Biophotolysis-based Hydrogen Production by Cyanobacteria and Green Microalgae

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Biological hydrogen evolution provides a sustainable and environmentally friendly way to produce clean energy from renewable resources. Cyanobacteria and green microalgae split water into molecular hydrogen and oxygen using sunlight under special conditions. This mini review examines the technology status of direct and indirect biophotolysis for hydrogen production. Progress has been made in solving the intrinsic incompatibility of the simultaneous evolution of hydrogen and oxygen gas in photoautotrophic cells, particularly the adverse impact on the key hydrogen enzymes (hydrogenase and nitrogenase). Technical challenges for sustained hydrogen production are outlined.

Key words biophotolysis; biohydrogen; cyanobacteria; green microalgae; energy productivity

1. Introduction

Hydrogen gas is seen as an ideal future energy carrier because it is easily converted to electricity in fuel cells, liberates a large amount of energy per unit mass, and generates no air pollutants. Although hydrogen is one of the most abundant elements on this planet, its pure form (H_2) exists at extremely low levels (< 1 ppm) in the atmosphere [1]. Fuel hydrogen must therefore be produced from hydrogen-rich substances [2]. Growing concerns about global warming and peak oil have increased interest in renewable energy. Biologically-produced hydrogen or biohydrogen is considered a renewable, CO_2-neutral energy form [3]. A large number of microbial species evolve hydrogen when growing on renewable feedstocks under special anaerobic conditions with low hydrogen pressure [4]. Depending on carbon and energy sources, three different mechanisms are involved in microbal hydrogen evolution, presenting unique technical challenges for hydrogen production [5]. Heterotrophic obligate or facultative anaerobes (e.g., *Clostridium*) obtain both carbon and energy from carbohydrates such as glucose and deposit the excess reducing power in fermentative products and hydrogen [6]. The hydrogen yield is therefore dependent on the profile of fermentative products, ranging from 0.3 to 4 moles of hydrogen per mole of glucose utilized [7]. Photosynthetic bacteria (e.g., *Rhodobactor*) can use broad organic substrates including lactic and acetic acids as the energy and carbon source under light irradiation. Light energy is essential to hydrogen evolution by photosynthetic cells [8]. Photoautotrophic green microalgae and cyanobacteria use sunlight and CO_2 as the sole sources for energy and carbon. The reducing power for cellular photosynthesis and/or biophotolysis comes from water oxidation under light irradiation [9, 10]. Biophotolysis is the action of light on biological systems that results in dissociation of water into molecular hydrogen and oxygen, $H_2O \rightarrow H_2 + ½ O_2$. This review focuses on biophotolysis-based hydrogen production by green microalgae and cyanobacteria.

2. Photosynthesis and Biophotolysis

Photoautotrophic microorganisms, either prokaryotic cyanobacteria or eukaryotic green microalgae, possess chlorophyll a and other pigments to capture sunlight energy and use photosynthetic systems (PSII and PSI) to carry out plant-like oxygenic photosynthesis [11,12]. The pigments in PSII (P680) absorb the photons with a wavelength shorter than 680 nm, generating a strong oxidant capable of splitting water into protons (H^+), electrons (e^-) and O_2 as shown in Fig. 1. The electrons or reducing

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equivalents are transferred through a series of electron carriers and cytochrome complex to PSI. The pigments in PSI (P700) absorb the photons with a wavelength under 700 nm, which further raises the energy level of the electrons to reduce the oxidized ferredoxin (Fd) and/or nicotinamide adenine dinucleotide phosphate ($NADP^+$) into their reduced forms. The proton gradient formed across the cellular (or thylakoid) membrane drives adenosine triphosphate (ATP) production via ATP synthase. CO_2 is reduced with ATP and NADPH via a reductive pentose phosphate pathway or Calvin cycle for cell growth. The excess reduced carbon is stored inside the cells as carbohydrates (CH_2O) and/or lipids.

