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Morphological, molecular and biochemical characterization of cyanobacteria from rice field cultivated for last 75 years

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ABSTRACT

The present study investigated the diversity of cyanobacteria population existent in the rice fields of National Rice Research Institute, Cuttack, India. Rice is being continuously grown in two seasons annually for last 75 years with different crop management practices. A total of 20 different isolates of cyanobacteria were collected based on morphological and molecular markers and were characterised for growth pattern, primary metabolites, different pigments and nitrogen fixing enzymes. *Anabaena variabilis, Nostoc* sp. (1) and *Scytonema* sp. (2) had significantly higher content of cell dry weight, protein and carbohydrate as compared to others. Similarly, different pigments such as chlorophyll *a*, carotenoids and phycobiliproteins were maximum in *Nostoc* sp. (1), *Westiellopsis* sp. (2) and *Niveispirillum cyanobacteriorum,* respectively. The nitrogen fixing ability was checked by studying nitrate reductase and glutamine synthatase and they were significantly higher in *Nostoc* sp. (1) and *Anabaena variabilis,* respectively. Based on principal component analysis and heat map study, *Anabaena variabilis* and *Nostoc* sp. (1) could be used as biofuel producer and biofertilizer; *Westiellopsis* sp. (2), *Fischerella* sp., *Synechocystis* sp. and *Anabaena variabilis* could be promoted in cosmetic/commercial industries. There is a great scope for further utilisation of these characterised isolates of cyanobacteria in different industries.

1. Introduction

Cyanobacteria (Blue Green Algae) are the largest group of gram-negative, oxygen-evolving photoautotrophic prokaryotes, which belong to the kingdom Eubacteria. This group is highly diverse based on ecological, biological and morphological characters (Flores and Herrero, 2010; Komárek, 2010). Morphologically, they are differentiated as filamentous, non-filamentous, unicellular, planktonic or benthic and colonial (coccoid) forms (Burja *et al.,* 2001). Ecologically, they are widely distributed in almost every habitats of the world (Sao and Kritika, 2015). They can easily be found in the diverse habitats such as terrestrial areas, desert, freshwater and hypersaline environments because of their specialized features. They are present as both free-living form as well as in syntrophic/symbiotic association with algae, fungi, bryophytes, pteridophytes, gymnosperms, angiosperms, and animals like ascidians (Adams, 2000).

Cyanobacteria play a crucial role in different ecosystem functions. They can fix nitrogen, mobilize phosphorus and can metabolise CO_2, H_2 and O_2 (Wilson, 2006). They can act as a bio-resource group for different industries such as biofuels, biofertilizers, vitamins, bioremediants, natural colouring agents, pharmaceutical drugs, biopolymers, neutraceuticals, cosmetics and feed, etc. (Gupta *et al.,* 2013; Singh *et al.,* 2016). Cyanobacteria are also helpful in enhancing the plant growth, crop yields, crop weight, microbial biomass carbon, soil fertility, water holding capacity, the availability of nutrients and provides oxygen to the rhizosphere (Wilson, 2006; Rana *et al.,* 2015).

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The environment of rice fields is most suitable for the growth of cyanobacteria as it provides convenient temperature, nutrient facility with continuous water supply and high level of CO_2. Comprehensive work on cyanobacterial diversity of paddy fields have been carried out in several parts of the world i.e., Odisha (Singh, 1973; Adhikary, 2002; Dash *et al.,* 2011; Dash *et al.,* 2020), Vietnam (Pham *et al.,* 2017), Srilanka (Amarawansa *et al.,* 2018), Indonesia (Purbani, 2019), Assam (Thajamanbi *et al.,* 2016), Kerala (Vijayan and Ray, 2015), Karnataka (Basavaraja and Naik, 2018). Abiotic attributes viz., light, pH, temperature, water content, nutrient availability, cropping pattern and different types of management practices are able to influence the diversity of cyanobacteria (Kirrolia *et al.,* 2012). For example, the alkaline soil contains more Cyanobacterial sp. (Prasanna and Nayak, 2007) and they are more abundant in rainy season. The soil of the rice field, confronts with both anoxic and aerobic conditions, gives a diversified microbial community (Mohanty *et al.,* 2017).

Indian Council of Agricultural Research-National Rice Research Institute (20° 252 N, 85° 552 E) is doing research on different aspects of rice with an experimental farmland of 60 hectares. Rice is cultivated for last 75 years with different management practices in two different seasons, i). *Kharif* (rainy season, June to November) and ii). *Rabi* (Dry season, December to May). Various management practices (such as organic field, long term pesticide applied field, short term pesticide applied fields) are being practiced here. In the current scenario, pesticides are an integral part of agriculture. They protect crops from various pests, weeds, and diseases by securing high yield. On the other hand, they are adversely affecting the growth of non-target soil microbes including cyanobacteria (Meena *et al.,* 2020). Herbicides and insecticides affects soil microbes and cyanobacteria by reducing their growth and diversity whereas some insecticides have hermetic effects on cyanobacterial populations of rice fields (Das *et al.,* 2015; Dash and Mohapatra, 2018). One-time pesticide application may not have substantial effect on the soil microbiology but continuous application of different pesticides (organophosphorous, organochlorine etc.) in the same field may alter the soil micro-biota (Kumar *et al.,* 2017). Here, the long term pesticide field trial was initiated around 10 years ago with pretilachlor, cartap hydrochloride, chlorpyriphos and carbendazim as treatments. The organic fields (maintained for more than 10 years) were supplemented with FYM, biofertilizers, green manure, etc. Therefore, the above stated management conditions may affect the microbial and cyanobacteria population in the field.

The main objective of this study was to isolate and identify the paddy field cyanobacteria having different physiological characteristics. This investigation was carried out to find out the morphological and biochemical variations of cyanobacteria present in paddy soil. Based on different growth attributes and biochemical parameters of cyanobacteria, they are grouped as potential biofertilizer, biofuel as well as different other industrial applications.

2. Materials and Methods

2.1 Collection of soil samples

The soil and cyanobacteria samples were collected from the ICAR-NRRI paddy fields during both the seasons. They were randomly collected from the top surface of soil up to a depth of 1 cm. The places were selected on the basis of their different management practices i.e., long term and short term pesticide applied fields, organic fields and other experimented fields, so that the diversity of cyanobacteria can be ascertained in ICAR-NRRI paddy fields. Collections were made from the sites of the fields where there were visible growth of cyanobacterial colonies. No specific comparison of the cyanobacteria diversity among different management practices was made.

2.2 Physiochemical properties of soil samples

The physiological properties of soil such as, pH and Electric Conductivity (EC) were measured as per standard protocol. The pH meter (CyberScanpH 510, Eutech Instruments, Oakton, Singapore) electrode contained KCl was calibrated by using pH buffer 4.0 and 9.2. The 10 g of soil samples were dissolved in 25 ml of distilled water and the content was shaken for 30 minutes. Measurement was done in three replications. The electric conductance of different soil samples was measured by using EC meter (PCSTestr™35, Eutech Instruments, Oakton, Singapore).

2.3 Purification and maintenance of culture

The purification of cyanobacterial strains was done by serial dilution and pour plate spreading technique (Andersen and Kawachi, 2005). The isolated and purified strains were maintained in nitrogen free BG11 liquid medium (Rippka *et al.,* 1979) at pH 7.2, 28±2°C and light intensity of 50±5.83 µE/m^2 s with a 14/10 h light/dark cycle. The cultures were hand homogenized (to break the clumps and obtain uniform suspension) for further analysis.

