Polyphasic taxonomic identification of marine cyanobacteria of the Indian Sundarbans and their biotechnological potential

SYNOPSIS SUBMITTED FOR

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By

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Chapter 1. Introduction

Thorough investigation of underexplored ecosystems conducted to identify innovative and environmentally friendly sources of medicines, agricultural chemicals, clean energy, and colors has shown the promise of cyanobacteria, or blue-green algae, being producers of these materials. Mangroves constitute an area of significant biodiversity which is severely challenged by abiotic influences and susceptible, located in the intertidal zones of tropical and subtropical climates, supporting various microorganisms. Nevertheless, compared with other prokaryotic organism there is presently a lack of information on the taxonomic categorization of cyanobacteria and the investigation of secondary metabolites that are comprised of new compounds for mangrove cyanobacteria. Comprehensive study conducted for many years has revealed that cyanobacteria, particularly those sourced from intertidal and marine habitats, are high producers of both established as well as new bioactive molecules and natural products (NPs) of commercial significance, with broad applicability throughout multiple industries. Despite their significance for environmental benefits as well as the industrial production of several products, there are presently very few articles on cyanobacterial NPs, particularly from mangrove and coastal environments. Only few organisms which described below have been the subject of in-depth studies on bioactive chemicals obtained from mangrove cyanobacteria. This is likely because of the disordered nature of the conventional method of screening naturally existing cyanobacterial populations in mangrove habitats for desired compounds, which does not use polyphasic taxonomy. The polyphasic characterization of cyanobacteria in mangroves improves our knowledge of the cyanobacterial diversity structure in these ecosystems, which in turn helps us to know their potential uses in various biotechnological industries.

Chapter 2. Materials and methods

2.1. Study area and sample collection

The cyanobacterial samples utilized in this investigation were collected from distinct locations (Purba Gurguria, Maipit, Pathar Pratima, Namkhana and Kakdwip) of the Indian Sundarbans. All the samples were collected during low tide in November 2018. Green or brownish-green colored phototrophic biofilms were collected from the upper surface of the soil using a trowel. They were transferred into plastic sample collection bags and carried to the laboratory for further investigation.

2.2. Sample processing, isolation, and purification of cyanobacteria

A small portion of the biofilm samples (about 1.0 g) was put into a 100ml Erlenmeyer flask containing 30ml of ASN-III liquid medium (Rippka *et al*. 1979). The flasks were illuminated for 12h a day by fluorescent lamps (50 µmol photons $m^{-2}s^{-1}$), bubbled with 0.1% CO_2 in air, and incubated at 25 ± 1°C (Chakraborty *et al*. 2019). Cyanobacteria cultures were seen mixed with other phototrophs as well as heterotrophs under light microscopes. To obtain the pure, axenic filamentous cyanobacterial strain, several strategies taken. In the first step, to prevent eukaryotic growth, 1.0 mL cycloheximide from a stock solution (50 mg· L^{-1} in methanol at 75%) was added to 1000 mL of ASN-III liquid medium (Urmeneta *et al*. 2003) and to prevent the growth of other heterotrophs, a triple antibiotic solution was used. 100 mg of penicillin G (Na salt), 50 mg of streptomycin sulphate dissolved in 10 mL distilled water, and 10 mg of chloramphenicol dissolved in 1 ml of 95% ethanol were mixed and filtered by syringe filtration (Acrodisc syringe filter; Pall Corporation, NY, USA) following Guillard (2005). A small amount of biofilm, about 1 ml was added to 100ml Erlenmeyer flask containing 30 ml of ASN-III culture medium, to which different

volumes (0.125, 0.250, 0.500, 1.0, 1.5, 2.0, 2.5, 3.0 ml) of the triple antibiotic solution were added and were inoculated for 48 to 72 hrs to obtain the optimal level that allowed contamination free cyanobacterial growth (Pramanik *et al*.2011). The second step of isolation was done by repeated transfers between liquid culture and solid agar plate culture. The method is called spread plate method. A small amount of cyanobacterial biomass was spread on agar solidified ASN-III medium petri plate. Alternating transfers between solid and liquid media were carried out until uni-cyanobacterial strains were obtained. It is possible to pick up single filaments of cyanobacteria from solid agar plate culture using inoculating loop and then transferring them into a 100 ml Erlenmeyer flask having 30 ml of ASN-III media. After 7-10 days, axenic cyanobacterial strains were obtained from the flask by confirming under microscopic analysis.

2.3. Morphological analysis

The morphological features of all the purified strains were examined using a light microscope. A small amount of 8-10 day-old cyanobacterial isolates was placed on a glass slide and temporarily mounted with a cover slip aseptically. The slides were then observed under 400-1000X magnification using DM750 light microscope (Leica Microsystems, Buffalo Grove, Illinois, USA). Images of the six cyanobacterial strains were captured using an ICC5HD camera attached to the microscope.

Scanning electron microscopy was performed in 10-days old sample of each strain. The cell biomass of each sample was centrifugated (8000 rpm) and fixed in 3% glutaraldehyde for 2 hrs. Then the fixed cell biomass was washed with the distilled water followed by its dehydration using ethanol gradients. At last, samples were dried by placing into grids and then observed under scanning electron microscope.

2.4. Ultrastructural analysis

Cellular ultrastructure was studied by the help of transmission electron microscopy. Samples from fresh log-phase cultures were centrifuged and cell biomass was pre-fixed using 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.8) for 5-6 hrs at 4 ºC. This step was followed by post-fixation by 1% solution of osmium tetroxide incubated for 1 hr. After post-fixation, dehydration was carried out by gradients of ethanol followed by the ultrathin sections of the embedded block using microtome. These ultrathin sections were then visualized under the transmission electron microscope.

2.5. Molecular and Phylogenetic analysis

Genomic DNA extraction for each strain was performed by harvesting the fresh filaments from the log-phase of the culture, homogenizing and centrifuging it at 6000 rpm for 10 min at room temperature. The cell pellet was then utilized for the gDNA extraction using Gene JET^{TM} Genomic DNA Purification Kit following the manufacturer's protocol. The purity of the extracted DNA was checked by running the samples in DNA gel electrophoresis and also in UV-Vis spectrophotometer. Extracted DNA was used as a template for the amplification of 16S rRNA gene and 16S-23S ITS (Internal Transcribed Spacer) gene sequence. 16S rRNA gene sequence was amplified by using specific cyanobacterial forward primer (CYA106F by Nubel et al. 1997) and universal reverse primer (1492R by Lane 1991) using standardized conditions. ITS sequences were amplified by 16SF and 23SR (Iteman et al. 2000). After amplification, the PCR products were screened for the acceptable amplicon size. The selected PCR products were cloned using pGEM®-T Easy Vector System I (Cat No. A1360, Promega Corp., Madison, Wisconsin, USA) Cloning Kit into a suitable vector. This ligated product was transformed into *E. coli* DH5α cells and successively positive transformants were selected by blue-white screening method. Then the cloned products were

sequenced using the primer pUC/M13 forward (5′-CGCCAGGGTTTTCCCAG TCACGAC-3′) and pUC/M13 reverse (5′-CAGGAAAC AGCTATGAC-3′). After obtaining the sequences, they were aligned with the closest related members based on the similarity values in BLAST. The aligned data set was used to construct a consensus phylogenetic tree to interpret the evolutionary relationship among the isolated as well as reference strains of the phylogenetic tree. Bayesian analysis, Maximum Likelihood, Maximum Parsimony and Neighbor-Joining analyses were utilized for the construction of the phylogenetic tree.

