STUDIES ON PRESERVATION AND CONSERVATION OF CYANOBACTERIA

BY

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STATEMENT

I, **Amrita Bhattacharjee**, hereby declare that the subject matter of this thesis is the record of the work done by me, that the content of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

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This thesis is dedicated to my ma for always standing beside me and to the ever-inspiring loving memories of my baba.

LIST OF CONTENTS

2.4.2 Protein estimation

2.4.2.1 Extraction of protein

2.4.2.2 Estimation of protein

2.5 Oxygen exchange

2.6 Heterocyst and akinete frequency

2.7 Enzyme assays

2.7.1 Nitrogenase activity

2.7.2 Glutamine synthetase (transferase) activity

2.7.2.1 Extraction of enzyme

2.7.2.2 Assay of glutamine synthetase (transferase) activity

2.7.3 Nitrate reductase activity

2.7.3.1 Nitrite estimation

2.7.4 Nitrite reductase activity

2.8 Immobilization

2.8.1 In calcium alginate beads

2.8.2 In foam cubes

2.8.3 In agar cubes

2.9 Preservation using cryoprotectants

2.9.1 Preservation in 15% glycerol

2.9.2 Preservation in 5% DMSO

2.10 Testing for viability of the immobilized cyanobacteria

2.11 Culture condition for akinete differentiation

2.12 Culture condition for akinete germination

2.13 Germination of rice seeds

2.14 Co-cultivation of rice and cyanobacteria

2.14.1 Screening for symbiotic competence with rice plantlets

2.14.2 Associative nitrogen fixation study

2.15 DNA fingerprints

2.16 Metal sorption studies by cyanobacteria

2.17 Scanning electron microscopy

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ABBREVIATIONS

ADP	Adenosine 5'- diphosphate
AAS	Atomic absorpsion spectroscopy
ATP	Adenosine 5'- triphosphate
°C	Degree centigrade
C_2H_2	Acetylene
C_2H_4	Ethylene
Chl *a*	Chlorophyll *a*
d	Day(s)
DMSO	Di methyl sulfoxide
g	Gram
h	Hour(s)
HF	Heterocyst frequency
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid
kDa	Kilo Dalton
M_r	Molecular weight
l	Litre
min	Minute(s)
ml	Mililitre
mM	Mili molar
µM	Micro molar
µmol	Micro mole
N	Nitrogen
N_2	Dinitrogen
nmol	Nano mole
PCR	Polymerase chain reaction
PS	Photosystem
psi	Pounds per square inch
rpm	Revolutions per minute
Tris	2-amino-2-hydroxymethyl propane-1,3-diol
wt	Weight
v	Volume
%	Percentage
‰	Parts per million

LIST OF TABLES

LIST OF FIGURES

Fig: 7.4: Chlorophyll *a* concentration (μg ml^{-1}) of the regenerated cyanobacterial samples as compared to their free-living batch cultures.

Fig 7.5: Heterocyst frequency of the regenerated cyanobacterial samples as compared to their free-living batch cultures.

Fig 7.6: Nitrogenase activity of the regenerated cyanobacterial samples as compared to their free-living batch cultures.

Chapter 1

Introduction

1.1 Cyanobacteria

Cyanobacteria are an ancient group of photosynthetic, Gram negative, photoautotrophic prokaryotes, which are cosmopolitan in nature. They inhabit every conceivable habitat on earth ranging from terrestrial to aquatic, from frigid regions to tropical areas (Carr and Whitton 1982; Stal 1995; Bergman 1996; Capone et al 1997). Cyanobacteria are also known to exist in deserts where they remain dormant for most of the time (Hu et al 2003). They occur in soil, on rocks, in fresh as well as in salt water (Hoffmann 1989; Kulasooriya 1998; Nayak and Prasanna 2007). They exist as free-living and are also capable of symbiotic associations with a wide variety of organisms, ranging from protists, plants, animals and fungi (Rai 1990; Bergman et al 1996; Adams 2000; Whitton and Potts 2000; Rai et al 2000; 2002).

Cyanobacteria are remarkably capable of efficiently combining two seemingly mutually exclusive processes–oxygen-evolving oxygenic photosynthesis and oxygen-sensitive nitrogenase dependent nitrogen fixation. In aquatic environments, cyanobacteria usually form thick microbial mats, serving as crucial components in such ecosystems. They are known to increase oxygen concentration and also improve the other physio-chemical parameters of the environment in which they grow and flourish (Mandal et al 1998). One of their most significant contribution to all ecosystems in which they occur is to supply combined nitrogen owing to their ability to fix atmospheric nitrogen. Their role in the soil ecosystem and in paddy fields is manifold, they being the most important contributors of fixed nitrogen and carbon, besides promoting release of nutrients and reducing the rate of

loss of water and soil through erosion. In paddy fields, their relative occurrence varies within large limits, ranging from 0 to 76-85%. They secrete polysaccharides that bind soil, and thus, help in maintaining stability and also control erosion and runoff. In nature, cyanobacteria are generally abundant in areas where there is nitrogen deficiency.

Cyanobacteria's trophic independence from carbon and nitrogen, together with a tremendous adaptability to environmental variations, enables these organisms to be ubiquitous. Their successful existence in a wide range of environments including the most extreme ones such as high temperatures, high levels of UV light, and high salinity may be attributed to their structural-functional flexibility which provides them with great versatility (Nayak and Prassana 2007). However, environmental conditions prevailing in diverse terrestrial and aquatic ecosystems are known to affect cyanobacterial diversity and abundance. Soil pH in particular is known to have a selective effect on cyanobacteria and their succession and abundance in soil. (Kaushik 1991; 1994; Prasanna and Nayak 2007).

Cyanobacterial species include unicellular, filamentous, branched-filamentous and non-filamentous colonial forms (Rippka 1979). They form the only representative of true multicellular organisms, among prokaryotes (Adams 1992; Tandeau de Marsac and Houmard 1993). Many of the members are capable of multiple cellular differentiations and may exist as vegetative cells (sites for photosynthesis), heterocysts (sites for nitrogen-fixation), akinetes/spores (perennating bodies) and hormogonia (motile trichomes) (Tandeau de Houmard 1993; Adams and Duggan 1999).

1.2 Vegetative cells

Vegetative cells are the sites that house the entire photosynthetic machinery in cyanobacteria. The chlorophyll *a* protein complexes, photosynthetic reaction centres,

carotenoids and the electron transport system are all contained within the thylakoids present in the vegetative cells. Light harvesting pigments called the phycobilliproteins located in the phycobilisomes, which are attached to the surface of thylakoids serve as sites of stored N sources under conditions of N-deficiency (Tandeau de Marsac and Houmard 1993; Byrant 1994). The vegetative cells are also known to contain a number of inclusion bodies namely the cyanophycean starch (glycogen) as C reserve, carboxysomes containing Rubisco (ribulose 1, 5 bisphosphate carboxylase /oxygenese), cyanophycin (a polymer of aspertate and arginine) as N-reserve, and polyphosphate bodies as P-reserve. ATP is produced in the vegetative cells by oxidative phosphorylation as well as by photophosphorylation coupled to cyclic and non-cyclic photosynthetic electron transport chain. Carbon dioxide fixation occurs through the Calvin cycle (Carr and Whitton 1982).

In nitrogen fixing non-heterocystous cyanobacteria, the enzyme nitrogenase which plays the crucial role in nitrogen fixation is expressed in vegetative cells under unique conditions. The enzyme is extremely sensitive to oxygen (Fay 1992), and this is manifested both at the level of synthesis and activity (Gallon and Chaplin 1987; Gallon 1992; Rai et al 1992; Durner et al 1996). In these organisms nitrogenase is protected from inactivation by the oxygen produced during photosynthesis by a temporal separation of nitrogen fixation and photosynthesis (Gallon 1992; Bergman et al 1997). Some of them, e.g., *Gleothece* (Mullineaux et al 1981), *Cyanothece* (Schneegurt et al 1994) and *Oscillatoria* (Stal and Heyer 1987) fix nitrogen mainly during the dark period of a light/dark photosynthetic cycle. *Plectonema boryanum* and *Phormidium* fix nitrogen under anaerobic or microaerobic conditions (Stewart and Lex 1970; Weisshar and Boger 1983; Rai et al 1992).

In heterocystous cyanobacteria in response to low nitrogen availability several structural, biochemical and genetic changes take place in some vegetative cells in order to convert them into specialized nitrogen fixing cells called heterocyst. Such changes include synthesis of multilayered cell envelope, loss of PS II activity (Petterson et al 1981), presence of uptake hydrogenase, and high rates of respiration (Wolk et al 1994) to facilitate maintaining a microaerobic interior conducive for nitrogen fixation.

1.3 Heterocysts

Heterocysts are morphologically distinct cells present in filamentous heterocystous cyanobacteria, and are responsible for the vital process of nitrogen fixation. Absence of nitrogen sources triggers 5-10% of the vegetative cells to differentiate into heterocyst. The differentiation of a heterocyst from a vegetative cell is a nitrogen-regulated process. In the presence of exogenous N sources such as nitrate, nitrite, ammonia and some amino acids the differentiation of heterocyst is repressed in all heterocyst forming cyanobacteria (Wolk et al 1996). Heterocysts are regularly spaced in a filament and contain the enzyme nitrogenase (Flores and Herrero 1994; Wolk et al 1994; Adams and Duggan 1999).

The nitrogenase enzyme complex is made up of two different proteins: Mo-Fe protein (dinitrogenase) and Fe-protein (dinitrogenase reductase). The dinitrogenase is a $\alpha_2\beta_2$ tetramer (M_r 226.8 kDa) and its α and β units are encoded by the *nif* D (Lammers and Hasselkorn 1983; Golden et al 1985) and *nif* K (Mazur and Chui 1982) genes respectively. It also contains two molecules of Mo-Fe cofactor. The dinitrogenase reductase (Mr 66 kDa) is a dimer of two identical subunits encoded by *nif* H gene. In heterocyst, the *nif* HDK is contiguous but in vegetative cells an 11kb DNA fragment interrupts *nif* D gene (Golden et al 1985). A second rearrangement involving deletion of a 55 kb fragment located in *fdx* N gene

(Golden et al 1987) has also been seen to occur during heterocyst differentiation. These rearrangements events involve site-specific excisases encoded by *xis* A (Lammers et al 1986) and *xis* F (Carrasco et al 1994) respectively. The only exception to this is a characteristic *nif* gene rearrangement found in *Mastigocladus laminosus,* where the structural *nif* genes are contiguous both in vegetative cells and heterocysts (Singh and Stevens 1992).

Conventionally nitrogenase is a Mo-dependent enzyme (Nif 1) and functions exclusively in heterocysts under aerobic conditions (Elhai and Wolk 1990; Thiel et al 1995). Thiel and Pratte in 2001 showed that *Anabaena variabilis* ATCC 29413 also possesses another Mo-dependent nitrogenase (Nif 2) which functions in vegetative cells under anaerobic conditions. A vanadium-dependent nitrogenase encoded by *vnf* DGK genes and a Fe-only nitrogenase has also been reported in *Anabaena variabilis* (Kentemich et al 1991; Thiel 1993).

The process of nitrogen –fixation is a highly energy intensive process and requires ATP, reductants and low oxygen concentration. The requirements of ATP are fulfilled by photophosphorylation, oxidative phosphorylation, substrate level phosphorylation and/or uptake hydrogenase activity (Bottomley and Stewart 1976; Maryan et al 1986; Daday and Smith 1987). Oxidative pentose phosphate pathway is a major contributor of reductants (Smith 1982; Wolk et al 1994). The immediate electron donor to nitrogenase is ferredoxin, a product of *fdx* H gene, (Bohme and Haselkorn 1988), whereas flavodoxin can take up this role under conditions of iron deficiency (Fillat et al 1991).

Genes responsible for regulation of heterocyst formation had been characterized (Wolk et al 1994; Wolk 1996; Adams and Duggan 1999). In response to nitrogen step down,

an autoregulatory gene *het* R is induced in regularly spaced cells (pro heterocysts) within 2-3.5 h. The Het R protein is an unusual serine type protease, which may be degrading the repressor of genes to be switched on and activators of genes to be switched off during heterocyst differentiation (Zhou et al 1998a; b). Transcription of *het* R is indirectly controlled by the product of *ntc* A gene, which is found to be wide spread in cyanobacteria (Frias et al 1993; Herrero et al 2001). The *ntc* A gene encodes a global nitrogen regulatory protein named Ntc A (a cyclic AMP-binding protein) required for utilization of nitrate and for heterocyst differentiation. *ntc* A from *Anabaena* PCC 7120 interacts with the promoter regions of *xis* A (an exciases gene necessary for the formation of heterocysts), *gln* A (gene encoding glutamine synthetase), *rbcl* S (encoding Rubisco), *nif* H (encoding dinitrogenase reductase) and *ntc* A (encoding Ntc A itself) (Frias et al 1994; Wei et al 1994). *ntc* A also binds to the genes encoding glutathione reductase (*gor* gene). Thus, in addition to the global response to N-deprivation (Frias et al 1994) *ntc* A also responds to redox status (Jiang et al 1997). Many genes essential for heterocyst differentiation and development have been identified and characterized on the basis of various phenotypes exhibited by heterocyst formation defective mutants. Two other important genes for heterocyst development are *hep* P (Fernandez- Piñas et al 1994) and *het* C (Khudyakov and Wolk 1997). Insertional inactivation of *het* P prevents the formation of pro heterocysts and strains with multiple copies of *het* P form multiple contiguous heterocysts in absence of combined nitrogen sources. The synthesis of innermost glycolipid layer that is important in protection of nitrogenase from oxygen requires *het* M (also known as *hgl* B) and *hgl* K. *hgl* K gene encodes a protein for transport of heterocyst glycolipids in *Anabaena* PCC 7120 (Black et al 1995; Bauer et al 1997). Three genes named *hep* A, *hep* B and *hep* C, are required for the

synthesis and stabilization of heterocyst envelop (Wolk 1996). Genes involved in heterocyst spacing has also been identified. Pat S gene product (Pat S-5; a pentapeptide) diffuses along the filaments and creates a gradient of inhibitory signal for maintaining the pattern of spaced heterocyst (Yoon and Golden 1998).

1.4 Akinetes

Some members of *Nostocaceae, Rivulariaceae* and *Stigonemataceae* are known to form akinetes, which serve as a means of perrenation in these organisms (Wolk 1965; 1973; Nichols and Carr 1978; Adams and Carr 1981; Nichols and Adams 1982; Herdman 1987, 1988; Adams 1992) and provide the capacity for growth into new filaments by germination under favourable conditions even after long-term exposure to extreme environmental conditions (Livingstone and Jaworski 1980; Sili et al 1994). Akinetes are larger than vegetative cells, with a thickened cell wall and a multilayered extracellular envelop (Nichols and Adams 1982) and their shape varies considerably (e.g., spherical in *Anabaena* CA but elongated in *A. cylindrica*). A variety of factors trigger the differentiation of a vegetative cell into an akinete. The environmental factors which have been implicated as trigger for akinete formation includes limitation of nitrogen, carbon, iron, trace elements, light, phosphate (Wolk 1965; Sinclair and Whitton 1977; Suthetland et al 1979; Nichols and Adams 1982; Fay et al 1984; Wyman and Fay 1986; Herdman 1987; 1988) and sulfate (Kyndiah and Rai 2007). Akinetes do not structurally resemble bacterial endospore and are not heat resistant but are resistant to cold and desiccation. They are suggested to be the evolutionary precursors of heterocysts (Wolk et al 1994). In recent years, akinetes have been projected as inoculum for cyanobacterial biofertilizer in rice paddy cultivation (Kyndiah and Rai 2007).

1.5 Hormogonia

Hormogonium represents a transient morphological stage in developmental cell cycle of some filamentous cyanobacteria and is observed upon the transfer of stationary phase cultures to fresh medium. Hormogonia differentiation occurs from vegetative cells in filamentous cyanobacteria (both heterocystous and non-heterocystous). They are distinguishable from vegetative cells by cell shape and in some species by cell motility and presence of gas vesicles. Various environmental factors such as change in temperature, light spectral quality and altered N-metabolism are involved in hormogonia differentiation. Removal of $NaNO_3$ from the medium has been shown to trigger the formation of hormogonia in *Nostoc muscurom* (Armstrong et al 1983), and in *Calothrix* PCC 7601 and PCC 7504 (Herdman and Rippka 1988). The ability to form hormogonia is of importance to those cyanobacterial strains that enter into symbiosis with plants. Hormogonia are the infecting units in many cyanobacteria-plant symbioses (Bergman et al 1996; Rai et al 2000).

1.6 Importance of cyanobacteria

Cyanobacteria are now regarded to have enormous potential in serving humanity in more ways than one. Their diazotrophic nature, wide distribution pattern and capability to enter into N_2–fixing symbiosis make them attractive research interest. The importance of cyanobacteria in rice fields have been known for a long time and their abundance in rice fields was first reported by Fritsch in 1907. Cyanobacteria have a long history of usage in agriculture as biofertilizer (De 1939; Watanabe et al 1951; Singh 1961). The popularity of cyanobacteria as potential biofertilizer lies in the fact that the cyanobacteria are photoautotrophic biological nitrogen fixers that naturally populate rice fields. It has been estimated that cyanobacteria contribute 20-80 KgN/ha/crop on turnover of their biomass in

the rice fields (Venkataraman 1981; Albrecht et al 1991; Roger and Ladha 1992; Ladha and Reddy 1995). Recent research on biofertilizer has also shown that rice fields populated with free-living cyanobacteria have increased rice yield (Watanabe et al 1951; Venkataraman 1972; Roger and Kulasooriya 1980; Metting 1988; Whitton 2000). The growing realization about the harmful effects of the long-term use of chemical fertilizers, herbicides and pesticides on human health has intensified research on biofertilizers in recent times, especially in areas where rice is a prime cash crop. This is especially true in tropical Asia and India where there are water-logged, low lying rice fields that are favourable for both free-living and symbiotic cyanobacteria. Species of *Nostoc, Anabaena, Aulosira, Tolypothrix, Cylindrospermum, Gloeotrichia, Gloeocapsa, Anabaenopsis, Camptylonema, Scytonema and Westiellopsis* are widespread in Indian soils and rice fields and contribute greatly to soil fertility (Nayak and Prasanna 2007). Cyanobacteria have also been tried as biofertilizer in cultivation of other crops, including wheat, tomato, pulses and other vegetables (Gupta and Gupta 1972; Kaushik and Venkataraman 1979; Karthikeyan et al 2007).

Other potential applications of cyanobacteria in diverse fields of human welfare have also gained a lot of attention in recent years. This includes their use as food and animal feed, in bioremediation of toxic compounds, in biocontrol of pests, production of commercial and laboratory chemicals, restriction enzymes, pharmacological tools (Chapman and Gellenbeck 1989; Lem and Glick 1985; Becker 1994; Maquieira et al 1994; Wilde and Benemann 1993; Moore et al 1988; Houmard and Tandeau de Marsac 1988; Gorcham and Carmichael 1988; Patterson 1996) and potential therapeutic drugs for the management of diseases like cancer, asthma, diabetes (Skulberg 2000), and in waste and effluent water treatment including the

removal of harmful dyes from textile effluents (Fatma 1999; Prassana et al 2000; Shah et al 2001; Sadettin and Domez 2006) etc. In the last few years, these organisms have been identified as a rich source of different biologically active compounds with anticancer (Gerwick et al 1994), antifungal (Kajiyama et al 1998), antibacterial (Jaki et al 2000), antiviral (Patterson et al 1994), and antiplasmodial activities (Papendorf et al 1998, Bhadury et al 2004; Singh et al 2005; Dahms et al 2006). Further, several strains of cyanobacteria are known to accumulate poly-hydroxyalkanoates which can be used as a substitute for non-biodegradable petrochemical-based plastics (Doi 1990). Recent studies have shown that oil-polluted sites are rich in cyanobacterial consortia capable of degrading oil components and other complex organic compounds such as surfactants and herbicides (Yan et al 1998; Radwan and Al-Hasan 2000; Raghukumar et al 2001; Mansy and El-Bestway 2002). In addition, hydrogen produced by cyanobacteria is being considered as a very promising source of alternative energy (Dutta et al 2005). The advantages of using biological hydrogen as a fuel are its eco-friendly nature, efficiency, renewability and the absence of carbon dioxide emission during its production and utilization (Lindbald 1999). Thus, cyanobacteria are emerging as potential organisms having many fold applications for human and environmental benefits.

Many microbes including cyanobacteria act as natural bioremediating agents and hence are being employed in clean up operations of contaminated sites. Contamination of the natural environment by toxic metals is a serious problem worldwide due to their continued persistence in the ecosystem and also because of their incremental accumulation in the food chain (Chen and Pan 2005). Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state,

or to levels below concentration limits established by regulatory authorities (Mueller et al 1996). As such, it uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and can often be carried out on site. Bioremediation has become one of the developing fields of environmental restoration programmes, which utilizes microorganisms to reduce concentration and toxicity of heavy metals, dyes, pesticides, various chemical pollutants etc. It is an option that offers the possibility to destroy or render harmless various contaminants using biological activity. The use of natural biological resources in reducing the pollution load in our environment is widely accepted idea and alternative, innovative treatment techniques have focused attention on use of biological materials such as algae, fungi, yeast, and bacteria for the removal and recovery technologies. This has gained significance in recent years owing to their better performance and low cost (Veglio and Beolchini 1997; Volesky 2001). Also, they can be integrated with other processes e.g. aquaculture/ pisciculture, feed production, soil conditioner production, fine chemical production and recovery of metals (Prasanna et al 2008). Major metal pollutants which are commonly found in industrial wastes are Cu, Zn, Ni, Co, Pb, Cr and Cd. Among the photoautotrophs, cyanobacteria are relatively more tolerant to heavy metals (Fiore and Trevors 1994). Recent studies also suggest the efficiency of cyanobacterial strains in bioremediation (Gupta et al 2000; Vidali 2001; Mallick 2002; De Philippis et al 2003; Rangsayatorn et al 2004; Awasthi and Rai 2004 Awasthi and Rai 2006; Prasanna et al 2008; Kiran et al 2008). Effluents from industries producing textile dyes are a cause of great concern because of their toxic nature, mutagenicity and carcinogenicity, in addition to their low biodegradability leading to environmental pollution. Several reports exist indicating the potential application of diverse cyanobacterial strains in remediating such pollution (Arnon

1977; Zhu et al 1979; Jinqi and Houtian 1992; Shah et al 2001; Sadettin and Donmez 2006). Thus, cyanobacteria have emerged as potential organisms serving various beneficial purposes in diverse areas of human and environmental welfare. However, all strains of cyanobacteria may vary in their ability to contribute towards these diverse fields of applications. Hence different cyanobacterial strains need to be characterized for their specific potential application(s) and once evaluated they have to be preserved for future research and use.

1.7 Maintenance and preservation of cyanobacteria

To categorically place various cyanobacterial strains according to their potential applications, naturally existing cyanobacteria needs to be systematically isolated, purified and characterized. Isolation and purification procedures are time consuming and tedious in nature. Possible threats of contaminations in such purified cultures have led researchers to pay serious attention to methods of preservation of microorganisms, in order to enable successful and effective maintenance of these organisms in the laboratory conditions.

The most common procedure for maintaining cyanobacteria is by serial transfer of actively growing cultures to a fresh media on a regular schedule under suboptimal conditions (Lorenz et al 2005). Some of the disadvantages associated with this method are the high risk of contamination and loss of characters, as well as, possible mis-labeling or loss of cultures (Acreman 1994). Also there are reports of loss of phenotypic characters in several micro algal cultures during serial sub culturing (Day et al 2005). Moreover, the genetic and phenotypic stability of the strains cannot be guaranteed over years of routine maintenance (Day and Brand 2005). When maintained at optimal conditions strains do not need to express many of their characters as stresses of any form is not presented to them

during routine maintenance in the laboratory (Smith 2004). Other drawbacks of this method of serial sub culturing is that it is labour intensive and requires consumables that are expensive and thus, limits the capacity of researchers to maintain large number of strains.

Another widely used method for laboratory maintenance of cultures is by storing them in agar slants. This is done by inoculating pure cultures into a nutrient agar media which are solidified in sterile vials. Agar was first suggested for microbiological purposes in 1881 by Fannie Hesse. It is a phycocolloid extracted from a group of red-purple algae, usually *Gelidium* sp. By the early 1900s, agar became the gelling agent of choice over gelatin because agar remains firm at growth temperature for many pathogens and agar is generally resistant to breakdown by bacterial enzymes. The use of agar in microbiological media significantly contributed to the advance of microbiology, paving the way to study pure cultures. Agar is a gel at room temperature, remaining firm at temperatures as high as 65°C. Agar melts at approximately 85 - 91°C, a different temperature from solidification at 34 - 36°C. This property is known as hysteresis. It is generally resistant to shear forces; however, different agar may have different gel strengths or degrees of stiffness.

However, this method of culture maintenance also suffers from the limitation of having a relatively short shelf-life and the jelly-like consistency of agar changes over a period of time thus, subjecting the entrapped cultures to ample chances of contamination(s). In addition, such agar slants need to be stored in culture room or in refrigerators where they occupy valuable space.

Lyophilization has also been attempted as a method of preservation of cyanobacteria. However, successful results were obtained under stringent conditions only using specific suspending substances viz, lamb serum, beef serum etc (Corbett and Parker 1976). Freeze

drying has not been found to be a successful biostorage method for microalgae resulting in very low viabilities (< 1% of original population) (Day 2006).

Cryopreservation of cultures is another dependable method of preservation. Cryopreservation is employed for a wide range of algal species with optimum results. High success rates are obtained with cyanobacterial cultures. These procedures can be categorized in two step freezing protocols. The first step consists of addition of a cryoprotectant and then cooling to a specified subzero temperature to facilitate dehydration or cryodehydration of the sample. Next it is cooled rapidly to storage temperature (Day 2006). These can then be stored for indefinite periods.

Entrapment is by far the most frequently used method in laboratory culture maintenance and there are some examples of industrial process based on entrapped cells. Entrapment methods are based on the confinement of the cells in a three-dimensional gel lattice. The cells are free within their compartments and the pores in the material allow substrates and products to diffuse to and from the cells. Several synthetic (acrylamide, polyurethane, polyvinyl, etc.) and natural polymers (collagen, agar, agarose, cellulose, alginate, carrageenan, etc.) are used for this purpose. However, for algal immobilization the most frequently used natural gels are alginate and carrageenan. The gel is generally formed into useful biocatalyst beads by first adding the cells as a suspension to an aqueous solution of the gelling material. This material is then formed into droplets by forcing it drop wise through a nozzle or orifice to an interacting salt solution. The droplets are subsequently stabilized into biocatalyst beads with entrapped organisms *via* polymerization or other types of cross-linking. For example, alginate droplets can be stabilized with divalent ions such as Ca^{2+} and carrageenan droplets are typically cross-linked with K^{+} (Mallick 2002).

Entrapment of cells (prokaryotic and eukaryotic) in hydrogel supports is a simple and nondestructive method that preserves the metabolic and physiological properties of the cells Jeanfils 1986, Garbisu et al 1990, Willaert and Baron 1993). Tamponnet et al. (1985) and Hertzberg and Jensen (1989) showed that immobilized microalgae maintained ultra structural integrity and physiological activities for several months, suggesting its application in algal storage.

Dormant resting cells of some microbes can be maintained at ambient temperatures for many years (Day 2006; Kyndiah and Rai, 2007). Hence spores may be considered to be a method of preservation in species capable of undergoing sporulation.

1.8 Present study

A laboratory working with microbes accumulates a large number of strains that needs to be maintained for considerable period of time, even when not under active research. Isolation, purification and maintenance of strains in their axenic forms are a tedious and time consuming process. Routine batch culturing popularly engaged in maintaining microbial cultures is cumbersome with disadvantages such as the chances of contamination, mis-nomenclature and space limitation within the culture rooms as well as increased chances of loss of characters that are not under selective pressure (Smith 2004). Keeping in mind the different applications of cyanobacteria, it becomes essential to carry out in depth investigation on these organisms which holds promise to alleviate many of our present day problems in environment, health and agriculture. And such explorations also opens new avenues towards eco-friendly and sustainable development, at a time when natural resources are depleting and alternative sources of food and energy are being sought. Considering this

objective, it becomes crucial to develop techniques that enable preservation of strains without affecting their viability or characters.

This work is dedicated to studying various existing methods of preservation of cyanobacteria. Detailed study was carried out on techniques of conventional maintenance and preservation of cyanobacteria such as batch culturing, maintaining on agar slants as well as preservation in calcium alginate beads, and preservation using cryoprotectants. All methods were evaluated on the basis of their suitability in retaining desired characters of the cyanobacterial strains for long and short term storage in the laboratory. Each of the techniques were individually evaluated exhaustively and then compared with each other. Introduction of modifications for improvements in few of the existing techniques were possible due to in depth information collected during the study period. Extending the knowledge of preservation to its putative application in the field of biotechnology, high nitrogen fixing strains were immobilized on locally available, biodegradable matrices such as betel nut cover, in order to offer them as affordable and convenient biofertilizer inoculum in rice cultivation. All such preserved cyanobacteria were regenerated periodically to assess their viability and retention of characters on such matrices. Time period up to which such carrier material can be used indisputably without compromising cyanobacterial attributes and viability was also determined.

Chapter 2

Materials and Methods

2.1 Collection of cyanobacterial strains

Cyanobacterial samples were collected from different terrestrial and aquatic environments including rice fields, tree barks, stagnant water holes, river banks, irrigation canals as well as, water bodies adjacent to rice fields. Water samples were also collected from roadsides. Some samples were collected from extreme ecosystems, *viz.* Jakrem hot spring in West Khasi Hills, Meghalaya and Garampani hot spring in Karbi Anglong district of Assam.

Fig 2.1: Collection of cyanobacterial samples.

2.2 Isolation and purification of the collected samples

The samples were inoculated in BG-11_0 media (Table 1a) and allowed to grow under culture room conditions at 25±2°C and at a photon fluence rate of 50 µmol m^{-2} s^{-1}. After 8-10 days, the visible cyanobacterial filaments were purified using repeated pour plate and serial dilution method on 1.2% nutrient agar until single colonies of axenic cultures were obtained. The cyanobacterial colonies were identified by light microscopic observation using Olympus BX 51 light microscope. The purified cyanobacteria were grown in $BG11_0$,

$BG11_0$ + 5mM nitrate media (for heterocystous and non-heterocystous cyanobacteria respectively) and maintained under culture room conditions (Rippka et al 1979). *Mastigocladus laminosus* which was isolated from the hot spring of Jakrem, Meghalaya, India was found to be thermophilic in nature and hence, this was maintained inside a BOD incubator (BOD incubator super deluxe automatic. NSW. India) at 45°C in D- medium (Table 1b) in batch cultures and with a photon fluence rate of 50 μmol m^{-2} s^{-1} (Castenholz 1981). The pH of the medium was adjusted to 7.5 before autoclaving.

Table 1 a: Composition of BG 11_0-medium

Macronutrients	Grams/ L
$K_2HPO_4.3\ H_2O$	40.0
$MgSO_4.7H_2O$	75.0
$CaCl_2.\ 2H_2O$	36.0
Citric acid	6.0
Ferric ammonium citrate	6.0
Na_2CO_3	20
EDTA (disodium salt)	1.0

Micronutrients	Grams/ L
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.22
$Na_2MoO_4.2\ H_2O$	0.39
$CuSO_4.\ 5H_2O$	0.079
$Co(NO_3)_2.\ 6\ H_2O$	0.0494

For NO_3^- medium. 5 mM $NaNO_3$ and for NH_4^+ medium, 2mM NH_4Cl was used.

Table 1 b: Composition of D-medium

Macronutrients	Grams/L
Nitrilotriacetic acid (NTA)	10
$NaNO_3$	70
KNO_3	10
Na_2HPO_4	11
$MgSO_4.7H_2O$	10
$CaSO_4.2\ H_2O$	6
NaCl	0.8
$FeCl_3$	0.03

Micronutrients	Grams/ L
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.22
$Na_2MoO_4.2\ H_2O$	0.39
$CuSO_4.\ 5H_2O$	0.079
$Co(NO_3)_2.\ 6\ H_2O$	0.0494

For N_2 medium, NaCl and KCl were used in place of $NaNO_3$ and KNO_3.

2.3 Sterilization

All glassware were washed thoroughly using laboratory detergent and rinsed with double distilled water before drying in the hot air oven. Sterilization of glassware and culture media was done by autoclaving at 121°C (15 psi) for 15 minutes.

2.4 Growth parameters

2.4.1 Chlorophyll *a* measurement

Growth of the cyanobacterial cultures was measured as increase in chlorophyll *a* (MacKinney 1941). 5 ml of each sample was centrifuged and equal volume of 100% methanol was added to the pellet. Chlorophyll *a* was extracted in these tubes by incubation at 4°C overnight. The solution in the tubes were mixed thoroughly using a vortex mixer and then centrifuged. The resultant supernatant was used for determination of chlorophyll *a* content. Chlorophyll *a* concentration was measured spectrophotometrically at 663 nm by using the formula: Chlorophyll *a* (µg/ml) = Absorbance at 663 nm x 12.63.

2.4.2 Protein estimation

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

2.4.2.1 Extraction of protein

5ml of cyanobacterial culture was centrifuged and the pellet resuspended in 1 ml distilled water. Cells were disrupted by ultrasonication using Sonics Vibra cell sonicator fitted with a microprobe. Supernatant was collected for protein determination after centrifugation at 3000 rpm for 5 minutes.

2.4.2.2 Estimation of protein

Reagents

A. 2% Na_2CO_3 in 0.1 N NaOH.

B. 1% sodium potassium tartarate solution.

C. 0.5% $CuSO_4$ solution.

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

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

F. Standard protein solution: Bovine Serum Albumin (BSA) solution was prepared in the range of 10- 100 $\mu g\ ml^{-1}$.

Procedure

5ml of reagent D is mixed with 1ml of cyanobacterial protein extract and mixed gently. This was incubated for 10 minutes at room temperature and then 0.5 ml of reagent E was added and mixed rapidly. After 30 minutes of incubation, the mixture was centrifuged and the absorbance of the supernatant read at 750 nm. A calibration curve was prepared by using BSA solution as standard for determination of cyanobacterial protein content.

2.5 Oxygen exchange

Oxygen evolution and consumption was measured by using a Clark-type oxygen electrode installed in a 3ml Plexiglass container with magnetic stirring (Rank Brothers,

England). Measurements involved: adding 3 ml cyanobacterial culture to the sample chamber of the non-polarized electrode and allowing each sample to equilibrate for 5 minutes with stirring. The electrode was then polarized and a linear rate of oxygen evolution was obtained in light supplied by a 100W tungsten filament bulb, which was shielded from the sample by water bath acting as heat filter. Oxygen consumption was measured in dark with the chamber wrapped in aluminum foil. The rate of oxygen evolution and consumption were expressed as nmol O_2 evolved/consumed.min^{-1}.μg^{-1} chlorophyll *a*.

2.6 Heterocyst and akinete frequency

Heterocyst and akinete frequency was calculated as percentage of total cell populations by light microscopic observations. At least 1000 cells were counted for each study.

2.7 Enzyme assays

2.7.1 Nitrogenase activity

Nitrogenase activity (EC 1.18.6.1) was measured *in vivo* using acetylene reduction assay method (Stewart et al 1967). 5 ml of cyanobacterial culture was placed in a 10 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 for 1 hour in light at a photon fluence rate of 50 $\mu mol\ m^{-2}s^{-1}$, at 25°C with constant shaking. 1ml gas sample from the vial 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 a Porapak T of mesh size 80/100) and a flame ionization detector. Nitrogenase activity was expressed as nmol C_2H_4 produced μg^{-1}chlorophyll *a*.h^{-1}.

2.7.2 Glutamine Synthetase (transferase) activity

2.7.2.1 Extraction of enzyme

Cyanobacterial cultures were harvested in their exponential phase by centrifugation. These were then washed twice in 50 mM Tris-HCL buffer, pH 7.5 and resuspended in the same buffer. Enzyme extraction was done by ultrasonication using Sonics vibra cell sonicator. Glutamine synthetase transferase activity was then analyzed *in situ* using the sonicated cells.

2.7.2.2 Assay of glutamine synthetase (transferase) activity

This was essentially done using the method described by Stewart et al 1967. The reaction mixture contained in a final volume of 3 ml, 1 ml enzyme extract, 40 mM Tris-HCL buffer (pH 7.5), 3 µmol $MnCl_2$, 20 µmol potassium arsenate, 0.4 µmol ADP (Sodium salt), 60 µmol hydroxylamine and 30 µmol glutamine. This reaction mixture was incubated in the dark for 10 minutes at 30 °C. The reaction was terminated by the addition of 2ml of stop mixture (4ml $FeCl_3$, 1 ml of 24% TCA, 0.5 ml 6 N HCL and 6.5 ml of water). The absorbance of the supernatant was read at 540 nm after 10 minutes of centrifugation at 2000 rpm. The concentration of γ-glutamyl hydroxymate formed was estimated from a standard curve that was prepared in the range of 100- 1000 nmol γ-glutamyl hydroxymate $.ml^{-1}$.

2.7.3 Nitrate reductase activity

Nitrate reductase (NR) activity was measured *in situ* (Manzano et al 1976) using sonicated 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 buffer (pH 7.5), 0.1 M NaCl, 0.3 M sucrose, 1 mM KNO_3, 1mM EDTA and 5mM $MgCl_2$). The cells were then lysed by sonication. The reaction mixture contained in addition to permeabilized cells

in a final volume of 1ml: 20 mM KNO_3, 100mM Glycine-KOH (pH 10.5), 4 mM methyl viologen, 10mM sodium dithionate freshly dissolved in 0.1 ml of 0.23 M $NaHCO_3$. After 10 minutes of incubation in dark at 30 °C, the reaction was terminated by adding 0.2 ml of 1M zinc acetate. Subsequently nitrite formed was determined by the method described by Snell and Snell, 1949.

2.7.3.1 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 in the range of 10-100 nmol/ml. This was used as standard.

Procedure

To 1 ml of sample, 1 ml sulphanilamide and 1 ml of NED was added. The solution was mixed thoroughly and absorbance read at 540 nm after 15 minutes. A calibration curve was prepared with KNO_2 as standard.

2.7.4 Nitrite Reductase activity

Nitrite Reductase (NIR) activity was assayed using the method of Arizmendi and Serra, 1990. 5 ml of cyanobacterial culture was centrifuged and thoroughly washed in 50 mM Tris-HCL buffer (pH 7.5). This was then subjected to sonication for disruption of cells and release of the enzymes in solution. The reaction mixture contained in addition to permeabilized cells in a final volume of 1ml: 2.5 mM KNO_2, 90 mM Tris-HCl buffer (pH 7.5), 3 mM methyl viologen, 20mM sodium dithionate freshly dissolved in 0.3M $NaHCO_3$.

After 5 minutes of pre-incubation at room temperature, the reaction was carried out at 30 °C for 10 minutes. The reaction is terminated by vigorous shaking to oxidize excess reductant. Subsequently the remaining nitrite was estimated by the method described by Snell and Snell, 1949.

2.9 Immobilization

2.9.1 In Agar cubes

2.5% agar solution was prepared in suitable media. 30 ml of exponentially growing culture (chlorophyll *a* content of 10 μg ml^{-1}) was centrifuged to a final volume of 10 ml. The resultant 10 ml was then added into the lukewarm agar solution, mixed thoroughly and left to solidify at room temperature. Uniform sized cubes (0.5 cm cubes) were cut using a sterile scalpel and left to air dry inside the culture room.

2.9.2 In foam cubes

Ordinary packing foam was cut into uniform sized cubes ($1cm^3$) and washed a number of times with sterile distilled water, dried and then soaked in media and autoclaved. Excess media was drained in the laminar flow. 30 ml exponentially growing culture (chlorophyll *a* content of 10 μg ml^{-1}) was concentrated to 10 ml by centrifugation. 0.5 ml of the concentrated cyanobacterial culture was injected into each of the twenty foam cubes. These were then left to air dry in sterile condition inside the culture room.

2.9.3 In calcium alginate beads

Immobilization was carried out as described by Musgrave et al (1982). 40 ml of 1.5% (w/v) sodium alginate solution was prepared in suitable medium by warming the solution. 30 ml exponentially growing culture (chlorophyll *a* content of 10 μg ml^{-1}) was concentrated to 10 ml by centrifugation and added to the sodium alginate solution at room

temperature. After mixing thoroughly the solution was added drop wise using a syringe canula, into 100 ml of 0.1M calcium chloride solution in a laminar flow cabinet. Calcium alginate beads thus formed were left in the same solution for hardening at 4°C for 1 hour. The beads were then harvested and washed with sterile water followed by media. These were then left to air dry on a petri plates inside the laminar flow cabinet.

2.10 Preservation using cryoprotectants

2.10.1 Preservation in 15% glycerol

15% v/v (final concentration) glycerol solution was made in 40 ml of suitable media (depending on the cyanobacterium being preserved). 10 ml (chlorophyll *a* content of 30 µg ml^{-1}) of concentrated cyanobacterial culture was added to the glycerol solution and mixed gently. The flasks were kept at -80 °C in Heto ultrafreeze as well as inside the freezer of a domestic refrigerator.

2.10.2 Preservation in 5% DMSO

30 ml of exponentially growing culture (chlorophyll *a* content of 10 µg ml^{-1}) was concentrated to 10 ml. Whole 10 ml culture was added to 5% DMSO solution in suitable medium (final volume = 50 ml). The solution was mixed and then stored refrigerated as in the case of preservation in 15% glycerol.

2.11 Testing for viability of the immobilized cyanobacteria

At every three months interval immobilized samples were reintroduced in the growth media. Media were closely monitored for growth of cyanobacterial filaments. These filaments were used for growth, heterocyst frequency counting and nitrogenase activity studies to ascertain suitability of the preservation techniques in keeping the cyanobacterial cells viable for a period of time.

2.12 Culture condition for akinete differentiation

Conditions such as nutrient variation, temperature alterations, duration of light/dark exposure were used to study initiation of sporulation in cyanobacterial cultures. Sporulating media deficient in sulfate were supplemented with equimolar concentration of $MgCl_2$ to counter balance the reducing concentration of $MgSO_4$ (Wolk 1965; Kyndiah and Rai 2007).

2.13 Culture condition for akinete germination

The spores (2×10^6 ml^{-1}) were plated on 1.2% nutrient agar to induce germination.

2.14 Germination of rice seeds

Rice seeds (varieties RCPL-1-87-8, DR-92, VL Dhan-81 and *Synteng*) were surface sterilized by washing with distilled water, followed by 1% (v/v) sodium hypochlorite solution for 5 minutes. Sterilized seeds were thoroughly rinsed in sterile distilled water. Seed germination was carried out on autoclaved perlite in glass beakers. The perlite was irrigated with a 10-fold dilution of autoclaved $BG\text{-}11_0$ medium containing 2mM $NaNO_3$ (nitrate medium) which was buffered with equimolar concentration of HEPES. Germination was carried out in culture room under fluorescent light (photon fluence rate of 50 $\mu mol\ m^{-1}\ s^{-1}$) at 25°C, at saturating relative humidity (Syiem 2005).

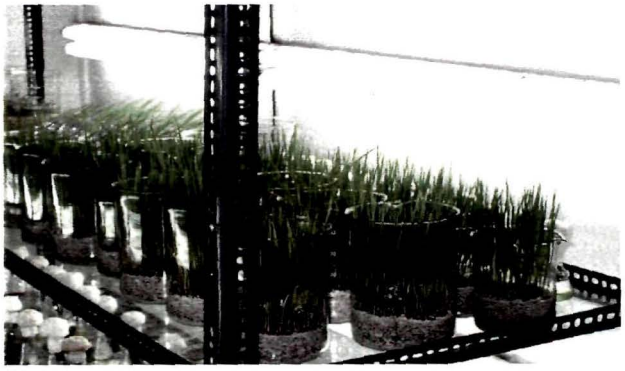


Fig 2.2: Rice seedlings being grown inside culture room.

2.15 Co-cultivation of rice and cyanobacteria

10 days old seedlings of rice were uprooted from perlite and the roots were carefully washed with distilled water. For colonization experiments, five rice seedlings were introduced into each of the 30 ml capacity culture tubes containing 20 ml media. The cyanobacterial cells used for inoculation were grown in batch cultures, harvested by centrifugation, washed several times, and finally inoculated in the tubes containing rice seedlings at a concentration of 1µg ml^{-1}. Co-cultivation was carried out at 25°C with the plant roots exposed to light.

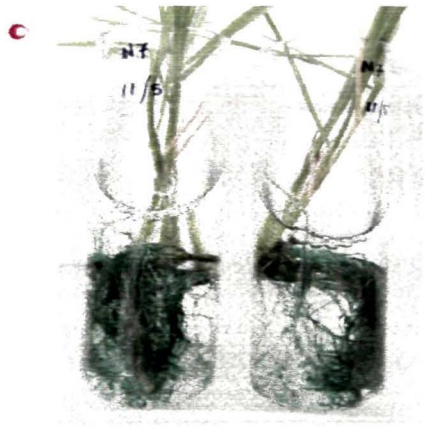


Fig 2.3: Co-cultivation of rice and cyanobacteria.

2.15.1 Screening for symbiotic competence with rice plantlets

After ten days of co-culturing, roots were excised from the experimental rice seedlings and washed for 1 minute in an ultrasonic bath (Power sonics 450) to remove loosely associated cyanobacteria. Roots were immersed in 3 ml of methanol and kept at 4°C overnight to extract chlorophyll *a* from the cyanobacteria that remained tightly associated with the roots. The roots were then dried at 25°C in an oven and root dry weight was determined. Chlorophyll *a* estimated in this experiment was expressed as µg chlorophyll *a* $.g^{-1}$ root dry weight.

2.15.2 Associative nitrogen fixation study

Nitrogenase activity of the tightly associated cyanobacteria to rice roots was measured using acetylene reduction technique as described by Stewart et al 1967. The roots used in the above section were used for associative nitrogenase activity study before they were subjected to chlorophyll extraction. Nitrogenase activity was expressed as nmol C_2H_4 produced. μg^{-1} chlorophyll *a*. h^{-1}.

2.16 DNA fingerprints

PCR-based DNA fingerprint profiles were generated using STRR 1A as primer (3'-CCCCTRACCCCTRACC-5') for the strains to establish the genetic identity of the organisms (Rasmussen U and Svenning M M 1998).

2.17 Metal sorption studies by cyanobacteria

For metal absorption studies, the cyanobacteria were grown in light in presence of half lethal dose (LD_{50}) of the heavy metal in the growth medium. The experimental flasks were shaken for 24 h in a shaker and then centrifuged at 10,000g. The residual heavy metal in the medium was determined by Perkin Elmer 3110 Atomic Absorption Spectrophotometer (Zakaria 2001).

2.18 Scanning electron microscopy

Scanning electron microscopic studies were conducted at the Sophisticated Analytical Instrumentation Facility, NEHU, Shillong. Samples were prepared for SEM using the standard protocol for biological samples. The samples were subjected to primary fixation in 2.5- 3% glutaraldehyde. These were then washed in 0.1 M sodium cacodylate buffer. Samples were dehydrated at 4°C by the following steps

30% acetone	15 minutes × 2 changes
50% acetone	15 minutes × 2 changes
70% acetone	15 minutes × 2 changes
80% acetone	15 minutes × 2 changes
90% acetone	15 minutes × 2 changes
95% acetone	15 minutes × 2 changes
100% acetone	15 minutes × 2 changes

The dried samples were mounted on brass stubs and coated using gold (Fine coat ion sputter JFC 1100). Samples were viewed in JEOL-JSM- 6360 Scanning Electron Microscope.

Chapter 3
Collection, isolation and purification of cyanobacteria

3.1 Introduction

This thesis involved studying the impact of different preservation methods on the various groups of cyanobacteria. The first part of the work started with the collection of cyanobacterial samples. This short chapter details collection, isolation, purification and identification of different cyanobacteria. Collection of cyanobacteria was undertaken to obtain representative members of different groups of cyanobacteria *viz.* unicellular, filamentous non-heterocystous, filamentous heterocystous and filamentous branched heterocystous cyanobacteria. Extensive collection was done from different locations within the state of Meghalaya. Some samples were also collected from other states in India. Owing to different altitudes, distinctive rainfall pattern and varied climatic and other ecological conditions, the pool of cyanobacterial isolates was expected to be diverse in nature. Favorable growth conditions, such as the water logged rice fields are known to host a wide variety of cyanobacteria and therefore many rice fields were included in the list of sites from which samples were to be collected. As cyanobacteria are ubiquitous in almost every terrestrial and aquatic environment, many of the samples were isolated from different water bodies, including stagnant water holes, river banks, irrigation canals as well as, water bodies adjacent to rice fields and roadsides. Since many cyanobacteria that are capable of entering symbiotic associations are known to undergo extensive morphological, physiological and biochemical modifications, two symbiotic forms of cyanobacteria were isolated from colloroid roots of cycas plants as well as from the *Anthoceros punctatus* gametophytic thalli to assess efficiency of preservation methods in preserving such changes. Jakrem hot springs

in West Khasi Hills, Meghalaya and Garampani hot springs in Karbi Anglong of Assam were also chosen for collection as they represent extreme environments.

3.2 Isolation and purification of cyanobacterial samples

Collection was done in autoclaved sample containers. These samples were inoculated in media under culture room conditions. Samples collected from hot springs were inoculated in BG 11_0 as well in D-media and were kept at 25±2°C and also at 40°C to isolate any cyanobacteria that are thermophilic in nature. After 8-10 days, visible cyanobacterial filaments were then picked from the medium and purified using repeated pour plate and serial dilution method until axenic cultures were obtained (Fig 3.1).

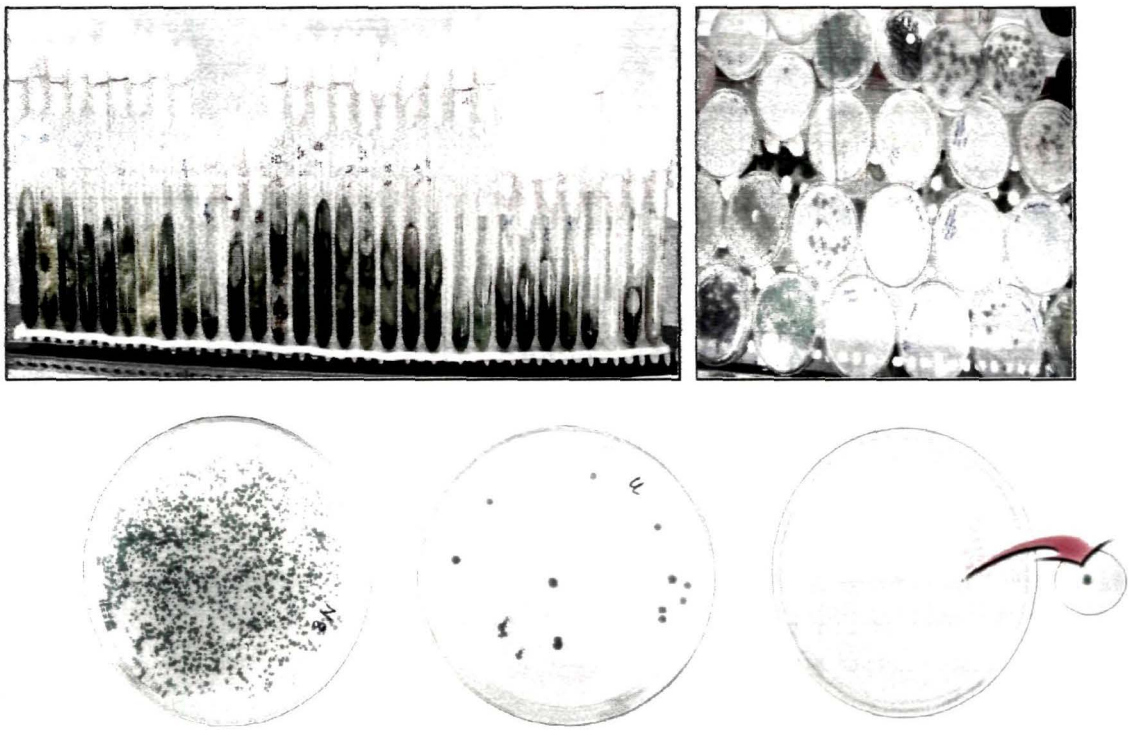
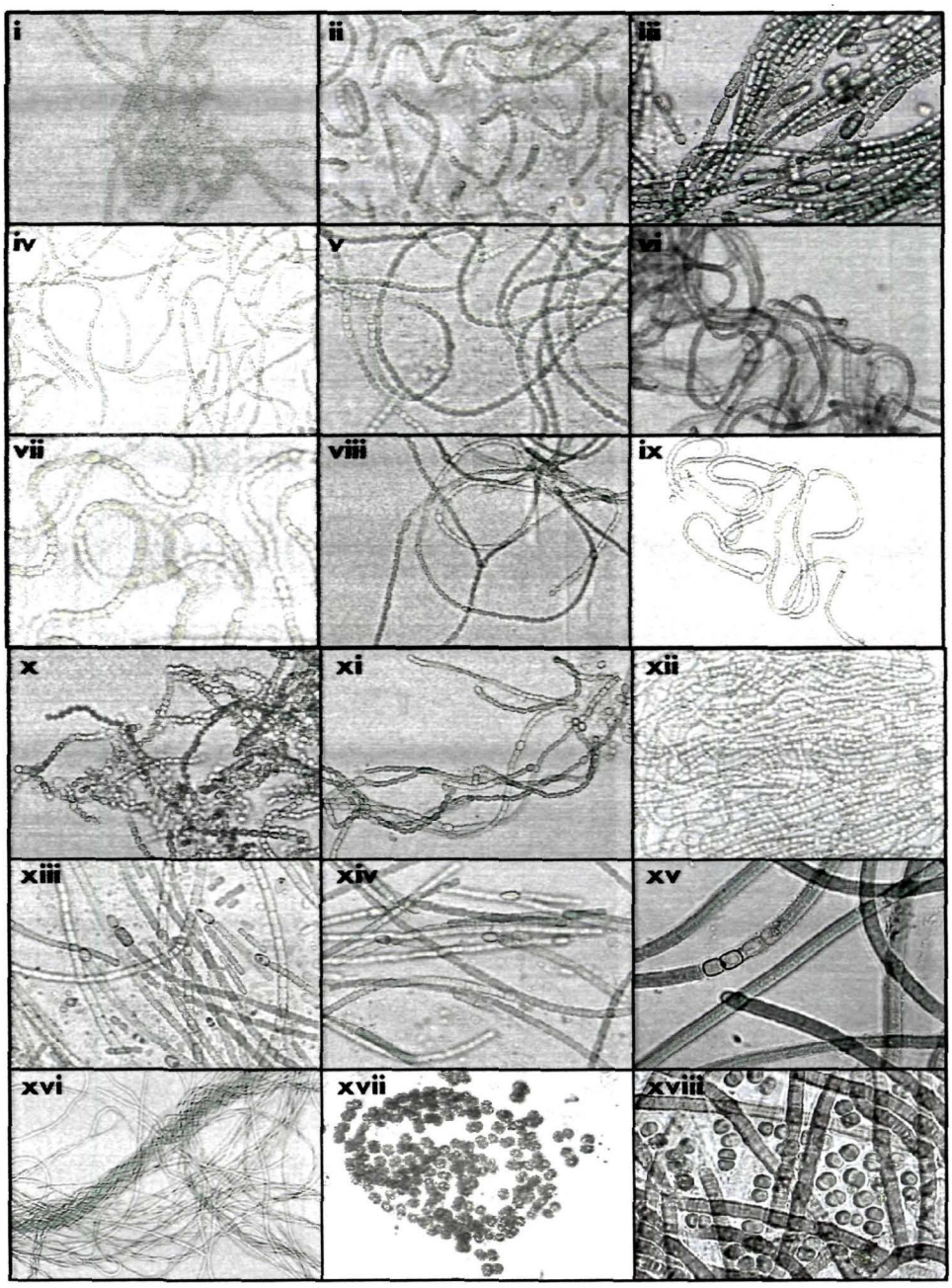


Fig 3.1: Purification of cyanobacterial samples by pour plate and serial dilution method

3.3 Identification of cyanobacterial samples

The cyanobacterial colonies were identified by light microscopic observation using Olympus BX 51 light microscope. Purified cultures were used for the purpose of identification.



