NREL
NATIONAL RENEWABLE ENERGY LABORATORY

Biological Systems for Hydrogen Photoproduction

Maria L. Ghirardi

Key personnel:

M. Seibert, P. King,
K. Ratcliff,
S. Smolinski

National Renewable Energy Laboratory

2010 Annual Merit Review and Peer Evaluation Meeting, 10 June 2010, Washington, D.C.

NREL/PR-560-48068

Project ID # 37

This presentation does not contain any proprietary, confidential, or otherwise restricted information

Overview

Timeline

Project start date: FY00

Project end date: FY18

Percent complete: N/A

Barriers

Production barriers addressed

- Continuity of H_2 production (AI)
- Feedstock cost in an integrated system (AT)
- Rate of H_2 production (AH)

Budget

Funding received in FY09: $800K

Funding allocated for FY10: $600K

Partners

Drs. Anatoly Tsygankov and Sergey Kosourov, Institute of Basic Biological Problems, RAS, Pushchino, Russia

Dr. Michael Flickinger, North Carolina State University

Dr. Eric Johnson, Johns Hopkins University

Drs. Iftach Yacoby and Shuguang Zhang, MIT

* New tasks in orange

Objectives/Relevance

General: Develop photobiological and integrated photobiological/fermentative systems for large-scale H_2 production.

- **Task 1**: Address the O_2-sensitivity of hydrogenases, which prevents continuity of H_2 photoproduction under aerobic, high solar-to-hydrogen (STH) conditions.
- **Task 2**: Utilize a limited STH H_2-producing method (sulfur deprivation) as a platform to address other factors limiting commercial algal H_2 photoproduction.
- **Task 3**: Integrate photobiological and fermentative systems in different configurations for less costly H_2 production in the short term.

Parameters	Current Status	2013 Targets	Maximum Potential
Duration of continuous photoproduction • Aerobic, high STH (O_2-tolerant) • Aerobic, limited STH (S-deprivation) • Anaerobic, limited STH (S-deprivation)	 0 10 days 90 days	 30 min	 12 hours indefinite indefinite
O_2 tolerance (half-life in air) • Oxidized conditions • Reduced conditions	 4 min 40 min		
Cost ($/kg H_2) • Aerobic, high STH (O_2-tolerant) • Anaerobic, limited STH (S-deprivation) • Integrated (photo + fermentative)			 $2.99 $6.02 $3.21

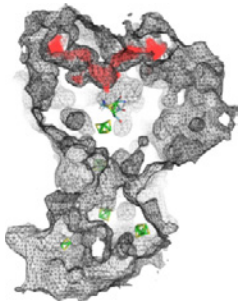
Task 1 – O_2 Sensitivity/Rate of Hydrogenases Objectives, Approaches, and Collaborations

Objectives: (1) Develop and optimize *aerobic, high-STH* photobiological systems for the production of H_2 from water by engineering a H_2-producing catalyst ([FeFe]-hydrogenase) that has an extended half-life following exposure to O_2.

(2) Explore fusions between hydrogenase and ferredoxin to increase photosynthetic electron flow to the hydrogenase (this is unrelated to O_2 sensitivity, but it addresses the rate of H_2-production barrier).

Approaches:

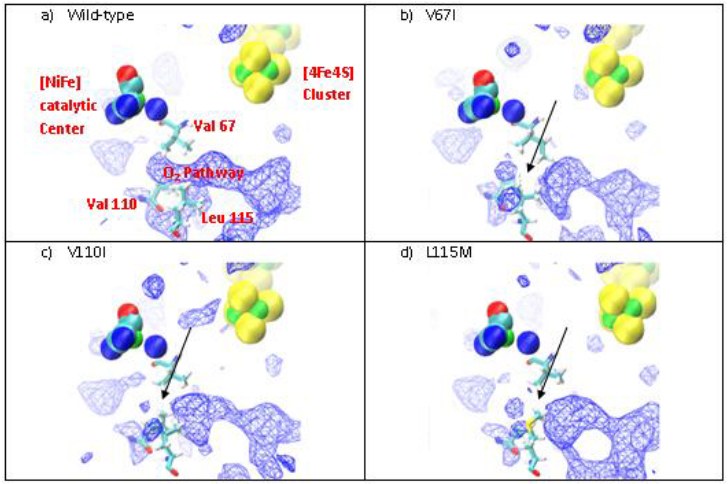
- Use computational simulations to identify pathways by which O_2 accesses the catalytic site and use site-directed mutagenesis to molecularly engineer the enzyme to prevent O_2 access.
- Use random methods to generate mutants with higher O_2 tolerance.
- Introduce a more O_2-tolerant bacterial hydrogenase into algae.
- Evaluate the feasibility of creating fusions between hydrogenases and ferredoxin to increase electron flux to the hydrogenase.



