Chapter 2
2. ISOLATION, SCREENING, AND SELECTION OF AMMONIA RELEASING CYANOBACTERIAL STRAINS

Introduction

Biological nitrogen fixation, limited in nature to prokaryotic organisms such as bacteria and cyanobacteria, helps to maintain soil fertility in tropical countries. It is a natural process that converts the most abundant element—nitrogen in the atmosphere, into a form that can be utilised by plants.

Cyanobacteria constitute a diverse group of photosynthetic prokaryotes capable of inhabiting a great variety of natural habitats. They are often abundant in fresh water, sea water, water logged paddy fields and terrestrial environments of tropical countries. Cyanobacteria form symbiotic associations with all types of plant groups namely algae, fungi, bryophytes, pteridophytes, gymnosperms and angiosperms (Fogg et al., 1973). They exhibit a wide morphological diversity of the thallus which ranges from unicellular organisms to heterotrichous branched forms. Due to the ability to undergo cellular differentiation in some of the forms, formation of heterocysts and akinetes are observed. Heterocysts are specialised cells and confer the ability to fix dinitrogen, thus making them photoautotrophic diazotrophs—a property that has fascinated many investigators. Their ability to
combine the two most vital life processes on our planet - oxygenic photosynthesis as well as the oxygen sensitive nitrogen fixation has earned them a unique status.

The role of cyanobacteria in maintaining the soil fertility in the tropics was proposed as early as 1939 by De in our country. India has been the pioneer in the field of biofertilizers and has generated valuable information on practical utilisation of this important input in crop production. Nitrogen fixing biological agents in soil and water have been hailed as natural 'fertilizer factories' and promoting their growth and nitrogen fixing activity is an important strategy for sustaining crop production. Extensive algalization of the fields was advocated under sustainable agriculture with a view to supplement the costly chemical fertilizers that were beyond the reach of marginal farmers. The goal of agricultural scientists was to substitute synthetic fertilizers with natural biofertilizers. One approach towards reaching this goal was to identify, isolate and develop cyanobacterial strains which are not only efficient nitrogen fixers but are also capable of releasing much of the fixed nitrogen as ammonia, for the benefit of crop plants. Hence, the search for ammonia releasing strains of cyanobacteria which can be utilised as natural nitrogen photobiofertilizer for crops.

As early as 1974, Fogg observed that as much as 60% of the total nitrogen fixed may be released into the medium. Puri and Grover (1981) reported the release of 46.3% of the fixed nitrogen by Anabaena G 102 into the
extracellular medium. A positive correlation between total and extracellular nitrogen indicated that as soon as the nitrogen was fixed a part of it was liberated into the medium. Bergman (1984) observed photorespiratory ammonium release by cyanobacterium *Anabaena cylindrica* in the presence of methionine sulfoximine. Boussiba *et al.* (1984) reported that the ammonia released by *Anacystis nidulans* R-2 was due to protein degradation when glutamine synthetase was inhibited with methionine sulfoximine.

Uninduced ammonia release both in the presence and absence of combined nitrogen source in the medium was observed in several strains of *Anabaena* by Subramanian and Shanmugasundaram (1986). Anand and Parameswaran (1992) screened 12 strains of heterocystous forms collected from the paddy fields of Kerala and Tamilnadu and found that all of them released ammonia, some starting from the fifth day in culture and the rest from the tenth day. In *Trichodesmium* sp., fixed nitrogen was released into the medium when the organism experienced nitrogen sufficiency (Siddique *et al.*, 1992). Release of fixed nitrogen has been reported from non-heterocystous bloom forming colonies of *Trichodesmium thiebautii* (Capone *et al.*, 1994). Nitrogenase activity occurs only during the day and more than one half of the fixed nitrogen is released as glutamine or glutamate. Rates of release of dissolved organic nitrogen by marine diazotrophic *Trichodesmium* sp. showed diel variation (Glibert and Bronk, 1994). Highest rates of release occurred from mid to late afternoon. Anand and Gayathri
(1999) reported ammonia release by non-heterocystous, filamentous cyanobacterium *Lyngbya spiralis* in nitrogen free medium as well as nitrate amended BG₁₁ medium.

