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Molecular detection of microcystin synthetase genes (*mcy* genes) and semi-quantitative immunological detection of the production of microcystin toxin in *vitro-grown* pure cultures of cyanobacteria

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Abstract

Laboratory mass cultures were established for cyanobacterial strains M. aeruginosa, O. laetevirens var. minimus, A. fertilissima, P. uncinatum, and S. elongatus. The growth of these cultures was assessed by monitoring turbidity, chlorophyll concentration, and protein content. After an 18-day inoculation period, the maximum growth of pure cultures was observed. Well-developed cultures were concentrated using centrifugation and subsequently lyophilized to preserve them in powdered form. DNA extraction was performed on the lyophilized cultures, resulting in clear DNA bands just below the wells. The quality of the extracted DNA, as determined by the A260/280 ratio, ranged from 1.6 to 1.8. The genes mcyABDE were successfully amplified in M. aeruginosa and O. laetevirens var. minimus, while A. fertilissima and P. uncinatum showed amplification of mcyABD and mcyABE genes, respectively. No amplification was observed in S. elongatus. Using a semi-quantitative ELISA technique, a significant concentration of Microcystin was detected only in Microcystis aeruginosa, at a level of 0.5 ppb, whereas the other cultures produced trace amounts below 0.5 ppb.

Keywords: Cyanobacterial strains, Microcystin, Semi-quantitative ELISA, mcyABDE genes.

Introduction

Cyanobacteria serve as photosynthetic agents in aquatic ecosystems, but their excessive proliferation can lead to irritations. These proliferations, known as blooms, form colorful mats on the water's surface. Blooms cause low oxygen levels in water bodies, resulting in hypoxia, which can lead to increased mortality rates among invertebrates, shellfish, fish, and plants. Additionally, blooms can obstruct light from reaching the benthic region of the ecosystem, reducing productivity. Some blooms produce harmful toxins that pose risks to aquatic creatures, livestock, and humans, and are referred to as Harmful Algal Blooms[1-3].

The most commonly reported cyanobacterial toxin is microcystin, which belongs to a family of cyclic pentapeptides with over 90 structural variants. Microcystin's general structure is cyclo (D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha), where X and Z represent variable L amino acids. Microcystin-producing genera in freshwater cyanobacteria include Microcystis, Planktothrix, and Anabaena[4]. Terrestrial strains like Nostoc and Hapalosiphon[5,6] and benthic Phormidium[7] have also been identified as microcystin producers.

Microcystin forms an irreversible covalent bond with the cysteine of eukaryotic protein phosphatase type 1 and 2A[8]. Specifically, the Mdha moiety in microcystin binds covalently to cysteine 273 of the protein phosphatase active site[9,10]. Microcystin acts as a tumor promoter, while nodularin functions as a carcinogen[11,12]. It can cause damage to cell tissue and organs, particularly affecting the mitochondrion, leading to the loss of cytochrome-C, calcium ions, and inter-membrane proteins[13,14]. The release of cytochrome-C triggers caspase cascades, including caspase-3 via caspase-9. Microcystin also plays a role in mitosis regulation[15] and induces oxidative stress in vitro conditions in rat hepatocytic cells and fish tissues[16]. In plants, microcystin inhibits the activity of Protein Phosphatase 1 and Protein Phosphatase 2A, affecting antioxidant enzyme peroxidases and superoxide dismutase, leading to oxidative stress, apoptosis, and hepatotoxicity[17].

The diversity of chemotypes observed in various cyanobacterial taxa is due to microcystin-producing cyanobacteria. On average, individual genera have been found to produce four distinct microcystins, but up to 27 have been identified so far. Furthermore, certain cyanobacterial strains do not produce microcystins, and studies have shown that microcystin-producing and non-producing strains coexist in natural populations.

While research on microcystins has predominantly focused on terrestrial mammals, there is growing recognition of their effects on aquatic organisms. Fish, being at the top of the aquatic food chain, are particularly susceptible to cyanobacterial toxins, which can be ingested or absorbed through their gills. This

poses risks not only to fish but also to humans through the food chain[18].

Harmful cyanobacterial blooms have adverse ecological and environmental impacts, as well as posing risks to animals and humans. Future climate change scenarios predict an increase in bloom-forming cyanobacteria due to changes in hydrological cycling, rising water temperatures, and nutrient loading. As a result, harmful blooms are expected to become more frequent and last longer.

This study investigates diverse cyanobacteria that produce toxins and form blooms, with a focus on their cultivation. In-depth studies have been conducted using both in vivo and in vitro approaches to analyze and compare the concentration of the toxins produced. Furthermore, a comprehensive molecular analysis has been carried out to better understand the characteristics of these toxins.

Materials and Methods

Culture conditions: The axenic pure cultures Microcystis aeruginosa[19], *Oscillatoria laetevirens var. minimus*[20], *Anabaena fertilissima*[21], *Phormidium uncinatum*[22], *Synechococcus elongatus*[23], were provided by Cyanobacterial Research Lab, Dept. of P.G. Studies and Research in Biological Science, R. D. University, Jabalpur, Madhya Pradesh, India. The cyanobacterial strain was fully grown and maintained in BG-11 medium and its composition is given below –

Composition of macronutrient: Independent stock solution each of g/L – Potassium phosphate (4.0), Magnesium sulphate (7.5), Calcium chloride (3.6), Citric acid (0.6), Ferric ammonium citrate (0.6), EDTA (0.1), Sodium carbonate (0.2), Sodium nitrate (85.0).

