A new approach for sustained and efficient H₂ photoproduction by *Chlamydomonas reinhardtii*

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Photobiological water splitting to molecular hydrogen (H₂) and oxygen (O₂), also known as direct water biophotolysis, has been considered as one of the most promising and environmentally friendly approaches for generating bulk quantities of clean H₂ biofuel. Many species of cyanobacteria and eukaryotic green algae, including the model organism *Chlamydomonas reinhardtii*, are capable of catalyzing this reaction. In green algae, water biophotolysis proceeds in two steps:

\[
2H₂O → 4H⁺ + O₂ + 4e⁻ \quad \text{Step 1}
\]

\[
4H⁺ + 4e⁻ → 2H₂ \quad \text{Step 2}
\]

involving the photosystem II (PSII) water-oxidizing complex at step 1 and the [FeFe]-hydrogenase (H₂ase) enzyme at step 2 being interconnected via the photosynthetic electron-transport chain (PETC) (Scheme 1, a pathway from A to C). While the splitting of water in PSII results in the release of O₂, the process catalyzed by the H₂ase is O₂-sensitive. The O₂ sensitivity issue has been recognized as a major challenge to efficient H₂ production in green algae. Unfortunately, there is no apparent solution allowing the simultaneous production of O₂ and H₂ in algal cultures at full PSII capacity.

H₂ photoproduction in *C. reinhardtii* also occurs through a mechanism independent of water oxidation. In the indirect process, the reductants derived from the degradation of stored organic substrates, such as starch and proteins, are incorporated into the PETC by a type II NADPH dehydrogenase (Nda2) at the level of the plastoquinone (PQ) pool, thus bypassing the water splitting at PSII (Scheme 1, a pathway from B to C). Similar to the direct process, this pathway requires PSI activity to donate electrons to the H₂ase. Since both pathways are linked to the H₂ase via PSI and Fd (Scheme 1, the C pathway), their contribution to the overall H₂ production yield in algal cultures may vary depending on physiological conditions.

Efficient H₂ photoproduction in green algae occurs in the light after a period of dark anaerobic incubation. The reaction is transient due to a rapid, within seconds, inhibition of H₂ase by O₂, which is co-produced in the water-splitting reaction. One of the approaches to achieve sustained H₂ photoproduction in *C. reinhardtii* cultures is to deprive them of sulfur. Sulfur-deprivation prevents the efficient repair of the light-damaged D1 reaction center protein of PSII, thus leading to a gradual loss of the water-splitting activity in algal cells over time. As a consequence, the actively respiring algae establish an
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water oxidation-dependent flow of electrons to the H2ase,
resulting in low overall efficiency of the process. Sulfur-
deprivation requires extensive and time-consuming centri-
fugations, which make this protocol difficult for application
even in laboratory scale projects (yet a few alternatives have
been suggested10,11).

In the current work, we demonstrate that efficient H2
photoproduction can be sustained in growing C. reinhardtii
cultures for at least three days by switching the algal suspens-
sions from continuous light to a train of short strong light
dottomed on either darkness or permanent low light
illumination. The protocol is very simple, non-damaging to algae
and reproducible even under strict autotrophic conditions.

Theoretical considerations
At the current state, H2 photoproduction in algal cultures is
only possible via a temporal separation of the O2 evolving and
H2 producing reactions. C. reinhardtii cultures, dark-adapted in
anoxic conditions, produce H2 upon exposure to light, before
the onset of O2 evolution (Fig. 1A), while sulfur-deprived cells
show the opposite behavior (Fig. 1B). Although the maximum
specific H2 photoproduction activity is higher in dark-adapted
cells than in sulfur-deprived algae, the latter produce H2 much
longer and yield more H2 gas. For sustaining the H2 production
process in dark-adapted algae, one could suggest the low light/
high cell density condition that prevents O2 accumulation in
cultures due to active respiration, but at the expense of
efficiency. 12 Alternatively, H2 production can be driven at high
light intensities by funneling photosynthetic electrons to the
H2ase (Scheme 1, the C pathway), instead of the Calvin–Benson-
Bassham (CBB) cycle (Scheme 1, the D pathway), with simulta-
neous control of the intracellular O2 level. Although Rubisco
deficiency has been reported to promote H2 evolution in green
algae, the yield of H2 in the Rubisco-deficient mutant culture
was not particularly high, most probably due to the down-
regulation of the photosynthetic electron flow to the H2ase in
this strain. 13 Nevertheless, the partial inactivation of the CBB
cycle did improve the H2 photoproduction yield. 14

