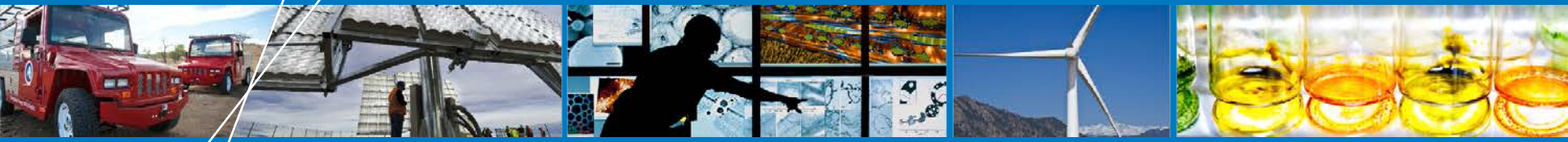
NREL
NATIONAL RENEWABLE ENERGY LABORATORY

Fermentation and Electrohydrogenic Approaches to Hydrogen Production



2014 Annual Merit Review and Peer Evaluation Meeting; June 19, 2014

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: FY 05 (not funded in FY 06)
- Project End Date: 10/2014*

Barriers

Barriers addressed

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

Budget

- FY13 DOE Funding: $410K
- Planned FY14 DOE Funding: $470K
- Total Project Value: $3.13M (includes $554K subcontract)

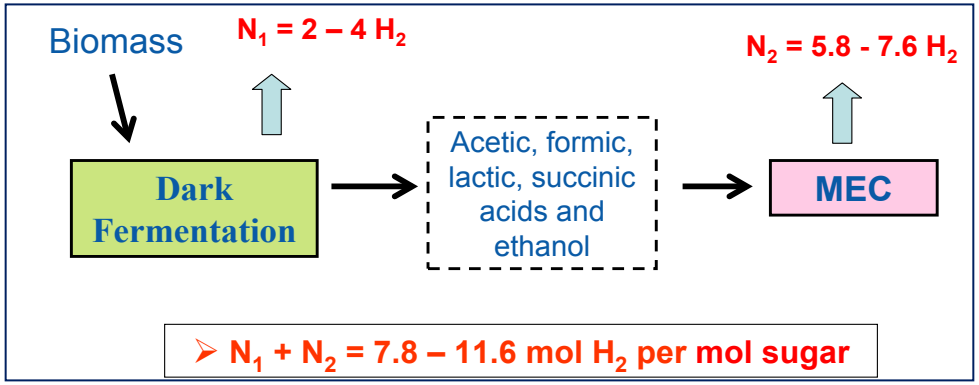
Partners

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

*Project continuation and direction determined annually by DOE

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).
- Hydrogen molar yield (AX) (N_1 & N_2: mol H_2/mol hexose): via genetic engineering (Task 2) and integration with Microbial Electrolysis Cell (MEC) (Task 3)

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.

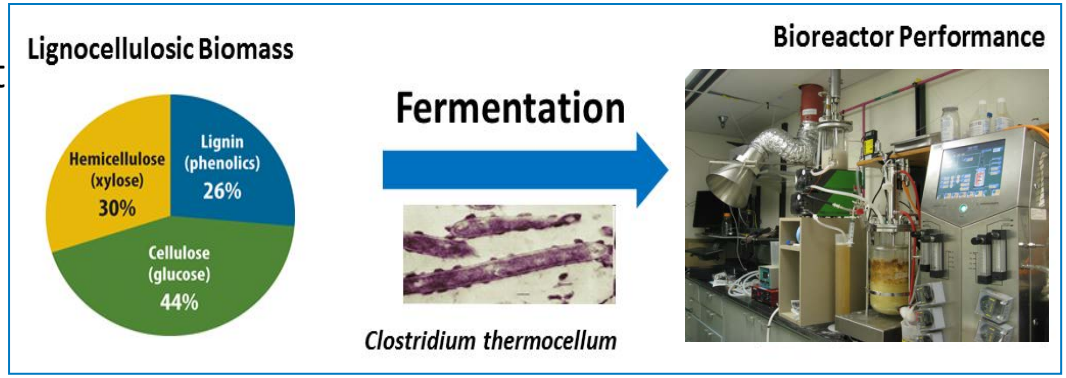
b. Low carbon substrate loading (1 g/L) led to high H_2 molar yield.

Approach/Milestone

Task 1: Bioreactor Performance

Lauren Magnusson

- **Approach:** Optimize bioreactor in fed-batch mode by testing parameters such as lignocellulose loadings, hydraulic retention time (HRT), and liquid volume replacement and frequency, using the cellulose-degrading bacterium *Clostridium thermocellum*, the fastest cellulose-degrader.
- Leverage the DOE BETO investment in biomass pretreatment technologies.

