STUDIES ON NITROGEN METABOLISM AND SPORULATION IN THE THERMOPHILIC CYANOBACTERIUM *MASTIGOCLADUS* SPECIES

NONIBALA KHUMANTHEM

DEPARTMENT OF BIOCHEMISTRY

NORTH EASTERN HILL UNIVERSITY
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NORTH EASTERN HILL UNIVERSITY
DEPARTMENT OF BIOCHEMISTRY
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DECLARATION

I, **Nonibala Khumanthem**, hereby declare that the subject matter of this thesis 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 that 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**.

Prof. A. N. Rai
Head
Deptt. Biochemistry
NEHU Shillong-8

Prof. A. N. Rai
(Supervisor)

Nonibala Khumanthem
(Candidate)

Dedicated

to

my Baba and

loving memory of my Mama

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ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
C	Carbon
°C	Degree centrigrade
C_2H_4	Ethylene
Chl	Chlorophyll
d	Day (s)
h	Hour (s)
HEPES	4- (2- hydroxyethyl) - 1- piperazine ethane sulphonic acid
HF	Heterocyst frequency
GS	Glutamine synthetase (transferase)
kDa	Kilodalton
l	Litre
m	metre
M_r	Molecualr weight
mg	Milligram
µg	Microgram
min	Minute (s)
ml	Millilitre

mM	Millimolar
µmol	Micromole
µM	Micromolar
Mo	Molydenum
N	Nitrogen
N_2	Dinitrogen
Nir	Nitrite reductase
NR	Nitrate reductase
nm	Nanometer
nmol	Nanomole
nrt	Nitrate/Nitrite transport
ntc	Nitrogen control
N_2ase	Nitrogenase
PCR	Polymerase chain reaction
PS	Photosystem
psi	Pounds per square inch
rpm	Revolution per minute
s	Second (s)
TCA	Trichloroacetic acid
Tris	2- amino- 2- hydroxymethyl propane-1, 3- diol
v	Volume
%	Percent

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CHAPTER 1

Introduction

Microorganisms populate every habitable environment on earth and for billions of years they have been affecting physical and chemical properties of their surroundings by their metabolic activities such as oxygenic photosynthesis, nitrogen fixation and carbon sequestration (Kasting & Siefert, 2002; Newman & Banfield, 2002). Harvesting light to produce energy and oxygen (photosynthesis) is the signature of all land plants. This ability was co-opted from a precocious and ancient form of life known as cyanobacteria (Kasting & Siefert, 2002). Among microorganisms, cyanobacteria are universally believed to have been responsible for the initial rise of atmospheric O_2 around 2-3 billion years ago [Ga] (Holland, 1994; Farquhar *et al.*, 2000). Moreover, cyanobacteria can fix atmospheric nitrogen using nitrogenase enzyme. Nitrogen is one of the essential and elemental constituents of all living matter. It is an integral component of proteins, enzymes, nucleic acids, chlorophylls and other related compounds and is essential for maintaining structural and functional integrity of living systems. Although molecular nitrogen constitute about 78 % of the earth's total atmosphere, it cannot be directly used as nitrogen source by vast majority of organisms. Instead they require combined nitrogen. The most common nitrogen source for plants and microbes is ammonia. Some prokaryotic microorganisms can convert molecular nitrogen into ammonia under normal physiological conditions. Among such organisms, one of the largest sub-groups of gram-negative prokaryotes.

cyanobacteria, are of special interest as they have simple growth requirements, oxygenic photosynthesis and nitrogen fixation (Singh, 1961; Sprent, 1979; Stewart, 1980; Carr & Whitton, 1982; Gallon & Chaplin, 1987; Fay & van Ballen, 1987; Rai, 1990). Cyanobacteria have a wide ecological distribution, and they occupy a range of habitats, which includes vast oceanic areas, temperate soils, and freshwater lakes, and even extreme habitats like arid deserts, frigid lakes, or hot springs (Singh, 1961; Carr & Whitton, 1982; Bergman *et al.*, 1997; Whitton & Potts, 2000).

1.1 Cyanobacteria

Cyanobacteria are a structurally diverse assemblage of gram-negative eubacteria characterized by their ability to perform oxygenic photosynthesis. Their morphological diversity ranges from a unicellular to a branched filamentous organization (Rippka *et al.*, 1979). The filamentous cyanobacteria possess multiple cellular types, which include vegetative cells (site for photosynthesis), heterocysts (sites for dinitrogen-fixation), akinetes/spores (perennating bodies) and motile trichomes called hormogonia (Tandeau de Marsac & Houmard, 1993; Adams & Duggan, 1999). This structural-functional plasticity confers great versatility, enabling cyanobacteria to adapt and inhabit a wide range of environments and niches (Gallon, 1992; Tandeau de Marsac & Houmard, 1993; Bryant, 1994; Bergman *et al.*, 1997).

1.1.1 Vegetative cells

These cells are the site of photosynthesis and CO_2 fixation. CO_2 fixation occurs primarily through Calvin cycle (Stanier, 1977; Stewart, 1977; Allen, 1984). Oxidative

pentose phosphate pathway is the main catabolic route. Oxidative phosphorylation and cyclic and non-cyclic photophosphorylation generate ATP. Vegetative cells contain a number of nitrogen and carbon reserves such as cyanophycin, cyanophycean starch (glycogen) (Simon, 1971), carboxysomes (polyhedral bodies) that contain ribulose 1, 5-bisphosphate carboxylase/oxygenase (Stewart & Codd, 1975; Codd & Marsden, 1984), and polyphosphate bodies (Grillo & Gibson, 1979). The chlorophyll *a* protein complexes, the photosynthetic reaction centers, the carotenoids and the electron transport system are all contained within the thylakoids. The light harvesting accessory pigments (phycobiliproteins) are located in phycobilisomes attached to the surface of thylakoids, which are also used as nitrogen sources under conditions of N-deficiency (Tandeau de Marsac & Houmard, 1993; Bryant, 1994).

1.1.2 Heterocysts

The correlations that cyanobacteria capable of aerobic dinitrogen fixation have heterocysts, and that ammonium which apparently represses nitrogenase, inhibits the formation of heterocysts (Fogg, 1949), led Fay and co-workers (1966, 1968) and Prisgsheim (1968*a*, *b*) to suggest that heterocysts are important in dinitrogen fixation. A number of subsequent studies have conclusively shown that nitrogenase is confined to heterocysts, under aerobic conditions, in heterocystous cyanobacteria (Stewart, 1980; Murry *et al.*, 1984; Bergman *et al.*, 1986; Rai *et al.*, 1989; Janson *et al.*, 1993, 1995). Thus, heterocysts are differentiated cells, which are specialized for aerobic dinitrogen fixation. Because of their role in dinitrogen fixation, heterocysts are of

agronomic importance (Singh, 1961; Peter, 1977; Singh, 1977; Watanabe *et al.*, 1977) and because their differentiation is easily followed in the microscope, and involves intercellular controls, they have assumed increasing importance as developmental models (Wolk, 1975, 1979*a*, *b*; Haselkorn, 1978; Carr, 1979). Wolk (1982) has suggested that structural and metabolic differences between heterocysts and vegetative cells may enhance the effectiveness of heterocysts as sites for dinitrogen fixation. The most obvious morphological change brought about in the branching, filamentous cyanobacterium *Mastigocladus laminosus* by nitrogen starvation was the differentiation of vegetative cells to heterocysts (Stevens *et al.*, 1985). Contiguous heterocysts occur naturally in *M. laminosus* (Nierzwicki-Bauer *et al.*, 1984*a*; Stevens *et al.*, 1985). The morphological and ultrastructural aspects of heterocysts differentiation in this cyanobacterium examined with light and electron microscopy revealed cytoplasmic changes including rapid degradation of carboxysomes and polysaccharide granules, and accumulation of electron-dense ribosomal or protein material (or both) (Nierzwicki-Bauer *et al.*, 1984*a*). Nitrogen starvation also led to the accumulation of polyphosphate bodies and lipid bodies in the vegetative cells, and the intracytoplasmic membranes vesiculated to form vacuole like structures and, eventually, led to the formation of large empty regions in the cytoplasm of mature heterocysts (Stevens *et al.*, 1985). Mature heterocysts of *M. laminosus* contain small, spherical granules of varying electron density (Nierzwicki-Bauer *et al.*, 1984*a*).

The heterocysts envelope is discontinuous at the point of contact between the heterocysts and its neighbouring vegetative cells and a pore channel is formed. A polar plug consisting of cyanophycin (a storage polymer consisting of alternating

residues of arginine and aspartic acid) often occurs near the pore channels. There are considerable physiological and biochemical differences between vegetative cells and heterocysts (Wolk, 1982). Heterocysts retain significant amounts of chlorophyll and carotenoid pigments but have less phycobiliproteins compared to the vegetative cells. They also lack the Mn^{+2} containing components of photosystem II and the lack of a functional PSII contributes to the protection of nitrogenase from O_2 damage. Heterocysts lack glutamate synthase (GOGAT) (Thomas *et al.*, 1977; Rai *et al.*, 1982) and nitrate reductase (Kumar *et al.*, 1985; Rai & Bergman, 1986; Rai *et al.*, 1992) and hence avoid competition for molybdenum and reductant (reduced Fd) with nitrogenase. They also lack ribulose 1, 5-bisphosphate carboxylase enzyme and therefore do not fix CO_2 (Codd & Stewart, 1977; Codd *et al.*, 1980; Cossar *et al.*, 1985). The demand for fixed carbon is met from the neighbouring vegetative cells (Wolk, 1968; Stewart, 1980; Bothe *et al.*, 1984; Stewart *et al.*, 1985; Bergman *et al.*, 1986). Ammonia produced via nitrogenase is assimilated by the action of glutamine synthetase (GS), which is present in the heterocysts at a level twofold higher than that in vegetative cells (Dharmawardene *et al.*, 1973; Stewart *et al.*, 1975; Thomas *et al.*, 1977; Rai *et al.*, 1989). High level of GS is probably required in heterocysts for the assimilation of N_2 derived ammonia (Renstrom-Kellner *et al.*, 1990). Glutamine thus produced in the heterocysts is transported to adjacent vegetative cells where it is converted to glutamate by glutamate synthase (GOGAT) (Stewart *et al.*, 1975; Stewart, 1977). Part of the glutamate produced in the vegetative cells through the activity of GOGAT is transported back to the heterocysts to provide substrate for glutamine synthesis by GS (Thomas *et al.*, 1977).

1.1.3 Akinetes

Akinetes are thick-walled reproductive units produced by many cyanobacteria that aid survival under unfavourable growth conditions. They are generally found in members of *Nostocaceae*, *Rivulariaceae* and *Stigonemateceae* (Rippka *et al.*, 1979) and these specialized single cells serve as means of perennation in these organisms (Wolk, 1965*a*, 1973; Nichols & Carr, 1978; Adams & Carr, 1981). The akinetes are usually larger, its cell wall thicker and its cytoplasm more granular than the vegetative cells. The structural and cytoplasmic changes associated with the differentiation of an akinete from a vegetative cell have been studied by using electron microscopy (Wildon & Mercer, 1963; Leak & Wilson, 1965; Miller & Lang, 1968; Clark & Jensen, 1969; Jensen & Clark, 1969; Wildman *et al.*, 1975; Sutherland *et al.*, 1979; Grilli Caiola & de Vecchi, 1980). Mature akinetes have a low respiration rate and lack photosynthetic pigments, photosynthetic oxygen evolution and the activities of nitrogenase, nitrate reductase and glutamine synthetase (GS) in *Anabaena doliolum* (Rao *et al.*, 1984; Rai *et al.*, 1985, 1989). The loss of nitrogenase activity and glutamine synthetase activity during development of the akinetes preceded both cessation of diazotrophic growth and the initiation of akinete development. This was followed by complete cessation of oxygenic photosynthesis (Rao *et al.*, 1987). Rao *et al.* (1984) proposed that glutamine synthetase activity could be used as a marker of akinete development and maturity. Glycogen accumulation in cells undergoing akinete formation has been demonstrated in *Anabaena* sp. (Sarma & Kanta, 1979). Wolk and coworkers have determined the nature of the polysaccharides in the cellular envelopes of akinetes of *Anabaena cylindrica* (Dunn & Wolk, 1970; Cardemil &

Wolk, 1976, 1979). Profuse akinete formation occurs in *Fischerella musciola* (*Mastigocladus laminosus*) at the end of diazotrophic growth, and mature akinetes have low rate of respiration and lack photosynthetic pigments, photosynthetic O_2-evolving activity and the activities of the primary enzymes of inorganic nitrogen assimilation. The C:N ratio steadily increases during akinetes differentiation due to the fact that the loss of activities of nitrogenase and glutamine synthetase precedes the loss of CO_2 fixation (Singh & Kashyap, 1988).

1.1.3.1 Factors controlling akinete formation

The factors that control the onset of akinete differentiation are poorly understood, although many nutrients have been implicated. A deficiency of fixed-nitrogen has been reported to induce akinete formation (Harder, 1917; Demeter, 1956) and these observations have been supported by more recent findings, which indicate that the addition of nitrogen (nitrate, nitrite or ammonium) had the opposite effect (Singh & Srivastava, 1968; Tyagi, 1974, 1978). Phosphate has been considered important, since the deficiency of this nutrient favoured akinete formation (Wolk, 1965*b*; Gentile & Maloney, 1969). This might seem an attractive candidate since akinetes do not appear to contain polyphosphate bodies. Sutherland *et al.* (1979) also found that with *Nostoc* 7524, removal of phosphate caused cessation of growth and although cyanophycin accumulated, akinetes were never produced. Harder (1917) and Sutherland *et al.* (1979) found that akinete production was inhibited by sucrose, while Tyagi (1974, 1978) reported a stimulation by glucose. Sodium chloride (Canabaeus, 1929) and drying have been shown to induce or increase akinete formation. Various amino acids

(tryptophan, asparctic acid, phenylalanine, proline and isoluecine) have been shown to increase akinete production in *Cylindrospermum licheniforme* (Hirosawa & Wolk, 1979*a*). Nitrogen starvation stopped growth and cyanophycin production, and arrested akinete formation, even in cultures, which had commenced differentiation. Akinete formation has been reported to occur towards the end of, or after the cessation of exponential growth in nitrogen-fixing batch cultures in *Anabaena cylindrica* (Fay, 1969*b*, Nichols *et al.*, 1980). Light is one of the most important factors that influence akinete formation. In both *Nostoc* 7524 (Sutherland *et al.*, 1979) and *A. cylindrica* (Nichols *et al.*, 1980) changes in light intensity alter the cell density at which akinete formation occurs. Sarma and Kanta (1982) studied the variations in enzyme levels during akinete formation in *Anabaena torulosa*, and reported that there is increase in acid and alkaline phosphatase activities during akinete initiation and maturation.

1.1.3.2 Pattern of akinete formation

Akinete forming cyanobacteria could be classified into two categories according to the spatial relationship between heterocysts and akinetes:

(i) Cyanobacteria where akinetes occur adjacent to the heterocysts.

(ii) Cyanobacteria where akinetes occur away from the heterocysts.

Akinetes have been found to developed in complete absence of heterocysts (Eberly, 1966; Hill, 1970) even in the organisms, which form akinetes adjacent to or between the heterocysts. Sutherland *et al.* (1979) found that in ammonium-supplemented cultures of *Nostoc* PCC 7524, where no heterocysts development occurred, akinetes

developed at random positions. In nitrogen fixing conditions, where heterocysts developed, akinete developed near the center of the inter-heterocysts interval suggesting that although heterocysts were not necessary for akinetes development, their presence exerted some influence on akinete pattern. Chains of akinetes are produced in *Anabaena* CA and *Fischerella* and they are slightly larger than the vegetative cells. Such akinetes are most easily recognized under the phase-contrast optics and reveal the numerous refractile cyanophycin granules, which they contained (Sutherland *et al.*, 1979).

1.1.3.3 Germination of akinete

Akinetes are propagating bodies, which show some resistance to adverse conditions and germinate under growth favourable conditions in nature. The factors, which induce akinete germination, are many including light, temperature and various nutrients. Increased light intensity, and phosphorous and nitrogen availability have been implicated as major factors for akinete germination (Herdman, 1987, 1988; van Dok & Hart, 1997). The energy demands of akinete differentiation are initially met from aerobic oxidation of carbon reserve (Rai *et al.*, 1988). During germination, all the metabolic activities related to C and N metabolism reappear sequentially, allowing the cells to continue their normal growth (Sutherland *et al.*, 1985*a*; Rai *et al.*, 1988).

1.1.2 Nitrogen Fixation

Many cyanobacteria are able to grow at the expense of atmospheric N_2 under aerobic conditions and many more are able to perform N_2 fixation under microaerobic/anaerobic conditions. Due to the widespread distribution of these microorganisms in nature, it is believed that cyanobacteria contribute significantly to the global biological N_2 fixation. The machinery for fixing N_2 is present in all the currently recognized taxonomic groups of cyanobacteria. A phylogenetically–coherent group of filamentous cyanobacteria, that includes genera such as *Anabaena*, *Nostoc*, and *Fischerella* of Section IV and V of the taxonomic classification of Rippka *et al.* (1979), has developed a complex and efficient mechanism for performing N_2 fixation under aerobic conditions. This consists of the development of specialized cells (the heterocysts) under conditions of aerobiosis and combined-N deprivation. Heterocysts differentiate from vegetative cells at semi-regular intervals in the filament and bear a series of modifications devoted to the protection of the N_2-fixation apparatus from O_2. Heterocysts contain the enzyme nitrogenase, which catalyses conversion of molecular nitrogen into ammonia (Flores & Herrero, 1994; Wolk *et al.*, 1994; Adams & Duggan, 1999). The enzyme nitrogenase catalyzes the ATP-dependent reduction of N_2 to two molecules of ammonium. This requires the transference of six electrons to N_2. It consists of two different protein components: dinitrogenase (the Mo-Fe protein) and the dinitrogenase reductase (the Fe protein). Dinitrogenase is an α_2 β_2 tetramer (Mr 226.8 kDa), and its α and β subunits are encoded by the *nif*D and *nif*K genes, respectively. It also contains two molecules of Mo-Fe cofactor. Dinitrogenase reductase (Mr 66 kDa) is a dimer of identical subunits encoded by *nif*H gene. Dinitrogenase reductase mediates the ATP-dependent transfer

of electrons from external electron donors, such as ferredoxin or flavodoxin, to the 'P' clusters of dinitrogenase. Dinitrogenase binds N_2 through its Mo-Fe cofactors and catalyzes the reduction of N_2 to ammonia. Studies involving genetic and immunological experiments have revealed the cyanobacterial nitrogenase protein to be similar to other N_2-fixing organisms (Bergman *et al.*, 1986, 1997; Dean & Jacobson, 1992; Flores & Herrero, 1994; Kim & Rees, 1994).

The reduction of N_2 to NH_4^+ catalyzed by nitrogenase is a highly endergonic reaction requiring metabolic energy in the form of ATP. The enzyme nitrogenase shows extreme sensitivity to oxygen which is manifested both at the level of synthesis and activity (Gallon & Chaplin, 1987; Gallon, 1992; Rai *et al.*, 1992; Durner *et al.*, 1996). Several structural, biochemical and genetic changes such as synthesis of multi-layered cell envelope, loss of PSII activity, presence of uptake hydrogenase, and high rates of respiration take place in the heterocysts of filamentous cyanobacteria in order to maintain a microaerobic interior (Wolk *et al.*, 1994).

In the heterocyst, reduced ferredoxin (Fd) donates electrons to nitrogenase. The identification and cloning of the gene *fdx*H, that encodes a Fd specific to the heterocyst has been achieved. The *fdx*H gene is present in the *nif* gene cluster of the *Anabaena* sp. strain PCC 7120 genome (Böhme & Haselkorn, 1988). Extensive genetic study has been done on *Anabaena* PCC 7120 providing sufficient information about the genetic aspect of heterocysts differentiation and its regulation. Differentiation of vegetative cells is a highly complex process, involving significant changes in the gene expression. Several characterized genes that are induced in response to nitrogen deprivation (*hep*A, *hep*C, *het*R and *nif*H) are expressed in well-

spaced cells that are probably presumptive heterocysts. During the course of development, several genes are regulated at transcriptional level. These include *tln*2 and *tln*6 (Wolk, 1991), *cpc*BA and *apc*AB (encoding the major phycobiliproteins; Wealand *et al.*, 1989), *het*R (required early in heterocyst differentiation; Black *et al.*, 1993), *hep*A and *hep*C (required for polysaccharide biosynthesis; Wolk *et al.*, 1988), *nif*HDK (for the expression of nitrogenase; Elhai & Wolk, 1990), *gln*A (encoding gltutamine synthetase; Tumer *et al.*, 1983), and *sig*B and *sig*C (encoding for sigma factors, modular components for recognition of promoter sequences; Helmann & Chamberlain, 1988).

The operons associated with nitrogen fixation in heterocysts include *nif*HDK (encodes for structural proteins for dinitrogenase and dinitrogenase reductase which together constitute the nitrogenase complex), *nif*B (for synthesis of FeMoCo-the active site of dinitrogenase), *fdx*N (for heterocyst specific ferredoxin), *nif*S (probably for the maturation of dinitrogenase), *fdx*H (encodes for heterocyst specific ferredoxin that acts as terminal electron donor to nitrogenase), *hup*SL (encodes for heterocyst specific periplasmic permease which facilitates the transport of the *hgl*K and *hep*A products), and *hgl*K (encodes for heterocyst specific glycolipid which forms internal layer to the polysaccharide). The organization of nitrogen fixation (*nif* cluster) genes comprises of several open reading frames (ORFs), two interrupting DNA elements and four operons dispersed in around 80 Kb of DNA (Mazur *et al.*, 1980; Rice *et al.*, 1982). Two other genes *xis*A and *xis*F are there which reside on the interrupting DNA elements encoding the site-specific recombinases and are very essential for nitrogen fixation in *Anabaena* 7120. In *Anabaena* 7120, *nif*HDK operon is interrupted by an

11 Kb DNA sequence in the *nif*D gene (Golden *et al.*, 1985). Other interruptions are a 55 Kb element in the *fdx*N gene (Golden *et al.*, 1988) and a 10.5 Kb element in the *hup*L gene (Carrasco *et al.*, 1995). These interrupting sequences are removed late during heterocyst differentiation by three independent site-specific recombinations resulting in three functional operons (Apte & Prabhavathi, 1994). These gene rearrangements in *Anabaena* PCC 7120 are brought about by three different excisases *xis*A (Lammers *et al.*, 1986), *xis*F and *xis*C (Carrasco *et al.*, 1994, 1995) encoded by the three independent genes. The only exception to the characteristic *nif* gene rearrangement is found in *Mastigocladus laminosus*, where the structural *nif* genes are contiguous both in vegetative cells and heterocysts (Singh & Stevens, 1992).

Genes, which code for the heterocyst specific proteins include *het*R (controls heterocyst development and is autoregulatory), *pat*A (ntrC like transcriptional regulator), *pat*B (involved in pattern formation) (Liang *et al.*, 1992, 1993) and *pat*S (function as a diffusible signal produced pro-heterocyst to inhibit neighbouring cells from differentiating) (Yoon & Golden, 1998). Yoon & Golden, (1998) have suggested the role of a diffusible inhibitor Pat S in the regulation of heterocyst pattern formation. *dev*R and *pat*B genes are necessary for the completion of heterocyst development (Campbell *et al.*, 1996). A *pat*B mutant shows delayed and incomplete heterocyst development (Liang *et al.*, 1993). *het*C, which encodes a protein similar to the bacterial ABC protein exporters, is involved in relatively early regulation of heterocyst differentiation (Khudyakov & Wolk, 1997). *sig*B and *sig*C (alternate sigma factor during nitrogen step down) and *ntc*A (transcriptional regulator) are involved in heterocyst development. Over expression of *het*R produces multiple heterocysts even

in the presence of nitrate while its deficiency blocks heterocyst formation (Buikema & Haselkorn, 1991; Black *et al.*, 1993). A *nif*J like gene has also been described in *Anabaena* 7120, which is essential for nitrogen fixation under Fe-deficient but not under Fe-replete conditions (Bauer *et al.*, 1993).

1.2 Inorganic nitrogen metabolism by cyanobacteria

1.3.1 Nitrate and nitrite metabolism

The nitrogen sources commonly used by cyanobacteria are nitrate, ammonium, urea and dinitrogen (Flores & Herrero, 1994). Nitrate assimilation involves uptake of nitrate and its two step reduction (via nitrite) to ammonium, catalyzed by ferredoxin dependent nitrate reductase and nitrite reductase. An ATP-binding cassette (ABC) type transporter constituted by the products of the *nrt*A, *nrt*B, *nrt*C, and *nrt*D genes is involved in nitrate/nitrite uptake by the fresh water *Synechococcus* sp. strain PCC 7942 and *Anabaena* sp. strain PCC 7120 (Omata *et al*, 1993; Luque *et al.*, 1994; Cai & Wolk, 1997; Frias *et al.*, 1997; Maeda & Omata, 1997), whereas a carrier belonging to the major facilitator super family mediates nitrate/nitrite uptake in the marine cyanobacteria *Synechococcus* sp. strain PCC 7002 (Sakamoto *et al.*, 1999) and *Trichodesmium* sp. strain WH 9601 (Wang *et al.*, 2000). The gene encoding this carrier has been named *nrt*P (Sakamoto *et al.*, 1999) and the permease named as Nrt P.

In *Synechococcus* sp. strain PCC 7942 and *Anabaena* sp. strain PCC 7120, the genes encoding nitrate/nitrite transporter are clustered together with the structural genes for nitrite reductase (*nir*A) and nitrate reductase (*nar*B) ((Luque *et al.*, 1993;

Rubio *et al.*, 1996) constituting an operon (nir operon) with the structure nirA-nrtABCD-narB (Luque *et al.*, 1992; Omata *et al.*, 1993; Suzuki *et al.*, 1993; Cai & Wolk, 1997; Frias *et al.*, 1997). The nir operon is inhibited by ammonium and activated by nitrate and nitrite (Kikuchi *et al.*, 1996; Frias *et al.*, 1997). A number of genes whose mutation results in different degrees of impairment of expression of the nitrate and nitrite reductases have been found upstream from the nir operon, in the opposite DNA strand (Suzuki *et al.*, 1995; Frias *et al.*, 2000). The ammonium repressible *ntc*B is one of the genes that are involved in coding for a protein which belongs to the Lys R family of transcriptional regulators. Ntc B mediates the positive effect of nitrate (via nitrite) on the expression of the nir operon in *Synechococcus* sp. strain PCC 7942 (Aichi & Omata, 1997), but in *Anabaena* sp. strain 7120, it is required for expression of the nir operon irrespective of the presence of nitrate (Frias *et al.*, 2000). In the genome of *Synechococcus* sp. strain PCC 6803, the *nrt*ABCD and *nar*B genes are clustered together whereas the *nir*A gene is found at a different location (Kaneko *et al.*, 1996).

The assimilation of nitrate involves it's uptake and reduction (via nitrite) to ammonium. Ammonium is then incorporated into organic compounds. The uptake of nitrate in cyanobacteria takes place through a high affinity transport system (Flores *et al.*, 1983*a*) that permits efficient uptake even at low external concentrations of nitrate. The nitrate uptake is sensitive to the protonophore and F_oF_1-ATPase inhibitor, DCCD (Ohmori *et al.*, 1977; Flores *et al.*, 1983*a*). A cytoplasmic-membrane protein of about 48 kDa was identified in *Synechococcus* sp. strain PCC 7942 and its expression was altered in mutant strains pleiotropically impaired in nitrate assimilation (Madueno *et*

al., 1988) suggesting an involvement of this protein in nitrate transport. Nitrite uptake takes place in two different ways in cyanobacteria: i) active transport, which is sensitive to DCCD and exhibits high affinity for nitrite (Ks, 6-22 μM) (Flores *et al.*, 1987; Martin-Nieto *et al.*, 1989); ii) diffusion of nitrous acid whose contribution of the net uptake of nitrite decreases as the pH of the medium is raised (Flores *et al.*, 1987; Martin-Nieto *et al.*, 1989). A competition for uptake between nitrate and nitrite has been observed suggesting that the same system operates the active transport of both substrates (Madueno *et al.*, 1987; Rodriguez *et al.*, 1992). Disruption of the *nrt*D gene in *Synechococcus* sp. strain PCC 7942 impaired nitrate transport and abolished active nitrite transport, thus showing that nitrate and nitrite are transported by the Nrt ABCD permease (Luque *et al.*, 1994). Bhattacharya *et al.* (2002*b*) have recently shown that in *Nostoc* ANTH there is a separate nitrite transport system quite distinct from the common nitrate/nitrite transport system.

Intracellular nitrate is reduced to nitrite in a two-electron reaction catalyzed by nitrate reductase (Nar), the resulting nitrite being then reduced to ammonium in a six-electron reaction catalyzed by nitrite reductase (Nir). Both Nar and Nir use reduced Fd as the physiological electron donor (Manzano *et al.*, 1976; Mendez *et al.*, 1981; Arizmendi & Serra, 1990). Reduced flavodoxin can serve as an alternative to reduced Fd during growth under iron-limited conditions (Manzano, 1977). Both Nar and Nir are conveniently assayed *in vitro* by using methyl viologen as electron donor (Manzano *et al.*, 1976; Martin-Nieto *et al.*, 1989). Cyanobacterial nitrate reductases are single polypeptides of about 75 kDa (Candau, 1979; Mikami & Ida, 1984; Martin-Nieto *et al.*, 1992). They contain molybdenum (Peschek, 1979; Herrero & Guerrero,

1986; Martin-Nieto *et al.*, 1990), which is integrated into a Mo-cofactor (Martin-Nieto *et al.*, 1990), and non-heme iron and acid-labile sulfide (Candau, 1979; Mikami & Ida, 1984). Nitrate reductase from *Plectonema boryanum* contains two Fe_2S_2 clusters (Mikami & Ida, 1984). Expression of the genes encoding the proteins involved in the uptake and reduction of nitrate, i.e., *nrt*ABCD or *nrtP* for the nitrate/nitrite transport (NRT), *nar*B for nitrate reductase (NR), and *nir*A for nitrite reductase (Nir), in cyanobacteria, are negatively regulated by ammonium (Elhai & Wolk, 1988; Suzuki *et al.*, 1993; Merchan *et al.*, 1995; Frias *et al.*, 1997; Sakamoto *et al.*, 1999). In addition, cyanobacteria have a number of other ammonium-repressible genes related to nitrogen metabolism. Expression of these genes commonly requires a Crp-type transcriptional regulator protein, Ntc A (Vega-Palas *et al.*, 1992; Herrero *et al.*, 2001). In addition to ammonium-promoted negative regulation, positive regulation of the nitrate assimilation operon by nitrite has been found in *P. boryanum* and *Synechococcus* sp. strain PCC 7942 (Kikuchi *et al.*, 1996).

1.3.2 Ammonium transport and assimilation

Ammonia (NH_3/NH_4^+) is generated within the cell by a variety of processes, including N_2-fixation, nitrate/nitrite reduction, protein turn over/amino acid catabolism etc. Ammonia can also be directly taken up from the outer medium and cyanobacteria are in general able to grow with ammonia as N-source. While NH_3 diffuses across the membrane and can be trapped in the cell by protonation, movement of exogenous NH_4^+ into the cell requires transport. [^{14}C]-methylammonium can be used as a probe to study the ammonium transport system. In

Synechococcus sp. strain PCC 7942 and *Anabaena variabilis* strain ATCC 29413, time-cource experiments showed a rapid accumulation of [^{14}C]- methylammonium within the cells followed by the formation, which proceeds at a slower rate, of a product of [^{14}C]-methylammonium metabolism via GS (Boussiba *et al.*, 1984*a*; Rai *et al.*, 1984). The accumulation of [^{14}C]-methylammonium is hampered by a number of metabolic inhibitors including some ionophores (Boussiba *et al.*, 1984*a*; Rai *et al.*, 1984; Bhattacharya *et al.*, 2002*a*). Ammonium transporters have been characterized in numerous organisms. They are mono-component permeases necessary for uptake of ammonium when it is present at low concentrations (i.e., below 1μM) in the extracellular medium or when the organisms grow in a rather acidic medium. Three genes encoding ammonium permeases have been found in the chromosomes of *Synechocystis* sp. strain PCC 6803 (Kaneko *et al.*, 1996; Montesinos *et al.*, 1998). These three genes are expressed at the highest levels when *Synechocystis* cells are incubated in a medium lacking a source of nitrogen. One of them (*amt*1) is responsible for most of the [^{14}C]-methylammonium transport activity when this substrate is provided at micromolar concentrations (Montesinos *et al.*, 1998). A homologue of Amt has been found responsible for ammonium-methylammoium uptake in *Synechococcus* sp. strain PCC 7942, and *amt* homologues are present in heterocyst-forming cyanobacteria and unicellular marine strains whose genomes have been sequenced (Kazusa DNA Research Institute; DOE Joint Genome Institute). The ammonium/methylammonium transport system is repressed in ammonium-supplemented cultures (Boussiba *et al.*, 1984*a*; Vega-Palas *et al.*, 1990), and under these conditions, passive diffusion of NH_3 followed by its protonation in the cell

allows net uptake. The ammonium transport system plays a role in recapturing of NH_3 leaked from cells growing on nitrate or N_2 (Boussiba *et al.*, 1984*a*; Rai *et al.*, 1984). A biphasic [^{14}C]-methylammonium uptake pattern consisting of a fast initial phase equilibrating within 60 sec, followed by a slower second phase has been found in *Anabaena cycadeae* (Singh *et al.*, 1985), *Anabaena variabilis* (Rai *et al.*, 1984), *A. nidulans* (Boussiba *et al.*, 1984*a*) and *Nostoc* ANTH (Bhattacharya *et al.*, 2002*a*). The first phase of the uptake corresponds with [^{14}C]-methylammonium accumulation in the cells while the second phase is linked with the GS activity (Boussiba *et al.*, 1984*a*; Rai *et al.*, 1984; Boussiba & Gibson, 1985). Ammonium transport studies on a GS defective mutant of *Anabaena cycadeae* showed that the second phase of ammonium transport may be a separate ammonium transport system (ATS) thus suggesting the occurrence of two ATS. A faster MSX-insensitive ATS and a slower MSX-sensitive one (Singh *et al.*, 1985).

Ammonium is incorporated into organic constituents of the cell as glutamate by means of glutamate dehydrogenase (GDH) or by a cycle of reactions catalyzed by GS and GOGAT. The notion that ammonium assimilation in cyanobacteria takes place primarily via the GS/GOGAT cycle originated from the observation that, whereas GDH activity is very low or even undetectable in some cyanobacteria (Hoare *et al.*, 1967; Neilson & Doudoroff, 1973), high levels of GS activity could be found in *Anabaena cylindrica* (Dharmawardene *et al.*, 1972). MSX, an inhibitor of GS activity was observed to depress severely the assimilation of newly fixed ammonium in N_2-fixing cultures of *A. cylindrica* (Stewart & Rowell, 1975), and GOGAT was detected in the heterocyst-forming cyanobacteria (Lea & Miflin, 1975). Wolk *et al.* (1976)

established that GS/GOGAT cycle is the major pathway for the assimilation of ammonium in cyanobacteria while working with *A. cylindrica*. When $^{13}N_2$ or $^{13}NH_4^+$ was made available to *A. cylindrica* and other N_2-fixing cyanobacteria, the first labeled organic compound was found to be glutamine followed by glutamate (Wolk *et al.*, 1976; Meeks *et al.*, 1977, 1978). In the presence of MSX, the formation of [^{13}N] glutamine and [^{13}N] glutamate was inhibited (Wolk *et al.*, 1976; Meeks *et al.*, 1977). Thus, the GS/GOGAT cycle is established to be the major pathway of NH_4^+assimilation in N_2-fixing and non-N_2-fixing cyanobacteria (Flores *et al.*, 1983*b*; Boussiba *et al.*, 1984*b*, Boussiba, 1989; Merida *et al.*, 1991) and this is reported to be independent of the N-source used for growth (Wolk *et al.*, 1976; Meeks *et al.*, 1977; Flores *et al.*, 1983*b*). Specific activity of GOGAT is similar in cells grown on nitrate or N_2 (Meeks *et al.*, 1977; Flores *et al.*, 1987) and its activity in ammonium-grown cultures is only reduced to half of that found in N_2-or nitrate-grown cultures (Meeks *et al.*, 1977; Rowell *et al.*, 1977; Stacey *et al.*, 1977; Vega-Palas *et al.*, 1990).

GS accounts for about 0.52 % of the total cell protein and has been purified from a number of cyanobacteria (Stacey *et al.*, 1977; Sampio *et al.*, 1979; Orr *et al.*, 1981; Florencio & Ramos, 1985; Blanco *et al.*, 1989; Merida *et al.*, 1990). The *gln*A gene (encoding GS) from *Anabaena* sp. strain PCC 7120 has been cloned by hybridization using the *gln*A gene from *Escherichia coli* as a probe (Fisher *et al.*, 1981) and sequenced (Tumer *et al.*, 1983). Other cyanobacterial *gln*A genes have been cloned either by using the *Anabaena* sp. strain 7120 *gln*A gene as a probe (Elmorjani *et al.*, 1992; Merida *et al.*, 1992) or by complementation of *E. coli* glutamine auxotrophs (Riccardi *et al.*, 1985; Wagner *et al.*, 1993). In *Anabaena* sp. strain PCC 7120,

transcription of *gln*A from the *E. coli*-like promoter takes place in ammonium-grown cells (Tumer *et al.*, 1983). Increased level of *gln*A mRNA is observed after N-starvation and this is consistent with the increased GS activity found in N_2-grown cells as compared to ammonium-grown cells. A second GS, encoded by the *gln*N has been identified in the *Synechocystis* sp. strain PCC 6803 (Reyes & Florencio, 1994). In general, the expression of *gln*A (Flores *et al.*, 1999) and *gln*N (Reyes *et al.*, 1997; Crespo *et al.*, 1998; Sauer *et al.*, 2000) is lower in cells grown with ammonium than in nitrate or in absence of combined nitrogen sources. In both *Plectonema boryanum* and *Synechocystis* sp. strain PCC 6803, the genes encoding a ferredoxin dependent (*gls*F) and an NADH-dependent (*glt*B and *glt*D) glutamate synthase have been characterized (Kaneko *et al.*, 1996; Okuhara *et al.*, 1999). The contribution of ferredoxin-glutamate synthase to the photoassimilation of nitrogen is dominant over that of NADH-glutamate synthase when high level of carbon is available (Okuhara *et al.*, 1999). *Gls*F encoding a ferredoxin-dependent enzyme has been found in *Anabaena* sp. strain PCC 7120 (Martin-Figueroa *et al.*, 2000). 2-oxoglutarate is the carbon skeleton used for incorporation of ammonium through the glutamine synthetase-glutamate synthase cycle and is provided by $NADP^+$-isocitrate dehydrogenase (Muro-Pastor & Florencio, 1992, 1994). *icd* gene encoding the isocitrate dehydrogenase expression is highest under nitrogen stress in *Synechocystis* sp. strain PCC 6803 or under N_2-fixing conditions in *Anabaena* sp. strain PCC 7120 (Muro-Pastor *et al.*, 1996). 2-oxoglutarate is an appropriate sensor of the C:N balance of the cyanobacterial cells (Forchhammer, 1999) and its cellular level influenced by the nitrogen source (Merida *et al.*, 1991; Coronil *et al.*, 1993; Tapia *et al.*, 1996). The

presence of ammonium in the culture causes inactivation of the type I-glutamine synthetase in *Synechocystis* sp. strain PCC 6803, with reactivation taking place in response to ammonium withdrawal (Merida *et al.*, 1991). Inactivation involves the noncovalent binding of a phosphorylated compound (Merida *et al.*, 1991) or binding of one or two of the inactivating factors IF7 and IF17, the product of *gif*A and *gif*B genes respectively (Garcia-Daminguez *et al.*, 1999). These genes are quickly induced in response to the addition of ammonium in *Synechocystis* cells. An ammonium-triggered decrease (other than repression) in glutamine synthetase has been observed in *Synechocystis* PCC 6803 but not in other cyanobacteria (Merida *et al.*, 1990, 1992; Rowell *et al.*, 1979).

1.4 Amino acid transport and assimilation

Cyanobacteria are autotrophs, and can use amino acids as nitrogen or carbon sourses (Neilson & Larsson, 1980; Vaishampayan, 1982; Rawson, 1985; Spence & Stewart 1986). Amino acids like Arg, Asn, Gln can be used as sources of nitrogen by a number of cyanobacteria (Thiel & Leone, 1986; Herrero & Flores, 1990; Singh *et al.*, 1991; Flores & Herrero, 1994) while Arg has been described to serve a source of carbon for *Synechocystis* (*Aphanocapsa*) sp. strain PCC 6803 (Weathers *et al.*, 1978). Some amino acids like glutamate, histidine and lysine are reported to be growth inhibitory (Chapman & Meeks, 1983; Flores & Muro-Pastor, 1990; Prakasham *et al.*, 1991).

