TWO-PHASE PHOTOBIOLOGICAL ALGAL H_2-PRODUCTION SYSTEM

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Abstract

Continuous production of large volumes of H_2 by algal cells has been achieved by depleting the cells of sulfur (Melis et al., 2000). The operation of this novel algal H_2-production system occurs in the light with acetate-supplemented medium. Investigations are under way to simplify the system and determine the metabolic pathways involved in the process.

Current year results include the observation that: (i) depletion of nutrients other than sulfur will also inactivate O_2 evolution but at slower rates, (ii) light and acetate are required for rapid inactivation of O_2 evolution, suggesting an energy-dependent process, and (iii) inhibitors of Photosystem II also inhibit H_2 evolution, indicating that residual water-oxidation activity is an important source of reductant for H_2 production.

An automated photobioreactor experimental system was also developed at NREL. We report the design being used and the parameters found important to monitor sulfur deprivation. The automated system will be utilized in the future to measure the effect of a variety of parameters on the H_2-evolution activity of sulfur-depleted cells.

Introduction

Microbial H_2 photoevolution is catalyzed either by nitrogenases or hydrogenases, enzymes that can only function under anaerobic conditions due to their extreme sensitivity to O_2. Since O_2 is a by-product of photosynthesis, nitrogenase-containing organisms have developed the following spatial and temporal strategies to protect the enzyme from inactivation by O_2: (a) heterocyst-containing cyanobacteria physically

separate O_2 evolution from nitrogenase activity by segregating oxygenic photosynthetic activity in vegetative cells and nitrogenase activity in heterocystis with reduced O_2-permeability (Fay, 1992) and (b) non-heterocystous cyanobacteria separate O_2-evolution from nitrogenase activity by performing these functions during, respectively, light and dark periods (Bergman et al., 1997).

Similar strategies cannot be found in phototrophic hydrogenase-containing organisms in nature. In order to sustain H_2 production by green algae in the light, researchers have used a variety of methods to keep the cultures free of O_2. These include addition of O_2 scavengers such as chromous chloride (Healey, 1970) or dithionite (Randt and Senger, 1985), or purging the cultures with inert gases such as nitrogen (Gfeller and Gibbs, 1984) or helium (Greenbaum et al., 1999). Benemann (1996) has advocated the use of the principle of temporal separation of H_2 and O_2 evolution in green algae ("indirect biophotolysis"), triggered by an unspecified reversible inactivation of Photosystem II (PSII) O_2-evolution activity. In his proposed model, H_2 photoevolution in the absence of PSII would require the break-down of starch to provide reductants for Photosystem I (PSI) through the chlororespiratory pathway. These reductants would then be used by ferredoxin to reduce protons to H_2, in a reaction catalyzed by the hydrogenase enzyme. Based on his proposal, we identifed and used sulfur depletion to reversibly inactivate PSII (Melis et al., 2000), achieving apparent temporal separation of O_2 and H_2 evolution in the green alga *Chlamydomonas reinhardtii*. In the absence of sulfur and in the presence of acetate, the cells shut off most (but not all) of their O_2-evolving activity, respire all measurable remaining O_2 in a closed environment, and induce the expression of the hydrogenase enzyme. At this point, they can evolve H_2 in the light for up to 4 days. Subsequently, if sulfur is added back to the cultures, they will recover PSII activity and return to a normal growth mode. Cycles of O_2 and H_2 production can be repeated at least 3 times without significant loss of activity. Our results also showed that protein degradation, rather than starch breakdown, correlated with H_2 production by the algal cells. The rates of H_2 evolution by our system were much lower than its potential for electron transport (Melis et al., 2000), suggesting that the system is being limited by some factor other than enzyme activity. Possible limitations include: (a) the rate of substrate degradation, (b) redox control of the rate of electron transport by reducing conditions, (c) limited supply of electrons from residual water oxidation, and (d) competition between the hydrogenase and other physiological pathways.

Currently, both light and acetate are present during the H_2-production phase. However, the development of a commercial system for algal H_2 production using sulfur-depleted cells will require the elimination of superfluous nutrients and/or procedures to bring down the cost. Light is required for H_2 evolution. Acetate, however, is not consumed during the time in which H_2 is actually produced, and is thus not necessary for that step (Melis et al., 2000). In the current report, we have examined the requirement for light and acetate during the O_2-inactivation phase, before H_2 evolution commences. We also examined the effect of inhibiting the residual water oxidation activity (using DCMU) on the H_2-evolution activity of the sulfur-depleted cells. Finally, we present initial results on a new automated photobioreactor system that can continuously monitor 5 key parameters in algal cultures during sulfur-depletion treatment.