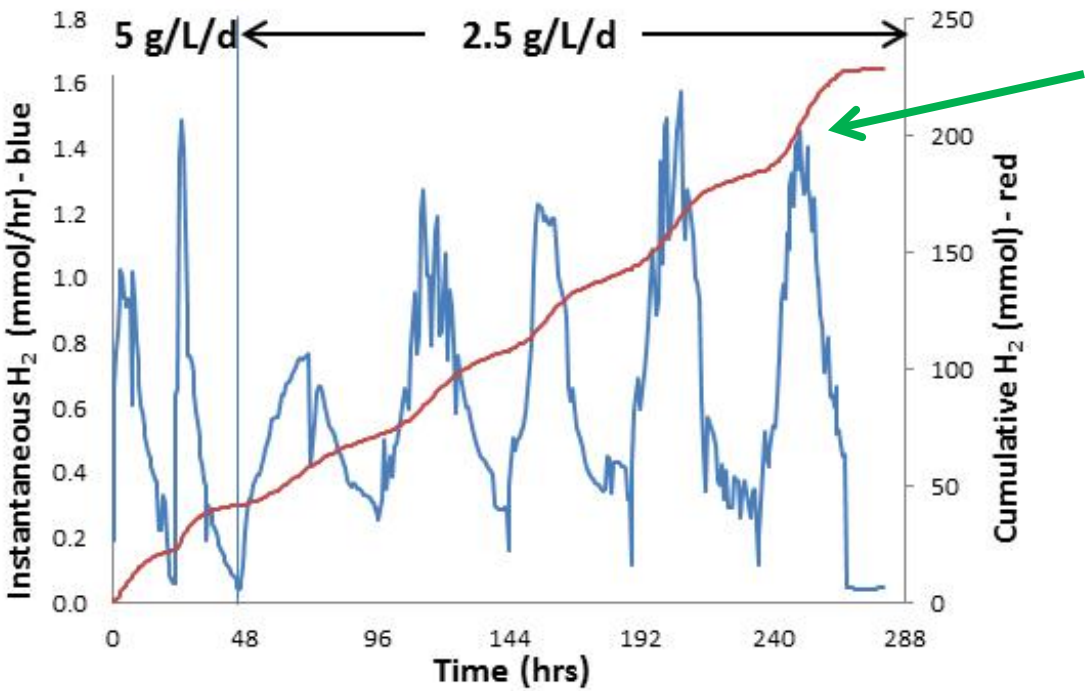


	FY14 Milestone (all regular)	Completion Date	Status
Q1	Demonstrate the potential to overcome fermentation inhibition from accumulation of lignin and other by-products by obtaining a H_2 production rate of 300 mL H_2/L/day by fermentation of corn stover lignocellulose (at 5 g/L loading based on cellulose content) after at least 6 rounds of feeding in a sequencing fed-batch bioreactor, compared to a 2010 baseline rate of 871 mL H_2/L/d in batch mode with no repeated feeding, where inhibition was not an issue, via optimizing hydraulic retention time and frequency of liquid replacement.	12/13	Complete
Q3	Increase the rate of H_2 production by fermentation 50% (based on 300 mL/L/d base rate) by using microbe that have been acclimated to degrade lignocellulose in the presence of lignin to address potential inhibition by the residual lignin.	6/14	Complete

Task 1 – Technical Accomplishments

H_2 from Corn Stover Lignocellulose via Fed-batch Fermentation

- Corn stover lignocellulose: 59% glucan, 3.9% xylan, and 27.5% lignin
- HRT 48 h and 50% liquid replacement every 24 h, with lignocellulose loading of 8.5 g/L (5 g/L **cellulose**) at 24 h (cycle 1-2) and 48 h (cycle 3-7).
- HRT: the length of time to replace the working volume (2 L) in a bioreactor.



By the 7th cycle, lignin content is ~16.3 g/L in the bioreactor, yet no inhibition was observed.

Cellulose g/L/d	Rate of H_2 Production (mL/L/d)	
	Average	Maximum
5	193	**476**
2.5	239	474.3

➢ **Summary: Complete FY14 Q1 milestone, using corn stover lignocellulose as the feedstock and verified no inhibitory effect from lignin buildup.**

Task 1 – Technical Accomplishments

H_2 from Corn Stover Lignocellulose –Reverse Feeding Regime

- Previous experiment led to higher average rate of H_2 when using lower lignocellulose loading, contrary to earlier findings
- Repeat with same HRT 48 h and 50% liquid replacement every 24 h, except with lignocellulose loading of 4.2 g/L (2.5 g/L **cellulose**) in cycles 1-4, followed by 8.5 g/L (5 g/L **cellulose**) in cycles 4-8 for acclimation.

Cellulose g/L/d	Rate of H_2 Production (mL/L/d)	
	Average	Maximum
2.5	266	566
5.0	466	**1102**

By the 8th cycle, lignin content is ~14 g/L in the bioreactor, yet no inhibition.

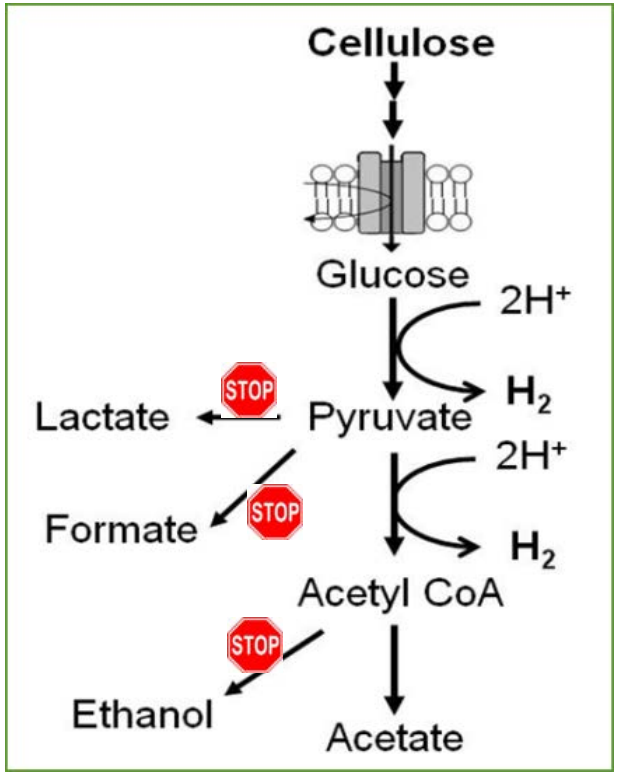
Settled lignocellulose retained acclimated microbes

- **Summary: Complete FY14 Q3 milestone: obtained average rate more than 450 mL/L/d benchmark with no inhibitory effect from lignin buildup.**
- **Yet total H_2 production is 62% of that produced by pure cellulose (5 g/L), suggesting some of the cellulose in "pretreated" corn stover is not fermentable, likely due to binding to lignin.**

Approach

Task 2 – Generate Metabolic Pathway Mutant in *C. thermocellum*

- **Approach:** Redirect metabolic pathways to improve H_2 molar yield via developing genetic methods.
- The goal in FY14 is to delete ethanol production in a mutant already lacking the pyruvate-to-formate step.
  - **A major breakthrough:** NREL developed proprietary tools and generated mutants: we are one of the three labs that can transform *C. thermocellum*.
  - The lactate and ethanol steps consume NADH, which could be directed toward H_2 production.
  - Goals in FY13-14 are to block the competing pathways for formate and ethanol production and demonstrate increased H_2.



Katherine Chou

Approach/Milestones

Task 2 – Develop Genetic Methods for Metabolic Engineering

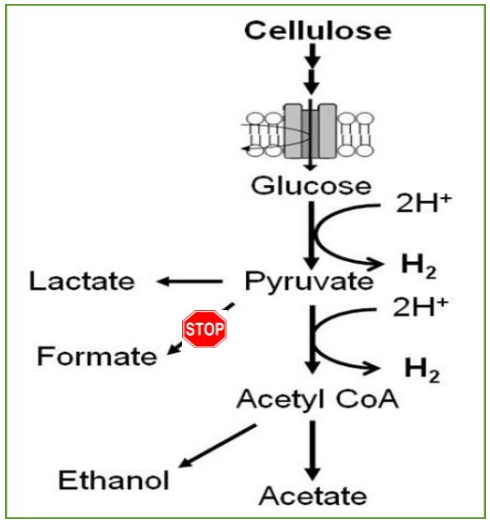
	FY13 Milestone – regular	Com-pletion Date	Status
3.2.2-2	Use Δ*hpt* mutant as the platform strain to further knockout the **pyruvate-to-formate** competing pathways. This approach aims at increasing H_2 molar yield by redirecting resources such as carbon and electrons towards H_2 production by reducing side-products production.	9/13	Complete
	FY14 Milestone – all regular		
Q2	Design and construct a plasmid followed by its transformation in the *Clostridium thermocellum* Delta(*hpt*) background aimed to delete the lactate dehydrogenase (LDH) competing pathway. This approach aims at increasing H_2 molar yield by re-directing resources such as electrons toward H_2 production while reducing the lactate side-product production	3/14	Interim milestone; Complete
Q3	Generate a delta(*hpt*) delta(*pfl*) delta(*adhE*) triple mutant of *Clostridium thermocellum*, missing the *adhE* gene encoding the ethanol pathways, and demonstrate 50% reduction in ethanol production, with genetic deletion verified by PCR and metabolite analysis. This approach aims at increasing H_2 molar yield by redirecting resources such as electrons towards H_2 production while reducing ethanol side-product production.	6/14	Delayed from Q2; on track
Q4	Increase H_2 output by 10% during fermentation in the delta(*hpt*) delta(*pfl*) delta(*adhE*) triple mutant of *Clostridium thermocellum*, missing the gene for the ethanol pathway, compared to the wild-type strain to verify that blocking competing pathway is a feasible approach toward increasing H_2 molar yield.	9/14	On Track

