Int.J.Curr.Microbiol.App.Sci (2018) *7*(12): 2822-2834

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 12 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.712.321

Exploration and Characterization of Cyanobacteria from Different Ecological Niches of India for Phycobilins Production

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Keywords

Cyanobacteria, Phycobilin, Carotenoid, Chlorophyll, Heterocystous, Non-heterocystous

Article Info

Accepted:
20 November 2018
Available Online:
10 December 2018

A B S T R A C T

Cyanobacteria are photosynthetic microorganisms capable of producing high value pigments like phycoerythrin, phycocyanin, allophycocyanin, chlorophyll and carotenoids. These high value pigments have immense commercial value as they have applications in food, pharmaceutical and cosmetic industries. In the present study, using soil and water samples collected from different ecological niches of India, 10 isolates of heterocystous and 09 isolates of non- heterocystous cyanobacteria were obtained. Morphological identification revealed that the isolates belonged to *Nostoc*, *Anabaena*, *Phormidium*, *Lyngbya*, *Westiellopsis*, *Aphanotheca*, *Oscillatoria*, *Tolypothrix* and *Chroococcus*. When these isolates were screened for phycoerythrin, *Nostoc* sp. BG1 was recorded with highest (870 µg/mL) production. Determination of phycocyanin revealed *Nostoc* sp. RD1 to be the highest (450 µg/mL) producer. *Westiellopsis* sp. BG2 was found to produce maximum (530 µg/mL) of Allophycocyanin. Likewise, *Phormidium* sp. SB6 was recorded as highest Chlorophyll a (270 µg/mL) producer. In case of carotenoids, *Lyngbya* sp. SB2 showed maximum (13 µg/mL) production. These isolates can be potential candidates for high value pigment production and could be exploited for commercial use in future.

Introduction

Cyanobacteria are photosynthetic, gram negative microorganisms capable to fix nitrogen and produce high value products like phycobilins (PBP), carotenoides and polyunsaturated fatty acids which have immense application in pharmaceutical, food, cosmetic industry (Begum *et al.*, 2016). These photosynthetic organisms are present in diverse ecological niches ranging from extreme hot, cold and hypersaline environment, aquatic to terrestrial, ultra-oligotrophic and hypereutrophic (Bhatnagar and Bhatnagar, 2005). Cyanobacteria are morphologically diverse phyla of prokaryotes showing 323 genera with more than 2000 species (Sharma *et al.*, 2014; Nabout *et al.*, 201; Guiry, 2012). Large amount of diversity exists in cyanobacteria comprising unicellular to filaments, branched to unbranched and colonial to complex cell structure. High growth rate and wider adaptability makes cyanobacteria excellent candidate for mass production of various industrially important compounds (Encarnacao *et al.*, 2015). In this

fast growing era, synthetic chemicals have been utilized heavily as colorants in food and cosmetic industries which are carcinogenic and have detrimental effect on health (Amchova *et al.,* 2015). Cyanobacteria is good source of natural colorant known as PBP free from toxic effects and have numerous health benefits. Cyanobacterial pigments are gaining an importance as natural colorant over synthetic colorant in food and biotechnological industries (Dasgupta, 2015). Apart from natural colorants, PBP could be used as fluorescent label in immunoassays and in microscopy for diagnostic and biomedical research for cancer daignostic, due to their spectroscopic and fluorescence properties (CQVB, 1988; Hill, 1988; Soni *et al.,* 2006). Phycocyanin of *Spirulina platensis* as a natural colorant has been used in products such as dairy products and jellies (Santiago−Santos et al., 2004), fermented milk products, ice creams, deserts (Sekar and Chandra-Mohan, 2008).

Many multinational companies have been using colorants made from red algae and cyanobacteria among them Fujifilm corporation with Astalift whitening essence a astaxanthin based product for antiaging, similarly Japanese firm Kose used astaxanthin based product AstaBlanc as anti-wrinkle, C-phycocyanin by Prozyme (PhycoPro^{TM}), C-phycocyanin by Cyanotech (Phycolink^{TM}) and other companies involved are Europa bioproducts Ltd, Sigma Aldrich, Fishcer scientific etc. (Chakdar and Pabbi 2017, Chakdar *et al.,* 2012).

