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Research Article

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Isolation, purification and identification of cyanobacterium *Tolypothrix* sp. KJE1

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Abstract

A cyanobacterial strain, *Tolypothrix* sp. KJE1, was isolated from rice fields of Banaras Hindu University, Varanasi, during the monsoon of 2019. Morphological analysis and 16S rRNA phylogenetic studies identified it as *Tolypothrix* sp., known for nitrogen fixation. The partial 16S rRNA gene sequence (1283 bps) was submitted to NCBI (Accession Number OP353555). This study underscores *Tolypothrix*'s role in sustainable agriculture, contributing to soil fertility and reducing dependency on synthetic fertilizers, with potential applications in biofertilization.

Keywords: Rice field, Cyanobacteria, Strain, Light Microscope, Heterocyst, Hormogonia, BLAST.

1. Introduction

Cyanobacteria are Gram-negative, oxygen-producing, and widespread prokaryotes that have existed on Earth for over two billion years[1]. Approximately 2.8 billion years ago, they were the primary contributors to an oxygenic atmosphere, thriving in fossilized forms like stromatolites and oncolites[2]. These organisms exhibit diverse life forms, ranging from free-living species to those in symbiotic relationships with plants, fungi, and animals, adapted to various environments and showing significant morphological variation[3, 4, 5]. Cyanobacteria play a crucial role in maintaining soil health by enhancing nutrient availability, soil porosity, pH balance, water retention, and reducing soil salinity. Additionally, they contribute to essential plant physiological and biochemical processes. Paddy fields, in particular, serve as a key ecological habitat for cyanobacteria, where they perform vital functions such as nitrogen fixation and photosynthesis, supporting various physicochemical processes[6, 7].

Rice, an annual grass belong to the Poaceae family, includes 20 wild species and two cultivated varieties. Of these, *Oryza sativa* is the most widely cultivated, while *Oryza glaberrima* is primarily restricted to West Africa[8, 9].

The cultivation of rice is highly dependent on climatic conditions and seasonal factors. As a staple food, it provides a significant source of calories for over 60% of the global population. In India, around 45 million hectares of arable land are dedicated to rice cultivation, relying heavily on chemical fertilizers[10].

Currently, the world faces two critical challenges: the changing climate and rapid population growth. Recent estimates suggest that the global population could exceed 9 billion by 2050, putting increased pressure on food production with limited land resources.

Isolating, purifying, identifying, and screening cyanobacterial isolates is a vital step in leveraging their potential for bio-fertilizer production. The isolation process involves separating cyanobacterial species from their natural habitats, using techniques like serial dilution and streak plating to obtain individual species from various sources. Studying mat-forming cyanobacteria is crucial due to their ecological and biotechnological importance, providing insights into their survival strategies and potential applications across different fields. These cyanobacteria typically thrive in environments with fluctuating water availability, such as terrestrial soils, biological crusts, and specific aquatic settings. Therefore, understanding the processes of isolation, identification, purification, and screening of mat-forming cyanobacteria is essential for uncovering the mechanisms that enable their resilience in challenging environmental conditions. This research work aims to systematically investigate mat-formation in one or more strains of cyanobacteria using a comprehensive approach.

The isolation process begins with collecting samples from agricultural fields. Identification techniques, including microscopic examination and molecular methods, facilitate accurate taxonomic classification and confirmation of the isolated cyanobacterial strains. Purification steps refine these cultures to obtain pure isolates for further analysis. Additionally, the screening process evaluates the characteristics of mat-forming cyanobacteria. These screening efforts not only enhance our understanding of cyanobacterial mat formation but also hold potential for identifying unique candidates with beneficial mat-forming traits for biotechnological applications, such as soil enhancement and environmental restoration.