Ammonia is used as a nitrogen source by all cyanobacteria and it is taken up preferentially even if compounds such as nitrate, nitrite or dinitrogen are present (Ohmori, 1972, 1974). Ammonia is a small uncharged molecule which can be expected to move relatively freely through lipid bilayers. A possible mechanism for ammonia uptake might therefore, involve simple diffusion followed by trapping through protonation, given a membrane permeable to NH₃ but impermeable to NH₄⁺. Ammonia uptake involves a transport system that is energy dependent, eliminated in dark, anaerobic conditions (Boussiba *et al.*, 1984). Such systems function in the retention of internally generated ammonia as well as in their acquisition from the surrounding medium. Alternative nitrogen sources are all reduced to ammonia intracellularly before incorporation into aminoacids. Cyanobacterial cells maintain a large intracellular pool of ammonia. Even so, a continuous loss of ammonia from the cell must be occurring since high internal concentrations of the unprotonated ammonia would be toxic to the cell (Gibson, 1988).

Ammonia exists in a protonated and an unprotonated form. Equilibration between both forms is always spontaneous and extremely fast.
Ammonia can diffuse across the membrane whereas ammonium can do so only via a carrier mediated transport. The presence of ammonium transport system in nitrogen fixing cyanobacteria is of importance for the retention of ammonium produced during nitrogen fixation and for the utilization of ammonium from the external medium which relieves the highly energy demanding process of nitrogen fixation (Rai et al., 1984). Such an ammonium transport system is encountered in the symbiotic *Anabaena azollae* also, though ammonium provided in the medium does not affect nitrogenase activity in them. Ammonium transport systems have been detected in many prokaryotes (Rowell et al., 1977; Kerby et al., 1986; Rai et al., 1986). ATS is repressed when the bacteria grow with abundant ammonium as nitrogen source i.e., the carrier is repressed by its own substrate. Diffusion of ammonia into the cell is sufficient for nitrogen supply as long as the concentration gradient is large enough. This diffusion must also occur in the opposite direction when the gradient is reversed, i.e., during growth with nitrogen sources other than ammonium, which generally are catabolised via ammonia. Ammonia diffusion is six times faster than glutamine formation. This means that each ammonia molecule formed from nitrogen fixation passes the cycle of diffusion and recapture six times before being trapped by binding. This cycle leads to the net import of six protons which have to be ejected to maintain the proton motive force. Cyclic retention mechanism is therefore necessary to counteract nitrogen depletion.
Mutants deficient in ammonium transport constantly excrete ammonia into the medium and they exhibit a markedly reduced growth rate (Kleiner, 1994).

Ammonia release could also be artificially induced by the addition of a glutamate analogue methionine sulfoximine (MSX) which is an inhibitor of glutamine synthetase activity. Considerable amount of work has been done on continuous photoproduction of ammonia by different nitrogen fixing cyanobacteria in the presence of MSX (Stewart and Rowell, 1975; Musgrave et al., 1982; Ramos et al., 1984; Subramanian et al., 1985, 1994). Anabaena sp.strain 2B, a facultatively heterotrophic cyanobacterium liberated ammonia when treated with MSX under nitrogen fixing conditions and DCMU enhanced ammonia liberation (Newton and Cavins, 1985).

Ammonia releasing mutant strains (ED81 and ED92) of Anabaena variabilis were isolated by Hien et al. (1988). The mutants had normal levels of GS protein and GS transferase activity but much reduced GS biosynthetic activity. In mutants of Anabaena siamensis, the unassimilated ammonium is consequently released into the medium due to reduced GS activity (Thomas et al., 1990).

The ability of symbiotic cyanobacteria in fixing atmospheric N₂ and the transfer of fixed N₂ to the partner has been documented by Bergman and Rai (1989), Rai (1994), Nierzwicki – Bauer and Bushnell (1998) and Kannaiyan, (1990). High heterocyst frequency with increased nitrogenase activity has been
recorded in symbiotic cyanobacteria. Fixed N₂ is transferred to the eukaryotic partner in the form of N₂ derived ammonia (Rai, 1994). Generally cycad symbionts are known to release aminoacids. However, in the present study *Anabaena cycadeae* was found to release varying quantities of amino acids infrequently.

