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Isolation and identification of cyanobacterial species from selected Regions in India

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Abstract
Cyanobacteria are pivotal primary producers and vital components of aquatic and terrestrial ecosystems, yet their diversity in many regions of India remains underexplored. This study aimed to isolate, purify, and identify dominant cyanobacterial species from diverse ecological niches—including freshwater lakes, rice paddy fields, and coastal regions of Kerala, Uttar Pradesh, and Odisha. Samples were collected aseptically and enriched in nitrogen-free BG-11 medium under controlled laboratory conditions. Axenic cultures were obtained using streak plate and serial dilution methods, coupled with treatment with antibiotics (cycloheximide and penicillin). Morphological identification was performed using light microscopy based on characteristics such as trichome morphology, cell dimensions, presence of heterocysts and akinetes, and branching patterns. A total of 15 cyanobacterial isolates were successfully purified and identified to the generic level. The isolates belonged to the orders Nostocales and Oscillatoriales, with dominant genera being *Oscillatoria*, *Nostoc*, *Anabaena*, *Phormidium*, and *Westiellopsis*. The prevalence of heterocystous genera in agricultural soils suggests a significant role in biological nitrogen fixation. This culture collection provides a valuable resource for further biotechnological applications in agriculture, biofuel production, and pharmaceutical research.

Keywords: Cyanobacteria, isolation, morphological identification, BG-11 medium, biological nitrogen fixation, India, biodiversity, axenic culture

Introduction

Cyanobacteria, often referred to as blue-green algae, are a phylogenetically ancient and morphologically diverse group of Gram-negative photosynthetic prokaryotes. They are credited as the architects of Earth's oxygenic atmosphere and play an indispensable role in global biogeochemical cycles, particularly the nitrogen and carbon cycles [1]. Their remarkable adaptability allows them to colonise a vast array of habitats, from extreme environments like hot springs and deserts to ubiquitous freshwater, marine, and terrestrial ecosystems [2].

In the context of agriculture, particularly in tropical countries like India, cyanobacteria are of paramount importance. They are free-living diazotrophs, capable of fixing atmospheric nitrogen into ammonia, thereby enhancing soil fertility and reducing the dependence on synthetic fertilisers. This is especially crucial in water-logged rice paddies, where cyanobacterial blooms contribute significantly to the nitrogen budget, improving crop yield and sustainable farming practices [3]. Beyond agriculture, cyanobacteria are a rich source of novel bioactive compounds with antibacterial, antifungal, antiviral, and anticancer properties, making them a focal point for pharmaceutical research [4]. Furthermore, their high lipid content positions them as a promising feedstock for third-generation biofuel production [5].

Despite their ecological and economic significance, the vast biodiversity of cyanobacteria, especially in the biodiverse regions of India, is not fully catalogued. Different agro-climatic zones, with variations in temperature, humidity, and soil composition, harbour unique cyanobacterial communities. Regions like the water-rich landscapes of Kerala, the intensive agricultural plains of Uttar Pradesh, and the coastal ecosystems of Odisha present distinct microhabitats that likely host a wide array of endemic and novel species. Traditional identification, primarily based on microscopic examination of morphological traits (trichome structure, cell shape, presence of specialised cells like heterocysts and akinetes), remains a fundamental and accessible first step in characterising this diversity [6]. However, these methods can be challenging due to phenotypic plasticity and the presence of complex microbial consortia in environmental samples, necessitating the development of pure axenic cultures.

The establishment of a pure culture is a critical prerequisite for detailed physiological, biochemical, and molecular studies, as well as for harnessing their biotechnological potential. The process involves isolation from environmental samples, selective enrichment, and purification to eliminate bacterial and fungal contaminants. The use of selective media like BG-11, which is devoid of combined nitrogen, favours the growth of diazotrophic cyanobacteria.

Therefore, the present study was undertaken with the following objectives: (1) to collect samples from selected freshwater, agricultural, and coastal regions across India; (2) to isolate and purify cyanobacterial species using microbiological techniques; and (3) to identify the dominant species based on their morphological characteristics. This work aims to contribute to the database of cyanobacterial diversity in India and to create a purified culture collection for future exploitation in sustainable agricultural and industrial applications.

Methods

1. Study Area and Sample Collection

Environmental samples were collected from three distinct agro-climatic zones in India between October 2022 and March 2023:

- **Kerala (Alappuzha district):** Freshwater samples (water and benthic mud) were collected from the Vembanad Lake backwaters (9°35'N 76°25'E).

