E-ISSN: 2788-9297
P-ISSN: 2788-9289
https://www.agrijournal.org
SAJAS 2023; 3(1): 125-132
Received: 07-12-2022
Accepted: 19-01-2023

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Isolation and characterization of *Azotobacter* sp. for plant growth promotion and abiotic stress tolerance in Telangana's agro-climatic regions: Towards sustainable development

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DOI: https://doi.org/10.22271/27889289.2023.v3.i1b.79

Abstract
Current study was done to understand and improve plant survival and growth under biotic and abiotic stress tolerance was considered. The primary goal of this work was to isolate azotobacterial species from indigenous places where the agriculture community has been practicing traditional methods along with regular places, also examined the areas of stressed agriculture areas which are seasonally cultivated and environments in their capacity to enhance drought stress tolerance brought from different crop fields such as Maize, sweet Sorghum, Sugarcane, corn, and wheat to increase the yield.
These crops soil samples were taken from few drought areas tolerant places, i.e. under different temperature, salinity, pH and moisture conditions of those particular areas in Telangana. The different soil samples isolated for azotobacter strains from different regions in Telangana were collected and analyzed for growth in media to get the high yield for the agriculture purpose which will benefit the agricultural community and leads to sustainable development. Morphological, biochemical, and molecular characteristics were used to evaluate the Azotobacter species. Azotobacter beijerinckii strain and *Azotobacter tropicalis* strain were identified by biochemical testing and 16s rRNA sequencing. Azotobacter beijerinckii strain (BKPOU06TS) and Azotobacter tropicalis strain (BKPOU08TS) 16S rRNA sequences were submitted to GenBank with accession numbers OP536202 and OP536206, respectively.

Keywords: Sustainable, drought, microbes, *azotobacter*, abiotic

1. Introduction
In order to improve plant-microbe interaction and practise sustainable agriculture, soil samples were taken from various farmed areas in the Telangana state according to the agro-climatic region. These samples were then examined for plant growth promotion capabilities. The regions of North Telangana, Central Telangana, and South Telangana were the ones from which soil samples were collected. The study's goal was to classify the soil samples according to their texture, percentage of organic carbon (OC), pH, electron conductivity (EC), moisture (M), phosphorus (P), potassium (K), and nitrogen (N) content. Telangana's location on the Deccan Plateau grants it access to a handful of India's major rivers, but it also has a unique climate that is mostly dry and semi-arid, with monsoonal rainfall giving aboard spectrum of Agricultural community to explore their cultivation sector in different crops pattern to grow and help in state and National GDP on economic path. Priority is placed on the use of natural microbial biota in agriculture to promote sustainable practises in the current study (NMB). Azotobacter is one of the soil bacteria that is difficult to locate because of its high efficacy in enhancing soil fertility and crop yield. It can fix atmospheric nitrogen aerobically, which benefits plants and improves agricultural productivity. According to Chennappa G *et al.* (2016) [10] and O'Callaghan *et al.* 2022 [14], the agricultural community uses chemical fertilisers abhorrently while ignoring their negative impacts. Urban technology has supplanted traditional wisdom, resulting in decreased production and changes in soil bacteria (Sumbul *et al.*, 2020) [11]. Bio-fertilizer is produced commercially by a relatively small number of firms. Although commercially viable and having no effect on the soil, bio-fertilizer really helps to preserve the natural soil biome that results from interactions between soil microbes. (International Soil Day Honors Soil Biodiversity's Contribution to Increasing Food Production and Nutrition Mirage News | 04-Dec-2020. Yahoo India Search Results, 2023.

There are numerous nitrogen sources that can be used to supplement crops' nitrogen needs. In addition to inorganic fertilisers, organic nitrogen through the nitrogen cycle plays a very important role. This nitrogen comes from animal manure and other waste products, which then leads to superficial nitrogen fixation by leguminous crops, which then supplies enough nitrogen for the best crop production with the aid of microbes (Naveed *et al.*, 2013; JIA *et al.*, 2020) [2, 8]. According to Bergey's Manual of Systematic Bacteriology, the Azotobacteriaceae family (Jensen, 1954) [43] belongs to a physiologically more homogeneous group than other families.