In cyanobionts, repression of GS levels leads to N₂ derived ammonia being liberated by the cyanobiont (Rai *et al.*, 1996). In symbiosis, the two processes of GS enhancement and nitrogenase derepression are uncoupled whereby nitrogenase derepression is not accompanied by increase in GS levels in heterocysts.

In the present study, cyanobacterial strains originally isolated from the paddyfields were screened for their ability to release fixed nitrogen into the extracellular medium. Isolates capable of good growth and consistent release of ammonia were selected for further studies. *Anabaena cycadeae* isolated from the coralloid roots of *Cycas circinalis* was also investigated as symbiotic cyanobacteria are known to release much of the fixed nitrogen.
Materials and Methods

The following cyanobacterial isolates were obtained from the Culture Collection of Algae, Centre for Advanced Studies in Botany, University of Madras: *Lyngbya spiralis*, *Oscillatoria foreaui*, *Phormidium ambiguum*, *Anabaena oscillarioides*, *Anabaena variabilis*, *Calothrix elenkinii*, *Cylindrospermum stagnale*, *Nostoc muscorum*, *Scytonema millei* and *Westiellopsis prolifica* (details are in Table 1). Another isolate, *Anabaena cycadeae* Reinke was isolated from the coralloid roots of *Cycas circinalis*. The coralloid roots of *Cycas circinalis* were collected from Women's Christian College, Chennai. The roots were thoroughly washed in running tap water and then rinsed in distilled water. The root bits were surface sterilised in 1% mercuric chloride for 30 minutes. They were again washed several times in distilled water and sterilised water. The roots were then crushed with a clean pestle and mortar and filtered through muslin cloth to remove root tissue. The green suspension contained the cyanobacterial symbiont. It was centrifuged at 2856 xg for 10 minutes in a Beckman centrifuge with J2-21 rotor. The pellet was washed with sterile water twice and finally suspended in BG₁₁ medium. The cyanobacterial symbiont was made axenic by lysozyme treatment (Kim *et al.*, 1999). It was plated on BG₁₁ agar medium and incubated under culture conditions with continuous light. Each colony was transferred to BG₁₁ liquid medium in test tubes. Axenicity of the culture was tested by plating one mL of culture on nutrient agar plates. Microscopic observation of the culture revealed
filaments of *Anabaena*. Identification was done with Starmach (1959). Geitler (1932) and Desikachary (1959).

All the isolates were maintained in BG\textsubscript{11} medium under a light intensity of 40 µE m\textsuperscript{2} s\textsuperscript{-1} and 25±1° C. The culture room was provided to give alternatingly 12 hours light and 12 hours dark cycle with Sangamo Weston S650 313F automatic model timer. Batch cultures of the isolates were maintained in both BG\textsubscript{11} –N and BG\textsubscript{11} + N medium in 250 mL conical flasks.

**Table 1 : List of Cyanobacterial isolates**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of the isolate</th>
<th>Acc.No.</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Lyngbya spiralis</em> Geitler</td>
<td>A6</td>
<td>UBL, Madras</td>
</tr>
<tr>
<td>2.</td>
<td><em>Oscillatoria foreaui</em> Fremy</td>
<td>A1340</td>
<td>Paddy field isolate</td>
</tr>
<tr>
<td>4.</td>
<td><em>Anabaena oscillarioides</em> Bory</td>
<td>A801</td>
<td>Czech.Hind 64/20</td>
</tr>
<tr>
<td>5.</td>
<td><em>Anabaena variabilis</em> Kütz.</td>
<td>A514</td>
<td>CCAP1403/12</td>
</tr>
<tr>
<td>8.</td>
<td><em>Nostoc muscorum</em> Agardh</td>
<td>A509</td>
<td>CCAP, Cambridge,UK</td>
</tr>
<tr>
<td>9.</td>
<td><em>Scytonema millei</em> Born.</td>
<td>A 294</td>
<td>Lucknow, Dr.S.N.Srivastava</td>
</tr>
<tr>
<td>10.</td>
<td><em>Westiellopsis prolifica</em> Janet</td>
<td>A865</td>
<td>UBL, Madras</td>
</tr>
</tbody>
</table>
Composition of the BG₁₁ medium (Rippka et al., 1979)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>1.5</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.04</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.075</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>0.036</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.006</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.006</td>
</tr>
<tr>
<td>EDTA (Na₂)</td>
<td>0.001</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>0.020</td>
</tr>
<tr>
<td>*Trace metal solution</td>
<td>1 mL</td>
</tr>
<tr>
<td>Glass distilled water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

