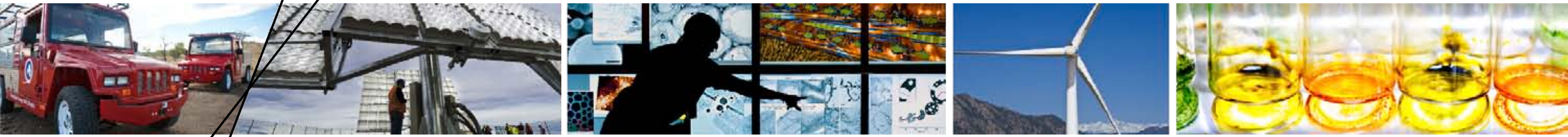
NATIONAL RENEWABLE ENERGY LABORATORY

Bench Scale Integration WBS 2.4.1.100



2015 DOE BioEnergy Technologies Office (BETO) Project Peer Review

Date: March 25, 2015

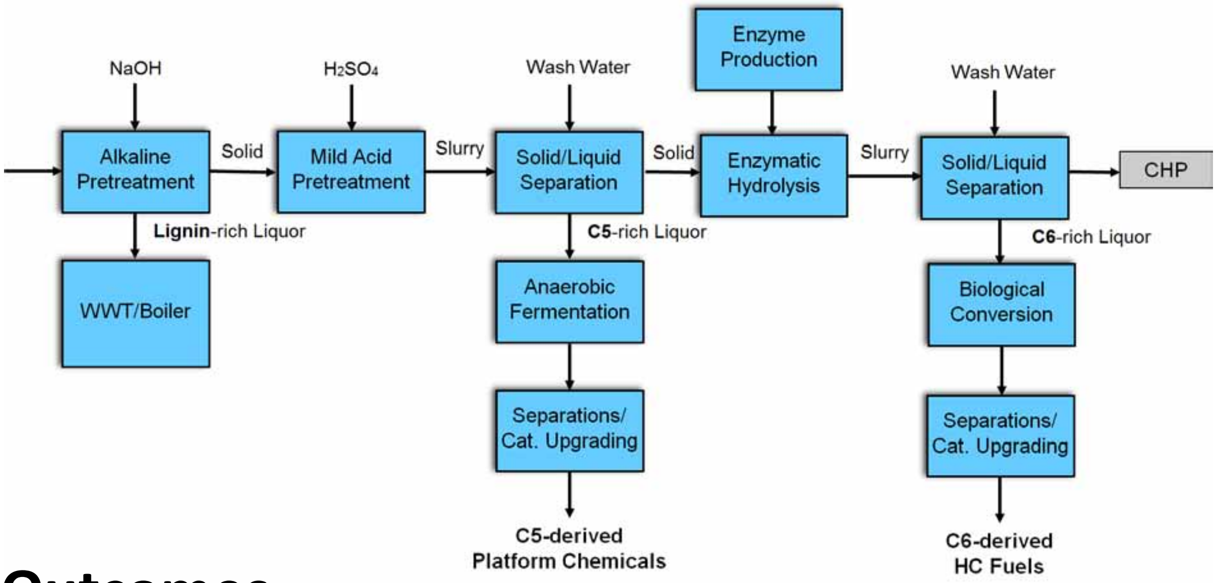
Technology Area Review: Biochemical Conversion

Principal Investigator: Nancy Dowe (Rick Elander, presenter)

Organization: National Renewable Energy Laboratory

Goal Statement

Goal is to demonstrate, at bench scale, an integrated process to produce HC fuel and a model chemical co-product from biomass to meet BETO cost target of $5/GGE by 2017 and $3/GGE by 2022



Project Outcomes

- Bench scale demonstrations will **reduce risk in scaling** technology to pilot scale
- Bench scale data will be used in yearly State-of-Technology reports to **track research progress**
- Project **works closely with industry** to incorporate new technology into process when possible
- Process development will be made public to **enable commercial scale up**

