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Sustained hydrogen photoproduction by phosphorus-deprived Chlamydomonas reinhardtii cultures

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ABSTRACT

This study demonstrates that, besides sulfur deprivation, sustained H_2 photoproduction in *Chlamydomonas reinhardtii* cultures can be generated by incubating algae under phosphorus-deprived (−P) conditions. However, phosphorus deficiency in algal cells could not be obtained by resuspension of algae in −P medium, evidently due to a significant reserve of phosphorus in cells. In this study, phosphorus deficiency was accomplished by inoculating the washed algae into the −P medium at low initial cell densities (below 2 mg Chl l^{-1}). After the initial growth period, where cells utilize intracellular phosphorus, algae established anaerobic environment followed by the period of H_2 photoproduction. The maximum H_2 output (∼70 ml l^{-1}) was obtained in cultures with the initial Chl content ∼1 mg l^{-1}. Cultures with Chl above 2 mg l^{-1} did not produce H_2 gas. The physiological response of algal cultures to phosphorus deprivation demonstrated significant similarities with the response of algae to sulfur depletion.

1. Introduction

Chlamydomonas reinhardtii cultures deprived of inorganic sulfur are capable of prolonged H_2 photoproduction [1]. Sulfur deprivation causes a gradual and reversible inhibition of the PSII-dependent O_2-evolving activity in algal cells due to their inability to re-synthesize the D1 protein in the absence of sulfur [2], an overreduction of plastoquinone pool [3], and a decrease in the amount of RuBisCo enzyme [4]. When the capacity for O_2 evolution in algae decreases below that of respiration, cultures respire all available O_2 and become anaerobic in the light if photobioreactors are sealed [1,5]. The enzyme catalyzing H_2 photoproduction in algae is a reversible [Fe−Fe]-hydrogenase that (i) accepts electrons from ferredoxin, a terminal acceptor of the photosynthetic electron transport chain, and (ii) reduces protons of water to molecular H_2. The absence of O_2 induces the expression of two [Fe−Fe]-hydrogenases in *C. reinhardtii* cells [6,7]. Hydrogen evolution in sulfur-deprived suspension cultures lasts normally for about 100 h, and depends both on the residual PSII activity remaining in algal cells [3,8] and on the catabolism of starch accumulated during the first 18−24 h of sulfur deprivation [9−11].

Besides sulfur, the depletion of phosphorus can also inhibit O_2-evolving activity in algae [2]. However, the inhibition process by phosphorus deprivation is slower than that by sulfur and cultures establish anaerobiosis later [2]. Similar to sulfur deprivation, phosphorus deprivation causes a decline in the *in vivo*, light-saturated rate of photosynthesis in high plants [12−15], but unlike sulfur deprivation the decline is

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mainly attributed to a limitation in the rate of CO_2 fixation because of depletion in the pool of phosphorylated intermediates of the reductive pentose phosphate cycle [13,15]. The partial inhibition of O_2-evolving activity in phosphorus-deprived algae suggests the possibility of H_2 photoproduction under these conditions, but to our knowledge, the efficient H_2 production in phosphorus-deprived cultures has not been demonstrated. Nevertheless, the effect of phosphorus deprivation on H_2 photoproduction was studied in combination with sulfur deprivation [16] or sulfur/nitrogen limitation [17].

In the present work, we show that bulk amounts of H_2 gas in green alga, C. reinhardtii can be obtained under phosphorus-deprived conditions, but only after depriving the intracellular pools of reserved phosphorus in cells. Algae withdrawn from a regular growth medium and washed of phosphorus using a standard technique have enough intracellular phosphorus allowing them to grow continuously for few days after removal of phosphates from the medium. As a result, the cultures reach more than 30 mg Chl $(a + b)$ l^{-1} and do not produce H_2 gas despite the fact that they have a high hydrogenase activity. Continuous H_2 production in phosphorus-deprived algae was generated using a dilution approach first introduced for sulfur deprivation [18]. Cultures washed of phosphorus and diluted to below 2 mg Chl l^{-1} in the phosphate-free medium were able to establish anaerobiosis in the photobioreactor after the growth period and produce H_2 gas in the same quantities as sulfur-deprived cultures.