Amino acid transport systems present in cyanobacteria have been characterized in detail only for the unicellular *Synechocystis* sp. strain PCC 6803 (Labarre *et al.*,

1987; Flores & Muro-Pastor, 1990) and the filamentous heterocyst-forming *Anabaena* sp. strain PCC 7120 (Flores & Muro-Pastor, 1988; Herrero & Flores, 1990; Montesinos *et al.*, 1995; Xu & McAuley, 1990). Three amino acid transport systems, one specific for basic amino acids and glutamine, one specific for neutral amino acids (excluding glutamine), and another one specific for the glutamate and glutamine have been described in *Synechocystis* PCC 6803 (Labarre *et al.*, 1987). In *Anabaena* PCC 7120, three high affinity amino acid transport systems have been defined: one for basic amino acids (Herrero & Flores, 1990) and two for neutral amino acids (Montesinos *et al.*, 1995). Some of the transport systems have a role in the recapture of amino acids that have leaked from the cells as well as in the intercellular transfer of amino acids that takes place in the *Anabaena* filaments during diazotropic growth (Montesinos *et al.*, 1995). Two genes, *nat*A and *nat*B, encoding elements of an ABC type permease for neutral amino acids (conserved component Nat A, and periplasmic binding protein, Nat B) were identified by insertional mutagenesis in *Synechocystis* PCC 6803 open reading frames from the recently produced genomic DNA sequence of this cyanobacterium (Montesinos *et al.*, 1997). *Fischerella muscicola* UTEX 1829, a heterocyst forming cyanobacterium that belongs to the taxonomic section V (Rippka *et al.*, 1979), showed neutral and acidic amino acid uptake activities which were ca. 30% and 50% of those exhibited by *Anabaena* PCC 7120, respectively. Uptake activities of the basic amino acids Arg and Lys in strain *F. muscicola* UTEX 1829 were rather low, and at 6 and 13% of the values observed in *Anabaena* PCC 7120, respectively (Montesinos *et al.*, 1997). Arginine was shown to be taken up and concentrated within the cells of *Synechocystis* PCC 6803 and *Anabaena* PCC 7120 by

means of transport systems specific for basic amino acids rather than arginine only (Labarre *et al.*, 1987; Flores & Muro-Pastor, 1990; Herrero & Flores, 1990). This basic amino acid transport system is of binding protein-dependent type (Flores & Muro-Pastor, 1990). Two kinetic components, one corresponding to a high affinity system that catalyzes a concentrative (active) transport, and another one corresponding to a low affinity system that mediates a non concentrative (passive) transport are observed for Arg transport in *Anabaena* PCC 7120 (Herrero & Flores, 1990). In cells of *Anabaena,* a high affinity transport system for Gln (Ks, 14-33 μM) is evident (Rowell *et al.*, 1977; Chapman & Meeks, 1983; Flores & Muro-Pastor, 1988) and it is a broad specificity system that is able to transport neutral amino acids and Glutamate (Chapman & Meeks, 1983; Flores & Muro-Pastor, 1988). A second low affinity glutamine transport activity (Ks, 1.1 mM) is observed in *Anabaena variabilis* ATCC 29413 (Chapman & Meeks, 1983). Glutamine transport in cells of *Synechocystis* PCC 6803 have two kinetic components (Ks, 0.01 & 5 mM, respectively), the low affinity activity being attributable to the amino acid transport system (Labarre *et al.*, 1987; Flores & Muro-Pastor, 1990).

1.3 Thermophilic cyanobacteria

Blue green algae (cyanobacteria) can be classified as thermophilic (high temperature), mesophilic (room temperature), or psychrophilic (low temperature), according to the temperature of the culture media in which these organisms grow. Temperature is one of the most important environmental factors controlling the activities and evolution of organisms, and is one of the easiest variables to measure. High temperature

environments are of especial interest, in that they reveal the extremes to which evolution has been pushed. The high temperature environments most useful for study are those associated with volcanic activity, such as hot springs, since these natural habitats have probably existed throughout most of the time in which organisms have been evolving on earth (Brock, 1967). Geothermal environments (hot springs) are home to a diverse biota of thermophilic organisms. Scientific exploration for life in geothermal environments has accelerated in recent decades, and dramatically new forms of life have been discovered in recent years. The biotechnology revolution has been profoundly accelerated by discovery of heat-stable enzymes such as the DNA polymerases produced by the hot spring bacterium, *Thermus aquaticus* (Brock, 1978). Thermophiles use numerous strategies that enable them to survive fluctuating temperatures in the environment, where exposure to supra optimal temperatures is probably the normal rather than the exception (Reysenbach & Shock, 2002). In order to thrive, they use mechanisms like thermal stable designs of proteins, heat shock adaptations, and efficient DNA repair (Trent *et al.*, 1991). At high temperature DNA stability is reduced and mutational frequencies increase, providing many dilemmas for thermophiles. Many produce proteins that help stabilize DNA such as reverse gyrases (Forterre *et al.*, 2000) and histones (Sandman & Reeve, 2000). Phycocyanin, a major biliprotein extracted from the cyanobacteria, can be used as a model to investigate the effect of variations in environmental stress on the structure and function of proteins (Chen & Berns, 1978).

A number of thermophilic cyanobacteria have been isolated and cultured under laboratory conditions (Castenholz, 1969a, 1970). Experimental use has been made of

thermophilic *Oscillatoria* (Castenholz, 1968, 1971), *Mastigocladus* (Holton, 1962), and several strains of *Synechococcus* (Dyer & Gafford, 1961; Berns & Scott, 1966; Peary & Castenholz, 1964; Edward *et al.*, 1968; Sheridan & Castenholz, 1968). *Mastigocladus laminosus* is a cosmopolitan thermophilic cyanobacterium found in thermal waters on every continent (Castenholz, 1969*a*). High temperature and low combined nitrogen source in the hot springs, favour the growth of such heterocystous cyanobacteria as *Chlorogloeopsis* and *Mastigocladus* (Ward & Castenholz, 2000). *M. laminosus* is a remarkably hardy organism capable of cell division and growth from 5°C to about 64°C (Holton, 1962; Castenholz, 1969*b*; Stevens *et al.*, 1985) and from a pH of 4.8 to 9.8 (Brock & Brock, 1970; Binder *et al.*, 1972). It is also the most thermophilic nitrogen-fixing cyanobacterium with an upper temperature limit for nitrogen-fixation of up to 60°C (Stewart, 1970). In addition, *M. laminosus* is among the morphologically most complex microorganisms (Nierzwicki *et al.*, 1982, 1984*a*, *b*; Balkwill *et al.*, 1984*a*; Stevens *et al.*, 1985; Hernandez-Muniz & Stevens, 1987). The morphological complexity of this cyanobacterium has been documented through use of light microscopy (Schwabe, 1960; Marcenko, 1961; Rippka *et al.*, 1979) and scanning and transmission electron microscopy (Nierzwicki *et al.*, 1982; Nierzwicki-Bauer *et al.*, 1984*a*). Under nitrogen-fixing conditions, *M. laminosus* contains narrow and wide vegetative cells that may differentiate into heterocysts (Nierzwicki-Bauer *et al.*, 1984*a*, *b*). Typically, the heterocysts retain the general shape of the vegetative cells from which they differentiate (Nierzwicki-Bauer *et al.*, 1984*b*). Wide cells serve as the starting point for new narrow-celled trichomes and may also give rise to true branches in the vegetative trichome, which are also of the narrow-celled morphology

(Balkwill *et al.*, 1984*b*; Nierzwicki *et al.*, 1982). The narrow celled trichomes produced by the wide cells, may in turn, differentiate into motile hormogonia that apparently move by gliding motility (Gorbunova, 1975; Castenholz, 1982; Hernandez-Muniz & Stevens, 1987). *M. laminosus* has been investigated from an ecological standpoint (Castenholz, 1976, 1977; Fagerberg & Arnott 1979) and for its potential use in biophotolysis of water (Miyamoto *et al.*, 1979*a*, *b*; Miura *et al.*, 1980). The ability of *M. laminosus* to utilize gaseous nitrogen was first documented by Fogg (1951). This species is ecologically important as a component of algal-bacterial mats in neutral to alkaline thermal springs. It is one of the few thermophilic cyanophytes recognized as truly cosmopolitan (Castenholz, 1973, 1978). Field studies have shown that *in situ* populations of *M. laminosus* possess active nitrogenase even at 55°C (Billaud 1967; Stewart 1970; Wickstrom & Wiegert, 1980).

Immobilized filaments of *M. laminosus* can function as an anodic photoelectrode and serve as an electron donor for production of hydrogen (Ochiai *et al.*, 1980). ATP can be generated photosynthetically using a live *Mastigocladus* sp. cells without the need for chromatophore preparation and immobilization (Sawa *et al.*, 1982). The purified reaction center of PSI in *M. laminosus* retains its temperature stability and remains active in cytochrome c photooxidation (Nechushtai *et al.*, 1983). There is immunological cross reactivity between PSI reaction centers of *M. laminosus* and of higher plants and green algae indicating a common evolution of the photosystem I reaction centers (Nechushtai *et al.*, 1983). The isolated PSII complex from the thermophilic cyanobacterium *Synechococcus elongatus* also remains stable in terms of both protein composition and function, exhibiting no release of extrinsic proteins,

or proteolytic degradation in any of its subunits. These properties are highly useful for various types PSII studies (Sagiura & Inoue, 1999). An important milestone in research on photosynthesis was achieved from the study of PSII isolated from *S. elongatus* (Zouni *et al.*, 2001). The structural feature of PSII and spectroscopic studies provided novel and much deeper insights into the functioning of PSII than was previously available. Isolated PSII from *S. elongatus* was studied, as proteins from thermophilic organisms are better suited for crystallographic studies than those from mesophilic organisms and since PSII isolated from higher plants could not be crystallized in a form suitable for high resolution X-ray diffraction studies. The stable nature of the PSII complex from the thermophilic cyanobacterium *S. elongates* has been successfully used in building stable and high sensitivity PSII-based biosensors for herbicides (Koblizek *et al.*, 1998).

1.6 **Present Study**

High temperature organisms remained curiosities until the molecular, phylogenetic, and genomic studies during the past two decades moved them to the center stage in the context of the mechanisms of evolutions, the depth of the biosphere, mineral-microbe relations, the origins of ecosystems, the emergence of life, and the potential for life on other planets. In addition to *in-situ* ecological experiments, studying microbial isolates or corsortia under laboratory conditions provide further clues to their growth, survival, and ecological adaptation in the environment. Genomes of many thermophilic have been sequenced over the past 6 years and these databases

provide a source for exploring ecological potential and for linking gene expression with environmental parameters (Reysenbach & Shock, 2002).

Molecular, physiological, and ecological studies of thermophiles have had ramifications for fundamental processes and questions in biology. Environmental conditions are some of the strongest forces that drive evolution. Because hydrothermal system have prevailed throughout Earth's history, the extant organisms in these systems and their genomes are living records of changes over geological times (Klenk, 1999; Philippe & Forterre, 1999).

Studies of microbial isolates from hot spring may provide further clues to the growth, survival, and ecological strategies in the adverse environment. N_2-fixing cyanobacteria are important contributors to N-economy of rice fields, however mostly mesophilic strains are employed as biofertilizers. A thermophilic strain capable of N_2-fixation over a wide range of temperatures will be of great use in rice fields of tropics. Keeping this in view, the thermophilic cyanobacterium *Mastigocladus laminosus* (growing in the hot spring of Jakrem, Meghalaya) was chosen for the present study. I have studied the morphological and physiological features of this cyanobacterium including N_2-fixation, transport and assimilation of nitrate, ammonium and amino acids, and akinete differentiation and germination at normal and elevated temperatures. In addition, DNA fingerprinting was done to generate a genetic identity for this strain. It is expected that the information generated through this study would enable exploitation of *M. laminosus* as an efficient biofertilizer in tropical rice cultivation.

CHAPTER 2

Materials and Methods

2.1 Isolation and purification of *Mastigocladus* sp

The cyanobacterium *Mastigocladus* species used in the present study was isolated from the hot spring at Jakrem, West Khasi Hills, Meghalaya. The cyanobacterial samples were collected from the hot spring and brought to the laboratory. The samples were examined using light microscope, washed with double distilled water and then homogenized using glass beads. The homogenized samples were then plated on sterilized nitrogen-free D-medium (Castenholz, 1981) with 1.5 % agar. The plates were incubated at 45°C under continuous light (photon fluence rate of 50 μmol. m^{-2}. s^{-1}). When colonies appeared, these were picked up from the plates and viewed under the microscope before subsequent re-plating on D-medium. The process was repeated several times until well-separated colonies were obtained. The colonies were then selected and purified by plating on solidified D-medium, containing polymixin-B sulphate (10 μg. ml^{-1}) and cycloheximide (100 μg. ml^{-1}). The individual *Mastigocladus* colonies were picked up under aseptic conditions and transferred to sterilized D-medium in test tubes. The procedures were repeated till axenic cultures of *Mastigocladus* sp. were obtained. Stocks were maintained on solidified D-medium in test tubes in the culture room.

2.2 Identification of the isolated *Mastigocladus*

The organism was identified as *Mastigocladus laminosus* by observations under light microscope. PCR fingerprints using STRR-1A primer was obtained as described by Rasmussen & Svenning (1998) to generate a genetic identity.

2.3 Culture methods

2.3.1 Sterilization

All glass wares and nutrient media were autoclaved at 121°C (15 psi) for 15 min in an autoclave. The heat labile chemicals were sterilized by ultrafiltration using Whatman membrane filters of pore size 0.45 μm. Such chemicals were then added to previously sterilized nutrient medium.

2.3.2 Culture conditions

Mastigocladus laminosus was maintained on agar slants as well as in liquid D-N_2 medium or D-nitrate medium or D-NH_4-medium. Cultures were maintained at 25°C (culture room) or 45°C (inside a B.O.D. incubator) and light was provided at a photon fluence rate of 50 μmol. m^{-2}. s^{-1} on the surface of the vessels.

2.3.3 Culture media

D-nitrate medium (Castenholz, 1981).

Nutrients	Concentration
Nitrilotriacetic acid	0.52 mM
$NaNO_3$	8.24 mM
KNO_3	0.99 mM
Na_2HPO_4	0.78 mM
$MgSO_4.7H_2O$	0.41 mM
$CaSO_4.2H_2O$	0.35 mM
NaCl	0.14 mM
$FeCl_3$	0.002 mM
H_3BO_3	4.00 μM
$Co(NO_3)_2.6H_2O$	0.10 μM
$CuSO_4.5H_2O$	0.05 μM
$MnCl.4H_2O$	6-7 μM
$Na_2MoO_4.2H_2O$	0.05 μM
$ZnSO_4.7H_2O$	0.87 μM

D-N_2 medium: $NaNO_3$ and KNO_3 replaced by NaCl and KCl, respectively.

D-ammonium medium: 2 mM NH_4Cl added to the D-N_2 medium, buffered with 2 mM HEPES.

In experiments involving addition of amino acids, 1 mM L-amino acids were added and the medium was buffered with equimolar concentration of HEPES. In all cases, pH was adjusted to 7.5 before autoclaving.

2.4 **Morphology**

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

2.5 **Growth parameters**

The growth was measured using the following parameters:

2.5.1 **Chlorophyll *a***

Chlorophyll *a* was measured using the method as described by Mackinney (1941). Cells from 5 ml culture were centrifuged and resuspended in 5 ml of 100 % methanol and incubated for 10 min at 4°C in darkness. The chlorophyll *a* concentration was measured spectrophotometrically in the supernatant of the centrifuged extract at 663 nm by using the formula:

Chlorophyll *a* ($\mu g.\ ml^{-1}$) = Absorbance at 663 nm x 12.63.

2.5.2 **Phycobiliprotein**

The phycobiliprotein content [phycocyanin (PC), allophycocyanin (APC),

phycoerythrin (PE)] was determined according to Bennett and Bogorad (1973). Cultures were harvested, washed and resuspended in saline buffer (0.15 mM, pH 7.0). The cells were disrupted by using an ultrasonicator (Sonics & Materials, Inc. USA) fitted with a microprobe. The phycobiliprotein concentration was measured spectrophotometrically in the supernatant of the centrifuged extract by using the formula:

$$[PC] = \frac{OD_{615} - 0.475\ (OD_{652})}{5.34}\ mg/ml$$

$$[APC] = \frac{OD_{652} - 0.208\ (OD_{615})}{5.09}\ mg/ml$$

$$[PE] = \frac{OD_{562} - 2.41[PC] - 0.844\ [APC]}{9.62}\ mg/ml$$

2.5.3 **Measurement of protein content**

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

2.5.3.1 **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 an ultrasonicator (Sonics & Materials, Inc. USA) fitted with a microprobe. The

supernatant was collected after centrifugation at 3000 rpm for 5 minute and used for protein determination.

2.5.3.2 **Estimation of protein**

Reagents:

A: 2 % Na_2CO_3 in 0.1 N NaOH.

B: 1 % sodium potassium tartarate solution

C: 0.5 % $CuSO_4$ solution

D: 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 minute at room temperature and then 0.5 ml of reagent E 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.

2.6 **C:N Ratio**

The cells were harvested, rapidly washed with distilled water and dried by keeping them at 45°C for four days inside an oven. The dried samples were collected, and Carbon to Nitrogen (C:N) ratio of samples was determined using Vario III CHNS analyzer fitted with autosampler (Elementary Analysensysteme, GmbH, Germany).

2.7 **Oxygen exchange**

Oxygen evolution and consumption was measured by using a Clark-type oxygen electrode installed in a 3 ml Plexiglass container with magnetic stirring (Rank Brothers, England). Measurement involved adding 3 ml cyanobacterial culture to the sample chamber of the non-polarised electrode and allowing each sample to equilibrate for 5 minute while stirring. The electrode was then polarized and the linear rate of oxygen evolution was obtained in light supplied by tungsten filament bulb, which was shielded from the sample by water bath acting as heat filter (photon fluence rate at the surface of the sample chamber: 50 μmol. m^{-2}. s^{-1}). Oxygen consumption was measured in darkness (sample chamber wrapped with aluminium foil).

2.8 **Enzyme assays**

2.8.1 **Nitrogenase activity**

Nitrogenase activity was measured using acetylene reduction assay (Stewart *et al.*, 1967). 5 ml of cyanobacterial culture was placed in 15 ml serum stoppered vials. Acetylene gas was injected to a final concentration of 10 % (v/v) of air phase in the vials. The vials were incubated in light (photon fluence rate of 50 μmol. m^{-2}. s^{-1}) at

45°C. After 1 hour, 1 ml gas sample was analyzed for ethylene produced by using a Chemito gas chromatograph fitted with a poropak-T column (80-100 mesh; ⅛" x 2 m stainless steel) and a flame ionization detector. The oven temperature of the GC was maintained at 60°C during the operation.

2.8.2 **Glutamine synthetase (transferase) activity**

2.8.2.1 **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 disrupted by ultrasonication using an ultrasonicator (Sonics & Materials, Inc. USA) fitted with a microprobe. Glutamine synthetase (tranferase activity) was then assayed using the ultrasonicated cultures.

2.8.2.2 **Assay of Glutamine synthetase (transferase) activity**

This method for GS activity was essentially described by Sampio *et al.*, (1979). The reaction mixture contained in a final volume of 3 ml: 1 ml enzyme extract (ultrasonicated culture suspension), 40 mM Tris-HCl buffer pH 7.5, 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 45°C or at 25°C depending upon the temperature where the cultures were grown. 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 fomed was estimated from a standard curve that was prepared in the range of 0-200 nmol γ-glutamyl hydroxamate. ml^{-1}.

2.8.3 **Nitrate reductase activity**

Nitrate reductase (NR) activity was measured (Manzano *et al.*, 1976) using the ultrasonicated culture suspension. 5 ml of cyanobacterial culture was taken and centrifuged. The pellet was thoroughly washed 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 $MgCl_2$). The reaction mixture contained, in addition to ultrasonicated cultures, 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 0.1ml of 0.23 M $NaHCO_3$. After 10 min of incubation in darkness at 45°C or at 25°C depending upon the culture temperature, the reaction was terminated by adding 0.2 ml of 1 M zinc acetate. Subsequently nitrite formed was determined by the method of Snell and Snell (1949).

2.8.4 **Nitrite reductase activity**

Nitrite reductase (NIR) activity was measured using the ultrasonicated culture suspension (Arizmendi & Serra, 1990). 5 ml of cyanobacterial culture was centrifuged and the pellet was thoroughly washed with buffer containing 50 mM Tris (pH 7.5). The reaction mixture contained, in addition to ultrasonicated culture suspension, in a final volume of 1ml: 2.5 mM KNO_2, 90 mM Tris-HCL (pH 7.5), 3 mM methyl viologen and 20 mM sodium dithionate freshly prepared in 0.3 M

$NaHCO_3$. After 5 min of pre-incubation at room temperature without KNO_2, the reaction was started by addition of KNO_2. After 10 min at 45°C or 25°C (for cultures grown at 45°C and 25°C, respectively), the reaction was stopped by vigorous shaking to oxidize excess reductant. Subsequently remaining nitrite was determined by the method of Snell and Snell (1949).

2.9 **Nitrate and nitrite uptake**

Nitrate and nitrite uptake by cyanobacterial cultures was measured by following the disappearance of nitrate and nitrite (100 μM) from the external medium, respectively. The concentration of nitrite and nitrate were determined by the methods of Snell and Snell (1949) and Cawse (1967), respectively.

2.9.1 **Nitrate estimation**

2.9.1.1 **Reagents**

A: 2 % sulphamic acid.

B: 6.67 % $HClO_4$.

C: Sodium nitrate solution was prepared in the range of 100-500 nmol. ml^{-1}. This was used as standard.

2.9.1.2 **Procedure**

To 1 ml of sample, 1 ml of sulphamic acid and 3 ml of $HClO_4$ was added. The solution was mixed thoroughly and the absorbance was read at 210 nm. A calibration

curve was prepared by using sodium nitrate solution as standard for estimation of nitrate.

2.9.2 **Nitrite estimation**

Nitrite was estimated calorimetrically as described by Snell and Snell (1949).

2.9.2.1 **Reagents**:

A: 1 % (w/v) sulphanilamide in 3 M HCl.

B: 0.02 % (w/v) N-(1-Naphthylethylene 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.

2.9.2.2 **Procedure**

To 1 ml of sample, 1 ml of sulphanilamide solution and 1 ml of 1-Naphthyl ethylene dihydrochloride solution 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.

2.10 **Ammonium transport**

Ammonium uptake was studied using ^{14}C-methylammonium, an analogue of ammonium (Rai *et al.*, 1984). Exponentially grown cells of *M. laminosus* were harvested, washed in D-N_2-medium and then resuspended in fresh D-N_2-medium for 48 h at 45°C or 25°C. The cells were then harvested, resuspended in 10 mM HEPES-

NaOH buffer, pH 7.0, to a concentration of 10 µg Chl *a*. $ml^{-1}$and equilibrated for 1 h at 45°C or 25°C under light (photon fluence rate: 50 µmol. m^{-2}. s^{-1}) inside a B.O.D. incubator. The ^{14}C labeled methylammonium was added to a final concentration of 50 µM (specific activity 350 KBq. μmol^{-1}). Wherever needed, dichloro phenyl dimethyl urea (DCMU, 10 µM) or carbonyl cyanide chlorophenyl hydrazone (CCCP, 25 µM) were added to the cell suspension 30 min prior to the addition of labeled methylammonium, and were present during the experiments. At different time intervals, 400 µl samples were taken out rapidly and the cells were separated from their bathing medium by microcentrifugation through silicon oil/dinonyl phthalate (45/55, v/v) into perchloric acid/water (15/85, v/v) as previously described (Scott & Nicholls, 1980; Rai *et al.*, 1984). The ^{14}C in perchloric acid fraction was measured using a liquid scintillation counter (Model 1801, Beckman Instruments).

2.11 **Amino-acid transport**:

Glutamine, asparagine, alanine and arginine uptakes were measured using ^{14}C labeled glutamine, asparagine, alanine and arginine, respectively. Cells from NO_3-grown culture were harvested during the exponential growth phase, washed, resuspended and then incubated in fresh D-N_2-medium and in D-N_2-medium supplemented with 1 mM glutamine, asparagine, alanine or arginine for 48 h at 45°C or 25°C. After incubation, the cells were harvested and resuspended in 10 mM HEPES-NaOH buffer, pH 7.0, to a concentration of 10 µg Chl *a*. $ml^{-1}$and equilibrated for 1 h at 45°C or 25°C under light (photon fluence rate: 50 µmol. m^{-2}. s^{-1}) inside a B.O.D. incubator. The ^{14}C labeled glutamine (specific activity 256 KBq. μmol^{-1}),

asparagine (specific activity 63 KBq. μmol^{-1}), alanine (specific activity 71 KBq. μmol^{-1}) and arginine (specific activity 65 KBq. μmol^{-1}) were added to a final concentration of 50 μM. Wherever needed, chloramphenicol (1 $\mu g. ml^{-1}$), dichloro phenyl dimethyl urea (DCMU, 10 μM) or carbonyl cyanide chlorophenyl hydrazone (CCCP, 25 μM) were added to the cell suspension 30 min prior to the addition of labeled amino acid, and were present during the experiments. At different time intervals, 400 μl samples were taken out rapidly and the cells were separated from their bathing medium by microcentrifugation through silicon oil/dinonyl phthalate (45/55, v/v) into perchloric acid/water (15/85, v/v) as previously described (Scott & Nicholls, 1980; Rai *et al.*, 1984). The ^{14}C in perchloric acid fraction was measured using a liquid scintillation counter (Model 1801, Beckman Instruments).

2.12 **Chemicals**

All bio-chemicals were purchased from Sigma chemical company, USA. All glass wares used were borosil make. General chemicals and solvent were from Qualigen or Glaxo. Electrophoresis requirements were procured from Bangalore Genie and alcohol from Bengal Chemicals, Kolkata. Gases used were of highest purity from Sigma Gases and Services, New Delhi (helium) and Assam Air Products, Assam (N_2, O_2, H_2). Ethylene was obtained from Eurasian Associates, West Bengal.

CHAPTER 3

General characterization of *Mastigocladus laminosus*

3.1 Introduction

Temperature is one of the most important environmental factors controlling the activities and evolution of organisms, and is one of the easiest variables to measure. High temperature environments are of special interest, in that they reveal the extremes to which evolution has been pushed. The high temperature environments, such as hot springs, have probably existed throughout most of the time in which organisms have been evolving on earth (Brock, 1967). Such environments are home to a diverse biota of thermophilic organisms including N_2-fixing cyanobacteria. High temperature and low combined nitrogen source in the hot springs, favour the growth of heterocystous cyanobacteria e. g., *Chlorogloeopsis* and *Mastigocladus* (Ward & Castenholz, 2000).

Mastigocladus laminosus is a cosmopolitan thermophilic cyanobacterium found in thermal waters on every continent (Castenholz, 1969*a*, 1973, 1978). This species is ecologically important as a component of algal-bacterial mats in neutral to alkaline thermal springs (Castenholz, 1976, 1977; Fagerberg & Arnott, 1979). *M. laminosus* is reported to be a remarkably hardy organism capable of cell division and growth from 5°C to about 64°C (Holton, 1962; Castenholz, 1969*b*; Stevens *et al.*, 1985) and from a pH of 4.8 to 9.8 (Brock & Brock, 1970; Binder *et al.*, 1972). It is also the most thermophilic nitrogen-fixing cyanobacterium with an upper temperature limit of 60°C for nitrogen-fixation (Billaud, 1967; Stewart, 1970; Wickstrom & Wiegert, 1980). In

addition, *M. laminosus* is among the most morphologically complex microorganisms (Balkwill *et al.*, 1984a; Hernandez-Muniz & Stevens, 1987; Nierzwicki *et al.*, 1982; Nierzwicki-Bauer *et al.*, 1984*a*, *b*; Stevens *et al.*, 1985). *M. laminosus* may differentiate from narrow vegetative cells into wide vegetative cells (Nierzwicki *et al.*, 1982). Under nitrogen-fixing conditions, both narrow and wide vegetative cells may differentiate into heterocysts (Nierzwicki-Bauer *et al.*, 1984*a*, *b*). Typically, the heterocysts retain the general shape of the vegetative cells from which they differentiate (Nierzwicki-Bauer *et al.*, 1984*b*). Wide cells serve as the starting point for narrow-celled morphology and true branches in the vegetative trichome (Balkwill *et al.*, 1984*b*; Nierzwicki *et al.*, 1982). The narrow celled trichomes produced by the wide cells, may in turn, differentiate into motile hormogonia that apparently move by gliding motility (Gorbunova, 1975; Castenholz, 1982; Hernandez-Muniz & Stevens, 1987).

While mesophilic cyanobacteria have been extensively studied, studies on thermophilic cyanobacteria are far fewer. In the present study, thermophilic cyanobacterium *Mastigocladus laminosus* was isolated from a local thermal spring and characterized using such parameters as morphological features, growth, heterocyst frequency, enzymes of nitrogen metabolism (activities of nitrogenase, nitrate reductase, glutamine transferase synthetase), photosynthesis, respiration, and phycobiliproteins in cultures grown at different temperatures (25°C, 45°C) and growth media.

3.2 Materials and Methods

3.2.1 Culture conditions

Mastigocladus laminosus was grown in axenic aerated batch cultures in D-medium (Castenholz, 1981) at 45°C inside a B.O.D. incubator or at 25°C in a sterile culture room (photon fluence rate of 50 μmol. m^{-2}. s^{-1} on the surface of the vessels). The cultures were maintained on agar slants as well as in liquid media (D-N_2 medium or D-nitrate medium or D-NH_4-medium).

3.2.2 Morphological observations

Morphological features were studied using light microscope.

3.2.3 Physiological characterization

Growth (chlorophyll *a*, protein), heterocyst frequency, enzymes of nitrogen metabolism (activities of nitrogenase, nitrate reductase, glutamine transferase synthetase), photosynthesis, respiration, and phycobiliprotein content were studied in cultures grown on different nitrogen media at 25°C and 45°C. See Chapter-2 (Material and Methods) for full details.

3.2.4 DNA fingerprints

PCR-based DNA fingerprints using STRR 1A as primer (3′-CCCCTRACCCCTRACC-5′) were obtained for *M. laminosus* to generate a genetic identity for this organism. For comparison fingerprints of a few *Nostoc* strains were also obtained. The protocols used were identical to those described by Rasmussen & Svenning (1998).

3.3 Results and Discussion

3.3.1 Morphology and DNA fingerprints

Extensive cyanobacterial mats occurs at Jakrem hotsprings (Fig.3.1a). Examination of these mats under light microscope revealed that *Mastigocladus laminosus* is the only cyanobacterium present. This organism is a heterocystous branched-filamentous cyanobacterium belonging to Section V as per Rippka's classification of cyanobacteria (Rippka *et al.*, 1979). It exhibits true branching and three cell types: vegetative cells, heterocysts, akinetes (Fig.3.1b).

3.3.2 Growth, heterocyst and nitrogen fixation

When grown in N_2-medium at different temperatures, *M. laminosus* grew best at 45°C. I selected this temperature for all subsequent studies and for comparative purpose experiments were also run at room temperature (25°C). Studies on growth of *Mastigocladus laminosus* at 25°C and 45°C in media containing N_2, NO_3^- or NH_4^+ as inorganic nitrogen-source are presented in Figs 3.3 and 3.4, respectively. As in the case of other heterocystous cyanobacteria (Herrero & Flores, 1990; Flores & Herrero, 1994; Herrero *et al.*, 2001; Bhattacharya *et al.*, 2002*a*), *M. laminosus* was able to utilize all the three inorganic nitrogen sources for growth at 25°C. Nitrate served as the best source of nitrogen for growth (measured as increase in Chl *a*), followed by ammonium and then N_2. However, unlike most heterocystous cyanobacteria, *M. laminosus* also grew at 45°C. While the trend of relative growth performance in different nitrogen-media remained similar to that at 25°C, the growth was

significantly higher at 45°C than that at 25°C in all cases. A similar trend was observed with regard to protein content (Fig. 3.5).

In N_2-medium, *M. laminosus* differentiated heterocysts and showed nitrogenase activity (Table 3.1). Both heterocyst frequency and nitrogenase activity were higher at 45°C than that at 25°C. No heterocyst formation or nitrogenase activity was detected in NO_3^- or NH_4^+-medium at either of the temperatures. These observations are consistent with reported growth of *M. laminosus* in hotsprings with temperatures varying between 45-60°C (Binder *et al.*, 1972) and the repressive effect of NO_3^- and NH_4^+ on heterocyst formation and nitrogenase in heterocystous cyanobacteria (see Stewart, 1980; Bhattacharya *et al.*, 2002*a*).

3.3.3 Activities of nitrate reductase and glutamine synthetase (transferase)

The nitrate reductase activity in cyanobacteria is reported to vary in response to the nitrogen sources in the growth medium (Bagchi & Singh, 1984; Bagchi *et al.*, 1985*a*, *b*). Nitrate reductase activity was studied in *M. laminosus* cells grown in media containing different nitrogen sources at 25°C and 45°C (Fig 3.6). Cells grown at 25°C in N_2-medium showed a NR activity of 8.5 nmol. NO_2^- formed. min^{-1}. mg^{-1} protein. The activity was similar in cells grown in NO_3^--medium (9 nmol. NO_2^- formed. min^{-1}. mg^{-1} protein) but this was repressed by 54 % in cells grown in NH_4^+-medium (3.9 nmol. NO_2^- formed. min^{-1}. mg^{-1} protein). A similar pattern of NR activity was found in cells grown at 45°C in media containing different nitrogen sources. Furthermore, in all the media, NR activity in cells grown at 45°C was higher than that in corresponding cells grown at 25°C. These results indicated that in *M. laminosus*, NR

is ammonium-repressible that is derepressed in absence of ammonium. The ammonium-repressible nature of *M. laminosus* NR is consistent with findings in other cyanobacteria (Bagchi & Singh, 1984; Herrero *et al.*, 1981, 1985; Bagchi *et al*, 1985*a*, *b*; Martin-Nieto *et al.*, 1989; Rai *et al.*, 1992; Bhattacharya *et al.*, 2002*a*). The derepressible nature of *M. laminosus* NR is consistent with observations of Bagchi *et al.* (1985*a*) on *Nostoc muscorum* NR but in contrast to the NO_3^--inducible nature of NR in *Anabaena cycadeae* (Bagchi *et al*, 1985*b*).

Glutamine synthetase (transferase) activities were also studied in *M. laminosus* cells grown in media containing different nitrogen sources at 25°C and 45°C (Fig.3.7). In different nitrogen media, GS activities in cells grown at 45°C were higher or similar to that in corresponding cells grown at 25°C. GS activity was significantly higher in N_2-grown cells than in nitrate- or ammonium-grown cells both at 25°C and 45°C. The lower GS activity in nitrate- and ammonium-grown cells is consistent with earlier observations that sources of combined-nitrogen repress GS activity in cyanobacteria (Merida *et al.*, 1991; Frias *et al.*, 1994; Bhattacharya *et al.*, 2002*a*). The observations that NR and GS remain active, and infact show higher activity, at 45°C indicate that *M. laminosus* is indeed a thermophile and possesses mechanisms for NR and GS to function at higher temperatures.

3.3.4 Photosynthesis and respiration

Photosynthesis is very intimately linked to nitrogen status of the cells since nitrogen is a vital constituent of several photosynthetic components. Nitrogen deficiency is known to cause impairments of photosynthesis (Apte, 1996). N_2-fixing cultures

grown at 25°C showed O_2 evolution rates of 240 nmol O_2 evolved. μg^{-1} Chl *a*. h^{-1}. The rates were higher in NO_3^-- and NH_4^+-grown cultures (Fig.3.8). This is probably due to the increased nitrogen status leading to higher levels of photosynthetic accessory pigments (phycobiliproteins) of the cells grown in media containing combined-N sources such as nitrate and ammonium (See Section 3.3.5). While the trend of photosynthetic O_2 evolution by cells grown in N_2-, NO_3^--, and NH_4^+-media at 45°C was similar to those obtained at 25°C, the rates were higher at 45°C than the corresponding rates at 25°C.

Respiration rates (respiratory O_2 consumption) of cells grown in different nitrogen media showed a trend that was reverse of photosynthesis. At 25°C, higher rate of O_2 consumption occurred in N_2-grown cells (196 nmol O_2 consumed. μg^{-1} Chl *a*. h^{-1}), followed by NO_3^--grown cells (142 nmol O_2 consumed. μg^{-1} Chl *a*. h^{-1}) and NH_4^+-grown cells (122 nmol O_2 consumed. μg^{-1} Chl *a*. h^{-1}). Similar trend was observed at 45°C but cells grown in all the three media (N_2-, NO_3^-- and NH_4^+-media) showed rates of O_2 consumption that were more than 50 % higher than the corresponding rates at 25°C (Table 3.2). Higher photosynthetic activity coupled with lower respiration rates may explain the better rates of growth in media containing nitrate and ammonia (See Section 3.3.2).

These observations show that *M. laminosus* not only carries out photosynthesis and respiration at elevated temperatures (45°C) but it does so more effectively than that at 25°C.

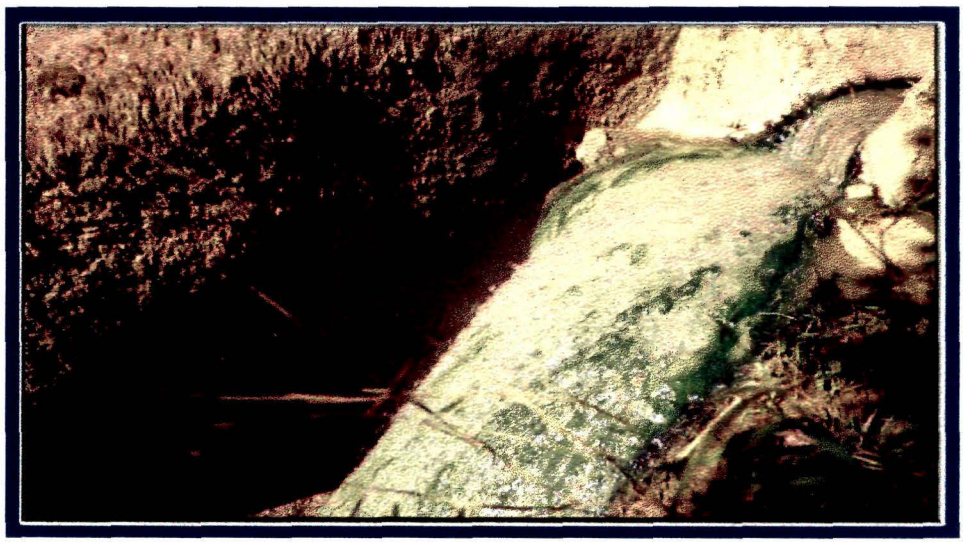
3.3.5 Phycobiliprotein content

The phycobiliproteins [phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE)] are accessory photosynthetic pigments in cyanobacteria. Phycobiliprotein content of *M. laminosus* cells grown in NO_3^-- and NH_4^+-media was higher than that in N_2-grown cells (Table 3.3). Furthermore, cells grown in NO_3^-- and NH_4^+-media at 45°C had significantly higher phycobiliprotein content than cells grown at 25°C. Higher phycobiliprotein content in cells grown with combined-N (NO_3^-- or NH_4^+-media) is consistent with earlier observations (Stewart, 1980). However, higher phycobiliprotein content at 45°C is unique to *Mastigocladus*, consistent with its thermophilic nature and observed better growth, activities of nitrogen metabolizing enzymes, photosynthesis and respiration at elevated temperatures.

Fig 3.1 (a) *Mastigocladus laminosus* mats in the hot spring at Jakrem (Meghalaya, India).

(b) A filament of *Mastigocladus laminosus* with heterocyst (h), akinete (a), vegetative cells (v) and branches (b). Magnification 40X.

a



b

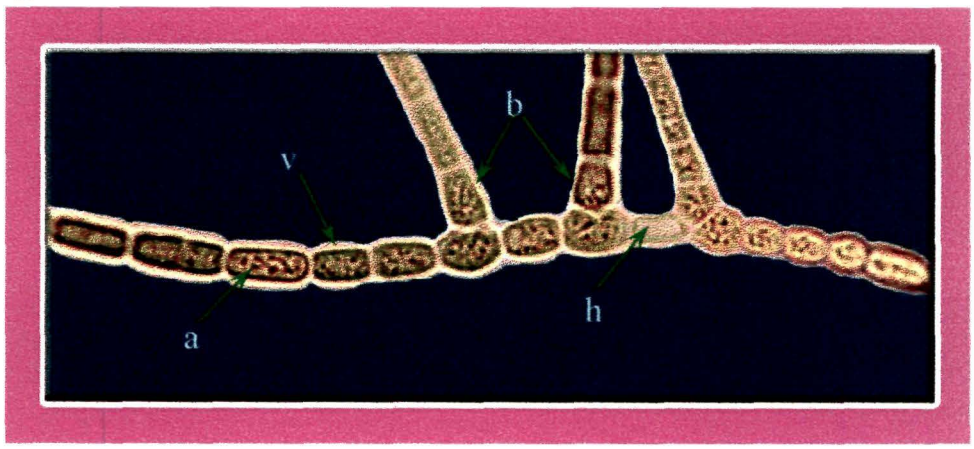


Fig 3.2 STRR 1A PCR-based DNA fingerprints of *Mastigocladus laminosus*. The fingerprints were obtained using short tandemly repeated repetitive sequences (STRR 1A) as primer and whole filaments of *Mastigocladus laminosus* as templates. The primer (STRR 1A) had the following sequence: 3'-CCCCTRACCCCTRACC-5'. Note that the *Mastigocladus* fingerprints are quite distinct from the 5 different *Nostoc* strain included for comparative purpose. M represents molecular weight markers (bp).

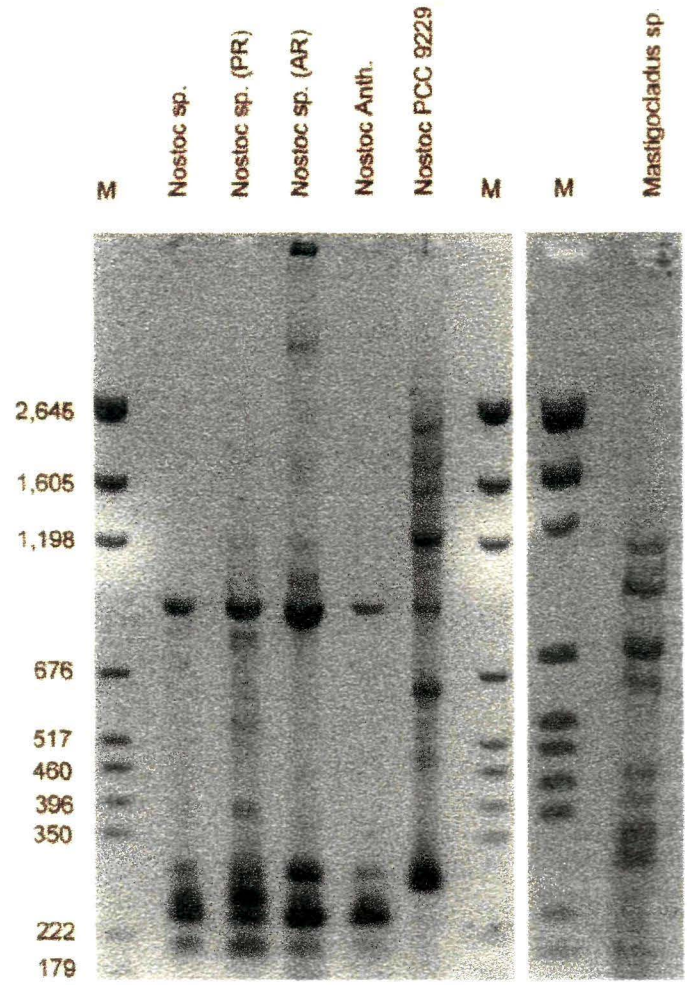


Fig 3.3 Growth of ***Mastigocladus laminosus*** in media containing different nitrogen sources at 25°C.

