Biological Systems for Hydrogen Photoproduction

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NREL
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Team members:
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Sergey Kosourov, Christine
English, Sharon Smolinski

Project ID# PDP 26

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Overview

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

Barriers
- Barriers addressed
  - Production Barrier: Continuity of H₂ production.

Budget
- Funding received in FY08: $2,000K
- Funding allocated for FY09: $0

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Current Status</th>
<th>2013 Target</th>
<th>Maximum Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of continuous photoproduction</td>
<td>90 days (-S, anaerobic)</td>
<td>10 min (aerobic)</td>
<td>12 hours (aerobic)</td>
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<td></td>
<td>6 days (-S, aerobic, immobilized)</td>
<td></td>
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<tr>
<td>O₂ tolerance (half-life in air)</td>
<td>~ 4 min after exposure to air (clostridial enzyme)</td>
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</table>

Partners
- Dr. Anatoly Tsygankov, Russian Academy of Sciences, Pushchino, Russia.
- Dr. Michael Flickinger, North Carolina State University.
- Dr. Klaus Schulten, The Beckman Institute, University of Illinois.
- Dr. Mace Golden, GoldenBioenergy, CO
Objectives

Develop and optimize aerobic photobiological systems for the production of H₂ from water (subtask 1); utilize the sulfur-deprivation platform to address biochemical and engineering issues related to photobiological H₂ production (subtask 2); integrate photobiological with fermentative organisms to more efficiently utilize the solar spectrum and the substrates/products from each reaction (subtask 3).

FY2007/08:
Subtask 1: Engineer a H₂-producing catalyst ([FeFe]-hydrogenase) that has an extended half-life, following exposure to O₂.
Subtask 2: Demonstrate improvements in the light conversion properties of a H₂-producing anaerobic algal system by immobilizing the cultures at high cell density on a thin-film matrix;
Subtask 3: Test the ability of H₂-producing, fermentative organisms to consume algal biomass; optimize photosynthetic bacterial H₂ production.
## Milestones

<table>
<thead>
<tr>
<th>Milestone</th>
<th>Description</th>
<th>Completion Date</th>
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<tbody>
<tr>
<td>3.3.1.</td>
<td>Participate in DOE’s annual program review</td>
<td>06/08</td>
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<tr>
<td>3.3.2</td>
<td>Generate and test 5 new mutants for O₂ tolerance</td>
<td>01/08</td>
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<td>3.3.3</td>
<td>Determine the light conversion efficiency of stacked photobioreactors vs. single photobioreactors</td>
<td>03/08</td>
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<td>3.3.4</td>
<td>Test the performance of immobilized ATPase mutants</td>
<td>06/08</td>
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<tr>
<td>3.3.5</td>
<td>Test the performance of immobilized antenna mutant</td>
<td>10/08</td>
</tr>
<tr>
<td>3.3.6</td>
<td>Introduce and test the expression of a clostridial hydrogenase gene in <em>Chlamydomonas</em></td>
<td>08/08</td>
</tr>
<tr>
<td>3.3.7</td>
<td>Design and test a new DNA construct for E. coli expression of hydrogenases</td>
<td>12/08</td>
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</table>
Subtask 1:
1. Apply site-directed mutagenesis to a bacterial hydrogenase; express mutant enzymes in *E. coli* and test mutants for increased tolerance to O$_2$;
2. Initiate random mutagenesis/higher-throughput approaches for generation of hydrogenases with increased tolerance to O$_2$; express mutant enzymes in *E. coli* and test mutants for increased tolerance to O$_2$;
3. Develop DNA plasmids to express heterologous wild-type or mutated hydrogenases in *Chlamydomonas reinhardtii* in order to test O$_2$-tolerance in the alga.

Subtask 2:
1. Determine the rates and light conversion efficiency of H$_2$ production by *C. reinhardtii* cultures immobilized in alginate films;
2. Test the performance of truncated antenna and proton channel mutants under the same conditions;

Subtask 3: the continuation of this subtask will be determined by the results of a techno-economic analysis to be performed in FY08-09
1. Test the advantages of using single or stacked reactors of green algae and photosynthetic bacteria for increased H$_2$ production per illuminated area;
2. Determine whether a H$_2$-producing, fermentative consortium can utilize spent algal and photosynthetic bacteria biomass;
3. Find a consortium of thermophilic organisms that is able to ferment organic wastes, produce organic acids to support photosynthetic bacteria growth, and produce H$_2$ at high efficiency.
Subtask 1. Site-directed mutagenesis (C. English P. King, and M. Seibert)

Two pathways for O$_2$ diffusion from the surface of the hydrogenase protein to its catalytic site were previously identified by computational simulations (molecular dynamics and solvent accessibility). Single and double mutants along the two pathways were generated to sterically hinder O$_2$ diffusion to the catalytic site. All mutants tested showed loss of activity and increased sensitivity to O$_2$.

