Biological Systems for Hydrogen Photoproduction

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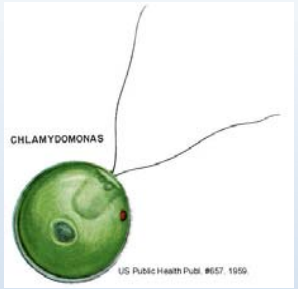

National Renewable Energy Laboratory

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Overview

Timeline

Project start date: FY00
Project end date: continuing

Percent completed: N/A

Barriers

- Barriers addressed

Production Barrier Z: Continuity of H_2 photoproduction.

Targets

Parameters	Current Status	2010 Target	Maximum potential
Duration of continuous photoproduction	80 days (sulfur-deprived)	30 minutes (aerobic)	12 hours (aerobic)
O_2 tolerance (half-life in air)	2-4 min (*Clostridium* hydrogenase *in vitro*)	10 minutes	12 hours

Budget

- Funding received in FY05: $785K ($20K for subcontract).
- Funding for FY06: $345K.

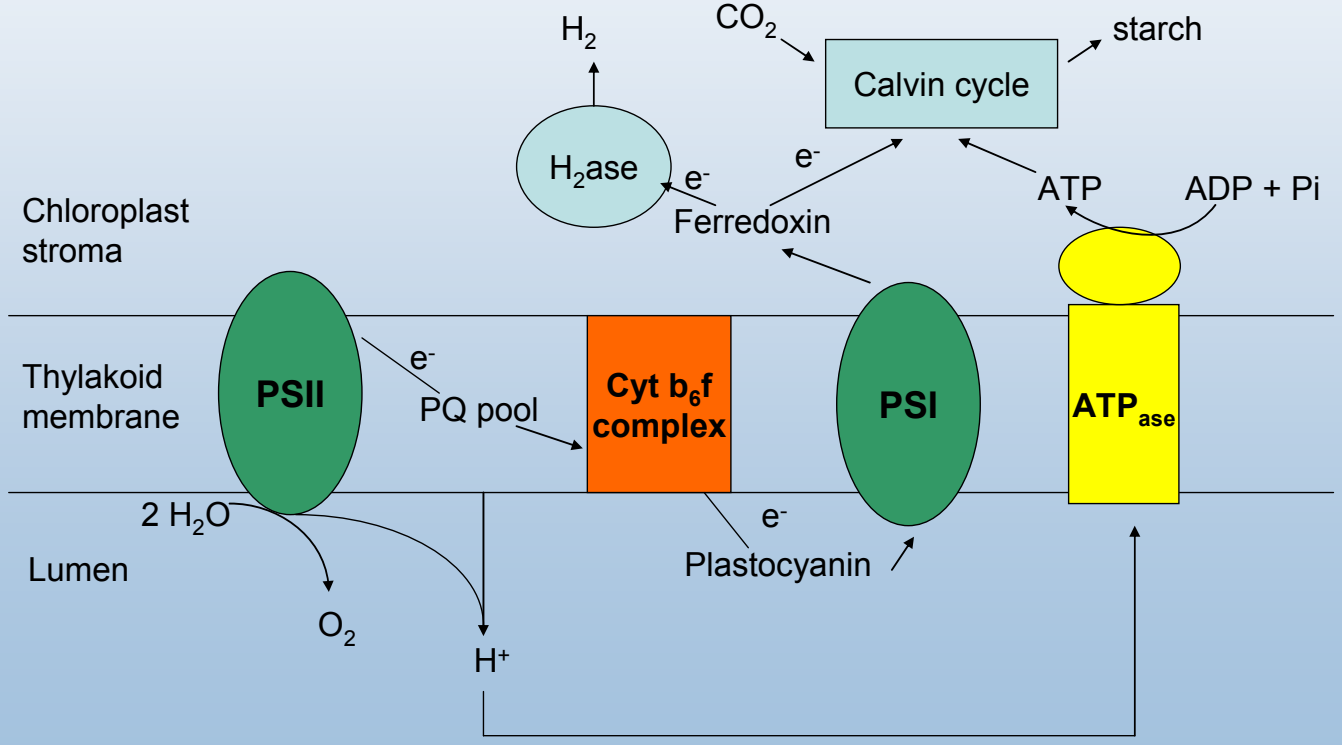
Partners

- Interactions/collaborations

Dr. Klaus Schulten, Beckman Institute, University of Illinois; Dr. Juan Fontecilla-Camps, CEA/CNRS, Grenoble, France; Dr. Giovanni Finazzi, Paris; Dr. Anatoly Tsygankov, Russian Academy of Sciences, Pushchino, Russia

