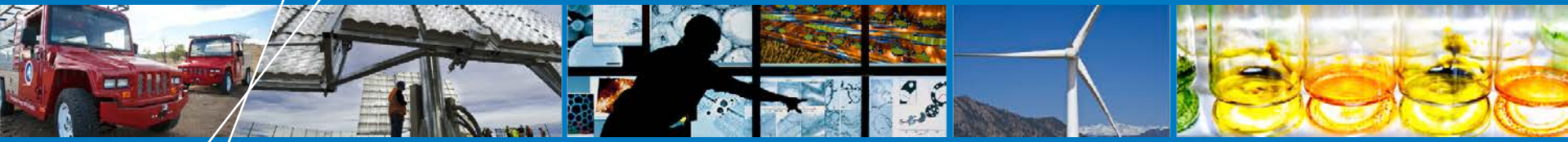
NREL
NATIONAL RENEWABLE ENERGY LABORATORY

# Biological Systems for Hydrogen Photoproduction

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**National Renewable Energy Laboratory**


**May 16, 2013** **PD037**

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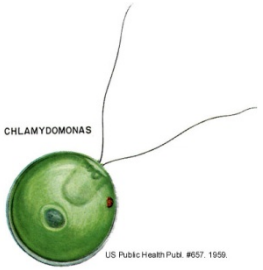
# Overview

## Timeline

- Project start date: FY00
- Project end date: 9/30/2013*
- Percent complete: 85%

*Project continuation and direction determined annually by DOE

## Barriers




- Barriers addressed:
  - Rate of $H_2$ production (AO)
  - Oxygen Accumulation (AP)
- Targets:
  - Duration of production
  - Solar to $H_2$ (STH) energy conversion

## Budget

- Total project funding: $10,551K
- Funding received in FY12: $600K
- Planned funding for FY13: $480K

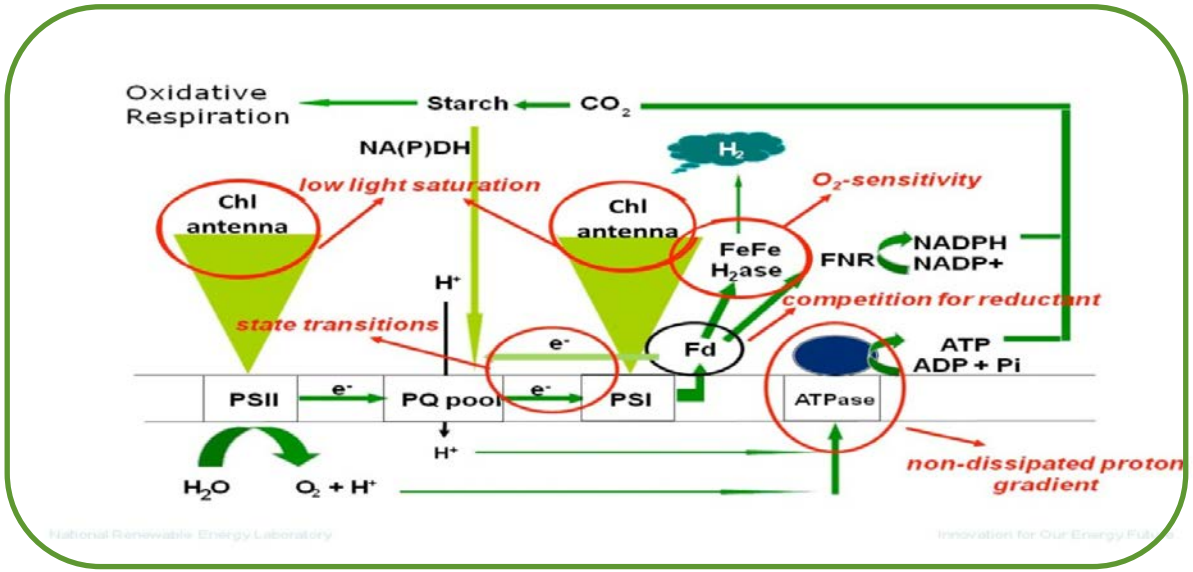
## Partners (in FY12)

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

# 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,
- the competition for reductant with $CO_2$ fixation and cyclic electron flow,
- the down-regulation of photosynthesis due to non-dissipation of the proton gradient and state transitions, and
- the low light-saturation of photosynthesis.



# Objectives

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

| Technical Targets: Photolytic Biological Hydrogen Production | | | | | |
|---|---|---|---|---|---|
| **Characteristics** | **Units** | **2012 Status** | **2015 Target** | **2020 Target** | **Ultimate Target** |
| **Duration of continuous $H_2$ production at full sunlight intensity** | **Time units** | **N/A** | **30 min** | **4 h** | **8 h** |
| **Solar to $H_2$ (STH) energy conversion ratio** | **%** | **N/A** | **2** | **5** | **17** |

- **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) 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 – ***discontinued in FY13 due to budget restrictions***

# Approach/Milestones – Task 1

**Task 1:** Address the $O_2$ sensitivity of hydrogenase by introducing the gene encoding for a hydrogenase from *Clostridium acetobutylicum* that is more $O_2$-tolerant *in vitro* into the photosynthetic alga *Chlamydomonas reinhardtii*; measure its linkage to water oxidation and *in vivo* $O_2$ tolerance.

