Turkish Online Journal of Qualitative Inquiry (TOJQI)
Volume 12, Issue 10, October 2021: 1855-1866

SCREENING AND SYSTEMATIC EVALUATION OF ACTIVE PHYTOCHEMICAL CONSTITUENT OF CYANOBACTERIAL ISOLATES

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ABSTRACT

Cyanobacteria drive biogeochemical cycle and produces active metabolites viz., carbohydrates, proteins, lipids, alkaloids, flavonoid, phenols and saponins. These phytochemical metabolic constituents have played a crucial role to retain cellular structure, cell to cell communication, cellular defence, healthcare and household applications. The present work was done to screen out and systematic evaluation of active phytochemical constituent of Cyanobacteria isolated from rice field and pond. Two hundred samples were screened for cyanobacteria strains and *Anabaena* sp. (45%) and *Cylindrospermum* sp. (42%) observed to occupy maximum density in the samples. The maximum carbohydrate content (33.7 %) and protein (25.48±0.29) content was observed in *Anabaena* sp. while lipid (9.93 %) and amino acid content (4.82±0.14) in *Geitlerinema* sp. *Anabaena* sp., *Cylindrospermum* sp., *Cyclotella* sp. and *Diatoma* sp were found to retain most active fractions. The combination of Column chromatography, HPLC and FTIR were used to identification these active fractions. The active fractions i.e., α-cyclocitral, limonene, inositol and hexa-decanoic acid were identified. These active phytochemical constituent could further be explored for their utilization in healthcare, household and industrial applications.

Keywords: cyanobacteria, isolation, screening, phytochemical constituents,

1. INTRODUCTION

Cyanobacteria are well known for the conversion of carbon dioxide (CO_2) into its monomer that has played a crucial role in the synthesis of metabolites during the central metabolic process necessary to survive living organisms to life. Cyanobacteria most often synthesize organic metabolites (primary and secondary) to drive the biogeochemical cycle (Fiore et al., 2015; Stuart et al., 2016). Diverse group of phyochemical viz., primary (carbohydrates, proteins and lipids) and secondary (alkaloids, flavonoid, phenols, saponins) metabolites is the major constituent of cyanobacterial culture. These phytochemical constituents are crucial for structural organisation of cell, communication between cells and defence against certain ecological (biotic and abiotic) factors (Stuart et al., 2016; Kujawinski, 2011; Yadav et al., 2011). Cyanobacteria e.g. *Anabaena* sp., *Aphanocapsa* sp., *Calothrix* sp., *Chlorogloea* sp., *Chroococcus* sp., *Cyanobacterium* sp., *Cyanocystis* sp., *Gloeocapsopsis* sp.,

*Hapalosiphon*sp., *Leptolyngbya* sp., *Lyngbya* sp. *Oscillatoria* sp. *Phormidium* sp., *Porphyrosiphon* sp., *Microcystis* sp., *Nostoc* sp. and *Scytonema* sp.have been most often reported to present in the freshwater bodies (Saha et al., 2007). Cyanobacteria (e.g. *Anabaena, Nostoc, Porphyridium* and etc.) have now become more prevalently applicable to serve pharmaceuticals as food fortification and nutritional agent (Nutraceutical) due to their capability to synthesize amino acids, essential micronutrient elements, fatty acids (Omega 6 and Omega 3) and vitamins (Bishop *et al*., 2012). The present course of investigation was focused on the screening and systematic evaluation of the phyto-content of Cyanobacterial isolates to explore their potential for applicability in pharmaceuticals and nutraceutical sector.

2. MATERIALS AND METHODS

2.1 Sample collection, Isolation and Identification of cyanobacteria

The water samples were collected from lakes, water reservoirs and rice crop fields of Bilaspur, Chhattisgarh region. The water samples were collected in polyethylene terephthalate (PET) bottles aseptically. The samples were collected from surface; discrete depth and depth using sterile rope. The protocol for the isolation and purification of cyanobacteria from soil were adopted from Ferris and Hirsch (1991). Samples were preserved in Lugol's iodine solution at the time of sampling and brought to laboratory and stored in ambient temperature at slightly dark light. The sample was mixed with 100 ml of double distilled water and filtered via Millipore Membrane Filter. The filter was then placed onto BG-11 medium for 30 days to stimulate the cyanobacterial growth for further microscopic examinations and as cyanobacterial colonies appeared, they were transferred to fresh BG-11 medium (Stanier et al., 1971). Pure cultures were prepared by subsequent sub-culturing and streaking (incubation condition was 25± 2^0C under 16 h light (4000 lux) and 8 h dark). Each pure culture of cyanobacterial strains was harvested and processed for biochemical analysis.

