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Isolation and Characterization of Two Cyanobacterial Strains *Calothrix* sp. and *Microchaete* sp. from Rice Fields of Karimganj District, Assam, North East India

MOIRANGTHEM THAJAMANBI[1], JAYASHREE ROUT[1]* and NOORUDDIN THAJUDDIN[2]

[1]Department of Ecology and Environmental Science, Assam University, Silchar-788011, Assam, India.
[2]Department of Microbiology, Bharathidasan University, Tiruchirappalli-620024, Tamil Nadu, India.
*Corresponding author E-mail: routjaya@rediffmail.com

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ABSTRACT

Studies on various nitrogen fixing microalgal strains found in the rice paddy field soils are carried out in different parts of the world. In the present study two cyanobacterial strains belonging to the order nostocales, *Calothrix* sp. and *Microchaete* sp. were isolated from the rice fields of Karimganj district, South Assam, India and characterized based on their morphological, biochemical and molecular analysis. For the phenotypic characterization - growth, pigments (chlorophyll *a*, total carotenoid content, phycobiliproteins) and biochemical properties (total carbohydrate and soluble proteins) were studied. The study showed that both strains contain lower phycoerythrin content as compared to the other pigments. The *Microchaete* strain contain a higher total carotenoid content while chlorophyll *a* accumulation was higher in the *Calothrix* strain. Phylogenetic compairision was made using 16S rRNA gene sequences including other sequences of *Calothrix, Microchaete* and *Tolypothrix* species from GenBank. The results showed that polyphasic approach provides necessary information for the identification of cyanobacterial species using morphological analysis in combination with molecular techniques.

Keywords: Assam, Biochemical, *Calothrix*, *Microchaete*, Molecular, Morphology.

INTRODUCTION

Cyanobacteria are oxygen evolving photoautotropic prokaryotes known to cohabitate with rice and exploited in agriculture for their specific inoculation as nitrogen supplementing biofertilizers in paddy fields[1]. They have received much attention in soil due to their nitrogen fixing ability and significant contribution in primary production. The rice field ecosystem provides an environment favourable for the growth of cyanobacteria with respect to their requirements for light, water, high temperature and nutrient availability[2]. The classification system of cyanobacteria usually depends on morphological attributes, which however, are not always consistent as they may show disparity with change in culturing conditions[3]. Therefore a polyphasic approach involving traditional morphology, biochemical and molecular data has become necessary in recent years.

According to the traditional classification system, the genus *Calothrix* as described by C. Agardh (1824) belong to the order Nostocales and family Rivulariaceae[4-5]. It is a polymorphic genus with the general features including hormogonia giving rise to young filaments with terminal heterocyst at only one end of the trichome, mature trichome tapers from base, which bears a terminal heterocyst to apex, vegetative cells disc-shaped, isodiametric or cylindrical[5]. Recent studies have suggested that a polyphasic approach (using both morphological and molecular data) is required to determine *Calothrix* taxa[6]. The Rivulariaceae are considered amongst

the most morphologically complex cyanobacteria with tapered trichomes, apart from short phases of hormogonium formation that has a terminal heterocyst, although in some species intercalary heterocyst is also present and cell division is largely localized to a region near the heterocyst[7]. Species of the genus *Calothrix* are blue green, filamentous with a basal heterocyst and occur in both salt and freshwater environments as well as sub- aerially and aerially[8-9].

The other genera *Microchaete*, [Thuret 1875] Bornet et Flahault 1886, is a filamentous, heterocystous cyanobacterium that belongs to the family Microchaetaceae, Division Cyanophyta generally found in ponds, rice paddy fields, fresh water lakes, and marine environments[10-12]. Usually filaments are attached by one end to a substratum, single or with colonies of many filaments which are arranged irregularly or forming a turf. Trichome surrounded by a very distinct sheath, mostly narrow and tapered towards the end, heterocyst basal and also often intercalary. The two genera differ morphologically where cell division in *Calothrix* occurs mainly in the part of the trichome near the heterocyst while cell division in *Microchaete* apparently takes place towards the apex and cell elongation takes place away from this region[13]. Therefore, the objective of the present study was to isolate the two cyanobacterial strains from the rice fields of Karimganj district, South Assam, India and characterize them for morphological, biochemical and molecular analysis.