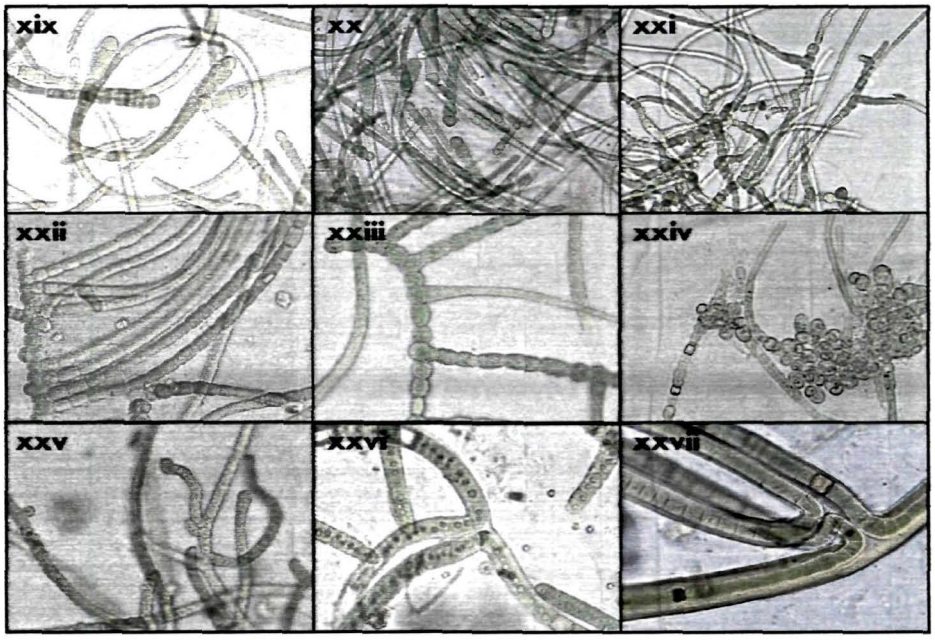


Fig 3.2: Light microscopic pictures of some cyanobacterial isolates that were purified from the collection. (i, iii, iv, vii, ix -*Anabaena* sp.; ii, v, vi, xii - *Nostoc* sp.; viii- *Anabaena cycadeae*; x- *Nostoc muscorum*; xi- *Nostoc* ANTH; xiii, xiv- *Cylindrospermum* sp.; xv- *Tolypothrix* sp.; xvi-*Plectonema boryanum*; xvii- *Microcysis* sp.; xviii- *Gloeocapsa* sp.; xix, xx- *Calothrix* sp.; xxi- *Mastigocladus laminosus*; xxii, xxiii- *Fischerella* sp.; xxiv- *Westieollopsis* sp.; xxv- *Stigonema* sp., xxvi, xxvii- *Scytonema* sp.).

3.4 Maintenance of cyanobacterial cultures

The purified cultures were maintained in BG 11_0 media as batch cultures at 25±2°C and at a photon fluence rate of 50 µmol m^{-2} s^{-1} (Rippka et al 1979) (Fig. 3.3). The medium was supplemented with 5mM sodium nitrate for the non-heterocystous cyanobacteria. Thermophilic cyanobacteria *Mastigocladus laminosus* isolated from the hot spring of Jakrem, was maintained at 45°C in D-medium in batch cultures and with a photon fluence rate of 50 µmol m^{-2} s^{-1} (Castenholz 1981). This temperature was chosen after growing the organism at different temperatures to find its ideal growing temperature.

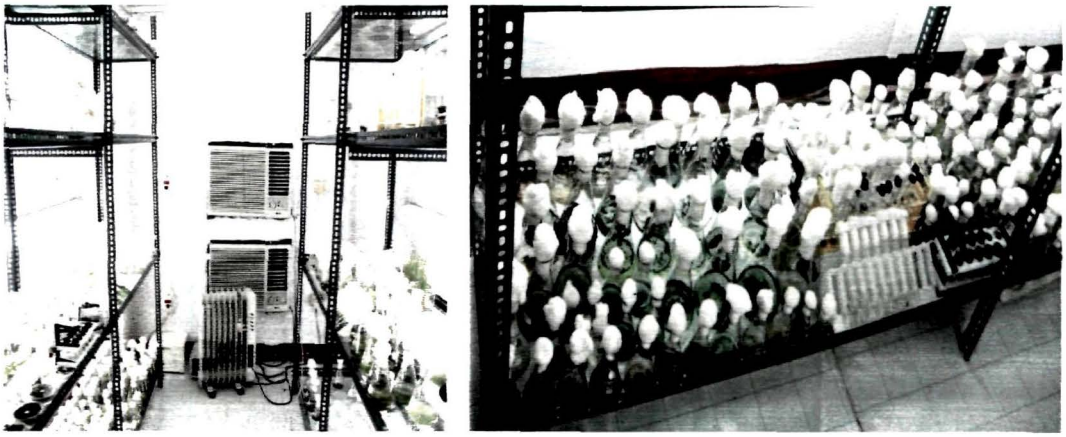


Fig 3.3: Maintenance of cyanobacterial cultures inside the culture room

3.5 Results and discussion

Cyanobacteria were randomly collected from forty one locations of ten different states (Table: 3.1).

Table: 3.1 State wise number of collection sites of cyanobacteria

Sl. No.	States	Number of collection sites	pH Range
1	Meghalaya	16	5.2 –7.0
2	Assam	10	4.2 – 5.8
3	Manipur	2	5.4 – 6.8
4	West Bengal	3	5.5 – 6.8
5	Uttar Pradesh	3	6.5 – 9.5
6	Uttarakhand	2	5.6 –7.1
7	Delhi	1	7.5 – 83
8	Rajasthan	1	7.0 – 8.5
9	Punjab	1	7.8 – 8.5
10	Haryana	2	7.0 – 8.5

Number of locations was kept large to accommodate different ecological regions to ensure diversity in the collection. A total of ninety eight isolates were purified which

include members of all groups *viz.* unicellular, filamentous non-heterocystous, filamentous heterocystous, filamentous branched heterocystous and thermophilic cyanobacteria. Table 3.2 gives details of cyanobacterial collection for the study.

Table 3.2: Cyanobacterial samples collected from different ecosystems during the course of study

Sl. No	Sample (s)	Origin of sample (s)	Sample identified as
1.	*RF 1*	Rice fields. Near NEHU Campus. Meghalaya	*Anabaena oryzae*
2.	*RF 2*	Rice fields. Near NEHU Campus. Meghalaya	*Plectonema boryanum*
3.	*RF3*	Rice fields. Near NEHU Campus. Meghalaya	*Nostoc* sp.
4.	*RF4*	Rice fields. Near NEHU Campus. Meghalaya	*Anabaena oryzae*
5.	*RF5*	Rice fields. Near NEHU Campus. Meghalaya	*Aphanothece* sp.
6.	*RF6*	Rice fields. Near NEHU Campus. Meghalaya	*Nostoc* sp.
7.	*RF7*	Rice fields. Near NEHU Campus. Meghalaya	*Anabaena* sp.
8.	*RF8*	Rice fields. Near NEHU Campus. Meghalaya	*Calothrix* sp.
9.	*RF9*	Rice fields. Near NEHU Campus. Meghalaya	*Nostoc* sp.
10.	*RF10*	Rice fields. Near NEHU Campus. Meghalaya	*Nostoc* sp.
11.	*RF11*	Umkhen. Jaintia Hills. Meghalaya.	*Tolypothrix* sp.
12.	*RF12*	Umium. Ri Bhoi. Meghalaya.	*Nostoc* sp.
13.	*RF13*	Nongstoin. West Khasi Hills. Meghalaya.	*Anabaena* sp.
14.	*RF14*	Mawphlang. East Khasi Hills. Meghalaya.	*Nostoc* sp.
15.	*RF15*	Syntu Ksiar. Jaintia Hills. Meghalaya.	*Anabaena* sp.
16.	*RF16*	Syntu Ksiar. Jaintia Hills. Meghalaya.	*Nostoc* sp.
17.	*RF17*	Syntu Ksiar. Jaintia Hills. Meghalaya.	*Anabaena* sp.
18.	*RF18*	Syntu Ksiar. Jaintia Hills. Meghalaya.	*Nostoc* sp.
19.	*RF19*	Syntu Ksiar. Jaintia Hills. Meghalaya.	*Nostoc* sp.
20.	*RF20*	Syntu Ksiar. Jaintia Hills. Meghalaya.	*Gloeocapsa* sp.
21.	*RF21*	Syntu Ksiar. Jaintia Hills. Meghalaya.	*Anabaena variabilis*
22.	*RF22*	Rice fields. Guwahati, Assam	*Nostoc* sp.
23.	*RF23*	Rice fields. Guwahati, Assam	*Nostoc* sp.
24.	*RF24*	Rice fields. Sonapur, Assam	*Anabaena* sp.
25.	*RF25*	Rice fields. Sonapur, Assam	*Plectonema* sp.
26.	*RF26*	Rice fields. Sonapur, Assam	*Nostoc* sp.

27.	*RF27*	Rice fields. Bagori, Assam	*Nostoc* sp.
28.	*RF28*	Rice fields. Bagori, Assam	*Calothrix* sp.
29.	*RF29*	Rice fields. Dergaon, Assam	Westiellopsis sp.
30.	*RF30*	Rice fields. Dergaon, Assam	*Cylindrospermum fertilisma*
31.	*RF31*	Rice fields. Golaghat, Assam	*Anabaena* sp.
32.	*RF32*	Rice fields. Golaghat, Assam	*Plectonema* sp.
33.	*RF33*	Rice fields. Nambor, Assam	*Anabaena variabilis*
34.	*RF34*	Rice fields. Nambor, Assam	*Nostoc* sp.
35.	*RF35*	Rice fields. Nambor, Assam	*Nostoc* sp.
36.	*RF36*	Rice fields. Jorhat, Assam	*Fiscerella* sp.
37.	*RF 37*	Rice fields, Jowai, Meghalaya	*Anabaena variabilis*
38.	*RF 38*	Rice fields, Jowai, Meghalaya	*Nostoc* sp.
39.	*RF39*	Rice fields. Imphal. Manipur	*Tolypothrix* sp.
40.	*RF40*	Rice fields. Imphal. Manipur	*Fischerella* sp.
41.	*RF41*	Rice fields. Imphal. Manipur	*Fischerella* sp.
42.	*RF42*	Rice fields. Imphal. Manipur	*Anabaena oryzae*
43.	*RF43*	Rice fields. Imphal. Manipur	*Tolypothrix* sp.
44.	*RF44*	Rice fields. Imphal. Manipur	*Nostoc* sp.
45.	*RF45*	Rice fields. Imphal. Manipur	*Nostoc* sp.
46.	*RF39*	Rice fields. Imphal. Manipur	*Nostoc puntiformi*
47.	*RF40*	Rice fields. Manipur	*Cylindrospermum muscicola.*
48.	*RF41*	Rice fields. Manipur	*Cylindrospermum* sp.
49.	*CM1*	Coal mining site. Cherrapunjee. East khasi hills. Meghalaya.	*Anabaena* sp.
50.	*CM2*	Coal mining site. Cherrapunjee. East khasi hills. Meghalaya.	*Nostoc* sp.
51.	*CM3*	Coal mining site. Jowai. Jaintia Hills. Meghalaya.	*Nostoc* sp.
52.	*CM4*	Coal mining site. Ladrymbai. Jaintia Hills. Meghalaya.	*Nostoc* sp.
53.	*CM5*	Coal mining site Ladrymbai. Jaintia Hills. Meghalaya.	*Nostoc* sp.
54.	*CM6*	Coal mining site Khliehriat. Jaintia Hills. Meghalaya.	*Anabaena* sp.
55.	*CM7*	Coal mining site Khliehriat. Jaintia Hills. Meghalaya.	*Anabaena* sp.
56.	*CM8*	Coal mining site Khliehriat. Jaintia Hills. Meghalaya.	*Anabaena* sp.
57.	*CM9*	Coal mining site Jowai. Jaintia Hills. Meghalaya.	*Nostoc* sp.
58.	*T1*	Tree bark. Shillong	*Nostoc* sp.
59.	*T2*	Tree bark. Shillong	*Nostoc* sp.
60.	*T3*	Tree bark. Assam.	*Scytonema hofmanai*

61.	*T4*	Tree bark. Assam.	*Scytonema* sp.
62.	*UP1*	Mustard field Meerut, (Uttar Pradesh)	*Haphalosiphon laminosus*
63.	*UP2*	Mustard field Meerut, (Uttar Pradesh)	*Nostoc* sp.
64.	*UP3*	Adjoining the highways, Roorkee, Uttar Pradesh	*Nostoc* sp.
65.	*UP4*	Wheat fields on the Delhi-Agra highway	*Anabaena sporides*
66.	*Ganga 1*	From waters close to the banks of the river. Haridwar. Uttarakhand.	*Anabaena fertilissma*
67.	*Ganga 2*	From waters close to the banks of the river. Haridwar. Uttarakhand.	*Nostoc* sp.
68.	*Ganga 3*	From waters close to the banks of the river. Haridwar. Uttarakhand.	*Nostoc* sp.
69.	*Ganga 4*	From waters close to the banks of the river. Rishikesh. Uttarakhand.	*Nostoc* sp.
70.	*Ganga 5*	Wet soils on river bank. Rishikesh. Uttarakhand.	*Nostoc* sp.
71.	*K1*	Stagnant water, Road side, Kolkata.	*Fischerella* sp.
72.	*K2*	Stagnant water, Residential location, Kolkata.	*Fischerella* sp.
73.	*K 3*	Water sample from ornamental water body in a residential area, Kolkata.	*Tolypothrix tenuis*
74.	*K 4*	Water sample from ornamental water body in a residential area, Kolkata.	*Fischerella* sp.
75.	*K 5*	Water sample from ornamental water body in a residential area, Kolkata.	*Nostoc* sp.
76.	*K 6*	Water sample from ornamental water body in a residential area, Kolkata.	*Nostoc* sp.
77.	*K 7*	Water sample from ornamental water body in a residential area, Kolkata.	*Nostoc* sp.
78.	*H1*	Mustard fields adjoining the highways. Haryana	*Anabaena* sp.
79.	*H2*	Mustard fields adjoining the highways. Haryana	*Nostoc* sp.
80.	*H3*	Water holes adjoining highways. Haryana	*Nostoc commune*
81.	*H4*	Water holes adjoining highways. Haryana	*Nostoc* sp.
82.	*P1*	Mustard fields adjoining the highways. Punjab	*Anabaena* sp.
83.	*Raj1*	Soil Sample. BITS Campus. Pilani, Rajasthan.	*Microcystis* sp.
84.	*Raj2*	Water sample BITS Campus. Pilani, Rajasthan.	*Microcystis* sp.
85.	*Raj3*	Soil Sample BITS Campus. Pilani,	*Microcystis* sp.

		Rajasthan.	
86.	*Raj4*	Water sample. Heritage park. Pilani, Rajasthan.	*Microcystis* sp.
87.	*Raj5*	Water sample Heritage park. Pilani, Rajasthan.	*Microcystis* sp.
88.	*Raj6*	Soil Sample Heritage park. Pilani, Rajasthan.	*Microcystis* sp.
89.	*Raj7*	Water sample Heritage park. Pilani, Rajasthan.	*Microcystis* sp.
90.	*NA1*	From *Anthoceros punctatus* (NEHU)	*Nostoc* sp.
91.	*NA2*	From *Anthoceros punctatus* (NEHU)	*Nostoc* ANTH
92.	*AC1*	From Roots of cycas tree (A. G Office, Shillong)	*Anabaena cycadeae*
93.	*NA3*	From *Anthoceros punctatus* (Mawphlang)	*Nostoc* sp.
94.	*HS1*	Jakrem hot spring. West Khasi Hills. Meghalaya.	*Mastiglcladus laminosus*
95.	*HS2*	Garampani hot spring. Garampani wild life sanctuary. Karbi Anglong. Assam.	*Oscillatoria* sp.
96.	*HS 3*	Garampani river bank. Garampani wild life sanctuary. Karbi Anglong. Assam.	*Nostoc* sp.
97.	*HS 4*	Garampani hot spring. Garampani wild life sanctuary. Karbi Anglong. Assam.	*Phormidium* sp.
98.	*NM*	From Banaras Hindu University	*Nostoc muscorum*

Sixteen different genera of cyanobacteria were present in the collection. Most genera were limited in number except members of the genera *Nostoc* and *Anabaena*. The genus *Nostoc* was found to be predominant in all locations. 43.87% of the total collection belonged to this genus. Next most abundant genus was *Anabaena*. It was close to 22% in the collection and thus a large difference in abundance was seen between these two genera even though most of our collection sites were rice fields where both these genera have been reported to be plentiful in all stages of crop growth and fertilizer treatments (Nayak et al 2001; 2004). However, in our case maximum sample collection was done from Meghalaya and Assam where the soil and water pH was recorded in the acidic range (Table 3.1). Thus, pH seems to be the factor determining the relative abundance and diversity of cyanobacteria in any given location. That *Nostoc*sp. and *Anabaena* sp. are highly competitive and versatile

diazotrophic cyanobacteria and inhabit all types of environments is a well established observation. The overwhelming abundance of *Nostoc* sps. in all sites indicated their fierce competitiveness and strength of adaptability to a range of environmental variations that may surpass even that of *Anabaena* sp. Finding *Microcystis* sp. as the next largest contributor in our collection was misleading as we found all samples collected from Pilani (Rajasthan) contained only *Microcystis* sp., and this genus was completely absent in all other samples. This may represent a unique scenario where profuse *Microcystis* bloom completely wiped out all other forms of cyanobacteria in the collection sites. The overall generic diversity and percent abundance of various cyanobacteria is depicted in Fig. 3.4.

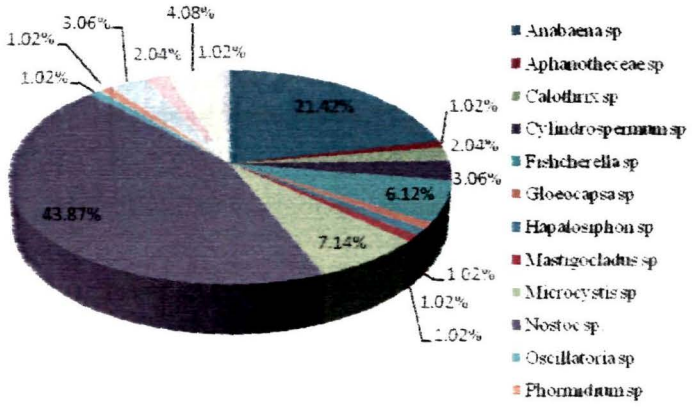


Fig.3.4: Overall generic diversity and percent abundance of various cyanobacteria in the collection

The main objective behind isolation of cyanobacteria from different regions was to obtain a set of diverse cyanobacterial strains whose composition has been shaped by different environmental conditions. Whether preservation techniques employed in the following section could ensure retention of inherent and acquired characters was studied by regenerating preserved cyanobacteria from time to time and selected attributes were evaluated and compared with that of free-living control cultures. For this, representatives of various cyanobacterial groups *viz.* unicellular (*Gloeocapsa* sp.), non-heterocystous

(*Plectonema boryanum*), heterocystous (*Nostoc muscorum*; *Anabaena variabilis*), branched heterocystous and thermophilic cyanobacteria (*Mastigocladus laminosus*) were selected from the collection. That any given method of preservation holds good for one or all the cyanobacterial groups was evaluated by preserving various representative members using the method. Five established methods were tried to preserve the various members. Following chapters describes in detail the results of experiments conducted to study the efficiency of preservation methods and compares their performance with respect to each other.

3.6 Salient findings

- Samples were collected from forty one distinct environmental locations from ten different states.
- A total of ninety eight cyanobacteria were isolated and purified.
- These include members of both aquatic and terrestrial habitats.
- Three members of thermophilic cyanobacteria were isolated from hot springs in Meghalaya and Assam.
- All groups of cyanobacteria were represented in the collection.
- *Gloeocapsa* sp., *Plectonema boryanum, Nostoc muscorum*; *Anabaena variabilis* and *Mastigocladus laminosus* were selected as members representing unicellular, filamentous non-heterocystous, filamentous heterocystous, filamentous branched heterocystous thermophilic cyanobacteria respectively for further study involving preservation.

Chapter 4

Preservation of cyanobacteria using different laboratory methods

4.1 Introduction

Microalgae, including cyanobacteria, are employed in a broad range of biotechnological applications (Cohen 1999; Richmond 2004). Diverse areas in which cyanobacteria find putative applications include agriculture, health, in production of various bioactive compounds with anti-plasmodic (Papendorf et al 1998), anti microbial (Patterson et al 1994; Kajiyama et al 1998; Jaki et al 2000), antioxidant and anticancer (Gerwick et al 1994) activities. In the field of agriculture, cyanobacteria are currently being used as a biofertilizer in rice crops in many Asian countries (Mishra and Pabbi 2004; Chunleuchanon et al 2003), with potential applications in Spain (Fernández-Valiente et al 2000) and more recently in South America (Irisarri et al 2001). They are involved in the production of many commercial and laboratory chemicals, in waste and effluent water treatment (Fatma 1999; Shah et al 2001; Sadettin and Domez 2006; Prassana et al 2000) and in bioremediation of toxic compounds. Recent studies have shown that oil-polluted sites are rich in cyanobacterial consortia capable of degrading oil components and other complex organic compounds such as surfactants and herbicides (Yan et al 1998; Radwan and Al-Hasan 2000; Raghukumar et al 2001; Mansy and El-Bestway 2002). Hydrogen produced by cyanobacteria has been considered as a very promising source of alternative energy (Dutta et al 2005). The advantages of using biological hydrogen as fuel are its eco-friendly nature, efficiency, renewability and the absence of carbon dioxide emission during its production and utilization (Lindbald 1999). In addition, several strains of cyanobacteria are known to

accumulate poly-hydroxyalkanoates, which can be used as a substitute for non-biodegradable petrochemical-based plastics (Doi 1990). In the last few years, cyanobacteria have gained much attention as a rich source of many bioactive compounds and have been considered as one of the most promising groups of organisms to produce them (Bhadury et al 2004; Dahms et al 2006; Singh et al 2005).

Considering the immense application potential of cyanobacteria, large number of laboratories across continents is involved in elucidating various applicable traits of cyanobacteria. However, not all cyanobacterial strains are equally competent for a particular application. Thus, a range of cyanobacterial strains must be evaluated for each application to short list the best possible candidates. This requires elaborate collection, purification and maintenance of different cyanobacterial strains. The most common method for maintenance is serial sub-culturing of actively growing cultures in batches (Lorenz et al 2005). However, keeping large number of purified strains as batch cultures requires sizeable space. And keeping many cultures in close proximity in culture rooms leads to increased chances of contamination as well as mislabeling (Acreman 1994). In addition, maintaining microbes for a long time under optimal conditions may lead to loss of crucial characters that an organism might have acquired under exposure to unique environmental conditions over a period of time (Smith 2004; Day et al 2005). Thus, the phenotypic and genetic stability of any strain cannot be guaranteed over years of routine maintenance under optimal conditions (Day and Brand 2005). Serial sub culturing is also labor intensive requiring input of significant number of working hours.

Another popular method of preservation of cyanobacteria is maintaining them on agar slants whenever they are required to be stored for a considerable period of time. Yet

other methods of preservation include lyophilization, cryopreservation and immobilization of cyanobacterial cells on three-dimensional gel matrices.

The above mentioned methods of cyanobacterial preservation have been found to be reliable under laboratory condition to a certain extent with their associated problems mentioned earlier. However, time period up to which a strain can be maintained without loss of any phenotypic and genetic characters using these preservation techniques has not been worked out in detail. Also, no systematic study has been published recording any alteration(s) in and/or reduction of expression (if any) of any significant character of a strain under preservation. This work was aimed at studying efficiency of five popular methods in preservation of cyanobacteria. A thorough and extensive study was carried out on representative members of five distinct groups of cyanobacteria. Each member was preserved using all five methods. Preserved material was regenerated in defined interval of time over a period three years and various vital characters were studied in the regenerated samples and the results were compared with their free-living counterparts. To improve their suitability, modifications were introduced in some of these techniques. At the end of the study, the preservation techniques were also compared to each other to list them according to their suitability, performance, time period upto which they can be employed, ease of handling and storage of the material preserved.

This section of the thesis gives details of experiments carried out on preservation of cyanobacteria. The methods that have been used for preservation are:

1. Preservation on agar: improvisation of agar slant method into dehydrated agar cubes for longer and convenient storage.
2. Immobilization

(a) In calcium alginate beads

(b) In foam cubes

3. Cryopreservation

(a) Preservation by maintaining in 15% glycerol

(b) Preservation by maintaining in 5% DMSO

Five cyanobacterial strains *viz. Nostoc muscorum* and *Anabaena variabilis* (filamentous heterocystous), *Mastigocladus laminosus* (filamentous branched heterocystous; thermophilic), *Plectonema boryanum* (filamentous non-heterocystous) and *Gloeocapsa* sp. (unicellular) were preserved in the above mentioned methods and selected characters of these organisms were studied in the regenerated samples. The preservation methods were systematically evaluated for their efficiency in preserving organisms under study.

4.2 Preservation in modified agar

Cyanobacterial cells were kept on slants prepared in nutrient agar (1.5%). As slants require substantial space for maintaining large number of cultures, a modification was introduced in preparation of nutrient agar. The concentration of agar was increased (2.5%) to achieve a denser agar solution. Agar cubes containing embedded cyanobacteria were prepared as described in chapter 2. These cubes were left to air dry for 24 h inside laminar flow cabinet. Agar flakes thus obtained after drying were stored in sterile glass vials at room temperature.

Samples were regenerated every three months up to a period of three years and different parameters were studied to evaluate if any noticeable changes had occurred in the preserved organism(s).

4.2.1 Results

Growth, heterocyst frequencies, nitrogenase activities, photosynthetic oxygen evolution and consumption and activities of enzymes – glutamine synthetase (transferase), nitrate reductase and nitrite reductase were studied and results are discussed below.

4.2.1.1 Growth

Growth was measured as increase in their chlorophyll *a* content. All regenerated samples showed comparable chlorophyll *a* content to their free-living batch cultures during their exponential phase of growth.

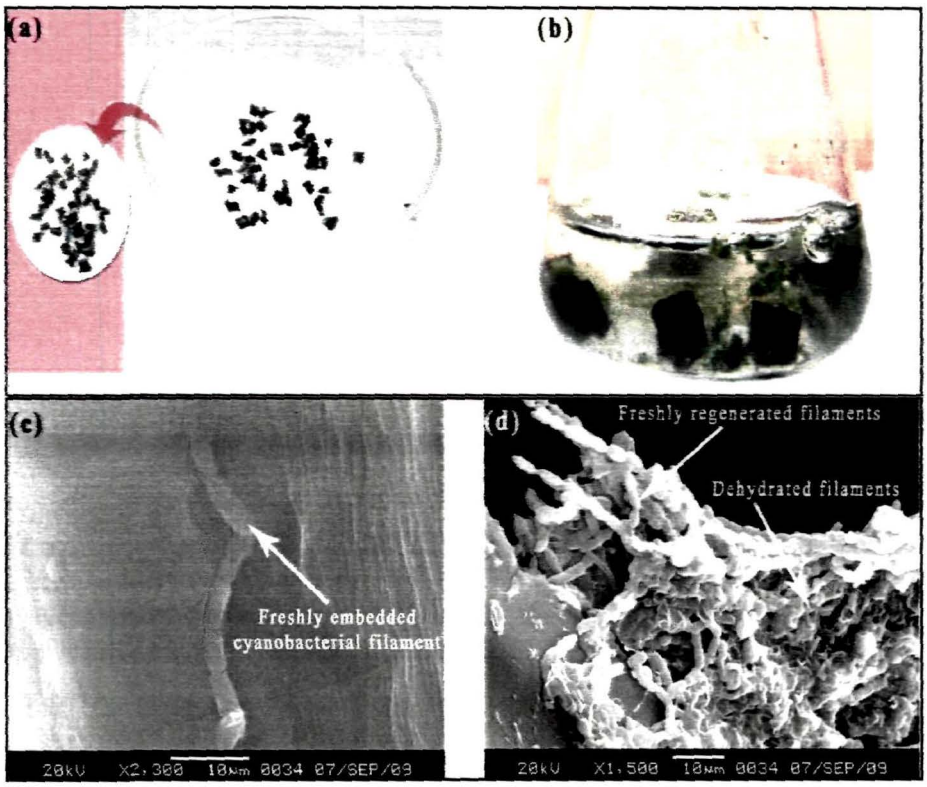


Fig 4.1: Cyanobacterial cultures immobilized in agar cubes and their subsequent regeneration. (a) Dried agar flakes (b) cyanobacterial cells growing out of agar cubes after flakes were introduced into fresh medium (c) freshly embedded *Nostoc* filament in agar as seen under scanning electron microscope (d) Scanning electron micrograph showing regeneration of *Nostoc* filaments from agar flakes after one year of immobilization.

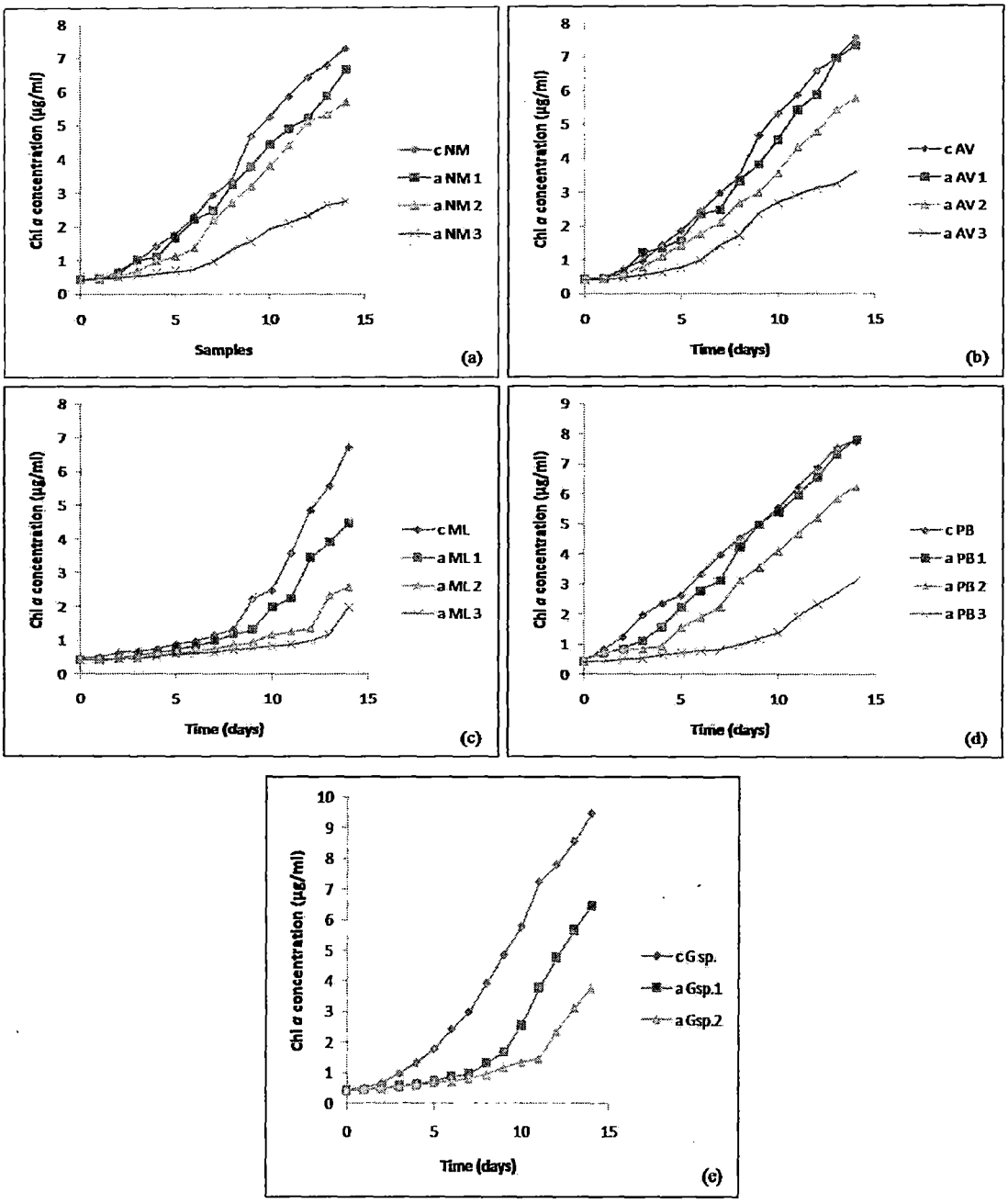


Fig 4.2: Growth curves of (a) *Nostoc muscorum* (NM) (b) *Anabaena variabilis* (AV) (c) *Mastigocladus laminosus* (ML) (d) *Plectonema boryanum* (PB) and (e) *Gloeocapsa* sp. (G sp.) regenerated from immobilization in agar flakes as compared to their free-living counterpart. [*In the figures 'c' denotes control free-living culture; 'a' refers to agar flakes and '1, 2, 3' indicates samples regenerated after first, second and third years of preservation. Mastigocladus laminosus was kept at 45°C for all studies. An initial inoculum of 0.4µg/ml was used in all cases.*]

Cyanobacterial filaments were seen emerging from agar flakes within 5-7 days of inoculation to nutrient media (Fig 4.1 b). A comparative growth profile is given in the Fig 4.2. Growth of *Nostoc muscorum* and *Anabaena variabilis* regenerated after one year of preservation quickly matched that of their controls maintained in batch cultures. A comparison of chlorophyll *a* content on day ten among the cultures regenerated after 1st, 2nd and 3rd years of preservation showed that growth was decreased by ~15%, 27% and 62% respectively in *Nostoc muscorum*. For *Anabaena variabilis* these values were ~15%, 33% and 50% respectively. However, upon subsequent sub-culturing all cultures showed growth equal to their controls. The 1st and 2nd year cultures needed only two stages of sub-culturing to match the chlorophyll *a* value to their free-living counterparts. In case of *Mastigocladus laminosus* an extended lag period was seen whenever these cultures were transferred to fresh media. The same trend was followed by all its regenerating samples. Growth was reduced in regenerated cultures of *Mastigocladus laminosus* as well, however complete recovery was seen upon subsequent transfers. A comparison of chlorophyll *a* among all groups of cyanobacteria under study regenerated after one year of preservation showed best regeneration capability in *Plectonema boryanum,* closely followed by *Anabaena variabilis.* In all cultures, regeneration took longer time as the period of preservation increased (2nd and 3rd years). These cultures showed a long lag phase of growth. This extended lag period was seen only in cultures regenerated directly from preserved material. Free-living regenerates however, matched the growth pattern of their control cultures upon successive sub culturing.

4.2.1.2 Heterocyst frequency and nitrogenase activity

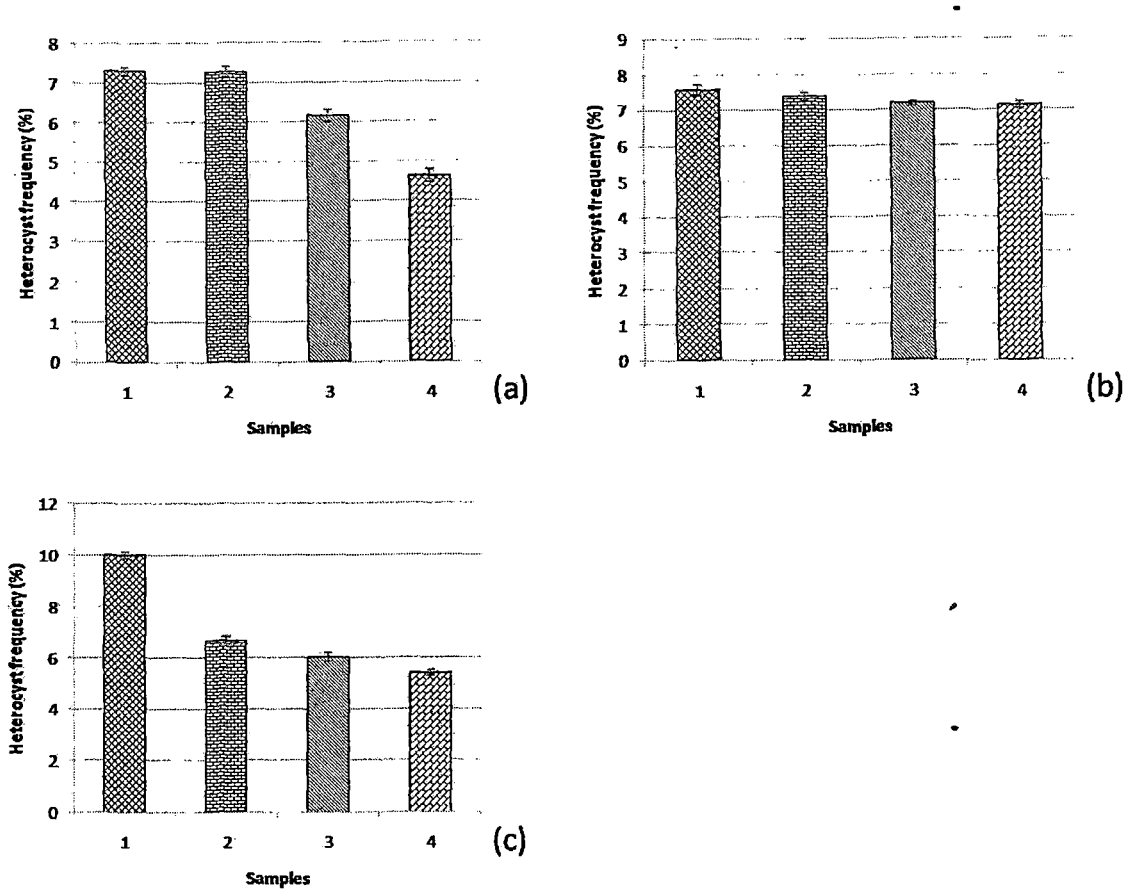


Fig 4.3: Heterocyst frequency of samples regenerated from agar flakes (a) *Nostoc muscorum* (b) *Anabaena variabilis* (c) *Mastigocladus laminosus [In fig. column 1 represents the control, column 2 ,3 and 4 represents samples regenerated at the end of one, two and three years respectively.*]

Fig 4.3 shows a comparison of the heterocyst frequency of the experimental samples regenerated at the end of first, second and third years of immobilization in agar flakes. In each case, the regenerated samples were compared to the free-living counterparts taken as control. It was seen that in case of *Nostoc muscorum* heterocyst frequency was comparable to its control in cells regenerated after one year of preservation (Fig 4.3 a). This was true for *Anabaena variabilis* as well (Fig 4.3 b). Heterocyst frequency was completely maintained in all *Anabaena variabilis* cells regenerated even after three years of preservation (Fig 4.3 b).

But, occurrence of double and multiple heterocysts were common in many regenerated filaments of *Anabaena variabilis*. In *Nostoc muscorum* and *Mastigocladus laminosus* regenerates a successive drop in the heterocyst frequency was seen as the period of preservation increased (Fig 4.3 a, c).

Fig 4.4 shows the nitrogenase activities of the regenerated cultures of the experimental cyanobacterial samples under study. A feature common to all the regenerated samples was the gradual drop in the nitrogenase activity with increase in the duration of the preservation time. While in *Nostoc muscorum* decline in the nitrogenase activity was between 30-65% (Fig 4.4 a), it was between 20-35% in *Anabaena variabilis* in the samples regenerated during the three years of preservation (Fig 4.4 b). Best nitrogenase activity was maintained in regenerated cells of *Plectonema boryanum* followed by *Gloeocapsa* sp. The decline of nitrogenase activity was between 4-25% in *Plectonema boryanum* when maintained in 12h light/dark cycle (Fig 4.4 d). This reduction was between 7-45% in *Gloeocapsa* sp. (Fig 4.4 e). Thus, looking at values of chl *a* and nitrogenase activity in regenerated cultures, *Plectonema boryanum* among the cyanobacteria studied performed best when preserved in agar flakes.

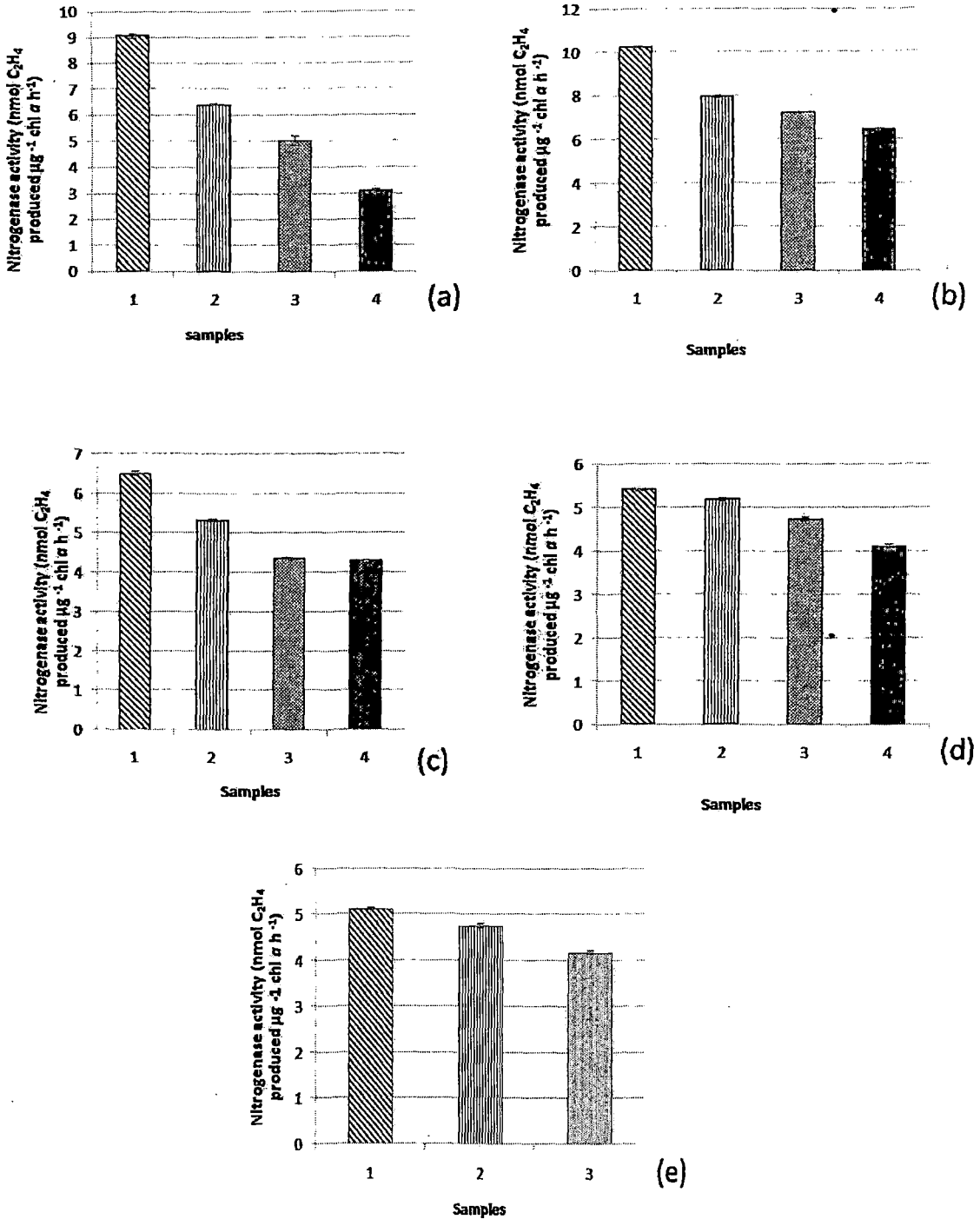


Fig 4.4: Nitrogenase activity of samples regenerated from agar flakes (a) *Nostoc muscorum*, (b) *Anabaena variabilis* (c) *Mastigocladus laminosus* (d) *Plectonema boryanum* and (e) *Gloeocapsa* sp. *[In fig. column 1 represents the control, column 2 ,3 and 4 represents samples regenerated at the end of one, two and three years respectively.]*

4.2.1.3 Photosynthesis and respiratory activities

Table 4.1 a: Photosynthesis and respiration of *Nostoc muscorum* immobilized in agar cubes.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	437 ± 3.1	427 ±2.9	413 ±3.2	395 ±2.5
2nd year	439 ±2.8	403 ±2.6	425 ±3.3	388 ±2.5
3rd year	446 ±2.6	397 ±2.6	417 ±3.1	317 ±1.9

Table 4.1 b: Photosynthesis and respiration of *Anabaena variabilis* immobilized in agar cubes.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	442 ±3.3	421 ±3.2	417 ±2.5	402 ±2.4
2nd year	440 ±3.5	421 ±3.2	415 ±2.5	375 ±2.4
3rd year	440 ±4.1	387 ±3.4	413 ±2.7	354 ±2.3

Table 4.1 c: Photosynthesis and respiration of *Mastigocladus laminosus* immobilized in agar cubes.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	281 ±4.1	247 ±3.5	398 ±2.3	346 ±2.5
2nd year	278±4.3	239 ±3.3	394 ±2.5	342 ±2.5
3rd year	281 ±4.2	229 ±3.3	390±2.7	332 ±2.7

Table 4.1 d: Photosynthesis and respiration of *Plectonema boryanum* immobilized in agar cubes.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	402 ± 2.8	387 ± 3.1	378 ±2.1	369 ±2.6
2nd year	400 ±2.8	385 ±3.0	373 ±2.1	354 ±2.4
3rd year	400 ±2.5	379 ±2.8	374 ±2.5	323 ±2.2

Table 4.1 e: Photosynthesis and respiration of *Gloeocapsa* sp. immobilized in agar cubes.

	Control	**Regenerated sample**	**Control**	**Regenerated sample**
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	396 ±2.1	393 ±2.5	342 ±3.2	345 ±3.3
2nd year	395 ±2.4	385 ±2.5	340 ±3.5	351 ±3.4

Table 4.1 a, b, c, d and e represents the oxygen evolution and consumption activities of the regenerated samples. As the duration of the storage period increased, a subtle drop in these activities was noticed in all the regenerated cultures. A comparison of these activities on day six in regenerated samples at the end of the third year of preservation revealed that in *Nostoc muscorum* photosynthetic activity was compromised by a value of ~10% while a ~25% decrease in respiratory oxygen consumption was seen in the same organism. In *Anabaena variabilis* both these activities were compromised by a ~12% decrease in oxygen evolution and ~14% in oxygen consumption activities. Similar trend was followed by regenerated cells of *Mastigocladus laminosus* (~18 % in oxygen evolution and ~15% in oxygen consumption activities) and *Gloeocapsa* sp. (~11% and ~7% in oxygen evolution and oxygen consumption activities respectively). *Plectonema boryanum* cells showed greater difference in these two activities (~5 % and ~14% respectively) thus, following the pattern as in *Nostoc muscorum*. Thus, in immediate regenerates of all samples, photosynthetic activity recovered better than the respiratory activity. Respiratory oxygen evolution in immediately regenerated cells was most affected in *Nostoc muscorum* when preserved in agar cubes.

4.2.1.4 Glutamine synthetase (transferase) (GS), nitrate reductase (NR) and nitrite reductase (NIR) activities

Table 4.2 a: Enzyme activities of *Nostoc muscorum* regenerated from agar cubes.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/ mg protein)	Control Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/m g protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	767 ± 5.5	2.4 ±0.1	547 ± 3.2	722 ±5.3	1.9 ±0.04	530 ±3.2
2nd year	763 ±5.3	2.3 ±0.13	542 ±3.1	715 ±5.1	1.2 ±0.03	529 ±3.3
3rd year	763 ±5.3	2.3 ±0.1	546 ±3.3	635 ±5.1	1.1 ±0.03	520 ±3.3

Table 4.2 b: Enzyme activities of *Anabaena variabilis* regenerated from agar cubes.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/ mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/m g protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	765 ± 5.6	2.5 ±0.11	554 ±3.4	712 ±5.6	1.6 ±0.07	532 ±2.5
2nd year	757 ±5.7	2.3 ±0.12	547 ±3.3	707 ±5.8	1.4 ±0.05	524 ±2.7
3rd year	763 ±5.5	2.3 ±0.11	545 ±3.1	654 ±5.8	1.4 ±0.05	521 ±2.4

Table 4.2 c: Enzyme activities of *Mastigocladus laminosus* regenerated from agar cubes.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/ mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/m g protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	2346 ± 123	8.79 ±1.3	157.43 ±1.4	2113 ±122	8.23 ±1.1	151.16 ±1.2
2nd year	2337 ± 122	8.71 ±1.2	152.42 ±1.2	2112 ±126	8.12 ±1.1	143.78 ±1.2
3rd year	2343 ± 125	8.71 ±1.5	151.21 ±1.2	1987 ±125	7.11 ±1.2	123.41 ±1.4

Table 4.2 d: Enzyme activities of *Plectonema boryanum* regenerated from agar cubes.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	***Control*** Nitrate reductase activity (nmol NO_2^- formed/min/ mg protein)	***Control*** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	***Regenerated sample*** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	***Regenerated sample*** Nitrate reductase activity (nmol NO_2^- formed/min/m g protein)	***Regenerated sample*** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	729 ±3.2	2.1 ±1	521 ±2.3	721 ±3.1	2.1 ±0.7	518 ±2.1
2nd year	729 ±3.4	2.1 ±0.8	520 ±2.2	720 ±2.9	1.8 ±0.9	505 ±2.1
3rd year	725 ±3.4	2.1 ±0.8	517 ±2.1	689 ±2.8	1.0 ±0.9	478 ±2.1

Table 4.2 e: Enzyme activities of *Gleocapsa sp.* regenerated from agar cubes.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/ mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/m g protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	329 ±3.1	1.1 ±0.5	221 ±1.1	320 ±3.8	1.1 ±0.4	220 ±1.1
2nd year	329 ±2.9	1.1 ±0.5	220 ±1.1	320 ±2.7	1.2 ±0.3	217 ±0.7

Table 4.2 a, b, c, d and e shows the enzyme activities of all the regenerated samples. Like all other biochemical characters preservation did affect various enzyme activities. A slight reduction in their activities was recorded in all samples directly regenerated from preserved agar. However, this decline in activities was not permanent as subsequent generations of cells showed comparable enzyme activities to their free-living controls.

4.3 Immobilization in calcium alginate beads

Exponentially growing cultures of cyanobacteria was used for immobilization in calcium alginate beads (details given in chapter 2). The alginic acid solution was made in BG-11_0 for entrapment of the heterocystous cyanobacteria, and supplemented BG-11_0 media (with 5mM $NaNO_3$ for the non-heterocystous ones). For *Mastigocladus laminosus*, D-medium was used). The dried beads were stored in sterile vials in room temperature and in dark (Fig 4.5 a). As in the case of agar, cells were regenerated from alginate beads every three months to study the performance of calcium alginate as preserving material for cyanobacteria.

a b

Fig 4.5: Immobilization of cyanobacteria in calcium alginate beads (a) large number of dried beads containing different cyanobacterial strains were stored in eppendorf tubes (b) regeneration of cyanobacteria from one year old dried beads. The picture was taken after eight days of transferring the beads to fresh medium.

4.3.1 Results

All cyanobacteria under study exhibited almost complete retention of characters when regenerated from immobilization in calcium alginate. Freshly regenerated samples showed slightly reduced activities which completely recovered upon transfer to new media. No significantly perceptible changes were observed in the preserved cells. The results presented here are for cultures that were regenerated after one, two and three years of preservation.

4.3.1.1 Growth

Filaments were seen emerging out of the alginate beads within 5-10 days after preserved beads were introduced into fresh media (Fig 4.5 b). The length of time under storage determined the time the cells took to regenerate into viable filaments. Beads introduced into medium after one year of preservation took ~5- 6 days to regenerate while it

took 8-10 days for two years old sample. Almost 12-14 days was required in case of samples that were kept for three years in the preserved state.

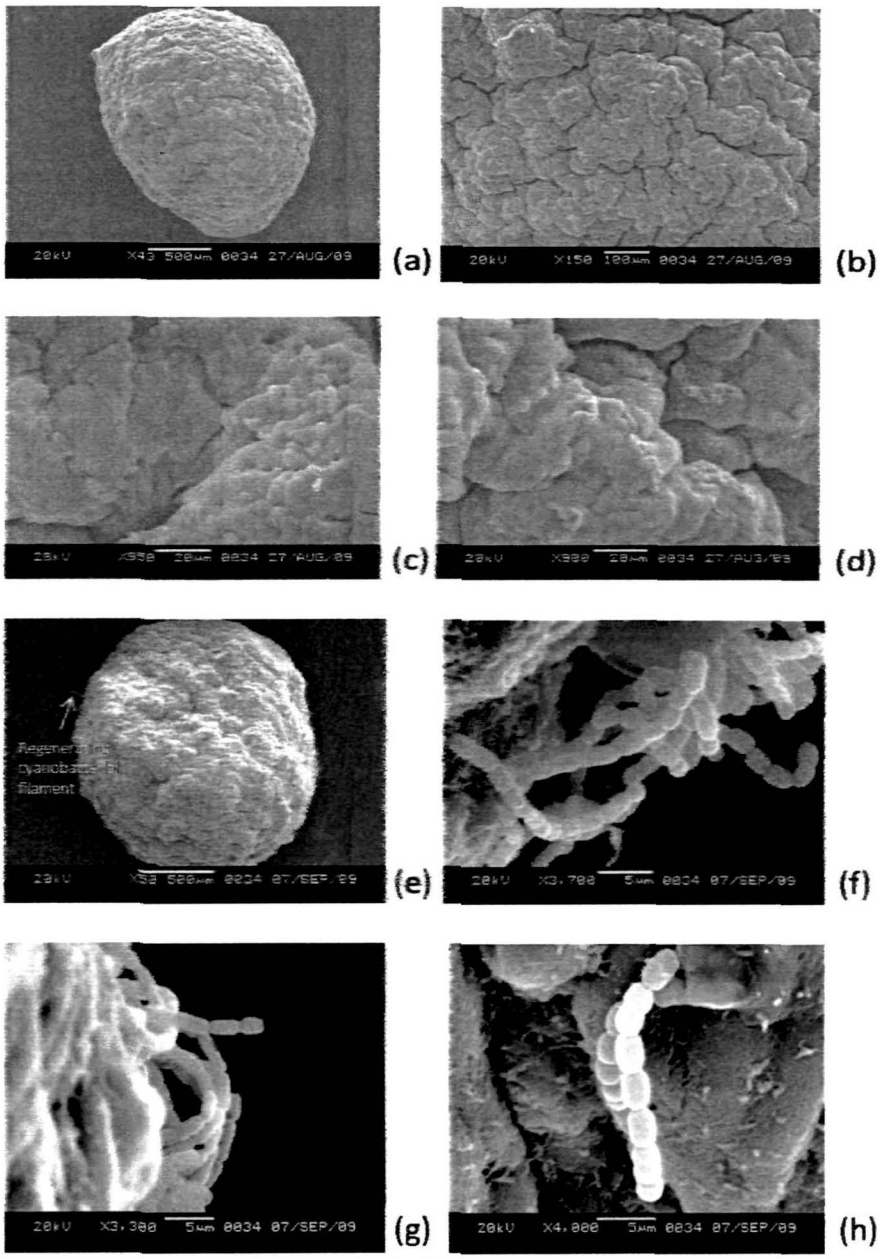


Fig 4.6: Scanning electron micrograph (JEOL JSM 6360, SAIF, NEHU) of cyanobacterial cells immobilized in calcium alginate bead (1 year old sample): (a, b) SEM of dried calcium alginate bead, (c, d) cyanobacterial filaments embedded within the matrix of a calcium alginate bead, (e) emerging cyanobacterial filament regenerating from a calcium alginate bead reinoculated into fresh media, (f, g, h) healthy regenerating cyanobacterial filaments seen emerging from calcium alginate bead.

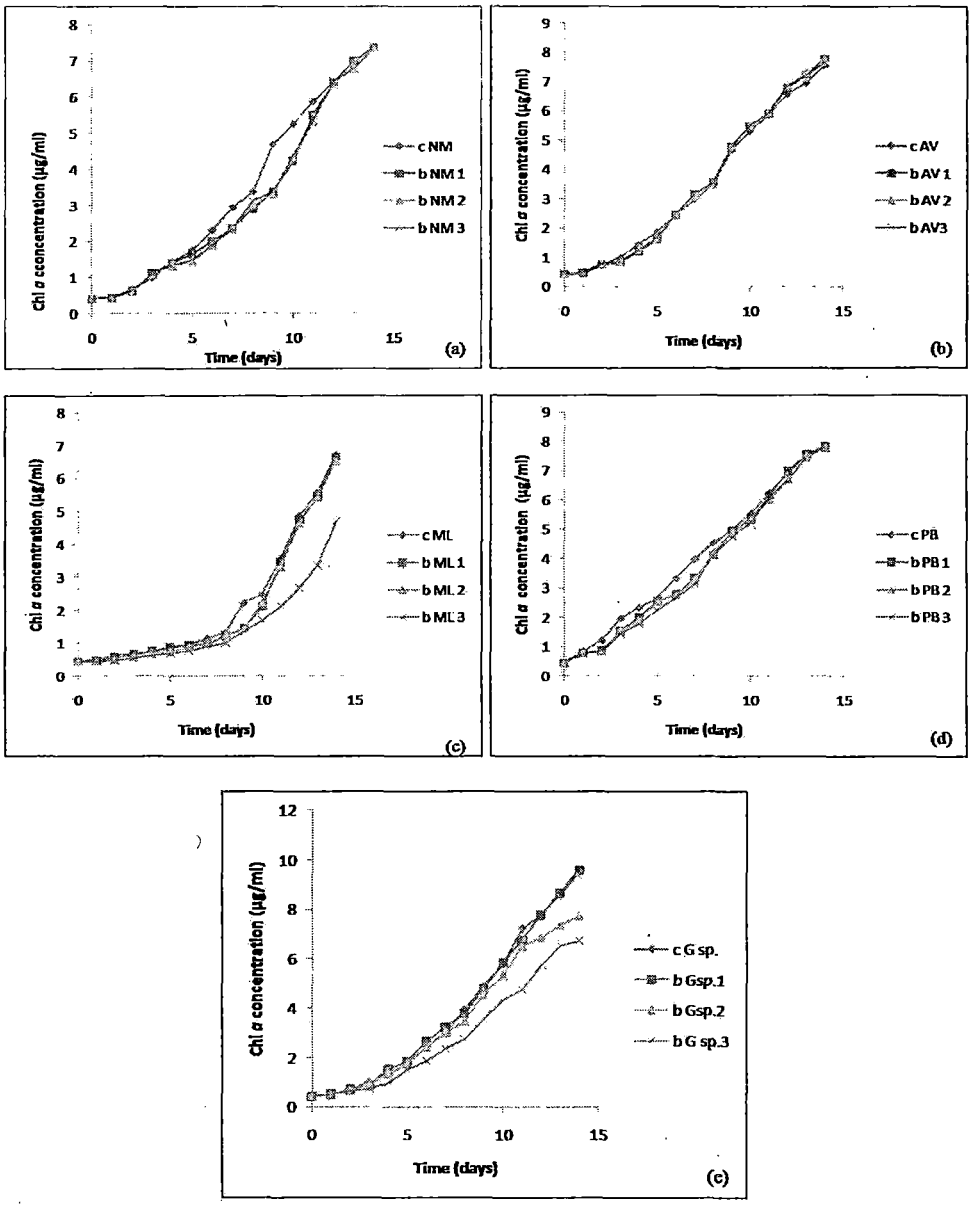


Fig 4.7: Growth curves of (a) *Nostoc muscorum* (NM) (b) *Anabaena variabilis* (AV) (c) *Mastigocladus laminosus* (ML) (d) *Plectonema boryanum* (PB) and (e) *Gloeocapsa* sp. (Gsp.) regenerated from immobilization in calcium alginate beads as compared to their free-living counterpart. [*In fig, "b" refers to calcium alginate beads and "1, 2, 3" indicates samples regenerated after first, second and third years of preservation. An initial inoculum of 0.4µg/ml was used*].