Composition of micronutrient: Composition of combined stock solution in gm/L – Boric acid (2.86), Manganese chloride (1.81), Zinc sulfate (0.22), Sodium molybdate (0.39), Copper sulfate (0.079), Cobalt nitrate (0.0494).

For the preparation of one liter of medium 10ml, each macronutrient and 1mL of combined micronutrient stock solution was taken, the pH of the medium was adjusted to 7.5 and this was autoclaved at 15 lbs pressure for 20 min at 121˚C.

Growing condition: The culture was kept for growth in an air-conditioned growth chamber at a temperature of 28 ± 2˚C under the cool white fluorescent light of a continuous light intensity of approximately 1500 lux. The culture was manually shaken twice a day for aeration**.**

Estimation of growth: An increase in the density in the pure culture was taken as a growth parameter and increasing total chlorophyll A and protein content was considered for a more precise estimation of growth. For growth studies, aliquots of 3 ml of culture were taken aseptically at regular intervals and optical density (OD) at 750nm was measured for growth, using a spectrophotometer (digital spectrophotometer, model ME 802). Absorbance of the density of the culture was taken against the BG-11 medium blank. Thereafter another growth parameter was chlorophyll concentration, this was done according to the method of MacKinney[24]. The final growth parameter was protein concentration within all pure cultures which were done according to the method of Lowry et al.[25].

Standard curve of Bovine serum albumin: A standard curve was prepared by using the increasing concentration of bovine serum albumin in the different ranges of 100µg - 1000µg per ml according to Lowry et al.[25]. Absorbance was taken of each concentration against the blank at 750 nm by using a digital spectrophotometer (digital spectrophotometer, model ME 802).

Preservation of cyanobacterial cultures: Pure cultures were centrifuged for 10 min at 6000×g at 25ºC to collect the supernatant and the pellets sediment at the bottom surface of the centrifuge tubes. Culture materials were transferred to lyophilization tubes and kept in a pre-freezer and maintained at –20ºC for 1 hour before lyophilization. Lyophilization tubes (Borosil, India) were fitted at the appropriate position of freeze-drier (NSW, India) and the vacuum was set at –20ºC. The process was carried out till the material completely dried in the form of a powder which was transferred into airtight storage vials and kept in the refrigerator for further experimental use.

Isolation of genomic DNA from cyanobacteria cultures: The culture material was lyophilized at –20ºC until it turned into powder and became brittle. It was stored in cryo-vials at 4ºC. Samples collected at different times or locations were dried and stored. Two methods were standardized for the isolation of genomic DNA from dry culture material. In the first method described by Jungblunt and Neilan[26], 25mg of lyophilized cyanobacteria culture material was heated at 65ºC for 2h in 3.0 ml of DNA extraction buffer containing 800 mM ammonium acetate, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 1 % SDS and 1% lysozyme (fresh). Thereafter, 50µl of RNase from a stock of 10 mg ml-1 was added and further incubation was done at 37ºC for 30 min. To stop the reaction, the mixture was chilled in an ice bath for 10 min and centrifuged at 12000×g for 10 min at 4ºC. To one volume of cell extract was added one volume of ice-cold isopropanol and 0.1 volume of 4 M ammonium acetate and centrifuged at 12000×g for 10 min at 4ºC to precipitate the DNA.

DNA quantification and purity assessment: Approximately 10µl of DNA samples obtained from different protocols was added to 990µl of sterile double distilled water. Their purity was checked by taking the ratio of their absorbance at A260/A280 nm. The yield of each sample was also calculated by using the following formula:

A260 × dilution factor × 50µg ml-1

Agarose gel electrophoresis of cyanobacterial genomic DNA: One percent agarose gel was prepared in 0.5X TBE running buffer (45mM Tris-borate/1mM EDTA). To 100 ml warm (70ºC – 80ºC) sterilized TBE buffer, agarose was slowly added followed by gentle stirring so that the agarose dissolves completely. Thereafter, 50µl of ethidium bromide from the stock of 10mg ml^{-1} was added. The temperature of the solution was decreased to 50ºC and was poured onto a sealed gel caster. A comb was placed in the solidifying gel. After solidification, agarose gel was submerged in a running buffer (0.5X TBE buffer). DNA extracted as above (15µl) was mixed with 3µl of bromophenol blue (0.5mg/ml) and glycerol (5µl). This was applied to the wells along with the DNA ladder (HI media, India). Electrophoresis was carried out at 70 V and 25 mA for 1 h in a horizontal slab gel electrophoresis apparatus (Bangalore Genei) until the bromophenol blue reached 3/4th of the bottom of the gel. The tank was filled with 0.5X TBE and the gel was kept submerged throughout.