Yousefi and colleagues (2016) [12]; Onyancha (2022) [6] Azotobacter microbes which show fragile characteristics and some strains require special diluents for their enumeration towards micro and macronutrient such as NaCl (0.9%) is lethal diluents but the salt components of standard media for Azotobacter are satisfactory for plant growth promoting traits. Azotobacter tends to be sensitive towards acid pH values, high phosphate concentrations and temperature above 32 ºC ± 3 ºC because due to change in climatic variations the microbes getting adapted (Sonnleitner, 2000; Curá *et al.*, 2017; Nag *et al.*, 2018; Pillai, 2021) [5, 9, 15].

Azotobacter is found in the rhizosphere's regions of leguminous crop roots of some plants which helps in production of growth hormone stimulant (Brown *et al.*, 1962) [13]. Azotobacter naturally fixed atmospheric nitrogen in the rhizosphere to overcome the nutrient deficiency against biotic and abiotic stress (Doe, 1966) [4].

Due to different strains of Azotobacter shows varied chemical, biological and other plant growth promoting characters (Glick, 2014; Sumbul *et al.*, 2020) [43, 11]. Variation in strains have shown greater higher nitrogen fixing ability than others rhizosphere bacteria. The research in the university primarily focuses on problem solving in a multi-disciplinary approach to deliver cost effective, remunerative and location specific technologies catering to the operational needs of farm holdings of all sizes. Our research development goals in the core areas of Agriculture, Agricultural Engineering & Technology and Community/ Home Science ensure sustainable use of natural resources while enhancing livelihood security and wholistic empowerment of rural community (Azotobacter Chroococcum (Nitrogen-fixing Bacterium)," 2022) [16]. Agriculture is the mainstay of Indian culture and economy since time immemorial. As the world transformed to a global village, our farmers too transformed from family food providers to agri innovators and shrewd agripreneurs competing with the rest of the world, proving their resilience and quick sense of adaptability. Sustainability is an embodiment of this very spirit, constantly striving to provide transformative education, remunerative and sustainable research solutions to the contemporary challenges of the state's farmers through committed one to-one digital and personal connect. (Ashraf, 2010) [1].

While reinforcing traditional wisdom and interdisciplinary support in research to resolve complex problems with multiple dimensions related to agriculture community. The discovery of the fixation of free nitrogen by leguminous plants was announced by two German scientists in 1886. The plants, however, have not this power in themselves, because a certain bacterium named azotobacter must be present in the soil, (Ahemad & Kibret, 2014) [45].

2. Materials and Methods

2.1 Collection of Soil Sample

The soil sample was collected from the sub-surface. The surface litter were removed at the sampling spot. A 'V' shaped cut to a depth of 15 cm in the sampling spot was done using spade.

2.2 Physiological characteristics of soil sample

Some of the physico-chemical characterization was carried out for the soil sample collected from different soil regions of Telangana against abiotic stress tolerance.

2.2.1 pH

The pH of the soil sample was analyzed by potentiometric method (ASTM, 1995; Takamoto *et al.*, 2023) [46, 18].

2.2.2 Moisture Content

Moisture content was done by "Oven dry method" (ASTM D2216-19, 2019).

2.2.3 Organic Carbon

Organic Carbon content was estimated by Walkley-Black method (Walkley & Black, 1934) [47]. The methodology involves wet combustion of organic matter with mixture of Potassium dichromate and Sulphuric acid at 125 ºC. The residual dichromate is titrated with Ferrous sulfate. An empirical correction factor of 1.3 is applied to compensate incomplete destruction.

2.2.4 Carbonates

The quantification of carbonates was estimated by "Rapid Titration method" or alternatively called as "Acid Neutralization method". The soil sample is treated with dilute acid, the residual acid (not neutralized by carbonates) was titrated (Maulood *et al.*, 2012) [21].