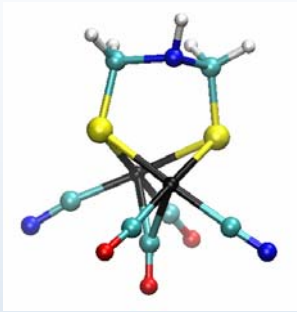
Project Goal

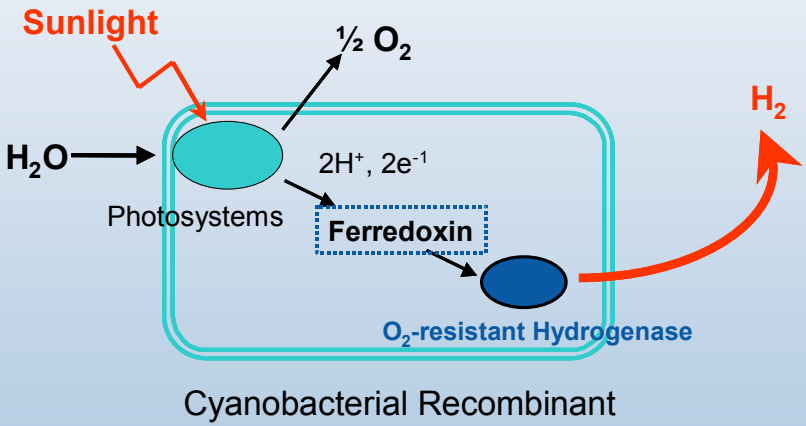
Develop photolytic H_2-production technologies based on microbial H_2O-splitting processes that are not inhibited by O_2.



Technical Approaches

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





Subtask 2. Introduce a 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 (sulfur deprivation) to promote culture anaerobiosis and subsequent H_2-production activity in algae (**discontinued in Jan 06 due to lack of funds**).

Objectives for 2005

- **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 hydrogenases from different organisms for continuity of H_2 production in the presence of O_2; start mutagenesis work to implement identified changes.
- **Subtask 2.** Demonstrate the feasibility of linking photosynthetically-produced reductants in cyanobacteria to H_2 production by an O_2-tolerant bacterial [NiFe]-hydrogenase.
- **Subtask 3.** Extend H_2 production in the continuous system by adjusting algal culture parameters; demonstrate continuous H_2 photoproduction using immobilized algae.

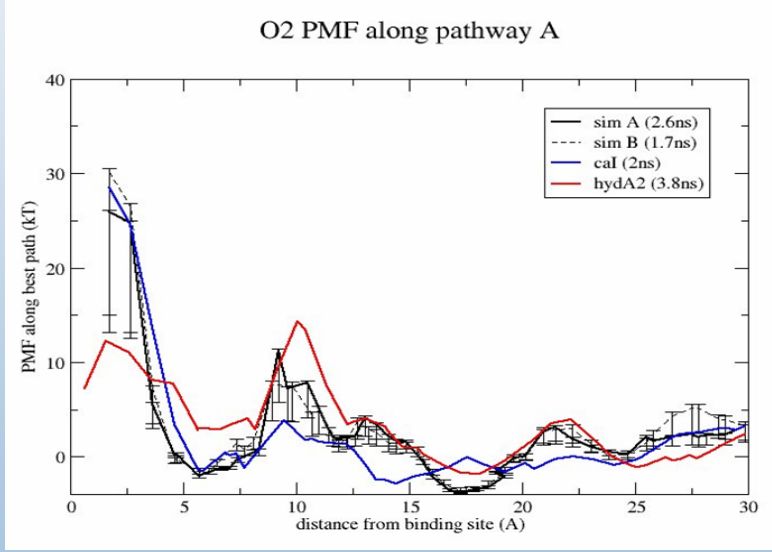
Objectives for 2006

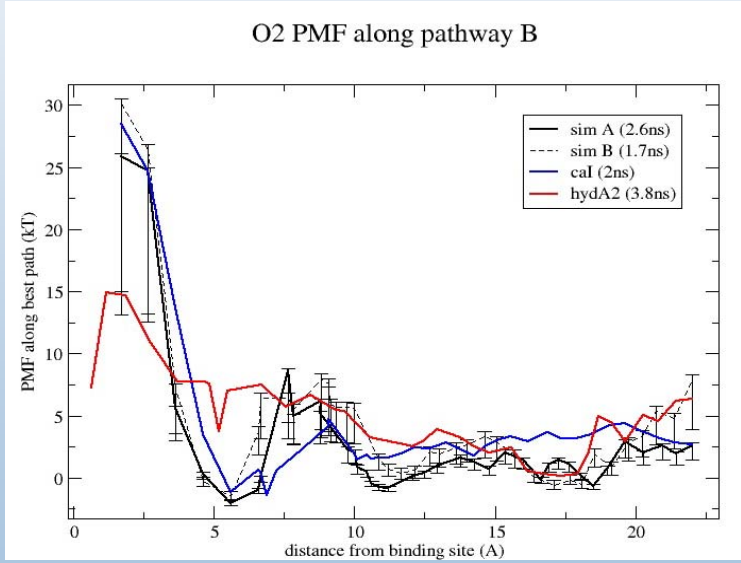
- **Subtask 1.** Continue to identify targets for mutagenesis by means of computational simulations; implement some of the identified changes; use the mutants to test for continuity of H_2 photoproduction in the presence of O_2.
- **Subtask 2.** Start cloning the genes that encode for both an O_2-tolerant bacterial [NiFe] hydrogenase and its maturation proteins and then express them in the bacterium, *E. coli* (some of this work will be done with the Florida International University).
- **Subtask 3.** Examine possible electron transport limitation factors; demonstrate photoautotrophic H_2 production activity in sulfur deprived cultures. *THE PROJECT WAS DISCONTINUED IN JANUARY OF 2006 DUE TO LACK OF FUNDS.*

Technical Accomplishments/Progress
Subtask 1

FY06 Results

Three different modeling methods were used to identify pathways for H_2 and O_2 gas diffusion through the CpI [FeFe]-hydrogenase protein structure: molecular dynamics (FY05), solvent accessibility maps (FY05), and potential energy profiles (FY06, see figures below).



