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International Journal of Hydrogen Energy 31 (2006) 1574–1584
International Journal of HYDROGEN ENERGY
www.elsevier.com/locate/ijhydene

# Hydrogen production by sulfur-deprived *Chlamydomonas reinhardtii* under photoautotrophic conditions

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Available online 20 July 2006

**Abstract**

Thus far, all experiments leading to $H_2$ production by sulfur-deprived cultures of microalga have been done with photoheterotrophic cultures in the presence of acetate, which increases the cost of the $H_2$ produced. This study demonstrates that sustained $H_2$ photoproduction by a sulfur-deprived green alga, *Chlamydomonas reinhardtii*, is possible under strictly photoautotrophic conditions in the absence of acetate or any other organic substrate in the medium. To accomplish this, we used cultures pre-grown with 2% $CO_2$ under low light conditions ($25\,\mu E\,m^{-2}\,s^{-1}$) and also supplemented with $CO_2$ during S-deprivation, along with a special light regime. Maximum $H_2$ production ($56.4 \pm 16.7\,ml\,l^{-1}$ culture, equal to $56.4 \times 10^{-3}\,m^3\,m^{-3}$ culture) was observed with photoautotrophic cultures: (a) supplied with carbon dioxide for the first 24 h of sulfur deprivation, (b) exposed during the $O_2$-producing stage to high light ($110 - 120\,\mu E\,m^{-2}\,s^{-1}$), and (c) then exposed to low light ($20 - 25\,\mu E\,m^{-2}\,s^{-1}$) during the $O_2$-consumption and $H_2$-production stages.


*Keywords:* Hydrogen production; Green algae; Photoautotrophic; Sulfur-deprivation

## 1. Introduction

Prolonged exposure of green algae to anaerobic conditions in the dark leads to the expression of the hydrogenase enzyme and subsequent $H_2$ evolution in the light. This phenomenon was first reported over 60 years ago in the pioneering work of Gaffron and Rubin [1], but until recently algal $H_2$ production remained a laboratory curiosity, despite its fundamental importance and practical potential. Recent studies reported that $H_2$ photoproduction in anaerobically adapted green algae proceeds at higher rates compared to other microalgae [2] and that the light conversion efficiency of the process can be very high [3,4]. Nevertheless, the generation of bulk quantities of $H_2$ by green algal cultures was considered impractical, due to the fact that hydrogenase in green algae is very sensitive to the $O_2$ co-evolved during photosynthesis. This problem was deemed unsolvable [5]. As a consequence of its sensitivity to $O_2$, $H_2$ photoproduction in anaerobically adapted cells can be sustained for only short periods of time in the absence of $O_2$ scavengers [2,6]. Recent studies revealed that not

*Abbreviations:* PhBR, photobioreactor; HL, high-light conditions; LL, low-light conditions; TAP, Tris-acetate-phosphate containing medium; PAR, photosynthetically active radiation; $pO_2$, partial oxygen pressure of dissolved oxygen; PSII, photosystem II

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0360-3199/$30.00 
doi:10.1016/j.ijhydene.2006.06.024

only the hydrogenase enzyme *per se* is irreversibly inhibited by molecular $O_2$ but also the expression of the genes associated with $H_2$ metabolism is down regulated in the presence of $O_2$ [7–10].

A few years ago, a team at UC Berkeley and NREL devised a system for bulk production of molecular $H_2$ in *Chlamydomonas reinhardtii* cultures [11,12]. The system is based on the partial inactivation of photosystem II $O_2$-evolving activity in algal cells in response to sulfur-deprivation stress [13]. The inhibition of photosystem II (PSII) activity results in the transition of the cultures to anaerobic conditions, expression of the hydrogenase enzyme, and $H_2$ gas production in the light for several days [11,12]. In the first report of the sulfur-deprived $H_2$-producing system [11], it was proposed that protein degradation was the main process feeding electrons to PSI, since starch degradation during $H_2$ production stage was not significant in the algal strain used. Later, it was shown that electrons for $H_2$ evolution were derived mostly from residual PSII activity [14–16], and that the relative contribution of PSII to $H_2$ photoproduction depends on the stage of sulfur deprivation [17]. It is now accepted that both water oxidation and the endogenous catabolism of starch and/or protein contribute electrons to $H_2$ production [16,18]. Moreover, organic substrate degradation fuels the respiratory consumption of $O_2$ produced by residual PSII activity during the $H_2$-production stage and is responsible for maintaining culture anaerobicity [12,16,19,20]. Finally, substrate degradation during the $H_2$-production stage is also required to maintain the appropriate intracellular redox potential to control the expression of the hydrogenase gene in *C. reinhardtii* [10].

All experiments on $H_2$ production by sulfur-deprived cultures have been done so far with photoheterotrophic cultures using TRIS-acetate-phosphate (TAP) medium. When photoheterotrophic cultures are sulfur-deprived, acetate is consumed during the $O_2$-producing and $O_2$-consuming, aerobic stages, but not during the $H_2$-production anaerobic stage [11]. Indeed, some acetate is even produced during $H_2$-production stage [16]. The use of acetate in the growth medium increases the expense associated with maintenance of the system and, as a consequence, the cost of the $H_2$ gas produced [21]. Indeed, the molar ratio of $H_2$ produced per mole of acetate consumed during the aerobic phase is 1.0 at an initial pH of 7.7 and lower at other pH values [16]. In contrast, purple bacteria use light in combination with organics to produce $H_2$ much more efficiently [22], and are able to achieve molar ratios of 2.67 $H_2$/acetate [23] compared to a maximum theoretical ratio of 4 [24]. These observations raised the question of the feasibility of using photoautotrophic instead of photoheterotrophic sulfur-deprived green algae for $H_2$ production.

The present study demonstrates that sustained $H_2$ photoproduction by the sulfur-deprived green alga, *C. reinhardtii*, is possible under strictly photoautotrophic conditions, in the absence of acetate or any other organic substrate in the medium. We accomplished this by pre-cultivating cells under a special light regime and $CO_2$ supplementation during S-deprivation of the culture.