Cyanobacteria was identified and characterized as per their morphological variation and taxonomic characteristics (Jensen and Solndergaard, 1985). Morphological traits were examined under polarizing light microscope (Olympus CX31-P-HD) and further specified under inverted microscope using micropipette (Rai and Rajashekhar, 2016). The final identification was carried out as per keys and monographs described by Prescott (1951) and classification system adopted from Desikachary (1959). The microphotographs of 40x and 100x magnifications of each identified cyanobacteria were done using Nikon-HD Camera.

Morphological characteristics were recorded as filamentous or unicellular type of cellular appearance. The dimension (widths and heights) of cyanobacterial cells were measured by micrometry. Systematics (naming and relationships between species) was done as per algae datasheet of the department.

2.2. Quantitative analysis of cyanobacterial constituent biomolecules

2.2.1. Total carbohydrate content

A 100 mg of dried cyanobacterial sample was mixed with 2.5N hydrochloric acid at 100ºC for one hour for hydrolysis. The total carbohydrate content in the sample was determined as mentioned by Dubois et al. (1956). A 0.2 ml of hydrolyzed sample transferred with 5% of phenol (1.0 ml) and

concentrated sulphuric acid (H_2SO_4) (5.0 ml) and kept in a water bath at 25^0C for 30 minutes. The absorbance of the mixture was then assessed at 490 nm using UV-Visible Spectrophotometer (Systronics India Limited) against the blank. The standard was prepared using glucose at a range of 10 to 100 µg ml^{-1}.

2.2.2. Total Protein content

The total protein content was estimated by Lowry's method (Lowry et al., 1951). The 6% Trichloro Acetic Acid (TCA) was used to precipitate cyanobacterial proteins. The protein was extracted using 2% alkaline Na_2CO_3 reagent (4.5 ml at 55^0C) and $CuSO_4$- $KNaC_4H_4O_6$ for three min. The filtrate was then collected and diluted to 5.0 ml with alkaline reagent. The Folin-Ciocalteu reagent (0.5 ml) was added, mixed vigorously and kept at room temperature for ten min for development of color. The absorbance of the filtered mixture was taken at 750 nm using UV-Visible Spectrophotometer (Systronics India Limited) against the blank. The standard was prepared using Bovine Serum Albumin (BSA) standards at a range of 40 to 200 µg ml^{-1}.

2.2.3. Free amino acid content

The free amino acid content was estimated using Moore and Stein's ninhydrin reaction assay (Moore and Stein, 1948). A 50 mg of dried cyanobacteria was mixed with 80% ethanol (10 ml) for hydrolysis. The hydrolyzed sample (1.0 ml) was mixed with Ninhydrin reagent (3.8 ml) and kept on Boiling Water Bath (BWB) for 12 min. The sample was then assessed at 570 nm using UV-Visible Spectrophotometer (Systronics India Limited) against the blank. The standard was prepared using leucine solution at a range of 10 to 100 µg ml^{-1}.

2.2.4. Total lipid content

Total lipid content was determined using Folch's method (Folch *et al.,* 1957). To evaluate total lipids content, the dried cyanobacteria (100 mg) was hydrolyzed using Chloroform:Methanol solvent (2:1, v/v) and the 1.0 ml of filtrate of hydrolyzed sample was transferred to a pre-weighed glass weighing bottle followed by evaporator drying. The final weight was noted. The final weight was deducted by initial weight to get total lipid content.

2.3. Assessment of bioactive secondary metabolites of Cyanobacterial isolates

Bioactive secondary metabolite profiles of cyanobacterial strains were performed by standard protocol (Harborne, 1973; Kokate, 2005).

2.3.1. Alkaloids

Alkaloid's content was estimated by Wagner's test. Aqueous extract (1 ml) of cyanobacterial isolates were mixed with a few drops of Wagner's reagent and the appearance of reddish-brown color confirmed the presence of alkaloid.

2.3.2. Cardiac Glycosides

Cardiac Glycosides content was evaluated using Keller Kelliani's Test. A one milliliter of aqueous extract of cyanobacterial isolates were mixed with glacial acetic acid (1 ml) and few drops of 5%

ferric chloride solution. The mixtures were then added with 0.5 ml of conc. H_2SO_4. The appearance of a brown ring at the interface confirmed the presence of cardenolides.

2.3.3. Flavonoids

Flavonoid content was determined using alkaline reagent (NaOH). A one milliliter of aqueous extract of cyanobacterial isolate was mixed with few drops of 20% Sodium Hydroxide (NaOH). The appearance of a yellowish color that becomes colour-less upon the addition of dilute HCl (0.5 ml) was the characteristic feature of the presence of flavonoids in the sample.

2.3.4. Phenols

Phenol content was evaluated using Ferric Chloride ($FeCl_3$). A one milliliter of aqueous extract of cyanobacterial isolate was mixed with few drops of aqueous $FeCl_3$ (5%) and the appearance of deep blue or black color indicates the presence of Phenols in the sample.

