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III- PHOTOPRODUCTION OF HYDROGEN: MECHANISM AND PROBLEMS

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Abstract

Fossil fuels as energy sources are coming, sooner or later, to end. Hence, trials to find c alternatives including chemical, photochemical and biological production of hydrogen are continually running but each of them has its problems. Hydrogen gas as a fuel is extremely fascinating not least because it is absolutely clean with water being the sole combustion product. Also, it produces the highest energy output compared with other fuels. The capability to photoevolve hydrogen has been recorded in several photosynthetic bacteria, blue green algae and algae. The basic requirements for biological hydrogen evolution can be summarized, in general terms, into an electron source and an enzyme system. Photosynthesis, respiration and fermentation have been proved to supplement electrons for proton reduction.

Almost 60 years ago, it was first observed that the anaerobically adapted unicellular alga *Scendesmus* is capable of evolving H_2 gas in a photochemical reaction. Since that time, algae and blue green algae are used in experiments to produce hydrogen gas on a large scale.

The major problem in the photoproduction of hydrogen is the sensitivity of the catalysing enzymes (nitrogenases and hydrogenases) to oxygen. Another problem is that the evolved hydrogen comes out in a mixture with other gases such as nitrogen, oxygen and carbon dioxide.

Introduction

Photosynthesis, respiration and fermentation have been proved to supplement electrons for proton reduction Photosystem II, in particular, catalyzes water oxidation very efficiently, very simply and absolutely cost-less: it needs only water and sunlight. The amount at oxygen thus produced *via* photosynthesis is sufficient to compensate for all the global consumption of this gas and subsequently its atmospheric concentration is sustained constant allover history. The accompanying electrons should suffice, on a theoretical basis, the production of hydrogen (following proton reduction) in a volume double that of oxygen, which can be imagined, then, as a promising renewable energy. However, oxygen diffuses freely out of cells into the atmosphere while protons and electrons can not instead they are consumed in cellular activities such as NADPH synthesis, CO_2 fixation, nitrate-and nitrite reduction... etc. Protons represent another electron sink to form molecular hydrogen that can be either evolved as a gas or re-oxidized yielding ATP molecules *via* the activity of hydrogenase(s). Almost 60 years ago. Gaffron (1940), Gaffron and Rubin (1942) first observed that the anaerobically adapted unicellular alga *Scendesmus* is capable of evolving H_2 gas in a photochemical reaction.

The amount of solar energy at disposal on earth is gigantic accounting to about 14×10^{23} J (after excluding both reflection by clouds and gases of the atmosphere and the long-wavelength low-energy radiations). Only one type of organism -the photoautotrophes- is capable of converting this abiotic energy into biomass. Three to four billion years ago, the blue green algae invented the outstanding process of light-driven oxidation of water. Before the invention of water oxidation process, life depended on

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electrons from inorganic salts. Therefore, Greenbaum (1988) considered the photosynthetic oxidation of water as "the ultimate triumph of biology over an inorganic world". The extremely stable water molecule needs electric current or high temperatures of about 2000 °C or more to be technically oxidized. Photosynthetic organisms, however, perform oxygenic photosynthesis under physiological conditions at room temperature utilizing sunlight the miracle of the "oxidation" of "water" can be attributed to two major factors being present at the time:

1. The oxidation process happened in a reducing atmosphere constituted of nitrogen, carbon dioxide with possibly some hydrogen, no oxygen was present.
2. The photosynthetic oxidation of water by principle requires catalytic amounts of oxygen. In absolutely anaerobic atmosphere the oxygen-evolving complex (OEC) does not operate (Bader and Schmid, 2000). Photolytic reactions by e.g. UV-light might have played the role to generate the necessary traces of oxygen.

Mechanism of hydrogen evolution:

The basic requirements for hydrogen evolution can be simplified into an electron source and an enzyme system. As a general characteristic of the two enzyme systems (nitrogenases and hydrogenases) catalyzing photoproduction of H_2 is their sensitivity to oxygen.