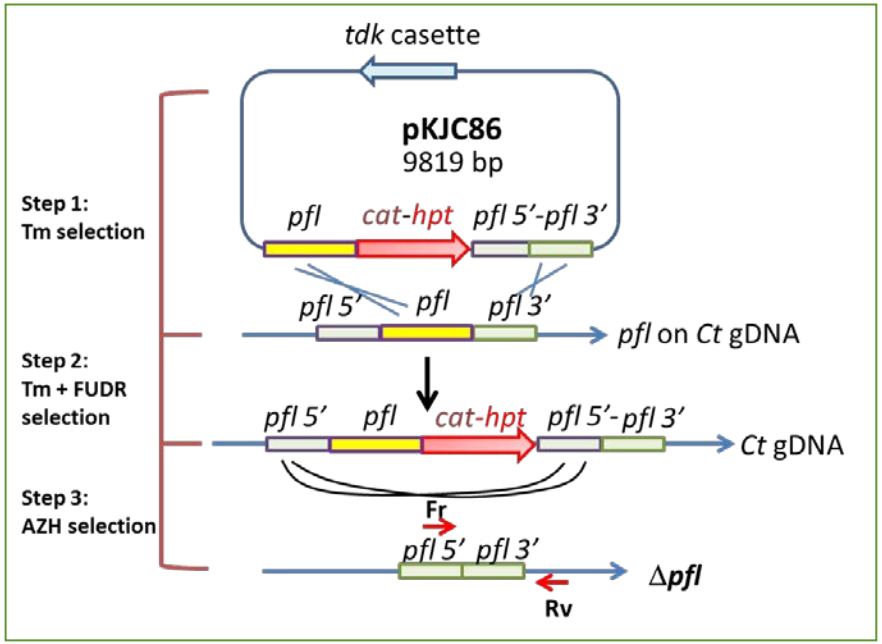
Task 2 – Technical Accomplishments: FY13 AMR

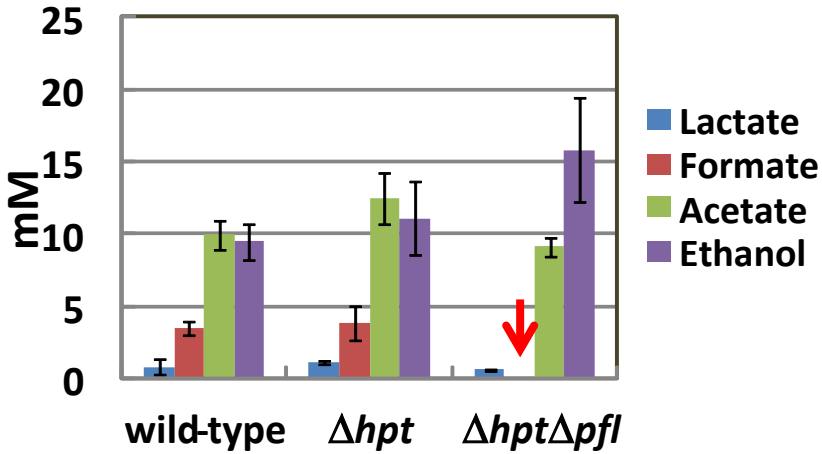
Generated Pyruvate-to-formate (PFL) Pathway Mutants

Generated Δ*pfl* mutants, verified by PCR and lack of formate production.

- pKJC86 plasmid design features both *hpt* (phosphoribosyl transferase) and *tdk* (thymidine kinase) genes. *hpt* leads to cellular toxicity in 8-azahypoxanthine (AZH) and *tdk* leads to cellular toxicity in fluoro-deoxyuracil (FUDR) – a powerful double selection strategy.





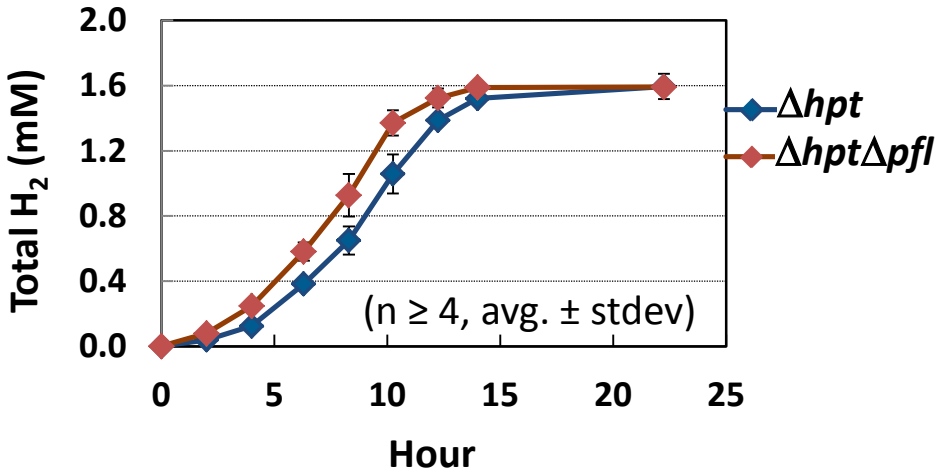


➢ **Summary:** Δ*pfl* mutant generated and it produced **1.6-fold more ethanol** than control.