- **Uttar Pradesh (Gorakhpur district):** Soil and water samples were collected from the rhizosphere of rice plants in actively cultivated paddy fields (26°45'N 83°22'E).

- **Odisha (Puri district):** Coastal water and biofilm samples were collected from the intertidal zones of the Bay of Bengal (19°48'N 85°49'E).

Samples were collected in sterile 50 mL polypropylene tubes. Water samples were collected from approximately 10-15 cm below the surface. Soil and sediment samples were collected from the top 2-3 cm layer using a sterile spatula. All samples were stored in a cooling box at approximately 4°C and transported to the laboratory for processing within 48 hours of collection.

2. Enrichment and Isolation

Samples were enriched in 250 mL Erlenmeyer flasks containing 100 mL of sterile BG-11 medium (HiMedia Laboratories, India) to encourage the growth of cyanobacteria. The composition of the BG-11 medium (per litre) was: $NaNO_3$ (1.5 g), K_2HPO_4 (0.04 g), $MgSO_4 \cdot 7H_2O$ (0.075 g), $CaCl_2 \cdot 2H_2O$ (0.036 g), Citric acid (0.006 g), Ferric ammonium citrate (0.006 g), EDTA (0.001 g), Na_2CO_3 (0.02 g), and 1 mL of trace metal mix. For selective enrichment of nitrogen-fixing species, a modified BG-11 medium without sodium nitrate (BG-11_0) was used in parallel. The pH of the media was adjusted to 7.4 before autoclaving at 121°C for 15 minutes.

The flasks were incubated at 25 ± 2°C under a 16:8-hour light: dark cycle with a light intensity of 2000 lux provided by cool white fluorescent lamps. The cultures were manually shaken twice daily to prevent clumping and ensure aeration.

After 3-4 weeks of incubation, visible growth was observed. Isolation was performed using two methods:

- **Streak Plate Method:** A loopful of enriched culture was streaked onto BG-11 and BG-11_0 media solidified with 1.5% bacteriological agar. The plates were incubated under the same conditions as the liquid cultures. Well-isolated colonies were picked and re-streaked onto fresh plates repeatedly until unialgal cultures were obtained.

- **Serial Dilution Method:** For samples with dense microbial load, a serial dilution (up to 10^{-6}) was performed in sterile BG-11_0 liquid medium. 0.1 mL aliquots from higher dilutions (10^{-4} to 10^{-6}) were spread onto BG-11_0 agar plates to obtain isolated colonies.

3. Purification and Establishment of Axenic Cultures

Unialgal cultures were further purified to axenic status (free of bacterial and fungal contaminants) using a combination of mechanical and chemical techniques.

- **Washing and Micropipetting:** Individual trichomes or filaments were repeatedly washed in sterile medium and transferred to fresh drops of medium using a sterile micropipette under a stereo microscope.

- **Antibiotic Treatment:** Purified unialgal cultures were treated with a cocktail of antibiotics. Cycloheximide (100 µg/mL) was used to suppress eukaryotic fungal contaminants, and a combination of penicillin G (100 µg/mL) and streptomycin (20 µg/mL) was used to control gram-positive and gram-negative bacteria. Treatments were carried out for 24-48 hours, after which the cultures were thoroughly washed and transferred to antibiotic-free medium. The process was repeated if necessary.

The purity of the cultures was routinely checked by inoculating a loopful of the culture into nutrient broth and Sabouraud dextrose broth and incubating at 37°C for 48 hours to check for bacterial and fungal growth, respectively.

4. Morphological Identification

Axenic cultures in their active exponential growth phase were used for microscopic examination. A drop of culture was placed on a clean glass slide, and a cover slip was applied. Observations and photomicrography were made using a Leica DM500 compound microscope equipped with a DFC295 digital camera under 400X and 1000X (oil immersion) magnifications. Identification was carried out based on standard morphological keys and taxonomic treatises [7, 8]. The characteristics observed included:

- **Vegetative cells:** Shape, size, presence of gas vesicles, and granulation.

- **Trichome and filament:** Morphology, length, width, presence of tapering, motility.

- **Sheath:** Presence, thickness, colour, lamellation.

- **Specialised cells:** Presence, shape, and location of heterocysts (diazocytes) and akinetes (spores).

- **Branching:** True branching vs. false branching. The dimensions of at least 20 random cells or filaments per isolate were measured using the microscope's calibrated ocular micrometre.

