3.1. Bacteria

Azotobacter sp. was isolated from garden soil. The soil sample was collected from Thazhathangady, Kottayam District. 1 g soil was added to 90 ml of sterile distilled water and shaken by rotation of the flask. Sample was streaked on to Jenson's agar plates. Growth was observed after 48 h. The bacteria was identified based on Bergy's manual of systematic bacteriology.84

Composition of Jenson's agar medium:5

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Potassium monohydrogen phosphate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Sodium molybdate</td>
<td>0.005 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>DGDW*</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>

*Deionised glass distilled water was used to ensure that the medium is devoid of any metal
3.1.1 Metal treatment of bacterial culture and measurement of Nitrogenase activity

Jensens' broth culture was prepared by the inoculation of bacteria from Jensens' agar plates into the Jensens' broth medium. Broth cultures were shaken for uniform growth. After 48 h, i.e., at logphase, the culture was subjected to metal treatments.

Sterile metal salt solutions were added to 20 ml of 48 h old bacterial culture in 100 ml glucose bottles, so that concentration of metal in the culture was 0.5, 1, 5, 10 and 25 ppm. Chlorides of nickel, cadmium, copper and mercury (NiCl₂, CuCl₂, CdCl₂ and HgCl₂) were used.

10% of air in the bottle was replaced by acetylene using a sterile gas tight glass syringe. After incubation period 0.5 ml of gas mixture was taken from the glass bottle using microsyringe through rubber stopper and injected into the gas chromatograph at intervals. Nitrogenase activity was measured after 24, 48 and 72 h of metal treatment.⁸⁵

GC operational parameter for ARA

<table>
<thead>
<tr>
<th>Detector</th>
<th>FID (Flame Ionisation detector)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrier gas</td>
<td>N₂ 45 ml/min</td>
</tr>
<tr>
<td>Detector gas</td>
<td>H₂ and air</td>
</tr>
<tr>
<td>Injection port temperature</td>
<td>100°C</td>
</tr>
<tr>
<td>Oven temperature</td>
<td>75°C</td>
</tr>
<tr>
<td>Detector temperature</td>
<td>120°C</td>
</tr>
<tr>
<td>Column used</td>
<td>Poro pack (stainless steel)</td>
</tr>
<tr>
<td>Company</td>
<td>Shimadzu</td>
</tr>
</tbody>
</table>
The amount of ethylene formed was calculated by the equation and expressed as nanomoles C\textsubscript{2}H\textsubscript{4} produced per mg protein per min.

\[
\text{Area of sample ethylene} \times \frac{Gv-VCF \times 105}{\text{Area of standard ethylene}} = 22.4 \ (T_1 - T_0)h
\]

\begin{align*}
Gv &= \text{Gas volume of the container} \\
VCF &= \text{Vacuum correction factor} \\
T_1 - T_0 &= \text{Difference in sampling interval} \\
22.4 \ L &= \text{Volume of 1 mol of gas at standard temperature and pressure.}
\end{align*}

3.1.2 Preparation of extracts for enzyme assays

Bacterial culture was treated with metal chloride solutions as described above. The bacterial culture was centrifuged at 1500 rpm for 20 min at 4°C and cell paste was washed with double distilled water and later with 0.01 M imidazole HCl buffer (pH 7.0) (containing 2\% mercaptoethanol). The cells were disrupted in 10 ml of the buffer by sonication at 4°C. The clear supernatant fluid, after centrifugation was used as the enzyme source.

Enzyme assays

(a) Glutamine synthetase (GS)\textsuperscript{86}

Assay mixture contained:

\[
\begin{align*}
\text{Imidazole HCl buffer (0.2 M)} & \quad 0.2 \ ml \\
\text{Glutamine (0.1 M)} & \quad 0.4 \ ml \\
\text{Manganese chloride (0.1 M)} & \quad 0.6 \ ml \\
\text{Adenosine diphosphate (0.01 M)} & \quad 0.2 \ ml \\
\text{Potassium arsenate (1 M)} & \quad 0.4 \ ml \\
\text{DGDW} & \quad 2.2 \ ml
\end{align*}
\]

Standard: Prepared a range of standards containing 100-500 \(\mu\)g \(\gamma\)-glutamyl hydroxamate (Sigma Co.) in the buffer.
0.5 ml of assay mixture was added to 0.21 ml of the extract. Added 0.2 ml distilled water and 0.1 ml hydroxylamine solution (2 M \( \text{NH}_2\text{OH}.\text{HCl} \) and 2 N \( \text{NaOH} \) mixed in equal volume). Incubated at 30°C for 30 min. Reactions were terminated and colour was developed after the addition of 2 ml of stopping reagent (10% FeCl₃, 2.4% trichloroacetic acid, 6 N HCl and distilled water mixed in the ratio 8: 2: 1: 13). Absorbance was recorded at 540 nm with Schimadzu 1601A UV-visible spectrophotometer. Enzyme activity was expressed as nanomoles of hydroxamate formed per mg protein per min.

