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Isolation Identification and Partial Characterization of Nitrogen Fixing Bacteria from Soil and Then the Production of Biofertilizer –

Debojyoti Roychowdhury[1], Manibrata Paul[2], Sudip Kumar Banerjee[3]
[1]*Corresponding Author, Debojyoti Roychowdhury, Department of Biochemistry, Techno India University .Em-4 Sector-5,Salt Lake,Kolkata-700091.*
[2,3] *Department Of Biochemistry Techno India University. Em-4 sector- 5Salt Lake, Kolkata-700091*

Abstract: Nitrogen is a very essential nutrient for the growth and development of the plants. But it has been observed that the chemical fertilizers that are applied in the fields to gain a maximum yield output has destructive effects on the environment and more specifically to the crops.So in this research paper an effort has been made to isolate identify and partially characterize the Azotobacter spstrains from the soil and then the preparation of starter culture and mass production of the Azotobacter sp strains and then finally to use it as nitrogen fixing bacterial strain which can be further utilized as biofertilizer after mixing with proper carrier materials and then applying them in the fields.
Keywords- Nitrogen fixing bacteria, Azotobacterspp strains, Biofertilizer, Gram staining, Mass production.

I. INTRODUCTION

Nitrogen is regarded as one of the most important nutrient for the growth and development of plants as all the vital process is carried out by proteins where nitrogen is the essential constituent.[1] However the application of artificial synthetic or chemical fertilizers to increase the productivity of crops are the major cause of soil destruction and leads to other environmental problems like ground water contamination ,loss of soil fertility and other vital agricultural and health problems.[2].In this case the use of biofertilizers are the most important alternative to be used in the agricultural fields. Bio fertilizers are actually the preparations of latent or living cells that are encapsulated in proper carrier based materials which when applied to the soils improves the nutrient uptake by the action in the rhizosphere of the plants.[3]. Biofertilizers may also be stated as the living formulations of the beneficial microbes that enriches the quality of nutrient in the soil that can be further utilized by plants.[4]. In this case one of the most important nitrogen fixing microorganism that can be used as biofertilizers are Azotobacter species strains[5].Azotobacter species are non nodule forming are free living and utilizes the biological nitrogen fixation and converts the atmospheric nitrogen by binding to them and them utilizable by plants which can be demonstrated either by increase in number of leaves or panicles weight height of roots and shoots.[6].Moreover these bacteria are found in alkaline or the neutral soils in large quantities and also has properties of fixing an average of 20kg/hacter of nitrogen in the soils though they are non symbiont[7].It has also been found that the Azotobacter spp strains are present in around 104 g^{-1} of soil .[8].It can be also stated that the proper methodology of the Azotobacteriaspp in enhancing the plant growth has not been fully understood.Thus the N2 fixation has been one of the methods for providing the nutrients to the plants has been thought to be the important process.[9].

Thus in this research paper an attempt has been made to isolate identify and partially characterize the Azotobacter spp strains from the soil and then production of biofertilizer was made after mass production and mixing with proper carrier based materials were done to be used finally in the soil as organic fertilizers.

II. MATERIALS AND METHODS

A. *Collection of soil samples*

Soil samples from agarden of about 10 grams which were obtained after sampling out 0-15 cm of soils. The soils were then brought to the laboratory in zip lock plastic packets for the isolation purpose of the *Azotobacter* sp. strains.

B. *Serial dilution of the soil samples*

About 1gm of the soil was taken from the plastic bag and then the soil was diluted to 10 ml of water in a test-tube, which served as stock solution. Remaining 9 test ubes were filled with 9 ml of water. Transferring of 1 ml of water from the stock solution to 9 ml of

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sterilized distilled water with the help of pipettes yielded 10^{-1} dilutions and the series continued upto 10^{-9} dilutions. Sterility is the hallmark of any bacteriological isolation so the entire process was carried in the laminar airflow (Model: Envir-05, Made in India).

C. Bacterial colony growth on the nitrogen free mannitol agar media (Ashby media)

In this method the spread plate method, the development of bacterial colonies were noted and then their external morphology and identification was done. For the development of Azotobacter sp. strains the selective media known as nitrogen free mannitol media or Ashby media was prepared. Then the serial dilutions of 10^{-2}, 10^{-4} and 10^{-8} were selected and from that 0.1ml of culture was transferred from the test tubes to the petridishes containing the Ashby media, which were four in number. Then by the help of spreader the culture was spreaded in the media contained petridishes and they were placed in the incubator at 37^{o}C for 7 days. After incubation the plates were observed for the growth of the bacterial colonies.

