Appl Biochem Biotechnol (2010) 162:208–220
DOI 10.1007/s12010-009-8741-6

Hydrogen Generation Through Indirect Biophotolysis in Batch Cultures of the Nonheterocystous Nitrogen-Fixing Cyanobacterium *Plectonema boryanum*

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Received: 14 May 2009 / Accepted: 29 July 2009 /
Published online: 21 August 2009

Abstract The nitrogen-fixing nonheterocystous cyanobacterium *Plectonema boryanum* was used as a model organism to study hydrogen generation by indirect biophotolysis in nitrogen-limited batch cultures that were continuously illuminated and sparged with argon/CO_2 to maintain anaerobiosis. The highest hydrogen-production rate (i.e., 0.18 mL/mg day or 7.3 μmol/mg day) was observed in cultures with an initial medium nitrate concentration of 1 mM at a light intensity of 100 μmol/m^2 s. The addition of photosystem II (PSII) inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) did not reduce hydrogen-production rates relative to unchallenged controls for 50 to 150 h, and intracellular glycogen concentrations decreased significantly during the hydrogen generation period. The insensitivity of the hydrogen-production process to DCMU is indicative of the fact that hydrogen was not derived from water splitting at PSII (i.e., direct biophotolysis) but rather from electrons provided by intracellular glycogen reserves (i.e., indirect biophotolysis). It was shown that hydrogen generation could be sustained for long time periods by subjecting the cultures to alternating cycles of aerobic, nitrogen-limited growth and anaerobic hydrogen production.

Keywords Indirect biophotolysis · Cyanobacteria · Glycogen · DCMU

Introduction

Declining crude oil supplies and the threat of global climate change necessitate the rapid development of carbon-neutral biofuels [1], such as renewable hydrogen for use in fuel cells and for transportation. Photobiological hydrogen production by microalgae has been

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investigated since the early 1970s [2]. The fundamental concept is to use microalgae to catalyze the conversion of solar energy and water into hydrogen with oxygen as a by-product. Most research in this field has focused on the so-called direct biophotolysis approach in which water is split into H_2 and O_2 without intermediate CO_2 fixation [3–7]. During direct biophotolysis, electrons flow from water via photosystem II (PSII) through photosystem I (PSI) to reduce ferredoxin which then in turn reduces the hydrogenase enzyme, which transfers these electrons to protons and thus causing the release of hydrogen gas. Despite its simplicity and conceptual attractiveness, direct biophotolysis faces several major hurdles in the development of a practical and commercially viable hydrogen-production process: (a) the photosynthetically evolved oxygen strongly inhibits the hydrogenase enzyme; (b) the resulting H_2–O_2 mixtures are highly explosive; and (c) the required closed photobioreactors are likely cost prohibitive [8]. Although O_2-resistant hydrogenases have been claimed to have been isolated from some microorganisms or by genetic technologies, there is no evidence that these can actually function in the presence of any significant quantities of free O_2.

These various problems of direct biophotolysis led to proposals for indirect, two-stage light-driven processes with CO_2 fixation and O_2 release occurring in the first stage followed by H_2-production reactions in the second stage [9]. In the indirect biophotolysis process, reduced substrates (i.e., carbohydrates, such as starch in microalgae or glycogen in cyanobacteria) accumulate during the photosynthetic O_2-production and CO_2-fixation stage, and these are then used in a second stage for H_2 production under anaerobic conditions with CO_2 evolution. By temporarily separating the photosynthetic oxygen-production phase from the hydrogen-production phase, indirect biophotolysis overcomes two of the major problems associated with direct biophotolysis, namely, oxygen inhibition of the hydrogenase and the generation of potentially highly explosive H_2–O_2 mixtures. It also greatly reduces the third problem, since the second hydrogen-production stage would require much smaller photobioreactors, and it may be possible to dispense with them completely.

The nonheterocystous nitrogen-fixing blue–green cyanobacterium *Plectonema boryanum* has been previously used as a model organism to study the photoproduction of hydrogen by biophotolysis [10–13]. As in other cyanobacteria, nitrogen-limited culture conditions causes *P. boryanum* to slow down photosynthetic oxygen production and to accumulate intracellular glycogen reserves [14–16]. Furthermore, anaerobiosis ensues as soon as dark respiration rates exceed photosynthetic oxygen evolution rates, as the O_2 dissolved in solution would be rapidly utilized. If the culture is both nitrogen limited and anaerobic, the nitrogenase enzyme is induced to "fix" N_2, oxidizing 8 mol of reduced ferredoxin, hydrolyzing 16 mol of adenosine triphosphate ([ATP] to adenosine diphosphate), and producing 1 mol H_2 and 2 mol of ammonia, for each mole of N_2 reduced [17–19]. In the absence of N_2 gas (achieved by sparging the culture with an inert gas such as argon), the nitrogenase continues to oxidize ferredoxin and hydrolyze ATP, but now protons, rather than N_2, are the exclusive substrate and H_2 the sole product, generating 1 mol of this gas for each 2 mol of ferredoxin oxidized and 4 mol of ATP hydrolyzed. This very high-energy requirement of the nitrogenase enzyme for H_2 production (or N_2 fixation) is supported by the metabolism of glycogen to supply the reducing power (reduced ferredoxin) with the ATP produced by either a light-driven PSI-only reaction ("cyclic photophosphorylation") or in the dark by oxidative phosphorylation via the respiratory electron transport chain. In the latter case, some small amount of O_2 must be present (typically 10% or less of atmospheric levels or somewhat less than 1 mg/L O_2). In cyanobacteria, substrate level phosphorylation (e.g., glycolysis) is not sufficient to provide significant amounts of ATP for dark anaerobic H_2 production (or N_2 fixation) [13].

