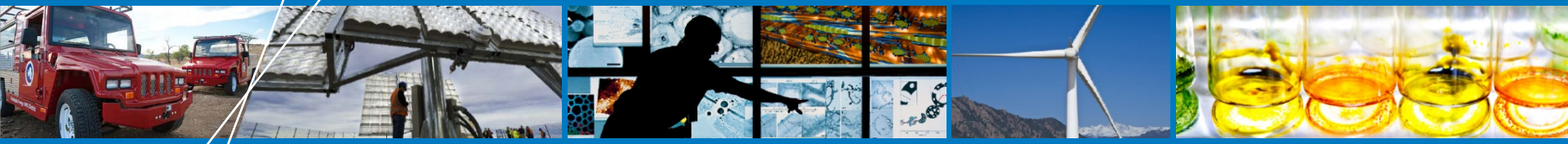
NREL
NATIONAL RENEWABLE ENERGY LABORATORY

Biological Systems for Hydrogen Photoproduction



Maria L. Ghirardi, P.I.
National Renewable Energy Laboratory
June 19, 2014

PD037

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Overview

Timeline and Budget

Project start date: FY00
Project end date: 9/30/2014

FY13 DOE Funding: $480K

Planned FY14 DOE Funding: $480K

Total DOE project value: $11M

Barriers

- **Barriers addressed**

Rate of H_2 production (AO)

Oxygen Accumulation (AP)

- **Targets:**

Duration of production

Solar to H_2 (STH) energy conversion

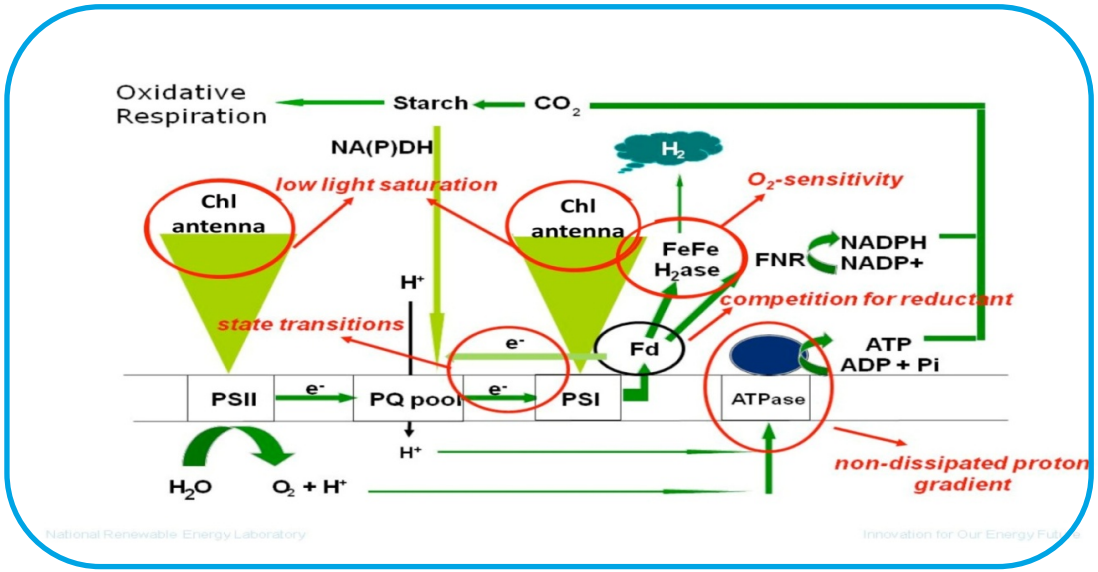
Partners

- **Interactions/ collaborations:** none

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.



Relevance – Objectives

- **General goal:** Develop photobiological systems for large-scale, low cost and efficient H_2 production from water (barriers AO and AP); reach the Programmatic milestones listed below.

Characteristics	Units	2015 Target	2020 Target	Ultimate target
Duration of continuous H_2 production at full sunlight intensity	minutes or hours	30 min	4 h	8 h
Solar to H2 (STH) energy conversion ratio	%	2	5	17

- **Specific tasks:**

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

  Task 2: Genetically add various desirable traits to an algal straining expressing and O_2-tolerance hydrogenase to achieve higher STH and longer duration of H_2 photoproduction.

