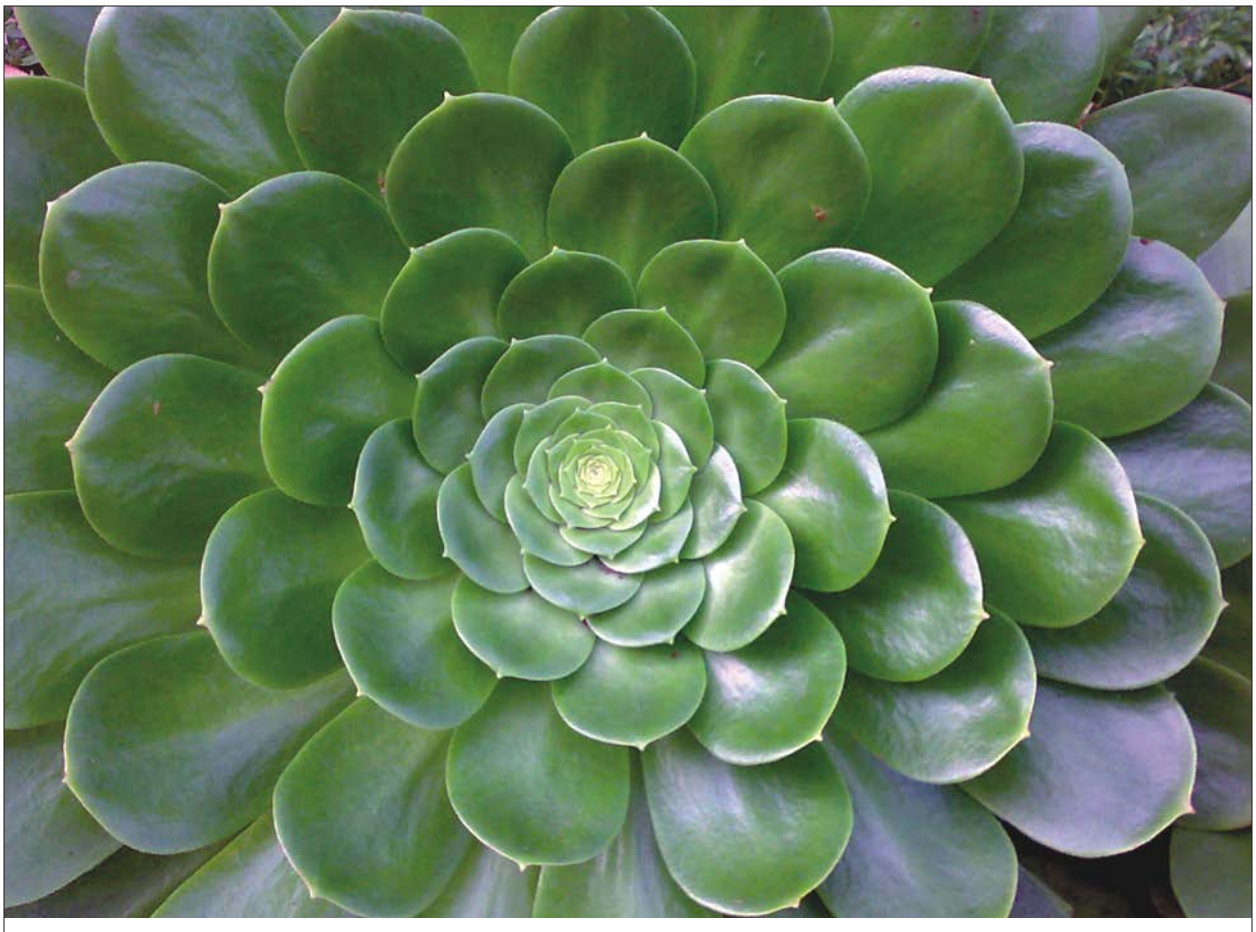

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Hydrogen Generation from Algae: A Review

[1]K. Vijayaraghavan, [1]R. Karthik and [2]S.P. Kamala Nalini
[1]Biotechnology Research Division,
Prathyusha Institute of Technology and Management,
Department of Biotechnology, Thiruvallur District, Tamil Nadu, India
[2]Department of Biotechnology, D.G. Vaishnav College, 833,
EVR Periyar Highroad, Arumbakkam, Chennai-600106, Tamil Nadu, India

Abstract: The study focuses on the research involved in generating hydrogen using algae as a renewable energy resource. Due to the decline in fossil fuel resource, the energy derived from biomass seems to be the only major source of world's renewable energy. The hydrogen derived from algae is promising due to its sustainability, no green house gases emission during the combustion of hydrogen and security of its supply even at remote places. The novel approach of generating hydrogen at commercial scale from algae has been a curiosity among many researchers till today. This review study updates the research involved in hydrogen generation from algae based on light intensity and its photoperiod, nitrogen and sulfur content, fermentative metabolism and symbiosis. The following algal species had been widely investigated for hydrogen production namely: *Chlamydomonas*, *Anabaena*, *Chorella*, *Oscillatoria*, *Scenedesmus* and their mutant. The generation of hydrogen from algae is still at research level. Hence, this review would be an eye opener for researchers who are interested in generating hydrogen from algae.

