II.H.1 Biological Systems for Hydrogen Photoproduction

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Fiscal Year (FY) 2011 Objectives

Primary Objectives

- Develop and optimize aerobic, high solar-to-hydrogen (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.
  - Introducing a more O_2-tolerant hydrogenase into the green alga, *Chlamydomonas reinhardtii.*
- Design a fusion between hydrogenase Ca1 and ferredoxin to re-direct a larger portion of photosynthetic electron transport to hydrogenase rather than to CO_2 fixation under aerobic conditions; advise Massachusetts Institute of Technology (MIT) regarding *in vitro* testing of the fusion.
- 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 a leaky chloroplast adenosine triphosphate (ATP) synthase on the rates of H_2 photoproduction.
- Integrate fermentative H_2 production using potato waste or sulfur-deprived, alginate-immobilized algal biomass as feedstock, with photosynthetic H_2 production by anaerobic, purple non-sulfur bacteria.

Technical Barriers

This project addresses the following technical barriers from the Production section of the Fuel Cell Technologies (FCT) Program Multi-Year Research, Development and Demonstration Plan.

(AH) Rate of H_2 Production
(AI) Continuity of H_2 Production
(AT) Feedstock Cost

Technical Targets

TABLE 1. Progress toward Meeting Technical Targets for Photobiological Hydrogen Production

Parameters	Current Status	2013 Targets	Maximum Potential
Duration of continuous photoproduction:			
• Aerobic, high STH (O_2-tolerant)	0	30 min.	12 hours daylight
• Anaerobic, low STH (S-deprivation)	90 days		Indefinite
• Aerobic, low STH (S-deprivation)	10 days		Indefinite
Cost[1] ($/kg H_2)			
• Aerobic, high STH			$2.99
• Anaerobic, low STH			$6.02
• Integrated (photobiological and fermentative)			$3.21

[1] Cost estimates based on B.D. James, G.N. Baum, J. Perez, K.N. Baum, 2009, http://nrel.gov/docs/fy09osti/46674.pdf

FY 2011 Accomplishments

- Used computational methods to identify differences in the geometries and energies of the gas diffusion barrier protecting the H-cluster in two [FeFe]-hydrogenases that display a 1,000-fold difference in the level of O_2 sensitivity.
- Used a previously-developed random mutagenesis technique to randomize the amino acid residues that comprise the areas identified above.
- Demonstrated the presence of double O_2-inactivation kinetics in green algal transformants expressing a clostridial hydrogenase, suggesting that the more O_2-tolerant clostridial enzyme Ca1 is active in wild-type green algae.

- Demonstrated an up to 2.6-fold increase in the H_2-photoproduction rates upon increases in the photobioreactor gas-to-liquid ratio, using either sulfur/phosphorus-deprived cell suspensions or alginate-immobilized, sulfur/phosphorus-deprived cultures. (The effect was shown to be due to inhibition of the reaction rates by H_2 that accumulated in the headspace.)
- Found that pre-treatment of alginate films with 0.1% polyethylenimine stabilized the films, resulting in a 14% increase in the H_2 yields but no significant increase in the period of performance.
- Showed a 40% improvement in the H_2 photoproduction rates by ATP synthase mutants due to dissipation of the proton gradient that limits photosynthetic electron transport in non-mutated cells.
- Demonstrated successful induction of a marker gene behind a chloroplast inducible promoter, a preliminary step for introduction of mutated ATP synthase subunits into *Chlamydomonas.*
- Obtained a 55% yield (based on 12 moles H_2/glucose) following fermentation of 2% starch (from potato waste), and subsequent utilization of the organic acid effluent using non-purple photosynthetic bacteria.

Introduction

Green algae can extract electrons from water and generate H_2 under illumination, using the concerted activities of the photosynthetic electron transport chain and the enzyme [FeFe]-hydrogenase. This pathway evolves O_2 as a by-product, which irreversibly inhibits the hydrogenase enzyme's catalytic center. The continuity of H_2 photoproduction is one of the major technical barriers to developing photobiological H_2-production systems that use water as the source of electrons (technical barrier AI).

