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Review

Cyanobacterial hydrogen production

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Summary

With the global attention and research now being focussed on looking for an alternative to fossil fuel, hydrogen is the hope of future. Cyanobacteria are highly promising microorganisms for biological photohydrogen production. The review highlights the advancement in the biology of cyanobacterial hydrogen production in recent years. It discusses the enzymes involved in hydrogen production, viz. hydrogenases and nitrogenases, various strategies developed by cyanobacteria to limit nitrogenase inactivation by atmospheric and photosynthetic O_2, different biochemical and physicochemical parameters influencing the commercial cyanobacterial hydrogen production and the methods opted by different researchers for eliminating them to obtain maximum and sustained hydrogen production. Integrating the existing knowledge, techniques and expertise available, much future improvement and progress can be made in the field.

Introduction

With the rapid industrialization taking place in the last two decades, we have exploited, damaged and neglected the very environment which forms the very basis of our existence. Massive energy and material consumption have disrupted the delicate balance of the environment.

Present energy requirements are based on utilization of fossil fuels. Fuels such as oil, natural gas and coal supply more than 90% of the world's energy needs (Kalia 1995). Due to the escalated use of these fossil fuels, according to 'Geological survey of United States', 80% of the coal reserve will be utilized roughly within 300 years from 2000 AD and oil production will last less than a century. Also associated with the use of fossil fuels are problems of environmental pollution, global warming, acid rain and other multiplicative effects. In view of this crisis, more efforts are being focussed on development of clean and sustainable energy resources; which are renewable, non polluting and capable of meeting the global need.

Of the alternatives available, molecular hydrogen [H_2] can supplement and substitute fossil fuels. It is the most abundant element in the universe and has maximum energy per unit weight (122 KJ g^{-1}). On a weight basis, it is calculated that the heating value of H_2 is 141.65 MJ kg^{-1} which is the highest amongst known fuels (Ali & Basit 1993). It is easy to collect, therefore can be used during peak periods of demand, easy to transport and can be stored as gas-metal hydride or as liquid. It has greater energy conversion efficiency than petroleum. Hydrogen when used as a fuel does not pollute the environment, because its only conversion by-product is water. Besides its use as a fuel, hydrogen is useful in industrial applications like iron ore reduction and ammonia production (fertilizer). It has successfully been tested in aviation and automobiles (Ogden & Williams 1989).

Hydrogen can be produced in a number of ways:

1. Production from fossil fuels and biomass: coal gasification, steam reforming, partial oxidation of oil. Although these processes involve non-renewable sources and expensive techniques, these are still practised due to the abundant availability of low cost coal and oil.

2. Production from water through non-biological methods: thermal and thermochemical processes, electrolysis and photolysis. Owing to the heavy utilization of fossil and non-fossil fuels and other problems the possibility of these methods of reaching a commercial scale is very meagre.

3. Biological hydrogen production:

   a. *Fermentative hydrogen production*: Several members of the Enterobacteriaceae family generate reducing equivalents (e^-) during the degradation of organic carbon and cells evolve H_2 to dispose of these excess reductants by the action of the enzyme hydrogenase (Vos *et al.* 1983). However the disadvantages associated with these methods are the strict requirement of anaerobic conditions, energy sources and generation of CO_2 gas.

b. *Photobiological hydrogen production*: Harvesting solar energy is one of the approaches to develop clean and renewable energy resources. Solar energy is considered to be our largest and ultimate non-fossil, non-nuclear energy resource. In photobiological energy conversion photosynthetic bacteria, cyanobacteria, and green algae act under solar radiation to convert H_2O, reduced sulphur compounds and organic compounds into hydrogen. The present review is limited to photobiological conversion of water to H_2 using cyanobacteria. Cyanobacteria are oxygen-evolving, photosynthetic prokaryotes that can grow in air (N_2 and CO_2 as N and C source), water (electrons and reductant source) and simple mineral salts with light as the energy source.

The biggest natural process of conversion of light energy to chemical energy is through photosynthesis. Cyanobacteria posses photosynthetic systems having pigment molecules that capture solar quanta in the visible portion of the electromagnetic spectrum and channel this excitation energy into one of the two specialized reaction centres (PS-II) producing molecular O_2 by splitting water (Luque *et al*. 1994).

The other (PS-I) generates reducing equivalents which eventually reduce atmospheric CO_2 to organic compounds. The precise molecular distribution of the fixed carbon compounds, as well as local physiological growth conditions, will vary from species to species. However, all the reducing equivalents are ultimately derived from water. This is to say, the photosynthetic process is capable of synthesizing energy-rich biomolecules from H_2O, salts, CO_2 and sunlight.

Enzymes involved in hydrogen metabolism

The biological capacity to take up or evolve molecular hydrogen probably occurs only in the microbial system of the biosphere. These phenomena are especially more prevalent among photosynthetic microorganisms. Most photosynthetic bacteria, cyanobacteria and eucaryotic algae carry out both hydrogen consumption and evolution functions (Kosaric & Lyng 1988; Miyamoto *et al*. 1990).