Dianipon Ink Corporation from Japan commercially marketing Linablue, a phycocyanin product as a natural colorant from *Spirulina platensis* (Chakdar *et al.,* 2012). Antioxidant activity of PBP extracted from *Lyngbya* sp. A09DM have been reported (Sonani *et al.,* 2014). PBP are water soluble light harvesting pigment-protein complexes present in cyanobacteria, red algae and cryptophytes (Apt *et al.,* 1995; Glazer and Apell, 1977). Antiallergic and anti-inflammatory activity of cyanobacteria has also been reported by many researchers (Hayashi *et al.,* 1996; Egorova *et al.,* 2005). Phycobilins have been grouped into blue phycocyanin (λ_{max} ~ 610-625 nm, pink phycoerythrin (λ_{max} ~ 490-570 nm) and allophycocyanin (λ_{max} ~ 650-660 nm) (Kuddus *et al.,* 2013; Singh *et al.,* 2015; Manirafash *et al.,* 2016). Phycocyanin producing strain *Arthrospira platensis* (Lee *et al.,* 2016), *Anabaena* (Chakdar *et al.,* 2014), *Nostoc* (Lee *et al.,* 2017) are currently exploited for commercial production. Commercial value of analytical grade phycocyanin purity more than 4.0 can be estimated as USD 15 per mg (Cisneros and Rito-Palomeares, 2004). Despite the diversity among cyanobacteria very few strains have been reported as potential pigment producer. Pigment production from cyanobacteria varies from species to species and mostly depends on environmental factors (Chaneva *et al.,* 2007; Sarda *et al.,* 1999). Present study deals with isolation of cyanobacteria from diverse ecological habitat and their potential for pigments production.

Materials and Methods

Sample collection

Soil and water samples were collected from different ecological niches of India including the districts of Odisha (Kendrapada, Puri, Ganjam), Jammu and Kashmir (Panggong lake, Nubra valley), Uttarakhand (Valley of Flower), Uttar Pradesh (Ballia) and Assam (Guhawati). Details of the samples are presented in table 1. Water and soil samples were stored in autoclaved plastic bottles and brought to the laboratory for isolation and purification of the cyanobacteria. pH and EC of the samples were also measured with standard method.

Isolation, identification and purification

10 g of soil from each sample was taken and processed for serial dilution. 1 ml of aliquots was spread on BG 11 agar plates with nitrogen source for non heterocystous strain and without nitrogen source for heterocystous strain. Waters samples were processed as such in the BG 11 broth. The flasks were incubated at 27 ± 1°C, 50-55 µmol photons m^{-2} s^{-1} light intensity and 16:8 light and dark cycle and axenic cultures were obtained by the procedure given by Stanier *et al.,* (1971). The pure cultures were maintained in BG11 broth in culture room for further experiment.

Morphological identification through microscopy

Microscopic observations of cyanobacterial isolates were taken by bright field with ×40 (Olympus BX41, Japan) microscope and identification was done by taxonomic keys provided by Desikachary (1959). Among the morphological characteristics filament color, heterocyst formation and position on filament, trchichome end etc. were documented.

Extraction and estimation of PBP

Phycobililn pigments (PC, PE and APC) were extracted according to the method given by Bennet and Bogorad (1973). 10 ml of algal culture was centrifuged at 7000 rpm for 10 minutes. The pellets were suspended in 5 ml of 0.1 M phosphate buffer. The pigments were extracted by repeated freezing (-20 °C) and thawing at room temperature. The supernatants were collected and absorbance was measured at 562 nm, 561 nm and 652 nm for phycocyanin, allophycocyanin and phycoerythrin respectively using UV-visible spectrophotometer (Shimadzu 1700). Phycocayninan, phycoerythrin and allophycicyanin was estimated by following equations and expressed in µg/mL.

$PC = [A_{615}-(0.474 \times A_{652})]/5.34$
$APC = [A_{652}-(0.208 \times A_{615})]/5.09$
$PE = [A_{562}-(2.41 \times PC)-(0.849 \times APC)]/9.62$

Estimation of total carotenoides

Total carotenoids were determined by the method given by Jensen (1978). 10 ml of homogeneized algal suspension was centrifuged at 6500 rpm for 10 minutes. Supernatants were discarded and 3 ml 85% acetone was added in to the pellets. The contents then subjected to repeated freezing and thawing until the pellets become colorless. Final volume of the content was made up to 10 ml with 85% acetone and absorbance was measured at 450 nm using 85% acetone as blank. Carotenoid concentration was estimated as per the equation given below

Carotenoids (µg/mL) = $[A_{461}-(0.046 \times A_{664})] \times 4$

Estimation of chlorophyll

Chlorophyll a and b was estimated by the method described by Mckinney (1941). 10 ml of algal culture was centrifuged at 7000 rpm for 10 minutes. The pellets were suspended in 10 ml of 90% methanol in test tubes and covered with aluminium foil and placed in water bath at 60°C for 30 minute. The absorbance of the supernatant was measured at 650 and 665 nm.