A: Enzyme Systems

1. **Nitrogenase:** A side reaction of nitrogenase is the evolution of hydrogen, as an inherent property of the enzyme mechanism, according to either reaction of the following:

A): In the presence of nitrogen

$$N_2 + 8\ H^+ + 8\ Fd_{red} + 16\ Mg\text{-}ATP \rightarrow 2\ NH_3 + H_2 + 8\ Fd_{ox} + 16\ Mg\text{-}ADP + 16\ P_i$$

B): In the absence of nitrogen

$$2\ H^+ + 2Fd_{red} + 4Mg\text{-}ATP \rightarrow H_2 + 2\ Fd_{ox} + 4\ Mg\text{-}ADP + 4\ P_i$$

2. **Hydrogenases:** Hydrogenases are a heterogenous group of enzymes now known to be widespread in prokaryotes and eukaryotes catalyzing consumption or evolution of hydrogen and thus they are subdivided into "uptake" and "bi-directional" hydrogenases. Uptake hydrogenases are Nihydrogenases that possess a Ni catalytic centre and at least two [4Fe-4S] cluster. They are membrane bound enzymes re-oxidize hydrogen molecules and feeding electrons thus produced into the electron transport chain *via* the quinone pool of the thylakoid membrane which finally donated to O_2 in the dark (Boichenko and Hoffman, 1994). This reaction is sensitive to CO and CN^- and is coupled to oxidative phosphorylation (Eisbrenner and Bothe 1979). Fe-hydrogenases are bidirectional (with one exception, Boichenko and Hoffmann, 1994) catalyzing both H_2 oxidation and proton reduction. These are monomeric soluble enzymes contain a catalytic site center termed the H-cluster and two-four [4Fe-4S] or Fe-clusters which transfer electrons between the H-cluster and ferredoxin. Reversible hydrogenase(s) however, catalyze hydrogen metabolism in both directions i.e. oxidation (the same as the uptake enzyme) or proton reduction

(H_2 evolution). The structural gene sets coding for hydrogenase are *NifD*, *fdxN*, *hupL* and *hox* gene sets.

B. Electron sources:

Photosynthesis, respiration and fermentation act as possible sources of electrons for proton reduction into molecular hydrogen. Artificial electron donors (e.g. methyl viologen or reduced DCPIP) efficiently supplement electrons also for H_2 evolution through saturation of redox components under non-photosynthetic conditions (e.g. inhibited PS II). With respect to hydrogen production being finally mediated by ferrodoxin, it is generally accepted that PS I and PS II function separately The close interaction of hydrogenases with photochemical centers is evidenced by kinetic analysis of H_2 photoproduction under a short flash excitation. The halftime of 25 ms was recorded earlier (Efimtsev *et al.*, 1976) or later as 6 ms (Unpublished, cf., Boichenko and Hoffmann, 1994).

PS I: Specific inhibitors, mutants lacking some pigment protein complexes and monochromatic radiation confirm the independence of PS I to evolve hydrogen with a very high maximum quantum efficiency. The immediate source of electrons for PS I-driven H_2 photoproduction is a pool of reduced carriers between the two photosystems, mainly plastoquinone molecules. These probably belong to cyt b_6/f/plastocyanin and plastoquinone subpools or two fractions of PS I complexes. Depending on irradience, the PS I donor pool is competitively re-reduced by an electron flow from fermentative metabolism *via* the NADH-plastoquinone oxidoreductase and chloroplastic succinate dehydrogenase or by the PS II-driven electron flux from the water oxidizing system. Although some PS I-deficient mutants have been reported to be capable of evolving molecular hydrogen (Greenbaum *et al.*, 1995, careful examination of these mutants revealed that they are PS I-contaminated (Boichenko and Bader, 1998). True PS I-acking mutants of *Chlamydomonas* with the normal dark hydrogenase activity were incapable of H_2 photoreduction.

PS II: Unless competed with other electron acceptors (CO_2, NO_2, NO_3) a prolonged steady-state rate of H_2 photoproduction is sustained mainly by the electron flux from water-splitting reactions of PS II. In *Oscillatoria chalybea* however, no decrease in the light-induced hydrogen evolution (m/e=2) was recorded with the light induction of the dark-inactive Calvin cycle (Abdel-Basset and Bader, 1998) Thus, carbon dioxide and protons do not compete for photosynthetic electrons as it has been proposed previously by Greenbaum *et al.* (1995). Beside the long-range electron flow from PS II to hydrogenase *via* PS I, there is a possibility of a short circuit in PS II-driven electron flow immediately to potential acceptors via photoreduced pheophytin. There is doubt, however, on the idea of a direct participation of PS TI in photoreduction of the ferredoxin-hydrogenase system.