Materials and Methods

Cell Growth and Sulfur Depletion

Wild-type *C. reinhardtii* $C137^{+}$ cells were initially grown photoheterotrophically in Tris-acetate-phosphate (TAP) medium, pH 7, and bubbled with 3% CO_2 in air at about 25° C. The photobioreactors consisted of flat bottles with stirring capability and placed under continuous cool-white fluorescent illumination at about 100-200 $\mu E \bullet m^{-2} \bullet s^{-1}$. The cultures were grown to late log phase, harvested by centrifugation, washed three times in TAP minus sulfur medium, and resuspended in 1.2 l of the same medium to a concentration of 11-18 μg Chl /ml. The sulfur-depleted cell suspension was placed back in the light for up to 150 h.

Oxygen and Hydrogen Evolution Measurements

Oxygen- and hydrogen-evolution activities of the cultures were measured as previously described (Melis et al., 2000) with two different Clark-type electrodes, each poised for the optimal measurement of each gas.

Gas Collection Measurements

The reactor bottles were fitted with a #25 Ace thread and with smaller side-ports for liquid sampling. A threaded glass stopper with capillaries for gas sampling was fitted with a Viton O-ring and used to seal the reactor. Threaded side-arm and gas sampling ports were sealed with rubber-laminated Teflon septa. Figure 1 shows how teflon tubing (HPLC, Aminco), attached to one of the gas ports, was used to conduct gas evolved by the algae in the culture bottles to an upside-down graduated cylinder filled with H_2O. The gas collection tubing was detached from the culture bottle during liquid and gas sampling to avoid disturbance of gas volume readings in the graduated cylinder.

Results

Effect of depletion of nutrients other than sulfur on inactivation of O_2 evolution

It is known that, besides sulfur, depletion of nutrients such as phosphorus (Wykoff et al., 1998) or nitrogen (Kumazawa and Mitsui, 1981) from the medium also inactivates the photosynthetic O_2-evolution activity of algae. However, inactivation by phosphorus depletion is slower than that by sulfur (Wykoff et al., 1998). We investigated the effect of depleting algal cultures of nutrients other than sulfur on the rate of inactivation of PSII activity. Figure 2 (left side) shows the half-life for inactivation of O_2 evolution from algal

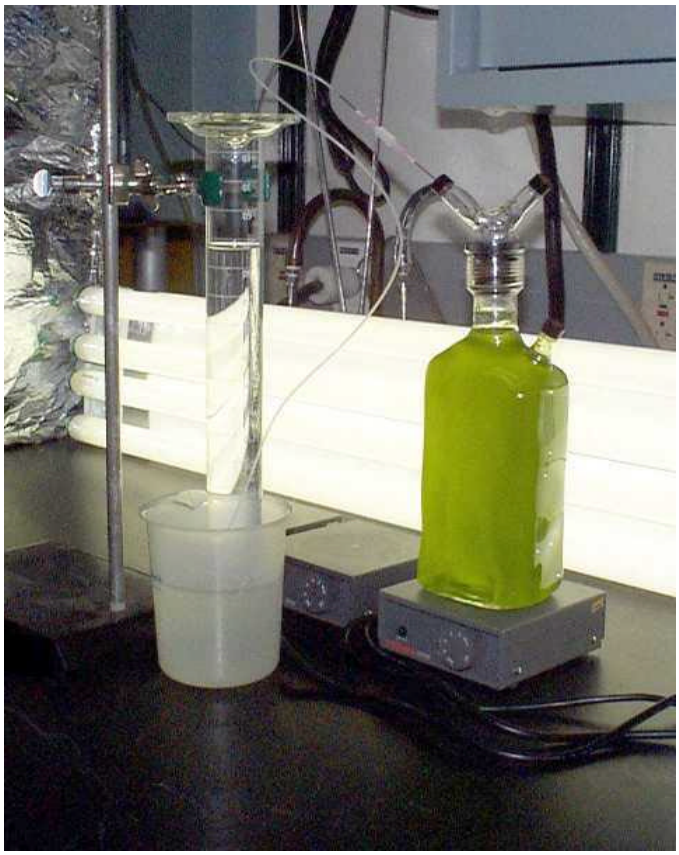


Figure 1. Photobioreactor for algal H_2-production and system for gas collection

cultures resuspended in media depleted of different nutrients. It is clear that PSII can be inactivated by removing either S, Fe, or Mn from the medium. However, the inactivation is 3 to 4 times faster when sulfur is removed. Figure 2 also shows the effect of combining sulfur depletion with Fe or Mn depletion. The combined depletions do not result in faster inactivation of O_2 evolution, and gave similar rates of subsequent H_2 production (not shown).