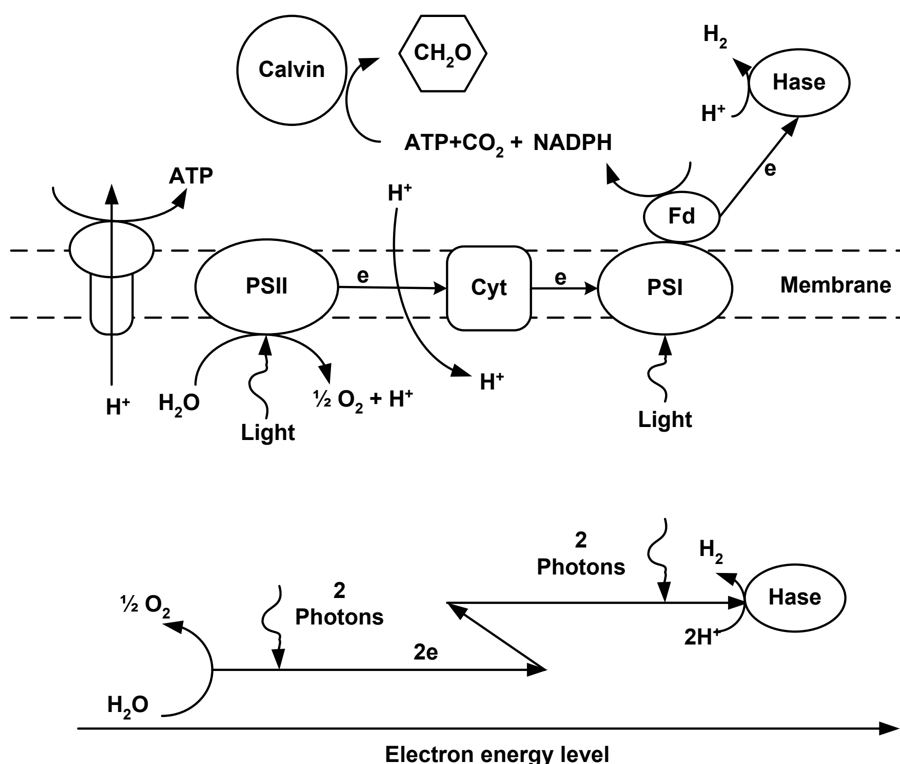

Fig. 1. Schematic mechanisms of photosynthesis and biophotolysis of photoautotrophic microbes [13-16]. The energy level of electrons or reducing equivalents from water oxidation is raised by the adsorbed photons at PSII and PSI. The reducing equivalent (NADPH) is used for CO_2 reduction in photosynthesis and carbohydrates (CH_2O) are accumulated inside the cells. The reducing power (Fd) could also be directed to hydrogenase (Hase) for hydrogen evolution.

Under special conditions, the reducing equivalents (ferredoxin) can also be used by hydrogenase or nitrogenase to reduce protons for evolution of molecular hydrogen ($2H^+ + 2Fd^- \Leftrightarrow H_2 + 2Fd$) [17]. Gaffron and Rubin first reported that *Scenedesmus,* a green microalga, evolved molecular hydrogen under light conditions after being kept in anaerobic and dark conditions [18]. The responsible enzyme for hydrogen evolution is a reversible hydrogenase because it catalyzes the reaction in both directions [19]. Benemann and Weare reported that a nitrogen-fixing cyanobacterium, *Anabaena cylindrica,* evolved hydrogen and oxygen gas simultaneously in an argon atmosphere for several hours [20]. The enzyme involved in hydrogen evolution is nitrogenase that is responsible for the reduction of nitrogen (N_2) into ammonia (NH_3) [21]. The reversible hydrogenase and nitrogenase, however, are sensitive to the oxygen evolved in biophotolysis and promptly deactivated at quite low O_2 partial pressures (<2% v/v) [22,23], which results in a transient hydrogen evolution [24,25]. This intrinsic incompatibility of biophotolysis is a major barrier for sustained hydrogen evolution [9,26]. Technologies have been extensively researched to overcome the hurdle. Examined will be the current status of biophotolysis-based hydrogen production

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by cyanobacteria and green microalgae via direct and indirect means as identified by whether or not light is irradiated during hydrogen evolution [3].

3. Direct biophotolysis: sustained hydrogen evolution

Direct biophotolysis refers to sustained hydrogen evolution under light irradiation. The light energy is absorbed by the pigments at PSII, or PSI or both, which raises the energy level of electrons from water oxidation when they are transferred from PSII via PSI to ferredoxin. A portion of the light energy is directly stored in hydrogen gas.