The main advantage of using PS TI particles *in vitro* experiments is the exclusion of electron sinks and economize electrons for only one purpose (H_2 gas evolution, in this context).

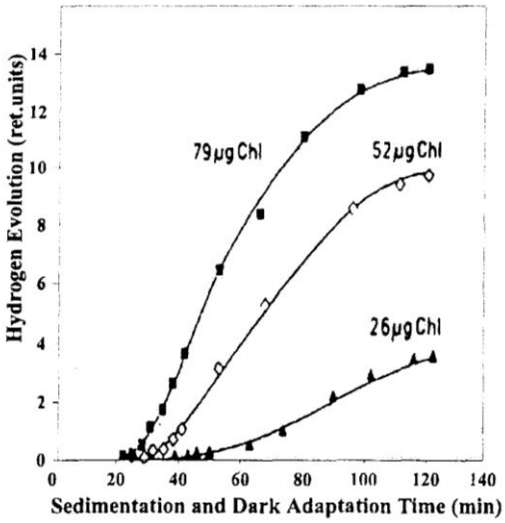

Fig. 1: Effect of time on the mass spectrometrically recorded hydrogen gas exchange in *Oscillatoria chalybea*. Independence of the respective chlorophyll concentration, the maximum signals were observed only after 2 h (Abdel-Basset and Bader, 1999).

Condition of hydrogen evolution:

Since hydrogenases are very sensitive to oxygen, strict anaerobic conditions are a prerequisite. Furthermore, a prolonged adaptation period of anaerobiosis is also important for the cells to photoevolve hydrogen in its maximum rates. By means of mass spectrometry, Abdel-Basset *et al.* (1999) analyzed in detail, the time-dependent increase (TDI) during the anaerobic dark adaptation period in *Oscillatoria chalybea* (Fig. 1) since the amplitude of hydrogen signal was found to increase with the lapse of time up to *Ca* 2h. These included the settlement of cells on the membrane of the cell of the mass spectrometer, transition from aerobic conditions into anaerobiosis, time course and the duration of anaerobic conditions. Neither sedimentation nor anaerobiosis *per se* can he responsible for the elevating the amplitudes of hydrogen signals by extended adaptation periods. Although sedimentation and anaerobiosis are prerequisites of hydrogen evolution, they can be ruled out from signal maximization since the cells are actually subjected for 45 mm to vacuum of about 10^{-6} mbar under dark conditions along with continuous flushing of nitrogen. These are assumed to be more than sufficient for the cells to sediment and the anaerobic conditions to be installed. Rather the activation or the *de novo* synthesis of hydrogenases during extended adaptation period might be the effective factors. Hydrogen evolution by *A. variables* was dependent on the degree of vacuum while decreased by addition of N_2. In this case, Markov *et al.* (1993) observed that vacuum degassing leads to partial removal of molecular nitrogen and creates conditions. which are favorable for nitrogenase-catalyzed hydrogen production. On the long run. however, hydrogen content has been recorded to decrease due to tile development of an

uptake hydrogenase activity. The lack of CO_2 and N_2 lowered the steady state rates of hydrogen evolution, due to inhibition of photosynthesis and nitrogen fixation. Thus, a two-stage hydrogen production system is suggested. In the first stage, the blue green algal cells consume CO_2 from the gas phase and synthesize the products that will be used for high rates of H_2 in the second phase.