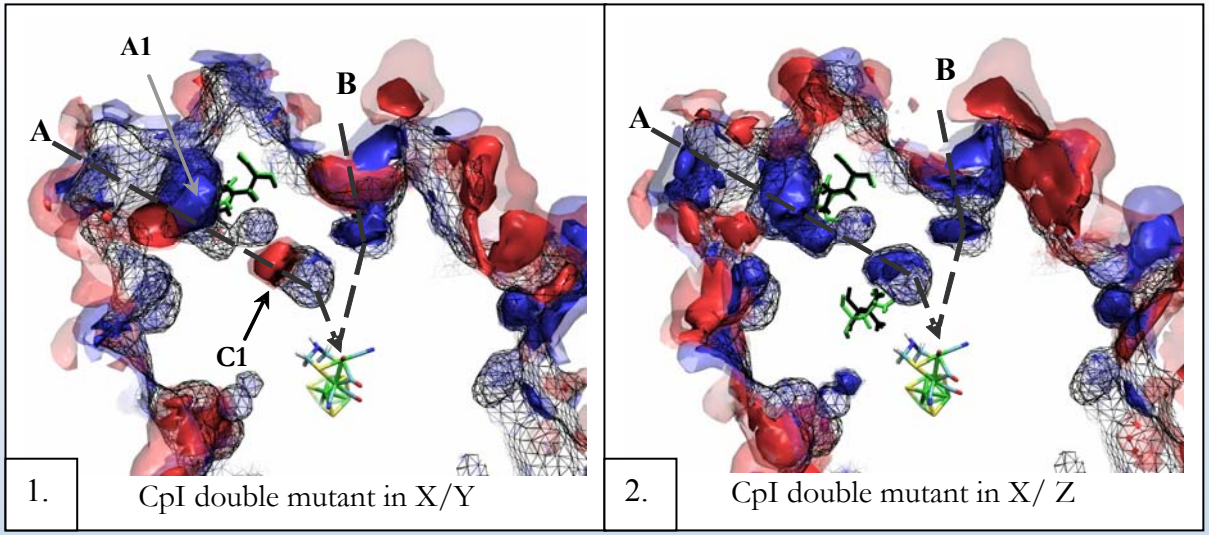


All three methods identified amino acid residues along two pathways in the protein structure that, if replaced by bulkier residues, could in principle block O_2 diffusion to the catalytic site.

Technical Accomplishments/Progress Subtask 1

FY06 Results

We identified residues X (pathway A), Y (pathway B) and Z (center cavity C1) as potential targets for mutagenesis. Single and double mutants were constructed. One of the single mutants, X showed an increase in O_2 tolerance and maintained high enzymatic activity. The other mutated enzymes showed a significant loss of activity and displayed increases in O_2 sensitivity, suggesting a more porous structure and lack of assembly. Future work will focus on pathway B residues further away from the catalytic site, in combination with X.

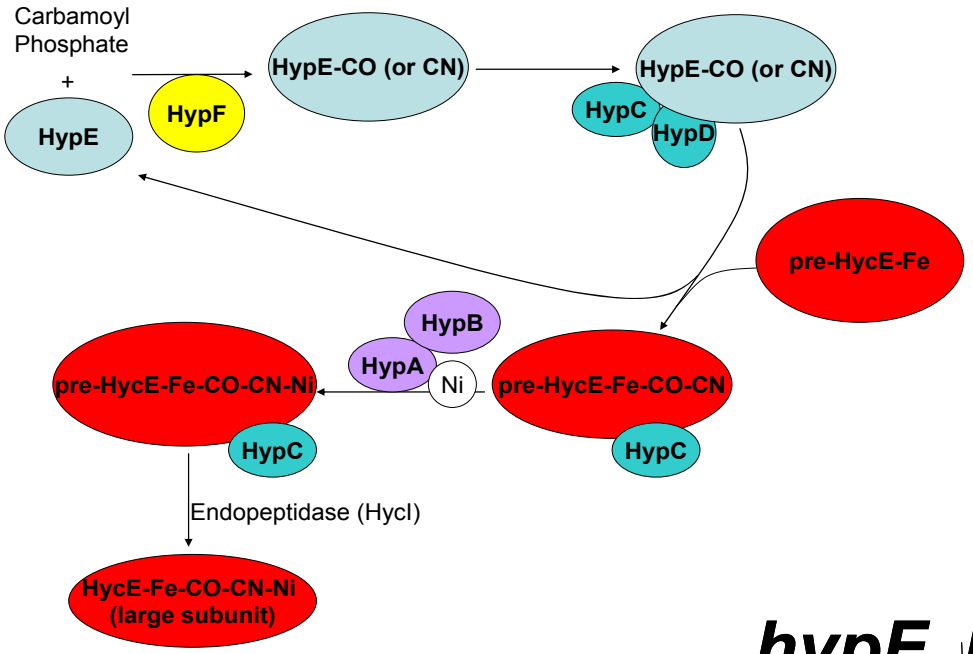


CpI	Inactivation Rate Constant in Air (k)	Normalized H_2-evolution Activity in Whole-Cell Extracts	Fold-change in the Inactivation Rate (negative values reflect increased tolerance to O_2)
Wild-Type	0.14	100	1
X	0.12	71	-1.2 (increased tolerance)
Z	0.82	7.6	+5.9
Y	0.68	7.3	+4.9
X/Z	0.39	8	+2.8
X/Y	0.48	2	+3.4

