# II.G.1 Biological Systems for Hydrogen Photoproduction

Maria L. Ghirardi (Primary Contact), Paul W. King, Kathleen Ratcliff and David Mulder
National Renewable Energy Laboratory (NREL)
1617 Cole Blvd.
Golden, CO 80401
Phone: (303) 384-6312
Email: maria.ghirardi@nrel.gov

DOE Manager
Eric Miller
Phone: (202) 287-5829
Email: Eric.Miller@hq.doe.gov

Subcontractors:
- Dr. Sergey Kosourov, Institute of Basic Biological Problems, RAS, Pushchino, Russia
- Dr. Eric Johnson, Johns Hopkins University, Baltimore, MD

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

## Fiscal Year (FY) 2012 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*
- 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 an altered chloroplast adenosine triphosphate (ATP) synthase on the rates of $H_2$ photoproduction.

## Technical Barriers

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

(AH) Rate of $H_2$ production

(AI) Continuity of $H_2$ production

## 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) | 4.5 min | 30 min | 12 hours |
| – Anaerobic, low STH (S-deprivation) | 50-150 days | | Indefinite |
| – Aerobic, low STH (S-deprivation) | 10 days | | Indefinite |
| Cost ($/kg $H_2$) | | | |
| – Aerobic, high STH | | | $2.99 |
| – Anaerobic, low STH | | | $6.02 |

## FY 2012 Accomplishments

- Successfully expressed a more $O_2$-tolerant clostridial hydrogenase (Ca1) in a double hydrogenase knock-out mutant of *Chlamydomonas reinhardtii* and observed in *vivo* $H_2$ photoproduction.
- Reached a No-Go decision concerning a mutagenesis approach to generate targeted random mutants in *Chlamydomonas.*
- Demonstrated long-term $H_2$ photoproduction by sulfur-deprived algal cultures immobilized into alginate films, using cycles of +S/-S or continuous flow of medium with low concentrations of sulfate.
- Successfully generated an inducible system for expression of chloroplast genes and tested its efficacy in expressing an Orange Fluorescent Protein.
- Tested transformants containing altered atpE genes using immobilized cells under sulfur-deprivation conditions.

## 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 [FeFe]-hydrogenase 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 $H_2$ production is the low rate of the reaction (technical barrier AH), which is

dependent on many intracellular regulatory factors, including the down-regulation of photosynthetic electron transport from water under $H_2$-producing conditions.

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.

## Approach

### Task 1. Molecular Engineering Approaches to Increase the $O_2$ Tolerance of $H_2$ Photoproduction

This task has two 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; and (b) the expression of a functional, more $O_2$-tolerant clostridial [FeFe]-hydrogenase in *Chlamydomonas reinhardtii*.

The targeted mutagenesis approach is guided by extensive computational studies of gas diffusion in the *Clostridium pasteurianum* CpI [FeFe]-hydrogenase (that has a solved crystal structure). These studies previously identified a hydrophobic cavity separated from the catalytic H-cluster by a high-energy barrier. However, site-directed mutagenesis of barrier residues designed to reduce $O_2$ transport did not yield enzymes with higher $O_2$-tolerance. Alternative computational and mutagenesis approaches were devised in order to target residues present in the regions around the diffusion barriers (but not in the barrier *per se*), and high-throughput assays were developed in FY 2012 to test the hypothesis. However, due to the complexity of the $O_2$ effect on hydrogenases as observed by our previous efforts, combined with a limited budget for FY 2012, we concluded that, in order to meet programmatic milestones for increased oxygen tolerance it would be more feasible to focus on introducing known, more $O_2$-tolerant enzymes into *Chlamydomonas*. If an additional budget is available in the future, we will return to pursuing the targeted random mutagenesis approach for engineering.