The H2ase enzyme, induced in algae under dark anaerobic
conditions, 15,16 acts as an alternative electron sink upon illu-
mination and promotes the activity of oxygenic photosynthesis
by eliminating the accumulation of excess electrons in PETC. 17
The light activation of the CBB cycle requires time affecting
photosynthetic productivity under fluctuating light. 18 We propose
that a train of very short light pulses should arrest the algal
photosynthesis in the H2 photoproduction stage, provided the
duration of each light pulse is short enough to minimize the
electron flow to the CBB cycle and to prevent O2 accumulation.
To test this hypothesis, we subjected C. reinhardtii to a train of
short (1–5 s) light pulses interrupted by longer (3–9 s) dark
phases. These experiments were subsequently repeated under
low background illumination (3 μmol photons m–2 s–1) in
place of dark phases.

Materials and methods
All experiments were performed with unstressed, actively growing
C. reinhardtii cultures either on TAP (photomixotrophic growth) or
on a modified TAP medium without acetate (photoautotrophic
growth). CC-124, CC-4533 and CC-5128 (hydEF) strains were pre-
grown under a 14 h photoperiod at 75 μmol photons m–2 s–1
photosynthetic active radiation (PAR) and 25 °C. H2 photoproduc-
tion was analyzed during the active period of photosynthesis,
within 5 to 10 h from the beginning of the photoperiod.
No centrifugation steps were applied. Growing algal cultures were

Scheme 1 A schematic representation of the H2 photoproduction (red
arrows) and competing (blue arrows) metabolic pathways in the chloro-
plasts of green alga C. reinhardtii. From A to C: direct water biophotolysis;
from D to C: indirect water biophotolysis; D: NADPH production and
Calvin–Benson–Bassham cycle; E: light-dependent oxygen reduction.
Abbreviations: TM, thylakoid membrane; OEC, oxygen-evolving complex;
PSII and PSI, photosystems II and I, respectively; NDA2, type II NADPH
dehydrogenase; PO, plastoquinone; Cyt, cytochrome; PC, plastocyanin; Fd,
ferredoxin; [FeFe]-H2ase, [FeFe]-hydrogenase; FNR, ferredoxin-NADP+
dereductase; FlvA and FlvB, flavodiron protein A and B, respectively; CBB,
Calvin–Benson–Bassham; RuBP, ribulose-1,5-bisphosphate; ATPase, ATP
synthase.

Fig. 1 Available protocols for the induction of H2 production in
C. reinhardtii cultures. (A) The dark adaptation protocol was first intro-
duced by Gaffron and Rubin in 1942 and re-produced in our experimental
set-up. (B) The sulfur-deprivation protocol was repeated according to
Melis and co-authors.
pipetted into a gas-tight 23 mL GC vial equipped with H₂ and O₂ microsensors (H₂-NP and OX-NP, Unisense A/S) connected to an amplifier. The electrodes were pierced inside the vial through a Teflon-coated rubber septum. Cells in the vial were sparged with argon (Ar) for 2–3 min in the dark, followed by incubation in the dark for another 1–5 min. Subsequently, a train of light pulses was applied to the culture and the H₂ and O₂ levels were monitored by the OxyHydrogen software via the STM32F103 microcontroller board connected to a high precision 24-bit ADC (ADS1256, Texas Instruments). The white LED light pulses (420 μmol photons m⁻² s⁻¹) were synchronized through the same microcontroller board. The gas exchange was measured by membrane inlet mass spectrometry (MIMS) using a modified DW1 (Hansatech Instruments) electrode chamber as previously described.¹⁹