Although the prior studies of Weare and Benemann [13] (see also [11]) suggested that the process of H_2 production in *P. boryanum* was an indirect one, as also evidenced by the effect of the PSII inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), this has not been confirmed and relatively little is known about the mechanisms of hydrogen generation and how to optimize these in this organism. The objectives of this study were (a) to confirm that hydrogen generation in *P. boryanum* proceeds by indirect biophotolysis, (b) to identify culturing conditions that maximize hydrogen-production rates, and (c) to demonstrate hydrogen production in alternating aerobic–anaerobic cycles.

Material and Methods

Culture Conditions

The cyanobacterium, *P. boryanum* (ATCC 18200), was grown in autoclaved 1-L (flat) Roux bottles filled with sterile-filtered 950-mL Chu #10 medium [11] containing, per liter, 10 mg K_2HPO_4, 25 mg $MgSO_4{\cdot}7H_2O$, 20 mg Na_2CO_3, 3.5 mg ferric citrate, 5.4 mg sodium citrate, 58.9 g $Na_2SiO_3{\cdot}9H_2O$, and varying amounts of $Ca(NO_3)_2{\cdot}4H_2O$, as indicated for each specific experiment (1 mM=232 mg/L). The pH of the medium was adjusted to 7.5 using hydrochloric acid. All Roux bottles were placed on magnetic stirrers and illuminated continuously (i.e., 24 h/day) from one side with a 500-W halogen (Regent PN PQS45). The light intensity was adjusted using neutral-density filters (i.e., door screen). All experiments were performed at room temperature (approximately 22 °C). Culture growth was initiated by adding 50 mL of inoculum to the Roux bottle. Further details about each specific experiment, including the composition for the sparging gas, are given in the respective figure captions.

Addition of DCMU

DCMU (Diuron™) inhibits photosynthesis by blocking the plastoquinone-binding site of PSII [20]. Thus, cultures challenged with DCMU are unable to carry out direct biophotolysis. Where indicated, 100 μL of a 0.02-g/mL solution of DCMU (100 μmol) dissolved in dimethyl sulfoxide was added to the culture.

Gas Composition Analysis

The gas composition of the Roux bottle headspace was analyzed using an SRI Instruments 8610C Gas Chromatograph equipped with a thermal conductivity detector. Argon was used as the carrier gas and the oven temperature was controlled at 35 °C. In all experiments where the cultures were kept anaerobic by sparging with a mixture of argon and CO_2, the gas flow rate was reduced prior to sampling to approximately 10 mL/min which assured that the concentration of hydrogen in the headspace was significantly above the detection limit. An equilibration period of approximately 2 h was permitted prior to the first headspace gas analysis. The cumulative volume of hydrogen (V_{H2}) was calculated as:

$$V_{H2} = \sum X_{H2} \times F_g \times \Delta t$$

where X_{H2} is the fraction of hydrogen in the bioreactor off-gas, F_g is the gas flow rate, and Δt is the time interval between measurements.

Biomass Measurement

The biomass in the algal cultures was measured both photospectrometrically as either optical density and/or gravimetrically as ash-free dry weight (AFDW, in milligrams per liter). For optical density (OD_{590}), the absorbance of the cell suspension at 590 nm was determined using a Unico 1100 photospectrometer, diluting the sample as needed to obtain an $OD_{590}<0.5$.

For AFDW, between 10 and 25 ml (V) of cell culture were vacuum filtered through a previously rinsed and ashed 55 mm Whatman GF/F glass microfiber filter placed in an aluminum foil pouch, heated overnight in an oven at 100–105 °C, followed by weighing (M_1) and then heating in a 550 °C muffle furnace for 4 h, followed by reweighing (M_2). The AFDW concentration was calculated as $(M_1-M_2)/V$.

Nitrate Measurement

The concentration of nitrate in the culture was determined using a cadmium reduction assay, using a Hach Company kit (http://www.hach.com). Approximately 11 mL of culture was removed using a sterile pipette and vacuum filtered through a glass fiber filter. The contents of one Hach NitraVer 5 packet, along with 10 mL of filtrate, were placed in a threaded test tube, which was then sealed and continually mixed for exactly 5 min. A 2.5-mL subsample of this reaction mixture was then placed in a cuvette and analyzed at 500 nm with a Unico 1100 spectrophotometer previously blanked with deionized water. The absorbance value was converted to the corresponding nitrate concentration using a calibration curve.

Chlorophyll-a Measurement

Chlorophyll-a was extracted from 1 mL of cell culture with boiling methanol following the procedure described by Tett et al. [21]. A 1-mL culture sample was transferred to a 35-mL amber glass centrifuge tube and 0.2 mL of 1% (w/v) $MgCO_3$ together with 9 mL of 90% (v/v) methanol/water were added. After boiling the vortexed sample in a water bath at 90 °C for 2 min, the extract was centrifuged at 1,500 rpm for 4 min to remove particulates. After reconstituting the volume of the cooled extract with 90% (v/v) methanol/water back to 10 mL, the absorbance of both the extract and a 90% (v/v) methanol/water blank was measured with a Beckman DU-8 Spectrophotometer at 663 and 750 nm. If necessary, the extract was diluted with 90% (v/v) methanol/water to assure that the absorbance reading was in the linear range, i.e., less than 0.8. The chlorophyll-a (Chl-a) concentration (in milligrams per liter) was then calculated as:

$$\text{Chl}-\text{a} = 13 \times \text{DF} \times ((C_{663} - B_{663}) - (C_{750} - B_{750}))$$

where DF is the dilution factor, C_{663} and C_{750} are the absorbance of the extract and B_{663} and B_{750} are the absorbance of the blank at 663 and 750 nm, respectively.