Collaborator: MIT (currently unfunded)

Task 1 – O_2 Sensitivity of Hydrogenases Accomplishments and Milestones

1. **Computational modeling**: We extended analysis of pathways to [NiFe]-hydrogenases; identified 3 key residues as potential targets for mutagenesis to decrease O_2 diffusion to catalytic site:Val67, Val110 and Leu115 in *D. gigas*.



Task	Due date	Status
Use implicit ligand sampling method to map the pathways in [NiFe]-hydrogenases	January 2010	completed

2. **Site-directed mutagenesis**:
(a) We attributed the multiphasic kinetics of O_2 inactivation to the existence of three states of [FeFe]-hydrogenases, each with different tolerance toward O_2 (the reduced state is more O_2-tolerant than the oxidized one; the third state is O_2-insensitive);
(b) we are re-assessing our strategy for controlling O_2 diffusion to the catalytic site of [FeFe]-hydrogenases; a manuscript is in preparation (see future work).

Previous results showed that the clostridial H_2ase is 100X more tolerant to O_2 than the algal enzyme; *re-directed resources toward expressing the clostridial hydrogenase in Chlamydomonas to assess the effect of a more O_2-tolerant hydrogenase on H_2 production in vivo (see next slide).*

Task 1 – O_2 Sensitivity of Hydrogenases Accomplishments and Milestones

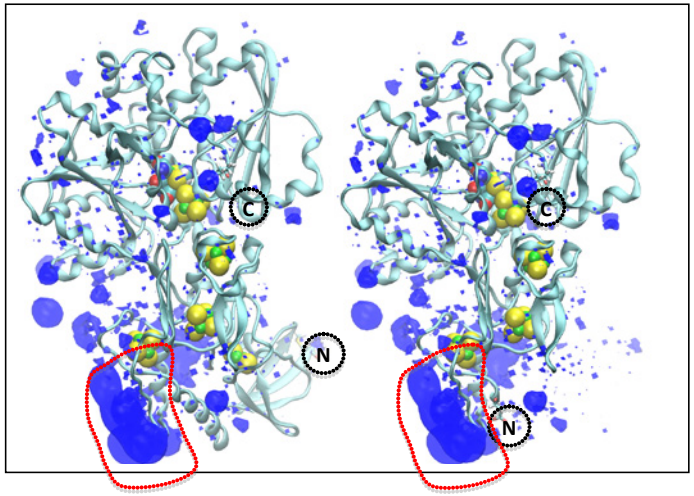
3. **Random mutagenesis**: No new results to report.

4. **Expression of the clostridial hydrogenase in Chlamydomonas**: Inconclusive activity results with one transformant; evaluation of additional transformants show expression in Chlamydomonas; activity is being evaluated.

Task	Due date	Status
Demonstrate that CaI is active in *C. reinhardtii*	February 2010	*Inconclusive; postponed*
Measure the O_2 sensitivity of H_2ase activity in *C. reinhardtii* transformants	April 2010	In progress

5. **Create fusions between hydrogenases and ferredoxin to improve reductant flux to the hydrogenase**: We simulated the docking between the Ca1 H_2ases with the algal ferredoxin to guide MIT's engineering efforts. Results suggest that the interaction could be facilitated if the clostridial hydrogenase were truncated, to reposition the N-terminus for fusion with Fd.