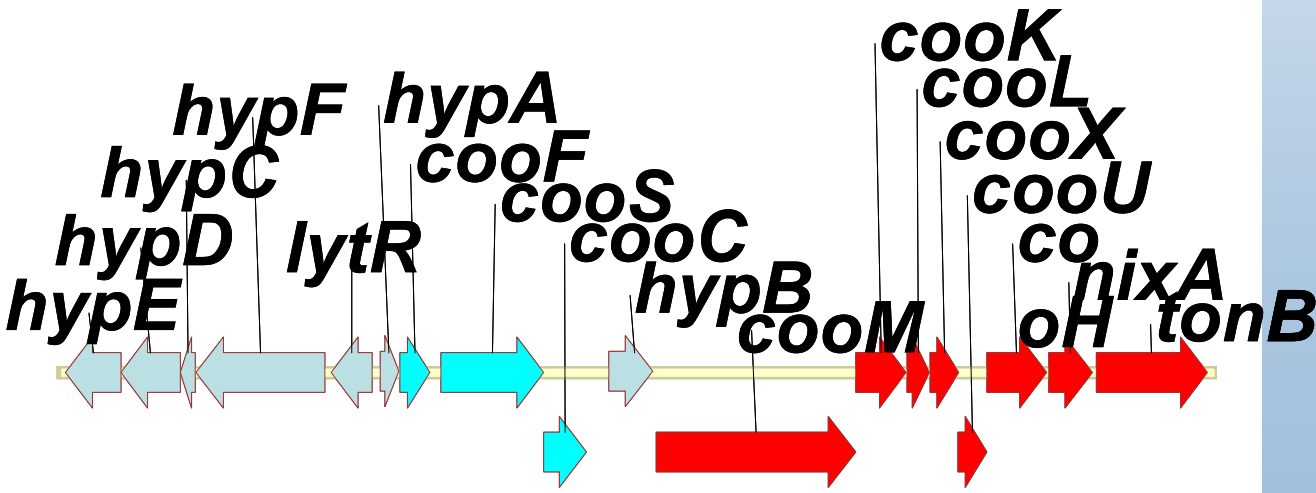
Table 1. Air inactivation rates of H_2-evolution activities in whole-cell extracts expressing either wild-type or mutant CpI enzymes.

Technical Accomplishments/Progress
Subtask 2

FY06 Results



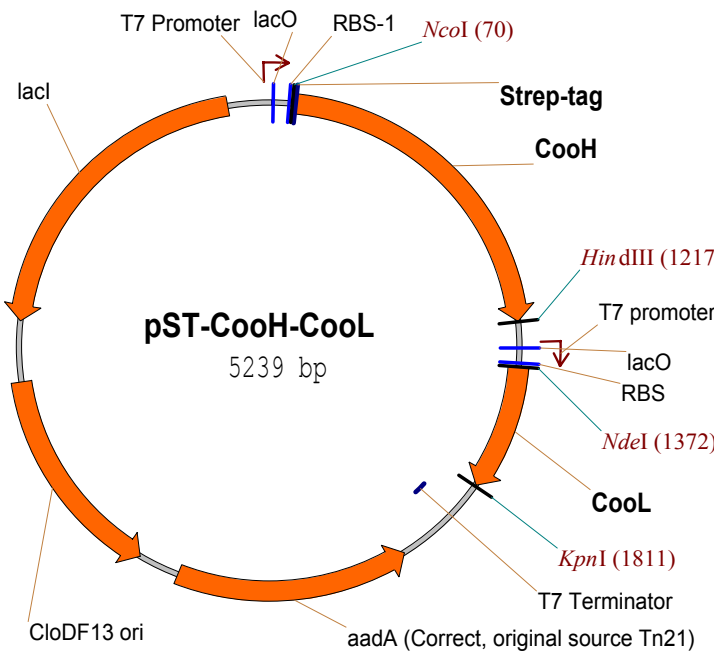
The O_2-tolerant [NiFe]-hydrogenase consists of two subunits, whose maturation requires a suite of other proteins (left figure). We cloned the operon encoding all these genes (bottom figure, FY05) and we are currently transferring each gene to the host organism, *E. coli,* to determine which and how many genes are required for activity of the enzyme in the host organism.

