Review Paper

STRATEGIES FOR ENHANCEMENT OF BIOLOGICAL HYDROGEN PRODUCTION

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ABSTRACT

Hydrogen gas with the highest gravimetric energy density of all known fuels produces energy without giving rise to the notorious carbon emissions that damage the environment. A promising next-generation non-conventional resource, hydrogen yields energy to the tune of about 122kJ/g. Biological processes for hydrogen production proffer distinct advantages such as clean and green energy that may be economically attractive. Among several known processes, two processes viz. direct and indirect biohydrogen production are reported in algae and cyanobacteria, respectively. In the direct process, photosystem II (PSII) dependent and PSII independent reaction cascades operate, out of which the PSII independent process appears to be more efficient and economical. Here, under anoxic conditions (induced by blockage of PSII) electrons derived from endogenous substrates such as proteins and carbohydrates are channelized via cytochrome b_{6f} to PSI. The absorbance of light by reactive center of PSI subsequently allows transfer of the electrons to ferrodoxin and then to an active hydrogenase enzyme on the outer face of the thylakoid membrane. Under oxygen limiting conditions the hydrogenase is active and utilizes the electrons to reduce protons that are released out of the thylakoid membrane during ATP (Adenosine Triphosphate Synthesis). Attractive as it may seem, recent breakthroughs in H_{2} photoproduction have been able to tap only about 15% of the theoretical maximum, suggesting room for substantial improvement in the energy yield. Urgent steps need to be taken in order to develop the technology for commercial scale production of biohydrogen. Certain approaches appear to be promising in this regard, viz. (i) reducing antenna size to minimize shading effect and to avoid loss of energy during transfer of photons from antenna to the reaction center with the overall effect of increasing conversion efficiency, (ii) enriching the quality of endogenous substrate that would enhance generation of electrons, (iii) enhancing the release of hydrogen ions entrapped in thylakoid membrane by using protonophores. Additionally in a fourth approach, efforts need to be taken for designing photobioreactors that will minimize loss of photons and ensure availability of specific wavelengths of light for hydrogen production. Experimental data pertaining to some of these approaches will be discussed in the presentation.

Key Words: Biohydrogen, Photosystem II, Protonophores, Photobioreactor, Hydrogenase, Adenosine Triphosphate Synthesis (ATP)

INTRODUCTION

Energy is vital to global prosperity, yet dependence on fossil fuels as our primary energy source contributes to global climate change, environmental degradation and health problems. Hydrogen (H_{2}) offers tremendous potential as a clean, renewable energy currency with the highest gravimetric energy density (122kJ/g) of any known fuel.1 It is compatible with electrochemical and combustion processes for energy conversion without producing carbon-based emissions that contribute to environmental pollution and climate change. H_{2} may be produced by a number of processes, including electrolysis of water, thermo-catalytic reformation of hydrogen-rich organic compounds and biological processes. At present 40% of H_{2} is produced from natural gas, 30% from heavy oils and naphtha, 18% from coal, 4% from electrolysis of water and 1% from biomass.2 However, these processes could turn out to be
expensive due to recurring cost of chemicals, non-renewability, requirement of inventory and energy intensive nature. Biological production of H2 (biohydrogen), using (micro)organisms, is an exciting new area of technology development that offers the potential for production of usable H2 from a variety of renewable resources. Different physiological processes operating in the biological systems can be utilized for biohydrogen production viz. i) direct photolysis of water by algae ii) indirect biophotolysis of water by cyanobacteria iii) photofermentation by photosynthetic bacteria and iv) dark fermentation by anaerobic fermentative bacteria. Among these methods, the direct and indirect photolysis methods are regarded as cleaner methods since the major product is H2 (90-95%) with traces (2-3%) of oxygen and carbon dioxide. On the other hand, other methods require higher inputs of carbohydrates and electron donors and produce high levels of carbon dioxide and other acids.34 In spite of some of the advantages of photolytic H2 production using micro-organisms, the conversion rates have not exceeded 15% of the theoretical maximum. There is a room for substantial improvement in the yield of the process in order to make it practicable on a commercial scale.

