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RESEARCH ARTICLE

Characterization and comparative analysis of *Anabaena* strain of rice fields of Manipur, India

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Abstract

Thirty two different *Anabaena* strains were isolated from rice fields of Manipur, India, successfully cultured and characterized based on morphological and biochemical features under semi controlled conditions. *Anabaena* sp. BTA04 and BTA903 were investigated as high biomass in terms of chlorophyll-a producing and acetylene reduction activity expressing strains respectively. *Anabaena* sp. BTA03 was observed for the production of good content of total carotenoids and BTA35 for phycocyanin and allophycocyanin. *Anabaena* sp. BTA84 yielded high content of phycoerythrin in culture conditions. Biochemically potent *Anabaena* strains were deeply studied using amplified 16S rRNA gene analysis and compared with morphological characters. All strains were deposited to the National fresh water Cyanobacterial and Microalgal repository of DBT-IBSD, Imphal, Manipur with accession numbers. The sequences of the 16S rRNA gene of the five highly potent strains selected from this experiment have been submitted in the NCBI GenBank through GenBank submission tool Sequin software version 12.30 and got accession number as KM010233, KM010234, KJ562184, KF953534, KJ652540 for BTA03, BTA04, BTA35, BTA84, BTA903 respectively.

INTRODUCTION

Cyanobacteria a group of gram negative photoautotrophic bacteria are one of the most primitive component of earth that were responsible for creating oxygenic atmosphere through their photosynthetic activities. Rice fields are temporary wetland ecosystems with variable biodiversity and cyanobacteria are known to be an integral component of waterlogged rice fields. The rice field's ecosystems with its optimum level of light, water, temperature, humidity and nutrient availability provide a favourable environment for the luxuriant growth of cyanobacteria. Members of the order Nostocales are broadly characterized by the production of three kinds of differentiated cells. The genus *Anabaena* is characterized by unbranched trichomes that possess intercalary heterocysts (Whitton, 2002). *Anabaena* species are nitrogen-fixing cyanobacteria belonging to the family Nostocaceae in the order Nostocales (Castenholz and Waterbury, 1989). Heterocysts differentiate in response to the lack of combined nitrogen in the environment and are the sites of nitrogen fixation. *Anabaena* species also produce relatively short, motile filaments called hormogonia and this characteristic, in part, distinguishes them from members of the closely related genus *Nostoc*. In addition,

Anabaena species differentiate spore-like structures termed akinetes in response to nutrient limitation other than nitrogen (Sarma et al., 2004). *Anabaena* species are widely distributed in portions of the biosphere, including fresh waters and tropical, temperate and polar terrestrial systems; they are rarely found in marine habitats (Shah *et al*., 2003). Many *Anabaena* species occur in symbiotic associations with fungi to form lichens and with representatives of each of the major phylogenetic groups of plants (Meeks, 1998). *Anabaena* species are terrestrial and benthic cosmopolitan microorganisms, which form extended mucilaginous layers on soil and in the aquatic environment on stones and mud. Many secondary metabolites with new structures have been isolated from these organisms.

Materials and methods:

Study area and sampling: Manipur is landlocked, hilly and mountainous state within the north eastern part of India. It has 22, 327 sq. km area, which constitutes 0.7 per cent of the total land surface of India (fig. 1). The state has a valley area of about 1843 sq. km, which is 8 per cent of the total area of the state with two main seasons separated by two transitions; the winter season and the monsoon season. Visible samples were collected from various rice fields covering 6 districts using spatula and knifes. Water samples were also taken from each site for analyzing pH.

Morphological analysis: Strains were identified according to Komarek and Anagnostidis (2013) and Desikachary (1959). Morphological studies and photomicrography were performed per strain using a Carl Zeiss-A10 fluorescence microscope. The isolated strains examined in this study comprises of vegetative cells, heterocysts and akinetes and shape of apices under the microscope using 40 and 100 times objective lenses (list-1).

Growth and maintenance of cyanobacteria:

Thirty two *Anabaena* strains were collected from different rice fields of Manipur, India (list-1; fig. 1) were cultured as unialgal in 100 ml BG-11 medium (Stanier et al., 1971) in Erlenmeyer flasks (capacity 250 ml) and strains were identified by the keys of Desikachary (1959) and Komarek and Anagnostidis (2013).