2.3.5. Saponins

A one milliliter of aqueous extract of cyanobacterial isolate was shaken vigorously and the formation of persistent foam indicates the presence of saponins.

2.3.6. Tannins

Tannin's content was estimated using Braymer's test. A one milliliter of aqueous extract of cyanobacterial isolate was mixed with 10% alcoholic ferric chloride solution (1 ml) and the appearance of blue or greenish color.

2.3.7. Terpenoids

Terpenoids content was determined using Salkowski's test. A one milliliter of aqueous extract of cyanobacterial isolate was mixed with chloroform (0.5 ml) and few drops of conc. H_2SO_4. The appearance of reddish brown precipitate indicates the presence of Terpenoids in the sample.

2.4. Characterization of Cyanobacterial Isolates

2.4.1. Column Chromatography (CC)

The aqueous extract of cyanobacterial isolate was passed with the Sephadex G-25 column with 0.2 M of NaCl as eluting agent. The fractions were collected sequentially and again tested for phytochemicals.

2.4.2. High Performance Liquid Chromatography (HPLC)

The purified samples were re-assessed by HPLC (CHEMITO) integrated with KNAUER high pressure liquid pump and UV-Vis-2501 detector. The sample was mixed with methanol and 10 µl of aliquot was injected to the port. The C-18 column was used. The Methanol and Distilled water at a ratio of 70:30 (v/v) was used a solvent at a flow rate of 1 ml/min at 240 nm. The peak retention time was recorded.

2.4.3. Fourier Transform Infrared Spectroscopy (FTIR)

The HPLC purified fraction was eluted and nylon membrane (0.25 μm, Axiva Sichem, Biotech) filtered. Filtered fraction was further subjected to vacuum drying (hotplate, Remi, India at 40^0C) in combination with vacuum evaporator. The dried fraction was processed with KBr to make sample for FTIR (FTIR-8400S, DRS mode, Shimadzu Scientific Instruments) analysis.

2.5. Statistical analysis

The results were processed and analyzed using Microsoft excel and the quantitative values were expressed with standard deviation (±).

3. RESULTS AND DISCUSSION

3.1.1. Sample collection, Isolation and Identification of cyanobacteria

A total of two hundred (200) water samples were collected from Bilaspur, Chhattisgarh. Lakes, Pond (Tifra talab and Karbala pond) and Rice fields were selected as the major sampling sites to collect water samples. Out of 200 water samples, 53 samples consisted of cyanobacteria strains. Among them, 35 cyanobacterial isolates were isolated**.** Nostocales viz., *Anabaena* sp. (45%) and *Cylindrospermum* sp. (42%) were present dominantly in the samples. Chorococcales viz., *Cyclotella* sp.(2%), *Diatoma* sp.(7%) and *Chroococcus* sp.(2%) mordantly while Oscillatoriales viz., *Oscillatoria* (1%) present in the least amount.

Upon the examination of morphological characteristics of cyanobacterial isolates under light microscope, the *Anabaena* sp. (NCS/BGA/19/AM/521), *Cylindrospermum* sp. (NCS/BGA/19/AM/522), *Cyclotella* sp. (NCS/BGA/19/AM/523), *Diatoma* sp. (NCS/BGA/19/AM/524), *Chroococcus* sp. (NCS/BGA/19/AM/525), *Geitlerinema* sp. (NCS/BGA/19/AM/526) and *Oscillatoria* sp. (NCS/BGA/19/AM/527) were observed in the samples (Fig.1). The labeled cultures (NCS/BGA/19/AM/521 to NCS/BGA/19/AM/521) have been deposited in the NCS Green Earth Private Limited, Nagpur (M.S.).

3.1.2. Quantitative analysis of cyanobacterial constituent biomolecules

The maximum carbohydrate content (dry weight) was observed in *Anabaena* sp. (33.7 %) while least in *Geitlerinema* sp. (18.04%). But, lipid content was recorded higher in *Geitlerinema* sp. (9.93 %). The protein content was distributed among cyanobacterial isolates at a range between 24.63±0.09 to 31.58±0.08. The *Anabaena* sp. (25.48±0.29) contained least protein content while *Geitlerinema* sp. (31.58±0.08) consisted of higher amount of proteins. *Oscillatoria* sp. (2.7±0.07) was observed with lower amino acid contents while *Geitlerinema* sp. (4.82±0.14) recorded with higher amino acid content. The graphical representation of quantitative profile of cyanobacterial constituent biomolecules is shown in fig. 2. Quantitative analysis of cyanobacterial constituent is tabulated in Table No. 1.

