STUDIES ON PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF SPORULATION IN NITROGEN–FIXING CYANOBACTERIUM *NOSTOC* SPECIES

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STATEMENT

I, Omarlin Kyndiah, hereby declare that this thesis entitled " Studies on physiological and biochemical aspects of sporulation in N_2-fixing cyanobacterium *Nostoc* sp." is the record of work done by me, that the content of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and the thesis has not been submitted by me for any research degree in any other University/ Institute.

This is being submitted to the North Eastern Hill University for the degree of **Doctor of Philosophy in Biochemistry**.

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This thesis is dedicated to
my beloved late parents

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ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
C	Carbon
^0C	Degree centigrade
C_2H_2	Acetylene
C_2H_4	Ethylene
Chl	Chlorophyll
cm	Centimeter
d	Day (s)
h	Hour (s)
HEPES	4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid
HF	Heterocyst frequency
kDa	Kilodalton
M_r	Molecular weight
mg	Milligram
µg	Microgram
min	Minute (s)
ml	Millilitre
mM	Millimolar
µmol	Micromole
µM	Micromolar

nm	Nanometer
nmol	Nanomole
N_2ase	Nitrogenase
PCR	Polymerase chain reaction
PS	Photosystem
s	Second (s)
tRNA	Transfer ribonucleic acid
TCA	Trichloroacetic acid
Tris	2-amino-2-hydroxymethyl propane-1,3-diol
UV	Ultra violet
W	Watt
wt	Weight
v	Volume
%	Percent

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CHAPTER 1

INTRODUCTION

The nutritional needs of the growing world population depends mainly on the success of modern agriculture that in turn depends heavily on the availability of fixed nitrogen in the form of chemical fertilisers. The demand for nitrogen fertilisers has further increased with the introduction of high yielding varieties of crop plants. Modern farming depends on the petroleum based chemical inputs such as fertilisers, herbicides and pesticides. The cost of such chemical nitrogen fertilisers rise proportionately with the rise in the cost of fossil fuels. Thus, the provision of chemical nitrogen fertilisers is gradually becoming out of reach for developing countries. In addition, there is an increasing concern over their adverse effects on aquatic and terrestrial ecosystem by rendering them eutrophic. These realisations have led to research for developing alternative sources of nitrogen supply in agricultural farming. The most obvious candidates in this regard are biological nitrogen fixers called diazotrophs which play a vital role in maintaining soil fertility and sustaining the crop yield even in the absence of any added nitrogenous fertilisers (Venkataraman, 1981).

1.1. Cyanobacteria:

Cyanobacteria are an ancient and diverse group of gram-negative eubacteria characterised by their ability to perform higher-plant type oxygenic photosynthesis (Stanier and Cohen-Bazire, 1977). They occupy a wide range of habitats and colonise many terrestrial and aquatic habitats from polar to tropical areas (Bergman, 1996; Capone *et al.*, 1997; Carr and Whitton, 1982; Dodds *et al.*, 1995; Stal, 1995). Many cyanobacteria are also found in extreme environments which include arid deserts, frigid lakes or hot springs and salt marshes (Carr and Whitton, 1982; Dodds *et al.*, 1995; Potts, 1996). They form symbiotic associations with a wide range of organisms, ranging from protists, animals, plants and fungi (Adams, 2000; Bergman *et al.*, 1996; Rai, 1990; Rai *et al.*, 2000, 2002). Cyanobacterial species include unicellular, filamentous, branched filamentous and non-filamentous colonial forms. Among prokaryotes, cyanobacteria are the only representative of true multicellular organisms (Adams, 1992; Tandeau de Marsac and Houmard, 1993). The filamentous forms may produce four kinds of structurally and functionally different cells. They are the vegetative cells, heterocysts, hormogonia and akinetes.

1.2. Vegetative cells:

Photoautotrophy, fixing carbondioxide through the Calvin cycle and using light as energy source, is the dominant mode of growth in cyanobacteria. Vegetative cells are the sites for photosynthesis. The chlorophyll *a* protein complexes, the photosynthetic reaction center, the carotenoids and the electron transport system are all contained within the vegetative cells. The light harvesting accessory pigments (phycobiliproteins) are located in phycobilisomes attached to the surface of thylakoids, which under conditions of nitrogen-deficiency, also serve as nitrogen source (Bryant, 1994; Tandeau de Marsac and Houmard, 1993). The vegetative cells also contain a number of storage bodies such as cyanophycean starch (glycogen) as C reserve, carboxysomes that contain ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), cyanophycin (a polymer of aspartic acid and arginine) as N-reserve, and polyphosphate bodies as P-reserve.

1.3. Hormogonia:

The differentiation of hormogonia from vegetative cells occurs in filamentous cyanobacteria (both heterocystous and non-heterocystous). Hormogonia are distinguishable from vegetative cells by cell shape and in some species by cell motility and presence of gas vesicles. Hormogonium differentiation represents a transient morphological stage in the

developmental cell cycle of some filamentous heterocystous and non-heterocystous cyanobacteria. The differentiation of hormogonia from vegetative cells takes place upon transfer of stationary phase cultures to fresh medium. Various environmental factors, such as altered N-metabolism, change in temperature and light spectral quality affect hormogonia differentiation. In *Nostoc muscurom* (Armstrong *et al.*, 1983), and in *Calothrix* PCC 7601 and PCC 7504 (Herdman and Rippka, 1988) removal of $NaNO_3$ from the medium triggers the formation of hormogonia. In some *Calothrix* sp. strains, hormogonia are produced upon addition of iron to iron-deficient cultures (Douglas *et al.*, 1986). In various heterocystous strains grown under phosphorous limitation, hormogonium induction occurs upon transfer to phosphorous rich medium (Castenholz and Waterbury, 1989; Mahasneh *et al.*, 1990; Whitton, 1992; Wood *et al.*, 1986). The ability to form hormogonia is of much 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). It has been reported that in *Gunnera-Nostoc* Symbiosis, the acid mucilage secreted by the *Gunnera* induced the hormogonium differentiation, which is essential for infection (Johansson and Bergman, 1992; Liaimer *et al.*, 2001; Rasmussen *et al.*, 1994).

1.4. Heterocyst:

Heterocysts are microoxic cells for N_2-fixation in filamentous heterocystous cyanobacteria. The enzyme nitrogenase which converts molecular nitrogen into ammonia is localised inside the heterocyst. The differentiation of heterocyst from a vegetative cell is a nitrogen-regulated process in cyanobacteria. Presence of exogenous nitrogen sources such as nitrate, nitrite, ammonia and some amino acids repress heterocyst differentiation in all heterocyst forming cyanobacteria (Wolk *et al.*, 1994) whereas in the absence of such nitrogen sources 5-10% of the vegetative cells differentiate into heterocysts.

The nitrogenase enzyme complex consists of two different proteins: Mo-Fe Protein (dinitrogenase) and Fe-Protein (dinitrogenase reductase). The dinitrogenase is $\alpha_2\beta_2$ tetramer (M_r 226.8 kDa) and its α and β units are encoded by the *nif D* (Golden *et al.*, 1985; Lammers and Haselkorn, 1983) and *nif K* (Mazur and Chui, 1982) genes, respectively. It also contains two molecules of Mo-Fe cofactor. The dinitrogenase reductase (M_r 66 kDa) is a dimer of two identical subunits encoded by *nif H* gene. The chromosome region harboring *nif HDK* genes in *Anabaena* sp. PCC 7120, undergoes DNA rearrangement during differentiation of vegetative cells into heterocysts. In heterocyst *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 55 kb fragment located in *fdxN* gene (Golden *et al.*, 1987) has also been shown to occur during heterocyst differentiation. These rearrangement events involve site-specific excisases encoded by *xisA* (Lammers *et al.*, 1986) and *xisF* (Carrasco *et al.*, 1994), respectively.

The above mentioned nitrogenase is the conventional Mo-dependent nitrogenase (Nif 1) which functions exclusively in heterocysts under aerobic conditions (Elhai and Wolk, 1990; Thiel *et al.*, 1995). *Anabaena variabilis* ATCC 29413 also possesses another Mo-dependent nitrogenase (Nif 2) which functions in vegetative cells under anaerobic conditions (Thiel and Pratte, 2001). A vanadium-dependent nitrogenase encoded by *vnfDGK* genes and an Fe-only nitrogenase have also been reported in *Anabaena variabilis* (Kentemich *et al.*, 1991; Thiel, 1993). In N_2-fixing non-heterocystous cyanobacteria (Bergman *et al.*, 1997), there is a temporal separation of nitrogen fixation and photosynthesis (Bergman *et al.*, 1997; Gallon, 1992). Some of them, e.g. *Gloeothece* (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 cycle. *Plectonema boryanum* and *Phormidium* fix nitrogen under anaerobic or microaerobic conditions (Rai *et al.*, 1992; Stewart and Lex, 1970; Weisshar and Boger, 1983). *Symploca* PCC 8002 fixes nitrogen in the light under aerobic conditions (Fredriksson,

et al., 1998). In marine non-heterocystous filamentous cyanobacterium *Trichodesmium* sp., N_2-fixation takes place during the day when the photosystem II is fully operative.

The process of N_2-fixation is metabolically expensive requiring ATP, reductant and microaerobic conditions. Photophosphorylation, oxidative phosphorylation, substrate level phosphorylation and/or uptake hydrogenase are the main sources of ATP (Bottomley and Stewart, 1976). The oxidative pentose phosphate pathway is the main source of reductant for heterocyst.

The enzyme nitrogenase is extremely sensitive to oxygen (Fay, 1992), and both the dinitrogenase and dinitrogenase reductase are inactivated upon exposure to O_2. Several structural, biochemical and genetic changes take place during differentiation of a vegetative cell into heterocyst in order to maintain a microearobic interior. Such changes include synthesis of multilayered cell envelope which acts as a specific barrier for O_2, loss of PS II activity (Peterson *et al.*, 1981), presence of uptake hydrogenase and high rate of respiration (Wolk *et al.*, 1994).

Genes responsible for regulations of heterocyst formation have started to be characterised (Adams and Duggan, 1999; Wolk *et al.*, 1994; Wolk, 1996). In response to nitrogen-step-down, an autoreglatory gene *hetR*, is induced in regularly spaced cells within 2-3.5 h. The presence and

expression of *hetR* in non-heterocystous cyanobacterium *Symploca* PCC 8002 have also been reported (Janson *et al.*, 1998). The HetR 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.*, 1998 a, b). Transcription of *hetR* is indirectly controlled by the product of *ntcA* 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 NtcA (a cyclic AMP-binding protein) required for utilisation of nitrate and for heterocyst differentiation. NtcA from *Anabaena* PCC 7120 interacts with promoter regions of *xisA* (an excisases gene necessary for the formation of heterocysts), *gln*A (gene encoding glutamine synthetase), *rbcLS* (encoding Rubisco), *nifH* (encoding dinitrogenase reductase) and *ntcA* (encoding NtcA itself) (Frias *et al.*, 1994; Wei *et al.*, 1994). In addition NtcA also binds to the genes encoding glutathione reductase (*gor* gene). Thus, in addition to global response to N-deprivation (Frias *et al.*, 1994) *ntcA* also responds to redox status (Jiang *et al.*, 1997). Many genes essential for heterocyst differentiation and development have been identified and characterised on the basis of the various phenotypes exhibited by heterocyst formation defective mutants. Two more important genes for heterocyst development are *hepP* (Fernandez-Piñas *et al.*, 1994) and *het*C (Khudyakov and Wolk, 1997).

Insertional inactivation of *hetP* prevents the formation of proheterocysts and strains with multiple copies of *hetP* 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 *hetM* (also known as *hglB*) and *hglK* encodes a protein for transport of heterocyst glycolipids in *Anabaena* PCC 7120 (Bauer *et al.*, 1997; Black *et al.*, 1995). Three genes named *hepA*, *hepB* and *hepC*, are required for the synthesis and stabilisation of heterocyst envelop (Wolk, 1996). A Fox$^-$ mutant that is defective in heterocyst envelope and has impaired N_2-fixation ability under aerobic conditions has been isolated (Ernst *et al.*, 1992). Genes involved in heterocyst spacing has also been identified. *PatS* gene product (PatS-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).

1.5. Akinete:

Akinetes are formed by some members of Nostocaceae, Rivulariaceae and Stigonemataceae. They serve as a mean of perennation in these organisms (Adams and Carr, 1981; Nichols and Adams, 1982; Nichols and Carr, 1978; Wolk, 1965, 1973) and provide the capacity for growth by germinating under favourable conditions even after long-term exposure to extreme

environmental conditions (Livingstone and Jaworski, 1980; Sili *et al.*, 1994). Akinetes are suggested to be the evolutionary precursors of heterocysts (Wolk *et al.*, 1994). A variety of environmental factors trigger the differentiation of a vegetative cell into an akinete. Akinetes are larger than vegetative cells, with a thickened cell wall and a multilayered extracellular envelop (Herdman, 1987, 1988 Nichols and Adams, 1982) and their shape varies considerably (e.g., spherical in *Anabaena CA* but elongated in *A. cylindrica*). Akinetes do not resemble the bacterial endospore structurally, and are not heat resistant but are resistant to cold and desiccation. Akinetes germinate to produce new filaments after resumption of favourable growth conditions (Adams, 1992; Herdman, 1987, 1988; Nichols and Adams, 1982).

1.5.1. **Pattern formation**:

The akinetes of filamentous cyanobacteria develop at distinct positions in relation to heterocyst:

(a) akinetes occur adjacent to heterocyst (e.g., in *A. cylindrica, Cylindrospermum* and *Rivularia*).

(b) akinetes occur away from the heterocyst, near midpoint of interheterocysts interval (e.g., in *Anabaena CA* and *Nostoc* PCC 7524).

Akinetes have also been found in complete absence of heterocysts even in the organisms which form akinete adjacent to or

between the heterocyst. Therefore, heterocysts seem to impose regularity on the pattern of akinete development but their presence is not essential for formation of akinetes. A number of amino acid analogues (e.g., arginine analogues canavanine and cyanoalanine and the serine analogue α-aminobutyric acid) increase akinete frequency as well as alter akinete pattern in *Anabaena cylindrica.* The amino acid analogues 7-azatryptophan and canavanine have been shown to alter akinete pattern in *Nostoc* PCC 7524 (Sutherland *et al.*, 1979).