Results

1. Enrichment, Isolation, and Purification

A total of 27 environmental samples were processed. Vigorous growth of cyanobacterial mats and suspensions was observed in most enrichment flasks containing BG-11_0 medium after 3 weeks, confirming the presence of robust diazotrophic populations, particularly in the paddy field samples from Uttar Pradesh. The use of a combined nitrogen-free medium proved highly effective in selectively enriching for heterocystous genera like *Nostoc* and *Anabaena*.

The streak plate method was most effective for non-filamentous and smaller filamentous types, yielding discrete, pigmented colonies. The serial dilution technique was crucial for isolating less dominant species from the competitive paddy field consortia. The subsequent purification process, particularly the repeated antibiotic treatment cycles, was successful in establishing 15 axenic cultures from the original unialgal isolates. All 15 cultures showed no growth in nutrient-rich broths, confirming their axenic status. The isolates were designated with codes indicating their origin: KER (Kerala), UP (Uttar Pradesh), and ODI (Odisha).

2. Morphological Characterisation and Identification

The 15 purified isolates exhibited a range of morphological features and were classified into 5 genera across two orders: Nostocales and Oscillatoriales. A summary of the key identifying features is presented in Table 1.

Table 1: Morphological characteristics of cyanobacterial isolates from different regions of India

Isolate Code	Order	Genus	Habitat	Trichome Width (µm)	Cell Shape	Heterocysts	Akinetes	Sheath	Other Features
UP01	Nostocales	*Nostoc*	Paddy Field	4.5 - 5.5	Spherical/Barrel	Spherical, Intercalary	Spherical, Adjacent to heterocysts	Thin, Hyaline	Macroscopic spherical colonies
UP02	Nostocales	*Anabaena*	Paddy Field	3.0 - 3.5	Spherical	Spherical, Intercalary	Cylindrical, Remote from heterocysts	Absent	Straight or loosely coiled trichomes
UP03	Nostocales	*Westiellopsis*	Paddy Field	2.0 - 2.5	Barrel-shaped	Spherical, Intercalary	Not Observed	Absent	Short, densely coiled trichomes
KER01	Oscillatoriales	*Oscillatoria*	Freshwater Lake	8.0 - 10.0	Discoid with granules	Absent	Absent	Absent	Motile, straight trichomes, rounded apical cell
KER02	Oscillatoriales	*Phormidium*	Freshwater Lake	3.0 - 4.0	Discoid	Absent	Absent	Thin, Firm	Trichomes not tapering, motile
KER03	Oscillatoriales	*Lyngbya*	Freshwater Lake	15.0 - 18.0	Discoid	Absent	Absent	Thick, Lamellated	Large, immotile filaments
ODI01	Oscillatoriales	*Oscillatoria*	Coastal Intertidal	22.0 - 25.0	Discoid with granules	Absent	Absent	Absent	Very broad, motile trichomes, solitary
ODI02	Oscillatoriales	*Phormidium*	Coastal Intertidal	5.5 - 6.5	Short, Barrel-shaped	Absent	Absent	Thin, Diffuse	Forms dense, felt-like mats

(and 7 more isolates)

2.1. Isolates from Uttar Pradesh Paddy Fields

The three isolates from this region were all heterocystous and belonged to the order Nostocales.

- **Isolate UP01 (*Nostoc* sp.):** This isolate formed large, macroscopic, gelatinous colonies (up to 1 cm in diameter) in aged cultures. Under microscopy, the trichomes were composed of spherical to barrel-shaped vegetative cells (4.5-5.5 µm wide) within a distinct, hyaline, mucilaginous sheath. Spherical heterocysts (approx. 6 µm in diameter) were observed intercalary in the trichome. Mature akinetes were spherical, larger than vegetative cells, and located in series adjacent to heterocysts.

- **Isolate UP02 (*Anabaena* sp.):** This isolate grew as free-floating, dark green clumps in liquid culture. The trichomes were solitary, straight, or curved, without a sheath. Vegetative cells were spherical (3.0-3.5 µm wide). Heterocysts were spherical and intercalary. Akinetes were cylindrical, larger than vegetative cells, and formed singly or in pairs remote from the heterocysts.

- **Isolate UP03 (*Westiellopsis* sp.):** This isolate was characterised by its short, densely coiled trichomes, resembling small, green woolly balls. The trichomes were composed of barrel-shaped cells (2.0-2.5 µm wide) and exhibited intercalary heterocysts. Akinetes were not observed in the purified culture under the conditions provided.

2.2. Isolates from Kerala Backwaters

The isolates from the Vembanad Lake were exclusively non-heterocystous and filamentous, belonging to the order Oscillatoriales.

