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RESEARCH ARTICLE

Pigment production and growth metabolites amongst *Nostoc* strains of unexplored areas of Manipur, India falling under Indo-Burma biodiversity hotspots

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Abstract

In the present investigation, nine (09) *Nostoc* strains were morphologically and molecularly characterized. These strains were also examined for production of pigments, total carbohydrates, total soluble proteins and nitrogenase activity under growth and culture conditions. *Nostoc muscorum* BTA27 yielded highest chlorophyll-a and total carotenoids followed by *Nostoc parmelioides* BTA29. Highest total soluble proteins was yielded by *Nostoc carneum* BTA38 which also had good amount of phycoerythrin and allophycocyanin, however, phycocyanin was found to be highest in *Nostoc commune* BTA67 along with comparable amount of acetylene reduction activity. The investigated strains were molecularly characterized using 16S rRNA and obtained NCBI GenBank accession numbers. The present finding depicted that there could be variation in production of pigments, metabolites and nitrogenase activity amongst the strains of same genera.

INTRODUCTION

Manipur is one of the states of the northeast India, having 24º44´N latitudes and 93º58´E longitudes covering an area of 22,327 sq. km. The semitropical climatic condition augmented with high annual rainfall has played a crucial role enhancing the biodiversity richness that supports luxuriant growth of cyanobacteria as well. Cyanobacteria are impressive ecosystem engineers with an evolutionary history stretching back at least 2.15 billion years (Hayes et al., 2007). Owing to their tremendous adaptability to varying environmental conditions, they successfully colonize almost all kinds of terrestrial and aquatic habitats, including those with extreme conditions (Ward and Castenholz, 2000; Oren, 2000). The role of nitrogen fixing cyanobacteria in enhancing soil fertility has been long known and is well documented (Singh, 1961; Venkataraman, 1981). Cyanobacteria also have the ability to store reserve materials that are sources of lipids, proteins, vitamins and minerals (Rastogi and Sinha, 2009). The potential or actual applications of cyanobacteria in agriculture, aquaculture, nutraceuticals, bioenergy and pollution control (bioremediation) are well-known (Abed et al., 2009). The pigments contained in the cells of cyanobacteria are currently used by the food, cosmetic and pharmaceutical industries. These pigments are added to other products, such as colourants or antioxidants, or used alone as drugs. Carotenoids exhibit pro-vitamin A activity in addition to strengthening the immune system and reducing the risk of degenerative diseases, such as cancer, cardiovascular diseases, cataract and macular degeneration (Muller et al., 2003). Cyanobacterial phycobiliproteins have gained considerable importance in the commercial sector. Phycobiliprotein is a common light-harvesting protein and are regarded as nontoxic and non-carcinogenic natural food colorants alternative to the widely used synthetic food

colourants/ additives having potential toxicity and carcinogenicity (Chaneva et al., 2007). The amazing versatility of cyanobacteria has attracted huge scientific interest in recent years (Zhou and Li, 2010). At present, cyanobacteria generally remain as potential sources for further investigations as prospective and excellent sources of biologically active constituents produced during primary and especially secondary metabolism (Skulberg, 2000). Among the cyanobacteria, *Nostoc*, a genus of filamentous cyanobacteria which form macro and microscopic colonies was found to have immense industrial interest. *Nostoc* is an ecologically, morphologically and physiologically diverse genus of microorganisms inhabiting soils, and represents large reservoir of potentially valuable natural compounds (Dembitsky and Rezanka, 2005). The ecological significance of the *Nostoc* species extends beyond the compounds which they primarily known to produce, as many of these organisms are capable of modifying their habitats through the synthesis of biologically active products (Ehrenreich et al., 2005). Work and publications on this group of microorganisms from northeast India is sporadic despite the fact that this region falls within Indo-Myanmar biodiversity hot spot (Myres et al., 2000). Considering the above potentialities, the present study is taken up to characterize the *Nostoc* strains of different habitats of Manipur, India for selection of most promising strains and also to identify the strains through 16S rRNA gene sequence.

