Available online at www.sciencedirect.com
ScienceDirect
Journal of Biotechnology 128 (2007) 776–787
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

A comparison of hydrogen photoproduction by sulfur-deprived *Chlamydomonas reinhardtii* under different growth conditions

Sergey Kosourov [a,*], Elena Patrusheva [a], Maria L. Ghirardi [b], Michael Seibert [b], Anatoly Tsygankov [a]

[a] *Institute of Basic Biological Problems RAS, Pushchino, Moscow Region 142290, Russia*
[b] *National Renewable Energy Laboratory, 1617 Cole Boulevard, Golden, CO 80401-3393, USA*

Received 30 October 2006; accepted 15 December 2006

Abstract

Continuous photoproduction of H_2 by the green alga, *Chlamydomonas reinhardtii*, is observed after incubating the cultures for about a day in the absence of sulfate and in the presence of acetate. Sulfur deprivation causes the partial and reversible inactivation of photosynthetic O_2 evolution in algae, resulting in the light-induced establishment of anaerobic conditions in sealed photobioreactors, expression of two [FeFe]-hydrogenases in the cells, and H_2 photoproduction for several days. We have previously demonstrated that sulfur-deprived algal cultures can produce H_2 gas in the absence of acetate, when appropriate experimental protocols were used (Tsygankov, A.A., Kosourov, S.N., Tolstygina, I.V., Ghirardi, M.L., Seibert, M., 2006. Hydrogen production by sulfur-deprived *Chlamydomonas reinhardtii* under photoautotrophic conditions. Int. J. Hydrogen Energy 31, 1574–1584). We now report the use of an automated photobioreactor system to compare the effects of photoautotrophic, photoheterotrophic and photomixotrophic growth conditions on the kinetic parameters associated with the adaptation of the algal cells to sulfur deprivation and H_2 photoproduction. This was done under the experimental conditions outlined in the above reference, including controlled pH. From this comparison we show that both acetate and CO_2 are required for the most rapid inactivation of photosystem II and the highest yield of H_2 gas production. Although, the presence of acetate in the system is not critical for the process, H_2 photoproduction under photoautotrophic conditions can be increased by optimizing the conditions for high starch accumulation. These results suggest ways of engineering algae to improve H_2 production, which in turn may have a positive impact on the economics of applied systems for H_2 production.

Keywords: *Chlamydomonas reinhardtii*; Hydrogen photoproduction; Sulfur deprivation; Acetate; CO_2; Growth conditions

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; $\Delta F/F'_m$, measure of the photochemical activity of PSII; F'_m, the maximum fluorescence level under the ambient light induced by a saturating light pulse; F_t, the steady-state level of fluorescence measured under ambient light prior to a saturating light pulse; HS, high salt medium; PAM, pulse amplitude modulated; PAR, photosynthetically active radiation; PQ, plastoquinone; PSI, photosystem I; PSII, photosystem II; Q_B, the secondary quinone acceptor of PSII; TAP, Tris–acetate–phosphate medium

* Corresponding author. Tel.: +7 4967 732791; fax: +7 4967 300532.
E-mail address: sergks@ibbp.psn.ru (S. Kosourov).

0168-1656/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2006.12.025

1. Introduction

Hydrogen photoproduction in green algae can be sustained by depriving the cells of sulfate (Melis et al., 2000; Ghirardi et al., 2000). Sulfur deprivation causes the partial and reversible inhibition of photosystem II (PSII) water-oxidation activity in algae (Wykoff et al., 1998), has little affect on cellular respiration, and results in culture transition from an aerobic to an anaerobic state (Melis et al., 2000; Ghirardi et al., 2000; Kosourov et al., 2002; Zhang et al., 2002). The establishment of anaerobiosis in a photobioreactor induces the expression of two [FeFe]-hydrogenases in algal cells (Happe and Kaminski, 2002; Forestier et al., 2003). These enzymes redirect the flow of electrons coming from the photosynthetic electron-transport chain in the chloroplast from carbon fixation towards proton reduction. As a result, sulfur-deprived algae produce H_2 for several days (Melis et al., 2000; Ghirardi et al., 2000). During sulfur deprivation, the algal cultures progress through the following five phases: the aerobic, O_2-consumption, anaerobic, H_2-production, and termination phases (Kosourov et al., 2002).

Several approaches have been examined to increase the yield of H_2 in *Chlamydomonas reinhardtii* under these conditions, including optimization of the light and pH regimes in the photobioreactors (Kosourov et al., 2003; Hahn et al., 2004; Laurinavichene et al., 2004), addition of small amounts of sulfate back to the culture medium during sulfur deprivation (Kosourov et al., 2002, 2005; Zhang et al., 2002), optimization of the medium composition (Jo et al., 2006), synchronization of cell division (Tsygankov et al., 2002), increasing the duration of H_2 production (Fedorov et al., 2005), and coupling of H_2 production to a fuel cell for direct electricity generation (Rosenbaum et al., 2005). Recently, Kruse et al. (2005) reported a significant increase in the rate and duration of H_2 photoproduction in sulfur-deprived mutants that are starch over-accumulators and blocked in state transition. Another advance related to the sulfur-deprived process came with the recent discovery of a mutant affected in sulfate permease activity, which is required to transport sulfate into the chloroplast (Chen et al., 2005). This mutant may be a candidate for H_2 photoproduction without the need to deplete the culture medium of sulfate.

It should be noted that all the results reported above were done with algae grown in the presence of acetate. The use of acetate for industrial H_2 production may not be very practical because it can increase the cost of the H_2 produced. Therefore, optimization of the system for photoautotrophic H_2 production, where acetate is replaced with low cost or waste CO_2, would represent a significant advance. Several attempts have been made to generate H_2 under photoautotrophic conditions. However, in all the reported experiments, the cultures either did not establish anaerobiosis in the photobioreactor (Kosourov et al., 2001), required the addition of DCMU (an inhibitor of O_2 evolution) for H_2 photoproduction (Fouchard et al., 2005), or the output of H_2 was too low (a few microlitres) (Guan et al., 2004). Recently, we showed that photoautotrophic cultures can produce H_2 continuously under sulfur-deprived conditions when supplied with CO_2 gas instead of acetate (Tsygankov et al., 2006). The rates of H_2 production and the total yields of H_2 under constant light regime, however, were still low compared to our previously reported data obtained in the presence of acetate.