Relevance – Impact

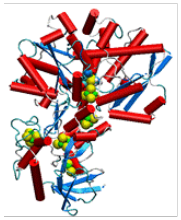
Progress since the 2013 AMR towards the 2015 programmatic targets:

1. Benchmarked the STH conversion efficiency of WT and Ca1-expressing Chlamydomonas at, respectively, 0.75% and 0.12%, using the Clark electrode chamber as the photobioreactor.
2. Demonstrated similar final H_2 yield of the Ca1-expressing Chlamydomonas compared to the wild-type strain over a 30-minute illumination period.

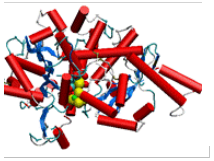
Approach – Subtask 1

- **Technical approach:** Address the O_2 sensitivity of hydrogenase by stably 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 and productivity compared to the wild-type strain.

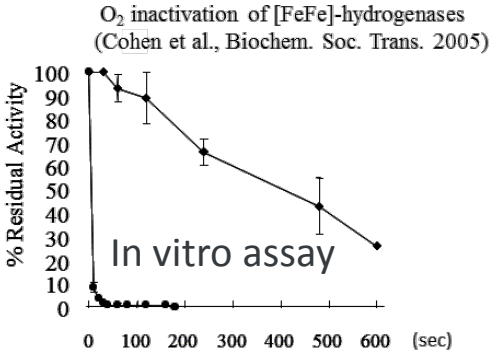
Dr. Paul King, NREL



Clostridial (bacterial) H2ase



Chlamydomonas (algal) H2ase



Seth Noone, NREL

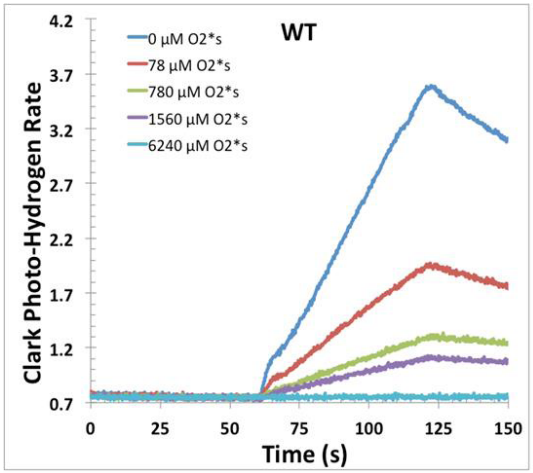
Construct 1: psad promoter and terminator (constitutive expression) with ferredoxin transit peptide/Ca1 optimized by geneart.

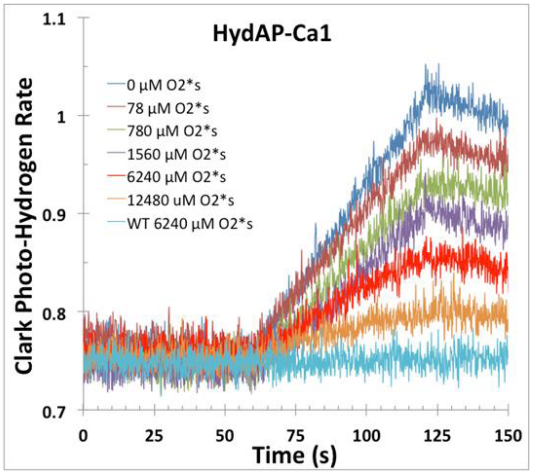
Construct 2: native algal hydrogenase promoter and terminator with ferredoxin transit peptide/Ca1 optimized by geneart.

Note: besides being more O_2-tolerant, Ca1 also has lower affinity for H_2 (K_m = 460 μM vs. 190 μM for the algal enzyme), which may decrease the uptake reaction.

Approach – Subtask 1 - Previous Accomplishments

The Ca1 hydrogenase was stably expressed in *Chlamydomonas reinhardtii* and it showed 2% of the wild-type strain's rate and increased (5-19 fold) *in vivo* O_2-tolerance.





