Discussions

5.1. Isolation, identification and chemotaxonomical characterization of Oscillatoriales (cyanobacteria) of North Eastern region of India

Oscillatoriales cyanobacteria encountered from north eastern region of India showed diverse characters even within the same strains of the species. Lyngbya, Plectonema, Oscillatoria and Phormidium were found to be distributed and dominated in the entire north eastern region of India and observed cosmopolitan in distribution. Genus Oscillatoria showed highest diversity as shown by the Shannon index (1.92) and Simpson index (0.83). It may be due to the species richness and high evenness in the entire north eastern region of India. It may be also due to their adaptability and presence of laminated sheath for their growth at the temperate to tropical climate with the temperature ranges from 1-35°C with annual rainfall ranges from 210 cm to 250 cm. Species richness was more in genus Phormidium in the north eastern region of India but the diversity was less due to uneven abundance of species proportion resulting to less evenness.

Occurrence of Hydrocoleum, Porphyrosiphon and Trichodesmium showed least diversity index and confined to certain areas of Manipur. It may be due to average rainfall of 150 cm and temperature ranges from sub-zero to 36°C with altitude ranges from 780-792 MSL. Soils with high retention of water and rich in phosphate may also be the key factor for the survival percentage of different genus and species belong to non heterocystous filamentous cyanobacteria. Microcoleus showed occurrence only in Sikkim which may be due to the cold climate and humid as rainfall occurs in each month with temperature ranges from 05-28°C.

Leptolyngbya, Limnothrix and Pseudanabaena showed sparse distribution and it was observed that the climate of Assam was suitable for the growth of all the strains mentioned above so the ideal climate for their growth may be tropical monsoon rainforest climate temperate with
heavy rainfall and humidity with temperature ranges from 07-35°C. From the results obtained based on the altitudes, 11 genera viz. *Phormidium*, *Limnothrix*, *Microcoleus*, *Oscillatoria*, *Leptolyngbya*, *Lyngbya*, *Hydrocoleum*, *Trichodesmium*, *Porphyrosiphon*, *Pseudanabaena* and *Plectonema* were found to occur from low lying plains of 2 MSL to altitude of 700-1200 MSL. The diversity of Oscillatoriales in entire north eastern region of India was found to be highest in Assam. It may be due to high species richness and high evenness of the species.

Species richness was observed high in Manipur but with least evenness resulting to less diversity compare to Assam. The diversity of Oscillatoriales in entire north eastern region of India was found to be lowest in Sikkim which may be due to less species richness along with less abundance of the species or strains. It may be also due to dominance of 1 genus i.e. *Phormidium* from the rest of the genus resulting to uneven abundance. It may be due to highly hot and humid climate at the lower altitude and valley while too cold in the higher altitude and abundant rainfall which may lead to leaching of nutrients. It may be also due insufficient nitrogen available at the high altitudes of these regions.

Earlier, Devi et al. (2008, 2010) recorded *Oscillatoria leavittae* from Thoubal, Manipur and Nohkalikai falls, Meghalaya; *Oscillatoria okeni* from Imphal West, Manipur; *Oscillatoria salina* from Dimapur, Nagaland; *Oscillatoria willei* from Senapati, Manipur; *Phormidium acidophilum* from Imphal West, Manipur; *Phormidium fragile* from Imphal West, Manipur and Loktak lake, Bishnupur, Manipur; *Phormidium hamelii* from Senapati, Manipur. *Phormidium tenue* was recorded from 12 different places viz. Chandel, Manipur; Imphal East, Manipur; West Tripura; Cherrapunjee, Meghalaya; Arunachal Pradesh; Nohkalikai falls, Meghalaya; Dibrugarh, Assam; Dimapur, Nagaland; Silchar, Assam; *Phormidium valderianum* from Imphal East, Manipur and West Tripura. Five species of *Plectonema* were from different
parts of north eastern region of India vis-a-vis *Plectonema corium* from Meghalaya; *Plectonema indica* from Shillong, Meghalaya and Loktak lake, Bishnupur, Manipur; *Plectonema notatum* from Churachandpur, Manipur; Imphal West, Manipur; Shillong, Meghalaya and Cherrapunjee, Meghalaya; *Plectonema nostocorum* from Shillong, Meghalaya and Nohkalikai falls, Meghalaya. Hindak (2001) and Krienitz *et al.* (2003) were reported *Phormidium* cf. *terebriformis* from hot springs at the shore of lake Bogoria. Sankaran, 2001 reported cyanobacteria from sea level to high altitudes and abundant in temple tanks, ponds in the hill ranges and water lakes like Kodaikanal lake, Ooty lake and Yercaud lake situated at altitudes up to 2200 MSL. Choudhary and Singh, 2013 reported *Gloeocapsa alpina, Oscillatoria tenuis, Phormidium fragile* and *Phormidium uncinatum* at 300 MSL; *Phormidium tenue, Phormidium autumnale* and *Oscillatoria* sp. at 3500 MSL from NE region of India. Nongbri and Syiem (2012) found the highest diversity of cyanobacteria from Mawlai where richness was only 12 strains as against 17 strains that were isolated from Sung Valley and Syntuksiar thereby indicating richness may not be a function of diversity in this region.