## 2. Materials and methods

### 2.1. Cell growth conditions and sulfur-deprivation procedure

Wild-type *C. reinhardtii* Dang 137C (*mt*+) was grown photoautotrophically in flat glass bottles containing 1.5 l of high salt (HS) medium, pH 7.0 [25], at 28 ± 1 °C. Algal cultures were bubbled continuously with 2% $CO_2$ in air. During growth, the algae were illuminated from two sides with cool-white fluorescence lamps providing an average incident light intensity of about 25 $\mu E\,m^{-2}\,s^{-1}$ PAR (low light conditions, LL) or 120 $\mu E\,m^{-2}\,s^{-1}$ PAR (high light conditions, HL) on each surface of the culture bottles. If not specially indicated, cells were grown to about 13–16 $\mu g\,Chl\,ml^{-1}$ (13–16 × $10^{-3}$ $kg\,m^{-3}$) under LL and to about 18–22 $\mu g\,Chl\,ml^{-1}$ (18–22 × $10^{-3}$ $kg\,m^{-3}$) under HL. This required about 72 h cultivation for the algae placed under HL conditions and about 96 h for the algae placed under LL conditions. For sulfur deprivation, cultures were harvested and washed two times in HS-minus-sulfur medium by centrifugation (5 min at 2800*g*), and re-suspended in the same medium to a final concentration of about 14–16 $\mu g\,Chl\,ml^{-1}$ (14–16 × $10^{-3}$ $kg\,m^{-3}$). In the HS-minus-sulfur medium all sulfates were replaced with chloride salts at the same concentrations. Experiments with batch cultures in the absence of pH control were done in HS-minus-sulfur medium with or without addition of 40 mM Tris-HCl. The pH of the medium was adjusted to 7.0 with concentrated HCl. Prior to the experiment, filter-sterilized 10–40 mM $NaHCO_3$ was introduced into the PhBRs to optimize the pH of the culture for $H_2$ production.

Sulfur-deprived cultures were transferred either to sealed 500 ml cylindrical bottles with an inner diameter of 60 mm [17] or to the automated PhBRs described in the next section.

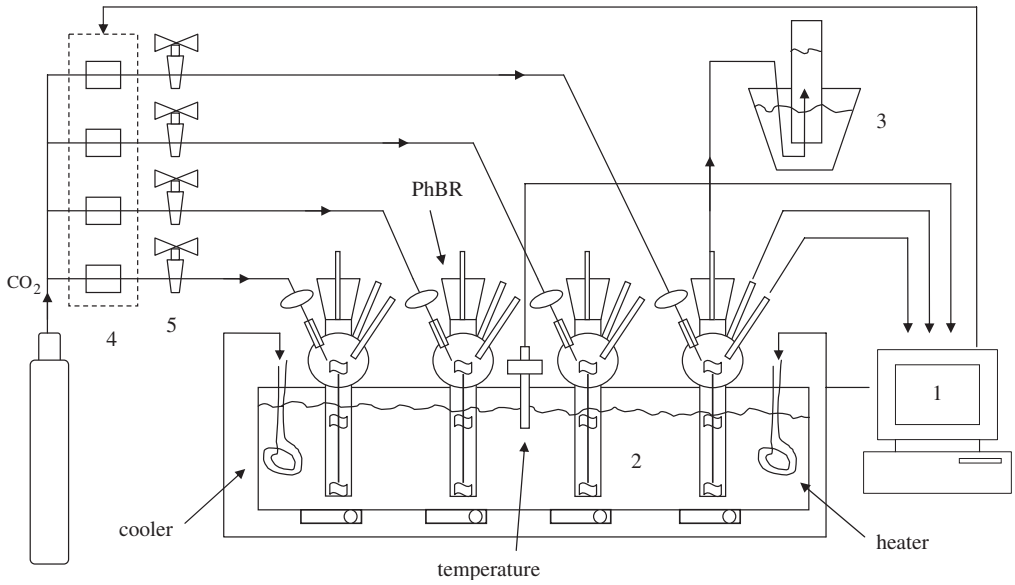


Fig. 1. Schematic drawing of the automated PhBR system used for studying $H_2$ production under photoautotrophic conditions: (1) microprocessor system, (2) thermostated water bath, (3) upside-down graduated cylinder for $H_2$ collection, (4) electromagnetic valves, and (5) manual valves.

### 2.2. Photobioreactor system

Fig. 1 is a schematic of the PhBR system used for studies of $H_2$ production under photoautotrophic conditions. Four specially fabricated glass PhBRs ($\sim$ 0.55 l culture volume; [14,15]) with pH and $pO_2$ sensors [26] were installed in a water bath and illuminated from two sides with cool-white fluorescent lamps. The light intensity could be adjusted as required. In our experiments, we used two light intensities: 20 $\mu E\,m^{-2}\,s^{-1}$ (mentioned in the text below as low light intensity, LL), and 110 $\mu E\,m^{-2}\,s^{-1}$ (mentioned as high light intensity, HL). A magnetic stirring bar attached to a glass rod with three mounted agitators was placed inside each PhBR for mixing capability. The pH and $pO_2$ sensors were connected to a PC with pre-installed software via analog-digital converters and special controller cards [26]. The temperature in the water bath was controlled using a temperature sensor and maintained at 28±0.2 °C with a heater and a cooler operating under the control of the same computer. The PhBRs were sealed with threaded silicon stoppers and attached FEP tubing (Cole–Parmer) for gas collection. The gas produced by the cells was collected in an upside-down graduated cylinder filled with water. The $H_2$ measured was expressed in the figures and tables as the collected gas, but the dissolved $H_2$ (app. 18 $ml\,l^{-1}$ at $H_2$ saturation, 28 °C, equal to $18 \times 10^{-3}$ $kg\,m^{-1}$) in the liquid was ignored. The pH of the medium was maintained at 7.4 during the photosynthetic $O_2$-evolution stage of sulfur deprivation by adding sterile carbon dioxide. At the start of sulfer deprivation experiment, sterile sodium bicarbonate solution was introduced into the cultures to bring the pH to 7.4. The quantity of added $CO_2$ was monitored by the system.