Growth in terms of μg Chl *a*. ml^{-1} was determined after intervals of five days. The initial inoculum concentration was 1.1 μg Chl *a*. ml^{-1}. N_2 refers to medium D without any N (•), NO_3^- to medium D with 10 mM $NaNO_3$, (▲) and NH_4^+ to medium D with NH_4Cl as N source (2 mM, ▼).

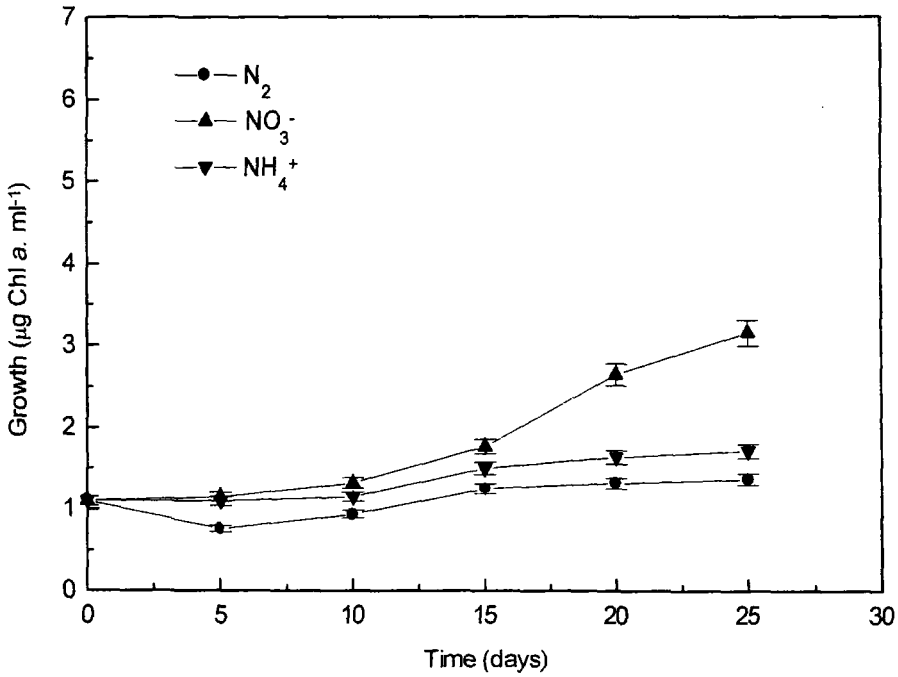


Fig 3.4 Growth of ***Mastigocladus laminosus*** in media containing different nitrogen sources at 45°C.

Growth in terms of μg Chl *a*. ml^{-1} was determined after intervals of five days. The initial inoculum concentration was 1.1 μg Chl *a*. ml^{-1}. N_2 refers to medium D without any N (•), NO_3^- to medium D with 10 mM $NaNO_3$ (▲) and NH_4^+ to medium D with NH_4Cl as N source (2 mM, ▼).

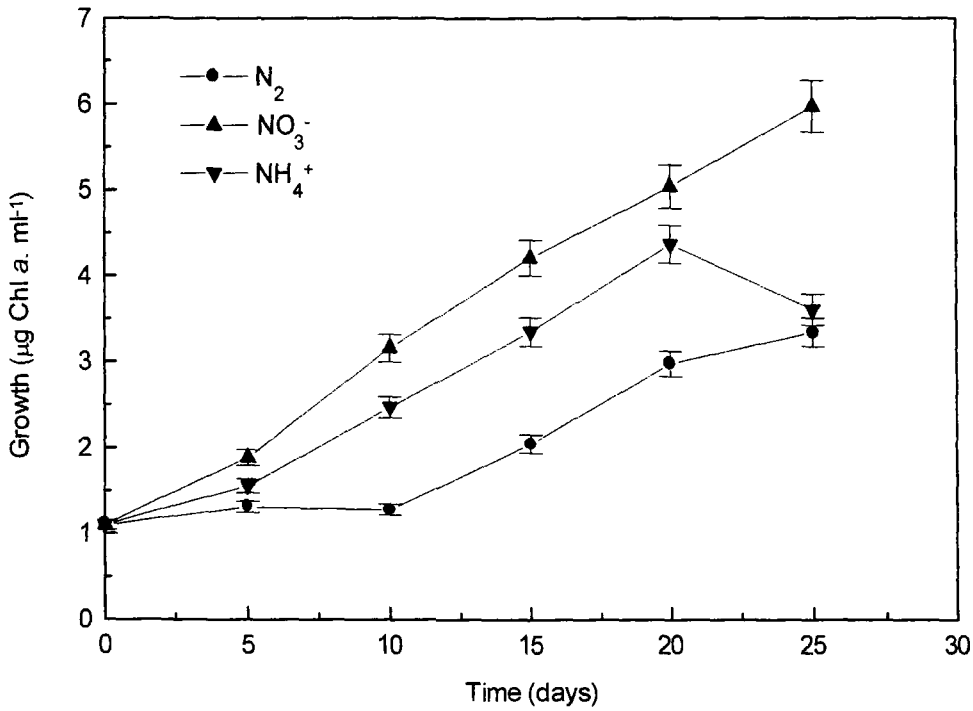


Fig 3.5 Protein content of ***Mastigocladus laminosus*** cells grown in media containing different nitrogen sources at 25°C and 45°C.

Protein concentration (μg. ml^{-1}) was determined after 4 days of inoculation to the fresh D-media (with and without N). N_2 refers to medium D without any N, NO_3^- to medium D with 10 mM $NaNO_3$ and NH_4^+ to medium D with NH_4Cl (2 mM) as N source.

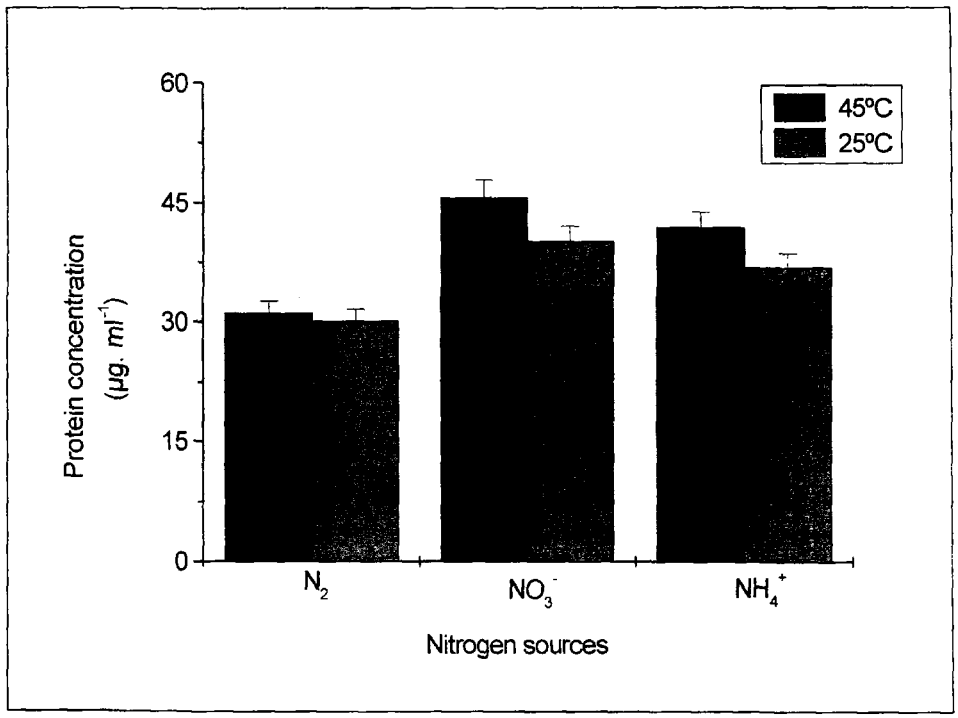


Table 3.1 Heterocyst frequency and nitrogenase activity of ***Mastigocladus laminosus*** cells grown in media containing different nitrogen sources at 25°C and 45°C.

Heterocyst frequency (%) and nitrogenase activity (nmol of C_2H_4 formed. μg^{-1} Chl *a*. hr^{-1}) were determined after 4 days of inoculation to the fresh medium-D with and without N (Castenholz, 1981). The initial inoculum concentration was 1.5 µg Chl *a*. ml^{-1}. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates. N_2 refers to medium D without any N, NO_3^- to medium D with 10 mM $NaNO_3$ and NH_4^+ to medium D with NH_4Cl (2 mM) as N source.

Medium	Heterocyst frequency (%)		Nitrogenase activity	
	45°C	25°C	45°C	25°C
N_2	10 ± 0.5	3 ± 0.2	4.2 ± 0.2	2 ± 0.1
NO_3^-	0.0	0.0	0.0	0.0
NH_4^+	0.0	0.0	0.0	0.0

Fig 3.6 Nitrate reductase (NR) activity of ***Mastigocladus laminosus*** cells grown in media containing different nitrogen sources at 25°C and 45°C.

Nitrate reductase (nmol NO_2^- formed. min^{-1}. mg^{-1} protein) was determined after 4 days of inoculation to the fresh D-media (with and without N). N_2 refers to medium D without any N, NO_3^- to medium D with 10 mM $NaNO_3$ and NH_4^+ to medium D with NH_4Cl (2 mM) as N source.

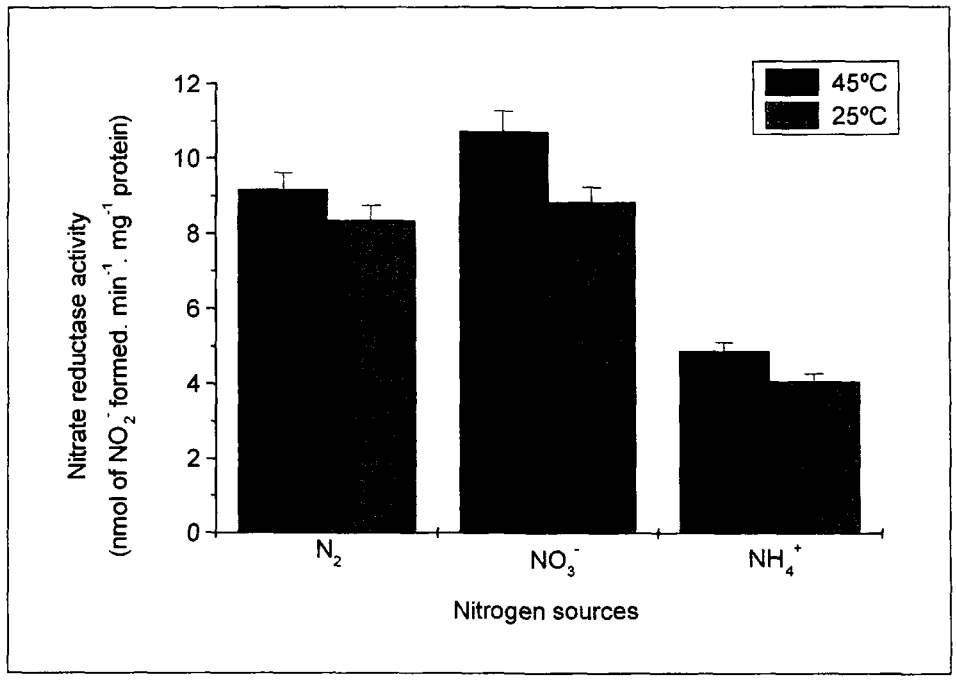


Fig 3.7 Glutamine synthetase (transferase) activity of ***Mastigocladus laminosus*** cells grown in media containing different nitrogen sources at 25°C and 45°C.

Glutamine synthetase (transferase) activity (nmol γ-glutamyl hydroxamate formed. min^{-1}. mg^{-1} protein) was determined after 4 days of inoculation to the fresh D-media (with and without N). N_2 refers to medium D without any N, NO_3^- to medium D with 10 mM $NaNO_3$ and NH_4^+ to medium D with NH_4Cl (2 mM) as N source.

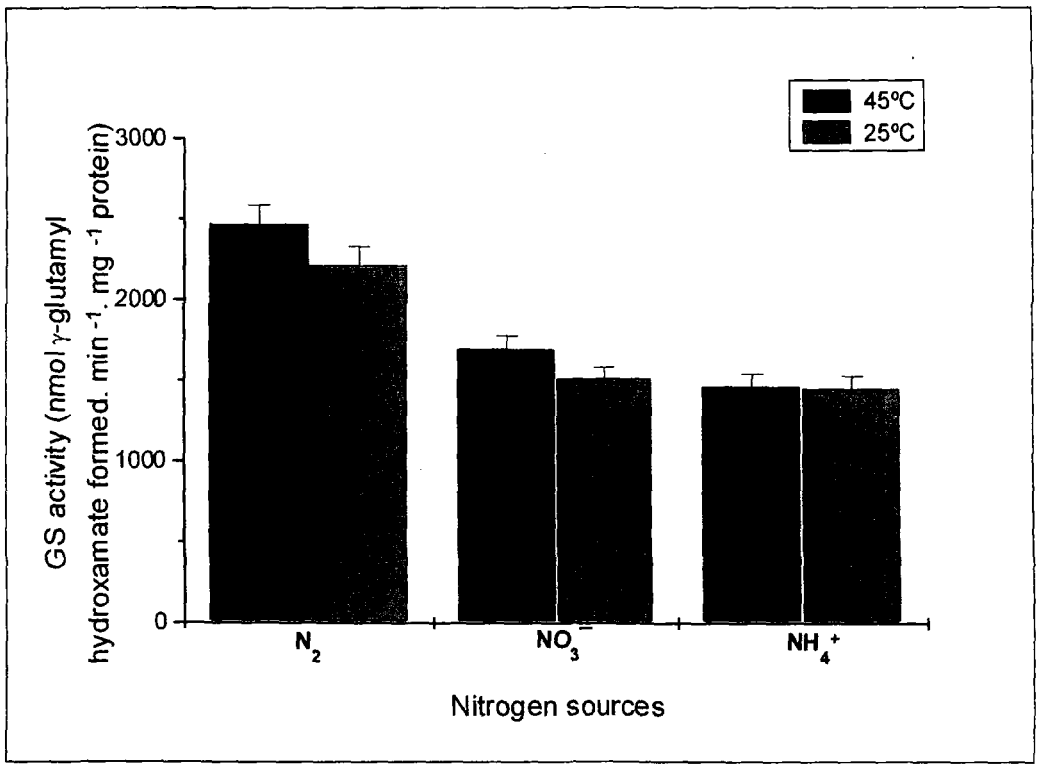


Fig 3.8 Rates of photosynthesis (oxygen evolution) in ***Mastigocladus laminosus*** cells grown in media containing different nitrogen sources at 25°C and 45°C.

Rate of photosynthesis (nmol of O_2 evolved. µg Chl *a*. hr^{-1}) was determined after 4 days of inoculation to the fresh D-media (with and without N). The initial inoculum concentration was 1.5 µg Chl *a*. ml^{-1}. N_2 refers to medium D without any N, NO_3^- to medium D with 10 mM $NaNO_3$ and NH_4^+ to medium D with NH_4Cl (2 mM) as N source.

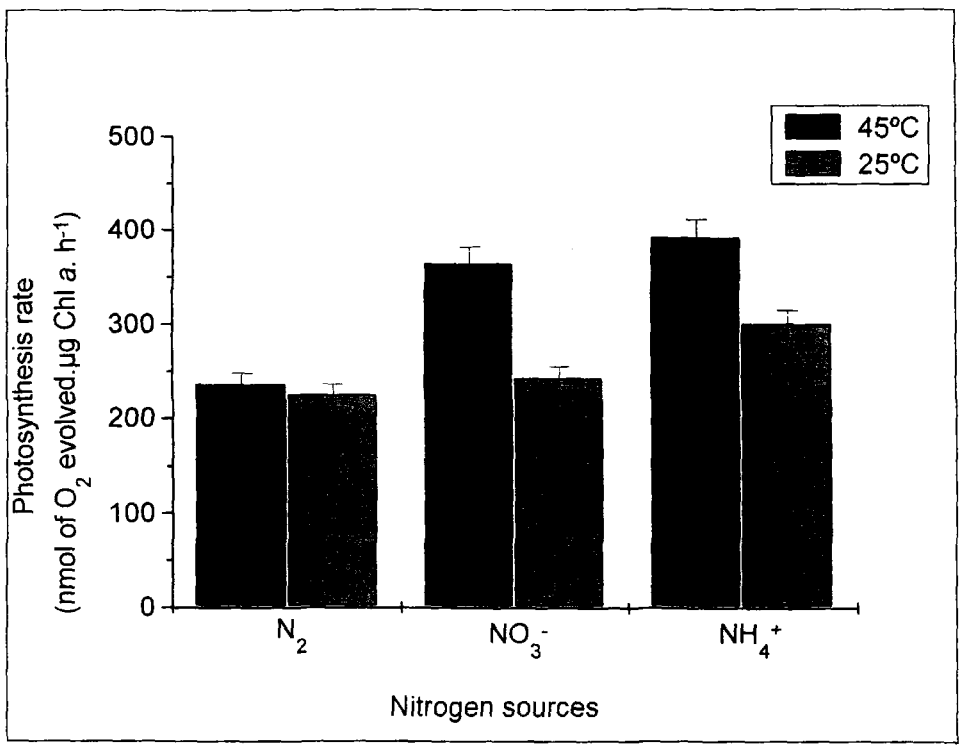


Table 3.2 Rates of respiratory O_2 consumption by ***Mastigocladus laminosus*** cells grown in media containing different nitrogen sources at 25°C and 45°C.

Respiration rate (nmol of O_2 consumed. μg Chl *a*. hr^{-1}) was determined after 5 days of inoculation to the fresh D-media (with or without N). The initial inoculum concentration was 0.72 μg Chl *a*. ml^{-1}. N_2 refers to medium D without any N, NO_3^- to medium D with 10 mM $NaNO_3$ and NH_4^+ to medium D with NH_4Cl (2 mM) as N source. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates.

Growth-medium	**Respiration rates** (nmol of O_2 consumed. μg Chl *a*. hr^{-1})	
	25°C	**45°C**
N_2	196.50 ± 9	293.50 ± 11
NO_3^-	142.87 ± 7	235.20 ± 11
NH_4^+	122.41 ± 6	187.76 ± 9

Table 3.3 Phycobiliprotein (phycocyanin, allophycocyanin, phycoerythrin) content of ***Mastigocladus laminosus*** cells grown in media containing different nitrogen sources at 25°C and 45°C.

Phycobiliprotein [phycocyanin (PC), allophycocyanin (APC), phycoerythrin (PE)] content (μg. ml^{-1}) was determined after 4 days of inoculation to the fresh D-media (with and without N). The initial inoculum concentration was 1.5 μg Chl ***a***. ml^{-1}. N_2 refers to medium D without any N, NO_3^- to medium D with 10 mM $NaNO_3$ and NH_4^+ to medium D with NH_4Cl (2 mM) as N source. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates.

Growth-medium	PC		APC		PE	
	25°C	45°C	25°C	45°C	25°C	45°C
N_2	2.12 ± 0.1	2.13 ± 0.1	2.01 ± 0.1	2.36 ± 0.1	1.48 ± 0.1	1.44 ± 0.1
NO_3^-	2.89 ± 0.1	3.65 ± 0.2	2.55 ± 0.1	2.74 ± 0.1	1.85 ± 0.1	2.17 ± 0.1
NH_4^+	2.79 ± 0.1	6.08 ± 0.3	2.40 ± 0.1	5.57 ± 0.3	1.79 ± 0.1	1.94 ± 0.1

CHAPTER 4

Nitrogen transport and assimilation in the thermophilic cyanobacterium, *Mastigocladus laminosus*

4.1 Introduction

Cyanobacteria are an ancient, large and diverse group of gram-negative, photoautotrophic prokaryotes characterized by their ability to perform oxygenic photosynthesis and biological N_2 fixation (Stewart, 1980; Bryant, 1994; Rai *et al.*, 2000; Whitton & Potts, 2000). Most cyanobacteria assimilate inorganic nitrogen compounds (nitrate, nitrite, dinitrogen and ammonia) as well as organic sources like urea and some amino acids (Herrero & Flores, 1990; Flores & Herrero, 1994; Herrero *et al.*, 2001). Nitrate assimilation involves active transport of nitrate into the cell, followed by its reduction to ammonium by sequential action of ferredoxin-dependent nitrate reductase and nitrite reductase, and finally the incorporation of ammonium into amino acids by the GS-GOGAT pathway (Flores & Herrero, 1994). An ATP-binding cassette (ABC) type transporter constituted by the products of the *nrt*A, *nrt*B, *nrt*C, and *nrt*D genes is involved in nitrate/nitrite uptake by cyanobacteria (Omata *et al.*, 1993; Luque *et al.*, 1994; Cai & Wolk, 1997; Frias *et al.*, 1997; Maeda & Omata, 1997; Sakamoto *et al.*, 1999; Wang *et al.*, 2000). In *Synechococcus* sp strain PCC 7942 and *Anabaena* sp strain 7120, the transporter genes are clustered together with the structural genes for nitrite reductase, *nir*A (Luque *et al.*, 1993) and nitrate reductase, *nar*B (Rubio *et al.*, 1996) constituting an operon (*nir* operon), with the

structure *nir*A-*nrt*ABCD-*nar*B (Luque *et al.*, 1992; Omata *et al.*, 1993; Suzuki *et al.*, 1993; Cai & Wolk, 1997; Frias *et al.*, 1997).

The nitrate uptake in cyanobacteria takes place through a high affinity nitrate transport system (Flores *et al.*, 1983*a*). Nitrate and nitrite are reported to share a common transport system (Madueno *et al.*, 1987; Rodriguez *et al.*, 1992). However, Bhattacharya *et al.* (2002*b*) recently showed that in *Nostoc* ANTH, there is a separate nitrite transport system quite distinct from the common nitrate/nitrite transport system. Regulation of cyanobacterial nitrogen metabolism involved inducible as well as repressible-derepressible systems. Nitrate is an inducer of nitrate uptake and assimilation (Bagchi *et al.*, 1985*a*, *b*; Martin Nieto *et al.*, 1989; Frias *et al.*, 1994, 1997). Ammonium represses uptake and assimilation of nitrate and nitrite (Bagchi *et al.*, 1985*a*, *b*; Martin Nieto *et al.*, 1989; Frias *et al.*, 1994, 1997), glutamine (transferase) synthetase (Flores & Herrero, 1994; Frias *et al.*, 1994), and ammonium transport (Rai *et al.*, 1984; Prakasham & Rai, 1991), whereas in the absence of ammonium, these proteins are derepressed (Rai *et al.*, 1984; Bagchi *et al.*, 1985*a*, *b*; Martin Nieto *et al.*, 1989; Prakasham & Rai, 1991; Frias *et al.*, 1994, 1997).

Ammonia (NH_3/NH_4^+) is generated within the cell by a variety of processes, including N_2-fixation, nitrate reduction, amino acid catabolism etc. Ammonia can also be directly taken up from the outer medium and cyanobacteria are in general able to grow with ammonia as N-source. NH_3 diffuses across the membrane and can be trapped in the cell by protonation, but the movement of exogenous NH_4^+ into the cell requires transport. [^{14}C]-methylammonium, an analogue of NH_4^+ can be used as a probe to study the ammonium transport system (Rai *et al.*, 1984).

Amino acids like Arg, Asn, Gln can be used as sole source of nitrogen by a number of cyanobacteria (Thiel & Leone, 1986; Herrero & Flores, 1990; Singh *et al.*, 1991; Flores & Herrero, 1994). The relative ability of different amino acids to support cyanobacterial growth varies greatly and requires further study. For example, *Synechocystis* PCC 6803 grows as well on arginine as on nitrate, but *Anabaena* sp PCC 7120 grow more slowly on arginine than on nitrate (Flores & Muro-Pastor, 1990; Herrero & Flores, 1990). Amino acid transport systems present in cynobacteria have been characterized in detailed only for the unicellular *Synechococcus* sp strain PCC 6803 (Labarre *et al.*, 1987; Flores & Muro-Pastor, 1990) and the filamentous heterocyst-forming *Anabaena* sp strain PCC 7120 (Flores & Muro-Pastor, 1988; Herrero & Flores, 1990; Xu & McAuley, 1990; Montesinos *et al.*, 1995). Studies on amino acid uptake and utilization of various nitrogen sources by *Nostoc* ANTH (an isolate from *Anthoceros*) showed that glutamine, asparagine, and arginine were good sources of fixed nitrogen and uptake of these amino acids were substrate-inducible and energy-dependent. The induction by substrate required *de novo* protein synthesis (Bhattacharya *et al.*, 2002*a*).

M. laminosus is a heterocystous, branched filamentous cyanobacterium frequently encountered in hot springs throughout the world (Castenholz, 1969*a*; Gloaguen *et al.*, 1999). In the present study, uptake and assimilation of nitrate, nitrite, ammonium (methylammonium) and amino acids were investigated in *Mastigocladus laminosus* isolated and purified from the hot spring of Jakrem in Meghalaya, India.

4.2 Materials and Methods

4.2.1 Strain and culture condition

Axenic cultures of the diazotrophic, thermophilic cyanobacterium *Mastigocladus laminosus* were grown in batch cultures using D-nitrate medium (10 mM $NaNO_3$) (Castenholz, 1981) at 25°C (culture room) or 45°C (inside a B.O.D. incubator) with a photon fluence rate of 50 μmol. m^{-2}. s^{-1}. Whenever needed, the N_2-medium (D-medium without any N source) was supplemented with 2 mM NH_4Cl (NH_4^+-medium), or 1 mM L-glutamine, asparagine, arginine or alanine. The medium was buffered with equimolar concentration of HEPES and its pH was adjusted to 7.5 before autoclaving.

4.2.2 Growth, heterocyst frequency and nitrogenase activity

Growth was measured as increase in concentration of Chl *a* (Mackinney, 1941). Heterocyst frequency was calculated as percentage of total cells by light microscopic observations after 96 hr of incubation in different media. Acetylene reduction assay was used to measure nitrogenase activity (Stewart *et al.*, 1967).

4.2.3 Nitrate and nitrite uptake assays

The N_2-, NO_3^--, and NH_4^+-grown cultures of *Mastigocladus laminosus* were harvested during the exponential growth phase, washed and resuspended in Tricine-$NaHCO_3$ buffer (25 mM, pH 8.1). Uptake of nitrate and nitrite was measured by determining the rates of their depletion from the external buffer. Uptake was started by addition of $NaNO_3$ (100 μM) or KNO_2^- (100 μM) to the cell suspension. The

choice of 100 μM external concentration was based on earlier studies in *Anabaena* sp. PCC 7120 and *Synechococcus* sp. strain PCC 7942 (Frias *et al.*, 1997; Maeda & Omata, 1997). Samples were withdrawn after 3 h of incubation, subjected to rapid centrifugation and the cell-free supernatants analyzed for residual nitrate or nitrite. Nitrate concentration was measured by its absorbance at 210 nm in acid solution (Cawse, 1967) and nitrite concentration was measured by the method of Snell and Snell (1949).

4.2.4 Nitrate reductase, nitrite reductase and glutamine synthetase (transferase) activities

These activities were measured in cells sonicated by Vibra-Cell Ultrasonic Processor (Sonics & Materials, Incoporation). Glutamine synthetase (transferase) activity was measured as described by Sampio *et al* (1979). Ferredoxin-dependent nitrate reductase and nitrite reductase activities were measured using dithionite-reduced methyl viologen as reductant (Arizmendi & Serra, 1990; Manzano *et al.*, 1976). There was slight modification in case of the *Mastigocladus laminosus* as the incubation for the enzyme assays was performed at higher temperature (45°C) for the cultures grown at 45°C, while for cultures grown at 25°C, the incubation was performed at 30°C. Nitrite was estimated calorimetrically as described by Snell and Snell (1949). Protein concentration was measured according to Lowry *et al.* (1951).

4.2.5 Ammonium and amino acid transport assays

Ammonium transport assay was done using the radioactive analogue of ammonium, [^{14}C]-methyammonium (sp.activity 370 KBq. μmol^{-1}). Glutamine, arginine, and asparagine uptakes were measured using ^{14}C-labeled glutamine (sp.activity 256 KBq. μmol^{-1}), arginine (sp.activity 65 KBq. μmol^{-1}), and asparagine (sp.activity 63 KBq. μmol^{-1}). The NO_3-grown cultures of *Mastigocladus laminosus* were harvested during the exponential growth phase, washed in N_2-medium and then incubated in N_2-medium, NO_3^--medium and N_2-medium supplemented with 1mM glutamine, arginine, or asparagine for 48 h at 25°C or 45°C. After incubation in different nitrogen-media, the cells were harvested, washed and resuspended in 10 mM HEPES-NaOH buffer (pH 7.0) to a concentration of 10 μg Chl *a*.ml^{-1}. After equilibration for 1 h at 25°C or 45°C, radiolabeled methylammonium or amino acids were added to a final concentration of 50 μM. The uptake experiments were carried out at 25°C or 45°C at a photofluence rate of 50 μmol. m^{-2}. s^{-1}. Whenever needed, dichlorophenyldimethylurea (DCMU, 10 μM), or carbonyl cyanide chlorophenyl hydrazone (CCCP, 25 μM) were added to the cell suspension 30 minute prior to the addition of labeled amino acid and were present during the experiments. At different time intervals, 400 μl samples were taken out rapidly and the cells were separated from their suspension medium by centrifugation through silicon oil DC 550/ dinonyl phthalate (40/60, v/v) into perchloric acid/water (15/85, v/v) (Scott & Nicholls, 1980). The [^{14}C] in perchloric acid fraction was measured using a liquid Scintillation counter (Model 1801, Beckman Instruments). Non-specific binding of [^{14}C]-

methylmmonium was determined by measuring its incorporation in toluene-treated cells (Rai *et al.*, 1984; Prakasham & Rai, 1991).

4.3 Results and Discussion

4.3.1 Growth, heterocyst frequency and nitrogenase activity

As seen from the data in table 4.1, *M. laminosus* was able to utilize glutamine, asparagine, arginine and alanine as sole N-sources for growth in addition to N_2, NO_3^- and NH_4^+, when added in the growth medium. These findings are consistent with earlier reports that some amino acids can serve as sole N-source for growth in heterocystous cyanobacteria (Wolk, 1973; Kapp *et al.*, 1975; Neilson & Larson, 1980; Rawson, 1985; Theil & Leone, 1986; Flores & Muro-Pastor, 1990; Herrero & Flores, 1990; Singh *et al.*, 1991; Bhattacharya *et al.*, 2002*a*). Among the amino acids tested, best growth was found in asparagine-supplemented medium followed by glutamine-, arginine- and alanine-supplemented media (Table 4.1). In all cases, higher growth was found at 45°C grown cultures than at 25°C.

When grown in medium lacking combined N (N_2-medium), *M. laminosus* developed heterocysts and nitrogenase, but not in media supplemented with NO_3^-, NH_4^+, or amino acids (Table 4.1). These data indicate that *M. laminosus* was able to use N_2 as N-source for growth by developing heterocysts and nitrogenase when other utilizable nitrogen sources were absent and that NO_3^-, NH_4^+, glutamine, asparagine, arginine or alanine were used as sole source of N (instead of N_2) when available leading to complete repression of heterocyst formation and nitrogenase. This is consistent with reports in other heterocystous cyanobacteria (Theil & Leone, 1986; Bhattacharya *et al.*, 2002*a*). Heterocyst and nitrogenase activity was 2-3 fold higher at 45°C than that at 25°C. Complete repression of heterocyst development and nitrogenase activity in arginine-medium is in contrast to the partial repression

reported in *Anabaena* PCC 7120 (Herrero & Flores, 1990) and *Nostoc* ANTH (Bhattacharya *et al.*, 2002*a*).

4.3.2 Nitrate and nitrite uptake

Nitrate and nitrite uptake was studied in *M. laminosus* grown in media containing different nitrogen sources at 45°C and 25°C. As in cases of other heterocystous cyanobacteria (Bagchi *et al.*, 1985*a*, *b*; Martin-Neito *et al.*, 1989; Frias *et al.*, 1994), nitrate acted as an inducer of nitrate and nitrite uptakes in *M. laminosus* (Table 4.2). Nitrate and nitrite uptake rates were approximately 20 % and 50 % higher, respectively, in cells grown in NO_3^--medium than that in cells grown in N_2-medium (Table 4.2). The nitrate uptake activity in NH_4^+-grown cells was 32 % lower than that in N_2-grown cells, indicating repression of nitrate uptake by NH_4^+ (Table 4.2). Cells grown at 45°C showed a nitrate uptake activity of 27.04, 32.94 and 8.81 μmol nitrate taken up. min^{-1}. mg^{-1} Chl *a* in N_2, NO_3^-- and NH_4^+-medium, respectively. In cells grown at 25°C, the nitrate uptake rates in N_2, NO_3^-- and NH_4^+-media were 9.52, 12.29 and 3.96 μmol nitrate taken up. min^{-1}. mg^{-1} Chl *a*, respectively. Thus in all cases, nitrate uptake in cells grown at 45°C were significantly higher (2-3 fold) than those in corresponding cells grown at 25°C.

The nitrite uptake rates in cells grown at 45°C in N_2, NO_3^-- and NH_4^+-medium were 21.74, 30.41 and 17.29 μmol nitrite taken up. min^{-1}. mg^{-1} Chl *a*, respectively (Table 4.2). The corresponding nitrite uptake rates in cells grown at 25°C were 8.72, 11.17 and 7.93 μmol nitrite taken up. min^{-1}. mg^{-1} Chl *a* (Table 4.2). Thus, nitrite uptake rate in cells grown in NO_3^--medium was 28 % higher than that in cells grown

in N_2-medium. These results are consistent with earlier reports that nitrate induces nitrite uptake in cyanobacteria (Bagchi *et al.*, 1985*a*, *b*; Martin-Neito *et al.*, 1989; Frias *et al.*, 1994, 1997; Flores & Herrero, 1994). Again as in the case of nitrate uptake, nitrite uptake rates were significantly higher (2-3 fold) in cells grown at 45°C than those in cells grown at 25°C. It is interesting to note here that NH_4^+ repressed nitrate uptake only partially and had even a far lesser impact on nitrite uptake (Table 4.2). This is in contrast to a complete repression of nitrate and nitrite uptake by NH_4^+ in other cyanobacteria and would merit further study.

4.3.3 Nitrate reductase, nitrite reductase and glutamine synthetase (transferase) activities

Cells grown in N_2-medium at 45°C showed nitrate reductase activity of 9.16 nmol nitrite formed. min^{-1}. mg^{-1} protein. The nitrate reductase activity was repressed by 16 % in cells grown in presence of glutamine (7.65 nmol nitrite formed. min^{-1}. mg^{-1} protein), 21 % in presence of asparagine (7.20 nmol nitrite formed. min^{-1}. mg^{-1} protein), 36 % in presence of arginine (5.82 nmol nitrite formed. min^{-1}. mg^{-1} protein) and 24 % in presence of alanine (6.98 nmol nitrite formed. min^{-1}. mg^{-1} protein). In NH_4^+-grown cells, the NR activity was repressed by 47 % and in NO_3^--grown cells the activity increased by approximately 10 % (Table 4.3). Thus the maximum repression of NR occurred in cells grown in NH_4^+-medium, substantial NR activity remained in cells grown in various nitrogen-containing media and NO_3^- had little effect on NR induction. It appears therefore that NR in *M. laminosus* is an NH_4^+ repressible-derepressible system. The corresponding activities of NR in cells grown at

25°C in various nitrogen-containing media followed a similar pattern to that at 45°C, but the actual activities were lower (Table 4.3). These results are in agreement with earlier reports of partial repression of nitrate reductase activity by arginine in *Anabaena* 7120 (Herrero & Flores, 1990) and in *Nostoc* ANTH (Bhattacharya *et al.*, 2002*a*), but in contrast to the reported induction of NR activity by arginine in *Oscillatoria chalybea* (Bednarz & Schmid, 1991). The fact that there was partial inhibition of NR activity by glutamine and arginine is also in contrast with earlier reports that indicated strong inhibition of NR activity by glutamine and arginine in *Nostoc* ANTH (Bhattacharya *et al.*, 2002*a*). The fact that nitrate reductase activity was repressed by ammonium and derepressed in its absence is consistent with earlier reports that in some cyanobacteria NR is NH_4^+ repressible-derepressible (Rai *et al.*, 1984; Bagchi *et al.*, 1985*a*, *b*; Martin-Neito *et al.*, 1989; Prakasham & Rai, 1991; Flores & Herrero, 1994; Frias *et al.*, 1994, 1997).

Nitrite reductase (NIR) activity in dinitrogen grown cultures of *M. laminosus* was 162.53 nmol NO_2^- consumed. min^{-1}. mg^{-1} protein. NIR activity was repressed by 60 % in cells grown in presence of glutamine (65.12 nmol NO_2^- consumed. min^{-1}. mg^{-1} protein), 59 % in presence of asparagine (67.22 nmol NO_2^- consumed. min^{-1}. mg^{-1} protein), 66 % in presence of arginine (55.63 nmol NO_2^- consumed. min^{-1}. mg^{-1} protein) and 57 % in presence of alanine (69.46 nmol NO_2^- consumed. min^{-1}. mg^{-1} protein). The NIR activity increased to 175.03 nmol NO_2^- consumed. min^{-1}. mg^{-1} protein in cells grown in nitrate-medium, and decreased to 152.65 nmol NO_2^- consumed. min^{-1}. mg^{-1} protein in cells grown in ammonium-medium (Table 4.3). The corresponding activities of NIR in cells grown at 25°C in various nitrogen-containing

media followed a pattern similar to that at 45°C except that the actual activities were siginificantly lower (Table 4.3). Thus, while addition of amino acids in the growth media caused substantial repression in NIR in *M. laminosus* cells, NO_3^- and NH_4^+ had little or no effect (< 10 %) on NIR activity. Together with the observation on effect of ammonium on nitrite uptake, these data indicate that nitrite uptake and assimilation in *M. laminosus* is not subject to NH_4^+ repression.

Cells grown in N_2-medium at 45°C showed glutamine synthetase actvity of 2464 nmol γ-glutamyl hydroxamate formed. min^{-1}. mg^{-1} protein. This activity was repressed by 50 % in cells grown in presence of glutamine (1222.23 nmol γ-glutamyl hydroxamate formed. min^{-1}. mg^{-1} protein), 45 % in presence of asparagine (1355 nmol γ-glutamyl hydroxamate formed. min^{-1}. mg^{-1} protein), 60 % in presence of arginine (979 nmol γ-glutamyl hydroxamate formed. min^{-1}. mg^{-1} protein) and 68 % in presence of alanine. The corresponding activities of GS in cells grown at 25°C in various nitrogen-containing media followed a pattern similar to that at 45°C, but the actual activities were significantly lower, particularly in cells grown in amino acid containing media (Table 4.3).

These results are in contrast to the earlier reports that there was no significant repression of GS activity in presence of amino acids in *Nostoc muscorum* (Singh *et al.*, 1991) and *Nostoc* ANTH (Bhattacharya *et al.*, 2002*a*). However, it is noteworthy that cells grown in amino acid-containing media retained 32-50 % of the GS activity. This is not surprising since *M. laminosus* is able to use these amino-acids as sole N-source for growth (see Section 4.3.1) and GS is essential for them to do so (Singh *et al.*, 1991).

4.3.4 Ammonium transport

Using ^{14}C-methylammonium as probe, an energy-dependent and NO_3^- and NH_4^+-repressible ammonium transport system, that is essential for cyclic retention of ammonia in the cell, has been characterized in several cyanobacteria (Rai *et al.*, 1984; Boussiba *et al.*, 1984*a*; Singh *et al.*, 1985; Prakasham & Rai, 1991). Typically, in all cases, ^{14}C-methylammonium uptake shows a biphasic curve, a rapid first phase of accumulation lasting 60 s and a slower second phase linked to ^{14}C-methylammonium assimilation. As seen in Fig 4.1, ^{14}C-methylammonium uptake in N_2-grown *M. laminosus* also showed the characteristic biphasic pattern representing a rapid initial phase of accumulation lasting 60 s followed by a slower second phase. In N_2-medium, the methylammonium uptake rate in cultures grown at 45°C were 42.00 and 15.77 nmol. mg^{-1} Chl *a*. min^{-1} and at 25°C, 36 and 3.7 nmol. mg^{-1} Chl *a*. min^{-1}, during the first and second phase, respectively. Higher rates at 45°C than that at 25°C reflect that *M. laminosus* is a thermophile. As in other cyanobacteria (Prakasham & Rai, 1991; Singh *et al.*, 1991; Bhattacharya *et al.*, 2002*a*), the ^{14}C-methylammonium uptake was repressed in cells grown in nitrate- and NH_4^+-media (Fig 4.1).

The effect of DCMU (an inhibitor of non-cyclic photosynthetic electron transport) and CCCP (an uncoupler) was studied on ^{14}C-methylammonium uptake in *M. laminosus*. The results show that DCMU and CCCP inhibited rates of intracellular accumulation by 64 % and 62 %, respectively. This implies that the cellular uptake of ammonium is energy-dependent (Table 4.4). Thus, these results are consistent with characteristics of ATS in other cyanobactera (Boussiba *et al.*, 1984*a*; Rai *et al.*, 1984;

Bhattacharya *et al.*, 2002*a*) and indicate operation of an energy-dependent ATS that is repressed by NH_4^+ and NO_3^-.

4.3.5. Amino acid transport

Uptake of amino acids (glutamine, asparagine, arginine and alanine) was studied in *M. laminosus* grown in media containing different nitrogen sources and at different temperatures. The glutamine uptake by the cells was biphasic in nature consisting of an initial phase representing intracellular accumulation followed by a slower second phase representing assimilation. This pattern was independent of nitrogen-source used and temperature at which the cells were growth (Fig 4.2). Cells grown in N_2-medium at 25°C, showed a glutamine uptake rate of 57.61 and 5.25 nmol. mg^{-1} Chl *a*. min^{-1}, during the first and second phase, respectively. When grown in glutamine-containing medium, the uptake rate by the cells during the first phase increased by nearly 24 % while the second phase was largely unaffected. Thus, growth in glutamine-medium resulted in increased rate of glutamine accumulation. Glutamine uptake rates by cells grown at 45°C responded similarly; however, the rates were much higher than those in cells grown at 25°C (33 % and 85 % higher during first phase in N_2 and gln-grown cultures, respectively and nearly 100 % higher during second phase in both N_2 and gln-grown cultures).

