2.1. Isolation and purification of *Nostoc* sp:

Soil samples were collected during the month of September from North Eastern Hill University campus, Shillong and brought to the laboratory. The samples were examined under phase contrast microscope. *Nostoc* colonies were identified, washed thoroughly with doubled distilled water and then homogenised using glass beads. The homogenised samples were then plated on sterilised nitrogen free BG-11 medium (BG-11o; Rippka *et al.*, 1979) with 1.5 % agar. The plates were incubated at 25°C under light (photon fluence rate 50 μmol photons.m⁻².s⁻¹). When colonies appeared, these were picked up and viewed under the microscope before subsequent re-plating on BG-11o medium. The process was repeated several times until well separated colonies were obtained. These colonies were then purified by plating on solidified BG-11o medium containing Polymixin-B sulphate (10 μg.ml⁻¹) and Cycloheximide (100 μg.ml⁻¹). The individual *Nostoc* colonies were picked up under aseptic conditions and transferred to liquid sterilised BG-11o medium in test tubes. The procedures were repeated till axenic cultures of *Nostoc* sp. were obtained. Stocks were maintained on solid BG-11o medium in test tubes.
2.2. **Identification of the isolated *Nostoc* strain:**

tRNA\textsubscript{Leu} (UAA) intron sequence analysis (done in the laboratory of Prof. P. Lindblad, Uppsala University, Sweden) and PCR fingerprints using STRR-1A primer (done in the laboratory of Prof. B. Bergman, Stockholm University, Sweden) were obtained to determine the identity of this *Nostoc* strain. The results (Figure 2.1 and 2.2) show that this *Nostoc* strain is virtually identical to *Nostoc* ANTH isolated by us earlier from *Anthoceros* (Prakasham and Rai, 1991). Both strains showed identical fingerprints and tRNA\textsubscript{Leu} (UAA) intron sequences of both strains were similar except for a difference of just two bases (at positions 111 and 139). For full details protocols for STRR-1A PCR and tRNA\textsubscript{Leu} (UAA) intron sequence analysis, see Rasmussen and Svenning (1998) and Paulsrud and Lindblad (1998), respectively.

2.3. **Culture medium:**

The cyanobacterium *Nostoc* ANTH was grown from axenic stock cultures in N\textsubscript{2}-medium (BG-11\textsubscript{0} medium; Rippka *et al.*, 1979). The concentrations of macronutrients in N\textsubscript{2}-medium were (mM): K\textsubscript{2}HPO\textsubscript{4}.3H\textsubscript{2}O, 0.18; Na\textsubscript{2}CO\textsubscript{3}, 0.19; MgSO\textsubscript{4}.7H\textsubscript{2}O, 0.30; CaCl\textsubscript{2}.2H\textsubscript{2}O, 0.25; EDTA (disodium salt), 0.003; Citric acid, 0.029; Ferric ammonium citrate, 0.030. The concentrations of micronutrients in N\textsubscript{2}-medium were (\mu M): H\textsubscript{3}BO\textsubscript{3}, 46; MnCl\textsubscript{2}.4H\textsubscript{2}O, 9.2; ZnSO\textsubscript{4}.7H\textsubscript{2}O, 0.77; Na\textsubscript{2}MoO\textsubscript{4}.2H\textsubscript{2}O, 1.6; CuSO\textsubscript{4}.5H\textsubscript{2}O, 0.32; Co(NO\textsubscript{3})\textsubscript{2}.6H\textsubscript{2}O, 0.17. As and when
required, the N$_2$-medium was supplemented with combined nitrogen sources such as potassium nitrate (nitrate-medium) or ammonium chloride (ammonium-medium). The medium was always buffered with equimolar concentration of HEPES. The pH of the medium was adjusted to 7.5 before autoclaving.

2.4. **Growth conditions:**

Cultures of *Nostoc* ANTH were routinely grown in a culture room maintained at 25°C with a light intensity (photon fluence rate) of 50 μmol photons.m$^{-2}$.s$^{-1}$.

