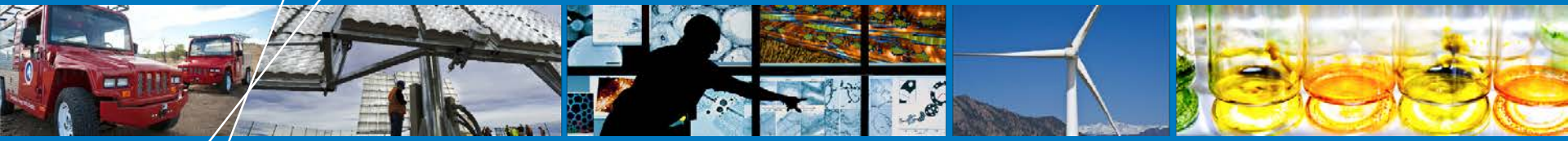
NREL
NATIONAL RENEWABLE ENERGY LABORATORY

Fermentation and Electrohydrogenic Approaches to Hydrogen Production



2015 Annual Merit Review and Peer Evaluation Meeting; June 11, 2015

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

Bruce Logan (Presenter); Penn State University

Project ID #: PD038

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

Overview

Timeline

- Project Start Date: FY 05 (not funded in FY 06)
- Project End Date: 10/2015*

Barriers

Barriers addressed

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

Budget

- FY14 DOE Funding: $470K
- FY15 planned DOE Funding: $750K
- Total DOE funds received to date: $3.78M (includes $704K Penn State subcontract)

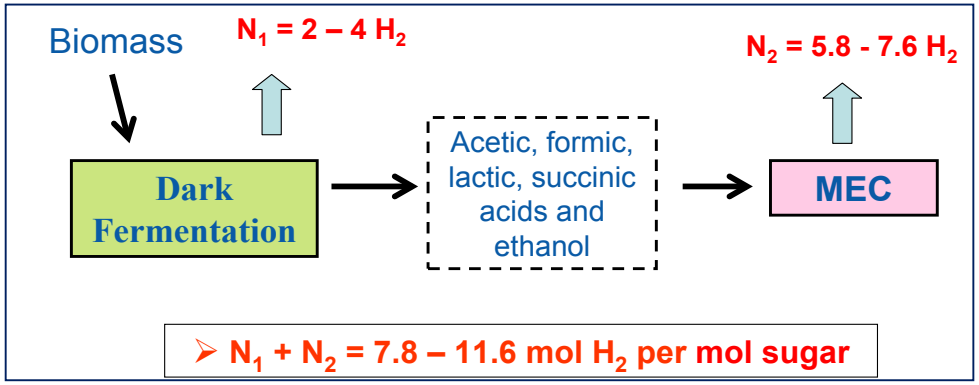
Partners

- 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

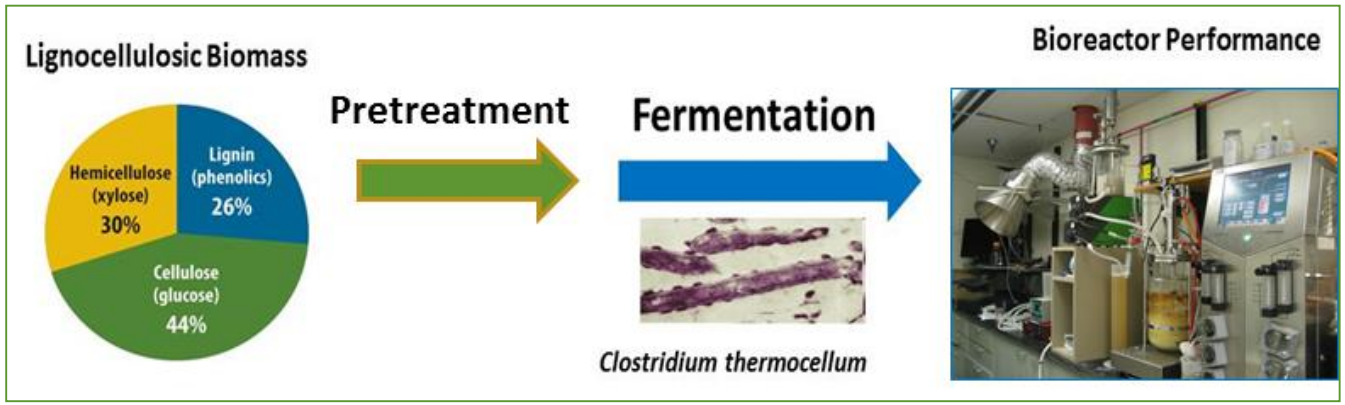
a. Status and target of the DOE Bioenergy Technology Office (BETO) – leverage BETO funding.

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

Approach

Task 1: Bioreactor Performance

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



Pretreated Corn Stover – Acid Hydrolysis (PCS)

1% H_2SO_4

160-190°C

Solid:
59% cellulose
27.5% lignin
→ H_2

Liquid:
90% xylan
9% glucan

More sugar loss, more inhibitors.

Pretreatment – De-acetylated and Mechanically Refined (DMR)

0.4% NaOH

70°C

Solid: 42% cellulose
25% xylan
16% lignin
→ H_2

Liquid: 79% acetate
2.9% xylan

Less sugar loss, less inhibitors.

Approach/Milestone

Task 1: Bioreactor Performance

- Corn stover lignocellulose: ~44% cellulose, 30% hemicellulose, and 26% lignin.
- Rationale of DMR (vs. PCS): milder pretreatment, better sugar recovery with less inhibitors: hydroxymethylfurfural (HMF, loss from glucose), and furfural (loss from xylose). It also reduces processing cost.
- FCTO can leverage BETO's investment in developing DMR technology.