Estimation of chlorophyll-a: Estimation of chlorophyll-a was determined by adopting the method described by McKinney (1941). 10 ml of homogenized algal suspension was taken in centrifuge tube and centrifugation at 7000 rpm for 10 min and then discarded the supernatant and transferred the algal pellet to a test tube and added 10 ml of 90% methanol. Shaked the contents and placed the tubes covered with aluminium foil in a water bath at 60˚C for 30 min. The absorbance of supernatant was measured at 665 nm against methanol blank in UV-visible spectrophotometer (Shimadzu 1800).

Acetylene reduction activity determination: Acetylene reduction activity was determined by the method described by Hardy et al. (1973). A known volume of algal biomass was taken into 13 ml capacity vial. Stopper the vial and remove the gas phase equivalent to 10% of the remaining volume of the vial and injected equivalent volume of acetylene (C_2H_2). Vials were incubated for 120 min under light conditions (4 Klux) at 28±2°C and interval shake was done. A gas sample of known volume (0.1 to 1.0 ml) was withdrawn with gas tight syringe and injected into injection port of the gas chromatograph (Thermo Chemito Ceres 800 plus software version 2.6).

Phycobiliproteins estimation: Estimation of phycobiliproteins was determined by the method described by Bennett and Bogorad (1973). 10 ml algal suspension was centrifuged at 7000 rpm for 10 min (refrigerated centrifuge Eppendorf 5430 R). The pellets were suspended in 5 ml phosphate buffer. The contents were repeatedly freezed in 4ºC and thawed at room temperature. The supernatants were pooled and the absorbance was measured at 562 nm, 615 nm and 652 nm for phycocyanin, allophycocyanin and phycoerythrin respectively using UV-visible spectrophotometer (Shimadzu 1800).

Total carotenoids estimation: Estimation of total carotenoids was determined by the method described by Jensen (1978). 10 ml homogenized algal suspension was taken and centrifuged at 6500 rpm for 10 min (refrigerated centrifuge Eppendorf 5430 R). Discarded the supernatant and added 3 ml of 85% acetone and subjected to repeat freezing and thawing until the pellet becomes colorless. Measured the volume of the extract and make up the final volume upto 10 ml with 85% acetone and read the O.D. at 450 nm using 85% acetone as blank and calculated the total amount of carotenoids in µg/ml using UV-visible spectrophotometer (Shimadzu 1800).

16S rRNA PCR amplification: Amplification was carried out for 50 µl of reaction mixture using 2 µl (50 ng) of extracted DNA using PCR master mix made up of 5 µl of 1X *Taq* buffer with 1.5 mM $MgCl_2$, 5 µl of 200 µM each of dNTPs solution, 0.25 µl of 1.25U *Taq* polymerase along with 1.5 µl of 0.3 µM each of cyanobacterial universal primer for 16S rRNA like (536f-GTGCCAGCAGCCGCGGTRATA) and reverse (1488R-GGTTACCTTGTTACGACTTCACC) with 34.75 µl of sterile double distilled water. After DNA amplification, 3 µl of PCR sample was mixed with 1 µl of loading dye by pipetting in and out. Later on loaded the sample in 2% agarose gel in gel electrophoresis (Elchrome Scientific SEA 2000) unit and run for 45 mins at 60V. 200 bp DNA

ladder was used as marker. After gel electrophoresis, gel was illuminated in gel documentation system and observed DNA bands.

Sequence alignment and phylogenetic analysis: Sequences were aligned using the CLUSTALW to produce working alignment of 16S rDNA sequences for the target strains. The final alignments were obtained by manual refinement. Phylogenetic tree was constructed including the available cyanobacterial gene sequences along with the sequences determined in this study using the neighbour-joining method (Saitou and Nei, 1987; Thompson et al., 1994) was done by using Kimura 2-parameter model (Kimura, 1980) method contained in the MEGA 4.0 software. The analysis of phylogenetic tree was done. Statistical significance level of interior nodes was determined by bootstrap analysis (1,000 data re-samplings) (Felsenstein, 1985) and values above 50% were reported.

Results and discussion:

Cyanobacterial forms represent a large group of structurally complex and ecologically significant gram negative prokaryotes which flourish in rice fields and also known to sustain the fertility of the agro ecosystem. Rice field ecosystem with its optimum levels of light, water, temperature, humidity and nutrient provided a favorable environment for the luxuriant growth of cyanobacteria. Total 32 *Anabaena* strains were isolated from rice fields of six districts of Manipur, India, namely; Imphal East (11 strains), Imphal West (08 strains), Bishnupur (06 strains), Senapati (02 strains), Thoubal (04 strains) and Churachandpur (01 strain) and summarized in list-1. Maximum number represented from Imphal East (11 nos.) and least number represented from Churachandpur (01 no.). Species of the genus *Anabaena* have traditionally been distinguished based on morphological characteristics. One of the many characteristics used to differentiate species is the position of the akinete relative to the heterocyst. This characteristic is useful when identifying samples collected from nature, but in cultured strains, it is likely that either sporulation is delayed or that heterocyst formation occurs in response to the nutrients available in the medium. Out of 32 *Anabaena* spp. five (05) strains namely: *Anabaena* sp. BTA03 produced high content of total carotenoids (41.8 µg/ml), chlorophyll-a produced high by *Anabaena* sp. BTA04 (21.4 µg/ml), *Anabaena* sp. BTA35 produced high content of phycocyanin and allophycocyanin (150.4 µg/ml and 130.7 µg/ml) respectively. High content of phycoerythrin was recorded by *Anabaena* sp. BTA84 however *Anabaena* sp. BTA903 showed high acetylene reduction activity (14.2 nmole C_2H_4/µg of Chl-a/hr) (table 1).

Since the neighbor-joining (NJ) and maximum-parsimony (MP) trees were similar, only the NJ tree is presented to show the phylogenetic relationship of the cyanobacterial strains for each gene (fig. 2 and 3). A phylogenetic 16S rRNA gene trees were reconstructed from an alignment of 22 sequences, including 5 *Anabaena* sp. isolates, representing the major lineage of cyanobacteria with *Synechococcus elongatus* as the outgroup (fig. 2). The cyanobacteria studied were divided into two main groups, where the first consisted of filamentous cyanobacteria, *Anabaena* and *Nostoc*, capable of forming heterocysts and akinetes, the second contained non-heterocystous, filamentous *Oscillatoria*, *Lyngbya*, and *Arthrospira* strains and *Synechococcus elongatus* as outgroup. The sequences of *Anabaena* strains were compared with the retrieved cultures from NCBI GenBank database and the published 16S rDNA sequences. Trees were constructed by neighbor joining (fig. 2) (Saitou *et al*., 1987) maximum-parsimony (Eck and Dayhoff, 1966) (fig. 3) and methods in MEGA 4.0 (Tamura *et al*., 2007). These five sequences shared greater than 99.8% sequence similarity and were supported by 99% bootstrap re- samplings in both NJ and MP analysis. The identifications of genomic approached by hierarchical clustering analysis allowed the assessment of intra-specific diversity for the representative species under present study and also revealed the approach to be a useful tool for traceability purposes within a rice field ecosystem. Moreover, in culture, the biometric characteristics of vegetative cells, heterocysts and akinetes can vary from those of natural specimens. Therefore, using morphological characteristics to classify cultured strains may give inaccurate results. The present study was also determined whether the morphological characteristics on which the taxonomic identity is based are genetically strong and stable.