2. Materials and Methods

2.1. Sampling of cyanobacterial strain

Cyanobacteria are widely distributed across diverse ecosystems globally. Rippka et al.[11] classified these organisms into five subsections based on their morphology and cell differentiation characteristics. In the present study, we have focused on collecting heterocystous cyanobacteria from subsection IV. These cyanobacteria are crucial for nitrogen management due to the presence of heterocysts, specialized cells capable of nitrogen fixation.

The robustness of this cyanobacterial system is evidenced by the formation of akinetes under nutrient stress and the production of hormogonia, which act as reproductive filaments. Samples were collected in pre-sterilized Falcon tubes from the rice fields of BHU, Varanasi, India (25º15′50″N 82º59′13″E and 25º15′20″N 82º59′25″E). Sampling took place between August and October 2019, when cyanobacterial colonies are most abundant, thriving in temperatures of 25-35°C[12, 13]. These specific areas within BHU's agricultural fields were chosen for their consistent water availability and minimal exposure to prolonged desiccation.

2.2. Growth medium for cyanobacteria

Axenic cultures of cyanobacterial strains were carefully maintained in 120 ml of nitrate-free basal growth medium (BG-11N^{-}) within 250 ml borosilicate flasks, which lacked any combined nitrogen source[11]. The culture conditions were regulated at a pH of 7.5 and a temperature of 25 ± 2ºC, with 16 h light and 8 h dark cycle at an intensity of around 55 µmol photons $m^{-2}s^{-1}$. The specific composition of the culture medium is outlined below:

Table 1. Composition of BG-11 medium[11]

Macronutrients	gL^{-1}	Micronutrients	gL^{-1}
$K_2HPO_4.3H_2O$**	40.0	$MnCl_2.4H_2O$	1.810
$MgSO_4.7H_2O$	75.0	$ZnSO_4.7H_2O$	0.222
$CaCl_2.2H_2O$	36.0	$Na_2MoO_4.2H_2O$	0.390
Na_2CO_3	20.0	$CuSO_4.2H_2O$	0.079
Na_2-Citrate	6.0	$CoCl_2.2H_2O$	0.0494
$Fe(III)(NH_4)_3$citrate**	6.0		
EDTA	1.0		

$NaNO_3$ was not added to the medium.

** Fe $(NH_4)_3$ citrate and $K_2HPO_4.3H_2O$ were autoclaved separately and added to the precooled sterilized liquid medium to avoid precipitation. The pH of the medium was maintained to 7.5 with 0.1 N NaOH and 0.1 N HCl.

2.3. Isolation, identification and maintenance of the cyanobacterial strain

Samples were collected from the campus of Banaras Hindu University campus in Varanasi, Uttar Pradesh, India. The dilution plate method[14] was used to isolate the *Tolypothrix* from rice field soil. A 1 mL aliquot of soil suspension was spread on solidified (1.5%) BG-11N$^-$ medium. The plates were incubated at 25 ± 2°C with a 16-hour light: 8-hour dark[11] photoperiod under white fluorescent light with an intensity of 55 µmol photons $m^{-2}s^{-1}$. Morphological characteristics such as cell shape, length, and breadth of intercalary cells and heterocysts were observed for taxonomic identification based on cell or colony morphology[15]. Visible cyanobacterial colonies growing on the agar surface were aseptically picked and transferred to 100 mL of BG-11N$^-$ medium in flasks. Cyanobacterial suspensions were prepared in BG-11N$^-$ medium and spread on petri-plates. Purified colonies were isolated and transferred to fresh BG-11N$^-$ plates. Bacterial and fungal contamination, as well as cyanobacterial growth, was monitored using a light microscope. Repeated transfers between surface colonies and suspension cultures were conducted to establish axenic cultures. This iterative process continued until consistently purified cyanobacterial colonies were obtained. The cultures were shaken three times daily to enhance growth. Purified colonies were then transferred to 100 mL of BG-11N$^-$ broth, regularly checked for contamination, and maintained for further experimentation. These samples were used for mass cultivation of isolates and for analyzing growth behavior, biomass production, and generation time.