Glycogen Measurement

Glycogen concentration was measured using the method described by Gfeller and Gibbs [22]. A 1-mL culture sample was collected and centrifuged at 13,000 rpm for 2.0 min. The supernatant was discarded and replaced with 1 mL methanol. The sample was then sonicated for 10 s using a Branson Sonifier to suspend the pellet. Following a second

centrifugation, the sample was washed thrice more—once with 1.0 mL methanol and twice with 100 mM sodium acetate (1.0 mL×2) previously adjusted to pH4.5 using hydrochloric acid. Following each wash, the sample was centrifuged, and after the final wash, the pellet was resuspended in 1.7 mL fresh sodium acetate. α-Amylase (20 μL, Sigma Aldrich, A4582) was then added to the sample, which was placed in an autoclave for 10 min to solubilize the glycogen, after which amyloglucosidase (50 μL, Sigma Aldrich, S9144) was added and the sample placed in a 55 °C incubator for approximately 14 h to allow the glycogen to be hydrolyzed into glucose. The sample was then centrifuged, the volume was adjusted to 2.0 mL with distilled water, and the glucose assay reagent (2.0 mL, Sigma Aldrich, G3666, D2679, containing glucose oxidase, horseradish peroxidase, and *o*-dianisidine) was added followed by incubation for 30 min at 37 °C. The glucose oxidase oxidized the glucose into gluconic acid and hydrogen peroxide. The hydrogen peroxide, in conjunction with the horseradish peroxidase, oxidized the *o*-dianisidine to yield a brown-colored product [23]. The reaction was stopped by addition of 2 mL of 12 N sulfuric acid, which transformed the oxidized *o*-dianisidine into a more stable reddish-pink solution. After the sample was thoroughly mixed, the absorbance was measured at 540 nm using a Unico 1100 spectrophotometer, previously blanked with distilled water. The glucose concentration was determined using a previously measured standard curve.

Light Intensity Measurement

At various times during the culture experiments, the light intensity (micromoles per square meter second) was measured at five locations (i.e., at the four corners and in the middle) in front of and behind each Roux bottle using a Hansatech Instruments (Norfolk, UK) Quantitherm light meter.

Results and Discussion

Effect of DCMU at Low-Light Intensity

Three cultures were induced to produce hydrogen by growing them at moderate light intensities (100 μmol/m^2 s) until nitrogen was completely depleted and subsequently sparging them with argon–CO_2 to induce anaerobic conditions to allow for nitrogenase biosynthesis. Two cultures were then exposed to two low-light intensities (10 and 20 μmol/m^2 s) with the third culture exposed to both 20 μmol/m^2 s light and DCMU. As shown in Fig. 1, the rates and extents of hydrogen production were similar in all three cultures, reaching about 100–120 mL H_2/L after 210 h. Throughout the hydrogen-production phase, there was a continuous decline in biomass concentration (approximately 30% of initial). Most likely, the small amounts of oxygen that were still produced by photosynthesis at these low-light intensities by the nitrogen-depleted cultures were immediately removed by respiration, thereby ensuring the anaerobic conditions required for the functioning of the nitrogenase. The lack of effect of DCMU indicates that hydrogen was generated by indirect biophotolysis. As no other exogenous electron donors were added, the only plausible source would have been the intracellular glycogen reserves formed during nitrogen limitation. While not determined during this experiment, such reductions in intracellular glycogen reserves were measured during the hydrogen-production phase in subsequent experiments (see Table 1).

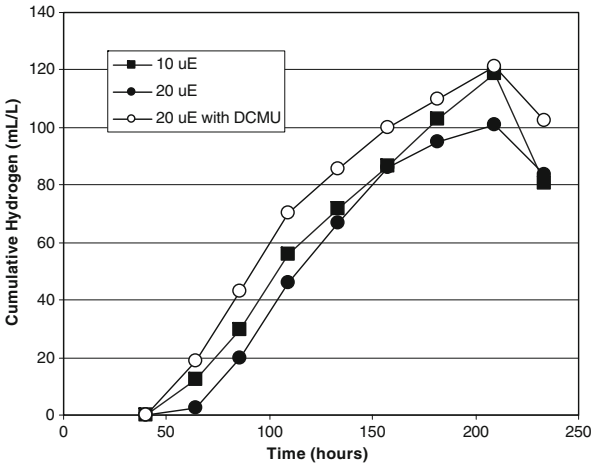

Fig. 1 Cumulative hydrogen production (in milliliters per liter) as a function of time. Three Roux bottles, filled with medium containing 0.5 mM N, were inoculated at time 0, mixed, sparged with CO_2-enriched air (99.7% air/0.3% CO_2 *v*/*v*), and continuously illuminated at 100 μmol/m^2 s. When the cultures had reached stationary growth due to nitrogen limitation (i.e., an OD_{590} of about 0.5, corresponding to approximately 150 mg/L AFDW, after 40 h), all Roux bottles were sparged with argon/CO_2 (99.7%/0.3% *v*/*v*) for 10 min to remove oxygen and generate anaerobic conditions necessary for the induction of the nitrogenase and hydrogenase enzymes. One culture was then illuminated at low-light intensity (10 μmol/m^2 s) while the two other ones were illuminated at twice that light intensity, i.e., 20 μmol/m^2 s with DCMU added to one of these cultures to inhibit the formation of oxygen via water splitting. The gas generated by each culture was collected in an inverted graduated cylinder submerged in a water bath and analyzed for hydrogen as described in the "Material and Methods" section. The decline in hydrogen concentrations in all three cultures at the end of the experiment was due to a headspace air leak as O_2 was detected in the gas sample in approximate air-level ratios