Key words: Algae, hydrogen, phototrophic, *Chlamydomonas* sp., *Anabaena* sp., *Chorella* sp., *Oscillatoria* sp., *Scenedesmus* sp.

INTRODUCTION

The renewable technology has garnered great importance due to high raise in oil price and global warming. The energy derived from biofuels especially algae in receiving more and more attraction in recent years. Algae can grow in places even where no agricultural activities can be carried out. Researchers have found out that the metabolic switch in algae allows the primitive plants to produce hydrogen gas. Hydrogen can be used as a clean burning fuel in cars and power plants, which is virtually limitless in availability, because it is part of the water molecule. Moreover, it is one of the probable candidates to become the world's primary fuel in coming decades. But until now, it was obtainable in quantity only through relatively expensive extraction procedures involving the electrolysis of water or processing natural gas.

Photobiological production of hydrogen gas can be achieved by green algae and cyanobacteria in the presence of hydrogenase enzyme using water as the only electron donor. The hydrogenases can be either of [FeFe] or [NiFe] enzymes which are

Corresponding Author: S.P. Kamala Nalini, Department of Biotechnology, D.G. Vaishnav College, 833, EVR Periyar Highroad, Arumbakkam, Chennai-600106, Tamil Nadu, India Tel: 91-44-24756655, 24754349, 24753008

phylogenitically distinct but perform the same catalytic reaction symptomatic of convergent evolution, which was due to the presence of different metallo-cluster in the two classes of hydrogenases.

Discovery of hydrogen from algae dates back to 1939 when a German researchers named Hans Gaffron identified the switching over capability of algae between oxygen and hydrogen production. In 1997, Prof. Anastasios Melis found out that sulfur deprived algae switched from producing oxygen to hydrogen in the presence of hydrogenase enzyme. During 2006 genetically modified *Chalamydomonas reinhardtii* named as *stm6* exhibited the capability of producing hydrogen five times more than the wild strain on volume basis. Subsequently in 2007 Anastasios Melis investigated the efficiency of solar to chemical energy conversion using *tla1*, a mutant variety of *Chalamydomonas reinhardtii.* The findings showed that by truncating a chlorophyll antenna size the wasteful dissipation of sunlight by individual cells were minimized resulting in 15% more efficiency in solar to chemical energy conversion. In 2007, the algal switching over from oxygen to hydrogen production was investigated by the addition of copper (Wikipedia, 2010; Melis and Happe, 2001; Ghirardi *et al*., 2000).

FACTORS AFFECTING HYDROGEN EVOLUATION FROM ALAGE LIGHT

The investigation on hydrogen metabolism in algae showed that the algae utilized hydrogen in dark when subjected to anaerobic incubation (Gaffron, 1940). Moreover, the algae which were capable of consuming hydrogen exhibited the capability of hydrogen evolution in dark. The capability to include hydrogen in algal metabolism appeared after anaerobic adaptation, but was lost in the presence of small quantity of oxygen (Kosourov *et al*., 2007). The hydrogenase was detected based on the hydrogen uptake and evolutions by correlating with the related reactions in the intact algae. The successes of this measurement are limited due to extreme sensitivity of enzyme to oxygen.

Hydrogen evolution from algae can be achieved by two different pathways: (1) using either hydrogen or carbohydrate as electron donor (Adams *et al*., 1981) and (2) direct coupling between photosynthetic activity of oxygen and hydrogen evolving mechanism which occur during the initial period of light exposure (Bishop *et al*., 1977; Greenbaum, 1982; Pow and Krasna, 1979). The cellular carbon provides the reducing equivalent for hydrogen evolution, while H^+ served as electron acceptors there by heading to a simple redox reaction. The removal of excess internal reducing power should be present in the redox reaction pathway, as it is essential especially under lower oxygen tension in anaerobic bacteria (Adams *et al*., 1981; Klein and Betz, 1978a; Vinayakumar and Kessler, 1975).

The overall gas exchange reaction which occurred in hydrogenase containing algae is as follows. The light dependent reaction consisted of photosystem (II), photoreduction and hydrogen photoproduction. The dark enzymatic reactions consisted of respiration, dark hydrogen production, oxygen hydrogen reduction and hydrogenase. In the absence of phosphorylation due to the presence of inhibitors, the energy requiring reactions like photosynthesis, photoreduction and hydrogenase comes to an end. While the light dependent evolution of hydrogen based on photohydrogen production becomes prominent and long lasting (Stuart, 1971; Stuart and Kaltwasser, 1970).

