CHAPTER 4: RESULTS

Ten cyanobacterial strains from genus *Spirulina/Arthrospira* were procured from culture collection of CCUBGA, IARI, New Delhi-110012. Some of these strains were isolated from soils of IARI fields and other geographical locations and are maintained at CCUBGA, IARI. *Arthrospira* sp. (CCC538 and CCC539) which were originally procured from Canary Islands (Spain) are presently maintained in the germplasm of CCUBGA. The *Spirulina* sp. CCC540 was an isolate from Loktak Lake (Manipur), which is a wetland recognized under Ramsar Convention, 1971 and this isolate was provided by Dr. O.N. Tiwari, scientist from Institute of Bioresources and Sustainable Development, Imphal, Manipur.

These cyanobacterial strains were examined microscopically to check the purity and were made axenic through repeated sub culturing, streaking and antibiotic treatment. After ascertaining the purity of cultures, such bacteria free cyanobacterial strains were utilized for further work.

4.1. Cultural characteristics

The cultural behavior of the selected strains of *Spirulina/Arthrospira* was assessed at different stages of growth in nitrogen enriched modified Z- medium. Most of the strains exhibited uniform suspension in liquid medium and the remaining were planktonic/free floating. In solid medium, almost all the strains show spreading growth behavior. Variation in the colour of the thallus was distinct and the colour ranged from blue green to pale green. *Spirulina platensis* (CCC479) and *Spirulina maxima* (CCC481) showed green colored thalli. Most of these strains were non-mucilaginous except few which depicted mucilaginous growth in the liquid medium resulting in the formation of clumps.

4.2. Morphometric parameters

All the strains of *Spirulina/Arthrospira* were studied microscopically for morphological parameters in terms of shape of trichomes, number of cells per filament, size and shape of vegetative cells. *Spirulina/Arthrospira* strains showed spirals which were regular or irregular. However, on an average these could be
categorized as short (8-75 cells), medium (76-150 cells) and long (>150 cells) filaments. All the three types of filaments were recorded in the selected strains. The filaments were non-heterocystous and exhibited vegetative cells which were cylindrical, rectangular, barrel shaped or oblong. The length of the vegetative cells ranged from the lowest of 1.21µm to the highest of 7.67µm. The largest length of the vegetative cell was recorded in CCC539 (*Arthrospira* sp.) and the smallest cell was recorded in CCC481 (*Spirulina maxima*). The smallest breadth of vegetative cells varied from 1.12 µm (CCC540) to the largest size of 7.56µm which was observed in CCC480. No heteropolarity was recorded in the filaments of *Spirulina/Arthrospira* used in the present study. (Table-5, Plate-5)

4.3. Physiological parameters

There was a significant difference in chlorophyll content (mg g⁻¹ dry wt.) amongst the selected strains of *Spirulina/Arthrospira* strains. Strain CCC540 showed highest chlorophyll content (1.609 mg g⁻¹ dry wt.) at 28th day of incubation; however CCC477 depicted lowest chlorophyll content (0.086 mg g⁻¹ dry wt.) on 7th day of incubation (Fig.1.A). Highest carotenoids content (0.101 mg g⁻¹ dry wt.) was shown by CCC540 at 7th day of incubation and lowest (0.012 mg g⁻¹ dry wt.) in CCC480 on 28th day of incubation (Fig.1.B). Total phycobiliproteins (1.998 mg g⁻¹ dry wt.) were maximum in CCC540 on 21st day of incubation and these were lowest (0.168 mg g⁻¹ dry wt.) in CCC538 on 7th day of incubation (Fig.2.A). Total soluble proteins exhibited significant differences amongst the studied cultures. These were very low (2.35 mg g⁻¹ dry wt.) in CCC478 on 7th day of incubation and highest (10.24 mg g⁻¹ dry wt.) in CCC540 at 14th day of incubation (Fig.2.B). Nitrate reductase activity ranged from the lowest of 64.94 µmole C₂H₄/mg proteins in CCC479 on 7th day of incubation to the highest of 432.67 µmole C₂H₄/mg proteins in CCC440 on 14th day of incubation (Fig.3.B). Glutamine synthetase (GS) activity which is a key enzyme for ammonia fixation differed significantly in the present study. The activity was highest (21.698 µ mole γ-glutamyl hydroxamate/ mg protein) in CCC540 on 14th day of incubation and lowest (1.496 µ mole γ-glutamyl hydroxamate/ mg protein) in CCC477 on 7th day of incubation (Fig.3.A). The total sugars showed a linear enhancement with incubation time and the content was maximum (10.167 mg g⁻¹ dry wt.) on 21st day of incubation in CCC538. However, CCC478 showed the lowest total sugar content (3.002 mg g⁻¹ dry wt.) on 21st day of
incubation Mean dry weight of ten strains of *Spirulina* calibrated during the incubation time till log phase (Fig.4.B). Dry weight was highest (11.231 mg/mL) in CCC540 on 28th day of incubation and lowest (2.043 mg/ml) in CCC477 on 7th day of incubation (Fig.4.A).

