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Screening and characterization of cyanobacterial species isolated from Loktak Lake, Manipur, India with emphasis on biofortification

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Abstract

The present investigation deals with the 150 algal and soil samples which were collected from the Loktak Lake, Manipur, India and its periphery. Forty three (43) heterocystous unialgal cyanobacteria were isolated, successfully cultured, purified and deposited to the National fresh water cyanobacterial and Microalgal repository of IBSD, Imphal, Manipur with accession numbers. These strains belong to seven genera namely: *Cylindrospermum* (02), *Nostoc* (13), *Anabaena* (10), *Westiellopsis* (04), *Microchaete* (05), *Calothrix* (08) and *Hapalosiphon* (01). All these strains were morphologically studied and biochemically characterized particularly for extracellular ammonium excretion, chlorophyll-a production and expression of ARA activity during 7th, 14th 21st and 28th days growth in defined culture conditions. *Cylindrospermum* sp. BTA1113 determined high ARA activity followed by *Anabaena* sp. BTA1097 from the bunch of characterized strains. DNA sequences of the 16S rRNA gene has compared with the retrieved cultures from NCBI GenBank database. Molecular phylogenetic analysis confirmed the distinction between the studied strains established on morphological ground.

Keywords: Accession, Cyanobacteria, Heterocystous, Loktak Lake, Repository, 16S rRNA.

Introduction

The cyanobacteria are prokaryotic, mostly photoautotrophic organisms capable of fixing dinitrogen. These organisms provide good examples of elaborate morphological and physiological differentiation of one cell type to another. Dominant nitrogen-fixer cyanobacteria are *Anabaena*, *Nostoc*, *Aulosira*, *Calothrix*, *Cylindrospermum*. Cyanobacteria have the abilities of photosynthesis as well as biological nitrogen fixation. The majority of the algae contain three types of cell i.e., vegetative cell, heterocyst and akinete (spore). Heterocysts and akinetes are the structures with specific structural and biochemical properties suggestive of their role as organs of nitrogen fixation and perennation respectively (Wolk and Wojciuch, 1971; Yamamoto, 1975; Thomas et al., 1977). Heterocyst structure and metabolic activity function together to accommodate the oxygen-sensitive process of nitrogen fixation. Organisms composed of multiple differentiated cell types can possess structures, functions and behaviors that are more diverse and efficient than those of unicellular organisms. They have evolved multiple specialized cell types of nitrogen-fixing heterocysts, spore-like akinetes and the cells of motile hormogonia filaments.

Cyanobacteria mainly use two mechanisms to separate these activities: a biological circadian clock to separate

them temporally and multicellularity and cellular differentiation to separate them spatially. In the absence of combined nitrogen, it produces heterocysts, which are terminally differentiated nitrogen-fixing cells that form at semi regular intervals between stretches of vegetative cells to produce a multicellular pattern of single heterocysts every ten to twenty vegetative cells along filaments. Cyanobacteria are known to be one of the promising supplements to nitrogenous fertilizer, but the process biological nitrogen fixation mediated through the enzyme nitrogenase may be inhibited in presence of readily available nitrogen sources. Nitrogen-fixing cyanobacteria are being used as nitrogen biofertilizers in rice fields in countries where rice is the major staple diet. Although some strains which thrive in rice fields release small quantities of the major fertilizing product, ammonia, during active growth, most of the fixed products are made available mainly through autolysis and microbial decomposition. Under these circumstances, it is difficult to control the flow of nitrogen compounds needed for the development of rice plants.

The excessive use of chemical fertilizers has generated several environmental problems. These problems can be tackled by use of biofertilizers (Choudhury and Kennedy, 2005; Rai, 2006). Cyanobacteria play an important role in maintenance and build-up of soil fertility consequently increasing rice growth and yield as a natural fertilizer (Song et al., 2005). Among the different groups of cyanobacteria, the filamentous nitrogen-fixing species are particularly attractive for the production of biomass and chemicals, since they are able to use atmospheric nitrogen as the sole nitrogen source. Although cyanobacteria are being used as biofertilizer but research is still focused on standard model cultures which where obtain from freshwater rather than exploring potential strains from unusual sites. The clear advantages and their potential significance to biotechnology as bio-fertilizer, there has been very little applied research carried out with filamentous nitrogen-fixing cyanobacteria and few strains there has been successfully grown outdoors, with high biomass productivities (Morena et al., 1995). The present research areas for unveiling the untapped resourceful species for bio-fertilizer from Loktak Lake, Manipur, India still remain largely unexplored. Few works have been done but still need to explored more. The approach for study is the determination of nitrogenase activity with estimation of extracellular ammonium excretion and chlorophyll-a and identification of potent strains through molecular approach. Such an approach can clearly identify the best strains for algal bio-fertilizer and accurate molecular identification.

