CYCLIC PHOTOBIOLOGICAL ALGAL H_2-PRODUCTION

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Abstract

We have achieved continuous photoproduction of large volumes of H_2 by down-regulating O_2 evolution activity in algal cells (Ghirardi, *et al.* 2000a). Trends in Biotechnol. 18: 506-511). This was accomplished by temporarily depleting the cells of sulfur (Melis, *et al.* 2000). Investigations are underway to simplify the system, accelerate the transition to the H_2-production mode upon sulfur depletion, and to determine the metabolic pathways involved in the process.

Current year results include the observations that: (i) the rates of H_2 production are not limited by the level of enzyme activity nor by the residual *capacity* of the algal cells to extract reductants from H_2O but are directly related to the steady-state rate of photosynthetic electron transport, (ii) synchronization of the cultures by light/dark phases results in a higher total output of H_2 but lower specific activity; and (iii) rigorous sulfur depletion and controlled sulfur re-addition increase the total amount of H_2 produced, increase the specific rate of H_2 production, and shorten the transition from the aerobic to the anaerobic, H_2-production phase.

We conclude that the used of light/dark growth cycles, as required for cultivation of algal cells under outdoor conditions does not have any adverse effects on subsequent H_2 photoproduction (under continuous illumination) when the cultures are re-supplemented with low concentrations of sulfate. In fact, some results indicate increased yields of H_2 gas upon re-addition of sulfate.

Introduction

Photosynthetic production of H_2 from water is a biological process that can convert sunlight into useful, stored chemical energy. The basic phenomenon has been known for 60 years (Gaffron 1940; Gaffron and Rubin 1942), but little progress has been reported on the biotechnology of the process. Hydrogen production is a property of many phototrophic organisms (Weaver *et al*. 1980; Appel and Schulz 1998; Asada and Miyake 1999; Boichenko *et al*. 2001), and the list of H_2 producers includes several hundred species from different genera of both prokaryotes and eukaryotes (Boichenko and Hoffmann 1994). The enzyme mediating H_2 production in green algae is the reversible (or bidirectional) hydrogenase that catalyzes the following ferredoxin (Fd)-linked reaction in the absence of ATP input (Boichenko and Hoffman, 1994):

$$2\,H^+ + 2\,e^- \xleftrightarrow{\text{hydrogenase}} H_2$$

Hydrogenase activity appears after several hours of anaerobic induction in the dark (Gaffron and Rubin 1942; Roessler and Lien 1984; Happe *et al*. 1994), and H_2-photoproduction depends on low potential electrons supplied to ferredoxin by the photosynthetic electron-transport chain (Appel and Schulz 1998; Wünschiers *et al*. 2001). Unfortunately, the reversible hydrogenase in green algae is highly sensitive to O_2, which irreversibly inactivates the enzyme's activity within minutes (Ghirardi *et al*. 1997). As a consequence, the direct photoproduction of H_2 from water in algal cultures is difficult to sustain. The sensitivity of hydrogenase to O_2 generated by normal photosynthesis has until now precluded consideration of green algae for possible use in applied H_2-producing systems.

Both chemical and mechanical methods have been developed to remove O_2 produced by the photosynthetic activity of the algal cells. These have included the addition of O_2 scavengers (Healey 1970; Randt and Senger 1985), the use of added reductants (Randt and Senger 1985), and the purging the cultures with inert gases (Greenbaum 1982; Gfeller and Gibbs 1984). However, all these methods are expensive upon scale-up and realistically may not be applicable to applied systems. To keep the cost of H_2 production low, applied systems will have to operate under ambient outdoor conditions (Borodin *et al*. 2000). In attempting to achieve continuous H_2 production in green algae under these conditions, we subjected the cultures to sulfur (in the form of sulfate) depleted conditions (Melis *et al*. 2000). In the absence of sulfur but in the presence of light, *C. reinhardtii* cells lose PSII activity reversibly. After about 20 – 24 hours, the rate of O_2 evolution by PSII decreases to the rate of O_2 uptake by respiration. After this point, the algae respire all remaining dissolved O_2, rapidly making their environment anaerobic. Under these conditions, the cells induce the reversible hydrogenase and produce H_2 for up to 4 days (Melis *et al*. 2000; Ghirardi *et al*. 2000a). Subsequently, if sulfate is re-added to the spent cultures at high concentration, addition cycles of cell growth and H_2 production can be observed (Ghirardi *et al*. 2000a).

