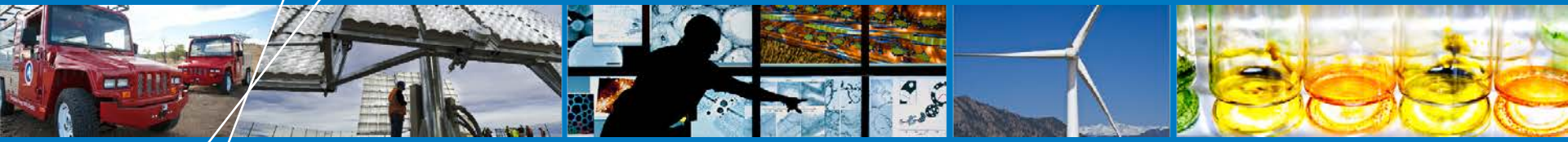
NREL
NATIONAL RENEWABLE ENERGY LABORATORY

Biological Systems for Hydrogen Photoproduction



2012 Annual Merit Review and Peer Evaluation Meeting

Maria L. Ghirardi, PI
National Renewable Energy Laboratory

May 16, 2012

NREL/PR-5200-54467

PD037

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Overview

Timeline

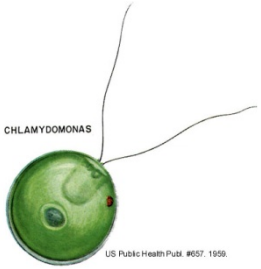
- Project start date: FY00
- Project end date: 9/30/2012*
- Percent complete: 80%

*Project continuation and direction determined annually by DOE

Budget

- Total project funding: $9,951K
- Funding received in FY11: $750K
- Planned funding for FY12: $600K

Barriers



- Barriers addressed:
  - Rate of H_2 production (AH)
  - Continuity of H_2 production (AI)
  - Engineering issues (AJ)
- Targets (see next page)
  - light conversion efficiency
  - rates of production
  - duration of production

Partners

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

Relevance/Objectives

- **General goal:** Develop photobiological systems for large-scale, low cost and efficient H_2 production from water (barriers AH, AI and AJ).

Characteristics	Units	2003	2006	2013 Target	2018 Target
Utilization efficiency of incident solar energy	%	10	15	15	20
Efficiency of incident light energy conversion of water to hydrogen	%	0.1	0.1	2	5
Duration of continuous H_2 photoproduction	Time units	NA	NA	30 min	4 h
O_2 tolerance (half-life in air)	Time units	1 sec	1 sec	10 min	2 h

- **Specific tasks:**

  Task 1: Address the O_2 sensitivity of hydrogenases that prevent continuity of H_2 photoproduction under aerobic, high solar-to-hydrogen (STH) light conversion efficiency conditions.

  Task 2: Utilize a limited STH H_2-producing method (sulfur deprivation) as a platform to address or test other factors limiting commercial algal H_2 photoproduction, including low rates due to biochemical and engineering mechanisms.

Approach/Milestones – Task 1

Task 1: Address the O_2 sensitivity of hydrogenase by (a) using targeted random mutagenesis to generate O_2-tolerant hydrogenases; and (b) introducing the gene encoding for a more O_2-tolerant hydrogenase from *Clostridium acetobutylicum* into the photosynthetic alga *Chlamydomonas reinhardtii*; measure its linkage to water oxidation and *in vivo* O_2 tolerance.

Task 1	Milestone	Due date	Status
3.3.2	Go/NoGo: Assess progress in the random mutagenesis approach to evaluate whether to further pursue this approach in FY12.	12/31/11	Completed: NoGo
3.3.5	Demonstrate expression of an active Ca1 in a *C. reinhardtii* hydrogenase-less background and characterize O_2-sensitivity of light-driven H_2 production.	9/30/12	80% completed

Dr. Paul King, NREL

Dr. Kath Ratcliff, NREL

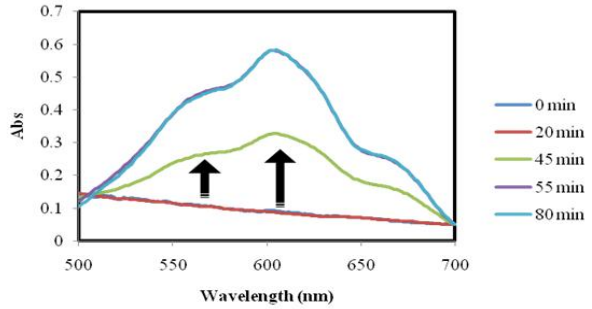
Dr. David Mulder, NREL

Accomplishments and Progress – Task 1

Task 1 – Random mutagenesis – completion of milestone 3.3.2 – NoGo.