Quad Chart

Timeline

- **Project start: October 2013**
- **Project end date: September 2017**
- **Percent complete: 40%**

Budget

	Total Costs FY 12 ($MM)	FY 13 Costs ($MM)	FY 14 Costs ($MM)	Total Planned Funding (FY 15-Project End Date
DOE Funded	$1.20	$1.27	$1.22	$4.16
Project Cost Share (Comp.)*	------	------	------	------

Barriers

Barriers addressed

- Bt-K Biochemical Conversion Process Integration
- Bt-L Biochemical/Thermochemical Processing Integration
- Bt-G Cellulase Enzyme Loading

Partners

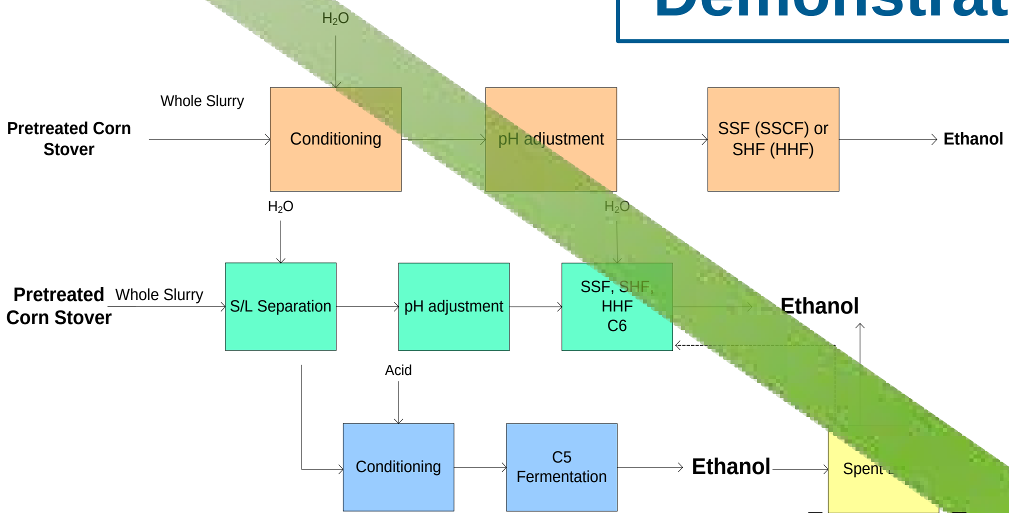
Collaborators

- NREL Projects
  - Biological Upgrading of Sugars
  - Pilot Scale Integration
  - Separations Development & Applications
  - Pretreatment and Process Hydrolysis
  - Analytical Methods Development
  - Biochemical Platform Analysis
- University of Pretoria
- UC Davis Phaff Yeast Culture Collection
- Novozymes
- DuPont
- PNNL

