0273-2289/97/63-65—0141$10.75

Oxygen Sensitivity of Algal H_2-Production

MARIA L. GHIRARDI,*,[1] ROBERT K. TOGASAKI,[2] AND MICHAEL SEIBERT[1]

[1]*National Renewable Energy Laboratory, Golden, CO 80401; and*
[2]*Department of Biology, Indiana University, Bloomington, IN 47405*

ABSTRACT

Photoproduction of H_2 by green algae utilizes electrons originating from the photosynthetic oxidation of water and does not require metabolic intermediates. However, algal hydrogenases are extremely sensitive to O_2, which limits their usefulness in future commercial H_2-production systems. We designed an experimental technique for the selection of O_2-tolerant, H_2-producing variants of *Chlamydomonas reinhardtii* based on the ability of wild-type cells to survive a short (20 min) exposure to metronidazole in the presence of controlled concentrations of O_2. The number of survivors depends on the metronidazole concentration, light intensity, preinduction of the hydrogenase, and the presence or absence of O_2. Finally, we demonstrate that some of the selected survivors in fact exhibit H_2-production capacity that is less sensitive to O_2 than the original wild-type population.

Index Entries: Hydrogen; green algae; *Chlamydomonas*; oxygen; metronidazole.

Abbreviations: ATP, adenosine triphosphate; cw15, cell wall-less strain of *Chlamydomonas reinhardtii*; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Fd, ferredoxin; I_{50}, inhibitor concentration that decreases the rate of an enzymatic reaction to 50% of the rate measured in the absence of the inhibitor; MNZ, metronidazole, [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole]; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of NADP; V_0, initial rate of an enzymatic reaction; WT, wild-type.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

There are three major classes of organisms that photoproduce H_2: photosynthetic bacteria, cyanobacteria, and green algae. Photosynthetic bacteria utilize reductants other than water to produce H_2, in a reaction catalyzed by the enzyme nitrogenase. Whereas this reaction is energy intensive, requiring at least 4 ATP/H_2 produced, it is also quite functional when waste reductant is available *(1)*. Cyanobacteria also produce H_2 via a nitrogenase enzyme system. In this case the source of reductant is H_2O, but the reaction involves the formation of a metabolic intermediate. As a result, the quantum yield of H_2 production is rather low, in the order of one H_2/9–10 quanta *(2)*. Green algae do not synthesize nitrogenase; rather, H_2 production in these organisms is catalyzed by the hydrogenase enzyme. Algal H_2 production does not require ATP input nor the generation of metabolic intermediates. Consequently, a higher theoretical quantum yield of one H_2/4 quanta is possible *(2)*. Recent work with mutants of *Chlamydomonas reinhardtii* lacking Photosystem I suggests that an even higher quantum yield of one H_2/2 quanta may be attainable with green algae *(3)*.

The desired characteristics of a photobiological H_2-production system are:

1. Use of water as the source of reductant,
2. Solar-driven,
3. High efficiency of solar energy conversion,
4. Durable and self-replicating,
5. H_2 production at high equilibrium pressure, and
6. Cost-competitiveness *(4)*.

By these criteria, the use of green algae appears to be a promising alternative. On the other hand, the current practical limitations of green algae in a photobiological H_2-evolving system include:

1. The sensitivity of its hydrogenase to O_2,
2. The occurrence of a dark back reaction between O_2 and H_2 (i.e., the oxyhydrogen reaction),
3. Competition between the CO_2-fixation and the H_2-production pathways for electrons from H_2O,
4. The low equilibrium pressure of H_2 release, and
5. Saturation of H_2 production capacity at low light intensity.