2.6. 16S-23S ITS secondary structure analysis

The sequences of ITS regions obtained were also utilized to compare among the investigated strains and the reference strains in terms of sequence length and the secondary structures. The sequences of the species to compare were aligned and compared. The secondary structures were folded by using M-fold web server (Zuker, 2000) and were redrawn using Adobe Illustrator.

2.7. Uncorrected p-Distance analysis for16S and 16S-23S ITS

Sequences of 16S rRNA and ITS obtained for the isolated strains were aligned with the reference strains in MEGA version 6.0 and percentage similarity and dissimilarity were calculated based on the p-distance analysis.

2.8. Whole genome analysis of S1 strain

Small amount of biofilm from a 6-7-day-old unicyanobacterial culture of the S1 sample was subjected to centrifugation and then rinsed with nuclease-free water. Following the manufacturer's instructions, the genomic DNA was extracted from the rinsed sample employing the DNeasy plant mini kit (Catalog No. 69104, Qiagen, Hilden, Germany). The library was prepared utilizing the KAPA HyperPlus Kit (Catalog No. 07962428001, Roche, Basel, Switzerland). Sequencing

performed using Illumina MiSeq platform (Illumina, CA, USA). The quality evaluation of the raw FASTQ readings from the sample was conducted using FastQC v.0.11.9 with default settings (Andrews, 2017). The raw FASTQ readings underwent pre-processing using Fastp v.0.23.4 (Chen *et al*. 2018). Processed data were re-evaluated with FastQC. The processed readings were assembled de novo via Unicycler v.0.4.4 (Wick *et al*. 2017) with default settings. The final genome was derived using the metagenomic binning approach, which ensured that it was free of any microbial and viral contaminants, as genomic DNA was obtained from a mono-specific non-axenic culture of isolate S1. The draft genomes were subsequently binned using MaxBin2 version 2.2.7 to retrieve the genomes from the draft assemblies. (Wu *et al*. 2016). The complete genomic sequence of S1 has been submitted to DDBJ/ENA/GenBank with the accession number JBHZOL000000000. The version referenced in this document is JBHZOL010000000.

2.9. Anti-UV compound production by strain S19

Biofilms of growing cyanobacteria (S19) from a culture that was 6 days old were put on clear, germ-free Petri dishes and placed in an incubation chamber. The incubation chamber included two types of light: one is ultraviolet (UV) lamp (Philips, Netherlands), and the other is a white fluorescent light (Philips, Netherlands). Exposure to UV light at approximately 280-315 nm was achieved using the UV-B lamp, while photosynthetically active radiation (PAR) at 400-700 nm was exposed using the white light. Continuous exposure was given to the cyanobacterial biofilms for 12 hrs.: 12hrs light: dark photoperiod for 5 days. MAAs were isolated then partly purified following the procedures reported by Ahmed *et al*. 2021, with minor changes. After UV treatment, cyanobacterial cells were collected by centrifugation at 8000 × g for 10 minutes (Eppendorf 5810R, Rotor F-34-6-38, Hamburg, Germany). A total of about 0.5 grams of dry powder cells were dissolved in 5.0 ml of 100% methanol (HPLC grade), and the mixture was then incubated at a

temperature of 4 ℃ uninterrupted overnight. Following the incubation period, the cells were homogenized employing a glass homogenizer, and then it was subjected to sonication for 5 minutes. After that, the solution was centrifuged at room temperature for 10 minutes at 8000 × g. Following centrifugation, the supernatants were put in to a fresh centrifuge tube, and the resulting pellet was removed. The supernatants underwent spectroscopic investigation within the wavelength range of 250-700 nm with a UV-VIS spectrophotometer (Perkin Elmer, Lamda 25, Shelton, Connecticut, USA). Software connected to the spectrophotometer (Lamda 25) was used to analyze the raw spectra (peaks). A rotary evaporator was then used to evaporate the methanolic extracts at 35 °C (Eyela, model no. N-1200A, Tokyo, Japan). The residual substances were dissolved in 0.5 µl of distlled water. This solution was mixed with 75 µl of chloroform, centrifuged at 8000 × g for 10 minutes, and then gently vortexed. Following centrifugation the aqueous phase (uppermost layer) was delicately moved into new centrifuge tube to exclude contamination with lipophilic photosynthetic pigments in the MAAs (water-soluble). Absorption spectra were once again captured. The MAAs were partially purified by passing the materials through microcentrifuge filters with pore sizes of 0.2 mm. The HPLC analysis was conducted on the partially purified MAAs.

The MAAs that were partially filtered, were subjected to additional analysis and purification utilizing a high-performance liquid chromatography (HPLC) system (Shimadzu Corp, Serial no. L203054, 61426, Kyoto, Japan). This system consisted of with a reverse-phase semi-preparative C18 column made by Shimadzu Corp., which was linked to a guard column that had a photodiode array detector (SPD-M20A). Approximately 50 µl of partly purified MAA sample was injected using a Hamilton syringe (Switzerland) onto the HPLC column. The mobile phase consisted of 0.2% acetic acid, and the recognition wavelength was 334 nm and the flow rate was 1.0 ml/min

The PDA check wavelength ranges from 280 nm to 370 nm. The distinct peak, exhibiting a retention duration (RT) of around 2.9 minutes, was eluted and obtained using a fraction collector connected to the HPLC machine. Additionally, the purified MAA was categorized using liquid chromatography–electrospray ionization mass spectrometry in the positive electrospray ionization (ESI) mode, with a scan range of m/z 200–600. The specific MAA were determined using UV/Vis and mass spectrometry analysis.

The function of MAAs in UV-protection was evaluated by applying on the bacteria *Escherichia coli* (MTCC 1195). The MAAs-mixed (1 mg/ml) and pure *E. coli* cells were subjected to UV-B radiation for about 30 minutes and then inoculated into sterile Petri plates with solid LB medium. Following a 12-hour incubation period at 37 °C, the amount of bacterial colonies on every Petri plate was determined, and the formula was used to calculate the percentage survival rates following Rastogi *et al*. 2013.

The percent survival rate (SR%) = $\frac{N \times 100}{NC}$

N represents the quantity of colonies subjected treated (without MAAs, only UV-B without MAAs and MAAs with UV-B) cells and NC represents the quantity of control colonies (without MAA mixture).

2.10. Biodiesel production by strain S1

Cyanobacterial biofilm cells that were initially developing exponentially were obtained from the pure axenic culture. The biofilms were then rinsed twice with distilled water to remove excessive media before being reinoculated into ASN-III medium with varying nitrogen contents. As a nitrogen source, sodium nitrate ($NaNO_3$) was used in ASN-III medium. In order to maximize lipid

synthesis, we used five distinct concentrations of $NaNO_3$ (0.25, 0.5, 0.75, 1.0, and 1.25 g/l) in ASN-III medium. The control medium for growth conditions was 0.75 g/l $NaNO_3$ in ASN III media.

