NATIONAL RENEWABLE ENERGY LABORATORY

Biomass to Hydrogen (B2H2)

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National Renewable Energy Laboratory

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PD038

This presentation does not contain any proprietary, confidential, or otherwise restricted information.

Overview

Timeline

- Project start date: FY16
- Project end date: 10/2017*

*Project continuation and direction determined annually by DOE

Budget

- FY16 DOE Funding: $1M
  - Include $300K to partners
- Planned FY17 DOE Funding: $700K
  - Include $150K to partner
- Total DOE funds received to date: $1.85M

Barriers

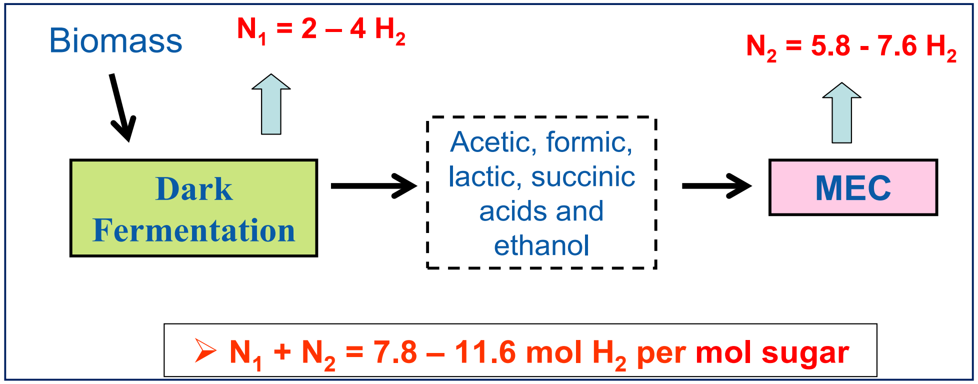
- H_2 molar yield (AX)
- Feedstock cost (AY)
- System engineering (AZ)

Partners

- Dr. Bruce Logan
  Pennsylvania State University
- Drs. Steven Singer, Lawrence Berkeley National Lab (LBNL) and Ken Sale, Sandia National Lab (SNL)
- Dr. James Liao at UCLA (no cost)

Relevance

Overall Objective: Develop ***direct*** fermentation technologies to convert renewable lignocellulosic biomass resources to H_2.



Directly Address Barriers

- Feedstock cost (AY): via bioreactor development using lignocellulose (Task 1), and biomass pretreatment via ionic liquid (Task 2).
- Hydrogen molar yield (AX) (N_1 & N_2: mol H_2/mol hexose): via genetic engineering (Task 3) and integration with Microbial Electrolysis Cell (MEC) (Task 4)

Address Key DOE Technical Targets

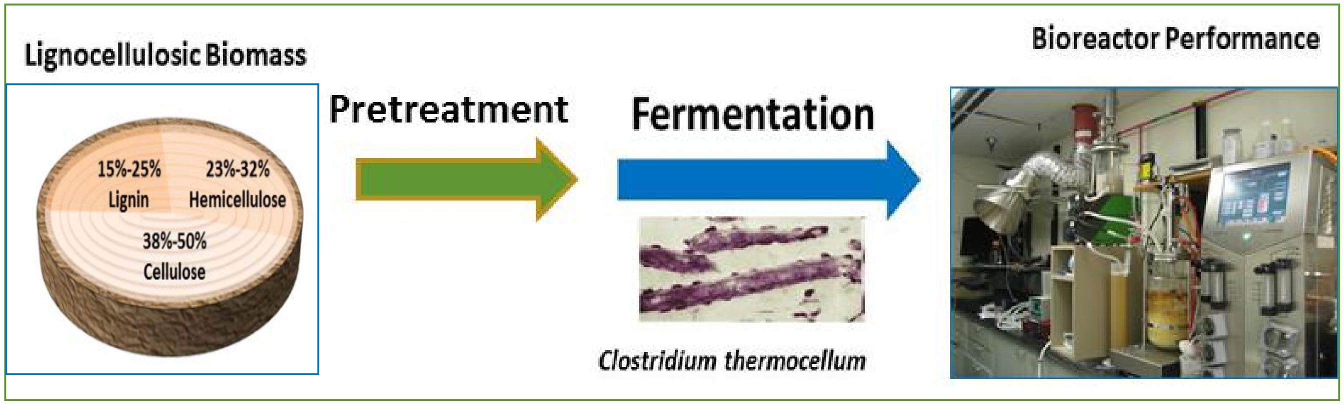
Characteristics	Units	2011 Status	2015 Target	2020 Target
Feedstock cost[a]	Cents/lb sugar	13.5	10	8
Yield of H_2 production from glucose	Mol H_2/mol glucose	**3.2**[b]	4	6
MEC production rate	L-H_2/L-reactor-day	-	1	4

a. Status and target of the DOE Bioenergy Technology Office (BETO) – leverage BETO funding.
b. Low carbon substrate loading (1 g/L) led to high H_2 molar yield.

Approach

Task 1: Bioreactor Performance

- **Approach:** Optimize bioreactor in batch and fed-batch modes by testing parameters such as corn stover lignocellulose loadings (DMR), hydraulic retention time (HRT), and liquid volume replacement and frequency, using the cellulose-degrading bacterium *Clostridium thermocellum*, one of the fastest cellulose-degraders.

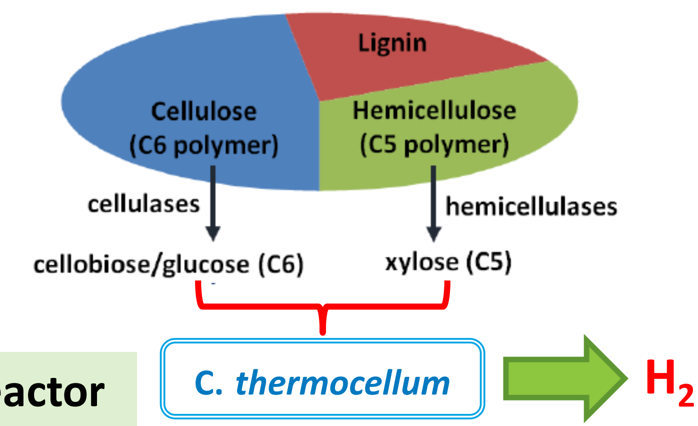


Pretreatment – De-acetylated and Mechanically Refined (DMR)

0.4% NaOH → 70°C

- **Solid:** 44% cellulose, 23% xylan, 17% lignin, Ash, etc. → H_2
- **Liquid:** 79% acetate, 2.9% xylan

Engineer Cellulose Microbe to Co-metabolize C5 Sugars



Ferment all the sugars to H_2 in one bioreactor

High Rate of H_2 Production from Fermenting DMR Corn Stover

- DMR corn stover retains more intact biomass structure, hence more recalcitrant. Yet it can be fermented directly to H_2 by *C. thermocellum* without adding expensive enzyme cocktail.

Carbon Concentration ($g\ L^{-1}\ d^{-1}$)	Cycle	Average H_2 Production Rate ($mL\ L^{-1}\ d^{-1}$) Avicel	DMR	Average cellulose consumption (g/L) Avicel	DMR
10	1	1119	738	7.8	4.8
	2	1140	1064	9.1	12.6
	3	1621	763	7.6	8.3
	4	1098	1733	10.8	10.6
Average		1244	**1075**	8.83	9.06
stdev		22%	**43%***	17%	37%

* High SD likely from incomplete biomass hydrolysis in cycles 1&3.

Work is ongoing to increase DMR loadings to attain an average rate of 2.5 $LH_2/L/d$ – FY17 Q4 Milestone.