The axenic nature of the cultures were evaluated using several methods, including phase-contrast microscopic examination and plating samples on caseinate-glucose agar. This specific agar was prepared with a nutrient solution containing casamino acids (0.05%), glucose (0.5%), and agar-agar (1.2%). To check for potential bacterial contamination, a properly diluted suspension of the cyanobacterial culture was streaked onto the nutrient agar. After a week of incubation, bacteria-free micro-colonies were identified, marked with a sterile toothpick, excised along with the agar, and transferred to a 10 ml nutrient solution. The medium was sterilized, and agar slants were prepared in several sterilized tubes. The sample was inoculated into these tubes, with some incubated under light and others in darkness. After 14 days, the cultures were microscopically examined, confirming the absence of bacteria. Bacteria-free cyanobacterial colonies were selected and maintained on different agar slants, with the purity of the suspension regularly checked. Liquid cultures were manually shaken three times daily. Further investigations were postponed until a pure clonal culture of various cyanobacterial strains was successfully established and verified using taxonomic keys by Geitler[16], Desikachary[15], and Rippka et al.[11].

2.4. Identification of cyanobacterial strain

Identification of isolates by light microscope

The isolates were initially examined under light microscope by preparing temporary slides. During taxonomic characterization, various features were considered, including the shape, size, and color of the thallus, the width and length of the trichomes, the presence and location of heterocysts, filament branching, as well as the presence of hormogonia and akinetes[16, 17]

2.5. Genomic DNA Isolation and PCR Conditions

To further identify the cyanobacterial strains, partial sequencing of the 16S rRNA gene was performed. Genomic DNA was extracted from the cyanobacterial isolate using the conventional xanthogenate method[18]. The 16S rRNA gene was partially amplified with a forward primer (359F, 5'-GGG GAA TYT TCC GCA ATG GG-3') and a reverse primer (781R, 5'-GAC TAC TGG GGT ATC TAA TCC CAT T-3') (Nübel et al., 1997). The PCR reaction was carried out in 25 µl volumes, containing 30-50 µg of DNA template, 200 µM dNTPs, 0.4 µM of each primer, 1 U/µl Taq Polymerase, and 1.5 µM MgCl2 using a BioRad DNA Engine Peltier Thermal Cycler. The thermal cycler was used to amplify the DNA[19], The PCR products were sequenced using Sanger's method[20], and the resulting sequence was compared to the NCBI database using the BLAST tool.

2.6. Nucleotide sequence analysis

The partial 16S rRNA sequences obtained from DNA sequencing were thoroughly analyzed using the NCBI sequence database. The nucleotide Basic Local Alignment Search Tool (BLAST) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi was used for comparison, aligning these sequences with already existing gene sequences from various cyanobacterial strains found in the database. Additionally, the partial 16S rRNA sequences of the test cyanobacterium were formally submitted to the NCBI database.

3. Results and discussion

3.1. Collection, isolation and purification of cyanobacterial isolate

A cyanobacterial strain was collected from the agricultural fields of Banaras Hindu University (BHU) in Varanasi, Uttar Pradesh, India, as a part of an extensive study aimed at isolating and identifying naturally occurring cyanobacteria from local rice fields. Cyanobacteria, known for their ability to fix atmospheric nitrogen and thrive in a variety of environments, are crucial in agricultural settings like rice paddies, where they contribute to soil fertility. The collection was carried out between mid-August and October 2019, a period chosen strategically as the monsoon season leads to optimal growth conditions for cyanobacteria in waterlogged environments like rice fields. Specific rice fields targeted for this study were chosen for their historical use in traditional farming practices, as cyanobacteria tend to proliferate in such environments. Rice paddies, with their semi-aquatic conditions and nutrient-rich soil, provide an ideal habitat for cyanobacterial growth. The water stagnation and nutrient availability create conditions in which cyanobacteria flourish, making them excellent candidates for studies on agricultural microbiomes and their potential in biofertilization.