MATERIALS AND METHODS

Isolation of strains from soil samples

The soil samples were collected from selected rice fields of Karimganj district, South Assam. The strain *Calothrix* sp. (AUS-JR/MT/NT-036) was isolated from the rice field of Deodhar (24°69'862'' N, 92°45'115''E) and *Microchaete* sp.(AUS-JR/MT/NT-037) from the rice field of Sone beel (24°42'059''N, 92°27'164''E) respectively. Ten gram of the soil sample was transferred to a 250ml flask containing 90ml sterile distilled water and shaken (120 rpm) for 30 mins[14]. Serial dilution (10^{-2}, 10^{-1}) was made and 1ml aliquots were spread on agar plates containing BG 11_0 N-free medium[15]. The resulting axenic cyanobacterial isolates were maintained in their isolation media at 24°C±1 in laboratory conditions with a light intensity of 2000-3000lux. Repeated subculturing was performed a number of times until pure axenic culture were obtained.

Phenotypic characterization of the cyanobacterial isolates

The morphological characterization of the cyanobacterial isolates was determined. Microscopic observation and microphotography of the pure cultures were made with the aid of Leica DM1000 microscope and morphological characteristics such as structure and colour of thallus, filaments length and width; attenuation, shape and size of vegetative cells, constriction, position, shape and number of heterocysts, colour, thickness and distance of sheath, and presence or absence of spores. Identification of the strains was done using taxonomic keys[16].

Growth characteristics and biochemical estimation

Growth pattern, biochemical constituents (soluble protein and total carbohydrate) and pigment profile (chlorophyll *a*, total carotenoid and phycobiliprotein) of the two cyanobacterial isolates were examined. The cyanobacterial growth pattern was determined by analyzing the species biomass in terms of chlorophyll *a* concentration[17]. Soluble protein was measured with the modified Lowry method[18] and total carbohydrate was estimated by the Anthrone method[19]. Phycobiliproteins and total carotenoid content were also estimated[20-21].

16S rDNA gene amplification and sequencing

Genomic DNA of the two isolated cyanobacterial strains was extracted from exponentially growing cultures following standard method[22]. Amplification of 16S rDNA genes were done using CYA106 f (5'-GGACGGGTGAGTAACGCGTGA-3') and CYA781r (a) (5'-GACTACTGGGGTATCTAATCCCATT-3') as forward and reverse primers respectively in the Applied Biosystem thermal cycler with the following temperature as initial denaturation at 94°C for 5min, 30cycles of 94°C for 1min, 58°C for 45s, 72°C for 1min and final extension at 72°C for 7min[23]. The 50µl of reaction mixture was prepared using 2µl (50 ng) of extracted DNA and the PCR reaction mixture

consisting of 2x Genet Bio Premix pH 9.0 (Prime Taq TM DNA Polymerase 1 unit 10 µl, 20 mM Tris-HCl, 80 mM KCl, 4 mM $MgCl_2$, enzyme stabilizer sediment, loading dye and 0.5mM of each dATP, dCTP, dGTP, dTTP) 25 µl, 1µl of each forward and reverse primers, and 21µl MiliQ water.

Phylogenetic analysis and construction of tree

The obtained sequences were checked for homology with other sequences deposited in the available databases using Basic Local Alignment Search Tool (BLAST) search (http://www.ncbi.nim.nih.gov/BLAST). The gene sequences were then submitted to National Centre for Biotechnology Information (NCBI) GenBank, US., under their respective accession numbers. Phylogenetic tree was constructed using the MEGA6 analysis platform[24] including the available cyanobacterial gene sequences along with the sequences determined in this study using the neighbour-joining method[25]. Sequences were aligned using the CLUSTALW aligning utility to produced working alignment of 16S rDNA sequences for the target strains. The evolutionary distances were computed and expressed as number of base substitutions per site. Statistical significance level of interior nodes was determined by bootstrap analysis (1000 data re-samplings)[26].

