REVIEW OF LITERATURE
Cyanobacteria are the largest and most diverse group of photosynthetic prokaryotes, found in wide variety of aquatic and terrestrial habitats in nature as well as in association with other organisms. In view of their tropical independence to carbon and also nitrogen in a number of cases, their cultivation and exploitation prove to be the least expensive.

**Enzymes involved in hydrogen metabolism:**

Three different types of enzymes involved in hydrogen metabolism of cyanobacteria are:

i. Nitrogenase, which catalyses hydrogen evolution unidirectionally;

ii. A membrane bound 'uptake' hydrogenase which provides reductant to the photosynthetic and respiratory electron transport chains and

iii. A soluble hydrogenase ('Reversible' hydrogenase) which catalyses either hydrogen evolution or hydrogen uptake when provided with suitable electron donors or acceptors.

These three enzymes occur in several different combinations depending on the organism examined and the growth conditions (Lambert and Smith, 1987; Bothe, 1982; Houchins, 1984; Benemann and Hallenbeck, 1978).

**Nitrogenase:**

Nitrogenase of all the organisms are very similar in their physical and catalytic properties (Winter and Burris, 1976; Eady and Smith, 1979). The nitrogenase complex is comprised of two protein compounds. The larger (MoFe)
protein contains Mo, Fe and acid labile sulphide with a molecular weight of around 2,20,000 daltons and the smaller (Fe) protein has only Fe and acid labile sulphide with a molecular weight of around 68,000 daltons. Weights may vary with the organism.

The larger component called as dinitrogenase (Mo-Fe protein or Component 1) is responsible for the catalytic reduction of substrate molecules. Second component is nitrogenase reductase (Fe protein or Component II) accepts electron from donors such as ferredoxin or flavodoxin, or dithionite and transfer these electrons to dinitrogenase with the concomitant hydrolysis of two molecules of ATP per electron transferred. The six electron reduction of N₂ to 2NH₃. therefore, requires a minimum of 12 ATP molecules making nitrogen fixation an energetically expensive process.

\[
\text{Mo-Nitrogenase} \\
N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2
\]

In addition to reducing nitrogen to ammonia, dinitrogenase can reduce a number of substrates such as proton, acetylene, cyanide, nitrous oxide and azide.

In addition to the conventional molybdenum-based nitrogenase an alternative vanadium-based nitrogenase has also been reported. *Anabaena variabilis* can express a second nitrogenase when grown under molybdenum deficiency (Bothe and Kentemich, 1990). This nitrogenase contains vanadium in the prosthetic group. Although many similarities exist between the two nitrogen fixing systems, compared to molybdenum-nitrogenase, vanadium
nitrogenase is less efficient in carrying out nitrogen fixation relative to hydrogen evolution and is less efficient in reducing acetylene. Both the nitrogenases are rapidly and irreversibly inactivated by oxygen especially, vanadium-nitrogenase is more sensitive. Organisms that fix nitrogen in aerobic environments, therefore, must be provided with some means of protecting nitrogenase from oxygen. Cyanobacteria are the only organisms capable of simultaneous oxygen evolution and nitrogen fixation.

Like the reduction of nitrogen, reduction of protons by nitrogenase requires ATP and a strong reductant. Reductant can be provided as reduced ferredoxin, flavodoxin or as commonly employed in laboratory experiments, dithionite. The evolution of hydrogen by nitrogenase always occurs during nitrogen fixation and a minimum of 25% of electron flux through nitrogenase is divided to the reduction of H\(^+\) even at a very high partial pressures of nitrogen (Rivera-Ortiz and Burris, 1975) and optimal levels of ATP and reductant (Hageman and Burris, 1975). If the rate of dinitrogenase turnover is slowed owing to limitation in the supply of ATP or reductant, the relative proportion of electrons diverted to H\(^+\) reduction increases (Hageman and Burris, 1975). The evolution of hydrogen by nitrogenase, therefore, consumes a substantial amount of the reductant and ATP utilized by nitrogenase.

The vanadium-nitrogenase produces more hydrogen than the conventional Mo-nitrogenase.

\[
\text{Va-nitrogenase} \\
N_2 + 12H^+ + 12e^- \rightarrow 2NH_3 + 3H_2
\]
Initial studies of nitrogenase activity in cyanobacteria suggested that the enzyme was found in all heterocystous organisms, but only in a very limited number of non-heterocystous forms. However, it is now known that substantial numbers of nonheterocystous organisms in every major taxonomic group are capable of synthesizing nitrogenase under anaerobic conditions (Rippka et al., 1979; Rippka and Waterbury, 1977).

Heterocystous organisms are generally capable of nitrogen fixation under aerobic conditions, although laboratory generated mutants exist which possess heterocysts and yet do not express nitrogenase activity (Currier et al., 1977). Heterocysts are exclusive sites of nitrogen fixation during aerobic growth, although nitrogenase activity may also appear in vegetative cells under anaerobic or microaerobic conditions (Haselkorn, 1978; Rippka and Stanier, 1978). Heterocysts possess a number of morphological and biochemical modifications designed to protect nitrogenase from oxygen inactivation (Haselkorn, 1978; Stewart, 1980).