**4.4. Influence of environmental/cultural variables**

The environmental variables can influence growth and pigment production which in turn may change the composition of the biomass by affecting the metabolism. Varied light intensity exhibited differential influence on the accumulation of chlorophyll, carotenoids and phycobilins. Results indicated that a light intensity of 55µmolm⁻² s⁻¹ was optimum for maximum chlorophyll and that of 85µmolm⁻² s⁻¹ was inhibitory in most cases, except in case of CCC538 (Fig.5.A). Variation in the temperature influenced the pigment profile with 35°C being the optimum for enhanced chlorophyll (Fig.7.A). In general, chlorophyll content enhanced with increase in carbon dioxide concentration and a concentration of 750 ppm was optimum (Fig.9.A). Effect of pH was distinct with 10.5 pH being the optimum for enhanced chlorophyll production (Fig.11.A). Sodium chloride concentration of 2 g L⁻¹ depicted highest chlorophyll (Fig.13.A). Carotenoids and phycobiliproteins were highest at 70 µmol m⁻² s⁻¹ light intensity (Fig.5.B and Fig.6.a,b,c) and 10.5 pH was optimum for higher carotenoids and phycobiliproteins (Fig.11.B and Fig.12.a,b and c). In conventional cultivation, light absorption is proportional to the phycocyanin and chlorophyll content of the cells and a slight reduction in phycocyanin may be accompanied by an increase in chlorophyll. Carotenoids were maximum at 35°C temperature (Fig.7.B) and enhanced CO₂ resulted in their increase with 750 ppm to be an optimum concentration (Fig.9.B). Sodium chloride concentration of 1.5 g L⁻¹ showed highest carotenoids (Fig.13.B). Phycobiliproteins enhanced significantly at 35°C in some cultures (Fig.8.a,b and c), whereas decline was observed in others. Enhancement in CO₂ concentration increased the phycobiliproteins (Fig.10.a, b and c.), and sodium chloride concentration of 2 g L⁻¹ was most suitable for phycobiliprotein production (Fig.14.a,b and c). Study clearly showed that the pigments from *Spirulina*, which have the potential applications in biotechnology can be enhanced by manipulating cultural conditions and environmental variables. Standardized protocol for enhanced production of pigments involved an optimized light intensity: 70 µmol m⁻² s⁻¹ for chlorophyll and carotenoids, and 85 µmol m⁻² s⁻¹ for phycobiliproteins, temperature:
35°C for chlorophyll and phycobiliproteins, and 25°C for carotenoids, CO₂ concentration: 500 ppm and 750 ppm, pH:10.5 and NaCl concentration: 2 g L⁻¹ (Table-6,7 and 8).