Project Overview – Bench Scale Integration “Ancient” History

2007

Different Process Configurations



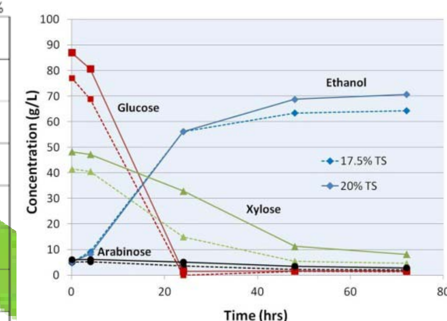
Cellulosic EtOH Demonstration

Target Table

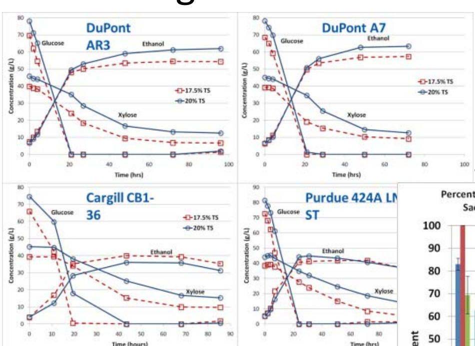
	2007	2008	2009	2010	2011 Targets	2012 Targets
Minimum Ethanol Selling Price ($/gal)	$3.64	$3.56	$3.19	$2.77	$2.62	$2.15
Feedstock Contribution ($/gal)	$1.12	$1.04	$0.95	$0.82	$0.76	$0.74
Conversion Contribution ($/gal)	$2.52	$2.52	$2.24	$1.95	$1.86	$1.41
Yield (Gallon/dry ton)	69	70	73	75	78	79
Feedstock						
Feedstock Cost ($/dry ton)	$77.20	$72.90	$69.65	$61.30	$59.60	$58.50
Pretreatment						
Solids Loading (wt%)	30%	30%	30%	30%	30%	30%
Xylan to Xylose (including enzymatic)	75%	75%	84%	85%	88%	90%
Xylan to Degradation Products	13%	11%	6%	8%	5%	5%
Conditioning						
Ammonia Loading (mL per L Hydrolyzate)	50	50	38	23	25	25
Hydrolyzate solid-liquid separation	Yes	Yes	Yes	Yes	Yes	No
Xylose Sugar Loss	2%	2%	2%	2%	1%	1%
Glucose Sugar Loss	1%	1%	1%	1%	1%	0%
Enzymes						
Enzyme Contribution ($/gal EtOH)	$0.39	$0.38	$0.36	$0.36	$0.36	$0.34
Enzymatic Hydrolysis & Fermentation						
Total Solids Loading (wt%)	20%	20%	20%	17.5%	20%	20%
Combined Saccharification & Fermentation Time (d)	7	7	7	5	5	5
Corn Steep Liquor Loading (wt%)	1%	1%	1%	1%	0.60%	0.25%
Overall Cellulose to Ethanol	86%	86%	84%	86%	86%	86%
Xylose to Ethanol	76%	80%	82%	79%	85%	85%
Arabinose to Ethanol	0%	0%	51%	68%	80%	85%

Bench Scale Demonstrations

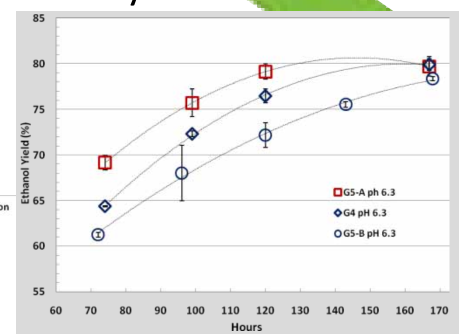
	20%	17.5%
EH Glucose Yield	90%	93%
EtOH Process Yield	94%	95%
EtOH Yield from Gluc	97%	97%
EtOH Yield from Xylose	94%	96%
EtOH Yield from Arabinose	51%	63%
Final EtOH titer (g/L)	70	64
Produced EtOH (g/L)	65	59
Xylose Utilization	81%	91%
Arabinose Utilization	53%	67%