In the present study, we compared the rates and yields of H_2 photoproduction in sulfur-deprived cultures of *C. reinhardtii* under photoautotrophic, photoheterotrophic, and photomixotrophic conditions. Experiments were performed in an automated photobioreactor system with the pH set at 7.4 during the aerobic phase (i.e., the first 24–25 h of sulfur deprivation) by either addition of CO_2 gas (photoautotrophic and photomixotrophic cultures) or phosphoric acid (photoheterotrophic cultures) (Kosourov et al., 2002; Tsygankov et al., 2006). It is also important to note that all experiments were performed under the same physiological conditions with cultures having the same initial Chl concentration. We show that, despite the fact that acetate increases the H_2-photoproduction capacity of sulfur-deprived algae, its presence in the medium is not critical for H_2 evolution per se. This observation could contribute to the future development of more cost-effective H_2-production systems based on photoautotrophic growth conditions.

2. Materials and methods

2.1. Growth conditions

Stock cultures of *C. reinhardtii* (Dang 137c) were grown photoheterotrophically on a standard

Tris–acetate–phosphate (TAP) medium (Harris, 1989) in 250 ml erlenmeyer flasks at room temperature under cool-white fluorescent light (~20 μE m^{-2} s^{-1} PAR), and maintained by weekly dilution. The stock culture (~10 ml) was inoculated into 1.5 l flat glass bottles; and the algae were grown at 28 ± 1 °C under photoautotrophic, photoheterotrophic, or photomixotrophic conditions. For photoautotrophic growth with CO_2 as the only carbon source, the cells were placed in standard high salt (HS) medium (Harris, 1989) and bubbled with ~2% CO_2 in air. For photoheterotrophic and photomixotrophic growth, cells were placed in TAP medium, containing 17.4 mM acetate. In the case of photomixotrophic growth, cultures were additionally bubbled with air containing ~2% CO_2. The CO_2 content in the air flow was analyzed with a DX6100-01 gas analyzer (RMT Ltd., Russia), and it was maintained at 2 ± 0.5% using a TPM1 microprocessor system (Oven, Russia). During growth on sulfur replete media, the algae were illuminated from two sides with cool-white fluorescence lamps, which provided an average incident light intensity of about 25 μE m^{-2} s^{-1} PAR on each surface of the culture bottles.

2.2. Sulfur deprivation procedure

After reaching the late logarithmic phase (14–18 μg Chl ml^{-1} in photoautotrophic and photoheterotrophic cultures or 18–24 μg Chl ml^{-1} in photomixotrophic cultures), the cells were harvested by centrifugation at 2,800 × *g* for 5 min. Depending on the growth conditions, the algae were washed once by centrifugation in either HS-minus-sulfur medium (photoautotrophic cultures) or TAP-minus-sulfur medium (photoheterotrophic or photomixotrophic cultures) and then re-suspended in the same medium to a final concentration of about 14–16 μg Chl ml^{-1}. All experiments on H_2 production in sulfur-deprived cultures were done with a bioreactor system that was described previously (Tsygankov et al., 2006). The system consists of four photobioreactors equipped with pH and pO_2 sensors. Both were connected to a personal computer loaded with pre-installed software (written in Pushchino) via analog–digital converters and special controller cards. The pH of the medium was maintained at 7.4 for the first 24–25 h of sulfur deprivation by the automated addition of either sterile carbon dioxide (photoautotrophic and photomixotrophic cultures) or 0.2 M phosphoric acid (photoheterotrophic cultures). The gas that was produced was collected by fluid displacement in upside-down graduated cylinders filled with water. The photobioreactors were exposed to an average incident light intensity of about 110 μE m^{-2} s^{-1} PAR. The change of light intensity from 25 μE m^{-2} s^{-1} during growth to 110 μE m^{-2} s^{-1} during sulfur deprivation was shown to provide measurable outputs of H_2 in photoautotrophic cultures (Tsygankov et al., 2006).

2.3. Pulse-amplitude modulated (PAM) chlorophyll *a* fluorescence and O_2-evolution measurements

Chlorophyll a fluorescence yields were obtained *in situ* with a MINI PAM fluorometer (Walz, Germany). An optical fiber probe was affixed onto the surface of the illuminated glass photobioreactor midway between the top and bottom of the bottle. As mentioned above, the average ambient light intensity was 110 μE m^{-2} s^{-1} PAR. The steady-state level of the fluorescence under ambient light (F_t) was excited with dim red light (~0.3 μE m^{-2} s^{-1} at the end of the fiber probe), modulated at 0.6 kHz (Antal et al., 2003). The maximum fluorescence emission under ambient light (F'_m) was induced by an 800 ms pulse of intense white light (~15,000 μE m^{-2} s^{-1} at the end of the fiber probe). Finally, the photochemical activity of the algal cells was calculated as $\Delta F/F'_m = (F'_m - F_t)/F'_m$ and recorded every 20 min.

Photosynthetic O_2 evolution was measured with a Clark-type O_2 electrode at 28 °C. Four millilitres aliquots of the culture were taken from the photobioreactor at the indicated times, equilibrated with air, and then placed in an electrode chamber (CB1-D, Hansatech Instruments Ltd., Kings Lynn, England). The algal cultures were supplemented with 80 μl of 0.5 M $NaHCO_3$ and adapted in the dark for 2 min. Oxygen evolution was initiated by illumination with saturating steady-state light at about 1,900 μE m^{-2} s^{-1}. The rate of O_2 evolution was calculated from the initial linear part of the kinetic curve and corrected for the rate of dark respiration, measured for 4 min after the end of illumination.