One such strain, exhibiting heterocystous filamentous cyanobacteria, was designated as KJE1. This strain was isolated and identified based on its distinct morphological features observed under a light microscope. These features included the filamentous arrangement of cells, the presence of heterocysts at regular intervals along the filaments, and a characteristic structure that helped differentiate it from other cyanobacterial strains. To further support the identification of strain KJE1, detailed cross-referencing with classical taxonomic literature, particularly the monograph of Desikachary[15], was performed. This monograph is a key reference in the study of cyanobacteria, providing detailed descriptions of various genera and species. The morphological features of the strain KJE1 matched closely with those described for filamentous heterocystous cyanobacteria, leading to the confirmation of its taxonomic identity. Figure 1 visually represents the site of collection, providing a geographical context for the study.

Figure 1. Collection of cyanbacterial isolates from different agricultural fields at Banaras Hindu University.

3.2. Purification of cyanobacterial isolates

When we work with motile filamentous cyanobacteria, such as those that exhibit gliding motility, a slightly different approach is required. Filamentous cyanobacteria possess the ability to move across surfaces by gliding, a process facilitated by specialized structures called trichomes or hormogonia. These motile cells can be difficult to purify using standard methods, as their movement may cause them to intermingle with other microorganisms, complicating the isolation process. To address this challenge, the purification of motile filamentous cyanobacteria involves transferring individual gliding cells to fresh agar plates. During this process, a single motile filament or trichome was carefully transferred onto a new agar plate. The transferred cells were given the opportunity to glide across the fresh surface, which helped them move away from potential contaminants. As the cyanobacterial cells glided, they effectively separated themselves from unwanted microorganisms, allowing for a more precise purification of the strain. This technique was repeated multiple times, each time transferring only the desired motile cells to fresh plates, ensuring the gradual isolation of a pure, uncontaminated cyanobacterial culture (Figure 2).

3.3. Morphological identification

Morphological analysis of the cyanobacterial isolates was conducted using a light microscope to closely examine their distinctive structural features and differentiate between the various strains. This process involved a careful observation of key morphological traits, such as the shape and size of cells, the arrangement of filaments, and the presence of specialized structures known as heterocysts—cells that play a vital role in nitrogen fixation in certain cyanobacteria. These characteristics were scrutinized to help in the identification of the strains. In addition to direct microscopic observation, the morphological features were cross-referenced with the seminal monograph of Desikachary[15], a comprehensive taxonomic guide widely regarded as one of the most authoritative resources on cyanobacterial taxonomy. This monograph includes detailed descriptions and illustrations of a wide variety of cyanobacterial genera and species, making it an essential tool for accurately identifying strains based on their morphology. The process of comparison with Desikachary's work ensured that the identification was not only thorough but also scientifically grounded in established classification systems. One of the critical aspects observed in the study was the presence of heterocysts.