Hydrogen evolution by the chlorophyllous algae during light period was due to the electron transport through the photosystem associated with hydrogenase. Moreover, the chlorophyllous algae also produced hydrogen at dark period with a lower rate. Two mechanisms had been proposed to account for hydrogen release in light. The first method is based on the photolysis of water coupled with electron transport through photosystem

(II) and photosystem (I). The metabolic pathway resulted in simultaneous production of hydrogen and oxygen with a molar ratio of 2 and exhibited inhibition when exposure to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Bishop *et al*., 1977; Pow and Krasna, 1979; Spruit, 1958). The second mechanism is based on the oxidative carbon metabolism and photosystem (I) which characterized by the release of hydrogen and carbon dioxide and was insensitive to DCMU (Bishop, 1966; Bishop *et al*., 1977; Gaffron and Rubin, 1942; Frenkel, 1952; Healey, 1970a, b). The DCMU sensitive pathway are well documented but only limited information is available regarding the reactions involved in oxidation of organic compound in the generation of carbon dioxide and the reductant for the evolution of hydrogen in darkness and light. *Cyanobacterial* heterocysts when grown under a lower partial pressure of nitrogen, the sugar supplied by the vegetative cell served as a reductant, while the proton was utilized during the nitrogenase reaction to generate hydrogen (Benemann and Weare, 1974; Hall *et al*., 1995).

Electron transport for hydrogen generation through the algae occurs in the following sequence. Initially energy is derived from the sunlight by the algae for extracting electron from water molecules through the photosystem (II). The potential energy of electron increased in the photosystem (II) and subsequently in photosystem (I) due to the sequential light driven reaction. Thereby the electrons released during oxidation of water are transported to the Fe-S (protein ferridoxin) in photosystem (I). The reversible hydrogenase reaction $2H^{+}+2Fd^{-} \leftrightarrow H_2+2Fd$ accept electron from the reduced ferridoxin and transfer them to the $2H^{+}$ for generating hydrogen molecule. On theoretical basis the biophotolysis can yield hydrogen to oxygen ratio of 2:1 on mol basis. In practice under ambient condition it is difficult to arrive at this ratio as the hydrogenase reaction is extremely sensitive to oxygen and moreover the hydrogenase will be deactivated at a partial pressure of 2% oxygen (Ghirardi *et al*., 1997). ATP generation occurs in the thylakoid membrane due to the electron transport reaction in the hydrogenase pathway and photosynthetic phosphorylation (Arnon *et al*., 1961). The ATP plays a major role in the maintenance and repair of cell (Melis, 1991).

During biophotolysis by heterocystous blue green algae a minimal inhibition on hydrogen production occurred in spite of coproduction of oxygen, when compared to photohydrogen production using green algae (Bishop and Gaffron, 1963; Gaffron and Rubin, 1942; Healey, 1970a; Stuart and Gaffron, 1972; Stewart and Pearson, 1970; Weare and Benemann, 1973; Weissman and Benemann, 1977). Heterosytous filamentous blue green algae when cultured in nitrogen free medium produced hydrogen for 7 to 19 days with an efficiency of 0.4%. The results showed that algal filament exhibited the tendency to break up and decrease in hydrogen production till the nitrogen supply was restored to the cells. The addition of ammonium chloride did not inhibit hydrogen evolution. While in contrast the nitrogen starved cultures lost its activity after 1 day of starvation (Weissman and Benemann, 1977).

Hydrogen evolving capability of green algae was investigated by subjecting them to anaerobic incubation under dark condition (Greenbaum, 1982; Roessler and Lien, 1984; Happe and Naber, 1993; Schulz, 1996). Photooxidation of water by *Chlorella* species (Spruit, 1954, 1958) and *Scenedesmus obliquus* (Bishop and Gaffron, 1963) evolved hydrogen and oxygen. The release of hydrogen gas along with carbon dioxide occurred in *Chalamydomonas moewusii* (Frenkel, 1952) and *Scenedesmus obliquus* (Kaltwasser *et al*., 1969. Organic substrate stimulated the photohydrogen evolution in *Ankistrodesmus braunii* (Kessler, 1962) and *Chlamydomonas eugametos* (Abeles, 1964).

NITROGEN

The role of electron acceptor like NO_3^- and NO_2^- on hydrogen production was investigated using anaerobically adapted cells of *Chlamydomonas reinhardii.* The results showed that in the presence of oxidized nitrogen compound hydrogen production was suppressed depending on the oxidizing state of nitrogen released into the medium (NO_2^- and/or NH_4^+). Ammonium ion (NH_4^+) when served as a nitrogen source enhanced the evolution of hydrogen (Aparicio *et al*., 1985). The enzymes NAD (P) H-nitrate reductase reduced NO_3^- to NO_2^- while the ferredoxin-nitrite reductase reduced NO_2^- to NH_4^+ (Guerrero *et al*., 1981).