Table 1 Biomass concentrations, chlorophyll-a concentrations and contents, glycogen concentrations and contents, and hydrogen-production rates at t=188 h and t=408 h in three batch cultures illuminated at 50, 100, and 200 μmol/m^2 s, respectively.

Light intensity (μmol/m^2 s)	50		100		200	
Sampling time (h)	188	408	188	408	188	408
AFDW (mg/L)	390	195	340	190	560	235
Chlorophyll-a (mg/L)	NA	1.5	NA	0.65	NA	0.35
Chlorophyll-a content (mg/mg AFDW)	NA	0.78%	NA	0.34%	NA	0.15%
Glycogen (mg/L)	75	19	75	22	77	35
Glycogen content (mg/mg AFDW)	19%	10%	22%	11%	14%	15%
H_2-production rate						
mL/L h	0.61	0.89	2.51	1.25	2.67	1.23
mL/mg AFDW day	0.04	0.11	0.18	0.16	0.11	0.13
μmol/mg AFDW day[a]	1.54	4.51	7.31	6.51	4.72	5.19
mL/mg Chl-a h	NA	0.59	NA	1.92	NA	3.56
μmol/mg Chl-a h[a]	NA	24	NA	79	NA	147
μmol/μmol photons	NA	1.1%	NA	0.9%	NA	0.5%

[a] Using the ideal gas law approximation, 1 mL of H_2 equals 41.3 μmol at 22 °C

In order to determine whether there was any supersaturated hydrogen remaining in the liquid culture medium at the end of the experiment, the small headspace of all Roux bottles was sparged with nitrogen and each bottle was then shaken vigorously by hand for about 5 min. A subsequent analysis of the headspace revealed about 4–6 mL H_2/L of culture (approximately 0.25 mmol/L). Although not a large amount in this case, it is important to correct for any such dissolved liquid phase H_2, as these can easily accumulate many-fold above saturation, resulting in possibly significant errors if the slow out-gassing of supersaturated hydrogen is not accounted for [24].

Effect of Medium Nitrate Concentration

As expected, the concentration of nitrate in the medium was directly correlated to the biomass concentration reached at the end of the photosynthetic growth phase under these aerobic growth conditions, since, unlike heterocystous cyanobacteria, *P. boryanum* can fix N_2 only under at most microaerobic conditions, i.e., O_2 concentration <2% of saturation [13]. The 0.5, 1, and 2 mM N-containing cultures reached a final optical density (OD_{590}) of about 1, 1.75, and 3, corresponding to approximately 305, 480, and 770 mg AFDW/L, respectively, after 70 to 90 h of incubation. After confirming that the nitrate concentration in all stationary growth cultures was below the assay detection limit, DCMU (10 µmol) was added to a duplicate 1 mM N culture at t=70 h, and the off-gasses of all Roux bottles were periodically monitored for hydrogen (Fig. 2). The onset of hydrogen production was detected first in the culture containing 0.5 mM N, later in the culture with 1.0 mM N, and finally in the culture with 2 mM N, which took longer to achieve the nitrogen-limited conditions necessary for the induction of the nitrogenase enzyme. The highest rate and extent of hydrogen production was observed in the 1-mM N culture without DCMU added with 480 mL H_2 produced per liter of culture in 480 h (r_{H2}=1 mL/L h). The 0.5-mM N culture produced about 25% less total hydrogen, possibly because it had a lower biomass concentration. Surprisingly, hydrogen generation in the 2-mM N culture not only started

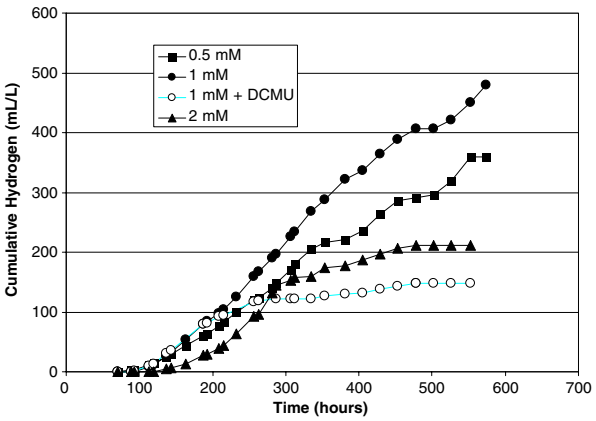

Fig. 2 Cumulative hydrogen production (in milliliters per liter) as a function of time. Four Roux bottles, filled with 950 mL Chu #10 medium containing 0.5, 1.0, 1.0, and 2.0 mM N, respectively, were inoculated at time 0, sparged with argon/CO_2 (99.7%/0.3% *v*/*v*) at 50–100 mL/min under continuous illumination of 100 µmol/m^2 s. As soon as biomass growth slowed down due to nitrogen limitation, one of the 1-mM cultures was spiked with DCMU (10 µmol at t=70 h; see the "Material and Methods" section), and the off-gasses from all four Roux bottles were periodically monitored for hydrogen concentrations (see the "Material and Methods" section)