Nucleotide accession numbers

The sequences of the 16S rRNA genes of the strains have been deposited in the NCBI Gen Bank and respective accession numbers were obtained.

RESULTS AND DISCUSSION

Morphological characterization of strains

The morphology of the isolated cyanobacterial strains were studied under light microscope. The morphological features of the two strains are as follows.

***Calothrix* sp. (AUS-JR/MT/NT-036)**

The structure of the thallus on petri plate was filamentous; blue green or brownish in colour; slightly bent, single, 7-10µm broad at the base with thin colourless sheath; trichome brownish green, constricted at the cross walls; cells quadratic, shorter or longer than broad, 4-7µm long and 3-7µm broad; heterocyst single, basal and spherical, 4-5µm broad. **(Fig. 1 a, c and e).**

***Microchaete* sp. (AUS-JR/MT/NT-037)**

The culture on the petri plate were filamentous, blackish brown in colour, forming a wooly type thin film; trichomes solitary, slightly attenuated towards the end; cells quadrate 8-9µm broad, 9-11µm long; cell contents granular at the cross walls; basal heterocyst globose, intercalary heterocyst quadrate; sheath distinct, thick, colourless and lamellated, 7-8µm broad and 6-7µm long. **(Fig. 1 b, d and f).**

Growth characteristics, pigments and biochemical attribute

The growth curve analysis for both the strains in terms of chlorophyll *a* showed that the *Microchaete* strain with a longer exponential phase was found to be a slow- grower as compared to the *Calothrix* strain that has a longer lag phase (Fig. 2). The pigment analysis and biochemical attributes of the strains are shown in Table 1. Higher chlorophyll *a* (13.84 µg mL^{-1}) and phycobiliproteins (103.35 mg g^{-1}) content was found in *Calothrix* strain while the other *Microchaete* strain was higher in total carotenoid content (3.11 µg mL^{-1}). The soluble protein concentration by *Calothrix* sp. isolated from Deodhar rice field was 79.80 µg $mL^{-1}$and the lowest protein content was found in *Microchaete* sp.(42.47 µg mL^{-1}). The total carbohydrate content accumulated by *Microchaete* sp. (115.71 µg mL^{-1}) was higher whereas *Calothrix* sp. showed only 50.14 µg mL^{-1}

Molecular and phylogenetic analysis

A partial 16S rRNA gene sequences was obtained for both the strains and compared with closely related other nostocalean members comprising of *Calothrix*, *Microchaete* and *Tolypothrix* species from India and other parts of the world retrieved from the GenBank database. The Neighbor-Joining method was utilized to infer the evolutionary history. A phylogenetic tree reflecting the relationship among the strains and the related sequences of various nostocales strains has been presented in **Fig. 3**. The optimal tree with the sum of branch length = 0.14439346 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates)

is shown next to the branches. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated resulting in a total of 567 positions in the final dataset.

In the phylogenetic tree, *Calothrix* sp. (AUS-JR/MT/NT-036) was most closely related to the sequences of *Tolypothrix* sp. PCC 7504 from Mexico and *Calothrix brevissima* from Japan positioned in a large clade with other *Calothrix* species. This is congruent with recent molecular studies which place the genus in the clade with *Tolypothrix* and other closely related genera[27]. However, the *Microchaete* sp. (AUS-JR/MT/NT-037) formed a robust clade with other *Calothrix* species and *Tolypothrix* species from Japan and is closely related *Microchaete diplosiphon* CCALA 811 from Mexico with high boostrap values.

