CHAPTER 2

Materials and Methods

2.1 Isolation and purification of *Mastigocladus* sp

The cyanobacterium *Mastigocladus* species used in the present study was isolated from the hot spring at Jakrem, West Khasi Hills, Meghalaya. The cyanobacterial samples were collected from the hot spring and brought to the laboratory. The samples were examined using light microscope, washed with double distilled water and then homogenized using glass beads. The homogenized samples were then plated on sterilized nitrogen-free D-medium (Castenholz, 1981) with 1.5 % agar. The plates were incubated at 45°C under continuous light (photon fluence rate of 50 µmol. m⁻². s⁻¹). When colonies appeared, these were picked up from the plates and viewed under the microscope before subsequent re-plating on D-medium. The process was repeated several times until well-separated colonies were obtained. The colonies were then selected and purified by plating on solidified D-medium, containing polymixin-B sulphate (10 µg. ml⁻¹) and cycloheximide (100 µg. ml⁻¹). The individual *Mastigocladus* colonies were picked up under aseptic conditions and transferred to sterilized D-medium in test tubes. The procedures were repeated till axenic cultures of *Mastigocladus* sp. were obtained. Stocks were maintained on solidified D-medium in test tubes in the culture room.
2.2 Identification of the isolated *Mastigocladus*

The organism was identified as *Mastigocladus laminosus* by observations under light microscope. PCR fingerprints using STRR-1A primer was obtained as described by Rasmussen & Svenning (1998) to generate a genetic identity.

2.3 Culture methods

2.3.1 Sterilization

All glass wares and nutrient media were autoclaved at 121°C (15 psi) for 15 min in an autoclave. The heat labile chemicals were sterilized by ultrafiltration using Whatman membrane filters of pore size 0.45 μm. Such chemicals were then added to previously sterilized nutrient medium.

2.3.2 Culture conditions

*Mastigocladus laminosus* was maintained on agar slants as well as in liquid D-N2 medium or D-nitrate medium or D-NH4-medium. Cultures were maintained at 25°C (culture room) or 45°C (inside a B.O.D. incubator) and light was provided at a photon fluence rate of 50 μmol. m⁻²·s⁻¹ on the surface of the vessels.
2.3.3 Culture media

D-nitrate medium (Castenholz, 1981).

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrilotriacetic acid</td>
<td>0.52 mM</td>
</tr>
<tr>
<td>( \text{NaNO}_3 )</td>
<td>8.24 mM</td>
</tr>
<tr>
<td>( \text{KNO}_3 )</td>
<td>0.99 mM</td>
</tr>
<tr>
<td>( \text{Na}_2\text{HPO}_4 )</td>
<td>0.78 mM</td>
</tr>
<tr>
<td>( \text{MgSO}_4.7\text{H}_2\text{O} )</td>
<td>0.41 mM</td>
</tr>
<tr>
<td>( \text{CaSO}_4.2\text{H}_2\text{O} )</td>
<td>0.35 mM</td>
</tr>
<tr>
<td>( \text{NaCl} )</td>
<td>0.14 mM</td>
</tr>
<tr>
<td>( \text{FeCl}_3 )</td>
<td>0.002 mM</td>
</tr>
<tr>
<td>( \text{H}_3\text{BO}_3 )</td>
<td>4.00 (\mu\text{M})</td>
</tr>
<tr>
<td>( \text{Co(NO}_3\text{_2)2.6H}_2\text{O} )</td>
<td>0.10 (\mu\text{M})</td>
</tr>
<tr>
<td>( \text{CuSO}_4.5\text{H}_2\text{O} )</td>
<td>0.05 (\mu\text{M})</td>
</tr>
<tr>
<td>( \text{MnCl}_4\text{H}_2\text{O} )</td>
<td>6-7 (\mu\text{M})</td>
</tr>
<tr>
<td>( \text{Na}_2\text{MoO}_4.2\text{H}_2\text{O} )</td>
<td>0.05 (\mu\text{M})</td>
</tr>
<tr>
<td>( \text{ZnSO}_4.7\text{H}_2\text{O} )</td>
<td>0.87 (\mu\text{M})</td>
</tr>
</tbody>
</table>

D-\( \text{N}_2 \) medium: \( \text{NaNO}_3 \) and \( \text{KNO}_3 \) replaced by \( \text{NaCl} \) and \( \text{KCl} \), respectively.

