

## IV.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 to function with aerobic H<sub>2</sub> production systems being developed in collaboration with Oak Ridge National Laboratory (ORNL) and the University of California, Berkeley (UC Berkeley).
- Generate a recombinant photosynthetic cyanobacterial system for H<sub>2</sub> production that utilizes an [NiFe]-hydrogenase with increased O<sub>2</sub> resistance.
- Develop and optimize a physiological method to promote culture anaerobiosis and subsequent H<sub>2</sub> production activity in algae.

### Technical Barriers

This project addresses the following technical barrier from the Hydrogen Production section of the Hydrogen, Fuel Cells & Infrastructure Technologies 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 min
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.

### Approach

#### Subtask 1 (Molecular Engineering of the [FeFe]-Hydrogenase)

- Use a rational, iterative process to generate an O<sub>2</sub>-tolerant algal system for H<sub>2</sub> photoproduction. The process includes (a) O<sub>2</sub>-gas-diffusion/solvent-accessibility studies through computational simulations and (b) experimental generation and testing of the O<sub>2</sub> resistance of hydrogenase mutants expressed in *E. coli*.

### Subtask 2 (Recombinant Cyanobacterial H<sub>2</sub> Production)

- Generate a recombinant cyanobacteria that lacks a native NiFe hydrogenase but photoproduces H<sub>2</sub> through the O<sub>2</sub>-tolerant CBS (Casa Bonita Strain) hydrogenase.

### Subtask 3 (Biochemical and Process Engineering)

- Identify possible limitations in the slow H<sub>2</sub> production rates observed currently with sulfur-deprived cultures and, if necessary, overexpress the limiting factors in the alga.
- Further study the advantages of cell immobilization on the rates of H<sub>2</sub> photoproduction using different types of low-cost matrices.

## **Accomplishments**

- Identified only two well-defined pathways for O<sub>2</sub> diffusion and multiple pathways for H<sub>2</sub> diffusion into the *Clostridium pasteurianum* [FeFe]-hydrogenase CpI using molecular dynamics modeling and solvent-accessibility maps.
- Developed a highly flexible heterologous [FeFe]-hydrogenase expression system in *E. coli*.
- Discovered that [FeFe]-hydrogenases from anaerobic, nonphotosynthetic bacteria can be synthesized using the same NREL-discovered genes that are responsible for the assembly of algal hydrogenases.
- Demonstrated that the O<sub>2</sub> tolerance of these bacterial hydrogenases is 100–400 times higher than that of algal hydrogenases, which makes them better candidates for further mutagenesis improvement.
- Studied the effect of *in silico* mutations on the accessibility of the catalytic site to O<sub>2</sub> and implemented some of the site-directed mutations *in vitro* using the CpI hydrogenase; mutations affecting amino acid residues close to the catalytic site interfere with its assembly; mutations that narrow only one of the O<sub>2</sub> pathways cause only small increases in O<sub>2</sub> tolerance.
- Demonstrated an *in vitro* linkage between the photosynthetic electron transport pathway of *Synechocystis* and the O<sub>2</sub>-tolerant hydrogenase from *Rubrivivax gelatinosus*.
- Knocked out the host *Synechocystis* native hydrogenase and generated a hydrogenase-free background in the host for future transformation with the *R. gelatinosus* enzyme.
- Found the optimal operating conditions for a continuous algal suspension H<sub>2</sub>-producing system and obtained rates of 15 mL L<sup>-1</sup> d<sup>-1</sup>.
- Achieved rates of H<sub>2</sub> production of 300 mL L<sup>-1</sup> d<sup>-1</sup> by immobilizing algal cultures onto fiberglass and running the reactor continuously for 3 months in the presence of 10 μM sulfate.

## **Future Directions**

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

- Continue the iterative process of computational simulations and experimental mutagenesis studies to generate mutants affected in both O<sub>2</sub> pathways.
- Develop an *in vitro* light-dependent system to study mutated hydrogenase activity in the presence of a component that generates O<sub>2</sub>.
- Crystallize the algal HydA1 and/or HydA2 hydrogenases for future use in computational simulations as has been done with the CpI bacterial hydrogenase.
- Use *in vitro* random mutagenesis to generate additional hydrogenase mutants; develop a high-throughput assay to screen positive transformants for O<sub>2</sub> tolerance.

