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Green alga hydrogen production: progress, challenges and prospects

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1. Prologue

The ability of green algae to photosynthetically generate molecular hydrogen has captivated the fascination and interest of the scientific community for the past 60 years due to the fundamental and practical importance of the process. In nature—under relevant physiological conditions—the photosynthetic activity of the green alga "hydrogenase" was only transient in nature. It lasted from several seconds to a few minutes due to the fact that photosynthesis and H_2O-oxidation entail the release of molecular O_2. Oxygen is a positive suppressor of hydrogenase gene expression, and a powerful inhibitor of the [Fe]-hydrogenase. Given the acute oxygen sensitivity of the hydrogenase and the prevailing oxidative environmental conditions on earth, questions have been asked as to whether the hydrogenase is anything more than a relic of the evolutionary past of the chloroplast in green algae, and whether this enzyme and the process of photosynthesis can ever be utilized to generate hydrogen for commercial purposes [1].

Deprivation of sulfur-nutrients in green algae causes a reversible inhibition in the activity of oxygenic photosynthesis. In the absence of sulfur from the growth medium, protein biosynthesis is impeded, and the green algae cannot perform the required turnover of the photosystem-II (PSII) D1/32 kDa reaction center protein [2]. Under S-deprivation, the photochemical activity of PSII declines, and rates of photosynthetic oxygen evolution drop below those of oxygen consumption by respiration [3]. In consequence, sealed cultures of the green alga *Chlamydomonas reinhardtii* become anaerobic in the light. Following anaerobiosis, they spontaneously induce a novel "hydrogenase pathway" of electron transport in the chloroplast and photosynthetically produce hydrogen. For the first time, substantial rates of hydrogen production were steadily sustained for about 60 h in the light, but gradually declined thereafter. In the course of such hydrogen production, cells consumed significant amounts of internal starch and protein [4]. Such catabolic reactions may sustain, directly or indirectly, the hydrogen-production process. The use of green algae in this "two-stage photosynthesis and H_2-production" method does not entail the generation of toxic or environmentally disturbing byproducts, and it may even offer the advantage of value-added products as a result of the mass cultivation of green algae. The work discusses the physiology, biochemistry and molecular biology that underline this sustained green alga hydrogen production process.

In spite of the recently achieved significant breakthrough in H_2-photoproduction, rates were only about 15% of the biological theoretical maximum, suggesting room for substantial improvement in the yield of the process. Similarly, other improvements must be made to maintain the continuity of production and to optimize the solar conversion efficiency of the algae under mass culture conditions. The continuity of the process needs to be addressed, as H_2-production by S-deprivation of the algae is time limited. The yield begins to level off after about 60 h of production. After about 100 h of S-deprivation, the algae need to go back to normal photosynthesis in order to be rejuvenated by replenishing endogenous substrate [5]. Furthermore, optical problems associated with the size of the chlorophyll antenna and the light-saturation curve of photosynthesis must be addressed [6] before the relevant green algae can achieve high photosynthetic solar conversion efficiencies in mass culture. Additional challenges that must be successfully addressed include ways for the recycling of photobioreactor components and minimizing the cost of the alga growth nutrients, as these two items constitute 80–85% of the overall cost of a commercial H_2-production operation. There is a need to form a working relationship between the scientists who develop microalga-based applications and the energy-related industries. In each of these areas, the work summarizes the

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progress achieved so far, provides an assessment of the state of the art in photobiological H_2-production, and offers a discussion of the challenges that have been encountered. Finally, it offers a view of the prospects and promises of Bio-Hydrogen in general, and photobiological hydrogen in particular, as important players in the coming Hydrogen Age.

2. Perspective

Hydrogen metabolism is primarily the domain of bacteria and microalgae. Within these groups, it involves many taxonomically diverse species, a variety of enzymes and metabolic pathways and processes [7–9]. The photosynthetic metabolism of hydrogen in green algae was discovered by Hans Gaffron [10–14] who observed that, under anaerobic conditions, green algae can either use H_2 as electron donor in the CO_2 fixation process in the dark, or evolve H_2 in the light. Gaffron's original observations were extended to many green algae, including *Scenedesmus obliquus* [14–16], *Chlorella fusca* [17–20], and *Chlamydomonas reinhardtii* [21–24], among others.

Historically, hydrogen evolution activity in green algae was induced upon a prior *anaerobic incubation* of the cells *in the dark* [17,25–27]. A hydrogenase enzyme [9] was expressed under such incubation and catalyzed, with high specific activity, a light-mediated H_2-evolution. The monomeric form of the enzyme, reported to belong to the class of [Fe]-hydrogenases [28–36], is nuclear encoded. However, the mature protein is localized and functions in the chloroplast stroma of the unicellular green algae [31]. Light absorption by the photosynthetic apparatus is essential for the generation of molecular hydrogen, since light-energy facilitates the endergonic transport of electrons to ferredoxin. Photosynthetic ferredoxin is the physiological electron donor to the [Fe]-hydrogenase and, therefore, links the soluble [Fe]-hydrogenase to the electron transport chain in the green alga chloroplast [35,37]. The absence of CO_2 enhanced the light-driven H_2-production, suggesting a competition for electrons between the CO_2-fixation and the H_2-production processes [38].