After the samples had run to a desired distance the gel was taken out from the electrophoresis unit and placed over a UV transilluminator (Biotch R & D laboratories Yercaud). The orange fluorescent bands of DNA were detected under a UV cut-off filter. These bands were photographed using a digital camera (Sony, Japan).

PCR amplification for desired genes of dry cyanobacteria cultures materials: For PCR amplification of mcyABDE genes, the preparation of the reaction mixture and amplification cycle was carried out as described by Jungblunt and Neilan[26] and Kumar[27]. A reaction mixture was prepared containing necessary reagents for PCR amplification of 23µl was prepared using the PCR amplification kit containing a final concentration: 12.5µl of autoclave distilled water, 2.5µl of 10 x taq polymerase buffer, 1µl of 25mM $MgCl_2$, 4µl of 200µM of dNTPs mixture solution, 1µl of 20 pmol forward and reverse primers, 1µl of 1U taq DNA polymerase enzyme, 2µl of template DNA sample 50 ng in each case.

Thermal cycling was performed using a gradient-type thermal cycler (Merck Genei) with an initial denaturation step at 94ºC for 5 min, followed by 40 cycles of 94ºC for 1 min, 60.8ºC for 1 min, and 72ºC for 1 min and a final extension of 10 min at 72ºC. The amplified product was then analyzed using agarose gel electrophoresis as above. The forward and reverse primer pairs[28] as follows were procured from (Imperial Life Sciences, India):

*mcy*A
Forward 5'-AAAATTAAAAGCCGTATCAAA-3'
Reverse 5'-AAAAGTGTTTTATTAGCGGCTCAT-3'
*mcy*B
Forward 5'-CTATGTTATTTATACATCAGG-3'
Reverse 5'-CTCAGCTTAACTTGATTATC-3'
*mcy*D
Forward 5'-GATCCGATTGAATTAGAAAG-3'
Reverse 5'-GTATTCCCCAAGATTGCC-3'
*mcy*E
Forward 5'-TTTGGGGTTAACTTTTTTGGGCATAGTC-3'
Reverse 5'-AATTCGCCGGTATTAGACGTT-3'

Preparation of pure cyanobacterial culture medium for ELISA test: Cyanobacterial pure cultures cultivated at mass scale upto 2 liters in specific growth mediums in the laboratory. Thereafter cyanobacterial cultures were filtered with different kinds of filtration methods used to possibly remove all the finest particles present in the growth medium, as particles may chock ODS cartridges. First of all cyanobacterial growth mediums were filtered with a muslin cloth to remove coarse particles and, thereafter again filtered with Whatman filter paper no. 42 to remove fine impurities. Later, growth mediums were again filtered with the vacuum filtration method using cellulose nitrate filters with pore size 5µm (Axiva Sichem Biotech, India). In the last step, the growth medium was filtered with fine cellulose nitrate membrane filters of 0.45µm (Millipore Corporation, Bedford) under a vacuum. In totality, 1 liter of above-filtered lake water was passed through ODS cartridges (Merck LiChrolut ® RP-18).

Concentrating the cyanobacterial growth medium for the detection of Microcystin: The ODS cartridges were fitted on top with syringes using tubing and manual pressure was applied to the contents of the cartridges. Firstly, the cartridges were pre-washed with 10ml of 100% methanol in order to equilibrate and subsequently, they were washed with 10ml of distilled water. Gradually the entire 1 liter of pure culture was passed through the cartridges. The bound material was first washed with 10 ml of 20% methanol which was discarded. Microcystin was eluted using 10 ml of 100% methanol. The eluted 100% methanolic extract was kept for air drying for 1 to 2 days until methanol was evaporated. The dried matter was suspended in 1 ml of water containing 10% methanol and stored under refrigeration until used for ELISA test.

Pure cultures for ELISA: Two grams of lyophilized pure culture material and pelleted cyanobacterial cultures were used for the detection of the microcystin amount. The first step of extraction was done with 50ml of 100% methanol.

A mixture of dried cyanobacteria powder and methanol was stirred using a magnetic stirrer for 1h. After decanting off the methanol the residues were again extracted under stirring with an additional 50ml of methanol for 1 hour. Both extracts were pooled and kept in solvent until dry. The residues were dissolved in 20% methanol and kept at 4ºC until subjected to ELISA test.

ELISA test for Microcystin: Envirologix inc., (USA) provided the completely available detection ELISA kit. The QualiTube kit (Envirologix Inc., USA) was used for microcystin analysis. In this test, microcystin in a sample competes with enzyme (horseradish peroxidase)-labeled microcystin for a limited number of antibody binding sites on the inside surface of the test tubes. Following list of the chemicals were provided in the kit. Procedure, result and interpretation were done according to the manufacturer's instructions.

Results and Discussion

Standard curve of protein: The protein standard curve was generated using Bovine serum albumin (BSA) with known concentrations, following the method described by Lowry's. Absorbance measurements were taken at 750 nm using a Digital spectrophotometer, comparing each concentration against the blank. Microsoft Excel was used to create a graph with a regression equation, yielding an R2 value of 0.986. This indicates a confident limit exceeding 98% and confirms the acceptability of the standard curve (Figure-1).