Sequencing Fed-Batch fermentation of 10 g/L/d of DMR feeding at a 48 h HRT generated an **average** (4-day period) H_2 production rate of **<u>1075 mL $H_2/L_{reactor}/d$</u> at a 48 h HRT (max rate = 1.7 L $H_2/L_{reactor}/d$, 1-day period).**

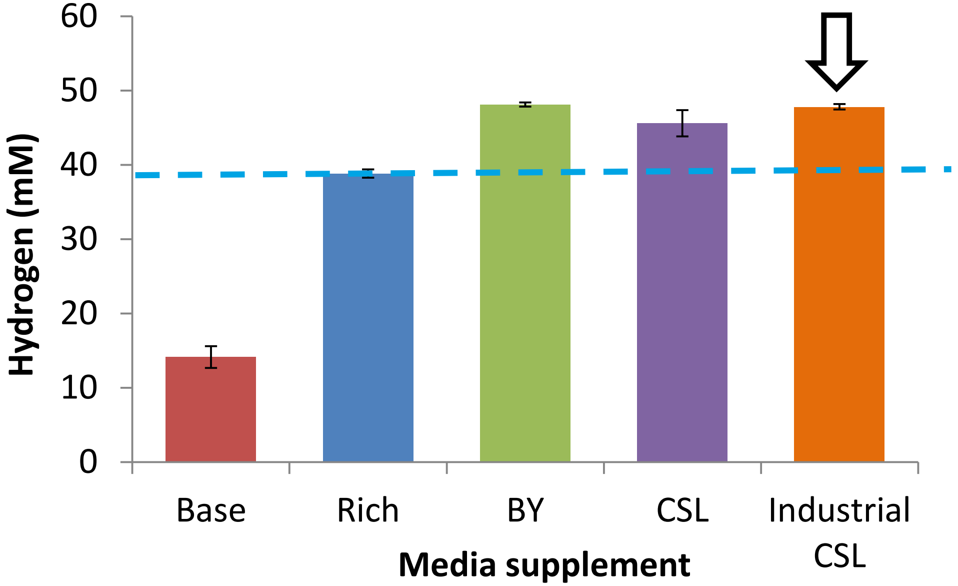

Lauren Magnusson

FY16 Q4 Milestone (regular) - NREL		
Optimize the hydraulic/solid retention time with respect to H_2 production and media utilization by testing HRT between 12 and 48 h in a sequencing fed-batch reactor, and obtain a continuous average H_2 production rate of $1L/L_{reactor}/d$ using DMR.	9/2016	Complete

Task 1 – Accomplishments/Progress

Industrial Waste Reduced Growth Medium Cost by 49%

- In FY16 AMR, we reduced the medium cost by ~89% via replacing/eliminating three expensive ingredients (MOPS buffer, cysteine, and resazurin) from the rich medium without impacting H_2 production.
- The FY2017 goal is to replace yeast extract (0.45%; $201.5/kg) in the medium with an industrial waste.
- Corn steep liquor (CSL; $49.4/kg, Industrial CSL; $0.61/kg) or brewers yeast (BY; $114.5/kg) could replace yeast extract as they contain the needed vitamins and amino acids to boost growth and H_2 production.



	H_2 (mM)	Medium* Cost ($/L)
Rich	38.8	1.85
BY	48.1	1.38
CSL	45.6	1.39
Industrial CSL	47.8	0.95

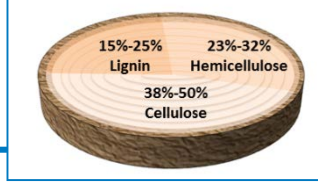
We realized a 49% reduction ($0.95/$1.85) in medium cost when yeast extract is replaced with industrial waste corn steep liquor with increased H_2 production.

*already excluding MOPS, cysteine, and resazurin.

FY17 Q2 Milestone (regular) - NREL		
Develop an industrially relevant growth medium for *C. thermocellum*, by evaluating and supplementing either commercially available components or industrial waste products that will maintain similar level of cell fitness and rate of H_2 production compared to the growth medium currently used with the aim to reduce medium cost (NREL).	3/2017	Complete

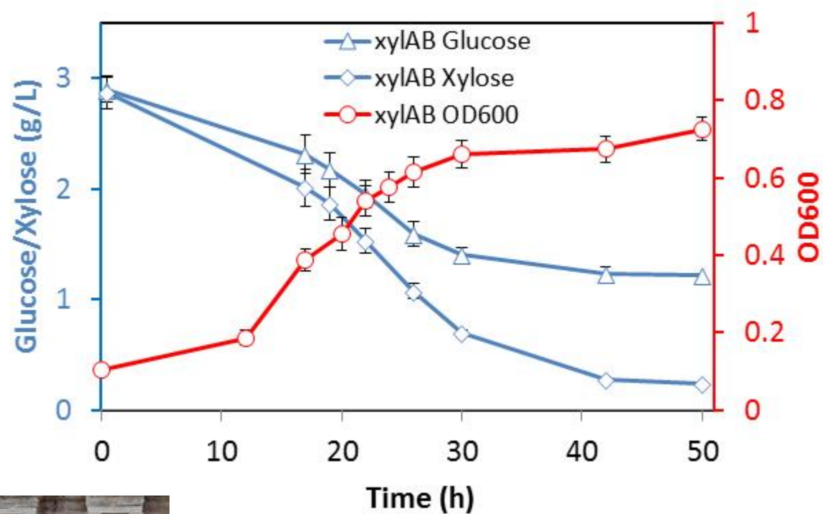
Task 1 – Accomplishments/Progress

Engineered *C. thermocellum* Can Co-utilize C6/C5 Sugar

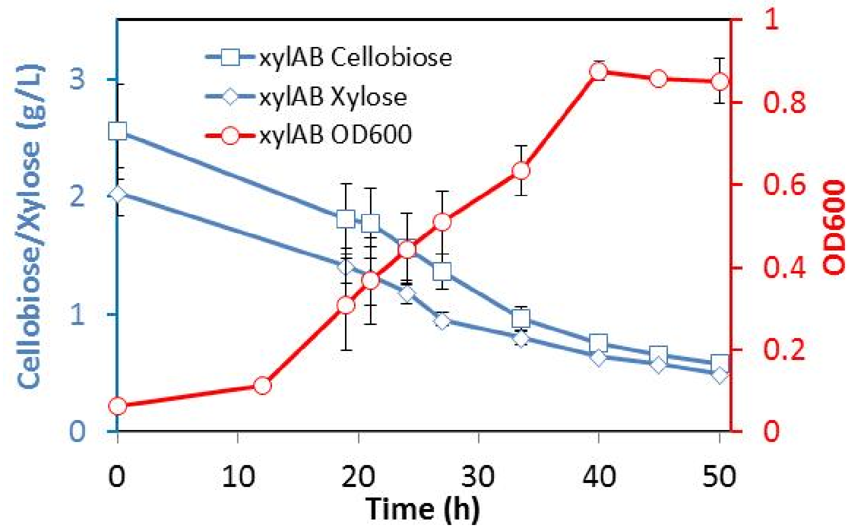


- Up to 32% of biomass is hemicellulose, a polymer of C5 sugar (mostly xylose)
- We engineered *C. thermocellum* (*xylAB* mutant strain) to co-utilize C5 sugar while degrading cellulose – leveraging NREL LDRD success.
- C5 (xylose) and C6 sugars (glucose or cellobiose) were metabolized <u>simultaneously</u> **without** cross inhibition – a breakthrough finding.

