BioHydrogen Generation

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Stanford University
Can this be One Component of our Sustainable Energy Future?

Solar Energy

Engineered Photosynthetic Microorganisms

A Clean Sustainable Cycle

H₂O

O₂

H₂

Fertilizer, Cement, Chemicals, Fuel Enrichment, Electricity, Transportation

STANFORD ENGINEERING

GCEP

DOE
What is the Potential for Solar Biohydrogen?

Current U.S. Consumption Of Liquid Fuels plus Nat. Gas
\[ \approx 60 \times 10^{15} \text{ Btu / yr} \]

Assume: Solar Incidence of 7 kWh/m\(^2\)-day

and 15\% Energy Efficiency (Overall)

Need 17,000 Square Miles

= 2.5\% of Current Cropland

(But Do Not Need to Convert Current Crop Land)
Lacking Effective Storage and Transport, What are the Initial Markets?

Current World Market for Hydrogen is about 50 million metric tons (about $135 Billion/yr)

In US, we use about 9 million metric tons/yr (95% from CH$_4$) Releasing about 60 million metric tons of CO$_2$ per year Equivalent to 34 million cars (DOE) Most is used for fertilizer and refineries

<table>
<thead>
<tr>
<th>Current Markets:</th>
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<tbody>
<tr>
<td>1. NH$_3$ Fertilizer – Uses about half of the hydrogen</td>
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<tr>
<td>2. Refineries – Increase fuel quality</td>
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<td>3. Chemical Synthesis</td>
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<table>
<thead>
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<th>Potential Markets:</th>
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<tbody>
<tr>
<td>1. Portland cement – about 100 million tons/yr in US $(\approx 10\times10^6$ tons CO$_2$/yr)</td>
</tr>
<tr>
<td>2. Short term solar energy storage for electricity</td>
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</table>
Direct BioConversion of Sunlight to H₂

- Incident Sunlight
- Large Surface Area Collector/Reactor
- Engineered Organisms
- Rejuvenate Culture
- Low Partial Pressure H₂ & O₂ Harvest
- Temp. Control Fluid In
- Temp. Control Fluid Out
- Transparent Cover
- Transparent Gas Permeable Membrane
- Cooling Fluid
- Organism Suspension
- Low Pressure*

Estimated Economics Allow About $20-30/ft²

One Candidate Collector/Reactor Cross Section

*or Purge Gas (N₂)
Direct Photobiological Hydrogen Production: Building New Electron and Proton Pathways

Goal: Engineered *Synechocystis* Bacterium
BUT – Oxygen is a Side Product and it Inactivates the Hydrogenase We Need

Initial and Primary Challenge:
EVOLVE AN OXYGEN-TOLERANT HYDROGENASE
[Fe-Fe] Hydrogenases are the Fastest H₂ Producers – But They Are VERY Complex

Hydrogenase CpI from *Clostridium pasteurianum*¹

\[ 2H^+ + 2\text{Fd}^{\text{red}} \leftrightarrow H_2 + 2\text{Fd}^{\text{oxid}} \]

Active Iron-Sulfur Site

Electron transfer metal centers

Active Site “H-Cluster” Has 6 Irons, 6 Sulfurs, 3 CO, 2 CN and a Bridging Dithiol

A Structural Dynamic Model Suggests Two Dominant Gas Channels for Oxygen Entry

PROTEIN EVOLUTION HYPOTHESIS

Before

After

This is Our Evolution (Screening) Protocol

Generate Diversity

PCR
- Error Prone PCR
- DNA Shuffling
- Rational Design

Isolate Mutants by Dilution

Anaerobic Chamber, 2% H₂

Amplify Mutants

Cell-Free Protein Synthesis:

Gene Library → Protein Library

Oxidized Methyl Viologen Hydrogen Consumption Assay

2MV<sub>ox</sub> + H₂ → 2MV<sub>red</sub> + 2H<sup>+</sup> (Blue)