3.1.3. Assessment of bioactive secondary metabolites of Cyanobacterial isolates

Cyanobacterial isolates i.e. *Anabaena* sp. *Cylindrospermum* sp. *Cyclotella* sp. and *Diatoma* sp. were observed for the significant secondary metabolite content (Table 2.). Phenols, Flavonoid, Saponins and Alkaloids were recorded in all selected cyanobacterial isolates. Besides, Tannins and Steroids were not observed in any of the cyanobacterial isolates.

3.1.4. Characterization of Cyanobacterial Isolates:

Aqueous extract of*Anabaena* sp., *Cylindrospermum* sp., *Cyclotella* sp. and *Diatoma* sp. was purified by column chromatography in 40 fractions. Among forty (40) fractions, fraction 5 to14, 9 to 13, 11 to 16 and 25 to 30 achieved for *Diatoma* sp., *Anabaena* sp., *Cylindrospermum* sp. and *Cyclotella* sp. respectively. These fractions showed the presence of phytochemicals upon re-assessment. These fractions were further subjected to HPLC analysis. The fractions obtained through column chromatography were processed in HPLC. Varieties of peaks were received as HPLC graph of *Anabaena* sp., *Cylindrospermum* sp., *Cyclotella* sp. and *Diatoma* sp. (Fig. 3). Among them, significant and sharp peaked fraction (active fraction) was further selected for FTIR assessment. Active fractions from *Anabaena* sp., *Cylindrospermum* sp., *Cyclotella* sp. and *Diatoma* sp. were then assessed by FTIR to reveal presence of function groups so that they could further be characterized for suitable applicability in pharmaceuticals and nutraceuticals. Hydroxy, aromatic, aldehyde, ketone and substituted benzene were observed with Anabaena. Acetyl, aldehyde, alkyl, aryl ether and alkane group divulged with Cylindrospermum. Carbonyl, methylene and alkane group were observed with Cyclotella. Amino-aryl ether, amino-alkyl and hydroxyl group Diatoma. As per the final correlation of active factions of *Anabaena* sp., *Cylindrospermum* sp., *Cyclotella* sp. and *Diatoma* sp. database obtained from HPLC and FTIR, the purified fractions were identified as α-cyclocitral, limonene, inositol and hexa-decanoic acid respectively.

Discussion

Results exhibited the screening of cyanobacteria from rice fields and ponds. To systematically evaluate phyto-content of Cyanobacterial isolates, the secondary metabolites of cyanobacterial strains were purified by TLC and silica gel column chromatography and purified fractions were identified by HPLC and FTIR. The extracellular biomolecules were also estimated. Hosmani and Anitha (1998) revealed significant carbohydrate and protein contents in *Microcystis aeruginosa.* The extracellular secretions of *Oscillatoria* sp., *Schizothrix* sp. and *Synechocystis* sp. have been revealed to have significant carbohydrate and protein content (Kawaguchi and Decho, 2000; Kawaguchi et al., 2003). Walach (1987) reported that carbohydrate synthesis was inversely related to the nitrogen availability while carbon content directly proportional towards the same. The carbohydrate and protein content of cyanobacteria serve as nutraceutical in food processing sector. Another application of extracellular carbohydrate and protein content of cyanobacteria was documented for metal removal (Shah et al., 2000). As the cyanobacterial cells are well-known for their adaptation to adverse environmental conditions, thereby applicability of cyanobacteria in metal removal is well-justified. The cyanobacterial cell surface consisted of polysaccharide with numerous binding sites for metal ions (MBP-Metal Binding Proteins) such as cysteine ligands for heavy metals (Bind et al., 2019). Growth promoting agents were also divulged on cyanobacterial extracellular secretions (Safonova and Reisser, 2005). The maximum 31.58% of protein and 9.93 % lipid content (calculated against dry weight) was recorded in *Geitlerinema* sp. Ansari and Fatma (2016) conducted an investigation on different *Spirulina* sps. and almost similar findings were reported as seems in present analytical work. Contradictory, the Shashikumar and Madhyastha (2002) found 18.4% of amino acid content in the *Synechococcus aquatilis.* Whereas Rajeshwari and Rajashekhar (2011) reported that *Phormidium tenue* exhibited 5.24% of amino acids content this could be correlated with present findings. A cyanobacterium also secretes growth promoting bioactive substances for efficient

growth of plants (Misra and Kaushik, 1989). The variations among biochemical profile of cyanobacteria very on basis of species to species and physiochemical condition of culture medium (Subhashini et al., 2004; Rosales et al., 2005). Rosales et al. (2005) observed high chlorophyll, carotenoids, carbohydrates and proteins constituent in cell under optimum nutritional feeding. *Anabaena* sp., *Cylindrospermum* sp., *Cyclotella* sp. and *Diatoma* sp consisted of α-cyclocitral (a food additive), limonene (cleaning agent with aroma), inositol (vitamin like sugar that participate in cell signalling) and hexa-decanoic acid (fatty acid having medical applications) respectively as active ingredients. Similar cyanobacterial active ingredients were earlier documented in other cyanobacterial species that was helped to correlated the authenticity of our data (National Center for Biotechnology Information, 2021; Shetty et al., 2006; Chittora et al., 2020; Prihantini et al., 2018). However, the aspect and application of these ingredients were different in these literatures.

