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Screening and Molecular Characterization of High Phycocyanin Producing Selected Freshwater Cyanobacteria for Commercial Application

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ABSTRACT

Phycobiliproteins [PBPs] is a potent substance among others with the ability to replace synthetic, toxic and are carcinogenic chemical products. In the present study, the cyanobacteria procured from freshwater sites were isolated in laboratory-controlled conditions. Four out of 16 high phycocyanin-producing cyanobacterial isolates were selected and characterized based on a specific growth rate of chlorophyll *a*, phycocyanin concentration and the high irradiance tolerance in response of hill activity was also determined. It was found that the NSSP6 showed the highest tolerance and survival percentage in response to hill activity (DCPIP reduction) and 50% growth inhibition (I_{50}) which was followed by isolates 5MNS2014, 13MNS2014 and NSSP33. The molecular identification of selected cyanobacterial isolates was also made using a molecular method like DNA extraction. The extracted DNA was amplified by using 16S r RNA region-specific primers using PCR. Phylogenetic identification and analysis of species was made by evaluating obtained sequence using the maximum likelihood method based on the Tamura-Nei model. Evolutionary analyses were conducted in molecular evolutionary genetics analysis (MEGA) software version X. The high light intensity tolerant and high phycocyanin-producing cyanobacterial isolate suggested that they had enormous potential to produce phycocyanin as producers in commercial industries.

Key words : Cyanobacteria, molecular identification, hill activity, percentage of survival, phycocyanin

INTRODUCTION

Cyanobacteria are a group of photosynthetic gram-negative bacteria. The morphology of cyanobacteria can be colonial or filamentous, a complex form (Karan *et al.,* 2017). Three main light-harvesting systems are involved in the formation of the photosynthetic bacteria : two photo-systems and phycobilisomes assembled on the photosynthetic thylakoid membrane. Different potential photosynthetic pigments are present in cyanobacteria, such as chlorophyll-*a*, phycobiliproteins, β-carotene and xanthophylls, etc. (Pagels *et al.,* 2019; Kannujiya *et al.,* 2021). Certain cyanobacteria are classified based on their morphological features. Morphological characters are possibly altered due to changes in environmental and physico-chemical conditions. Selective culture conditions can reserve the bio-diversity in culture. Conventional taxonomic classification of filamentous and colonial cyanobacteria is based on morphology, but this classification is often revised through phylogenetic analyses based on molecular sequence data (Komárek *et al.,* 2014). 16S r-RNA gene is a marker gene for dual purpose that is identifications of micro-organism and to study evolutionary relationship between them. Many evolutionary conserved sequences are present in the 16S r-RNA gene, but some species-specific variable sequences also exist in the 16S r-RNA gene. Polymerase chain reaction amplifies the species-specific variable region of 16S r-RNA gene of strain for identification at species level. Previously reported literature states that many cyanobacterial strains were molecularly identified using 16S r-RNA as markers such as Cylindrospermum sp. and 45 taxa of Cylindrospermum identified using 16S r-RNA (Johansen *et al.,* 2014). Karan *et al.* (2017) also used the cyanobacterial specific primer to amplify 16S r RNA and phycocyanin gene using PCR to identify seven cyanobacterial isolates and their evolutionary relationship sequence analysis computer software which isolated

from the freshwater site throughout Tokat province of Turkey. The molecular identification of cyanobacterial isolate would be required for aqurate identification. In the present study, based on the study of fresh water cyanobacteria for commercial application, therefore, it is important for aqurate identification to avoid selection of any harmful protein producing cyanobacteria. This study was aimed at isolation, purification, and screening of potent phycobiliprotein producing cyanobacterial isolates and their molecular identification.

MATERIALS AND METHODS

The sample for the study belonged to Prof. Namita Singh personal culture collection, Microbial Biotechnology Laboratory, Department of Bio and Nano Technology, Guru Jambheshwer University of Science and Technology, Hisar (Haryana), India from different freshwater sites of north India (Table 1).

Isolation and purification of cyanobacteria was done using a spread plate and streak plate method at 27±2°C and 16 : 8 hrs dark and light regime (Karan *et al.*, 2017). The isolated strains were transferred to a new plate. After several transfers, strains were inoculated into the appropriate nutritional liquid medium BG-11 (Hi-media Labs, Mumbai, India) by using the line incubation method. These steps were repeated several times until a single pure strain was isolated.

