Cyanobacteria are morphologically diverse group of photoautotrophic bacteria showing oxygenic photosynthesis whose classification in accordance with the Botanical (Anagnostidis and Komarek 1985) and Bacterial (Castenholz 1989; Rippka et al., 1979) codes is almost entirely based on phenotypic traits. The classification of these organisms is complicated owing to the misidentification of the strains, the lack of isolates and genetic information for many morphophytes as well as inadequate morphological data on many genetically characterized strains (Wilmotte and Herdman, 2001). The phenotype of cyanobacteria is known to change occasionally during prolonged laboratory cultivation (Gugger et al., 2002a; Lehtimaki et al., 2000), which makes their identification sometimes difficult. Ten cyanobacterial strains from genus *Spirulina/Arthrospira* were procured from culture collection of CCUBGA, IARI, New Delhi-110012. These were examined microscopically to check the purity and were made axenic through repeated sub culturing, streaking and antibiotic treatment for further studies.

5.1 Cultural characteristics and morphological parameters

Most of the strains of *Spirulina/Arthrospira* were non-mucilaginous when growth was seen in liquid medium. Few showed mucilaginous growth resulting in the formation of clumps. The increased mucus production by some of the strains studied may be due to certain biochemical activities as reported by some workers (Yamamoto et al., 1974). The cultural behavior was assessed at different stages of growth in nitrogen enriched modified medium. Most of the *Spirulina* strains exhibited uniform suspension in liquid medium and other genera in general showed planktonic growth. Variation in the color of the thallus was distinct and color ranged from blue green to pale green.

Microscopic examination of *Spirulina/Arthrospira* showed spirals which were regular and/or irregular. The spiral trichome of *Spirulina/Arthrospira* could transform to straight filaments but the reversal could not be observed in these two genera. These observations were in accordance with the results of earlier workers (Baker, 1997). Reversible change from loose helix to tight helix has also been reported (Li et al., 2001). Helix orientation in the cyanobacterium *Arthrospira* has been extensively...
studied and this feature is not found to be stable and is controlled by environmental conditions, particularly temperature (Muhling et al., 2003). *Arthrospira* was distinct from *Spirulina* for having clear septate filaments when observed under light microscope and the *Spirulina* filaments showed distinct pores. Komarek and Lund (1990), Desikachary and Jeeji-Bai (1992; 1996) have described different species of *Arthrospira* (*A. platensis, A. fusiformis, A. jenneri, A. maxima* and *A. indica*) considering various morphological characters and habitats. In the case of *Arthrospira* and *Spirulina* which were merged by Geitler (1932), several morphological studies have shown differences between the genera in terms of helicity, trichome size, cross-wall structure and pore pattern and gas vesicles (Tomaselli et al., 1996; Tomaselli 1997).

Overall number of cells per filament ranged from 10 to 226 and on an average, and the filaments could be categorized as short, medium and long. All the three types of filaments were recorded in the cyanobacterial genera which were examined. Vegetative cells were cylindrical, rectangular, barrel shaped and oblong and their length ranged from the lowest of 1.21µm (CCC481) to the highest of 7.67µm (CCC539). The breadth varied from 1.12 µm (CCC540) to 7.56 µm (CCC480). On the basis of these observations and related parameters, the identification was authenticated based upon the keys given by Desikachary (1959). Morphological changes can be problematic in establishing species definitions because they are usually defined based on cell dimensions and ecology and as a result additional criteria are warranted for definite and accurate classification of cyanobacteria. Since morphological attributes may not be controlled by genetics alone, a combination of morphology and molecular data would help to alleviate the discrepancies in taxonomic assignments (Saker et al., 1999). According to Komarek and Anagnostidis (1989), about 50% of the cyanobacterial strains available in culture collections were misidentified.

### 5.2 Physiological parameters

In addition to morphological diversity and widespread distribution, cyanobacteria reflect a broad spectrum of physiological properties and tolerance to environmental stress (Tandeau de Marsac and Houmard 1993). Cyanobacteria are good source of pigments, vitamins, polysaccharides, proteins, pharmaceuticals and other biologically active
compounds of high commercial value (Thajuddin and Subramanian 2005). Little attention has been given to the general features of physiology of cyanobacteria with the specific attention given to nitrogen fixation and some aspects of photosynthesis (Brown and Webster 1953; Fog 1947). A number of studies have used these organisms as reliable tools for studies involving growth and other related parameters (Kratz and Myres 1955). Some of the physiological parameters such as growth, composition of pigments, N-assimilatory enzymes and certain metabolites are considered important for differentiation of cyanobacterial strains (Rippka et al. 1979).

