S37-009

Sustained Hydrogen Photoproduction by *Chlamydomonas reinhardtii* – Effects of Sulfur Re-Addition

S. Kosourov[1], A. Tsygankov[1,2], M.L. Ghirardi[1] and M. Seibert[1]

[1]*National Renewable Energy Laboratory, 1617 Cole Blvd., Golden CO 80401, U.S.*
Fax: 1-303-384-6312; e-mail: mike_seibert@nrel.gov
[2]*Institute of Basic Biological Problems, Russian Academy of Sciences, Pushchino, Moscow Region, 142290, Russia*

Keywords: Chlamydomonas reinhardtii, hydrogen production, photobioreactor, sulfur-deprivation, oxygen sensitivity

Introduction

Photosynthetic production of H_2 from water is a biological process that can convert sunlight into useful, stored chemical energy. The enzyme mediating H_2 production in green algae is the reversible (or bidirectional) hydrogenase. Hydrogenase activity appears after several hours of anaerobic induction in the dark (Gaffron and Rubin, 1942; Roessler and Lien, 1984; Happe *et al*., 1994). Unfortunately, the hydrogenase is highly sensitive to O_2, a by-product of photosynthesis that irreversibly inactivates the enzyme's activity within minutes (Ghirardi *et al*., 1997). As a consequence, photoproduction of H_2 in algal cultures is difficult to sustain.

In attempting to achieve prolonged H_2 production in green algae under these conditions, we developed an approach based on the partial inactivation of photosynthetic O_2 evolution. This can be accomplished physiologically by depleting the cultures of sulfur in the form of sulfate (Melis *et al*., 2000). In the absence of sulfur, *Chlamydomonas reinhardtii* cells lose PSII activity reversibly. After about 20 – 24 hours of sulfur deprivation, O_2 evolved by PSII decreases to the level of O_2 uptake by dark respiration. After this point, the algae respire all dissolved O_2, rapidly making the photobioreactor environment anaerobic. Once the system becomes sufficiently anaerobic, the cells induce the reversible hydrogenase and produce H_2 for up to 4 days (Melis *et al*., 2000; Ghirardi et al., 2000). Subsequently, if sulfate is re-added to the cultures at an initial concentration of 1 mM, additional cycles of cell growth and H_2 production can be observed (Ghirardi et al., 2000).

We have investigated the effects of re-addition of micromolar concentrations of sulfur to sulfur-depleted medium on H_2 photoproduction by *C. reinhardtii* cells. To do this, we built an automated photobioreactor system that can simultaneously and continuously monitor H_2 production and several biophysical and electrochemical parameters.

Materials and Methods

Cell growth

C. reinhardtii, strain cc124, was grown photoheterotrophically on Tris-acetate-phosphate (TAP) medium, pH = 7.2, under continuous cool white fluorescent illumination (~ 200 µE • m^{-2} • s^{-1} PAR) and bubbled with 3% CO_2 in air. Cells were

harvested by centrifugation at 2000 g for 5 min, washed 5 times in TAP-minus-sulfur medium, and resuspended in the same medium to a final concentration of about 9 – 12 μg Chl • ml^{-1} (equivalent to 4-5 • 10^6 cells • ml^{-1}).

Bioreactor system

Sulfur-deprived cell suspensions were placed in glass photobioreactors (4-cm optical path, 1.2 L culture volume). The algal cells were cultured under continuous two-sided cool white fluorescent illumination (~300 μE • m^{-2} • s^{-1} PAR) at 28 ± 1.5ºC for up to 140 h. Four biophysical and electrochemical parameters were monitored simultaneously: dissolved O_2 (pO_2), redox potential (eH), pH, and temperature. The quantity of gas produced by the algal cultures was measured by the displacement of water. The specific rates of H_2 production were calculated at the beginning of the H_2-production phase (during the first 10–15 h) and expressed on the basis of chlorophyll content in the cultures measured at that time.