3. Media Composition: Ashby nitrogen free media modified

Mannitol 15.0 g, MgSO4.7H2O 0. 2 g K2HPO4 0.2 g Ferric chloride (10% aqueous solution) 0.5 ml Molybdenum trioxide (10% aqueous solution) 1 drop, Agar 15.0 g Distilled water to 1000 ml pH 7.2.

3.1 Isolation of Bacteria sample from contaminated soil sample

The Target microbial strain (Bacteria) were from few sites of agriculture practice is going on near industrial areas. The bacterial colonies were isolated initially using the two methods:

1. Serial dilution method
2. Spread plate technique

3.1.1 Serial dilution

A serial dilution is the stepwise dilution of a substance in solution. Usually, the dilution factor at each step is constant, resulting in a geometric progression of the concentration in a logarithmic fashion. A ten-fold serial dilution. Serial dilutions are used to accurately create highly diluted solutions as well as solutions for experiments resulting in concentration curves with a logarithmic scale (Cullen & MacIntyre, 2016) [19].

3.1.2 Spread plate technique

Spread plate technique is a method employed to plate a

liquid sample for the purpose of isolating or counting the bacteria present in that sample. The technique is most commonly applied for microbial to isolate and identify variety of microbial flora present in the environmental samples e.g. soil (Sanders, 2012) [48].

3.1.3 Colony Morphology
Bacteria grows as colonies on solid media. A colony is defined as a visible mass of microorganisms all originating from a single mother cell. Key features of these bacterial colonies serve as an important criterion for characterization of bacterial colonies. Different descriptors for bacterial colonies are described as to such properties as form, size, margin, consistency, colour, shape, texture, elevation, pigmentation, opacity, etc. (Bae *et al.*, 2015) [49].

3.1.4 Cell staining
Cell staining technique is used for better visualization of cells and cell components under a light microscope. Different stain and staining techniques are being used for better visibility of the cells such as fixed or non-living cells. The cells may also be stained for study of metabolic processes or to differentiate between live and dead cells in given bacteriological sample. The staining of cells can also be used to determine biomass in an environment of interest (Alturkistani *et al.*, 2015) [22].

3.1.5 Gram staining
The gram stain procedure is a technique that can be used for differentiating bacterial species. It is very useful and commonly performed staining technique used for identification of bacterial species based on cell wall, although some bacteria don't possess cell wall (Popescu & Doyle, 1996) [23].

3.1.6 Pure Culture
Pure culture is a technique used for enumeration of single cell/organism from a mixed group of culture (one containing many species). It is done by transferring a small sample into new sterile growth medium in such a manner that the cells disperse (Lagier *et al.*, 2015) [24].

3.2 Identification of bacteria by different Biochemical test
The identification of bacteria is essential for any scientific co-relations. The unidentified bacteria were identified by various biochemical tests that are exhibited by the bacteria (Giuliano *et al.*, 2019) [25]. The bacteria are first identified based on their Gram stain and colony morphology and later identified by their responses to the different biochemical test as suggested in Bergey Manual for Determinative Bacteriology (Bergey *et al.*, 1939; Buchanan & Gibbons, 1974) [26, 27].

3.2.1 Indole Test
Indole test determines the ability of the bacteria to produce tryptophanase enzyme to that convert Tryptophan (amino-acid) into indole. Thus, the production of Indole production is detected by Kovac's or Ehrlich's reagent (4(p)-dimethyl amino benzaldehyde). The Kovac reagent (an aldehyde compound) thus reacts with product indole to produce a red coloration in the alcoholic layer (Isenberg & Sundheim, 1958) [33].

3.2.2 Methyl Red Test
Methyl Red (MR) test has been used to determines whether the microbe enables for the production of mixed acids by anaerobic fermentation supplied with glucose as the carbon source. And helps to differentiate various genera of enteric bacteria.
Mixed acid fermentation is one of the two broad patterns, 2, 3-butanediol fermentation and mixed acid fermentation (acetic, lactic and succinic are formed). As the mixed acid fermentation takes place, significant number of acids is formed results significant decrease in the pH of the medium below 5.0. Methyl red (p-dimethylamino-benzene-O-carboxylic acid) is a pH indicator indicates that indicates the production of acid (Barry *et al.*, 1970) [34].

