Renewable Bio-solar Hydrogen Production from Robust Oxygenic Phototrophs
AFOSR MURI Progress Update: January 2007

-BioSolar\(\text{H}_2\) Team

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http://www.princeton.edu/~biosolar/
The Approach:
Algal & Cyanobacteria cell factories that produce chemical energy w/o sacrificing the microbe

Sunlight
\( \text{CO}_2 \text{H}_2\text{O} \)
Nutrients
\[ \text{Cell Factories} \]

Chemical energy
\( \text{H}_2, \text{alcohols, acids, hydrocarbons} \)

Native & GMOs that produce more energy
The Goal

\[ 2 \text{H}_2\text{O} \rightarrow \text{O}_2 + 2 \text{H}_2 (\text{H}^+ / \text{e}^+) \]
Instrumentation Development

Rapid H₂ & Fluorescence Screening
CSM/NREL H₂ gas sensor

Microarrayed chemostats for directed evolution

Fast repetition rate fluorimeter

Dissolved H₂ & O₂
LED + Clark cells

Metabolomics & Proteomics

Two LC-MS Engines
Active February 2007

Mass Spectrometry
Sampling of Culture Collections in Progress

**Marine Microbial Ecology and Diversity Center, University of Hawaii**

- **Patterson Collection.**
  - Largest collection of cyanobacteria in the world
  - Approx. 1800 viable strains
  - Difficult to assemble a library of this scope due to sampling rights.

- **NREL Collection.**
  - 180 strains collected from the US southwest for NREL biodiesel program.

- **Mitsui collection.**
  - 165 strains marine cyanobacteria.

- Actively expanding collection

- Bioprospecting
  - Great Salt Lake & Yellowstone NP
The time profiles reveal the kinetics of induction of photo-H$_2$ production capacity that occurs following initiation of anaerobiosis at time zero of cultures grown on light photosynthetically. Six strains of *Chlamydomonas*...
Micronutrients, growth & bioreactor optimization has produced large improvements in H₂ production

1. Sufficiency of Nickel during growth. **17X H₂ increase**
2. Duration of prior photoautotrophic growth. **oldies but goodies**
3. Anaerobicity & darkness during fermentation. **strong respiring strains**
4. Selection of salt-tolerant strain. **synthesis of fermentable sugars**
5. Mechanical agitation used for photoautotrophic growth. **minimize shearing**
6. Higher fermentation temperature. **2x (23 → 30 C)**
7. Lower light intensity 3x after attaining steady-state. **Fermentation adaptation**
A. $[\text{Ni}^{2+}]$ causes chlorosis during initial stage of photoautotrophic growth (130µE/m²sec).

B. After chlorosis induced lag, cells recover & grow photoautotrophically to normal cell density. NO Effect.

C. $\text{Ni}^{2+}$ stimulates cell’s capacity to evolve hydrogen by 20 fold!
2006 rates & yields of $H_2$ by cyanobacteria & algal strains

Future target
Cell free $H_2$ase
28 ml $H_2$ L$^{-1}$h$^{-1}$

$H_2$ build-up, 30 C

$H_2$ vented, 23 C

2006 Arthrospira Maxima $H_2$ yield in the dark
250 ml $H_2$ L$^{-1}$ in 4 days batch culture, 1 growth cycle.

2006 NREL immobilized algae*
0.35%

2004 NREL batch algae*
0.15%

photo-$H_2$ by $C.\ reinhardtii$ under Sulfur depletion

without Nickel

% Efficiency Solar-to-$H_2$
Osmotic Stress by Dilution of Growth Medium Boosts H₂ Yield by 18 X!