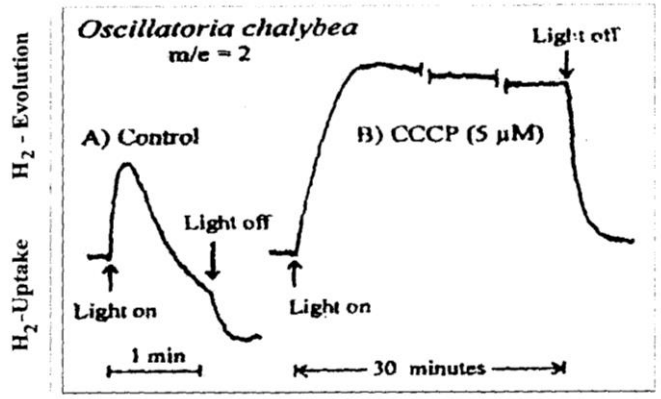

Fig. 2: Mass spectrometric recording (m/e=2) of the photoevolution of molecular hydrogen with whole filaments from the non-heterocystous cyanobacterium *Oscillatoria chalybea*. (A): Control measurment during 1 min without additions. The initial evolution rate corresponds to 9 *p*M molecular hydrogen. (B): experiment in the presence of the protonophore carbonyl cyanide *m*-chlorophenyllydrazone (CCCP) (5 µM). Note that following the addition of CCCP, the hydrogen gas exchange consisted exclusively of an evolution and that the evolution rate of 4 µM (mg Chl)$^{-1}$h^{-1} was stable for 30 min or more (Abdel-Basset *et al.*, 1998).

Oscillatoria chalybea, in particular, is a very active cyanobacterium that exhibits a strong hydrogen signal after a period of anaerobic dark adaptation (Abdel-Basset and Bader, 1997). Hydrogen evolution however appeared unstable under standard conditions with the overall balance of the hydrogen gas exchange turning back to rather small reaction rates after minutes (Fig. 2a). Following the scrutinized screening of the effect of various electron transport effectors, the phase of H_2-oxidation could be completely inhibited (Abdel-Basset and Bader, 1998, Abdel-Basset *et al.*, 1998) by the application of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP). Under these conditions the steady state evolution of molecular hydrogen could be substantially enhanced and moreover, this evolution is stable for a long time (30-60 min or more) reaching a steady-state level (Fig 2b). In this case, the action of CCCP seems to be far from its properties both as an ADRY-reagent (Acceleration of the Deactivation Reactions of the water-splitting enzyme system Y; deactivating the higher S-state) and as un-coupler of photophosphorylation. Rather, it inhibits the photosynthetic electron transport at the level of cytochrome b_6/f complex and/or in the vicinity of the oxygen-evolving complex (compare also Barsky *et al.*, 1991). Later, it was discussed that CCCP catalyses a cyclic electron flow by the oxidation of cyt b_{559} (Samson and Fork 1992) or the membrane pool of plastoquinone (Samuilov and Kitashov 1996). Thus CCCP functions as a redox component that is oxidized and reduced by the respective component of the electron transport chain. Moreover, it has been suggested that CCCP might supply protons for

photosynthetic reactions (Samuilov *et al.*, 1995). Moreover, the artificial electron donor dichlorophenolindophenol (DCPIP/ascorbate) could induce photosystem I (PS I) to evolve H_2 gas without the participation of PS II (Fig. 3) which casts light upon the debatable role of PS II/PS I in hydrogen evolution (e.g. Greenbaum *et al.*, 1995).

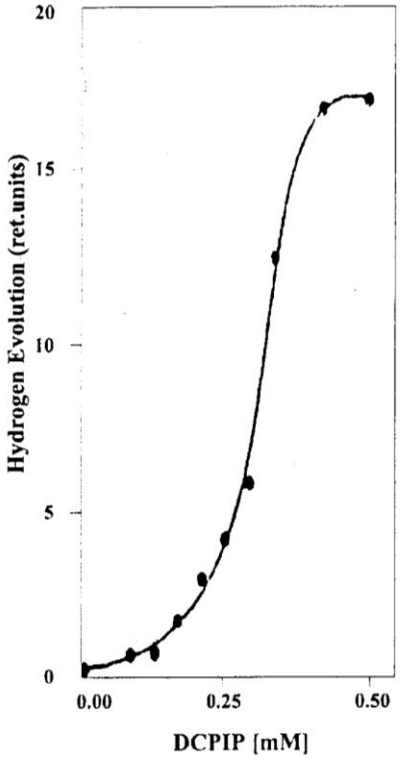

Fig. 3: Dependence of the photoevolution of molecular hydrogen on the concentration of reduced dichloro-phenol-indophenol (ascorbate) in the mass spectrometric assay with *Oscillatoria chalybea*. (Abdel-Basset *et al.*, 1998).