### 2.3. $H_2$ photoproduction in short-term experiments

Two milliliters of the culture were taken anaerobically from the PhBR at different times after sulfur deprivation and injected into 13 ml vials filled with pure argon. The vials were then evacuated, refilled with argon three times, and placed in a water bath at 30 °C. During the experiment, the culture aliquots were illuminated from one side with two cool white fluorescent lamps and continuously shaken. The light intensity on the surface of vials was varied from 8 to 150 $\mu E\,m^{-2}\,s^{-1}$ with neutral density screens. The quantity of $H_2$ accumulated in the gas phase was measured by gas chromatography on an hourly basis for a total period of 4 h.

### 2.4. Other analytical procedures

The chlorophyll a + b content was assayed spectrophotometrically in 95% ethanol extracts [25]. Samples for starch determination (2 ml) were taken from the PhBR and prepared as described by Gfeller and Gibbs [27]. The glucose level in the samples was measured

according to the Trinder's technique (Glucose GOD FS kit, DiaSys, Germany). The starch data are presented as millimoles of glucose equivalent. The $H_2$ concentration in the gas produced by the culture was determined by gas chromatography [14]. Light intensities were measured with a Li-Cor quantum photometer (Model LI-250, Lincoln, USA).

## 3. Results

### 3.1. Demonstration of photoautotrophic $H_2$ production

$H_2$ production by sulfur-deprived *C. reinhardtii* cultures under photoautotrophic conditions can indeed be demonstrated in HS medium with added bicarbonate. Initially, photoautotrophic cultures, pre-grown under HL conditions (120 μE $m^{-2}$ $s^{-1}$ PAR), were sulfur-deprived and incubated under 150 μE $m^{-2}$ $s^{-1}$ PAR in a non-automated PhBR system. At the beginning of S-deprivation, 10–40 mM $NaHCO_3$ was introduced into each PhBR as the sole carbon source, and the PhBRs were sealed on the second day of sulfur deprivation. Under these conditions, sulfur-deprived algae did not produce $H_2$ gas (Fig. 2, closed circles), because the pH was too high. Even in the presence of only 10 mM $NaHCO_3$, the pH of the cultures were higher than 8.8 at the end of incubation under S-deprivation. Since previous work with photoheterotrophic cultures showed that high pH inhibits $H_2$ production in algal cells [16], we added Tris-HCl buffer to the medium. The buffering capacity of 40 mM Tris-HCl in the HS-minus-sulfur medium was enough to protect the cultures from the rapid increase in pH during the $O_2$-production stage (when the algae assimilated bicarbonate ions).

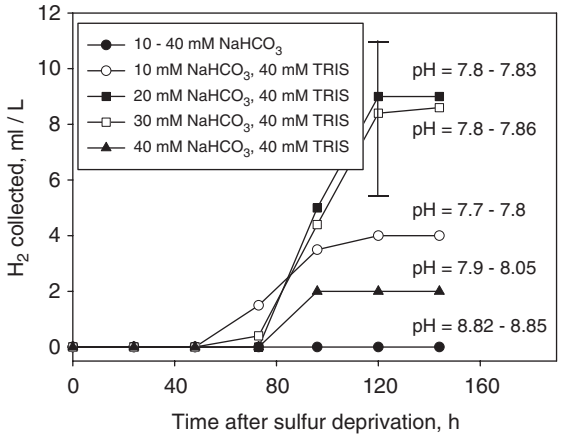


Fig. 2. Photoautotrophic $H_2$ production by sulfur-deprived cultures in modified HS medium with added 10–40 mM bicarbonate and 40 mM Tris-HCl. Cells were pre-grown under high-light conditions (120 μE $m^{-2}$ $s^{-1}$ PAR), sulfur deprived and incubated under 150 μE $m^{-2}$ $s^{-1}$ of light throughout the experiment. Typical experimental error for this series of trials (standard deviation of six experiments) is given for one point. One ml of $H_2$ produced per l of culture corresponds to $10^{-3}$ $m^3$ $H_2$ $m^{-3}$ of culture.

As shown in Fig. 2, $H_2$ gas appeared in the system after about 60 h of sulfur deprivation, and $H_2$ photoproduction stopped after 120 h. In this case, the pH of the medium was about 7.7–8.05 at the end of incubation period, depending on the quantity of added bicarbonate. The maximum output of $H_2$ (about 9 ml per liter of culture) was obtained when 20–30 mM $NaHCO_3$ was introduced in the medium at the beginning of the experiment. The decreased yield of $H_2$ in cultures with 40 mM bicarbonate can be explained either by the high pH (above 8.0) or by the high ionic strength of the medium reached during the photosynthetic stage under these experimental conditions. These results were confirmed in three independent experiments. Unfortunately, reproducibility in the above-described experiments was still low (Fig. 2), possibly due to the lack of strict pH control of the medium. We then developed an automated PhBR system that allowed us to monitor and control the pH of the medium by adding $CO_2$ (see Methods and Fig. 1).

In the next series of experiments, the algae were pre-grown in HS medium under HL conditions (120 μE $m^{-2}$ $s^{-1}$ PAR). After sulfur deprivation, they were transferred to the automated PhBRs and exposed again to HL intensity (110 μE $m^{-2}$ $s^{-1}$ PAR). The pH in the reactors was set at 7.4 and controlled by $CO_2$ gas addition for 20 h during the $O_2$-production stage. The cultures became anaerobic and photoproduced $H_2$ in a manner similar to that observed with photoheterotrophic cultures. Fig. 3 shows that sulfur-depleted photoautotrophic cultures transition consecutively through the same physiological stages as photoheterotrophic cultures [26] and exhibited aerobic/photosynthetic (I), $O_2$-consumption (II), anaerobic (III), $H_2$-production (IV) and termination (V) stages. The major differences between the two types of cultures were the presence of a delay in the transition to anaerobiosis and a comparatively low output of $H_2$ from the photoautotrophic cultures. Under these experimental conditions, anaerobiosis in the PhBRs normally occurred after 80 h of sulfur deprivation (compared to 20–40 h with photoheterotrophic cultures; see [11,16]), and the total output of $H_2$ gas in the system did not exceed 10 ml $l^{-1}$ of culture (less than 0.5 mmol $l^{-1}$, equal to 0.5 × $10^{-3}$ mmol $m^{-3}$, compared to 5–6 in photoheterotrophic cultures at pH 7.3; see [16]). The adaptation of photoautotrophic cells to sulfur-deprived conditions is also accompanied by the accumulation

