RESEARCH ARTICLE

Identification and Characterization of *Azotobacter sp.* Screened from Different Agro-climatic Zones in Telangana.

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Abstract

Declining soil quality due to overexploitation of chemical-based fertilizers, pesticides, and insecticides has a deleterious effect on soil health. This has resulted in decreased productivity. Using *Azotobacter* sp. as an alternative to chemical-based agricultural practices can be a remedial step to increase soil healthiness and productivity. In this research work, soil samples from the rhizosphere of Pigeon peas cultivated at Mahabubnagar and Medchal agroclimatic zone of Telangana were collected. The *Azotobacter* spp. were isolated and maintained on Ashby's mannitol agar medium. Morphological, biochemical, and molecular attributes evaluated the *Azotobacter* spp. Biochemical tests and 16s rRNA sequencing revealed the identification of strains as *A. beijerinckii* strain (BKPOU06TS) and *A. tropicalis* strain (BKPOU08TS). The 16s rRNA sequences of *A. beijerinckii* strain (BKPOU06TS) and *A. tropicalis* strain (BKPOU08TS)were submitted to GenBank with accession ID- OP536202 and OP536206, respectively.

Keywords: *Azotobacter* sp., agroclimatic zone, sustainable agriculture practice, 16s rRNA sequencing.

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Introduction

Azotobacter sp. is one of the Plant Growth Promoting Rhizobacteria (PGPR) that are high-living, heterotrophic, and free-living, which can be beneficial for the overall growth and development of different plants. They also involve the development of the health of the soil (Gandora *et al.*, 1998). The bacteria utilize free nitrogen, and it converts to cell proteins. Further, these cells are permineralized, which helps in the availability of nitrogen to plants and, ultimately, growth promotion in plants. *Azotobacter* helps increase nutrient mineralization in the soil, increase thereby increasing the bioavailability of micro and macro minerals such as Carbon, Nitrogen, Phosphorus, and Sulphur to plants (M.avoidrigues *et al.*, 2020).

They also help in avoiding the uptake of inorganic heavy metals from soil to cellular components of plants (Lévai *et al.*, 2008). *Azotobacter* releases growth regulators and synthesis esters. Besides nitrogen fixation, it also synthesizes different Phyto-hormones that regulate cellular processes (Hennequin *et al.*, 1966; Azcorn & Barea, 1975). *Azotobacter* also has the capability for an anti-pathogenic effect against plant pathogens.

They secrete siderophores that bind to the available form of iron (Fe^{+3}), making them unavailable for pathogens. They also secrete fungicidal antibiotics like anisomycin (Zuhaib M *et al.*, 2019**;** Sagar *et al.*, 2022). *Azotobacter* balances nutrient conditions, thereby increasing leaf area, thereby increasing photosynthetic capacity leading to better assimilation and yield (Estiyar *et al.*, 2014). *Azotobacter sp.* also helps in the development of overall physiological development, such as rooting, shooting, flowering, and fruiting. These bacteria also help to increase the tolerance of plants towards water scarcity conditions. (Gandora *et al.*, 1998;A.M Sorty et al, (2018).

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Materials and Methodology

Collection of soil sample

The soil sample was collected from the rhizosphere soil region (root nodules of *Cajanus cajan* (Pigeon pea) in agricultural land (Mc Pherson *et al.*, 2018) of different agro-climatic regions such as Mahabubnagar and Medchal location in Telangana (Fig 1).

The loose soil was collected from the plant's root nodules by gently shaking the uprooted plant. The loose soil thus collected was kept in a zipper bag for further analysis.

Physiochemical Assessment of Soil Sample

The soil sample was first assessed for physical and chemical properties. Some physiochemical analysis was pH, Moisture content, Electrical conductivity, soil texture, Total Organic matter, Dry Bulk density, Total nitrogen, Nitrates (NO_3^-), Phosphorus, Potassium (Tziachris *et al.*, 2022).