### 5.2. Evaluation and screening of carotenoids from selected oscillation cyanobacteria of North Eastern region of India

One hundred (100) fast grower cyanobacterial strains from 11 genera were screened for carotenoids production, showed strain dependent. Wide variations between the particular species of the genus for the carotenoids production may be due to the fact that they were biotypes in which their original environmental factors may affect the physiology and biosynthesis of carotenoids. The total carotenoids production by the *Phormidium animale* BTA 258 showed higher (21.53 μg/mg) than the previously reported carotenoids from the *Phormidium* sp. (Asadi *et al.*, 2011). *Leptolyngbya* sp. BTA 287 showed carotenoids (25.38 μg/mg) at log phase which
was found to be lower than the carotenoids production by *Spirulina platensis* (Saleh *et al.*, 2011). The β-carotene showed a typical sigmoid behavior observed during exponential phase of growth in *Spirulina* strains (Saleh *et al.*, 2008).

Earlier, Singh *et al.* (2012) reported that *Phormidium tenue* of north eastern region of India contains chl-a (3.24±0.04 μg/ml), total soluble proteins (72.00±0.03 μg/ml), total carotenoids (14.03±0.00 μg/ml), ammonia excretion (69.15±0.00 μg/ml), total carbohydrates (73.00±0.02 μg/ml), PE (3.81±0.00 μg/ml), PC (31.44±0.00 μg/ml) and APC (15.18±0.00 μg/ml). Patel *et al.* (2005) reported good quantity of C-PC exists in *Spirulina* sp. [17.5% (w/w)] as compared to *Phormidium* sp. [4.1% (w/w)] and *Lyngbya* sp. [3.9% (w/w)], while APC and PE were present at lower quantities. Previous workers were reported that some *Nostoc* and *Anabaena* strains had much higher content of phycobiliproteins compared to *Spirulina* strains SJ and SS (Simeunovic *et al.*, 2012, Devanathan and Ranganathan, 2012).

Chemotaxonomical identification for the selected strains was made using fatty acids as a marker. It was observed that genus *Phormidium* produced good amount of carotenoids also contains myristic acid methyl ester (C14:0) and myristoleic acid methyl ester (C14:1) in sufficient amount. Total 32 fatty acids were profiled from *Phormidium animale* (BTA 258) which contains 20.61% of total fatty acid myristic acid methyl ester (C14:0) and can be used in identification of this particular strain. Earlier it was reported that *Phormidium animale* KNUA026 strain produced hexadecadienoic acid (C16:2ω4), hexadecenoic acid (C16:1ω7), palmitic acid (C16:0), linoleic acid (C18:2ω6), oleic acid (C18:1ω9) and stearic acid (C18:0) as its major fatty acids (Chang *et al.*, 2013). Total 30 fatty acids were profiled from *Phormidium tenue* BTA 803 and myristoleic acid methyl ester (C14:1) was found to contains 27.99% of the total fatty acids and can be referred as chemotaxonomical marker. Murakami *et al.* (1990a) and
Yamada et al. (1993) reported that *Phormidium tenue* showed high percentage of linoleic (C18:2) and linolenic (C18:3) by extracting lyophilized cells with acetone but with inactive myristic acid (C14:0) and palmetic acid (C16:0).

Genus *Leptolyngbya* showed high percentage of pentadecanoic acid methyl ester (C15:0) in all the 3 strains studied. The fatty acids profiled from *Leptolyngbya* were different among the same genus and therefore strain dependent. It was found that the polyunsaturated fatty acids produced by the Oscillatoriales were most of linoleic (C18:2) and α-linolenic (αC18:3) acids. Occasionally, however, γ-linolenic (γC18:3), palmitdienoic (C16:2) or octadecatetraenoic (C18:4) acids may partially or totally replace these acids (Ikawa, 2004).

### 5.3. PCR based molecular characterizaton of oscillatory cyanobacteria having rich Carotenoids

Good amount of carotenoids produced strains namely; *Phormidium animale* BTA 258, *Leptolyngbya* sp. BTA 287 and *Leptolyngbya* sp. BTA 477, *Leptolyngbya tenerrima* BTA 766 and *Phormidium tenue* BTA 803 along with 05 others viz. *Leptolyngbya* sp. BTA 356, *Phormidium terebriforme* BTA 503, *Phormidium terebriforme* BTA 507, *Phormidium tenue* BTA 605, *Limnothrix redekei* BTA 657 were identified based on the molecular profiling by pairwise alignment analysis using BLASTN showed highest similarity with the corresponding sequences from NCBI GenBank database.

