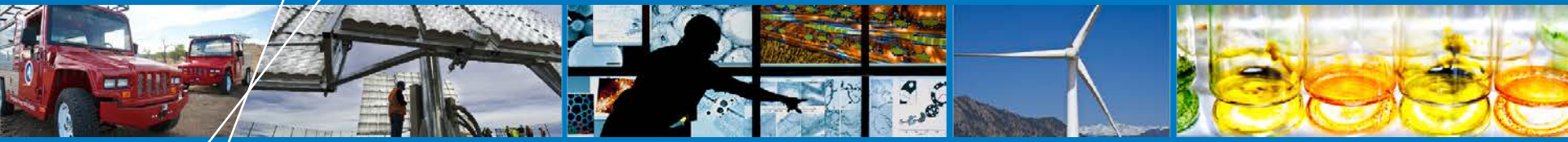
NREL
NATIONAL RENEWABLE ENERGY LABORATORY

Fermentation and Electrohydrogenic Approaches to Hydrogen Production



2013 Annual Merit Review and Peer Evaluation Meeting; May 16, 2013

Pin-Ching Maness (PI; Presenter); National Renewable Energy Laboratory

Bruce Logan (Presenter); Penn State University

Project ID #: PD038

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Overview

Timeline

- Project start date: FY05
- Project not funded in FY06
- Project end date: 10/2013*
- Percent complete: N/A

Barriers

Barriers addressed

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

Budget

- Total project funding: $2,720K (includes $387K subcontract)
- Funding received in FY12: $350K
- Planned funding for FY13: $410K

Partners

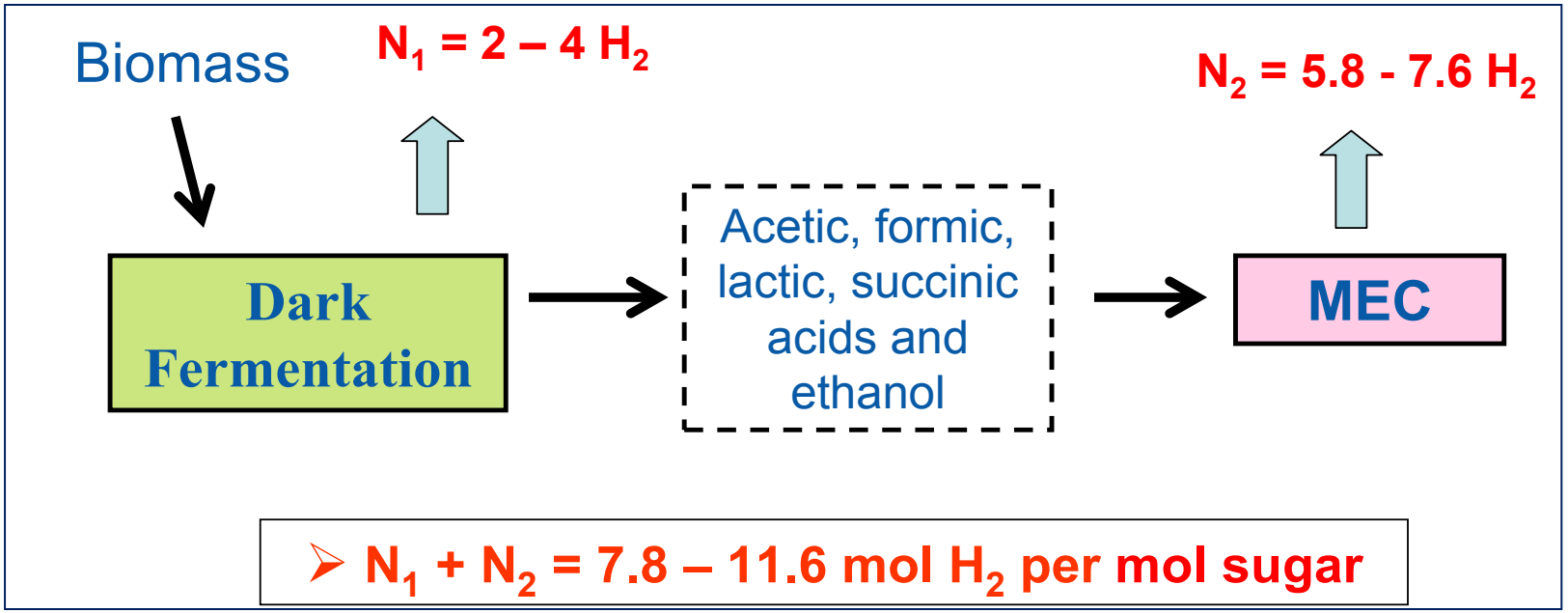
- Dr. Bruce Logan
  Pennsylvania State University
- Drs. David Levin and Richard Sparling, University of Manitoba, Canada

*Project continuation and direction determined annually by DOE

Objectives/Relevance

Project Overview

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



Integrating fermentation with MEC dramatically improves combined H_2 molar yield while reducing fermentation waste.

- MEC: microbial electrolysis cell; N_1 and N_2: H_2 molar yield (mol H_2/mol hexose).

Relevance

Relevance: The project addresses directly two DOE barriers (feedstock cost and H_2 molar yield) aimed at improving the techno-economic feasibility of H_2 production via biomass fermentation.

- **Task 1. Bioreactor Performance:** Use cellulose (in lieu of sugars) and optimize parameters in sequencing fed-batch bioreactor to lower feedstock cost (Barrier AY).
- **Task 2. Metabolic Engineering:** Develop genetic tools to block competing pathways aimed at improving H_2 molar yield (Barrier AX).
- **Task 3. Electrochemically Assisted Microbial Fermentation:** Integrate microbial electrolysis cell (MEC) reactor with fermentation to improve H_2 molar yield (Barrier AX).

Technical Targets

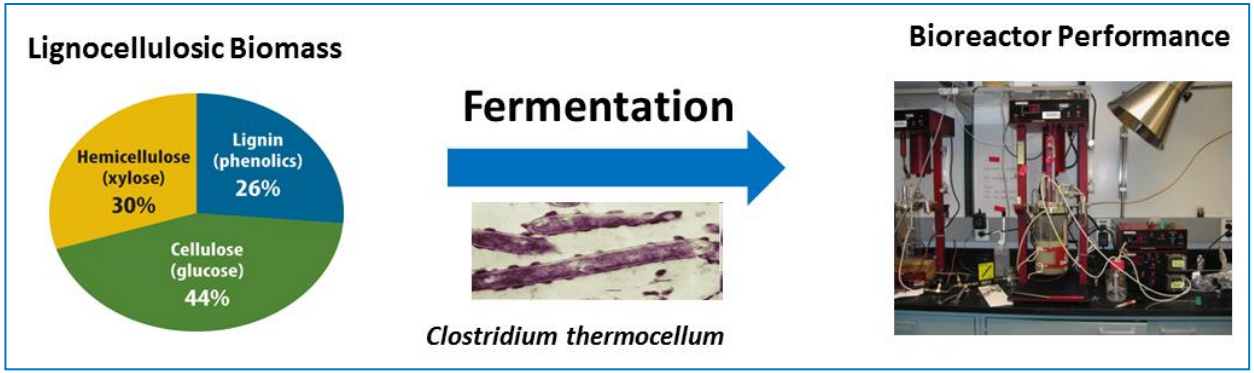
Characteristics	Units	2011 Status	2015 Target	2020 Target
Feedstock cost[a]	Cents/lb sugar	13.5	10	8
Yield of H_2 production from glucose	Mol H_2/mol glucose	**3.2**[b]	4	6
MEC production rate	L-H_2/L-reactor-day	-	1	4

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

Approach/Milestone

Task 1: Bioreactor Performance

- **Approach:** Optimize bioreactor in sequencing fed-batch mode by testing parameters such as cellulose loadings, hydraulic retention time, and liquid volume replacement and frequency using the cellulose-degrading bacterium *Clostridium thermocellum*.