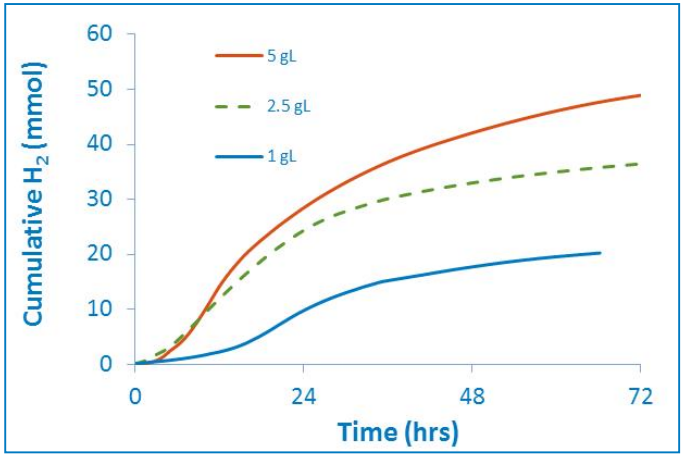
	FY14 Milestone (all regular)	Completion Date	Status
Q3	Increase the rate of H_2 production by fermentation 50% (based on 300 mL/L/d base rate) by using microbe that have been acclimated to degrade lignocellulose in the presence of lignin to address potential inhibition by the residual lignin.	6/14	Complete
Q4	Provide technical input for development of an H2A case study for a biological fermentation pathway to ultimately produce hydrogen at $2-4/gge (with SA, Inc.)	9/14	Complete
	FY15 Milestone (regular)		
Q2	In sequencing fed-batch reactor, use de-acetylated corn stover lignocellulose as the substrate and obtain a H_2 production rate of 450-550 mL H_2/L/d and use an 80% total H_2 output as that of avicel cellulose based on the same amount of cellulose loading (5 g/L) – a similar rate as with the current pretreated corn stover feedstock but a higher total output (currently at 62% of the avicel output). The outcome will demonstrate the potential to overcome the effect of lignin on blocking cellulose accessibility to bacterial fermentation.	3/15	Complete

Task 1 – Technical Accomplishments

H_2 from DMR Corn Stover in batch Fermentation

Lauren Magnusson

- DMR corn stover retains intact biomass structure, hence more recalcitrant. Its direct fermentation by microbes has not been tested (without adding enzyme cocktail).
- Similar to avicel and PCS, higher DMR carbon loading leads to faster rate of H_2, and lower carbon loading leads to higher H_2 molar yield.
- Mass analysis indicated 88% and 96% of total DMR solid and cellulose solid were solubilized (may not be completely metabolized), respectively, by the microbe.



*Cellulose (g/L)	H_2, Amount (mM)			H_2, Maximum Rate (mmol L^{-1} hr^{-1})			Molar Yield (mol H_2 mol^{-1} hexose)		
	1	2.5	5	1	2.5	5	1	2.5	5
Avicel	19.46	32.23	50.33	0.58	0.89	0.98	3.15	2.09	1.63
PCS	17.35	30.22	35.77	0.51	1.06	1.21	2.47	1.70	1.16
DMR	**13.50**	**24.65**	**33.14**	**0.60**	**0.88**	**1.56**	**2.19**	**1.60**	**1.07**

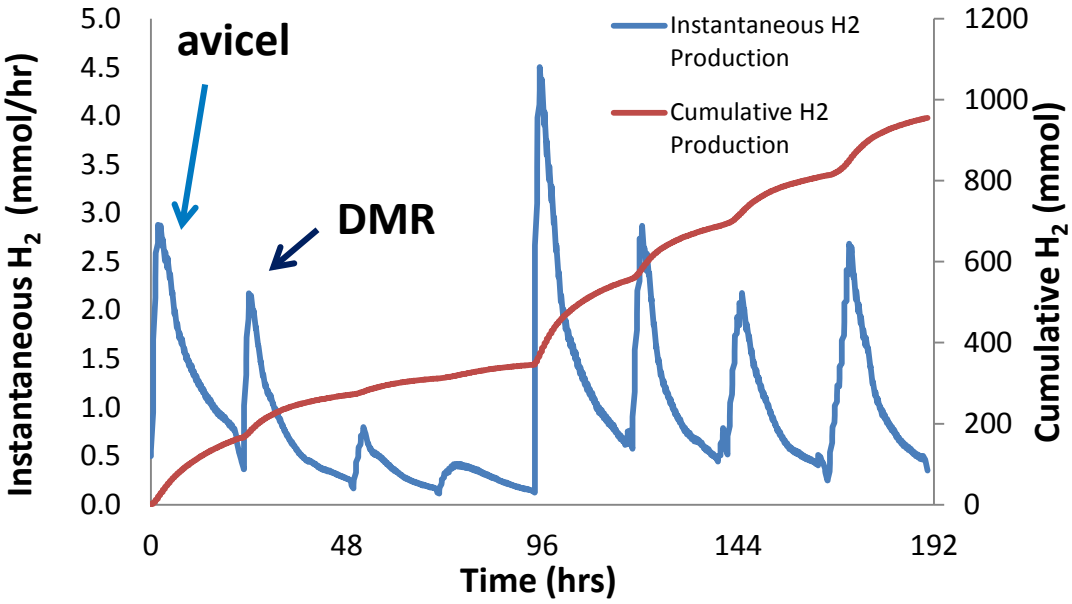
* Loading based on cellulose content.

➢ **Summary: First demonstration that DMR biomass can be fermented by a cellulose-degrader, with rates and yield closely resemble those of PCS.**

Task 1 – Technical Accomplishments

H_2 from DMR Corn Stover – Sequencing Fed Batch

- Avicel in first cycle, followed by 7 cycles of DMR corn stover (5 g/L as cellulose), HRT 48 h, and 50% liquid replacement every 24 h.
- HRT: the length of time to replace the working volume (2 L) in a bioreactor.
- Microbes require adaptation with the more recalcitrant DMR corn stover, shown in cycles 2-4. Future work will feed DMR biomass in cycle 1.



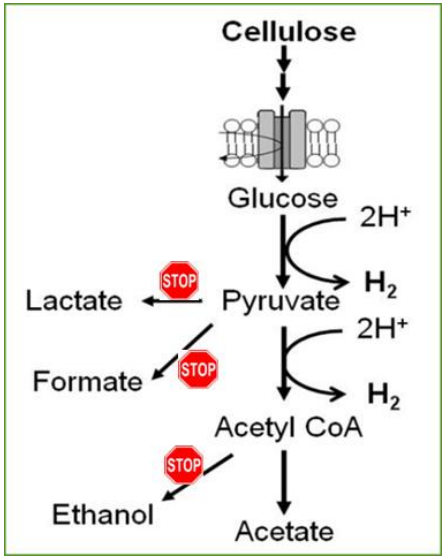
Cellulose 5 g/L/d	Rate of H_2 Production (mL/L/d)	
	Average	Maximum
Avicel	938	2108
PCS	550	1154
DMR	**757**	**1373**

➢ **Summary:** Complete FY15 Q2 milestone: obtained an average rate of 757 mL H_2/L/d (more than the 450-550 mL/L/d benchmark), with an H_2 output ~81% that of avicel.