*Composition of trace metal solution A5+ Co (after Rippka et al., 1979)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₃BO₃</td>
<td>2.86</td>
</tr>
<tr>
<td>MnCl₂.4H₂O</td>
<td>1.18</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>0.222</td>
</tr>
<tr>
<td>NaMoO₄.2H₂O</td>
<td>0.390</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>0.079</td>
</tr>
<tr>
<td>Co(NO₃)₂.6H₂O</td>
<td>0.0474</td>
</tr>
<tr>
<td>Glass distilled water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Before autoclaving the medium, pH was adjusted to 7.5. Sodium nitrate was omitted to prepare combined nitrogen free BG₁₁ medium.
Sterilization

Medium was sterilized in a ‘Natsteel’ autoclave at 121°C and 15 lbs pressure for 20 minutes.

Estimation of growth

Growth was estimated by determining the chlorophyll $a$ (MacKinney, 1941) content every fifth day upto 30 days. Aliquots of 5mL of uniform suspension of the culture were taken for the estimation of chlorophyll $a$. After centrifugation at 4112 xg for 10 minutes in a J2-21 Beckman centrifuge, the pellet was suspended in 5 mL of 80% acetone and allowed to stand overnight at 4°C and incubated in dark by covering the tubes in aluminium foil. After 24 hours, the suspension was again centrifuged and the optical density of the supernatant was read at 663nm in a Beckman DU-40 spectrophotometer. Chl $a$ content was calculated with the formula given below.

$$ \text{Chl } a \ (\mu g/mL) = \text{OD} \times 12.63 \times \frac{\text{volume of acetone extract}}{\text{volume of culture}}.$$  

Estimation of Ammonia

Ammonia in the culture filtrate was estimated by the Phenol-hypochlorite method (Solorzano, 1969). The culture filtrate obtained after pelleting the cells for chlorophyll $a$ estimation was collected into clean, broad test
tubes. To 5 mL of the culture filtrate, 0.2 mL of Phenol-alcohol reagent and 0.2 mL of sodium nitroprusside were added. Finally, 0.5 mL of the freshly prepared oxidising solution was added and the contents were mixed well. The test tubes were incubated in dark for one hour for complete colour development. Blank was prepared with BG_{11}-N and/or BG_{11}+N medium. Optical Density was measured at 640nm in a Beckman DU-40 spectrophotometer. Analytical grade ammonium chloride in the range of 10-100\mu M was used as standard.

**Reagents**

- **Phenol-Alcohol Solution**: 10 g of Phenol (reagent grade) was dissolved in 100mL of 95% ethanol.

- **Sodium nitroprusside**: 1 g of sodium nitroprusside was dissolved in 200 mL of glass distilled water and stored in a dark bottle.

- **Alkaline solution**: 100g of trisodium citrate and 5g of sodium hydroxide were dissolved in 500mL of glass distilled water.

- **Sodium hypochlorite solution**: (commercial grade)

- **Oxidising solution**: 100mL of alkaline solution and 25 mL of sodium hypochlorite were mixed. This was always prepared fresh.
Results

Growth of all the isolates was studied in both BG$_{11}$-N medium and BG$_{11}$ +N medium and ammonia release during the growth was closely monitored.

Lyngbya spiralis recorded a decrease in Chl a content in BG$_{11}$ -N medium with a consequent bleaching whereas in nitrate amended BG$_{11}$ medium, 3.334 µg Chl a /mL was recorded on the 20$^{th}$ day when it reached the exponential phase. During the active growth phase in nitrate amended medium 14.5 µM/mL ammonia was released and in nitrogen free medium, ammonia was released during the first week and no further release was recorded (Figs. 1a & 1b).