Since the first demonstration of biological H2 production by Scenedesmus obliquus, there have been numerous attempts to investigate the pathways of H2 production in algae and cyanobacteria. Attempts have been made in order to enhance the H2 yield using purified cultures of algae and cyanobacteria, viz. Chlamydomonas reinhardtii,5 Scenedesmus obliquus, Clorella pyranoisosa, Clamydomonas moewusi,6 Clamydobotrys stellata,7 Anabena cylindrica, Nostoc punctiforme, Platymonas subcordiformis8 and others. Most work has been carried out with Chlamydomonas reinhartii apparently on account of lower exacting requirement for growth as compared to cyanobacteria and the harmless metabolic byproduct.

AIMS AND OBJECTIVES
This paper presents an overview of algal biohydrogen production and strategies to improve the yield of this important energy source.

DISCUSSION
Photosynthesis and its relation to biohydrogen production
The production of H2 in plants, algae and cyanobacteria can be tracked down to the photosynthetic reactions. The organisms utilize sunlight to produce energy rich organic molecules from carbon dioxide and water. Six carbon dioxide molecules are reduced to form one hexose molecule, the hydrogen required for reduction being taken from water. Since water is a poor reducing agent, light energy is required to boost up the reducing potential. In green algae and higher plants, photosynthesis occurs in chloroplasts that enclose the stroma (liquid rich in metabolic enzymes). Thylakoids (membrane enclosed sacs stacked up to form grana) present in the stroma, harbour most of the components of the electron transport chain necessary for generation of reducing equivalents and proton motive force. To make the uphill task possible, the transport is stimulated at two points, viz. Photosystem I (PSI) and Photosystem II (PSII) by the absorption of light. In the light dependent reactions, water molecule in the lumen of thylakoid is split into protons, electrons, and oxygen atom by PSII. PSII consists of P680 reaction center in association with D1 and D2 proteins (water splitting) and the Mn-Ca cluster.9 Radiant energy from the sun is absorbed by the Light Harvesting Complex II (LHCII) containing chlorophyll a/b pigments and transferred to reaction centre P680. The redox potential of oxidized form of P680 is now sufficient to split water molecules. The excited electrons are transferred through the electron transport chain i.e. mobile electron carrier plastoquinone and membrane integral cytochrome b563-cytochrome f complex followed by copper containing mobile carrier plastocyanin to reduce PSI. During this process, protons formed during water splitting are transported across the inter-membrane space forming proton gradient for ATP synthesis (photophosphorylation). Light absorption a second time by the light harvesting complex of PSI and its subsequent transfer to reaction
center P700 elevates the redox potential of electrons to the redox equivalent of ferrodoxin. The electrons then transferred through ferrodoxin to NADP⁺ in the stroma generating NADPH + H⁺ which is subsequently used for CO₂ fixation (Fig. 1).

![Diagram of photosynthesis](image)


**Fig. 1**: Process of photosynthesis

The stroma of chloroplasts contains hydrogenase enzyme which is active under anoxic conditions. The enzyme is capable of associating protons with available electrons to generate hydrogen. The high level of protons leaking into stroma during photosynthesis offers the opportunity of utilizing hydrogenase enzyme to generate H₂ if sufficient level of anaerobiosis is maintained.

**Strategies for biological hydrogen production**

**Direct hydrogen production**

In oxygen limiting conditions, photosynthetic organisms stop carbohydrate synthesis and switch over to carbohydrate metabolism for generation of energy. This cellular respiration occurs in the mitochondria while photosynthetic processes are significantly reduced. The Fe-hydrogenase enzyme which is sensitive to oxygen and is inhibited by oxygen becomes active under oxygen deprived conditions. Catabolism of endogenous substrate and oxidative carbon metabolism generates electrons for photosynthetic apparatus. These electrons are directed towards cytochrome-b₆/f and transferred to PSI. In presence of light the electrons are excited and hence transferred to ferrodoxin. From here, electrons are transferred not to ferrodoxin reductase but to an active hydrogenase enzyme that acts as a competitor. Hydrogenase then donates the electrons to protons acting as terminal electron acceptors generating molecular hydrogen (Fig. 2).

