II.H.2 Biological Systems for Hydrogen Photoproduction

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Projected End Date: Project continuation and direction determined annually by DOE

Objectives

- Engineer an [FeFe]-hydrogenase that is resistant to O_2 inactivation as part of an aerobic algal H_2-production system being developed with the University of California, Berkeley (UCB).
- Optimize a physiological method to promote culture anaerobiosis and H_2-production activity in algae.
- Address one of the 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 Hydrogen Production section of the HFC&IT Program Multi-Year Research, Development and Demonstration Plan:

(AI) Continuity of Photoproduction

Technical Targets

Characteristics	Current Status	2013 Target
Duration of continuous photoproduction*	180 days (sulfur-deprived)	30 min (O_2-tolerant hydrogenase)
O_2 tolerance (half-life in air)	2–4 min (*Clostridium* hydrogenase *in vitro*)	10 min

*Duration reflects continuous production in the light, not necessarily at peak efficiencies.

Accomplishments

- Substituting larger, bulkier residues for smaller residues along the hydrogenase gas channel has been partially successful. New computational results have presented evidence that O_2 diffusion through a protein correlates well with the occurrence of specific amino acid residues, but not as well with the occurrence of a specific protein fold in the tertiary structure.
- Immobilized sulfur-deprived algal cultures on new substrate materials led to a significant increase in the stability of H_2-production activity, increase in the light conversion efficiency, and increase in tolerance to O_2 compared to suspension cultures.
- Observed H_2 production from a consortium of fermentative organisms (collected from sewage sludge) fed with algal biomass that had been sulfur-deprived for 24 hours (time point at which starch accumulation achieves its peak).

Future Directions

Task 1. Molecular Engineering of the Algal Hydrogenase

- Utilize the new results obtained from the computational simulations to identify new targets and mutational strategies for engineering of the *Clostridium pateurianum* CpI hydrogenase.
- Transform, express and measure the activity of a more O_2-tolerant clostridial hydrogenase in *C. reinhardtii*.
- Use other mutagenesis approaches to generate additional hydrogenase mutants; utilize a high-throughput assay to screen positive transformants for O_2 tolerance.
- Initiate molecular gas-diffusion work using other model hydrogenases of interest to DOE.

Task 2. Biochemical and Process Engineering

- Evaluate the H_2-production activity of sulfur-deprived algal cultures when immobilized on polymeric films.
- Test the performance of truncated-antenna mutants under sulfur deprivation.
- Perform techno-economic analyses of the immobilized, sulfur-deprived algal system.

Task 3. Integrated System

- Compare the light conversion efficiencies of single vs. stacked reactors of algae and photosynthetic bacteria.
- Optimize microbial fermentation of algal cell biomass with concomitant H_2-production.
- Optimize H_2 production by photosynthetic bacteria.

Introduction

Eukaryotic green algae can photoproduce H_2 from water, and this property requires the coordinated operation of the photosynthetic water oxidation machinery (which generates O_2, reductants, and protons from water) and the hydrogenase enzyme (which recombines protons and electrons to produce H_2 gas). The catalytic center of green algal [FeFe]-hydrogenases is composed of a unique 2Fe2S center that is sensitive to O_2, a by-product of photosynthetic water oxidation. O_2 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 identified by the *HFC&IT Program Multi-Year Research, Development and Demonstration Plan.*

Our current project addresses two different strategies for surmounting the O_2 sensitivity of H_2-producing algae: (a) molecular engineering efforts to alleviate the O_2 sensitivity of the [FeFe]-hydrogenase (Task 1), and (b) use of a physiological switch to separate O_2 and H_2 production (Task 2). Our project also proposes to develop a novel system that integrates photobiological H_2 production with fermentative processes (Task 3).

Approach

Task 1. Molecular Engineering of the Algal Hydrogenase

Our hypothesis is that inactivation of the algal [FeFe]-hydrogenase depends on access of O_2 to the enzyme's catalytic site through a hydrophobic channel connecting the surface to the catalytic center. In collaboration with the Beckman Institute of the University of Illinois and the National Renewable Energy Laboratory's (NREL) Computational Sciences Center, we have utilized computational simulation models to help us develop a strategy to prevent O_2 from diffusing into the catalytic site, while maintaining high H_2 diffusion from the site. Different approaches are currently being tested experimentally and validated with respect to the types of residues that confer O_2 tolerance to [FeFe]-hydrogenases.

