II.I.3 Biological Systems for Hydrogen Photoproduction

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- Golden BioEnergy Corp., Highlands Ranch, CO

Project Start Date: October 1, 2000
Project End Date: Project continuation and direction determined annually by DOE

Objectives

- Engineer an [FeFe]-hydrogenase that has an extended half-life following exposure to O_2, as part of an aerobic algal H_2-production system being developed with other Hydrogen Program-sponsored groups.
- Optimize and use a platform for testing algal mutants with improved H_2-production properties and higher light-conversion efficiencies.
- Address individual 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 barriers from the Production section of the Hydrogen, Fuel Cells and Infrastructure Technologies (HFCIT) Program Multi-Year Research, Development and Demonstration Plan (MYRDDP):

(AI) Continuity of Photoproduction by Green Algae

(AT) Feedstock Cost in an Integrated System

Technical Targets

The technical targets for this project are listed in Table 1.

TABLE 1. Photolytic Biological Hydrogen Production from Water

Characteristics	Units	2003 Status	2009 Status	2013 Target	2018 Target
Duration of Continuous Photoproduction	Time units	N/A	180 days (-S, anaerobic) 6 days (-S, aerobic, immobilized)	30 min (aerobic)	4 h
O_2 Tolerance (half-life in air)	Time units	1 s	4 min (clostridial enzyme)	10 min (aerobic)	2 h

N/A - not applicable

Accomplishments

- Demonstrated that purified [FeFe]-hydrogenases show different sensitivity to O_2 inactivation, depending on the presence or absence of reductant during the purification procedure.
- Designed deoxyribonucleic acid (DNA) constructs for expression of recombinant [FeFe]-hydrogenases in *E. coli* upon anaerobic induction; integrated these constructs into appropriate vectors; successfully expressed recombinant hydrogenase in *E. coli* using these vectors; and designed a high-throughput chemochromic assay for screening random mutagenesis libraries for O_2-tolerant hydrogenases.
- Designed a DNA construct for expression of the clostridial, more O_2-tolerant [FeFe]-hydrogenase in the green alga *Chlamydomonas reinhardtii*; demonstrated expression of the gene by reverse transcription polymerase chain reaction (RT-PCR).
- Demonstrated 30-45% higher H_2-gas production by an ATPase mutant (where electron transport is not limited by the formation of a proton gradient) compared with the parental strain, using the sulfur-deprivation, alginate-immobilized platform.
- Showed higher and sustained rates of H_2 production by a sulfur-deprived, truncated-antenna mutant, immobilized in alginate at $1/10^{th}$ sun, when compared with the parental strain.
- Demonstrated H_2 production by fermentative organisms using starch-rich potato waste, accompanied by production of organic acids; showed H_2 production by photosynthetic bacteria using organic acids from the fermentation of potato wastes.

Introduction

Green algae can photoproduce H_2 using water as the source of electrons. This property requires the coordinated operation of the photosynthetic apparatus (splits water, producing O_2, electrons, and protons) and [FeFe]-hydrogenases (recombines protons and electrons, producing H_2 gas). The catalytic center of [FeFe]-hydrogenases is composed of a unique 2Fe2S metallocenter that is sensitive to O_2, a by-product of photosynthetic water oxidation. This inactivation prevents sustained H_2 production by the organism in the light. The continuity of H_2 photoproduction is one of the major technical barriers to developing photobiological H_2-production systems, as listed in the HFCIT Program MYRDDP (Barrier AI).

Our current project addresses (a) the O_2 sensitivity of H_2-producing algae by using molecular engineering (both site-directed and random mutagenesis) to alleviate this effect; (b) the further development of a platform, based on the induction of H_2 production by sulfur deprivation, to test biochemical and reactor engineering factors required to improve the rates and light-conversion efficiencies of algal H_2-photoproduction; and (c) the performance of different components of a proposed system that integrates fermentative with photobiological processes for more cost-effective, biological H_2 production. The latter addresses the MYRDDP Barrier AT (feedstock cost in an integrated system).