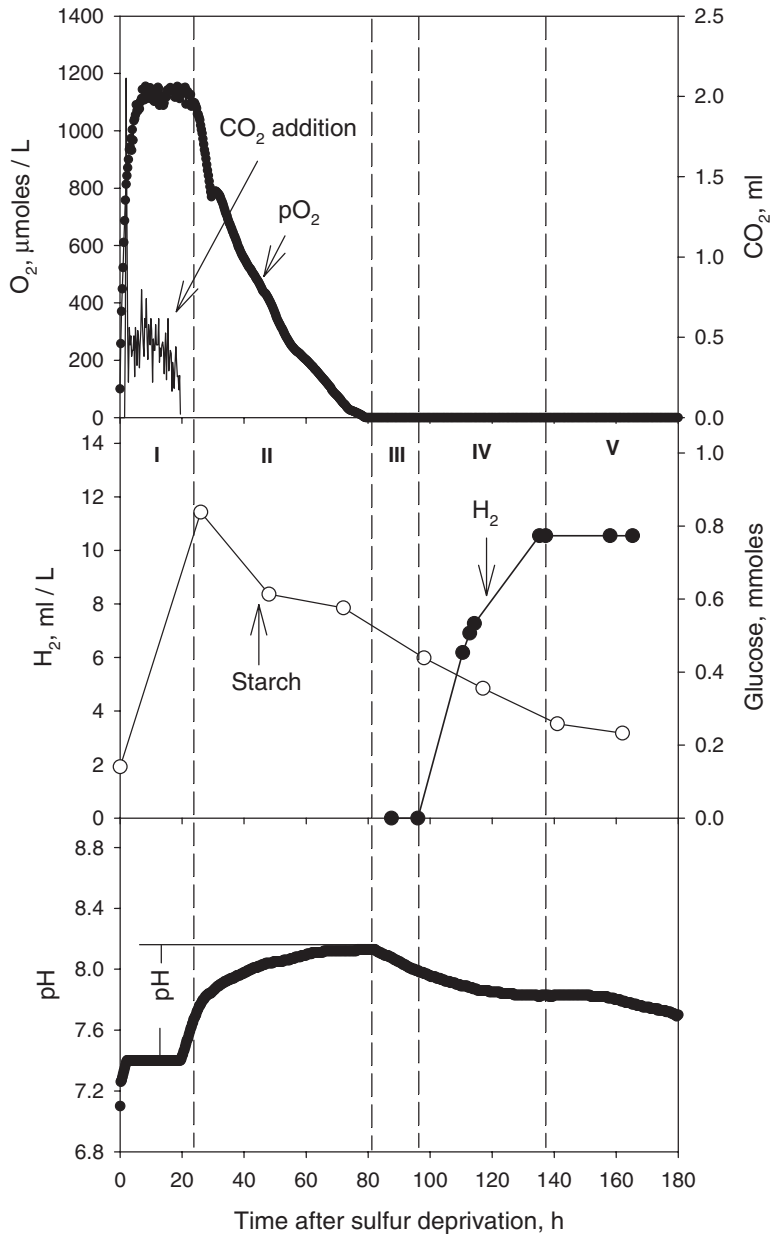


Fig. 3. The changes in dissolved $O_2$, pH and volume of the $H_2$ produced during the cultivation of sulfur-deprived algae under photoautotrophic conditions. This is one of the experiments presented in Table 1. The culture was pre-grown under HL conditions (120 $\mu E\,m^{-2}\,s^{-1}$ PAR), depleted of sulfate, and then placed again under HL conditions (110 $\mu E\,m^{-2}\,s^{-1}$ PAR). The pH was controlled by $CO_2$ additions over the first 20 h of the experiment. Delta ($\Delta$) pH indicated in the figure is the shift in pH occurring immediately after the pH stat operation ceased. One ml of $H_2$ produced per l of culture corresponds to $10^{-3}\,m^3\ H_2\,m^{-3}$ of culture.

of starch during the $O_2$-production stage as well as its degradation during the $O_2$-consumption, anaerobic and $H_2$-production stages (Fig. 3, middle panel). Starch accumulation occurs because of increased carbon dioxide fixation during the initial stages of sulfur deprivation. Accumulation was maximal during the $O_2$-production stage, but then degradation of starch during the $O_2$-consumption stage began (Fig. 3) after the termination of $CO_2$ addition. The subsequent continuing decrease in starch content during the $H_2$-production stage supports the fact that starch catabolism contributes to the process, either directly or indirectly, as seen with

Table 1
Effects of the duration of pH control on $H_2$ production, starch accumulation, and starch degradation in sulfur-deprived *C. reinhardtii* cultures

| Duration of pH-stat function (h) | ΔpH, units | Starch accumulated as glucose ($mmol\,l^{-1}$) | Average time for maximum starch accumulation (h) | Start of anaerobiosis (h) | Starch degraded as glucose ($mmol\,l^{-1}$) | Total $H_2$ gas produced ($ml\,l^{-1}$) |
|---|---|---|---|---|---|---|
| 20 | 0.47–0.73 | $0.59 \pm 0.12$ | $56 \pm 24$ | 58–95 | $0.40 \pm 0.19$ | $4.4 \pm 4.7^*$ |
| 25–35 | 0.44–0.60 | $0.60 \pm 0.18$ | $48 \pm 22$ | 65–118 | $0.40 \pm 0.12$ | $10.8 \pm 2.6$ |
| 40–45 | 0.02–0.44 | $0.56 \pm 0.24$ | $33 \pm 11$ | 45–107 | $0.47 \pm 0.15$ | $18.3 \pm 7.8$ |
| > 65 | 0–0.22 | $0.69 \pm 0.08$ | $31 \pm 7$ | 76–91 | $0.36 \pm 0.09$ | $5.7 \pm 4.2$ |

All experiments were done with cells pre-grown in HS medium under high light conditions ($120\,\mu E\,m^{-2}\,s^{-1}$ PAR) and incubated under high light intensities ($110\,\mu E\,m^{-2}\,s^{-1}$ PAR) during sulfur deprivation. The pH of the medium was controlled by $CO_2$ bubbling for different periods of time (first column). The change in pH observed when the pH stat was stopped is recorded in the second column (see Fig. 3 for an example).
*Under these experimental conditions, two cultures out of five did not produce $H_2$.

photoheterotrophic cultures [16]. Under these particular conditions the reproducibility of $H_2$ production was still not acceptable: five experiments resulted in an average $H_2$ production volume of $4.4 \pm 4.7$ ml (Table 1).