Uptake of asparagine, arginine and alanine (Figs 4.3, 4.4, 4.5) showed a pattern similar to that observed for glutamine uptake described above. Their uptake was also induced by the presence of respective amino acids in the growth medium and the uptake rates were higher in cells grown at 45°C than those at 25°C. However, unlike

the glutamine uptake where the second phase was largely unaffected by presence of glutamine in the growth medium, presence of alanine, asparagine and arginine in the growth medium increased their respective uptake rates by cells grown at 45°C during first and second phase of uptake. In other words, these amino acids not only induced their accumulation but induced their assimilation also. This phenomenon was observed only in cells grown at 45°C and not in those grown at 25°C. The induction of glutamine uptake (accumulation during first phase) in cells grown in glutamine-medium required *de novo* protein synthesis since chloramphenicol prevented such induction (Fig 4.2). Similarly, the induction of alanine, arginine and asparagine by the respective substrates also required *de novo* protein synthesis (data not shown).

The inhibitory effects of DCMU and CCCP on the amino acid uptake (accumulation during first phase) was also studied in *M. laminosus*. DCMU inhibited glutamine accumulation by 61 % and CCCP by 83 % (Table 4.5), indicating that the cellular accumulation of glutamine was energy-dependent. Similar results were found in cases of alanine, asparagine and arginine accumulation (data not shown). These results are consistent with energy-dependent amino acid uptake in *Anabaena* PCC 7120, *A. variabilis* and *Nostoc* ANTH (Theil, 1988; Herrero & Flores, 1990; Montesinos *et al.*, 1995; Bhattacharya *et al.*, 2002*a*).

Overall, the results show that *M. laminosus* was able to take up and utilize exogenously supplied glutamine, asparagine, arginine and alanine in addition to inorganic nitrogen sources (N_2, NO_3^-, NH_4^+) as sole sources of fixed-N for growth. Heterocyst formation and nitrogenase activity were completely repressed and GS activity was lower in cultures grown in presence of combined nitrogen sources.

Presence of ammonia or amino acids in the medium caused partial repression of nitrate/nitrite uptake and reductase activities while NO_3^- partially increased the activities. Ammonium and amino acid uptakes showed a biphasic pattern, were energy-dependent and the induction of uptake required *de novo* protein synthesis. The ammonium transport was substrate (NH_4^+)-repressible. In keeping with the thermophilic nature of this cyanobacterium, the activities/levels of various parameters measured in this study were higher in cells grown at 45°C than those at 25°C.

Table 4.1 Growth (Gr), heterocyst frequency (HF) and nitrogenase (N_2ase) activity of *Mastigocladus laminosus* cells grown in media containing different nitrogen sources at 25°C and 45°C.

Growth-medium	Gr		HF		N_2ase	
	45°C	25°C	45°C	25°C	45°C	25°C
N_2	2.3 ± 0.1	1.6 ± 0.1	10.0 ± 0.5	3.3 ± 0.2	4.6 ± 0.2	2.6 ± 0.1
NO_3^-	3.1 ± 0.2	1.8 ± 0.1	0.0	0.0	0.0	0.0
NH_4^+	2.6 ± 0.1	1.7 ± 0.1	0.0	0.0	0.0	0.0
Gln	4.2 ± 0.2	2.2 ± 0.1	0.0	0.0	0.0	0.0
Asn	4.3 ± 0.2	2.3 ± 0.1	0.0	0.0	0.0	0.0
Arg	4.1 ± 0.2	1.8 ± 0.1	0.0	0.0	0.0	0.0
Ala	3.0 ± 0.2	1.3 ± 0.1	0.0	0.0	0.0	0.0

Nitrate–grown exponential cultures were washed and transferred to fresh media containing different nitrogen sources. The growth (μg Chl *a*. ml^{-1}), frequency of heterocysts (per 100 vegetative cell), and nitrogenase activity (nmol C_2H_4 formed. μg^{-1} Chl *a*. h^{-1}) were determined after 4 days of incubation. Chl *a* concentration at the start of incubation was 1.5 μg Chl *a*. ml^{-1}. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates. N_2 refers to medium D without any N, NO_3^- to medium D (+ 10 mM $NaNO_3$) and NH_4^+ to medium D with 2 mM NH_4Cl. Gln, Asn, Arg and Ala refer to N_2-medium with 1 mM glutamine, asparagine, arginine and alanine, respectively.

Table 4.2 Nitrate and nitrite uptake by *Mastigocladus laminosus* cells grown in media containing different nitrogen sources at 45°C and 25°C.

Growth-medium	Nitrate uptake		Nitrite uptake	
	45°C	25°C	45°C	25°C
N_2	27.04 ± 0.4	9.52 ± 0.4	21.74 ± 1.0	8.72 ± 0.4
NO_3^-	32.92 ± 1.6	12.29 ± 0.6	30.41 ± 1.5	11.17 ± 0.5
NH_4^+	8.81 ± 0.4	3.96 ± 0.2	17.29 ± 0.8	7.93 ± 0.3

Nitrate–grown exponential cultures were washed and transferred to fresh growth media containing different nitrogen sources. After 48 h of growth, the cultures were harvested, washed and resuspended in Tricine/NaOH buffer (for nitrate uptake experiment) or in N_2 medium (for nitrite uptake experiment) at a concentration of 7 μg Chl *a*. ml^{-1}. Nitrate ($NaNO_3$) or nitrite (KNO_2^-) was added to a final concentration of 100 μM, and its disappearance from the buffer or medium was monitored. The uptake rates are expressed as μmol nitrate taken up. min^{-1}. mg^{-1} Chl *a* (nitrate uptake) or μmol nitrite taken up. min^{-1}. mg^{-1} Chl *a* (nitrite uptake). The values presented are means ± standard error (SE) from two independent experiments, each with two replicates. N_2, NO_3^- and NH_4^+ refer to respective growth media (see legends to Table 4.1).

Table 4.3 Nitrate reductase (NR), nitrite reductase (NIR) and glutamine synthetase (transferase) activities of *Mastigocladus laminosus* cells grown in media containing different nitrogen sources at 45°C and 25°C.

Growth-medium	NR		NIR		GS	
	45°C	25°C	45°C	25°C	45°C	25°C
N_2	9.16 ±0.4	8.33 ± 0.4	162.53 ± 8	127.80 ± 6	2464 ± 123	2217± 110
NO_3^-	10.72 ±0.5	8.81± 0.4	175.03 ± 8	133.03 ± 6	1689 ± 84	1507 ± 75
NH_4^+	4.87 ± 0.2	4.07 ± 0.2	152.65 ± 7	112.00 ± 5	1466 ± 73	1456 ± 72
Gln	7.65 ± 0.4	6.35 ± 0.3	65.12 ± 3	48.23 ± 2	1222 ± 61	693 ± 34
Asn	7.20 ± 0.4	6.25 ± 0.3	67.22 ± 3	52.00 ± 2	1355 ± 67	701 ± 35
Arg	5.88 ± 0.3	5.16 ± 0.3	55.63 ± 2	43.86 ± 2	979 ± 48	684 ± 34
Ala	6.98 ± 0.3	6.12 ± 0.3	69.46 ± 3	54.81 ± 3	788 ± 39	546 ± 27

Nitrate–grown exponential cultures were washed and transferred to fresh growth media containing different nitrogen sources. Activities of nitrate reductase (nmol NO_2^- formed. min^{-1}. mg^{-1} protein), nitrite reductase (nmol NO_2^- consumed. min^{-1}. mg^{-1} protein) and glutamine synthetase (nmol γ-glutamyl hydroxamate formed. min^{-1}. mg^{-1} protein) were determined after 4 days of incubation in the different growth media as indicated. Chl *a* concentration at the start of incubation was 1.50 μg Chl *a*. ml^{-1}. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates. For details of different growth media see legend to Table 4.1.

Fig 4.1 [^{14}C]-methylammonium uptake in *Mastigocladus laminosus* grown in N_2-medium (25°C, ●; 45°C, ▲), NO_3^--medium (45°C, ▼), and NH^+_4-medium (45°C, ♦). Nitrate–grown cultures (exponential phase) were washed and transferred to different nitrogen media and incubated for 48 h. The cultures were then harvested, washed and resuspended in HEPES buffer, and used for [^{14}C]-methylammonium uptake as described in Materials and Methods. The values presented are means from two independent experiments, each with two replicates. The values shown are corrected for non-specific binding of ^{14}C-methylammonium using toluene-treated cells.

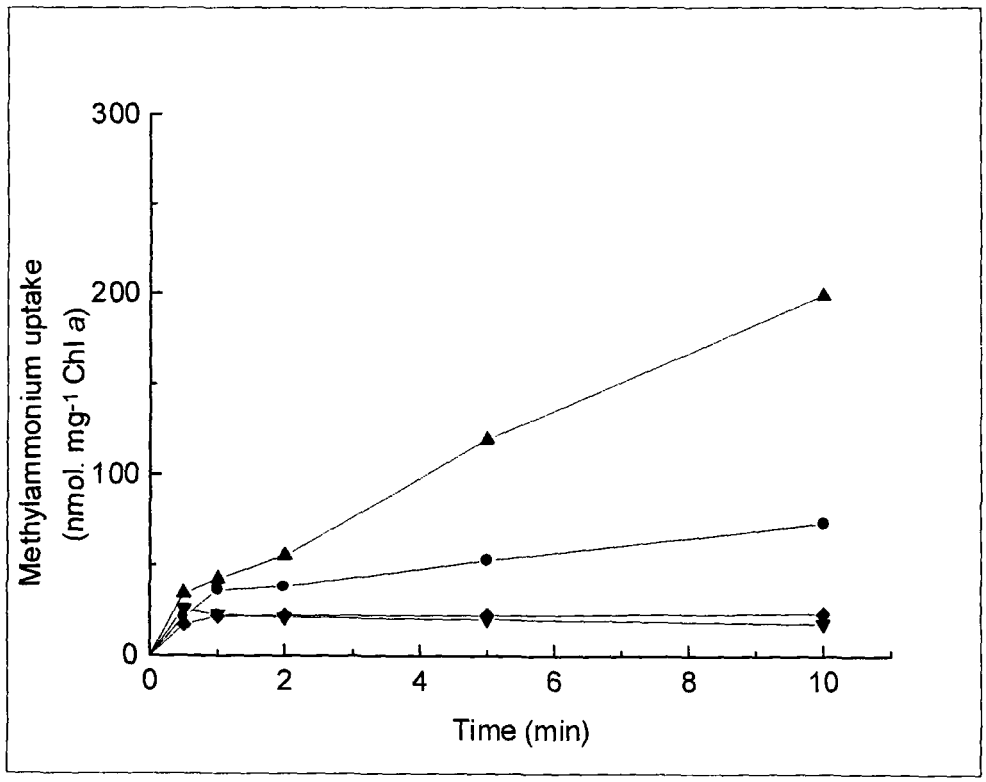


Table 4.4 Effect of inhibitors (DCMU and CCCP) on [^{14}C]-methylammonium uptake by *Mastigocladus laminosus*.

Treatment	Uptake rate (nmol. mg^{-1} Chl *a*. min^{-1})
Control (N_2-medium)	42.00 ± 2.1
Control + DCMU	15.15 ± 0.8
Control + CCCP	16.12 ± 0.8

Nitrate–grown exponential cultures were washed and transferred to N_2-medium at 45°C. After 48 h of incubation, cultures were harvested, washed and resuspended in 10 mM HEPES buffer. [^{14}C]-methylammonium was added after 60 min of incubation in light at 45°C. DCMU (10 μM) and CCCP (25 μM) were added 30 min prior to the addition of [^{14}C]-methylammonium. The uptakes rates were measured after 60 s of ^{14}C accumulation into the cells. The values presented are means ± standard error from two independent experiments, each with two replicates.

Fig. 4.2 [^{14}C]-glutamine uptake in *Mastigocladus laminosus* grown in N_2-medium (25°C, ■; 45°C, ●), glutamine-medium (25°C, ▲; 45°C, ▼) and chloramphenicol treated glutamine-medium (45°C, ♦).

Nitrate–grown exponential cultures were washed and transferred to N_2- or glutamine-medium (N_2-medium + 1mM glutamine) and incubated for 48 h at 45°C or 25°C. Chloramphenicol (1 μg. ml^{-1}) was added at the beginning of incubation to one set of glutamine-medium (at 45°C). After incubation, the N_2- and glutamine-grown cells were washed, resuspended in HEPES buffer and used for [^{14}C]-glutamine uptake as described in Material and Methods. Values are means from two independent experiments, each with two replicates.

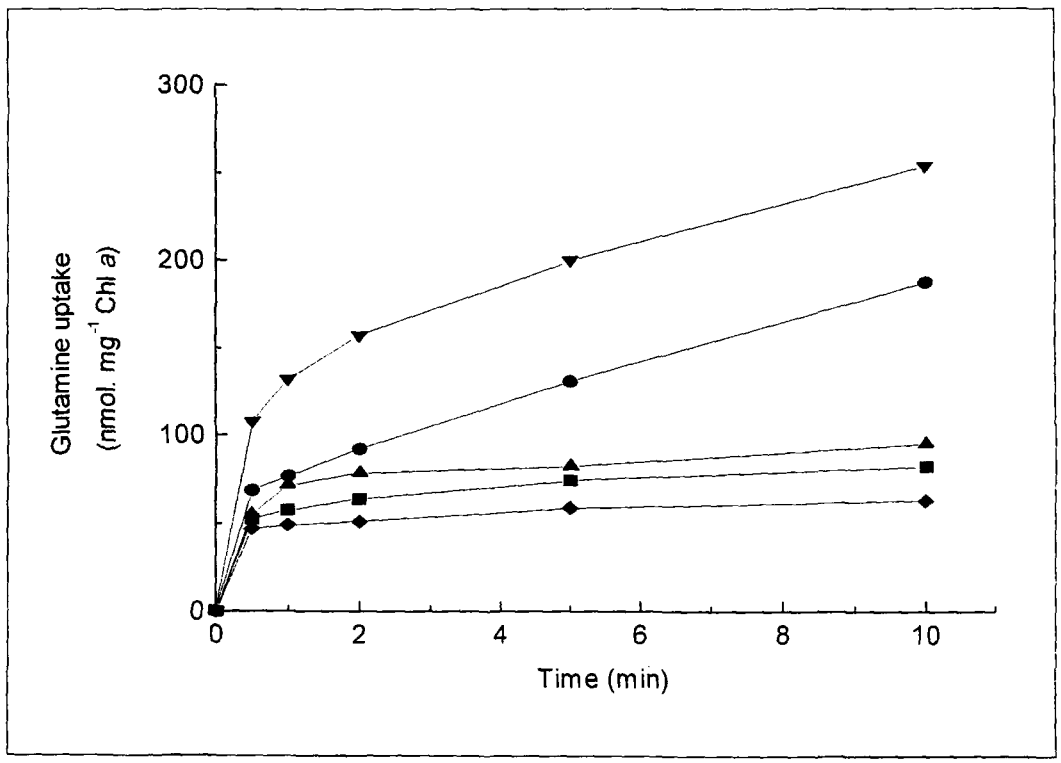


Fig 4.3 [^{14}C]-Asparagine uptake in *Mastigocladus laminosus* grown in N_2-medium (25°C, ■; 45°C, ●), and asparagine-medium (45°C, ▼; 25°C, ▲).

Nitrate–grown exponential cultures were washed and transferred to N_2- or asparagine-medium (N_2-medium + 1mM asparagine) and incubated for 48 h at 45°C or 25°C. After incubation, the N_2- and asparagine-grown cells were washed, resuspended in HEPES buffer and used for [^{14}C]-asparagine uptake as described in Material and Methods. Values are means from two independent experiments, each with two replicates.

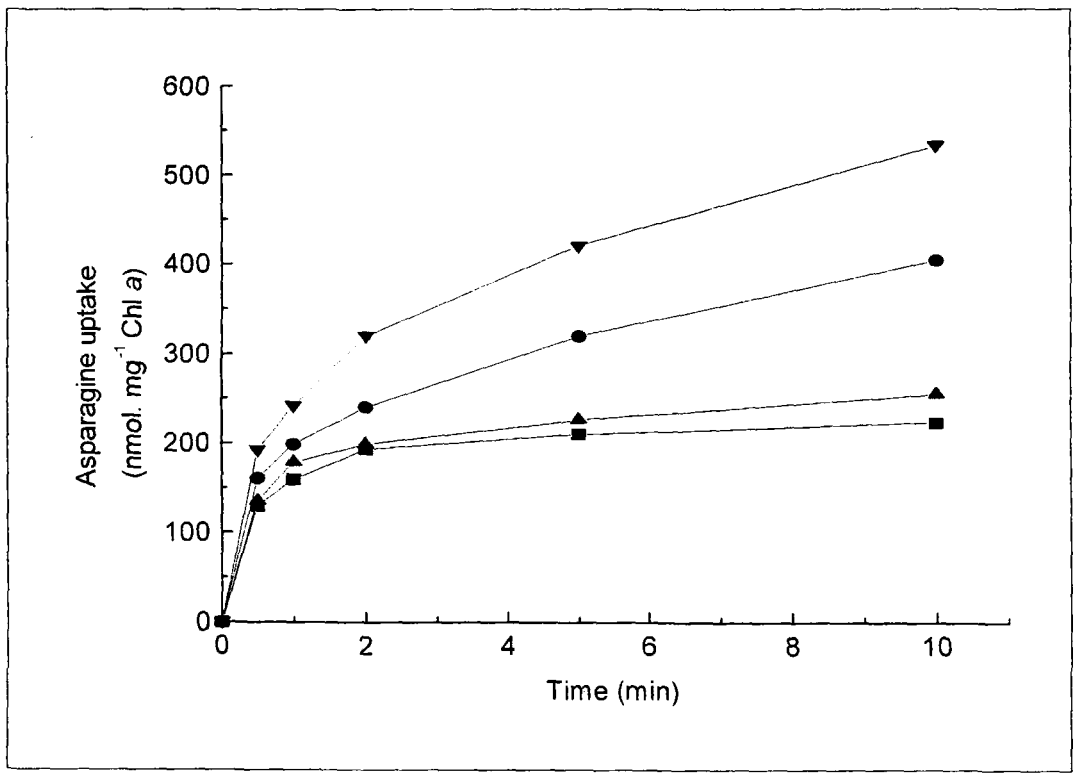


Fig 4.4 [^{14}C]-Arginine uptake in *Mastigocladus laminosus* grown in N_2-medium (25°C, ■; 45°C, ●) and arginine-medium (45°C, ▼; 25°C, ▲).

Nitrate–grown exponential cultures were washed and transferred to N_2- or arginine- medium (N_2-medium + 1mM arginine) and incubated for 48 h at 45°C or 25°C. After incubation, the N_2- and arginine-grown cells were washed, resuspended in HEPES buffer and used for [^{14}C]-arginine uptake as described in Material and Methods. Values are means from two independent experiments, each with two replicates.

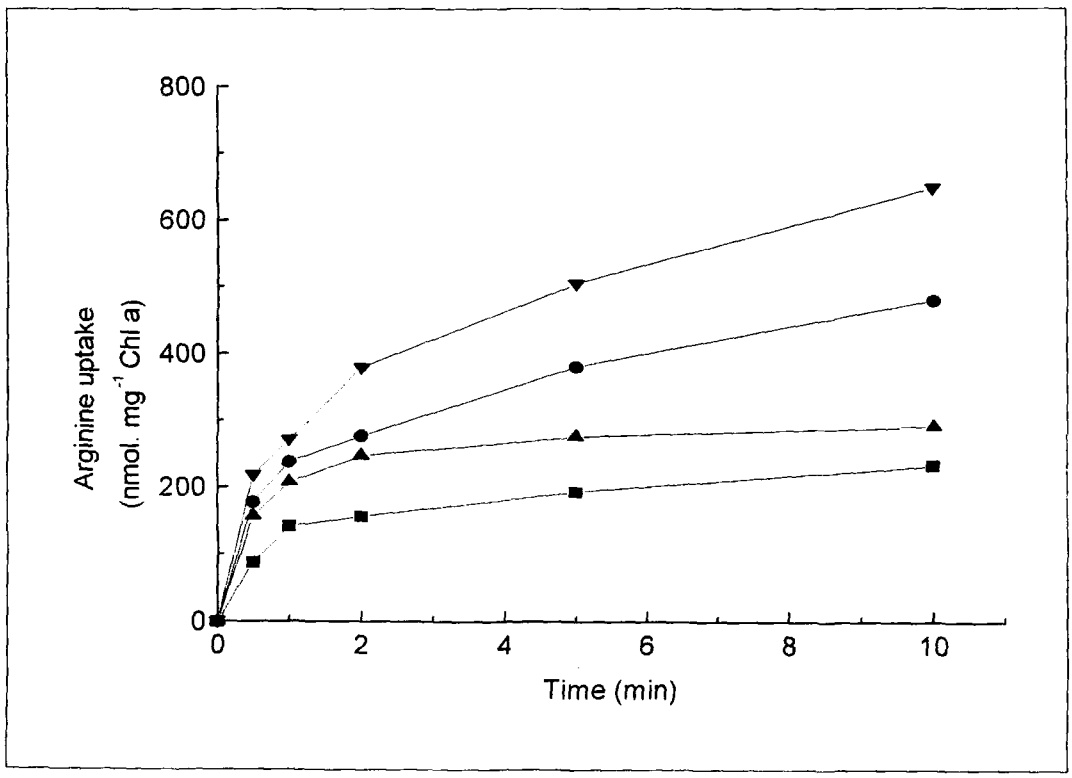


Fig 4.5 [^{14}C]-Alanine uptake in *Mastigocladus laminosus* grown in N_2-medium (25°C, ■; 45°C, ●) and alanine-medium (25°C, ▲; 45°C, ▼).

Nitrate–grown exponential cultures were washed and transferred to N_2- or alanine-medium (N_2-medium + 1mM alanine) and incubated for 48 h at 45°C or 25°C. After incubation, the N_2- and alanine-grown cells were washed, resuspended in HEPES buffer and used for [^{14}C]-alanine uptake as described in Material and Methods. Values are means from two independent experiments, each with two replicates.

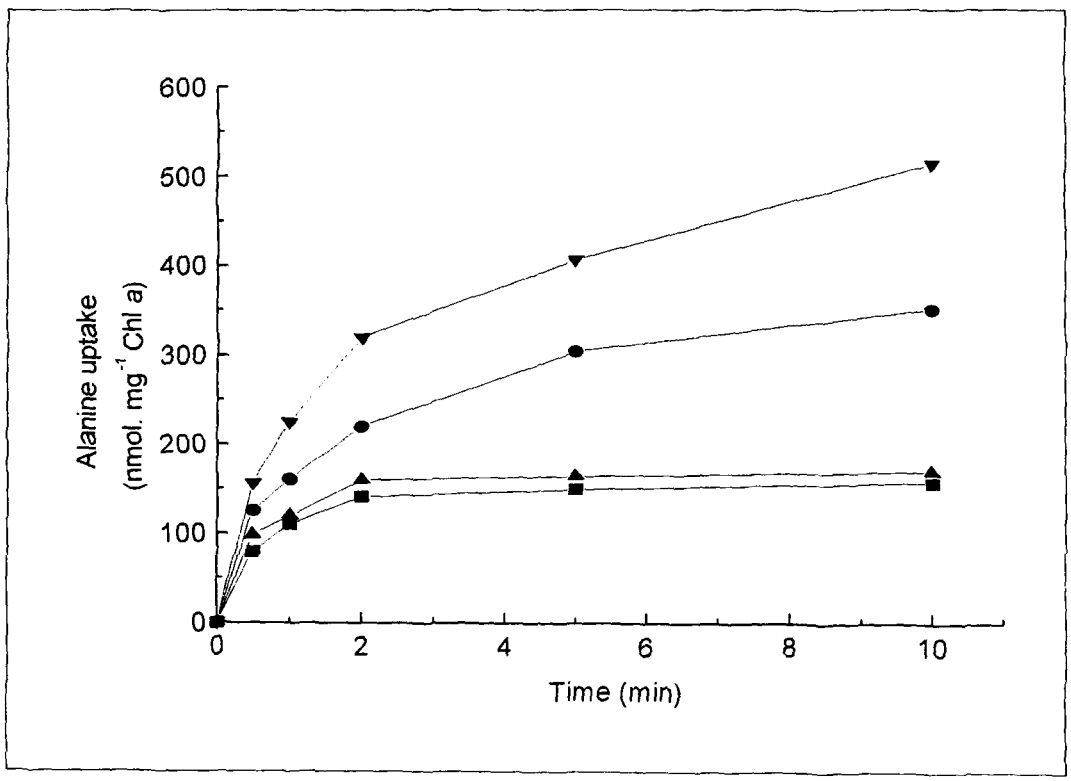


Table 4.5 Effect of inhibitors (DCMU and CCCP) on [^{14}C]-glutamine uptake by *Mastigocladus laminosus.*

Treatment	Uptake rate (nmol. mg^{-1} Chl *a*. min^{-1})
Control (N_2 + 1 mM Gln)	131.71 ± 6
Control + DCMU	51.21 ± 2
Control + CCCP	21.87 ± 1

Nitrate–grown exponential cultures were washed and transferred to glutamine-medium (N_2 + 1 mM Gln) medium at 45°C. After 48 h of incubation in the glutamine-medium, cultures were harvested, washed and resuspended in 10 mM HEPES buffer. [^{14}C]-glutamine was added after 60 min of incubation in light at 45°C. DCMU (10 μM) and CCCP (25 μM) were added 30 min prior to the addition of [^{14}C]-glutamine and were present during the experiment. The uptakes rates were measured after 60 s of ^{14}C accumulation into the cells. The values presented are means ± standard error from two independent experiments, each with two replicates

CHAPTER 5

Akinete differentiation and germination in the thermophilic cyanobacterium, *Mastigocladus laminosus*

5.1 Introduction

Akinetes are thick-walled reproductive units produced by many cyanobacteria that belong to the groups *Nostocaceae*, *Rivulariaceae* and *Stigonemateceae* (Rippka *et al.*, 1979). These specialized single cells serve as means of perennation in these organisms (Wolk, 1965*a*, 1973; Nichols & Carr, 1978; Adams & Carr, 1981). There are a number of published reports on factors controlling akinete formation, and akinete germination in mesophilic cyanobacteria (See Chapter I, Section 1.1.3). However, information regarding akinete formation in thermophilic cyanobacteria is lacking.

The present study was aimed at investigating the processes of akinete differentiation and akinete germination in the thermophilic cyanobacterium *Mastigocladus laminosus* (an isolate from the hot springs of Jakrem, Meghalaya, India). Parameters such as growth, heterocyst frequency, enzymes of nitrogen metabolism (nitrogenase, nitrate reductase, glutamine synthetase), phycocyanin, protein, photosynthesis, respiration and C:N ratios were studied during akinete formation and germination at different temperatures (25°C, 45°C).

5.2. Materials and Methods

5.2.1 Culture conditions for akinete differentiation

5.2.1.1 Sulfate limitation

M. laminosus was grown in D-N_2 medium, harvested by centrifugation, washed with D-N_2 medium lacking $CaSO_4$ and $MgSO_4$ and then transferred and grown in D-N_2 medium lacking $CaSO_4$ and $MgSO_4$. The medium was supplemented with equimolar concentration of $CaCl_2$ and $MgCl_2$ respectively, to counter the effect of removing $CaSO_4$ and $MgSO_4$ so that the ionic balance of the medium remained unchanged. As and when required, the medium was supplemented with 10 mM $NaNO_3$ and buffered with equimolar concentration of HEPES.

5.2.1.2 Phosphate limitation

M. laminosus was grown in D-N_2-medium, harvested by centrifugation, washed with D-N_2-medium lacking Na_2HPO_4 and transfered and grown in D-N_2-medium lacking Na_2HPO_4. The medium was supplemented with equimolar concentration of NaCl to counter the effect of removing Na_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 10 mM $NaNO_3$ and buffered with equimolar concentration of HEPES.

5.2.1.3 Other conditions

Other factors whose effects were studied on akinete formation included: additions of NH_4Cl (2mM), amino acids (glutamine, arginine, asparagine or alanine, 1mM), sugars (glucose, fructose or sucrose, 10-50 mM) and NaCl (100-400 mM) to the

culture medium, changing the pH of culture medium, removal of $FeCl_3$ from the culture medium, and incubation in darkness. *M. laminosus* was grown in D-N_2-medium with the required additions, omissions or alterations as mentioned above.

5.2.2 Culture conditions for akinete germination

Akinete population was washed twice and resuspended in fresh D-N_2-medium at a concentration of 1.23 x 10^6 akinetes. ml^{-1} and incubated at a photon fluence rate of 50 µmol Photons. m^{-2}. s^{-1} at 25°C or 45°C. Whenever necessary $NaNO_3$ (10 mM) and NH_4Cl (2 mM) were added as sources of combined nitrogen.

5.2.3 Akinete frequency

Akinete frequency was calculated as percentage of total cell population by light microscopic observations using an Olympus BX-51 light microscope fitted with a JVC digital video camera. Fluorescence microphotographs were taken using excitation filter BP 545-580.

5.2.4 Growth, heterocyst frequency and nitrogenase activity

Chlorophyll *a* was measured using the method described by Mackinney (1941). Heterocyst frequency was calculated as percentage of total cell populations by light micrsoscopic observations. Nitrogenase activity was measured using acetylene reduction assay (Stewart *et al.*, 1967).

5.2.5 Photosynthetic oxygen evolution and consumption

Oxygen evolution and consumption were measured using a Clark-type oxygen electrode (Rank Brothers, England) installed in a 3 ml Plexiglass container with magnetic stirring. See Chapter 2 (Materials & Methods) for full details.

5.2.6 Absorption spectra of photosynthetic pigments

Chlorophylls and carotenoids present in the cells were extracted in methanol and phycobiliproteins in 0.15 mM phosphate buffer, pH 7.0 by ultrasonication. The solutions containing chlorophylls, carotenoids or phycobiliproteins were placed in 1 cm light path cuvettes and absorption spectra of the samples were determined in the wavelength range 400 -700 nm using Beckman DU- 530 UV/VIS spectrophotometer.

5.2.7 Phycobiliprotein and protein content

The phycobiliprotein content [phycocyanin (PC), allophycocyanin (APC), phycoerythrin (PE)] was determined according to Bennett and Bogorad (1973). Protein content was measured according to Lowry *et al*, (1951).

5.2.8 Nitrate reductase and glutamine synthetase activities

Nitrate reductase (NR) activity was measured according to Manzano *et al.* (1976). GS activity measurement was essentially as described by Sampio *et al.* (1979).

5.2.9 C:N Ratio:

The cells were harvested, rapidly washed with distilled water and dried by keeping them at 45°C for four days inside an oven. The dried samples were collected, and

Carbon to Nitrogen (C:N) ratio of samples was determined using Vario III CHNS analyzer fitted with autosampler (Elementary Analysensysteme, GmbH, Germany).

5.3 Results and Discussion

5.3.1 Akinete differentiation

5.3.1.1 Time course, pattern and factors affecting akinete differentiation

As noted in other cyanobacteria (Nichols & Carr, 1978; Nichols & Adams, 1982; Rai *et al.*, 1985; Herdman, 1987, 1988), there was cell enlargement and accumulation of cyanophycin granules in vegetative cells preparatory to the initiation of akinete differentiation in *M. laminosus*. Akinetes were first noticed towards the end of growth phase. The culture changed colour, from blue-green to brown, as akinete differentiation proceeded and most cells became akinetes (Fig 5.1). The initiation of akinete differentiation was noted adjacent to heterocyst and/or at the $2^{nd}/3^{rd}$ cell from the heterocyst. These results are in keeping with the observations made in *Aphanizomenon flos aquae* (Wildman *et al.*, 1975); *Nostoc* PCC 7542 (Sutherland *et al.*, 1979), *Anabaena* CA (Nichols & Adams, 1982) and *A. cylindrica* (Nichols *et al.*, 1980).

A numbers of factors that trigger or enhance akinete formation in cyanobacteria are mentioned in Chapter I (see section 1.1.3). There are no earlier studies on sporulation in *Mastigocladus laminosus*. Various factors that may trigger akinete formation in *M. laminosus* were tested and the observations are summarized in Table 5.1. When *M. laminosus* cultures were grown in N_2-medium, akinete formation started at the end of growth phase (after 10 days at 25°C and 40 days at 45°C). Akinete formation also occurred when *M. laminosus* cultures was grown in NH_4^+-medium (by 20 days at 25°C and 40 days by 45°C), but no akinetes were formed in NO_3^--medium. Altering the pH of the medium (from normal 7.5 to pH 5, 9 or 11), darkness, omitting iron from the growth medium, and increasing salt concentration

also triggered akinete formation (Table 5.1). However, addition of carbon sources or amino acids in the growth medium proved ineffective in triggering akinete formation although *M. laminosus* did utilize amino acids (Chapter 4).

In N_2-media lacking sulphate, akinete formation started within 5 days at 25°C, but after 20 days at 45°C (Fig 5.2; Table 5.1, 5.2). Transfer of *M. laminosus* cultures to N_2-medium lacking phosphate also triggered akinete formation although after an initial growth period. Futhermore, the akinete differentiation was delayed starting after 10 days at 25°C and 40 days at 45°C. The initial growth of *M. laminosus* in medium lacking phosphate can be explained by the fact that repeated subculturing of cyanobacteria in laboratories lead to accumulation of phosphate (polyphosphate bodies) that can be mobilized under phosphate limiting conditions (Stewart, 1980). Therefore, under phosphate limiting conditions they continue to grow as long as internal reserves of phosphate last. Thus, the cells grew in phosphate limiting medium using internal reserves of phosphate, after which the growth ceased and akinete formation started. There are no such reserves known for sulphate in cyanobacteria, therefore the effect of sulphate–limitation is quicker on cessation of growth and triggering of akinete formation.

While akinetes did not differentiate in the NO_3^--medium, removal of sulphate or phosphate from the NO_3^--medium allowed akinete differentiation in such media (Fig 5.2b, d; Table 5.2). The facts that akinete differentiation occurred in NH_4^+-medium and in NO_3^--medium lacking sulphate/phosphate, even though no heterocysts developed, indicate that presence of heterocysts are not necessary for akinete differentiation in *M. laminosus*. 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. Addition of HEPES in the nitrate-supplemented medium lacking sulphate does not relieved the sulphate limitation, indicating that HEPES did not serve as source of sulphate for *M. laminosus.*

Some of the above observations on akinete differentiation in *M. laminosus* are consistent with the observations on akinete differentiation in other cyanobacteria by earlier workers. Limitation of light, iron limitation, increase in concentration of NaCl (Canabaeus, 1929; Fay, 1969*a*, *b*; Sinclair & Whitton, 1977; Sutherland *et al.*, 1979; Nichols & Adams, 1982; Fay *et al.*, 1984; Wyman & Fay, 1986; Herdman, 1987, 1988) and phosphate limitation (Nichols & Adams, 1982; van Dok & Hart, 1996; Herdman, 1987, 1988) have all been reported as major triggers for akinete differentiation in various cyanobacteria. However, a number of features regarding akinete differentiation in *M. laminosus* are unique and/or in contrast to the features of akinete differentiation in other cyanobateria. Addition of exogenous sources of fixed carbon prolonged the growth phase, but in contrast to the *Nostoc* PCC 7542 (Sutherland *et al.*, 1979), no akinete were formed in *M. laminosus*. The triggering of akinete differentiation in *M. laminosus* under sulphate limitation reported in the present study is also in contrast to the report by Sinclair and Whitton (1977) that sulphate limitation had no effect on akinete differentiation in *Anabaena cylindrica.*

The cessation of growth of *M. laminosus* (both under phosphate and sulphate limitation) before akinete differentiation is consistent with similar observations on *A. cylindrica* (Fay, 1969*a*; Nichols *et al.*, 1980; Simon, 1977), *Nostoc* PCC 7542

(Sutherland *et al.*, 1979) and *Anabaena doliolum* (Rao *et al.*, 1987). The time course of akinete differentiation over a 40 day experimental period is presented in Table 5.2. After 40 days in N_2-medium, 80 % of the vegetative cells became akinetes at 25°C while only 20 % became akinetes at 45°C. The corresponding figures in NH_4^+-medium were 75 % and 15 % only. In N_2-medium lacking sulphate, virtually all vegetative cells (98 %) became akinetes by day 40 at 25°C while only 62 % became akinetes at 45°C. The corresponding figures in N_2-medium lacking phosphate were 75 % and 25 % only. In NO_3^--media lacking sulphate, 84 % of vegetative cells became akinetes by day 40 at 25°C and 60 % at 45°C. The corresponding figures in NO_3^--media lacking phosphate were 40 % and 0 % only.

Overall, the data indicate that sulphate limitation is a powerful factor for akinete formation in *M. laminosus*. The best response of *M. laminosus* in terms of maximum and early akinete differentiation was in N_2-medium lacking sulphate at 25°C. There was no evidence of release of any substance by *M. laminosus* into the growth medium that may stimulate akinete formation. Filtrates of spent akinete-differentiating medium (N_2-medium lacking sulphate in which akinete formation has taken place) did not induce akinete formation when added to *M. laminosus* cultures growing in N_2-medium. This was in contrast to earlier reports on *Cylindrospermum licheniforme* (Fisher & Wolk, 1976; Hirosawa & Wolk, 1979*b*) and *Nostoc* PCC 7542 (Sutherland *et al.*, 1979).

5.3.1.2 Changes in contents of chlorophyll *a*, phycocyanin, protein and the photosynthetic and respiratory activities during akinete differentiation

Changes in Chl *a* , phycocyanin, soluble protein and photosynthetic oxygen evolution and respiratory O_2 consumption were monitored during the 40 day experimental period starting from the time *M. laminosus* was transferred to various growth media (Fig. 5.2; Tables 5.3-5). The Chl *a* and phycocyanin contents declined during akinete differentiation and became undetectable or negligible after 40 days in cases of cultures grown in N_2-medium, NH_4^+-medium, N_2-medium minus sulphate, NO_3^--medium minus sulphate and N_2-medium minus phosphate at 25°C (Fig 5.2; Table 5.3). This is because in these media, most cells became akinetes by this time. In NO_3^--medium where akinetes did not differentiate at all, no such decline in photosynthetic pigments occurred either at 25°C or 45°C. Furthermore, in cultures grown at 45°C, where akinete differentiation was delayed and all vegetative cells did not become akinetes (N_2-medium, NO_3^--medium, NH_4^+-medium, N_2-medium minus sulphate, N_2-medium minus phosphate, NO_3^--medium minus sulphate and NO_3^--medium minus phosphate) the Chl *a* and phycocyanin declined but did not disappeared altogether. These data indicate disappearance of photosynthetic pigments as vegetative cells became akinetes and such akinetes matured. This was further confirmed by taking absorption spectra of methanol-soluble and water-soluble pigments from 40 days old cultures grown in various growth media at 25°C and 45°C (Fig. 5.3a, b, c, d and 5.4a, b, c, d). Chlorophyll *a* and phycocyanin peaks were undetectable in extracts of cultures where most vegetative cells had become akinetes. Chlorophyll *a* and phycocyanin peaks were observed only in cultures where significant number of vegetative cells remained as such or akinetes did not form (Figs. 5.3, 5.4). Fluorescence microscopy confirmed the disappearance of

photosynthetic pigments (phycobiliproteins) in mature akinetes (Fig 5.5). While a strong fluorescence was detected from vegetative cells, akinetes did not fluoresce at excitation wavelengths associated with phycobiliproteins. The protein content also declined during akinete differentiation This is in consistent with the fact that cyanobacterial akinete are known to divert much of the nitrogen to reserve polymers (Fay, 1969*a*, *b*; Rai *et al.*, 1985; Herdman, 1987, 1988; Adams & Duggans, 1999).

Photosynthetic O_2 evolution showed a steady decline during akinete differentiation and stopped altogether by day forty (Table 5.4) at 25°C in N_2-medium, NH_4^+-medium, and in N_2-medium lacking sulphate or phosphate. This decline was lesser at 45°C where akinete differentiation was delayed and fewer cells became akinetes. In NO_3^--medium where akinetes differentiation did not occur there was no such decline. The decline in photosynthetic O_2 evolution was expected during akinete differentiation since photosynthetic pigments also declined. The two events virtually paralleled each other. The respiratory O_2 consumption also declined (Table 5.5) during akinete differentiation but did not disappeared altogether and significant respiratory activity still remained. Again comparatively higher respiratory activity remained at 45°C than at 25°C.

The decline in photosynthetic pigments, photosynthetic O_2 evolution and respiratory O_2 consumption during akinete development, and the lack of photosynthetic pigments in mature akinetes of *M. laminosus* are consistent with earlier findings on akinetes of *Anabaena cylindrica* (Fay, 1969*a*, *b*), *Nostoc* PCC 7542 (Sutherland *et al.*, 1979; Chauvat *et al.*, 1982), *Anabaena doliolum* (Rao *et al.*, 1984, 1987) and *Nostoc spongiaeforme* (Theil & Wolk, 1983).