Oscillatoria foreaui showed better growth in nitrate amended BG$_{11}$ medium and had 9.397 µg/mL Chl a on the 20$^{th}$ day. Growth was slow in BG$_{11}$-N medium which gradually increased after 15 days. Ammonia release was recorded throughout the growth phase in nitrate amended medium with maximum amount (78.189 and 83.997 µM/mL) coinciding with active growth between 10 – 20 days (Figs. 2a & 2b).

Phormidium ambiguum had the maximum Chl a value of 4.673 µg/mL on the 15$^{th}$ day in BG$_{11}$ -N medium. It was preceded by ammonia release on the 5$^{th}$ and 10$^{th}$ days (Fig. 3a). In BG$_{11}$ +N medium the maximum Chl a (10.483 µg/mL) was recorded on the 25$^{th}$ day and 1-15µM/mL of ammonia was released
Fig 1a Growth and ammonia release by *Lyngbya spiralis* in BG$_{11}$ -N medium

Fig 1b Growth and ammonia release by *Lyngbya spiralis* in BG$_{11}$ +N medium
Fig 2a Growth and ammonia release by *Oscillatoria foreaui* in BG$_{11}$ -N medium

Fig 2b Growth and ammonia release by *Oscillatoria foreaui* in BG$_{11}$ +N medium
throughout the growth period. Ammonia release varied up to 15 days, then there was little release and maximum release was on the 30\textsuperscript{th} day (Fig. 3b).

All the three non-heterocystous cyanobacteria released comparatively higher quantities of ammonia when grown in BG\textsubscript{11} +N medium. Of the three, \textit{Oscillatoria foreaui} released consistently higher quantities of ammonia.

The heterocystous filament, \textit{Anabaena oscillarioides} did not release ammonia into the external culture medium when grown in BG\textsubscript{11}-N medium. However, very small quantities of less than 1 \textmu M/mL of ammonia was released when it was grown in nitrate amended BG\textsubscript{11} medium. Also, higher content of Chl \textit{a} (9\textmu g/mL) was present in BG\textsubscript{11} +N medium (Figs. 4a & 4b).

\textit{Anabaena variabilis} showed good growth in both BG\textsubscript{11} -N medium and BG\textsubscript{11}+N medium and had highest chl \textit{a} content on the 25\textsuperscript{th} day in culture (9.738 \textmu g/mL and 10.9 \textmu g/mL respectively). Ammonia release was not only higher in BG\textsubscript{11}-N medium, it was liberated throughout the growth phase except the pre-exponential phase. Maximum release of 12.269 \textmu M/mL was recorded on the 10\textsuperscript{th} day (Figs. 5a & 5b).

\textit{Calothrix elenkinii} had maximum growth in BG\textsubscript{11}-N medium with 8.41 \textmu g Chl \textit{a}/mL on the 20\textsuperscript{th} day whereas the highest Chl \textit{a} content of 7.85 \textmu g/mL was attained a little earlier in BG\textsubscript{11}+N medium. In nitrate amended medium, \textit{C.}
Fig 3a. Growth and ammonia release by *Phormidium ambiguum* in BG$_{11}$ -N medium.

Fig 3b. Growth and ammonia release by *Phormidium ambiguum* in BG$_{11}$ +N medium.
Fig 4a Growth and ammonia release by *Anabaena oscillarioides* in BG₁₁⁻N medium

Fig 4b Growth and ammonia release by *Anabaena oscillarioides* in BG₁₁⁺N medium
Fig 5a  Growth and ammonia release by *Anabaena variabilis* in BG$_{11}$-N medium

Fig 5b  Growth and ammonia release by *Anabaena variabilis* in BG$_{11}$+N medium
*elkenii*. releases ammonia throughout the growth phase and maximum release (18.73 μM/mL) was recorded soon after the active growth phase, on the 20th day (Fig. 6a & 6b)

*Cylindrospermum stagnale* showed a gradual increase in Chl *a* content reaching a maximum of 7.073 μg/mL on the 30th day in nitrate amended medium. The growth in BG11–N was more stable with a short exponential phase. However, the maximum Chl *a* attained was 1.452 μg/mL. Ammonia liberation into the external medium was observed throughout the growth phase and a maximum of 9.9 μM/mL and 10.3 μM/mL were recorded on the 25th and 20th day in BG11–N and BG11+N medium respectively (Figs. 7a & 7b).