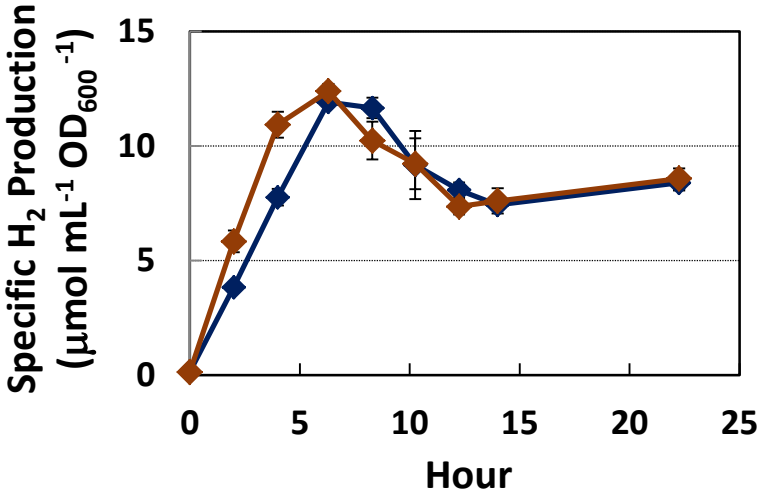
Task 2 – Technical Accomplishments

Increased H_2 and Lactate in the PFL (formate) Pathway Mutant

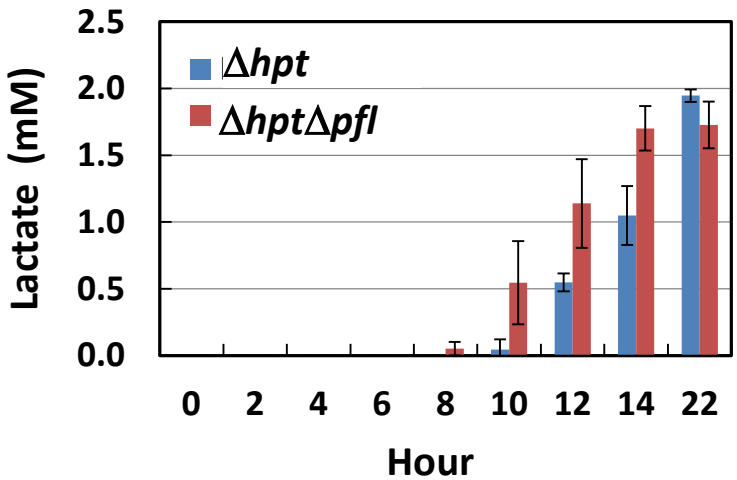
(A) Total H_2 Production



(B) Specific Rate of H_2 Production



(C) Lactate byproduct



PFL Mutant

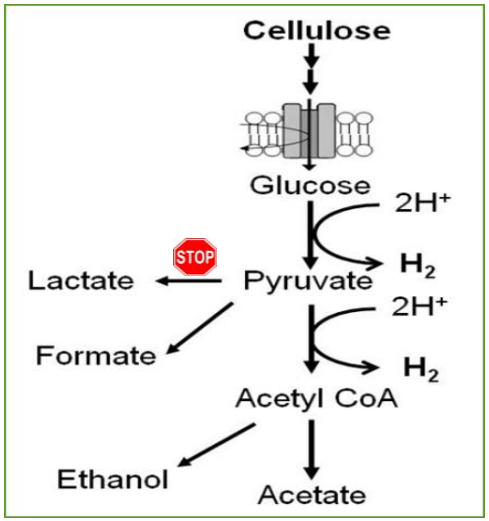
- Increased H_2 production (both total and specific rate) in log-phase culture.
- Significant increases in both lactate (> 2 fold) and ethanol (~1.6 fold).
- **Complete FY13 Q4 milestone.**

➢ **Summary:** Blocking both lactate and ethanol production could increase H_2 yield.

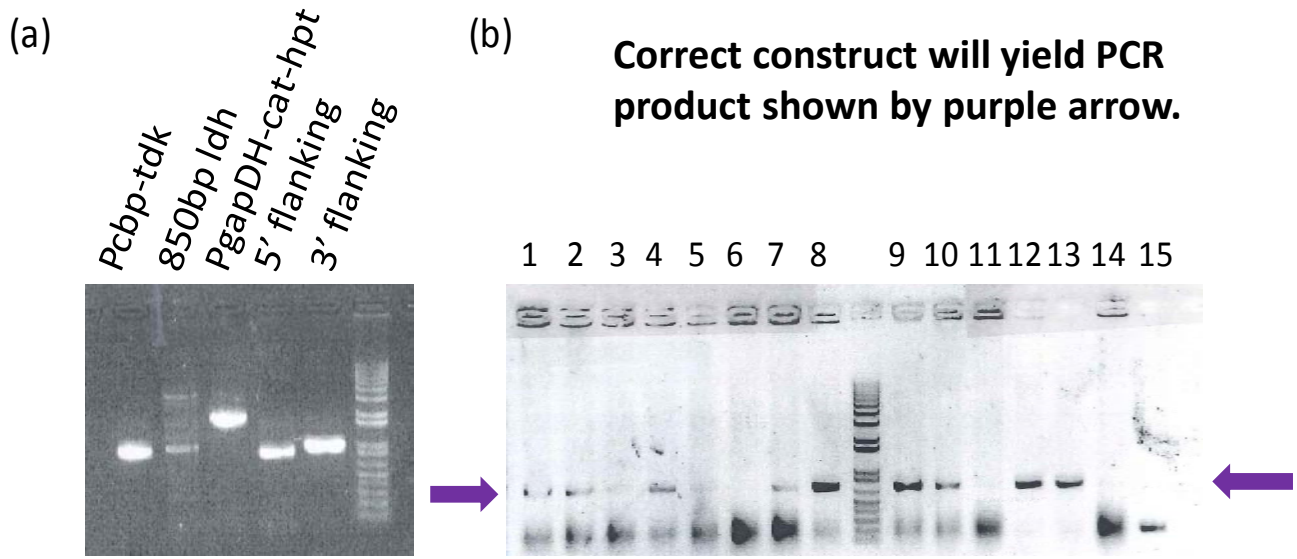
Task 2 – Technical Accomplishments

Design Plasmid to Knockout Lactate Production

- Constructed a plasmid aimed to knockout lactate production by deleting the gene encoding lactate dehydrogenase (LDH) – **complete FY14 Q2 Interim Milestone.**
- The plasmid features (1) *tdk* and *hpt* genes for counter-selection, (2) LDH gene and its 5' and 3' flanking regions for deletion, and (3) thiamphenicol antibiotic marker *cat* gene, all verified by PCR and commercial sequencing.
- *C. thermocellum* has been successfully transformed with counter-selections underway to generate LDH mutant.



PCR Analysis



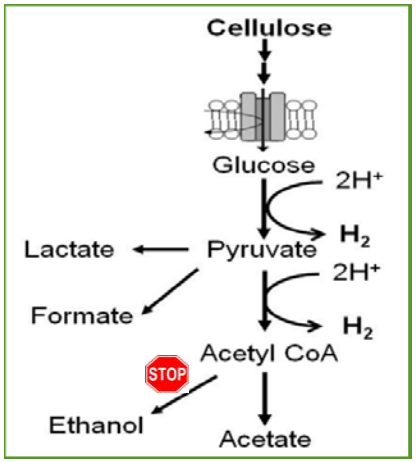
Correct construct will yield PCR product shown by purple arrow.

