CHAPTER-6

LARGE SCALE CULTIVATION OF CYANOBACTERIA AND FIELD TRIALS

6.1 INTRODUCTION

Cyanobacteria (Blue-Green Algae) are one of the major components of the nitrogen fixing biomass in paddy fields. The ability of cyanobacteria to fix atmospheric dinitrogen is implicated in maintaining the improved high-yielding rice varieties, which are highly responsive to fertilizers, and has become an integral part of cultivation practices.

After water, nitrogen is the second limiting factor for plant growth in many fields and deficiency of this element is met by fertilizers (Malik et al. 2001). The excessive use of chemical fertilizers has generated several environmental problems. These problems can be tackled by use of biofertilizers (Choudhury and Kennedy 2005, Rai 2006). The recent past studies on cyanobacteria have emphasized their important role in ecosystems. They grow at any place and in any environment where moisture and sunlight are available. However, specific algae grow in specific environment and therefore their distributional pattern, ecology, periodicity, qualitative and quantitative occurrences differ widely. The abundance and composition of blue green algal population in surface waters of ponds and lakes have been discussed by many workers (Fogg, 1975 and Seenayya, 1972). The interaction of heavy metals with microorganism has become an increasing global interest because of its potential as biotechnological method in removing heavy metals from polluted aqueous system. The possibility of separating metal saturated algae from its medium may provide an economic method for removing heavy metals.

Salination of agricultural lands is considered to be a grave problem that limits crop yields. In India alone, over 7 million hectares of arable land are inflicted by salinity (Kaushik, 1998). Sodium is the chief cation responsible for the deterioration of soil properties upon salination. Cyanobacteria have been recommended for reclamation of the saline and alkali soils (Singh, 1961, Kaushik and Ummat, 1992, Kaushik, 1995). Though pesticides were the miracle drugs for the plants during but their hazardous effects on the environment were a matter of concern. Despite improvement of crop plants through various breeding techniques, agriculture remains
heavily dependent on these agrochemicals (Gadkari, 1988). The paddy field soil can be a natural sink for these pesticides, especially when recalcitrant forms are used. Many of the herbicides used in paddy fields are known to inhibit photosynthesis. Inhibition in growth and cellular nitrogen level was reported in *Anabaena doliolum* (Kapoor and Sharma, 1980).

### 6.2 REVIEW OF LITERATURE

Commercial history of biofertilizers began with the launch of ‘Nitragin’ by Nobbe and Hiltnner, a laboratory culture of Rhizobia in 1895, followed by the discovery of Azotobacter and then the blue green algae and a host of other microorganisms. There are numerous reports on the beneficial use of cyanobacteria in increasing the growth and yield of rice (Ladha and Reddy 1995; Kannaian *et al*. 1997; Kennedy and Islam 2001). Cyanobacteria are very susceptible to sudden physical and chemical alterations of light, salinity, temperature and nutrient composition (Boomiathan, 2005; Semyalo, 2009). Cyanobacteria have a great deal of potential as a source of fine chemicals, as a bio-fertilizer and as a source of renewable fuel (Lem and Glck., 1985).

Blue greens have been shown to be highly effective as accumulators and degraders of different kinds of environmental pollutants, including metal ions (Bender *et al.*, 1994; Lujan *et al.*, 1994) salinity (Moisander *et al*. 2002) pesticides (Megharaj *et al.*, 1994).

Worldwide, cyanobacteria have been used efficiently as a low-cost method for remediating pollution by converting the dissolved nutrients into biomass (Lincoln *et al.*, 1996) and for biotreatment (removal) of dissolved inorganic nutrients (Duma *et al.*, 1998), to allow them to be used as economic and low-maintenance remediation technology for contaminated systems. The paddy field ecosystem provides a favorable environment for the growth of cyanobacteria with respect to their requirements for light, water, high temperature and nutrient availability. This could be the reason for more abundant cyanobacteria growth in paddy soils than in upland soils (Roger and Reynaud 1982, Kondo and Yasuda 2003) The incorporation of bio-fertilizers (N2fixers) plays major role in improving soil fertility, yield attributing characters and thereby final yield has been reported by many workers (Subashini *et al*. 2007a; Kachroo and Razdan, 2006; Son *et al*. 2007). In addition, their application in soil
improves soil biota and minimizes the sole use of chemical fertilizers (Subashini et al. 2007a). Blue green algae is, phototrophic in nature and produce Auxin, Indole acetic acid and Gibberllic acid, fix 20-30 kg N/ha in submerged rice fields as they are abundant in paddy, so also referred as ‘paddy organisms’. N is the key input required in large quantities for low land rice production. The 50-60% N requirement is met through the combination of mineralization of soil organic N and biofertilizer by free living and rice plant associated bacteria (Roger and Ladha, 1992). To achieve food security through sustainable agriculture, the requirement for fixed nitrogen must be increasingly met by biofertilizer rather than by industrial nitrogen fixation.

