PERGAMON

International Journal of Hydrogen Energy 27 (2002) 1239–1244

International Journal of HYDROGEN ENERGY

www.elsevier.com/locate/ijhydene

Hydrogen photoproduction under continuous illumination by sulfur-deprived, synchronous *Chlamydomonas reinhardtii* cultures

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

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

Abstract

Unsynchronized *Chlamydomonas reinhardtii* cells subsequently deprived of sulfur produce H_2 under continuous illumination in the laboratory for 3–4 days. However, cultures grown outdoors will be exposed to day-and-night cycles that may synchronize their growth and cell division. While it is clear that only insignificant amounts of H_2 can be produced by sulfur-deprived cells during the night period, little work has been done to examine the effects of the light/dark cycles preceding sulfur deprivation on subsequent H_2 photoproduction. We show that (a) *C. reinhardtii* cells exhibit synchronized growth and cell division in the presence of acetate, (b) cells with the highest specific rates of H_2 photoproduction also have the highest rates of biomass accumulation, and (c) the highest rates of starch and protein degradation coincide with the highest rates of formate and acetate accumulation, but not with H_2 photoproduction. This work shows that it is possible to maximize the production of H_2 by sulfur-depriving synchronized cultures at about 4 h after the beginning of the light period.

Keywords: Green algae; Hydrogen; Photoproduction; Synchronous growth; Sulfur deprivation

1. Introduction

Synchronous growth and cell division of green algae are achieved by alternating light and dark cycles [1]. All the cells in a synchronized culture simultaneously increase in size during the light period, and undergo cell division during the dark period [1,2]. Most commonly, light and dark (L:D) periods of 14 h : 10 h or 12 h : 12 h have been used to synchronize algal cells in a medium for photoautotrophs [1]. Several metabolic functions show periodicity in synchronous cultures. Respiratory O_2 uptake peaks during the early hours (< 4 h) of the light period [3], and photosynthesis, measured either as O_2 evolution or CO_2 fixation, peaks at about 6–8 h after the onset of the light period [4]. The peak of photosynthetic activity correlates with the peak of photosystem II (PSII) activity [5,6]. Starch accumulates in the light and is consumed in the dark, so starch content is highest at the end of the light period and lowest at the end of the dark period [7]. The periodicity of H_2 photoproduction activity in synchronized *C. reinhardtii* algal cells was studied extensively by Yanyushin [7–10]. He observed a peak in anaerobically induced hydrogenase activity during the early hours of the dark period, coinciding with the beginning of starch degradation [8].

The periodicity of several cellular functions in synchronized cultures is dependent either on metabolic events that are regulated by the cell cycle or on an internal circadian clock (see references in [2]). For example, the periodicity of the affinity of the photosynthetic apparatus for CO_2 can be maintained even after synchronized cells are transferred to continuous light [11]. On the other hand, many metabolic inhibitors, nutrient-deprived conditions, or other physiological interferences prevent the normal operation of the cell cycle by interrupting it at different control points [12].

* Corresponding author. Fax: +1-303-384-6150.
E-mail address: maria_ghirardi@nrel.gov (M.L. Ghirardi).

0360-3199/02/$ 22.00
PII: S0360-3199(02)00108-8

Sulfur deprivation arrests the normal algal cell cycle before the cell division stage at the end of the light period, and gives rise to cultures enriched in large cells that do not divide [13].

We have used sulfur deprivation of green algae to obtain cultures that photoproduce H_2 for several days before they require a sulfur-replete, regenerative period [14,15]. Our objective has been to develop a system for potential future applications under outdoor conditions, and the research has concentrated on optimizing H_2 production rates and accumulated amounts in sulfur-deprived cultures. In our previous studies, algal cells were grown photoheterotrophically to late-log phase, deprived of sulfate and then exposed to continuous light. We used 14 h : 10 h L:D synchronized cultures and observed that cells harvested 4 h after the start of the light period showed high yields of H_2 photoproduction when subsequently deprived of sulfate [16]. In the work presented here, we have expanded our investigations to include studies of H_2-photoproduction activity of cells harvested at other stages during the cell cycle.