Two general classes of enzymes, hydrogenase and nitrogenase, that catalyse hydrogen metabolism, are closely associated with the final H_2-evolving act in photosynthetic microorganisms (Benemann 1997). However, the primary functions of the enzymes are quite distinct. Nitrogenase is normally operative in biological nitrogen fixation whereas hydrogenase normally catalyses hydrogen uptake or hydrogen consuming reactions.

The major differences between the hydrogenase- and nitrogenase-mediated reactions is in the H_2 evolution reaction itself. H_2 evolution by nitrogenase requires considerable ATP for H_2 evolution in addition to reductants. The quanta required for H_2 evolution by nitrogenase would double those required for CO_2 fixation with an overall minimum quantum requirement of approx. 9–10 quanta/H_2. In principle, hydrogenase requires less than 1 quantum/H_2 and thus, should exhibit three times the efficiency of any nitrogenase-mediated system (Benemann 1994).

Hydrogenases

The term hydrogenase refers not to a single enzyme but a class of enzymes. Hydrogenases are enzymes that catalyse the oxidation of hydrogen to protons and the reduction of protons to hydrogen.

$$H_2 \rightleftarrows 2H^+ + 2e^-$$

This property makes them very simple enzymatic entities that could be used under a variety of environmental conditions either to get rid of excess electrons or to gather energy through the oxidation of hydrogen by different substrates. Hydrogenases are very diverse in their relative molecular mass, co-factor composition and spectroscopic properties (Krasna 1979). Hydrogenases in microorganisms have either a Fe-S centres, nickel/Fe-S centres (selanocysteine or non selanocysteine) or are metal-free. Cyanobacteria mostly have nickel/Fe–S–(selanocysteine) hydrogenases (Appel & Schulz 1998; Schlegel & Schneider 1985).

Uptake hydrogenase

Uptake hydrogenase is located at the cytoplasmic face of the cell membrane or thylakoid membrane, where it utilizes hydrogen evolved by nitrogenase. There is a considerable loss of energy through the production of hydrogen during nitrogen fixation. Some of this energy can be regained through the action of uptake hydrogenase. This enzyme splits the hydrogen and feeds the electrons back into the electron-transport chain. The reduction of a substrate with a relatively high redox potential like cytochrome through this hydrogenase seems to be a wasteful process. But since nitrogen-fixing cells maintain a highly reducing environment, it seems necessary to use part of the reductive power of hydrogen and saving reducing equivalents. The whole process is ultimately light-driven (Figure 1).

Hydrogen-utilizing uptake hydrogenase has several functions: (1) it serves as one of the mechanisms to protect oxygen-sensitive nitrogenase (Robson & Postgate 1980), (2) it generates ATP in the hydrogen-dependent respiratory oxygen uptake ('Knallgas' or oxyhydrogen reaction) and (3) it provides additional reducing equivalents to photosystem–I. Uptake hydrogenase has been found in all heterocystous cyanobacteria and some non-heterocystous cyanobacteria (Peschek 1979).

The structural genes encoding cyanobacterial uptake hydrogenases have been sequenced and characterized in only a few strains (Axelsson *et al*. 1999). The large subunit of the enzyme is encoded by *hup*L genes and small subunit is encoded by *hup*S genes. In the organisms studied so far, there is a high degree of homology in the gene sequence of *hup*SL (Tamagnini *et al*. 1997). But

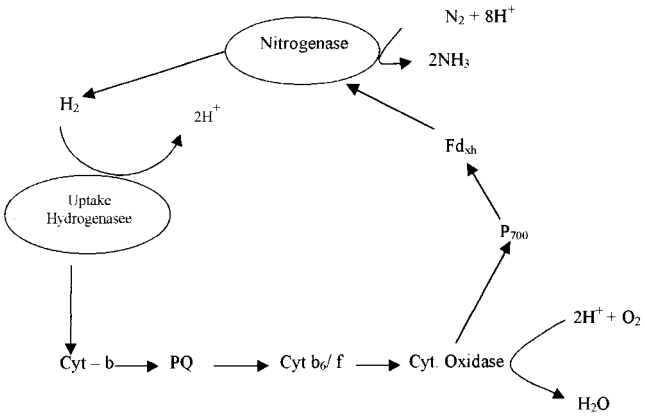

Figure 1. The uptake hydrogenase captures the hydrogen produced by the nitrogenase and feeds the electrons back into the electron transport system via the (PQ) plastoquinone pool, thereby accomplishing the oxyhydrogen reaction of cytochrome oxidase (Cyt. Oxidase). Alternatively it can pass electrons back to the nitrogenase through the action of P700 (PSI) and a specialised ferredoxin (Fd_xH). Cyt, cytochrome.

the mode or rearrangement of the genes varies from one organism to another (Axelsson *et al.* 1999).