After 15 days of continuous growth in ASN-III medium with different nitrogen condition, the cyanobacterial biomass was harvested and cleaned with distilled water. Lyophilization of the biomass was carried out in a freeze dryer (Eylea Pte. Ltd.) subsequent to its cleaning. Approximately 0.5 grams of biomass was ground in a mortar and pestle with 5.0 ml of a chloroform and methanol mix in a 2:1 ratio. To lyse the cyanobacteria cell walls, liquid nitrogen was applied simultaneously. Following the extraction process, the liquid solution was maintained at 25 ℃ under shaking conditions for a duration of 5 hours. Subsequently, the mixture was centrifuged at 7000 g for 10 minutes. Moreover, the pellet was disposed of after centrifugation, and the greenish supernatant was taken in a glass vial that had been previously weighed and incubated at 30 °C for the whole night. The glass vial was weighed once again to determine the lipid content after incubation.

$$\text{Lipid content (\%)} = \frac{\text{Final Lipid}}{\text{Biomass}} \times 100$$

Chapter 3. Results and discussion

3.1. Morphological analysis

Among the five strains, S1, S6, and S10 were identified as distinct strains of the same genus and species due to their physical and genetic resemblance, as compared to the reference genus *Euryhalinema mangrovii* (Chakraborty et al. 2019) based on 16S rRNA similarity. The other two strains, S19 and S23, were evaluated against their reference, *Thainema salinarum* (Rasouli-Dogaheh *et al*. 2022). All five strains exhibited a simple, filamentous, and non-heterocytic

morphology. The strains were identified using the taxonomic key by Komarek & Anagnostidis (2005) by morphological comparison. Light and scanning electron microscopy show different diagnostic features in the strains that were studied, which were thought to be apomorphic features. The isolated strains exhibit the following diagnostic features:

Strains S1, S6, and S10: Exhibit gliding movement, cells that are noticeably longer than broad, a thin mucilaginous sheath, and an apical cell with a conical shape.

Strains S19 and S23: Barrel-shaped, apical cell pointed, mucilage sheath present, gliding action, and cells much longer than their breadth.

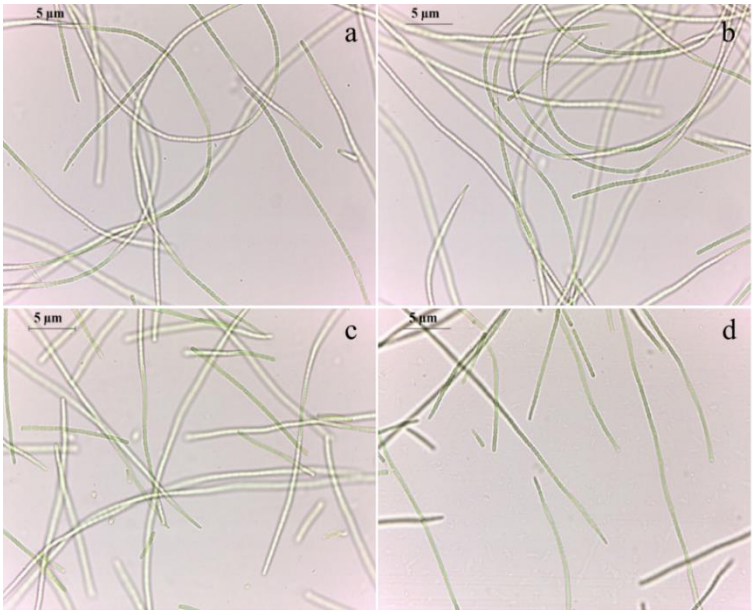

FIGURE 1. Light microscopic images of *Almyronema epifaneia* gen. nov. sp. nov. Image **a** and **b** show filaments of S1 and S6 strains, respectively. Image **c** and **d** show the straight and waving filaments of strain S10. Scale bar for all the images is 5 µm.

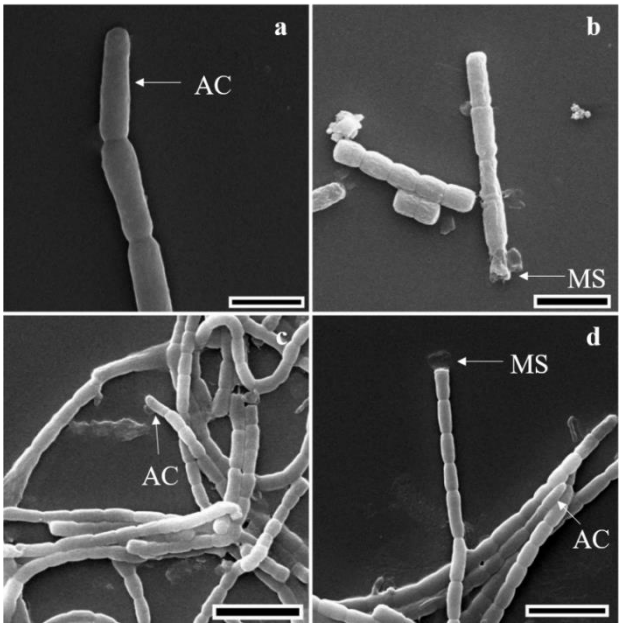

FIGURE 2. Scanning electron microscopic images of *Almyronema epifaneia* gen. nov. sp. nov. Image **a** and **b** show filaments of S1 and S6 strains, respectively. Image **c** and **d** show the filaments of strain S10. AC= Apical cell, MS= Mucilaginous sheath. Scale bar for image **a**, **b**, **c**, **d** is 1 µm, 3 µm, 5 µm, 5µm respectively.

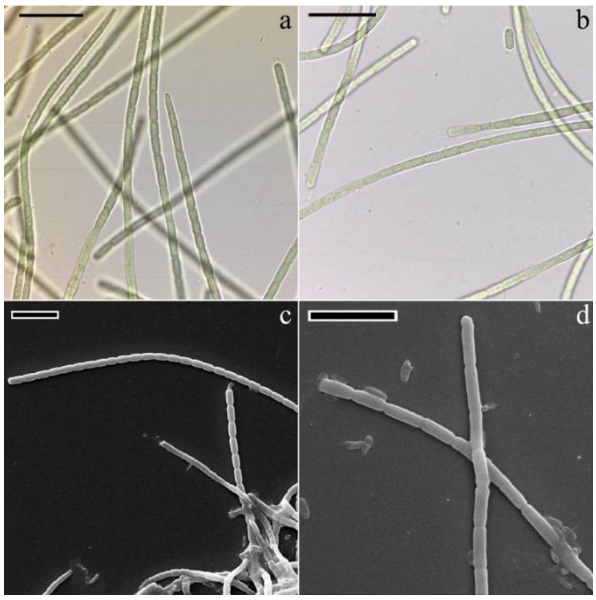

FIGURE 3. Image **a** and **b** showing light microscopic photograph of S19 and S23 strain respectively. Image **c** and **d** showing SEM photograph of S19 and S23 strain respectively. Scale bar for light microscopic and SEM images are 5 µm and 300 nm respectively.

3.2. Ultrastructural analysis

Isolated strains exhibited a basic thylakoidal architecture, coupled with additional cellular inclusions. According to Mares *et al*. (2019), the arrangement of thylakoids has remained consistent throughout evolution. Thus, the fundamental thylakoid pattern is regarded as a family characteristic. An ultrastructural analysis was conducted and compared using this data. The parietal arrangements of thylakoids in strains S1, S6, and S10 resembled those of their reference *Euryhalinema* sp., which belongs to the Nodosilineaceae family. Strains S19 and S23 exhibited parietal arrangement of thylakoids, a crucial characteristic of the family Nodosilineaceae (Strunecky *et al*., 2023). Strains S1, S6, and S10 have unique cellular features including cyanophycin granules, distinguishing them from other Nodosilineaceae genera. While S19 and S23 had ultrastructural characteristics that were more or less identical to those of their reference genus, *Thainema salinarum*.