5.3.1.3 Heterocyst frequency and activities of nitrogen metabolism enzymes (nitrogenase, nitrate reductase and glutamine synthetase) during akinete differentiation

From the time when *M. laminosus* cultures were transferred to different growth media for akinete differentiation, the heterocyst frequency and activities of various enzymes of nitrogen metabolism (N_2ase, NR and GS) were monitored for the next 40 days. Under aerobic condition, nitrogenase is located in heterocyst and these are the sites of aerobic N_2-fixation in filamentous heterocystous cyanobacteria (Bergman *et al.*, 1986; Rai *et al.*, 1989). The *M. laminosus* inoculum (NO_3^--grown) had no heterocysts. However, upon transfer into N_2-medium at 25°C, heterocysts developed and a frequency of 2 % was observed after 10 days. No new heterocysts developed thereafter and heterocyst frequency declined reaching undetectable levels by day 40 (Table 5.6). Nitrogenase activity paralleled appearance and disappearance of heterocysts (Table 5.7). In comparison a higher frequency of heterocysts and higher levels of nitrogenase activity (Table 5.6, Table 5.7) developed and remained operative at 45°C. Since N_2 is the only nitrogen source in N_2-medium, these observations indicate a more severe limitation of nitrogen at 25°C than at 45°C. This may explain earlier cessation of growth and onset of akinete differentiation at 25°C than at 45°C (Fig. 5.2; Table 5.2). A similar trend was found in N_2-medium lacking sulphate and phosphate. The lowest heterocyst frequency and nitrogenase activity occurred in N_2-medium lacking sulphate where cessation of growth and subsequent akinete differentiation was quickest and most profuse. Thus, heterocyst and nitrogenase

appeared only transiently restricting nitrogen availability that led to early cessation of growth under the conditions that favoured akinete differentiation (25°C in N_2-medium and in N_2-medium lacking sulphate or phosphate). These data further support the conclusion that akinete differentiation is linked to cessation of growth.

The activities of nitrate reductase during growth of *M. laminosus* in different growth media are presented in Fig 5.6. In NO_3^--medium, where no akinete differentiation occurred, NR activity increased both at 25°C and 45°C. In other media (N_2-medium, NH_4^+-medium, N_2-medium lacking sulphate or phosphate and NO_3^--medium lacking sulphate or phosphate), NR activity started to decline after day 20 in cultures maintained at 25°C and eventually reached undetectable levels when most cells became akinetes. A similar trend also occurred at 45°C in media that supported akinete differentiation (Fig. 5.6) although the rate and extent of decline was slower in keeping with the rate and extent of akinete differentiation. These data indicate that mature akinetes lack NR activity as do heterocysts (Kumar *et al.*, 1985). Thus, the levels of NR activity in various growth media (Fig. 5.6) followed exactly the same trend as the photosynthesis in the corresponding media (Table 5.4). Nitrate reductase in cyanobacteria is dependent on photosynthesis as source of reduced ferredoxin that serves as reductant for NR (Manzano *et al.*, 1976). Therefore, it is not surprising that when photosynthesis rates declined during akinete differentiation and totally disappeared in mature akinetes (Table 5.4), the nitrate reductase activities also declined and finally disappeared.

The changes in activities of GS during growth and akinete differentiation are presented in Fig 5.7. Glutamine synthetase is the primary ammonia assimilating

enzyme in cyanobacteria (Stewart, 1980) and is essential for assimilation of ammonia 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 level of GS activity varied according to the medium and temperature at which the *M. laminosus* was grown. Upon transfer to various growth media GS activity initially increased in all the cultures at both 45°C and 25°C. Subsequently GS activity started to decline with onset of akinete differentiation in all media except NO_3^--media where in no akinetes differentiated (Fig. 5.7). The rate and extent of decline in GS activities depended on the timing and extent of akinete differentiation in different media and at different temperatures. The decline started earlier and occurred at a faster rate where akinete differentiation started earlier and higher percentage of cells became akinetes. The GS level eventually became undetectable in cultures as and when most cells became akinetes (after 30 days at 25°C in media lacking sulphate; after 40 days at 25°C in N_2- and NH_4^+-medium and in media lacking phosphate; after 40 days at 45°C in media lacking sulphate). These data indicate lack of GS activity in mature akinetes of *M. laminosus*. The initial increase in GS activity may be of significance in macromolecular reorganization of the cell preparatory to akinete differentiation.

The lack of nitrogenase, nitrate reductase and glutamine synthetase activities in mature akinetes of *M. laminosus* is consistent with the earlier reports on mature akinetes of *Anabaena doliolum* (Rao *et al.*, 1984) and other cyanobacteria (Rai *et al.*, 1985).

5.3.1.4 Changes in C:N ratio during akinete differentiation

C:N ratio of *M. laminosus* cultures, grown in NO_3^--media with or without phosphate, varied between 4.67 to 6 at 25°C and 4.67 to 5.37 at 45°C depending on the age of the culture (Table 5.8). In these cultures there was little or no akinete differentiation. In contrast, C:N ratios substantially increased with time when NO_3^--grown cells were transferred to growth media where akinete differentiation occurred (N_2-medium with or without sulphate, NO_3^--medium lacking sulphate and NH_4^+-medium). The extent of increase paralleled the extent of akinete formation. For example at 25°C, in N_2-medium lacking sulphate, where maximum levels of akinete differentiation occurred, the increase in C:N ratio was maximum (from 4.67 to 14.45) while the increase was comparatively lower in N_2-medium (from 4.67 to 9.35), NH_4^+-medium (from 4.67 to 8.48) and NO_3^--medium lacking sulphate (from 4.67 to 9.23). At 45°C, the increase in C:N ratio was comparatively lesser, again in keeping with the fact that akinete differentiation was lower at 45°C. Thus, the increase in C:N ratio paralleled akinete differentiation and maximum increase occurred where maximum percentage of cells became akinetes (N_2-medium lacking sulphate, at 25°C).

Overall, the present study indicates that conditions that adversely affect growth such as darkness, alteration of pH, limitation of sulphate, phosphate or iron, and increased salt concentration induce akinete formation in *M. laminosus* particularly in N_2-medium at 25°C. During akinete differentiation, photosynthetic pigments, photosynthetic O_2 evolution, heterocyst frequency and activities of N_2ase, NR, and GS declined reaching undetectable levels in mature akinetes. Respiration rates also declined during akinete differentiation but substantial levels of respiratory activity

remained in mature akinetes. In contrast, C:N ratio increased during akinete differentiation.

5.3.2 Akinete germination

5.3.2.1 Germination frequency, germination timing and germination pattern

For akinete germination studies, mature akinetes were harvested, washed, and resuspended in akinete germination studies, mature akinetes were harvested, washed, and resuspended in media containing different nitrogen sources at 25°C and 45°C. The first sign of germination was noticed when the brown cellular contents of akinetes turned blue-green (Fig 5.8). For the purpose of germination frequency, the germination is defined as the emergence of germling after the rupture of akinete wall. Germination varied according to the nitrogen source in the growth media and temperature at which the akinetes were incubated. At 45°C, akinete germination started within 24 h in N_2-, NO_3^-- and NH_4^+-medium (Table 5.9a, b). In contrast, it took 3 days in NO_3^-- and NH_4^+-medium, and 20 days in N_2-medium for start of akinete germination at 25°C. Furthermore, the germination frequency was higher in NO_3^-- and NH_4^+-media.

When the akinetes were transferred to fresh N_2-medium, the first cell division occurred by 24 h and by 48 h, the first heterocyst appeared at intercalary position of 4-5 cell length germlings. The second heterocyst appeared at the terminal end when the germling had grown to 10-12 cell length. These results are in contrast to that observed during germination of akinetes in *Nostoc* PCC 7542 (Sutherland *et al.*, 1985*b*), *Anabaena* PCC 7937 and *Nostoc* PCC 6720 (Skill & Smith, 1987) and

Cyanospira capsulata (Sili *et al.*, 1994) as the first heterocyst appeared at terminal positions of 4-5 cell length germlings. In the NO_3^-- and NH_4^+-media, akinete germination was quicker. The first cell division occurred within 12-24 h in the NO_3^-- and NH_4^+-media, and a germination frequency of 92 % was reached by day 4. The results presented here suggest that presence of nitrate and ammonium in growth media accelerated the germination process, but they were not essential for akinete germination. Thus, the internal reserves of akinetes were sufficient to initiate the germination process and the subsequent growth was supported by the appearance of heterocysts and nitrogen fixation in N_2-medium.

There was no germination of *M. laminosus* akinetes under darkness. As reported earlier in cases of *A. cylindrica* (Fay, 1969*b*; Yamamoto, 1976), *Nostoc* PCC 7542 (Chauvat *et al.*, 1982), *Anabaenapsi arnoldi* and *Anabaena* sp. (Reddy *et al.*, 1975), and *Anabaena doliolum* and *Fischerella musciola* (Kaushik & Kumar, 1970), light was essential for germination of *M. laminosus* akinetes and no akinete germination occurred under darkness.

It is apparent that the germination of *M. laminosus* akinete population is asynchronous (Table 5.9a). At 45°C, germination frequency of 25 %, 55 % and 90 % was recorded after 48 h, 72 h and 96 h of incubation in N_2-medium under light at 45°C, respectively (Table 5.9a). Since the akinete formation process itself was asynchronous leading to a population that contained akinetes of different ages, the asynchronous germination was as expected.

5.3.2.2 Heterocyst frequency and nitrogenase activity during germination of akinetes.

The heterocyst frequency and nitrogenase activity were monitored during akinete germination in N_2-, NO_3^-- and NH_4^+-media. A heterocyst frequency of 2 % was observed after 48 h of incubation in N_2-medium, whereas none were found in NO_3^-- or NH_4^+-media. At 48 h, only 25 % of the total akinetes had germinated and heterocysts were present in 4-5 cell germlings only. As the time progressed, more akinetes germinated with emergence and growth of additional germlings that developed heterocysts (Fig. 5.9).

The appearance of nitrogenase activity coincided with the appearance of the first heterocyst at 48 h (Fig. 5.9). The activity however increased subsequently from 1 nmol C_2H_4 formed. μg^{-1} Chl *a*. h^{-1} to 5.8 nmol C_2H_4 formed. μg^{-1} Chl *a*. h^{-1} by 96 h of germination, paralleling the increase in heterocyst frequency from 2 % to 8.5 % (Fig. 5.9). As heterocysts were absent in NO_3^-- and NH_4^+-media, there was no nitrogenase activity in either media.

5.3.2.3 Appearance of photosynthetic pigments, and photosynthetic and respiratory activities during germination of akinetes

The akinetes of *M. laminosus* lacked both phycocyanin and Chlorophyll *a*. Timing of Chlorophyll *a* and phycocyanin appearance during akinete germination depended on the growth medium used. At 45°C, in the NO_3^-- and NH_4^+-medium, both Chlorophyll *a* and phycocyanin appeared within 24 h of incubation, whereas in N_2-medium, it took 48 h. In contrast, Chlorophyll *a* and phycocyanin appeared after 72 h of

incubation in NO_3^-- or NH_4^+-medium when akinete germination was carried out at 25°C (Table 5.10). 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 at 45°C (Fig 5.10). As expected, this coincided with the appearance of photosynthetic pigments phycocyanin and Chl *a*. Thus, when the akinetes were transferred to NO_3^-- and NH_4^+-medium for germination, the appearance of photosynthetic pigments and photosynthetic O_2 evolution were quicker and became evident by 24 h. Akinetes had a low rate of respiration as mentioned earlier. During germination, the respiratory activity of the germinating akinete population increased steadily with time as more and more akinetes germinated and germlings grew (Fig 5.10). From a respiratory rate of 40 nmol. μg^{-1} Chl *a*. h^{-1} at start of the akinete germination, the respiratory rate reached to the level of 200 nmol. μg^{-1} Chl *a*. h^{-1} after 96 h of start of germination (Fig. 5.10).

5.3.2.4 Activities of nitrate reductase and glutamine synthetase during germination of akinetes

NR and GS activities are absent in mature akinetes. These activities reappear during akinete germination. The nitrate reductase activity appeared within 48 h of initiation of germination in N_2-medium and it coincided with the appearance of photosynthesis (Fig.5.11). However, in NO_3^--medium, the NR activity was significantly higher and appeared earlier (24 h after the initiation of germination), again coinciding with the appearance of photosynthetic pigments and photosynthetic O_2 evolution. The NR

activity increased till 72 h, and remained stable thereafter. NR activity also appeared during akinete germination in NH_4^+-medium with levels 50 % lower than that in N_2-medium, indicating that NR in *M. laminosus* is NO_3^- -inducible and partially repressed by NH_4^+.

The activity of GS during germination and germling growth is presented in Fig 5.12. GS appeared within 36 h of initiation of akinete germination in N_2-medium, and reached a steady level by 96 h. A similar pattern of GS appearance occurred during akinete germination in NO_3^-- and NH_4^+- media, however the levels of GS were lower than those in N_2-medium. Appearance of GS before heterocyst and nitrogenase was probably needed for the reassimilation of ammonia generated from mobilization of reserves for the synthesis of new macromolecules including various pigments, enzymes and proteins preparatory to akinete germination.

5.3.2.5 Changes in C:N ratio during germination of akinetes

Changes in C:N ratio and soluble protein content occurred during akinete germination and the rate of such changes varied in line with the rate of akinete germination and growth in various media (Table 5.11 and 5.12). During the 96 h study period there was no akinete germination at 25°C in N_2-medium and accordingly there was little or no change in C:N ratio or soluble protein content. However, in N_2-medium at 45°C, and in NO_3^-- and NH_4^+- media at both 25°C and 45°C, C:N ratios decreased and soluble protein contents increased as akinetes germinated and germlings grew. The decrease in C:N ratio was mainly due to an increase in nitrogen content. The initial increase in soluble protein content prior to the appearance of N_2-fixation, NR or GS

was surely due to mobilization of internal reserves. However, the subsequent increase in protein content can be explained by the fact that N_2-fixation, NR and GS became operational and photosynthesis appeared that served as source of carbon. The carbon content on the other hand remained more or less same during the akinete germination except that there was a small decrease during 24-48 h. This was consistent with the finding on *Anabaena doliolum* (Rai *et al.*, 1988) that respiration of stored carbon serves as source of energy for akinete germination till the appearance of photosynthesis.

Fig 5.1 Change in colour of *Mastigocladus laminosus* during akinete differentiation.
(a) culture containing vegetative cells and
(b) culture at the end of akinete differentiation

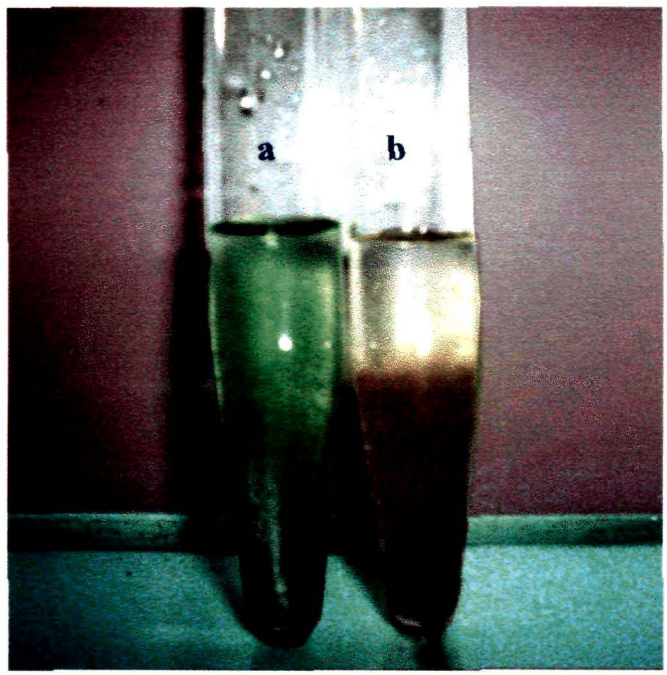


Table 5.1. Factors affecting akinete differentiation in the cyanobacterium *Mastigocladus laminosus*.

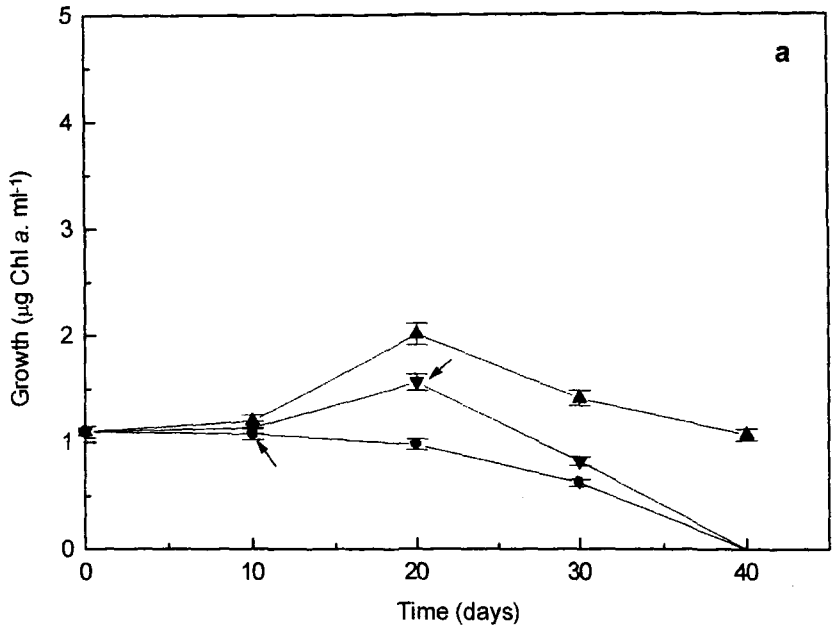
Growth conditions	Observations	[a]Start of akinete differentiation Time (days)	[b]End of akinete differentiation Time (days)
[c]Inorganic nitrogen sources (N_2-,NO_3^-- and NH_4^+-media)	Akinetes were found in the non-exponential growth phase in N_2- and NH_4^+-medium.	N_2-medium: 10 at 25°C 40 at 45°C NH_4^+-medium: 20 at 25°C 40 at 45°C NO_3^--medium: —	N_2-medium: 40 at 25°C 70 at 45°C NH_4^+-medium:40 at 25°C 60 at 45°C NO_3^--medium: —
pH shift of culture medium from 7.5 to 5, 6, 9 or 10	Akinetes observed	4 (pH 9)	20 (pH 9)
Carbon sources (addition of 10-50 mM glucose, fructose or sucrose to the culture medium)	No akinetes observed	—	—
Amino acids (addition of 1 mM glutamine, asparagine, arginine or alanine to the growth medium)	No akinetes observed	—	—
Sulphate limitation	Akinetes observed	5 at 25°C 20 at 45°C	30 at 25°C 40 at 45°C
Phosphate limitation	Akinetes observed	10 at 25°C 40 at 45°C	40 at 25°C 60 at 45°C
D-N_2-medium lacking $FeCl_3$	Akinetes observed	4	50
Salt (NaCl)	Akinete frequency increased with addition of NaCl (100-400 mM)	3	10
Dark incubation	Akinetes observed	7	45

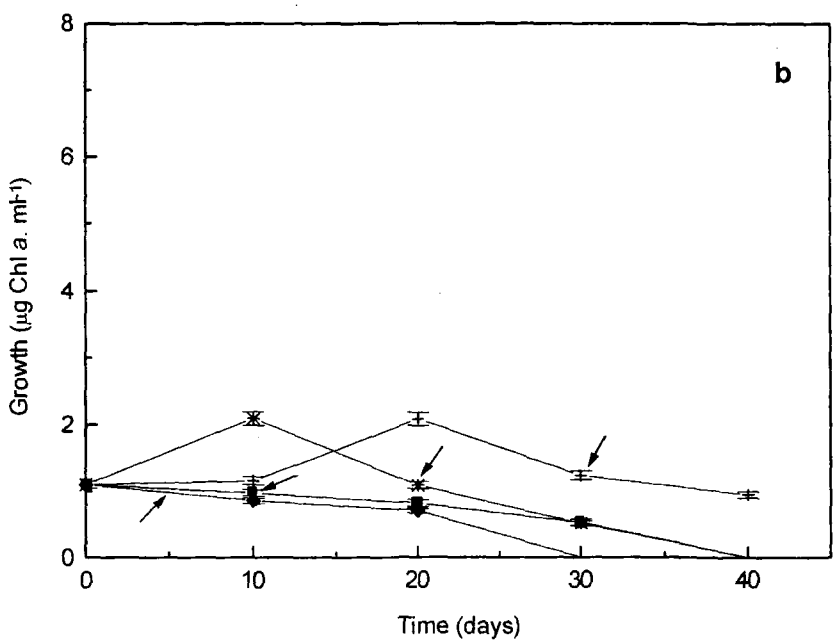
[a] Time when akinetes first appeared.

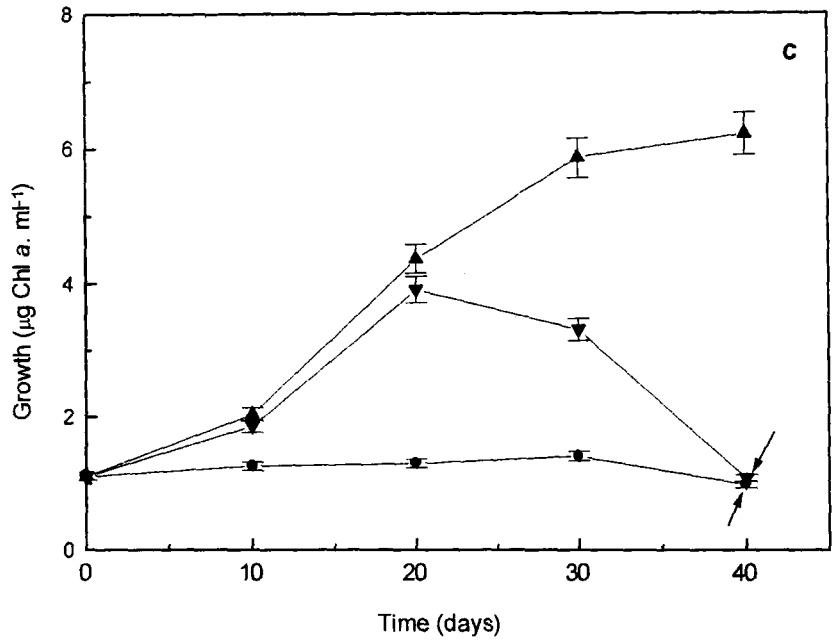
[b] Time when maximum number of cells had become akinetes and no further akinete differentiation occurred.

[c] N_2 refers to medium D without any N, NO_3^- to medium D containing 10 mM $NaNO_3$ and NH_4^+ to medium D with 2 mM NH_4Cl.

Fig 5.2 Growth of *Mastigocladus laminosus* at 25°C (a, b) and 45°C (c, d). N_2-medium (●), NO_3^--medium (▲), NH_4^+-medium (▼), N_2-medium minus sulphate (♦), NO_3^--medium minus sulphate (*), N_2-medium minus phosphate (■) and NO_3^--medium minus phosphate (+). Arrows indicate the start of akinete differentiation.






c
Growth (µg Chl a. ml-1)
Time (days)
0
2
4
6
8
0
10
20
30
40

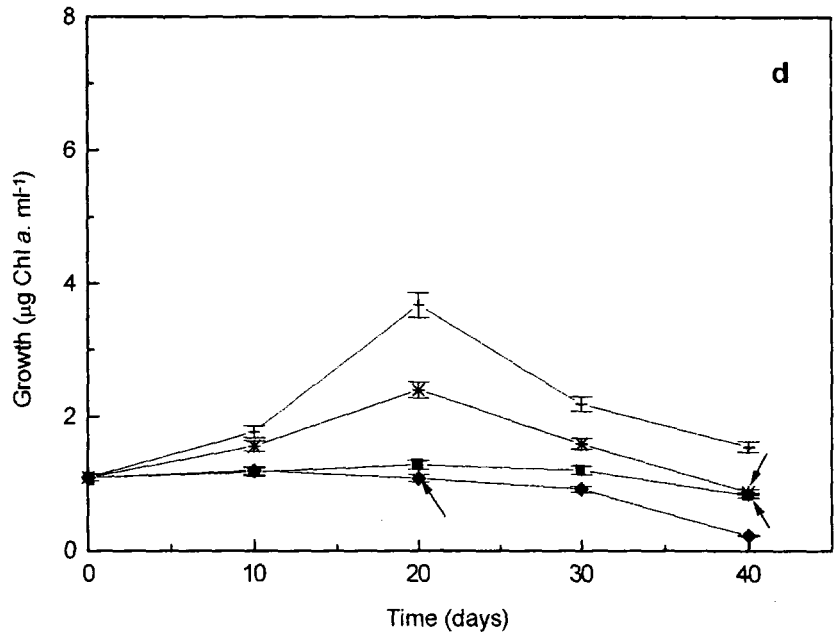

d
Growth (µg Chl a. ml-1)
Time (days)
0
2
4
6
8
0
10
20
30
40

Table 5.2 Time course of akinete differentiation in *Mastigocladus laminosus* on transfer to different growth media at 25°C and 45°C. (A) In N_2-, NO_3^-- and NH_4^+-media; (B) In N_2-medium minus sulphate, N_2-medium minus phosphate, NO_3^--medium minus sulphate and NO_3^--medium minus phosphate.

Exponentially growing cultures of *M. laminosus* were washed and transferred to media containing different nitrogen sources at 25°C and 45°C. At different time interval, akinete frequency was calculated as percentage of total vegetative cells. One thousand cells were counted in each sample. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates. N_2 refers to medium D without any N, NO_3^- to medium D (+ 10 mM $NaNO_3$) and NH_4^+ to medium D with 2 mM NH_4Cl. $-SO_4$ refers to N_2-medium minus sulphate, $-SO_4$ + NO_3^- to NO_3^--medium minus sulphate, $-PO_4$ to N_2-medium minus phosphate and $-PO_4$ + NO_3 to NO_3^--medium minus phosphate.

A.

Time (days)	Akinete frequency (%)					
	N_2		NO_3^-		NH_4^+	
	25°C	45°C	25°C	45°C	25°C	45°C
0	0.0	0.0	0.0	0.0	0.0	0.0
5	0.0	0.0	0.0	0.0	0.0	0.0
10	12.0	0.0	0.0	0.0	0.0	0.0
20	30.0±1.5	0.0	0.0	0.0	30.0±1.5	0.0
30	52.0±2.6	0.0	0.0	0.0	50.0±2.5	0.0
40	80.0±4.0	20.0±1.0	0.0	0.0	75.0±3.0	15.0±0.8

B.

Time (days)	Akinete frequency (%)							
	$-SO_4$		$-SO_4 + NO_3^-$		$-PO_4$		$-PO_4 + NO_3^-$	
	25°C	45°C	25°C	45°C	25°C	45°C	25°C	45°C
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	15.0±0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	50.0±2.5	0.0	0.0	0.0	28.0±1.4	0.0	0.0	0.0
20	60.0±3.0	25.0±1.2	24.0±1.2	0.0	36.0±1.8	0.0	0.0	0.0
30	80.0±4.0	50.0±2.5	68.0±3.4	40.0±2.0	50.0±2.5	0.0	28.0±1.4	0.0
40	98.0±4.9	62.0±3.1	84.0±4.2	60.0±3.0	75.0±3.7	25.0±1.2	40.0±2.0	0.0

Table 5.3 Change in phycocyanin content during akinete differentiation in *Mastigocladus laminosus* cultures grown in media containing inorganic nitrogen sources (A) and in media lacking sulphate (B) or phosphate (C) at 25°C and 45°C.

Exponentially growing *M. laminosus* cultures in NO_3^--medium were washed and transferred to different growth media at 25°C and 45°C. At different time intervals, phycocyanin content (μg. ml^{-1}) was determined. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates. N_2 refers to medium D without any N, NO_3^- to medium D (+ 10 mM $NaNO_3$) and NH_4^+ to medium D with 2 mM NH_4Cl. $-SO_4$ refers to N_2-medium minus sulphate, $-SO_4 + NO_3$ to NO_3^--medium minus sulphate, $-PO_4$ to N_2-medium minus phosphate and $-PO_4 + NO_3$ to NO_3^--medium minus phosphate.

A.

Time (days)	Phycocyanin (μg. ml^{-1})					
	N_2		NO_3^-		NH_4^+	
	25°C	45°C	25°C	45°C	25°C	45°C
0	2.58±0.1	2.58±0.1	2.58±0.1	2.58±0.1	2.58±0.1	2.58±0.1
10	2.58±0.1	3.16±0.2	4.57±0.2	7.25±0.4	6.39±0.3	7.47±0.4
20	2.40±0.1	3.36±0.2	5.83±0.3	15.91±0.8	4.19±0.2	12.41±0.6
30	2.13±0.1	3.74±0.2	5.52±0.3	27.14±1.4	3.60±0.2	9.27±0.5
40	0.13±0.1	2.77±0.1	4.95±0.2	16.27±0.8	0.65± 0.1	4.99±0.2

B

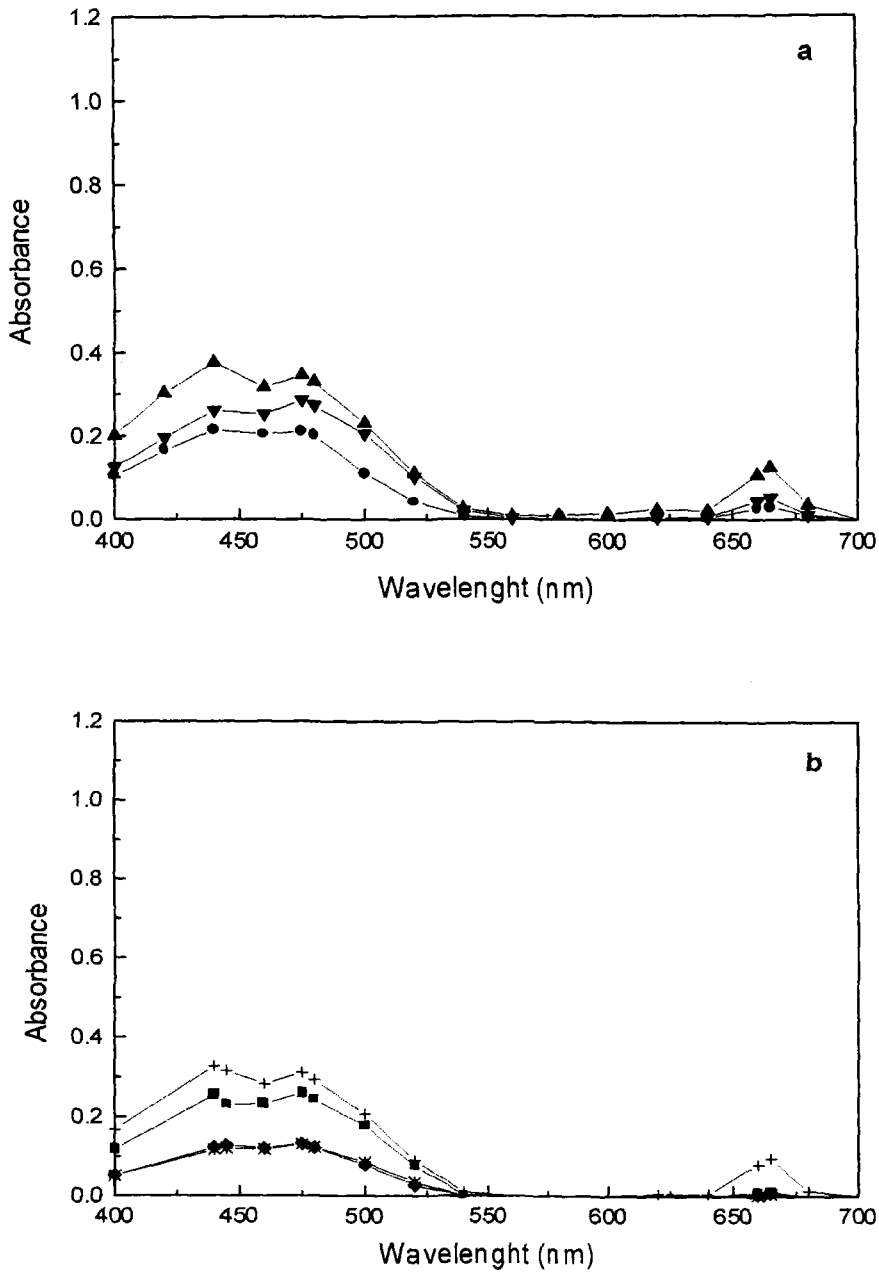
.

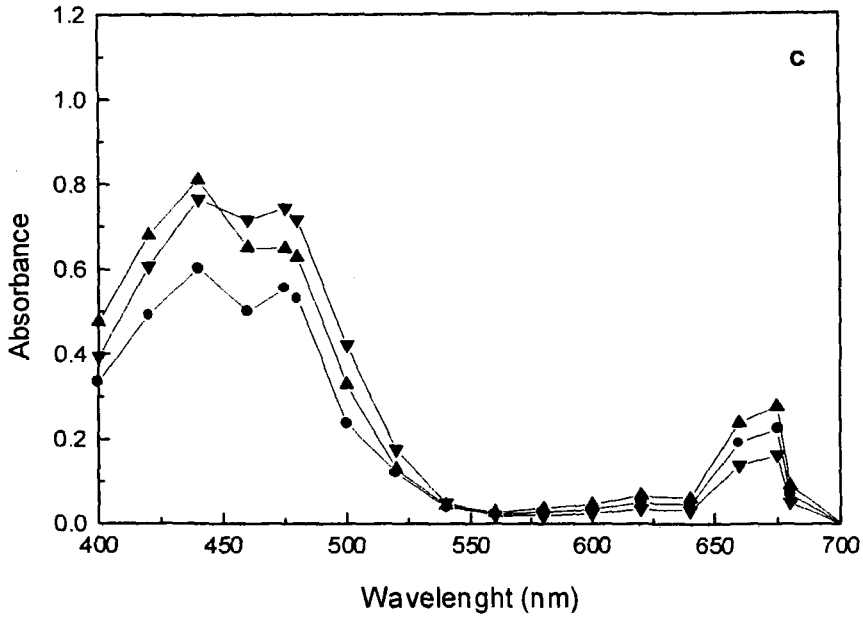
Time (days)	Phycocyanin (µg. ml^{-1})			
	$-SO_4$		$-SO_4 + NO3^-$	
	25°C	45°C	25°C	45°C
0	2.58 ± 0.1	2.58 ± 0.1	2.58 ± 0.1	2.58 ± 0.1
10	1.60 ± 0.1	3.02 ± 0.2	2.48 ± 0.1	4.99 ± 0.2
20	1.20 ± 0.1	2.32 ± 0.1	2.32 ± 0.1	8.20 ± 0.4
30	0.56 ± 0	1.80 ± 0.1	0.32 ± 0.0	3.84 ± 0.2
40	ND	0.01± 0	ND	2.12 ± 0.1

C

Time (days)	Phycocyanin (µg. ml^{-1})			
	$-PO_4$		$-PO_4 + NO_3^-$	
	25°C	45°C	25°C	45°C
0	2.58 ± 0.1	2.58 ± 0.1	2.58 ± 0.1	2.58 ± 0.1
10	2.40 ± 0.0	3.72 ± 0.2	2.62 ± 0.1	4.12 ± 0.2
20	2.23 ± 0.1	3.42 ± 0.2	3.12 ± 0.2	12.03 ± 0.6
30	0.56 ± 0.0	3.82 ± 0.2	2.85 ± 0.1	7.23 ± 0.4
40	ND	2.23 ± 0.1	2.40 ± 0.1	4.99 ± 0.2

Fig 5.3 Absorption spectra of methanol extract pigments from cultures of *Mastigocladus laminosus* grown at 25°C (a, b) and 45°C (c, d). N_2-medium (●), NO_3^--medium (▲), NH_4^+-medium (▼), N_2-medium minus sulphate (♦), NO_3^--medium minus sulphate (*), N_2-medium minus phosphate (■) and NO_3^--medium minus phosphate (+).

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Absorbance
Wavelenght (nm)
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1.0
0.8
0.6
0.4
0.2
0.0
400
450
500
550
600
650
700

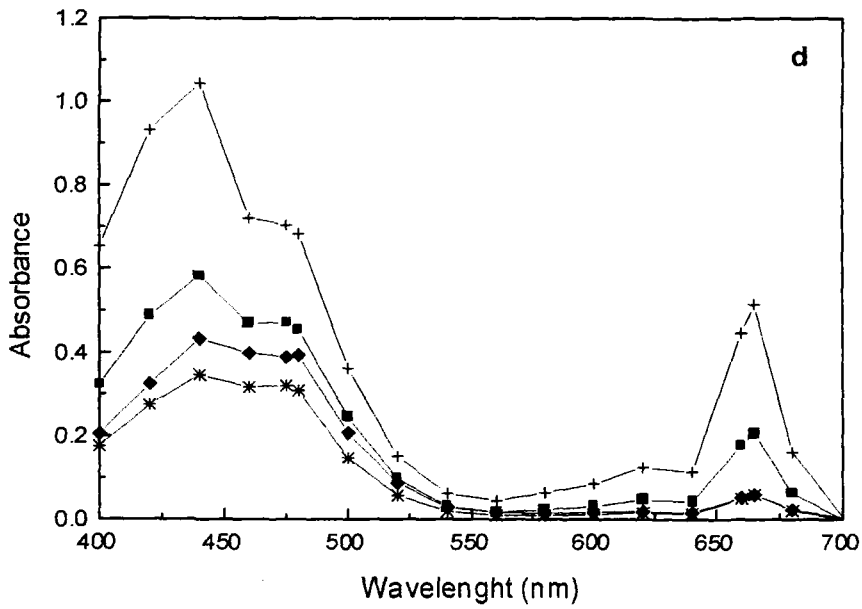
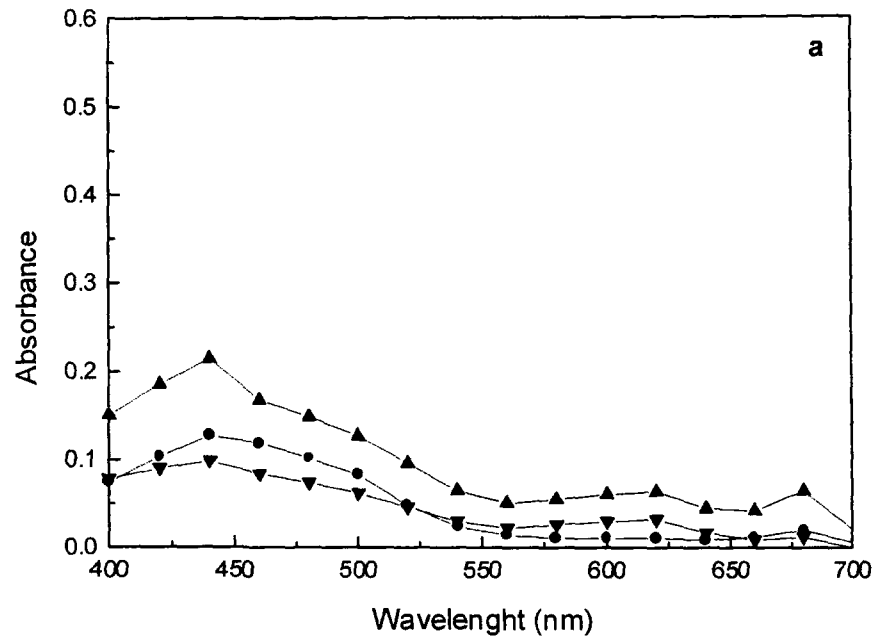
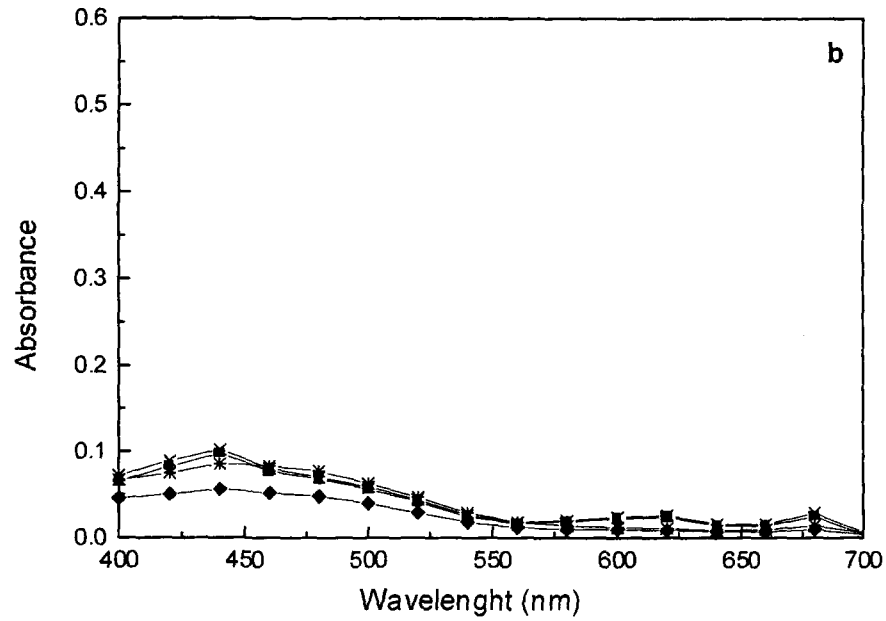
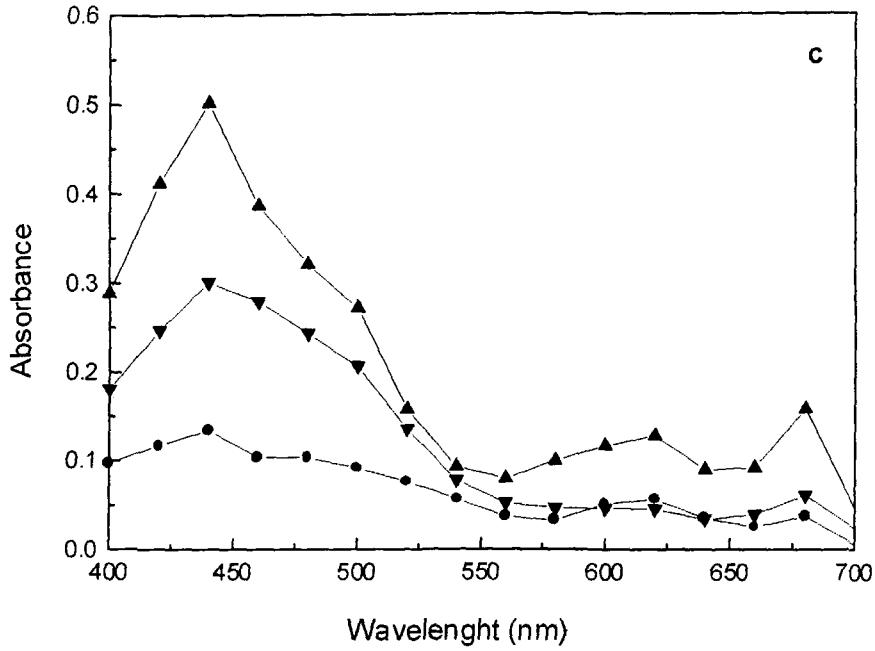

d
Absorbance
Wavelenght (nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
400
450
500
550
600
650
700

Fig 5.4 Absorption spectra of phosphate buffer extract pigments from cultures of *Mastigocladus laminosus* at 25°C (a, b) and 45°C (c, d). N_2-medium (●), NO_3^--medium (▲), NH_4^+-medium (▼), N_2-medium minus sulphate (♦), NO_3^--medium minus sulphate (*), N_2-medium minus phosphate (■) and NO_3^--medium minus phosphate (+).






c
Absorbance
Wavelenght (nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
400
450
500
550
600
650
700

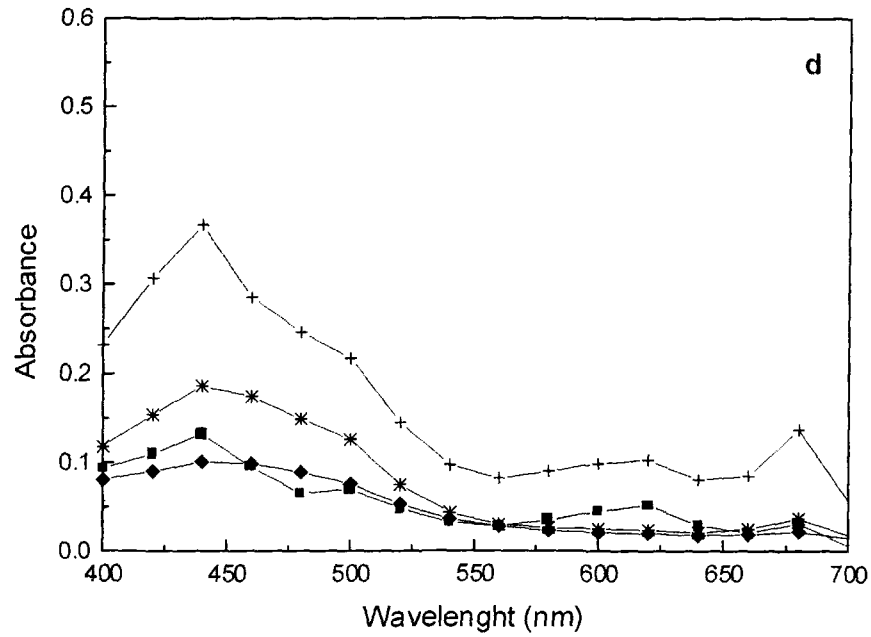

d
Absorbance
Wavelenght (nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
400
450
500
550
600
650
700

Fig 5.5 Light photomicrographs of *Mastigocladus laminosus*. a, filament showing heterocyst (H) and vegetative cells (V). b, the same filament as in 'a' under fluorescence (excitation λ 545-580 nm). Absence of fluorescence from heterocyst due to lack of phycobiliproteins is evident. c, filament showing chain of akinetes (A) and a vegetative cell (V). d, the same filament as in 'c' under fluorescence (excitation λ 545-580 nm). Absence of fluorescence from akinetes due to lack of phycobiliproteins is evident. Only the lone vegetative cell shows fluorescence.
Magnification- 40 X (a, b), 100 X (c, d).