Reversible hydrogenase

The reversible hydrogenase is located at the cytoplasmic membrane (Kentemich *et al.* 1991a). It has the dual function of catalysing hydrogen evolution and hydrogen uptake (Lambert & Smith 1981). It has been suggested that this enzyme functions as a valve for low potential electrons generated during the light reaction of photosynthesis, thus preventing the slowing down of the electron transport chain (Appel *et al.* 2000). It is available in the majority of the nitrogen-and non-nitrogen-fixing cyanobacteria (Eisbrenner *et al.* 1978).

Reversible hydrogenase is a heterotetrameric, NAD^+-reducing enzyme, consisting of a hydrogenase (encoded by *hox*Y and *hox*H genes) and a diaphorase part (encoded by *hox*F and *hox*U genes). The reversible hydrogenase of *Synechococcus* PCC 6301 was partially purified by Schmitz & Bothe (1996). The genes encoding reversible hydrogenase have been cloned by Boison *et al.* (1996) in *Anacystis nidulans*, by Schmitz *et al.* (1995) in *Anabaena variabilis* and by Appel & Schulz (1996) in *Synechocystis* sp. PCC 6803.

Nitrogenase

All nitrogenases studied so far are catalysts for H_2 production as they liberate H_2 during the reduction of nitrogen to ammonia. A minimum of 25% of the electron flux through nitrogenase is utilized in the reduction of protons to H_2.

$$N_2 + 8\,H^+ + 8\,e^- + 16\,ATP \rightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,Pi$$

ATP, reductant and electrons are provided by photosynthesis or by degradation of sugars in cyanobacteria.

Nitrogenase is a metalloenzyme complex consisting of dinitrogenase (MoFe protein: $\alpha_2\beta_2$) and dinitrogenase reductase (Fe protein: γ_2). The Mo-Fe protein or component-I is a larger component is responsible for the catalytic reduction of substrate molecules. The Mo-Fe protein from all sources examined are O_2 labile, have molecular weights of approximately 220,000 daltons. Approximately 2 mol of molybdenum and 24–32 mol of iron and sulphide are found per mol of protein (Kim & Rees 1994).

The second protein dinitrogenase reductase or component II accepts electrons from donors such as ferredoxin or flavodoxin, or dithionite and transfers these electron to dinitrogenase with the concomitant hydrolysis of two molecules of ATP per electron transferred. The six electron reduction of N_2 to $2NH_3$, therefore requires a minimum of 12 ATP molecules making nitrogen fixation an energetically expensive process. The Fe protein is also O_2 labile and has an average molecular weight of about 60,000 daltons. The protein consists of two subunits of equal weight (Kim & Rees 1994).

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

Apart from the conventional molybdenum-based nitrogenase, an alternative vanadium-based nitrogenase has also been reported (Kentemich *et al.* 1988). *A. variabilis* can express a third nitrogenase when grown under vanadium and molybdenum deficiency (Kentemich *et al.* 1991b). This nitrogenase contains vanadium in the prosthetic group. A novel mutant of *Azotobacter* which has a tungsten-based nitrogenase has also been isolated (Kajii *et al.* 1994).

In photosynthetic bacteria and cyanobacteria, photohydrogen production is mainly associated with nitrogenase rather than hydrogenase and coupled with ferredoxin or flavodoxin (Kosaric & Lyng 1988) It requires ATP and is inhibited by N_2 or NH_4^+. In this case, ferredoxin is reduced (1) directly by a light-driven reaction, (2) indirectly by ATP-driven reversed electron tranport, or (3) by dehydrogenation or oxidative decarboxylation reactions of intermediary metabolism not involving electron transport chains (Kosaric & Lyng 1988). Figure 2 shows the various pathways of reducing equivalents and hydrogen flowing towards nitrogenase during nitrogen fixation.

Nitrogenase is an extremely common, if not universal, enzyme in photosynthetic bacteria (Stewart 1973). It is difficult to ascertain its prevalence in cyanobacteria since oxygenic photosynthesis in these microbes is inherently incompatible with the nitrogenase protein. Cyanobacteria have evolved several mechanisms to overcome the O_2 incompatibility of nitrogenase.

Strategies developed by microbes to limit nitrogenase inactivation by atmospheric and photosynthetic O_2

Nitrogenases are highly sensitive to oxygen (Fay 1992). Various strategies have evolved in different nitrogen-