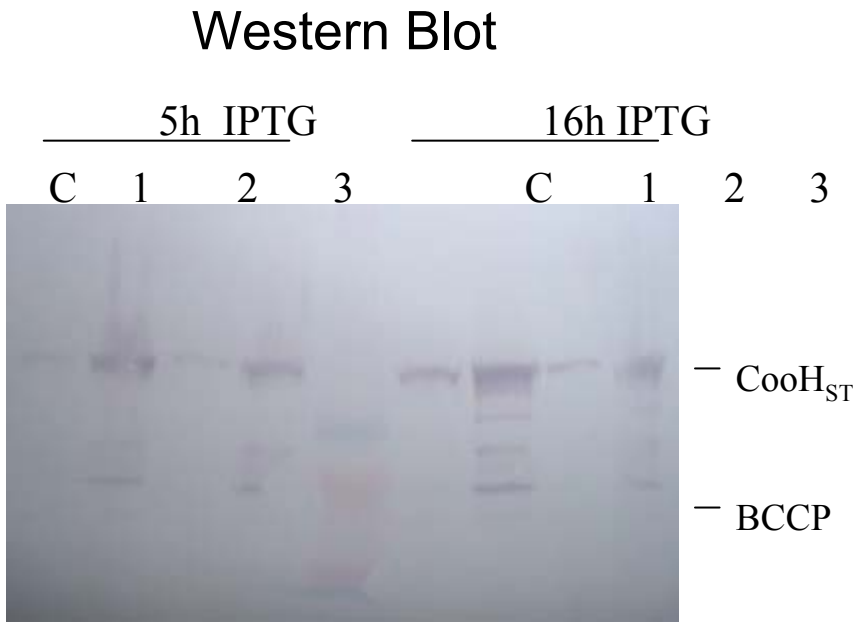


Technical Accomplishments/Progress
Subtask 2

FY06 Results

Preliminary results demonstrate the successful expression of an inactive hydrogenase in *E. coli*, when only the catalytic subunit gene (CooH) is incorporated into the expression plasmid (left figure, below).

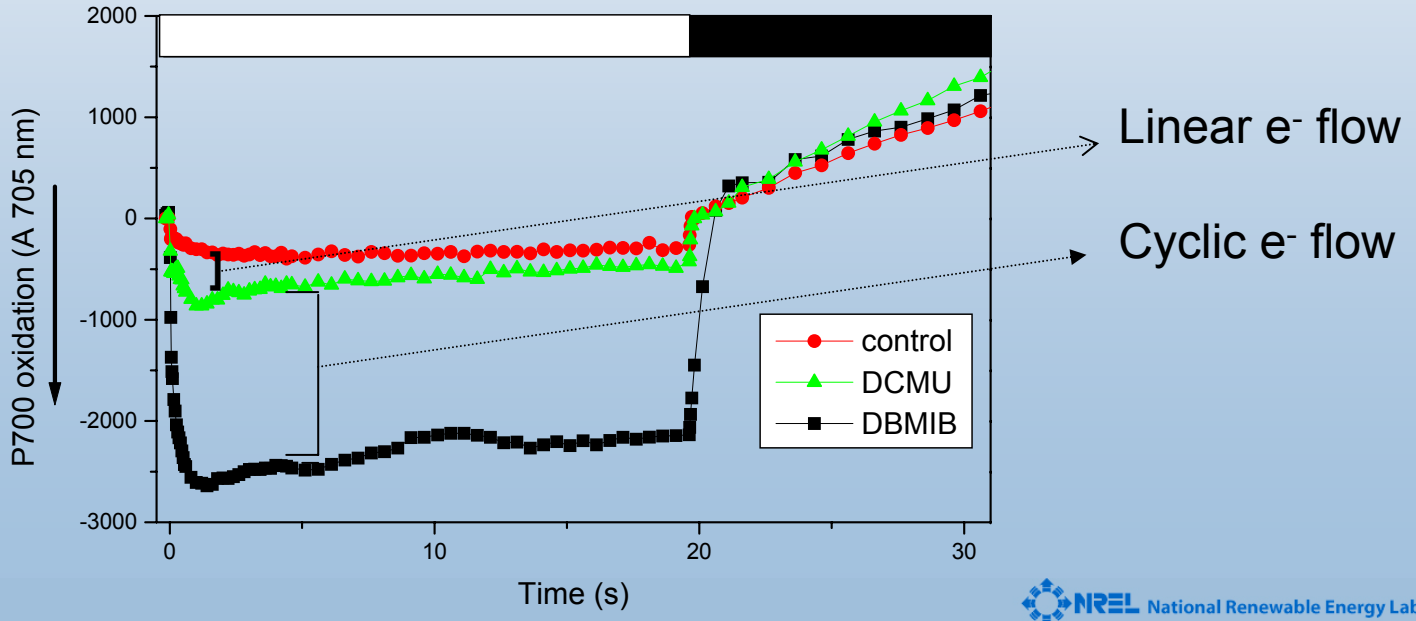




Technical Accomplishments/Progress
Subtask 3

FY06 Results

Spectroscopic measurements done in collaboration with Prof. Giovanni Finazzi (Institute de Biologie Physico Chimique, Paris) demonstrate that most reductants (about 90%) originating from water oxidation are not utilized for H_2 production because of the wasteful electron transport cycle around Photosystem I in sulfur-deprived cells (State II). Sulfur-deprived algal mutants unable to achieve State II have been shown to produce H_2 at 3-4 times higher rates than wild-type algae (Kruse et al., 2005), though their parental wild-type strain exhibited very low rates of H_2 production.



Technical Accomplishments/Progress Subtask 3

FY06 Results

The H_2-production performance of sulfur-deprived algal cells immobilized onto glass fibers was compared to that of cell suspensions. We demonstrated that cell immobilization (a) decreases the specific rate of H_2 production by 20-25%, (b) increases the light conversion efficiency of the cultures from 0.12% to 0.36% (**the maximum expected is 1%**), and (c) increases the duration of the process from 3-4 to 21 days/cycle. Additionally, continuous H_2-production (instead of cycles) could be maintained with immobilized cultures for a total of 80 days. Further increases in rates and efficiencies could conceivably be achieved using a mutant similar to that referred to in the previous slide

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	308,160 J/m^2	92,448 J/m^2
Efficiency of incident light energy conversion into H_2	0.12%	0.36% (**Three times improvement**)

Technical Accomplishments/Progress
Subtask 3

FY06 Results

The production of H_2 gas by photoautotrophic, sulfur-deprived algal cultures was demonstrated and conditions were optimized for cell suspensions. These results may lead to the elimination of the requirement for added acetate, an important milestone from the H_2-cost perspective.

