

## II.E.2 Biological Systems for Hydrogen Photoproduction

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Projected End Date: Project continuation and direction determined annually by DOE

### Objectives

- Engineer an [FeFe]-hydrogenase that is resistant to O<sub>2</sub> inactivation as part of an aerobic H<sub>2</sub>-production system being developed with Oak Ridge National Laboratory (ORNL) and University of California, Berkeley (UCB).
- Develop and optimize a physiological method to promote culture anaerobiosis and H<sub>2</sub>-production activity in algae.
- Address two components of an innovative H<sub>2</sub>-production system based on integrating fermentative and photosynthetic H<sub>2</sub>-producing organisms.

### Technical Barriers

This project addresses the following technical barrier from the Biological Hydrogen Production section (3.1.4.2.5) of the Hydrogen, Fuel Cells and Infrastructure Technologies (HFCIT) Program Multi-Year Research, Development and Demonstration Plan:

(Z) Continuity of Photoproduction

### Technical Targets

Characteristics	Current Status	2010 Target
Duration of continuous photoproduction*	180 days (sulfur-deprived)	30 minutes (O <sub>2</sub> -tolerant hydrogenase)
O <sub>2</sub> tolerance (half-life in air)	2–4 min ( <i>Clostridium</i> hydrogenase <i>in vitro</i> )	10 min

\*Duration reflects continuous production in the light, not necessarily at peak efficiencies.

### Accomplishments

- Used a new computational simulation method to identify the specific location of high and low energy barriers for O<sub>2</sub> diffusion along specific pathways.
- Generated site-specific mutations at amino acid residues identified as being potential targets for mutagenesis aimed at preventing O<sub>2</sub> access to the catalytic site of the [FeFe]-hydrogenase. A single mutation along one of the O<sub>2</sub> channels increased the enzyme's tolerance to O<sub>2</sub>, but the increase was limited by the lack yet of a successful mutant on the other channel. Most of the other mutations were disruptive to the enzyme and resulted in decreases of hydrogenase activity.
- Conducted spectroscopic measurements (with Prof. Finazzi, Institute de Biologie Physico Chimique, Paris) that provided evidence for a wasteful cycle of electrons around Photosystem I under sulfur-deprived conditions. This may in part explain the current low rates of H<sub>2</sub> production by these cultures.
- Achieved light conversion efficiencies of 0.36% (50% higher than in suspension cultures) by immobilizing the cultures onto glass fibers and running the reactor continuously in the presence of a limiting concentration of sulfate (with Dr. Tsygankov, Russian Academy of Sciences, Russia).
- Demonstrated significant rates of H<sub>2</sub> production by photoautotrophic cultures, providing evidence that added acetate might be eliminated from the process.

## Introduction

Eukaryotic green algae can photoproduce  $H_2$  from water, and this property requires the coordinated operation of the photosynthetic water oxidation machinery (which generates  $O_2$ , reductants, and protons from water) and the hydrogenase enzyme (which recombines protons and electrons to produce  $H_2$  gas). The catalytic center of green algal [FeFe]-hydrogenases is composed of a unique 2Fe2S center that is sensitive to  $O_2$ , a by-product of photosynthetic water oxidation.  $O_2$  inactivation prevents sustained  $H_2$  production by the organism in the light. The continuity of photoproduction is one of three major technical barriers (Barrier Z) to developing photobiological  $H_2$ -production systems, as identified by the *HFCIT Program Multi-Year Research, Development and Demonstration Plan*.

Our current project addresses two different strategies for surmounting the  $O_2$  sensitivity of  $H_2$ -producing algae: (a) molecular engineering efforts to alleviate the  $O_2$  sensitivity of the [FeFe]-hydrogenase (Task 1) and (b) use of a physiological switch to separate  $O_2$  and  $H_2$  production (Task 2). Our project also proposes to initiate studies to develop a novel system that integrates photobiological  $H_2$  production with fermentative processes (Task 3).

## Approach

### Task 1 (Molecular Engineering of the Algal Hydrogenase)

We proposed that inactivation of the algal [FeFe]-hydrogenase depends on access of  $O_2$  to the enzyme's catalytic site through a hydrophobic channel connecting the surface to the catalytic center. In collaboration with the Beckman Institute of the University of Illinois and the National Renewable Energy Laboratory's (NREL) Computational Sciences Center, we generated computational simulation models of  $O_2$  and  $H_2$  gas diffusion through [FeFe]-hydrogenase and volumetric solvent-accessibility maps. These maps confirmed that  $O_2$  diffusion occurs through a series of hydrophobic cavities separated by energy barriers, and allowed us to propose the possibility of affecting  $O_2$  access to the hydrogenase's catalytic site without necessarily affecting the outward diffusion of  $H_2$  gas. This year we used a third computational analysis method to more specifically identify the low and high energy barriers along the  $O_2$  diffusion pathways. Based on the three analyses described above, we started implementing site-directed mutagenesis techniques to attempt to prevent  $O_2$  molecules from reaching the catalytic site of the [FeFe]-hydrogenase.

