DISCUSSION
5 DISCUSSION

5.1 Substrate Induced Changes in Nostoc:

In the literature heterocysts, have been assigned a number of roles previously. One of the earliest was by Borzi (1878) who thought that heterocysts served in vegetative reproduction by promoting the fragmentation of filaments. Senescent and degenerating heterocysts certainly bring about the breakage of the filament but any disintegrating and dying vegetative cell also called necridium can bring fragmentation of a trichome, usually accompanied by hormogonium production.

The latest finding, that heterocysts are involved in nitrogen fixation and may be the actual sites of effective nitrogenase activity (Fay et al., 1968) provides a major metabolic role for these structures, and is in accord with much of our knowledge of heterocyst occurrence and physiology.

Fogg (1949) propounded that changes in heterocyst numbers may be brought about in two ways.

I. By the formation of new heterocysts from normal cells.

II. By the germination of heterocyst to give normal cells.

In the present study heterocyst frequency is found to be induced by many substances organic (Glucose, sucrose and fructose) (Fig.1.3) as well as inorganic (Cations) (Fig.2.3). Glucose, fructose and sucrose are also previously reported to enhance the efficiency of heterocyst production (Smoker et al., 1990, Weaver et al., 1981, Wolk et al., 1976), but in Nostoc using 40mM Fructose maximum induction about 3 times more than the control is found (Fig.1.3). Reddy et al., 1996 have reported that about 2-2.5 fold increase in the frequency by supplementing fructose. But they could not provide any plausible explanation for the rise, and linked it to the
need to transport carbon compounds as carriers of reductants across the vegetative cell-heterocyst barrier, helpful in increasing the hydrogen evolution of the *Anabaena* cultures.

Fogg (1949) hypothesized that heterocyst formation is dependent by the concentration of some substances e.g. an intermediate in the carbon metabolism, generally inversely proportional to the combined nitrogen. Since heterocysts contain a sizeable amount of cellulose, the availability of such substances is undoubtedly an important factor in their growth. However, the evidence at present available indicates that the role of nitrogenous compounds is the more direct one, since substances such as ammonia at low concentration exert more marked effects. From this theory it is to be expected that a compound containing nitrogen would inhibit heterocyst formation to an extent depending on the readiness with which it is converted by the alga to the inhibitory substance. In the case of nitrate, glycine and asparaginate the inhibition is transient; only in the case of ammonium salt does the suppression continue so long as an appreciable concentration of the substance remains in the medium. This hints that the active substance is either ammonia or some substance derived from it. He again emphasized that, since in all the types of cultures except those supplied with the ammonium nitrogen in the medium, potassium eventually reaches the same, relatively constant value of unity, irrespective of the concentration of available combined nitrogen in the medium, it seems that adaptation of the balance of metabolic reactions must tend to maintain a particular concentration of ammonium nitrogen within the cell.

Induction of heterocyst by the addition of glucose or fructose to the algae already grown on an inorganic nutrient medium may be explained as
due to a temporary preponderance of intermediates of carbon metabolism to which ammonium nitrogen may be linked. Consequently until the balance of metabolic reactions becomes adjusted to their presence, the concentration of ammonium nitrogen falls below its normal level and heterocyst formation increases.

In the present study inorganic substances like Ca, Mg and Na have also been found to induce the heterocyst formation, in *Nostoc* (Fig. 2.3). Smith (1988) also reported that $2 \times 10^{-3}$ M CaCl$_2$ increases the heterocyst frequency. He supported the concept that cyanobacteria maintain a low intracellular concentration of Ca, by employing an energy-dependent efflux. There are many examples recorded in which the artificial manipulation of this equilibrium results in changes in the metabolic and physiological responses of cyanobacterial cultures. Heterocyst frequency and the intracellular content of Ca in *Nostoc* are inversely related to the incident irradiance. It provides explanation for Ca mediated regulation in the form of a response to an environmental variable. Thus the capability is present in cyanobacteria for both transient regulatory phenomenon, such as the triggering of motility by Ca influx and long term regulation, such as the increase in heterocyst frequency.

Na is also reported to influence the heterocyst frequency through affecting the carbohydrate metabolism of blue-green algae (Erdmann *et al.*, 1992; Kuwada and Ogta, 1989).