3.2.3 Voges Proskauer Test
Voges-proskauer Test (VP Test) is also performed in MR-VP broth as similar to the broth taken in Methyl Red test where both the tests are used to detect the end products from bio-metabolism of glucose as the sole carbon source. Voges Proskauer test is used to detect acetoin as end product which can be detected by 5% α-napthol reagent in alkaline condition. The microbes that can metabolize glucose into acetoin (i.e., acetyl methyl carbinol or 3-hydroxybutanone) via pyruvic acid as an intermediate. This acetoin is further reduced to 2, 3-butanediol (Levine, 1916) [35].

2 pyruvate = acetoin + $2CO_2$ acetoin + NADH + H^+ = 2, 3-butanediol + NAD^+

In the presence of alkali (KOH) and atmospheric oxygen, acetyl methyl carbinol is oxidized to diacetyl, a reaction which is catalyzed by alpha- naphthol. Diacetyl formed reacts with guanidine-containing compounds such as arginine contributed by peptone in the medium, to form a red colored product. The resultant red color is indicative of a positive VP test. The second reagent, potassium hydroxide, absorbs carbon dioxide present in the medium and acts as an oxidizing agent thereby hastening the critical reaction that converts acetoin to diacetyl (Benjaminson *et al.*, 1964) [50].

3.2.4 Citrate Utilization Test
Citrate utilization test is used to select and identify microbes capable to utilize citrate as sole source of carbon. Bacteria capable of producing citrate permease enzyme converts citrate to Pyurvate via synthesis of Oxaloacetate (Lara & Stokes, 1952).

$$\text{Citrate} \xrightarrow{\text{Citrate Permease}} \text{Oxaloacetate} + \text{Acetic Acid} \longrightarrow \text{Pyurvate} + CO_2$$

The carbon dioxide (CO_2) thus produced combines with sodium and water form sodium carbonate that make the media alkaline.

$$CO_2 + 2Na^+ + H_2O \longrightarrow Na_2CO_3$$

The ammonium salts present in Simmons citrate agar media is the sole nitrogen sources that can be utilized to produce ammonia that increases the alkalinity of the media. The production of ammonia and Sodium carbonate are indicated by the increase in pH of the media that are indicated by change in coloration of media from green color to blue color

due to the presence of Bromothymol Blue (pH indicator) (Vaughn *et al.*, 1950) [28].

3.2.5 Carbohydrate fermentation test
Carbohydrate fermentation test is a procedure that are used to determine the ability of the micro-organism to use sugars (mono-, di-, oligo saccharide) as sole carbon source and carry out fermentation as because certain bacterial group can ferment specific sugar moieties and this pattern can be helpful in differentiation of the bacterial group/species. Lactic acid, formic acid, acetic acid, butyric acid, butyl alcohol, acetone, ethyl alcohol, carbon dioxide and hydrogen are some of the common end-products of bacterial fermentation process. Phenol red is commonly used as a pH indicator in carbohydrate fermentation tests. The fermentation of certain carbohydrates may result in production of acids that decreases the pH of the media, thereby changing the color of the media from red color to orange to yellow coloration (Reddick, 1975; McDade & Weaver, 1959) [29, 30].

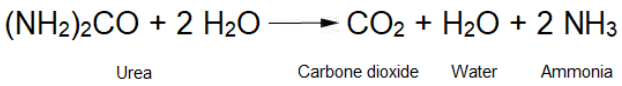
3.2.6 Starch hydrolysis test
Starch is a long chain polysaccharide that exists as a mixture of two forms, linear (amylose) and branched (amylopectin), predominant being the branched configuration. D-glucose molecules in both amylose and amylopectin are bonded by 1, 4-a-glycosidic (acetal) linkages (Pfister & Zeeman, 2016) [31].

