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# STUDY ON IAA PRODUCING *AZOTOBACTER TROPICALIS STRAIN RBS.* ISOLATED FROM NORTH GUJARAT, INDIA

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**ABSTRACT:**

A total of 21 free living diazotrophs were isolated from different rhizospheric Soils of North Gujarat. Out of them one individual isolate was selected for the production of indole acetic acid (IAA) in a medium with 1, 2 and 5 mg/ml of tryptophan. The isolate showed high level (31.4 mg/l) production of IAA at 5 mg/ml of tryptophan while at 1 and 2 mg/ml the production was 8.76 and 18.92 mg/l respectively. Pot experiment study indicates increased root length and shoot height indicates efficient IAA production by the isolate. The isolate was also analyzed for its nitrogen fixing ability. The DNA of bacterial isolate was isolated and amplified using 16S r-DNA primers. Amplified PCR product was purified and sequenced for its identification. The sequence of bacterial isolate was used to identify the species of organism using BLAST. The organism was identified as a *Azotobacter troerpicalis*. The sequence of isolate was submitted to NCBI gene bank data and the accession number provided by NCBI is JN591767.

**KEYWORDS:** Bacterial inoculation, IAA, plant growth promoting Azotobacter *tropicalis,* Pot experiment, 16S r-DNA sequencing.

## INTRODUCTION:

The source of soil nitrogen is the atmosphere where Nitrogen gas occupies about 79% of the total atmospheric gases. Living organisms that are present in the soil have profound effect on $N_2$ transformation, which provide food and fibre for an expanding world population. Although Nitrogen is very abundant in nature; it often limits plant productivity because atmospheric Nitrogen is only available to a very narrow range of organisms belonging to Diazotrophs, symbiotically and non-symbiotically.

The free-living bacteria having the ability to fix molecular nitrogen can be distinguished into obligate aerobic, facultative aerobic and anaerobic organisms. Obligate aerobic bacteria belong to genera *Azotobacter, Beijirinckia, Achromobacter, Mycobacterium, Arthrobacter, Enterobacter* and *Bacillus*. Among the facultative anaerobic bacteria are genera *Aerobacter, Klebsiella,* and *Pseudomonas*. Anaerobic nitrogen fixing bacteria are represented by genera *Clostridium*, *Chromatium*, *Rhodopseudomonas, Rhodospirillum, Desulfovibrio* and *Methanobacterium*. Azotobacter and Azospirillium are non-symbiotic bacteria they belong to *Azotobacteriaceae* family. They produce growth promoting substances which improve seed germination and growth of extended root system. They produce polysaccharides which improve soil aggregation. *Azotobacter* suppresses the growth of saprophytic and pathogenic micro-organism near the root system of crop plants. Effects of IAA on plants are significant and some of them are apical dominance (apex dominates lateral meristems), phototrophism, gravitrophism, prevention of leaf and fruit abscission and induction of adventitious roots. Therefore IAA has profound influence on crops. The present study was conducted to study IAA producing *Azotobacter tropicalis* isolated from North Gujarat, India.

## MATERIALS AND METHODS:

### Sample collection and Processing

Rhizosphere soil at a depth of 0 to 15 cm was sampled from an area of 100 $m^2$ during 2010 from Sabarkantha, Banaskantha, Patan and Mehsana districts of North Gujarat. Soil samples were subjected to selected physico-chemical properties.

### Isolation of pure culture

Discrete well-developed and separated colonies on the surface of Ashby's Mannitol agar plate were each picked up with a sterile niddle and transferred separately on Ashby's Mannitol Agar medium slant. Each of these new slant cultures represents the growth of a single bacterial species. The colonies, which are different in appearances and characters were picked and purified.

### Characterization and Identification of the isolate.

### Morphological analysis

A dried fixed smear was covered with oxalate crystal Violet reagent for sixty seconds. The stain was then rapidly washed off with clean water. All the water was then tipped off and the smear was covered with Lugol's Iodine for sixty seconds. The Iodine was then washed off with clean water. The smear was then decolorized rapidly for 10 seconds with acetone alcohol and was washed immediately with clear water. Finally, the smear was covered with

Safranine for 30 seconds. The slide was then washed thoroughly in water and blotted dry. The smear was examined under microscope by using immersion oil objective.