Though no report of MgSO$_4$ having any affect on the heterocyst frequency has been found, Shanmugasundaram *et al.* (1995) found that when *Nostoc* sp. MKU125 was starved for various divalent cations and monovalent cations, starvation for magnesium was found to reduce periplasmic DNAase activity. Even though Mg starvation marginally affected
growth, the cells become metabolically normal with in 24 to 48 hrs after getting shifted to medium containing magnesium salt.

All these facts lead us to certain assumptions.

1. Heterocysts are triggered by both organic and inorganic substances.

2. Differentiation takes place only in the active growth phase.

3. Induction can be due to suppression of ammonium level, leading to enhanced carbohydrate metabolism, in case of organic substances.

4. It may be due to some energy imbalance in case of inorganic substances like Ca and Mg, as Mg is a constituent of ATP and Ca is related to the signal pathway. Thus it can be said that the induction of heterocysts is multichannel. It could be through general metabolism level or at the higher gene level.

5. Increased production of heterocysts in low light intensity could be attributed to the low nitrogen fixation due to lower rate of carbohydrate production. So a reduced concentration of ammonia in the organism results, this explains the induction at low light.

To identify whether the effect occurred due to the anion or the cation, nitrogenase (EC-1.18.2.1) activity of the cultures was checked. The Cyanobacterial cells were grown in equiconcentrations of both the forms of the substance i.e. MgCl$_2$ and MgSO$_4$. It was found that nitrogenase activity was almost equal in both the cases, (Fig. 2.7), even slightly more in the MgCl$_2$ grown cells than the MgSO$_4$, this clearly indicates that Mg is the major factor responsible for the induction. In other words cations are playing a crucial role in bringing these changes.
5.2 Efficiency of Hydrogen Evolution by Enrichment

The question arises now is, whether the heterocyst frequency is linked to the hydrogen evolution activity of the algae? Does the increased heterocyst frequency will increase the evolution of H₂ too? The question seems to be logical as both the hydrogenase (EC-1.18.3.1) and nitrogenase enzymes are localized in the heterocyst, though it has been postulated that vegetative cells too possess a hydrogenase which is not functional or works under very limited conditions (Spiller, et.al., 1983).

The efficiency of hydrogen evolution has been noticed to vary directly with the heterocyst frequency of the enriched Nostoc cultures. In cations magnesium enriched Nostoc showed about 2 to 2.5 fold increase in the heterocyst frequency (Fig. 2.3). A corresponding 2 to 4 fold increase in hydrogen evolution has been observed in dark adapted (Fig.1.7) as well as in the presence of different electron donors supplemented (Fig. 2.8) cation grown algae.

Though the nitrogenase activity (Fig.2.6) has not shown any consistent proportionality either with the evolution or the heterocyst frequency in cation grown cultures. It could be due to more activity of hydrogenase, which shadowed the nitrogenase activity in the cultures. As hydrogen evolution in the dark adapted culutres and in the presence of Na-dithionite has been found to be the maximum and both these activities are contributed by hydrogenase. But in sugar grown cultures the nitrogenase activities are very high than control (Fig.1.6). Hydrogen uptake activity is also found to be more in the cation enriched cultures (Fig.2.9) indicating that enrichment has induced both the forms of hydrogenase i.e. reversible and uptake. In carbohydrate enriched cultures, too heterocyst frequency (Fig.1.3)
has corresponded with the hydrogen evolution in the dark adapted cultures (Fig.1.6) and with different electron donors (Fig.1.7). Glucose did not show much hydrogen evolution it could be due to more oxygen evolution in the glucose enriched cultures, as seen by the very high Na-dithionite dependent activity only. Since it quenches the oxygen radicals, evolution is higher. Nitrogenase activity has been found to be more or less equal to the control value (Fig.1.5) and uptake activity (Fig.1.8) is also same as in the control, showing thereby that only reversible hydrogenase has been effected by the carbohydrate enrichment.

In trace elements since not much change in the heterocyst frequency has been noticed, except for molybdenums where the growth as well as frequency was slightly more, the very high hydrogen evolution (Fig. 3.6) and nitrogenase activities (Fig.3.5) are due to very low chlorophyll content of the enriched cultures as compared to the control. Hydrogen uptake activity (Fig.3.7) has also increased owing to the same reason.