The bacterial cells don't take the starch from the media as the Starch molecule are too large to pass through the bacterial cell membrane. Therefore, for metabolism the starch molecule must have to be reduced to smaller molecules/fragments so that then can easily take up by the cell, converted and metabolized in the cell. The microbial cells that are able to metabolize starch, produce extracellular enzymes a-amylase and oligo-l,6-glucosidase that are to hydrolyze starch by breaking the glycosidic linkages between the sugar subunits (Brust *et al.*, 2020) [32].

Because both the starch and its sugar subunits are soluble (Clear) in the medium, the reagent iodine is used to detect the presence or absence of starch in the vicinity around the bacterial growth. Iodine reacts with starch and produces a blue or dark brown color; therefore, any microbial starch hydrolysis will be revealed as a clear zone surrounding the growth. (Harrigan & McCance, 1976) [40].

3.2.7 Catalase test
Catalase test is based on production of catalase enzyme that mediates the breakdown of hydrogen peroxide into oxygen and water. The catalase enzyme are produced by the bacteria to protect it from the lethal effect of Hydrogen peroxide that get accumulated during the carbohydrate metabolism. The catalase reaction is enabled when a small inoculum of bacterial isolate was introduced into hydrogen peroxide, formation of air bubbles indicate positive for catalase test (Murray *et al.*, 2003) [41].

3.2.8 Urease test
Urea is hydrolyzed to release of end products such as ammonia and carbon dioxide by the help of urease enzyme. The urease enzyme in the presence of water, splits Urea and releases ammonia and carbon dioxide. The ammonia thus produced combines with carbon dioxide and water to form ammonium carbonate. The ammonium carbonate thus produced turns the medium alkaline. The change in pH is indicated by phenol red (pH indicator) that changes the color of the media from yellow color to red/pink/orange color (Dahlén, *et al.*, 2018; Pathak, *et al.*, 2004; Heikrujam *et al.*, 2020) [36-38].



3.2.9 Sequence similarity search and Phylogenetic analysis
The National Center for Biotechnology Information (NCBI) database (Sayers *et al.*, 2022) [42] was used to conduct the online Basic Local Alignment Search Tool (BLAST) tool-based sequence similarity searches (Altschul *et al.*, 1990; Lorenz, 2012) [7, 17] searches. The 16S rRNA gene sequences of the type strains and other strains closely related to our 5–15 comparable isolates were collected from the NCBI database in order to create a phylogenetic tree (Zhang *et al.*, 2018;) [8].

4. Results and Discussion
4.1. Physio-chemical properties of soil sample

SL. No.	Description	Analysis Method	Units	Device	Values of soil sample collected	
					Mahabubnagar	Medchal
1	pH	pH meter	-	pH meter	7.1±0.01	7.9±0.25
2	Moisture content	Oven drying method	In Percentage	Oven drying method	21.30	24.01
3	Electrical conductivity	Digital portable water analyzer kit (model 161 E)	m/mhos	Digital portable water analyzer kit (model 161 E)	0.486±0.163	0.537±0.12
4	Soil texture	Robinsons pipette method	-	Robinsons pipette method	Deep dark brown loamy	Deep black clayey
5	Total Organic matter	Titrimetric method (Walkley and Black, 1934) [47] % Soil organic matter=% organic carbon × 1.724	In Percentage	Titrimetric method (Walkley and Black, 1934) [47] % Soil organic matter=% organic carbon × 1.724	0.89	1.282 0.231
6	Dry Bulk density	Core sampling method	Gm/cm^3	Core sampling method	0.98±0.08	1.21±0.38
7	Total nitrogen	Micro kjeldhal Method	Kg/ha	Micro kjeldhal Method	159	200
9	Phosphorus	Spectrophotometric method	Kg/ha	Spectrophotometric method	98.43±35.67	97.8±31.05
10	Potassium	Flame photometer method (1986)	Kg/ha	Flame photometer method (1986)	402.8±18.99	242.05±62.01

4.2 Enumeration of Azotobacter from soil sample

The Soil sample was collected from different geographical locations.