Task	Due date	Status
Use computational modeling to design fusions between [FeFe]-hydrogenases and ferredoxin	December 2009	completed
Create genetic constructs of CaI and PetF (by MIT)	March 2010	completed



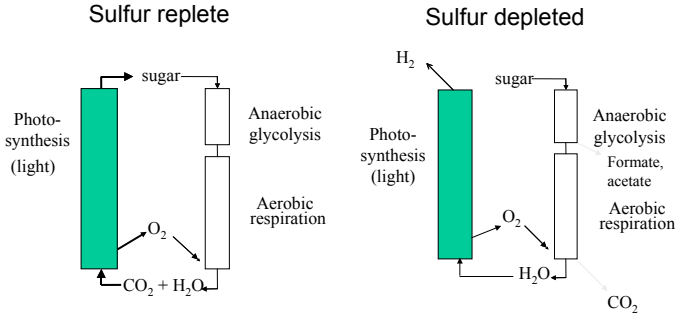
Models of docking between complete (left) or truncated (right) Ca1 H_2ase with algal ferredoxin.

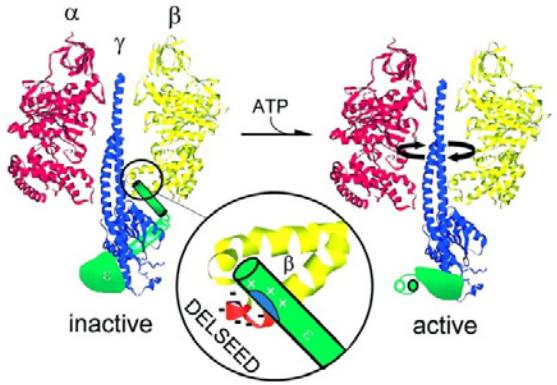
Task 1 – O_2 Sensitivity of Hydrogenases Future Work

1. **Computational simulation**: We will compare the geometry and energetics of the catalytic center and adjacent structures of [FeFe]-hydrogenases with different sensitivity to O_2. We are re-assessing how O_2 accesses the enzyme's catalytic center and to what extent this depends on channel structure/configuration.
2. **Site-directed mutagenesis**: A manuscript will be submitted summarizing current observations regarding the redox states effects on H_2ase O_2 inactivation; the approach involving gas channels is on hold until expression studies clarify whether higher hydrogenase O_2 tolerance as measured *in vitro* translates into higher O_2 tolerance *in vivo*.
3. **Random mutagenesis**: New personnel are being hired to restart the research. We will determine a new strategy based on new results from Subtask 1.
4. **Expression of clostridial hydrogenase in Chlamydomonas**: We will characterize additional constructs and, if required, design new Ca1 constructs or alternative approaches to increase H_2 production *in vivo*.
5. **Hydrogenase/ferredoxin fusions**: NREL will continue to provide guidance to MIT's work and will test their transformants in house if additional funding is available.

Task 2 – Sulfur-Deprivation Platform Objectives, Approaches, and Collaborations

Objectives: Further optimize and utilize an anaerobic, limited-STH working platform to study biochemical and engineering factors that affect H_2 photoproduction by biological organisms; focus on the effect of an inactive, leaky ATP synthase on the rates.





Approaches:

- Continue to improve the H_2-production yields by alginate-immobilized algae RAS).
- Test and optimize the performance of immobilized, photoautotrophic cultures (RAS).
- Generate inducible ATP synthase mutants and test them with the immobilized system.

Collaborators: Johns Hopkins University, the Institute of Basic Biological Problems, Russian Academy of Sciences (RAS)

Task 2 – Sulfur-Deprivation Platform Accomplishments and Milestones

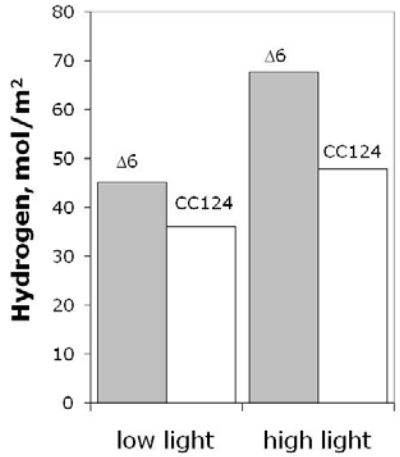
1. **Improve H_2 rates and yields using immobilized films**: Lower thickness improves rates and yields; higher thickness improves protection against O_2 inactivation under aerobic conditions and prevents acetate diffusion.