Only a very limited number of nonheterocystous cyanobacteria are capable of aerobic nitrogen fixation. All strains tested of the unicellular genus *Gleothece* were found to fix nitrogen during aerobic growth (Rippka and Waterbury, 1977). *Aphanothece pallida* (Singh, 1973) which is now believed to be *Synechococcus* sp. can also fix nitrogen aerobically.

Aerobic nitrogenase activity has also been reported in two filamentous nonheterocystous organisms which form colonial aggregates *Microcoleus* (Pearson et al., 1979) and *Trichodesmtum* (Carpenter and Price, 1976).
A large number of nonheterocystous cyanobacteria are capable of nitrogen fixation under anaerobic or microaerobic conditions (Stewart and Lex, 1970; Rippka et al., 1979; Rippka and Waterbury, 1977; Haystead et al., 1970; Nagatani and Haselkorn, 1978). The enzyme nitrogenase was least common in the Chroococcacean cyanobacteria (Houchins, 1984).

**Source of ATP and reductant:**

Several potential pathways for the generation of reductant for nitrogenase have been identified although the relative contribution of each in vivo is uncertain (Houchins, 1984).

The immediate electron donor to dinitrogenase reductase in cyanobacteria is probably ferredoxin since this protein has been shown to effectively mediate electron flow to nitrogenase in vitro (Haystead and Stewart, 1972; Gallon et al., 1973; Smith et al., 1971). Any pathway capable of generating reduced ferredoxin therefore is a potential source of reductant for nitrogenase.

Efforts to determine whether electron derived from the oxidation of H₂O directly support nitrogenase activity have failed to produce conclusive evidence supporting this possibility (Gallon, 1980). The photosystem II inhibitor DCMU causes a transient drop in acetylene reduction with full recovery occurring within 5 minutes explains that alternative processes can contribute reductant at sufficient rates to support the observed activity (Gallon, 1980). Furthermore, in the majority of the nonheterocystous nitrogen fixers, nitrogenase activity was induced under anaerobic conditions in the presence of DCMU, thus eliminating H₂O as a source of electrons, and activity required the
presence of an endogenous or exogenous supply of carbohydrates (Rippka and Waterbury, 1977).

Since photosystem II is absent in heterocysts it is clearly evident that electrons from water do not directly support nitrogen fixation in these cells (Stewart, 1980). The ultimate source of reductant for nitrogenase in heterocystous organisms is fixed carbon imported from the vegetative cells (Wolk, 1968); however, the nature of the transported compound is still uncertain.

Oxidative pentose phosphate pathway is the principal pathway of carbon degradation by both heterocystous and nonheterocystous cyanobacteria during nitrogen fixation (Winkenbach and Wolk, 1973; Lex and Carr, 1974; Apte et al., 1978; Gallon, 1980). The inhibition of the enzymes of this pathway by light in vegetative cells was also observed (Apte et al., 1978) allowing suspension of this pathway during periods when photosynthetic electron transport could provide an alternative source of ATP and NADPH. However no light inhibition was observed in heterocysts (Apte et al., 1978). The NADPH produced by the oxidative pentose phosphate pathway donates electrons to nitrogenase indirectly via ferredoxin NADP oxidoreductase (Apte et al., 1978; Houchins and Hind, 1982).

Other NADPH generating reactions that support acetylene reduction include isocitrate dehydrogenase (Eisbrenner and Bothe, 1979; Gallon et al., 1973; Smith et al., 1971; Lockau et al., 1978) and possibly malate dehydrogenase (Gallon et al., 1973).
Hydrogen is another possible source of reductant for nitrogen fixation (Bothe et al., 1978). The most rapid rates of acetylene reduction by isolated heterocysts have been measured with hydrogen as the electron donor (Elsbrenner and Bothe, 1979; Houchins and Hind, 1982; Peterson and Wolk, 1978). In light hydrogen may generate reduced ferredoxin by donating electron to the photosynthetic electron transport chain prior to photosystem I (Elsbrenner et al., 1978).

Reports concerning the NADH-generating enzymes at the glycolytic pathway in heterocysts have been contradictory. Early reports found no glyceraldehyde-3-phosphate dehydrogenase in heterocysts (Winkenbach and Wolk, 1973; Lex and Carr, 1974). A more recent report, however, claims that the enzyme is present in equal activities in heterocysts and vegetative cells (Bothe et al., 1980). The presence of a membrane-bound NADH dehydrogenase in isolated heterocysts (Houchins and Hind, 1982) suggests that some NADH-generating reaction or possibly an NAD:NADP transhydrogenase must be present. Rates of NADH-supported acetylene reduction were approximately 40% of those measured with dithionite or NADPH as electron donor. Like hydrogen, NADH donated electrons to nitrogenase only in the light and this reaction required electron flow through photosystem I (Houchins and Hind, 1982).

Another enzyme that may provide reduced ferredoxin to nitrogenase is pyruvate:ferredoxin oxido-reductase (Bothe et al., 1980). This enzyme has been found in extracts from heterocysts and vegetative cells and occurs with 5-fold higher activity in nitrogen-fixing cultures than in ammonia-
grown cultures. However, very low rates of acetylene reduction were observed upon provision of pyruvate to isolated heterocysts.