Enzyme/ Strain	O_2 Inactivation Rate Constants (τ_1 and τ_2) ($\mu M\ O_2^{-1} \bullet s^{-1}$) 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	1.4×10^{-3}	$1 \times 10^{-4} \pm 2 \times 10^{-5}$	19	17
HydAP-Ca1	3×10^{-3}	3.5×10^{-4}	9	5

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

Approach – Subtask 1

Milestones

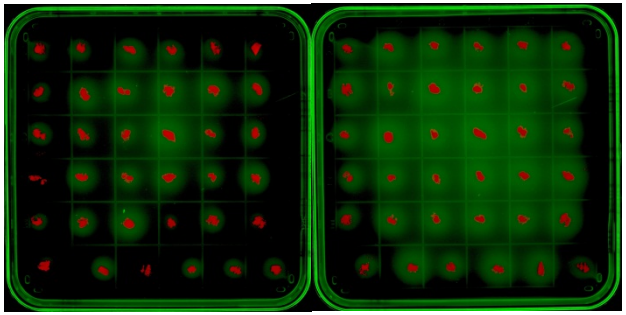
	FY13 Milestones	Due date	Status
3.3.1-3	Generate new transformants with 3 variations of added introns from, respectively, RbcS2, HYDA1 and HYDA2	4/13/13	Completed
3.3.1-4	Test at least 100 strains from the first generation of intron-containing transformants for H_2-production activity through the plate assay	5/13/13	Completed
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.	7/13/13	Postponed to 11/13 (see Q1-1 below);
	FY 14 Milestones		
Q1-1 Regular	Demonstrate that the addition of introns increases the H2 photoproduction activity and stability of Ca1 by at least 3 times compared to HYDA1- of PsaD-based constructs. Go: Use the strain for further improvements; No-Go: Propose a new plan for DOE's approval to re-direct the work.	11/13	NoGo; new approach proposed (see Q3-1 milestone)
Q1-2 Regular	Benchmark sunlight to hydrogen conversion efficiency and duration of H_2 production of wild-type and best H_2-producer against 2015 Programmatic Targets	12/2013	Completed
Q2-1 Go/NoGo	Achieve continuous H_2 photoproduction at an initial rate of 11 µmol H_2 mg Chl^{-1} h^{-1} with a minimum *final* rate of 0.06 µmol H_2 mg Chl^{-1} h^{-1} for at least 30 minutes (10x longer than wild type), *and a final net yield (normalized to chlorophyll concentration) equal to or higher than that of the wild type during the first 30 minutes of illumination*, by whole cells of a Ca1-expressing mutant strain of the algae *Chlamydomonas reinhardtii* under full solar-light intensities, in the presence of the uncoupler FCCP, measured continuously by the Clark electrode.	2/2014	Postponed to 4/2014; *not completed*.
Q3-1 Regular	Demonstrate an at least two-fold increase in H_2 production rates using the Clark electrode in cultures of a new *C. reinhardtii* mutant strain over the current *C. reinhardtii* Hyd(-) Ca1-expressing strain 55.	5/2014	On track; 7 transformants are being analyzed.

Accomplishments – Subtask 1

FY13 Milestones 3.3.1.3 and 3.3.1.4

3.3.1.3: Completed. However, due to cost limitations, we worked with constructs containing only the RBCS2 intron. The construct consisted of the codon-optimized Ca1 sequence cloned into the pChlamy HsP70A/RbcS2 promoter 5'-end intron-containing commercial vector.

3.3.1.4: Completed. Variable H_2 production of 100 transformants was demonstrated by the GFP (green fluorescence protein-based) plate assay.



Conclusion: transformation of Chlamydomonas with an intron-containing gene is achievable and the intron-containing Ca1 gene is expressed by the cells, resulting in verifiable H_2 production by the recombinant organism.

Accomplishments – Subtask 1

FY14 Milestone Q1-1

Previous constructs tested:

	Parent strain	Vector	Introns	Promoter/ terminator
D66 WT	Is WT	n.a.	Native HYDA introns	n.a.
[Hyd(-)]	D66 WT	n.a.	n.a.	n.a.
PsaD Ca1 (strain 55)	Hyd-	pSL18	None	PsaD
pChlamy1 Ca1	Hyd-	pChlamy1	One in 5' UTR of Ca1 gene	Hsp70A/RbcS2 (high constitutive expression levels, according to Invitrogen)
pChlamy1 + Ca1 ORF introns	Hyd-	pChlamy1	One in 5' UTR, three in Ca1 ORF	Hsp70A/RbcS2 (see above)
PsaD + Ca1 ORF introns	Hyd-	pSL18	Three in Ca1 ORF	PsaD

