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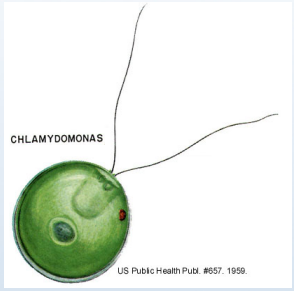
Office of Energy Efficiency & Renewable Energy

National Renewable Energy Laboratory

Innovation for Our Energy Future

Biological Systems for Hydrogen Photoproduction



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National Renewable Energy Laboratory

DOE Hydrogen, Fuel Cells & Infrastructure Technologies Program Review

May 24, 2005

Project ID# PD16

This presentation does not contain any proprietary or confidential information.

Overview

Timeline

- Project start date: FY00
- Project end date: continuing
- Percent completed: N/A

Budget

- Funding received in FY04: $710K ($20K for subcontract).
- Funding for FY05: $785K ($20K for subcontract).

Barriers

• **Barriers addressed:** Production Barrier Z: Continuity of H_2 photoproduction

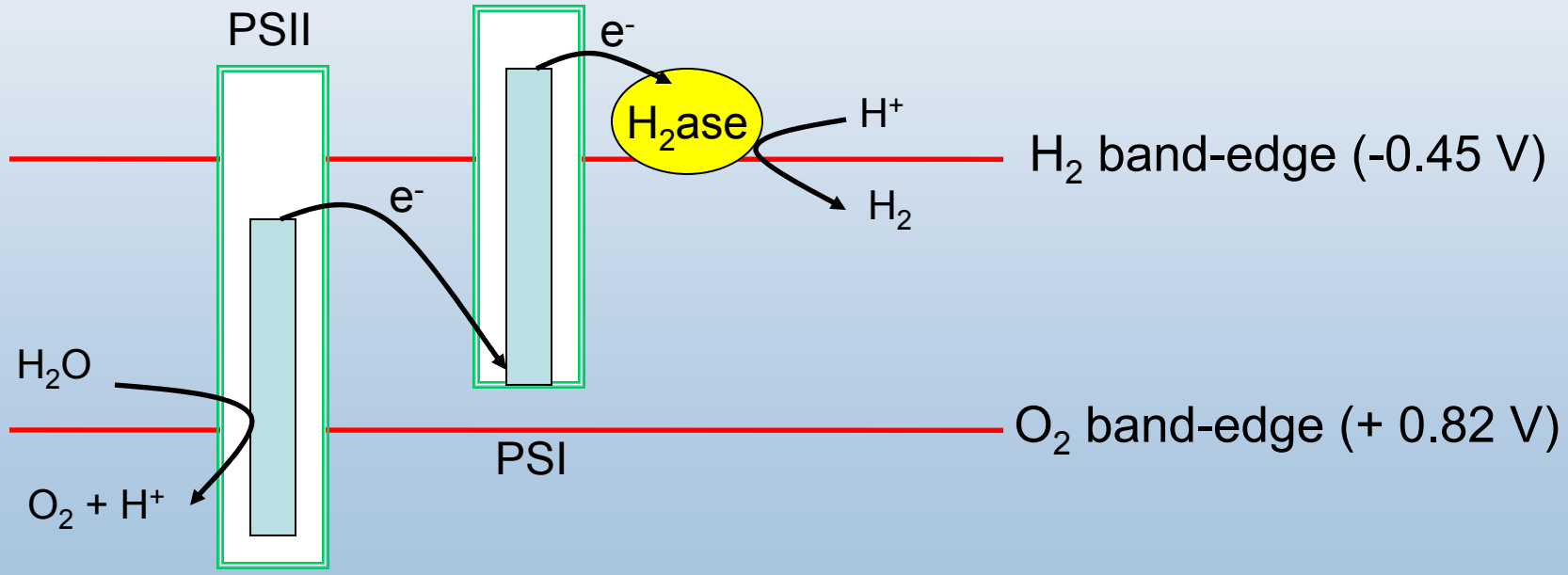
Partners

• Interactions/ Collaborations:
Dr. Klaus Schulten, Beckman Institute, University of Illinois; Dr. Juan Fontecilla-Camps, CEA/CNRS, Grenoble, France; Dr. Michael Flickinger, University of Minnesota; Dr.Hamilton Smith, J. Craig Ventner Institute, Rockville, MD; Drs. Matthew Posewitz and Dianne Ahmann, Colorado School of Mines, Golden CO.