The rates of H_2 production by sulfur-depleted algal cells are still too low at this time to warrant the development of commercial systems. To address this issue, we have investigated biochemical factors that might limit the rates. We have also studied the effects of

synchronization of cell division and re-addition of micromolar concentrations of sulfur to sulfur-depleted medium on H_2 photoproduction by *C. reinhardtii* cells.

Materials and Methods

Cell growth

Chlamydomonas reinhardtii, strain cc124, was grown photoheterotrophically on Tris-acetate-phosphate (TAP) medium, pH = 7.2, in flat glass bottles with stirring at about 25°C. During growth, the cultures were continuously illuminated with cool-white fluorescent light (~ 200 $\mu E \cdot m^{-2} \cdot s^{-1}$) and bubbled with 3% CO_2 in air. The gas mixture was sterilized using membrane filters with a 0.2 μm pore size (Acro 37 TF, Gelman Sciences, Inc., Ann Arbor, MI). Algal cells were grown to the mid-logarithmic phase ($2 – 5 \times 10^6$ cells • ml^{-1}), harvested by centrifugation at 2000 g for 5 min, washed five times in TAP-minus-sulfur medium, and resuspended in the same medium to a final concentration of about 9 – 12 μg Chl•ml^{-1} ($4\text{-}5 \times 10^6$ cells•ml^{-1}).

The synchronous division of *C. reinhardtii* cells was achieved by alternating light and dark (14-h light: 10-h dark) periods and maintained by daily dilution of the cultures to a starting density of about $0.5 – 1 \times 10^6$ cells•ml^{-1}. In this study, the synchronized cultures were harvested 4 h after the beginning of light cycle, washed and re-suspended in TAP-minus-sulfur medium as described above. This particular time in the cell cycle was chosen based on preliminary experiments indicating higher rates of H_2 evolution following sulfur deprivation at this point.

Bioreactor system

Both synchronized and unsynchronized sulfur-deprived cell suspensions were placed in each of four specially fabricated, glass photobioreactors (4-cm optical path, 1.2 L culture volume; Ghirardi et al. 2000b). The algal cells were cultured under continuous two-sided illumination of ~300 $\mu E \cdot m^{-2} \cdot s^{-1}$ (twelve 40-W cool-white fluorescent lamps) at 28 ± 1.5ºC for up to 140 h. Four biophysical and electrochemical parameters in addition to the volume of H_2 gas produced were monitored simultaneously and independently in each of the four photobioreactors: dissolved O_2 (pO_2), redox (eH), pH, and temperature. The quantity of gas produced by algal cultures was measured by the displacement of water from a gas-to-liquid conversion vessel to a liquid-accumulating bottle. The latter was weighed to determine the amount of gas produced. Finally, an integrated microprocessor system, consisting of two computers, was used for data accumulation, storage and processing. Before each experiment, the photobioreactors (with the electrodes in place) were washed several times with deionized double-distilled water and sterilized in an autoclave. 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 measured in the cultures at that time.

Biochemical assays

Hydrogenase activity was assayed amperometrically by measuring the initial rate of light-induced H_2 production (Ghirardi *et al.* 1997), or chemically using reduced methyl viologen as the electron donor. Sealed 13.5 ml glass vials, containing 1 ml of 10 mM oxidized methyl viologen

in 50 mM potassium-phosphate buffer (pH = 6.9) and 0.2% w/v Triton X 100, were flushed for 20 min with ultra pure Ar gas. Then, 100 μL of anaerobic 100 mM Na dithionite solution was added to the vials to reduce the methyl viologen. The reaction was performed at 37°C in the dark and started by the injection of 1 ml of anaerobic cell suspension taken directly from the bioreactor. The rate of H_2 production was measured with a Hewlett Packard gas chromatograph (Model 1050, Hewlett-Packard, Palo Alto, CA) and expressed on the basis of the sample chlorophyll content.

Photosynthetic O_2 evolution and dark respiration were measured at 25°C with a Clark-type O_2 electrode as described previously (Melis *et al.* 2000). The chlorophyll *a* + *b* content was assayed spectrophotometrically in 95 % ethanol extracts by the method of Spreitzer (Harris 1989). The protein concentration in the cells on a ml of culture basis was determined according to the method of Lowry et al. (1951). Light intensities on the surfaces of growth bottles and photobioreactors were measured with a Li-Cor quantum photometer (Model LI-250, Lincoln, NE).