3.1 Direct biophotolysis by cyanobacteria

Encompassing a wide diversity in morphology and physiology, cyanobacteria are potential microbial species for hydrogen production via direct biophotolysis [27]. By using nitrogenase and/or bi-directional hydrogenase, both heterocystous nitrogen-fixing strains and unicellular non-nitrogen-fixing strains are able to evolve hydrogen under special conditions [28,29]. *Anabaena* strains are the representative nitrogen-fixing cyanobacteria in hydrogen research as shown in Table 1. Fig. 2 illustrates how a heterocyst provides an oxygen-free environment to the oxygen-sensitive nitrogenase that reduces molecular nitrogen into NH_3 as well as protons into H_2 (Eq. 2) [30]. In air or N_2-containing atmosphere, nitrogen fixation is the predominant reaction while hydrogen is a minor byproduct. More hydrogen could be formed in the absence of molecular nitrogen (Eq.3). The reducing power for hydrogen evolution is derived from the energy-rich carbohydrate (CH_2O) stored in the heterocyst or transferred from neighbor cells [30]. Because of the high energy demand (4 ATP per hydrogen), the energy conversion efficiency from light to H_2 by nitrogenase is quite low (<1%) [31].

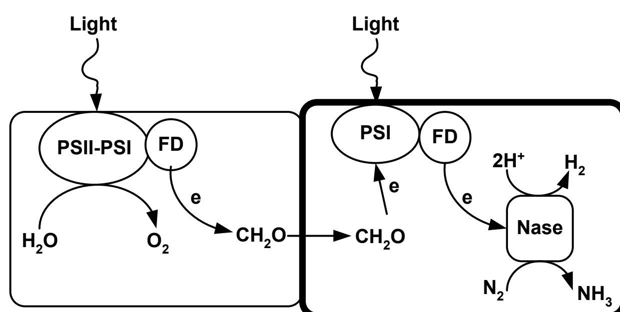
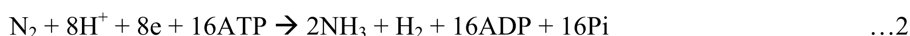


Fig. 2. Nitrogenase(Nase)-mediated hydrogen evolution in a heterocyst of nitrogen-fixing heterocystous cyanobacteria [10, 30, 32]. The oxygen and hydrogen evolution are carried out separately and the energy-rich carbohydrate (CH_2O) is used as the electron source in the oxygen-free heterocyst.



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

$$8H^+ + 8e + 16ATP \rightarrow 4H_2 + 16ADP + 16Pi \quad \ldots 3$$

Table 1 compares the representative nitrogen-fixing cyanobacteria with their maximum specific hydrogen evolution rate per gram of cell mass or chlorophyll a (the pigment content accounts for 2 to 3% of cell mass). Hydrogen production is also compared based on the volumetric productivity of the strain in laboratory photobioreactors. A major cost factor in large-scale hydrogen production is the photobioreactor that is usually characterized by surface area for light irradiation [15, 33, 34]. A hydrogen productivity based on reactor surface area can be found from the volumetric productivity and a critical optical length. The latter has a quite narrow range from 2 to 6 cm [15], depending on many factors including cell density, cell size, light intensity and light saturation [13, 35-37]. Hydrogen production is also compared in terms of energy productivity, a general performance parameter for energy generation based on the energy output per volume per time. The energy productivity is calculated by multiplying the volumetric productivity (mmol H_2/L/hr) by the heat of combustion of hydrogen at 25 ^oC

($\Delta H_{c,}$ H_2 = - 0.24 kJ/mmol). Biological hydrogen production is often conducted in two stages under different atmospheric conditions, the first stage for cell growth followed by the second stage for hydrogen evolution. Nitrogen starvation is often applied at the end of the growth stage as an efficient metabolic stress to induce the activity of nitrogenase. Table 1 also gives the light intensity and different atmospheres for cell growth and hydrogen evolution, respectively.

Table 1. Hydrogen evolution via direct biophotolysis by cyanobacteria in laboratory photobioreactors.

Organism	Maximum evolution rate (mmol/g /hr)[a]	Maximum productivity (mmol/L/hr)[b] (kJ/L/hr)[b]	Gas for growth; Light intensity (w/m^2)[c]	Gas for H evolution; Light intensity (w/m^2)[c]	Ref
Anabaena cylindrica	1.33	0.93 (0.22)	99.7% air 0.3% CO_2; 20	97% Ar 3% CO_2; 60	[38]
Anabaena variabilis	0.7	0.085 (0.02)	25% N_2 2% CO_2 73% Ar; 20	5% N_2 2% CO_2 93% Ar; 20	[39]
Anabaena variabilis PK84	3.06	0.35 (0.08)	25% N_2 2% CO_2 73% Ar; 20	5% N_2 2% CO_2 93% Ar; 20	[39]
Anabaena variabilis PK84	0.21	0.26 (0.06)	98% air 2% CO_2; 72 (L/D)[d]	98% air 2% CO_2; 72 (L/D)[d]	[40]
Anabaena AMC414	(12)[a]	0.084 (0.02)	98% air 2% CO_2; 48	98% air 2% CO_2; 99	[28]
Gloebacter PCC7421	(1.38)[a]	-	Air; 4	Ar/CO/C_2H_2; 4-6	[29]
Synechococcus PCC602	(0.66)[a]	-	Air; 4	Ar/CO/C_2H_2; 4-6 or dark	[29]
Aphanocapsa montana	(0.4)[a]	-	Air; 4	Ar; 4-6	[29]