The ability of green algae to photosynthetically generate molecular H_2 has captivated the fascination and interest of the scientific community because of the *fundamental* and *practical* importance of the process [1]. Below is an itemized list of the properties and promise of photosynthetic H_2-production, and the challenges that are encountered in the process:

- Photosynthesis in green algae can operate with a photon conversion efficiency of $\geqslant 80\%$ [39].
- Microalgae can evolve H_2 photosynthetically, with a photon conversion efficiency of $\geqslant 80\%$ [40].
- Molecular O_2 acts as a powerful and effective switch by which the H_2-production activity is turned off.
- This incompatibility in the simultaneous O_2 and H_2 photoproduction remained a problem in 60 years of related research.

3. Photobiological H_2-production

3.1. $2H_2O \rightarrow 2H_2 + O_2$

3.1.1. State of the art

The process of photosynthetic H_2-production with electrons derived from H_2O (also referred to as "biophotolysis" [41,42]) entails H_2O-oxidation and a light-dependent transfer of electrons to the [Fe]-hydrogenase, leading to the synthesis of molecular H_2. Electrons are generated upon the photochemical oxidation of H_2O by PSII. These are transferred through the thylakoid membrane electron-transport chain and, via PSI and ferredoxin, are donated to the HC cluster of [Fe]-hydrogenase [35]. Protons (H^+) are the terminal acceptors of these photosynthetically generated electrons in the chloroplast. The process does not involve CO_2-fixation or energy storage into cellular metabolites. This process results in the simultaneous production of O_2 and H_2 with a H_2: $O_2 = 2$:1 ratio [24,43]. This mechanism holds the promise of generating hydrogen continuously and efficiently through the solar conversion ability of the photosynthetic apparatus.

3.1.2. Challenges

In the absence of provision for the active removal of oxygen, this mechanism can operate only transiently, as molecular oxygen is a powerful inhibitor of the enzymatic reaction and a positive suppressor of [Fe]-hydrogenase gene expression. At present, this direct mechanism has limitations as a tool of further research and for practical application, mainly due to the great sensitivity of the [Fe]-hydrogenase to O_2, which is evolved upon illumination by the water-oxidizing reactions of PSII [5]. An additional problem, assuming that the mutual incompatibility of O_2 and H_2 co-production is overcome, entails the separation of the two gases, a costly and technologically challenging feat.

3.1.3. Prospects

Nevertheless, it has been shown that such O_2 and H_2 co-production can be prolonged under conditions designed to actively remove O_2 from the reaction mixture. Indeed, Greenbaum and co-workers [24,25,40] have sustained a photosynthetic $H_2O \rightarrow H_2$ process continuously for days upon sparging the reaction mixture with helium, thus removing from the vicinity of the cells the photosynthetic gas products (O_2 and H_2). Along this line, efforts are under way to mutagenize the [Fe]-hydrogenase with the objective of altering or removing the oxygen sensitivity of the enzyme [5], thereby permitting a light-driven O_2 and H_2 co-production in the green algae.

3.2. Endogenous substrate → H_2

3.2.1. State of the art

Apart from the above described PSII-dependent H_2-photoproduction, which involves H_2O as the source of electrons and, in the absence of CO_2, produces 2:1 stoichiometric amounts of H_2 and O_2, an alternative source of electrons has been described in the literature. Catabolism of endogenous substrate and the attendant oxidative carbon metabolism in green algae may generate electrons for the photosynthetic apparatus [44]. Electrons from such endogenous substrate catabolism feed into the plastoquinone pool between the two photosystems [45,46]. An NAD(P)H-plastoquinone oxidoreductase that feeds electrons into the plastoquinone pool has recently been identified in many vascular plant chloroplasts [47–52] but so far only from the green alga *Nephroselmis olivacea* [53]. Light absorption by PSI and the ensuing electron transport elevates the redox potential of these electrons to the redox equivalent of ferredoxin and the [Fe]-hydrogenase. In this case, protons (H^+) act as the terminal electron acceptor [44,54], thus permitting the generation of molecular H_2 [55]. In the presence of DCMU, a PSII inhibitor, this process generates 2:1 stoichiometric amounts of H_2 and CO_2 [56]. Thus, following a dark-anaerobic incubation of the culture (induction of the [Fe]-hydrogenase), initially substantial rates of H_2-production can be detected upon illumination of the algae in the presence of DCMU [31,35].

3.2.2. Challenges and prospects

The regulation of endogenous substrate catabolism and the attendant supply of electrons to the electron transport chain of photosynthesis are not well understood. Whereas rates of H_2O oxidation by the photosynthetic apparatus can be measured continuously and precisely, measurements of electron transport supported by endogenous substrate catabolism and NAD(P)H-plastoquinone oxidoreductase activity are more difficult to make. H_2-photoproduction with anaerobically incubated and DCMU-poisoned chloroplasts [35] suggests that, initially, substantial rates of H_2-production can be detected. However, this process could not be sustained for significant periods of time [4], suggesting limitation(s) in the capacity of the electron transport reactions associated with the NAD(P)H-plastoquinone oxidoreductase activity. Nevertheless, the prospect of endogenous starch, protein and lipid catabolism feeding electrons into the plastoquinone pool—and thus contributing to H_2-photoproduction—is important enough to warrant further investigation to fully assess its potential. In this respect, the tools of molecular biology could come to bear in efforts to increase the capacity of this important process.

