: The growth of Azotobacter on Ashby's agar plate after the secondary culture E

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF *AZOTOBACTER*

Table 1: Shows the morphological and biochemical characteristics of *Azotobacter*. From the table it can be seen that the isolated organism was of the same specie regarding and that the *Azotobacter* was a gram negative organism, catalase positive, indole positive and methyl red positive.

Table 1: Morphological and biochemical characteristics of *Azotobacter*

Morphological and biochemical test	of*Azotobacter*species
Colony appearance	1-10µ long, rod shaped. Formed thick-walled cysts and large quantities of capsular slime with cream light chains.
Gram reaction	-
Catalase test	+
Indole test	+
Methyl red test	+
Starch hydrolysis test	+

Key:

+ = Positive reaction

- = Negative reaction

Nitrogen contents, pH and electrical conductivity of the samples

Table 2: Shows the pH, electric conductivity and nitrogen contents of Agbani, Amuri and Esut farmlands. The table indicated that out of the three soil samples, that the conductivity of Agbani farm land was higher than the two other samples (2.50) while Amuri conductivity was (1.60) and Esut farmland (1.00). Also the pH of Agbani farmland was strongly alkaline or basic (8.981) while Amuri (6.072) and Esut (6.032) which are moderately acidic in nature. The nitrogen content of Esut farmland was observed to be the highest percentage (1.491%) while Amuri was (1.243%) and Agbani (1.312%). From the result, Amuri farmland has the lowest nitrogen content and Esut has the highest content followed by Agbani farmland.

Table 2: Nitrogen contents, pH and electric conductivity of the samples

S/N	SAMPLE I.D	PH (M ±SD)	EC(mS/m) (M ±SD)	N CONTENT (M ±SD)
1	Agbani farmland	8.981	2.50	1.312%
2	Amuri farmland	6.O72	1.60	1.242%
3	Esut farmland	6.032	1.00	1.491%

IV. Discussion

Azotobacter are nitrogen fixing bacteria which have great potentials in enhancing the growth and development of crops on the farmlands and could be isolated from nitrogen rich soils as seen in this study. The higher growth on the plates indicated that the only organisms that were capable of fixing atmospheric nitrogen such *Azotobacter* could grow on the soils. Azotobacter species have full range of enzymes which include fendoxin, hydrogenase and nitrogenase which coverts ammonia needed to perform the nitrogen fixation (Amutha *et al.*, 2014). Being a majour group of soil-borne bacteria, it plays different beneficial roles by producing different types of secondary metabolites in the soil such as vitamins, plant hormones, antifungal substances, hydrogen cyanide and sidrophores. These secondary metabolites have influence on the growth of shoots, roots and seed germination of many agricultural plants. Among the genera, *Azotobacter* species can break down different types of pesticide compounds such as phenols, substituted phenolics and hazardous compounds (Elsyaed, 2013).The colonial appearance and the biochemical characteristics of the isolates showed that the nitrogen-fixing bacteria is a gram negative organism and showed a positive result to catalase test, indole test and methyl red test; and hydrolyzed starch. This agrees with the report of Vahist *et al*, (2013). The biochemical test distinguishes the members of the family *Enterobacteriacea*. Indole test indicates the ability of the organism to degrade tryptophan to produce indole, starch hydrolysis helps to ascertain the ability of the organism to utilize starch by producing the amylase enzyme, while the catalase test confirms the ability of the organism to convert hydrogen peroxide to oxygen and water. Methyl red test on the other hand is performed to show the ability of the organism to produce stable acids by the mechanism of mixed acid fermentation when supplied with glucose. Soil pH is a useful indicator of the various chemical activities within the soil. It is a useful tool in making management decisions concerning the type of plants suitable for location, the possible need to modify soil pH (up or down) and a rough indicator of availability of nutrients in the soil to plants (Jones *et al*, 2011). Soil pH directly affects the activity of microbes. Research conducted by Jones *et al*, (2011) showed that once the pH has decreased to 4.7 or below, the ability of microbes to convert atmospheric nitrogen is greatly reduced. This is further worsened in the presence of aluminum as the normal metabolisms of plants are affected. The pH of Agbani farmland (8.91) indicated that the soil pH was strongly alkaline and may contain a great deal of sodium. This alkaline pH as earlier pointed out enhances the ability of microbes to convert atmospheric nitrogen. Amuri and Esut soil samples respectively had a pH of 6.072 and 6.032.The conductivity of the soil is a measure of the amount of salts in the soil. It is an important indicator of health status of the soil. It affects' yields, susceptibility, plant nutrient availability and activity of microorganisms which influence key soil

processes including the emission of greenhouse gases such as nitrogen oxides, methane and carbondioxide (Advieno *et al*, 2010). Excess salt hinders plant growth by affecting soil-water balance. Soil containing excess salt occurs in arid and semi-arid climates. Salts levels can increase as a result of increased cropping, irrigation and land management. Although electrical conductivity does not provide a specific measurement of ions or salt compounds, it has a co-relationship to concentrations of nitrates, potassium, sodium, chloride, suphate and ammonia. For certain non-saline soils, determining electrical conductivity can be convenient and economical way of to estimate the amount of nitrogen available for plant growth (Patriquin *et al*, 1993). From their electrical conductivities Agbani farm land was moderately saline, Amuri farm land slightly alkaline and ESUT farm land non-saline. This implies that Agbani and Amuri farm land impact microbial processes such as nitrogen cycle, production of nitrous and other nitrogen oxides, respiration and decomposition (Rayment *et al*, 2011). The nitrogen contents of the soil samples determined showed that Agbani soil sample had the lowest percentage (1.312 %) followed by Amuri (1.242 %) and ESUT farmland with a percentage (1.491 %). Thus, ESUT farmland is rich in nitrogen and other nutrients which are necessary for plant growth.

V. Conclusion

The results obtained from this study revealed that *Azotobacter* species as a nitrogen fixing microorganism can be isolated from these farmlands considered and could further be exploited in biofertilizer processes.

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I.I.Ujah, et. al. "Isolation and Characterization of Azotobactr Species from Three Soil Samples in Enugu State Nigeria." *IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)*, 7(5), (2021): pp. 14-18.