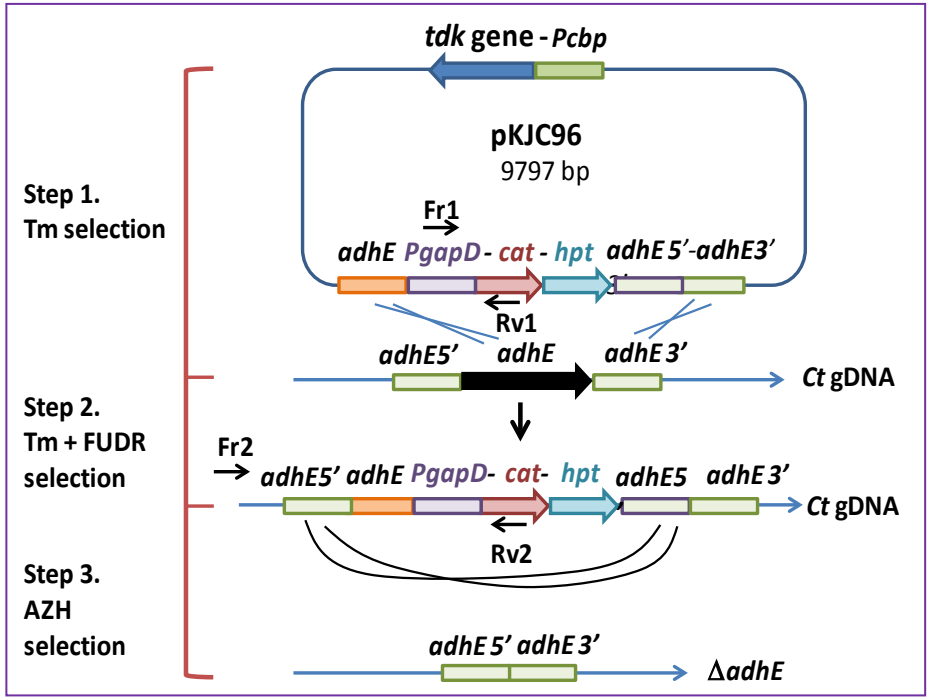
➢ **Summary:** Generation of LDH mutant is underway

Task 2 – Technical Accomplishments

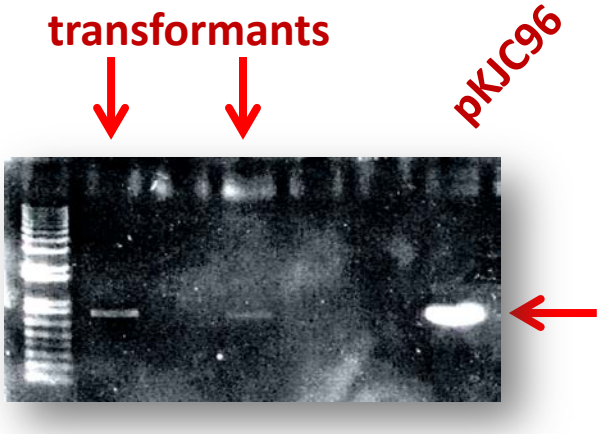
Generate the Ethanol-competing Pathway Mutant – on going

- Constructed pKJC96 plasmid aimed to knockout ethanol production by deleting the gene encoding the bifunctional ethanol dehydrogenase (*adhE*).
- Transformation (step 1) has been verified by PCR. Tm + FUDR dual selection (step 2), followed by AZH counter selection (step 3) is underway.





Verified Step 1 via PCR using primers Fr1/Rv1



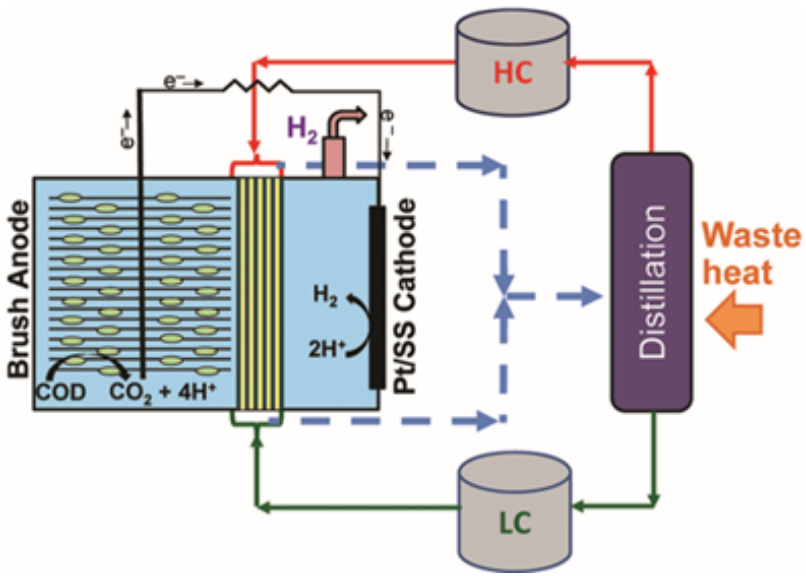
➢ **Summary:** Ethanol mutant is underway, Q3 and Q4 milestones on track.

Approach/Milestone

Task 3 – Electrochemically Assisted Microbial Fermentation

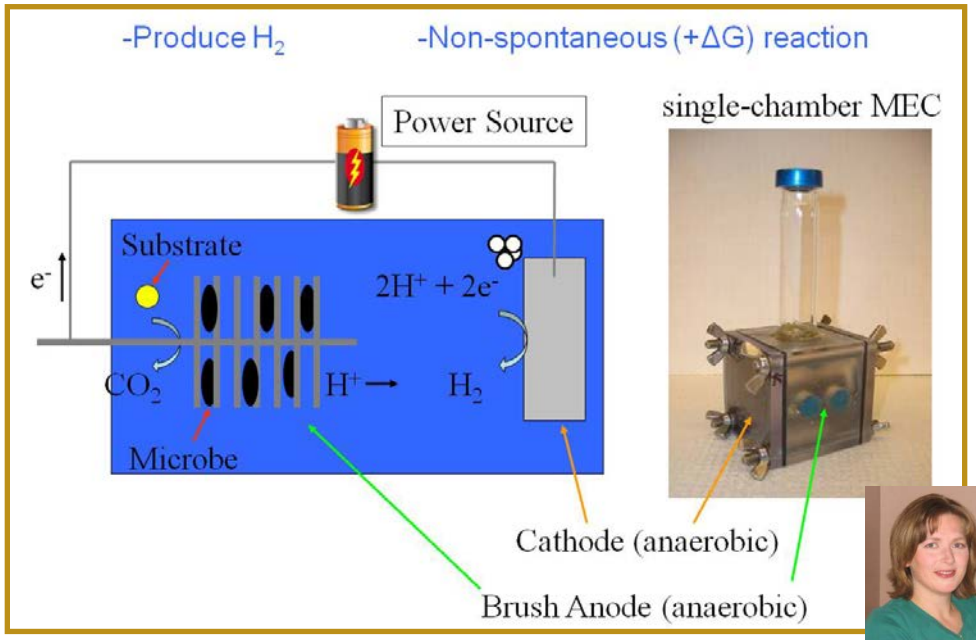
Microbial Reverse-electrodialysis Electrolysis Cell (MREC)