Growth curve: The growth curve was determined through spectrophotometric analysis, which involved monitoring the increasing density of pure cultures of *M. aeruginosa, O. laetevirens var. minimus, A. fertilissima, P. uncinatum*, and *S. elongatus.* The density of the cultures was assessed by measuring the absorbance of light at 750nm against the blank of the growth medium. These cyanobacterial pure cultures are naturally occurring photosynthetic microorganisms.

The growth pattern of the cyanobacterial culture resembled that of other bacteria, exhibiting distinct phases such as the lag phase, log phase, exponential growth phase, stationary phase, and decline phase. In order to support normal growth, cyanobacteria synthesize proteins, chlorophyll, and various enzymes. Estimating the concentration of protein and chlorophyll is an important parameter for measuring growth.

The growth, chlorophyll, and protein concentrations were estimated over a period of 15 to 18 days from the day of inoculation (Figure-2).

Concentration of chlorophyll: Photosynthetic microorganisms contain the green chlorophyll pigment in their cellular structure, which contributes to the intensity of the green color observed in cyanobacterial cultures. The concentration of chlorophyll pigment was estimated using the spectrophotometric method at 665nm, with absorbance readings taken against the methanol blank. The maximum chlorophyll concentration was observed on the eighteenth day of growth in pure cultures of *M. aeruginosa, O. laetevirens var. minimus, A. fertilissima, P. uncinatum*, and *S. elongatus*, approximately measuring 27.21, 26.13, 25.12, 27.97, and 24.21µg/ml, respectively (Figure-3).

Concentration of protein: The protein concentration was estimated in fully grown cyanobacterial cultures following the method described by Lowry et al (1951)[25]. A digital spectrophotometer was used at 750nm, and absorbance readings were taken against the blank. Cyanobacterial cultures not only synthesized proteins but also various enzymes to utilize the different nutrients present in the growth medium. The highest protein concentration was estimated on the eighteenth day of growth in cyanobacterial cultures of *M. aeruginosa, O. laetevirens var. minimus, A. fertilissima, P. uncinatum*, and *S. elongatus*, approximately measuring 62.88, 64.34, 56.60, 61.42, and 59.11µg/ml, respectively (Figure-4).

Microcystin-producing genotypes in isolated pure cultures of cyanobacteria: Pure cultures of cyanobacteria were examined for the presence of microcystin-producing genotypes. Five pure cultures, namely *Microcystis aeruginosa, Oscillatoria laetevirens var. minimus, Anabaena fertilissima, Phormidium uncinatum*, and *Synechococcus elongatus*, were obtained from the laboratory-grown culture collection. These cultures were isolated from different water bodies and identified through molecular analysis (16S rDNA analysis). They were maintained in specific culture media for over three years (Table-1).

Isolation of total DNA and its purity analysis in pure culture of cyanobacterium: DNA extraction was performed from freeze-dried cells of the pure cultures, and the purity of the extracted DNA was assessed by determining the A260, A280, and the A260/A280 ratio. The A260/A280 ratio ranged between 1.6 and 1.8, indicating good DNA purity. The DNA concentration in the cyanobacterial pure cultures ranged from 75 to 150µg/ml. Agarose gel electrophoresis confirmed the presence of intact genomic DNA, showing a single sharp band just below the starting point without any smearing or lower molecular weight DNA bands (Figure-5).

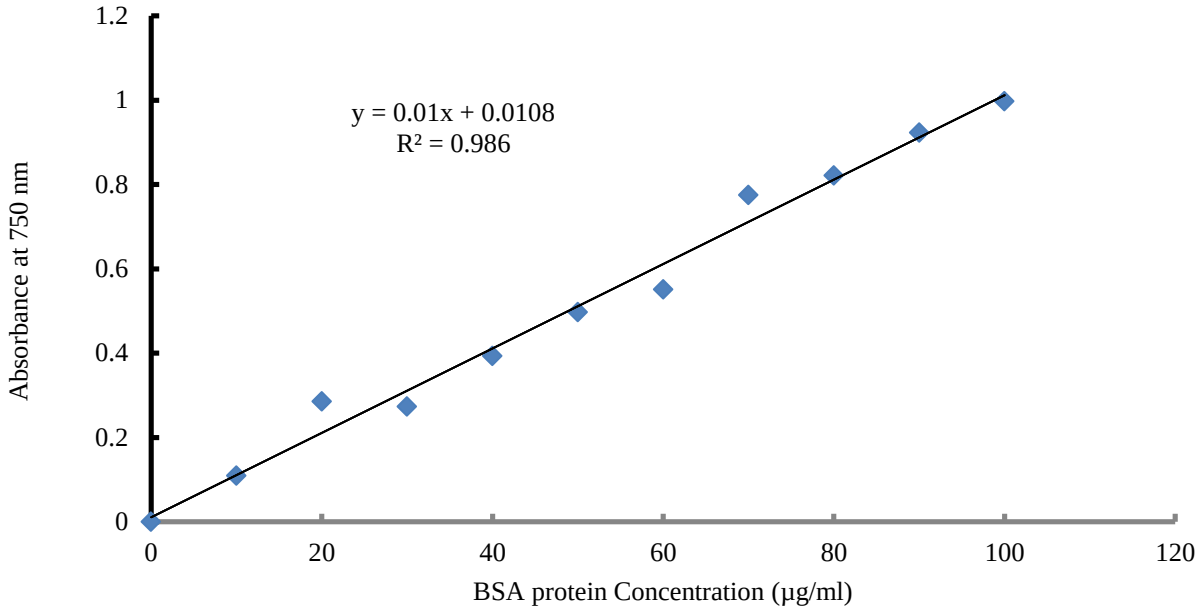
Occurrence of mcy genes in pure cultures: Amplification of microcystin synthetase genes was observed in *M. aeruginosa* and *O. laetevirens var. minimus*, with the presence of *mcyA*, *mcyB*, *mcyD*, and *mcyE* genes. In *A. fertilissima*, three genes *(mcyA, mcyB, and mcyD)* were amplified, while in *P. uncinatum*, the *mcyA, mcyB, and mcyE* genes were amplified. No mcy genes were detected in the DNA sample of *S. elongatus*. The amplified amplicons of *mcyABDE* exhibited sizes of 291-297 bp, 800 bp, 818 bp, and 472 bp, respectively (Figure-6).