### Subtask 2 (Recombinant Cyanobacterial H<sub>2</sub> Production)

- Further characterize the O<sub>2</sub> tolerance of the bacterial [NiFe]-hydrogenase.
- Optimize the CBS hydrogenase expression system using *E. coli* as the initial host; the outcome will guide us in expressing the CBS hydrogenase in the cyanobacterial host of choice in the future.

### Subtask 3 (Biochemical and Process Engineering)

- Identify possible limitations in electron carriers as a partial cause for the slow H<sub>2</sub> production rates observed with the sulfur-deprived system and overexpress the identified limiting component to increase the rates.
- Study the feasibility of using different types of low-cost matrices for cell immobilization.
- Develop a cyclic (i.e., +S/-S) immobilized system and test for improved average rates of H<sub>2</sub> production.
- Test the feasibility of H<sub>2</sub> production using sulfur-deprived photoautotrophic systems.
- Perform an economic analysis of immobilized algal systems.

### Subtask 4 (Integrated System)

- Address the efficiency of algal biomass fermentation by fermentative organisms and the efficiency of the combined H<sub>2</sub> production by S-deprived algal cultures and H<sub>2</sub>-producing photosynthetic bacteria in two separate photobioreactors illuminated from only one surface.
- Screen the NREL collection of photosynthetic bacteria for those that produce hydrogen most efficiently using fermentation waste by-products.

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## **Introduction**

Both eukaryotic green algae and prokaryotic cyanobacteria can photoproduce H<sub>2</sub> from water. This property requires the coordinated operation of the photosynthetic water oxidation machinery (which generates O<sub>2</sub>, reductants, and protons from water) and the hydrogenase enzyme (which recombines protons and reductants to produce H<sub>2</sub> gas). The catalytic center of green algal [FeFe]-hydrogenases is composed of a unique 2Fe2S center [1-3]; cyanobacterial hydrogenases contain Ni, Fe and S in their catalytic centers [4, 5]. Both classes of hydrogenases are sensitive to O<sub>2</sub>, a by-product of photosynthetic water oxidation, and O<sub>2</sub> inactivation prevents sustained H<sub>2</sub> production by either organism in the light. The continuity of photoproduction is one of the three technical barriers (Barrier Z) to the development of photobiological H<sub>2</sub> production systems, as identified by the Hydrogen, Fuel Cells and Infrastructure Technologies Program Multi-Year Research, Development and Demonstration Plan.

Our current project addresses three different strategies for surmounting the O<sub>2</sub> sensitivity of photobiological H<sub>2</sub>-producing organisms: (a) molecular engineering efforts to alleviate the O<sub>2</sub> sensitivity of the [FeFe]-hydrogenase (Subtask 1); (b) generation of a recombinant cyanobacterium expressing a previously discovered O<sub>2</sub>-tolerant [NiFe]-hydrogenase from a CO-utilizing bacterium

(Subtask 2); and (c) use of a physiological switch to separate O<sub>2</sub> and H<sub>2</sub> production (Subtask 3).

## **Approach**

### **Subtask 1 (Molecular Engineering of the Algal Hydrogenase)**

Previous work on bacterial [NiFe]-hydrogenases demonstrated the existence of a hydrophobic channel connecting the surface of the enzyme to its catalytic site [6, 7]. This observation led us to hypothesize that inactivation of the algal [FeFe]-hydrogenase depends on access of O<sub>2</sub> to the enzyme's catalytic site through a similar channel. Last year, in collaboration with the Beckman Institute of the University of Illinois and NREL's Computational Sciences Center, we generated a computational simulation model of O<sub>2</sub> gas diffusion through a [FeFe]-hydrogenase enzyme. This confirmed that O<sub>2</sub> diffusion occurs mainly through a series of hydrophobic cavities separated by energy barriers and validated our mutagenesis approach. This year, we further refined the simulation and included studies of H<sub>2</sub> gas diffusion from the catalytic site of the enzyme, as well as volumetric solvent-accessibility maps to determine the size of the cavities around the catalytic site [8]. The results allowed us to hypothesize that it is possible to affect O<sub>2</sub> accessibility to the hydrogenase's catalytic site without necessarily affecting the outward diffusion of H<sub>2</sub> gas produced by the enzyme. Ongoing studies

are aimed at implementing the information obtained from the computational simulations to generate and test actual mutants.