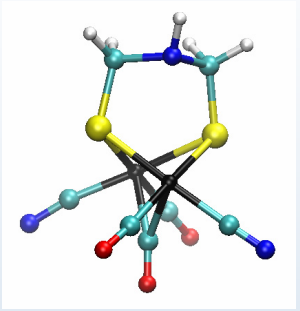
• Subcontractors:
Dr. Anatoly Tsygankov, Institute of Basic Biological Problems, Pushchino, Russia.

Project Goal

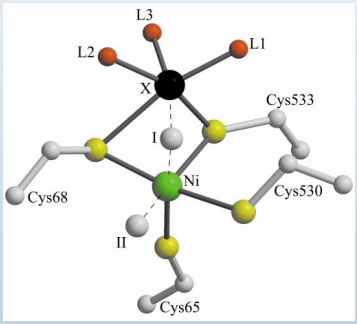
Develop photolytic H_2-production technologies based on microbial H_2O-splitting processes that are not inhibited by O_2.



Technical Approaches



Subtask 1. Engineer an algal [FeFe]-hydrogenase that is resistant to O_2 inactivation;



Subtask 2. Introduce the gene encoding for a [NiFe]-hydrogenase with increased O_2 resistance into a water-splitting, photosynthetic cyanobacterial system;

Subtask 3. Develop and optimize a physiological method to promote culture anaerobiosis and subsequent H_2-production activity in algae.

Objectives for this past year

- **Subtask 1.** Conduct computational simulations of O_2 and H_2 gas diffusion in [FeFe]-hydrogenases and identify targets for site-directed mutagenesis aimed at decreasing O_2 access to the catalytic site; test for **continuity of H_2 photoproduction in the presence of O_2**; start mutagenesis work to implement identified changes.

- **Subtask 2.** Demonstrate the feasibility of linking cyanobacterial photosynthetically-produced reductants to H_2 production by an O_2-tolerant bacterial [NiFe]hydrogenase to allow **continuity of H_2 photoproduction**;

- **Subtask 3. Extend H_2 production** in the continuous system by adjusting algal culture parameters; demonstrate continuous H_2 photoproduction using immobilized algal cultures.

Technical Accomplishments/Progress
Subtask 1

FY05 Results

[FeFe]-hydrogenases from anaerobic, non-photosynthetic bacteria can be synthesized using the same NREL-discovered genes that are responsible for the assembly of algal hydrogenases. The O_2-tolerance of these bacterial hydrogenases is significantly higher than that of algal hydrogenases, which makes them better candidates for further mutagenesis improvement.

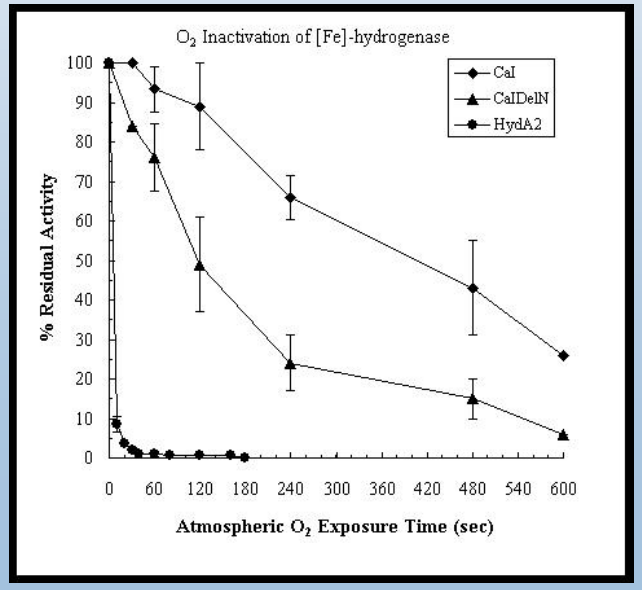


Table 1. Comparison of algal and bacterial [FeFe]-hydrogenase O_2 sensitivities

[FeFe]-hydrogenase	I_{50} value (s)
C. reinhardtii HydA1 and HydA2	<1
Clostridium pasteurianum CpI	120–300
Clostridium acetobutylicum HydA	415±115
Clostridium acetobutylicum HydAN	145±45