Materials and methods

Organisms and culture conditions

Nine (09) unialgal *Nostoc* strains were obtained from the National Repository for Cyanobacteria and Microgreen algae (Freshwater) at DBT-IBSD, Imphal, Manipur, India. The strains were grown in 250 ml Erlenmeyer flasks which contained 100 ml of BG-11 medium (Stainer et al., 1971) without nitrogen source. Experimental cultures were incubated at 28±2ºC under a light intensity of 54-67 µmol photon m^2s^{-1} with 14/10 h light and dark period provided by cool white fluorescent tubes fitted with photoperiodic timer (Saveer Biotech Limited, India) coupled with room temperature controller.

Microscopic identification

Trinocular and fluorescence microscope were used to study the morphology of the strains. The miniscule amount of biomass were viewed under trinocular research microscope (Nikon Eclipse 80*i*) and Carl Zeiss fluorescence microscope, Axio Scope A1 coupled with Carl Zeiss Imaging Systems 32 software AxioVision 4.7.2 followed by taxonomical characterization following standard keys of Desikachary (1959).

Estimation of Chlorophyll-a

Absorbance of the 90% methanol extract was recorded at 665 nm and the amount of Chlorophyll-a was determined using extinction coefficient of Mackinney (1941).

Estimation of total carotenoids

Carotenoids were estimated by optical density (O.D.) at 450 nm using acetone as blank (Jensen, 1978).

Estimation of phycobiliproteins

Phycobiliproteins was determined spectrophotometrically at 562 nm, 615 nm and 652 nm for phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC) respectively using the equation given by Bennett and Bogorad (1973).

Total soluble proteins estimation

The total soluble proteins content of extracts was measured as described by Herbert et al. (1971) using bovine serum albumin as standard.

Estimation of total carbohydrates

Total carbohydrate was estimated by Spiro (1966) using glucose as standard.

Estimation of nitrogenase activity

Nitrogenase activity of the strains was determined by the method described by Hardy et al. (1973).

16S rRNA gene sequence

The authentic identification of the experimental strains was performed using 16S rRNA gene sequence. Unialgal biomass was subjected for isolation of genomic DNA according to the Xanthogenate-SDS (XS) extraction modified protocol (Avijeet et al., 2013). Partial 16S rRNA gene sequence was amplified employing universal primers (Integrated DNA Technology, India) namely 536F 5'-GTGCCAGCAGCCGCGGTRATA-3' and 1488R 5'-CGGTTACCTTGTTACGACTTCACC-3' (Nubel et al., 1997). A total of 50 µl of PCR reaction mixture was prepared having 5 µl of 1X *Taq* buffer, 5 µl of 200 µM of each deoxynucleotides, 1.5 µl of 0.3 µM of each forward and reverse primer, 0.25 µl of 5U *Taq* DNA polymerase and 2 µl of DNA extract. The PCR conditions were set for 28 cycles with initial denaturation at 95˚C for 5 min then final denaturation of 95˚C for 1min, annealing at 55˚C for 1 min and final extension at 72˚C for 2 min using Thermal cycler (Mastercycler gradient, Eppendorf, Germany). The PCR product was detected with standard agarose gel electrophoresis (Elchrom Scientific GEPS 200/2000, Switzerland) and quantification of PCR product was done with Biospectrometer (Eppendorf, Germany). Sequences

of the quantified 16S rRNA PCR products were submitted to the NCBI GenBank database to obtain accession number.