### Subtask 2 (Recombinant Cyanobacterial H<sub>2</sub> Production)

A complementary strategy for overcoming the O<sub>2</sub>-sensitivity problem in photooxygenic organisms is to construct a recombinant cyanobacterial system in which the O<sub>2</sub>-sensitive hydrogenase of the cyanobacterial host is replaced with an O<sub>2</sub>-tolerant hydrogenase from another bacterium. Work conducted at NREL in the past led to the discovery of a [NiFe], O<sub>2</sub>-tolerant hydrogenase from the CO-oxidizing bacterium *Rubrivivax gelatinosus* CBS [9]. Because cyanobacteria already have the complex machinery necessary to synthesize, assemble, and activate [NiFe]-hydrogenases [10], they should express the recombinant CBS enzyme as well. This year, we did preliminary work to knock out the native cyanobacterial hydrogenase structural genes, creating a clean background for insertion of the CBS hydrogenase. We have also successfully demonstrated a linkage between the photosynthetic electron transport system and the CBS hydrogenase *in vitro*, a prudent strategy before initiating research to express the latter in the host.

### Subtask 3 (Biochemical and Process Engineering)

In 2000, NREL and UC Berkeley jointly developed a shorter-term approach to circumvent the O<sub>2</sub>-sensitivity issue of biological H<sub>2</sub> production. This work was based on the metabolic shift from O<sub>2</sub> to H<sub>2</sub> production induced by depriving algal cultures of sulfate [11]. The original system, which was designed to operate in cycles of +S and -S, was later converted into a continuous H<sub>2</sub>-producing system at NREL, which resulted in a decrease by a factor of 3 in the estimated cost of H<sub>2</sub> production [12]. The continuous system was optimized for operation but still yields very low rates of H<sub>2</sub> production [13]. We hypothesized that the observed low rates could be due in part to the low levels of a critical electron transport component and are currently investigating this hypothesis.

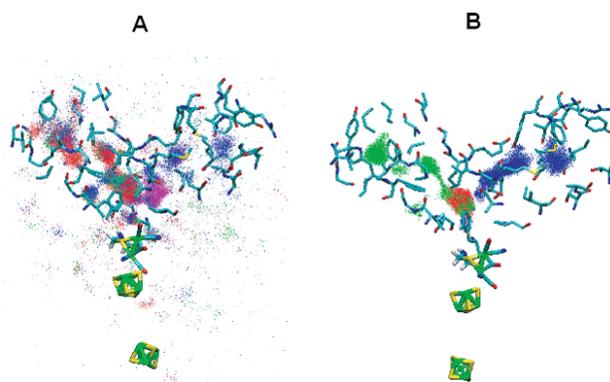
We also started assessing the feasibility of immobilizing algal cells at high density onto different classes of matrices to facilitate the cycling

of the cultures between +S and -S conditions. Our results indicate that immobilization does not affect the specific activity of the cultures and allows the cells to produce H<sub>2</sub> for much longer periods of time. On a volumetric basis, immobilized cultures produce 20X more H<sub>2</sub> than suspended dilute cultures [14]. Economic analyses will indicate whether this approach is desirable for future applications.

## Results

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

A high degree of homology exists between the algal hydrogenase, which has not been crystallized, and the *Clostridium pasteurianum* CpI hydrogenase [15], which has. This homology is allowing us to use the latter's solved X-ray structure to gain information about the algal enzyme. We performed molecular dynamics simulations of O<sub>2</sub> and H<sub>2</sub> gas diffusion pathways through CpI, using an improved variant of the locally enhanced sampling (LES) method [16], denoted TC-LES [8]. Figure 1 shows the results from an average of 3 to 5 separate simulations. Figure 1A demonstrates that the hydrogenase is porous to H<sub>2</sub>, which can penetrate a broad region of the protein on short time scales. In contrast, Figure 1B shows that the diffusion of O<sub>2</sub> molecules is restricted to two very well-defined pathways and that the movement of O<sub>2</sub> molecules is restricted by the