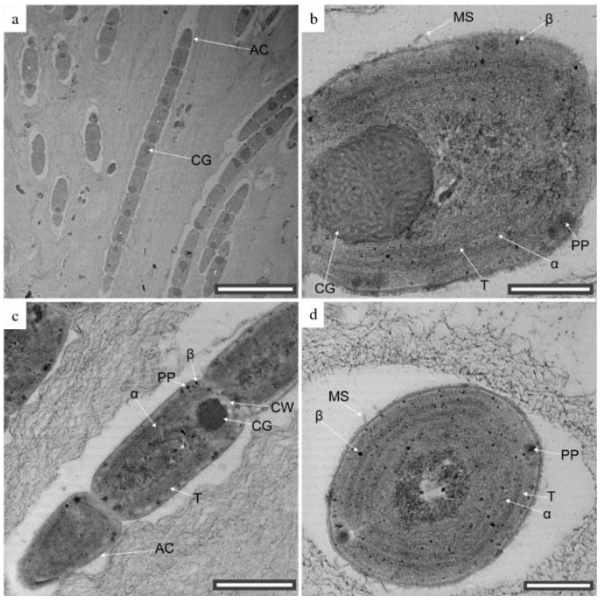

FIGURE 4. a) longitudinal section of a full filament of Strain S1. b) Cross section of S6 strain c) Longitudinal section of apical cell of Strain S10. d) Cross section of Strain S10. AC= Apical cell, Glycogen granules, lipid granules, CG= Cyanophycean granules, CW= Cross wall, MS=

Mucilaginous sheath, PP= Polyphosphate granules, T= thylakoid bands. Scale bar for image a, b, c, d is 5 µm, 250 nm, 700 nm, 300nm respectively.

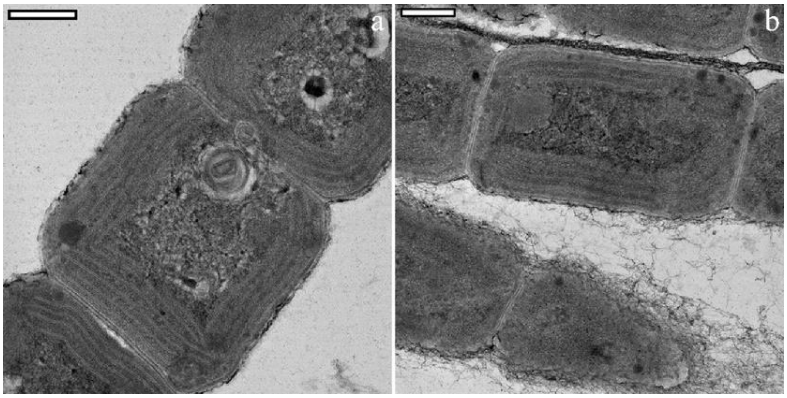

FIGURE 5. Image **a** and **b** showing cross section of S19 and S23 strain under transmission electron microscopy. Scale bar for both images were 700 nm.

3.3. Molecular and Phylogenetic analysis

According to the 16S rRNA sequence similarity, strains S1, S6, and S10 had the highest affinity for several unidentified strains that were submitted to the NCBI, and less than 95% similarity for the strains belonging to the genus *Euryhalinema mangrovii* (Chakraborty *et al*. 2019). The strains S19 and S23 exhibited a similarity of around 99% with the genus *Thainema salinarum* (Rasouli-Dogaheh *et al*. 2022). This finding led to the construction of a phylogenetic tree with strong bootstrap values. The phylogenetic tree revealed that S1, S6, and S10 were distinct genus sisters to the reference clade. The newly formed cluster appeared different from the clade that included the genus *Euryhalinema* which was considered the most similar in terms of both ecology and morphology. In the other phylogenetic tree for strains S19 and S23, it is closely associated with *Thainema salinarum* in the Nodosilineaceae family.

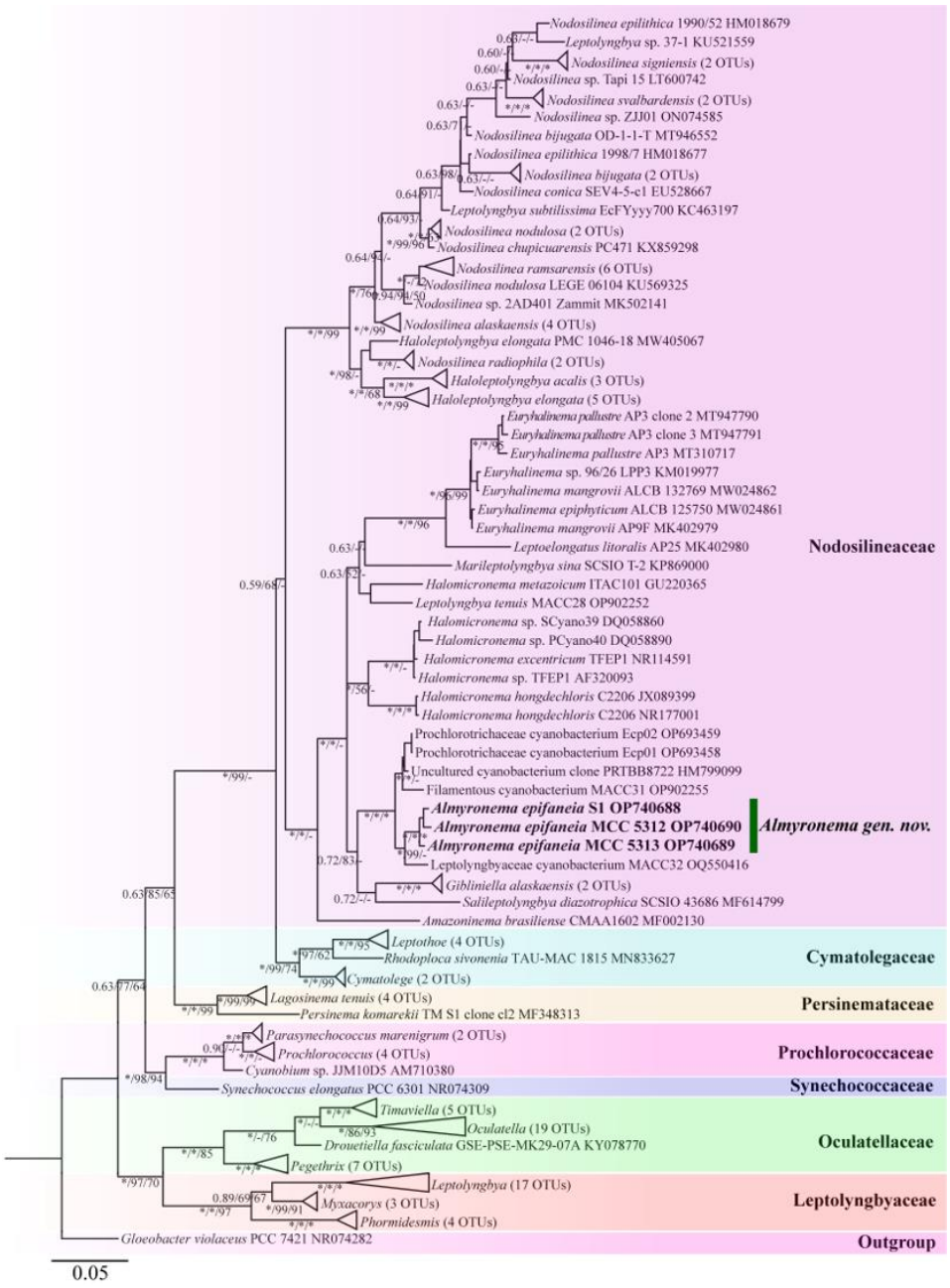

FIGURE 6. A phylogenetic tree was created using 16S rRNA gene sequences of 146 OTUs, containing *Gloeobacter violaceus* as the outgroup. The structure of the tree for Bayesian analysis. Scores for ML and MP trees were plotted in the tree. Bootstrap values based on 1000 resampling are given as for BI/ML/MP analysis at the nodes. Nodes indicated with an asterisk (*) were supported by both posterior probability and bootstrapping, achieving values 1.0 and 100%, respectively. Scores indicated by a dash (-) for any node demonstrated support of less than 50% for that branch.

