# II.H.2 Biological Systems for Hydrogen Photoproduction

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- Dr. Eric Johnson, Johns Hopkins University, Baltimore, MD
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### **Objectives**

- Engineer an [FeFe]-hydrogenase that has an extended half-life following exposure to O<sub>2</sub>, as part of an aerobic algal H<sub>2</sub>-production system being developed with other Fuel Cell Technologies Program-sponsored groups.
- Optimize and use a platform for testing algal mutants with improved H<sub>2</sub>-production properties and higher light-conversion efficiencies.
- Address individual components of an innovative H<sub>2</sub>production system based on integrating fermentative and photosynthetic H<sub>2</sub>-producing organisms.

### **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:

- (AI) Continuity of Photoproduction
- (AH) Rate of Hydrogen Production
- (AT) Feedstock Cost

### **Technical Targets**

TABLE 1. Photolytic Biological Hydrogen Production from Water

Characteristics	Units	2003 Status	2010 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 <sub>2</sub> Tolerance (half-life in air)	Time units	1 s	4 min (clostridial enzyme, oxidized) 40 min (clostridial enzyme, reduced)	10 min (aerobic)	2 h

N/A - not available

#### Accomplishments

- Extended the computational modeling techniques used to identify gas diffusion to the *Desulfovibrio* gigas [NiFe]-hydrogenase.
- Confirmed that the reduced state of the [FeFe]hydrogenase is more tolerant to O<sub>2</sub> in vitro than the oxidized state; detected an "O<sub>2</sub>-insensitive" state.
- Identified positive *Chlamydomonas* transformants transcribing the Ca1 hydrogenase gene.
- Simulated fusions between the petF ferredoxin and algal/clostridial hydrogenases to test optimal interactions and performed in vitro tests of fusions.
- Observed that increased thickness of the alginate film improves O<sub>2</sub> tolerance but decreases H<sub>2</sub>-production rates.
- Designed adenosine triphospate (ATP) synthase inducible mutants.
- Demonstrated that an anaerobic clostridial consortium ferments algal biomass, pure algal lipids, pure proteins and unpolymerized alginate.
- Optimized fermentative H<sub>2</sub> production from potato waste.
- Demonstrated sequential H<sub>2</sub> production from darkand light-driven processes.

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#### 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 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 upon many factors: 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: (a) the  $O_2$  sensitivity of  $H_2$ -producing algae and the low rates of  $H_2$ production by using molecular engineering (both sitedirected and random mutagenesis) to alleviate these barriers; (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 technical barrier AT (feedstock cost in an integrated system).

# Approach

Task 1. Molecular Engineering of [FeFe]-hydrogenases

This task has three objectives: (a) the engineering of increased O<sub>2</sub> tolerance in [FeFe]-hydrogenase through site-directed or random mutagenesis of region(s) that control  $O_2$  access to the catalytic site; (b) the functional expression of clostridial [FeFe]-hydrogenases in Chlamydomonas reinhardtii; and (c) the evaluation of strategies for decreasing the competition between the CO<sub>2</sub>-fixation and the H<sub>2</sub>-production pathways, which are being implemented in cooperation with a research group at MIT. 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<sub>2</sub> migration into the catalytic site. Up until now, our experimental strategy had been to increase the energy required for  $O_2$  to migrate through this barrier by changing its amino acid composition. Targets for mutagenesis were selected based on static and dynamic computational simulations

of gas diffusion and identification of energy barriers. A random mutagenesis approach, though more laborand 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.

In order to control the flow of photosynthetic reductant away from  $CO_2$  fixation and towards  $H_2$  photoproduction, we are working with Prof. Zhang's research group at MIT to engineer fusions between ferredoxins, the final electron acceptor in photosynthesis, and the HYDA1 algal hydrogenase. This work should prove the hypothesis that it is possible to decrease the competition between these two electron transport pathways and thus increase  $H_2$  photoproduction even in the presence of  $CO_2$ .

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, 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<sub>2</sub>-Production System

The FCT 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 two particular configurations being pursued at NREL

involve: (a) stacked reactors of sulfur-deprived green algae and photosynthetic bacteria that produce  $H_2$  in the light, followed by a fermentative component consisting 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; and (b) fermentors that utilize potato waste to produce  $H_2$ and organic acids, followed by organic acid-dependent photosynthetic  $H_2$  production by photosynthetic purple non-sulfur bacteria.

### Results

Task 1. Molecular Engineering of [FeFe]-hydrogenases

Our initial approach to engineer an O<sub>2</sub>-tolerant [FeFe]-hydrogenase focused on site-directed mutagenesis of the amino acids that comprise a single barrier region controlling O<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> sensitivity in [FeFe]-hydrogenases. This year, we showed that the ability of the enzyme to transition between redox states is critical for O<sub>2</sub> tolerance, and that hydrogenases isolated in the reduced state are more tolerant to O<sub>2</sub> than those isolated in the oxidized state. We also observed the presence of an apparently "O<sub>2</sub>-insensitive" state that may be related to similar states described for Desulfovibrio [FeFe]-hydrogenases. We are preparing a manuscript describing these observations.