6.3 MATERIALS AND METHODS

6.3.1 Incubation and maintenance of culture

Cultures of Nostoc muscorum and Anabena variabilis in BG-11 medium were kept in an air-conditioned culture room, which was illuminated by three 40W fluorescent tubes at a distance of 50 cm for 12 hrs daily. The cultures received 2500-3000 Lux light intensity and a temperature of 27 ± 2°C.

6.3.2 Stress application

6.3.2.1 Metal stress

Acclimatized cells were obtained by successive cultivation (4–5 times) at concentrations of heavy metal, Pb,Cd,Co,Mg,Mn,Zn in their chloride form in 10µM and a set of separate cells under the same metal in combination of NaCl stress(100mM), as described by Attaway and Schmidt (2001).

6.3.2.2 Pesticide stress

Acclimatized cells were obtained by successive cultivation (4–5 times) at concentrations of cypermethrin (20µM) and dimethoate (20µM).

6.3.2.3 Herbicide stress

Acclimatized cells were obtained by successive cultivation (4–5 times) at concentrations butachlor (20µM) and isoproturon (20µM).

6.4 Metal uptake studies

The metal uptake studies were undertaken in an atomic absorption spectrophotometer Model 2380 Perkin Elmer and calculated using the simple concentration difference methods (Arunakumara, Zang nas Song, 2008).
6.4.1 Digestion of sample

Samples were harvested and then transferred to 50 ml centrifuged tubes. The samples were pellet at 10,000 rpm for 25 minutes. Each pellet was transferred to a micro centrifuge tube and was again centrifuged at 10,000 rpm in an Ependorof microfuge TM for 5 minutes to remove more water. The wet biomass was collected and weighed for metal uptake experiments glassware were washed with 5% nitric acid and auto claved. After the reaction period of metal inoculation, the collected supernatant was placed in a beaker on a hot plate and was evaporated to about 15-20 ml, it was made sure that the sample did not boil. The solution was cooled and 3 ml concentrated nitric acid was added. The sample was again heated to a gentle reflux and evaporated to near dryness, without baking. The addition of 3 ml concentrated nitric acid was repeated until the digest was light in colour or did not change in appearance with continued refluxing. The sample was again evaporated to near dryness and 2.5 ml of 1:1 nitric acid and warm distilled deionized water was added to dissolve any precipitate or residue resulting from evaporation. The beaker walls and the watch glass covered were washed down with distilled de ionized water and filtered through a Whatman # 42 filter paper to remove silicates and other insoluble materials. The final volume was adjusted to 15 ml and this was analyzed by flame atomic absorption spectroscopy.

6.5 GC Analysis for pesticide and herbicide

Pesticide and herbicide concentrations were determined by gas chromatography (GC) at the organic chemistry laboratory at the Indian Institute of toxicology research (IITR), Lucknow, Uttar Pradesh, India.

6.6 Field trial

6.6.1 Seed germination

Rice seeds were soaked with water as control and wet cyanobacteria in the containers separately. After 20 days, seedlings height and root length were observed.

6.6.2 Large scale cultivation

Large scale cultivation of the stress tolerant cyanobacterial strains was carried out in Bakshi ka Talab, Lucknow, Uttar Pradesh, India under collaboration with UPCST(Uttar Pradesh council of science and technology), Lucknow, Uttar Pradesh, India. Beds of size 5 x 1 m are prepared in a ploughed land bonded on all sides and water is let into the field to a height of 2.5 cm and maintained for 1-2 cm depth. 0.5
kg of Algal inoculum were sprinkled for one cent plot. After 30 days, without drainage of water, plot was dried and hence algal mat settles over the soil. Drying peel of like flakes was collected and distributed for rice field application. To assess the effectiveness of test algal biomass as biofertilizer, field trials at the selected locations in Uttar Pradesh (India) regions were carried out on rice.