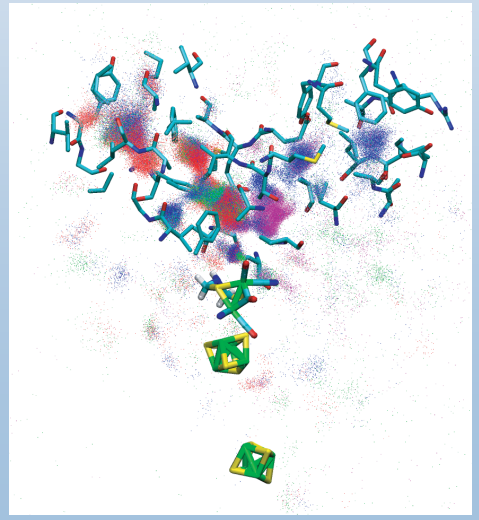
Technical Accomplishments/Progress
Subtask 1

FY05 Results

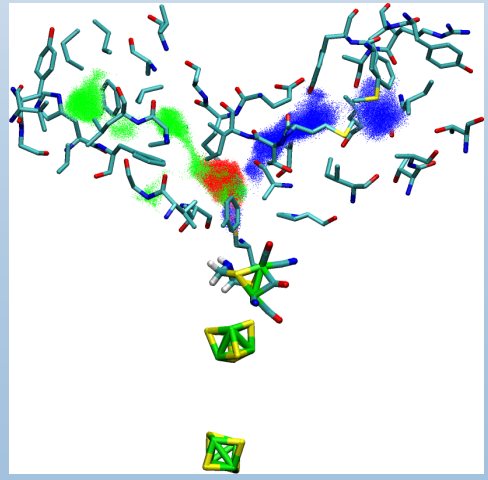
Molecular dynamics modeling of gas diffusion into the *Clostridium pasteurianum* [FeFe]-hydrogenase CpI identified only two well-defined pathways for O_2 diffusion and multiple pathways for H_2 diffusion. These results suggest that it is possible to affect O_2 accessibility to the hydrogenase's catalytic site without necessarily affecting the outward diffusion of H_2 gas produced by the enzyme.

Collaborators: K. Schulten, University of Illinois, and the NREL Computational Sciences Center.