A. Xylose and Glucose Co-metabolism

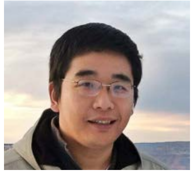


B. Xylose and Cellobiose Co-metabolism



Katherine Chou

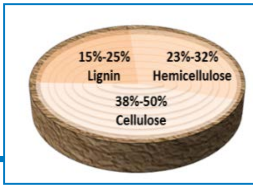
C6 and C5 sugars co-metabolism improves biomass utilization and lowers H_2 selling price.



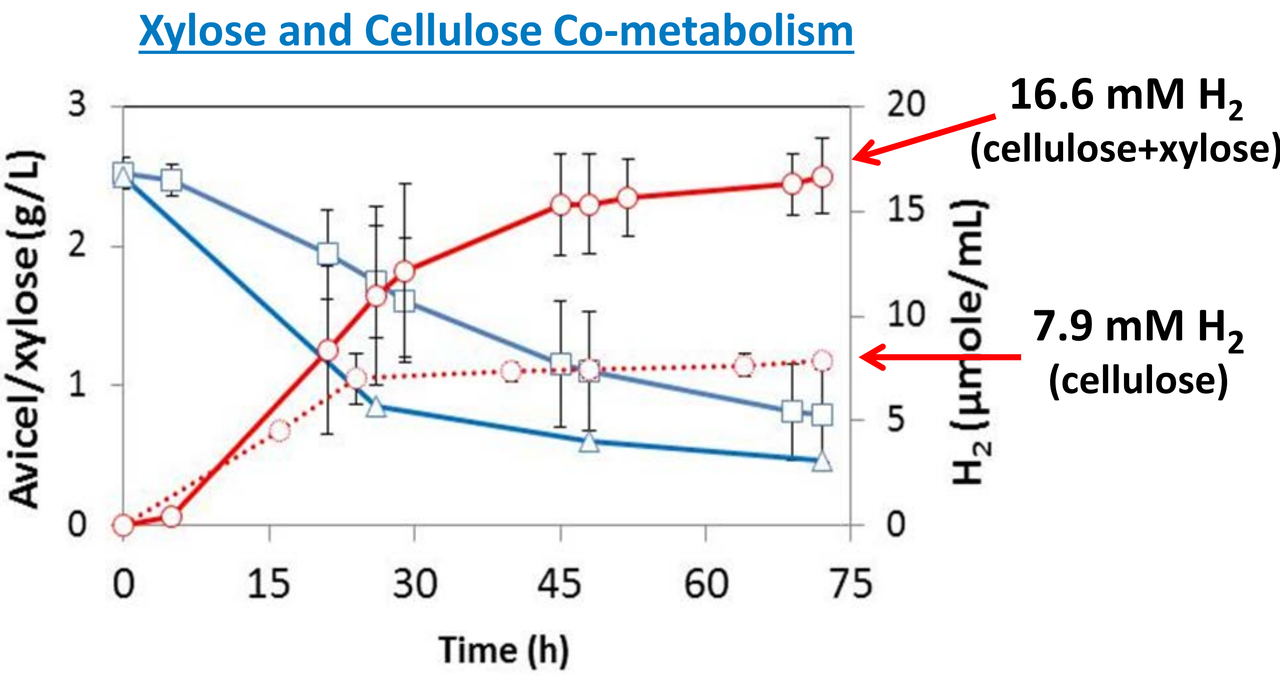
Wei Xiong

Task 1 – Accomplishments/Progress

Xylose and Cellulose Co-utilization Increases H_2 by 2.1-fold



- *C. thermocellum* mutant was cultured in 2.5 g/L cellulose (Avicel), +/- 2.5 g/L xylose.
- Engineered strain can also co-metabolize xylose with xylo-oligomer, and glucose with xylan, the latter is a modeled hemicellulose.



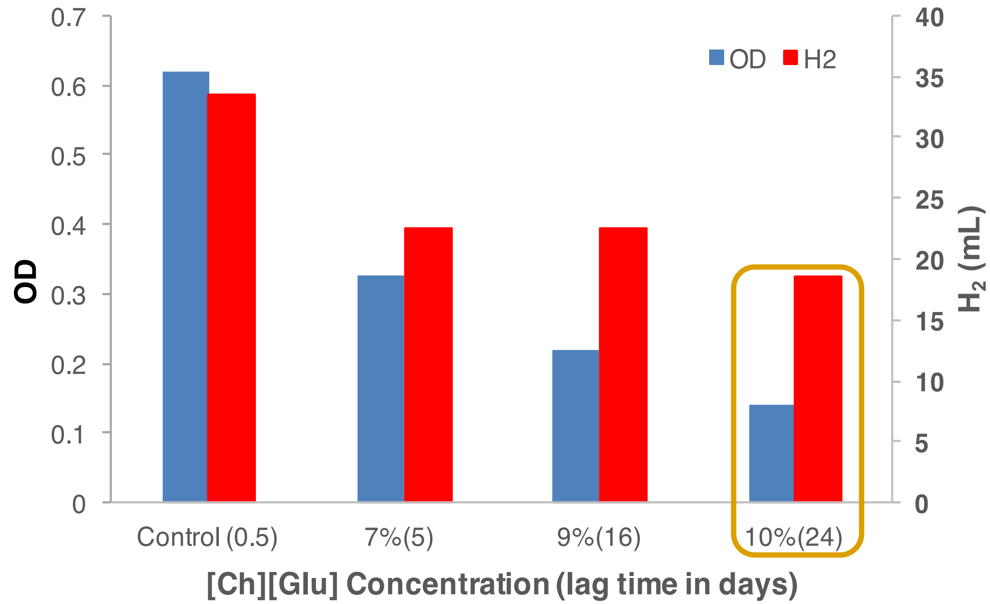
Cellulose and xylose co-utilization increased H_2 output by 2.1-fold

FY17 Q1 Milestone (regular) - NREL		
Profile H_2 production, substrate consumption, and metabolite profiles in an engineered C. thermocellum mutant using xylose alone, and in co-metabolism of xylose with xylan, xylose with cellobiose, and xylose with avicel cellulose, aimed at developing microbes fermenting all the sugars in biomass to improve biomass utilization (NREL).	12/ 2016	Complete

Approach/Accomplishments/Progress

Task 2. Fermentation of Pretreated Biomass using Ionic Liquid (LBNL/SNL)

- Ionic liquid (IL) pretreats biomass via electrostatic/hydrogen-bonding interactions, which compliments NREL's approach using DMR-pretreated biomass.
- Task 2 goal is to conduct IL pretreatment and fermentation in the same reactor to save cost, hence the GNG target of tolerating 10% IL.
- *C. thermocellum* displayed a lag phase of 24 days in 10% cholinium glutamate ([Ch][Glu]), with growth yield at **23%** that of the control(Go/No-go criterion is 85%).



Steve Singer
LBNL

Ken Sale
SNL

Task 2 was closed-out in FY17/Q1, not meeting the GNG Decision.

