Sweet Hydrogen: High-Yield Production of Hydrogen from Biomass Sugars Catalyzed by in vitro Synthetic Synthetic Biosystems

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6/7/2016 (5:15:00 PM)
Overview

Timeline

• Project Start Date: 06/15/2015
• Project End Date: 06/30/2017 (Two years)
• Project renewal and continuation will be determined by DOE EERE’s Go/No-Go milestones

Budget

• Total Project Budget: $937,602 (two years)
  • Total Recipient Share: $187,602
  • Total Federal Share: $750,000
  • Total DOE Funds Spent*: $320,522

* As of 3/31/2016

Barriers

Hydrogen production from Biomass

A. Reformer (Bioreactor) Capital Costs and Efficiency

B. Operations and Maintenance (O&M)

C. Biomass Feedstock Issues (starch and biomass sugars)

Collaborators

• Virginia Tech (lead)
• University of Georgia
Relevance

Fuel Cell Technologies Office Objective -- Develop advanced biological generation technologies to produce hydrogen with a projected cost of $10.00/gge at the plant gate by 2020.

Our novel approach – use renewable sugars (e.g., biomass sugars or starch) to split water to produce H₂ catalyzed by enzyme cocktails

\[ C_6H_{10}O_5 + 7 H_2O = 12 H_2 + 6 CO_2 \]

- Low-carbon or even nearly carbon-neutral production in terms of entire life cycle
- High-purity hydrogen generated (no CO)
- Mild reaction conditions (1 atm, 20-90°C, pH 7.0, aqueous phase)
- Local resources for distributed hydrogen production
- Highest biological hydrogen generation rates

<table>
<thead>
<tr>
<th>Targets</th>
<th>Units</th>
<th>June 2016 Target</th>
<th>June 2017 Target (estimated)</th>
<th>Year 2020 Target (Plant gate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production cost</td>
<td>$/kg H₂</td>
<td>1000</td>
<td>10</td>
<td>10.00 (year 2020)</td>
</tr>
<tr>
<td>Productivity</td>
<td>mmole H₂/L/h</td>
<td>300</td>
<td>750</td>
<td>2,000</td>
</tr>
<tr>
<td>Reactor volume</td>
<td>L of reactor</td>
<td>0.01</td>
<td>1</td>
<td>2,777*</td>
</tr>
</tbody>
</table>

*200 kg H₂ per day
Production of H₂ from biomass sugars (overview)

Sugars (e.g., starch, cellulose)

- Hydrolysis
- Glucose
  - Dark fermentation
    - 2 acetate → 4 H₂
    - 2 EtOH → 6 H₂
  - Partial oxidation reforming
    - 2 acetate → ~9 H₂
    - 2 EtOH → ~9 H₂
  - Gasification, (fast or flash) pyrolysis
    - ~12 H₂
  - Aquous phase reforming
    - ~12 H₂
  - In vitro synthetic enzymatic biosystems
    - ~3 H₂

Theoretical Practical

Microbes versus in vitro enzymatic biosystems

(a) Microbial cell factory

- Closed natural biosystems
- Dominant bio-manufacturing platform
- Enhanced performance by synthetic biology and systems biology

(b) Enzymatic systems

- Open man-made biosystems
- Emerging low-cost bio-manufacturing platform
- Surpass mother nature of cells, such as, bioenergetics, mass transfer rate, etc.
  - High yield,
  - Fast reaction rate,
  - Easy product separation,
  - Broad reaction conditions
Increasing volumetric rate (Rxn)

New Moore’s law? – Doubling speed every two years

Planned Solutions
- Better pathway design
- Better enzymes (complexes)
- High temperature
- Kinetic modeling
- More addition of rate-limiting enzymes
- High substrate levels
- More enzyme loadings

Rxn development history
- Highest Rxn of i.e., 153 mmole H₂/L/h
- 750-fold increase in past 10 years

This project Rxn goals
- 300 mmole H₂/L/h @Jun 2016
- 750 mmole H₂/L/h @Jun 2017
Decreased $\text{H}_2$ production costs

Quantitative indicator of enzymes and coenzyme: **total turn-over number (TTN)**, mole product/mole enzyme

**Preconditions for low-cost hydrogen production**
- Low cost enzyme production ($10-20$/kg)
- High stability of enzymes (TTN = $10^{8-9}$ mole product/mole enzyme)
- High stability of coenzymes (TTN = $10^{6-7}$ mole product/mole enzyme)
- Low-cost of coenzymes ($100$/kg)

**Key directions:** (1) discovery of better enzymes *(Task 1.3)*, (2) engineering of dehydrogenases on biomimetic cofactors *(Task 1.2)*, and (3) mass production of enzymes *(Tasks 3.1 & 3.2)*.