Effect of light and acetate on inactivation of O_2 evolution

We also investigated the need for both acetate and light during inactivation of O_2 evolution. The right side of Figure 2 shows that both acetate and light accelerate the inactivation of PSII. In the presence of acetate but in the dark (D+A), the cultures are inactivated two times slower than in the light; in the absence of acetate but in the light (L-A), inactivation takes four times as long. These results support the notion of an energy-dependent protease contributing to the inactivation of O_2 evolution. Energy-dependent proteases are involved in specific inactivation of various proteins in *E. coli* and other

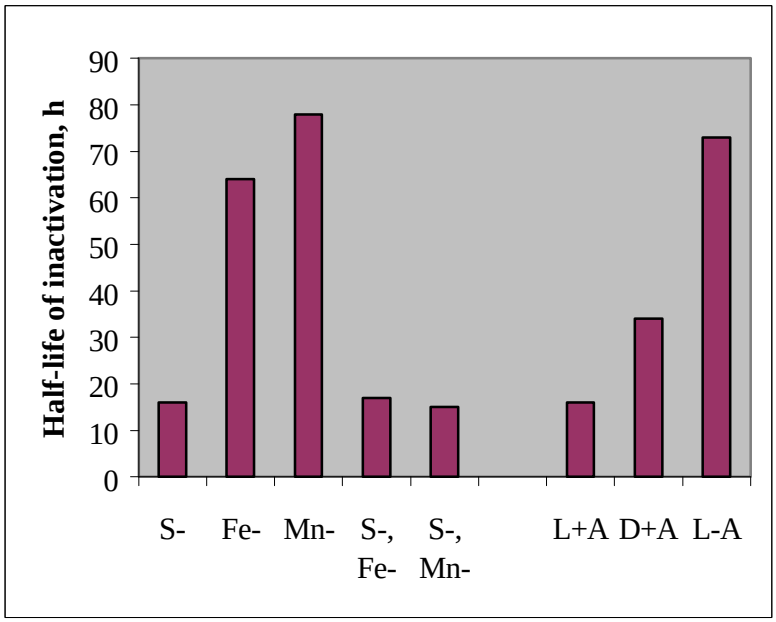


Figure 2. Effect of different nutrient and light parameters on the rate of inactivation of O_2 evolution.

organisms (Wilson et al., 2000; Wang et al., 1999; Hilliard et al., 1998; Laachouch et al., 1996).