The absorption of light energy by cyanobacteria is based upon the occurrence of one or two forms of chlorophyll with chlorophyll-a as pivotal pigment along with other accessory pigments such as carotenoids and phycobiliproteins. Chlorophylls are the key compounds for trapping light energy for photosynthesis and thus, their quantitative determination is of great importance in the study of photosynthesis, biomass production and biodiversity analysis. There was a wide difference observed in chlorophyll content amongst the strains and the variation was recorded between the lowest of (0.086 mg g\(^{-1}\) dry wt.) to the highest of (1.609 mg g\(^{-1}\) dry wt.) The variations in the patterns of carotenoid were also reported to be useful for species identification of cyanobacteria (Hertzberg et al. 1971). Similar carotenoid pattern were observed with *Spirulina*/Arthrospira strains. Carotenoids protect the cyanobacterial cell from photooxidation damage and are found associated with proteins. Carotenoids have commercial applications as food coloring and feed additives to enhance flesh color of salmonoid fish, as well as the colour of the egg yolk. These also improve the health and fertility of cattle (Borowitzka 1988). The variation in the patterns of carotenoids composition was reported to be useful for species identification of cyanobacteria (Hertzberg et al. 1971). Most of the carotenoids commercially available are chemically synthesized but there is increasing demand for natural carotenoids as nutritional supplements (Jin et al. 2003). Other set of pigments (phycobiliproteins) which function for driving the photosynthetic reactions are organized into granules (phycobilosomes) localized on the outer surface of thylakoids (Gantt 1975).

Total soluble proteins also differed and it was highest (10.24 mg g\(^{-1}\) dry wt.) in CCC540 at 14\(^{th}\) day of incubation in *Spirulina* strain isolated from Loktak Lake of Manipur. Variability in total soluble proteins has also been reported by other workers in cyanobacterial genera (Mishra et al. 2004), which was four times higher than that
of the protein content (40.33µg/ml) recorded in CCC480. The ratio of protein (µg) per unit of chlorophyll (µg) was also highest in same *Spirulina* strain. Variability in total soluble proteins has also been reported by other workers in cyanobacterial genera (Mishra *et al.* 2004). The cyanobacterial strains isolated from the arid zones of Rajasthan, India, depicted variability with respect to total soluble proteins and other parameters (Tiwari *et al.* 2005).

Nitrate reductase activity differed amongst *Spirulina* strains and it ranged from the lowest 64.94 µmoleC₃H₄/mg proteins by CCC479 to the highest of 432.67 µmoleC₃H₄/mg proteins by CCC540. A significant feature of cyanobacterial nitrate reductase is its close relationship with photo synthetically active thylakoid membrane and dependence upon ferrodoxin as a sole natural electron donor (Bagchi 1994; Lara *et al.* 1987). Meeks and coworkers (1983) have observed that *Anabaena* strains have a high nitrate reductase activity (26-30µmole) as compared to *Synechocystis* (1-4µmole). A significant feature of cyanobacterial nitrate reductase is its close relationship with photo synthetically active thylakoid membrane and dependence upon ferrodoxin as a sole natural electron donor (Bagchi 1994; Lara *et al.* 1987).

Glutamine synthetase is a key enzyme in nitrogen metabolism in prokaryotes and this enzyme is subjected to a regulated control which has been studied extensively. In cyanobacteria GS/GOGAT pathway has been shown to be major ammonia assimilatory route under nitrogen fixing conditions (Thomas *et al.* 1975). The activity was highest (21.698 µ mole γ-glutamyl hydroxamate/ mg protein) in CCC540 and lowest (1.496 µ mole γ-glutamyl hydroxamate/ mg protein) in CCC477. The glutamine synthetase activity can be controlled by feedback inhibition, reversible covalent modification and transcriptional regulation of structural gene. In the presence of abundant carbon sources, nitrogen deficiency results in a high level of glutamine synthetase activity and when the nitrogen source is abundant; its activity is down regulated (Garcia-Dominguez *et al.* 1999). High nitrate reductase enzyme may not be responsible for enhancement in the glutamine synthetase activity and the later parameter may be under different physiological and/or genetic control. These two enzymes performed a key role in nitrate assimilatory systems and their chemical characteristics reflect their response to changing environmental conditions.