2. Materials and methods

2.1. Cell growth

Chlamydomonas reinhardtii, strain cc124 (a wild-type strain originally from Levine's laboratory [1]), was grown photoheterotrophically on Tris-acetate-phosphate (TAP) medium, pH = 7.2, in 1.2 l flat glass bottles with stirring at about 28°C [16]. The synchronous growth and cell division of *C. reinhardtii* cells was achieved by alternating light (12 h) and dark (12 h) periods for at least 7 days and maintained by daily dilution of the cultures to a starting density of about $0.5–1 \times 10^6$ cells ml^{-1}. The achievement of synchronous cell division was checked by microscopic observations, as before [16]. We chose a shorter light period in this work (12 h, compared to 14 h in Ref. [16]) in order to conform with most of the previous synchronization literature. Synchronous and unsynchronous cultures were aerated with 3% CO_2 in air (approximately 90 ml min^{-1}). Synchronous algal cells were grown to a final concentration of about $2–5 \times 10^6$ cells ml^{-1}, harvested at different stages during the cell cycle by centrifugation at 2000 g for 5 min, washed five times in TAP-minus-sulfur medium, and then resuspended in the same medium at a final concentration of about 9 μg Chl ml^{-1} ($4–5 \times 10^6$ cells ml^{-1}).

2.2. Bioreactor system

Both synchronized and unsynchronized sulfur-deprived cell suspensions were placed in each of four glass photobioreactors (4-cm optical path, 1.2 l culture volume). The algal cells were cultured under continuous two-sided illumination of ~ 300 μE m^{-2} s^{-1} (12 40-W cool-white fluorescent lamps) at 28 ± 1.5°C for up to 140 h as described before [16]. Four physical, biophysical and electrochemical factors (pH, E_h, pO_2, and temperature), as well as the volume of gas produced, were computer-monitored simultaneously, continuously and independently in each of the four photobioreactors, using the system described in Kosourov et al. [16]. The maximum specific rates of H_2 production were calculated from the accumulated H_2-production curves, usually at 20–24 h after the start of gas collection. The results were expressed on the basis of chlorophyll concentrations in the cultures measured at the relevant times.

2.3. Biochemical assays

The chlorophyll $a + b$ content was assayed spectrophotometrically in 95% ethanol extracts by the method of Spreitzer [1]. The samples for starch, protein, acetate and formate analysis were taken directly from the photobioreactors and centrifuged for 5 min at ~ 2000 g. The pellets and supernatants were separated and stored frozen at −80°C until all the samples were ready for assay. The amount of starch and protein accumulated inside the cells was determined in the pellet on a per ml of culture basis according to the methods of Gfeller and Gibbs [17] and Lowry et al. [18], respectively. The levels of acetate and formate in the medium were determined by HPLC (Model 1050, Hewlett-Packard, Palo Alto, CA) using an ion-exchange column (Aminex HPX-87H, Bio-Rad, Hercules CA) and 4 mM H_2SO_4 as the mobile phase.

3. Results

Using the computer-monitored photobioreactor system, we have previously reported [16] that sulfur-deprived *C. reinhardtii* cultures progress through five well-defined physiological phases: (a) an aerobic phase (during which photosynthetic O_2 evolution activity is gradually inactivated); (b) an O_2-consumption phase (when the photosynthetic O_2 evolution activity falls below the respiratory activity of the cells, leading to net O_2 consumption); (c) an anaerobic phase (after all O_2 in the culture medium is removed by respiration); (d) a H_2-production phase (after establishment of anaerobiosis and induction of hydrogenase enzyme activity); and (e) a termination phase (when all cellular activity is gradually inactivated).

