SUMMARY

The present study deals with Nostocalean cyanobacteria of north eastern region of India which comprises the states of Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura. The region is one of the 12 mega-biodiversity rich zones of the world and forms a distinctive part of the Indo-Burma biodiversity hotspots that ranks 8th among the 34 biodiversity hotspots of the world. Cyanobacteria as a whole and Nostocalean forms in particular thrive exuberantly in the natural habitats of north eastern region of India which falls under the Himalayan region of Indo-Burma biodiversity hotspots. Despite its abundant occurrence in the north eastern region of India, few studies have been done so far as most of the previous studies were concentrated on state or region specific. Therefore, the present study was aimed to explore, isolate and identify by classical and modern PCR based molecular methods and characterization of the Nostocalean cyanobacteria of the north eastern region of India. With the establishment of correct identification of the potent strains through molecular approaches, application as eco-friendly biofertilizer in various cultivable lands can help in increasing the productivity without polluting the soil environment which is a burning problem of the present scenario.

The thesis of the present study constitute of eight different chapters. Chapter-1 emphasized on general introduction, chapter-2 on review of literature, chapter-3 on geographical location of the study sites, chapter-4 on isolation and identification of Nostocalean cyanobacteria from north eastern region of India, chapter-5 on maintenance and preservation of Nostocalean diversity through modern tools and techniques, chapter-6 on PCR based molecular characterization for phylogenetically study, chapter-7 on development of database of selected Nostocalean cyanobacteria of north eastern region of India and chapter-8 on general discussion.
The algal, water and soil samples were collected from different ecological habitats of north eastern region of India with locations recorded using GPS. Isolation and identification were made of Nostocalean cyanobacteria and unialgal cultures were maintained and preserved in the Fresh water Cyanobacterial and Microalgal Repository at IBSD, Imphal, Manipur, India (a state-of-the-art-facility of national importance created by the Department of Biotechnology, Govt. of India with reference no. BT/PR11323/PBD/26/171/2008 dated 31st March, 2009) at IBSD-DBT, Imphal, Manipur.

Two hundred forty seven (247) Nostocalean cyanobacteria were isolated from the 08 states of the north eastern region of India and classically identified by conventional methods. Manipur (110 unialgal strains) has the highest number of isolates followed by Assam (42 unialgal strains), Mizoram (35 unialgal strains), Meghalaya (28 unialgal strains), Arunachal Pradesh (13 unialgal strains), Tripura (08 unialgal strains) Nagaland (06 unialgal strains) and Sikkim (05 unialgal strains). By comparative analysis with the available literature, these above mentioned Nostocalean strains were identified and found to belong to 12 genera viz. *Anabaenopsis* (Wolosz.) Miller sensu strict. (01 strain), *Cylindrospermum* Kutz. (04 strains), *Wollea* Born. et Flah. (01 strain), *Nostoc* Vauch. (87 strains), *Anabaena* Bory (97 strains), *Aulosira* Kirchner (08 strains), *Scytonema* Ag. (06 strains), *Tolypothrix* Kutzing (04 strains), *Microchaete* Thuret (14 strains), *Calothrix* Ag. (23 strains), *Dichothrix* Zanardini (01 strain) and *Rivularia* (Roth) Ag. (01 strain).

The genus *Anabaena* was found abundantly in all 08 north eastern states while *Nostoc* occurred in all the states except Nagaland whereas the other genera were found sparingly in the different states of the north eastern region of India. Morphological characteristics viz. filament/trichome; branching pattern; appearance of sheath; cells shape and size; heterocysts positions, frequency and shape; akinetes position, frequency and shape; and geographical details of the collection sites and habitats viz. location, altitude, latitude, longitude etc. were
recorded. Natural habitats of the Nostocalean cyanobacterial colonies were recorded and photomicrographs of different Nostocalean genera were also recorded for morphological studies. Thallus behaviour of the selected strains was studied and photographs recorded.