5.3 Environmental variables
High temperatures are the key factors for large scale *Spirulina* cultures outdoors with the optimal temperature reported in range of 35–38°C. The temperature below 20°C and above 40°C retarded the growth considerably. The highest biomass at 30°C than at 35°C may be due to higher partial pressure of CO₂ in the medium leading to enhanced concentration of bicarbonates with consequent increase in the rate of photosynthesis. Further, a longer dark cycle respiratory activity in which cells use reserve material for respiration accordingly can decrease the cell weight. *Spirulina* requires relatively high pH between 9.5 and 9.8, which inhibit the contamination by most algae in the culture. Therefore, high amounts of sodium bicarbonate is essential in the culture medium to sustain high pH. High alkalinity is mandatory for the growth and bicarbonate is used to maintain the high pH. The significant pigment production may be the result of an increase in pH due to the formation of a CO₂/H₂CO₃/HCO₃⁻/CO₃²⁻ system, which may function as useful buffer system for maintaining the alkaline pH. This is important for the optimum growth and helps to prevent the carbon depletion.

Several species of *Spirulina* exhibit inhibition of growth in hostile environments of saline lakes. Exposure of *S. platensis* to an enhanced sodium concentration resulted in a 30% increase in intracellular accumulation of Na⁺ accompanied by small changes in dry mass and chlorophyll content.

### 5.4 Estimation of β-carotene and antioxidant activity

Statistically significant differences in the contents of β-carotene depending on the studied strain of cyanobacteria were observed. The highest concentration of β-carotene was found in CCC540 (260.39±4.07 mg/100 g DM). The lowest concentration was shown by CCC477 (183.71±4.07 mg/100 g DM). This fact was explained by Tjahjono *et al.* (1994), who discovered that the presence of iron ions favours the generation of hydroxyl radicals (H₂O₂ + Fe²⁺ → Fe³⁺ + HO⁻ + HO•), which stimulate carotenoid synthesis in a cell. Other studies (Bhosale 2004) prove that the addition of copper salts and zinc salts to the growth medium significantly increases the production of carotenoids by *Rhodotorula* yeast. This is probably caused by the generation of free radicals that stimulate the cells to increase the production of compounds as anti-radical protection. Probably the shortage of microelements is the cause of such a significant difference in the quantity of β-carotene synthesised by the
same strain, depending on the growth medium applied. The contents of carotenoids in strains may fluctuate in a range: from (183.71±1.17 mg/100g) to (260.39±4.07 mg/100 g DM), the contents of β-carotene being very variable. Studies carried out on cyanobacteria cells of Synechococcus sp. strain PCC7942 showed that β-carotene constituted 52% of the total quantity of carotenoids (Prasanna et al. 2010). Rao et al. (2010) studied the characteristics of carotenoids of three microorganisms, Arthospira platensis, Haematococcus pluvialis, and Botryococcus braunii, showing that β-carotene made up respectively 69.5, 1.7, and 1.5% of the total carotenoids content. In the powder made from cyanobacteria A. platensis, the content of β-carotene was 211 mg/100 g (Belay 2008).

Antiradical and antioxidant activities of selected Spirulina/Arthospira extracts (absolute methanol, 50% aqueous methanol) were determined using both DPPH and ABTS radical scavenging methods. Therefore, considering the classification of the microalgae antioxidant powers in four categories reported by Wong et al. (2006), most of the microalgae studied in this work were classified in the medium category (10–100 μmol Fe (II) g−1). Therefore, similar to a report by Li et al. (2007), it could be concluded that plants are richer sources of natural antioxidants in comparison to these studied microalgae. The trend in DPPH assay was similar to that observed in ABTS assay. Interestingly, the overall values of total antioxidant activity in DPPH were lower than those obtained in ABTS assay. Matrix interference with DPPH has been shown to account for lower values in carotenoid rich extracts. This suggests that ABTS is more appropriate than DPPH assay for measuring antioxidant activity in high carotenoid containing algae such as spirulina. New studies have made similar conclusions (Floegel, Kim, Chung, Koo, & Chun, 2011). Consequently, our results were not consistent with replicates. There are conflicting reports about the applicability of FRAP assay for evaluating the lipophilic fractions. Earlier reports of Pulido, Bravo, and Saura-Calixto (2000) also confirm the inability of carotenoids to reduce ferric chloride in FRAP reagent. In a recent publication Halvorsen and Blomhoff (2011) have also referred to the solubility issue of pure carotenoids in FRAP assay. Thus, it seems that ABTS is better assay for measuring antioxidant activity in comparison to DPPH and FRAP assay.

With DPPH method, methanol extract of S. platensis recorded higher antiradical and antioxidant activity which may be mainly due to the great content of phycobiliprotein
pigments (8.23 mg/g) which was famously known by its potent antiradical activity. Aqueous methanol extract either with DPPH or ABTS methods, recorded moderate antiradical and antioxidant activities which were attributed its moderate contents of phycobilin pigments, total phenolic content, phytochemical substances which coincided with the lower % yield of this extract (2.5%). On the other hand, using ABTS method, greater antiradical and antioxidant activities were recorded in absolute methanol (water extract in the second order) extract. These results may be due to the pronounced contents in this extract of total phenolic content and phenolic compounds (HPLC) which were characterized by its great free radical scavenging, hydrogen donating and metal chelating efficiencies.