later but also had a lower rate and stopped after reaching only 210 mL/L. It was previously observed (Benemann, unpublished) that, if the cultures were too dense at the onset of nitrogen limitation, nitrogenase activity was poorly expressed, possibly due to limited glycogen accumulation under light limitation. It is interesting to note that the 0.5-mM N culture and, to a lesser extent, also the 1-mM N culture, exhibited alternating periods of fast and slow hydrogen production (i.e., wavy curves). This cyclical hydrogen production may be indicative of a synchronized culture in which periods of glycogen synthesis alternate with nitrogenase-based hydrogen production, as was also reported previously [13].

The addition of PSII inhibitor DCMU to the 1-mM N culture did not have any significant effect on hydrogen production for about the first 150 h, after which hydrogen generation slowed down considerably, reaching only 150 mL/L. As in the low-light experiment (see Fig. 1), the initial insensitivity of hydrogen generation to DCMU is indicative of the fact that hydrogen is not derived from water splitting at PSII (i.e., direct biophotolysis) but rather from intracellular glycogen reserves (i.e., indirect biophotolysis). It apparently took about 150 h for these glycogen reserves to become depleted, at which point hydrogen production slowed down and ceased, as no further glycogen could be synthesized in the presence of DCMU. By comparison, the 1-mM culture that was not inhibited by DCMU continued to generate hydrogen, most likely in alternating cycles of glycogen synthesis and hydrogen production (as noted more clearly with the 0.5-mM culture).

Effect of Light Intensity

Following inoculation, all cultures, containing initially 1 mM N, reached similar stationary biomass concentrations (OD_{590}) of 1.75 (±0.25) after about 48 h in the 100- and 200-μmol/m^2 s illuminated cultures and 70 h in the 50-μmol/m^2 s illuminated culture. Following the onset of nitrogen limitation, DCMU (10 μmol) was added to one of the 100-μmol/m^2 s cultures and hydrogen was monitored in the off-gasses. As shown in Fig. 3,

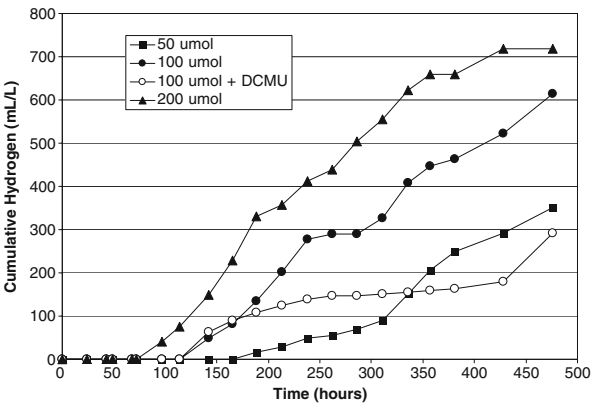

Fig. 3 Cumulative hydrogen production (in milliliters per liter) as a function of time. Four Roux bottles, filled with 950 mL Chu #10 medium containing 1.0 mM N, were inoculated at time 0, sparged with argon/CO_2 (99.7%/0.3% *v*/*v*) at 50–100 mL/min under continuous illumination of 50, 100, 100, and 200 μmol/m^2 s, respectively. As soon as biomass growth slowed down due to nitrogen limitation, one of the 100-μmol/m^2 s cultures was spiked with DCMU (10 μmol at t=48.5 h; see the "Material and Methods" section), and the off-gasses from all four Roux bottles were periodically monitored for hydrogen concentrations as described in the "Material and Methods" section

hydrogen production started first in the high-light (200 μmol/m^2 s) culture and last in the low-light (50 μmol/m^2 s) culture, which was expected, as this culture was the last to reach nitrogen-limited conditions due to slower growth. Hydrogen generation in the 50-μmol/m^2 s culture was relatively slow for the first 150 h but then increased to a rate comparable to those in the 100- and 200-μmol/m^2 s cultures. As was also observed in the prior experiment (Fig. 2), all cultures, particularly the 100-μmol/m^2 s culture, exhibited a pattern of cyclical hydrogen generation, suggesting again partially synchronized cultures in which periods of glycogen synthesis alternate with nitrogenase-based hydrogen production. And, again, the addition of DCMU to one of the 100-μmol/m^2 s cultures did not adversely affect hydrogen-production rates for the first 50 h, indicating hydrogen generation by indirect biophotolysis from intracellular carbohydrate (i.e., glycogen) reserves. However, as soon as these become depleted, hydrogen synthesis slows down considerably. (The last data point is plausibly a measurement error or artifact since it is unlikely that DCMU would have lost effectiveness at that point.)

As shown in Table 1, the biomass concentration at all three light intensities during the hydrogen-production period of about 220 h (between hour188 and 408) declined by ca. 50%. Some biomass decline may be expected as the cultures used their glycogen reserves and, indeed, glycogen declined, on a per liter basis, between 55% and 74%, with the DCMU-inhibited culture exhibiting the largest loss, at 88% (data not shown). The cellular glycogen content also declined about 50% in the 50- and 100-μmol/m^2 s cultures (and 70% in the DCMU-spiked culture), but not in the 200-μmol/m^2 s culture. This suggests more directly that glycogen served as an electron donor for nitrogenase/hydrogenase-mediated hydrogen generation by indirect biophotolysis. However, glycogen alone cannot explain the large decline in biomass, which was likely mainly due to cell lysis occurring during this nongrowth H_2-production period.

