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Review

Recent Achievements in Microalgal Photobiological Hydrogen Production

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Abstract: It is well known that over the last 60 years the trend of long-lived greenhouse gas emissions have shown a strong acceleration. There is an increasing concern and a mounting opposition by public opinion to continue with the use of fossil energy. Western countries are presently involved in a so-called energy transition with the objective of abandoning fossil energy for renewable sources. In this connection, hydrogen can play a central role. One of the sustainable ways to produce hydrogen is the use of microalgae which possess two important natural catalysts: photosystem II and hydrogenase, used to split water and to combine protons and electrons to generate gaseous hydrogen, respectively. For about 20 years of study on photobiological hydrogen production, our scientific hopes were based on the application of the sulfur protocol, which indisputably represented a very important advancement in the field of hydrogen production biotechnology. However, as reported in this review, there is increasing evidence that this strategy is not economically viable. Therefore, a change of paradigm for the photobiological production of hydrogen based on microalgae seems mandatory. This review points out that an increasing number of microalgal strains other than *Chlamydomonas reinhardtii* are being tested and are able to produce sustainable amount of hydrogen without nutrient starvation and to fulfill this goal including the application of co-cultures.

Keywords: biohydrogen; microalgae; *Chlamydomonas reinhardtii*; *Chlorella* sp.; photobioreactors; light conversion efficiency

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1. Introduction

Microalgae are capable of converting light energy into chemical energy. Biofuels such as biodiesel, biohydrogen, and bioethanol can be derived from microalgae [1]. Photosynthesis in microalgae is coupled to the splitting of water and the evolution of oxygen (O_2). This process is catalyzed by the membrane-bound multi-protein complex photosystem II (PSII) [2].

It has has been known since 1942, when Gaffron and co-workers noticed that under anaerobic conditions *Scenedesmus obliquus* cells can transiently produce hydrogen (H_2) upon illumination when deprived of oxygen [3]. In microalgae, hydrogenase enzyme catalyzes H_2 production in a light-dependent process [4]. Upon illumination, after a dark incubation period, due to light-driven electron transport from ferredoxin to hydrogenase, H_2 production is observed. H_2 production in microalgae can be divided into direct or indirect processes [5]. A direct process occurs when electrons (e^-) from water splitting are transferred via PSII and ferredoxin to hydrogenase. An indirect process occurs when e-

are derived from the metabolism of carbohydrates, previously accumulated during the (light) aerobic phase, and then utilized for H_2 production via both a photo-fermentation process involving photosystem I (PSI) and in a process in the dark, involving the enzyme pyruvate:ferredoxin oxidoreductase (PFR). In *Chlamydomonas reinhardtii* (hereafter *C. reinhardtii*), PFR enzyme catalyzes the reduction of ferredoxin (Fdx) and the transfer of e^- to hydrogenase in a similar pathway to that utilized by bacteria (Figure 1) [6]. In *C. reinhardtii,* up to 92% of the final H_2 output comes from the direct photolysis coupled to the water oxidation operated by PSII [7]. Contribution of dark fermentation to the overall H_2 output is considered negligible (about 4%) in *C. reinhardtii*, but it can be significant in other microalgae such as *Chlorella,* as recently shown [8]. Microalgal hydrogenase enzymes are inactivated by the presence of molecular oxygen, and their expression is induced under anaerobic conditions.

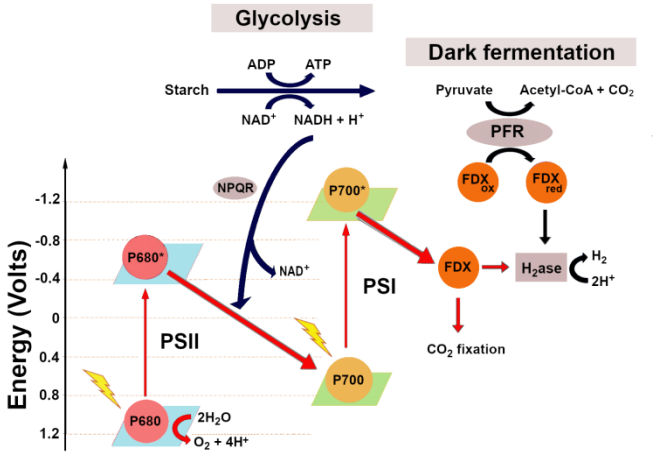


Figure 1. Metabolic hydrogen production pathways used by *Chlamydomonas reinhartii*.FDX: ferredoxin; H2ase: hydrogenase; NPQR: NADPH–plastoquinone oxidoreductase; PFR: pyruvate:ferredoxin oxidoreductase; PSI: photosystem I; PSII: photosystem II.

In recent years, energy-related H_2 demands have prompted scientists to develop methods that greatly enhance the H_2-evolving ability of microalgae. The most promising approach has been the so-called "two-stage process" of photosynthesis (stage 1) and H_2 production (stage 2) [9]. In this process, there is a separation of the reactions of oxygen and hydrogen production. This bypasses the sensitivity of the hydrogenase enzyme to oxygen. Under such conditions, it was possible to produce significant volumes of H_2 by *C. reinhardtii* in a sustained process.

Several microalgae species have been studied for H_2 production, especially *C. reinhardtii*, *Chlorella vulgaris*, and *Chlorella pyrenoidosa* [10–13]. Among them, *C. reinhardtii* is a model microorganism widely recognized as an H_2 producer, presenting a hydrogenase with an enzymatic activity 10 to 100 times higher than other species [14]. H_2 production requires many optimization steps in order to reach a sustainable process [8,14–17]. Some of these parameters include choosing a proper microalgae strain and selecting appropriate culture conditions (growth media, light, pH, temperature, chlorophyll concentration) and proper photobioreactor (PBR) designs [18–20].

