Biological Systems for Hydrogen Photoproduction

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Key personnel:
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K. Ratcliff,
S. Smolinski

National Renewable Energy Laboratory


NREL/PR-560-48068

Project ID # 37
Overview

Timeline
Project start date: FY00
Project end date: FY18
Percent complete: N/A

Barriers
Production barriers addressed
- Continuity of H₂ production (AI)
- Feedstock cost in an integrated system (AT)
- Rate of H₂ production (AH)

Budget
Funding received in FY09: $800K
Funding allocated for FY10: $600K

Partners
Drs. Anatoly Tsygankov and Sergey Kosourov, Institute of Basic Biological Problems, RAS, Pushchino, Russia
Dr. Michael Flickinger, North Carolina State University
Dr. Eric Johnson, Johns Hopkins University
Drs. Iftach Yacoby and Shuguang Zhang, MIT

* New tasks in orange
Objectives/Relevance

**General:** Develop photobiological and integrated photobiological/fermentative systems for large-scale H$_2$ production.

- **Task 1:** Address the O$_2$-sensitivity of hydrogenases, which prevents continuity of H$_2$ photoproduction under aerobic, high solar-to-hydrogen (STH) conditions.
- **Task 2:** Utilize a limited STH H$_2$-producing method (sulfur deprivation) as a platform to address other factors limiting commercial algal H$_2$ photoproduction.
- **Task 3:** Integrate photobiological and fermentative systems in different configurations for less costly H$_2$ production in the short term.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Current Status</th>
<th>2013 Targets</th>
<th>Maximum Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of continuous photoproduction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>● Aerobic, high STH (O$_2$-tolerant)</td>
<td>0 (10 days)</td>
<td>30 min</td>
<td>12 hours indefinite</td>
</tr>
<tr>
<td>● Aerobic, limited STH (S-deprivation)</td>
<td>90 days</td>
<td></td>
<td>indefinite</td>
</tr>
<tr>
<td>● Anaerobic, limited STH (S-deprivation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O$_2$ tolerance (half-life in air)</td>
<td>4 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>● Oxidized conditions</td>
<td>40 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>● Reduced conditions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cost ($/kg H$_2$)</td>
<td></td>
<td>$2.99</td>
<td>$3.21</td>
</tr>
<tr>
<td>● Aerobic, high STH (O$_2$-tolerant)</td>
<td></td>
<td>$6.02</td>
<td></td>
</tr>
<tr>
<td>● Anaerobic, limited STH (S-deprivation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>● Integrated (photo + fermentative)</td>
<td></td>
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</tbody>
</table>
Task 1 – \( \text{O}_2 \) Sensitivity/Rate of Hydrogenases

Objectives, Approaches, and Collaborations

**Objectives:**

1. Develop and optimize aerobic, high-STH photobiological systems for the production of \( \text{H}_2 \) from water by engineering a \( \text{H}_2 \)-producing catalyst ([FeFe]-hydrogenase) that has an extended half-life following exposure to \( \text{O}_2 \).

(2) Explore fusions between hydrogenase and ferredoxin to increase photosynthetic electron flow to the hydrogenase (this is unrelated to \( \text{O}_2 \) sensitivity, but it addresses the rate of \( \text{H}_2 \)-production barrier).

**Approaches:**

- Use computational simulations to identify pathways by which \( \text{O}_2 \) accesses the catalytic site and use site-directed mutagenesis to molecularly engineer the enzyme to prevent \( \text{O}_2 \) access.
- Use random methods to generate mutants with higher \( \text{O}_2 \) tolerance.
- Introduce a more \( \text{O}_2 \)-tolerant bacterial hydrogenase into algae.
- Evaluate the feasibility of creating fusions between hydrogenases and ferredoxin to increase electron flux to the hydrogenase.

**Collaborator:** MIT (currently unfunded)
Task 1 – O₂ Sensitivity of Hydrogenases
Accomplishments and Milestones

1. **Computational modeling:** We extended analysis of pathways to [NiFe]-hydrogenases; identified 3 key residues as potential targets for mutagenesis to decrease O₂ diffusion to catalytic site: Val67, Val110 and Leu115 in *D. gigas*.

2. **Site-directed mutagenesis:**
   (a) We attributed the multiphasic kinetics of O₂ inactivation to the existence of three states of [FeFe]-hydrogenases, each with different tolerance toward O₂ (the reduced state is more O₂-tolerant than the oxidized one; the third state is O₂-insensitive);
   (b) we are re-assessing our strategy for controlling O₂ diffusion to the catalytic site of [FeFe]-hydrogenases; a manuscript is in preparation (see future work).

