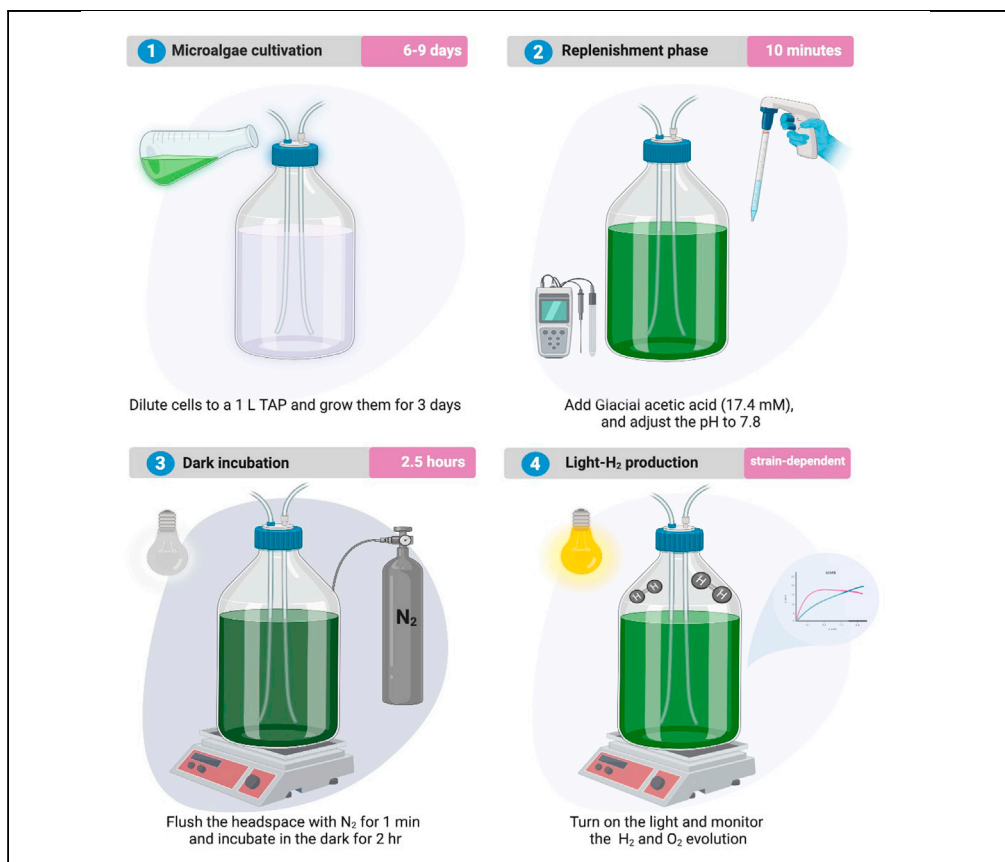


## Protocol

# A two-phase protocol for ambient hydrogen production using *Chlamydomonas reinhardtii*



H<sub>2</sub> production from green-microalgae, for energy purposes, is the ultimate goal of large-scale production. Here, we present a two-phase protocol for hydrogen production assay under ambient conditions using *Chlamydomonas reinhardtii*, which eliminates steps used previously, including centrifugation and resuspension with sulfur-deprived media. We detail steps for *Chlamydomonas reinhardtii* culture, acetate supply replenishment, anaerobic induction, and H<sub>2</sub> quantification. This protocol enables large-scale experiments in an easy and cost-effective method while maintaining cells vital, crucial factors for transition to industrial scales.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Tamar Elman, Iftach Yacoby

iftachy@tauex.tau.ac.il

### Highlights

2-step protocol for ambient hydrogen production in green algae

Detailed culture steps of *Chlamydomonas reinhardtii*

Acetate supply via addition of glacial acetic acid as a carbon source

H<sub>2</sub> production assay without centrifugation or sulfur-deprivation requirements

Elman & Yacoby, STAR  
Protocols 3, 101640  
September 16, 2022 © 2022  
The Author(s).  
<https://doi.org/10.1016/j.xpro.2022.101640>