Sixty two (62) Nostocalean cyanobacteria exhibiting fast and exuberant growth were selected and subjected for ammonia excretion, chlorophyll-a production and nitrogenase activity under culture conditions. 06 strains which comparatively showed good nitrogenase activity, also produced good amount of fine chemicals and would be useful for biofertilizer were chosen and undergone for detailed morphological as well as further biochemical characterization for available total soluble proteins and total carbohydrates. These strains were also analysed for lipid profiling and fatty acid composition by GC-FID method. The selected 06 strains are as follows:

* **Anabaena** sp. BTA650

**Thallus behaviour:** Typical blue green, scattered at the bottom, later floating on surface

**Filaments behaviour:** Straight, cells barrel-shaped 3.72-4.48 µm breadth and 1.94-3.56 µm length, heterocysts also barrel-shaped 4.37-6.32 µm breadth and 4.05-8.04 µm length with spherical spores, sheath not distinct

* **Calothrix** sp. BTA265

**Thallus behaviour:** Brownish colour, floccose, attached at the bottom initially and later aggregated into clumps

**Filaments behaviour:** Single and slightly bent, cells broader than the length 4.51-10.28 µm breadth and 3.65-7.34 µm length and heterocysts spherical 3.3-7.69 µm breadth and 4.63-9.81 µm length, sheath lamellated and distinct
* Nostoc sp. BTA197

**Thallus behaviour:** Yellowish green, initially attached at the bottom later floating

**Filaments behaviour:** Flexuous, cells partly cylindrical and partly spherical

4.81-5.93 µm breadth and 3.43-5.43 µm length, heterocysts spherical 2.71-3.14 µm radii, sheath diffuent

* Scytonema hofmanni BTA124

**Thallus behaviour:** Blue green, bushy growth attached at the bottom later floating

**Filaments behaviour:** Irregularly bent, cells broader than length 4.69-6.29 µm breadth and 3.32-4.22 µm length, rectangular heterocysts

6.22-7.38 µm breadth and 9.71-14.38 µm length, thick and hyaline sheath

* Rivularia sp. BTA510

**Thallus behaviour:** Dark blue green, initially attached at the bottom later floating at the surface

**Filaments behaviour:** Very closely pressed together, longer than broad at base 3.77-5.70 µm breadth and 4.5-8.22 µm length and broader than length at apex 2.75-4.18 µm breadth and 1.60-2.78 µm length, heterocysts conical 5.28-5.93 µm breadth and 7.34-8.54 µm length, sheath lamellated

* Anabaena sp. BTA281

**Thallus behaviour:** Blue green, occurring as floccose biomass later getting clumps

**Filaments behaviour:** Straight with constrictions at the cross walls, barrel-shaped cells 3.14-3.33 µm breadth and 4.4-6.83 µm length, barrel-shaped heterocyst 3.94-4.42 µm breadth and 6.6-9.19 µm length with ellipsoidal spores, sheath not distinct
Out of the 06 selected strains, *Anabaena* sp. BTA281 excreted highest ammonia (10.95±0.01 µg ml⁻¹) and the least by *Scytonema hofmannii* BTA124 (19.30±5.40 µg ml⁻¹). *Anabaena* sp. BTA650 showed the highest ARA activity (111.08±13.26 nmole C₂H₄ µg⁻¹ Chl-a h⁻¹) and the least by *Anabaena* sp. BTA281 (21.47±0.45 nmole C₂H₄ µg⁻¹ Chl-a h⁻¹). *Nostoc* sp. BTA197 produced the highest chl-a (0.99±0.11 µg ml⁻¹) and the least by *Calothrix* sp. BTA265 (0.07±0.06 µg ml⁻¹). *Anabaena* sp. BTA650 produced the highest total soluble proteins (120.67±2.31 µg ml⁻¹) and the least by *Rivularia* sp. BTA510 (11.00±3.46 µg ml⁻¹). *Anabaena* sp. BTA281 produced the highest total carbohydrates (22.67±0.58 µg ml⁻¹) and the least by *Calothrix* sp. BTA265 (5.00±0.00 µg ml⁻¹).

For fatty acid composition and lipid profiling, Capric Acid Methyl Ester (C10:0), Lauric Acid Methyl Ester (C12:0), Tridecanoic Acid Methyl Ester (C13:0), Myristoleic Acid Methyl Ester (C14:1), Pentadecanoic Acid Methyl Ester (C15:0), Palmitoleic Acid Methyl Ester (C16:1) and Heptadecanoic Acid Methyl Ester (C17:0) were found common in all the selected 06 Nostocalean strains except in *Anabaena* sp. BTA281 which lacked Palmitoleic Acid Methyl Ester (C16:1). Pentadecanoic Acid Methyl Ester (C15:0) was found highest in all the selected Nostocalean strains. *Rivularia* sp. BTA510 (79.05%) produced highest Pentadecanoic Acid Methyl Ester (C15:0) followed by *Nostoc* sp. BTA197 (68.34%), *Scytonema hofmannii* BTA124 (62.29%), *Anabaena* sp. BTA281 (55.02%), *Calothrix* sp. BTA265 (51.21%) and *Anabaena* sp. BTA650 (43.58%).