Results

Biochemical factors limiting algal H_2 photoproduction during sulfur depletion

The production of H_2 by sulfur-depleted cells occurs at fairly high steady-state rates in the early stages of sulfur depletion (Ghirardi *et al.* 2000b), but halts after about 3-4 days. In order to study factors that might limit the rates of H_2 production, we measured the hydrogenase activity of the organism following incubation for different periods of time in sulfur-depleted medium and compared it to the rate of H_2 gas collection. Two assays were used to determine enzyme activity. The first used a Clark electrode to amperometrically measure the amount of H_2 released by the cells upon illumination (electrode). The second measured the dark enzymatic reduction of protons by dithionite-reduced methyl viologen. Trixon X-100 was added to facilitate the penetration of the reductant into the cells, and the amount of H_2 produced was measured by gas chromatography (GC). Figure 1 shows the changes in hydrogenase activity, measured by the two assays (closed and open circles, respectively) as a function of the incubation time in sulfur-depleted medium. For comparison, the open triangles show the rate of H_2 collection in the same units. The capacity for hydrogenase activity measured with either of the two assays is substantially higher than the rate of actual gas collection at every time point, suggesting that factors other than enzyme activity are involved in limiting the reaction.

Earlier work (Ghirardi *et al.* 2000b) suggested that at least 80% of the reductant used for H_2 production by sulfur-depleted cells originated from the oxidation of H_2O (with concomitant O_2 evolution) by Photosystem II (PSII). We then investigated the possibility that the rate of PSII-catalyzed O_2 evolution limited H_2 production. Figure 1 (closed inverted triangles) also shows that the residual capacity of the cells for photosynthetic O_2 evolution (a 4-electron reaction) is about twice as high as the rate of H_2 production (a 2-electron reaction) and decreases as a function of incubation time in sulfur-depleted medium. These data further confirm the close link between photosynthetic electron transport and H_2 production in sulfur-depleted cells. They also suggest that steady-state H_2 evolution is possibly limited by factors that lower the effective capacity of photosynthetic electron transport under steady-state conditions.

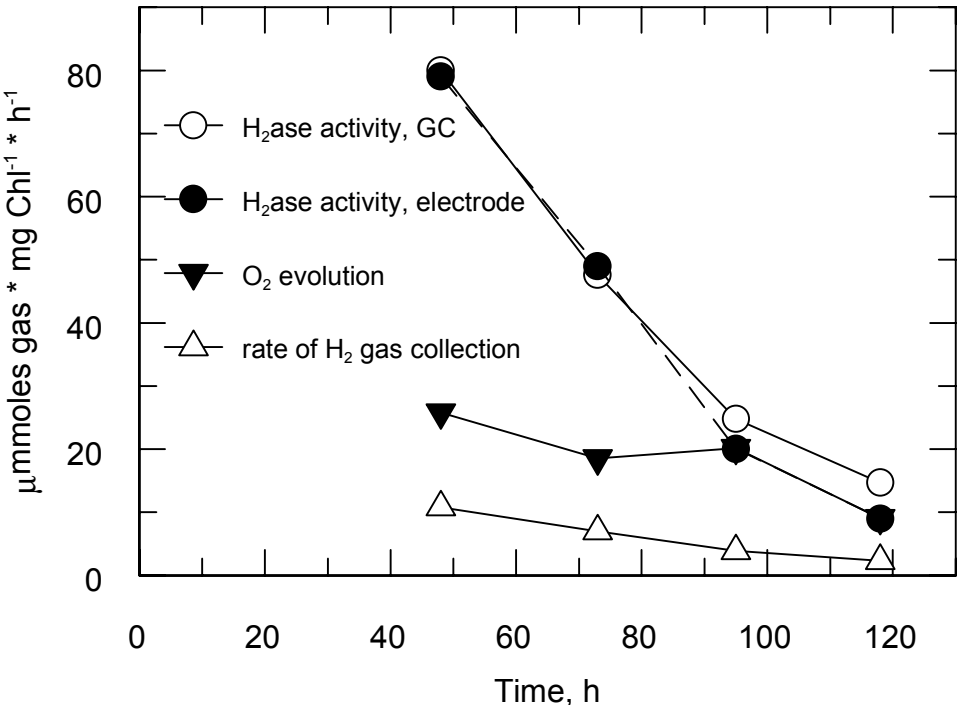

Figure 1. Measurements of hydrogenase activity, capacity for photosynthetic O_2 evolution and the rate of H_2 gas collection by algal cells as a function of incubation time under sulfur-depleted conditions.