3.3. Two-stage photosynthesis and H_2-production

3.3.1. Progress

Recent progress has shown that lack of sulfur nutrients from the growth medium of *C. reinhardtii* causes a specific but reversible decline in the rate of oxygenic photosynthesis [2], but does not affect the rate of mitochondrial respiration [3]. In sealed and S-deprived cultures, the absolute activity of photosynthesis becomes less than that of respiration. Such imbalance in the photosynthesis–respiration relationship by S-deprivation resulted in net consumption of oxygen by the cells causing anaerobiosis in the growth medium. It was shown that *expression of the* [*Fe*]*-hydrogenase is elicited in the light* under these conditions, autonomically leading to H_2-production by the algae [3,5]. For the first time, under S-deprivation, it was possible to photoproduce and to accumulate bulk amounts of H_2 gas, emanating as bubbles from the green alga cultures, a sustainable process that continued for a few days. Thus, progress was achieved by circumventing the sensitivity of the [Fe]-hydrogenase to O_2 through a temporal separation of the reactions of O_2 and H_2 photoproduction, i.e., by the so-called "two-stage photosynthesis and H_2-production" process [3]. Application of this novel two-stage protocol revealed the occurrence of hitherto unknown metabolic, regulatory and electron-transport pathways in the green alga *C. reinhardtii* [4]. This method may serve as a tool for the elucidation of the green alga photosynthesis/respiration relationship and biochemistry of hydrogen-related metabolism. Upon further refinement, it may also serve in the generation of H_2 gas for the agriculture, chemical and fuel industries. Briefly, the temporal sequence of events in this two-stage photosynthesis and H_2-production process is as follows:

(a) Green algae are grown photosynthetically in the light (normal photosynthesis) until they reach a density of 3–6 million cells/ml in the culture.
(b) Sulfur deprivation is imposed upon the cells in the growth medium, either by carefully limiting sulfur supply so that it is consumed entirely, or by permitting cells to concentrate in the growth chamber prior to medium replacement with one that lacks sulfur nutrients. Cells respond to this S-deprivation by fundamentally altering photosynthesis and cellular metabolism in order to survive [57–59]. Noteworthy in this respect is the 10-fold increase in cellular starch content during the first 24 h of S-deprivation [4,60].
(c) S-deprivation exerts a distinctly different effect on the cellular activities of photosynthesis and respiration (Fig. 1A). The capacity of oxygenic photosynthesis declines quasi-exponentially, with a half-time of 15–20 h, to a value about 10% of its original rate [2]. However, the capacity for cellular respiration remains fairly constant over the S-deprivation period [3]. In consequence, the absolute activity of photosynthesis drops below the level of respiration after about 24 h of S-deprivation (Fig. 1A). Following this cross-point between photosynthesis and respiration, sealed cultures of S-deprived *C. reinhardtii* quickly consume all dissolved oxygen and become anaerobic [5], even though they are maintained under continuous illumination.

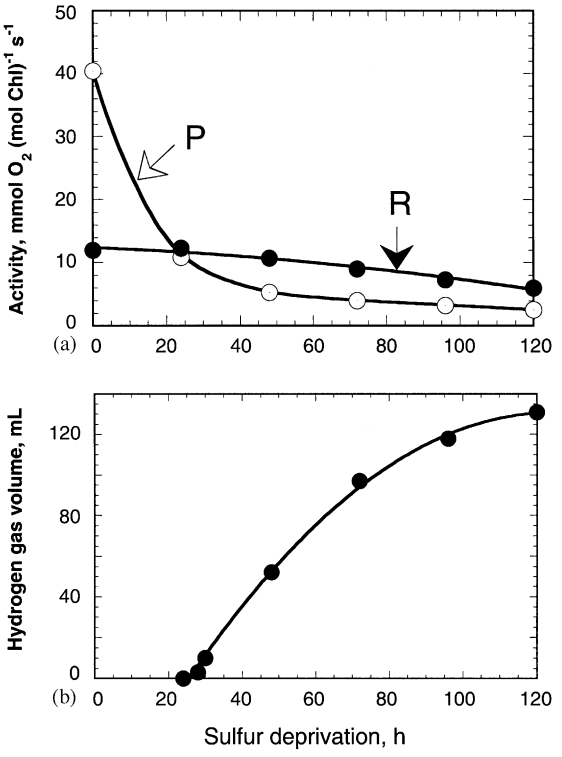

Fig. 1. Photosynthesis, respiration and H_2-production as a function of sulfur-deprivation in *C. reinhardtii*. (A) Absolute activity of oxygenic photosynthesis (P, open circles) and respiration (R, solid circles) in *C. reinhardtii* suspended in media lacking a source of sulfur. The rate of cellular respiration (R) was recorded in the dark, followed by measurement of the light-saturated rate of photosynthesis (P). Cultures at 0 h contained 2.2×10^6 cells/ml. (B) H_2 gas production and accumulation by *C. reinhardtii* cells suspended in media lacking sulfur. Gases were collected in an inverted burette and measured from the volume of water displacement.

(d) Under S-deprivation conditions, sealed (anaerobic) cultures of *C. reinhardtii* induce the [Fe]-hydrogenase in the light and produce H_2 gas (Fig. 1B). Induction of the [Fe]-hydrogenase will take place in the light or dark upon S-deprivation. However, H_2-production is strictly a light-dependent process. The rate of photosynthetic H_2-production was about 2 ml/l culture/h and was sustained in the 24–96 h period. The rate gradually declined thereafter.

(e) In the course of such H_2 gas production (S-deprivation), cells consumed significant amounts of internal starch and protein [4]. Such catabolic reactions apparently sustain, directly or indirectly, the H_2-production process.