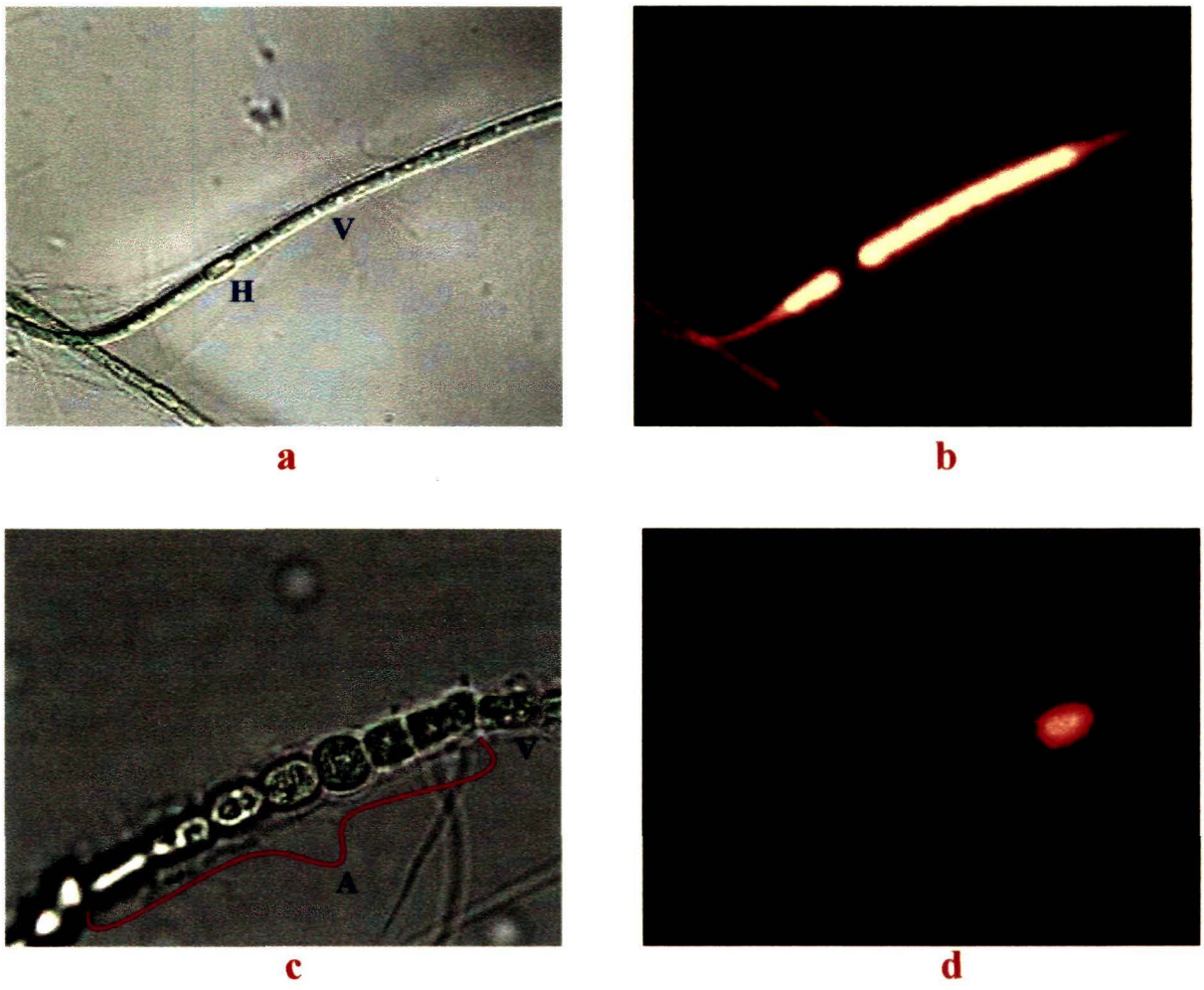


Table 5.4 Photosynthetic O_2 evolution during akinete differentiation in *Mastigocladus laminosus* cultures grown in media containing different nitrogen sources (A) and in media lacking sulphate or phosphate (B) at 25°C and 45°C.

Exponentially growing *M. laminosus* cultures in NO_3^--medium were washed and transferred to different growth media at 25°C and 45°C. Photosynthetic oxygen evolution (nmol O_2 evolved. μg^{-1} Chl *a*. h^{-1}) was determined at different time intervals. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates. N_2 refers to medium D without any N, NO_3^- to medium D containing 10 mM $NaNO_3$ and NH_4^+ to medium D with 2 mM NH_4Cl. $-SO_4$ refers to N_2-medium minus sulphate, and $-PO_4$ to N_2-medium minus phosphate.

A.

Time (days)	Photosynthetic oxygen evolution					
	N_2		NO_3^-		NH_4^+	
	25°C	45°C	25°C	45°C	25°C	45°C
0	180.0 ± 9	180.0 ± 9	180.0 ± 9	180.0 ± 9	180.0 ± 9	180.0 ± 9
10	265.0 ±13	540.0 ± 27	265.0 ±13	360.0 ±18	190.0 ± 9.5	320.0 ±16
20	360.0 ±18	760.0 ± 38	320.0 ±16	475.0 ± 23	220.0 ±11	370.0 ±18
30	80.0 ± 4	420.0 ± 21	190.0 ± 9	650.0 ± 32	110.0 ± 5.5	420.0 ±21
40	0.0	120.0 ± 6	160.0 ± 8	920.0 ± 46	0.0	170.0 ± 8

B.

Time (days)	Photosynthetic oxygen evolution			
	$-SO_4$		$-PO_4$	
	25°C	45°C	25°C	45°C
0	180.0 ± 9	180.0 ± 9	180.0 ± 9	180.0 ± 9
10	190.0 ± 9	265.0 ±13	256.0 ±12	420.0 ± 21
20	120.0 ± 6	120.0 ± 6	190.0 ± 9	540.0 ± 27
30	0.0	110.0 ± 5	0	280.0 ± 14
40	0.0	0.0	0	60.0 ± 3

Table 5.5 Respiration rates during akinete differentiation in *Mastigocladus laminosus* cultures grown in media containing different nitrogen sources (A) and in N_2-media lacking sulphate or phosphate (B) at 25°C and 45°C.

Exponentially growing *M. laminosus* cultures in NO_3^--media were washed and transferred to different growth media at 25°C and 45°C. Respiration rates (nmol O_2 consumed. μg^{-1} Chl *a*. h^{-1}) were determined at different time intervals. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates. N_2 refers to medium D without any N, NO_3^- to medium D containing 10 mM $NaNO_3$ and NH_4^+ to medium D with 2 mM NH_4Cl. $-SO_4$ refers to N_2-medium minus sulphate and $-PO_4$ to N_2-medium minus phosphate.

A.

Time (days)	Respiration rate (nmol O_2 consumed. μg^{-1} Chl *a*. h^{-1})					
	N_2		NO_3^-		NH_4^+	
	25°C	45°C	25°C	45°C	25°C	45°C
0	120 ± 6	120 ± 6	120 ± 6	120 ± 6	120 ± 6	120 ± 6
10	160 ± 8	280 ± 14	180 ± 9	320 ± 16	160 ± 8	220 ± 11
20	180 ± 9	315 ± 15	230 ± 11	412 ± 21	180 ± 9	270 ± 14
30	90 ± 4	210 ± 11	160 ± 8	602 ± 30	90 ± 5	300 ± 15
40	30 ± 2	80 ± 4	120 ± 6	730 ± 36	30 ± 2	110 ± 5

B.

Time (days)	Respiration rate (nmol O_2 consumed. μg^{-1} Chl *a*. h^{-1})			
	$-SO_4$		$-PO_4$	
	25°C	45°C	25°C	45°C
0	120 ± 6	120 ± 6	120 ± 6	120 ± 6
10	130 ± 7	190 ± 9	190 ± 10	320 ± 16
20	60 ± 3	80 ± 4	110 ± 5	460 ± 23
30	30 ± 2	60 ± 3	70 ± 3	210 ± 10
40	20 ± 1	40 ± 2	30 ± 1	40 ± 2

Table 5.6 Heterocyst frequency (HF) of *Mastigocladus laminosus* cultures grown in N_2-medium with and without sulphate and phosphate at 25°C and 45°C.

Exponentially growing *M. laminosus* cultures in NO_3^--media were washed and transferred to fresh N_2-media with and without sulphate or phosphate at 25°C and 45°C. Heterocyst frequency (%) was determined at different time intervals. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates. N_2 refers to medium D without any N, $-SO_4$ to N_2-medium minus sulphate, and $-PO_4$ to N_2-medium minus phosphate. ND, not detectable.

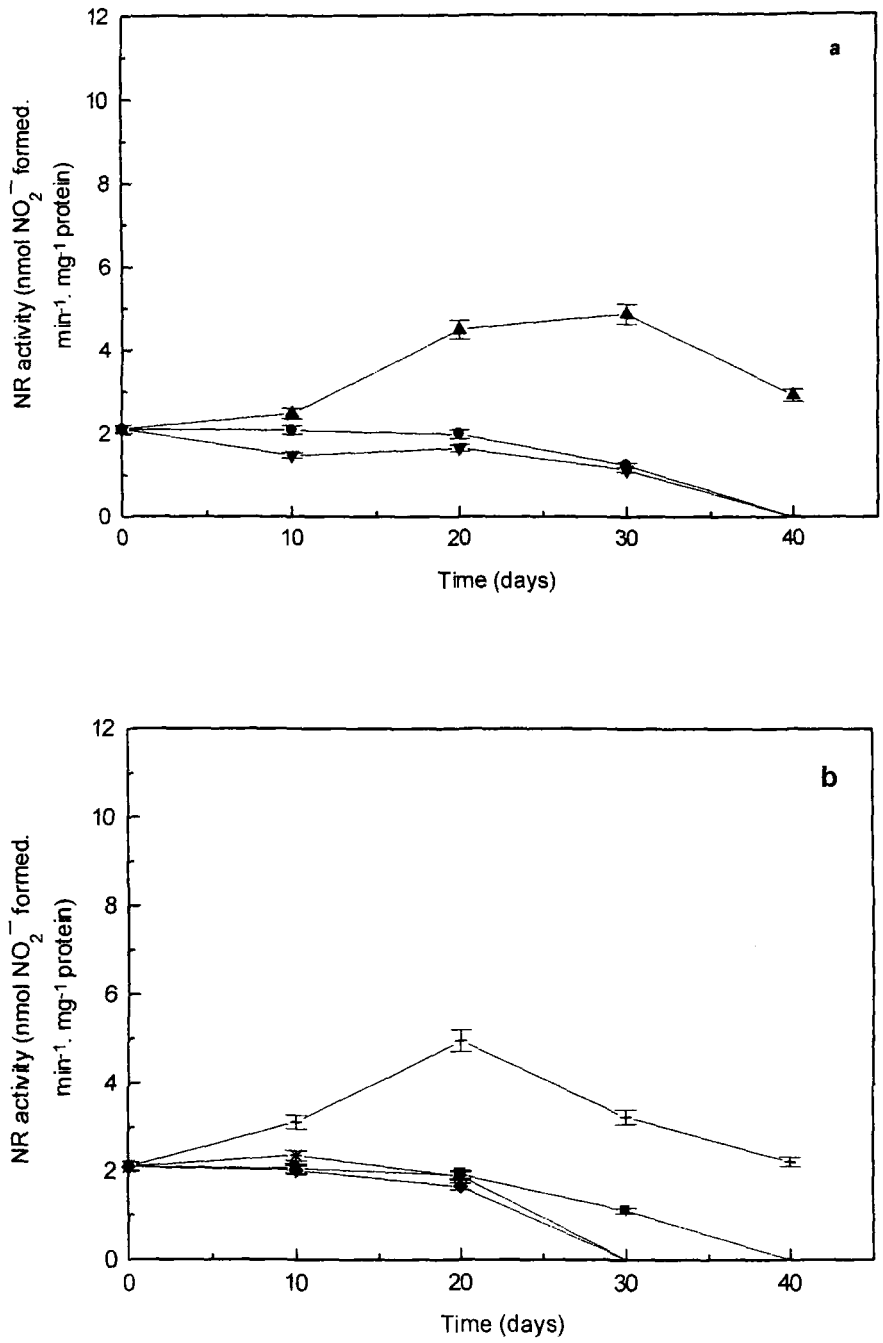
Time (days)	Heterocyst frequency (%)					
	N_2		$-SO_4$		$-PO_4$	
	25°C	45°C	25°C	45°C	25°C	45°C
0	ND	ND	ND	ND	ND	ND
10	2.0 ± 0.1	10.0 ± 0.5	0.8 ± 0.0	1.9 ± 0.1	1.0 ± 0.1	8.0 ± 0.4
20	1.2 ± 0.1	12.0 ± 0.6	ND	0.8	0.8 ± 0.0	10.0 ± 0.5
30	0.8 ± 0.0	8.0 ± 0.4	ND	ND	0.6 ± 0.0	6.0 ± 0.3
40	ND	1.5 ± 0.0	ND	ND	ND	0.8

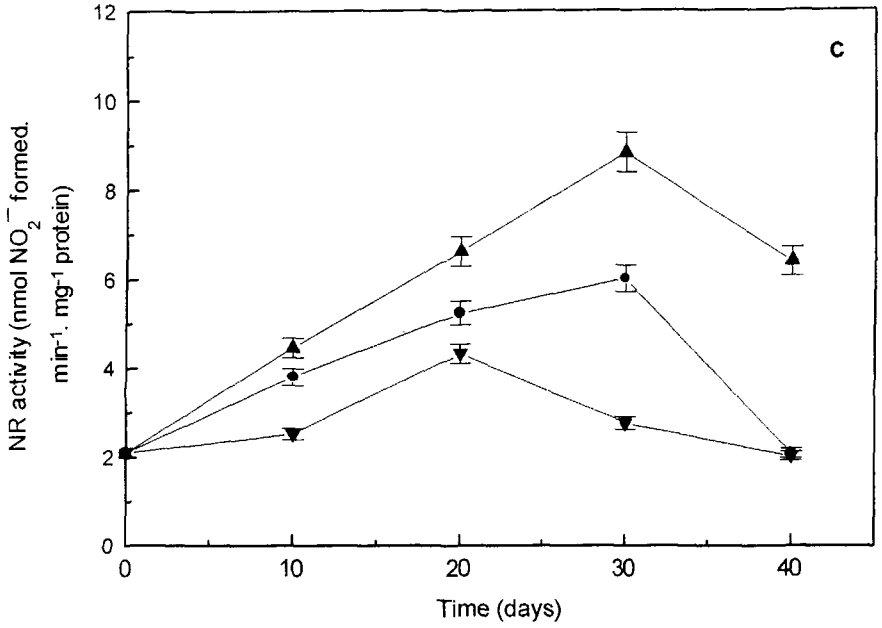
Table 5.7 Nitrogenase activity of *Mastigocladus laminosus* cultures grown in N_2-medium with and without sulphate and phosphate at 25°C and 45°C.

Exponentially growing *M. laminosus* cultures in NO_3^--media were washed and transferred to fresh N_2-media with and without sulphate or phosphate at 25°C and 45°C. Nitrogenase activity (nmol C_2H_4 formed.μg^{-1} Chl *a*. h^{-1}) was determined at different time intervals. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates. N_2 refers to medium D without any N, $-SO_4$ to N_2-medium minus sulphate, and $-PO_4$ to N_2-medium minus phosphate.

Time (days)	Nitrogenase activity					
	N_2		$-SO_4$		$-PO_4$	
	25°C	45°C	25°C	45°C	25°C	45°C
0	ND	ND	ND	ND	ND	ND
10	1.4 ± 0.7	7.2 ± 0.4	0.3 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	5.4 ± 0.3
20	0.8 ± 0.0	8.5 ± 0.4	ND	0.6 ± 0.0	0.6 ± 0.0	2.6 ± 0.1
30	0.2 ± 0.0	5.8 ± 0.3	ND	0.2 ± 0.0	0.2 ± 0.0	1.2 ± 0.1
40	ND	0.8 ± 0.0	ND	0.5 ± 0.0	ND	0.6 ± 0.0

Fig 5.6 *Nitrate reductase activity during akinete differentiation in Mastigocladus laminosus* grown in different media at 25°C (a, b) and 45°C (c, d). N_2-medium (●), NO_3^--medium (▲), NH_4^+-medium (▼), N_2-medium minus sulphate (♦), NO_3^--medium minus sulphate (•), N_2-medium minus phosphate (■) and NO_3^--medium minus phosphate (+).




c
NR activity (nmol NO_2^- formed. min^{-1}. mg^{-1} protein)
Time (days)
0
2
4
6
8
10
12
0
10
20
30
40

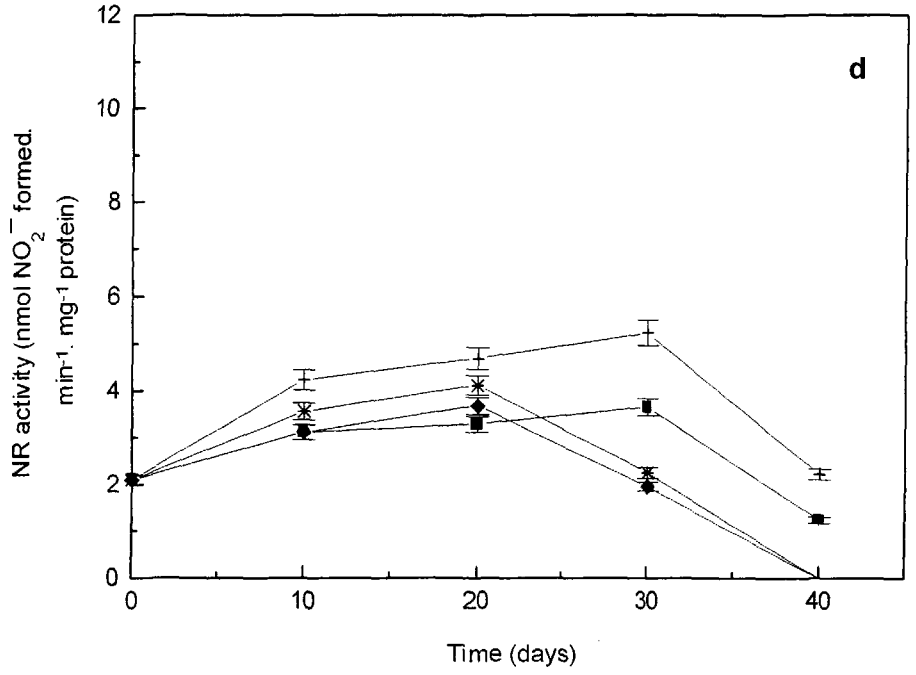
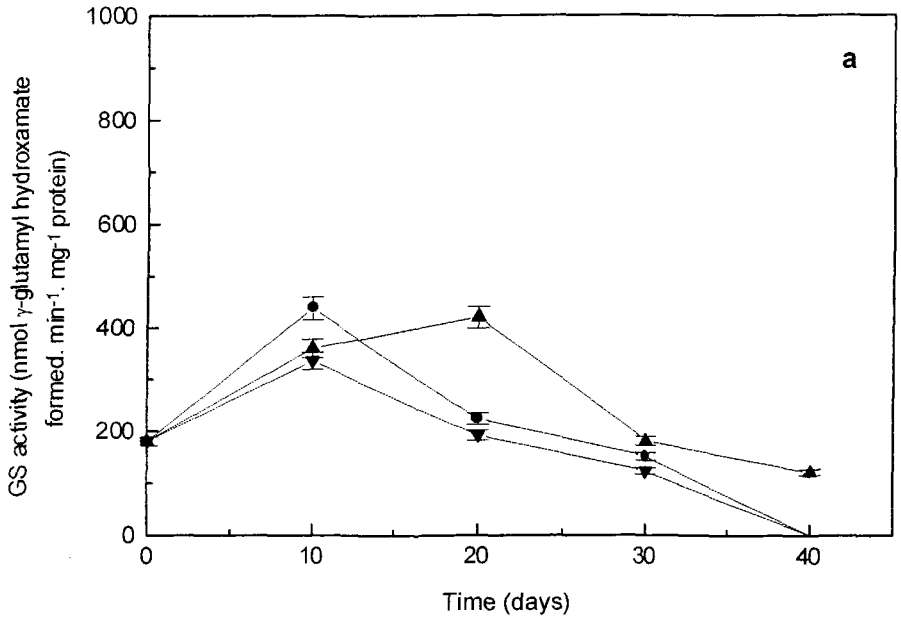
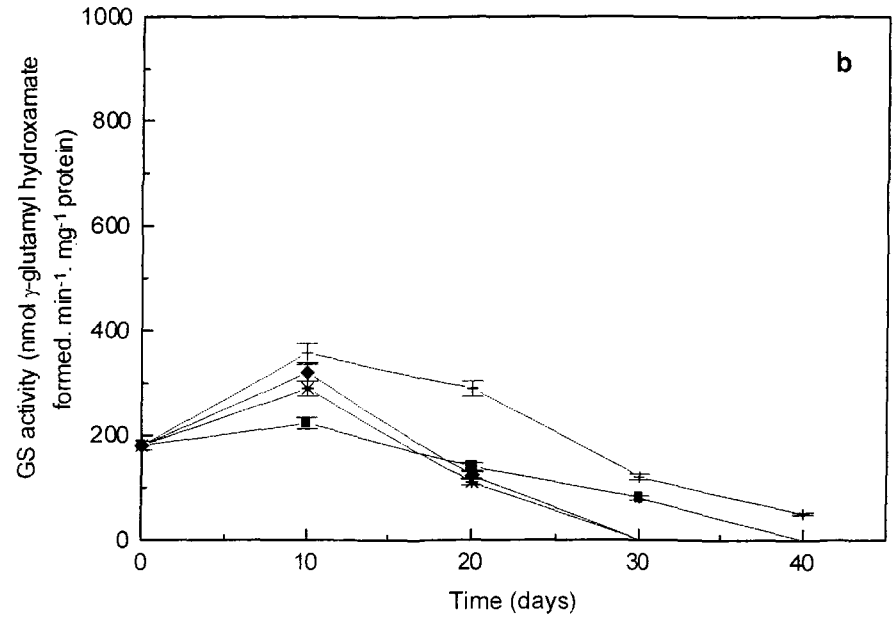
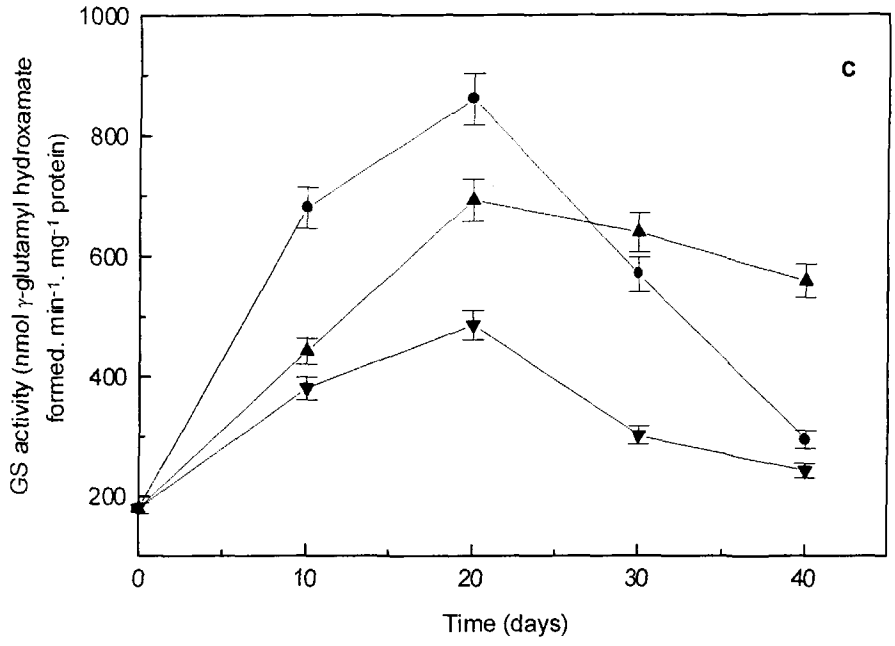

d
NR activity (nmol NO_2^- formed. min^{-1}. mg^{-1} protein)
Time (days)
0
2
4
6
8
10
12
0
10
20
30
40

Fig 5.7 Glutamine synthetase (transferase) activity during akinete differentiation in *Mastigocladus laminosus* grown in different media at 25°C (a, b) and 45°C (c, d). N_2-medium (●), NO_3^--medium (▲), NH_4^+-medium (▼), N_2-medium minus sulphate (♦), NO_3^--medium minus sulphate (*), N_2-medium minus phosphate (■) and NO_3^--medium minus phosphate (+).






c
GS activity (nmol γ-glutamyl hydroxamate formed. min-1. mg-1 protein)
1000
800
600
400
200
0
10
20
30
40
Time (days)

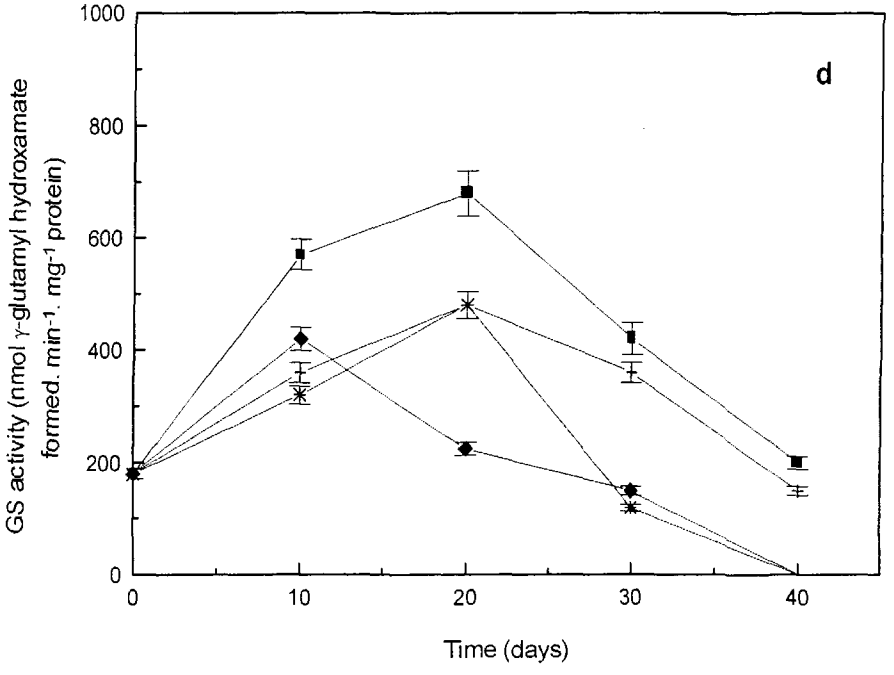

d
GS activity (nmol γ-glutamyl hydroxamate formed. min-1. mg-1 protein)
1000
800
600
400
200
0
10
20
30
40
Time (days)

Table 5.8 Changes in C:N ratio during akinete differentiation in *Mastigocladus laminosus* grown in media containing inorganic nitrogen sources (A) and in media lacking sulphate (B) or phosphate (C) at 25°C and 45°C.

Exponentially growing *M. laminosus* cultures in NO_3^--media were washed and transferred to different growth media at 25°C and 45°C. C:N ratio at different time intervals was determined. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates. N_2 refers to medium D without any N, NO_3^- to medium D containing 10 mM $NaNO_3$, and NH_4^+ to medium D with 2 mM NH_4Cl. $-SO_4$ refers to N_2-medium minus sulphate, $-SO_4 + NO_3^-$ to NO_3^--medium minus sulphate, $-PO_4$ to N_2-medium minus phosphate and $-PO_4 + NO_3$ to NO_3^--medium minus phosphate.

A.

Time (days)	C:N ratio					
	N_2		NO_3^-		NH_4^+	
	25°C	45°C	25°C	45°C	25°C	45°C
0	4.67 ± 0.2	4.67 ± 0.2	4.67 ± 0.2	4.67 ± 0.2	4.67 ± 0.2	4.67 ± 0.2
10	8.11 ± 0.4	7.19 ± 0.4	4.72 ± 0.2	4.32 ± 0.2	5.84 ± 0.3	5.55 ± 0.3
20	9.11 ± 0.5	7.60 ± 0.4	5.12 ± 0.3	5.05 ± 0.2	6.23 ± 0.3	6.03 ± 0.3
30	9.48 ± 0.5	7.99 ± 0.4	6.58 ± 0.3	5.30 ± 0.3	8.38 ± 0.4	7.12 ± 0.4
40	9.35 ± 0.5	8.69 ± 0.4	6.03 ± 0.3	5.37 ± 0.3	8.48 ± 0.4	8.12 ± 0.4

B.

Time (days)	C:N ratio			
	$-SO_4$		$-SO_4 + NO_3^-$	
	25°C	45°C	25°C	45°C
0	4.67 ± 0.2	4.67 ± 0.2	4.67 ± 0.2	4.67 ± 0.2
10	9.70 ± 0.5	8.58 ± 0.4	7.52 ± 0.4	6.93 ± 0.3
20	11.49 ± 0.6	9.23 ± 0.5	8.69 ± 0.4	7.12 ± 0.4
30	13.17 ± 0.7	9.49 ± 0.5	8.41 ± 0.4	7.46 ± 0.4
40	14.45 ± 0.7	10.61 ± 0.5	9.23 ± 0.5	8.89 ± 0.4

C.

Time (days)	C:N ratio			
	$-PO_4$		$-PO_4 + NO_3^-$	
	25°C	45°C	25°C	45°C
0	4.67 ± 0.2	4.67 ± 0.2	4.67 ± 0.2	4.67 ± 0.2
10	8.64 ± 0.4	7.12 ± 0.4	5.80 ± 0.3	5.42 ± 0.3
20	8.95 ± 0.4	7.20 ± 0.4	5.97 ± 0.3	5.39 ± 0.3
30	8.97 ± 0.4	7.58 ± 0.4	6.10 ± 0.3	5.67 ± 0.3
40	10.12 ± 0.5	8.70 ± 0.4	6.37 ± 0.3	6.12 ± 0.3

Fig 5.8 Light micrograph of *Mastigocladus laminosus* showing akinete germination. (Magnification 100 X).

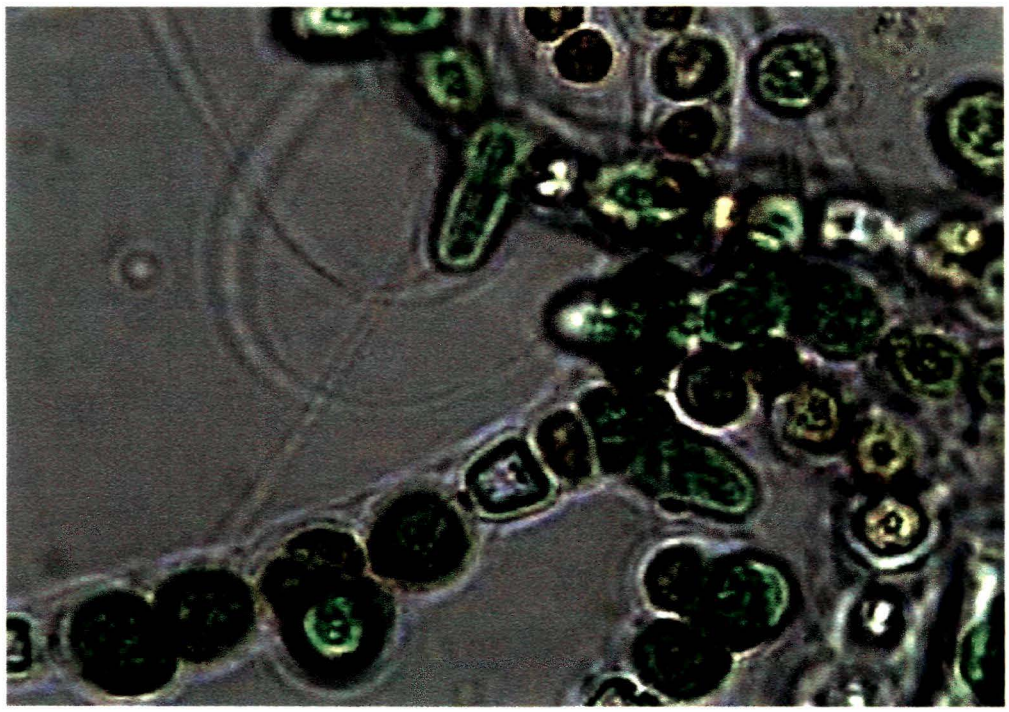


Table 5.9 Germination of *Mastigocladus laminosus* akinetes: germination frequency (A) and increase in Chl *a* concentration (B) in media containing different inorganic nitrogen sources at 25°C and 45°C.

Akinetes of *Mastigocladus laminosus* were suspended in media containing different inorganic nitrogen sources at 25°C and 45°C to a concentration of 1.23 x 10^6 akinetes.ml^{-1} at time zero. Germination frequency (%) and growth (µg Chl *a*. ml^{-1}) were determined in samples withdrawn at different time intervals. N_2 refers to medium D without any N, NO_3^- to medium D containing 10 mM $NaNO_3$ and NH_4^+ to medium D with 2 mM NH_4Cl. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates.

A

Time (hours)	Germination frequency (%)					
	N_2		NO_3^-		NH_4^+	
	25°C	45°C	25°C	45°C	25°C	45°C
0	0.0	0.0	0.0	0.0	0.0	0.0
24	0.0	1.0 ± 0.1	0.0	7.7 ± 0.4	0.0	6.0 ± 0.3
48	0.0	25.0 ± 1.2	0.0	30.0 ± 1.5	0.0	32.0 ± 1.6
72	0.0	55.0 ± 2.7	2.5 ± 0.1	60.0 ± 3.0	3.0 ± 0.1	75.0 ± 3.7
96	0.0	90.0 ± 4.5	8.5 ± 0.4	92.0 ± 4.6	6.2 ± 0.3	92.0 ± 4.6

B

Time (hours)	Increase in Chl *a* concentration (µg. ml^{-1})					
	N_2		NO_3^-		NH_4^+	
	25°C	45°C	25°C	45°C	25°C	45°C
0	0.03	0.03 ± 0.0	0.03 ± 0.0	0.03 ± 0.0	0.03 ± 0.0	0.03 ± 0.0
24	0.03	0.10 ± 0.0	0.05 ± 0.0	0.29 ± 0.0	0.06 ± 0.0	0.27 ± 0.0
48	0.03	0.24 ± 0.1	0.06 ± 0.0	0.34 ± 0.0	0.07 ± 0.0	0.32 ± 0.0
72	0.03	0.41 ± 0.1	0.12 ± 0.0	0.45 ± 0.0	0.16 ± 0.0	0.52 ± 0.0
96	0.03	0.95 ± 0.2	0.24 ± 0.0	1.05 ± 0.1	0.19 ± 0.0	1.10 ± 0.1

Fig 5.9 Heterocyst frequency (●), and nitrogenase activity (▲) during germination of *Mastigocladus laminosus* akinetes in N_2-medium at 45°C.

Akinetes of *Mastigocladus laminosus* were suspended in N_2-medium at 25°C and 45°C to a concentration of 1.23 x 10^6 akinetes. ml^{-1} at time zero. Heterocyst frequency (%) and nitrogenase activity (nmol C_2H_4 formed. μg^{-1} Chl *a*. h^{-1}) were determined in samples withdrawn at different time intervals.

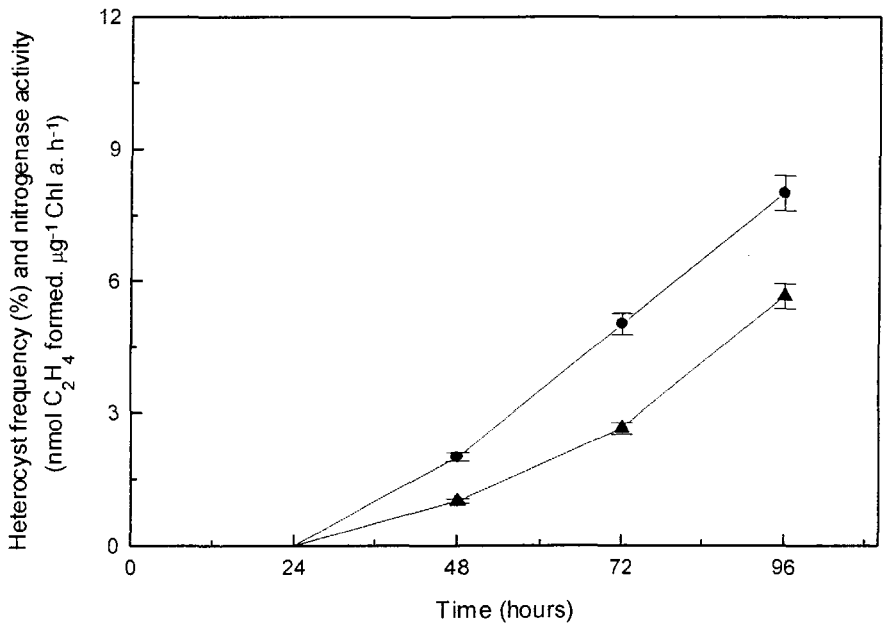


Table 5.10 Changes in phycocyanin content during germination of *Mastigocladus laminosus* akinetes.

Akinetes of *Mastigocladus laminosus* were suspended in media containing different inorganic nitrogen sources at 25°C and 45°C to a concentration of 1.23 x 10^6 akinetes. ml^{-1} at time zero. Phycocyanin content ($\mu g.\ ml^{-1}$) was determined in samples withdrawn at different time intervals. N_2 refers to medium D without any N, NO_3^- to medium D containing 10 mM $NaNO_3$, and NH_4^+ to medium D with 2 mM NH_4Cl. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates.

Time (hours)	Phycocyanin ($\mu g.\ ml^{-1}$)					
	N_2		NO_3^-		NH_4^+	
	25°C	45°C	25°C	45°C	25°C	45°C
0	0.0	0.0	0.0	0.0	0.0	0.0
24	0.0	0.0	0.0	0.65 ± 0.0	0.0	0.56 ± 0.0
48	0.0	1.14 ± 0.1	0.0	2.27 ± 0.1	0.0	2.24 ± 0.1
72	0.0	3.16 ± 0.2	0.56	4.09 ± 0.2	0.85 ± 0.0	4.69 ± 0.2
96	0.0	5.63 ± 0.3	1.53 ± 0.1	5.16 ± 0.3	1.14 ± 0.1	6.29 ± 0.3

Fig 5.10 Photosynthetic oxygen evolution (●) and respiratory oxygen consumption (▲) during germination of *Mastigocladus laminosus* akinetes in N_2-medium at 45°C.

Akinetes of *Mastigocladus laminosus* were suspended in N_2-medium at 45°C to a concentration of 1.23 x 10^6 akinetes. ml^{-1} at time zero. Photosynthetic O_2 evolution and consumption were determined in samples withdrawn at different time intervals.