2.4. Determination of starch and acetate

Samples (1 ml of algal suspension) for starch and acetate determination were taken directly from the photobioreactors at the indicated times. Cells and the media were separated by centrifugation and stored at −70 °C until all samples were ready for analysis. Starch accumulated in the cells was determined as glucose with a Glucose GOD FS kit (DiaSys, Germany) after enzymatic hydrolysis, according to Gfeller and Gibbs (Gfeller and Gibbs, 1984). For acetate analysis, samples (0.8 ml) were acidified to pH below 2.0 using approximately 50–100 μl of 50% H_2SO_4, dissolved in 0.5 ml ethyl ether, and centrifuged briefly to separate the ether and aqueous phases. The samples were frozen and then the ether layers were decanted into small test tubes. Anhydrous Na_2SO_4 was added to each tube in the amount of about one-half of the volume of the ether in order to dry the ether. The levels of acetate in the ether-extracted samples were determined with a gas chromatograph (Tsvet 800, Russia) and a flame ionization detector (FID), using a 2 m glass column (2 mm i.d.). The column was filled with 10% SP-1000/1% H_3PO_4 on 100/120 Chromosorb WAW (Cat. #1841, Supelco, Inc., USA). For better resolution, we used the following conditions: initial temperature, 100 °C for 60 s; final temperature, 155 °C for 250 s; and rate of temperature increase, 25 °C min^{-1}. Argon was used as the carrier gas with a flow rate of 30 ml min^{-1}.

2.5. Other analytical procedures

The chlorophyll ($a+b$) content was assayed spectrophotometrically in 95% ethanol extracts by the method of Spreitzer (Harris, 1989). Light intensities were measured with a Li-Cor quantum photometer (Model LI-250, Lincoln, USA).

2.6. Statistical analysis

All measurements were replicated 4–8 times with different cultures. Any sampling was done in triplicate. Deviation of the measurements within the triplicates was less than 5%. Thus, the main factor responsible for the errors was differences attributable to the independent cultures.

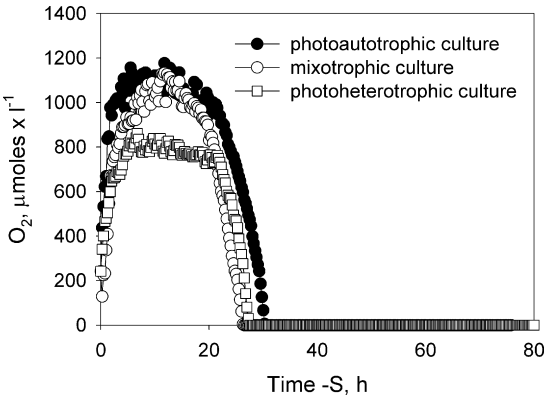

Fig. 1. The time courses for the establishment of anaerobiosis in sulfur-deprived *C. reinhardtii* cultures under different growth conditions. The level of O_2 in the photobioreactors was monitored with a Clark-type O_2 sensor. The results of typical experiments are presented, but each experimental condition was replicated 6–9 times. The pH of the medium inside photobioreactors in this and in all other experiments was controlled during the first 24–25 h at 7.4 by the automated addition of either CO_2 gas (photoautotrophic and photomixotrophic cultures) or phosphoric acid (photoheterotrophic cultures).

3. Results and discussion

3.1. Photosystem II activity and establishment of anaerobiosis in the photobioreactors

It is well documented that the depletion of sulfur from the growth medium inactivates photosynthetic O_2 evolution in algal cells reversibly (Wykoff et al., 1998; Melis et al., 2000). As mentioned before, the inhibition of photosynthetic O_2 evolution in sulfur-deprived algae results in the transition of the culture to anaerobic conditions due to cellular respiration. Since the establishment of anaerobiosis in algal cultures is important for the expression of the [FeFe]-hydrogenases and H_2 gas production, we first investigated how different growth conditions (photoautotrophic, photoheterotrophic, and photomixotrophic) affect the rates of PSII inactivation and transition to anaerobiosis.

Following the removal of sulfate from the medium, algal cultures transition to anaerobiosis under all the growth conditions tested (Fig. 1). The average time for the establishment of anaerobic conditions in the photobioreactors was 31 ± 4 h in photoautotrophic cultures, 26 ± 5 h in photoheterotrophic cultures, and 25 ± 4 h in photomixotrophic cultures. Note that we

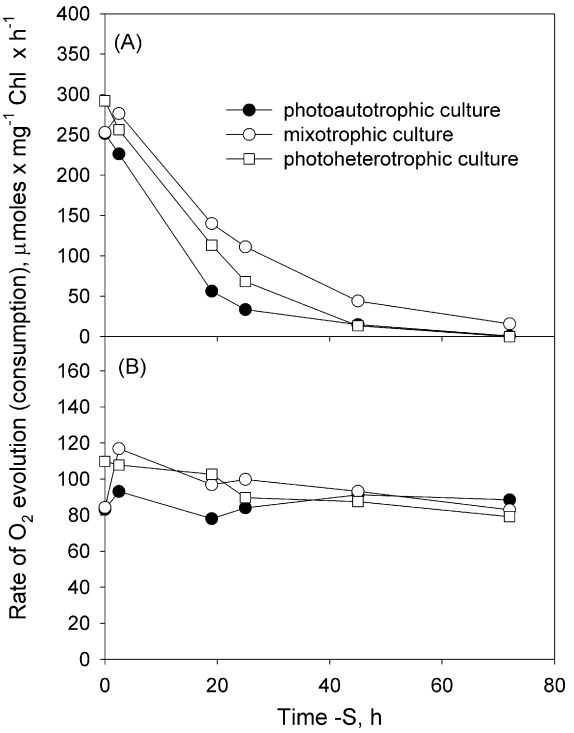

Fig. 2. Changes in photosynthetic O_2 evolution (A) and respiratory capacity (B) in photoautotrophic, photomixotrophic and photoheterotrophic cultures after sulfur deprivation. Rates were measured in a Clark-type O_2 electrode chamber with samples taken from photobioreactors as described in the Materials and methods. Note that all samples were aerobic during the assays.