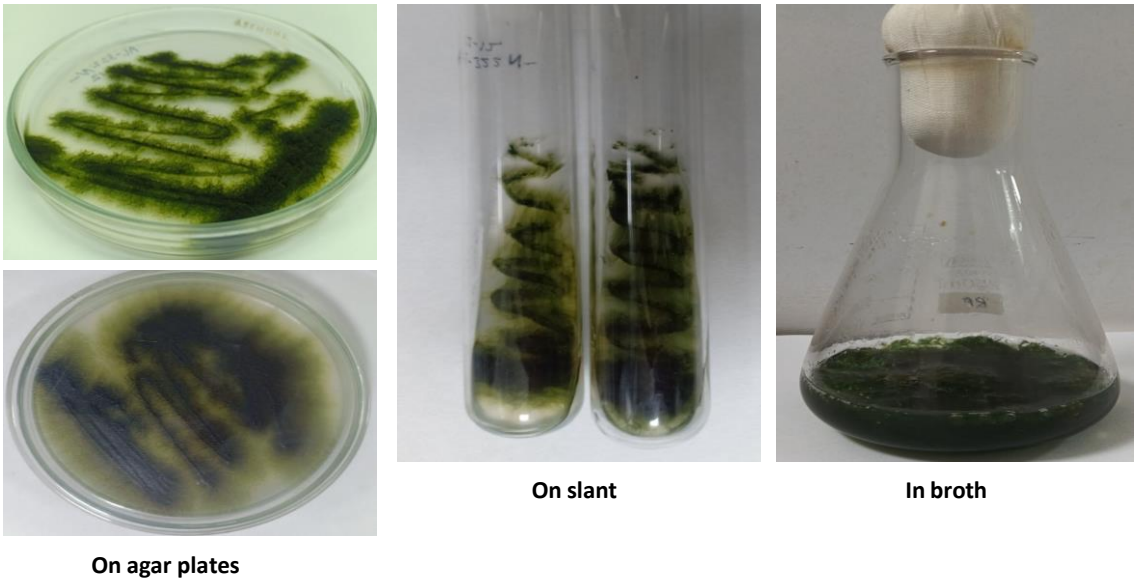

Figure 2. Pure cyanobacterial isolate KJE1 on agar plate, slant and in broth $BG^{-}11$ N^{-} media

The arrangement and frequency of heterocysts along with the filamentous structure provided important clues to the taxonomic identity of the cyanobacteria. These filaments, along with other key features such as cell dimensions, the nature of branching, and the surface texture, were meticulously compared with the descriptions provided in the monograph. The detailed examination and taxonomic matching led to the conclusive identification of the cyanobacterial strain as belonging to the *Tolypothrix* genus. *Tolypothrix* species are particularly significant in agricultural settings due to their nitrogen-fixing capacity, which contributes to soil fertility and can reduce the need for synthetic fertilizers. This makes them valuable from an ecological and agricultural standpoint, especially in regions where sustainable farming practices are being explored. The identification of this strain adds to the growing knowledge regarding cyanobacterial diversity in agricultural fields.

Figure 3, provides a visual representation of the *Tolypothrix* sp. strain under the microscope, highlighting its distinct structural features. The image serves as an important reference, visually confirming the morphological traits that were essential in its classification. These traits include the filamentous structure, the positioning of heterocysts, and the overall morphology that matches the descriptions outlined in the taxonomic literature. Through this detailed morphological analysis and taxonomic validation, the *Tolypothrix* sp. has been accurately identified, providing a foundation for further studies on its potential applications in biofertilization and sustainable agriculture.

3.4. Molecular and phylogenetic analysis of cyanobacterial isolates

Modern molecular techniques have become essential tools in studying cyanobacterial phylogenies and exploring population-level dynamics. These techniques provide insight into the evolutionary relationships and genetic diversity within cyanobacteria. Among the molecular markers used for phylogenetic studies, the 16S ribosomal RNA (16S rRNA) gene is one of the most reliable and informative markers. It is a highly conserved gene found universally across bacteria and cyanobacteria, making it an ideal target for taxonomic and phylogenetic analyses. The 16S rRNA gene contains both highly conserved regions, which are useful for broad taxonomic classification, and variable regions, which can distinguish closely related species. In the context of cyanobacterial phylogenetics, the 16S rRNA gene has been extensively utilized to assess evolutionary relationships and resolve taxonomic uncertainties[21, 22].