Fig 4.6 shows scanning electron micrographs of the different stages of regeneration of *Nostoc* from filaments immobilized in calcium alginate beads after one year of storage. A closer look at the dried bead showed dehydrated cyanobacterial filaments embedded in the inner matrix of the calcium alginate bead (Fig 4.6 c, d). When introduced in the suitable liquid media the dried beads swelled up and green cyanobacterial filaments were seen emerging out of the beads into the media (Fig 4.6 e). Profuse growth of healthy filaments was noticed within a week to ten days after the beads were introduced in fresh media (Fig 4.6 f, g h).

Chlorophyll *a* was considered as a parameter in measuring growth in cyanobacteria. Fig 4.7 gives detailed growth profiles of all the cyanobacteria used for the study. In *Anabaena variabilis* and *Plectonema boryanum*, chlorophyll *a* values were identical in samples regenerated after one, two and three years of preservation to their control cultures. In *Nostoc muscorum*, the lag period was slightly longer but the growth was similar to its control culture by day 12. In *Mastigocladus laminosus* and in *Gloeocapsa* sp. growth of regenerated samples after 1st and 2nd years of preservation was similar to their free-living cultures. However, a minor decline was seen in the growth of cultures after three years of preservation. All regenerated cultures showed complete recovery in the very next sub culture.

4.3.1.2 Heterocyst frequency and nitrogenase activity

Fig 4.8 gives an idea about heterocyst frequency of the regenerated cultures. The heterocyst frequency was maintained at the value of free-living cultures in *Anabaena variabilis* and *Nostoc muscorum*. In *Mastigocladus laminosus*, a drop of ~10% was noticed in the sample regenerated after three years.

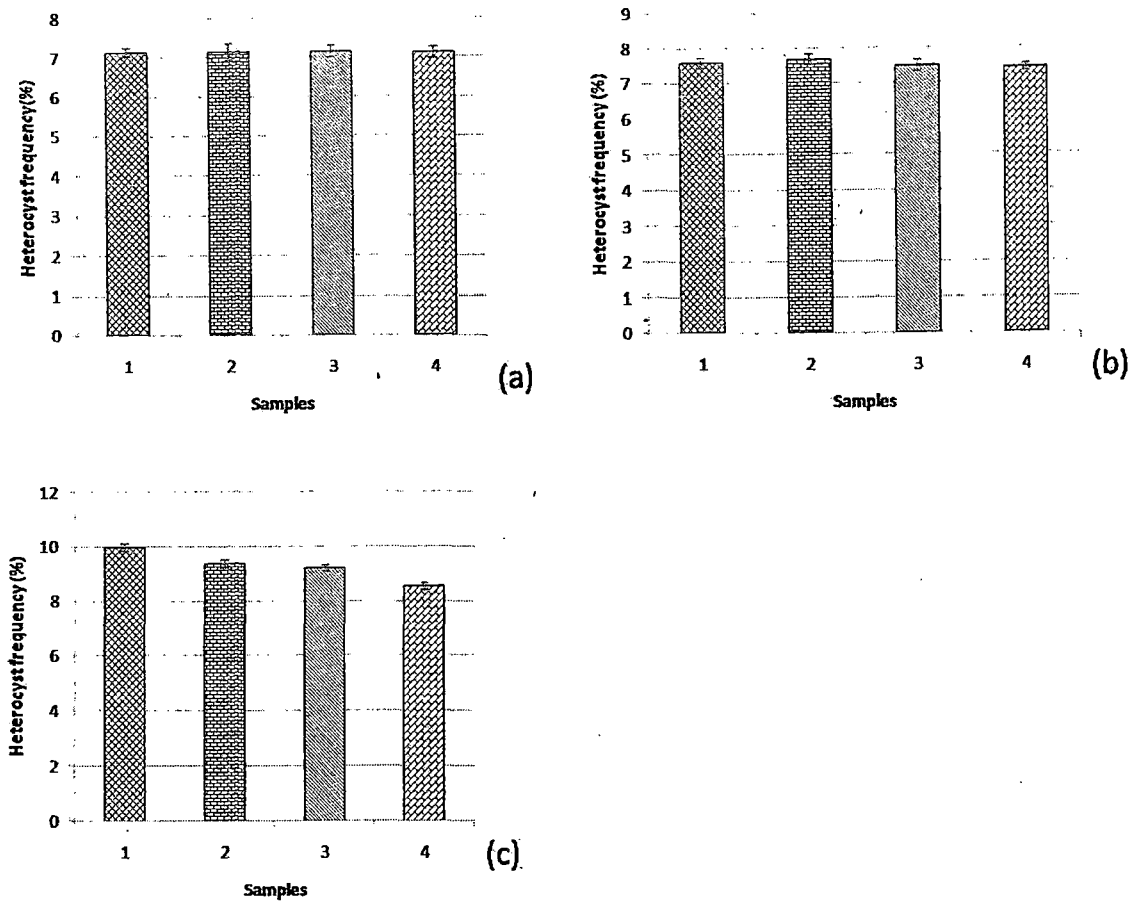


Fig 4.8: Heterocyst frequency of samples regenerated from calcium alginate beads (a) *Nostoc muscorum* (b) *Anabaena variabilis* (c) *Mastigocladus laminosus [In fig. column 1 represents the control, column 2 ,3 and 4 represents samples regenerated at the end of one, two and three years respectively.]*

Nitrogenase activity was maintained at their control value in regenerates of *Nostoc muscorum* and *Mastigocladus laminosus.* Decline in nitrogenase activities of *Anabaena variabilis, Plectonema boryanum* and *Gloeocapsa* sp. were ~5%. It seems that immobilization in calcium alginate beads has similar stabilizing effects on all the cyanobacteria and calcium alginate as a preserving material present less stress on the immobilized organisms (Fig 4.9).

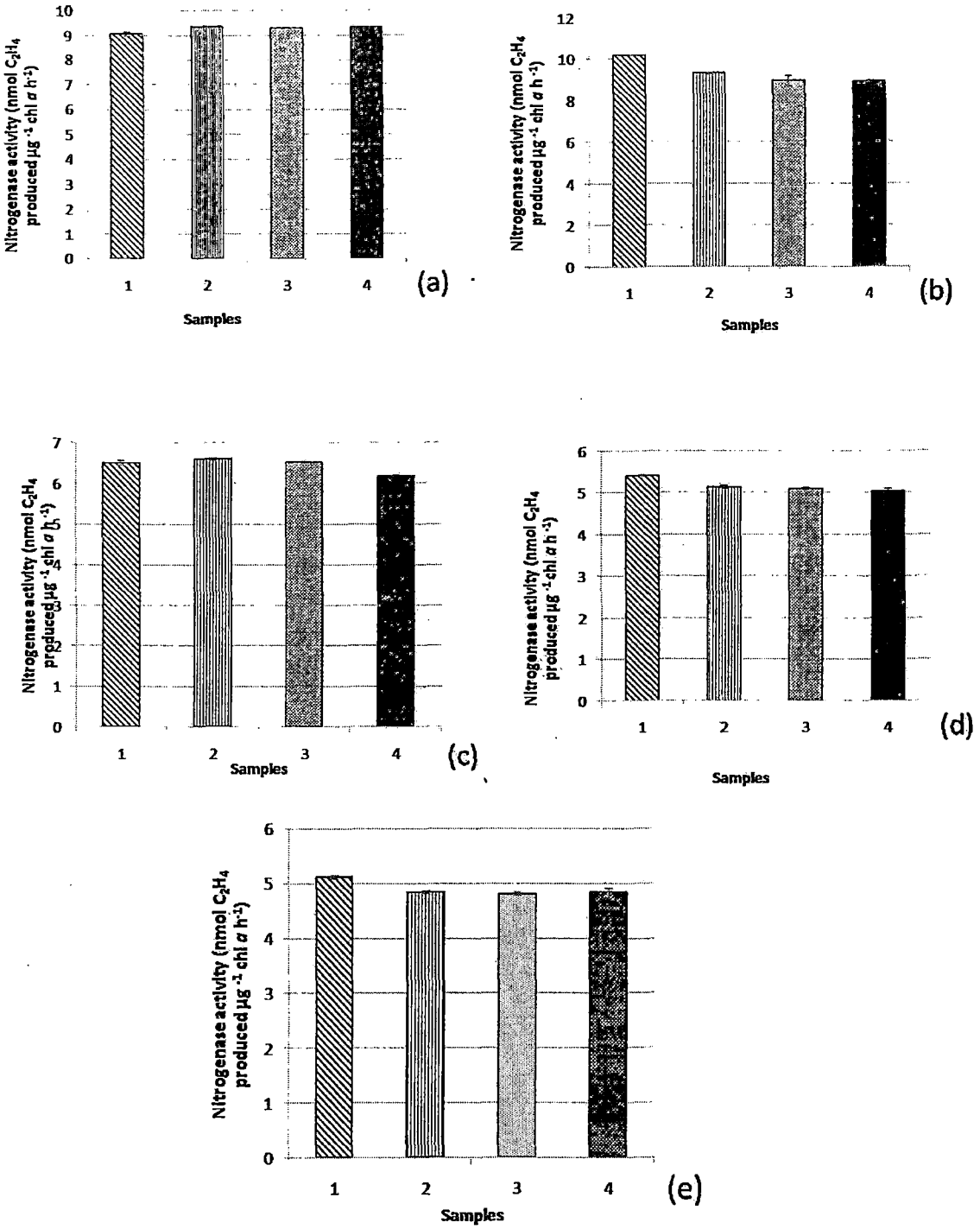


Fig 4.9: Nitrogenase activity of samples regenerated from calcium alginate beads (a) *Nostoc muscorum* (b) *Anabaena variabilis* (c) *Mastigocladus laminosus* (d) *Plectonema boryanum* and (e) *Gloeocapsa* sp. *[In fig. column 1 represents the control, column 2 ,3 and 4 represents samples regenerated at the end of one, two and three years respectively.]*

4.3.1.3 Photosynthetic and respiratory activities

When photosynthetic and respiratory activities of the immediately regenerated samples were compared with their free-living counterparts, following observations were noticed. In *Nostoc muscorum* respiration was more affected than its photosynthesis (Table 4.3 a). In *Anabaena variabilis* both photosynthetic oxygen evolution and respiratory oxygen consumption values were maintained close to their control cultures in all three years (Table 4.3 b). In *Plectonema boryanum* the photosynthetic activity was more affected than its respiratory activity (Table 4.3c). In *Mastigocladus laminosus* both respiration and photosynthesis exhibited a drop close to ~15% (Table 4.3 d). In *Gloeocapsa* sp. a reduction was observed only on the third year of preservation (Table 4.3 e). Even though we observed decline in these activities in the immediate cultures regenerated from dehydrated state after prolonged storage, we must mention that none of these activities were compromised in a long term basis. All activities approached their original values in succeeding sub cultures.

Table 4.3 a: Photosynthesis and respiration of *Nostoc muscorum* immobilized in calcium alginate beads.

	Control	**Regenerated sample**	**Control**	**Regenerated sample**
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	437 ±3.1	432 ±3.1	413 ±3.2	397 ±2.3
2nd year	439 ±3.0	427 ±2.7	415 ±3.2	388 ±2.3
3rd year	439 ±2.8	420 ±2.8	413 ±3.3	380 ±2.1

Table 4.3 b: Photosynthesis and respiration of *Anabaena variabilis* immobilized in calcium alginate beads.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	440 ±3.1	435 ±3.2	423 ±2.3	425 ±2.2
2nd year	435 ±3.3	437 ±3.1	420 ±2.2	423 ±2.2
3rd year	435 ±3.4	432 ±3.1	420 ±2.1	424 ±2.1

Table 4.3 c: Photosynthesis and respiration of *Plectonema boryanum* immobilized in calcium alginate beads.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	402 ±2.7	385 ±3.1	378 ±2.1	377 ±2.5
2nd year	400 ±2.6	379 ±3.1	373 ±2.3	369 ±2.4
3rd year	400 ±2.6	377 ±2.7	374 ±2.3	370 ±2.2

Table 4.3 d: Photosynthesis and respiration of *Mastigocladus laminosus* immobilized in calcium alginate beads.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	280.55 ±4.1	242.11 ±3.3	397.56 ±2.5	346.17 ±2.7
2nd year	277.56 ±4.2	231.12 ±3.1	394.15 ±2.6	333.37 ±2.7
3rd year	280.14 ±4.3	218.16 ±3.1	390.42 ±2.5	324.14 ±2.7

Table 4.3 e: Photosynthesis and respiration of *Gloeocapsa* sp. immobilized in calcium alginate beads.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	396 ±2.1	387 ±2.3	342 ±3.2	340 ±3.3
2nd year	395 ±2.0	385 ±2.5	340 ±3.3	354 ±3.5
3rd year	395 ±2.0	379 ±2.6	340 ±3.3	323 ±3.5

4.3.1.4 Glutamine synthetase (transferase) (GS), nitrate reductase (NR) and nitrite reductase (NIR) activities

Table 4.4 illustrates the retention of enzymatic activities such as GS, NR and NIR in regenerated samples from calcium alginate beads. The stress due to prolonged dehydration as a result of being immobilized manifested in partial reduction in these enzyme activities. The values presented in the table are those of the cultures regenerated directly from one, two and three years of immobilization. As the cells started to regenerate into viable filaments under optimum conditions, the enzyme activities recorded were yet to reach their maximum value of free-living cultures. These activities were estimated on day eight. All activities were lower (10-20%) at this point of time, however, they reached their original values in subsequent sub cultures. Thus, the adverse effects of immobilization were not irreversible in nature in these cyanobacteria under study. NR activity was most stable and showed least effect of immobilization in almost all the samples. Maintaining NR activity to its original value may be important as a survival tactic as this enables the organism (s) to utilize available nitrate in the surrounding as immediate source of nitrogen for growth and cell division. This feature was not seen in *Anabaena variabilis*. There was 20-40% inhibition of

NR activity in this organism. In *Mastigocladus laminosus*, NR activity was 3-4 fold higher than rest of the organisms studied.

Table 4.4 a: Enzyme activities of *Nostoc muscorum* regenerated from calcium alginate beads.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	767 ±5.3	2.4 ±0.1	547 ±3.2	735 ±5.2	2.4 ±0.04	540 ±2.8
2nd year	763 ±5.2	2.3 ±0.1	542 ±3.2	727 ±5.3	2.1 ±0.07	532 ±3.1
3rd year	763 ±5.2	2.3 ±0.8	546 ±3.1	727 ±5.2	2.1±0.07	532 ±3.1

Table 4.4 b: Enzyme activities of *Anabaena variabilis* regenerated from calcium alginate beads.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	765 ±5.5	2.5 ±0.11	554 ±3.2	726 ±5.6	1.8 ±0.07	541 ±2.3
2nd year	757 ±5.3	2.3 ±0.12	547 ±3.2	724 ±5.5	1.5 ±0.09	540 ±2.5
3rd year	763 ±5.3	2.3 ±0.12	545 ±3.3	720 ±5.4	1.4 ±0.09	540 ±2.5

Table 4.4 c: Enzyme activities of *Plectonema boryanum* regenerated from calcium alginate beads.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	729 ±3.3	2.1 ±1.1	521 ±2.6	720 ±3.1	2.1 ±0.7	515 ±2.0
2nd year	729 ±3.5	2.1 ±0.8	520 ±2.5	720 ±2.7	2.1 ±0.6	510 ±1.8
3rd year	725 ±3.5	2.1 ±0.8	517 ±2.5	715 ±2.8	1.7 ±0.5	510 ±1.7

Table 4.4 d: Enzyme activities of *Mastigocladus laminosus* regenerated from calcium alginate beads.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	2346 ±124	8.79 ±1.1	157.43 ±1.5	2326 ±122	8.54 ±1.1	150.14 ±1.2
2nd year	2337 ±125	8.71±1.3	152.42 ±1.3	2324 ±125	8.52 ±1.3	150.12 ±1.3
3rd year	2343 ±122	8.71±1.3	151.21±1.3	2320 ±122	8.52 ±1.1	143.54 ±1.3

Table 4.4 e: Enzyme activities of *Gleocapsa* sp. regenerated from calcium alginate beads.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	329 ±3.1	1.1±0.5	221 ±1.1	323 ±3.1	1.1 ±0.8	220 ±2.1
2nd year	329 ±2.8	1.1±0.5	220 ±1.1	320 ±2.8	1.1 ±0.5	215 ±2.1
3rd year	325 ±2.5	1.1±0.3	217 ±0.8	289 ±2.8	1.0 ±0.5	216 ±2.1

4.4 Immobilization in foam cubes

Immobilization of cyanobacteria in foam has been reported in 1989 by Brouers et al. Kannaiyan and co workers have reported the use of polyurethane as a immobilizing base for cyanobacteria with evidence of better performance by such immobilized cyanobacterial cells (Kannaiyan et al 1992; Mahesh and Kannaiyan 1993; Kannaiyan et al 1994; Kannaiyan et al 1997). Therefore, we have included foam in our preservation study to see if immobilization on foam is a better option for long time storage of cyanobacteria. Preparation for preservation on foam was carried out as described in the chapter 2 (Fig 4.10a). After drying, the foam cubes along with cyanobacteria were stored in sterile containers. At every interval of three months, some of the cubes were regenerated in the liquid medium (Fig 4.10b) and various characters were assessed and compared to the control culture to establish the performance of foam as preserving material for cyanobacteria.

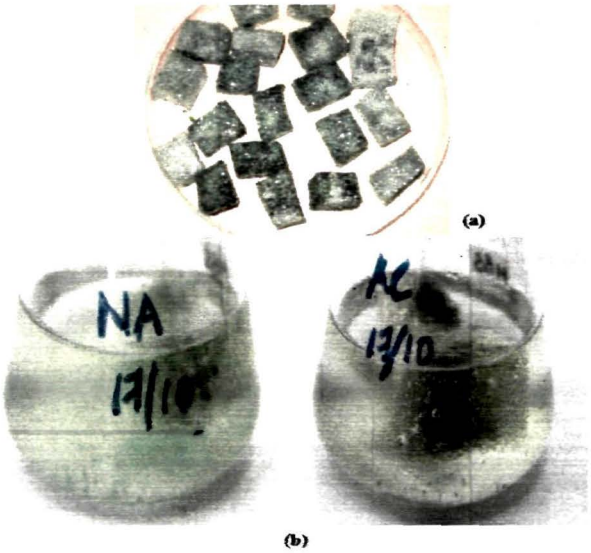


Fig 4.10: Immobilization in foam cubes. (a) cubes inoculated with fresh cyanobacterial culture (b) regeneration of cyanobacteria from one year old dried foam. The picture was taken after two days of transferring the cubes to fresh medium.

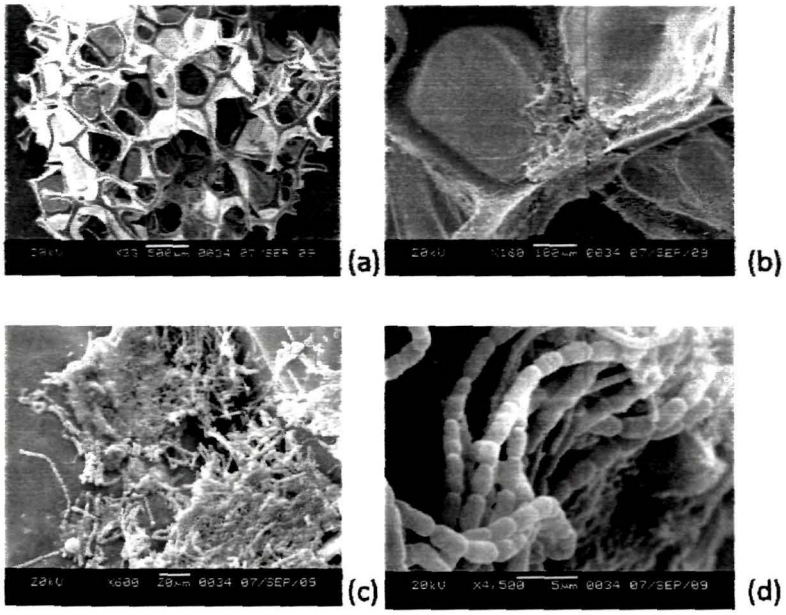


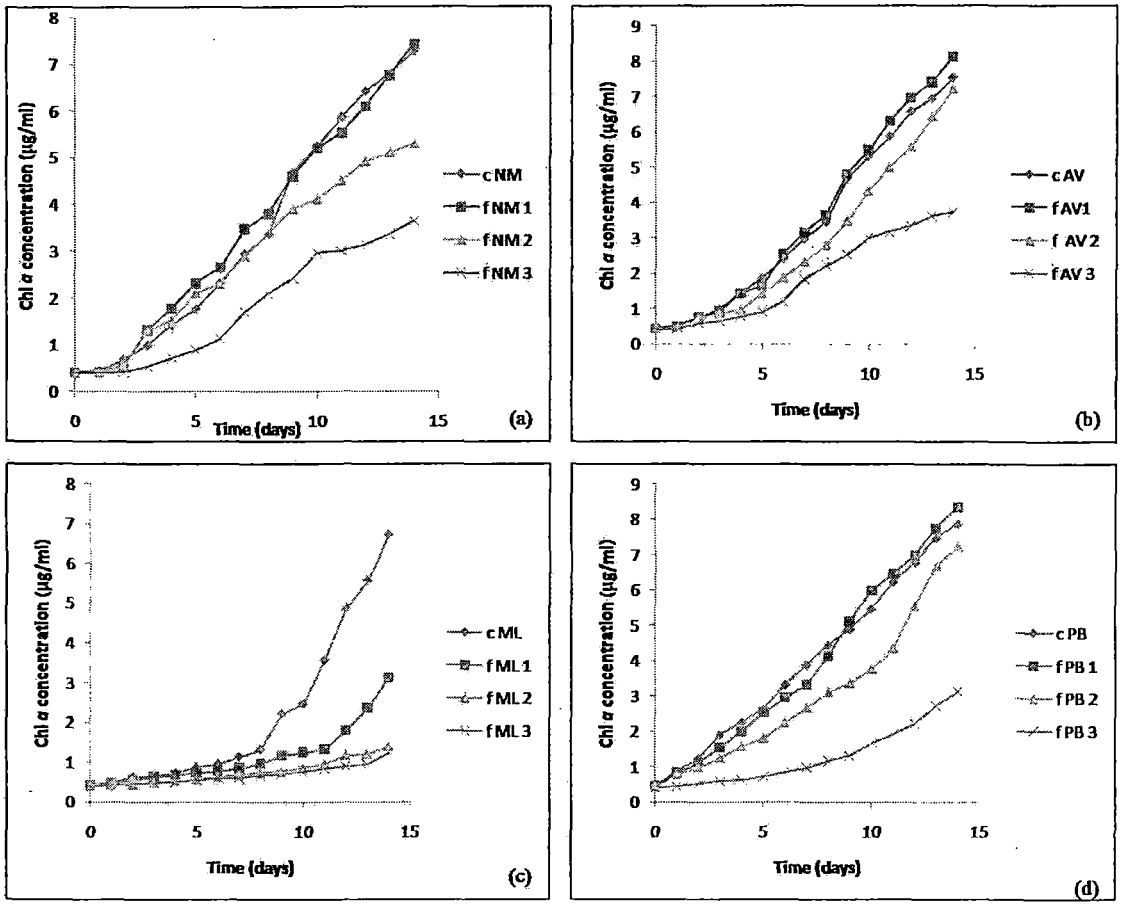
Fig 4.11: Scanning electron micrograph (JEOL JSM 6360, SAIF, NEHU) of cyanobacterial filaments immobilized on foam cubes (a) matrix of a foam cube as seen under SEM (b,c) adhered cyanobacterial filaments seen regenerating upon return of favorable growth conditions (d) healthy cyanobacterial filaments obtained filaments.

Fig 4.11 shows scanning electron micrographs of the various stages of cyanobacterial regeneration from foam cubes after one year of storage. The foam matrix has

large pores (Fig 4.11a) and cyanobacteria can only adhere to the foam material in concentrated amount (Fig 4.11b). When reintroduced into liquid media dried cells immediately started to grow into viable filaments. Fig 4.11c shows both viable and dried filaments on the foam matrix one day after the foam cubes were put in fresh medium. Morphologically healthy filaments were found regenerating from foam after four days.

4.4.1 Results

4.4.1.1 Growth



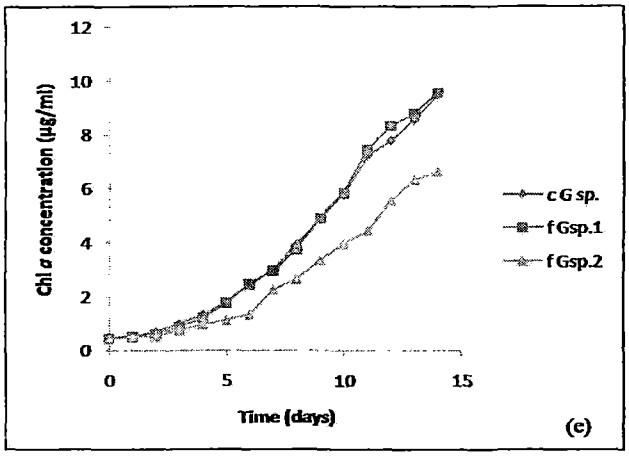


Fig 4.12: Growth curves of (a) *Nostoc muscorum* (NM) (b) *Anabaena variabilis* (AV) (c) *Mastigocladus laminosus* (ML) (d) *Plectonema boryanum (PB)* and (e) *Gloeocapsa* sp. (Gsp) regenerated from immobilization in foam cubes as compared to their free-living counterpart. *[In fig., "f" refers to foam cubes and "1, 2, 3" indicates samples regenerated after first , second and third years of preservation. An initial inoculum of 0.4µg/ml was used.]*

Growth was measured as increase in chlorophyll *a* content in all regenerated samples. When compared, cells growing out after one year of preservation showed a growth comparable to control cultures in all cases except *Mastigocladus laminosus*. Growth was also very close to control culture after second year of preservation in these cyanobacteria. Cells regenerated after third year showed reduced growth, however upon subsequent sub-culturing they showed complete recovery. In growing *Mastigocladus laminosus*, the lag period was always more extended than in other cyanobacteria. Foam did not do very well in preserving *Mastigocladus laminosus* cells. There was definitive reduction in growth of *Mastigocladus laminosus* even after first year of preservation. But it is worth mentioning here that *Mastigocladus laminosus* was stored at room temperature as was the case with other cyanobacterial samples, as well as at 45°C as it was a thermophile. Storing *Mastigocladus laminosus* at different temperatures however did not have any effect on the regeneration pattern of *Mastigocladus laminosus*. *Gloeocapsa* sp. showed comparable

growth after one year of preservation and reduction in growth was similar to other cyanobacteria after second year. However, in this cyanobacterium, cells did not grow out at all after three years of storage. Thus, foam can be regarded as potentially good preserving material for atleast a period of two years for the cyanobacteria that were studied except for *Mastigocladus laminosus*.

4.4.1.2 Heterocyst frequency and nitrogenase activity

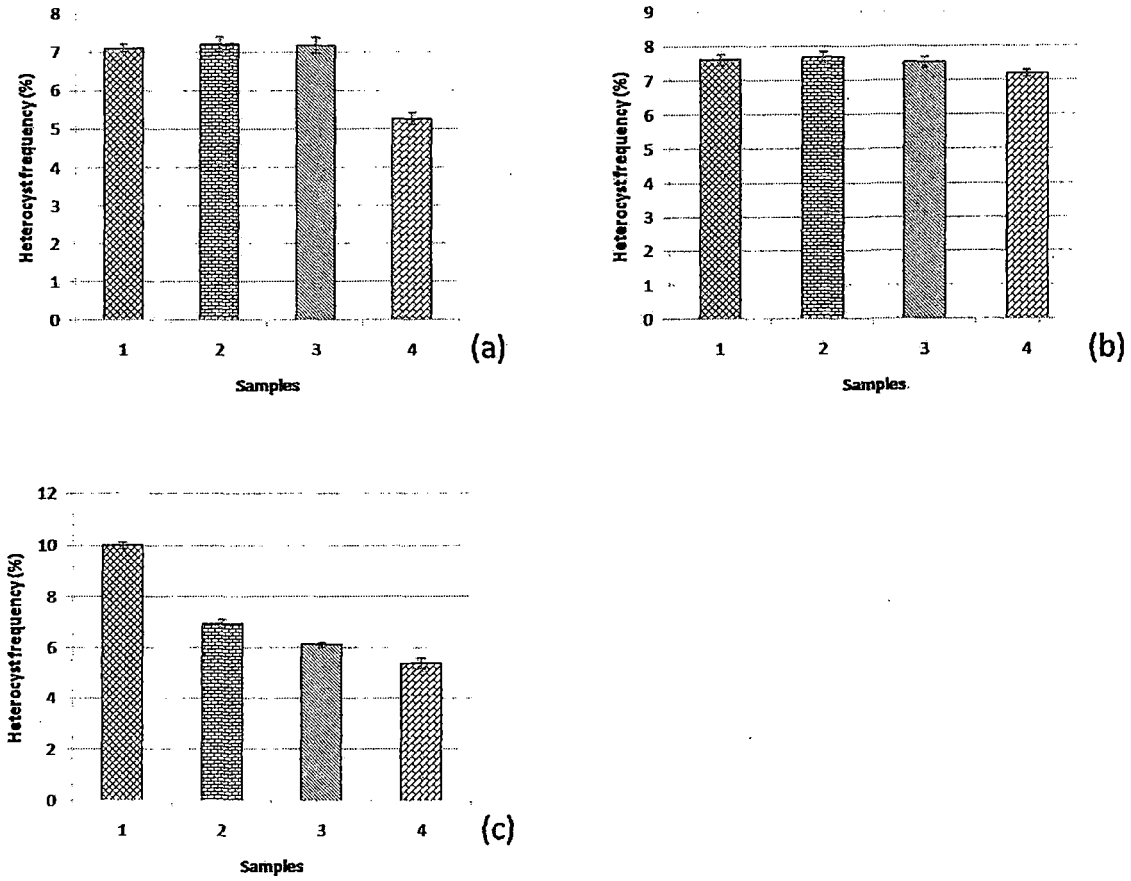
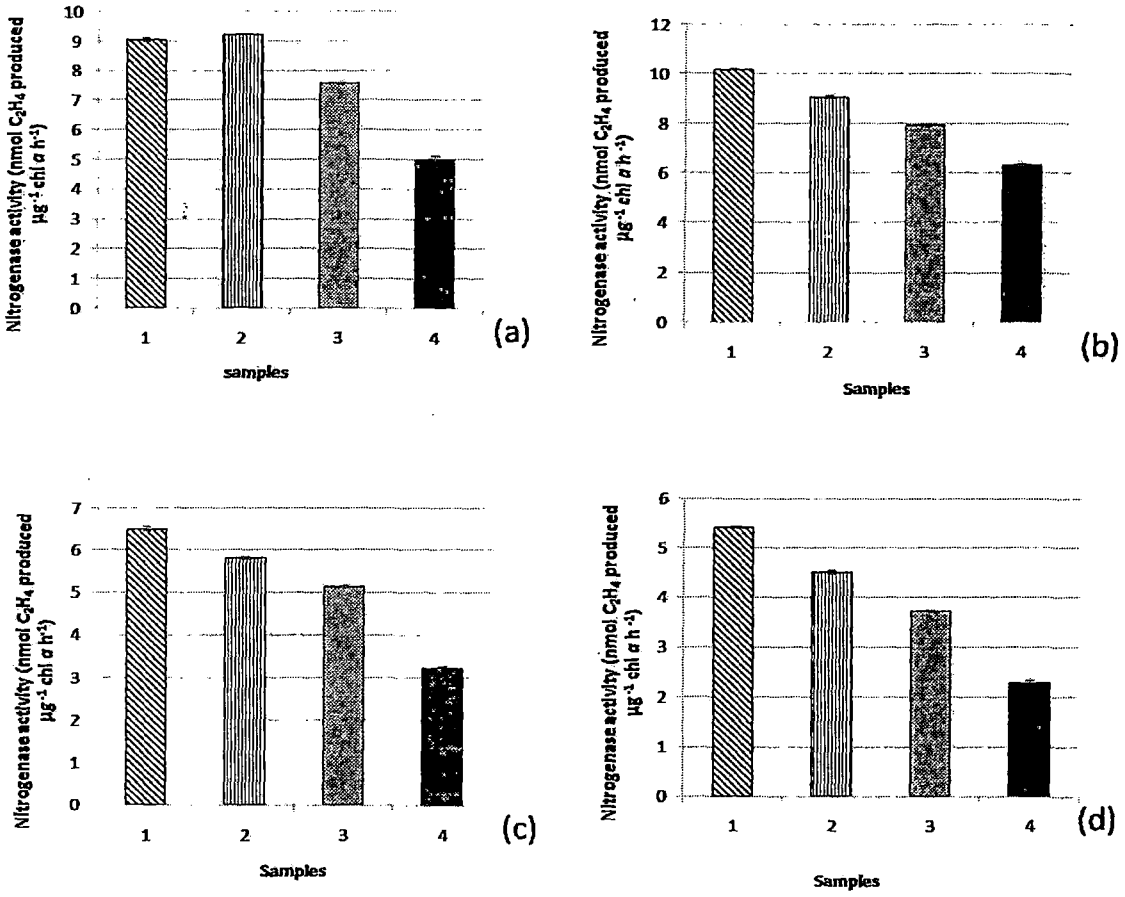


Fig 4.13: Heterocyst frequency of samples regenerated from foam cubes (a) *Nostoc muscorum* (b) *Anabaena variabilis* (c) *Mastigocladus laminosus [In fig. column 1 represents the control, column 2 ,3 and 4 represents samples regenerated at the end of one, two and three years respectively.]*

Heterocyst frequency in *Nostoc muscorum* and *Anabaena variabilis* were maintained in the level of their control cultures in the regenerated samples both after first and second

year of storage (Fig. 4.13). However, in case of *Mastigocladus laminosus*, heterocyst frequency was reduced by ~31% after first year and by ~39% after second year. Nitrogenase activity in immediate regenerates showed a gradual decrease in all cyanobacteria except in *Nostoc muscorum* where this activity was a complete match to that of control in the cells regenerated after first year of storage. This drop in nitrogenase activity in these samples may account for the reduction of growth seen in these cultures. Both heterocyst frequency and nitrogenase activity fully recovered in subsequent cultures (Fig. 4.14).



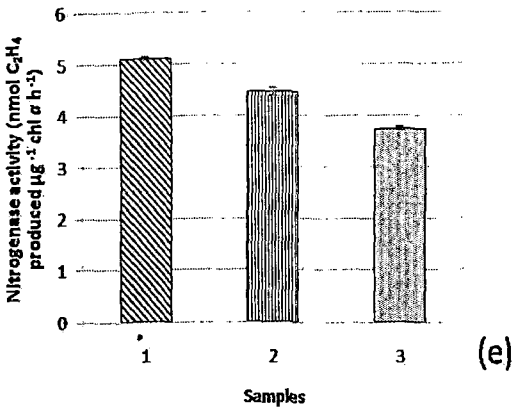


Fig 4.14: Nitrogenase activity of samples regenerated from foam cubes: (a) *Nostoc muscorum* (b) *Anabaena variabilis* (c) *Mastigocladus laminosus* (d) *Plectonema boryanum* and (e) *Gloeocapsa* sp. *[In fig. column 1 represents the control, column 2 ,3 and 4 represents samples regenerated at the end of one, two and three years respectively.]*

4.4.1.3 Photosynthetic and respiratory activities

A comparison of photosynthetic oxygen evolution and respiratory oxygen consumption between regenerated and control culture showed results similar to the ones obtained in case of agar and calcium alginate preservation. There are very small fluctuations in these activities between the two sets of samples. However in the case of *Mastigocladus laminosus*, there was a reduction by ~13% to 23% in photosynthetic activity and ~13% to 17% in respiratory activity from first year through to third year in regenerated samples (Table 4.5).

Table 4.5 a: Photosynthesis and respiration of *Nostoc muscorum* immobilized in foam cubes.

	Control	**Regenerated sample**	**Control**	**Regenerated sample**
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl a h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl a h^{-1})	
1st year	437 ±3.3	444 ±2.7	413 ±3.5	411 ±2.2
2nd year	439 ±2.8	424 ±2.3	415 ±3.3	397 ±2.1
3rd year	439 ±2.8	420 ±2.3	413 ±3.1	378 ±2.1

Table 4.5 b: Photosynthesis and respiration of *Anabaena variabilis* immobilized in foam cubes.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	442 ±3.2	437 ±3.2	417 ±2.3	410 ±2.1
2nd year	440 ±3.1	425 ±3.3	415 ±2.5	395 ±2.2
3rd year	440 ±3.1	415 ±3.3	413 ±2.7	367 ±2.4

Table 4.5 c: Photosynthesis and respiration of *Plectonema boryanum* immobilized in foam cubes.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	402 ±2.5	374 ±2.7	378 ±2.1	377 ±2.5
2nd year	400 ±2.3	357 ±2.3	373 ±2.1	346 ±2.3
3rd year	400 ±2.3	332 ±2.1	374 ±2.2	321 ±2.3

Table 4.5 d: Photosynthesis and respiration of *Mastigocladus laminosus* immobilized in foam cubes.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	280.55 ±3.9	242.11 ±3.5	397.56 ±2.3	346.17 ±2.1
2nd year	277.56 ±3.3	231.12 ±3.2	394.15 ±2.5	333.37 ±2.2
3rd year	280.14 ±3.4	218.16 ±3.3	390.42 ±2.1	324.14 ±2.2

Table 4.5 e: Photosynthesis and respiration of *Gloeocapsa* sp. immobilized in foam cubes.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	396 ±1.7	393 ±2.3	342 ±3.4	345 ±3.3
2nd year	395 ±1.6	385 ±2.3	340 ±3.3	351 ±3.2

4.4.1.4 Glutamine synthetase (transferase) (GS), nitrate reductase (NR) and nitrite reductase (NIR) activities

Table 4.6 a: Enzyme activities of *Nostoc muscorum* regenerated from foam.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	767 ±5.3	2.4±0.1	547±3.1	745 ±5.1	2.4 ±0.07	531 ±3.1
2nd year	763 ±5.2	2.3±0.13	542±3.1	635 ±5.0	2.0 ±0.07	520 ±3.1
3rd year	763 ±5.2	2.3±0.9	546±2.8	600 ±5.0	1.5 ±0.08	500 ±3.0

Table 4.6 b: Enzyme activities of *Anabaena variabilis* regenerated from foam.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	765 ±5.5	2.5 ±0.98	554 ±3.3	762 ±5.5	2.2 ±0.9	523 ±3.1
2nd year	757 ±5.4	2.3 ±0.11	547 ±3.2	727 ±5.6	1.6 ±0.78	542 ±3.1
3rd year	763 ±5.5	2.3 ±0.14	545 ±3.2	654 ±.5	1.1 ±0.79	500 ±3.0

Table 4.6 c: Enzyme activities of *Plectonema boryanum* regenerated from foam.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	729 ±3.1	2.1 ±0.7	521 ±2.5	720 ±3.2	2.1 ±0.5	517 ±2.1
2nd year	729 ±3.3	2.1 ±0.7	520 ±2.4	703 ±3.5	1.5 ±0.5	479 ±2.0
3rd year	725 ±3.5	2.1 ±0.5	517 ±2.5	654 ±3.5	1.0 ±0.3	432 ±2.0

Table 4.6 d: Enzyme activities of *Mastigocladus laminosus* regenerated from foam.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	2346 ±125	8.79 ±1.5	157.43 ±1.5	2124 ±122	8.12 ±1.2	142.16 ±1.2
2nd year	2337 ±123	8.71±1.3	152.42 ±1.5	2017 ±125	8.12 ±1.1	137.78 ±1.1
3rd year	2343 ±123	8.71±1.3	151.21 ±1.5	1794 ±127	7.53 ±1.2	126.41 ±1.5

Table 4.6 e: Enzyme activities of *Gloeocapsa sp.* regenerated from foam.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	329 ±3.3	1.1 ±0.3	221 ±1.1	321 ±3.1	1.1 ±0.5	220 ±1.1
2nd year	329 ±2.9	1.1 ±0.5	220 ±1.3	319 ±3.5	1.1 ±0.5	215 ±1.1

Table 4.6 illustrates the retention of GS, NR and NIR activities in cyanobacterial samples regenerated from foam. There is partial reduction in these enzyme activities that could be due to dehydration when immobilized cells were dried on foam and stored for a prolonged period. Eighth day observations are given in the table 4.6. All activities were lower (7-15%) but the downward plunge in these activities was not permanent and all these activities reached their original values on subsequent sub culturing. NR activity in *Gloeocapsa* sp. showed least effect of immobilization and prolonged storage.

4.5 Cryopreservation using 15% glycerol and 5% DMSO

A number of low molecular weight neutral solutes have been identified as potential cryoprotectants since the early 1950's.The most common among them being glycerol and dimethylsulfoxide (DMSO). Such compound lower the temperature at which freezing occurs and can alter the crystal habit of ice when it separates. It is thought that the colligative property of the cryoprotectants minimizes the deleterious action of excessive concentration resulting from removal of water and conversion of water to ice (Nash 1966). The cryoprotectants generally used for freezing biological specimens fall into two catagories, permeating and non- permeating. DMSO and glycerol are two most frequently used

permeating additives. DMSO permeates into cells more rapidly than glycerol and thus requires shorter treatment duration. However, in regards to toxicity, glycerol is less toxic than DMSO at equimolar concentrations, while at the same time, DMSO is superior to glycerol in cryoprotection of living tissues. Generally a concentration of 5-10% DMSO and 10-20% glycerol is adequate for most materials (Kartha and Engelmann 1994).

For the present study, cyanobacterial isolates were preserved in 15% glycerol and 5% DMSO. When kept inside Heto ultrafreeze below -80°C, all cyanobacteria under study stayed preserved for three years. Initially, all regenerated samples showed the characteristics reduction in activities in their various attributes that were investigated. These reached their optimum value in subsequent generations and became comparable to their free-living cultures. These were maintained in freezer of domestic refrigerators. This was done in an attempt to deduce whether ordinary refrigeration systems can be employed for long term storage and preservation of cyanobacteria using cryoprotectants. This would make preservation user friendly by reducing the cost required for maintaining samples in expensive ultra cooling systems.

4.5.1 Results

4.5.1.1 Growth, heterocyst frequency and nitrogenase activity

Growth was measured as increase in chlorophyll *a* concentration. With the temperature that was employed along with the use of cryoprotectants, the observations showed that the lag period of growth increased with subsequent increase in the years of preservation (Fig. 4.15). Samples regenerated after three years of preservation had an extended lag period of almost 15 days for all the cyanobacteria studied. *Mastigocladus* recorded the worst performance. When preserved using glycerol as cryoprotectant, growth

was reduced by ~64% on the 10th day in samples regenerated after one year of preservation. This had increased to ~69% when preserved for yet another year. Almost similar effects were seen when 5% DMSO was used as cryoprotectants (Fig 4.16).

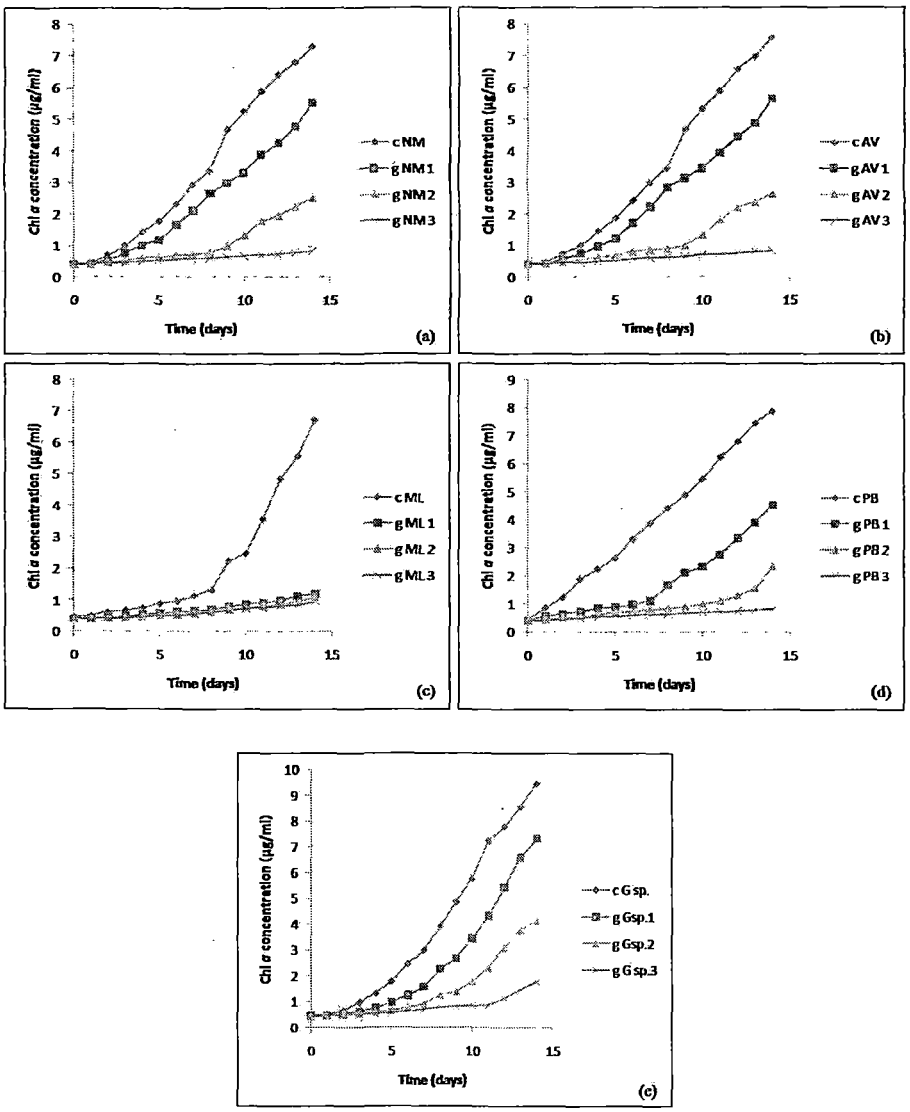


Fig 4.15: Growth curves of (a) *Nostoc muscorum* (NM) (b) *Anabaena variabilis* (AV) (c) *Mastigocladus laminosus* (ML) and (d) *Plectonema boryanum* (PB) and (e) *Gloeocapsa* sp.(Gsp) regenerated from preservation in 15% glycerol as compared to their free-living counterpart. [*In fig. "g" refers to 15% glycerol and "1, 2, 3" indicates samples regenerated after first , second and third years of preservation. An initial inoculum of 0.4µg/ml was used.]*

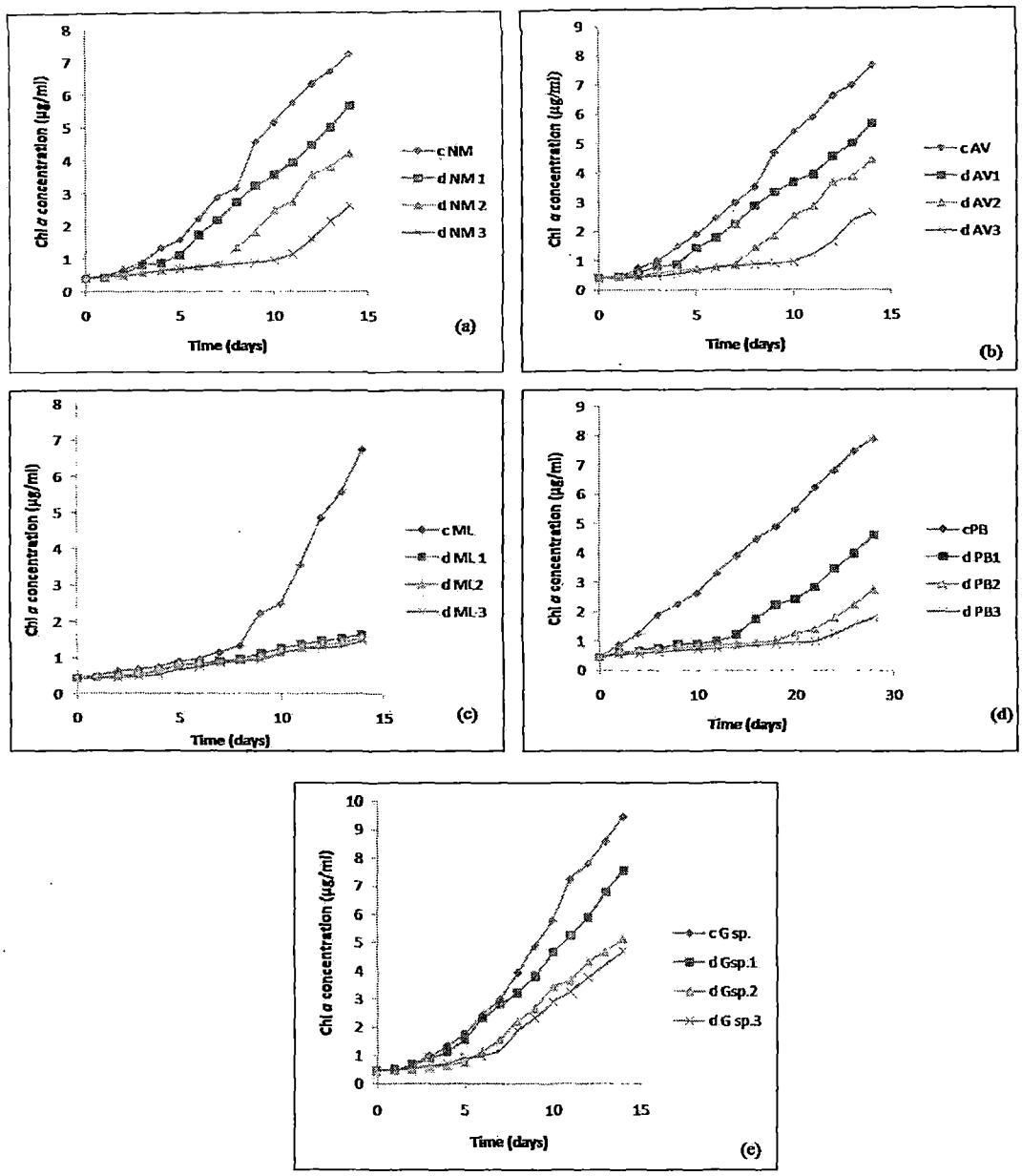


Fig 4.16: Growth curves of (a) *Nostoc muscorum* (NM) (b) *Anabaena variabilis* (AV) (c) *Mastigocladus laminosus* (ML) (d) *Plectonema boryanum* (PB) and (e) *Gloeocapsa* sp. (Gsp) regenerated from preservation in 5% DMSO as compared to their free-living counterpart. [*In fig., "d" refers to samples regenerated from 5% DMSO and "1, 2, 3" indicates samples regenerated after first , second and third years of preservation. An initial inoculum of 0.4µg/ml was used.*]

A study of heterocyst frequency of the regenerated samples of the heterocystous cyanobacteria recorded a decline in the heterocyst frequency with increase in the duration of

preservation. Here again it was noticeable that retention of the ability of achieving a heterocyst frequency comparable to the control was better in samples preserved in 5% DMSO. In case of preservation using both cryoprotectants, *Mastigocladus laminosus* showed a reduction of ~45 % in the samples regenerated after one year of preservation. However, there was no further prominent drop in this in the subsequent years of preservation (Fig 4.17 c). *Anabaena variabilis* appeared to better adapted to preservation in 5% DMSO and the reduction noticed in its heterocyst frequency was less than that of *Nostoc muscorum* and *Mastigocladus laminosus*. When compared to its control, *Anabaena variabilis* showed a reduction of~20%, ~25% and ~40% in the heterocyst frequency of its samples regenerated after one, two and three years of preservation in 15% glycerol (Fig 4.17 b). *Nostoc muscorum* and *Mastigocladus laminosus* however recorded a more prominent drop of ~ 27%, ~ 36%, 58% and ~ 45%, ~ 53%, ~55% respectively after one, two and three years of preservation in 15% glycerol.

In 5% DMSO was used as cryoprotectant, *Anabaena variabilis* showed a reduction in heterocyst frequency of ~16 %, ~15% and ~28% after one, two and three years of preservation (Fig 4.18 b). This was ~8%, ~15% and ~22% for *Nostoc muscorum*. *Mastigocladus laminosus* showed a higher reduction of ~42%, ~47% and ~48% in samples regenerated after one, two and three years of preservation (Fig 4.18 a, c).