Discussion: In this study, laboratory cultures of *Microcystis aeruginosa, Oscillatoria laetevirens var. minimus*[28], *Anabaena fertilissima*[29], *Phormidium uncinatum*[30], and *Synechococcus elongatus*[31] were grown under mass cultivation conditions. The growth of these cultures was estimated based on parameters such as absorbance, protein concentration, and chlorophyll concentration. Visual observations revealed that fully grown cultures of these cyanobacteria produced various biochemical substances, including proteins and enzymes, for cellular metabolic processes, as well as secondary metabolites and microcystin. Microcystin is a toxin synthesized by a multi-enzyme complex system without mRNA translation.

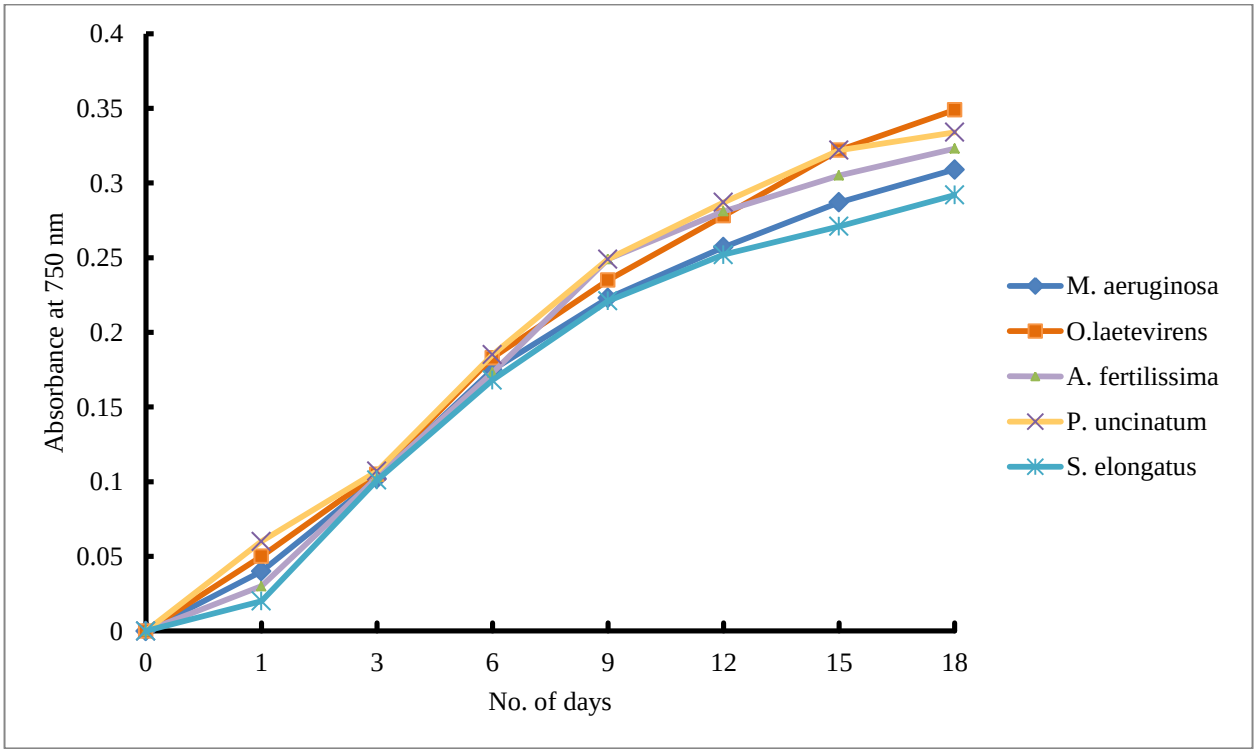
In the current study, *M. aeruginosa* and *O. laetevirens var. minimus* showed amplification of genes *mcyABDE*, while *A. fertilissima* exhibited amplification of genes *mcyABD*, and *P. uncinatum* carried out amplification of genes *mcyABE*. In contrast, *S. elongatus* did not show any amplification of mcy genes. These differences between natural and laboratory growing conditions can lead to variations in the activation and

deactivation of microcystin synthase genes, resulting in changes in microcystin toxicity[32,33].