## Protocol

A two-phase protocol for ambient hydrogen production using *Chlamydomonas reinhardtii*Tamar Elman<sup>1,2</sup> and Iftach Yacoby<sup>1,3,\*</sup><sup>1</sup>School of Plant Sciences and Food Security, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel<sup>2</sup>Technical contact: [tamar90@gmail.com](mailto:tamar90@gmail.com)<sup>3</sup>Lead contact\*Correspondence: [iftachy@tauex.tau.ac.il](mailto:iftachy@tauex.tau.ac.il)  
<https://doi.org/10.1016/j.xpro.2022.101640>

## SUMMARY

H<sub>2</sub> production from green-microalgae, for energy purposes, is the ultimate goal of large-scale production. Here, we present a two-phase protocol for hydrogen production assay under ambient conditions using *Chlamydomonas reinhardtii*, which eliminates steps used previously, including centrifugation and resuspension with sulfur-deprived media. We detail steps for *Chlamydomonas reinhardtii* culture, acetate supply replenishment, anaerobic induction, and H<sub>2</sub> quantification. This protocol enables large-scale experiments in an easy and cost-effective method while maintaining cells vital, crucial factors for transition to industrial scales.

For complete details on the use and execution of this protocol, please refer to Elman et al. (2022).

## BEFORE YOU BEGIN

The following protocol describes the specific steps for long-term hydrogen (H<sub>2</sub>) production assay from the green microalgae *Chlamydomonas reinhardtii* (*Cr*) under ambient conditions in a 1 L photobioreactor (PB) but can be applied to other strains of interest or in alternating volumes. Importantly, different conditions, particularly at the H<sub>2</sub> production phase, will likely need to be recalibrated to adapt the protocol optimally to other strains.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
<i>Chlamydomonas reinhardtii</i> CC-5101	Chlamy collection	<a href="https://www.chlamycollection.org/product/cc-5101-sequence-verified-clone-of-strain-t222/">https://www.chlamycollection.org/product/cc-5101-sequence-verified-clone-of-strain-t222/</a>
<i>Chlamydomonas reinhardtii</i> pgr5	LMJ.RY0402.166681 Clip Library	<a href="https://www.chlamylibrary.org/showMutant?mutantName=LMJ.RY0402.166681">https://www.chlamylibrary.org/showMutant?mutantName=LMJ.RY0402.166681</a>
Chemicals, peptides, and recombinant proteins		
Sodium hydroxide (NaOH)	Sigma	1310-73-2
Acetone ((CH <sub>3</sub> ) <sub>2</sub> CO)	Sigma	67-64-1
K <sub>2</sub> HPO <sub>4</sub>	Sigma	7758-11-4
KH <sub>2</sub> PO <sub>4</sub>	Sigma	7778-77-0
NH <sub>4</sub> Cl	Sigma	12125-02-9
MgSO <sub>4</sub> ·7H <sub>2</sub> O	Sigma	10034-99-8
CaCl <sub>2</sub> ·2H <sub>2</sub> O	Sigma	10035-04-8

(Continued on next page)



<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
EDTA disodium salt	Sigma	139-33-3
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	Sigma	6381-92-6
H <sub>3</sub> BO <sub>3</sub>	Sigma	10043-35-3
MnCl <sub>2</sub> ·4H <sub>2</sub> O	Sigma	13446-34-9
CoCl <sub>2</sub> ·6H <sub>2</sub> O	Sigma	7791-13-1
CuSO <sub>4</sub> ·5H <sub>2</sub> O	Sigma	7758-99-8
NH <sub>4</sub> -6Mo7O <sub>24</sub> ·4H <sub>2</sub> O	Sigma	12054-85-2
FeSO <sub>4</sub> ·7H <sub>2</sub> O	Sigma	7782-63-0
Tris base	Sigma	77-86-1
Glacial acetic acid ≥ 99%	Sigma	64-19-7
<b>Software and algorithms</b>		
BlueVIS 4.0 bioprocessor	BlueSens	<a href="https://www.bluesens.com/products/bioprocess-software/bluevis">https://www.bluesens.com/products/bioprocess-software/bluevis</a>
<b>Other</b>		
BlueVcount gas sensor	BlueSens	<a href="https://www.bluesens.com/products/gas-analyzers/bluevcount">https://www.bluesens.com/products/gas-analyzers/bluevcount</a>
Thermal conductivity detectors (H <sub>2</sub> /O <sub>2</sub> )	BlueSens	N/A
Photometer	LI-COR	LI-250A <a href="https://www.licor.com/env/products/light/light_meter">https://www.licor.com/env/products/light/light_meter</a>
Light filter	Lee Filters	209 0.3ND <a href="https://leefilters.com/colour/209-0-3nd/">https://leefilters.com/colour/209-0-3nd/</a>
White light led strip 6000-6500k	Avtek	200668W
Closures, silicone sponge	Chemglass	CLS-1490-028 <a href="https://chemglass.com/closures-silicone-sponge">https://chemglass.com/closures-silicone-sponge</a>
1.15 L photobioreactor	BluSens	N/A
Magnetic stirrer with hotplate	witeg Germany	MSH-20D
Septum cap	Starna Scientific	N/A
pH meter	Carl Roth	pH 50 VioLab Set
Gas Chromatograph	Agilent Technologies	5890 Series II
GC Columns	Agilent Technologies	5A Mol-Sieve