Note:

a. The specific hydrogen evolution rate based on per gram of dry cell mass or chlorophyll a (in blanket).
b. Hydrogen productivity per liquid volume of photobioreactor during hydrogen evolution stage, not including the time and space required for cell growth and enzyme induction. The value in blankets is the energy productivity (kJ/L/hr) based on the heat of combusion of hydrogen (0.24 kJ/mmol) at 25 °C.
c. 1 W/m^2 = 4.6 $\mu molE/m^2/s$ (APR). APR: photosynthetically active radiation that includes light energy of 400-700 nm in wavelength.
d. 12 hour light and 12 hour dark.

Comparing the recent data with those published 30 years ago by Weissman and Benemann [38], it is clear that a necessary technology breakthrough has not yet been attained for hydrogen productivity by nitrogen-fixing cyanobacteria. For instance, in a typical laboratory photobioreactor operated continuously at a dilution rate of 0.03 hr^{-1}, *Anabaena variabilis* (ATCC 29413) was maintained at steady

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state in the exponential growth phase to have a high nitrogenase activity [39]. The dry cell mass concentration reached 151 mg/L containing 1.8 wt% of chlorophyll a, 22.8 wt% of protein, and 6.4% of heterocysts under normal nitrogen supply, resulting in a maximum specific hydrogen evolution of 0.37 mmol/g dry wt/hr. Under nitrogen starvation, the hydrogen evolution rate was increased to 0.7 mmol/g dry wt/hr (Table 1) with an increase of heterocysts to 10.2%.

Progress has been made with the genetic engineering of nitrogen-fixing cyanobacteria. In wild type strains, the formed hydrogen is quickly re-oxidized by uptake hydrogenase, an enzyme found in all known nitrogen-fixing cyanobacteria [41]. The uptake hydrogenase saves the reducing power for the cell's benefits, but reduces hydrogen evolution. By using *A. variabilis* PK84, a mutant impaired in utilization of molecular hydrogen, the hydrogen evolution rate is increased to 3.1 mmol/g dry wt/hr, more than a four fold increase in comparison with the wild type as shown in Table 1 [39]. It has been reported that the energy conversion efficiency of photosynthetically active radiation (PAR) could be increased from lower than 0.005% of a wild type to above 1% for a mutant with impaired uptake hydrogenase [21].

The atmosphere plays an important role in hydrogen evolution by cyanobacteria and could be a cost factor in large-scale hydrogen production. A N_2-free gas phase such as argon plus CO_2 gives a high hydrogen evolution rate [38]. In the presence of nitrogen, nitrogenase uses the reducing power for nitrogen reduction, rather than hydrogen evolution. *A. variabilis* PK84, for instance, produces 14.5 times more hydrogen in CO_2-enriched argon than in CO_2-enriched air [40]. When the strain was cultivated in a tubular photobioreactor subject to an irradiation cycle of 12 hr light and 12 hr dark, little hydrogen was produced during the dark time, implying that nitrogenase, instead of bi-directional hydrogenase, was responsible for hydrogen evolution under light [40]. A wild type cyanobacterium, *Anabaena* PCC 7120, has three enzymes involved in hydrogen metabolism, a Mo-nitrogenase, an uptake hydrogenase and a NiFe bi-directional hydrogenase. It does not evolve hydrogen at all in CO_2-enriched air. A hydrogen uptake deficient mutant, *Anabaena* AMC414, is able to produce hydrogen at 0.084 mmol/L/hr in CO_2-enriched air, which is about 28% of the hydrogen evolved in argon [28]. Its maximum hydrogen evolution rate based on chlorophyll a is about 12 mmol/g Chl/hr (Table 1) or 0.34 mmol/g dry wt/hr as the pigment accounts for about 2.8% of dry cell mass. In general, the energy productivity of cyanobacteria via direct biophotolysis is relatively low, ranging from 0.02 kJ/L/hr in air to 0.22 kJ/L/hr in argon.