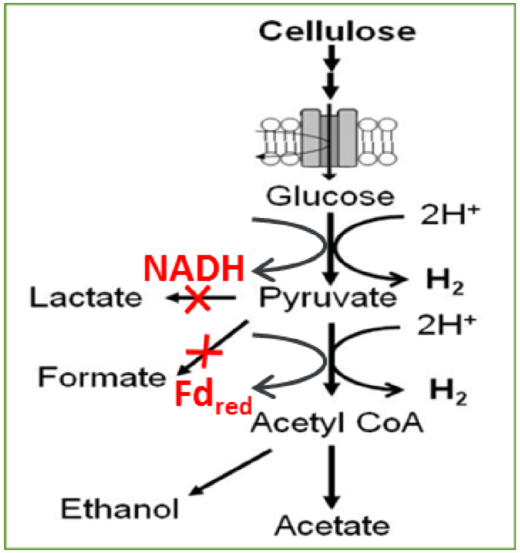
FY16 Q4 Go/No-go (GNG) Decision – LBNL/SNL	Comple-tion Date	Status
Demonstrate biocompatibility of at least one ionic liquid with C. thermocellum by comparing cell growth with and without the presence of 10% selected ionic liquid in DSM 1191 growth medium with cellobiose as carbon source and achieving a similar growth rate (>85%) with and without ionic liquid.	9/2016	Incomplete

Approach/Milestones

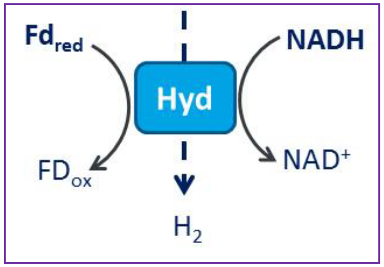
Task 3 – Generate Metabolic Pathway Mutant in *C. thermocellum*

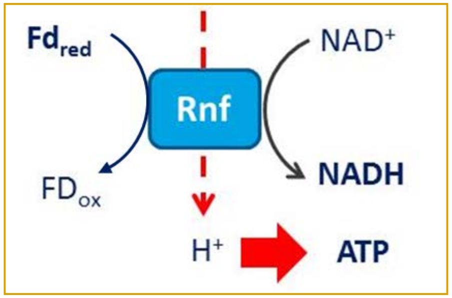
Approach: Redirect metabolic pathways to improve H_2 molar yield via developing genetic methods.

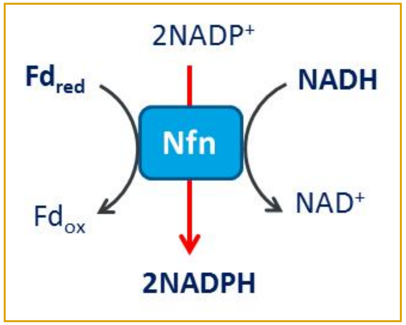
- Blocking the lactate and formate carbon-competing pathways led to **86% increase** in rate of H_2 production (2016 AMR).
- A new strategy in FY17 is to directly manipulate enzymes (Nfn and/or Rnf) to increase the pools of NADH and reduced ferredoxin (Fd_{red}), both are electron donors for H_2 production.



Hydrogenase (Hyd)





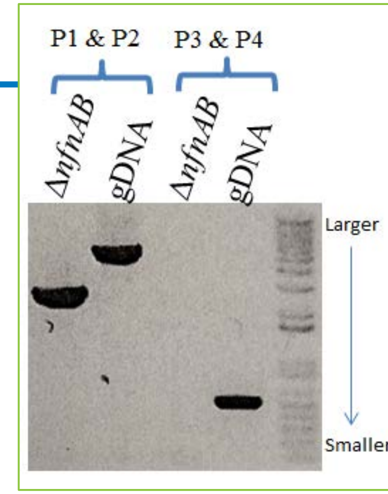


FY17 Q3 Milestone (Regular) - NREL	Com-pletion Date	Status
Generate a mutant lacking either ferredoxin: NAD^+ oxidoreductase (Rnf) and/or NADH-ferredoxin: $NADP^+$ oxidoreductase (Nfn) and profile H_2 and carbon metabolites production. This mutant will then serve as the host for deleting the ethanol competing pathway using H_2 production as the new electron sink aimed at maintaining redox balance to yield stable mutants with increased H_2 production	6/2017	Complete

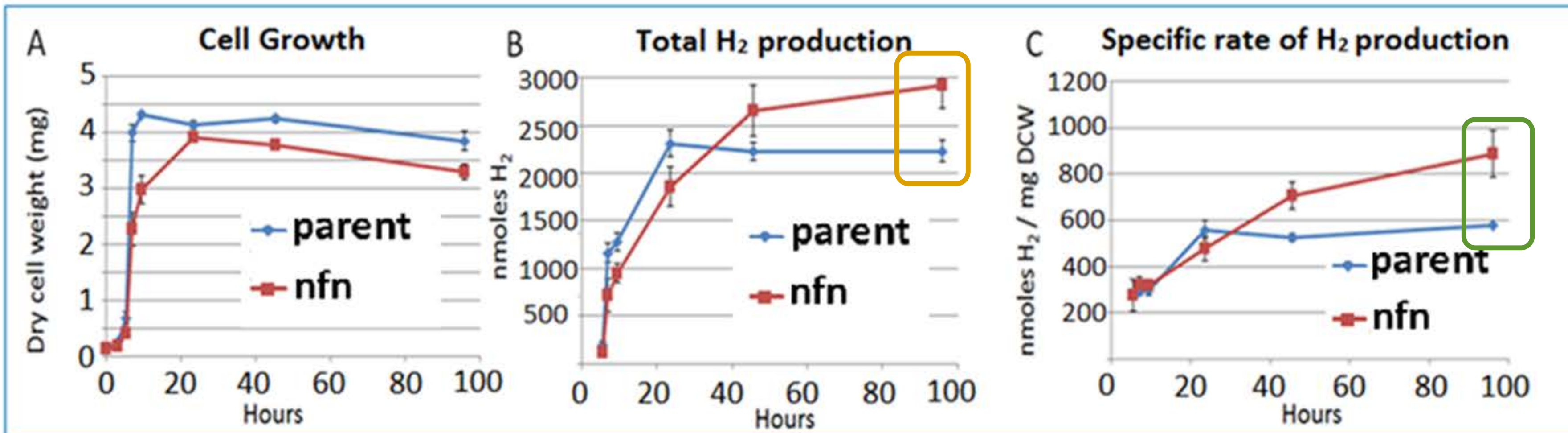
Task 3 – Accomplishments/Progress

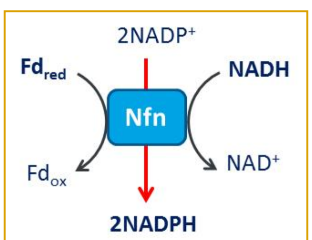
Nfn Mutant Produced 29% More H_2

$$NADH + Fd_{red} + NADP^+ \xrightarrow{Nfn} NAD^+ + Fd_{ox} + NADPH$$



- Nfn: transhydrogenase; its deletion should yield more Fd_{red} and NADH for H_2 production.
- PCR data confirm deletion of *nfnAB* genes from *C. thermocellum* genome: Δ*nfn* mutant yields a lower band (lane 1) or no band (lane 3) vs. the parental control (gDNA); P1-P4: PCR primers.

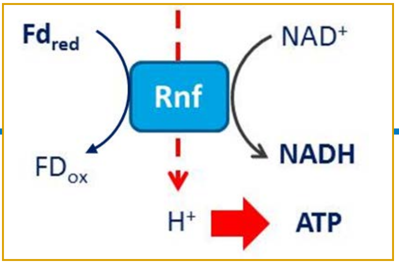




Nfn mutant: total H_2 production was increased by **29%**, and specific rate of H_2 production by **55%**.