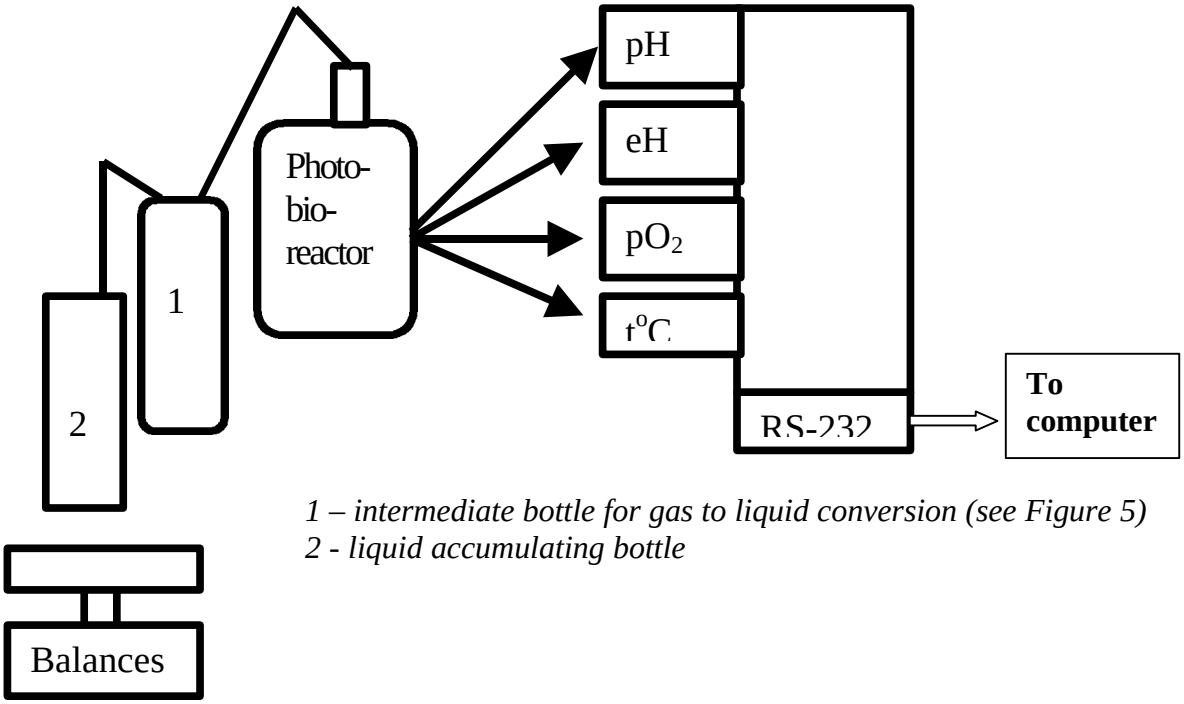
Source of reductant for H_2 evolution

Sulfur depletion for 24 h inhibits more than 90% of the O_2-evolution activity in algal cells (Melis et al., 2000). Our previous observation that protein consumption alone could potentially provide the reductant needed for the amount of H_2 evolved by sulfur-depleted algal cells seemed to indicate that at least some of the reductant for H_2 evolution originated from protein degradation. However, given that the subsequent rate of H_2 evolution is only 10% of the capacity of electron transport chain (based on the O_2 evolution rate measured at the beginning of the sulfur-deprivation experiment), it is also possible that the electrons used to reduce protons to H_2 come from residual PSII activity. In order to test this idea, we added DCMU, a specific inhibitor of PSII activity, to the algal cultures following onset of H_2 evolution. This treatment resulted in inhibition of about 80% of the rate of H_2 evolution, suggesting that most of the reductant required for H_2 production by the algal hydrogenase originated from residual water-oxidation activity.

Automated Photobioreactor Experimental System

We have developed an automated system that allows us to continuously monitor a series of physical and electrochemical parameters in our sulfur-depleted, algal H_2-production system, as shown in Figure 3. Preliminary experiments showed that pH, eH (redox

potential), pO_2 (dissolved O_2), temperature, and quantity of evolved gas are important parameters to record.



1 – intermediate bottle for gas to liquid conversion (see Figure 5)
2 - liquid accumulating bottle

Figure 3. Schematic of an automated system to monitor algal H_2-production

In order to test this design, photobioreactors with additional ports for each of the sensors were fabricated. Each photobioreactor has 3 ports for the sensors, one for culture sampling or chemical injection, and one for gas outlet, as shown in Figure 4.

The method adopted for gas-to-liquid volume conversion is described in Figure 5. It consists of an intermediate bottle (bottle 1 in Fig. 3) full of water that collects the gas evolved by the photobioreactor cultures. The collected gas displaces the liquid in the intermediate bottle, which in turn is syphoned to a second bottle (bottle 2 in Fig. 3). Bottle 2 is located on an electronic balance. The changes in the weight of bottle 2 are a measure of the rate of gas evolution by the algal cultures. We found that the inside diameter of the connecting tubes had to be no less than 0.5 mm, otherwise the time response of the system was excessively high. This type of gas-to-liquid conversion and measurement system is temperature and pressure sensitive but under properly controlled conditions gives an error of less than 3%.

Finally, an integrated microprocessor system that simultaneously monitors four separate algal culture vessels was assembled, according to the schematic shown in Figure 6.

Preliminary experiments have been conducted in order to confirm consistent changes in all the monitored parameters in all four vessels (not shown).