Strain/Construct	Rate: μmole H_2 mg Chl^{-1} h^{-1}	% WT (induced in the light)
D66 WT	133	na
PsaD Ca1 (strain 55)	8.8	6.6
pChlamy1 Ca1	0.7	0.3
pChlamy1 + Ca1 ORF introns	3.3	1.3
PsaD Ca1+ ORF introns	0	0

Conclusion: None of the intron-containing constructs were expressed at higher rates than that of the PsaD strain 55. A different transformation strategy to improve Ca1 expression was proposed (see next slide).

Accomplishments – Subtask 1

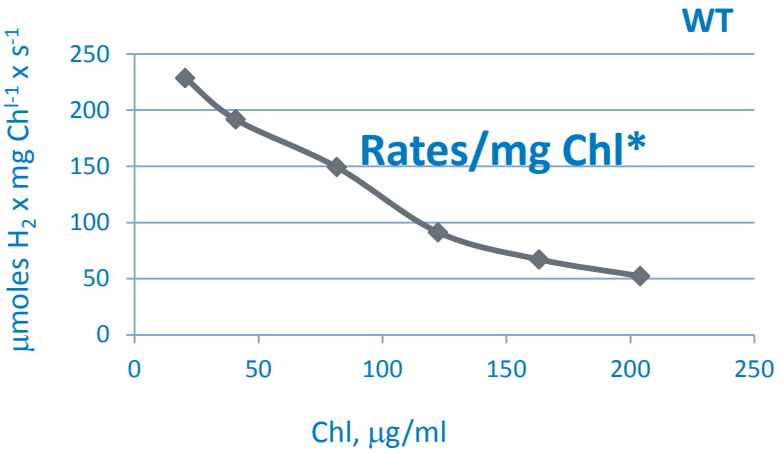
FY14 Milestone Q1-1

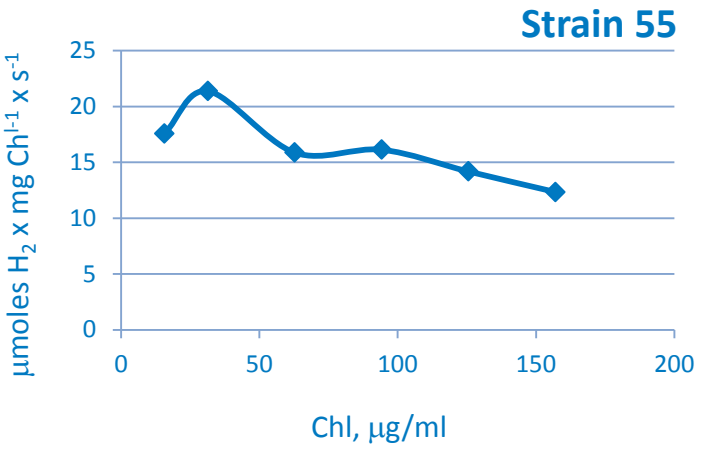
The work will be re-directed towards investigating the effect of using a linear vs. a circular plasmid to introduce the Ca1 gene in Chlamydomonas (FY14 Milestone Q3-1; due May 2014). Strain 55 was generated with a circular plasmid.

Accomplishments – Subtask 1

Milestone Q1-2: Benchmark STH and duration to document strain improvements. Experimental Setup:

The H_2-photoproduction rates of WT and Strain 55 at various Chl concentrations was measured with a Clark electrode under the equivalent of sunlight intensity. The electrode chamber had an illuminated surface area of 1 cm^2 and a volume of 2 ml. Light was provided by PAR (photosynthetically active radiation) LEDs at 2,000 µE m^{-2} s^{-1} (or 500 J/hour, equivalent to the PAR region of the solar spectrum). Solar to Hydrogen efficiencies (STHs, next slide) were estimated assuming the energy content of sunlight as 1,111 kJ/hour.