List 1**:** Comparative morpho-taxonomical identification of *Anabaena* strains

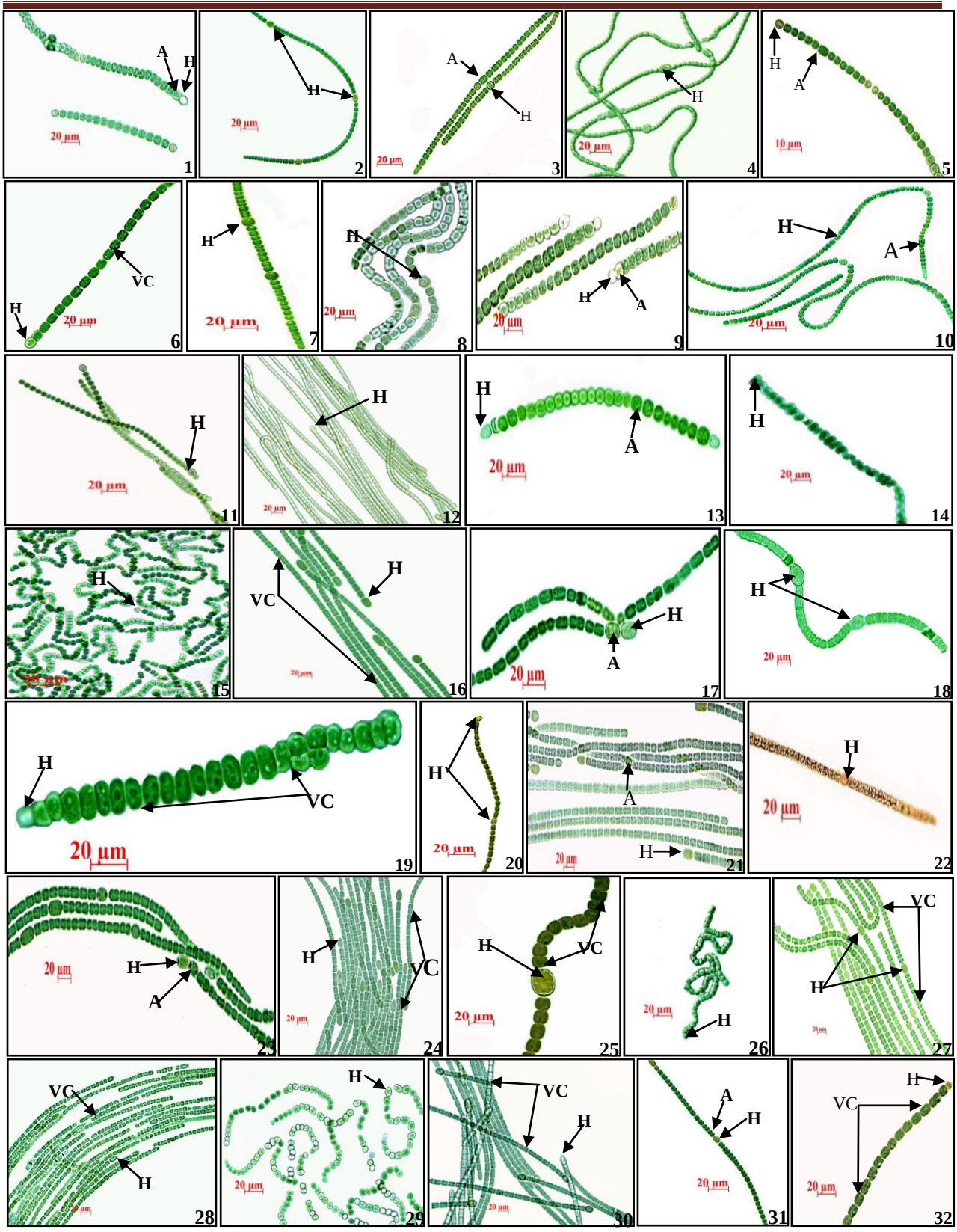
Name of strains	Heterocyst position/ shaped	Akinete	Filament/ Vegetative cells
Anabaena sp. BTA03 DOC:03-07-2004	intercalary, spherical	solitary	broad, solitary
Anabaena sp. BTA04 DOC:06-04-2004	intercalary, barrel	adjacent to heterocystous	tapering ends
Anabaena sp. BTA06 DOC:06-04-2004	intercalary, spherical	solitary	straight, pointed end

Anabaena sp. BTA08 DOC:06-04-2004	intercalary, barrel	solitary	slightly attenuated
Anabaena sp. BTA14 DOC:06-04-2004	intercalary, terminal cylindrical	rounded flattened	straight and flattened
Anabaena sp. BTA17 DOC:06-04-2004	terminal, barrel	solitary	straight, cylindrical
Anabaena sp. BTA18 DOC:06-04-2004	intercalary, cylindrical	very rare	end faintly attenuated
Anabaena sp. BTA19 DOC:06-04-2004	terminal, intercalary spherical	joined to heterocyst	cluster or mucilaginous
Anabaena sp. BTA21 DOC:21-04-2004	terminal, subspherical	spherical	flexuous, constricted
Anabaena sp. BTA23 DOC:21-04-2004	intercalary, slightly oval	aside of heterocyst	long straight
Anabaena sp. BTA30 DOC: 21-04-2004	terminal, spherical	adjacent to heterocyst	elongated
Anabaena sp. BTA31 DOC: 21-04-2004	terminal, little pointed	rare	solitary and entangled
Anabaena sp. BTA34 DOC: 21-04-2004	terminal, subspherical	adjacent to heterocyst	flexuous solitary
Anabaena sp. BTA35 DOC: 21-04-2004	intercalary, subspherical	not observed	single, spiral
Anabaena sp. BTA36 DOC: 12-05-2004	intercalary, subspherical	cylindrical	circinate not straight
Anabaena sp. BTA41 DOC: 12-05-2004	terminal, semi rounded	rare	flexuous, mucilaginous
Anabaena sp. BTA43 DOC: 12-05-2004	terminal, spherical	adjacent to heterocyst	cell broad
Anabaena sp. BTA50 DOC:12-05-2004	intercalary, ellipsoidal	rare	single not straight
Anabaena sp. BTA84 DOC:16-06-2004	terminal, spherical	adjacent to heterocyst	broad flexuous, solitary