Previous results showed that the clostridial H₂ase is 100X more tolerant to O₂ than the algal enzyme; **re-directed resources toward expressing the clostridial hydrogenase in Chlamydomonas** to assess the effect of a more O₂-tolerant hydrogenase on H₂ production in vivo (see next slide).

<table>
<thead>
<tr>
<th>Task</th>
<th>Due date</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Use implicit ligand sampling method to map the pathways in [NiFe]-hydrogenases</td>
<td>January 2010</td>
<td>completed</td>
</tr>
</tbody>
</table>
Task 1 – O$_2$ Sensitivity of Hydrogenases Accomplishments and Milestones

3. **Random mutagenesis**: No new results to report.

4. **Expression of the clostridial hydrogenase in Chlamydomonas**: Inconclusive activity results with one transformant; evaluation of additional transformants show expression in Chlamydomonas; activity is being evaluated.

<table>
<thead>
<tr>
<th>Task</th>
<th>Due date</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demonstrate that CaI is active in C. reinhardtii</td>
<td>February 2010</td>
<td>Inconclusive; postponed</td>
</tr>
<tr>
<td>Measure the O$_2$ sensitivity of H$_2$ase activity in C. reinhardtii</td>
<td>April 2010</td>
<td>In progress</td>
</tr>
</tbody>
</table>

5. **Create fusions between hydrogenases and ferredoxin to improve reductant flux to the hydrogenase**: We simulated the docking between the Ca1 H$_2$ases with the algal ferredoxin to guide MIT’s engineering efforts. Results suggest that the interaction could be facilitated if the clostridial hydrogenase were truncated, to reposition the N-terminus for fusion with Fd.

Models of docking between complete (left) or truncated (right) Ca1 H$_2$ase with algal ferredoxin.
Task 1 – O₂ Sensitivity of Hydrogenases

Future Work

1. **Computational simulation**: We will compare the geometry and energetics of the catalytic center and adjacent structures of [FeFe]-hydrogenases with different sensitivity to O₂. We are re-assessing how O₂ accesses the enzyme’s catalytic center and to what extent this depends on channel structure/configuration.

2. **Site-directed mutagenesis**: A manuscript will be submitted summarizing current observations regarding the redox states effects on H₂ase O₂ inactivation; the approach involving gas channels is on hold until expression studies clarify whether higher hydrogenase O₂ tolerance as measured *in vitro* translates into higher O₂ tolerance *in vivo*.

3. **Random mutagenesis**: New personnel are being hired to restart the research. We will determine a new strategy based on new results from Subtask 1.

4. **Expression of clostridial hydrogenase in Chlamydomonas**: We will characterize additional constructs and, if required, design new Ca1 constructs or alternative approaches to increase H₂ production *in vivo*.

5. **Hydrogenase/ferredoxin fusions**: NREL will continue to provide guidance to MIT’s work and will test their transformants in house if additional funding is available.
**Task 2 – Sulfur-Deprivation Platform**

**Objectives, Approaches, and Collaborations**

**Objectives:** 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 inactive, leaky ATP synthase on the rates.

**Approaches:**
- Continue to improve the H$_2$-production yields by alginate-immobilized algae (RAS).
- Test and optimize the performance of immobilized, photoautotrophic cultures (RAS).
- Generate inducible ATP synthase mutants and test them with the immobilized system.

**Collaborators:** Johns Hopkins University, the Institute of Basic Biological Problems, Russian Academy of Sciences (RAS)
1. **Improve H\textsubscript{2} rates and yields using immobilized films**: Lower thickness improves rates and yields; higher thickness improves protection against O\textsubscript{2} inactivation under aerobic conditions and prevents acetate diffusion.

