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Molecular and Morphological Characterization of a Mat-forming Cyanobacterium and Evaluation of their Valuable Pigments

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ABSTRACT

The mat forming microbial communities are shaped by the presence of certain dominant species of cyanobacterium. In the present study, a filamentous cyanobacterial strain was isolated from the moist surface of a wall. Molecular characterization of the isolated cyanobacterium using the 16S ribosomal RNA (rRNA) gene (1397 bp) sequencing followed by phylogenetic analysis showed that the strain represented a member of the cyanobacterial genus *Microcoleus* from the order Oscillatoriales. The morphology of the cyanobacterium *Microcoleus* sp. strain RSA1 was determined using a light and fluorescence microscopy. Furthermore, growth and biochemical characterization of the isolated cyanobacterium were also performed to investigate the growth kinetics and occurrence of certain cellular pigments such as carotenoid and phycobiliprotein. Significant increase in growth was marked after 12 h up to 72 h. There was 2-3 fold increase in chlorophyll (chl-a) content after 24 h of growth. Results revealed that the isolated *Microcoleus* sp. produced significant amount of carotenoid (0.24 mg/g DW), and phycobiliproteins such as phycocyanin (6 mg/g DW) and phycoerythrin (14 mg/gDW). The results indicated that the isolated cyanobacterial strains may be explored as a viable candidate for the industrial production of value-added biomolecules.

Key words: Cyanobacteria, *Microcoleus* sp., microbial mat, phycobiliprotein, carotenoid

INTRODUCTION

Cyanobacteria are one of the most ancient group of photosynthetic prokaryotes with a cosmopolitan distribution ranging from fresh to marine water, hot springs to the Arctic and Antarctic regions (Stanojkovic *et al.,* 2022). *Microcoleus* sp. is one of the largest genera in the family Microcoleaceae and filamentous cyanobacterium is widely existing in various ecological niches. *Microcoleus sp.* has been characterized with filaments, densely packed trichomes, isodiametric vegetative cells, strongly constricted cross walls and crosswise cell division (Geng *et al.,* 2021). Cyanobacteria are also important biomass producers in both aquatic and terrestrial ecosystems. Several species of cyanobacteria form a thick layer of microbial mat in diverse habitats (Bouma-Gregson *et al.,* 2022). Besides inherent capacity to fix atmospheric nitrogen, cyanobacteria are excellent sources of several natural products of ecological and economic importance (Rastogi *et al.,* 2015; Nowruzi *et al.,* 2020). Cyanobacteria synthesize chlorophyll (Chl-a) photosynthetic pigment and carotenoids, which helps in photosynthesis. Carotenoids are terpenoids pigments and can be divided into two major classes; carotenes, such as α and β-carotene, and xanthophylls (oxygenized derivatives of carotenes) or such as zeaxanthin and echinenone. Cyanobacteria also contain high amount of phycobiliproteins (PBPs), a family of coloured proteins (Saini *et al.,* 2018) that are associated with light-harvesting complex in photosystem called phycobilisome (PBS). Besides immense role in photosynthesis, these pigments are considered as valuable ingredients in the food, medicine, biotechnological, pharmaceutical and cosmetic processes (Sonani *et al.*, 2016; Basheva *et al.*, 2018) and are biologically active substances with anticancer, antioxidant, antimicrobial, anti-inflammatory, nephroprotective effects (Mysliwa-Kurdziel and Solymosi, 2017). The aim of this study was to access molecular and morphological characterization of isolated cyanobacterial strain which was identified as *Microcoleus* sp. and furthermore, its potential was evaluated for the production of

photosynthetic pigments and value-added pigments that have immense industrial importance.

MATERIALS AND METHODS

The cyanobacterial mat sample was collected from the moist surface of wall at Kurukshetra University, Kurukshetra, (Haryana), India. Collected samples were washed with sterilized water and inoculated in BG-11 solid media in a petri plate and allowed to grow at room temperature. After repeated inoculating process, an isolated colony of the culture grown on the solid medium after 2-3 weeks of inoculation was transferred to the sterilized liquid BG-11 culture media (100 ml) in 250 ml conical flask. The organisms were grown in a culture room at 28±2°C, illuminated with fluorescent white light (12 W/m^2) under 16:8 h light/dark cycles. Thus, the pure cyanobacterial cultures obtained through repeated serial dilution and plating methods were grown in a mass volume. All experiments were done using the organism growing in exponential phase.