Results and Discussion

Nine (09) unialgal *Nostoc* strains from different habitat of Manipur, India were morphologically and biochemically characterized (table 1 and 2). The studied strains were *Nostoc* sp. BTA12, *Nostoc muscorum* BTA27, *Nostoc parmelioides* BTA29, *Nostoc carneum* BTA38, *Nostoc* sp. BTA131, *Nostoc commune* BTA67, *Nostoc muscorum* BTA950, *Nostoc* sp. BTA972 and *Nostoc ellipsosporum* BTA1075. *Nostoc* strains of this study were from different habitats comprising of rice field, water logged area of lake, foothill, seepage area etc. Occurrence of several species of cyanobacteria in the biological crusts on different substrata comprising of building facades, stone monuments, bricks, bark of trees and soil surfaces in temperate and tropical region of the globe are reported in different studies (Crispim et al., 2003, 2004; Gaylarde and Gaylarde, 2000; Johansen and Shubert, 2001; Ortega-Morales et al., 2005; Tirkey and Adhikary, 2006). The growth and morphological features of the different strains of *Nostoc* varies and was shown in Fig. 1 and 2. The investigated strains possess different filament structures, cell shapes, sheaths along with various shapes of heterocyst and akinete. The morphological structure enumerated in our study is very much supported by earlier report of Pereira et al. (2005). Cyanobacteria have been identified as one of the most promising group of organisms from which novel and biochemically active natural products are isolated. In our study, different *Nostoc* strains were investigated for production of chlorophyll-a, total carotenoids, total soluble proteins and total carbohydrates. Out of the nine strains, *Nostoc muscorum* BTA27 was found to have highest chlorophyll-a production (10.60±0.03 μgml^{-1}) followed by *Nostoc parmelioides* BTA29 (8.97±0.06 μgml^{-1}) and the least production in *Nostoc* sp. BTA972 (1.22±0.01 μgml^{-1}). The chlorophyll-a content in all the investigated strains showed more amount than *Nostoc spongiaeforme* (1.76±0.00 μgml^{-1}) in earlier study by Ojit et al. (2012). *Nostoc carneum* BTA38 produced maximum amount of total carotenoids (30.90±0.12 μgml^{-1}) and the minimum in *Nostoc ellipsosporum* BTA1075 (4.18±0.30 μgml^{-1}). *Nostoc carneum* BTA38 ranked highest in total soluble protein contents (77.00±0.16 μgml^{-1}) and the lowest in *Nostoc commune* BTA67 (10.00±0.00 μgml^{-1}). Total carbohydrates were recorded highest in *Nostoc ellipsosporum* BTA1075 as 43.00±0.01 μgml^{-1} comparing to other investigated strains. The above mentioned results of our investigated strains were found to be in the range of previous study by Narayan et al. (2006). Our work revealed that the content of phycobiliprotein varied from strains to strains. The highest value of phycocyanin (88.00±0.25 μgml^{-1}) was observed in *Nostoc commune* BTA67 and *Nostoc carneum* BTA38 showed highest amount of allophycocyanin and phyoerythrin as 58.70±0.39 μgml^{-1} and 20.00±0.49 μgml^{-1} respectively. The amounts of phycocyanin, allophycocyanin and phyoerythrin obtained in our study were found more than the findings reported by other workers. The content of phycobiliproteins varied to a great extent depending on the species, indicating them to be species-specific biochemical features. The inter-specific variation in phycobiliprotein content and composition, as found in our study, has been reported by other workers (Moreno et al., 1995). The levels of phycocyanin, allophycocyanin and phycoerythrin in total phycobiliproteins content in cyanobacteria vary not only with species but are also influenced by environmental factors. Cyanobacterial species with high content of phycobiliproteins can be considered as their prospective source for commercial use. The filamentous cyanobacteria, particularly nitrogen-fixing heterocystous species, are regarded as attractive organisms for the production of phycobiliproteins and other important chemicals (Borowitzka, 1988; Moreno et al., 1995). Further, the study has demonstrated that *Nostoc commune* BTA67 exhibited the highest nitrogenase activity (24.65±0.11 nmole C_2H_4 μg^{-1}chl-a hr^{-1}). Earlier study by Nilsson et al. (2002) also shows that many competent *Nostoc* strains colonize rice root surfaces and intercellular spaces. Such *Nostoc* strains are shown to have higher nitrogenase activity compared to their free-living counterparts. The nitrogenase activity of *Nostoc commune* BTA67 was recorded higher than the activity of *Nostoc* isolates of another earlier study of Mayashree et al. (2010). The higher nitrogenase activity may be because of higher heterocyst frequency. The findings of Mayashree et al. (2010) also supported our assumption of the correlation between increased heterocyst frequency and greater nitrogenase activity. The results of our finding showed that *Nostoc* strains were the prominent components in the soil crust only when sufficient moisture is available in the substratum.