Initially more than 40 samples of cyanobacteria were analyzed for potentially pigment production and better growth for selecting 16 pure strains (Table 1) for screening of prebable pigment-producing isolates. Screening of the potent isolates was done based on growth in terms of chlorophyll-*a* content, protein concentration and concentration of phycocyanin.

To estimate specific growth rates, macromolecules concentration was elevated like that of protein, chlorophyll-*a,* and phycocyanin. A fast rise in the growth and biomass during the exponential phase was considered for calculating the specific growth rate as mentioned below :

$$\text{Specific growth rate } (\mu) = \frac{3.32 \times (\log N_2 - \log N_1}{t_2 - t_1}$$

Here, N_1 = Initial concentration of Chl *a* or phycocyanin concentration at time T_1; N_2 = Final concentration of Chl *a* or phycocyanin concentration at time T_2.

A plot of V_0/V of 2 was used to calculate the 50% growth inhibitory light intensity (I_{50}).

Where, V_0 = value of the specific growth rate in terms of phycocyanin concentration at control condition and V = specific growth rate in terms of phycocyanin concentration at different light intensity.

The extraction of chlorophyll-*a* of each sample was done following modified Lee *et al.* (2021). The absorbance of the extract was measured at 650 and 665 nm in UV-V is spectrophotometer (Agilent technologies CARY100) using 95% methanol as blank. Chlorophyll estimation was done using the following formula :

Table 1. List of cyanobacterial samples with geographical location

S. No.	Samples	Geographical area	Coordinates
1.	NSSP6	Out door pond after summer season, Israel	31.779°N35.1923°E
2.	NS07	Out door pond, Isreal	31.00°N35.00°E
3.	NSSP12	Bhoda, Fatehbad (Haryana)	29.51°N75.45°E
4.	NS32	Dr. Namita Singh personal laboratory (Culture collection)	29.10°N75.45°E
5.	NSSP33	Dr. Namita Singh personal laboratory (Culture collection)	29.17°N75.72°E
6.	1MNS2014	Mangali, Hisar (Haryana)	29.04°N75.73° E
7.	2MNS2014	Jalmahal, Jaipur (Rajasthan)	26.95°N75.84°E
8.	5MNS 2014	Jalmahal, Jaipur (Rajasthan)	26.96°N75.85°E
9.	9MNS2014	Sorkhi (Haryana)	29.05°N76.11° E
10.	10MNS2014	Dadi Gori temple, Hisar (Haryana)	29.04°N75°54'39"E
11.	12MNS2014	Amer, Jaipur (Rajasthan)	26.98°N75.86° E
12.	13MNS2014	Sambhar lake, Jaipur (Rajasthan)	26.97°N75.08° E
13.	14MNS2014	TNAU, Tamil Nadu, South India (Culture collection)	11.015°N76.93° E
14.	15MNS2014	TNAU, Tamil Nadu, South India (Culture collection)	11.01° N76.93° E
15.	16MNS2014	IARI, New Delhi (BGA culture collection)	28.63°N77.15° E
16.	17MNS2014	IARI, New Delhi (BGA culture collection)	28.63°N77.15° E

Chlorophyll-*a* (mg/ml) = $2.55 \times 10^{-2}A_{650} + 0.4 \times 10^{-2}A_{665}$

Here, A_{650} = Absorbance at 650 nm; A_{665} = Absorbance at 665 nm

The phycobiliproteins were extracted from the biomass of cyanobacterial isolates using 0.05M phosphate buffer (pH–6.8) proceeded by freezing-thawing method (-20°C and 4°C) followed by centrifugation. The phosphate extract was spectrophotometrically analyzed using the method described by Zahra *et al.* (2018). The content of phycocyanin was calculated as :

$$\text{Phycocyanin (PC; mg/ml)} = \frac{A_{620} - 0.74 \times A_{652}}{7.38}$$

Here, A_{620} = the absorbance of extract at 620 nm; A_{652} = the absorbance of extract at 652 nm.

Protein content in the isolates was estimated by modified Lowry's method. Hill reaction activity was measured in terms of photo-reduction of DCPIP (50 µM). Firstly, sphaeroplasts of cyanobacteria cells were prepared by using lysozyme (0.5 mg/ml). Then, sphaeroplasts of cyanobacterial cells were suspended in HEPES buffer (10 mM, pH 7.0) containing DCPIP (50 µM) and incubated in light. Samples were withdrawn at 15 min intervals. The decrease in colour of the dye was measured at 595 nm.