1.5.2. Structural and genetic similarities between Heterocysts and Akinetes:

Akinetes and heterocysts are generally larger than vegetative cells, with a thickened cell wall, and a multilayared extracellular envelope. A lot of structural similarities exist between heterocysts and akinetes although both are known to be functionally different from each other. In *Anabaena* species, the envelopes of heterocyst and akinete contain equivalent polysaccharides (Cardemil and Wolk, 1976, 1979, 1981). Soriente *et al.* (1993) reported the presence of glycolipids envelope in akinetes. Akinete formation has been little studied genetically and most studies on the genes involved in akinete development are obtained from the work on heterocyst formation. The formation of both akinetes and heterocysts in *Anabaena variabilis* ATCC

29413 requires a common gene *hepA*, which encodes envelop polysaccharides (Leganés, 1994). Presence of functional *hetR* has also been shown to be essential for both akinete as well as heterocyst formation (Leganés *et al.*, 1994; Wolk *et al.*, 1994). In *Nostoc ellipsosporum*, mutation in *hetR* blocks the differentiation of both heterocysts and akinetes at an early stage. Studies on the relationship between the differentiation of heterocysts and akinetes have been carried out in a genetically manipulated cyanobacterium, *Nostoc ellipsosporum* (Leganés *et al.*, 1994). Recently, a marker gene that is found to be expressed primarily in akinetes have been identified (Zhou and Wolk, 2002).

1.5.3. Factors controlling akinete formation in cyanobacteria:

A variety of environmental factors have been reported to frequently trigger the akinete formation in filamentous cyanobacteria. The environmental factors which have been implicated as trigger for akinete formation includes limitation for nitrogen, carbon, iron, trace elements, light and phosphate (Wolk, 1965).

1.5.3.1. **Light limitation:** Light limitation and spectral quality strongly influence cyanobacterial growth and differentiation. Direct correlations have been observed between the light intensity at which *A. cylindrica* is

grown and the cell density at which akinete formation is initiated (Nichols *et al.*, 1980). Sutherland *et al.* (1979) demonstrated that addition of utilisable carbon sources such as sucrose to the exponentially growing culture of *Nostoc* PCC 7524 (facultative photoheterotrophy) delay akinete formation implying a direct role for light availability in control of akinete formation. Fay *et al.* (1984) also advocated that light limitation was the most important factor triggering akinete differentiation in *Anabaena circinalis* (KÜtz Hansgirg). However, van Dok and Hart (1996) ruled out the possibility of light availability alone triggering akinete differentiation in *Anabaena circinalis.*

1.5.3.2. **Nutrient Limitation**: Among the major nutrients used by cyanobacterial strains for growth and multiplication, phospahte limitation has been implicated as a major trigger for akinete formation (Herdman, 1987, 1988; Nichols and Adams, 1982). In *A. variabilis, Nostoc linckia* (Reddy, 1983), *A. cylindrica* (Wolk, 1965), *Fischerella muscicola* (Kaushik *et al.*, 1971) and *A.circinalis* (Van dok and Hart, 1996) phosphate limitation has been reported to be the major trigger. However, in contrast to such reports, Fay *et al* (1984) demonstrated that presence of phosphate stimulated akinete differentiation in *A. circinalis.*

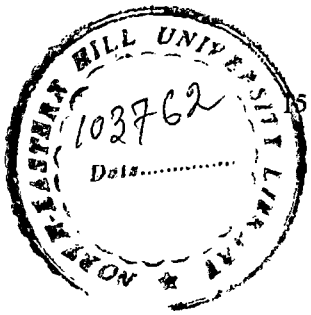
Limitation of fixed-nitrogen such as nitrate, nitrite and ammonium has been reported to trigger akinete differentiation (Dementer,

1956; Harder, 1917). Singh and Srivastava (1968), and Tyagi (1974) has reported that presence of combined nitrogen sources inhibit akinete differentiation. Conversely, Sutherland *et al* (1979) and Van dok and Hart (1996) have independently reported that N-availability had no effect on akinete differentiation of *Nostoc* PCC 7524 and *Anabaena circinalis,* respectively.

Various other compounds have been reported for their impact on akinete differentiation. Sucrose (Sutherland *et al.*, 1979), glucose (Tyagi, 1974, 1978), Sodium glutamate (Dementer, 1956), NaCl (Cannabaeus, 1929) and high concentration of sulphate have been reported to induce akinete differentiation. Wolk (1965) has reported that acetate and calcium glucuronate increased akinete differentiation in *A. cylindrica.* In this organism, Sinclair and Whitton (1977) have shown that deficiency of iron increased akinete frequency, whereas deficiencies of trace ions showed the opposite effect (Mg^{2+} and Ca^{2+}) or no effect (Mo and SO_4^{2-}). Conversely, in *Anabaena circinalis* limitation of Fe^{3+} did not cause akinete differentiation (Van Dok and Hart, 1996). In *C. licheniforme* amino acids tryptophan, aspartic acid, phenylalanine, proline and isoleucine have been reported to increase akinete differentiation whereas cyclic nucleotides had little or no effect (Hirosawa and Wolk, 1979a,b).

1.5.3.3. **Metabolic changes during akinete differentiation**: There are conflicting reports on the metabolic activities of akinetes. This may be due to the difficulty in obtaining pure akinetes or asynchronous akinete differentiation. However, studies with *Nostoc* PCC 7524 (Sutherland *et al.*, 1979) and *Anabaena variabilis* (Braune, 1980) have shown that akinete differentiation can be synchronised.

The most striking changes in cellular composition during akinete differentiation are increase in glycogen, cyanophycin, carbon content and dry weight (Fay, 1969; Simon, 1977a, b). In *Anabaena cylindrica* the mature akinetes have little or no chlorophyll and lack photosystem I. In contrast, Sutherland *et al* (1979) reported that the chlorophyll *a* content of *Nostoc* PCC 7524 was higher than that of the vegetative cells, while the phycocyanin content was unchanged. In *Anabaena doliolum*, the metabolic changes associated with akinete differentiation include reduction in respiratory activity and loss of nitrogenase, nitrate reductase, glutamine synthetase, aspartate dehydrogenase and photosynthetic activity along with photosynthetic pigments (Rao *et al.*, 1984). The finding that the respiratory activity is reduced in akinetes of *A. doliolum* is in uniformity with *Nostoc* PCC 7524 (Chauvat, 1982) and *Nostoc spongiaeforme* (Thiel and Wolk, 1983). Singh and Kashyap (1988) reported that the metabolic activities of the akinetes of *Fischerella muscicola* are similar to those of



Anabaena doliolum and unlike those of *Nostoc* PCC 7524 (Sutherland *et al.*, 1979). However, inactivation of glutamate synthase, partial inactivation of nitrogenase, and unaltered glutamine synthetase activity have been reported in *Clostridium pasteurianum* akinetes (Vallespinos and Kleiner, 1980). The decreased activity of superoxide dismutase in akinetes and heterocysts has been reported in *Anabaena cylindrica* (Grilli Caiola *et al.*, 1991).

1.5.3.4. **Akinete germination:** In response to favourable growth conditions, akinetes germinate and produce vegetative cells. Increased light intensity, phosphorous and nitrogen availability have been implicated as major triggers for akinete germination (Herdman, 1987, 1988; van Dok and Hart, 1997). However, in *Nodularia spumigena* akinete germination has been reported to occur at low light intensity (Huber, 1985). The energy demands of akinete differentiation are initially met from aerobic oxidation of carbon reserve (Rai *et al.*, 1988). However, the action of both photosystem I and II are required for efficient and rapid germination (Herdman, 1987, 1988). *Nostoc* PCC 7524 akinetes do not germinate in dark, although they show respiratory O_2 uptake (Chauvat *et al.*, 1982). During germination, all the metabolic activities related to C and N metabolism reappear sequentially

allowing the cells to continue their normal growth (Rai *et al.*, 1988; Sutherland *et al.*, 1985a).

1.6. Spores (akinetes) in biofertilisers technology:

The abundance of cyanobacteria in rice fields was first reported by Fritsch (1907a, b) and their importance in nitrogen economy of rice fields has been well documented (De, 1939; Singh, 1961; Watanabe *et al.*, 1951). It has been estimated that cyanobacteria contribute 20-80 Kg N ha^{-1} $crop^{-1}$ on turnover of their biomass in the rice fields (Albrecht *et al.*, 1991; Ladha and Reddy, 1995; Roger and Ladha, 1992; Venkataraman, 1981). The use of cyanobacteria (algalization) in rice fields has been shown to increase the rice yield (Metting, 1988; Roger and Kulasooriya, 1980; Venkataraman, 1972; Watanabe *et al.*, 1951). However the potential benefits of cyanobacteria as biofertilisers in rice fields has been limited due to low viability of inocula and their susceptibility towards adverse environmental stresses. The strategy frequently employed by cyanobacteria to face such environmental stresses is to form structurally and metabolically distinct cells called spores (akinetes), which can germinate to produce new filaments when growth conditions are favourable. If the inocula can be provided to the farmers in form of spores, this would be easier to handle and can withstand adverse environmental conditions.

1.7. Present Study:

When applied as biofertiliser in rice fields free-living cyanobacteria release nitrogen into the soil after death and decay. Thus the nitrogen released is not directly transferred to the crop plants. Instead, all soil organisms including the crop plants share this nitrogen. Infact some of it is even lost by denitrification. The benefits of cyanobacterial biofertilisers can be drastically enhanced if there is direct and continuous N-transfer from cyanobacteria to crop plants. Direct and continuous transfer of fixed nitrogen to rice plants could be achieved by creation of artificial associations involving symbiotically competent N_2-fixing cyanobacterial strains and rice plants (Nilsson *et al.*, 2002; Rai *et al.*, 2000; Whitton, 2000). The potential of cyanobacteria as biofertilisers in rice field has also been limited due to the use of herbicides and chemical nitrogen fertilisers that limit the growth and N_2-fixing capacity of cyanobacteria, respectively (Whitton, 2000) and lack of high viability inocula.

The overall aim of the present study is to understand the processes of akinete formation and germination, identify factors which can trigger profuse akinete formation, study the viability of these akinetes (in term of longevity and efficiency of germination), and finally select a simple but efficient process by which inocula can be prepared in the form of akinetes with high viability and efficient germination. A *Nostoc* sp. strain was

isolated locally from soil samples. This strain was identified, by fingerprinting and tRNA ^{Leu} intron analysis, as *Nostoc* ANTH. This strain was chosen for the present study because it has been shown to be symbiotically competent, it has been screened for resistance to the herbicide paraquat, it colonises roots of rice plants and carries out associative nitrogen fixation, and its chlorate-resistant strain fixes N_2 in presence of nitrate (Bhattacharya, 2002; Bhattacharya *et al.*, 2002b; Nilsson *et al.*, 2002).

CHAPTER 2

MATERIALS AND METHODS

2.1. Isolation and purification of *Nostoc* sp:

Soil samples were collected during the month of September from North Eastern Hill University campus, Shillong and brought to the laboratory. The samples were examined under phase contrast microscope. *Nostoc* colonies were identified, washed thoroughly with doubled distilled water and then homogenised using glass beads. The homogenised samples were then plated on sterilised nitrogen free BG-11 medium ($BG\text{-}11_0$; Rippka *et al.*, 1979) with 1.5 % agar. The plates were incubated at 25^0 C under light (photon fluence rate 50 μmol photons.$m^{-2}.s^{-1}$). When colonies appeared, these were picked up and viewed under the microscope before subsequent re-plating on $BG\text{-}11_0$ medium. The process was repeated several times until well separated colonies were obtained. These colonies were then purified by plating on solidified $BG\text{-}11_0$ medium containing Polymixin-B sulphate (10 μg.ml^{-1}) and Cycloheximide (100 μg.ml^{-1}). The individual *Nostoc* colonies were picked up under aseptic conditions and transferred to liquid sterilised $BG\text{-}11_0$ medium in test tubes. The procedures were repeated till axenic cultures of *Nostoc* sp. were obtained. Stocks were maintained on solid $BG\text{-}11_0$ medium in test tubes.

2.2. Identification of the isolated *Nostoc* strain:

$tRNA^{Leu}$ (UAA) intron sequence analysis (done in the laboratory of Prof. P. Lindblad, Uppsala University, Sweden) and PCR fingerprints using STRR-1A primer (done in the laboratory of Prof. B. Bergman, Stockholm University, Sweden) were obtained to determine the identity of this *Nostoc* strain. The results (Figure 2.1 and 2.2) show that this *Nostoc* strain is virtually identical to *Nostoc* ANTH isolated by us earlier from *Anthoceros* (Prakasham and Rai, 1991). Both strains showed identical fingerprints and $tRNA^{Leu}$ (UAA) intron sequences of both strains were similar except for a difference of just two bases (at positions 111 and 139). For full details protocols for STRR-1A PCR and $tRNA^{Leu}$ (UAA) intron sequence analysis, see Rasmussen and Svenning (1998) and Paulsrud and Lindblad (1998), respectively.

2.3. Culture medium:

The cyanobacterium *Nostoc* ANTH was grown from axenic stock cultures in N_2-medium (BG-11_0 medium; Rippka *et al.*, 1979). The concentrations of macronutrients in N_2-medium were (mM): $K_2HPO_4.3H_2O$, 0.18; Na_2CO_3, 0.19; $MgSO_4.7H_2O$, 0.30; $CaCl_2.2H_20$, 0.25; EDTA (disodium salt), 0.003; Citric acid, 0.029; Ferric ammonium citrate, 0.030. The concentrations of micronutrients in N_2-medium were (μM): H_3BO_3, 46; $MnCl_2.4H_2O$, 9.2; $ZnSO_4.7H_2O$, 0.77; $Na_2MoO_4.2H_2O$, 1.6; $CuSO_4.5H_2O$, 0.32; $Co(NO_3)_2.6H_2O$, 0.17. As and when

required, the N_2-medium was supplemented with combined nitrogen sources such as potassium nitrate (nitrate-medium) or ammonium chloride (ammonium-medium). The medium was always buffered with equimolar concentration of HEPES. The pH of the medium was adjusted to 7.5 before autoclaving.

2.4. Growth conditions:

Cultures of *Nostoc* ANTH were routinely grown in a culture room maintained at 25^0 C with a light intensity (photon fluence rate) of 50 μmol photons.$m^{-2}.s^{-1}$.

2.5. Culture conditions for akinete differentiation:

2.5.1. By Sulphur limitation:

Nostoc ANTH was grown and allowed to sporulate in $BG\text{-}11_0$ medium minus $MgSO_4$. The medium was supplemented with equimolar concentration of $MgCl_2$ to counter the effect of reducing the concentration of $MgSO_4$ so that the combined cation and anion concentrations remained the same in all cultures. As and when required, the medium was supplemented with 5 mM potassium nitrate or 2 mM ammonium chloride and buffered with equimolar concentration of HEPES.