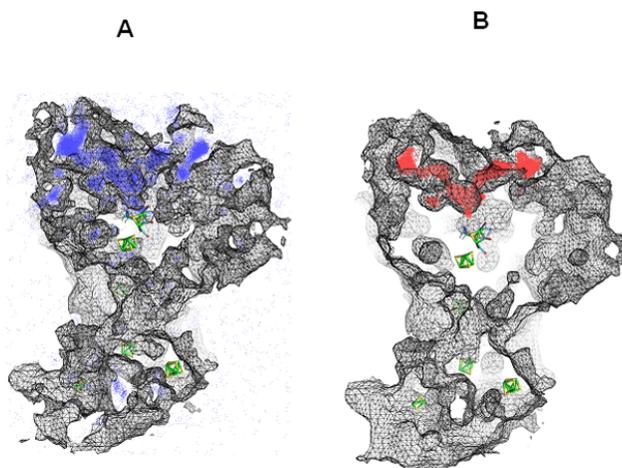


**Figure 1.** Four-ns TC-LES simulations of 1,000 copies of H<sub>2</sub> (A) and O<sub>2</sub> (B) molecules diffusing out from the H cluster of the CpI hydrogenase enzyme. The iron sulfur clusters and the H cluster are indicated in green and yellow and the gas-diffusion trajectories are shown in red, blue, and green.

presence of cavities in these pathways. The results are consistent with our hypothesis that it should be possible to close the O<sub>2</sub> diffusion pathways into the catalytic site without significantly affecting H<sub>2</sub> diffusion from the catalytic site.

We confirmed the existence of cavities that trap the O<sub>2</sub> molecules in the CpI hydrogenase by mapping the regions of the protein that would be accessible to a particle of a given radius. Figure 2A demonstrates that a molecule of the size of H<sub>2</sub> (1.35 Å) can occupy an almost continuous area from the surface of the protein to its catalytic site, including the pathways identified by gas-diffusion simulations (blue superimposed area). The presence of cavities large enough to contain O<sub>2</sub> molecules (1.6 Å) is indicated in Figure 2B. Only a very few cavities along the two main diffusion pathways are large enough, and these cavities correspond to those identified by the gas-diffusion simulations (red superimposed area). Moreover, these cavities are transient and arise from the protein's natural equilibrium dynamic motion.

One of our group's major accomplishments from last year [18; partially supported by the DOE Office of Science] allowed us to use an *E. coli* expression system to obtain large amounts of algal hydrogenase by co-expressing it with a set of assembly genes. This year, we demonstrated that the heterologous system can be improved by incorporating the clostridial assembly genes instead of the algal ones [17], and that it can also express [FeFe]-hydrogenases from a variety of bacteria (Table 1).



**Figure 2.** Solvent-Accessibility Maps Predicted from the Equilibrium Simulation of CpI in the Absence of Gas for a Particle Size of H<sub>2</sub> (A) or O<sub>2</sub> (B)

When tested for O<sub>2</sub> tolerance, all the bacterial enzymes exhibited significantly increased resistance to O<sub>2</sub> inactivation compared to the algal enzymes. As a result, we have shifted our mutagenesis work to the CpI hydrogenase, which is also the model for our computational simulation work. We focused our initial mutagenesis efforts on the pathways indicated in green on Figure 1B and attempted to reduce the size of the cavities forming that diffusion pathway or to increase the energy required for O<sub>2</sub> to migrate through the transition barriers separating the cavities. The results show that mutations too close to the catalytic site may result in either defective protein folding or lack of assembly of the catalytic site, or may yield enzyme structures that are more permeable to gases. To avoid these pitfalls, our future work will focus on mutations of sites closer to the enzyme surface and will include mutations of the second identified O<sub>2</sub> pathway (indicated in blue in Figure 1B) as well

### Subtask 2 (Recombinant Cyanobacterial H<sub>2</sub> Production)

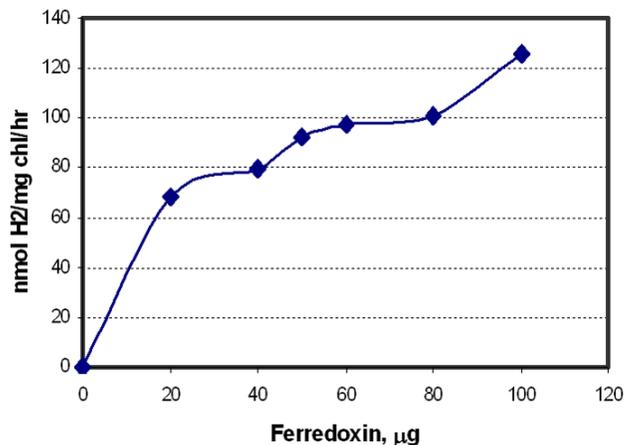
The deletion of the cyanobacterium *Synechocystis* [NiFe]-hydrogenase (encoded by the *hoxH* gene [19]) will create a background free of hydrogenase activity and facilitate the transfer of the