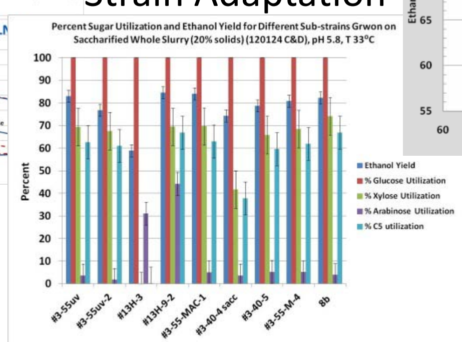
Ethanologen Evaluations



Enzyme Evaluations



Strain Adaptation



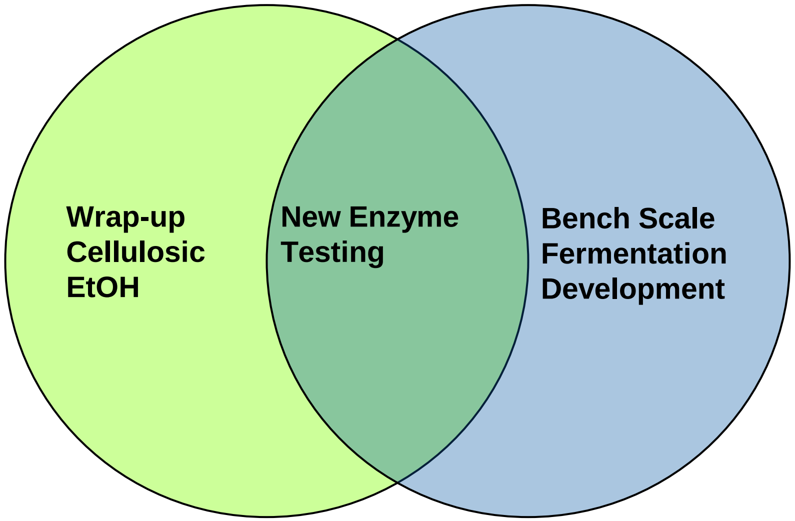
- Inoculum development
- Bench scale methods development
- Yield calculations

2012

$2.15 MESP

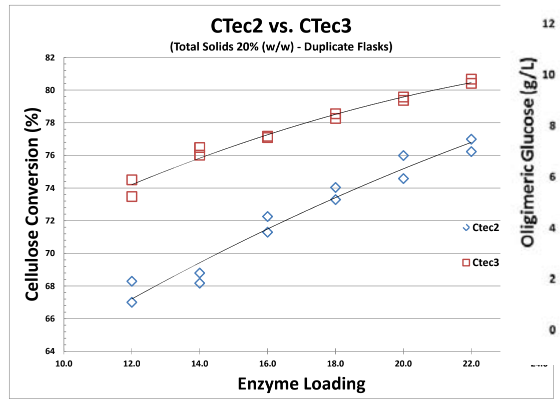
Project Overview – BSI Recent History

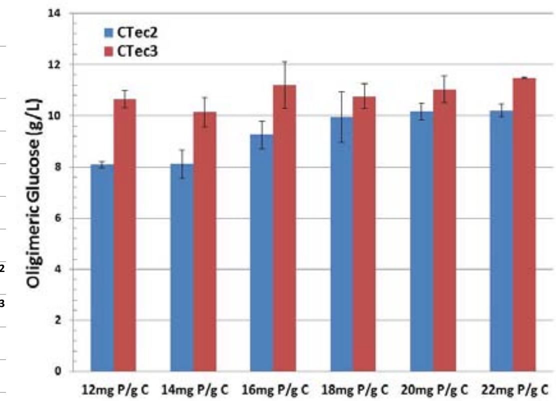
FY13 Was a Transition Year



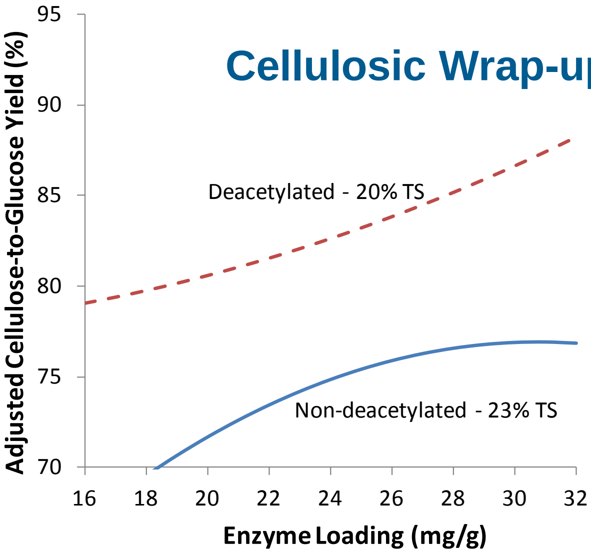
New Enzyme Testing

- CTec2 and CTec3
- 14 mg CTec3 equivalent to 20 mg CTec2
- Improved oligomeric sugar conversion