Chlorophyll a= $2.55 \times 10^{-2} A_{650} + 0.4 \times 10^{-2} A_{665}$

Results and Discussion

Soil and water samples collected from different ecological niches of India showed diversity among cyanobacterial genera. Table 2 presents different cyanobacteria isolated from respective ecological niches. 50% of the cyanobacterial isolates were heterocystous

while the rest were non-heterocystous. Among heterocystous cyanobacteria, *Anabaena* sp., *Nostoc* sp., *Westielopsis* sp., *Tolypothrix* sp., *Cylindrospermum* sp. were isolated (Plate 1). While non heterocystous cyanobacteria included *Lyngbya* sp., *Phormidium* sp., *Oscillatoria* sp., *Aphanothece* sp., and *Chroococcus* sp. (Plate 2). Morphological characteristics of the isolates are presented in Table 2. All the pure cultures were accessioned and submitted to National Agriculturally Important Microbial Culture Collection (NAIMCC), Mau, Uttar Pradesh, India (Table 3). These isolates were investigated for pigment production. Among all the isolates *Nostoc* sp. BG1 was recorded with highest (870 µg/mL) phycoerythrin while lowest (21 µg/mL) was recorded in *Chroococcus* sp. SB 4.

Determination of phycocyanin revealed *Nostoc* sp. RD1 to be the highest (450 µg/mL) producer however, lowest was recorded (10 µg/mL) in *Chroococcus* sp. HC1. *Westeillopsis* sp. BG2 was found to produce maximum (530 µg/mL) of Allophycocyanin. Tiwari *et al.,* (2015) also obtained similar kind of results and found *Phormidium arthurensis* BTA042 and *Nostoc muscorum* BTA 087 were rich in phycoerythrin. Simeunovic *et al.,* (2012) studied phycobilin pigments of filamentous cyanobacteria and found *Anabaena* strain C2 could produce 22.62 µg/ml of phycocyanin and *Anabaena* LC1B producing 24.87 µg/ml of phycoerythrin. PC concentration of *Synechococcus* sp. and *Nostoc spumigena* was reported as 0.79 µg/cm^{-3} and 20.22 µg/cm^{-3} respectively by Sasim *et al.*, (2014).

Horvath *et al.,* (2013) reported higher extraction efficiency of phycocyanin with freezing and thawing along with sonication in *Cylindrospermopsis raciborskii*. Khatoon *et al.,* (2018) reported highest phycobilin yield of 237 mg/g in *Pseudoanabaena mucicola* when cultivated in waste water and white light. Hemlata and Fatma (2009) extracted phycobilin from *Anabaena* NCCU-9 and reported 91.54 mg/g dry weight.

Khazi *et al.,* (2018) investigated cyanobacteria for phycobilin production under different nitrogen sources and found *Phormidium* sp. and *Pseudoscillatoria* sp could yield phycobilin pigments of 19.38% and 19.99 % of dry weight in presence of ammonium chloride, however, sodium nitrate was found best for *Arthrospira platensis* which could produce 22.27% of dry weight. Among cyanobacterial pigments allophycocyanin is naturally found in lower amount approximately 10% of the total cell biomass than phycocyanin and phycoerythrin.

Phormidium sp. SB6 was recorded as highest Chlorophyll a (270 µg /mL) producer and lowest was recorded in *Tolypothrix* sp. RC1 (23 µg/mL). In case of carotenoids *Phormidium* sp. SB6 showed maximum (13.3 µg/mL) production (Fig. 1). Tiwari *et al.,* (2015) recorded carotenoid yield of 80.3 µg/mL in *Nostoc muscorum* BTA087 isolated from Manipur region of India.

Shukla and Kashyap (2003) investigated Antartic and tropical cyanobacterial strains for carotenoides production and reported that *Nostoc* sp. could produce 36.4 µg/mg dry weight of carotenoid and *Phormidium* sp. resulted in 22.6 µg/mg dry weight. Cyanobacteria including *Phormidium laminosum* (Fresendo *et al.,* 1991), *Synechococcus* sp. PCC 7942 (Linden *et al.,* 1990) and *Nostoc commune* (Olie and Potts 1986) have been reported to be rich source of carotenoids. Various environmental factors are also responsible for cyanobacterial pigment production and composition. Amount of phycobilins production depends on light, temperature, pH and nutrient source (Hemlata and Fatma 2009) (Fig. 2 and 3).