5.5 Lipid Extraction

*Spirulina* contains 6–13% lipids, half of which is TFA16. The average value of TFA in raw *Spirulina* was 4.86% of the dry weight. This value was used as the basis for the calculation of TFA recovery. In the single-stage extraction, an increase in sample-solvent ratio led to an increase in both lipid and TFA yields in the solvent. When the sample-solvent ratio increased from 1:3 to 1:4, 1:4 to 1:5, and 1:5 to 1:6 (w/v), the yield of extracted lipids increased by approximately 60%, 50%, and 11%, respectively, and the yield of TFA in the solvent also increased by 33%, 33%, and 12.5%, respectively. Increases in sample-solvent ratios from 1:6 to 1:8, 1:8 to 1:10, and 1:10 to 1:12, resulted in a slight increase in the yield of lipids and TFA in the solvent. A samplesolvent ratio of 1:10 was found to be the initial ratio necessary for a stable TFA yield in the solvent (2.2% of dry weight). A sample-solvent ratio of 1:5 resulted in a yield of lipid and TFA in the solvent of 3.6% and 1.6% of dry weight, respectively. In spite of the fact that a sample-solvent ratio of 1:50 obtained a yield of lipids (7.9% of dry weight) and TFA yield in the solvent (2.6% of dry weight) higher than a 1:5 ratio, a 10-fold increase in the amount of solvent was needed for extraction. In the single-stage extraction method, TFA remained in the residue despite the use of large quantities of solvent. Therefore, a multistage method using cross-current extraction was applied in order to increase the yield of TFA and to decrease the amount of solvent used. With the same volume of solvent, results showed that TFA recovery in solvent from a sample solvent ratio of 1:5 using a two-stage extraction technique was no different to that of a single-stage technique using a ratio of 1:10.
When cells were extracted using a three-stage extraction method with a 1:5 ratio, TFA recovery in the solvent was found to be higher than that using a single-stage extraction method with a ratio of 1:15. However, it was found that TFA recovery in solvent from a sample solvent ratio of 1:5 using a three-stage extraction method (10 min each) was no different to that using a single-stage method and a ratio of 1:15 for 30 min. The total time taken for extraction using a sample-solvent ratio of 1:5 with a three-stage extraction of 30 min/stage was 10% less than for TFA recovery in solvent using 10 min/stage. Therefore, the sample-solvent ratio of 1:5 was used.

The percentages of the major FAMEs in the Spirulina cultivated by us were in accordance with previous works by other authors (Olguín et al., 2001; Quoc et al., 1994; Cohen et al., 1987), the principal fatty acids present were palmitic, gamma-linolenic and linoleic acid. However, it may be possible to increase the content of gamma-linolenic acid, because Olguín et al. (2001) obtained 26D31% of gamma-linolenic acid (C18:3) in a study on the effect of low light flux and nitrogen deficiency on the chemical composition of Spirulina cultivated in sea-water supplemented with anaerobically digested pig waste. The analyses of variance shown that the variation of temperature and nitrogen content caused significant effects on the concentration of palmitoleic (C16:1) and linoleic (C18:2) acids. Changing the temperature from 30°C to 35°C had a positive effect and increased the concentration of both palmitoleic and linoleic acid, but increasing the concentration of sodium nitrate had a negative effect on the concentration of palmitoleic acid while the interaction of both factors (sodium nitrate concentration and temperature) was significant (p < 0.01) in the case of linoleic acid and should be taken into account instead of the individual effects of the two factors. The relationship between the concentration of the nitrogen source in the culture medium and the fatty acid content of Spirulina has been studied by Rijn and Shilo (1986), who have shown that reserve compounds accumulate during nitrogen depletion. However, Piorreck et al. (1984) showed that such reserve compound accumulation occurs mainly in green algae metabolism, while in the cyanobacterium Spirulina the fatty acids of the lipid polar fraction remain constant for potassium nitrate concentrations between 0.001% and 0.1%, similar results have been found by Tedesco and Duerr (1989). Even though, Olguín et al. (2001) have observed an increase in the concentration of linolenic acid in Spirulina growing in a nitrogen deficient medium compared to Spirulina growing in Zarrouk’s medium. Besides the effect of temperature on linoleic acid content, we also verified with that increasing the
incubation temperature the amount (p < 0.10) of palmitic acid increased, but palmitoleic acid and stearic acid decreased the amount of GLA. It has been reported that the content and composition of the fatty acids are temperature-dependent in *S. platensis*, an increase in temperature reduces the composition of polyunsaturated fatty acids in membrane lipids. This regulation of fatty acid saturation by desaturase enzymes is known as homeoviscous adaptation (i.e. the adjustment of the membrane fluidity needed to maintain the optimal function of biological membranes), although another explanation for this change in lipid saturation with temperature is that at lower temperatures more dissolved oxygen is available in the culture medium for desaturase enzymes that are oxygen dependent (Cohen et al., 1987). Because of the high costs of extraction of polyunsaturated fatty acids (especially GLA) from *Spirulina* it seems that the best way to use *Spirulina* is by its direct consumption as a nutritional supplement, especially because in this way the consumer will benefit not only from the beneficial properties ascribed to GLA but also to other nutritionally active components. *Spirulina* can be used either as a food supplement or taken in capsule form, capsules appearing to be the preferred form at present. It is important to know the fatty acid profile *Spirulina* preparations, our results (Table II) showing that different *Spirulina* preparations have different fatty acid profiles. The fatty acid profile of the *Spirulina* cultivated by us demonstrate that palmitic acid is the most abundant followed by linolenic and linoleic acids. Temperature was more important than sodium nitrate concentration, with greater amounts of GLA being obtained at 30 ∞C. It seems that the *Spirulina* produced under the culture conditions described in this paper is a potential source of GLA for use as a food additive or in capsule form as a nutritional supplement.