Approaches/Milestones

(*As of 3/31/2016)

Task 1.1. Co-expression of multiple enzymes in one host (FY16 Q1) 100%
Task 1.2. Two redox enzymes on biomimics at 0.1 U/mg (FY16 Q3) 80%
Task 1.3. Discovery of four novel enzymes (FY16 Q1) 100%
Task 1.3. Discovery of eight novel enzymes (FY16 Q3) 100%

Task 2.1. Optimization of enzyme ratios by modeling (FY16 Q1) 100%
Task 2.2. Data fitting and validating of rate-limiting steps (FY16 Q4) 100%
Task 2.3. Construction of artificial electron transport chains (FY16 Q2) 100%
Task 2.4. Construction of synthetic enzyme complexes (FY16 Q3) 60%

Task 3.1. High-density of protein expression in E. coli (FY16 Q3) 90%
Task 3.2. Mass production of hydrogenase (SH1) (UGa) (FY16 Q3) 80%
Task 3.3. 10-mL level demonstration (FY16 Q3) 80%

This reporting period (June 2016, Go/No-Go milestones)

- 10-fold volume scale-up (10 mL reactor) with 2-fold increase in H₂ peak production rate (i.e., 300 mmol H₂/L/h)
Accomplishments and Progress:
Responses to Previous Year Reviewers’ Comments

- This project started on June 15, 2015. No previous year reviewers’ comments are available.
Accomplishments: Multi-enzyme expression in one host

Two enzymes in one plasmid

- Case 1 (pET20b backbone)
- Case 2 (pET20b backbone)
- Case 3 (pET20b backbone)

Four enzymes in two plasmids
- pET20b backbone
- pACYCduet-1 backbone

Four enzymes in one plasmid

We can adjust enzyme expression ratios by changing plasmid design.

We can control multiple (two-four) enzyme expression levels by novel strategies. High-level expression of more than four heterologous enzymes is technically difficult.
Accomplishments: Discovery of better enzymes

Desired enzyme characteristics: expected reaction, thermostability, high activity, easy expression and purification in *E. coli*

Technical approaches:
- Data mining thermostable enzyme candidates from megagenomic DNA databases (KEGG, NCBI, JGI)
- Compare available enzyme characteristics from Brenda database and literature
- Clone, express and characterize enzyme candidates

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Abb.</th>
<th>EC</th>
<th>Original enzyme Source</th>
<th>New Enzymes Discovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-glucan phosphorylase</td>
<td>aGP</td>
<td>2.4.1.1</td>
<td><em>C. thermocellum</em></td>
<td><em>T. maritima</em> (Q1)</td>
</tr>
<tr>
<td>4-glucanotransferase</td>
<td>4GT</td>
<td>2.4.1.25</td>
<td>Thermococcus litoralis</td>
<td><em>Thermococcus kodakarensis</em> (Q2)</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>PGM</td>
<td>5.4.2.2</td>
<td><em>C. thermocellum</em></td>
<td><em>Thermococcus kodakarensis</em> (Q2)</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>G6PDH</td>
<td>1.1.1.49</td>
<td><em>G. stearothermophilus</em></td>
<td><em>T. maritima</em> (Q1)</td>
</tr>
<tr>
<td>6-phosphoglucoolactonase</td>
<td>6PGL</td>
<td>3.1.1.31</td>
<td><em>T. maritima</em></td>
<td></td>
</tr>
<tr>
<td>6-phosphoglucanate dehydrogenase</td>
<td>6PGDH</td>
<td>1.1.1.44</td>
<td><em>M. thermoacetica</em></td>
<td><em>T. maritima</em> Q1</td>
</tr>
<tr>
<td>Aldolase</td>
<td>ALD</td>
<td>4.1.2.13</td>
<td><em>T. thermophilus</em></td>
<td><em>Sulfolobus tokodaii STK_03180</em> (Q2)</td>
</tr>
<tr>
<td>Fructose-1,6-bisphosphatase</td>
<td>FBP</td>
<td>3.1.3.11</td>
<td><em>T. maritima</em></td>
<td><em>Sulfolobus tokodaii STK_03180</em> (Q2)</td>
</tr>
<tr>
<td>Phosphoglucone isomerase</td>
<td>PGI</td>
<td>5.3.1.9</td>
<td><em>C. thermocellum</em></td>
<td><em>Thermus thermophilus</em> (Q1)</td>
</tr>
<tr>
<td>NAD(P)H: rubredoxin oxidoreductase</td>
<td>NROR</td>
<td>1.18.1.4</td>
<td><em>P. furiosus</em></td>
<td><em>P. furiosus</em> (PF1197) used for electron transfer from NADPH to EM (Q2)</td>
</tr>
</tbody>
</table>