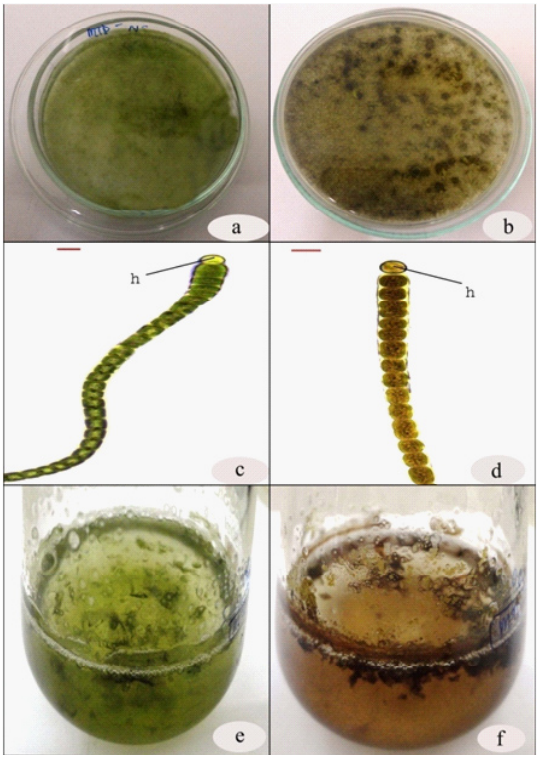

Fig. 1: Microphotographs of the studied strains. Plate view - a) *Calothrix* sp. (AUS-JR/MT/NT-036) and b) *Microchaete* sp. (AUS-JR/MT/NT-037); Individual trichomes showing heterocyst (h) - c) *Calothrix* sp., d) *Microchaete* sp.; liquid culture – e) *Calothrix* sp. (AUS-JR/MT/NT-036), f) *Microchaete* sp. (AUS-JR/MT/NT-037)

Nucleotide accession numbers

The sequences obtained were submitted to NCBI with the respective accession numbers *Calothrix* sp. (AUS-JR/MT/NT-036) - KM252910 and *Microchaete* sp. (AUS-JR/MT/NT-037) - KM252911.

Systematic account on blue green algae occurring as biological soil crust from different regions of India were studied and detailed morphological study of *Calothrix* species were studied[28]. The present study revealed that the isolated *Calothrix* strain showed high chlorophyll *a* accumulation and soluble protein content, unlike the *Calothrix* species isolated from Manwar and Pokharan region, Rajasthan that, these were poor in terms of chlorophyll and

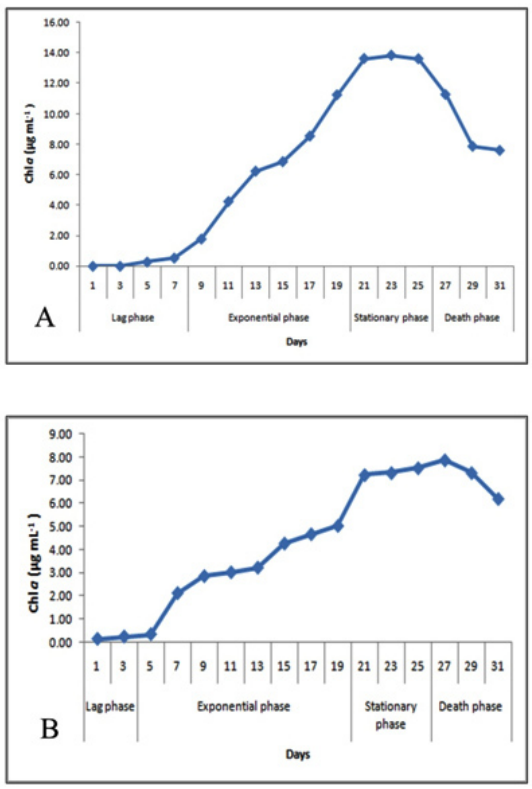

Fig. 2: Growth curve analysis in terms of chlorophyll *a* concentration A. *Calothrix* sp. (AUS-JR/MT/NT-036) B. *Microchaete* sp. (AUS-JR/MT/NT-037)