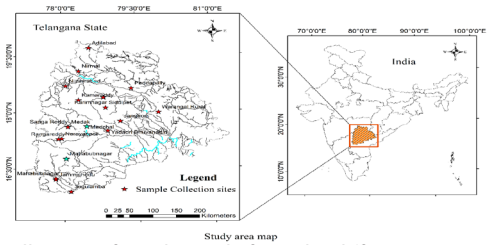

Fig 1: Collection of a soil sample from the different geographical areas in Telangana.

Isolation of *Azotobacter* from a Rhizosphere Soil Sample

The Azotobacter strains were isolated from the soil sample using Ashby's mannitol agar medium (Wz Yusminah Halal, Alimuddin Ali(2019); Wakarera *et al*., 2022). The micro-organism was isolated by serial dilution (Ben-David & Davidson 2014; Hala(2020) of the soil sample collected from the different geographical area spread plate (Hartman, 2011) onto Ashby's mannitol agar medium and were incubated at 28°C for seven days in the standardized bacteriological incubator until colonies appear on the plates.

Identification of *Azotobacter sp.* by Biochemical Tests

The isolated bacterial colonies (sticky and glistening colonies) were first characterized by colony morphology (Sousa *et al*., 2013) and different biochemical tests (Dadook *et al*., 2014; Jiménez *et al*., 2011) in accordance with Bergey's Manual for Determinative bacteriology method (Cowan, 1948) were performed to identify *Azotobacter sp.*

Genomic DNA Isolation

The isolated cultures of azotobacter were first taken and inoculated into sterile Luria Bertini (LB) broth (Yamamoto *et al*., 2021) and kept in a shaking incubator at 20°C for 48 hours at 150 r.p.m.

For isolation of genomic DNA from azotobacter, 1.5 ml of the overnight *azotobacter* culture was centrifuged (8000 r.p.m for 10 mins at 4°C). After centrifugation, the pellet was collected and then resuspended in 1000µl 1X Trizma-EDTA buffer (10 mM Tris-HCl, 1-mM EDTA). The mixture was again centrifuged, and the pellet was again resuspended in 500 µl 1X Trizma-EDTA buffer. 05µl of lysozyme enzyme (standard concentration-50microgram/milliliter) and 2 µL of RNase A (standard concentration- 10 microgram/milliliter) were added to the above mixture and were kept at 37°C for 10 minutes. Then after incubation, 04 µL of proteinase K (standard concentration-20 microgram/mL) and 15µl of 20% SDS was again added and kept for incubation at 60°C for 30 minutes. Then Phenol: Chloroform: Isoamyl alcohol (25:24:1) mix was added, shaken well, and allowed to stand for 15-20 minutes. The organic layer was carefully separated, and to this layer, an equal volume of Chloroform: Isoamyl alcohol (24:1) was added and allowed to stand for 30 minutes. Ice-cold isopropanol (3 times the volume of the mixture) was added. DNA appears to be a suspended cloud-like structure. The solution was then centrifuged, and pellet was collected and resuspended in 500µl of 1X Trizma-EDTA buffer and stored at -5°C for further use.

The DNA was separated in 1.0% agarose gel and visualized on UV trans-illuminator. The quantitative and qualitative analyses for the isolation was DNA were done using a Spectrophotometer method. (Khare *et al*., 2014).

Pcr Amplification And 16s RRNA Sequencing

The PCR reaction was carried out in a Thermal cycler (Perkin Elmer) using two different universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3'). These primers complement the conserved regions of the bacterial 16S rRNA gene (Lorenz, 2012). The PCR conditions for were is specific reaction was followed as initial denaturation step: 95°C with time = 3 minutes, 35 cycles of denaturation at 94°C with time= 45 seconds, annealing- 51.8°C with time=45 seconds, elongation- 72°C with time= 2 minutes and final extension- 72°C with time=5 minutes (Dos Santos *et al*., 2019). The PCR hold time to at –4°C (Chan *et al*., 2016; Rosselli *et al*., 2016).