In heterocyst cyanobacteria the nitrogenase utilizes the reduced product of CO_2 fixation in the vegetative cell and the 5'-triphosphate (ATP) synthesized by oxidation or photophosphorylation in the heterocyst for reducing N_2 to NH_3 (Benemann and Weare, 1974; Jeffries *et al*., 1978; Weissman and Benemann, 1977; Bottomley and Stewart, 1976; Jones and Bishop, 1976). The reductant and ATP formation are light dependent, while the diffusion of reductant from the vegetative cell to the heterocyst is a slow and light independent process (Fay, 1976; Wolk, 1968). As the rate of ATP generation in the heterocyst exceeded the rate of reductant supply from the vegetative cell, an improved hydrogen yield was observed during intermittent illumination. The reason behind the increase in hydrogen yield was due to the diffusion of the reductant into the heterocyst during the dark phase (Jeffries *et al*., 1976).

The cyanobacterium heterocystous blue-green algae was investigated for its hydrogen producing capability, during nitrogen limiting condition. (Jeffries *et al*., 1978; Hallenbeck *et al*., 1978; Jeffries and Leach, 1978; Lambert and Smith, 1977; Mitsui and Kumazawa, 1977; Miyamoto *et al*., 1979a; Spiller *et al*., 1978). In heterocyst the oxygen production occurred in the vegetative cell (Weissman and Benemann, 1977; Benemann and Hallenbeck, 1978; Benemann and Weissman, 1976).

Anabaena cylindrica 629 a heterocyst blue green algae produced hydrogen and oxygen under nitrogen limiting condition with H_2 to O_2 ratio of 1:7 (Benemann and Weare, 1974; Fay and Cox, 1967; Haystead *et al*., 1970). The light saturated and unstarved culture of *Anabaena cylindrica* did not exhibit oxygen inhibition at atmospheric oxygen tension (Weare and Benemann, 1972, 1973). The nitrogenase activity localized in the heterocyst lacks oxygen evolving photosystem (II), which resulted in a high respiration rate due to the thick cell wall surrounding them leading to a reduced intracellular environment (Donze *et al*., 1972; Tel-Or and Stewart, 1975; Thomas, 1970; Fay and Walsby, 1966; Lang and Fay, 1971; Stewart *et al*., 1969). In heterocyst reductant flow occurred from the vegetative cell due to the energy supplied by the photo and oxidative phosporylation, while the fixed nitrogen flowed out of the heterocyst into the vegetative cell (Weare and Benemann, 1972). The heterocystous cyanobacteria produce hydrogen under anaerobic condition in the absence of nitrogen in a pathway mediated by the nitrogenase enzyme. The cyanobacteria exhibited the capability of nitrogen fixation from oxygen (Benemann and Weare, 1974; Bothe *et al*., 1977a, b; Daday *et al*., 1977; Jones and Bishop, 1976; Fogg *et al*., 1973; Oshchepkov *et al*., 1974).

The nitrogen gas is a competitive inhibitor of the hydrogen evolution reaction of nitrogenase and oxygen gas inactivator of nitrogenase, even though the aerobic nitrogen-fixers have various protection mechanisms against the inhibitory effect of oxygen gas (Houchins, 1984; Lambert and Smith, 1981; Stewart, 1980). The hydrogen producing capability of cyanobacteria was investigated by numerous researchers (Daday *et al*., 1977; Asada *et al*., 1979, 1985; Kumazawa and Mitsui, 1981, 1985; Lambert *et al*., 1979a , b; Mitsui,

1980; Miura *et al*., 1980-1982; Miyamoto *et al*., 1979a-c, 1984; Philips and Mitsui, 1983; Reddy and Mitsui, 1984; Stewart *et al*., 1982; Weissman and Benemann, 1977; Zhang *et al*., 1983). The evolution of hydrogen from the nodules of soyabean and cowpeas was due to the nitrogenase reaction (Hoch *et al*., 1957, 1960; Dart and Day, 1971). The negative impact of hydrogen evolution from the nodules was related to the decreased nitrogen fixation capability, (a) due to the utilization of ATP and reductant by the nitrogenase in hydrogen evolution (Schubert and Evans, 1976, 1977) and (b) due to the reduced energy supply from the photosynthate to the nodules (Hardy and Havelka, 1975).