Our studies aimed at expressing a more $O_2$-tolerant hydrogenase from *Clostridium acetobutylicum* (Ca1) in *C. reinhardtii* have benefited enormously from recent findings from a DOE Office of Science project under Dr. Ghirardi, which provided us with a *Chlamydomonas* strain that lacks the two native algal hydrogenases. This strain, together with our efforts at optimizing Ca1 gene expression in *Chlamydomonas* during FY 2012, has been used in our current efforts to demonstrate *in vivo* $H_2$ photoproduction by a transformant expressing only Ca1. The availability of this transformant will allow us to observe the effects of a more $O_2$-tolerant hydrogenase in algal $H_2$ production and overall physiology and guide further studies aimed at developing hydrogenases with even higher $O_2$ tolerance.

### Task 2. Use of the Sulfur-Deprivation Platform to Test Additional Biochemical and Engineering Barriers to $H_2$ Photoproduction

To induce sustained $H_2$ photoproduction, we collaborated with the University of California in 2000 and developed 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. In FY 2012, our efforts focused on (a) increasing the duration of $H_2$ photoproduction using immobilized algal cells; and (b) developing inducible mutants that express an altered ATP synthase gene that prevents down-regulation of electron transport rates during $H_2$ photoproduction.

## Results

### Task 1. Molecular Engineering Approaches to Increase the $O_2$ Tolerance of $H_2$ Photoproduction

Computational simulations identified differences in the geometries and energies of the gas diffusion barriers protecting the H-cluster of [FeFe]-hydrogenases, indicating that diffusion itself may not be the limiting step for inactivation. Based on these studies, we identified targets for mutagenesis that may increase the energy barriers and allow diffusion to become the rate-limiting step, without affecting the maturation of these hydrogenases. In order to test the validity of this hypothesis, we developed a chemochromic, high-throughput assay, based on the oxidation of methyl viologen by $H_2$-producing algae and adapted it to a microwell format. The assay was tested with *Escherichia coli* strains expressing hydrogenase mutants that are unable to produce $H_2$ and it was further optimized. The selected *E. coli* strain, Rosetta-2 (DE3) was chosen as a host for mutated hydrogenases due to its low background endogenous hydrogenase activity (which is catalyzed by native [NiFe]-hydrogenases) and it was shown to successfully express the mutated [FeFe]-hydrogenases upon IPTG (isopropyl β-D-1 thiogalactopyranoside) induction.

More recently, we successfully expressed the clostridial Ca1 hydrogenase in wild-type *Chlamydomonas*, using a psaD promoter and terminator based-expression system. The data obtained from these transformed strains, however, have been challenging to interpret. Low expression levels of Ca1 relative to the native algal hydrogenases (HYDA1 and HYDA2) made testing the resulting effects of CaI expression on $O_2$ sensitivity highly variable, and thus difficult to interpret. However, $O_2$-inhibition assays revealed double decay kinetics, suggesting that the more

$O_2$-tolerant Ca1 enzyme was expressed and active in the transformed alga. At the beginning of FY 2012, we shifted our transformation experiments to a new host, a double hydrogenase knock-out strain of *C. reinhardtii* that has recently been developed (Meuser et al. 2012. Biochem. Biophys. Res. Commun. 417:704) through a project funded by the DOE's Office of Science Basic Energy Sciences Program. We have since demonstrated successful genome incorporation and expression of Ca1 (Figure 1). At this point, we have postponed our random mutagenesis efforts in order to allocate our resources to pursue this more promising approach.

Although successful Ca1 transformants have been obtained to date, the expression levels are low and may been less stable. As a consequence, we have been unable to accumulate enough cells to test their $O_2$ tolerance. We have since generated additional transformants and will be measuring their $O_2$ tolerance to complete milestone 3.3.5 by the end of September 2012.