Photophosphorylation, oxidative phosphorylation and substrate level phosphorylation probably all contribute to the ATP supply during periods of nitrogenase activity (Bottomley and Stewart, 1976). In heterocysts of Anabaena cylindrica and Anabaenopsis circularis cyclic phosphorylation is the major source of ATP in the light and can support maximum rates of acetylene reduction provided there is an adequate supply of fixed carbon (Bottomley and Stewart, 1976). Heterocysts are incapable of non-cyclic photophosphorylation, because they lack a functional photosystem II.

ATP for nitrogen fixation may be supplied by oxidative phosphorylation at a rate which is usually about half that seen in the light. The level and duration of acetylene reduction in the dark can be extremely variable and depends on the size of the endogenous carbon reserve that has accumulated during the preceding period in the light (Fay, 1976). If carbon starvation has occurred, acetylene reduction in the dark will be almost negligible. It is under these conditions that supplementary electron donors such as hydrogen have the most pronounced effect in enhancing nitrogenase activity. If an abundant carbon reserve is present, nitrogenase activity will continue for several hours in the dark and hydrogen has little stimulatory effect (Houchins, 1984) but in Anabaena cylindrica and Anabaenopsis circularis the anaerobic synthesis of ATP was apparently not important in supporting nitrogen fixation. ATP production was limited in the dark, in the absence of oxygen
(Bottomley and Stewart, 1976) and under these conditions nitrogenase activity was low or undetectable (Stewart, 1977).

Substrate-level phosphorylation apparently can support substantial nitrogenase activity in Gloeothece (Gallon, 1980). After a 4 hour dark pretreatment under nitrogen, acetylene reduction was 40% of the illuminated anaerobic control rate.

Because of the high demand of nitrogenase for ATP and reductant, reactions that compete for these substrates can inhibit nitrogenase activity (Houchins, 1984).

**Hydrogenases:**

Unlike nitrogenase, an enzyme which shows remarkable similarity in molecular properties in all organisms from which it has been isolated, hydrogenases are very diverse in their relative molecular mass, co-factor composition and spectroscopic properties (Adams et al., 1981; Mayhew and O’connor, 1982). Therefore, the term "Hydrogenase" refers not to a single enzyme but a class of enzymes. Hydrogenases are found in many species of bacteria, both aerobic and anaerobic, and in pro and eukaryotic algae.

'Uptake' hydrogenase:

This enzyme catalyses hydrogen consumption associated with both photosynthetic and respiratory electron transport, although it is uncertain that whether the same enzyme is involved in both pathways or whether two separate enzyme activities occur in the membranes (Bothe, 1982). In most cases, uptake hydrogenase is unable to directly reduce low potential electron
acceptors such as ferredoxin, pyridine nucleotide and viologen dyes. When provided with low potential electron donors, it usually evolves hydrogen very slowly.

Uptake hydrogenase has been found in all heterocystous cyanobacteria so far examined. The level of activity varies greatly with growth conditions (Lambert and Smith, 1980) and in some strains of *Anabaena cylindrica*, activity is nearly undetectable under certain growth conditions (Bothe et al., 1978; Jones and Bishop, 1976).

Uptake hydrogenase is yet to be found in any of the non-heterocystous nitrogen fixers although these organisms have not been intensively studied (Houchins, 1984). The only non-heterocystous organism so far shown to possess uptake hydrogenase is the non-nitrogen fixing unicell *Anacystis nidulans* (Peschek, 1979a; Peschek, 1979b). Uptake hydrogenase has been intensively studied in this organism as well as the heterocystous species *Anabaena* and *Nostoc* (Bothe et al., 1978; Houchins and Burris, 1981; Lambert and Smith, 1980; Peterson and Burris, 1978; Tel-or et al., 1978).

**Physiological reaction involving uptake hydrogenase:**

The donation of electron from hydrogen to nitrogenase was shown clearly by Bothe et al. (1977) who found acetylene-dependant hydrogen uptake in the absence of any other electron acceptors. They found this reaction to be dependant on photosystem I, although, it was not clear whether light was
required for ATP synthesis, electron transport or both. The light requirement for electron transfer was clearly shown by Houchins and Hind, (1982).

The rate of electron donation from hydrogen to acetylene can vary considerably, depending on the degree to which alternative electron donors contribute. In one experiment using whole filaments of *A. cylindrica*, Bothe et al., (1977) found that 90-95% of the electrons for acetylene reduction came from hydrogen. In a similar experiment, Houchins and Burris, (1981) reported that approximately 60% of the electron supporting acetylene reduction came from sources other than hydrogen. This variation in results are due to differences in the carbohydrate pool available to heterocysts. If partial carbon starvation occurs, the cells rely more heavily on hydrogenase as a source of reductant. This was demonstrated by Benemann and Weare (1974) who found that hydrogen had only a small effect on acetylene reduction unless reductant starvation was induced by addition of DCMU.

Smith et al., 1976 used a combination of inhibitors to demonstrate in *Azotobacter chroococcus* and *Klebsiella pneumoniae* that the hydrogen evolved by nitrogenase was quantitatively consumed by hydrogenase. Hydrogen evolution was observed only after addition of carbon monoxide, which blocks all reactions of nitrogenase except hydrogen evolution and acetylene, which prevented hydrogen recycling by hydrogenase. Similar results were reported by Bothe and co-workers (1972) for *A. cylindrica*.