4. CONCLUSION

The present course of investigation was carried out to screen and systematic evaluation of phytochemical constituents of Cyanobacterial species isolated from rice fields and ponds of Chhattisgarh region. *Anabaena* sp., *Cylindrospermum* sp., *Cyclotella* sp., *Diatoma* sp., *Chroococcus* sp. and *Oscillatoria* are present in the samples. Among them *Anabaena* sp., *Cylindrospermum* sp., *Cyclotella* sp. and *Diatoma* sp were evaluated for the assessment of active ingredients and α-cyclocitral, limonene, inositol and hexa-decanoic acid were divulged using the combination of Column chromatography, HPLC and FTIR based analytics respectively. These ingredients have been document for their application in food additives, household cleaning, and cell signaling and medical applications. The future prospect of the present study could be to explore more supporting database of the identified active ingredients for their applicability.

5. ACKNOWLEDGEMENT

The authors express their gratitude to MATS school of Biological and Chemical Sciences, MATS University, for providing central laboratory facilities.

6. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

Table 1. Quantitative analysis of cyanobacterial constituent bimolecules

S.N.	Name of Algae	Protein (±SD)	Carbohydrate (±SD)	Amino Acid (±SD)	Lipid (±SD)
1.	*Anabaena* sp.	25.48 ±0.29	33.7 ±0.52	3.68 ±0.09	7.21 ±0.11
2.	*Cylindrospermum* sp.	27.63 ±0.31	27.23 ±0.28	3.91 ±0.05	8.26 ±0.17
3.	*Cyclotella* sp.	30.32 ±0.22	26.3 ±0.07	3.9 ±0.08	8.3 ±0.20
4.	*Diatoma* sp.	27.9 ±0.18	30.28 ±0.30	2.32 ±0.07	5.69 ±0.06
5.	*Chroococcus* sp.	24.63 ±0.09	28.01 ±0.11	4.03 ±0.10	7.63 ±0.02
6.	*Geitlerinema* sp.	31.58 ±0.08	18.04 ±0.09	4.82 ±0.14	9.93 ±0.11
7.	*Oscillatoria* sp.	26.38±0.15	27.43±0.25	2.7±0.07	8.02±0.05

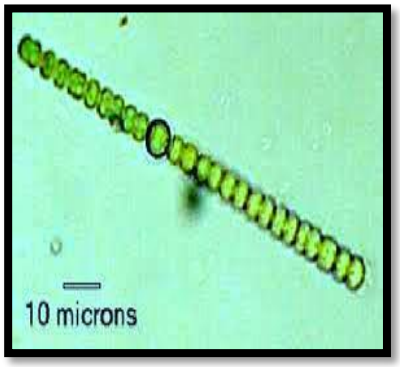
Table 2. Assessment of bioactive secondary metabolites of Cyanobacterial isolates

S.N.	Secondary Metabolites	*Anabaena* sp.	*Cylindrospermum* sp.	*Cyclotella* sp.	*Diatoma*sp.
1.	Phenols	+	++	+	+
2.	Flavonoid	+	+	+	+
3.	Terpenoids	+	-	+	-
4.	Tannins	-	-	-	-
5.	Saponins	++	+	++	++
6.	Alkaloids	++	++	++	++
7.	Steroids	-	-	-	-
8.	Glycosides	-	+	-	-

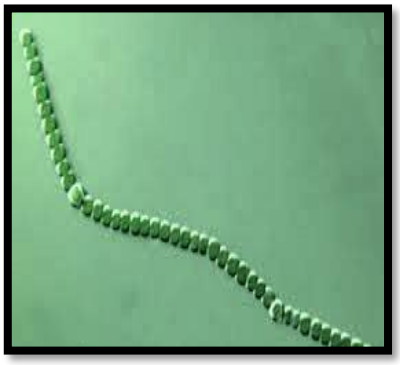
+ Sign represents presence and - sign represents absence.