We re-evaluated the effects of O_2 on the H_2-production activity of the more O_2-tolerant *Clostridium acetobutylicum* Ca1 hydrogenase and concluded that (a) a targeted random mutagenesis effort would require more funding than currently available through the FCT Program; (b) we need to first validate that higher in vitro O_2 tolerance translates into higher *in vivo* O_2 tolerance. We decided, instead, to focus on the expression of Ca1 in *Chlamydomonas reinhardtii* (see next slide).

Progress before the Go/NoGo milestone included:



(a) Development of a chemochromic high-throughput assay for H_2 production in micro-wells, based on methyl viologen reduction by H_2.

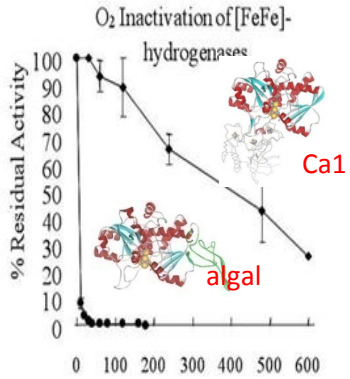
Strain	Ca1 whole cell activity (nmol H_2/min/mL)
Rosetta-2 (DE3)	1625
MC4100 (DE3)	1194
MW1001 (DE3)	173

Background control rate: 16 nmol H_2/min/mL

(b) Selection of a *E. coli* strain lacking native hydrogenase activity and expressing Ca1 at high levels.

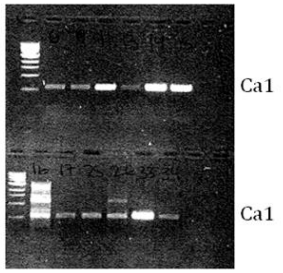
Accomplishments and Progress – Task 1

Task 1 – Expression of Ca1 in *C. reinhardtii* and measurement of *in vivo* O_2 tolerance – towards completion of milestone 3.3.5.

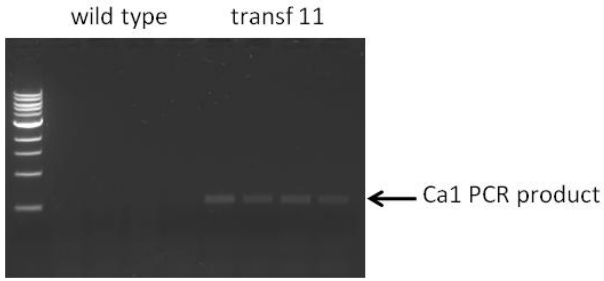


Justification: the Ca1 hydrogenase is 2 orders of magnitude more O_2-tolerant than the algal hydrogenase *in vitro*.

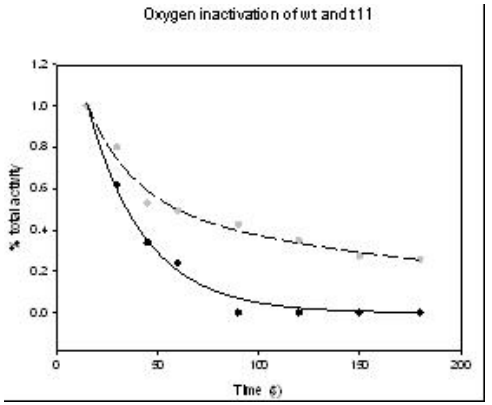
Previous work: we successfully expressed Ca1 in a wild-type strain of *C. reinhardtii* that contained native hydrogenase activity; O_2-inactivation kinetics were biphasic, suggesting the presence of a more O_2-tolerant enzyme in combination with the native hydrogenases.