H_2 pathways



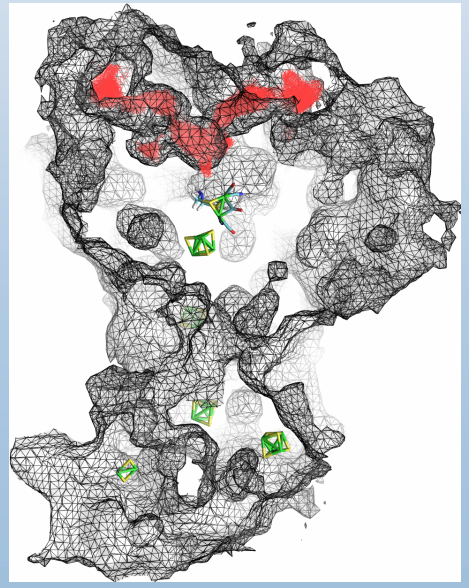
O_2 pathways



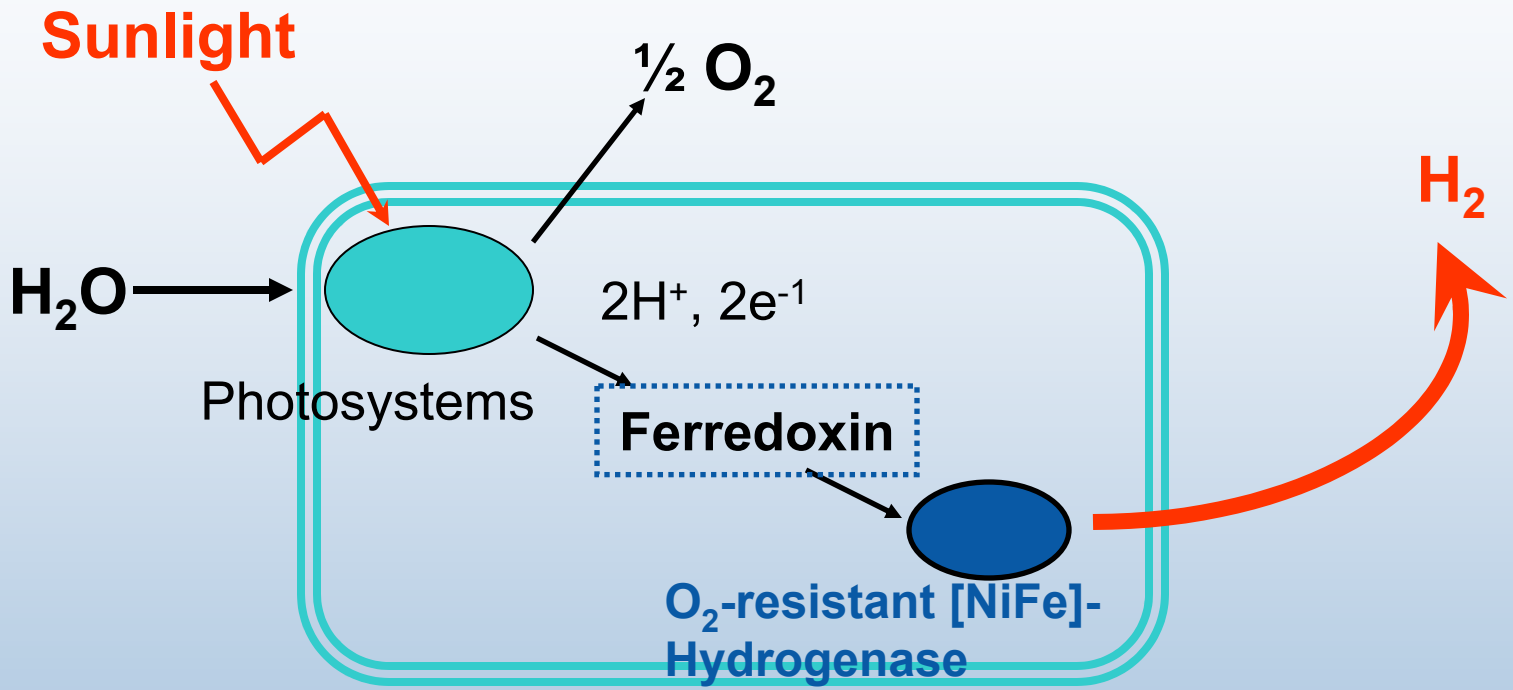
Technical Accomplishments/Progress Subtask 1

FY05 Results

Volumetric solvent accessibility maps were shown to confirm the O_2 pathways revealed by molecular dynamics simulations. These data were used to find the effect of *in silico* mutations on the accessibility of the catalytic site to O_2. Some of the site-directed mutations have been implemented *in vitro*; mutations of sites very near the catalytic site resulted in reduced enzymatic activity. Mutations of a single O_2 pathway resulted in only small increases in O_2 tolerance.



Technical Accomplishments/Progress
Subtask 2



Cyanobacterial Recombinant

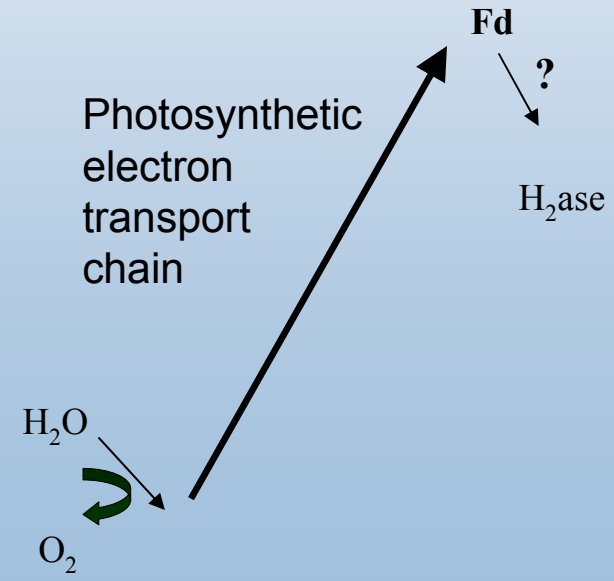
- A complementary approach to surmount the O_2-sensitivity issue

Technical Accomplishments/Progress Subtask 2

FY05 Results

The linkage between photosynthetic H_2O oxidation (using spinach photosystems), *Synechocystis* ferredoxin and CBS hydrogenase was demonstrated *in vitro*.