### 3.2. Dependence of $H_2$ photoproduction on added $CO_2$

Having demonstrated that photoautotrophic cultures of *C. reinhardtii* are capable of $H_2$ photoproduction under sulfur-deprived conditions, we addressed the question of the low yield of $H_2$ gas in the system and the poor reproducibility of the process. One of the reasons for the low output of $H_2$ may be a limitation in starch accumulation during the $O_2$-production stage of sulfur deprivation, caused by the limited amount of added $CO_2$. In other words, 20 h of $CO_2$ addition during the $O_2$-production stage may not be enough for sufficient starch accumulation to support subsequent $H_2$ evolution. Indeed, we observed that in some experiments the algal cultures accumulated very little starch and did not produce $H_2$ at all (data not shown). This gave rise to the wide variation in the average $H_2$-output data reported above. Therefore, in the next series of experiments, we checked the dependence of starch accumulation and $H_2$ production on the duration of pH-stat function (i.e., the duration of the $CO_2$ addition period). For these experiments, we again utilized the automated PhBR system. The pH in the PhBRs was maintained at 7.4 for, respectively, 20, 25–35, 40–45 and more than 65 h after the beginning of sulfur deprivation. All data were obtained in cultures pre-grown under HL conditions ($120\,\mu E\,m^{-2}\,s^{-1}$ PAR) and exposed to HL ($110\,\mu E\,m^{-2}\,s^{-1}$ PAR) during sulfur deprivation. The highest output of $H_2$ gas (about 18 ml $H_2$ per liter of culture, equal to $18 \times 10^{-3}\,m^3\,m^{-3}$ of the culture) occurred when the pH was controlled for about 40–45 h (Table 1). A decrease or an increase in the duration of $CO_2$ addition resulted in a decrease in $H_2$ production. The best reproducibility was also observed when the pH-stat functioned for 25–45 h. In contrast to $H_2$ production, starch accumulation did not depend on the duration of pH-stat function. The total amount of starch accumulated during the $O_2$-production stage, measured in millimoles of glucose equivalent, was about 0.6–0.7 $mmol\,l^{-1}$ in all cultures. Although the levels of accumulated starch were the same in all algal cultures, the time required to reach the peak value declined with the duration of pH-stat conditions. We also found that the amount of starch degraded during the $H_2$-production stage did not depend on the duration of pH-stat operation (it was about 0.4 $mmol\,l^{-1}$ in all cases). Interestingly, the shift in pH (ΔpH in Fig. 3) depended on the duration of $CO_2$ addition (Table 1). The pH of the medium rises quickly after switching off the pH control (Fig. 3, bottom panel). However, the increase in the pH was more pronounced when the addition of $CO_2$ to the medium was terminated during the earlier stages of sulfur deprivation. Since the shift in the pH is due to the photosynthetic assimilation of dissolved $CO_2$, we concluded that the decline in the ΔpH with the duration of pH-stat function is the result of the gradual decrease of $CO_2$ assimilation in algal cells at longer times of sulfur deprivation. These data are consistent with the findings of Zhang and co-workers [18] who reported a gradual decrease in the levels of the key enzyme of the Calvin cycle, Rubisco, in sulfur-deprived photoheterotrophic algae.

### 3.3. The effect of light intensity on $H_2$ production

In the next series of experiments, we checked the dependence of $H_2$ production by photoautotrophic cultures on the light intensity during both pre-growth and sulfur-deprivation. For this purpose, algae were pre-grown under two different light conditions

Table 2
Hydrogen photoproduction, starch accumulation, and starch degradation in sulfur-deprived *C. reinhardtii* cultures incubated under different light conditions

| Light intensity ($\mu E\,m^{-2}\,s^{-1}$) | | Starch accumulated as glucose ($mmol\,l^{-1}$) | Start of anaerobiosis (h) | Starch degraded as glucose ($mmol\,l^{-1}$) | Total $H_2$ gas produced ($ml\,l^{-1}$) |
|---|---|---|---|---|---|
| Pre-growth | Sulfur-deprivation | | | | |
| 120 | 20 | $0.36 \pm 0.07$ | 91–nt | $0.19 \pm 0.10$ | 0 |
| 120 | 110 | $0.59 \pm 0.12$ | 58–95 | $0.40 \pm 0.19$ | $4.4 \pm 4.7$ |
| 25 | 20 | $0.24 \pm 0.03$ | 49.5–nt | $0.16 \pm 0.04$ | $7.3 \pm 10.4$ |
| 25 | 110 | $0.86 \pm 0.34$ | 22.5–69 | $0.77 \pm 0.27$ | $31.8 \pm 10.8$ |
| 25 | 110 during photosynthetic and early oxygen consumption stages, and 20 thereafter | $0.78 \pm 0.11$ | 22–65 | $0.71 \pm 0.11$ | $56.4 \pm 16.7$ |

Culture conditions were the same as in Table 1. In all cases, the pH was controlled during the first 20–24 h of sulfur deprivation. nt—transition to anaerobiosis was not observed.

(25 and 120 $\mu E\,m^{-2}\,s^{-1}$ PAR), depleted of sulfur, and re-suspended in HS-minus-sulfur medium. Cell suspensions were transferred into the automated PhBRs and incubated again under two different light intensities: about 20 (LL) and 110 (HL) $\mu E\,m^{-2}\,s^{-1}$ PAR. The pH in the PhBRs was set at 7.4 and controlled for 20–24 h by adding $CO_2$ gas.