A biological approach to address the hydrogenase O_2-sensitivity issue in green algae was developed about 20 yr ago by McBride et al. *(5)*. The approach used a positive selection technique based on the reversible H_2 uptake reaction catalyzed by algal hydrogenases. Mutants were selected under this photoreductive pressure and increasing concentrations of O_2. These conditions require algal cells to utilize H_2 as a source of reducing

equivalents to fix CO_2 in the presence of DCMU, an inhibitor of electron flow from Photosystem II and hence, from water. Consequently, cells with hydrogenase sensitive to low oxygen concentration starve to death, whereas cells with an O_2-tolerant hydrogenase grow. Oxygen-tolerant mutants of *C. reinhardtii* obtained by this technique could produce H_2 in the presence of up to 8% O_2. Unfortunately, the mutants exhibited high levels of the oxyhydrogen back reaction *(5)* and were not maintained in culture for further study.

It is clear that novel approaches will be required to obtain algal hydrogenases that are stable in the presence of O_2. We have addressed this problem by developing and examining a new technique based on the application of selective pressure under H_2-producing, rather than H_2-utilizing conditions. One of the potential advantages of this approach may be in elimination of the high rates of oxyhydrogen reaction observed in the old experiments. In this paper the authors describe the new selection procedure and report on the results of preliminary experiments done to test its validity in selecting for O_2-tolerant, H_2-producing organisms. Results suggest that application of this type of selective pressure, combined with mutagenesis, has the potential for yielding H_2-producing algal mutants with increased tolerance to O_2. These organisms may prove useful in future commercial photobioreactors for the continuous production of H_2 under aerobic conditions.

MATERIALS AND METHODS

Cell Growth

Wild-type (WT) *C. reinhardtii* (137mt$^+$) was obtained from Prof. S. Dutcher at the University of Colorado, Boulder. A cell wall-less strain, cw15 (CC-400 mt$^+$), was acquired from Dr. E. Harris at the Chlamydomonas Genetics Center, Duke University. Wild-type cells were grown photoautotrophically in Sager's minimal medium *(6)*. The cw 15 strain required Sueoka's high salt medium, modified according to Vladimirova and Markelova *(7)*. Both cultures were grown in a chamber at 25°C under 8 W/m^2 fluorescent illumination and continuous bubbling with a mixture of 1.7% CO_2 and air. Cells were harvested by centrifugation at 1000*g* for 10 min. Wild-type and cw15 cells were also grown on plates containing 1.5 and 0.8% agar, respectively.

H_2-Production Selection Technique

Harvested cells were resuspended in a small volume of resuspension buffer (50 m*M* potassium phosphate buffer, pH 7.2, containing 3 m*M* $MgCl_2$) *(8)* at a final concentration of about 200 μg Chl · mL^{-1} *(9)*. Chlorophyll concentrations were determined by extracting the pigment using 95% ethanol and assaying spectrophotometrically *(6)*. In order to induce the hydrogenase enzyme, the cell suspension was made anaerobic by bubbling argon for 30 min. Maintenance of anaerobic condition was

insured by the addition of a glucose/catalase/glucose oxidase oxygen-scrubbing system *(10)*. This two-enzyme system reduces any available O_2 to water. *C. reinhardtii* cells cannot use glucose as a carbon source for growth *(6)*. The mixture was incubated at room temperature for 4 h in the dark *(11)* and transferred to 4°C for overnight storage.

For H_2-production selection, anaerobically-treated cells were added to a selective medium containing different concentrations of metronidazole and 1 m*M* sodium azide *(12)*. The azide inhibits endogenous catalase activity. All procedures were done under sterile conditions. The selection medium was also made anaerobic by argon bubbling before introduction of the cells. Oxygen was then added to the medium by syringe through a gas-tight septum to achieve final concentrations of O_2 in the gas phase (ranging 0–10%), as required. The final cell suspension was exposed to light of controlled intensity (Fiber-Lite High Intensity Illuminator, model 170-D, Dolan-Jenner Industries) for 20 min. The cells were pelleted out using an IEC clinical centrifuge, washed once with phosphate buffer, and then once with the resuspension buffer (5 m*M* potassium phosphate buffer containing 1 m*M* $CaCl_2$ and 1 m*M* Mg_2SO_4). Undiluted and sequential dilutions of each sample were plated on minimal medium and incubated in a growth chamber under low light levels. Survival rates were determined by counting the number of colonies detected on each plate following the treatment, and estimating the percentage of survivors with respect to the number of cells used in the MNZ treatment.