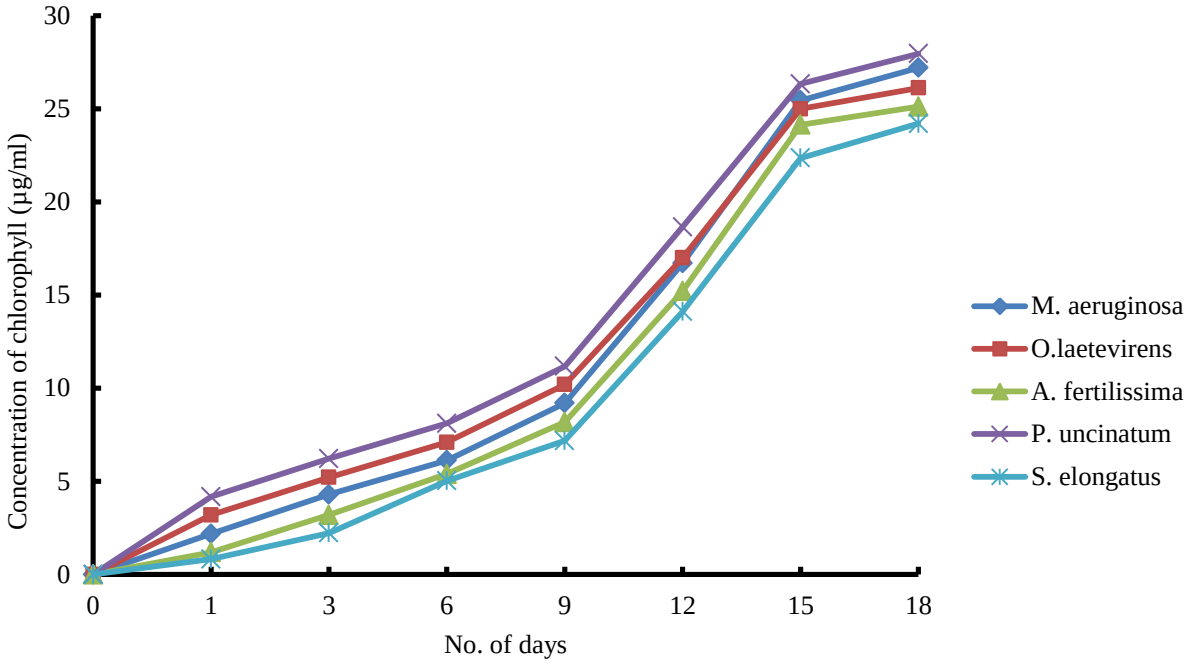
In eutrophicated lakes, harmful cyanobacterial blooms thrive rapidly when there are elevated levels of nitrate and phosphate due to industrial, agricultural, sewage, and human activities. These algal blooms have a detrimental impact on the natural ecology and disrupt the food chain[34-36].



Figure–1: Standard curve of protein prepared from BSA protein against blank by spectrophotometeric analysis.



Figure–2: Growth curve of cynobacterial pure cultures on the basis of turbidity development.



Figure–3: Growth curve of cynobacterial pure cultures on the basis of chlorophyll concentration.