Distribution of hydrogen photoproducers

Many photosynthetic prokaryotes as well as eukaryotes are reported to exhibit the ability to evolve hydrogen. According to Boichenko and Hoffmann (1994), these include species from at least 50 genera of prokaryotes and 33 genera of eukaryotes.

Photosynthetic bacteria: Purple photosynthetic bacteria, which contain only one photosystem generate ATP *via* a cyclic electron flow and thus they are incapable of direct photochemical reduction of ferredoxin. In these species, fi_2 is ultimately a by-product of nitrogenase activity, which is largely accumulated reaching up to 25% of total soluble proteins under conditions of deprived nitrogen. However, a unidirectional uptake activity of hydrogenase negatively affects the rate of H_2 production. In green photosynthetic bacteria, however, H_2 evolution utilizes inorganic sulphur compounds as electron donors. Unlike purple bacteria, the photogenerated potential of reaction centers in green bacteria is sufficiently low for a direct reduction of ferredoxin.

Blue green algae: Blue green algae possess prokaryotic cellular organization but contain, same as eukarvotic algae and higher plants, two photosystems capable of oxidizing water and reducing ferredoxin. Many blue green algae display activity of a membrane-bound uptake hydrogenase and/or a soluble reversible hydrogenase. In unicellular blue green algae, the uptake hydrogenase is a thylakoid-bound enzyme, whereas the reversible hydrogenase is associated with cytoplasmic membranes and in filamentous blue green algae both of the enzymes occur in both heterocysts and vegetative cells.

Although several classes of microorganisms are actually hydrogen producers, blue green algae are the best candidates for hydrogen production purposes on a large scale because:

1. Blue green algae are photosynthetic prokatyotes lacking cell organelles like chloroplasts and mitochondria by principle so that all electron transport reactions have to be carried out (and regulated!) within the same thylakoid membrane system (Fig 4). The electron supply is substantially maintained under *in vivo* conditions by the interaction of various electron transport systems possibly sharing the same redox components. Respiratory processes are without any doubt involved in this reaction and in fact there is an impact of respiration inhibitors on the light-induced liberation of molecular hydrogen. In that sense all the electron-generating processes (photosynthetic and respiratory) constitute finally a common electron pool within the common membranous system. This fact *per se* implies the possibility to shift part of this electron pool for either activity depending on the targeted purpose, e.g. hydrogen production, in this case.
2. Blue green algae can be easily grown for a long time as immobilized cultures which are more hydrogen evolving than the flee living ones.
3. Blue green algae are highly adaptive to wide variations of environmental conditions and, subsequently, they can survive under extremely stressing conditions due to their evolutionary history.

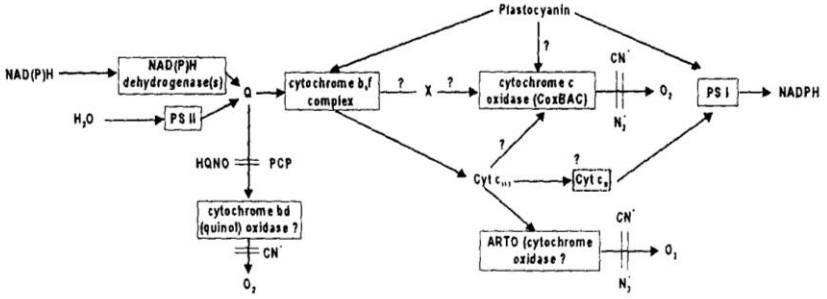

Fig. 4: Photosynthetic and respiratory electron transport sharing redox components in green algae.

Only some blue green algae are capable of hydrogen photoproduction under aerobic conditions in spite of oxygen sensitivity of nitrogenase (and hydrogenase), which suggests the existence of some protective mechanisms within the cells, which may be

promising for biotechnological applications. Significance of hydrogen metabolism in a particular cyanobacterium *Prochiorm* having chloroplast-like type of organization of thylakoid membranes and light-harvesting chlorophyll a/b-protein complexes are still lacking.