Approach/Milestones

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

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



- **A major breakthrough:** NREL developed proprietary tools in *C. thermocellum* that very few labs can rival.
- The goal in FY14/FY15 is to delete competing pathways that make ethanol (pathway uses 4 e^-) and lactate (pathway uses 2 e^-) in a mutant already lacking the pyruvate-to-formate step (conserving pyruvate flux; FY14 accomplishment).

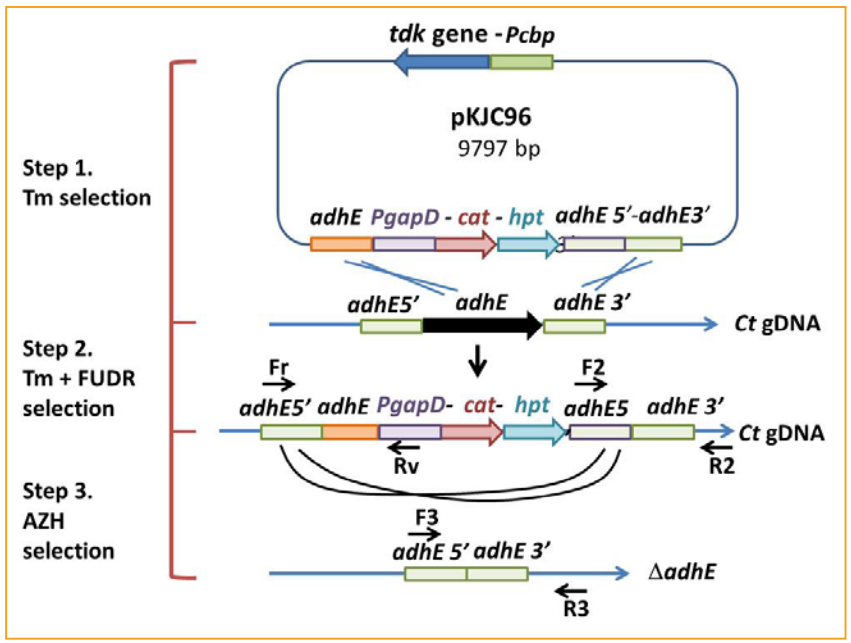
	FY15 Milestone – Regular	Com-pletion Date	Status
Q3	Generate a delta(*hpt*) delta(*pfl*) delta(*adhE*) triple mutant of *Clostridium thermocellum*, missing the *adhE* gene encoding the ethanol pathways, and demonstrate 50% reduction in ethanol production, with genetic deletion verified by PCR and metabolite 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.	7/15	On Track (Delayed from FY14 Q4)
Q3	Use the lactate dehydrogenase knockout design generated in FY2014 and transform *Clostridium thermocellum*, in the background of delta(hpt) delta(pfl) delta(adhE). The outcome will lead to generation of mutants lacking the lactate competing pathway.	6/15	On Track

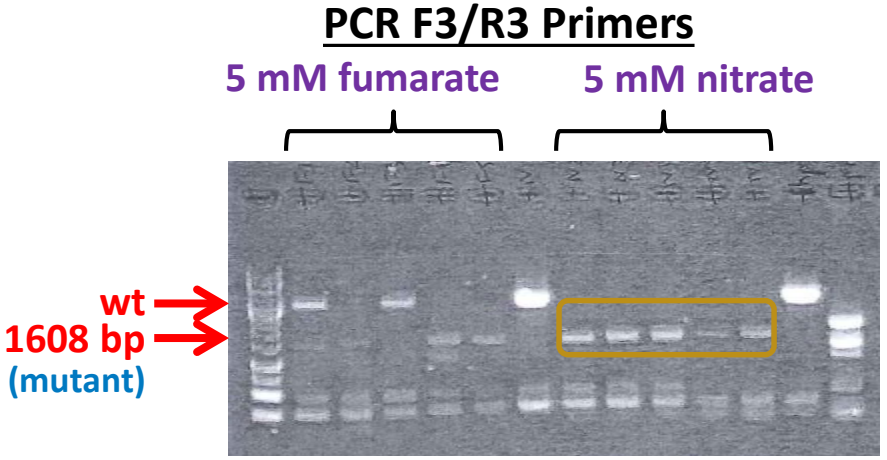
Task 2 – Technical Accomplishments:
Generated Ethanol Pathway Mutant Genotype


Katherine Chou

Ethanol production is encoded by a bifunctional acetaldehyde/ethanol dehydrogenase (*adhE*) and consumes four electrons (2 NADH).

- pKJC96 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.





- Deleting ethanol pathway might cause an imbalance of NAD(H) pool.
- Adding an oxidant (nitrate) during screening led to mutants with Δ*adhE* genotype.

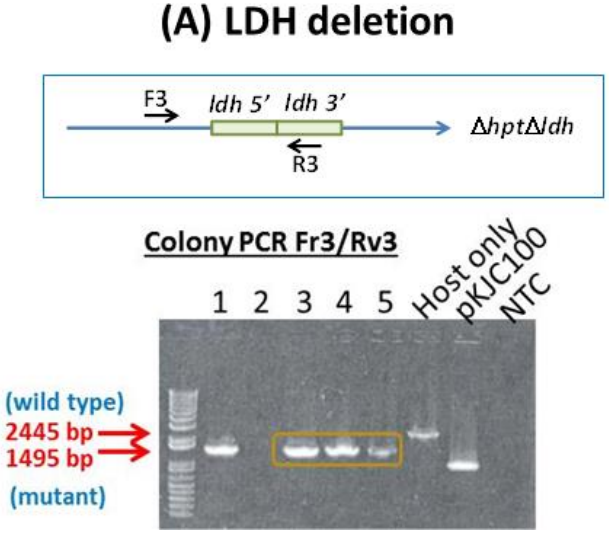
➢ **Summary:** Obtained Δ*adhE* mutant genotype, single colony selection is ongoing using the oxidant strategy (Q4 Milestone).