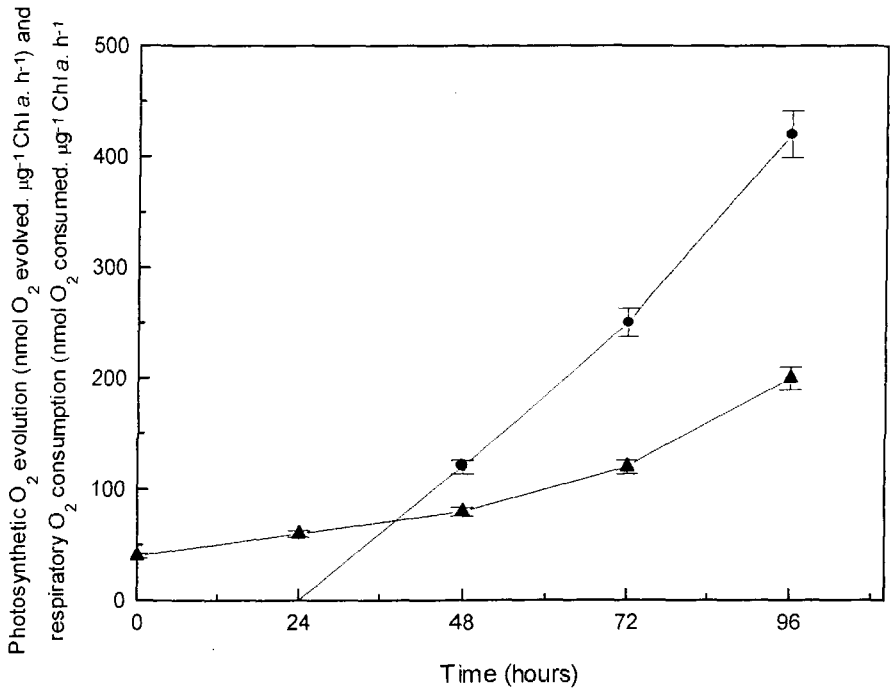


Fig 5.11 Nitrate reductase activity during germination of *Mastigocladus laminosus* akinetes in different growth media at 45°C.

Akinetes of *Mastigocladus laminosus* were suspended in media containing different inorganic nitrogen sources (N_2, •; NO_3^-, ▲; NH_4^+, ▼) at 45°C to a concentration of 1.23 x 10^6 akinetes. ml^{-1} at time zero. Nitrate reductase activity was determined in samples withdrawn at different time intervals. N_2 refers to medium D without any N, NO_3^- to medium D containing10 mM $NaNO_3$, and NH_4^+ to medium D with 2 mM NH_4Cl.

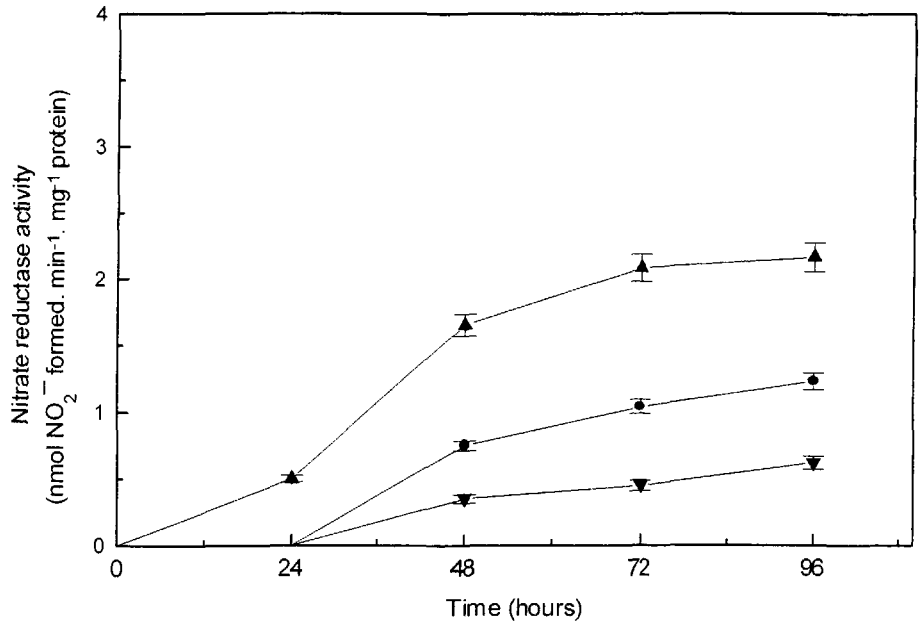


Fig 5.12 Glutamine synthetase (transferase) activity during germination of *Mastigocladus laminosus* akinetes in different growth media at 45°C.

Akinetes of *Mastigocladus laminosus* were suspended in media containing different inorganic nitrogen sources (N_2, •; NO_3^-, ▲; NH_4^+, ▼) at 45°C to a concentration of 1.23 x 10^6 akinetes. ml^{-1} at time zero. Glutamine synthetase (transferase) activity was determined in samples withdrawn at different time intervals. N_2 refers to medium D without any N, NO_3^- to medium D containing 10 mM $NaNO_3$, and NH_4^+ to medium D with 2 mM NH_4Cl.

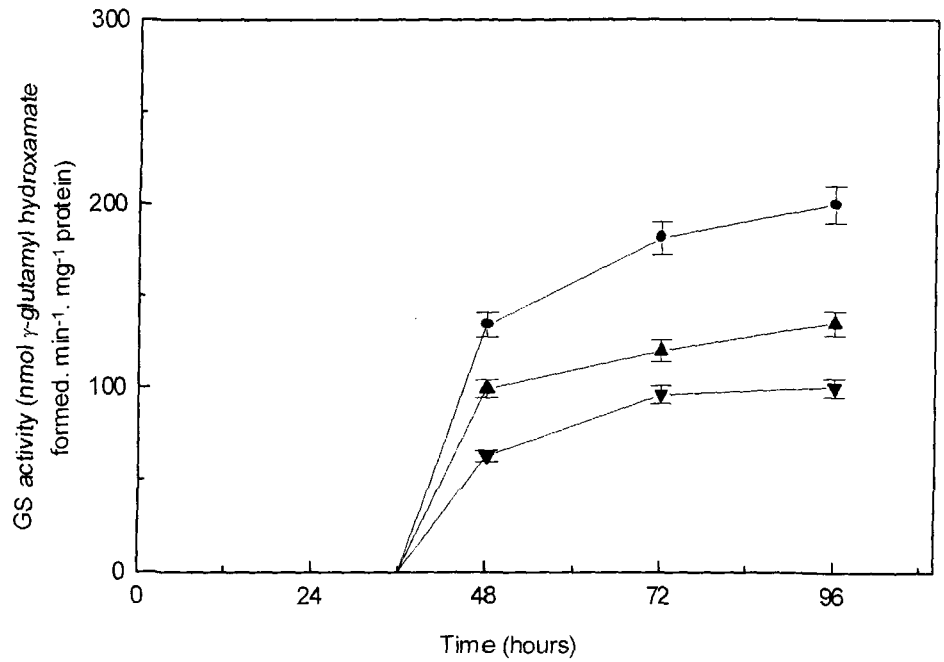


Table 5.11 Changes in C:N ratio during germination of *Mastigocladus laminosus* akinetes in different growth media at 25°C and 45°C.

Akinetes of *Mastigocladus laminosus* were suspended in media containing different inorganic nitrogen sources at 25°C and 45°C to a concentration of 1.23 x 10^6 akinetes. ml^{-1} at time zero. C:N ratio was determined in samples withdrawn at different time intervals. N_2 refers to medium D without any N, NO_3^- to medium D containing 10 mM $NaNO_3$, and NH_4^+ to medium D with 2 mM NH_4Cl. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates.

Time (hours)	C: N ratio					
	25°C			45°C		
	N_2	NO_3^-	NH_4^+	N_2	NO_3^-	NH_4^+
0	9.23 ± 0.0	9.23 ± 0.5	9.23 ± 0.5	9.23 ± 0.5	9.23 ± 0.5	9.23 ± 0.5
24	9.23 ± 0.0	9.12 ± 0.5	9.13 ± 0.4	8.79 ± 0.4	7.23 ± 0.4	7.45 ± 0.4
48	9.23 ± 0.0	8.99 ± 0.4	9.02 ± 0.4	8.23 ± 0.4	6.57 ± 0.3	6.69 ± 0.3
72	9.23 ± 0.0	8.23 ± 0.4	8.69 ± 0.4	7.89 ± 0.4	5.99 ± 0.3	6.12 ± 0.3
96	9.23 ± 0.0	7.22 ± 0.4	7.89 ± 0.4	7.75 ± 0.4	5.29 ± 0.3	5.21 ± 0.3

Table 5.12 Changes in protein contents during germination of *Mastigocladus laminosus* akinetes in different growth.

Akinetes of *Mastigocladus laminosus* were suspended in media containing different inorganic nitrogen sources at 25°C and 45°C to a concentration of 1.23 x 10^6 akinetes. ml^{-1} at time zero. Protein content was determined in samples withdrawn at different time intervals. N_2 refers to medium D without any N, NO_3^- to medium D containing 10 mM $NaNO_3$, and NH_4^+ to medium D with 2 mM NH_4Cl. The values presented are means ± standard error (SE) from two independent experiments, each with two replicates.

Time (hours)	Protein content (μg. ml^{-1})					
	N_2		NO_3^-		NH_4^+	
	25°C	45°C	25°C	45°C	25°C	45°C
0	19.95 ±1.0	19.95 ±1.0	19.95 ±1.0	19.95 ±1.0	19.95 ± 1.0	19.95 ±1.0
24	19.98 ±1.0	20.23 ±1.0	22.35 ±1.0	24.23 ±1.0	22.29 ± 1.0	25.32 ±1.0
48	20.12 ±1.0	24.43 ±1.0	24.50 ±1.0	26.59 ±1.0	24.00 ± 1.0	28.31 ±1.0
72	20.98 ±1.0	29.02 ±1.0	26.11 ±1.0	30.11 ±1.0	25.32 ± 1.0	31.02 ±2.0
96	21.22 ±1.0	32.25 ±1.0	28.18 ±1.0	34.23 ±1.0	26.11 ± 1.0	33.42 ±2.0

CHAPTER 6

Summary

Micro-organisms populate every habitable environment on earth and for billions of years they have been affecting physical and chemical properties of their surroundings by their activities such as oxygenic photosynthesis, nitrogen fixation and carbon sequestration. Among microorganisms, cyanobacteria are of special interest as they have simple growth requirements, oxygenic photosynthesis and nitrogen fixation. Cyanobacteria have a wide ecological distribution, and they occupy a range of habitats, which includes vast oceanic areas, soils, freshwater lakes, and even extreme habitats like arid deserts, frigid lakes, or hot springs. They can be classified as thermophilic (high temperature), mesophilic (room temperature) or psychrophilic (low temperature), according to the temperature of the culture media in which these organisms grow.

Little is known about thermophilic cyanobacteria, since very few studies have been conducted on them. Keeping this in view, the thermophilic cyanobacterium *Mastigocladus laminosus*, an isolate from the hot spring of Jakrem, Meghalaya was chosen for the present study. *M. laminosus* is a cosmopolitan thermophilic cyanobacterium found in thermal waters on every continent. The capability of *M. laminosus* to fix atmospheric nitrogen makes it ecologically important as a component of algal bacterial mats in neutral to alkaline thermal springs. Such an organism may be of greater use in agricultural fields of high temperature zones.

During the present study, the morphological and physiological features, which include N_2-fixation, uptake and assimilation of nitrate, ammonium and amino acid, and akinete formation and germination were investigated at 25°C and 45°C. DNA fingerprinting was done to generate a genetic identity. The results are summarized below:

1. *M laminosus* utilized the inorganic nitrogen sources (N_2, NO_3^-, NH_4^+) for growth (increase in Chl *a* or protein content). Among these, nitrate served as the best N-source for growth followed by ammonium and N_2. The growth was significantly higher at 45°C than at 25°C while the trend of relative growth performance in different nitrogen media remained similar. *M. laminosus* was also able to take up and utilize exogenously supplied glutamine, asparagine, arginine and alanine as sole sources of fixed-N for growth. Among these, best growth was found in asparagine–supplemented media followed by glutamine-, arginine- and alanine-supplemented media. In all cases, higher growth was found at 45°C than at 25°C.
2. Heterocyst and nitrogenase activity were higher in cultures grown in N_2-medium at 45°C than at 25°C. There was no heterocyst formation and nitrogenase activity in NO_3^-- or NH_4^+-media at either of the temperatures. Heterocyst and nitrogenase were completely repressed in presence of amino acids glutamine, asparagine, arginine and alanine also.
3. Nitrate and nitrite uptake activities were higher in NO_3^--grown cells than the N_2-grown cells by 20 % and 28 %, respectively. Presence of NH_4^+ in the

medium only partially repressed the nitrate and nitrite uptake activities. Nitrate and nitrite uptake in cells grown at 45°C was significantly higher (2-3 fold) than those in corresponding cells grown at 25°C.

4. Nitrate and nitrite reductase activities were repressed partially by ammonia/amino acids in the medium while NO_3^- partially increased the activities. The corresponding activities of nitrate and nitrite reductases in cells grown at 25°C in various nitrogen containing media followed a pattern similar to that at 45°C, but the actual activities were lower at 25°C than those at 45°C.
5. Glutamine synthetase activity was significantly higher in N_2-grown cells than in cells grown in NO_3^--, NH_4^+- or amino acid containing media both at 25°C and 45°C. In all these growth media, higher activity of GS occurred in cells grown at 45°C than at 25°C indicating that *M. laminosus* is indeed a thermophile and possesses mechanism for functioning of NR and GS at higher temperatures. It is noteworthy that 32-50 % of the GS activity still remains in cells grown in amino acid containing media. This is not surprising since *M. laminosus* is able to use these amino acids as sole N-source for growth and GS is essential for them to do so.
6. Photosynthesis rate (photosynthetic O_2 evolution) and phycobiliprotein content were higher in NO_3^-- and NH_4^+-grown cultures than those in N_2-grown cultures both at 25°C and 45°C. The rates of photosynthesis and phycobiliprotein content were higher at 45°C than those at 25°C in all the growth media.

7. Respiration rates of cells grown in the different nitrogen media showed a trend that was reverse of photosynthesis. Higher respiration rates occurred in cells grown in N_2-medium than in NO_3^-- or NH_4^+-media. As in the case of photosynthesis, O_2 consumption at 45°C was higher than that at 25°C in N_2-, NO_3^-- as well as NH_4^+-media.
8. Ammonium and amino acid uptakes showed a biphasic pattern, were energy-dependent and the induction of uptake required *de novo* protein synthesis. The ammonium transport was substrate (NH_4^+)-repressible, while the amino acids uptakes were substrate-inducible.
9. Limitation of fixed nitrogen and iron, removal of sulphate or phosphate from the growth medium, increased concentration of NaCl, altered pH, darkness and temperature shift all induced akinete differentiation in *M. laminosus* to various degrees. The best response was observed in media supplemented with NaCl and in N_2-media lacking sulphate at 25°C. In the latter case virtually all vegetative cells became akinetes by day 40. Profuse akinete differentiation occurred during the post-log phase of growth. The akinete differentiation was associated with cessation of growth and was quicker at 25°C than at 45°C.
10. Akinete differentiation did not occur in cultures grown in NO_3^--media. However, akinetes did develop in NO_3^--media lacking sulphate or phosphate although, such akinete formation was much delayed. Presence of heterocysts was not essential for akinete formation since akinetes did develop in absence of heterocysts (in NO_3^--media lacking phosphate or sulphate and in ammonium-media).

11. Chl *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 heterocyst frequency and activities of nitrogenase, nitrate reductase and glutamine synthetase declined during the akinete differentiation and completely disappeared in mature akinetes. The C:N ratio increased during akinete differentiation.
12. Germination of *M. laminosus* akinetes occurred in presence of light and varied according to the nitrogen sources and temperature. Best germination response was in NO_3^--medium at 45°C and the slowest in N_2-medium at 25°C. In other words, conditions favouring better growth favoured akinete germination. The first cell division occurred after 24 h in N_2-medium and 10-20 h in NO_3^-- or NH_4^+-medium at 45°C. At 25°C, the first cell division occurred after 72 h in NO_3^-- or NH_4^+-medium and after 20 days in N_2-medium.
13. The first cell division was followed by emergence of germling that grew with further cell division followed by heterocyst differentiation. The appearance of nitrogenase activity coincided with the appearance of the first heterocyst at 48 h in N_2-medium at 45°C. Only 25 % of the akinetes had germinated by this time and by 72 h, 90 % of the akinete germinated.
14. Phycocyanin and Chlorophyll *a* appeared within 24 h of start of akinete germination at 45°C in NO_3^-- or NH_4^+-medium, whereas in N_2-medium they appeared within 48 h. There was a steady increase in the levels of both these pigments as more and more germlings appeared and grew. Appearance of

photosynthetic oxygen evolution and nitrate reductase activity coincided with the appearance of phycocyanin and Chl *a*. Appearance of phycocyanin, Chl *a*, photosynthetic O_2 evolution and nitrate reductase activity was delayed when akinetes were germinated at 25°C.

15. Glutamine synthetase activity appeared within 36 h of initiation of akinete germination in N_2-medium at 45°C. Appearance of GS before heterocyst formation and nitrogenase activity (48 h) was probably needed for the reasimilation of ammonia generated from mobilization of reserves for the synthesis of new macromolecules including various pigments, enzymes and proteins preparatory to akinete germination. The C:N ratio decreased during akinete germination and germling growth while protein content increased.

References

Adams, D. G. and Carr, N. G. 1981. The developmental biology of heterocyst and akinete formation in cyanobacteria. Crit. Rev. Microbiol. 9, 45-100.

Adams, D. G. and Duggan, P. S. 1999. Heterocyst and akinete differentiation in cyanobacteria. New Phytol. 144, 3-33.

Aichi, M. and Omata, T. 1997. Involvement of Ntc B, a Lys R family transcription factor, in nitrite activation of nitrate assimilation operon in the cyanobacterium *Synechococcus* sp. strain PCC 7942. J. Bacteriol. 179, 4671-4675.

Allen, M. M. 1984. Cyanobacterial cell inclusions. Annu. Rev. Microbiol. 38, 1-25.

Apte, S. K. and Prabhavathi, N. 1994. Rearrangements of nitrogen fixation (*nif*) genes in the heterocystous cyanobacteria. Molec. Microbiol. 9, 77-84.

Apte, S. K. 1996. Inter-relationship between photosynthesis and nitrogen fixation in cyanobacteria. J. Sci. Industrial. Res. 55, 583-595.

Arizmendi, J. M. and Serra, J. L. 1990. Heterocyst and akinete differentiation in cyanobacteria. New Phytol. 144, 3-33.

Bagchi, S. N. and Singh, H. N. 1984. Genetic control of nitrate reduction in the cyanobacterium *Nostoc muscorum*. Mol. Gen. Genet. 193, 82-84.

Bagchi, S. N., Rai, A. N. and Singh, H. N. 1985a. Regulation of nitrate reductase in cyanobacteria. Repression-derepression control of nitrate reductase apoprotein in the cyanobacterium *Nostoc muscorum*. Biochem. Biophys. Acta. 838, 370-373.

Bagchi, S. N., Rai, U. N., Rai, A. N. and Singh, H. N. 1985b. Nitrate metabolism in the cyanobacterium *Anabaena cycadeae*: Regulation of nitrate uptake and reductase by ammonia. Plant Physiol. 63, 322-326.

Balkwill, D. L., Nierzwicki-Bauer, S. A. and Stevens, S. E. Jr. 1984a. Modes of cell division and branch formation in the morphogenesis of the cyanobacterium *Mastigocladus laminosus*. J. Gen. Microbiol. 130, 2079-2088.

Balkwill, D. L., Nierzwicki-Bauer, S. A. and Stevens, S. E. Jr. 1984b. Ultrastructure of the cyanobacterium *Mastigocladus laminosus* before and during reactivation of a stationary culture. Cytobios. 39, 135-149.

Bauer, C. C., Scappino, L. and Haselkorn, R. 1993. Growth of the cyanobacterium *Anabaena* on molecular nitrogen: NifJ is required when iron is limited. Proc. Natl. Acad. Sci. U. S. A. 90, 8812-8816.

Bednarz, J. and Schmid, G. H. 1991. Induction of nitrate reductase activity by arginine in the filamentous cyanobacterium *Oscillatoria chalybea*. Z. Naturforsch. 46c, 591-596.

Bennett, A. and Bogorad, L. 1973. Complementary chromatic adaptation in a filamentous blue-green alga. J. Cell. Biol. 58 (2), 419-435.

Bergman, B., Lindblad, P. and Rai, A. N. 1986. Nitrogenase in free-living and symbiotic cyanobacteria: immunoelectron microscopic localization. FEMS. Microbiol. Lett. 35, 75-78.

Bergman, B., Gallon, J. R., Rai, A. N. and Stal, L. J. 1997. Nitrogen fixation by non-heterocystous cyanobacteria. FEMS. Microbiol. Rev. 19, 139-185.

Berns, D. S. and Scott, E. 1966. Protein aggregation in a thermophilic protein.

Phycocyanin from *Synechococcus lividus*. Biochemistry. 5, 1528-1533.

Bhattacharya, J., Singh, A. K. and Rai, A. N. 2002a. Nitrogen nutrition in the cyanobacterium *Nostoc* ANTH, a symbiotic isolate from *Anthoceros*: uptake and assimilation of inorganic-N and amino acids. Indian. J. Biochem. Biophys. 39, 163-169.

Bhattacharya, J., Singh, A. K. and Rai, A. N. 2002b. Isolation and characterization of a chlorate-resistant mutant (Clo-R) of the symbiotic cyanobacterium *Nostoc* ANTH: Heterocyst formation and N_2-fixaion in the presence of nitrate, and evidence for separate nitrate and nitrite transport systems. Curr. Microbiol. 45, 99-104.

Billaud, V. A. 1967. Aspects of the nitrogen nutrition of some naturally occurring populations of blue-green algae. In: Bartch, A. F. (ed). Environmental Requirements of Blue-Green Algae. Federal Water Pollution Control Administration, Corvallis, Oregon. 35-55.

Binder, V. A., Locher, P. and Zuber, H. 1972. Concerning the large-scale cultivation of the thermophilic cosmopolitan *Mastigocladus laminosus* in Icelandic hot springs. Arch. Hydrbiol. 70, 541-533.

Black, T. A., Cai, Y. and Wolk, C. P. 1993. Spatial expression and autoregulation of *het*R, a gene involved in the control of heterocyst development in *Anabaena.* Mol. Microbiol. 9, 77-84.

Blanco, F., Alaña, A., Liama, M. J. and Serra, J. L. 1989. Purification and properties of glutamine synthetase from the non-N_2-fixing cyanobacterium *Phormidium laminosum*. J. Bacteriol. 171, 1158-1165.

Böhme, H. and Haselkorn, R. 1988. Molecular cloning and nucleotide sequence

analysis of the gene coding for heterocyst ferredoxin from the cyanobacterium *Anabaena* sp. strain PCC 7120. Mol. Gen. Genet. 214, 278-285.

Boussiba, S., Dilling, W. and Gibson, J. 1984a. Methylammonium transport in *Anacystis nidulans* R-2. J. Bacteriol. 160, 204-210.

Boussiba, S., Resch, C. M. and Gibson, J. 1984b. Ammonia uptake and retention in some cyanobacteria. Arch. Microbiol. 138, 287-292.

Boussiba, S. and Gibson, J. 1985. The role of glutamine synthetase activity in ammonium and methylammonium transport in *Anacystis nidulans* R-2. FEBS. Lett.180, 13-16.

Boussiba, S. 1989. Ammonium uptake in the alkalophilic cyanobacterium *Spirulina plantensis*. Plant Cell Physiol. 32, 303-314.

Bothe, H., Nelles, H., Hager, K. P., Papen, H. and Neuer, G. 1984. Physiology and Biochemistry of N_2-fixation Research. Nijhoff, Junk, Puduc. The Hague and Wageningen. 199.

Brock, T. 1967. Life at high temperatures. Science. 158, 1012-1019.

Brock, T. D. and Brock, M. L. 1970. J. Phycol. 6, 371-375.

Brock, T. D. 1978. Thermophilic Microorganisms and Life at High Temperatures. Springer-Verlag, New York.

Bryant, D. A. 1994. The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Buikema, W. and Haselkorn, R. 1991. Characterisation of a gene controlling

Heterocyst differentiation in the cyanobacterium *Anabaena* 7120. Genes. Devel. 5, 321-330.

Cai, Y. and Wolk, C. P. 1997. Nitrogen deprivation of *Anabaena* sp. strain PCC 7120 elicits rapid activation of a gene cluster that is essential for uptake and utilization of nitrate. J. Bacteriol. 179, 258-266.

Campbell, E. L., Hagen, D. K., Cohen, F. M., Summers, L. M. and Meeks, C. J.1996.The *dev*R gene is characteristic of receivers of two component regulatory systems and essential for heterocyst development in the filamentous cyanobacterium *Nostoc* sp. strain ATCC 29133. J. Bacteriol. 178, 2037-2043.

Canabaeus, L. 1929. Über die Heterocysten und Gasvakuolen der Blaualgen und ihre Beziehungen zueinander. Pflanzenforschung. 13, 1-48.

Candau, P. 1979. Purificatión y propiedades de la ferredoxinanitrato reductasa de la cyanobacteria *Anacystis nidulans*. Ph.D Thesis, Universidad de Sevilla, Sevilla.

Cardemil, L. and Wolk, C. P. 1976. The polysaccharides from heterocyst and spore envelopes of a blue-green alga. Methylation analysis and structure of the backbones. J. Biol. Chem. 251, 2967-2975.

Cardemil, L. and Wolk, C. P. 1979. The polysaccharides from heterocyst and spore envelopes of a blue-green alga. Structure of the basic repeating unit. J. Biol. Chem. 254, 736-741.

Carr, N. G. 1979. Differentiation in filamentous cyanobacteria. In: Parish, J.H. (ed) Developmental Biology of Prokaryotes. Blackwell, Oxford. 167-201.

Carr, N. G. and Whitton, B. A. 1982. The biology of cyanobacteria. Blackwell Scientific Publications, Oxford, UK.

Carrasco, C. D., Ramaswamy, S. K., Ramasubramaniam, S. T. and Goldon, W. J. 1994. *Anabaena xis*F gene encodes a developmentally regulated site specific recombinase. Genes. Devel. 8, 74-83.

Carrasco, C. D., Buettner, A. J. and Goldon, W. J. 1995. Programmed DNA rearrangement of a cyanobacterial *hup*L gene in heterocysts. Proc. Natl. Acad. Sci. U. S. A. 92, 791-795.

Castenholz, R. W. 1968. The behavior of *Oscillatoria terebriformis* in hot springs. J. Phycol. 4, 132-139.

Castenholz, R. W. 1969a. Thermophilic blue-green algae and the thermal environment. Bacteriol. Rev. 33, 476-504.

Castenholz, R. W. 1969b. J. Phycol. 5, 360-368.

Castenholz, R. W. 1970. Laboratory culture of thermophilic Cyanophytes. Schweig. Z. Hydrol. 32, 538-551.

Castenholz, R. W. 1971. Low temperature acclimation and survival in thermophilic *Oscillatoria terebeisformis*. In: T. V. Desikachory (ed). Pro. 1st Internat. Symp. On Taxonomy and Biology of Blue-green algae. (in press).

Castenholz, R. W. 1973. Ecology of blue-green algae in hot springs. In: Carr, N. G. and Whitton, B. A. (eds). The Biology of blue-Green Algae. University California Press, Berckeley, California. 379-414.

Castenholz, R. W. 1976. The effect of sulfide on the blue-green algae of hot springs.

I. New Zealand and Iceland. J. Phycol. 12, 54-68.

Castenholz, R. W. 1977. The effect of sulfide on the blue-green algae of hotsprings. II. Yellowstone National Park. Microb. Ecol. 3, 79-105.

Castenholz, R. W. 1978. The biogeography of hot springs algae through enrichment cultures. Mitt. Int. Ver. Limnol. 21, 296-315.

Castenholz, R.W. 1981. The Prokaryotes. In: Starr, M. P., Stolp, H., Trüper, H. G., Balows, A. and Schlegel, H. G. (eds). Springer-Verlag, Berlin. 1, 236.

Castenholz, R. W. 1982. The biology of Cyanobacteria. In: Carr, N. G. and Whitton, B. A. (eds). Blackwell-Scientific publications, Oxford. 413-440.

Cawse, P. A. 1967. The determination of nitrate in soil solution by ultraviolet spectrophotometry. Analyst. 92, 311-315.

Chauvat, F., Corre, B., Herdman, M. and Joset-Espardellier, F. 1982. Energetic and metabolic requirements for the germination of akinetes of the cyanobacterium *Nostoc* PCC 7524. Arch. Microbiol. 133, 44-49.

Chapman, J. S. and Meeks, J. C. 1983. Glutamine and glutamate transport by *Anabaena variabilis*. J. Bacteriol. 156, 122-129.

Chen, C. H. and Berns, D. S. 1978. Comparison of the stability of phycocyanins from thermophilic, mesophilic, psychrophilic, and halophilic algae. Biophys. Chem. 8, 203-213.

Clark, R. L. and Jensen, T. E. 1969. Ultrastructure of akinete development in a blue-green alga, *Cylindrospermum* sp. Cytologica. 34, 439-448.

Codd, G. A. and Stewart, W. D. P. 1977. D-ribulose 1, 5-diphosphate carboxylase from the blue-green alga *Aphanocapsa* 6308. FEMS. Microbial. Lett. 2, 247-249.

Codd, G. A., Okabe, K. and Stewart, W. D. P. 1980. Cellular compartmentation of photosynthetic and photorespiratory enzymes in the heterocystous cyanobacterium *Anabaena cylindrica*. Arch. Microbiol. 124, 149-154.

Codd, G. A. and Marsden, W. J. N. 1984. The carboxysomes (polyhedral bodies) of autotrophic prokaryotes. Biol. Rev. 59, 389.

Coronil, T., Lara, C. and Guerrero, M. G. 1993. Shift in carbon flow and stimulation of amino-acid turnover induced by nitrate and ammonium assimilation in *Anacystis nidulans*. Planta. 189, 461-467.

Cossar, J. D., Rowell, P., Darling, A. J., Murry, S., Codd, G. A. and Stewart, W. D. P. 1985. Localization of ribulose 1, 5-bisphosphate carboxylase/oxygenase in the nitrogen fixing cyanobacterium *Anabaena cylindrica*. FEMS. Microbiol. Lett. 28, 65-68.

Crespo, J., García-Domínguez, M. and Florencio, F. J. 1998. Nitrogen control of the *glnN* gene that codes for Gs type III, the only glutamine synthetase in the cyanobacterium *Psuedanabaena* sp. PCC 6903. Mol. Microbiol. 30, 1101-1112.

Dean, D. R. and Jacobson, M. R. 1992. Biochemical genetics of nitrogenase. In: Stacey, G., Burris, R. H. and Evans, H. J. (eds). Biological nitrogen fixation. Chapman and Hall, New York, U. S. A. 763-834.

Demeter, O. 1956. Über Modifikationen bei Cyanophyceen. Arch. Microbiol. 24, 105-133.

Dharmawardene, M. W. N., Stewart, W. D. P. and Stanley, S. O. 1972. Nitrogenase activity, amino acid pool patterns and amination in blue-green algae. Planta. 108, 133-145.

Dharmawardene, M. W. N., Haystead, A. and Stewart, W. D. P. 1973. Glutamine synthetase of the nitrogen-fixing alga *Anabaena cylindrica*. Arch. Microbiol. 90, 281-295.

Dunn, J. H. and Wolk, C. P. 1970. Composition of the cellular envelopes of *Anabaena cylindrica*. J. Bacteriol. 103, 153-158.

Durner, J., Bohm, I., Knorzer, O. C. and Boger, P. 1996. Proteolytic degradation of dinitrogenase reductase from *Anabaena variabilis* (ATCC 29413) as a consequence of ATP depletion and impact of oxygen. J. Bacteriol. 178, 606-610.

Dyer, D. L. and Gafford, R. D. 1961. Some characteristics of a thermophilic blue-green alga. Science. 134, 616-617.

Eberly, W. R. 1966. Notes on some new and rare Myxophyceae in laboratory culture. Trans. Am. Microsc. Soc. 85, 130-138.

Edwards, M. R., Berns, D. S., Ghiorse, W. C. and Holt, S. C. 1968. Ultrastructure of the thermophilic blue-green alga, *Synechococcus lividus* Copeland. J. Phycol. 4, 283-289.

Elhai, J. and Wolk, P. C. 1988. Conjugal transfer of DNA to cyanobacteria. Methods of Enzymol. 167, 747-754.

Elhai, J. and Wolk, P. C. 1990. Developmental regulation and spatial pattern of expression of the structural genes for nitrogenase in the cyanobacteria

Anabaena. J. EMBO. 9, 3379-3388.

Elmorjani, K., Liotenberg, S., Houmard, J. and Taneau de Marsac, N. 1992. Molecular characterization of the gene encoding glutamine synthetase in the cyanobacterium *Calothrix* sp. PCC 7601. Biochem. Biophys. Res. Commun. 189, 1296-1302.

Fagerberg, W. R. and Arnott, H. J. 1979. Seasonal changes in structure of submerged blue green algal/bacterial community from a geothermal hotspring. J. Phycol. 15, 445- 452.

Farquhar, J., Bao, H. and Thiemens, M. 2000. Atmospheric influence of Earth's earliest sulfur cycle. Science. 289, 756-259.

Fay, P. and Walsby, A. E. 1966. Metabolic activities of isolated heterocysts of the blue-green alga *Anabaena cylindrica*. Nature (London). 209, 94-95.

Fay, P. 1969a. Cell differentiation and pigment composition in *Anabaena cylindrica*. Arch. Microbiol. 67, 62-70.

Fay, P. 1969b. Metabolic activities of isolated spores of *Anabaena cylindrica*. J. Exp. Bot. 20, 100-109.

Fay, P., Stewart, W. D. P., Walsby, A. E. and Fogg, G. E. 1968. Is the heterocyst the site of nitrogen fixation in blue-green algae. Nature (London). 220, 810-812.

Fay, P. and van Baalen, C. 1987. The Cyanobacteria. Elsevier, Amsterdam, New York, Oxford. 173.

Fay, P., Lynn, J. A. and Majer, S. C. 1984. Akinete development in the planktonic blue -green alga *Anabaena circinalis*. J. Brit. Phycol. 19, 163-173.

Fisher, R. W. and Wolk, C. P. 1976. Substance stimulating the differentiation of spores of the blue-green alga *Cylindrospermum licheniforme*. Nature. 259, 394-395.

Fisher, R. W., Tuli, R. and Haselkorn, R. 1981. A cloned cyanobacterial gene for glutamate synthetase functions in *Escherichia coli*, but the enzyme is not adenylated. Proc. Natl. Acad. Sci. U. S. A. 78, 3393-3397.

Florencio, F. J. and Ramos, J. L. 1985. Purification and characterization of glutamate synthetase from the unicellular cyanobacterium *Anacystis nidulans*. Biochem. Biophys. Acta. 838, 39-48.

Flores, E., Guerrero, M.G. and Losada, M. 1983a. Photosynthetic nature of nitrate uptake and reduction in the cyanobacterium *Anacystis nidulans*. Biochem. Biophys. Acta. 722, 408-416.

Flores, E., Ramos, J. L., Herrero, A. and Guerrero, M. G. 1983b. Nitrate assimilation by cyanobacteria. In: Papageotgiou, G.C. and Packer, L (eds). Photosynthetic Prokaryotes: Cell Differentiation and Function. Elsevier, New York. 363-387.

Flores, E., Herrero, A. and Guerrero, M.G. 1987. Nitrate uptake and its regulation in the cyanobacterium *Anacystis nidulans*. Biochem. Biophys. Acta. 896, 103-108.

Flores, E. and Muro-Pastor, M. I. 1988. Uptake of glutamine and glutamate by the dinitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120. FEMS. Microbiol. Lett. 56, 127-130.

Flores, E. and Muro-Pastor, A. M. 1990. Mutational and kinetic analysis of basic transport in the Cyanobacterium *Synechocystis* sp. PCC 6803. Arch.

Microbiol. 154, 512-527

Flores, E. and Herrero, A. 1994. Assimilatory nitrogen metabolism and its regulation In: D.E. Bryant (ed). The molecular biology of cyanobacteria. Kluwer Academic Publications Dordrecht, The Netherlands. 487-517.

Flores, E., Muro-Pastor, A. M. and Herrero, A. 1999. Cyanobacterial nitrogen assimilation genes and NtcA-dependent control of gene expression. In: Peschek, G. A., Löffelhardt, W. and Schmetterer, G. (eds). The phototrophic prokaryotes. Plenum Publishing Corporation, New York, N.Y.

Fogg, G. E. 1949. Growth and heterocyst production in *Anabaena cylindrica* Lemn. Ann. Bot. 13, 241-259.

Fogg, G. E. 1951. Studies on nitrogen fixation by blue-green algae. II. Nitrogen fixation by *Mastigocladus laminosus* Cohn. J. Exp. Bot. 2, 117-120.

Forchhammer, K. 1999. The P_{II} protein in *Synechococcus* PCC 7942 senses and signals 2-oxoglutarate under ATP-replete conditions. In: Peschek, G.A., Löffelhardt, W. and Schmetterer, G. (eds). The phototrophic prokaryotes. Plenum Publishing Corporation, New York, N.Y. 549-553.

Forterre, P., Bouthier De La Tour, C., Phillipe, H. and Duguet, M. 2000. Reverse gyrase from hyperthermophiles: probable transfer of a thermoadaptation trait from archaea to bacteria. Trends Genet. 16, 152-154.

Frias, J. E., Flores, E. and Herrero, A. 1994. Requirement of the regulatory protein NtcA for the expression of nitrogen assimilation and heterocyst development genes in the cyanobacterium *Anabaena* sp. Strain PCC 7120. Mol. Microbiol. 14, 823-832.

Frias, J. E., Flores, E. and Herrero, A. 1997. Nitrate assimilation gene clusters from the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. 179, 477-486.

Frias, J. E., Flores, E. and Herrero, A. 2000. Activation of the *Anabaena* nir operon promoter requires both Ntc A (CAP family) and Ntc B (Lys R family) transcription factors. Mol. Microbiol. 38, 613-625.

Gallon, J. R. and Chaplin, A. E. 1987. An introduction to nitrogen fixation. Cassell Educational, London, UK.

Gallon, J. R. 1992. Reconciling incompatible nitrogen fixation and oxygen. New Phytol. 122, 571-609.

García-Domínguez, M., Reyes, J. C. and Florencio, F. J. 1999. Glutamine synthetase inactivation by protein-protein interaction. Proc. Natl. Acad. Sci. U. S. A. 96, 7161-7166.

Gentile, J. H. and Maloney. 1969. Toxicity and environmental requirements of a strain of *Aphanizomenon flos-aquae* (L.) Ralfs. Can. J. Microbiol. 15, 165-173.

Gloaguen, V., Morvan, H., Hoffmann, L., Plancke, Y., Wieruszeski, J-M., Lippens, G. and Strecker, G. 1999. Capsular polysaccharide produced by the thermophilic cyanobacterium *Mastigocladus laminosus*: Structural study of an undecasaccharide obtained by lithium degradation. Eur. J. Biochem. 266, 762-770.

Golden, J. W., Robinson, J. S. and Haselkorn, R. 1985. Rearrangement of nitrogen fixation genes during heterocyst differentiation in the cyanobacterium *Anabaena*. Nature. 314, 419-423.

Golden, J. W., Carrasco, C. D., Mulligan, M. E., Schneider, G. J. and Haselkorn, R. 1988. Deletion of a 55-kilobase-pair DNA element from the chromosome during heterocyst differentiation of *Anabaena* sp. strain PCC 7120. J. Bacteriol. 170, 5034-5041.

Golden, J. W. and Yoon, S. H. 1998. Heterocyst formation in *Anabaena*. Curr. Opin. Microbiol. 1, 623-629.

Gorbunova, R. W. 1975. Vestn. Mosk. Uni. Ser. VI Biol. Pochnoved. 30, 57-64

Grilli Caiola, M. and de Vecchi. 1980. Akinete ultrastructure of *Nostoc* species isolated from cycad coralloid roots. Can. J. Bot. 58, 2513-2519.

Grillo, J. F. and Gibson, J. 1979. Regulation of phosphate accumulation in the unicellular cyanobacterium Synechococcus. J. Bacteriol. 140, 508-517.

Harder, R. 1917. Ernährungsphysiologische Untersuchungen an Cyanophycean, hauptsächlich dem endophytischen *Nostoc punctiforme*. Z. Bot. 9, 145-242.

Haselkorn, R. 1978. Heterocyst. Annu. Rev. Plant Physiol. 29, 319-344.

Haystead, A., Dharmawardene, M. W. N. and Stewart, W. D. P. 1973. Ammonia assimilation in a nitrogen-fixing blue-green alga. Plant Sci. Lett. 1, 439-445.

Helmann, J. D. and Chamberlain, J. M. 1988. Structure and function of bacterial s-factors. Ann. Rev. Biochem. 57, 839-872.

Herdman, M. 1987. Akinetes: structure and function. In: Fay, P. and Van Baalen, C. (eds). The Cyanobacteria. Amsterdam, Elsevier, The Netherlands. 227-250.

Herdman, M. 1988. Cellular differentiation: Akinetes. In: Packer, L. and Glazer, A. N. (eds). Methods in enzymology. Academic Press, London, UK. 167. 222-232.

Hernandez-Muniz, W. and Stevens S. E. Jr. 1987. Characterization of the motile hormogonia of *Mastigocladus laminosus*. J. Bacteriol. 169, 218-223.

Herrero, A., Flores, E. and Guerrero, M. G. 1981. Regulation of nitrate reductase level in *Anacystis nidulans*, *Anabaena* sp. Strain 7119, and *Nostoc* sp. Strain 6719. J. Bacteriol. 145, 175-180.

Herrero, A., Flores, E. and Guerrero, M. G. 1985. Regulation of nitrate reductase cellular levels in the cyanobacteria *Anabaena variabilis* and *Synechocystis* sp. FEMS. Microbiol. Lett. 26, 21-25.

Herrero, A. and Guerrero, M. G. 1986. Regulation of nitrite reductase in the cyanobacterium *Anacystis nidulans*. J. Gen. Microbiol. 132, 2463-2468.

Herrero, A. and Flores, E. 1990. Transport of basic amino acids by the dinitrogen-fixing cyanobacterium *Anabaena* PCC 7120. J. Biol. Chem. 265, 3931-3935.