Algae: The ability for H_2 photoproduction has been recorded in 30 genera of green algae two species of yellow-green algae, and one species of diatoms and in only three primitive multicellular algae *Tribonema, Ulothrix* and *Volvox*. This ability was never observed in green, red, and brown macroalgae or in some unicellular algae as *Porphyridum cruentum, Euglena gracilis, Dunaliella salina* which belong to the hydrogenase-containing species. Several species of macroalgae, *osmmarium* and *Cryptomonas* exhibit only dark hydrogen production.

The maximum radiant energy saturated rate in the most powerful H_2 producers such as *Chlamydomonas reinhardti* and *Scenedesmus obliquus* under optimal conditions reaches 55.6-83.3 mmol (H_2) $Kg^{-1}(Chl)$ S^{-1}, that is close to maximum steady state rate of CO_2-dependent O_2 evolution in the first seconds of irradiation which then rapidly decayed to a sustained level. Steady state rates of H_2 photoproduction in green algae usually do not exceed 0.6-5.6 mmol $kg^{-1}(Chl)$ S^{-1}. The main reason for this low steady state rate can he stimulation of a competitive ferrodoxin-mediated cyclic electron flow around PSI since the turnover time of electron transfer from plastoquinone pool to the cytochrome b_6f complex is comparable to the turnover time of hydrogenase.

Physiological significance:

The functions of hydrogenase(s) in cell metabolism are altogether debatable. It is getting more complicated in non-heterocystous blue green algae where the enzyme exists in oxygen-evolving cells while all known hydrogenases are sensitive to oxygen (oxygen insensitive hydrogenases are scarcely reported and genetical studies are running to clone oxygen insensitive hydrogenases (e.g. Ghirardi *et al.*, 1998). In heterocysts, there is most probably very little opportunity for oxygen-related inhibition of hydrogenases. The question why such enzymes exist in green photosynthetic cells (seemingly with no physiological contribution and is subjected to oxygen inhibition) urges a molecular comparison between heterocystous- and green cell hydrogenases. Nevertheless) several postulations have been proposed for physiological importance of hydrogenase, some of them are:

* After prolonged periods of anaerobiosis, endogenous donors over reduce photosynthetic electron transport chain from fermentative metabolism. Moreover, the process is probably even activated by hydrogenase. The ATP level in cells of algae without hydrogenase (and some hydrogenase-containing algae) is lowered under these conditions. Simultaneously, anaerobic conditions inhibit the functioning of water-oxidizing system that sometimes cannot be removed by external oxidants. In this non-fundamental state, PS II possibly mediates a cyclic electron flow with the participation of cyt b-559 and reduced plastoquinone pool (via Q_a and Q_B). H_2 photoproduction in hydrogenase-containing algae promotes rapid reoxidation of carriers between the two photosystems and increases the ATP level due to coupled photophosphorylation and indirectly stimulates substrate level phosphorylation that triggers CO_2 fixation and the mechanism of positive feedback of evolving O2 on operate ion of water-splitting

system. Obviously this important function of hydrogenase/PSI couple gives to algae a selective ecological advantage to survive and grow under natural environment with occasional anaerobic conditions (for example, during a mass multiplication of accompanying heterotrophic microorganisms). In addition, the ability of reversible hydrogenase to derive reductants without participation of PS II by directing the available radiant energy into PS I-dependent cyclic phosphorylation could prove beneficial in light-limiting anaerobic environments. An unidentified phycobilisome-bound hydrogenase interacts with a protein kinase, regulating the distribution of excitation energy between the two photosystems. Therefore, some workers postulated that hydrogen metabolism simulates a safety valve at either direction (H_2 oxidation or proton reduction) depending on the energy status of the cell.

* There is an intriguing correlation between hydrogenase content in green algae and their enhanced growth and chlorophyll synthesis under unfavorable conditions.
* Hydrogenases cooperate with nitrogenase complex recycling the H_2 lost during the N_2-fixing cycle, and/or protecting the latter against oxygen inactivation by an oxyhydrogen reaction.
* An interaction exists between H_2-metabolizing enzymes and other oxidoreductases
* A light modulation of the dark fermentative H_2 production in greening mutants of *Chlorella* through competition of hydrogenase with NADPH-photochlorophyllide photoreductase and an unidentified photoreductase of Mg-protochlorophyrin for common electron donors
* Only blue green algae and algae are net energy producers from the viewpoint of H_2 photoproduction since at least 4 mol ATP per mol evolved H_2 are consumed in the reaction of blue green algal nitrogenase, but not in the hydrogenase-mediated reaction.