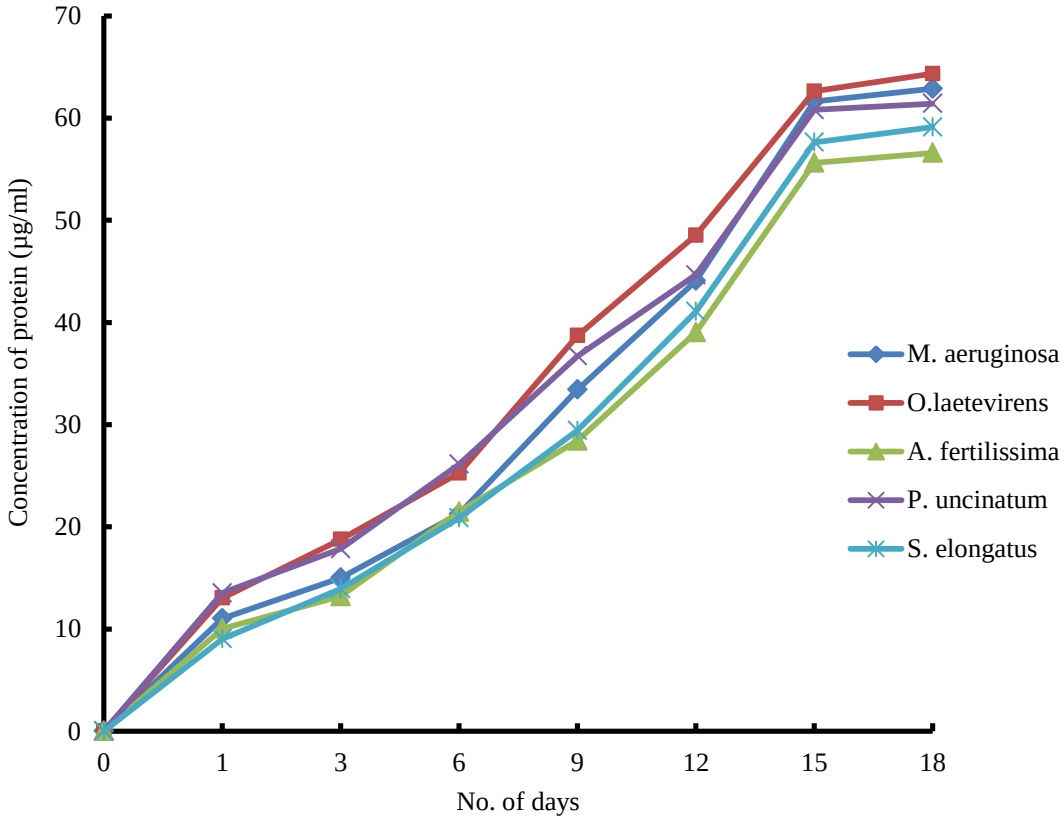


Figure-4: Growth curve of cynobacterial pure cultures on the basis of protein concentration.

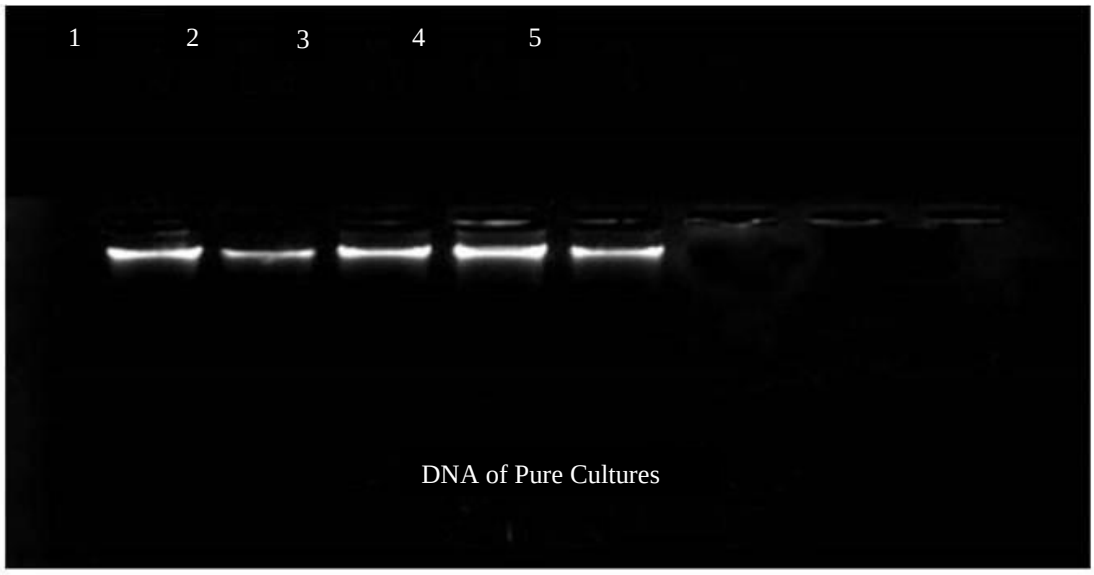


Figure-5: DNA bands in Lane (1) *M. aeruginosa*, (2) *O. laetevirens var. minimus*, (3) *A. fertilissima*, (4) *P. uncinatum* and (5*) S. elongatus* of pure cultures.