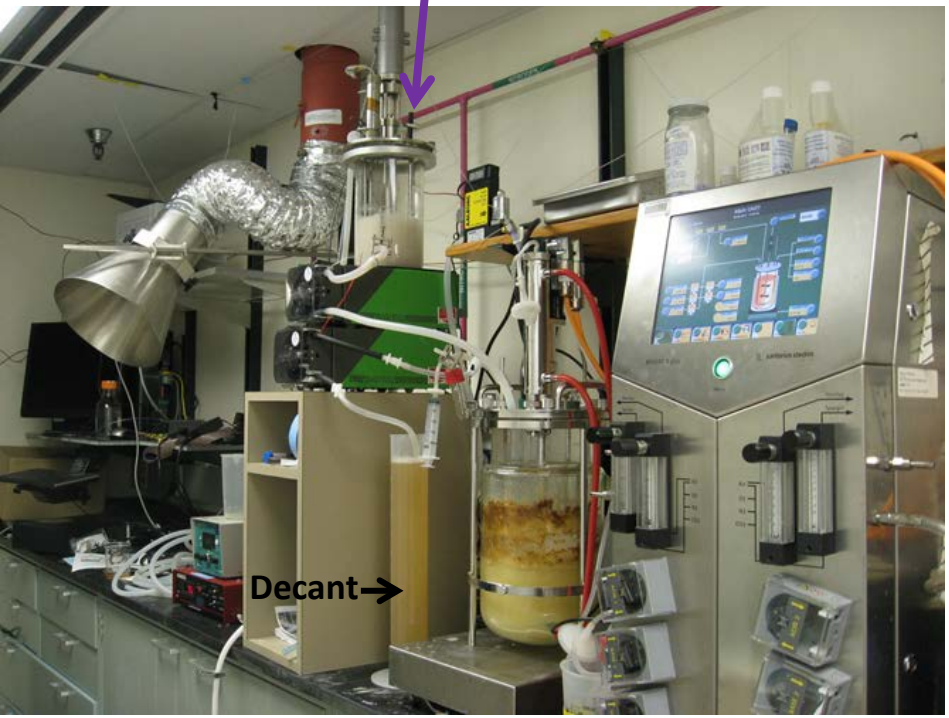
Lauren Magnusson

	Milestone	Completion Date	Status
3.2.1-1	Design and build a feed tank capable of delivering a constant concentration of cellulose over the course of fermentation under variable volume conditions. A successful design will be defined by a standard deviation of less than 10%, evaluated by dry weight of cellulose exiting the vessel.	1/13	Completed
3.2.1-2	In order to demonstrate feasibility of H_2 production from biomass, improve H_2 molar yield by 20% compared to the current baseline (1.1 mol H_2/mol hexose at 5 g/L/day cellulose feeding) by decreasing hydraulic retention time from 48 hr to 12-24 hr with more frequent liquid replacement using a constant cellulose loading of 5 g/L/day (*CPS Agreement Milestone*)	5/13	On track

Task 1 – Technical Accomplishments

Scale Up "Automated" Sequencing Fed-Batch Bioreactor

- Automation features: Fermentation module executes the Feed, React, Settle, and Decant stages in a 5-liter bioreactor (2-liter working volume).
- New feed-tank design (impeller, high RPM) affords better stirring and reliable cellulose delivery.



Feed, React, Settle, Decant

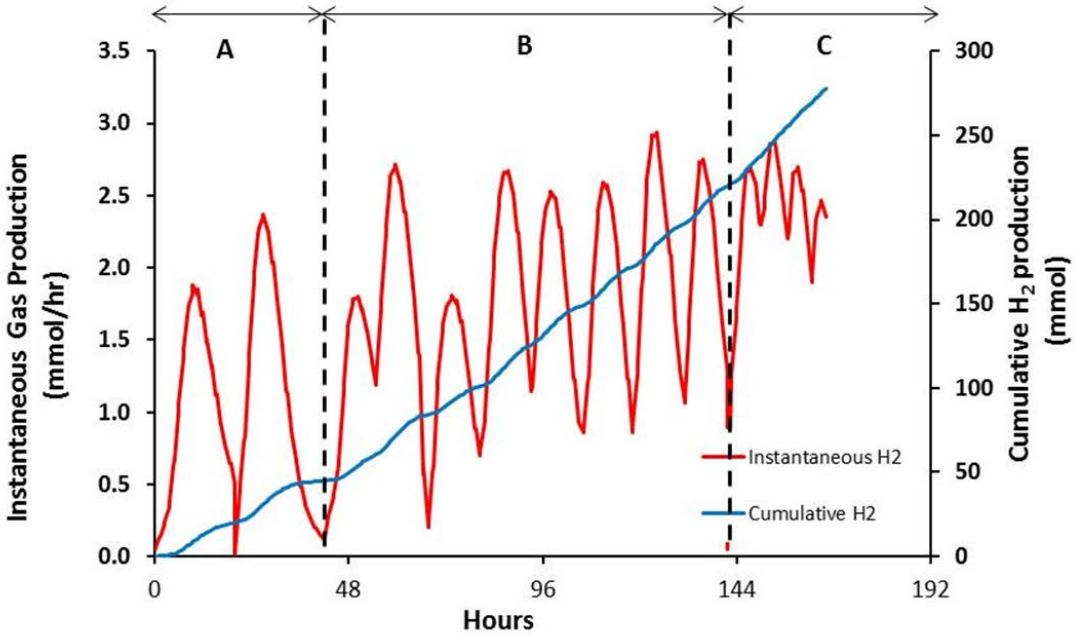
Cellulose settling retained acclimated microbes

C. thermocellum **are attached to cellulose, hence allowing the bulk of the growth medium to be replenished while retaining the fully acclimated microbes.**

Task 1 – Technical Accomplishments

Increased Rate of H_2 Production by Two-fold

- Cellulose loading at 2.5 g/L/day with varying hydraulic retention time (HRT) and amount and frequency of liquid medium replacement.
- HRT: the length of time to replace the working volume (2 L) in a bioreactor.