Many works have reported improved H_2 production in many microalgal strains by using sulfur, phosphorus, or nitrogen-depleted media [12,21–23]. In such culture conditions, microalgae sustain H_2 production only for some days since macro/micro-nutrient depletion in the culture compromises cell viability. This is the major drawback in microalgal H_2 production processes carried out by nutrient deprivation. Microalgae-based H_2 production requires anaerobic conditions due to the sensitivity of hydrogenase to O_2 [24]. O_2 sensitivity of hydrogenase is a major issue for H_2 production; therefore, there are many studies on oxygen suppression in order to improve H_2 production yield. Genetic

and metabolic engineering of microalgae [25,26], nutrient stresses [27,28], light conditions optimization [29], and elimination of competing pathways for electrons [30] are examples of strategies used to improve H_2 evolution in microalgae.

This review provides an overview of the most relevant achievements in the photobiological production of H_2 by microalgae, and proposes a change of paradigm for the future research in the field.

2. Genetic Modification

Krishna et al. reported that sustained H_2 production is achieved by altering the ratio between PSI and PSII [31]. In this work, a *C. reinhardtii* C3 mutant with a modified PSI/PSII ratio (0.33) produced H_2 with a rate of 3mL H_2/L/d for 42 days. Chen et al. identified a *C. reinhardtii* mutant strain hpm91 lacking proton gradient regulation 5, with 30-fold H_2 production yield compared to wild type (WT) [32]. Characterization of the hpm91 strain revealed an increased reactive-oxygen-species-scavenging capacity. This translates into an enhanced stability of PSII complex and increased H_2 production yield. Steinbeck et al. investigated the capacity of *C. reinhardtii pgr5* and *pgr5 pgrl1* double mutant to produce H_2 [33]. The *pgr* mutants showed four times higher maximal enhanced H_2 production rate (7 mL/L/h) than the WT. Pinto et al. studied a *Chlamydomonas* mutant with reduced rubisco levels, activity, and stability [34]. This mutant was used to reduce carbon fixation by Calvin cycle activity, which is the main competitor for the reducing power required by the hydrogenase. In this work, the rubisco mutant presented 15 times higher H_2 production than the WT. Eilenberg et al. studied the in vivo H_2 production efficiency of a *C. reinhardtii* strain Fd-HydA containing ferredoxin fused to HydA. H_2 production rate was 4.5 times higher than that of the native HydA in vivo [35]. Torzillo et al. showed that the in vivo H_2 production of the *C. reinhardtii* mutant strain L159I-N230Y was up to 5-fold higher (16nmoles $H_2/\mu g_{chl}$/h) than that of *C. reinhartii* CC 124 [36,37]. Batyrova et al. developed a genetically modified *C. reinhardtii* strain that activates photosynthesis in a cyclical manner. In this strain, the low O_2 production benefits H_2 production [38]. In comparison with the WT, this genetically modified strain presented higher H_2 production levels. Kosourov et al. showed that a truncated light antenna *C. reinhardtii* mutant could produce six times more H_2 compared to the WT strain [39]. Xu et al. introduced a catalase gene from *Synechococcus elongatus* PCC7942 and an *Escherichia coli* pyruvate oxidase gene, both driven by a HSP70A/RBCS2 promoter, into the chloroplast of *C. reinhardtii* [40]. Under low light, these microalgal cells consumed more O_2 than WT, resulting in a lower O_2 content and increased H_2 production [40]. Kruse et al. used the *Chlamydomonas* strain Stm6, which has a modified respiratory metabolism and large starch reserves compared with the WT [41]. *Chlamydomonas* strain Stm6 presented 5–13 times increased H_2 production rate (540mL $H_2/L_{culture}$) compared to the WT [41]. Later, Volgusheva et al. obtained similar results by using the *Chlamydomonas* Stm6 mutant [42]. They attained an anaerobic condition much faster in the Stm6 strain than in the WT. This was a result of the higher respiration rate and lower initial O_2 production rate. H_2 production was four times higher in the Stm6 strain compared to the WT. Oey and co-workers reported the knock-down of the LHCMB 1, 2, and 3 proteins in the *C. reinhardtii* strain Stm6Glc4 [43]. The produced *C. reinhardtii* mutant exhibited increased light-to-H_2 and biomass conversion efficiencies of 180% and 165%, respectively. Wu et al. introduced a leghemoglobin gene (lba) into chloroplasts of *C. reinhardtii*. The genetically modified *Chlamydomonas* with lba gene consumed O_2 faster than WT, thus improving H_2 production [44]. Noone et al. introduced the clostridial hydrogenase gene into *C. reinhardtii* that contains insertionally inactivated hydrogenase genes. The presence of the more O_2-tolerant clostridial hydrogenase led to more sustained H_2 production [45].

Nowadays, the primary current challenge of such a process is the development of an oxygen-resistant hydrogenase. However, other bottlenecks may also be of significant importance, such as the oxygen sensitivity of hydrogenases. In this case, a number of other scientific and engineering issues are very likely to arise. They may include: (1) maximizing

photosynthetic light-conversion efficiency (LCE); finding the proper redox potential balance in the organism to facilitate H_2 production; (2) preventing the effect of the buildup of high relative H_2 partial pressure restricting the process by feedback inhibition; (3) addressing inefficient metabolic processes such as unneeded ATP generation during H_2 production in microalgae; (4) examining issues associated with the generation of destructive, active-oxygen species; and (5) minimizing the production of alternative, carbon-containing products that drain usable reducing power from the system. Recently, an increased H_2 output was attained by bioengineering photosynthesis [46].

In the following paragraphs, some of the most recent strategies used for sustained photobiological H_2 production by microalgae are summarized.