Morphological characters were observed under the light microscope (Leica Microsystems Ltd., Germany). All the experiments were performed in triplicate, and the results were expressed as means (±) (n = 3) plus the standard deviation of the means. Statistical analysis was performed using Microsoft Excel.

Genomic DNA was isolated as per the standred method with slight modification. The size of the extracted DNA was determined by using 0.8% agarose gel electrophoresis. The DNA library preparation and genomic DNA sequencing were performed as mentioned by Mironov *et al.* (2016). Extracted genomic DNAs was amplified using universal primers (5MNS2014, NSSP33 and NSSP6) having sequences for forward primer as 536F : 5'-GTGCCAGCA GCCGCGGTRATA-3' and reverse primer as 1488R : 5' -CGGTTACCTTGTTACGA CTTCACC-3' and a different set of primer was used for cyanobacterial isolate (13MNS2014) which had a sequence for forward primer as CY106F 5' -CGGACGGGTGAGTAACGCGTGA-3' and for reverse primer CYA781R 5' -GACTACTGGGGT ATCTAATCCCATT-3'. Taq DNA polymerase kit (Thermo Fisher Scientific, USA) was used for PCR amplification. PCR reaction mixture was prepared in 0.2 ml sterile PCR tube by addition of following chemicals : 1.25 µl 10X taq DNA polymerase assay buffer; 0.5 µl $MgCl_2$ (3.2 mM); 0.5 µl dNTPs (2.5 mM); 1.0 µl Template DNA (100 ng/µl); 0.1 µlTaq DNA polymerase enzyme (3 U/µl); 1.0 µl Forward primer (10 pM/µl); 1.0 µl Reverse primer (10 pM/µl). The total mixture volume was made up to 12.5 µl by adding sterile double distill water. Template DNA amplification was done using a thermal cycler (Biotron/Thermo Fisher Scientific, USA) with following step for 35 cycle in given condition : initial denaturation at 94°C for 3 min; denaturation at 94°C for 1 min; annealing at 58°C for 1 min; extension at 72°C for 2 min. Eventually, a final extension at 72°C for 7 min was applied. PCR products were subjected to electrophoresis inside 0.8% agarose gel by applying 100 V current for 45 min. They were further stained by ethidium bromide and analyzed under UV trans-illuminator cabin (Karan *et al.*, 2017).

The amplified PCR product of 16s rRNA was sequenced and edited by Sanger di-deoxy method (Genosys Informatics, New Delhi) using ABI 3130 Genetic Analyzer A (Thermo Fisher Scientific, U. S. A). The data analysis was performed using Seq Scape_ v 5.2 with Big Dye terminator version 3.1cycle sequencing kit (Thermo-fisher scientific U. S. A.).

RESULTS AND DISCUSSION

The criterion for selection of cyanobacterial isolate was the high phycocyanin concentration for the targeted product. Specific growth rate pattern in terms of chlorophyll-*a*, phycocyanin and protein content of cyanobacteria under controlled condition has been shown in Fig. 1.

Isolate NSSP6 showed significantly high growth rate in the term of chlorophyll-*a*, phycocyanin and protein content and this was followed by 5MNS2014, 13MNS2014 and NSSP33. On the basis of specific growth rate measurements (µ/h), cyanobacteria isolates NSSP6, NSSP33, 13MNS2014 and 5MNS2014 were selected because of their high growth

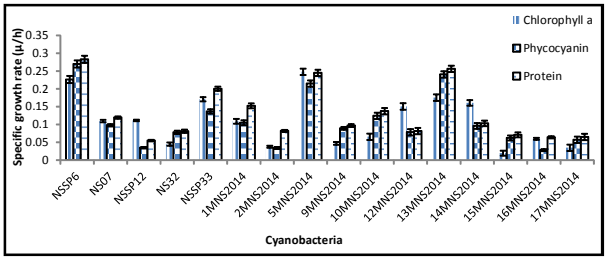

Fig. 1. Specific growth rate (µ/h) in terms of chlorophyll *a*, phycocyanin and protein content in different isolates of cyanobacteria. Data show mean values as an average of triplicate determination. Error bars with 5% values.

rates in comparison to other isolates. The results indicated that the specific growth of selected isolates was approximately two times higher than the other isolates due to their high efficient photo-system and better growth which was indicated by high chlorophyll-*a*, phycocyanin and protein content. This finding was in accordance with Khatoon *et al.* (2018) who reported that the growth of cyanobacteria was directly proportional to the concentration of chlorophyll-a, phycobili proteins and light until a saturation level.