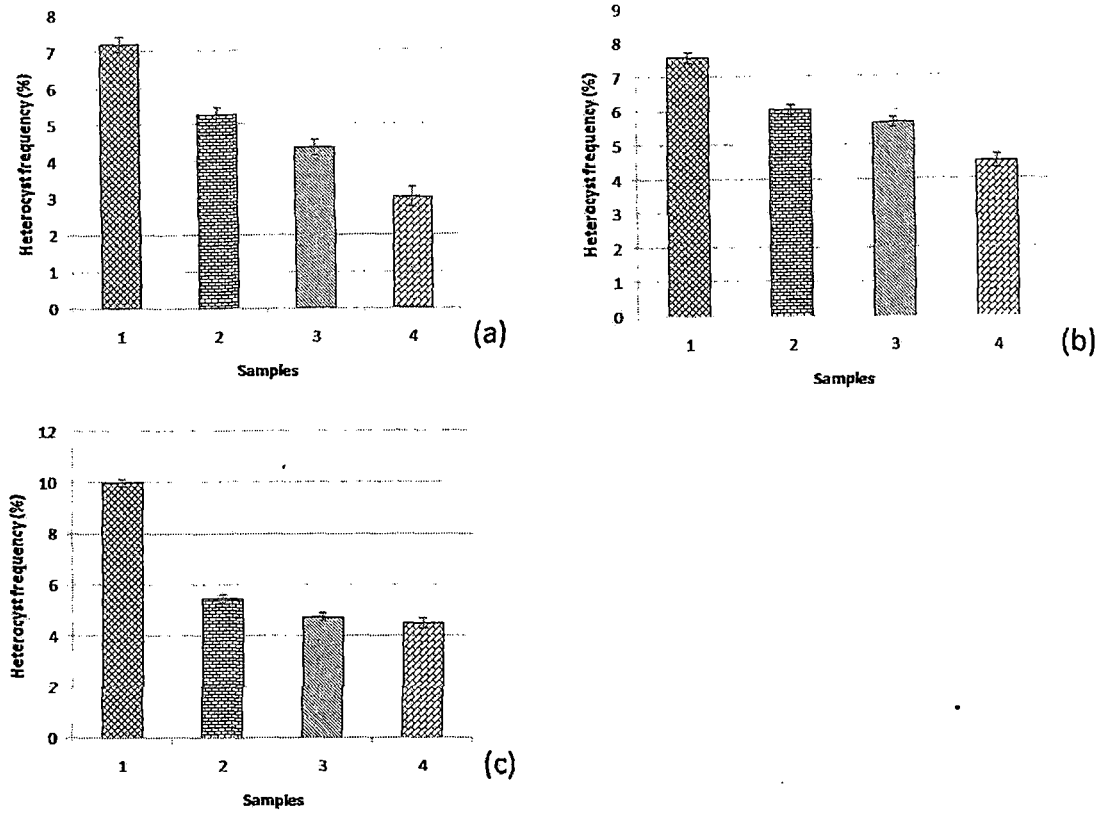


Fig 4.17: Heterocyst frequency of samples regenerated from 15% glycerol (a) *Nostoc muscorum*, (b) *Anabaena variabilis*, (c) *Mastigocladus laminosus[In fig, column 1 represents the control in each case; columns 2, 3 and 4 represent samples regenerated after 1st, 2nd and 3rd years of preservation respectively]*

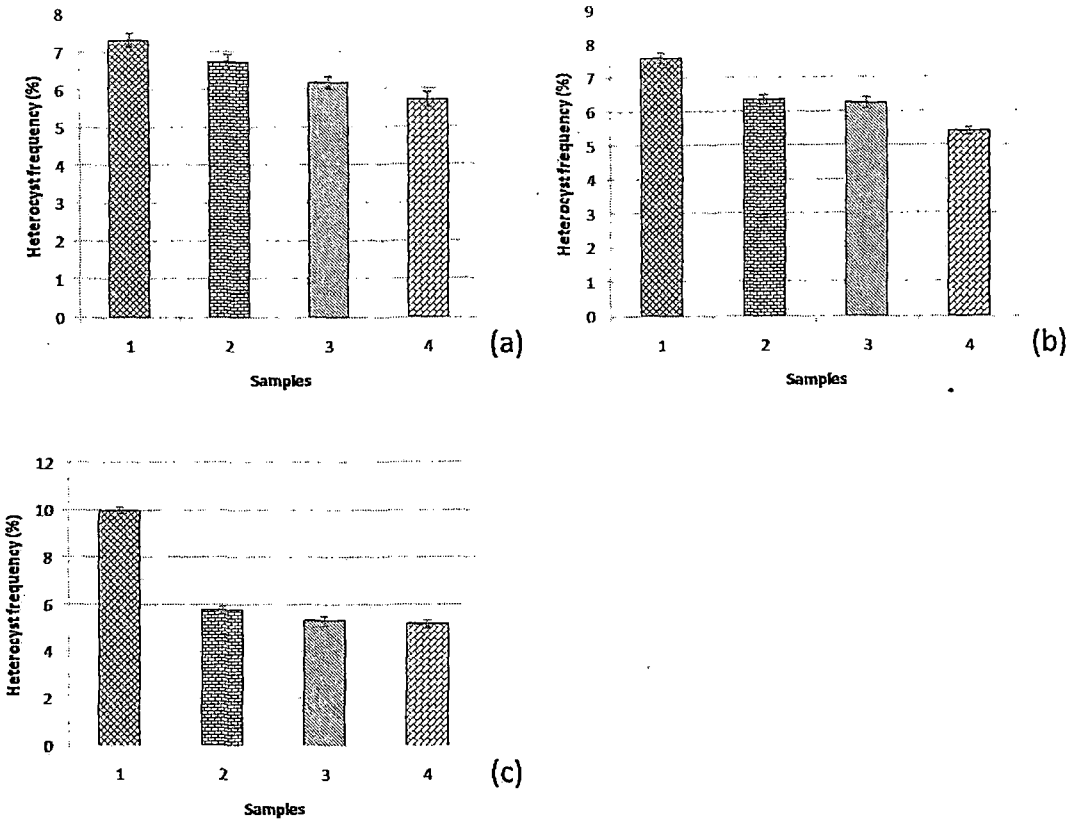
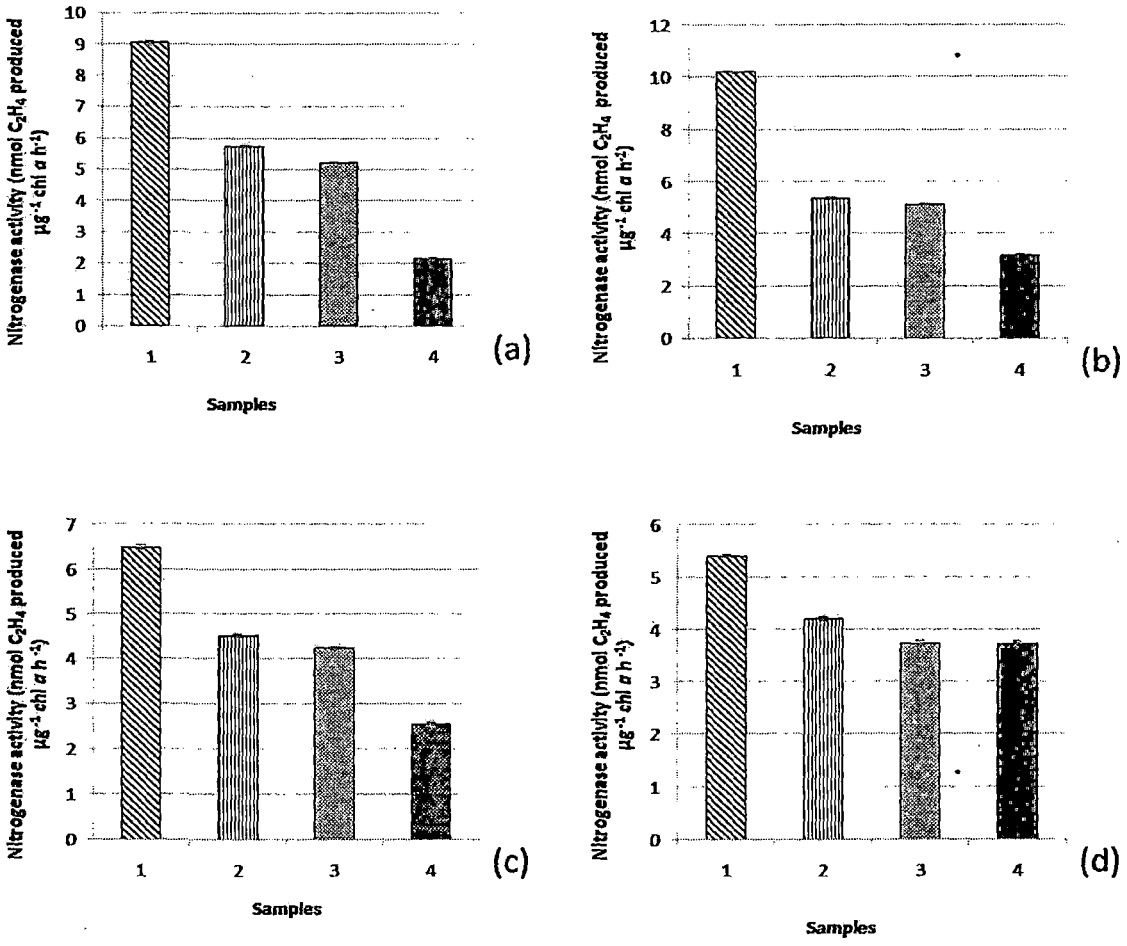


Fig 4.18: Heterocyst frequency of samples regenerated from 5% DMSO (a) *Nostoc muscorum*, (b) *Anabaena variabilis*, (c) *Mastigocladus laminosus. [In fig, column 1 represents the control in each case; columns 2, 3 and 4 represent samples regenerated after 1st, 2nd and 3rd years of preservation respectively]*

Fig 4.19 and 4.20 shows the nitrogenase activities of cyanobacterial samples regenerated from preservation in 15% glycerol and 5% DMSO respectively. A close look at these figures reveals that this activity is also severely affected by preservation in this method. In case of *Nostoc muscorum*, the nitrogenase activity declined by ~ 37-89 % during the 1st to the 3rd years of preservation in 15% glycerol. For *Anabaena variabilis* it was between 47%-70%, for *Mastigocladus laminosus*, 30%-61% and for *Plectonema boryanum* and *Gloeocaosa* sp. it was between 22%-31% and 37%-48% respectively. Thus it is evident that a prominent drop of nitrogenase activity occurs during preservation and storage at ~0°C

using 15% glycerol. However, a less severe but similar drop was also noticed when 5% DMSO was used as cryoprotectant. For instance, in *Nostoc muscorum*, the nitrogenase activity declined by ~37%-45%; in *Anabaena variabilis* between 38%-50%; for *Mastigocladus laminosus*, 29%-58%, and for *Plectonema boryanum* and *Gloeocaosa* sp. it was between 6%-20% and 14%-34% respectively when regenerated samples were compared to their control cultures from first year to third year of preservation.



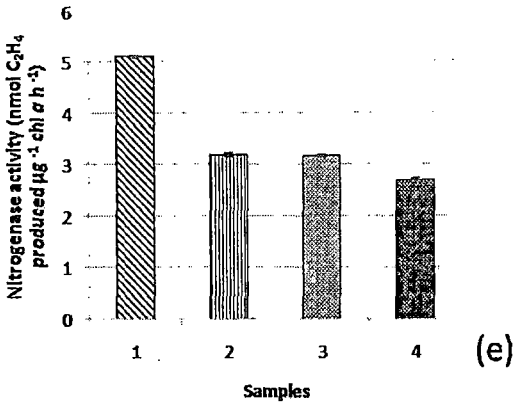
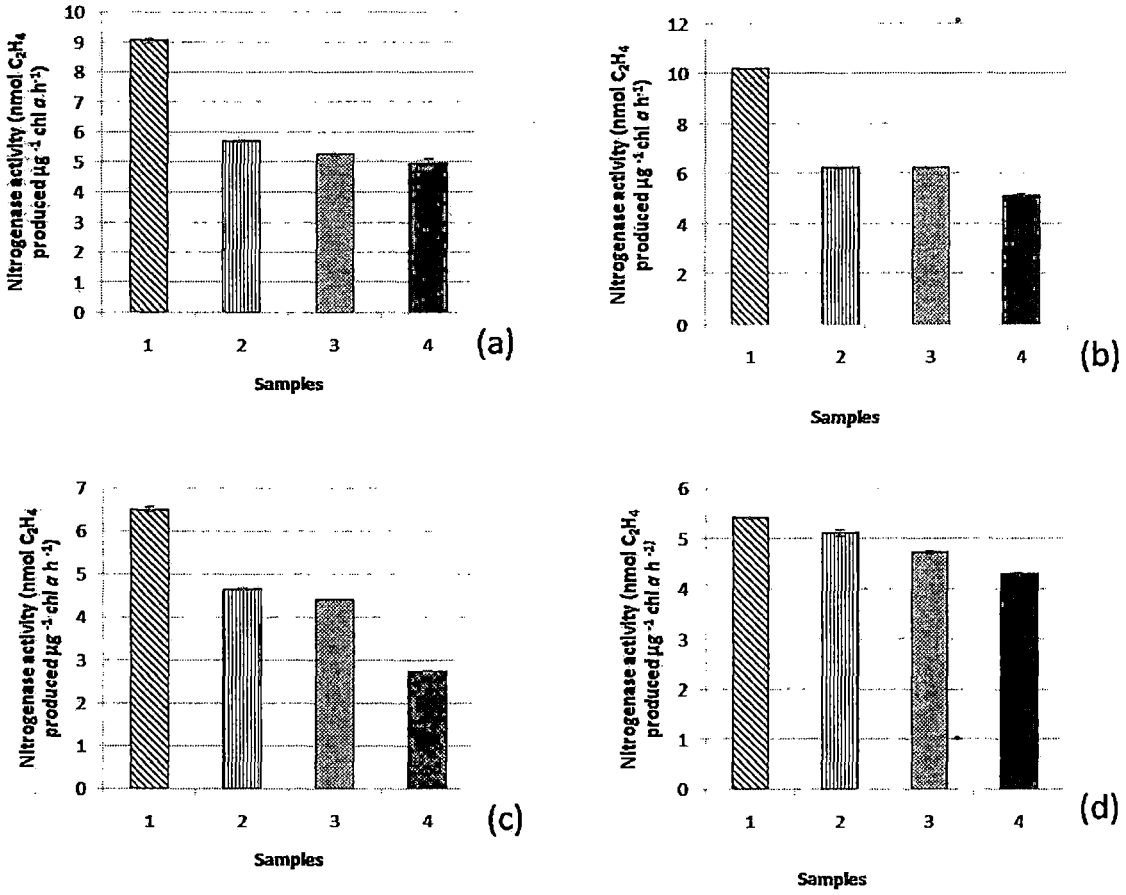


Fig 4.19: Nitrogenase activity of samples regenerated from 15% glycerol (a) *Nostoc muscorum* (b) *Anabaena variabilis* (c) *Mastigocladus laminosus* (d) *Plectonema boryanum* and (e) *Gloeocapsa* sp. *[In fig, column 1 represents the control in each case; columns 2, 3 and 4 represent samples regenerated after 1st, 2nd and 3rd years of preservation respectively]*



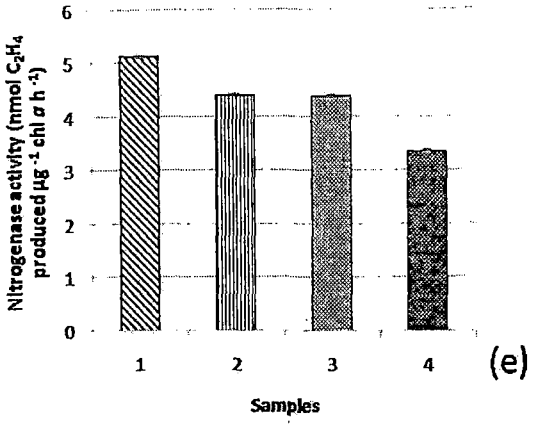


Fig 4.20: Nitrogenase activity of samples regenerated from 5% DMSO (a) *Nostoc muscorum* (b) *Anabaena variabilis* (c) *Mastigocladus laminosus* (d) *Plectonema boryanum* and (e) *Gloeocapsa* sp. *[In fig, column 1 represents the control in each case; columns 2, 3 and 4 represent samples regenerated after 1st, 2nd and 3rd years of preservation respectively]*

15% glycerol and 5% DMSO are well recognized cryoprotectants. They are among the most popular cryoprotectants that are currently being employed. Such cryoprotectants are commonly used to store organisms for extended periods under ultra low temperature of ~80 ° C. However, it was evident that for all the cyanobacteria that were used for this study, preservation using both 15% glycerol and 5% DMSO did not prove to be an efficient method of considerable importance at ~zero degree temperature as the viability of the preserved samples declined drastically over the period of preservation. Although this did not result in death of all the organisms that were preserved, this method certainly reduced the viability and performance of the preserved organisms. Even after subsequent sub-culturing the biomass yield remained low. The preservation under these conditions may be dependable only upto a period less than 12 months for *Nostoc muscorum*, *Anabaena variablis* and *Gloeocaosa* sp. Even during this period there was noticeable decrease in growth. These methods did not prove to be reliable at all for *Mastigocladus laminosus* (a thermophile) and *Plectonema boryanum* (non-heterocystous cyanobacteria). 5% DMSO seemed only a

fraction better that 15% glycerol as cryoprotectant in our case. However, we must emphasize that all other earlier reports mention DMSO and glycerol as cryoprotectant at ultra low temperature. And we have used temperature only close to zero. This was tried to find user friendly methods of cyanobacterial preservation that could be put to use even by laboratories that are not highly equipped. Even though these methods were not highly efficient, they could still be safely used for preservation for at least six to twelve months. *Mastigocladus laminosus* however, did not work well in either of these methods. Interestingly, *Gloeocaosa* sp. which is a unicellular cyanobacterium gave encouraging results as growth was much better in this cyanobacterium compared to the others used for this study.

4.5.1.2 Photosynthetic and respiratory activities

Table 4.7 a: Photosynthesis and respiration of *Nostoc muscorum* preservation in 15% glycerol.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	437 ±3.3	321 ±2.8	413 ±3.2	311 ±2.5
2nd year	439 ±3.1	317 ±2.7	415 ±3.3	298 ±2.5
3rd year	439 ±3.1	293 ±2.5	413 ±3.3	217 ±1.8

Table 4.7 b: Photosynthesis and respiration of *Anabaena variabilis* preservation in 15% glycerol.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	442 ±3.3	330 ±3.2	417 ±2.7	311 ±2.4
2nd year	440 ±3.5	323 ±3.2	415 ±2.7	300 ±2.4
3rd year	440 ±4.1	310 ±3.4	413 ±2.5	231 ±2.3

Table 4.7 c: Photosynthesis and respiration of *Plectonema boryanum* preservation in 15% glycerol.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	402 ±2.8	354 ±2.8	378 ±2.5	345 ±2.5
2nd year	400 ±2.8	351 ±2.9	373 ±2.6	340 ±2.4
3rd year	400 ±2.3	339 ±3.2	374 ±2.6	321 ±2.2

Table 4.7 d : Photosynthesis and respiration of *Mastigocladus laminosus* preservation in 15% glycerol.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	280.55 ±4.1	227.13 ±3.3	397.56±2.3	331.16 ±2.3
2nd year	277.56 ±4.2	220.12 ±3.5	394.15 ±2.5	329.37 ±2.3
3rd year	280.14 ±4.2	220.16 ±3.5	390.42 ±2.6	320.14 ±2.7

Table 4.7 e: Photosynthesis and respiration of *Gloeocapsa* sp. preservation in 15% glycerol.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	402 ±2.1	354 ±2.3	378 ±2.3	345 ±2.9
2nd year	400 ±1.8	351 ±2.3	373 ±2.3	340 ±2.8
3rd year	400 ±1.7	339 ±2.2	374 ±2.1	321 ±2.8

Table 4.8 a: Photosynthesis and respiration of *Nostoc muscorum* preservation in 5% DMSO.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	437 ±3.0	382 ±2.7	413 ±3.1	327 ±2.4
2nd year	439 ±3.1	377 ±2.5	415 ±3.2	320 ±2.5
3rd year	439 ±3.2	354 ±2.5	413 ±3.1	270 ±2.7

Table 4.8 b: Photosynthesis and respiration of *Anabaena variabilis* preservation in 5% DMSO.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	442 ±3.5	397 ±3.2	417 ±2.5	354±2.3
2nd year	440 ±3.6	381 ±3.3	415 ±2.3	331±2.2
3rd year	440 ±3.3	372 ±3.4	413 ±2.1	325±2.1

Table 4.8 c: Photosynthesis and respiration of *Plectonema boryanum* preservation in 5% DMSO.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	402 ±2.5	387 ±3.1	378 ±2.1	369 ±2.4
2nd year	400 ±2.7	385 ±3.0	373 ±2.1	354 ±2.3
3rd year	400 ±2.5	379 ±2.7	374 ±2.4	323 ±2.3

Table 4.8 d: Photosynthesis and respiration of *Mastigocladus laminosus* preservation in 5% DMSO.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	280.55 ±3.9	236.13 ±3.7	397.56 ±2.5	342.16±2.3
2nd year	277.56 ±4.1	232.12 ±3.5	394.15 ±2.7	339.37 ±2.3
3rd year	280.14 ±4.1	232.16 ±3.5	390.42 ±2.7	320.14 ±2.1

Table 4.8 e: Photosynthesis and respiration of *Gloeocapsa sp.* preservation in 5% DMSO.

	Control	Regenerated sample	Control	Regenerated sample
	(Photosynthetic oxygen evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})		Respiratory oxygen consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
1st year	396 ±2.1	387 ±2.7	342 ±3.1	354 ±3.3
2nd year	395 ±2.3	385 ±2.5	340 ±3.3	354 ±3.2
3rd year	395 ±2.3	379 ±2.5	340 ±3.3	333 ±3.1

Effect of long term cryopreservation on photosynthetic and respiratory activities using 15% glycerol and 5% DMSO manifested as a slight decrease in these activities, which

as in previous cases recovered almost to the values obtained for control cultures. However, this recovery needed at least three to four subsequent transfers to fresh medium under optimum conditions. Samples preserved in 5% DMSO the degree of reduction in these activities were comparatively less than that in the case of 15% glycerol. In *Nostoc muscorum* preserved in 15% glycerol, the drop in photosynthetic activity was ~27 %-34% and in respiration was ~25%-47%. For *Anabaena variabilis* this reduction was ~25%-29% for photosynthetic activity and ~25-44% for respiratory activity. In *Plectonema boryanum* photosynthesis was down by ~12%-16% and respiration was reduced by ~9%-15%. Photosynthesis was lowered by ~19%-21% and respiration by ~16%-19% in *Mastigocladus laminosus*. For *Gloeocapsa* sp. photosynthesis exhibited a drop of ~12%-17%, while its respiration declined by ~9%-15%. Hence reduction in the activities of photosynthesis and respiration was more pronounced in *Nostoc muscorum, Anabaena variabilis* and *Mastigocladus laminosus. Plectonema boryanum* and *Gloeocapsa* sp. were affected to a lesser extent.

Preservation in 5% DMSO appeared to better to some extent as it was seen that regeneration of preserved cyanobacteria was better and also the retention of properties. Here too reduction in photosynthetic and respiratory activities was evident, although to a slightly less extent. In *Nostoc muscorum* the drop in photosynthetic activity was ~13%-19%, while respiration reduced by ~21%-35 %. *Anabaena variabilis* exhibited an almost similar reduction of ~10-15% in photosynthesis and ~15-22% in respiratory activity. In *Mastigocladus laminosus* reduction was recorded in the range ~16-17% in photosynthesis and ~14-18% in respiration. *Plectonema boryanum* showed a meager decline in photosynthesis and respiration that were in the range of ~4-6% and ~3-14% respectively.

However, *Gloeocapsa* sp. recorded a minute increase of ~3%-5% in case of respiration in the samples regenerated directly after storage for 1st and 2nd years.

4.5.1.3 Glutamine synthetase (transferase) (GS), nitrate reductase (NR) and nitrite reductase (NIR) activities

A study of the enzyme activities revealed that a prominent reduction in the GS, NR and NIR enzyme activities resulted in the cyanobacteria that were stored at ~0°C using 15% glycerol and 5% DMSO. Samples regenerated from 5% DMSO showed lesser reduction in these activities when compared to the samples regenerated from 15% glycerol after storage for similar period of time. While all preserved cyanobacteria showed a reduction of ~15-35% in GS, NR and NIR activities, NR activities in case of *Plectonema boryanum* and *Gloeocapsa* sp. were least affected.

Table 4.9 a: Enzyme activities of *Nostoc muscorum* regenerated from preservation in 15% glycerol

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	767 ±5.3	2.4 ±0.11	547 ±3.1	627 ±5.3	1.1 ±0.14	420 ±3.3
2nd year	763 ±5.2	2.3 ±0.15	542 ±3.3	590 ±5.5	0.95 ±0.13	407 ±3.4
3rd year	763 ±5.2	2.3 ±0.13	546 ±3.3	524 ±5.5	0.82 ±0.13	400 ±3.4

Table 4.9 b: Enzyme activities of *Anabaena variabilis* regenerated from preservation in 15% glycerol.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	765 ±5.6	2.5 ±0.14	554 ±3.3	631 ±5.7	1.1 ±0.13	479 ±3.1
2nd year	763±5.5	2.3 ±0.17	547 ±3.2	592 ±5.6	0.98 ±0.12	462 ±3.1
3rd year	757 ±5.7	2.3 ±0.17	545 ±3.2	531 ±5.6	0.82 ±0.12	437 ±3.2

Table 4.9 c: Enzyme activities of *Plectonema boryanum* regenerated from preservation in 15% glycerol.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	Control Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	729 ±3.2	2.1 ±0.7	521 ±2.5	700 ±3.1	1.8 ±0.5	507 ±2.3
2nd year	729 ±3.2	2.1 ±0.5	520 ±2.3	678 ±3.1	1.5 ±0.4	507 ±2.2
3rd year	725 ±3.4	2.1 ±0.5	517 ±2.3	642 ±3.2	1.0 ±0.4	482 ±2.2

Table 4.9 d: Enzyme activities of *Mastigocladus laminosus* regenerated from preservation in 15% glycerol.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	2346 ±125	8.79 ±1.5	157.43 ±1.4	2001 ±121	7.4 ±1.2	137.16 ±1.3
2nd year	2337 ±126	8.71 ±1.4	152.42 ±1.4	1987 ±120	6.5 ±1.2	121.78 ±1.2
3rd year	2343 ±122	8.71 ±1.3	151.21±1.2	1947 ±121	5.1±1.1	106.41 ±1.2

Table 4.9 e: Enzyme activities of *Gloeocapsa* sp. regenerated from preservation in 15% glycerol.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	329 ±3.0	1.1 ±0.3	221 ±1.1	300 ±3.1	1.1 ±0.1	201 ±1.0
2nd year	329 ±3.1	1.1 ±0.3	220 ±1.2	289 ±3.3	0.85 ±0.1	195 ±1.1
3rd year	325 ±3.1	1.1 ±0.5	217 ±1.1	267 ±3.3	0.93 ±0.1	195 ±1.1

Table 4.10 a: Enzyme activities of *Nostoc muscorum* regenerated from preservation in 5% DMSO.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	Regenerated sample Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	767 ±5.4	2.4 ±0.1	547 ±3.2	689 ±5.3	1.4 ±0.07	520 ±3.2
2nd year	763 ±5.6	2.3 ±0.15	542 ±3.3	672 ±5.4	1.1 ±0.07	514 ±3.3
3rd year	763 ±5.6	2.3 ±0.15	546 ±3.3	632 ±5.5	1.1 ±0.09	500 ±3.3

Table 4.10 b: Enzyme activities of *Anabaena variabilis* regenerated from preservation in 5% DMSO.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	765 ±5.5	2.5 ±0.11	554 ±3.3	679 ±5.6	1.7 ±0.07	543 ±2.5
2nd year	757 ±5.7	2.3 ±0.13	547 ±3.4	654 ±5.3	1.5 ±0.05	527 ±2.7
3rd year	763 ±5.7	2.3 ±0.13	545 ±3.4	632 ±5.3	1.3 ±0.05	518 ±2.4

Table 4.10 c: Enzyme activities of *Plectonema boryanum* regenerated from preservation in 5% DMSO.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	729 ±3.4	2.1 ±1.0	521 ±2.7	723 ±3.1	2.1 ±0.7	518 ±2.2
2nd year	729 ±3.2	2.1 ±0.6	520 ±2.4	720 ±2.8	1.8 ±0.8	505 ±2.2
3rd year	725 ±3.5	2.1 ±0.6	517 ±2.2	689 ±2.8	1.0 ±0.8	478 ±2.2

Table 4.10 d: Enzyme activities of *Mastigocladus laminosus* regenerated from preservation in 5% DMSO.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	2346 ±125	8.79 ±1.3	157.43 ±1.5	2217 ±122	8.6 ±1.1	143.16 ±1.2
2nd year	2337 ±124	8.71 ±1.3	152.42 ±1.4	2215 ±126	8.5 ±1.1	143.78 ±1.2
3rd year	2343 ±122	8.71±1.3	151.21 ±1.4	2109 ±125	7.8 ±1.1	137.41 ±1.4

Table 4.10 e: Enzyme activities of *Gloeocapsa* sp. regenerated from preservation in 5% DMSO.

	Control Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Control** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Control** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)	**Regenerated sample** Glutamine synthetase transferase activity (nmol γ-glutamyl hydroxymate formed/min /mg protein)	**Regenerated sample** Nitrate reductase activity (nmol NO_2^- formed/min/mg protein)	**Regenerated sample** Nitrite reductase activity (nmol NO_2^- consumed/min/ mg protein)
1st year	329 ±3.3	1.1 ±0.5	221 ±1.1	320 ±3.5	1.1 ±0.4	220 ±1.1
2nd year	329 ±3.2	1.1 ±0.7	220 ±1.1	320 ±2.7	1.2 ±0.5	217 ±1.1
3rd year	325 ±3.1	1.1 ±0.4	217 ±1.1	297 ±2.9	1.1 ±0.5	217 ±1.1

4.6 Calculating the retained viability index (RVI_{10})

The tolerance of filamentous heterocystous cyanobacteria to the stress afforded by preservation methods on different solid matrices was evaluated using the retained viability index (RVI_{10}) as describe by Silva and Silva 2003. Cell viability was estimated by the RVI_{10} using the following expression: $(B_{10}-B_0)/B_0$ where B_0 is the initial biomass dry weight and B_{10} the biomass dry weight after 10 days of incubation. Positive values of RVI_{10} indicate net yields of actively growing biomass, while negative values correspond to cases with partial survival or total death of cyanobacterial biomass. RVI_{10} gives meaningful insights on the viability status of cyanobacteria after prolonged periods of preservation and storage.

4.6.1 Results

The concept of RVI_{10} as described by Silva and Silva 2003; 2007 is for devising a method of cyanobacterial biomass preservation method for production of economically convenient stable formulations, that would ensure use of viable biofertilizer stored in a dry

form. Hence, the main thrust of application of this concept is to evaluate the impact of desiccation and preservation on particular cyanobacteria of agronomical importance. In the present study, RVI_{10} was calculated for the two members belonging to filamentous heterocystous group, *Nostoc muscorum* and *Anabaena variabilis.*

Biomass was estimated by dry weight determinations in cyanobacterial cells washed twice with distilled water and dried at 30°C until constant weight. For determination of RVI_{10}, 20 mg of powder biomass were suspended in 20 ml of medium and poured onto Petri dishes followed by incubation for 10 days at 30°C under illumination. The biomass was then washed twice with distilled water and dried at 30°C until constant weight. RVI_{10} was then calculated using the expression given above. Positive values of RVI_{10} indicate net yields of actively growing biomass, while negative values correspond to cases with partial survival or total death of cyanobacterial biomass.

In the case of the cyanobacterial cells immobilized in agar cubes, alginate beads and in foam, the growth of released biomass was calculated until it equaled the control sample at ~14 days of incubation. Expressed as a percentage, the viability recovery was calculated after 14 days incubation by dividing the cell concentrations in the preserved samples by the control sample concentration (Montaini et al 1995).

Table 4.11 a: Effect of preservation on *Nostoc muscorum* as indicated by retained viability index (RVI_{10}).

Sl. no.	Preservation method(s)	Storage condition	Retained viability index (RVI_{10})			
			6 months	*12 months*	*24 months*	*36 months*
1	Serial subcultures (control)	Continuous illumination, 25±2°C	3.8	3.8	3.8	3.8
2	Calcium alginate beads	Desiccated, room temperature, dark	2.7	2.7	2.6	2.6

3	Agar cubes	Desiccated, room temperature, dark	2.2	2.1	1.0	0.87
4	Foam cubes	Room temperature, Dark	2.0	1.8	1.0	0.75

Table 4.11 b: Effect of preservation on *Anabaena variabilis* as indicated by retained viability index (RVI_{10}).

Sl. no.	Preservation method(s)	Storage condition	Retained viability index (RVI_{10})			
			6 months	*12 months*	*24 months*	*36 months*
1	Serial subcultures (control)	Continuous illumination, 25±2°C	4.3	4.2	4.1	4.0
2	Calcium alginate beads	Desiccated, room temperature, dark	3.6	3.5	3.4	3.3
3	Agar cubes	Desiccated, room temperature, dark	2.2	2.2	1.5	1.3
4	Foam cubes	Room temperature, Dark	3.3	2.5	2.0	1.3

Table 4.11 a and b shows the RVI_{10} values obtained for *Nostoc muscorum* and *Anabaena variabilis*. In both cases it is evident that viability was retained better and for a longer duration when the cyanobacteria were preserved in calcium alginate beads. When the RVI_{10} values for samples regenerated from agar cubes and foam are compared it becomes apparent that samples were better preserved in agar than in foam. Between these three solid matrices calcium alginate beads appears to be the best immobilizing material for both the cyanobacteria studied. This observation is consistent with the other experiments conducted which also revealed that calcium alginate beads preserve cyanobacteria better than most other matrices. A reduced value of RVI_{10} was obtained in samples regenerated from foam and agar cubes after 12 months of preservation. Hence the finding here from RVI_{10} studies is

that preservation in calcium alginate is definitely a better method for long term cyanobacterial preservation.

4.7 Assessment of genetic stability of preserved cyanobacteria

To establish whether preservation had any noticeable alteration in the genetic profile of the preserved cyanobacteria, a STRR 1A fingerprint profile was generated using the free-living cyanobacteria as control. As preservation in calcium alginate emerged as the method of choice for long term laboratory storage of purified cyanobacteria, PCR profiles were generated using cyanobacterial cells that were regenerated from calcium alginate beads. Experiments were conducted using regenerated samples after every one year interval. Fig 4.21 was generated using the oldest samples (>three years) stored in calcium alginate beads.

4.7.1 Results

PCR profiles of the cyanobacteria studied showed no apparent changes in the banding pattern thus indicating conservation of their genetic material during preservation (Fig 4.21).

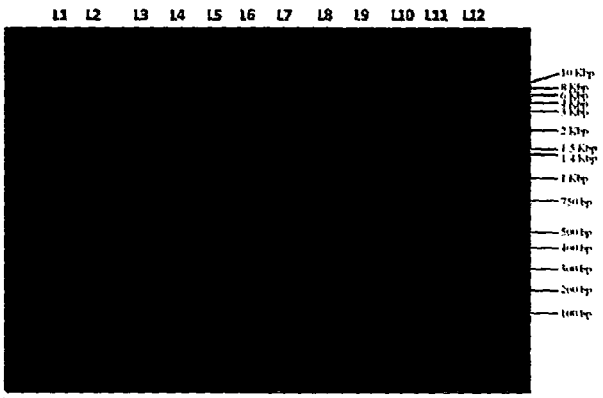


Fig 4.21: PCR profile of cyanobacteria regenerated from calcium alginate beads. [*In fig., lanes L1,L2,L3 and L4 represent fingerprint profiles of Nostoc muscorum; L5,L6 of Plectonema boryanum; L7,L8 of Mastigocladus laminosus; L9,L10 of Anabaena variabilis; and L11 and L12 that of Gloeocapsa* sp. *L1,L3,L5,L7,L9 amd L11 indicate profiles of the control free-living batch cultures.]*

4.8 Discussion

This chapter dealt with detailed assessment of efficiency of various existing methods of cyanobacterial preservation. The target of present work was to identify the time scale up to which a designated method could be relied upon completely to preserve cyanobacterial cells, thus eliminating the need of routine sub-culturing. In addition to listing them according to their performance in long term preservation of cyanobacteria, this study was also directed towards modifying some methods to develop protocols of preservation that are user friendly and less expensive. Hence, efforts were aimed at storing cyanobacterial cultures in dry form in which metabolic activities in the cell would be minimal due to dehydration. This has added advantage of least alterations in desired characters as the cell division is kept at lowest possible. For every experimental procedure, investigations were conducted to evaluate the performance of the technique in preserving cyanobacteria as well as to assess the performance of the preserved organism(s) when regenerated from long term dormancy due to preservation.

The first among various modifications brought about in preservation protocols involved the creation of agar flakes. Maintaining large number of cyanobacterial cultures as agar slants suffers few drawbacks as mentioned before. Making of agar flakes substantially overcame not only the problem of reducing the space required for storage, it also ensured the storage of axenic strains in dried form for a much longer duration with least occurrence of contamination. Cultures preserved in agar flakes had longer shelf life and could be stored in limited space. This treatment did not lead to any severe effect on the viability and survival of the immobilized cyanobacteria. There was minor reduction in expression of most characters in the cyanobacterial cells as they were regenerated from the agar flakes. This apparent

reduction in chlorophyll *a* content, heterocyst frequency, nitrogenase activity, photosynthetic and respiratory activities and the different enzyme activities were recovered in subsequent sub-culturing. All the distinct cyanobacterial groups that were immobilized in this modified method using agar gave encouraging results with respect to retention of viability and characters. Amongst the cyanobacteria that were included for study, *Anabaena variabilis* recorded best performance, followed by *Nostoc nuscorum*, *Plectonema boryanum*, *Mastigocladus laminosus* and *Gloeocapsa* sp. It may be concluded that cyanobacteria may be safely stored/preserved using this method at least for a period of 24 months.

A major finding of this work was the excellent performance of calcium alginate in long term preservation of cyanobacteria. It became evident that this technique holds good for all the distinct groups of cyanobacteria. Significantly, preservation in this matrix did not lead to any significant reduction or loss of viability of cyanobacteria. Cyanobacterial cells were well stabilized in this matrix and immediately regenerated samples matched all characters to the level of the free-living cultures. This held true even for samples that were stored for more than 36 months. The beads could be dried in a short duration of time (24 h) thus reducing chances of contamination. Large number of dehydrated beads could be stored in small vials. This method has proved to be very convenient for long term preservation of cultures in axenic conditions without losing any viability or essential characters in the samples preserved. This gives liberty to a researcher from maintaining batch cultures that has associated problems of being labor intensive, expensive as there is a need for regular sub-culturing, increased chances of contamination due to routine handling inside a culture room that hosts many other similar organisms and mis-labelling or loss of cultures due to overcrowded racks. This not only reduces the work load involved in regular batch culturing

and maintenance, this method practically negates the chances of cross contaminations between various cyanobacterial cultures kept in close proximity. Also, the method is reliable in retaining the viability of immobilized cells which is of paramount importance in long term maintenance of cyanobacteria.

Another base for preservation of cyanobacteria that was attempted in the present work was immobilization in foam. This is a known method of preservation. In this study ordinary packing foam was used as they are readily available in any laboratory that buys usual consignment of research equipments and thus this supply of foam material is cost effective. Our investigation demonstrated that the ordinary packing foam can effectively be employed as a preservation matrix for cyanobacteria at least for a period of 24 months. Nevertheless, like in almost every other protocol, the initial amount of inoculum plays a determining role in deciding the duration of viability of the immobilized cyanobacteria. It must be mentioned here that this load had to be much more in case of foam due to its porous nature. The dried foam cubes can be conveniently stored in sterile containers at room temperature in dark.

A proven method of long term preservation of cyanobacteria is storage at ultra low temperature (-80°C) using cryoprotectants. However, this requires sophisticated ultra refrigeration systems. Since domestic refrigerators are cost effective and are part of most laboratories, the role of these refrigerators in storing cyanobacterial samples preserved for a period of time was evaluated during this project. The duration up to which such cyanobacterial samples can be reliably stored was also established. This could be of interest in case of laboratories with modest resources and also to researchers who collect samples from remote locations and need time to start work on the collection. Two commonly used

cryoprotectants DMSO and glycerol were used for this study. Results obtained illustrated that under such modification in the established method of preservation, cyanobacteria could be preserved for at least 9-12 months without loss of viability. DMSO was found to be a better cryoprotectant than glycerol, and this was true for all the different groups of cyanobacteria. Nevertheless, it was apparent that viability and growth was most affected when cyanobacterial samples were preserved using this method as compared to all others methods that were studied in the present work.

Retained viability index (RVI_{10}) gives an indication of viability of dried cyanobacterial samples after extended periods of storage. However, RVI_{10} is generally estimated for samples which are projected as biofertilizer. Thus, retained viability indices for desiccated stored *Nostoc muscorum* and *Anabaena variabilis* samples were calculated keeping in mind that these two cyanobacteria are widely distributed in paddy fields and are under intense research as possible biofertilizer candidates. Results obtained showed that preservation in alginate was a superior method for long term preservation of cyanobacteria.

A notable observation that surfaced from this study was that preservation of the non-heterocystous cyanobacteria always necessitated the addition of supplementary amounts of nitrate to the preserving medium. Initial experiments conducted without this further supplementation of nitrate resulted in inefficient preservation of the cyanobacteria with reduced duration of viability in the preserving matrix.

4.9 Salient findings

- This chapter dealt with evaluation and assessment of some existing methods of cyanobacterial preservation. The emphasis was to find the suitability of the methods

and also to find the time period up to which cyanobacterial samples may be safely stored using matrices and protectants described in the methods.

- Certain changes were introduced in some of the protocols in order to make them more readily useable and less expensive.
- The existing method of making agar slants was modified to make agar flakes for convenient storage of cyanobacteria. This modification has the advantages of ease of handling, and ensuring storage of immobilized cyanobacterial samples in dry form. This also substantially reduces the chances of contamination between cultures which are maintained in close proximity in culture rooms. All groups of cyanobacteria could be stored on agar flakes for at least a period of 18-24 months.
- Calcium alginate proved to be most promising and dependable in long term preservation of cyanobacteria. Cyanobacterial samples thus stored retained their viability for very long periods without any loss of characters that are indispensable for independent survival. Also, large quantities of samples could be stored as calcium alginate beads in small vials thus bringing down the space required for maintenance. Safe period up to which the cyanobacteria can be stored using this protocol is at least 36 months. Cells also regenerated from these beads even after 5 years of storage at room temperature and in dark.
- The use of ordinary packing foam in immobilization of cyanobacteria was another major achievement of this work. It became obvious that packing foam can be used in storing cyanobacteria at least for a period of 18 months. Foam is often a waste material readily found in laboratories as they come as protective packing material

around instruments. Thus, use of such material is highly cost effective and is convincingly efficient in preservation of cyanobacteria for substantial period of time.

- It was also observed that cyanobacterial samples can be stored in ordinary refrigerators using cryoprotectactants for about 9-12 months. This would enable researchers to store cyanobacteria for at least this period in laboratories that lack expensive ultra refrigeration systems.
- The initial amount of inoculum played a very determining role in deciding the duration of survival and performance of regenerated samples from the different preservation matrices and materials. A higher concentration of initial inoculum (at least 10 µg/ml chl *a)* was a prerequisite to ensure longer duration of viability of preserved cyanobacteria.
- Non-heterocystous cyanobacteria needed addition of supplementary nitrate in the media.

Chapter 5

Development of alternative methods of preservation:

Relevant to field applications of cyanobacteria

5.1 Introduction

Cyanobacteria have immense applications in various fields of biotechnology. These include their uses as biofertilizer, in reclamation of soil, in bioremediation of toxic compounds from polluted environments, waste and effluent water treatments and in biocontrol of pests, among many other applications. For the above mentioned applications however, efficient strains of cyanobacteria have to be released to the desired sites of interest. For cyanobacteria to be effective for these applications they have to be viable during packaging and transport. At present cyanobacteria are distributed for such purposes as live cells which perish within a short period of time under packaged conditions. Therefore, immobilized cyanobacteria in dry form are an attractive alternative to wet live cells for transport and distribution. This increases their shelf-life significantly. However, the matrices on which the cyanobacteria are to be immobilized for such purposes must be environment friendly, biodegradable and economical. In the recent past, a significant variety of carrier materials have been experimented with for the purpose of obtaining a suitable carrier for efficient transfer of cyanobacterial biofertilizers to the fields. Included among them are thermocol, riverbed sand, saw dust, rice straw, paddy straw (Kaushik and Prasanna 1998), sugarcane waste/rice husk and coconut coir (Kannaiyan 2000; Malliga et al 1996) etc. Tobacco waste-based cyanobacterial biofertilizer has also been attempted (Jha and Prasad 2006). A cheap and easily adaptable method for the production of soil based algal inoculum, was developed for the benefit of small and marginal farmers which involved very

little capital investment (Venkataraman 1981). The production technology has now been substantially improved with the introduction of new and cheap carrier materials such as multani mitti (Fuller's earth) and wheat straw that support higher algal load with longer shelf life (Kaushik 1998; Goyal et al 1997; Prasanna et al 1998).

This Ph.D. work mainly concerns preservation and conservation of cyanobacteria. Effective preservation and conservation would certainly benefit future researchers as the preserved material could be the starting point for cyanobacterial research and valuable time, effort, energy and cost of chemicals involved in isolation, purification and maintenance could be reduced to a great extent. However, during the course of work one question that constantly came to mind is whether preservation could have any further function apart from simply preserving the material for a period of time. Keeping various field applications of cyanobacteria in view, we tried to find economically feasible immobilizing bases for cyanobacterial preservation that are environment friendly. Emphasis was given to locate materials that could keep cyanobacterial cells viable for a significant period of time, long enough to transfer cyanobacteria to desired sites with little or no loss of viability. Based on the above requirements we experimented with garden soil, charcoal chips, dried fibrous *Luffa cylindrica* and the outer cover of mature betel nut (*Areca catechu*) as carrier material. Rationale for choosing these materials was that they are biodegradable natural substances that are locally available. This chapter deals with performance assessment of various eco-friendly materials that were sampled as possible carrier materials.

5.2 Methods

5.2.1 Organisms

The cyanobacteria used for the present study were *RF7* (*Anabaena* sp.) and *CM9* (*Nostoc* sp.) from chapter 3, table 3.2. *RF7* showed high degree of association with rice roots and high associative nitrogenase activity and therefore, could be used as biofertilizer inoculum. *CM9* was efficient in heavy metal removal.

5.2.2 Cultivation and concentration of cyanobacteria

Pure cultures of the two cyanobacterial isolates were raised in BG 11_0 media. 100 ml each of exponentially growing (15 days old) cyanobacterial culture with chlorophyll *a* concentration of 10 $\mu g\ ml^{-1}$ was concentrated to 5 ml by centrifugation.

5.2.3 Immobilization on luffa (*Luffa cylindrica*)

The fibrous placenta of *Luffa cylindrica* was used for entrapment of cyanobacterial cultures (Fig 5.1 a). These were cleaned, deseeded and washed several times in water, rinsed with suitable media and then dried in the oven at 35°C. Once dried these were cut into small pieces (Fig 5.1 b). The concentrated slurry of cyanobacterial was then poured over and mixed properly using sterilized forceps and left to dry under aseptic conditions inside the laminar flow cabinet (Fig 5.1c, d). Once the cyanobacterial cultures dried, the fibres were packed into polybags.

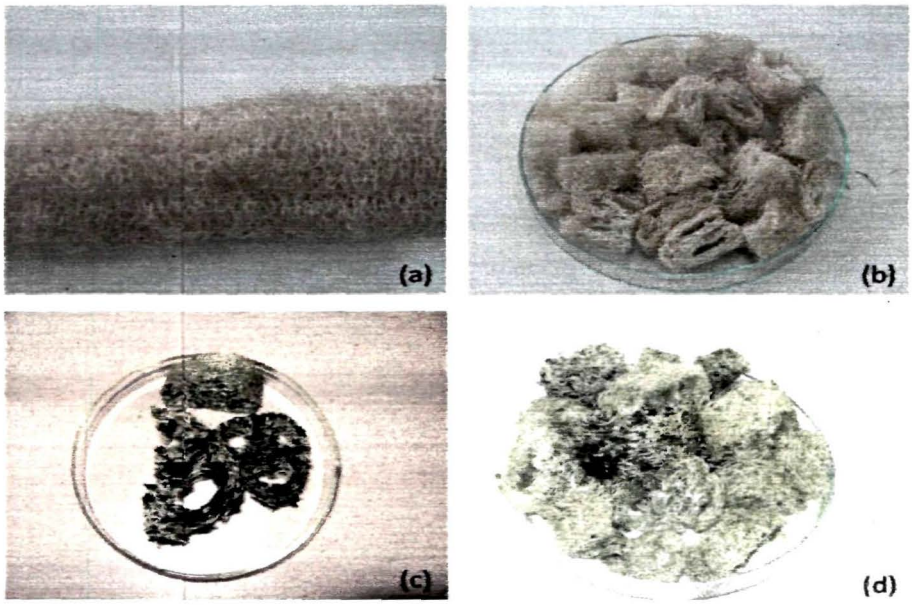


Fig 5.1: Immobilization of cyanobacteria in *Luffa cylindrica* (a) dried *Luffa cylindrica*, (b) washed and cleaned cubes of *Luffa cylindrica*, (c, d) *Luffa cylindrica* cubes containing cyanobacterial cultures.

5.2.4 Immobilization on betel nut covers ('snep kwai')

Betel nut (*Areca catechu*), 'snep kwai' (Fig 5.2 a) were cut into uniform sized pieces and the fibers were loosely separated from each other (Fig 5.2 b). These were washed thoroughly several times, rinsed with suitable media and then dried at 35°C in the oven (Fig 5.2 c). Inoculation and packaging were done as in the case of luffa (Fig 5.2 d).

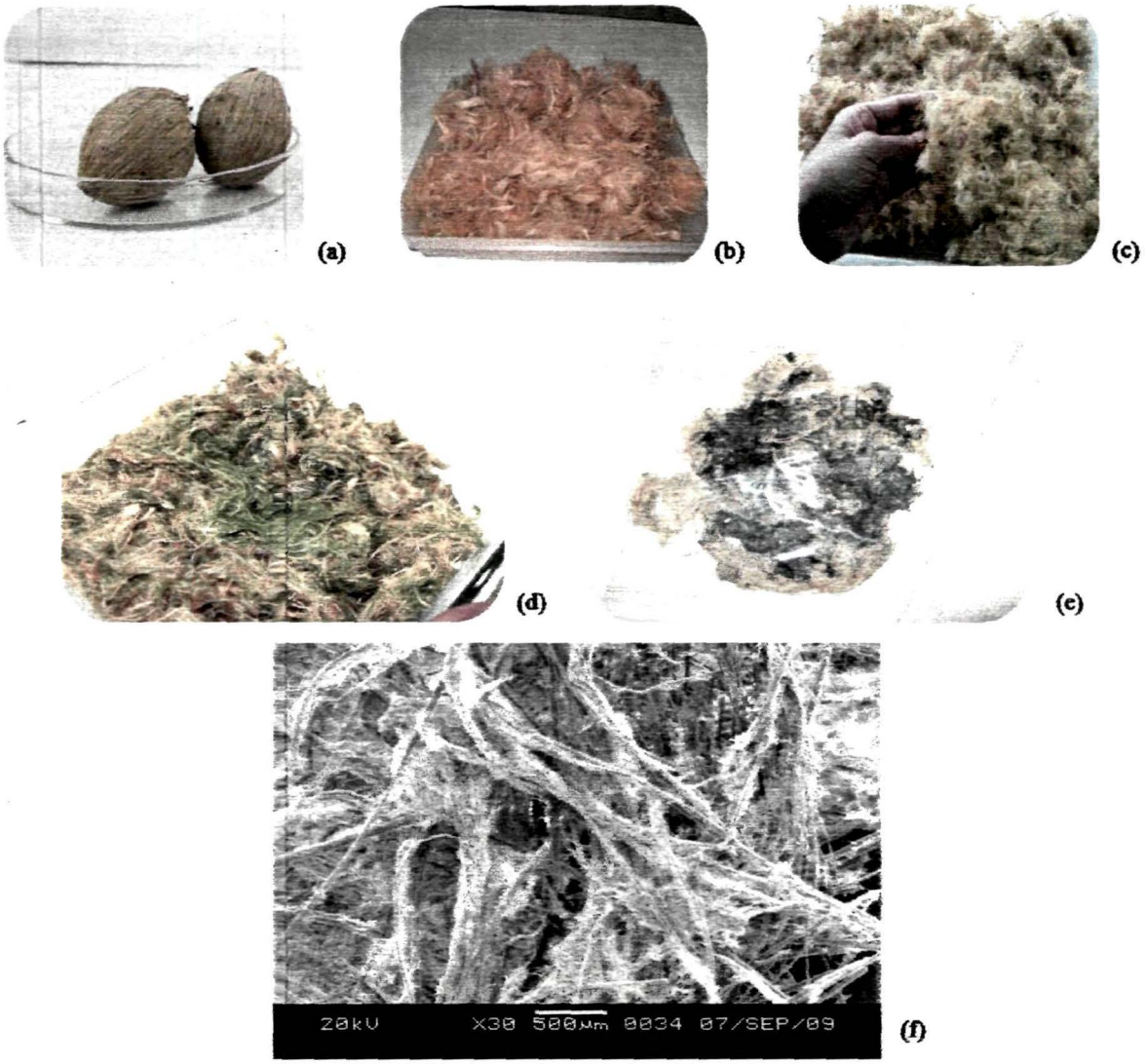
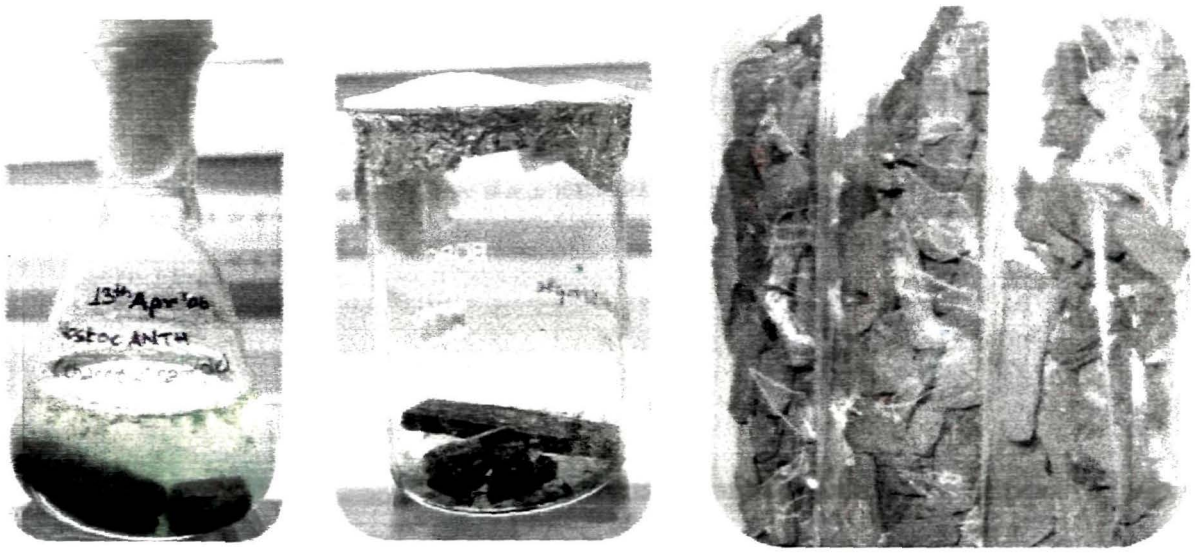


Fig 5.2: Immobilization of cyanobacteria in fibres of *Areca catechu* (a) Dried *Areca catechu* (betel nut) (b) fibres of betel nut (c) washed and cleaned fibres of betel nut (d) cyanobacterial cultures inoculated on to fibres of betel nut (e) dried immobilized cyanobacteria packed in sterile plastic bag (f) Scanning electron micrograph of immobilized cyanobacteria on fibres of betel nut cover.

5.2.5 Immobilization on charcoal pieces

Small size charcoal pieces were washed and dried before autoclaving. Concentrated cyanobacterial slurry was poured on these pieces as in the case of 'luffa' and 'snep kwai'. However, cyanobacterial filaments were not retained effectively on the charcoal pieces. To

overcome this limitation, cyanobacterial cultures were grown for 15 days to a chlorophyll *a* concentration of 10 µg ml^{-1}. Autoclaved charcoal pieces were then dipped in to the conical flasks containing the cultures (Fig 5.3 a). These were retrieved after being left for four days in the flasks. The excess media was drained off and the chips were left to dry. *This adjustment in the method of immobilization of cyanobacterial cells led to adherence of substantial amount of cyanobacterial filaments on the cracks and crevices on the surface of the charcoal.* The dried pieces of charcoal containing the immobilized cyanobacteria were then stored in sterile polybags and kept at room temperature (Fig 5.3 b, c).



(a) **(b)** **(c)**

Fig 5.3: Immobilization of cyanobacteria on charcoal pieces (a) charcoal pieces inoculated in cyanobacterial culture (b) dried cyanobacteria on charcoal pieces (c) packets containing charcoal immobilized cyanobacteria.

5.2.6 Enrichment of soil with cyanobacterial cultures

Soil was collected from the university campus. The soil was sieved to remove pebbles and other organic substances such as roots, leaves etc. The fine soil particles were autoclaved at 121°C (15 psi) for 30 minutes. Following this inoculation was done with dense

slurry of cyanobacterial cultures (10 μg ml^{-1}). This was dried and then lumps were broken to obtain finer soil particles. These were then stored in sterile containers.

5.2.7 Regeneration studies on the immobilized samples

At every three months interval the dehydrated samples were reintroduced in appropriate growth media to ascertain viability of the immobilized cells. Fig 5.4 shows regenerated filaments from 'snep kwai' after one year of preservation.

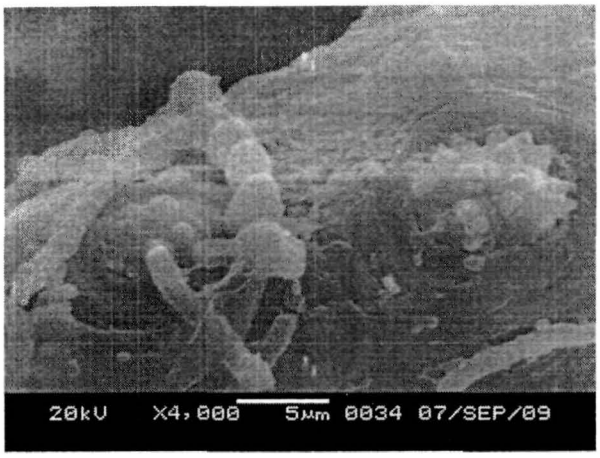


Fig 5.4: Sacnning electron micrograph of cyanobacteria regenerating from immobilized fibres of betel nut

Filaments growing out of the matrices were then used to measure growth, heterocyst frequency and nitrogenase activity. Growth in the regenerated samples was measured as an increase in chlorophyll *a* (*MacKinney* 1941). Their heterocyst frequency was counted under a light microscope and expressed as percentage of total cells. Acetylene reduction activity by nitrogenase enzyme (*EC1.18.6.1*) was estimated *in vivo* by gas chromatography (Stewart et al 1967). Since this work was dedicated to finding suitable carrier material for cyanobacterial inoculum to be transferred for field applications, the potential of these regenerated samples in colonizing rice roots and their *efficiency in removing heavy metals have also been assessed.* For all evaluations, preserved cyanobacteria were allowed to grow

out form the bases and then reinoculated into fresh media at a constant concentration. This was done to enable us to compare the various characteristic under study with free-living batch culture of the same organism.

5.3 Results and Discussion

5.3.1 Growth

Fig 5.5 and 5.6 compares the growth expressed as increase in chlorophyll *a* content of *Nostoc* sp. (*CM9*) and *Anabaena* sp. (*RF7*) in the four methods mentioned above.