The pool of protons in the stroma which was formed in aerobic phase by photolysis of water and ATP synthesis is thus utilized by hydrogenase for hydrogen production. In other words, blocking the PSII for a short duration in the dark (by inducing anoxic conditions) allows a direct transfer of electrons from endogenous substrates to PSI and availability of hydrogenase in the active form is responsible for photoproduction of hydrogen instead of the reducing power, i.e. NADPH + H⁺. This method, viz. direct photoproduction of H₂ has been employed for biohydrogen generation.
Oxygen deprived condition for hydrogen production has been achieved mainly by two methods. In the first, pre-grown culture of algae/cyanobacteria is transferred to a bioreactor with continuous argon or nitrogen bubbling in the dark for short duration. Due to argon or nitrogen, dissolved oxygen is removed, fermentative reaction are activated to maintain NAD/NADH balance and ATP supply. This condition triggers hydrogenase synthesis. When the culture is shifted to light, NADPH formation is inhibited and hydrogen is formed. However, an important concern in this method could be the cost of maintaining an argon environment.

In a variation of this method, a two-stage process is used. In this method, cells are grown in TAP medium with bubbling of carbon dioxide as a carbon source or by adding a carbonate to the medium. Under optimized growth conditions, the cells increase the internal starch and protein content in the first phase. The cell biomass is then exposed to anaerobic conditions in the dark for 24 hour. Further, the active PSII is partially blocked or its activity lowered by using PSII blocking agents such as 3- (3, 4-dichlorophenyl) - 1, 1 - dimethylurea (DCMU) or by using sulfur-deprived medium or copper containing medium responsible for reducing the PSII photolysis activity even in the presence of light (D1 protein blocking). A combination of methods involving blocking of PSII for reducing the oxygen level in the culture and providing dark condition for lowering photosynthetic activity has also been used for enhanced hydrogen production by increasing cellular respiration and endogenous substrate utilization. In an alternative method, a 24h dark incubation is followed by brief exposure of cells to light while maintaining anaerobic condition. The partially blocked PSII splits water in very low amounts resulting in a low amount of oxygen that is utilized by mitochondria for respiration. This ensures total anoxic conditions favorable for hydrogen production. There are concerns that a high rate of respiration which leads to utilization of endogenous substrate and consequent scarcity of NADPH coupled with the loss of chlorophyll pigment may cause cell death. Death of cells can be prevented by exposing them briefly to aerobic conditions.

The two stage method appears promising due to possibility of enhanced biohydrogen production of high purity.
Reduction of antenna size to minimize shading effect

Wild algal species have a tendency to assemble large arrays of light absorbing chlorophyll antenna molecules in their photosystems. This has been reported as a survival strategy that provides competitive advantage in the wild where light is often limiting. However, at higher solar intensities, the rate of photon absorption far exceeds the rate of photon utilization for photosynthesis. This imbalance often results in dissipation and loss of excess photons in the form of florescence or heat. This effect is detrimental to the yield and productivity of mass culture. Moreover, it has also been noted that if wild culture is maintained in culture bottles, due to excessive chlorophyll content, light cannot pass efficiently through two or three layer of algal culture. Thus, inner layers of cells masked away from light due to cells at the exposed surface of culture bottle. This shading effect causes unequal distribution of sunlight, with surface cells getting maximal sunlight and inner cells being deprived of the light.  

![Diagram of sunlight and chlorophyll pigments](image)

**Fig. 3**: Shading effects of cells due to large chlorophyll antenna

Shading effect reduces biomass production with consequent reduction in the yield of hydrogen produced. Enhanced photon conversion efficiency is necessary for building up the endogenous substrate levels for higher and sustained production of hydrogen. It has been observed that truncated or smaller chlorophyll -antenna (with reduced chlorophyll concentration) minimized the excessive absorption and wasteful dissipation of energy. Higher H₂ output has been achieved with high starch accumulation during aerobic phase, which is used up during respiration phase. Recently, a mutant species of *C. reinhardtii* (CC4169) with truncated light harvesting antenna showed increased photosynthetic capacity and higher photon conversion efficiency at high light intensities of particular importance is the fact that, not only was the H₂ production higher, but the evolution of the gas occurred for longer duration of time as compared to the parental strain CC-425.  