Four potential cyanobacterial isolates (NSSP6, 5MNS2014, 13MNS2014 and NSSP33) were incubated in liquid medium and at a different range of light irradiance (1000 to 4000 Lux) during the measurement effect of light intensity on the survival of cyanobacterial cells. The results showed that light depended on inhibition of growth rate in term of phycocyanin in all potential cyanobacterial isolates (Fig. 2A, B).

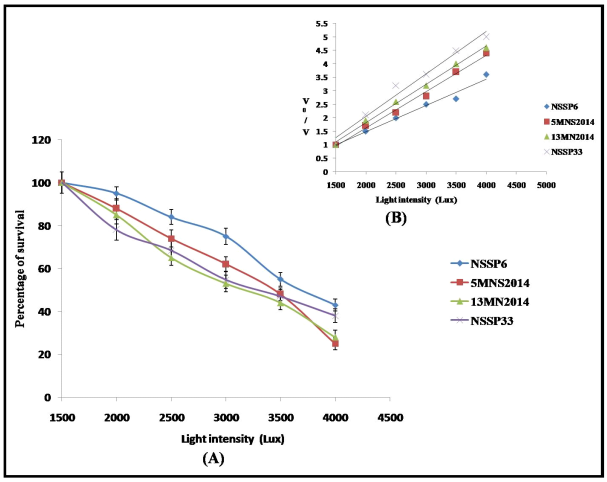

Fig. 2 (A) shows the percentage of survival of selected cyanobacterial isolates against the different intensity of light; (B) Plot of V_0/V of 2 for data derived from figure (A) indicating I_{50} intensity of light for the all selected cyanobacterial isolates cells.

The specific growth rate of isolate NSSP 6 was higher than the other isolates in high light intensity tolerance. A plot of V_0/V of 2 (Fig. 2B) indicated that the intensity of light required for the 50% growth inhibition (I_{50}) of the selected cyanobacterial isolates NSSP6, 5MNS2014, 13MNS2014 and NSSP33 was 2900, 2700, 2600 and 2400 lux, respectively. Light, played an important role in growth and accumulation of pigments in cyanobacterium. Cyanobacteria had the ability to maintain the amount of their pigments. The change in the intensity of light exposure upon cyanobacterial cells, its dramatically change in its pigmentation composition and growth rate were to optimize their light-harvesting mechanism (Ojit *et al.*, 2015).

Sphaeroplasts of four potential cyanobacterial isolates were incubated at different range of light irradiance (1000 to 3000 lux) during the measurement of Hill activity as DCPIP reduction. The results showed (Fig. 3) that the DCPIP reduction (Hill activity) in the potential isolates NSSP6, 5MNS2014, 13MNS2014 and NSSP33 reached a maximum 0.072, 0.063, 0.058 and 0.048 nmol/mg, respectively, at 2500 lux light intensity and were followed by a sharp decline in activity of DCPIP with further increase in light intensity. According to Ojit *et al.* (2015), availability of increase in light decreased the concentration of chlorophyll-*a* and phycocyanin. Due to the high efficient photosystem of cyanobacteria, it was able to perform better under different light stress conditions and showed better growth in the low temperature and high light intensity during the winter session as a better defence mechanism against oxidative stress. Under low light harvesting antenna complex pigment increased due to high concentration of

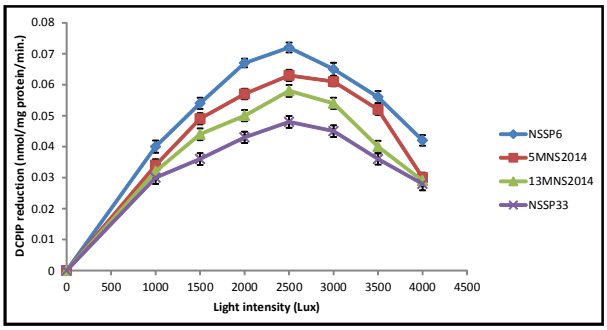

Fig. 3. Rate of DCPIP reduction (Hill activity) in the potential cyanobacterial isolates incubated under different light intensities (1000-4000 Lux).

phycocyanin indicating good option for dessert, semi-arid region where day light flucation was very high. These isolates can be commercially exploited to produce pigments that already indicate high Chl-*a* and phycobiliproteins concentration.