Evidently, the absence of sulfur nutrients from the growth medium of algae acts as a metabolic switch, one that selectively and reversibly inhibits photosynthetic O_2 production. Thus, in the presence of S, green algae do normal photosynthesis (H_2O-oxidation, O_2-evolution and biomass accumulation). In the absence of both S and O_2, photosynthesis in *C. reinhardtii* slips into the H_2-production mode. Reversible application of the switch (presence/absence of S) permits the algae to alternate between O_2- and H_2-production (cycling of the stages, Fig. 2), thus bypassing the incompatibility and mutually exclusive nature of the O_2- and H_2-producing reactions. Interplay between oxygenic photosynthesis, mitochondrial respiration, catabolism of endogenous substrate, and electron transport via the hydrogenase pathway is essential for this light-mediated H_2-production process. The release of H_2 gas serves to sustain baseline levels of oxygenic photosynthesis, which feeds electrons into the [Fe]-hydrogenase for the generation of H_2. This residual oxygenic photosynthesis, via the molecular O_2 released, is coupled to mitochondrial respiration (Fig. 3), which in effect scavenges the baseline amounts of photosynthetic O_2. The bioenergetic purpose of the two organelles is the generation of ATP [61], needed for the survival of the organism under the protracted sulfur-deprivation stress conditions.

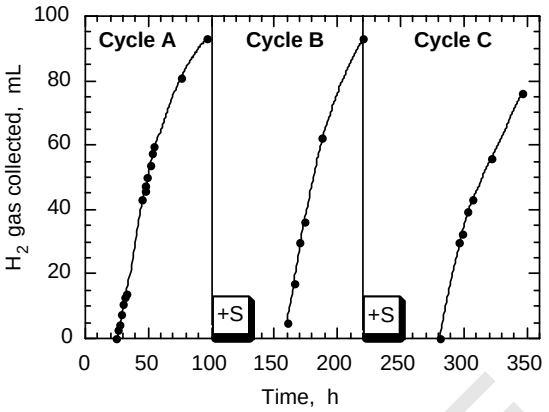

Fig. 2. Cycling of Stages in *C. reinhardtii*. Reversibility and reproducibility of the S-deprivation and H_2-production sequence of events was demonstrated by cycling a single *C. reinhardtii* culture between the two stages (oxygenic photosynthesis in the presence of S and H_2-production in its absence) for up to three full cycles. At the end of H_2-production in cycle A, the culture was supplemented with inorganic S ($t = 100$ h). The latter caused prompt inhibition in H_2-production (beginning of cycle B), because of the ensuing activation of oxygenic photosynthesis ($100 < t < 130$ h). The culture was driven to anaerobiosis upon a subsequent S-deprivation ($130 < t < 160$ h) and H_2-production ($160 < t < 220$ h). Cycle C shows a third temporal cycling of the Stage 1 → Stage 2 process. When indicated, sulfur was added as sulfate salts in the growth medium to a final concentration of 0.4 mM. (From [5].)

3.3.2. Challenges

There are specific improvements that need to be made to increase the likelihood of successful commercial exploitation of the method. Foremost, the continuity of the process needs to be addressed, as H_2-production by S-deprivation

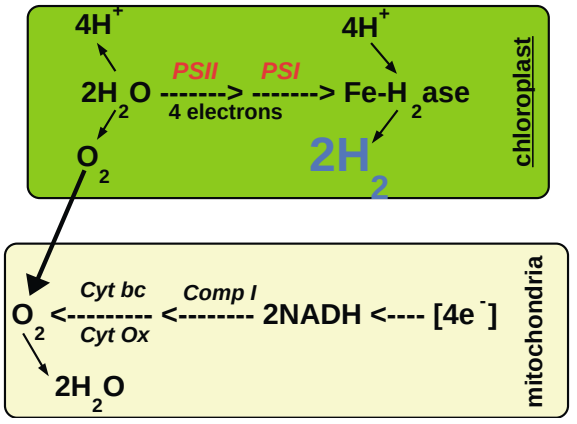

Fig. 3. Coordinated photosynthetic and respiratory electron transport and coupled phosphorylation during H_2-production. Photosynthetic electron transport delivers electrons upon photooxidation of H_2O to the hydrogenase, leading to photophosphorylation and H_2-production. The oxygen generated by this process serves to drive the coordinate oxidative phosphorylation during mitochondrial respiration. Electrons for the latter ([4e]) are derived upon endogenous substrate catabolism, which yields reductant and CO_2. Release of molecular H_2 by the chloroplast enables the sustained operation of this coordinated photosynthesis–respiration function in green algae and permits the continuous generation of ATP by the two bioenergetic organelles in the cell. (Adapted from [1].)

of the algae is time limited. As evident from the results of Fig. 2 [5], the yield begins to level off after about 80 h of S-deprivation. After about 100 h of S-deprivation, the algae need to go back to normal photosynthesis in order to be rejuvenated by replenishing endogenous substrate. Moreover, the yield of H_2 gas accumulation (about 2 ml/h/l culture) represents about 15% of the photosynthetic capacity of the cells, when the latter is based on the capacity for O_2 evolution under physiological conditions [3]. The relatively slow rate of H_2-production suggests a rate-limiting step in the overall process, one that needs to be identified and overcome.

3.3.3. *Prospects*

The discovery of sustainable H_2-production that bypasses the sensitivity of the [Fe]-hydrogenase to O_2 and produces essentially pure H_2 [3] is a significant development in this field. It may serve as a tool in the elucidation of the four-way interplay between the processes of oxygenic photosynthesis, mitochondrial respiration, regulation of cellular metabolism, and electron transport via the [Fe]-hydrogenase pathway for the generation of H_2. It may also lead to the commercial exploitation of green algae for the manufacturing of a clean and renewable fuel. Importantly, it raises to a meaningful level of questions about the optical properties of the cells in mass culture (maximizing green alga solar conversion efficiencies under mass culture conditions) and the engineering of the process (photobioreactors) to an industrial facility level.

