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Biochemical composition and chemotaxonomy of cyanobacteria isolated from Assam, North-East India

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Abstract:

Six strains of two closely related genera – *Nostoc* and *Cylindrospermum* were isolated from the biodiversity hotspot zone of Assam, North-East India. A detailed study was made on the biochemical composition and chemotaxonomy of the strains based on Fatty acid profile and Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR). The strains were found to be rich in biochemical composition (pigments, total carbohydrate and soluble proteins). Fatty acid methyl ester (FAME) analysis differentiated the strains into two groups supporting the morphological classification. Despite the reports available ATR - FTIR analysis tested for the same cyanobacteria was found to be a weak tool for strain differentiation.

Keywords: Assam, ATR-FTIR, biochemical, cyanobacteria, Fatty acid methyl ester (FAME)

Introduction:

Cyanobacteria (also known as blue- green algae) are a group of extraordinarily diverse Gram –ve prokaryotes that originated 3.5 billion years ago. These organisms are unicellular to multicellular, coccoid to branched filaments, nearly colourless to intensely pigmented, autotrophic to heterotrophic, psychrophilic to thermophilic, acidophilic to alkylophilic, planktonic to barophilic, freshwater to marine including hypersaline (Thajuddin and Subramanian, 2005). With morphological, physiological and ecological characteristics recently, cyanobacterial taxonomy is also based on biochemical and molecular approaches (Wilmotte, 1994). Cyanobacteria obtain their characteristic colour from the chlorophylls, carotenoids and abundant phycobiliproteins (Henrikson, 1989; Sarada *et al.,*1999; Biswas *et al.,*2010). Lipids are useful chemotaxonomic markers for classification of prokaryotes (Ratledge and Wilkinson, 1988). In particular, for cyanobacteria, it was proposed that the fatty acid profiles permit to divide these microorganisms in groups (Cohen *et al.,* 1995 ; Kenyon, 1972). Cyanobacteria have been classified into four groups on the basis of fatty acid composition (Kenyon, 1972). The first group include cyanobacterial strains devoid of polyunsaturated fatty acids (PUFA), containing only saturated and mono-unsaturated fatty acids. The second and third groups consist of strains containing either linolenic acid [α, 18: 3(ω_3)] or linolenic acid [γ, 18 : 3 to 6], respectively, while strains belonging to group four also contain octadecatetraenoic acid [18:4 (ω3)] as mentioned by Kenyon (1972).

With the knowledge of a wide spectrum of fatty acid profiles of lipids, polyunsaturated fatty acids, including the essential fatty acids, *viz.* linoleic acid, a-linolenic acid (ala), γ-linolenic acid (GLA), arachidonic acid (aa), and eicosapentaenoic acid (epa), essential fatty acids are becoming increasingly important in the pharmaceutical industry (Borowitzka, 1988; Becker, 1994). GLA is well recognized as a promising therapeutic agent for numerous health disorders with potential applications in heart disease, Parkinson disease, multiple sclerosis, inflammatory diseases, premenstrual syndrome, plasma cholesterol levels, and other disorders (Borowitzka, 1988; Henrikson, 1989; Richmond, 1990). Holton *et al*., (1968) demonstrated close relation between biochemical study and morphological complexity. Biochemical diversity allowed the generic distinction in between *Anabaena* and *Nostoc* (Rippka *et al*., 1979).

Mycosporine-like amino acids (MAAs) were initially identified in fungi (Leach, 1965). This water soluble pigments having UV photoprotection property with cyclohexenone or cyclohexenimine chromophore conjugated with the nitrogen substituent of an amino acid or its imino alcohol are having absorption maxima ranging from 310 to 362 nm (Nakamura *et al.,*1982; Carreto *et al.,*1990).

Attenuated total reflectance- Fourier transform infrared spectroscopy (ATR-FTIR) is also used for discrimination of cyanobacterial strains (Bounphanmy *et al.,* 2010) based on the distribution of functional groups. Biochemical and chemotaxonomy studies of the cyanobacteria from the Dima Hasao district is limited except some sporadic records (Rout *et al.,* 2012 ; Borah *et al*., 2014). Hence present study was carried out to study the biochemical composition and discriminate the selected two closely related genera- *Nostoc* and *Cylindrospermum* based on Fatty acid profile and ATR – FTIR.

Materials and methods:

Isolation and characterization of cyanobacteria:

Table 1 showed the list of abbreviation, location and isolation source of the selected cyanobacteria. Fresh soil samples (10g) were shaken with sterile water (90ml) for 2 h, according to Lukešoúa (1993). Plates were incubated at a temperature of 22°C±1 under a 16:8 (light: dark) photoperiod at a light intensity of 2000-3000 lux. Cyanobacteria were made unialgal by repeated streaking on agar plates with or without combined nitrogen. The morphological parameters such as shape of the cells and tips, length and breadth of intercalary cells as well as heterocysts and akinetes, presence and absence of constriction of the cross wall, sheath and its colour were taken into account. For each biometrical character repeated measurements were obtained from different cells, heterocysts and filaments (Singh *et al.*, 2008). The taxonomic identification of the cyanobacteria was done based on the cell or colony morphology (Desikachary, 1959).