All the 247 Nostocalean cyanobacteria which belong to 12 genera were maintained in BG-11 (-N) agar slant quadruplicate tubes and separately one BG-11 (-N) broth medium. Out of these, 06 strains which expressed comparatively good amount of nitrogenase activity were subjected for cell immobilization and cryopreservation for future study without losing their viability. The viability level after six months of preservation has been studied. The post-thaw viability varied depending on the preservation methods. The selected strains comprised of
Anabaena sp. BTA650, Nostoc sp. BTA197, Rivularia sp. BTA510, Scytonema hofmanni BTA124, Anabaena sp. BTA281 and Calothrix sp. BTA265 were revived completely after six months of cryopreservation and immobilization. In case of cryopreservation with cryoprotectant DMSO, all the 06 strains were revived fairly well in one to two weeks time and yielded chlorophyll-a as follows: Nostoc sp. BTA197 (9.76±0.17 µg ml⁻¹), Anabaena sp. BTA650 (7.91±2.15 µg ml⁻¹), Scytonema hofmanni BTA124 (5.71±0.44 µg ml⁻¹), Anabaena sp. BTA281 (2.12±0.15 µg ml⁻¹), Rivularia sp. BTA510 (1.90±0.12 µg ml⁻¹) and Calothrix sp. BTA265 (1.29±0.23 µg ml⁻¹).

With cryoprotectant glycerol, comparatively all the 06 strains performed poorly under same conditions and time period but revival was observed in all of them. Chlorophyll-a estimation result shown were Scytonema hofmanni BTA124 (4.27±0.18 µg ml⁻¹), Anabaena sp. BTA281 (2.92±0.13 µg ml⁻¹), Anabaena sp. BTA650 (2.14±0.24 µg ml⁻¹), Rivularia sp. BTA510 (2.12±0.21 µg ml⁻¹), Nostoc sp. BTA197 (1.82±0.13 µg ml⁻¹) and Calothrix sp. BTA265 (1.14±0.12 µg ml⁻¹). For the immobilization technique, all the 06 strains at 18±2°C under photon flux rate of around 20 µmol m⁻² s⁻¹ revived very exuberantly but at 4°C Calothrix sp. BTA265 and Rivularia sp. BTA510 showed no revival inspite of subjection to the same culture conditions.

Under storage condition of 18±2°C, Scytonema hofmanni BTA124 yielded the highest chl-a (14.95±1.66 µg ml⁻¹) followed by Nostoc sp. BTA197 (13.29±2.19 µg ml⁻¹), Anabaena sp. BTA650 (6.44±0.17 µg ml⁻¹), Rivularia sp. BTA510 (5.37±0.99 µg ml⁻¹), Calothrix sp. BTA265 (2.38±0.22 µg ml⁻¹) and Anabaena sp. BTA281 (2.10±0.03 µg ml⁻¹). Under storage condition of 4°C, Scytonema hofmanni BTA124 yielded the highest chl-a (8.87±0.49 µg ml⁻¹), followed by Nostoc sp. BTA197 (1.89±0.37 µg ml⁻¹), Anabaena sp. BTA281 (1.88±0.18 µg ml⁻¹) and Anabaena sp. BTA650 (0.32±0.03 µg ml⁻¹).
For the molecular characterization, total genomic DNA of twelve (12) Nostocalean cyanobacteria including 03 strains of UTEX, The Culture Collection of Algae were used as templates. The genomic DNA absorbance $A_{260}/A_{280}$ falling in the ratio 1.8 - 1.9 were considered good and use for further PCR amplifications. The size of the genomic DNA ranges from 18,000-20,000 bp. Using the 12 genomic DNA as templates RAPD analysis for the 06 primers was carried out, a total of 96 bands were generated, out of which 60 were polymorphic and 36 were monomorphc bands. The total number of bands per strain ranged from 4 to 13. Each primer produced from 8 to 25 bands. For Primer-1 (P1), the DNA bands observed for the analysed strains ranges from 320-1,931 bp; for P2 ranges from 716-1,370 bp; for P3 ranges from 827-1,497 bp; for P4 ranges from 949-1,861 bp; for P5 ranges from 303-1,814 bp and for P6 ranges from 432-1,956 bp. The size of the amplified products ranged from 303 to 1,956 bp. The reproducibility of the results was assessed by repeating the experiments thrice.