Film thickness, μm	Total Chl concentration, μg/cm^2 film	Maximum specific rate of H_2 production in argon, μmole mg Chl^{-1} h^{-1}	Maximum specific rate of H_2 production in air, μmole mg Chl^{-1} h^{-1} (% of rate in argon)	Total yield of H_2 gas in argon, mol m^{-2}	Total yield of H_2 gas in air, mol m^{-2} (% of rate in argon)
180	71.37	13.5	3.4 (25%)	0.55	0.094(17%)
260	101.6	7.8	2.8 (36%)	0.43	0.096 (22%)
290	117.74	6.1	2.6 (43%)	0.42	0.113 (27%)
310	110.89	5.9	2.3 (39%)	0.41	0.093 (23%)

2. Test and improve the performance of photoautotrophic, immobilized cultures: No results to report; work just getting started.

Task 2 – Sulfur-Deprivation Platform Accomplishments and Milestones

3. **Design ATP synthase conditional mutants**: A C-terminus-mutated ε-subunit of the ATP synthase will be expressed in the chloroplast of Chlamydomonas behind a promoter that induces expression upon anaerobiosis. Specific mutations have been identified and transformants are being screened in an immobilized environment.



Site-directed alteration of the C-terminus to remove positive charges should further stimulate H_2.

Task	Due date	Status
Design ATPase conditional mutants	December 2009	completed
Test immobilized ATPase mutants under sulfur-deprived conditions	August 2010	completed

Task 2 – Sulfur-Deprivation Platform Future Work

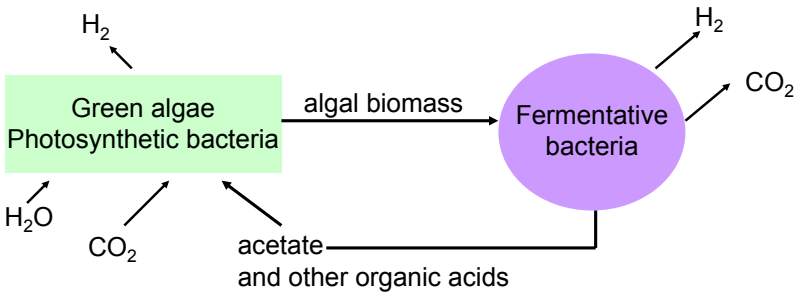
1. **Improve H_2 rates and yields using immobilized films:** Test the effect of the volume of the photobioreactor's headspace on the H_2-production properties of algal cultures.

2. **Test and improve the performance of photoautotrophic, immobilized cultures:** Adapt and improve on the methods previously used to induce photoautotrophic cultures to produce H_2 in the absence of added acetate.

3. **Construct and test the performance of Chlamydomonas inducible transformants carrying a leaky ATP synthase ε-subunit gene**: Transformants will be tested for growth, photosynthetic activity ,and H_2 production capability.

Task 3 – Integrated Systems
Objectives, Approaches, and Collaborations

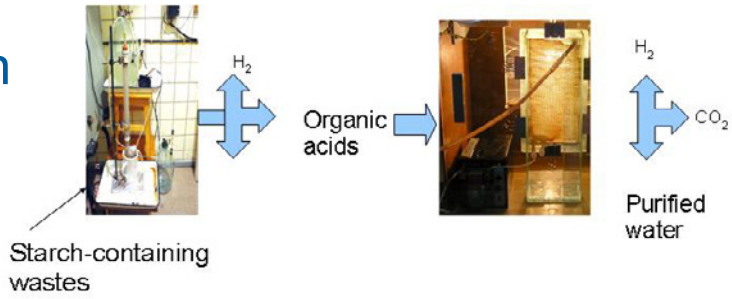
Objectives: Integrate photobiological with fermentative organisms to more efficiently utilize the solar spectrum and the substrates/products from each reaction for H_2 production.



Approaches:

- Integrate sulfur-deprived, alginate-immobilized algal H_2 production to fermentative H_2 production by an anaerobic consortium isolated from wastewater sludge.

- Integrate fermentative H_2 production from potato waste to photosynthetic H_2 production by anaerobic, purple non-sulfur bacteria (RAS).



Collaborator: Institute of Basic Biological Problems, RAS

Task 3 – Integrated Systems Accomplishments and Milestones

1. **Complete small-scale experiments on fermentability of algal biomass feedstock by the anaerobic consortium:** The consortium ferments algal biomass with a molar yield >4 , which suggests that other cell components are being utilized.