Anabaena sp. BTA205 DOC:10-12-2006	terminal, intercalary	not observed	slightly curved, flexuous
Anabaena sp. BTA246 DOC:10-12-2006	terminal, rounded end	not distinct	constricted and straight
Anabaena sp. BTA561 DOC:09-09-2009	intercalary, spherical	not distinct	faintly attenuated at end
Anabaena sp. BTA564 DOC:09-09-2009	intercalary, solitary	solitary, cylindrical	solitary, flexuous, deeply constricted
Anabaena sp. BTA600 DOC:09-09-2009	terminal, cylindrical	not observed	straight, constriction
Anabaena sp. BTA653 DOC:12-06-2010	intercalary, subspherical	not observed	broad cell
Anabaena sp. BTA880 DOC:21-01-2011	intercalary, semi spherical	adjacent to heterocyst	curved
Anabaena sp. BTA881 DOC:21-01-2011	intercalary, ellipsoid	adjacent	solitary, straight

Anabaena sp. BTA883 DOC:21-01-2011	intercalary, terminal,	not seen	thin, constriction
Anabaena sp. BTA903 DOC:21-01-2011	intercalary, irregular shaped	germinated	loosely arranged
Anabaena sp. BTA919 DOC:15-03-2011	terminal, semi rounded	rare	entangled
Anabaena sp. BTA927 DOC:15-03-2011	terminal, intercalary	rare	thin, straight
Anabaena sp. BTA990 DOC:12-09-2011	terminal	adjacent to heterocyst	straight, deeply constricted

Abbreviation: (DOC) Date of collection; (BTA) Biotechnological Algae



Photoplate1: Light photomicrograph images of *Anabaena* strains (magnification=40x and 100x, scale bars=20 µm (A: akinete, H: heterocyst, VC: vegetative cell). *Anabaena* spp. 1-BTA03; 2-BTA04; 3-BTA06; 4-BTA08; 5-BTA14; 6-BTA17; 7-BTA18; 8-BTA19; 9-BTA21; 10-BTA23; 11-BTA30; 12-BTA31; 13-BTA34; 14-BTA35; 15-BTA36; 16-BTA41; 17-BTA43; 18-BTA50; 19-BTA84; 20-BTA205; 21-BTA246; 22-BTA561; 23-BTA564; 24-BTA600; 25-BTA653; 26-BTA880; 27-BTA881; 28-BTA883; 29- BTA903; 30-BTA919; 31-BTA927; 32-BTA990

Table 1: Pigment analysis and nitrogenase activity of different *Anabaena* strains isolated from rice fields of Manipur

Name of strain	Geographic record of strains	Biomass production and ARA activity		Carotenoids (µg/ml)	Phycobiliproteins (µg/ml)		
		Chlorophyll-a (µg/ml)	Nitrogenase activity nmole C_2H_4/µg of Chl- a/hr		PE	PC	APC
Anabaena sp. BTA003	Alt: 775 m N24°49'26.4" E093°57'52.0"	2.32±0.15	0.80±0.01	41.8±2.41	11.2±1.57	32.6±0.01	19.8±2.41
Anabaena sp. BTA004	Alt: 773 m N24°15'11.1" E093°57'50.5"	24.1±0.19	0.24±0.03	26.5±1.91	13.2±0.62	31.3±1.92	36.1±1.61
Anabaena sp. BTA006	Alt: 775 m N24°49'26.4" E093°57'52.0"	6.19±2.12	0.40±0.09	39.3±1.76	12.3±1.32	22.4±1.32	24.7±1.69
Anabaena sp. BTA008	Alt: 775 m N24°49'26.4" E093°57'52.0"	2.54±0.38	0.15±0.06	53.7±1.30	23.4±2.00	55.3±1.00	18.2±0.01
Anabaena sp. BTA014	Alt: 775 m N24°49'26.4" E093°57'52.0"	2.45±0.27	0.08±0.01	6.22±2.84	7.78±1.09	48.2±1.73	15.3±1.76
Anabaena sp. BTA017	Alt: 745 m N24°46'06.7" E093°54'14.5"	2.09±0.36	0.75±0.05	8.87±1.21	3.02±0.67	7.81±2.19	6.50±0.96
Anabaena sp. BTA018	Alt: 769 m N24°46'06.7" E093°54'26.5"	5.15±0.90	0.31±0.01	18.6±3.46	17.6±0.91	29.8±1.73	9.59±0.77
Anabaena sp. BTA019	Alt: 772 m N24°46'06.7" E093°54'11.5"	4.33±0.43	4.48±0.01	2.83±0.33	3.40±0.78	19.3±3.53	8.47±2.65
Anabaena sp. BTA021	Alt: 789 m N24°49'37.0" E093°43'35.2"	3.23±0.08	0.13±0.09	2.81±0.28	9.32±0.20	21.0±1.03	19.2±1.42
Anabaena sp. BTA023	Alt: 780 m N24°49'36.0" E093°53'25.5"	8.58±0.75	0.22±0.01	24.2±0.76	4.14±0.36	55.8±4.09	24.1±2.97
Anabaena sp. BTA030	Alt: 782 m N24°48'14.3"	1.70±0.86	3.08±±0.01	19.5±1.30	22.0±1.40	30.3±1.07	18.7±1.80