Materials and Methods

Sample collection: Loktak Lake is the largest freshwater lake in North-East India is famous for the phumdis floating over it located near Moirang in Manipur state, India. This lake was designated as wetland of international importance under Ramsar convention in 23rd March 1990 of its biological richness where naturally occurring Phumdis (heterogeneous mass of vegetation, soil, and organic matters at various stages of decomposition) floating over it. Samples were collected from different sides of Loktak Lake and its adjoining areas where cyanobacteria grown as mat, thin film on surface or from water samples, from the leaf of lotus plant attached, on water hyacinth and attached on the periphery of the lake side. All the samples were collected by using clean spatula and knife and specimen container for water samples and store in room temperature for further action.

Strain isolation and growth condition: The samples were inoculated in prepared broth BG-11 medium without addition of sodium nitrate as the target was fixed for isolation of heterocystous cyanobacteria. Enrichment and isolations were carried out using culture medium till unialgal forms of various species were obtained (Castenholz, 1988). Unialgal biomass was inoculated in Erlenmeyer flask containing BG-11 (-N) broth medium (Stanier et al., 1971). The flasks were kept in culture room under light: dark cycles of 14:10h conditions maintained at 28±2ºC under illumination provided by cool white fluorescent tubes of 54-67µmol photons $m^{-2}s^{-1}$.

Morphological study: The trinocular Carl Zeiss microscope with Axio Vision Viewer 4.8 software was used for image analysis. The length and width of vegetative cells, positions, frequency, size and shape of heterocysts and akinetes and thallus morphology and behaviour were the major parameters for morphological characterization.

Estimation of chlorophyll-a: Estimation of chlorophyll-a was determined by adapting the method described by Mckinney (1941). Cyanobacteria possess chlorophyll-a and it is important for evaluation of growth and photosynthetic rate and participate directly in the light requiring reactions of photosynthesis. 10 ml of homogenized algal suspension was taken in centrifuge tube and done centrifugation at 7000 rpm

for 10 mins and then discarded the supernatant and transferred the algal pellet to a test tube and added 10 ml 90% methanol. Shaked the contents and placed the tubes covered with aluminium foil in a water bath at 60˚C for 30 mins. The absorbance from supernatant was measured at 665 nm against methanol blank.

Determination of acetylene reduction activity: Nitrogenase activity was measured by acetylene reduction technique described by Hardy et al., (1973). Activity was performed in calibrated triplicate serum bottles. A known volume of algal biomass was taken into 13 ml capacity serum bottles. Stopper the bottle and remove the gas phase equivalent to 10% of the remaining volume of the tubes and injected equivalent volume of acetylene (C_2H_2). Serum bottles were incubated for 90 mins under light conditions 54-67 µmol photons $m^{-2}s^{-1}$ at 28±2°C interval shake was done and reaction was terminated by injecting 0.8ml of 15% trichloroacetic acid. Ethylene produced in the bottle was analyzed in gas chromatograph (Ceres 800 Plus Thermo scientific) using Porapak-R column.

Estimation of extracellular ammonium excretion: Ammonia excretion by cyanobacteria is important to study the nitrogen contribution to the soil *i.e.* the amount of nitrogen trapped from the atmosphere. The more ammonia excreted, the more free nitrogen fixed. In present investigation, ammonia excretion was determined by the method described by Solorzano, (1969). Culture filtrate of 05 ml obtained by filtration of homogenized algal suspension filtered through Whatmann's filter paper was taken. Added 0.2 ml mixed phenol (2 g of reagent grade phenol dissolved 100 ml of 95% ethyl alcohol) thereafter added 0.2 ml reagent-A (0.15 g of sodium nitroprusside dissolved in 30 ml of distilled water and stored in amber colour bottle). Finally 0.5 ml reagent-B (10 g of trisodium citrate and 0.5 g of NaOH in 50 ml of distilled water with 20 ml of 1.5 N sodium hypochlorite soln), mixed thoroughly with the aid of vortex shaker and kept for 1 hour for development of blue colour in dark place. The absorbance was measured at 640 against blank.

Genotypic characterization: Mechanical disruption of cell was done in present experiment by Xanthogenate method (Tillett & Neilan 2000).

Phylogenetic analysis: 16S rRNA sequences of cyanobacteria accession no. HQ419202.1, AF091110.1, HQ419204.1, HQ832941.1, GQ206140.1, KJ56186.1, JX827162.1, FJ839360.1, AM398777.1, KJ652539.1, KJ562185.1, EF088335.1, KM438179.1, AJ505943.1, AY218831.2, DQ897365.1, NR_125686.1, NR_125684.1, KJ652540.1, JQ237772.1, FJ705808.1, KJ511808.1, KJ652540.1, KJ511808.1, HQ847577.1, KM010235.1, KM010230.1, KF953528.1, KM982553.1, AB074504.1, HE797729.1, KC971090.1, KM982553.1, KF953526.1, KC971095.1, AM711533.1, AM711532.1, HQ847577.1, HM573458.1, AB093490.1, AB325908.1 and AM711534.1 were obtained from Gen Bank. Nucleotide sequence obtained from DNA sequence was compared with the sequence available in the NCBI database using BLAST (http://www.ncbi.nml.nih.gov /BLAST). Trees based on 16S rRNA were constructed using the available cyanobacterial gene sequences along with the sequence determined in this study using the neighbour-joining method (Saitou et al., 1987; Thompson et al.,1994**)** by using Kimura 2-parameter model (Kimura, 1980) contained in the MEGA 4.0 software (Tamura et al., 2007). Sequences were aligned using CLUSTALW to produced working alignment of 16S rDNA sequences for the target strains. The final alignments were obtained by manual refinement. The analysis of similarity matrix and phylogenetic tree was done using 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