The strains were found to cluster with the *Phormidium*, *Leptolyngbya* and *Limnothrix* as supported by the neighbour joining and maximum parsimony phylogenetic tree. Evolution relation of the selected strains also showed divergent between the same species which may be due to influence of environment on their genes. *Phormidium* sp. BTA 605 showed divergent from the rest of strains which can be considered as less evolved with least substitution.
Phormidium tenue BTA 803 showed divergent and found to be less substitution from the rest of the clusters. It is evident that Leptolyngbya sp. BTA 356, Leptolyngbya sp. BTA 287 and Leptolyngbya sp. BTA 477 showed highest similarity in their characters and genetic distance though they belong to different locations and habitats. Phormidium cf. terebriforme BTA 503 and Phormidium sp. BTA 507 was found to be closely related sharing same ancestor with same genetic distance. Leptolyngbya tenerrima BTA 766 was found to be related with Phormidium animale BTA 258 and found to have less substitution as determine by the phylogenetic tree. It may be due to the ongoing taxonomic refinements of this group besides adaptive mutations leading to ecotypes (Premanandh et al., 2009).

The recent establishment of the genus Leptolyngbya (Anagnostidis and Komarek, 1988) was the result of reassignment of many species with thin filaments into this genus. Hence, it can be speculated that morphological simplicity of this group may perhaps have resulted in two or more ecological groups. Comparisons of cyanobacteria in freshwater, marine and terrestrial environments (Marqardt and Palinska, 2007; Palinska and Marquardt 2008) showed that the distribution of populations follows patterns that correlate with ecological determinants rather than with the formal morphotypic description of the genus “Phormidium”.

These results were consistent with similar conclusions derived from the phylogenetic assessment of the formal genus Oscillatoria, which also proved to be polyphyletic (Wilmotte and Herdman, 2001). Within a broader genotypic framework, the studied morphotypic characters were subject to nutritional and other environmental impacts and that changes in environmental conditions modified phenotypes in a predictable fashion at light-microscopic and ultrastructural level (Marqardt and Palinska, 2007).
The 05 strains viz. *Phormidium animale* BTA 258, *Leptolyngbya* sp. BTA 287, *Leptolyngbya* sp. BTA 477, *Leptolyngbya tenerrima* BTA 766 and *Phormidium tenue* BTA 803 produced good percentage carotenoids did not form in a cluster which showed that high carotenoids production did not determine their phylogenetic relatedness. *Phormidium* cf. *terebriforme* BTA 503 (16.92 μg/mg), *Phormidium* sp. BTA 507 (12.88 μg/mg) showed 100% similarity with each other in their 16S rRNA sequences but different carotenoids production resulted to the fact that *Phormidium* sp. BTA 507 may be *Phormidium* cf. *terebriforme* with difference in the carotenoids production influenced by environmental factors.

*Phormidium animale* BTA 258 (21.53 μg/mg) and *Leptolyngbya tenerrima* BTA 766 (18.02 μg/mg) showed different carotenoids production as their branch length were different though they fall on the same clade. *Limnothrix* sp. BTA 657 (13.16 μg/mg) and *Leptolyngbya* clusters i.e. BTA 287, BTA 356 and BTA 477 showed high difference in carotenoids production. According to 16S rRNA, the variations in the production of carotenoids may be explained for *Leptolyngbya* sp. due to wide variation (50%) similarity and their difference in habitat and location leading to genetic changes for biosynthesis of carotenoids.

It has been reported by Thacker and Paul (2004) that variation in 16S rRNA gene sequences often was not correlate with chemical variability among samples from the genus *Lyngbya* collected in Guam. These data suggest that other mechanisms, such as responses to environmental conditions or faster rates of genetic change in biosynthetic genes, may contribute to the chemical variations observed among them.

From the RAPD analysis, it was found that diversity of the genus *Phormidium* was found to be diverse based on their locations as in the case *Phormidium animale* BTA 258 from Arunachal Pradesh, *Phormidium* sp. BTA 507 from Mizoram, showed similarity of 20%. The
diversity within the same genus were also largely different as *Leptolyngbya* sp. BTA 287 and *Leptolyngbya tenerrima* BTA 766 belong to Tripura and Mizoram which showed 33.3% similarity and 25% with *Leptolyngbya* sp. BTA 356. It was earlier reported that some degree of variation is likely to occur even among clonally related isolates (Hyhtia *et al.*, 1999; Roberts and Crawford, 2000) had polymorphic profiles generated by RAPD analysis suggest considerable degree of intra species heterogeneity despite morphological similarity. Since morphology might not be strictly controlled by genetics (Saker *et al.*, 1999), genotypic variations were virtually indistinguishable by morphological means. The failure of morphological features to distinguish cyanobacterial strains agrees with previous findings on *Phormidium retzii* using RAPD markers (Casamatta *et al.*, 2003) and *Leptolyngbya* utilizing 16S rRNA gene sequences (Payne *et al.*, 2001).