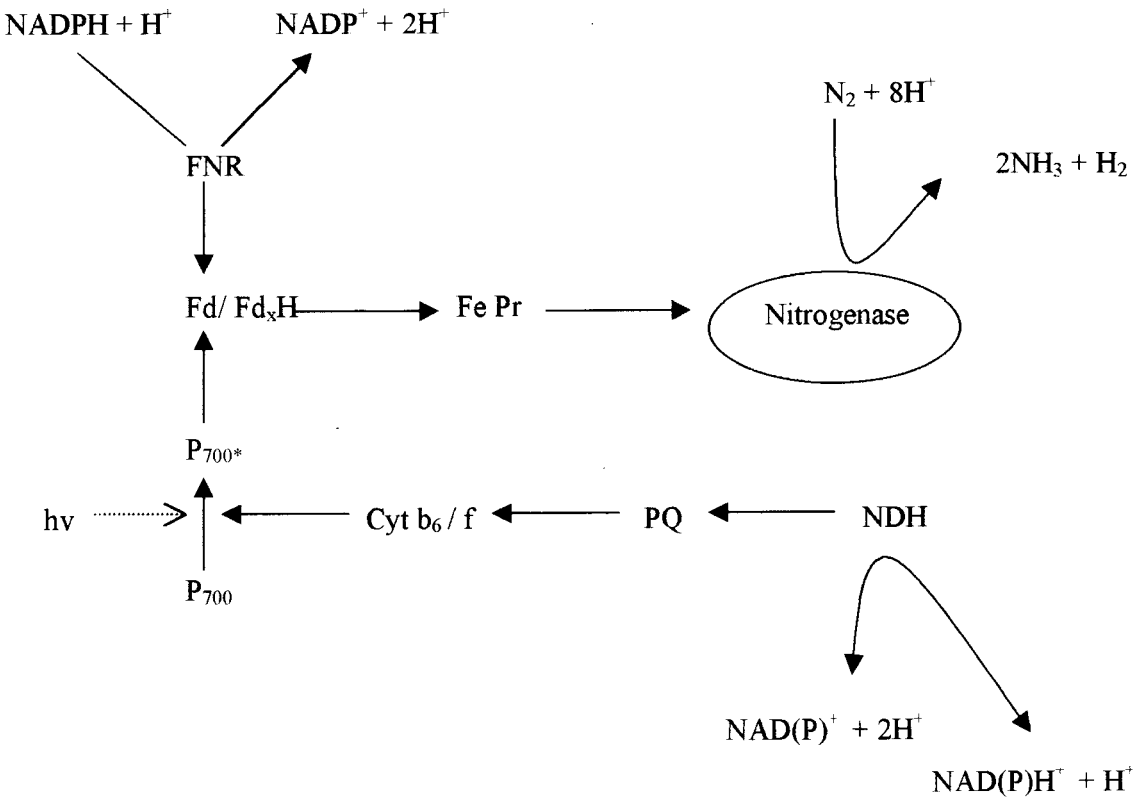

Figure 2. Schematic representation of the various sources of reducing equivalents and hydrogen to nitrogenase during nitrogen fixation. Electrons could be passed on to the nitrogenase by a reversed action of ferredoxin – NAD reductase (FNR) or the complex I (NDH). Cyt, cytochrome; Fd, ferredoxin; PQ, plastoquinone pool.

fixing cyanobacteria to overcome this problem. Frequently an organism may employ more than one strategy to protect the nitrogenase from oxygen.

1. Many cyanobacteria develop some form of physical barriers to limit oxygen diffusability and protect nitrogenase. Filamentous cyanobacteria like *Nostoc*, *Anabena* and many other cyanobacteria produce non-photosynthetic cells called heterocysts where nitrogen fixation takes place (Gallon 1992). This cells have a thick, laminated cell envelope that functions to limit the entry of atmospheric oxygen. Also they do not evolve O_2 through photosynthesis, allowing nitrogenase to fix nitrogen (Murry *et al.* 1984).

2. A number of cyanobacteria have been reported to produce exopolysaccharide (Adhikary 1998; Philippis *et al.* 1998; Shah *et al.* 1999; Shah *et al.* 2000b). Although not yet confirmed, these capsules may function as an barrier to the ingress of oxygen.

3. In non-heterocystous cyanobacteria, nitrogenase activity and photosynthetic activity are temporally separated (Chen 1986; Reedy *et al.* 1993; Misra & Tuli 2000). In this organisms, during the light phase a high rate of photosynthesis is exhibited, while nitrogen fixation takes place only in the dark. This circadian cycle remains unaltered in many organisms even in the presence of continuous light (Huang *et al.* 1990; Mitsui *et al.* 1986).

4. Under natural conditions, cyanobacteria are found associated with many other organisms. This association may contain several species of cyanobacteria and other heterotrophic organisms (Ferris *et al.* 1997). In such systems, the depletion of oxygen by heterotrophic bacteria and oxygen-consuming reactions of cyanobacteria allow nitrogen fixation by nitrogenase (Paerl & Carlton 1988; Paerl & Prufert 1987).

5. A switch on/off type of nitrogenase protection mechanism against conditions which are not conducive for nitrogenase function, exists in several cyanobacteria. Here, a reversible inactivation of nitrogenase by modification of the Fe-protein is employed (Apte 1996). A modification of the Fe-protein of nitrogenase at a time when the incident light intensity is greater than the photosynthetic compensation point is a means of energy conservation for the cyanobacterium *Synechocystis*. This reflects regulation that responds to fluctuation in diurnal light climate (Brass *et al.* 1992).

6. In organisms such as *Plectonema boryanum*, stimulation of PS-I and depression of PS-II were observed during the nitrogen fixation period. This results in low levels of O_2 evolution, a sharp fall in the amount of light-harvesting pigments and an uncoupling between the electron transfer from the PS-II complex to the acceptor leading to the depression of light-dependent O_2 evolution during N_2 fixation.