Total 43 heterocystous cyanobacteria belong from 7 genera representing from 3 families and 3 orders of the class cyanophyceae were recorded namely: *Cylindrospermum*, *Nostoc*, *Anabaena*, *Westiellopsis*, *Microchaete*, *Calothrix* and *Hapalosiphon*. Maximum species were found under the genus *Nostoc* followed by *Anabaena*. After screening, 9 strains were found high determination of nitrogenase activity during 21st day growth cultures which were considered as the potent strains for nitrogen fixation and can be used as biofertilizers in the rice fields. The photomicrographs of selected 9 strains of cyanobacteria under 63X magnification were captured and are placed in (photoplates-1) along with valued of acetylene reduction activity. *Anabaena* sp. BTA1097 determined the high content of ethylene (123.3 nmole C_2H_4/µg of Chl-a hr^{-1}) followed by *Cylindrospermum* sp. BTA1113 (113.3 nmole C_2H_4/µg of Chl-a hr^{-1}) (table-1) also estimated ammonium excretion and chlorophyll-a. Nine strains were done PCR based molecular characterization and NCBI accessions acquired (table-2). The identity and diversity of the dominant diazotrophs in typical biological soil of

Table-1: Biomass production, ARA activity and ammonia excretion by cyanobacterial strains of Loktak Lake, Manipur, INDIA

Strain name with sample code	Chlorophyll-a (µg ml $^{-1}$)				Nitrogenase activity (nmole C_2H_4/µg of Chl-a hr^{-1})				Ammonia excretion (µg ml^{-1})			
	7th	14th	21st	28th	7th	14th	21st	28th	7th	14th	21st	28th
Westiellopsis prolifica Janet BTA51	0.79±0.05	5.60±2.54	0.18±0.03	1.44±0.05	2.53±0.02	1.81±0.12	47.7±0.22	6.04±0.01	3.50±1.48	5.60±2.54	7.20±0.84	4.40±0.84
Nostoc paludosum Kutzing ex Born.et Flah BTA53	2.02±0.94	1.23±0.08	2.66±0.10	3.74±0.38	7.12±0.06	10.8±0.15	8.60±0.09	0.40±0.02	2.19±1.41	4.20±0.00	3.90±0.21	3.60±0.42
Westiellopsis sp. BTA55	1.87±0.75	6.10±0.21	0.46±0.07	1.51±0.26	1.71±0.01	1.90±0.10	60.6±0.10	56.78±0.12	4.80±1.69	6.10±0.21	2.80±0.42	4.20±1.69
Nostoc paludosum Kutzing ex Born.et Flah BTA56	3.50±0.53	3.67±0.17	3.81±0.09	5.48±0.21	2.91±0.03	1.37±0.08	7.63±0.00	2.93±0.03	2.89±0.17	4.40±1.27	3.90±0.21	1.80±0.42
Calothrix sp. BTA57	1.42±0.41	1.70±0.48	1.45±1.04	1.82±0.28	0.35±0.02	0.23±0.01	84.5±0.03	0.82±0.01	2.00±0.42	18.5±0.63	10.5±2.33	13.8±1.69
Hapalosiphon welwitschii W.et G.S. West BTA58	1.84±0.75	9.40±0.42	0.44±0.04	1.13±0.00	6.57±0.10	19.5±0.09	84.5±0.20	33.2±1.32	3.20±2.12	9.40±0.42	7.50±1.06	5.20±0.84
Microchaete sp. BTA59	2.12±0.14	3.78±0.69	7.92±2.52	6.10±0.29	0.04±0.00	0.04±0.00	0.01±0.00	0.04±0.00	11.3±3.60	12.1±1.90	14.2±4.24	12.7±4.87
Nostoc sp. BTA60	0.88±0.00	9.50±1.90	0.45±0.05	1.17±0.05	23.5±0.10	29.8±0.01	**108.2±0.01**	41.4±1.22	0.88±0.00	9.50±1.90	3.40±0.84	6.10±1.48
Nostoc sp. BTA61	0.89±0.04	12.8±0.00	0.22±0.02	1.72±0.11	3.59±0.12	3.60±0.12	**111.0±0.23**	67.41±1.24	6.80±1.27	12.8±0.00	8.30±0.21	5.80±1.27
Nostoc commune Vaucher ex Born. et Flah BTA67	3.34±1.57	2.34±1.81	4.01±0.89	7.55±1.09	17.4±0.13	9.46±0.07	**93.9±0.04**	0.55±0.00	2.49±0.96	3.80±1.69	3.30±0.21	2.80±2.54
Westiellopsis sp. BTA68	0.82±0.16	5.90±1.06	0.24±0.03	1.44±0.09	10.9±1.22	9.80±0.10	64.5±0.02	11.4±0.22	1.30±1.06	5.90±1.06	6.50±0.21	4.10±1,06
Calothrix marchica Lemmermann BTA70	0.93±0.03	0.48±0.05	1.15±0.46	1.18±0.06	14.2±1.31	30.4±0.01	11.5±0.23	10.8±1.12	1.10±0.21	14.9±3.18	8.50±1.48	13.0±1.69
Calothrix sp. BTA73	3.15±0.83	0.43±0.00	7.23±1.03	7.67±0.49	11.4±0.02	80.7±0.10	**98.5±0.01**	4.57±0.22	0.80±0.00	10.3±2.75	9.80±0.84	9.50±1.06
Anabaena iyengarii Bharadwaja BTA73	13.8±2.54	0.30±0.05	0.92±0.12	1.67±0.67	0.03±0.00	3.00±0.09	4.83±1.14	12.7±0.10	1.70±0.21	2.80±0.41	1.00±0.00	1.00±0.42