*Nostoc muscorum* exhibited a gradual increase in Chl *a* content upto 20 days in BG11–N medium and maximum Chl *a* content was recorded on the 25th day (5.027 μg/mL). A similar trend in growth was observed in BG11+N medium also with maximum Chl *a* content at 8.487 μg/mL on the 30th day. Ammonia liberated into the external medium was in the range of 1-3 μM/mL in nitrogen free BG11 medium and slightly higher (1-5 μM/mL) in nitrate amended BG11 medium (Figs. 8a & 8b).

In *Scytonema millei*, maximum Chl *a* (0.935 μg/mL) was recorded on the 25th day in BG11–N medium and 1.213 μg Chl *a* /mL on the 20th day in BG11+N medium. In BG11–N medium, 9.8 μM/mL of ammonia was released at the
Fig 6a  Growth and ammonia release by *Calothrix elenkinii* in BG$_{11}$-N medium

Fig 6b  Growth and ammonia release by *Calothrix elenkinii* in BG$_{11}$+N medium
Fig 7a  Growth and ammonia release by *Cylindropermum stagnale* in BG$_{11}$-N medium

Fig 7b  Growth and ammonia release by *Cylindropermum stagnale* in BG$_{11}$+N medium
Fig 8a  Growth and ammonia release by *Nostoc muscorum* in BG$_{11}$-N medium

Fig 8b  Growth and ammonia release by *Nostoc muscorum* in BG$_{11}$+N medium
beginning of logarithmic growth. In BG\textsubscript{11} –N medium, maximum release was of ammonia was observed on the 10\textsuperscript{th} day, while in BG\textsubscript{11} +N medium, maximum release of 3.8 \(\mu M/mL\) was observed on the 25\textsuperscript{th} day. Ammonia release in BG\textsubscript{11} –N medium was more than twice that in BG\textsubscript{11} –N medium (Figs. 9a & 9b).

*Westiellopsis prolifica* did not release ammonia into the culture medium when grown in nitrogen free medium. It exhibited a gradual increase in Chl a for the first two weeks in culture and a quick growth thereafter to reach 5.658 \(\mu g\) Chl a/mL on the 30\textsuperscript{th} day. In nitrate supplemented medium, faster growth rate was recorded with a maximum of 6.568 \(\mu g\) Chl a/mL on the 30\textsuperscript{th} day. In BG\textsubscript{11} +N medium, 0.808 \(\mu M/mL\) of ammonia was released on the 5\textsuperscript{th} day and 5.46 \(\mu M/mL\) on the 25\textsuperscript{th} day (Figs. 10a & 10b).

*Anabaena cycadeae*, isolated from the corallloid roots of *Cycas*, grew well in BG\textsubscript{11} –N medium as well as BG\textsubscript{11} +N medium. Exponential phase was reached on the 15\textsuperscript{th} day with 2.134 \(\mu g\) Chl a/mL in nitrate amended medium whereas it was delayed in BG\textsubscript{11}–N medium by 5 days when the Chl a value was 1.061 \(\mu g/mL\). Ammonia release was observed in both –N and +N medium throughout the growth period. Higher quantities (40.768 and 35.076 \(\mu M/mL\)) were recorded on the 20\textsuperscript{th} and 25\textsuperscript{th} days in BG\textsubscript{11} +N medium, though the highest release was on the 30\textsuperscript{th} day (Figs.11a & 11b).
Fig 9a  Growth and ammonia release by *Scytonema millei* in BG$_{11}$-N medium

Fig 9b  Growth and ammonia release by *Scytonema millei* in BG$_{11}$+N medium
Fig 10a  Growth and ammonia release by *Westiellopsis prolifica* in BG$_{11}$-N medium

Fig 10b  Growth and ammonia release by *Westiellopsis prolifica* in BG$_{11}$+N medium
**Fig 11a** Growth and ammonia release by *Anabaena cycadeae* in BG11-N medium