FERMENTATIVE METABOLISM

Hydrogen evolution in green algae occurred as a part of fermentative metabolism. The unicellular green algae *Chlamydomonas moewusii* exhibited the capability of utilizing or evolving molecular hydrogen depending on the photoperiod. Under dark or light condition the hydrogen evolution occurred after the cells have been exposed to anaerobiosis in the presence of nitrogen or inert gas (Frenkel, 1952; Healey, 1970a; Klein and Betz, 1978b). The hydrogen evolution in *Scenedesmus* occurred due to the dark fermentation of glucose into lactic acid (Gaffron and Rubin, 1942). Similar observation was found with *Chlorella pyrenoidosa* during glucose fermentation (Damascfhke, 1957). The hydrogen evolution by *Chlamydomonas* species under dark condition followed citric acid cycle fermentative pathway. This mechanism suggested that during dark condition the surplus NADH either reduced some unknown acceptor or bring its electrons to a higher redox level at the expense of ATP which resulted in hydrogen evolution. During light condition the NADH supplied electron through the light driven reaction into the electron transport chain (Healey, 1970a). The light driven electron transport from NADH to hydrogenase occurred either due to NADH oxidation or plastoquinone (King *et al*., 1977), whereas in the case of *Scenedesmus* species the electron transfer occurred through NADH pathway (Kaltwasser *et al*., 1969). In the case of fermentative photodissimilation of acetate by *Chlamydomonas reinhardtii* F-60, the hydrogen metabolism followed anaerobic and light-driven cycles like citric acid and glyoxylate (Gibbs *et al*., 1986). It was suggested that apart from carbon dioxide, ATP was also generated during the fermentative process. ATP increased the redox potential of the electron from the reductant NADH to a higher state for producing hydrogen. During dark period the uncouplers of photophosphorylation reaction increased the release of hydrogen and carbon dioxide (Gaffron and Rubin, 1942). The capability of fresh water algal biomass in hydrogen production was investigated under sulphur limiting condition. The results showed that when the reactor was operated at varying photoperiod namely 2, 3 and 4 h of alternating light and dark period, the gas generation was found to be 40±3, 74±4 and 68±4 mL h^{-1}, while the corresponding hydrogen content was 49, 85 and 88%, respectively. Functional components of hydrogen generation reaction centres were also analysed, which showed that the PS(I) reaction centres were involved in hydrogen production pathway, as the light absorption by PS(I) was prerequisite for hydrogen generation under sulphur deprived photoautotrophic condition (Vijayaraghavan and Karthik, 2010).

SYMBIOSIS

A symbiotic relationship occurred between *Anabaena* and *Azolla* in which *Anabaena* supplied nitrogen to *Azolla*. The nitrogenase present during the symbiotic relationship

reduced C_2H_2 to H_2 in an ATP dependent pathway with the liberation of ammonia (Peters, 1976; Peters *et al*., 1976; Peters and Mayne, 1974a, b). The C_2H_2 measurement revealed indirectly nitrogen fixation capability. The reduction of N_2 to $2NH_3$ and C_2H_2 to C_2H_4 require six and two electrons respectively. To reduce one mole of fixed nitrogen three molecules of C_2H_2 was required. The nitrogenase when provided with an ATP source as a reductant, the rate of electron flow through the enzyme was found to be independent of the substrate (Hadfield and Bulen, 1969). At lower concentration of C_2H_2 an increase in hydrogen production occurred, as the electron were utilized for reducing protons to hydrogen when nitrogen served as a substrate. The ratio of C_2H_2/N_2 in the *in vitro* and *in vivo* studies showed a value of 4 and 5±3 for nitrogenase. The wide difference in ratio was due to the variation in experimental condition and the type of organism (Rrvear-Ortiz and Bums, 1975; Stiefel *et al*., 1977). Metabolic flux in *Synechocysis* sp. PCC6803 was investigated during the hydrogen production by Navarro *et al*. (2009).

***Chlamydomonas* sp.**

Chlamydomonas reinhardtii, *Chlamydomonas noctigama* (freshwater) and *Chlamydomonas euryale* (brackish water) produced hydrogen gas under sulfur deprived and photoheterotrophic condition (Skjånes *et al*., 2008). *Chlamydomonas reinhardtii* exhibited hydrogen production during photoautotrophic conditioning in a sulfur deprived medium when subjected to carbon dioxide exposure for 24 h followed by light and dark phase (Tsygankov *et al*., 2006). In the presence of acetate *Chlamydomonas reinhardtii* exhibited synchronized growth and cell division. The fermentative method of photohydrogen generation yielded products like formate and acetate during starch and protein degradation (Tsygankova *et al*., 2002). Hydrogen production by microalga was verified at an optimal light intensity of 238 $\mu/Em^2/sec$ using a discrete multi-state model. The model considers concentration of metabolism and intensity of light as the continuous variable while specific nutrient served as discrete variable (Wonjun and Moon, 2005).

Sulfur deprived *Chlamydomonas reinhardtii* exhibited partial and reversible inactivation of photosynthetic oxygen evolution in algae. The light induced anaerobic condition led to the evolution of photohydrogen due to the [Fe-Fe] hydrogenase system (Kosourov *et al*., 2007). In the closed cultures of *Chlamydomonas reinhardtii* the respiratory oxygen consumption was found to be below the photosynthetic oxygen evolution rate, which leads to intracellular anaerobiosis due to reversible inhibition of photosystem (II). In contrast the algal metabolism switched to a kind of photofermentation which enabled the white cells of *Scenedesmus obliquus* to survive under anaerobic condition. *Scenedesmus obliquus* did not produce significant amount of hydrogen even in the presence of [Fe] hydrogenase gene under sulfur deprived conditions (Winkler *et al*., 2002).