The characterization of cyanobacterial isolates was done manually with the help of a Desikacharya based on shape, morphology, types of filaments, and branching pattern (Fig. 4). Respective physical features were observed with the help of compound microscope and found that all selected isolates belonged to order *Oscillatoriales* with filamentous present in colonies. The shape of cells was mostly found to be sub-spherical or barrel-shaped, not elongated to the ends (Karan *et al.*, 2017).

DNA was isolated with slight modifications as previously discussed (Fig. 5). This was followed by PCR amplification of extracted genomic DNA using cyanobacterial specific universal primer 536 F' and 1488R' for isolates 5MNS2014, NSSP33 and NSSP06; CYA106F' and CYA781R' for 13MNS2014 isolate. Size of PCR product obtained from primers 536 F' and 1488R' was approx. 1500 bp and CYA106F' and CYA781R' was approx. 1000 bp (Fig. 5.2).

Amplified PCR products were subjected for sequencing and the nucleotide sequences were submitted to Gen Bank (*https://www.ncbi.nlm.nih.gov/genbank/*). The 16S r-RNA sequences of isolates were retrieved from NCBI using BLASTn (Sinetova *et al.,* 2017; Tiwari *et al.,* 2019) and then submitted to Gene Bank specific accession number. The gene accession numbers of selected potent cyanobacterial isolate based on 16S r-RNA sequencing for *Desertifilumtharense* (5MNS2014), *Oscillatoria earlei* (NSSP6), *Oscillatoria tenuis*

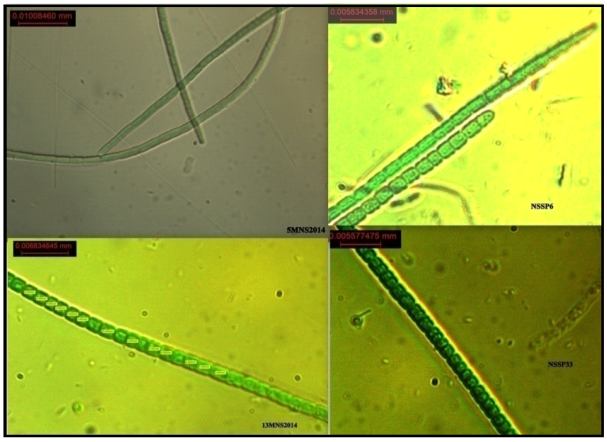

Fig. 4. Light micrographs of cyanobacterial isolates : 5MNS2014, NSSP6, 13MNS2014 and NSSP33 (scale bar = 0.005 mm).

(13MNS2014) and *Arthrospira platensis* (NSSP33) were MW687121.1, MW661176.1, MW546279.1 and MW649132.1, respectively, and the sequence was aligned with 10 to 15 cyanobacterial 16S rDNA sequences with highest homology score, which were retrieved using BLASTn. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model. The Maximum Likelihood phylogenetic tree was constructed (Figs. 6, 7 and 8) for cyanobacterial isolates NSSP33, 5MNS2014, NSSP6 and 13MNS2014 using the BLASTn hits for its 16S r-DNA sequences. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio-NJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with a superior log-likelihood value. The tree was