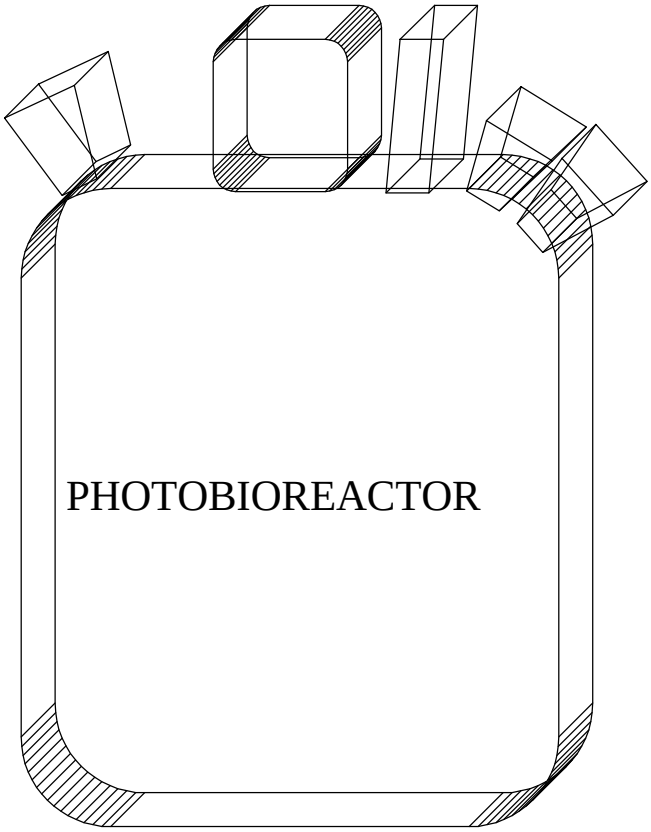


Figure 4. Design of a photobioreactor for the automated algal H_2-production system.

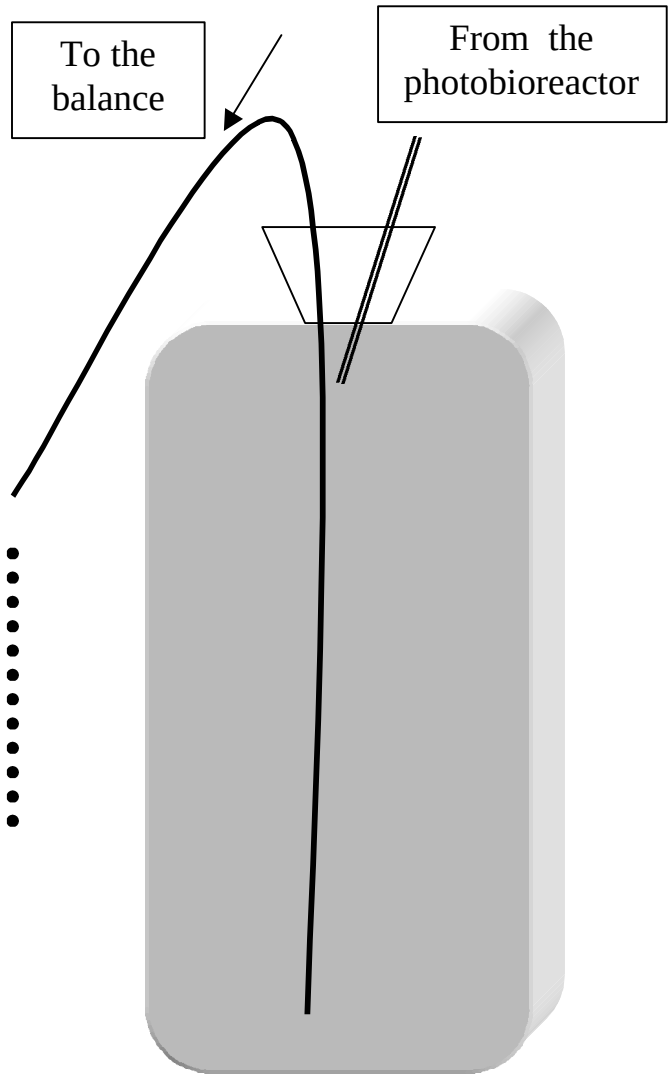


Figure 5. Vessel for gas-to-liquid conversion.