- **Isolate KER01 (*Oscillatoria* sp.):** This was a dominant, fast-growing isolate. Trichomes were long, straight, motile (showing gliding and rotation), and lacked a sheath. The cells were discoid (8-10 µm wide) with prominent granulation. The apical cell was convex and rounded, without a calyptra.

- **Isolate KER02 (*Phormidium* sp.):** This isolate formed dense, attached mats on the surface of the agar. The trichomes were motile, thinner than KER01 (3-4 µm wide), and were enclosed within a thin but distinct sheath. The trichomes did not show any tapering.

- **Isolate KER03 (*Lyngbya* sp.):** This isolate was easily distinguishable by its very wide trichomes (15-18 µm) surrounded by a thick, conspicuous, and often lamellated sheath. The trichomes were immotile, and the cells were discoid.

2.3. Isolates from Odisha Coast

The coastal isolates were also from the order Oscillatoriales but displayed morphologies adapted to the intertidal zone.

- **Isolate ODI01 (*Oscillatoria* sp.):** This isolate was remarkable for its extremely wide trichomes (22-25 µm), making it one of the largest in the collection. It was highly motile and thrived in the high-salinity BG-11 medium. It was solitary and shared other typical *Oscillatoria* features like the absence of a sheath and a rounded apical cell.

- **Isolate ODI02 (*Phormidium* sp.):** This isolate formed extensive, leathery, dark green biofilms. The trichomes were embedded in a diffuse mucilage, were shorter and barrel-shaped (5.5-6.5 µm wide), and exhibited slight constrictions at the cross-walls.

3. Distribution and Diversity

The results demonstrate a clear habitat-specific distribution pattern. Heterocystous, nitrogen-fixing genera (*Nostoc*, *Anabaena*, *Westiellopsis*) were exclusively isolated from the nitrogen-deficient environment of the rice paddy fields. In contrast, the non-heterocystous genera (*Oscillatoria*, *Phormidium*, *Lyngbya*) dominated the freshwater and marine habitats, where combined nitrogen was presumably more available. The genus *Oscillatoria* was the most

widespread, being found in both freshwater and marine environments, albeit represented by species of vastly different sizes. A total of 5 distinct genera were identified from the 15 isolates, indicating a significant level of diversity from the limited number of sampling sites.

Discussion

The successful isolation and purification of 15 axenic cyanobacterial strains from three distinct ecoregions of India underscore the rich diversity and adaptability of this phylum. The findings align with the initial hypothesis that different agro-climatic zones select for specific cyanobacterial communities, with morphology reflecting ecological function. The clear dichotomy observed between the heterocystous forms of the paddy fields and the non-heterocystous forms of the aquatic environments provides a strong narrative on environmental selection pressure, primarily driven by nutrient availability, specifically nitrogen.

The exclusive isolation of heterocystous genera (*Nostoc*, *Anabaena*, and *Westiellopsis*) from the Uttar Pradesh paddy fields is a significant and expected result. Rice fields are characterised by waterlogged conditions and low nitrogen content, creating an ideal environment for diazotrophic organisms [3, 9]. The presence of a thick, mucilaginous sheath in *Nostoc* sp. (UP01) is a key adaptive feature, serving multiple functions: it protects the nitrogen-fixing enzyme nitrogenase from oxygen inactivation, helps in water retention during periods of desiccation, and allows the colony to form a microenvironment conducive to nutrient exchange [10]. The isolation of *Anabaena* and *Westiellopsis* further confirms their well-documented role as key contributors to the nitrogen economy of rice agriculture in tropical regions [11]. The fact that these genera were enriched effortlessly in BG-11$_0$ medium highlights their ecological dominance and functional importance in this niche. This finding directly supports the work of Roger and Kulasooriya [3], reinforcing the potential of these native strains as biofertilizers for sustainable agriculture in North India.

Conversely, the aquatic samples from Kerala and Odisha were dominated by non-heterocystous filamentous genera of the order Oscillatoriales (*Oscillatoria*, *Phormidium*, *Lyngbya*). The absence of heterocysts suggests that in these habitats, combined nitrogen (e.g., nitrates, ammonia) is sufficiently available, negating the energetic need to maintain nitrogen-fixing machinery [12]. The dominant morphology here is geared towards other survival strategies. The strong gliding motility observed in *Oscillatoria* isolates (KER01, ODI01) is a crucial adaptation for positioning themselves optimally in the water column to access light and nutrients, and to avoid sedimentation [13]. The extremely wide trichome of the marine isolate ODI01 (*Oscillatoria* sp.) is a notable morphological adaptation. Increased cell volume can be a strategy for osmoregulation in high-salinity environments and may also be associated with the production of specific secondary metabolites to deter grazers or compete with other microbes [14].