	E093°54'18.3"						
Anabaena sp. BTA031	Alt: 782 m N24°48'14.3" E093°54'18.3"	9.21±1.04	0.57±0.02	29.2±2.00	24.2±7.78	76.8±2.41	10.2±4.21
Anabaena sp. BTA034	Alt: 776 m N24°43'15.5" E093°50'27.5"	3.09±0.03	2.07±0.01	38.4±2.30	10.6±6.20	112.7±1.46	20.6±7.84
Anabaena sp. BTA035	Alt: 776 m N24°43'15.5" E093°50'27.5"	11.6±3.59	1.43±0.03	31.7±1.00	21.1±5.40	150.4±1.12	130.7±1.46
Anabaena sp. BTA036	Alt: 776 m N24°43'15.5" E093°50'27.5"	18.2±1.96	7.01±0.02	31.3±2.60	4.29±0.76	63.7±7.75	3.85±1.94
Anabaena sp. BTA041	Alt: 765 m N24°30'12.3" E093°46'46.4"	19.7±0.42	10.0±0.02	27.4±1.22	10.2±1.38	66.3±1.03	45.0±1.00
Anabaena sp. BTA043	Alt: 764 m N24°32'21.4" E093°45'27.6"	3.24±0.14	6.77±0.08	21.3±1.42	13.2±1.09	57.2±1.03	36.8±0.09
Anabaena sp. BTA050	Alt: 769 m N24°29'25.1" E094°00'43.7"	11.5±1.60	5.31±0.06	32.3±1.30	53.4±1.50	72.7±1.25	39.6±3.64
Anabaena sp. BTA084	Alt: 769 m N24°29'25.1" E094°00'43.7"	10.4±3.55	0.25±0.05	40.3±2.30	73.9±2.70	61.3±2.11	1.38±2.14
Anabaena sp. BTA205	Alt: 776 m N24°43'15.5" E093°50'27.5"	1.11±0.36	5.00±0.07	17.5±1.42	4.28±0.45	11.6±1.08	4.47±0.06
Anabaena sp. BTA246	Alt: 783 m N24°52'54.7" E093°55'01.4"	1.75±0.16	1.62±0.12	10.5±0.84	11.6±4.48	42.3±1.50	24.2±1.06
Anabaena sp. BTA561	Alt: 780 m N24°53'54.9" E093°59'03.8"	1.64±0.74	2.65±0.03	29.6±1.40	5.12±1.22	18.3±1.42	6.22±1.30
Anabaena sp. BTA564	Alt: 933 m N25°02'20.2" E093°55'36.0"	1.22±0.12	8.68±0.01	12.9±0.99	12.4±2.30	24.4±3.91	8.32±1.93
Anabaena sp. BTA600	Alt: 780 m	0.20±0.20	3.66±0.06	5.60±1.40	7.32±3.54	7.22±1.36	8.32±3.40

	N24°53'54.9" E093°59'03.8"						
Anabaena sp. BTA653	Alt: 933 m N24°02'19.1" E094°18'00.1"	1.10±0.19	1.30±0.07	16.8±4.66	32.7±4.50	22.6±5.90	10.2±2.20
Anabaena sp. BTA880	Alt: 765 m N24°50'49.7" E093°56'22.7"	2.45±1.16	2.72±0.02	18.7±9.90	4.67±1.10	21.2±1.29	26.6±2.26
Anabaena sp. BTA881	Alt: 765 m N24°50'49.7" E093°56'22.7"	1.24±0.30	8.52±0.02	13.1±0.86	7.05±1.31	18.8±1.62	16.8±1.47
Anabaena sp. BTA883	Alt: 765 m N24°50'49.7" E093°56'22.7"	1.08±0.06	6.86±0.08	1.06±0.04	4.36±1.80	19.5±6.96	14.5±4.67
Anabaena sp. BTA903	Alt: 769 m N24°46'06.7" E093°54'14.5"	2.11±0.41	14.2±0.01	0.57±0.16	7.51±0.20	16.5±3.34	21.2±6.30
Anabaena sp. BTA919	Alt: 761 m N24°30'12.3" E093°46'46.4"	2.90±0.78	13.4±0.02	2.66±0.19	12.2±1.60	20.7±1.27	7.75±3.46
Anabaena sp. BTA927	Alt: 782 m N24°39'18.5" E093°59'18.6"	3.85±0.56	13.7±0.06	11.4±1.57	28.3±5.70	52.9±5.90	22.1±3.20
Anabaena sp. BTA990	Alt: 805 m N24°29'28.7" E094°00'24.1"	2.64±0.04	6.50±0.18	7.79±1.60	10.0±1.60	36.8±1.93	41.9±1.83