2.5.2. By Phosphorous limitation:

Nostoc ANTH was grown and allowed to sporulate in BG-11_0 medium minus K_2HPO_4. The medium was supplemented with equimolar concentration of K_2SO_4 to counter the effect of reducing the concentration of K_2HPO_4 so that the combined cation and anion concentration remained the same in all cultures. As and when required, the medium was supplemented with 5 mM potassium nitrate or 2 mM ammonium chloride and buffered with equimolar concentration of HEPES.

2.5.3. By addition of various carbon sources:

Nostoc ANTH was grown in BG-11_0 medium supplemented with glucose, sucrose, or fructose. Four different concentrations (10, 20, 30 and 50 mM) of each sugar were used. During growth, the appearance of any akinetes was closely monitored.

2.6. Culture condition for akinete germination:

Akinete population was washed twice and resuspended in fresh BG-11_0 medium at a concentration of 2×10^6 akinetes ml^{-1} and incubated in the presence of light at 50 µmol photons.$m^{-2}.s^{-1}$. Whenever necessary KNO_3 (5 mM) and NH_4Cl (2 mM) were added as sources of combined nitrogen.

2.7. Akinete and heterocyst frequency:

Heterocyst and akinetes frequency was calculated as percentage of total cell populations by light microscopic observations.

2.8. Estimation of akinete viability:

The percentage of germinating akinetes was determined by examination of at least 1000 akinetes under the light microscope. Akinetes that did not lead to emergence of germling and remained in the single cell stage were considered to be inviable.

2.9. Growth measurements:

Growth was measured as increase in concentrations of chlorophyll *a*.

2.10. Chlorophyll *a* determination and culture density measurement:

Chlorophyll *a* was extracted into 90 % methanol in darkness at 4^0 C. The absorbance at 663 nm was measured using a Beckman DU-530 Spectrophotometer and chlorophyll *a* concentration calculated according to Mackinney (1941). The culture density was measured by measuring absorbance of the culture at 650 nm.

2.11. Light micrography:

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

2.12. C:N Ratio:

Carbon to Nitrogen ratio of samples was determined using Vario III CHNOS analyser fitted with autosampler (Elementar Analysensysteme, GmbH, Germany).

2.13. Protein Estimation:

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

2.13.1. Extraction of protein:

5 ml of cyanobacterial culture was centrifuged and the pellet was resuspended in 1 ml of distilled water. The cells were disrupted by ultrasonication using a Soniprep 150 (MSE) fitted with an utrasonic microprobe. The supernatant was collected after centrifugation at 3000 g for 5 min and used for protein determination.

2.13.2. **Estimation of protein:**

Reagents:

A. 2 % $Na_2 CO_3$ in 0.1 N NaOH.

B. 1 % sodium potassium tartarate solution.

C. 0.5 % $Cu SO_4$ solution.

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

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

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

Procedure:

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

2.14. Oxygen exchange:

Oxygen evolution and consumption was measured polarographically by using a Clark-type oxygen electrode installed in a 3 ml Plexiglass container with magnetic stirrer (Rank Brothers, England). Three ml cyanobacterial culture was added to the sample chamber of the non-polarised electrode and allowed to equilibrate for 5 min while stirring. The electrode was then polarised and the linear rate of oxygen evolution was obtained in light supplied by a 100 W tungsten filament bulb, which was shielded from the sample by a water bath acting as heat filter. The light intensity at the surface of the sample chamber was 50 μmol photons.m^{-2} .s^{-1}. Oxygen consumption was measured in dark with the chamber wrapped in aluminium foil. The rate of oxygen evolution and consumption were expressed as nmol O_2 evolved/consumed.min^{-1}.mg $^{-1}$ protein.

2.15. Absorption spectra of photosynthetic pigments:

Chlorophylls and carotenoids were extracted in methanol. The phycobiliproteins were extracted in 0.05 M phosphate buffer, pH 7 by sonication. The solutions containing chlorophylls and carotenoids or phycobiliproteins were placed in 1 cm light path cuvettes and absorption spectra of the samples were determined in the wavelength range 400 nm-700 nm using Beckman DU-530 UV/Visible spectrophotometer.

2.16. Enzyme assays:

2.16.1. Nitrogenase:

Nitrogenase activity was measured as ethylene production using acetylene reduction assay (Stewart *et al.*, 1967). 8 ml of cyanobacterial culture was placed in a 18 ml stoppered serum vial. Acetylene gas was injected to a final concentration of 10 % (v/v) of the air phase in the vials. The vials were incubated in light (photon fluence rate of 50 µmol photons.m^{-2}.s^{-1}) at 25 °C on a magnetic shaker. After 1 hour, 1 ml gas sample 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 Porapak T of mesh size (80/100) and a flame ionization detector.

2.16.2. Glutamine synthetase:

2.16.2.1. **Extraction of enzyme:** Cultures were harvested by centrifugation, washed twice in 50 mM Tris-HCl buffer (pH 7.5) and resuspended in the same buffer. The cells were treated for 10 min with alkyltrimethylammonium bromide (CTAB) at a final concentration of 100 µg.ml^{-1} (Frias *et al.*, 1994). Glutamine synthetase transferase activity was then assayed *in situ* using CTAB permeabilised cells.

2.16.2.2. **Glutamine synthetase transferase assay:** This was essentially as described by Sampio *et al* (1979) except that CTAB permeabilised cells were used. The reaction mixture contained in a final volume of 3 ml, 1 ml enzyme extract (CTAB-permeabilised cell suspension), 40 µmol Tris-HCl buffer pH 7, 3 µmol $MnCl_2$, 20 µmol Potassium arsenate, 0.4 µmol ADP (Na^+salt), 60 µmol hydroxylamine and 30 µmol glutamine. The reaction mixture was incubated in the dark for 10 min at 30 °C. The reaction was terminated by the addition of 2 ml of stop mixture (4 ml of $FeCl_3$, 1 ml of 24 % TCA, 0.5 ml of 6 N HCl and 6.5 ml of water). The absorbance of the supernatant was read at 540 nm after 10 min of centrifugation at 2000 rpm. The concentration of γ-glutamyl hydroxamate formed was estimated from a standard curve that was prepared in the range of 0-0.2 µmol γ-glutamyl hydroxamate. ml^{-1}.

2.16.3. **Nitrate reductase:**

Nitrate reductase (NR) activity was measured *in situ* (Manzano *et al.*, 1976) using CTAB permeabilised 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 (pH 7.5), 0.1 M NaCl, 0.3 M sucrose, 1 mM KNO_3, 1 mM EDTA and 5 mM $MgCl_2$). CTAB was added at a final concentration of 100 $\mu g.ml^{-1}$ and the suspension incubated for 10 min at room temperature with vigorous shaking. The reaction mixture contained, in

addition to permeabilised cells, in a final volume of 1 ml: 20 mM KNO_3, 100 mM glycine-KOH (pH 10.5), 4 mM methyl viologen, and 10 mM sodium dithionite freshly dissolved in 0.1 ml of 0.23 M $NaHCO_3$. After 7 min of incubation in dark at 30^0 C, the reaction was terminated by adding 0.2 ml of 1 M zinc acetate. Subsequently, the nitrite formed was determined by the method of Snell and Snell (1949).

2.16.3.1. **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 was prepared in the range of 10-100 $nmol.ml^{-1}$. This was used as standard.

Procedures:

To 1 ml of sample, 1 ml of sulphanilamide and 1 ml of 1-Napthyl ethylene diamine dihydrochloride was added. The solution was mixed thoroughly and the absorbance was read at 540 nm after 15 min. A calibration curve was

prepared by using potassium nitrite solution as standard for estimation of nitrite.

2.17. SDS-PAGE of proteins of vegetative cells and akinetes:

Cyanobacterial cells and akinetes were harvested by centrifugation (3000g) for 5 minute. The pellet was then resuspended in sodium dodecyl sulphate (SDS) sample buffer (1:1, v/v). The sample buffer consisted of 10 mM Tris-HCl (pH 8.8), 1 mM Ethylene diamine tetra acetic acid (EDTA), 2.5 % (w/v) SDS, 5 % β-mercaptoethanol, and 0.01 % (w/v) bromophenol blue. These samples were boiled for 5 min and then centrifuged at 15,000g for 5 min. The supernatants were subjected to standard SDS-PAGE (10 % polyacrylamide gel; 100 mA current). The electrophoresis was stopped when the tracking dye (Bromophenol blue) reached near the bottom edge of the gel. The proteins were stained with Coomassie brilliant blue R-250 overnight and then destained.

2.18. Chemicals, Glasswares and Gases:

All gases used were highest purity grade from Indian Oxygen Company Ltd. All glasswares used were Borosil make. All biochemicals were purchased from Sigma Chemicals Company, USA. General chemicals and solvent were

from Qualigen or Glaxo. Electrophoresis requirements were procured from Bio Rad and Alcohol from Bengal Chemicals, Kolkatta.

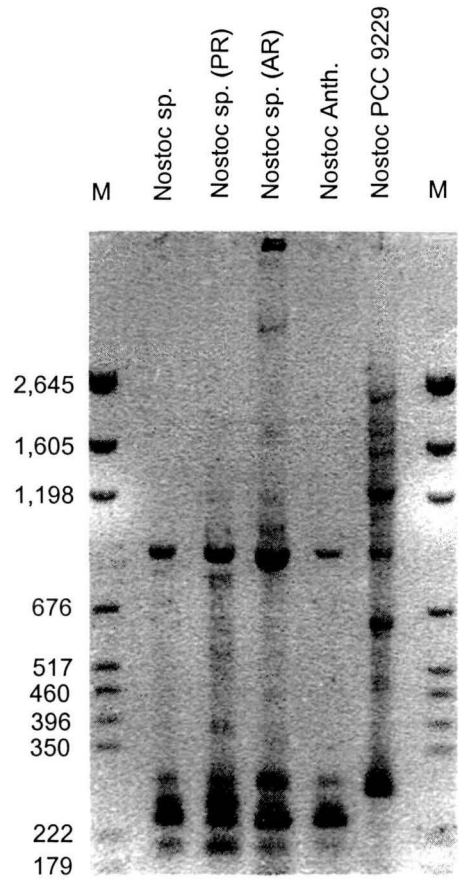


Figure 2.1: STRR 1A-PCR-based DNA fingerprints of *Nostoc* sp., *Nostoc* sp. (PR: Paraquat-resistant mutant), *Nostoc* sp. (AR: Azetidine-2-carboxylate-resistant mutant), *Nostoc* ANTH and *Nostoc* PCC 9229.

These fingerprints were obtained using short tandemly repeated repetitive sequences (STRR 1A) as primer and whole filaments of *Nostoc* as templates. The STRR 1A primer used here had the following sequence: 3′-CCCCTRACCCCTRACC-5′. Note that the fingerprint of *Nostoc* sp. and its mutant are similar to that of *Nostoc* ANTH and quite distinct from *Nostoc* PCC 9229 (included here as control).

Lane M represents DNA molecular weight standard (bp).

Sample				
Nostoc ANTH	AAATAATTGA	GCCTTAAAGA	AGAAATTCTT	30
Nostoc sp.	AAATAATTGA	GCCTTAAAGA	AGAAATTCTT	30
Nostoc ANTH	TAAGTGGATG	CTCTCAAACT	CAGGGAAACC	60
Nostoc sp.	TAAGTGGATG	CTCTCAAACT	CAGGGAAACC	60
Nostoc ANTH	TAAATCTGTT	CGCAGACATG	GCAATCCTGA	90
Nostoc sp.	TAAATCTGTT	CGCAGACATG	GCAATCCTGA	90
Nostoc ANTH	GCCAAGCCCA	AGATAATTGG	AAAGGTGCAG	120
Nostoc sp.	GCCAAGCCCA	AGATAATTGG	GAAGGTGCAG	120
Nostoc ANTH	AGACTCGACG	GGAGCTACCC	TAACGTCAAG	150
Nostoc sp.	AGACTCGACG	GGAGCTACTC	TAACGTCAAG	150
Nostoc ANTH	ACGAGGGTAA	AGAGAGAGTC	CAATTCTCAA	180
Nostoc sp.	ACGAGGGTAA	AGAGAGAGTC	CAATTCTCAA	180
Nostoc ANTH	AGCCATTAGG	CAGTAGCGAA	AGCTGCGGGA	210
Nostoc sp.	AGCCATTAGG	CAGTAGCGAA	AGCTGCGGGA	210
Nostoc ANTH	GAATG			215
Nostoc sp.	GAATG			215

Figure 2.2: Comparison of nucleotide sequences of tRNA ^{Leu} (UAA) intron from cyanobacterium *Nostoc* ANTH and *Nostoc* sp.

The bold letters (at positions 111 and 139) indicate the difference of bases between *Nostoc* ANTH and *Nostoc* sp.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Akinete Differentiation

3.1.1. Factors affecting akinete differentiation:

In the past, several authors have reported that a number of factors trigger or enhance akinete formation in cyanobacteria. These include light limitation, phosphate limitation, iron limitation, limiting fixed nitrogen, providing amino acids or increasing concentrations of NaCl in the growth medium (see section 1.5.3 of Introduction). There have been no earlier studies on sporulation in *Nostoc* ANTH. Therefore, to start with, I tested various factors that may trigger akinete formation in *Nostoc* ANTH. When *Nostoc* ANTH was grown in BG-11_0 medium (N_2-medium), no akinete formation was evident at any stage of the growth. Akinete differentiation was not triggered even after altering the pH of the medium (from normal 7.5 to pH 5, 9 or 11) or the temperature at which the cells were cultured (25, 30, 35, 45 or 50^0 C). Even when *Nostoc* ANTH, grown in NH_4^+- or NO_3^--supplemented BG-11_0 medium (NH_4^+- and NO_3^--medium, respectively), was transferred to BG-11_0 medium (N_2-medium), akinete formation was not triggered. Thus, limitation of combined nitrogen did not cause akinete differentiation (Table 3.1).