7. Reactive species of oxygen are formed within a cell (Bagchi *et al.* 1991). Cyanobacterial cells have been shown to have enzymes that prevent the formation of the toxic radicals and destroy those that are formed.

Some of these enzymes are superoxide dismutase, catalase, ascorbate peroxidase etc. (Gallon 1992; Grilli *et al*. 1991; Mackey & Smith 1983; Miyake *et al*. 1991).

8. Compounds such as carotenoids (Fresnedo *et al*. 1991) and tocopherols (Newton *et al*. 1977) are proposed to play a role in the protection of nitrogenase from oxidative damage.

9. In cyanobacteria which continually synthesize and degrade their nitrogenase, daily synthesis of nitrogenase occurs at relatively high rates. Such mechanisms have been reported from *Gleothece* (Gallon & Hamadi 1984), *Oscillatoria limosa* (Stal & Krumbein 1985b), *Anabena flos-aquae* (Bone 1971).

10. Recently Schneegurt *et al*. (2000) have reported that in *Cyanothece* sp. there is a burst of respiration in the organism to reduce intracellular oxygen tensions to protect nitrogenase.

11. Organisms in aquatic environments exhibit negative aerotaxis. In addition, aggregation of filaments/unicells limits the diffusion of O_2 in the centre of the aggregate. Sometimes these aggregates are of heterogeneous forms which balance O_2 availability in the aggregate (Barak *et al*. 1982).

12. In filamentous nonheterocystous cyanobacteria like *Lyngbya aestuarii* and *Trichodesmium*, it has been seen that only some cells of the filaments synthesize nitrogenase. In *Trichodesmium* approximately 15% of cells synthesize nitrogenase even during maximal nitrogen-fixing conditions (Fredriksson & Bergman 1995). These cells were evenly distributed in the filaments. In *Lyngbya* the nitrogen fixation was confined to cells present in the terminal regions of the filaments (Paerl *et al*. 1991). The results suggest spatial portioning of photosynthesis and nitrogen fixation.

Constraints in hydrogen photoproduction

The main problem in commercially exploiting cyanobacterial cells for hydrogen production is its efficiency which involves a complex of viables associated with photosynthesis and cell metabolism.

Metabolic potential

The metabolic potential of microorganisms is associated with their photosynthetic capabilities, which in turn determine their hydrogen-producing potentials. Different species exhibit different metabolic potentials. The efficiency of conversion of light energy to chemical energy of hydrogen via cyanobacteria and other photosynthetic organisms so for reported is very low (Hall *et al*. 1995).

Proper environmental conditions

The attainment of maximum metabolic potential (H_2-producing potential) is dependent on the existence of proper environmental conditions like light, temperature, salinity, nutrient availability, gaseous atmosphere etc.

Light. The effect of light on nitrogenase-mediated hydrogen production by most cyanobacteria has been well documented (Stal & Krumbein 1985). *A. cylindrica* purged with argon gas produced hydrogen continuously for 30 days under light-limited conditions (6.0 Wm^{-2}) and for 18 days under elevated conditions (32 Wm^{-2}) in the absence of exogenous nitrogen (Hallenback *et al*. 1978). It has been noted that nitrogenase function is saturated at much higher intensities than are required for optimal growth and hence, hydrogen production rates could be doubled in shift-up experiments in which cultures were switched from (20 Wm^{-2}) to high (60 Wm^{-2}) light intensities (Hallenback *et al*. 1978). In our study, *Phormidium valderianum* when coupled with *Halobacterium halobium* gave an increase in hydrogen production with increase in light intensity from 1 Klux to 4 Klux (Patel & Madamwar 1994). Even though an initial increase in light intensity improved rate of the production of hydrogen however, the duration of hydrogen evolution was reduced, resulting in low total hydrogen yield at higher light intensity.

Temperature. The optimum temperature for hydrogen production varies considerably with the microorganism. Ernst *et al*. (1979) observed higher rates of hydrogen evolution by *Nostoc* after culturing at 22 °C but not at 32 °C. *Oscillatoria* sp Miami BG7 (Philips & Mitsui 1983) and *Anabaena variabilis* SPU 003 (Shah *et al*. unpublished data) showed 30 °C as optimum for hydrogen production whereas *Nostoc muscorum* SPU 004 showed an optimum temperature of 40 °C for maximum hydrogen production (Shah *et al*. unpublished data). Short term hydrogen production was high in the thermophilic cyanobacterium *M. laminosus* grown at the optimal temperature of 45 °C. Hydrogen production could be doubled by incubation subsequently at 49 °C rather than 45 °C (Miyamoto *et al*. 1979).

Salinity. In general, fresh water cyanobacteria show lower hydrogen production with increasing salinity. This may be attributed to the diversion of energy and reductants for extrusion of Na^+ ions from within the cells or prevention of Na^+ influx (Rai & Abraham 1995). However, marine cyanobacteria are not affected by salinity upto a certain level for hydrogen production. Salinity is one of the most important stress factors demanding the synthesis of osmoregulators (Apte & Bhagwat 1989).