Dr. Paul King, NREL

Seth Noone, NREL

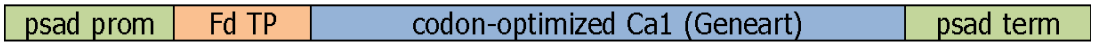
| Task 1 | FY12 Milestone | Due date | Status |
|---|---|---|---|
| 3.3.5 | Demonstrate expression of an active Ca1 in a *C. reinhardtii* hydrogenase-less background and characterize $O_2$-sensitivity of light-driven $H_2$ production (PsaD-Ca1 construct) | 9/30/12 | Completed |

| | FY13 Milestones | Due date | Status |
|---|---|---|---|
| 3.3.1-1 | Re-test the $H_2$ photoproduction activity of the PsaD-Ca1 transformant strain 55 by the Clark electrode | 12/12 | Completed |
| 3.3.1-2 | Screen 300 HYDA-Ca1transformants for the presence of the Ca1 gene and test positive transformants for $H_2$ production activity through the plate assay and Clark electrode | 2/13 | Completed |
| 3.3.1-3 | Generate new transformants with 3 variations of added introns from, respectively, RbcS2, HYDA1 and HYDA2 | 4/13 | On track |
| 3.3.1-4 | Test at least 100 strains from the first generation of intron-containing transformants for $H_2$-production activity through the plate assay (contingent upon finding a new post-doc to perform the work) | 5/13 | On track |
| 3.3.1-5 | Go/NoGo: if addition of introns increases the $H_2$ photoproduction activity and stability of Ca1 by at least 3 times compared to HYDA1 of PsaD-based constructs, use the strain for further improvements; if not, propose a new plan for DOE's approval to re-direct the work (*CPS Agreement Milestone*) | 7/13 | On track |

# Accomplishments and Progress – Task 1

**Task 1 –** Expression of the more $O_2$-tolerant clostridial Ca1 hydrogenase in *C. reinhardtii*

**Last year's Progress:** A double hydrogenase knock-out mutant (⁻hydA1/hydA2) was isolated under BES funding and served as a host for expression of CaI hydrogenase gene behind the PsaD promoter (which requires light for optimal anaerobic induction).




Construct 1: psad promoter and terminator (constitutive expression) with ferredoxin transit peptide/Ca1 optimized by geneart.


Construct 2: native algal hydrogenase promoter and terminator with ferredoxin transit peptide/Ca1 optimized by geneart.

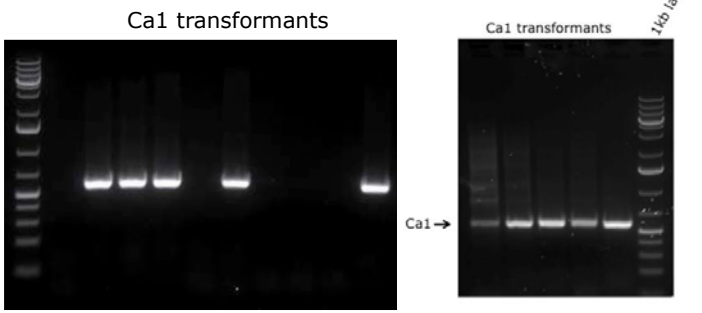


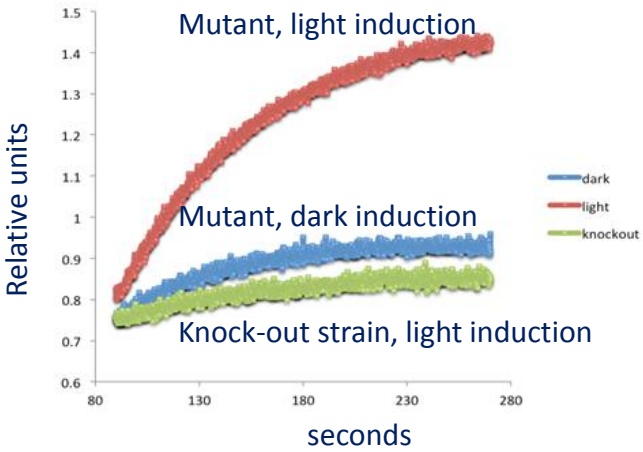

**Figure 1.** PCR of genomic DNA from various Ca1transformants

PCR (genomic DNA)

RT-PCR (mRNA)

The clostridial hydrogenase was inserted into the Chlamydomonas genome and transcribed.