usually observed a 5–6 h delay in the establishment of anaerobiosis in photoautotrophic cultures compared to photoheterotrophic and photomixotrophic cultures. As expected, the transition to anaerobiosis was accompanied by the gradual decrease in PSII O_2-evolving capacity in the cells (Fig. 2A). In contrast, cellular respiration was not dramatically affected by sulfur deprivation (Fig. 2B). The relatively high rate of respiration in photoautotrophic cultures as compared with photomixotrophic and photoheterotrophic cultures is in agreement with early observation of Heifetz et al. (2000), who showed that the level of acetate in the growth medium does not affect the rate of cellular respiration in long-term experiments. The maximum capacity of algal cells to photoevolve O_2 during the aerobic phase of sulfur deprivation was highest in the cultures grown on acetate. This parameter, however, was measured under saturating light ($\sim$1,900 $\mu E\,m^{-2}\,s^{-1}$ PAR) in samples removed from the photobioreactors and equilibrated at atmospheric O_2 pressure. Although this is the maximum potential water-splitting activity of PSII in the algae, it does not directly reflect the *in situ* photochemical activity of PSII in the photobioreactors (Antal et al., 2003). Indeed, under moderate light (110 $\mu E\,m^{-2}\,s^{-1}$ PAR) and high levels of O_2 in the bioreactors, dissolved O_2 levels were always highest in photoautotrophic algae during this phase (Fig. 1). Note that photoautotrophic algae produce more O_2 per photobioreactor compared to photomixotrophic and photoheterotrophic cultures, but the former exhibit lower PSII capacity. This could be due to the different light and O_2 regimes in the photobioreactors compared to those in the Clark-type electrode chamber.

To estimate the actual photochemical activity of PSII *in situ*, we measured chlorophyll a fluorescence in cultures inside the photobioreactors using a pulse-amplitude-modulated (PAM) fluorometer (Antal et al., 2003). Fig. 3A shows that photochemical activity of all algal cultures obtained under the ambient light ($\Delta F/F'_m$) declined during sulfur deprivation. The gradual decrease in $\Delta F/F'_m$ during the first 20–25 h of the experiment (the aerobic phase) was more pronounced in the photomixotrophic cultures, while the photoautotrophic and photoheterotrophic cultures maintained higher photochemical activities over most of this time period. The decrease in $\Delta F/F'_m$ during the aerobic phase was the result of the larger increase in F_t compared to F'_m observed with all cultures (Fig. 3B and C). The prominent rise of the steady-state fluorescence yield (F_t) under the ambient light, especially in photomixotrophic cultures, can reflect the gradual increase in the reduction state of the plastoquinone (PQ) pool. It is not clear at this point where the extra reductants accumulating in the PQ pool originate. It is possible that the extra reductants reflect increased cyclic electron transport (state 2), or an accumulation of NADPH in the chloroplast, even in the presence of higher starch synthesis (see Section 3.3 below). This could happen since NADPH is not being used for anabolic processes, which are down regulated in the absence of cell growth under sulfur-deprived conditions. During the aerobic phase of sulfur deprivation, starch content in photoheterotrophic algae can increase more than eight-fold (Tsygankov et al., 2002; Zhang and Melis, 2002; Zhang et al., 2002). Our current experiments show

Table 1
The effect of different growth conditions (A: photoautotrophic, H: photoheterotrophic and M: photomixotrophic) on H_2 photoproduction in *C. reinhardtii* cultures and on the utilization of acetate and starch in cells during their adaptation to sulfur deprivation

	Aerobic phase			O_2-consumption phase			Anaerobic phase[a]		
	A	M	H	A	M	H	A	M	H
Acetate uptake (+) or release (−) (mmol l^{-1})	0.00	9.21 ± 0.82	6.95 ± 1.78	−0.05 ± 0.02	2.02 ± 0.49	2.32 ± 1.23	−0.75 ± 0.29	0.43 ± 0.62	0.68 ± 0.50
Starch accumulation (+) or degradation (−) (mmol glucose l^{-1})	0.97 ± 0.06	1.51 ± 0.12	1.00 ± 0.16	−0.17 ± 0.07	0.16 ± 0.04	0.18 ± 0.06	−0.42 ± 0.17	−1.02 ± 0.2	−0.72 ± 0.22
Proposed yield of starch conversion to hydrogen[b] (%)							∼21 (52)[c]	∼37 (32)[c]	∼10 (8)[c]
Hydrogen photoproduction (mmol l^{-1})							1.1 ± 0.4	4.5 ± 1.6	0.9 ± 0.8

Values represent an average of 4–6 experiments for acetate and starch measurements and 6–8 experiments for H_2 yields (± is the standard error).

[a] Defined from the beginning of anaerobiosis until the end of H_2 production.

[b] Assuming that 1 mmol glucose can generate 12 mmol H_2.

[c] Efficiency in parenthesis was calculated with the correction for acetate uptake or release.

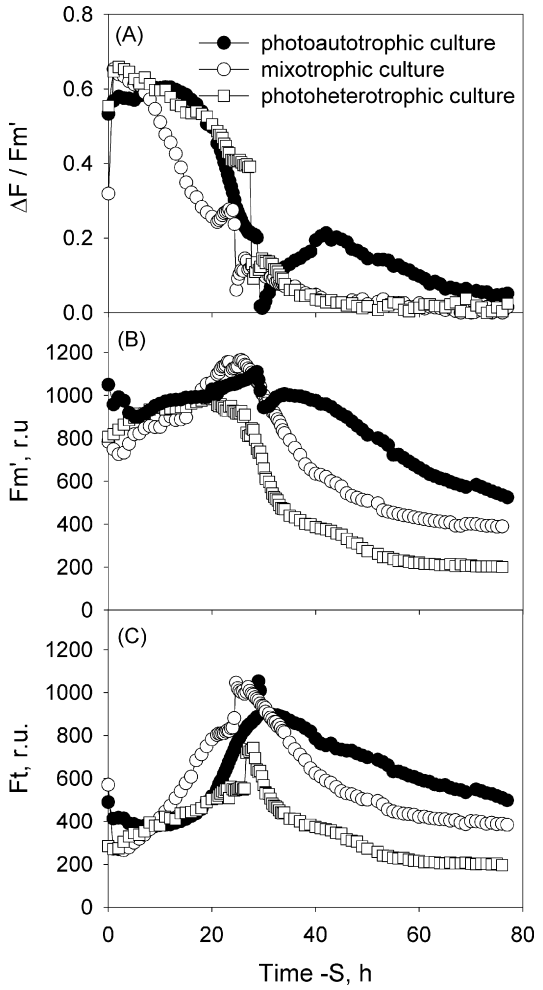

Fig. 3. Changes in the photochemical activity of PSII ($\Delta F/F'_m$), F'_m and F_t in photoautotrophic, photomixotrophic, and photoheterotrophic cultures after sulfur deprivation. All parameters were measured *in situ* with a MINI PAM fluorometer.

that the accumulation of starch is most pronounced in photomixotrophic cultures (Table 1). This also correlates with the faster inactivation of PSII photochemical activity (Fig. 3A).

