Producing Fuels the Old-Fashioned Way: Using Biology

Direct Biological Conversion of Sunlight to Hydrogen

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Energy History: Planet Earth

Solar Energy

Microorganisms, Plants

H₂O CO₂

Biological Ecosystems

O₂ Biomass

CO₂

Consumption

Energy (Fossil fuels)
Solar Energy

Sustainable Energy Future: Planet Earth

Engineered Photosynthetic Microorganisms

Energy (H₂ Economy)

Fuel Cells, Power Plants, Automobiles

H₂O → H₂O

O₂ → O₂

Our Overall Proposal
Can Solar Energy Help Provide The Answer?
The solar resources for generating power from concentrating solar power systems is plentiful. For instance, enough electric power for the entire country could be generated by covering about 9 percent of Nevada – a plot of land 100 miles on a side* – with parabolic trough systems.

* Equivalent to 1.7% of Current Cropland

http://www.energylan.sandia.gov/sunlab/overview.htm#solres
How Can We Harness Biology?

-- AGAIN
The Direct Conversion Concept

Incident Sunlight

Large Surface Area Collector/Reactor

Engineered Organisms

Rejuvenate Culture

Transparent Cover

Transparent Gas Permeable Membrane

Low Pressure H₂ & O₂ Harvest

Vacuum

Organism Suspension

Cooling Fluid

2H₂O \rightarrow O₂ + 2H₂
Direct PhotoBiological Hydrogen Production: Building a New Electron Pathway

**Aerobic Process**

2 H₂O → O₂ + 4 H⁺

Sunlight → Reduced Ferredoxin → Growth

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**New Pathway**

**Anaerobic Process**

**Clostridium pasteurianum** Catabolism

Glucose → Pyruvate → Reduced Ferredoxin → Hydrogenase → 4 H⁺ → 2H₂
Direct PhotoBiological Hydrogen Production: Building a New Electron Pathway

Solar Energy

Photolysis Center

H₂O → O₂ → H₂

H⁺ e⁻ Reduced Ferredoxin → Oxidized Ferredoxin → Hydrogenase (Clostridial)

Goal: Engineered *Synechocystis* Bacterium
Can Reduced Ferredoxin Drive Significant Hydrogen Accumulation?
Equilibrium is Determined by Gibbs Free Energy of Reaction

\[ aA + bB \rightarrow cC + dD \]

\[ \Delta G = \Delta G^\circ + RT \left( \frac{[C]^c[D]^d}{[A]^a[B]^b} \right) \]

For Hydrogen Production:

\[ 2 \ H^+ + 2 \ Fd^{\text{red}} \rightarrow H_2 + 2 \ Fd^{\text{ox}} \]

\[ \Delta G = \Delta G^\circ + RT \left( \frac{[H_2][Fd^{\text{ox}}]^2}{[Fd^{\text{red}}]^2} \right) \]

(at pH = 7)
20\(\mu\)M Reduced Methyl Viologen as an Electron Source*

pH 6.9 Reactions in a **5% Hydrogen** Environment

\[
2 \text{MV}^{\text{red}} + 2 \text{H}^+ \rightarrow \text{H}_2 + 2 \text{MV}^{\text{ox}}
\]

**Reverse Reaction**

\[
\approx 20\mu\text{M MV}^{\text{red}}
\]

**Reverse Control**

*Methyl Viologen Redox Potential = - 440 mV
*Synechocystis ferredoxin Redox Potential = - 412 mV
How Does Photosynthesis Work?
Redox Biochemistry Associated with Light Capture and Conversion

Glycolysis
Tricarboxylic acid cycle

Need to Control Relative Electron Flux Rates

Note: This is the Diagram for Chlamydomonas*, Synechocystis is similar

Phycocyanin

Light Harvesting Structures in Synechocystis

Cytoplasm
Cell Wall
DNA
Thylakoids

Phycobilisome

Phycocyanin
Phycoerythrin and phycocyanin are phycobilins associated with phycobiliproteins in phycobilisomes. They fill the gap in the absorption spectra of the other pigments.
Will This Work Economically?
Rough Economic Analysis:

(Major Cost is Related to the Capital Required for the Collector/Reactor)

1. Estimate that 50% of the Light Energy is Usable Biologically
   This Calculates to be about 71 moles of photons/m²-day
   We should get about 1 mole of H₂ per 8 moles of photons
   A 1000 Acre Energy Farm Will Yield About $220 Million per Year

3. Assume:
   20% Profit per Year ($44 million)
   $5 million/yr Labor Costs
   $5 million/yr Capital Costs for Processing Plant
   $10 million/yr Maintenance Costs
   $5 million/yr Raw Material Costs

4. This Leaves $150 Million/Year To Service the Collector/Reactor Debt
   At 7% Interest, Can Invest $2.16 Billion
   or:
   Can Spend About $50/ft² For the Collector/Reactor

Given Economies of Scale, This Seems Reasonable
Now, What is the BIG Problem?

and

How Do We Solve It?
Direct PhotoBiological Hydrogen Production: Building a New Electron Pathway

Goal: Engineered *Synechocystis* Bacterium
Comparison of Different Fe-S Hydrogenases