Strain name with sample code	Chlorophyll-a (µg ml^{-1})				Nitrogenase activity (nmole C_2H_4/µg of Chl-a hr^{-1})				Ammonia excretion (µg ml^{-1})			
	7^{th}	14^{th}	21^{st}	28^{th}	7^{th}	14^{th}	21^{st}	28^{th}	7^{th}	14^{th}	21^{st}	28^{th}
Westiellopsis sp. BTA76	2.14±0.16	7.10±1.48	0.14±0.00	4.60±1.69	0.51±0.11	2.11±0.02	39.2±0.00	2.75±0.11	1.00±0.42	7.10±1,48	1.70±0.21	4.60±1.06
Calothrix ghosei Bharadwaja BTA77	3.65±1.55	0.69±0.05	3.32±0.17	3.06±0.27	11.2±0.10	60.8±0.20	13.5±1.15	14.8±0.12	1.30±0.21	11.5±2.33	13.7±5.30	7.90±3.60
Microchaet uberrima Carter N. BTA78	3.15±0.19	4.10±0.19	8.63±0.01	6.84±0.37	3.58±0.10	3.24±0.01	1.59±0.01	2.17±0.01	12.6±0.00	8.60±2.96	9.10±0.21	14.0±4.66
Nostoc sp. BTA80	1.75±0.06	1.58±0.03	1.64±1.29	2.68±0.28	10.2±0.12	3.31±0.04	**91.0±0.03**	0.89±0.01	6.30±0.30	5.00±0.00	5.80±0.42	0.20±0.00
Anabaena circinalis Rabenhorst ex Born. et Flah BTA945	1.50±0.09	2.58±0.41	4.35±0.09	10.2±1.82	0.55±0.01	9.06±0.10	2.06±0.03	4.03±0.05	ND	2.30±0.63	ND	0.90±0.21
Nostoc muscorum Ag. Ex Born. et Flah. BTA950	0.59±0.13	1.56±0.06	1.82±1.24	1.19±0.39	27.1±0.06	13.1±0.77	**95.6±1.34**	4.36±0.04	4.69±1.13	3.30±1.06	1.70±0.63	2.20±2.00
Cylindrospermun muscicola Kutzing ex Born.et Flah BTA963	2.50±0.49	10.4±0.42	0.30±0.00	6.10±0.63	4.76±0.13	6.94±0.12	4.94±0.01	19.1±0.10	5.30±1.06	10.4±0.42	3.10±0.63	6.10±0.63
Anabaena sp. BTA964	1.04±0.60	3.26±0.00	3.83±0.97	5.15±0.16	1.31±0.02	19.0±0.15	**123.3±0.03**	9.94±0.10	0.70±0.63	2.60±0.42	3.83±0.97	2.20±0.42
Nostoc sp. BTA978	1.18±0.17	1.71±0.07	3.04±0.55	3.67±1.19	25.3±0.10	16.5±0.44	12.1±0.12	9.26±0.14	3.49±0.34	4.30±0.21	5.50±0.21	1.60±0.00
Nostoc sp. BTA979	0.82±0.20	1.91±0.17	2.87±0.15	3.52±0.00	ND	0.34±0.02	38.9±0.03	5.36±1.95	6.90±0.79	6.40±0.00	4.50±0.21	7.80±0.42
Anabaena sp. BTA980	0.56±0.27	2.10±0.21	4.29±0.09	5.93±1.40	0.07± 0.02	0.90±0.08	3.22±0.04	6.81±0.23	0.10±0.02	2.10±0.21	ND	1.00±0.42
Anabaena ambigua Rao, C.B. BTA983	1.04±0.12	2.76±1.13	4.19±0.47	5.55±0.35	0.35±0.03	3.58±0.11	2.34±0.04	5.65±0.12	1.00±0.42	2.10±0.21	0.70±0.21	1.40±0.00
Nostoc calcicola Brebisson ex Born. et Flah BTA984	2.37±0.30	1.12±0.27	3.47±0.9	6.28±1.58	10.7±0.04	5.14±1.22	0.92±0.11	0.52±0.05	8.10±1.82	2.70±0.63	4.80±0.00	3.80±0.42
Anabaena sp. BTA988	0.89±0.01	3.08±0.27	5.36±1.69	1.23±0.36	0.16±0.02	2.92±0.01	2.05±0.03	25.1±0.16	0.50±0.63	1.80±0.84	3.50±1.06	1.30±0.21