**Biochemical analysis**

The physiological activities of the selected isolate tested through Oxidase, Catalase Motility Indole, Urease (MIU), Methyl red (MR), Acetoin production (Voges-Proskauer), Nitrate reduction, Citrate utilization, Hydrogen Sulfide ($H_2S$) production, Gelatin liquification and carbohydrate fermentation methods as demonstrated by Collee and Miles (1989).

**Genetic analysis**

**Characterization of isolate using 16S r-DNA sequencing**

**Culture medium and growth Conditions**

The pure culture was grown in a 100 ml medium of Ashby's Mannitol broth and incubated in an incubator shaker (120 rpm) at 30 °C for 48 hrs. The pellet of culture was obtained by centrifugation of broth.

**Genomic DNA extraction from isolate**

Genomic DNA was extracted from the isolate using a MEDOX-Bio $^{TM}$ Ultra pure Genomic DNA Spin Miniprep kit, following the manufacturer's instructions. The cell pellet was re-suspended in 200µl cold TE buffer. 400 µl of Digestion solution was added; mixed and 3 µl of proteinase K solution was added and incubated for 5 minutes. Then 260 µl of ethanol was added and thoroughly mixed. The mixture was applied to column placed in 2 ml of collection tube. The mixture was then centrifuged at 8000 rpm for 1 minute. 500 µl of wash solution was added and spin at 8000 rpm for 1 minute. This step was repeated. The flow through and collection tube was discarded. The mini spin column was carefully removed and placed in 1.5 ml micro-centrifuge tube; 30 µl of elution buffer was added and was incubated for 2 minutes at room temperature and then centrifuged at 1000 rpm for 1 minute. The DNA sample was then stored at -20 °C and thawed at room temperature as required.

**Determination of DNA concentration, yield and purity**

DNA yield was measured by determining the absorption of the elute at 260 nm wavelength. The purity of the DNA was calculated by the ratio of the absorbance at 260 nm and 280 nm, which provided an estimate of the purity with respect to contaminants that absorb UV light, such as protein. Pure DNA has an A260/A280 ratio of 1.8-2.0. DNA concentration was measured using spectrophotometric reading at 260 nm (Shimazdu)

**Amplification of 16S r-DNA by polymerase chain reaction (PCR)**

In PCR the isolated DNA was amplified by using 16S r-DNA primers (F-5'AGAGTTTGATCCTGGCTCAG 3' and R-5'AAGGAGGTGATTCCAGCC3'), and by using the Taq and GO PCR master mix; which was obtained from MP Bio. The Master mix contained Taq polymerase, divalent cations $Mg^{+2}$, dNTPs. The reaction for 50 µl was prepared according to standard protocol provided by MP Biomedicals for amplification. The isolated DNA was added in range of 100pg-100ng. The DNA was amplified by using Thermal cycler (eppendroff) using parameters (viz:- Denaturation temperature : 94 °C for 20 s, Annealing temperature 55 °C for 40s, Extension temperature 72 °C for 2 min) for 32 cycles. The PCR product was analyzed by gel electrophoresis.

**Gel Electrophoresis of PCR product and photographing of the gel**

Standard electrophoresis protocol was used using a Gel electrophoresis device. PCR product 5µl was mixed with 2µl of gel loading dye. The reaction was loaded in to the well of a 1% agarose gel prepared in TAE buffer. Etbr (10mg/ml) was added in gel. The gel was photographed using a Gel Documentation System.

**Extraction of PCR Product and DNA sequencing**

The PCR Product was extracted by a low melting agarose gel method. Sequencing was carried out by Ocimum Biosolution. The forward and reverse partial 16S r-DNA sequence along with chromatogram of isolate was obtained from Ocimum Biosolution in FASTA format.

**Analysis of $N_2$ fixing activity of isolates**

For this, well grown culture of bacteria was aseptically transferred into sterilized glass vials with the help of sterile pipettes. Each glass vials have total volume of 20ml, 10ml of culture was added to the vials and mouth of the vials were sealed. 10% of the air in the vessel was replaced with equivalent volume of acetylene ($C_2H_2$) gas and vessels were shaken and incubated for 30 minutes at appropriate temperature. 10 µl of Sample was withdrawn regularly after every 24 hrs for analysis of ethylene produced till 96 hrs of incubation. Ethylene concentration was assayed on a Shimadzu 2010 Gas Chromatography equipped with a flame ionization detector and a Porapack Q (Internal diameter 2.2 mm, length 6'). The reduction of acetylene to ethylene was depicted in the form of peak (graph). The efficient acetylene reducer was carried forward for characterization and further for mass production.