Table 1 List of abbreviation, location and isolation source of the selected cyanobacteria

Sl. No.	Name of the species	Abbreviation	Location	Source of isolation
1	*Nostoc carneum* Ag. ex Born. et Flah	CYA 1	25°11′07.0″N 93°06′06.1″ E	Terrace paddy field
2	*Nostoc hatei* Dixit	CYA 2	25°10′29.5″N 93°07′08.5″E	Terrace paddy field
3	*Nostoc muscorum* Ag. ex Born. et Flah.	CYA 3	25°10′22.9″N 93°07′10.9″E	Jhum land
4	*Cylindrospermum muscicola* Kützing ex.Born. et Flah (Strain A)	CYA 4	25°10′29.5″N 93°07′08.5″E	Terrace paddy field
5	*Cylindrospermum muscicola* Kützing ex.Born. et Flah (Strain B)	CYA 5	25°10′41.9″N 93°04′25.1″E	Terrace paddy field
6	*Cylindrospermum indicum* Rao	CYA 6	25°10′29.5″N 93°07′08.5″E	Terrace paddy field

Estimation of chlorophyll *a* and total carotenoid content (TCC), phycobiliproteins (PBPs) and Mycosporine-like amino acids (MAAs):

Chlorophyll *a* was estimated by hot extraction method in 80% methanol following McKinney (1941). Total carotenoid content (TCC) was determined by extracting the pigment in 85% acetone as mentioned by Jensen (1978). Phycobiliproteins of the dry lyophilized biomass were extracted in 0.05M phosphate buffer (pH 6.8) using the equation mentioned by Bennett and Bogorad (1973). The Mycosporine - like amino acids (MAA) pigment the cyanobacterial biomass was extracted with water according to Matsui *et al*., (2011).

Estimation of lipid, Fatty acid profiling, total carbohydrate and soluble proteins:

Extraction of lipid was done following the method mentioned by Folch *et al*., (1957) and quantified gravimetrically. Identification and quantification of fatty acids were done by modified method of Miller and Berger (1985) as mentioned in Saha *et al.,* (2003). The converted Fatty acid Methyl Esters (FAME) was chromatographed with GC-MS using a Flame Ionisation Detector (FID). The oven temperature was 140°C to 240°C, carrier gas: helium, detector temperature: 260°C. Fatty acids were identified by comparing the area and retention time of the authenticated standards.

Total carbohydrate was estimated by Anthrone method (Spiro, 1966). Soluble protein was measured by modified Lowry method (Lowry *et al*., 1951).

Attenuated total reflectance- Fourier transform infrared spectroscopy (ATR-FTIR):

For determination of functional group, the ATR-FTIR spectra was obtained for fine lyophilized cyanobacterial biomass powder placed on the Selenium crystal and scanned in the region of 4,000 to 600cm^{-1} on Perkin Elmer Spectrum 2 (Perkin Elmer, USA.)

Statistical analysis:

The preferred cyanobacterial strain with biochemical properties was evaluated by Multi-criteria analysis (MCA) with Graphical Analysis for Interactive Aid (GAIA) using Preference Ranking Organization Method for the Enrichment of Evaluations (PROMETHEE) according to Brans and Mareschal (2005).

Hierarchical Cluster Analysis was performed using SPSS 15 software package.

Results and Discussion:

The selected strains were from two morphologically distinct closely related genera viz. *Nostoc* and *Cylindrospermum* (**Fig 1**). Three strains were selected from each genus. The genus *Cylindrospermum* was clearly distinguishable from *Nostoc* due to the presence of terminal heterocysts followed by akinetes at the both ends (Rout *et al*., 2012). Growth curve obtained **(Fig 2)** for the isolates were specific to each strain. The isolates entered into their stationary phase of growth period after 20days except CYA 1 where stationary phase was quite earlier than the others. Shortest lag phase was observed for CYA 1, CYA 2 and CYA 3(4 days). CYA 5 and CYA 6 were observed to have highest lag phase (8 days). A long lag phase might be attributed to the adjusting time required by the organisms to the new environment (Madigan *et al.*, 2000) and repairing of macromolecular damage accumulated during last stationary phase (Dukan *et al.*, 1998). However size of the inoculums is another factor for determining the time duration of lag phase (Spencer, 1954). Log phase is the phase of the growth curve where cells increase logarithmically and is crucial phase for determining specific growth rate and generation time. Stationary phase obtained in the growth curve was due to the nutrient limitations of the batch cultures and the subsequent accumulation of the metabolic waste products which ultimately lead to the death phase indicated by the decline in chlorophyll *a* concentration in the isolates.