In the nitrogen-deficient cultures, the cellular chlorophyll-a content decreased from 0.78% to 0.34% to 0.15% as the light intensity increased from 50 to 100 to 200 μmol/m^2 s, respectively. This response suggests that, even under such nongrowing conditions, the cells responded by increasing the number of chlorophyll antennae to harvest more photons, while at high-light intensity, the cells adapted by reducing chlorophyll content.

Table 1 also presents the specific hydrogen-production rates based on biomass or chlorophyll-a to allow for easy comparison with data reported in the literature. Light intensity did not have any significant effect on AFDW-normalized hydrogen-production rates, which ranged from 0.04 to 0.18 mL/mg day (1.54 to 7.31 μmol/mg day) in all three cultures, but chlorophyll-a normalized hydrogen-production rates increased from 0.59 to 1.92 to 3.56 mL/mg h as light intensity increased from 50 to 100 to 200 μmol/m^2 s, respectively. Finally, the photosynthetic energy conversion efficiency, as micromoles of H_2 generated per micromole of photons absorbed, decreased from 1.1% at 50 μmol/m^2 s to 0.9% at 100 μmol/m^2 s to 0.5% at 200 μmol/m^2 s. This decline in energy conversion efficiency may be caused by the light saturation effect [25].

The hydrogen-production rates listed in Table 1 are comparable to those reported in the literature for other hydrogen-generating cyanobacteria and microalgae. Kashyap et al. [11] observed rates of about 2.9 μmol H_2/mg day in nitrogen-limited cultures of *P. boryanum*, a rate that increased fivefold by addition of 2 mM of the reducing agent dithionite. Kumazawa and Mitsui [26] measured a hydrogen-production rate of 0.14 mL/mg day using *Oscillatoria* sp. Miami BG7, although this may have been a heterocystous species after all. Markov et al. [27] used a strain of *Anabaena variabilis* without uptake hydrogenase which evolved H_2 at 0.22 mL/mg day. Finally, the rate of hydrogen production in one of the most

frequently studied species of green algae, *Chlamydomonas reinhardtii*, was found to be 0.34 mL/mg day [28] or 9.4 μmol/mg Chl-a h [29] under sulfur-depleted culture conditions, although hydrogen was generated mostly by direct biophotolysis (contrary to initial reports that this was indirect biophotolysis; [30]). The rates reported herein are comparable to these other studies.

Hydrogen Generation in Alternating Aerobic–Anaerobic Cycles

Figure 4 shows the results from two replicate cultures that were subjected to alternating cycles of aerobic photosynthesis and anaerobic hydrogen production. Following the initial