2.4 Microscopic characterisation of isolates

The morphological examination was conducted by viewing under a compound microscope (Zeiss, Germany) equipped with digital camera. Isolates were identified based on the shape and size of vegetative cells, heterocysts and akinetes, using morphological keys of Rippka *et al.,* (1979).

2.5 Genomic characterisation

The genomic DNA was isolated from cyanobacteria using Valerio *et al.* (2009) method. Cyanobacteria-specific primers such as CYA106F, CYA359F, CYA781R (a), CYA781R (b) and CYA1281R were used to amplify the cyanobacteria specific site and evident the presence of cyanobacteria community. The primer sequences used for PCR are listed in Table 1. The PCR conditions were as follows: 1 cycle at 95^0C for 10 min; 35 cycles of 94^0C for 45 secs, 55^0C for 45 secs, and 72^0C for 1 min, 1 cycle at 72^0C for 5 min, and a final step at 4^0C. For the ITS primers, the PCR cycling program was the same, except that the initial denaturation temperature was optimized at 95^0C for 6 min. PCR were performed in 50 µl containing 1X PCR buffer (Invitrogen, Thermo fisher scientific, USA), 0.4 mM of each of the four dNTPs (Invitrogen, Thermo fisher scientific, USA), 0.5 mM of each primer, 10–15 ng genomic DNA, 2.5 mM $MgCl_2$, 0.5 mg BSA ml^{-1}, 1% triton X, 0.1% gelatin and 1 U Taq DNA polymerase (Invitrogen, Thermo fisher scientific, USA). The amplifications were performed in a PCR Thermal cycler (BIORAD, T100 Thermal Cycler). The PCR products were verified by observing the PCR bands in a 1.4% (w/v) agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) and electrophoresis at 90 V for about 1.5 h using a gel electrophoresis system (HU25 Maxi-plus standard horizontal, SCIE-PLAS LTD). DNA ladder (1Kb plus) was assigned to determine the band size. The gels were documented by gel documentation unit. PCR reactions were repeated twice for each primer to check the reproducibility of the banding patterns. The purified PCR products were further used for Sanger sequencing. Only good quality DNA sequences based on the chromatogram data were used for further phylogenetic analysis.

The sequence of twenty isolates were manually edited using BioEdit software (Hall, 1999). The phylogentic tree was inferred using the UPGMA method in MEGA X (Sneath and Sokal, 1973). The tree has been drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

Table 1

Primers and their sequences used for genetic characterisation

Sl No.	Name Of the Primer	Sequences(5'-3')
1	CYA106F	CGG ACG GGT GAG TAA CGC GTG A
2	CYA359F	GGG GAA TYT TCC GCA ATG GG
3	CYA781R(a)	GAC TAC TGG GGT ATC TAA TCC CAT T
4	CYA781R(b)	GAC TAC AGG GGT ATC TAA TCC CTT T
5	CYAN1281R	GCA ATT ACT AGC GAT TCC TCC
6	ITSCYA236F	CTG GTT CRA GTC CAG GAT
7	ITSCYA225R	TGC AGT TKT CAA GGT TCT

2.6 Growth attributes and biochemical characteristics

The growth (as cell dry weight) of cyanobacteria isolates was determined as per the standard procedure (Sorokin, 1973). The pigments of cyanobacteria were extracted using ice cold methanol (98%) from 2 mL of culture. The chlorophyll *a* (Ritchie, 2006), carotenoids (Jensen, 1978), Phycobiliprotein contents (Bennett and Bogoard, 1973), proteins (Lowry *et al.,* 1951), total carbohydrates (Herbert *et al.,* 1971), nitrate reductase (NR) (Lowe and Evans, 1964) and glutamine synthetase (GS) (Shapiro and Stadtman, 1970) were measured as per the standard protocols.

2.7 Statistical Analysis

The data were taken in triplicates for each characteristic. Statistical analyses were performed by using the Statistical Analysis Software (SAS) of Indian Agricultural Statistics Research Institute (IASRI), New Delhi through the portal www.iasri.res.in/sscnars/-. All the data were subjected to one-way classified analysis of variance (ANOVA) and means of treatments were compared based on Tukey's honestly significant difference test (HSD) at 0.05 probability level using SAS.

To check the potential of different isolates, we considered for principal component analysis (PCA) and heat map analysis using *Clustvis*: a web tool for visualizing clustering of multivariate data (Metsalu and Vilo, 2015). Nitrate reductase (NR) and glutamine synthatase data from different isolates were used to identify potential biofertilzer.

Similarly, dry weight and carbohydrate content were used to understand the potential biofuel producers. Parameters namely; chlorophyll *a*, carotenoids, phycobiliprotein (phycocyanin (PC), phycoerythrin (PE) and allophycocyanin (APC)) were taken to identify the potential pigment producer isolates. In *Clustvis* tool, data were transformed to unit variance scaling, and singular value decomposition (SVD) with imputation was used to calculate principal components. In heat maps, rows and columns were clustered using correlation distance and average linkage.

3. Results

3.1 Characteristics of field soil

The abiotic environment of the sample collection sites varied in their physicochemical characters in terms of pH and Electrical Conductivity (EC) (Table 2). The soil pH of sampling sites ranged from 6.31-7.25. The isolates, *Scytonema* sp. (3) was obtained from soil with the highest pH value (7.25); whereas *Scytonema* sp. (2) was isolated from soil with the lowest pH value (6.31). The EC values of different sampling sites varied from 141.61 µS/cm (*Aphanizomenon* sp.) to 296.31 µS/cm (*Fischerella* sp.) (Table 2).

3.2 Morphological characterization

The heterocystous isolates were grown in nitrogen free BG11 liquid medium, whereas the non heterocystous isolates were grown in BG11 (N+) medium. They were planktonic and having coloured thalli i.e., pale green, bluish green, dark green, olive green and brownish violet (Fig. 1). The non-filamentous isolates were found in homogenized form whereas the filamentous isolates were having thick masses. *Scytonema* spp. (isolates 1-3), *Brasilonema* sp., and *Westiellopsis* sp. (1) were dark green in colour with mucilaginous balls and they were distributed either randomly or settled at the bottom. *Hapalosiphon* spp. (isolates 1&2) were pale greenish brown with mucilage balls. The non-filamentous isolates, *Synechocystis* sp., *Aphanothece* sp. and *Gloeothece* sp. were found as colonial form. The filaments of *Scytonema* sp. showed false branching, while

Table 2

Molecular identification of collected isolates and physiochemical properties of the collected sites