$H_2O \longrightarrow$ **Ferredoxin (Fd)** $\longrightarrow H_2$



Electron Mediator	Rate of H_2 Production (µmol H_2/mg chl/h)
Methyl viologen	484.1
***Synechocystis* Ferredoxin**	**16.2**
Clostridium Ferredoxin	22.4
Red algal Ferredoxin	17.6

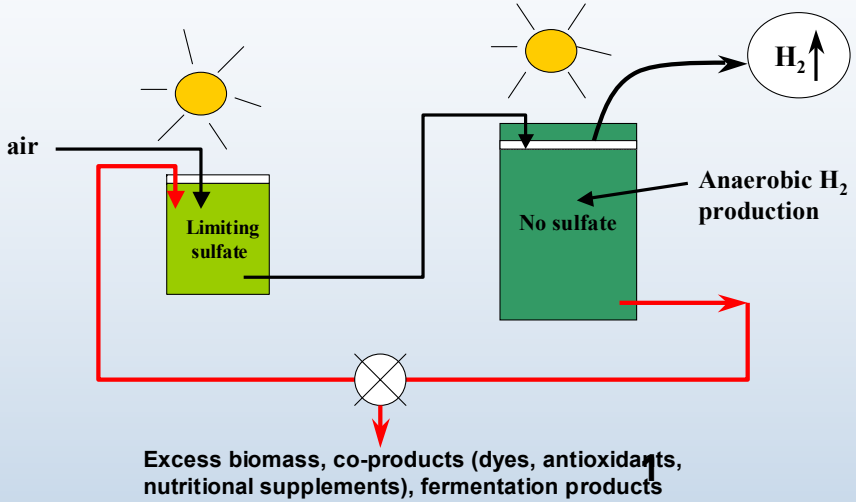
Technical Accomplishments/Progress
Subtask 2

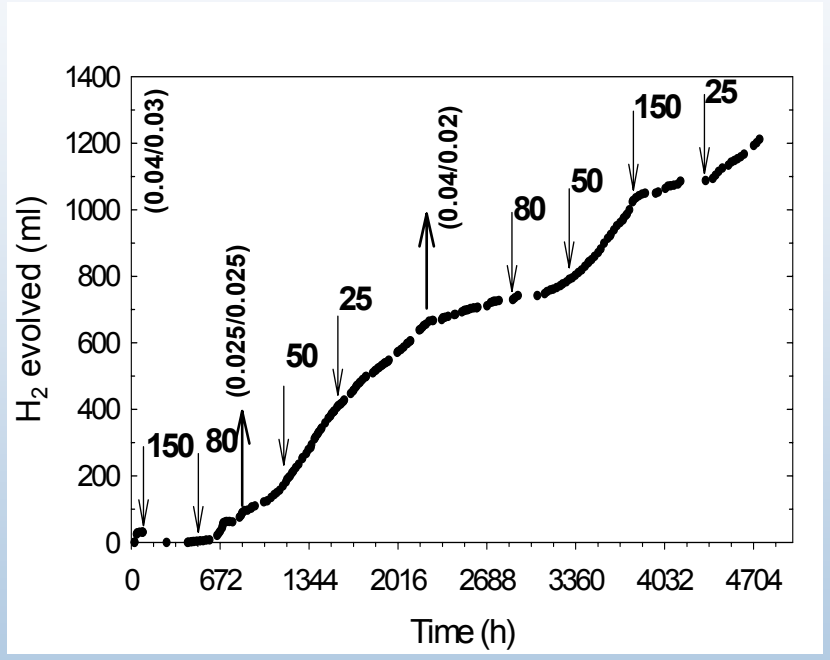
FY05 Results

The host's native O_2-sensitive hydrogenase was knocked-out by mutagenesis, yielding a clean background strain for introduction of the CBS hydrogenase genes.