These two enzymes (nitrogenase and hydrogenase) are the two key factors for hydrogen evolution, they too must have some effect of the altered frequency of heterocysts. Reddy et al. (1996) has also calculated Hydrogen evolution in terms of number of heterocysts, in *Anabaena* and quoted that increasing functional heterocyst frequency should enhance the rate of hydrogen production. Troshina et al. (1996) also reported in *Anabaena variabilis* that the development of nitrogenase activity and hydrogen uptake activities coincided with an increase in heterocyst frequency by fructose addition. Further emphasizing that both activities reached maximal levels at the same time as the cells exhibited the highest heterocyst frequency. Thereafter, nitrogenase activity being dependent on the glycogen content
(Ernst et. al., 1984) decreased significantly, whereas hydrogen consumption showed a slower response. He concluded that the stimulatory effect of fructose appears to be due to an induction of higher heterocyst frequencies as well as to increased rate of respiration resulting in a lower oxygen tension (Ernst et. al. 1985, Haury et. al. 1981).

In Nostoc sp, it is noticed that stimulation in heterocyst frequency by various substances (inorganic as well as organic) corresponded with the increased hydrogen evolution, (Fig.1.6) i.e. two fold rise in heterocyst frequency 1m fructose and magnesium enriched cultures resulted in similar two fold stimulation in hydrogen generation, indicating that these two functions are linked at some stage.

5.3 Hydrogen Evolution under anaerobic adaptation in dark and light conditions

Hydrogenase in the algae is activated by anaerobic incubation either under argon or nitrogen. Anaerobic adaptation is a variable process and largely differs with the organism, its growth conditions and the enzyme involved. In the present work, the maximum rate of evolution is found in low light adapted anaerobic cultures of Nostoc (Fig.1.6), and is attributed to the reductive activation of hydrogenase. Reductive activation of oxidised, inactive hydrogenase has been reported by Gaffron (1940), Fisher et. al. (1954), Hartman and Krasna (1961) and Klein Betz (1978).

5.4 Hydrogen Evolution in the Presence of PSI and PSI Electron Donors and Inhibitors

In cells of Nostoc incubated in an aerobic condition, nitrogenase
(EC-1.18.2.1) is also appreciably enhanced though it already exists in the active state, further incubation of the filaments under nitrogen or argon increases the activity (Haselkorn, 1978). Pre-incubation of Nostoc muscorum in nitrogen or argon lead to enhanced nitrogenase dependent hydrogen formation (Scherer et. al. 1980). Incubation under carbon monoxide, an inhibitor of nitrogenase and acetylene an inhibitor of hydrogenase (EC-1.8.3.1) was shown to enhance nitrogenase mediated hydrogen formation in A. cylindrica (Bothe et. al., 1977). So increased evolution of hydrogen in Nostoc under anaerobic adaptation (Fig.6) in light for 5hrs. can be explained by more activation of the nitrogenase and reductive activation of the hydrogenase.

5.4.1 H₂ Evolution in the Presence of Dithionite

The stable dithionite mediated H₂ evolution in Nostoc cells indicates greater stability of PSI mediated reactions. Mg at 2mM showed maximum activity (Fig.2.8). This could be due to two reasons that either PS I under magnesium has become more active or the enzyme hydrogenase is acting in a Mg dependent manner and is responsible for about four fold activity under magnesium enrichment of the cultures.

Apart from acting as a source of electrons for PSI, dithionite also activates H₂ase by reduction. Reductive activation of H₂ase with dithionite has been reported in cell free H₂ase preparation (Hartmann and Krasna; 1963, Mortenson and Chen; 1976, Adams and Hall; 1979a,b, Van-Dijk et. al.; 1986, Adams et. al.; 1981) and crude cell extracts of Anabaena (Houchins and Burris, 1981) and Anacystis (Peschek 1979). It is suggested by Adams et. al., 1981 that more electrons to the enzyme at the first instance itself is
not sufficient for H₂ evolving reactions by hydrogenase, and the rate limiting step appears to be further addition of electrons.

5.4.2 BQ Mediated H₂ Evolution

BQ mediated H₂ evolution (Fig.2.8) is attributed to a plastoquinone H2ase reaction. PQ-H₂ase reaction is established and worked out by many (Gaffron and Bishop; 1963, Ben-Amotz and Gibbs; 1965, Mahro et. al.; 1986, Maione and Gibbs; 1986). Among cyanobacteria it is reported in NH₄⁺ grown filaments of *Anabaena* (Houchins; 1981) and *Anacysti* (Peschek; 1979). BQ being a lipophilic electron acceptor can easily penetrate the cells, binds with in the lipid phase of the photosynthetic membranes of cyanobacteria and higher plant chloroplasts and rapidly extracts electrons via PQ (Trebst and Reimer; 1973). Consequently, the reduced BQH2 is formed. This BQH2 then might act as the major electron source for the hydrogenase. BQH₂, hence formed may interact with the H₂ase either directly or through an intermediate electron carrier, the exact mechanism, however, is not worked out.