Cellulosic Wrap-up



Enzymatic Hydrolysis Benchmarking

- Deacetylated and Non-Deacetylated PCS
- Cellulose loading equivalent, %TS varied
- EH glucose yields 7% higher from deacetylated PCS

Bench Scale Fermentation

Farnesene from r*Zymomonas*

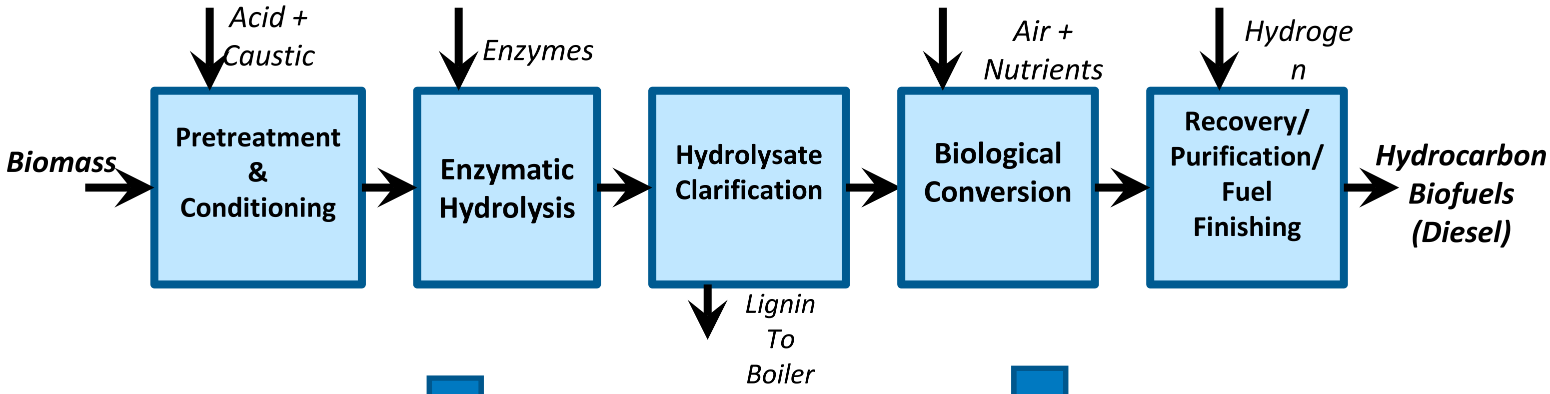
- Farnesene in the mg/L range
- EtOH production not affected
- Hydrolysate inhibition