Several other reactions utilizing ferredoxin or NADPH as electron donors can support hydrogen uptake in the light. Rapid rates of hydrogen dependant nitrite reduction were observed in *Anaerostis* membranes (Peschek,
Nitrate and nitrite, both reduced by ferredoxin linked membrane proteins, also supported low rates of hydrogen uptake in *Anabaena* 7120 (Houchins and Burris, 1981).

Carbon di-oxide, which is reduced in an NADPH-dependant pathway, supported very low rates of hydrogen uptake in *A. nidulans* (Peschek, 1979). The reason for low activity is not evident in this case, since the CO₂-fixing enzymes of the Calvin cycle are present in the cell and the capacity for hydrogen dependent NADP reduction far exceeded the observed CO₂ fixation rate. Uptake hydrogenase does not support CO₂ reduction in heterocystous organisms because hydrogenase is confined to the heterocysts and the Calvin cycle enzymes are not present in the cell type (Codd et al., 1980).

The oxyhydrogen reaction provides several benefits to nitrogen fixing cyanobacteria. The intracellular oxygen level is lowered as a result of this reaction, thereby providing additional protection to nitrogenase against oxygen inactivation. In nitrogen fixing filaments and in isolated heterocysts, higher levels of oxygen are required to inhibit acetylene reduction if hydrogen is included in the gas phase (Bothe et al., 1978). In addition to its irreversible inhibition of nitrogenase, oxygen can act by competing for reductant that would otherwise be used for acetylene reduction. The oxyhydrogen reaction, by consuming oxygen, can prevent diversion of electron from the NADPH pool into the respiratory chain. The oxyhydrogen reaction is not the only means of lowering the oxygen concentration in heterocysts but supplements the activities of other respiratory and nonrespiratory oxygen-scavenging reactions. Its role in scavenging oxygen becomes more important in carbon limited cultures where
other sources of electron for oxygen consumption are unavailable (Houchins, 1984).

Electron transport from hydrogen to oxygen supports ATP synthesis and thereby meets part of the energy requirement of nitrogenase. This has been demonstrated by a variety of methods in Azotobacter, Rhzobium and in isolated heterocysts from Anabaena (Peterson and Burris, 1978; Walker and Yates, 1978; Emerich et al., 1979). Acetylene reduction in the dark by Anabaena filaments is enhanced by hydrogen (Houchins and Burris, 1981; Bothe et al., 1977). The reaction requires oxygen and enhancement is most pronounced in carbon-limited culture. Since hydrogen can not provide electron to nitrogenase without the mediation of photosystem I, the observed enhancement is probably attributable to ATP synthesis from the oxyhydrogen reaction rather than direct donation of electron to nitrogenase (Houchins, 1984).

An additional proposed function of uptake hydrogenase is to consume hydrogen, thereby preventing the competitive inhibition of nitrogen fixation by hydrogen (Dixon, 1972). At atmospheric levels of nitrogen, hydrogen is a weak inhibitor of nitrogen reduction (25% inhibition at 0.3 atm. hydrogen (Hwang et al. 1973)). Since hydrogen is a highly diffusible molecule it is unlikely that concentration of hydrogen sufficient to inhibit nitrogen fixation could ever accumulate within a cell.

It is clear that uptake hydrogenase is not exclusively responsible for any of those functions in vivo, but, rather that it acts to supplement other reactions.
Although this enzyme could catalyze hydrogen evolution the rate of evolution would be very slow because of the large energy input required for electron transfer from the enzyme back to H+. This property in effect makes the hydrogenase irreversible. Because the heterocyst is a highly reducing environment, a reversible hydrogenase would probably function largely in the direction of hydrogen evolution in the environment, thereby undermining the purpose of the enzyme which is to recapture hydrogen evolution by nitrogenase. The initial energy release, though it precludes utilization of the third coupling site, is necessary to prevent further loss of reduction (Houchins, 1984).

Reversible hydrogenase:

The term reversible hydrogenase usually refers to a protein that is soluble or loosely membrane-associated and is capable of catalysing hydrogen evolution and hydrogen uptake at similar rates (Lambert and Smith, 1981; Benemann and Hallenbeck, 1978; Hallenbeck and Benemann, 1979). Methyl viologen is chemically reduced by dithionite, support hydrogen evolution by all cyanobacterial reversible hydrogenases so far tested and usually is used as the electron donor in the assay of this enzyme.

The very existence in cyanobacteria of a soluble, reversible hydrogenase distinct from uptake hydrogenase has been a matter of some controversy. Elsbrenner et al. (1981) argue that there was no easily explainable physiological role for a hydrogen evolving hydrogenase in photoautotrophically grown cyanobacteria. They present evidence that reversible hydrogenase activity is an artifact of disruption and solubilization
treatments and they conclude that uptake hydrogenase is responsible for the catalysis of viologen dependent hydrogen evolution. However, the recent separation, partial purification and biochemical characterization of the two hydrogenases (Houchins and Burris, 1981 a and b; Hallenbeck and Benemann, 1978) provide convincing evidence for the existence of a reversible hydrogenase in cyanobacteria.