The long-term H₂ photoproduction experiments were performed with a 10 mL cell suspension in 70 mL gas-tight vials under an Ar atmosphere. The pulses of white light (280 μmol photons m⁻² s⁻¹) interrupted by dark periods or the constant light of the same intensity were provided by the growth chamber (AlgaeTron AG 130-ECO, PSI). The vials were continuously shaken and H₂ production yields were measured using a gas chromatograph (Clarus 500, PerkinElmer) equipped with a thermal conductivity detector and a molecular sieve 5A column (60/80 mesh). The total Chl content and hydrogenase activity were measured as described previously.⁹

The average energy of the incident light in the PAR (400–700 nm) region was determined at the surface of the liquid with the STS-VIS spectrometer (Ocean Optics, Inc.). Light energy to hydrogen energy conversion efficiency (LHCE) was calculated using eqn (1), which considers the partial pressure of H₂ gas in the vial headspace at the moment of calculation:²⁰

\[
\eta(\%) = \frac{\frac{\Delta G^o - RT \ln \left(\frac{P_{\text{H}_2}}{P}\right)}{E_{\lambda A}}}{V_{\text{H}_2}} \times 100
\]  

where \(\Delta G^o\) is the change of the standard Gibb’s free energy for the water-splitting reaction (237 200 J mol⁻¹ at 25 °C and 1 atm), \(R\) is the universal gas constant, \(T\) is the absolute temperature, \(P^*\) and \(P\) are the standard and observed H₂ pressures (atm), \(V_{\text{H}_2}\) is the amount of H₂ photoproduced (mol), \(E_{\lambda}\) is the energy of the incident light radiation (J m⁻² s⁻¹), and \(A\) is the illuminated surface area (m²) and \(t\) is the sum of the illumination periods (s).

For protein analysis, cells were harvested and rapidly frozen in lysis buffer (50 mM Tris pH 8, 2% SDS, 10 mM EDTA, protease inhibitors from Sigma). After thawing, the total protein fraction was isolated and separated in a 12% SDS-PAGE without urea, transferred to a polyvinylidene difluoride membrane (Millipore) and blocked with a 5% blotting grade blocker (Bio-Rad). The samples were loaded on an equal protein basis as determined using a Direct Detect¹⁶ infrared spectrometer (Merck) and visualized as control with Coomassie Brilliant Blue (Bio-Rad). The accumulation of HydA1/A2 was analyzed by using a specific antibody (Agrisera). As a secondary antibody, anti-rabbit horseradish peroxidase was used in 1:10 000 dilution and HydA1/A2 was visualized with ECL.

Results and discussion

A train of light pulses sustains H₂ production in algal cultures

As shown in Fig. 2, a train of 1 s light pulses interrupted by 9 s dark periods induces continuous H₂ photoproduction in algal cultures. The procedure shows reproducibility even at a very low cell density (6–7 mg total Chl L⁻¹) and in the absence of acetate (Fig. 3) but requires pre-established anaerobic conditions. Trace quantities of H₂ could be observed almost immediately after starting the light pulse illumination of anaerobic cultures, and thereafter the H₂ level gradually increased with time. The experiments performed in the DW1/AD electrode chamber (Hansatech Instruments) but under high light intensity (~800 μmol photons m⁻² s⁻¹) pulses produced similar results. The H₂ase-deficient hydEF mutant did not show the presence of H₂ gas throughout the experiment (Fig. 2A, magenta line), as expected. H₂ photoproduction also occurs in algae exposed to pulses superimposed on low background illumination (Fig. 2A, green line).

During the H₂ production phase, no net O₂ evolution could be detected by either the O₂ electrode (Fig. 2A) or MIMS (Fig. 2C).