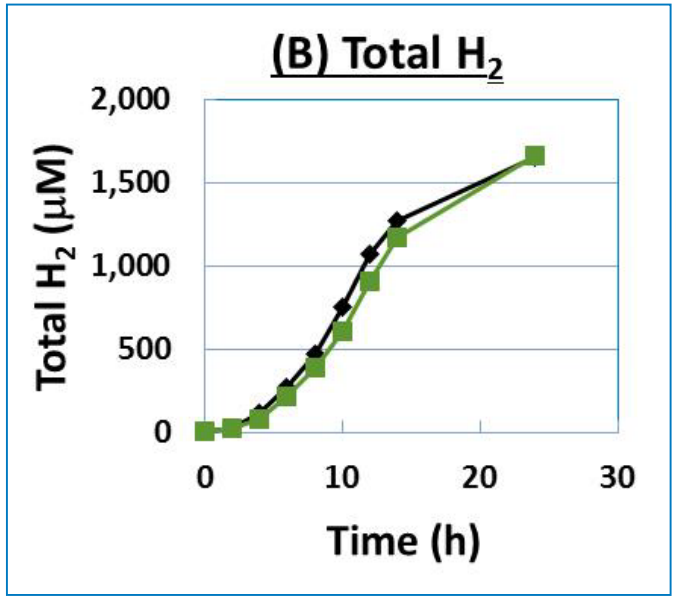
Task 2 – Technical Accomplishments

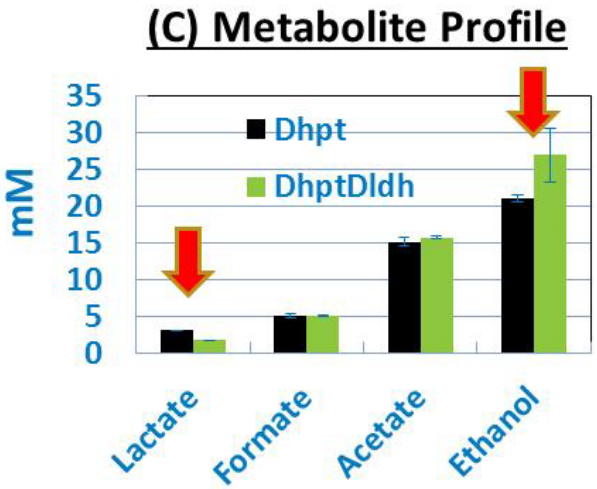
Generated Lactate Pathway Mutant

Lactate production consumes 2 e^- (NADH), encoded by lactate dehydrogenase (LDH).

- Successfully deleted *ldh* gene, verified by colony PCR (A).
- No change of H_2 in the LDH mutant (B), with 40% less lactate, and 24% **more** ethanol (C).
- A putative LDH/malate dehydrogenase (clo1313_1878) might also produce lactate.





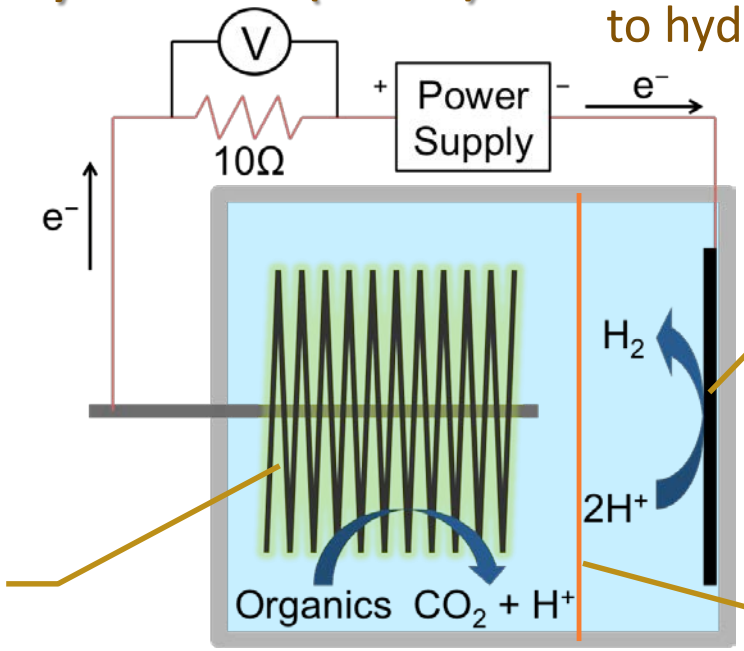


➢ **Summary:** Generated lactate pathway mutant, yet no change in H_2. The 24% increase in ethanol motivates the deletion of ethanol pathway to boost H_2 (Q3 Progress Measure).