<table>
<thead>
<tr>
<th>Film thickness, µm</th>
<th>Total Chl concentration, µg/cm\textsuperscript{2} film</th>
<th>Maximum specific rate of H\textsubscript{2} production in argon, µmole mg Chl\textsuperscript{-1} h\textsuperscript{-1}</th>
<th>Maximum specific rate of H\textsubscript{2} production in air, µmole mg Chl\textsuperscript{-1} h\textsuperscript{-1} (% of rate in argon)</th>
<th>Total yield of H\textsubscript{2} gas in argon, mol m\textsuperscript{-2}</th>
<th>Total yield of H\textsubscript{2} gas in air, mol m\textsuperscript{-2} (% of rate in argon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>180</td>
<td>71.37</td>
<td>13.5</td>
<td>3.4 (25%)</td>
<td>0.55</td>
<td>0.094 (17%)</td>
</tr>
<tr>
<td>260</td>
<td>101.6</td>
<td>7.8</td>
<td>2.8 (36%)</td>
<td>0.43</td>
<td>0.096 (22%)</td>
</tr>
<tr>
<td>290</td>
<td>117.74</td>
<td>6.1</td>
<td>2.6 (43%)</td>
<td>0.42</td>
<td>0.113 (27%)</td>
</tr>
<tr>
<td>310</td>
<td>110.89</td>
<td>5.9</td>
<td>2.3 (39%)</td>
<td>0.41</td>
<td>0.093 (23%)</td>
</tr>
</tbody>
</table>

2. **Test and improve the performance of photoautotrophic, immobilized cultures**: No results to report; work just getting started.
Task 2 – Sulfur-Deprivation Platform
Accomplishments and Milestones

3. **Design ATP synthase conditional mutants**: A C-terminus-mutated ε-subunit of the ATP synthase will be expressed in the chloroplast of Chlamydomonas behind a promoter that induces expression upon anaerobiosis. Specific mutations have been identified and transformants are being screened in an immobilized environment.

Site-directed alteration of the C-terminus to remove positive charges should further stimulate H₂.

<table>
<thead>
<tr>
<th>Task</th>
<th>Due date</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Design ATPase conditional mutants</td>
<td>December 2009</td>
<td>completed</td>
</tr>
<tr>
<td>Test immobilized ATPase mutants under sulfur-deprived conditions</td>
<td>August 2010</td>
<td>completed</td>
</tr>
</tbody>
</table>
Task 2 – Sulfur-Deprivation Platform

Future Work

1. **Improve H₂ rates and yields using immobilized films**: Test the effect of the volume of the photobioreactor’s headspace on the H₂-production properties of algal cultures.

2. **Test and improve the performance of photoautotrophic, immobilized cultures**: Adapt and improve on the methods previously used to induce photoautotrophic cultures to produce H₂ in the absence of added acetate.

3. **Construct and test the performance of Chlamydomonas inducible transformants carrying a leaky ATP synthase ε-subunit gene**: Transformants will be tested for growth, photosynthetic activity, and H₂ production capability.
**Task 3 – Integrated Systems**

**Objectives, Approaches, and Collaborations**

**Objectives:** Integrate photobiological with fermentative organisms to more efficiently utilize the solar spectrum and the substrates/products from each reaction for \( \text{H}_2 \) production.

**Approaches:**
- Integrate sulfur-deprived, alginate-immobilized algal \( \text{H}_2 \) production to fermentative \( \text{H}_2 \) production by an anaerobic consortium isolated from wastewater sludge.
- Integrate fermentative \( \text{H}_2 \) production from potato waste to photosynthetic \( \text{H}_2 \) production by anaerobic, purple non-sulfur bacteria (RAS).

**Collaborator:** Institute of Basic Biological Problems, RAS
Task 3 – Integrated Systems
Accomplishments and Milestones

1. Complete small-scale experiments on fermentability of algal biomass feedstock by the anaerobic consortium: The consortium ferments algal biomass with a molar yield >4, which suggests that other cell components are being utilized.

2. Optimize fermentative H₂ production from potato waste.
Factors that increase rates/yields: exclusion of ammonium, addition of Fe ions, peptone and zinc; high phosphate buffering capacity; best yield: 1.6 mol H₂/mol glucose.

3. Demonstrate sequential H₂ production from integrated dark and light-driven processes.
Maximum demonstrated yield from sequential process using potato waste as feedstock is 5.6 mol H₂/mol glucose.

<table>
<thead>
<tr>
<th>Biomass</th>
<th>mol H₂/mol glucose (from starch)</th>
<th>mg glucose (from starch)/100 mg biomass dry wt</th>
<th>µmol H₂/mg biomass dry wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>142h-S (fresh)</td>
<td>1.86</td>
<td>8.7</td>
<td>0.60</td>
</tr>
<tr>
<td>142h-S (frozen)</td>
<td>2.11</td>
<td>3.5-8.7</td>
<td>0.64</td>
</tr>
<tr>
<td>+S (frozen)</td>
<td>6.30</td>
<td>1.9</td>
<td>0.52</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Feedstock</th>
<th>mol H₂/mol feedstock</th>
<th>µmol H₂/mg feedstock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid</td>
<td>0.09</td>
<td>0.20</td>
</tr>
<tr>
<td>Protein</td>
<td>6.56</td>
<td>0.10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Task</th>
<th>Due date</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Determine the fermentability of alginate films</td>
<td>March 2010</td>
<td>completed</td>
</tr>
<tr>
<td>Design and test connections between fermentors and photobioreactors</td>
<td>March 2010</td>
<td>completed</td>
</tr>
<tr>
<td>Report on the carbon mass balance and H₂ yields of a scaled-up fermentative system</td>
<td>September 2010</td>
<td>In progress</td>
</tr>
</tbody>
</table>
1. Scale up and further optimize fermentation of suspended and immobilized algal biomass by the fermentative consortium using new fermenters.