6.7 RESULTS AND DISCUSSION

Fig 6.1: Metal accumulations by Nostoc muscorum in presence of metal and salinity stress

Table 6.1: Concentration of metal uptake (µg/ml) in presence of metal and salinity stress in Nostoc muscorum

<table>
<thead>
<tr>
<th>Metal concentration</th>
<th>Metal concentration(µg/ml) in metal stress</th>
<th>Metal concentration(µg/ml) in metal and NaCl stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg</td>
<td>1.972</td>
<td>0.804</td>
</tr>
<tr>
<td>Mn</td>
<td>1.901</td>
<td>0.782</td>
</tr>
<tr>
<td>Co</td>
<td>1.804</td>
<td>0.805</td>
</tr>
<tr>
<td>Cd</td>
<td>1.842</td>
<td>0.341</td>
</tr>
<tr>
<td>Zn</td>
<td>1.503</td>
<td>0.589</td>
</tr>
<tr>
<td>Pb</td>
<td>1.431</td>
<td>0.241</td>
</tr>
</tbody>
</table>
Fig: 6.2 Metal accumulations by *Anabena variabilis* in presence of metal and salinity stress

Table 6.2: Concentration of metal uptake (µg/ml) in presence of metal and salinity stress in *Anabena variabilis*

<table>
<thead>
<tr>
<th>Metal</th>
<th>Metal concentration(µg/ml) in metal stress</th>
<th>Metal concentration(µg/ml) in metal and NaCl stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg</td>
<td>2.034</td>
<td>0.724</td>
</tr>
<tr>
<td>Mn</td>
<td>2.179</td>
<td>0.827</td>
</tr>
<tr>
<td>Co</td>
<td>1.824</td>
<td>0.415</td>
</tr>
<tr>
<td>Cd</td>
<td>1.101</td>
<td>0.232</td>
</tr>
<tr>
<td>Zn</td>
<td>1.193</td>
<td>0.669</td>
</tr>
<tr>
<td>Pb</td>
<td>1.234</td>
<td>0.322</td>
</tr>
</tbody>
</table>

6.7.1 Metal absorption concentration under various stress

Cyanobacteria accumulate metal by both adsorption and uptake method (Bender *et al.* 1994). Many of the reported biosorption studies are conducted in cyanobacteria with single metal ion species, in aqueous solutions while industrial effluents invariably contain more than one metal ion (Modak and Natrajan, 1996.) result observed from this study reveals that cyanobacteria accumulate variety of metals, in detectable concentration from culture medium (Slotton, Goldman, and Frank (1989) when grown under various metal stress condition. The accumulated amount of metal showed significant differences. Accumulation of metal in presence of NaCl stress showed less uptake of metal by the cyanobacterial cells in comparison with only metal contamination present in the
culture medium, less bioconcentration factor (BCF) was observed in Fig: 6.1 and 6.2.

Highest concentration factor was observed in magnesium (1.972µg/ml) in *N. muscorum* (Table:6.1) and manganese (2.179µg/ml), and the lowest in lead (1.43µg/ml) in *N. muscorum* and cadmium (1.101µg/ml) in *A. variabilis* (Table:6.2) respectively under metal stress only, whereas metal and osmotic stress showed significant variation in the results, highest concentration shared by magnesium (0.804µg/ml) manganese (0.782µg/ml) and cobalt (0.805µg/ml) as shown by the approximate values and lowest by lead (0.232µg/ml as observed in *N. muscorum*. Accumulation of manganese (0.827µg/ml) and cadmium (0.232µg/ml) was highest and lowest in *A. variabilis* Cyanobacteria possess metal binding sites for metallopro-teins and phytochelatins (Lombardi and Vieira 2000). Metals were removed by adsorption and precipitation in extra-cellular and cellular compartments in algae and cyanobacteria.