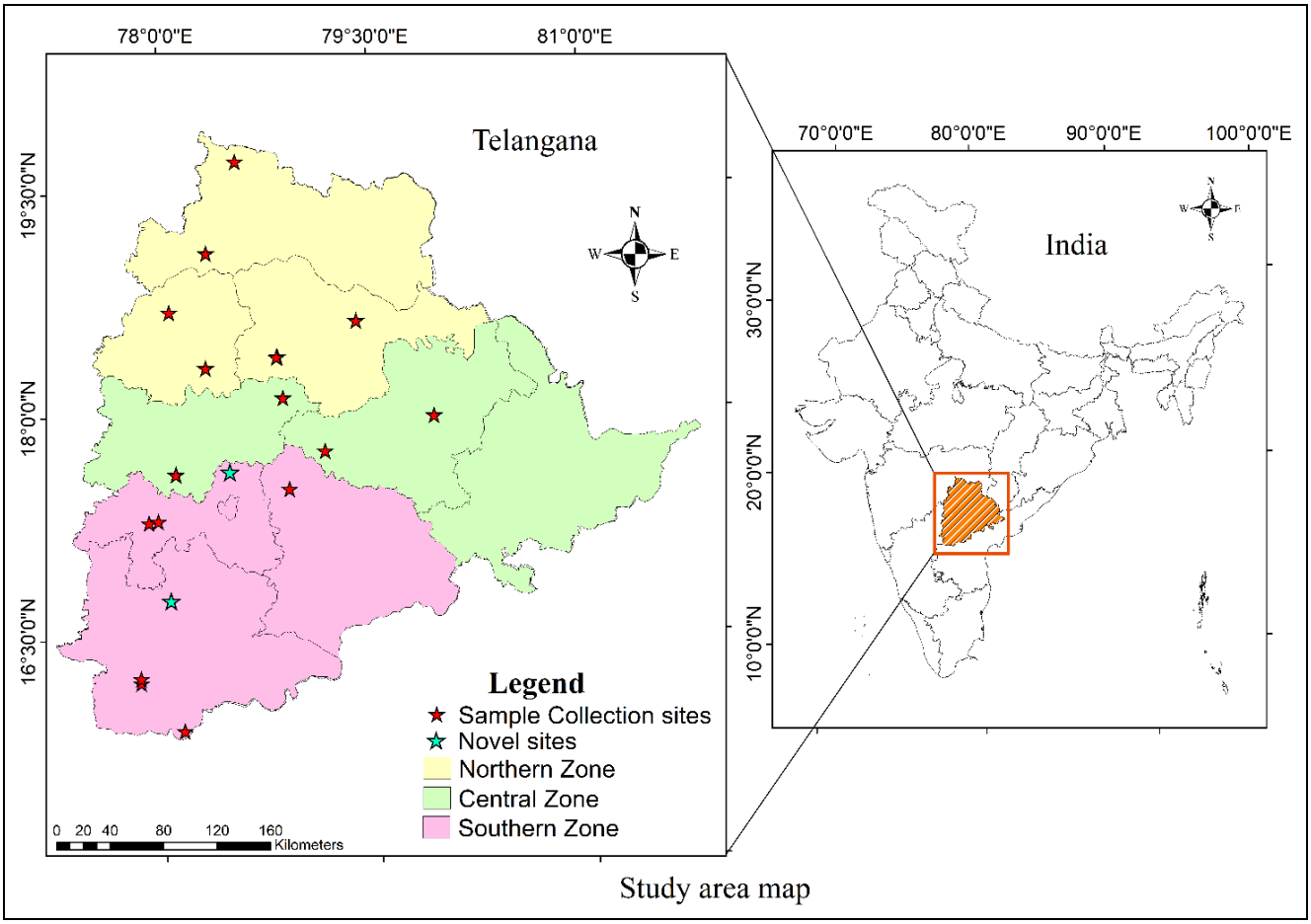
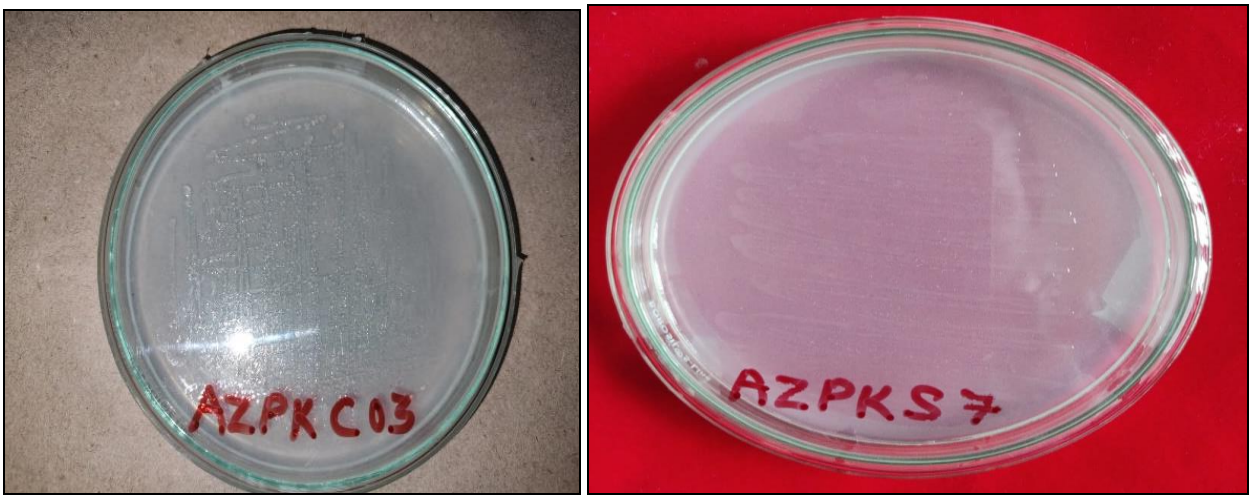