Hydrogen evolution is also observed in unicellular non-nitrogen fixing cyanobacteria when the cells are exposed to a reducing atmosphere consisting of CO and C_2H_2 under a low light intensity (4-7 w/m^2) (Table 1). In the absence of nitrogenase, a bi-directional hydrogenase is most likely responsible for hydrogen evolution in those strains, including *Gloebacter* PCC7421, *Synechococcus* PCC602, and *Aphanocapsa montana* [29,42]. Compared with the heterocystous nitrogen-fixing strains, the unicellular non-nitrogen-fixing cyanobacteria exhibit a relatively low hydrogen evolution rate and need a highly reducing gas to protect the hydrogenase from the inhibitive oxygen.

3.2 Direct biophotolysis by green microalgae

Chlamydomonas reinhardtii, a facultative photoautotrophic and photoheterotrophic microalga, is the representative of green microalgae for biohydrogen research [43-45]. Other algal species such as *Chlorococcum littorale* and *Platymonas subcordiformis* are also investigated for hydrogen evolution [17, 46]. Gaffron and Robins first observed that unicellular microalga *Scenedesmus obliquus* could either use H_2 as an electron donor in CO_2-fixation or evolve H_2 under anaerobic conditions in dark or light [18]. Anaerobic adaptation is needed for the cells to induce reversible Fe-hydrogenase. The period of hydrogen evolution, however, is transient upon illumination because of the high sensitivity of Fe-hydrogenase to the oxygen generated from water splitting on PSII [47]. In order to overcome the inhibitive effect of oxygen on hydrogenase, various methods have been investigated with limited success, such as spatial separation of oxygen from hydrogen, immobilization of chloroplasts, electron carrier and hydrogenase, oxygen scavenging, and gas purging [48-50].

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Sustained photobiological hydrogen evolution by *C. reinhardtii* was observed when a sulfur-deprived culture medium was used [51]. The essential anaerobiosis in cells under light is achieved by depriving sulfur from the medium (<0.45mM) to reduce the oxygenic PSII activity, and equally important to remove the generated oxygen by keeping the cell's respiration rate on exogenous acetate and/or endogenous carbohydrates [9]. Acetate is a good carbon substrate for *C. reinhardtii* cells to maintain a high respiration rate [52]. After the photosynthetic oxygen generation rate is lower than the rate of O_2-uptake by respiration, the cells culture gradually becomes anaerobic, which induces the activity of reversible hydrogenase [53]. It takes up to 40 hrs for the cells to reach the hydrogen production state that can last 60 to 70 hrs [54]. The specific hydrogen evolution rate based on chlorophyll and the maximum photobioreactor productivity (Table 2) are similar to those of cyanobacteria (Table 1).

Table 2. Direct biophotolysis hydrogen production by green microalgae in laboratory photobioreactors.

Organism	Maximum hydrogen evolution (mmol/g Chl/hr)[a]	Maximum hydrogen productivity (mmol/L/hr)[b] (kJ/L/hr)[b]	Gas for growth; Carbon source; Light intensity (w/m^2)[c]	H_2 evolution medium; Light intensity (w/m^2)[c]	Ref
Chlamydomonas reinhardtii cc124	5.94	0.094 (0.022)	97% air 3% CO_2; Acetate (17mM); 43	Argon; S-free acetate (17mM); 65	[54]
Platymonas subcordiformis	(0.001)[a]	0.002 (0.0005)	Air; Seawater nutrients; 22(L/D)[d]	*N_2*; S-free seawater; 35	[46]
Chlamydomonas reinhardtii cc1036	5.91	0.48 (0.12)	Air; Acetate (17mM); 22	Argon; S-free acetate (17mM); 26	[55]

Note:

a. The specific hydrogen evolution based on per gram of chlorophyll or 10^9 cells (in blanket).
b. See Table 1.
c. See Table 1.
d. 14-hour light and 10-hour dark.