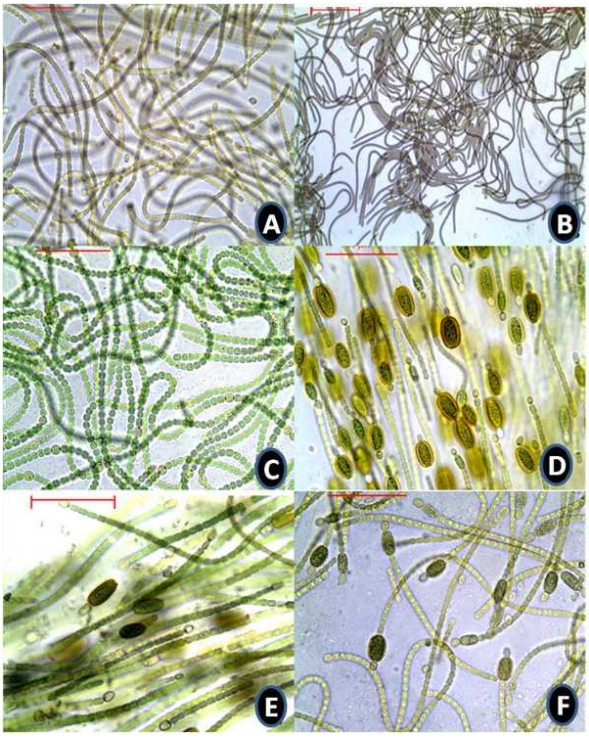

Fig 1. **Microphotographs of cyanobacteria isolated from the agro-ecosystems. CYA1 (A), CYA2(B), CYA3(C), CYA4(D), CYA 5(E), CYA6(F). Scale bar -50µm, except B (100µm)**

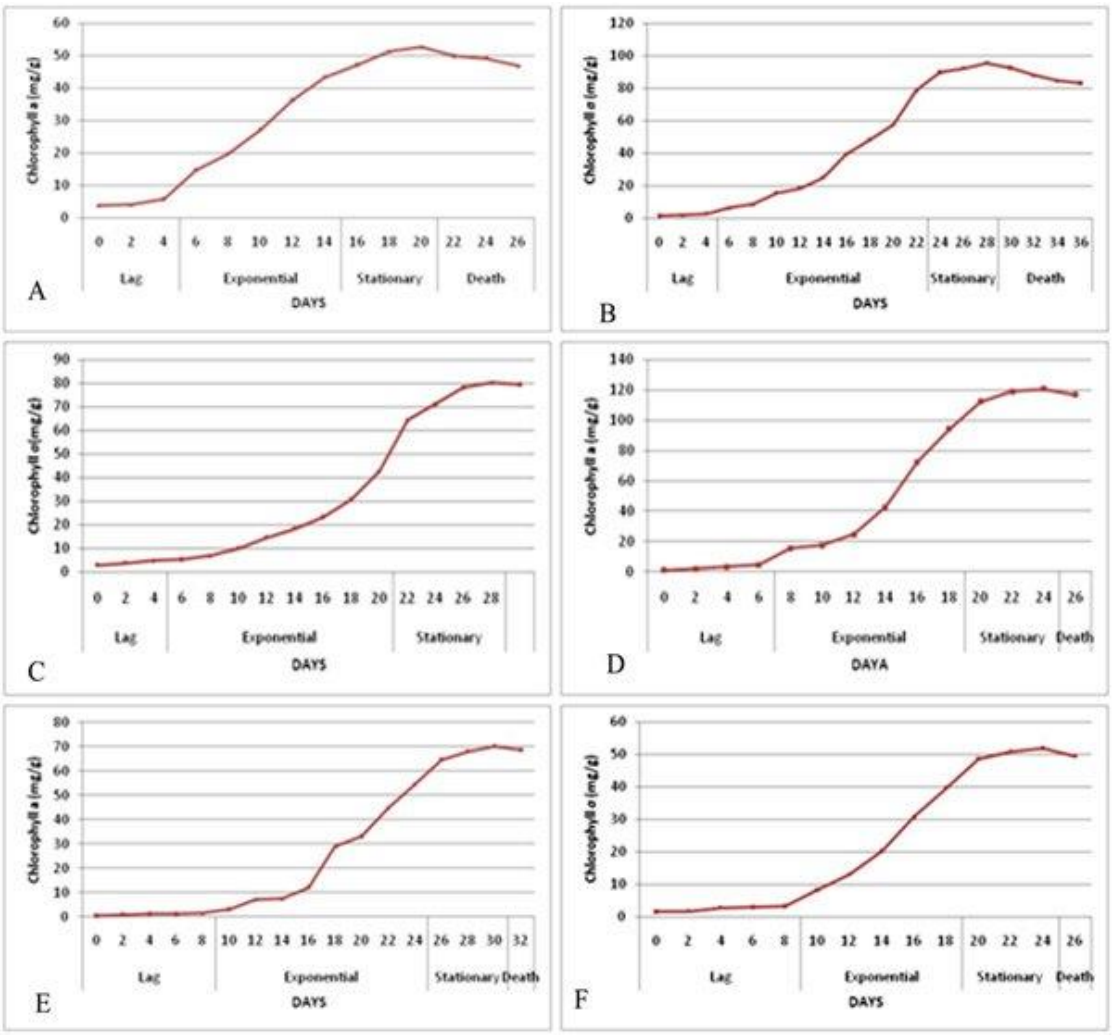

Fig 2. Growth curve obtained for the cyanobacterial isolates. CYA1 (A), CYA2(B), CYA3(C), CYA4(D), CYA 5(E), CYA6(F)