Light-dependent $H_2$ production was detected!



# Accomplishments and Progress - Task 1

**Task 1 –** Expression of the more $O_2$-tolerant clostridial Ca1 hydrogenase in *C. reinhardtii* and measurement of its $O_2$ tolerance - *completed FY12 milestone 3.3.5.*

| Strain | Rate of $H_2$ production in Clark electrode (μmol $H_2$ mg $Chl^{-1}$ $h^{-1}$) | % WT |
|---|---|---|
| WT | 260 | 100 |
| Double knock-out | 0.09 | 0.035 |
| Double knock-out/Ca1 | 5.1 | 2 |

Strain 55 expressing Ca1 photoproduces $H_2$ at around 2% of WT rate

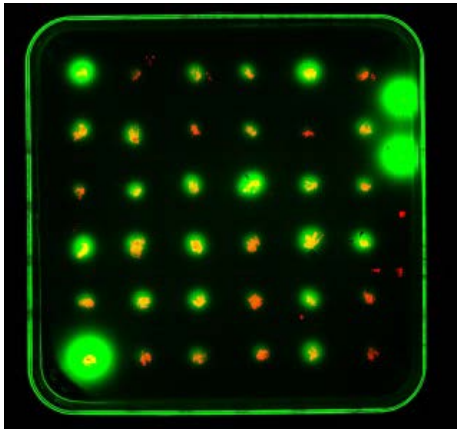
Ca1 activity is not lost upon subsequent cycles of illumination, suggesting higher tolerance to photosynthetically-produced $O_2$

| Strain | Cycle of illumination | Rate of $H_2$ production in Clark electrode (μmol $H_2$ mg $Chl^{-1}$ $h^{-1}$) | Change in rate from 1st light cycle |
|---|---|---|---|
| WT | 1 | 269 | N/A |
| | 2 | 205 | -23% |
| | 3 | 179 | -33% |
| Double knock-out/Ca1 | 1 | 5.1 | N/A |
| | 2 | 5.6 | +10% |
| | 3 | 6.2 | +25% |

# Accomplishments and Progress - Task 1

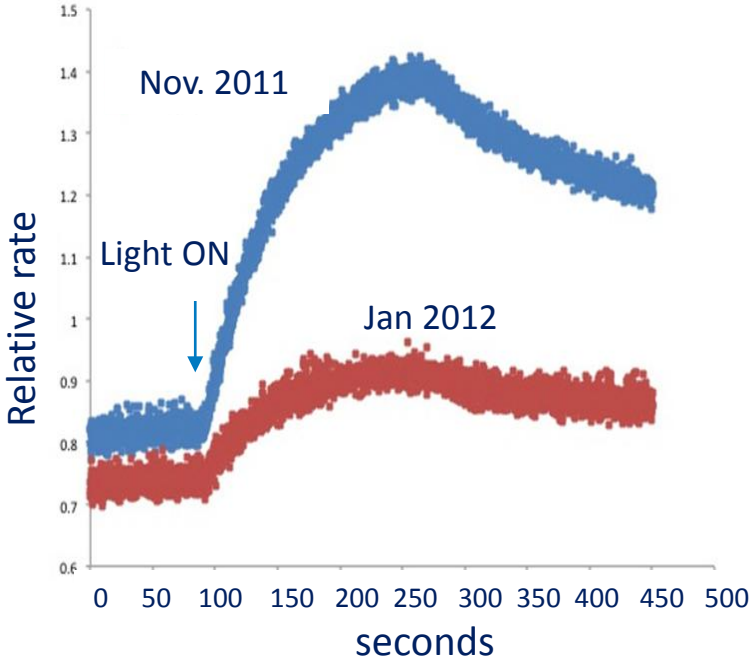
**Task 1 –** Expression of the more $O_2$-tolerant clostridial Ca1 hydrogenase in *C. reinhardtii* and measurement of its $O_2$ tolerance

However, GFP plate screen shows considerable heterogeneity of $H_2$ production levels among cells grown from a liquid culture of the Ca1-expressing transformant ...



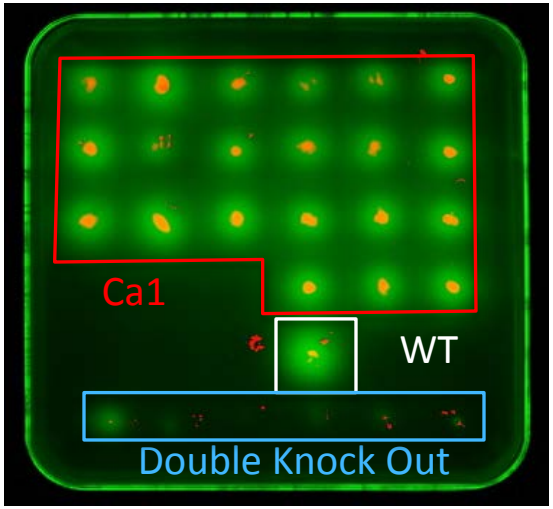
PsaD-Ca1 Strain 55

... and activity is not stable over time if culture is serially propagated.