The effect of light intensity on the hydrogen production was investigated using *Chlamydomonas reinhardtii* under sulfur deprived condition. The results showed a maximum hydrogen production and specific production of 225 mL H_2/L and 2.01 mL H_2/g cells/h at an intensity of 200 $\mu/Em^2/sec$. The photosystem (II) was subjected to damage when the light intensity was increased up to 300 $\mu/Em^2/sec$ (Kim *et al*., 2006). The hydrogen producing capability of *Chlamydomonas reinhardtii* was also investigated with respect to photosystem (II) and oxygen consumption (Antal *et al*., 2003). *Chlamydomonas reinhardtii* when subjected to a light intensity of 2000 $\mu mol/m^2/sec$ for 30 min suppressed the photosynthetic oxygen evolution, while at a light intensity 15 $\mu mol/m^2/sec$ a maximum hydrogen production occurred (Markov *et al*., 2006). *Chlamydomonas reinhardtii* when grown under sulfur limiting medium produce 45 mL H_2/day (Laurinavichene *et al*., 2008).

The effect of Tris-Acetate-Phosphate (TAP) medium on prolonged hydrogen production (90 days) was investigated under sulfur limiting condition. The sulfur deprivation was carried out by repeated dilution of algal cultures at a ratio of 1:10 on volume basis (Laurinavichene *et al*., 2002). The effect of intense light and oxidative stress on the genetic impairment of *Chlamydomonas reinhardtii* was investigated by Förster *et al.* (2005). The inhibitory studies on nonphotochemical plastoquinone reduction and hydrogen photoproduction in *Chlamydomonas reinhardtii* revealed that the plasticidal NDH-2 in photosystem (II) was independent of hydrogen production (Mus *et al*., 2005). *Chlamydomonas reinhardtii* immobilized on silica particle in a sulfur rich medium, proved the capability of producing hydrogen (Hahn *et al*., 2007).

Hydrogen producing metabolic pathway in *Chlamydomonas reinhardtii* was investigated using power law analysis. The model considered photosynthetic efficiency (proton produced due to the photolysis of water during hydrogen production) and ATP consumption (due to the cellular functions). The experiment proved that the *Chlamydomonas reinhardtii* when grown on sulfur deprived medium yielded hydrogen gas for 70 h (Horner and Wolinsky, 2002).

Hydrogen production by *Chlamydomonas reinhardtii* and *Dunaliella salina* was studied based on truncated chlorophyll antenna size of photosystem. The findings revealed that the photosynthetic productivity was dependent on antenna size and photosystem (II) than photosystem (I) (Polle *et al*., 2002). The adaptation of *Chlamydomonas* sp. MGA161 (marine green algae) to light dependent hydrogen evolution based on photosystem (I) and electron donation was compared with *Chlamydomonas reinhardtii*. The hydrogen production in the illuminated cells of *Chlamydomonas* sp. MGA161 was little more than in dark as the metabolism was dependent on cellular starch for an electron source instead of water (Miyamoto *et al*., 1990).

Scale-up of photohydrogen production was investigated using mixed cultures of *Chlamydomonas* sp. MGA161 a marine green algae and *Rhodopseudomonas* sp. W-1S a photosynthetic bacteria (Miura *et al*., 1995). The ability of *Chlamydomonas reinhardtii* to produce hydrogen and oxygen was monitored by subjecting them to anaerobiosis and carbon dioxide deprivation followed by irradiation for 160 h. The results showed that the stability of hydrogen and oxygen photoproduction was greater in the 5th cycle that in any previous cycle (Greenbaum and Reeves, 1985).

Hydrogen producing strains of *Chlamydomonas reinhardtii* and its oxygen tolerant phenotype was screened based on chemical mutagenesis (DBMIB) (Flynn *et al*., 2002). Effect of oxygen tolerance on algal hydrogen production was investigated using *Chlamydomonas reinhardtii* mutant (Seiberta *et al*., 2001). The effect of temporal phenomena on hydrogen production was investigated in *Chlamydomonas reinhardtii* based on Fourier analysis (Dante *et al*., 2004). *Chlamydomonas moewusii* when investigated under aerobic and autotrophic conditions produced hydrogen to carbon dioxide at ratio <0.5. At low light intensity the hydrogen production was pronounced without any change in the production of carbon dioxide level. In the absence of carbon dioxide the rate of hydrogen production was dependent on the light intensity. At a light intensity corresponding to oxygen consumption during the normal photosynthesis and respiration period, the hydrogen production dropped to zero (Frenkel, 1952). Sulfur deprived *Chlamydomonas reinhardtii* when grown under different conditions namely photoautotrophic, photoheterotrophic and photomixotrophic condition. The results showed that acetate and carbon dioxide were required for rapid inactivation of photosystem (II) with a higher yield of H_2 (Kosourova *et al*., 2007). The effect of light intensity and nitrogen sources on hydrogen production was studied