Isolate code	Species	Genetic Similarity (%)	Location (GPS)	pH	EC (µS/cm)
PP1	*Anabaena variabilis*	100%	20°26'56.0"N 85°56'24.4"E	6.45 ±1.31	154.66±2.31
PP2	*Scytonema* sp.(1)	98.97%	20°26'58.2"N 85°56'11.1"E	6.65±0.25	279.27±11.81
PP3	*Scytonema* sp. (2)	98.99%	20°26'57.7"N 85°56'20.2"E	6.31±1.21	251.07±0.97
PP4	*Hapalosiphon* sp. (1)	98.90%	20°27'10.8"N 85°56'20.4"E	6.74±0.72	158.94±2.14
PP5	*Brasilonema* sp.	98.99%	20°27'11.1"N 85°56'17.9"E	7.19±0.28	214.18±23.11
PP6	*Nostoc* sp. (1)	99.47%	20°27'08.1"N 85°55'59.6"E	7.24±0.17	242.21±11.21
PP7	*Synechocystis* sp.	98.09%	20°27'10.6"N 85°55'57.6"E	6.32±1.84	208.94±11.43
PP8	Uncultured cyanobacterium (1)	98.00%	20°27'09.5"N 85°55'58.3"E	6.58±1.13	244.70±12.1
PP9	*Aphanothece* sp.	98.27%	20°27'03.5"N 85°56'01.3"E	7.21±0.43	249.49±25.12
PP10	*Gloeothece* sp.	98.03%	20°26'55.4"N 85°56'01.4"E	6.33±1.65	203.11±2.31
PP11	*Westiellopsis* sp. (1)	99.00%	20°27'04.8"N 85°56'24.0"E	6.85±1.39	244.82±12.08
PP12	*Hapalosiphon* sp. (2)	96.90%	20°27'08.4"N 85°56'27.6"E	7.22±0.59	248.67±11.3
PP13	*Westiellopsis prolifica*	100%	20°27'12.5"N 85°56'15.0"E	6.44±1.31	146.32±6.56
PP14	Uncultured cyanobacterium (2)	95.05%	20°27'09.4"N 85°56'17.9"E	7.24±0.19	189.12±8.25
PP15	*Niveispirillum cyanobacteriorum*	98.91%	20°26'56.7"N 85°56'21.7"E	7.16±0.18	217.54±9.59
PP16	*Nostoc* sp. (2)	98.99%	20°26'49.4"N 85°56'25.2"E	6.46±1.81	155.63±1.18
PP17	*Fischerella* sp.	98.89%	20°26'57.3"N 85°56'28.0"E	6.38±1.52	296.31±48.39
PP18	*Aphanizomenon* sp.	99.12%	20°27'13.0"N 85°56'14.9"E	6.85±1.28	141.61±1.31
PP19	*Westiellopsis* sp. *(2)*	93.88%	20°27'10.7"N 85°56'20.3"E	7.21±0.33	255.02±11.24
PP20	*Scytonema* sp. *(3)*	99.19%	20°26'58.2"N 85°56'09.7"E	7.25±0.12	181.59±2.06

Remarks: The species mentioned are the most abundantly distributed species of that particular site.

other (*Hapalosiphon* sp. (1), *Westiellopsis* sp. (1&2), *Westiellopsis prolifica* and *Fischerella* sp.) showed true branching. The main and lateral branches of *Hapalosiphon* sp. (1&2) could not be distinguished morphologically. Shape of vegetative cells was different among the isolates (Table-3).

The shape and size of heterocysts varied significantly among the isolates (Table 3). Among the heterocystous cyanobacteria, the minimum size of vegetative cell was noted in *Scytonema* sp. (1) and the maximum one was found in *Aphanizomenon* sp. whereas, in non-filamentous and non-heterocystous strains the minimum size of vegetative cells was observed in *Synechocystis* sp. and the maximum size was observed in *Aphanothece* sp. (Table 3). The maximum size of akinetes was observed in *Aphanizomenon* sp. and minimum size was observed in uncultured cyanobacterium (1).

The akinetes help the species to survive under unfavorable conditions and maintain its fundamental metabolic activity. The length and width of akinetes were 1.5 and 1.2 times more than that of the vegetative cells as they were produced by enlarging of the vegetative cells (Table 3). *Nostoc* sp. (2) and *Aphanizomenon* sp. exhibited distinctly large sized oval and barrel shaped akinetes, respectively as compared to other isolates in the present case, while the akinetes of uncultured cyanobacterium and *Hapalosiphon* sp. (2) were much smaller in size.

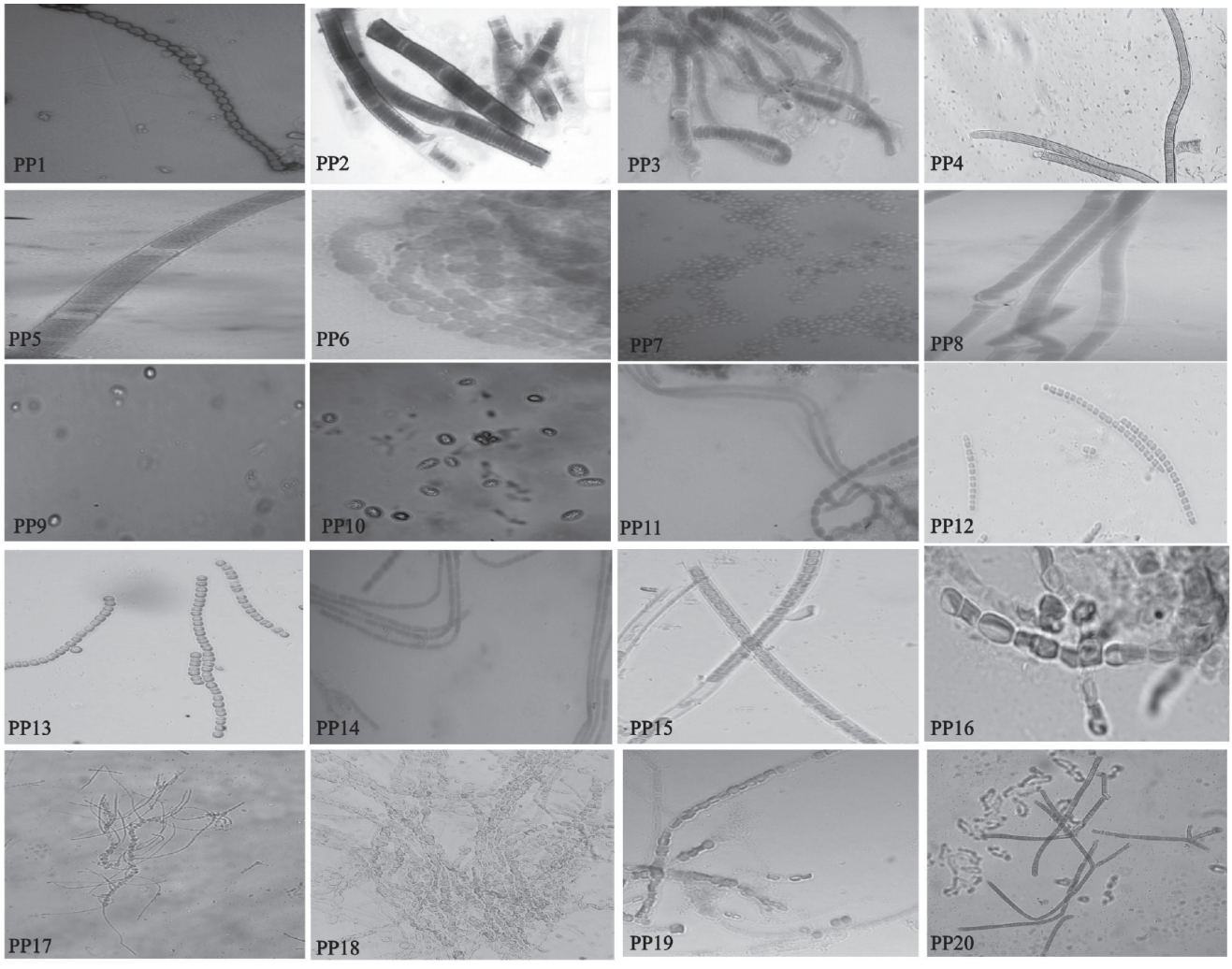


Fig 1. Images of 20 isolates of cyanobacteria (PP1:*Anabaena variabilis*, PP2:*Scytonema* sp.(1), PP3:*Scytonema* sp. (2), PP4:*Hapalosiphon* sp. (1), PP5:*Brasilonema* sp., PP6:*Nostoc* sp. (1), PP7:*Synechocystis* sp., PP8:Uncultured cyanobacterium (1), PP9:*Aphanothece* sp., PP10:*Gloeothece* sp., PP11:*Westiellopsis* sp. (1), PP12:*Hapalosiphon* sp. (2), PP13:*Westiellopsis prolifica*, PP14:Uncultured cyanobacterium (2), PP15:*Niveispirillum cyanobacteriorum*, PP16:*Nostoc* sp. (2), PP17:*Fischerella* sp., PP18:*Aphanizomenon* sp., PP19:*Westiellopsis* sp. (2), PP20:*Scytonema* sp. (3))

Table 3

The morphological characteristics of different isolates obtained from the study sites.