# Accomplishments and Progress – Task 1

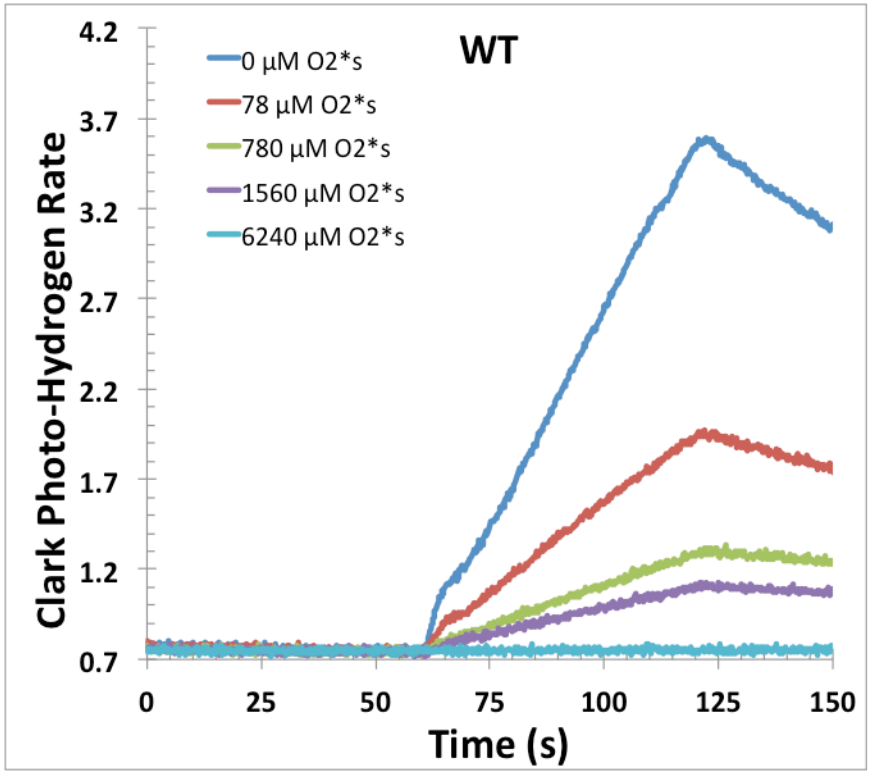
**Task 1.** Recover original high $H_2$-producing strain from plates and re-test it – *completion of milestone 3.3.1-1*

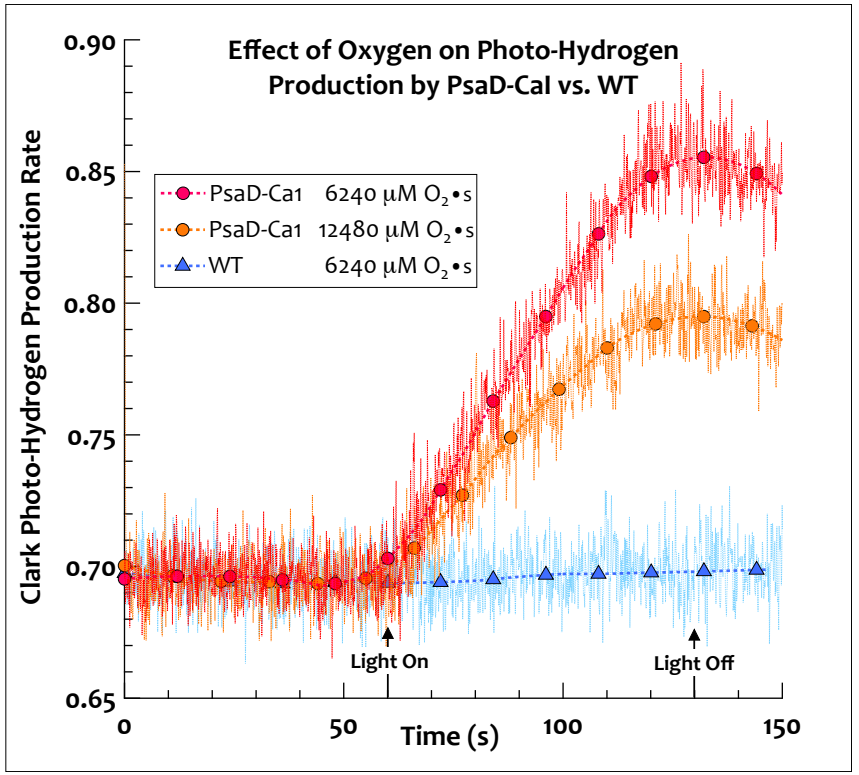



The strain shown as having the highest $H_2$ production in the previous slide, strain 55 was re-plated, retested by GFP, and showed more uniform $H_2$ production as shown above.