A second major barrier to efficient algal H_2 photoproduction is the low rate of the reaction (technical barrier AH), which is dependent on many intracellular regulatory factors, including the competition for photosynthetic reductant between the H_2-production and the CO_2-fixation pathways; the down-regulation of photosynthetic electron transport from water under H_2-producing conditions; and the predominance of cyclic, unproductive electron transport over linear electron transfer under anaerobiosis.

Our current project addresses the O_2 sensitivity and low rates of algal H_2-production by using molecular engineering to alleviate these barriers and testing the results through the sulfur-deprivation platform. The latter allows us to measure the effects of molecular engineering on sustained hydrogen production, although at low STH conversion levels. Our project also addresses the performance of an integrated fermentative/photobiological H_2-producing system that holds the potential for lowering the costs and increasing the yields of photobiological H_2.

Approach

Task 1. Molecular Engineering Approaches to Increase the O_2 Tolerance and the Rates of H_2 Photoproduction

This task has three major objectives: (a) the engineering of increased O_2 tolerance in [FeFe]-hydrogenases through random mutagenesis, targeted to regions that control O_2 access to the catalytic site; (b) the expression of a functional, more O_2-tolerant clostridial [FeFe]-hydrogenase in *Chlamydomonas reinhardtii*; and (c) the design and testing of bacterial hydrogenase-ferredoxin fusions for activity and interaction with Photosystem I (NREL acted as a consultant for MIT).

The targeted mutagenesis approach is being guided by extensive computational study of gas diffusion in the clostridial *Clostridium pasteurianum* CpI hydrogenase (that has a solved crystal structure). These studies previously identified a cavity, present next to the H-cluster of [FeFe]-hydrogenases, as a high-energy barrier for O_2 diffusion to the cluster. However, site-directed mutagenesis of residues that comprise the barrier have not yielded mutants with higher O_2-tolerance. Alternative computational and mutagenesis approaches have been devised and are being pursued.

Our studies aimed at expressing a more O_2-tolerant hydrogenase from *Clostridium acetobutylicum* (Ca1) in *C. reinhardtii.* Linking it functionally to photosynthetic electron transport involved: (i) designing genetic constructs for expression, activation and translocation of the hydrogenase into the stromal compartment of algal chloroplast; (ii) testing transformants for the presence of recombinant hydrogenase enzyme and activity; (iii) testing for O_2-tolerance of H_2 photoproduction under different physiological conditions. The availability of an algal strain expressing a more O_2-tolerant enzyme will provide us with the opportunity to examine physiological effects of such an enzyme in algal metabolism.

Sustained H_2 photoproduction, which is currently achieved through sulfur deprivation (see task 2), requires that the algal cultures be anaerobic, which, in turn, inactivates CO_2 fixation. However, once an O_2-tolerant enzyme becomes available, H_2 photoproduction will compete with the CO_2 fixation pathway (at the level of the electron transport mediator, ferredoxin) for photosynthetic reductant. In order to direct the flux of reductant towards the hydrogenase, we worked with Prof. Shuguang Zhang's research group at MIT to design (NREL), construct and test (MIT) the performance of bacterial hydrogenase-ferredoxin fusions. The collaboration produced some computational models but experimental work was not done due to limited funding.

Task 2. Use of the Sulfur-Deprivation Platform to Test Biochemical and Engineering Factors

To induce sustained H_2 photoproduction, we collaborated with the University of California in the development of a physiological switch that is based on removing sulfate from the algal growth medium. This procedure has become a platform for testing the performance of a variety of algal mutants, growth conditions, immobilization surfaces and other engineering factors that may affect the overall H_2 yield.

Task 3. Integrated H_2 Production System

The FCT Hydrogen Biological Production working group identified a system for biological H_2 production that depends on the integrated activity of photosynthetic (oxygenic and non-oxygenic) and fermentative organisms. The potential advantages of this system are (a) the wider absorption spectrum or oxygenic and non-oxygenic phototrophs, (b) the utilization of spent algal and bacterial biomass as feedstocks by fermentative organisms for increased H_2 production, and (c) the utilization of the fermentative effluent for improved growth of algae and photosynthetic bacteria.