using *Chlamydomonas reinhardtii*. The results showed that high intensity impaired the hydrogen evolution at an average for 50 h (Aparicio *et al*., 1985). Hydrogen metabolism in photosynthetic organisms was investigated under dark condition using *Chondrus crispus* and mosses (Ben-Amotz *et al*., 1975). *Chlamydomonas reinhardtii* when grown in sulfur deprived photoautotrophic condition resulted in maximum hydrogen production (63±7 mL h^{-1}) when subjected to alternating photoperiod for 3 h (Vijayaraghavan *et al*., 2009). Reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii* and the effect of sulfur deprivation was investigated with respect to photobiological hydrogen production (Melis *et al*., 2000; Zhang *et al*., 2002).

***Anabaena* sp.**

The effect of nutrient and medium composition in photohydrogen production was investigated using *Anabaena variabilis*. The results showed that the specific hydrogen production rate in Allen-Arnon medium, BG-11 and BG-110 was found to be 4.5×10^{-4}, 8.0×10^{-5} and 7.2×10^{-5} kg H_2/kg dry cell/h (Berberoğlu *et al*., 2008). The photohydrogen producing capability was investigated using varying nitrogen fixing culture like *Anabaena* (Jones and Bishop, 1976). *Anabaena cylindrica* (Neil *et al*., 1976; Jeffries and Leach, 1978) and marine blue green algae (Lambert and Smith, 1977). The effect of 2-methyl-5-nitroimidazole-1-ethanol (Metronidazole) on hydrogen evolution was studied using *Anabaena* and *Scenedesmus* (Tetley and Bishop, 1979). *Anabaena* N-7363 immobilized in k-carrageenan gel resulted in a hydrogen production rate of 3.2 mmol/h/g dry gel at a light intensity of 6000 lux in a nitrogen free medium (Karube *et al*., 1986). During intermittent illumination the hydrogen production was found to be improved due to the ATP generation, which enhanced the diffusion of reductant into the heterocyst during the dark reaction (Jeffries *et al*., 1976).

***Chlorella* sp.**

The photohydrogen evolution by *Chlorella shellata* was investigated during the transition from autotrophic to photoheterotrophic nutritional condition based on the light harvesting antenna size of photosystem (I) (Boichenko *et al*., 1992; Polle *et al*., 2001). The *Chlorella pyrenoidosa* produced 0.7 kg H_2 m^{-3} under optimum condition. *Chlorella pyrenoidosa* when subjected to inhibition by the addition of 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU) a decrease in hydrogen evolution occurred by 75%. The mechanism behind this inhibition was due to the blockage of electron flow through the photosystem (II), which indicated that water was a main electron donor for hydrogen production (Kojima and Yamaguchi, 1988). The effect of pH and temperature was investigated on *Chlorococcum littorale* (marine green algae) based on photosynthesis and photohydrogen production. The results showed that for 5% CO_2 at pH 7.5 and 25°C a maximum photosynthetic oxygen evolution and photohydrogen generation occurred. At higher pH a decrease in oxygen evolution occurred due to partial inhibition of the water splitting complex. The photosynthesis and hydrogen evolution was found to be unstable at high temperature (Schnackenberg *et al*., 1996).

***Oscillatoria* sp.**

Photohydrogen producing capability of *Oscillatoria* sp. Miami BG7 was investigated based on the nutrient (nitrogen) limiting condition. The result showed a maximum hydrogen production of 260 μmol mg^{-1} chlorophyll/h. The enhancement in hydrogen production occurred under nitrogen limiting condition due to the increased nitrogenase synthesis which

declined the photosystem (II) acidity and resulted in the accumulation of electron donor substance (Kumazawa and Mitsui, 1981). A hydrogen production rate of 13 μL H_2 mg^{-1} dry wt/hr was obtained using immobilized *Oscillatoria* sp. Miami BG 7 (Philips and Mitsui, 1986).