Similarly, limiting light intensity or omitting iron from the growth medium did not trigger akinete formation (data not shown). Addition of glucose, fructose or sucrose (each at concentrations of 10, 20, 30 and 50 mM) to the growth medium prolonged the exponential growth phase but proved to be ineffective in triggering akinete formation (Table 3.1). Similarly, addition of amino acids or NaCl proved ineffective (data not shown) although the *Nostoc* ANTH is known to take up and utilize amino acids (Bhattacharya *et al.*, 2002a). When N_2-grown cultures of *Nostoc* ANTH were transferred to fresh N_2-medium from which sulphate was omitted (BG-11_0 minus $MgSO_4$), there was no growth and akinete formation started within 3 days of the transfer to medium lacking sulphate. By day 24, most cells had become akinetes and no further akinetes formation occurred (Table 3.1; Figure 3.1). Transfer of *Nostoc* ANTH from N_2-medium to N_2-medium lacking phosphate (K_2HPO_4) also triggered akinete formation. However, the akinete differentiation was delayed compared to that in medium lacking sulphate (after 8 days of transfer instead of just 3 days). In addition, the akinetes continued to differentiate for a longer period than that in the medium lacking sulphate (35 days instead of 24 days). In the medium lacking phosphate, the *Nostoc* ANTH also showed significant level of growth during the initial 7-8 days in contrast to the sulphate lacking medium where no growth was observed (Table 3.1; Figure 3.1). The initial growth of *Nostoc* ANTH in medium lacking

phosphate can be explained by the fact that repeated subculturing of cyanobacteria in laboratories leads to accumulation of phosphate (polyphosphate bodies) that can be mobilised under phosphate limiting conditions (Stewart, 1980). Therefore under phosphate-limiting conditions they continue to grow as long as internal reserves of phosphate last. Thus, for the first 7-8 days the cells grew in phosphate-limiting medium using internal reserves of phosphate, after which the growth ceased and akinete formation started. In cyanobacteria, there are no such reserves known for sulphate, therefore the effect of sulphate limitation is quicker on cessation of growth and triggering akinete formation. Even after 5 years of repeated culturing in BG-11_0 medium, there was no change in the above response of *Nostoc* ANTH to sulphate limitation. In medium lacking both phosphate and sulphate, the growth and akinete formation response of *Nostoc* ANTH remains similar to the medium lacking only the sulphate (i.e. there is no quickening of cessation of growth or earlier triggering of akinete formation).

The lack of growth in medium lacking sulphate (mentioned above; Table 3.1 and Figure 3.1) persisted even when such medium was supplemented with nitrate or ammonia (Figure 3.2). While no akinetes were formed in ammonium-supplemented medium, akinete formation did occur in the nitrate-supplemented medium. In the latter case, the start of akinete differentiation was delayed by 2-3 days however, and it took that much

longer for maximum number of cells to become akinetes (Table 3.2; Figure 3.3). Nitrate did not cause such a delay in akinete formation when I used a chlorate-resistant mutant of *Nostoc* ANTH (Bhattacharya *et al.*, 2002b) that is defective in nitrate uptake and utilization. This indicates that the delay in triggering akinete formation was due to nitrate *per se* or a product of its metabolism. These data also suggest that presence of heterocysts is not necessary for akinete differentiation in this cyanobacterium since *Nostoc* ANTH did not form heterocysts in nitrate medium but still differentiated akinetes.

An interesting phenomenon was noticed when these experiments were repeated using medium lacking sulphate that was buffered with 5 mM HEPES (Figure 3.3). There was growth but no akinete differentiation in unbuffered BG-11_0 medium, while akinetes differentiated but no growth occured in unbuffered BG-11_0 medium lacking sulphate. However, growth was observed and no akinete differentiation occurred when the medium lacking sulphate was buffered with HEPES. Similarly, in nitrate-supplement unbuffered BG-11_0 medium lacking sulphate, there was no growth but akinete differentiation occurred. However, when this medium was buffered with HEPES, there was growth but no akinete differentiation. HEPES (4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid) contains sulphonic acid and *Nostoc* ANTH must be using it as a source for S, at least under the

sulphate limiting conditions, as indicated by growth of *Nostoc* ANTH in HEPES-buffered medium lacking sulphate. These results clearly show that the cessation of growth and triggering of akinete differentiation was due to sulphate limitation and that the addition of HEPES relieved this sulphate limitation.

Some of the above observations on akinete differentiation in *Nostoc* ANTH are consistent with the observations on akinete differentiation in other cyanobacteria by earlier workers. However, a number of features regarding akinete differentiation in *Nostoc* ANTH are unique and/or in contrast to the features of akinete differentiation in other cyanobacteria. Limitation of light due to the increase in culture density during growth that results in self shading has been suggested as a trigger for akinete development (Fay, 1969a; Fay *et al.*, 1984; Herdman, 1987, 1988; Nichols and Adams, 1982; Sutherland *et al.*, 1979; Wyman and Fay, 1986). Furthermore, there have been reports that iron limitation (Sinclair and Whitton, 1977), limitation of fixed nitrogen (Harder, 1917; Dementer, 1956), increase in concentration of NaCl (Cannabaeus, 1929), and provision of amino acids trigger or increase akinete differentiation in various cyanobacteria. However, the results of the present study indicate that this is not true in the case of *Nostoc* ANTH. As reported for *Nostoc* PCC 7524 (Sutherland *et al.*, 1979), addition of exogenous sources of fixed carbon prolonged the growth phase

of *Nostoc* ANTH, but in contrast to the *Nostoc* PCC 7524 no akinete were formed in *Nostoc* ANTH. The akinete formation in *Nostoc* ANTH under phosphate limitation is consistent with earlier reports implicating lack of phosphate as a major trigger of akinete development (Herdman, 1987, 1988; Nichols and Adams, 1982; van Dok and Hart, 1996).

The triggering of akinete differentiation in *Nostoc* ANTH under sulphate limitation reported in the present study is the first report of its kind, and is in contrast to the report by Sinclair and Whitton (1977) that sulphate limitation has no effect on akinete differentiation in *Anabaena cylindrica.* Both under phosphate limitation and sulphate limitation, the akinete differentiation was associated with cessation of growth of *Nostoc* ANTH. This is consistent with similar observations on *Anabaena cylindrica* (Fay, 1969a; Nichols *et al.*, 1980; Simon. 1977b), *Nostoc* PCC 7524 (Sutherland *et al.*, 1979) and *Anabaena doliolum* (Rao *et al.*, 1987). Overall, the data indicate that sulphate limitation is a powerful trigger, and better than phosphate limitation, for akinete formation in *Nostoc* ANTH. In contrast to the reports on *Cylindrospermum licheniforme* (Fischer and Wolk, 1976; Hirosawa and Wolk, 1979b), but in keeping with the observations on *Nostoc* PCC 7524 (Sutherland *et al.*, 1979), I did not find any evidence of *Nostoc* ANTH releasing any substances into the growth medium that stimulate akinete formation. Filtrates of spent akinete-differentiating medium (BG-11_0 lacking

sulphate in which akinete formation has taken place) did not induce akinete formation when added to *Nostoc* ANTH cultures growing in N_2-medium ($BG\text{-}11_0$).

Table 3.1: Factors affecting akinete differentiation in the cyanobacterium *Nostoc* ANTH.

Growth conditions	Observations	[a]Start of akinete differentiation Time (days)	[b]End of akinete differentiation Time (days)
BG-11_0	No akinete were observed during or at the end of exponential phase of growth.	-	-
pH	No akinete were observed in culture grown at pH 5, 7.5, 9 and 11.	-	-
Temperature	No akinete were observed in culture grown at 20, 25, 30, 35, 45, or 50^0 C.	-	-
BG-11_0+ exogenous C sources (glucose, fructose or sucrose; 10-50 mM	Exogenous C sources prolonged the exponential phase, but no akinete were observed during or at the end of exponential phase of growth.	-	-
BG-11_0 minus sulphate	Cessation of growth followed by akinete differentiation.	3 ± 1	24 ± 2
BG-11_0 minus phosphate	Akinete differentiation were observed during the exponential growth phase.	8 ± 1	35 ± 2

[a] Time when akinetes first appeared

[b] Time when maximum numbers of cells had become akinetes and no further akinete differentiation occurred.

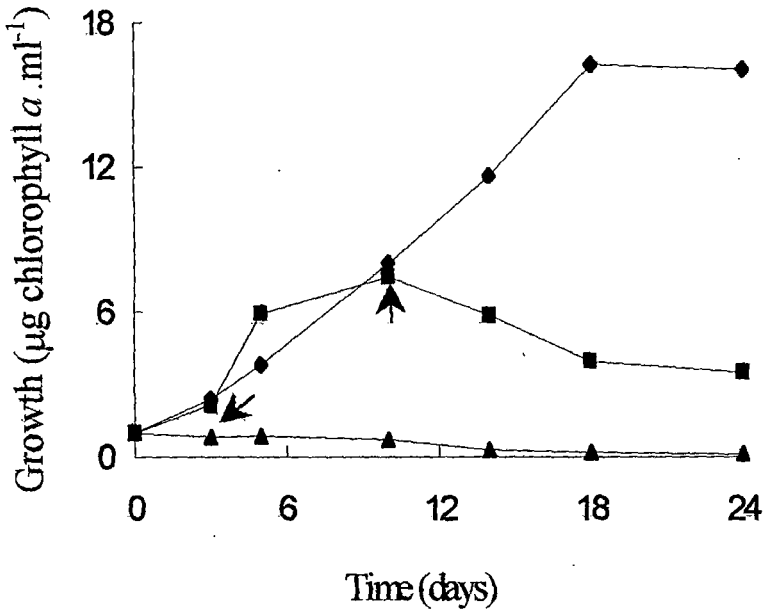


Figure 3.1: Growth of *Nostoc* ANTH in BG-11_0 (◆); BG-11_0 minus K_2HPO_4 (■) and BG-11_0 minus $MgSO_4$ (▲) media. Arrows indicate the start of akinete differentiation (i.e. when akinetes first appeared).

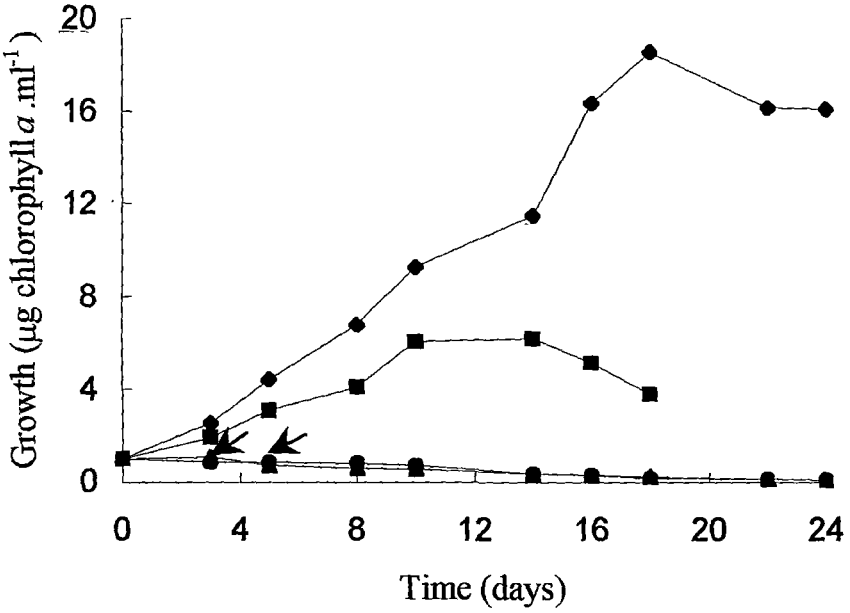


Figure 3.2: Akinete differentiation in *Nostoc* ANTH: effect of nitrate on akinete differentiation induced by sulphate limitation. (◆), BG-11_0 (N_2-medium; (■), BG-11_0 + 5 mM KNO_3 (NO_3^--medium); (●), N_2-medium minus $MgSO_4$ and (▲), NO_3^--medium minus $MgSO_4$. The arrows indicate the time when akinete differentiation started (i.e. when akinetes first appeared).

Table 3.2: Effect of inorganic nitrogen sources on akinete differentiation induced by sulphate limitation in *Nostoc* ANTH.

The values presented are mean ± SE of two independent experiments. The terms "start" and "end" of akinete differentiation are as defined in legends to Table 3.1.

	Akinete Differentiation Time (days)	
Nitrogen sources	**Start**	**End**
N_2-grown		
BG-11$_0$ medium minus $MgSO_4$	3 ± 1	24 ± 2
NO_3^--grown		
BG-11$_0$ medium minus $MgSO_4$ + 5 mM KNO_3	5 ± 1	26 ± 1
BG-11$_0$ medium minus $MgSO_4$ + 20 mM KNO_3	8 ± 1	28 ± 2
NH_4^+-grown		
BG-11$_0$ medium minus $MgSO_4$ + 2 mM NH_4Cl	-	-

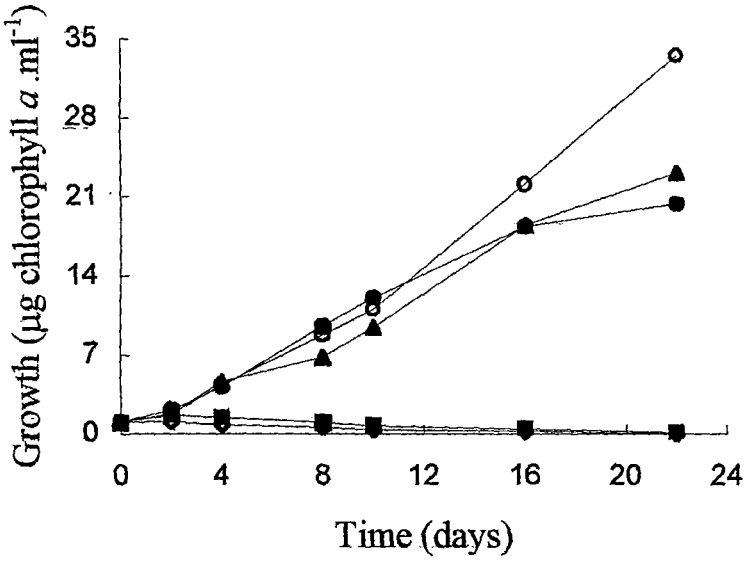


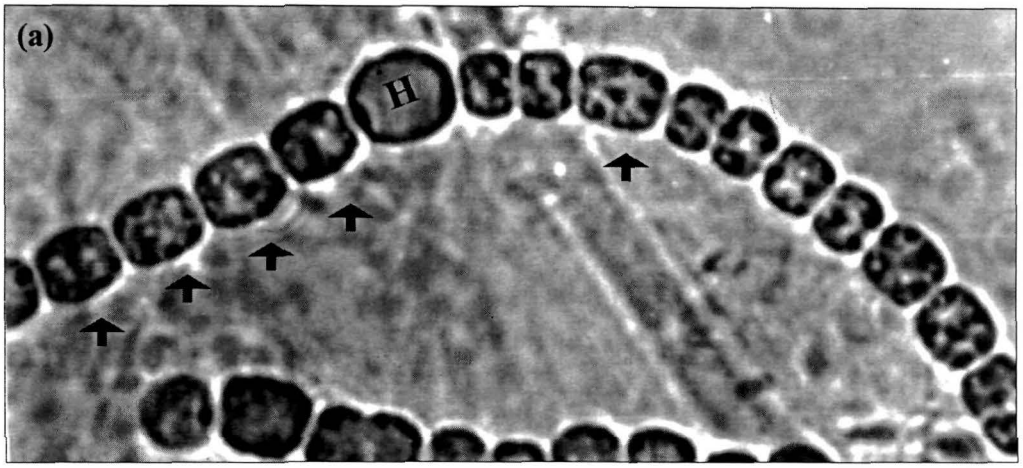
Figure 3.3: Growth of *Nostoc* ANTH in (▲), BG-11_0; (■), BG-11_0 minus $MgSO_4$; (O), BG-11_0 minus $MgSO_4$ + 5 mM HEPES; (◊), BG-11_0 minus $MgSO_4$ + 5 mM KNO_3; (●), BG-11_0 minus $MgSO_4$ + 5 mM KNO_3 + 5 mM HEPES.