Sulfur deprivation is a reversible metabolic stress and re-addition of sulfur can regenerate the depleted algal cells for another run of H_2 production [53]. In order to facilitate the sulfur deprivation and re-addition cycles, a non-motile mutant of *C. reinhardtii*, CC-1036 pf18 mt+, was immobilized by cell attachment on matrices of glass fibers at about 0.5 g Chl per m^2 of matrix [55]. The cell immobilization results in about a five fold increase in the maximum hydrogen productivity of the photobioreactor (Table 2). It is interesting to note that the immobilized and suspended algal cells show the same maximum specific hydrogen evolution rate based on chlorophyll (Table 2). This fact implies little effect of cell immobilization on *C. reinhardtii* and good mass transfer under continuous argon bubbling. Hydrogen evolution, however, was significantly reduced after argon bubbling was stopped [55]. Gas sparging of liquid medium with nitrogen and CO_2 creates an atmosphere of low hydrogen partial pressure that favors hydrogen evolution [56]. Hydrogen evolution in sulfur-deprived seawater is also observed with a marine alga *Platymonas subcordiformis* after a long anaerobic dark adaptation [46]. The hydrogen evolution rate, however, is very low in comparison with photoheterotrophic *C. reinhardtii* (Table 2). The energy productivity of green microalgae in laboratory photobioreactors ranges from 0.02 to 0.12 kJ/L/hr.

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4. Indirect biophotolysis: dark fermentation on endogenous carbohydrates

In photosynthesis, the reduced carbon is stored as endogenous carbohydrates, such as starch in microalgae and glycogen in cyanobacteria [57]. Following Gaffron and Rubin's discovery [18], studies on the mechanisms involved in hydrogen evolution have found that the electrons or reducing equivalents of hydrogenase and nitrogenase do not always come from water, but may sometimes originate from the intracellular energy reserve including carbohydrates [58,59]. The stored energy is released through fermentation of the endogenous carbohydrates in dark conditions, and the excess reducing power can be deposited by hydrogenase on protons (H^+) forming molecular hydrogen [60]. Hydrogen evolution from endogenous carbon reserve under dark anaerobic conditions looks very similar to the conventional anaerobic hydrogen fermentation, but the endogenous carbon reserve is made in vivo during photosynthesis. In this sense, the electrons or reducing equivalents in indirect biophotolysis are derived from water by photoautotrophic cells (see Fig. 1). This indirect biophotolysis, therefore, consists of two stages in series: photosynthesis for carbohydrate accumulation, and dark fermentation of the carbon reserve for hydrogen production. In this way, the oxygen and hydrogen evolutions are temporally and/or spatially separated [61]. This separation not only avoids the incompatibility of oxygen and hydrogen evolution (e.g., enzyme deactivation and the explosive property of the gas mixture), but also makes hydrogen purification relatively easy because CO_2 can be conveniently removed from the H_2/CO_2 mixture [62]. Miura and coworkers proposed a process of hydrogen production via indirect biophotolysis by using natural light/dark cycles [50]. According to this proposal, CO_2 is reduced to starch by photosynthesis in daytime, and the starch thus formed, is fermented to hydrogen gas and organic acids under anaerobic conditions during nighttime. The organic acids and other fermentative products can be further used for hydrogen evolution by photosynthetic bacteria under light irradiation [63].

4.1 Indirect biophotolysis by green microalgae

Few studies have been conducted on fermentative hydrogen production by green microalgae. Two representative ones are listed in Table 3 for comparison purpose. Gfeller and Gibbs grew *Chlamydomonas reinhardtii* F-60 on acetate in air under a very low light intensity (0.6 w/m^2), and the starch content reached 5.5 g glucose/g Chl or about 15.3% of dry cell mass [60]. In darkness and nitrogen atmosphere, the maximum utilization rate of starch was 2.2 mmol glucose/g Chl/hr and hydrogen evolution 0.95 mmol/g Chl/hr (Table 3). The hydrogen evolution in dark anaerobic fermentation is about one-sixth of direct biophotolysis (Table 2). This fact implies that a large portion of hydrogen energy in direct biophotolysis is originated from the light energy absorbed at PSII and/or PSI, which raises the energy level of reducing power or electrons. The fermentative hydrogen yield is quite low, about 0.43 mole of H_2 per mole of glucose utilized, and other major fermentative products include 2.07 moles of formate, 1.07 moles of acetate, 0.91 mole of ethanol and 0.04 mole of glycerol [60].