### Task 2 (Biochemical and Process Engineering)

In 2000, NREL and UCB jointly developed a shorter-term approach to circumventing the  $O_2$ -sensitivity issue of biological  $H_2$  production. This approach is based on the metabolic shift from  $O_2$  to  $H_2$  production induced by depriving algal cultures of sulfate. The original system, which was designed to operate in cycles of +S and -S, was later converted into a continuous  $H_2$ -producing system at NREL, which resulted in a decrease by a factor of 3 in the estimated cost of  $H_2$  production. The latter was optimized for continuity of operation, but yields and rates of  $H_2$  production were too low for scale-up. We hypothesized that the observed low rates could be due in part to the low levels of a critical electron transport component, but last year we showed that this was not the case. This year, we studied the possibility of wasteful cyclic electron transport around Photosystem I that does not result in  $H_2$  production.

We are also attempting to decrease the cost of a  $H_2$ -producing immobilized culture reactor using two different approaches: (a) cultivating the algae under photoautotrophic conditions (no added acetate), and (b) studying the feasibility of using more cost-effective matrices for algal cell immobilization.

### Task 3 (Integrated $H_2$ -Production System)

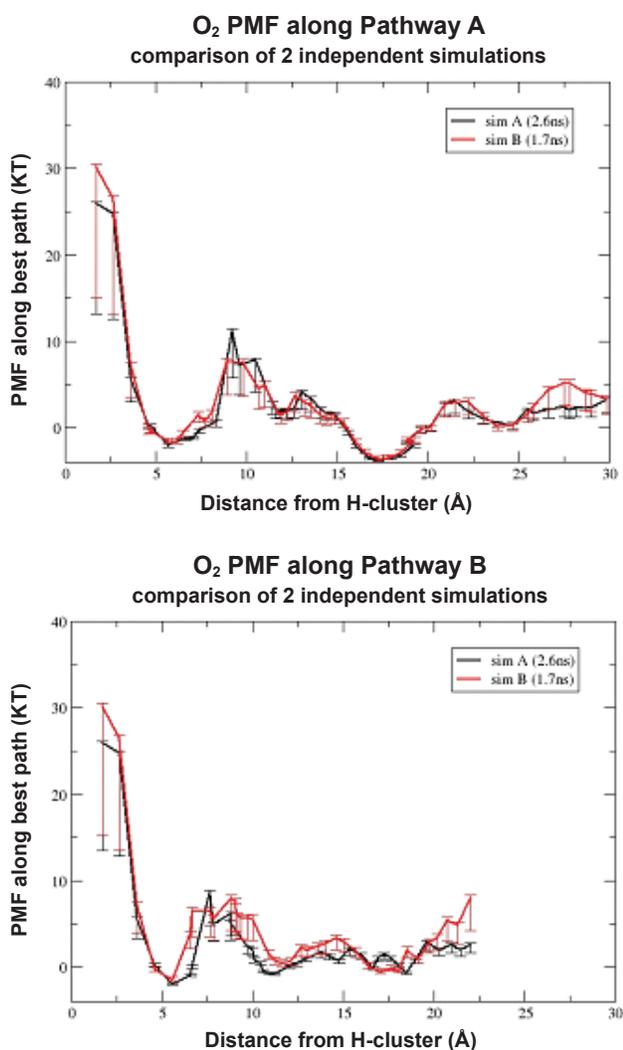
No activities are reported on this task due to lack of funding in FY 2006.

## Results

### Task 1 (Molecular Engineering of the Algal Hydrogenase)

Our previous results demonstrated that, while [FeFe]-hydrogenases are very porous to  $H_2$ , the diffusion of  $O_2$  molecules is restricted to two very well-defined pathways and their movement is restricted by the presence of transient cavities in these pathways.

We precisely identified specific amino acid residues involved in forming the barriers between the cavities by determining the potential mean free energy (PMF) along each of the two  $O_2$  pathways. Figure 1 plots the PMF along pathways A and B, respectively. It is clear that (a) there are a few high energy barriers along both pathways, and (b) the energy barrier found in the *Clostridium acetobutylicum* hydrogenase next to the catalytic site is much higher than the one found in the algal hydrogenase HydA2, which may explain the much higher sensitivity of the latter to  $O_2$ .