4.5 Extraction and estimation of β-carotene

β-carotene concentration (mg/100 gdwt.) differed significantly amongst *Spirulina/Arthrospira* strains tested and the levels ranged from the highest of in CCC540 (260.39 mg/100 g dwt.) to the lowest of 183.71 mg/100 gdwt.) in CCC477 (Table-9). The standard beta carotene peak was achieved around the retention time of 6.7 minutes (RT = 6.7) in all the strains. The concentrations of the beta carotene standards were plotted against the peak area to obtain a straight line. The peak was automatically identified and quantified by comparing its retention time of all the strains with the standard retention time (Fig.15.a, b, c, d and e).

4.6 Antioxidant activity

The levels of antioxidants in hexane extract ranged from 0.52±0.03μmolTroloxg⁻¹ in CCC481 to 3.64±0.03μmol Trolox g⁻¹ in CCC540 based upon FRAP assay, in hexane extract. The obtained data also showed a diverse antioxidant capacity in the various fractions of the extracellular substances of the studied strains.

Water extract fractions depicted better antioxidant levels in comparison to hexane and ethyl acetate fractions based upon DPPH assay. Aliquot volume used for water extract was 10µL which was 50 times less than hexane or ethyl acetate extract and water was shown to be very strong solvent for antioxidant extraction. The highest radical-scavenging power with DPPH assay was in CCC540 (11.29±0.05 μmol Trolox g⁻¹) and it was lowest in CCC538 (2.61±0.02 μmol Trolox g⁻¹). The selected strains of *Spirulina/Arthrospira* showed high antioxidant activity with DPPH assay and were also supported with FRAP assay.

Antioxidant activity shown by ABTS assay is higher than FRAP and DPPH assay. The scavenging capacity was highest (13.29±0.06 μmol Trolox g⁻¹) in CCC540 and lowest (3.22±0.05 μmol Trolox g⁻¹) in CCC477. The trend observed in DPPH assay was seen to be similar to that of ABTS assay and interestingly, the overall value of total antioxidant activity in DPPH were lower than those obtained in ABTS assay (Table-9).
4.7 Total lipids extraction

Total lipids were determined on dry weight basis using homogenized suspension of *Spirulina/Arthrospira* and palmitic acid was used as standard. Overall mean showed that the total lipids were maximum (11.4±0.02) in *Spirulina platensis* CCC540 and these were lowest (3.6±0.04) in *Spirulina maxima* CC481 (Table-10).

Fatty acid profile of all strains was studied at GC-MS equipment with triple axis detector. The lipids extracted from these strains were tranesterified before analysis of the various major and minor constituents. Fatty acid profile chromatogram (Fig.16.a, b,c,d and e) showed the presence of saturated as well as unsaturated fatty acids. In *Spirulina*, the identified compounds included saturated hydrocarbons namely Methyl 9,12,15-octadecatrienoate (Linolenic acid), Methyl- hexadecanoate (Palmitic acid), Methyl 9,12-octadecadienoate (α-Linoleic acid ), Methyl 6,9,12-octadecatrienoate (gamma-linolenic acid), Methyl methacyrloctadecyl ester (Methacrylic acid), Methyl cis-8,11,14-Eicosatrienoate (Eicosatrienoic acid), Methyl 9,12,15-octadecatrienoate (Linolenic acid), Methyl 4,7,10,13-hexadecatetraenoate (Hexadecatrienoic acid), Methyl 9,12 octadecadienoate (α-Linoleic acid) Methyl 4,7,10-hexadecatrienoate (Hexadecatrienoic acid), Methyl-heptadecanoate (Heptadecanoic acid), Methyl 7,10-hexadecadienoate (Hexadecadienoic acid), Methyl 9-hexadecenoate, (Palmitelaidic acid) Methyl 9,12,15-octadecatrienoate (Linolenic acid) and Methyl tetracosanoate (Lignoceric acid). The unsaturated hydrocarbons were tetracosahexaene. In all the strains of *Spirulina/Arthrospira*, Lignoceric acid was the major fatty acid which accounted for 22.64% of the total on the basis of FAME analyses and total hydrocarbons were more (37.55%) than the acid constituents (26.95%) (Table-11).