Reversible hydrogenase has been detected in representatives of nearly every major cyanobacterial group. Reactions catalyzed by reversible hydrogenase were demonstrated in the unicellular organisms Synechococcus, Synechocystis (Frenkel et al., 1950) and Aphanocapsa (Belkin and Padan, 1978); in filamentous nonheterocystous strains such as Spirulina (Llama et al., 1979) and Oscillatoria (Lambert and Smith, 1980; Belkin and Padan, 1978) and in heterocystous organisms including several strains of Anabaena, Nostoc and Mastigocladus (Houchins and Burris, 1981; Lambert and Smith, 1980; Tel-or et al., 1978; Rieder and Hall, 1981). In most cases, reversible hydrogenase was demonstrated by measuring methyl viologen-dependent hydrogen evolution and reversible hydrogenases been partially purified from some cyanobacterial sources (Houchins and Burris, 1981; Hallenbeck and Benemann, 1978; Llama et al., 1979; Belkin et al., 1981; Rieder and Hall, 1981). However, for Synechococcus and Synechocystis, the measurement of hydrogen-dependent photoreduction of CO$_2$ provides presumptive evidence for the existence of reversible hydrogenase in these cyanobacteria (Frenkel et al., 1950).
Effect of inhibitors

Various metabolic inhibitors have been used in an attempt to increase hydrogen production.

[3-(3,4-dichlorophenyl)-1,1-dimethyl urea]: (DCMU)

Presence of the photosynthetic inhibitor DCMU decreased photosystem II - dependent photosynthetic electron transport (oxygen evolution) by more than 95% and in turn nitrogenase activity was lowered between 30 and 50% only and this decrease in nitrogenase activity was dependent on the endogenous substrate (Weisshaar et al., 1983). When the substrate levels were low, DCMU inhibition of nitrogenase activity was comparatively high. No inhibition was observed in carbon-enriched cultures. Hallenbeck et al. (1979) have also showed in the cultures of A. cylindrica, that has been carbon starved for days or weeks were more susceptible to DCMU inhibition than the fresh cultures. Many workers have also noticed that DCMU inhibits hydrogen evolution in A. cylindrica and Mastigocladus laminosus; and in M. laminosus oxygen co-production was completely nil at 1mM concentration (Daday et al., 1977; Bothe et al., 1977).

However, DCMU has also been reported to stimulate hydrogen production in Nostoc muscorum (Spiller et al., 1979) and Mastigocladus laminosus (Miyamoto et al., 1979). The stimulation has been explained due to the inhibition of hydrogen uptake resulting in a net increase in hydrogen evolution.
Houchins and Burris (1984) have reported a direct DCMU stimulation of reversible hydrogenase.

**Carbon mono oxide and Acetylene: (CO and C2H2)**

Carbon mono oxide is an inhibitor of nitrogen fixation and acetylene reduction by nitrogenase but not of nitrogenase-mediated hydrogen production (Bradbeer and Wilson, 1963) and acetylene is an inhibitor of hydrogenase (Smith et al., 1976).

Addition of carbon mono oxide and acetylene together was shown to stimulate hydrogen production in *A. cylindrica* in an argon gas phase (Bothe et al., 1977). *Anabaena cylindrica* (Daday et al., 1977) and marine cyanobacteria (Lambert and Smith, 1977) showed hydrogen production even in air in the presence of these inhibitors. Similar observations were recorded by Lambert et al. (1979) after a lag phase of 2-3 hours. Bothe and Co-workers (1977) provided evidence that acetylene inhibited hydrogen recycling and that this inhibition increased with carbon mono oxide. Daday et al. (1979) also have shown that hydrogenase activity was inhibited by carbon mono oxide but was little affected by acetylene.

Using the inhibitors carbon mono oxide and acetylene, Mitsui et al. (1979) have found that hydrogen production in *Oscillatoria* sp miami BG7 was catalyzed by both nitrogenase (80-90%) and hydrogenase (10-20%) enzyme.

Production of hydrogen using cyanobacteria seems more preferable among the biological hydrogen production systems because of it's effectiveness. Hence it is imperative to optimize the conditions for maximal and
sustained hydrogen production. Some work on the influence of different environmental factors especially on nitrogenase which has been shown to be the major hydrogen producing enzyme has already been reported in literature.

Factors influencing Hydrogen Production:

Light:

The light dependence of nitrogenase-mediated hydrogen production by most cyanobacteria is well documented (Benemann and Weare, 1974; Jones and Bishop, 1976; Berchtold and Bachofen, 1979; Kerfin and Boger, 1982).