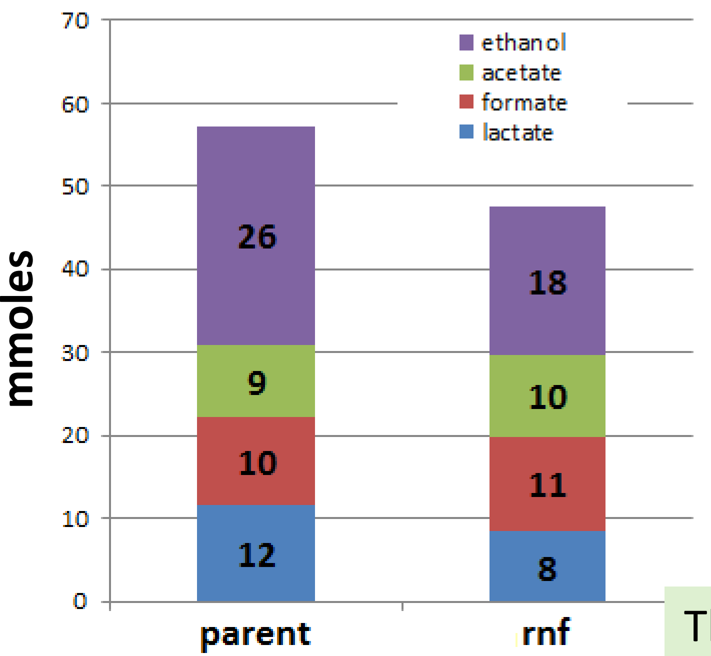
Jonathan Lo

Task 3 – Accomplishments/Progress
Rnf Mutant Produced 35% More H_2

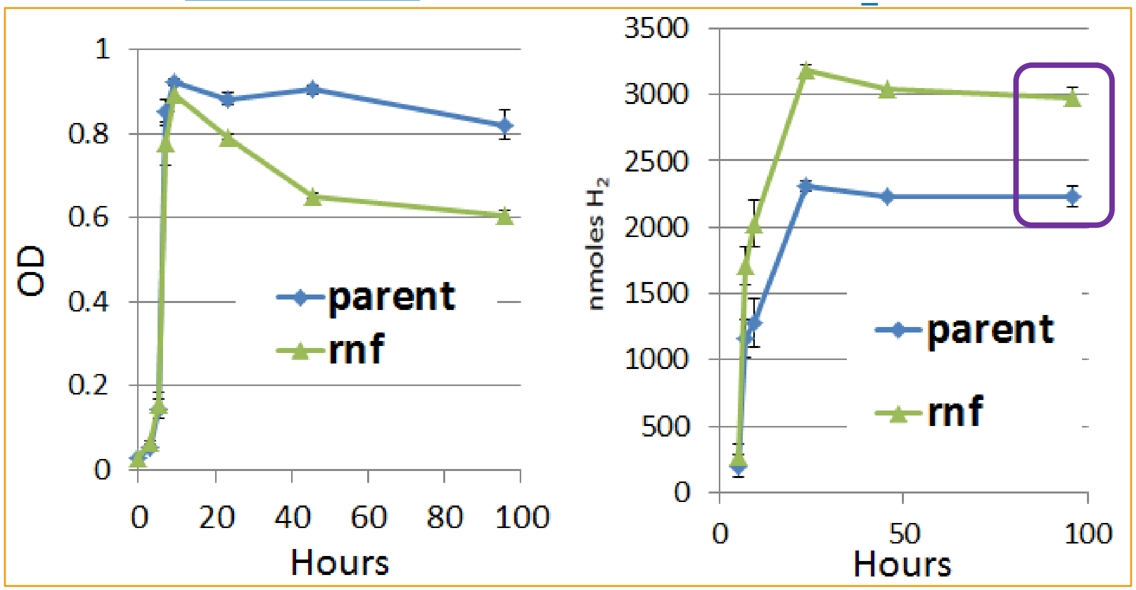


- Rnf: Fd_{red}-NAD^+ oxidoreductase
- The enzyme Rnf converts Fd_{red} to NADH which yields energy.
- Its deletion should increase the pool of Fd_{red} toward more H_2 production.
- Generated Rnf mutant that produced **35% more H_2.**
- Rnf mutant also produced ~30% less ethanol, suggesting electron redirection.

Carbon Metabolite Profile



Cell Growth / Total H_2 Production



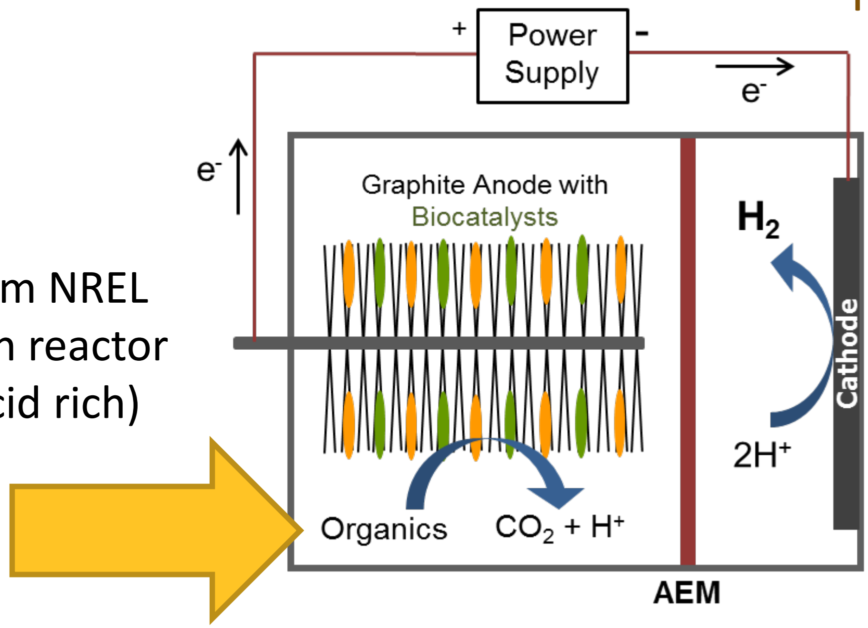
The 35% increase in H_2 production validates that redirecting electron pool is an effective strategy to boost H_2 production.

Approach/Milestone

Task 4 – Electrochemically Assisted Microbial Fermentation

Microbial Electrolysis Cell (MEC) — Conversion of Organic Waste to Hydrogen Gas

Effluent from NREL fermentation reactor (Organic acid rich)



Goal:
Achieving high rate of H_2 production with non-Pt based cathode using NREL fermentation effluent.

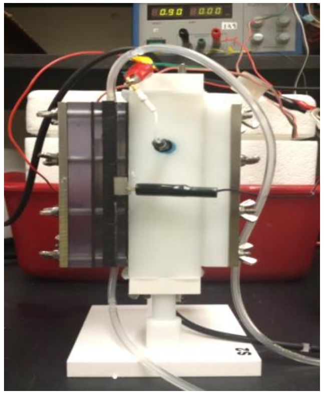
Bruce Logan

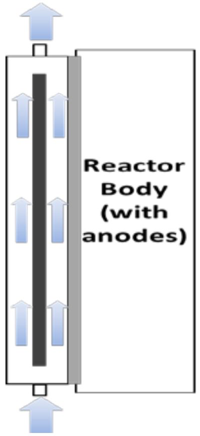
	Milestones (PSU)	Completion Date	Status
FY16	Design MEC cathodes with reduced width to increase H_2 production rate to a maximum H_2 rate of 1.2 L/Lreactor/day based on overall reactor volume reduction	9/2016	Complete
FY17	Investigate alternative cathode materials and increase electrode loading to double reactor performance to 2.4 L/reactor-d using synthetic fermentation effluent.	9/2017	On track