A marine green alga, *Chlamydomonas* MGA 161, was grown under air and CO_2 with light irradiation of 25 w/m^2 [64]. The starch content as glucose was about 22% of dry cell mass. Induced in dark and nitrogen gas, hydrogen evolution lasted for more than 9 hours at 0.1 mmol/g dry wt/hr or 3.7 mmol/g Chl/hr (Table 3). These values are similar to those of *Chlamydomonas reinhardtii* in direct biophotolysis (Table 2). For one mole of hydrogen evolved, the cells also released 0.8 mole of CO_2, 0.73 mole of acetate, 0.33 mole of ethanol, 0.13 mole of glycerol and a negligible amount of formate. Further utilization of the fermentative products for hydrogen evolution has been investigated by using a mixed culture of *Chlamydomonas* MGA 161 and a marine photosynthetic bacterium *Rohodovulum sulfidophilum* W-1S in photobioreactors [50]. Unfortunately, the hydrogen evolution was very low in both the dark fermenter and photobioreactor. It was attributed to formation and accumulation of polyhydroxybutyrate (PHB) from the fermentative products by *R. Sulfidophilum* [50]. It was recently reported that a photosynthetic bacterium, *Rhodobacter sphaeroid*, evolved little hydrogen under light when it was grow on fermentative products in the presence of nitrogen-containing compounds [65].

Table 3. Fermentative hydrogen evolution by cyanobacteria and microalgae in dark and anaerobic fermenters.

Organism	Maximum hydrogen evolution (mmol/g dry wt /hr)[a]	Maximum hydrogen productivity (mmol/L/hr)[b] (kJ/L/hr)[b]	Gas for growth/ Carbon/ nutrient; Light intensity (w/m^2)[c]	H evolution gas; Induction time; Carbohydrate storage (g/L)	Ref
Chlamydomonas reinhardtii	(0.96)[a]	0.13 (0.032)	Air/Acetate; 0.6	*N_2;* ~5hr dark; Starch 0.77	[60]
Chlamydomonas MGA 161	0.1	0.2 (0.048)	95% air/ 5% CO_2; 25	*N_2;* 12 hr dark; Starch 0.22	[64]
Spirulina platensis	0.11	0.18 (0.043)	Air/ N-limited; 8	*N_2;* 12-24 hr dark; Glycogen 0.81	[66]
Gloeocapsa alpicola	1.02	1.6 (0.38)	98% air/ 2% CO_2/ N-limited; 36	Argon; 24 hr dark Glycogen 1.4	[67]
Gloeocapsa alpicola	(~4.5)[a]	0.0072 (0.002)	96% air/ 4% CO_2/ S-deprived; 5	Argon; 12 hr dark Glycogen 0.024	[58]
Synechocystis PCC6803	(~3)[a]	0.0048 (0.001)	96% Air/ 4% CO_2/ S-deprived; 5	Argon; 12 hr dark Glycogen 0.02	[58]

Note: see Table 1.

4.2 Indirect biophotolysis by cyanobacteria

Compared with green microalgae, unicellular non-nitrogen-fixing cyanobacteria have attracted more research interest for hydrogen production via indirect biophotolysis. *Spirulina platensis* is a filamentous cyanobacterium cultivated at large commercial scales as a food supplement with high proteins, antioxidants and other nutrients. *S. platensis* NIES-46 accumulates a high glycogen content (50 % dry mass) in nitrogen-limited conditions and evolves molecular hydrogen at a moderate rate of 0.11 mmol/g dry wt/hr after induction in dark and nitrogen atmosphere [66] (Table 3). For one mole of hydrogen evolved, the cells also release 1.4 mole of acetate, 0.65 mole of ethanol, 0.4 mole of formate and 0.1 mole of lactate as electron acceptors.

A unicellular non-nitrogen-fixing cyanobacterium *Gloeocapsa alpicola* CALU743 showed a high activity of reversible hydrogenase induced in dark anaerobic conditions [42]. In nitrogen-limited medium, accumulation of glycogen reached 1.12 g glucose per gram of cellular proteins. High hydrogen productivity per volume of bioreactor was observed at 1.6 mmol H_2/L/hr and maintained for more than 8 hours [67] (Table 3). The maximum specific hydrogen evolution of 1.02 mmol/g dry wt/hr is similar to those of heterocystous nitrogen-fixing cyanobacteria, but much higher than those of direct biophotolysis by no-nitrogen-fixing strains in reducing gases (Table 1). In dark fermentation of one mole glucose, the strain produces 3.92 moles of H_2, 1.83 moles of CO_2, 1.97 moles of acetate, and small amounts of lactate and ethanol. The hydrogen yield is very close to the theoretical value when acetate is the sole fermentative product. Fig. 3 illustrates the homoacetic pathway that produces the maximum amount of hydrogen from anaerobic glucose (glycogen) fermentation. This metabolic pathway is quite rare among

chemoheterotrophic bacteria, but reported to occur in several cyanobacterial species [32]. The energy efficiency of the overall glucose fermentation ($C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 2CO_2$) can be roughly estimated from the heats of combustion of glucose, hydrogen and acetic acid. The energy obtained in 4 moles of hydrogen is about 40% of the energy in one mole of glucose. The remainder of glucose energy is mainly left in 2 moles of acetic acid. The overall energy efficiency would therefore be significantly improved if acetate could be reused to form endogenous carbon reserve by facultative photoautotrophic cells in photosynthesis.