Computational simulations of the mean potential energy required to move a molecule of O$_2$ from the surface of the protein (right side of the graph) to the catalytic cluster (left side of the graph) suggested the presence of a high energy barrier a few Å from the cluster, at the junction of the two gas pathways (see red region in the top figure). We attempted to engineer hydrogenases in which this barrier was increased by substitution with larger residues.
Amino acid residues line a cavity that is likely to form the high-energy barrier described above in WT strains (see graph). One of the residues was mutated to a much larger residue. The mutated enzyme was expressed in E. coli, and its activity was measured in membrane-free cell extracts. For this work, we prepared enzyme without the addition of chemical reductant. Both wild-type and mutant disclosed more than one level of O_2 tolerance, characteristic of a distribution of redox states of the enzyme. These distribution of states differ for the mutant compared to wild-type, affecting the measured rate of O_2 inactivation. It is not immediately clear how this new discovery could impact the HFC&IT project.

These new results underscore the complexity of the hydrogenase system with respect to factors that control O_2 tolerance (redox state of the enzyme, O_2 diffusion, nature of the ligands to the catalytic center), and point out the need for further research to deconvolute the influence of these factors on the measurement of O_2 tolerance of the enzyme.
Subtask 1. Random mutagenesis/higher throughput assay (P. King, M. Golden)

Rationale: to improve the probability of finding O$_2$-tolerant mutants

Mutagenesis
Error-prone PCR amplification of a partial gene sequence: introduces random errors

Expression in E. coli

Screening
An automated gas chromatograph assay will be developed to screen for O$_2$-tolerant mutants

The hydDA gene (encoding for the structural hydrogenase protein from C. acetobutylicum) and the gene encoding for one of its assembly proteins, hydE, will be incorporated into the multiple cloning site (MCS) of the pETDuet-1 plasmid (left), and the hydF and hydG genes, encoding for the other two assembly proteins will be incorporated into the MCS of the CDFDuet-1 plasmid (right); the hya E. coli promoter will be used instead of the T7 promoter to allow anaerobic induction of all heterologous genes.
Technical Accomplishments

Subtask 1. Expression of clostridial hydrogenase in C. reinhardtii (P. King, C. English)

Rationale: the clostridial [FeFe]-hydrogenase is highly homologous to the algal hydrogenase, and it is ~ 400X more tolerant to O₂

Protocol

Current status: the DNA construct is ready; we are now attempting to clone in ble resistance gene.
Technical Accomplishments


Cell suspensions
Immobilized onto glass fibers
Immobilized into alginate films

- Low cell-density, short duration
- Higher cell density, longer duration
- Non-degradable matrix
- High cell density
  Long duration
  Degradable matrix

Graphs:
- Hydrogen production over time
- Rate of H₂ production over days
- Hydrogen production vs. time
- pH 7.3 vs. pH 7.7

Images:
- Cell suspensions
- Immobilized onto glass fibers
- Immobilized into alginate films