### 5.6 Estimation of purified phycocyanin

Phycobiliproteins are major constituents of the cells in *S. platensis*, representing up to 60% of the total cellular protein content; thus, *S. platensis* is commonly used as a source for PBP purification. These are also considered as an ideal nutritious food and has been cultivated in a large scale throughout the world. The gross mass grown in China is about 1,000 t year⁻¹, accounting for one third of the world’s total production. Thus, *S. platensis*, as a starting material for PBP purification, is both convenient and available on a large scale. The common methodologies for separation
of PBP in the previous publications involved ammonium sulfate precipitation, gel filtration chromatography, hydroxyapatite chromatography, ion exchange chromatography, hydrophobic interaction chromatography, expanded bed adsorption chromatography, aqueous two-phase systems, and the rivanol sulfate procedure. There are great differences in the efficiency, recovery, and final purity of the PBPs obtained using these methods. Some purification methods can be allied to obtain pure PBPs through multistep procedures, and some methods can efficiently purify C-PC alone. High cost of pure PBP, as much as US $50 mg⁻¹ (Haugland 1996; Market Corporation 2005), could be reduced if more convenient methods for purification of PBPs are developed. Anion exchange chromatography with ionic strength gradient elution was usually employed to efficiently separate C-PC (Boussiba and Richmond 1979; Zhang and Chen 1999; Patel et al. 2005). Our study showed that the PBPs after anion exchange chromatography with pH gradient elution were purer than that with ionic strength gradient elution. Anion exchange chromatography with pH gradient elution is an efficient and high-performance method for PBP purification because different PBPs can be accurately eluted using different pH value eluents according to their individual isoelectric points (3.7–5.3; Glazer 1981; Liu et al. 2005). Anion exchange chromatography with pH gradient elution has shown its advantage in the purification of R-phycoerythrin (Liu et al. 2005). It is the first time here that we introduce and modify this method for the purification of C-PC and APC from *S. platensis*. Moreover, one-step ion exchange chromatography with pH gradient elution for purification of C-PC and APC from *S. platensis* is more efficient than previously reported methods. In previously published methods, APC could not be obtained simultaneously when purifying C-PC, resulting in a waste of the PBP. Rivanol sulfate method was introduced to purify C-PC and APC simultaneously, but the purities of obtained PBPs were relatively low (Minkova et al. 2007). In this report, two types of pure PBPs could be simultaneously obtained with high purity and yield. Purity and recovery are the two most important indices of PBP purification. Analytical grade PBPs (\(A_{\text{max}}/A_{280}> 4.0\)) are more suitable to be used as fluorescence marker and pharmaceuticals (Siegelman and Kycia 1978; Rito-Palomares et al. 2001). One point of view is that high recovery is even more important than high purity because recovery will decrease as purity increases due to the extra purification steps required. The recovery of C-PC obtained using this method reached as high as 111.83 mg g⁻¹ lyophilized biomass, which is 25-fold higher than previously reported data (Soni et al.
APC purification is more difficult and less efficient because of its lesser abundance in *S. platensis* in comparison with C-PC. Su et al. (2010) proposed hydroxyapatite chromatography combined with anion exchange chromatography for the efficient purification of APC. The method reported here for simultaneous purification of C-PC and APC shows higher efficiency and lower production costs than previously reported methods (Zhang and Chen 1999). In conclusion, C-PC and APC with high purity and yield could be efficiently purified from *S. platensis* using one-step anion exchange chromatography with pH gradient elution. In terms of purity, recovery, simplicity, and efficiency, this method proved to be a good method for PBP purification.