Photobioreactors:

Photobioreactors are sophisicated type of continuous culture designed to use solar energy for the production of molecular hydrogen coupled with the uptake of carbon dioxide. Figure (5) shows a schematic diagram of a photobioreactor for continuous production of H_2 by immobilized blue green algae on hydrophilic and hydrophobic hollow fibers (Markov *et al.*, 1993). Cell attachment to hydrophylic cellulosic hollow fibers was greater than to the hydrophobic polysulphone fibers. The two-phase photobioreactor was run continuously for a period of several months with a blue green algal suspension.

CO_2 uptake phase:

$CO_2 + H_2O$ photosynthetic products + O_2
Maximum CO_2 consumption rate = 150-170 ml g^{-1} dry wt h^{-1}

H_2 photoproduetoin phase:

Photosynthetic products $\rightarrow$ H_2
Maximum H_2 production rate = 20 ml g^{-1} dry wt h^{-1}

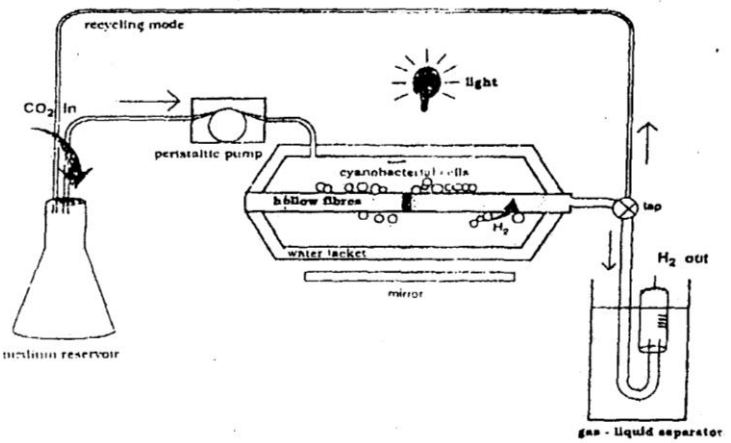

Fig. 5: Schematic diagram of hollow fiber photobioreactor for continuous production of hydrogen by immobilized cyanobacteria (Markov *et al.*, 1995).

In the CO_2 uptake phase, the cells take up CO_2 from the gas phase and synthesize the products that will subsequently be used for H_2 photoproduction in the H_2 production phase. Such two-stage systems of photosynthetic accumulation of starch followed by anaerobic dark fermentation with H_2 production in algae as well as in mixed cultures of algae and photosynthetic bacteria demonstrated a stable but rather moderate yield. Use of previously fixed carbon (carbohydrates) through the oxidative pentose phosphate pathway (which generates the reductant for nitrogenase and hence H_2 production) occurs in blue green algae with the release of CO_2. Improvement of this system is limited also by ATP - dependence of the dark H_2 production. Incorporation of a third stage with dark fermentation of the exhausted and dead algal cells by cellolytic bacterial association.

The addition of N_2 to this system is essential for long-term H_2 production because N_2 fixation is required to maintain cell metabolism. Also, it is significant to operate the photobioreactor at higher CO_2 concentrations than the normal level in air. (e.g. from industrial plants and thermal power stations exhaust gases which contain 10-20% CO_2). The immobilization materials (cellulosic fibers) are relatively cheap because they are made from waste products of the cotton industry. The problem of 02 sensitivity of algal hydrogenase can be overcome, at least to some extent, by screening for particular species or mutant strains with a more resistant enzyme, similarly as the problem of prolonged tolerance to anaerobiosis of the whole algal cells.

Incorporating heterocystous blue green algae that posses an active H_2 uptake system can be operated under a partial vacuum and with continuous flow of medium through the system, thus avoiding H_2 consumption.

The major problem for hydrogen evolution on a large scale is the sensitivity of hydrogenases (and nitrogenases) to oxygen urging to separate the oxygen evolution pathway from that of hydrogen which is a big dilemma in the concept of photosynthesis Another problem is that hydrogen evolved in a mixture of other gases such as nitrogen, oxygen and carbon dioxide.

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