Approach

Task 1. Molecular Engineering of [FeFe]-Hydrogenases

This task has two objectives: (a) the engineering of increased O_2 tolerance in [FeFe]-hydrogenase through selective, random mutagenesis of region(s) that control O_2 access to the catalytic site; and (b) the functional expression of clostridial [FeFe]-hydrogenases in *Chlamydomonas reinhardtii*. The efforts being conducted under the first objective have been guided by an extensive computational study of gas diffusion in the clostridial CpI [FeFe]-hydrogenase, which identified four amino acids that form a "barrier" for O_2 migration into the catalytic site. The current experimental strategy is to increase the energy required for O_2 to migrate through this barrier by changing its amino acid composition. A random approach, though more labor- and time-intensive, will create a more comprehensive library of mutants, increasing the likelihood of finding one with improved tolerance. The efforts being conducted under the second objective involve the design of a genetic construct for expression, activation, and translocation of a clostridial [FeFe]-hydrogenase into the stromal compartment of the algal chloroplast. Clostridial [FeFe]-hydrogenases are ~100x more O_2 tolerant than algal hydrogenases, yet both undergo the same activation process. The progress made through this approach will lead to the development of expression constructs and techniques that will be essential to expressing engineered [FeFe]-hydrogenases in *C. reinhardtii*, and will provide data on the effects of a more O_2-tolerant enzyme on the kinetics and metabolism of photo-hydrogen production.

Task 2. Optimization of the Sulfur-Deprivation Platform to Test the Performance of Various Algal Mutants

With our collaborators at University of California, Berkeley (UCB), we developed a method, based on depriving algal cultures of sulfate, to induce continuous H_2 photoproduction. This procedure has become a platform for testing the performance of a variety of algal mutants, as well as to study process engineering parameters that affect the light-conversion efficiency of the system. These will become important once an O_2-tolerant hydrogenase system (see Task 1) becomes available.

Task 3. An Integrated Biological H_2-Production System

The HFCIT Hydrogen Biological Production working group identified a novel system for biological H_2 production that depends on the coordinated activity of photosynthetic (oxygenic and non-oxygenic) and fermentative organisms. An integrated system has the potential for circumventing the shortcomings of each of the individual H_2-producing components in terms of limitations in their overall light-conversion efficiencies and substrate dependence. The particular configuration being pursued at NREL involves stacked reactors of sulfur-deprived green algae and photosynthetic bacteria that produce H_2 in the light. The fermentative component consists of anaerobic bacteria that degrade the algal and photosynthetic bacteria biomass and produce H_2 and acetate as products. The latter is the source of reductant for H_2 production by the photosynthetic bacteria.

Results

Task 1. Molecular Engineering of [FeFe]-Hydrogenases

Our initial approach to engineer an O_2-tolerant [FeFe]-hydrogenase focused on *site-directed mutagenesis* of the amino acids that comprise a single barrier region controlling O_2 access from the hydrogenase's central cavity to its catalytic site. One mutation of this region in the clostridial Ca1 [FeFe]-hydrogenase possessed high O_2 tolerance when expressed and purified from *E. coli* in the absence of reducing agents. However, this property

was also found to be shared by the wild-type enzyme when purified under similar conditions, suggesting that gas accessibility alone may not be the sole determinant of O_2 sensitivity in [FeFe]-hydrogenases. The ability of the enzyme to transition between redox states may also be critical. Moreover, mutations of barrier residues may affect this property as well as gas accessibility. It is possible that this redox effect is similar to one previously described for the "inactive, O_2-resistant" state of the periplasmic [FeFe]-hydrogenases from *Desulfovibrio*, and until now a property known only to those [FeFe]-hydrogenases.

In order to increase the probability of generating O_2-tolerant hydrogenase mutants, we initiated a *random mutagenesis*/high-throughput (HTP) screening approach. It involves the design of expression vectors that can be used in combination with a gas-chromatography-based HTP assay. Work is being performed under subcontract to Golden BioEnergy.

Current site-directed and random mutagenesis efforts were performed with the Ca1 hydrogenase protein, which has a higher tolerance to O_2 inactivation in vitro than either of the two *C. reinhardtii* hydrogenases. To determine how this higher O_2-tolerance affects activity when coupled to algal photosynthesis, we developed a transformation vector to introduce the *Ca1* hydrogenase gene into the *C. reinhardtii* genome in a manner that is intended to result in the expression of an active hydrogenase. We completed the transformation of the *Ca1* gene into the *C. reinhardtii* strain CC-849 genome and identified by PCR approximately 15 transformants that show the presence of the entire *Ca1* codon-optimized gene. The presence of the *Ca1* ribonucleic acid transcript was demonstrated by RT-PCR in two transformants, and one of these appears to be anaerobically regulated (as is the case for the endogenous *HydA1* gene). In addition, preliminary biochemical evidence was obtained that suggests one *C. reinhardtii* transformant is expressing the codon-optimized Ca1 [FeFe]-hydrogenase protein. Future efforts will be directed at confirming expression of the Ca1 [FeFe]-hydrogenase protein. In addition, preliminary O_2-sensitivity results will be confirmed and physiological analyses will be pursued.