3. O_2 Removal

The use of inert gas (such as N_2 or Ar) is another type of strategy to remove the O_2 in microalgal cultures [47,48]. Alternatively, O_2 scavengers can be employed to remove the O_2 in order to induce anaerobiosis in the culture. Paramesh and Chandrasekhar screened three O_2 scavengers individually in order to improve H_2 production in *Chlorococcum minutum* [49]. In the presence of all three O_2 scavengers, efficient H_2 generation was found. They found that sodium sulfite was the best one for enhancement of H_2 production. Nagy et al. showed that the simultaneous addition of glucose, glucose oxidase, and ascorbate to the *C. reinhardtii* culture resulted in reduced O_2 content in the headspace and tenfold-increased H_2 production [30]. Su et al. created an O_2-consuming sandwich-like layer by using tannic acid, polydopamine, and laccase, in order to generate anaerobiosis around the *Chlorella pyrenoidosa* cells [50]. This layer enabled the encapsulated cell to switch from O_2 production to H_2 production. Márquez-Reyes et al. found that the chemical reducing agent cysteine induced anaerobic H_2 production in cultures of *Chlamydomonas gloeopara* and *Scenedesmus obliquus* cultures [51]. In the presence of cysteine, H_2 production was 5 times higher compared to the sulfur-starvation protocol. Chen and coworkers found that *C. reinhardtii* produce H_2 at a rate of 0.44 μmol H_2/h/mg_{chl} per month by using a chemoenzymatic cascade system (CEC). The CEC system contained four components: glucose oxidase, catalase, glucose, and $Mg(OH)_2$. In this CEC, they combined O_2 consumption, cell aggregation, and pH maintenance to activate hydrogenase [52]. Nagy et al. showed that the application of an iron-based O_2 absorbent ($O20_{TM}$) in *C. reinhardtii* cultures, in which the activation of the Calvin–Benson–Bassham cycle in the light was prevented, presented a H_2 production yield of 2.58 mL/L/h, to which corresponded a mean LCE (light to H_2) of 0.27% [30].

4. Co-Cultures

Another approach to create an anaerobic environment is the addition of living aerobic bacteria to the microalgae cultures (co-culture) [53]. Many works have proven the possibility of increasing H_2 production by co-culturing microalgae and bacteria [28,54–57]. The main advantage of co-culturing microalgae with heterotrophic bacteria is the efficient removal of the O_2 from the growth media. Simultaneously, the CO_2 released during bacterial fermentation of an organic substrate can support microalgae growth. Moreover, many metabolites can be exchanged between these microorganisms, such as carbon, nitrogen, phosphorous, and sulfur sources, and vitamins [58,59]. The presence of bacteria inside the microalgal culture enhances starch accumulation [60]. Different *Chlamydomonas* WT co-cultures incubated in sulfur depleted TAP medium employing *Pseudomonas* sp. or *Bradirizhobium japonicum* have achieved high H_2 production rates (165–170mL H_2/L) [54,60]. Fakhimi et al. evaluated H_2 production by *C. reinhardtii* in co-cultures with different bacteria strains [28]. They found that co-culturing *Pseudomonas* spp. with *Chlamydomonas* significantly improved microalgal H_2 production. Interestingly, the integration of the photobiological and the fermentative H_2 production in *Chlamydomonas* and *Escherichia coli* co-cultures resulted in H_2 production 60% higher than the sum of the respective monocultures [28]. *Chlamydomonas* co-cultures with *Pseudomonas* sp. and

Bradyrhizobium japonicum (not H_2-producing bacteria) in sulfur-depleted TAP medium improved H_2 production by 22.7 times and 32.3 times compared to the pure microalgal cultures, respectively [57]. Furthermore, the production of H_2 by *C. reinhardtii* in nutrient-replete cultures is strongly limited by the O_2 release, unless it is performed under very low light irradiance (lower than 20 μmol photons/m^2/s), but it may become feasible under higher light irradiance by using different consortia, which allow the maintenance of anaerobiosis conditions, thus creating an opportunity to use full medium and much higher light irradiance, enhancing the H_2 output. Finally, it must be pointed out that large-scale production of H_2 with *Chlamydomonas* and other microalgae will be necessarily carried out not with axenic cultures, but rather a microalgae–bacteria consortium, therefore understanding the complex interplay between microalgae H_2 producers and bacteria is important for the economic exploitation of an industrial H_2 production process.

5. Immobilization

Microalgae immobilization can increase the H_2 production yield [61]. The main reason for their higher performance is that such experiments are usually carried out by using a much higher chlorophyll concentration compared to liquid cultures. The process of switching between oxygenic photosynthesis (aerobiosis) and H_2 production (anaerobiosis) can be facilitated by using cell immobilization systems [62]. One of the most used materials for microalgae encapsulation is calcium alginate [63]. Immobilization in calcium alginate matrix allows high cell density and protection from mechanical stress and contamination and is easy to scale-up [64]. Immobilization of microalgal cells could increase their LCE [62]. Using *Calothrix*, *Anabaena,* and *Chlamydomonas* cells immobilized on thin calcium alginate films gave an LCE of 2.5% of the photosynthetically active radiation [48,65]. *C. reinhardtii* immobilized on calcium alginate films in nutrient-depleted cultures (-P,-S) presented a H_2 production rate of 12.5μmol/mgChl/h [66]. Ruiz-Marin et al. proposed immobilization of *Chlorella vulgaris* and *Scenedesmus obliquus* cells in calcium alginate for the production of H_2 [11]. These microalgae were grown in urban wastewater under sulfur starvation and blue or purple light conditions. The maximum H_2 production obtained under red light was 204.8mL H_2/L/d for *Scenedesmus obliquus* and 39.1mL H_2/L/d for *Chlorella vulgaris* [11]. Maswanna et al. studied H_2 production by *Tetraspora* sp. CU2551 cells immobilized in a 4% w/v calcium alginate matrix in their recent work. They obtained a maximum H_2 production rate of 182±20 nmol/mg of cell dry weight/h [67].

Our group recently tested the capability of immobilized *Chlorella vulgaris* (BEIJ G-120 strain) cells in a calcium alginate (3%w/v) gel matrix to produce H_2 in a direct light-driven process under continuous illumination. Calcium alginate beads were stable, showing minimal cell leakage, and they measured 4.69±0.02mm in diameter and 54.01±0.03μL in volume, carrying 145.5±8.9μg of microalgal cells (biomass dry weight/bead) (Figure 2). Immobilized cells retained their viability for more than 30 days (Figure 3A). Immobilized *Chlorella* cells were capable of generating H_2 without nutrient deprivation with a maximum rate of 162mL/L (Figure 3B). Anaerobiosis was maintained by the presence of glucose and the high respiration rate of the strain.