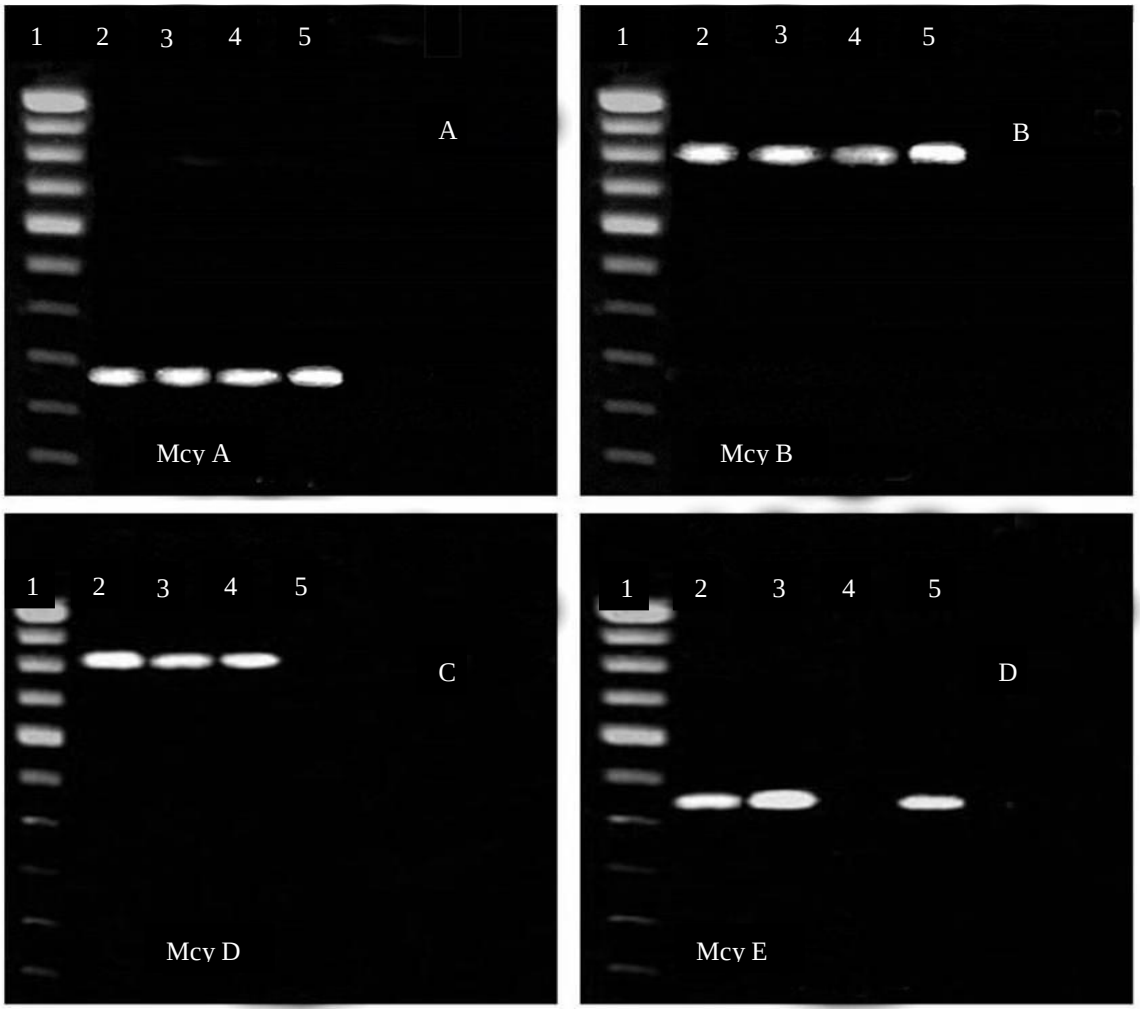


Figure-6: PCR gene amplification (1) *M. aeruginosa* (2), *O. laetevirens var. minimus*, (3) *A. fertilissima*, (4) *P. uncinatum* and (5) *S. elongatus* of pure cultures and *mcyABDE* resolved at 291 – 297, 800, 818 and 472 bp of amplicons in size respectively.

Table-1: Level of cell bound microcystin in pure culture.

S.No.	Pure Culture in Laboratory	Microcystin level (ppb)
*	0.5 ppb calibrator	0.5 ppb
*	3.0 ppb calibrator	3.0 ppb
01.	*M. aeruginosa*	≥0.5 ppb ; ≤3.0 ppb
02.	*O.laetevirens var. minimus*	≤ 0.5 ppb
03.	*A. fertilissima*	≤ 0.5 ppb
04.	*P. uncinatum*	≤ 0.5 ppb
05.	*S. elongatus*	≤ 0.5 ppb

In a eutrophicated environment, many harmful and toxic cyanobacterial bloom and mat-forming species compete with normal cyanobacteria, bacterial species, microbial flora, coliforms, and water-borne bacteria present in the lake's natural aquatic environment. To maintain an antagonistic relationship with other microbes, these cyanobacteria produce higher levels of microcystin. Natural conditions activate *mcy* genes to a greater extent, allowing for maximum microcystin synthesis[33,37-40].

Pure cultures of *M. aeruginosa* cultivated in controlled laboratory conditions, with a defined and limited nutrient supply and no antagonistic relations with other organisms, result in lower amounts of microcystin compared to natural bloom samples collected from various lakes, ponds, and reservoirs in previous surveys[41-43]. *O. laetevirens var. minimus*, *A. fertilissima*, and *P. uncinatum* produced trace amounts of microcystin under laboratory conditions due to the absence of other microorganisms for an ecological antagonistic relationship. Similarly, earlier studies have reported higher microcystin levels in natural scums and mat samples containing these genera[44, 45].

Conclusion

The laboratory-cultivated cyanobacterial pure cultures were subjected to controlled growth conditions in a specific growth medium, allowing for continuous monitoring within a defined time frame. Three key parameters, namely turbidity, chlorophyll concentration, and protein concentration, were measured to assess the growth. The eighteenth day of growth was found to exhibit maximum growth. Subsequently, the well-developed cultures were concentrated and preserved for DNA extraction. High-quality DNA was successfully extracted from these pure cultures and utilized for PCR amplification of mcy gene(s). Interestingly, most cyanobacterial DNA samples displayed amplification of mcy genes, except for S. elongatus which did not show any gene amplification. Despite the presence of amplified mcy genes, the majority of cyanobacterial species produced only trace amounts of microcystin, measuring below 0.5 ppb. Notably, *M. aeruginosa* K1 exhibited relatively higher levels of microcystin production ranging between 0.5 - 3.0 ppb.

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