6.7.2 Analysis of pesticide and herbicide stress present in *N. muscorum* and *A. variabilis* by Gas chromatography

![Graph](image)
Fig: 6.3 GC analysis representing the chromatogram for cypermethrin present in
(a) standard (b) *N.muscorum* (c) *A.variabilis*
The Chromatogram (Fig 6.3b) represents the result obtained during the GC Analysis of *N. muscorum* cultures containing cypermethrin as the stress. From the above result it was evident on comparing with reference standard (Fig 6.3a) that the retention time of cypermethrin in the column was 64.0 min, 71.46 min, 72.19 min, 72.7 min and 73.0 min. 5 peaks in the above chromatogram further indicate that cypermethrin was broken down into its isomers by the *Nostoc muscorum* cells. Also the percentage of cypermethrin detected in the sample was less than 9% as seen on the y-axis of the graph. *A. variabilis* (Fig 6.3c) showed dissociation of cypermethrin into different organic compound as shown by eight peaks in the chromatogram which is much similar to standard.
Fig: 6.4 GC analysis representing the chromatogram for dimethoate present in (a) standard (b) *N.muscorum*  (c) *A.variabilis*
In the above chromatogram it is evident that the retention time for dimethoate in the column was 13.5 min after which it was eluted out. Single peak was observed in *N. muscorum* (Fig: 6.4b) which is similar to the reference standard (Fig 6.4a) whereas *A. variabilis* (Fig: 6.4c) showed high degree of degradation as multiple peaks can be observed with their respective retention time.

![Chromatogram](image)

**Fig: 6.5. GC analysis representing the chromatogram for butachlor present in (a) standard (b) *N. muscorum* (c) *A. variabilis***
The chromatogram indicates that the Retention time for butachlor was 32.4 in *N.muscorum*, (Fig 6.5b) thus it is clear that butachlor was eluted out from the column after 32.4 min. duration. The y-axis of this chromatogram indicates the percentage from which it is evident that the percentage of butachlor at 32.4min was ±9.5%. A single peak in the chromatogram also indicates that no other isomers of butachlor were found in sample after comparing with the reference standard (Fig 6.5a). *A.variabilis* (Fig 6.5c ) showed great effect on the butachlor as degradation of herbicide can be observed by the various peaks shown in the chromatogram, these peaks are found absent in reference standard. Trouble shooting gas chromatogram was found due to high baseline probably due to electronic or mechanical failure, contamination in critical areas, such as detectors. Incorrect or inappropriate setpoints, leaks, column or septum bleed (Troubleshooting Gas Chromatograph Baseline Problems A16089).

* GC for isoproturon (herbicide) could not be done due to non availability of the column in the research center.

**Table: 6.3 Concentration present before and after the accumulation of pesticide and herbicide by cyanobacteria**

<table>
<thead>
<tr>
<th></th>
<th>Cypermethrin (20µM)</th>
<th>Dimethoate (20µM)</th>
<th>Butachlor (20µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amount added at the time of culturing</strong></td>
<td>300µg/ml</td>
<td>300µg/ml</td>
<td>100µg/ml</td>
</tr>
<tr>
<td><strong>Amount detected in the sample during GC (N.muscorum)</strong></td>
<td>0.05µg/ml</td>
<td>21.40µg/ml</td>
<td>0.130µg/ml</td>
</tr>
<tr>
<td><strong>Amount detected in the sample during GC (A.variabilis)</strong></td>
<td>0.11µg/ml</td>
<td>29.05µg/ml</td>
<td>0.250µg/ml</td>
</tr>
</tbody>
</table>

The above Table 6.3 indicate that there was a drastic reduction in the amount of pesticide/herbicide added to the *N.muscorum* and *A.variabilis* cultures. Thus, it can be concluded that the cyanobacterial cells present in the culture utilized the pesticide/herbicide present in the media and converted it to other compounds.

The pesticide most readily utilized was cypermethrin as its amount detected in the sample was the lowest. However, dimethoate was least utilized, among the 3
pesticide/herbicides analyzed, as its amount detected in the sample was the highest. It has been observed that cyanobacterial forms used in biofertilizers are capable of tolerating pesticides levels recommended for fields applications. Insecticides are generally less toxic to cyanobacteria than their pesticides (Manoharan C, Subramanian G., 1993; Vijayakumar S., 2005) Cyanobacteria have been reported to accumulate very high concentration of insecticides. *Synechococcus elongates, Anacystis nidulans* and *Microcystes aeruginosa* have been able to degrade many organophosphorus and organochlmine insecticides from the aquatic system. (Li J, Wang J, Zhang J.,1991; Manoharan C, Subramanian G.,1992)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period of time germination (days)</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Seedlings height (cm)</td>
<td>4.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Root length (cm)</td>
<td>0.8</td>
<td>3.0</td>
</tr>
</tbody>
</table>