The isolates showed diverse pigment profiles (**Table 2**). TCC was highest in CYA 6 (5.43 mg g-1) whereas the lowest was obtained for CYA 1 (0.899 mg g-1). TCC was in order CYA6 > CYA4 > CYA2 > CYA5 > CYA1. CYA2 was highest (35.67%) in phycobiliprotein yield of total protein. Among the six strains studied two strains (CYA1 and CYA2) were found to be rich in phycoerythrin (PE) with 77.65% and 63.89% of total phycobiliprotein. The *Cylindrospermum* strains- CYA4, CYA5 and CYA6 were mainly phycocyanin (PC) producers. CYA 4 (71.68%) was the highest in phycocyanin yield followed by CYA5 (71.54%) and CYA6 (67.60%). Highest allophycocyanin(APC) was found in CYA3(36.66%) with the least in CYA1(2.29%). Most interestingly, UV-absorbing pigment mycosporine like amino acids (MAA) were present in all *Cylindrospermum* strains but absent in all *Nostoc* strains studied.

Table 2 Pigment properties of the cyanobacteria isolated from the agro-ecosystems (Mean±SD, n=3)

Sl. No.	Name of the species	TCC mg/g dw	PBP(%)			PBP of Tot al protein (%)	MAA
			PC	APC	PE		
1	CYA1	0.899 ± 0.10	20.06	2.29	77.65	7.13	-
2	CYA2	2.94 ± 0.002	24.42	11.69	63.89	35.67	-
3	CYA3	0.093 ± 0.02	36.91	36.66	26.44	1.21	-
4	CYA4	3.01 ± 0.003	71.68	14.15	14.16	14.46	+
5	CYA5	1.04 ±0.002	71.54	11.88	16.58	14.88	+
6	CYA6	5.43 ± 0.032	67.60	24.87	7.52	4.85	+

The cyanobacterial strains were rich in biochemical properties **(Table 3).** Highest lipid accumulation was obtained for CYA2 (12.26%) and the least for CYA5 (3.95%). The order of lipid accumulation was found as CYA2 > CYA1 > CYA4 > CYA6 > CYA3 > CYA5. Rich carbohydrate was found in CYA3 (219.59 mg g^{-1}, 21.96%). Sallal *et al.,* (1990) reported the total lipid content of the four cyanobacterial species ranged between 10.7 and 12.3 % of dry weight. CYA2 was found to contain the lowest carbohydrate (72.69 mg g^{-1}, 7.27%). Soluble protein was highest in CYA6 (366.95 mg g^{-1}, 36.70%). It was the least in CYA1 (10.98%).

Table 3 Biochemical properties of the isolated cyanobacterial strains (Mean ± SD, n=3)

Sl. No.	Name of the species	Lipid (%)	Total Carbohydrate		Soluble protein	
			mg/g dw	(%)	mg/g dw	(%)
1	CYA 1	11.15	72.69 ± 0.294	7.27	109.80 ± 0.02	10.98
2	CYA2	12.26	154 ± 1.905	15.40	176.68 ± 0.832	17.67
3	CYA3	7.89	219.59 ± 0.61	21.96	280.23 ± 4.104	28.03
4	CYA4	11.07	90.86 ± 0.247	9.09	196.98 ±0.147	19.70
5	CYA5	3.95	194.31 ± 0.108	19.43	141.10 ± 0.077	14.10
6	CYA6	10.48	167.73 ± 0.37	16.77	366.95 ± 1.35	36.70

A multi-criteria decision method (MCDM) software PROMETHEE-GAIA was used to make objective selections for most potential strain based on pigment and biochemical properties (**Fig 3, Table 4**). The decision vector indicates the most preferable species, i.e., those that align with the direction of this vector and the outermost criteria in the direction of the decision vector are the most preferable (Brans and Mareschal, 2005; Anahas and Muralitharan, 2015). The length of the criteria vectors indicates their influence on the decision vector and therefore the ranking (Brans and Mareschal, 2005). Very short criteria vectors indicate that the microalgal species showed little to no variance in these important biodiesel quality parameters, thus they do not influence the length and direction of the decision vector. Thus it can be concluded that CYA6 is the most potential strain in terms of its pigment and biochemical profile.