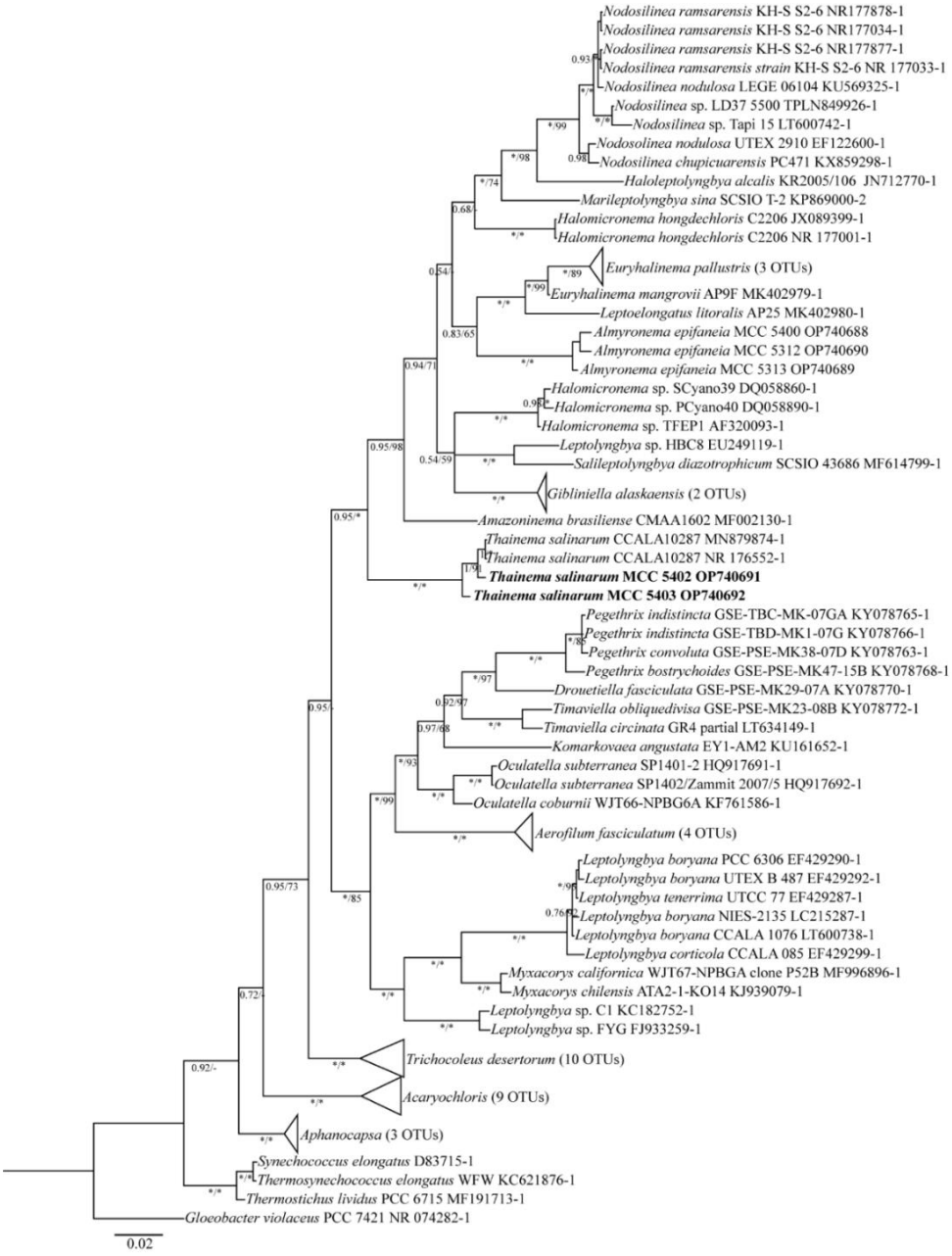

FIGURE 7. Phylogenetic tree of *Thainema salinarum* MCC 5402 generated from 84 OTUs of 16S rRNA gene sequences, having *Gloeobacter violaceus* serving as the outgroup. Supported values were BI/ML values given to the BI tree. The nodes shown by a star (*) obtained confirmation from both the posterior probability and the bootstrap, achieving values of 1.0 and 100%, accordingly. Scores indicated by a dash (-) for each node reflected less than 50% support for that branch.

3.4. 16S-23S ITS secondary structure analysis

Significant variations in the number of nucleotides were observed in the sequence length of the ITS regions of the S1, S6, and S10 strains (*Almyronema epifaneia*) when compared to the reference members of Nodosilineaceae. These variations were sufficient to identify the strains as novel genus. Table 1 displayed the total comparative account. All 16S and 23S ITS sequences consist of a single operon with $tRNA^{Ile}$ and $tRNA^{Ala}$. Furthermore, the secondary structures, including the D1-D1' helix, Box-B helix, and V3 helix, were analyzed in comparison. S19 and S23 strains have 99.17% and 99.49% sequence similarity to *Thainema salinarum* CCALA 10287, respectively, according to NCBI BLAST results. The secondary structures of the D1-D1' and Box-B helices are identical to those of the reference genus *Thainema salinarum*.