2. Materials and methods

2.1. Cell growth

Green alga, C. reinhardtii Dangeard 137C mt^+, was grown photomixotrophically on a standard TAP medium, pH 7.2 in flat glass bottles at 28 ± 1 °C. Algal cultures were bubbled continuously with 2% CO_2 in air using autoclavable membrane filters with a 0.2 μm pore size (Pall, Ann Arbor, MI). During growth, the algae were illuminated from two sides with cool-white fluorescence lamps providing of about 40 μmol m^{-2} s^{-1} PAR on the bottle surface. Light intensity was measured with a quantum radiometer photometer (Model LI- 250, LI-COR, Lincoln, NE). Three-days-old cultures with a final Chl concentration of around 20–25 mg l^{-1} were used in all experiments.

2.2. H_2 photoproduction in sulfur and/or phosphorous-deprived cultures

Two methods were used to deprive algal cultures of nutrients. In the first, cells were harvested by centrifugation, washed once in either sulfur-free (TAP −S), phosphorus-free (TA −P) or sulfur/phosphorus-free (TA −S −P) medium and re-suspended in the same medium at required Chl $(a + b)$ concentrations (see [1] for details). In the second method that was used for phosphorus deprivation only, cultures grown in the standard TAP medium were washed once in the TA −P medium by centrifugation and inoculated into the same medium at different Chl concentrations: ~0.5–25 mg l^{-1} (see [18] for details).

Nutrient-deprived cultures obtained in either of two ways were incubated in 500-ml cylindrical PhBRs (~50 mm ID) under continuous illumination of 45 μmol m^{-2} s^{-1} PAR from two sides at 28 °C. Stirring was provided as described earlier [3]. The gas produced by the culture was collected by the displacement of water from a graduated 50 ml glass syringe connected to the sealed PhBR with Tygon® tubing.

In the final experiment, H_2 photoproduction by phosphorus-deprived algae was studied in the microprocessor-controlled PhBR system developed in our laboratory in Pushchino, Russia [19]. In this case, phosphate-deprived cell suspensions diluted to 1.5 ± 0.2 mg Chl l^{-1} (~0.67 × 10^6 cells ml^{-1}) with the TA −P medium were placed in four glass PhBRs and cultured under continuous one-sided illumination (~80 μmol m^{-2} s^{-1} PAR) with cool-white fluorescence lamps. Unlike the experiment done by Melis and co-authors [1], the PhBRs were sealed 80 h after transferring cultures into the TA −P medium. The H_2 photoproduction activities were monitored independently in each of four photobioreactors by the displacement of water, as described above. The H_2 contents in the gas phase of PhBRs were determined by a gas chromatography (GC).

2.3. Other analytical procedures

Chlorophyll concentrations were measured spectrophotometrically in 95% ethanol cell extracts by the method of Spreitzer [20]. The samples for starch, Chl and acetate contents were taken directly from PhBRs with a sterile syringe and pelleted by centrifugation at ~13000 rpm (MiniSpin, Eppendorf, NY) for 3 min. The pellets and supernatants were separated and stored frozen at −20 °C until all samples were ready for processing. The amount of starch accumulated inside the cells was determined in the pellet according to the method developed by Gfeller and Gibbs [21], except that methanol used for cell disruption and pigments extraction was replaced with ethanol. The levels of acetate in supernatants were determined by a GC as described elsewhere [22].

Hydrogenase activity was measured by the rate of H_2 evolution from methyl viologen reduced with dithionite. Culture samples (1 ml) were taken anaerobically from the PhBR at different stages of H_2 production and injected into 13-ml anaerobic vials, containing 0.25 ml of 40 mM oxidized methyl viologen and 0.75 ml 50 mM potassium-phosphate buffer (pH 6.9) and 0.2% w/v Triton X 100. The vials were then evacuated and refilled with argon three times, and placed in a water bath at 30° C. The reaction was started by the addition of 100 μl of anaerobic, 100 mM Na dithionite. The H_2 evolution rate was measured in the dark using a GC.