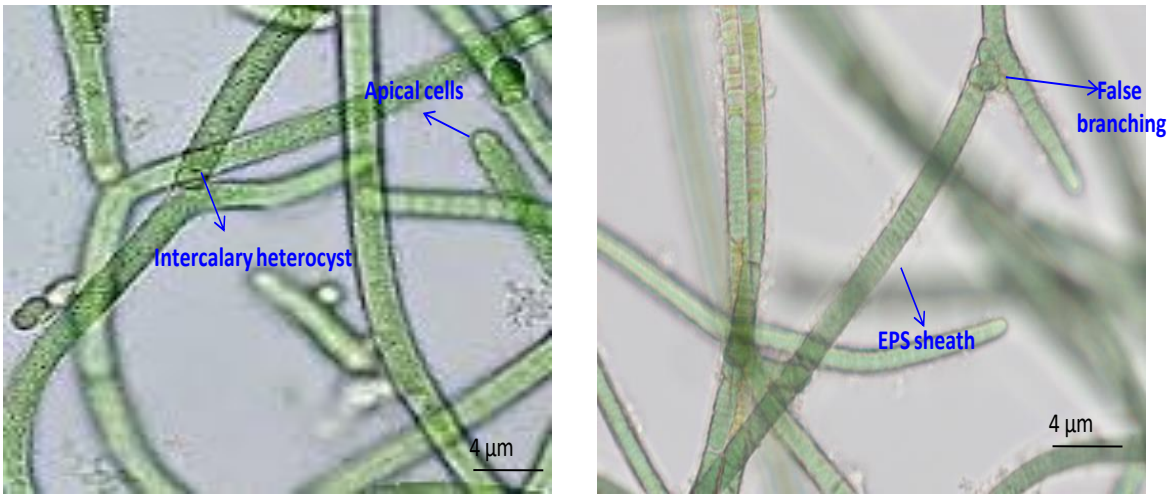

Figure 3. Light microscopic image of isolated cyanobacterial at 40X magnification *Tolypothrix* sp. KJE1

In this study, maximum likelihood phylogenetic analysis was conducted using the 16S rRNA gene sequence to determine the closest phylogenetic relatives of the cyanobacterial isolate KJE1. The results revealed that the closest species to strain KJE1 was *Tolypothrix* sp., with a nucleotide similarity of 96.72% (Figure 4.). This high level of sequence similarity confirms the close relationship between KJE1 and *Tolypothrix* sp. The partial sequence of the 16S rRNA gene for *Tolypothrix* sp. strain KJE1, consisting of 1283 base pairs (bps), was submitted to the National Center for Biotechnology Information (NCBI) gene bank database to make the sequence publicly accessible for future research and comparative studies. The deposited DNA sequence was assigned with a Gene Bank Accession Number OP353555, under the name *Tolypothrix* sp. KJE1. Cyanobacterial *Tolypothrix* sp. KJE1nucleotide sequence (16S rRNA gene) has been given below:

ACTTGCTTACCATGCAAGTCGAACGGTCTCTT
CGGAGATAGTGGCGGACGGGTGAGTAACGCG
TGAGAATCTAGCTTCAGGTCGGGGACAACCAC
TGGAAACGGTGGCTAATACCGGATGTGCCGAA
AGGTGAAAGATTTATTGCCTGAAGATGAGCTC
GCGTCTGATTAGCTAGTAGGTGTGGTAAGAGC
GCACCTAGGCGACGATCAGTAGCTGGTCTGAG
AGGATGATCAGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATTTTCCGCAATGGGCGAAAGCCTGACGGA
GCAATACCGCGTGAGGGAGGAAGGCTCTTGGT
TGTAAACCTCTTTTCTCAGGGAAGAAAAAAAT
GACGGTACCTGAGGAATAAGCATCGGCTAACT
CCGTGCCAGCAGCCGCGGTAATACGGAGGAT
GCAAGCGTTATCCGGAATGATTGGGCGTAAAG
CGTCCGCAGGTGGCTATGTAAGTCTGCTGTTA
AAGAGTGAGGCTCAACCTCATAAGAGCAGTG
GAAACTACACAGCTAGAGTGCGTTCGGGGCA
GAGGGAATTCCTGGTGTAGCGGTGAAATGCGT
AGAGATCAGGAAGAACACCGGTGGCGAAAGC
GCTCTGCTAGGCCGCAACTGACACTGAGGGAC
GAAAGCTAGGGGAGCGAATGGGATTAGATAC
CCCAGTAGTCCTAGCCGTAAACGATGGATACT
AGGCGTGGCTTGTATCGACCCGAGCCGTGCCG
TAGCTAACGCGTTAAGTATCCCGCCTGGGGAG
TACGCACGCAAGTGTGAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAGCGGTGGAGTATGTG
GTTTAATTCGATGCAACGCGAAGAACCTTACC
AAGACTTGACATGTCGCGAATCTTTTTGAAAG
GAAAGAGTGCCTTCGGGAGCGCGAACACAGG
TGGTGCATGGCTGTCGTCAGCTCGTGTCGTGA
GATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTCGTTTTATTTGCCAGCATTAAGTTGGGCAC
TCTAAAGAAACTGCCGGTGACAACCGGAGGA
AGGTGGGATGACGTCAGTCACATGCCCTTACG
CTTGGGCTACCACGTACTACATGCTACGACAA
GGGCACGAGCTACCATACAGCAATTTCTTAAC
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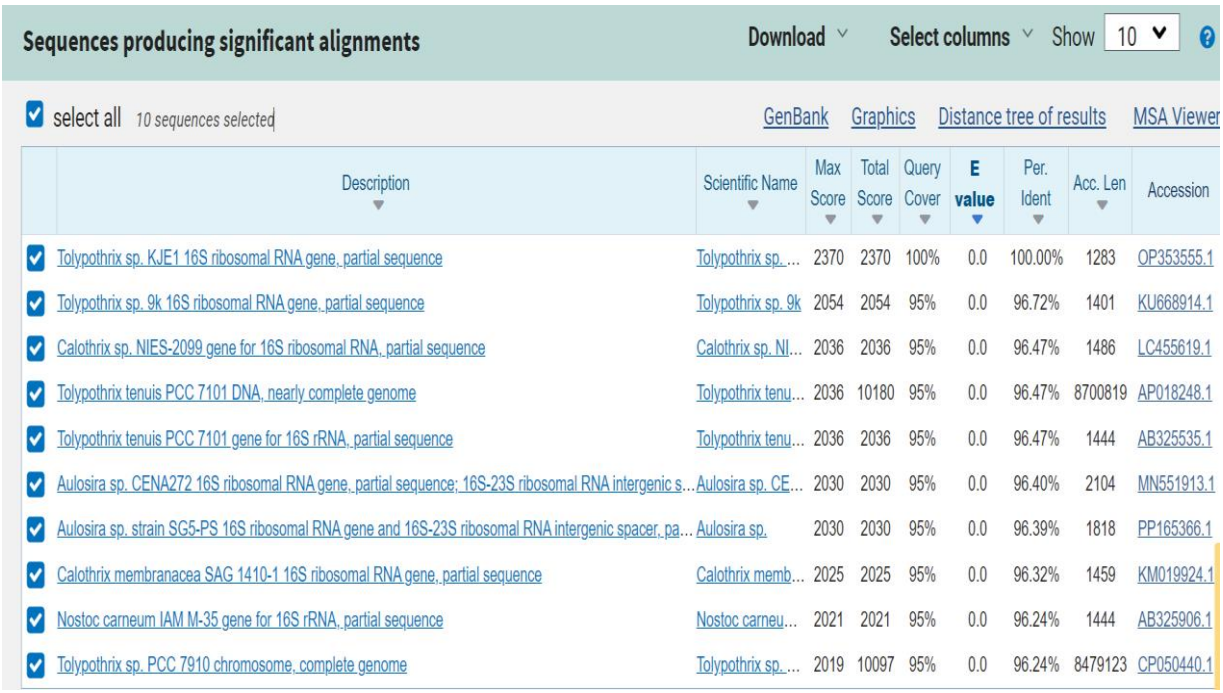
CTGAAGGAGAATCCTAGATTGCGGCCCCTATT
GCGGAATTCTCCGCCTTTCCGGCGGAGGAGGA
AAAAAAAAAAAG