Table.1 Details of geographical locations of samples collection sites in India with physicochemical characteristics of soil

Locations	Habitat	Latitude and Longitude (In degrees)	EC (µS/cm)	pH
Panggong lake, Leh (Jammu and Kashmir)	Water	33.75 N, 78.66E	216	7.8
Phephna, Ballia (Uttar Pradesh)	Arsenic contaminated soil	25.77 N; 84.03 E	107.8	6.7
Ekauna, Ballia (Uttar pradesh)	Arsenic contaminated Soil	25.71 N, 84.27 E	214	6.9
Chilka lake (Odisha)	Water	19.84 N, 85.47 E	840	6.6
Bhitarkanika (Odisha)	Water	20.71 N, 86.82 E	251	7.7
Brahamagiri (Odisha)	Soil	19.78 N, 85.61 E	270	7.9
Atri hot spring (Odisha)	Hot water	20.15 N, 85.30 E	233	7.4
Puri (Odisha)	Sand	85.83 N, 19.81 E	1200	8.0
Guwahati (Assam)	Soil	26.14 N, 91.73 E	210	5.5
Valley of Flower (Uttarakhand)	Soil	30.72 N and 79.60 E	57.9	9.5

Table.2 Morphological/taxonomical characteristics of cyanobacteria isolated from different ecological niches of India

Cyanobacterial strain	Habit	Taxonomical description		
		Heterocyst	**Cell/filament color**	**Trichome ends**
***Nostoc* sp. HC2**	Valley of Flower, Uttarakhand	Spherical,	Olive green, entatangled	Spherical
***Nostoc sp.* BG1**	Brahamagiri. Odisha	Oblong	Blue green	Elongated
***Anabaena* sp. BG1**	Brahamagiri, Odisha	Oval, terminal, intercalary	Dark blue green	Elongated
***Lyngbya* sp. SB2**	Atri, Odisha	Absent	Blue green	Flat
***Anabaena* sp. SB1**	Bhitarkanika, Odisha	Spherical, terminal, intercalary	Yellowish green	Round
***Oscillatoria* sp. SK2**	Leh, Jammu and Kashmir	Absent	Olive green	Flat, conical
***Westeillopsis* sp. BG2**	Brahamagiri, Odisha	Oblong intercalary	Dark green, Branched	Round, conical
***Chroococus* sp. HC1**	Valley of flower, Uttarakha	Absent	Group of 4 cells	-
***Westeillopsis* sp. SB10**	Puri, Odisha	Spherical, Intercalary	Blue green, branched	Round
***Chroococcus* sp. SB4**	Atri, Odisha	Absent	Group of 8-16 cells	-
***Aphanothece* sp. SB3**	Guhawati, Assam	Absent	Barrel shape	-
***Nostoc* sp. SK1**	Leh, Jammu and Kashmir	Spherical, terminal, intercalary	Dark green	Round
***Tolypothrix* sp. RC1**	Ballia, Uttar Pradesh	Square, Terminal, intercalary	Yellowish green	Round
***Nostoc* sp. RD1**	Ballia Uttar Pradesh	Barrel, intercalary	Dark green	Oval
***Lyngbya* sp. Rh1**	Ballia Uttar Pradesh	Absent	Blue green	Conical
***Phormidium* sp. RC2**	Ballia Uttar Pradesh	Absent	Dark green	-
***Phormidium* sp. SB9**	Chilka, Odisha	Absent	Dark green	-
***Phormidium* sp. SB6**	Leh, Jammu and Kashmir	Absent	Dark green	-

Table.3 Cyanobacterial accessions submitted to NAIMCC

Sr. No.	Strain name	NAIMCC accession No.
1	*Nostoc* sp. HC2	NAIMCC--C-C-00239
2	*Nostoc sp.* BG1	-
3	*Anabaena* sp. BG1	NAIMCC-C-C-00236
4	*Lyngbya* sp. SB2	NAIMCC-C-C-00241
5	*Anabaena* sp. SB1	NAIMCC-C-C-00234
6	*Oscillatoria* sp. SK2	NAIMCC-C-C-00244
7	*Westeillopsis* sp. BG2	NAIMCC-C-C-00237
8	*Chroococus* sp. HC1	NAIMCC-C-C-00238
9	*Westeillopsis* sp. SB10	NAIMCC-C-C-00235
10	*Chroococcus* sp. SB4	NAIMCC-C-C- 00242
11	*Aphanothece* sp. SB3	NAIMCC-C-C-00243
12	*Nostoc* sp. SK1	-
13	*Tolypothrix* sp. RC1	NAIMCC-C-C-00229
14	*Nostoc* sp. RD1	NAIMCC-C-C-00231
15	*Lyngbya* sp. Rh1	NAIMCC-C-C-00232
16	*Phormidium* sp. RC2	NAIMCC-C-C-00230
17	*Phormidium* sp. SB9	NAIMCC-C-C-00233
18	*Phormidium* sp. SB6	NAIMCC-C-C-00240

Fig.1 Estimation of total phycobilin from cyanobacteria isolated from different ecological niches of India

