Biological Systems for Hydrogen Photoproduction

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National Renewable Energy Laboratory
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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 H₂ production (AO)
  Oxygen Accumulation (AP)

• Targets:
  Duration of production
  Solar to H₂ (STH) energy conversion

Partners

• Interactions/ collaborations: none
Photobiological water splitting coupled to hydrogenase-mediated H₂ production has the potential to convert about **10% of incident solar energy** into H₂. Various barriers have been identified as currently limiting green algal H₂ production, including:

- the O₂ sensitivity of the hydrogenase enzyme,
- the competition for reductant with CO₂ 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 H₂ production from water (barriers AO and AP); reach the Programmatic milestones listed below.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Units</th>
<th>2015 Target</th>
<th>2020 Target</th>
<th>Ultimate target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of continuous H₂ production at full sunlight intensity</td>
<td>minutes or hours</td>
<td>30 min</td>
<td>4 h</td>
<td>8 h</td>
</tr>
<tr>
<td>Solar to H₂ (STH) energy conversion ratio</td>
<td>%</td>
<td>2</td>
<td>5</td>
<td>17</td>
</tr>
</tbody>
</table>

• **Specific tasks:**
  
  **Task 1:** Address the O₂ sensitivity of hydrogenases that prevent continuous H₂ photoproduction under aerobic, high solar-to-hydrogen (STH) conversion efficiency conditions.
  
  **Task 2:** Genetically add various desirable traits to an algal straining expressing and O₂-tolerance hydrogenase to achieve higher STH and longer duration of H₂ 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 O₂ sensitivity of hydrogenase by stably introducing the gene encoding for a hydrogenase from *Clostridium acetobutylicum* that is more O₂-tolerant *in vitro* into the photosynthetic alga *Chlamydomonas reinhardtii*; measure its linkage to water oxidation and *in vivo* O₂ tolerance and productivity compared to the wild-type strain.

  ![In vitro assay](image)

  Note: besides being more O₂-tolerant, Ca1 also has lower affinity for H₂ (Kₘ = 460 µM vs. 190 µM for the algal enzyme), which may decrease the uptake reaction.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fd TP</th>
<th>codon-optimized Ca1 (Geneart)</th>
<th>psad term</th>
</tr>
</thead>
<tbody>
<tr>
<td>psad prom</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hyda prom</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

  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.

  ![O₂ inactivation of [FeFe]-hydrogenases](image)
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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rate of $H_2$ production in Clark electrode ($\mu$mol $H_2$ mg Chl$^{-1}$ h$^{-1}$)</th>
<th>% WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>260</td>
<td>100</td>
</tr>
<tr>
<td>Double knock-out</td>
<td>0.09</td>
<td>0.035</td>
</tr>
<tr>
<td>Double knock-out + Ca1</td>
<td>5.1</td>
<td>2</td>
</tr>
</tbody>
</table>

### Enzyme/Strain

<table>
<thead>
<tr>
<th>Enzyme/Strain</th>
<th>$O_2$ Inactivation Rate Constants ($\tau_1$ and $\tau_2$, $\mu$M $O_2^{-1}$•s$^{-1}$)</th>
<th>$O_2$ Tolerance (Ratio to WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fast $2.7 \times 10^{-2} \pm 2 \times 10^{-3}$</td>
<td>Slow $1.7 \times 10^{-3} \pm 5.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>WT</td>
<td>$2.7 \times 10^{-2} \pm 2 \times 10^{-3}$</td>
<td>$1.7 \times 10^{-3} \pm 5.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>PsaDP-Ca1</td>
<td>$1.4 \times 10^{-3}$</td>
<td>$1 \times 10^{-4} \pm 2 \times 10^{-5}$</td>
</tr>
<tr>
<td>HydAP-Ca1</td>
<td>$3 \times 10^{-3}$</td>
<td>$3.5 \times 10^{-4}$</td>
</tr>
</tbody>
</table>
## Approach – Subtask 1

### Milestones

<table>
<thead>
<tr>
<th></th>
<th>FY13 Milestones</th>
<th>Due date</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.1-3</td>
<td>Generate new transformants with 3 variations of added introns from, respectively, RbcS2, HYDA1 and HYDA2</td>
<td>4/13/13</td>
<td>Completed</td>
</tr>
<tr>
<td>3.3.1-4</td>
<td>Test at least 100 strains from the first generation of intron-containing transformants for ( \text{H}_2 ) production activity through the plate assay</td>
<td>5/13/13</td>
<td>Completed</td>
</tr>
<tr>
<td>3.3.1-5</td>
<td>Go/NoGo: if addition of introns increases the ( \text{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.</td>
<td>7/13/13</td>
<td>Postponed to 11/13 (see Q1-1 below);</td>
</tr>
</tbody>
</table>