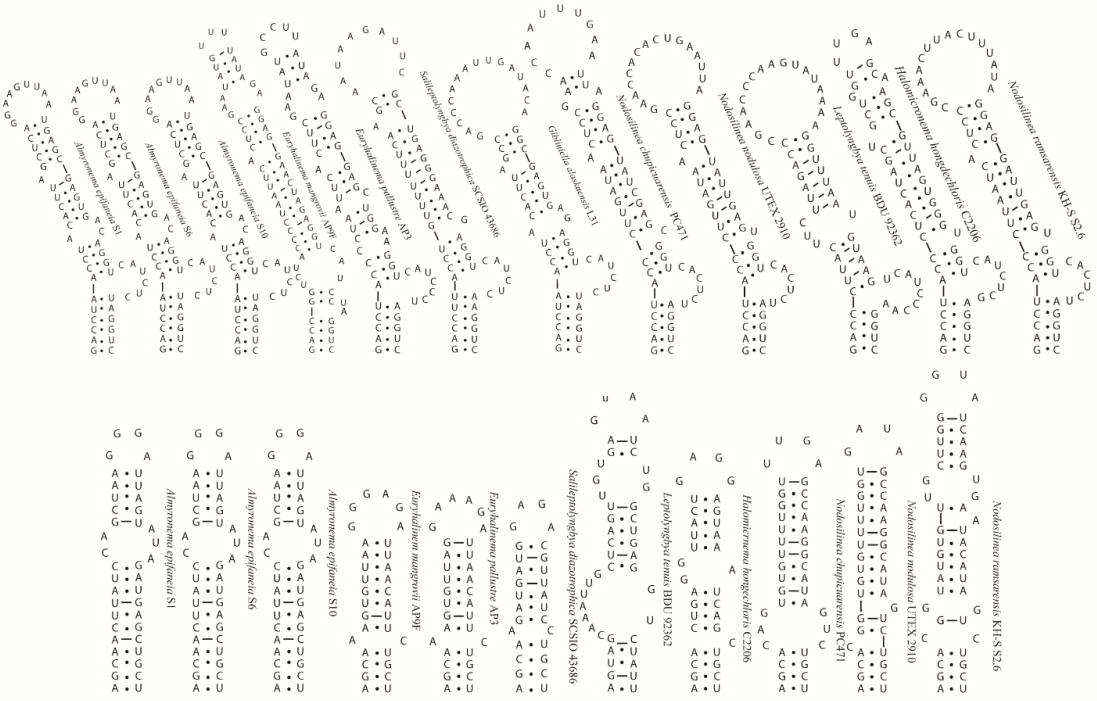

FIGURE 8. Comparative analysis of helix D1-D1′ (Upper row) and Box-B (Lower row) of the 16S-23S ITS domain of *Almyronema epifaneia* alongside closely related phylogenetic members of the family Nodosilineaceae.

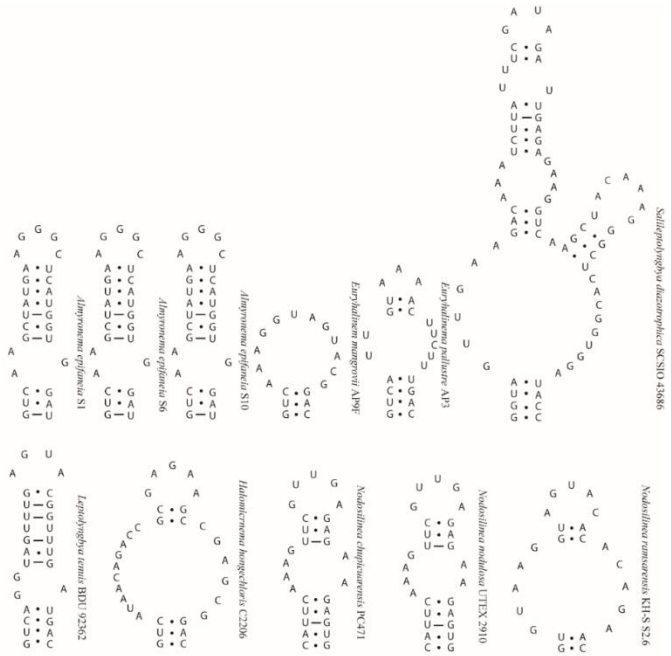

FIGURE 9. Characterization of the V3 helices of the 16S–23S ITS sections of *Almyronema epifaneia* gen. nov. sp. nov. strains S1, S6, and S10, alongside closely related phylogenetic relatives within the family Nodosilineaceae.

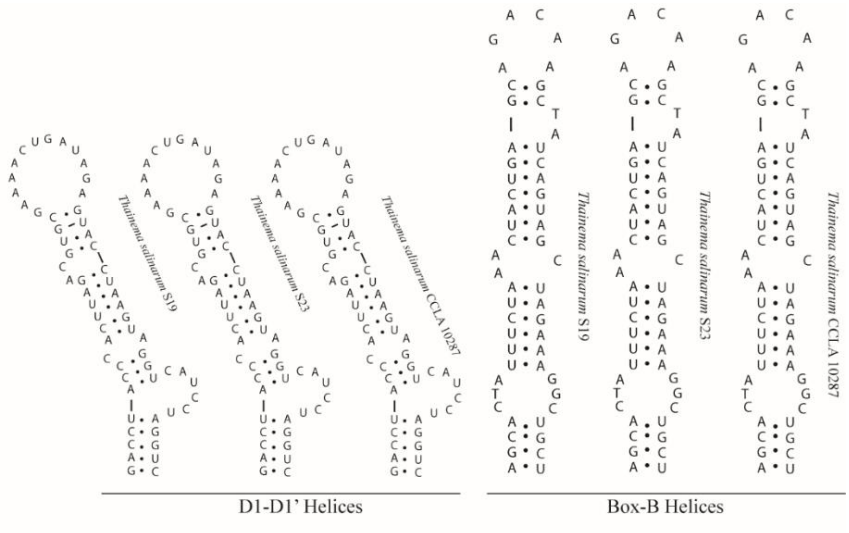

FIGURE 10. A comparative analysis examining the folded secondary structures of D1-D1′ and Box-B helices of 16S-23S ITS of *Thainema salinarum* strains S19 and S23 in relation to Thainema salinarum CCLA 10287 (Rasouli-Dogaheh et al. 2022).

Table 1. Study comparing the nucleotide sequences of the ITS sections of the strains of *Almyronema epifaneia* (S1, S6, and S10) to those of *Euryhalinema mangrovii* and other closely related Nodosilineaceae family members.

Strains	Leader	D1-D1'helix	Spacer + D2 with spacer	D3 with spacer	$tRNA^{ile}$ gene	V2 spacer	$tRNA^{Ala}$ gene	Pre-Box B spacer	Box B	Post Box B spacer	Box A	D4	V3	D5
***Almyronema epifaneia* S1**	8	**59**	**35**	**12**	74	**5**	73	**36**	**41**	19	11	**7**	**28**	**15**
***Almyronema epifaneia* S6**	8	**59**	**35**	**12**	74	**5**	73	**36**	**41**	19	11	**7**	**28**	**15**
***Almyronema epifaneia* S10**	8	**59**	**35**	**12**	74	**5**	73	**36**	**41**	19	11	**7**	**28**	**15**
Euryhalinema mangrovii AP9F	8	**63**	**36**	**12**	74	**7**	73	**34**	**32**	19	11	**7**	**20**	**16**
Euryhalinema pallustre AP3	8	**63**	**39**	**9**	74	**15**	73	**37**	**32**	24	11	**11**	**24**	**18**
Salileptolyngbya diazotrophica SCSIO 43686	8	**62**	**40**	**7**	74	**6**	73	**24**	**28**	24	12	**7**	**72**	**17**
Gibliniella alaskaensis L31	8	**62**	**38**	**7**	74	**6**	73	**-**	**-**	-	-	**-**	**-**	**-**
Leptolyngbya tenuis MACC28	8	**64**	**34**	**12**	74	**5**	73	**34**	**45**	11	11	**7**	**29**	**19**
Halomicronema hondechloris C2206	8	**60**	**34**	**7**	74	**72**	73	**17**	**36**	18	11	**7**	**23**	**6**
Nodosilinea nodulosa UTEX 2910	8	**62**	**33**	**71**	74	**6**	73	**24**	**40**	18	11	**38**	**27**	**34**

3.5. Whole genome analysis of S1 strain

The de novo assembly of the S1 raw genomic sequence reads resulted in an optimal assembly approximately 4.6 Mb genome size had a completeness of 94.05% with low contamination 0.43%. The genome comprising of 144 contigs, with lengths ranging from a minimum of 1,510 bp to a maximum of 265,967 bp, and a N50 value of 78,042 bp (Table No. 5). The experimental *Almyronema* sp. (S1) was found to have a G+C concentration of 50.6%. The RAST server utilized for genome annotations identified approximately 40 genes encoding RNA, which includes one copy of the 16S rRNA gene measuring 1,494 bp. The S1 genome annotation report indicates the presence of 4,595 protein-coding genes distributed across 209 subsystems.