Table 3 showed the NCBI GenBank accession numbers of the investigated strains. The 16S rRNA gene sequences of the strains will be informative for molecular based study. We believed that the result of the current investigation would be helpful in future diversity study for reference as well as in researching strains for biotechnological applications. Since these strains were found to be potent in various aspects, they can be commercialized. The present study focused mainly on the cultural studies and biochemical characterization of the *Nostoc* strains from Manipur, India. Till date only few information are available on the diversity and cultural behaviour of different genera from the North-Eastern region of India as studied by Tiwari and Singh (2005) and Deepa et al. (2010). Knowledge of cyanobacterial diversity of a region may help in selecting appropriate cyanobacterial inocula to be applied as

biofertilizer consortia in crop fields as well as help in finding strains with other biotechnological potentials. The present findings demonstrated that there is difference in the cellular production among species of same genera and this in-depth information would be helpful in selection of biotechnologically potent strains. Intensive research is thus needed to understand many of the basic aspects pertaining to the production of a metabolite with the concurrent evolution of applied research towards the large production of the products. Also, further studies are essential from this region over the long term and the use of products of biological, non-toxic products from these strains which is still a long way down the road.

Table-1: Morpho/taxonomical characterization of *Nostoc* strains of Manipur, India

Name of the strains	Habitat of the strains	Location	Taxonomical enumeration				
			Filament/ Trichome	**Sheath**	**Cell shape**	**Heterocyst**	**Akinete**
Nostoc sp. BTA12	Rice field, Imphal East, Manipur, India	775m N 24˚49′26.4′′ E093˚57′52.0′′	Irregular curved	Not distinct	Barrel	Spherical	Not observed
Nostoc muscorum Ag. ex Born. et Flah. BTA27	Rice field, Imphal West, Manipur, India	792m N 24˚50′33.6′′ E093˚56′23.4′′	Densely entangled	Distinct at periphery	Short barrel	Spherical	Oblong
Nostoc parmelioides Kutz. ex Born. et Flah. BTA29	Rice field, Imphal West, Manipur India	792m N 24˚50′33.6′′ E093˚56′23.4′′	Entangled	Distinct at periphery	Subspherical	Spherical	Oval
Nostoc carneum Ag. ex Born. et Flah. BTA38	Rice field, Bishnupur, Manipur, India	776m N 24˚43′15.5′′ E093˚50′27.8′′	Loosely contorted	Not distinct	Oblong cylindrical	Oblong	Spherical
Nostoc commune Vaucher ex Born. et Flah. BTA67	Loktak Lake, Bishnupur, Manipur, India	772m N 24˚30′57.8′′ E093˚47′36.0′′	Flexuous	Lamellated	Barrel	Spherical	Oblong
Nostoc sp. BTA131	Foot hill, Imphal East, Manipur, India	89m N 27˚26′23.6′′ E094˚57′02.3′′	Flexuous and loosely entangled	Diffluent	Partly cylindrical and partly barrel-shaped	Spherical	Oblong
Nostoc muscorum Ag. ex Born. et Flah. BTA950	Loktak Lake, Bishnupur, Manipur, India	765m N 24˚30′33.3′′ E093˚47′10.1′′	Densely entangled	Distinct at periphery	Short barrel	Spherical	Oblong
Nostoc sp. BTA972	Running water stream, Singda, Senapati, Manipur, India	850m N 24˚52′39.6′′ E093˚48′23.4′′	Densely entangled	Distinct at periphery	Short barrel	Spherical	Oblong
Nostoc ellipsosporum (Desm.) Rabenh. ex Born. et Flah. BTA1075	Loktak Lake, Bishnupur, Manipur, India	765m N 24˚30′28.1′′ E093˚47′03.0′′	Flexuous and loosely entangled	Not distinct	Cylindrical	Subspherical	Ellipsoidal