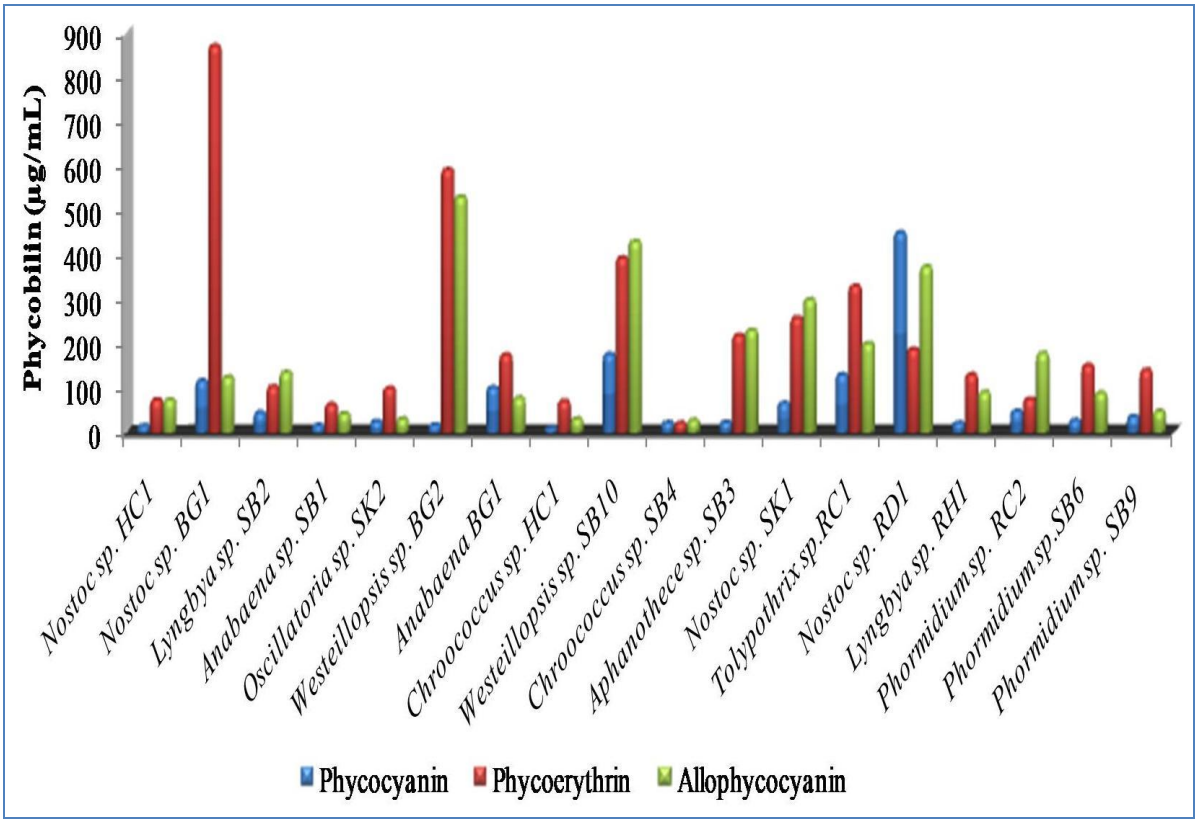


Fig.2 Estimation of chlorophyll from cyanobacteria isolated from different ecological niches of India