### FY 14 Milestones

<table>
<thead>
<tr>
<th></th>
<th>FY 14 Milestones</th>
<th>Due date</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1-1 Regular</td>
<td>Demonstrate that the addition of introns increases the ( \text{H}_2 ) 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.</td>
<td>11/13</td>
<td>NoGo; new approach proposed (see Q3-1 milestone)</td>
</tr>
<tr>
<td>Q1-2 Regular</td>
<td>Benchmark sunlight to hydrogen conversion efficiency and duration of ( \text{H}_2 ) production of wild-type and best ( \text{H}_2 )-producer against 2015 Programmatic Targets</td>
<td>12/2013</td>
<td>Completed</td>
</tr>
<tr>
<td>Q2-1 Go/NoGo</td>
<td>Achieve continuous ( \text{H}_2 ) photoproduction at an initial rate of 11 ( \mu \text{mol} ) ( \text{H}_2 ) ( \text{mg} ) ( \text{Chl}^{-1} ) ( \text{h}^{-1} ) with a minimum ( \text{final} ) rate of 0.06 ( \mu \text{mol} ) ( \text{H}_2 ) ( \text{mg} ) ( \text{Chl}^{-1} ) ( \text{h}^{-1} ) for at least 30 minutes (10x longer than wild type), and a ( \text{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 <em>Chlamydomonas reinhardtii</em> under full solar-light intensities, in the presence of the uncoupler FCCP, measured continuously by the Clark electrode.</td>
<td>2/2014</td>
<td>Postponed to 4/2014; not completed.</td>
</tr>
<tr>
<td>Q3-1 Regular</td>
<td>Demonstrate an at least two-fold increase in ( \text{H}_2 ) production rates using the Clark electrode in cultures of a new <em>C. reinhardtii</em> mutant strain over the current <em>C. reinhardtii</em> Hyd(-) Ca1-expressing strain 55.</td>
<td>5/2014</td>
<td>On track; 7 transformants are being analyzed.</td>
</tr>
</tbody>
</table>
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 H₂ 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 H₂ production by the recombinant organism.
Accomplishments – Subtask 1

FY14 Milestone Q1-1

Previous constructs tested:

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

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

The $\text{H}_2$-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 $1 \text{ cm}^2$ and a volume of $2 \text{ ml}$. Light was provided by PAR (photosynthetically active radiation) LEDs at $2,000 \mu\text{E m}^{-2} \text{s}^{-1}$ (or $500 \text{ J/hour}$, 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 $1,111 \text{ kJ/hour}$.

*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

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 H₂ production under full solar-light intensities

- The WT strain produces H₂ for only about 3 min, followed by net H₂ uptake (due to a combination of gas diffusion and hydrogenase-mediated H₂ uptake).
- Strain 55 is able to accumulate H₂ 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 H₂ production under full solar-light intensities

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chl Concentration (µg Chl/ml)</th>
<th>Initial rate* (µmoles H₂ x mg Chl⁻¹ x h⁻¹)</th>
<th>Final rate (µmoles H₂ x mg Chl⁻¹ x h⁻¹)</th>
<th>Final Net H₂ yield at 30 min (µmoles H₂ x mg Chl⁻¹)</th>
<th>Duration of production (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milestone target</td>
<td>Not defined</td>
<td>11</td>
<td>0.06</td>
<td>Mutant equal to or 2X higher than WT</td>
<td>30</td>
</tr>
<tr>
<td>Wild-type</td>
<td>12-42</td>
<td>146 ± 26*</td>
<td>-2.52 ± 3.15</td>
<td>0.41 ± 0.17</td>
<td>2.63 ± 0.47</td>
</tr>
<tr>
<td>Strain 55</td>
<td>14-32</td>
<td>15.3 ± 7.66 *</td>
<td>0.05 ± 0.15</td>
<td>0.31 ± 0.1</td>
<td>≥ 30 in 5 experiments</td>
</tr>
</tbody>
</table>

- 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 (H₂ uptake) and further improve STH.
  o For example, use a different reactor configuration, with larger headspace (see Kosourov et al., Int. J. Hydrogen Energy, 2012) – shown for sustained H₂ production by algal cultures on immobilized films under sulfur-deprived conditions.
Approach – Subtask 2

Genetically combine traits addressing other barriers to H₂-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

<table>
<thead>
<tr>
<th>Milestone</th>
<th>Description</th>
<th>Fiscal Year</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q2-1</td>
<td>Obtain at least 200 zygotes from crossing strain tla3 with hyd-, and germinate at least 30 spores for subsequent genetic characterization.</td>
<td>3/2014</td>
<td>Completed</td>
</tr>
<tr>
<td>Q3.2</td>
<td>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.</td>
<td>3/2014</td>
<td>Postponed to 5/2014 due to delays in receiving the microscope; further postponed to 06/2014 due to loss of personnel</td>
</tr>
<tr>
<td>Q4-1</td>
<td>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</td>
<td>7/2014</td>
<td>Postponed to 9/2014 due to loss of personnel</td>
</tr>
</tbody>
</table>

**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 \( \text{H}_2 \)-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 O₂, while O₂-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 H₂ production and CO₂ 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 O₂-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 CO₂ 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 H₂ photoproduction by the Ca1-expressing strain lasts for up to 30 minutes, and, at the end of this period, the final net H₂ 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 H₂ 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.
Technical Back-Up Slides
PAR spectrum of LEDs

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