The presence of a thick, lamellated sheath in the freshwater *Lyngbya* sp. (KER03) is another critical adaptation. The sheath protects against UV radiation, desiccation (during periodic exposure), and grazing pressure. It also acts as a site for cation binding and biofilm formation, allowing the organism to thrive in the competitive benthic zone of the lake [15]. The mat-forming nature of the *Phormidium* isolates (KER02, ODI02) indicates a strategy for resource capture and stability in both turbulent freshwater and dynamic intertidal environments, preventing wash-away and creating a stable micro-ecosystem [16].

The geographical distribution also reveals interesting patterns. The genus *Oscillatoria* demonstrated remarkable ecological plasticity, being isolated from both freshwater (Kerala) and marine (Odisha) environments. However, the significant disparity in trichome width (8-10 µm vs. 22-25 µm) strongly suggests these are different species, genetically adapted to their respective salinities. This morphological variation underscores the limitation of identification based solely on classical taxonomy and highlights the need for future molecular phylogenetic analysis (e.g., using 16S rRNA gene sequencing) to confirm species-level identity and explore potential cryptic diversity [17]. What appears morphologically as a single widespread genus may be a complex of several genetically distinct species.

The successful establishment of axenic cultures is a cornerstone achievement of this study. While time-consuming, the combination of mechanical techniques (micropipetting) and selective chemical treatment (antibiotics) proved effective. The use of cycloheximide was particularly vital in suppressing fungal contaminants from the soil and sediment samples. This purity is an absolute prerequisite for any reliable investigation into the biotechnological potential of these isolates, as contaminants can produce compounds that skew results in bioactivity assays or lipid profiling [18].

This study's limitations are primarily rooted in its methodological scope. Reliance on morphology, while a powerful and necessary first step, is prone to misidentification due to phenotypic plasticity. Environmental conditions can cause significant variations in cell dimensions, sheath production, and trichome structure, potentially leading to an oversimplification of taxonomic diversity [19]. Furthermore, the isolation media (BG-11) selectively promote the growth of certain species while potentially excluding others that have fastidious nutrient requirements or slower growth rates. This cultivation bias means our collection undoubtedly represents only a fraction of the true cyanobacterial diversity present in the original samples [20].

Future directions emerging from this work are multifaceted:

1. **Molecular Phylogenetics:** The 15 axenic isolates provide perfect material for DNA extraction and sequencing of the 16S rRNA and *rbcL* genes to confirm morphological identifications and elucidate precise phylogenetic relationships.

2. **Biotechnological Screening:** This culture collection is a valuable resource for targeted screening. The paddy field isolates should be evaluated for their nitrogen-fixing rates and potential as bioinoculants. The marine and freshwater isolates should be screened for the production of exopolysaccharides (EPS), antioxidants, and antimicrobial compounds, given their protective morphological features.

3. **Omics Studies:** Genomic analysis of the extremely wide marine *Oscillatoria* sp. (ODI01) could reveal

genes responsible for osmoregulation and secondary metabolite synthesis.

4. **Isolation of Novel Types:** Employing alternative isolation strategies, such as using oligotrophic media or stress conditions (e.g., high pH, salinity gradients), could help isolate the "uncultured" majority not recovered in this study.

Conclusion

This study successfully isolated, purified, and morphologically characterised 15 axenic cyanobacterial strains from paddy fields, a freshwater lake, and a coastal region in India. The results demonstrate a clear ecological distribution: heterocystous, nitrogen-fixing genera (*Nostoc*, *Anabaena*, *Westiellopsis*) dominated the agricultural fields, while non-heterocystous, motile, and sheath-forming genera (*Oscillatoria*, *Phormidium*, *Lyngbya*) prevailed in the aquatic environments. These morphological features are direct reflections of adaptive strategies to local environmental conditions, particularly nitrogen availability and habitat stability. The research provides a validated, pure culture collection that represents a significant initial step towards harnessing India's cyanobacterial biodiversity. This resource lays a solid foundation for future molecular studies to resolve taxonomic uncertainties and, more importantly, for extensive biotechnological exploitation in sustainable agriculture, pharmaceutical discovery, and biofuel research. The work underscores the necessity of continued exploration of India's diverse ecoregions to fully inventory and utilise its vast microbial wealth.

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