D-ammonium medium: 2 mM \( \text{NH}_4\text{Cl} \) added to the D-\( \text{N}_2 \) medium, buffered with 2 mM HEPES.
In experiments involving addition of amino acids, 1 mM L-amino acids were added and the medium was buffered with equimolar concentration of HEPES. In all cases, pH was adjusted to 7.5 before autoclaving.

2.4 Morphology

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

2.5 Growth parameters

The growth was measured using the following parameters:

2.5.1 Chlorophyll $a$

Chlorophyll $a$ was measured using the method as described by Mackinney (1941). Cells from 5 ml culture were centrifuged and resuspended in 5 ml of 100 % methanol and incubated for 10 min at 4°C in darkness. The chlorophyll $a$ concentration was measured spectrophotometrically in the supernatant of the centrifuged extract at 663 nm by using the formula:

\[ \text{Chlorophyll } a \ (\mu g. \ ml^{-1}) = \text{Absorbance at 663 nm} \times 12.63. \]

2.5.2 Phycobiliprotein

The phycobiliprotein content [phycocyanin (PC), allophycocyanin (APC),
phycoerythrin (PE)] was determined according to Bennett and Bogorad (1973). Cultures were harvested, washed and resuspended in saline buffer (0.15 mM, pH 7.0). The cells were disrupted by using an ultrasonicator (Sonics & Materials, Inc. USA) fitted with a microprobe. The phycobiliprotein concentration was measured spectrophotometrically in the supernatant of the centrifuged extract by using the formula:

\[
[\text{PC}] = \frac{\text{OD}_{615} - 0.475 \times \text{OD}_{652}}{5.34} \text{ mg/ml}
\]

\[
[\text{APC}] = \frac{\text{OD}_{652} - 0.208 \times \text{OD}_{615}}{5.09} \text{ mg/ml}
\]

\[
[\text{PE}] = \frac{\text{OD}_{562} - 2.41 \times \text{PC} - 0.844 \times \text{APC}}{9.62} \text{ mg/ml}
\]

2.5.3 Measurement of protein content

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

2.5.3.1 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 an ultrasonicator (Sonics & Materials, Inc. USA) fitted with a microprobe. The
supernatant was collected after centrifugation at 3000 rpm for 5 minute and used for protein determination.

2.5.3.2 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 minute at room temperature and then 0.5 ml of reagent E 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.

2.6 C:N Ratio
The cells were harvested, rapidly washed with distilled water and dried by keeping them at 45°C for four days inside an oven. The dried samples were collected, and Carbon to Nitrogen (C:N) ratio of samples was determined using Vario III CHNS analyzer fitted with autosampler (Elementary Analysensysteme, GmbH, Germany).

2.7 Oxygen exchange

Oxygen evolution and consumption was measured by using a Clark-type oxygen electrode installed in a 3 ml Plexiglass container with magnetic stirring (Rank Brothers, England). Measurement involved adding 3 ml cyanobacterial culture to the sample chamber of the non-polarised electrode and allowing each sample to equilibrate for 5 minute while stirring. The electrode was then polarized and the linear rate of oxygen evolution was obtained in light supplied by tungsten filament bulb, which was shielded from the sample by water bath acting as heat filter (photon fluence rate at the surface of the sample chamber: 50 μmol. m⁻². s⁻¹). Oxygen consumption was measured in darkness (sample chamber wrapped with aluminium foil).