The PCR products (amplified region) were then observed by agarose gel electrophoresis using 1.0% agarose gels and 1X Trizma-acetate EDTA buffer (TAE buffer), pH = 8.0. (Khare *et al*., 2014).

Sequence Similarity Search And Phylogenetic Analysis

The similarity search for the sequence was performed by the online BLAST tool (Altschul *et al*., 1990) maintained by NCBI, and the line sequences to the query sequences obtained from other microbial sequences in the Global NCBI nucleotide database (Sayers *et al*., 2022).

The phylogenetic tree (Zhang *et al*., 2018) was constructed with respect to the available sequence in NCBI databases. The 16S rRNA gene sequences of type strains and other strains closely related to our 5-15 similar isolates were obtained from the NCBI nucleotide database. Sequence alignments were performed by the Clustal Omega program (Thompson *et al*., 1994).

Submission to NCBI Database

The rRNA sequence of isolated *Azotobacter* strains was submitted to the NCBI database through the concerned submission portal available.

Results

Physiochemical Properties Of Soil Sample

The Soil sample was collected from geographical locations such as Mahabubnagar and Medchal in Telangana. The physicochemical properties of the soil sample thus collected were analyzed as described in Table 1.

Enumeration of *Azotobacter* from soil sample

The spread plates were incubated at 28°C for 7 days. The *Azotobacter sp*. were concluded by the presence of sticky and glistening colonies around the grains. These sticky and glistering colonies were then isolated by inoculation loop and again streaked on sterilized Ashby Mannitol agar, incubated to maintain the pure culture for the isolates (Fig 2).

Table 1: Physiochemical properties of soil sample.

Sl. No.	Description	Analysis Method	Units	Values of soil sample collected	
				Mahabubnagar	Medchal
1	pH	pH meter	-	7.1 ± 0.01	7.9 ± 0.25
2	Moisture content	Oven drying method	In Percentage	21.30	24.01
3	Electrical conductivity	Digital portable water analyzer kit (model 161 E)	m/mhos	0.486 ± 0.163	0.537 ± 0.12
4	Soil texture	Robinsons pipette method	-	Deep dark brown loamy	Deep black clayey
5	Total Organic matter	Titrimetric method (Walkley and Black, 1934) %Soil organic matter=% organic carbon × 1.724	In Percentage	0.89	1.282 0.231
6	Dry Bulk density	Core sampling method	Gm/cm3	0.98±0.08	1.21±0.38
7	Total nitrogen	Micro kjeldhal Method	Kg/ha	159	200
9	Phosphorus	Spectrophotometric method	Kg/ha	98.43±35.67	97.8±31.05
10	Potassium	Flame photometer method (1986)	Kg/ha	402.8±18.99	242.05±62.01

Identification and Characterization of *Azotobacter sp*

The *Azotobacter sp.* that were isolated from the geographical area was coded as BPKOU06TS (Mahabubnagar), and BPKOU08TS (Medchal) were further identified initially by their colony morphology (Table 2) and characterized by different staining and Biochemical tests. Different staining and biochemical procedures that were carried out for different azotobacter isolates were described in Table 3.

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

The isolated A*zotobacter sp.* strains were further characterized by PCR and 16s ribosomal RNA sequencing. The DNA was extracted from the microbial population and was purified. For qualitative estimation, the ratio of A_{260nm}/ A_{280nm} was 1.76 (~1.8), suggesting that the DNA extracted was purified DNA. Quantitatively, the amount of purified DNA extracted was 142.5 µg/ml.

BPKOU06TS (Mahabubnagar) **BPKOU08TS (Medchal)**

Fig 2: Pure culture of isolated *Azotobacter sp.* on Ashby Mannitol agar.

Table 2: Colony morphology of *Azotobacter sp.* isolated from different agro-climatic zones.