	Hydrogenase Activity
Wild Type	650
Hydrogenase Knockout Mutant	0

Technical Accomplishments/Progress
Subtask 3





FY05 results

The running conditions for the continuous H_2 production system were optimized and the algal cultures now produce 15 ml H_2 L^{-1} d^{-1}. We are investigating biochemical (not engineering) rate limitations.

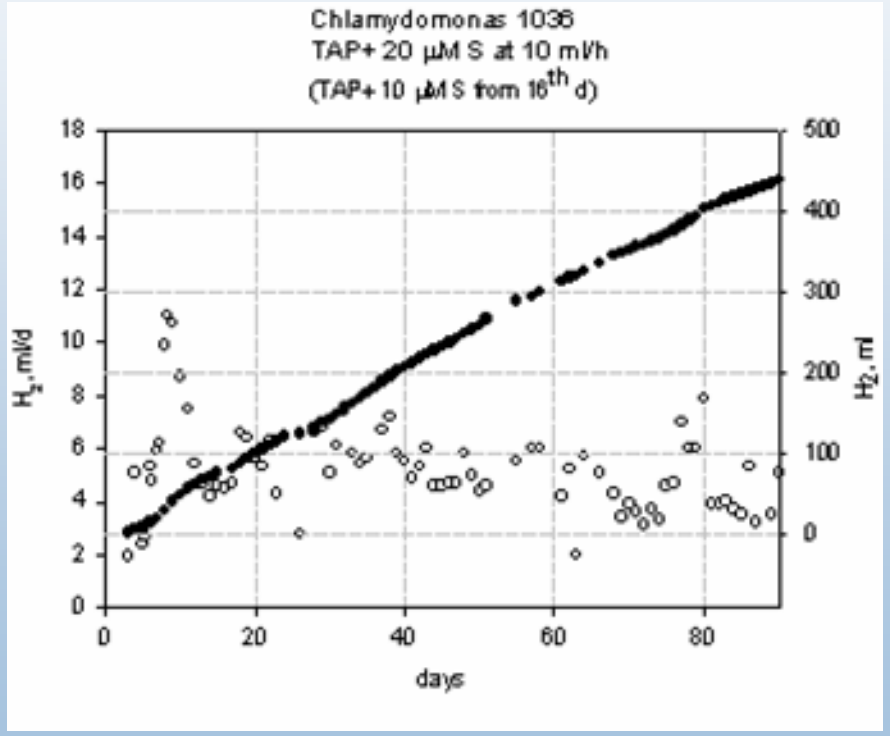
Technical Accomplishments/Progress
Subtask 3

FY05 Results

Algae were immobilized on fiberglass surfaces and subjected to continuous flow of 10 μM sulfate at 6 ml/h. The average rate of H_2 production was about 300 ml $L_{immobilized\ cells}^{-1}d^{-1}$, 20X higher than the average rate obtained with suspended cultures.

Collaborators:

Dr. Anatoly Tsygankov
and colleagues at the
Institute of Basic Biological
Problems, Pushchino, Russia.



Volume of immobilized cells: 18 ml

Responses to Previous Year Reviewers' Comments

1. *"Difficult to relate project to overall DOE objectives"; "The long-term aspect of the work means it is not critical to the President's H_2 Initiative, but is relevant to the long-term vision of DOE"; "Relevant, but progress has been extremely slow".* **The National Academy of Sciences recommended that the Program fund long-term projects related to the production of renewable H_2. The HFC&IT Program is committed to fully support long-term, high potential photobiological research. The progress has not been slow for a long-term project, and many important tools have been developed and tested since the project's inception in FY2000.**

2. *"Might it be possible to utilize a synthetic analog of hydrogenases to perform the $e^- + H^+ <-->H_2$ reaction, even in the presence of O_2?"* **At present, there are no inexpensive synthetic analogs of hydrogenases that perform the reaction in the presence of O_2, although there is on-going research to achieve that. Besides the catalyst, however, one needs an efficient source of light-generated reductants, which the microorganisms can easily provide.**

3. *"Current subtask 2 is important to extend to other species, but is hydrogenase knock-out the first best step?"* **Following the reviewers' suggestions, our work has focused on two first steps: the generation of a hydrogenase knock-out mutant and the demonstration of linkage between cyanobacterial photosynthesis and O_2-tolerant hydrogenase activity *in vitro.* This work will be followed closely by the introduction of the O_2-tolerant hydrogenase gene(s) into the cyanobacterial host for *in vivo* linkage in FY06 and beyond.**