**Fig 11b** Growth and ammonia release by *Anabaena cycadeae* in BG11+N medium
Discussion

It has generally been observed that fixed nitrogen is released by cyanobacteria only after death and decay of their biomass (Fogg et al., 1973). Several cyanobacteria are known to release fixed nitrogen either as ammonia or as organic nitrogen into the extracellular medium during their growth phase. Ammonia release induced artificially by adding glutamine synthetase inhibitors such as MSX could be sustained for short periods only and the much expected continuous photoproduction of ammonia in bioreactors is the current necessity. Biotechnological exploitation was aimed at genetic engineering of some of the strains to derepress nitrogenase to escape the adverse effect of fertilizers in the field and to develop mutants with minimal glutamine synthetase activity (Spiller et al., 1986; Lattore et al., 1986). These attempts are besieged with hurdles such as poor growth of the modified organism or reversion of mutants in the field conditions (Vaishampayan et al., 1998).

Hence, the present attempt is to identify and optimize conditions for naturally occurring strains of cyanobacteria that release ammonia during their growth phase either continuously or discontinuously into the surrounding medium and which can be utilised by crop plants. In the present study, of the ten isolates screened for ammonia release, seven were heterocystous filaments and three were non-heterocystous filaments. While all the three non-heterocystous filaments
release ammonia into the external medium. only five of the heterocystous filaments exhibited ammonia release in BG_{11}-N medium. *Anabaena oscillarioides* and *Westiellopsis prolifica* did not release ammonia when grown in BG_{11}-N medium. This may be due to the efficient coordination of nitrogenase activity and ammonia assimilating mechanism in these forms. Also, fixed nitrogen released into the medium, may be subsequently reassimilated as reported in *Gloeothecce* by Flynn and Gallon (1990). Intermittent release was recorded in three of the forms. *Anabaena variabilis*, *Nostoc muscorum*, and *Scytonema millei* whereas *Cylindrospermum stagnale* and *Calothrix elenkinii* released ammonia throughout the growth phase. Subramanian and Shanmugasundaram (1986) observed three patterns of ammonia release in different strains of *Anabaena* during the growth period – a release pattern parallel to the growth curve, a continuous increase in release and a release showing a bimodal curve. Intermittent as well as continuous ammonia release by heterocystous filaments in culture was reported earlier by Anand and Parameswaran (1992).

Except *Anabaena variabilis* and *Scytonema millei*, all the other forms released more ammonia when grown in BG_{11}+N medium. This may be explained by the fact that ammonia release can be a consequence of nitrogen sufficiency in the cells as reported by Siddique *et al.* (1992) in *Trichodesmium* sp. Non-heterocystous filaments released higher quantities of ammonia into the culture.
medium. Of the heterocystous forms, *Calothrix elenkinii* and *Cylindrospermum stagnale* gave consistent release and comparatively higher quantities.

The isolates selected for detailed studies were *Calothrix elenkinii* - a heterocystous filament and *Oscillatoria foreaui* - a non-heterocystous filament. Though continuous release of ammonia by *Anabaena cycadeae* was recorded in both BG11-N medium and BG11 +N medium, growth and biomass accumulation was just about 25% when compared to free-living isolates. Growth of symbiotic cyanobacteria was significantly diminished in culture. Whereas the cyanobionts in Lichen, hornworts and *Azolla* liberate nitrogen derived ammonia to the partner, the form of fixed nitrogen transferred to the partner in algal, cycad and *Gunnera* symbiosis is not known (Rai *et al.*, 1996). *Anabaena cycadeae* was very sensitive to changes in pH and pesticide amendments, resulting in overnight bleaching of the culture. Bharati and Angadi (1981) studying the effect of fungicides on nitrogen fixing BGA reported that the growth of *A. cycadeae* isolated from the coralloid roots of *Cycas revoluta* was inhibited in all concentrations of Captan and Agallol 3’. Therefore, this isolate could not be used for experiments described in Chapter 4. Nevertheless, it was included for experiments on regulation of nitrogenase, ammonia assimilation and glutamine synthetase activity described in Chapter 5.
Ammonia release has been studied for more than three decades now. The interest in this area of research is sustained by the potential application of cyanobacteria as low cost biofertilizer for a variety of crops. Photoproduction of ammonia can be successfully exploited if a suitable cost effective technology is developed.