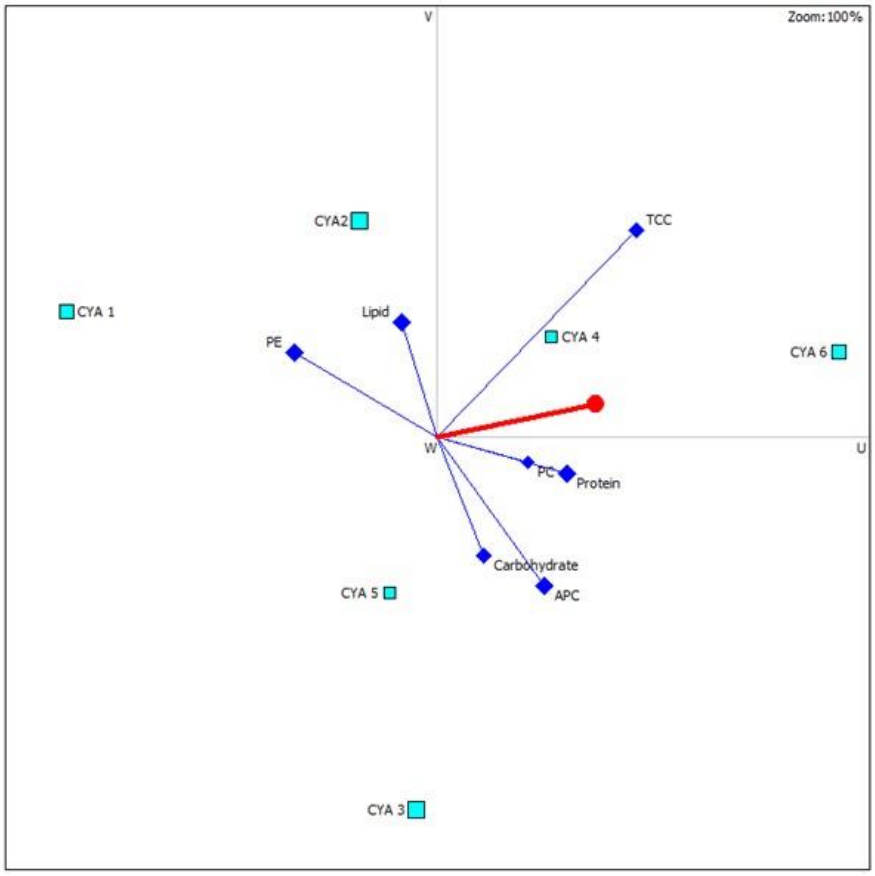

Fig 3. Graphical Analysis for Interactive Assistance (GAIA) visual analysis of six cyanobacterial strains on the criterion of pigment and biochemical properties

Table 4 Ranking of the cyanobacteria based on their outranking flow

Rank	Name of the organism	Phi
1	CYA 6	0.2688
2	CYA 2	0.0831
3	CYA 3	0.0258
4	CYA 4	-0.0126
5	CYA 5	-0.1510
6	CYA 1	-0.2141

As mentioned by previous workers (Sallal *et al*., 1990; Cohen *et al.,* 1995) all cyanobacteria under study contained saturated C16 and C18 fatty acids (Table 5). Following the classification of Murata *et al.,* (1992) CYA3 was found to be belonging to group 1. This strain was devoid of polyunsaturated fatty acids (PUFA) and contained only saturated and monounsaturated fatty acids (MUFA). CYA1 was found to be under group 2 containing α-linolenic acid. However, this classification system excludes the occurrence of cyanobacterial strains containing PUFA with only two double bonds, such as 16:2 or 18:2 (Cohen *et al.,* 1995). Three double bonds in 18-carbon linolenic acid present in CYA1, the most abundant fatty-acyl chains of plant thylakoid membranes, render these membranes highly fluid despite environmental low-temperatures (Yashroy, 1987). Presence of α-linolenic acid and the commercial importance in *Nostoc* strains was already reported by Temina *et al*., (2007). The authors also reported the presence of low molecular, hydroxy, dioic, saturated and unsaturated fatty acids in *Nostoc*. Tetradecanoic acid (C14:0) and Pentadecanoic acid (C15:0) were found only in *Cylindrospermum* strains whereas was absent in *Nostoc* strains. Similarly, 12-Hydroxy-9-Octadecenoic acid (C18:1 12-OH) was present in all the *Cylindrospermum* strains but not in *Nostoc* strains. Tridecanoic acid (C13:0) was found in *Nostoc* strains but absent in *Cylindrospermum*. 6-Octadecenoic acid (C18:1 cis-6) was found only in CYA 3. Similarly, Butanoic acid (C4:0), Pentanoic acid(C5:0), Octanoic acid(C8:0), Heptadecanoic acid (C17:0), Triacontanoic acid (C30:0), 6-Octadecenoic acid (C18:1 cis-6), Cis Vaccinic acid (C18:1 cis-11), Cis-13-Octadecenoic acid (C18: 1 cis-13),

10-Nonadecenoic acid (C19:1), 13-docosenoic acid (C22:1) and 5,8,11,14-Eicosatetraenoic acid (C20:4) were found only in CYA 5, CYA 1, CYA 3, CYA 2, CYA 4, CYA 3, CYA 5, CYA 4, CYA 4, CYA 2, CYA 4 strains respectively.