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Subtask 2. continued

<table>
<thead>
<tr>
<th></th>
<th>Light Intensity (μE•m⁻²•s⁻¹)</th>
<th>Maximum specific rate of H₂ production (μmoles•mg Chl⁻¹•h⁻¹)</th>
<th>Average specific rate of H₂ production (μmoles•mg Chl⁻¹•h⁻¹)</th>
<th>Maximum light conversion efficiency into H₂ (%)</th>
<th>Average light conversion efficiency into H₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspension</td>
<td>2x 150 (two illuminated surfaces)</td>
<td>9.44</td>
<td>3.79</td>
<td>0.53</td>
<td>0.21</td>
</tr>
<tr>
<td>Glass fibers</td>
<td>120</td>
<td>6.4</td>
<td>2.37</td>
<td>0.36</td>
<td>n.a.</td>
</tr>
<tr>
<td>Alginate films</td>
<td>29</td>
<td>4.71</td>
<td>2.43</td>
<td>2.17</td>
<td>1.28</td>
</tr>
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<thead>
<tr>
<th>Cell biomass in formulation, g</th>
<th>Average total Chl concentration, μg per 6 cm² screen</th>
<th>The total yield of H₂ in an atmosphere of argon, mol m⁻² (A)</th>
<th>The total yield of H₂ in an atmosphere of air, mol m⁻² (B)</th>
<th>Hydrogen production in (B) as a % of (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.125</td>
<td>43 +/- 3</td>
<td>0.06 +/- 0.01</td>
<td>0.04 +/- 0.01</td>
<td>67</td>
</tr>
<tr>
<td>0.25</td>
<td>90 +/- 12</td>
<td>0.14 +/- 0.05</td>
<td>0.08 +/- 0.02</td>
<td>57</td>
</tr>
<tr>
<td>0.5</td>
<td>188 +/- 20</td>
<td>0.26 +/- 0.06</td>
<td>0.14 +/- 0.02</td>
<td>54</td>
</tr>
<tr>
<td>1</td>
<td>278 +/- 44</td>
<td>0.30 +/- 0.02</td>
<td>0.14 +/- 0.02</td>
<td>47</td>
</tr>
<tr>
<td>2</td>
<td>402 +/- 38</td>
<td>0.27 +/- 0.03</td>
<td>0.02 +/- 0.01</td>
<td>7</td>
</tr>
</tbody>
</table>

Substantially increased light conversion efficiencies

Tolerance to O₂ depends on the amount of immobilized biomass

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Rationale: Green algae absorb visible light (400-700 nm); photosynthetic bacteria have absorption peaks in the infra-red (750-900 nm); by stacking two photobioreactors one is able to extend the portion of the absorbed spectrum and thus increase light conversion efficiency to H₂.

Increased rates of H₂ photoproduction were observed when the stacked cultures were illuminated with a combination of fluorescent (below 700 nm) and incandescent (including infra-red) lamps.
Technical Accomplishments

Subtask 3. Fermentative H₂-production from algal biomass (S. Smolinski, P.C. Maness, S. Kosourov)

The amount of starch present in algal biomass harvested at the end of sulfur-deprivation experiments was determined, and different amounts of biomass were added to a fermentor. Fermentative H₂ production was demonstrated and the total amount of H₂ produced was a function of the amount of biomass added.

The high molar yield of H₂/glucose observed (see last column of the table) was probably due to the capability of the fermentative organism to degrade other biomass components, such as proteins and/or lipids.
Subtask 3. H₂-production by photosynthetic bacteria (A. Tsygankov, M. Seibert)

- Samples were taken from a variety of sites in Russia, and the ability of fermentative consortia from each sample to simultaneously ferment various starch-containing wastes and produce H₂ was tested (stage 1, below). Conditions were optimized for H₂ production at 37°C;
- The inhibitory effects of organic acids on photosynthetic bacterial growth and H₂ production is being studied in order to better define the type of photosynthetic bacteria that will be used in stage 2 (below);
- A quick procedure for immobilization of photosynthetic bacteria on glass fibers for stage 2 was devised.

Stage 1: Starch-containing wastes → Organic acids → H₂

Stage 2: H₂ + CO₂ → Purified water
Summary

- **Subtask 1:** (1) We discovered the existence of different states of the clostridial enzyme with differences in O\textsubscript{2} sensitivity and whose distribution varies between wild-type and mutants; a procedure for random mutagenesis/high throughput screening is being developed; DNA constructs for expression of clostridial hydrogenases in *C. reinhardtii* are being made.

- **Subtask 2:** (1) High light conversion to H\textsubscript{2} efficiencies have been demonstrated when sulfur-deprived algae are immobilized into alginate films; (2) the film was shown to protect the cultures from inactivation by atmospheric O\textsubscript{2} and partial rates of H\textsubscript{2}-production were measured even under aerobic conditions.

- **Subtask 3:** (1) Increased rates of H\textsubscript{2} photoproduction were observed when stacked cultures of green algae and photosynthetic bacteria were illuminated with a combination of fluorescent (below 700 nm) and incandescent (including infra-red) lamps; (2) fermentative H\textsubscript{2}-production was detected when algal biomass was used as the substrate; (3) high yields of H\textsubscript{2}/glucose suggest that other cell components (besides glucose) are also being fermented.
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