The molecular identification of isolated cyanobacterium was carried out at the sequencing facility of National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune, India (Sample ID: PRN B_NOV_20_454). At the facility, genomic DNA was isolated by the standard phenol/chloroform extraction method, followed by PCR amplification of the 16S rRNA gene using cyanobacterial universal primers pA-F [5'-AGAGTTTGATCCTGGCTCAG-3'] and B23S-R [5'- CTTCGCCTCTGTGTGCCTAGGT -3']. The amplified 16S rRNA gene PCR product was purified by PEG-NaCl precipitation and directly sequenced on an ABI®3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA) as per manufacturer's instructions. Assembly was carried out using Lasergene package followed by identification using the EzBioCloud database (Yoon *et al.,* 2017). The sequences of 16S rRNA gene fragment were submitted at GenBank database of National Centre for Biotechnology Information (NCBI) (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*) and the strain was determined as *Microcoleus* sp. RSA1 with Gene Bank accession no. OP662618. The phylogenetic analysis was inferred using the Neighbour-Joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Maximum Composite Likelihood method in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1410 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al.*, 2021).

The morphology and axenicity of the isolated cyanobacterium were determined using a fluorescent light microscope (Fluorex, SUSWOX OPTIK, INDIA, Model: 190715453) equipped with a green and blue filters (WG) to get the auto-fluorescence of photosynthetic pigments (Chl-a and accessory pigments). Photographs were taken using a digital camera (PRO Series, 1080P HDMI Camera, Model-MICAPS FERLAF050) coupled to a PC and processed using MICAPS-MicroView software (version: x64, 3.7.12967.20180920). The morphology of the cyanobacterium was also studied using the standard taxonomic keys and monographs.

Absorption spectra of methanolic extracts were taken between 250 and 750 using a UV/VIS spectrophotometer (UV-1900, Shimadzu). The raw data were transferred to a microcomputer and peaks were analyzed with software provided by the manufacturer.

The cultures were grown continuously for 96 h under fluorescent light. Equal volumes of homogenized cultures were harvested at desired time intervals (6, 12, 24, 48, 72 and 96 h). The growth of the organism was determined by measuring the optical density (at 730 nm) and chlorophyll (chl-a) content. The Chl-a content was estimated using the method as described earlier (Zavrel *et al.*, 2015) with slight modification, In brief, the homogenized cyanobacterial cells were harvested using centrifugation (6000 g x 10 min). The supernatant was discarded and pellet was suspended in methanol (≥99.9%, v/v) and incubated overnight in the refrigerator at 4°C. The extract was centrifuged and clear supernatant was obtained. The absorbance (A) was taken at 665 (A_{665}) nm and 720 nm (A_{720}). The chl-a content was quantified using the following equation:

$$\text{Chlorophyll-a} = 12.9447\ (A_{665} - A_{720})\ \mu g/ml.$$

Exponentially growing homogenized cyanobacterial culture was used for biochemical analysis of pigments carotenoid and phycobiliproteins. Estimation of carotenoids was done following (Ghosh *et al.*, 2019). To quantify the carotenoids, absorbance of final extract was measured at 450 nm using acetone (85% v/v) as a blank. Following equation was used to quantify the content of carotenoids:

$$\text{Carotenoid content} = (D \times V \times F) \times 10/2500 \text{ (mg/ml)}.$$

Where, D, V and F stand for optical density (at 450 nm), volume of the extract and dilution factor, respectively.

The phycobiliproteins (PBPs) were extracted and purified as described earlier by Khatoon *et al.* (2018) from the exponentially growing cells of *Microcoleus* sp. using the freeze (-25°C) and thaw (4°C) method in an extraction buffer (20 mM potassium phosphate buffer, pH 7.2) followed by ammonium sulfate precipitation. The purity ratio of PBPs at each step of purification was measured using the absorbance ratio at A_{615}/A_{280} and A_{564}/A_{280} for PC and PE, respectively. Following equations were used to quantify the PBPs:

$$PC = (A_{615} - 0.474 \times A_{653})/ 5.34$$
$$PE = [(A_{564} - (2.41\ (PC) - (0.849 \times APC)/ 9.62$$

All data were evaluated as mean values of three replicates. One-way analysis of variance was applied to evaluate the significance of the data. Multiple comparisons were made by using the Turkey test (SPSS 15.0, Chicago, IL, USA) to assess the differences among treatments.

RESULTS AND DISCUSSION

The cyanobacterial mat collected from the moist surface of wall at Kurukshetra University, Kurukshetra was dominated with a filamentous cyanobacterium *Microcoleus* sp., which was isolated using the standard microbial techniques (Fig. 1). It was observed that the isolated cyanobacterium formed fine mats and occurred in diverse habitats.