4. Maximizing solar conversion efficiencies under mass culture conditions

Cultures growing under full sunlight, when productivity ought to be at a maximum, have disappointingly low solar conversion efficiencies. The reason for this inefficiency is that green algae have a genetic tendency to assemble large arrays of light-absorbing chlorophyll (Chl) antenna molecules in their photosystems. At high solar intensities, the rate of photon absorption by the Chl antennae of the *first few layers of cells* in the culture, or pond, far exceeds the rate at which photosynthesis can utilize them, resulting in dissipation and loss of the excess photons as fluorescence or heat. Up to 95% of absorbed photons could thus be wasted, reducing solar conversion efficiencies and cellular productivity to unacceptably low levels. In addition to the wasteful dissipation of excitation, and due to the high rate of photon absorption by the photosynthetic apparatus, cells at the surface of the mass culture are subject to severe photoinhibition of photosynthesis [62,63], a phenomenon that compounds losses in productivity. Moreover, cells deeper in the culture are deprived of much needed sunlight, as this is strongly attenuated due to filtering [6,64,65]. A genetic tendency of the algae to assemble large arrays of light-absorbing Chl antenna molecules in their photosystems is a survival strategy and a competitive advantage in the wild, where light is often limiting. Obviously, this property of the algae is detrimental to the yield and productivity of a mass culture.

Theoretically, a smaller, or truncated, Chl antenna size of the photosystems in the chloroplast of the microalgae could alleviate the optical shortcomings associated with a fully pigmented Chl antenna, because it will minimize the overabsorption of bright incident sunlight by the photochemical apparatus of the algae. A truncated Chl antenna will diminish the overabsorption and wasteful dissipation of excitation energy by the cells, and it will also diminish photoinhibition of photosynthesis at the surface of the culture. Moreover, a truncated Chl antenna size will alleviate the rather severe gradient of light and mutual cell shading and it will permit a more uniform illumination of the cells in the mass culture. Such altered optical properties of the cells would result in much greater photosynthetic productivity and better solar utilization efficiency in the culture. Indeed, actual experiments [6,66] showed that a smaller Chl antenna size results in a relatively higher light intensity for the saturation of photosynthesis in individual cells but, concomitantly, in a 3-fold greater productivity of the mass culture. Thus, approaches by which to genetically truncate the Chl antenna size of photosynthesis in green algae merit serious consideration.

The Chl antenna size of the photosystems is not constant. Rather a parameter known as “excitation pressure” regu-

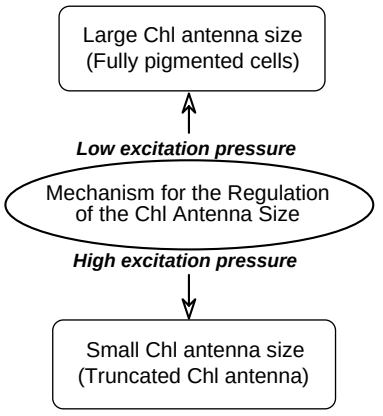

Fig. 4. Molecular mechanism for the regulation of the Chl antenna size in the photosynthetic apparatus. A sensory and signal transduction pathway, highly conserved in all photosynthetic organisms, regulates the Chl antenna size of the photosystems. Within limits, defined by genetic and structural considerations, the response is a compensation reaction to the level of irradiance.

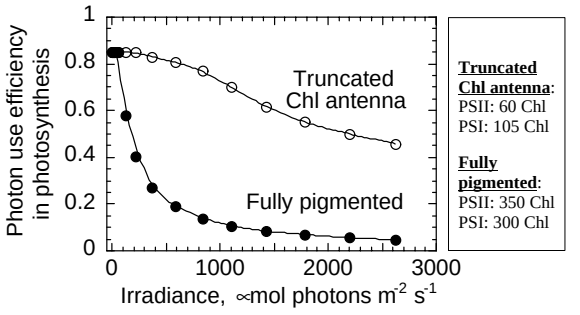

Fig. 5. Photosynthetic solar photon use efficiency as a function of irradiance in fully pigmented and truncated Chl antenna *D. salina*. Under bright sunlight (2000–2500 μmol photons/m^2/s), fully pigmented cells (PSII=350 Chl; PSI=300 Chl) show a mere 5–10% solar conversion efficiency. Under the same conditions, cells with a truncated Chl antenna size show a 45% solar conversion efficiency. (Adapted from [6].)

lates the size and composition of the light-harvesting Chl antenna during chloroplast development [67–70]. "Excitation pressure" is generated whenever there is imbalance between the supply and consumption of light energy in the photosynthetic apparatus. Such persistent imbalance is communicated to the metabolic machinery of the chloroplast via the redox state of the plastoquinone pool [70–72]. In general, low light intensity during growth results in low excitation pressure, a condition that promotes a large Chl antenna size for both PSII and PSI. Growth under high light intensities results in higher excitation pressure, a condition that elicits the assembly of a smaller Chl antenna size. This regulatory mechanism is known to function in all organisms of oxygenic and anoxygenic photosynthesis [68,73,74]. The function of this highly conserved mechanism is depicted in Fig. 4. An example of the operation of this molecular mechanism was provided in work from this and other laboratories. In these reports, the Chl antenna size of PSII was shown to be as large as 460 Chl molecules in fully pigmented green algae and as small as 60 Chl molecules in cells stressed upon continuous excitation pressure [39,75,76]. Adjustments of the Chl antenna size in response to irradiance are essentially a compensation reaction of the chloroplast as they are inversely related to the incident intensity. In principle then, it should be possible to genetically interfere with the relevant regulatory mechanism (Fig. 4) and, in transformant green algae, to direct the chloroplast biosynthetic and assembly activities toward a permanently truncated Chl antenna size.