Approach/Milestone

Task 3 – Electrochemically Assisted Microbial Fermentation

Microbial Electrolysis Cell (MEC) — conversion of organic waste to hydrogen gas



Anode – Graphite brush & attached microbial community

Cathode – Pt/C on SS Mesh

Anion exchange membrane

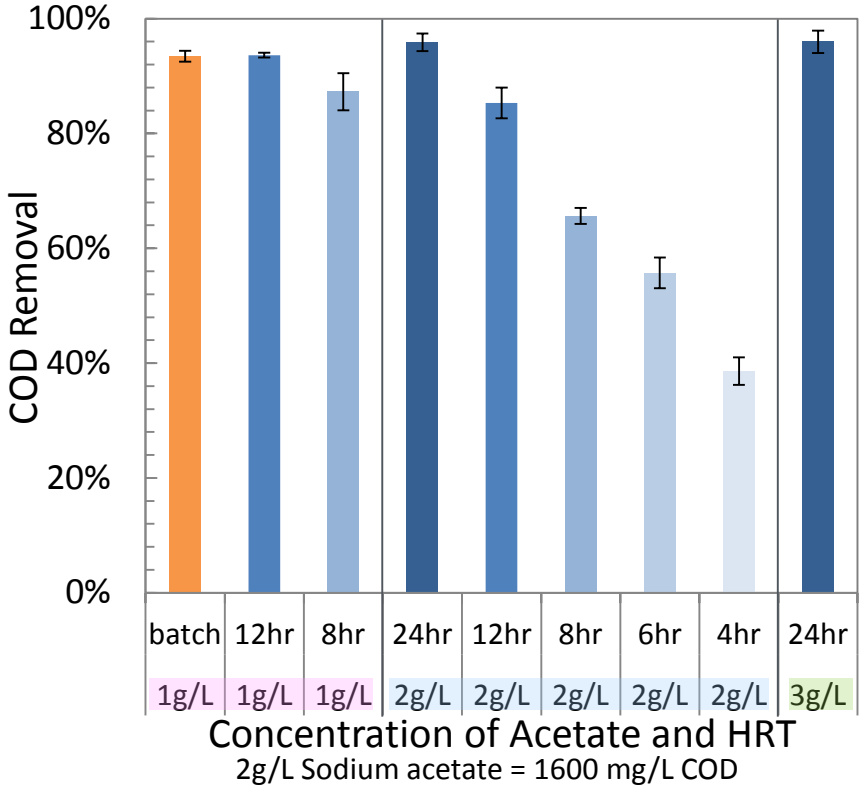
	Milestones	Completion Date	Status
FY14	Conduct continuous flow test with MEC individually acclimated to protein and acetate, and demonstrate > 80% protein removal, 0.5 L $H_2/L_{reactor}/d$ over 3 hydraulic retention times.	12/14	Complete
FY15	Optimize the design of the cathode chamber to increase the volumetric hydrogen production rate to 1.2 L $H_2/L_{reactor}/d$ (over 3 HRT, using NREL fermentation effluent) in a continuous flow MEC, using Pt/C cathodes and improved configurations.	9/15	On Track

Task 3 – Technical Accomplishments FY14

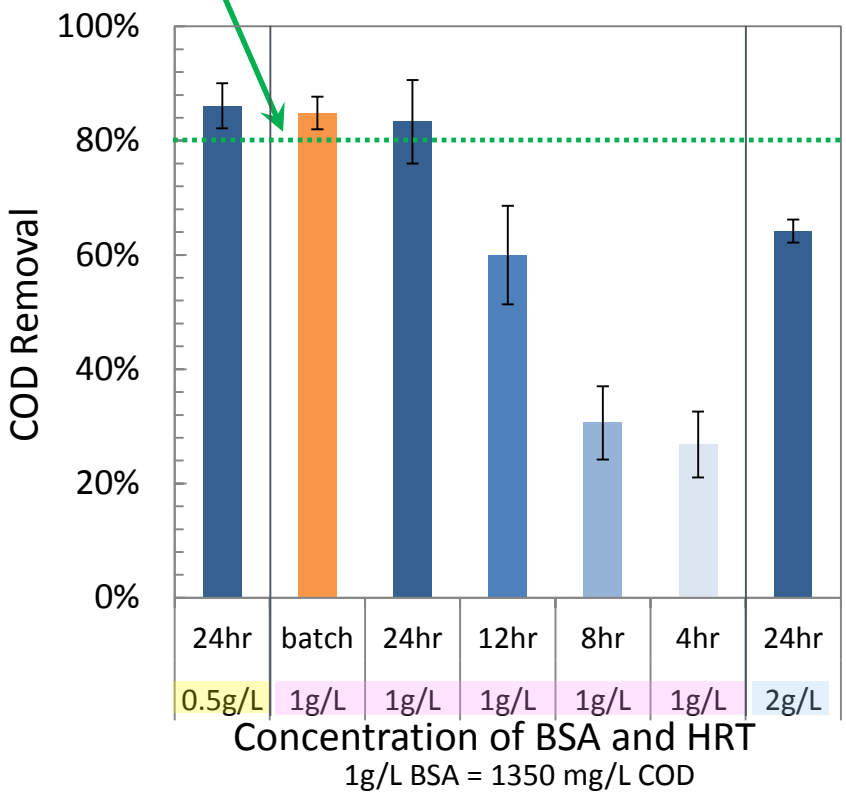
COD removal in MECs fed acetate or BSA

Milestone: >80% protein removal

Acetate MECs



BSA MECs



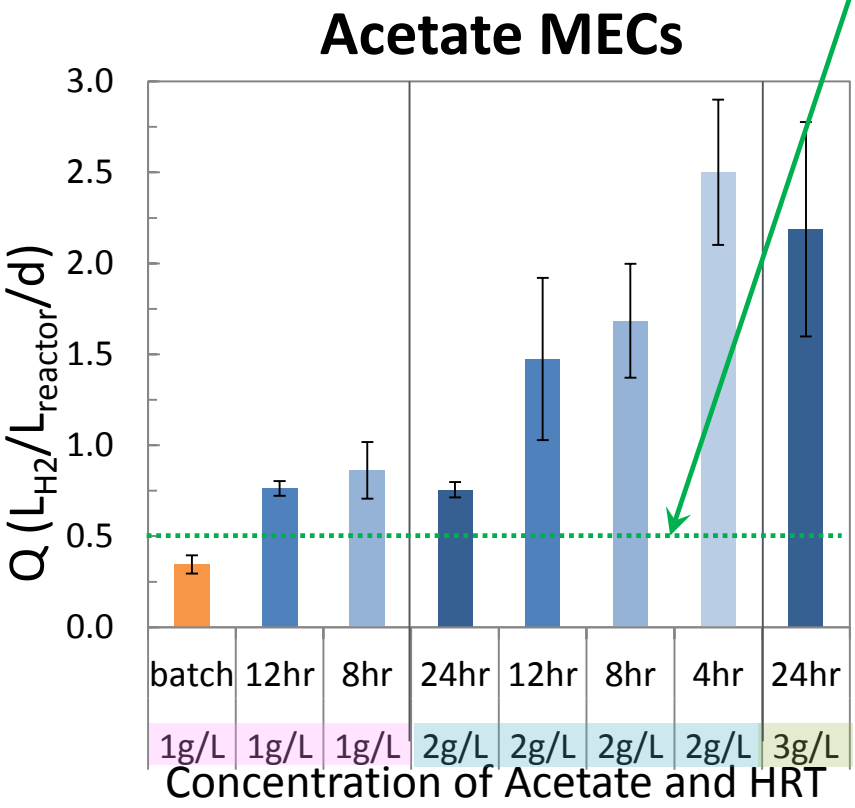
- COD removal decreases as the hydraulic retention time (HRT) in the anode chamber is decreased
- Fermentation effluent: protein = 1.3 ± 0.5 g/L (20 – 30 % of COD). BSA: bovine serum albumin.