(b) \textit{Glutamate synthase (GOGAT)}

In this case cells were disrupted in 0.5 M tris-HCl buffer (pH 7.8).

Assay mixture contained:

- Cell extract: 0.05 ml
- Tris-HCl buffer (0.5 M pH 7.8): 0.1 ml
- 2-Oxoglutarate (0.1 M): 0.05 ml
- DGDW: 0.5 ml

The contents were mixed in a tube and absorbance was read at 340 nm in spectrophotometer. 0.05 ml NADPH (0.25 mM) and 0.1 ml glutamine (0.1 M) were added to the above solution and absorbance was measured. Enzyme activity was expressed as nanomoles of NADPH oxidised per mg protein per min.

(c) \textit{Glutamate dehydrogenase (GDH)}

\textbf{Reagents}

- Tris-HCl buffer (pH 7.5): 0.1 M
- 2-Oxo-glutarate: 5 mM
- NADPH: 0.25 mM
- Ammonium chloride: 3 M
Reaction mixture containing 0.3 ml 2-oxo-glutarate, 0.12 ml NADPH and 0.5 ml NH₄Cl was prepared in 2.7 ml final volume of tris-HCl buffer pH 7.6. 0.3 ml of the extract was added to it.

Incubated at 37°C for 30 min. The change in absorbance was measured at 340 nm in a spectrophotometer. Enzyme activity was expressed as nanomoles of NADPH oxidised per mg protein per min.

3.2. **Lyngbya sp. and Nostoc sp.**

Soil samples were collected from Ambaloor in Ernakulam District, Manampur in Thiruvananthapuram District and Kumaranalloor in Kottayam District and Lyngbya sp. and Nostoc sp. were isolated using Fogg's medium.

**Medium for isolation and culture**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>0.2 g/L</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.2 g/L</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.1 g/L</td>
</tr>
<tr>
<td>Fe.EDTA solution</td>
<td>1 ml/L</td>
</tr>
<tr>
<td>As solution</td>
<td>1 ml/L</td>
</tr>
</tbody>
</table>

Fe.EDTA solution-26.1 g EDTA was taken in 268 ml of 1 N potassium hydroxide solution and to it was added 24.9 g of FeSO₄·7H₂O. The contents were dissolved by stirring. Aerated the solution overnight till a dark straw colour was developed, and stored in amber coloured bottles.

**Preparation of As stock solution**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manganese chloride</td>
<td>1.81 g/L</td>
</tr>
<tr>
<td>Sodium molybdate</td>
<td>0.39 g/L</td>
</tr>
<tr>
<td>Boric acid</td>
<td>2.86 g/L</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>0.222 g/L</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>0.079 g/L</td>
</tr>
</tbody>
</table>
The media prepared was autoclaved at 121°C for 20 min at 15 lbs. Potassium dihydrogen phosphate was autoclaved separately. This sterilised media was used for culturing the algae. The organism containing soil flakes were washed thoroughly in distilled water and used for primary culture.

The algae obtained from primary culture was centrifuged using sterile Fogg's medium 3-4 times. The pellets obtained was washed in antibiotic solution containing streptomycin and ampicillin, 6000 and 3000 µg/L respectively to remove surface contamination. This pellet was homogenised to obtain short filaments. These filaments were subsequently used for culturing on Fogg's agar medium containing antibiotics. Fogg's medium containing antibiotics was prepared as follows.

Fogg's medium - 100 ml
Agar - 2 g
Ampicillin - 400 µg
Streptomycin - 200 µg

The algae were inoculated onto the solidified media in petridishes using a wire loop. After 3-4 days, they were transferred to an antibiotic-less medium. The purity of culture was tested by streaking them on nutrient agar. The algae isolated were identified using standard procedure.

3.2.1 Nitrogenase assay

Algal culture at its log phase was washed several times with distilled water and was transferred to freshly prepared Fogg's medium. 20 ml of the culture was dispensed into the glucose bottles so that each bottle contains 100 mg fresh weight of alga. Then added metal stock solutions so that the final concentration of the cultures were 0.5, 1, 5, 10 and 25 ppm.

Cultures were subjected to nitrogenase assay after 15, 30 and 50 days of metal treatment. Nitrogenase activity was measured by acetylene
reduction assay (ARA) as described in the case of *Azotobacter* sp. Values are expressed as nanomoles C\textsubscript{2}H\textsubscript{4} produced per mg protein per min.