![Fig. 2](image-url) Induction of H₂ photoproduction in C. reinhardtii cultures by a train of light pulses. (A) The cultures were flushed with Ar for 3 min in the dark, and H₂ photoproduction was initiated by a train of 1 s light pulses interrupted by 9 s dark periods. (B) A typical trace of H₂ photoproduction shown at higher magnification. (C) Simultaneous monitoring of H₂, O₂, and CO₂ exchange in algal cultures by MIMS. The sawtooth wave could not be seen in the MIMS due to background noise. Green line (panel A) shows H₂ photoproduction in algae, where 3 μmol photons m⁻² s⁻¹ of white light was applied in the background instead of darkness. The kinetics of H₂ diffusion out of the medium (panels A and B, black line) and the suspension of the H₂ase-deficient hydEF mutant (panels A and B, magenta line) are shown after the injection of a few μL H₂-saturated medium into the chamber. Downward arrows indicate the injection points.
The exposure of algae to a similar train of light pulses in DUAL-PAM, but at a background of measuring light, demonstrated a slight decline of the PSI photochemical efficiency in the course of the experiment (Fig. S1, ESI†). The exposure of the pulse-illuminated cells to continuous light induced O₂ evolution, occurring with some delay (Fig. S2, ESI†). The accumulation of O₂ could also be observed on shortening the dark phase to ≤3 s between the light pulses (Fig. S3, ESI†), thus confirming the presence of functional PSII in algal cells under the illumination system applied here.

Gas exchange measurements performed with MIMS showed no signs of CO₂ fixation upon a standard train of light pulses (Fig. 2C). CO₂ fixation occurred only upon accumulation of O₂ in the cultures as a consequence of shortening the dark phase to ≈3 s between light pulses (Fig. S3, ESI†). This provides compelling evidence that in the newly established protocol, the algal cells function as a biocatalyst funneling photosynthetic electrons directly to the H₂ase without the activation of the CBB cycle.

Pulse-illumination shows the presence of H₂ uptake in algae

As shown in Fig. 2B, transient H₂ production peaks regularly appear upon pulse-illumination of *C. reinhardtii*, whilst noticeable H₂ consumption takes place between the light pulses. The amplitude of the sawtooth wave, which occurs both in photoheterotrophic (Fig. 2B) and photoautotrophic (Fig. 3A, inset) cultures, became more pronounced in the course of H₂ accumulation in the system. This behavior can be explained by the dependence of the H₂ uptake reaction on the H₂ partial pressure,21 as well as by the gradual induction of the H₂ase activity in cells (Fig. 3B). The involvement of passive processes in the overall H₂ uptake, such as a leak of H₂ from the system or activity in cells (Fig. 3B), can be explained by the gradual induction of the H₂ase in the overall H₂ uptake, such as a leak of H₂ from the system or activity in cells (Fig. 3B). The involvement of passive processes in the overall H₂ uptake, such as a leak of H₂ from the system or activity in cells (Fig. 3B), can be explained by the gradual induction of the H₂ase in the overall H₂ uptake, such as a leak of H₂ from the system or activity in cells (Fig. 3B). The involvement of passive processes in the overall H₂ uptake, such as a leak of H₂ from the system or activity in cells (Fig. 3B), can be explained by the gradual induction of the H₂ase in the overall H₂ uptake, such as a leak of H₂ from the system or activity in cells (Fig. 3B).

A switch of pulse-illumination to continuous low light, however, did not lead to any noticeable H₂ consumption in the cultures (Fig. 2A, green line).

Since the reaction balance catalyzed by the reversible H₂ase is shifted towards H₂ release in the course of pulse-illumination, the involvement of the oxyhydrogen reaction in H₂ uptake is very unlikely or its contribution to the process is minor. A similar conclusion could be applied also to H₂ uptake during the dark phase after the period of pulse illumination (Fig. 2 and 3). Otherwise, flavodiiron proteins might be involved in the oxyhydrogen reaction by donating electrons to O₂ under illumination (Scheme 1, the E pathway).22-23 In principle, H₂ uptake in algae may occur without O₂ consumption:

\[ \text{H}_2 + 2\text{OH}^- \rightarrow 2\text{H}_2\text{O} + 2\text{e}^- \]