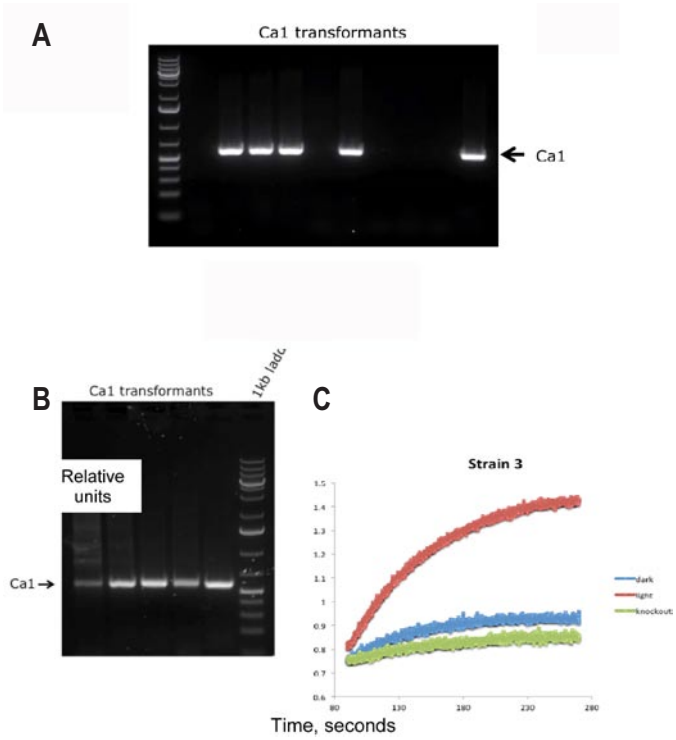


**FIGURE 1.** (A) Polymerase chain reaction of positive transformants demonstrating the incorporation of the Ca1 gene into the *Chlamydomonas* genome; (B) Reverse-transcriptase polymerase chain reaction of ribo nucleic acid extracted from positive transformants for Ca1, demonstrating that the Ca1 gene is transcribed in the transformants; (C) Initial rates of $H_2$ production by one of the transformants (upper curve) upon illumination, as measured by the Clark electrode; control curves represent, respectively, $H_2$-production activity of the transformant in the dark, and $H_2$ production by the parental strain, the double hydrogenase knock-out mutant upon illumination.

## Task 2. Use of the Sulfur-Deprivation Platform to Test Additional Biochemical and Engineering Barriers to $H_2$ Photoproduction

Sulfur-deprived algal cells immobilized into alginate films in the presence of 0.1% polyethylenimide (PEI) have previously been shown to photoproduce $H_2$ for a total period of about 10 days. In order to increase the period of $H_2$ photoproduction, we used two approaches that had been proven successful in extending $H_2$ production by cells in suspension: (a) perform cycles of +S/-S; and (b) continuously add low concentrations of sulfate to the medium. Figure 2 demonstrates that approach (a) resulted in $H_2$ photoproduction for a total period of about 150 days, with a total $H_2$ accumulation of ~0.56 mol/$m^2$. Approach (b) proved more successful when applied to strain EJ12F3 (Figure 3A), a mutant on the ATP synthase subunit atpE that is constitutively expressed in the host strain (see following). The process resulted in $H_2$ photoproduction for a period of about 53 days, with a total $H_2$ accumulation of 0.8 moles/$m^2$ (for comparison, the wild-type accumulated 0.32 moles $H_2$/$m^2$). The longest period of $H_2$ photoproduction was detected in the wild-type strain supplied continuously with TAP–S–P medium containing only the residual amounts of sulfates and phosphates. In this case, the alginate film produced 0.27 moles $H_2$/$m^2$ for about 92 days (Figure 3B). Under these conditions, EJ12F3 also demonstrated the highest $H_2$ photoproduction rate (Figure 3B). In the absence