Strain name with sample code	Chlorophyll-a (µg ml^{-1})				Nitrogenase activity (nmole C_2H_4/µg of Chl-a hr^{-1})				Ammonia excretion (µg ml^{-1})			
	7^{th}	14^{th}	21^{st}	28^{th}	7^{th}	14^{th}	21^{st}	28^{th}	7^{th}	14^{th}	21^{st}	28^{th}
Nostoc sp. BTA995	1.05±0.01	4.07±1.29	6.43±0.60	6.97±0.58	0.39±0.01	5.11±0.09	2.75±0.01	7.17±0.15	0.90±0.21	2.10±0.21	2.80±1.27	6.97±0.58
Anabaena sp. BTA997	0.25±0.06	0.23±0.00	2.52±0.39	2.70±0.68	12.4±0.31	54.7±0.02	17.0±0.02	16.0±0.14	1.30±0.21	11.5±0.63	10.7±0.63	13.0±2.54
Calothrix geitonos Skuja BTA998	1.19±0.25	3.29±0.08	6.79±0.28	4.33±0.54	1.34±0.11	0.88±0.02	90.6±0.04	78.06±0.03	8.60±0.84	13.4±3.39	9.90±1.90	14.0±0.42
Calothrix clavata West, G. S. BTA1002	1.11±0.33	2.76±0.12	4.73±1.55	2.44±0.05	1.26±0.01	0.54±0.02	0.06±0.00	0.12±0.03	26.2±0.84	8.20±2.12	7.80±0.84	10.6±0.84
Anabaena sp. BTA1006	1.20±0.55	0.44±0.00	1.44±0.85	1.48±0.12	6.25±0.09	23.6±0.10	1.44±0.10	8.11±0.01	1.20±0.42	9.20±0.84	7.70±2.75	10.5±1.06
Anabaena anomala Fritsch BTA1007	1.15±0.09	0.52±0.00	1.48±0.00	1.04±0.27	0.34±0.02	2.30±0.03	45.0±0.11	52.57±0.03	1.40±0.00	6.80±2.12	8.60±0.84	5.20±0.42
Calothrix marchica Lemmermann BTA1014	1.60±0.90	3.11±0.55	7.88±0.64	5.29±0.18	1.00±0.12	0.54±0.11	0.02±0.00	0.04±0.01	10.0±2.12	11.8±1.27	8.70±3.18	8.30±4.03
Calothrix ghosei Bharadwaja BTA1019	1.26±0.01	3.36±1.09	4.13±1.95	7.72±1.03	1.11±0.00	0.53±0.01	0.05±0.01	0.05±0.01	9.20±1.27	15.2±0.42	13.6±1.69	5.30±0.21
Anabaena oryzae Fritsch BTA1026	1.63±0.27	0.49±0.30	3.61±0.18	4.40±2.54	0.43±0.04	2.24±0.02	0.60±0.01	0.85±0.02	1.10±0.21	7.20±0.00	11.0±2.12	4.40±2.54
Microchaete grisea Thuret ex Born. et Flah. BTA1041	0.83±0.09	1.96±0.39	3.40±0.07	4.39±0.18	2.40±0.01	1.58±0.02	0.08±0.02	0.68±0.01	8.40±1.27	9.20±2.90	9.80±0.84	12.0±1.27
Microchaete uberrima Carter, N. BTA1043	1.10±0.17	2.52±0.44	4.80±0.31	2.32±0.42	2.11±0.12	0.95±0.01	0.06±0.01	0.17±0.02	6.80±1.27	14.0±1.69	8.50±0.63	13.0±0.42
Nostoc spongiaeforme Agardh ex Born. et Flah BTA1056	4.17±0.78	3.09±1.67	4.48±0.00	6.21±1.84	ND	ND	0.76±0.02	1.80±0.09	0.70±0.21	3.60±1.69	4.40±1.27	1.10±0.21
Microchaete grisea Thuret ex Born. et Flah. BTA1081	2.53±0.60	10.5±0.63	0.51±0.22	5.20±2.54	0.59±0.10	1.37±0.05	9.41±0.10	3.57±0.13	4.80±2.54	10.5±0.63	3.00±1.27	5.20±2.54
Nostoc sp. BTA1097	0.51±0.03	10.5±0.31	0.43±0.07	5.60±1.27	15.8±0.10	9.91±0.13	**98.2±3.24**	32.59±0.09	3.90±1.06	10.5±0.31	0.43±0.7	5.60±1.27
Cylindrospermum sp. BTA1113	3.30±0.20	16.0±0.02	19.3±0.03	32.1±0.23	5.23±0.11	7.34±0.11	**113.6±2.22**	56.1±0.11	3.30±0.07	12.7±0.66	24.1±0.40	32.0±0.22