Light intensity, μE • m^{-2} • s^{-1}		Starch accumulated as glucose, mmole / L	Start of anaerobiosis, h	Starch degraded as glucose, mmole / L	Total H_2 gas produced, ml / L
Pre-growth	Sulfur-deprivation				
120	20	0.36 ± 0.07	91 – nt	0.19 ± 0.10	0
120	110	0.59 ± 0.12	58 – 95	0.40 ± 0.19	4.4 ± 4.7
25	20	0.24 ± 0.03	49.5 – nt	0.16 ± 0.04	7.3 ± 10.4
25	110	0.86 ± 0.34	22.5 – 69	0.77 ± 0.27	31.8 ± 10.8
25	110 during photosynthetic and early oxygen consumption stages, and 20 thereafter	0.78 ± 0.11	22 – 65	0.71 ± 0.11	56.4 ± 16.7

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

Future Work

Subtask 1: Continue the 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*. If additional funding becomes available, we would like to initiate random mutagenesis protocols to target less obvious but advantageous mutations.

Subtask 2: Transfer additional CBS hydrogenase subunit genes and genes involved in the maturation of the NiFe active site into *E. coli* (in collaboration with Florida International University) and later into the cyanobacterial host. Continue to investigate the coupling between cyanobacterial membranes and the [NiFe]-hydrogenase *in vitro*.

Subtask 3: *The project has been discontinued this year due to lack of funding resulting from the effect of earmarks.* If reinstated next year, we will continue to optimize photoautotrophic H_2 production and start testing the process in immobilized cultures. We will expand our examination of different types of matrices for cell immobilization, as well as photobioreactor material and design. We will also attempt to examine the performance of Kruse's mutant (see slide 10) under our experimental conditions.

Summary

- **Subtask 1:** We developed a third computational tool to help identify targets for site-directed mutagenesis aimed at increasing the O_2-tolerance of [FeFe]-hydrogenases; initial mutagenesis work demonstrated that a mutant, X in the O_2 pathway A had increased O_2 tolerance, while others resulted in a more porous and less active hydrogenase. Further work will focus on mutations located further away from the catalytic site along pathway B, in combination with X. Random mutagenesis approaches will be initiated to improve the chances of achieving the desired results on a more timely basis.

- **Subtask 2:** We successfully expressed the catalytic subunit of an O_2-tolerant [NiFe]-hydrogenase in the bacterium, *E. coli*, but in an inactive form. We are currently testing the appropriate combination of genes (structural and accessory) that are required for expression of an active form of the enzyme in *E. coli* (with Florida International University). After this is done, we will attempt to express the O_2-tolerant [NiFe]-hydrogenase in cyanobacteria.

- **Subtask 3:** The presence of a significant wasteful electron transport cycle around PSI under sulfur-deprivation was demonstrated by spectroscopic measurements. To circumvent this wasteful cycle, we will attempt to obtain a mutant unable to perform the cyclic pathway (see slide 10). Additionally, we demonstrated significant H_2 production in the absence of added acetate, which may positively affect the economics of the sulfur-deprived system, particularly when combined with cell immobiization. This year we showed an increase in the light conversion efficiency of H_2 production by a factor of 3.

Responses to Previous Year Reviewers' Comments

We are grateful to the reviewers for the careful way in which they reviewed our project last year, and we thank them for the mostly positive comments. In the next slides, we address some of the more specific questions raised by them.

1. ***"not clear why a biological means of hydrogen production should be a better or cheaper long term alternative to other means"; "discussion of feasible target yields of hydrogen, even theoretical limits would be helpful".*** The potential maximum light conversion efficiency of photosynthetic organisms (the theoretical limit) to split water directly into H_2 and O_2 is about 13% and, in contrast with other H_2-production methods, biological systems do not require expensive catalysts. Indeed, biological systems are self-assemblying and self-repairing, and require very little maintenance. The major contributor to the cost of a biological H_2-producing system (once the biochemical issues are solved) will be photobioreactor material, a problem that is common to all other long-term alternative systems. Additionally, photobiological systems will require the development of cost-effective O_2/H_2 separation technologies. Target yields are shown in the Program's Multi-Year Plan.

2. ***"A clearer set of incremental goals and plans would be helpful".*** The information on the long-term goals has been incorporated into slide 2, and can be found in the Multi-Year Program Plan. The Summary slide describes plans to achieve the desired targets.

Response to Previous Year Reviewers' Comments

3. ***"More comprehensive study of other possible stability determinants may be in order if closing both predicted O_2 channels does not greatly enhance stability".*** Approaches based on random enzyme mutagenesis and/or gene shuffling technologies were proposed to the Program in previous years and again in FY06. However, there has never been enough funding to pursue these efforts. They are being proposed again as future work (see slide13).