Results and Discussion

Behavior of green algae after removal of sulfur from the culture medium

C. reinhardtii cultures undergo dramatic changes in dissolved oxygen (pO_2), pH, and redox potential (eH) during adaptation to sulfur-deprived conditions. As seen in Figure 1, sulfur-deprived algal cultures transition first from an aerobic to an anaerobic environment and then start to produce H_2. The aerobic phase (I) is characterized by photosynthetic O_2 evolution and a positive redox potential. During this period, the pH of the algal suspension increases, due to photosynthetic consumption of dissolved CO_2 and utilization of acetate. After about 21-24 h of sulfur deprivation, the pO_2 falls rapidly (II) and the cultures become anaerobic within several hours. When the O_2 concentration in the medium reaches zero, the redox potential starts to decrease rapidly, going from approximately +400 mV to between -100 to -300 mV (determined vs. the H_2 electrode). The anaerobic phase (III) is followed by the H_2-production phase (IV). During the H_2-production phase, the redox potential in the growth medium reaches a maximum negative value, and the pH starts to decrease due to CO_2 evolution resulting from respiratory oxidation of stored cellular material (Melis *et al*., 2000; Ghirardi et al., 2000). When the H_2-production phase terminates (V), the starved *Chlamydomonas* cells still exhibit the residual O_2-evolving and respiratory activities. Adaptation to sulfur deprivation is also accompanied by changes in levels of intracellular and extracellular protein, as well as on the amount of starch (Figure 1B). Sulfur deprivation is known to induce the degradation of intracellular proteins and extrusion of arylsulfatases (Wykoff et al. 1998; Leustek et al. 2000), proteins that are responsible for scavenging organic sulfate from the environment. We show that this treatment also results in the mobilization of stored starch.

Effects of re-addition of micromolar concentrations of sulfur on H_2 photoproduction

Additions of small quantities of sulfate (12.5 – 50 μM $MgSO_4$ final concentrations) to rigorously sulfur-depleted cell suspensions resulted in larger initial increases in the culture density, measured on the basis of both cellular chlorophyll and protein contents (not shown), compared to control cultures without added sulfate. Significantly, these increases during the aerobic phase are accompanied by only slight increases in the cell number. This means that re-addition of sulfate to the medium affects culture density mainly through acceleration of cell growth, but not cell division.