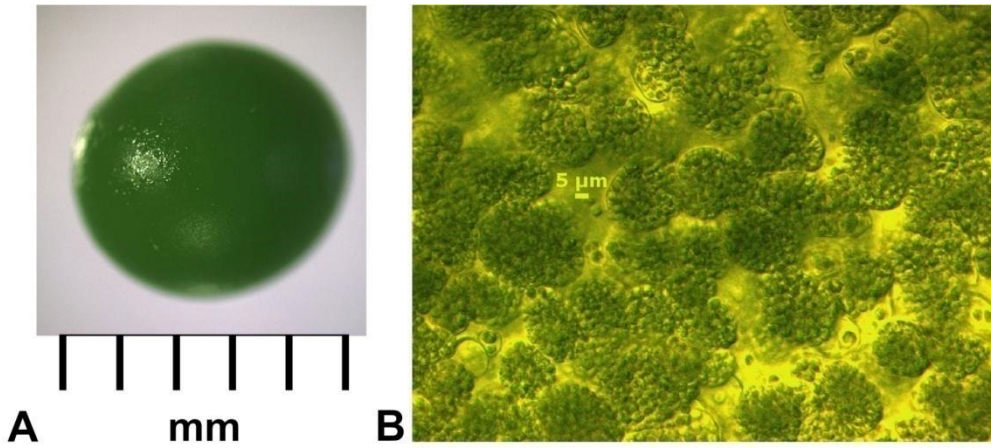


Figure 2. *Chlorella vulgaris* (BEIJ G-120 strain) immobilized in calcium alginate beads. (**A**) Calcium-alginate bead; (**B**) *Chlorella vulgaris* cells inside the calcium-alginate beads.

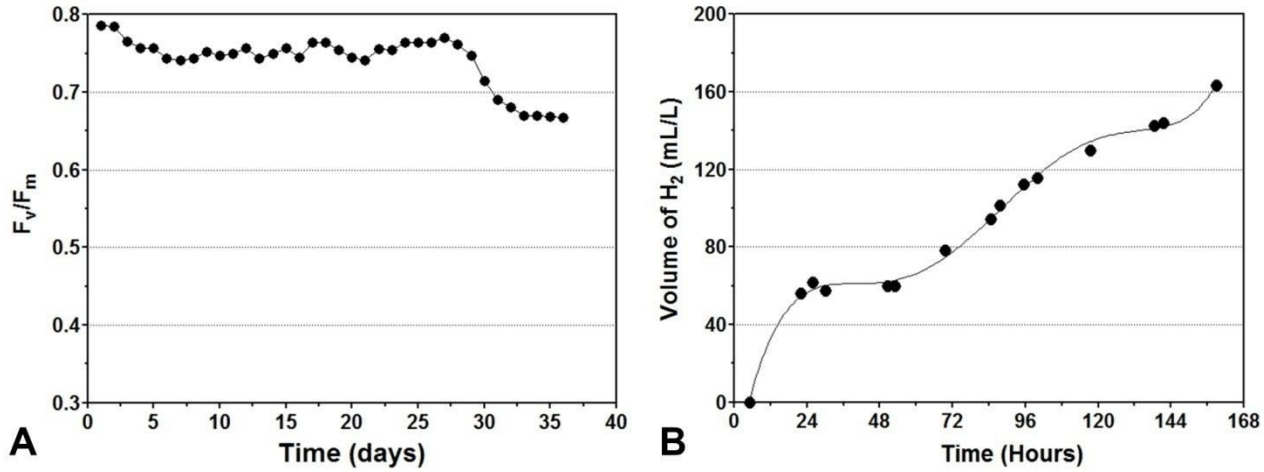


Figure 3. (**A**) F_v/F_m of immobilized *Chlorella vulgaris* cells as a function of time; (**B**) H_2 production of the immobilized *Chlorella vulgaris* cells as a function of time.

6. Hydrogen Production without Nutrient Starvation

The H_2 production protocol by Melis and coworkers based on sulfur starvation greatly improved light-driven, algal H_2 production, and particularly the possibility for researchers to study the process [68]. However, in the recent years it has become clear that it is not adequate for an industrial development of the process since it requires one to eliminate sulfur residues. Moreover, the severe reduction of PSII activity caused by the sulfur deprivation greatly reduces the H_2 production and thus the viability of the process. Awareness of these limits has prompted several workers to eliminate the sulfur deprivation phase by selecting strains with high respiration-to-photosynthesis ratios.

Liu et al. presented a work on H_2 production of *Chlorella pyrenoidosa* using $NaHCO_3$ as a carbon source and N′-(3,4-Dichlorophenyl)-N,N-dimethylurea (DCMU) [69]. In this work, *Chlorella pyrenoidosa* cells showed an overall H_2 production of 93.86mL/L. In a recent work, Li et al. constructed a transgenic *C. reinhardtii* strain (amiRNA-D1) with a heat-inducible expression system targeting D1 gene (*psbA*). After a heat-shock, the transgenic *C. reinhardtii* strain presented a 73% decrease of *psbA* gene expression and a 60% increase of H_2 content compared to the WT strain [70]. Ben-Zvi et al. explored the in vivo H_2 production of HydA–SOD fusion phenotype in *C. reinhardtii* and found that expression of an active hydrogenase superoxide dismutase fusion protein resulted in sustained H_2 production with a rate of 20mL H_2/L/d for 8 days [71]. Hwang et al. showed that the over-expression of the hydrogenase gene in *Chlorella vulgaris* resulted in H_2 production under

aerobic conditions with continuous illumination using CO_2 as the sole source of carbon [72]. Under 5% O_2 and 10% CO_2, *Chlorella vulgaris* strains YSL01 and YSL16 produced 1.9mL H_2/h and 1.2mL H_2/h in 3 and 4 days, respectively. In another of their works, this group studied and compared the photosynthetic activities of *C. reinhardtii* and *Chlorella sorokiniana* with different acetate/Cl^- ratios [73]. They found that maintaining acetate/Cl^- ratios greater than 60–100 led to continuous O_2 depletion. Using fermenter effluents, at an acetate/Cl^- ratio of 150, *Chlorella sorokiniana* and *C. reinhardtii* presented an H_2 production rate of 0.25–0.33mmol/L/min and 0.20–0.38mmol/L/min, respectively. Kosourov et al. demonstrated sustained H_2 production by *C. reinhardtii* by shifting the culture light conditions from continuous illumination to a set of light pulses interrupted by longer dark phases [29]. In a recent work, Sirawattanamongkol et al. demonstrated that *Chlorella* sp. strain KLS Sc59 was able to produce up to 750mL H_2/L in the presence of reducing agents such as ethanol and dithionite [74]. H_2 production rates in various microalgae strains are summarized in Table 1.