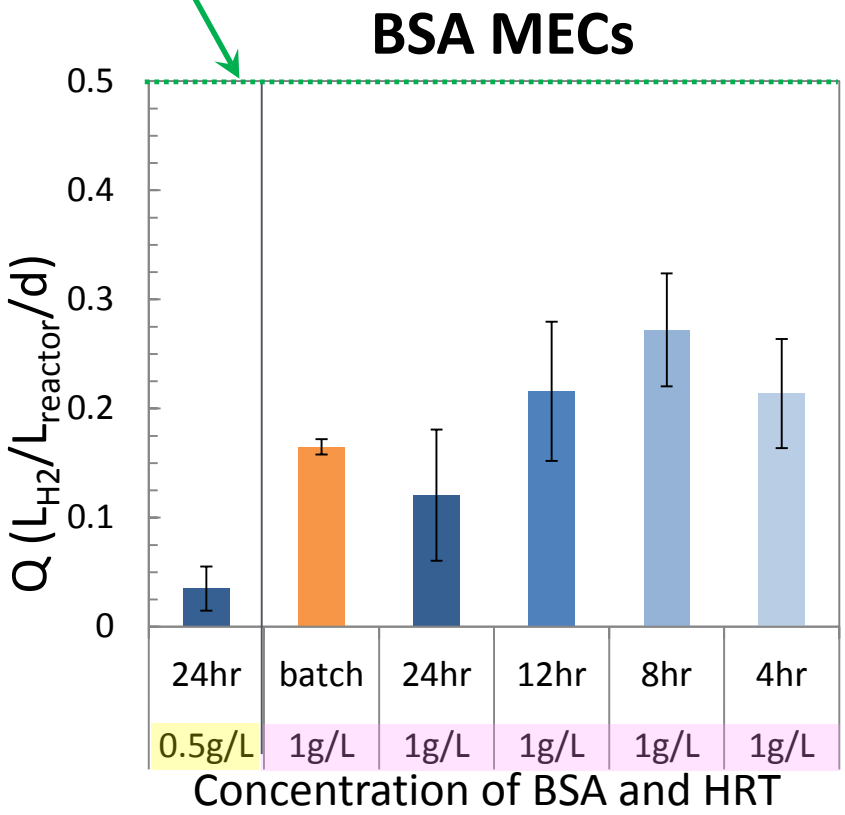
➢ **Summary:** Acetate is removed faster and more completely than BSA (protein) even when the MEC is conditioned specifically to that substrate, so HRT of each treatment stage will depend on COD removal goal for the target organic.

Task 3 – Technical Accomplishments FY14

Hydrogen production in MECs fed acetate or BSA

H_2 Production Milestone: 0.5 $L_{H2}/L_{reactor}/d$



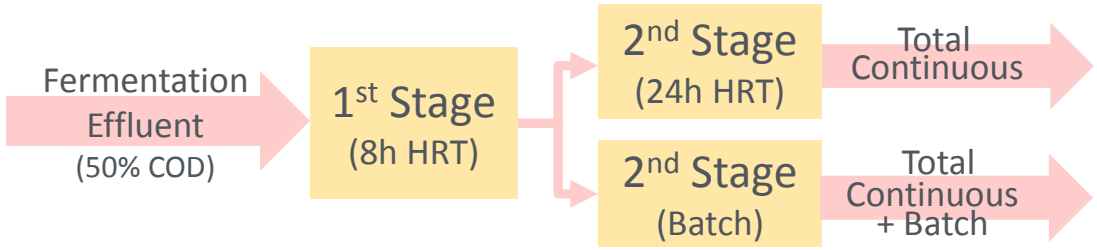


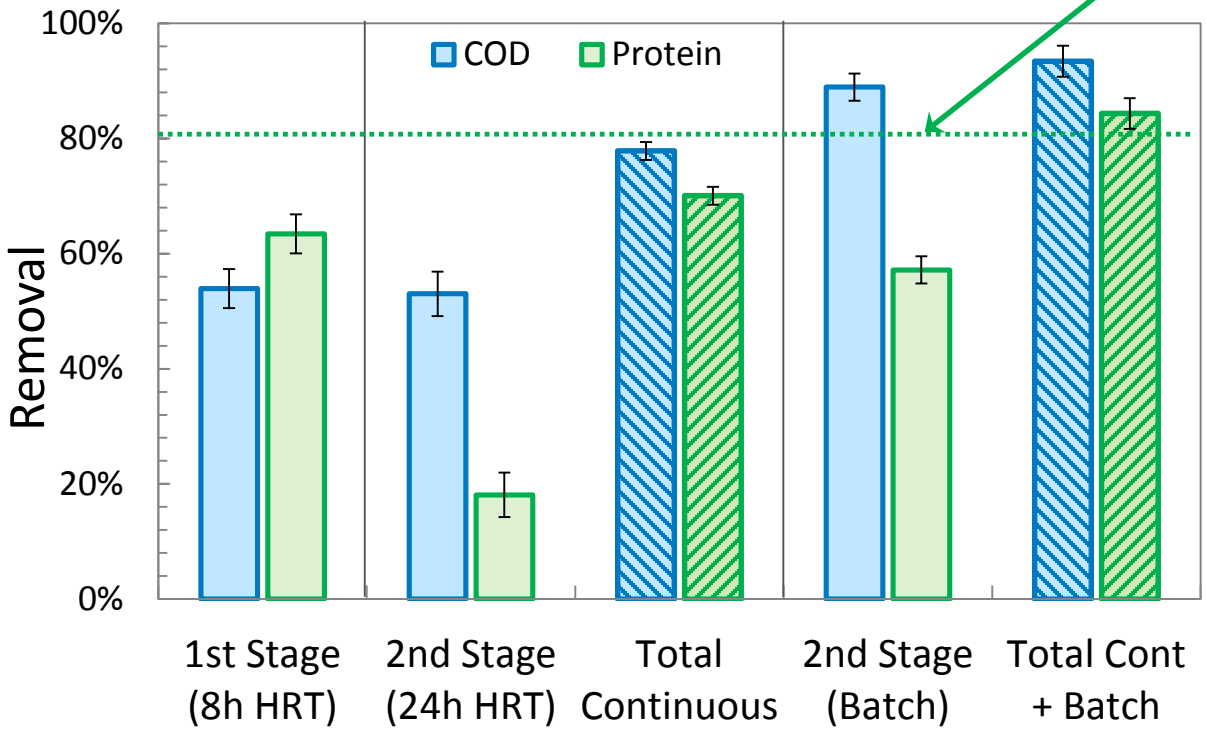
- When treating acetate, shorter HRT or higher concentration leads to increased H_2 production rates , there is a tradeoff between hydrogen production rate and COD treatment.
- Protein oxidation does not directly transfer to current production