Exposé to Oxygen by Addition of Air Saturated Buffer

PCR

Jim Stapleton, Jon Kuchenreuther, Phil Smith, Alyssa Bingham
We Could Choose from Two [Fe-Fe] H₂ases and Began with the Simpler One
We Found a Faster One (But not with Ferredoxin)
None of the Mutants was more Oxygen Tolerant

Jim Stapleton
Jon Kuchenreuther
We Then Switched to The More Complex Enzyme – And Had to Reoptimize Expression

C. reinhardtii HydA1

C. pasteurainum HydA (Cpl)
Alyssa Bingham Found a More Oxygen-Tolerant Mutant !! With Three Mutations

- Mutants were expressed *in vivo* in *E. coli* and purified
- Added 5 ul of air saturated buffer to 95 ul of enzyme solution
- Incubated 20 minutes (uncovered in anaerobic glove box)

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Percent Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0</td>
</tr>
<tr>
<td>I197V N160D</td>
<td>5</td>
</tr>
<tr>
<td>I197V A280V</td>
<td>10</td>
</tr>
<tr>
<td>I197V N289D</td>
<td>15</td>
</tr>
<tr>
<td>I197V N160D</td>
<td>20</td>
</tr>
<tr>
<td>I197V N289D</td>
<td>25</td>
</tr>
<tr>
<td>I197S N160D</td>
<td>30</td>
</tr>
<tr>
<td>I197S N289D</td>
<td>35</td>
</tr>
</tbody>
</table>

Alyssa Bingham, Kunal Mehta, Rachel Bent
Two of the Mutations were in An Unexpected Location -- Near an Electron Conducting Center

We Then Needed a Method to Test for Oxygen Tolerance During Hydrogen Production
We Wanted a Continuous Electron Supply from Ferredoxin – The Natural Electron Source

We Assembled This Pathway Using Purified Proteins

\[
glucose-P \xrightarrow{\text{Glucose-P Dehydrogenase}} \text{NADPH} \xrightarrow{\text{FNR}*} \text{Fd}^{\text{Red}} \xrightarrow{\text{H}_2\text{ase}} \text{H}_2
\]

*FNR = Ferredoxin NADPH Reductase

In Spite of Thermodynamic Concerns, It Worked

Phil Smith, Alyssa Bingham
Unfortunately, The “Oxygen-Tolerant” Mutant Was NOT Tolerant When Making Hydrogen

- Simple gas channel hypothesis is not the whole story

- We will now conduct protein evolution by screening for mutants that are more oxygen tolerant when making hydrogen using ferredoxin as the electron source – the natural way

- We will use tungsten oxide/palladium coated glass plates to sense hydrogen production
BUT – We also realized that our enzyme pathway might be useful to make \( H_2 \) from Glucose

To be commercially viable the process must deliver:
1. High conversion efficiencies, and
2. High Productivities

It must also have an inexpensive source of enzymes. (We can’t afford to separately produce and purify 15 enzymes!)

Could We Conduct Cell-free Metabolic Conversions Using Crude Cell Extracts?
We can use the Pentose Phosphate Pathway to Get High Conversion Yields

**Basic Pathway**

\[
glucose-P \rightarrow 6\text{ NADP}^+ + 6\text{ H}_2\text{O} + 12\text{ NADPH} + 6\text{ CO}_2 + 12\text{ H}^+
\]

**Complete Pathway**

(Can also use xylose; i.e. cellulose hydrolysates)

**Oxidative Phosphorylation**

\[
\text{Glucose} \rightarrow \text{Glucose-6-P} \rightarrow \text{H}_2\text{O} + \frac{1}{2}\text{O}_2
\]

This Predicts Very High Conversion Efficiencies (92%)
The process flow diagram would look like this; We will need to express high levels of the H₂ase.