NREL has been testing two integrated system configurations. The first one is based on using potato waste as the feedstock for H_2-producing fermentative organisms, which co-generate organic acids that are used to support subsequent H_2-photoproduction by photosynthetic bacteria. The second configuration takes advantage of spent algal biomass (harvested at the end of a photobiological H_2-production process) as feedstock for fermentative H_2-producing microbes.

Results

Task 1. Molecular Engineering Approaches to Increase the O_2 Tolerance and the Rates of H_2 Photoproduction

Computational simulations identified differences in the geometries and energies of the gas diffusion barriers protecting the H-cluster in two [FeFe]-hydrogenases with a 1,000-fold difference in the level of O_2 sensitivity. We randomized the regions around the diffusion barriers (not the barrier residues *per se*) to test the information provided by the simulations. The resulting mutants will be expressed and screened in a new high-throughput technique previously developed at our laboratory.

Concomitantly, we have proceeded with the expression of the more O_2-tolerant, clostridial [FeFe]-hydrogenase in wild-type *C. reinhardtii*. Plasmids for constitutive or inducible expression of Ca1 hydrogenase were constructed and used to transform wild-type Chlamydomonas. The presence of the codon-optimized Ca1 gene was confirmed by polymerase chain reaction (PCR), and its transcription was detected by reverse-transcriptase-PCR. Measurement of hydrogenase activity was initially inconsistent, perhaps due to differences in gene expression from sample to sample. However, O_2-inhibition assays revealed double kinetics, suggesting that the more O_2-tolerant Ca1 gene was active. Optimization of the conditions for Ca1 expression will continue in order to unambiguously determine its contribution to overall hydrogenase activity in recombinant strains.

Our collaborative work with MIT helped to support the publication of a Proceedings of the National Academy of Sciences' manuscript, detailing the evidence for improved photosynthetic reductant flux to the hydrogenase *in vitro* using an algal hydrogenase-ferredoxin fusion. These results hold the promise to improve H_2 production rates *in vivo* as well.

Task 2. Use of the Sulfur-Deprivation Platform to Test Biochemical and Engineering Factors

Algal H_2-production is catalyzed by a reversible hydrogenase that is capable of H_2 production and H_2 oxidation, depending on the prevailing physiological conditions, such as the availability of photosynthetic reductant (favors H_2 production) and high headspace H_2 pressure (favors H_2 oxidation). We investigated the magnitude of the effect of different gas-to-liquid (or solid) ratios in the rates of H_2 production by suspension and immobilized algal cell reactors. The expectation is that, by diluting the H_2 produced by the cultures by increasing the headspace volume, it is possible to sustain high rates of H_2 production during the sulfur-deprivation process. Table 1 shows that increases in the gas-phase volume result in higher rates of H_2 photoproduction, with the highest reported rate of 12.5 μmoles mg Chl^{-1} h^{-1} being observed with a headspace volume of 925 mL. On the other hand, although the rates of H_2 photoproduction by cultures immobilized in alginate increase as the headspace volume is raised, they saturate at about 10 μmoles mg Chl^{-1} h^{-1}, perhaps due to limited diffusion of released H_2 within the alginate matrix. Also, the inhibitory effect of H_2 was quantified by measuring the yields of H_2 photoproduction by suspension cultures in the presence of different concentrations of added H_2, as shown in Figure 1. These combined results imply that the added cost of gas purging and gas separation must be balanced with the added benefit of increased H_2 productivities in order to achieve the most economical system.