The establishment of anaerobiosis in the photobioreactors is followed by a sharp decline in $\Delta F/F'_m$ under all growth conditions (Fig. 3A). The decline in $\Delta F/F'_m$ under photoheterotrophic conditions occurred immediately upon the onset of anaerobiosis in the photobioreactors, and took less than 20 min in agreement with Antal et al. (2003). The loss of photochemical activity in photoautotrophic and photomixotrophic cultures, on the other hand, usually took more than 20 min to occur. Antal et al. (2001) suggested that the sharp drop in photochemical activity reflects the over-reduction of the PQ pool as a result of anaerobiosis in sulfur-deprived cells. PQ pool over-reduction is known to cause the temporal inactivation of PSII under different stress conditions (Chemeris et al., 1996). Additionally, the decline in $\Delta F/F'_m$ can indicate transition of the photosynthetic machinery from states 1 to 2, which indeed occurs under anaerobic conditions (Finazzi et al., 1999). State transition, a reversible migration of a fraction of the light harvesting antenna from PSII to PSI, is driven by a protein kinase, which is activated under reducing conditions (Bennett, 1991). In *Chlamydomonas* the transition to state 2 induces a switch from linear to cyclic electron flow and can even result in the physical isolation of PSII from PSI (Finazzi et al., 2002). Increased cyclic electron transfer has been confirmed recently in the sulfur-deprived cc124 strain (Finazzi, personal communication), and may also occur in the 137C strain used in this work. After the sharp decline, $\Delta F/F'_m$ started to increase again (Fig. 3A). The restoration of increased levels of photochemical activity may indicate the appearance of a terminal acceptor and the partial restoration of the linear electron transport flow from PSII (Antal et al., 2003) due to a partial shift back to state 1. Since the expression of hydrogenases only begins after the sharp decline in photochemical activity and it takes time for the culture to attain maximum H_2-production activity, the utilization of reductants at this point is most probably driven by competitive processes. After a short period of increase, the photochemical activity ($\Delta F/F'_m$) started to decline again. This is probably caused by the continued degradation of PSII complexes under sulfur deprivation and anaerobiosis. It is interesting to note that the photochemical activity of photoautotrophic cultures remains higher than that of the other two cultures during this period, although no dissolved O_2 was detected in the medium (Fig. 1) in either case.

3.2. Hydrogen photoproduction

In the past, studies of H_2 metabolism in sulfur-replete green algae revealed the stimulatory effect of acetate on H_2 photoproduction (Healy, 1970; Bamberger et al., 1982; Gibbs et al., 1986). As pointed

out by Gibbs et al. (1986), the effect of acetate is rather complex and dependent upon the reactions of the glyoxylate and the citric acid cycles. Both cycles can conceivably provide reductants to PSI through a dicarboxylic acid shuttle and the NADPH-plastoquinone oxido-reductase enzyme. Since the glyoxylate and the citric acid cycles are located in the cytoplasm and mitochondria, respectively, the evolution of H_2 in the chloroplast may represent an effective way for releasing the metabolism of the whole cell from the excess reducing power observed under anaerobic conditions.

In sulfur-deprived algae, the effect of acetate on H_2 production is even more complicated. Previous experiments showed that *C. reinhardtii* cells uptake acetate only during the aerobic and O_2-consumption phases but suggest that the cells do not utilize it during the H_2-production phase (Melis et al., 2000; Tsygankov et al., 2002; Kosourov et al., 2003). Despite this observation, the rates of H_2 production by sulfur-deprived algae were assumed to depend significantly on the presence of acetate in the medium. Several attempts to generate measurable amounts of H_2 under photoautotrophic conditions met with little success (Zhang and Melis, personal communication; Kosourov et al., 2001). Nevertheless, other researchers (Guan et al., 2004) showed that the sulfur-deprived marine green alga, *Platymonas subcordiformis*, can produce H_2 under photoautotrophic conditions after 30 h of dark anaerobic incubation. However, as expected, the rates of H_2 production were very low (a few $\mu l\,h^{-1}$). More recently, Fouchard et al. (2005) observed H_2 photoproduction in photoautotrophic cultures of *C. reinhardtii* treated with DCMU, an inhibitor of electron transport and O_2 evolution from PSII. All these results suggested that perhaps appropriate conditions needed to be found to optimize photoautotrophic H_2 production under physiological conditions and led us to develop a protocol that resulted in much higher rates of H_2 production than previously reported (Tsygankov et al., 2006). For better transition to anaerobiosis and improved H_2-production rates, the protocol requires the proper set of light and CO_2-supply regimes in the photobioreactors. In the present study we applied this protocol to photoautotrophic, photoheterotrophic and photomixotrophic cultures (see Section 2), except that CO_2 gas was not supplied to photoheterotrophic algae during the aerobic phase. In all cases, the pH was controlled at 7.4 by the micro-

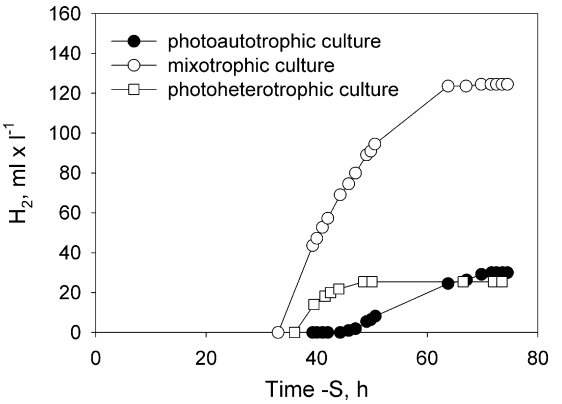

Fig. 4. The effect of photoautotrophic, photomixotrophic, and photoheterotrophic growth conditions on H_2 photoproduction in sulfur-deprived cultures. The results of typical experiments are presented (for average amounts of H_2 produced, see the data in Table 1).

processor system during the first 24–25 h of sulfur deprivation.