# Accomplishments and Progress – Task 1

**Task 1.** Recover original high $H_2$-producing PsaD-CaI strain from plates and re-test – *completion of milestone 3.3.1-1*

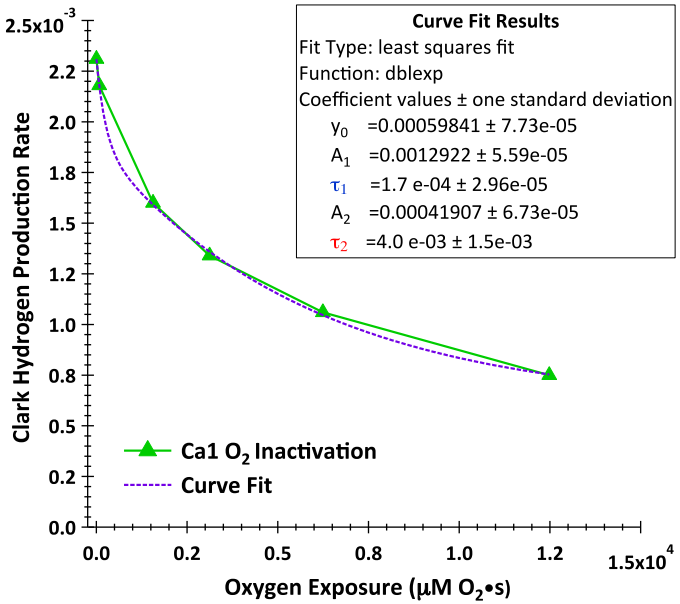


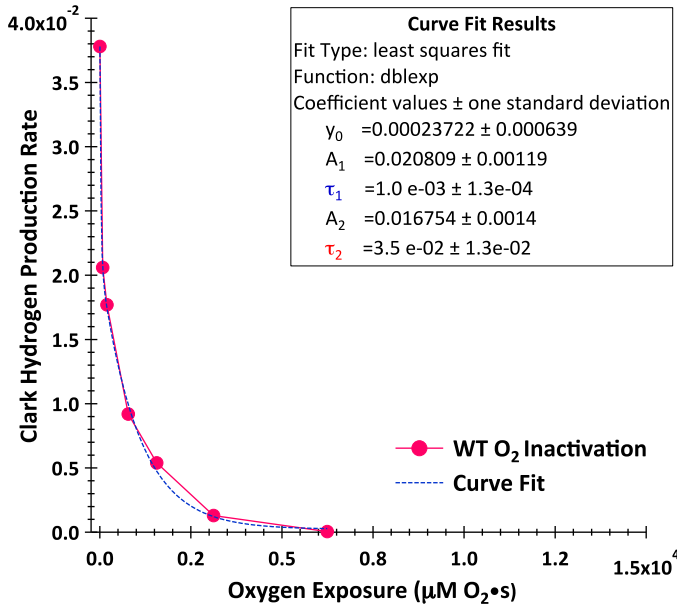




Light-induced $H_2$ photoproduction by WT and the PsaD-Ca1 transformant were measured with a Clark electrode, following a 1-min dark incubation in the presence of different amounts of added $O_2$. Relative rates are shown above.

Equivalent to exposure to atmospheric $O_2$ concentration = 15,600 µM $O_2$•s

# Accomplishments and Progress – Task 1




Clark $H_2$-Production Rate versus µM $O_2$•s were plotted, and the results fitted to a bi-exponential-decay curve, according to the equation below. The two inactivation rate constants, $\tau_1$ and $\tau_2$, were estimated for WT and Ca1 cells.

$$y = y_0 + A_1 e^{-x\tau_1} + A_2 e^{-x\tau_2} \qquad x = \frac{(260\ \mu\text{M})(vol_{O_2\ buffer})}{vol_{total}}(Incubation\ time, s)$$

| Enzyme/ Strain | $O_2$ Inactivation Rate Constants ($\tau_1$ and $\tau_2$) (µM $O_2^{-1}$•$s^{-1}$) Fast | Slow | $O_2$ Tolerance (Ratio to WT) Fast | Slow |
|---|---|---|---|---|
| WT | $2.7 \times 10^{-2} \pm 2 \times 10^{-3}$ | $1.7 \times 10^{-3} \pm 5.2 \times 10^{-4}$ | NA | NA |
| PsaDP-Ca1 | $1.4 \times 10^{-3}$ | $1 \times 10^{-4} \pm 2 \times 10^{-5}$ | 19 | 17 |
| HydAP-Ca1 | $3 \times 10^{-3}$ | $3.5 \times 10^{-4}$ | 9 | 5 |