Task 4 – Accomplishments/Progress

Cathode Chamber Optimization: replaced Pt with alternative materials

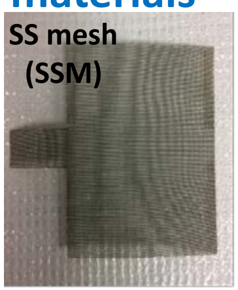
MEC with cathode flow chamber

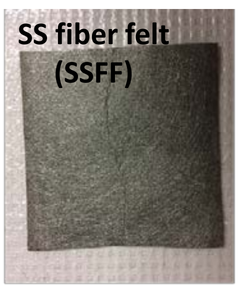


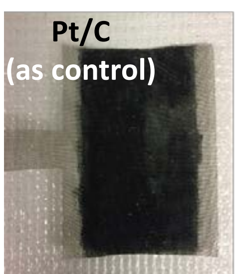


Anion exchange membrane
Cathode

Cathode materials





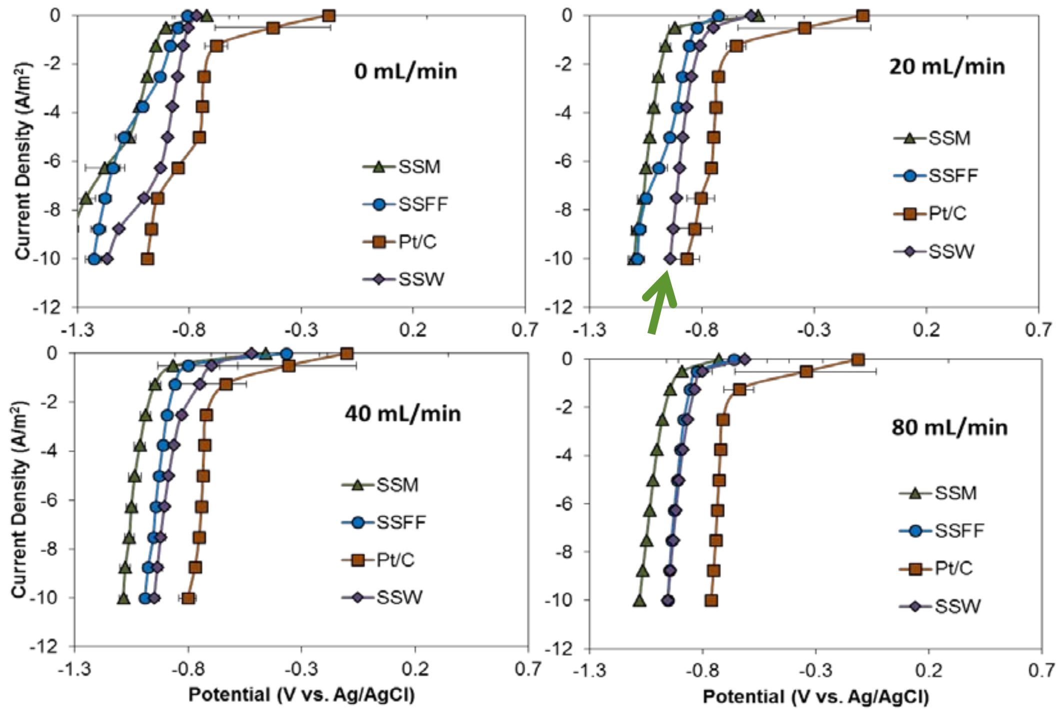


SS: stainless steel

Kyoung-Yeol Kim

Abiotic electrochemical tests (chronopotentiometry) at catholyte flow rates from 0 to 80 mL/min

(mean ± SD, n=3)



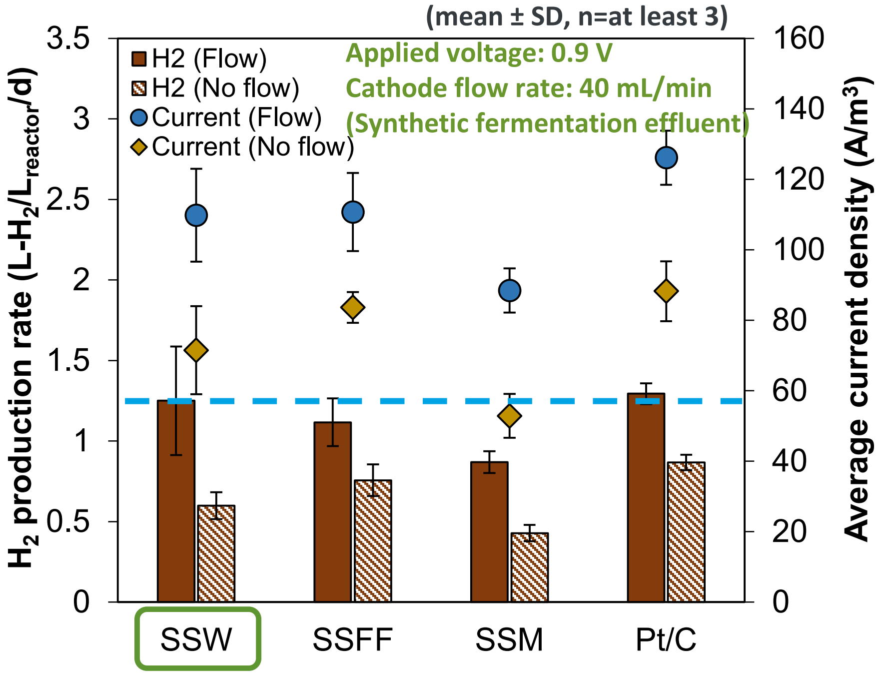
- **Higher variability at low current densities or with no flow.**
- **Higher current produces specific trends with materials.**
- **Performance ranking: Pt/C > SSW>SSFF>SSM in abiotic tests.**

The low cost stainless steel wool is the best performer to replace Pt

Task 4 – Accomplishments/Progress

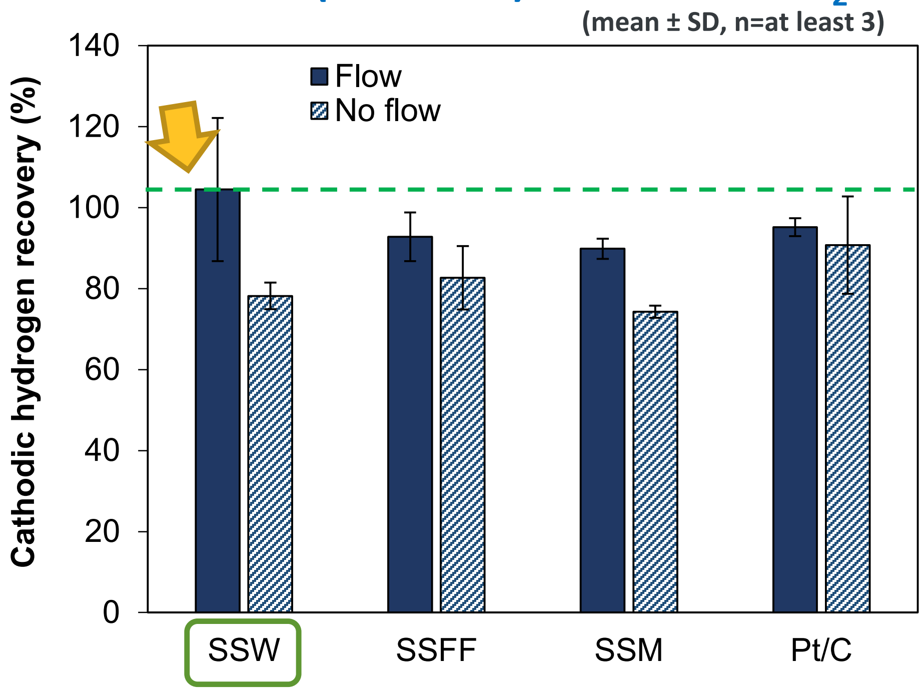
Achieved 1.3 L H_2/L-d with low cost non-Pt Cathode

H_2 production rates and current densities with different SS materials



- Hydrogen production rates ranged from 1.3±0.3 L/L-d with SSW, to 0.9±0.1 L/L-d SSM.
- Pt/C only marginally better than SSW
- 4.5 ±0.5 A/m²with SS wool (versus 5.1±0.3 A/m² with Pt/C; per cathode projected area)

Cathodic hydrogen recoveries (CR) = percent of coulombs (electrons) converted to H_2



- CRs with flow: 105±18 % for SSW; 95±2 % with Pt/C
- With no flow, some H_2 lost: CR=71±13 % for SSW

H_2 production rate meets 2016 milestone (1.2 L/L-d) with low cost SS wool (a non-Pt based cathode), and with ~100% H_2 recovery from current

Responses to Previous Year Reviewers' Comments

Utilization of xylose by C. thermocellum is likely diauxic, so incorporation of this into the fermentation process will be an engineering challenge.