PCR (genomic DNA)



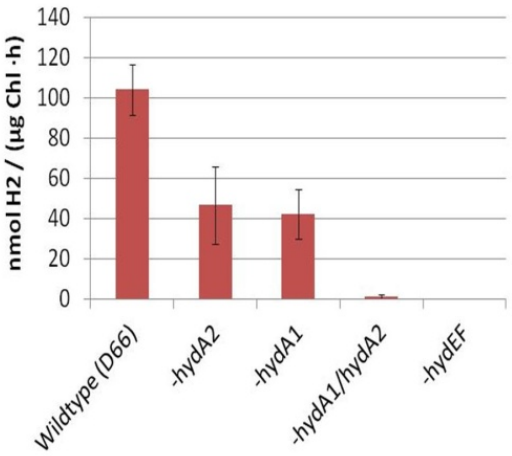
RT-PCR (mRNA)



Accomplishments and Progress – Task 1

Task 1 (cont.) – Expression of Ca1 in *C. reinhardtii* and measurement of *in vivo* O_2 tolerance – towards completion of milestone 3.3.5.

Current Progress: A double hydrogenase knock-out mutant was isolated under BES funding and served as a host for expression of Ca1 behind the psaD promoter.



Meuser et al., Bioch. Biophys. Res. Comm. 417, 704

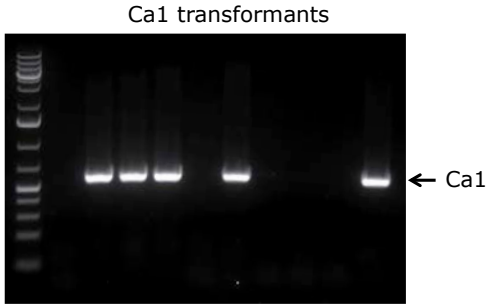
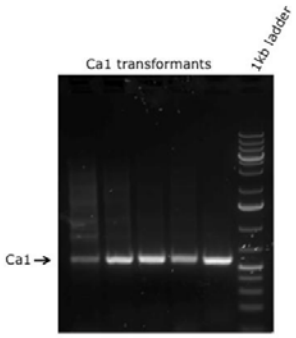


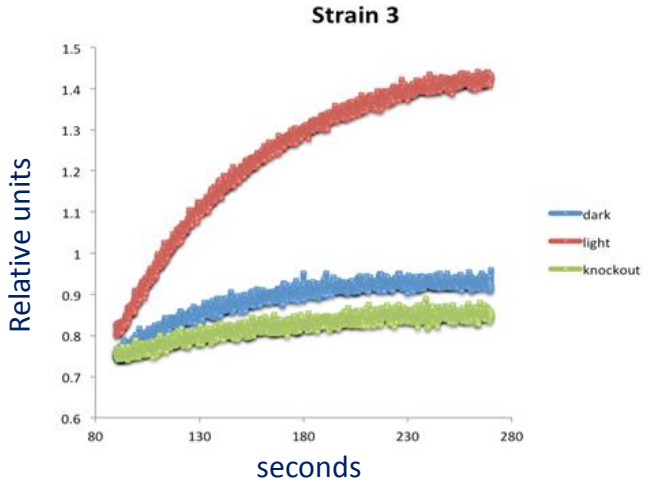
Figure 1. PCR of genomic DNA from various Ca1transformants

PCR (genomic DNA)



RT-PCR (mRNA)

Light-dependent H_2 production was detected!



Approach/Milestones – Task 2

Task 2: Utilize the limited STH sulfur-deprivation method to test (a) the rates of H_2 production by inducible ATP synthase mutants that are not limited by the non-dissipation of a proton gradient; and (b) the long-term performance of immobilized algal cultures.

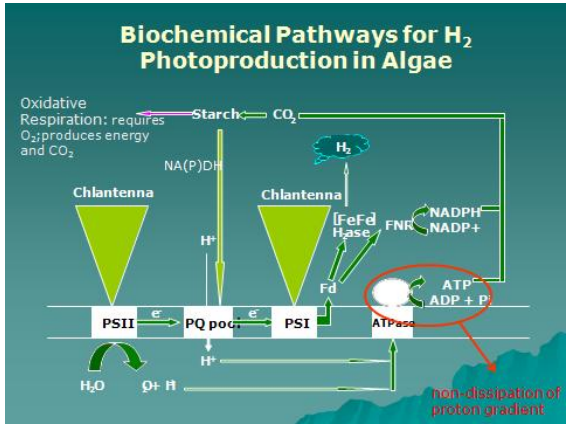
Task 2	Milestone	Due date	Status
3.3.3	Physiologically characterize mutant strains with a defective AtpE gene under the regulation of an inducible promoter	3/30/12	Completed
3.3.4 (CSP Agreement milestone 51536)	Demonstrate continuous operation of the sulfur-deprivation process for a total of 2 months upon addition of phosphate/sulfate to alginate-immobilized cultures	9/30/12	50% completed