➢ **Summary:** H_2 production rate increases with decreasing HRT and increased substrate concentration in acetate fed MECs, but this trend is not observed with protein fed MECs

Task 3 – Technical Accomplishments FY14

COD & protein removal: MECs in series fed fermentation effluent





Milestone: >80% protein removal

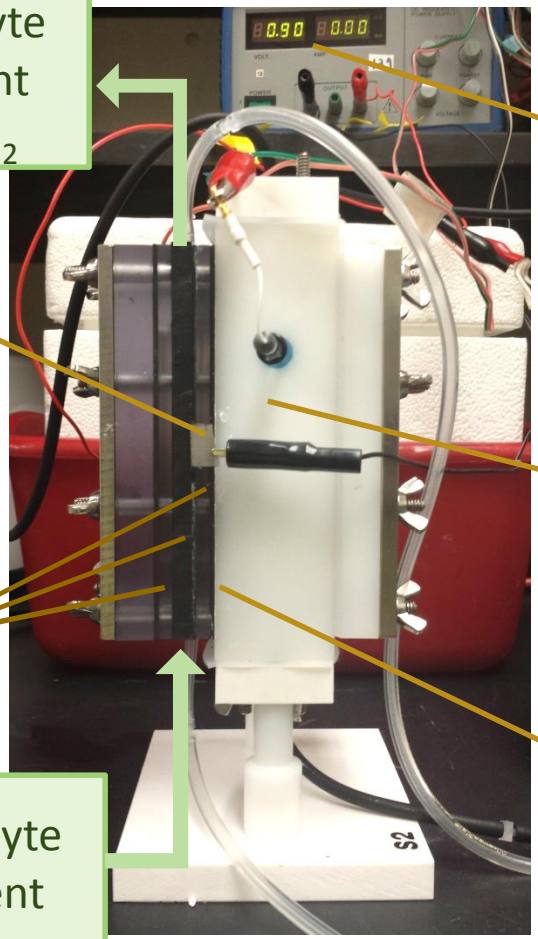
- 1st stage: H_2 = **2.1±0.4 $L_{H2}/L_{reactor}/d$**
- 1st stage + 2nd stage batch: H_2 = **0.3±0.1 $L_{H2}/L_{reactor}/d$** and **84±3% protein removal**
- 2nd stage reactor time is large to achieve sufficient protein removal
- Combined milestones for H_2 production and protein removal for 2 stages, not met…
- Solution: Reduce goal for protein removal in MEC to maximize H_2 production rate.
- Use a secondary process for overall COD removal (no H_2 production) such as an anaerobic fluidized bed membrane bioreactor (AFMBR)

➢ **Summary:** There is a tradeoff between maximum H_2 production rate and maximum COD & protein removals using a series of MECs

Task 3 – Technical Accomplishments FY15

MEC cathode chamber optimization

MEC with continuous flow adjustable volume cathode chamber



➢ **Summary:** Current goal - Increase cathode performance and H_2 production rate by optimization of cathode chamber volume and catholyte flow rate

Response to Previous Year Reviewers' Comments

It is not clear whether there has been consideration of what to do with the C5 sugars, which are a substantial fraction of the feedstock.

- **Response:** NREL has already generated mutants that can utilize xylose (ROI-15-42) to address this issue. The outcome will dramatically improve biomass utilization.

An assessment of what the targets need to be to reach cost-effective hydrogen production

- **Response:** Ongoing work of a techno-economic analysis by Strategic Analysis, Inc. will set targets and guide research directions.

Metabolic flux analysis is needed (to guide metabolic engineering)

- **Response:** We will propose this in FY16 research and determine how carbon pathway redirection affecting the flux, and then use flux analysis to predict the most fruitful mutation to guide metabolic engineering, using in-house expertise in C^{13} flux analysis.

It was unclear from the presentation what, if any, separations methods are being tested or will be tested for cleaning up the fermentation effluent before introduction into the MEC.

- **Response:** Thus far MEC can produce additional H_2 directly from the fermentation effluent, suggesting no inhibitors are present and a separation method is not necessary. Penn State will explore Anaerobic Fluidized Bed Membrane Bioreactor (AFMBR) to treat MEC effluent to lower its COD further in order to close the water loop.

Collaborations

- **Task 1 (Bioreactor):**

  Drs. Ali Mohagheghi and Melvin Tucker, National Bioenergy Center at NREL, provide acid-pretreated and DMR corn stover and their 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 leveraged funding by other projects of Dr. Logan.

Remaining Challenges and Barriers

Task 1. Bioreactor Performance

- De-acetylated biomass is more recalcitrant due to the milder biomass pretreatment process.
  - Start with DMR biomass in fed-batch fermentation (instead of avicel) to accelerate the initial H_2 production kinetics in bioreactor.

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

- Deleting ethanol pathway might cause a redox imbalance (excess NADH), hence the difficulty in isolating pure colony.
  - Supplement exogenous oxidants (nitrate, acetate) during screening to balance the NAD(H) pools to obtain mutant.