- Our data have proved to the contrary, that xylose and cellulose are co-utilized **without** cross inhibition (i.e., xylose usage is not inhibited by the presence of cellulose). This breakthrough finding warrants continued research to realize more complete biomass conversion to increase H_2 production.

The reasoning for using IL-derived sugars is not clear, what it adds to the project.

- The IL Task was closed out in FY17/Q1, due to not meeting the GNG Decision.

It is unclear how novel the mutations are completed…overexpression of hyd2 is good

- Mutating two carbon-competing pathways led to near 86% increase in H_2 production (end point data). This combined mutation has not been reported in literature. Work is ongoing to over-express either native or foreign hydrogenases to improve H_2 production while also maintaining redox and energy balance to maximize cell fitness.

The rate for the MEC should be normalized to biomass not reactor volume, state rate as initial, peak or average, and give the amount of energy or net energy yield.

- The biomass concentration is not known in the MEC, so that cannot be done, and it is not needed as the critical factor is current normalized to electrode area or volume (we used volume here). The rate given is an average rate (now stated), and the energy input is calculated as energy efficiency (energy in the H_2 produced relative to the energy input in the electrolyzer). As noted in our previous work, this electrolyzer energy could be substituted with a reverse electrodialysis stack that would be powered using waste heat.

Collaborations

- **Task 1 (Bioreactor)**

  Drs. Ali Mohagheghi and Melvin Tucker, National Bioenergy Center at NREL: provide DMR pretreated corn stover and their characterizations - leveraging DOE BETO funding.

- **Task 2 (Ionic Liquid)**

  Drs. Steve Singer (LBNL) and Kent Sales (SNL): conducted biomass pretreatment using ionic liquid as a complementary pretreatment approach to lower feedstock cost.

- **Task 3 (Genetic Methods)**

  Dr. James Liao of UCLA in pathway engineering of *C. thermocellum* – leveraging DOE Office of Science funding, leading to a joint publication in PNAS, 2016.

- **Task 4 (MEC)**

  Dr. Bruce Logan at Penn State University: microbial electrolysis cells to improve H_2 molar yield.

Remaining Challenges and Barriers

Task 1. Bioreactor Performance

- High solid-substrate loading (175 g/L) is needed to lower H_2 selling price, which might present a challenge to ensure sufficient mixing.
  - Impeller design with high power/low torque will address this challenge.

Task 2. Fermentation of Pretreated Biomass using Ionic Liquid (LBNL/SNL)

- This task was closed out in FY17/Q1.

Task 3. Generate Metabolic Pathway Mutant in *C. thermocellum*

- Deleting carbon-competing pathways to increase H_2 molar yield might cause a redox imbalance (excess NADH or reduced ferredoxin) and compromise microbial fitness.
  - Over-express hydrogenase-encoding genes to maintain redox balance.

Task 4. Electrochemically Assisted Microbial Fermentation of Acetate (PSU)

- Maximizing current generation with non-precious metal catalysts
  - Already demonstrated good performance with SS wool; no others have yet shown better and more promising MECs.
- Further reducing reactor size, which will increase overall reactor production rates; examining new anode configurations which may help.

Proposed Future Work

Task 1 (NREL)

- Optimize sequencing-fed batch reactor using DMR corn stover to obtain average rate of 2.5 L $H_2/L_{reactor}/d$ (FY17 Q4 Milestone).
- Work with Argonne NL in a Life Cycle Analysis of the fermentative H_2 technology
- Optimize cellulose and hemicellulose co-fermentation to improve biomass utilization to achieve a rate of 2.5 L $H_2/L_{reactor}/d$ and higher (FY17/18).

Task 2 (LBNL/SNL)

- None

Task 3 (NREL)

- Over-express native or heterologous hydrogenases to increase H_2 production and to maintain redox balance for cell fitness in either Rnf or Nfn mutant host (FY17/18).
- Profile expression pattern to identify the most important ferredoxin(s) to improve H_2 production as *C. thermocellum* contains multiple ferredoxins with unknown functions (FY17/18).

Task 4 (Penn State)

- Further improve anode and cathode chamber (increasing electrode loading) to double performance of MEC to produce 2.4 L-$H_2/L_{reactor}/d$ (FY17) using synthetic effluent.
- Examine alternative materials and catalysts for the cathode (FY17).
- Design a multi-chamber MEC module (FY17/18).

Technology Transfer Activities

Technology-to-market or technology transfer plan or strategy

- Air Product and Chemicals, Inc.
  - Main interest in H_2 from biomass can be low carbon or even potentially carbon neutral; have funded the Logan lab in the past for work on MECs and RED for H_2 production from wastewaters
  - Large-scale process of greatest interest, but currently there are no larger reactors.
  - Cost needs to be near to, or lower than, making H_2 from alternative sources (natural gas).

Plans for future funding

- Network with biofuels industry to expand the use of H_2.
- Advocate the advantages of “green” H_2 rather than fossil-fuel derived H_2

Patents, licensing

- A Record of Invention (ROI-14-70) is filed for developing the proprietary genetic tools tailored for *C. thermocellum*.
- A second ROI-15-42 has been filed for generating xylose-metabolizing strain, leading to enhanced biomass utilization.

Summary

Task 1

- Achieved an average (4 days) H_2 production rate of <u>**1075 mL H_2/$L_{reactor}$/d**</u> fermenting DMR-pretreated corn stover directly to H_2.
- Lowered growth medium cost by **49%** by replacing the costly yeast extract with industrial <u>waste</u> corn steep liquor, without impacting H_2 production.
- Engineered *C. thermocellum* to co-metabolize **C5 sugar** with cellulose, without cross inhibition, leading to **2.1-fold increase** in total H_2 production, which improves biomass utilization and lowers the cost of H_2.

Task 2

- Closed out in FY17/Q1, not meeting GNG.

Task 3

- Generated a *nfn* mutant that produced **29% more** H_2 and with a 55% increase in specific rate of H_2 production.
- Generated a *rnf* mutant that produced **35% more** H_2. Either *nfn* or *rnf* mutant can serve as the host to over-express hydrogenases.

Task 4

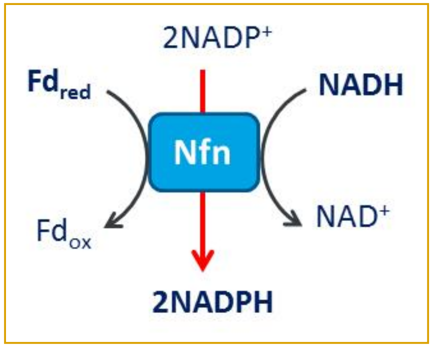
- SS cathodes improved with flow, with the SS wool the best performer to replace Pt.
- H_2 production rate meets 2016 milestone (**1.2 L/L-d**) with SS wool (a non-Pt based cathode) with ~**100% H_2 recoveries from current**.