Table-2: Composition of pigments, nitrogenase activity, proteins and carbohydrates of *Nostoc* strains of Manipur, India

Name of the strains	**Chl-a (µgml^{-1})**	**Nitrogenase activity (nmole C_2H_4 µg^{-1} chl-a h^{-1})**	**Carotenoids (µgml^{-1})**	**Total soluble proteins (µgml^{-1})**	**Total carbohydrates (µgml^{-1})**	**PE (µgml^{-1})**	**PC (µgml^{-1})**	**APC (µgml^{-1})**
Nostoc sp. BTA12	5.28±0.01	1.46±0.01	19.90±0.12	42.30±0.03	11.00±0.16	2.56±0.71	77.50±0.74	25.7±0.60
Nostoc muscorum Ag. ex Born. et Flah. BTA27	**10.60±0.03**	0.07±0.02	**30.90±0.12**	75.20±0.21	36.60±0.04	2.52±0.20	79.20±0.20	20.10±0.54
Nostoc parmelioides Kutz. ex Born. et Flah. BTA29	8.97±0.06	1.57±0.01	26.40±0.05	67.00±0.05	24.30±0.04	15.50±0.30	4.64±0.44	15.30±0.73
Nostoc carneum Ag. ex Born. et Flah, BTA38	2.40±0.05	11.30±0.07	19.40±0.24	**77.00±0.16**	24.60±0.04	**20.00±0.49**	82.70±0.35	**58.70±0.39**
Nostoc commune Vaucher ex Born. et Flah. BTA67	8.18±0.05	**24.65±0.11**	2.96±0.05	10.00±0.00	12.24±0.12	5.20±0.94	**88.00±0.25**	40.71±0.72
Nostoc sp. BTA131	3.97±0.02	3.10±0.04	24.68±0.06	43.00±0.01	10.33±0.52	16.10±0.03	5.30±0.01	7.52±0.02
Nostoc muscorum Ag. ex Born. et Flah. BTA950	3.81±0.02	21.25±0.07	4.49±0.90	27.60±0.17	17.66±0.15	13.11±0.89	20.45±0.80	3.80±0.98
Nostoc sp. BTA972	1.22±0.01	5.10±0.02	1.62±0.12	51.33±0.01	6.66±0.57	2.02±0.66	3.98±0.21	5.74±0.94
Nostoc ellipsosporum (Desm.) Rabenh. ex Born. et Flah. BTA1075	5.19±0.04	15.00±0.18	4.18±0.52	67.33±0.04	**43.00±0.01**	11.68±0.12	31.53±0.06	2.44±0.04

All the experiments were performed in triplicate and results were presented as mean±SD.

Table-3: NCBI GenBank accession number of the *Nostoc* strains of Manipur, India

SN	Name of the strains	GenBank accession number
1	*Nostoc* sp. BTA12	KM010236
2	*Nostoc muscorum* BTA27	KM435246
3	*Nostoc parmelioides* BTA29	KM435247
4	*Nostoc carneum* BTA38	KM435249
5	*Nostoc commune* BTA67	KF953518
6	*Nostoc* sp. BTA131	KF953508
7	*Nostoc muscorum* BTA950	KF953521
8	*Nostoc* sp. BTA972	KJ511801
9	*Nostoc ellipsosporum* BTA1075	KJ652545