Lipid Production from *Lipomyces*

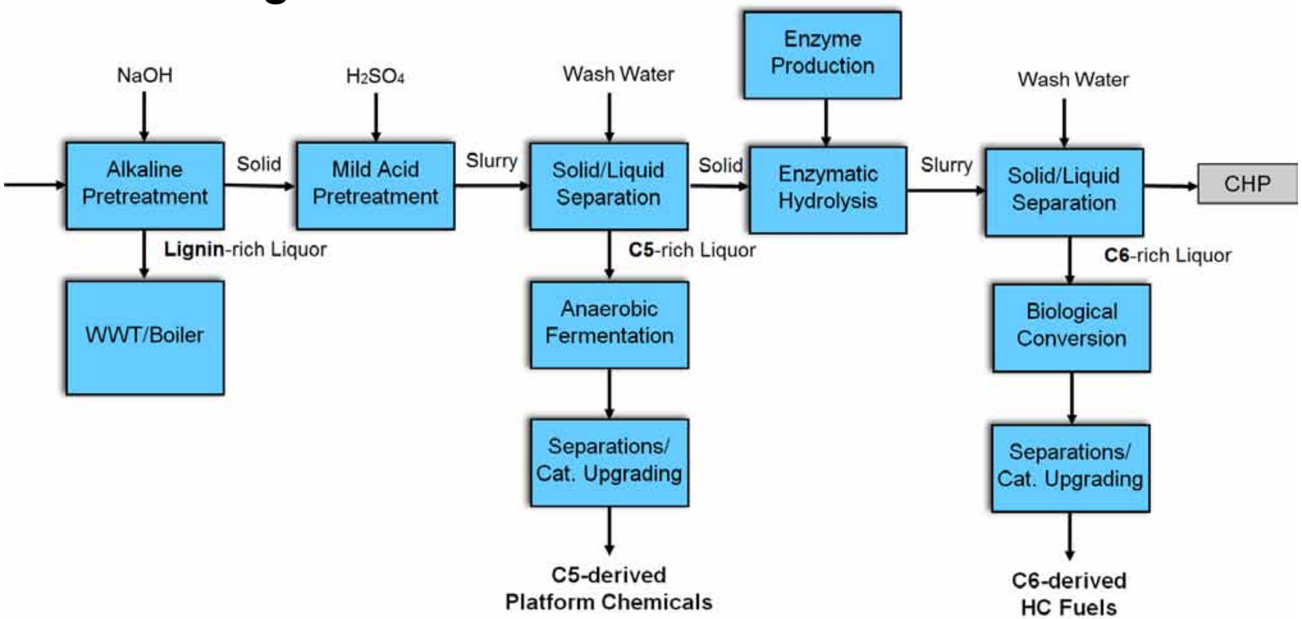
- Wild-type strain makes 60% lipid
- Slow growth rate in shake flasks
- Improved productivity by moving to fermentors (~6.5 g/L lipids from 100 g/L gluc in 72h, 0.1 g/L-hr)

Path to 2017 Cost Target Changed

2013 Davis et al. Design Report



2017 Design Case



Advantages

- Utilizes near-term demonstrated technology
- Product versatility
- Avoids C5 utilization to fuels for now

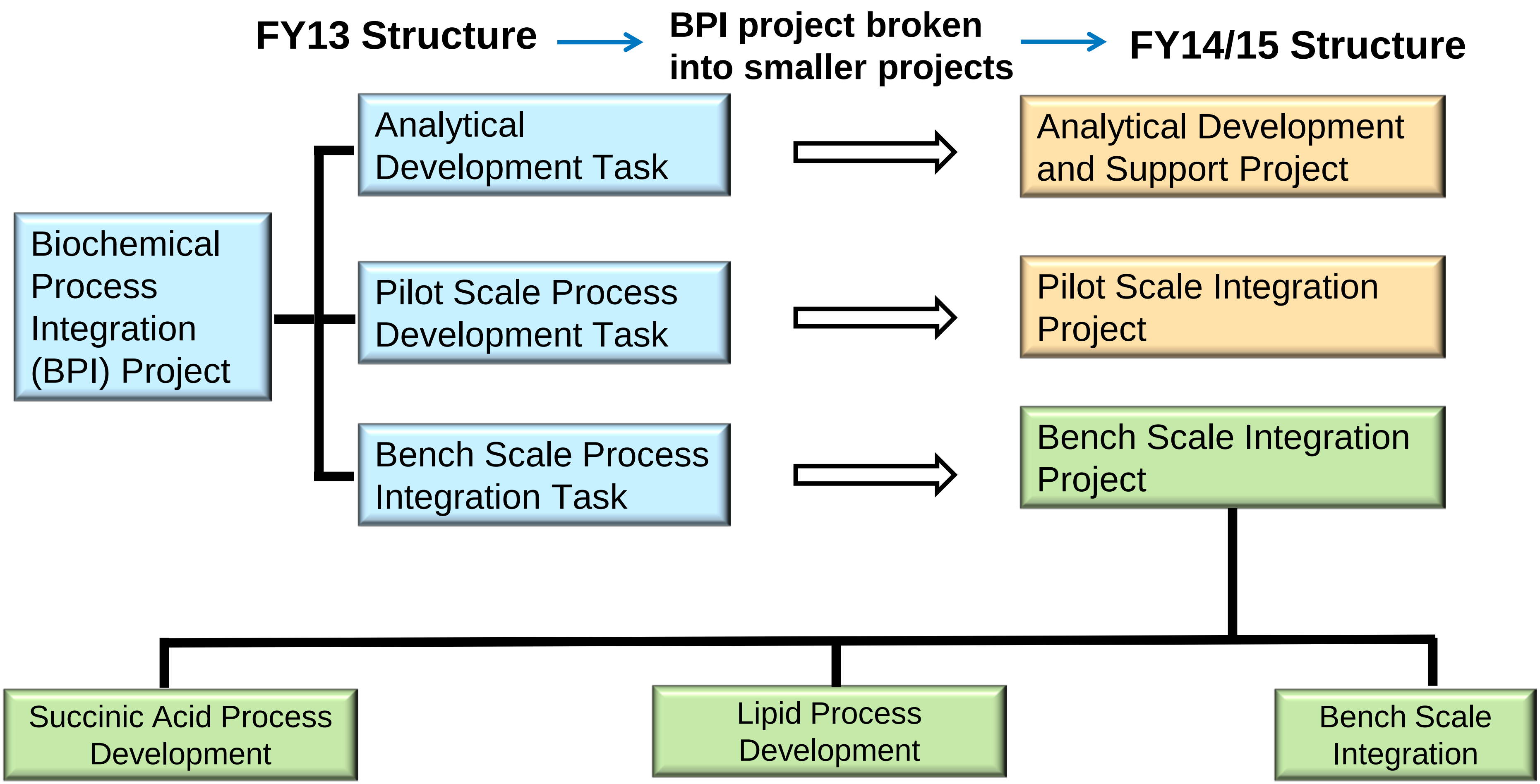
Technical Targets for 2017 Cost Demonstration