D. Preparation of pure culture slants from the respective petriplates

After the bacterial colonies were identified by external morphology few well developed colonies were selected to isolate the pure culture of *Azotobacter* sp. strains slants. In this purpose, again the Ashby medium was prepared of about 50ml only for three testubes. After that the media was poured in the respective test tubes and allowed for solidification in slant position. After solidification by the help of an inoculating loop selected colonies were transferred from the plated o the pure culture slants. Then the test tubes were kept inside the incubator at 37^{o}C for 48hrs. After incubation the test tubes had developed bacterial species strain slants.

E. Microscopic examination preparation of strains

The strains that were isolated in the test tubes were to be studied for morphological characteristics and confirmation. The studies were conducted according to Collee et al. Actually the gram staining is generally done for the bacterial morphology study. In here with the help of inoculating needle the loopful strains were picked from each test tubes from the incubated pure culture slants, which were grown in 37^{o}C and made a smear on the slides and heat fixed by passing the slides over the bunsen burner flame. Before all these the slides were washed with ethanol. Then each colony was we remarked on the slides.

F. Gram Staining Of Isolated Bacterial Cultures

The heat fixed smears on the slide were then taken to the staining room for staining purpose. Over here the on the smear the first applied reagent was crystal violet on each six slides kept for 30secs. Distilled water wash. Iodine on the slides as mordant (1 min) then 95% alocohol wash and then washed with distilled water and safranin was applied on the slides and then washed with distilled water and air dried the slides. The bacterial morphologies were shown in the microscope. All the species strains were gram negative and pink in colour.

G. Screening of the acterial species strains: Biochemical tests and partial characterization of the nitrogen fixing bacteria

Furthermore, after the strains were studied in the microscopic slides, it was morphologically confirmed to be *Azotobacter sp.* strains but for further confirmation several biochemical test were performed for the partial characterization of the *Azotobacter* sp. strains from the pure culture slants. The biochemical tests included methyl red test, VP test, indole test, ctalase test, starch hydrolysis test, casein hydrolysis test, nitrate reduction test and hydrogen sulphide test were carried out.

H. Preparation of production media for the nitrogen fixing bacteria (Azotobacter sp. strains)

After the partial characterization and Gram staining of the *Azotobacter* sp. strains, the production media also known as starter culture was to be made for the final production of biofertilizer. The production media may be defined as the media in which the number of viable bacterial cells of a particular species increases rapidly as because the bacterial species are grown in the selective media in which it obtained its nutrients. Thus, a 100ml conical flask was taken. The production media or the starter culture used was of Ashby medium. Then inside the laminar airflow by the inoculating loop a loopful of bacterial culture from the pure culture slants were transferred to the Ashby production media conical flasks by the help of sterilized inoculating loop. Then the conical flasks were put in the rotary BOD shaker incubator at 37^{0}C for 7 days. These starter cultures were further transferred to large conical flasks for mass production.

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I. Mass production of Azotobacter sp. strains for inoculum preparation

The starter cultures or production media, maintained in BOD shaker for 1 week were further needed to be transferred to larger conical flask for further viable cell production. Thus, 1000ml flasks were taken in which again the Ashby media was prepared for appropriate volumetric measurements. The starter culture about 10ml to be transferred to the large conical flasks, which was sufficient for the present study. Then again the conical flasks for mass productions were kept inside the BOD rotary shaker for 1 week. The viable count in the lrger conical flask was checked for time to time because of no fungus development within the flask. The conical flask was also checked for the viable number of cells and when it reached to 10^8-10^9 CFU/ml, it was taken out of the rotary shaker. Then the broth in conical flasks was mixed with carrier materials like activated charcoal, peat in proportions. The mixture was then stored in cool placesand after 7 days it was applied in the field.