Other than variations in the intensity of bands, no major variation was observed in the banding profile. A UPGMA phylogenetic tree was constructed. The highest percentage of similarity was observed between strains Nostoc sp. BTA197 and Anabaena doliolum BTA281, Calothrix sp. BTA265 and Anabaena sp. BTA650, Rivularia sp. BTA510 and Nostoc sp. BTA676 (24% each); 20% similarity was observed between strains Nostoc sp. BTA197 and Anabaena sp. BTA650; 15% similarity was observed between strains Rivularia sp. BTA510 and Calothrix anomala UTEX1319, Anabaena doliolum BTA280 and Nostoc sp. UTEXB2211. 14% similarity was observed between strains Scytonema hofmanni BTA124 and Anabaena doliolum BTA280, Nostoc sp. BTA197 and Nostoc sp. UTEXB2211. 13% similarity was observed between strains Nostoc sp. BTA197 and Scytonema sp. UTEX1163, Rivularia sp. BTA510 and Scytonema sp. UTEX1163, Anabaena sp. BTA281 and Calothrix
anomala UTEX1319, Nostoc sp. BTA676 and Calothrix anomala UTEX1319, Anabaena bergii BTA284 and Calothrix anomala UTEX1319.

The selected 09 Nostocalean strains exhibiting high nitrogenase activity were preliminarily characterized morphologically and for authentic identification, molecular approach using 16S rRNA was adopted. The size of the amplified 16S rRNA PCR products of the 09 Nostocalean strains fall around 1,200 bp and were sequenced at NCCS-DBT, Pune.

Pair-wise similarity alignment by BLASTN of the obtained 09 Nostocalean sequences was subjected to identify the strains by comparing with the sequences available at NCBI GenBank database. Scytonema hofmanni BTA124 showed 93% identity with Scytonema hofmanni PCC 7110 GenBank accession no. AF132781 and E value (0.0), Nostoc sp. BTA197 showed 99% identity with Nostoc sp. HA4356-MV1 GenBank accession no. HQ847577 and E value (0.0). Calothrix sp. BTA265 showed 99% identity with Calothrix sp. PCC 7715 GenBank accession no. AM230701 and E value (0.0). Anabaena sp. BTA281 showed 96% identity with Anabaena sp. CH1 GenBank accession no. DQ294214 and E value (0.0). Rivularia sp. BTA510 showed 89% identity with Rivularia sp. MU24 UAM-305 GenBank accession no. EU009149 and E value (0.0). Anabaena sp. BTA650 showed 97% identity with Anabaena sp. 08-05 GenBank accession no. FN691915 and E value (0.0). Nostoc sp. BTA676 showed 96% identity with Nostoc sp. UIC 10274 GenBank accession no. JX1880019 and E value (0.0). Anabaena dolioolum BTA280 showed 99% identity with Anabaena dolioolum BF4 GenBank accession no. GU396094 and E value (0.0). Anabaena bergii BTA284 showed 79% identity with Anabaena bergii ANA283A GenBank accession no. FJ234897 and E value (9e-72).

16S rRNA gene sequences of the 09 Nostocalean strains generated by NCCS-DBT, Pune were deposited to the NCBI GenBank through Sequin Application Version 12.30 - NCBI stand alone software tool for obtaining accession numbers of the said strains. The
GenBank accession nos. of the studied strains obtained were Scytonema hofmanni BTA124 (KF779147), Nostoc sp. BTA197 (KF779148), Calothrix sp. BTA265 (KF779149), Anabaena sp. BTA281 (KF779150), Rivularia sp. BTA510 (KF779151), Anabaena sp. BTA650 (KF779152), Nostoc sp. BTA676 (KF779153), Anabaena doliolum BTA280 (KF779154) and Anabaena bergii BTA284 (KF779155). Anabaena bergii BTA284 (KF779155) and excluded from the phylogenetic tree construction due to short sequence length.

From the neighbour-joining phylogenetic tree of the 08 Nostocalean strains i.e. Scytonema hofmanni BTA124 (KF779147), Nostoc sp. BTA197 (KF779148), Calothrix sp. BTA265 (KF779149), Anabaena sp. BTA281 (KF779150), Rivularia sp. BTA510 (KF779151), Anabaena sp. BTA650 (KF779152), Nostoc sp. BTA676 (KF779153) and Anabaena doliolum BTA280 (KF779154), it can be inferred that Scytonema hofmanni BTA124 was the first to diverge among the strains studied and found to be less evolved from the rest of the strains sharing same ancestor with Anabaena sp. BTA650, Calothrix sp. BTA265, Rivularia sp. BTA510, Nostoc sp. BTA197, Anabaena sp. BTA281, Anabaena doliolum BTA280 and Nostoc sp. BTA676 belong to the same cluster. However, Calothrix sp. BTA265 and Rivularia sp. BTA510 were in one clade which showed the close relatedness and substitution supported by the bootstrap value 99%, Rivularia sp. BTA510 was more diverged and less evolved than Calothrix sp. BTA265.