As shown in Fig. 4, *C. reinhardtii* cultures were able to produce H_2 under all growth conditions. Hydrogen-gas production in the photobioreactors, measured by the displacement of water in inverted graduated cylinders, appeared around 8 h after the establishment of anaerobiosis in photomixotrophic cultures, around 10 h in photoheterotrophic cultures, and almost 15 h in photoautotrophic cultures. In fact, photoheterotrophic algae had been shown previously to start to produce H_2 soon after the establishment of anaerobiosis (Antal et al., 2003). However, it takes time to saturate the culture liquid with H_2 (the solubility of H_2 in water under the atmosphere of pure H_2 is 754 nmol ml^{-1} at 28 °C) and build enough pressure to displace water in the collecting system. Therefore, the 7 h delay in the start of visible H_2 photoproduction in photoautotrophic cultures compared to photomixotrophic cultures could be explained by their lower rate of H_2 production. The initial rates of visible H_2 production were about 2.8 ml $(h\,l)^{-1}$ in photoautotrophic cultures, ~4.0 ml $(h\,l)^{-1}$ in photoheterotrophic cultures, and ~6.9 ml $(h\,l)^{-1}$ in photomixotrophic cultures. The highest output of H_2 (4.5 ± 1.6 mmol l^{-1}) was observed in cultures grown both on acetate and CO_2 (photomixotrophic growth) (Table 1). Photoautotrophic cells produced about 1.1 ± 0.4 mmol l^{-1} under these conditions. The H_2 output of about 4.5 mmol l^{-1} (~100 ml l^{-1}) in photomixotrophic cul-

tures is close to the previously reported yield of H_2 in experiments done with photomixotrophic cultures, grown photosynthetically in the presence of acetate and CO_2 under moderate light intensities, but without CO_2 (photoheterotrophically) during the initial stages of sulfur deprivation (Melis et al., 2000; Zhang et al., 2002; Kosourov et al., 2002). It is important to emphasize that the photoheterotrophic cells in our current work derived CO_2 only from the respiration of acetate and, thus, were carbon dioxide limited both during growth and the aerobic phase of sulfur deprivation. Carbon dioxide deficiency is known to reduce linear electron transport in chloroplasts and causes inhibition of the Calvin cycle (Demeter et al., 1995). The latter may be one of the reasons for the lower H_2 output in these cultures (about 1 mmol l^{-1}) compared to the photomixotrophic ones, because less starch is synthesized. The photoautotrophic cultures in the current experiments were placed under the same conditions as in our previous work (Tsygankov et al., 2006) and exhibited similar H_2-production yields.

3.3. Starch accumulation, acetate uptake and hydrogen photoproduction

Hydrogen photoproduction in green algae depends on two metabolic pathways, which provide reductants for the hydrogenase-catalyzed reaction. The first involves the photosynthetic water-splitting process of PSII and subsequent transport of electrons from water to the [FeFe]-hydrogenases through PSI. The second mechanism depends on the metabolic oxidation of organic substrates that are coupled to PSI and the [FeFe]-hydrogenases through the plastoquinone pool (Gfeller and Gibbs, 1984; Gibbs et al., 1986). According to Gibbs, acetate and starch could be the main substrates providing electrons for H_2 production in green algae. Under sulfur-deprived conditions, however, it appears that algae do not uptake acetate (Melis et al., 2000), and sustained H_2 production requires some catabolism of starch either for the removal of O_2 produced by PSII or for the direct donation of electrons to the process (Ghirardi et al., 2000; Zhang et al., 2002; Kosourov et al., 2003). Therefore, the accumulation of starch in the cells at the beginning of sulfur-deprivation seems to be a key factor for sustaining H_2 photoproduction, especially in photoautotrophic cultures (Fouchard et al., 2005; Tsygankov et al., 2006).

The high output of H_2 in photomixotrophic cultures was accompanied by high levels of starch accumulation in the cells. Indeed, there was a clear correlation between starch accumulation and H_2-production levels among all independent experiments conducted (data not presented). Table 1 shows that starch in photomixotrophic and photoheterotrophic algae not only increased during the aerobic phase but also during the O_2-consumption phase (however, at much slower rates). In contrast, photoautotrophic cultures transition to anaerobiosis after the start of starch degradation. Assuming that the highest possible solubility of O_2 in water at 28 °C is about 1,152 μmol l^{-1}, the establishment of anaerobiosis in a 1 l photobioreactor will require the consumption of about 0.19 mmol of glucose. Comparison of this value with data presented in Table 1 shows that the transition to anaerobiosis in photoautotrophic cultures could be driven by the respiration of stored starch alone during the O_2-consumption phase. On the other hand, the establishment of anaerobiosis in photomixotrophic and photoheterotrophic algae must depend mostly on the respiration of acetate during this phase. Taking these results into account, we conclude that the low efficiency of H_2 production in photoautotrophic cultures compared to cultures supplied with acetate is due to the need to start respiring starch earlier, during the O_2-consumption phase. Utilization of acetate as the major substrate for respiration allows the cells to accumulate extra starch. This can be seen as an increase in the level of starch during the O_2-consumption phase, and it also explains the highest accumulation of starch in photomixotrophic cultures. It should be noted also that the overall uptake of acetate during the aerobic and O_2-consumption phases was higher in photomixotrophic cultures compared to photoheterotrophic cultures. The high utilization of acetate under photomixotrophic conditions can be attributed to the high photosynthetic activity observed in these algae (Fig. 2A). The latter can provide algal cells with extra ATP, which is important for assimilation of acetate (Gibbs et al., 1986).