Jeffris et al., (1978) observed that Anabaena cylindrica sparged with argon gas produced hydrogen continuously for 30 days under light limited conditions (6.0 W m$^{-2}$) and for 18 days under elevated light conditions (32 W m$^{-2}$) in the absence of exogenous nitrogen. The dependence of hydrogen production on light intensity has also been studied for A. cylindrica by Kosyak et al., (1978). Hydrogen production increased with light intensity to about 4 x 10$^4$ erg cm$^{-2}$ S$^{-1}$, and no photoinhibition of hydrogen production occurred up to the maximum light intensity used (8 x 10$^4$ erg cm$^{-2}$ S$^{-1}$). Noteworthy was the fact that, oxygen production continued to increase above 4 x 10$^4$ erg cm$^{-2}$ S$^{-1}$. Possibly, hydrogen production was limited either by rate of transfer of, or by metabolism of photosystem II - generated reductant, or by a saturated photosystem I activity in heterocysts. It is worth noting that nitrogenase function is saturated at much higher intensities than are required for optimal growth. Hence, hydrogen production rates could be doubled in shift-up
experiments in which cultures were switched from medium \((2 \times 10^4 \text{ erg cm}^{-2} \text{ S}^{-1})\) to high \((6 \times 10^4 \text{ erg cm}^{-2} \text{ S}^{-1})\) light intensities (Hallenbeck et al., 1979). Asada et al., (1979) observed that in argon atmosphere hydrogen evolution by *Anabaena* N-7363 was saturated beyond approximately 10K Lux.

Hydrogen production could also be observed in some species in the dark. Weare and Benemann, (1974) have reported that *A.cylindrica* produced hydrogen in dark, but the quantity was very meagre. *Plectonema boryanum* also showed considerable acetylene reduction and hydrogen production in the dark (Weare and Benemann, 1974) presumably using reductant previously accumulated in light. Brechtold and Bachofen (1979) found varying dark/light ratios of hydrogen production in six cyanobacterial strains tested. It was suggested that the level of dark hydrogen production reflected to a large extent the ability of the organism to metabolize glycogen in dark.

Intermittent illumination (10s light/10s dark cycle) was found to result in an increased efficiency of hydrogen production in *A.cylindrica* (Jeffries et al 1978). Miura et al., (1980) have shown that hydrogen production by thermophillic cyanobacterium *Mastigocladus laminosus* was strongly dependent on the light intensity up to 10 K lux. Miyamoto et al., (1979) have shown that a light intensity of \(1 \times 10^4 \text{ erg cm}^{-2} \text{ S}^{-1}\) was saturating for acetylene reduction activity in *M.laminosus*.

Hydrogen production by *Oscillatoria* sp. Miami BG 7 was found to be proportional to light intensity at low levels of irradiance, but at higher intensities hydrogen production was inhibited by increased production and
accumulation of oxygen (Mitsui et al., 1979). This has been suggested to be due to the consumption of oxygen at low light intensities which could exceed oxygen production and consequently oxygen accumulation did not take place (Mitsui et al., 1979). Cyanobacterium *Synechococcus* sp. Miami BG 043511 showed hydrogen evolution in low light, whereas oxygen evolution significantly got reduced (Reddy and Mitsui, 1984). Spiller and Shanmugam, (1987) have observed that *Synechococcus* sp. strain SFl showed no nitrogenase activity in dark whereas, at low light intensities as low as 3 uE m$^{-2}$ S$^{-1}$, the activity was noticed and increased with increasing flux with an inhibition at 80 uE m$^{-2}$ S$^{-1}$. This result has been explained in terms of the rate of reductant diffusion from vegetative cells to heterocysts which was rate-limiting for hydrogen production under continuous light condition. It should be noted that, although the efficiency of conversion of light energy to hydrogen was higher under intermittent illumination, the hydrogen formed for unit time was still less than in the case of continuous illumination.

Jones and Bishop (1976) studied the relative effectiveness of different wavelength regions of light for simultaneous hydrogen and oxygen photoproduction. 615 nm (Photosystem II light) proved better for oxygen production than hydrogen production in both *Anabaena cylindrica* and *A. flos-aquae*. Whereas, it was found to be reverse at 686 nm (photosystem I light).

**Temperature:**

The optimum temperature for hydrogen production varied considerably with the organism. *Anabaena* sp. 7367 produced hydrogen maximally at 30°C (Asada et al., 1979). Mitsui et al., 1979 have found that the
optimum temperature for hydrogen production was around 30°C for Oscillatoria sp. Malami BG 7. Ernst et al., (1979) observed higher rates of hydrogen evolution by Nostoc after culturing at 22°C but not at 32°C. The reason for the higher activities at lower growth temperature could not be explained by them.

Striking data on the effect of temperature on short term hydrogen production have been obtained in the thermophilic cyanobacterium Mastigocladus laminosus grown at the optimal growth temperature of 45°C. Hydrogen production could be doubled by incubation subsequently at 49°C rather than 45°C (Miyamoto et al., 1979). Miura et al., (1980) also observed that M.laminosus produced maximum hydrogen at temperatures ranging from 44 to 49°C.

pH:

In general, hydrogen production was found to be optimum at neutral or weak alkali conditions. In the case of Anabaena sp pH 8.0 was found to be optimum for hydrogen production (Asada et al., 1979) and Mastigocladus laminosus it was 7.0-7.5 (Miura et al., 1980). pH variation did not show any significant difference in hydrogen production in Oscillatoria sp. Miami BG 7 in the pH range from 6.5 to 9.0 (Ramachandran and Mitsui, 1984).