We have obtained nine new thermostable enzymes, all of whose activities are higher than previous ones. They can be purified by easy heat precipitation.
Progress: Replace NAD(P) of dehydrogenases with NR

Wild-type dehydrogenases (i.e. glucose 6-phosphate dehydrogenase, \textbf{G6PDH}, and 6-phosphogluconate dehydrogenase, \textbf{6PGDH}, cannot work on biomimetic coenzymes.

**Comparison of coenzymes**

<table>
<thead>
<tr>
<th>Coenzyme</th>
<th>Structure</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP⁺</td>
<td><img src="image" alt="NADP⁺ Structure" /></td>
<td>$4,500/kg</td>
</tr>
<tr>
<td>NAD⁺</td>
<td><img src="image" alt="NAD⁺ Structure" /></td>
<td>$1,500/kg</td>
</tr>
<tr>
<td>NR⁺</td>
<td><img src="image" alt="NR⁺ Structure" /></td>
<td>$100/kg</td>
</tr>
</tbody>
</table>

**Coenzyme engineering**

- **A**: Rational design
- **B**: Directed evolution
- **C**: Module swap

Nicotinamide riboside (NR) is better than NAD(P)
- Less costly
- More stable
- Small size – better mass transfer

- We have three strategies to change coenzyme preference of dehydrogenases
- New area – very high risk.
- Directed evolution of enzymes is preferred.
We established high-throughput screening for dehydrogenases working on biomimetic coenzyme with minimum influences from inherent coenzymes (NAD(P)).

Engineered glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) can work on NR for the first time.
Accomplishments: Identification of key rate-limiting steps by modeling

**Standard Conditions**

| Green line, experimental data |
| Red line, model data after data fitting by using a genetic algorithm |
| Blue line, model robustness (leave one out) cross-validation |

**#1.** Hydrogenase (SH1) is No. 1 rate-limiting step.

**#2.** NADPH regeneration from NADP⁺ is No. 2 rate-limiting step catalyzed by G6PDH.

**#3.** ALD for FDP synthesis from GA3P and DHAP is the no. 3 rate-limiting step.

**Planned solutions**

- **#1,** to be addressed by artificial electron transport chains (Task 2.3), biomimetic coenzyme (Task 1.2); enzyme complexes (Task 2.4)
- **#2,** to be addressed by redesigned pathway, better enzymes & biomimic (Task 1.2)
- **#3,** to be addressed by better enzymes (Task 1.3)
Accomplishments: Increasing Rxn by using artificial electron transport chains

**Previous pathway design**

1. Glucose 6-phosphate → G6PDH → NADP⁺ → 6-Phosphogluconolactone → NADPH → H₂
2. NADPH (-320 mV) → H₂ (-420 mV)
3. Big concerns:
   - Very slow reaction rate from NADPH to H₂
   - Thermodynamics unfavorable (from -320 to -420 mV)
   - Very high activation energy barrier

**New artificial electron transport chain**

1. Glucose 6-phosphate → G6PDH → NADP⁺ → 6-Phosphogluconolactone → NADPH → NROR → SH₁
2. Electron mediator (oxidized) → Electron mediator (reduced)
3. Electron mediator (reduced) → NADPH (-320 mV) → H₂ (-420 mV)
4. Electron mediator (oxidized) → NADPH (NR, -330 mV) → H₂ (MV, -450 mV)
5. Electron mediator (oxidized) → NADPH (BV, -360 mV) → H₂ (SH₁, -420 mV)
6. Key idea: the use of an electron mediator bridging NAD(P)H and H₂, decreasing activation energy and increase Rxn.
Accomplishments: Increasing Rxn by using the artificial electron transport chain

Better pathway

- We achieve the highest biohydrogen generation rates (i.e., 310 mmole H₂/L/h, 0.62 g H₂/L/h or 6.6 L H₂/L/h) by using BV and NROR, far higher than anaerobic microbial fermentation, electrochemically-assisted microbial fuel cells, algal hydrogen production.