Table 5 Distribution of Fatty acids (%) among the cyanobacterial strains

Sl. No.	Fatty acids	CYA1	CYA2	CYA3	CYA4	CYA5	CYA6
1	Butanoic acid (C4:0)	-	-	-	-	2.30	-
2	Pentanoic acid(C5:0)	2.54	-	-	-	-	-
3	Octanoic acid(C8:0)	-	-	22.63	-	-	-
4	Dodecanoic acid (C12:0)	-	-	-	-	11.84	18.44
5	Tridecanoic acid (C13:0)	4.11	0.85	0.99	-	-	-
6	Tetradecanoic acid (C14:0)	-	-	-	1.68	4.61	7.82
7	Pentadecanoic acid (C15:0)	-	-	-	2.58	10.53	17.32
8	Hexadecanoic acid (C16:0)	24.70	31.28	57.58	28.51	2.63	12.29
9	Heptadecanoic acid (C17:0)	-	2.68	-	-	-	-
10	Octadecanoic acid (C18:0)	20.96	4.01	8.27	6.85	3.95	3.35
11	Triacontanoic acid (C30:0)	-	-	-	1.12	-	-
12	cis-9-hexadecenoic acid (C16:1 cis-9)	-	3.27	4.73	6.96	-	-
13	6-Octadecenoic acid (C18:1 cis-6)	-	-	5.81	-	-	-
14	(9*Z*)-Octadecenoic acid (C18:1 cis-9)	31.56	14.29	-	9.88	-	-
15	Cis Vaccinic acid (C18:1 cis-11)	-	-	-	-	35.86	-
16	Cis-13-Octadecenoic acid (C18: 1 cis-13)	-	-	-	6.85	-	-
17	12-Hydroxy-9-Octadecenoic acid (C18:1 12-OH)	-	-	-	7.97	24.01	35.20
18	10-Nonadecenoic acid (C19:1)	-	-	-	2.01	-	-
19	13-docosenoic acid (C22:1)	-	4.04	-	-	-	-
20	9,12-octadecadienoic acid (9,12-C18:2)	3.68	39.58	-	24.47	4.28	5.59
21	9,12,15-octadecatrienoic acid or α-linolenic acid (C18: 3ω3)	12.45	-	-	-	-	-
22	5,8,11,14-Eicosatetraenoic acid (C20:4)	-	-	-	1.12	-	-

Hierarchical cluster analysis (**Fig 4**) on the basis of fatty acid profile of the selected cyanobacteria revealed the generic difference establishing fatty acids as a chemotaxonomic marker. Two distinct clusters, one for *Nostoc* (CYA1, CYA2 and CYA3) and *Cylidrospermum* were obtained. Most interestingly, CYA1 and CYA2 that were rich phycoerythrin clustered together. However CYA4 and CYA5 that were the two strains of same species did not form any strong cluster.

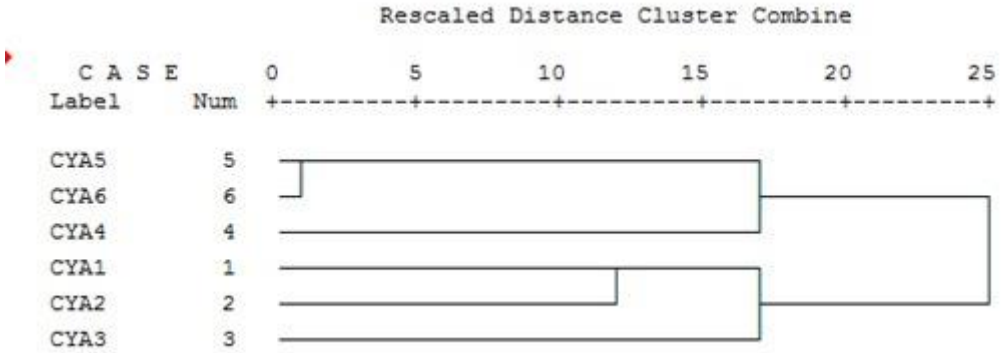

Fig 4. Hierarchical cluster analysis of Cyanobacteria based on Fatty acid analysis

UV-Vis spectrum for water extract of lyophilized biomass of the cyanobacteria revealed the presence of UV absorbing pigment- Mycosporine like amino acids (MAAs) in *Cylindrospermum* strains (**Fig 5**). To the best of our knowledge this is the first report of occurrence of MAAs in *Cylindrospermum* strains. According to the

database from Sinha *et al.,* (2007), the peaks obtained at 327 nm might be due to the presence of Mycosporine-methylamine-serine or Mycosporine-methylamine-threonine.