Fig 1: Map of Collected samples Study area from Telangana state, India

4.3 Identification and Characterization of *Azotobacter sp.*

The Azotobacter sp. that were isolated from the area were classified as BPKOU06TS (Mahabubnagar) and BPKOU08TS (Medchal), and they were further recognized by the morphology of their colonies (Table 1) before being subjected to various stains and biochemical tests to determine their characteristics. Table 2 lists the various biochemical and staining techniques used for various azotobacter isolates.



BPKOU06TS (Mahabubnagar) BPKOU08TS (Medchal)

Fig 1: Pure culture of isolated Azotobacter sp. on Ashby Mannitol agar

Table 1: Morphological characteristic of cultures isolated from rhizosphere soils grown on Ashby nitrogen modified media at 32 °C.

Isolate label	Colony Form	Colony Margin	Colony Elevation	Colony Colour	Optical density	Cell shape
AZPKN 1	Circular	Entire	Convex	Brownish	Opaque	Cocci shaped
AZPKN 4	Circular	Entire	Convex	Creamy	Opaque	Rod shaped
AZPKC 3	Circular	Entire	Raised	Creamy	Opaque	Rod shaped
AZPKC 7	Circular	Entire	Convex	Creamy	Opaque	Rod shaped
AZPKS 9	Circular	Entire	Convex	Creamy	Opaque	Rod shaped
AZPKS 13	Irregular	Entire	Raised	Creamy	Opaque	Rod shaped