Table 1. Comparison of H_2 production rates in various microalgae strains.

Microalgal Species	Growth Mode	H_2 Production	References
C. reinhardtii	TAP	25 μmol/mg_{Chl}/h	[29]
Chlorella sp. AARL G014	TAP-S	0.49 mmol/mg_{Chl}/h	[75]
C. reinhardtii CC-503	TAP co-culture	255 mmol/mg_{Chl}	[55]
Chlorella vulgaris strains YSL01	BBM-EDTANa_2	1.9 mL/L	[72]
Chlorella lewinii KU201	TAP-S	13.03 mL/L	[18]
Chlorella sp. IOAC707S	TAP-NaCl	38.00 mL/L	[38]
Chlorella sorokiniana KU204	TAP-P	69.00 mL/L	[18]
Chlorella protothecoides	TAP-NS	82.50 mL/L	[76]
Chlorella sorokiniana KU204	TAP-S	89.64 mL/L	[18]
Chlorella pyrenoidosa	TCP + DCMU	93.86 mL/L	[69]
C. reinhardtii Stm6	TAP-S	540 mL/L	[41]
C. reinhardtii C3	TAP	3.0 mL/L/d	[31]
C. reinhardtii (HS-14)	TAP	20 mL/L/d	[71]
Immobilized *Chlorella vulgaris*	Artificial wastewater-S	39.1 mL/L/d	[11]
Chlorella vulgaris MACC360	TAP co-culture	56.0 mL/L/d	[77]
Immobilized *Scenedesmus obliquus*	Artificial wastewater-S	204.8 mL/L/d	[11]
Chlorella salina Mt	TAP-S	0.5 mL/L/h	[78]
C. reinhardtii CC124	TAP-S	0.6 mL/L/h	[79]
C. reinhardtii CC-124	TAP-S	3.3 mL/L/h	[80]
C. reinhardtii pgr5/pgrl1	TAP-S	7.0 mL/L/h	[33]
C. reinhardtii L159I-N230Y	TAP-S	11.1 mL/L/h	[37]
Chlorella vulgaris BEIJ (G-120)	HM + glucose	5.0 mL/L/h.	[8]
Immobilized Chlorella vulgaris NIER-10003	MA-S + glucose	238 mL/L/h	[81]
Chlorella sorokiniana	150 of acetate/Cl^- ratio	0.33 mmol/L/min	[73]
C. reinhardtii	150 of acetate/Cl^- ratio	0.38 mmol/L/min	[73]
C. reinhardtii (ΨH1)	TAP	3.6 mL/L/h	[46]
C. reinhardtti	HSM + O_2 absorbent	2.58 mL/L/h	[30]

In our recent work, we reported H_2 production by *Chlorella vulgaris* (strain BEIJ G-120) without the use of nutrient deprivation [8]. This *Chlorella* strain presents two main properties: high respiration rate and high light compensation point. By exploiting these two properties, it was possible to efficiently consume the photosynthetically produced O_2, thus maintaining anaerobiosis, even under light conditions. In this work, *Chlorella* cells presented a maximum H_2 production rate of 12mL/L/h and an average rate of 4.98mL/L/h.

The strain was capable of producing H_2 in the dark as well, by fermentation of glucose. The excessive accumulation of byproducts of the fermentation (e.g., acetate, formate, lactate, ethanol) may inhibit H_2 production. However, the possibility of also producing H_2 in the dark by microalgae is desirable for the development of the process under natural light/dark cycle. On the other hand, some of the byproducts of dark fermentation, such as acetate, can be used as substrate for mixotrophic grown during the following light phase.

7. Theoretical Limit for Biological Hydrogen Production

Only a small fraction of the total solar light radiation (>1,100,000 EJ per year) can potentially be transformed into H_2 energy using the process of photosynthesis, according to the following general equation:

$$2H_2O + 8\ photons = O_2\uparrow + 4e^- + 4H^+ = 2H_2\uparrow$$

Step 1 indicates the total incident radiation received at the surface of the culture (100%) (Figure 4). It follows that:

1. Approximately 10% is lost by reflection and scattering (90% of initial remaining).
2. Approximately 55% of radiation is not available to drive photosynthesis since it falls outside of the photosynthetically active radiation (400–700 nm) and thus is not utilized by photosynthetic pigments. As a result, the total amount of available light drops to 41%.
3. About 20.4% of the radiation is lost as heat [82].
4. Assuming as quantum requirement that 8 photons are required to produce 2 mol of H_2, and considering that 1mol of H_2 is 286 KJ, and the mean energy for charge separation at PSII and PSI is 173.5KJ/mol, it follows that the efficiency of the process will be the following: (286KJ/mol×2)/(173.5KJ/mol×8)×100=41.2%, with a corresponding loss of energy of 59%. Consequently, the theoretical LCE for H_2 production, attainable by direct biophotolysis is about 13.4% of incident solar light [83].
5. With a LCE of about 10%, assuming that approximately 20% of the energy can be lost for cell maintenance, it might be possible to produce about 600.000m^3/ha/y of H_2 in sunny areas.