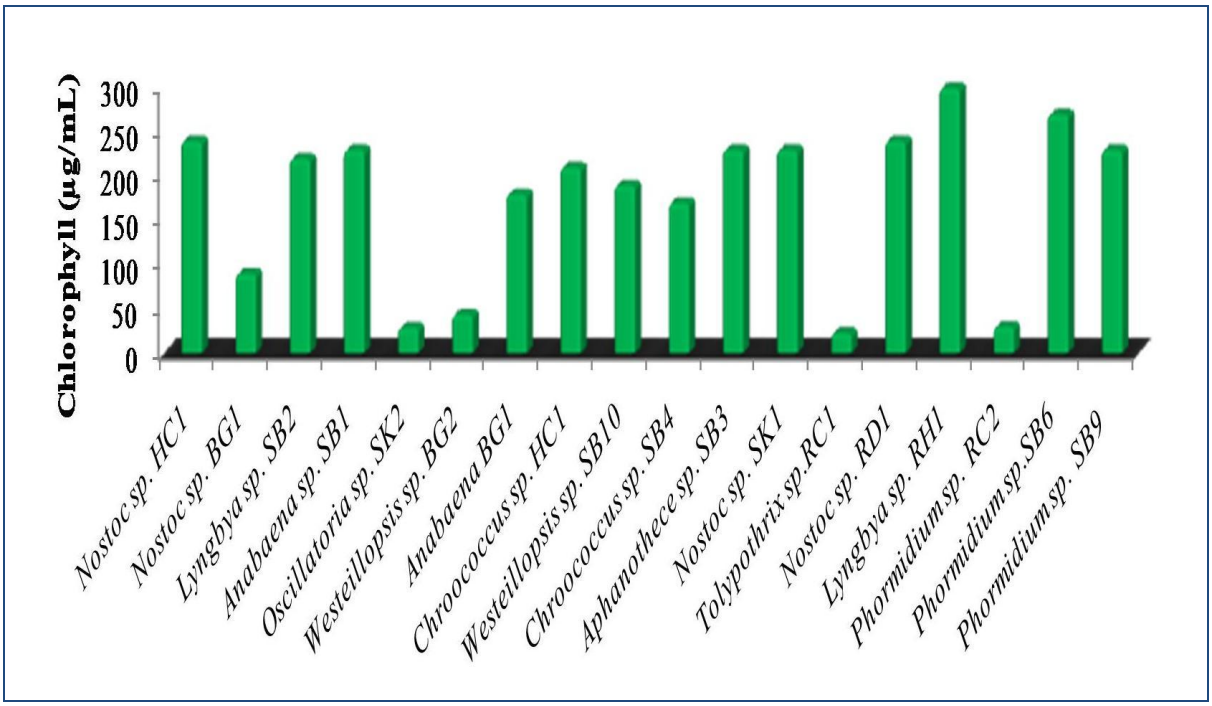


Fig.3 Estimation of total carotenoids from cyanobacteria isolated from different ecological niches of India

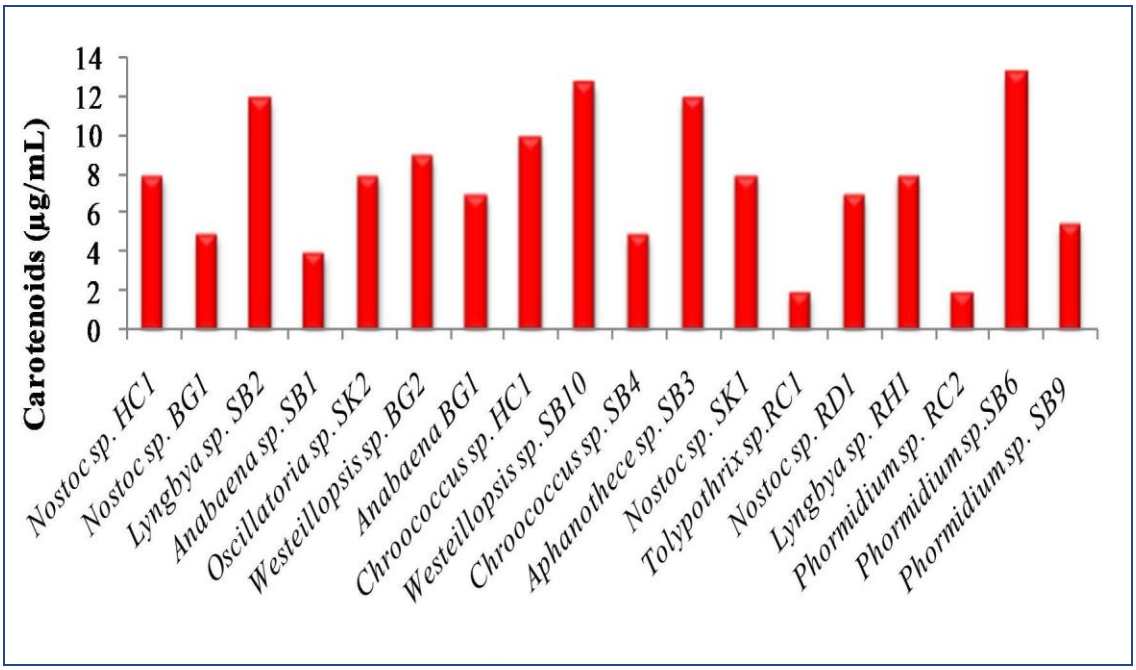


Plate.1 Microphotographs of heterocyst forming cyanobacteria isolated from different ecological niches of India; 1: *Anabaena* sp. SB1; 2: *Anabaena* sp. BG1; 3: *Nostoc sp*. RD1; 4: *Nostoc sp*. SK1; 5: *Nostoc sp*. HC2; 6: *Nostoc sp*. BG1; 7: *Westeillopsis* sp. BG2; 8: *Westeillopsis* sp. SB10; *Tolypothrix* sp. RC1