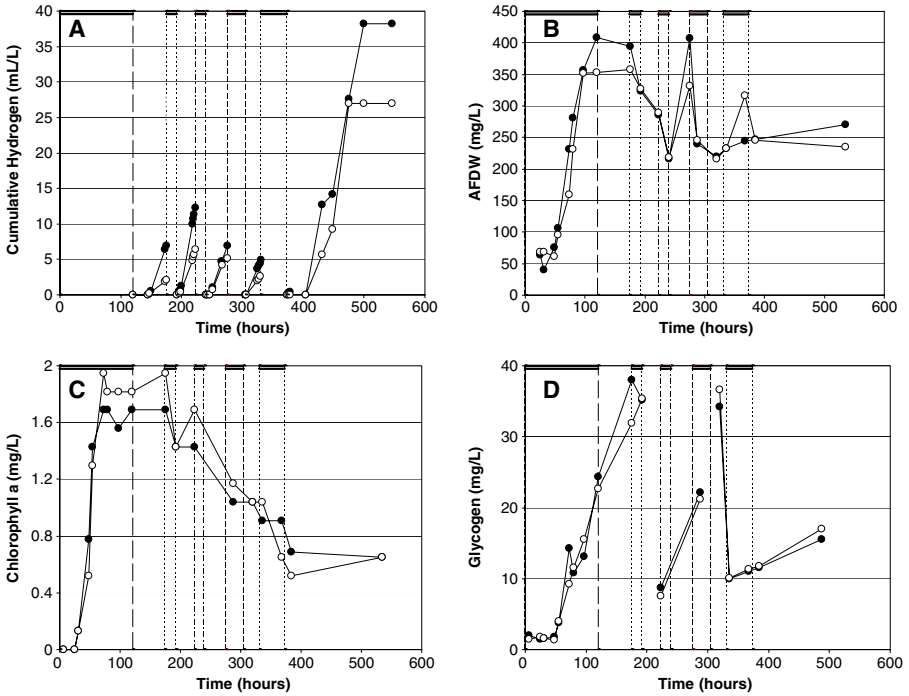

Fig. 4 **a** Cumulative hydrogen production (in milliliters per liter) and concentrations of **b** biomass (in milligrams of ADFW per liter), **c** chlorophyll-a (in milligrams per liter), and **d** glycogen (in milligrams per liter) as a function of time in two replicate cultures (**a** ●, **b** ○) subjected to alternating aerobic–anaerobic cycles. The duration of the aerobic phases are indicated by a *double line at the top* of each plot. During the aerobic photosynthetic CO_2-biofixation phase, the Roux bottles were sparged with carbon dioxide-enriched air (99.7% air/0.3% CO_2 *v*/*v*), while during the subsequent anaerobic hydrogen-production phase, the cultures were sparged with 100% argon. Specifically, two replicate Roux bottles with Chu #10 medium containing 1 mM N were inoculated at time 0 and allowed to grow in CO_2-enriched air under continuous illumination of 100 μmol/m^2 s. As soon as nitrogen limitation was detected, the cultures were sparged with argon and monitored for hydrogen production. Once started, hydrogen production was allowed to continue during this anaerobic phase for about 1.5 days. Subsequently, the cultures were replenished with nitrate by adding fresh medium, containing 1.0 mM N, equal to 10% of the volume in each Roux bottle. The cultures were then switched aerobic for the following 1/2 day by sparging with CO_2-enriched air to allow for biomass growth. At the end of this aerobic CO_2-biofixation phase, the cultures again became nitrogen limited, at which point they were switched anaerobic to induce hydrogen production. After five such aerobic–anaerobic cycles, the experiment was terminated by adding DCMU (10 μmol, see the "Material and Methods" section) to each Roux bottle. The culture volumes were recorded throughout the experiment to allow for the calculation of specific hydrogen-production rates, i.e., in milliliters of H_2 per liter of culture

period of biomass growth (see Fig. 4b), the cultures were switched to anaerobic conditions after becoming nitrogen limited at around t=120 h and monitored for hydrogen production. After a period of hydrogen production, the cultures were subjected to another period of aerobic conditions followed by a hydrogen-production cycle, and this was repeated two more times. As shown in Fig. 4a, hydrogen production was observed in the four anaerobic periods in both cultures. The highest rate was observed in culture A in the first cycle, i.e., 0.44 mL/L h, equivalent to 0.034 mL/mg ADFW day (1.41 μmol/mg AFDW day). The rate of hydrogen generation slowly decreased in successive cycles to the point that no hydrogen was produced in either culture at the beginning of the fifth cycle (t=373 h). It is likely that the cultures became increasingly stressed by the nitrogen-limited conditions and the alternating aerobic–anaerobic cycling, as evidenced by significant declines in both biomass (Fig. 4b) and chlorophyll-a (Fig. 4c) concentrations during the duration of the experiment.

Following addition of DCMU (10 μmol) at the end of the unproductive fifth anaerobic cycle (t=403 h), hydrogen generation unexpectedly started in both cultures and continued for about 70 h (t=475 h) at which point it completely stopped. It is not clear why the addition of DCMU triggered renewed hydrogen production but it is possible that inhibiting photosynthetic oxygen evolution maintained the anaerobic conditions required for the activity of nitrogenase. Hydrogen production in the presence of DCMU in prior experiments (see above) is likely due to respiration by these cultures, which removed the residual oxygen produced, but in the degraded cultures at the end of the experiment in Fig. 4, respiration was likely severely reduced. The fact that hydrogen generation occurred, the presence of DCMU confirms, as in earlier experiments (see Figs. 1, 2, and 3), that indirect biophotolysis, rather than direct biophotolysis, was the responsible mechanism. The glycogen concentration (Fig. 4d) declined during the fourth anaerobic hydrogen generation period from 35 to 10 mg/L, suggesting that the decline in glycogen reserves is directly associated with hydrogen generation. On the other hand, glycogen concentrations increased significantly during the first anaerobic hydrogen-production phase and did not decline after the addition of DCMU, which is not what we anticipated.

Additional research is needed to further elucidate the mechanisms of hydrogen generation in this nonheterocystous nitrogen-fixing cyanobacterium. However, we have demonstrated that this alga could be used as a model system for indirect biophotolysis on which an economic industrial-scale renewable hydrogen manufacturing process could be based [31].

Acknowledgements Funding for this project was provided by the Department of Energy National Energy Technology Laboratory and two SULI stipends from the Department of Energy Office of Science to Blaine Carter and Jared Gerschler, respectively.

References

1. Huesemann, M. H. (2006). Can advances in science and technology prevent global warming? A critical review of limitations and challenges. *Mitigation and Adaptation Strategies for Global Change, 11*, 539–577.
2. Prince, R. C., & Kheshgi, H. S. (2005). The photobiological production of hydrogen: Potential efficiency and effectiveness as a renewable fuel. *Critical Reviews in Microbiology, 31*, 19–31.
3. Healey, F. P. (1970). Hydrogen evolution by several algae. *Planta, 91*, 220–226.