Effects of synchronization of growth prior to removal of sulfur from the culture medium on H_2 photoproduction

The simultaneous and continuous monitoring of a number of biophysical and electrochemical parameters in *C. reinhardtii* cultures shows that the algae exhibit dramatic changes in dissolved oxygen (pO_2), pH and redox potential (eH) during adaptation to sulfur-deprived conditions (Kosourov *et al.* submitted). Light/dark synchronized, sulfur-deprived *Chlamydomonas* cultures transition first from an aerobic to an anaerobic phase and then start to produce H_2. Unsynchronized cultures exhibit similar behavior.

The kinetic parameters of the response to sulfur depletion by the two types of cultures, however, show significant differences, as seen in Table 1. Synchronized cells become anaerobic earlier and thus begin producing H_2 earlier. We also observed that the specific rate of H_2 production, calculated at the beginning of H_2 production phase was lower in synchronized cells compared to unsynchronized cells (3.27 ± 0.83 vs. 5.74 ± 0.30 µmoles H_2•mg Chl^{-1}•h^{-1}, respectively). On the other hand, the final yields of H_2 collected over the 140-h experiment were similar in both types of cultures (about 80–86 ml).

In summary, during adaptation to sulfur-depletion conditions, synchronized cells transition from one phase to the next earlier than unsynchronized cells, and thus start producing H_2 sooner, but with a lower specific rate.

Table 1. Kinetic Parameters for H_2 Photoproduction by Sulfur-Depleted Algal Cells

Sulfur concentration, μM	Start of the anaerobic phase, h	Start of H_2 photoproduction, h	Specific initial rate of H_2 photoproduction[1]	Total yield of H_2 at 140 h
Synchronized cultures				
0	25-27	39-43	3.27 ± 0.83[2]	80 ± 31
12.5	33-36	38-39	5.15 ± 0.26	219 ± 43
25	35-38	38-41	5.94 ± 0.56	241 ± 56
50	35-37	39-40	3.96 ± 0.30	109 ± 9
100	-	68[3]	-	56[3]
Unsynchronized cultures				
0	31-40	41-49	5.74 ±0.30	86 ± 19
12.5	30-37	39-47	6.40 ± 0.39	127 ± 14
25	35-37	44-47	5.31 ± 0.16	152 ± 11
50	32-38	43-49	3.99 ± 0.26	191 ± 27
100	-	144[3]	-	43[3]

[1]μmoles H_2•mg Chl^{-1}•h^{-1}
[2]Values are means ± standard deviations of three to five independent experiments
[3]One experiment

Effects of addition of micromolar concentrations of sulfur on H_2 photoproduction by sulfur-depleted cells

Preliminary experiments showed that the total yield of H_2 produced by sulfur-deprived algal cultures depends on the stringency of the sulfur-deprivation procedure. We suspected that small quantities of sulfur remaining in the medium after incomplete washing might still affect the metabolic pathways responsible for H_2 evolution. Therefore, we studied the effect of re-adding micromolar concentrations of sulfur to algal cultures that had been rigorously depleted of sulfur. In these experiments, *C. reinhardtii* cells were washed five times in TAP-minus-sulfur medium, resuspended in the same medium, and then placed into the photobioreactors.

Additions of small quantities of sulfate (12.5–50 μM $MgSO_4$ final concentrations) to sulfur-depleted cell suspensions resulted in an initial increase in the culture density, measured on the basis of both cellular chlorophyll and protein contents (not shown). These increases were accompanied by only slight initial increases in the cell number, suggesting that re-addition of sulfate to the growth medium affects culture density mainly through an acceleration of cell growth, but not of cell division. As a result of increases in culture density (to a limited point), we also observed increases in the total H_2 output in both synchronized and unsynchronized algal cultures (Table 1).

The maximum effect of sulfur re-addition on H_2 production was observed in synchronized cell suspensions supplemented with about 25 μM sulfate. The length of the H_2-production phase increases to above 100 h (the cells still produced H_2 by the end of the 140-h experiment), and the final yield of H_2 gas produced per liter of culture increased from about 80 ml to 241 ml (more than 3 times) compared to cells without added sulfate. Re-addition of sulfate at 100 μM results in a significant delay in the start of the H_2-photoproduction phase and in a decrease in the H_2 output in both synchronized and unsynchronized cells.