### 6.8 Field trial

**Table: 6.4 Effect of *N.muscorum* on germination of rice seeds**

![Fig: 6.5 Rice seeds soaked with water (left), rice seeds soaked with water and *N.muscorum* (right)]
Table: 6.6 Yield data of field demonstration of *Nostoc muscorum* as biofertilizer conducted in paddy field

<table>
<thead>
<tr>
<th>Demonstration location in Uttar Pradesh</th>
<th>Demonstration Area (in square meter)</th>
<th>Average yield of paddy (Kg/50 Square meter)</th>
<th>Net increase in yield (kg/50 Square meter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With cyanobacteria</td>
<td>Without cyanobacteria</td>
</tr>
<tr>
<td>Kalyanpur, Bareily</td>
<td>50</td>
<td>25.10</td>
<td>24.800</td>
</tr>
<tr>
<td>BKT, Lucknow</td>
<td>50</td>
<td>25.30</td>
<td>25.050</td>
</tr>
<tr>
<td>Chinhat, Lucknow</td>
<td>50</td>
<td>25.57</td>
<td>25.150</td>
</tr>
<tr>
<td>Barabanki, Lucknow</td>
<td>50</td>
<td>25.45</td>
<td>25.075</td>
</tr>
</tbody>
</table>

Table: 6.7 Effect of *A. variabilis* on germination of rice plant

<table>
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<tbody>
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<td>Period of time germination (days)</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Seedlings height (cm)</td>
<td>3.0</td>
<td>5.5</td>
</tr>
</tbody>
</table>
Significant changes were observed in seeds soaked with water as control and the treatment seed species. *N. muscorum* treated seeds showed enhanced root length and the seedling height than the control as observed from the result (Table: 6.4, Fig: 6.6). the same pattern of observation was recorded in *Anabena variabilis* treated seeds (Table 6.6, Fig: 6.7). Large-scale cyanobacterial biofertilizer was produced with the strains showing high rates of growth in the selected location for field trials. Increased in plant yield as recorded from the field data (Table:6.5 and 6.7) is attributed to the production of growth substance and vitamins induced by cyanobacteria for better yield (Venkataraman and Neelakantan, 1967). (S.Bocchi and A. Malgioglio, 2010). Three out of five strains of cyanobacteria tested survived the winter, with an increase in biomass from March to May producing approximately 30–40 kg/hectare of nitrogen. One of these strains, named “Milan”, emerged as the most resistant to herbicide and the most productive. Of the herbicides tested, Propanil permitted the survival of growing Azolla. Results of the field trials showed that cyanobacterial biofertilizer improve the fertility status and may supplement 25–35% N for rice cultivation in these soils.

### 6.9 CONCLUSION

The data generated from these experiments have provided the wide use of cyanobacteria to improve the paddy crop resulting in improved quantitative and qualitative rice crop as it has the tendency to withstand under various stress conditions cyanobacteria can be mass cultured to degrade pollution load and to meet the requirement on nitrogenous fertilizers with minimal investment and one of the good alternative for synthetic fertilizer. It is easy to cultivate cyanobacteria on large scale.

<table>
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<th>Demonstration location in Uttar Pradesh</th>
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<td>Kalyanpur, Bareily</td>
<td>50</td>
<td>22.20</td>
<td>22.00</td>
</tr>
<tr>
<td>BKT, Lucknow</td>
<td>50</td>
<td>21.54</td>
<td>20.75</td>
</tr>
<tr>
<td>Chinhat, Lucknow</td>
<td>50</td>
<td>27.17</td>
<td>26.53</td>
</tr>
<tr>
<td>Barabanki, Lucknow</td>
<td>50</td>
<td>26.63</td>
<td>26.03</td>
</tr>
</tbody>
</table>
The need of the hour is only to identify ‘super’ strains, performing the desired functions, grow them on large scale and make available quality inocula on demand. Cyanobacteria possess high metal absorption capacity and very high multiplication rate. Such characters have encouraged the application of this microbial biomass in detoxification.

6.10 REFERENCES