Table 3. Functional group revealed by FTIR

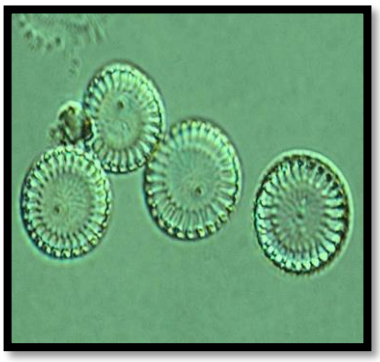
Functional Group			
Anabaena sp. **(Anabaena-1)**	***Cylindrospermum*** **sp. (Cylindrospermum-1)**	***Cyclotella*** **Sp. (Cyclotella-1)**	***Diatoma*** **Sp. (Diatoma-1)**
Hydroxy Aromatic (α-ionone) Aldehyde Conjugated Ketone α- β UnsarturatedKetons Substituted benzene Aldehyde	Acetyl Aldehyde Alkyl group Aryl ether group Alkane	Carbonyl group Methyleen group Alkane	Amino functional group with aryl ether group Amino functional group with alkyl group Hydroxyl group



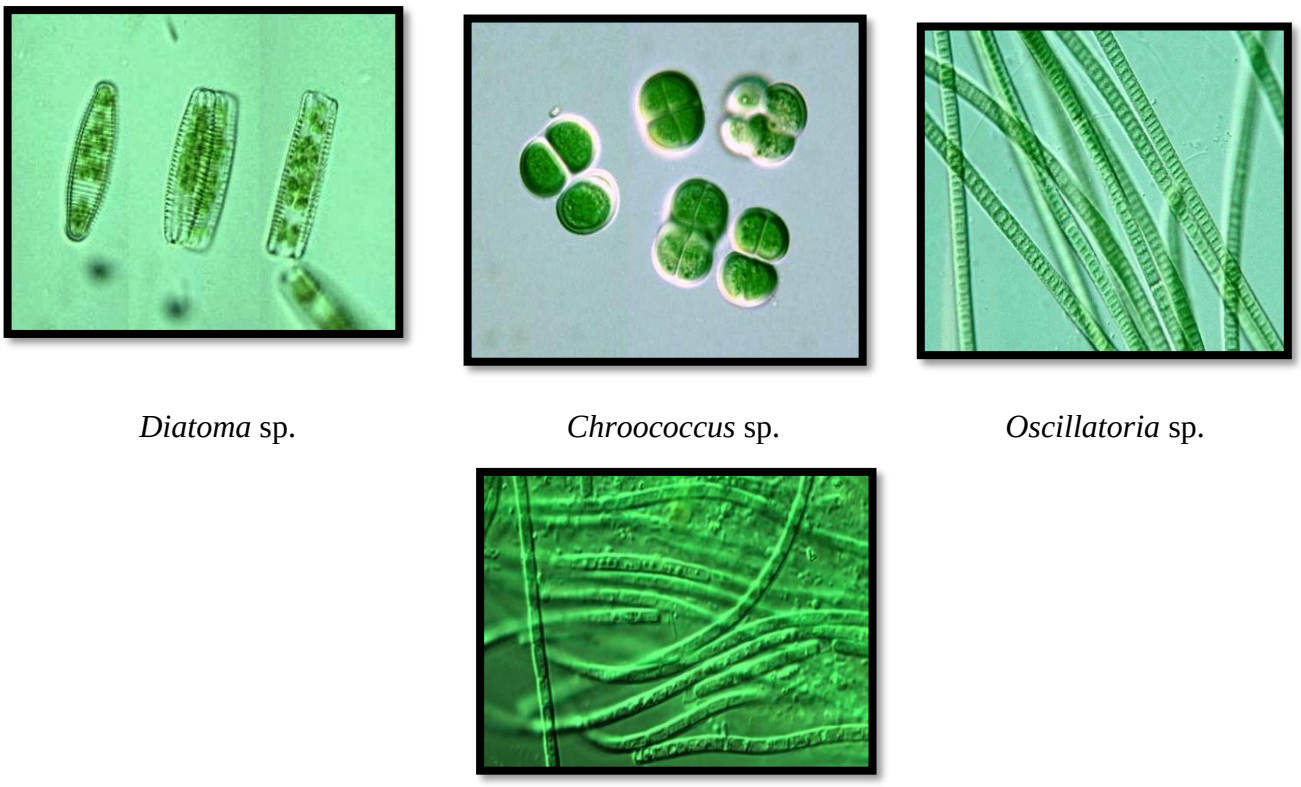
Anabaena sp.



*Cylindrospermum*sp.



Cyclotella sp.



Diatoma sp. *Chroococcus* sp. *Oscillatoria* sp.

Geitlerinema sp.