Biomass	mol H_2/mol glucose (from starch)	mg glucose (from starch)/100 mg biomass dry wt	µmol H_2/mg biomass dry wt
142h-S (fresh)	1.86	8.7	0.60
142h-S (frozen)	2.11	3.5-8.7	0.64
+S (frozen)	6.30	1.9	0.52

Feedstock	mol H_2/mol feedstock	µmol H_2/mg feedstock
Lipid	0.09	0.20
Protein	6.56	0.10

2. **Optimize fermentative H_2 production from potato waste.**
   Factors that increase rates/yields: exclusion of ammonium, addition of Fe ions, peptone and zinc; high phosphate buffering capacity; best yield: 1.6 mol H_2/mol glucose.

3. **Demonstrate sequential H_2 production from integrated dark and light-driven processes.**
   Maximum demonstrated yield from sequential process using potato waste as feedstock is 5.6 mol H_2/mol glucose.

Task	Due date	Status
Determine the fermentability of alginate films	March 2010	completed
Design and test connections between fermentors and photobioreactors	March 2010	completed
Report on the carbon mass balance and H_2 yields of a scaled-up fermentative system	September 2010	In progress

Task 3 – Integrated Systems
Future Work

1. Scale up and further optimize fermentation of suspended and immobilized algal biomass by the fermentative consortium using new fermenters.

2. Optimize the integration of the fermentative/photobiological H_2-production system using potato waste as the feedstock.

Summary

Task 1:

- Extended the computational modeling techniques used to identify gas diffusion to the *Desulfovibrio gigas* [NiFe]-hydrogenase.
- Confirmed that the reduced state of the [FeFe]-hydrogenase is more tolerant to O_2 *in vitro* than the oxidized state.
- Identified positive Chlamydomonas transformants containing the Ca1 hydrogenase gene.
- Simulated fusions between the petF ferredoxin and algal/clostridial hydrogenases to test optimal interactions.

Task 2:

- Observed that increased thickness of the alginate film improves O_2 tolerance but decreases H_2-production rates.
- Designed ATP synthase inducible mutants.

Task 3:

- Demonstrated that an anaerobic clostridial consortium ferments algal biomass, pure algal lipid,s and pure proteins.
- Optimized fermentative H_2 production from potato waste.
- Demonstrated sequential H_2 production from dark- and light-driven processes.

Supplemental Slides

Responses to Previous Year's Reviewer Comments

Reviewer's comment: "The project milestones should be better defined; The near term milestones are inadequate for this project. The milestones should include some performance targets; there are no milestones for the high-volume screening process development; milestones should be added."

Response: The new milestones (FY 2010) are now better defined, but it is much harder to set specific, quantitative performance targets for longer-term research projects, given their discovery nature. Actually, discoveries made in longer-term projects lead to redirection of projects. Regarding the high-volume screening, see next comment.

Reviewer's comment: "The team has been developing the high-volume throughput screening tests since 2007. More details on their progress are needed; they have been working on developing high-volume throughput screening for several years and it is not clear what progress has been made. The challenges and progress should be better identified."

Response: Due to budget uncertainties in the last two years, we elected to focus our work on the site-directed mutagenesis/expression approach. Moreover, the proposed new computational simulations may redirect the particular localized random mutagenesis approach that we chose to take.

Reviewer's comment: "The team's antenna and sulfur deprivation work seem very similar to what was done by Professor Melis at UC Berkeley."

Response: The team collaborated with Professor Melis in the discovery of the sulfur deprivation approach and NREL subsequently optimized the process. We are now testing truncated antenna mutants generated at UC Berkeley in an attempt to integrate the different research areas in photobiology. Prof. Melis had not reported on the H_2-production properties of his truncated antenna mutants before.

Responses to Previous Year's Reviewer Comments

Reviewer's comment: "…more details about pretreatments regarding the integrated system are needed."

Response: No pre-treatment was required. The algal biomass was previously frozen or fed directly to the fermentor. We will be addressing the effect (if any) of pre-treatment next.

Reviewer's comment: "The PI should identify areas that the partners collaborated more clearly. It is difficult to determine what was done by partners and what was done by NREL."