Table 5. Characteristics and summary of the assembled draft genome obtained of S1 sample.

Size of the Genome (Mb)	Genome completeness (%)	Contamination (%)	Length of the genome (bp)	Contig number	G+C (%)	N50 (bp)	L50	CDS
4.6	94.05	0.43	4,661,909	144	50.6	78042	19	4595

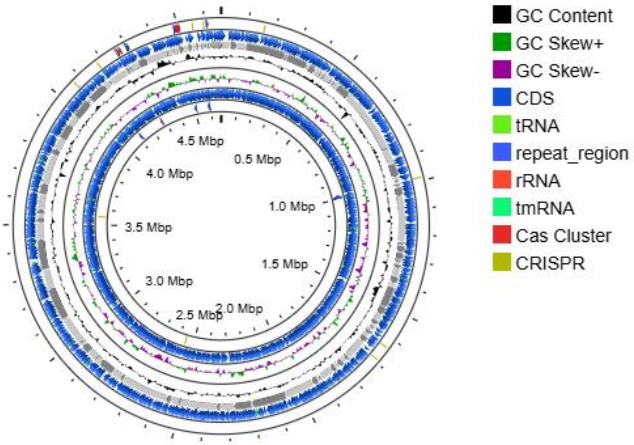

FIGURE 11. Circular chromosome map of *Almyronema epifaneia* (S1) showing the distribution of CDS, tRNAs, rRNAs, GC content skew and CRISPER/Cas genes. The map was generated using Proksee server.

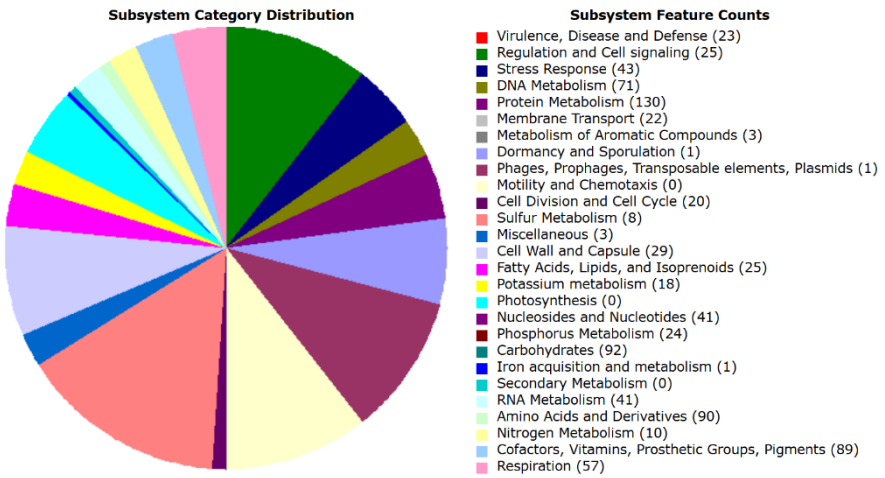

FIGURE 12. Number of genes present on the total CDS of the annotated *Almyronema epifaneia* (S1) genome.

3.6. Anti-UV compound production by *Thainema salinarum* (S19)

To identify if *Thainema salinarum* (S19) contains UV-absorbing MAAs, spectrophotometric examination was first performed on the raw methanolic extract. After 7 days of incubation, the cyanobacterial extract that was mixed with 100% methanol showed absorption peaks at various wavelengths. These wavelengths indicated various photosynthesis pigments and other molecules in the S19 sample (Fig. 13). The absorption spectra confirmed that *Thainema salinarum* (S19) contains MAAs because it absorbs light with a wavelength of about 334 nm. Additionally, after the partial purification of MAAs, the absorption spectra validated the existence of UV-absorbing molecules, MAAs (Fig. 14). The HPLC examination of partly purified MAAs revealed a distinct single peak with a retention period of 2.9 minutes, with a λmax of 334 nm (Fig. 15). The fraction was collected and then subjected to ESI-MS analysis for characterization. The ESI-MS examination of *Thainema salinarum* (S19) produced a significant ion peak of protonated molecules [M+H] + at m/z 333.1 (Fig. 16). UV-VIS spectrophotometric study, HPLC analysis, and ESI-MS analysis indicated Shinorine to be the only MAA present in the sample.

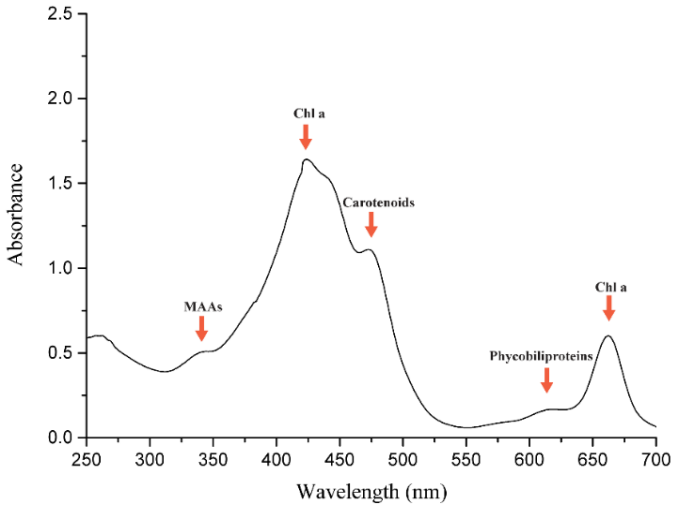

FIGURE 13. Ultraviolet-visible spectrum of a 100% methanolic solution of *Thainema salinarum* (S19) showing peaks for MAAs (332 nm), Chlorophyll a (420 nm and 665 nm), carotenoids (470 nm) and phycobiliproteins (616 nm).

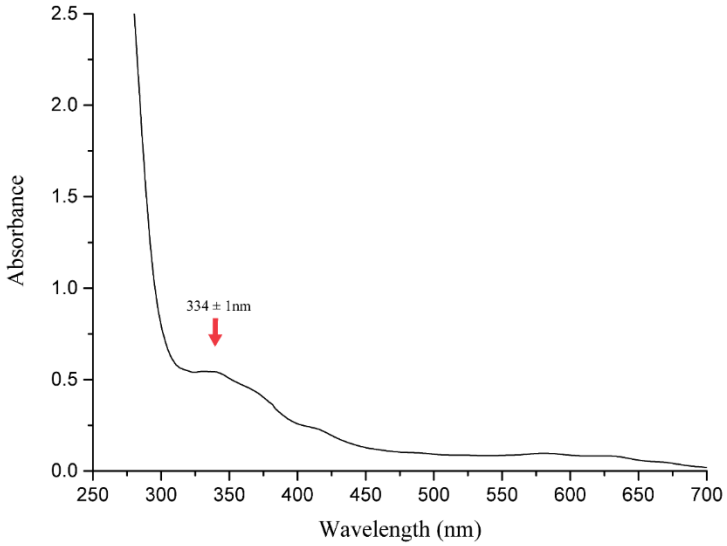

FIGURE 14. Ultraviolet light absorption spectra of *Thainema salinarum* (S19) MAAs in water after partial purification.