2.5. **Culture conditions for akinete differentiation:**

2.5.1. **By Sulphur limitation:**

*Nostoc* ANTH was grown and allowed to sporulate in BG-11$_0$ medium minus MgSO$_4$. The medium was supplemented with equimolar concentration of MgCl$_2$ to counter the effect of reducing the concentration of MgSO$_4$ so that the combined cation and anion concentrations remained the same in all cultures. As and when required, the medium was supplemented with 5 mM potassium nitrate or 2 mM ammonium chloride and buffered with equimolar concentration of HEPES.
2.5.2. **By Phosphorous limitation:**

*Nostoc ANTH* was grown and allowed to sporulate in BG-110 medium minus K$_2$HPO$_4$. The medium was supplemented with equimolar concentration of K$_2$SO$_4$ to counter the effect of reducing the concentration of K$_2$HPO$_4$ so that the combined cation and anion concentration remained the same in all cultures. As and when required, the medium was supplemented with 5 mM potassium nitrate or 2 mM ammonium chloride and buffered with equimolar concentration of HEPES.

2.5.3. **By addition of various carbon sources:**

*Nostoc ANTH* was grown in BG-110 medium supplemented with glucose, sucrose, or fructose. Four different concentrations (10, 20, 30 and 50 mM) of each sugar were used. During growth, the appearance of any akinetes was closely monitored.

2.6. **Culture condition for akinete germination:**

Akinete population was washed twice and resuspended in fresh BG-110 medium at a concentration of 2 $\times$ 10$^6$ akinetes ml$^{-1}$ and incubated in the presence of light at 50 $\mu$mol photons.m$^{-2}$.s$^{-1}$. Whenever necessary KNO$_3$ (5 mM) and NH$_4$Cl (2 mM) were added as sources of combined nitrogen.
2.7. **Akinete and heterocyst frequency:**

Heterocyst and akinetes frequency was calculated as percentage of total cell populations by light microscopic observations.

2.8. **Estimation of akinete viability:**

The percentage of germinating akinetes was determined by examination of at least 1000 akinetes under the light microscope. Akinetes that did not lead to emergence of germling and remained in the single cell stage were considered to be inviable.

2.9. **Growth measurements:**

Growth was measured as increase in concentrations of chlorophyll a.

2.10. **Chlorophyll a determination and culture density measurement:**

Chlorophyll a was extracted into 90 % methanol in darkness at 4° C. The absorbance at 663 nm was measured using a Beckman DU-530 Spectrophotometer and chlorophyll a concentration calculated according to Mackinney (1941). The culture density was measured by measuring absorbance of the culture at 650 nm.
2.11. **Light micrography:**

The cultures were studied by light microscopy and where necessary, light micrographs were taken using the Jenaval (Carl Zeiss Jena) Research Microscope.

2.12. **C:N Ratio:**

Carbon to Nitrogen ratio of samples was determined using Vario III CHNOS analyser fitted with autosampler (Elementar Analysensysteme, GmbH, Germany).

2.13. **Protein Estimation:**

Protein content was measured according to Lowry *et al.*, (1951) as per details given below:

2.13.1. **Extraction of protein:**

5 ml of cyanobacterial culture was centrifuged and the pellet was resuspended in 1 ml of distilled water. The cells were disrupted by ultrasonication using a Soniprep 150 (MSE) fitted with an ultrasonic microprobe. The supernatant was collected after centrifugation at 3000 g for 5 min and used for protein determination.
2.13.2. Estimation of protein:

Reagents:

A. 2% Na₂CO₃ in 0.1 N NaOH.

B. 1% sodium potassium tartarate solution.

C. 0.5% CuSO₄ solution.

D. 100 ml of reagent A mixed with 1 ml each of reagent B and C (freshly prepared before use).

E. 1 N Folin and Ciocalteu's phenol reagent.

F. Standard protein solution: Bovine Serum Albumin (BSA) solution was prepared in the range of 10-100 μg.ml⁻¹.

Procedure:

To 1 ml of cyanobacterial protein extract, 5 ml of reagent D was added and mixed gently. This was incubated for 10 min at room temperature and then 0.5 ml of Folin reagent was added rapidly. After 30 min the mixture was centrifuged and the absorbance of the supernatant was read at 750 nm. A calibration curve was prepared by using BSA solution as standard for determination of cyanobacterial protein content.
2.14. **Oxygen exchange:**

Oxygen evolution and consumption was measured polarographically by using a Clark-type oxygen electrode installed in a 3 ml Plexiglass container with magnetic stirrer (Rank Brothers, England). Three ml cyanobacterial culture was added to the sample chamber of the non-polarised electrode and allowed to equilibrate for 5 min while stirring. The electrode was then polarised and the linear rate of oxygen evolution was obtained in light supplied by a 100 W tungsten filament bulb, which was shielded from the sample by a water bath acting as heat filter. The light intensity at the surface of the sample chamber was 50 μmol photons.m⁻².s⁻¹. Oxygen consumption was measured in dark with the chamber wrapped in aluminium foil. The rate of oxygen evolution and consumption were expressed as nmol O₂ evolved/consumed.min⁻¹.mg⁻¹ protein.

2.15. **Absorption spectra of photosynthetic pigments:**

Chlorophylls and carotenoids were extracted in methanol. The phycobiliproteins were extracted in 0.05 M phosphate buffer, pH 7 by sonication. The solutions containing chlorophylls and carotenoids or phycobiliproteins were placed in 1 cm light path cuvettes and absorption spectra of the samples were determined in the wavelength range 400 nm-700 nm using Beckman DU-530 UV/Visible spectrophotometer.
2.16. Enzyme assays:

2.16.1. Nitrogenase:

Nitrogenase activity was measured as ethylene production using acetylene reduction assay (Stewart et al., 1967). 8 ml of cyanobacterial culture was placed in a 18 ml stoppered serum vial. Acetylene gas was injected to a final concentration of 10 % (v/v) of the air phase in the vials. The vials were incubated in light (photon fluence rate of 50 μmol photons.m⁻².s⁻¹) at 25 °C on a magnetic shaker. After 1 hour, 1 ml gas sample was analyzed for ethylene produced by using a Tracor 540 gas chromatograph fitted with a porapak T column (stainless steel column 6' x 1/8", packed with Porapak T of mesh size (80/100) and a flame ionization detector.

2.16.2. Glutamine synthetase:

2.16.2.1. Extraction of enzyme: Cultures were harvested by centrifugation, washed twice in 50 mM Tris-HCl buffer (pH 7.5) and resuspended in the same buffer. The cells were treated for 10 min with alkyltrimethylammonium bromide (CTAB) at a final concentration of 100 μg.ml⁻¹ (Frias et al., 1994). Glutamine synthetase transferase activity was then assayed in situ using CTAB permeabilised cells.
2.16.2.2. **Glutamine synthetase transferase assay:** This was essentially as described by Sampio et al. (1979) except that CTAB permeabilised cells were used. The reaction mixture contained in a final volume of 3 ml, 1 ml enzyme extract (CTAB-permeabilised cell suspension), 40 μmol Tris-HCl buffer pH 7, 3 μmol MnCl₂, 20 μmol Potassium arsenate, 0.4 μmol ADP (Na⁺ salt), 60 μmol hydroxylamine and 30 μmol glutamine. The reaction mixture was incubated in the dark for 10 min at 30 °C. The reaction was terminated by the addition of 2 ml of stop mixture (4 ml of FeCl₃, 1 ml of 24 % TCA, 0.5 ml of 6 N HCl and 6.5 ml of water). The absorbance of the supernatant was read at 540 nm after 10 min of centrifugation at 2000 rpm. The concentration of γ-glutamyl hydroxamate formed was estimated from a standard curve that was prepared in the range of 0-0.2 μmol γ-glutamyl hydroxamate ml⁻¹.