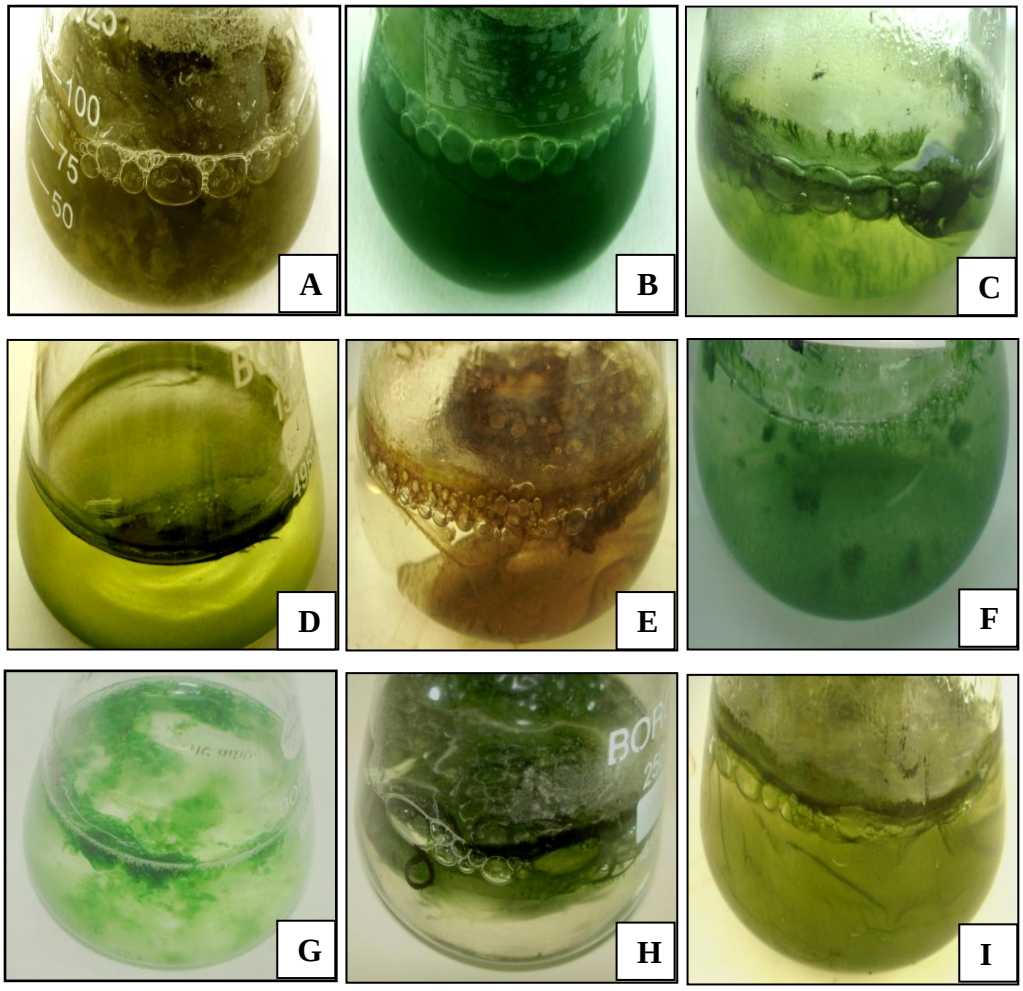

Fig 1. Growth of *Nostoc* strains in conical flasks- A: *Nostoc* sp. BTA12; **B:** *Nostoc muscorum* BTA27; **C:** *Nostoc parmelioides* BTA29; **D:** *Nostoc carneum* BTA38; **E:** *Nostoc* sp. BTA131; **F:** *Nostoc commune* BTA67; **G:** *Nostoc muscorum* BTA950; **H:** *Nostoc* sp. BTA972 and **I:** *Nostoc ellipsosporum* BTA1075