-salinity gradient energy drives the reaction



Microbial Electrolysis Cell (MEC)

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

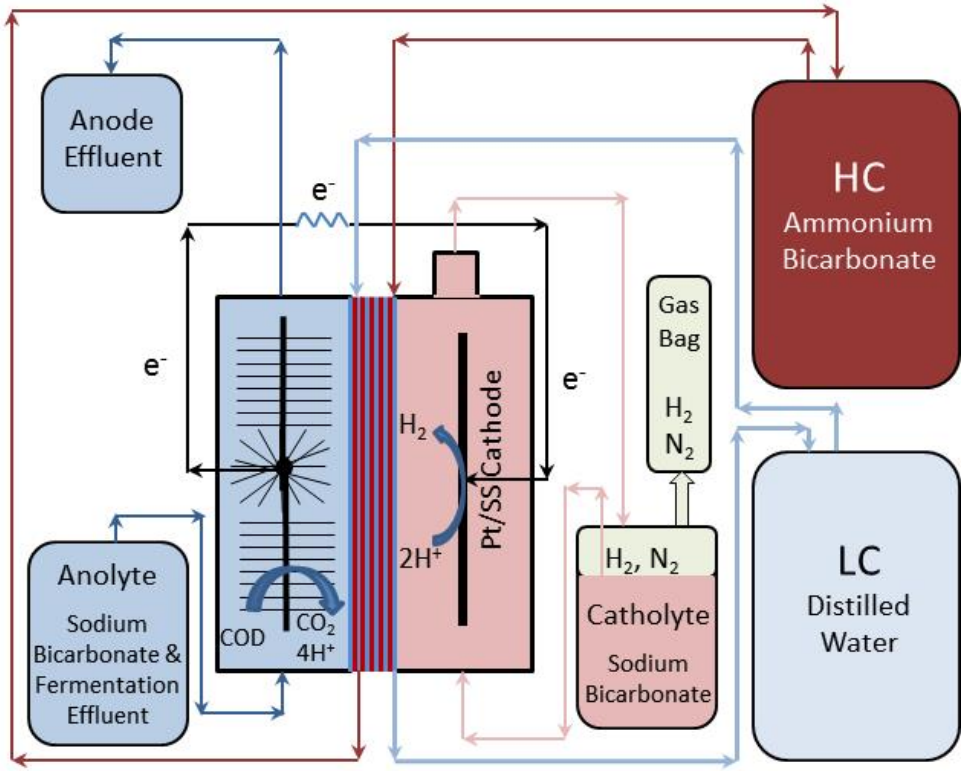


Valerie Watson

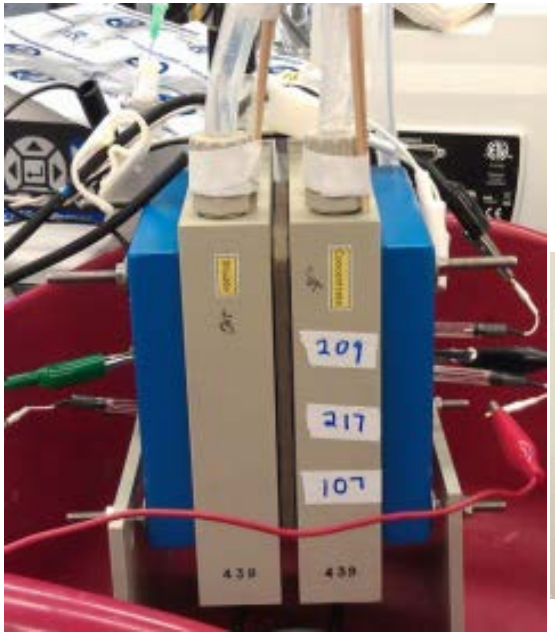
	Milestones (all regular)	Completion Date	Status
3.2.3-1 (FY13)	Build prototype MREC reactor and evaluate H_2 production using NREL fermentation effluent with zero electrical grid energy and demonstrate a H_2 production rate of at least 0.5 L $H_2/L_{reactor}$/day over 3 hydraulic retention times.	11/13	Complete; (delayed from 9/13)
Q4 (FY14)	Conduct continuous flow test with MEC individually acclimated to protein and acetate, and demonstrate > 80% protein removal, 0.5 L $H_2/L_{reactor}$/day over 3 hydraulic retention times.	9/14	On Track

Task 3 – Technical Accomplishments

MREC - Design and Current Production



MREC Assembled



Anode Design

– 8 carbon fiber brush electrodes wired together to fill the chamber

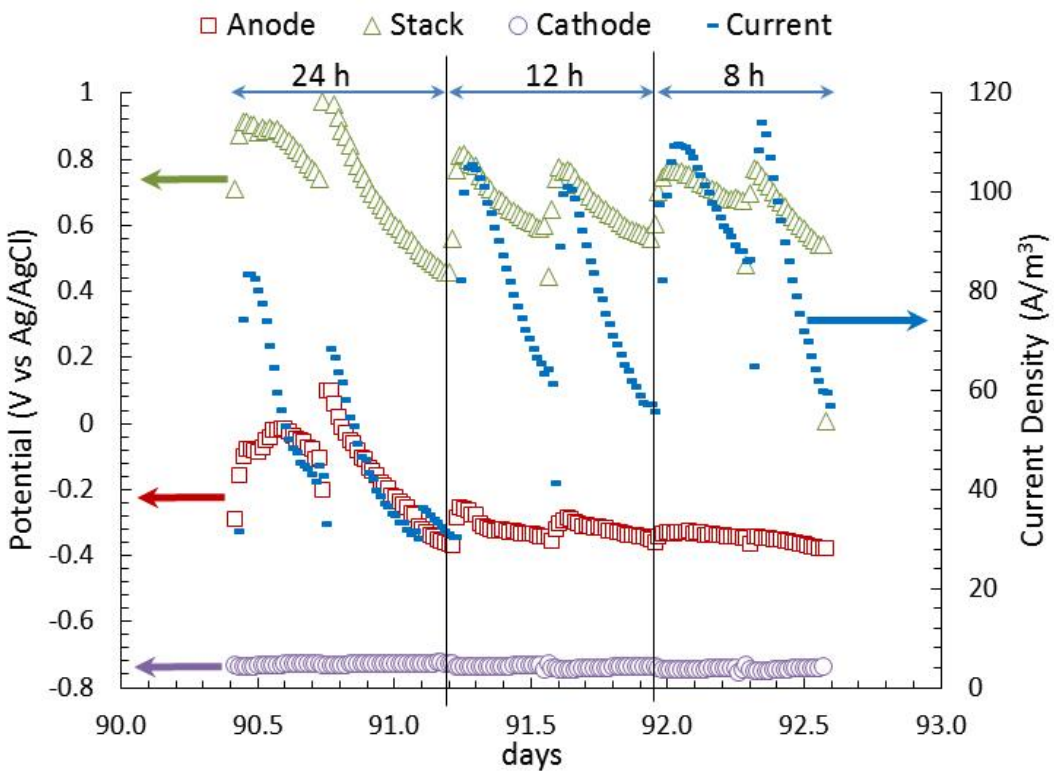
- Reactor driven by ammonium bicarbonate/distilled water salinity gradient – recycled until low current/minimal H_2 production
- Anode=150 mL, Cathode=165 mL, Membrane=64 cm^2, 10 cell pairs
- Inoculated with acetate-fed MFC effluent, acclimated to a synthetic fermentation effluent w/ influent COD = 1.2 g/L. Varied **HRT=hydraulic retention time**