Task 2. Biochemical and Process Engineering

In 2000, NREL and UCB jointly developed a nearer-term approach to circumventing the O_2-sensitivity issue of biological H_2 production. This approach is based on the metabolic shift from O_2 to H_2 production induced by depriving algal cultures of sulfate. The original system, which was designed to operate in cycles of +S and –S, was later converted into a continuous H_2-producing system at NREL, and resulted in a decrease by a factor of three in the estimated cost of H_2 production. The latter was optimized for continuity of operation, but yields and rates of H_2 production were too low for scale-up. We subsequently increased the efficiency of the algal system by immobilizing sulfur-deprived cells on to different matrices. However, an evaluation of the overall effect of cell immobilization on the cost of the H_2 produced has not been done yet. The latter will provide us with guidance regarding further necessary improvements. Given the inherent low light-conversion efficiency of sulfur-deprived systems, we are thus using sulfur-deprived, immobilized cultures as a means to study critical process engineering parameters that will become important once an O_2-tolerant hydrogenase system (see Task 1) becomes available.

Task 3. Integrated H_2-Production System

The HFC&IT Hydrogen Production Team 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 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 from, respectively, H_2O and added acetate. The fermentative component consists of anaerobic bacteria that are able to degrade the algal and photosynthetic bacterial biomass and produce H_2 and acetate as products. The latter is the source of reductants for H_2 production by the photosynthetic bacteria.

Results

Task 1. Molecular Engineering of the Algal Hydrogenase

Our previous theoretical results demonstrated that, while [FeFe]-hydrogenases are very porous to H_2, the diffusion of O_2 molecules is restricted to two very well-defined pathways (or, more accurately, a bifurcated pathway) and their movement is restricted by the presence of transient cavities in these pathways. From these studies emerged a hypothesis that the kinetics of

O_2 diffusion, and therefore enzyme inactivation, should be amenable to modification through the molecular engineering of the gas pathways. Targets for mutagenesis have been selected and evaluated computationally for an effect on limiting O_2 access to the catalytic site. An important consideration obtained from this evaluation phase was that H_2 diffusion in hydrogenase was not significantly altered by any of the pathway mutations. Based on the guidance provided by the theoretical results, we have generated clostridial hydrogenase mutants that were tested experimentally for kinetics of O_2 inactivation. One of the single mutants showed both an increase in O_2 tolerance and normal levels of enzymatic activity. However, other hydrogenase pathway mutants showed a significant loss of activity and, unexpectedly, an increase in O_2 sensitivity. This suggests that in contrast to results of the computational simulations, some mutations result in a more accessible catalytic site and might possibly effect maturation or catalysis. With these experimental results, the computational theory has been improved leading to a second generation of mutations that will be evaluated experimentally.

Task 2. Biochemical and Process Engineering

Due to lack of funds from DOE, our group was supported by the U.S. Air Force Office of Scientific Research (USAFOSR) for part of FY 2006 and the initial five months of FY 2007. With USAFOSR support, we were able to show that immobilization of sulfur-deprived cultures onto new substrate materials significantly increase the stability of the H_2-production activity, resulting in close to a 1% incident light conversion efficiencies. Finally, we also observed that the matrices used in this work permit H_2 production to occur even in the presence of O_2 in the headspace.

Task 3. Integrated H_2-producing System

We assessed the capability of a consortium of fermentative organisms, isolated from sewage sludge to produce H_2 upon utilization of algal biomass. The initial experiments were done using algal cells that were isolated from the sulfur-deprivation photobioreactor at the maximum point of starch accumulation (about 24 hours following re-suspension in sulfur-deprived medium). Preliminary results demonstrate that high H_2-production rates correlated well with high levels of algal starch storage. Further experiments will be conducted to determine whether the consortium can also produce H_2 when fed algal biomass from the end of the sulfur-deprivation process, when starch levels are low, but polysaccharide cell wall may become accessible.

Conclusions

- Recent computational simulations indicate that substituting a different class of amino acid residues (identified in the gas pathways of gas-transport proteins) might have a more definitive effect in reducing the O_2 accessibility of the hydrogenase catalytic site than our previous approach.
- The incident light conversion efficiency of H_2-producing algae, the capability of the cultures to produce H_2 in the presence of O_2 in the headspace, and the length of the H_2-producing phase were all enhanced by cell immobilization in polymeric matrices.
- A consortium of fermentative organisms was able to produce H_2 by fermenting starch-enriched algal biomass.