H_2-Evolution Measurements

Anaerobically-treated cells were added to a small volume of the assay buffer (50 m*M* MOPS, pH 6.8) *(9)* to a final concentration of about 15 μg Chl · mL^{-1}. The medium, in the assay chamber of a two-electrode apparatus (Clark-type, YSI 5331), was adjusted to different initial concentrations of O_2 before introduction of the cells. H_2 evolution was induced by illumination with saturating light from the same lamp described above except that a heat filter consisting of a 1% solution of $CuSO_4$ was used. The data were recorded on a strip-chart recorder and initial rates were calculated from the initial slopes of each curve. Initial O_2 concentrations were also determined from the recorded O_2 concentration measured at the time when the cells were introduced into the chamber. Gas concentrations were corrected for their decreased solubility in aqueous solution at Golden, CO, located 1580 m above sea level.

RESULTS AND DISCUSSION

The hydrogen-production selective pressure that we have employed in this work is based on the toxic effect of metronidazole (MNZ) on photosynthetic organisms. Metronidazole is a heterocyclic compound with a low redox potential (E_m = –325 mV at pH 6.9) *(13)* that is normally used to treat

infections caused by protozoa and anaerobic bacteria *(14)*. In these organisms, MNZ is a strong oxidizer of ferredoxin, in a reaction catalyzed by the reversible hydrogenase *(14)*. Its toxic effect is a result of one of its reduced intermediate states. The site of action of MNZ in photosynthetic organisms is restricted to chloroplasts, where it oxidizes light-reduced ferredoxin *(13)*. Schmidt et al. *(13)* have proposed that the subsequent reoxidation of reduced MNZ by molecular O_2 yields a superoxide radical (*15*), which then disproportionates into H_2O_2 *(16)*. Consequently, the toxicity of MNZ was attributed to the generation of H_2O_2. Long exposure (up to 24 h) to MNZ has been used to select for *Chlamydomonas* mutants defective in photosynthetic electron transport function *(13)*.

Ferredoxin (Fd) is a key electron carrier, located on the reducing side of photosystem I, and it provides the reducing equivalents to a variety of pathways. The most prevalent pathway, of course, is through NADP, which in turn is coupled to CO_2 fixation. Among the other Fd-dependent pathways is hydrogenase-catalyzed H_2 production. This pathway, of course, is inoperative during phototrophic growth because of inactivation of the enzyme by O_2 produced during the water-splitting process. It is reasoned that, since MNZ toxicity depends on the accumulation of reduced Fd, an organism with an active hydrogenase will be less sensitive to MNZ toxicity in the absence of CO_2, because an alternative pathway for electrons from Fd is available.

Based on the aforementioned information, a treatment to select for potential O_2-tolerant, H_2-producing organisms was designed. Cells were first incubated in the presence of MNZ at different concentrations of O_2 for a short period of time in the light and then grown on minimal agar medium to determine survival rates. The authors examined the effect of the following treatment parameters on the rate of survival: MNZ concentration, light intensity (which limits the rate of accumulation of reduced Fd), preinduction of the hydrogenase enzyme, and O_2 concentration in the selection medium. The authors tested both WT and a cell wall-less strain (cw15) of *C. reinhardtii*, taking into consideration that future work may require the use of genetic transformation, if they succeed in isolating a mutant containing an O_2-tolerant hydrogenase. The choice of the cw15 strain was based on the observation that the absence of the cell wall increases the efficiency of genetic transformation *(17)*.