## MATERIALS AND EQUIPMENT

### Recipe of Tris-Phosphate-Acetate medium

Reagent	Final concentration [mM]
K <sub>2</sub> HPO <sub>4</sub>	0.62
KH <sub>2</sub> PO <sub>4</sub>	0.41
NH <sub>4</sub> Cl	7.48
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.406
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.34
EDTA disodium salt	0.171
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.076
H <sub>3</sub> BO <sub>3</sub>	0.184
MnCl <sub>2</sub> ·4H <sub>2</sub> O	0.025
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.006
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.006
NH <sub>4</sub> -6Mo7O <sub>24</sub> ·4H <sub>2</sub> O	0.0009
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.018
Tris base	20
Glacial acetic acid ≥ 99%	17.4

- Add DDW to a final volume of 1 L
- pH should stabilize between 7.2–7.6
- Autoclave at 121°C for 20 min
- Store at 20°C–25°C for maximum 6 months



**Figure 1. Algal growth in BlueSens photobioreactors**

(A) Cells at the mid-log phase ( $\sim 5 \mu\text{g Chl mL}^{-1}$ ) were diluted into 950 mL TAP. Two outlets of the photobioreactors were capped with silicone sponge enclosures to allow air diffusion.

(B) The cells were taken for further protocol steps after three days of growth under high white light ( $\sim 250 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) at a Chlorophyll concentration of  $15 \mu\text{g mL}^{-1}$ .

## STEP-BY-STEP METHOD DETAILS

### Culture of *Chlamydomonas reinhardtii*

⌚ Timing: 6–9 days

1. Inoculate strains of *Chlamydomonas reinhardtii*: 4 biological replicates of *pgr5* mutant (efficient  $\text{H}_2$  producer, i.e., positive control), a wild-type strain CC-5101 (negative control) and your strain of interest from 1.5% AGAR plates into 50 mL Tris Acetate Phosphate (TAP) medium [see Materials] (inside four 100 mL Erlenmeyer flasks), capped with silicone sponge enclosures (also seen in Figure 1).
2. Grow the cells at  $25^\circ\text{C}$  under constant illumination ( $\sim 90 \mu\text{mol m}^{-2} \text{s}^{-1}$ , cool white led strips; measure with photometer) and constant agitation (magnetic stirrers or vertical shaker).
3. To estimate the culture's density, determine the chlorophyll (Chl) concentration according to (Jeffrey and Humphrey, 1975):
  - a. Take 1 mL from the culture and centrifuge for 2 min at max speed (14,000–16,000 g).
  - b. Remove the supernatant and resuspend with 1 mL 80% Acetone.
  - c. Incubate at  $4^\circ\text{C}$  for 10 min and then centrifuge for 2 min at max speed (14,000–16,000 g). Keep the supernatant.
  - d. Spectrophotometrically, measure the supernatant's absorption (abs) at 663, 645 and 750 nm.
  - e. Place the reads in the following equation to extract the Chl concentration:

$$\text{Chl}[\mu\text{g} / \text{mL}] = 6.43 \times (\text{abs}(664) - \text{abs}(750)) + 18.07 \times (\text{abs}(647) - \text{abs}(750))$$

4. When reaching the mid-log phase, at approximately  $5 \mu\text{g Chl mL}^{-1}$ , dilute the cells into 950 mL TAP to receive a final volume of 1 L culture inside a 1.15 L PB (in Figure 1A, an example of a 90 mm diameter PB).