**Figure 1.** Mean Free Potential Energy Profiles along the Putative  $O_2$  Channels A and B, using the Cal [FeFe]-hydrogenase from *Clostridium Acetobutylicum*

We identified three amino acid residues from the computational analyses as possible targets for mutagenesis. The three residues (X, Y, and Z), were mutated *in silico*, either singly or in combination with each other. The solvent accessibility maps suggested that the mutations should substantially close both pathways to  $O_2$ . Upon implementation of the mutations *in vitro*, we observed that mutant X on pathway A resulted in a hydrogenase with high  $H_2$ -production activity (71% of the non-mutated enzyme) and higher tolerance to  $O_2$  (20% more than the non-mutated enzyme). The other mutations, however, yielded hydrogenases with very low activity, suggesting that the mutated residues did not allow the proper assembly of the enzyme's catalytic site, and that the resulting enzyme became more porous to  $O_2$ .

## Task 2 (Biochemical and Process Engineering)

The current rate of  $H_2$  production by sulfur-deprived algae on a chlorophyll basis is still about  $1/8^{\text{th}}$  of the expected maximum. This year, Prof. Finazzi conducted spectroscopic measurements of sulfur-deprived *Chlamydomonas reinhardtii* cultures for us. He demonstrated that most of the reductant originated from water oxidation cycles around Photosystem I, instead of being used for  $H_2$  production. This limitation is consistent with a recent report of a *C. reinhardtii* mutant that is unable to transition to cyclic electron transport and that produces  $H_2$  at five times higher rates than its wild-type (non-mutated) parent (Kruse et al., J. Biol. Chem. 280, 34170-34177, 2005). We will be investigating the performance of this type of mutant in our  $H_2$ -producing system next.

Suspension cultures show low light conversion efficiency to  $H_2$  under limiting light intensities, as a result both of their less than optimal rates of  $H_2$  production and because of their low cell density. To circumvent this problem, we immobilized algal cultures onto glass fibers, determined that they produce  $H_2$  for longer periods of time (with only a slight decrease in their specific rates), and compared the light conversion efficiency of suspension vs. immobilized cultures. Table 1 shows that, when the same number of cells are present in both reactors, immobilized cells will convert 0.36% of the incident light intensity to  $H_2$ , compared to only 0.24% for suspension cultures. This corresponds to  $1/3$  of the 1% maximum light conversion efficiency expected with sulfur-deprived cultures. We expect that combining the above type of mutant with cell immobilization will bring us closer to this maximum value next year.

**TABLE 1.** Physical and biochemical parameters measured during the sulfur-deprivation,  $H_2$ -production stage of algal systems using either suspension or immobilized cultures

Parameters	Cell suspension	Immobilized cells
Illuminated reactor surface (in cm)	$2 \times 261 \text{ cm}^2 = 522 \text{ cm}^2$	$200 \text{ cm}^2$
Rate of $H_2$ production per reactor during the time of operation (ml/h and $\mu\text{moles/h}$ );	2.5 and 82.5	0.7 and 31.22
Energy of incident light per $\text{m}^2$ per hour	$154,080 \text{ J/m}^2$	$92,448 \text{ J/m}^2$
Efficiency of incident light energy conversion into $H_2$	0.24%	0.36% ( <b>50% improvement</b> )

Finally, in order to further decrease the cost of  $H_2$  produced by sulfur-deprived cultures, we tested the capability of the algae to produce  $H_2$  photoautotrophically in the absence of added acetate.

We demonstrated that acetate is not strictly required for H<sub>2</sub> production. Future work will involve immobilization of photoautotrophically-grown algal cultures for H<sub>2</sub> production.

## Conclusions and Future Directions

### Conclusions

- The development of computational simulations to identify O<sub>2</sub>-diffusion pathways into the [FeFe]-hydrogenases is uncovering targets for site-directed mutagenesis aimed at restricting O<sub>2</sub> access to the catalytic site. Preliminary experimental results have shown that effective mutations must be selected judiciously in order to preserve the assembly of the catalytic site within the protein structure.
- We clearly demonstrated the advantages of cell immobilization as a means of significantly increasing the light conversion efficiency of sulfur-deprived, H<sub>2</sub>-producing algal systems. Moreover, we identified one of the limiting factors that may prevent higher rates of H<sub>2</sub> production. Future work will involve assessing H<sub>2</sub>-producing capabilities by circumventing this limitation.

### Future Directions

- Task 1 (Molecular Engineering of the Algal Hydrogenase)
  - Continue the iterative process of computational simulations and experimental mutagenesis studies to generate mutants affected on both O<sub>2</sub> pathways.
  - Develop a new in vitro assay to test for O<sub>2</sub> tolerance of hydrogenases expressed in the bacterium, *E. coli*.
  - Continue efforts to crystallize and solve the x-ray structure of the algal [FeFe] hydrogenases (in collaboration with Dr. Juan Fontecilla-Camps, CEA/CNRS Grenoble, France).
  - Use other mutagenesis approaches to generate additional hydrogenase mutants; develop a high-throughput assay to screen positive transformants for O<sub>2</sub> tolerance.
  - Extend computational simulation studies to [NiFe]-hydrogenases, in support of the work being conducted in cyanobacteria and funded by the HFCIT Program.
- Task 2 (Biochemical and Process Engineering)
  - Investigate the H<sub>2</sub>-production capabilities of a mutant that is unable to conduct wasteful electron transport.