Results based on RAPD showed diverse relationship between *Phormidium* strains from different geographical places. *Phormidium, Leptolyngbya* and *Limnothrix* strains were genetically changed under pressure of different environments. This was also reflected in their morphology and most pronounced in pigment composition. The production of carotenoids was also deferred within the same genus where *Leptolyngbya* sp. BTA 287 showed high presence of carotenoids. *Leptolyngbya* sp. BTA 477 of Assam showed highest similarity with *Phormidium tenue* BTA 803 of Mizoram showed 47.6%. Molecular and phylogenetic studies showed pigment analysis data that did not correlate with their phylogenetic relationship. *Phormidium, Leptolyngbya* and *Limnothrix* strains from this study survived under different environments and the adaptation/acclimation strategies were deferred between different strains. It was found that chemotaxonomic features had no correlation between position of the strains in the phylogenetic tree as supported by Marquardt and Palinska, 2007. Structural properties were insufficient in
themselves for identification at the genus or species level since some phylogenetically distant members also showed similar morphological traits. However, genetically similar strains came from similar ecosystems and were similar in cell size and shape. Nevertheless, these traits were insufficient in themselves for classification since some distantly groups showed similar traits.

5.4. Optimization of carotenoids production and other fine chemicals for industrial application

The carotenoids production under different pH from acidic to alkaline showed strain dependent. Most of the strains showed high carotenoids productions with increase of pH and also supported by Otero and Vincenzini (2004) that the increase of pH was related to an inhibition of nitrate uptake which may lead to high carotenoids synthesis. The strains investigated under present study showed strain dependent and majority of the investigated strains produced highest carotenoids in N and 2N nitrate concentration except *Leptolyngbya* sp. BTA 287 which produced highest in high nitrate concentration (5N) thereby supported that decreased nitrogen concentrations showed positive impact on the production of carotenoids. Previous workers (Santhose *et al.*, 2011; El-Sayed *et al.*, 2010; Fresnedo *et al.*, 1991) reported that the increase of carotenoids under high nitrate (5N) concentration might be attributed to the changing N: P ratio and supported that nitrogen starvation play a negative effect on the carotenoids synthesis as observed in *Phormidium laminosum*.

Production of carotenoids was found to be strain dependent and increased as the concentration of phosphate increased upto 5N except in *Leptolyngbya* sp. BTA 477 and *Leptolyngbya tenerrima* BTA 766. Also similar findings were reported by Celekli *et al.* (2009) that the phosphates supply increased biomass and carotenoids production in non heterocystous cyanobacteria like *Spirulina platensis*. The productions of phycobiliproteins at different level of
pH were found to be strain dependent. PE, PC and APC were produced maximum at both acidic and high alkalinity depending on the type of strains. Phycobiliproteins in *Anabaena* NCCU-9 was optimized by sodium phosphate buffer of pH 7.5 (Hemlata *et al.*, 2011). Soni *et al.* (2006) and Silveira *et al.* (2007) reported that sodium phosphate buffer of pH 7.0 as optimum for phycocyanin extraction in *Oscillatoria* and *Spirulina*.

The effect of nitrate on the phycobiliproteins of the 05 selected strains showed strain dependent. High dose of nitrate lead to declined the phycobiliproteins production except in case of *Leptolyngbya* sp. BTA 477 (PC at 5N) and *Phormidium tenue* BTA 803 (PE at 5N). Rai and Tiwari (2001) and Moore *et al.* (2002) reported that cyanobacteria cultivation generally include nitrate added as a nitrogen source when grown in batch culture. The use of nitrate in cultivation causes an increase in the biomass production as well as pigment content. The metabolism of nitrate reducing nitrate to nitrite and then to ammonium and by subsequent ammonium incorporation, to carbon skeletons yielding amino acids, has been demonstrated for *Spirulina platensis* and *Synechococcus* sp. WH 7803 (Boussiba, 1989; Chadd *et al.*, 1996). The BG-11 medium which was increased at 0.32 gm/L phosphate showed maximum phycobiliproteins which was increased 400% i.e. from 4.9 to 25.9 mg/L was reported (Hong and Lee, 2008). Phycobiliproteins production at high dose of phosphate may be also due to requirement of reconstitution of phycobiliproteins at high phosphate concentrations (Glick and Zilinskas, 1982).