➢ **Summary:** With this design, current production at stack potentials between 0.5 – 0.75 V ranged from 60 – 130 A/m^3 using synthetic fermentation effluent and an 8 h HRT.

Task 3 – Technical Accomplishments

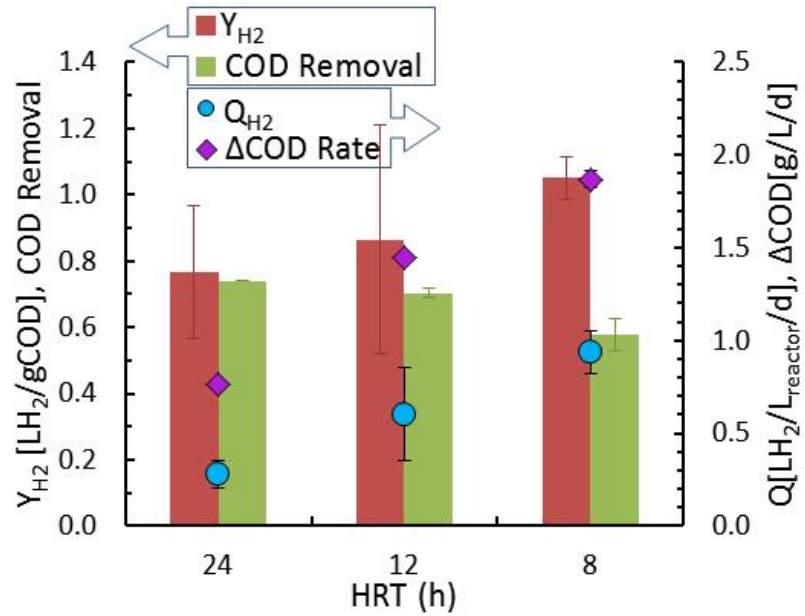
NREL Fermentation Effluent – Current, H_2 Production, and COD Removal

Reducing HRT increases current production



- Anode potential best (lowest) when COD is more uniform, which occurs at shorter HRTs of 12 and 8 h
- Current production at stack potentials between 0.6 – 0.75 V for different HRTs ranged from: 35 – 45 A/m³ (24 h), 65 – 100 A/m³ (12 h), 78 – 110 A/m³ (8 h)

Lower HRT increases H_2 production rate



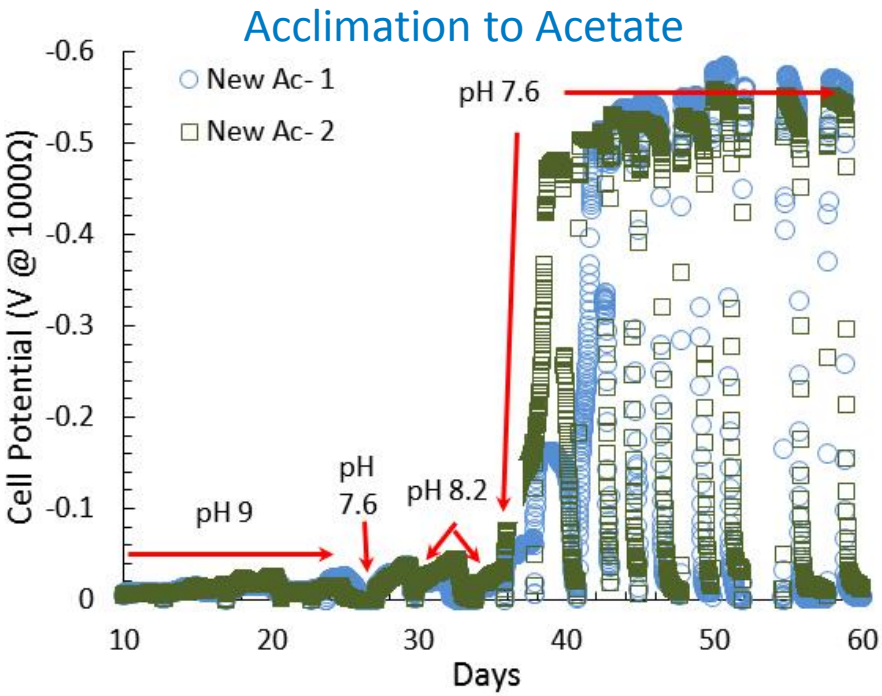
- H_2 production increased from 0.3 to 0.9 $L/L_{reactor}/d$ with decreased HRT
- H_2 yields increased from 0.8 to 1.0 L/g COD with decreased HRT
- COD removal decreased from 73% to 60% with decreased HRT, but rate of COD removed increased from 0.8 to 1.9 g/L/d

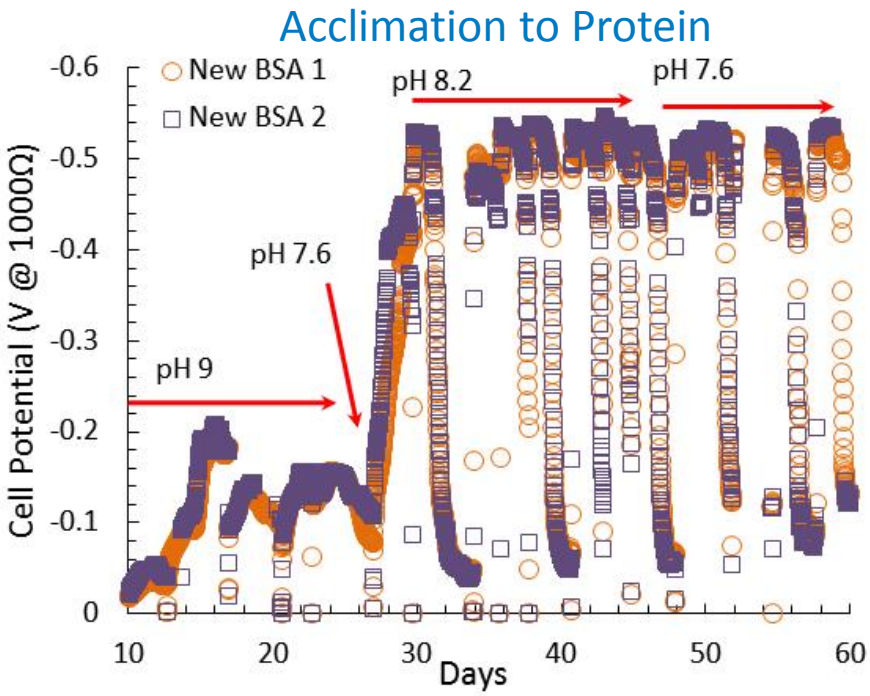
➢ **Summary:** H_2 production rate met goal of 0.5 L $H_2/L_{reactor}$/day at both 8 and 12 h HRTs.