5.7 Study on the basis of macromolecules

Macromolecules like proteins and nucleic acids are the copies of translation of the genetic information and thus, may be the best tools for the characterization of enzymes. These can be studied by sequencing DNA or directly by electrophoresis, hybridization or immunological methods. In the present investigation, a polyphasic approach including morphological parameters, cultural characteristics, 16S rRNA gene sequence and phycocyanin operon locus (cpcB-IGS-cpcA) was applied to understand the phylogenetic relationship.

Lot of work has been done in the phylogenetic characterization of the 16S rRNA analysis (Ludwig and Klenk 2001; Pace *et al.* 1997; Woese 1987). This gene is being focused because they are functionally and evolutionarily homologues in all the organisms (Stackebrandt and Woese 1981). Numerical analysis of protein patterns and RFLP of 16S rRNA gene provides broader taxonomic application, as these methods are quite suitable for all cyanobacteria as the number of strains for sequencing of the entire 16S rRNA gene can be limited (Rippka and Herdman 1992). Also sequence analysis may not be necessarily a foolproof criterion to guarantee species identity, because closely related species may not be recognizable with this tool (Fox *et al.* 1992). Thus, complete sequencing of the 16S rRNA gene along with RFLP analysis may clarify cyanobacterial relationships further and in more depth.

DNA was extracted from selected strains of *Spirulina/Arthospira*. 16S rRNA gene was amplified with standardized protocol using polymerase chain reaction.
with FD1 and RP2 primers. The amplified band of approx. 1500bp was observed in all the cases and the recorded size of 16S rRNA gene was in accordance to the findings of Lyra et al. (1997). Nubel et al. (1997) developed a set of oligonucleotide primer for the specific amplification of 16S rRNA segment from a variety of cyanobacteria. A diagnostic system using the sequence polymorphism within the 16S rRNA variable region V6, V7, V8 for individual strain characterization and identification of toxin producing organisms belonging to Nostoc, Anabaena, Microcystis and Planktothrix has been developed (Rudi et al. 1997). Rudi et al. (2000) also developed a method to analyse genetically the cyanobacterial composition in natural water bodies using multiple sequence. A cyanobacterial specific oligonucleotide probe which distinguished prior to cloning and sequencing at least between 16S rRNA genes from those of cyanobacterial contaminants was constructed (Nierzwicki-Bauer and Haselkorn 1986). After PCR amplification of the 16S rRNA gene from the non axenic cultures and their separation by agarose gel electrophoresis, the probe was used to identify cyanobacterial PCR products. The selected strains showed Jaccard’s similarity coefficient which was in accordance with the previously published data based on partial 16S rRNA gene sequence analysis (Giovannoni et al. 1988; Wilmotte and Herdman 2001). More than 50% of the strains in the culture collections have taxonomic names which do not match with morphological descriptions of taxon (Komarek and Anagnostidis 1989). Similar observations were recorded in other genera where reference strains PCC 73102 and PCC 7422 (originally isolated from Cycas sp.) showed a low level of genetic relatedness (Lyra et al. 1997). Deviations of closely related genera occupying separate subcluster was probably due to Horizontal Gene Transfer (HGT) (Manen and Falquet 2002). Heterogeneity was also observed amongst planktonic strains of Anabaena and Aphanizomenon by using PCR/RFLP of phycocyanin locus with intergenic spacer (Neilan et al. 1995). Interestingly Spirulina platensis, mutant (CCC483) showed 100% similarity with Arthrospira (CCC538).

DNA sequences play an essential role in the reconstruction of evolutionary relationships among organisms and have led to new genetic classifications that may confirm or conflict with traditional taxonomy. Application of molecular techniques to amplify some portions of the genome in order to characterize and deduce phylogenetic relationships has increased considerably (Orcutt et al. 2002; Taton et al.
2003) at the molecular level. At molecular level the rRNA genes are the most widely used markers for the identification of cyanobacteria due to their conserved function and universal presence. These conserved regions have been exploited and have added potentially significant information for phylogenetic studies (Crosbie et al. 2003; Olsen and Woese 1993; Woese 1987). Most of the studies of cyanobacterial phylogeny with 16S rRNA sequences were based on isolates available as axenic cultures (Giovannoni et al. 1988; Nierzwicki-Bauer and Haselkorn 1986). Dwivedi et al (1996) developed a rapid method of DNA sequencing of rRNA gene amplified by PCR without the need for cloning of the gene for sequence.