4. *"An economic analysis would be an appropriate next step."* **Due to lack of funding, this task has been postponed for next year.**

Future Work

Subtask 1

- Continue iterative process of (a) O_2-gas-diffusion/solvent accessibility computational simulations and (b) experimental generation and testing of the O_2-resistance of hydrogenase mutants expressed in *E. coli*. Generate double mutants affecting both O_2 pathways (**milestone FY06**).
- Further refine the computational simulations and initiate studies of the effect of *in silico* mutations on gas diffusion, concomitant with *in vitro* mutations;
- Crystallize the algal HydA1 and HydA2 hydrogenases for future use as model systems in computational simulations as well.

Subtask 2

- Further characterize the O_2 tolerance of the bacterial [NiFe]-hydrogenase (**milestone FY06**);
- Genetically transfer the CBS hydrogenase gene(s) initially to the host, *E. coli* and later to the cyanobacterial host.

Subtask 3

- Identify possible limitations in electron carriers as the cause for the slow H_2 production rates observed with the H_2-production system (**milestone FY05**);
- Study the feasibility of using low-cost matrices for cell immobilization (**milestone FY06**);
- Perform an economic analysis of immobilized algal systems.

Integrated System

- Collaborate with UCB and ORNL on the development of a system that integrates photosynthetic H_2 production by oxygenic and non-oxygenic organisms and fermentation.

Publications

Published

1. Ghirardi, ML and W. Amos. **2004**. Hydrogen photoproduction by sulfur-deprived green algae – status of the research and potential of the system. *Biocycle* 45, 59.
2. Hahn, JJ, ML Ghirardi and WA Jacoby. **2004**. Effect of process variables on photosynthetic algal hydrogen production. *Biotechnol. Progr*. 20, 989-991.
3. Seibert, M, PC Maness and ML Ghirardi. **2004**. Algal hydrogen production – an innovative approach. *Fuel Cell Catalyst* 4, 3.
4. Posewitz, MC, PW King, SL Smolinski, L Zhang, M Seibert and ML Ghirardi. **2004**. Discovery of two novel Radical SAM proteins required for the assembly of an [Fe]-hydrogenase. *J. Biol. Chem*. 279, 25711-25720.
5. Melis, A., M. Seibert, and T. Happe **2004** Genomics of Algal Hydrogen Production. *Photosynthesis Research*. 82, 277-288.
6. Ghirardi, ML, PW King, MC Posewitz, PC Maness, A Fedorov, K Kim, J Cohen, K Schulten and M. Seibert. **2005**. Approaches to developing biological H2-photoproducing organisms and processes. *Biochem. Soc. Transact*. 33, 70-72.
7. Cohen, J, K Kim, M Posewitz, ML Ghirardi, K Schulten, M Seibert and P King. **2005**. Molecular dynamics and experimental investigation of H_2 and O_2 diffusion in [Fe]-hydrogenase. *Biochem. Soc. Transact*. 33, 80-82.
8. Invited cover for Biochemical Society Transactions, vol. 33. 2005.

In press and submitted

9. Fedorov, A, S Kosourov, M Seibert and ML Ghirardi. **2005**. Continuous hydrogen photoproduction by *Chlamydomonas reinhardtii* using a novel two-stage, sulfate-limited chemostat system. *Appl. Biochem. Biotechnol*., in press.
10. Kosourov, S, V Makarova, AS Fedorov, A Tsygankov, M Seibert and ML Ghirardi. **2005**. The effect of sulfur re-addition on hydrogen photoproduction by sulfur-deprived green algae. *Photosynth. Res*., in press.
11. Ghirardi, ML, P King, S Kosourov, M Forestier, L Zhang and M Seibert. **2005**. Development of algal systems for hydrogen photoproduction – addressing the hydrogenase oxygen-sensitivity problem. In: *Artificial Photosynthesis*,(Collings, ed), Wiley – VCH Verlag, Weinheim, Germany, in press.
12. Maness, P. C., J. Huang, S. Smolinski, V. Tek, and G. Vanzin **2005** "Energy Generation from the CO Oxidation: Hydrogen Production Pathway in *Rubrivivax gelatinosus*.. *Appl. Envir. Microbiol*., in press.
13. Cohen, J, K Kim, P King, ML Ghirardi, M Seibert and K Schulten. "Finding gas diffusion pathways in proteins: O_2 and H_2 gas transport in CpI hydrogenase and the role of packing defects." Submitted.
14. Ghirardi, ML, A Melis, JW Lee, E Greenbaum and M Seibert. **2004**. Photobiological H_2 production in the USA. *Proc. World Renewable Energy Congress*, *Denver, 2004*, in press.