High pH requirement for hydrogenase activity has been reported in Anacystis (Peschek, 1979) Anabaena and Nostoc (Tel-or et al., 1978). Benzoquinone mediated oxygen and hydrogen evolution in Anacystis sp. showed maximum rate at neutral or weak alkaline pH range (Kulandaivelu et al., 1988).
The low level of aerobic hydrogen evolution observed at both acidic and alkaline pH might due to the fact that both extreme pH ranges inhibit the water oxidation and subsequent electron flow (Yocum et al., 1984).

Salinity:

The effect of salinity on hydrogen production has been studied only to a limited extent. The rates of hydrogen production in different salinities by Oscillatoria sp. BG 7 did not show significant difference between 5 to 35 ppt. Even at the higher salinity of 45 ppt, the activity remained 60% of the maximum (Ramachandran and Mitsui, 1984).

The most interesting result obtained by Mitsui et al. (1979) was the diversity of salinity preferences for nitrogenase activity in different cyanobacterial strains. The optimum salinity range for maximum nitrogenase activity varied depending upon the strains between 5 ppt. to 50 ppt.

Micronutrients:

The influence of micronutrients on hydrogen production by cyanobacteria has been reported only from one group. Ramachandran and Mitsui (1984) have shown the effect of trace elements cobalt, copper, molybdenum, zinc and nickel on hydrogen production on Oscillatoria sp. Malami BG 7. Of the 5 elements molybdenum and cobalt exerted pronounced enhancement in hydrogen production. Production was enhanced to 160% at a concentration of 0.5 mg l$^{-1}$ by molybdenum and to 140% at 0.2 mg l$^{-1}$ by Co. This increase was attributed to the effect of these metals on the enzyme
nitrogenase which catalyses hydrogen production in that organism. It was also shown that the hydrogen production was not affected by Hg and Cd upto 0.1 mg l\(^{-1}\) concentration.

**Carbon source:**

Influence of organic compounds on nitrogenase activity was reported in a number of cyanobacteria including heterocystous species capable of photoheterotrophic growth (Rippka, 1972; Spiller et al., 1986).

Several simple organic carbon compounds stimulated acetylene reduction in heterocysts. Among these erythrose was found to be the most active one in *Anabaena* 7120 heterocysts (Privale and Burris, 1984) and *Anabaena* whole filaments (Prevalle, 1984). Whereas, Neuer and Bothe, (1985) have reported that erythrose as well as lactose, galactose, and cellobiose were inactive in heterocysts of *Anabaena cylindrica*.

In *Synechococcus* sp. strain SF1, bicarbonate, pyruvate, glucose, fructose and sucrose were found to show pronounced activity of nitrogenase (Spiller and Shanmugam, 1987). Bicarbonate enhanced nitrogenase activity has been suggested to occur in cases where endogenous carbon reserves were not a major contributing factor for nitrogen fixation (Spiller and Shanmugam, 1987).

With regard to uptake hydrogenase the presence of glucose was reported to repress its activity in *Rhizobium* (Maier et al., 1979) and *Azotobacter*
(Patridge et al., 1980) and such a repression also was reported in *Anabaena cycadeae* within 24 hours of glucose addition (Kumar et al., 1986).

**Nitrogen Source:**

Cyanobacterial nitrogen metabolism has been widely investigated with regard to nitrogen fixation (Allen and Arnon, 1955; Gallon, 1980) and the assimilation of inorganic and organic nitrogen sources (Haystead et al., 1973; Batt and Brown, 1974; Rowell et al., 1977).

A marine, non-heterocystous cyanobacteria *Trichodesmium* sp. NIBB 1067 showed no inhibition of nitrogenase activity for 7 hours with the addition of 2mM NaNO₃ or 0.02mM NH₄Cl. However, continued growth in these nitrogen sources completely removed nitrogenase activity (Ohki et al., 1991).

Nitrite inhibited the nitrogenase activity in *Anabaena variabilis* (Bohme, 1986). The action of nitrite was envisaged on different levels of the overall process of nitrogen fixation:

i. Competition of nitrite reductase with nitrogenase for reduced ferredoxin;

ii. inhibition of cyclic photophosphorylation;

iii. build-up of an excessive proton motive force and
iv. direct inhibition of nitrogenase by reduction of nitrite to nitric oxide. But it was shown that nitrite primarily exerted its inhibitory effect by inactivating irreversibly (Bohme, 1986).

In addition to NO₃ and NO₂ inhibitions, NH₄⁺ also inhibited acetylene reduction and hydrogen production in *A.cylindrica* (Lambert et al., 1979). The effect of NH₄⁺ could be explained, at least partly, in terms of a redirection of reductant, ATP or both, away from nitrogenase by competing biosynthetic pathways involving NH₄⁺ (Ohmori and Hattori, 1974, 1978). In aerobic cultures of *A. cylindrica* nitrate (upto 10mM) has no adverse effect on hydrogen production, while nitrite at 1 to 10mM, eliminated acetylene reduction and hydrogen production (Lambert and Smith, 1981).

Aminoacid uptake by cyanobacteria has been reported in several studies (Lee-kaden and Simonis, 1982; Thomas et al., 1982). Studies by Rowell et al., (1977) and Stacey et al., (1979) showed that feedback inhibition by aminoacids of the enzymes involved in ammonia assimilation occurred in cyanobacteria.