A summary of the data obtained in these experiments is presented in Table 2. The transition to anaerobic conditions was variable in algal cultures placed under LL conditions during sulfur deprivation, independent of the light intensity used during pre-growth of the cultures. In the case of such cultures pre-grown under HL intensity, anaerobiosis was never achieved, and hence no $H_2$ gas was ever produced. On the other hand, sulfur-deprived cultures pre-grown under LL exhibited large variations in but unimpressive amounts of $H_2$ produced. Algae pre-grown under low light conditions and exposed to high light intensities during S-deprivation (LL/HL) produced the highest quantities of $H_2$ gas and displayed the lowest degree of variability in productivity. Additionally, these cultures transitioned more rapidly to anaerobiosis than cultures exposed to the other light regimes tested. In most cases, anaerobic conditions were established inside the PhBRs after 25–30 h of sulfur deprivation and, in this sense, LL/HL cultures behaved like photoheterotrophic cultures that were pre-grown and sulfur-deprived under 200–300 $\mu E\,m^{-2}\,s^{-1}$ PAR (i.e., HL/HL; see). The pH of the LL/HL photoautotrophic culture medium declined soon after the pH stat operation ceased (data not shown). This indicates significant inhibition of $CO_2$ assimilation and an immediate shift of intracellular metabolism towards respiration and fermentation. The cultures submitted to all other light regimes usually exhibited an increase in pH after the pH control was terminated reflecting the sustained presence of $CO_2$ fixation activity. Finally, the early inhibition of $CO_2$ assimilation in photoautotrophic LL/HL algae cultures also correlates with high accumulation of starch in the cells (Table 2).

Two groups showed that $H_2$ production during sulfur-deprivation in photoheterotrophic *C. reinhardtii* is maximal at rather low light intensity (30–40 $\mu E\,m^{-2}\,s^{-1}$; see [17,28]. However, our current results with photoautotrophic cultures (Table 2) indicate that $H_2$ production is higher in sulfur-deprived cells exposed to HL intensity. Considering that photoautotrophic cultures produce lower quantities of $H_2$ than photoheterotrophic ones, we reasoned that light might play a different role at different stages of sulfur deprivation. Furthermore, light might have a different effect on photoautotrophic and photoheterotrophic cultures. Photoheterotrophic cultures accumulate starch during the $O_2$-producing stage and use both starch and acetate as substrates for respiration during the $O_2$-producing and the $O_2$-consuming stages. In contrast, photoautotrophic cultures are only able to utilize starch during the same two stages. We reasoned then, that HL (which favors starch accumulation,see Table 2) during the initial stages of sulfur deprivation, will have a more significant effect on subsequent $H_2$ production by photoautotrophic than photoheterotrophic cultures, but the presence of high light during the $H_2$-production stage may not be necessary and in fact detrimental.

To further characterize $H_2$ photoproduction by photoautotrophic cultures, we studied the effect of light intensity on $H_2$ photoproduction in short-term experiments. Algal samples were withdrawn from the PhBRs at different time points after $H_2$ production started as described in the Materials and Methods section, and

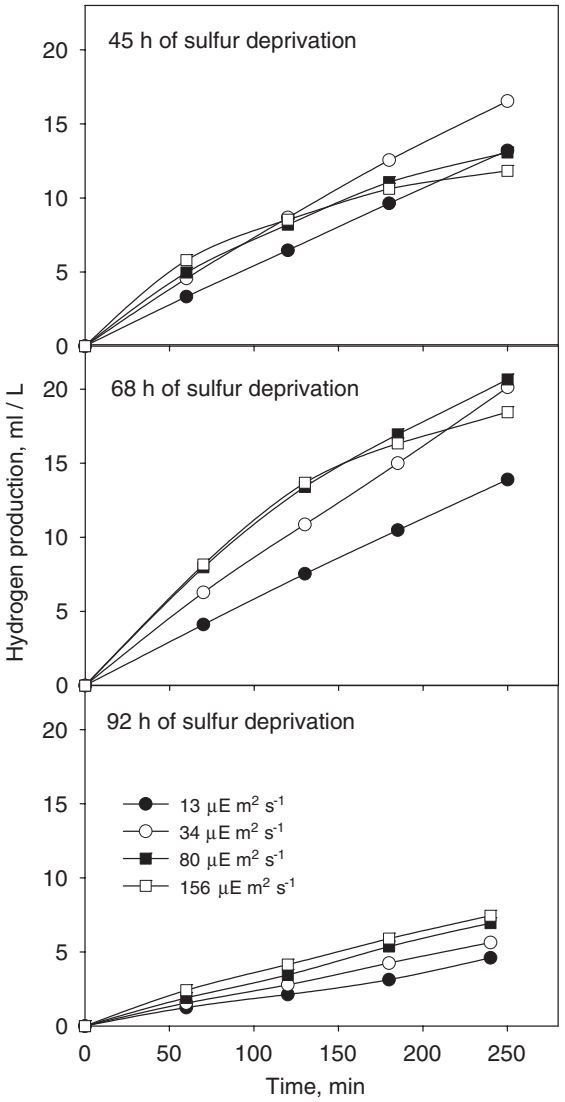


Fig. 4. The effect of light intensity on $H_2$ production in sulfur-deprived algal cells at different times after sulfur deprivation. Two-ml samples were taken anaerobically from the bioreactor at different times after the beginning of $H_2$ production and injected into 13-ml vessels with pure argon. The quantity of $H_2$ produced was measured by gas chromatography in the gas phase each hour for the total period of 4 h. One ml of $H_2$ produced per l of culture corresponds to $10^{-3}$ m$^3$ $H_2$ m$^{-3}$ of culture.