Partial 16S rRNA gene sequence for *Spirulina* sp. was submitted in NCBI GenBank (EU434883) and in addition to this the other partial 16S rRNA sequences have been submitted for *Spirulina platensis*. According to cut off points, 97.5% and 95% in 16S rRNA gene sequence similarity has been suggested for bacterial species and genus definition (Ludwig et al. 1998; Stackebrandt and Goebel 1994). Li et al (2001) reported 100% sequence similarity when investigating 16S rDNA sequence of *Arthrospira maxima* and *Arthrospira fusiformis*. There was a similarity of 99.7 percent in 16S rRNA sequence amongst *Arthrospira* strains according to Castenholz et al (2001). Such a high molecular similarity makes it likely that all *Arthrospira* strains are representative of only one species and if the similarity is more than 97.5 percent, the two strains can be considered belonging to same species (Stackebrandt and Goebel 1994). However, Fox et al (1992) have mentioned that 16S rRNA sequences are not necessarily a foolproof criterion to guarantee the identity. Rajaniemi et al (2005) also showed a higher closeness at genus or species level between related genera based on sequence similarity. 16S rDNA sequence similarity of *Anabaena* and *Aphanizomenon* strains was higher than 97.5% which indicates that the strains of two genera belonged to same species (Stackebrandt and Goebel 1994). Margheri et al (2003) were able to clearly distinguish *Spirulina* and *Geitlerinema* strains from different alkaline, saline and fresh water habitats and found that hypersaline and alkaline strains are genetically distinct from other marine habitats. The closeness of *Leptolyngbya* with *Phormidium* on the basis of 16S sequence profile may suggest a possible gene transfer between the taxa (Barker et al. 1999). The distinction between *Arthrospira* and *Spirulina* was confirmed by early studies of Gomont (1892). However Geitler’s revision (1932) of cyanobacteria reunited the
members of these two genera and Geitler actually split the genus into two generic subtaxa (section 1- *Arthrospira* and section 2- *Euspirulina*) on the basis of criterion earlier used by Stizenberger (1852) to separate *Spirulina* and *Arthrospira* i.e, visible or non visible cross walls. The forms with septa were classified under *Arthrospira* and those without septa were put under *Euspirulina*. Studies conducted by Williame et al (2006) have indicated the relation between phenotypic characters and partial 16S rRNA data and the first sequence of *Komvopheron* appeared to be poorly related to other available cyanobacterial sequences. *Spirulina platensis* showed 98% similarity with *Arthrospira platensis* indicating the ambiguity regarding the differentiation between these two genera.

The phylogenetic investigations using 16S sequences have shown that many unicellular and filamentous non-heterocystous cyanobacterial genera are probably polyphyletic and cannot be grouped as natural taxa whereas heterocystous strains form a monophyletic group (Rippka et al. 2001; Wilmotte and Herdman 2001). The cyanobacterial orders/subsections have not been supported by the 16S rRNA gene sequence analysis (Giovannoni et al. 1988; Gugger and Hoffmann 2004; Ishida et al. 2001; Turner 1997). Only heterocystous cyanobacteria belonging to the two orders/subsections appear to be monophyletic in the 16S rRNA gene analysis (Gugger and Hoffmann 2004; Wilmotte and Herdman 2001). This incongruence between phylogenetic analysis and the classification of cyanobacteria was taken into account in the most recent classification proposal (Hoffmann et al. 2005). The phylogenetic clustering of strains of several cyanobacterial genera seem to be incongruent with the cyanobacterial morphology and does not follow their current classification [e.g., *Anabaena* and *Aphanizomenon* (Gugger et al. 2002a; Lyra et al. 2001), *Oscillatoria* (Suda et al. 2002) and picocyanobacterial genera such as *Synechococcus* and *Synechocystis* (Wilmotte and Herdman 2001)]. In some cases strains of a genus or species formed a monophyletic cluster in the 16S rRNA gene analysis, for example *Planktothrix agardhii* (Lyra et al. 2001), *Nodularia* (Lyra et al. 2005), and *Microcystis* (Lyra et al. 2001; Otsuka et al. 1998). However, the morphologically distinguished *Microcystis* species were found to be genetically very closely related to each other (Otsuka et al. 1998) and unification of different *Microcystis* species into a single species has been proposed (Otsuka et al. 1998, 2001).
With respect to the terminal branching patterns, the results suggested a high degree of congruence between the 16S rRNA genes and the cpcBA-IGS-based phylogenies of the nonmarine members of the picophytoplankton clade, yet with less well delineated subgroups in the more conserved 16S rRNA gene phylogeny (Robertson et al. 2001, Crosbie et al. 2003). In a previous report, 16S rRNA gene data and cpcBA-IGS data also indicated that several taxa were genetically heterogeneous and that the taxonomy of cyanobacteria needed to be reconsidered. Manen and Falquet (2002) suggested that 16S rRNA gene data and cpcBA-IGS data were often in agreement. Furthermore, another study investigated the use of the cpcBA-IGS region of the phycocyanin operon, flanked by cpcB and cpcA, as a marker for the subgenus characterization of cyanobacteria (Neilan et al. 1995, Bolch et al. 1996). The phylogenetic analyses based on the 16S rRNA gene and cpcBA-IGS sequences showed no division between A. platensis and A. maxima, plus the 16S rRNA gene and cpcBA-IGS clusters did not exhibit any well-defined geo0 graphical distributions, and instead overlapped in a rather interesting way. Thus, it is now widely accepted that S. laxissima SAG 256.80 is distinct and relatively distant from Arthrospira strains.