We also observed that, based on the recently published crystal structure of the algal hydrogenase by the Peters' group, our targets for mutagenesis could interfere with the appropriate folding and assembly of the enzyme's catalytic center, resulting mostly in inactive mutants, which agrees with our results. Thus, while considering different mutagenesis strategies for generation of O<sub>2</sub>-tolerant Ca1 mutants, we also initiated efforts 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. Since Ca1 is already more O<sub>2</sub> tolerant than the algal hydrogenase, these studies will provide evidence that increased in vitro O2 tolerance does result in increase in vivo tolerance. For these purposes, we introduced the Ca1 gene into the C. reinhardtii strain CC-849 genome and identified by polymerase chain reaction (PCR) transformants that show the presence of the entire Ca1 codon-optimized gene. The presence of the Ca1 transcript was demonstrated by reverse transcriptase

polymerase chain reaction in at least one transformant, and it is anaerobically induced in a similar manner as expression of the endogenous *C. reinhardtii HYDA1*. Future efforts will be directed towards confirming that in these transformants the Ca1 protein is also expressed, localized and active. In addition, once CaI is confirmed to be active, preliminary  $O_2$ -sensitivity results will be confirmed and physiological characterization will be pursued.

In order to understand the partitioning of photosynthetic reductant among different metabolic pathways, we measured H<sub>2</sub> evolution and its competitive reaction, reduced nicotinamide adeninine dinucleotide phosphate (NADPH) production (required for CO<sub>o</sub> fixation), in a series of *in vitro* reactions with purified thylakoids, Ferredoxin (Fd), FD/NADPH oxidoreductase (FNR) NADP<sup>+</sup> and hydrogenases. In agreement with previous studies, we found that lightmediated H<sub>2</sub> evolution by algal hydrogenase with thylakoid membranes is blocked in the presence of FNR, Fd and oxidized NADP. When free hydrogenase was replaced with a Fd-hydrogenase fusion, H<sub>2</sub> was evolved at near maximal rates with NADPH as a co-product. Some of the NADPH was also recycled back into H<sub>2</sub>. This tunable, catalytic complex led to partitioning of photosynthetic electron transport to H<sub>2</sub> even under conditions that support CO<sub>2</sub> fixation and will be investigated as a way to improve light-conversion efficiencies in algae under photosynthetic growth for scale-up development.

#### 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 the discoveries that: (a) a decrease in film thickness improves maximum specific rates and yields of  $H_2$  production under anaerobic conditions but decreases  $H_2$  production rates under aerobic conditions due to protection from  $O_2$  inactivation; (b) the addition of acetate to alginate-immobilized algal cells stimulates  $H_2$  production; (c) alginate-immobilized ATP synthase mutants show increased  $H_2$  production under both low and high illumination, as predicted from uncoupled preparations; and (d) new ATP synthase mutants have been designed and are being introduced in *C. reinhardtii* behind an inducible promoter to allows us to regulate its expression.

# Task 3. An Integrated Biological $\rm H_2\mathchar`-Production$ System

We demonstrated that our anaerobic bacterial consortium metabolizes algal biomass with a  $H_2$ /glucose ratio higher than 4, suggesting that components other than carbohydrates are being utilized. This

hypothesis was confirmed by the observation that the same consortium is capable of degrading pure algal lipid and protein. Finally, we demonstrated that the consortium also metabolizes un-polymerized alginate, thus underlining its usefulness in consuming residual immobilized algal biomass from an integrated photobiological/fermentative  $H_2$ -production system.

Working with a different integrated system that links fermentation of potato waste to photosynthetic  $H_2$  production by purple non-sulfur bacteria, our collaborators in Russia optimized the fermentative component by examining factors such as exclusion of ammonium; addition of Fe ions, peptone and zinc; and increased phosphate buffering capacity. They reported final yields of 1.6 moles  $H_2$ /glucose. Finally, they demonstrated sequential  $H_2$  production from the integrated system with maximum yields at this point of 5.6 moles  $H_2$ /glucose if the fermentation effluent is feed to the non-sulfur bacteria.

# **Conclusions and Future Directions**

**Task 1:** (a) Continue to characterize positive algal transformants expressing the bacterial Ca1 gene; (b) measure the *in vivo*  $O_2$  tolerance of those transformants; (c) devise a different mutagenesis approach to generate  $O_2$ -tolerant [FeFe]-hydrogenase mutants; and (d) complete *in vitro* studies of Fd/hydrogenase fusions in collaboration with MIT and attempt *in vivo* expression of the fused proteins.

**Task 2:** (a) Test the effect of the volume of the photobioreactor's headspace on the  $H_2$ -production properties of algal cultures; (b) adapt and improve on the methods previously used to induce photoautotrophic cultures to produce  $H_2$  in the absence of added acetate; (c) test more advanced truncated antenna mutants from the University of California, Berkeley; and (d) construct and test the performance of an ATP synthase gene expressed in *C. reinhardtii* behind an inducible promoter.