	HRT (h)	Liquid Displace-ment (%)	Interval (h)	Rate of H_2 Production (ml/L/d)
A	48	50%	24	**308 (1X)**
B	24	50%	12	**508 (1.7X)**
C	24	25%	6	**637 (2.1X)**

- New feed-tank design delivered consistent amount of cellulose, resulting in <10% variations in H_2 production in each feeding cycle (completed Milestone 3.2.1-1).
- **Increased rate of H_2 production by >2 fold with shorter HRT and more frequent liquid medium replacement.**

Task 1 – Technical Accomplishments

Increased Hydrogen Molar Yield by 53%

- Cellulose loading at 5 g/L/day with varying HRT and amount and frequency of liquid medium replacement.

HRT (h)	Liquid Displacement (%)	Interval (h)	H_2 Molar Yield (mol H_2/mol hexose)	Rate of H_2 Production (ml/L/d)
48	75%	36	0.79 (1X)	**764.6 (1x)**
48	25%	12	1.09 (1.38X)	**1057 (1.38X)**
12	50%	6	**1.21 (1.53X)**	**1179 (1.54X)**

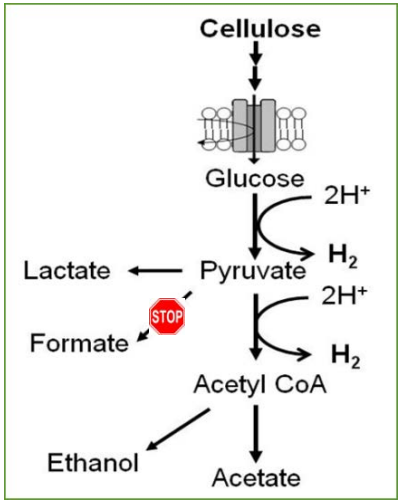
- **Increased both H_2 molar yield (by 53%) and the rate of H_2 production (by 54%; by 3.8-fold if compared to rate in the previous slide).**
- At fixed HRT, more frequent liquid replacement (interval) is key to improvement.
- HRT of 12-24 h may be more optimal since shorter HRT increases water usage.

	Milestone	Completion Date	Status
3.2.1-2	Improve H_2 molar yield by 20% compared to the current baseline (1.1 mol H_2/mol hexose at 5 g/L/day cellulose feeding) by decreasing HRT from 48 hr to 12-24 hr with more frequent liquid replacement (*CPS Agreement Milestone*)	5/13	On Track

Approach/Milestone

Task 2 – Develop Genetic Methods for Metabolic Engineering

- **Approach:** Redirect metabolic pathways to improve H_2 molar yield via the development of genetic methods. The immediate goal is to delete the pyruvate-to-formate step.



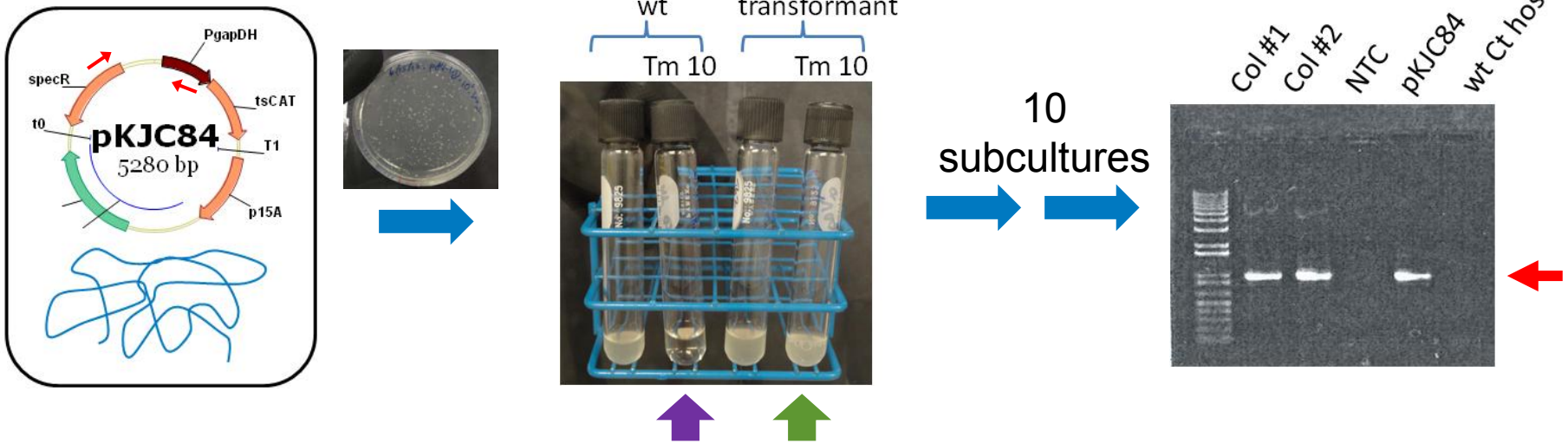
Katherine Chou

	Milestones	Completion Date	Status
3.2.2 (FY12)	Produce at least one plasmid for future use in knock-out strain production and other genetic manipulations, and demonstrate successful transformation into *C. thermocellum* via electroporation, with plasmid transfer confirmed by antibiotic selection and PCR (CPS Agreement Milestone 51458)	9/12	Completed
3.2.2-1 (FY13)	Produce a *C. thermocellum* mutant lacking its *hpt* gene to serve as the host for metabolic pathway mutant	4/13	Completed
3.2.2-2 (FY13)	Use Δ*hpt* mutant as the platform strain to further knockout the **pyruvate-to-formate** competing pathways. This approach aims at increasing H_2 molar yield by redirecting resources such as carbon and electrons towards H_2 production by reducing side-products production.	9/13	Completed

Task 2 – Technical Accomplishments

Generated a Stable Plasmid for Genetic Transformation

- NREL developed proprietary plasmid and stably transformed *C. thermocellum*.
  - We are one of the only two labs we know of that can transform *C. thermocellum*.
- PCR analysis verified presence of the plasmid (red arrow) which conferred growth in the antibiotic thiamphenicol (Tm, 10 µg/ml) (green arrow) whereas wild type (WT) cannot.