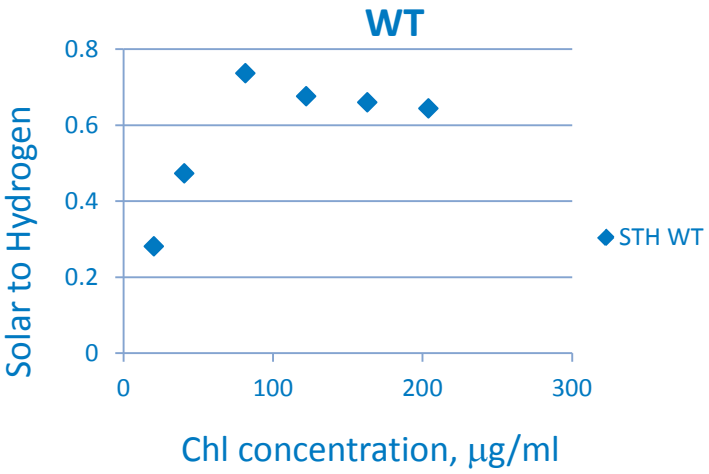


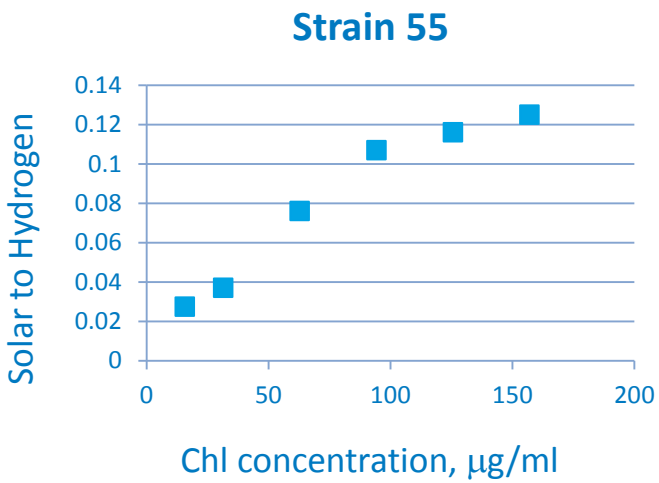


*Rates decrease at higher [Chl] concentrations due to shading of the cultures

Accomplishments – Subtask 1

Milestone Q1-2: Benchmark STH and duration - Results on STH





STH values are estimated on a per surface area basis; they increase as the cell density increases, and they reach a plateau due to culture shading and loss of the ability of denser cultures to capture more sunlight.