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

- There is a tradeoff between maximum H_2 production rate and maximum chemical oxygen demand (COD) & protein removals using a series of MECs
  - Reduce goal for protein removal in MEC to maximize H_2 production rate.
  - Use a secondary process for overall COD removal (no H_2 production) such as an anaerobic fluidized bed membrane bioreactor (AFMBR)

Proposed Future Work

Task 1 (NREL):

- Perform carbon mass balance of fermentation using de-acetylated corn stover to identify the solubilized forms of cellulose, hemicellulose, and lignin, if they are present as monomeric, oligomeric, or complex forms, which might reveal rate-limiting step(s) of fermentation (FY15).
- Test "pretreatment" of de-acetylated biomass with *C. thermocellum* cellulosomes (cellulase enzyme cocktail) to accelerate the initial fermentation kinetics (FY15, FY16).

Task 2 (NREL):

- Optimize culturing conditions (medium supplements, growth phase) to knockout ethanol competing pathway in mutants lacking lactate and formate pathways (FY15).
- Profile the above mutants for H_2, ethanol, and other metabolites (FY15, FY16).
- Over-express hydrogenases and determine H_2 molar yield and metabolite profile (FY16).

Task 3 (Penn State):

- Increase cathode performance and H_2 production rate by optimization of cathode chamber volume and catholyte flow rate (FY15).
- Examine cathode performance using new catalyst materials and flow configurations to replace the platinum catalyst (FY16).

Technology Transfer Activities

Technology-to-market or technology transfer plan or strategy

- Air Product and Chemicals, Inc.
  - Main interest in H_2 that is carbon neutral
  - Large-scale process
  - Cost near to or lower than making H_2 from alternative sources.

Plans for future funding

- Responding to FCTO FOA opportunities
- Network with biofuels industry to expand the use of 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 is filed for generating xylose-metabolizing strain, leading to enhanced biomass utilization.

Summary

Task 1:

- Demonstrate for the first time that C. *thermocellum* can ferment the more recalcitrant DMR-corn stover without adding the expensive cellulase enzyme cocktail – a cost saver.
- Obtain an average H_2 production rate of 757 mL/L/d in sequencing fed-batch bioreactor fermenting DMR corn stover, with an H_2 output 81% that of avicel cellulose - complete Q2 milestone.

Task 2:

- Obtained genotype of ethanol pathway mutant when an oxidant is added to balance redox state. Will further optimize this strategy to obtain ethanol knockout mutant.
- LDH mutant is generated and it produced only 40% less lactate, suggesting other putative pathway might also yield lactate. The increase in ethanol (24%) in this mutant motivates the need to delete the ethanol-competing pathway.

Task 3:

- Two stage treatment with MECs in series: 1st stage (8h HRT) produced 2.1±0.4 $L_{H2}/L_{reactor}/d$; 1st stage + 2nd stage (batch) produced 0.3±0.1 $L_{H2}/L_{reactor}/d$ and 84±3% protein removal.
- There is a tradeoff between maximum H_2 production rate and maximum COD & protein removals using a series of MECs.
- Solution: Reduce goal for protein removal in MEC to maximize H_2 production rate and use a secondary process for overall COD removal (no H_2 production) such as an anaerobic fluidized bed membrane bioreactor (AFMBR).

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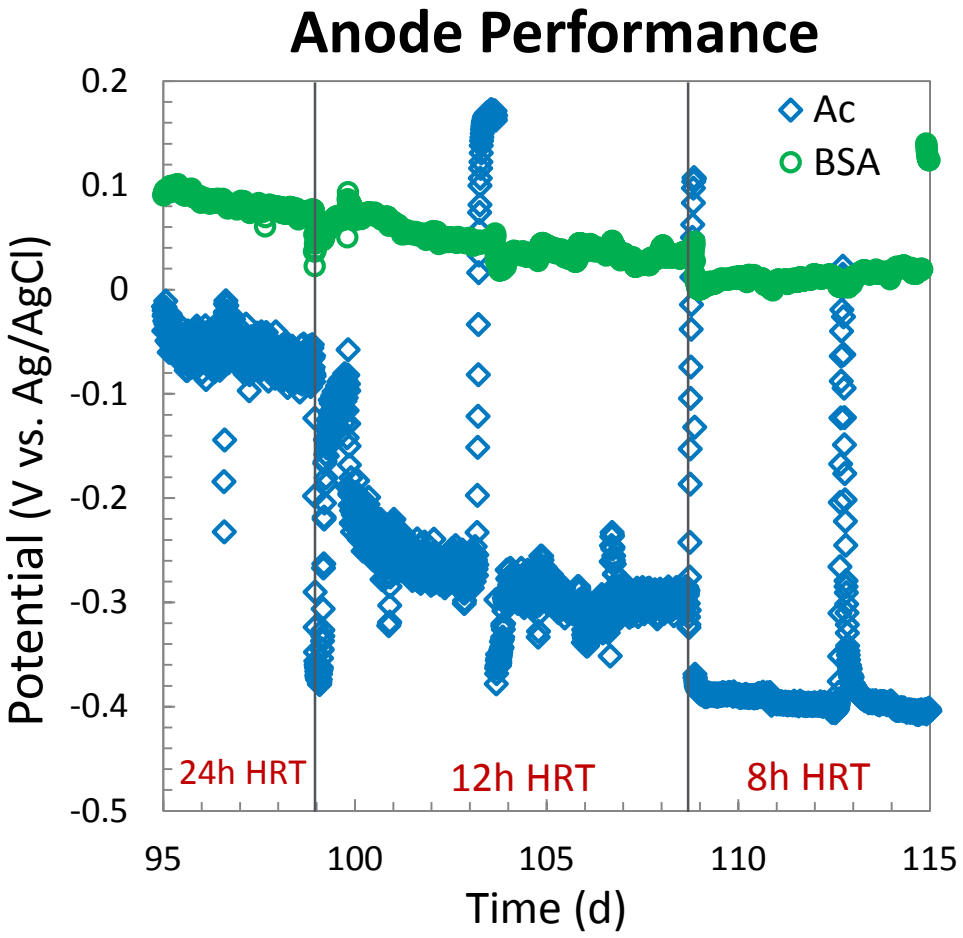
Technical Back-Up Slides

Task 3 – Technical Back-Up

Effect of HRT on anode potential of MECs fed acetate or protein



- As the hydraulic retention time (HRT) is reduced, the performance of the anode and current production increases
- HRT effect on anode potential is more noticeable for MECs fed acetate.

➢ **Summary:** HRT effects anode potential, which effects H_2 production rate.