**Note:** Large volumes such as 1 L should be preferably mixed from this step on with a magnetic bar (2–3 cm) and a stirring device.

- Place the PBs under high white light;  $\sim 250 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

**Note:** As a variable parameter, light intensity should be determined according to each strain of interest (lighting preferences or limitations, growth rates, etc.). In addition, technical specifications, such as the PB's diameter (for the light path distance) and the light source heat output, should be taken into consideration.

- For a 1 L assay, cells should be grown to at least  $6\text{--}7 \mu\text{g Chl mL}^{-1}$  (usually within 48–72 h of growth after 50 mL dilution, [Figure 1B](#)). Based on the desired cell density for the  $\text{H}_2$ -producing phase of the experiment, either return cells for further growth or continue.

**Note:** A higher concentration of cells may allow higher light intensities because cells shadow one another. As a result, each cell can benefit from high amounts of photons for short periods of time, thereby avoiding photoinhibition. Higher light intensities promote higher photosynthetic activity and, therefore, higher yields of  $\text{H}_2$  production. The minimal cell density against each light intensity should be calibrated to prevent photoinhibition.

### Replenishment of acetate supply

⌚ Timing: 10 min

In this step, prior published protocols ([Nagy et al., 2018](#); [Steinbeck et al., 2015](#)) involved a centrifugation step followed by resuspension with either sulfur depleted TAP or fresh TAP. This was done both to keep the cells in an anaerobic state, maintained by sulfur deficiency, and in view of the fact that the culture consumed the growth medium's nutrients, and therefore it was necessary to renew it. We discovered, however, that by adding glacial acetic acid as a carbon source, the culture maintains a healthy physiological profile and can also produce  $\text{H}_2$ .

- Add 1 mL of pure glacial acetic acid (>99%) into the PB to obtain a final concentration of 17.4 mM.

**Note:** Depending on the initial pH of the culture, after the addition of acetic acid, the pH should drop in-between 5.5–6.

**Note:** Before the  $\text{H}_2$  production phase, pH is elevated from 7.2 (during the growth phase) to 7.8 based on ([Kosourov et al., 2003](#)) findings, that higher pH is ideal for the activity of the  $\text{H}_2$  producing enzyme – hydrogenase, resulting in increasing yields of  $\text{H}_2$  photoproduction.

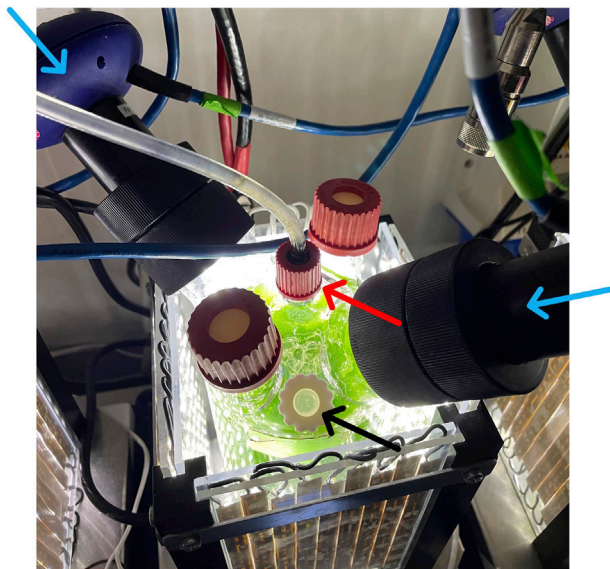
- Using a pH meter or a pH-indicator strip, adjust the pH to 7.8 using NaOH 10 M, dropwise (roughly 1 mL).
- Make sure all outlets are hermetically closed to prevent air from entering. Ensure that one outlet is closed with a needle-permeable cap ([Figure 2](#), black arrow).