3.2.2 Preparation of extracts for enzyme assays

Metal treated cultures were prepared as in the case of nitrogenase. Extract used for assays of GS, GOGAT and GDH was prepared as follows.

20 ml metal treated cultures were centrifuged and suspended the algal pellet in about 10 ml of tris buffer (50 mM 2% β-mercaptoethanol) pH 7.8. Sonication was done in 6 cycles of 20 sec at 4°C. centrifuged for removing the cell debris at 1500 rpm for 10 min at 4°C. The resultant supernatant was used for enzyme assays.

(a) Glutamine synthetase (GS)

GS was estimated using transferase assay. Assay mixture was prepared as follows.

\[
\text{Imidazole buffer 0.2 M} \quad 0.2 \text{ ml} \\
\text{Glutamine 0.2 M} \quad 4 \text{ ml} \\
\text{Manganese chloride 0.1 M} \quad 0.6 \text{ ml} \\
\text{Adenosine diphosphate 0.01 M} \quad 0.2 \text{ ml} \\
\text{Potassium arsenate 1 M} \quad 0.4 \text{ ml} \\
\text{DGDW} \quad 2.2 \text{ ml} \\
\text{Stop mixture -10% FeCl}_3, 24\% \text{TCA, 6N HCl and DW in ratio of 8:2:1:13}
\]

0.5 ml of the assay mixture was added to 0.2 ml of extract, to this 0.2 ml distilled water and 0.1 ml hydroxylamine solution (2 M NH\textsubscript{2}OH.HCl and 2N NaOH mixed in equal volume) were added and incubated at 30°C for 30 min. Reaction was stopped and colour was developed by the addition of 2 ml of stop mixture. Absorbance was recorded at 540 nm. Enzyme
activity was expressed as nanomoles of hydroxamate formed per mg protein per min.

(b) **Glutamate synthase (GOGAT)**

GOGAT was assayed by monitoring oxidation of NADPH. Contents of the assay mixture were:

- Cell extract: 0.05 ml
- Tris buffer (0.5 M, pH 7.8): 0.1 ml
- α-Ketoglutarate (0.1 M): 0.05 ml
- DGDW: 0.5 ml

The contents were mixed in a tube and absorbance was read at 340 nm. 0.05 ml of NADPH (5 mM) and 0.1 ml glutamine (0.1 M) were added to the above solution and absorbance recorded. Controls were also incubated in all cases. Values of GOGAT activity were expressed as nanomoles of NADPH oxidised per mg protein per min.

(c) **Glutamate dehydrogenase (GDH)**

**Reagents**

- Tris HCl buffer (pH 7.5): 0.1 M
- Ammonium chloride: 3 M
- 2-Oxoglutarate: 5 mM
- NADH: 10 mg/ml

Reaction mixture contains 1 ml of tris HCl buffer, 0.3 ml 2-oxoglutarate, 0.5 ml of NH₄Cl, 0.12 ml NADH. 0.2 ml of extract was added to it. Then made up to 3 ml with buffer.

Incubated for 15 min. Recorded the change in absorbance at 340 nm.
Protein estimation (Lowry's method)\footnote{Protein estimation (Lowry's method)\footnote{Protein estimation (Lowry's method)}}

**Reagents**

A - 2% sodium carbonate in 0.1 N NaOH.

B - 0.5% copper sulphate in 1% potassium sodium tartrate.

C - Alkaline copper solution. Mixed 50 ml of A and 1 ml of B.

D - Folin-Ciocalteau reagent. Diluted the reagent with DW in 1:1 ratio.

**Standard:** Bovine Serum Albumin (1 mg/ml) was taken as standard.

**Procedure:** Pipetted 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes. Pipetted 0.1 ml and 0.2 ml of the extract in two other tubes. Made up the volume to 1 ml with 0.1 N NaOH. Added 5 ml of reagent C mixed well and allowed to stand for 10 min. Then added 0.5 ml reagent D, mixed well and incubated at room temperature in the dark for 30 min. Absorbance was read at 660 nm.

3.3. Metal absorption study

3.3.1 Sample preparation

**Azotobacter sp.**

250 ml of Jensen's broth culture of *Azotobacter sp.* at its log phase was treated with metal chloride solutions so that the final concentration in the medium was 0.5 ppm and 1 ppm. After 24 hours of treatment, the bacteria was separated by centrifugation at 1500 rpm for 20 min at 4°C. The bacterial precipitate was sonicated by applying 5-10 cycles of 20 sec burst of energy at an intensity of 100-110 mv. Cell debris were removed by centrifugation at 6000 rpm. The supernatant solution was used for further analysis.
Lyngbya sp. and Nostoc sp.