Presentations and Others

Visitors

National Advisory Council, NREL's Technology Day participants, the National Association of State Universities and Land Grant Colleges, Dr. Michael Nobel (head of the Nobel Foundation), Kathi Epping and Dr. Tom Sheahen (DOE HFC&IT Program), Dr. Steve Schlasner (a consultant for the HFC&IT Program), Dr. Ray Stults (Director of the Office of Science at Los Alamos National Lab), Dr. Jungmeier (Johanneum Research Institute, Austria).

Meetings and Presentations

- German 298 Symposium on Protein-Cofactor Interactions in Biological Processes (Berlin, May 2004);
- 26th Symposium on Biotechnology for Fuels and Chemicals and special session on Hydrogen Research organized by Dr. James Lee, ORNL (Chattanooga TN, May 2004);
- 7th International Hydrogenase meeting (England, August 2004);
- World Renewable Energy Conference (Denver CO, September 2004);
- NREL's Power Lunch presentation (Golden CO, February 2005);
- National Hydrogen Association meeting (Washington D.C., March 2005);
- Invited seminar at Penn State University (College Park PA, March 2005);
- Class on Photobiological Hydrogen Production at the European Genetics Foundation Course (Italy, March 2005).

Workshops and Panels

- Participation in an NSF advisory panel on Hydrogen research (June 2004);
- Participation in a workshop to create a joint institute between NREL and the Colorado School of Mines to support hydrogen and fuel cell research (Golden, July 2004);
- Co-organization of a workshop with NREL's Computational Sciences Center on the role of computation in biology (September 2004);
- Participation in the ACS-Bio-Chief Technical Officer's summit (Washington D.C., October 2004);
- Reviewer for the Natural Sciences and Engineering Research Council of Canada (January 2005);
- Meeting with DOE's Hydrogen Production Technical Team (January 2005);
- Participation in the revision of the Outcome Map for biological H_2 production (January 2005).

Patents

"Process and Genes for Expression and Overexpression of Active [Fe]-Hydrogenases.", patent application filed (Jan 05).

"Hydrogen Production Using Hydrogenase-containing Oxygenic Photosynthetic Organisms", provisional patent allowed (Feb. 05).

Hydrogen Safety

The most significant hydrogen hazards associated with this project are:

(a) Hydrogen pressure build-up, potentially explosive mixtures of H_2 and O_2, and H_2 leaks from anaerobic chambers and from the enclosed photobioreactors;

(b) Use of genetically-modified organisms (GMOs);

(c) Use of radioactivity to monitor gene expression

(As stated in the Safety Evaluation Report submitted to the HFC&IT Program by the Safety Review Team in June, 2005).

Hydrogen Safety

Our approaches to deal with these hazards are:

(a) Anaerobic gas chambers: addressed in Maness' presentation (Project PD 18).

(b) Photobioreactors: A catastrophic leak might release up to 0.5 liters of H_2 at one time, which again is negligible in a large laboratory air volume. Warning signs have been posted in the laboratories, and an appropriate ventilation system to ensure dispersion of any H_2 leaked are described above.

(c) Currently, the GMOs being generated for our project do not have any survival advantages over their unmodified parental strains, and in fact are far less equipped to survive if released accidentally into Nature than the parental wild-type strains. However, the following procedures are implemented routinely to prevent the transmittance of a mutation into other laboratory or outside strain: (i) containment (mutant strains are grown in glass containers or agar plates and not mixed with wild-type strains) and (ii) safe disposal (all GMOs are killed by heat or bleaching before being disposed of into the local water system).

(d) We have just transitioned from using radioactivity to measure gene expression to using a non-radioactive method (real-time PCR).

(As stated in the Safety Evaluation Report submitted to the HFC&IT Program by the Safety Review Team in June, 2005).