The morphology of the cyanobacterium was studied using the light fluorescent microscope and certain characteristic features such as presence of sheath, trichome/filament shape, cell dimension and wall constrictions, presence of apical cell, granulation of cells, etc. were focused during the morphological evaluation. The morphological variability of cyanobacteria was studied in both natural and isolated strains (Fig. 2). The isolated cyanobacterium was filamentous and surrounded by the polysaccharide sheath, which is a characteristic feature of several Cyanobacteria (Fig. 3). Conklin *et al.* (2020) also described the molecular and morphological characteristic of a distinct new member of the cyanobacterial genus *Microcoleus* from the Russian river in Northern California (USA).

Molecular identification of the studied cyanobacterium was carried out by means 16S rRNA gene (1397 bp) sequencing followed by phylogenetic analysis. The molecular data for the *Microcoleus* sp. species under study were consistent with morphology. Based on

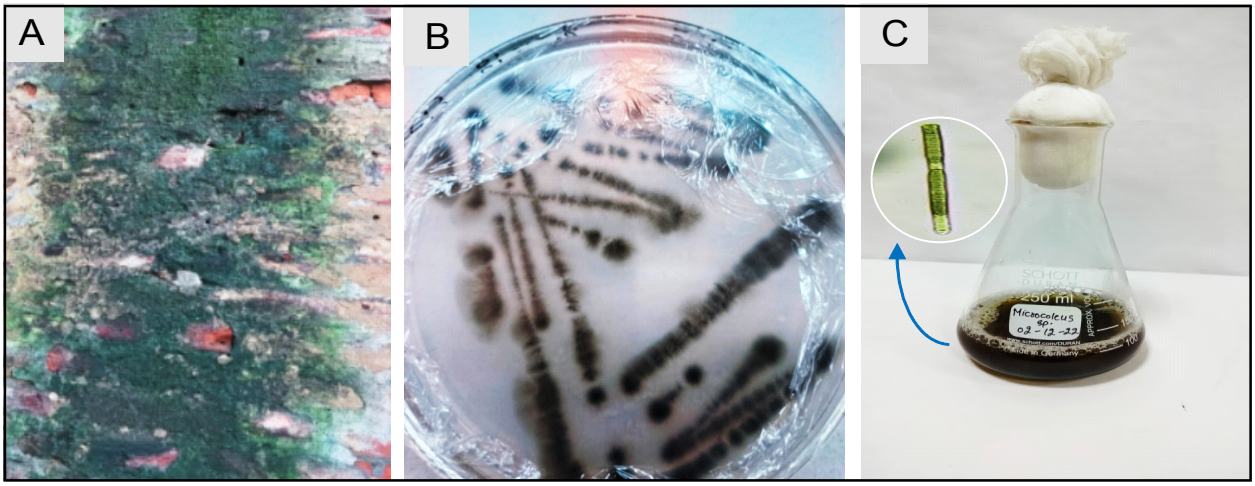

Fig. 1. Cyanobacterial mat sample growing on a wall surface (A), growing on a solid agar medium (B) and sterilized liquid growth medium (C) with pure culture of *Microcoleus* sp. (in inset).