Fig. 1. Microscopic structure of cyanobacterial isolates

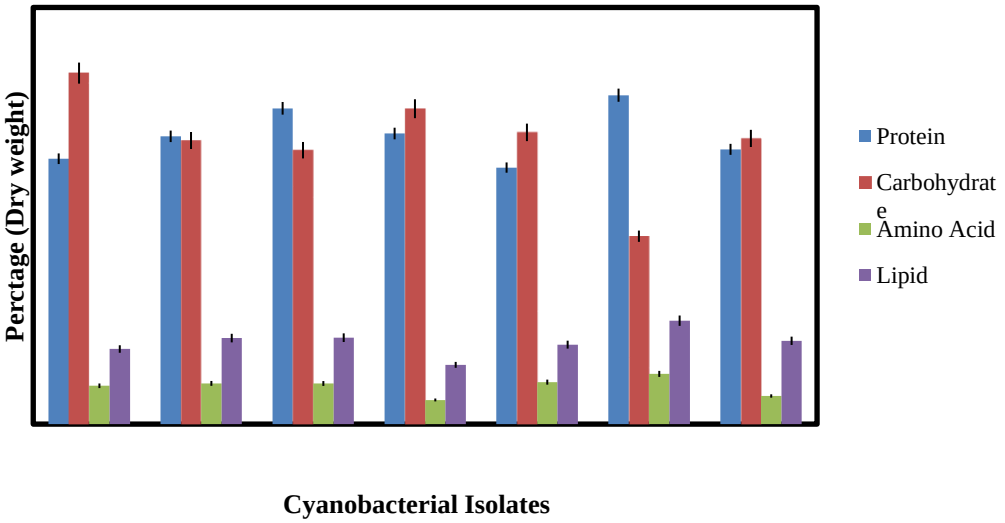
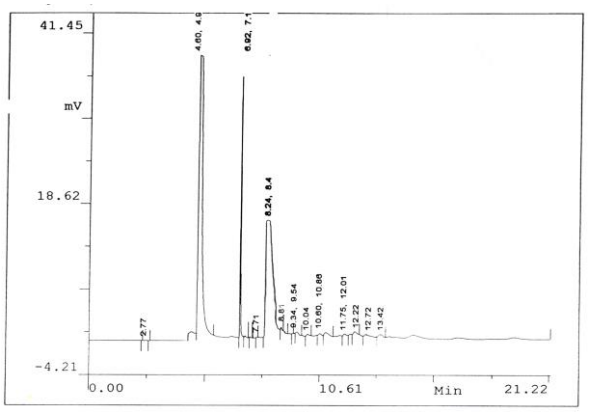
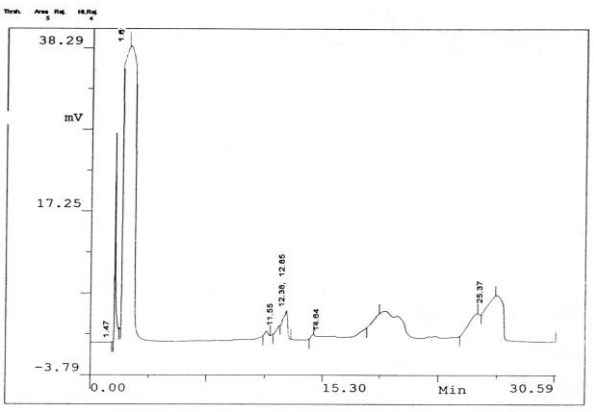


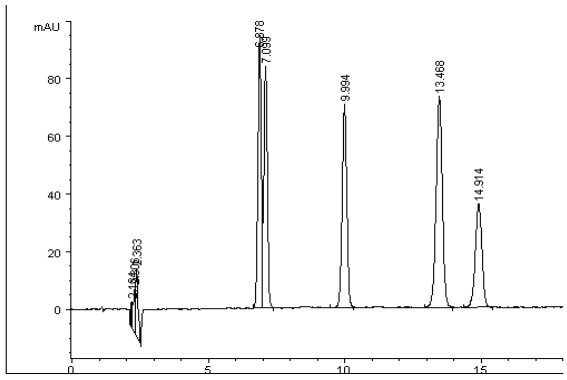
Fig. 2 Cellular constituents of cyanobacterial isolates from different aquatic habitats expressed in % of dry weight



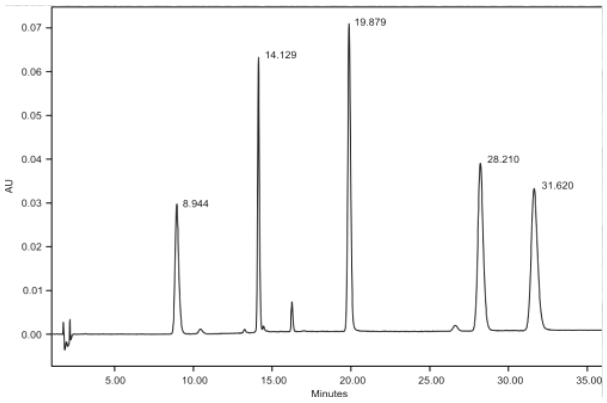
Anabaena sp.