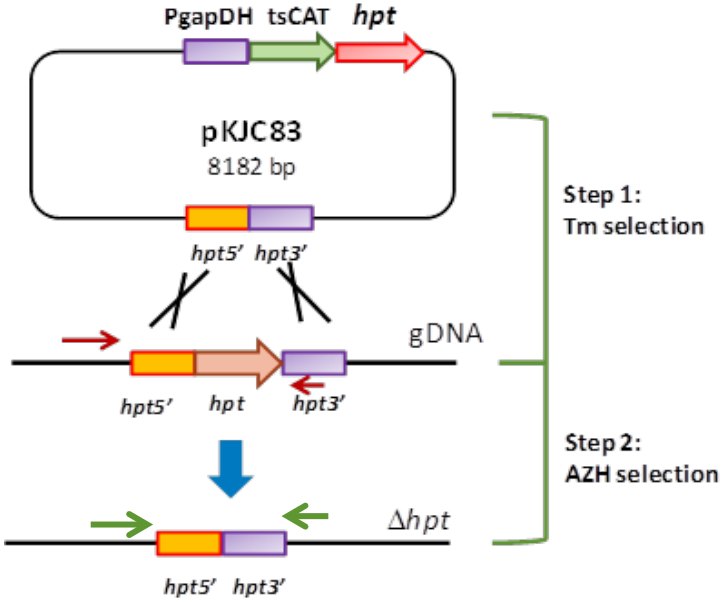


	Milestones	Completion Date	Status
3.2.2 (FY12)	Produce at least one plasmid for future use in knock-out strain production and other genetic manipulations, and demonstrate successful transformation into *C. thermocellum* via electroporation, with plasmid transfer confirmed by **antibiotic selection** and **PCR analysis** (*CPS Agreement Milestone*)	9/12	Completed

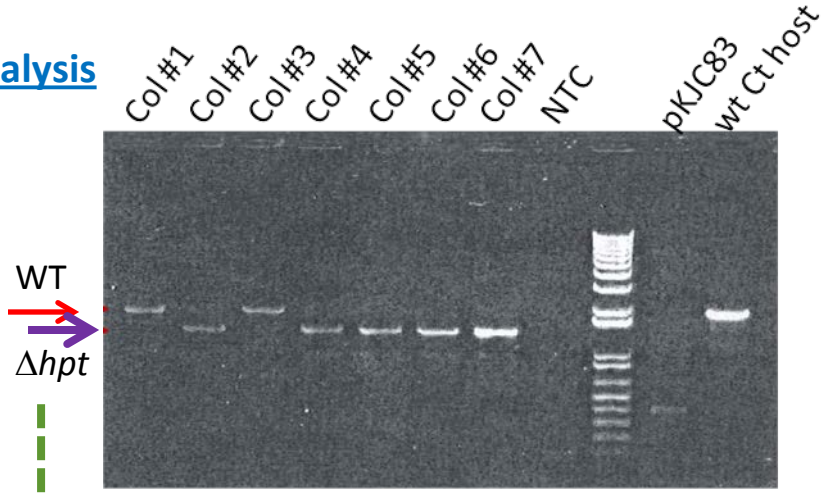
Task 2 – Technical Accomplishments

Generated Five *hpt* Knockout Mutants using Proprietary pKJC83 Plasmid

- *hpt* encodes hypoxanthine phosphoribosyl transferase, which converts AZH (8-azahypoxanthine) to a toxic product that kills the wild-type cells.
- Δ*hpt* mutants survive in AZH, which forms the basis for a Δ*hpt* counter-selection strategy.
- **Five Δ*hpt* mutants were verified by PCR (purple arrow, right figure) and DNA sequencing.**
- Δ*hpt* mutant will serve as the host to generate metabolic pathway mutants.



PCR Analysis



Only Δ*hpt* mutants will yield this PCR product (lower band; lanes 2, 4-7).

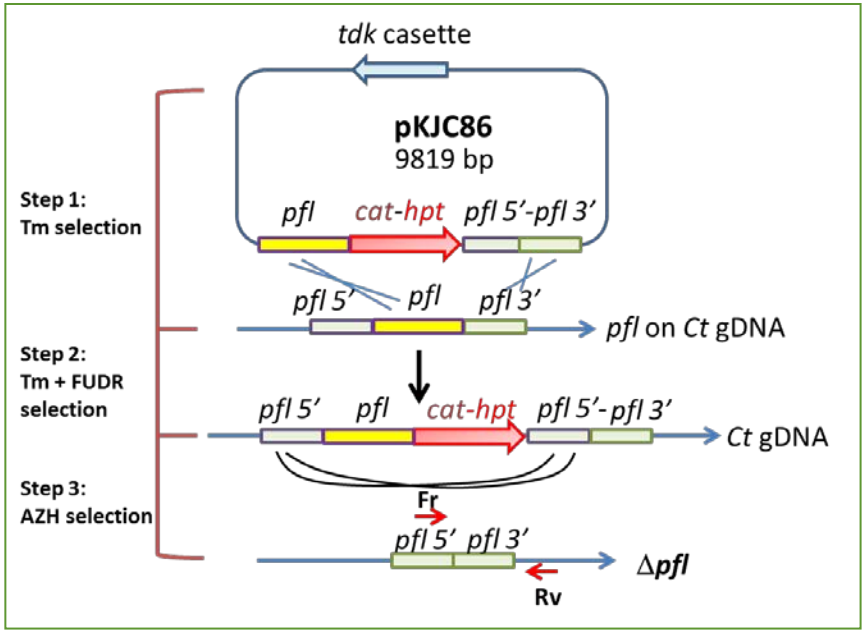
	Milestone	Completion Date	Status
3.2.2-1 (FY13)	Produce a *C. thermocellum* mutant lacking its *hpt* gene to serve as the host for metabolic pathway mutant	4/13	Completed

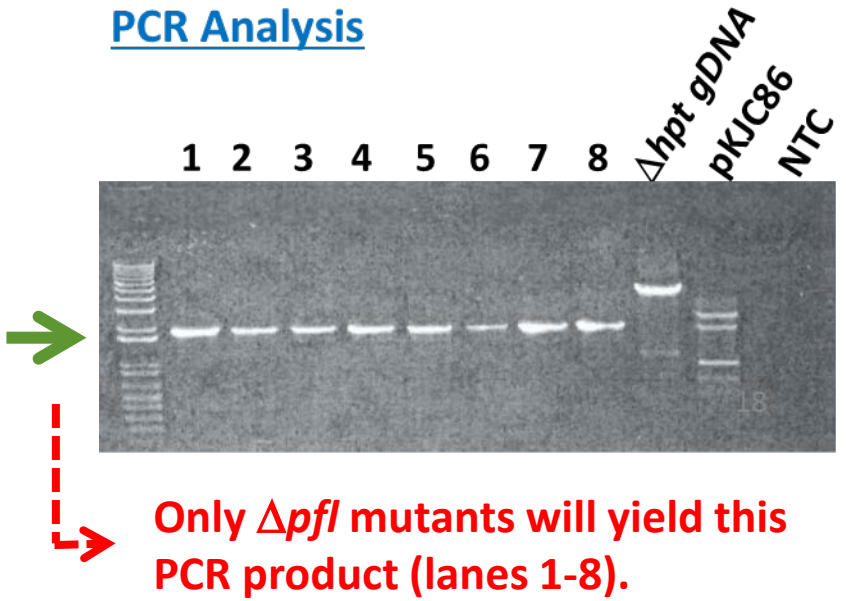
Task 2 – Technical Accomplishments

Generated Eight Pyruvate-to-formate (PFL) Pathway Mutants

Generated **eight** Δ*pfl* mutants, verified by PCR using Δ*hpt* mutant as the host (green arrow in the right figure).