4. ***"The long term possibilities of utilization growth under sulfur deprivation are difficult to predict. Developing strategies for metabolic engineering could be more fruitful"; "The unclear justification of the future efficacy of sulfur deprivation as a growth variation for hydrogen production (is a weakness of the project)".*** An economic analysis of the sulfur-deprivation method, done at NREL for DOE, supports the feasibility of the sulfur-deprivation system for future applications, assuming that the biochemical issues can be solved, thus allowing maximum light conversion efficiencies of 1%. Indeed, our current results demonstrate increased efficiencies by a factor of 3 this year (from 0.12% to 0.36%, see slide 11) by optimizing photobioreactor engineering through cell immobilization. Other laboratories (such as U.C. Berkeley and ORNL) are looking into engineering different metabolic pathways to achieve yields higher than 1% with this system. Finally, in order to achieve conversion efficiencies closer to 1%, we propose investigating Kruse's mutant as one of our tasks for next year (see slides 10, 13 and 14).

Response to Previous Year Reviewers' Comments

5. ***"current work shows that this (coupling hydrogen production to photosynthetic water oxidation using native cyanobacterial electron carriers) may not be very efficient. Is there a practical plan for overcoming this barrier?"*** The reviewer is referring to the *in vitro* work that we presented last year. We are currently investigating the reason for the low coupling in the assay, which could simply be due to the loss of an intermediate electron carrier (cytochrome c6) during the isolation of cyanobacterial membranes. This is currently being investigated but at a lower priority due to budget cuts. Alternative approaches will depend on what is the cause of the limitation.

6. ***"improvements from previous year results are not specified in the presentation so it is difficult to assess results".*** We attempted to address this in the current presentation (see slides 6, 8 and 11). Usually the Program has very specific instructions regarding the content of each slide, and previous results is not one of them.

7. ***"Participation from a microbiologist with expertise in the appropriate metabolic systems (algal H_2 production) could also be useful".*** Reduction in funding this year prevented us from hiring a new post-doc for the project, as initially planned.

Response to Previous Year Reviewers' Comments

8. ***"The work is very exploratory and, possibly as a consequence, not much in the way of contingency planning is articulated"; "the multiple approach has a higher likelihood for success".*** These two statements are contradictory, since we do have three different approaches to maximize the potential for success in the ultimate goal, i.e., the development of photolytic H_2-production technologies based on microbial H_2O splitting processes that are not inhibited by O_2 (slide 3).

9. ***"Work in places appears to aim at solving difficult engineering goals before the basic science is firmly in place".*** The reviewer is probably referring to the sulfur-deprivation project, where we are attempting to demonstrate increased yields by immobilizing algal cells onto different matrices. If this is the case, we must emphasize that this work has allowed us to demonstrate, this year, a 3 times increase in light conversion efficiency simply by using a particular engineering approach (see slide 11), which is 30% of the maximum expected efficiency for this particular system (see also bullet 4, in slide 16).

10. ***"The responses to previous reviewer's comments could have been addressed in a more specific and substantive manner".*** Last year there was a limit to the number of slides that we were allowed to present, which precluded us to from responding in a more detailed manner. This year the limitation was lifted, since the response to reviewers' slides are not being presented during the public sessions.

Response to Previous Year Reviewers' Comments

11. ***"Is there a plan to study and "improve" O_2 tolerance of semi-O_2-stable NiFe hydrogenase?"*** There is a plan to apply the same computational simulation/mutagenesis approach being used with the algal [FeFe]-hydrogenase, if necessary. However, preliminary measurements indicate that the purified enzyme still maintains high levels of tolerance to O_2 (half-life of about 6 hours in air), as determined from the H/D exchange method.

Publications

Published

1. 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*., 121-124, 403-412.
2. 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*., 85, 295-305.
3. 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*, *from Basic Biology to Industrial Application* (eds. A.F. Collings and C. Critchley), Wiley-VCH Verlag, Weinheim Germany, pp. 213-227
4. 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*. 71,2870-2874.
5. Cohen, J, K Kim, P King, M Seibert and K Schulten **2005.** Finding gas diffusion pathways in proteins: O_2 and H_2 gas transport in CpI hydrogenase and the role of packing defects. *Structure* 13, 1321-1329.
6. Makarova VV, SNKosourov, TE Krendeleva, GP Kukarshkikh, ML Ghirardi, M Seibert and AB Rubin. **2006**. Photochemical activity of Photosystem II and Hydrogen Photproduction in Sulfur-Deprived *Chlamydomonas reinhardtii* mutants D1-R232D and D1-R323L. *Biophysics* 50 no 6, 90-96.
7. Laurinavichene, TV, AS Fedorov, ML Ghirardi, M Seibert and AA Tsygankov. **2006**. Demonstration of sustained hydrogen photoproduction by immobilized, sulfur-deprived *Chlamydomonas reinhardtii* cells. *Int. J. Hydrogen Energy*,.31, 659-667.
8. King, PW, MC Posewitz, ML Ghirardi and M Seibert. **2006**. Functional studies of [FeFe] hydrogenase maturation in an *Escherichia coli* biosynthetic system. *J. Bacteriol*. 188, 2163-2172.