Response: The collaborator's contribution was more clearly delineated in this presentation.

Reviewer's comment:"The project team's future plans are the same as in previous years. Since progress has been made, it would seem reasonable to adjust the plans."

Response: Two new projects were added in FY2010: examine fusions between hydrogenase and ferredoxin, and study the effects of a "leaky" ATP synthase on H_2 production rates under sulfur deprivation. Readjustments in the direction of the molecular-engineering project have also been made to reflect results on the heterogeneity of redox states of hydrogenases.

Reviewer's comment: "There is no indication, at this point, how this work could be scaled and no real understanding of how to increase the hydrogen production to a point that will be useful at scale."

Response: The technoeconomic analysis performed by DTI has addressed some of those issues and did come up with a concept of a process for large-scale H_2 production.

Responses to Previous Year's Reviewer Comments

Reviewer's comment: "The PI has reported making an impressive number of presentations – 19. This is over one conference a month, which is a lot of travel. Their resources and time would be better utilized if they limited their conference attendance to only the premiere conferences."

Response: The team consists of 3 PIs; the presentations made this year were divided among them as follows: 7 presentations by Seibert, 2 by King, and 6 by Ghirardi. The number of invitations reflects the high standing of the PIs in the research community.

Publications

Belokopytov, B.S., K.S. Laurinavichius, T.V. Laurinavichene, M.L. Ghirardi, M.Seibert, and A.A. Tsygankov. **2009**. “Towards the integration of dark- and photo-fermentative waste treatment. 2. Optimization of starch-dependent fermentative hydrogen production". *Int. J. Hydrogen Energy*, 34: 3324-3332.

Maness, P.C., J. Yu, C. Eckert and M.L. Ghirardi**. 2009**. “Photobiological hydrogen production – prospects and challenges”. *Microbe* 4: 275-280.

Seibert, M. **2009**. "Applied Photosynthesis for Biofuels Production", *in* Photobiological Sciences Online (K. C. Smith, Ed.) Am. Soc. Photobiol. Website: http://www.photobiology.info/Seibert.html#TOP

Ghirardi, M.L., S. Kosourov, P.C. Maness, S. Smolinski and M. Seibert. **2009**. “Algal H_2 Production” in *Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation and Cell Technology* (ed. M.C. Flickenger), John Wiley and Sons, Inc., xx-xxx.

Long H., P.W. King, M.L. Ghirardi and K. Kim**. 2009**. “Hydrogenase/ferredoxin charge-transfer complexes: effect of hydrogenase mutations on the complex associatio”n. *J. Phys. Chem. A.* 113:4060-7.

English C.M., C. Eckert, K. Brown, M. Seibert and P.W. King. **2009**. “Recombinant and *in vitro* expression systems for hydrogenases: new frontiers in basic and applied studies for biological and synthetic H_2 production”. *Dalton Trans.* 45:9970-78.

Ghirardi, M.L. and Mohanty, P. **2010**.” Oxygenic hydrogen photoproduction – current status of the technology”. *Current Science Indi*a, in press.

Laurinavichene, T.V., B.F. Belokopytov, K.S. Laurinavichius, D.N. Tekucheva, M. Seibert and A.A. Tsygankov. **2010**. “Towards the integration of dark- and photo-fermentative waste treatment. 3. Potato as substrate for sequential dark fermentation and light-driven H_2 production.” *Int. J. Hydrogen Ener*gy, in press.

Smolinski, S., S.N. Kosourov, P.C. Maness and M.L. Ghirardi. “Hydrogen production from the fermentation of algal biomass by a bacterial consortium isolated from wastewater sludge”. Submitted.

Tekucheva, D.N., T.V. Laurinavichene, M.L. Ghirardi, M. Seibert, A.A. Tsygankov (2010) "Immobilization of purple bacteria for light-driven H_2 production from starch and potato fermentation effluents." Submitted.