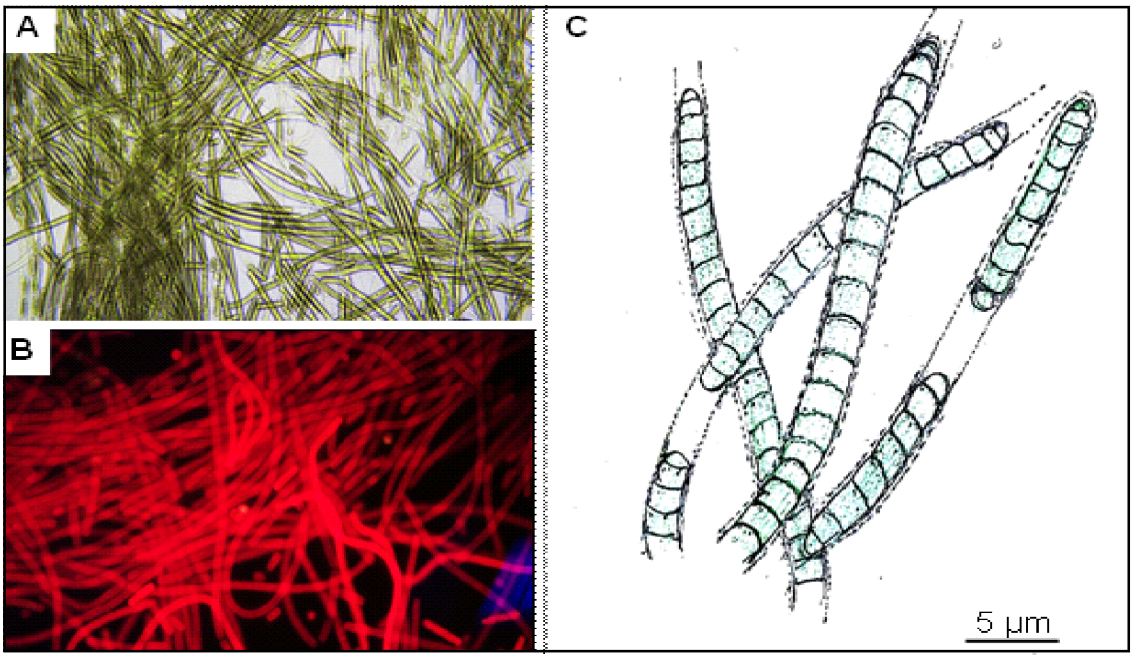

Fig. 2. The cyanobacterium *Microcoleus* sp. showing the presence of vegetative cells/filaments within the sheath (A), outside the sheath (B) and only sheath (C).

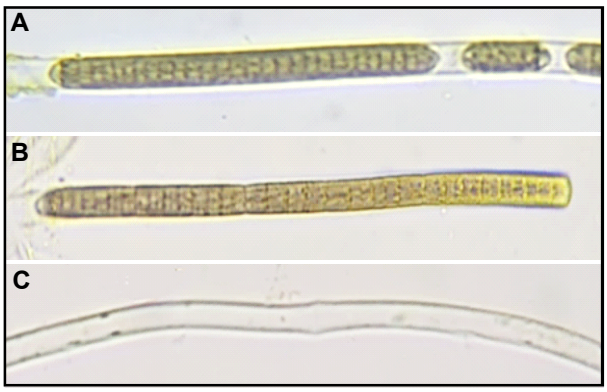

Fig. 3. The cyanobacterium *Microcoleus* sp. showing the presence of vegetative cells/filaments within the sheath (A), outside the sheath (B) and only sheath (C).

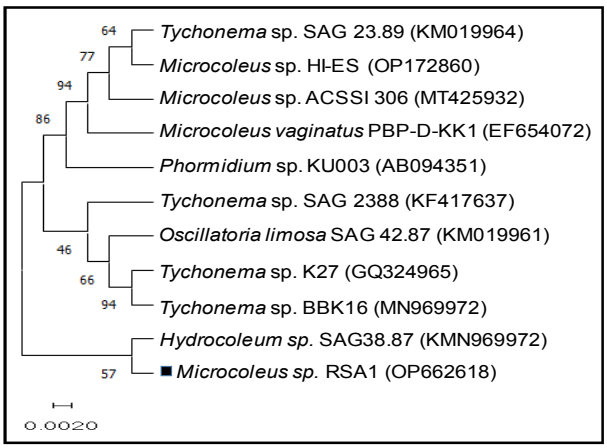

Fig. 4. The evolutionary history of the isolated cyanobacterium *Microcoleus* sp. Strain RSA1 and its close homologues, on the basis of partial 16S rRNA gene sequences. The phylogenetic analysis was confined using the 11 nucleotide sequences. Sequence obtained in the present study is indicated by a black rectangle.

morphological as well as molecular analysis, the cyanobacterium (Fig. 2) was identified as *Microcoleus* sp. and named as *Microcoleus* sp. strain RSA-1 (GenBank accession No. OP662618). The close relationship of the cyanobacterium isolated from mat sample with other cyanophycean species/strain is supported by the results from bootstrap analysis. The 16S rRNA sequence was used in alignments prior to applying neighbour-joining analyses for phylogenetic tree determination (Fig. 4). The phylogenetic analyses show the close relationship of *Microcoleus* sp. RSA-1 with other strains of the genus *Microcoleus* (e.g., *Microcoleus* sp. HI-ES, *Microcoleus* sp. ACSSI 306, *Microcoleus vaginatus* PBP-D-KK1, etc.), *Tychonema* (e.g., *Tychonema* sp. SAG 23.89), *Phormidium* (*Phormidium* sp. KU003) and *Oscillatoria* (e.g., *Oscillatoria limosa* SAG 42.87) by high bootstrap values (Fig. 4). The distance between each cyanobacterial group in the phylogenetic tree is very close, and a little difference in the base sequence may lead to a separation into diverse taxonomic groups or clusters. Bouma-Gregson

et al. (2019) used metagenomics to define four potentially novel *Microcoleus* species within the Eel river, Northern California, USA.