Another mutant L159I-N230Y that has low chlorophyll content showed higher production of H₂ (98-99%) with levels of contaminants (0.5%-1% CO₂). Lower amount of CO₂ signifies involvement of PSII which is protected by zeaxanthin (formed from violaxanthin) from rapid degradation leading to prolonged PSII based hydrogen
production. The authors opine that low chlorophyll content in the mutants allow such strains to be grown under conditions of higher light intensities for higher biomass generation. A number of approaches have been enlisted for the development of truncated antennae, viz. (i) exposing the cells to continuous irradiance stress with manipulation of medium, (ii) DNA insertional mutagenesis and other genetic manipulations and (iii) use of mutant algae. According to available literature, these approaches may contribute in a big way for commercial H₂ production.

Enriching the quality of endogenous substrate

Although hydrogen producing organisms are the ones that do not require exogenous organic carbon sources, it has been recognized that the quality of endogenous carbon sources can be altered by addition of certain organic carbon supplements to the growth medium. For a two stage system, presence of sufficient levels of starch enhances the hydrogen production. It has been reported that growth of autotrophic algae in TAP (Tri-Acetate Phosphate) medium in the presence of added carbon source results in higher growth of the cells and accumulation of the carbon in the cells.

Addition of extraneous carbon and nitrogen sources such as glucose, NaHCO₃ at different stages of growth has reportedly enhanced the hydrogen yield. The method essentially consists of an initial growth stage in which algal culture is grown aerobically in presence of sunlight, bicarbonates gaseous or non-gaseous form) and nitrogen source. Once the biomass has built up, glucose is added in the second stage. Glucose is rapidly consumed in this heterotrophic stage since respiration rate is higher than in photosynthesis. Biohydrogen evolution has been to be enhanced by 30 to 50% in a two stage process with the addition of acetate and glucose in the second stage.

Use of protonophores for enhanced release of protons from thylakoid membrane

As described earlier, PSII carries out two important roles for photosynthetic organisms, one is transfer of electrons generated by photolysis of water through ETC (Electron Transport Chain) and the other is generation of a proton gradient across the thylakoid membrane. Protons accumulating in the lumen of thylakoids are drained into the stroma of chloroplast with production of ATP. Partial blocking of PSII lowers the oxygen level and increases respiration as well as induces the production of hydrogenase enzyme which subsequently uses protons to produces H₂.

Transfer of protons from thylakoid lumen into stroma is depend upon the activity of ATP synthase. H₂ production can be enhanced by increasing the release of protons in the stroma. This can be achieved by using uncouplers or protonophores that disrupt the proton motive force and cause a rapid leakage of protons across the thylakoid membrane. The released protons may be then used by hydrogenase enzyme to produce larger amounts of H₂, however, at the expense of proton motive force which is lost. Protonophores could be particularly useful in the two step hydrogen production method described earlier. Here, PSII is required to be partially blocked for maintaining anoxic conditions. As mentioned earlier DCMU or sulphur deprived conditions have been used. Use of protonophores which are also called ADRY agent (agent accelerating the deactivation reactions of water-splitting enzyme system Y) would rapidly inhibit PSII activity of cells, resulting in a marked decline in the coupled oxygen evolution. However, since DCMU irreversibly inhibits PSII, protonophores which are reversible inhibitors could be more advantageous for create anoxic conditions. Some protonophores that can be used for biohydrogen production are ANT-2p [2-(3-chloro-4-trifluoromethyl) anilino-3,5-dinitrothiophene], CCCP [carbonylcyanidem-chloro-phenylhydrazone], FCCP [carbonyl-cyanide p-trifluoro – ethoxyphenyl-hydrazone] and acridones (9-azaanthracene-10-ones). Most research work have been carried out using CCCP and FCCP and the researchers have demonstrated higher H₂ production (243-fold) in the marine alga Platymonas subcordiformis. In one interesting report, cultures of P. subcordiformis were rejuvenated by CCCP (Carbonyl Cyanide m-Chlorophenyl Hydrazone) removal and replenishing fresh micronutrients for repeated hydrogen
Five cycles of carbon dioxide fixation (photosynthesis) and CCCP-regulated hydrogen photoproduction were successfully established with an average yield of $46.2 \pm 2.8 \text{ ml } H_2/\text{l}$. Demerits of using excess dosages of protonophores are the inhibition of mitochondrial oxidative respiration and blocking of PSII.