- Optimize photoautotrophic systems for H<sub>2</sub> production and test cell immobilization.
- Study the feasibility of lower cost matrices to immobilize H<sub>2</sub>-producing algae.
- Task 3 (Integrated System)
  - Address the efficiency of algal biomass fermentation by bacteria and the efficiency of H<sub>2</sub> production by S-deprived algal cultures integrated with H<sub>2</sub>-producing photosynthetic bacteria.
  - Screen the NREL collection of photosynthetic bacteria for those that produce H<sub>2</sub> most efficiently using fermentation waste by-products.

## FY 2006 Publications/Presentations

### Publications In Press

1. Blake, D., Amos, W., Ghirardi, M.L., and Seibert, M. Materials requirements for photobiological hydrogen production. In *Materials for the Hydrogen Economy*, CRC Press.
2. Ghirardi, M.L. *Ind. J. Biochem. Biophys.*
3. Ghirardi, M.L., Maness, P.C., and Seibert, M. In *Solar Generation of Hydrogen*, (McConell, ed.) Springer Verlag.
4. Melis, A., Ghirardi, M.L., and Seibert, M. In *Transgenic Microalgae as Green Cell Factories* (Leon, Fernandez, and Galvan, Eds.) Landers Bioscience Publ., Georgetown, TX.
5. Tsygankov, A.A., Kosourov, S.N., Tolstygina, I.V., Ghirardi, M.L., and Seibert, M. *Int. J. Hydrogen Energy*.

### Publications

1. Cohen, J., Kim, K., King, P., Ghirardi, M. L., Seibert, M., and Schulten, K. (2005). *Structure* 13, 1321-1329.
2. Ghirardi, M. L., King, P., Kosourov, S., Forestier, M., Zhang, L., and Seibert, M. (2005). In *Artificial Photosynthesis*, (Collings, ed.), Wiley – VCH Verlag, Weinheim, Germany, 213-227.
3. King, P., Posewitz, M. C., Ghirardi, M. L., and Seibert, M. (2006). *J. Bacteriol.* 188, 2163-2172.
4. Kosourov, S., Makarova, V., Fedorov, A. S., Tsygankov, A., Seibert, M., and Ghirardi, M. L. (2005). *Photosynth. Res.* 85, 295-305.
5. Laurinavichene, T. V., Fedorov, A. S., Ghirardi, M. L., Seibert, M., and Tsygankov, A. A. (2006). *Int. J. Hydrogen Energy* 31, 659-667.
6. Makarova, V.V., Kosourov, S.N., Krendeleva, T.E., Kukarshkikh, G.P., Ghirardi, M.L., Seibert, M. and Rubin, A.B. (2006). *Biophysics* 50, 90-96.

## Presentations

1. Briefing to the Jason group; invited presentations at the Photosynthesis Gordon Conference, to DOE's HFCIT headquarters, at the International Hydrogen Energy Congress in Istanbul, at the BES Solar Energy Utilization Workshop, as the Raiziss lecturer on "Frontiers in Biochemistry and Biophysics" at the University of Pennsylvania, to Dr. Ray Orbach, to the Secretary of Energy, Dr. Samuel Bodman, at the International Partnership for Hydrogen Energy workshop in Seville, Spain, and many others.
2. Chair of the COST meeting and participant at the close-out IEA Annex 15 session in Istanbul. Invited participant at the American Academy of Microbiology's colloquium on "Microbial Production of Energy."
3. Interviews by Bridget Enis for the MicrobeWorld radio program; by SciCentral for a video; by Discover magazine and selection of our technology as one of Discover's 25 "Frontiers of Science," by New Scientist; by Dragonfire, an online publication; by Wired News; by the Discovery Channel for a video on renewable energy technologies.

## Special Recognitions & Awards/Patents Issued

1. HENAAC award granted to Maria Ghirardi for outstanding achievement as a Hispanic scientist (October 2005); Internal NREL grant (DDRD) award to Drs. Howard Branz and Maria Ghirardi to conduct research on a novel DNA hybridization technique (October 2005); Melis, A., L. Zhang, J. R. Benemann, M. Forestier, M. Ghirardi, and M. Seibert (2003) "Photosynthetic Hydrogen by Microalgal Cultures," U.S. Patent #6,989,252.