**Note:** Steps 7–9 should preferably be performed under sterile conditions (inside a biological hood). However, if this is impossible, it could be performed quickly in an unsterile working environment.

### Setting up your $\text{H}_2$ measuring system

⌚ Timing: 10 min

Once the PB has been hermetically enclosed, place it into the experimental setup. It is recommended to connect a continuously discharging one-way outlet, especially when measuring large volumes of culture. Commercial options, such as BlueVcount gas sensor (BlueSens), as well as self-built



**Figure 2. BluSens 1-L photobioreactor system – upper view**

The PB is equipped with thermal conductivity detectors (TCD) for O<sub>2</sub> and H<sub>2</sub> (recorded by BlueVIS software, blue arrows) and multiple air outlets. All outlets are capped and sealed during the experiment, except for one outlet, which is connected to the gas flow counter (red arrow). At least one cap is needle permeable for the inert gas flush (black arrow).

counters, as described in (Melis et al., 2000), can be used to count gas flow [mL]. From this point on, any changes in air pressure observed in the PB's headspace result from an exchange of gases initiated by the culture. Higher air pressures from microalgae, for example, are most likely due to the production of oxygen (O<sub>2</sub>), H<sub>2</sub>, or CO<sub>2</sub>.

This step involves setting different experimental conditions, such as lighting, temperature, and stirring speed.

- Adjust the light intensity before the PBs are placed in the experiment position.

**Note:** The setup should take into account the confluency of the culture, the light path length of the PB, and the physiological background of the strain tested.

**Example:** For *Chlamydomonas* strain CC-5101 (known as t222+), a light intensity of 350 μmol m<sup>-2</sup> s<sup>-1</sup> can be used for a concentration of as little as 6 μg Chl mL<sup>-1</sup> in a 90 mm diameter PB.

- Shut down the light source and place the PBs into their experimental position.
- Set the magnetic stirring plate to 800 rpm.

**Note:** It is recommended to use a hot magnet stirring plate.

- Set the temperature at 34°C (for room temperatures between 22°C–24°C). As the temperature rises, the H<sub>2</sub> solubility decreases, and gas output increases.

**Note:** Various algal strains may require calibration of this condition.

- Open one of the air outlets of the PB, and connect it to the gas flow discharger and counter (Figure 2, red arrow).

**CAUTION:** perform this step quickly to prevent contaminations.

### Promoting anaerobic conditions for ideal H<sub>2</sub> production

⌚ Timing: 2.5 h

The next steps involve an anaerobic induction, during a 2 h dark incubation, necessary to express and mature the H<sub>2</sub> evolving enzyme hydrogenase (Grossman et al., 2011).

15. For about 1 min, inject into the headspace of the PB a constant stream of an inert gas through an inlet needle, such as Argon or Nitrogen. As a safety measure, inject an additional outlet needle along with the built-in gas discharger; gas flow should still be noticed on the gas flow counter. Replacing the composition of gases in the PB's headspace with 100% inert gas will ensure that any change in air pressure after the experiment has begun is a consequence of a gas generated by the culture.

**CAUTION:** Wear protective eyewear.

**Note:** This step is not biologically mandatory. The inert gas flush helps removing O<sub>2</sub> from the headspace. When this is done, for a 1 L culture, 2 h of dark incubation is sufficient to remove the necessary amount of O<sub>2</sub> to induce Hydrogenase. If not, O<sub>2</sub> will still be removed, however this will be exclusively by the cells mitochondrial respiration. Yet, this process might take longer than 2 h since the O<sub>2</sub> from the headspace might extend the overall respiration time required. If a gas flush could not be performed due to technical limitations, the dark incubation period should be calibrated for each volume of PB and culture.

16. When using a gas analyzer system such as BluVIS (BlueSens), monitor the O<sub>2</sub> levels, and stop gas purging when O<sub>2</sub> levels reach zero.

**Example:** When working without an online gas monitor, for a headspace volume of 150 mL, 5–10 min of an inert gas flush are sufficient.