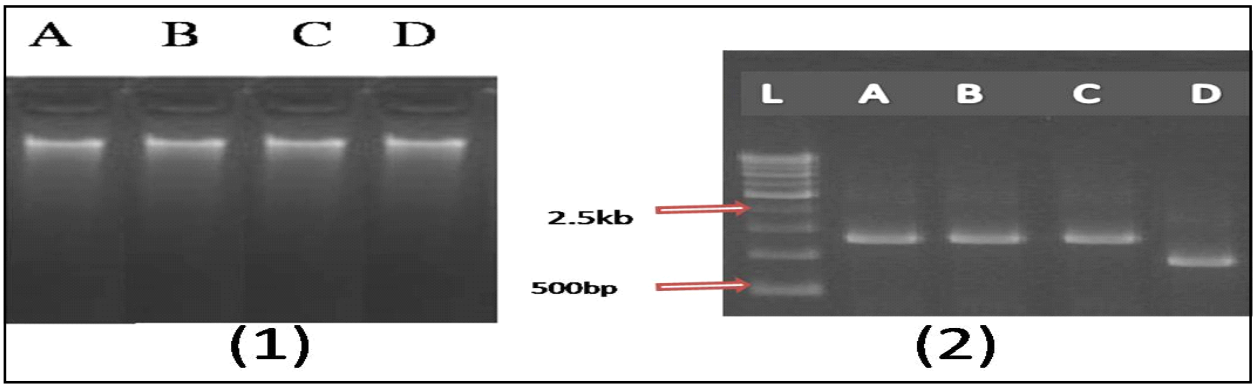

Fig. 5. Agarose gel visualization of (1) DNA isolation of samples (2) 16S r-RNA gene fragment amplified product where, L stands for Ladder; A for isolate 05 MNS2014; B for isolate NSSP06; C for isolate NSSP33 and D for isolate 13MNS2014.