- Meet the rate target of the Go/No-Go milestones of Phase I project -- Increase H₂ production rate by 2-fold to 300 mmole H₂/L/h.

- Use of another new enzyme 6-phosphogluconolactonase (6PGL) accelerates the reaction rates of oxidative phase of pentose phosphate pathway.
Progress: Complete starch utilization for H₂ generation

**New pathway for better substrate (starch) utilization**

- Use isoamylase to debranch branched starch (amylopectin, dominant corn starch component) (increasing H₂ yield by 30%)
- Use 4-glucanotransfer (4GT) to utilize maltose, hydrolytic product of starch (increase H₂ yield about 4%)
- Polyphosphate glucokinase (PPGK) to utilize glucose (increasing yield about 4%)

All enzymes have been purified. The experiment will be finished in the middle of May 2016.
We will utilize all energy stored in starch to generate theoretical yield H₂.

(Note 1: We have converted all glucose and xylose from biomass to H₂ before this project.)

(Note 2: We have utilized all glucose units of starch for the production of another product.)
Progress: Scale-up of reactor volume

VT glass shop-made bioreactors

Now we conduct most experiments in the small bioreactor (1-2 mL working volume) for optimization of reaction condition, new pathway design, etc.

10X scale-up. We will scale up enzymatic hydrogen production in 10-mL working volume bioreactor by the end of June 2016 (the end of Phase I).
(Now we are scaling up enzyme production capacity by 10 (large flasks) - 1000 fold (e.g., 6-L fermenter.))

1000X scale-up. We will scale up enzymatic hydrogen production in 1000-mL working volume bioreactor by the end of June 2017 (the end of Phase II).
Accomplishments: Scale-up of high density *E. coli* fermentation

1000 X scale-up

<table>
<thead>
<tr>
<th>200 mL/flask</th>
<th>4000 mL/ reactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD = 3.0</td>
<td>OD = 150</td>
</tr>
</tbody>
</table>

Recombinant enzyme production cost analysis

- Cell density (OD = 150, 50 g DCW/L)
- Protein expression level = 20-30% of cellular protein
- Fermentation costs = $200-300/m³ in 20-50 m³ fermenters (including low-cost industrial medium and OpEx) from a few Chinese contract research outsourcing (CRO) enzyme companies and Korean companies.

Enzyme production cost = $40-50/kg enzyme (enzyme order scale of 10 tonnes/yr)

Enzyme production cost = $400-500/kg (enzyme order scale of 10-100 kg/yr)

Thermostable enzyme production

1. High-density fermentation
2. Cell lysis by heat treatment
3. Centrifugation
4. Ultra-filtration
5. Spray drying
6. Recombinant Enzymes
7. Combi-CLEA Immobilization
Accomplishments: Over-expression of Soluble Hydrogenase I (SHI)

NADPH + 2H⁺ → H₂ + NADP

P₅₈₉ promoter: constitutive, high level expression; Affinity tagged Strep- or His₉-PF0891

<table>
<thead>
<tr>
<th>Host</th>
<th>Affinity tag</th>
<th>Purification Steps</th>
<th>SHI yield (mg/10 g cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native P. furiosus</td>
<td>-</td>
<td>4</td>
<td>0.25</td>
</tr>
<tr>
<td>Overexpressed P. furiosus</td>
<td>Strep tag II</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Overexpressed P. furiosus</td>
<td>His tag</td>
<td>1</td>
<td>13.5</td>
</tr>
</tbody>
</table>

We increased SH1 expression level by 54 fold, making 340 mg SH1 per batch.