3. Results and discussion

Besides light and CO_2, growth of phototrophic microalgae depends significantly on the availability of essential macronutrients in the environment, such as: sulfur, nitrogen or phosphorus [23]. In the absence of any of these nutrients, cell division is arrested and cultures stop growing [24]. Acclimation of algal cells to the nutrient-deprived conditions at the early stages of the stress is usually accompanied by the significant changes in cell physiology, including changes in

protein, lipid and starch biosynthesis/degradation, a reduction of photosynthetic activity, an increase in the rate of cellular respiration and many other responses. One of the most interesting is the reduction of a linear photosynthetic electron transport from PSII [2,3]. Previous reports demonstrated that sulfur starvation progressively inhibits the PSII-dependent O_2-evolving activity in algal cells [2] that under certain conditions results in the establishment of anaerobic conditions and continuous H_2 photoproduction in algal cultures [1]. The reduction of O_2-evolving activity was also shown under Fe, Mn [25] and phosphorus [2] deprivation, but H_2 photoproduction has not been studied under these conditions, most probably due to a significant delay in the PSII inactivation process.

We first checked how exclusion of phosphorus from the medium affects H_2 photoproduction in algal cultures in addition to sulfur starvation. Unlike Jo and co-authors [17], we did not notice any significant effect of additional withdrawing of phosphorus from the sulfur-deprived medium on H_2 photoproduction by *C. reinhardtii* cells. As shown in Fig. 1, cultures deprived of sulfur or sulfur and phosphorus behaved similar in many aspects. The final outputs of H_2 gas were almost the same. The difference in the kinetics of H_2 photoproduction was not significant and varied slightly from one experiment to another in both cultures. Similar results were obtained in immobilized cultures, where cells produce H_2 gas at equal efficiencies both under sulfur-deprived and sulfur/phosphorus-deprived conditions [16].

Cultures washed of phosphorus by centrifugation and resuspended in the phosphorus-free medium did not produce any noticeable amounts of H_2 gas (Table 1). In contrast to sulfur-deprived cells, algae grew more significantly under these conditions, as can be seen from an increase in the total Chl concentration (Table 1). Interestingly, the overall hydrogenase activity in these cultures was rather high and almost identical to sulfur-deprived cells (Table 1). Since cultures washed of phosphorus reached rather high Chl concentrations, the appearance of hydrogenase activity could be caused by an establishment of the partial anaerobiosis in the PhBR dark zone [26]. Therefore, we checked if sulfur/phosphorus-
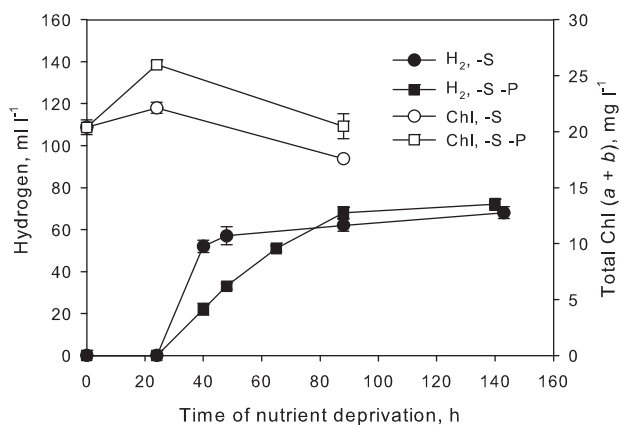
replete cells have hydrogenase activity at high cell densities in the same PhBR setup. Indeed, as shown in Table 1 sulfur/phosphorus-replete *C. reinhardtii* cultures had high hydrogenase activity, but produced only trace amounts of H_2 gas, which can be detected in the PhBR headspace by GC. Therefore, the high hydrogenase activity in algal cultures washed of phosphorus by centrifugation was caused by a high cell density that allows dark fermentation processes to occur in the inner layers of the PhBR. In this case, the efficient H_2 production was not possible due to the presence of stirring, since the photosynthetic surface layers produce O_2 inhibiting the H_2 photoproduction activity. In addition, under this condition other metabolic pathways may compete more effectively for the photosynthetic reductant. Since microorganisms, including green algae, synthesize polyphosphates as a reserve material [27], one could suggest that exclusion of phosphorus from the medium does not necessary lead to the immediate stress response and cultures may not experience phosphorus starvation for a prolonged time period. Therefore, the first step in this research was to determine the point where algae experience phosphorus starvation.