J. Preparation of the carrier materials and then the mixing of the Azotobacter spp strains inoculums with these carrier materials-

In the preparation of the activated charcoal about 1kg of black coal was commercially brought from different places and with the help of mortar and pestle they were very deeply crushed in the first time. Next with the help of grinder and mixer the entire crushed coal was meshed to fine granules. To it certain amount of already prepared activated charcoal was mixed and the fine powdery material was kept in a big metallic tray. As the charcoal is slightly acidic so certain amount of Hcl/Naoh was added and it was neutralized further by addition of Calcium Carbonate. The pH was adjusted and neutralized and also the maximum care was taken to maintain the moisture content to 45%.After this the Azotobacter spp strains the conical flasks of 1000ml containing the *Azotobacter* sp. strain in the nitrogen free mannitol media broth is to be mixed with the sterile carrier material in a ratio of 2:1. But in this case about 750-800ml of the liquid broth is to be mixed with 1000gm carrier material, which was separated in three parts and sterilized. Moreover, the most important part is the broth count of the *Azotobacter* sp. strains and was calculated and a high broth was mixed with the carrier materials in the big metallic tray by wearing hand gloves .

K. Packaging and storage of the inoculates

Finally the composite biofertilizers were picketed in 250gm and then these were left for curing under the room temperature. Furthermore, in the case of packaging the each of the individual biofertilizer and the triple composite biofertilizer, inoculate packets were stored in cool place and these were kept away from the sunlight. These packets were stored in the room temperature maintaining the cool conditions.

III. RESULTS AND DISCUSSION

A. Isolation and partial characterization of the nitrogen fixing bacterial strains from the soil (Azotobacter sp. strain) for the preparation of Biofertilizers

In the Table 1, the appearance of the *Azotobacter* sp. strain and the identification of the bacterial species strain were done by observations on the respective petriplates that were incubated in the incubator. The strains all appeared on the nitrogen free mannitol media, which were gummy fucoid dark pigmented sometimes yellowish brown and also slimy and flat.

Table 1 Identification of Azotobacter sp. colonies on the basis of the external morphology

Plates as per dilution	Number of Colonies Observed	Colour of the Colony	Character of the Colony
10^{-2}	1	Yellowish Brown	Slimy/Flat
	2	Dark	Slimy/flat

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	3	Dark	Slimy/flat
10^{-4}	4	Yellow	Slimy/flat
	5	Yellow	Slimy/flat
10^{-8}	6	Dark	Slimy/flat

B. Isolation of the NFB (Azotobacter sp. strain on the pure culture slants)

After the colonies or the strains were isolated on the petriplates and were identified by external morphology the strains were grown on respective selective media slants by transferring a loopful of the inoculums from the petriplates to the Ashby agar media in the test tubes (Fig 1).



Fig1-Pure culture slant of the Azotobacter spp strains.

C. Identification of the Azotobacter sp. strains through Gram staining

After the bacterial strains developed in the pure culture slants loopful of the strain were utilized for the gram staining purpose. Microscopically, it was found that the strains of 1, 2, 3, 4 were all pink in staining and according to the shape they were all spherical or oval with a texture that resembled in the Table 2 showed the gram staining characteristics of the bacterial species strains.

Table 2. Gram staining of the Azotobacter sp. strains

Colonies in Petriplates	Colour of the Colonies	Shape of the Colonies	Generic name of the bacteria
Strain 1	Pink	Spherical	Azotobacter sp.

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Strain 2	Pink	Spherical	Azotobacter sp.
Strain 3	Pink	Spherical/oval	Azotobacter sp.
Strain 4	Pink	Spherical	Azotobacter sp.

D. Screening and partial characterization of the Azotobacter sp. strains

All the strains after gram staining were further screened for the partial characterization and confirmation as to whether they were Azotobacter sp. strains or not. In this case, all the strains were subjected to different biochemical test like that of the methyl red test, VP test, indole test, catalase test, starch hydrolysis, caesin test and nitrate reduction test.In the table 3 the biochemical tests of the following strains were confirmed.

Table3. Biochemical tests of the Azotobacter sp. strains

Parameters	Strains/Colonies	Observations
Methyl red test	1, 2, 3, 4	Positive (+)
VogaesProskaur test	1, 2, 3, 4	Negative (-)
Indole test	1, 2 ,3, 4	Positive (+)
Catalase test	1, 2, 3, 4	Positive (+)
Hydrogen sulphide test	1, 2, 3, 4	Positive (+)
Nitrate reduction test	1, 2, 3, 4	Negative (-)
Starch hydrolisis test	1, 2, 3, 4	Positive (+)
Caesin test	1, 2, 3, 4	Positive (+)

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Finally after the biochemical tests as partial characterization and gram staining the viable cells of the Azotobacter spp strains were mixed with carrier materials and then packeted in 250 gm. packets and stored in cool place.Then the carrier based biofertilizers were ready to be applied in the fields.