The specific rates of H_2 photoproduction calculated at the beginning of the H_2-production phase vary, as a function of added sulfate concentration. Re-addition of small quantities of sulfate (12.5 – 25 μM) to the sulfur-depleted cultures resulted in an increase in the initial specific rates of H_2 photoproduction in both synchronized and unsynchronized cell cultures. However, the relative effect of sulfur re-addition was also greater in synchronized cells, particularly in the concentration range below 25 μM. Further increases in the initial sulfate concentration have a clearly negative effect on the specific rates as mentioned before. The decrease in the specific rate of H_2 production at above 25 μM was shown to be most likely the result of increased light limitations (not shown), since the addition of inorganic sulfur at these levels leads to a significant increase in the optical density of the cultures.

Effects of re-addition of sulfur on residual water-oxidation capacity

Since we have shown above that the rate of H_2 photoproduction by sulfur-depleted cells is dependent on the capacity of the culture for photosynthetic electron transport, we hypothesized that the increase in the specific rates of H_2 production observed upon re-addition of sulfate could be due to a specific effect of sulfur on the residual activity of PSII. We measured O_2-evolution and respiratory capacity of the cells directly with a Clark-type O_2 electrode. Table 2 shows that re-addition of sulfate at the beginning of the experiment indeed increases the residual O_2 evolution capacity, but it also increases the respiratory activities of the cells measured at the start of the H_2-production phase. These results suggest that addition of controlled concentrations of sulfate to sulfur-depleted cells protects the residual PSII activity against inactivation.

Table II. Effect of sulfur addition on rates of photosynthetic and respiratory electron transport by sulfur-depleted synchronized algal cultures

Sulfur concentration, μM	Rate of Photosynthetic O_2 Evolution[1]	Rate Respiration[1]
0	1.5	19
12.5	12	24
25	15	31
50	20	34

[1] μmoles $O_2 \bullet$ mg $Chl^{-1} \bullet h^{-1}$

Discussion

The algal reversible hydrogenase is extremely sensitive to O_2, and H_2 production does not occur in this class of organism under normal photosynthetic conditions. Recently, we reported a new physiological approach that enables sustained H_2 photoproduction with the green alga, *C. reinhardtii*, based on the removal of inorganic sulfur from the culture medium (Melis *et al*. 2000; Ghirardi *et al*. 2000a). Under sulfur-deprived conditions, algae lose photosynthetic O_2-evolution activity with time, transition to an anaerobic phase as the result of respiratory activity, and then start to produce H_2 for up to 4 days.

We have investigated H_2 photoproduction by both synchronized and unsynchronized cultures of *C. reinhardtii* under sulfur-deprived conditions. Oxygen consumption and the establishment of anaerobiosis is critical for subsequent H_2 production, because a delay in the transition from an aerobic to an anaerobic phase also results in a delay in the onset of H_2 evolution (Table 1). The use of synchronized cell suspensions, which are induced by light/dark cycles simulating natural photoperiodic conditions outdoors, decreases the time until the start of H_2 production by about 4 hours compared to unsynchronized cultures, and thus promote earlier onset of H_2 photoproduction (Table 1). At the same time, in the absence of inorganic sulfur in the medium, both types of cultures demonstrate similar total amounts of H_2 output (Table 2) over the 140 hours of the experiment (but synchronized cells show lower initial specific activity). These findings demonstrate that, in principle, synchronized cell suspensions generated by light/dark cycles could be used in an outdoor H_2-production system.

Moreover, we also found that re-addition of micromolar quantities of inorganic sulfur as magnesium sulfate to the sulfur-deprived cell suspension significantly affects both the initial specific rate of H_2 evolution and the total output of H_2. These data raise the question as to why the re-addition of sulfate affects H_2 production in sulfur-deprived cultures. We checked the influence of re-added sulfur on both the residual O_2-evolving activity of PSII and the rate of respiration measured at the start of the H_2-photoproduction phase. Based on these data we conclude that sulfur does indeed affect H_2 production in this system by increasing the residual activity of PSII.

In conclusion, optimization of H_2 photoproduction in this system 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 depend. 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. Cell synchronization is also an important parameter. In this context, it is clear that additional experiments are necessary in order to determine if there are other physical and biochemical parameters that might be manipulated to increase the yield of H_2 produced.

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