4.8. Estimation of purified phycocyanin

Extraction and purification of phycocyanins involved four major steps namely, Crude extract preparation (Step I), Ammonium sulfate precipitation (Step II), Dialyses (Step III) and Anion exchange chromatography (Step IV). The purity of the extracted phycocyanin was based upon (OD$_{620}$/OD$_{280}$) value. After ammonium sulphate precipitation the purity of phycocyanin was 1.5 and after dialyses purity was enhanced to 2.93. The final purity after anion exchange chromatography was 4.58 (Table-12, and Plate-6). The systematic purification steps until ion exchange
chromatography enhanced the purity nearly six times. During the chromatographic separation, phycocyanin was eluted with maximum purity as a bright blue coloured solution at pH 3.76 (Fig. 17.a). The stringed protocol indicated the efficiency of method to obtain high purity phycocyanin. The absorption spectra of the purified phycocyanin showed a prominent peak at 620 nm and the purity was also confirmed by the presence of single band during SDS-PAGE electrophoresis of α-subunit (16kDa) and β-subunit (17kDa) respectively (Fig. 19). Reverse phase HPLC was performed using C₅ column to further characterize purified phycocyanin from *Spirulina* (Fig. 18.A). When the PDA detector was set at 620 nm and 226 nm, two major peaks were recorded at 25.612 minutes and 27.024 minutes for purified phycocyanin (Fig. 18.B and C). Absorption spectrum of individual peak is presented in (Fig. 17.b). When absorption spectrum of these two chromatogram peaks was critically analyzed, it was found that A620:A280 for the first peak (RT = 25.612 minutes) was approximately one which is due to the presence of one phycocyanobilin (PCB) chromophore. This indicated that the peak corresponded to α subunit of PC. On the other hand, A620:A280 for the second peak (RT = 27.024 minutes) was approximately two which is due to the presence of two PCB chromophores, therefore, this peak corresponded to β subunit of PC.

4.9 Molecular Characterization

4.9.1 DNA isolation and gene amplification

DNA of selected *Spirulina* strains was isolated from DNeasy Tissue Kit Manufacturer’s Protocol (Qiagen, Cat. No. 69504), and checked by gel electrophoresis (Plate-7). After PCR amplification, 16S rRNA and cpcB-IGS-cpcA gene was also run in gel electrophoresis. Size of amplified product was 1500bp (16S rRNA) and 600 bp (cpcB-IGS-cpcA) respectively (Plate-8 and 9). Sequences of all strains given in Plate-10 and Plate-11.

4.9.2 Phylogenetic analysis

The 16S rRNA gene and *cpcBA*-IGS sequences obtained from the *Spirulina* strains were initially compared with sequences available in the National Center for Biotechnology Information database using BLAST network services (http://www.ncbi.nlm.nih.gov/BLAST) to determine their approximate phylogenetic
affiliations (Altschul et al. 1997). The sequences were aligned using PHYDIC 3.0, and unambiguously aligned nucleotide positions then used for phylogenetic analyses using MEGA.5. The similarity values between the sequences were calculated from distance matrices by reversing the Jukes-Cantor distance formula (Jukes and Cantor 1969). Phylogenetic trees were then inferred by neighbour joining (NJ) (Saitou and Nei 1987) using the Kimura two-parameter model. The resulting NJ tree was evaluated by bootstrap analyses based on 1,000 resembling. Due to the spacer variability, a phylogenetic analysis of the matrix was also performed using just the two coding regions. Finally, an overview of the phylogenetic position of *Spirulina* in cyanobacteria was created by comparing the 16S rRNA gene and *cpcBA-IGS* sequences to corresponding cyanobacterial sequences available in databases and the sequences obtained in this study for *Spirulina laxissima* SAG 256.80 and *Oscillatoria sancta* NIER 10027.