Problem: >30% of the SHI protein lacks the PF0894 catalytic subunit (E3).
Progress: Identification of limiting factors for SHI expression

- Eight accessory genes (hypABCDEF, frxA and slyD) are required for SHI biosynthesis
- The accessory proteins construct and insert the NiFe catalytic site into PF0894 of SHI
- FrxA, HypCD and HypF are regulated with SHI in the native organism

FrxA, HypCD and HypF expression will be optimized for better SHI.
• Over-expression of *frxA* increased almost 200-fold in strain MW519 & three-fold higher H₂ evolution activity.
• Over-production of HypC/D in the FrxA over-expression strain did not further increase SHI production

Over expression of HypF in OE-FrxA and OE-FrxA/HydC/D strains is underway
Collaboration

- Subcontract – Co-PI (Prof. Mike Adams) at University of Georgia

- Focus on low-cost mass production of hyperthermophilic Fe-Ni soluble hydrogenase I (*P. furiosus* SH1) without a discount of specific activity of SH1

- Related to **Objective 1** (decrease hydrogen production costs for enzymes) and **Objective 3** (scale-up of enzymatic hydrogen production)
Remaining Challenges and Barriers

- Increase catalytic efficiencies \( \frac{k_{\text{cat}}}{k_M} \) dehydrogenases on biomimic (NR) to comparative levels of to those on their natural coenzyme (NADP).

- Decrease SH1 production costs – better expression levels without a decrease in specific activity (i.e., coordinated co-expression of enzyme components).

- Construct G6PDH-6PGDH-SH1 enzyme complexes featuring electron transport channeling.

- Scale up recombinant enzyme production from gram scales at labs to kg or ton scale in pilot plants and demonstration plants.
Proposed future work

• Decrease H₂ production costs by 1000 fold to $10/kg, where production cost will be estimated by using H2A model
  Task 1.2. Replace costly NADP by biomimic -- NR
  Task 1.4. Detailed economic analysis of H2 production

• Increase H₂ production rate from 300 to 750 mmole H₂/L/h
  Task 2.3. Construction of artificial electron transport chains
  Task 2.4. Construction of synthetic metabolons (enzyme complexes)

• Scale up H₂ reaction volume from 10 mL to 1000 mL
  Task 3.1. High-density of recombinant protein expression in E. coli
  Task 3.2. Mass production of hydrogenase (SH1) (UGa)
  Task 3.3. Liter level demonstration
Technology Transfer Activities

• Before this EERE award, Virginia Tech and Oak Ridge National laboratory received issued patent US 8,211,681. Biohydrogen production by an artificial enzymatic pathway. (2012).

• PI. Prof. Dr. Percival Zhang, President, Cofounder, and CSO of a start-up company, Cell Free Bioinnovations (CFB). CFB optioned to license this hydrogen technology from Virginia Tech but this company is focusing on high-end applications – sugar-powered biobatteries (direct sugar fuel cells). Both hydrogen and bio-batteies projects share most enzymes used.

• CFB is seeking for capital investment and SBIR/STTR grants.

• Several provisional patent disclosures will be filed about engineered dehydrogenases, biomimetic coenzymes, artificial electron transport chains, artificial enzyme complexes.
We achieved the highest biological hydrogen generation rate (310 mmole H₂/L/h, 0.62 g H₂/L/h) by using sugar energy to split water, reaching the rate target of the Go/No-Go milestone (June 2016).

We demonstrated the feasibility of changing coenzyme preference of engineered dehydrogenases from NADP to NAD and biomimic.

We can produce 12 H₂ each sugar unit of starch regardless of linear or branched starch.

We scaled up recombinant *E. coli* enzyme production by 1000-fold and recombinant *P. furiosus* SH1 (hydrogenase) production by 50-fold.

**Summary**

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<tr>
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<td>mmole H₂/L/h</td>
<td>310 (achieved)</td>
<td>750</td>
<td>2,000</td>
</tr>
<tr>
<td>Reactor volume</td>
<td>L of reactor</td>
<td>0.01 (to be finished)</td>
<td>1</td>
<td>2,777*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*200 kg H₂ per day</td>
</tr>
</tbody>
</table>
Technical Back-Up Slides
Appraisal of enzymes as biocatalysts

**Basic facts**
- Most enzymes are proteins
- Biocatalysis catalyzed by enzymes has highly chemical selectivity (no side product)
- Most enzymes work at mild reaction conditions (low temperature, 1 atm, neutral pH, and aqueous phase)
- Enzymes do not require costly precise metals

**Conflicting concepts (academic researchers versus industrial enzyme experts)**
- × For academic researchers, enzymes are VERY costly (e.g., **billion dollars per kg**)
  √ For industrial enzyme experts, bulk enzymes are less costly (e.g., **10 dollars per kg**)
- × For academic researchers, enzymes are very labile, losing activities within hours or days
  √ For industrial enzyme experts, some enzymes (e.g., immobilized or engineered) are very stable, lasting months and years (e.g., glucose isomerase for HFCS production, protease in detergent, glucose dehydrogenase in blood sugar test strips).
- × For academic researchers, enzymes have narrow reaction conditions in terms of pH, temperature and solutions.
  √ For industrial enzyme experts, some enzymes (engineered or discovered from extremophiles) can work on a large temperature range from 0 – 100ºC, pH from 1 to 14, from aqueous solution to 100% organic solvent.
Enzyme costs are NOT showstopper for economically viable production of high-volume products (> 1 million tons per year) via in vitro synthetic enzymatic biosystems.
## Enzyme stability