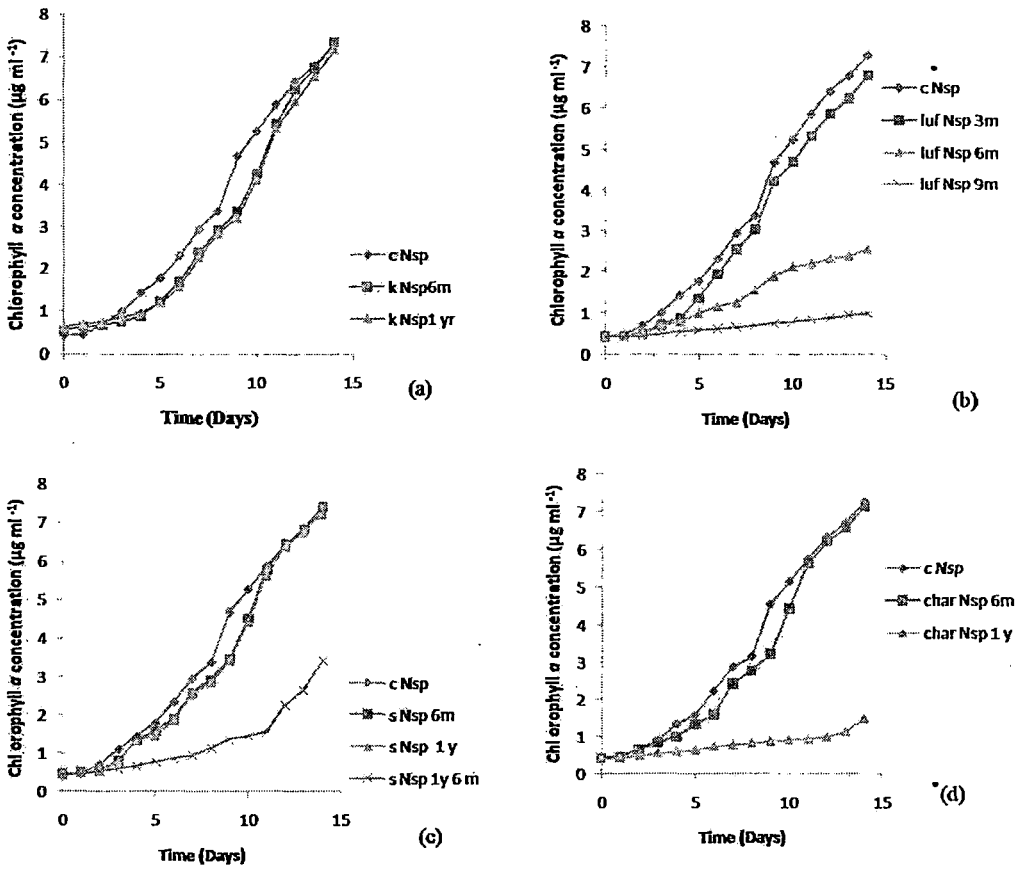


Fig 5.5: Growth curve of *Nostoc* sp. (Nsp) regenerated from (a) 'snep kwai', (b) *Luffa cylindrica*, (c) soil and (d) charcoal. *[In fig, 'c' refers to control samples; 'k', 'luf', 's' and 'char' refers to samples regenerated from immobilization in kwai, luffa, soil and charcoal respectively. 'm' indicates the age (in months) and 'y' the age in years of the preserved samples.]*

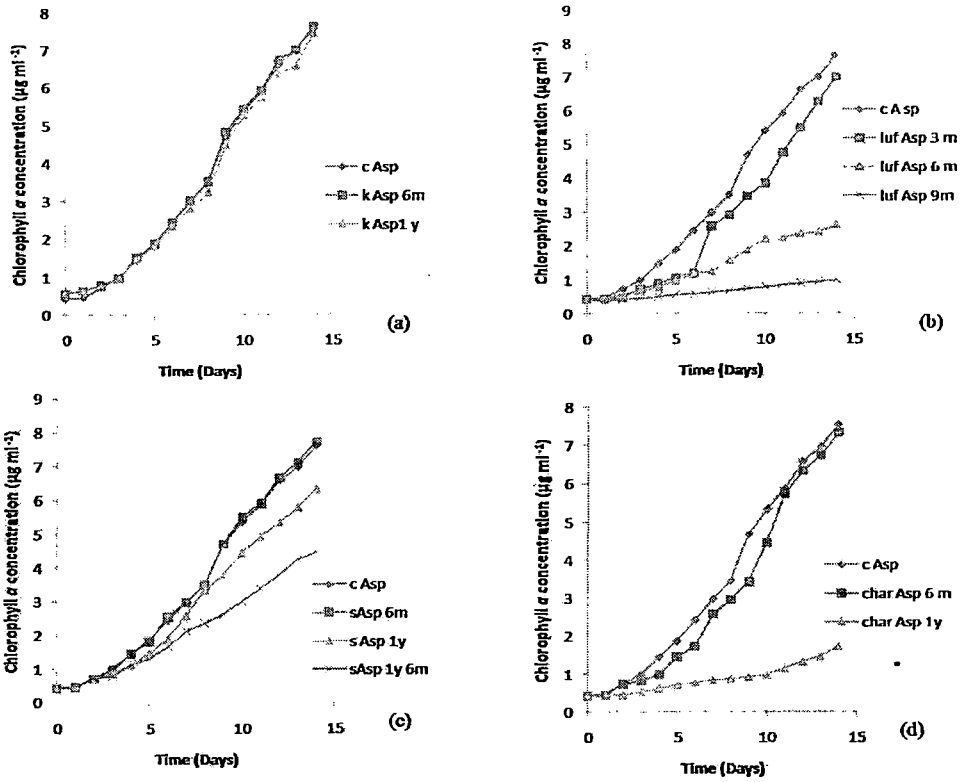


Fig 5.6: Growth curve of *Anabaena* sp. (Asp) regenerated from, (a) "snep kwai", (b) *Luffa cylindrica*, (c) soil and (d) charcoal. *[In fig, 'c' refers to control samples; 'k', 'luf', 's' and 'char' refers to samples regenerated from immobilization in kwai, luffa, soil and charcoal respectively. 'm' indicates the age (in months) and 'y' the age in years of the preserved samples.]*

Experiments to standardize initial concentration of cyanobacteria needed for immobilization resulted in finding 10µg ml^{-1} of cyanobacteria being optimum for getting high number of viable cells in regenerated cultures. *Nostoc* sp. and *Anabaena* sp. remained viable up to 9-12 months in all the immobilizing bases used to varying degrees. The outer cover of betel nut ('snep kwai') and soil seemed to be the best immobilizing materials for *Nostoc* sp and *Anabaena* sp. The growth of regenerated cells after a year of preservation on these bases was similar to the control cells. *Nostoc* cells could survive even longer duration of immobilization of soil up to a period of 18 months, even though growth was reduced by ~72% (Fig 5.5 c). Similar trend was observed in cells of *Anabaena* sp. (Fig 5.6). However,

negligible regeneration of cells was seen from 'snep kwai' samples after a period of 1 year. The medium turned blue when preserved 'snep kwai' samples were introduced for regeneration indicating lysis of the preserved cells. Thus, 'snep kwai' can be safely used with a good regeneration potential upto a period of one year. The growth in cells regenerated from luffa and charcoal seem to follow a parallel trend of being inversely proportional to the period of preservation. Growth from charcoal was comparable in 6 months to their control cultures. Drastic drop in regeneration and growth was seen after this period. Luffa as carrier was good only for about three month. After this, the regeneration of cyanobacterial cells from luffa was poor. This may be due the texture of luffa that does hold substantial amount of cyanobacteria on itself even though an initial concentration of 10 μg ml^{-1} of cyanobacteria was maintained for immobilization. This brings to the fact that initial amount of inocula plays a definitive role in regeneration potential of immobilized cultures. This was found to be true for all methods employed for preservation and storage.

5.3.2 Heterocyst frequency and nitrogenase activity

Heterocyst frequency of regenerated *Nostoc* and *Anabaena* sps. were compared to their free living cultures. Figs 5.7 and 5.8 clearly project the percent heterocyst differences between the regenerated specimens and their controls. *Nostoc* cells regenerated from soil samples maintained the original heterocyst count up to a period of one year. Cells regenerated from 'snep kwai' after 6 months and one year displayed a reduction of 27 to 31% in heterocyst frequency. However, in the case of *Anabaena* cells, almost complete retention of heterocyst frequency was seen in samples regenerated from 'snep kwai' (and not from the samples regenerated from soil) for the same period. The correlation between heterocyst frequency and nitrogenase activity held true for the *Nostoc* cells regenerated from

all the immobilizing bases (Fig 5.7; 5.9). However, nitrogenase activity did not match its heterocyst frequency in regenerated *Anabaena* cells in case of 'snep kwai' (Fig 5.8; 5.10). This may have been due to the noticeable occurrence of double and multiple heterocysts in many of the regenerated filaments of *Anabaena* where these heterocysts present in a row are in short supply of carbohydrates from the neighboring vegetative cells. This feature disappeared upon subsequent sub-culturing of the regenerated samples and the correlation between heterocyst frequency and nitrogenase activity was restored. In all cases, subsequent cultures of the preserved samples matched the parameters of their free-living control cultures. Soil as an immobilizing base was best for both the organisms under study. The duration of preservation on soil was as long as eighteen months without any significant loss in heterocyst frequency and nitrogenase activity. The nitrogenase activity of immediately regenerated *Nostoc* cells after a year of preservation in soil was 92% of that of its control cultures.

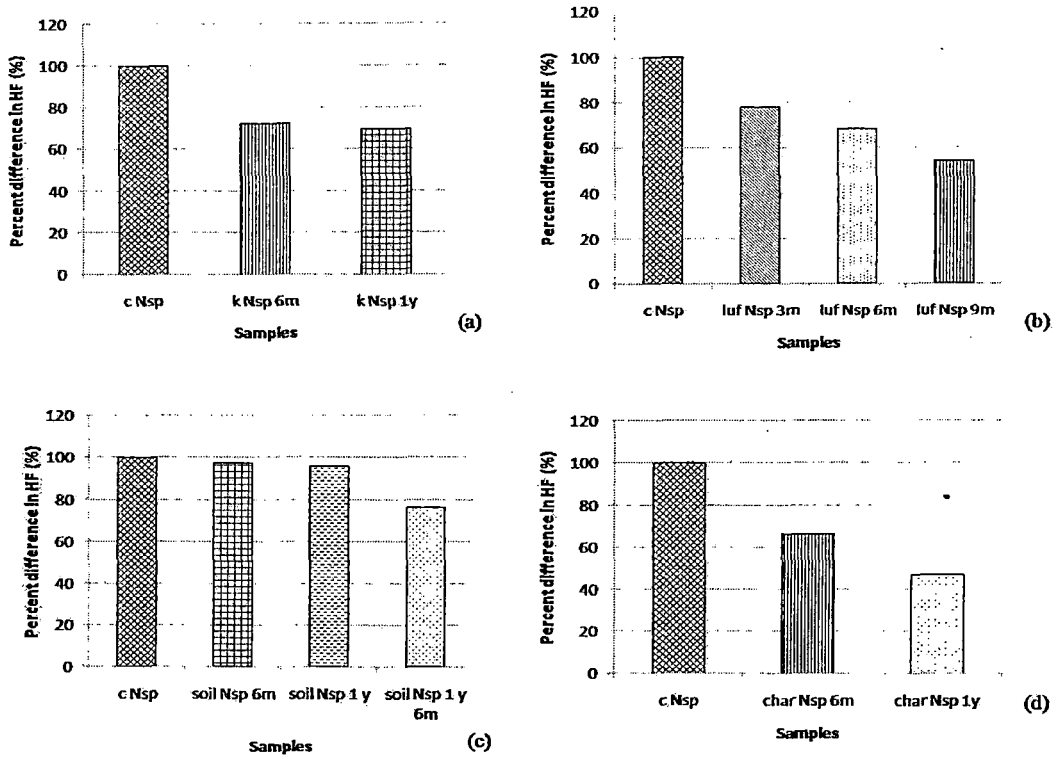


Fig 5.7: Percent difference in heterocyst frequency of *Nostoc* sp. (Nsp) regenerated from (a) "snep kwai", (b) luffa, (c) soil and (d) charcoal. *[In fig, 'c' refers to control samples; 'k', 'luf', 's' and 'char' refers to samples regenerated from immobilization in kwai, luffa, soil and charcoal respectively. 'm' indicates the age (in months) and 'y' the age in years of the preserved samples.]*

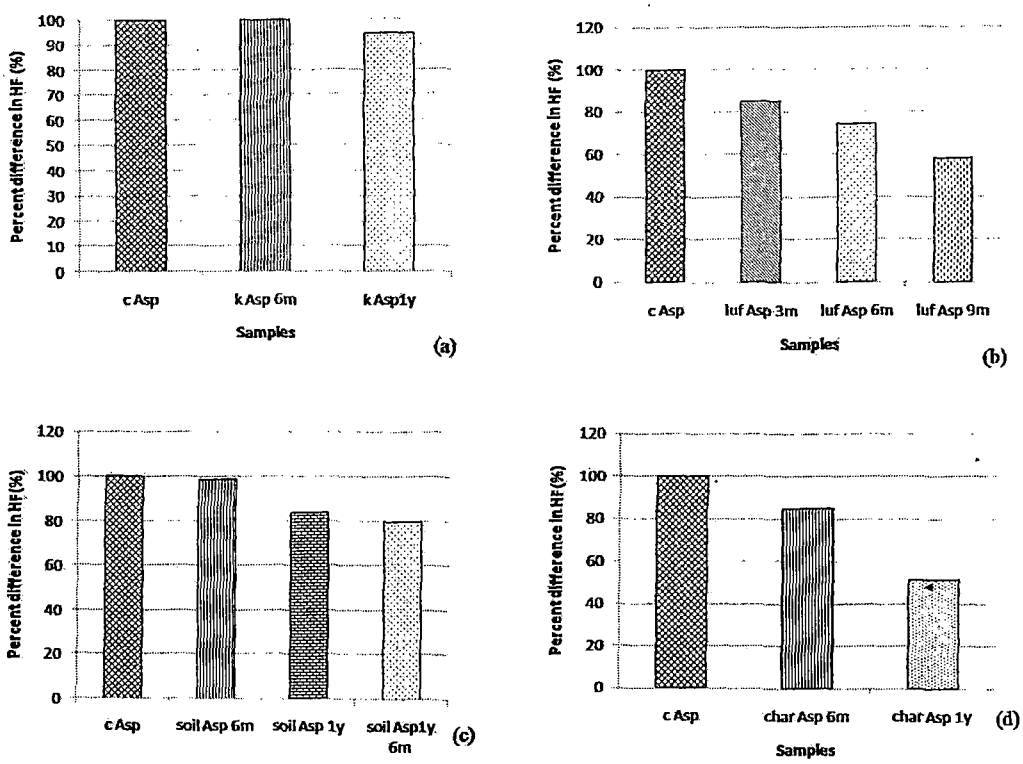


Fig 5.8: Percent difference in heterocyst frequency of *Anabaena* sp. (Asp) regenerated from (a) "snep kwai", (b) luffa, (c) soil and (d) charcoal. *[In fig, 'c' refers to control samples; 'k', 'luf', 's' and 'char' refers to samples regenerated from immobilization in kwai, luffa, soil and charcoal respectively. 'm' indicates the age (in months) 'y' the age in years of the preserved samples.]*

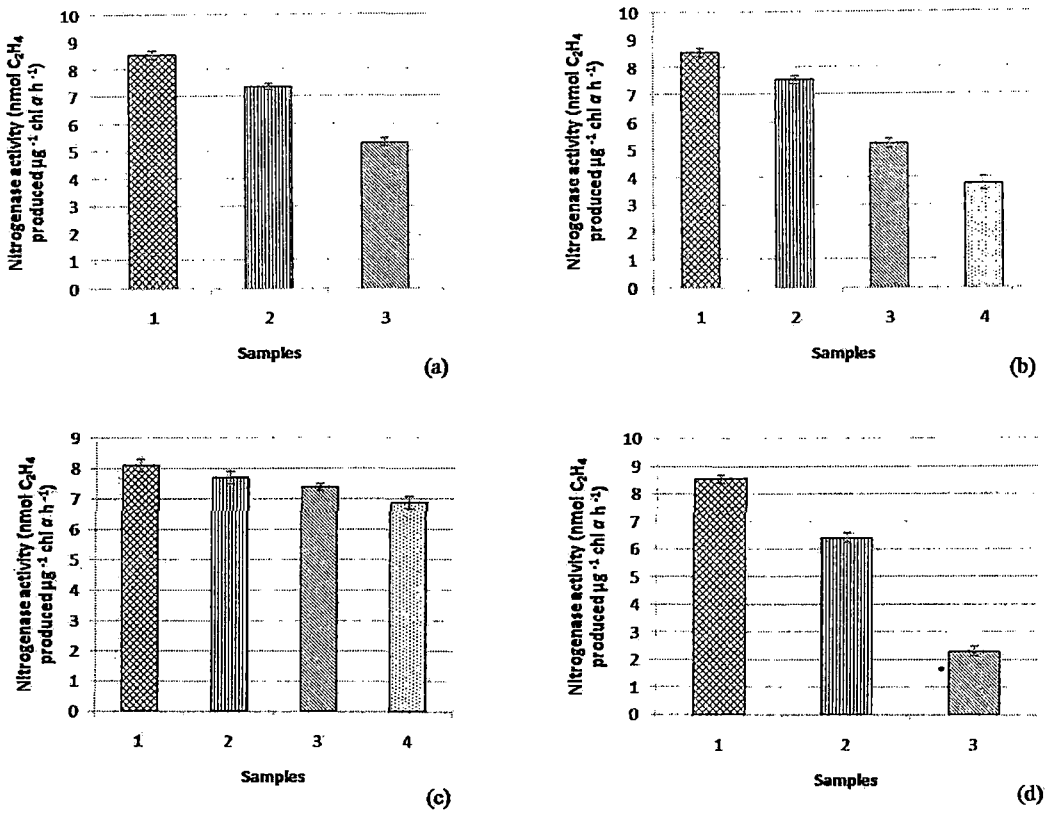


Fig 5.9: Nitrogenase activity of *Nostoc* sp. (Nsp) regenerated from, (a) 'snep kwai' after one year of preservation, (b) from luffa after nine months of preservation, (c) from soil after one year and six of preservation and (d) from charcoal after one year of preservation. *(Note: column1 refers to the control sample in each case; for fig a, column 2 and 3 represents the nitrogenase activity of regenerated samples at the end of 6 months and 1 year respectively; for fig b, column 2, 3 and 4 represents the nitrogenase activity of regenerated samples at the end of 3, 6 and 9 months respectively; for fig c, column 2, 3 and 4 represents the nitrogenase activity of regenerated samples at the end of 6 months, 1 year and 1 year 6 months respectively; for fig d, column 2 and 3 represents the nitrogenase activity of regenerated samples at the end of 6 months and 1 year respectively).*

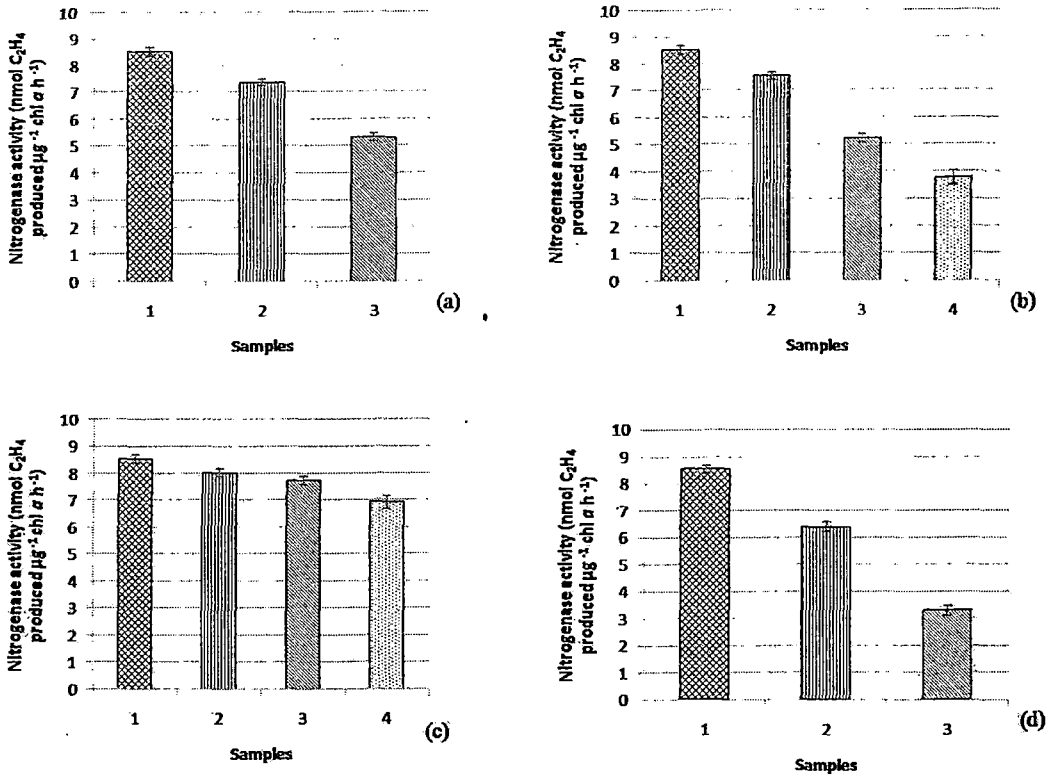


Fig 5.10: Nitrogenase activity of *Anabaena* sp. (Asp) regenerated from (a) "snep kwai" after one year of preservation and (b) luffa after nine months of preservation, (c) soil after one year and six months of preservation and (b) of charcoal after nine months of preservation. *(Note: column1 refers to the control sample in each case; for fig a, column 2 and 3 represents the nitrogenase activity of regenerated samples at the end of 6 months and 1 year respectively; for fig b, column 2, 3 and 4 represents the nitrogenase activity of regenerated samples at the end of 3,6 and 9 months respectively; for fig c, column 2, 3 and 4 represents the nitrogenase activity of regenerated samples at the end of 6 months, 1 year and 1 year 6 months respectively; for fig d, column 2 and 3 represents the nitrogenase activity of regenerated samples at the end of 6 months and 1 year respectively).*

5.4 Conclusion

This chapter dealt with unconventional methods of preservation that could be useful for application of cyanobacteria in agriculture and in environmental restoration programmes. As mentioned earlier, soil has already been attempted as a base for carrying cyanobacterial inoculum for field applications. The use of betel nut covers for immobilizing cyanobacteria was a significant step forward towards development of suitable and economical carrier of cyanobacteria for such applications. Our experiments suggested that cyanobacteria could temporarily be preserved on betel nut covers for duration up to twelve months. This period is sufficient for bulk production, immobilization, storage and transfer of viable cyanobacterial inoculum to intended destinations. Betel nut covers are waste generated locally in substantial amount and has the potential to be used as inexpensive carrier of cyanobacteria as it fulfils various requirements of an ideal carrier including its biodegradability. Washed and cleaned fibres of betel nut are relatively inert and could hold sufficient amount of inoculum on itself. Introduction of favourable growth conditions resulted in profuse proliferation of the immobilized cyanobacterial cells from these fibres and no alteration of any vital characters such as nitrogenase activity was apparent in the regenerated cells.

Therefore, this initial study provides an insight into some short term preservation methods relevant in application of cyanobacteria and could have important contribution towards development of appropriate carriers for future biofertilizer and bioremediation programmes. The following chapter describes the experiments conducted to establish betel nut covers as successful carrier of cyanobacteria for field applications. Charcoal as carrier of

cyanobacteria for potted plants specifically for growing orchids has also been tested with encouraging results.

5.5 Salient findings

- Betel nut covers, soil, *Luffa cylindrica*, charcoal chips were used for short term preservation of cyanobacteria.
- *Luffa cylindrica* was found unsuitable as the holding capacity was much less due to its porous texture.
- Initial inoculum load was found to have a definitive role in influencing the duration up to which dry samples could be stored.
- Among the bases considered, soil was found to be the best for preservation of cyanobacteria in terms of duration as well as retention of native characters.
- Fibres of betel nut covers came in close second as base for preserving cyanobacteria. It is a biodegradable waste material and thus a technology utilizing these could be an environment friendly and economically feasible option.
- Charcoal could maintain cyanobacteria viable for at least a period of six months upon immobilization and thus, could be projected as a rich potting material for plants such as orchids.

Chapter 6

Use of fibres of betel nut cover as carrier for biofertilizer and bioremediator inoculums: *screening of local isolates of cyanobacteria for potential applications in agriculture and in improvement of polluted environment and their immobilization on 'snep kwai'.*

6.1 Potential applications of cyanobacteria in agriculture and environment

Inclusion of cyanobacteria as biofertilizer for improving soil quality and fertility in agricultural practices has long been known (De 1939; Venkataraman 1981; Roger and Ladha 1992; Roger and Kulasooriya 1980; Metting 1988; Whitton 2000). Besides in recent times, considerable research has been done on application of cyanobacteria in cleaning up of polluted environment. Live cyanobacterial cells are ideal for such applications as they are capable of photosynthesis and nitrogen fixation and thereby require very little chemical inputs for their growth and cell division. These qualities could project them as cheap and affordable alternatives to agrochemicals as well as to expensive environmental remediation protocols. Being biological, they have little or no adverse affect on the environment in which they are introduced. However, not all strains are equal in their performance. Thus, isolates have to be assessed for their ability to perform desired function(s). In this chapter we put forward some finding relating to potential applications of indigenous cyanobacterial isolates as biofertilizer in rice cultivation and as natural agents capable in heavy metal removal from the surrounding. We also present possible alternative carrier solution of these strains to site of applications.

6.2 Screening of cyanobacteria for biofertilizer application

Thirty five cyanobacteria isolated from various places of Meghalaya and the neighbouring state Assam was screened for colonization of rice roots. Most isolates were from rice fields as we felt that cyanobacteria present in these ecosystems are already accustomed to being in close proximity to rice seedlings as well as to prevailing conditions of rice fields. One hot spring isolate was also tried for colonization study in anticipation of presenting it as biofertilizer for tropical rice fields. In addition, *Nostoc* cyanobionts were isolated from three independent *Anthoceros* thalli collected from different locations. Their inclusion in the study could be justified as these cyanobionts already existed in symbiotic association and therefore are well acquainted with symbiotic life style. Thus, we presumed that they could be efficient in forming successful association with rice seedlings in a short duration of time.

Table 6.1: Cyanobacterial isolates selected for the study

Serial no.	Sample	Origin of sample	Sample identified as
1	*RF 1*	Rice fields. Near NEHU Campus. Meghalaya	*Anabaena oryzae*
2	*RF 2*	Rice fields. Near NEHU Campus. Meghalaya	*Plectonema boryanum*
3	*RF3*	Rice fields. Near NEHU Campus. Meghalaya	*Nostoc* sp.
4	*RF4*	Rice fields. Near NEHU Campus. Meghalaya	*Anabaena oryzae*
5	*RF5*	Rice fields. Near NEHU Campus. Meghalaya	*Aphanothece* sp.
6	*RF6*	Rice fields. Near NEHU Campus. Meghalaya	*Nostoc* sp.
7	*RF7*	Rice fields. Near NEHU Campus. Meghalaya	*Anabaena* sp.
8	*RF8*	Rice fields. Near NEHU Campus. Meghalaya	*Calothrix* sp.
9	*RF9*	Rice fields. Near NEHU Campus. Meghalaya	*Nostoc* sp.
10	*RF10*	Rice fields. Near NEHU Campus. Meghalaya	*Nostoc* sp.
11	*RF11*	Umkhen. Jaintia Hills. Meghalaya.	*Tolypothrix* sp.
12	*RF12*	Umium. Ri Bhoi. Meghalaya.	*Nostoc* sp.
13	*RF13*	Nongstoin. West Khasi Hills. Meghalaya.	*Anabaena* sp
14	*RF14*	Mawphlang.East Khasi Hills. Meghalaya.	*Nostoc* sp.
15	*RF15*	Syntu Ksiar. Jaintia Hills.	*Anabaena* sp.

		Meghalaya.	
16	*RF16*	Syntu Ksiar. Jaintia Hills. Meghalaya.	*Nostoc* sp.
17	*RF17*	Syntu Ksiar. Jaintia Hills. Meghalaya.	*Anabaena* sp.
18	*RF18*	Syntu Ksiar. Jaintia Hills. Meghalaya.	*Nostoc* sp.
19	*RF19*	Syntu Ksiar. Jaintia Hills. Meghalaya.	*Nostoc* sp.
20	*RF20*	Syntu Ksiar. Jaintia Hills. Meghalaya.	*Gloeocapsa* sp.
21	*RF21*	Syntu Ksiar. Jaintia Hills. Meghalaya.	*Anabaena variabilis*
22	*RF22*	Rice fields. Guwahati, Assam	*Nostoc* sp.
23	*RF23*	Rice fields. Guwahati, Assam	*Nostoc* sp.
24	*RF24*	Rice fields. Sonapur,Assam	*Anabaena* sp.
25	*RF25*	Rice fields. Sonapur,Assam	*Plectonema* sp.
26	*RF26*	Rice fields. Sonapur,Assam	*Nostoc* sp.
27	*RF27*	Rice fields. Bagori,Assam	*Nostoc* sp.
28	*RF28*	Rice fields. Bagori,Assam	*Calothrix* sp.
29	*RF29*	Rice fields. Dergaon,Assam	*Westiellopsis* sp.
30	*RF30*	Rice fields. Dergaon, Assam	*Cylindrospermum fertilisma*
31	*RF31*	Rice fields. Goiaghat, Assam	*Anabaena* sp.
32	*HS1*	Jakrem hot spring. West Khasi Hills. Meghalaya.	*Mastigocladus laminosus*
33	*NA1*	From *Anthoceros punctatus* (NEHU)	*Nostoc* sp.
34	*NA2*	From *Anthoceros punctatus* (NEHU)	*Nostoc* ANTH
35	*NA3*	From *Anthoceros punctatus* (Mawphlang)	*Nostoc* sp

A visual screening established that all 35 isolates showed colonization of rice roots to varying degrees (Fig 6.1).

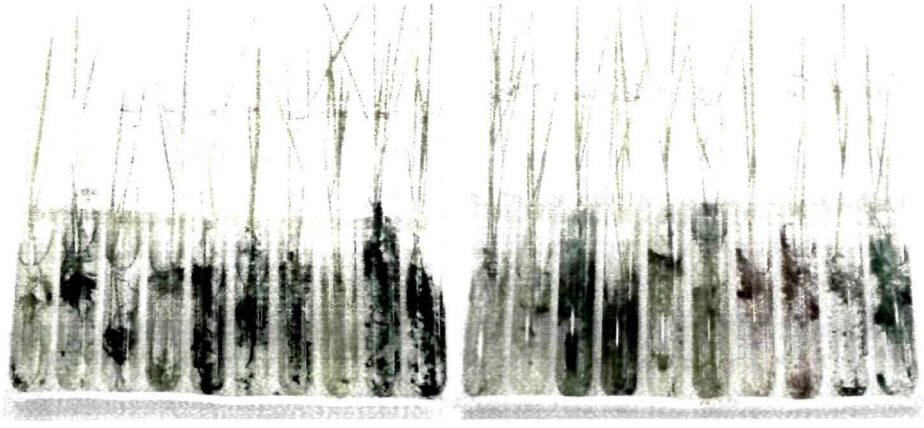


Fig 6.1: Rice root colonization by cyanobacteria

Of these, 21 showed tight association with rice roots (Table 6.2). This was determined by calculating chlorophyll *a* content and associated nitrogenase activity in the excised roots. The excised roots were subjected to ultrasonic bath for two minutes to remove loosely associated cyanobacteria. The values obtained for chlorophyll *a* and nitrogenase activity were those of the tightly associated cyanobacteria. A notable observation of the colonization study was that the nitrogenase activity was higher in the cyanobacterial filaments associated with roots than their free-living counterparts.

Table 6.2: Successful colonization of rice roots by different cyanobacteria. (*Values are mean of three independent sets of experiments*).

Serial no.	Sample number	Sample identified as	Chlorophyll *a* (µg/g root dry weight)	Nitrogenase activity nmol C_2H_4 produced/ µg chl *a*/h (free-living)	Associated nitrogenase activity nmol C_2H_4 produced/ µg/g root dry weight/h
1	*RF 1*	*Anabaena oryzae*	432	5.1	7.32
2	*RF3*	*Nostoc* sp.	505	6.3	8.22
3	*RF4*	*Anabaena oryzae*	412	4.3	5.91
4	*RF6*	*Nostoc* sp.	378	3.8	5.12
5	*RF7*	*Anabaena* sp.	682	7.0	10.62
6	*RF8*	*Calothrix* sp.	565	6.4	8.60
7	*RF9*	*Nostoc* sp.	398	4.9	5.72
8	*RF13*	*Anabaena* sp.	518	6.2	8.43
9	*RF14*	*Nostoc* sp.	501	6.0	7.87
10	*RF16*	*Nostoc* sp.	619	7.8	9.76
11	*RF19*	*Nostoc* sp.	583	6.8	8.74
12	*RF21*	*Anabaena variabilis*	467	4.9	6.13
13	*RF22*	*Nostoc* sp.	453	5.2	6.87
14	*RF23*	*Nostoc* sp.	466	5.9	7.59
15	*RF24*	*Anabaena* sp.	379	4.9	5.73
16	*RF26*	*Nostoc* sp.	475	5.9	6.91
17	*RF 27*	*Nostoc* sp.	498	6.5	7.40
18	*NA 1*	*Nostoc* sp.	663	7.4	10.33
19	*NA 2*	*Nostoc* ANTH	493	7.1	7.84
20	*NA 3*	*Nostoc* sp.	488	6.4	7.74
21	*HS1*	*Mastigocladus laminosus*	324	3.1	4.66

The main objective of finding suitable cyanobacteria to be used as biofertilizer is to provide crop plants with higher fixed nitrogen in its vicinity. In our colonization study, sample numbers *RF7*, *RF16* and *NA1* exhibited higher associative nitrogenase activity and

therefore, were selected as potential biofertilizer strains to be preserved for future studies. A point to note is that two of these isolates were collected from rice fields that recorded pH in the acidic range (6.2 for isolate *RF7* and 6.8 for *RF16*). Thus, these cyanobacteria are adapted to low pH and could be kept in mind as biofertilizer for acidic rice fields.

Co-cultivation showed positive influence on the health of rice plants as well. Cyanobacterial association had significant beneficial impact on the overall growth of rice seedlings (Fig 6.2).

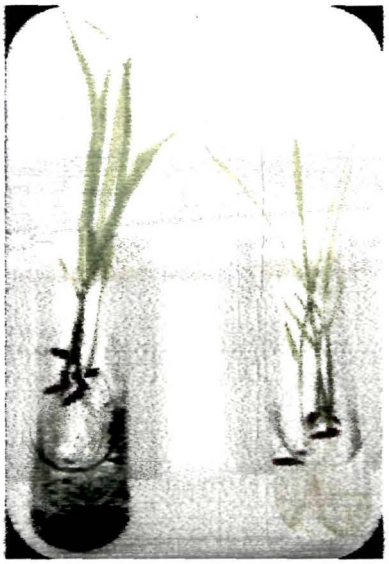


Fig 6.2: Positive influence of cyanobacteria on the growth of rice plants

Seven experimental set ups each comprising ten seedlings were used to study the effect of rice-cyanobacteria co-cultivation on the overall growth of rice plants. Rice variety DR-92 and cyanobacteria *RF7* was used for the experiment. Root and shoot lengths and their respective dry weights of the associated rice seedlings were calculated after co-cultivation period of 30 days. A parallel experiment was also run with rice seedlings growing without cyanobacteria to compare the difference brought about in growth of rice seedlings in presence and absence of cyanobacteria in their vicinity. Table 6.3 gives the details of the

experimental results. A distinct encouraging difference was seen in rice seedling grown along with cyanobacteria.

Table 6.3: Comparative root and shoot lengths and their respective dry weights of rice seedlings grown in presence and absence of cyanobacteria. *(Values are mean of three independent sets of experiments each containing ten seedlings).*

Sl no	Root lengths (cm)		Shoot lengths (cm)		Root dry weight (g)		Shoot dry weight (g)	
	- C	+ C	- C	+ C	- C	+ C	- C	+ C
1	5.5	8.9	11.3	17.7	0.076	0.089	0.059	0.143
2	5.2	9.3	10.2	16.8	0.079	0.093	0.049	0.121
3	4.6	10.5	10.6	17.3	0.083	0.102	0.052	0.155
4	3.9	10	11.1	17.8	0.073	0.083	0.038	0.136
5	4.2	10.6	9.8	20.1	0.087	0.123	0.063	0.122
6	6.1	11.2	11.6	18.6	0.084	0.109	0.044	0.149
7	5.3	10.3	9.9	18.9	0.082	0.118	0.046	0.155
	Average	Average	Average	Average	Average	Average	Average	Average
	4.97	11.11	10.64	18.18	0.08	0.102	0.051	0.140

[-C = seedlings grown in absence of cyanobacteria; +C = seedlings grown in presence of cyanobacteria]

6.3 Preservation of *RF7*, *RF16* and *NA1* on fibres of betel nut cover

As illustrated in the chapter 5, betel nut covers were able to retain viable cyanobacterial cells without compromising characters such as nitrogenase activity for atleast a period of 12 months. This is crucial for regenerated cyanobacteria if they are to be used as inoculums for biofertilizer programme. Betel nut covers have already been shown to be economically feasible option as carrier of cyanobacteria in the previous chapter. The cyanobacterial isolates *RF7*, *RF16* and *NA1* were immobilized on betel nut covers (5 ml cyanobacterial culture with chlorophyll *a* content of 10 μg ml^{-1} was immobilized on 5 gram betel nut fibres). These were stored in sterile plastic bags and kept at room temperature in dark. Every three months samples were regenerated and tested for retention of colonization ability. The fact that nitrogenase activity of associative cyanobacteria was found to be higher than that of the free-living cells (Table 6.2), associative nitrogenase activity of regenerated

sample that were used for colonization study was also measured to assess if this character has been compromised during long term preservation on these fibres.

Table 6.4: Chlorophyll *a*, nitrogenase activity and associated nitrogenase activity of cyanobacteria (*RF7*, *RF16* and *NA1*) regenerated after one year of preservation on betel nut fibres. *(Values are mean of three independent sets of experiments).*

Serial no.	Sample number	Sample identified as	Chlorophyll *a* (µg/g root dry weight)	Nitrogenase activity nmol C_2H_4 produced/ µg chl *a*/h (free-living)	Associated nitrogenase activity nmol C_2H_4 produced/ µg/g root dry weight/h
1	*RF 7*	*Anabaena* sp.	696	7.32	10.55
2	*RF16*	*Nostoc* sp.	612	7.56	9.48
3	*NA1*	*Nostoc* sp.	668	7.23	10.19

6.4 $\delta^{15}N$ analysis as evidence of N-transfer from associated cyanobacteria (*RF7*) to rice seedlings

The knowledge of symbiotic association where there is beneficial exchange of metabolites between the partners has been extended to the practice of co-cultivation of rice with cyanobacteria. Better growth of rice seedlings in presence of cyanobacteria (Fig 6.2) prompted the experiment to study the transfer of fixed nitrogen from the cyanobacteria to the rice plants. A CHNS analyser (Vario EL 111 Elementar) was used to investigate the ^{15}N transfer between the two partners. Following formula was used for calculation of $\delta^{15}N$:

$$\delta^{15}N\ (‰) = [(R_{sample}/R_{standard})-1] \times 100$$

Where R = $^{15}N/\ ^{14}N$ ratio and the standard is the dinitrogen of air, defined as having a δ value of zero.

It is well established that soil N has higher abundance of ^{15}N than atmospheric N owing to ^{15}N being selectively retained because of its heavier mass. ^{15}N enrichment of soil being the net result (Turner et al 1983; Azam and Farooq 2003). Consequently processes such as nitrogen fixation, N losses and plant uptake continuously causes ^{15}N enrichment of

soil N and depletion of atmospheric N (Azam and Farooq 2003). Because of the difference in natural ^{15}N abundance, N_2 fixing plants that depends on soil N and biological nitrogen fixation, have low ^{15}N abundance than a non-fixing plant that obtains nitrogen from soil alone (Kohl and Shearer 1980; Danso et al 1993). This difference of natural ^{15}N abundance among plants can be used for assessing the contribution of biological nitrogen fixation in a diazotrophic association (Shearer and Kohl 1986).

For this experiment 10 days old rice seedling were co-cultured with cyanobacterial isolate *RF7* for four days ,the loosely associated cyanobacteria removed and the seedlings transferred to 10 fold diluted BG 11_0 medium. The plants were subjected to a 12h light/dark cycle. After three weeks, the plants were uprooted, cleaned and dried in a hot air oven at 25°C. The dried plants were finely ground and the powdered sample used for analysis. In two parallel experiments, rice plants were grown without association with cyanobacteria and with regenerated cyanobacterial samples. In Table 6.5, natural $\delta^{15}N$ abundance of rice seedlings grown with or without *RF7* are presented to compare cyanobacterial contribution to the nitrogen budget of rice plants.

Table 6.5: $\delta^{15}N$ values of N_2-fixing *RF7* (free-living and regenerated) in association with rice seedlings compared to unassociated rice plants. *(Values are mean of three independent sets of experiments).*

Samples	$\delta^{15}N$ value (‰)
Unassociated Rice seedlings	4.82±0.12
Associated Rice seedling with free-living RF7	1.65±0.18
Rice seedling associated with regenerated RF7 (six month under preservation)	1.98±0.24

6.5 Results and Discussion

All thirty five cyanobacterial isolates were able to colonize rice roots to varying degrees (Fig 6.1). Thus it appears that colonization is favoured by both organisms and there seems to be no adverse reaction to such close proximity by either of them. Twenty one of

these cyanobacteria could be distinguished as being able to form tight association with the roots (Table 6.2). And such association led to visible improvement in the health of the rice seedlings (Fig 6.2; Table 6.3). A study of nitrogenase activity of the associated roots showed that the cyanobacteria adhering tightly to these roots had increased activity than their free-living counterparts. Therefore, it may be concluded that there could be possible exchange of metabolites between the associated partners to bring about positive influence on growth of rice plants. Of the twenty one tightly associating cyanobacteria, *RF7*, *RF16* and *NA1* showed distinctly higher associative nitrogenase ability, thereby making them targets for research involving their potential as biofertilizers in acidic rice fields. Isotopic analysis using the isolate *RF7* revealed that $\delta^{15}N$ value of unassociated plants was 4.82‰ while that of the plants associated with free-living *RF7* was 1.65‰ (Table 6.5). This indicated that significant amount of nitrogen was derived from nitrogen fixation by the associated cyanobacteria. $\delta^{15}N$ value of 1.98‰ obtained from rice seedlings associated with *RF7* sample regenerated from immobilization on fibres of betel nut outer cover after six month of preservation suggested that these immobilized cells retained their ability not only to regenerate into viable cells but also were able to retain nitrogen fixation faculty and nitrogen transfer ability.

6.6 Assessing heavy metal removal ability of indigenous cyanobacteria

Many substances known to have toxic properties are introduced into the environment through human activity. Such anthropogenic activities many a times lead to release of heavy metals in to the environment. These substances ultimately come in contact with soil and accumulate over time in the exposed area. In recent times, technology offers bioremediation as alternative method to clean up contaminated environments. The science of bioremediation engages microbes. Among microbes, considerable amount of research has been done to

reduce heavy metals from wastewater by using cyanobacteria (Anjana et al 2007; El-Enany and Issa 2000; Cain et al 2008; Inthorn et al 2002). In addition, some reports mention that cyanobacteria have been used as an absorbent agent in removing heavy metal like cadmium, lead, chromium from water (Anjana et al 2007; El-Enany and Issa 2000; and Cain et al 2008; Inthorn et al 2002). Microbial biomass can passively bind large amounts of metal(s), a phenomenon commonly referred to as biosorption, thus providing a cost-effective solution for industrial wastewater management (Volesky and Holan 1995).

This section details the experiments and the results obtained from a study engaging cyanobacteria in order to isolate potential indigenous strains with metal removal ability. Results of experiments in this section also envisage betel nut cover fibres as suitable, convenient and economical storage material and as biodegradable carrier for application of cyanobacteria in clean up operations.

6.7 Screening of some indigenous cyanobacterial isolates for heavy metal removal potential

Samples were collected from areas adjacent to coal mines in the state of Meghalaya (Table 6.6).

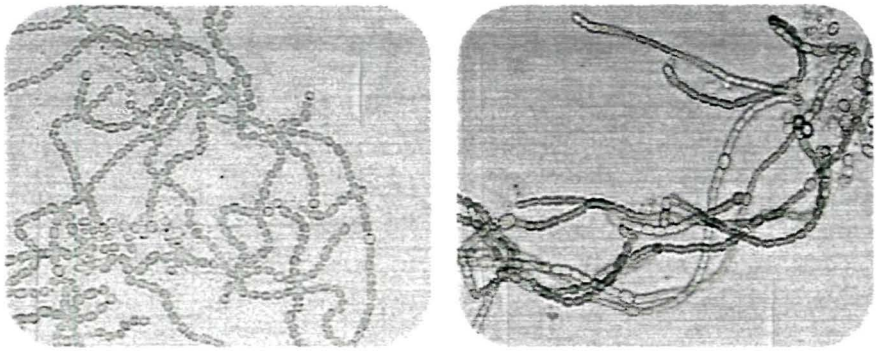
Table 6.6: Cyanobacterial isolates collected from coal mining sites in Meghalaya.

Sl.no.	Sample no.	Origin of sample(s)	Sample identified as
1	CM1	Coal mining site. East khasi hills. Meghalaya.	*Anabaena* sp.
2	CM2	Coal mining site. East khasi hills. Meghalaya.	*Nostoc* sp.
3	CM3	Coal mining site. Jowai. Jaintia Hills. Meghalaya.	*Nostoc* sp.
4	CM4	Coal mining site. Ladrymbai. Jaintia Hills. Meghalaya.	*Nostoc* sp.
5	CM5	Coal mining site Ladrymbai. Jaintia Hills. Meghalaya.	*Nostoc* sp.
6	CM6	Coal mining site Khliehriat. Jaintia Hills. Meghalaya.	*Anabaena* sp.
7	CM7	Coal mining site Khliehriat. Jaintia Hills. Meghalaya.	*Anabaena* sp.
8	CM8	Coal mining site Khliehriat. Jaintia Hills. Meghalaya.	*Anabaena* sp.
9	CM9	Coal mining site Jowai. Jaintia Hills. Meghalaya.	*Nostoc* sp.

Being isolated from coal mining areas, these cyanobacteria were already exposed to pollutants and their survival in such areas indicated that they had developed strategies to overcome toxic impact of such pollutant to certain degree. Amongst heavy metals Cd is known for its potential toxicity to microbes, plants animals and humans (Jose et al 2002; Yao et al 2003). Thus, all isolates were screened for their tolerance to presence of cadmium in the surrounding growth medium. Additionally, we had included zinc in our study as zinc is a common heavy metal pollutant in the environment. Among the isolates, two ubiquitous cyanobacterial strains (*Anabaena* sp. and a *Nostoc* sp.) were found to be most efficient in metal removal. These two strains were selected for the study. Atomic absorption spectroscopy (Perkin Elmer 3110 Atomic Absorption Spectrophotometer, Sophisticated Analytical Instrumentation Facility, NEHU, Shillong) was used to determine presence and concentrations of heavy metals in different fractions of experimental samples. After assessment, potential strains were immobilized in calcium alginate beads and stored. The cyanobacterial cells were regenerated from the storage matrices periodically to study retention of heavy metal removal property in these organisms.

6.8 Results and Discussion

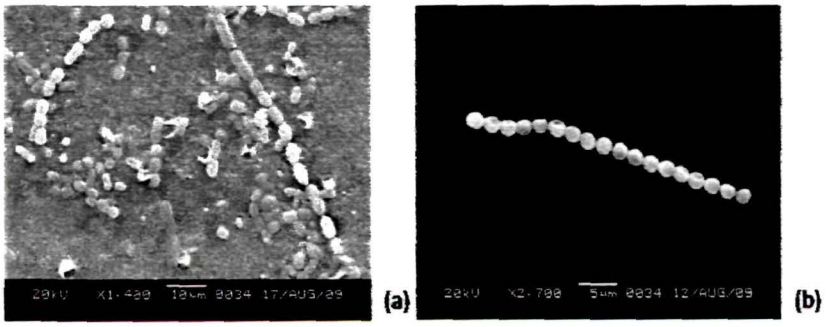
The state of Meghalaya has significant amount of coal deposits and thus large area within the state is under mining activities. This results in production of mine tailings and sludge that pollutes adjoining water bodies. Since role of cyanobacteria in removal of heavy metals from contaminated water has been well established by a number of researchers (Anjana et al 2007; El-Enany and Issa 2000; and Cain et al 2008), we have for metal removal potential study, shortlisted an *Anabaena* (*CM1*) and a *Nostoc* (*CM9*) sps. that were ubiquitous in almost all collection sites.



(a) (b)

Fig 6.3: Light microscopic pictures of *Anabaena* sp. (a) and *Nostoc* sp. (b) selected for the study

For evaluating metal removal potential of these cyanobacterial strains, exponentially growing cyanobacterial cultures were concentrated by centrifugation and then were resuspended in BG 11_0 medium containing 0.02 mM cadmium sulphate or zinc sulphate. Increasing concentration of Cd and Zn in the growth medium negatively affected the growth and other metabolic characters in both the cyanobacteria sps. Presence of 0.02 mM Cd led to visible fissures on many cyanobacterial cells without altering the cell shape (Fig 6.4 a). However, same concentration of Zn in the medium had completely different effect on the morphology of the cells (Fig 6.4 b). The cells in Zn supplemented medium showed osmotic type of stress where many cells had become rounded and deflated. The connections between the cells were more fragile in Cd treated cells than in the cells exposed to Zn stress.



(a) (b)

Fig 6.4: Scanning electron micrograph showing the effect of heavy metal (a) Cd stress and (b) Zn stress on cyanobacteria

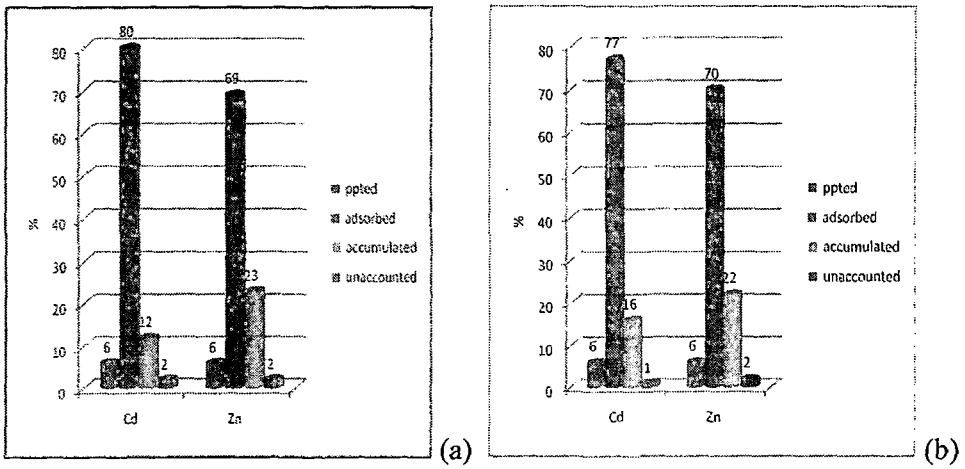
To study heavy metal removal ability, the treated cells were harvested by centrifugation at 5000Xg after 48 h of heavy metal exposure and remaining Cd or Zn was estimated in the supernatant using atomic absorption spectroscopy (AAS). This value was subtracted from the initial concentration to arrive at the amount of metal ions removed by the cyanobacterial cells. Further, distribution of the removed ions on and within the cells was calculated using the following method. To find out the amount of heavy metals precipitated on the cell surface 3 ml of 0.2 M phosphate buffer (pH 7.5) was added to the pellet and the tubes were centrifuged at 5000Xg. The precipitated metal ion concentration was estimated by AAS in the supernatant. The pellet was further washed with 3 ml of 10 mM EDTA for desorption of metal ions and centrifuged at 5000Xg. Adsorbed concentration of metal ions was determined in this supernatant. Further, the pellet resuspended in growth medium and cells were disrupted by sonication. The amount of intracellular accumulation of heavy metal was determined by measuring the metal ion concentration in the supernatant obtained after centrifugation at 5000Xg. The values obtained are expressed in percentage in the table. The % concentration of heavy metal was calculated as follows: (amount of metal ion present in the supernatant) / (amount of initial metal ion) X100.

Table 6.7: Metal ions removal by cyanobacteria. (*Values are mean of three independent sets of experiments*).

	Nostoc sp.		*Anabaena* sp.	
%Cd and Zn removal in 48 h	**Cd**	**Zn**	**Cd**	**Zn**
(A) Total metal ions removed from the medium	61	75	53	62
(B) Precipitated on the surface (of removed metal)	6	6	6	6
(C) Adsorbed on the surface (of removed metal)	80	67	77	70
(D) Accumulated intracellularly (of removed metal)	12	23	16	22
(E) Unaccounted metal ions	2	2	1	2

Atomic absorption studies showed that 61% (i.e. 0.0122 mM) of the Cd available in the medium was removed by the *Nostoc* cells within 48 h. The removal was saturating at 72h. Of the removed Cd 6% was precipitated on the cell surface, 80% was adsorbed and

12% was intracellularly accumulated. Remaining 2 % was unaccounted for. This may due to some Cd being adhered to the glass surface of the test tubes. However, *Nostoc* cells removed much higher percentage of Zn (75%) within 48 h. It is interesting to note that the *Nostoc* cells accumulated almost two fold higher amount (12% vs. 23%) of Zn intracellularly than it did for Cd. This may be the reason for the expressed osmotic stress on the morphology of the cells in Zn supplemented medium (Fig 6.4b). The ability to remove heavy metals from the surroundings is lower in *Anabaena* cells (it removed 53% Cd and 62% Zn as against 61% Cd and 75% Zn by *Nostoc* cells respectively). As in case of *Nostoc* cells, high amount of the heavy metals were adsorbed on the cell surfaces (77%). Intracellular accumulation of Zn was similar in both the organisms (23% and 22%) while more Cd was accumulated by the *Anabaena* cells (16%) as compared to *Nostoc* cells (12%).

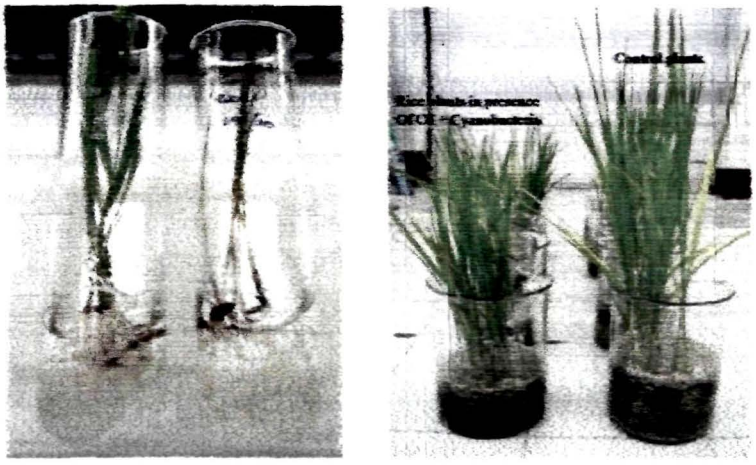


(a) (b)

Fig 6.5: Metal ions removal by (a) *Nostoc* sp. and (b) *Anabaena* sp.

With the above knowledge of cyanobacteria's potential in reducing heavy metal load in the surrounding, experiments were set up to study their potential in reducing cadmium and zinc toxicity to rice plants. If so then cyanobacteria could serve dual purpose of acting as biofertilizer as well as protecting rice plants from heavy metal toxicity in the fields containing such pollutants. For this, experiments were set up with rice plants growing under

controlled conditions, in presence of 0.02 mM cadmium or zinc, as well as in combination of one heavy metal (0.02 mM) and cyanobacteria (*CM9*) at a concentration of 10 μg ml^{-1}. 0.02 mM cadmium and zinc was found to be toxic to rice plants. Fig 6.7a shows that exposure to this concentration of $CdSO_4$ led to death of rice seedlings within 6-8 days. However, inclusion of cyanobacteria significantly reduced the toxicity (Fig 6.6 b).



(a) **(b)**

Fig 6.6: (a) Effect of cadmium toxicity on rice seedlings; (b) protection to toxicity offered by presence of cyanobacteria

Table 6.8: Comparative root and shoot length of rice plantlets grown in cadmium supplemented medium, with and without cyanobacteria. (*Values are mean of three independent sets of experiments*).

Root length (cm)			Shoot length (cm)		
Control (A)	***0.02 mM cadmium (B)***	***(B) + cyanobacteria***	***Control***	***0.02 mM cadmium (B)***	***(B) + cyanobacteria***
8.9	5.5	5.6	17.7	11.3	14.7
9.3	5.2	7.3	16.8	10.2	13.6
10.5	4.6	6.5	17.3	10.6	13.9
10.0	3.9	5.9	17.9	11.1	14.2
10.6	4.2	5.5	20.1	12.8	15.0
11.2	6.1	7.3	18.6	11.6	14.5
10.3	5.3	7.3	18.9	11.9	13.5
Average	**Average**	**Average**	**Average**	**Average**	**Average**
10.11	4.97	6.5	18.18	11.24	14.2

When root and shoot length of the rice seedlings were compared in absence and presence of cyanobacteria, the positive effect of cyanobacteria in their vicinity is clearly

visible (Table 6.8). Presence of 0.02mM cadmium in the medium in absence of cyanobacteria led to 50.85% and 38.11% growth inhibition in the root and shoot length respectively. However, presence of cyanobacteria reduced this inhibitory effect to 35.70% and 21.89% in the growing root and shoot lengths. Thus, presence of cyanobacteria in the vicinity of rice seedlings had definite beneficial consequences.