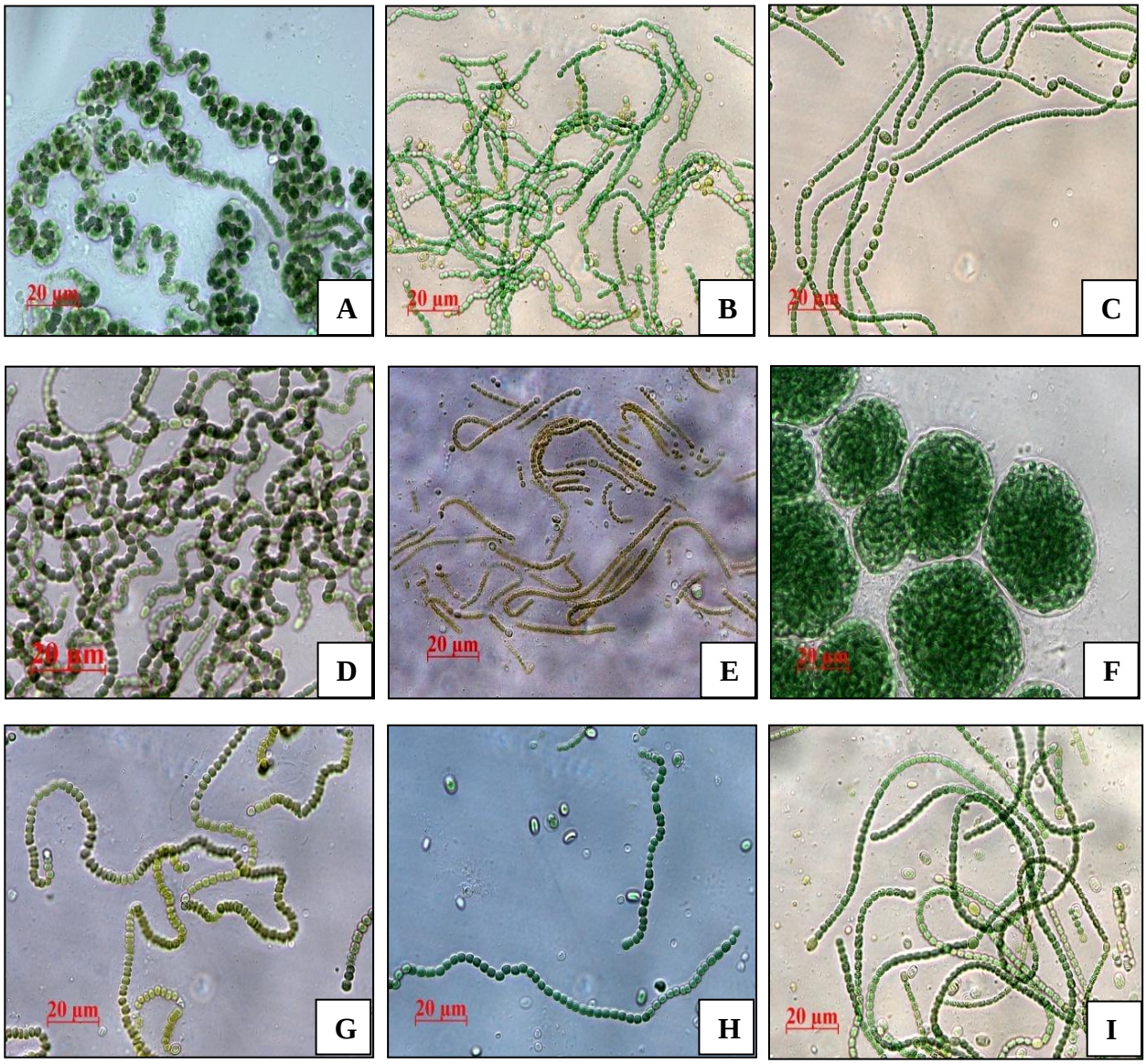

Fig 2. Photomicrographs of *Nostoc* strains- A: *Nostoc* sp. BTA12; **B:** *Nostoc muscorum* BTA27; **C:** *Nostoc parmelioides* BTA29; **D:** *Nostoc carneum* BTA38; **E:** *Nostoc* sp. BTA131; **F:** *Nostoc* commune BTA67; **G:** *Nostoc muscorum* BTA950; **H:** *Nostoc* sp. BTA972 and **I:** *Nostoc ellipsosporum* BTA1075

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Conflict of interest
The authors declare that there is no conflict of interest.

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