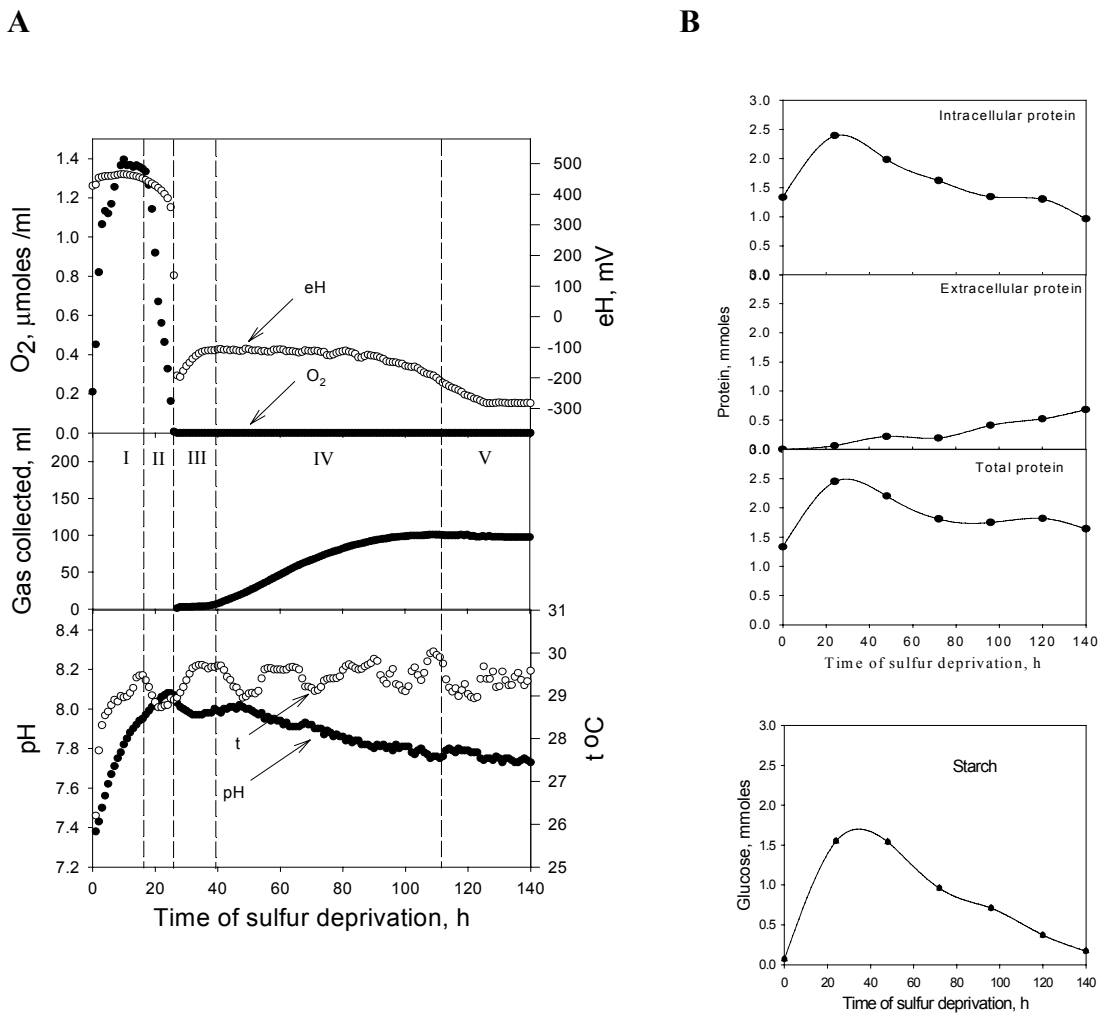

Figure 1. (A) Change in culture parameters measured upon transfer of *Chlamydomonas reinhardtii* cells to sulfur-deprived medium. (B) Changes in the levels of protein (intracellular, extracellular and total) and starch (measured as glucose) during sulfur deprivation.

The effect of re-added sulfur on H_2 photoproduction is shown in Table I. The maximum H_2 yield observed was 6.30 mmoles (191 ml) in cultures supplemented with 50 µM sulfate, compared to 2.84 mmoles (86 ml) in control cultures that lacked re-added sulfate. The maximum increase in initial specific activity was detected upon addition of 12.5 µM sulfate (from 5.74 to 6.40 µmoles • mg Chl^{-1} • h^{-1} or 174 to 194 µl• mg Chl^{-1} • h^{-1}). The decrease in the initial specific rate of H_2 production seen upon addition of higher levels of sulfate is most likely the result of increased light limitations, since high sulfate levels lead to a significant increase in the optical density of the cultures. Finally, addition of sulfate at 100 µM results in a significant delay in the start of the H_2-production phase (to > 144 h) and in a decrease in the H_2 output to 1.42 mmoles or 43 ml.

In order to examine the increase in specific activity obtained upon addition of 12.5 µM sulfate, we measured hydrogenase activity in algal cultures as a function of added sulfate. We found that sulfur addition increases the enzyme activity only when re-added at concentrations of 50 µM or higher (not shown), and thus cannot explain

Table I. Kinetic parameters measured upon re-addition of sulfur. Values are means ± standard deviations of three to five independent experiments.

Sulfur Concentration, µM	**Beginning of the anaerobic phase, h**	**Beginning of the H_2-production phase, h**	**Specific initial rate of H_2 production*, µmoles • mg Chl^{-1} • h^{-1}**	**Total yield of H_2 after 140 h*, mmoles (per 1.2 l of culture)**
0	31 - 40	41 - 49	5.74 ± 0.30	2.84 ± 0.63
12.5	30 - 37	39 - 47	6.40 ± 0.36	4.19 ± 0.46
25	35 - 37	44 - 47	5.31 ± 0.10	5.02 ± 0.36
50	32 - 38	43 - 49	3.99 ± 0.26	6.30 ± 0.89
100	-	144**	-	1.42**

*33 µmoles H_2 = 1 ml H_2 under our experimental conditions and altitude.
** One experiment.

the observed increase in specific activity with 12.5 µM sulfate. However, we also observed that re-addition of even low concentrations of sulfate enhances both the residual O_2-evolving capacity of PS II and the respiratory capacity of the cells (not shown). This effect could be responsible for the increase in specific activity seen upon addition of 12.5 µM sulfate to the cells. An enhanced water-splitting process can both supply electrons directly to the hydrogenase for H_2 production and supply the O_2 needed to maintain the higher levels of respiration.

In conclusion, optimization of H_2 production can be achieved by carefully controlling the amount of sulfur in the medium at the time of sulfur deprivation. On the one hand, the presence of micromolar concentrations of inorganic sulfur stimulates the residual activity of PSII upon which most of the electrons for H_2 production depends. But on the other, the addition of too much sulfur (above 50 µM) results in the over-expression of this activity, which in turn delays the onset of H_2 production and lowers the final yield of H_2 produced (Table I).

References

Leustek T., Martin MN, Bick, J-A, Davies JP. 2000. *Ann. Rev. Plant Physiol. Plant Mol. Biol*. **51**: 141-165.

Gaffron H, Rubin J. 1942. *J. Gen. Physiol.* **26**: 219-240.

Ghirardi ML, Togasaki RK, Seibert M. 1997. *Appl. Biochem. Biotech.* **63**: 141-151.

Ghirardi ML, Zhang L, Lee JW, Flynn T, Seibert M, Greenbaum E, Melis A. 2000. *Trends Biotechnol.* **18**: 506-511.

Happe T, Mosler B, Naber JD. 1994. *Eur. J. Biochem.* **222**: 769-774.

Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M. 2000. *Plant Physiol.* **122**: 127-136.

Roessler PG, Lien S. 1984. *Plant Physiol.* **76**: 1086-1089.

Wykoff DD, Davies JP, Melis A, Grossman AR. 1998. *Plant Physiol.* **177**: 129-139.