Task 3 – Technical Accomplishments

Acclimation of Anodes to Protein or Acetate – pH Influence

- Anodes from MREC acclimated to fermentation effluent were removed from the reactor and put into individual MFCs
- "Old" anodes were fed 1 g/L acetate or BSA and effluent was used to inoculate "New" anodes





- Previous MFC research: highest power production at pH = 9 using sodium bicarbonate buffer & acetate
- Finding here: Establishment of electrogenic microbes on new anodes required lower pH of 7.6 for acetate or protein degradation
- Current: Higher current production by new anodes fed protein (vs. acetate) may be due to availability of soluble mediators (cysteine) in proteins

➢ **Summary:** Protein removal now being investigated with specially acclimated electrodes

Response to Reviewers' Comments

The linkage between tasks and where the whole project is going is not clear. Lignocellulose is the apparent target feedstock, but it is not very prominent in the plans. This project is also apparently slow-moving overall.

- **Response:** Work in FY13 was aimed to optimize bioreactor parameters using cellulose as the model substrate, which guides fermentation in FY14 using corn stover lignocellulose. This project uses a dual approach to improve H_2 molar yield (Tasks 2 & 3), which will be scaled up based on progress of Task 1. Overall all the tasks are well integrated with steady and solid progress of the individual task. Both Tasks 2 &3 have also made major breakthroughs (genetic tool and MREC).

However, the genetic engineering component may have benefitted from a collaboration with specific researchers who are proficient in working with C. thermocellum…

- **Response:** We had initiated multiple contacts with a specific researcher yet with no response. This constraint has prompted NREL to develop in-house tool and claimed success.

This project has too large a scope with limited resources. The MEC reactor could be considered a separate project altogether with its own set of difficulties that may warrant a larger effort…this is a very fragmented project…

- **Response:** Despite the large scope and limited resources, the project has made impressive progress with the three-prong synergistic approaches, with the best collaboration as commented by other reviewers. More funding is needed to consider MEC a separate project.

Collaborations

- **Task 1 (Bioreactor):**

  Drs. Ali Mohagheghi and Melvin Tucker, National Bioenergy Center at NREL (provide pretreated corn stover biomass and its characterization) - leveraging DOE BETO funding.

- **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* - leveraging Canadian funding.

- **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 Dr. Logan.

Remaining Challenges and Barriers

Task 1. Bioreactor Performance

- A small portion of lignin is known to re-deposit on cellulose after the acid-hydrolysis/thermochemical pretreatment process, hence likely decreasing the amount of readily fermentable cellulose.
  - Ferment "de-acetylated" biomass, a new pretreatment technology using mild-alkaline, which does not re-deposit lignin onto cellulose. NREL has shown that the resultant cellulose has good or higher sugar yields upon cellulase enzyme hydrolysis.

Task 2. Generate Metabolic Pathway Mutant in *C. thermocellum*

- Generate ethanol pathway mutant and evaluate improvement in H_2 molar yield.
  - Effort is ongoing in ethanol and lactate competing pathway mutants.
  - Over-expressing hydrogenases in the out years may also improve H_2.

Task 3. Electrochemically Assisted Microbial Fermentation of Acetate (PSU)

- Effluent from MEC treatment includes significant COD including protein at highest current and H_2 production levels.
  - Use anodes individually acclimated to acetate or protein to produce H_2 at maximum rate followed by improved COD/protein removal.

Proposed Future Work

Task 1 (NREL):

- Complete H_2 production and carbon mass balance profiles from corn stover lignocellulose (rate, yield) to quantify the conversion of lignocellulose to H_2 (FY14).
- Perform fermentation test using de-acetylated corn stover, a new NREL pretreatment technology that does not result in lignin re-depositing on cellulose (FY15).

Task 2 (NREL):

- Generate triple knockout mutants lacking formate and ethanol competing pathways (FY14).
- Test the above mutants for H_2, ethanol, and other metabolites (FY14, FY15).
- Over-express hydrogenases to increase H_2 molar yield in the triple mutant (FY15).

Task 3 (Penn State):

- Install individually acclimated anodes into MECs and evaluate H_2 production and COD removal when fed acetate or protein in both batch and continuous modes (FY14).
- Evaluate H_2 production and COD removal in continuous flow MECs with fermentation effluent produced from NREL (FY14).
- Examine improved cathode performance using new catalyst materials and flow configurations (FY15).

Task 4 (H2A Case Study, NREL with Strategic Analysis Inc.)

- Initiated recently, no results to report yet.

Summary

Task 1:

- Fed-batch fermentation using corn stover lignocellulose yielded maximum H_2 production rates of 475 and 1102 mL/L/d, exceeding the benchmark rates of 300 mL/L/d and 450 mL/L/d, respectively, during numerous cycles of lignocellulose feedings.
- The accumulation of lignin up to 16 g/L in the bioreactor did not inhibit H_2 production. Yet we discovered that certain fraction of cellulose is more recalcitrant and have devised strategy for more complete fermentation.

Task 2:

- Mutant lacking the pyruvate-to-formate pathway displays a faster kinetics of H_2 production along with increases in both ethanol (1.6 fold) and lactate (> 2 fold) production. The latter prompted the deletion of the lactate competing pathway which is underway.
- The deletion of the ethanol pathway is underway with initial transformants confirmed.

Task 3:

- New MREC reactor produced 60 – 130 A/m^3 at stack potential between 0.5 – 0.75 V with acetate (12 h HRT) and synthetic fermentation effluent (8 h HRT)
- Achieved H_2 production rate of 0.9 L/$L_{reactor}$/day and current production of 78 – 110 A/m^3 (stack potential 0.6 – 0.75 V, 8 h HRT) over more than 3 HRTs using NREL fermentation effluent. This exceeded the project goal of 0.5 L H_2/$L_{reactor}$/day with zero electrical grid energy.
- Testing of anodes acclimated individually to acetate or protein in MECs is underway.