The maximum STHs obtained for WT and Strain 55 are 0.75% and 0.12%

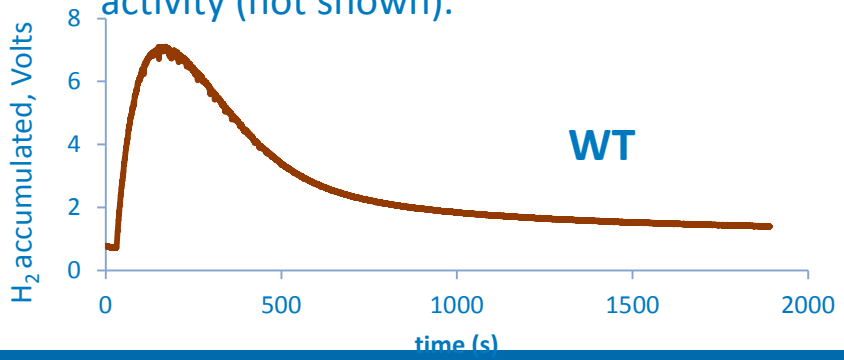
Estimated maximum potential STH: 10-13%

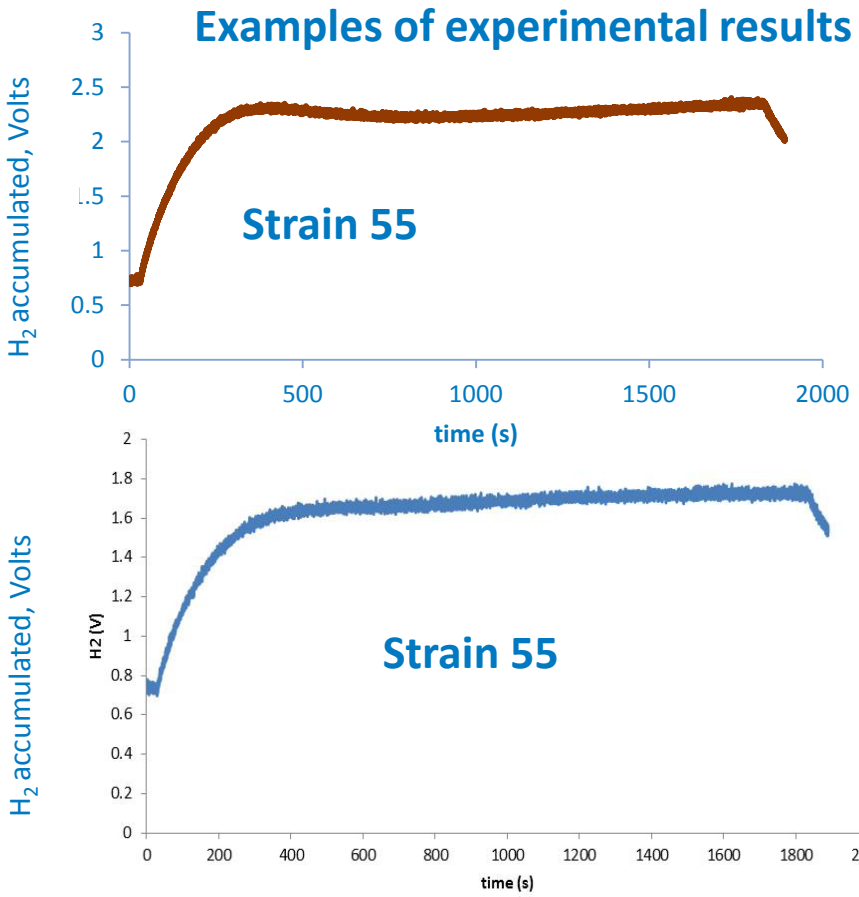
1. Both WT and strain 55 STH are much lower than the FY2015 target of 2%; further work is needed;
2. The estimated STH for strain 55 was 6.25-fold lower than that of the WT strain, closer to the ratio of 10 for the *in vitro* hydrogenase activity for the two strains;
3. The source of variability for strain 55 rates is not currently known;
4. *Benchmark regarding duration is shown under milestone Q2-1 (next slide).*

Accomplishments – Subtask 1

Milestone Q2-1: Achieve continuous H_2 production under full solar-light intensities

- The WT strain produces H_2 for only about 3 min, followed by net H_2 uptake (due to a combination of gas diffusion and hydrogenase-mediated H_2 uptake)
- Strain 55 is able to accumulate H_2 for about 30 minutes (with some variation).
- At the end of the experiment, the WT hydrogenase is almost completely inactive (not shown).
- At the end of the experiment, strain 55 showed more than 20-40% residual hydrogenase activity (not shown).





Accomplishments – Subtask 1

Milestone Q2-1: Achieve continuous H_2 production under full solar-light intensities

Strain	Chl Concentration (μg Chl/ml)	Initial rate* (μmoles H_2 x mg Chl^{-1} x h^{-1})	Final rate (μmoles H_2 x mg Chl^{-1} x h^{-1})	Final Net H_2 yield at 30 min (μmoles H_2 x mg Chl^{-1})	Duration of production (minutes)
Milestone target	Not defined	11	0.06	Mutant equal to or 2X higher than WT	30
Wild-type	12-42	146 ± 26*	-2.52 ±3.15	0.41±0.17	2.63±0.47
Strain 55	14-32	15.3 ± 7.66 *	0.05±0.15	0.31±0.1	≥ 30 in 5 experiments

- Achieved milestone targets for initial rates and duration *on average*, but variability results in very high standard deviations – Not completed.

*Note: standard deviation of 6 experiments for WT and 9 experiments for strain 55.

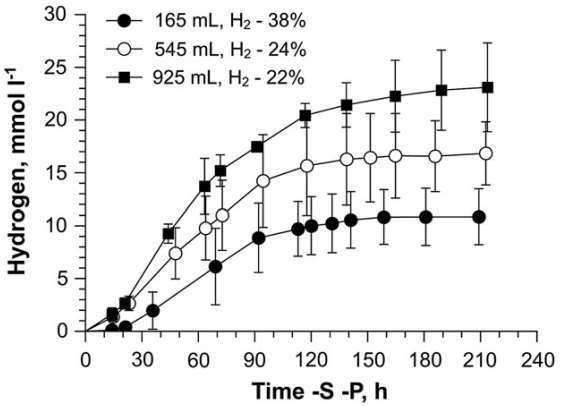
Proposed Future Work – Subtask 1

INCREASE RATES

- Complete upcoming milestone for 2-fold increased rate (circular vs. linear transformation vector);
- Identify the subsequent rate limiting step (transcriptional vs. translational vs. physiological).