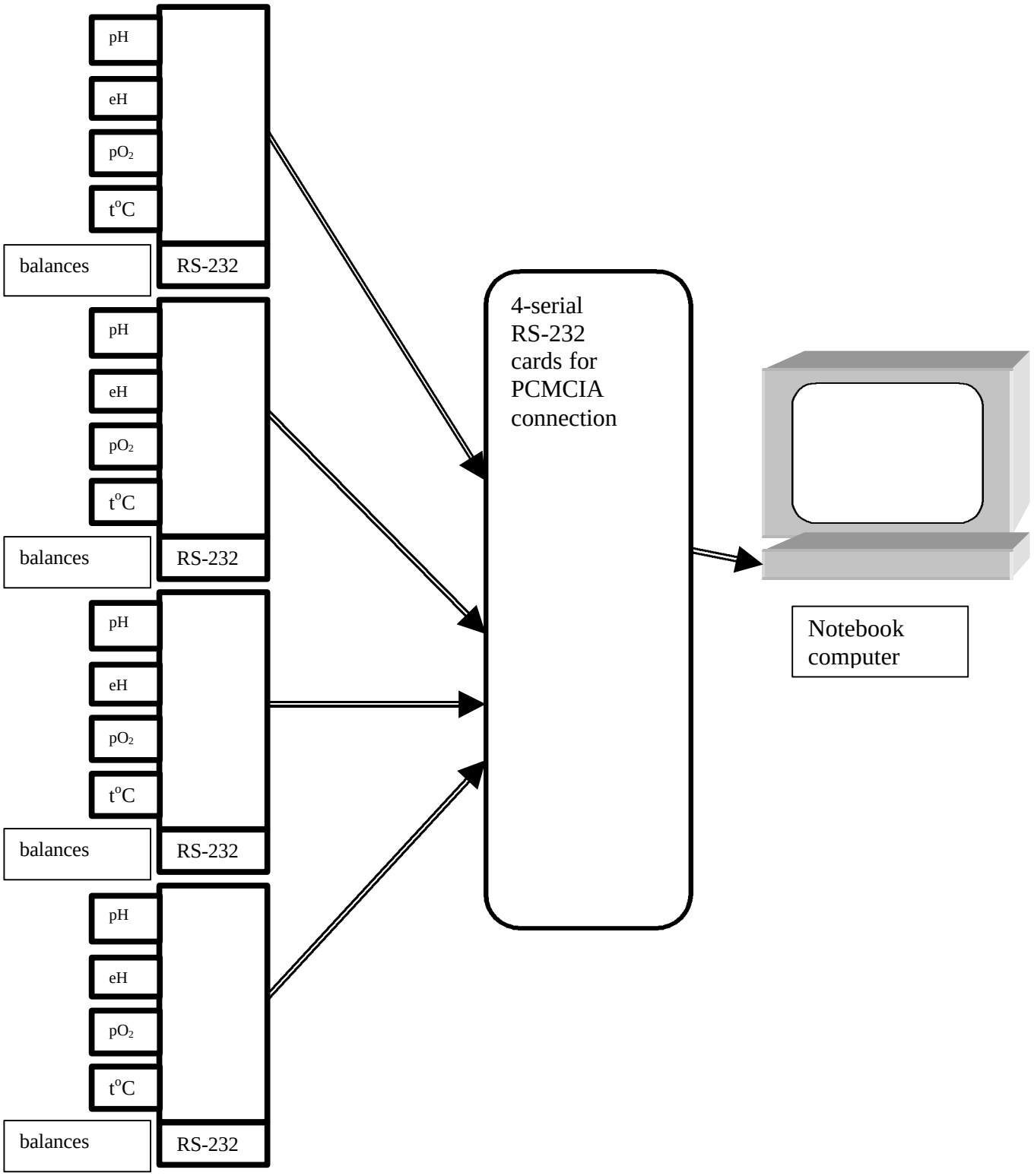


Figure 6. Schematic of an integrated system for simultaneously monitoring five parameters from four photobioreactors in an algal H_2-production system.

Summary and Conclusions

Since algal H_2 photoproduction from water was discovered almost 60 years ago (Gaffron and Rubin, 1942), H_2 could be collected only if O_2, produced by photosynthesis, was removed continuously by chemical or mechanical means. Earlier this year, we described a physiological method (sulfur depletion) to reversibly inactivate O_2-evolution activity in an algal culture; this inactivation leads to the induction of hydrogenase activity and subsequent production of large quantities of H_2 for 3-4 days (Melis et al., 2000). We now report that, while depletion of a number of other nutrients besides sulfur can also inactivate O2-evolution, their rate of inactivation is slower (Fig. 2). We also provide evidence that this inactivation must involve energy-dependent proteases (Fig. 2), since it occurs much more slowly in the dark and/or in the absence of acetate. Finally, we present preliminary evidence suggesting that residual water-oxidation activity may be the source of most of the reductant for the H_2 evolution process (Fig. 3). Clearly, much more detailed work will be required to clarify the metabolic pathways involved in the process, and the inter-relationship between H_2 evolution and protein degradation.