Isolate	Vegetative cell					Heterocyst				Akinetes		
	Shape	Size		P/A	Position	Shape	Size		P/A	Position	Shape	Size	
		Length (µm)	Width (µm)				Length (µm)	Width (µm)				Length (µm)	Width (µm)
PP1	S	4.26±0.15	4.21±0.15	P	I/T	S	5.51±0.36	5.51±0.36	P	I	O/B	7.91±0.32	6.00±0.55
PP2	E	3.51±0.31	4.96±0.40	P	I	B/C	9.03±0.50	6.16±0.55	P	I	B	7.76±0.55	7.00±0.36
PP3	B	3.86±0.55	4.23±0.41	P	I	B	6.16±0.35	8.83±0.51	P	I	C	6.76±0.47	7.73±0.15
PP4	B	6.33±0.55	8.91±0.52	P	I/T	B/S	7.56±1.07	10.56±0.32	P	I/T	Ss	9.11±0.88	10.93±0.37
PP5	C/R	5.66±0.40	6.16±0.35	P	I	C/D	8.23±0.30	5.93±0.56	P	I	S	6.33±0.15	6.33±0.15
PP6	R	5.32±0.43	4.96±0.49	P	I/T	R	10.16±0.45	9.73±0.40	P	I/T	O	10.66±0.56	10.56±0.20
PP7	S	3.93±0.15	3.93±0.15	A	NA	NA			A	NA	NA	NA	NA
PP8	O/C	7.46±1.25	5.52±1.17	p	I	C	8.76±0.45	6.71±1.34	P	I/T	Re	5.61±1.11	6.33±0.64
PP9	Ov	6.33±1.05	5.71±1.90	A	NA	NA	-	-	A	NA	NA	NA	NA
PP10	O	4.13±0.60	4.63±0.73	A	NA	NA	-	-	A	NA	NA	NA	NA
PP11	C	6.21±0.65	4.63±0.45	P	I/T	O	5.63±0.55	8.73±0.35	P	I/T	B	6.81±0.52	8.43±0.90
PP12	B	5.23±0.70	5.36±0.25	P	I/T	B	5.61±0.71	6.41±0.31	P	I/T	O	5.83±0.50	6.46±0.96
PP13	S	4.53±0.92	4.73±0.47	P	I/T	O	5.86±0.45	7.42±0.88	P	I/T	B	6.63±0.41	7.96±0.58
PP14	C	6.13±0.65	4.76±0.40	P	I/T	R/C	6.00±0.55	8.83±0.35	P	I/T	E	6.73±0.77	8.43±1.00
PP15	C	6.36±0.55	4.91±0.31	P	I/T	O	5.73±0.58	8.53±0.25	P	I/T	B	6.71±0.45	8.26±0.97
PP16	C	4.73±0.85	4.93±0.37	P	I	O	5.73±0.30	3.86±0.30	P	I/T	O	11.43±0.25	9.11±0.42
PP17	S/B	5.210±0.3	5.93±0.15	P	I/T	O	6.73±0.55	6.16±0.41	P	I	S	6.81±0.42	6.81±0.42
PP18		9.06±0.75	8.43±0.86	P	I	C	10.71±0.51	8.33±0.15	P	I/T	B	11.31±0.22	8.26±0.35
PP19	B	5.33±0.40	5.63±0.37	P	I/T	C	6.63±0.30	6.23±0.45	P	I/T	O	7.86±0.30	8.13±0.20
PP20		6.30±0.15	6.63±0.20	P	I/T		8.93±0.45	7.51±0.36	P	I/T	O	9.26±0.55	9.26±0.32

Note: Shape: B-Barrel, C- Cylindrical, D-discoid, E- Elliptical, Ob-Oblong, O-Oval, Ov-Ovoid, Re-Rectangular, R-Rounded, S-Spherical, Ss-Sub-spherical, P/A- Present/ Absent; I-Intercalary; T-Terminal

3.3 Genetic characterization

Gel pictures of 20 cyanobacterial isolates were presented in Fig 2. Based on the genetic analysis, different isolates were identified on percent similarity from NCBI database and is given in (Table 2). The dendrogram divided the whole 20 cyanobacteria isolates into six main groups (I-VI) (Fig. 3). The Uncultured cyanobacterium (1) might own enough similarity to *Nostoc* sp. isolate. *Niveispirillum cyanobacteriorum,* which was found to be distinct from the rest of the cyanobacteria, was grouped the major group VI. The results of this dendrogram concluded that the isolates which had genetically kindred but they might be differed in their other parameters.

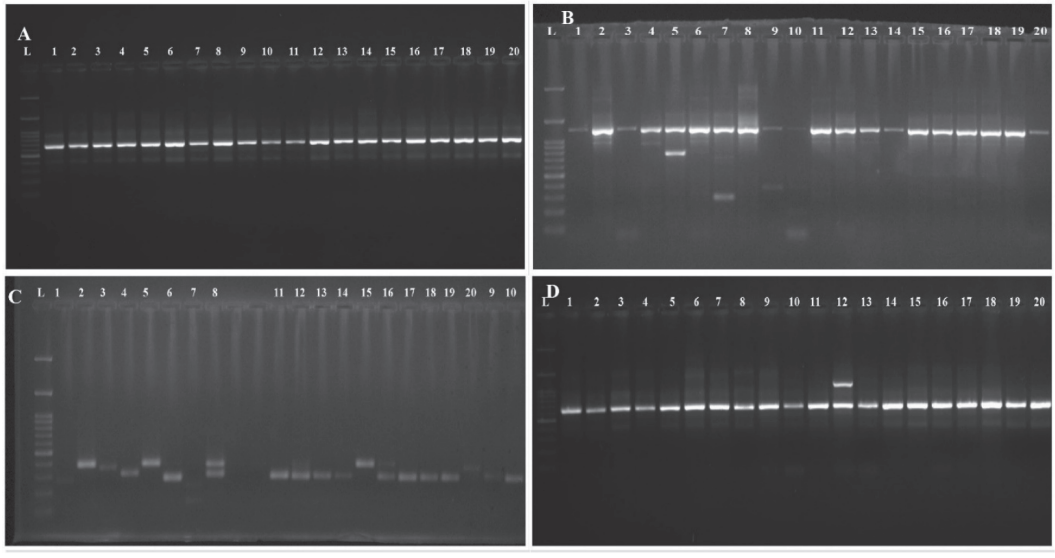


Fig. 2. Documented gel picture of 20 Cyanobacterial isolates (A. CYA106F + CYA781R(a) + CYA781R(b), B. CYA359F + CYA781R(a) + CYA781R(b), C. CYA106F + CYAN1281R, D. ITSCYA236F + ITSCYA225R) (Left to Right)