Micronutrients. Ramanchandran & Mitsui (1984) have reported the effect of the trace elements cobalt, copper, molybdenum, zinc, and nickel on hydrogen production. Many of these metals have shown pronounced enhancement of hydrogen production. This may be due to their involvement in the enzyme nitrogenase which catalyses hydrogen production in those organisms. In our study with *Anabaena variabilis* SPU 003 we found that the culture is highly sensitive to Mn^{+2}, Ni^{+2}, Cu^{+2}, Co^{+2}, Zn^{+2} or Fe^{+3} at concentrations of 10 mM and no hydrogen production was seen (Shah *et al*. unpublished data).

Carbon source. Several organic compounds have been shown to influence nitrogenase activity. The presence of simple organic carbon compounds increases hydrogen production, probably due to increased electron donation by these compounds to nitrogenase (Neuer & Bothe 1985).

Nitrogen source. Several inorganic nitrogenous compounds have been found to influence hydrogen production. Nitrite, nitrate and ammonia inhibited nitrogenase activity in *Anabaena variabilis* SPU 003 and *A. cylindrica* (Lambert *et al.* 1979; Shah *et al.* unpublished data). Cyanobacterial nitrogen metabolism has been widely investigated with regard to nitrogen fixation. In general all nitrogen sources added externally inhibit nitrogenase synthesis (Rawson 1985).

Oxygen. The major difficulty in the biophotolysis scheme is the evolution of oxygen by the photosynthetic component during biophotolysis of water, ultimately affecting long term hydrogen production by inhibiting the oxygen-sensitive enzymes nitrogenase/hydrogenase. The key to successful construction of a biophotolytic hydrogen production system will be the stabilization of photosynthetic particles and enzymes (hydrogenase/nitrogenase) against oxygen inactivation. Some experiments have shown that addition of sodium dithionate to the reaction produced a considerable stimulation of hydrogen photoproduction, mainly because it acts as an effective oxygen-removing agent. Other effective scavengers like deoxyhaemoglobin, deoxymyoglobin and a variety of chelators of cobalt are known for their ability to enhance hydrogen production (Kojima & Yamaguchi 1988; Mitsui *et al.* 1985).

Molecular nitrogen. The removal of dinitrogen from the atmosphere is essential for hydrogen production, since it is a competitive inhibitor. It has been shown that hydrogen production is inhibited in large number of strains by dinitrogen (Lambert & Smith 1977) though micro quantities of dinitrogen are essential to support metabolism.

Competing pathways for electrons

There are numerous pathways, which can take excited electrons from the reduced low potential electron donor ferredoxin (Gupta & Narasimharao 1987). For maximum hydrogen yield it is necessary to inhibit these pathways by some means.

Rate-limiting reactions

Certain steps in the electron flow between PS-I and PS-II, hydrogenase and nitrogenase are rate-limiting (Gupta & Narasimharao 1987).

Environmental concerns

Many cyanobacteria produce toxic metabolites which are dangerous to terrestrial and aquatic flora. The production of large scale microalgal biomass may also pollute the environment (Rao & Hall 1996).

Strategies for increased H_2 production

Several decades of research on photobiological hydrogen production have highlighted the need for enhanced/continuous/stable production of H_2, overcoming the constraints involved in the process. Various groups of researchers have developed strategies for this purpose.

I. Development of combination cultures or co-culturing for biological hydrogen systems

Single cultures have been used for a long time for H_2 production. Both cyanobacteria and photosynthetic bacteria have been well studied. Miyake *et al.* (1984) have demonstrated that different enzymes from two different cultures can be used. The hydrogenase of *Cl. butyricum* and nitrogenase of photosynthetic bacteria work cooperatively through degradation of carbohydrates. *Cl. butyricum* evolves H_2 from carbohydrate at a high rate. However the yield is limited because it produces organic acids besides H_2. Immobilization of this co-culture resulted in 7 mol H_2/mol of glucose utilized.

Another approach receiving attention recently involves a coupled system of Halobacteria and cyanobacteria (marine) (Patel & Madamwar 1994). The extreme halophile *Halobacterium halobium* has attracted attention in recent years from the point of view of solar energy conversion and bioenergetics (Stoeckenius *et al.* 1979). *H. halobium* synthesizes distinct purple-coloured patches in its cell membrane when grown under conditions of light and low oxygen. A single species of protein, bacteriorhodopsin, is present within the purple membrane (Dancher 1988). Bacteriorhodopsin has been shown to function as a light-driven proton pump (Srivastava *et al.* 1984), which creates an electrochemical gradient across the plasma membrane of the intact cell. The purple membrane is oriented in such a manner that the release of protons will occur into the surrounding medium. The function of bacteriorhodopsin in a proton pump is coupled to the formation of ATP.