The most direct and evident way to prove that microalgae experience phosphorus starvation is to find the region where their growth is limited by the amount of added phosphorus. In general, phosphorous is introduced into the culture by two ways: with the medium and with the inoculum. Since the direct exclusion of phosphorus from the medium did not lead to the immediate phosphorus starvation effect, we applied a dilution approach that was first designed for obtaining sulfur-deprived microalgae [18]. In this approach, cells utilize the limited nutrient during the growth phase in the nutrient-deprived medium. To get the different initial Chl concentrations in the cultures, different volumes of the inoculum were introduced into the phosphorus-deprived medium. However, in contrast to sulfur-deprived algae the cells were washed of phosphorus by centrifugation in the beginning of the experiment and the inoculum was prepared in the TA −P medium. As shown in Fig. 2, the cell growth (defined as an increase in the maximum Chl $(a + b)$ concentration in the culture) was limited by the amount of phosphorus, introduced into the culture with the inoculum, only when the initial concentration of the total Chl in the culture was below 2 mg Chl l^{-1}. At higher initial Chl concentrations, the maximum Chl contents did not change significantly with an increase in the initial cell density meaning that in this region cultures are not phosphorus-deprived and limited by other factors. Initially, all cultures produced oxygen followed by oxygen consumption (data not shown). After 80–100 h of growth, cultures with low initial Chl contents started to accumulate H_2 gas. Fig. 2 shows that there was a narrow range in initial Chl concentrations leading to the efficient H_2 photoproduction under phosphorus-deprived conditions. The maximum volume of H_2 gas (68 ml l^{-1}) was obtained in the culture with the initial Chl content ∼1 mg l^{-1}. Cultures with the initial Chl concentration above 2 mg l^{-1} did not produce H_2 gas since they are not phosphorus-deprived.

Finally, we used a microprocessor-controlled photo-bioreactor system [19] to demonstrate the ability of phosphorus-deprived cultures to establish anaerobiosis in the PhBR and produce H_2 gas. Fig. 3 shows that cultures washed

Fig. 1 — Changes in hydrogen photoproduction activities and Chl contents in sulfur-deprived (−S) and sulfur/phosphorus-deprived (−S −P) cultures. Nutrient-deprived cultures were obtained by the centrifugation method.

Table 1 – Changes in the total (a + b) Chl content, H₂ photoproduction and hydrogenase (H₂-ase) activities in sulfur-deprived (−S), phosphorus-deprived (−P) and sulfur/phosphorus-replete (+S +P) cultures. Nutrient-deprived cultures were obtained by the centrifugation method.