Figure 1 shows the effect of increasing concentrations of MNZ and light intensity on the number of cw15 *Chlamydomonas* cells surviving the selective treatment. The light intensities examined were 17.5, 50 (Fig. 1A, first experiment), 200, and 400 (Fig. 1B, second experiment) W/m^2. The selective pressure was applied for 20 min to anaerobically-treated cells (containing an initially active hydrogenase), and O_2 was added at 2.8%. Oxygen inhibits most of the hydrogenase activity in both WT and cw15 cells at this concentration. The maximum concentration of MNZ used in the treatment (58 m*M*) was determined by the solubility of MNZ in aqueous

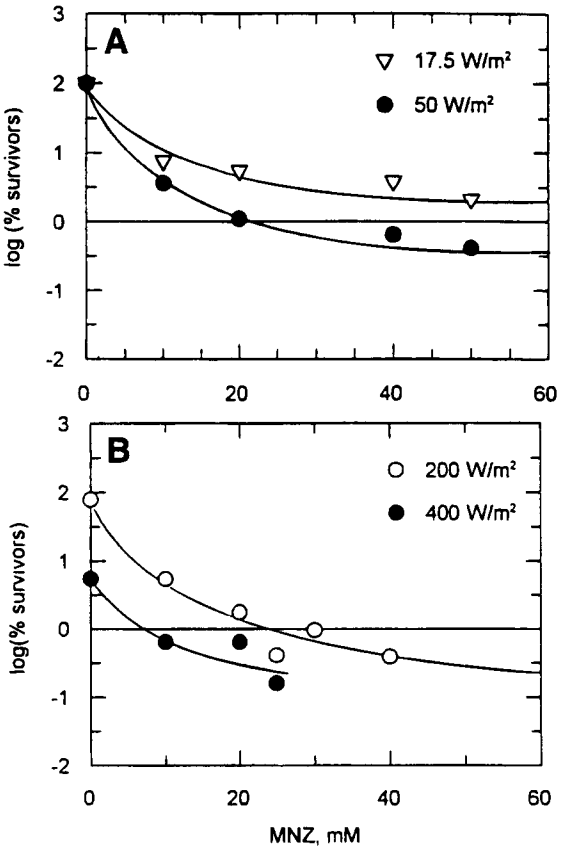

Fig. 1. Effect of MNZ concentration and light intensity on the number of survivors from two selective experiments. **(A)** Selection was done using cw15 cells in the presence of varying concentrations of MNZ and 2.8% O_2, at either 17.5 or 50 W/m^2. **(B)** Selection was done as above, under either 200 or 400 W/m^2. Symbols represent individual data points.

medium. It is clear from Fig. 1 that MNZ toxicity is a function of the light intensity, consistent with the theory that the extent of killing depends on the rate of electron transport. It is interesting that the curves in Fig. 1 do not fit an exponential function, which suggests that factors other than MNZ concentration are limiting the rate of killing (*see* the hydrogenase induction experiment as shown in Fig. 2). Figure 1 also shows the degree of variability of the results obtained from different experiments. The data obtained by exposure of cells to 50 W/m^2 in the first experiment (Fig. 1A) are very similar to the data obtained with 200 W/m^2 in the second experiment (Fig. 1B). It is possible that the phase of growth at which cells were harvested for each experiment is responsible for the variability. Treatment done in the presence of light intensities above 200 W/m^2 (Fig. 1B) caused an increase in temperature that may have affected the number of survivors detected even in control samples not exposed to MNZ.

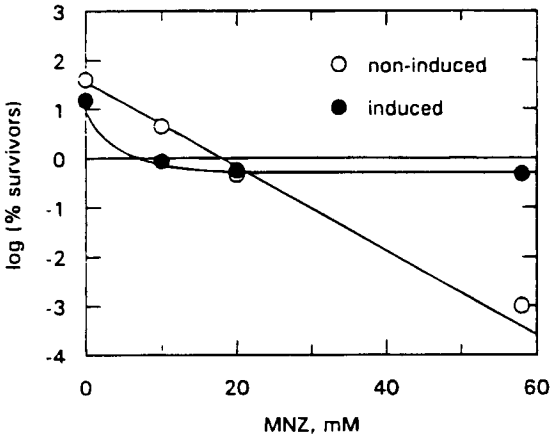

Fig. 2. Effect of anaerobic pre-induction of hydrogenase on the number of cw15 survivors. Cells submitted to the MNZ selective treatment were either preinduced by anaerobic incubation or not induced, as described in the Materials and Methods section. The treatment was done in the presence of 2.8% O_2 and 200 W/m^2.