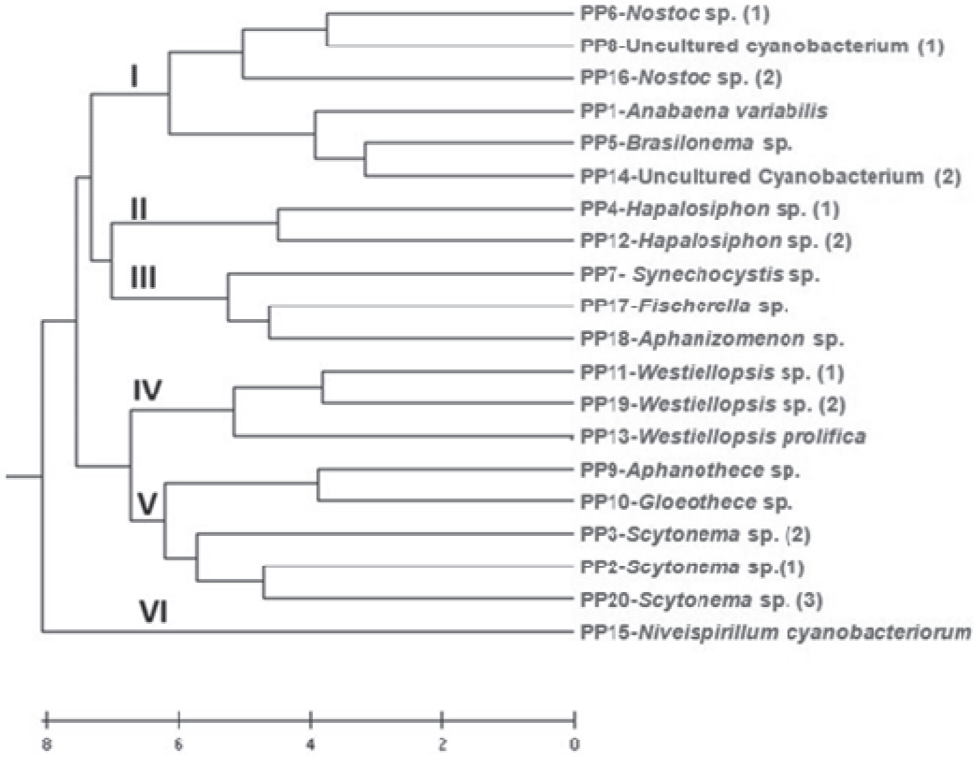


Fig 3. Cluster analysis of twenty cyanobacteria isolates using UPGMA methods.

3.4 Growth attributes

Cell dry weight, protein and carbohydrate contents varied significantly among the isolates (Table 4). A broad range of cell dry weight (3.47-17.20 mg/mL) was observed. The maximum cell dry weight was observed in *Scytonema* sp. (2), which was nearly same as in *Nostoc* sp. (1) and *Anabaena variabilis* and significant difference among the species was observed. Both protein and carbohydrate contents were maximum in *Anabaena variabilis,* which was significantly higher than of all other strains. The lowest value of protein content was found in *Hapalosiphon* sp. (1). Among the isolates, *Anabaena variabilis*, *Nostoc* sp. (1) and *Westiellopsis* sp. (1) had maximum carbohydrate accumulation whereas, the carbohydrate accumulation was minimum in *Brasilonema* sp. and *Hapalosiphon* sp. (1).

Table 4

Growth attributes (Cell Dry weight, Protein and Carbohydrate content) of different isolates

Species	Dry wt (mg/mL)	Protein (µg/mL)	Carbohydrate (µg/mL)	Chl a (µg/mL)	Car (µg/ mL)	PE (µg/mL)	PC (µg/mL)	APC (µg/mL)
Anabaena variabilis	12.37^{abc}	34.53^{a}	67.23^{a}	20.02^{ab}	7.98^{ab}	2.73^{b}	3.85^{b}	5.90^{a}
Scytonema sp. (1)	7.67^{cdefg}	19.88^{de}	36.76^{bcde}	6.01^{gh}	3.57^{hi}	1.56^{def}	1.94^{fg}	3.01^{cde}
Scytonema sp. (2)	17.20^{a}	29.05^{b}	45.09^{bcd}	17.37^{abcd}	5.58^{defg}	2.05^{c}	2.86^{c}	4.14^{b}
Hapalosiphon sp. (1)	8.60^{cdefg}	1.68^{j}	3.10^{j}	7.94^{fgh}	2.97^{i}	0.60^{ij}	0.84^{ij}	1.25^{fg}
Brasilonema sp.	3.47^{g}	1.72^{j}	2.92^{i}	4.88^{h}	1.25^{j}	0.55^{j}	0.84^{ij}	1.09^{ghi}
Nostoc sp. (1)	14.43^{ab}	22.34^{d}	50.24^{b}	22.23^{a}	7.27^{abcd}	1.75^{cde}	2.62^{cde}	4.08^{b}
Synechocystis sp.	7.30^{cdefg}	6.08^{ij}	11.53^{ghi}	18.17^{abcd}	6.20^{cdef}	2.68^{b}	3.89^{b}	6.06^{a}
Uncultured (1)	6.70^{cdefg}	20.93^{de}	47.10^{bc}	12.99^{def}	3.80^{hi}	1.58^{de}	2.35^{def}	3.62^{bc}
Aphanothece sp.	4.63^{fg}	14.16^{fg}	24.03^{efg}	8.59^{fgh}	3.24^{hi}	0.95^{hi}	1.39^{h}	2.12^{ef}
Gloeothece sp.	5.53^{efg}	5.05^{ij}	8.33^{hi}	5.31^{h}	1.26^{j}	1.05^{h}	1.47^{gh}	2.17^{ef}
Westiellopsis sp. (1)	8.40^{cdefg}	25.06^{c}	45.11^{bcd}	12.82^{def}	6.37^{bcde}	1.71^{cde}	2.16^{ef}	3.32^{bcd}
Hapalosiphon sp. (2)	7.33^{cdefg}	13.87^{fg}	30.52^{def}	11.42^{efg}	4.65^{fghi}	1.67^{de}	2.32^{def}	3.23^{bcd}
Westiellopsis prolifica	7.53^{cdefg}	15.96^{f}	35.09^{bcde}	13.17^{cdef}	7.11^{abcd}	1.90^{cd}	2.66^{cd}	3.79^{bc}
Uncultured (2)	7.97^{cdefg}	13.87^{fg}	27.76^{ef}	18.73^{abc}	8.08^{a}	1.55^{def}	2.08^{f}	2.62^{de}
N. cyanobacteriorum	5.80^{defg}	18.95^{e}	32.21^{cdef}	19.97^{ab}	7.92^{ab}	3.11^{a}	4.50^{a}	6.26^{a}
Nostoc sp. (2)	10.87^{bcde}	13.65^{fg}	23.51^{efg}	15.62^{bcde}	7.49^{abc}	1.43^{efg}	1.59^{gh}	1.16^{gh}
Fischerella sp.	11.40^{bcd}	13.16^{g}	18.31^{fgh}	14.71^{bcde}	8.21^{a}	1.07^{gh}	0.56^{ij}	0.31^{hi}
Aphanizomenon sp.	9.73^{bcdef}	9.64^{h}	18.76^{fgh}	8.28^{fgh}	4.09^{ghi}	0.31^{j}	0.37^{j}	0.26^{hi}
Westiellopsis sp. *(2)*	11.17^{bcde}	13.07^{g}	23.49^{efgh}	15.23^{bcde}	8.42^{a}	1.21^{fgh}	0.64^{ij}	0.63^{ghi}
Scytonema sp. *(3)*	7.37^{cdefg}	8.66^{h}	17.26^{fghi}	10.49^{efgh}	4.93^{efgh}	0.32^{j}	0.29^{j}	0.24^{i}
HSD	5.7972	2.5626	15.163	5.6772	1.7003	0.3719	0.4701	0.9178