3.1.2. Time Course and pattern of akinete differentiation in *Nostoc* ANTH:

Enlargement of the vegetative cells and the accumulation of cyanophycin granules by them are taken as the signs of the start of akinete differentiation (see Herdman, 1987, 1988; Nichols and Carr, 1978; Nichols and Adams, 1982; Rai *et al.*, 1985). The same criteria was adopted here. Cell enlargement and cyanophycin accumulation became apparent by day 3 when *Nostoc* ANTH was transferred to N_2-medium lacking sulphate (see Figure 3.4). The culture changed colour, from blue-green to brown, as akinete differentiation proceeded and most cells became akinetes (Figure 3.5). The initiation of akinete differentiation was noted first in cells next to heterocyst and/or in the 2nd/3rd cell from the heterocyst. In the nitrate supplemented medium, randomly located cells started differentiating into akinetes (Figure 3.4). Thus, there was not a strict correlation between location of heterocysts and the location of the start of akinete differentiation. Furthermore, presence of heterocysts was not essential for akinete differentiation as indicated by the fact that akinetes developed even in nitrate-containing medium (provided sulphate was lacking). This is not unique to *Nostoc* ANTH. In fact similar observations have been made in *Aphanizomenon flos aquae* (Wildman *et al.*, 1975), *Nostoc* PCC 7524 (Sutherland *et al.*, 1979), *Anabaena* CA (see Nichols and Adams, 1982) and *Anabaena cylindrica* (Nichols *et al.*, 1980). These data

are consistent with the view expressed by Rai *et al* (1985) that heterocysts may impose regularity on the pattern of akinete development in some cyanobacteria but their presence is not essential for akinete formation.

The akinete differentiation started within 3 days of the transfer of *Nostoc* ANTH to the N_2-medium lacking sulphate (Table 3.3). At the onset of akinete differentiation about one fifth (21%) of the cells began to differentiate into akinetes and by day 28, 97-98% of the cells had become akinetes. When this experiment was repeated in the presence of nitrate, akinete differentiation was delayed by two days but at the onset of akinete differentiation the number of cells that started to differentiate into akinetes were almost twice as many (46% as against 21%). By day 28, 92-93 % of the cells had become akinetes. Thus, the maximum akinete frequency reached was higher (97-98%) when N_2 served as nitrogen source than nitrate (92-93%).



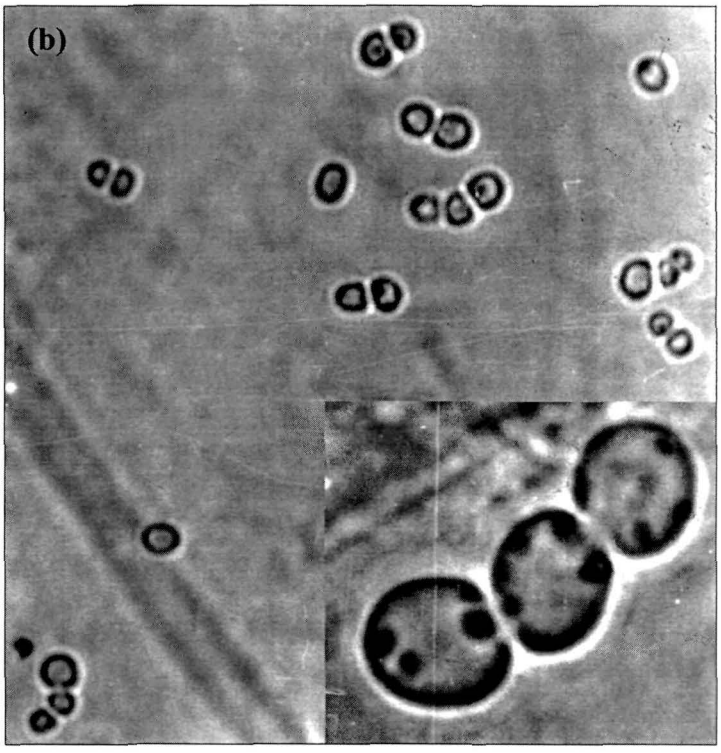


Fig. 3.4: Light micrograph (a) A filament containing a heterocyst (H) and showing accumulation of cyanophycin granules and enlargement of cells (arrows) adjacent to the heterocyst (Magnification 100 X), (b) mature akinetes (40 X; Inset 100 X).

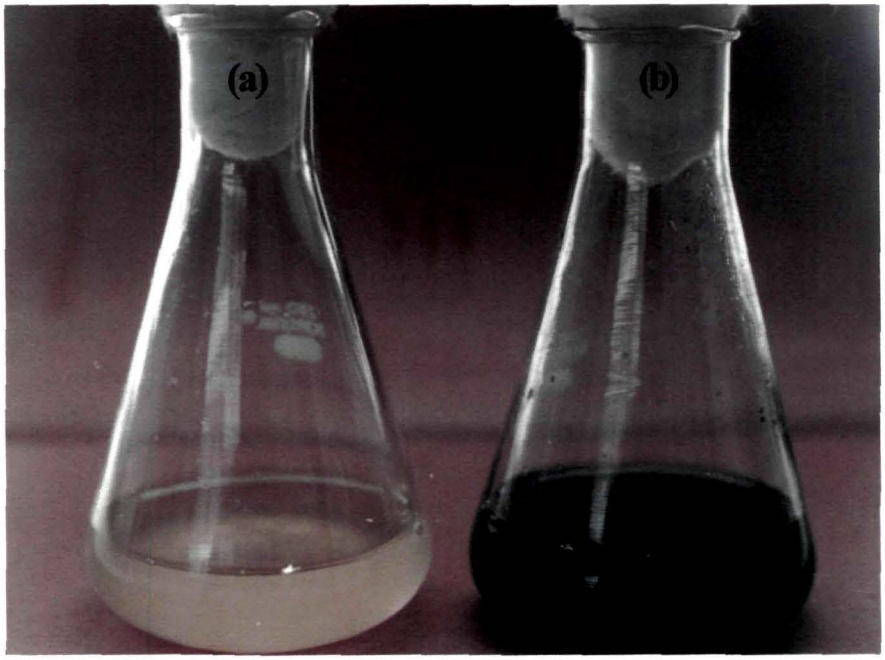


Figure 3.5: Changes in colour of *Nostoc* ANTH culture during akinete differentiation. (a), akinetes; (b) vegetative cells.

Table 3.3: Time course of akinete differentiation induced by sulphate limitation in *Nostoc* ANTH and effect of nitrate on this process.

N_2-grown (BG-11_0 medium) cultures of *Nostoc* ANTH were centrifuged, washed and resuspended in BG-11_0 minus $MgSO_4$ medium and in BG-11_0 minus $MgSO_4$ + 5 mM KNO_3 medium. From 2nd day onwards one ml samples were withdrawn and examined under microscope. Akinete frequency was calculated as percentage of total vegetative cells. One thousand cells were counted in each sample. The values presented are mean ± SE from two independent experiments.

Akinete frequency (% of Total cells)		
Time (days)	**In BG-11_0 minus $MgSO_4$**	**In BG-11_0 minus $MgSO_4$ + 5 mM KNO_3**
2	0.0	0.0
3	21 ± 1	0.0
5	46 ± 1	46 ± 1
10	57 ± 1	65 ± 1
15	72 ± 1	70 ± 2
24	84 ± 1	90 ± 2
28	97 ± 1	92 ± 2
32	98 ± 1	93 ± 2

3.1.3. Changes in contents of chlorophyll *a*, phycocyanin and protein, and the photosynthetic and respiratory activities during akinete differentiation in *Nostoc* ANTH:

From the time *Nostoc* ANTH was transferred to the akinete-differentiating BG-11_0 medium lacking sulphate (zero time), changes in chlorophyll *a*, phycocyanin, soluble protein, and photosynthetic oxygen evolution were monitored for the next twenty four days. Respiratory oxygen consumption and SDS-PAGE protein profile of mature akinetes were also obtained and compared with the exponentially growing cultures from BG-11_0 medium. The chlorophyll *a* and phycocyanin contents declined during akinete differentiation and disappeared altogether by day twenty four when virtually all cells except heterocysts had become akinetes (Figures 3.6, 3.7). This indicated that mature akinetes lack photosynthetic pigments. The lack of photosynthetic pigments in mature akinetes was further confirmed by taking absorption spectra of extracted photosynthetic pigments from akinetes and from N_2-grown filaments of *Nostoc* ANTH (Figure 3.8). While absorption spectra of extracts from N_2-grown filaments of *Nostoc* ANTH showed peaks of chlorophyll and phycocyanin, no peaks were observed in extract from mature akinetes. The protein content, measured in cell-free extracts of samples taken at different times during akinete differentiation, remained stable for the first three days and then declined becoming half by

day twenty four. This may reflect the fact that some of the soluble proteins may have been broken down and the nitrogen diverted to synthesise reserve polymers. A SDS-PAGE profile of protein bands obtained from cell-free extracts of mature akinetes (Figure 3.9) clearly shows fewer and fainter bands in comparison to those in N_2-grown cells or in cells from sulphate deficient medium prior to the start of akinete differentiation. This is consistent with the fact that cyanobacterial akinetes are known to divert much of the nitrogen to reserve polymers (see Adams and Duggan, 1999; Fay, 1969a,b; Herdman, 1987, 1988; Rai *et al.*, 1985).

Photosynthetic O_2 evolution showed a steady decline during akinete differentiation and stopped altogether by day twenty (Figure 3.7). This decline in photosynthetic O_2 evolution was expected since during akinetes differentiation photosynthetic pigments also declined. The two events virtually paralleled each other. The respiratory O_2 consumption also declined during akinete differentiation but did not disappear altogether. In mature akinetes a respiration rate of 13 nmol O_2 consumed. $min^{-1}.mg^{-1}$ protein was observed. This contrasts with a respiratory rate of 74 nmol O_2 consumed.$min^{-1}.mg^{-1}$ protein in N_2-grown filaments of *Nostoc* ANTH. Thus, in mature akinetes the respiration rate was only 17.6% of that in N_2-grown filaments.

The decline in photosynthetic pigments, photosynthetic O_2 evolution, and respiratory rates during akinete differentiation, and the lack of

photosynthetic pigments and photosynthetic O_2 evolution in mature akinetes of *Nostoc* ANTH are consistent with the findings in akinetes of *Anabaena cylindrica* (Fay, 1969a,b), *Nostoc* PCC 7524 (Chauvat *et al.*, 1982; Sutherland *et al.*, 1979), *Anabaena doliolum* (Rao *et al.*, 1984, 1987), and *Nostoc spongiaeforme* (Thiel and Wolk, 1983). Thiel and Wolk (1983) did report protein synthesis and photosynthetic O_2 evolution by akinetes at a rate approximately 7 % of that found in vegetative cells. This small activity could have been due to contamination by some vegetative cells or due to the fact that some akinetes in the population may be getting ready for germination since the akinete population was not synchronous (see Rai *et al.*, 1985).

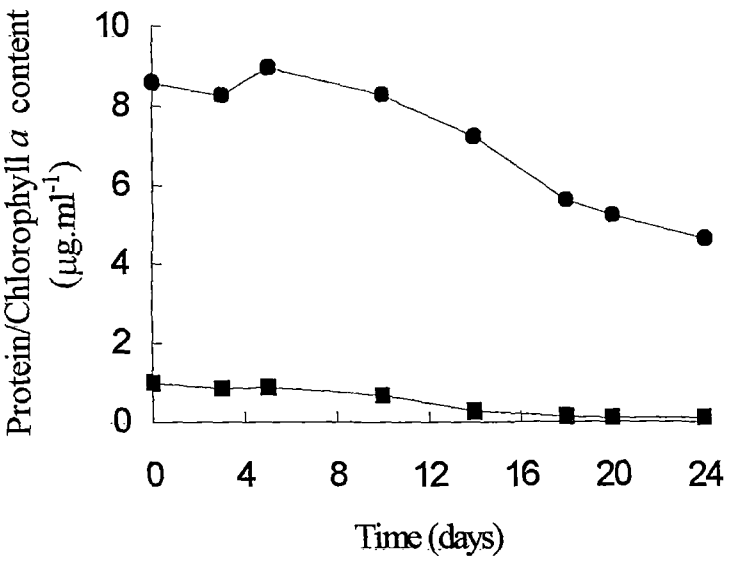


Figure 3.6: Changes in protein (●) and chlorophyll *a* (■) contents of *Nostoc* ANTH during akinete differentiation in BG-11_0 medium lacking sulphate with N_2 as sole nitrogen source.