- pKJC86 plasmid design included both *hpt* and *tdk* (thymidine kinase) genes. *tdk* leads to toxicity in fluoro-deoxyuracil (FUDR) – a double selection strategy (combining with AZH).



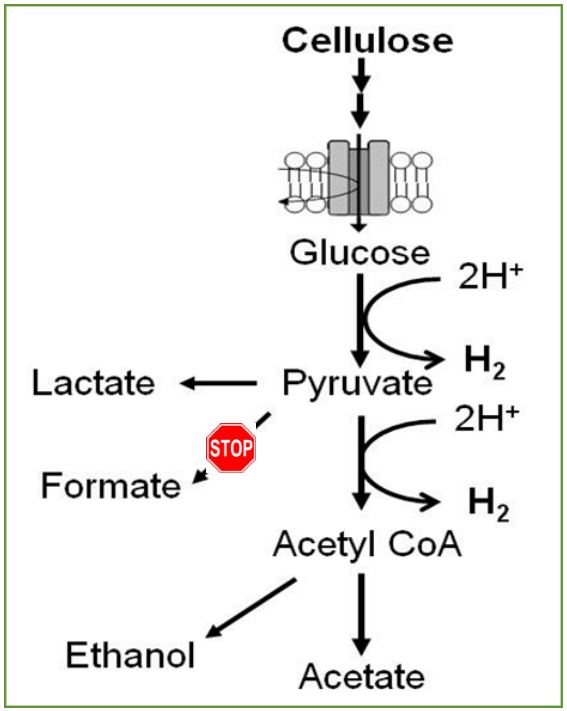


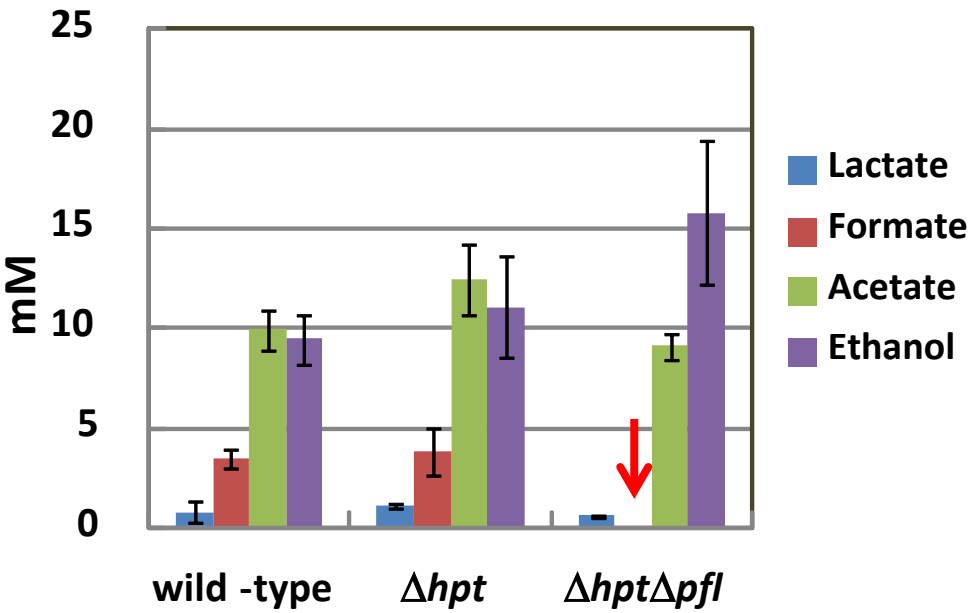
	Milestone	Completion Date	Status
3.2.2-2 (FY13)	Use Δ*hpt* mutant as the platform strain to further knockout the pyruvate-to-formate competing pathways aims at increasing H_2 molar yield by redirecting carbon and electrons toward H_2 production.	9/13	**Completed**

Task 2 – Technical Accomplishments

Confirmed Δ*Pfl* mutant by Metabolite Analysis

- Δ*pfl* mutant did not produce formate as expected based on deletion of *pfl* gene encoding the pyruvate-to-formate pathway.
- Δ*pfl* mutant produced ~ **60% more ethanol** than the parent strains.
- Knocking out the ethanol pathway may increase H_2 molar yield in future experiments since both pathways compete for the reductant NAD(P)H.



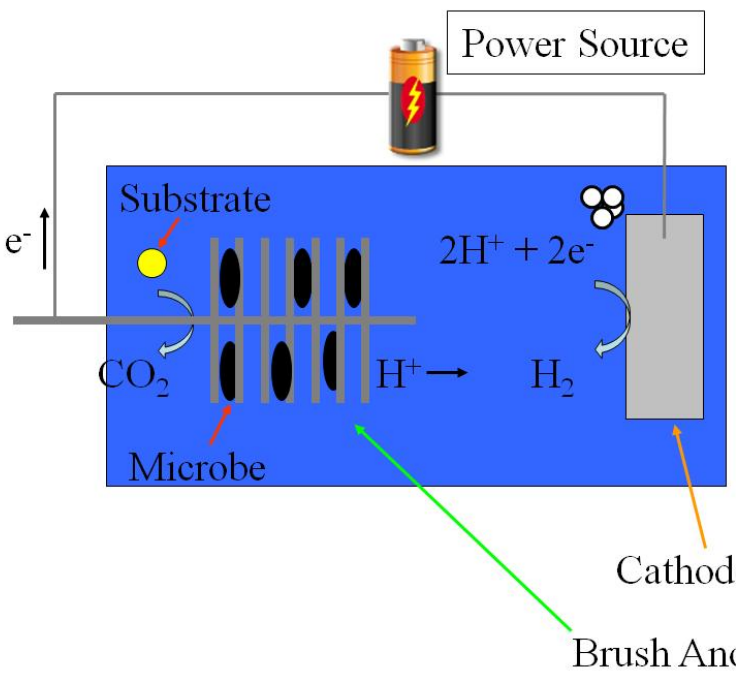


- Work is underway to determine H_2 production from various strains in bioreactors.