2. Optimize the integration of the fermentative/photobiological H₂-production system using potato waste as the feedstock.
Summary

Task 1:
• Extended the computational modeling techniques used to identify gas diffusion to the *Desulfovibrio gigas* [NiFe]-hydrogenase.
• Confirmed that the reduced state of the [FeFe]-hydrogenase is more tolerant to O$_2$ *in vitro* than the oxidized state.
• Identified positive Chlamydomonas transformants containing the Ca1 hydrogenase gene.
• Simulated fusions between the petF ferredoxin and algal/clostridial hydrogenases to test optimal interactions.

Task 2:
• Observed that increased thickness of the alginate film improves O$_2$ tolerance but decreases H$_2$-production rates.
• Designed ATP synthase inducible mutants.

Task 3:
• Demonstrated that an anaerobic clostridial consortium ferments algal biomass, pure algal lipids and pure proteins.
• Optimized fermentative H$_2$ production from potato waste.
• Demonstrated sequential H$_2$ production from dark- and light-driven processes.
Supplemental Slides
Responses to Previous Year’s Reviewer Comments

Reviewer’s comment: “The project milestones should be better defined; The near term milestones are inadequate for this project. The milestones should include some performance targets; there are no milestones for the high-volume screening process development; milestones should be added.”

Response: The new milestones (FY 2010) are now better defined, but it is much harder to set specific, quantitative performance targets for longer-term research projects, given their discovery nature. Actually, discoveries made in longer-term projects lead to redirection of projects. Regarding the high-volume screening, see next comment.

Reviewer’s comment: “The team has been developing the high-volume throughput screening tests since 2007. More details on their progress are needed; they have been working on developing high-volume throughput screening for several years and it is not clear what progress has been made. The challenges and progress should be better identified.”

Response: Due to budget uncertainties in the last two years, we elected to focus our work on the site-directed mutagenesis/expression approach. Moreover, the proposed new computational simulations may redirect the particular localized random mutagenesis approach that we chose to take.

Reviewer’s comment: “The team’s antenna and sulfur deprivation work seem very similar to what was done by Professor Melis at UC Berkeley.”

Response: The team collaborated with Professor Melis in the discovery of the sulfur deprivation approach and NREL subsequently optimized the process. We are now testing truncated antenna mutants generated at UC Berkeley in an attempt to integrate the different research areas in photobiology. Prof. Melis had not reported on the H₂-production properties of his truncated antenna mutants before.
Responses to Previous Year’s Reviewer Comments

Reviewer’s comment: “…more details about pretreatments regarding the integrated system are needed.”
Response: No pre-treatment was required. The algal biomass was previously frozen or fed directly to the fermentor. We will be addressing the effect (if any) of pre-treatment next.

Reviewer’s comment: “The PI should identify areas that the partners collaborated more clearly. It is difficult to determine what was done by partners and what was done by NREL.”
Response: The collaborator’s contribution was more clearly delineated in this presentation.

Reviewer’s comment: “The project team’s future plans are the same as in previous years. Since progress has been made, it would seem reasonable to adjust the plans.”
Response: Two new projects were added in FY2010: examine fusions between hydrogenase and ferredoxin, and study the effects of a “leaky” ATP synthase on H₂ production rates under sulfur deprivation. Readjustments in the direction of the molecular-engineering project have also been made to reflect results on the heterogeneity of redox states of hydrogenases.

Reviewer’s comment: “There is no indication, at this point, how this work could be scaled and no real understanding of how to increase the hydrogen production to a point that will be useful at scale.”
Response: The technoeconomic analysis performed by DTI has addressed some of those issues and did come up with a concept of a process for large-scale H₂ production.
Reviewer’s comment: “The PI has reported making an impressive number of presentations – 19. This is over one conference a month, which is a lot of travel. Their resources and time would be better utilized if they limited their conference attendance to only the premiere conferences.”