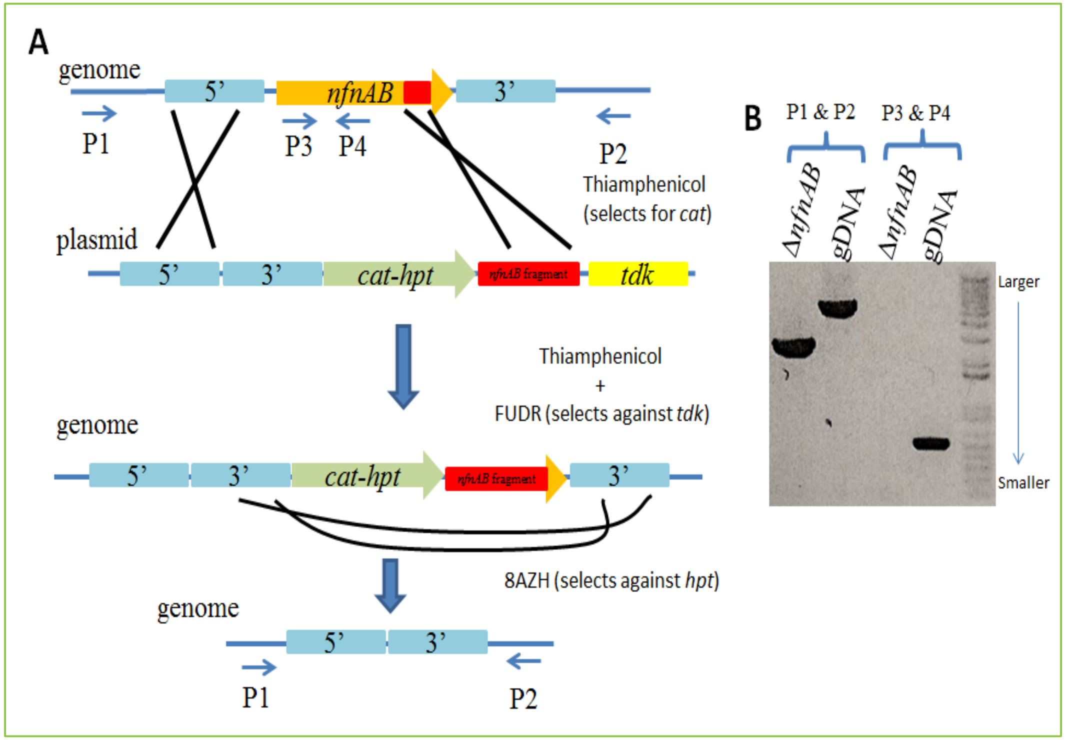
Technical Back-Up Slides

Task 3 – Accomplishments/Progress

Genetic Protocol to Generate the Nfn or Rnf Mutant

- The parental strain lacks *hpt* (phosphoribosyl transferase) gene, which allows counter selection with the antimetabolite 8AZH (8-azahypoxanthine) to lose the antibiotic marker thiamphenicol.
- The integration plasmid contains *tdk* (thymidine kinase) gene, which allows counter-selection with the antimetabolite FUDR (fluoro-deoxyuracil) to cure the plasmid.
- P1 – P4 are PCR primers to validate the mutagenesis steps.





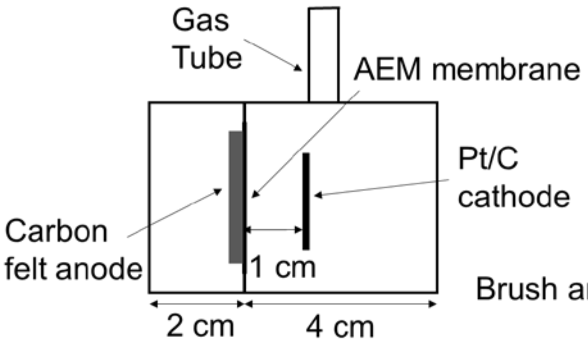
Task 4 – Accomplishments/Progress

Anode Chamber Optimization: Minimizing Anode Size

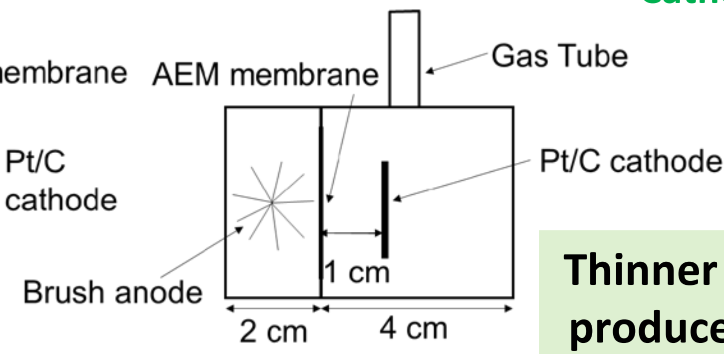
Goal: Reduce anode chamber volume by using flat anodes or smaller brushes

Minimizing anode chamber

Carbon felt anode (0.6 cm thickness)

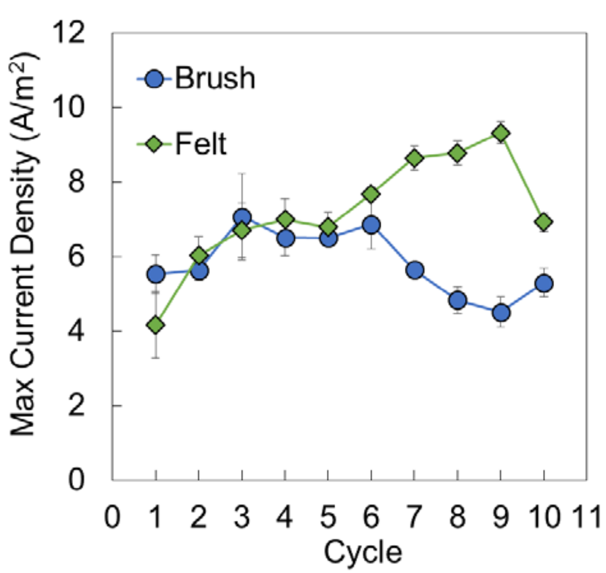


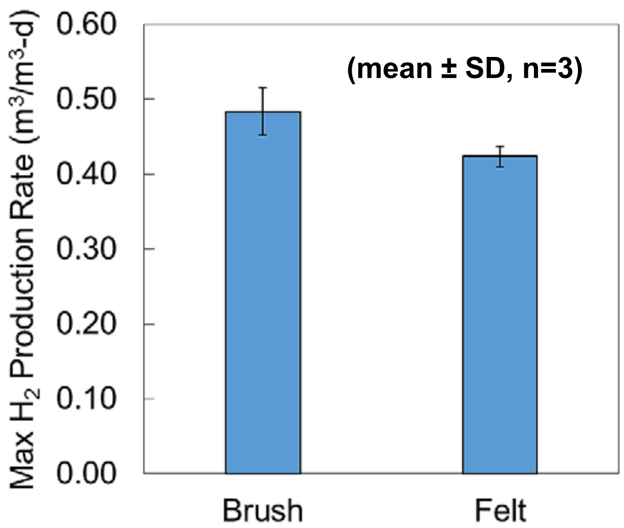
Brush anode (1.5 cm diameter)



Applied voltage: 0.9 V
Substrate: Acetate
Catholyte: 50 mM PBS

Thinner flat anodes could produce a more compact MEC reactor and increase H_2 production rates.





- Ongoing results (no flow): only slightly reduced H_2 production rates for thinner carbon felt electrodes (0.6 cm thick) than the brush electrode (1.5 cm diameter).