_Arthospira maxima_ acclimated to NaCl

<table>
<thead>
<tr>
<th>Carbonate medium</th>
<th>250 mM</th>
<th>250 mM</th>
<th>500 mM</th>
<th>750 mM</th>
<th>NaCl</th>
</tr>
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Dilution of salt

- 0.75 M to 85 mM
- 1 M to 85 mM
- 1 M
- 0.75 M
Arthrospira exhibits large increases in fermentative H₂ production by application of stresses or by carbon supplementation:

1. Osmotic stress of salt-tolerant strains. **18X increase**
2. Nitrate removal during fermentation (if sole N source). > **5-20X**
3. Add fermentable carbon substrates. > **10X with glucose**

These results with the Princeton microbioreactor indicate promising Applications for larger scale fermentation trials in progress.
Genetic Opportunities Ahead

- Develop a genetic system for *Arthrospira* sp.
- Determine the optimal conditions for H$_2$ase expression in cyanobacteria
- Over-express H$_2$ase(s) in cyanobacteria
- Introduce foreign H$_2$ase(s) into cyanobacteria
- [NiFe]-hydrogenase engineering of NAD vs NADP selectivity.
- [NiFe]-hydrogenase gene shuffling
- Engineer cyanobacterial strains with reduced amounts of light-harvesting antenna
- Use comparative genomics, molecular genetics, and strain engineering to optimize H$_2$ production
Obtain genome sequences from robust, hydrogen-producing oxyphototrophs.

Arthrospira maxima

Develop genetics in this excellent H₂-producer; genome will be sequenced in 2007 by JGI-DOE.

Synechococcus sp. PCC 7002

Apply "-omics" Transcriptomics Proteomics Metabolomics

Metabolic Engineering

hoxE hoxF hoxU hoxY hoxH

hypE hyp3 hoxW hypA hypB hypF hypC hypD

In *Synechococcus* sp. PCC 7002, the genes encoding the Ni-Fe hydrogenase uniquely form an operon of 13 genes.
Improving H₂ production with proven genetics tools

Full genome available for Synechococcus sp. PCC 7002

- **Identify, and then delete or inactivate, genes for pathways that compete for electrons with H₂ase; enhance glycogen storage**

- **Over-express genes for modified hydrogenases**

- **Insert hydrogenase structural genes in pAQ1**

- **Insert hydrogenase accessory genes in pAQ3**

- **Synechococcus sp. PCC 7002 strains for enhanced H₂ production**

- **Actual copy numbers for chromosome and plasmids in Synechococcus sp. PCC 7002 cells in mid-exponential and early stationary phase**
3 Fermentative Gene Knockout Targets based on Genome Sequences

glycogen → G6P

2 NAD⁺ + 2 ADP, Pᵢ
glycolysis

2 NADH + 2 ATP

pyruvate

D-lactate dehydrogenase

CH₃CH₂OH
Ethanol

H₂ ase

H₂

Acetate

Acetyl phosphate

Acetyl-CoA synthetase

Acetate kinase

CH₃COO⁻

Acetyl-CoA + CO₂

Acetaldehyde

Aldehyde dehydrogenase

Alcohol dehydrogenase

Acetyl-CoA

Phosphotransacetylase

Acetyl-CoA synthetase

Synechocystis
6803 only

Synechococcus
6803 & 7002

Kelsey McNeely & Bryant Lab
In vitro Hydrogenase activation: a robust platform to investigate hydrogenase maturation

The hydrogenase “H cluster”

- In vitro activation of heterologously expressed hydrogenase structural protein HydA (HydAΔEFG) by the addition of co-expressed accessory proteins HydE, HydF and HydG.

- A robust system amenable to in depth study and characterization of hydrogenase maturation.

In vitro activation is the first step in the characterization of the biochemical reactions involved in “H Cluster” biosynthesis and hydrogenase maturation. These studies are critical to the effective genetic engineering of organisms expressing [FeFe] hydrogenase.
Analysis of C. reinhardtii transcriptome under H₂ producing conditions

- Levels of over 500 transcripts change significantly.
- Several hundred are of unknown function.
- Novel targets potentially influencing hydrogenase activity have been identified.

Analysis of the transcriptome during H₂ production is essential to understand hydrogenase activity in the context of whole-cell metabolism. Pathways of electron transfer are being analyzed and targets to enhance H₂ production identified.
Application of gene-shuffling for the rapid generation of novel [FeFe]-hydrogenases

- Gene shuffling protocol was identified, optimized and used to rapidly generate libraries of unique [FeFe]-hydrogenases
- Generates a high percentage of active enzymes in *E. coli*
- User friendly and requires a single set of maturases