Sl. No.	Colony Characteristics	Staining and Biochemical test results	
		BPKOU06TS Mahabubnagar	BPKOU08TS Medchal
1	Shape	Circular	Circular
2	Size	Moderate	Moderate
3	Elevation	Raised	Raised
4	Margin	Entire	Entire
5	Opacity	Translucent	Opaque
6	Color	White	Pale white
7	Texture	sticky and glistering	sticky and glistering

Table 3: Biochemical tests for identification of different Azotobacter strains isolated from different geographical locations in Telangana.

Sl. No.	Description of Staining and Biochemical Tests	Staining and Biochemical test results	
		BPKOU06TS Mahabubnagar	BPKOU08TS Medchal
1	Staining (Gram stain)	Positive rods	Negative rods
2	Indole test	Positive	Positive
3	Methyl Red test	Positive	Positive
4	Voges Proskauer test	Positive	Positive
5	Citrate test	Positive	Positive
6	Catalase test	Positive	Positive
7	Nitrate Reduction	Positive	Positive
8	H2S production	Positive	Positive
9	Oxidase test	Positive	Positive
10	Gelatin Hydrolysis	Positive	Negative
11	Pigment production test	Positive	Positive
12	Ortho nitro phenyl β-D-galactopyranoside test	Negative	Negative
13	Lysine decarboxylase test	Negative	Negative
14	Urease test	Negative	Negative
15	Starch hydrolysis	Positive	Positive
16	Motility	Motile	Motile
17	Carbohydrate Fermentation		
17.1	Glucose	Positive	Positive
17.2	Fructose	Positive	Positive
17.3	Sucrose	Positive	Positive
17.4	Rhamnose	Negative	Negative
17.5	Maltose	Negative	Negative
17.6	Mannitol	Positive	Negative
Identified *Azotobacter sp.*		*Azotobacter beijerinckii*	*Azotobacter tropicalis*

The 16s rRNA sequencing was carried out using the specified primers. The amplified genomic fragments are then analyzed by 0.8% agarose gel (Fig 3).

The consensus sequence for the isolates *of Azotobacter* strains was

>Consesus_BPKOU06TS

TCTAAGAATAGGGGACAACGTTTCGAAAGGAACGCTAATAC-
CGCATACGTCCTACGGGAGAAAGTGCTCGCGGACCTCAC-
GCTATCCCATATGGACTAGCCTAGGTCGGCATTTATAGC-
TAGTTGGTGGGGTAAAGGCCCCGATCCGTAACTGGTCT-
GAGAGGATGATNNGTCACACTGGAACTGAGACACGGTC-
CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA-
CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGT-
GAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAG-
GAAGGGCTGTAAGCGAATACCTTGCAGTTTTGACGTTACC-
GACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCG-
GTAATACGAAGGGTGCAAGCGTTAATCGGAATTTACTGGGC-
GTAAAGCNNGCGTAGGTGGTTTGGTAAGTTGGATGT-
GAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAANNCT-
GCCTGACTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGT-
GTAGCTTGGTGAAACGTAGATATAGGAAGGAACACCAGTG-
GCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGC-
GAAAGCGTGGGGAGCAAACTATTATAGATACCCTGGTAGTC-
NACGCCGTAAAGTATGTCGACTAGCCGTTGGGCTCCTT-
GAGAGCTTAGTGGCGCAGCTAACGCATTAAGTCGACC-
GCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATT-
GACGGGGGCCCGCACAAGCGGCTATAGGCATGTGGTTAT-
TAATTCGAAGCAACGCGACCTTACCTGGCCTTGACATCCTGC-
GAACTGGAAGATACCTGGGTGCCTTCGGGAGCGCAGAGA-
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGAT-
GTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGT-
TACCAGCACGTCATGGTGGGCACTCTAAGGAGACTGCCG-
GTGTTTAGCCGGAGGAAGGTGGGGATGACGTCAAGTCAT-
CATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATG-
GTCGGTACAGAGGGTTGCCAAGTCGCGAGGCGGAGC-
TAATCCCAGAAAACCGATCGTAGTCCGGATCGCAGTCTG-
CAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCAAAT-
CAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACAC-
CGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGC-
TAGTCTAACCTTCGGGGGGACGGTTACCACGGAGTGATTCAT-
GACTGGGGTGAAGTCGTAACAAGGTAGTCGGCGAACCAATG