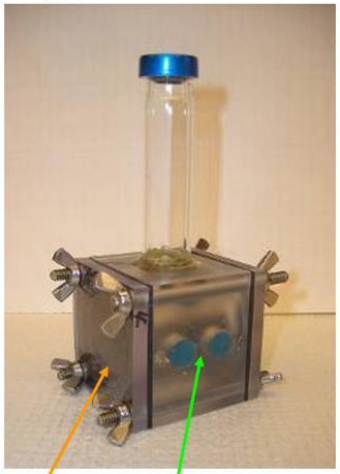
Approach/Milestone

Task 3 – Electrochemically Assisted Microbial Fermentation

-Produce H_2 -Non-spontaneous (+ΔG) reaction



single-chamber MEC

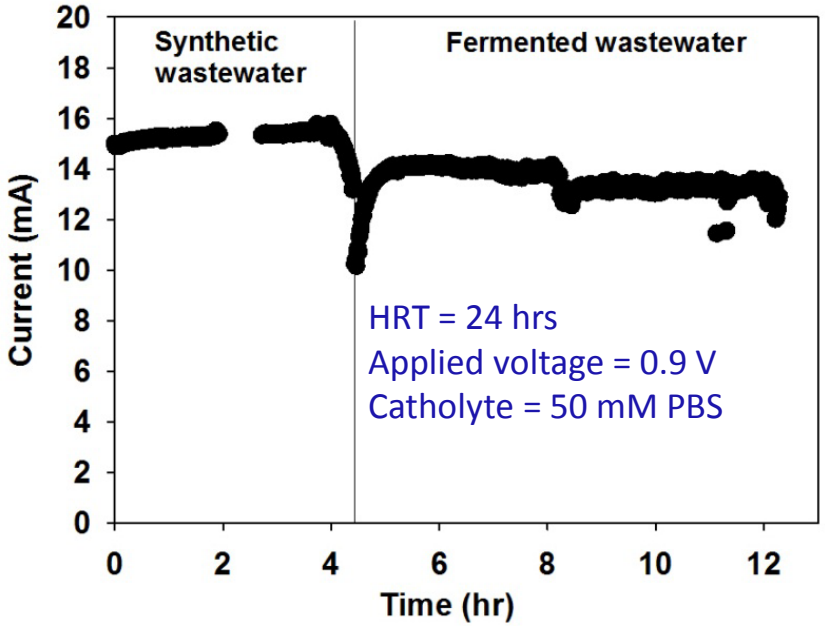


Jooyoun Nam

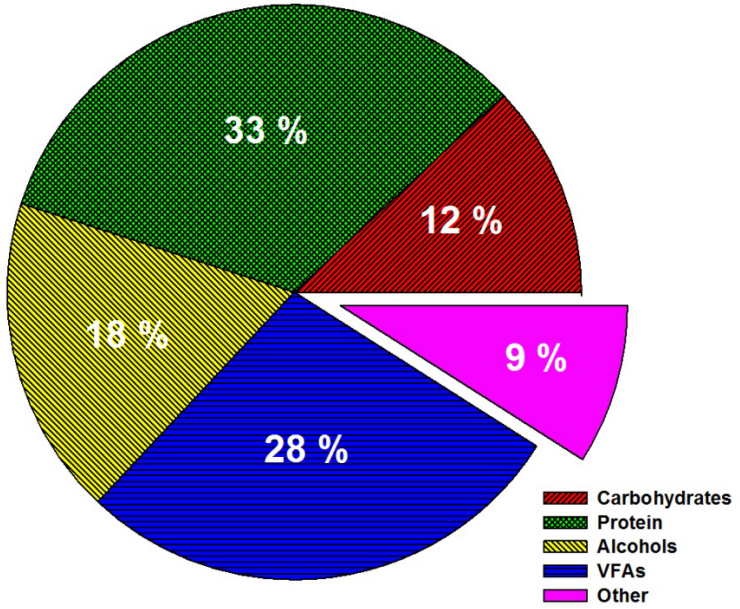
	Milestones	Completion Date	Status
3.2.3 (FY12)	Correlate removal of the subcomponents of the NREL fermentation effluent with current density and H_2 production	9/12	Completed
3.2.3-1 (FY13)	Build prototype MREC reactor and evaluate H_2 production using NREL fermentation effluent with zero electrical grid energy; demonstrate production rate of $\geq$0.5 L H_2 L^{-1} reactor day $^{-1}$ over 3 hydraulic retention times with continuous flow	9/13	On Track

Task 3.1 – Technical Accomplishments

Hydrogen Generation from Fermentation Wastewater



Fermentation Effluent Composition

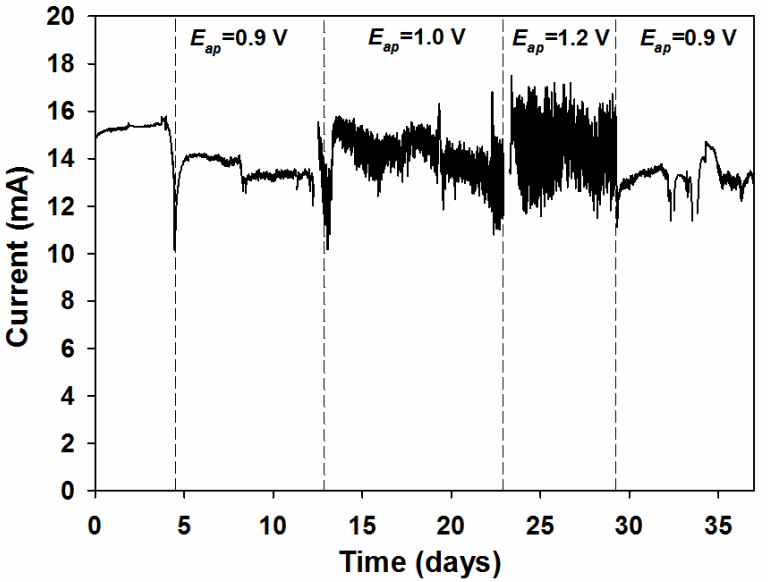


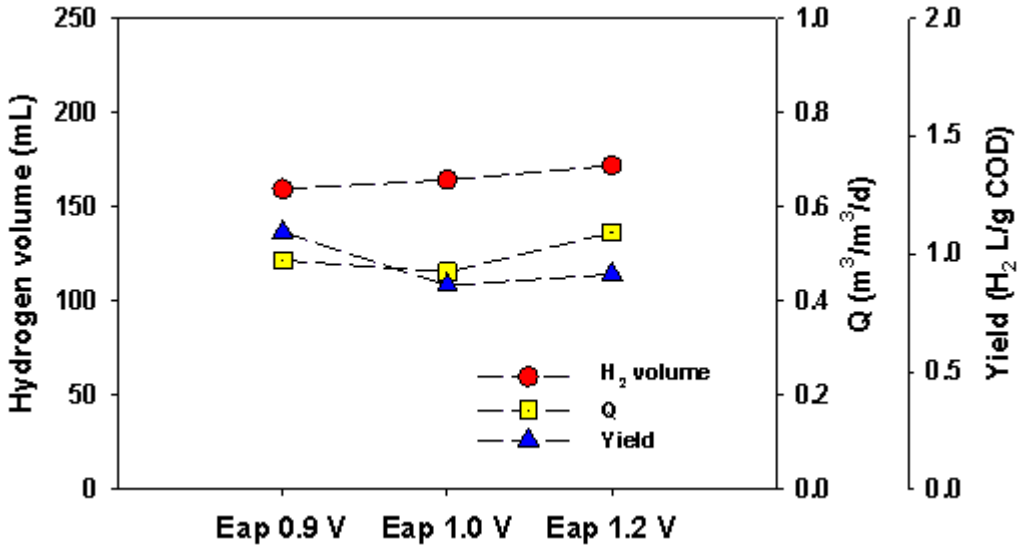
- Lower performance with fermentation wastewater (ww) due to substrate complexity
- **Current**: Synthetic ww = 51 A/m^3; Fermented ww = 44 A/m^3 (no protein in synthetic ww).
- **COD (chemical oxygen demand) removal**: Synthetic ww = 87%; Fermented ww = 73%.
- **Gas volume**: Synthetic = 183 mL; Fermented = 159 mL
- Gas production rates: Synthetic, 0.6 L-H_2 L^{-1} d^{-1} ; Fermented, 0.5 L-H_2 L^{-1} d^{-1}.