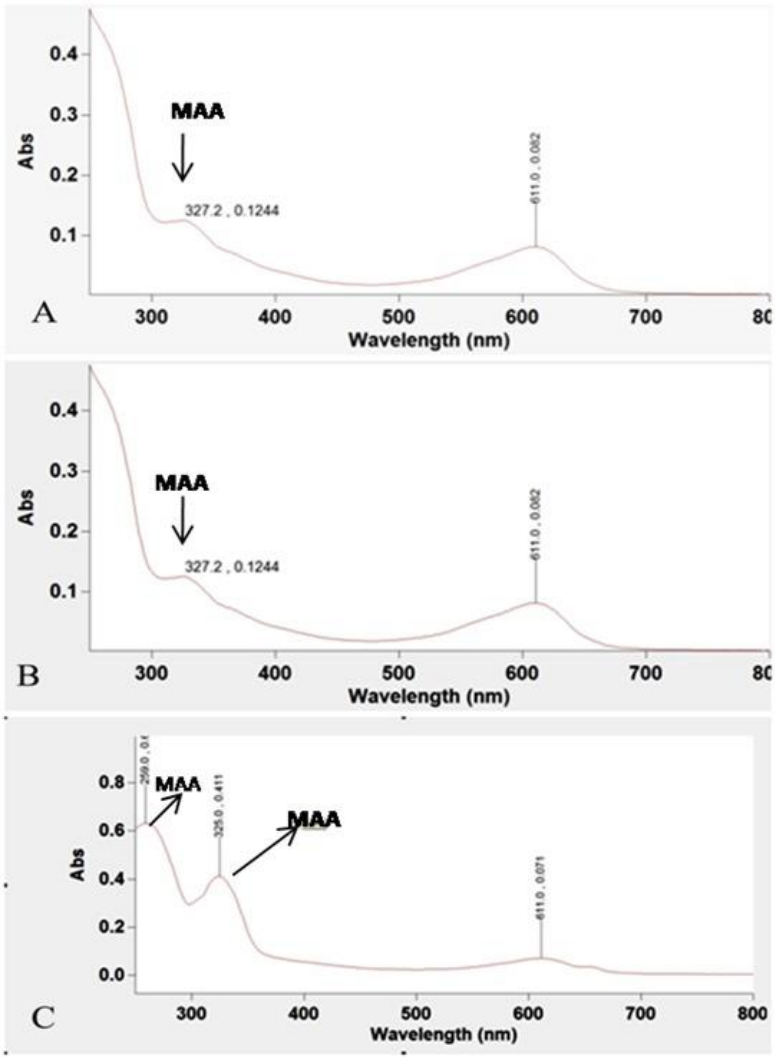

Fig 5. Distribution of Mycosporine like amino acids (MAA) among the cyanobacterial strains – CYA 4(A), CYA5 (B), CYA6(C)

Fig 6 & 7 presents ATR – FTIR spectra of lyophilized biomass of cyanobacteria strains. The presence of functional groups and corresponding bands (Kansiz *et al.,* 1999; Giordano *et al.,*2001; Stehfest *et al.,* 2005; Parikh and Madamwar, 2006; Kenne and van der Marwe, 2013) are mentioned in **Table 6.** Unlike the Fatty acid profiling of the strains here by using ATR-FTIR spectra it was not able to differentiate the two genera (**Fig 8**). Based on the position of the bands it was observed that CYA1, CYA3, CYA4, CYA5 and CYA6 formed distinct cluster which was separate from CYA2. Though any generic differentiation was not observed for ATR-FTIR, CYA4 and CYA5 which were the different strains of same species clustered together. Differentiation of green algae and cyanobacteria using ATR – FTIR was mentioned by Kenne and van der Marwe, (2013).

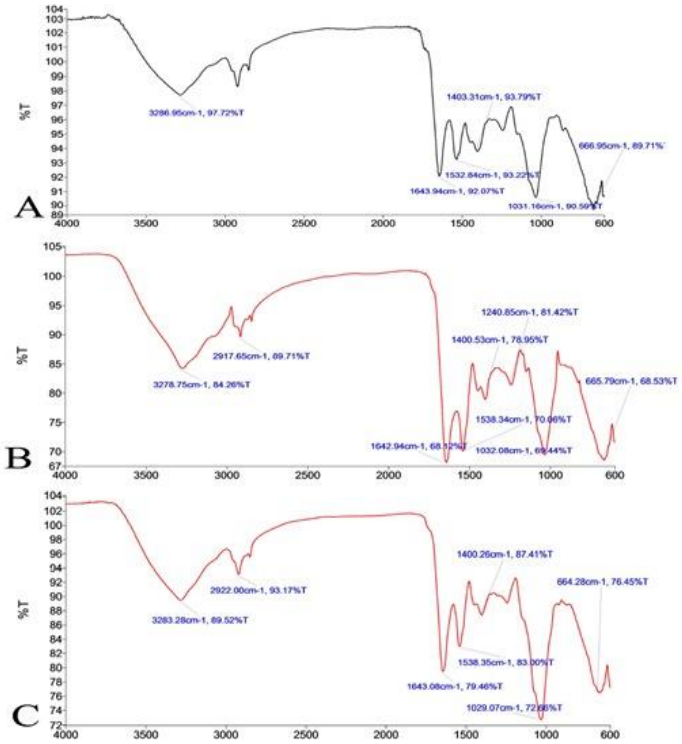

Fig 6. ATR-FTIR banding patterns of cyanobacterial strains. CYA 1 (A), CYA2 (B), CYA 3(C)