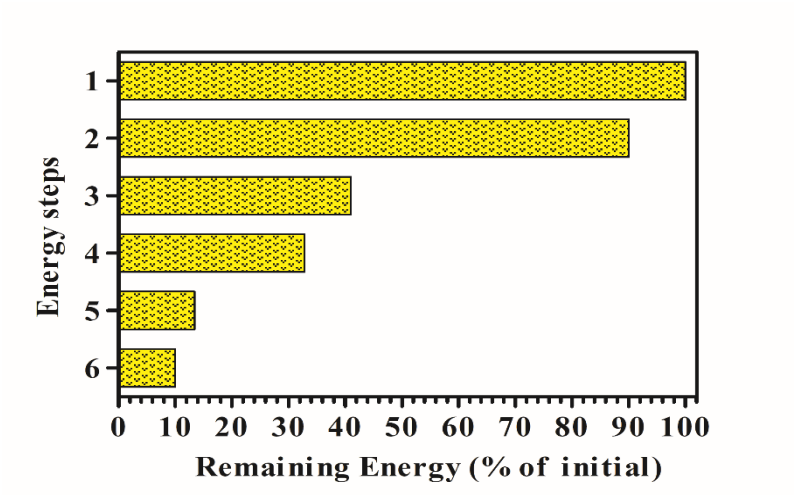


Figure 4. The energy losses of the incident solar light during the different steps of the photobiological H_2 production process.

8. Chlorophyll Fluorescence Measurements as a Tool for Monitoring Changes of Photochemical Efficiency during the Hydrogen Production Process

Chlorophyll fluorescence is a fast and non-invasive tool for monitoring residual photosynthetic activity during the H_2 production process through changes in the

maximum quantum yield of PSII (F_v/F_m) and the effective quantum yield ($\Delta F/F'_m$) [38]. In particular, it has been observed that in *C. reinhardtii*, at the occurrence of anaerobiosis, the value of $\Delta F/F'_m$ rapidly declined, and this drop could be ascribed to the state 1 to state 2 transition, controlled by the redox state of plasto-quinone (PQ)-pool [36,84–86]. This mechanism regulates the migration of the light-harvesting complex (LHC) from PSII (state 1) to PSI (state 2), and it is induced under high level of PQ-pool reduction and excess of light energy. The start of the H_2 production induces a partial oxidation of the photosynthetic electron chains, comprising PQ-pool, with a partial recovery of $\Delta F/F'_m$. In *C. reinhardtii*, migration of LHC can involve up to 80% of the total LHC. The redox potential of the cells represents another important parameter related to the cell physiology under anaerobiosis, as it is the result of a balance between starch degradation, the capacity of PSII to perform photosynthesis, and the ability of cell to dissipate electrons from PQ-pool. Indeed, after establishing anaerobiosis, the value of the redox potential changes from a positive initial value to a very low value (about –550mV in *C. reinhardtii*). The changes of the values of the redox potential lag behind the changes in the yield and are less rapid that the chlorophyll fluorescence changes. In Figure 5, an example of the typical kinetics of chlorophyll fluorescence yield and redox potential in the different phases of the H_2 production process is reported.

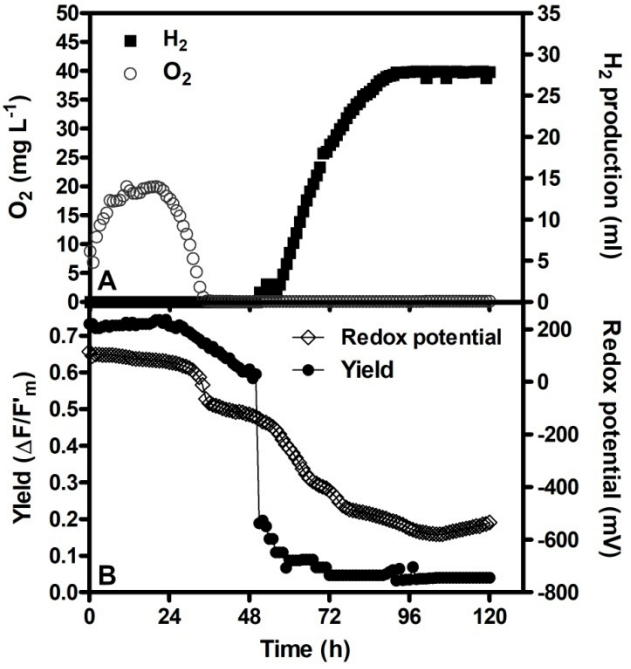


Figure 5. *C. reinhardtii* kinetics of chlorophyll fluorescence and redox potential during induction of H_2 production under sulfur deprivation . (**A**) Time courses in dissolved oxygen (empty circle) and output of hydrogen (H_2) (filled square). (**B**) The time courses in the effective quantum yield of PSII ($\Delta F/F'_m$) (filled circle) and the redox potential (Eh) (empty diamond) in *C. reinhardtii* under sulfur deprivation with 70μmol photons/m^2/s, supplied on both sides of the reactor.

Other important information on the changes of the photosynthetic efficiency can be provided by the chlorophyll fluorescence rise kinetics (OJIP curve), strictly reflecting the progressive reduction of the photosynthetic electron transport chain [60,87], which can indicate and quantify the reduction of electrons transport for each step [88]. The most evident change occurs at the J-step level, indicating the reduced transfer of electrons further than Q_A, measured by V_J parameter, and thus, an accumulation of reduced Q_A^- [88]. An example of the changes of the shape of the OJIP curve during the occurrence of anaerobiosis in *C. reinhardtii* is reported in Figure 6.

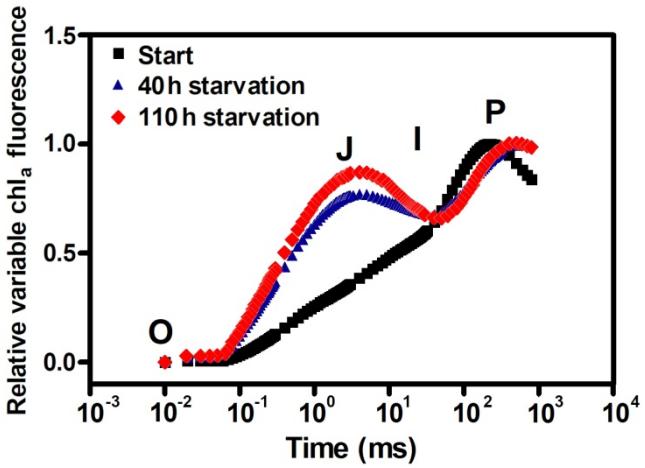


Figure 6. Effect of sulfur deprivation on the chlorophyll a fluorescence transient in *C. reinhardtii* cultures. Start (dark squares); 40h of sulfur deprivation (blue triangles); 110h of sulfur deprivation (red diamonds). Relative variable fluorescence ($V_t=(F_t-F_0)/(F_m-F_0)$) [89].