Analyses of the 16S rRNA gene and cpcBA-IGS sequences of the Spirulina/Arthrospira strains showed a high similarity and did not confirm the taxonomic validity of these two genera. Phylogenetic analyses based on 16S rDNA sequences have already been widely used. However, the 16S rRNA gene is often ineffective for resolving bacterial strains, due to its slow rate of evolution. In addition to analyzing the 16S rDNA sequences (Neilan et al. 1997; Otsuka et al. 1998; Honda et al. 1999), the genes encoding the major light-harvesting accessory pigment proteins, particularly the phycocyanin operon (cpc), including the intergenic spacer (IGS) between cpcB and cpcA and the corresponding flanking regions (cpcBAIGS), have been targeted for phylogenetic studies of Cyanobacteria (Barker et al. 1999). Phycocyanins are specific accessory pigments in Cyanophyta, Rhodophyta, Glaucophyta, and Cryptophyta. In a previous report (Teneva et al. 2005), the length of the IGS separating cpcB and cpcA was found to exhibit a strong relationship with the phylogenetic groups. However, there were generally lower in the cpcBA-IGS tree than in the 16S rRNA gene tree, due to a smaller number of cpcBA-IGS data bases. Moreover, to define and delimit the genus Anabaena and Trichormus, this study also included other cyanobacteria, and a further goal was to identify specific
molecular markers to fingerprint different strains of *Anabaena* and *Trichormus* and enable the development of tools for strain-specific identification. The phylogenetic analysis of the 16S rDNA sequences further reinforced the polyphyletic nature of this taxon. The difference between the molecular and morphological results may have reflected the existence of species-variant populations or ecotypes adapted to different environmental conditions. Recently, Baker *et al.* (2001, 2002) employed a PCR amplification method for the analysis of *cpcBA* from environmental samples using a primer set previously designed by Neilan *et al.* (1997) and found a limited cyanobacterial diversity. Although the primer set was originally designed with 6 *cpcBA* sequences to study the genetic diversity of several pure culture strains, *cpcBA* sequence information from various other cyanobacteria has also been deposited in public databases for potential enhanced primer design. Furthermore, since the IGS length of each group of cyanobacteria is different, the *cpcBA* gene amplification protocol could be directly coupled to length polymorphism analysis techniques, such as TRFLP, to facilitate rapid monitoring of the cyanobacterial community. In conclusion, *Trichormus* and *Anabaena* strains, which were isolated from Korean waters, were separated by morphological and phylogenetic analyses. However, the difference remained between morphology and phylogeny of *Trichormus* and *Anabaena* strains.

Because of the low sequence divergence of the *cpcB-IGS-cpcA* region in the studied strains, the use of distance methods for tree building might not be optimal. Therefore, maximum parsimony and maximum likelihood analyses were used. Results from analyses of both molecular datasets (*cpcB-IGS-cpcA* and 16S rRNA) strongly support the conclusion that order *Oscillatoriales* is polyphyletic despite the differences in the branching patterns. Hence, the morphological criteria used for taxonomic identification of the oscillatorian cyanobacteria within the “LPP-group” should be reexamined. The differences between morphotypes and genotypes may also result from the variation induced during culturing (Otsuka, 2000), or differential selection may favor the maintenance of different phycocyanin genotypes in the population. Despite the low sample numbers, the genetic data support the recent classical taxonomic revisions of Komárek and Anagnostidis (2005). Additional genetic studies are planned using other markers such as 16S rRNA, *hetR* and *rpo* genes to resolve the relationships between *Spirulina/Arthrospira* and other *Oscillatoriales*. In conclusion,
the molecular analysis of *cpcBA*-IGS and the 16S rRNA region supports the polyphyletic nature of *Spirulina* and other Oscillatoriales species. More studies comprising different gene regions in combination with well-characterized morphology from natural and cultivated populations may provide a better understanding of the systematics of order *Oscillatoriales.*