

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

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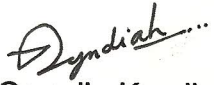
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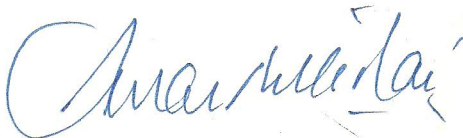
STATEMENT

I, **Omarlin Kyndiah**, hereby declare that this thesis entitled " Studies on physiological and biochemical aspects of sporulation in N₂-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
°C	Degree centigrade
C ₂ H ₂	Acetylene
C ₂ H 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	OF TABLES
nmol	Nanomole	
N ₂ ase	Nitrogenase	
PCR	Polymerase chain reaction	Page 42
PS	Photosystem	
s	Second (s)	Page 45
tRNA	Transfer ribonucleic acid	
TCA	Trichloroacetic acid	Page 51
Tris	2-amino-2-hydroxymethyl propane-1,3-diol	
UV	Ultra violet	Page 63
W	Watt	
wt	Weight	Page 64
v	Volume	
%	Percent	Page 67

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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 carbon dioxide 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 N₂-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 *vnfD* and *vnfG* genes and an Fe-only nitrogenase have also been reported in *Anabaena variabilis* (Kentemich *et al.*, 1991; Thiel, 1993). In N₂-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; Weissbar 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., N₂-fixation takes place during the day when the photosystem II is fully operative.

The process of N₂-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 O₂. Several structural, biochemical and genetic changes take place during differentiation of a vegetative cell into heterocyst in order to maintain a microaerobic interior. Such changes include synthesis of multilayered cell envelope which acts as a specific barrier for O₂, 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 autoregulatory 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 *ntcA* 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), *glnA* (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 *hetC* (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 N₂-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 multilayered 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, phosphate 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 (Demeter,

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 O₂ 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 N₂-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 N₂-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 N₂ in presence of nitrate (Bhattacharya, 2002; Bhattacharya *et al.*, 2002b; Nilsson *et al.*, 2002).