Yet, in such a case, neither the final electron acceptor nor any intermediate players are known. The occurrence of H₂ uptake in the green alga, *Scenedesmus* sp. was first demonstrated more than 70 years ago.24 Since that time only a little follow-up progress has been made in resolving the metabolic pathways participating in the H₂ uptake reaction. H₂ oxidation has been proposed to provide reducing equivalents for CO₂ fixation, but the reaction requires either a very low level of O₂ (up to 1%) or light illumination in complete anaerobiosis for ATP re-generation.25

The absence of CO₂ fixation either during a train of light pulses or during the dark phase after termination of the protocol (Fig. 2C) suggests that H₂ uptake in algae exposed to pulse-illumination and thereafter is not linked to CO₂ reduction. The presence of the H₂ consumption pathway was also confirmed in sulfur-deprived *C. reinhardtii* cells,21,26 harbouring the inactivated Rubisco enzyme.13,27 Since H₂ uptake in both cases occurs upon a shift to darkness, the process seems to be driven by the same catabolic pathway. It is clear that more research is needed to completely understand the mechanism(s) of H₂ consumption in green algae, yet the elimination of this process should dramatically improve the H₂ photoproduction yield in algal cultures.

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Fig. 3 H₂ photoproduction in *C. reinhardtii* cultures requires anaerobiosis. (A) Accumulation of H₂ under a train of light pulses occurred only after the establishment of anaerobiosis in algal cultures. The pulse-illumination protocol was initiated at a very low O₂ level in the medium, allowing the spontaneous establishment of anaerobiosis in autotrophic algal cultures exposed to a train of light pulses. (B) The activity assay showed an induction of H₂ase in algal cells under pulse-illumination, but the HydA1/A2 protein level did not change within 15 min. (C) Long-term incubation of autotrophic algal cultures under pulse-illumination led to the accumulation of H₂ in the vial headspace (as detected with GC) and HydA1/A2 proteins in cells, but not under continuous light that caused degradation of H₂ase enzymes in cells due to the accumulation of O₂ in the vials (not shown). Immunoblots were performed using anti-HydA antibody (Agrisera) that recognizes both H₂ase proteins in *C. reinhardtii* cells.
Pulse-illumination demonstrates a fast activation of [FeFe]-hydrogenase by anaerobiosis

Recently, Liran and co-authors\textsuperscript{28} concluded that the entire pool of cellular H\textsubscript{2}ase remains active in air-grown cells, thus allowing algae to produce H\textsubscript{2} even under aerobic conditions, and in particular, on switch from low to high light conditions. On the other hand, there is extensive literature showing the extreme sensitivity of algal H\textsubscript{2}ase to molecular O\textsubscript{2}. For resolving this contradiction, Liran and co-authors suggested the existence of anaerobic niches inside the cells with a high rate of local respiration that protects the H\textsubscript{2}ase from O\textsubscript{2} inactivation.

Our experimental data show that the activation of the H\textsubscript{2}ase enzyme in air-grown cells and the production of H\textsubscript{2} (Fig. 3) occur only after the establishment of anaerobiosis in the culture. As shown in Fig. 3A, the photoautotrophic \textit{C. reinhardtii} culture is capable of spontaneous establishment of anaerobiosis in the medium under the pulse-illumination if the initial level of O\textsubscript{2} is lowered to below 10 μmol L\textsuperscript{-1} by Ar purging. Algae start producing H\textsubscript{2} almost immediately after consuming the residual O\textsubscript{2} in the chamber. The reaction, thus, requires strong anaerobiosis and it does not occur in an aerobic environment. The cells pre-grown in air contain HydA1/A2 proteins and the amount does not increase within 15 min of the pulse-illumination (Fig. 3B, inset). Nevertheless, the H\textsubscript{2}ase activity (measured in the presence of reduced methyl viologen) rises gradually during this time (Fig. 3B) and correlates with the induction of H\textsubscript{2} photoproduction in the cells (Fig. 3A). The amount of the HydA1/A2 proteins increases later (Fig. 3C, inset). During the long-term cultivation under the train of light pulses, we could detect the rise of H\textsubscript{2}ase in the cells, but continuous high light causes the opposite effect (Fig. 3C). In the latter case, no H\textsubscript{2} production is observed. These experimental data prove that algae express H\textsubscript{2}ase during aerobic growth under moderate light. H\textsubscript{2}ase activation, however, requires strong anaerobiosis, which contradicts the suggestion of Liran and co-authors\textsuperscript{28} about the functional [FeFe]-H\textsubscript{2}ase enzyme in an aerobic environment.