Cylindrospermum sp.

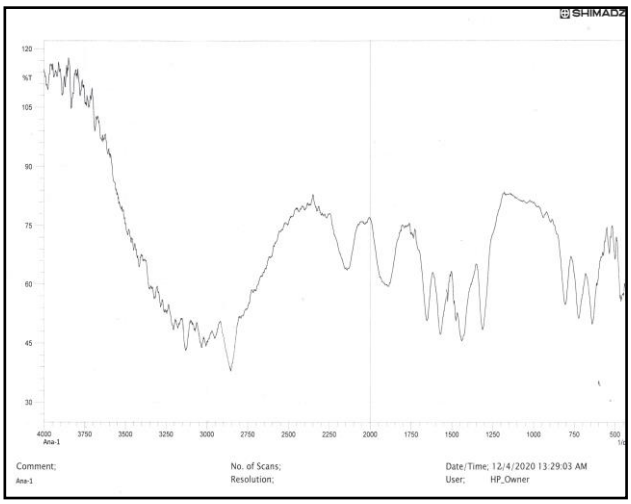


Cyclotella sp.

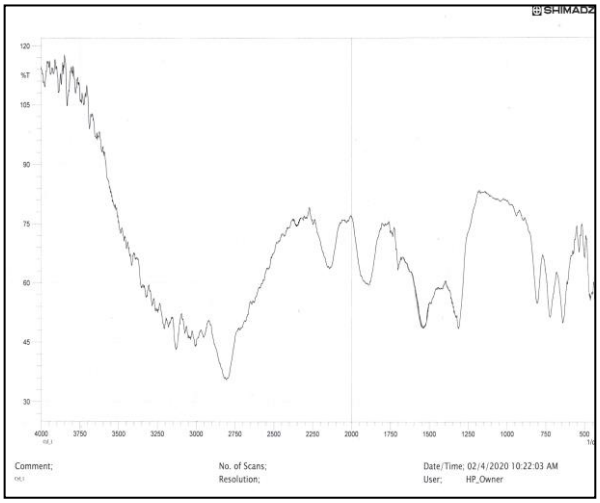


*Diatoma*sp.

Fig. 3. HPLC chromatogram of *Anabaena* sp., *Cylindrospermum, Cyclotella* sp. and *Diatoma* sp.



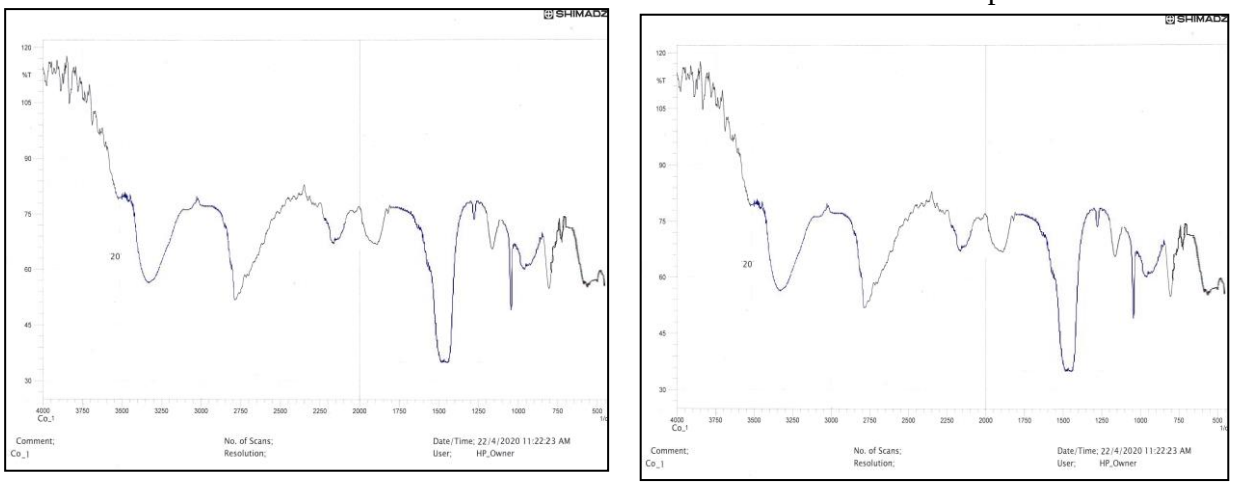
Anabaena Sp.



Cylindrospermum Sp.

screening and systematic evaluation of active phytochemical constituent of cyanobacterial isolates

Diatoma sp.



Cyclotella sp.

Fig. 4. FTIR spectra of *Anabaena* sp., *Cylindrospermum*, *Cyclotella* sp. and *Diatoma* sp.

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