Abbreviation: (PC) Phycocyanin; (PE) Phycoerythrin; (APC) Allophycocyanin

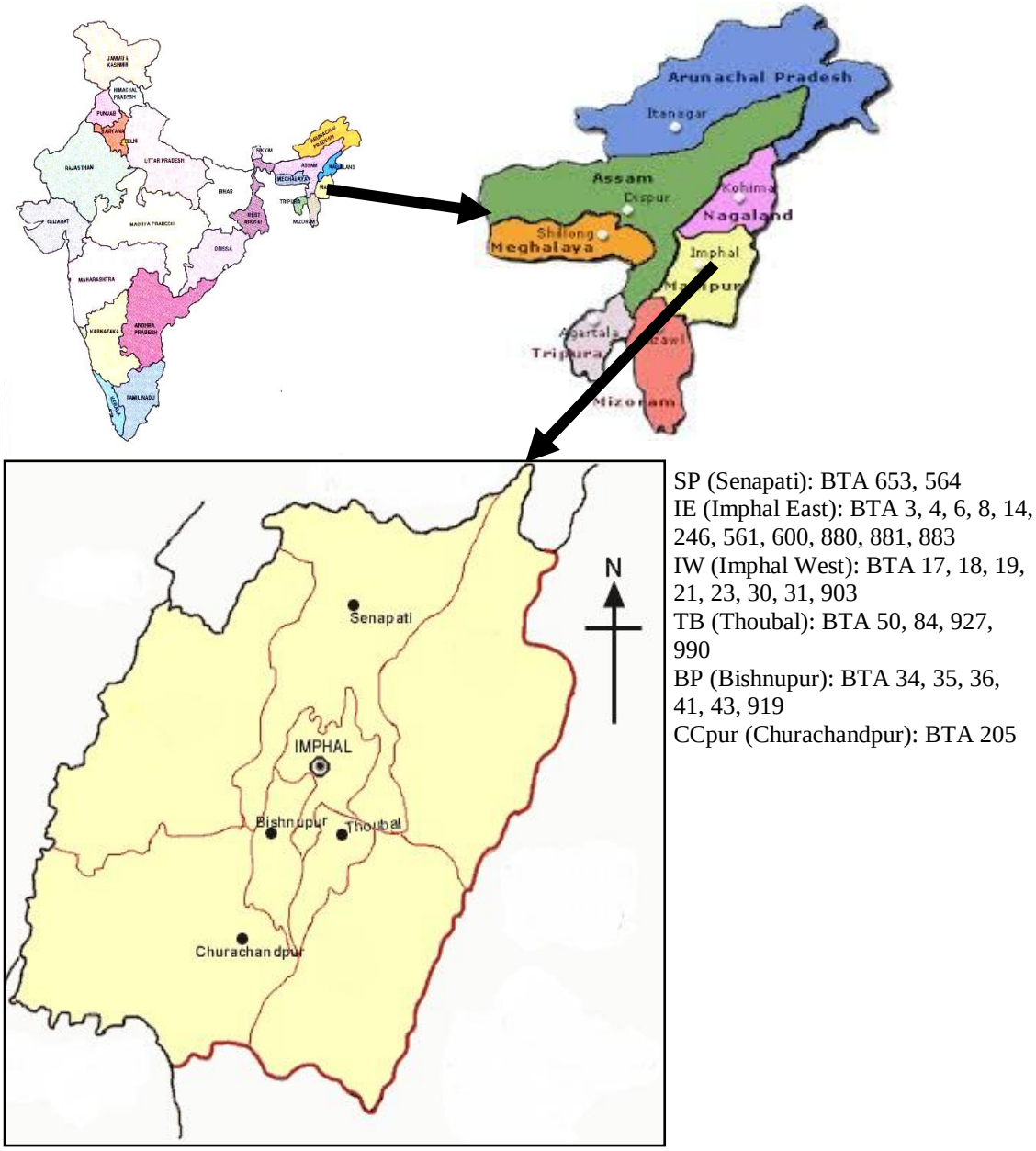


Figure 1: Sample collection sites of rice fields of Manipur, India

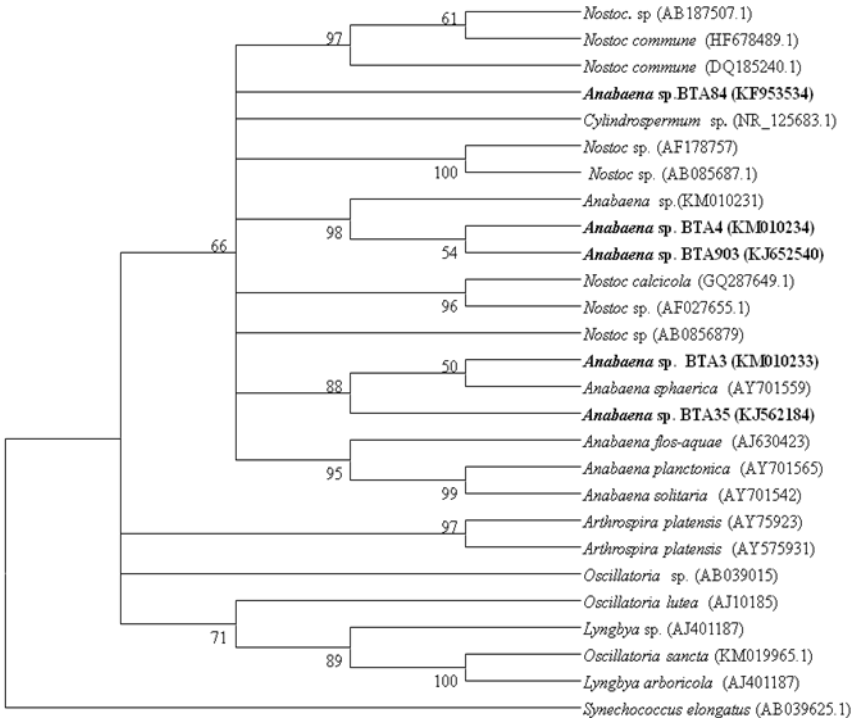


Figure 2: Phylogenetic tree showing clustering of five biochemically potent *Anabaena* strains. Sequences obtained during the present study are indicated in bold. Numbers near nodes indicate bootstrap values over or equal to 50% for NJ analysis. Sequences from GenBank are indicated by accession numbers. Outgroup was *Synechococcus elongatus* (AB039625.1)