17. Reset the gas flow counter.
18. Allow 2 h of dark incubation by turning off the surrounding lights in the room.
19. When the dark incubation phase is complete, turn the lights back on (Figure 3A). H<sub>2</sub> production should be noticed within minutes (Figure 3B).

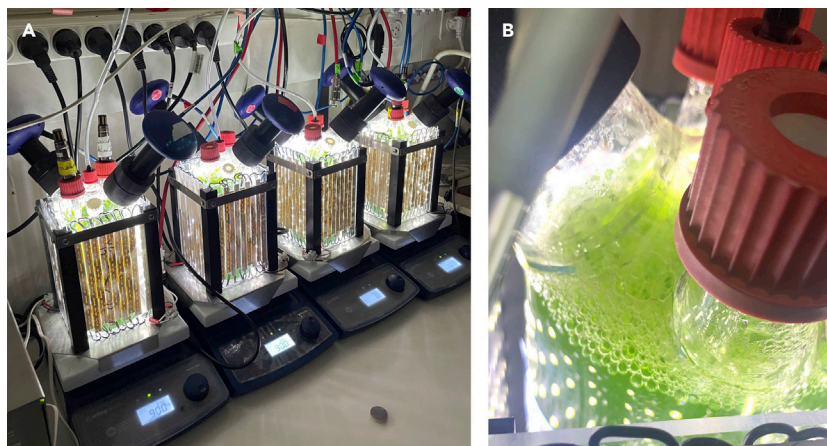
### H<sub>2</sub> quantification throughout the experiment

When online analysis of the gas composition in the PB is unavailable, to confirm H<sub>2</sub> as the sole contributor to the gas output, a continuous sampling from the headspace should be taken to a Gas Chromatograph (GC) for air composition analysis. This method is described by (Eilenberg et al., 2016).

Additionally, to an H<sub>2</sub> gas accumulation data (in volume units), an absolute amount of H<sub>2</sub> in μmol can be calculated:

20. When H<sub>2</sub> is recorded online as percentages, use the following conversion equation:
  - a. Based on the equation of  $PV = nRT$ .
  - b.  $P[atm] \times \text{Headspace vol.}[L] = n \times 0.082 \times 297[K]$ .
  - c. The pressure is presented as the percentage of H<sub>2</sub> in the air composition:

$$P[atm] \times H_2[\%] = H_2/100.$$



**Figure 3. H<sub>2</sub> production in BluSens 1-L photobioreactors under continuous illumination**

(A and B) (A) After 10 min of inert gas flushing and 2 h of dark incubation, a continuous white light at  $350 \mu\text{mol m}^{-2} \text{s}^{-1}$  is applied, and H<sub>2</sub> production is observed (B) as bubbles on the culture surface within minutes.

**Example:** following 1 h of lighting, if the TCD detectors reported a 5% H<sub>2</sub> in a 1 L BluSens photobioreactor headspace with a volume of 150 mL:

$$d. [5]/100 \times 0.15[L] = n \times 0.082 \times 297.$$

$$n = H_2[\text{mol}] = 0.000308 = 308 H_2[\mu\text{mol}].$$

21. When H<sub>2</sub> is recorded manually by gas chromatography, the percentage of H<sub>2</sub> is demonstrated by the chromatograph area where H<sub>2</sub> leaves the column. To convert the measurement of the area to nmol, a calibration curve of H<sub>2</sub> should be generated, followed by a similar  $PV = nRT$  calculation. For a detailed method, see (Eilenberg et al., 2016).

### EXPECTED OUTCOMES

For large volume assays (>1 L), wild type strains should cease producing H<sub>2</sub> within 2–3 days but at yields of no more than tens of mL, while effective and prolonged producers of H<sub>2</sub>, such as *pgr5*, could potentially maintain a productive activity for over a week and produce significant amounts of H<sub>2</sub> (600–800 mL), see results at Elman et al. (2022).