Sequences producing significant alignments — Download — Select columns — Show 10

select all 10 sequences selected — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Tolypothrix sp. KJE1 16S ribosomal RNA gene, partial sequence	Tolypothrix sp. ...	2370	2370	100%	0.0	100.00%	1283	OP353555.1
Tolypothrix sp. 9k 16S ribosomal RNA gene, partial sequence	Tolypothrix sp. 9k	2054	2054	95%	0.0	96.72%	1401	KU668914.1
Calothrix sp. NIES-2099 gene for 16S ribosomal RNA, partial sequence	Calothrix sp. NI...	2036	2036	95%	0.0	96.47%	1486	LC455619.1
Tolypothrix tenuis PCC 7101 DNA, nearly complete genome	Tolypothrix tenu...	2036	10180	95%	0.0	96.47%	8700819	AP018248.1
Tolypothrix tenuis PCC 7101 gene for 16S rRNA, partial sequence	Tolypothrix tenu...	2036	2036	95%	0.0	96.47%	1444	AB325535.1
Aulosira sp. CENA272 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic s...	Aulosira sp. CE...	2030	2030	95%	0.0	96.40%	2104	MN551913.1
Aulosira sp. strain SG5-PS 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic spacer, pa...	Aulosira sp.	2030	2030	95%	0.0	96.39%	1818	PP165366.1
Calothrix membranacea SAG 1410-1 16S ribosomal RNA gene, partial sequence	Calothrix memb...	2025	2025	95%	0.0	96.32%	1459	KM019924.1
Nostoc carneum IAM M-35 gene for 16S rRNA, partial sequence	Nostoc carneu...	2021	2021	95%	0.0	96.24%	1444	AB325906.1
Tolypothrix sp. PCC 7910 chromosome, complete genome	Tolypothrix sp. ...	2019	10097	95%	0.0	96.24%	8479123	CP050440.1

Figure 4. BLAST analysis of 16s r-RNA sequence of cyanobacterial isolate KJE1.

The phylogenetic analysis of isolate KJE1 was constructed using maximum likelihood methods, which are considered a robust approach for inferring evolutionary relationships. This analysis allowed for a detailed understanding of the position of KJE1 within the broader cyanobacterial phylogeny. The high sequence similarity to *Tolypothrix* suggests that isolate KJE1 shares many genetic and possibly ecological characteristics with this genus. These findings highlight the utility of the 16S rRNA gene in resolving cyanobacterial relationships and provide a foundation for further investigation into the ecological roles and metabolic potential of isolate KJE1.

4. Conclusion

The isolation and identification of the cyanobacterial strain KJE1 from rice fields of Banaras Hindu University (BHU) in Varanasi, India, revealed its close association with the genus *Tolypothrix* species. Detailed morphological analyses, complemented by comparisons with Desikachary's monograph (1959), confirmed its filamentous structure, presence of heterocysts, and other characteristic features. These findings were further substantiated by molecular phylogenetic analysis using the 16S rRNA gene sequence, which showed a 96.72% similarity to *Tolypothrix* species. The partial 16S rRNA sequence (1283 bp) was submitted to the NCBI GenBank with Accession Number OP353555.

This study highlights the ecological and agricultural significance of *Tolypothrix* due to its nitrogen-fixing abilities, which contribute to soil fertility and promote sustainable farming practices. The identification of strain KJE1 adds to the growing body of knowledge on cyanobacterial diversity in agricultural ecosystems and provides a basis for further research into its biofertilizer potential. These findings underscore the importance of combining traditional taxonomic methods with modern molecular tools for comprehensive microbial studies.

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6. Authors Contribution

Research Scholar Jalaluddin is responsible for conducting fieldwork, collecting cyanobacterial samples, performing morphological and molecular analyses, and drafting the manuscript. Prof. Rajan Kumar Gupta, as the supervisor, provided guidance throughout the study, reviewed the results, and contributed to the finalization of the manuscript.

All authors have read and approved the final version of the research article.

Conflicts of interest

Not Applicable.

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