The growth of isolated cyanobacterium was determined by measuring the optical density (O.D) and Chl-a content. Fig. 5 shows the optical density of the isolated cyanobacterium growing at different time intervals. An increase in absorbance (at 720 nm) of growing cultures was observed, however, no significant difference in OD was observed up to 24 h of growth condition. Further, increase in Chl-a content was also observed at successive intervals of growth period (Fig. 6).

Besides, molecular and morphological

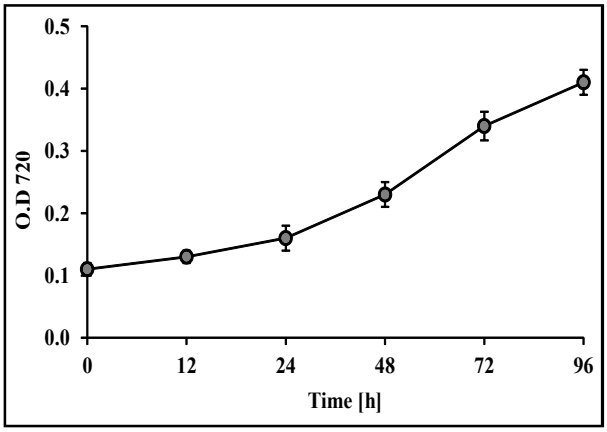

Fig. 5. Growth measurements based on increases in optical density of the biomass of *Microcoleus* isolates.

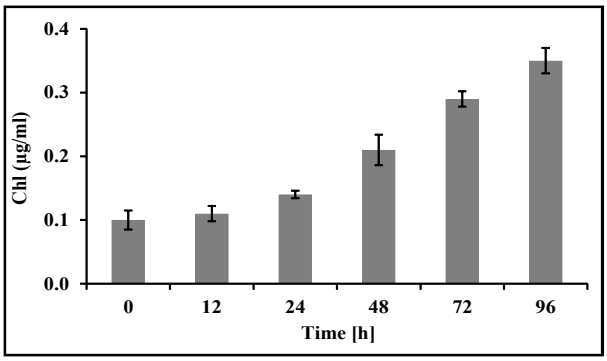

Fig. 6. Growth measurements based on increases in chlorophyll-*a* content of *Microcoleus* isolates.

identification, the biochemical characterization of certain pigment molecules such as carotenoids and phycobiliproteins (PBPs) such as phycocyanin (PC) and phycoerythrin (PE) was also assessed in the isolated cyanobacterial strain. It was observed that the studied cyanobacterium synthesized significant amount of carotenoids and PBPs. Total carotenoid content of 0.24 mg/g DW (dry weight) was found in the studied cyanobacterium (Fig. 6). In was observed that some species of cyanobacteria produced four different types of carotenoids such as myxoxanthophyll, isozeaxanthin, zeaxanthin, canthaxanthin, echinenone and ~β-carotene. In the present study, individual carotenoids were not characterized and needs further study. Synthesis of both PC and PE was found, however, the cyanobacterium *Microcoleus* sp. RSA-1 had high potential to produce PE (14 mg/g DW) in comparison to PC content (6 mg/g DW) as shown in Fig. 7. Both carotenoids and PBPs were considered high value compounds due to their significant role in production of cosmeceuticals and pharmaceutical products (Paliwal *et al.*, 2016; Sonani *et al.*, 2016). Besides carotenoids and PBPs, some other secondary bioactive molecules were also reported from *Microcoleus* sp. Overall, the studied cyanobacterium could be used as a potential candidate in different pharmaceutical and biotechnological industries.

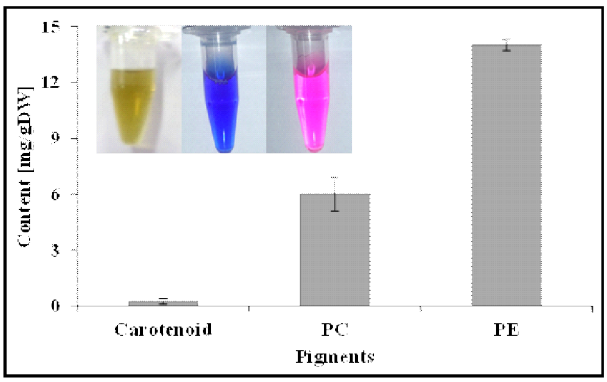

Fig. 7. Synthesis of pigment carotenoids, phycocyanin (PC) and phycoerythrin (PE) in the isolated cyanobacterium *Microcoleus* sp.

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