2.8 Enzyme assays

2.8.1 Nitrogenase activity

Nitrogenase activity was measured using acetylene reduction assay (Stewart et al., 1967). 5 ml of cyanobacterial culture was placed in 15 ml serum stoppered vials. Acetylene gas was injected to a final concentration of 10 % (v/v) of air phase in the vials. The vials were incubated in light (photon fluence rate of 50 μmol. m⁻². s⁻¹) at
45°C. After 1 hour, 1 ml gas sample was analyzed for ethylene produced by using a Chemito gas chromatograph fitted with a poropak-T column (80-100 mesh; ½" x 2 m stainless steel) and a flame ionization detector. The oven temperature of the GC was maintained at 60°C during the operation.

2.8.2 Glutamine synthetase (transferase) activity

2.8.2.1 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 disrupted by ultrasonication using an ultrasonicator (Sonics & Materials, Inc. USA) fitted with a microprobe. Glutamine synthetase (transferase activity) was then assayed using the ultrasonicated cultures.

2.8.2.2 Assay of Glutamine synthetase (transferase) activity

This method for GS activity was essentially described by Sampio et al., (1979). The reaction mixture contained in a final volume of 3 ml: 1 ml enzyme extract (ultrasonicated culture suspension), 40 mM Tris-HCl buffer pH 7.5, 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 45°C or at 25°C depending upon the temperature where the cultures were grown. 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 0-200 nmol γ-glutamyl hydroxamate. ml⁻¹.

2.8.3 Nitrate reductase activity

Nitrate reductase (NR) activity was measured (Manzano et al., 1976) using the ultrasonicated culture suspension. 5 ml of cyanobacterial culture was taken and centrifuged. The pellet was thoroughly washed 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). The reaction mixture contained, in addition to ultrasonicated cultures, 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 10 min of incubation in darkness at 45°C or at 25°C depending upon the culture temperature, the reaction was terminated by adding 0.2 ml of 1 M zinc acetate. Subsequently nitrite formed was determined by the method of Snell and Snell (1949).

2.8.4 Nitrite reductase activity

Nitrite reductase (NIR) activity was measured using the ultrasonicated culture suspension (Arizmendi & Serra, 1990). 5 ml of cyanobacterial culture was centrifuged and the pellet was thoroughly washed with buffer containing 50 mM Tris (pH 7.5). The reaction mixture contained, in addition to ultrasonicated culture suspension, in a final volume of 1 ml: 2.5 mM KNO₂, 90 mM Tris-HCL (pH 7.5), 3 mM methyl viologen and 20 mM sodium dithionate freshly prepared in 0.3 M
NaHCO₃. After 5 min of pre-incubation at room temperature without KNO₂, the reaction was started by addition of KNO₂. After 10 min at 45°C or 25°C (for cultures grown at 45°C and 25°C, respectively), the reaction was stopped by vigorous shaking to oxidize excess reductant. Subsequently remaining nitrite was determined by the method of Snell and Snell (1949).

2.9 Nitrate and nitrite uptake

Nitrate and nitrite uptake by cyanobacterial cultures was measured by following the disappearance of nitrate and nitrite (100 μM) from the external medium, respectively. The concentration of nitrite and nitrate were determined by the methods of Snell and Snell (1949) and Cawse (1967), respectively.

2.9.1 Nitrate estimation

2.9.1.1 Reagents

A: 2 % sulphamic acid.

B: 6.67 % HClO₄.

C: Sodium nitrate solution was prepared in the range of 100-500 nmol. ml⁻¹. This was used as standard.

2.9.1.2 Procedure

To 1 ml of sample, 1 ml of sulphamic acid and 3 ml of HClO₄ was added. The solution was mixed thoroughly and the absorbance was read at 210 nm. A calibration
curve was prepared by using sodium nitrate solution as standard for estimation of nitrate.

2.9.2 Nitrite estimation

Nitrite was estimated calorimetrically as described by Snell and Snell (1949).

2.9.2.1 Reagents:

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

B: 0.02% (w/v) N-(1-Naphthylethylene 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.

