II.E.2 Biological Systems for Hydrogen Photoproduction

Maria L. Ghirardi (Primary Contact), Paul King and Michael Seibert
National Renewable Energy Laboratory (NREL)
1617 Cole Blvd.
Golden, CO 80401
Phone: (303) 384-6312; Fax: (303) 384-6150
E-mail: maria_ghirardi@nrel.gov

DOE Technology Development Manager:
Roxanne Garland
Phone: (202) 586-7260; Fax: (202) 586-9811
E-mail: Roxanne.Garland@ee.doe.gov

Subcontractor:
Anatoly Tsygankov
Institute of Basic Biological Problems
RAS, Pushchino, Russia

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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_2 inactivation as part of an aerobic H_2-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_2-production activity in algae.
- Address two components of an innovative H_2-production system based on integrating fermentative and photosynthetic H_2-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_2-tolerant hydrogenase)
O_2 tolerance (half-life in air)	2–4 min (*Clostridium* hydrogenase *in vitro)*	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_2 diffusion along specific pathways.
- Generated site-specific mutations at amino acid residues identified as being potential targets for mutagenesis aimed at preventing O_2 access to the catalytic site of the [FeFe]-hydrogenase. A single mutation along one of the O_2 channels increased the enzyme's tolerance to O_2, 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_2 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_2 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.

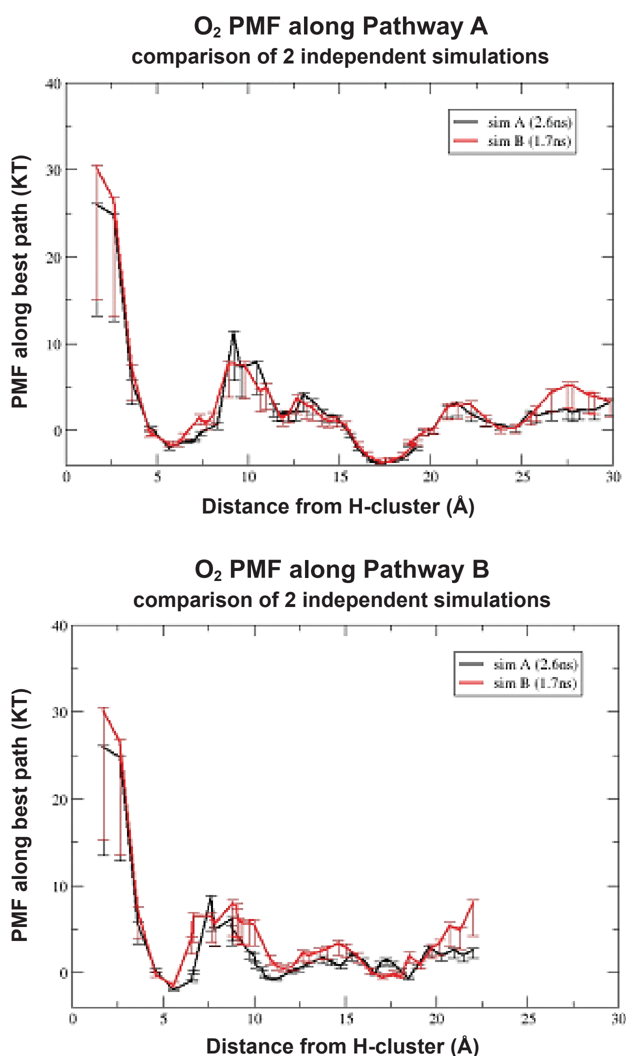

Figure 1. Mean Free Potential Energy Profiles along the Putative O_2 Channels A and B, using the CaI [FeFe]-hydrogenase from *Clostridium Acetobutylicum*

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 $1/8^{th}$ 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 x 261 cm^2 = 522 cm^2	200 cm^2
Rate of H_2 production per reactor during the time of operation (ml/h and μmoles/h);	2.5 and 82.5	0.7 and 31.22
Energy of incident light per m^2 per hour	154,080 J/m^2	92,448 J/m^2
Efficiency of incident light energy conversion into H_2	0.24%	0.36% **(50% improvement)**

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_2 production. Future work will involve immobilization of photoautotrophically-grown algal cultures for H_2 production.

Conclusions and Future Directions

Conclusions

- The development of computational simulations to identify O_2-diffusion pathways into the [FeFe]-hydrogenases is uncovering targets for site-directed mutagenesis aimed at restricting O_2 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_2-producing algal systems. Moreover, we identified one of the limiting factors that may prevent higher rates of H_2 production. Future work will involve assessing H_2-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_2 pathways.
  - Develop a new in vitro assay to test for O_2 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_2 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 H2-production capabilities of a mutant that is unable to conduct wasteful electron transport.
  - Optimize photoautotrophic systems for H_2 production and test cell immobilization.
  - Study the feasibility of lower cost matrices to immobilize H_2-producing algae.
- Task 3 (Integrated System)
  - Address the efficiency of algal biomass fermentation by bacteria and the efficiency of H_2 production by S-deprived algal cultures integrated with H_2-producing photosynthetic bacteria.
  - Screen the NREL collection of photosynthetic bacteria for those that produce H_2 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. Biophy.*

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.