Fermentor A: Make FNR (2X Volume)
Niacin
Iodoacetamide
RNase

Homogenizer

Glucose
Gas Out

H₂ Reactor (7X Volume)
Semi-permeable Membrane

Fermentor B: Make H₂ase and Fd (5X Volume)

O₂ Slow Feed

N₂

H₂ Off

CO₂ Recovery

SDS Page Gel of UNPURIFIED Cell Lysates

A New Cell Line and New Process Produces High Level of the H₂ases

Jon Kuchenreuther
What Productivities Might Be Possible?  
-- Use Ethanol Production as a Benchmark

<table>
<thead>
<tr>
<th>Vessel</th>
<th>[FNR] (µM)</th>
<th>[Fd] (µM)</th>
<th>[H$_2$ase] (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentor A</td>
<td>505</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fermentor B</td>
<td>---</td>
<td>1166</td>
<td>120</td>
</tr>
<tr>
<td>H$_2$ Reactor</td>
<td>144</td>
<td>833</td>
<td>86</td>
</tr>
</tbody>
</table>

Ethanol:  \[
\frac{100 \text{ g EtOH}}{L} \left( \frac{1}{60 \text{ hr}} \right) \left( \frac{0.025 \text{ MJ}}{g \text{ EtOH}} \right) = 0.04 \text{ MJ/L-hr}
\]

Proposed Process:  \[
\frac{86 \mu\text{mole H}_2\text{ase}}{L} \left( \frac{5 \mu\text{moles H}_2}{\mu\text{mole H}_2\text{ase} \cdot \text{sec}} \right) \left( \frac{3600 \text{ sec}}{\text{hr}} \right) \left( \frac{2 \text{ g H}_2}{1 \text{ mole H}_2} \right) \left( \frac{0.13 \text{ MJ}}{g \text{ H}_2} \right) = 0.40 \text{ MJ/L-hr}
\]
**We Are Making Good Progress in Increasing Fuel Value Productivities**

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<tbody>
<tr>
<td>Fuel value productivity (kJ L⁻¹ hr⁻¹)</td>
<td>0.052</td>
<td>0.195</td>
<td>1.446</td>
<td>2.245</td>
<td>6.385</td>
</tr>
<tr>
<td>Molar Productivity (µmole L⁻¹ hr⁻¹)</td>
<td>200</td>
<td>750</td>
<td>5562</td>
<td>8633</td>
<td>24556</td>
</tr>
<tr>
<td>H’ase Turnover number (sec⁻¹)</td>
<td>0.12</td>
<td>0.005</td>
<td>1.54</td>
<td>0.24</td>
<td>3.41</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>40</td>
<td>30</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>[FNR] (µM)</td>
<td>--</td>
<td>--</td>
<td>10⁺</td>
<td>50⁺</td>
<td>30ᵇ</td>
</tr>
<tr>
<td>[Fd] (µM)</td>
<td>--</td>
<td>--</td>
<td>50ᶜ</td>
<td>50ᶜ</td>
<td>80ᶜ</td>
</tr>
<tr>
<td>[Hydrogenase] (µM)</td>
<td>0.47ᵈ</td>
<td>39.4ᵈ</td>
<td>1ᵉ</td>
<td>10ᵉ</td>
<td>2ᵉ</td>
</tr>
<tr>
<td>[NADPH] (mM)</td>
<td>0.5</td>
<td>2</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

³E. coli FNR; ᵇSpinach FNR; ᶜSynechocystis [2Fe2S] ferredoxin; ᵈPyrococcus furiosus hydrogenase; ᵉClostridium pasteurianum hydrogenase I

Phillip Smith
Conclusions and Future Work

Conclusions:

- Cell-free H$_2$ase Production Enables Effective Protein Evolution
- Screen Needs to EXACTLY Replicate Desired Enzyme Use
- Cell-free Metabolism Offers Great Promise for Hydrogen Production from Glucose and from Cellulose Hydrolysates

Future Work:

- Evolve Oxygen Tolerant Hydrogenase
- Optimize Hydrogen Production from Sunlight in Cyanobacteria
- Find/Evolve More Active FNR Enzyme
- Implement Cell-free Hydrogen Production from Biomass
- Develop High Cell Density Protein Production Processes
Questions ?