The maximum parsimony tree which is a character based method also was in agreement with the above method along with good bootstraps values. In this case also, Scytonema hofmanni BTA124 was the most diverged strains among all. Calothrix sp. BTA265 and Rivularia sp. BTA510 were supported with 100% bootstrap value indicating the closeness and relatedness of the family rivulariaceae. Further, distance matrix of the studied strains was also in support of the above two phylogenetic trees.
Twenty two (22) sequences including one outgroup were retrieved from NCBI GenBank database for reconstruction of phylogenetic trees using neighbour-joining and maximum parsimony methods to study the relatedness of the strains. In case of neighbour-joining method, *Scytonema hofmanni* BTA124 forms homolog with *Scytonema hofmanni* PCC7110 which was supported by consensus of 68% falling under the family scytonemataceae cluster. *Nostoc* sp. BTA676 was found to be diverged from *Nostoc* sp. UIC 10274 and *Nostoc* sp.UIC 10279 clade as the substitution rate was more than *Nostoc* sp. UIC 10274. *Anabaena doliolum* BTA280 forms homolog with *Anabaena doliolum* BF4 showed 95% bootstrap value supporting the similarity shown by BLASTN analysis. *Nostoc* sp. BTA197 showed the same rate of substitution with *Nostoc* sp. HA4356-MV1 that was found to be 99% similarity in BLAST test. *Anabaena* sp. BTA281 showed less substitution than *Anabaena* sp. CH1 forming separate cluster. *Anabaena* sp. BTA650 showed less substitution rate than *Anabaena* sp. 08-05 thereby forming separate clade from the cluster of *Anabaena* sp. *Rivularia* sp. BTA510 was found to be divergent from the rest of the rivulariaceae cluster due to least substitution rate from the rest of the rivulariaceae cluster. *Calothrix* sp. BTA265 and *Calothrix* sp. PCC175 shared same node and was found to be sister homolog supported by 99% bootstrap value.

In case of maximum parsimony method, *Scytonema hofmanni* BTA124 and *Scytonema hofmanni* PCC7110 showed same node and showed 100% bootstrap supporting the BLASTN analysis. *Anabaena* sp. BTA281 showed divergent from *Anabaena* sp. CH1. *Anabaena doliolum* BTA280 and *Anabaena doliolum* BF4 showed same node probably same ancestor supported by 100% bootstrap value. *Nostoc* sp. BTA197 showed same node with *Nostoc* sp. HA4356-MV1 supported by 95% bootstrap value with same characteristics. *Rivularia* sp. BTA510 was found to be most diverged from rivulariaceae cluster which was least evolved and supported by 100%. *Anabaena* sp. BTA650 was found to be more evolved
from *Anabaena* sp. 08-05. *Nostoc* sp. BTA676 showed more evolved than *Nostoc* sp. UIC 10274 which was found to be diverged from each other.

With the support of the distance based and character based methods adopted for the 16S rRNA analysis the correct identification of the selected Nostocalean strains have been established which would find use as biofertilizer in future. However, the 16S rRNA tree topology did not match with that generated via RAPD analysis.

Database of sixty two (62) potent Nostocalean cyanobacteria of north eastern region of India were prepared. Two strains were accommodated per datasheet. Therefore, a total of 31 datasheets were prepared in present study. Details of each strains and taxonomical information and the maintenance conditions being used as well as biochemical characterization viz. NH$_3$ excretion, chlorophyll-a and acetylene reduction activity were presented in the datasheet. Partial sequences of 16S rRNA along with NCBI GenBank accession numbers of nine (09) Nostocalean cyanobacteria viz. *Scytonema hofmanni* BTA124 (KF779147), *Nostoc* sp. BTA197 (KF779148), *Calothrix* sp. BTA265 (KF779149), *Anabaena* sp. BTA281 (KF779150), *Rivularia* sp. BTA510 (KF779151), *Anabaena* sp. BTA650 (KF779152), *Nostoc* sp. BTA676 (KF779153), *Anabaena doliolum* BTA280 (KF779154) and *Anabaena bergii* BTA284 (KF779155) which expressed high nitrogenase activity in culture condition were presented. Photomicrographs were also presented in the respective datasheet of each strain.