2.9.2.2 Procedure

To 1 ml of sample, 1 ml of sulphanilamide solution and 1 ml of 1-Naphthyl ethylene dihydrochloride solution 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.

2.10 Ammonium transport

Ammonium uptake was studied using ¹⁴C-methylammonium, an analogue of ammonium (Rai et al., 1984). Exponentially grown cells of M. laminosus were harvested, washed in D-N₂-medium and then resuspended in fresh D-N₂-medium for 48 h at 45°C or 25°C. The cells were then harvested, resuspended in 10 mM HEPES-
NaOH buffer, pH 7.0, to a concentration of 10 μg Chl a. ml⁻¹ and equilibrated for 1 h at 45°C or 25°C under light (photon fluence rate: 50 μmol. m⁻² . s⁻¹) inside a B.O.D. incubator. The ¹⁴C labeled methylammonium was added to a final concentration of 50 μM (specific activity 350 KBq. μmol⁻¹). Wherever needed, dichloro phenyl dimethyl urea (DCMU, 10 μM) or carbonyl cyanide chlorophenyl hydrazone (CCCP, 25 μM) were added to the cell suspension 30 min prior to the addition of labeled methylammonium, and were present during the experiments. At different time intervals, 400 μl samples were taken out rapidly and the cells were separated from their bathing medium by microcentrifugation through silicon oil/dinonyl phthalate (45/55, v/v) into perchloric acid/water (15/85, v/v) as previously described (Scott & Nicholls, 1980; Rai et al., 1984). The ¹⁴C in perchloric acid fraction was measured using a liquid scintillation counter (Model 1801, Beckman Instruments).

2.11 Amino-acid transport:

Glutamine, asparagine, alanine and arginine uptakes were measured using ¹⁴C labeled glutamine, asparagine, alanine and arginine, respectively. Cells from NO₃-grown culture were harvested during the exponential growth phase, washed, resuspended and then incubated in fresh D-N₂-medium and in D-N₂-medium supplemented with 1 mM glutamine, asparagine, alanine or arginine for 48 h at 45°C or 25°C. After incubation, the cells were harvested and resuspended in 10 mM HEPES-NaOH buffer, pH 7.0, to a concentration of 10 μg Chl a. ml⁻¹ and equilibrated for 1 h at 45°C or 25°C under light (photon fluence rate: 50 μmol. m⁻² . s⁻¹) inside a B.O.D. incubator. The ¹⁴C labeled glutamine (specific activity 256 KBq. μmol⁻¹),
asparagine (specific activity 63 KBq. μmol⁻¹), alanine (specific activity 71 KBq. μmol⁻¹) and arginine (specific activity 65 KBq. μmol⁻¹) were added to a final concentration of 50 μM. Wherever needed, chloramphenicol (1 μg. ml⁻¹), dichlorophenyl dimethyl urea (DCMU, 10 μM) or carbonyl cyanide chlorophenyl hydrazone (CCCP, 25 μM) were added to the cell suspension 30 min prior to the addition of labeled amino acid, and were present during the experiments. At different time intervals, 400 μl samples were taken out rapidly and the cells were separated from their bathing medium by microcentrifugation through silicon oil/dinonyl phthalate (45/55, v/v) into perchloric acid/water (15/85, v/v) as previously described (Scott & Nicholls, 1980; Rai et al., 1984). The ¹⁴C in perchloric acid fraction was measured using a liquid scintillation counter (Model 1801, Beckman Instruments).

2.12 Chemicals

All bio-chemicals were purchased from Sigma chemical company, USA. All glass wares used were borosil make. General chemicals and solvent were from Qualigen or Glaxo. Electrophoresis requirements were procured from Bangalore Genie and alcohol from Bengal Chemicals, Kolkata. Gases used were of highest purity from Sigma Gases and Services, New Delhi (helium) and Assam Air Products, Assam (N₂, O₂, H₂). Ethylene was obtained from Eurasian Associates, West Bengal.