>Consesus_BPKOU08TS

TAGCATTTAGGGCTCAGATTGAACGCTGGCGGCAGGCCTAA-
CACATGCAAGTCGAGCGGCAGCGGGACCTTCGGGTTGCCG-
GCGAGCGGCGGATAAGATAGTAATGCCTAGGAATCTGCCT-
GTTAGTGGGGGATAACGCGGGGAAACTCGCGCTAATACCG-
CATACGTCCTACGGGAGAAAGCGGGGGCTCTTCGGACCTC-
GCGCTAACTGATGAGCCTAGGTCGGATTAGCTAGTTG-
GTGGGCTAGTAGCCCACCAAGGCGACGATCCGTAACTG-
GTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACG-
GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG-
GACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGT-
GAAGACTAAAGTCGGATTGTAAAGCAGGAGGAAGGGCT-
GTAGGCTAATACCTTGCAGTTTTGACGTTCCGACAGAATA-
AGCGACCTAGCTATTACGTGCCAGCAGCCGCGGTAATAC-
GAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC-
GCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAAGCCCC-
GGGCTCAACCTGGGAACTGCATCCAAAACTACTGGGC-
TAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGT-
GAAATGCGTAGATACATGAAGGAACACCAGTGGCGAAGGC-
GACCACCTGGACTGATACTGACACTGAGGTGCGAAAGC-
GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC-
CGTAAACGATGTCGACTAGCCGTTGGCTCCTTGAGAGCTTA-
AGTGGCGCAGCTAACGCATTAAGTCGACCGCCTGGGGAG-
TACGGCCGCAAGGTTAAAACTCAAATGAATTAGTAGGATG-
TAGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC-
GCGAAGAACCTTACCTGGCCTTGACATCCTGCGAACTTG-
GTAGAGATACCTTGGTGCCTTCGGGAGCGCAGAGACAGGT-
GCTGCATGGCTGTCGTCAGCTTATGTCGTGAGATGTTGGGT-
TAAGCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGT-
CACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAAC-
CGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCT-
TACGGCCAGGGCTACACACGTGCTACAATGGTCGGTA-
CAGAGGGTTGCCAAGCCGCGAGGCGGAGCTAACATCCC-
GAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACT-
GCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTC-
GCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT-
CACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTC-
TAACCTTCGGGGGGACGGTTACCACGGAGTGATTCAT-
GACTGGGGTGTAGGATAGGATGTATTA

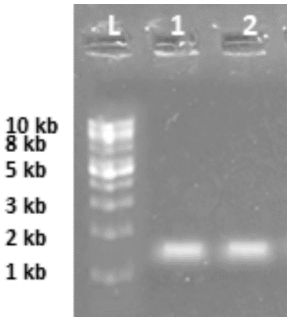

Fig 3: Agarose gel image (0.8% agarose) of gel electrophoresis. Lane L – LADDER DNA; Lane 1- BPKOU06TS; Lane 2- BPKOU08TS *(Approx size: ~13kb-15kb)*

The consensus sequences were then searched for the similar sequence in the global non-redundant databases (GenBank+EMBL+DDBJ+PDB+RefSeq) with the help of the BLAST program using blastn suite (Zheng et at., 2000). The phylogenetic tree was constructed using the NJ (using Neighbour Joining) method. From the analysis, it was identified that the isolated *Azotobacter sp.* BPKOU06TS and BPKOU08TS identified as *Azotobacter beijerinckii* (Fig 4) and *Azotobacter tropicalis* (Fig 5).

Submission of *Azotobacter sp.* into NCBI database

The sequences were then submitted to the NCBI database through the sequence submission tool. The gene bank accession ID for the *Azotobacter beijerinckii* strain (BKPOU06TS) is OP536202, and for *Azotobacter tropicalis* strain (BKPOU08TS) is OP536206.