**4.9.3 Phylogenetic tree analyses of 16S rRNA gene**

Near-complete 16S rRNA gene sequences were determined *Spirulina* strain received from culture collections across the world. A phylogenetic tree was then reconstructed using a NJ analysis based on aligning the all sequences with *Escherichia coli* K-12 as the outgroup and Nostocales (*Anabaena*) as other cyanobacterial group (Plate-12). The corrected sequence alignment, providing the basis of the phylogenetic analyses, corresponded to positions 8-1512 according to the *E. coli* numbering system and was 1,383 nucleotides (nt) in length after removing all gaps and ambiguous positions. The cluster analysis resolved the selected *Spirulina/Arthrospira* strains into two main genotypic clusters, designated Clade I and II. Clade I contained all nostocales members and Clade II contained all oscillatoriales namely CCC477 to CCC540. The bootstrap value between the Group I and Group II clusters was 60% in the phylogenetic tree, and the 16S rRNA gene similarity was 99.5%. *Escherichia coli* K-12 shows as out group in tree. Thus, the clusters were poorly supported by the bootstrap analysis. In the 16S rRNA gene sequences for the strains, the number of different nucleotides was less than 7 out of a total of 1,420 nt. In the geographical analysis, the strains in Group I originated from Chad, Namibia, and Kenya in Africa, while the strains in Group II originated from Chad in Africa and Peru in South America. In a previous report, a complete analysis of the dendrogram structure
grouped the strains into two well-separated genotypic groups. The genotypic diversity of several strains attributed to these two species was also previously investigated on the basis of morphological criteria using a very sensitive total DNA restriction profile analysis. In this case, the strains were also divided into two well-separated genotypic groups.

The similarity of the 16S rRNA genes between the *Spirulina* strains and *Lyngbya aestuarii* PCC 7419 was about 98%. However, the similarity of the 16S rRNA genes among the *Spirulina* strains and *Microcystis aeruginosa* NIES98 (U40337), *Synechococcus* sp. PCC7943 (AF216949), *Oscillatoria sancta* PCC7515 (AB039015) and *Lyngbya aestuarii* PCC 7419 (AB039013) is 99%. Thus, as shown by the 16S rRNA gene sequences (Nelissen et al. 1996, Ishida et al. 1997), it would seem that *Lyngbya aestuarii* PCC 7419 is also closely related to *Arthrospira* and a sister to the clade *Planktothrix/Arthrospira*. All the strains of *Spirulina/Arthrospira*, used in the present study showed nearly 99% similarity amongst themselves and were also nearly 98% similar with members of Clade I (Nostocales). When the selected strains were compared with fifty sequences of *Spirulina* taken from NCBI data base and subjected to NJ tree with the help of MEGA 5.0, a similarity of approximate 99% was depicted (Plate-13). All the sequences were subjected to Multiple Sequence Alignment with the help of T-Coffee (Version 7.38) and Bio-Edit (Version 7.1.9) softwares (Plate16,18).