Dr. Eric Johnson
JHU

Dr. Sergey Kosourov
RAS

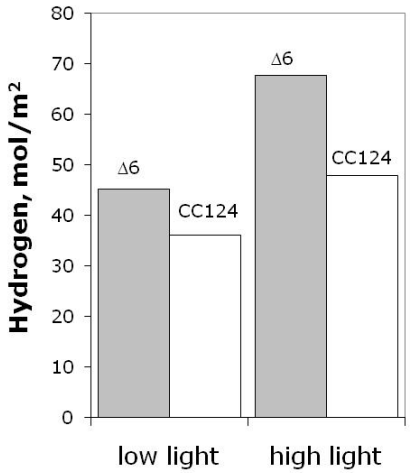
Accomplishments and Progress – Task 2

Task 2 – Generate, physiologically characterize and test the rate of inducible ATP synthase mutants that are defective in maintaining the proton gradient – completion of milestone 3.3.3.



Justification: electron transport from water to ferredoxin is accompanied by the formation of a proton gradient that drives the production of ATP (essential for CO_2 fixation). During H_2 photoproduction, ATP demand drops, impeding the efficient dissipation of the proton gradient and impairing electron transport (and thus of H_2 production).

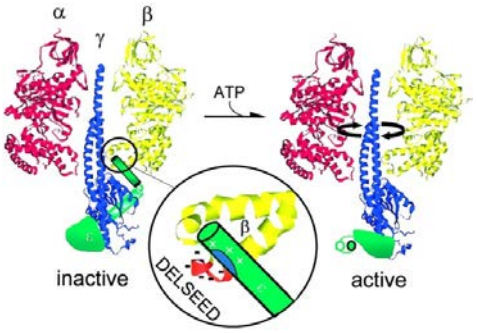
Previous results: *C. reinhardtii m*utants defective in one of the ATP synthase subunits, AtpE (Δ6, dark bars), grow more slowly but produce H_2 at higher rates than the corresponding wild-type (cc124, light bars) strains, particularly at high light intensities.



Accomplishments and Progress – Task 2

Task 2 (cont.) – Generate, physiologically characterize and test the H_2-production rate of inducible ATP synthase mutants that are defective in maintaining the proton gradient – milestone 3.3.3 completed.

Current progress:



(a) Generated six mutants in the C-terminal of the ATP synthase subunit ε ; four showed similar rates of H_2 production as their parental strain but two, EJ13 and EJ17, were unable to grow constitutively.

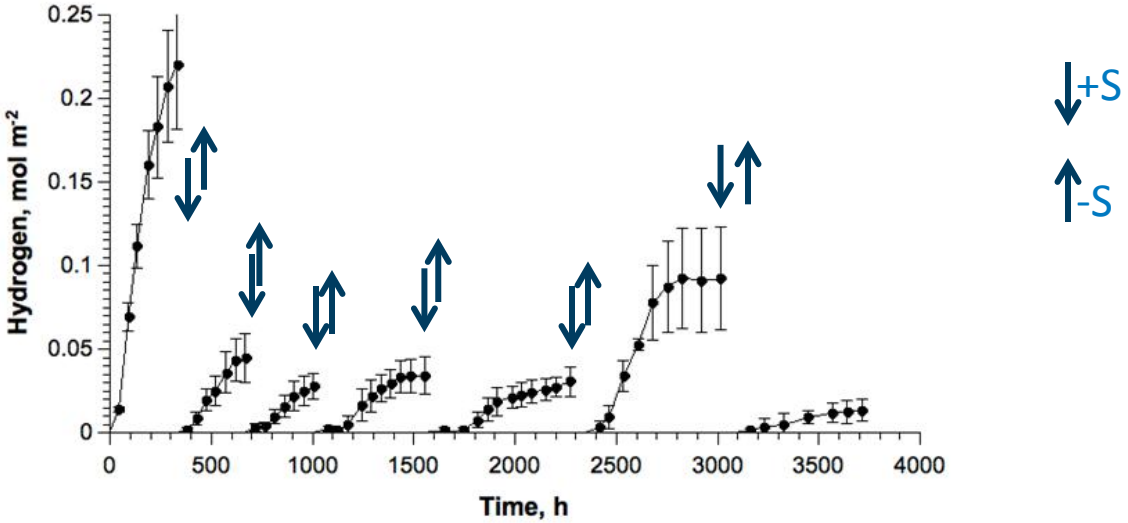
(b) The defective EJ17 gene was transformed behind the PsbD promoter into strain Ind41_15D. The transformed gene was shown to be stably incorporated into the chloroplast genome and to allow growth of the transformant under photo or heterotrophic conditions in the presence of spectinomycin - ***completion of milestone 3.3.3***.