The utility of the small Chl antenna size in maximizing solar conversion efficiencies was demonstrated in recent studies [6,65]. Excitation pressure was used as a tool to generate green algae (*Dunaliella salina*) with a truncated Chl antenna size. These cultures were used to obtain information on the photosynthetic efficiency and productivity of the cells under mass culture conditions. Fig. 5 shows measurements of the photon use efficiency as a function of incident irradiance in fully pigmented (solid circles) and truncated Chl antenna cells (open circles). It is evident that, at low intensities (< 100 μmol photon/m^2/s), both cell types performed with a relatively high photon use efficiency. At increasing incident intensities, however, photon use efficiencies for the fully pigmented cells declined sharply, reaching a value of ~ 0.05 (5%) at an irradiance corresponding to full sunlight (2500 μmol photon/m^2/s). The cells with the truncated Chl antenna size exhibited a smaller decline in photon use efficiency with irradiance. This decline was noticeable only at intensities greater than 500 μmol photon/m^2/s, reaching a value of ~ 0.45 at the intensity of full sunlight [6]. It is concluded that green algae with a truncated Chl antenna size are indispensable in efforts to substantially increase photosynthetic efficiencies and the yield of H_2-production in photobioreactors under mass culture conditions.

5. Genes for the regulation of the Chl antenna size

The foregoing clearly show that, for purposes of biomass or H_2-production under ambient sunlight conditions, it is important to identify genes that confer a truncated Chl antenna size in the model green alga *C. reinhardtii*. Once a library of such genes is at hand, overexpression or down-regulation of expression of these genes, as needed, can be applied to other green algae that might be suitable for commercial exploitation and H_2-production.

5.1. Progress

The chlorophyll *a* (Chl *a*) oxygenase (*CAO*) gene encodes a chloroplast enzyme that catalyzes the last step in

Table 1
Photosystem Chl antenna size in wild type and three *Chlamydomonas reinhardtii* mutant strains

	Wild type	*cbs3* (Chl *b*-less)	*npq2*/*lor1* (Lut, Vio & Neo-less)	*tla1*	Goal (minimum Chl antenna size)
Chl-PSII	230	90	125	115[a]	37
Chl-PSI	240	289	294	160[a]	95

The *cbs3* strain lacks Chl *b* and was isolated upon DNA insertional mutagenesis [78]. The *npq2*/*lor1* strain lacks all β,ε-carotenoids as well as the β,β-epoxycarotenoids. It contains zeaxanthin but lacks lutein, violaxanthin and neoxanthin from its thylakoid membranes [80]. The *tla1* strain was isolated upon DNA insertional mutagenesis [81]. Note that the *tla1* transformant has the smallest combined Chl antenna size of the three mutants described. Numbers show the Chl antenna size, i.e., the Chl (*a* and *b*) molecules specifically associated with each photosystem.

the Chl biosynthetic pathway, namely the conversion of Chl *a* into Chl *b*. A mutant with inactivated *CAO* would be unable to synthesize Chl *b*, thereby lacking this auxiliary light-harvesting pigment. The assembly, organization and function of the photosynthetic apparatus was recently investigated in wild type and a chlorophyll (Chl) *b*-less mutant of the unicellular green alga *C. reinhardtii*, generated by DNA insertional mutagenesis [77]. It was shown that lack of Chl *b* diminished the PSII functional Chl antenna size from 230 Chl (*a* and *b*) to about 95 Chl *a* molecules [78]. However, the functional Chl antenna size of PSI remained fairly constant at about 290 Chl molecules, independent of the presence of Chl *b* (Table 1).

[a]Polle, Kanakagiri and Melis, unpublished. This work provided evidence to show that transformation of green algae [79] can be used as a tool by which to interfere with the biosynthesis of specific pigments and, thus, to generate mutants exhibiting a permanently truncated Chl antenna size. In support of the role of *CAO* in the Chl antenna size of photosynthesis, recent work [72] showed that *CAO* gene expression is highly regulated in vivo according to the Chl antenna size needs of the organism. Thus, the *CAO* gene may be a target for a truncated Chl antenna size in PSII.

C. reinhardtii double mutant *npq2*/*lor1* lacks the β,ε-carotenoids lutein and loroxanthin as well as all β,β-epoxycarotenoids derived from zeaxanthin (e.g. violaxanthin and neoxanthin). Thus, the only carotenoids present in the thylakoid membranes of the *npq2*/*lor1* cells are β-carotene and zeaxanthin. The effect of these mutations and the lack of specific xanthophylls on the Chl antenna size of the photosystems was investigated [80]. In cells of the mutant strain, the Chl antenna size of PSII was substantially smaller than that of the wild type (Table 1). In contrast, the Chl antenna size of PSI was not truncated in the mutant. This analysis showed that the absence of lutein, violaxanthin and neoxanthin specifically caused a smaller functional Chl antenna size for PSII but not for that of PSI. Thus, xanthophyll-biosynthesis genes, such as *lycopene ε-cyclase* and *zeaxanthin epoxidase* may be targets for a truncated Chl antenna size in PSII.