Presentations

- Invited seminar at the CSIC Spanish National laboratory in Zaragoza, Spain, Apr 09 (Seibert).
- Presentation to the group of Dr. X. Zhang at MIT, Apr 2009 (King).
- Invited plenary talk at the Great Lakes Bioenergy Research Center (GLBRC) Hydrogenase Forum, May 09 (Seibert).
- Presentation at the American Society for Plant Biology meeting in Hawaii, Jul 09 (Ghirardi).
- Invited update on EERE BioHydrogen research at the U.S. Air Force Office of Scientific Research Annual Review Meeting, Aug 09 (Seibert).
- Presentation of the USA country report at the IEA Annex 21 Biohydrogen Experts Meeting in Jyväskylä, Finland, Sep 09 (Seibert).
- Invited presentation at the University of Washington, St. Louis, Sep 09 (Ghirardi).
- Invited presentation at the Rocky Mountain American Vacuum Society meeting, Denver, Sept 09 (Ghirardi).
- Invited presentation at the Center for Revolutionary Solar Photoconversion meeting in Denver, Oct 09 (Ghirardi).
- Invited presentation at the Fall Rocky Mountain Branch of the American Society for Microbiology in Denver, Nov 09 (Ghirardi).
- Oral presentation to the Solar Fuels 2009 Meeting, Sigtuna, Sweden, Oct 2009 (King).
- Invited presentation to the Microbiology Department, Colorado State University, Nov. 09 (Seibert).
- Presentation at NREL's Energy Bioscience Center monthly seminar, Jan 10 (Ghirardi).
- Presented USA country report at the IEA Annex 21 Biohydrogen Experts Meeting in Florence, Italy, March, 2010 (Seibert).
- Invited talk at the DiBA-UNIFI & ISE-CNR Workshop on BioHydrogen in Florence, Italy, March, 2010 (Seibert).

Other Activities

Ghirardi reviewed articles for *Nature, Journal of Photobiology, Applied Environmental Microbiology, Resources, Energy and Development, International Journal of Hydrogen Energy, Bioresource Technology*.

Ghirardi reviewed proposals for EPSCoR, University of Padua, National Science Foundation, the Joint Genome Institute, ARPA, USDA, and DOE.

Ghirardi was nominated a Fellow of the Renewable and Sustainable Energy Institute (RASEI).

Ghirardi hosted Dr. Alex Bradel, Chris Yeager (SRNL), Patrice Hamel (OSU), members of the European Commission, Dr. Glaucia Souza (Brazil's FAPESP).

Ghirardi serves as an advisor for the University of Tennessee's, NSF-funded STAIR (Sustainable Technology through Advanced Interdisciplinary Research) Program.

Seibert was elected the new Operating Agent for the IEA/HIA Task 21 (Biohydrogen).

Critical Assumptions

Assumptions:

Molecular engineering of O_2 accessibility to the [FeFe]- and [NiFe]-hydrogenases' catalytic sites will improve O_2 tolerance. This assumption was based on published reports demonstrating that significant changes in the O_2 sensitivities of other FeS proteins (ferredoxin and the H_2-sensing NiFe hydrogenase) were achieved solely by changing the accessibility of the FeS centers or catalytic sites to O_2 by substitution of critical amino-acid residues.

Since the clostridial hydrogenase is already more tolerant to O_2 than the algal enzyme, we expect to see increased activity under aerobic conditions when we express it in Chlamydomonas. Successful expression will also allow us to start addressing other barriers (such as competition for reductant) in a more effective manner.

Immobilizing dense algal cultures in very inexpensive matrices will drive the light-conversion efficiency up, while contributing very little to (and perhaps lowering) the cost of the H_2 produced by the system.

Integrating photobiological with fermentative systems will contribute beneficially to the overall cost of biological H_2 production if parameters such as biomass disposal are taken into consideration. This will be considered in a new technoeconomic analysis to be performed at the end of FY10.

Other Issues

Issues:

Inconsistent funding and the lack of understanding regarding the nature of the different O_2-tolerant states of the hydrogenase has prevented us from further developing the site-directed mutagenesis approach. The expression of a more O_2-tolerant clostridial hydrogenase in Chlamydomonas will give us a better handle on which enzyme characteristics are important for hydrogenase activity under O_2-evolving conditions, thus allowing us to redesign our strategy and screening assays.

Preliminary evidence gained by comparing the structure of different hydrogenases suggests that factors beyond gas diffusion may contribute significantly to O_2 sensitivity. We will further examine this through computational simulations, which may lead to a change in our mutagenesis strategy.