The same system was used to study the influence of the cell cycle on H_2 photoproduction by sulfur-deprived cells. Cells grown under 12 h : 12 h L:D cycles were harvested at different times during the cycle (4, 8, 12, 16, 20 and 24 h). These cells were transferred to fresh medium, which was deprived of sulfur, and exposed to continuous illumination. In the text, these will be designated as $t = 4, 8, 12, 16, 20$ and 24 h cells or cultures. Table 1 shows the average length of each of the first four phases mentioned above. The length of the aerobic phase varied from 23–53 h. It was shortest when

Table 1
Length of the physiological phases resulting from sulfur deprivation, and the total yield of H_2 production by cultures harvested at different stages during a 12 h : 12 h L:D cycle. The cultures were harvested, sulfur-deprived and then exposed to continuous illumination

Type of culture	Aerobic phase (h)	O_2-consumption phase (h)	Anaerobic phase (h)	H_2-production phase (h)	Total yield of H_2 (after 140 h) (ml)
Synchronized (h)					
4	35	14	11	79	102
8	22.5	17	16.5	71	29
12	23	14.5	10.5	64	33
16	25	11	14	60	44
20	53	4	13	62	50
24	47	8	13	72	74
Unsynchronized	23	13	9	63	86

the samples were taken at the 8 and 12 h time points (second half of the light period), and longest with samples at the 20 and 24 h time points (second half of the dark period). This trend reversed during the O_2-consumption phase, where the shortest duration was seen with $t = 20$–24 h cells and the longest duration with $t = 8$–12 h cells. The longest anaerobic phase was also observed with $t = 8$ h cells, but the duration of this phase varied little in cultures harvested at the other time points. Finally, the duration of the H_2-production phase peaked with $t = 4$ h cells and was shortest with $t = 16$ and 20 h cells. The behavior of the unsynchronized control cultures most closely resembled that of cells harvested at $t = 12$ h, with short aerobic and long O_2-consumption phases, and short anaerobic and H_2-production phases. However, in contrast with $t = 12$ h cultures, unsynchronized cultures produced larger volumes of H_2 gas and exhibited higher maximum specific rates of H_2 production (data not shown), which are characteristic of $t = 4$ h cultures.

Fig. 1 shows the accumulation of H_2 as a function of time in the sulfur-deprived medium. Each panel in Fig. 1 shows the total yield of H_2 (in ml) obtained with samples harvested at the indicated times. The upper panels correspond to cells that were harvested and sulfur-deprived during the light period and the lower panels represent samples from the dark period. Vertical dashed lines in each panel indicate the times at which the cultures became anaerobic. The total amount of H_2 produced by each culture and the maximum specific H_2-production rates were then plotted in Fig. 2A as a function of the time during the cell cycle at which the cells were harvested and deprived of sulfur. The maximum specific rates of H_2 production and the total accumulated amounts of H_2 were highest at $t = 4$ h, and declined sharply with cells harvested at $t = 8$ h. Fig. 1 also shows that protein and starch concentrations increase during the early stages of sulfur deprivation, as previously reported, and then decrease, concomitantly with H_2 production [16,19]. The concentration of chlorophyll follows a less predictable course. An initial increase in concentration, followed by a decrease concomitantly with H_2 production is only observed with $t = 4$ and 8 h cells. Cells from $t = 12$ and 16 h show a decrease in chlorophyll concentration throughout sulfur deprivation, and very small variations in chlorophyll concentration were seen during the experiment with $t = 20$ and 24 h cells. Fig. 2B shows that maximum protein and chlorophyll (but not starch) accumulation occurs during the early stages of sulfur deprivations in cells harvested at $t = 4$ and 24 h. This indicates that the peak of H_2 photoproduction and biomass accumulation (measured by increases in chlorophyll and protein contents) by sulfur-deprived cells coincides with the previously reported peak in photosynthetic activity of algal cells [4].