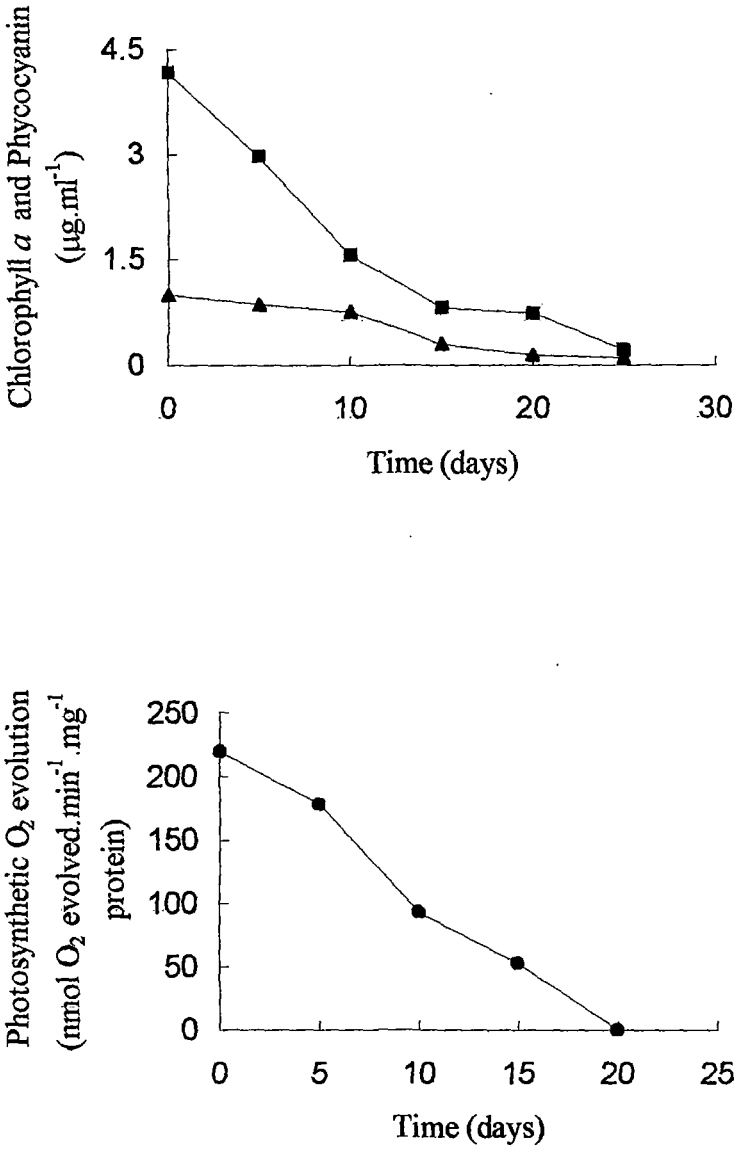


Figure 3.7: Chlorophyll a (■), phycocyanin (▲) and photosynthetic oxygen evolution rates (●) of *Nostoc* ANTH during akinete differentiation in BG-11_0 medium lacking sulphate with N_2 as sole nitrogen source.

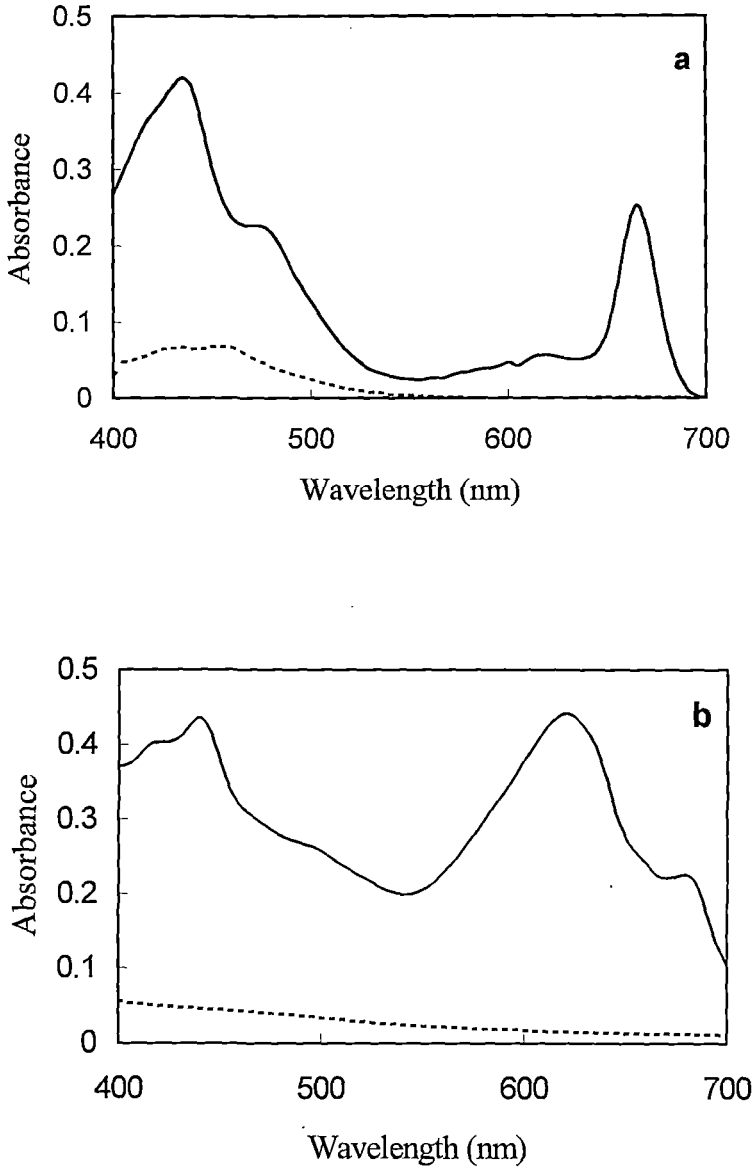


Figure 3.8: Absorption spectra of methanol extract pigments (a) and water extract pigments (b) mature akinetes (– –) and N_2-grown filaments (—) of *Nostoc* ANTH.

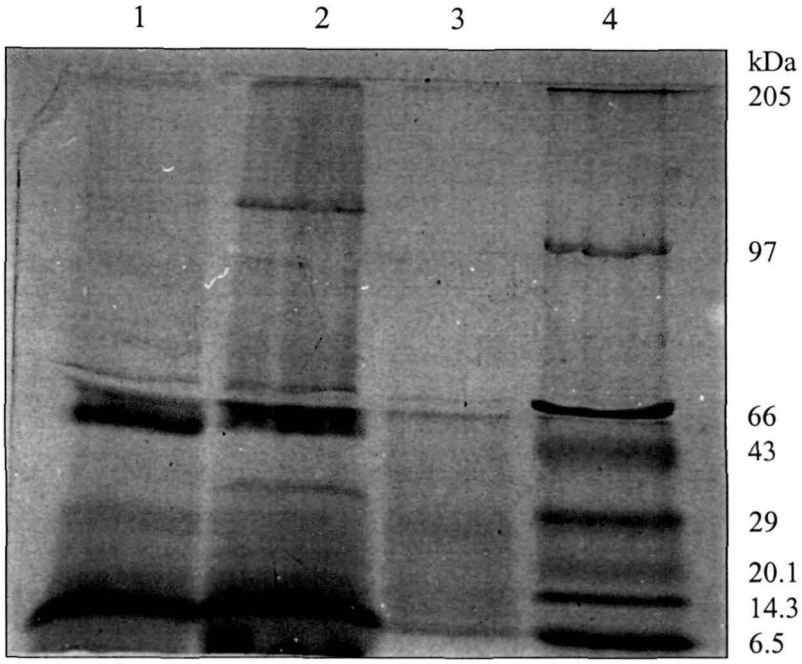


Figure 3.9: SDS-PAGE of soluble proteins in N_2-grown cells, cell from sulphate deficient medium prior to the start of akinete differentiation, and mature akinetes of *Nostoc* ANTH. Vertically arrayed numbers are sizes of molecular weight markers in kilodaltons. Samples: lane 1, cell free extract of N_2-grown cells; lane 2, cell free extract of cells from sulphate deficient medium prior to the start of akinete differentiation; lane 3, cell free extract of mature akinetes; and lane 4, molecular weight markers.

3.1.4. Heterocyst frequency and activities of nitrogenase, nitrate reductase and the primary ammonia assimilating enzyme glutamine synthetase during akinete differentiation in *Nostoc* ANTH:

Heterocyst frequency and activities of various enzymes of nitrogen metabolism (nitrogenase, nitrate reductase and glutamine synthetase) were monitored during akinete differentiation in *Nostoc* ANTH upon transfer to the akinete-differentiation medium (BG-11_0 lacking sulphate). For comparison these parameters were also monitored in cultures of *Nostoc* ANTH upon transfer to N_2-medium (BG-11_0). Under aerobic conditions nitrogenase is located in heterocysts and these are the sites of aerobic N_2-fixation in filamentous heterocystous cyanobacteria (Bergman *et al.*, 1986; Rai *et al.*, 1989). At the time of transfer to fresh media, the heterocyst frequency and nitrogenase activity of the *Nostoc* ANTH inoculum were 4.2% and 5.8 nmol C_2H_4 formed.μg^{-1} chlorophyll *a* .h^{-1}, respectively. During growth in BG-11_0 medium the heterocyst frequency and nitrogenase activity increased initially and then declined in older cultures (Table 3.4). This is a general trend found in batch cultures. When the same inoculum was transferred to the akinete differentiating medium (BG-11_0 lacking sulphate), there was an increase in heterocyst frequency and nitrogenase activity during the first four days but then there was a steep decline and by 14th day no heterocysts or

nitrogenase activity could be found (Table 3.4). The increases in heterocyst frequency and nitrogenase activity during the initial 4 days in sulphate-limiting medium indicate that although the culture did not grow, differentiation of functional heterocysts continued prior to the start of akinete differentiation. Further, the data indicate that akinetes lack nitrogenase activity. The decline and eventual disappearance of heterocysts after 4 days in sulphate-limiting medium raises a question as to what happened to the heterocysts that were present on the 4th day. In fact, as the akinete differentiation proceeded, filaments got fragmented to a size of one, two or three cells, all of which were akinetes. The heterocysts got detached, started to appear like void dead cells and became progressively more and more difficult to distinguish. Since no vegetative cells remained attached to heterocysts (i.e., all vegetative cells became akinetes), the latter became nonfunctional in terms of N_2-fixation (no supply of fixed carbon). This is consistent with the fact that vegetative cells next to heterocysts supply fixed carbon to heterocysts for N_2-fixation (see Wolk *et al.*, 1994).

The activities of nitrate reductase during growth of *Nostoc* ANTH in N_2-medium (BG-11_0) and during akinete differentiation in sulphate-limiting medium (BG-11_0 lacking sulphate) are presented in Table 3.5. The activity of nitrate reductase increased during the onset of akinete differentiation. The peak activity occurred on the 4th day, after which it steadily declined and

became undetectable by 20th day. Nitrate reductase in cyanobacteria is dependent on photosynthesis, the latter providing reduced ferredoxin as reductant (Manzano *et al.*, 1976). Since, photosynthesis parallels the decline in nitrate reductase activity (Figure 3.7), the decline in nitrate reductase activity may be attributable to the lack of reductant (reduced ferredoxin). Alternatively, or in addition, the synthesis of the enzyme nitrate reductase could have stopped in akinetes.

The changes in activities of glutamine synthetase (GS), during growth of *Nostoc* ANTH in N_2-medium ($BG\text{-}11_0$) and during akinete differentiation in sulphate-limiting medium ($BG\text{-}11_0$ lacking sulphate), are presented in Table 3.6. Glutamine synthetase is the primary ammonia assimilating enzyme in cyanobacteria (see Stewart, 1980) and is essential for assimilation of ammonia generated during N_2-fixation in heterocysts as well as for the assimilation of ammonia generated during turnover of proteins or amino acids (Singh *et al.*, 1991). The GS activity of *Nostoc* ANTH growing in N_2-medium showed an initial increase, after which it declined to a lower level and remained steady during the growth. At the end of the growth phase, the activity further declined but substantial activity remained (Table 3.6). On the otherhand, when *Nostoc* ANTH was transferred to the akinete-differentiating medium ($BG\text{-}11_0$ lacking sulphate), there was a two-fold increase in GS activity during the time of onset of akinete differentiation (day 4). The GS

activity steadily declined thereafter and was not detectable by day 20 (Table 3.6). The data indicate that mature akinetes lack GS activity and that preparatory to akinete differentiation, there is a large increase in GS activity of the cells.

The initial increases in heterocyst frequency, nitrogenase activity and activity of glutamine synthetase of *Nostoc* ANTH upon transfer to the akinete-differentiating medium (BG-11_0 lacking sulphate), despite lack of growth, mean increased N_2-fixation and nitrogen assimilation preparatory to akinete differentiation. This is consistent with the fact that massive macromolecular synthesis leading to reserve polymers occurs in cells differentiating into akinetes (see Herdman, 1987; Nichols and Adams, 1982; Rai *et al.*, 1985; Simon, 1977a; Sutherland *et al.*, 1979). The lack of nitrogenase, nitrate reductase and glutamine synthetase activities in mature akinetes of *Nostoc* ANTH is also in keeping with the findings on akinetes of *Anabaena doliolum* (Rao *et al.*, 1984) and of other cyanobacteria (see Rai *et al.*, 1985).

Table 3.4: Heterocyst frequency (HF) and nitrogenase (N_2ase) activity of *Nostoc* ANTH during growth and akinete differentiation.

Frequency of heterocysts is expressed as percent of total cells. N_2ase activity is expressed as nmol C_2H_4 formed. μg^{-1} Chlorophyll *a* $.h^{-1}$. The values presented are mean ± SE of two independent experiments.

	BG-11_0		**BG-11_0 minus $MgSO_4$**	
Time (days)	**HF**	**N_2ase**	**HF**	**N_2ase**
0	4.2 ± 0.2	5.8 ± 0.5	4.2 ± 0.2	5.8 ± 0.5
4	8.5 ± 0.2	12.7 ± 0.5	6.1 ± 0.3	7.4 ± 0.4
8	5.8 ± 0.3	6.1 ± 0.8	2.6 ± 0.1	2.4 ± 0.3
14	4.9 ± 0.4	5.1 ± 0.2	0.0	0.0
16	3.5 ± 0.2	3.8 ± 0.3	0.0	0.0

Table 3.5: Activity of nitrate reductase during growth and akinete differentiation in *Nostoc* ANTH in N_2-medium (BG-11_0) and in N_2-medium lacking $MgSO_4$.

Nitrate Reductase Activity[a] (nmol NO_2^- formed.min^{-1}.mg^{-1} protein)		
Time (days)	**Cells grown in BG-11_0**	**Cells grown in BG-11_0 minus $MgSO_4$**
0	2.19 ± 0.07	2.19 ± 0.07
4	2.69 ± 0.08	4.67 ± 0.16
8	2.71 ± 0.07	1.64 ± 0.12
12	2.59 ± 0.08	1.03 ± 0.06
20	1.96 ± 0.06	0.0

[a] The values presented are mean ± SE from two independent experiments.

Table 3.6: Activity of glutamine synthetase (GS) during growth and akinete differentiation in *Nostoc* ANTH grown in N_2-medium ($BG\text{-}11_0$) and in N_2-medium lacking $MgSO_4$.