In *Chlorella sorokiniana,* nutrient starvation conditions reduce photosynthetic activity and induce anaerobiosis and H_2 production, indicated by the decrease of both F_v/F_m and $\Delta F/F'_m$. Similarly, to what was observed in *C. reinhardtii*, the maintenance of a residual PSII activity provides electron to hydrogenase enzyme [90]. The same behavior was observed in *Chlorella vulgaris* without nutrient starvation with a strain able to reach anaerobiosis in complete medium, showing a decline of F_v/F_m and $\Delta F/F'_m$ within 24 h [8].

In conclusion, the use of fluorescence measurements to monitor changes in photosynthetic activity can help us to better understand the physiological status of microalgae during the H_2 production process, making it easier to interfere in the cell metabolism or enhance the production process. Moreover, the application of chlorophyll fluorescence helps in selecting strains more resistant to the stress imposed by anaerobic conditions, and with higher potential H_2 output.

9. Photobiological Hydrogen Production in Outdoor Photobioreactors

Until now, H_2 production experiments using *C. reinhardtii* have been carried out mostly under laboratory conditions. Mean LCE in sulfur-deprived laboratory cultures grown in well-mixed PBR has hardly surpassed 1% (light to H_2). The necessity to downregulate the PSII activity to the level of the respiration is considered the main reason for such a low efficiency. As a matter of fact, the LCE strongly increased when it was possible to use microalgal strains with high respiration-to-photosynthesis ratio. This was the case of the *Chlorella* strain G-120, which averaged 3.2%, over the 8-day period [8].

The utilization of solar energy is mandatory for the economical scale-up of the H_2 production process. However, under solar light, the light energy received by microalgae cells exceeds their ability of light conversion into valuable biomass. This leads to either energy dissipation as heat or to photodamage and cell death, which strongly reduce the LCE. To reduce the "saturation effect", a number of PBR designs have been proposed [91]. Torzillo and coworkers reported H_2 production of about 21% of that attained under laboratory in an outdoor 50L tubular PBR using *C. reinhardtii* under sulfur deprivation (Figure 7) [92]. The PBR consisted of ten glass tubes (2.0m length and 4.85cm internal diameter) placed horizontally and connected by polyvinylchloride U-bends (Figure 7). The PBR was placed in a stainless-steel container with temperature-controlled water. A polyvinyl chloride pump allowed the culture to circulate.

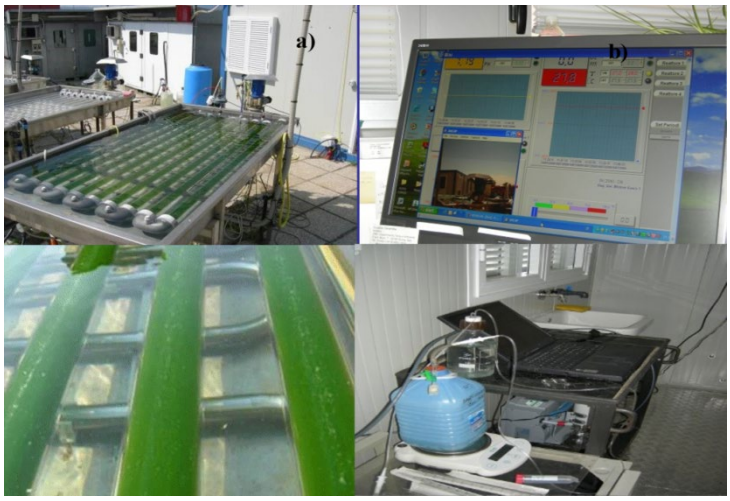
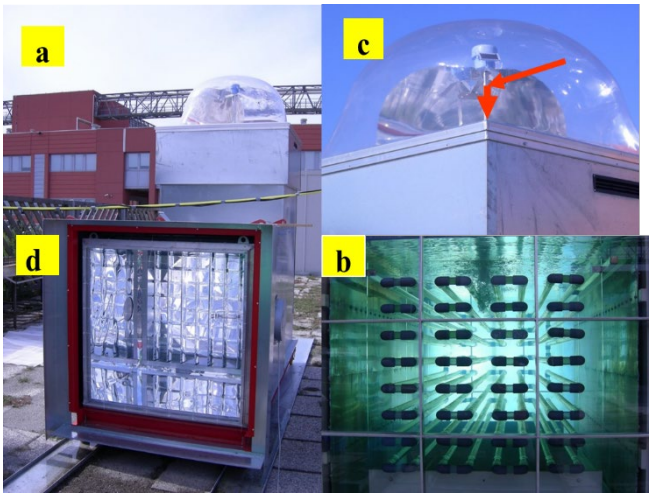


Figure 7. The tubular photobioreactor (working volume 50 L) used for the outdoor H_2 production experiments. The photobioreactor was equipped with probes for measurement and control of pH and temperature. Culture speed can be adjusted to reach the desired turbulence.

The low performance of the culture was explained by the rapid PSII inactivation by the high light irradiation, which during the experiments reached as much as 1850 μmol photons/m^2/s in the middle of day. In order to avoid the problem of light saturation, Giannelli and Torzillo (2012) [79] proposed a 110L PBR in which the culture tubes were immersed in water with light-scattering silica nanoparticles. The PBR contained 64 glass tubes (length 2.0m, internal diameter 27.5mm) arranged on an 8×8 square pitch cell and connected by polyvinylchloride U-bends. The PBR was immersed in a rectangular parallelepiped tank made of isotactic polypropylene, except for the opposite square faces, which were made of transparent Plexiglas. The culture was circulated with a peristaltic pump. The light scattering promoted by nanoparticles permitted a homogeneous distribution of light on the surface of the PBR (Figure 8). Solar light was collected by two sun-tracking mirrors, which delivered light to the opposite faces of the reactors through two light ducts (Figure 8c).