Fig. 1 also shows that significant starch and protein degradation takes place during the H_2-production phase of the sulfur-deprivation process. Both starch and protein degradation are important for H_2 photoproduction, because they are involved in the establishment and maintenance of anaerobic conditions in the photobioreactor vessel following the decrease in photosynthetic O_2-evolution activity of the cultures [16,19], and also because they contribute a small fraction of the reductants used for H_2 photoproduction [20]. The total amounts of starch and protein degraded during the later phases of the sulfur-deprivation process, estimated from Fig. 1, are shown in Fig. 2C. In contrast to chlorophyll and protein accumulation and H_2 photoproduction, starch and protein degradation under sulfur-deprived conditions are slightly higher with cells harvested during the dark period. We also measured the extracellular accumulation of fermentation products during sulfur deprivation that resulted from anaerobic starch and protein degradation. Fig. 3 shows that the acetate content of the medium decreases during the early phases of sulfur deprivation, due to acetate consumption when PSII inactivation is occurring. After the establishment of anaerobiosis, however, both acetate and formate are produced and excreted into the growth medium. Fig. 2C shows that the total production of formate and acetate also depends on the stage in the cell cycle at which the cells were sulfur-deprived and, as expected, the depen-

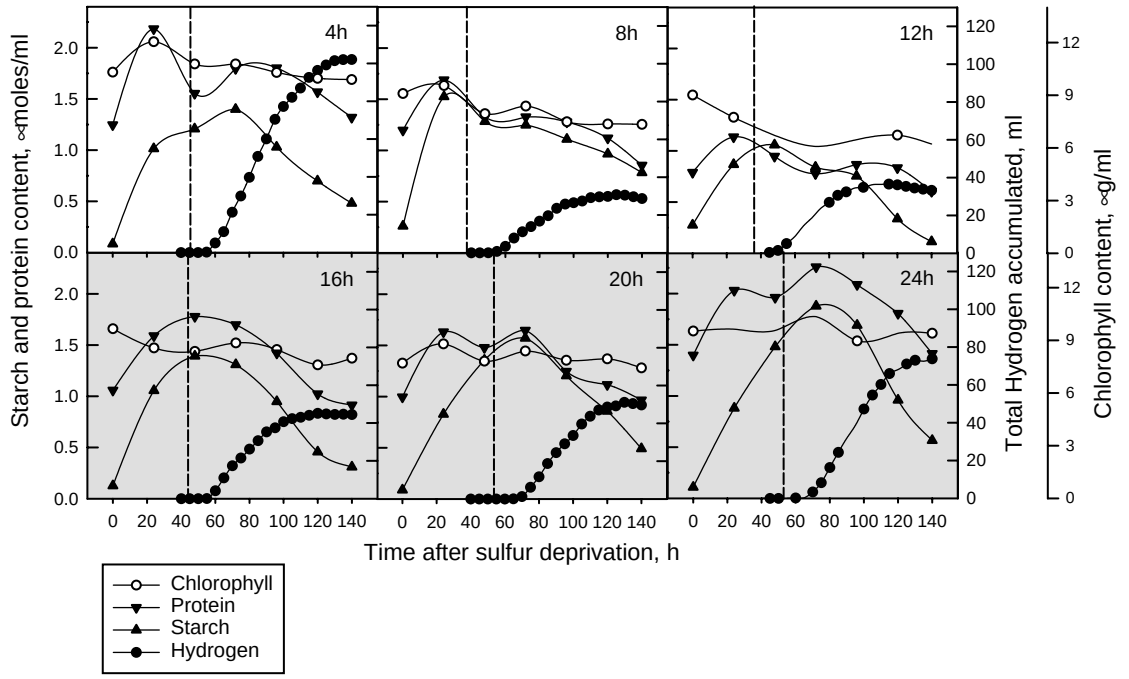

Fig. 1. Concentrations of chlorophyll $a + b$ ($\mu g\ ml^{-1}$), protein and starch ($\mu moles\ ml^{-1}$) and accumulated volume of H_2 (ml) produced during continuous illumination under sulfur-deprived conditions. Cells were harvested at indicated times during a 12 h : 12 h L:D cycle. Upper panels: cultures of cells harvested during the light period (at 4, 8 and 12 h); lower panels: cells harvested during the dark period (16, 20 and 24 h). Vertical dashed lines indicate the times at which the cultures became anaerobic.

dence on the cell cycle resembles that for starch and protein degradation.