2.16.3. **Nitrate reductase:**

Nitrate reductase (NR) activity was measured *in situ* (Manzano et al., 1976) using CTAB permeabilised cells. 5 ml of cyanobacterial culture was taken and centrifuged. The pellet was thoroughly washed with and resuspended in NR buffer (50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 0.3 M sucrose, 1 mM KNO₃, 1 mM EDTA and 5 mM MgCl₂). CTAB was added at a final concentration of 100 μg.ml⁻¹ and the suspension incubated for 10 min at room temperature with vigorous shaking. The reaction mixture contained,
addition to permeabilised cells, in a final volume of 1 ml: 20 mM KNO₃, 100 mM glycine-KOH (pH 10.5), 4 mM methyl viologen, and 10 mM sodium dithionite freshly dissolved in 0.1 ml of 0.23 M NaHCO₃. After 7 min of incubation in dark at 30°C, the reaction was terminated by adding 0.2 ml of 1 M zinc acetate. Subsequently, the nitrite formed was determined by the method of Snell and Snell (1949).

2.16.3.1. Nitrite estimation: Nitrite was estimated colorimetrically as described by Snell and Snell (1949).

Reagents:

A. 1 % (w/v) sulphanilamide in 3 M HCl.

B. 0.02 % (w/v) N-(1-Napthyl ethylene diamine dihydrochloride) in distilled water.

C. Potassium nitrite solution was prepared in the range of 10-100 nmol.ml⁻¹. This was used as standard.

Procedures:

To 1 ml of sample, 1 ml of sulphanilamide and 1 ml of 1-Napthyl ethylene diamine dihydrochloride was added. The solution was mixed thoroughly and the absorbance was read at 540 nm after 15 min. A calibration curve was
prepared by using potassium nitrite solution as standard for estimation of nitrite.

2.17. **SDS-PAGE of proteins of vegetative cells and akinetes:**

Cyanobacterial cells and akinetes were harvested by centrifugation (3000g) for 5 minute. The pellet was then resuspended in sodium dodecyl sulphate (SDS) sample buffer (1:1, v/v). The sample buffer consisted of 10 mM Tris-HCl (pH 8.8), 1 mM Ethylene diamine tetra acetic acid (EDTA), 2.5 % (w/v) SDS, 5 % β-mercaptoethanol, and 0.01 % (w/v) bromphenol blue. These samples were boiled for 5 min and then centrifuged at 15,000g for 5 min. The supernatants were subjected to standard SDS-PAGE (10 % polyacrylamide gel; 100 mA current). The electrophoresis was stopped when the tracking dye (Bromophenol blue) reached near the bottom edge of the gel. The proteins were stained with Coomassie brilliant blue R-250 overnight and then destained.

2.18. **Chemicals, Glasswares and Gases:**

All gases used were highest purity grade from Indian Oxygen Company Ltd. All glasswares used were Borosil make. All biochemicals were purchased from Sigma Chemicals Company, USA. General chemicals and solvent were
from Qualigen or Glaxo. Electrophoresis requirements were procured from Bio Rad and Alcohol from Bengal Chemicals, Kolkatta.
Figure 2.1: STRR 1A-PCR-based DNA fingerprints of *Nostoc* sp., *Nostoc* sp. (PR: Paraquat-resistant mutant), *Nostoc* sp. (AR: Azetidine-2-carboxylate-resistant mutant), *Nostoc* ANTH and *Nostoc* PCC 9229. These fingerprints were obtained using short tandemly repeated repetitive sequences (STRR 1A) as primer and whole filaments of *Nostoc* as templates. The STRR 1A primer used here had the following sequence: 3'‐CCCCTRACCCCTRACC‐5'. Note that the fingerprint of *Nostoc* sp. and its mutant are similar to that of *Nostoc* ANTH and quite distinct from *Nostoc* PCC 9229 (included here as control).

Lane M represents DNA molecular weight standard (bp).
### Sample

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**Figure 2.2: Comparison of nucleotide sequences of tRNA\textsuperscript{Leu} (UAA) intron from cyanobacterium Nostoc ANTH and Nostoc sp.**

The bold letters (at positions 111 and 139) indicate the difference of bases between Nostoc ANTH and Nostoc sp.