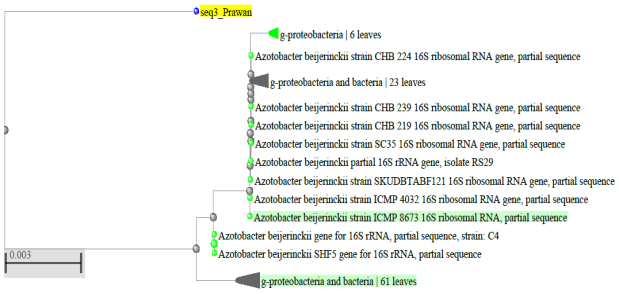

Fig 4: Phylogenetic analysis show isolated *Azotobacter sp.* (BPKOU06TS) identified as *Azotobacter beijerinckii*.

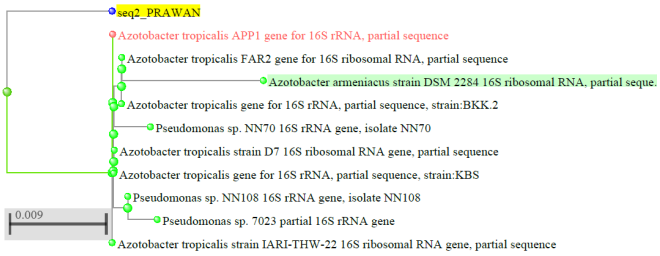

Fig 5: Phylogenetic analysis show isolated *Azotobacter sp.* (BPKOU08TS) identified as *Azotobacter tropicalis*

Conclusion

The Rhizobacteria (PGPR) are the most important entity for overall plant development. The study of isolation, identification, and characterization of Azotobacter sp. from various agro-climatic zones will help manage and facilitate healthy and sustainable agricultural systems, especially as a healthy alternative to bio-fertilizer. Through comprehensive screening processes, the researchers were able to isolate these beneficial bacteria, which are known for their nitrogen-fixing capabilities, making them potentially valuable for sustainable agricultural practices. This study successfully identified and characterized Azotobacter spp. strains from various agro-climatic zones in Telangana.

The findings of this research provide valuable insights into the diversity and distribution of Azotobacter sp. in different agro-climatic zones within the Telangana region. By understanding their characteristics and adaptability to various environmental conditions, farmers and agricultural practitioners can make informed decisions on the application of these strains to enhance soil fertility and crop productivity.

Moreover, the study's results may also open up opportunities for further research and biotechnological applications, such as developing biofertilizers or exploring their potential for biocontrol of plant pathogens. Harnessing the capabilities of Azotobacter sp. can contribute significantly to reducing the dependency on chemical fertilizers and promoting sustainable agriculture in the region. This research sheds light on the importance of microbial resources and their potential contributions to agricultural practices. It serves as a stepping stone for future studies in the field of microbial ecology and agriculture, and it has the potential to benefit farmers, environmentalists, and the overall agricultural community in Telangana.The research successfully identified and characterized two distinct Azotobacter strains, to confirm and gain deeper insights into the genetic identity of these strains, 16s ribosomal RNA sequencing was performed. The results of the sequencing aligned with the global non-redundant databases, confirming the accurate identification of Azotobacter beijerinckii and Azotobacter tropicalis in BPKOU06TS and BPKOU08TS, respectively.

The findings from this study contribute valuable data to the field of microbial ecology, particularly in understanding the diversity and distribution of Azotobacter sp. in the Telangana region. Moreover, by providing specific gene bank accession IDs (OP536202 for Azotobacter beijerinckii and OP536206 for Azotobacter tropicalis), these sequences have been made publicly available in the NCBI database for reference and future research.

Conflict of Interest

None

Acknowledgments

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Author Contributions

The project was conceptualized and carried out by Koppula Prawan under the supervision of Dr. B Kiran Kumar. Mohammad Mir Imran, Eshwar Chate, and Kandula Jayapaul assisted in sample collections and preparation.

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