DECREASE VARIABILITY AND INCREASE STH

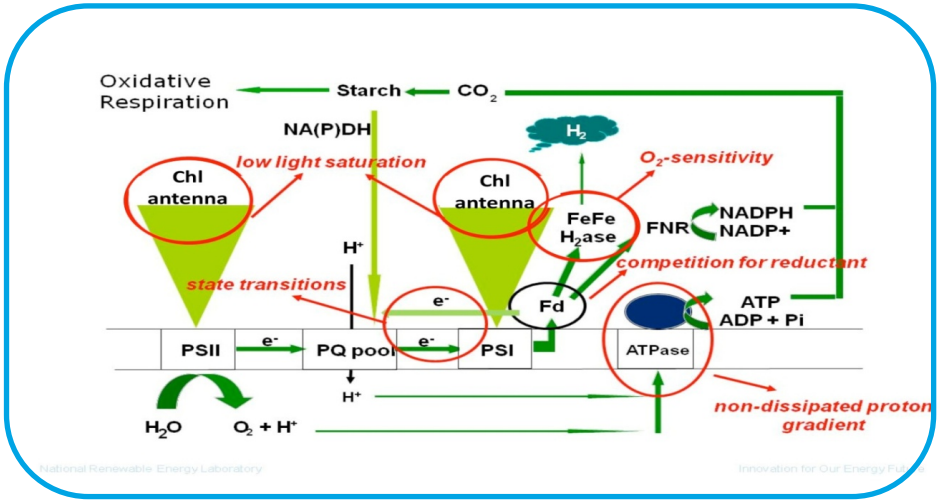
- Optimize induction and culture conditions to decrease variability in rates;
- Test the performance of Ca1 transformants in photobioreactors optimized for gas diffusion and light delivery to prevent back-reaction (H_2 uptake) and further improve STH.
  - For example, use a different reactor configuration, with larger headspace (see Kosourov et al., Int. J. Hydrogen Energy, 2012) – shown for sustained H_2 production by algal cultures on immobilized films under sulfur-deprived conditions.



Approach – Subtask 2

Genetically combine traits addressing other barriers to H_2-production into the double-knock-out hydrogenase Chlamydomonas strain; cross the triple strain with the best Ca1-expressing transformant.

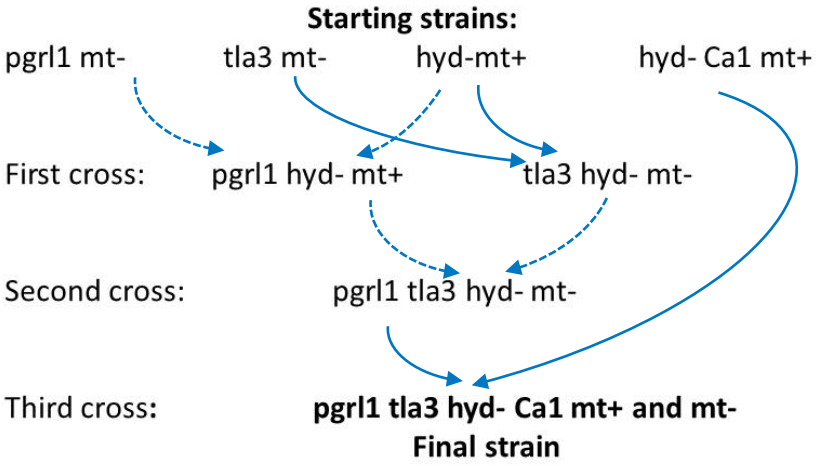
1. Truncated antenna (from Prof. Melis): tla3
2. Low proton-gradient formation and no state transitions (from Dr. Peltier): pgrl1
3. Hydrogenase knock-out (from Prof. Posewitz): hyd(-)



Accomplishments – Subtask 2

Milestones:

FY2014 Milestones			
Q2-1	Obtain at least 200 zygotes from crossing strain tla3 with hyd-, and germinate at least 30 spores for subsequent genetic characterization.	3/2014	Completed
Q3.2	Verify at least one successful genetic cross each between the pgrl1 and tla3 strains with the hyd-, yielding the pgrl1 hyd- mt+ and the tla3 hyd- mt- strains.	3/2014	Postponed to 5/2014 due to delays in receiving the microscope; further postponed to 06/2014 due to loss of personnel
Q4-1	Verify at least one successful genetic cross between the pgrl1 hyd- mt+ and tla3 hyd- mt- strains yield the strains pgrl1 tla3 hyd- mt+ and	7/2014	Postponed to 9/2014 due to loss of personnel



Accomplishments:

- First crosses have been successfully completed (34 spores germinated); their phenotype is being determined by PCR.

Proposed Future Work – Subtask 2

- Complete all three genetic crosses; Prof. Patrice Hamel from Ohio State University is providing unfunded help.
- Verify the final phenotype with respect to the FY 2015 Programmatic Targets (duration and STH).

- If appropriate, introduce other traits (e.g., fused Fd/H2ase – under investigation by Office of Science project) to further improve the H_2-production performance of the multiple-mutant strain.

Responses to Previous Year Reviewers' Comments

Reviewer Comment: "The rationale for pursuing a parallel (to cyanobacteria) track with green microalgae is not very clear."

Response: There are advantages in working with algae vs. cyanobacteria: (a) the [FeFe]-hydrogenase in Chlamydomonas is encoded by only one gene, while the [NiFe] hydrogenases in cyanobacterium are multiple-subunit complexes that include a membrane-embedded protein (much more difficult to express); and (b) the physiological electron donor to [FeFe]-hydrogenases is ferredoxin (Fd), while the donor to [NiFe]-hydrogenases is NAD(P)H, which is less reducing than Fd, shifting the reaction towards uptake. However, there are no known [FeFe]-hydrogenases with high tolerance to O_2, while O_2-tolerant [NiFe]-hydrogenases do exist.

Reviewer Comment: "The reduction in collaboration is the fault of funding shortages. The reviewer wonders if collaborations are not possible without providing funding"… "the weakness is the speed with which the work is progressing.. "This may simply reflect the reviewer's lack of knowledge of DOE funding decisions and guidelines."

Response: We are still conducting unfunded collaborations with the Ohio State University and with Dr. Peltier's group in France. The DOE's Office of Science funds much more basic work related to (a) understanding the regulation of hydrogenase expression and partitioning of reductant between H_2 production and CO_2 fixation (BES), and (b) deconvolution the ferredoxin interactome that provides electrons to hydrogenase in Chlamydomonas.

Remaining Challenges and Barriers

Meeting Programmatic FY2015 Targets:

- **Subtask 1:** Increase rates and STH of Ca1-expressing strains by (a) identifying current rate-limiting steps, (b) finding and decreasing data variability, and (b) optimizing the photobioreactor configuration to increase the headspace to volume ratio.

- **Subtask 2:** Combine traits addressing the O_2-sensitivity of the hydrogenase with (a) the down-regulation of photosynthesis due to non-dissipation of the proton gradient and state transitions (pgrl1 mutant), (b) the low light-saturation of photosynthesis (tla3 mutant); and (c) the competition for reductant with CO_2 fixation and cyclic electron flow (pgrl1 or other mutants).

Summary

- We have increased the activity of the Chlamydomonas strain expressing the Ca1 hydrogenase from 2% to about 11% of the native hydrogenase.
- The duration of H_2 photoproduction by the Ca1-expressing strain lasts for up to 30 minutes, and, at the end of this period, the final net H_2 yield is ~76% of the wild-type strain.
- Current limitations in rate, STH, and duration may be due in large part to the photobioreactor configuration, since there is very little headspace to allow effective gas diffusion and prevent H_2 uptake from becoming dominant.
- We expect that the incorporation of new genetic traits addressing additional physiological barriers, optimization of the photobioreactor, and increased Ca1 activity will yield an STH closer to the Programmatic Target of 2% for FY 2015.

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Technical Back-Up Slides

PAR spectrum of LEDs

- Lighting Spectrum of Photosynthetic Active Radiation (PAR) at 400-700nm.