Table-2: PCR based molecular characterization of selected cyanobacterial strains of Loktak Lake, Manipur, INDIA and NCBI accessions

Sample code	Location co-ordinates	Description	Accession	Accepted date from NCBI	Length	Highest match with NCBI database	% similarity
BTA60	N24º30′57.8″ E093º47′36.0″	*Nostoc* sp.	KF953516.1	04-MAR-2014	736bp	*Nostoc* sp. R76DM (KJ994254)	100
BTA61	N24º30′57.8″ E093º47′36.0″	*Nostoc* sp.	KF953517.1	04-MAR-2014	835bp	*Nostoc* sp. CCAP (HE974997)	98
BTA67	N24º30′57.8″ E093º47′36.0″	*Nostoc commune*	KF953518.1	10-DEC-2013	852bp	*Nostoc* sp. PCC7120 (KM019921)	99
BTA73	N24º30′57.8″ E093º47′36.8″	*Calothrix* sp.	KF953519.1	04-MAR-2014	684bp	*Calothrix marchica* MBDU602 (KC971090)	98
BTA80	N24º30′57.3″ E093º47′36.8″	*Nostoc* sp.	KJ511782.1	11-MAY-2014	915bp	*Nostoc entophylum* ISC32(JN605002)	99
BTA950	N24º30′33.3″ E093º47′10.1″	*Nostoc muscorum*	KF953521.1	04-MAR-2014	884bp	*Nostoc muscorum* CENA61 (AY218828)	99
BTA964	N24º31′09.6″ E093º47′56.0″	*Anabaena* sp.	KF953522.1	04-MAR-2014	760bp	*Anabaena azotica* FACHB-118 (AY422691)	99
BTA1097	N24º31′08.2″ E093º48′46.7″	*Nostoc* sp.	KM652629.1	17-DEC-2014	408bp	*Nostoc* sp. AH-12 (KC699844)	99
BTA1113	N24º31′11.6″ E093º48′00.1″	*Cylindrospermum* sp.	KM652630.1	17-DEC-2014	556bp	*Cylindrospermum musicola* Ind12 (HM573454)	95

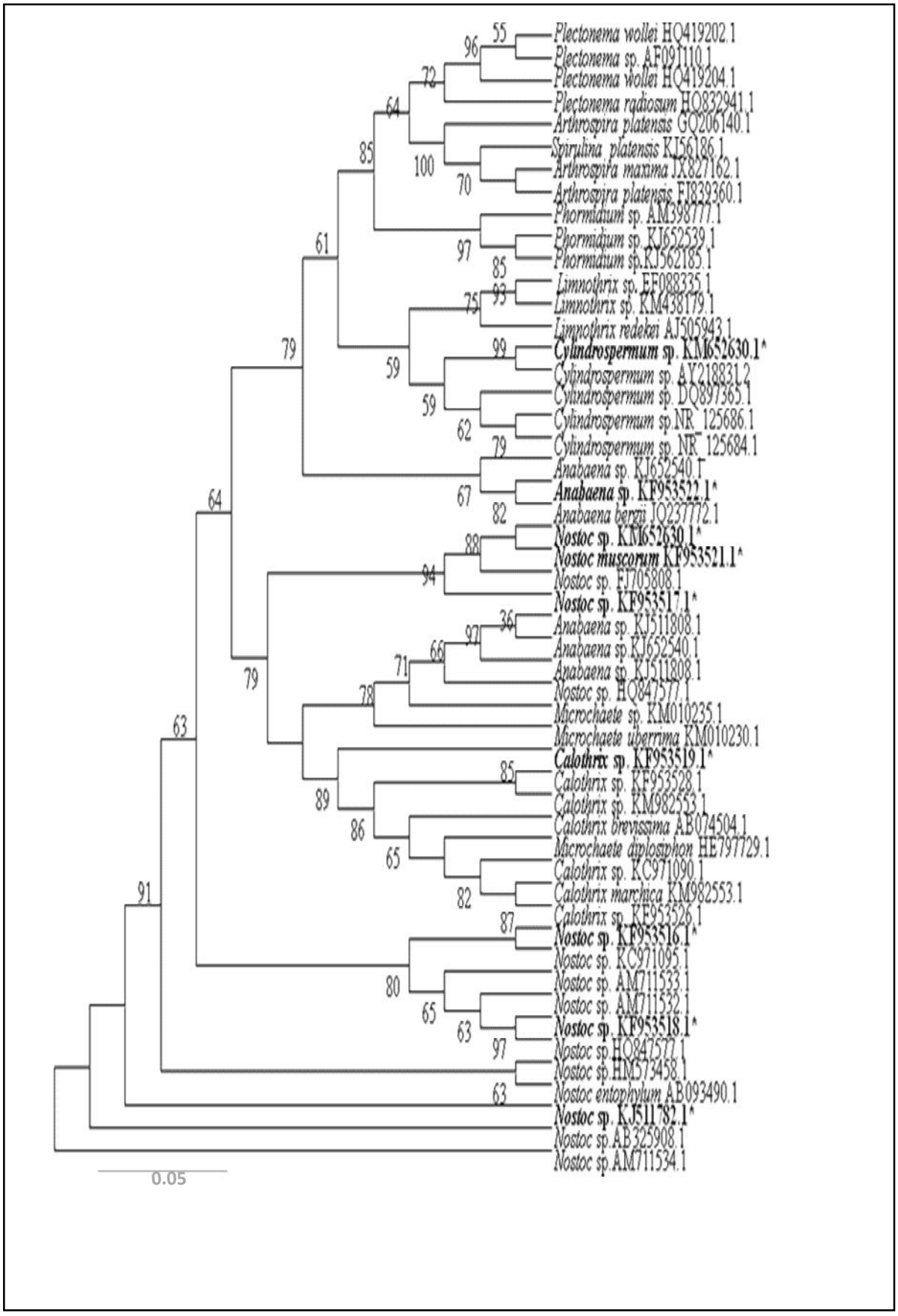
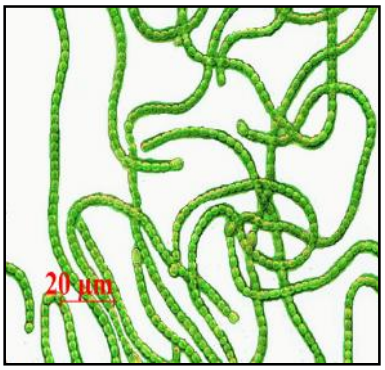
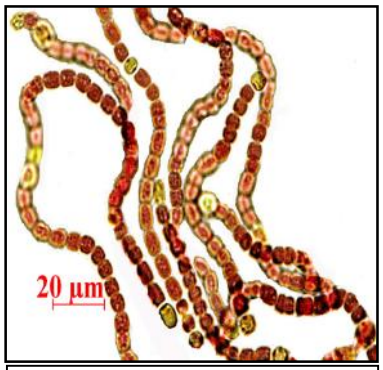