### LIMITATIONS

The main limitation of this protocol is the lack of distinction between strains with improved ability of the enzyme hydrogenase and strains with prolonged H<sub>2</sub> production phenotype. The limitation of continuous H<sub>2</sub> production is challenged simultaneously by (i) sensitivity to O<sub>2</sub> and (ii) lack of electron feed, directed in favor of the carbon fixation cycle (Tóth and Yacoby, 2019). The present protocol is intended to test the “bottom line” - whether a particular strain is capable of producing continuous H<sub>2</sub> on a large scale to be tested in the future on an industrial scale. However, some engineered strains focus on solving one challenge at a time so that the strain might fail the prolonged H<sub>2</sub> production test. Nevertheless, when other parameters and assays are examined in smaller volumes, those strains may display an interesting phenotype worth exploring further. Consequently, this protocol may overlook potential candidates and should preferably be applied to strains of interest after they have been tested in more assays or at least in parallel, and not as a preliminary “yes or no” test, especially for research purposes rather than industrial ones.



## TROUBLESHOOTING

### Problem 1

Inactivation of H<sub>2</sub> production by O<sub>2</sub>.

A potential holdback of this protocol is the inactivation of hydrogenase by rising O<sub>2</sub> levels. A rising amount of O<sub>2</sub> in the culture could be a consequence of two scenarios:

- The strain of interest is a high O<sub>2</sub> producer, and therefore, biologically, it is not capable of prolonged H<sub>2</sub> production.
- There is an air leak from the surrounding environment into the PB.

### Potential solution

- A positive and negative control should be tested regularly (see our controls strain suggestions [culture of Chlamydomonas reinhardtii](#)).
- Monitor the O<sub>2</sub> measurement during the two-hour dark incubation period (providing anaerobic conditions for H<sub>2</sub> production). The O<sub>2</sub> level will increase consistently when there is a leak.
- Detect air leaks prior to the experiment: immerse in water the empty PB when hermetically sealed. Alternatively, spray soapy water on any connection point or area in the system that may experience air leakage. In both methods, when the system experiences air leakages, bubbles will appear and hence, alternative caps should be replaced.

### Problem 2

Lagging H<sub>2</sub> production rate.

A prolonged H<sub>2</sub>-producing strain produces H<sub>2</sub> at a slow rate.

### Potential solution

When setting up your H<sub>2</sub> measuring system consider the following solutions:

- Raise the temperature. Higher temperature reduces the solubility of H<sub>2</sub> in water.
- Set the stirring device to a higher rpm. The mixing of the solution will help to reduce the H<sub>2</sub> solubility.

### Problem 3

Shorter H<sub>2</sub> production time than expected.

When a prolonged H<sub>2</sub> production strain ceases to produce H<sub>2</sub> earlier than expected.

### Potential solution

The acetic acid supply might be the limiting factor and an additional replenishment of acetate supply should be considered. This could be tested by injecting an additional dose of acetate (1 mL of 17.4 M glacial acetic acid into 1 L culture) coupled with NaOH injection for the solution titration to pH 7.8.

**Note:** A direct titration of acetate and NaOH to the PB is possible only when a continuant and direct pH measurement of the culture is available. If not, the amount of acetic acid supplied to the culture before the H<sub>2</sub> production phase should be calibrated by several concentrations.

### Problem 4

Slow cell growth after cells dilution into high volume PB.

During the transition to large volumes, the growth rate of the culture might be slowed by the limited air diffusion into the medium from the PB's headspace, as the availability of O<sub>2</sub> to the cells decreases.

### Potential solution

To enrich the culture with O<sub>2</sub>, integrate into the PB a filtered air bubbling system to inject air directly into the culture.

### Problem 5

Contaminations.

Growing cells in large volumes is often followed by an outbreak of culture infection.

### Potential solution

Working with sterile equipment under sterile conditions can significantly reduce the incidence of infections. If necessary, antibiotics against prokaryotic organisms such as carbenicillin or in case the utilized strain was genetically engineered with a resistance gene, an antibiotic against eukaryotic organisms can be used.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Iftach Yacoby ([iftachy@tauex.tau.ac.il](mailto:iftachy@tauex.tau.ac.il)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate/analyze [datasets/code].

## ACKNOWLEDGMENTS

Funding via the Israel Science Foundation ISF 1646/16. Graphical abstract was adapted from "sequence panels", by [BioRender.com](https://www.biorender.com) (2022). Retrieved from <https://app.biorender.com/biorender-templates>.