4. Ghirardi, M. L., Zhang, L., Lee, J. W., Flynn, T., Seibert, M., Greenbaum, E., et al. (2000). Microalgae: A green source of renewable H_2. *Trends in Biotechnology, 18*, 506–511.
5. Melis, A., & Happe, T. (2001). Hydrogen production: Green algae as a source of energy. *Plant Physiology, 127*, 740–748.
6. Benemann, J. R. (1996). Hydrogen biotechnology: Progress and prospects. *Nature Biotechnology, 14*, 1101–1103.
7. Benemann, J. R. (1997). Feasibility analysis of photobiological hydrogen production. *International Journal of Hydrogen Energy, 22*(10), 979–987.
8. Tredici, M., Zittelli, G., & Benemann, J. R. (1998). In O. Zaborsky, et al. (Eds.), *BioHydrogen* (pp. 391–402). New York: Plenum.
9. Hallenbeck, P. C., & Benemann, J. R. (2002). Biological hydrogen production: Fundamentals and limiting processes. *International Journal of Hydrogen Energy, 27*, 1185–1193.
10. Benemann, J. R., & Weare, N. M. (1974). Hydrogen evolution by nitrogen-fixing *Anabaena cylindrica* cultures. *Science, 184*, 174–175.
11. Kashyak, A. K., Pandey, K. D., & Sarkar, S. (1996). Enhanced hydrogen photoproduction by non-heterocystous cyanobacterium *Plectonema boryanum*. *International Journal of Hydrogen Energy, 21*(2), 107–109.
12. Rogerson, A. C. (1980). Nitrogen fixing growth by non-heterocystous cyanobacterium *Plectonema boryanum*. *Nature, 284*, 563–564.
13. Weare, N. M., & Benemann, J. R. (1974). Nitrogenase activity and photosynthesis in *Plectonema boryanum*. *Journal of Bacteriology, 119*(1), 258–265.
14. Burrows, E. H., Chaplen, F. W. R., & Ely, R. L. (2008). Optimization of media nutrient composition for increased photofermentative hydrogen production by *Synechocystis* sp. PCC 6803. *International Journal of Hydrogen Energy, 33*(21), 6092–6099.
15. Troshina, O., Serebryakova, L., Sheremetieva, M., & Lindblad, P. (2002). Production of hydrogen by the unicellular cyanobacterium *Gloeocapsa alpicola* CALU 743 during fermentation. *International Journal of Hydrogen Energy, 27*(11–12), 1283–1289.
16. Yoo, S. H., Keppel, C., Spalding, M., & Jane, J. L. (2007). Effects of growth condition on the structure of glycogen produced in cyanobacterium *Synechocystis* sp. PCC6803. *International Journal of Biological Macromolecules, 40*(5), 498–504.
17. Dutta, D., De, D., Chaudhuri, S., & Bhattacharya, S. K. (2005). Hydrogen production by cyanobacteria. *Microbial Cell Factories, 4*, 36.
18. Madamwar, D., Garg, N., & Shah, V. (2000). Cyanobacterial hydrogen production. *World Journal of Microbiology and Biotechnology, 16*, 757–767.
19. Rao, K. K., & Hall, D. O. (1996). Hydrogen production by cyanobacteria: Potentials, problems, and prospects. *Journal of Marine Biotechnology, 4*, 10–15.
20. Berry, S., Bolychevtseva, Y. V., Rogner, M., & Karapetyan, N. V. (2003). Photosynthetic and respiratory electron transport in the alkaliphilic cyanobacterium *Arthrospira (Spirulina) platensis*. *Photosynthesis Research, 78*, 67–76.
21. Tett, P., Kelly, M. G., & Hornberger, G. M. (1975). A method for the spectrophotometric measurement of chlorophyll-a and pheophytin a in benthic microalgae. *Limnology and Oceanography, 20*(5), 887–896.
22. Gfeller, R., & Gibbs, M. (1984). Fermentative metabolism of *Chlamydomonas reinhardtii*. *Plant Physiology, 7*(75), 212–218.
23. Bergmeyer, H. U., & Bernt, E. (1974). *Methods of enzymatic analysis* (2nd ed., pp. 1205–1212). New York: Academic.
24. Pauss, A., Andre, G., Perrier, M., & Guiot, S. R. (1990). Liquid-to-gas mass transfer in anaerobic processes: Inevitable transfer limitations of methane and hydrogen in the biomethanation process. *Applied and Environmental Microbiology, 56*(6), 1636–1644.
25. Huesemann, M. H., Hausmann, T. S., Bartha, R., Aksoay, M., Polle, J., Weissman, J. C., et al. (2009). Biomass productivities in wild type and a new pigment mutant of *Cyclotella* sp. (Diatom). *Applied Biochemistry and Biotechnology, 157*(3), 507–526.
26. Kumazawa, S., & Mitsui, A. (1981). Characterization and optimization of hydrogen photoproduction by a saltwater blue–green alga, *Oscillatoria* sp. Miami BG7. 1. Enhancement through limiting the supply of nitrogen nutrients. *International Journal of Hydrogen Energy, 6*, 339–348.
27. Markov, S., Weaver, P. F., & Seibert, M. (1996). Hydrogen production using microorganisms in hollow-fiber bioreactors. In T. N. Verziroglu, et al. (Eds.), *Hydrogen energy progress XI, Proceedings of the 11th World Hydrogen Energy Conference, Stuttgart, Germany, 23–28 June 1996, volume 3* (pp. 2619–2624). Frankfurt am Main: Schon and Wetzel.
28. Ghirardi, M. L., Kosourov, S., Tsygankov, A., & Seibert, M. (2000b). Two-phase photobiological algal H_2-production system. Proceedings of the 2000 DOE Hydrogen Program Review, NREL/CP-570-28890.

29. Kosourov, S., Seibert, M., & Ghirardi, M. L. (2003). Effects of extracellular pH on the metabolic pathways in sulfur-deprived, H_2-producing *Chlamydomonas reinhardtii* cultures. *Plant Cell Physiology, 44*(2), 146–155.
30. Melis, A., Zhang, L., Forestier, M., Ghirardi, M. L., & Seibert, M. (2000). Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green algae *Chlamydomonas reinhardtii*. *Plant Physiology, 122*, 127–136.
31. Benemann, J. R. (1998). Process analysis and economics of biophotolysis of water. Report to the International Energy Agency (IEA), IEA/H2/10/TR2-98, March 1998.