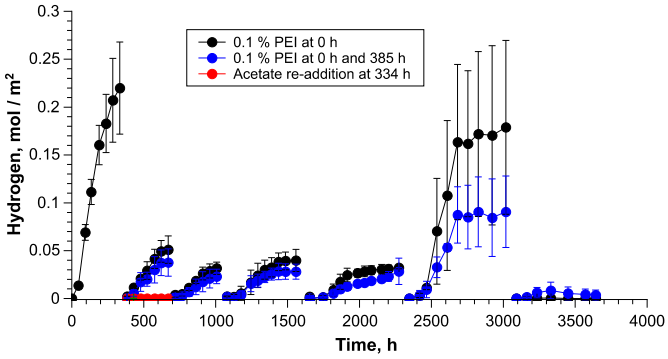


**FIGURE 2.** Continuous hydrogen photoproduction by sulfur/phosphorus-deprived *C. reinhardtii* cultures entrapped in alginate films. All films were pre-treated for 5 min with 0.1% PEI at the beginning of the experiment, washed in distilled water and transferred to anaerobic vials containing TA-S-P medium. At the beginning of each cycle (except the first one), alginate films were transferred into aerobic vials containing normal tandem affinity purification medium. After 3 days (~72 h), the films were transferred back to anaerobic vials with TA-S-P medium. For better stability, some films were additionally treated with 0.1% PEI for 5 min at the end of the first aerobic phase (~385 h, blue circles). In addition, acetate was re-added back to a third set of vials in the end of the first $H_2$ photoproduction cycle (at t = 334 h), and the vials were sparged with argon for 20 min. In these vials, the experimental medium was not replaced but acetate was re-added in the concentration equal to its initial concentration in the tandem affinity purification medium.

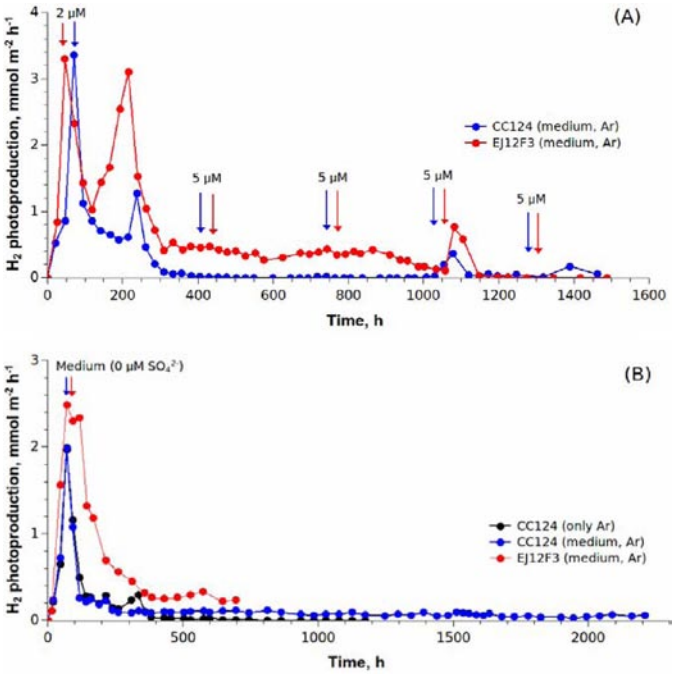


**FIGURE 3.** Continuous hydrogen photoproduction by sulfur-deprived *C. reinhardtii* wild-type (CC124) and ATP-synthase mutant (EJ12F3) cultures entrapped within thin alginate films. (A) Additional sulfate was added to the cultures at different points, as shown by the arrows. (B) Experiments were done under continuous flow of TAP –S –P medium without re-addition of sulfates, except the experiment marked «only Ar» where only argon was supplied to the bioreactor.

of continuous TAP –S –P flow, the alginate film produced only 0.17 moles/$m^2$.$H_2$ gas for about 48 days.

The sulfur-deprivation process is being used to test the performance of ATP synthase mutants designed to dissipate the proton gradient that is established during electron transfer from $H_2O$ to $H_2$ and that is known to down-regulate electron transfer (and thus $H_2$ production). Mutants in the atpE subunit defective in its C-terminal were expressed in Chlamydomonas strain FUD17 that lacks native E subunit. The expression of atpE in these initial strains was *constitutive*, which resulted in lower or no growth under photoautotrophic conditions. The $H_2$-photoproduction activity of a series of slow-growing mutants was tested under sulfur-deprivation conditions and two different light intensities, 90 and 150 µE $m^{-2}$ $s^{-1}$. The results demonstrate that (i) the host strain FUD, when complemented with a non-mutated atpE subunit (wtF) produces substantially more $H_2$ than the usual wild-type strain used in our laboratory, CC124; (ii) all mutants that were tested showed $H_2$-production levels similar to the wild-type when cultivated under 90 µE $m^{-2}$ $s^{-1}$ but lower $H_2$-production levels when exposed to 150 µE $m^{-2}$ $s^{-1}$; and (iii) the decrease in $H_2$ production at higher level intensity compared to wtF was a function of the number of amino acid residues that were mutated. The results suggest that the mutations may have affected other cellular activities as well.