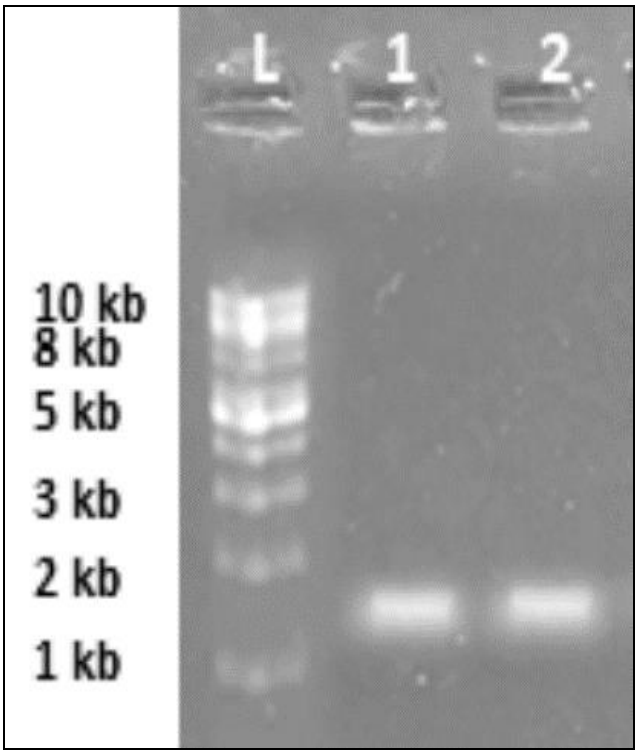
Table 2: Biochemical Characteristics of the cultures isolated from rhizosphere soils

Isolate label	Catalase	Oxidase	Starch	Citrate	Nitrate reduction	Motility	Indole	Methyl Red	Voges Proskauer
AZPKN 1	+	-	-	+	+	+	-	-	-
AZPKN 4	+	+	+	+	+	+	+	-	+
AZPKC 3	+	-		+	+	+	-	-	+
AZPKC 7	+	+	-	-	+	+	-	-	+
AZPKS 9	+	-	-	+	+	+	-	-	-
AZPKS 13	+	+	+	+	+	+	+	-	+

Note: (+: good activity, -: no activity).

4.4 Characterization of different *Azotobacter sp*. by 16s rRNA sequence

16s rRNA sequencing was used to further describe the azotobacter sp. strains. Purified DNA was taken from the microbial isolate. The ratio of A260nm/A280 nm was 1.76 (1.8) for qualitative estimate, indicating that the DNA extracted was pure DNA. Quantitatively, 142.5 g/ml of pure DNA were extracted. Using the designated primers, the 16s rRNA was sequenced. The bacterial species is identified through the amplification of a region utilising specific universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3'), which are complementary to the conserved sections of the bacterial 16S rRNA gene. Afterwards a 0.8% agarose gel is used to examine the genomic fragments that have been amplified (Figure 3).



Lane L - LADDER DNA; Lanes 1 and 2 are BPKOU06TS and BPKOU08TS, respectively.

Fig 3: Shows a gel electrophoresis image on an agarose gel (0.8% agarose)

4.5 Submission of *Azotobacter sp.* into NCBI database

Following that, the sequence submission tool was used to submit the sequences to the NCBI database. Azotobacter tropicalis strain (BKPOU08TS) has the gene bank accession ID OP536206, while Azotobacter beijerinckii strain (BKPOU06TS) has OP536202.

5. Conclusions

The current studies prove to be showing different Soil microbial biodiversity effects and it was found to be 2 new strains of azotobacterial species due to climatic variation in those areas due to change in the temp, Ph, salinity i.e. Abiotic factors. One of the most crucial elements for total plant development is the Plant Growth Promoting Rhizobacteria (PGPR). The management and facilitation of a healthy and sustainable agricultural system will benefit from the study of Azotobacter sp. isolation, identification, and characterization from various agro-climatic zones, particularly as a healthy alternative as bio-fertilizer.

6. Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7. Acknowledgements

The authors acknowledge Department of Botany, University college of Science, Osmania University Hyderabad, Telangana, India for the support and laboratory facilities. My gratitude to the founder, Nawab Osman Ali Khan, the seventh Nizam of Hyderabad who rather through a Farman or Royal Charter, brought the University into existence in 1918.

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