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\begin{align*}
2H₂O + 2BQ \rightarrow \text{PSII} \rightarrow 2BQH₂ + O₂ \\
BQH₂ + X \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow BQ + H₂X \\
H₂X + H₂ase \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow H₂ + X \\
X = \text{Coupling Electron Carrier}
\end{align*}
\]

If both the photosystems i.e. PSI and PSII are taking part in the reaction it may be possible that BQH₂ possess an electron via the oxidizing side of PSI and in that case the reaction becomes both PSII and PSI dependent as suggested by Kurita *et. al.*; 1976.
2H₂O + 2BQ ------PSII-------- 2BQH₂ + O₂
BQH₂ + X ------PSI-------- BQ + H₂
H₂X + H₂ase ------------- X + H₂

DCPIP and Ascorbate as PSI electron donors, showed evolution, though DCPIP alone could not show any change, due to its inability to penetrate in the cells.

Dithionite mediated H₂ evolution was always found to be more than the BQ dependent evolution, this could be due to more activity of PSI in the cultures, or more hydrogenase dependence of the reactions. As the dithionite is reported to react with PSI and through a hydrogenase.

The observed difference in the rate of H₂ formation in the three groups of sugar, cations and trace metals enriched cultures could be attributed to different reasons.

1. Difference in the amount of quinone under different enrichments.
2. Difference in the activity of photosystems.
3. Due to differential activities of the enzymes nitrogenase and hydrogenase.

Hydrogen uptake activity measured as methylene blue reduction in terms of uptake hydrogenase (EC-1.18.3.1). The rate was more for autotrophically grown cells compared to the heterotrophically grown cells.

5.5 Hydrogenase (EC-1.18.3.1) - Nitrogenase (EC-1.18.2.1) Relationship in the Blue-green Alga

Bothe et. al. (1977) through their studies on the inhibitors and with different nitrogen sources in the cyanobacterium Anabaena reported that there is a parallelism between C₂H₄ and H₂ formation; it is therefore unlikely that the H₂ is evolved by a hydrogenase only.

The ATP dependent H₂
evolution by nitrogenase is shown later on by many workers (Smith et al.; 1976, Schubert and Evans; 1976, Spiller et al.; 1983).

Andersen and Shanmugam (1977) while working on the nitrogen fixation in K. Pneumonie concluded that nitrogenase catalyzed \( \text{H}_2 \) production is of major importance in the overall efficiency of biological \( \text{N}_2 \) fixation in vivo and in the absence of \( \text{N}_2 \), \( \text{H}_2 \) production catalyzed by nitrogenase increased about three fold.

The results presented here give insight in to the natural role of the hydrogenase in \textit{Nostoc}. Since \( \text{H}_2 \) evolution is maximal only in the presence of anaerobic incubation and with dithionite addition (Fig.1.6,1.7). Uptake hydrogenase is not active in the first few min. because evolution is appreciably higher than the uptake during this period.

Nitrogenase activity (Fig. 1.5,2.6,3.5) is found to be lower than the hydrogenase activity and enrichment has also lowered the nitrogenase activity as compared to the control. This could be due to some energy balance between the two enzymes so that induction of one suppresses the other.