Even without a clear knowledge of all the pathways involved in the transport of reductant to hydrogenase in sulfur-depleted cells, it is evident that H_2 production can be sustained for up to 4 days (Melis et al., 2000). Moreover, the system can be recycled back and forth between photosynthetic growth and H_2 production (data not shown). This algal H_2-production system does not seem to be a pure "indirect biophotolysis" system, as proposed by Benemann (1996). Indeed, it is similar in concept to systems in which photosynthetically-produced O_2 is removed by addition of O_2 scavengers or by purging with neutral gases. The significant difference is that sulfur-depleted cells operate with only 10% of their normal oxygenic PSII activity, while previous systems (listed in Table I), presumably operate with 100% functional PSII. Nevertheless, this does not seem to make a large difference in terms of the actual rates of steady-state H_2 production, as shown in Table I. Indeed, sulfur-depleted cells produce H_2 at rates comparable to systems in which 100% of the PSII are operational, with the additional advantage that O_2 is removed by physiological means, not by the introduction of extraneous chemical reductants or inert gases.

Up until now, only nitrogenase-based systems were capable of sustained H_2 photoproduction without expensive O_2-removal systems (Benemann, 1996). Indeed, cyanobacteria, in the absence of fixed nitrogen sources can produce H_2 for months, particularly if their uptake hydrogenase activity is concomitantly inactivated (Markov et al., 1996). Table II shows a comparison of the rates of H_2 production by a variety of nitrogenase-containing cyanobacteria, and our sulfur-depleted algal system. Given the different pigment composition of cyanobacteria and green algae, we show the data on a per mg dry weight basis. The sulfur-depleted algal cells evolve H_2 at rates higher than most optimized cyanobacterial systems. This is not surprising, given that nitrogenases are known to be sluggish enzymes that, besides reductants, require ATP.

Table I. Comparison of Rates and Volumes of H_2 Collected from Different Algal Systems and Methods Used to Sustain High H_2 Evolution Rates

Organism	Initial rate (μmoles H_2 • mg Chl^{-1} • h^{-1})	Steady-state rate (μmoles H_2 • mg Chl^{-1} • h^{-1})	Total volume H_2 collected	Culture volume and Chl content
Chlamydomonas moewsuii (Healey, 1970)	-	5 [chromous chloride]*	-	-
Chlamydomonas reinhardtii F60 (Gfeller & Gibbs, 1984)	-	5.7 [N_2 purging]	0.25 ml in 3.5 h	0.3 mg Chl in 3 ml
Scenedesmus obliquus (Randt & Senger, 1985)	54	13 [Na dithionite]	-	-
Chlamydomonas reinhardtii (Greenbaum, personal communication)	50	≅ 10 [He purging]		
Chlamydomonas reinhardtii (this work)	90	≅ 10 [Sulfur depletion]	325 ml in 95 h	18 mg Chl in 1 liter

* Values in brackets indicate the means by which O_2 was removed from the cultures.

Table II. Comparison of the Rates of H_2 Evolution among Different Nitrogenase-Based Systems and our Sulfur-Depleted Green Algal System.

Organism	Rate of H_2 evolution (ml•mg dry $weight^{-1}$•d^{-1})	Reference
Anabaena cylindrica	0.09-0.03	Miyamoto et al., 1979
Oscillatoria sp. Miami BG7	0.14	Kumazawa and Mitsui, 1981
Anabaena variabilis (no uptake hydrogenase)	0.22	Markov et al., 1996
Sulfur-depleted *Chlamydomonas reinhardtii*	0.34	This work

In conclusion, we have shown that green algae can produce significant amounts of bulk H_2 gas at rates comparable to other oxygenic photosynthetic organisms, when their O_2-evolving capability is reduced by physiological means. Hydrogen production depends on the depletion of sulfur from the medium, is reversible, and results in the generation of pure H_2 (co-evolved CO_2 stays in solution). We are currently investigating in more detail the metabolic pathways involved in the evolution of H_2 under sulfur-depletion conditions.

Acknowledgements

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