**Table 1.** Measured Activities of Recombinant Hydrogenases Expressed in *E. coli*

Organism	Enzyme	Subunit composition	Activities in whole cell extracts (nmole H <sub>2</sub> ml <sup>-1</sup> min <sup>-1</sup> )	Specific activities of purified enzymes (nmole H <sub>2</sub> mg <sup>-1</sup> min <sup>-1</sup> )
<i>E. coli</i>			0.35	ND
<i>C. reinhardtii</i>	HydA1	Monomeric	61	887
<i>C. reinhardtii</i>	HydA2	Monomeric	12	699
<i>C. acetobutylicum</i>	HydA	Monomeric	96	2894
	HydAΔN	Monomeric	6	580
	HydB	Monomeric	3	682
<i>C. pasteurianum</i>	HydA	Monomeric	20	ND
	HydAΔN	Monomeric	7	ND

O<sub>2</sub>-tolerant hydrogenase from CBS to the cyanobacterium. Using polymerase chain reaction (PCR) amplification, we synthesized an inactive copy of the *hoxH* gene (interrupted with a kanamycin antibiotic marker) and swapped the inactive *hoxH* gene with the host's native gene using homologous recombination. Because *Synechocystis* harbors 8–10 copies of its chromosome, we gradually adapted the mutated *Synechocystis* to elevated concentrations of kanamycin (10 µg/ml to 70 µg/ml) to ensure that most copies of the *Synechocystis* chromosomes contain the inactive gene. The complete loss of hydrogenase activity in the mutated host was determined biochemically, and the biochemical analysis indicated the complete elimination of the host background hydrogenase activity. We can now transfer the O<sub>2</sub>-tolerant hydrogenase from CBS without interference, and any H<sub>2</sub> production activity detected from this recombinant will indicate that the CBS hydrogenase is expressed and functional.

Electron transport to the CBS hydrogenase is mediated by one or more Fe-S proteins (20). It is likely, then, that the recombinant CBS hydrogenase will utilize the accessory Fe-S proteins from the host for H<sub>2</sub> production, thus minimizing the number of foreign genes required for transfer to the new host. In FY 2005, we demonstrated that the CBS hydrogenase could indeed link to the *Synechocystis* host ferredoxin (PetF1) *in vitro* as a mediator to support H<sub>2</sub> photoproduction. Enhanced rates of H<sub>2</sub> photoproduction were detected only when the light was turned on and when the PetF1 ferredoxin was included in the reaction mixture that contained spinach photosynthetic membranes and CBS hydrogenase (Figure 3). The CBS hydrogenase could also link to ferredoxin from other sources, including *Clostridium pasteurianum*, *Spirulina*, and a red alga, at comparable rates. Based on this finding, it is likely that only the gene encoding CBS hydrogenase will need to be transferred, where it will use the host-reduced ferredoxin as the electron donor to mediate H<sub>2</sub> production. By minimizing the number of genes required for transfer, we will maximize our chances for constructing a successful cyanobacterial recombinant.

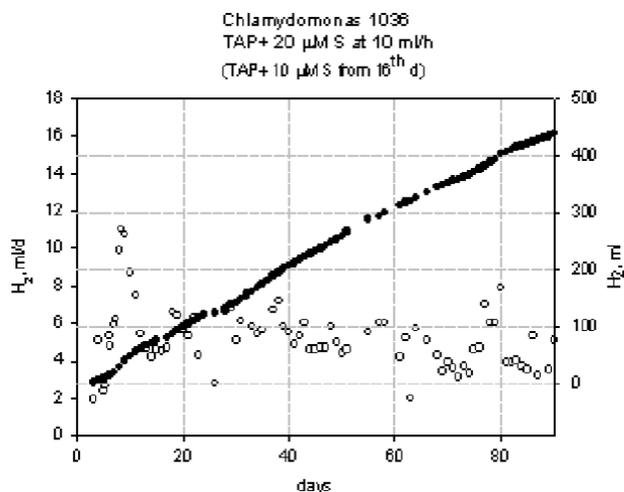


**Figure 3.** Ferredoxin Requirements for H<sub>2</sub> Production in an *in Vitro* System Linking Water-Oxidation Activity from Spinach with the Hydrogenase from CBS

### Subtask 3 (Biochemical and Process Engineering)

The continuous H<sub>2</sub> production chemostat system is comprised of two automated photobioreactors. In the first photobioreactor, the algal cultures are grown photoheterotrophically under limited sulfate to obtain photosynthetically competent cells. Active cells are then continuously delivered to the second photobioreactor, where H<sub>2</sub> production occurs under anaerobic, sulfur-deprived conditions. We tested the effects of varying the sulfate concentration in the medium delivered to the first photobioreactor, cell density, dilution rates, incident light intensity and pH on H<sub>2</sub> photoproduction in the second photobioreactor. We found that optimization of the above parameters yielded a maximal rate of 15 mL L<sup>-1</sup> d<sup>-1</sup> (the highest yet observed for the system).