**Task 3:** (a) Scale up and further optimize fermentation of suspended and immobilized algal biomass by the fermentative consortium using new fermentors; (b) optimize the integration of the fermentative/photobiological  $H_2$ -production system using potato waste as the feedstock.

# **Special Recognitions & Awards/Patents Issued**

**1.** Seibert was elected the new Operating Agent for the IEA/HIA Task 21 (Biohydrogen). Ghirardi was elected a Fellow of the Renewable and Sustainable Energy Institute (RASEI).

# FY 2010 Publications/Presentations

#### **Publications**

1. Belokopytov, B.S., K.S. Laurinavichius, T.V. Laurinavichene, M.L. Ghirardi, M.Seibert, and A.A. Tsygankov. 2009. "Towards the integration of darkand photo-fermentative waste treatment. 2. Optimization of starch-dependent fermentative hydrogen production". *Int. J. Hydrogen Energy*, 34: 3324-3332.

**2.** Maness, P.C., J. Yu, C. Eckert and M.L. Ghirardi. 2009. "Photobiological hydrogen production – prospects and challenges". *Microbe* 4: 275-280.

**3.** Seibert, M. 2009. "Applied Photosynthesis for Biofuels Production", *in* Photobiological Sciences Online (K.C. Smith, Ed.) Am. Soc. Photobiol. Website: http://www.photobiology.info/Seibert.html#TOP

**4.** Ghirardi, M.L., S. Kosourov, P.C. Maness, S. Smolinski and M. Seibert. 2009. "Algal H<sub>2</sub> Production" in *Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation and Cell Technology* (ed. M.C. Flickenger), John Wiley and Sons, Inc.

**5.** Long H., P.W. King, M.L. Ghirardi and K. Kim. 2009. "Hydrogenase/ferredoxin charge-transfer complexes: effect of hydrogenase mutations on the complex association". *J. Phys. Chem. A.* 113:4060-7.

**6.** English C.M., C. Eckert, K. Brown, M. Seibert and P.W. King. 2009. "Recombinant and *in vitro* expression systems for hydrogenases: new frontiers in basic and applied studies for biological and synthetic  $H_2$  production". *Dalton Trans.* 45:9970-78.

**7.** Ghirardi, M.L. and Mohanty, P. 2010." Oxygenic hydrogen photoproduction – current status of the technology". *Current Science India*, 98, 499-507.

8. Laurinavichene, T.V., B.F. Belokopytov, K.S. Laurinavichius, D.N. Tekucheva, M. Seibert and A.A. Tsygankov. 2010. "Towards the integration of darkand photo-fermentative waste treatment. 3. Potato as substrate for sequential dark fermentation and light-driven  $H_2$  production." *Int. J. Hydrogen Energy*, 16, 8536-8543.

**9.** Smolinski, S., S.N. Kosourov, P.C. Maness and M.L. Ghirardi. "Hydrogen production from the fermentation of algal biomass by a bacterial consortium isolated from wastewater sludge". Submitted.

**10.** 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." Submitted.

#### Presentations

**Invited presentations** at the CSIC Spanish National laboratory in Zaragoza, Spain, Apr 09 (Seibert); to the group of Dr. X. Zhang at MIT, Apr 2009 (King); plenary talk at the Great Lakes Bioenergy Research Center (GLBRC) Hydrogenase Forum, May 09 (Seibert); at the American Society for Plant Biology meeting in Hawaii, Jul 09 (Ghirardi); update on EERE BioHydrogen research at the U.S. Air Force Office of Scientific Research Annual Review Meeting, Aug 09 (Seibert); the USA country report at the IEA Annex 21 Biohydrogen Experts Meeting in Jyväskylä, Finland, Sep 09 (Seibert); invited presentation at the University of Washington, St. Louis, Sep 09 (Ghirardi); invited presentation at the Rocky Mountain American Vacuum Society meeting, Denver, Sept 09 (Ghirardi); invited presentation at the Center for Revolutionary Solar Photoconversion meeting in Denver, Oct 09 (Ghirardi); invited presentation at the Fall Rocky Mountain Branch of the American Society for Microbiology in Denver, Nov 09 (Ghirardi); presentation to the Solar Fuels 2009 Meeting, Sigtuna, Sweden, Oct 2009 (King); invited presentation to

the Microbiology Department, Colorado State University, Nov. 09 (Seibert); at NREL's Energy Bioscience Center monthly seminar, Jan 10 (Ghirardi); USA country report at the IEA Annex 21 Biohydrogen Experts Meeting in Florence, Italy, March, 2010 (Seibert); invited talk at the DiBA-UNIFI & ISE-CNR Workshop on BioHydrogen in Florence, Italy, March, 2010 (Seibert); visit J. Zhang's group at MIT, april 28–30<sup>th</sup> (King); departmental seminar presentation at the North Carolina State University, Raleigh, NC, May 2–4 (Seibert); invited talk at the Christian-Albrechts-University in Kiel, Germany, May 16–18 (Seibert); the Kendric C. Smith Lecture on Innovations in Photobiology, June 12–14 (Seibert); oral presentation at the Gordon Conference on Iron-sulfur Enzymes, Colby-Sawer College, NH, June 6–11 (King).