Use of cyanobacteria as biofertilizer as well as agents of bioremediation requires careful selection of efficient cyanobacterial strains and preserving them for timely use. In addition, field applications require transport to specified locations. Thus, appropriate carrier materials also have to be chosen keeping in mind their non-toxicity to the organism being carried on. They have to be cheap and locally available to be economically feasible, preferably carry the strains in dry state for convenience of transport and most of all should be biodegradable and non toxic to the environment. As pointed out in the chapter 4, preservation in calcium alginate beads was most suitable for storing selected cyanobacteria under laboratory conditions. Therefore, *CM1* and *CM9* were immobilized in alginate beads and marked for future work in relation to heavy metal removal. For field transfer, fibres of betel nut cover were found to fulfil all the requirements needed to be an ideal carrier.

6.9 Development of an innovative approach for using immobilized cyanobacteria as potting supplements for orchid cultivation

Use of cyanobacteria in rice cultivation has been extensively studied by various researchers (Watanabe et al 1951; Venkataraman 1972; Roger and Kulasooriya 1980; Venkataraman 1981; Metting 1988; Albretch et al 1991; Roger and Ladha 1992; Whitton 2000). However, potential of the cyanobacteria as biofertilizer in garden and potted plants has not yet been explored. For commercial purposes cyanobacterial biofertilizer have to be

distributed in powder form or on matrices that are suitable for potted plants. In addition, for effectively acting as biofertilizer in potted plants, similarly applied cyanobacteria have to be able to grow and proliferate quickly in the pots upon watering. Further, these cyanobacterial filaments should be able to fix atmospheric nitrogen at a rate close to its free-living counterparts.

The state of Meghalaya is home to almost 300 species of orchids (http://megtourism.gov.in/flora.html). Many exotic orchids are widely grown in Meghalaya as ornamental plants at homes as well as cultivated on commercial scales. Orchids are among the most highly prized of ornamental plants. Many orchids are tree dwellers. They are epiphytic in nature and they do not obtain any moisture or nutrient from the tree. Epiphytic orchids use the branches of the trees on which they grow as a place of anchorage where they gather humidity, shade and pollinating insects. Their adhering roots are thick (sometimes as thick as a pencil) and are modified to obtain water from the atmosphere and that which collects on the bark of the tree. Growing orchids as ornamental plants is challenging as traditional soil or peat based compost holds too much moisture and clog the roots, causing the thick roots to rot. Hence, what is needed as potting material is for the material to resemble tree branch but not retain excessive water. Few options for such a material are charcoal, brick and bark. Of these, charcoal has been in use for potted orchid cultivation in recent years. Charcoal is made from tree branches and due to the nature of varied texture and thickness provides a perfect surface for the roots to grow over. It holds relatively little moisture and hence does not get sodden. In the first part of this work we have studied the potential of charcoal as an immobilizing base for cyanobacteria (Fig 5.3). Such immobilized cyanobacterial strains remained viable for a period of six to nine months

on the dry charcoal. Upon return of favourable conditions of growth, these immobilized cyanobacteria proliferated in the medium and were found to retain their nitrogen fixing ability. Therefore, charcoal pieces along with cyanobacteria could be excellent potting material for orchids as the cyanobacteria would be able to continuously provide fixed nitrogen to the plants. This would eliminate the need for any added supplements to the plant for promoting better growth, shorter duration between flowering and longer floral lifespan. Based on our observations, we have studied performance of cyanobacteria immobilized on charcoal in supporting better growth etc of orchid plantlets.

Dendrobium aphylum plantlets were germinated from seeds in the culture room using medium (Murashige and Skoog 1962; Das et al 2007). The plants were hardened inside the culture room and finally transferred on to disposable plastic cups containing soil and charcoal chips (Fig 6.7).

Fig 6.7: Plantlets of orchid being grown on charcoal chips containing immobilized *Nostoc* filaments.

In another set, the charcoal chips used for orchid transfer were pre-inoculated with *Nostoc* sp. cultures. After a month, better growth was distinctly visible in the pots

containing charcoal with cyanobacteria cells (Fig 6.8 b). The pots were only watered every two days throughout the experiment. No other nutrient was added.

(a) (b)

Fig 6.8: (a) Orchid plantlets growing on un-supplemented charcoal (b) better growth of orchid plantlets growing in presence of immobilized cyanobacteria on charcoal.

On close inspection cyanobacterial cells were found adhered to the individual orchid roots (Fig 6.9).

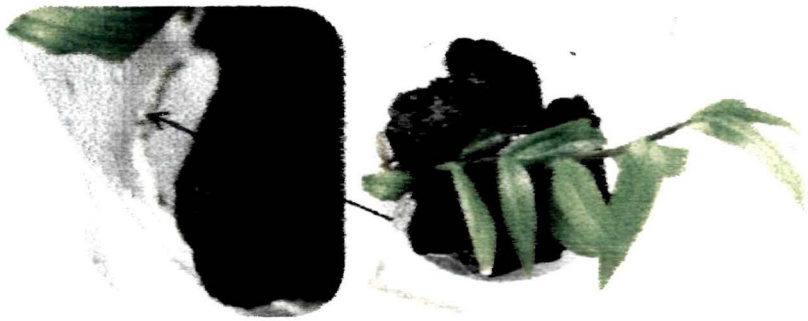


Fig 6.9: Cyanobacterial filaments adhering to the roots of orchid plantlets

The overall growth, the root size (length and thickness) and shoot appearance (the individual leaf size) was higher in the pots with *Nostoc* cells than in the plants used as reference without the immobilized cyanobacteria (Fig 6.10 a, b).

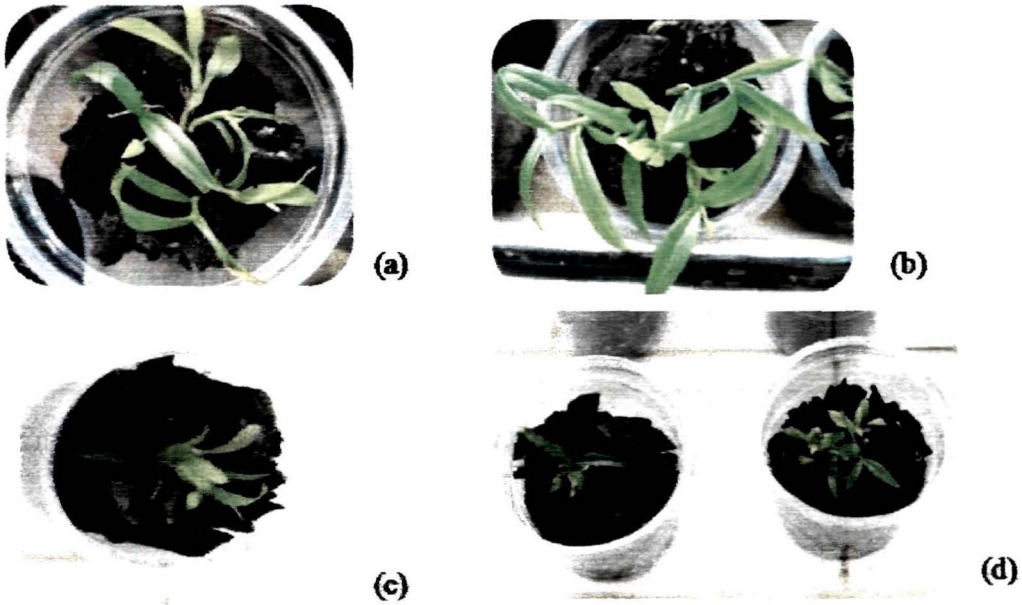


Fig 6.10: (a, b) Healthy orchid plantlets growing in presence of cyanobacteria; (c, d) poor growth of orchid plantlets on un-supplemented charcoal.

6.10 Salient findings

- Rice fields provide ideal conditions for growth of cyanobacteria. All collections from diverse rice fields yielded one of more cyanobacteria in the collection even though most rice fields in the state recorded acidic pH and cyanobacteria are known to prefer neutral to alkaline pH.
- All individual cyanobacterial isolates were capable of colonizing rice root *albeit* to varying degrees.
- Close association of cyanobacteria with rice roots resulted in better growth of these plants.
- Due to their abundance in acidic soils of Meghalaya coupled with higher associative nitrogenase activity when in close proximity to rice seedlings; *RF7*, *RF16* and *NA1* can be projected as likely candidates for biofertilizer programme in acidic rice fields.

- Evidence of biologically fixed nitrogen transfer was obtained using $\delta^{15}N$ analysis.
- Negative effects were seen on morphology and growth of cyanobacteria when subjected to increasing concentration of Cd and Zn. Atomic absorption studies showed that 61% of the Cd available in the medium was removed by the *Nostoc* cells within 48 h.
- *Nostoc* cells accumulated almost two fold higher amount (12% vs. 23%) of Zn intracellularly than it did for Cd.
- The ability to remove heavy metals from the surroundings was lower in *Anabaena* cells (it removed 53% Cd and 62% Zn as against 61% Cd and 75% Zn by *Nostoc* cells respectively).
- Presence of cyanobacteria in the vicinity of rice seedlings reduced inhibitory effect of cadmium by approximately 36% and 22% in the growing root and shoot lengths of rice plantlets.
- There was positive indication of better growth of orchid plantlets that were grown on charcoal containing immobilized cyanobacterial filaments. *Thus, use of such charcoal pieces carrying immobilized cyanobacteria can be exploited at commercial scale specifically for growing orchids.*

Chapter 7

Projecting spores as a means of preservation in many sporulating cyanobacterial strains: *standardization of procedures for bulk spore production in desired cyanobacteria for use as biofertilizer inoculum in rice cultivation.*

7.1 Cyanobacterial spores

Various genera of heterocystous cyanobacteria are known to differentiate morphologically and ultra-structurally into resting-state cells for survival under environmental stresses (Seckbach 2007). In nature, akinetes are believed to play an important role in perennating, allowing the producer strain to survive cold winters, and to withstand adverse environmental stresses (Sukenik et al 2007). These spores exhibit profuse growth and multiplication when favorable growth conditions returns. In 2007 Kyndiah and Rai had reported another aspect of sporulation. They have projected spores of *Nostoc* ANTH as an efficient candidate for cyanobacterial inoculum in rice cultivation. Also they have reported that spores of *Nostoc* ANTH retained viability after extended periods of storage. Thus, spores can be regarded as storage form of cyanobacterial strains from which a culture can be generated whenever necessary.

Keeping in mind this aspect of cyanobacterial spores, the present study was designed to find various modifications in growth conditions that can be introduced to bring about quick and efficient sporulation in cyanobacterial strains. Bulk quantities of spores thus generated have been stored for future use. The time period up to which these stored spores stay viable without losing any of the characters of the native strains have also been assessed.

Possible use of spores in the field of cyanobacterial application as inoculum for biofertilizer has been envisaged and experiments were conducted to establish this as a fact.

The following section in this chapter describes alterations in standard growth conditions that led to effective sporulation in the study samples. It also contains detailed accounts of experiments conducted to study spores' ability to germinate back into viable cells and express desirable characters after cyanobacterial cells have spend a considerable amount of time as spores. Rice root colonization ability and associative nitrogen fixation of the cells regenerated from spores were also assessed to ascertain whether spores could be projected as ideal biofertilizer inoculums for rice cultivation.

This study has given an insight to how spores can serve dual roles as efficient mode for preservation of cyanobacterial strains as well as being able to be putatively used as a biofertilizer inoculum to rice fields.

7.2 Methods

7.2.1 Organisms and growth conditions

Of the ninety eight cyanobacteria isolated and purified as described in the chapter 3, twenty samples were selected for the present study (Table 7.1). In selecting these twenty isolates, importance was given to climatic and environmental difference of the locations from which they were isolated, their prevalence in rice fields and to their ability to colonize rice roots. All isolates except *Mastigocladus* sp. were maintained at 25 ± 2°C and at a photon fluence rate of 50 μmol m^{-2} s^{-1}. *Mastigocladus* sp. was maintained at 45°C in D-medium in batch cultures and with a photon fluence rate of 50μmol m^{-2} s^{-1} inside a BOD incubator.

Table 7.1: Samples identified for the study and their associative capability with rice roots (on day 10) *(In table, + indicates positive association between rice roots and cyanobacteria and ++ indicates tighter association between rice roots and cyanobacteria.)*

Sl. No.	Samples	Sample no	Strains	Location from which collected	Association with rice roots
1	A	*RF 16*	*Nostoc* sp.	Meghalaya	++
2	B	*RF 2*	*Plectonema boryanum*	Meghalaya	-
3	C	*RF3*	*Nostoc* sp.	Meghalaya	+
4	D	*RF4*	*Anabaena oryzae*	Meghalaya	++
5	E	*RF5*	*Aphanothece* sp.	Meghalaya	-
6	F	*RF6*	*Nostoc* sp.	Meghalaya	++
7	G	*RF7*	*Anabaena* sp.	Meghalaya	+
8	H	*RF8*	*Calothrix* sp.	Meghalaya	+
9	I	*RF9*	*Nostoc* sp.	Meghalaya	++
10	J	*RF10*	*Nostoc* sp.	Meghalaya	+
11	K	*HS1*	*Mastigocladus laminosus*	Meghalaya	++
12	L	*RF24*	*Anabaena* sp.	Assam	++
13	M	*RF27*	*Nostoc* sp.	Assam	+
14	N	*K1*	*Fischerella* sp.	West Bengal	+
15	O	*RF40*	*Cylindrospermum muscicola*	Manipur	+
16	P	*Ganga 1*	*Anabaena fertilissma*	Uttarakhand	+
17	Q	*Ganga 2*	*Nostoc* sp.	Uttarakhand	++
18	R	*Ganga 4*	*Nostoc* sp.	Uttarakhand	+
19	S	*UP3*	*Nostoc* sp.	Uttar Pradesh	+
20	T	*UP2*	*Nostoc* sp.	Uttar Pradesh	++

7.2.2 Induction of sporulation

Different treatment in the form of change in temperature, duration of light, change in ideal media composition and a combination of all/or some was used to find the best possible parameters to induce sporulation in these isolates. Best results were obtained when N_2-grown cultures of different cyanobacteria were transferred to sulfate limited $BG11_0$ media and phosphate limited $BG11_0$ media and kept in light. Cultures kept in light and in media lacking sulfate produced spores within seven days of the treatment. Spore differentiation occurred after 15 days in phosphate limiting media (Fig 7.1). In some strains that were isolated from high temperature regions such as the *Mastigocladus laminosus* from hot

spring, Jakrem (Meghalaya) and *Nostoc* sps. collected from Uttar Pradesh, sporulation could be induced by simply maintaining the cultures at much lower temperature of 10±2°C. These cultures when maintained at lower temperature in sulfate deficient media resulted in quicker and profuse (~ 90- 95%) induction of sporulation within 7 -10 days (Fig 7.2).

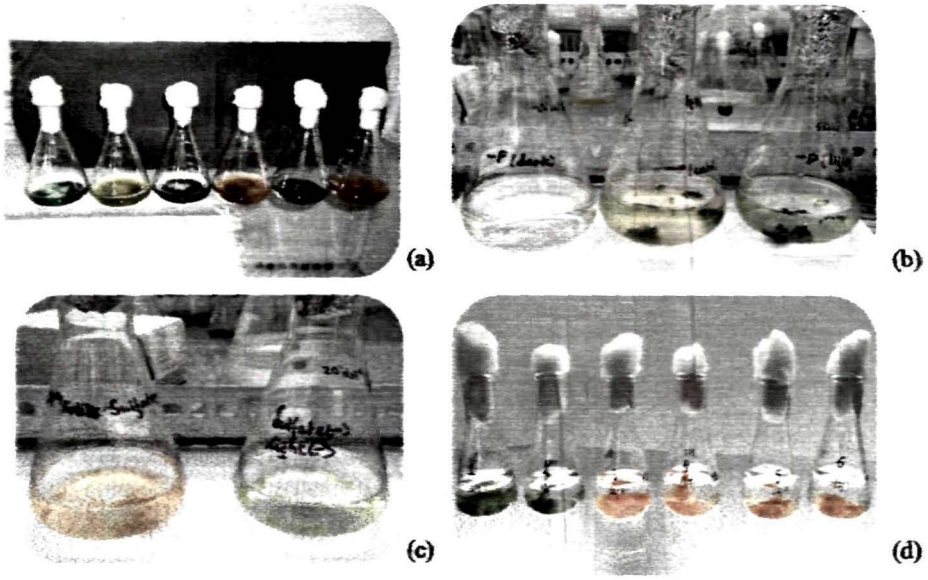


Fig 7.1: Sporulation in cyanobacteria (a) Differential degree of sporulation observed in experimental samples (b) effect of phosphate limitation on cyanobacterial cultures (c) effect of sulfate limitation on cyanobacterial cultures (d) complete sporulation in some cyanobacterial strains.

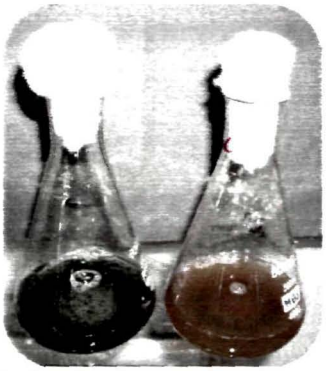


Fig 7.2: Sporulation in *Mastigocladus laminosus* due to temperature difference. The flask on left was kept at 45°C (ideal growth temperature) and the flask on the right was kept at 10°C.

7.2.3 Spore counting

Spore counting was done as percentage of total cell population by light microscopic observation using Olympus BX 51 light microscope.

7.2.4 Spore storage

The spore samples of the individual cyanobacterium were dried and stored in room temperature in sterile vials.

7.3 Results

After calculating the sporulation percentage and time required for induction of sporulation, sulfate limitation in growth medium was found to be the simplest and best protocol for generating large number of spores in a short duration of time. This observation is consistent with the earlier report of Kyndiah and Rai in 2007. Table 7.2 gives details of highest percentage of sporulation achieved in selected strains by application of different treatments. In case of sample A, L, Q and T maximum sporulation was achieved when the sample was sulfate deficient, and the flasks were incubated at an unfavorable growth temperature of 10±2°C. The hot spring isolate K (*Mastigocladus laminosus*) did not need any modification in media composition in order to sporulate. Simply incubation at 10±2°C was enough to trigger profuse sporulation in this cyanobacterium. 98% sporulation in *Mastigocladus laminosus* was observed within 30 days of incubation at this unfavorable temperature. In rest of the isolates, temperature did not play such a significant role in the induction of sporulation. Almost similar sporulation percentage was achieved when the strains were maintained in sulfate deficient media at 10±2°C or at 25±2°C. Five best sporulating strains (A, K, L, Q and T) that showed high colonizing ability were short-listed for further study (Table 7.3).

Table 7.2: Percent sporulation (on day 30) of the selected cyanobacterial isolates.

Sl. No.	Samples	Strains	Percent sporulation (%)
1	A	*Nostoc* sp.	98
2	C	*Nostoc* sp.	86
3	D	*Anabaena* sp.	87
4	F	*Nostoc* sp.	90
5	G	*Anabaena* sp.	86
6	H	*Calothrix* sp.	88
7	I	*Nostoc* sp.	94
8	J	*Nostoc* sp.	92
9	K	*Mastigocladus laminosus*	98
10	L	*Anabaena* sp.	96
11	M	*Nostoc* sp.	78
12	N	*Fischerella* sp.	80
13	P	*Anabaena fertilissma*	89
14	Q	*Nostoc* sp.	97
15	R	*Nostoc* sp.	88
16	S	*Nostoc* sp.	81
17	T	*Nostoc* sp.	97

Table 7.3: Best sporulating strains.

Sl no.	Samples	Strains	Location from which collected	Percent sporulation (%)
1.	A	*Nostoc* sp.	Meghalaya	98
2.	K	*Mastigocladus* sp.	Meghalaya	98
3.	L	*Anabaena* sp.	Assam	96
4.	Q	*Nostoc* sp.	Uttar Pradesh	97
5.	T	*Nostoc* sp.	Uttar Pradesh	97

7.3.1 Storage time and spore germination

To ascertain the effect of storage time on the germination of spores, desiccated spores were introduced into fresh BG11$_0$ media after every three months interval of storage at a final concentration of 2 X 10^6 spores ml^{-1}, to study spore germination. 10 µl of the above spore solution was also plated on nutrient agar to count the number of viable colonies germinated from such stored spores (Fig 7.3). The first apparent sign of spore germination was evident when green dots appeared in media containing the brown spores.

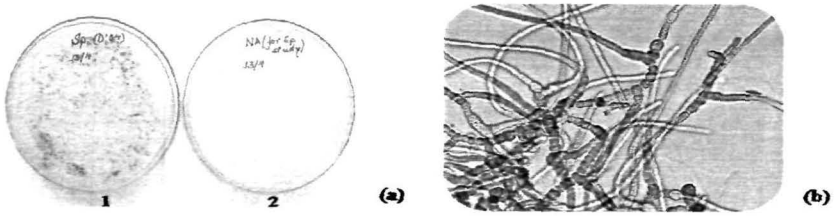


Fig 7.3: (a) Germination of spores on nutrient agar plates (*plate 1 shows spore germination while plate 2 represents a control plate*), (b) Profuse germination of *Mastigocladus laminosus* filaments from spores.

Within 24h of transfer to fresh media, the spore wall ruptured and filaments started to appear in the media. Fig 7.3a shows the high germination of spores in nutrient agar as compared to similar number of vegetative cells plated at the same time. The growth of the filaments was profuse for next 120 h (Fig 7.3 b). 100% spore germination was observed within 148h. Light was an important factor for spore germination as spores did not germinate in the dark at all. This confirmed the earlier reports by Fay 1969; Yamamoto 1976; Chauvat et al 1982; Reddy et al 1975.

7.3.2 Growth, Heterocyst frequency and Nitrogenase activity

Fig 7.4, 7.5 and 7.6 compares growth as increase in chlorophyll *a* content, heterocyst frequency and nitrogenase activity respectively of cultures regenerated from spores to their free-living counterparts after the spores were stored for one, two and three years in desiccated state. Measurements were taken on the 8th day. Hectic growth activities were seen in all strains when spores were returned to favorable growth conditions in the form of ideal media, light and temperature. Growth measured as increase in chlorophyll *a* content was always found higher than the free-living counterpart (Fig 7.4). Heterocyst frequency and nitrogenase activity followed a similar trend. Higher heterocyst frequency was observed in all cultures regenerated from spores in K (1st, 2nd and 3rd year samples) consistently. There was evidence of double and multiple heterocysts in many filaments as the spores started to

germinate into filaments. However, on subsequent sub culturing this phenomenon of multiple heterocyst occurrences disappeared. In the sample L isolated from Assam, consistently higher nitrogenase activity was recorded in the regenerated samples. The heterocyst frequency of this strain was slightly higher than its free-living counterpart but these heterocysts were well spaced and no double and multiple heterocysts were recorded in this strain.

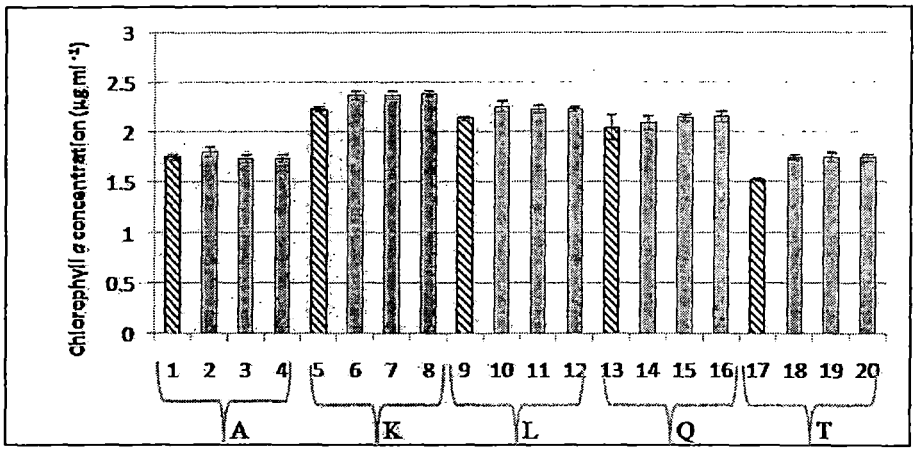


Fig: 7.4: Chlorophyll *a* concentration (µg ml $^{-1}$) of the regenerated cyanobacterial samples as compared to their free-living batch cultures.

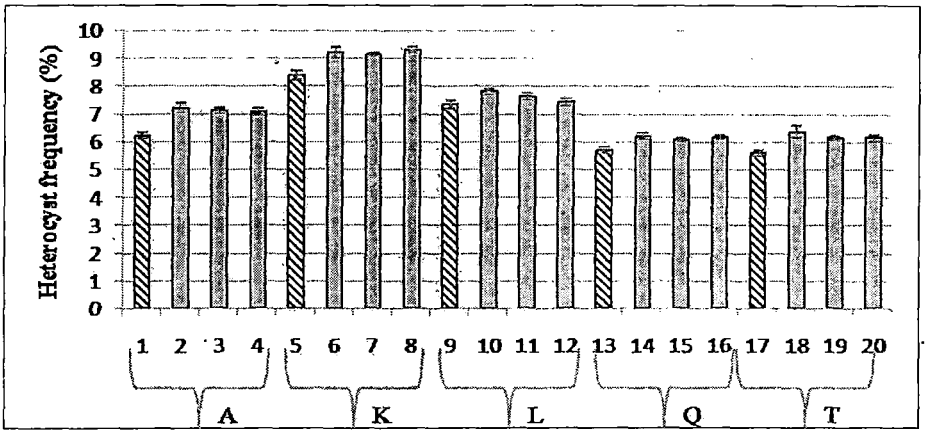


Fig 7.5: Heterocyst frequency of the regenerated cyanobacterial samples as compared to their free-living batch cultures.

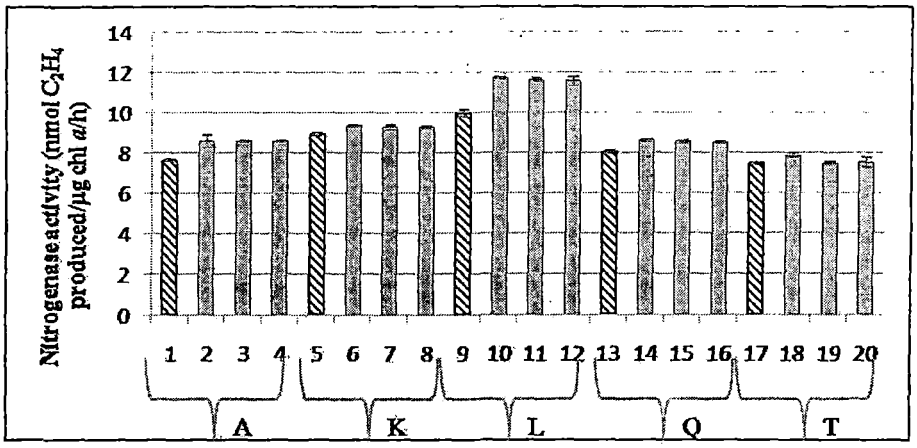


Fig 7.6: Nitrogenase activity of the regenerated cyanobacterial samples as compared to their free-living batch cultures.
[In figs 7.4, 7.5 and 7.6, columns 1, 5,9,13 and 17 represent the control cultures of samples A, K, L, Q and T respectively. Columns 2, 6, 10, 14 and18 represent samples regenerated at the end of one year of storage of each sample; columns 3, 7, 11, 15 and 19 are for regenerated samples at the end of 2nd year of storage, while columns 4, 8, 12, 16 and 20 represent regenerated samples of A, K, L, Q and T after three years of storage.]

7.3.3 Glutamine synthetase (transferase) and nitrate reductase activities

Other biochemical characters important in nitrogen metabolism of cyanobacteria such as glutamine synthetase (transferase) and nitrate reductase activities were studied in the regenerated spore samples. Table 7.4 compares these activities in cyanobacterial cultures regenerated from spores after 1st, 2nd and 3rd year of storage and their control free-living counterparts. As evident from the table, these biochemical parameters remained unaltered throughout the period of storage as spores.

Table 7.4: Comparison of glutamine synthetase (transferase) and nitrate reductase activities in cultures regenerated from spores of cyanobacteria.

	Glutamine synthetase (transferase) (nmol γ-glutamyl hydroxymate formed min^{-1} mg^{-1} protein)	Nitrate reductase (nmol product NO_2^- min^{-1} mg^{-1} protein)
Control A	756 ± 8	2.10 ± 0.14
A (1st year)	767 ± 7	2.16 ± 0.15
A (2nd year)	784 ± 5	2.20 ± 0.13
A (3rd year)	721 ± 5	2.14 ± 0.13
Control K	2442 ± 121	9.02 ± 0.6
K(1st year)	2450 ± 125	9.14 ± 0.5
K(2nd year)	2464 ± 123	9.16 ± 0.4

K(3rd year)	2121 ± 122	9.02 ± 0.6
Control L	789 ± 9	3.21 ± 1.1
L(1st year)	799 ± 9	3.34 ± 1.1
L(2nd year)	801 ± 8	3.23 ±0.9
L(3rd year)	782 ± 6	3.29 ±1.1
Control Q	781 ± 5	2.28 ±0.7
Q(1st year)	780 ± 6	2.35 ±0.8
Q(2nd year)	792 ± 8	2.48 ±0.7
Q(3rd year)	787 ± 9	2.29 ±0.7
Control T	756 ± 7	2.03 ±0.12
T(1st year)	762 ± 5	2.13 ±0.12
T(2nd year)	779 ± 5	2.07 ±0.11
T(3rd year)	771 ± 5	2.21 ±0.12

7.3.4 Rice root colonization studies

Retention of colonization ability of various cyanobacteria after storing them as spores was tested using rice variety DR 92 collected from ICAR Research Complex for NEH Region Umiam, Shillong, Meghalaya. Experiments were set up as described in section 2.16 of chapter 2. All samples were found to colonize roots and submerged shoots. Table 7.5 showed higher nitrogenase activities in the cyanobacteria associated with rice roots and submerged shoots of the co-cultured rice seedling than the free-living cultures of the same organism(s). In association with rice seedlings, the high sporulating strains exhibited enhanced nitrogenase activity (A =23%; K = 31%; L = 24%; Q = 32%; T =13 %) as compared to their free-living counterparts. Thus, the characteristic increase in nitrogenase activity seen in many cyanobacteria when in association with rice roots (chapter 6) is retained in all the regenerated cultures. This increase seen in nitrogenase activity was substantial and encouraging as this has feature has a potential biotechnological implication.

Table 7.5: Nitrogenase activity of selected high sporulating cyanobacterial strains in their free-living and in associated state with rice roots. *(The values presented here are average from 10 plants. ++ indicates tighter association between cyanobacteria and rice roots).*

Sl no.	Samples	Strains	Colonization ability	Nitrogenase activity of free-living cultures (nmol C_2H_4 produced/ µg chl *a*/ h)	Nitrogenase activity in association with rice roots (nmol C_2H_4 produced/ µg chl *a*/ h)
1.	A	*Nostoc* sp.	++	5.7	7.0
2.	K	*Mastigocladus laminosus*	++	7.4	9.7
3.	L	*Anabaena* sp.	++	6.2	7.7
4.	Q	*Nostoc* sp.	++	4.7	6.2
5.	T	*Nostoc* sp.	++	4.5	5.9

7.4 Discussion

Several authors have earlier reported that phosphate limitation is a major trigger to akinete formation in *Anabaena variabilis, A cylindrical, A circinalis* and *Nostoc linckia* (Wolk 1965; Herdman 1987). However, it is difficult to induce quick sporulation by phosphate limitation in cyanobacterial strains kept under laboratory conditions, since they accumulate several polyphosphate bodies which are reserves of phosphate and takes time to deplete (Kyndiah and Rai 2007).

In the present study sulfate limitation was found to be a more definitive trigger in inducing sporulation in the cyanobacteria used for the study. In addition to this, it was observed that maintaining the samples in temperatures lower than their ideal growing temperature led to the onset of sporulation in many cyanobacteria that were originally isolated from high temperature areas. In case of those samples that had originally been isolated from a higher temperature zones, e.g., the thermophile *Mastigocladus laminosus* that grows ideally at 45°C and above exhibited immediate sporulation (within 3-4 days) when maintained at 10°C or below. This was also true for the other *Nostoc* isolates (samples Q and T) which were collected from higher temperature regions. The sporulation efficiency

of these samples was further enhanced when the samples were maintained in sulfate deficient media, at a lower temperature (10-12°C). It was a consistent observation during the study that all cyanobacterial spores exhibited profuse and immediate germination and subsequent growth on return of favorable growth conditions. On evaluation, characters such as chlorophyll *a* content, heterocyst frequency nitrogen fixation, glutamine synthetase (transferase), nitrate reductase activities as well as associative nitrogen fixation was comparable to their free-living cultures and there was no evidence of loss or reduction of any of the activities in the cyanobacteria due to storage as spores for a long period of three years. Upon return of favorable conditions all spores germinated into viable filaments within a short period of time. This finding is consistent with earlier reports that the akinetes produced by *Anabaena cylindrica* were found to germinate after five years of desiccation (Yamamoto 1975).

Our studies showed that simple modifications in growth conditions could be used to induce quick and profuse sporulation in desired cyanobacteria. Spores can be desiccated and large quantities of spore samples can be stored in small vials for long period of time. This reduces the work load involved in maintaining strains in batch cultures in culture rooms. Viable cultures of these strains can be regenerated within a short period of time by returning the spores to ideal growth conditions.

The high sporulating strains that had retained association ability and demonstrated enhanced nitrogenase activity in association could be targeted as biofertilizer to rice fields. Distribution of cyanobacterial biofertilizer inoculum has been an area of concern to all those who advocate use of cyanobacteria as biofertilizer. Fresh cyanobacterial cultures packed in air tight packets have short life spans and therefore, the desired population of cyanobacteria

is not achieved in the fields. Delivery of potential biofertilizer strains as spores in a dry form could overcome such limitations associated with distribution of viable inoculum. Spores released to water logged rice fields would germinate profusely and immediately populate the fields as the environment prevailing in rice fields are highly conducive to growth of cyanobacteria. Thus, spores as inoculum could be more attractive alternative to fresh culture of cyanobacteria in biofertilizer programmes.

Our short study involving conditions leading to induction of sporulation, their germination, retention of characters and rice root colonization ability and the period up to which spore can be safely stored reflected that spores can be excellent preservation method of those cyanobacterial strains that are capable of sporulating. In addition, efficient biofertilizer strains can be distributed as spores for better and efficient management of biofertilizer inoculum in rice fields. Desired strains with high nitrogenase activity, better colonizing ability could be released into crop fields as spores to deliver higher benefits from cyanobacteria as biofertilizer.

7.5 Salient findings

This chapter investigated the possibility of considering spores as a natural means of preservation of certain cyanobacterial strains. Some salient findings of this study are:

- Many cyanobacterial strains produce spores under adverse conditions. In the laboratory simple modifications in media composition led to induction of sporulation.
- Change in ideal growth temperature seemed to be an immediate trigger for some of the high temperature isolates, with *Mastigocladus laminosus* showing immediate and profuse sporulation on subjecting the cultures to a drop in temperature.

- Spores are capable of germinating into viable filaments upon return of favorable conditions.
- They can be stored safely for at least a period of at least 3 years with no loss or reduction of characters that are crucial for their independent survival. Storage as spores also minimizes chances of contamination, mis-nomenclature etc. that are otherwise often encountered during routine batch culturing and maintenance of strains.
- Filaments regenerated from spores retain their ability to associate with rice roots and submerged shoots.
- In conclusion it may be stated that spores could be an attractive natural mode of preservation of those cyanobacterial strains which are capable of undergoing sporulation. Such spores can be considered an excellent preservation material of many cyanobacterial strains. They could also be projected as biofertilizer inocula to rice fields as they showed high efficiency of germination and thus, are capable of populating fields with vigor within a short span of time. These attributes would help tackle the various inoculum distribution problems of the current biofertilizer programme.

Summary

This present study incorporated details of studies conducted on the various existing methods of preservation of cyanobacteria. Different groups of cyanobacteria, including unicellular, filamentous heterocystous, filamentous non-heterocystous, filamentous branched heterocystous thermophilic cyanobacteria were selected for the study. Several modifications were introduced in the existing methods to make them more user-friendly and applicable. Some salient findings of the study are listed below:

- Samples were collected from forty one distinct locations from ten different states. A total of ninety eight cyanobacteria were isolated and purified, and these included members of both aquatic and terrestrial habitats. Three members of thermophilic cyanobacteria were isolated from Jakrem hot springs in Meghalaya and Garampani hot spring in Assam. *All groups of cyanobacteria were represented in the collection. Gloeocapsa* sp., *Plectonema boryanum, Nostoc muscorum, Anabaena variabilis and Mastigocladus laminosus were selected as members representing unicellular, filamentous non-heterocystous, filamentous heterocystous, filamentous branched heterocystous thermophilic cyanobacteria respectively for further study involving preservation.*
- In depth evaluation and assessment of some existing methods of cyanobacterial preservation was carried out. Since the emphasis was to find the suitability of the methods and also to find the time period up to which cyanobacterial samples may be safely stored using matrices and protectants, certain changes were introduced in some of the protocols in order to make them more readily useable and less

expensive. For doing this, *the existing method of making agar slants was modified to make agar flakes for convenient storage of cyanobacteria.* This modification has the advantages of ease of handling, and ensuring substantially long term storage of immobilized cyanobacterial samples in dry form. This also significantly reduces the chances of contamination between cultures which are maintained in close proximity in culture rooms. All groups of cyanobacteria could be stored on agar flakes for at least a period of 18-24 months. *Preservation in calcium alginate proved to be most promising and dependable for long term storage of cyanobacteria.* Cyanobacterial samples thus stored retained their viability for very long periods without any loss of characters that are indispensable for independent survival. Also, large quantities of samples could be stored as calcium alginate beads in small vials thus bringing down the space required for maintenance. Safe period up to which the cyanobacteria can be stored using this protocol is at least 36 months. Cells remained viable in the beads and regenerated even after 5 years of storage at room temperature and in dark. The use of ordinary packing foam in immobilization of cyanobacteria was another major achievement of this work. It became obvious that *packing foam can be used in storing cyanobacteria at least for a period of 18 months.* Foam is often a waste material readily found in laboratories as they come as protective packing material around instruments. Thus, use of such material is highly cost effective and is convincingly efficient in preservation of cyanobacteria for substantial period of time. *It was also observed that cyanobacterial samples can be stored in ordinary refrigerators using cryoprotectants for at least for 9-12 months.* Another prominent observation was that the *initial amount of inoculum played a very determining role in*

deciding the duration of survival and performance of regenerated samples from the different preservation matrices and materials. A higher concentration of initial inoculum (at least 10 µg/ml chl *a)* was a prerequisite to ensure longer duration of viability of preserved cyanobacteria. Also it was found that non-heterocystous cyanobacteria needed addition of supplementary nitrate in the media.

- Naturally available, biodegradable and environment friendly matrices were experimented to find suitable carrier material for short term preservation of cyanobacteria. Betel nut covers, soil, *Luffa cylindrica*, charcoal chips were used for preservation of cyanobacteria. Among these, dried *Luffa cylindrica* was found unsuitable as its holding capacity was much less due to its porous texture. It became evident that the initial inoculum load had a definitive role in influencing the duration up to which dry samples containing viable cyanobacteria could be stored. Among all the bases studied, soil was found to be the best for preservation of cyanobacteria in terms of duration as well as retention of native characters of immobilized cyanobacteria. *Fibres of betel nut covers came in close second as base for preservation as this also proved to be an efficient carrier and short term preserving material for cyanobacteria.* It is a biodegradable waste material and thus, a technology utilizing these could be an environment friendly and economically feasible option. *An interesting observation that resulted from this study was that charcoal also could maintain cyanobacteria viable for at least a period of six months upon immobilization and thus, such charcoal chips containing cyanobacteria could be projected as a rich potting material for plants such as orchids.*

- Since rice fields are known to host a diverse consortium of cyanobacteria, several cyanobacterial isolates were collected from diverse rice fields in the state as well as from neighboring states. Every collections from rice fields yielded one or more cyanobacteria in the collection even though most rice fields in the state of Meghalaya recorded acidic pH and cyanobacteria are known to prefer neutral to alkaline pH. All individual cyanobacterial isolates were capable of colonizing rice root *albeit* to varying degrees. It was seen that close association of cyanobacteria with rice roots resulted in better growth of these plants. Associated cyanobacteria showed increase in nitrogenase activity. *RF7, RF16 and NA1 were identified as likely candidates for biofertilizer programme in acidic rice fields as they were found abundantly in acidic soil of Meghalaya and showed higher associative nitrogenase activity.* Evidence of biologically fixed nitrogen transfer was obtained using $\delta^{15}N$ analysis. Negative effects were seen on morphology and growth of cyanobacteria when subjected to increasing concentration of Cd and Zn. Atomic absorption studies showed that 61% of the Cd available in the medium was removed by the *Nostoc* cells within 48 h. *Nostoc* cells accumulated almost two fold higher amount (12% vs. 23%) of Zn intracellularly than it did for Cd. The ability to remove heavy metals from the surroundings was lower in *Anabaena* cells (it removed 53% Cd and 62% Zn as against 61% Cd and 75% Zn by *Nostoc* cells respectively). Presence of cyanobacteria in the vicinity of rice seedlings reduced inhibitory effect of cadmium by about 36% and 22% in the growing root and shoot lengths of rice plantlets. There was positive indication of better growth of orchid plantlets that were grown on charcoal containing immobilized cyanobacterial

filaments. *Thus, use of such charcoal pieces carrying immobilized cyanobacteria can be exploited at commercial scale specifically for growing orchids.*

- It is known that many cyanobacterial strains produce spores under adverse conditions. In the laboratory simple modifications in media composition and/or alteration of ideal growth temperature led to induction of sporulation. These spores were capable of germinating into viable filaments upon return of favorable conditions. Such *spores could also be stored safely for at least a period of 3 years with no loss of or reduction in expression of characters that are crucial for their independent survival.* The regenerated filaments retained their ability to associate with rice roots and submerged shoots. Nitrogenase activity of these cyanobacteria was found to be higher in the associative state than in their free-living counterparts. In conclusion it may be stated that *spores could be an attractive natural mode of preservation of those cyanobacterial strains which are capable of undergoing sporulation. Thus, maintaining cyanobacteria as spores can be considered as an excellent means of preservation of many cyanobacterial stranis that are capable of sporulating. They could also be projected as biofertilizer inocula to rice fields with higher efficiency of germination and populating fields with vigorous cells within a short span of time. These attributes would help tackle the various inoculum distribution problems of the current biofertilizer programme.*

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1. "*Studies on preservation and conservation of cyanobacteria*" Abstract In: **Trends in Biochemical research. National seminar on Trends in Biochemical research.** Department of Biochemistry. NEHU Shillong. **(Oral Presentation).** 25-26 February 2005.

2. "*Immobilization as a tool for preservation of cyanobacterial diversity*" Abstract In: **Issues and Challenges for Conservation of Plants and Ecosystems in India.** Department of Botany. NEHU Shillong. **National seminar on Issues and Challenges for Conservation of Plants and Ecosystems in India. (Oral Presentation).** 2-3rd November 2006.

3. "*Maintenance and preservation of diverse cyanobacterial strains*" Abstract In: **Current Trends in Algal Bioresource Utilization. International conference on Current Trends in Algal Bioresource Utilization.** Department of Ecology and Environmental Science. Assam University. Silchar. **(Oral Presentation).** 4-6th December 2006.

4. "*Time frame for application of cyanobacteria as biofertilizer in rice cultivation*" Abstract In: **Microbes in Pharmaceuticals, Food and Agriculture. National seminar on Microbes in Pharmaceuticals, Food and Agriculture.** Department of Microbiology. Vidyasagar University. Midnapore, West Bengal. **(Oral Presentation).** 20-21st December 2006.

5. "*Preservation of cyanobacterial strains*" Abstract In: **Adaptation Biochemistry.** Department of Biochemistry. NEHU Shillong. **National seminar on Adaptation Biochemistry. (Oral Presentation).** 22-23rd March 2007.

6. "*Characterization and preservation of potential biofertilizer strains from north east India*" Abstract In: **Applied Phycology and Environmental Biotechnology. International symposium on Applied Phycology and Environmental Biotechnology ISAPEB'07.** Biological Sciences Group and Centre for Desert Development Technologies. BITS-Pilani. Rajasthan. **(Oral Presentation).** 29-31st October, 2007.

7. "*Engineering conditions for bulk spore production for application as biofertilizer inoculums in paddy cultivation*" Abstract In: **Applied Bioengineering. International conference on Applied Bioengineering ICAB'07.** Sathyabama University. Chennai. **(Oral Presentation).** 5-7th December, 2007.

8. "*A study on cyanobacterial compatibility with nitrogen based fertilizers and their transfer to paddy fields on a biodegradable material*" Abstract In: **Applied Bioengineering. International conference on Applied Bioengineering ICAB'07.** Sathyabama University. Chennai. **(Adjudged best paper presented).** 5-7th December, 2007.

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10. "*An ideal time span for cyanobacterial application as biofertilizer in rice fields*" Abstract In: **Advances in Medical and Microbial Biochemistry. National seminar on Advances in Medical and Microbial Biochemistry.**Department of Biochemistry, NEHU, Shillong. 13-14th March, 2008.

11. *"Assessment of cyanobacterial diversity of north east India and their preservation"* Abstract In: **Frontiers in Biocomplexity and Biodiversity of Plants**. Centre for Advanced Studies in Botany, NEHU, Shillong. **(Oral Presentation) (Adjudged best orally presented paper). National symposium on Frontiers in Biocomplexity and Biodiversity of Plants**. Centre for Advanced Studies in Botany, NEHU, Shillong. 14-15th March, 2008.

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14. *"Cyanobacterial Application in Rice Fields: Dual Benefits in Ecorestoration"* Abstract In: **31st All India Botanical Conference and International Symposium on Plant Biology and Environment: Changing Scenario.** Dept of Botany, University of Allahabad. Allahabad. **(Oral Presentation).** 17-19th December, 2008.

15. *"Intracellular Proline Accumulation during Stress Management in Cyanobacteria"* Abstract In: **96th Indian Science Congress section New Biology**. NEHU, Shillong. **(Poster Presentation). 96th Indian Science Congress.** 3-7th January, 2009.

16. *"Cadmium removal by an Anabaena sp. isolated from coal mining areas of Meghalaya, India."* Abstract In: **Changing Environmental Trends and Sustainable Development. International Symposium on Changing Environmental Trends and Sustainable Development CETAS 2009.** Dept of Environmental Science. Guru Jambheshwar University of Science and Technology. Hisar, Haryana. **(Oral Presentation).** 9-11th February, 2009.

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18. *"Potential application of 'snep kwai' (betel nut cover) as biofertilizer carrier to rice fields."* Abstract In: **UPE-Biosciences seminar on Assessment, Conservation and Value Addition of Biodiversity. School of Life Sciences. NEHU, Shillong. (Oral Presentation) UPE-Biosciences national seminar on Assessment, Conservation and Value Addition of Biodiversity. NEHU, Shillong.** 22-23rd April, 2010.

Workshops Attended:

1. National Workshop on **Microbial diversity database**. Department of Biotechnology and Bioinformatics, NEHU. 29 Oct 2009 – 30 Oct 2009.

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Publications:

1. Nitrogen metabolism, artificial association in two cyanobacterial isolates and the assessment of their potential as biofertilizer. **Indian Journal of Biotechnology. Vol. 6 July 2007**, pp 397-403.

2. Engineering conditions for bulk spore production for application as biofertilizer inoculums in paddy cultivation. Proceeding in **Applied Bioengineering.** Sathyabama University. Chennai. Dec 2007. pp 408-411.

3. A study on cyanobacterial compatibility with nitrogen based fertilizers and their transfer to paddy fields on a biodegradable material. Proceeding in **Applied Bioengineering.** Sathyabama University. Dec 2007. Chennai. pp 97-100.

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8. Efficiency of Cyaonobacterial preservation techniques. **Ecosystem & Plant Diversity**, Daya Publications, New Delhi.

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Significance of cyanobacterial diversity in different ecological conditions of Meghalaya, India

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Abstract: The present study deals with preliminary investigation of cyanobacterial diversity in Meghalaya. A total of 75 samples were collected from 10 different ecosystems and analyzed. 65 strains of cyanobacteria isolated under 11 genera include *Nostoc*, *Anabaena*, *Calothrix*, *Cylindrospermum*, *Gleocapsa*, *Fischerella*, *Plectonema*, *Tolypothrix*, *Stigonema*, *Loriella* and *Westiellopsis*. *Nostoc* was most abundant. Diversity analysis indicated maximum Shannon's diversity index (H) in Mawlai. Highest Simpson's diversity index was seen in Sung Valley (0.75). Both Shannon's and Simpson's diversity indices were lowest in Mairang. Richness was highest in Sung valley and Syntuksiar with both the sites supporting 17 strains each. Although, highest diversity was recorded from Mawlai, richness recorded at this site was only 11 strains thereby indicating richness need not be a function of diversity in this region. This study revealed the cyanobacterial strains, which can withstand acidic pH and prevail in the region. A study on colonization also identified some potential biofertilizer strains from the region such as *Nostoc punctiforme*, *Nostoc muscurum* and *Anabaena azollae* that could be effective in acidic crop fields.

Keywords: Northeast India, Biodiversity hot spots, Cyanobacterial diversity, Shannon's index and Simpson's diversity index

INTRODUCTION

Cyanobacteria are a group of gram negative, morphologically diverse, aerobic phototrophs whose distribution is ubiquitous in nature and are found everywhere including places with extreme climatic conditions such as in Antarctica, in hot springs (Whitton and Potts, 2000) and even in oxic and anoxic environments (Thajuddin and Subramanian, 2005). They flourish in places with nitrogen deficient environment. They are important as they contribute towards carbon and nitrogen economy of different ecological habitats and can grow in purely inorganic medium using light as energy and CO_2 and N_2 as sole carbon and nitrogen sources respectively (Wyatt and Silvey, 1969). The role of nitrogen fixing cyanobacteria in enhancing soil fertility has been long known and is well documented (Singh, 1961; Venkataraman, 1981). Cyanobacteria contribute to overall soil health not only by its ability to perform biological nitrogen fixation but also because of its ability to produce polysaccharides and other bioactive compounds which have growth stimulating effect on plants, as well as important in maintaining soil quality and preventing erosion. However, in nature cyanobacteria are constantly challenged by different factors and stresses including fluctuations in temperature, pH, moisture content (droughts and floods), salinity and so forth. These factors influence distribution and diversity of cyanobacteria in a region. Among these, pH is an important determinant as cyanobacteria are known to prefer neutral to alkaline pH for their optimum growth (Singh, 1961; Kaushik, 1994). In recent times, studies on cyanobacterial diversity has received due attention (Thajuddin and Subramanian, 2005; Prasanna *et al.* 2009). However, work and publications on this group of microorganisms from Northeast India is sporadic despite the fact that this region falls within Indo-Malayan biodiversity hot spot (Myres *et al.* 2000). Meghalaya is one of the 8 states of the northeastern India, lying between 25°5′N and 26°10′N latitudes and 89°47′E and 92°47′E longitudes covering an area of 22,429 sq. km. Physio-geographically the region consists of hilly terrains. Diverse terrestrial and aquatic ecosystems including extreme ones (hot springs) could be encountered within the state. The climate is moderate but humid. It also receives the highest rainfall in the world with average annual rainfall as high as 1200 mm making Meghalaya the wettest state of India. The natural ecosystems such as soil, freshwater bodies-streams, ponds, lakes, rivers, hot springs, and trees within extensive forest areas provide excellent habitats and favourable environments for the luxuriant and diverse growth of cyanobacteria. However, the region also exhibit neutral to acidic range of soil and water pH. It is the

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Table 1. Cyanobacterial Collection sites

Locality	Location		No. of samples	Type of ecosystems
	Longitude	Latitude		
Syntu Ksiar	92°12'39"E,	25°25'59"N	8	Rice field, soil, water
Mawphlang	91°45'37"E	25°27'02"N	7	Soil, water, tree barks
Sung Valley	92°06'15"E	25°30'09"N	8	Rice field, soil, water
Nongstoin	91°16'14"E	25°30'34"N	8	Rice field, soil, water
Mairang	91°38'26"E	25°33'55"N	7	Soil, water, tree barks
Mawlai	91°52'11"E	25°35'42"N	8	Tree barks, rice fields, soil, water
Mawroh	91°53'54"E	25°35'54"N	7	Rice field, soil, water, tree barks
Umkhen	92°07'51"E	25°36'18"N	7	Soil, water, tree barks
Umiam	91°54'11"E	25°39'59"N	8	Tree barks, soil, water, rice field
Umsning	91°54'10"E	25°42'59"N	7	Stagnant pond, rice fields, soil

Total number of samples collected = 75

interplay among such favourable and adverse conditions that has resulted in shaping the distinct biodiversity of the region. Keeping these facts in view, we have collected samples from diverse ecosystems to assess cyanobacterial diversity of the state. Since, cyanobacteria have immense potential for varied biotechnological applications, the present investigation was undertaken to get a database of available cyanobacteria (blue green algae) under diverse ecological conditions in different districts of Meghalaya.

MATERIALS AND METHODS

Study Area: Four districts of Meghalaya namely East Khasi Hills, West Khasi Hills, Jaintia Hills and Ri Bhoi

Table 2. Distribution of strains within genera in different localities.

Genus		Localities										Total no. of strains
		Mairang {5.8}	Mawlai {6.2}	Mawphlang {6.2}	Mawroh {6.3}	Nongstoin {6.0}	Sung valley {6.9}	Syntuksiar {6.8}	Umiam {5.9}	Umkhen {5.6}	Umsning {5.8}	
Anabaena	(24)	1	2	2	5	2	4	3	2	2	1	11
Nostoc	(64)	6	5	8	7	7	7	9	5	5	5	34
Calothrix	(08)	0	0	1	1	1	1	1	1	0	2	4
Westiellopsis	(01)	0	1	0	0	0	0	0	0	0	0	1
Gleocapsa	(04)	0	0	0	0	0	1	1	2	0	0	3
Fischerella	(09)	0	1	3	0	1	1	1	0	1	1	4
Tolypothrix	(01)	0	0	0	0	0	0	0	0	0	1	1
Stigonema	(01)	0	1	0	0	0	0	0	0	0	0	1
Cylindrospermum	(05)	1	0	0	0	1	1	1	0	1	0	3
Loriella	(01)	0	1	0	0	0	0	0	0	0	0	1
Plectonema	(07)	1	0	0	1	0	2	1	1	1	0	2
Total	(125)	9	11	14	14	12	17	17	11	10	10	65

The numbers in (brackets) indicates the total number of isolates.The numbers in {brackets} indicates the pH of the soil/water sample collected.

Table 3. Generic diversity indices and dominance for different localities.

Diversity indices	Mairang	Mawlai	Mawphlang	Mawroh	Nongstoin	Sung valley	Syntuksiar	Umiam	Umkhen	Umsning	All sites
Shannon-Weiner's Diversity Index	1.002	1.976	1.11	1.09	1.23	1.624	1.476	1.414	1.359	1.359	1.63
Simpson's Diversity index	0.52	0.71	0.60	0.61	0.61	0.75	0.67	0.71	0.68	0.68	0.68
Simpson's Dominance	0.48	0.29	0.40	0.39	0.39	0.25	0.33	0.29	0.32	0.32	0.32
Cyanobacterial richness	9	11	14	14	12	17	17	11	10	10	65

were chosen for the study. Samples were collected randomly from different types of ecosystems: terrestrial ground, terrestrial epiphyte and aquatic (Table.1). The sites were selected carefully to include diverse environments in terms of altitude, pH, and temperature, moist or water logged conditions as well as hilly or low lying terrain.

Aquatic samples were collected in specimen collection tubes. Soil samples were collected by scraping the surface of the soil and epiphytic samples were collected by scraping the upper surface of barks of different trees. pH of the collected samples was analysed using the method described by Black in 1992.

Isolation, purification and cultivation of cyanobacteria: Isolation and purification of cyanobacterial strains were carried out as described by Packer and Glazer (1988). The pure strains were maintained in BG-11_0 media (Rippka *et al.* 1979).

Growth, heterocyst frequency and nitrogenase activities: Growth was measured as increase in chlorophyll *a* content (McKinney, 1941). Heterocyst frequency was calculated as percentage of total cells by using an Olympus BX 51 light microscope (Wolk, 1965). The results are mean of three independent observations. Nitrogenase activity was estimated *in vivo* by gas chromatography using the acetylene reduction assay. 5 ml liquid culture was placed in a 15 ml serum vial and 1 ml air in the tube was replaced by 1 ml of pure acetylene. These vials were incubated for 1 hr at 25±1°C with constant shaking. The ethylene produced in each vial was determined using a Tracor 540 GC with a Porapak 'T' column and a flame ionization detector (Stewart *et al.* 1967).

Immobilization for preservation: The purified strains were preserved by immobilization in calcium alginate beads (Musgrave *et al.* 1982). The viability of the strains under preservation was established by regenerating the isolates periodically in liquid media. Regeneration of the immobilized filaments was confirmed visually. Nitrogenase enzyme was assayed in the regenerated cultures to ascertain no loss of this crucial character in the isolates due to preservation.