**Designing photobioreactor for enhanced biohydrogen production**

In natural conditions of growth of algal biomass is limited on account of several environmental factors. It has been observed that factors that limit growth of algae are also responsible for lower hydrogen yields. Hence, controlling the growth environment of algal cultures assumes importance in order to ensure high biomass yields. Optimization of growth for biohydrogen production by photosynthetic organisms can be achieved by using a photobioreactor - a device in to a fermenter with provision for providing appropriate lighting conditions. The design of a typical photobioreactor ensures that appropriate light intensity and wavelength, media components and hydrogen collection systems are provided to the growing cells. Moreover, a photobioreactor offers maximal surface area to the cells so that they are properly exposed to light and shading effects are minimized. The depth of photonic zone depends upon the reactor dimensions, operational parameters, algal concentration and the specific absorption coefficient of the algae. According to published literature, tubular and flat bed photo bioreactors have shown high photochemical efficiency with a high biomass density and net yield of biomass. The schematic representation of a flat bed photobioreactor (Fig. 4) and a tubular photobioreactor (Fig. 5) show that a large surface area is available for growth of algal cells and for light penetration. A typical flat bed bioreactor is a rectangular transparent box with width of only 1-5 cm and height of 1m with an inlet for sparging gas and an outlet for collecting hydrogen. Tubular photobioreactors are typically 10-100m long cylinders with 3-6 cm diameter.

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**Fig. 4 : Flat bed Photobioreactor**

It is important to note that the wavelength of light provided assumes importance in the operational design of a photobioreactor. Algal cultures differ in their requirements with respect to the incident light depending on the number and types of light absorbing pigments in the cells. Active centers of PSI and PSII which consist of light absorbing chlorophylls need specific radiations for activation. Since green algae have chlorophyll a, chlorophyll b and β-carotene as the light harvesting pigments present, the absorption spectra for these organisms are in the range of 400-500 nm and 620-680 nm. It has been observed that if high energy photons are absorbed from incident radiation (400-500 nm or 500-630 nm) the reaction centers need to lose the excess energy as heat before the light can be used in photosynthetic reactions that require 680-700 nm light. This mechanism of heat dissipation prevents damage to the light harvesting complexes that are sensitive to high energy radiation. The use of Light Emitting Diodes
(LEDs) having specific wavelengths could have a bright future in photobioreactor technology due to the fact that these light sources demand lesser energy and are capable of providing optimized wavelengths of light in the optimal dosage. Use of traditional monochromatic filters for selection of appropriate component of natural sunlight is also thought to have a bright future in the photobioreactor technology. According to some reports, cyclic variations in the wavelength of incident light improved the biomass yield of certain species of algae. In spite of latest developments in biohydrogen production, the best reactor design is still a distant dream. It needs to be mentioned that presently used designs of photobioreactors cannot prevent the loss of photons by reflection or heat dissipation (Fig. 6). Slight alterations in the design might be necessary to prevent such loss of radiation. Closed photoreactors with provision to prevent photon loss by reflection may be investigated for enhanced biomass production and hydrogen generation. Fig. 7 is a conceptual design of a closed photobioreactor and shows that specially modified surfaces may enhance biohydrogen yield.31,32

![Fig. 6](image_url)  Some part of light is absorbed and some is lost in open bioreactor

![Fig. 7](image_url)  Closed flat bed photobioreactor

**CONCLUSION**

A judicious use of appropriate combination/s of above mentioned strategies, viz. use of algal culture having truncated light harvesting antenna, effective media supplements, protonophores and photobioreactor design will increase H₂ production. The authors believe that research in these areas will reduce and probably remove our dependence on the fossil fuels.

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