II.F.5 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 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 barrier from the Production Section of the Hydrogen, Fuel Cells and Infrastructure Technologies (HFC&IT) Program Multi-Year Research, Development and Demonstration (MYRDD) Plan:

(AI) Continuity of Photoproduction

Technical Targets

TABLE 1. Photolytic Biological Hydrogen Production from Water

Characteristics	Units	2003 Status	2008 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

- Discovered a new redox-state of [FeFe]-hydrogenases that is more tolerant to O_2 inactivation; finished the construction of a plasmid for expression of clostridial hydrogenases in algae.
- Increased the light-conversion efficiency, O_2-tolerance and duration of H_2 production of -S algae through immobilization in biodegradable alginate films.
- Evaluated the increase in rates of H_2 photoproduction by using single or stacked photobioreactors of green algae or photosynthetic bacteria.
- Determined a stoichiometry of about 4 mol H_2/mole glucose using algal biomass as a substrate for fermentation by a consortium of anaerobic bacteria.
- Selected a strain of purple bacteria that is able to efficiently metabolize the effluent from a H_2-producing fermentor and identified the effect of mixed substrates and toxic agents on the efficiency of photosynthetic bacterial H_2 production.

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 (recombine 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 HFC&IT Program MYRDD Plan (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 -S, 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.

Approach

Task 1. Molecular Engineering of [FeFe]-Hydrogenases

Based on computational modeling work done in collaboration with the University of Illinois, Urbana-Champaign, we have been using site-directed mutagenesis to block O_2 access to the [FeFe]-hydrogenase's catalytic site. We have also initiated a random mutagenesis approach, to improve the probability of finding non-obvious O_2-tolerant mutants. Finally, we also initiated an approach to determine the effects of more O_2-tolerant hydrogenases (such as CpI, or CaI) on the *in vivo* H_2-production properties of the photosynthetic green alga, *Chlamydomonas reinhardtii*.

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

With our collaborators at the 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 HFC&IT Hydrogen 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

Site-direct mutagenesis efforts to generate an O_2-tolerant hydrogenase this year targeted one of the amino acid residues that comprise an energy barrier that separates a loosely packed region or cavity from the H-cluster. A mutant of a single residue in the CaI hydrogenase displayed increased O_2 tolerance when expressed in *E. coli* and assayed in membrane-free extract (MFE). However, when the hydrogenases were purified, the O_2 tolerance was lost. Further results confirmed that the apparent "increased O_2 tolerance" in MFE was observed only when the mutant was prepared in the absence of reductant. This same trait (lack of O_2 sensitivity) was also observed for native CaI purified in the absence of reductant. These results are novel and suggest, for the first time, that clostridial [FeFe]-hydrogenases can be isolated under (chemically) non-reducing conditions in a variety of redox states, where one of the states is O_2 insensitive. This had been previously described for [FeFe]-hydrogenases from species of *Desulfovibrio*, but believed to be restricted only to those [FeFe]-hydrogenases.

All our current mutagenesis efforts have been done with CaI, 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 have developed a transformation vector to introduce the *CaI* gene into the algal genome in a manner that will express an active hydrogenase. The construction of the vectors has been completed, and transformation will be initiated in Fiscal Year 2009.

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

Task 2. Optimization of the –S Platform to Test the Performance of Various Algal Mutants

Our major accomplishment this year was the characterization and optimization of H_2 production

by –S algal cells immobilized in alginate films. We demonstrated that, under these conditions, algal H_2 production is less sensitive to O_2 inactivation, it lasts longer, and it occurs with light-conversion efficiencies of about 0.93% under low photosynthetically-active radiation. Finally, we also demonstrated that alginate-immobilized algal adenosine triphosphate synthase (ATPase) mutants, which have impaired coupling of proton translocation to ATP synthesis, photoproduce H_2 under sulfur-deprived conditions at 50% higher rates than non-mutated *C. reinhardtii*. This observation, which addresses Technical Target AM in the MYRDD, will be further pursued in FY 2009.

Task 3. An Integrated Biological H_2-Production System

Our work, which addressed three different components of the integrated system demonstrated:

- A significant increase in the yield of H_2 photoproduction when two photobioreactors are stacked, with the green algae on top, when compared with the performance of either of the reactors separately.
- H_2 production at molar ratios of H_2/glucose (from starch) of about 4, when fed algal biomass. High molar yields suggest that biomass components other than starch are also being metabolized.
- The characterization of a strain of *Rhodobacter sphaeroides* that is able to utilize a variety of volatile fatty acids, and exhibited interesting selectivity for 2-, 3- and 4-C molecules.

Conclusions and Future Directions

Task 1: (a) Further investigate the biochemical nature of our observations regarding the different redox states of [FeFe]-hydrogenases to help us determine what controls redox state transitions into insensitive state(s) and, whether the transition process or the states themselves differ for mutant and native enzymes; (b) transform clostridial hydrogenases in *C. reinhardtii* and characterize expression and activity, and quantify the effects of a more O_2-tolerant hydrogenase on photosynthetic H_2 production; and (c) finish the design and start testing a new gene expression/higher throughput screen for generating and isolating desired O_2 tolerant hydrogenases.

Task 2: (a) Use the -S/cell immobilization platform to further investigate the increase in H_2-production rates using ATPase mutants and (b) use the same platform to examine the light-to-H_2 conversion efficiencies of antenna mutants (from UCB).