Exogenous glutamine has been shown to cause inhibition of nitrogenase in *A. cylindrica* (Rowell et al., 1977) while other major pool aminoacids did not (Stewart, 1980). Rawson, 1985 has reported reduction of nitrogenase activity accompanied by reduced growth by a number of aminoacids. Cultures supplemented with isoleucine, phenylalanine and valine at 1mM concentration were not significantly affected but had a pronounced depression of nitrogenase activity and growth at aminoacid concentration of 10mM (Rawson, 1985). Leucine, glycine, lysine and threonine at 1mM and
10mM concentrations and tyrosine, methionine and tryptophan at 1mM and 2mM concentrations showed a decreased nitrogenase activity resulting in major depression in growth at higher concentrations. The normal growth at low concentration suggested the utilization of aminoacids as nitrogen sources at these concentrations (Rawson, 1985).

Proline supplemented cultures (10mM), showed little sign of perturbation during the first 100 hours of culture, but by 190 hours nitrogenase activity fell to zero whereas, two aminoacids namely, cysteine and histidinie appeared to be toxic at both 1mM and 10mM concentrations (Rawson, 1985).

In Plectonema boryanum the nitrogenase synthesis was repressed by nitrate, ammonia, glutamine and arginine but not by aspartate even though this compound could enter the algal cells (Nagatani and Haselkorn, 1978).

Oxygen:

Nitrogenase is an oxygen labile enzyme (Haystead et al., 1970). However, light saturated, unsaturated cultures of A.cylindrica exhibit no significant oxygen inhibition at atmospheric oxygen tension on nitrogenase activity (Weare and Benemann, 1973, 1974). On the contrary they also reported in a predominantly argon gas phase added oxygen reduced hydrogen production in A.cylindrica (Weare and Benemann, 1973b). Similar results were also confirmed by Spiller et al., (1978) and Ernst et al., (1979) in A.variabilis. This could be either due to result of the oxygen sensitivity of nitrogenase, particularly in the case of older culture (Weare and Benemann, 1973 a, b; Ernst
et al., 1979) or by the facilitation of an oxyhydrogen reaction in the heterocyst which diminishes net hydrogen production (Spiller et al., 1978; Ernst et al., 1979). It was also believed to be likely that photorespiratory reactions in the vegetative cells stimulated by oxygen, resulting in a competition with and diminution of, nitrogenase activity in the heterocysts (Bergman, 1980 a, b).

Hydrogen evolution by *Anabaena* N-7363 was stimulated by adding about 5% of oxygen to the argon atmosphere, whereas, in *Anabaena cylindrica* it was inhibited by oxygen (Asada et al., 1979). In *Anabaena* N-7363 oxidative respiration might play an important role in supplying reducing power and/or ATP to the nitrogen-fixing system. This was supported by the fact that low illumination increased the extent of the oxygen stimulation on hydrogen production (Asada et al., 1979).

With *Mastigocladus laminosus*, Miyamato et al., (1979) have recorded no significant difference in oxygen inhibition with varied growth rates. Atmospheric oxygen (about 20%) was found to be slightly inhibitory (Miyamato et al., 1979). In the same organism treated with 20% O₂ during the dark periods of an imitated day and night cycle effectively suppressed the hydrogenase activity and thereby, enhanced the subsequent hydrogen evolution in the light periods (Miura et al., 1980).

**Molecular Nitrogen:**

The addition of gaseous nitrogen inhibits hydrogen production in argon atmosphere, due to the competition between nitrogen reduction and
proton reduction. Nitrogen at 4% of the gas phase has been found to inhibit hydrogen production in argon atmosphere by about 50% with complete inhibition at 25% nitrogen for A. cylindrica (Jones and Bishop, 1976).

**Hydrogen:**

In general hydrogen production in cyanobacteria has been assumed to be largely independent of the concentration of hydrogen in the gas phase (Benemann et al., 1980). In Anabaena cylindrica after addition of 2-20% hydrogen to an initial gas phase balanced by argon/CO₂ there was a vigorous hydrogen consumption in the light after several hours of incubation (Bothe et al., 1977; Lambert and Smith, 1981). Interestingly, Scherer et al., (1980) have shown that pre-incubation of N. muscorum in non-growth conditions in hydrogen in the light leads to a significant enhancement of nitrogenase activity, measured as hydrogen or acetylene reduction.

**Effect of immobilization:**

Immobilization of Oscillatoria sp. Miami BG7 cells in 1.5% agar matrix significantly enhanced the rate, yield and stability of hydrogen production, compared to free cell suspensions. Rate of hydrogen production in excess of 13ul mg drywt⁻¹ h⁻¹ were observed and hydrogen production was sustained for three weeks (Philips and Mitsui 1985)

Brouers and Hall, (1986) have showed that the hydrogen produced under argon by polyvinyl foam immobilized with A. azollae was twice that
produced by free-living cells; however, under argon/4% CO, the yield was similar in both samples. The increased yields under immobilization were suggested to be mainly due to an increase in hydrogenase mediated hydrogen production. Immobilization in alginate did not significantly increase the rate of hydrogen production but it maintained a net production for at least 16 hours instead of 8-10 hours in free living cells. This could be due either to a stabilization of the enzymes on immobilization or to a delay in the development of the uptake hydrogenase activity (Brouers and Hall, 1986).