Publications

In press or submitted

- Tsygankov, A.A., Kosourov, S.N., Tolstygina, I.V., Ghirardi, M.L. and Seibert, M. “Hydrogen production by sulfur-deprived *Chlamydomonas reinhardtii* under photoautotrophic conditions”, Int. J. Hydrogen Energy, in press.
- Ghirardi, M.L., Maness, P.C. and Seibert, M. “Photobiological Methods of Renewable Hydrogen Production” in *Solar Generation of Hydrogen* (M Conell, ed.), Springer-Verlag, submitted.
- Blake, D.M., Amos, W., Ghirardi, M.L. and Seibert, M. “Materials Requirements for Photobiological Hydrogen Production” *in* Materials for the Hydrogen Economy, (R. Jones and G. Thomas, Eds.) CRC Press, submitted.
- Ghirardi, M.L. “Hydrogen Production by Photosynthetic Green Algae”, Indian J. Biochem., submitted.
- Kosourov, S., Patrusheva, E., Ghirardi, M.L., Seibert, M. and Tsygankov, A. “A comparison of hydrogen photorpoduction by sulfur-deprived *Chlamydomonas reinhardtii* under different growth conditions” , submitted.

Awards and Others

- HENAAC (Hispanic Engineer National Annual Achievement Award Committee) award to M. Ghirardi for outstanding performance in 2005.
- Our Biohydrogen technology was chosen as one of Discover’s 25 “Frontiers of Science” in their 25th Anniversary Issue of October 2005

Visitors

Drs. Craig Kvien and Kim Cutchins (University of Georgia), Dr. G. Jungmeier (Johanneum Research Institute, Austria), a delegation of Brazilian scientists and program managers, David Thomassen (DOE), Dr. David Kyle (Advanced Bionutrition), members of the U.S. House Committee on Appropriations, members of the Energy and Minerals Government Field Institute, Dr. Samuel Bodman, (U.S. Secretary of Energy), Dr. Juan Fontecilla-Camps, Christine Cavazza and Frederic Garzoni (CEA/CNRS, Grenoble, France), Dr. Giuseppe Torzillo (CRN, Florence, Italy), Drs. Tom Moore, Ana Moore and Devens Gust (Arizona State University), Dr. John Peters (Montana State University), representatives from the 2nd International Conference for Integration of Renewable and Distributed Energy Sources, members of the New Mexico State University, Joe Gutierrez from the Biomass Program (Los Alamos), 4th and 6th graders from Boulder schools, Greenfuel representatives, Praxair representatives, Dr. Ray Orbach (DOE's Office of Science), students from the Colorado School of Mines and from the University of Colorado, Dr. Paula Moon (Argonne National Lab), Jackeline Morales (Society of Women Engineers, Cleveland State University), Jim Sandler (the Sandler Foundation), Niels van der Lelie (Brookhaven National Lab), Harlan Sands (Florida International University), Lisa Morgenthaler-Jones (venture capitalist, Arare).

Meetings and Presentations

Invited presentation at the Photosynthesis Gordon Conference (MLG), presentations at the International Hydrogen Conference in Istanbul (PCM and MLG), session chair at the COST meeting in Istanbul (MS), participant at the close-out IEA Annex 15 session in Istanbul (MS), invited presentation at Penn State University (PCM), overview presentation at the BES Solar Energy Utilization Workshop (MS), invited presentation at DOE HFC&IT's headquarters (MLG), presentation at the Beckman Institute, Illinois (MLG), interview with SciCentral from New York (MS), NREL's Chemical Sciences seminar (MLG), poster at the Biofuels meeting in Denver (Fedorov), invited seminar at the J. Craig Venter Institute (MS), interview with Bridget Ennis for the MicrobeWorld radio program (MS), poster at the Steenbock conference on FeS proteins (PK), invited talks at the U.S. AFOSR Biohydrogen kick-off meeting (MS and MLG), interview with Michael Robbins, Discovery Magazine (MS), briefing to the Jason group (PCM and MLG), graduate-level class taught in the Chemistry Department at the University of Colorado in Boulder (MS), invited Raiziss lecture presented at the University of Pennsylvania (MS), invited seminar at the Hawaii Natural Energy Institute (MS), talks at the IPHE workshop in Seville, Spain (MS and PCM), interview with New Scientist (MS), invited presentation at Brookhaven National Lab (PK), invited presentation at the Pacific Rim Summit, Hawaii (MLG), presentation at the Western Photosynthesis Conference, Monterey CA (MLG), video by the Discovery Channel International (MLG and PK), invited presentation at the University of Nevada (MS), invited participant at the American Academy of Microbiology 's colloquium on "Microbial Production of Energy (MS).

Critical Assumptions and Issues

- Molecular engineering O_2 access to the catalytic site of hydrogenases will improve their O_2 tolerance – this assumption is based on previous successful reports in the literature demonstrating changes in the tolerance of other FeS proteins (ferredoxin and the [NiFe] H_2-sensing hydrogenase) to O_2 achieved solely by modifications in the permeability of these proteins to O_2 gas.

- The [NiFe]-hydrogenase that we are working with is really tolerant to O_2 inactivation – this assumption is based on measurements of the H/D exchange properties of the hydrogenase done in the presence of O_2 gas.

- Other assumptions regarding the long-term development of an applied biological system with a 10% solar light-conversion efficiency include: (a) the separation of H_2 and O_2 gases will be achieved by the development of new materials; (b) a cost-effective photobioreactor material with appropriate properties will be found; and (c) light-harvesting antenna and electron transport limitation issues will be solved.