***Scenedesmus* sp.**

Photohydrogen production by *Scenedesmus obliquus* and *Chlorella vulgaris* was investigated with the addition of sodium dithionite. The results showed that the evolution of hydrogen occurred due to the removal of oxygen by dithionite during light dependent stage. In the case of 3-(3,4-dichlorophenyl)-1,1-dimethylurea addition, the photosystem (II) was subjected to inhibition which suppressed the evolution of hydrogen. Furthermore in sulfur containing medium, the hydrogen production did not follow photosystem (II) pathway even in the presence of dithionite, which confirmed that the production occurred via photosystem (I) and (II) with water as a source (Pow and Krasna, 1979; Senger and Bishop, 1979). *Scenedesmus* sp. also produced hydrogen from endogenous organic compound due to the cyclic photophosphorylation reaction which occurred through the photosystem (I) (Stuart and Kaltwasser, 1970). *Scenedesmus* sp. when subjected to heat and salicylaldoxime treatment, the electron transport and phosphorylation occurred through photosystem (I) which was independent of cyclic photophosporylation reaction due to the non-cyclic flow of electron from the organic substance to hydrogen for the release of molecular oxygen (Stuart, 1971). The turn over time and pool size of photosynthetic hydrogen production showed that the intrinsic kinetic rate of hydrogen photoapparatus was in pace with incidental rate and light quanta. The photogenerated electron followed the mainstream of the electron transport chain for hydrogen production (Greenbaum, 1979).

The hydrogen producing capability by *Chlamydomonas*, *Chlorella* and *Scenedesmus* was studied using organic substrate uncoupler namely carbonyl cyanide m-chlorophenylhydrazone (Cl-CCP) (Healey, 1970b). The effect of glucose and uncoupler Cl-CCP on *Scenedesmus obliquus D3* showed that at a concentration of 5×10^{-5}M Cl-CCP, a maximum rate of photohydrogen production occurred while hydrogen evolution, photo reduction and dark hydrogen evolution was fully inhibited. *Scenedesmus obliquus* produced hydrogen during light by utilizing organic matter as the oxygen evolution did not follow photosystem (II) (Kaltwasser *et al*., 1969).

Scenedesmus culture when incubated in dark with an inhibitor (Cl-CCP), the pool utilized by photosystem (I) vanished, but the hydrogen production occurred (0.5 mol of H_2 gas/mol.glucose) when illuminated (3.4×10^3 W.cm^{-2}) after the inhibitor addition (Stuart and Gaffron, 1971). The photohydrogen production by *Ankistrodesmus, Chlorella* and *Scenedesmus* followed photosystem (I) and did not follow photosystem (II), even in the presence of 10^{-5} M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The photohydrogen production was found to be independent of photophosporylation reaction. In the presence of Cl-CCP and SAL the photophosphorylation reaction was inhibited, while the hydrogen evolution was found to be stimulated (Kaltwasser *et al*., 1969). The hydrogen generating capability of *Scenedesmus* was proved to be potential source of fuel (Buvet *et al*., 1977; Mitsui *et al*., 1977; Schlegel and Barnea, 1976; Gaffron and Rubin, 1942) and its inhibition due to the photoproduction of O_2 was also investigated (Kessler, 1974; Zajic *et al*., 1978). The hydrogen producing capability during fermentative (*Clostridium strain*) and photosynthetic process (*Scenedesmus species*) resulted in a value of 1.65 mol H_2 mol^{-1} glucose in the pectin culture up to 2.45 in the mixed culture with a hydrogen content of 30% under fermentative condition (Ustak *et al*., 2007).

MUTAGEN

The biochemical and metabolic pathway that promoted hydrogen production in green algae was explored based on screening DNA insertional mutagenesis library from the strains which binds the ability to produce hydrogen after subjecting to anaerobic condition. The screening of DNA in mutagen library in *Chalamydomonas reinhardtii* played an important role in identifying genes involved in specific cellular pathway and process (Debuchy *et al*., 1989; Rochaix, 1995; Tam and Lefebvre, 1995; Niyogi *et al*., 1997; Adam and Loppes, 1998; Davies *et al*., 1999; Moseley *et al*., 2000; Van *et al*., 2001; Dame *et al*., 2002; Polle *et al*., 2003). Mutants that compromised in their ability to produce photohydrogen was subjected to screening and the characterization. One of the mutant *sta7-10* revealed that the gene with higher homology to the isoamylase gene family was found to be critical in the formation of insoluble starch in *Chalamydomonas reinhardtii*. Furthermore the insoluble starch content in the mutant was <3% when compared with the wild strain. The mutants exhibited the ability to produce hydrogen and maintained hydrogenase transcription even after subjecting to anaerobic condition. In the case of starch mutant *sta-6* (BAF J5) reduced hydrogen production rate and hydrogenase gene transcription ability was observed (Posewitz *et al*., 2004; Ball *et al*., 1996; Monille *et al*., 1996; Ball, 1998; Myers *et al*., 2000; Dauvillee *et al*., 2001).

CONCLUSION

The ability of algae to produce hydrogen based on photo and fermentative method has been reviewed in detail in this review. The characteristic of medium and its role on hydrogen generation are also presented with respect to nitrogen, sulfur and organic matter. The effect of physical parameters like light intensity, photoperiod and its influence on antenna size and metabolism pathyway in generating hydrogen are well addressed, moreover the optimum condition, maximum hydrogen yield and composition of medium for algal growth are presented. This review will be a handy research guide for researchers who opt to address the benefit of algae as a renewable energy resource.

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