FY 2007 Publications/Presentations

Publications

In press:

1. Blake, D., Amos, W., Ghirardi, M.L., and Seibert, M. Materials requirements for photobiological hydrogen production. In *Materials for the Hydrogen Economy*, CRC Press;

2. Hahn, J.J., Ghirardi, M.L. and Jacoby, W.A. 2007. *Biochem. Engin. J.*;

3. Turner, J., Sverdrup, G., Mann, M.K., Maness, P.C., Kroposki, B., Ghirardi, M., Evans, R.J. and Blake, D. Int. *J. Hydrogen Energy*.

4. Ghirardi, M.L., Maness, P.C., and Seibert, M. In *Solar Generation of Hydrogen*, (McConell, ed.) Springer Verlag.

Published:

1. Melis, A., Ghirardi, M.L., and Seibert, M. In *Transgenic Microalgae as Green Cell Factories* (Leon, Fernandez, and Galvan, Eds.) Landers Bioscience Publ., Georgetown, TX.

2. Tsygankov, A.A., Kosourov, S.N., Tolstygina, I.V., Ghirardi, M.L., and Seibert, M. *Int. J. Hydrogen Energy* 31: 1574-1584.

3. King, PW, D Svedruzic, J Cohen, K Schulten, M Seibert and ML Ghirardi. 2006. *Proc. of SPIE* 6340, 63400Y.

4. Ghirardi, ML, J Cohen, P King, K Schulten, K Kim and M Seibert. 2006. *Proc. of SPIE* 6340, 63400X.

5. Ghirardi, ML. 2006. *Indian J Biochem. Biophys.* 43, 201-210.

6. Ghirardi, ML, MC Posewitz, PC Maness, A Dubini, J Yu and M Seibert. 2007. *Ann. Rev. Plant Biol.* 58, 71-91.

7. Kosourov, S., Patrusheva, E., Ghirardi, M.L., Seibert, M. and Tsygankov, A. 2007. J. Biotechnol. 128: 776-787.

8. Nagy, LE, JE Meuser, S Plummer, M Seibert, ML Ghirardi, PW King, D Ahmann and MC Posewitz. 2007. *Biotechnol. Lett*. 29: 431-430.

Presentations

1. NREL visitors: Savanah River National lab representatives; Prof. D. Bagley, University of Wyoming; Matt Caspari from Aurora Biofuels; Upward Bound Program from Metro State, Denver; BASF Venture Capital America, Inc.; the National Development and Reform Commission of the People's Republic of China; Dave Thomassan, DOE's Office of Science; Mesa State Community College; the Minority Leader of the Senate Environment Public Works Committee; members of the Technology Information, Forescasting and Assessment Council from India; Dave Austgen from Shell Hydrogen; Julie Carruthers, the Science and Policy Advisor for DOE's Office of Science; Roger Dahlman, DOE's Climate Change Research Division; Gary Johnson, DOE's Advanced Scientific Computing Research Division; Ram Ramachandra, Vice-President Technology and Satish Tamhankar, Section Director PGS Technology for BOC/Linde; Ray Orbach, DOE Undersecretary for Science; John Hofmeister from Shell International; Exxon Mobil representatives.

2. Educational video for a Boulder TV station (Ghirardi); CU/NREL poster session in Boulder (all team members); interview by Self Reliance Foundation (Ghirardi).

3. Speaker at the Kavli Frontiers of Science (Ghirardi); at Kansas State University (Seibert); at Washington University, St. Louis MO (Ghirardi); attendance of the closed Asia Bio-Hylinks meeting in Taiwan (Seibert); session chair at the 2006 Asian Biohydrogen Symposium in Taiwan (Seibert); representative at the IEA Annex 21 Biohydrogen Experts meeting in Taiwan (Seibert); speaker at the Photosynthesis Gordon Conference (Seibert); at AFOSR Hydrogen symposium in Princeton (Seibert); session chair at the Western Photosynthesis Conference (Ghirardi); speaker at the Gordon Conference on Renewable Fuels (Ghirardi); at Ohio State University (Ghirardi); at PNNL (Seibert); at Rutgers University (Seibert); at Brookhaven National Laboratory (King); at Regis College, Denver (Seibert); at the Arrowhead 2006 meeting (Seibert); at NREL's Power Lunch (Ghirardi); at the Colorado School of Mines (Seibert).

Special Recognitions & Awards/Patents Issued

1. Nominated a Kavli Fellow (Ghirardi); Staff Award for Excellence (King).