TABLE 1. Rates of H_2 Photoproduction and Total Yield of H_2 as a Function of Headspace Volume

Gas space volume	Rate of H_2 production (mmoles x mg Chl^{-2} x h^{-1})	Total final yield of H_2
Suspension cultures		
165 mL	4.8	250 mL/L
545 mL	7.3	400 mL/L
925 mL	12.5	560 mL/L
Immobilized cultures		
9 mL	6.1	0.20 mol/m^3
20 mL	9.6	0.24 mol/m^3
35 mL	10	0.33 mol/m^3
54 mL	10	0.36 mol/m^3
119 mL	10	0.35 mol/m^3

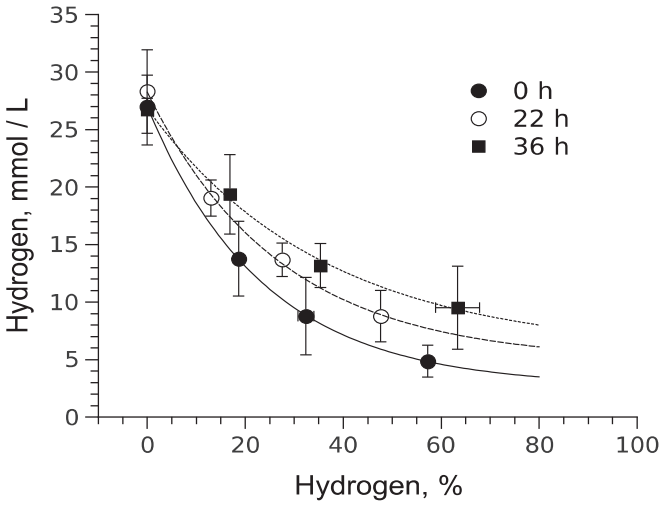

FIGURE 1. Final yield of H_2 as a function of the concentration of H_2 gas present in the bioreactor headspace at different time points (0, 22 and 36 hours) during the sulfur-deprivation process.

A second known limitation to the immobilized system is the low stability of alginate films over time. We examined the effect of treating the films with polyethyleneimine, a polymer that has been successfully used to increase the mechanical stability of a variety of films. We processed immobilized cultures through cycles of +S+P/-S-P and demonstrated that an increase in the polyetherimide (PEI) concentration up to 0.5% inhibits H_2 photoproduction but increases the mechanical stability of the film, allowing the cultures to undergo more cycles. The highest total H_2 yield was obtained in films pre-treated with 0.1% PEI, compared to untreated films. Our future studies will then be performed with cells immobilized in 0.1% PEI-treated films.

The sulfur-deprivation process was also used to test the performance of ATP synthase mutants designed to dissipate the proton gradient that is established during linear electron transfer (LEF) from H_2O to H_2 and that is known to down-regulate LEF (and thus H_2 production). Initial mutants, which were generated by Dr. Eric Johnson, Johns Hopkins University, had higher rates of H_2 photoproduction under high light but were affected in growth rates. New mutants were designed and are being tested to function under regulation of an inducible promoter, to allow the dissipation of the proton gradient to occur only under H_2-producing conditions.

Task 3. Integrated H_2 Production System

The integrated H_2-production system was tested, using a two-chamber reactor which separated the fermentative consortium from the photosynthetic bacteria. Unfortunately, many issues were found to affect the process, including (a) the low diffusion of volatile fatty acid feedstock; (b) the improper sealing of the membrane that did not prevent contamination; (c) inhibition of photosynthetic bacterial growth by factors present in the fermentative effluent; and (d) the nitrogen sources for photosynthetic bacteria need to be tightly controlled. This process was replaced by independent, successive cultivations with pH correction and addition of N_2 gas. The latter was tested with different amounts of feedstock, different dilutions of fermentative effluent before transfer to photobioreactor, and a new strain of *Rhodobacter capsulatus*. Yields of 55% (based on 12 moles H_2/glucose) were obtained with 2% starch (from potato waste), 75%–95% dilution of fermentative effluent before feeding the *R. capsulatus* strain N7. These yields are among the highest reported in the literature for an integrated system, and they serve as a baseline for our next step, where we will use spent algal biomass as the feedstock.