<table>
<thead>
<tr>
<th>EC</th>
<th>Enzyme name</th>
<th>Source</th>
<th>Form</th>
<th>Cond.</th>
<th>TTN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.1.44</td>
<td>6-phosphogluconate hydrogenase</td>
<td><em>T. maritima</em></td>
<td>Free</td>
<td>80°C</td>
<td>2.4 x 10^8</td>
</tr>
<tr>
<td>2.2.1.2</td>
<td>Transaldolase</td>
<td><em>T. maritima</em></td>
<td>Free</td>
<td>60°C</td>
<td>1.7 x 10^7</td>
</tr>
<tr>
<td>3.1.3.11</td>
<td>Fructose 1,6-Bisphosphatase</td>
<td><em>T. maritima</em></td>
<td>Free</td>
<td>60°C</td>
<td>2 x 10^7</td>
</tr>
<tr>
<td>5.4.2.2</td>
<td>Phosphogluomutase</td>
<td><em>C. thermocellum</em></td>
<td>Free</td>
<td>60°C</td>
<td>7.1 x 10^7</td>
</tr>
<tr>
<td>5.3.1.5</td>
<td>Xylose (glucose) isomerase</td>
<td></td>
<td>Immobilized</td>
<td>50-60°C</td>
<td>5.0 x 10^8</td>
</tr>
<tr>
<td>5.3.1.6</td>
<td>Ribose-5-phosphate isomerase</td>
<td><em>T. maritima</em></td>
<td>Free</td>
<td>60-70°C</td>
<td>2.2 x 10^8</td>
</tr>
<tr>
<td>5.3.1.9</td>
<td>Phosphoglucoisomerase</td>
<td><em>C. thermocellum</em></td>
<td>Free Immob.</td>
<td>60°C</td>
<td>3.2 x 10^7 1.1 x 10^9</td>
</tr>
</tbody>
</table>

**Our goal:**
1 kg of enzyme produces 300 kg of H2 @ TTN = 4 E7 ➔ $0.10/kg H2
Enzymatic H₂ costs

Cost goal = $2.00/kg H₂

Sugar = 81% @ $0.30/kg starch
Enzyme = 4%
Coenzyme = 1%
Others (capital, labor, etc.) = 13.4%

Expected enzyme expenditure = $0.10/kg H₂
Expected coenzyme = $0.025/kg H₂
1 kg enzyme produces 300 kg of H₂.

All estimates were conducted based on key inputs of Slide 7 – enzyme costs & TTN by the PI.
Progress: H$_2$ production cost analysis

Distributed H$_2$ Production

- Distributed SMR, Carbon Tax, No CCS: $2.53
- Distributed SMR, Carbon Tax, CCS: $2.49
- Distributed SMR, No Carbon Tax, CCS: $2.40
- Central Coal Gasification, Carbon Tax, No CCS: $2.33
- Central Coal Gasification, Carbon Tax, CCS: $2.14
- Central Coal Gasification, No Carbon Tax, CCS: $2.12
- Central Coal Gasification, No Carbon Tax, No CCS: $2.04
- Central SMR, Carbon Tax, No CCS: $1.79
- Distributed Enzymatic Biomass Hydrogen: $1.77
- Central SMR, No Carbon Tax, CCS: $1.75
- Central SMR, Carbon Tax, CCS: $1.72
- Central SMR, No Carbon Tax, No CCS: $1.59

Key inputs:
- CapEX= $6 MM

Other inputs
- $1.35/kg of hydrogen for sugar ($0.18/kg sugar)
- $0.10/Kg of hydrogen for enzyme
- $0.03/Kg of hydrogen for coenzyme

Ultimate cost
- = $1.77/kg H$_2$
- @8000 ton H$_2$ per year
- = $2.00/kg
- @200 kg H$_2$ per day

All estimates were conducted by Cell Free Bioinnovations and VT business school but will be redone by using H2A model.