4. Discussion

In the past, researchers claimed that synchronization of *C. reinhartdtii* cell growth and division required photoautotrophic conditions [1], and if acetate were present in the medium, cell division occurred unsynchronously [21]. For H_2 photoproduction from sulfur-deprived algal cultures, acetate and light are necessary for timely inactivation of O_2 evolution [20]. In our experiments, however, the presence of acetate in the growth medium did not prevent the establishment of synchronous growth in *C. reinhardtii* cell cultures [16]. Photomicrographs taken at three different points during the L:D cell cycle, at $t = 4, 9$ and 23 h, clearly showed the well-defined differences in cell structure [16] that correlate with previously-described changes occurring as the cells went through the cycle [1].

Following sulfur deprivation, synchronous cultures of another green alga, *Chlorella ellipsoidea,* continue to go through the cell cycle, until they are arrested at the stage of cell division [13]. At the cell division stage, early during the dark phase, *C. reinhardtii* cultures exhibit the highest inducible hydrogenase activity and lowest rate of photosynthetic O_2 evolution [7]. One might expect, then, that cell cultures sampled at different times during the cell cycle would all eventually show the same high H_2-production activity after sulfur deprivation. In reality, sulfur-deprived cultures harvested at different intervals in the cell cycle exhibited marked differences in H_2-production activity. Cultures from 4 and 24 h cells produced the highest amounts of H_2 and showed the highest specific rates of H_2 photoproduction after sulfur deprivation, and they also exhibited the maximum rates of growth (protein and chlorophyll accumulation) during the aerobic and O_2-consumption phases. In contrast, cultures harvested at 8 and 12 h produced the lowest amounts of H_2 and had the lowest rates of protein accumulation. While it is difficult to explain this observation, it may be that an initial high rate of growth allows sulfur-deprived cells to undergo more than one cell cycle, and thus to build up more biomass (measured as protein) before growth is arrested by the lack of sulfate. This would explain the higher quantity of biomass at $t = 4$ and 24 h, but would not explain the increased specific H_2-production activity of the same cultures. We must thus conclude that the level of cell growth and photosynthetic activity of the cultures during the early stages of sulfur deprivation (the aerobic phase) determines the specific H_2-production capacity of the cultures during the subsequent H_2-production phase.