Richness and diversity of cyanobacteria: Genera were

Table 4. Colonization study: Associated chlorophyll *a* content and comparative nitrogenase activities of associated *Nostoc* isolates to their free-living counterparts.

Sl. No.	Isolate no.	Associative chlorophyll *a* ($\mu g\ g^{-1}$ root dry wt)	Nitrogenase activity	
			Free-living isolates (nmol C_2H_4 produced μg^{-1} chl *a* h^{-1})	Associated isolates (nmol C_2H_4 produced μg^{-1} chl *a* h^{-1})
1	27	987	7.92	12.56
2	12	782	7.20	9.69
3	28	767	6.88	9.41
4	29	532	6.53	7.31
5	18	519	6.41	7.22
6	4	411	6.35	6.65
7	7	368	6.32	6.67
8	8	362	6.21	6.49
9	20	330	5.90	6.11

identified under microscope with the help of Desikachary (1959) and strains were sorted out on the basis of morphology. Total number of strains under each genus was counted for estimating diversity and richness of cyanobacteria in study area. Generic diversity was estimated by Shannon-Weiner Index (H) (Shannon and Weaver, 1949) and Simpson's diversity (1-D) (Simpson 1949) using the following formulae:

Shannon-Weiner Index, $H = -\Sigma pi \ln pi$

Simpson's Dominance, $D = \Sigma (pi)^2$

Simpson's Diversity Index, $1 - D = 1 - \Sigma (pi)^2$

where pi = Total number of strains of genus i/total number of all strains.

Rice-cyanobacterial colonization study: Rice plants (*Oriza sativa* L.) local variety 'Synteng' was collected from ICAR-NEH Region Complex (Umiam, Shillong, India). The seeds were washed and surface sterilized using 1% sodium hypochlorite solution for 5 minutes. They were then rinsed thoroughly with sterile water and grown on autoclaved perlite in glass beakers. These beakers were irrigated with 10 times diluted sterile BG-11_0 media supplemented with 2mM $NaNO_3$ from time to time. 10 days old rice seedlings were transferred to 15 ml capacity tubes after washing them with distilled water. 5 ml BG-11_0 medium was poured into the tubes carefully dipping the roots in the medium. *Nostoc* filaments from exponentially growing cultures were introduced into the tubes (initial chlorophyll *a* content of the inoculums was kept at 1 µg ml^{-1} per tube). Association was confirmed under microscope as well as by estimating chlorophyll *a* concentration in the rice roots after removing loosely associated filaments using a sonic bath.

RESULTS AND DISCUSSION

Role of cyanobacteria in improving soil health and fertility is well known. To exploit the full potential of cyanobacteria in management of soil quality and fertility coordinated efforts between laboratory and field level research is needed. Region-based specific cyanobacterial isolates could be more effective in such applications as they are pre-acclimatised to the existing environmental conditions. Hence a region-specific biodiversity study is important for deriving optimum benefits from indigenous strains. Knowledge of cyanobacterial diversity of a region may help in selecting appropriate cyanobacterial inocula to be applied as biofertilizer consortia in crop fields as well as help in finding strains with other biotechnological potentials. Earlier we have reported one such *Anabaena* isolate from an acidic rice field in Cherrapunjee, Meghalaya that can grow at high cadmium concentration and is efficient in removing cadmium (Syiem et al. 2009). Thus, this investigation of cyanobacterial diversity is ecologically significant as it enumerates diversity, abundance, dominance and richness of various cyanobacteria in a region with primarily acidic soil and water pH. Such findings are important as they pinpoint biological strains that can be used to improve quality and enhance fertility of acidic soil.

Seventy five samples were collected from ten independent sites located in different districts of the state (Table 1). Almost all collection sites recorded acidic pH (6-7 in the pH scale). Of the total 65 strains isolated (Table 2), 92% were heterocystous cyanobacteria. Of which 51% alone was *Nostoc* and 16% was *Anabaena*. Predominance of the genera *Nostoc* in all collection sites irrespective of pH and other influencing factors including terrain, water logging, temperature, altitude and moisture content indicates towards their versatility, competiveness and ability to occupy diverse ecological niches. Growth was estimated in all 34 *Nostoc* isolates at pH 6.0 and at pH 7.5. Low growth of all isolates in pH 6.0 indicated compromised growth in acidic field conditions. Heterocyst frequency of the *Nostoc* strains at pH 6.0 was found to be within the range of 4.2 – 8.3% (Fig. 1a). Corresponding nitrogenase activity ranged from 3.9-7.9 nmol C_2H_4 produced μg^{-1} chl *a* h^{-1} (Fig. 1b). When compared, the correlation between increased heterocyst frequency and nitrogenase activity in the *Nostoc* isolates was evident with isolate no. 27 showing highest heterocyst frequency and nitrogenase activity followed by isolate nos. 12, 28 and 29.

Syntuksiar and Sung valley in Meghalaya primarily constitute widespread rice cultivation areas in the state. Statistical analysis revealed high richness and high Shannon's diversity indices in Syntuksiar and Sung valley (Table 3). Such values suggest that rice fields provide ideal environments for cyanobacterial diversity and growth despite the frequent use of agrochemicals in rice cultivation in order to enhance yield. In our study richness of cyanobacterial genera was found to vary with respect to pH of a particular collection site (Table 2). Although all sites recorded acidic pH, greater diversity and larger number of isolates were gathered from places that showed pH close to neutral. Prevalence of *Nostoc* and *Anabaena* in all sites indicated their resilience and high adaptability in varied types of ecosystems. However, *Westiellopsis*, *Tolypothrix*, *Stigonema* and *Loriella* sps. of cyanobacteria were not common in the state as only one isolate of each was found in the total collection.

In diversity study, richness relates to abundance of different genera/species of a group of organisms in an area and stands as a measure of number of different kinds of genera/species in that particular area. Low generic richness was seen in Mairang, Mawphlang and Mawroh areas. On the other hand, high generic richness was recorded in Syntuksiar and Sung valley. That diversity of an area strongly depends on pH and on interplay of various environmental factors is evident from this study

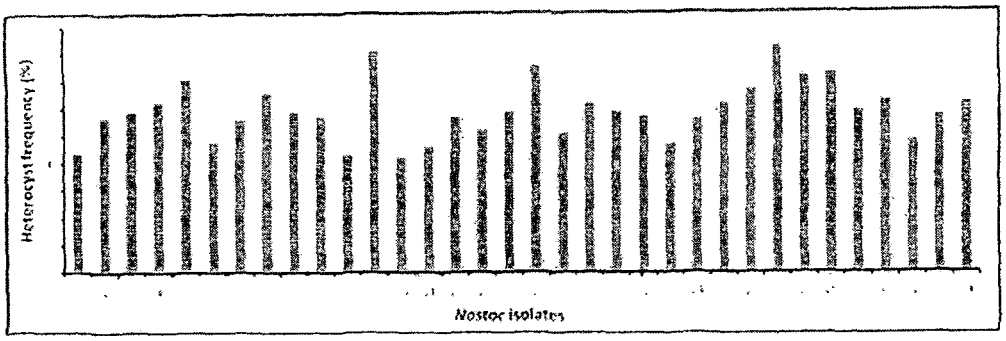

Fig. 1. (a) *Heterocyst frequency of Nostoc isolates (data is mean of three independent experiments).*

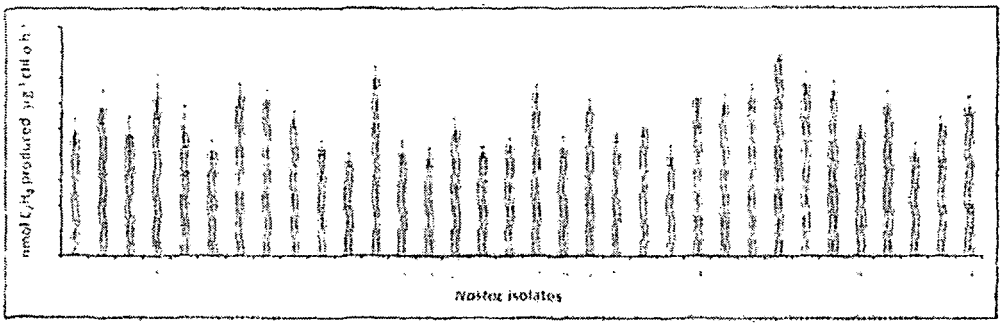

Fig. 1. (b) *Nitrogenase activity of Nostoc isolates.*

as Mairang, Mawphlang and Mawroh has lower pH (5.8-6.3) and lower temperature than warmer areas of Syntuksiar and Sung valley where pH was close to 7.0. The values of Shannon-Weiner's diversity, Simpson's Diversity and Simpson's Dominance indices recorded as a whole from all sites was found to be 1.630, 0.68 and 0.32 respectively. Richness was highest in Sung valley and Syntuksiar with both the sites supporting 17 strains each. Although higher diversity was seen in Sung valley, highest was recorded from Mawlai where richness was only 11 strains thereby indicating richness need not be a function of diversity in this region (Table. 3).

Nilsson et al (2002) have shown that many competent *Nostoc* strains colonize rice root surfaces and intercellular spaces. Such *Nostoc* strains were shown to have higher nitrogenase activity compared to their free-living counterparts. In pursuit of identifying indigenous *Nostoc* strains as potential biofertilizers in acidic rice fields, we have screened our *Nostoc* isolates with local rice variety 'Synteng'. All 34 *Nostoc* isolates showed root colonization to various degrees. Isolate no.27, 12 and 28 showed high degree of colonization and enhanced nitrogenase activity (Table.4) during association. These isolates could be marked for future soil and/or seedling inoculants in acidic rice fields.

Characters like nitrogen fixation have been found to be stable under immobilization in calcium alginate beads up to a period of two years. All our purified isolates have been preserved this way after characterization and cataloguing. We believe this would be helpful in future diversity study as reference as well as in researching strains for biotechnological applications. This would greatly reduce workload such as isolation, purification and characterization of unknown strains for such study as regenerated purified cultures from the preserved beads could be the starting point for future research.

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Cadmium Removal by *Anabaena sp.* Isolated from Coal Mining Areas of Meghalaya, India

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ABSTRACT

A ubiquitous cyanobacterial strain isolated from rice fields adjoining coal mining areas of Meghalaya was identified as *Anabaena sp.* using molecular technique involving partial sequence of 16S rRNA gene. The organism was grown in two different pH conditions (pH 7.5 that is optimum for cyanobacterial growth and in 6.5 to mimic the pH condition of the fields from which it was isolated). Various physiological and biochemical characters such as growth, heterocyst frequency, nitrogenase and glutamine synthetase activities, photosynthesis, respiration, nitrate uptake and nitrate reductase activities were assayed. Atomic absorption spectrophotometric (AAS) studies showed that comparatively more cadmium was removed from the medium at pH 7.5 than at pH 6.5. Cadmium sulphate was more toxic than cadmium nitrate. The removal was saturating within 72 h. For 20 μM cadmium nitrate in the medium, total cadmium removed was 9.72 μM, of which 68.13% was adsorbed on the cell surface and 22.67% was accumulated intracellularly within 48 h. By 48 h, of 20 μM cadmium sulphate in the medium, 58.7% was adsorbed on the cell envelope whereas at pH 7.5, 35% was intracellularly accumulated. These results were indicators of its potential application as biofertilizer and bioremediator in acidic rice fields.

Keywords: Acidic soil; Anabaena; molecular identification; biofertilizer; bioremediator.

I. INTRODUCTION

Climatic conditions prevailing in a region affect its floral, faunal and microbial diversity. Among the many factors viz. rainfall, temperature, pH, humidity, altitude etc. that govern the distribution of microbes over a region, pH plays a prominent role in determining the biodiversity in any given ecological niche. Microorganisms in nature are widely prevalent in almost all ecosystems. Their ubiquitous distribution renders them immensely susceptible to any unfavorable alterations in the environment in which they occur. Amongst microbes, cyanobacteria comprise an important group of ubiquitous photosynthetic nitrogen fixers that are found from arid deserts to Antarctic regions. De in 1939 first reported that fertility of rice fields are maintained due to the presence of cyanobacteria. The relative contribution of cyanobacteria as a percentage of total nitrogen fixed in the paddy fields varies widely and is mainly dependent on the chemical, climatic and biotic factors. It is now known that cyanobacteria contribute 20-80 Kg N/Ha/ crop on turnover of their biomass to rice fields. Tropical conditions ensure increased incidence of cyanobacteria in the rice field soils and high humidity, temperature and shade provided by the crop canopy favor the luxuriant growth of cyanobacteria (Roger & Reynaud, 1979). In addition, they secrete a number of important biologically active substances that improve soil structure (Misra & Kaushik 1989) and help in phosphate solubilization as well as in removal of toxic compounds.

Contamination of the natural environment by toxic metals is a serious problem worldwide due to their continued persistence in the ecosystem and also because of their incremental accumulation in the food chain (Chen & Pan, 2005). However, there are references of microbes acting as natural bioremediating agents in different polluted sites. The use of these natural biological resources in reducing the pollution load in our natural environment is widely accepted idea and alternative, innovative treatment techniques have focused attention on use of biological materials such as algae, fungi, yeast, and bacteria for the removal and recovery technologies. This has gained significance in recent years owing to their better performance and low cost (Veglio & Beolchini, 1997; Volesky, 2001). Microbes accumulate

metal ions by two well defined processes: (1) energy independent binding on the cell wall called 'Biosorption' and (2) energy dependent uptake of metal ions called 'Bioaccumulation' (Karna et al, 1999). Mucilage containing, polysaccharides producing cyanobacteria are highly efficient in passively absorbing high levels of dissolved metals using a charge mediated attraction (Macaskie, 1990). Over the years, many cyanobacteria have been studied for their role in bioremediation of polluted environments (Kumar et al, 1992; Leena & Fatma, 2000; Zakaria, 2001).

Among the heavy metals polluting soil, Cd is of serious concern because of its potentially harmful effects on humans and animals. It also has adverse effects on microbial biomass and their activities which play important roles in soil nutrients cycling and in maintaining soil fertility (Jose et al, 2002; Yao et al., 2003). Cadmium is widely in use as an anticorrosive agent, as a stabilizer in PVC products, as a colour pigment, a neutron-absorber in nuclear power plants, and in the fabrication of nickel-cadmium batteries. Phosphate fertilizers also show a large cadmium load. Although some cadmium-containing products can be recycled, a large share of the general cadmium pollution is caused by dumping and incinerating cadmium-polluted waste (Jarup, 2003).

The soils of Meghalaya are acidic in nature and more weathered in comparison to other North-eastern states of India, and hence cyanobacterial diversity is limited. However, certain stains of *Nostoc* and *Anabaena* are relatively common in rice fields of Meghalaya despite lower pH prevalent in these areas. We present here a study of cadmium removal potential of an indigenous *Anabaena* strain isolated from rice fields adjoining a coal mining site within the state of Meghalaya. This strain is ubiquitous in all rice fields from where the samples were collected. Meghalaya has a hilly terrain and low lying rice fields receive runoff water from adjoining coal mines, thus increasing concentrations of various pollutants in these fields. The logic of selecting a cyanobacterial species thriving in these fields was the fact that such strains are already exposed to increased pollutants in their surroundings and may have developed strategies to overcome inhibitory effects of such exposure.

2. RESEARCH METHODOLOGY

2.1 Collection sites

Water and soil samples were collected from various rice fields adjoining coal mining areas from the state of Meghalaya.

2.2 Isolation, purification and morphological identification of cyanobacteria

Cyanobacterium were isolated and purified by plating on 1% nutrient agar following pour plating method. Isolates were then grown and maintained in batch cultures in the same medium at 25 ± 2°C and at a photon fluence rate of 50 μmol m^{-2} s^{-1} (Rippka et al, 1979). Following this, initial morphological identification was done with the aid of Olympus BX 53 light microscope using description given by Desikachary (1959).

2.3 Molecular techniques for identification study

Genomic DNA was isolated from the cyanobacterium (isolate no. NEHU7) using MiniPrep of Bacterial Genomic DNA Method (Ausubel et al. 1999). For securing amplification of 16S rDNA homologous sequence, primer CF16S-7 was designed using Mac Vector software® based on cyanobacterial 16S rDNA sequences retrieved from GenBank. PCR amplification was carried out using primers CF16S-7 (forward, 5'- GGCTCAGGATGAACGCTG- 3') and FGPS-1490 (reverse, 5'- TGGAAAGCTTGATCCTGGCT- 3') following method described by Normand et al. (1996) in a GeneAmp 9700 Gold thermal cycler (Applied Biosystems). Purified amplicons were sequenced with the cycle sequencing method using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on a 3130 Genetic Analyzer (Applied Biosystems). Related sequences were retrieved from GenBank using BLAST search. Sequenced amplicon was aligned with retrieved sequences using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Phylogenetic analysis was performed with the PHYLIP version 3.66 (Felsenstein, 2008). The neighbour-joining algorithm based on a matrix of pair-wise distances corrected for multiple base substitutions by the method of Kimura (1980) was used to generate the phylogenetic tree with a bootstrap confidence value of 100.

2.4 Measurement of growth, heterocyst frequency and nitrogenase assay

Increase in chlorophyll a content was measured as a parameter of growth (McKinney 1941). The

heterocysts were counted using an Olympus BX 53 light microscope. Acetylene reduction activity by nitrogenase enzyme was estimated in vivo by gas chromatography using a Varian 3900 gas chromatograph fitted with a porapak 'T' column and a flame ionization detector. (Stewart et al. 1967).

2.5 Assay of electron transport activities

Photochemical and respiratory activities were measured using a Clark-type polarographic O2 electrode installed in a 3 ml Plexi glass container with magnetic stirrer (Robinson et al, 1982).

2.6 Nitrate uptake, nitrate reductase and glutamine synthetase (transferase) activities

Nitrate uptake and Ferredoxin-dependent nitrate reductase activities were measured using methods described by Cawse (1967); Snell & Snell (1949) respectively. Glutamine synthetase (transferase) activity was measured as described by Sampio et al. (1979). Protein was estimated according to Lowry et al. (1951).

2.7 Metal sorption by cyanobacteria

Cyanobacteria were inoculated in the nutrient media containing 20 µM $CdSO_4$ and 20 µM $CdNO_3$. The experimental flasks were shaken for 24 h and then centrifuged at 10,000g. The residual cadmium in the medium was determined by Perkin Elmer 3110 AAS.

3. RESULTS AND DISCUSSION

State of Meghalaya is rich in coal reserves. Heavy rainfall throughout the year in the state results in constant leaching from coal mining sites to the adjoining rice fields. In addition, the soil pH in Meghalaya is relatively low, ranging from 5.2 to 7.1. Such acidic conditions considerably restrict the cyanobacterial diversity since cyanobacteria prefer neutral to alkaline pH (De, 1939; Roger & Reynaud, 1979). A particular cyanobacterial strain was found to be prevalent in rice fields adjoining coal mining sites, suggesting its ability to populate and flourish under such non conducive conditions. 16S rRNA study confirmed that the organism was a member of the genus *Anabaena*. Sequencing yielded a partial sequence of 249 bases. Multiple alignments with the BLAST retrieved sequences showed homology of 94 & 92 percent with *Anabaena* sp. XPORK15F (Acc. No. EF568905.1) and *Nostoc* sp. (Acc. No. AB187508.1) respectively. The neighbour-joining phylogenetic tree constructed revealed that our cyanobacterial isolate named NEHU7 clustering into one group with the GenBank retrieved sequence Anabaena sp. XPORK15F and Nostoc sp. with a bootstrap confidence value of 100 and 80 respectively (Fig. 1).

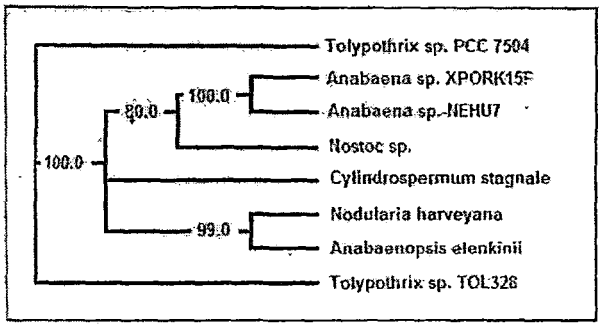

Fig.1: Phylogenetic tree derived from the aligned *Anabaena* sp. 16S rRNA sequences. The numbers at the nodes represents the bootstrap values out of 100 replicates.

The organism showed adaptability to rice field conditions as well as to acidic pH. Its heteroeyst frequency and nitrogenase activity was comparable to many other cyanobacterial species (Nilsson et al, 2002) suggesting its definite role in fixed nitrogen contribution to the rice field ecosystems. Table.1 summarizes the various parameters that were considered for characterizing the cyanobacterium. It has optimum photosynthetic and respiratory activities required for carbon dioxide fixation and generation of energy for important cellular activities such as nitrogen fixation. Nitrate uptake and reductase activities also indicated towards proper ability to utilize available nitrate in the medium. Nitrogenase activity of the organism in presence of nitrate indicates towards its possible application along with nitrate based fertilizer in rice fields. It also showed a value of 514 ± 2.0 and 493 ± 1.5 nmol r-glutamyl hydroxamate formed min-1 mg-1 protein for glutamine synthetase activity (in pH 7.5 and pH 6.5 respectively), suggesting its efficiency in incorporating available ammonia. All parameters that were studied in pH 6.5 were only slightly reduced in the organism when compared to its parameters at optimum pH 7.5.

Increasing concentration of cadmium in the medium had negative effect on the growth of Anabaena sp. Inhibition was higher at lower pH (Fig 2). Atomic absorption spectrophotometric study revealed that cadmium sulfate was found to be more toxic than cadmium nitrate. Comparatively more cadmium was

Table 1. Characteristic features and enzyme activities in the isolated Anabaena Sp.

Parameters	pH 7.5	pH 6.5
Growth (µg chlorophyll a ml^{-1} culture)	6.97 ± 0.3	6.12 ± 0.4
Heterocyst frequency (Percentage of total cells)	7 - 8%	6 - 7%
Nitrogenase activity (nmol C_2H_4 produced μg^{-1} chl a h^{-1})	9.19 ± 0.9	7.78 ± 0.6
Nitrogenase activity (nmol C_2H_4 produced μg^{-1} chl a h^{-1}) in presence of 1mM nitrate in growth medium	3.21 ± 0.4	2.78 ± 0.3
Photosynthetic oxygen evolved (nmol O_2 evolved μg^{-1} chl a h^{-1})	506 ± 0.6	473 ± 0.5
Respiratory oxygen consumed (nmol O_2 consumed μg^{-1} chl a h^{-1})	253 ± 0.5	216 ± 0.5
Glutamine synthetase (transferase) activity nmol r-glutamyl hydroxymate formed min^{-1} mg^{-1} protein	514 ± 2.0	493 ± 1.5
Nitrate uptake (µmol nitrate taken up min^{-1} mg^{-1} chl a)	4.8 ± 0.3	4.3 ± 0.2
Nitrate reductase activity (nmol nitrite formed min^{-1} mg^{-1} protein)	5.2 ± 0.1	4.9 ± 0.2

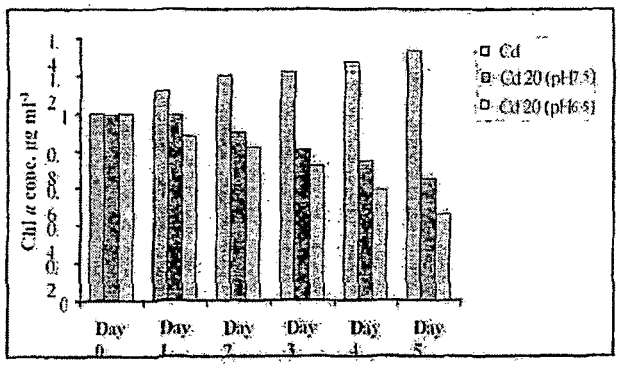

Fig.2: Effect of 20 µM cadmium sulphate on growth of Anabaena sp. in two different pH.

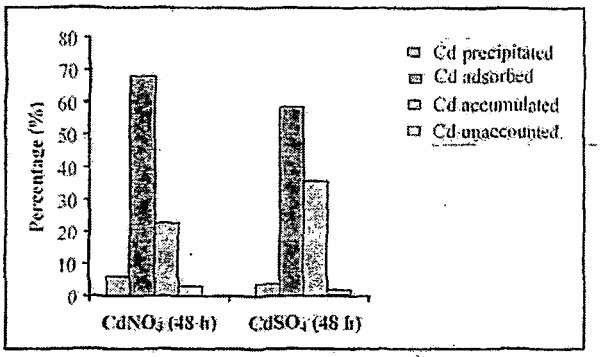

Fig.3: Percentage of cadmium nitrate and cadmium sulphate removed by Anabaena sp. at pH 7.5

removed by the organism from the medium at pH 7.5 than at pH 6.5. However, even at acidic pH considerable amount of Cd was removed from the medium. Total removed concentration out of 20 µM CdNO3 in the medium was between 9-10 µM at pH 7.5 whereas the value was between 6-7 µM at pH 6.5. The removal was saturating in 72 h. For 20 µM cadmium nitrate in the medium, total removed Cd was 9.72 µM, of which 68.13% was adsorbed on the cell surface and 22.67 % was accumulated intracellularly within 48 h (Fig 3). Of 20 µM cadmium sulfate, total removed was 6.37 µM, of which 58.7% was adsorbed on the cell surface and 35 % was accumulated intracellularly by 48 h at pH 7.5. Intracellular accumulation of $CdSO_4$ and $CdNO_3$ was found to be similar on comparison (2.22 µM as against 2.20 µM) even though the adsorbed concentration on the cell surface varied greatly (3.74 µM as against 6.62 µM respectively). Lesser toxic effect of $CdNO_3$ on cyanobacterial growth despite similar intracellular accumulation may point towards the role of nitrate in providing protection against such toxicity. This needs to be further investigated.

4. CONCLUSION

The Anabaena isolate from the rice fields adjoining coal mines was well adapted to the prevailing environmental and climatic conditions. It had substantial nitrogenase activity even in presence of nitrate in the surroundings. This makes it an attractive biofertilizer strain in fields supplemented with nitrate based fertilizers where it could continue to grow and add fixed nitrogen on their biomass turnover adding to fertility and soil quality. Its cadmium removal efficiency further contributes towards its usefulness in polluted rice fields with lower soil pH where in addition to atmospheric nitrogen fixation, it could reduce the heavy metal pollutant load in the fields.

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Role of cyanobacteria in cadmium bioremediation in rice fields

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Introduction:

Cyanobacteria are extremely diverse group of gram negative prokaryotes showing diversity in physiology, morphology, developmental characteristics and habitats[1-4]. In nature they are free-living as well as symbiotic[3,5]. Cyanobacteria as diazotrophs, have a long history of usage in agriculture as biofertilizer[6-8], and are known to enrich the nitrogen content of the rice fields[9]. Species of *Nostoc, Anabaena, Aulosira, Tolypothrix, Cylindrospermum, Gloeotchia, Gloeocapsa, Anabaenopsis, Camptylonema, Scytonema, Westiellopsis* widespread in Indian soils and rice fields contribute immensely to soil fertility. Cyanobacterial presence in rice field ecologies is of interest to scientists especially from rice growing countries as positive effects of cyanobacteria in rice fields are well established.

Mineral nutrients can be defined as all the inorganic elements required for life. In case of human inorganic nutrients include water, sodium, potassium, chloride, calcium, phosphate, sulphate, magnesium, iron, copper, zinc, manganese, iodine, selenium and molybdenum. Mineral toxicity is a condition where concentration of any one of the minerals in the body becomes abnormally high leading to adverse health effects. Apart from these, some common heavy metals that humans are exposed to are Al, As, Cd, Pb and Hg that can pose serious health hazards. Among these, Cd (atomic wt. 48) is a common bivalent metal naturally present in environment in different chemical forms such as cadmium sulfide, cadmium oxide, cadmium sulfate, and cadmium carbonate and cadmium chloride. It is used in production of colored inks and dyes, in metal plating, engraving, soldering, in plastics and in production of Ni-Cd batteries used in cell phones, portable computers and in many toys. Trace amount of Cd is found in most foods (higher in shell fish). Humans get exposed to Cd from sources that include drinking water, fertilizer, fungicides, pesticides, soil and pollution, refined grains, rice, coffee, tea, soft drinks and cigarettes. Cd accumulates in the body and replaces body's essential mineral zinc in the liver and kidney leading to serious kidney disease and liver damage.

Elevated levels of Cd result in hypertension, dulled sense of smell, anemia, joint soreness, hair loss, dry and scaly skin, loss of appetite, weakened immune system by reduced T cell production.

Agricultural soils are mainly contaminated with Cd from excessive use of fertilizers, dispersal of sewage sludge and atmospheric deposition. Cd is readily taken up by many crops including cereals, potatoes, vegetables (leafy and root) and fruits. Cd causes phytotoxicity in plants and its uptake and accumulation drives it further up the food chain. Negative effects of cadmium on RNA level, ribonuclease activity, phosphorolytic and

proteolytic enzymes in germinating rice seed are well documented[10-12]

Removal of metal ions from polluted waters using microbes is a well studied subject[13]. Microbes accumulate metal ions by two well defined processes: (1) energy independent binding on the cell wall called 'Biosorption' and (2) energy dependent uptake of metal ions called 'Bioaccumulation'[14]. Both living and non-living microbial biomass have been used for removing toxic metal ions[15-18]. Mucilage containing polysaccharides producing cyanobacteria are highly efficient in passively absorbing high levels of dissolved metals using a charge mediated attraction[19]. Many cyanobacteria have been studied for their role in bioremediation of polluted environments[20-23]. Proline accumulation in response to stress has been studied in many organisms including algae, protozoa, bacteria, marine invertebrates and plants[24]. In recent years, role of intracellular proline content in ameliorating environmental stress has been studied in cyanobacteria [22-23].

Rice field environment provides ideal conditions for cyanobacterial growth. Cyanobacteria are also efficient in metal uptake. Combining these two facts, the present study was undertaken to determine intracellular proline content of a heterocystous cyanobacterium belonging to *Nostoc* sp. in response to cadmium exposure and its role in decreasing the magnitude of the adverse effects of cadmium exposure to germinating rice seedlings.

Materials and Methods:

Sample collection:

***Cyanobacteria*:** Soil and water samples were collected from different rice fields during the month of August when the rice planted into the fields were about one and half months old (Table 1). These rice fields were water logged as there was continuous rain throughout the month of June, July and August.

***Rice seeds*:** Rice seeds of RCPL-1-87-8, DR-92 & Megha-I varieties were collected from ICAR, Umiam Complex, Meghalaya.

Cyanobacterial isolates from rice fields within the state of Meghalaya

Genus	Total strains	Collection sites							
		Umkhen (5.6)	Mairang (5.8)	Umiam (5.9)	Nongstoin (6.0)	Mawphlang (6.2)	Mawlai Mawroh (6.2)	Syntu Ksiar (6.5)	Sung Valley (6.9)
Anabaena	16	2	1	2	2	2	2	2	3
Nostoc	34	3	4	4	4	3	5	5	6
Cylindrosp-ermum	5	1	1	0	0	0	0	2	1
Calothrix	6	0	0	1	1	1	1	1	1
Fischerella	5	1	0	0	1	1	0	1	1
Gleocapsa	2	0	0	0	0	0	0	1	1
Plectonema	3	0	0	1	0	0	1	0	1
Total	71	7	6	8	8	7	9	12	14

Table 1. *Location of rice fields from which cyanobacteria were isolated. Values in the parenthesis indicate pH of the collection sites.*

Isolation and purification of cyanobacteria:

Cyanobacterial strains were isolated and purified after inoculating the samples in nitrogen free ($BG\text{-}11_0$) medium and subsequent plating on 1.2% nutrient agar. Isolates were grown and maintained in batch cultures at 25 ± 2°C and at a photon fluence rate of 50 μmol m^{-2} s^{-1} following the method described by Rippka *et al.*, 1979 [25]. Morphological identification was done under an Olympus BX 53 light microscope using description given by Desikachary (1959)[26]. A *Nostoc* species was selected for the present study.

Metal sorption by cyanobacteria:

For metal absorption studies cyanobacterium was grown in light in presence of half of the lethal dose (LD_{50}) i.e. 20μM cadmium sulphate in the growth medium. The concentration of 20μM of cadmium was carefully selected after determining the lethal dose (LD) of cadmium for the cyanobacterium under study (Fig 1). The experimental flasks were shaken for 24 h and then centrifuged at 10,000g. The residual cadmium in the medium was determined by Perkin Elmer 3110 Atomic Absorption Spectrophotometer [27]. The experiment was done in three independent sets.

Germination of Rice Seeds and co-cultivation of rice and isolated cyanobacteria:

Rice seeds were surface sterilized by washing with distilled water, followed by 1% (V/V) sodium hypochlorite solution for 5 minutes. These seeds were then thoroughly rinsed in sterile distilled water. Seed germination was carried out on autoclaved perlite in glass beakers[28]. The perlite was irrigated with a 10-fold dilution of autoclaved $BG\text{-}11_0$ medium containing 2mM $NaNO_3$ (nitrate medium) and varying concentration of cadmium sulphate (0, 5, 10, 15, 20, 25, 30, 35, 40 μM). The solution

was buffered with equi molar concentration of HEPES. Germination was carried out in culture room under fluorescent light (photon fluence rate of 50 µmol m^{-1} s^{-1}) at 25°C, at saturating relative humidity. DR-92 produced irregular results though out the study. Megha-I was found to be more susceptible to cadmium exposure and hence was selected. In another set of experiments, cyanobacterial suspension was also added to the germinating beakers in the beginning of the study.

Results and Discussion:

Coal mines of Sohra (Cherrapunji) village in Meghalaya leaches onto surrounding rice fields due to heavy rainfall thoughout the year. Two cyanobacterial strains were found to be most predominant in these rice fields. Out of this one *Nostoc* strain was more tolerant to cadmium exposure and hence was targeted for the present study. Varying concentration of cadmium sulphate (0, 5, 10, 15, 20, 25, 30, 35, 40 µM) was used to document the effect of cadmium on the germination of rice seeds. Presence of cadmium in the growth medium led to reduced seed germination. The affect was stronger in the rice variety Megha-I than in DR-92. 40µM cadmium sulphate in the germination medium was found to reduce seed germination up to 30% in the susceptible rice variety. At 20 µM concentration of cadmium, germination was reduced by 15-20% and presence of same amount of cadmium in the growth medium led to stunted plant growth.

When the cyanobacterium was grown in presence of increasing concentration of cadmium sulphate in the medium, it showed progressive reduction in its chlorophyll *a* content and growth (Fig 1). 40µM cadmium was lethal to the organism. However, intracellular proline showed marked increase at higher concentration of cadmium. Drop in proline concentration at 30 µM and 35µM cadmium in the medium probably was due to reduced number of viable cells at these concentrations although it does not correlate with the drop in chlorophyll amount (Fig1 & 2). It appears that there is an inverse relationship between chlorophyll *a* concentration and proline accumulation in the cyanobacterium under cadmium stress (Fig1 & 2).

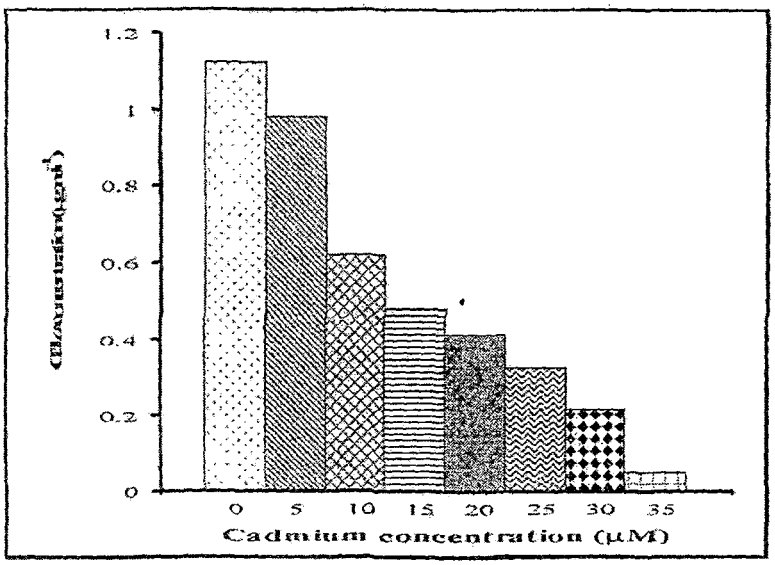

Fig. 1: Chlorophyll a content of Nostoc species at varying cadmium concentration

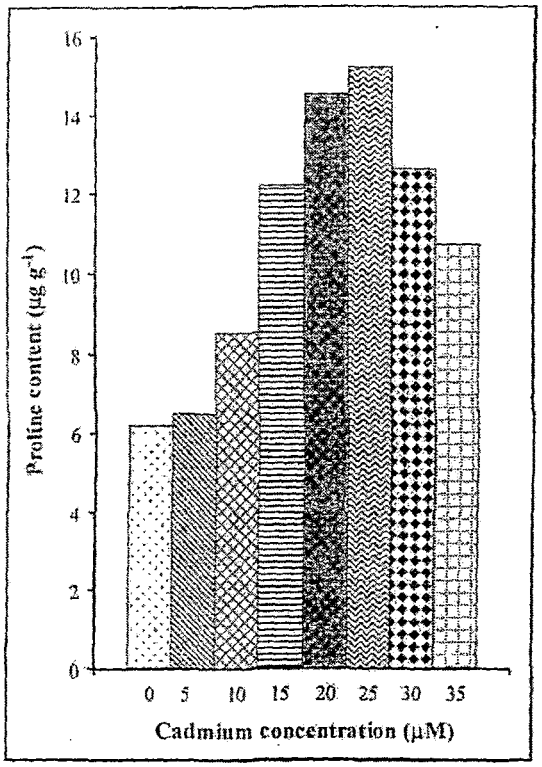

Fig. 2: Intracellular proline of Nostoc species at varying cadmium concentration.

A detailed study of chlorophyll concentration at LD_{50} in the medium at 24 h intervals showed a reduction in its value up to 182 h, after which there was no further noticeable decrease in the chlorophyll concentration (Fig 3). This implied that after the cyanobacterial cells have accumulated certain amount of cadmium from the medium, the level of toxicity was reduced in the surrounding medium enabling the cells to divide/grow better.

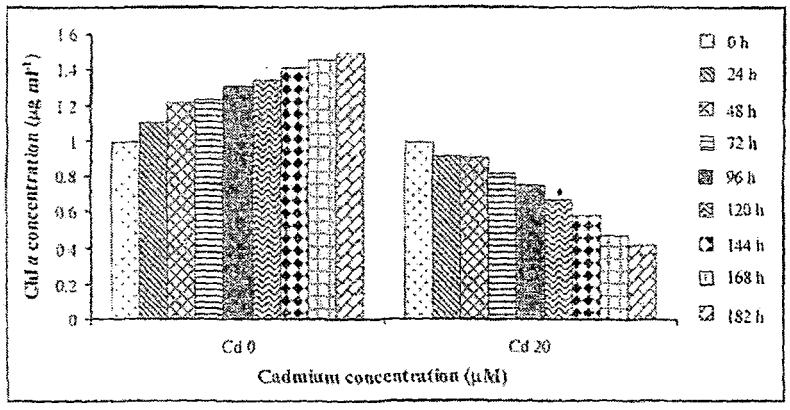

Fig. 3: Reduction in Chlorophyll a content in Nostoc species at LD_{50} of cadmium in comparison to control culture studied over eight days experimental period.

To study cadmium removal by the cyanobacterial cells, residual cadmium concentration in the medium was estimated by Atomic absorption spectrophotometry. The adsorption was saturating in 48 h. 62% of total Cd was removed from the medium with 24 h, which increased to 71% by 48 h. After this period there was no perceptible increase when the experiment was repeated after 72 h.

Experiments were set up to study the effect of cadmium and role of cyanobacterium in ameliorating negative effects of cadmium on growth of rice seedlings. Presence of 20 µM of cadmium in the growth medium led to 49% and 58% decrease in root and shoot length respectively (Table 2). Presence of cyanobacterium along with 20 µM of cadmium in the media showed an improvement of 34% and 40% in root and shoot length respectively over the value obtained for the seedling grown under the influence of cadmium in the medium.

Length of root & shoot of rice plants (in cm)

Root length (cm)			Shoot length (cm)		
Control (A)	0.02 mM cadmium (B)	(B) + cyano-bacteria	Control	0.02 mM cadmium (B)	(B) + cyano-bacteria
8.9	5.5	5.6	17.7	11.3	16.7
9.3	5.2	7.3	16.8	10.2	17.0
10.5	4.6	10.5	17.3	10.6	13.0
10.0	3.9	8.9	17.9	11.1	16.2
10.6	4.2	5.5	20.1	9.8	17.0
11.2	6.1	8.3	18.6	11.6	11.5
10.3	5.3	10.0	18.9	9.9	13.5
Average	Average	Average	Average	Average	Average
10.11	4.97	6.7	18.18	10.64	14.9

Table 2: Comparative root and shoot length of rice plantlets grown in cadmium supplemented medium, with and without cyanobacteria.

In conclusion, we found that presence of heavy metal cadmium led to decrease in growth and increase in intracellular proline content in cyanobacteria. Growing along cyanobacteria reduced ill effects of cadmium exposure in rice seedlings. There was immediate adsorption of cadmium by cyanobacteria leading to removal up to 71% of total cadmium present in the medium within 48 h thereby reducing available cadmium concentration and minimizing cadmium toxicity to rice plantlets.

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Role of Proline in Management of NaCl and pH Stress in Cyanobacteria

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Introduction

The role of nitrogen fixing cyanobacteria in enhancing soil fertility has been long known and is well documented[1-4]. Cyanobacteria contributes to overall soil health not only by its ability to perform biological nitrogen fixation but also because of its ability to produce polysaccharides and other bioactive compounds which has a growth stimulating effect on the vegetation/crop plants, as well as ensuring maintenance of soil quality and preventing erosion. Moreover it is also known that cyanobacteria increases the oxygen concentration of the environment in which they occur[5,6]. The general maintenance of soil fertility particularly in rice fields is attributed to the natural supply of assimilable nitrogen that is made available by the cyanobacteria through the process of biological nitrogen fixation[7]. Cyanobacteria generate cellular energy by harvesting light during the process of photosynthesis and utilizing this energy in the synthesis of carbon compounds required for their metabolic processes. Any alteration in the environment including change in pH and in salt concentration of the soil may have a direct negative impact upon photosynthesis and related metabolic processes of the cyanobacteria, including biological nitrogen fixation and overall performance of these organisms[8].

In nature microorganisms are constantly challenged by different factors and stresses including changes in temperature, moisture content (droughts and floods), salinity and so forth. Among these, salinity is known to be of major significance in limiting the growth and productivity of plants, eukaryotic microorganisms and bacteria[9]. Salinity posses to be a huge threat to agricultural productivity and approximately 7% of agricultural land are affected by high salt concentrations and the area is still increasing[10]. Owing to their considerable degree of salt tolerance, cyanobacteria have been applied in reclamation of saline and sodic lands with limited success[11,12]. Such a concept of biological soil reclamation using cyanobacteria was first advocated by Singh in 1961[4]. In trace amounts NaCl is known to act as a micronutrient to cyanobacteria and appears to be essential in certain metabolic processes[12-14]. However, elevated levels of the same may prove to be detrimental and result in inhibition of growth[15,16].

Cyanobacteria have evolved elaborate defense mechanisms in response to various environmental stresses that it encounters. Proline accumulation in response to stress is well documented in microbes, plants as well as marine invertebrates[17]. Proline is known to be an important indicator for stress tolerance and functions a stabilizer[18], a metal chelator[19], as an inhibitor of lipid peroxidation[20] and as a hydroxyl radical scavenger[21]. There are reports of proline being involved in ameliorating salinity stress as well as stress induced by

drought situation and also its role a singlet oxygen scavenger in higher plants[22,23]

Since cyanobacteria play a prominent role in the maintenance of soil fertility and productivity, any loss in cyanobacterial biomass may result in noticeable consequences. Moreover, these organisms are now regarded as promising agents in bioremediation and in cleaning up the environment. Hence, it becomes imperative to study and investigate the effects of stresses, either singly or in combination on the growth and characters of some naturally occurring cyanobacteria. This work investigates the effect of salinity, changes in pH and a combined effect of both on the growth and proline accumulation in cyanobacteria.

Materials and methods

The effects of salt stress as well as pH fluctuations were assessed on some of the filamentous heterocystous cyanobacteria which were isolated from various locations in Meghalaya. These were then grown as axenic cultures in batches in nitrogen free $BG11_0$ medium (Rippka et al, 1979)[24] at 25±2°C and under a photon fluence rate of 50 μmol m^{-2} s^{-1}. The exponential cultures were used for all the experiments.

Initially, three organisms that were sampled for the present study include two belonging to the *Nostoc* species (N1 and N2) and one to *Anabaena* species (A1). Three sets of these were grown in different concentrations of NaCl (50,100,150.....500 mM) in 50ml conical flasks to find out which amongst them is most tolerant to salinity stress. This was done by estimating the chlorophyll *a* concentration every 48 hours in each flask[25] to compare their growth to a control culture. One *Nostoc* species (N1) that showed highest tolerance to salt stress was selected for further experiments. The soil and water bodies of Meghalaya distinctly show a wide variation in pH. Therefore, effect of pH and combined effect of pH and salt stress was also measured in this cyanobacterium. For this, pH range of 5, 6, 7, 8 and 9 was selected. Also, a combination of a constant salt concentration (500 mM) and variable pH was used to assess their combined effect on the organism. The constant salt concentration was based on the fact that beyond this any further increase in the salt concentration was lethal to the organism. The changes in proline content in the organism in response to the different stress conditions was estimated on the 8th day using the method described by Bates et al 1973[26] . Chlorophyll *a* concentration on the 8th day was also considered.

Results and Discussion

In case of all the three organisms the chlorophyll *a* content was found to decrease with increasing salt concentrations (Fig 1, 2 and 3). However, the unfavorable concentration of salt was different for the individual cyanobacterium, with N1exhibiting higher tolerance. The *Anabaena* sp. that was used for the present study appeared to be highly susceptible to salinity stress with maximal reduction in chlorophyll *a* content at 500 mM by the 8th day (Fig 3).

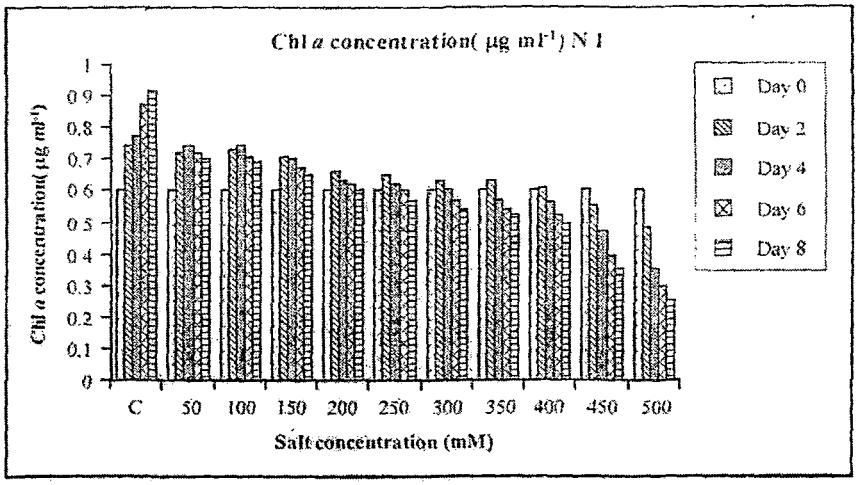

Fig 1: Effect of salt on growth of Nostoc sp.1. (C- Control condition)

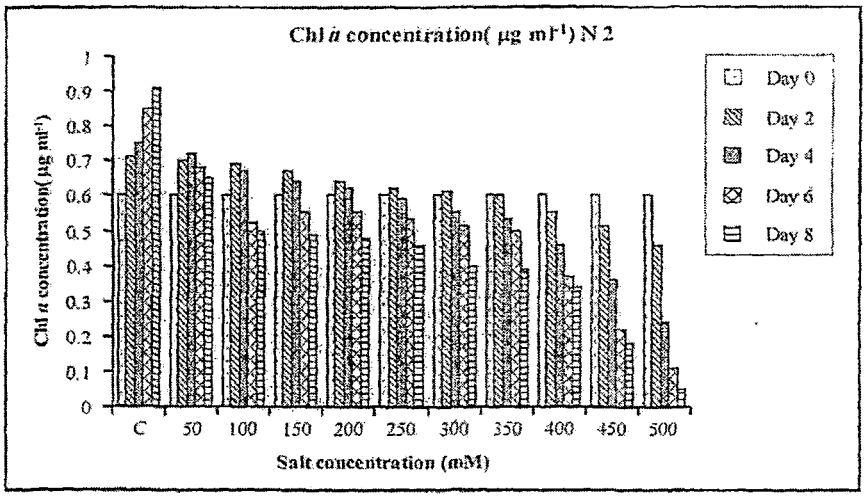

Fig 2: Effect of salt on growth Nostoc sp.2. (C- Control condition)

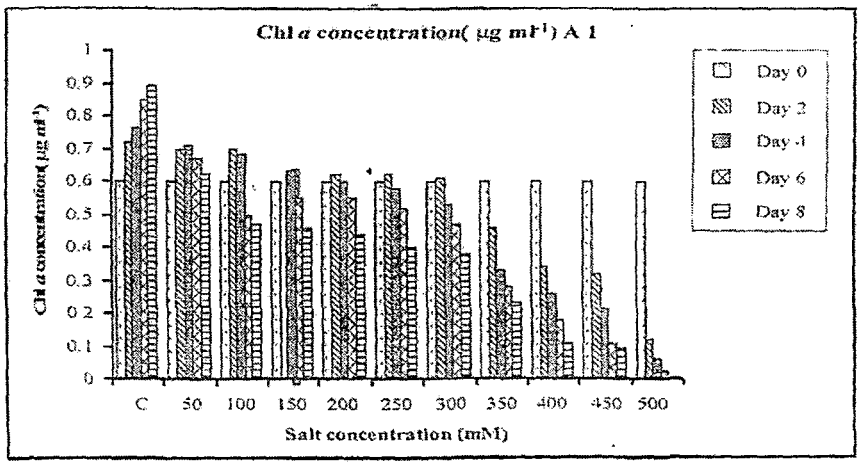

Fig 3: Effect of salt on growth Anabaena sp.1. (C- Control condition)

Studies with *N1*in different pH ranges revealed distinct changes in the growth pattern at pH 5 and 6. At higher pH of 8 and 9 there were no remarkable changes in growth. The cyanobacterium exhibited erratic growth at pH 5 and 6 and growth showed a decline in these pH with respect to time (Fig. 4). At pH 9, the exponential growth phase of the organism was shorter than the control (pH 7.6). However, the growth during this phase was comparable to the control. In combination with a constant salt concentration of 500mM, there was a steady decline in chlorophyll *a* concentration indicating low survival at pH 5 and 6. A similar behavior was also evident at the higher pH ranges of 8 and 9, although the effect was less drastic then that at lower pH (Fig. 5).

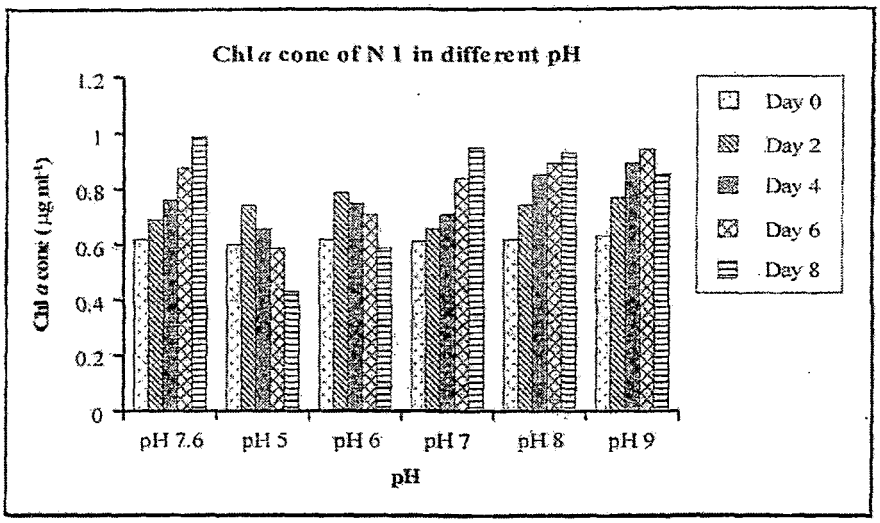

Fig 4: Effect of pH on growth of Nostoc sp.1. (C- Control condition)

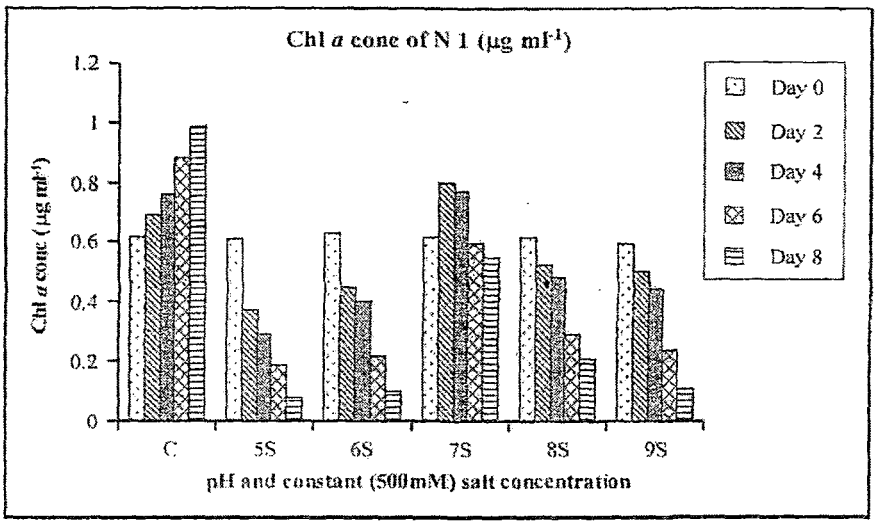

Fig 5: Combined effect of pH and salt on growth of Nostoc sp.1. (C- Control condition; S-500 mM NaCl)

Consistent with previous observations[8,27], it was found that with increase in stress, either as high salinity or unfavorable pH, there was an associated increase in proline content and a prominent decline in chlorophyll *a* concentration. High proline contents of 0.65 and 0.63µg g^{-1} were observed in pH range of 5 and 6 respectively followed by 0.63 and 0.61 µg g^{-1} for pH 9 and pH 8 as compared to 0.57 µg g^{-1} in unstressed condition in control culture (Fig 6). The proline content was found to be the highest in the case of the combined salt and pH stress (Fig 7). Proline was accumulated to an amount of 0.72 and 0.73 µg g^{-1} in pH 8 and 9 respectively which further increased in pH 5 and 6 to 0.92 and 0.76 µg g^{-1} respectively while in combination with salt.

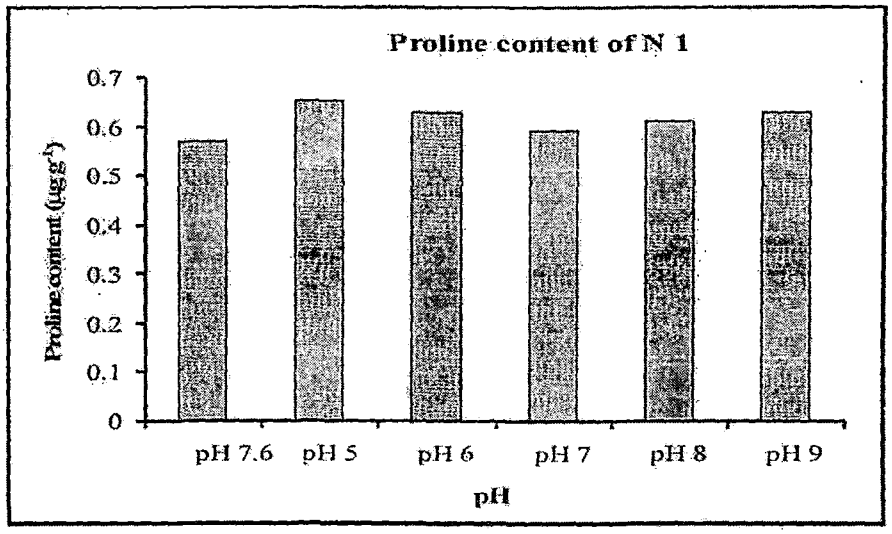

Fig 6: Proline content of Nostoc sp.1 at different pH. (C- control condition)

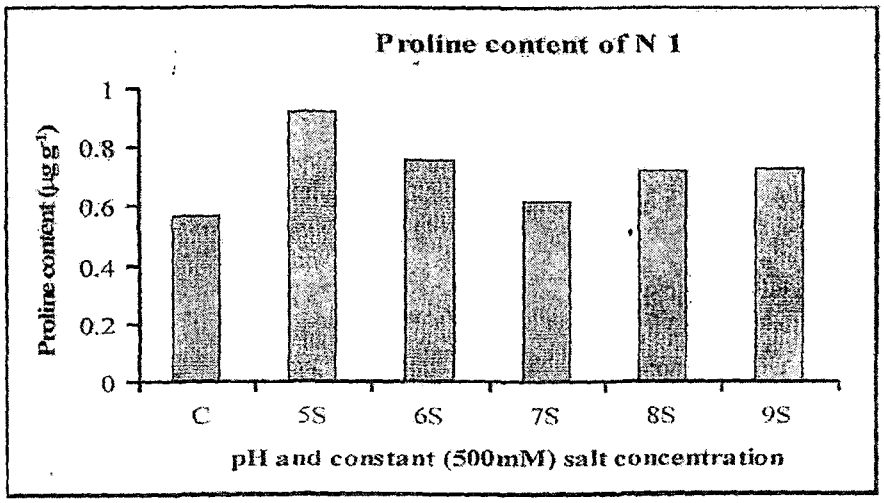

Fig 7: Proline content of Nostoc sp.1 under combined effect of salt and pH. (C- Control condition; S-500 mM NaCl)

There are extensive reports on stress induced proline accumulation in higher plants[28-32]. However, not much work has been done towards the assessment of the role of proline in cyanobacterial stress tolerance that may have a significant impact on their putative application in bioremediation as well as their use as biofertilizer in coastal areas. Wu et al (1998)[33] has reported increase in proline content under salt stress in *Chlorella*. It has been suggested that proline is involved in stabilizing folded protein structures[34]. Its role in stabilizing membranes by interacting with phospholipids[35] and being involved in osmotic adjustments[36] are also known. Stress induced by high salinity has a negative impact on the photosynthetic capacity and growth of cyanobacteria[37,38].