* Means with at least one letter common superscripts are not statistically significant using Tukey's honestly significant difference (HSD) test at $p \geq 0.05$

PE-phycoerythrin; PC-phycocyanin; APC-allophycocyanin

3.5 Pigment Content

The chlorophyll *a* content of all isolates ranged from 4.88 to 22.23 µg/mL (Table 5). The highest chlorophyll *a* was found in *Nostoc* sp. (1) and *Anabaena variabilis*. The carotenoids content among isolates ranged from 1.25 to 8.42 µg/mL (Table 5). *Westiellopsis* sp. (2) exhibited the maximum carotenoids content which was at par with *Fischerella* sp., and uncultured cyanobacterium (2). Likewise, the isolates also showed significant variability with respect to PC, PE and APC contents (Table 5). The total phycobiliproteins ranged from 0.85 µg/mL in *Scytonema* sp. (3) to 13.87 µg/mL in *Niveispirillum cyanobacteriorum*. The PC (4.50 µg/mL) and PE (6.26 µg/mL) contents were observed the highest in *Niveispirillum cyanobacteriorum* while the lowest content was in *Scytonema* sp. (3) (0.24 µg/mL). Among the isolates analyzed, the maximum quantity of APC was found in *Niveispirillum cyanobacteriorum* which was statistically at par with *Synechocystis* sp. and *Anabaena variabilis*.

3.6 Activities of enzymes

The maximum nitrate reductase (NR) activity was recorded in *Nostoc* sp. (1) (32.08 µmoles/ml h), while *Gloeothece* sp. produced the lowest activity (17.25 µmoles/ml h) (Fig. 4). Glutamine synthetase (GS) activity was found highest in *Anabaena variabilis* (106.17 µmoles/ml h). *Hapalosiphon* sp. (2) had the lowest GS activity of 55.63 µmoles/ml h among the cyanobacterial isolates (Fig. 4).

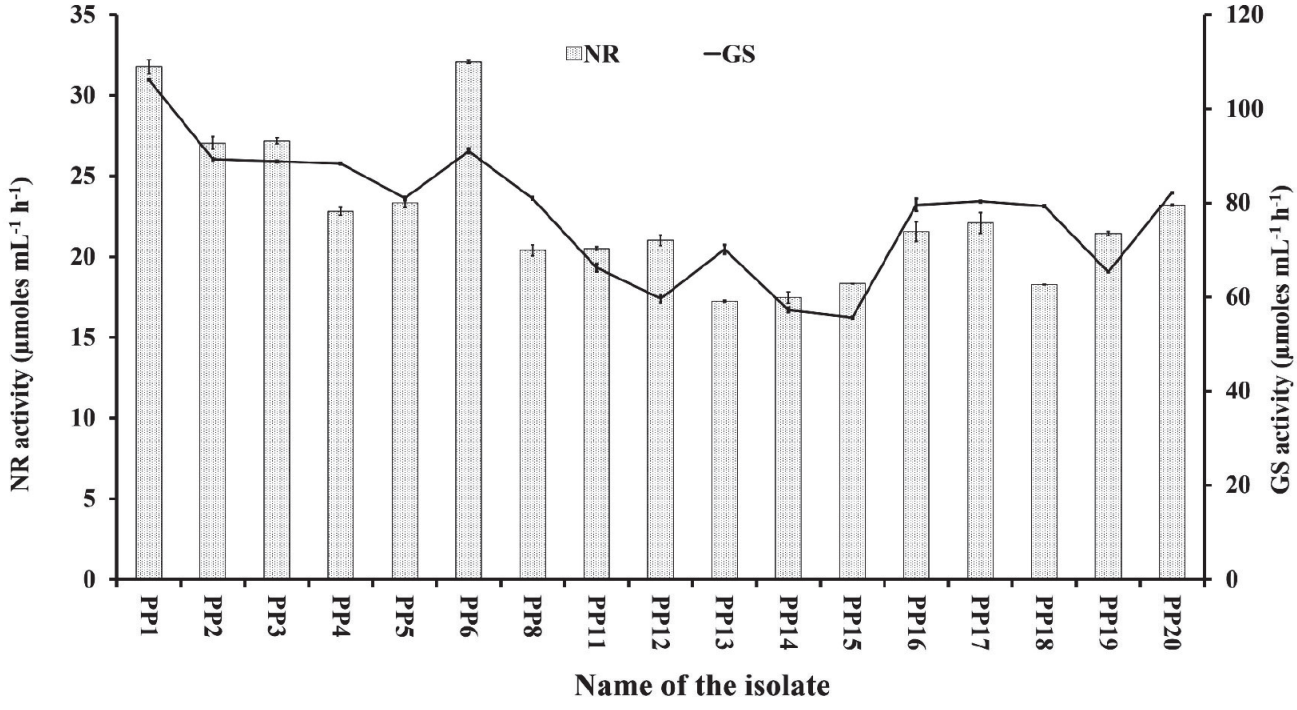


Fig 4. Nitrate Reductase (NR) and Glutamine Synthetase (GS) content of heterocystous cyanobacterial isolates

4. Discussion

Cyanobacteria are an oxygenic prokaryotic group and are present at all types of niches like terrestrial, sub-aerial, fresh water, saline water and hypersaline environments. Soil samples at experimental sites were slightly acidic to moderately alkaline. Cyanobacteria are ubiquitous in their distribution, but they prefer a neutral or alkaline pH for their best growth (Alghanmi and Jawad, 2019). Cyanobacteria such as *Anabaena, Scytonema, Hapalosiphon, Nostoc,* and *Westiellopsis* were profusely found in the soil within pH range of 6.5-7.5 and our finding was quite similar to these studies (Alghanmi and Jawad, 2019; Ghadage and Karanda, 2019). *Anabaena variabilis*, *Scytonema* sp. (1), *Scytonema* sp. (2), *Hapalosiphon* sp. (1), *Synechocystis* sp., Uncultured cyanobacterium (1), *Gloeothece* sp., *Westiellopsis* sp. (1), *Westiellopsis prolifica*, *Nostoc* sp. (2), *Fischerella* sp. and *Aphanizomenon* sp. were more abundantly found in slightly acidic soil pH. Whereas, *Brasilonema* sp., *Nostoc* sp. (1), *Aphanothece* sp., *Hapalosiphon* sp. (2), Uncultured

cyanobacterium (2), *Niveispirillum cyanobacteriorum*, *Westiellopsis* sp. (2), *Scytonema* sp. (3) were found in slightly alkaline paddy field soil.