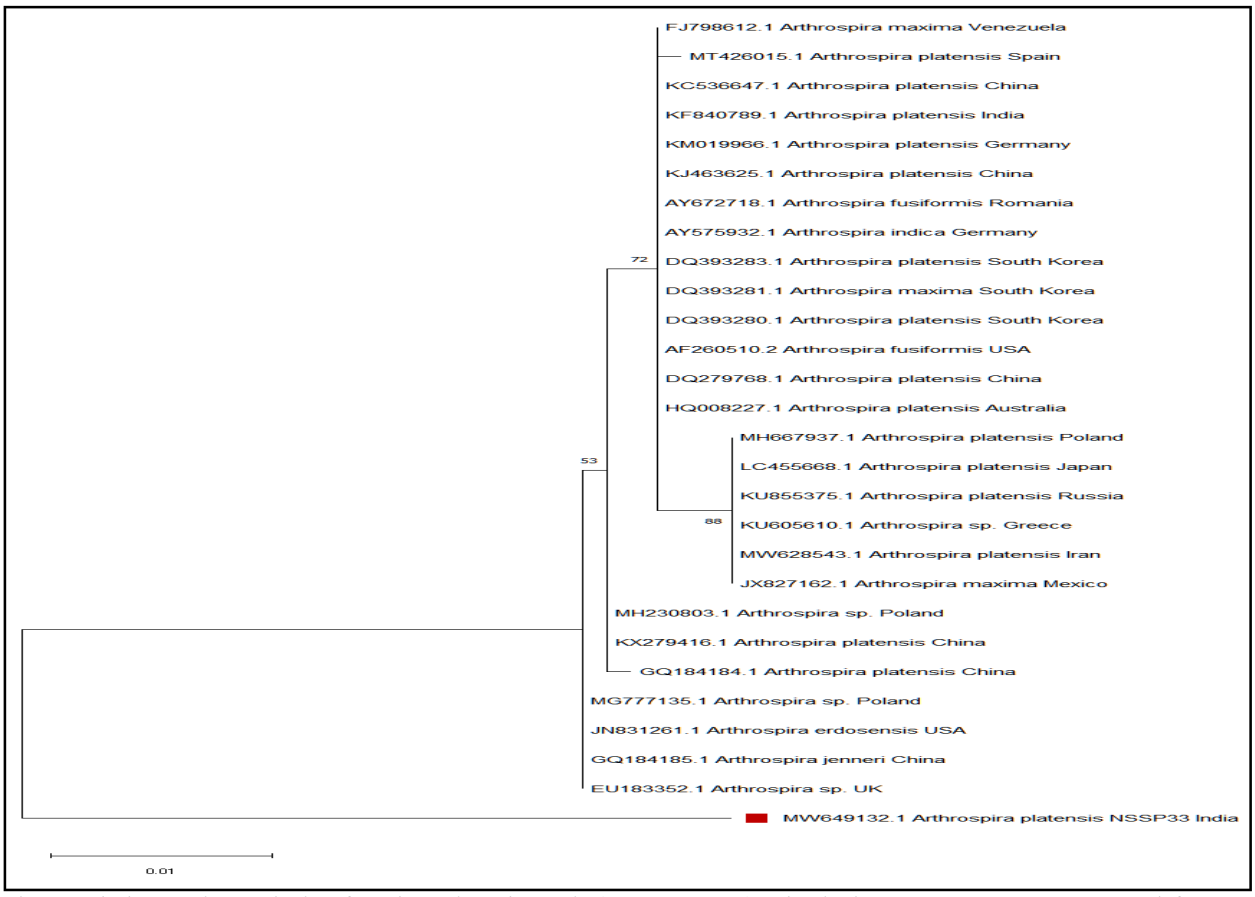

Fig. 6. Phylogenetic analysis of *Arthrospira platensis* (MW649132.1). The isolate NSSP33 was sequenced for 16s rRNA and identified as *Arthrospira platensis*. The tree with the highest log likelihood (-1482.79) was shown. This analysis involved 28 nucleotide sequences. There were a total of 895 positions in the final dataset. The isolate NSSP33 was clustering with Arthrospira sp. (EU183352.1), UK strain.

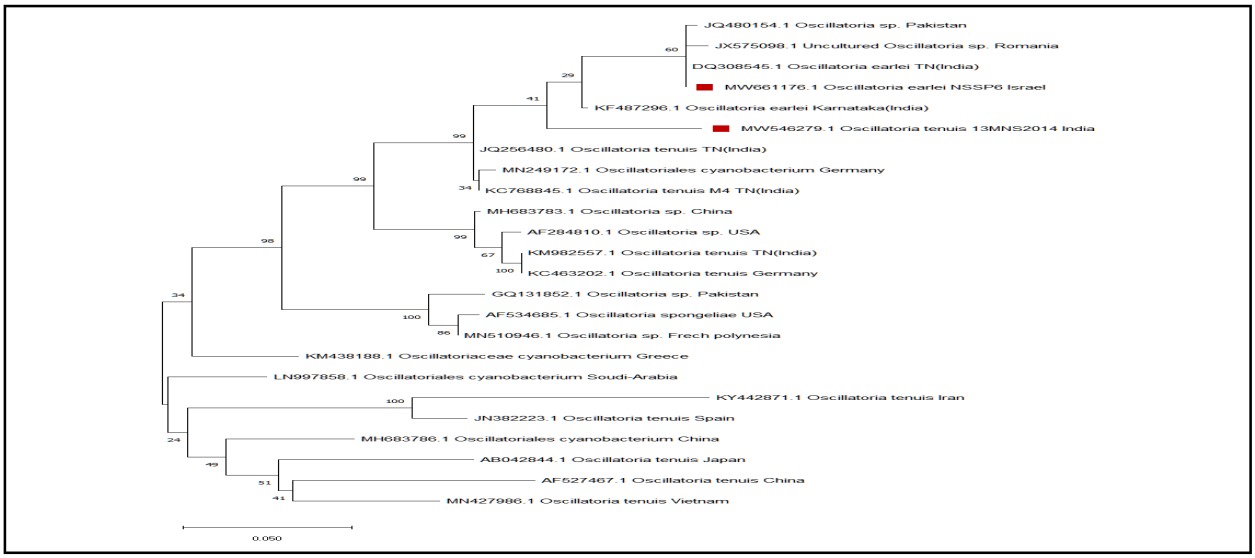

Fig. 7. Phylogenetic analysis of *Oscillatoria earlei* (MW661176.1) and *Oscillatoria tenuis* (MW546279.1). The isolates NSSP6 (Israel) and 13MNS2014 (India) were subjected to 16s rRNA sequencing and identified as *Oscillatoria earlei* (MW661176.1) and *Oscillatoria tenuis* (MW546279.1), respectively. The tree with the highest log likelihood (-6151.34) was shown. This analysis involved 24 nucleotide sequences. There were a total of 1518 positions in the final dataset. The isolate *Oscillatoria earlei* (MW661176.1) was clustering with *Oscillatoria earlei* (DQ308545.1), India, and *Oscillatoria tenuis* (MW546279.1) was clustering with *Oscillatoria earlei* (KF487296.1) Karnataka (India) strains.