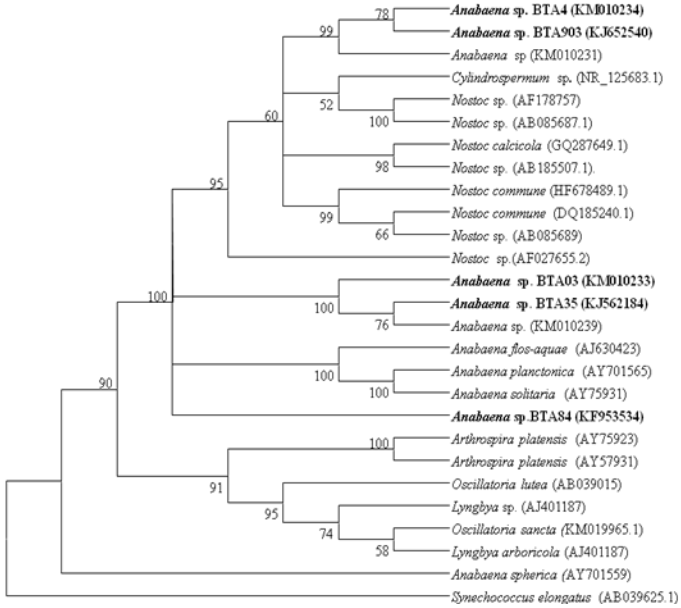


Figure 3: Phylogenetic tree showing clustering of five biochemically potent *Anabaena* strains. Sequences obtained during the present study are indicated in bold. Numbers near nodes indicate bootstrap values over or equal to 50% for maximum parsimony analysis. Sequences from GenBank are indicated by accession numbers. Outgroup was *Synechococcus elongatus* (AB039625.1)

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