Herrero, A., Muro-Pastor, M. A. and Flores, E. 2001. Nitrogen control in cyanobacteria. J. Bacteriol. 183, 411-425.

Hill, H. 1970. A new form of *Raphidiopsis mediterranae* Skuja found in Minnesota lakes. Phycologia. 9, 73-77.

Hirosawa, T. and Wolk, C. P. 1979a. Factors controlling the formation of akinetes adjacent to heterocysts in the cyanobacterium *Cylindrospermum licheniforme* Kütz. J. Gen. Microbiol. 114, 423-432.

Hirosawa, T. and Wolk, C. P. 1979b. Isolation and characterization of a substance which stimulates the formation of akinetes in the cyanobacterium *Cylindrospermum licheniforme* Kütz. J. Gen. Microbiol. 144, 433-441.

Hoare, D. S., Hoare, S. L. and Moore, R. B. 1967. The photoassimilation of organic compounds by autotrophic blue-green algae. J. Gen. Microbiol. 49, 351-370.

Holland, H. D. 1994. Early Life on Earth. In: Bergtson, S. (ed). Columbia Univ. Press. New York. 237-244.

Holton, R. W. 1962. Isolation, growth, and respiration of a thermophilic blue-green alga. Amer. J. Bot. 49, 1-6.

Janson, S., Rai, A. N. and Bergman, B. 1993. The marine lichen *Lichinia confinis* (O. F. Mull) C. Ag: ultrastructure and localization of nitrogenase, glutamine synthetase, phycoerythrin and ribulose 1, 5-bisphosphate carboxylase/oxygenase in the cyanobiont. New Phytol. 124, 149-160.

Janson, S., Rai, A. N. and Bergman, B. 1995. Intracellular cyanobiont *Richellia intracellularis*: ultrastructure and immunolocalization of phycoerythrin, nitrogenase, Rubisco and glutamine synthetase. Marine. Biol. 124, 1-8.

Jensen, T. E. and Clark, R. L. 1969. Cell wall and coat of the developing akinete of a *Cylindrospermum* species. J. Bacteriol. 97, 1494-1495.

Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, A., Yasuda, M. and Tabata. S. 1996. Sequence analysis of the genome of the unicellular cyanobacterium synechocystis sp. strain PCC 6803. II. Sequence determination of the entire genome and assignment of

potential protein -coding regions. DNA. Res. 3, 109-136.

Kapp, R., Stevens, S. E. and Fox, J. L. 1975. A survey of available nitrogen sources for the growth of the blue-green alga *Agmemellum quadruplicatum*. Arch. Microbiol. 104, 135-138.

Kasting, J. F. and Siefert, J. L. 2002. Life and the Evolution of Earth's Atmosphere. Science. 296, 1066-1068.

Kaushik, M. and Kumar, H. D. 1970. The effect of light on growth and development of two nitrogen-fixing blue-green algae. Arch. Microbiol. 74, 52-57.

Khudyakov, I. and Wolk, P. C. 1997. *het*C, a gene encoding for a protein similar to bacterial ABC protein exporters is involved in early regulation of heterocyst differentiation in *Anabaena* sp. strain PCC 7120. J. Bacteriol. 179, 6971-6978.

Kikuchi, H., Aichi, M., Suzuki, I. and Omata, T. 1996. Positive regulation by nitrite of the nitrate assimilation operon in the cyanobacterium *Synechococcus* sp. strain PCC 7942 and *Plectonema boryanum*. J. Bacteriol. 178, 5822-5825.

Kim, J. and Rees, D. C. 1994. Nitrogenase and biological nitrogen fixation. Biochem. 33, 387-397.

Klenk, H-P., Meier, T.D. and Duronic, P. 1999. RNA polymerase of *Aquifex pyrophilus*: Implications for the evolution of the bacterial *rpo*BC operon and extremely thermophilic bacteria. J. Mol. Evol. 48, 528-541.

Koblizek, M., Masojidek, J., Komenda, J., Kucera, T., Pilloton, R., Mattoo, A. K. and Giardi, M. T. 1998. A sensitive photosystem II-based behaviour for detection of a class of herbicides. Biotech. Bioeng. 60, 664-669.

Kumar, A. P., Rai, A. N. and Singh, H. N. 1985. Nitrate reductase activity in isolated heterocysts of the cyanobacterium *Nostoc muscorum*. FEBS. Lett. 179 (1), 125-128.

Labarre, J., Thurioux, P. and Chauvat, F. 1987. Genetic analysis of amino acid transport in the facultatively heterotrophic cyanobacterium *Synechocystis* sp. strain 6803. J. Bacteriol. 169, 4668-4673.

Lammers, P. J., Goldon, W. J. and Haselkorn, R. 1986. Identification and sequence of a gene required for a developmentally regulated DNA excision in *Anabaena*. Cell. 44, 905-911.

Lea, P. J. and Miflin, B. J. 1975. Glutamate synthase in blue-green algae. Biochem. Soc. Trans. 3, 381-384.

Leak, L. V. and Wilson, G. B. 1965. Electron microscopic observations on a blue-green alga, *Anabaena* sp. Can. J. Genet. Cytol. 7, 237-249.

Liang, J., Scappinoc, L. and Haselkorn, R. 1992. The *pat*A gene product, which contains a region similar to *che*Y of *Escherichia coli*, controls heterocyst pattern formation in the cyanobacterium *Anabaena* 7120. Proc. Natl. Acad. Sci. U. S. A. 89, 5655-5659.

Liang, J., Scappinoc, L. and Haselkorn, R. 1993. The *pat*B gene product required for growth of the cyanobacterium *Anabaena* sp. strain PCC 7120 under nitrogen limiting conditions, contains ferredoxin and helix-turn-helix domains. J. Bacteriol. 175, 1697-1704.

Lowry, G. H., Rosenbrough, J., Farr, A. L. and Landell, R. J. 1951. Protein measurement with Folin-phenol reagent. J. Biol. Chem. 244, 4436-4440.

Luque, I., Herrero, A., Flores, E. and Madueno, F. 1992. Clustering of gene involved in nitrate assimilation in the cyanobacterium *Synechococcus*. Mol. Gen. Genet. 232, 7-11.

Luque, I., Flores, E. and Herrero, A. 1993. Nitrate reductase gene from *Synechococcus* sp. PCC 7942: homology between cyanobacterial and higher plant nitrite reductases. Plant. Mol. Biol. 21, 1201-1205.

Luque, I., Flores, E. and Herrero, A. 1994. Nitrate and nitrite transport in the same permease. Biochem. Biophys. Acta. 1184, 296-298.

Mackinney, G. 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. 140, 315-322.

Madueño, F., Flores, E. and Guerrero, M. G. 1987. Competition between nitrate and nitrite uptake in the cyanobacterium *Anacystis nidulans*. Biochem. Biophys. Acta. 896, 109-112.

Madueño, F., Borris, W. E., van Arkel, G. A. and Guerrero, M. G. 1988. A cytoplasmic -membrane protein repressible by ammonium in *Synechococcus* R2: Altered expression in nitrate-assimilation mutants. FEBS. Lett. 239, 289-291.

Maeda, S. I. and Omata, T. 1997. Substrate binding lipoprotein of the cyanobacterium *Synechococcus* sp. strain PCC 7942 involved in the transport of nitrate and nitrite. J. Biol. Chem. 272, 3036-3041.

Manzano, C., Candau, P., Gómez-Moreno, C., Relimpio, A. M. and Losada, M. 1976. Ferredoxin-dependent photosynthesis reduction of nitrate and nitrite by particles of *Anacystis nidulans*. Mol. Cell. Biochem. 10, 161-169.

Manzano, C. 1977. La reduccion fotosintetica del nitrato en el alga verde-azulada *Anacystis nidulans*. Ph. D. Thesis, Uni. Sevilla, Sevilla.

Marcenko, E. 1961. Licht-und elektronenmikroskopische Untersuchungen an der Thermalalge *Mastigocladus laminosus*. Cohn. Acta. Bot Croatia. 20/21, 47-74.

Martín-Nieto, J., Herrero, A. and Flores, E. 1989. Regulation of nitrate and nitrite reductase in dinitrogen fixing cyanobacteria and Nif mutant. Arch. Microbiol. 151, 475-478.

Martín-Nieto, J., Flores, E. and Herrero, A. 1990. Mutants of *Anabaena variabilis* requiring high levels of molybdate for nitrate reductase and notrogenase activities. FEMS. Microbiol. Lett. 67, 1-4.

Martín-Nieto, J., Flores, E. and Herrero, A. 1992. Biphasic kinetic behaviour of nitrate reductase from heterocystous, nitrogen-fixing cyanobacteria. Plant Physiol. 100, 157-163.

Martín-Figueroa, E., Navarro, F. and Florencio, F. J. 2000. The GS-GOGAT pathway is not operative in the heterocysts. Cloning and expression of *glsF* gene from the cyanobacterium *Anabaena* sp. PCC 7120. FEBS. Lett. 476, 282-286.

Mazur, B. J., Rice, D. and Haselkorn, R. 1980. Identification of blue-green algal nitrogen fixation genes by using heterocystous DNA hybridization probes. Proc. Natl. Acad. Sci. U. S. A. 77, 186-190.

Meeks, J. C., Wolk, C. P., Thomas, J., Lockau, W. and Shaffer, P. W. 1977. The pathways of assimilations of $^{13}NH_4^+$ by the cyanobacterium *Anabaena cylindrica*. J. Bio. Chem. 252, 7894-7900.

Meeks, J. C., Wolk, C. P., Thomas, J., Lockau, W., Schilling, N., Shaffer, P. W. and Chien, W. S. 1978. Pathways of assimilations of ^{13}N -N_2 and $^{13}NH^{+}_{4}$ by cyanobacteria with and without heterocysts. J. Bacteriol. 134, 125-130.

Mendez, J. M., Herrero, A. and Vega, J. M. 1981. Characterization and catalytic properties of nitrate reductase from *Anabaena* sp. 7119. Z. pflanzenphysiol. 103, 305-315.

Merchan, F., Prieto, R., Kindle, K., Llama, M., Serra, J. and Fernandez, E. 1995. Isolation, sequence and expression in *Escherichia coli* of the nitrite reductase gene from the filamentous, thermophilic cyanobacterium *Phormidium laminosum*. Plant. Molec. Biol. 27 (5), 1037-1042.

Mérida, A., Leurentop, L., Chandu, P. and Florencio, F. J. 1990. Purification and properties of glutamine synthetases from the cyanobacteria *Synechocystis* sp. strain PCC 6803 and *Calothrix* sp. strain PCC 7601. J. Bacteriol. 172, 4732-4735.

Mérida, A., Candau, P. and Florencio, F. J. 1991. Regulation of glutamine synthetase activity in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 by the nitrogen source: Effect of ammonium. J. Bacteriol. 173, 4095-4100.

Mérida, A., Flores, E. and Florencio, F. J. 1992. Regulation of *Anabaena* sp. strain PCC 7120 glutamine synthetase activity in a *Synechocystis* sp. strain PCC 6803 derivative strain bearing the *Anabaena glnA* gene and a mutated host *gln*A gene. J. Bacteriol. 174, 650-654.

Mikami, B., and Ida, S. 1984. Purification and properties of ferredoxin-nitrate reductase from the cyanobacterium *Plectonema boryanum*. Biochem. Biophys. Acta. 791, 294-304.

Miller, M. M. and Lang, N. J. 1968. The fine structure of akinete formation and germination in *Cylindrospermum.* Arch. Microbiol. 60, 303-313.

Miura, Y., Yokayama, H., Konaoka, K., Saito, S., Iwasa, K., Okazaki, M. and Komemushi, S. 1980. Hydrogen evolution by a thermophilic blue-green algae *Mastigocladus laminosus.* Plant Cell Physiol. 21, 149-156.

Miyamoto, K., Hollenbeck, P. C. and Benemann, J. R. 1979a. Nitrogen fixation by thermophilic blue-green algae (cyanobacteria): temperature characteristics and potential use in biophotolysis. Appl. Environ. Microbiol. 37, 454-458.

Miyamoto, K., Hollenbeck, P. C. and Benemann, J. R. 1979b. Hydrogen production by the thermophilic algae *Mastigocladus laminosus*: effects of nitrogen, temperature, and inhibition of photosysnthesis. Appl. Environ. Microbiol. 38, 440-446.

Montesinos, M. L., Herrero, A. and Flores, E. 1995. Amino acid transport systems required for diazotrophic growth in the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. 177, 3150-3157.

Montesinos, M. L., Herrero, A. and Flores, E. 1997. Amino acid transport in taxonomically diverse cyanobacterium and identification of two genes encoding elements of a neutral amino acid permease putatively involved in recapture of leaked hydrophobic amino acids. J. Bacteriol. 179 (3), 853-862.

Montesinos, M. L., Muro-Pastor, A. M., Herrero, A. and Flores, E. 1998. Ammonium/methylammonium permeases of a cyanobacterium. Identification and analysis of three nitrogen- regulated *amt* genes in *Synechococcus* sp. PCC 6803. J. Biol. Chem. 273, 31463-31470.

Muro-Pastor, M. I. and Florencio, F. J. 1992. Purification and properties of $NADP^+$-

isocitrate dehydrogenase from the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. Eur. J. Biochem. 203, 99-105.

Muro-Pastor, M. I. and Florencio, F. J. 1994. $NADP^+$-isocitrate dehydrogenase from the cyanobacterium *Anabaena* sp. strain PCC 7120: purification and characterization of the enzyme and cloning, sequencing, and disruption of the *icd* gene. J. Bacteriol. 176, 2718-2726.

Muro-Pastor, M. I., Reyes, J. C. and Florencio, F. J. 1996. The $NADP^+$-isocitrate dehydrogenase gene (*icd*) is nitrogen regulated in cyanobacteria. J. Bacteriol. 178, 4070-4076.

Murry, M., Hallenbeck, P. and Benemann, J. 1984. Immunochemical evidence that nitrogenase is restricted to the heterocysts in *Anabaena cylindrica.* Arch. Microbiol. 137, 194-199.

Nechushtai. R., Muster, P., Binder. A., Liveanee. V. and Nelson, N. 1983. Photosystem I reaction center from the thermophilic cyanobacterium *Mastigocladus laminosus.* Proc. Natl. Acad. Sci. U. S. A. 80, 1179-1183.

Neilson, A. H. and Doudoroff, M. 1973. Ammonia assimilation in blue-green algae. Arch. Microbiol. 89, 15-22.

Neilson, A. H. and Larsson, T. 1980. The utilization of organic nitrogen for growth of algae: physiological aspects. Physiol. Plantarum. 48, 542-553.

Newman, D. K. and Banfield, J. F. 2002. Geomicrobiology: How molecular scale interactions underpin biogeochemical systems. Science. 296, 1071-1076.

Nichols, J. M. and Carr, N. G. 1978. Akinetes of cyanobacteria. Spores VII. Am. Soc.

Microbiol. Washington. D.C. 335-343.

Nichols, J. M., Adams, D. G. and Carr, N. G. 1980. Effect of canavanine and other amino acid analogues on akinete formation in the cyanobacterium *Anabaena cylindrica*. Arch. Microbiol. 127, 67-75.

Nichols, J. M. and Adams, D. G. 1982. Akinetes. In: Carr, N. G. and Whitton, B. A. (eds). The biology of cyanobacteria. Blackwell Scientific, Oxford, UK. 387-412.

Nierzwicki, S. A., Balkwill, D. L., Hardie, L. P., Mehta ,V. P. and Stevens, S. E. Jr. 1982. Ultrasturcture of the cyanobacterium, *Mastigocladus laminosus*. Arch. Microbiol. 133, 11-19.

Nierzwicki-Bauer, S. A., Balkwill, D. L. and Stevens, S. E. Jr. 1984a. Morphology and ultrastructure of the cyanobacterium, *Mastigocladus laminosus* growing under nitrogen- fixing conditions. Arch. Microbial. 137, 97-103.

Nierzwicki-Bauer, S. A., Balkwill, D. L. and Stevens, S. E. Jr. 1984b. Heterocyst in the cyanobacterium *Mastigocladus laminosus*. J. Bacteriol. 157 (2), 514-525.

Ochiai, H., Shibata, H., Sawa, Y. and Katoh, T. 1980. Immobilized filaments of the thermophilic cyanobacterium *Mastigocladus laminosus* can function as an anodic photoelectrode for periods up to 20 days and thus might be used as an electron donor for the production of hydrogen. Proc. Natl. Acad. Sci. USA. 77, 2442.

Ohmori, M., Ohmori, K. and Strotmann, H. 1977. Inhibition of nitrate uptake by ammonia in a blue-green alga, *Anabaena cylindrica*. Arch. Microbiol. 114, 225-229.

Okuhara, H., Matsumura, T., Fujita, Y. and Hase, T. 1999. Cloning and inactivation of genes encoding ferredoxin- and NADH-dependant glutamate synthases in the cyanobacterium *Plectonema boryanum*. Imbalances in nitrogen and carbon assimilations caused by deficiency of the ferredoxin dependent enzyme. Plant. Physiol. 120, 33-41.

Omata, T., Andriesse, X. and Hirano, A. 1993. Identification and characterization of a gene cluster involved in nitrate transport in the cyanobacterium *Synechococcus* sp. PCC 7942. Mol. Gen. Genet. 236, 193-202.

Orr, J., Keefer, L. M., Keim, P., Nguen, T. D., Wellems, T., Heinrikson, L. and Haselkorn, R. 1981. Purification, physical characterization, and NH_2-terminal sequence of glutamine synthetase from the cyanobacterium *Anabaena* 7120. J. Biol. Chem. 256, 13091-13098.

Peary, J. A. and Castenholz, R. W. 1964. Temperature strains of a thermophilic blue-green algae. Nature (Lond.). 202, 720-721.

Peschek, G. A. 1979. Nitrate and nitrite reductase and hydrogenase in *Anacystis nidulans* grown in Fe- and Mo-deficient media. FEMS. Microbiol. Lett. 6, 371-374.

Peter, G. 1977. The *Azolla-Anabaena azzolae* symbiosis. Basic Life. Sc. 9, 231-58.

Phillippe H. and Forterre, P. 1999. The rooting of the Universal Tree of life is not reliable. J. Mol. Evol. 49, 509.

Prakasham, R. and Rai, A. N. 1991. Evidence for the occurrence of specific methylammonium transport system in the cultured cyanobiont of *Anthoceros*

punctatus-Nostoc association. J. Gen. Microbiol. 137, 1783-1788.

Prakasham, R., Singh, A. K., Singh, H. N. and Rai, A. N. 1991. Inorganic nitrogen regulation of glutamate uptake in the cyanobacterium *Nostoc muscorum*. Palnt Physiol. 82, 257-260.

Pringsheim, E. G. 1968a. Cyanophyceen-Probleme. Planta, Berl. 79, 1-9.

Pringsheim, E. G. 1968b. Cyanophyceen-Studien. Arch. Microbiol. 63, 331-355.

Rai, A. N., Rowell, P. and Stewart, W. D. P. 1982. Interrelations of carbon and nitrogen metabolism in the N_2-fixing lichens *Peltigera canina* and *Peltigera aphthosa*. Proc. IV International Symp. On Photosynthetic Prokaryotes, Bombannes, France, abstract 39.

Rai, A. N., Rowell, P. and Stewart, W. D. P. 1984. Evidence for an ammonium transport system in free-living and symbiotic cyanobacteria. Arch. Microbiol. 137, 241-246.

Rai, A. N., Rao, V. V. and Singh, H. N. 1985. The biology of the cyanobacterial (blue- green algal) akinetes (spores). J. Plant Sci. Res. 1, 1-20.

Rai, A. N. and Bergman, B. 1986. Modification of NO_3^- metabolism in heterocysts of the N_2-fixing cyanobacterium *Anabaena* 7120 (ATCC 27893). FEMS. Microbiol. Lett. 36, 133-137.

Rai, A. N., Rao, V. V. and Singh, H. N. 1988. Metabolic changes associated with akinete germination in the cyanobacterium *Anabaena doliolum*. New Phytol. 109, 133-138.

Rai, A. N., Borthakur, M., Singh, S. and Bergman, B. 1989. *Anthoceros-Nostoc*

symbiosis: immunoelectronmicroscopic localization of nitrogenase, glutamine synthetase, phycoerythrin and ribulose 1,5-biphosphate carboxylase/oxygenase in the cyanobiont and cultured (free-living) isolate *Nostoc* 7801. J. Gen. Microbiol. 135, 385-395.

Rai, A. N. 1990. Handbook of symbiotic cyanobacteria. CRC Press, Boca Raton, Florida, USA.

Rai, A. N., Borthakur, M. and Bergman, B. 1992. Nitrogenase depression, its regulation and metabolic changes associated with diazotrophy in the non-heterocystous cyanobacterium *Plectonema boryanum* PCC 73110. J. Gen. Microbiol. 138, 481-491.

Rai, A. N., Soderback, E. and Bergman, B. 2000. Cyanobacterium-plant symbiosis. New Phytol. 147, 449-481.

Rao, V. V, Rai, A. N. and Singh, H. N. 1984. Metabolic activities of the akinetes of the cyanobacterium *Anabaena doliolum*: oxygen exchange, photosynthetic pigments and enzyme of nitrogen metabolism. J. Gen. Microbiol. 130, 1299-1302.

Rao, V. V, Ghosh, R. and Singh, H. N. 1987. Diazotrophic regulation of akinete development in the cyanobacterium *Anabaena doliolum*. New Phytol. 106, 161-168.

Rasmussen, U. and Svenning, M. M. 1998. Fingerprinting of cyanobacteria based on PCR with primers derived from short and long tandemly repeated repetitive sequences. Appl. Environ. Microbiol. 64, 265-272.

Rawson, D. M. 1985. The effects of exogenous amino acids on growth and nitrogenase activity in cyanobacterium *Anabaena cylindrica* PCC 7122. J.

Gen. Microbiol. 131, 2549-2554.

Reddy, P. M., Rao, P. S. N. and Talpasayi, E. R. S. 1975. Effect of red and far red illumination on the germination of spores of two blue-green algae. Curr. Sci. 44, 678-679.

Renstrom-Kellner, E., Rai, A. N. and Bergman, B. 1990. Correlation between nitrogenase, glutamine synthetase expression in the cyanobacterium *Anabaena cylindrica*. Plant Physiol. 80, 12-19.

Reyes, J. C. and Florencio, F. J. 1994. A new type of glutamine synthetase in cyanobacteria: The protein encoded by the *glnN* gene supports nitrogen assimilation in *Synechocystis* sp. strain PCC 6803. J. Bacteriol. 176, 1260-1267.

Reyes, J. C., Muro-Pastor, M. I. and Florencio, F. J. 1997. Transcription of glutamine synthetase genes (*glnA* and *glnN*) from the cyanobacterium *Synechocystis* sp. strain PCC 6803 is differently regulated in response to nitrogen availability. J. Bacteriol. 179, 2678-2689.

Reysenbach, A. L. and Shock, E. 2002. Merging genomes with geochemistry in hydrothermal Ecosystems. Science. 295, 1077-1082.

Riccardi, G., De Rossi, E., Della Valle, G. and Cifferi, O. 1985. Cloning of the glutamine synthetase gene from *Spirulina platensis*. Plant. Mol. Biol. 4, 133-136.

Rice, D., Mazur, J. B. and Haselkorn, R. 1982. Isolation and physical mapping of nitrogen fixation genes from the cyanobacterium *Anabaena* 7120. J. Biol. Chem. 257, 13157-13163.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. and Stanier, R. Y. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111, 1-61.

Rodriguez, R., Lara, C. and Guerrero, M. G. 1992. Nitrate transport in the cyanobacterium *Anacystis nidulans* R2. Kinetic and energetic aspects Biochem. J. 282, 639-643.

Rowell, P., Enticott, S. and Stewart, W. D. P. 1977. Glutamine synthetase and nitrogenase activity in the blue-green algae *Anabaena cylindrica*. New Phytol. 79, 41-54.

Rowell, P., Sampio, M. J. A. M., Ladha, J. K. and Stewart, W. D. P. 1979. Alteration of cyanobacterial glutamine synthetase activity in vivo in response to light and NH_4^+. Arch. Microbiol. 120, 195-200.

Rubio, L. M., Herrero, A. and Flores, E. 1996. A cyanobacterial *nar*B gene encodes a ferredoxin-dependent nitrate reductase. Plant Mol. Bio. 30, 845-850.

Saguira, M and Inoue Yorinao. 1999. Highly purified thermo-stable oxygen evolving photosystem II core complex from the thermophilic cyanobacterium *Synechococcus elongates* having his-tagged CP43. Plant Cell Physiol. 40(12), 1219-1231.

Sakamoto. T., Inoue-Skamoto, K. and Bryant, D. A. 1999. A novel nitrate/nitrite permease in the marine cyanobacterium *Synechococcus* sp. strain PCC 7002. J. Bacteriol. 181, 7363-7372.

Sandman, K. and Reeve, J. N. 2000. Structure and functional relationships of archael and eukaryal histones and nucleosomes. Arch. Microbiol. 173, 165-169.

Sampio, M. J. A. M., Rowell, P. and Stewart, W. D. P. 1979. Purification and some properties of glutamine synthetase from the nitrogen fixing cyanobacterium *Anabaena cylindrica* and *Nostoc* sp. J. Gen. Microbiol. 111, 181-191.

Sarma, T. A. and Kanta, S. 1979. Biochemical studies on sporulation in blue-green algae. I.Gycogen accumulation. Z. Allg. Mikrobiol. 19, 571-575.

Sarma, T. A. and Kanta, S. 1982. Variation in enzyme levels during sporulation in blue-green algae. Biochem. Physiol. Pflanzen. 177, 278-282.

Sauer, J., Dirmeier, U. and Forchhammer, K. 2000. The *Synechococcus* strain PCC 7942 *glnN* product (glutamine synthetase II) helps recovery from prolonged nitrogen chlorosis. J. Bacteriol. 182, 5615-5619.

Sawa, Y., Kanayama, K. and Ochiai, H. 1982. ATP can be generated photosynthetically using a living, thermophilic *Mastigocladus* sp. Biotech. Bioeng. 24, 305.

Schwabe, G. H. 1960. Über der thermobioten Kosmopoliten *Mastigocladus laminosus* Cohn. Schweiz. Z. Hydrobiol. 22, 759-792.

Scott, I. D. and Nicholls, D. G. 1980. Energy transduction in intact synaptosomes. Influence of plasma-membrane depolarization on the respiration and

membrane potential of internal mitochondria determined *in situ*. Biochem. J. 186, 21-33.

Sheridan, R. P. and Castenholz, R. W. 1968. Production of hydrogen sulfide by a thermophilic blue-green alga. Nature (London). 217, 1063-104.

Sili, C., Ena, A., Materassi, R. and Vincenzini, M. 1994. Germination of desiccated

aged akinetes of alkaliphilic cyanobacteria. Arch. Microbiol. 162, 20-25.

Simon, R. D. 1971. Cyanophycin granules from blue-green alga *Anabaena cylindrica*: a reserve material consisting of copolymers of aspartic acid and arginine. Proc. Natl. Acad. Sci. U. S. A. 68, 265-267.

Simon, R. D. 1977. Sporulation in the filamentous cyanobacterium *Anabaena cylindrica*: The course of spore formation. Arch. Microbiol. 111, 238-288.

Sinclair, C. and Whitton, B. A. 1977. Influence of nutrient deficiency on hair formation in the Rivulariaceae. J. Brit. Phycol. 12, 297-313.

Singh, A. K., Singh, H. N. and Rai, A. N. 1991. Evidence for a role of glutamine synthetase in assimilation of amino acids as nitrogen source in the cyanobacterium *Nostoc muscorum*. Biochem. Intl. 25, 887-894.

Singh, D. T., Rai, A. N. and Singh, H. N. 1985. Methylammonium (ammonium) uptake in a glutamine auxotroph of the cyanobacterium *Anabaena cycadae*. FEBS Letters. 186, 51-53.

Singh, H. N. and Srivastava. B. S. 1968. Studies on morphogenesis in a blue-green alga. I. Effect of inorganic nitrogen sources on developmental morphology of *Anabaena doliolum*. Can. J. Microbiol. 14, 1341-1346.

Singh, P. K. 1977. Multiplication and utilization of fern 'Azolla' containing nitrogen-fixing algal symbiont as green manure in rice cultivation. II Riso 26, 125-137.

Singh, R. K. and Stevens, S. E. Jr. 1992. Cloning of the *nif*HDK genes and their organization in the heterocystous cyanobacterium *Mastigocladus laminosus*. FEMS. Microbiol. Lett. 94, 227-234.

Singh, R. N. 1961. Role of Blue-green Algae in Nitrogen Economy of Indian Agriculture. Indian Council of Agricultural Research. New Delhi. 175.

Singh, S. and Kashyap, A. K. 1988. Metabolic characteristics of akinetes of the cyanobacterium *Fischerella muscicola*. New Phytol. 110, 97-100.

Skill, S. C. and Smith, R. J. 1987. Synchronous akinete germination and heterocyst differentiation in *Anabaena* PCC 7937 and *Nostoc* PCC 6720. J. Gen. Microbiol. 133, 299-303.

Snell, F. D. and Snell, C. T. 1949. Colorimetric methods of analysis. D. Van. Nostrand Co. New York, USA.3, 804-805.

Spence, D. W. and Stewart, W. D. P. 1986. Proline inhibits N_2-fixation in *Anabaena* 7120. Biochem. Biophys. Res. Commun. 139, 940-946.

Sprent, J. 1979. The Biology of Nitrogen Fixing Organisms. Mac. Graw-Hill. London. UK.

Stacey, G., Tabita, F. R. and Van Baalen, C. 1977. Nitrogen and ammonia assimilation in the cyanobacteria: Purification of glutamine synthetase from *Anabaena* sp. strain CA. J. Bacteriol. 132, 596-603.

Stanier, R.Y. 1977. The position of the cyanobacteria in the world of phototrophs. Carlsberg Res. Commun. 42, 77-98.

Stevens, S. E. Jr., Mehta, V. P. and Lane, L. S. 1985. Nitrogen Fixation and CO_2 metabolism. In: Ludden, P. W. and Burris, J. E. (eds). Elsevier Science Publishing, New York. 235-243.

Stewart, W. D. P., Fitzgerald, G. P. and Burris, R. H. 1967. *In situ* studies on N_2-fixing using acetylene reduction technique. Proc. Natl. Acad. Sci. USA. 58, 2071-2078.

Stewart, W. D. P. 1970. Nitrogen fixation by blue-green algae in Yellowstone thermal areas. Phycologia. 9, 261-268.

Stewart, W. D. P. and Codd, G. A. 1975. Polyhedral bodies (carboxysomes) of nitrogen-fixing blue-green algae. Br. Phycol. J. 10, 273-278.

Stewart, W. D. P. and Rowell, P. 1975. Effects L-methionine-DL-sulphoximine on the assimilations of newly fixed NH_3, acetylene reductions and heterocysts production in *Anabaena cylindrica*. Biochem. Biophys. Res. Commun. 65, 846-856.

Stewart, W. D. P., Haystead, A. and Dharmawardene, M. W. N. 1975. Nitrogen assimilation and metabolism in blue-green algae. In: Stewart, W.D.P. (ed.) Nitrogen fixation in free-living microorganisms. Cambridge Univ. Press. Cambridge. 129-158.

Stewart, W. D. P. 1977. A botanical ramble among the blue-green algae. Br. Phycol. J. 12, 89-115.

Stewart, W. D. P.1980. Some aspects of structure and function in N_2-fixing cyanobacteria. Ann. Rev. Microbiol. 34, 497-536.

Stewart, W. D. P., Rowell, P., Cossar, J. and Kerby, N. 1985. Physiological studies on N_2-fixing cyanobacteria. Nitrogen Fixation and CO_2 Metabolism.

Sutherland, J. M., Herdman, M. and Stewart, W. D. P. 1979. Akinetes of the cyanobacterium *Nostoc* PCC 7524: macromolecular composition, structure and

control of differentiation. J. Gen. Microbiol. 115, 273-287.

Sutherland, J. M., Reaston, J, Stewart, W. D. P. and Herdman, M. 1985a. Akinetes of the cyanobacterium *Nostoc* PCC 7524: macromolecular and biochemical changes during synchronous germination. J. Gen. Microbiol. 131, 2855-2863.

Sutherland, J. M., Stewart, W. D. P. and Herdman, M. 1985b. Akinetes of the cyanobacterium *Nostoc* PCC 7524: morphological changes during synchronous germination. Arch. Microbiol. 142, 269-274.

Suzuki, I., Sugiyama, T. and Omato, T. 1993. Primary structures and transcriptional regulation of the gene for nitrite reductase from the cyanobacterium *Synechococcus* PCC 7942. Plant Cell Physiol. 34, 1311-1320.

Suzuki, I., Horie, N., Sugiyama, T. and Omata, T. 1995. Identification and characterization of two nitrogen-regulated genes of the cyanobacterium efficiency of nitrogen assimilation. J. Bacteriol. 177, 290-296.

Tandeau de Marsac, N and Houmard, J. 1993. Adaptation of cyanobacteria to environmental stimuli: new step towards molecular mechanisms. FEMS. Microbiol. Rev. 104, 119-190.

Tapia, M. I., Ochoa de Alda, J. A. G., Llama, M. J. and Serra, J. L. 1996. Changes in intracellular amino acids and organic acids induced by nitrogen starvation and nitrate or ammonium resupply in the cyanobacterium *Phormidium laminosum*. Planta. 198, 526-531.

Thomas, J., Meeks, J. C., Wolk, C. P., Shaffer, P. W., Austin, S. M. and Chien, W. S. 1977. Formation of glutamine from [^{13}N]-ammonia, [^{13}N] dinitrogen, and [^{14}C] glutamate by heterocysts isolated from *Anabaena cylindrica*. J. Bacteriol. 129, 1545-1555.

Theil, T. and Wolk, C. P. 1983. Metabolic activities of isolated akinetes of the cyanobacterium *Nostoc spongaeforme*. J. Bacteriol. 156, 369-374.

Thiel, T. and Leone, M. 1986. Effect of glutamine on growth and heterocyst differentiation in the cyanobacterium *Anabaena variabilis*. J. Bacteriol. 168, 769-774.

Thiel, T. 1988. Transport of leucine in the cyanobacterium *Anabaena variabilis*. Arch. Microbiol. 149, 466-470.

Trent, J. D., Nimmesgern, E., Wall, J. S., Hartl, F. U. and Horwich, A. L. 1991. A molecular chaperone from a thermophilic archaebacterium is related to the eukaryotic protein t-complex polypeptide-1. Nature. 354 (6253), 490-493.

Tumer, N. E., Robinson, J. S. and Haselkorn, R. 1983. Different promoters for the *Anabaena* glutamine synthetase gene during growth using molecular or fixed nitrogen. Nature. 306, 337-342.

Tyagi, V. V. S. 1974. Some observations on the pattern of sporulation in a blue-green alga, *Anabaena doliolum*. Ann. Bot., N.S. 38, 1107-1111.

Tyagi, V. V. S. 1978. The pattern of sporulation in *Anabaena circinalis* and comments on the role of heterocysts in sporulation in blue-green algae. Z. allg. Mikrobiol. 18, 281-286.

van Baalen, C. 1987. In: The cyanobacteria, edited by P Fay and C van Baalen, Elsevier, Amsterdam, The Netherlands. 187-198.

van Dok, W. and Hart, B. T. 1996. Akinete differentiation in *Anabaena circinalis*

(Cyanophyta). J. Phycol. 32, 557- 565.

van Dok, W. and Hart, B. T. 1997. Akinete germination in *Anabaena circinalis* (Cyanophyta). J. Phycol. 33, 12-17.

Vaishampayan, A. 1982. Amino acid nutrition in the blue green algae *Nostoc muscorum*. New Phytologist. 90, 545-549.

Vega-Palas, M. A., Madueño, F., Herrero, A and Flores, E. 1990. Identification and cloning of a regulatory gene for nitrogen assimilation in the cyanobacterium *Synechoccocus* sp. strain PCC 7942. J. Bacteriol. 172, 643-647.

Vega-Palas, M. A., Flores, E. and Herrero, A. 1992. NtcA, a global nitrogen regulator from the cyanobacterium *Synechococcus* that belongs to the Crp family of bacterial regulators. Mol. Microbiol. 6, 1853-1859.

Wagner, S. J., Thomas, S. P., Kaufman, R. I., Nixon, B. T. and Stevens, S. E. 1993. The *glnA* gene of the cyanobacterium *Agmenellum quadruplicatum* PR-6 is nonessential for ammonium assimilation. J. Bacteriol. 175, 604-612.

Wang, Q., Li, H. and Post, A. F. 2000. Nitrate-assimilation genes of the marine diazotrophic, filamentous cyanobacterium *Trichodesmium* sp. strain WH 9601. J. Bacteriol. 182, 1764-1767.

Ward, D. and Castenholz, R. W. 2000. Cyanobacteria in geothermal habitates. In: Whitton, A. and Potts, M. (eds). The ecology of cyanobacteria, Kluwer Academic Publishers, Netherlands. 37-59.

Watanabe, I., Espinas, C. R., Berja, N. S. and Alimagno, B. V. 1977. Utilization of the *Azolla-Anabaena* complex as a nitrogen fertilizer for rice. IRRI Research

Paper Series. 11, 1-15.

Wealand, J. L., Myers, A. J. and Hirschberg, R. 1989. Changes in gene expression during nitrogen starvation in *Anabaena variabilis*. ATCC 29413. J. Bacteriol. 171, 1309-1313.

Weathers, P. J., Chee, H. L. and Allen, M. M. 1978. Arginine catabolism in *Aphanocapsa* 6308. Arch. Microbiol. 118, 1-6.

Whitton, B.A. and Potts, M. 2000. The ecology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Wickstrom, C. E. and Wiegert, R. G. 1980. Response of thermal algal-bacterial mat to grazing by brine flies. Microb. Ecol. 6, 303-315.

Wildon, D. C. and Mercer, F. N. 1963. The ultrastructure of the heterocyst and akinete of the blue-green algae. Arch. Microbiol. 47, 19-31.

Wildman, R. B., Loesher, J. H. and Winger, C. L. 1975. Development and germination of akinetes of *Aphanizomenon flos-aquae*. J. Phycol. 11, 96-104.

Wolk, C. P. 1965a. Control of sporulation in a blue-green alga. Devl. Biol. 12, 15-35.

Wolk, C. P. 1965b. Heterocyst germination under defined conditions. Nature (Lond). 205, 201-202.

Wolk, C. P. 1968. Movement of carbon from vegetative cells to heterocysts in *Anabaena cylindrica*. J. Bacteriol. 96, 2138-2143.

Wolk, C. P. 1973. Physiology and cytological chemistry of blue-green algae. Bacteriol. Rev. 37, 32-101.

Wolk, C. P. 1975. Differentiation and pattern formation in filamentous blue-green algae. In: Gerhardt P., Costilow, R. N. and Sadoff, H. L. (eds) Spores VI. Am. Soc. Microbiol., Washington, D.C. 619, 85-96.

Wolk, C. P., Thomas, J., Shaffer, P. W., Austin, S. M. and Galonsky, A. 1976. Pathway of nitrogen metabolism after fixation of ^{13}N-labeled nitrogen gas by the cyanobacterium *Anabaena cylindrica*. J. Biol. Chem. 251, 5027-5034.

Wolk, C. P. 1979a. Developmental biology of cyanobacteria: advances and challenges. In: Nichols, J.M. (ed.) Abst. Third Internat. Symp. Photosyn. Prokaryotes, Oxford. D_I. Univ. Liverpool, England.

Wolk, C. P. 1979b. Intercellular interactions and pattern formation in filamentous cyanobacteria. In: Subtelny, S. and Konigsberg, I.R. (eds). Determinants of Spatial Organization. 37th Symp. Soc. Developmental Biology.Academic Press, New York. 333, 247-266.

Wolk, C. P. 1982. Heterocysts. In: Carr, N. and Whitton, B. (eds). The Biology of Cyanobacteria. Oxford: Blackwell. 359-386.

Wolk, C. P., Cai, Y., Cardemil, L., Flores, E., Holn, B., Murry, M., Schmetterer, G., Schrautemeier, B. and Wilson, R. 1988. Isolation and complementation of mutants of *Anabaena* sp. strain PCC 7120 unable to grow aerobically on dinitrogen. J. Bacteriol. 170, 1239-1244.

Wolk, C. P. 1991. Genetic analysis of cyanobacterial development. Curr. Opin. Genes and Devel. 1, 336-341.

Wolk, C. P., Ernst, A. and Elhai, J. 1994. Heterocyst metabolism and development. In: D.A. Bryant (ed). The Molecular Biology of Cyanobacteria. Kluwer

Academic Publishers, Dordrecht. The Netherlands. 769-823.

Wyman, M. and Fay, P. 1986. Interaction between light quality and nitrogen availability in the differentiation of akinetes in the planktonic cyanobacterium *Gloeotrichia echinulata.* J. Brit. Phycol. 21, 147-153.

Xu, P. and McAuley P. J. 1990. Uptake of amino acids by the cyanobacterium *Anabaena* ATCC 27893. New Phytol. 115, 581-585.

Yamamoto, Y. 1976. Effect of some physical and chemical factors on the germination of akinetes of *Anabaena cylindrica.* J. Gen. Microbiol. 22, 311-323.

Yoon, H. S. and Golden, J. W. 1998. Heterocyst pattern formation controlled by a diffusible peptide. Science. 282, 935-938.

Zouni, A., Witt, H. T., Kern, J., Fromme, P., Krauss, N., Saenger, W. and Orth, P. 2001. Crystal structure of photosystem II from *Synechococcus elongates* at 3.8-angstrom resolution. Nature. 409, 739-743.

CURRICULUM VITAE

Name	: Nonibala Khumanthen
Date and place of birth	: 25-11-1971, Imphal, Manipur
Nationality	: Indian
Sex	: Female
Address	: Kwakeithel Thounoajam Leikai,
	Imphal- 795 001, Manipur, India.
	: e-mail- nonibala40@yahoo.com
Academic Qualifications	:
M. Sc. (Biochemistry, 1997)	: North Eastern Hill University, Shillong
	First Division
B. Sc. (Botany, 1995)	: Manipur University, Manipur
	First Division
P. U. Sc. (1991)	: Manipur University, Manipur
	Second Division
H. S. L. C (1988)	: Manipur Board of Secondary Education, Manipur
	First Division