In the present study, it was observed that stress due to enhanced salinity and changes in pH in the immediate vicinity resulted in induction and accumulation in intracellular proline in the cyanobacterium. This finding is similar to earlier report on proline accumulation under stress conditions in the cyanobacterium *Westiellopsis prolifica*-Janet Strain-NCCU331 by Fatma et al., 2007. In these organisms, proline appears to be the stress-induced substance that helps in generating tolerance to adverse environmental stresses.

The potential of cyanobacteria as biofertilizer is immense. However, their susceptibility to environmental changes limits their optimum use. Carefully selected cyanobacterial strains with high nitrogen fixing capability that are resistant and/or tolerant to fluctuations in the surrounding environment could be ideal for future biofertilizer programme and also as bioremediators for reclaiming saline and sodic lands for agricultural usage. One way to identify such strains could be to look at their proline accumulating capacity under stressful environment. These strains with high proline synthesizing potential may prove to be an ideal candidate for bioremediation.

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Nitrogen metabolism, artificial association study in two cyanobacterial isolates and assessment of their potential as biofertilizer

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Two strains of cyanobacteria, viz. *Nostoc* ANTH and *Mastigocladus* sp, were isolated from local separate temperature zones of Meghalaya, India. Both the strains showed preference for different temperatures for optimum growth [45°C for *Mastigocladus* sp.(thermophile) and 25°C for *Nostoc* ANTH (mesophile)]. The addition of nitrogen sources in the growth media (nitrate, ammonia and glutamine) supported their better growth but repressed heterocyst development and nitrogenase activity. Nitrate and nitrite uptake rates, NR and NiR activities increased by NO_3^- and decreased by NH_4^+ in *Nostoc* ANTH. However, such effects were only partial in *Mastigocladus* sp. The presence of fixed nitrogen sources in the media led to decreased GS activity and repressed methylammonium uptake in both the strains. Glutamine uptake was substrate inducible, energy-dependent and required *de novo* protein synthesis. Artificial association studies revealed successful establishment of association of rice roots with both cyanobacteria, including prolonged association of *Mastigocladus* sp. at high temperature (~45°C). Little modifications in growth temperature and growth media led to profuse akinete differentiation in target cyanobacteria. The replacement of normal cells by akinetes as field inoculants might have profound biotechnological implications in future biofertilizer programme.

Keywords: cyanobacteria, N_2-fixation, mesophile, thermophile, biofertilizer
IPC Code: Int. Cl.[8] C05F9/04

Introduction

Cyanobacteria, an extremely diverse group of Gram-negative prokaryotes, show diversity in physiology, morphology, developmental characteristics and habitats[1-4]. In nature, they are free-living as well as symbiotic[3,5]. Cyanobacteria, as diazotrophs, have a long history of usage as biofertilizer in agriculture[6-8] and are known to enrich the nitrogen content of the rice fields[9]. Species of *Nostoc*, *Anabaena*, *Aulosira*, *Tolypothrix*, *Cylindrospermum*, *Gloeotrichia*, *Gloeocapsa*, *Anabaenopsis*, *Camptylonema*, *Scytonema* and *Westiellopsis* are widespread in Indian soils and rice fields, contributing immensely to soil fertility. The current biofertilizer programme using free-living cyanobacteria poses many problems, including low survival rate of inoculum; adaptability of cyanobacteria inoculated in the fields with regards to competition with the preexisting natural populations; and incompatibility with chemical fertilizers and low nitrogen release. Also, most cyanobacteria have optimum N_2-fixing ability in a temperature range of 20-30°C. Therefore, the mesophilic cyanobacteria may not be the ideal source of biofertilizer in tropical rice fields where daytime temperature can soar anywhere 30-45°C, and this adversely affects their metabolic processes. Under these conditions, thermophilic cyanobacteria seem to be the better option as biofertilizer in mixed consortia. Information about thermophilic cyanobacteria that grow at such high temperatures is scanty and so far has not been looked into as the potential biofertilizer. Keeping this in mind, the mesophilic *Nostoc* ANTH and thermophilic *Mastigocladus* sp., both isolated from the state of Meghalaya, were compared with respect to selected metabolic processes with an aim to enumerate their use as potential biofertilizer in rice fields of different temperature zones.

Materials and Methods

Strains and Culture Conditions

Nostoc ANTH was isolated from the undersurface of the gametophytic thalli of the bryophyte

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Anthoceros, collected from the North Eastern Hill University campus, Shillong and purified under axenic conditions. Axenic cultures were grown in batches and maintained in BG-11$_0$ (N_2-medium)[10] at 25°C with a photon fluence rate of 50 µmol m^{-2} s^{-1}. Whenever needed, the N_2-medium was supplemented with 5 m*M* KNO_3 (NO_3-medium) or 2 m*M* NH_4Cl (NH_4-medium) or 1 m*M* L-glutamine (glutamine-medium).

Mastigocladus sp. was isolated from the hot spring of Jakrem, Meghalaya state, India and grown in axenic batch cultures in D-medium[11] under continuous light with a photon fluence rate of 50 µmol m^{-2} s^{-1} at 45°C in BOD incubator and also at 25°C in the culture room. Whenever needed, cultures were also grown in N_2-medium (D-medium without source of combined nitrogen), NH_4-medium (N_2-medium supplemented with 2 m*M* NH_4Cl) or in N_2-medium supplemented with 1 m*M* L-glutamine. All media for *Nostoc* ANTH and *Mastigocladus* sp. were buffered with equimolar concentration of HEPES, and *p*H was adjusted to 7.5 before autoclaving.

Growth, Heterocyst Frequency and Nitrogenase Activity

Growth was measured as increase in concentration of *Chl a*[12]. Heterocyst frequency in both the cyanobacteria was calculated as percentage of total vegetative cells after 96 h of incubation in different nitrogen media. Acetylene reduction assay was used to measure nitrogenase activity[13].

Nitrate and Nitrite Uptake Assay

The N_2-, NO_3- and NH_4-grown cultures of *Nostoc* ANTH and *Mastigocladus* sp. were harvested by centrifugation during the exponential growth phase (after 4 d growth), washed and resuspended in Tricine-$NaHCO_3$ buffer (25 m*M*, *p*H 8.1). Uptake experiments were initiated by the addition of $NaNO_3$ (100 µ*M*) or KNO_2 (100 µ*M*) to the cell suspension. Uptake of NO_3^- and NO_2^- was measured by the rates of their depletion from the medium. The choice of 100 µ*M* external concentration for both the combined N sources was based on earlier studies in *Anabaena* sp. PCC 7120 and *Synechococcus* sp. strain PCC 7942[14,15]. Samples were withdrawn after 3 h of incubation, subjected to rapid centrifugation at 5000 g and the cell-free supernatants analyzed for residual NO_3^- or NO_2^-. Nitrate and nitrite concentrations were measured by the method of Cawse[16] and Snell and Snell[17], respectively.

Nitrate Reductase, Nitrite Reductase and Glutamine Synthetase (Transferase) Activity

Ferredoxin-dependent nitrate reductase (NR) and nitrite reductase (NiR) activities were measured using dithionite reduced methyl viologen as reductant[18,19]. Glutamine synthetase (transferase; GS) activity was measured as described by Sampio *et al*[20]. However, in *Mastigocladus* sp., incubation for the enzyme assays was performed at higher temperature (45°C) for cultures grown at 45°C. In case of *Nostoc* ANTH, cultures were incubated at 30°C. Protein was measured according to Lowry *et al*[21].

Ammonium and Amino acid Transport Assay

Ammonium transport assay for *Nostoc* ANTH and *Mastigocladus* sp. was done using the radioactive [^{14}C]-methylammonium, an analogue of ammonium (sp. activity 370 KBq µmol^{-1})[22]. Glutamine uptake was measured using [^{14}C]-glutamine (sp. activity 256 KBq µmol^{-1}) for both the cyanobacterial strains. NO_3-grown cultures were harvested during the exponential growth phase, washed in N_2-medium and incubated in N_2-medium or N_2-medium supplemented with 1m*M* glutamine at 25°C for *Nostoc* ANTH and at 45°C for *Mastigocladus* sp. After 48 h, cells were harvested, washed and resuspended in 10 m*M* HEPES-NaOH buffer (*p*H 7.0) to a final concentration of 10 µg mL^{-1} *Chl a* for both the strains and equilibrated for 1 h under their respective growth conditions. Methylammonium or glutamine uptake experiments were started by adding [^{14}C]-methylammonium or [^{14}C]-glutamine to a final concentration of 50 µ*M* for both the cyanobacteria. Whenever needed, dichlorophenyldimethylurea (DCMU, 10 µ*M*) or carbonyl cyanide chlorophenyl hydrazine (CCCP, 25 µ*M*) was added to the cell suspension 30 min prior to the addition of labelled glutamine. At different time intervals, 400 µL sample was taken out quickly and cells separated by centrifugation through silicon oil DC 550/dinonylphthalate (40/60, v/v) into perchloric acid/water (15/85, v/v)[22] and [^{14}C] in the perchloric acid fraction measured using liquid scintillation counter (Beckman, Model 1801). The non-specific binding of [^{14}C]-methylammonium and glutamine was determined by measuring their incorporation in the toluene treated cells[22].

Cultures Conditions for Akinetes Differentiation

Nostoc ANTH cultures were induced to sporulate in BG-11$_0$ medium lacking $MgSO_4$ and replaced by equimolar $MgCl_2$. *Mastigocladus* sp., on the other

hand was found to sporulate spontaneously in N_2-medium at lower temperatures. Akinete frequency was calculated as the percentage of total cells in Olympus BX-51 light microscope fitted with a JVC digital video camera.

Co-cultivation of Rice and Cyanobacteria

Rice seedlings (10-d-old) grown on perlite were transferred to 15 mL culture tubes after washing with distilled water. D-N_2-medium and BG-11_0 medium (10 mL) was poured independently into these tubes and seedlings carefully placed with the roots dipped in the medium. *Mastigocladus* sp. and *Nostoc* ANTH were then inoculated in these test tubes in their respective growth media. Tubes with *Mastigocladus* sp. were kept in the BOD incubator at 45°C and with *Nostoc* ANTH in the growth chamber at 25°C.

Results

Growth, Heterocyst Frequency and Nitrogenase Activity

Both *Nostoc* ANTH and *Mastigocladus* sp. were grown initially in a temperature range of 20 to 60°C. Growth was measured as increase in *Chl a* content on 4th d. *Mastigocladus* sp. grew best at 45°C (2.3 µg mL^{-1} *Chl a* in D-N_2-media). However, *Nostoc* ANTH cells died at temperatures exceeding 35°C and showed optimal growth at 25°C (1.2 µg mL^{-1} *Chl a* in N_2-media). Growth was consistently higher in NO_3, NH_4 or glutamine supplemented media. Heterocyst frequency and nitrogenase activity were not detected in combined nitrogen supplemented media. The growth, heterocyst frequency and nitrogenase activity of *Mastigocladus* sp. were ~1.5-fold higher at 45°C than at 25°C.

Nitrate and Nitrite Uptake Activities

Both *Mastigocladus* sp. and *Nostoc* ANTH showed NO_3^- and NO_2^- uptake activities. The rate of NO_3^- uptake by *Mastigocladus* sp. grown in N_2-medium was 27.04 nmol min^{-1} mg^{-1} *Chl a* compared to 32.9 nmol min^{-1} mg^{-1} *Chl a* in NO_3-medium or 8.8 nmol min^{-1} mg^{-1} *Chl a* in NH_4-medium (Table 1). Further, NO_3^- uptake by *Nostoc* ANTH was 2.8 nmol min^{-1} mg^{-1} *Chl a* in N_2-medium, 3.6 nmol min^{-1} mg^{-1} *Chl a* in NO_3-medium and 0.4 nmol min^{-1} mg^{-1} *Chl a* in NH_4-medium. Hence, there was 21% and 28% increase in NO_3^- uptake by *Mastigocladus* sp. and *Nostoc* ANTH, respectively in the NO_3-medium as compared to N_2-medium. Interestingly, NO_3^- uptake rate of *Mastigocladus* sp. was ~10-fold higher compared to *Nostoc* ANTH under similar conditions of N_2- and NO_3-medium. However, the presence of NH_4^+ in the growth medium led to inhibition of NO_3^- uptake, which was more severe in case of *Nostoc* (88%) compared to *Mastigocladus* sp. (73%) than their respective N_2-grown cells.

On the other hand, NO_2^- uptake by *Mastigocladus* sp. and *Nostoc* ANTH was increased by 40 and 20%, respectively in NO_3-medium as compared to N_2-medium (Table 1). However, severe inhibition in NO_2^- uptake by *Nostoc* ANTH (99%) than in *Mastigocladus* sp. (39%) was noticed in the presence of NH_4^+.

NR, NiR and GS Activity

In NO_3-medium, NR activity showed a drastic increase (133%) in *Nostoc* ANTH (25°C) against a moderate increase (17%) in *Mastigocladus* sp. (45°C) in comparison to its activity in N_2-medium (Table 2). However, the NR activity declined by 88% in *Nostoc* ANTH and 46% in *Mastigocladus* sp. in the presence of NH_4^+ in the medium.

In N_2-medium, NiR activity in *Nostoc* ANTH was much higher (540 nmol NO_2^- consumed min^{-1} mg^{-1} protein) as against the enzyme activity in *Mastigocladus* sp. (163 nmol min^{-1} mg^{-1} protein). NiR activity showed a 38% rise in *Nostoc* ANTH in NO_3-medium in comparison to activity in N_2-medium, which by contrast was only 8% in *Mastigocladus* sp. However, the presence of NH_4^+ in the medium led to 57% inhibition of NiR activity in *Nostoc* ANTH, but the inhibition was only 6% in *Mastigocladus* sp. (Table 2).

A comparative study of GS activity revealed that *Mastigocladus* sp. had much higher enzyme activity (2464 nmol γ-glutamyl hydroxamate min^{-1} mg^{-1}

Table 1 Nitrate and nitrite uptake by *Nostoc* ANTH and *Mastigocladus* sp. pre-grown in media containing different nitrogen sources

Nitrogen sources in growth medium	Nitrate uptake (µmol nitrate taken up min^{-1} mg^{-1} *Chl a*)		Nitrite uptake (µmol nitrite taken up min^{-1} mg^{-1} *Chl a*)	
	Nostoc ANTH	*Mastigocladus* sp.	*Nostoc* ANTH	*Mastigocladus* sp.
N_2	2.8 ± 0.2	27.04 ± 0.4	24.72 ± 1.1	21.7 ± 1.9
NO_3^-	3.6 ± 0.1	32.92 ± 1.6	29.26 ± 0.9	30.11 ± 1.5
NH_4^+	0.4 ± 0.1	8.81 ± 0.4	0.2 ± 0.1	17.29 ± 1.8

N_2 = No combined nitrogen; NO_3 = + 10 m*M* $NaNO_3$; NH_4 = + 2 m*M* NH_4Cl

Table 2—*Effect of different nitrogen sources on nitrate reductase (NR), nitrite reductase (NiR), and glutamine synthetase (transferase) (GS) activities of Nostoc* ANTH and *Mastigocladus* sp.

Nitrogen sources in growth medium	NR activity (nmol NO_2^- formed min^{-1} mg^{-1} protein)		NiR activity (nmol NO_2^- consumed min^{-1} mg^{-1} protein)		GS activity (nmol γ-glutamyl hydroxamate formed min^{-1} mg^{-1} protein)	
	Nostoc ANTH	*Mastigocladus* sp.	*Nostoc* ANTH	*Mastigocladus* sp.	*Nostoc* ANTH	*Mastigocladus* sp.
N_2	1.8 ± 0.1	9.16 ± 0.4	540 ± 27	162.53 ± 8	610 ± 7	2464 ± 123
NO_3	4.2 ± 0.1	10.07 ± 0.5	745 ± 37	175.03 ± 8	598 ± 3	1689 ± 84
NH_4	0.2 ± 0.1	4.87 ± 0.2	230 ± 12	152.65 ± 7	376 ± 6	1466 ± 73

NR = Nitrate reductase; NiR = Nitrite reductase; GS = Glutamine synthetase
N_2 = No combined nitrogen, NO_3 = + 10 m*M* $NaNO_3$, NH_4 = + 2 m*M* NH_4Cl.

protein) compared to *Nostoc* ANTH (610 nmol γ-glutamyl hydroxamate min^{-1} mg^{-1} protein) grown in N_2-medium. However, GS activity was repressed by 31% in *Mastigocladus* sp. as against 2% in *Nostoc* ANTH when NO_3^- was added to the growth medium. The addition of NH_4^+ to the medium also showed similar inhibitory effect on the GS activity in *Mastigocladus* sp. (40%) and in *Nostoc* ANTH (38%) (Table 2).

Methylammonium and Glutamine Uptake

The status of ammonium uptake in *Nostoc* ANTH and *Mastigocladus* sp. was studied using [^{14}C]-methylammonium. Both the organisms showed a biphasic pattern of methylammonium uptake marked by an initial rapid phase lasting for 60 sec, followed by a slower second phase that remained linear during the next 10 min of the experimental period. Methylammonium uptake activity in *Nostoc* ANTH during the first and the second phase was 55 and 7.3 nmol mg^{-1} min^{-1} *Chl a*, respectively. The uptake rates for the same compound in *Mastigocladus* sp. cells were 42 and 15.77 nmol mg^{-1} min^{-1} *Chl a* during the first and second phase, respectively. The addition of NO_3^- and NH_4^+ in the growth-medium led to severe repression of the methylammonium uptake in both the cyanobacteria. Overall, the data showed that nitrogen starvation increases ammonium transport activity in both the strains.

Studies on the uptake of glutamine in *Nostoc* ANTH and *Mastigocladus* sp. also showed a biphasic nature where an initial rapid phase represented intracellular accumulation, followed by a slower second phase representing assimilation. The glutamine uptake rates were higher in glutamine-grown cells than in N_2-grown cells. Such an increase in the rates was significantly inhibited by the addition of chloramphenicol, an inhibitor of protein synthesis (Fig. 1). Also, DCMU and CCCP inhibited the glutamine uptake in both the strains (Table 3).

Table 3—The effect of DCMU and CCCP on [^{14}C]-glutamine uptake by *Nostoc* ANTH and *Mastigocladus* sp.

Growth-medium	[^{14}C]-Glutamine uptake (nmol [^{14}C]-glutamine taken up mg^{-1} min^{-1} *Chl a*)	
	Nostoc ANTH	*Mastigocladus* sp.
Control	44.0 ± 2.2	131.71 ± 6.5
Control + DCMU (10 μ*M*)	21.8 ± 1.0	51.21 ± 2.0
Control + CCCP (25 μ*M*)	3.8 ± 0.1	21.87 ± 1.5

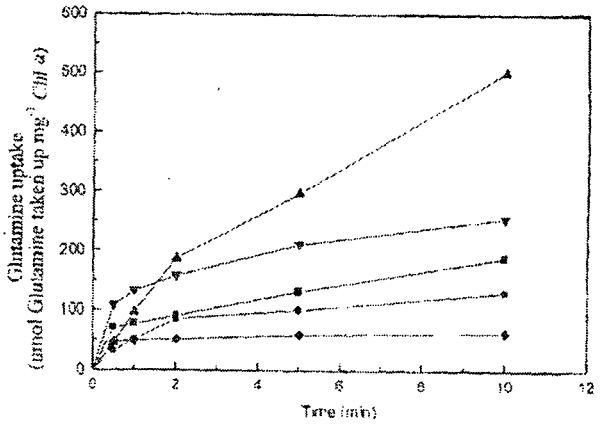

Fig 1—[^{14}C]-Glutamine uptake in *Nostoc* ANTH grown in N_2- (25 C, ●) and glutamine-medium (25°C, ▲), and *Mastigocladus* sp. grown in N_2- (45°C, ■), glutamine-medium (45°C, ▼) and glutamine-medium+chloramphenicol (♦). (NO_3-grown exponential cells were washed and transferred to N_2- and glutamine-medium (1m*M* glutamine) and incubated (48 h) at 25°C for *Nostoc* ANTH and at 45°C for *Mastigocladus* sp. Cells were then harvested, washed and resuspended in HEPES buffer and used for glutamine uptake as described in Materials and Methods. The values are means of two independent experiments, each with two replicates).

Akinete Differentiation

The interest in akinete was for its potential as inoculum to be used to populate rice fields with competent cyanobacterial strains. Akinete differentiation in *Nostoc* ANTH started only when the

N_2-medium was used to grow the cells lacked either sulphate or phosphate, the former limitation leading to quicker and higher extent of akinete differentiation (within 3-4 d as against 8-9 d). In contrast, akinetes in *Mastigocladus* sp. differentiated with in 10 d simply when grown in D-N_2-medium at lower temperature (25°C; Table 4). By 40th d, the akinetes percentage of *Nostoc* ANTH in sulfate limited N_2-medium and *Mastigocladus* sp. in normal D-N_2-medium were 98 and 80%, respectively at 25°C. Some degree of akinete differentiation (~20%) was also observed by 40th d in *Mastigocladus* sp. grown at 45°C probably due to exhaustion of media nutrients.

Table 4 — The time course of akinete differentiation in *Nostoc* ANTH (25°C) in sulfate limiting N_2-medium and *Mastigocladus* sp. in normal D-N_2 medium (25°C and 45°C)

Time (d)	Akinete frequency (%)		
	Nostoc ANTH	*Mastigocladus* sp.	
	25°C	25°C	45°C
0	0.0	0.0	0.0
5	48 ± 1.0	0.0	0.0
10	59 ± 1.0	12.0 ± 0.6	0.0
20	78 ± 1.0	30.0 ±1.5	0.0
30	92 ± 1.0	52.0 ± 2.6	0.0
40	98 ± 1.0	80.0 ± 4.0	20 ± 1.0

Artificial Association of Rice Plants with Cynobacteria

To test whether these two cyanobacteria can be used as biofertilizer, *Mastigocladus* sp. and *Nostoc* ANTH were incubated with rice seedlings. There was considerable association of both these cyanobacteria at room temperature (25°C). However, when incubated at 45°C, *Nostoc* ANTH died after 24 h. Interestingly, *Mastigocladus* sp. showed significant association with rice roots even at 45°C for prolonged period. N_2-fixation activity was measured after 7 d of incubation. It was higher in associated cells of both the cyanobacteria. This activity was 16.28 and 12 nmol C_2H_4 produced μg^{-1} h^{-1} *Chl a*, respectively in associated and free-living cells of *Nostoc* ANTH at 25°C. The corresponding values for *Mastigocladus* sp. at 45°C same were 6.27 and 4.62 nmol C_2H_4 produced μg^{-1} h^{-1} *Chl a*.

Discussion

Morphologically, *Nostoc* ANTH and *Mastigocladus* sp. are different cyanobacterial genera; *Nostoc* ANTH without branching and *Mastigocladus* sp. with true branching. Growth experiments conducted beyond 35°C led to cell death in *Nostoc* ANTH. However, there was increase in growth, heterocyst frequency and nitrogenase activity in *Mastigocladus* sp. at 45°C and beyond, indicating its thermophilic nature. Both the cyanobacteria were able to utilize, NO_3^-, NO_2^-, NH_4^+ and glutamine as sole N source for growth but these were inhibitory to heterocyst differentiation and nitrogenase activity. These observations are similar to earlier reports in other cyanobacteria[23-28]. In general, the appearance of the *Mastigocladus* sp. was more yellowish than of the *Nostoc* ANTH. This could be due to the fact that the media composition used for growth of *Mastigocladus* sp. lacked in one or more nutrient(s) that otherwise is available to the organism in the hot spring. A comparison of heterocyst frequency and nitrogenase activity in the two cyanobacterial genera at their respective growth temperatures showed that *Nostoc* ANTH had higher nitrogenase activity even though heterocyst frequency was higher in *Mastigocladus* sp. probably due to more efficient N_2-fixing and/or C-fixing machinery in the former.

Further, investigations into nitrogen metabolism aspects of the two isolates established that nitrate and nitrite uptake rates were inducible by NO_3^- and repressible by NH_4^+. However, nitrate uptake rates of *Mastigocladus* sp. were much higher (~10-fold) than of *Nostoc* ANTH. As is the case with other cyanobacteria[26], the severity of inhibition by NH_4^+ was more pronounced in *Nostoc* ANTH. However, in contrast to a complete repression of nitrate and nitrite uptake by NH_4^+ in other cyanobacteria[26,29], it was only partial in *Mastigocladus* sp.

Again, NR activity followed the trend of other cyanobacteria for being NH_4^+ repressible-depressible[30-33], and NO_3^- and NH_4^+ had visible inductive and repressive effects, respectively on the NiR activity of *Nostoc* ANTH. In contrast, these effects were negligible (<10%) in case of *Mastigocladus* sp. Even though this report on NiR activity in *Nostoc* ANTH is similar to other cyanobacteria[25], it is in complete contrast with regard to *Mastigocladus* sp. Such observations may be of importance, as *Mastigocladus* sp. under field conditions may be able to adapt in a much better way against the nitrate-based chemical fertilizers load in the fields.

Energy-dependent cellular accumulation of glutamine, as evident by inhibitory effects of DCMU or CCCP on glutamine uptake as well as the comparable effects on GS activity and

methylammonium uptake in the presence of fixed nitrogen sources in both the cyanobacteria, are consistent with the earlier reports on other cyanobacteria[24-27,34,35]. Thus, the comparative study of various aspects of nitrogen metabolism in *Nostoc* ANTH and *Mastigocladus* sp. showed that the two isolates from this region have similarities in the process apart from minor differences possibly due to their origin from different temperature and nutrient regimes.

Survival, nitrogen fixation and adaptability of various metabolic processes to different temperatures prompted further investigations into both the cyanobacteria for their ability to associate and also fix atmospheric N in the associated state with selected crop plants. As rice is the prime cash crop of the region and cyanobacteria are known to thrive well in the waterlogged rice fields, artificial association studies were carried out using these two cyanobacterial species with the rice seedlings. While *Nostoc* ANTH showed significant association with rice roots up to a temperature range of ~35°C and not beyond, *Mastigocladus* sp. showed association even at temperatures beyond ~35°C for prolonged period. The extent of nitrogen fixation was also almost one and half-fold higher in the associated cyanobacteria compared to the free-living cultures. This fact tempted us to presume that in future biofertilizer research, these two cyanobacterial isolates can be exploited as the potential biofertilizer candidates in varying temperature regimes.

Therefore, *Nostoc* ANTH with its high nitrogenase activity could be the effective biofertilizer for rice fields of temperate regions, while *Mastigocladus* sp. could be for tropical rice fields where day temperatures can go as high as 40-45°C. However, more studies are needed to ascertain the N-transfer potential of both these strains to rice plants as biofertilizer..

In the current biofertilizer programme, distribution of cyanobacterial inoculum is still a problem, as generally the inoculum comprises fresh cyanobacterium cells where most of these perish in the packing, storage and distribution. During the current study, one interesting observation was the ability and ease with which both these cyanobacteria differentiated high percentage of akinetes. As akienetes can withstand adverse environmental conditions, they can also probably adapt better than the fresh cyanobacterial inocula mainly owing to the stress during the packaging period. Therefore, akinetes can be viewed as the potential candidate to populate rice fields with N_2-fixing cyanobacteria. This could also tackle the low survival rate of inoculum in the rice fields. Also, once they are in the field conditions over a considerable period of time as akinetes, they probably would be better adapted to compete with the natural populations of other microbes in the fields. As reported earlier[36,37], *Nostoc* ANTH can fix nitrogen in associated state in NO_3-medium in dark and therefore, it can be used along with nitrate based chemical fertilizers in the fields. Currently, we are looking into various aspects of N_2-fixation in *Mastigocladus* sp. associated with rice roots in different N-media to ascertain the flexibility of N_2-fixing ability in the cyanobacterium and whether like *Nostoc* ANTH, *Mastigocladus* sp. can also be used as biofertilizer in rice fields in the presence of nitrate based chemical fertilizers. Further, we are looking into developing technology to induce quick sporulation in these cyanobacteria in an attempt to study the stability of the akinetes when stored over a longer period of time, their efficient germination into viable cyanobacterial cells and the retention of N_2-fixing ability of cultures. If the long-term storage is viable for akinetes, it will certainly open up the possibility of easy transportation and distribution of desired cyanobacterial inoculum as akinetes from the place of origin to the areas of application. Further, methods need to be developed to engineer direct delivery of effective strains endowed with enhanced N releasing capacity to the target crop plants for a fruitful N_2-fixing association.

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Engineering Conditions for Bulk Spore Production for Application as Biofertilizer Inoculum in Paddy Cultivation

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Abstract - With an attempt to alleviate the problems faced in the current biofertilizer programme, different alternatives are being looked into. In the present study, the potential use of spores as biofertilizer and their transfer to the fields on a biodegradable base (filter paper) has been evaluated.

Keywords- Biofertilizer, Cyanobacteria, Immobilization, Inoculum, Spores.

I. INTRODUCTION

Modern day agriculture faces the serious challenge of increasing the productivity to meet the food demands of the ever growing human population. As a result, current agricultural practice relies heavily on the use of agrochemicals to achieve higher crop yields. However, there is an increasing concern over the effects of such agrochemicals on the aquatic and terrestrial ecosystems. It is in this context, that a world wide scientific consensus is emerging for developing alternatives to chemical nitrogen fertilizers using biological systems that are cost effective, renewable and eco-friendly. The most obvious candidates in this regard are biological nitrogen fixers called diazotrophs, which play a vital role in maintaining soil fertility and sustaining crop yields even in the absence of any added nitrogenous fertilizers [1] [2] [3] [4]. Other potential benefits of cyanobacteria growing in crop fields are the production and release of inhibitors of plant pathogens and the amelioration of the negative effects of increased salt concentrations of soils, allowing saline and sodic soils to be brought into agricultural usage [5].

Cyanobacterial addition to paddy fields has been reported to increase stability and improve yields by 10-15% [6] [7] [5]. However, not all strains of cyanobacteria may be suited for all types of crop fields that vary in their geographical and climatic considerations. Hence, specific strains may be needed for particular crop fields to be used as biofertilizer depending upon these conditions [8]. Temperature also plays important role in deciding the optimum nitrogen fixing ability of a cyanobacterial strain. Most cyanobacteria have optimum N-fixing ability in a temperature range of 20°-30°C. Therefore, the mesophilic cyanobacteria may not be the ideal source of biofertilizer in tropical rice fields where day time temperatures can soar anywhere between 35-45°C as this adversely affects their metabolic processes [9]. In the current biofertilizer programme use of free-living cyanobacteria poses many problems including low survival rates of inoculum, adaptability of cyanobacteria inoculated in the fields with regards to competition with the preexisting natural population and incompatibility with chemical fertilizers and low nitrogen release. Therefore, in order to populate paddy fields with N-fixing cyanobacteria, alternatives to free-living cultures as inoculum need to be investigated. In this context, cyanobacterial spores could provide a definite solution since they are more adapted to withstand adverse conditions and are capable of immediate proliferation when conditions are favorable, provided protocol for quick and easy induction of sporulation in desired cyanobacteria is in place. This could also tackle the low survival rate of inoculum in the crop fields.

Keeping such factors into consideration, organisms have been isolated from different ecological niches with varied climatic conditions. These were then purified and compared with respect to their sporulation ability. Cyanobacterial strains which exhibited quick and profuse sporulation pattern were then immobilized and stored away on low grade filter paper. These were regenerated at regular intervals and assessed for their viability and regeneration potential as well as for retention of their ability to associate with rice roots, with an aim to enumerate their use as potential biofertilizer in rice fields of different temperature zones.

II. MATERIALS AND METHODS

A. Strains and Culture Conditions

Nostoc ANTH was isolated from the undersurface of the gametophytic thalli of *Anthoceros punctatus* collected from the North Eastern Hill University campus, Shillong and purified under axenic conditions. These were grown in batch cultures and maintained in BG-11$_0$ at 25°C with a photon fluence rate of 50 $\mu mol\ m^{-2}\ s^{-1}$ [10].

Mastigocladus sp was isolated from the hot spring of Jakrem, Meghalaya, India. This was also grown in batch cultures and maintained in D- medium at 45°C with a photon fluence rate of 50 μmol m^{-2} s^{-1} [10].

The other *Nostoc* isolates had been collected from soil samples of Uttar Pradesh and Uttarakhand. These were purified and maintained in batch cultures in BG-11_0 at 25°C with a photon fluence rate of 50 μmol m^{-2} s^{-1} [10].

Unless stated otherwise, 8 day old cultures were used in all the experiments.

B. Culture Condition for Sporulation

The cyanobacterial cultures were induced to sporulate in BG 11_0 media deficient in sulfur. However, the medium was supplemented with equimolar concentration of $MgCl_2$ to counter balance the reducing concentration of $MgSO_4$. These were then maintained in the culture room at 25±2°C [11] [12]. Further, some of these cultures were kept at lower temperature and in dark to assess the impact of these factors in inducing sporulation.

C. Chlorophyll Measurement

Chlorophyll *a* was measured according to MacKinney [13].

D. Heterocyst Frequency

Heterocyst frequency was calculated as percentage of total cells by light microscopy using an Olympus BX 60 light microscope.

E. Nitrogenase Assay

Nitrogenase enzyme (EC 1.18.6.1) activity was estimated *in vivo* by gas chromatography using the acetylene reduction assay [14]. 5 ml liquid culture was placed in a 15 ml serum vial and 1ml air was replaced by 1 ml of pure acetylene. These vials were incubated for 1 hour at 25±2°C with constant shaking. The ethylene produced in each vial was determined using a Tracor 540 GC with a Porapak T column and a flame ionization detector.

F. Co-cultivation of Rice and Cyanobacteria

10 days old rice seedlings were transferred to 15ml culture tubes after washing with distilled water. Suitable media was added into each tube and seedlings carefully placed with the roots dipped in the medium. Cyanobacterial cultures were then inoculated in these tubes. These were maintained under suitable growth conditions [9].

III. RESULTS

Cyanobacterial samples were collected from various locations within the country (Table 1). At the start of the experiment, sporulation was induced in the cultures by inoculating them in a sulfate deficient media. A 1000X diluted spore sample was then plated in a series of petri plates. Single spore colonies which developed in the plates were then inoculated in suitable liquid media and allowed to grow. All studies were performed on the 8th day after inoculation using a specific concentration of initial inoculum for both the free-living and regenerated spore sample(s). The immobilized spore samples were regenerated at regular intervals (1 month) and assessed for their viability and retention of associative ability.

N.B. * in the figures indicate the spores of the corresponding samples.

TABLE I
SAMPLES COLLECTED FROM DIFFERENT LOCATIONS (** INDICATES SAMPLES WHICH HAVE BEEN SELECTED FOR THE PRESENT STUDY)

Sl. No.	Samples	Strains	Collection sites
1	A**	*Nostoc* ANTH	Meghalaya
2	B	*Nostoc* sp	Meghalaya
3	C	*Nostoc* sp	Meghalaya
4	D	*Nostoc* sp	Meghalaya
5	E	*Nostoc* sp	Meghalaya
6	F	*Nostoc* sp	Meghalaya
7	G	*Nostoc* sp	Meghalaya
8	H	*Nostoc* sp	Meghalaya
9	I	*Nostoc* sp	Meghalaya
10	J	*Anabaena* sp	Meghalaya
11	K**	*Mastigocladus* sp	Meghalaya
12	L**	*Anabaena* sp	Assam
13	M	*Fischerella* sp	West Bengal
14	N	*Fischerella* sp	Manipur
15	O	*Nostoc* sp	Uttarakhand
16	P	*Nostoc* sp	Uttarakhand
17	Q**	*Nostoc* sp	Uttarakhand
18	R	*Nostoc* sp	Uttar Pradesh
19	S	*Nostoc* sp	Uttar Pradesh
20	T**	*Nostoc* sp	Uttar Pradesh

A. Sporulation

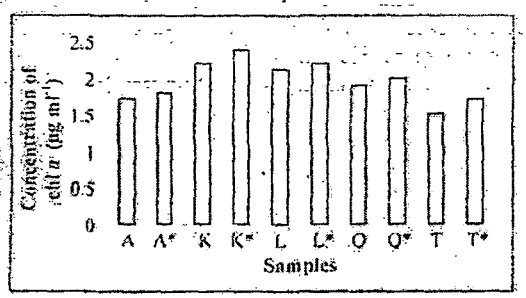
Sporulation in all the samples was confirmed by microscopic observations.

B. Growth

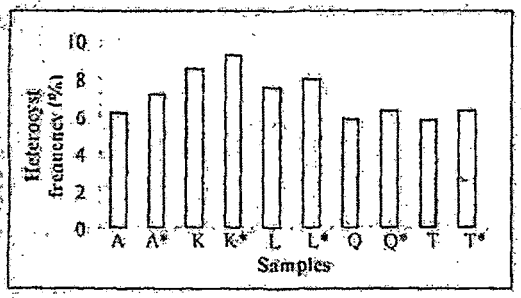
Figure 1 comparative growth of the free-living and regenerated spore samples. The growth rate was calculated in terms of increase in the chlorophyll *a* concentration and was found to be higher in the regenerated spore samples.

C. Heterocyst Frequency

Heterocyst frequency of the regenerated spore samples was also found to be higher then the free-living counterparts for all the samples studied (Figure 2).



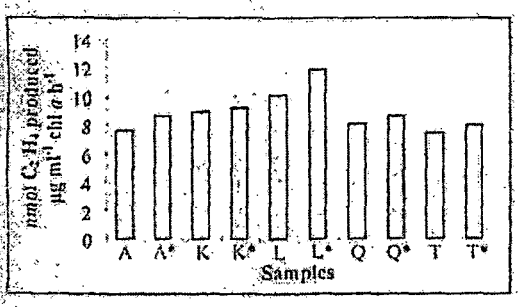
1: Comparison of growth of free-living samples and their regenerated spores.



ig 2: Comparison of heterocyst frequencies of free-living samples and their regenerated spores.

Nitrogenase Activity

Nitrogenase activities of all the free-living samples and their res exhibited a similar trend of increased activity in the spore ples (Figure 3).



ig 3: Comparison of nitrogenase activities of free-living samples and their regenerated spores.

rtificial Association of Cyanobacteria with Rice Plants

To test whether the cyanobacteria under study can be used as ofertilizer, the strains were incubated with rice seedling. All strains exhibited considerable association at room temperature (25±2°C) and their nitrogenase activity in the associated state was 2-3 folds higher than in case of free-living cultures. This result is similar to the earlier report on *Nostoc* ANTH [15]. Interestingly, *Mastigocladus* sp and two of the *Nostoc* species which had originally been isolated from higher temperature zones showed association with rice roots even at higher temperature. Regenerated spore sample(s) of the cyanobacterial strains also revealed associative ability with rice roots further supporting the idea that spores could be used as cyanobacterial inoculum to crop fields.

IV. Discussion

Current biofertilizer programme faces several challenges in the distribution of cyanobacterial inoculum owing to low survival rates of the inoculum during packing, transport and distribution of the same to crop fields. It is in this context that cyanobacterial spores promise to offer easier and effective alternative of viable inoculum to agricultural fields. Spores *per se* are adapted to withstand adverse environmental conditions and are capable of immediate proliferation on return of favorable conditions. Keeping this in mind, the present study was an attempt to explore the possibility of using cyanobacterial spores, as well as those immobilized on a biodegradable base (filter paper) as inoculum for possible N-fixing associations with rice plants in the water logged fields. It is worth mentioning here that this would be an economical as well as an eco-friendly approach, as filter paper being biodegradable would not accumulate in the rice fields and hence produce no ill effects on the overall soil quality. Also, it was observed during the course of study that immobilization of spore samples onto filter paper was a relatively easy and convenient method since it adsorbed on the strip of paper quickly and also dried up fast. This reduces the chances of contamination during drying the paper strips.

Induction of sporulation in a sulfate deficient media seemed effective for all the cyanobacterial samples studied. However, various other modifications were also experimented to evaluate their impact on induction of sporulation. These included maintaining the samples in temperatures other than their ideal growing temperature and also maintaining in dark conditions. These studies revealed that variation in temperature along with a nutrient deficiency did have a role in inducing faster sporulation, especially in those samples that had originally been isolated from a higher temperature zones. *Mastigocladus* sp growing ideally at 45°C and above exhibited immediate sporulation (within 3-4 days) when maintained at 25°C or below. This was true for two of the other *Nostoc* isolates which had also been collected from a higher temperature region. The sporulation efficiency of these samples was further enhanced when the samples were maintained

in sulfate deficient media, at a lower temperature (10-12 °C) and in dark.

When the spores of different cyanobacterial samples were studied with respect to their viability, regeneration, N-fixing and associative ability it was a consistent observation that they all exhibited profuse and immediate germination and subsequent growth on return of favorable growth conditions. This was an encouraging finding which supported our idea of the potential use of spores as inoculum. The growth, heterocyst frequency and nitrogenase activities of all spore samples studied revealed higher efficiencies as compared to their free-living counterparts. Thus, inoculation of spores to crop fields would not only ensure delivery of viable cyanobacteria and thus tackle the low survival problem offered by fresh inoculum; it would also be better adapted to compete with the preexisting micro flora of the fields.

From our experiments we observed that immobilization on filter paper did not have any adverse effects on the viability and regeneration of the spores up to at least 6-9 months. This provides a convenient and easy storage and transport possibility. An encouraging finding of this study was the assured retention of associative ability of the spore samples with rice roots. This also ensured proper anchorage of the samples to the rice roots and thus probably a better association when the spores eventually germinate and grow.

However, it needs to be mentioned that development of other effective protocols for induction of sporulation deserves further investigation. Currently we are looking into other alternative methods for inducing quick sporulation in cyanobacterial samples and also finding better immobilizing bases that would be easily available, biodegradable and appropriate for the purpose of storage, transport and delivery of biofertilizer.

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A Study on Cyanobacterial Compatibility with Nitrogen based Fertilizers and Their Transfer to Paddy Fields on a Biodegradable Material.

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Abstract-**Immobilization of potential cyano-bacterial biofertilizer strains on low cost filter paper has proved to be both economical and feasible. Since complete elimination of nitrogen based fertilizers from any cultivation is not possible, it becomes important to evaluate the compatibility of cyanobacteria to be used as biofertilizer with such chemical fertilizers. Present study indicates cyanobacterial compatibility with nitrate based fertilizers under laboratory conditions and survival of immobilized cyanobacteria up to 4-5 months on low cost filter paper.**

Keywords- Biofertilizer, Cyanobacteria, Immobilization, Inoculum, Nitrogen fixation

I. Introduction

Microorganisms are regarded to have immense potential to provide solutions to food, industrial, environment and health problems. Among these, cyanobacteria are microorganisms that have photosynthetic efficiencies comparable to those of higher plants and are the most prolific producers of biomass. Being autotrophs they are preferable to bacteria and fungi, for a variety of applications such as production of biomass and high value metabolites, clean up of oil spills and landfill pollutants, bioleaching and biofertilizers etc[1].

The existence of cyanobacteria in rice fields have been known for a long time and their abundance was first reported by Fritsch[2]. The potential use of cyanobacteria as biofertilizer particularly in rice fields have been looked into for many decades[3,4]. It has been estimated that cyanobacteria contribute 20-80 KgN/ha/crop on turnover of their biomass in the rice fields[5-8].Recent research on biofertilizer has also shown that rice fields populated with free living cyanobacteria have increased rice yield[9-13]. The popularity of cyanobacteria as potential biofertilizer lies in the fact that the cyanobacteria are photoautotrophic biological nitrogen fixers that naturally populate rice fields. However, distribution of cyanobacterial inoculum have serious drawback as fresh culture distributed as inoculum perish during packing and transportation. Hence, there is a need to develop proper form of inoculum that could withstand the stress presented during distribution and application period. Immobilization of cyanobacterial strains in porous and solid matrix has shown to be a convenient way of storing and transporting desirable cyanobacterial strains for various purposes including using them as inoculum in crop fields.

However, cyanobacteria alone cannot meet the total fixed nitrogen demand of high yielding modern varieties of crop and therefore complete elimination of nitrogen based fertilizers from any cultivation is not feasible. For the present study, cyanobacterial strains were isolated from rice fields, colloroid roots of cycas plants as well as from *Anthoceros punctatus* gametophytic thalli and were tested for their ability to colonize rice roots. Further, they were tested for their compatibility with different nitrogen based chemicals. The results are discussed in the following sections.

II. Materials And Methods

For the experimental purpose, different strains of cyanobacteria were isolated from rice fields, colloroid roots of cycas plants as well as from the *Anthoceros punctatus* gametophytic thalli. Batch cultures of *Nostoc* like strains were grown on $BG11_0$ at 25±2°C and at a photon fluence rate of 50 µmol $m^{-2}s^{-1}$ unless stated otherwise, 6 days old cultures were used in all the experiments[14].

A. Germination of Rice Seeds

Rice seeds (varieties RCPL-1-87-8, DR-92 & VL Dhan-81) were surface sterilized by washing with distilled water, followed by 1% (V/V) sodium hypochlorite solution for 5 minutes. These seeds were then thoroughly rinsed in sterile distilled water. Seed germination was carried out on autoclaved perlite in glass beakers. The perlite was irrigated with a 10-fold dilution of autoclaved $BG\text{-}11_0$ medium containing 2mM $NaNO_3$ (nitrate medium) which was buffered with equimolar concentration of HEPES. Germination was carried out in culture room under fluorescent light (photon fluence rate of 50 µmol m^{-1} s^{-1}) at 25°C, at saturating relative humidity.

B. Screening for Symbiotic Competence with Rice Plantlets

Rice seedlings grown for 10 days were uprooted from the perlite and washed with distilled water and resuspended in 50 ml capacity tubes containing 10 ml of autoclaved BG-11_0 medium containing 2mM $NaNO_3$ (+N) and BG-11_0 (-N) medium. The cyanobacteria used for inoculation to the media were grown for 6 days in batch cultures and harvested by centrifugation. The cyanobacterial inocula were added to a final concentration of 1 µg chlorophyll *a* (Chl *a* ml^{-1}). Co-cultivation was carried out for at 25°C with the plant roots exposed to light. After eight days of co-culturing, the rice seedlings were harvested and the roots were excised. These were washed for 1 minute in an ultrasonic bath to remove loosely associated cyanobacteria and then chlorophyll *a* in roots.

C. Associative Nitrogen Fixation Study

Nitrogenase activity was measured using acetylene reduction technique as described by Stewart *et al* [15] 10 days old rice seedlings were uprooted from the perlite and washed with distilled water and then suspended in 50 ml capacity tubes containing 30 ml of 10-fold diluted $BG11_0$ + 2mM $NaNO_3$ (Nitrate-media), $BG11_0$ + 2mM NH_4^+ (Ammonia-media), $BG11_0$ (-N) medium. The cyanobacteria used for inoculation to the media were grown for 6 days in batch cultures, and harvested by centrifugation. The cyanobacterial inocula were added to a final concentration of 1µg chlorophyll *a* (Chl *a*) ml^{-1}. Co-cultivation was carried out at 25°C with the plant roots exposed to light. After co-culture of eight days, the rice seedlings were harvested and the roots were excised. The roots were then washed for 1 minute in an ultrasonic bath to remove loosely associated cyanobacteria to measure nitrogenase activity.

D. Immobilization on Low Grade Filter

Low cost filter paper was cut into thin strips and cyanobacterial culture was immobilized on these strips. It was then dried in oven at ±20°C overnight and stored.

E. Growth Measurements

Growth was measured as increase in chlorophyll *a* content of the sample culture using the spectrophotometric method described by McKinney [16].

F. Heterocyst Frequency

Heterocyst frequency was calculated as percentage of total cells by using an Olympus BX 60 light microscope.

G. Nitrogenase Activity

Activity of nitrogenase enzyme (EC 1.18.6.1) was estimated *in vivo* by gas chromatography using the acetylene reduction assay. 5ml liquid culture was placed in a 15 ml serum vial and 1 ml air was replaced by 1 ml of pure acetylene. These vials were incubated for 1hr at 25±2°C with a Porapate 'T' column and a flame ionization detector [15].

H. Absorption Spectra of Phycobiliproteins

The phycobiliproteins were extracted in 0.05 M phosphate buffer saline pH 7.0 by sonication. The solution containing phycobiliproteins were placed in 1cm light path cuvette and absorption spectra of samples were determined in the wavelength 615 nm, 652 nm, and 562 nm for PC, APC, and PE respectively [17].

III. Results and Discussion

A. Isolation, Purification, Cultivation of Cyanobacteria and Their Colonization/ Association Study on Rice Roots

Ten different strains of cyanobacteria that were isolated and purified from rice fields, colloroid roots of cycas plants as well as from *Anthoceros punctatus* gametophytic thalli were studied for their colonization and association with rice roots. All ten strains show varying degree of colonization. The colonized roots were washed in ultrasonic bath for one minute and were microscopically observed for remains of any tightly associated cyanobacteria. Only four strains designated as NA, AC, RF1 and RF2 showed tight association with rice roots (Table I).

B .Associative Nitrogen Fixation Study

The four isolates that showed tight association was further studied in regard to their nitrogen fixing ability in associated state. This was done on 8th day and expressed as nmol C_2H_4 produced μg^{-1} Chl *a* h^{-1}.The four strains showed successful association in the presence of nitrate (2mM) and in the presence of ammonia (0.2mM) however, there is dissociation of the colonization in the presence of higher concentration of ammonia (2mM). Associative nitrogenase activity of the light exposed cyanobacteria in nitrate media was between 30 – 50% of the nitrogenase activity in nitrogen deficient media. The regenerated samples showed nearly the same results compared to the control samples (Fig. 1).

C. Growth Measurements

Growth was measured on the 8th day as increase in Chlorophyll *a* content of the four tightly associated isolates and was found to be highest for RF2 (control as well as regenerated sample) with an initial inoculum of 1µgml $^{-1}$ for all the samples (Table II).

D. Heterocyst Frequency

Heterocyst frequency ranged from 6.9 to 7% in the free-living samples. When immobilized samples were regenerated, prolific heterocyst differentiation was seen in all the regenerated cultures

with immobilized cells showing 100% recovery in heterocyst frequency by 8th day (Fig. 2). The recovery in heterocyst frequency was seen even after 5 months of immobilization on dried filter papers.

TABLE I
TEN DIFFERENT STRAINS OF CYANO- BACTERIA AND THEIR COLONIZATION POTENTIAL.

Sl. no	Samples	Strains	Colonization	Association
1	NA	*Nostoc* ANTH	+	+ -
2	AC	*Anabaena cycadea*	+	+ -
3	RF 1	*Nostoc*	+	+ -
4	RF 2	-Do-	+	+ -
5	RF 3	-Do-	+	
6	RF 4	-Do-	+	
7	RF 5	-Do-	+	
8	RF 6	-Do-	+	
9	RF 7	-Do-	+	
10	RF 8	-Do-	+	

TABLE. II
GROWTH EXPRESSED AS INCREASE IN CHL *A* CONTENT (µG/ML) OF CONTROL AND REGENERATED CULTURES.

Sl. no	Samples	Chl *a* (µg/ml) Control	Chl *a* (µg/ml) Regenerated
1.	NA	1.326	1.485
2.	AC	2.033	2.120
3.	RF 1	2.071	2.005
4.	RF 2	2.589	2.590

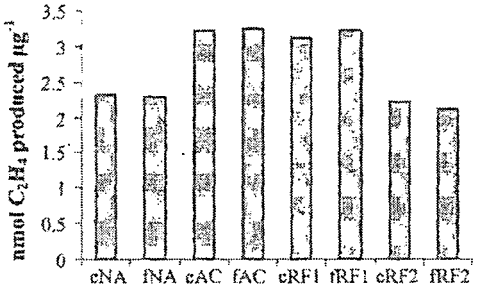

Fig. 1. Associative nitrogen fixation expressed as nmol C_2H_4 produced µg^{-1} chl *a* h^{-1}. c- control, f- filter paper, NA- *Nostoc* ANTH, AC- *Anabaena cycadeae*, RF-1 Rice field isolate 1, RF-2 Rice field isolate 2.

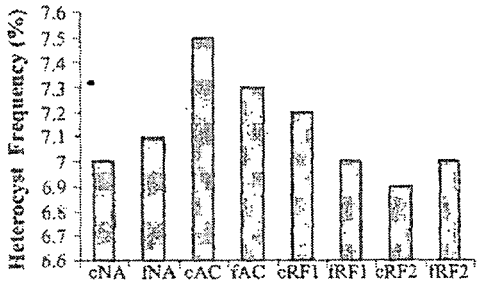

Fig. 2. Heterocyst frequency of free living control and regenerated samples from filter paper.

E .Nitrogenase Activity

Nitrogenase activity measured as nmoles C_2H_4 produced µgml^{-1}chl *a* h^{-1} of cultures regenerated from immobilized samples was roughly 70-85% of control cultures by 8th day. (Fig. 3)

F.Absorption spectra of phycobiliproteins

The phycobiliproteins are accessory pigments of photosynthesis. Phycocyanin, phycoerythrin as well as alophycocyanin were measured as an indicator of proper growth in the cyanobacteria taken for the study. When compared to phycobiliprotein content of free-living cultures, the immobilized samples were seen to retain the total phycobiliprotein content in the immobilized samples. The phycobiliproteins was expressed as mg ml^{-1} (Fig. 4).

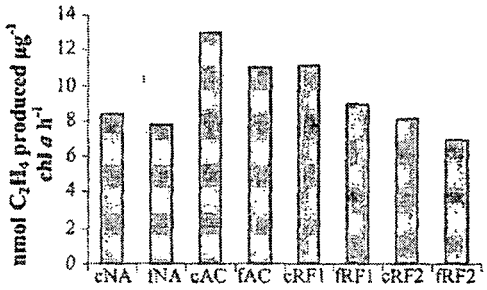

Fig. 3. Nitrogenase activity of free living control and regenerated samples from filter paper.

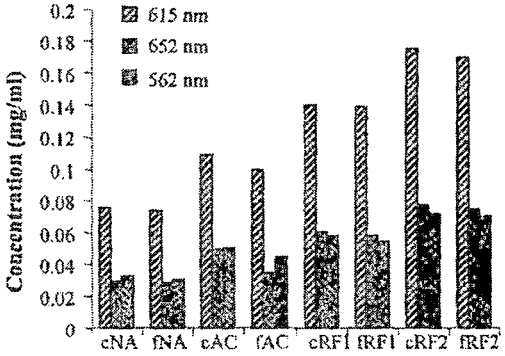

Fig. 4. Absorption spectra of phycobiliproteins of free living control and regenerated samples from filter paper.

IV. Discussion

Cyanobacteria thrive in water logged conditions that prevail in rice fields and their beneficial influence on soil quality and crop productivity has been known for decades. They are seen to colonize rice roots and submerged shoots. However, cyanobacterial nitrogen fixation is adversely affected by presence of any fixed nitrogen sources in the surrounding. But, to increase the rice productivity, cyanobacteria cannot be completely depended upon as they cannot meet the total nitrogen requirement of the whole crop and therefore, fixed nitrogen in the form of fertilizer has to be applied in the fields.

This study is an attempt to understand the cyanobacterial performance in the presence of nitrogen-based fertilizers in rice fields. The associative nitrogen fixation studies showed comparatively tight association between the four strains of cyanobacteria i.e. *Nostoc* ANTH (NA), *Anabaena cycadeae* (AC), rice field isolates 1 and 2 (RF1 and RF2) and the rice roots in the presence of nitrate (2mM). However, in presence of lower concentration of ammonia (0.2mM) colonization was seen but at higher concentration of ammonia there was dissociation and the cyanobacteria was seen to settle in the bottom of the tubes. The associative nitrogenase activity was found to be 30-50% of the nitrogenase activity in nitrogen deficient media.

Immobilizations on filter paper has proved to be both economical and desirable and being degradable in nature it is easily dispersed in the waterlogged conditions especially in rice fields. The comparative study between the free-living control samples with regenerated samples was shown to be similar with nearly 100% recovery of all characters. The heterocyst frequency, nitrogenase activity and phycobiliprotein content of the regenerated samples from preserved filter paper strips were comparable to the control cultures up to six months. The heterocyst frequency of RF1and RF2 was similar with RF2 having frequent occurrence of double or multiple heterocysts. This may account for lower nitrogenase activity of RF2 compared to RF1. This observation is similar to earlier report [18] where it was shown that restricted carbohydrate input to double or multiple heterocysts may be the cause of lower nitrogenase activity due to less availability of energy.

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