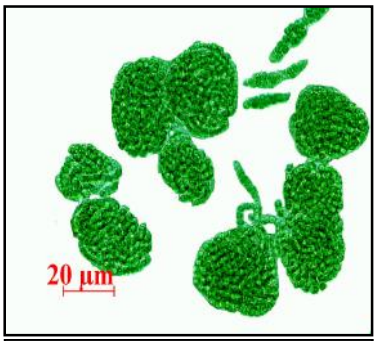
Figure. 1 Neighbour Joining (NJ) tree based on analysis of the 16S rRNA gene sequence showing the position of the sequences obtained from the present study (in bold). Number in nodes indicate bootstrap values ≥50% for analysis.



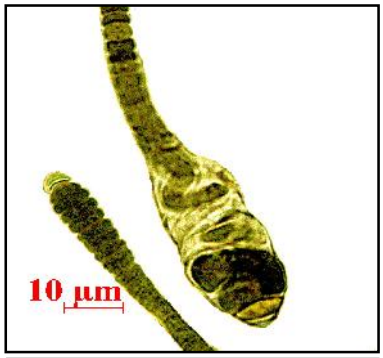
***Nostoc* sp. KF953516.1**
ARA: 108.2 nmole C_2H_4/µg of Chl-a hr^{-1}



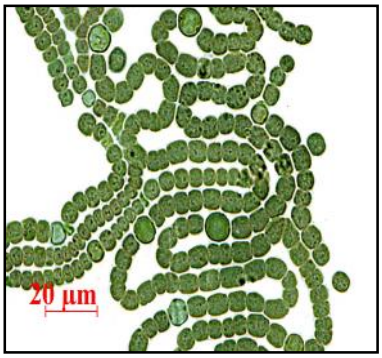
***Nostoc* sp. KF953517.1**
ARA: 111.1 nmole C_2H_4/µg of Chl-a hr^{-1}



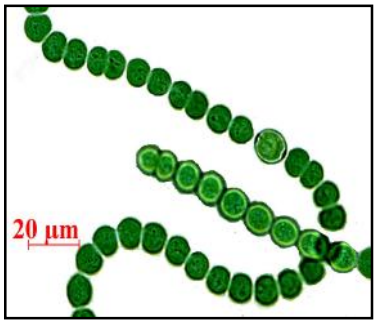
***Nostoc commune* KF953518.1**
ARA: 93.9 nmole C_2H_4/µg of Chl-a hr^{-1}