The establishment of anaerobiosis in the photobioreactors increases the consumption of starch under all growth conditions (Table 1). This provides the algal cells with energy through both the fermentation and

H_2-production pathways. Again, the overall consumption of starch was highest when acetate was added to the cultures, because cultures with acetate accumulate more starch during the aerobic and O_2-consumption phases. Earlier, we demonstrated that fermentation competes with H_2 photoproduction for reductants originating from starch degradation (Kosourov et al., 2003). Therefore, the high yield of H_2 has to be associated with high conversion efficiency of starch to H_2 and the low yields of fermentation products. Although we did not measure all the fermentation products in the present work, we can say that the high output of acetate in photoautotrophic algae during the anaerobic phase shows the dominance of fermentation over H_2 photoproduction in these cultures. Indeed, the calculated efficiency of starch to H_2 conversion in photoautotrophic algae almost doubles, if we subtract from this value that part of the starch converted to acetate (Table 1) This means that the efficiency of H_2 photoproduction under photoautotrophic conditions can be increased by either the artificial inhibition of competitive fermentation pathways or optimizing the ambient conditions favorable to H_2 photoproduction. It is clear at this point that additional experiments are required for totally understanding the mechanism of H_2 production under photoautotrophic conditions and to manipulate the different metabolic pathways to improve H_2 photoproduction.

4. Conclusions

This work demonstrates that sulfur-deprived *C. reinhardtii* are capable of prolonged H_2 photoproduction under photoautotrophic, photoheterotrophic, and photomixotrophic growth conditions. Thus, algal cells demonstrate similar responses to sulfur starvation, independent of the presence or absence of acetate in the medium. They accumulate starch during the short aerobic phase; inactivate PSII-driven, water-oxidation activity; establish anaerobiosis in photobioreactors; express [FeFe]-hydrogenases; and photoproduce H_2. Differences can be found only in the length of these physiological phases and some quantitative changes in metabolic responses.

We also showed that both acetate and CO_2 are required for the most rapid inactivation of PSII and the highest level of H_2 photoproduction (photomixotrophic cultures). While carbon dioxide is important for starch accumulation, acetate serves as the direct substrate for respiration during the aerobic phase of sulfur deprivation and, thus, contributes to the faster establishment of anaerobiosis in the photobioreactors. Additionally, utilization of acetate can provide the cells with extra carbon for starch accumulation (Gibbs et al., 1986; Ball et al., 1990). Although the metabolism of acetate in green algae is still poorly understood, there is evidence that photoassimilation of acetate occurs through the operation of the glyoxylate and citric acid cycles. While the citric acid cycle may increase the intracellular level of CO_2 favorable to its photoassimilation, the glyoxylate cycle is directly coupled to the conversion of succinate to carbohydrates. Despite the fact that acetate increases subsequent H_2 photoproduction in photomixotrophic cultures, its presence is not necessarily critical for the process to occur. Finally, the efficiency of H_2 photoproduction in sulfur-deprived photoautotrophic algae might be increased by optimizing the conditions for high starch accumulation during the aerobic phase and for more efficient utilization of starch during the O_2-consumption and H_2 production phases. The latter might be archived either by modifying the design of the photobioreactors to prevent the effect of shading and the over accumulation of O_2 in the system or by molecular engineering the metabolic processes in cells.

Acknowledgements

This work was supported in part by the Hydrogen, Fuel Cell, and Infrastructure Technologies Program, EERE, US Department of Energy (MLG and MS); by the Russian Foundation of Basic Research (Grant # 04-04-97205); and by Program # 7 of the Basic Research of the Russian Academy of Sciences (Project # 1.3.4).

References

Antal, T.K., Krendeleva, T.E., Laurinavichene, T.V., Makarova, V.V., Tsygankov, A.A., Seibert, M., Rubin, A.B., 2001. The relationship between the photosystem 2 activity and hydrogen production in sulfur deprived *Chlamydomonas reinhardtii* cells. Dokl. Biochem. Biophys. 381, 371–374.

Antal, T.K., Krendeleva, T.E., Laurinavichene, T.V., Makarova, V.V., Ghirardi, M.L., Rubin, A.B., Tsygankov, A.A., Seibert, M.,

2003. The dependence of algal H_2 production on Photosystem II and O_2 consumption activities in sulfur-deprived *Chlamydomonas reinhardtii* cells. Biochim. Biophys. Acta 1607, 153–160.

Ball, S.G., Dirick, L., Decq, A., Martiat, J.-C., Matagne, R.F., 1990. Physiology of starch storage in the monocellular alga *Chlamydomonas reinhardtii*. Plant Sci. 66, 1–9.

Bamberger, E.S., King, D., Erbes, D.L., Gibbs, M., 1982. H_2 and CO_2 evolution by anaerobically adapted *Chlamydomonas reinhardtii* F-60. Plant Physiol. 69, 1268–1273.

Bennett, J., 1991. Protein phosphorylation in green plant chloroplast. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 281–311.

Chemeris, Y.K., Venediktov, P.S., Rubin, A.B., 1996. Role of chloroplast respiration in the inactivation of photosystem II in *Chlorella*. Russian Plant Physiol. 43, 716–723.

Chen, H.C., Newton, A.J., Melis, A., 2005. Role of SulP, a nuclear-encoded chloroplast sulfate permease, in sulfate transport and H_2 evolution in *Chlamydomonas reinhardtii*. Photosynth. Res. 84, 289–296.

Demeter, S., Janda, T., Kovacs, L., Mende, D., Wiessner, W., 1995. Effects of in vivo CO_2-depletion on electron transport and photoinhibition in the green algae, *Chlamydobotrys stellata* and *Chlamydomonas reinhardtii*. Biochim. Biophys. Acta 1229, 166–174.

Fedorov, A.S., Kosourov, S., Ghirardi, M.L., Seibert, M., 2005. Continuous hydrogen photoproduction by *Chlamydomonas reinhardtii*: using a novel two-stage, sulfate-limited chemostat system. Appl. Biochem. Biotechnol. 121–124, 403–412.

Finazzi, G., Furia, A., Barbagallo, R.P., Forti, G., 1999. State transitions, cyclic and linear electron transport and photophosphorylation in *Chlamydomonas reinhardtii*. Biochim. Biophys. Acta 1413, 117–129.