We attempted to increase the current production rates by using cell immobilization techniques to concentrate the cultures. We had already demonstrated that immobilization of sulfur-deprived algal cultures on glass fibers allowed for prolonged H<sub>2</sub> photoproduction activity with little loss of specific activity [14]. This year, we devised a system for continuous photoproduction of H<sub>2</sub> by immobilized cells, which was dependent on the presence of very low amounts of sulfate in the medium. The results, which are shown in Figure 4,



**Figure 4.** Hydrogen Photoproduction Rates (open circles) and Total Amount of H<sub>2</sub> Accumulated (closed circles) by Algal Cells Immobilized on Glass Fibers and Washed Continuously with Tris-Acetate-Phosphate (TAP) Buffer Containing 10  $\mu\text{M}$  Sulfate

demonstrate that the cultures (18 mL) were able to produce H<sub>2</sub> continuously for a total of 3 months at a rate of 5 mL d<sup>-1</sup>. When compared to the rates of the cell suspension system (1 L volume), the immobilized algae are 20 times more productive, at 300 mL L<sup>-1</sup> d<sup>-1</sup>. It is clear that cell immobilization permits significant improvements in the light conversion efficiency and productivity of the cultures. Questions about the economics of an immobilized system and potential biochemical limitations on the rates of H<sub>2</sub> production remain to be addressed.

## **Conclusions**

- The development of computational simulations to identify O<sub>2</sub> diffusion pathways into the [FeFe]-hydrogenases is identifying targets for site-directed mutagenesis aimed at restricting O<sub>2</sub> access to the catalytic site. Preliminary experimental results have identified the need to concomitantly close the two pathways for O<sub>2</sub> diffusion.
- We have created a hydrogenase knockout mutant in the *Synechocystis* host that totally lacks hydrogenase background activity. With a clean background, any new activity in the recombinant will verify the successful transfer and expression

of a foreign hydrogenase. Moreover, we have demonstrated that CBS hydrogenase can use the *Synechocystis* host ferredoxin as the electron mediator for the photoproduction of H<sub>2</sub>. Minimizing the number of genes to be transferred will likely improve our chances of success in constructing a recombinant organism.

- We clearly demonstrated the advantages of cell immobilization as a means of significantly increasing culture productivity by the sulfur-deprived, H<sub>2</sub>-producing algal system. In the future, we will focus on optimizing cell immobilization matrices.

## **Special Recognitions & Awards/Patents Issued**

1. Structural, Functional and Integration Studies of Biocatalysts for Development of Solar-Driven, Bio-Hybrid H<sub>2</sub>-Production Systems, grant awarded by DOE's Office of Basic Energy Science.
2. Hydrogen from Water in a Novel Recombinant Oxygen-Tolerant Cyanobacterial System, grant awarded by the DOE HFCIT Program collaborating with the J. Craig Venter Institute.
3. Application of Large-Scale Genomic Analyses for Understanding Algal Hydrogen Metabolism, grant awarded by DOE's Office of Biological and Environmental Research, GTL Program.
4. Process and Genes for Expression and Overexpression of Active [Fe] Hydrogenases, provisional patent application filed (February 2005).
5. Hydrogen Production Using Hydrogenase-Containing Oxygenic Photosynthetic Organisms, provisional patent application allowed (March 2005).

## **FY 2005 Publications**

1. Boichenko, V. A., Greenbaum, E., and Seibert, M. (2004). Hydrogen production by photosynthetic microorganisms. In *Photoconversion of Solar Energy: Molecular to Global Photosynthesis* (M. D. Archer and J. Barber, eds.). Vol. 2. Imperial College Press, London, pp. 397–452.
2. Cohen, J., Kim, K., King, P., Ghirardi, M. L., Seibert, M., and Schulten, K. (2005). Finding gas diffusion pathways in proteins: O<sub>2</sub> and H<sub>2</sub> gas transport in Cpl hydrogenase and the role of packing defects. *Structure* 13, 1321-1329.
3. Cohen, J., Kim, K., Posewitz, M., Ghirardi, M. L., Schulten, K., Seibert, M., and King, P. (2005).