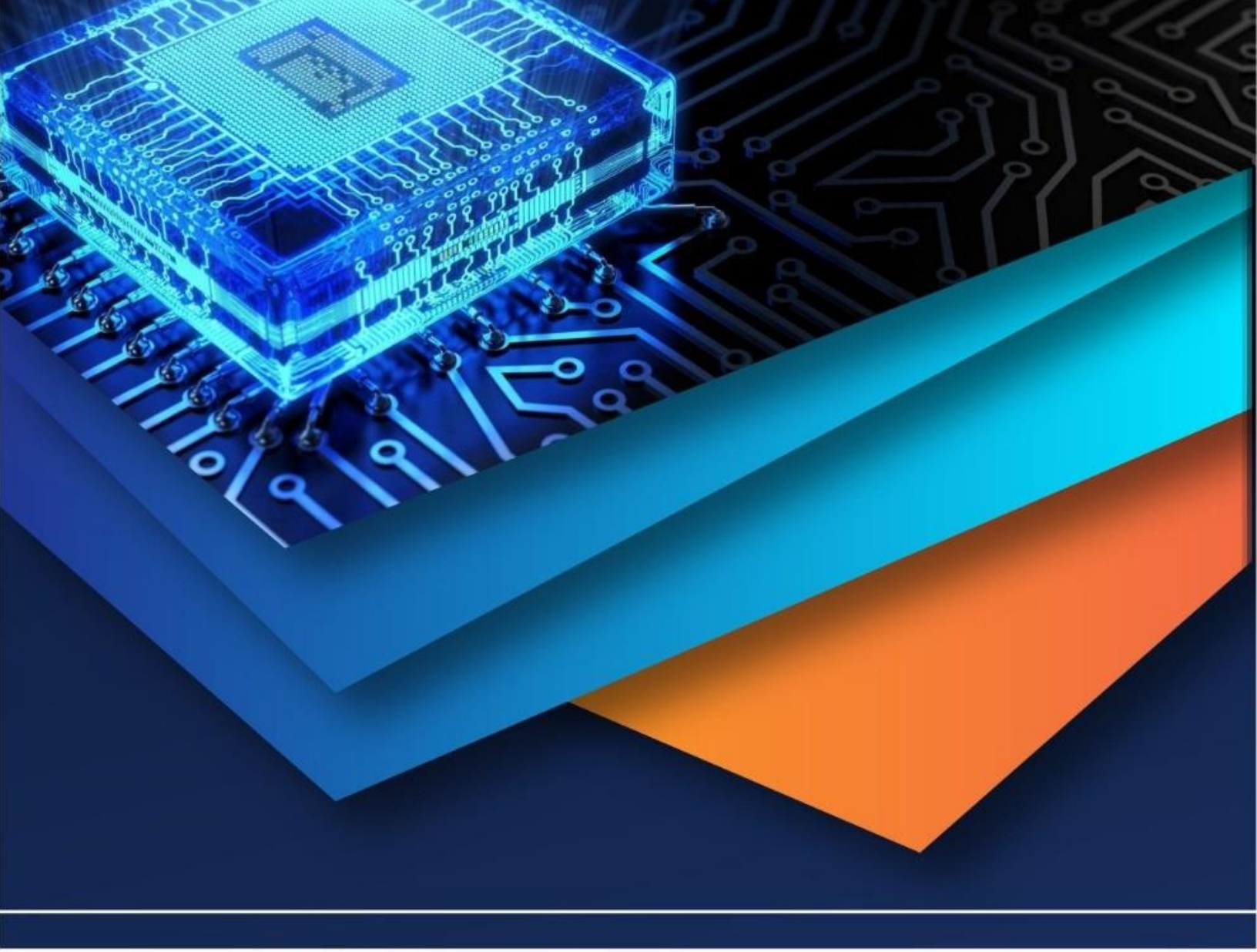
IV. CONCLUSION

Finally it can be concluded that the biofertilizers prepared from the Azotobacter spp strainscan be utilized in the fields as organic fertilizers to make an increase in productivity and reduce the use of chemical fertilizers.

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