incubated under different light intensities ranging from 13 to 156 μE m$^{-2}$ s$^{-1}$ PAR (one-side illumination) in the presence of argon gas. Only LL/HL cultures were used in this experiment. As seen in Fig. 4, the $H_2$-evolving capacity of algal cells not only depended on the stage of $H_2$ production at which samples were withdrawn from the bioreactor, but also on the time of incubation under the argon atmosphere. After 45 and 68 h of sulfur deprivation, the kinetics of $H_2$ production were non-linear over time under all the light intensities examined, except 13 μE m$^{-2}$ s$^{-1}$. In contrast, after 92 h of sulfur deprivation, $H_2$ production was nearly linear over time under all light intensities tested. As shown for photoheterotrophic cultures [17], non-linear kinetics may be the result of inhibition by over-accumulated $O_2$. Furthermore, we note that no $H_2$ is produced in the absence of light (data not shown), which is also the case with heterotrophic cultures [11]. From the results shown in Fig. 4, we conclude that the optimal light intensity for $H_2$ production in the PhBRs is the light intensity, which gives linear kinetics over the 4 h period in small vials. This conclusion is based on the fact that in small vials, algal cultures are exposed to a relatively large gas phase, which dilutes the $O_2$ evolved by photosynthesis and thus decreases the inhibition of $H_2$ production. In contrast, the very small gas phase of the PhBRs will not allow rapid dissipation of the $O_2$ gas evolved, and $H_2$ production will be inhibited more rapidly at the same light intensity.

These observations led us to test the effect of changes in the light intensity during the sulfur-deprivation phase. We pre-grew the photoautotrophic cultures at LL intensity, transferred the cultures to the sulfur-deprived medium under HL, and then decreased the light intensity again during the $H_2$-production stage. The data (Table 2, last row) show that the output of $H_2$ approximately doubled as compared to the best results observed under constant illumination, and that the results from different experiments were much more reproducible.

Finally, we tried to determine if there was indeed a correlation between starch accumulation (Fig. 5 or start of anaerobiosis (Fig. 6) and $H_2$-production activity in sulfur-deprived algal cultures submitted to changes in light intensity. The accumulation of starch was highest in LL/HL cells (Fig. 5, closed circles and squares). The correlation coefficient calculated from a linear regression analysis of all the data was 0.180 and ranged from 0.0150 to 0.6173 for sets of data obtained under different light intensities. Thus, if a correlation exists, it might be masked by other factors, for example, by the light regime. Indeed, the highest correlation coefficient (0.673) was observed with cultures that produced more hydrogen and were cultivated under the more optimal light regime of LL/HL.

The cultures, pre-grown under LL, reached anaerobiosis within 30–60 h of sulfur deprivation while the HL cultures establish anaerobiosis after more than 60 h (Fig. 6). In general, the cultures that established anaerobiosis earlier produced more $H_2$ (Fig. 6). However, that was not always the case. The correlation coefficient for

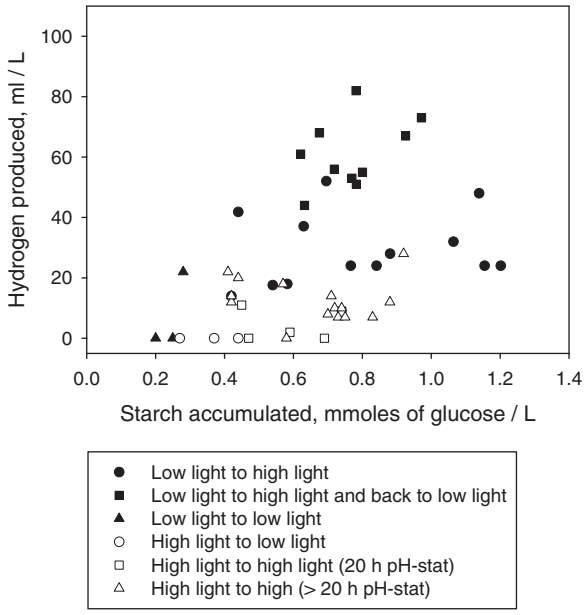


Fig. 5. The total amount of $H_2$ photoproduced as a function of accumulated starch. Each point represents an individual experiment performed under the light conditions indicated. One ml of $H_2$ produced per l of culture corresponds to $10^{-3}$ $m^3$ $H_2$ $m^{-3}$ of culture.

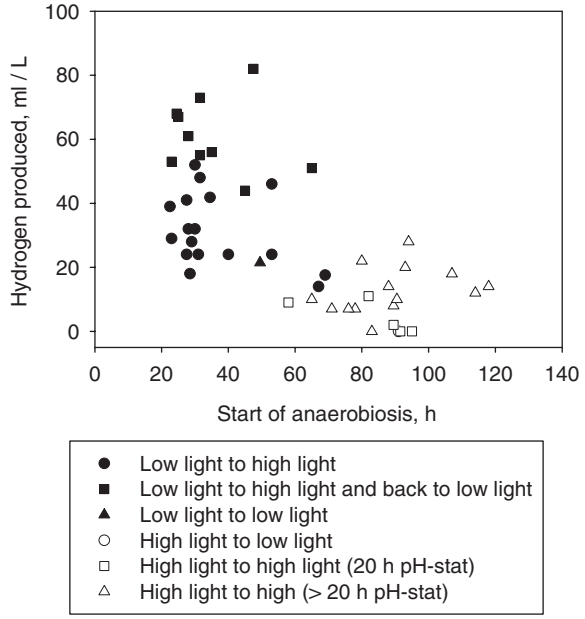


Fig. 6. The total amount of $H_2$ photoproduced as a function of the time that anaerobiosis was established in the medium. Each point represents an individual experiment performed under the light conditions indicated. One ml of $H_2$ produced per l of culture corresponds to $10^{-3}$ $m^3$ $H_2$ $m^{-3}$ of culture.

all the data was equal to 0.6602 (calculated from reciprocal regression analysis), indicating a better correlation between $H_2$ production and start of anaerobiosis than between $H_2$ production and starch accumulation under all tested conditions.

## 4. Discussion

Our first attempt to induce photoautotrophic production of $H_2$ in sulfur-deprived cultures of *C. reinhardtii* entailed simply excluding acetate from both the pregrowth and the sulfur-deprivation media [29]. However, in the absence of acetate, the algal cultures did not transition to anaerobiosis and, hence, they were unable to induce hydrogenase activity or photoproduce molecular $H_2$. In addition, those cultures did not accumulate starch during the $O_2$-producing stage of sulfur deprivation. Since starch plays an important role in cellular respiration during the $H_2$-production phase of sulfur deprivation, it is clear why these cultures were unable establish anaerobiosis in the PhBR.