- Molecular dynamics and experimental investigation of H<sub>2</sub> and O<sub>2</sub> diffusion in [Fe]-hydrogenase. *Biochem. Soc. Transact.* 33, 80–82.
4. Fedorov, A., Kosourov, S., Seibert, M., and Ghirardi, M. L. (2005). Continuous hydrogen photoproduction by *Chlamydomonas reinhardtii* using a novel two-stage, sulfate-limited chemostat system. *Appl. Biochem. Biotechnol.* 121–124, 403–412.
  5. Ghirardi, M. L., King, P., Kosourov, S., Forestier, M., Zhang, L., and Seibert, M. (2005). Development of algal systems for hydrogen photoproduction—addressing the hydrogenase oxygen-sensitivity problem. In *Artificial Photosynthesis* (Collings, ed.). Wiley – VCH Verlag, Weinheim, Germany, 213–227.
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  8. Invited cover for *Biochemical Society Transactions*, Vol. 33 (2005).
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  10. Kosourov, S., Makarova, V., Fedorov, A. S., Tsygankov, A., Seibert, M., and Ghirardi, M. L. (2005). The effect of sulfur re-addition on hydrogen photoproduction by sulfur-deprived green algae. *Photosynth. Res.*, in press.
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  12. Maness, P. C., Huang, J., Smolinski, S., Tek, V., and Vanzin, G. (2005). Energy generation from the CO oxidation: hydrogen production pathway in *Rubrivivax gelatinosus*. *Appl. Envir. Microbiol.* 71, 2870–2874.
  13. Melis, A., Seibert, M., and Happe, T. (2004). Genomics of algal hydrogen production. *Photosynthesis Research* 82, 277–288.
  14. Metting, F. B., Scott, M. J., Benemann, J., Greenbaum, E., Seibert, M., Spormann, A., Yukawa, H., and Houghton, J. (2004). Microbial and enzyme biotechnology: 21st century opportunities for greenhouse gas mitigation. In *Application of Biotechnology to Mitigation of Greenhouse Warming—Proceedings of the St. Michaels II Workshop, April 2003* (N. J. Rosenberg, F. B. Metting, and R. C. Izaurralde, eds.). Battelle Press, Columbus, OH, pp. 115–136.
  15. Seibert, M., Maness, P. C., and Ghirardi, M. L. (2004). Algal hydrogen production—an innovative approach. *Fuel Cell Catalyst* 4, 3.

### **FY 2005 Presentations**

1. Participation in the American Chemical Society (ACS) Bio-Chief Technical Officer's summit in Washington, DC (October 2004) by Seibert
2. Invited plenary talk entitled Hydrogen Photoproduction from Water in Green Algae at the 16th International Genome Sequencing and Analysis Conference in Washington, DC (September 2004) by Seibert
3. Seminar presentation at the Stanford University, Hopkins Marine Station in Pacific Grove, CA (June 2004) by Seibert
4. Presentation to the University of Minnesota delegation (October 2004) by Ghirardi and Maness
5. Presentation to the DOE Hydrogen Production TechTeam visit (January 2005) by Seibert and Maness
6. NREL's Power Lunch presentation (February 2005) by Ghirardi
7. National Hydrogen Association meeting in Washington, DC (March 2005) by Ghirardi
8. Invited seminar at Penn State University (March 2005) by Maness
9. Class on Photobiological Hydrogen Production at the European Genetics Foundation Course in Italy (March 2005) by Seibert
10. Invited presentation to DOE's Hydrogen, Fuel Cells, and Infrastructure Technologies (HFCIT) Program (May 2005) by Ghirardi
11. NREL's Chemical Sciences seminar (May 2005) by Ghirardi
12. Poster at the Biofuels meeting in Denver, CO (May 2005) by Fedorov
13. Poster presentations at the Steenbock meeting on FeS proteins (May 2005) by King, Kim, and Chang

14. Presentation to the Brazilian Delegation visit (May 2005) by Ghirardi and Maness
15. Secretary of Energy's visit to NREL (June 2005) by Ghirardi

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