20 ml of the algal culture (approximately 200 mg) at the log phase were treated with sterilised metal chloride solutions so that the final metal concentrations in the medium were 1 ppm and 20 ppm. After a period of 15 days, the algal mass was separated from the medium by centrifugation at 1500 rpm for 15 min, washed twice with distilled water. The algal mass was washed twice with Tris HCl buffer (0.1 M), pH 8.0 containing 20% phenanthroline (0.1 M) and was separated by centrifugation at 1500 rpm.

Algal mass in 10 ml of Tris buffer was sonicated 5-10 cycles of 20's burst of energy at 110 mv at 4°C. Centrifuged at 6000 rpm. Supernatant was used for metal absorption studies.

Resulting supernatant was applied to a column packed with Sephadex G-50 gel and eluted with Tris buffer (0.1 M) pH 8.0. In each case, 50 fractions were collected at a rate of 4 drops per minute. The absorbance of each fraction was read at 254 nm and 280 nm. The samples and the fractions were analysed for metal ion concentrations and for total thiols.

3.3.2 Analysis of metal concentrations by atomic absorption spectrophotometer (AAS) (Perkin Elmer 3110 AAS) Mercury analyser (ECIL)

Pretreatment

The samples and the fractions were subjected to nitric acid digestion for oxidisation of organic matter.

2 ml of each sample was treated with 2 ml conc. HNO₃ in a beaker and allowed to evaporate to the lowest volume. Necessary conc. HNO₃ was added until digestion was complete as shown by light-coloured, clear solution. Made up the solution to a maximum of 5 ml with conc. HNO₃. These samples were analysed for metal concentrations by AAS.
Standards

Nickel: 1000 mg/l

Dissolve 1 g of nickel metal in a minimum volume of (1:1) HNO₃. Dilute to 1 litre with 1% (v/v) HNO₃.

Copper: 1000 mg/l

Dissolve 1 g of copper metal in a minimum volume (1:1) HNO₃. Dilute to 1 litre with 1% (v/v) HNO₃.

Cadmium: 1000 mg/l

Dissolve 1 g of cadmium metal in a minimum volume (1:1) HCl. Dilute to 1 litre with 1% (v/v) HCl.

Mercury: 1000 mg/l

Dissolve 1.080 g mercury (II) oxide, in a minimum volume of (1:1) HCl. Dilute to 1 litre with deionised water.

Ni, Cu and Cd contents of the corresponding samples were estimated using AAS by directly aspiring the sample into air-acetylene flame. Metal concentration was determined in ppm levels.

Estimation of mercury using mercury analyser ECIL (by cold vapour technique)

Reagents

A - Reducing agent (Stannous chloride). Dissolved 20 g of SnCl₂ in 10 ml conc. HCl boiled and made upto 100 ml. B - 10% nitric acid.

Procedure

Added 5 ml B, 2 ml A and stirred for 5 minutes. Then added 1 ml of the sample and 4 ml distilled water. The metal solution was first reduced by the reducing agent and the hydride was then directed to the AAS for estimation of mercury.
3.3.3 *Estimation of thiols*

**Reagents**

0.5 M Tris HCl pH 6.5

10 mM DTNB (79.4 mg DTNB and 140.3 mg EDTA). Adjusted the pH to 6.5 with Tris buffer

Standard: 1 mM glutathione

**Procedure**

Centrifuged metal treated culture, washed the pellet with buffer. Sonicated the pellet in tris buffer centrifuged. Added 2 ml extract, 0.1 ml DTNB and 2 ml Tris bufer and read the absorbance at 412 nm. Thiol concentration was expressed as mM per ml of extract.

Total thiols in eluent fractions was also estimated by the same procedure. Instead of extract, 2 ml of eluent was added for estimation. Thiol concentration was expresed as mM per ml eluent.

**3.4. Statistical analysis**

The statistical analysis was done based on Microstat (Ecosoft Inc., USA, 1984) computer program. The values given are the average of six values in each case ± SD. Control (1) was compared with treatments 0.5 ppm (2), 1 ppm (3), 5 ppm (4), 10 ppm (5) and 25 ppm (6) for all the statistical analyses.

F ratio and significance at 0.05 level are given in the statistical table. Comparison between means of control and treatment are expressed by least significance difference (LSD).99

\[
LSD_a = (t_a) \text{ SEd}
\]

SEd is the standard error of the mean difference and \( t_a \) is the tabulated t value at 0.05 level of probability. Computed the mean difference between the means and LSD and expressed the significance at 0.05 level.