Response: The team consists of 3 PIs; the presentations made this year were divided among them as follows: 7 presentations by Seibert, 2 by King, and 6 by Ghirardi. The number of invitations reflects the high standing of the PIs in the research community.


Presentations

• Invited seminar at the CSIC Spanish National laboratory in Zaragoza, Spain, Apr 09 (Seibert).
• Presentation to the group of Dr. X. Zhang at MIT, Apr 2009 (King).
• Invited plenary talk at the Great Lakes Bioenergy Research Center (GLBRC) Hydrogenase Forum, May 09 (Seibert).
• Presentation at the American Society for Plant Biology meeting in Hawaii, Jul 09 (Ghirardi).
• Presentation of the USA country report at the IEA Annex 21 Biohydrogen Experts Meeting in Jyväskylä, Finland, Sep 09 (Seibert).
• Invited presentation at the University of Washington, St. Louis, Sep 09 (Ghirardi).
• Invited presentation at the Rocky Mountain American Vacuum Society meeting, Denver, Sept 09 (Ghirardi).
• Invited presentation at the Center for Revolutionary Solar Photoconversion meeting in Denver, Oct 09 (Ghirardi).
• Invited presentation at the Fall Rocky Mountain Branch of the American Society for Microbiology in Denver, Nov 09 (Ghirardi).
• Oral presentation to the Solar Fuels 2009 Meeting, Sigtuna, Sweden, Oct 2009 (King).
• Invited presentation to the Microbiology Department, Colorado State University, Nov. 09 (Seibert).
• Presentation at NREL's Energy Bioscience Center monthly seminar, Jan 10 (Ghirardi).
• Presented USA country report at the IEA Annex 21 Biohydrogen Experts Meeting in Florence, Italy, March, 2010 (Seibert).
Other Activities


Ghirardi reviewed proposals for EPSCoR, University of Padua, National Science Foundation, the Joint Genome Institute, ARPA, USDA, and DOE.

Ghirardi was nominated a Fellow of the Renewable and Sustainable Energy Institute (RASEI).

Ghirardi hosted Dr. Alex Bradel, Chris Yeager (SRNL), Patrice Hamel (OSU), members of the European Commission, Dr. Glaucia Souza (Brazil’s FAPESP).

Ghirardi serves as an advisor for the University of Tennessee’s, NSF-funded STAIR (Sustainable Technology through Advanced Interdisciplinary Research) Program.

Seibert was elected the new Operating Agent for the IEA/HIA Task 21 (Biohydrogen).
Assumptions:

Molecular engineering of \( \text{O}_2 \) accessibility to the [FeFe]- and [NiFe]-hydrogenases' catalytic sites will improve \( \text{O}_2 \) tolerance. This assumption was based on published reports demonstrating that significant changes in the \( \text{O}_2 \) sensitivities of other FeS proteins (ferredoxin and the \( \text{H}_2 \)-sensing NiFe hydrogenase) were achieved solely by changing the accessibility of the FeS centers or catalytic sites to \( \text{O}_2 \) by substitution of critical amino-acid residues.

Since the clostridial hydrogenase is already more tolerant to \( \text{O}_2 \) than the algal enzyme, we expect to see increased activity under aerobic conditions when we express it in Chlamydomonas. Successful expression will also allow us to start addressing other barriers (such as competition for reductant) in a more effective manner.

Immobilizing dense algal cultures in very inexpensive matrices will drive the light-conversion efficiency up, while contributing very little to (and perhaps lowering) the cost of the \( \text{H}_2 \) produced by the system.

Integrating photobiological with fermentative systems will contribute beneficially to the overall cost of biological \( \text{H}_2 \) production if parameters such as biomass disposal are taken into consideration. This will be considered in a new technoeconomic analysis to be performed at the end of FY10.
Issues:
Inconsistent funding and the lack of understanding regarding the nature of the different O$_2$-tolerant states of the hydrogenase has prevented us from further developing the site-directed mutagenesis approach. The expression of a more O$_2$-tolerant clostridial hydrogenase in Chlamydomonas will give us a better handle on which enzyme characteristics are important for hydrogenase activity under O$_2$-evolving conditions, thus allowing us to redesign our strategy and screening assays.

Preliminary evidence gained by comparing the structure of different hydrogenases suggests that factors beyond gas diffusion may contribute significantly to O$_2$ sensitivity. We will further examine this through computational simulations, which may lead to a change in our mutagenesis strategy.