EC is another important element that can affect the soil microbial community (Shariatmadari *et al.,* 2013). According to Singh *et al.*. (2014), *Anabaena constricta* were found abundantly in soil with high EC (801.8 µS cm^{-1}) whereas, in our study, *Fischerella* sp., *Scytonema* sp. (1) and *Westiellopsis* sp. (2) were found abundantly in soils with high EC and *Aphanizomenon* sp., *Westiellopsis prolifica* and *Anabaena variabilis* were more abundant in soils with low EC. The soils of this institute had generally unicellular *Aphanothece* sp. and filamentous *Gloeotrichia* sp. around 50 years ago (Singh, 1973; Pattnaik and Singh, 1978). This indicated a shift in the species distribution enforced by the agropractices and soil characteristics.

Cyanobacteria group occupy a high degree of morphological, physiological and developmental complexity. The size of *Aphanothece* sp., *Gloeothece* sp. and *Synechocystis* sp. were similar to the results obtained by Ghadage and Karande (2020). In our study, the cell width of cyanobacteria ranged from 4.23 to 8.9 µm. Similar results of *Scytonema* sp. (2) and *Westiellopsis* sp. were also found (Pattnaik and Samad, 2018). In certain cases, the morphological variability of cyanobacteria isolates was observed under the adverse environmental conditions.

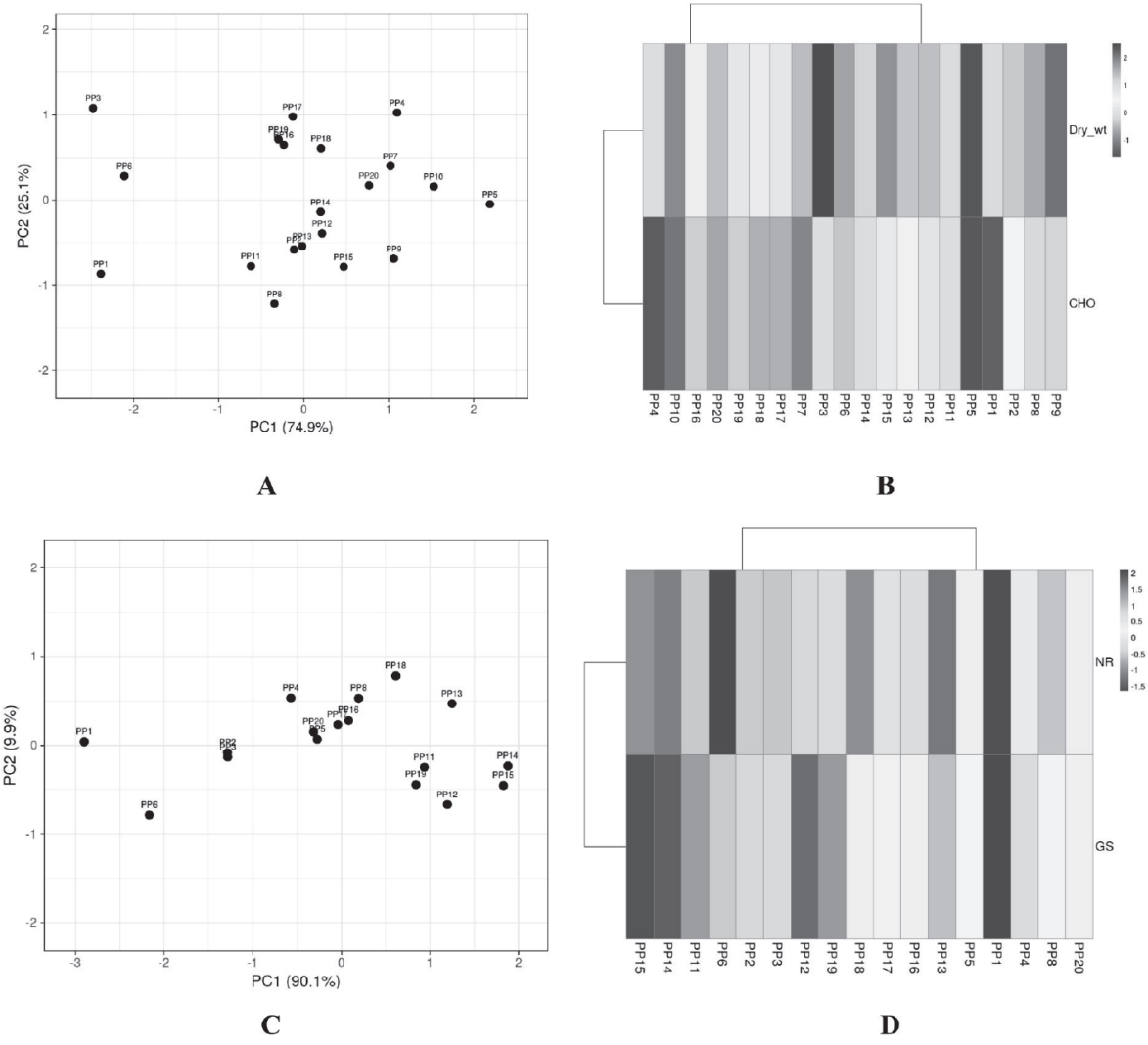
Most of the filamentous cyanobacterial species can generate heterocysts and akinetes with some exceptions like *Oscillatoria*. The abundant presence of heterocystous cyanobacteria may be indicating the lower nitrogen content at that particular location (Ghadage and Karande, 2019). The vegetative cell transformed into the heterocysts with certain morphological and physiological alterations in nitrogen deficient conditions. Heterocysts and vegetative cells are interdependent to each other for reduced carbon and nitrogen, respectively. These are the main attributes to compare the sizes between vegetative cell and heterocyst. The heterocysts size of *Aphanizomenon* sp. were larger as compared to vegetative cells as expected. Such features were recorded earlier in *Anabaena* species (Prasanna *et al.,* 2006). Heterocysts were mostly intercalary (Rippka *et al.,* 2015) but some exceptions were mentioned in Table 3, where both intercalary and terminal heterocysts were also observed. The large sized heterocysts may have greater potential to fix more nitrogen than smaller ones. Thus, the species with such attributes can be utilized for commercial purposes. The akinetes were found in away from the heterocyst with few exceptions. Similar results for the size of heterocysts and akinetes of *Anabaena* and *Nostoc* were also observed (Rajaniemi *et al.,* 2005).

The cell dry weight is a significant property to find out the growth rate of an organism. These broad range (14.52-9.43 mg/ml) of cell dry weight of different cyanobacterial species (*Lyngbya sp*., *Anabaena variabilis*) was also observed (Saxena *et al.,* 2007). As the cyanobacteria have more industrial value, the high growth rate gives a better opportunity to use them commercially. In cyanobacteria, carbohydrates are stored as small sized glycogens. This is the main reason behind the preference of cyanobacteria species in biofuel production. *Synechocystis* sp. and *Synechococcus elongatus* were able to produce biofuel along with other valuable chemicals (Machado and Atsumi, 2012). In our study, among the isolates, *Anabaena variabilis* (67.23 µg/ml) showed the highest content of carbohydrates followed by *Nostoc* sp. Principal component analysis and heat map study also depicted the similar picture (Fig. 5). Based on these *Anabaena variabilis*, *Nostoc* sp. (1) and *Scytonema* sp. (2). could be grouped together. The obtained results were similar to the results of several other workers (Prasanna *et al.,* 2006; Tiwari and Singh, 2005). The high protein content was observed in *Anabaena variabilis* and *Scytonema* sp. (2). The protein content of cyanobacteria was in the range from 6.1-497.8 µg/ml (Tiwari and Singh, 2005). The genera *Anabaena* had a wide range of protein content (31.17-447.69 µg/ml) because of a wide variation in their growth potential and biomass production (Prasanna *et al.,* 2006). Similarly, in our experiment the protein content of *Anabaena variabilis* was 34.53 µg/ml. The similar results were also reported by Narayan *et al.* (2006). There was a substantial variation in protein content among the 20 isolates. It may be because of the differences in the management practices of the crop fields resulting in different assimillable levels of nitrogen.