The third parameter that we checked was the effect of hydrogenase induction on the number of survivors. The experiment was done with preinduced and uninduced cw15 *Chlamydomonas* cells subsequently treated with different concentrations of MNZ in the presence of 2.8% O_2 and illuminated for 20 min at 200 W/m^2. Figure 2 shows that MNZ killing in the absence of a preinduced hydrogenase is exponential. However, preinduction of the hydrogenase causes a loss of exponential killing, consistent with the results in Fig. 1. This demonstrates that the effect of MNZ is limited in cells that have an active hydrogenase, confirming the hypothesis that hydrogenase-catalyzed H_2-production provides enough of an alternative sink for electrons from reduced Fd to mitigate the effects of MNZ.

Thus far the authors have found that their proposed selection technique depends on the concentration of MNZ, on the amount of accumulated reduced Fd (and thus on the electron transport rate), and on the presence of an active hydrogenase. They next examined the effect of O_2 concentration on the rate of survival. Oxygen is expected to inactivate the hydrogenase in a concentration-dependent manner, thus increasing the toxicity of MNZ. Figure 3 shows the result of adding 10% O_2 to the gas phase of the selection medium on the degree of survival of cw15 *Chlamydomonas* cells treated with MNZ and exposed to 200 W/m^2 light for 20 min. Whereas the increase in O_2 concentration in the gas phase appears to increase the sensitivity of MNZ-dependent killing, the magnitude of increase is not as pronounced as might be expected. One possibility is that internally generated photosynthetic O_2 production is affecting the MNZ treatment in part, and the impact of externally-set O_2 is superimposed on it.

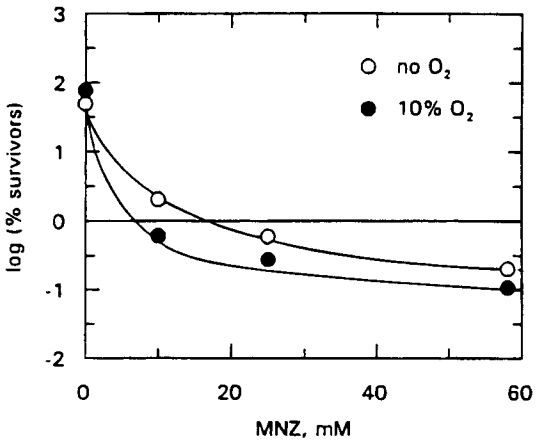

Fig. 3. Effect of O_2 concentration in the gas phase of the selective treatment vial on the number of survivors. MNZ incubation was done using cw15 cells, either under completely anaerobic conditions, or in the presence of 10% added O_2 in the gas phase of the selection medium. The light intensity was 200 W/m^2.

Finally, confirmation of the validity of the authors' proposed technique to select for O_2-tolerant, H_2-producing organisms comes from measurement of the O_2-sensitivity of H_2-production by some of the surviving isolates from two different selection experiments, compared to their parental strains. In order to establish baseline values, they initially determined the O_2 sensitivity of H_2 production by WT and cw15 cells. Initial rates of H_2 evolution were measured, as described in the Materials and Methods Section, as a function of the initial concentrations of O_2 in the assay medium. Given the variability of measuring rates of H_2 evolution from day to day (probably because of the condition of the cells at the time of harvest), the data from each experiment (from 5 to 10 points) were fitted to a single exponential decay equation from which the authors estimated V_0, the rate of H_2 evolution in the absence of O_2, and I_{50}, the initial O_2 concentration that causes a 50% decrease in the initial rate of H_2 evolution compared to V_0. The V_0s estimated from each experiment were then used to normalize the data obtained from all the experiments. The data in Fig. 4 show that as the initial concentration of O_2 increases, the rate of H_2 evolution by cw15 (Fig. 4A) or WT cells (Fig. 4B) decreases exponentially. The normalized V_0 values for the cw15 and WT strains were, respectively, 22 and 27 $\mu mol\ H_2 \cdot mg\ Chl^{-1} \cdot h^{-1}$ and the estimated I_{50}s were 0.300% ± 0.028% and 0.394% ± 0.068% O_2.