**Clostridium pasteurianum**

**Desulfovibrio desulfuricans**

**Scenedesmus obliquus**

**Chlamydomonas reinhardtii**
The 3-D Structure is Known for the Hydrogenase from *Clostridium pasteurianum*

\[ 2H^+ + 2Fd^{\text{red.}} \rightleftharpoons H_2 + 2Fd^{\text{oxid.}} \]
The Active Site is Most Complex
(and probably most oxygen sensitive)
It is Buried in the Interior of the Enzyme

Clostridium pasteurianum
CP1 Hydrogenase
We Will Use Rapid Directed Evolution to Produce Oxygen Tolerant Hydrogenases

Create Collection of Hydrogenase Genes with Genetic Diversity

Mutate Genes of Oxygen Tolerant Hydrogenases

Identify Candidates With Increased Oxygen Tolerance

Express Genes in Cell-Free System

Evaluate In Synechocystis

\[ 2H^+ + 2Fd^{\text{red.}} \rightleftharpoons H_2 + 2Fd^{\text{oxid.}} \]
Cell-Free Protein Synthesis (CFPS) – Can Easily Conduct Multiple Parallel Reactions

Combined Transcription/Translation: *E. coli*

1. Grow and Lyse *E. coli*
2. Prepare Extract
3. Add Substrates, Salts, and Folding Aids
4. Add Template
5. Incubate

Provides Direct Access and Control and Rapid Analysis
Using Cell-Free Synthesis to Evolve $\text{O}_2$ Tolerant Hydrogenases

1. Add a few DNA Molecules To Each Well of Plate and Amplify as Templates

1. Add cell extract and reagents for Cell-Free Synthesis, Express Hydrogenases

3. Assess Hydrogenase Activity (Begin with no $\text{O}_2$ Exposure, Increase $\text{O}_2$ for each screening cycle)

$$2\text{H}^+ + 2\text{Fd}^{\text{red.}} \rightleftharpoons \text{H}_2 + 2\text{Fd}^{\text{oxid.}}$$
We Began with Ferredoxin. It also Needs an Fe-S Center

Cell-Free Produced Ferredoxins are Fully Active

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Cell-Free
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Synechocystis Ferredoxin

Marcus Boyer
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![Activity of Ferredoxins on per Iron basis](image)

- In-Vivo
- KC1
- pRKISC

Fd Sample

- Cell-Free
- Cell-Free
Discovery of Two Novel Radical S-Adenosylmethionine Proteins Required for the Assembly of an Active [Fe] Hydrogenase*

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FIG. 5. Hydrogen production rates from purified HydA1 heterologously expressed in E. coli either alone or co-expressed with the indicated Hyd proteins.

Hydrogen production was measured using the methyl viologen-based assay. The data shown represent the average of four independent experiments; average deviations from the mean are shown.

*Genes Taken from Chlamydomonas reinhardtii
1. Built HydA and Cpl genes from synthetic oligonucleotides to avoid codons rarely used by *E.coli*.

4. In our hands *Chlamydomonas* helpers had very low activity (*in vivo & in vitro*).

6. Cloned helper protein genes from *Shewanella oneidensis* for expression in *E.coli* cell extract source cells.

4. Detected strong Hydrogenase activity with *E.coli* *in vivo* expression.

13. Produced cell extracts Anaerobically with specially equipped glove box.

15. Detected significant Hydrogenase activity from cell-free reactions.

17. Optimized Cell-Free reactions to produce 400x greater specific activity than in Posewitz paper.
Optimizing Extract Preparation and Cell-Free Reactions

Interestingly, Volumetric Hydrogenase Activity increased even though soluble hydrogenase polypeptide production decreased.

We Now Have Sufficient Activity To Begin Evolving For Oxygen Tolerance
Substrate Supply and Lower Temperatures Increase H₂ase Activity

- CpI Semi-continuous 30°C
- CpI batch 30°C
- sHydA1 batch 30°C
- CAT batch 30°C
- CPI batch 37°C
- sHydA1 batch 37°C
- CAT batch 37°C

Absorbance (578 nm)

Time (sec)

Feeding solution
Extract reaction mixture
Magnetic stir bars
Dialysis membrane
23°C Appears to be the Optimal Temperature for the Cell-Free Production of Active Hydrogenase

![Bar chart showing activity (µmol H₂ consumed per min mL cell-free reaction) at different temperatures and reaction times.

- Cpl
- sHydA1

Temperature: 37 °C, 30 °C, 23 °C, 18 °C

Time: 3 hours, 6 hours, 22 hours, 22 hours

At 23°C, the activity is significantly higher compared to other temperatures, indicating it is the optimal temperature for cell-free hydrogenase production.
We confirmed that the Cell-free produced Hydrogenases Produce Hydrogen

(Data obtained with the Cpi Hydrogenase)

1. 100 µl of cell-free rxn product added to 1 ml of 50µM reduced ferredoxin
2. 30 µl of cell-free rxn product added to 1 ml of 2mM reduced methyl viologen
3. 150 µl of cell-free rxn product added to 1 ml of 50µM reduced ferredoxin
We are Now Ready to Begin Hydrogenase Evolution!!

Generate Diversity

- PCR
  - Error-Prone PCR, Family Shuffling, Rational Design

Isolate Mutants by dilution

Amplify mutants

- PCR

An aerobic Chamber, 5% H₂

- Cell-Free Protein Synthesis
  - Gene Library → Protein Library

- Assess Remaining H₂ase Activity

“Airlock”

- Expose to Oxygen
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