Marine cyanobacteria (*Phormidium valderianum*) also absorb and convert solar energy to chemical free energy in a form useful for cellular metabolic reaction and show the processes of photosynthesis. Under the stimulation of light, the photosynthetic apparatus can provide electrons released from water at a reducing potential more negative than the hydrogen electrode. When the reducing potential of the electrons is coupled with the protons released from bacteriorhodopsin in presence of hydrogenase, molecular hydrogen is evolved. *Phormidium* sp. as such has the ability to produce hydrogen but with *H. halobium*, hydrogen production goes up several-fold due to the electron generation ability of its photosynthetic pigments along with protons generated by the bacteriorhodopsin (Patel & Madamwar 1994).

This system has been further stabilized for long term hydrogen production (15–30 days) by immobilization (Patel & Madamwar 1995).

Extensive work in our laboratory has also shown that mixed cultures are desirable. A useful combination using *Halobacterium halobium* with *E. coli* and *Phormidium valderianum* has resulted in continuous production up to at least 60 days. The halophile *Halobacterium halobium* possesses the pigment bacteriorhodopsin, which acts as a proton pump. *Phormidium valderianum* and *E. coli*, a salt-adapted strain are used. It is proposed that the protons and electrons available are combined in the presence of hydrogenase to liberate H_2.

II. Single or a combination of cultures have been used in bioreactors to achieve sustained production

Photobioreactors combine within them two phases required for H_2 production i.e. a growth phase of the cells either free or immobilized on a suitable matrix, followed by H_2 production. Depending upon the nature of the matrix, bioreactors have been designed. Usually bioreactors are made compact so that they are easy to handle/operate. H_2 production is started by inducing microaerobic or anaerobic conditions in the reactor for the necessary enzymes to function.

Bioreactors usually include points for entry of medium, sample collection and pH adjustment, along with a pump for medium recirculation, a column or flask containing the microorganisms and a suitable light source.

Various types of bioreactors ranging from lab scale to outdoor handling have been designed to achieve higher and stable production rates. (Markov *et al.* 1993) The photoreactor developed by Tsygankov *et al.* (1998) combined the computer processors with the reactors enhancing the control ability as well as precision in maintaining the environmental conditions.

III. Immobilized cells have also been used for H_2 production

Single cultures as well as combination cultures have been immobilized in matrices ranging from simply glass beads, glass sheets to agar beads, gels, composite agar layers, alginate and complex polymers such as PU (Polyurethane) and PVA (Polyvinyl alcohol) foams. The advantages of immobilization of whole cells in a matrix are protection of enzymes and cellular activities against denaturation and environmental disturbances respectively. Also the system can be used for a long period of time.

Philips & Mitsui (1986) have done a detailed study on immobilization of the blue green alga *Oscillatoria* for hydrogen production. Their results concluded that when *Oscillatoria* sp. Miami BG 7 was immobilized in 1.5% agar matrix, the rate, yield and stability of hydrogen production increased significantly compared to the free cell system. Dainty *et al.* (1986) have reported the immobilization of algal cells in alginate beads. In our laboratory using polyvinyl alcohol (PVA) for immobilizing a combined system of *Phormidium valderianum*, *E. coli and H. halobium*, prolonged production of hydrogen was seen. With intermittent supply of nitrogen, to retain cellular metabolic activities, hydrogen production was seen for more than 4 month (Bagai & Madamwar 1998). It was noticed that PVA concentration and cell concentration along with other physico-chemical parameters, play a very crucial role in the stability and productivity of the reactors (Bagai & Madamwar 1999). A lab scale photobioreactor has also been constructed using hollow fibres composed of semipermeable polymeric membranes (Markov *et al.* 1993). Filaments of *A. variabilis* are immobilized on the outer surface of the fibres. The design of the bioreactor is such that it allows movement of small molecules and gas through the fibres while restricting entry of cells, and thus separation is made easier. H_2 production rates ranging from 0.02 to 0.2 ml H_2/mg dry wt/h are observed and continuously run for 5 month.

IV. Continuous culture

Continuous cultivation has proved to be advantageous over batch cultivation in terms of stable production rates. Small capacity chemostat fermentors are able to increase H_2 production rates because the specific growth rate of the culture in the chemostat remains stable and therefore production rates of H_2 are stabilized.

Reports from Sode *et al.* (1994) have shown that pseudo-continuous culture of a marine cyanobacterium carried out under a light/dark cycle shows a steady state of cell concentration. Here small samples of culture medium are replaced with fresh medium every hour to maintain a steady state. Lichtl *et al.* (1997) reported four times higher production of hydrogen by *Nostoc flagelliforme* under chemostat conditions.

V. Hydrogen production using cell-free systems

Hydrogen production has been demonstrated in a cell-free system using ferredoxin and hydrogenase in combination with chemical reductants such as dithionate and pyruvate (Egan & Scott 1978). Cell free systems will have an advantage of overcoming the inherent framework of strict check and balances under which natural biological systems operate which might not function at an economically efficient rate of yield. Hall *et al.* (1981) and Ben Amotz *et al.* (1975) demonstrated the photoproduction of hydrogen from water using an illuminated suspension of spinach chloroplasts, hydrogenase, and ferredoxin. Each of these processes, one utilizing chemical reductants and the other utilizing the photochemical reducing potential of illuminated chloroplasts, involves the reduction of ferredoxin and protons to produce hydrogen gas.