Conclusions and Future Direction

- **Task 1.** (a) continue the characterization of a wild-type *C. reinhardtii* transformant library harboring the Ca1 expression construct, and characterize new transformant library of Ca1 in a *C. reinhardtii* Hyd^- strain (developed under Basic Energy Sciences funding) that eliminates native hydrogenase activity; (b) express and screen a random mutant library of the bacterial hydrogenase Ca1 in *E. coli* to identify active mutants for O_2 tolerance characterization.
- **Task 2.** (a) design a more efficient immobilized H_2-production system, suitable for long-term H_2 photoproduction (two months compared to 10 days using the current configuration); (b) finish the development and test the function of an inducible promoter that will turn the mutated ATP synthase ON only under certain environmental conditions, to allow us to control the expression of the proton channel dissipation trait; (c) revisit the use of photoautotrophic cultivation conditions for sulfur-deprived cultures, aimed at increasing H_2 yields and decreasing cost by eliminating organic carbon substrate.
- **Task 3.** Will be discontinued due to budget restrictions.

Patents Issued

1. U.S. 7,732,174 B2 by Kosourov et al., "Multi-stage microbial system for continuous hydrogen production."

FY 2011 Publications/Presentations

Publications

1. Ghirardi, M.L.; Mohanty, P. 2010. "Oxygenic Hydrogen Photoproduction – Current Status Of The Technology." *Current Science Indi*a 98, 499-507.

2. Laurinavichene, T.V.; Belokopytov, B.F.; Laurinavichius, K.S.; Tekucheva, D.N.; Seibert, M.; Tsygankov, A.A. 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." *International Journal of Hydrogen Energy* 16, 8536-8543.

3. Eckert, C.; Dubini, A.; Yu, J.; King, P.; Ghirardi, M.; Seibert, M.; Maness, P.C. 2010. "Hydrogenase Genes and Enzymes Involved in Solar Hydrogen Production." In *State of the Art and Progress in Production of Biohydrogen.* N. Azbar and D. Levin, eds., Bentham Science Publishers, U.S., in press.

4. King, P.W.; Brown, K.A.; Ratcliff K.; Beer, L.L. 2010. "Photobiological and Photobiomimetic Production of Solar Fuels." In *Carbon Neutral Fuels and Energy Carriers*, N. Muridov, ed., Taylor and Francis, New York.

5. Kosourov, S.N., M.L. Ghirardi and M. Seibert. 2011. "A truncated antenna mutant of *Chlamydomonas reinhardtii* can produce more hydrogen than the parental strain." *Int. J. Hydrogen Energy* 36, 2044-2048.

6. 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." *J. Biotechnol*., in press.

Presentations

1. Visited our collaborator S. Zhang's research group at MIT (Apr. 2010, PK).

2. Departmental seminar at the North Carolina State University (May 2010, MS).

3. Invited to talk at the Christian-Albrechts University in Kiel, Germany (May 2010, MS).

4. Oral presentation at the Gordon Conference on Iron-Sulfur Enzymes, Colby-Sawyer College, NH (June 2010, PK).

5. Kendric C. Smith Lecture on Innovations in Photobiology at the American Society for Photobiology National Meeting (June 2010, MS).

6. Oral presentation at the CIMTEC 5th Forum on New Materials, Montecatini-Terme, Italy (June 2010, PK).

7. Poster and invited presentations at the International Conference on Hydrogenases and H_2 Production, Uppsala, Sweden (June 2010, MS and MLG).

8. Presentation to teams of visiting scientists from the Taiwan Academia Sinica and from the Australian National University (July and Aug. 2010, MLG).

9. Poster presentation at the 15th International Congress on Photosynthesis, Beijing, China (Aug. 2010, MS).

10. Invited talk at the Shanghai Normal University, China (Aug. 2010, MS).

11. Oral presentation at the American Chemical Society – Fall Annual Meeting, Boston (Aug. 2010, PK).

12. Invited talk at the ICCE Meeting, Cyprus, Turkey (Sept. 2010, MS).

13. Invited talk at the National Chung Hsing University, Taiwan (Nov. 2010, MS).

14. Attendance at DOE"s ARPA-E workshop on Solar Fuels (Dec. 2010, MLG).

15. Oral presentation at the Fuel Cell and Hydrogen Energy Meeting, Washington, D.C. (Feb. 2010, MLG).