➢ **Protein removal (48%) was lower than alcohols and VFAs (>90%) and carbohydrate (89 %).**

Task 3.2 & 3.3 – Technical Accomplishments

Different Applied Voltages, Gas Production Rates



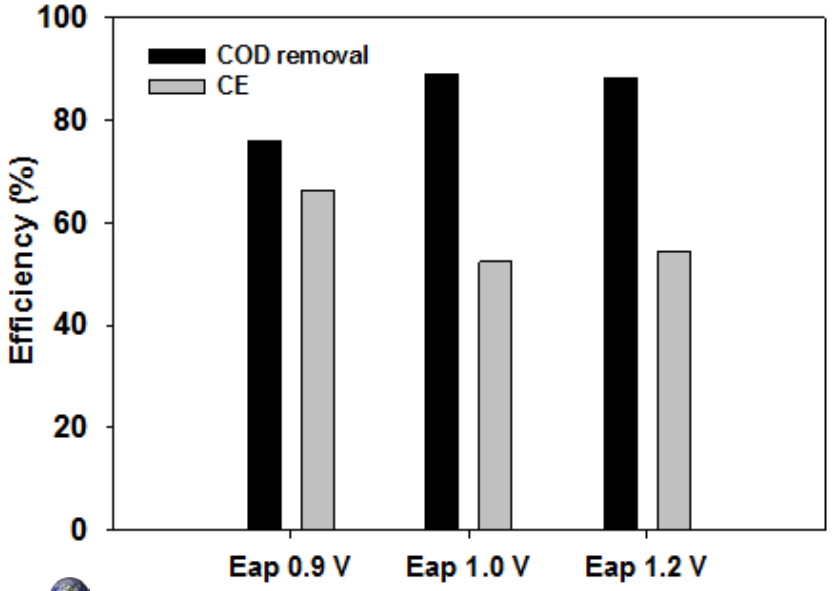


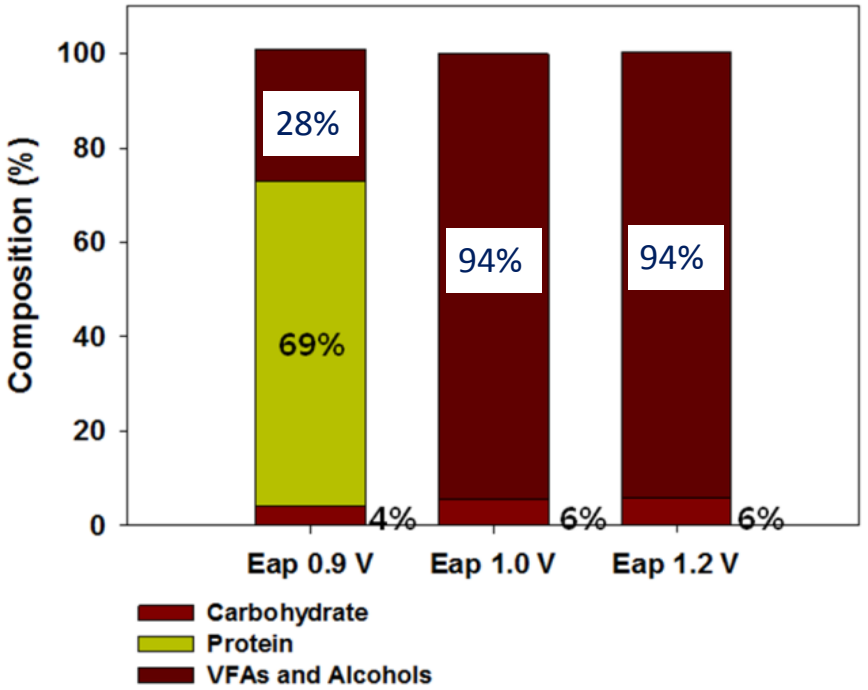
- Higher applied voltages resulted in more positive anode potentials which reduces performance
  - (P_{An}= –429±4 mV at E_{ap}= 0.9 V, P_{An}= –338±38 mV at E_{ap}= 1.0 V, and P_{An}= –113±38 mV at E_{ap}= 1.2 V).
  - Cathode potentials relatively constant (from –997 to –1031 mV).
- Current fluctuated at E_{ap}= ≥1.0 V. Likely due to substrate depletion near the reactor outlet
- Energy recovery based on electrical energy added and hydrogen gas recovered (η_E=223%) was highest at E_{ap}=0.9 V; overall energy recovery that includes the substrates (η_{E+S}) was 64%.

➢ **More hydrogen gas was generated at increased applied voltages, but H_2 yields were lower.** Highest H_2 yield was 1.1 H_2 L/g COD at E_{ap}= 0.9 V.

Task 3 – Technical Accomplishments

Removal of Subcomponents in Effluent at Different Applied Voltages





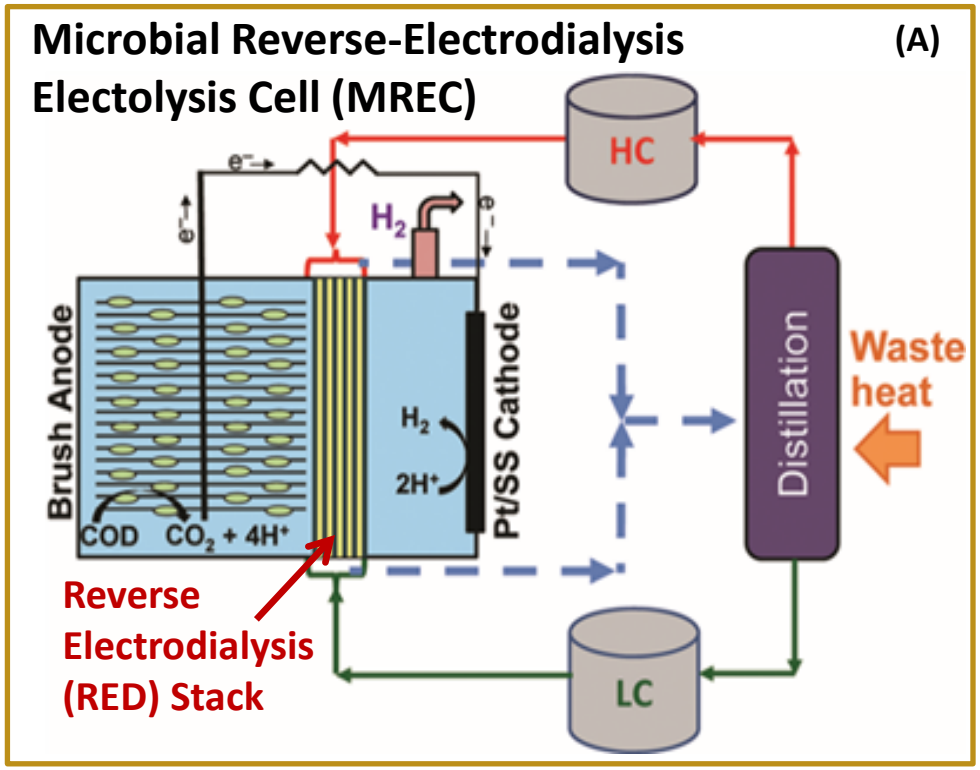
- Higher applied voltages increased COD removal, but lowered coulombic efficiencies (CE).
- Protein was almost completely removed at the higher applied voltages.