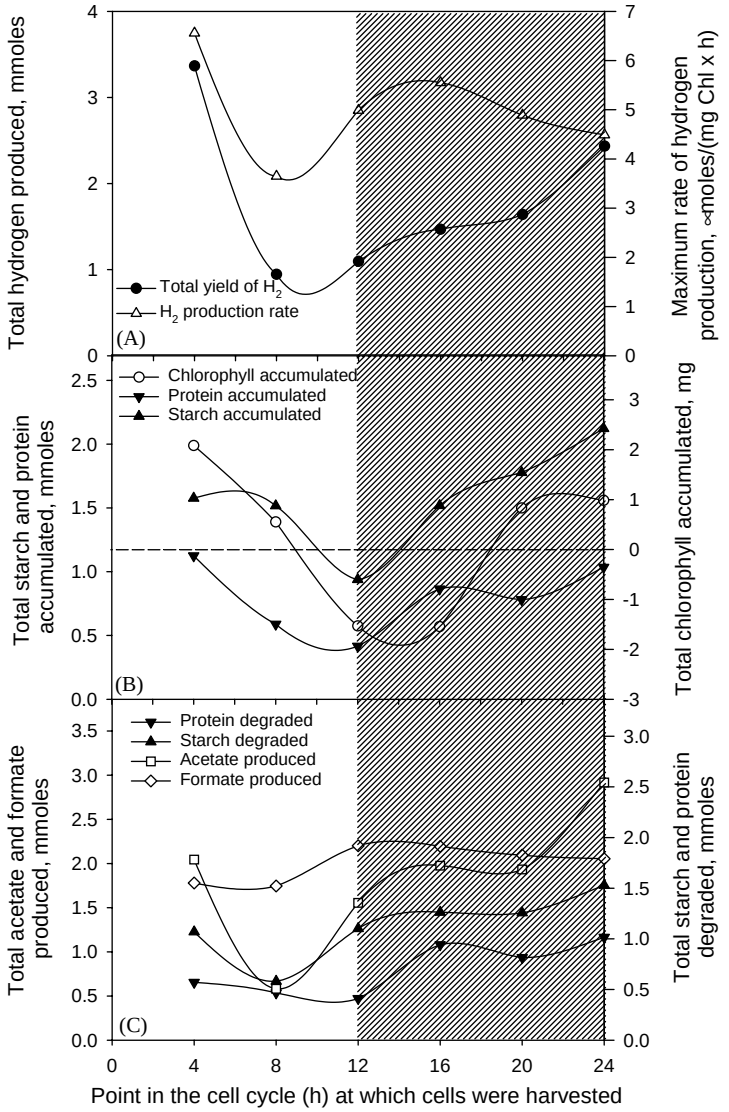

Fig. 2. Total production or degradation of different products during sulfur deprivation in 1.2 l of cells harvested at different times during the cell cycle: (A) amount of H_2 (mmoles) and maximum specific rate of H_2 produced (μmoles (mg Chl h)$^{-1}$); (B) changes in the total amount of chlorophyll $a+b$ (mg), starch and protein (mmoles) during the early phases of sulfur deprivation; and (C) amount of starch and protein degraded and formate and acetate produced (mmoles) during the late phases of sulfur deprivation. The conversion of H_2 from ml (Fig. 1) to mmoles at 28°C and 1600 m altitude is 1 ml = 0.033 mmoles.

We also observed that the amounts of starch and protein degraded during the sulfur-deprivation process are higher when the cells are harvested at $t = 16$–24 h, which coincides with the maximum accumulation of fermentation products. This confirms our observation that H_2 photoproduction is directly related to the photosynthetic water oxidation activity, and not to endogenous substrate degradation ([20]; S. Kosourov et al., unpublished results).

In summary, the rates and accumulated amounts of H_2 photoproduction by sulfur-deprived *C. reinhardtii* cultures is influenced by synchronous L:D growth and division of the cultures prior to sulfur deprivation. Furthermore, in contrast to previous reports, synchronous growth and cell division of *C. reinhardtii* can be achieved under photoheterotrophic conditions with acetate in the medium. Sulfur deprivation and subsequent continuous illumination of cells harvested during the early hours of the light cycle yield higher

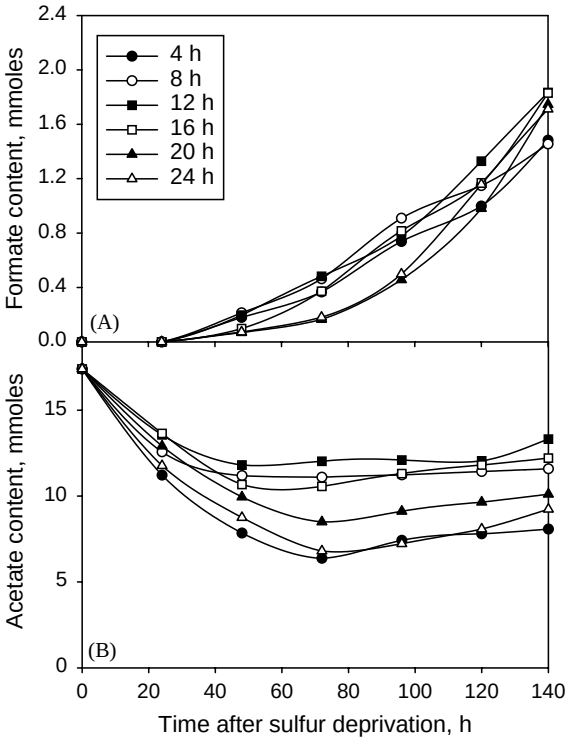

Fig. 3. Changes in the amount of formate (A) and acetate (B) in the extracellular medium (mmoles) during sulfur deprivation, using cells harvested at different times in the cell cycle.

production rates and volumes of H_2. This information may be useful in the design of algal H_2-production photobioreactor systems, which will have to utilize cells grown outdoors under day/night cycles.

Acknowledgements

This work was supported by the US DOE Hydrogen Program.