Task 2. Optimization of the Sulfur-Deprivation Platform to Test the Performance of Various Algal Mutants

Our major accomplishments on this task this past year were: (a) the demonstration of higher sustained rates of H_2 production at low light intensity ($1/10^{th}$ sun) by a truncated antenna mutant, following sulfur-deprivation and immobilization into alginate films; and (b) further confirmation that leaky ATPase mutants, which cannot accumulate a proton gradient across the thylakoid membrane, produce 30-45% more H_2 than the parental strain at high light intensities. These two results strengthen the hypotheses that H_2 production saturates at low light intensity due to (a) the presence of a large light-harvesting antenna; and (b) down-regulation of electron transport as a proton gradient accumulates across the thylakoid membrane. Our results also suggest genetic approaches to circumvent these limitations.

Task 3. An Integrated Biological H_2-Production System

We completed a series of small-scale batch experiments exploring the ability of a consortium to catabolize algal biomass and pure cellular components for H_2 production. The work demonstrated that the consortium is able to utilize both fresh and frozen (potentially damaged) biomass preparations, as well as biomass from algae that were cultured either in the presence or absence of sulfate (see Task 2). The molar yield of algal biomass fermentation varied from 6.24 H_2/glucose (when the algae were cultured under sulfur-replete conditions) to 0.94-2.30 H_2/glucose (when the algae were sulfur-deprived for different periods of time). The high molar yields of H_2/glucose observed with sulfur-replete algae suggest that the consortium is utilizing biomass components other than starch. In fact, we have demonstrated that the consortium is able to metabolize pure lipid (digalactosyl diacylglycerol) at molar ratios of 0.11 H_2/lipid and pure protein (generic bovine serum albumin) at 0.054 μmol H_2/mg protein, while co-producing organic acids.

Our collaborators in Russia successfully demonstratec H_2 production, concomitant with organic acid production by fermentative organisms using starch-rich potato wastes, and showed that the resulting organic acids were capable of being utilized by photosynthetic bacteria for additional H_2 generation.

Conclusions and Future Directions

Task 1: (a) Further investigate the biochemical properties of purified, wild-type [FeFe]-hydrogenase Ca1 to determine the inactivation kinetics of the O_2-insensitive state(s), the effects of CO, and the effects of H_2 on O_2 sensitivity; (b) characterize the biochemical and gene expression properties of *C. reinhardtii* transformants harboring the Ca1 expression construct; and (c) finish the design and start testing a new gene expression/HTP screen for generating and isolating desired O_2-tolerant hydrogenases.

Task 2: (a) Initiate a new collaboration with Johns Hopkins University to further investigate the properties of the ATPase mutants and exploit them for more efficient H_2 production; (b) test more advanced truncated antenna mutants from UCB; and (c) continue to improve the physical stability of alginate films.

Task 3: (a) Test whether the fermentative consortium can also metabolize photosynthetic bacterial biomass and alginate; (b) scale up fermentation of algal biomass; and (c) continue to optimize photosynthetic bacterial H_2 photoproduction using dilute fermentation products (organic acids) as feedstocks.

Special Recognitions & Awards/Patents Issued

Awarded travel funds by a Brazilian research funding agency (CNPq) to consult with members of the University of Bahia (Ghirardi).

FY 2009 Publications/Presentations

Publications

1. Turner, J, G Sverdrup, MK Mann, PC Maness, B Koproski, ML Ghirardi, RJ Evans, and D Blake. **2008**. *Int. J. Hydrogen Energy,* 32, 279-407.

2. Seibert, M., P. King, M.C. Posewitz, A. Melis, and M.L. Ghirardi. **2008.** *In* Bioenergy (J. Wall, C. Harwood, and A. Demain, Eds.) ASM Press, Washington, D.C., pp. 273-291.