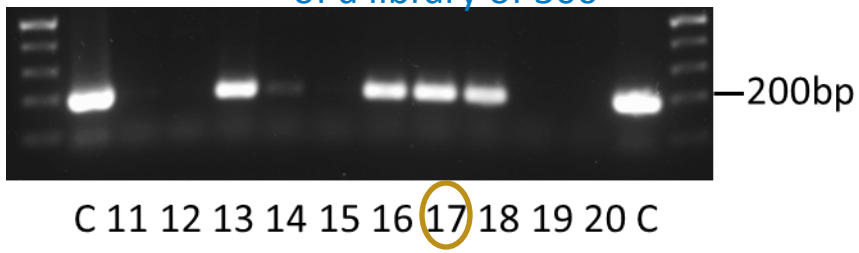
**Conclusions:**

- *In vivo* expression of Ca1 in *Chlamydomonas* shows higher $O_2$ tolerance (5-19 fold) for photo-$H_2$ production than wild-type;
- Level of increased $O_2$ tolerance of Ca1 compared to HYDA1 *in vivo* is similar to differences of the purified enzymes measured *in vitro*.

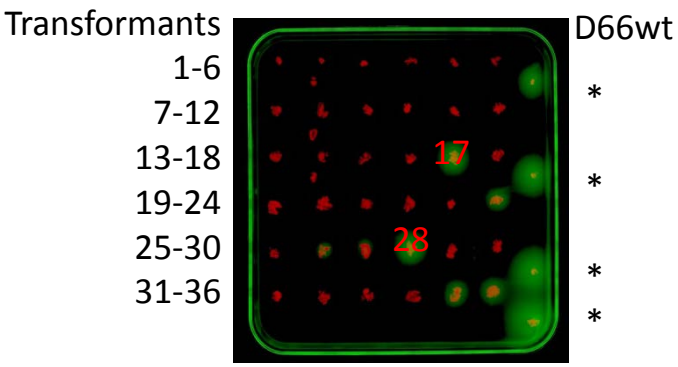
# Accomplishments and Progress - Task 1

**Task 1**. Screen 300 HYDA-Ca1transformants for the presence of the Ca1 gene and test positive transformants for $H_2$ production activity through the plate assay and Clark electrode – *completion of milestone 3.3.1-2*

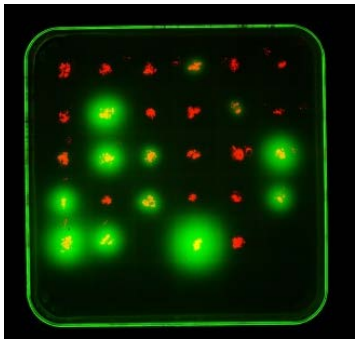
High-throughput PCR screen identified additional transformants carrying the Ca1 gene out of a library of 300



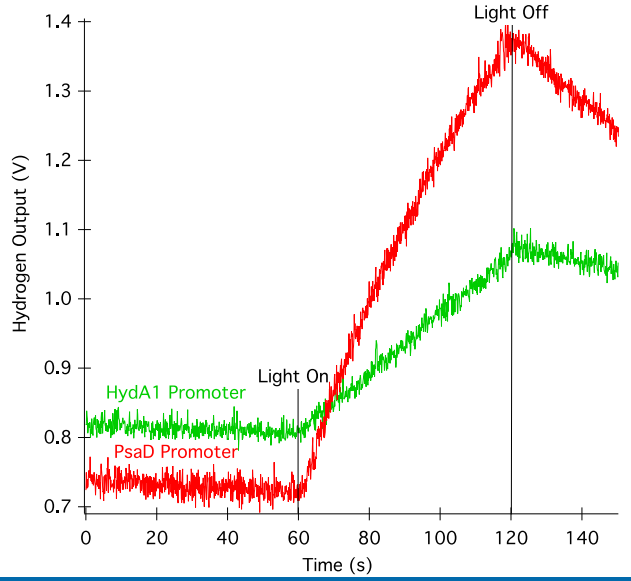

Plating and GFP screening showed two $H_2$ producing transformants (17, 28)




Strain 28 showed heterogeneity (left) in GFP $H_2$ plate assay, and photoproduced $H_2$ at lower rates (right) compared to strains 55