DNA insertional mutagenesis and screening resulted in the isolation of a regulatory mutant, the phenotype of which was Chl deficiency and elevated Chl *a*/Chl *b* ratio [81]. This *t*runcated *l*ight-harvesting Chl *a*ntenna (*tla1*) mutant apparently has a defect in the regulatory mechanism shown in Fig. 4, the result of which is inability to produce a large Chl antenna size under any growth conditions. Table 1 shows that both the Chl antenna size of PSII and PSI were smaller in the *tla1* strain. This work provided further evidence to show that transformation of green algae can also be used as a tool by which to genetically interfere with the molecular mechanism (Fig. 4) for the regulation of the Chl antenna size in green algae (Kanakagiri, Polle and Melis, unpublished).

5.2. Prospects

Identification of three genes that confer a truncated Chl antenna size in the photosynthetic apparatus is another significant development in the direction toward cost-effective commercialization of green algae for biomass and H_2-production. Most promising in this respect is the cloning of the *TLA1* regulatory gene. A complete genomic and cDNA sequence of *TLA1* as well as the amino acid sequence of the *Tla1* protein are currently at hand (Kanakagiri and Melis, in preparation). It should be noted that this is a first-time isolation and characterization of a "Chl antenna size" regulatory gene. The *TLA1* gene may serve as a molecular tool in the elucidation of the function of the above (Fig. 4) regulatory mechanism in photosynthesis. It may thus contribute to the identification of other genes that are important in this regulatory process (Fig. 4). Further, *TLA1* may serve in the truncation of the Chl antenna size in a variety of green algae and, potentially, in non-oxygenic photosynthetic bacteria.

The ultimate goal of this approach is to develop customized strains of green algae, which assemble only the minimum Chl antenna size of the PSII–core complex (37 Chl) and that of the PSI–core complex (95 Chl molecules) (Table 1).

6. Photobioreactors

The alga culture biotechnology has evolved over the recent past into a commercially viable sector, with many companies utilizing both open pond culture systems and

controlled closed photobioreactor-type facilities (see reviews in [82]). Recent biotechnological improvements have contributed in the expansion of this industry into commodity-scale products, high-value chemicals and fuels production. Of the latter, CO_2 mitigation efforts [83–86], algal biomass [87,88], and potentially hydrogen production [1] have received attention over the past 10 years. In any algal H_2-production process, however, the cost of the facility and its operation are most important factors.

6.1. Design and specifications

The photobioreactor design, whether semi-batch of continuous flow, flat plate or tubular, should be suitable for optimal light exposure of the algae. Useful features include some thermal control and provisions for monitoring flow rates, pH, and dissolved [O_2], [S] and [H_2]. A computer-controlled system for monitoring and automatic nutrient delivery and culture dilution is now a routine in the algal biotechnology industry. Continuous-flow or batch culturing of microalgae is also a routine. Combining this expertise with modifications to ensure a gas-tight system should be a straightforward technical development.

In order to gain maximum H_2 yields per unit volume, green alga biomass needs to be densely packed. Photobioreactors have been designed in the past with the goal of achieving a low-cost, fast-growth and high biomass density of the culture ($> 10^9$ cells/ml) (for review see [89,90]). Several photobioreactor designs could be suitable for green alga H_2-production, including flat plate, tubular, pond or pool-type, etc. For each of these designs, detailed cost analyses must be undertaken with a focus on minimizing the expense associated with materials and daily operation, and also for optimizing the yield and collection of H_2 gas (see below). Critical parameters in this respect include, but not limited to, the light environment, the regional climate and land area, photobioreactor construction materials, the mechanism of biomass mixing, photobioreactor maintenance and long-term operational stability with the maximum possible H_2-production output. Sufficient light supply for the cells is essential not only for high-density biomass generation but also for H_2-production through photosynthesis. Hence, light limitations must be kept to a minimum. The exact dimensions of the modular bioreactor also need to be determined for the most effective utilization of sunlight and surface area. In this respect, biomass mixing is important as it ensures uniform dispersion of nutrients, promotes a more uniform illumination of the culture, and prevents cell settling.

The modular experimental design must be intended for potential scale-up and use at the commercial and industrial levels and should allow for sustained long-term H_2-production with high yields in minimum volume. An important consideration in the design of the photobioreactor is that it must be constructed for the trapping and removal of H_2 gas. The two-stage photosynthesis and H_2-production protocol has the advantage of generating pure H_2 gas [3], thus it may serve as a model for the operation of pilot photobioreactors. Given the current state of the art in photobiological H_2-production, technical and economic strategies for cycling of the algal cultures between sulfur-deprivation and supply (Fig. 2) must be devised.

6.2. Cost analysis

6.2.1. Progress

An important aspect of the design that needs to be considered is that the reactor interior must be protected against contamination with alien microorganisms. Thus, the design and construction of the reactor needs to be modular to permit for periodic materials replacement and/or whole reactor de-contamination treatments. Moreover, various gas collection devices must be incorporated, which would remove H_2 from the gas-tight sealed bioreactor. Following a thorough design and evaluation process, a most economic and efficient scaled-up (500 l) modular unit was adopted and is being tested under field conditions (Candy and Melis, unpublished). This modular pilot photobioreactor was exposed to the elements continuously over a 6-month period with satisfactory materials performance. Significant experience was gained on the growth of the green algae and on the production and collection of H_2 under mass culture conditions in this scaled-up pilot reactor. Most important, the pilot photobioreactor permitted a realistic assessment of construction costs and analysis of performance. Such economic analysis showed the following cost distribution among the various facility categories, derived from the actual operation of this scaled-up modular unit.