GS Activity[a] (nmol γ-glutamyl hydroxamate formed.min^{-1}.mg^{-1} protein)		
Time (days)	**Cells grown in $BG\text{-}11_0$**	**Cells grown in $BG\text{-}11_0$ minus $MgSO_4$**
0	489 ± 8.4	489 ± 8.4
4	768 ± 9.1	1028 ± 4.9
8	403 ± 7.0	353 ± 5.6
12	419 ± 7.0	203 ± 7.7
15	447 ± 10	179 ± 4.9
20	309 ± 7.7	0.0

[a] The values presented are mean ± SE from two independent experiments.

3.1.5. Changes in C:N ratio during akinete differentiation in *Nostoc* ANTH:

In *Anabaena doliolum* (Rao *et al.*, 1987) and in *Anabaena torulosa* (Sarma and Khattar, 1993), C:N ratio of the cells seems to have an impact on akinete development. In the case of *Nostoc* ANTH, such a conclusion can not be drawn since addition of exogenous sources of fixed carbon or fixed nitrogen, which should have changed the C:N ratio of cells, failed to trigger akinete development (see section 3.1.1). However, C:N ratios did change during akinete development (Table 3.7). The *Nostoc* ANTH cells maintained a C:N ratio of approximately 5 when grown in N_2-medium ($BG\text{-}11_0$). However, when they were transferred to the akinete-differentiating medium ($BG\text{-}11_0$ lacking sulphate) the C:N ratios started increasing right from the onset of sporulation and continued to increase till the end of akinete development (day 24). In mature akinetes, the C:N ratio was nearly 60% higher than that in N_2-grown cultures (Table 3.7). This may be a reflection of the fact that during development and maturation of akinete from a vegetative cell, the metabolism is altered and geared more towards synthesis of carbon and nitrogen reserves as well as synthesis of additional cell wall layers.

Table 3.7: Changes in C:N ratio of *Nostoc* ANTH during diazotrophic growth and akinete differentiation.

C:N Ratio[a]		
Time (days)	**Cells grown in BG-11_0**	**Cell grown in BG-11_0 minus $MgSO_4$**
0	5.10 ± 0.007	5.10 ± 0.007
4	5.10 ± 0.007	6.48 ± 0.007
8	4.82 ± 0.007	6.19 ± 0.007
14	4.47 ± 0.007	7.80 ± 0.007
24	4.91 ± 0.021	8.19 ± 0.007

[a] The values presented are mean ± SE of two independent experiments.

3.2. Akinete germination

3.2.1 Germination frequency, germination timing and germination pattern of *Nostoc* ANTH akinetes:

Studies on germination of *Nostoc* ANTH akinetes were initiated by harvesting mature akinetes from sulphate-limiting medium (BG-11_0 lacking sulphate) at the end of akinete differentiation. The akinetes were washed and resuspended in fresh N_2-medium (BG-11_0) and incubated in light at 25^0 C in a culture room. The first sign of germination was noticeable when the brown cellular contents of akinetes turned blue-green. Thereafter, the akinete wall ruptured and a single cell germling emerged out and cell division followed. For the purpose of calculating germination frequency, the germination is defined here as the emergence of germling after the rupture of akinete wall. About 25% of the akinetes germinated within 24 h, 75% within 72 h and 95% by 96 h of incubation in N_2-medium under light (Figure 3.10). Germination of *Nostoc* ANTH akinetes did not occur under darkness. This is consistent with earlier reports showing light to be essential for germination of akinetes of *Anabaena cylindrica* (Fay, 1969b; Yamamoto, 1976), *Nostoc* PCC 7524 (Chauvat *et al.*, 1982), *Anabaenapsis arnoldii* and *Anabaena* spp. (Reddy *et al.*, 1975), and *Anabaena doliolum* and *Fischerella musicola* (Kaushik and Kumar, 1970). It is apparent that the germination of the *Nostoc* ANTH akinete population was asynchronous (Figure 3.10). While a quarter of the akinete population started

germination within 24 h of transfer to the fresh N_2-medium, it took nearly 96 h for all the viable akinetes to start germination. About 3-4% of the akinetes did not germinate even after 168 h and were considered nonviable. Since the akinete formation process itself was asynchronous (see section 3.1.2) leading to a population that contained akinetes of different ages, the asynchronous germination was as expected.

From the time akinetes were transferred to N_2-medium for germination, it took 20-22 h for germlings to emerge and first cell division to occur. By 48 h the first heterocyst appeared at terminal positions of 4-5 cell length germlings. The second heterocyst appeared, at the other terminal end, when the germling had grown to 10-12 cell length. Subsequently, intercalary heterocyst developed as the germination process proceeded further. These result are consistent with reports on *Nostoc* PCC 7524 (Sutherland *et al.*, 1985b), *Anabaena* PCC 7937 and *Nostoc* PCC 6720 (Skill and Smith, 1987), and *Cyanospira capsulata* (Sili *et al.*, 1994). Akinetes of *Nostoc* ANTH also germinated in presence of combined nitrogen. In the NO_3^--medium ($BG\text{-}11_0$ + 5 mM KNO_3), akinete germination was quicker. Almost 75% of akinetes germinated within the first 36 h of incubation and a germination frequency of more than 90% was achieved after 72 h. The first cell division occurred within 18-20 h of the start of germination. Presence of NH_4^+ (incubation in $BG\text{-}11_0$ + 2 mM NH_4Cl) also leads to quicker germination and the first cell

division as in the case of germination in nitrate medium. The results presented here suggest that presence of exogenous nitrogen sources accelerated the germination process but were not essential for akinete germination since equally high percentage of akinetes germinated even in the N_2-medium (i.e., in absence of combined nitrogen). Thus, the internal reserves of akinete were sufficient to initiate the germination process and the subsequent growth was supported by the appearance of heterocyst and N_2-fixation in N_2-media.

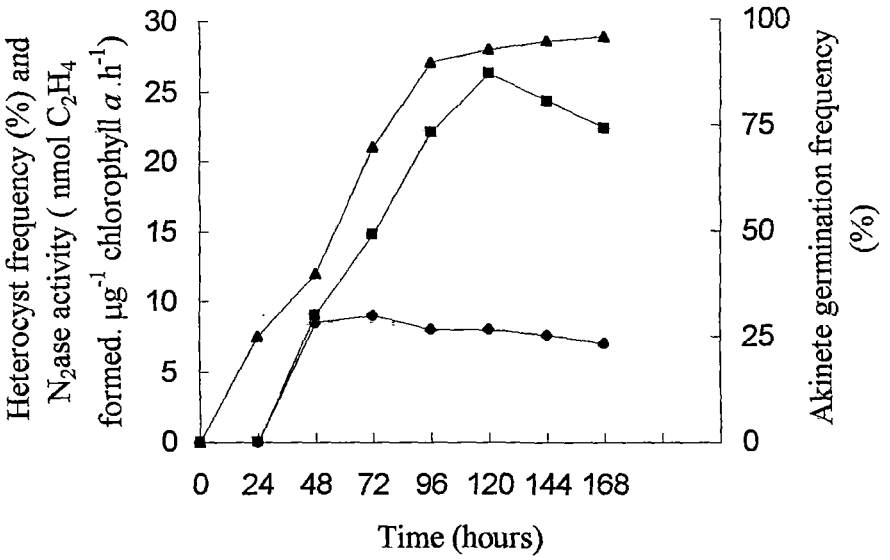


Figure 3.10: Germination of akinetes of *Nostoc* ANTH in BG-11_0 medium (N_2-medium). (▲), germination frequency; (■), nitrogenase activity; (●), heterocyst frequency (as % of total cell population including vegetative cells of germlings and as yet ungerminated akinetes). At time zero, a population of akinetes was inoculated in the BG-11_0 medium to a final concentration of 2×10^6 akinetes.ml^{-1}

3.2.2. Heterocyst frequency and nitrogenase activity during germination of *Nostoc* ANTH akinetes:

The heterocyst frequency and nitrogenase activity of the akinete population during germination in N_2-medium, is shown in Figure 3.10. After 48 h incubation, the heterocyst frequency (as % of the total cell population including vegetative cells of germlings and as yet ungerminated akinetes) was about 8%. This remain constant throughout the incubation period, however the distribution pattern of heterocyst changed during this period. It should be borne in mind that the germination of the akinete population was asynchronous. At 48 h, only 35% of the akinetes had germinated and heterocysts were present in 4-5 cells germling from these akinetes only. As the time progressed, more akinetes germinated with emergence and growth of additional germlings that developed heterocyst. Furthermore, while initially only a terminal heterocyst developed in each germling, as the germlings grew a second terminal heterocyst followed by intercalary heterocysts developed. While the heterocyst frequency remained constant as a percentage of total cells population, the heterocyst frequency of a germling was high at the start (over 24%, since a heterocyst develops at 4 cells stage itself) and declined to a level of 7-8 %.

The appearance of nitrogenase activity coincided with the appearance of the first heterocyst at 48 h (Figure 3.10). The activity however increased

subsequently upto 120 h and then declined partly. Since the heterocyst frequency remained constant at about 7-8% of the total cell population, the continued increase in nitrogenase activity may reflect the fact that at the early stage heterocyst were young and located at terminal ends of the germlings (i.e., connected to only one vegetative cell). As the germlings grew with time developing intercalary heterocysts (each connected to two vegetative cells, one at each end) and the young heterocysts matured, nitrogenase activity increased.

3.2.3. Appearance of photosynthetic pigments, and of photosynthetic and respiratory activities during germination of *Nostoc* ANTH akinetes:

Figure 3.11a shows the appearance of photosynthetic pigments during germination of akinetes of *Nostoc* ANTH with N_2 as sole nitrogen source ($BG\text{-}11_0$ medium). The akinetes lacked both phycocyanin and chlorophyll *a*. Phycocyanin appeared after 24 h of incubation, followed by chlorophyll *a* at 48 h. During the subsequent period, there was a steady increase in the levels of both these pigments as more and more germlings appeared and grew. The photosynthetic oxygen evolution became detectable 48 h after the akinetes were transferred to the N_2-medium. As expected, this coincided with the appearance of photosynthetic pigments phycocyanin and chlorophyll *a*.

When the akinete were transferred to NO_3^--medium for germination, the appearance of photosynthetic pigments was quicker. Both phycocyanin and chlorophyll *a* appeared, and photosynthetic O_2 evolution became evident by 24 h (see Table 3.8). Akinetes had a low rate of respiration as mentioned earlier (see section 3.1.3). During germination, the respiratory activity of the germinating akinete population increased steadily with time as more and more akinetes germinated and germlings grew (Figure 3.11b).

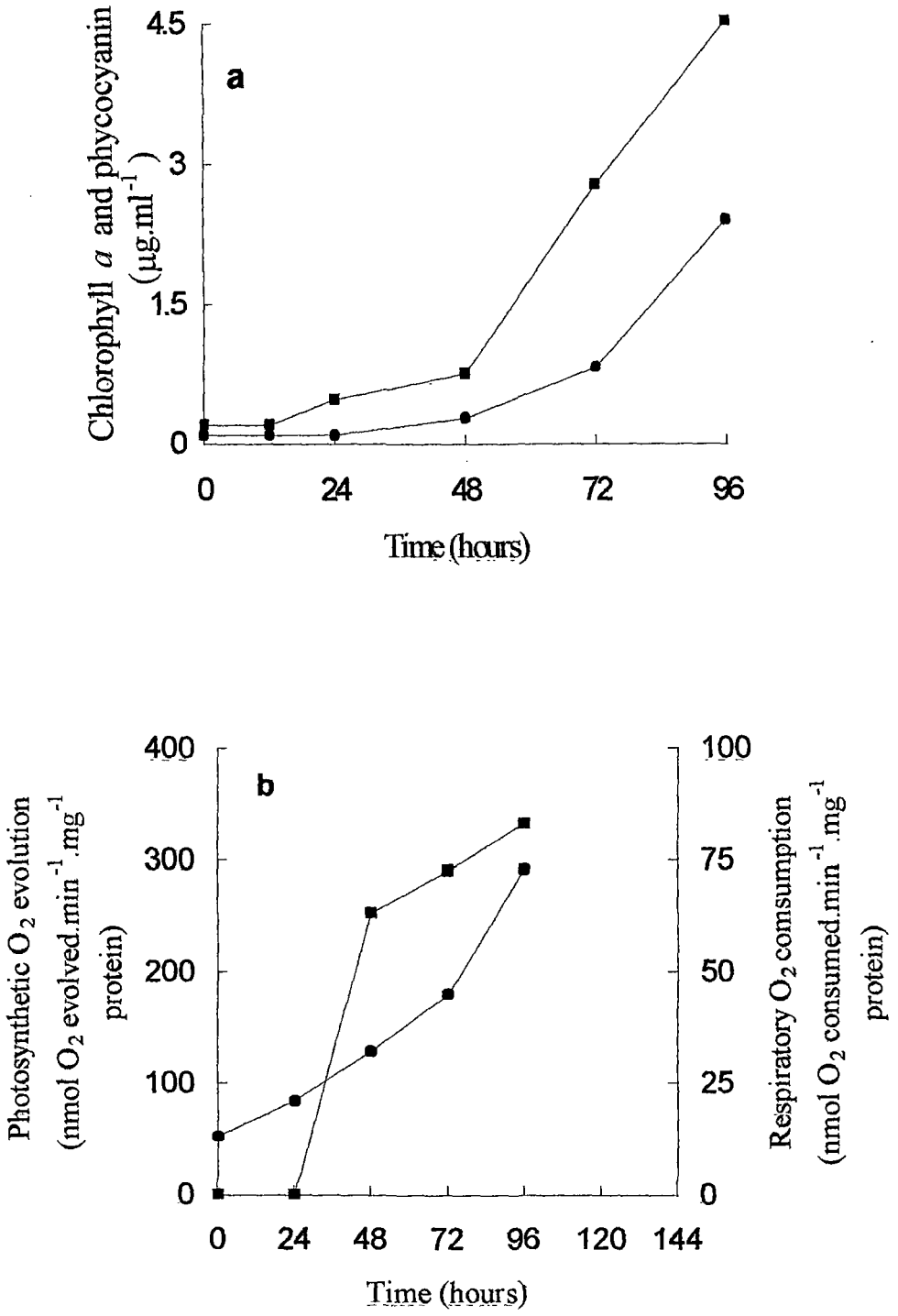


Figure 3.11: (a) Changes in chlorophyll *a* (●) and phycocyanin (■) contents during akinete germination with N_2 as sole nitrogen source (BG 11_0 medium). The inoculum at the start of germination was 2×10^6 akinetes ml^{-1}. (b) Photosynthetic oxygen evolution (■) and respiratory oxygen consumption (●) during akinete germination. The medium, and the inoculum at the start of germination, were same as in (a).