Finazzi, G., Rappaport, F., Furia, A., Fleischmann, M., Rochaix, J.D., Zito, F., Forti, G., 2002. Involvement of state transitions in the switch between linear and cyclic electron flow in *Chlamydomonas reinhardtii*. EMBO Rep. 3, 280–285.

Forestier, M., King, P., Zhang, L., Posewitz, M., Schwarzer, S., Happe, T., Ghirardi, M.L., Seibert, M., 2003. Expression of two [Fe]-hydrogenases in *Chlamydomonas reinhardtii* under anaerobic conditions. Eur. J. Biochem. 270, 2750–2758.

Fouchard, S., Hemschemeier, A., Caruana, A., Pruvost, J., Legrand, J., Happe, T., Peltier, G., Cournac, L., 2005. Autotrophic and mixotrophic hydrogen photoproduction in sulfur-deprived *Chlamydomonas* cells. Appl. Environ. Microbiol. 71, 6199–6205.

Gfeller, R.P., Gibbs, M., 1984. Fermentative metabolism of *Chlamydomonas reinhardtii*. I: analysis of fermentative products from starch in dark and light. Plant Physiol. 75, 212–218.

Ghirardi, M.L., Zhang, J.P., Lee, J.W., Flynn, T., Seibert, M., Greenbaum, E., Melis, A., 2000. Microalgae: a green source of renewable H_2. Trends Biotechnol. 18, 506–511.

Gibbs, M., Gfeller, R.P., Chen, C., 1986. Fermentative metabolism of *Chlamydomonas reinhardtii*. III: photoassimilation of acetate. Plant Physiol. 82, 160–166.

Guan, Y.F., Deng, M.C., Yu, X.J., Zhang, W., 2004. Two-stage photo-biological production of hydrogen by marine green alga *Platymonas subcordiformis*. Biochem. Eng. J. 19, 69–73.

Hahn, J.J., Ghirardi, M.L., Jacoby, W.A., 2004. Effect of process variables on photosynthetic algal hydrogen production. Biotechnol. Prog. 20, 989–991.

Happe, T., Kaminski, A., 2002. Differential regulation of the Fe-hydrogenase during anaerobic adaptation in the green alga *Chlamydomonas reinhardtii*. Eur. J. Biochem. 269, 1022–1032.

Harris, E.H., 1989. The *Chlamydomonas* Sourcebook: A Comprehensive Guide to Biology and Laboratory Use. Academic Press, San Diego, p. 780.

Healy, F.P., 1970. The mechanism of hydrogen evolution by *Chlamydomonas moewusii*. Plant Physiol. 45, 153–159.

Heifetz, P.B., Förster, B., Osmond, C.B., Giles, L.J., Boynton, J.E., 2000. Effects of acetate on facultative autotrophy in *Chlamydomonas reinhardtii* assessed by photosynthetic measurements and stable isotope analyses. Plant Physiol. 122, 1439–1445.

Jo, J.H., Lee, D.S., Park, J.M., 2006. Modeling and optimization of photosynthetic hydrogen gas production by green alga *Chlamydomonas reinhardtii* in sulfur-deprived circumstance. Biotechnol. Prog. 22, 431–437.

Kosourov, S., Ghirardi, M.L., Seibert, M., 2001. The effect of growth mode on hydrogen production by sulfur-depleted green algae. Abstr. 23th Symposium on Biotechnology for Fuels and Chemicals. Breckenrige, Colorado, May 6–9, pp. 2–86.

Kosourov, S., Tsygankov, A., Seibert, M., Ghirardi, M.L., 2002. Sustained hydrogen photoproduction by *Chlamydomonas reinhardtii*: effects of culture parameters. Biotechnol. Bioeng. 78, 731–740.

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 Physiol. 44, 146–155.

Kosourov, S., Makarova, V., Fedorov, A.S., Tsygankov, A., Seibert, M., Ghirardi, M.L., 2005. The effect of sulfur re-addition on H_2 photoproduction by sulfur-deprived green algae. Photosynth. Res. 85, 295–305.

Kruse, O., Rupprecht, J., Bader, K.P., Thomas-Hall, S., Schenk, P.M., Finazzi, G., Hankamer, B., 2005. Improved photobiological H_2 production in engineered green algal cells. J. Biol. Chem. 280, 34170–34177.

Laurinavichene, T., Tolstygina, I., Tsygankov, A., 2004. The effect of light intensity on hydrogen production by sulfur-deprived *Chlamydomonas reinhardtii*. J. Biotechnol. 114, 143–151.

Melis, A., Zhang, L.P., Forestier, M., Ghirardi, M.L., Seibert, M., 2000. Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. Plant Physiol. 122, 127–135.

Rosenbaum, M., Schröder, U., Scholz, F., 2005. Utilizing the green alga *Chlamydomonas reinhardtii* for microbial electricity generation: a living solar cell. Appl. Microbiol. Biotechnol. 68, 753–756.

Tsygankov, A., Kosourov, S., Seibert, M., Ghirardi, M.L., 2002. Hydrogen photoproduction under continuous illumination by sulfur-deprived, synchronous *Chlamydomonas reinhardtii* cultures. Int. J. Hydrogen Energy 27, 1239–1244.

Tsygankov, A.A., Kosourov, S.N., Tolstygina, I.V., Ghirardi, M.L., Seibert, M., 2006. Hydrogen production by sulfur-deprived *Chlamydomonas reinhardtii* under photoautotrophic conditions. Int. J. Hydrogen Energy 31, 1574–1584.

Wykoff, D.D., Davies, J.P., Melis, A., Grossman, A.R., 1998. The regulation of photosynthetic electron transport during nutrient deprivation in *Chlamydomonas reinhardtii*. Plant Physiol. 117, 129–139.

Zhang, L., Melis, A., 2002. Probing green algal hydrogen production. Philos. Trans. R. Soc. Lond. B Biol. Sci. 357, 1499–1511.

Zhang, L., Happe, T., Melis, A., 2002. Biochemical and morphological characterization of sulfur-deprived and H_2-producing *Chlamydomonas reinhardtii* (green alga). Planta 214, 552–561.