Strain 28



# Proposed Future Work

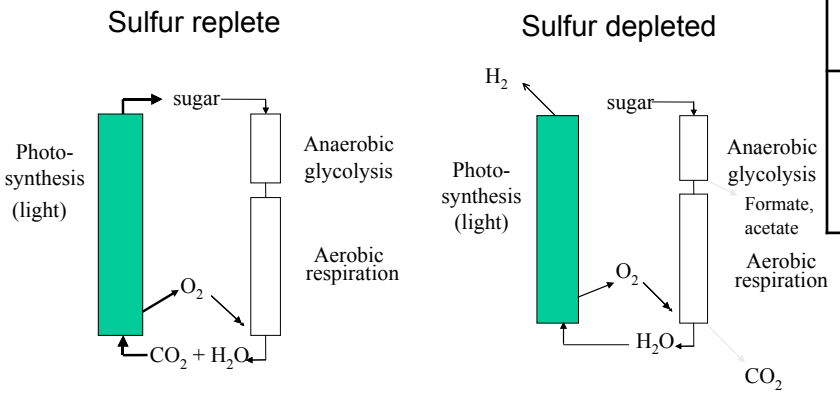
## 2013

**Task 1 –** Generate additional Ca1 expression constructs that contain Rubisco Small Subunit or HydA1 introns and test stability and levels of Ca1 activity by GFP plate assay and Clark Electrode; complete Go/NoGo milestone demonstrating at least 3X higher activity and stability of recombinant Ca1 expressed in the *C. reinhardtii* double knock-out background and characterize $O_2$ sensitivity of light-driven $H_2$ production.

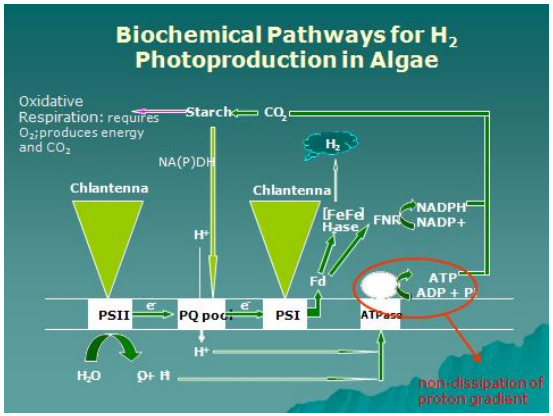
**Work beyond FY13:** Start to genetically combine selected traits into a strain expressing the Ca1 hydrogenase. Develop photobioreactor systems for cyclic or continuous $H_2$ production based on current optimization of gas space composition (shown last year) and alginate immobilization.

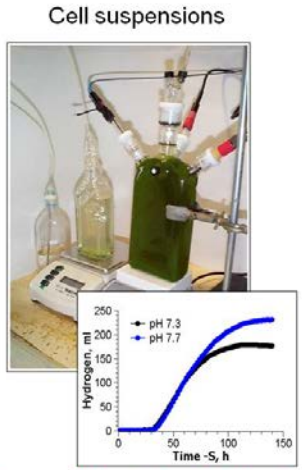
# Approach/Milestones – Task 2

**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. *Discontinued in FY13*

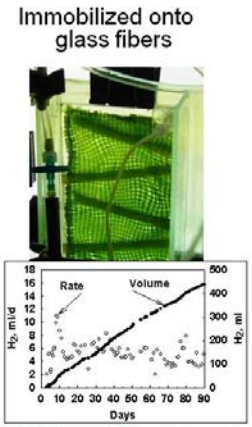



| Task 2 | FY12 Milestones | Due date | Status |
|---|---|---|---|
| 3.3.3 | Demonstrate continuous operation of the sulfur-deprivation process for a total of 2 months upon addition of phosphate/sulfate to alginate-immobilized cultures. | 9/30/12 | Completed |