**IAA production activity**

Test bacterial culture was inoculated in the respective medium (Jensen's broth) with tryptophan (1, 2, and 5 mg/ml) and incubated at 28 ± 2 °C for 07 days. Cultures were centrifuged at 3000 rpm for 30 min. Two millilitres of the supernatant was mixed with 2

drops of Orthophosphoric acid and 4 ml of Salkowaski's reagent (50 ml, 35% perchloric acid; 1 ml 0.5M FeCl3).

Development of a pink colour indicates IAA production. O.D. was read at 530 nm using spectrophotometer. The level of IAA produced was estimated by a standard IAA graph.

**Pot Experiment**

For Pot experiments, soil samples were collected, air dried, sieved and sterilized by repeated autoclaving before filling the pots. Cotton seeds were surface sterilized by exposing 95% ethanol and immersing in 0.2% $HgCl_2$ solution for 4 minutes. The seeds were then subjected to five washing with sterile distilled water.

One millilitre of overnight grown bacterial culture was applied on each seed for 10 minutes; seeds were dried and were transferred to pots containing sterile soil to a depth of 6 mm. 05 seeds were sown in each pot and the experiment was performed in triplicate. The pots were kept in sunlight and were observed every day.

**RESULT & DISCUSSION:**

Physical and chemical properties of the soil were indicated in Table 1. Biochemical characterization and carbohydrate utilization of *Azotobacter tropicalis* is indicated in Table-2 and 3 respectively. Nitrogen fixation activity of isolate was done indirectly by performing ARA and reduction of Acetrylene to Ethylene is shown in graphs after different incubation time. IAA production of isolate shown in Table 4.

The isolate is gram negative short rod, non-spore forming and motile. Isolate also found to be accumulating PHB granules inside the cell. The isolate found to be utilizing citrate as sole source of carbon. The isolate is having ability to utilize starch and protein gelatine along with that it also hydrolyses lipid. Nitrogen fixation activity of isolate was done indirectly by performing acetylene reduction assay. Reduction of acetylene to ethylene was found to be maximum after 96 hrs of incubation. The graph of acetylene reduction is shown in figure-1. IAA production by isolate is shown in Table-4. The 16S r-DNA partial sequence of isolated organism was aligned by using bioinformatics tool BLAST and it was found that the isolate is showing 99.2% similarity with *Azotobacter tropicalis*. The sequence submitted to NCBI Gene bank data. The accession number of the sequence provided by NCBI is **JN591767.**

**Table 1:Selected physical and chemical properties of the soil used in the experiment.**

| Physical properties | Values | Chemical properties | Values |
|---|---|---|---|
| Soil sampling depth | 0-15 cm | pH | 7.1 |

| | | | |
|---|---|---|---|
| Particle sizes: | 15 % | Organic carbon | 10.5 g $kg^{-1}$ |
| Sand | 59 % | Organic matter | 18.1 g $kg^{-1}$ |
| Silt | 26% | C/N ratio | 5.5 |
| Clay | Silt | Total Nitrogen | 1.9 g $kg^{-1}$ |
| | | Available Nitrogen | 25 mg $kg^{-1}$ |

**Table 2:Selected biochemical tests of isolate after 48 hours of incubation at 37±0.5ºC.**

| Name of the Test | Response of strain |
|---|---|
| Gram strain | - |
| $H_2S$ production | - |
| Indole production | - |
| Methyl Red reaction | - |
| Voges-Proskauer reaction | - |
| Citrate Utilization | + |
| Urease activity | - |
| Catalase | - |
| Oxidase | + |
| Gelatin liquefication | + |
| Starch hydrolysis | + |
| Lipid hydrolysis | + |
| Motility | + |

**Table 3:Selected carbohydrate utilization of isolate after 48 hours of incubation at 37±0.5 ºC.**

| Carbohydrate test | Response of isolate |
|---|---|
| Glucose | + |
| Maltose | + |
| Lactose | - |
| Mannitol | + |
| Mannose | + |
| Sucrose | + |

**Table: 4. IAA production activity**

| Concentration of Tryptotphan(mg/ml) | IAA produced mg/l |
|---|---|
| 1 | 8.76 |
| 2 | 18.92 |
| 5 | 31.4 |