VI. Integrated hydrogen production and waste water treatment

Cyanobacteria have shown to be effective in treating effluents from diversified industries and also to degrade xenobiotic compounds (Blier *et al.* 1995; Kuritz 1999; Shah *et al.* 1999; Shah *et al.* 2000b; Travieso *et al.* 1996).

Our investigations with *Phormidium valderianum* have revealed the ability of the organism to successfully treat textile dye wastes. We have successfully integrated the hydrogen production ability of this organism with its waste water treatment potentiality (Shah *et al.*, unpublished data).

Integrating this ability of cyanobacteria with hydrogen production will enhance the profitability and feasibility of the technique. If not the whole industrial unit, the waste water treatment plant can be energy self-sufficient with the hydrogen produced by the organisms.

VII. Integrated hydrogen production and production of commercially important compounds

To make hydrogen production by cyanobacteria economically feasible, the process can be linked with production of industrially important compounds such as pigments, enzymes, anti-microbial compounds etc. Ohtaguchi *et al.* (1997) have used *Synechococcus leopoliensis* and *Saccharomyces sake* IFO 2347 for production of ethanol. Here cyanobacteria fix CO_2 and produce organic carbon compounds which are then used by the yeast to produce ethanol. Matsunaga *et al.* (1995) have reported the production of palmitoleic acid from marine cyanobacteria.

VIII. Physiological and biochemical methods

Molybdenum is usually present in the dinitrogenase moiety of the nitrogenase enzyme system. If Mo can be replaced by vanadium by cultivating the cells in Mo-limited and V-enriched medium, hydrogen production by this enzyme is increased (Kentemich *et al.* 1988). To impair hydrogenase activity, the cells have been grown in Ni-limited medium. Nickel has been shown to be necessary for the synthesis and activity of uptake hydrogenase (Oxelfelt *et al.* 1995). Providing inhibitors of terminal respiratory electron transport systems also blocks the hydrogen uptake activity (Burris 1979). Proper selection of the age of cyanobacterial cultures (Ohta & Mitsui 1981) and obtaining synchronous cultures (Suda *et al.* 1992) increased the hydrogen production.

Dawar *et al.* (1999) have recently reported that the heterocyst frequency can be increased by increasing the magnesium and calcium concentration in the medium and thereby increasing hydrogen production. Also addition of fructose enhanced the heterocyst frequency and increased hydrogen production (Dawar *et al.* 1999; Reedy *et al.* 1996).

IX. Genetic engineering

Genetic engineering has become possible with the establishment of molecular biological tools and techniques for cyanobacteria (Thiel 1994). Efforts are being made to inactivate the hydrogenases in diazotrophic cyanobacteria, which might lead to mutants which are unable to recycle the nitrogenase-formed hydrogen (Happe *et al.* 2000; Lindblad *et al.* 1998). Also mutant varieties of existing organisms are being produced for enhanced hydrogen production (Tsygankov *et al.* 1998).

X. Isolation of new cyanobacterial cultures

In view of the versatility of the cyanobacteria and their ability to survive under diversified environmental conditions, new strains needs to be studied from such locations. New reports are being made about the isolation of organisms from various places (Nagarkar 1998; Shah *et al.* 2000a in press).

Future aspects

With the existing cultures and knowledge of bioengineering, approximately 63 g dry weight of cyanobacterial cell mass (producing 1 μl H_2/mg dry weight/h) is required to produce hydrogen that will give energy equivalent to 1 Kg of lignite, or 92 g dry weight cell mass is required to produce hydrogen that can substitute for energy obtained on combustion of 1 Kg of charcoal. Block & Melody (1992) have projected the costs of \$25/m^3 for the photobiological production of hydrogen that is far superior to that of \$170/m^3 for a photovoltaic system.

But still we are still far from fulfilling the wishes and dreams of using cyanobacteria as a future energy source. The solutions to the problem delineated should be looked into. New cultures need to be screened for better stabilized hydrogen-producing strains. Also, the cultures in various culture collection centres also need to be examined with respect to their biohydrogen production. To date, an ideal organism with continuous production and the optimal conditions for maximum production has not yet been identified. The technical and economic prospects involved in immobilization of microbes or enzymes should be solved. The most promising mode at present is the use of molecular biology and genetic engineering technology for construction of a biological system to produce molecular hydrogen. More oxygen-resistant enzymes should be constructed. McTavish *et al.* (1995) have reported a oxygen-insensitive hydrogenase obtained by substituting cysteine with serine in the nitrogenase small subunit using site-directed mutagenesis. Also an acetylene-resistant nitrogenase has been isolated and characterized (Christiansen *et al.* 2000).

Scientists working around the world in Japan, America, Europe and Asia are understanding, manipulating and applying the mechanism of photohydrogen production. But a need has arisen for a concentrated effort throughout the globe to further understand the hydrogen metabolism, the enzymes involved and the genes responsible for the components of this system. Only this can help us in realizing the goal of using the light-dependent production of molecular hydrogen by cyanobacteria.

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