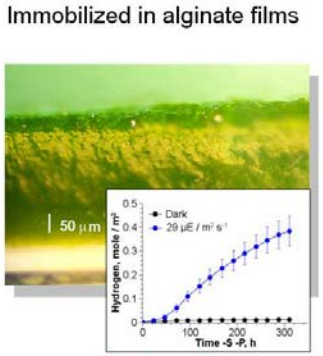



Low cell-density, short duration




Higher cell density, longer duration, non-degradable matrix

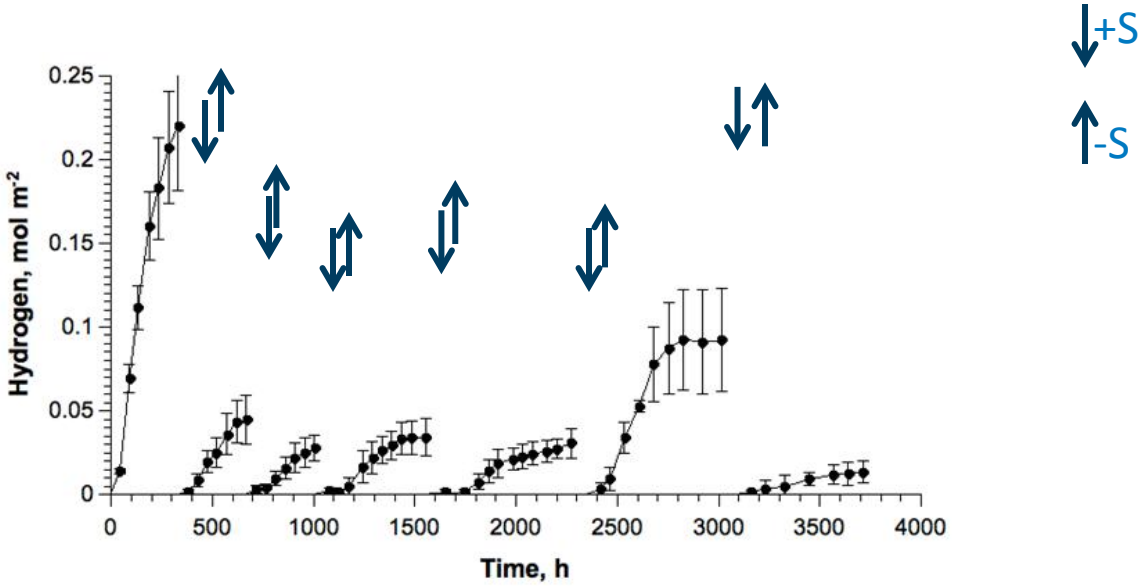



High cell density, long duration, controllable thickness, degradable matrix

# Accomplishments and Progress – Task 2

**Task 2 –** Demonstrate continuous $H_2$ production for 2 months (1440 hours) by sulfur-deprived, alginate-immobilized algae

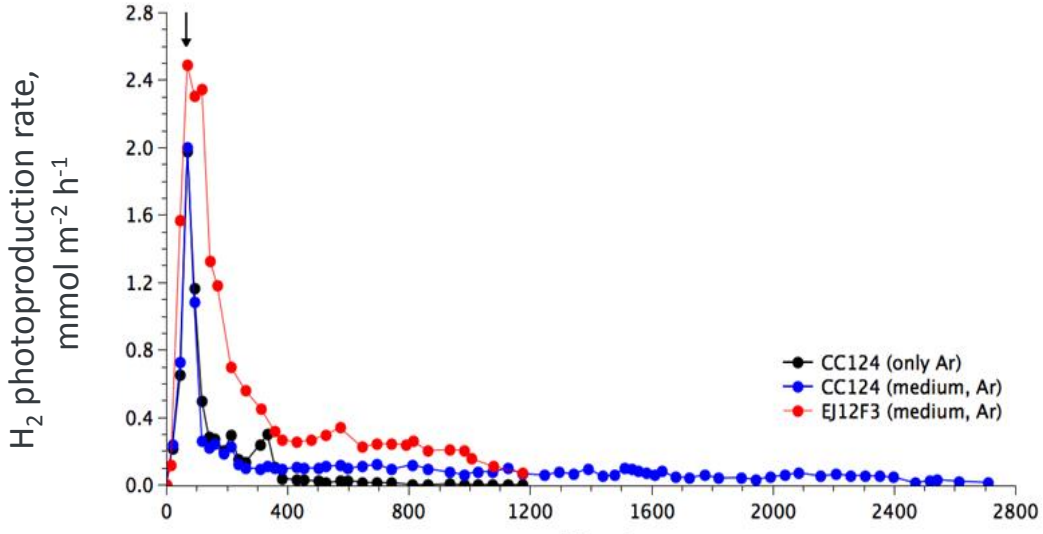
**Last year's results:** alginate-immobilized algae photoproduce $H_2$ at higher specific rates and light conversion efficiencies than cultures in suspension upon sulfur deprivation, and they show higher tolerance to aerobic environments; cycles of +S/-S resulted in prolongation of $H_2$ production for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




Prolonged $H_2$ photoproduction was demonstrated by cycles of +S/-S

# Accomplishments and Progress – Task 2

**Task 2:** Demonstrate continuous operation of the sulfur deprivation process for a total of 2 months (1440 hours) upon addition of phosphate/sulfate using alginate-immobilized cultures – *completed FY12 milestone 3.3.3. by 9/30/12.*




**Conclusions:**

- $H_2$ production was observed for up to 3.75 months using the wild-type CC124 strain
- The rates of production declined to very low levels after about 10 days
- A different strain of Chlamydomonas, EJ12F3 showed higher rates of $H_2$ photoproduction during the first 50 days.
- The economics of the process depend on the trade-off between longer $H_2$ production vs. lower rates

# Collaborations

## Partners (subcontractors) in FY12:

- 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).

# Summary Slide

**Relevance:** Photobiological systems required only water, $CO_2$ and minerals for cultivation; if optimized to collect additional wavelengths of light, they will have the potential of converting 17% of the solar energy into $H_2$ at a cost competitive with gasoline.

**Approach**: NREL is expressing a more $O_2$-tolerant bacterial hydrogenase in green algae and testing its *in vivo* tolerance to $O_2$.

**Technical Accomplishments and Progress:**

1. Successfully expressed a more $O_2$-tolerant, bacterial hydrogenase in a *Chlamydomonas* strain lacking native hydrogenase activity and detected more $O_2$-tolerant photoproduction of $H_2$ *in vivo*.

**Collaborations**: none in FY13.

**Proposed Future Research**: Increase the recombinant hydrogenase activity and stability in Chlamydomonas; start combining different traits to generate a more efficient $H_2$-producing strain of Chlamydomonas.