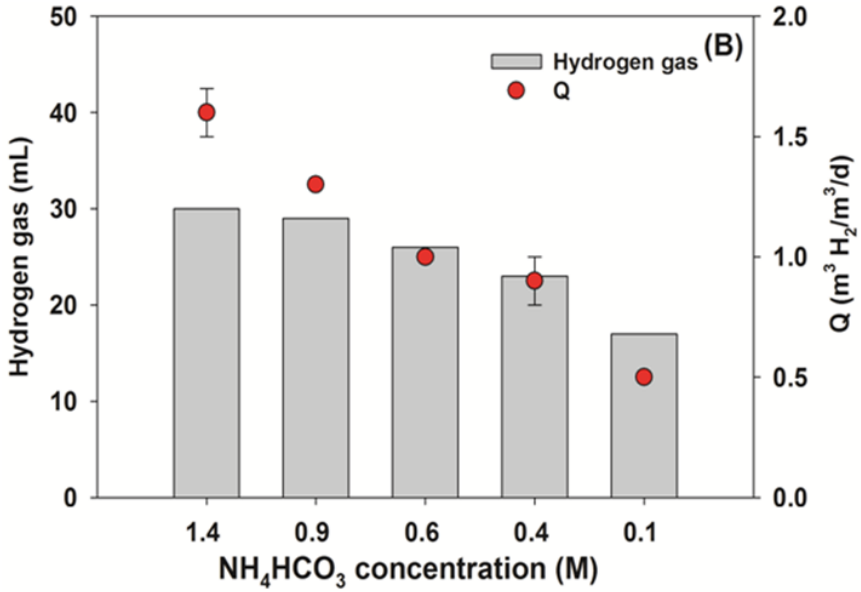
➢ **As CEs were lower at higher applied voltage, degraded protein seemed to be used more for cell growth than electricity generation.**

	Milestone	Completion Date	Status
3.2.3 (FY12)	Correlate removal of the subcomponents of the NREL fermentation effluent with current density and H_2 production	9/12	Completed

Task 3 – Technical Accomplishments

Hydrogen Generation in an MREC – No External Power Source





Decreasing HC concentration (1.4 M to 0.1 M) with fixed LC solution (distilled water)

- **Power source (A)**: RED stack using high concentration (HC) and low concentrations (LC) of Ammonium bicarbonate, NH_4HCO_3, which can be regenerated. (No electrical grid energy used).
- **Performance (B):** Gas recovery ranged from 30 mL-H_2 to 17 mL-H_2 (93–94% H_2, 6–7% CO_2) [bars].
- Gas production rate ranged from 1.6 m^3 H_2/m^3·d to 0.5 m^3 H_2/m^3·d [circles] [60 mL reactor, both chambers].
- Coulombic efficiency using acetate $'_{CE}$ = 72% to 50%. Maximum Yield = 3.4 mol H_2/mol acetate.
- **Current and H_2 were successfully generated in the MREC using NH_4HCO_3 solutions and no external power supply. Construction of a larger reactor (several hundred mL, with multiple anodes) for testing NREL fermentation effluent for H_2 production is ongoing toward meeting Milestone 3.2.3-1 (9/13).**

Collaborations

- **Task 1 (Bioreactor):**

  Dr. Ali Mohagheghi, National Bioenergy Center at NREL (biomass pretreatment and characterization).

- **Task 2 (Genetic Methods):**

  Drs. David Levin and Richard Sparling at the University of Manitoba, Canada. NREL is an international collaborator of the Genome Canada Grant award to co-develop genetic tools for pathway engineering in *C. thermocellum*.

- **Task 3 (MEC):**

  Dr. Bruce Logan, Penn State University (microbial electrolysis cells to improve H_2 molar yield). Task 3 was cost shared by other projects of the investigator.

Proposed Work

Task 1 (NREL):

- Complete the H_2 production profiles from cellulose (rate, yield) using shorter HRT, varying frequency and volume of medium replacement in sequencing fed batch reactor (FY13, FY14)
- Guided by the optimal parameters derived from above, determine H_2 production rate and H_2 molar yield using lignocellulosic biomass (FY14)

Task 2 (NREL):

- Test the pyruvate-to-formate pathway mutants for H_2 production rates, H_2 molar yield, and metabolites in bioreactors (FY13, FY14).
- Generate double knockout mutants by also deleting either lactic acid or the ethanol pathway (FY14).
- Using the optimized bioreactor paramters, test the above mutants for improved H_2 molar yield (FY14).

Task 3 (Penn State):

- Design, build, and test larger prototype MREC reactor with acetate solution for scalability (FY13).
- Evaluate performance of new MREC with NREL fermentation effluent as to H_2 yields, H_2 production rates, and gas production (FY13).
- Acclimate MECs separately to protein (bovine serum albumin) and acetate in batch-fed reactors, followed by continuous mode, and then with NREL fermentation effluent in terms of H_2 yields and gas production rates (FY14).

Summary

Task 1:

- Designed and built a feed tank that delivered cellulose reliably to the bioreactors, with less than 10% standard deviation of H_2 output in the bioreactor.
- Increased rate of H_2 production by 3.8-fold and H_2 molar yield by 53% via manipulating HRT, frequency, and liquid volume replacement, at either 2.5 g/L or 5 g/L cellulose loading.

Task 2:

- Generated proprietary plasmids and optimized protocols for reliable transformation in *C. thermocellum*.
- Produced five *C. thermocellum* mutants lacking the *hpt* gene, which served as the base strain for targeted pathway mutagenesis without leaving an antibiotic marker.
- Produced eight pyruvate-to-formate pathway mutants with ~60% increase in ethanol production.

Task 3:

- Evaluated performance of MEC using fermentation effluent in terms of H_2 yields, H_2 production rates, and gas composition.
- Verified boosted voltages can increase protein utilization in the fermentation effluent.
- Successfully produced H_2 gas without an external energy input in an MREC using salinity gradient energy.