Gest and Co-workerrs (1962,1965) postulatd that the \( \text{H}_2 \) formation in \textit{Rhodospirillum} regulates the balance between reducing power and energy pools. Excess of reducing power could be removed from the cells by means of this reaction. However, this explanation is not suited for cyanobacteria, because it should not take too long to remove the excess of electrons in the autotrophic algae. Alternative explanation that the hydrogen is recycled by \textit{Anabaena} is, therefore more likely, recycled \( \text{H}_2 \) could be used as an electron in nitrogen fixation in addition to pyruvate and NADPH (Bothe et al., 1974) and thereby enhance the efficiency of that process. For the past several
decades nitrogenase and/or hydrogenase have been known to catalyze hydrogen photoproduction in photosynthetic organisms (Kumazawa and Mitsui; 1984). In the green algae, hydrogenase is known to be the sole catalyst of hydrogen photoproduction, although minor hydrogenase participation may exist the enhancement of nitrogen fixing activity in blue green algae was noted to occur under nitrogen starvation conditions (Cobb and Myers, 1964). The cellular carbon to nitrogen (C/N) ratio and the levels of phycobilisome protein (Kulsooriya et.al., 1972) and cyanophycean granules (Neilson et.al., 1971; Vascancelos and Fay, 1974) were investigated in relation to nitrogenase activity. Increase in cellular C/N ratios during nitrogen limited growth tends to enhance nitrogenase synthesis. Some of the non-heterocystous blue-green algae are capable of fixing nitrogen but in most cases only under anaerobic conditions (Kenyan et.al., 1972). In these organisms, active nitrogenase synthesis has been shown to occur during anaerobic incubation with DCMU using nitrogen starved organisms. The accumulation of carbohydrate during nitrogen starvation (Allen and Smith, 1969) has been suggested as one of the important factors for the nitrogenase synthesis. In Miami BG7, nitrogenase synthesis (measured as hydrogen photoproduction capability) occurs when cells are aerobically cultured in medium containing low concentrations of combined nitrogen and subsequently incubated anaerobically in nitrogen free media (Rippka and Waterbury, 1977). Previously, it was shown that nitrogenase was the major enzyme participating in the hydrogen photoproduction in BG7 (Mitsui et.al., 1979). Increase of carbohydrate content and decrease of protein content play an important role in the nitrogenase synthesis through an increase of cellular C/N ratio.
5.6 Energetics of Hydrogen Production

A reduced ferredoxin (or flavodoxin during iron deficiency) serves as the ultimate electron donor to the Fe-protein of nitrogenase (Stewart, 1980). The mode by which the reduced ferredoxin is generated in different cyanobacteria is, however, different (Haselkorn, 1978). Cells of non-heterocystous cyanobacteria carry out oxygenic photosynthesis and nitrogen fixation in the same cell type. In cyanobacteria an electron flow through the electron transport chain interlinking the two photosystems (PSII--PSI) photoreduce NADP+ and consequently the ferredoxin, while the H+ gradient produces ATP by cyclic and non-cyclic photophosphorylation. In aerobically grown heterocystous cyanobacteria nitrogenase activity is restricted to heterocysts only (Stewart, 1980; Haselkorn, 1978). Heterocysts lack the Photosystem II (Thomas, 1970; Gorkom and Donze, 1971)) do not evolve oxygen and can not photoreduce NADPH or ferredoxin. Heterocysts are endowed with an unique ferredoxin (FdxH), a product of FdxH gene (Bohme and Haselkorn, 1988) which acts as the terminal electron donor to nitrogenase (Schrautmeier, 1985; Bohme, 1987). High NADPH/NADP+ ratios reversibly shut off G6PDH preventing over-production of NADPH and direct the flow of electrons from NADPH to FdxH. Lower NADPH/NADP+ ratios reactivate G6PDH in to producing more NADPH thus restoring electron supply to FdxH and thereby to nitrogenase. ATP can be generated by means of oxidative phosphorylation (Tel and Stewart, 1977).

H₂ evolution in most cyanobacteria being a part of nitrogen fixation, Andersen and Shanmugam (1977) calculated the energy requirement for N₂ fixation as whole in terms of ATP production in K. Pneumoniae. The
minimum energy requirement for N₂ fixation at a pH of 7 to 8 and below 25°C (optimum conditions) was about 4 mol glucose conserved per mol NH₄⁺ produced for the Nif derepressed strains. They calculated energy requirement for N₂ fixation in terms of ATP through fermentation of glucose. ATP requirement of 21 to 25 ATP/N₂ was calculated for N₂ fixation. Several different strains had H₂/NH₄⁺ molar ratios of 0.6 to 0.7 at pH 7.3, 25°C and 100% N₂. Even in the presence of 100% N₂ the H₂/NH₄⁺ ratios for purified nitrogenase from A. Vinelandii drops to only 0.5 to 0.8 (Hadfield and Bulenn; Hwang et. al., 1973). The ratio increases when N₂ was replaced by Argon.

In conclusion it can be said that energy lost through nitrogenase catalyzed H₂ production comprising at least 30% and under unfavorable conditions of temperature and pH, even more, is an important factor in the energy requirement for biological N₂ fixation.