3. Laurinavichene, T.V., D.N. Tekucheva, K.S Laurinavichius, M.L Ghirardi, M. Seibert, and A.A. Tsygankov. **2008.** *J. Hydrogen Energy 33, 7020-7026*.

4. Ghirardi, M.L., P.-C. Maness, and M. Seibert. **2008**. *In* Solar Generation of Hydrogen (K. Rajeshwar, R. McConnell, and S. Licht, Eds.) Springer, N.Y., Chapter 8, pp 229-271.

5. Blake, D.M., W. Amos, M.L. Ghirardi, and M. Seibert. **2008**. *In* Materials for the Hydrogen Economy (R. Jones and G. Thomas, Eds.) CRC Press, 123-145.

6. Posewitz, M.C., A. Dubini, J.E. Meuser, M. Seibert and M.L. Ghirardi. **2008**. *In* The Chlamydomonas Sourcebook, Vol 2: Organellar and Metabolic Processes (D. Stern, ed.) Elsevier Scientific 217-255.

7. Kosourov, SN, and M Seibert. **2008**. *Biotechn. Bioeng.102. 50-58*.

8. Ghirardi, ML, Dubini, A, Yu, J, and Maness, PC. **2009**. *Chem. Soc. Reviews* 38, 52-61.

9. Belokopytov, B.S., K.S. Laurinavichius, T.V. Laurinavichene, M.L. Ghirardi, M.Seibert, and A.A. Tsygankov. **2009**. *Int. J. Hydrogen Energy* 34, 3324-3332.

10. Meuser, J.E., G. Ananyev, L.E. Wittig, S. Kosourov, M.L. Ghirardi, M. Seibert, G.C. Dismukes, and M.C. Posewitz. **2009**. *J. Biotechnol.,* 143, 21-30.

Presentations

Invited presentations at the Energia de Portugal in Lisbon, Portugal (Ghirardi); International *Chlamydomonas* meeting in Hyeres, France (Ghirardi and Seibert); Gordon-Kenan Graduate Research Seminar at Mt. Holyoke, MA (English); Session chair – hydrogenases, GRC on Iron sulfur enzymes (King); Sustainable Energy Education Training (SEET) session, Golden, CO, July 2008 (Ghirardi); session chair at the Golden Research Conference on FeS enzymes, July 2008 (King); session chairs at the US AFOSR Biofuels Review, Arlington, VA, August 2008 (Seibert and Ghirardi); advisory board member for a University of Tennessee NSF-funded project (Ghirardi); Microbial Genomics meeting in Lake Arrowhead, CA, September 2008 (Ghirardi); co-organizer of an NREL Strategic Initiative Workshop on Novel Materials Synthesis for Renewable Energy Applications, Golden, CO, September 2008 (King); University of Wisconsin, Madison, September 2008 (Seibert); NREL Algal Group monthly meeting at NREL, October 2008 (Ghirardi); International Photosynthesis Conference in Indore, India, November 2008 (Seibert); presentation at the School of Life Sciences, Jawaharlal Nehru University, November 2008 (Seibert); session chair at the Co-Products at DOE's Bioalgal Fuels in Bethesda, MD, December 2008 (Ghirardi; Seibert also attended); the Joint U.S. AFSOR-NREL Biofuels meeting in Golden, CO, January 2009 (Seibert; Ghirardi also attended); Western Regional Photosynthesis conference in Asilomar, CA, January 2009 (Ghirardi); Gordon Conference on Solar Fuels in Ventura, CA, February 2009 (Ghirardi); CSIC, Zaragoza, Spain, April 2009 (Seibert); GLBRC Hydrogenase Forum, May 2009 (Seibert).

NREL visitors: Dr. Steven Chu (Director of the LBL); University of Wyoming professors; Andrew Gillette (Foundation for Computer Learning); Dr. Kevin Redding (Arizona State University); Jonathan Trent (NASA), Drs. Niels van der Lelie and Safiyh Taghavi (BNL), Dr. Plamen Atanassov (Director of the UNM Center for Emerging Energy Technologies); Dr. Patrice Hamel (Ohio State University); Savannah River National Lab researchers; Jacques Beadry-Losique (DOE's EERE); Prof. Harris (Georgia Institute of Technology); Drs. Andrew Hsu and Kyle Cline (Richard G. Lugar Center for Renewable Energy, Purdue University).