The maximum chlorophyll *a* content was found in *Nostoc* sp. (1) followed by *Anabaena variabilis*, *Niveispirillum cyanobacteriorum* and an uncultured cyanobacterium. Such results have also been observed by several workers in previous studies (Tiwari and Singh, 2005). The accessory pigments, carotenoids are abundantly found in cyanobacteria strains. Reports suggested that the cyanobacteria strains, that have high carotenoid content, possess more tolerance to high light intensity (Wilson *et al.,* 2006). Among the isolates the maximum carotenoids content was noted in *Westiellopsis* sp. (2) followed by *Fischerella* sp., an uncultured cyanobacterium and *Anabaena variabilis*. Thus, these isolates possessed high rate photosynthesis compared to other reported strains. The PC content varied from 0.29-4.49 µg/ml among the groups. The highest and lowest value has been exhibited by *Niveispirillum cyanobacteriorum* and *Scytonema* sp. (3), respectively. Similar reports by other workers suggested

that the APC content was higher than the PE and PC among the phycobiliproteins (Narayan *et al.,* 2006). Thus, our isolates *Niveispirillum cyanobacteriorum*, *Synechocystis* sp. and *Anabaena variabilis* be used as valuable resources for phycobiliprotein protein production as they have high content of this protein complexes. In some reports it was found that the carbohydrate accumulation and phycobiliprotein depletion had taken place at the same time (Mollers *et al.,* 2014).

The NR activity among these cyanobacteria varied from 17.48-32.08 µmoles/ml h. The minimum NR activities were found in *Westiellopsis prolifica*, uncultured cyanobacterium (2), *Niveispirillum cyanobacteriorum* and *Aphanizomenon* sp. whereas the maximum NR activity were shown by *Nostoc* sp. (1), *Anabaena variabilis* and *Scytonema* sp. (2). The activity of GS among these cyanobacteria ranged from 55.63-106.16 µmoles /ml h. Similar results have been obtained with *Anabaena* and *Nostoc* (Narayan *et al.,* 2006). Among the 20 isolates of the present experiment, the GS activity were found minimum in case of *Niveispirillum cyanobacteriorum*, Uncultured cyanobacterium (2) and *Hapalosiphon* sp. (2), whereas maximum GS activity was observed in *Nostoc* sp. (1), *Anabaena variabilis* and *Scytonema sp.* (2). Low GS with high NR enzyme activity found in *Westiellopsis* sp. (2) and *Hapalosiphon* sp. (2) are responsible for making of good quality biofertilizer (Shimkets, 2015). The attributes namely, heterocyst frequency and size, cell biomass and nitrogen fixation ability of cyanobacteria are the main determinants to qualify as biofertilizer. *Nostoc* sp. (1) and *Anabaena variabilis* can be used as biofertilizer as per the PCA and heat map analysis (Fig. 5). The isolate *Anabaena variabilis* showed a good result in all these growth attributes. This isolate can be utilized as biofertilizer and also in biofuel industry as a biofuel producer.



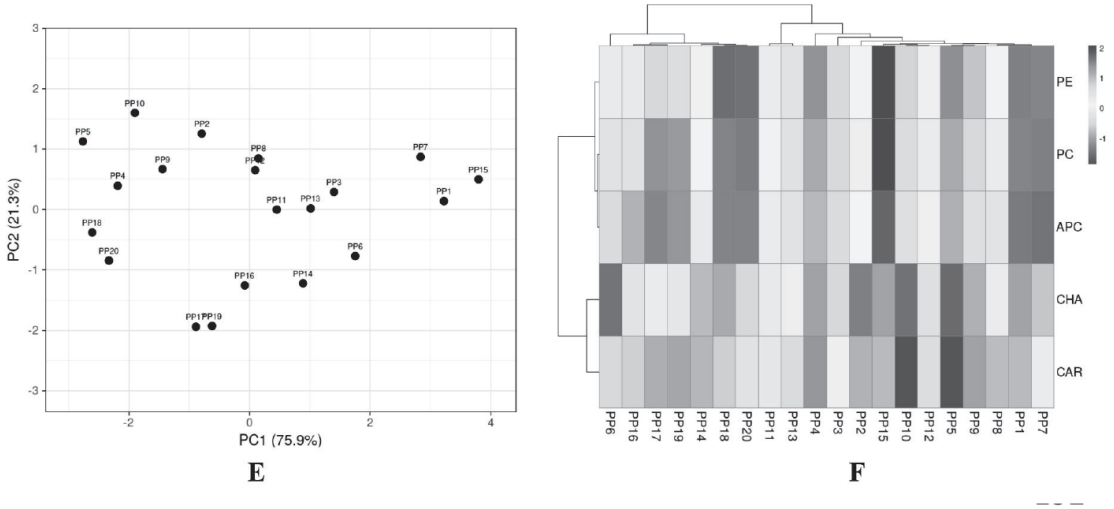


Fig. 5. Principal component analysis (PCA) and heat map (HM) study to differentiate cyanobacteria isolates as biofuel producer (A, PCA and B, HM), biofertilizer (C, PCA and D, HM) and pigment producer (E, PCA and F, HM) (Dry wt: dry weight; CHO: carbohydrate content; NR: Nitrate reductase; GS: Glutamine synthatase; CHA: chlorophyll *a*; CAR: carotenoids; PC: phycocyanin; PE: phycoerythrin and APC: allophycocyanin)

Reports suggested that the management practices like indiscriminate use of pesticides had detrimental effects on growth, photosynthesis, and nitrogen fixation of cyanobacteria (Shinde, 2018). The deleterious effect on photosynthesis may affect the nitrogen metabolism because photosynthesis supplies the energy to complete the process of N_2-fixation (Tiwari *et al.,* 2019). The nature, concentration and duration of expose to pesticides are found responsible for the toxicity to cyanobacteria. Thus, in our study, the different management practices were also playing the vital role as they have a duration based (long term and short term) pesticidal effect in the nitrogen fixation on the basis of the activity of enzymes i.e., NR and GS. This study also revealed that cyanobacteria can tolerate different groups of pesticides.

The rice ecosystem had both heterocystous and non-heterocystous cyanobacteria. The *Anabaena variabilis* and *Nostoc* sp. (1) may be utilized as biofertilizer and biofuel producer. Because of the high pigment content, *Westiellopsis* sp. (2), *Fischerella* sp., *Synechocystis* sp. and *Anabaena variabilis* may be used as valuable resources for cosmetic/ commercial industries. Based on the all parameters the *Anabaena variabilis* may be used as both agricultural and commercial sector. The long term and short term pesticide fields, organic fields and other experimented fields have various cyanobacterial strains which may have the ability to tolerate these stresses. These 20 abundant cyanobacterial isolates may be further studied for their role in bioremediation of pesticides. They can be analyzed for commercial suitability because of their huge genetic potential.

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