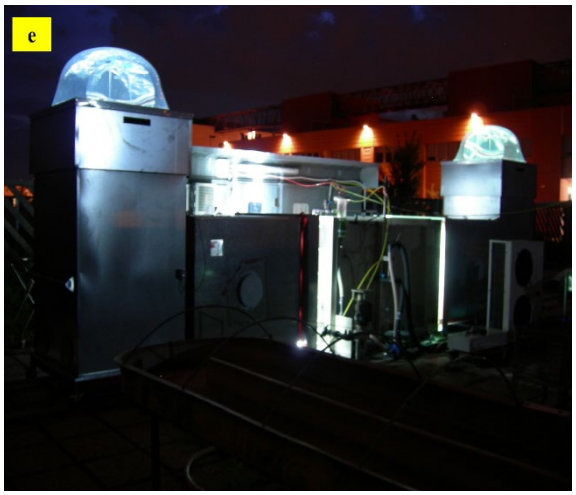


Figure 8. The 110 L photobioreactor utilized for the hydrogen production experiments. (**a**) General view of the photobioreactor; (**b**) frontal view of the photobioreactor; (**c**) sun-tracking mirrors collecting light; (**d**) light ducts delivering light to the photobioreactor from the opposite sides; (**e**) nocturnal view of the photobioreactor with two light bulbs (1000W each) placed in the light duct to provide illumination to the culture during the night.

The total amount of H_2 collected reached 3.5 L, which was almost 2-fold higher than that obtained with the 50L tubular PBR per unit of volume of reactor. Furthermore, in the scaled-up PBR, the LCE of the process increased from 0.055% in the 50L horizontal PBR to 0.213% in the 110L PBR, which was much closer to that attained in the laboratory with sulfur-deprived *C. reinhardtii* cultures. This still-low efficiency was the result of a number of factors: (i) the necessity to carry out the process according to the two-step protocol (sulfur starvation), which strongly reduces the contribution of PSII; (ii) the need to attain sulfur starvation by culture dilution, which normally yields lower H_2 output; and (iii) the longer mixing time of tubular PBRs, which delays reaching H_2-saturation levels in the cultures.

10. Optimal Photobioreactor Design for the Hydrogen Production

The experience acquired from experiments with different indoor and outdoor PBRs has provided some guidelines useful for the optimal design of future PBRs for H_2 production [93,94]. An in-depth discussion on the influence of the PBR design on the H_2 output can be found in a recent book on the subject of H_2 production [94].

The development of an economically feasible PBR system is the most important factor for successful H_2 production. Closed PBRs (such as flat-panel and tubular) are mandatory for H_2 production. Tubular PBRs contain one or more glass tubes arranged in various configurations and orientations. Flat-panel PBRs consist of one or more transparent panels containing the culture, which is circulated between the panels by a pump. Flat-panel PBRs probably meet most of the above-mentioned requirements for H_2 production. They present high area-to-volume ratio and good biomass productivity, and shorter mixing time compared to tubular PBRs, which reduces the risk of H_2 oversaturation in the reactor. A drawback is the difficulty of controlling the temperature, and the high power consumption for mixing. Although the setup costs of closed PBRs are high, they provide several advantages, such as optimal growth, minimizing the risk of culture contamination, and reduced water and CO_2 consumption.

The International Energy Agency established the commercial cost target for H_2 production at 0.3USD/kg. According to James et al., with 10% LCE, the cost could be 2.99USD per gallon of gasoline equivalent [95]. The authors estimated that by using robust microalgae strains presenting 1.5% LCE, the cost of H_2 would be 8.44USD per gallon of gasoline equivalent. Greater costs for PBR construction are materials, manufacturing, and personnel costs. H_2 production prices should be more promising than market prices. Show

et al. showed that the costs of fabrication materials and chemical nutrients are the main expenses (84% of the total cost) for PBR development [96]. Recycling metabolic products of PBRs (such as organic acids) and/or considering potentially cheaper nutrient sources are possible ways to reduce the chemical cost of nutrients necessary for microalgal growth [97]. Finally, production cost based on direct bio-photolysis, were estimated to be, 18.45 $/kg$H_2$, for the Netherlands, which is expected to drop significantly in the future (potential cost of 3.10 $/kg$H_2$) [98].

11. Concluding Remarks

This review provides evidence that photobiological H_2 production by microalgae and cyanobacteria might be a viable option. The discovery of the sulfur starvation method has allowed maintenance of H_2 production for several days, and thus, it has represented an opportunity to study the process beyond scientific curiosity.

Nowadays, however, a substantial change of paradigm in photobiological H_2 production is necessary. The opportunity to improve the economic feasibility of the process could come from employing strains which do not need sulfur starvation. These strains feature a high respiration-to-photosynthesis ratio and a higher level of saturation irradiance compared to the *C. reinhardtii* strains currently available. These characteristics are usually found in microalgal strains with reduced antenna size, which is a very important biotechnological condition to allow penetration of the light deep through the culture layers [99–102]. Important achievements could be expected from microalgal cultures growing both mixotrophically and heterotrophically in PBRs and fermenter, respectively, in the presence of glucose. A *Chlorella* strain with such characteristics was recently studied by us under laboratory conditions, but its better H_2 performance needs to be proved under solar light in PBRs. Of course, the use of an expensive source such as glucose as a respiratory substrate to maintain anaerobiosis, and thus the functioning of the hydrogenase, halves the efficiency of the process and strongly reduces its sustainability. Therefore, it will be important to consider the potential of much cheaper sources of organic substrates such as wastewater from sugar factories, baker's yeast, and breweries. The O_2 consumption, through respiration of organic substrates, produces H_2 with high purity (close to 98%), which strongly reduces the investment cost for H_2 purification. The use of molasses, which is very rich in glucose and sucrose, could represent an option.

In conclusion, until a hydrogenase resistant to oxygen is discovered, the selection of strains with higher resistance to oxygen and/or with high respiration-to-photosynthesis ratio represent nowadays the only realistic possibility for the success of photobiological H_2 production. This research should proceed in parallel with efforts to engineer organisms with O_2-resistant hydrogenases. Success in either direction will lead to expected improvements in technologies to: (1) increase effective conversion efficiency of photosynthesis; (2) reduce or possibly eliminate competing pathways, such as CO_2 fixation; (3) increase starch biosynthesis.

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