References

[1] Harris EH. The *Chlamydomonas* Sourcebook. New York, NY: Academic Press, 1989.

[2] Krupinska K, Humbeck K. Light-induced synchronous cultures, an excellent tool to study the cell cycle of unicellular green algae. J Photochem Photobiol 1994;26: 217–31.

[3] Ehara T, Osafune T, Hase E. Behavior of mitochondria in synchronized cells of *Chlamydomonas reinhardtii* (Chlorophyta). J Cell Sci 1995;108:499–507.

[4] Senger H, Bishop NI. Observations on the photohydrogen producing activity during the synchronous cell cycle of *Scenedesmus obliquus*. Planta 1979;145:53–62.

[5] Mell V, Senger H. Photochemical activities, pigment distribution and photosynthetic unit size of subchloroplast particles isolated from synchronized cells of *Scenedesmus obliquus*. Planta 1978;143:315–22.

[6] Kaftan D, Meszaros T, Whitmarsh J, Nedbal L. Characterization of Photosystem II activity and heterogeneity during the cell cycle of the green alga *Scenedesmus quadricauda*. Plant Physiol 1999;120:433–41.

[7] Yanyushin MF. Hydrogenase activity in a synchronous culture of *Chlamydomonas reinhardtii*. Sov Plant Physiol 1979;26:394–400.

[8] Yanyushin MF. Effects of electron transport inhibitors on photoevolution of hydrogen by a synchronous culture of *Chlamydomonas reinhardtii*. Sov Plant Physiol 1981a;28:542–7.

[9] Yanyushin MF. Hydrogen evolution and hydrogenase activity in synchronous culture of *Chlamydomonas reinhardtii* in connection with anaerobic degradation of starch. Sov Plant Physiol 1981b;29:121–6.

[10] Yanyushin MF. Hydrogenase activation and hydrogen photoevolution in a synchronous culture of *Chlamydomonas reinhardtii* during anaerobic adaptation under light. Sov Plant Physiol 1982;29:1126–33.

[11] Marcus Y, Schuster G, Michaels A, Kaplan A. Adaptation to CO_2 level and changes in the phosphorylation of thylakoid proteins during the cell cycle of *Chlamydomonas reinhardtii*. Plant Physiol 1986;80:604–7.

[12] Spudich JL, Sager R. Regulation of the *Chlamydomonas* cell cycle by light and dark. J Cell Biol 1980;85:136–45.

[13] Hase E, Otsuka H, Mihara S, Tamiya H. Role of sulfur in the cell division of *Chlorella*, studied by the technique of synchronous culture. Biochim Biophys Acta 1959;35: 180–9.

[14] Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M. Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. Plant Physiol 2000;122: 127–35.

[15] Ghirardi ML, Zhang L, Lee JW, Flynn T, Seibert M, Greenbaum E, Melis A. Microalgae: a green source of renewable H_2. Trends Biotechnol 2000a;18:506–11.

[16] Kosourov S, Tsygankov A, Seibert M, Ghirardi ML. Sustained hydrogen photoproduction by *Chlamydomonas reinhardtii*—effects of culture parameters. Biotechnol Bioeng 2002;78: 731–40.

[17] Gfeller RP, Gibbs M. Fermentative metabolism of *Chlamydomonas reinhardtii*; I. Analysis of fermentative products from starch in dark and light. Plant Physiol 1984;75:212–8.

[18] Lowry OH, Rosebrought NJ, Farr AL, Randall RJ. Protein measurements with the Folin phenol reagent. J Biol Chem 1951;194:267–75.

[19] Zhang L, Happe T, Melis A. Biochemical and morphological characterization of sulfur-deprived and H_2-producing *Chlamydomonas reinhardtii* (green alga). Planta 2002;214:552–61.

[20] Ghirardi ML, Kosourov S, Tsygankov A, Seibert M. Two-phase photobiological algal H_2-production system. Proceedings of the 2000 US DOE Hydrogen Program Review, 2000b, NREL/CP-570-28890.

[21] Surzycki S. Synchronously grown cultures of *Chlamydomonas reinhardtii*. Methods Enzymol 1971;23:67–73.