## AUTHOR CONTRIBUTIONS

Methodology, Investigation, and writing – Review & Editing, T.E. and I.Y.; Funding Acquisition and Supervision, I.Y.

## DECLARATION OF INTERESTS

The method described in this MS appears in a PCT Patent Application No. PCT/IL2021/051282 "PHOTOSYNTHETIC MICROALGAE AND USE THEREOF FOR HYDROGEN PRODUCTION" as a mean to produce algal- hydrogen under ambient, continuous growth conditions at cost-effective amounts. The patent was jointly submitted by the Tel Aviv University and the University of Munster. List of inventors: Iftach Yacoby, Tamar Elman, Oren Bez-zvi, Chris Philip Schwier, Thi Thu Hoai Ho, and Michael Hippler. Submitted on 28 Oct 2021.

## REFERENCES

- Eilenberg, H., Weiner, I., Ben-Zvi, O., Pundak, C., Marmari, A., Liran, O., Wecker, M.S., Milrad, Y., and Yacoby, I. (2016). The dual effect of a ferredoxin-hydrogenase fusion protein in vivo: successful divergence of the photosynthetic electron flux towards hydrogen production and elevated oxygen tolerance. *Biotechnol. Biofuels* 9, 182–210. <https://doi.org/10.1186/s13068-016-0601-3>.
- Elman, T., Thi Tho, H., Milrad, Y., Hippler, M., and Yacoby, I. (2022). Enhanced chloroplast-mitochondria crosstalk promotes ambient algal-H<sub>2</sub> production. *Cell Reports Physical Science*. <https://doi.org/10.1016/j.xcrp.2022.100828>.
- Grossman, A.R., Catalanotti, C., Yang, W., Dubini, A., Magneschi, L., Subramanian, V., Posewitz, M.C.,

and Seibert, M. (2011). Multiple facets of anoxic metabolism and hydrogen production in the unicellular green alga *Chlamydomonas reinhardtii*. *New Phytol.* 190, 279–288. <https://doi.org/10.1111/j.1469-8137.2010.03534.x>.

Jeffrey, S.W., and Humphrey, G.F. (1975). New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanz.* 167, 191–194. [https://doi.org/10.1016/s0015-3796\(17\)30778-3](https://doi.org/10.1016/s0015-3796(17)30778-3).

Kosourov, S., Seibert, M., and Ghirardi, M.L. (2003). Effects of extracellular pH on the metabolic pathways in sulfur-deprived, H<sub>2</sub>-producing *Chlamydomonas reinhardtii* cultures. *Plant Cell*

*Physiol.* 44, 146–155. <https://doi.org/10.1093/PCP/PCG020>.

Melis, A., Zhang, L., Forestier, M., Ghirardi, M.L., and Seibert, M. (2000). Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. *Plant Physiol.* 122, 127–136. <https://doi.org/10.1104/pp.122.1.127>.

Nagy, V., Vidal-Meireles, A., Podmaniczki, A., Szentmihályi, K., Rákhely, G., Zsigmond, L., Kovács, L., and Tóth, S.Z. (2018). The mechanism of photosystem-II inactivation during sulphur deprivation-induced H<sub>2</sub> production in *Chlamydomonas reinhardtii*. *Plant J.* 94, 548–561. <https://doi.org/10.1111/tpj.13878>.

Steinbeck, J., Nikolova, D., Weingarten, R., Johnson, X., Richaud, P., Peltier, G., Hermann, M., Magneschi, L., and Hippler, M. (2015). Deletion of Proton Gradient Regulation 5 (PGR5) and PGR5-Like 1 (PGR1) proteins promote sustainable light-driven hydrogen production in *Chlamydomonas reinhardtii* due to increased PSII activity under sulfur deprivation. *Front. Plant Sci.* 6, 892–911. <https://doi.org/10.3389/fpls.2015.00892>.

Tóth, S.Z., and Yacoby, I. (2019). Paradigm shift in algal H<sub>2</sub> production: bypassing competitive processes. *Trends Biotechnol.* 37, 1159–1163. <https://doi.org/10.1016/j.tibtech.2019.05.001>.