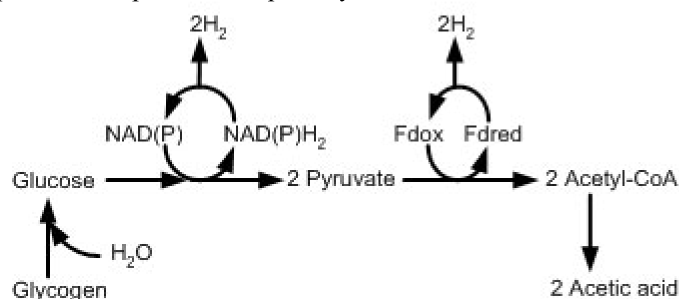

Fig. 3. A schematic homoacetic pathway in dark fermentation with a maximum hydrogen yield (4 mole H_2/mole glucose used) by cyanobacteria [32, 65, 67].

In a recent report by Antal and Lindblad [58], enhanced accumulation of glycogen in cyanobacteria, *Gloeocapsa alpicola* and *Synechocystis* PCC6803, was observed with sulfur deprivation. Hydrogen evolution increased in comparison with normal sulfur supply and glycogen content, and approached a plateau at glycogen content of 0.4 g glycogen/g protein or above. The maximum hydrogen evolution lasted 8 to 12 hours (Table 3). The cyanobacterial strains examined thus far switch immediately from photoautotrophy to fermentation when exposed to dark anoxic conditions [32], suggesting that the ability of fermentation is constitutive and that induction of new enzymes is not required. Different from green algae that only use Fe-hydrogenase and reduced ferredoxin as the substrate for hydrogen evolution [47], cyanobacteria can also induce NiFe-hydrogenase that use reduced NAD(P)H as the substrates in hydrogen evolution (Fig. 3) [67].

High volumetric hydrogen productivity is a prerequisite for high compact energy generators [68]. High cell density culture is a convenient and efficient way to increase the volumetric productivity of microbial fermentation. It results in, however, reduced hydrogen evolution [66]. This phenomenon is attributed to the reversed hydrogen oxidation under high hydrogen pressure in high cell density cultures. It implies that hydrogen evolution by hydrogenase in dark anaerobic conditions is thermodynamically unfavorable [69]. Gas sparging is widely used to maintain a low partial pressure of hydrogen in fermenter headspace, which favors hydrogen evolution [56, 67]. The highest energy productivity of cyanobacteria via indirect biophotolysis is 0.38 kJ/L/hr. It is also the highest biophotolysis-based energy productivity rate found in this review.

5. Conclusions

Hydrogen production from water photolysis under sunlight would be the cleanest and most direct energy conversion if the energy productivity rate can be improved. Direct biophotolysis, though limited by its relatively low hydrogen productivity, provides a working model for hydrogen production from water and sunlight energy. New knowledge and technical innovations in hydrogen enzymes, electron carriers, biomaterials and nanotechnology may lead to a bio-mimetic water photolysis system that overcomes the intrinsic incompatibility of simultaneous hydrogen and oxygen evolution and splits water into separated gas streams. Indirect biophotolysis, with the highest energy productivity of 0.38 kJ/L/hr found in this review, remains well behind the productivity rates of other biofuels. Ethanol fermentation, for example, has a rate of 15-30 kJ/L/hr. The relatively low energy productivity of biohydrogen can be improved if the energy stored in fermentative products, such as acetic acid, is reused. An efficient working strain could produce the maximum amount of hydrogen from endogenous carbohydrates via homoacetic dark fermentation, and then use the residual acetate for accumulation of endogenous carbon reserve in

photosynthesis. The hydrogen yield and productivity rate can therefore be significantly improved to make high compact energy generators for a future hydrogen economy.

Acknowledgements This work is partially supported by the US Department of Energy through the Hawaii Natural Energy Institute.

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