soluble protein content although showed remarkable potential for nitrogen fixation [29]. The dissimilarity may be due to the difference in the habitat from where they have been isolated. The variation in the range of chlorophyll content from 11.16 µg mL^{-1} to 3.51 µg mL^{-1} amongst cyanobacterial strains of *Calothrix* isolated from different geographical locations of India was also reported[30]. In another report, polyphasic characterization of *Nostoc commune* strains isolated from rice growing agroecosystems was also studied[31]. Similar to the findings of Berrendero *et al*., 2008[6] where the genera *Rivularia* and *Calothrix* from GenBank database were intermixed in the phylogenetic inferences, our study showed that representatives of the genera *Calothrix, Microchaete* and *Tolypothrix* were intermixed hence showing great genetic divergence[6]. Although 16S ribosomal gene analysis has been a method of choice for deducing phylogenies and establishing evolutionary relationships, morphological data have constantly been applied together with molecular data to

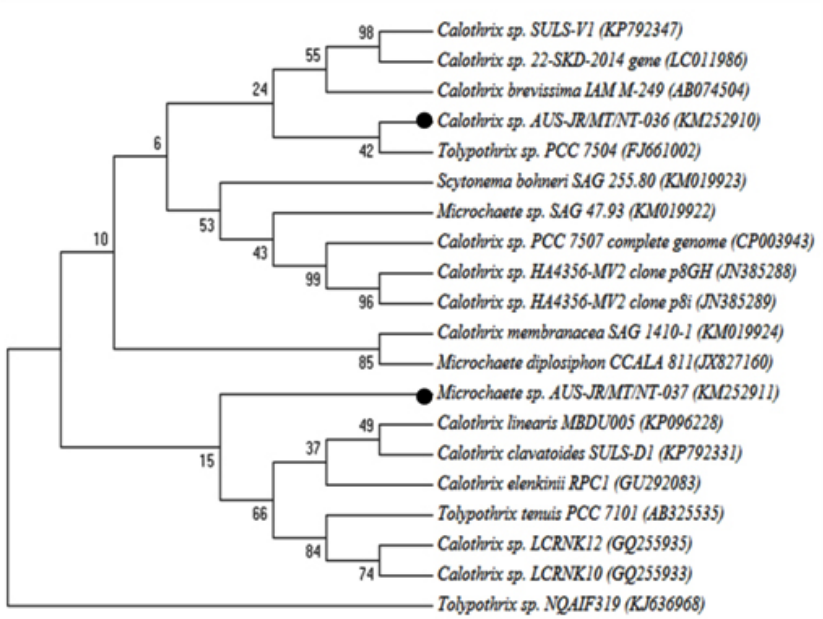

Fig. 3: Phylogenetic tree based on analysis of 16S rRNA genes showing the position of sequences obtained in this study (marked ones)

Table 1: Pigment analysis and biochemical attributes (mean± SD) of the studied strains

S. No	Biochemicalparameters		*Calothrix* sp. (AUS-JR/ MT/NT-036)	*Microchaete* sp. (AUS-JR/MT /NT-037)
1	Chlorophyll *a* (µg mL^{-1})		13.84±0.08	7.87±0.02
2	Total carotenoid content (µg mL^{-1})		1.12±0.003	3.11±0.34
3	Phycobiliproteins (mg g^{-1})	Phycoerythrin (PE)	8.57±0.052	3.11±0.052
		Phycocyanin (PC)	46.68±0.14	23.41±0.239
		Allophycocyanin (APC)	48.10±0.153	23.95±0.256
	Total phycobiliproteins(mg g^{-1})		103.35	50.47
4	Total carbohydrate(µg mL^{-1})		50.14±0.08	115.71±0.08
5	Soluble protein(µg mL^{-1})		79.80±0.20	42.47±0.31

obtain meaningful inferences concerning a more precise determination of the taxonomic status of cyanobacteria[32-33]. Thus, the polyphasic approach used in the present study involving morphological and molecular procedures for investigating the genetic diversity and ecological significance amongst the isolates of *Calothrix* and *Microchaete* proved to be powerful and helpful.

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