Photobioreactor materials	45%
Mineral nutrients	39%
Labor	4%
Water supply	4%
Land lease	2%
Power	1%
Miscellaneous	5%

As a point of reference, actual costs for the construction of this pilot unit photobioreactor were \$0.75 m^{-2}. These costs are substantially lower than the \$20–100 m^{-2} often quoted ([82], and references therein) and they probably reflect the simplified strip-down minimal design, which is necessary and sufficient for the growth of the algal biomass and the collection of H_2.

6.2.2. Prospects

The above preliminary analysis provides a glimpse into the relative cost of the components that are necessary and sufficient to assemble a commercially viable photobiological H_2-production facility. The analysis took into consideration the production phase of the operation in California, including facility maintenance, but does not include costs associated with the storage, transportation or H_2-conversion to

Table 2
Photobiological H_2-production: progress, challenges and prospects

Parameter	Basis of challenge	Approaches to overcome challenge	State of the art	Future prospects
Solar conversion efficiency of the photosynthetic apparatus	Under bright sunlight, solar conversion efficiency of green algae is low	Genetically truncate the Chl antenna size of photosynthesis to limit rates of light absorption	Successful truncation of the Chl antenna size by about 50% *TLA1*, a gene that regulates the Chl antenna size has been cloned	Additional genes that confer a truncated Chl antenna size should be identified
Generation of product (H_2)	Oxygen sensitivity of the process	Temporally separate the processes of photosynthetic O_2- and H_2-production	Development of anaerobic S-deprivation "two-stage" photosynthesis and H_2-production process	Improve sustainability of H_2-production
	Low light intensity for the saturation of H_2-production			Increase yield by overexpressing the [Fe]-hydrogenase in green algae
Photobioreactor	Cost of materials ($\$0.75\ m^{-2}$)	Recycling of materials	Testing of a pilot scale-up (500 L) photobioreactor of flexible, sturdy and transparent materials	Easy to obtain, off the shelf mechanical and chemical engineering technology
	Cost of nutrients ($\$0.65\ m^{-2}$)	Minimize use and/or recycle nutrients		
Land surface area	Large surface area is required due to the diffuse nature of solar insolation	Design optimal sized modular facility in preference to utilizing single-body gigantic area	Availability of sunny, arid climate with fresh, brackish, or seawater	Suitable domestic or international locations are available for plant construction

electricity. Nevertheless, it serves as an important guide in efforts to critically assess ways by which to lower the cost of photobiological H_2-production. For example, it is obvious from the above analysis that efforts should be directed toward the recycling and reutilization of photobioreactor materials and green alga mineral nutrients, in order to lower the cost of the overall operation.

A fringe benefit of the mass cultivation of microalgae is the generation of useful biomass. High-value bioproducts (such as vitamins, polyunsaturated fatty acids, carotenoids, and specialty proteins) could be extracted from the algal biomass. The residue could be processed for the further generation of H_2 via, for example, high temperature steam reforming.

7. The promise of photobiological hydrogen production

Hydrogen is recognized as an ideal energy carrier that does not contribute to air pollution or global warming. Hydrogen and electricity could team to provide attractive options in transportation and power generation. Interconversion between these two forms of energy suggests on-site utilization of hydrogen to generate electricity, with the electrical power grid serving in energy transportation, distribution, utilization and hydrogen regeneration as needed. A challenging problem in establishing hydrogen as a source of energy for the future is the renewable and environmentally friendly generation of large quantities of hydrogen gas.

The recently developed single-organism, two-stage photosynthesis and H_2-production protocol with green algae [3] is of fundamental importance because it revealed the occurrence of hitherto unknown metabolic, regulatory and electron-transport pathways in the green alga *C. reinhardtii* [3,4]. It is also of practical importance as it permitted, *for the first time*, the sustainable light-dependent production and accumulation of significant amounts of H_2 gas, generated from sunlight and water. This method may serve as a tool by which to probe and improve photobiological hydrogen production. A summary of the progress achieved, current challenges facing photobiological H_2, and the near-term prospects of the process are listed in Table 2. The long-term advantage of photobiological hydrogen production is that it does not entail the generation of any toxic

or polluting byproducts and it may even offer the advantage of value-added products as a result of the mass cultivation of green algae. These issues are of enormous consideration for the long-term success of a renewable hydrogen production process. Indeed, recent estimates and business models have shown that a typical industrial-size solar hydrogen production facility need occupy a minimum surface area of 500–1000 acres to permit the harvesting of sufficient solar irradiance for a cost-effective operation of the plant. The environmental impact of such a light-harvesting facility would be minimal with green algae, which may be viewed as a beneficial crop plant.

The process of hydrogen production also concerns global warming, environmental pollution, and the question of energy supply and demand. Projections of potential fossil fuel shortfall, toward the middle of the 21st century, make it important to develop alternative energy carriers that are clean, renewable and environmentally friendly. The advent of hydrogen in general will bring about technological developments in many fields, including agriculture, the transportation industry, power generation and other as yet unforeseen applications. Photosynthetic hydrogen, in particular, will increase the value of the US and world agriculture, as, in addition to hydrogen, scaled-up application of the method would produce substantial amounts of useful green algal biomass, and potentially several high value bioproducts.

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