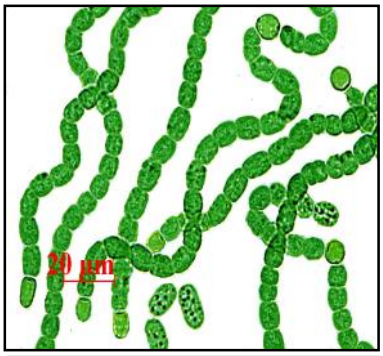
***Calothrix* sp. KF953519.1**
ARA: 98.5 nmole C_2H_4/µg of Chl-a hr^{-1}



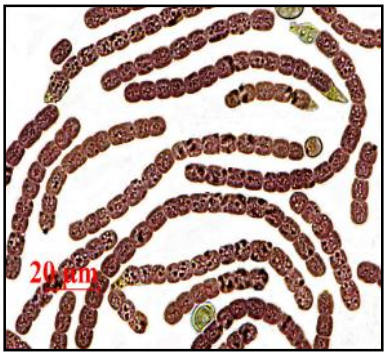
***Nostoc* sp. KJ511782.1**
ARA: 91.0 nmole C_2H_4/µg of Chl-a hr^{-1}



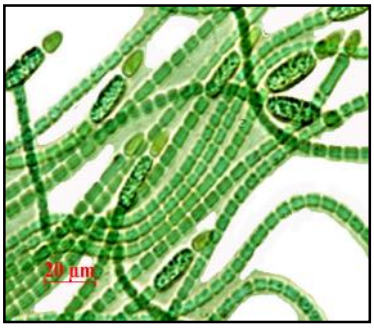
***Nostoc muscorum* KF953521.1**
ARA: 95.6 nmole C_2H_4/µg of Chl-a hr^{-1}



***Anabaena* sp. KF953522.1**
ARA: 123.3 nmole C_2H_4/µg of Chl-a hr^{-1}



***Nostoc* sp. KM652629.1**
ARA: 98.2 nmole C_2H_4/µg of Chl-a hr^{-1}



***Cylindrospermum* sp. KM652630.1**
ARA: 96.6 nmole C_2H_4/µg of Chl-a hr^{-1}

Photoplate-1: Photomicrographs of selected strains and determination of nitrogenase activity in respect of biomass production under culture conditions

Loktak Lake were determined by combining large scale environmental survey with morphological and molecular analysis of cultured cyanobacteria. N_2-fixing cyanobacterial strains found in the Loktak lake were obtained using BG-11 medium (Stanier et al., 1971) and a few permutation of temperature and light intensity. It was determined that a group of *Nostoc* strains that belong to the morphospecies *N. entophylum*, a phylogenetically and morphologically coherent group of strains well-represented by isolates of morphospecies *Calothrix*. *Nostoc* species are considered to be important components of the N_2-fixing community in nutrients poor, arid and semiarid soil worldwide (Dodds et al., 1995; Potts, 2000; Wynn-Williams, 2000; Bhatnagar & Bhatnagar, 2005).16SrRNA sequences obtained in this study consist of 1156-1159 bp nucleotides. To examine monophyly the nostocales 16SrRNA sequences of 41 other cyanobacteria and 9 studied strains. Phylogenetic analysis shown that heterocyst and akinete bearing cyanobacteria form monophyletic clades (Fig.1). Monophyly was supported by the NJ analysis with high bootstrap value (Felsenstein, 1985) of 97%. On the basis of phylogenetic analysis of the 16SrRNA gene, it has been found to be a very wide and heterogeneous group, which may contain more than one genus (Rajaniemi et al., 2005; Rehakova et al., 2007; Komarek, 2010). This study indicated that selected heterocystous cyanobacterial strains can play a very important role in nitrogen fixation in agro land soil. The present study highlights that the highest number of genera isolated was *Nostoc* sp. followed by *Anabaena* sp. The soils which harbored large number of algal species indicated their richness in soil fertility present of different nutrients and rich in soil properties. Algal species may allow the soil rich for cultivation of the rice plants thereby helping the plants for nitrogen and carbon source to the rice fields. However, chemical fertilizers cause pollution of water bodies as well as ground water, besides getting stored in crop plants. Therefore, environmentalists are pressing for switch over to biofertilizer farming. *Aulosira fertilissima* is considered to be the most active nitrogen fixer of rice fields in India (Aiyer et al., 1972).

Conclusion

Application of cyanobacterial biofertilizer to rice crop would bring an improvement in the soil physico-chemical properties and increased availability of phosphorus when used along with rock phosphate biologically fixed nitrogen. Some of the cyanobacteria would also provide growth-promoting substances and the quality of grain would be superior. Depletion of soil fertility, low fertilizer-use efficiency and growing environmental pollution are of major concern to agriculture in terms of crop productivity. Biofertilizers of cyanobacteria can provide a suitable supplement to the chemical fertilizers and organic farming can become a reality in the future. There is a definite need to deploy biofertilizers in combination with organic composts and minimal doses of chemical fertilizers for reaping cleaner and healthy harvests, securing food production and human health, protecting the environment and saving scarce natural resources.

Conflict of interest statement: The authors declare that they have no conflict of interest.

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