In the current study, we attempted to increase starch accumulation and subsequent rates of $H_2$-photoproduction by adding an inorganic carbon source to sulfur-deprived photoautotrophic cultures. However, simple addition of bicarbonate did not result in $H_2$ production (Fig. 2). It appears that, in addition to inorganic carbon, pH control of the medium is vital for $H_2$ photoproduction by S-deprived *C. reinhardtii*. Buffering the medium with Tris-HCl, together with bicarbonate addition, resulted in $H_2$ accumulation (Fig. 2).

We found only one previous report in the literature discussing $H_2$ production by photoautotrophic cultures of S-deprived microalga. Guan and co-workers [30] showed that sulfur-depriving the marine green alga, *Platymonas subcordiformis*, can lead to the production of $H_2$ under photoautotrophic conditions. However, the rates of $H_2$ production were very low (a few $\mu l\,h^{-1}$) compared to sulfur-deprived, photoheterotrophic *C. reinhardtii*, and the system required 30 h of dark, anaerobic incubation prior to $H_2$ evolution. This group did not use any buffer in the sulfur-deprived medium, which may explain the low productivity of the cultures. Their reported rates of $H_2$ production were about three orders of magnitude lower than the ones we observed in Fig. 2, which were measured in the presence of Tris buffer.

In order to further enhance starch synthesis during photoautotrophic sulfur deprivation, we investigated the effect of adding carbon dioxide gas (instead of bicarbonate) to the cultures and found that for cultures pre-grown under high light, a longer period of $CO_2$ supplementation was essential. After 40–45 h of incubation, more $H_2$ was produced and the reproducibility of the results improved dramatically. This suggested that

cultures pre-grown under high light need more inorganic carbon to accumulate critical quantities of starch and reproducibly produce $H_2$. Shorter periods of $CO_2$ supplementation result in less starch accumulation, but depending on the levels of accumulated starch, could result in either more efficient $H_2$ production or no production al all. This might explain the high variability in the observed rates of $H_2$ production following 20 h of $CO_2$ supplementation.

The interplay between starch accumulation during the photosynthetic phase of sulfur deprivation and the amount of $H_2$ gas produced during the later stages (see Fig. 5) suggests that it is possible to manipulate the culture conditions at different steps of the process as follows: (a) pre-grow the cultures photoautotrophically at LL in the presence of additional $CO_2$; (b) provide for high levels of starch accumulation during sulfur deprivation by adding $CO_2$ gas and illuminating with HL during the initial steps of the process at a pH of 7.4; and (c) maintain the anaerobic, sulfur-deprived cultures at LL during (and after) the beginning of the $H_2$ production phase in order to prevent $O_2$ accumulation.

It is well established that algae, like other photosynthetic organisms, adapt to light intensity. Green algae grown under low light during the pre-growth phase have higher chlorophyll content per dry weight, and decreased $O_2$ evolution and $CO_2$ fixation capacities per Chl, compared to algae grown under high light [31–33]. Assuming that damage to the PSII D1 protein takes place even under low light intensity [34] and that the rate of D1 repair is decreased under sulfur deprivation [13], it seems possible that algae adapted to low light intensity, experience additional photoinhibition when sulfur-deprived and placed under high irradiation. This may be the reason for the accelerated establishment of anaerobiosis and increase in subsequent $H_2$ production (Table 2).

In contrast to the enhancing effect of LL during pre-growth, we observed that the initial stages of sulfur deprivation required HL intensity, $CO_2$ addition and a stable pH of approximately 7.4 for about 40–45 h during the photosynthetic $O_2$-evolving stage for optimal subsequent $H_2$ production (Table 1). These parameters were found to permit higher starch accumulation and faster transition to anaerobiosis (Table 2).

Molecular $O_2$ produced by the residual PSII activity was shown to be one of the limiting factors for $H_2$ production in sulfur-deprived photoheterotrophic cultures [17]. A similar effect must also occur in photoautotrophic cultures. We showed that the inhibitory effect of $O_2$ produced by residual PSII activity was more pronounced during the initial stages of $H_2$ evolution and was higher at high light intensities (Fig. 4). To eliminate the inhibitory effect of $O_2$ on $H_2$ production in the PhBRs, we decreased the light intensity at the start of the $O_2$-consuming stage of sulfur deprivation. This approach increased the total output of $H_2$ in the system by twofold (Table 2). We thus concluded that light plays multiple functions during sulfur deprivation and that each phase of sulfur deprivation requires different optimal light intensities.

In conclusion, we have demonstrated for the first time in this paper that sustainable $H_2$ photoproduction in a batch, sulfur-deprived system is feasible under strictly photoautotrophic conditions, and that the conditions can be optimized to achieve rates comparable to those observed with photoheterotrophic cultures. In the presence of carbon dioxide, the system performs normal photosynthesis and accumulates significant amounts of starch during the initial, photosynthetic stage of sulfur deprivation. During the anaerobic stage, the algal cells can metabolize starch. This process could contribute to: (a) the direct transfer of reducing equivalents to the photosynthetic electron transport chain through the NADPH-plastoquionone oxidoreductase-like pathway and (b) the removal of $O_2$ generated by the residual PSII activity through respiration of the products of starch degradation as we have previously observed for photoheterotrophic cultures [14–16].

Optimization of photoautotrophic systems for $H_2$ production will require further research efforts, particularly with respect to a more detailed study of the light intensities required at each stage of sulfur deprivation. However, the current work opens the door to significant decreases in the costs associated with algal $H_2$ production by allowing for the elimination of acetate and expensive buffers from the algal medium. The extra cost of the $CO_2$ could probably be minimized by using waste stack gas from electrical power plants, so long as any sulfate was removed from the gas.

## Acknowledgments

This work was supported in part by the Hydrogen, Fuel Cells, and Infrastructure Technologies Program, EERE, US Department of Energy, and by the Russian Federation of Basic Research (Grant # 04-04-97205).

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