Table 3.8: Photosynthetic oxygen evolution during germination of *Nostoc* ANTH akinetes in N_2- and NO_3^--media.

The inoculum at the start of germination was 2×10^6 akinetes ml^{-1}. The values presented are mean ± SE of two independent experiments.

Photosynthetic O_2 Evolution (nmol O_2 evolved.min^{-1}.mg^{-1} protein)		
Time (hours)	**BG-11_0**	**BG-11_0+ 5mM KNO_3**
0	0.0	0.0
24	0.0	203.5 ± 10.6
48	252.5 ± 4.9	280.5 ± 3.5
72	291.0 ± 4.1	268.0 ± 4.2
96	333.0 ± 4.2	300.0 ± 2.8

3.2.4. Activities of nitrate reductase and glutamine synthetase during germination of *Nostoc* ANTH akinetes:

The activity of nitrate reductase during akinete germination is presented in Table 3.9. The nitrate reductase activity appeared 48 h after initiation of germination in nitrogen free medium and its appearance coincided with the appearance of photosynthesis (Figure 3.11b). However, in nitrate containing medium ($BG\text{-}11_0$ + 5 mM KNO_3), the activity appeared earlier (24 h after the initiation of germination), again coinciding with the appearance of photosynthetic pigments and photosynthetic O_2 evolution. The nitrate activity reached a maximum of 4.2 and 9.2 nmol NO_2^- formed.min^{-1}.mg^{-1} protein in nitrogen-free and nitrate-containing media, respectively. Subsequently, it declined to the level of whole filaments (2 and 2.61 nmol NO_2^- formed.min^{-1}.mg^{-1} protein), respectively.

The activity of glutamine synthetase during germination and germling growth is presented in Figure 3.12. Glutamine synthetase activity appeared within 24 h of the initiation of germination, remained steady upto 48 h, and then declined to a steady level normally found in N_2-grown whole filaments. The peak activity (at 24–48 h) was nearly 4 fold higher than that found in whole filaments. The appearance of glutamine synthetase before the heterocysts and nitrogenase is significant. Early appearance of glutamine synthetase was probably needed for reassimilation of ammonia generated

from mobilisation of reserves for the synthesis of new macromolecules including various pigments, enzymes and proteins preparatory to akinete germination. It is interesting to note that a similar peak of glutamine synthetase activity also appeared preparatory to akinete differentiation (Table 3.6).

Table 3.9: Activity of nitrate reductase during germination of *Nostoc* ANTH akinetes in BG-11_0 (N_2-medium) and in NO_3^--medium (BG-11_0 + 5 mM KNO_3).

The values presented are mean ± SE from two independent experiments. The inoculum at the start of germination was 2×10^6 akinetes ml^{-1}

Nitrate Reductase Activity (nmol NO_2^- formed.min^{-1}.mg^{-1} protein)		
Time (hours)	**In BG-11_0**	**In BG-11_0 + 5 mM KNO_3**
0	0.0	0.0
24	0.0	9.24 ± 0.37
48	4.20 ± 0.02	4.22 ± 0.05
72	1.79 ± 0.04	2.09 ± 0.14
96	1.30 ± 0.06	1.88 ± 0.10
120	2.03 ± 0.09	2.61 ± 0.10

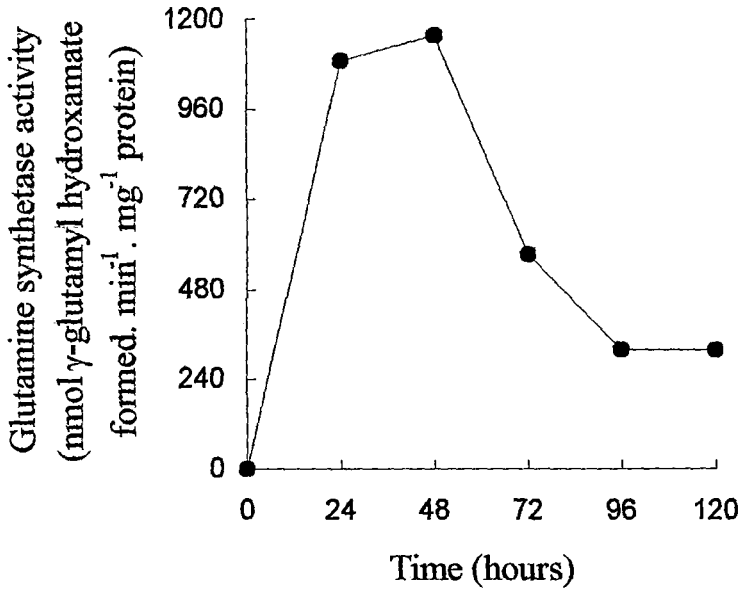


Figure 3.12: Activity of glutamine synthetase (transferase activity) during germination of *Nostoc* ANTH akinetes. Akinetes of *Nostoc* ANTH were suspended in BG-11_0 medium to a concentration of 2×10^6 akinetes ml^{-1} at time zero. GS activity was measured in samples withdrawn at different time intervals.

3.2.5. Changes in carbon, nitrogen, and protein contents and in C:N ratios during germination of *Nostoc* ANTH akinetes:

Changes in C:N ratio during akinete germination are shown in Table 3.10. The C:N ratio decreased during the akinete germination and germling growth, eventually reaching a level similar to that found in N_2-grown whole filaments of *Nostoc* ANTH. This decrease in C:N ratio was mainly because of an increase in nitrogen content. Indeed, the start of akinete germination is followed by development of heterocysts and increase in nitrogenase and other enzymes of nitrogen metabolism. The total nitrogen content remained constant during the initial 24 h of germination period but increased thereafter. Although, the total nitrogen content did not change during the first 24 h, the protein content started to increase right from the start of akinetes germination (Figure 3.13). Since akinetes germinated in nitrogen-free medium and N_2-fixation and nitrogen-assimilating enzymes had not appeared as yet, this initial increase in protein content was probably due to synthesis of new proteins by mobilising nitrogen and carbon from reserve polymers. The subsequent increase in protein content was higher and could of course be explained by the fact that photosynthesis and N_2-fixation became operational and served as sources of fixed carbon and nitrogen, respectively. The carbon content on the other hand remained more or less same during the akinete germination except that there was a small transient

decrease during 24-48 h. This is not surprising since at the start of germination, respiration of stored carbon must have been the source of energy for akinete germination till the time photosynthesis appeared. Similar conclusions were drawn by Rai *et al* (1988) in the case of *Anabaena doliolum*.

3.3. Long-term viability of the akinetes of *Nostoc* ANTH:

After dry storage of *Nostoc* ANTH akinetes at 4^0 C in the dark for 1 month, 95 % of the akinete germinated when transferred into fresh medium. In contrast, 95% of vegetative cells become inviable within 10 days of storage under similar conditions. When stored at higher temperatures (upto 35^0 C) there was no loss in viability of the akinetes and 95% of akinetes still germinated. However, the viability of akinete was reduced to less than 40% when stored at 50^0 C for 48 h. Storage of akinetes in dry state for prolonged periods at room temperature did not lead to a significant loss of viability. Nearly 90% of the akinetes germinated even after 4-5 years of storage.

Overall, the result presented in this study clearly show that sulphate limitation can be used as a trigger to induce quick and profuse akinete formation in *Nostoc* ANTH. The excellent germination rate, even after long-term storage at room temperature, makes these akinetes a good candidate for inoculum if *Nostoc* ANTH is used as biofertiliser in rice paddies. *Nostoc* ANTH is symbiotically competent, colonises rice roots and submerged parts

of the stem, carries out associative N_2-fixation and may provide fixed nitrogen to the rice plants (Bhattacharya, 2002; Nilsson *et al.*, 2002).

Table 3.10: Changes in C:N ratio during germination of *Nostoc* ANTH akinetes.

Akinetes of *Nostoc* ANTH were suspended in BG-11_0 medium to a concentration of 2×10^6 akinetes ml^{-1} at time zero. C:N ratio was determined in samples withdrawn at different time intervals.

Time (hours)	% Carbon	% Nitrogen	C:N Ratio
0	42.13	5.16	8.16
24	41.00	5.20	7.88
72	40.48	5.86	6.90
96	41.79	6.86	6.09
120	43.97	8.19	5.36
144	43.78	8.58	5.10

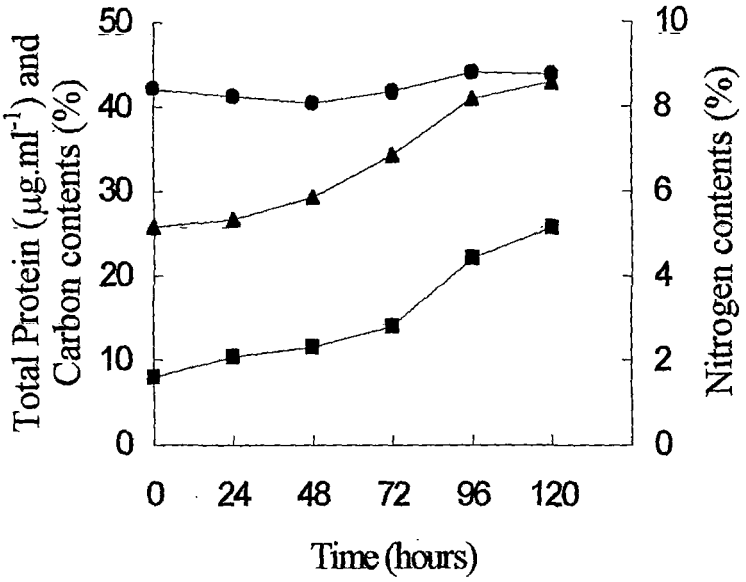


Figure 3.13: Changes in carbon (●), nitrogen (▲) and protein (■) contents during germination of *Nostoc* ANTH akinetes. Inoculum and germination medium was as given in legends to Figure 3.12.

CHAPTER 5

SUMMARY

N_2-fixing cyanobacteria are abundant in and important for the nitrogen economy of the rice paddies. The potential benefits of cyanobacteria as biofertilisers in rice fields is limited due to the use of herbicides and chemical nitrogen fertilisers that limit the growth and N_2-fixing capacity of cyanobacteria, respectively, and the lack of high viability inocula. The latter problem can be remedied if inocula can be prepared in the form of akinetes.

A *Nostoc* strain was isolated from soil and identified as *Nostoc* ANTH using STRR 1A-PCR-based DNA fingerprints and tRNA ^{Leu} intron analysis. This is a particularly useful strain as potential biofertiliser since it is known to colonise rice plants and carry out associative N_2-fixation. This strain is also amenable to creation of herbicide resistance and a chlorate-resistant mutant of this strain has been reported that fixes N_2 even in the presence of nitrate.

During the present study, akinete formation, akinete germination and akinete viability were investigated in *Nostoc* ANTH with a view to use these akinetes as inocula in rice paddies. The results are summarised below:

1. Factors such as light limitation, limitation of fixed nitrogen or iron, provision of amino acids, increased concentration of NaCl or altering pH and

temperature proved ineffective in triggering akinete differentiation in *Nostoc* ANTH. Profuse akinete differentiation occurred when the N_2-grown cultures of *Nostoc* ANTH were transferred to fresh medium from which phosphate or sulphate was omitted. The akinete differentiation in the latter case was quicker. In both cases, the differentiation of akinetes was associated with cessation of growth. This is the first report of akinete differentiation being triggered by sulphate limitation.

2. Addition of nitrate in the sulphate-limiting medium delayed the akinete differentiation in *Nostoc* ANTH by 2-3 days. Such a delay did not occur in the case of the chlorate-resistant mutant of *Nostoc* ANTH that was defective in nitrate uptake and utilisation.

3. No correlation was found between the pattern of akinete differentiation and the position of a heterocyst in the filaments of *Nostoc* ANTH. Furthermore, the presence of heterocysts was not essential for akinete differentiation.

4. The chlorophyll *a*, phycocyanin and photosynthetic oxygen evolution declined during akinete differentiation and were absent altogether in mature akinetes. Respiratory O_2 consumption also declined during akinete differentiation but did not disappear altogether. The mature akinetes had a respiratory O_2 consumption rate 17.6% of that in N_2-grown filaments.

5. The heterocyst frequency, and activities of nitrogenase, glutamine synthetase and nitrate reductase increased preparatory to akinete differentiation but then declined subsequently and disappeared. Mature akinetes of *Nostoc* ANTH lacked nitrogenase, nitrate reductase and glutamine synthetase activities.

6. Light was essential for germination of *Nostoc* ANTH akinetes. About 25% of the akinetes germinated within 24 h of akinetes being suspended in N_2-medium under light, and a germination frequency of > 95% was achieved after 96 h. At the onset of germination the brownish cellular contents of the akinete started to change colour to blue-green. Thereafter, the akinete wall ruptured and a single cell germling emerged out and cell division ensued by 24 h of start of germination. The photosynthetic pigments and photosynthetic O_2 evolution reappeared simultaneously within 48 h. Respiratory O_2 consumption rate started to increase right from the onset of germination and continued to increase till the end of germination. Addition of exogenous nitrogen sources (ammonium or nitrate) accelerated the germination process but was not essential for akinete germination.

7. The appearance of nitrogenase activity coincided with the appearance of the first heterocyst at 48 h. The nitrate reductase activity also appeared at 48 h after initiation of germination in nitrogen-free medium and its appearance coincided with the appearance of photosynthesis. The activity of

glutamine synthetase during germination and germling growth appeared within 24 h of initiation of germination. This early appearance of glutamine synthetase was probably needed for reassimilation of ammonia generated from mobilisation of reserves for the synthesis of new macromolecules including pigments, enzymes and proteins. Thus, photosynthetic O_2 evolution, nitrogenase, nitrate reductase and glutamine synthetase all became operative within 48 h of the start of germination of *Nostoc* ANTH akinetes. Both akinete differentiation and the germination were asynchronous.

8. The akinetes of *Nostoc* ANTH showed prolonged viability. Storage of akinetes in dry state for upto 5 years at room temperature did not lead to a significant loss of viability (> 90% akinetes still germinated).

Overall, this study has shown that sulphate-limitation can trigger profuse sporulation in *Nostoc* ANTH, a symbiotically compatible cyanobacterium that is known to associate with rice roots and carry out associative N_2-fixation. The akinetes show long-term viability and excellent germination frequency. This study would be of use for preparation of *Nostoc* ANTH inocula, in the form of akinetes, for application as biofertiliser in rice paddies.

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