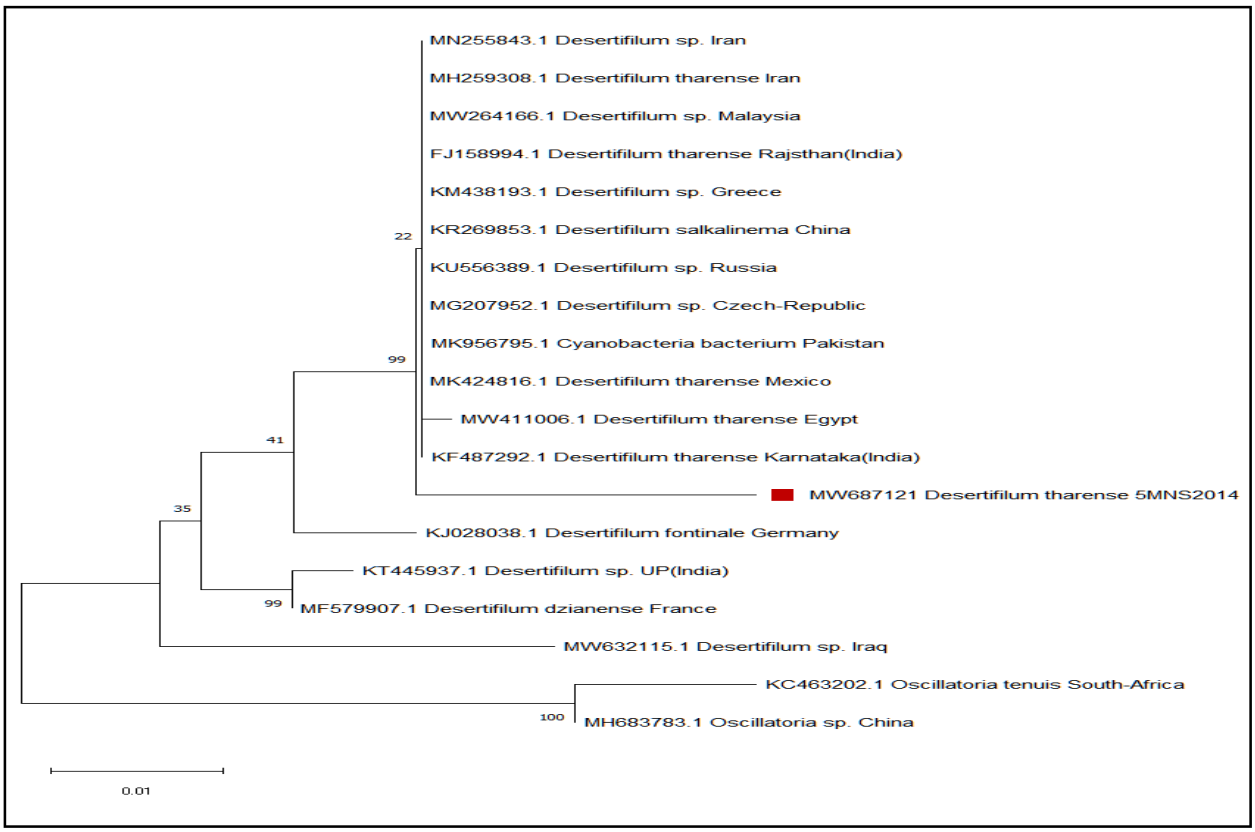

Fig. 8. Phylogenetic analysis of *Desertifilumtharense* (MW687121.1). The isolate 5MNS2014 was sequenced for 16S rRNA and identified as *Desertifilumtharense*. The tree with the highest log likelihood (-1209.82) was shown. This analysis involved 19 nucleotide sequences. There were a total of 568 positions in the final dataset. The isolate *Desertifilumtharense* (MW687121.1) was clustering with *Desertifilumtharense* (KF487292.1) Karnataka (India) strain.

drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in Molecular Evolutionary Genetics Analysis (MEGA) software version X (Stecher *et al.*, 2020).

CONCLUSION

The present study was important as a small contribution in updating the morphological and molecular characterization of freshwater cyanobacteria in literature. In this study, screening and characterization of high phycocyanin producing cyanobacterial isolates were procured from Dr. Namita Singh's unique culture collection, DBNT, GJUS&T, Hisar, Haryana and northern region of India. The cyanobacterial isolates NSSP6, NSSP33, 5MNS2014 and 13MNS2014 were selected on the basis of their specific growth rate in term of Chl *a*, phycocyanin and protein concentration. The phenotypic classification of selected isolates was conducted according to its morphological features. The molecular identification data of selected cyanobacterial isolates were collected by performing varoius characterization protocols. The results of molecular identification of selected isolates supported the result of morphological classification. The results came from molecular analysis and morphological classification conformed the same. These selected potent cyanobacterial species can be exploited for the commercial production of pigments.

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