4.9.4 Phylogenetic tree analyses of cpcBA-IGS gene

The analyses of the studied *Spirulina/Arthrospira* were conducted using both the coding sequences and the spacer and the outgroup was the *cpcBA-IGS* from the chloroplast of *Cyanidium caldarium*. The NJ tree derived from the translated *cpcB-IGS*-cpcA sequences clustered the *Spirulina/Arthrospira* into two clades, clade I (Oscillatoriales) and clade II (Nostocales) where clade I clustered with the clade II clusters in 100% of the bootstrap trees and the *cpcB-IGS*-cpcA similarity was more than 98% (Plate-14). In clade I two groups shown and Group I contained CCC477, CCC479, CCC483, CCC538, CCC540, *Arthrospira platensis* FACHB439 (AY244669) and *Arthrospira* sp. (AJ310554) and Group II exhibited CCC478, CCC480, CCC481, CCC482, CCC539, *Arthrospira* sp. PK (AJ401179), *Spirulina subsalsa* FACHB351 (AY244667 and *Arthrospira* sp. (FJ001915) and they represented 99% of the bootstrap replications.
All the strains of *Spirulina/Arthrospira*, which were used in the present study showed nearly 99% similarity amongst themselves and were also nearly 98% similar with members of Clade II (Nostocales). *Spirulina/Arthrospira* strains used in the present study showed 99% similarity with 50 sequences (cpcB-IGS-cpcA) of *Spirulina* strains from NCBI data base on the basis of NJ tree developed with the help of MEGA 5.0 (Plate-15). All the sequences were subjected to Multiple Sequence Alignment with the help of T-Coffee (Version 7.38) and Bio-Edit (Version 7.1.9) softwares (Plate-17, 19).

### 4.9.5 Sequence submission and homology search

The partial 16S rRNA gene sequences of *Spirulina/Arthrospira* were submitted in the NCBI GenBank and their Accession number was obtained. The gene sequences were subjected for Megablast search for sequence identity (through BLAST) and phylogenetic tree was prepared by Neighbor Joining method (MEGA 5) on NCBI website. The phylogenetic analysis based on percent sequence similarities of the submitted sequence with those of sequences already in the Gen Bank are provided as under: i) *Spirulina platensis* (CCC477, accession no. EU586048) exhibited 98% similarity with *Arthrospira platensis*, HZ01 (EU427543). This strain also exhibited 96% similarity with *Arthrospira fusiformis*, AICB 670 (AY672725) ii) *Spirulina platensis* (CCC 478, accession no. JX014313) exhibited 96% similarity with *Arthrospira platensis* (KC195869). *Spirulina lonar* (CCC482, accession no. EU586049) depicted 86% similarity with *Spirulina* sp, which was submitted under the accession number EU434883. These two strains occupied the same cluster in the dendrogram. *Spirulina lonar* also exhibited 84% sequence similarity with *Phormidium animale*, PMC 239.04 (AJ850919) iii) *Spirulina platensis* mutant (CCC480, accession no. EU586051) showed 94% similarity with *Phormidium animale*, PMC 239.04 (AJ 850919). This strain of *Spirulina platensis* also exhibited 92% similarity with sequence submitted for *Spirulina* strain (CCC540, EU434883), isolated from Loktal Lake, Manipur. These two also occupied same cluster in the dendrogram developed, Plate iv) *Arthrospira* sp. (CCC481, accession no. EU586050) showed 97% similarity with the partial 16S rRNA sequence of *Phormidium animale*, PMC 239.04 (AJ850919). There was also a similarity of 92% in partial 16S sequence data with *Oscillatoria* sp. (AV6115504). The sequence also depicted 92% closeness with the sequence submitted for *Spirulina* sp. (CCC540, EU434883). These also occupied the same tree in the dendrogram, v) *Spirulina* sp. (CCC540, accession no.
EU434883) was found to show 93% similarity with *Phormidium* animale, PMC 239.04 (AJ850919) vi). This also had a similarity of 91% with another sequence of 16S rRNA gene submitted for *Oscillatoria spongellae*, 310P1 (AY615504).

cpcB-IGS-cpcA gene sequences of *Spirulina/Arthrospira* strains were also submitted in NCBI and their Accession number (CCC477,JQ926188), (CCC478, JQ926189), (CCC479,JQ926190), (CCC480,JQ926191), (CCC481,JQ926192), (CCC482,JQ926193), (CCC482, JQ926194), (CCC483,JQ926195), (CCC538,JQ926196), (CCC539,JQ926197), (CCC540, JQ926198) was obtained. Through blast analyses it was seen that all strains show 100% similarity to *Arthrospira platensis* (AY244669) and about 98% similarity to each other.