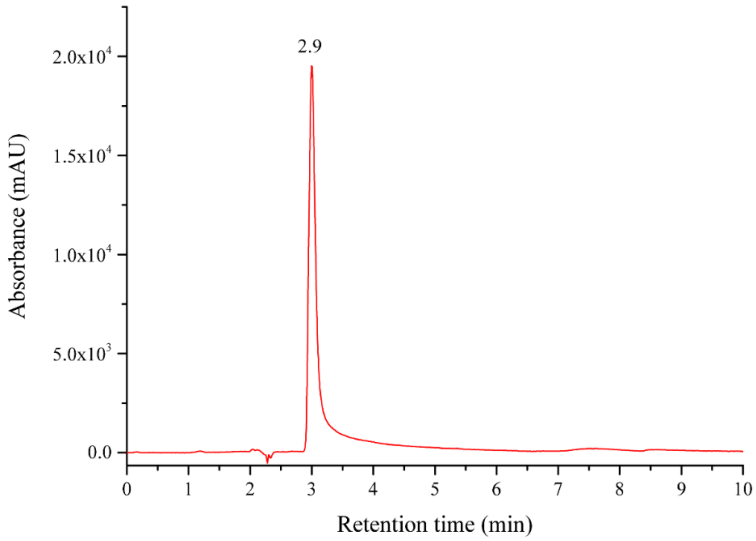

FIGURE 15. HPLC chromatogram of *Thainema salinarum* (S19) partially purified MAAs displays the characteristic peaks at retention time of 2.9 minute.

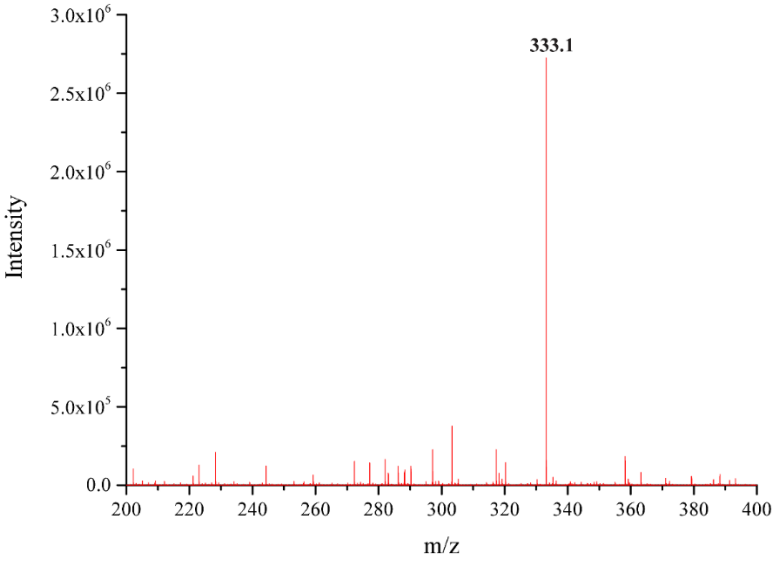

FIGURE 16. Mass spectrum of purified MAAs shows a noticeable peak at m/z 333.1 in the ESI-MS study, which indicates Shinorine.

3.7. Biodiesel compound production from *Almyronema epifaneia* (S1)

Almyronema epifaneia exhibited a lipid content of 11±2% when cultured in standard ASN-III media. It was regarded as a cyanobacteria that produced lipids with an average yield (5–15%) (Uma *et al*. 2020). In order to boost lipid production, the S1 strain was cultivated under four distinct nitrogen concentration ASN-III media conditions with control. Remarkably, as compared to 0.75 g/l $NaNO_3$ mixed controlled ASN-III media, the lipid content rose when the strain grown in 0.5, 1.0, and 1.25 g/l $NaNO_3$ mixed ASN-III medium. The *Almyronema* strain was cultivated in 0.5, 1.0, and 1.25 g/l $NaNO_3$ mixed ASN-III medium, yielding lipid contents of 13±2%, 19±2%, and 15±2%, respectively (Fig. 18). However, in 0.25 g/l mixed ASN-III medium, the lipid percentage dropped by 9.2±2%. In 1.0% NaNO3 mixed ASN-III medium, *Almyronema epifaneia* generated 19±2% of the lipid content, making it a high lipid yielder (above 15%) cyanobacteria (Uma *et al*. 2020).

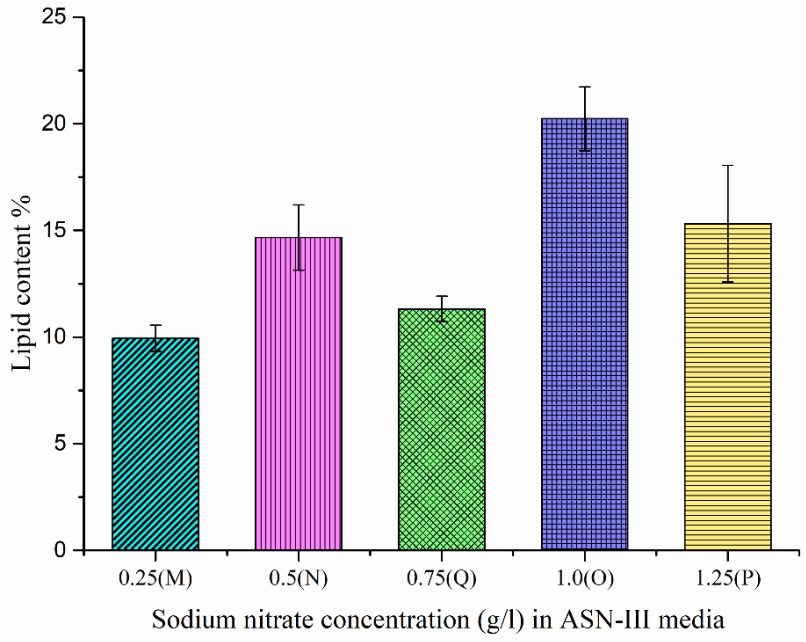

FIGURE 18. Lipid content (as percentage) of *Almyronema epifaneia* grown at various nitrogen concentrations.

Chapter 4. Conclusions and future prospect

The evaluation of the isolated strains using a comprehensive (polyphasic) methodology yields the following findings:

- Strains S1, S6, and S23 were classified as a new genus, *Almyronema epifaneia*, under the family Nodosilineaceae (Roy *et al*. 2024).
- Strains S19 and S23 were reported as *Thainema salinarum*, with the *Thainema* genus being reclassified within the Nodosilineaceae family and the Nodosilineales order in accordance with the most recent cyanobacterial classification by Strunecký *et al*. 2023.
- The draft genome analysis of *Almyronema epifaneia* (S1) was conducted using the latest Next Generation Sequencing technology (NGS). The de novo assembly of the genomic sequence represented the second genome within the Nodosilineaceae family, after the *Nodosilinea* genus.
- *Thainema salinarum* (S19), is a potential source of anti-UV compounds identified from mangrove cyanobacteria. Spectrophotometric, HPLC, and ESI-MS analyses verify that *T. salinarum* produces Shinorine, a mycosporine-like amino acid that which can use in the pharmaceutical and cosmetic industries in future.
- *Almyronerma epifaneia* (S1) strain produced 19% of lipid in 1.0 g/l NaNO3-supplemented ASN-III medium, making it high-yielding cyanobacterium. The lipid content isolated from *A*. *epifaneia* clearly shows that it may be utilized as biodiesel feedstock in future.

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