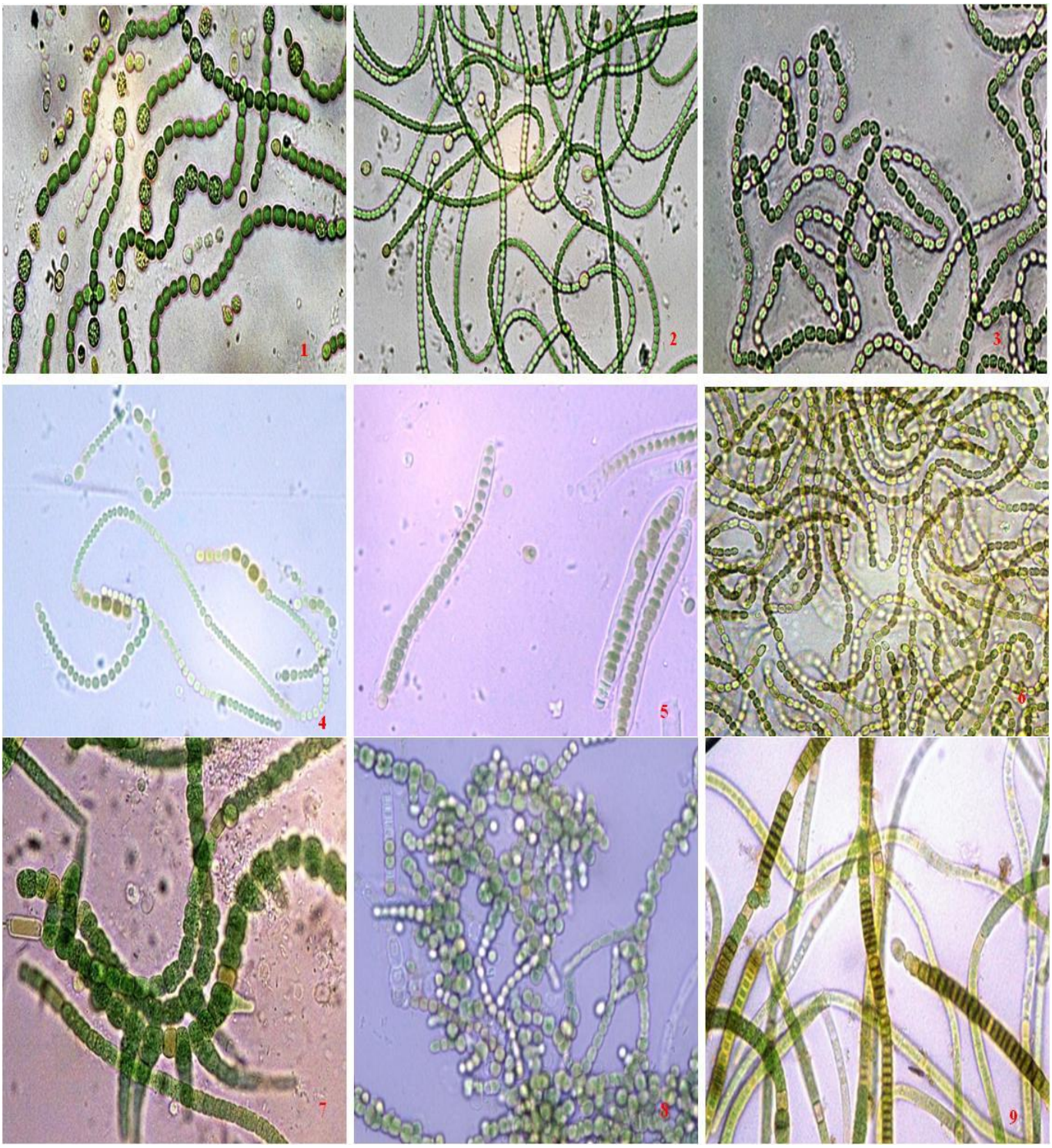
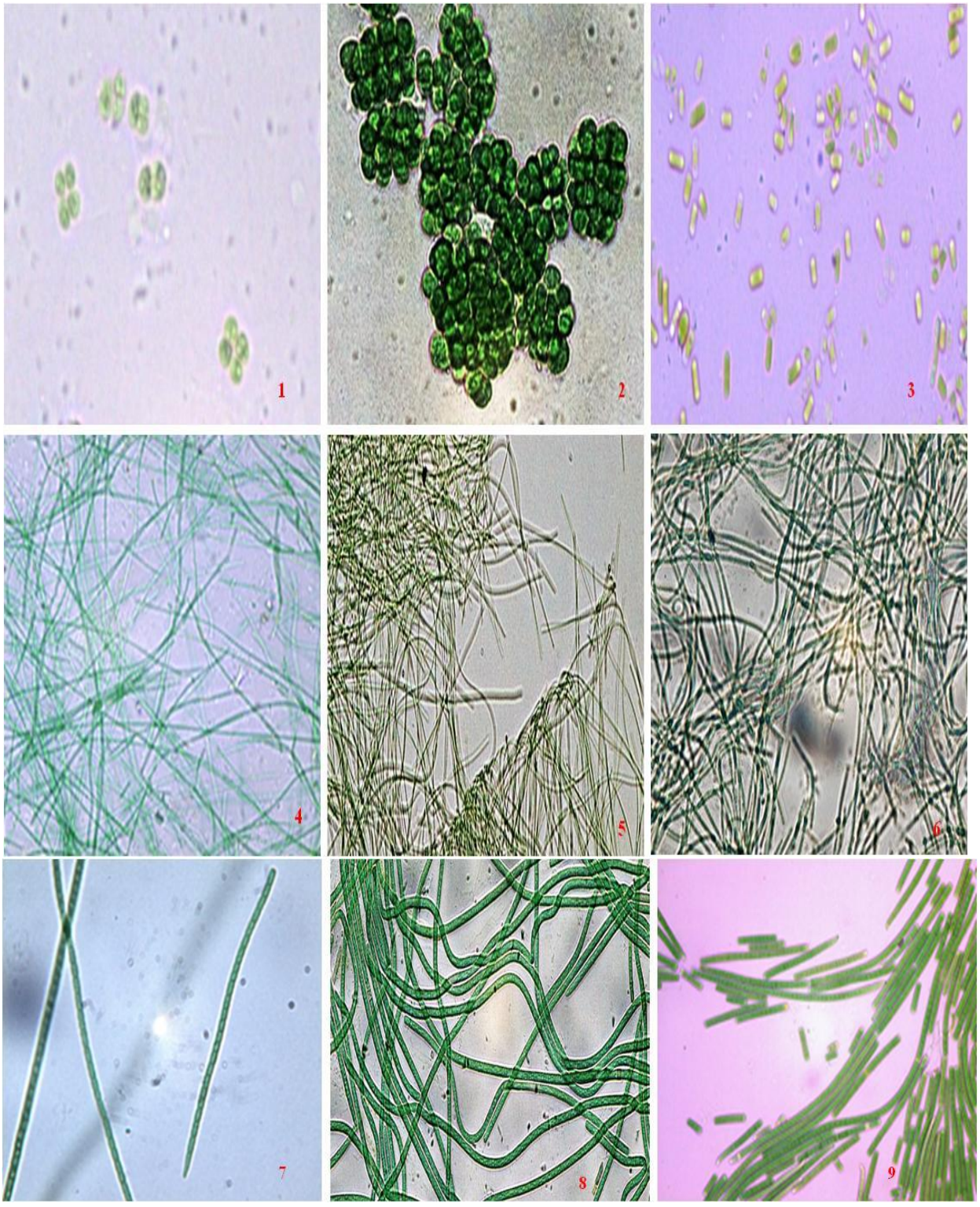