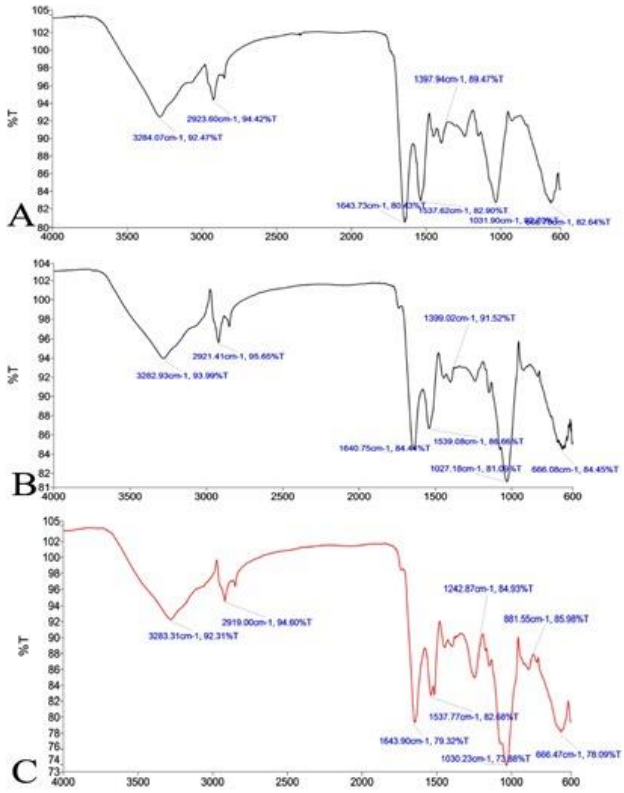

Fig 7. ATR-FTIR banding patterns of cyanobacterial strains. CYA4(A), CYA5 (B), CYA6 (C)

Table 6 ATR-FTIR band characteristic of cyanobacterial strains

Name of the species	Band characteristic (cm^{-1})								
	OH stretching	Lipid stretching	Phospholipids C= O Stretching	Amide I band	Amide II band	Polysaccharide C-H stretching	Polysaccharide O-H bending	Polysaccharide C-O stretch	Amide III band
CYA 1	3286.95	2915	~1650	1643.94	1532.84	~1460	1403.31	1031.16	1270
CYA 2	3278.75	2917.65	~1650	1642.94	1032.08	~1460	1400.53	1032.08	1240.85
CYA 3	3283.28	2922	~1650	1643.08	1538.35	~1460	1400.26	1029.07	1289
CYA 4	3284.07	2923.60	~1650	1643.73	1537.62	~1460	1397.94	1031.90	1282
CYA 5	3282.93	2921.41	~1650	1640.75	1539.08	~1460	1399.02	1027.18	1284
CYA 6	3283.31	2919	~1650	1643.90	1537.77	~1460	1398	1030.23	1242.87

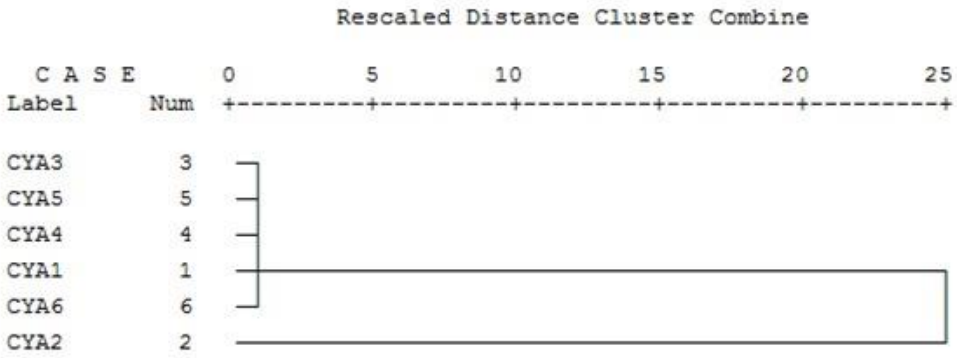

Fig 8. Hierarchical cluster analysis of cyanobacteria based on ATR-FTIR band characteristic

Conclusion

The strains were rich in pigments and biochemical properties. *Cylindrospermum muscicola* and *Cylinrospermum indica* were recorded for the first time for the presence of MAAs. Fatty acid profiling of the strain resulted into a chemotaxonomic marker at least for the generic classification. ATR-FTIR analysis of the strains was found as a week tool for chemotaxonomic studies.

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