(c) The inducibility of the Ind41_15D/psbD system under selective pressure was positively demonstrated by using the expression of an orange fluorescent protein as a marker.

Accomplishments and Progress – Task 2

Task 2 – Demonstrate continuous H_2 production for 2 months by sulfur-deprived, alginate-immobilized algae – towards completion of milestone 3.3.4.

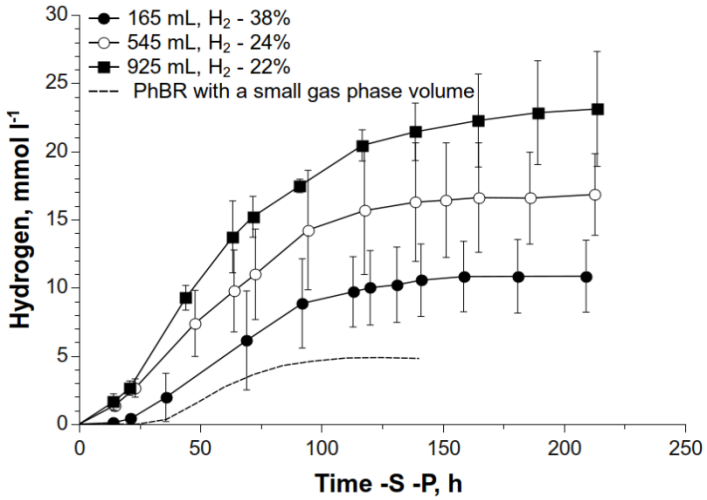
Previous results: alginate-immobilized algae photoproduce H_2 at higher specific rates and light conversion efficiencies than cultures in suspension upon sulfur deprivation, and they show higher tolerance to aerobic environments; cycles of +S/-S resulted in prolongation of H_2 production for an additional 6 cycles of about 500 hours each.



Current results: preliminary results by using continuous flow of S+ medium resulted in low rates of H_2 production; the experiment is being repeated under different operational conditions.

Accomplishments and Progress – Task 2

Task 2 – Demonstrate the effect of headspace volume on the H_2 photoproduction yield.



Current results: In suspension cultures, a 4x increase (from ~0.5 to ~2) in the ratio of gas/liquid volume results in a **2x increase in the total yield of H_2 gas**. Similar results were shown with immobilized cultures.

Remarkably, **565 ml of H_2 gas per liter of the suspension culture is the highest yield ever reported for a wild-type strain in a time period of less than 180 hours**. A control PhBR with a historically small gas phase volume of ~5 – 10 ml only produced up to 120 ml L^{-1} of H_2 gas.

Collaborations

Partners (subcontractors):

- Dr. Sergey Kosourov, Russian Academy of Sciences – applies sulfur deprivation to sulfur-immobilized *C. reinhardtii* cultures and tests their H_2-production capabilities (Task 2).

- Dr. Eric Johnson, Johns Hopkins University – generates ATP synthase mutants, develops transformation protocols and transforms Chlamydomonas reinhardtii; tests physiological properties of transformants (Task 2).