Time, h	−S			−P			+S +P		
	Chl[a]	H₂[b]	H₂-ase[c]	Chl[a]	H₂[b]	H₂-ase[c]	Chl[a]	H₂[b]	H₂-ase[c]
0	23.4 ± 0.8	0	0	22.5 ± 1.3	0	0	20.8	0	0
56	28.8 ± 2.1	22	156 ± 2	34.6 ± 0.4	0	117 ± 12	34.0	0	148 ± 21
104	20.5 ± 0.2	87	164 ± 12	26.4 ± 1.9	0	133 ± 71	–	–	–
128	19.9 ± 2.4	109	208 ± 29	31.5 ± 0.2	0	272 ± 93	–	–	–

a $(a + b)$ mg l^{-1}.
b ml l^{-1}.
c μmol H₂ (mg Chl)$^{-1}$ h^{-1}.

of phosphorus and diluted to the initial Chl concentration $1.5 ± 0.2$ mg l^{-1} (optimal for this particular PhBR configuration) pass through the same physiological stages as sulfur-deprived cultures: aerobic/photosynthetic, O₂-consumption, anaerobic, H₂ production and termination stages [5]. The major difference between these two cultures was that establishment of anaerobiosis in phosphorus-deprived cultures occurred 100 h after the beginning of phosphorus deprivation that is significantly later than in the sulfur-deprived cultures obtained by the centrifugation method (18–40 h) [1,5,8]. Nevertheless, the sulfur-deprived cultures obtained by the dilution method start to produce H₂ gas after about 75–100 h of growth in the sulfur-depleted medium [18]. As shown in Fig. 3, H₂ gas appeared in the system after 100 h of phosphorus deprivation and stopped after 300 h. The cell density in the culture increased gradually from about $0.67 × 10^6$ cells ml^{-1} at $t = 0$ h to about $5.16 × 10^6$ cells ml^{-1} at 155 h, and subsequently declined to $4.04 × 10^6$ cells ml^{-1} at 270 h (Fig. 3B). Concomitantly, the Chl content of the culture raised from $1.5 ± 0.2$ mg l^{-1} to $15.2 ± 3.1$ mg l^{-1} and declined steadily to $13.2 ± 2.8$ mg l^{-1}. These results showed that cell growth and division does occur during the first 150 h of phosphorus
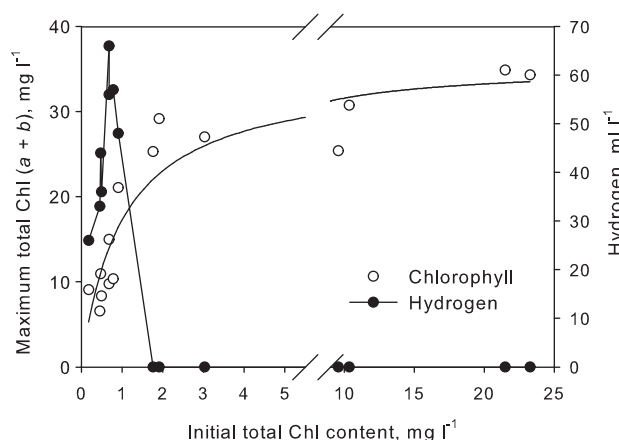

Fig. 2 – The effect of the initial culture density (measured as the total Chl concentration at the beginning of phosphorus deprivation) on accumulation of the biomass (measured as the maximum Chl concentration) in PhBR and the total yield of H₂ gas produced by the culture. The phosphorus deprivation effect was achieved by using the dilution method.
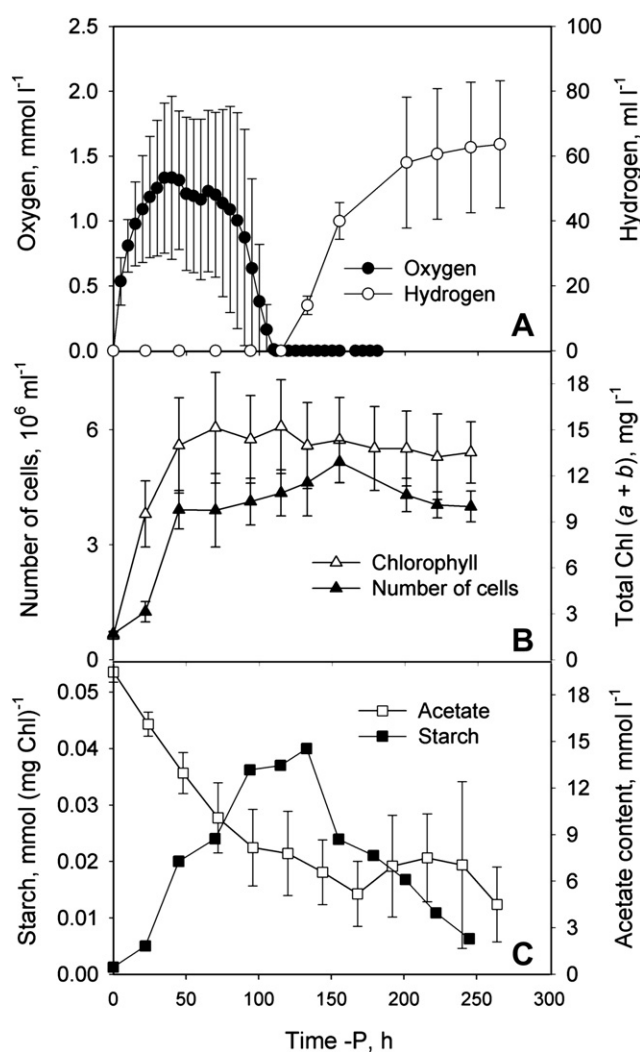