A pulse-illumination protocol sustains H\textsubscript{2} production for at least 70 hours and proves the competition between H\textsubscript{2} photoproduction and CO\textsubscript{2} fixation

Long-term experiments performed with \textit{C. reinhardtii} cultures in small anaerobic vials demonstrated that pulse-illuminated algae are capable of producing H\textsubscript{2} continuously for at least 3 days (Fig. 4). The reaction occurs in the absence of acetate and at an extremely low cell density (Fig. 4A), indicating that the self-shading in the suspension is not a reason for the induction of H\textsubscript{2} production in algal cells. \textit{C. reinhardtii} produces H\textsubscript{2} more efficiently during the first 6 h after which the rate gradually declines. The maximum specific rate exceeds the rate of H\textsubscript{2} photoproduction in sulfur-deprived algae\textsuperscript{21,29} and in the best case reaches up to 25 μmol H\textsubscript{2} (mg Chl h\textsuperscript{-1}). Under light conditions typical for the original sulfur-deprivation protocol (\textasciitilde200 μmol photons m\textsuperscript{-2} s\textsuperscript{-1}),\textsuperscript{6,27} pulse-illuminated cultures yield above 3 mmol H\textsubscript{2} L\textsuperscript{-1} during the first 48 h (Fig. S4, ESI\textsuperscript{†}), which is very close to the H\textsubscript{2} yield in sulfur-deprived algae.\textsuperscript{9} However, due to a much shorter illumination time (Fig. S4, ESI\textsuperscript{†}), the pulse-illuminated cultures produce H\textsubscript{2} more efficiently than the sulfur-deprived cells (0.5% vs. 0.24%,\textsuperscript{30} respectively). Sulfur-deprived algae also need an extra 24–48 h (without H\textsubscript{2} production) for PSII inactivation, which is not considered in LHCE calculations. Moreover, the pulse-illuminated algae are capable of producing H\textsubscript{2} at a maximum conversion efficiency of 1.6–1.7% (2–2.2% if the upper H\textsubscript{2} gas combustion energy is assumed) during the first 8 h.

It is important to note that algae do not accumulate biomass under pulse-illumination (Fig. 4C), in contrast to continuous light (Fig. 4D). The inhibition of biomass accumulation under a train of light pulses suggests the successful diversion of photosynthetic reductants from carbon fixation to H\textsubscript{2} photoproduction. These experimental data, thus, bring additional evidence that the re-direction of the photosynthetic electron flow to the [FeFe]-H\textsubscript{2}ase enzyme does improve the H\textsubscript{2} photoproduction activity in algal cells.\textsuperscript{31}

Conclusions

This research demonstrates that H\textsubscript{2} photoproduction in green algae can be sustained by a simple shift in the light conditions of growing algal cultures from continuous illumination to a train of light pulses interrupted by longer dark phases. In a low O\textsubscript{2} environment, such pulse-illuminated algae can spontaneously establish anaerobiosis and produce H\textsubscript{2} for up to three days. The appearance of H\textsubscript{2} gas in the cultures, almost immediately after the establishment of anaerobiosis, points to an important role of the [FeFe]-H\textsubscript{2}ase enzyme(s) in algal energy metabolism under anaerobic conditions. In addition, the pulse illumination protocol provides strong evidence that CO\textsubscript{2} fixation competes with the [FeFe]-H\textsubscript{2}ase enzyme for the photosynthetic electrons and demonstrates a direct means of eliminating this competition. All the findings together provide new opportunities for metabolic engineering and construction of efficient cell factories with a capacity to re-direct photosynthetic electrons to targeted metabolic pathways and biofuel products, instead of biomass.
Conflicts of interest

There are no conflicts to declare.

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