Task 3: (a) Test whether the fermentative consortium can also metabolize photosynthetic bacterial biomass and alginate; (b) scale up fermentation of algal biomass and finish investigation of biomass components being used as substrates for fermentation; and (c) continue to optimize photosynthetic bacterial H_2 photoproduction using fermentation products.

Special Recognitions & Awards/Patents Issued

1. Seibert, M.; Makarova, V.; Tsygankov, A.A.; Rubin, A.B. "Fluorescence Technique for On-Line Monitoring of the State of Hydrogen-Producing Microorganisms." U.S. Patent No. 7,229,785, June 6, 2007.

FY 2008 Publications/Presentations

Publications

1. Hahn, J.J.; Ghirardi, M.L.; Jacoby, W.A. *Biochem. Eng. J.*, Vol. 37, 2007. p. 75-79.

2. Makarova, V.V.; Kosourov, S.; Krendeleva, T.E.; Semin, B.K.; Kukarskikh G.P.; Rubin, A.B.; Sayre, R.T.; Ghirardi, M.L.; Seibert, M. *Photosynth. Res.* Vol. 94, 2007. p. 79-89.

3. McGlynn, S.E.; Ruebush, S.S.; Naumov, A.; Nagy, L.E.; Dubini, A.; King, P.W.; Broaderick, J.B.; Posewitz, M.C.; Peters, J.W. *J.Biol. Chem.* Vol. 12, 2007. p. 443.

4. Turner, J.; Sverdrup, G.; Mann, M.K.; Maness, P.C.; Koproski, B.; Ghirardi, M.L.; Evans, R.J.; Blake, D. *Int. J. Hydrogen Energy* Vol. 32, 2008. p. 279-407.

5. Laurinavichene, T.V.; Kosourov, S.N.; Ghirardi, M.L.; Seibert, M.; Tsygankov, A.A..*Journal of Biotechn*. Vol. 135, 2008. p. 275-277.

6. Jorquera, O.; Kiperstok, A.; Sales, E.C.; Embiruçu, M.; Ghirardi, M.L. *Int. J. Hydrogen Energy* Vol. 33, 2008. p. 2167-2177.

7. Seibert, M.: King, P.W.; Posewitz, M.C.; Melis, A.; Ghirardi, M.L. (2008). *Microbial Energy Conversion* (J. Wall, A. Demain, and C. Harwood, Eds.) ASM Press, pp. 273-291.

8. Posewitz, M.C.; Dubini, A.; Meuser, J.E.; Seibert, M.; Ghirardi, M.L. (2008). *Chlamydomonas Source Book* (E. Harris and D. Stern, Eds.), *in press*.

9. Ghirardi, M.L.; Maness, P.C.; Seibert, M. (2008). *Solar Generation of Hydrogen* (K. Rajeshwar, R. McConnell, and S. Licht, ed.), Springer Verlag, *in press*.

10. Blake, D.M.; Amos, W.; Ghirardi, M.L.; Seibert, M..(2008). *Materials for the Hydrogen Economy* (R. Jones and G. Thomas, Eds.) CRC Press, *in press*.

Presentations

1. Invited presentations at Colorado School of Mines (Seibert); Arizona State University (Seibert); Annual Meeting of the Society of Industrial Microbiology, Denver, CO (King and Seibert); SPIE Optics and Photonics Meeting, San Diego, CA (King); 8th International Hydrogenase Conference, Breckenridge, CO (King);

American Chemical Society Meeting, Boston, MA (Seibert); ARO Workshop on Base Camp Sustainability, Raleigh, NC (Seibert); University of California, Davis (Ghirardi); Universidade Federal da Bahia, Salvador, Brazil (Ghirardi); IEA Annex 21 meeting in Porto, Portugal (Seibert); Italian BioHydrogen Workshop in Florence (Seibert); Materials Research Society meeting, San Francisco, CA (Ghirardi); Thermal Biology Institute at Montana State University (King); Renewable energy event in Lisbon, Portugal (Ghirardi); International *Chlamydomonas* conference in France (Ghirardi and Seibert).

2. NREL visitors: Brazilian delegation of university and government representatives; Clay Sell, DOE; Gerry Tuskan, representative from DOE's LSP (Laboratory Sequencing Program); Dr. David Levin from University of Manitoba, Canada; Dr. Stephen Mayfield from Scripps; Dr. van der Lelie from BNL; GAO representatives; Hong Jin and Scott Mason from Conoco Philips; Dr. Arumugam from India; Dr. Steve Berman, JGI, visitors from Matsushita; Dr. Mark Gomelsky from the University of Wyoming.

3. Others: interview by *Science News for Kids* (Ghirardi); organization and running of 8th International Hydrogenase and Hydrogen Production Conference in Breckenridge, CO (Ghirardi) and poster presentations (Kosourov, Ghirardi, and Seibert); organization of the IEA Annex 21 Experts meeting and presentation of the USA report, Breckenridge, CO (Seibert); reviewer for the AFOSR Bio-Solar Hydrogen mid-term review (Seibert); panelist at the HNEI BioEnergy Forum (Seibert); reviewers and session chairs for the USAFOSR workshop on algal biodiesel (Ghirardi and Seibert); attendance of a NASA workshop on renewable energy at the Ames Center in California (Seibert and Ghirardi).