Fig. 3 – Changes in different parameters during phosphorous deprivation: (A) the level of dissolved O₂ in the medium and H₂ photoproduction; (B) the total $(a + b)$ Chl concentration and number of cells; (C) the amount of starch accumulated in algal cells and the acetate content in the medium. Cell suspensions washed of phosphate by centrifugation and diluted to the initial Chl concentration ~1.5 mg l^{-1} were placed in the microprocessor-controlled PhBR system and sealed at 80 h time point.

deprivation followed by a loss of cells at the end of the H_2 photoproduction stage.

Similar to sulfur-deprived algae, acclimation of algal cells to phosphorus-deprived conditions was accompanied by the accumulation of starch during the O_2-production stage and its degradation during the H_2 production stage (Fig. 3C). The decrease in the starch content during the H_2 production stage supports the fact that starch catabolism contributes to the process, either directly or indirectly, as seen by many researchers with sulfur-deprived cultures [8,10,11]. Fig. 3C also shows that cells consume acetate during the early stages of phosphorus deprivation, when PSII inactivation occurs. After the establishment of anaerobiosis, however, acetate is produced and excreted into the growth medium, evidently, due to fermentation of starch. The establishment of anaerobiosis under phosphorus deprivation is an energy-dependent process that requires a carbon substrate for respiration. The main substrate for respiration during the initial 150 h of phosphorus deprivation is acetate, as seen in Fig. 3C. As the culture becomes anaerobic, acetate consumption stops and anaerobiosis in cultures is supported by respiration due to starch degradation. Clearly, more work is needed to accurately define the metabolic pathways and functional regulations involved in the H_2 photoproduction process under phosphorus-deprived conditions: the role of PSII and plastoquinone pool are unclear at this point, the synthesis and activity of the RuBisCo enzyme should be clarified, other fermentation products should be measured. Nevertheless, the response of algal cells to phosphorus deprivation demonstrates significant similarities with the response of algae to sulfur depletion: cultures passed through the same physiological stages, including accumulation of starch, inactivation of PSII, establishment of anaerobiosis in cultures, H_2 photoproduction and fermentation.

4.   Conclusions

Sustained H_2 photoproduction by microalgae requires the maintenance of culture anaerobiosis in the light for prolonged periods of time. We demonstrate in the present work that this condition can be accomplished by incubating algae in the phosphorus-depleted medium. Similar to sulfur deprivation, phosphorus deprivation limits O_2-evolving activity in algal cells and causes other metabolic changes that are favorable for H_2 photoproduction. This approach can further be optimized for better H_2 photoproduction rates and used for generation of H_2 gas not only in fresh water but also in marine strains, where sulfur deprivation is impossible due to a high concentration of sulfates in seawater.

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