Plate.2 Microphotographs of non-heterocyst forming cyanobacteria isolated from different ecological niches of India; 1: *Chroococcus* sp. HC1; 2: *Chroococcus* sp.SB4; 3: Aphanothece sp.; SB3; 4; *Phormidium* sp. SB9; 5: *Phormidium* sp.; 6: *Phormidium* sp. RC2; 7: *Lyngbya* sp.; 8: *Lyngbya* sp. SB2; 9: *Oscillatoria* sp



Findings of the study could be useful for further exploitation of these pigment producing strains for commercial applications, however, further investigations are required for optimised conditions for cyaonbacterial strains and improved yield of pigments including phycobilins.

In conclusion, the pycobiliproteins have great potential and large number of applications. There is profound interest in the mass production of these pigments due to their food and pharmaceutical application such as natural colorant in foods, fermented dairy products, beverages etc and biomedical research. The results of the present study indicated that some of the isolates from diverse ecological niches of India have greater potential for phycobiliprotein production. Strains such as *Nostoc* sp SB7, RD1 and *Westeillopsis* sp. BG2, are potential candidates for pigment producers since they showed the highest content of phycoerythrin, phycocyanin and allophycocyanin among the studied strains. Still strenuous girth of research is required for high pigment production, process optimization and exploration of new ecological niches for potential pigment producing cyanobacterial strains.

Acknowledgement

Authors are thankful to Director ICAR-NBAIM, for providing financial support for the project. Authors also would like to thanks Dr. Sushil K Sharma, Dr. Renu, Dr. Sunil Kumar for providing soil and water samples.

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How to cite this article:

Samadhan Yuvraj Bagul, Sneha Tripathi, Hillol Chakdar, N. Karthikeyan, K. Pandiyan, Arjun Singh and M. Kumar. 2018. Exploration and Characterization of Cyanobacteria from Different Ecological Niches of India for Phycobilins Production. *Int.J.Curr.Microbiol.App.Sci.* 7(12): 2822-2834. doi: https://doi.org/10.20546/ijcmas.2018.712.321