Proposed Future Work

2012

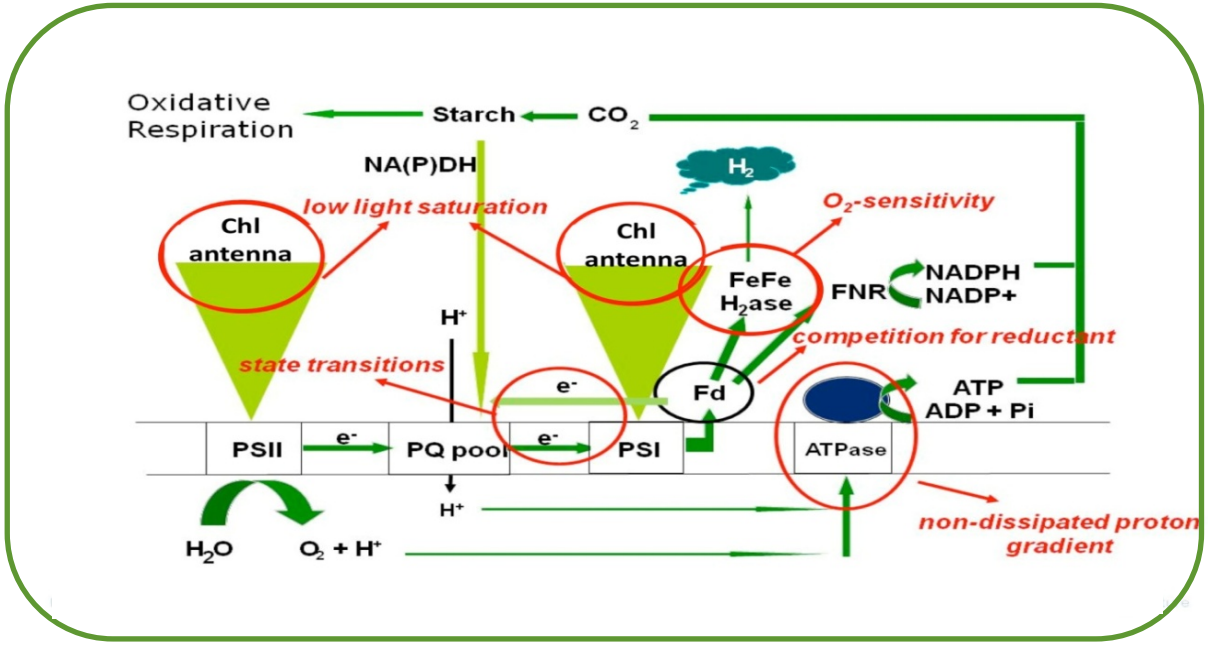
Task 1 – Complete milestone 3.3.5: Demonstrate expression of an active Ca1 in a *C. reinhardtii* hydrogenase-less background and characterize O_2 sensitivity of light-driven H_2 production.

Task 2 – Complete milestone 3.3.4: Demonstrate continuous operation of the sulfur-deprivation process for a total of 2 months upon addition of phosphate/sulfate to alginate-immobilized cultures.

Work beyond FY12: Start to genetically express mutated ATP synthase and truncated antenna regulatory genes into a strain expressing the Ca1 hydrogenase. Develop photobioreactor systems for cyclic or continuous H_2 production based on current optimization of gas space composition (not shown), alginate immobilization, and others.

Summary Slide

Relevance: Photobiological water splitting coupled to hydrogenase-mediated H_2 production has the potential to convert *about 10% of incident solar energy into H_2*. Various barriers have been identified as currently limiting green algal H_2 production, including the *O_2 sensitivity of the hydrogenase enzyme*, and *down-regulation of photosynthesis due to non-dissipation of the proton gradient*.



Summary Slide (cont.)

Approach: NREL is expressing a more O_2-tolerant bacterial hydrogenase in green algae and generating ATP synthase mutants that prevent down-regulation of photosynthesis with JHU. NREL and RAS use the low STH sulfur-deprivation process to test candidates that have been generated to address various barriers, as well as optimizing photobioreactor conditions for efficient and sustainable production of H_2 gas.

Technical Accomplishments and Progress:

1. Successfully expressed a bacterial hydrogenase in a Chlamydomonas strain lacking native hydrogenase activity and detected photoproduction of H_2.
2. Generated inducible ATP synthase mutants , tested for growth, and are testing their phenotype with respect to H_2 photoproduction.
3. Continues to optimize long-term H_2 photoproduction by alginate-immobilized Chlamydomonas.

Collaborations: Drs. Sergey Kosourov (RAS) and Eric Johnson (JHU).

Proposed Future Research: For 2012: complete milestones 3.3.4 (demonstrate continuous H_2 photoproduction by addition of sulfate) and 3.3.5 (measure the *in vivo* O_2 tolerance of mutants expressing Ca1); measure the inducible H2 photoproduction capability of the ATP synthase mutant. After 2012: genetically combine useful phenotypes into a single organism expressing Ca1.