Because of the variability in rates measured on different days, the authors always determined the O_2 sensitivity of H_2 production for survivors from different MNZ experiments and their respective parental strains on the same day. The results from two selection experiments are reported on Table 1. Experiment 1 was done with preinduced cw15 and WT cells treated with 58 m*M* MNZ in the presence of 2.8% O_2. There were

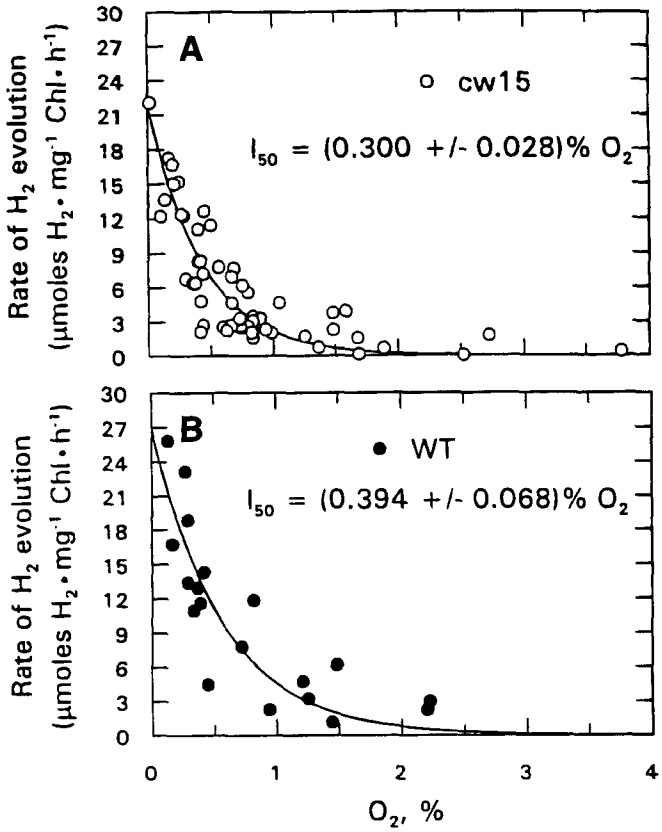

Fig. 4. Normalized rates of H_2 evolution measured with preinduced **(A)** cw 15 or **(B)** WT cells injected to an assay medium preset to the indicated initial concentrations of O_2. H_2 concentration in the assay medium was measured immediately after cell injection and the procedure included a 2-min interval prior to illumination to allow the cells to acclimate. No H_2 production was observed during this 2-min time. See the Materials and Methods section for a description of the experimental conditions and data analysis.

6 cw15 survivors, of which two were tested. Both showed increases in the I_{50} for O_2 compared to their parental strains. One particular survivor, identified as D5, had an estimated I_{50} 2.3 times higher than its parental strain. The other survivor, despite a higher I_{50}, had a decreased V_0 rate of H_2 evolution, perhaps indicating lower rates of electron transport. MNZ treatment of WT cells in Experiment 1 yielded five survivors. Two of them showed increases in I_{50} in the order of 50% compared to their parental strain. The rates of H_2 evolution by one of the survivors was very low, but the other had rates comparable to those of its parental strain. Experiment 2 was done only with WT cells following preinduction of the hydrogenase and MNZ treatment in the presence of 5% O_2. All of the four tested survivors out of a total of six had low rates of H_2 evolution. Two of them had I_{50}s in the order of their parental strain, whereas the other two exhibited small increases in their I_{50}s.