**Figure-1 Acetylene reduction assay**

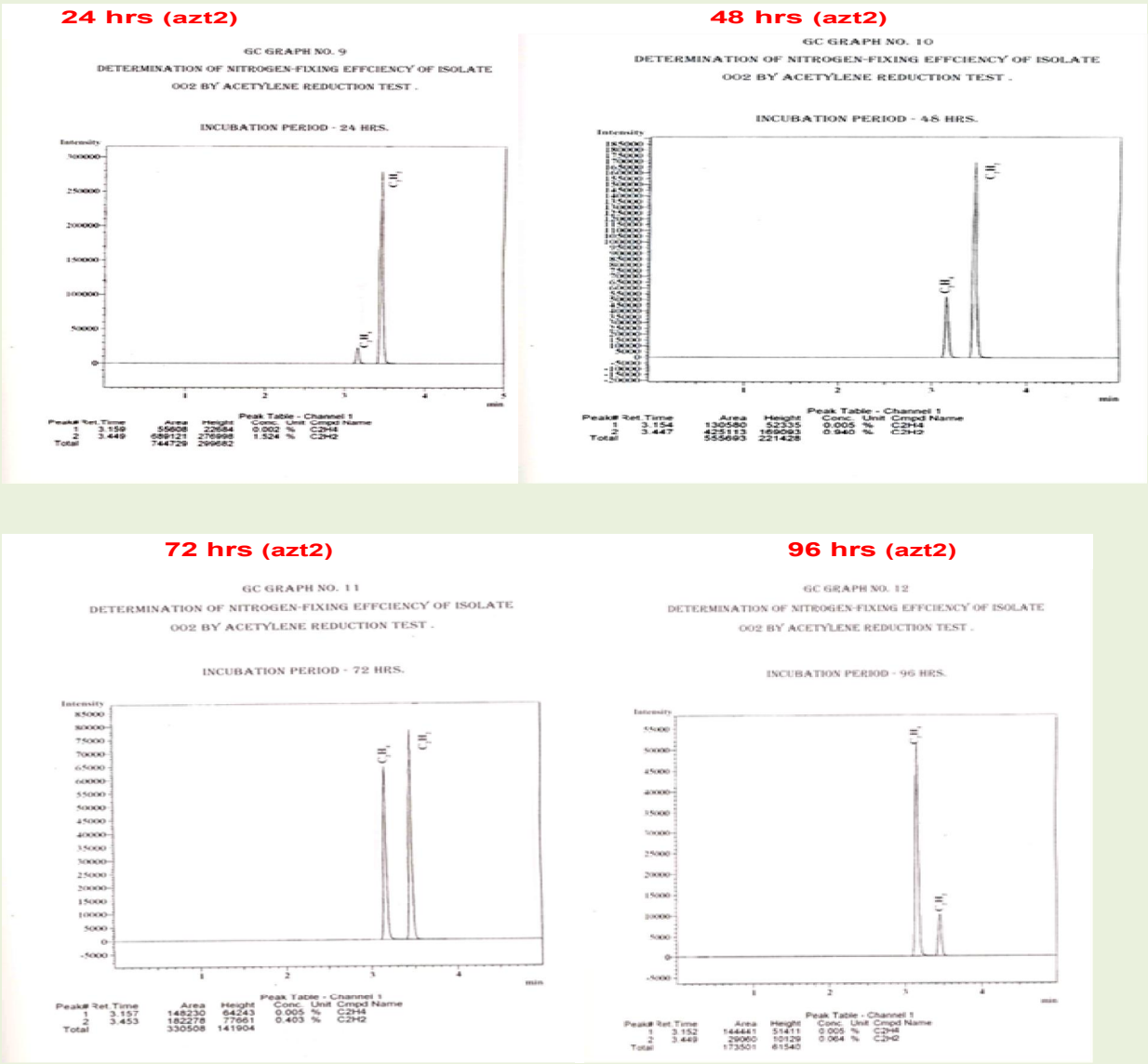


**Fig.2 Plant growth promotion study on cotton plant after 2 month.**

Left Pot: Inoculated with test organisms

Right Pot: control

**CONCLUSION:**

The isolated organism was identified as *Azotobacter tropicalis.* This species is a novel type of species isolated from this area first time. The organism has dual capacity of Nitrogen fixing as well as production of IAA, a very useful plant growth promoting hormone. The isolate has shown very promising results in ARA and in the pot culture study, shows its significance in fixation of atmospheric Nitrogen. So it can be used as potential biofertilizer.

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