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. The inducible promoter was tested with an Orange Fluorescent Protein and was shown to efficiently express this protein as a function of its expression level.

## Conclusions and Future Direction

**Task 1.** Continue the characterization of *C. reinhardtii* transformants harboring the Ca1 expression construct, and improve the expression of the heterologous gene through random mutagenesis of promoter, transit peptide and linker, as needed.

**Task 2.** Will be discontinued due to budget restrictions.

## FY 2012 Publications/Presentations

### Publications

**1.** Blankenship, R.E., D.M. Tiede, J. Barber, G.W. Brudvig, G. Fleming, M. Ghirardi, M.R. Gunner, W. Junge, D.M. Kramer, A. Melis, T.A. Moore, C.C. Moser, D.G. Nocera, A.J. Nozik, D.R. Ort, W.W. Parson, R.C. Prince and R.T. Sayre. **2011**. "Comparing Photosynthetic and Photovoltaic Efficiencies and Recognizing the Potential for Improvement". *Science* 332, 805-809.

**2.** Yacoby, I., Pochekailov, S., Toporik, H., Ghirardi, M.L., King, P.W. and Zhang, S. "Photosynthetic electron partitioning between [FeFe]-hydrogenase and ferredoxi:NADP+-oxidoreductase. Proc. Natl. Acad. Sci. USA, 108, 9396-9401.

**3.** Tekucheva, D.N., T.V. Laurinavichene, M. Seibert, A.A. Tsygankov (2011) "Immobilization of purple bacteria for light-driven $H_2$ production from starch and potato fermentation effluents." *Biotechnology Progress Journal* Online. http://dx.plos.org/10.1371/journal.pone.0025851 October 17.

**4.** Laurinavichene, T.V., Belokopytov B.F., Laurinavichius, K.S., Khusnutdinova, A.N., Seibert, M., Tsygankov, A.A**. 2012**. "Towards the integration of dark- and photo-fermentative waste treatment. 4. Fed-batch sequential fed-batch dark and photofermentation using starch as substrate". Int. J. Hydrogen Energy 37: 8800-8810.

**5.** Kosourov, S.N., Batyrova, K.A., Petushkova, E.P., Tsygankov, A.A., Ghirardi, M.L. and Seibert M. **2012**. "Maximizing the hydrogen photoproduction yields in *Chlamydomonas reinhardtii* cultures: the effect of the $H_2$ partial pressure". Int. J. Hydrogen Energy 37:8850-8858.

**6.** Johnson, EA. **2012**. "Monitoring foreign gene incorporation into the plastome of *Chlamydomonas reinhardtii* by multiplex qPCR" Photosynthesis Research, *submitted*.

**7.** Johnson, EA. **2012**. "Expression of monomeric Kusabira Orange in the chloroplast of *Chlamydomonas reinhardtii*". Photosynthesis Research, submitted.

## Presentations

**1.** Presentation to the research group at the Institute for Marine and Environmental Technology (IMET) at the University of Maryland (June 2011, Johnson).

**2.** Presentation at the Natural and Artificial Photosynthesis meeting (November 2011, King).

**3.** Seminar presentation at the Colorado State University, in Fort Collins, CO (November 2011, Ghirardi).

**4.** Oral presentation at the Western Photosynthesis Conference in Pacific Grove, CA, (January 2012, Ghirardi).

**5.** Invited talk to the Department of Life Sciences and Systems Biology at the University of Torino (March 2012, King).

**6.** Invited Talk at the Metal Hydrides in Biology Meeting-Oxford UK (March 2012, King).

**7.** Invited talk to the CEA Bioenergy Conference in Paris (February 2012, King and Ghirardi).

**8.** Organization and presentation at a joint symposium between NREL and French researchers working on algal biohydrogen and biofuels at NREL (April 2012, Ghirardi).