Table 1
H_2-Production Results from Two Selection Experiments done with cw15 and WT *Chlamydomonas reinhardtii cells*[a]

Experiment	Organism	I_{50} for O_2 % of control	V_o % of control
Experiment 1	cw 15 (D5)	233	100
(2.8% O_2)	cw15	153	20
	WT	152	100
	WT	163	12
Experiment 2	WT	100	23
(5% O_2)	WT	138	44
	WT	168	21
	WT	100	5

[a]The cells were preinduced and subsequently treated with 58 m*M* MNZ for 20 min under 200 W/m^2 illumination in the presence of either 2.8% O_2 or 5% O_2. Survivors from the two experiments were plated, and selected clones were transferred to liquid medium and grown photoautotrophically for 3 d. Preinduced cells were used in measurements of H_2-evolution rates in the presence of different initial O_2 concentrations as in Fig. 4. Estimated V_0 and I_{50} values for H_2 production obtained for each survivor were compared to values determined with control parental WT and cw15 cells on the same day.

The results using the selection technique described in this paper indicate that it is possible to select for *Chlamydomonas* isolates that exhibit H_2-production capability that is less sensitive to O_2 than the WT population. However, MNZ treatment can also select for mutants impaired in electron transport, as observed previously *(5,13)*. Although H_2-production selective pressure is a valid option for reaching the long-term goal, the authors conclude that they need to improve the screening procedure to deselect for electron transport mutants. Their assay for O_2 sensitivity, based on measuring the I_{50} of H_2 production in the presence of O_2 is time-consuming and subject to variability. Once this is done, H_2-production selection in combination with mutagenesis could lead to the identification of mutants that are tolerant to even higher concentrations of O_2. The ultimate goal is to develop a mutant that produces H_2 at ~1 atmosphere, with rates equivalent to the conversion of 10% of the incident light energy, and under atmospheric aerobic conditions. A mutant with these properties would demonstrate the commercial feasibility of this approach.

ACKNOWLEDGMENTS

This work was funded by the US Department of Energy Hydrogen Program. We thank Steve Toon for technical assistance.

REFERENCES

1. Weaver, P. F., Lien, S., and Seibert, M. (1979), in *Proceedings of the Fifth Joint US/USSR Conference of the Microbial Enzyme Reactions Project*, Jurmala, Latvia, USSR, pp. 461–479.
2. Benneman, J. R. (1994), in *Proceedings of the 10th World Hydrogen Energy Conference*, Cocoa Beach, FL.
3. Greenbaum, E., Lee, J. W., Tevault, C. V., Blankinship, S. L., and Mets, L. J. (1995), *Nature* **376**, 438–441.
4. Weaver, P. F., Lien, S., and Seibert, M. (1980), *Solar Energy* **24**, 3–45.
5. McBride, A. C., Lien, S., Togasaki, R. K., and San Pietro, A. (1977), in *Biological Solar Energy Conversion*, Mitsui, A., Miyachi, S., San Pietro, A., and Tamura, S. eds. Academic Press, New York, NY.
6. Harris, E. H. (1989), *The Chlamydomonas Sourcebook*, Academic Press, New York.
7. Vladimirova, M. G. and Markelova, A. G. (1980), *Sov. Plant Physiol.* **27**, 878–889.
8. Happe, T., Mosler, B., and Naber, J. D. (1994), *Eur. J. Biochem.* **222**, 769–774.
9. Roessler, P. and Lien, S. (1982), *Arch. Biochem. Biophys.* **213**, 37–44.
10. McTavish, H., Picorel, R., and Seibert, M. (1989), *Plant Physiol.* **89**, 452–456.
11. Roessler, P. G. and Lien, S. (1984), *Plant Physiol.* **76**, 1086–1089.
12. Asada, K. (1984), *Methods in Enzymology* vol. **105**, Academic Press, New York, NY, pp. 422–429.
13. Schmidt, G. W., Matlin, K. S., and Chua, N.-H. (1977), *Proc. Natl. Acad. Sci. USA* **74**, 610–614.
14. Church, D. L. and Laishley, E. J. (1995), *Anaerobe* **1**, 81–92.
15. Wardman, P. and Clarke, E. D. (1976), *Biochem. Biophys. Res. Comm.* **69**, 942–949.
16. Fridovich, I. (1974), in *Advances in Enzymology*, vol. **41**, Meister, A., ed., Wiley Interscience, New York, NY, pp. 35–97.
17. Kindle, K. L. (1990), *Proc Natl Acad Sci USA* **87**, 1228–1232.