	2014 SOT	2017 Target
Minimum Fuel Selling Price ($/GGE, 2011$)	**$12.97**	**$5.03**
Feedstock Contribution ($/GGE, 2011$)	$3.88 [1]	$1.87 [1]
Conversion Contribution ($/GGE, 2011$)	**$9.09 [1]**	**$3.16 [1]**
RDB Fuel Yield (GGE/dry ton)	18	22
Succinic Acid Yield (lb/dry ton)	197	270
Feedstock		
Feedstock Cost ($/dry ton) [2]	$130	$80
Feedstock Blend	Stover	Blend
Pretreatment/Separation		
Solids Loading (wt%)	30%	30%
Xylan to Xylose (including conversion in C5 train)	73%	78%
Hydrolysate solid-liquid separation	Yes	Yes
Xylose Sugar Loss (into C6 stream after acid PT separation)	5%	1%
Enzymes		
Enzyme Loading (mg/g cellulose)	14	10
Total Solids Loading to Hydrolysis (wt%)	15%	17.5%
Enzymatic Hydrolysis Time (d)	3.5	3.5
Hydrolysis Glucan to Glucose	77%	90%
Hydrolysis Residual Xylan to Xylose	30%	30%
Glucose Sugar Loss (into solid lignin stream after EH separation)	5%	1%
Expt'l bioconversion scale/method	Bench scale/ Batch	Pilot scale/ Fed-batch
Bioconversion Volumetric Productivity (g/L-hr)	0.29	0.4
Lipid Content (wt%)	57%	60%
Glucose to Product [total glucose utilization] [3]	75% [100%]	78% [100%]
Xylose to Product [total xylose utilization] [3]	74% [98%]	76% [98%]
C6 Train Bioconversion Metabolic Yield (Process Yield) (g/g sugars)	0.26 (0.26)	0.27 (0.27)
Intermediate Product Recovery	90%	90%
Carbon Yield to RDB from Biomass	10.4%	12.5%
Bioconversion Volumetric Productivity (g/L-hr)	0.3	2.0
C5 Train Bioconversion Metabolic Yield (Process Yield) (g/g sugars)	0.63 (0.59)	0.795 (0.74)
Succinic Acid Recovery Efficiency	80%	80%
Carbon Yield to Succinic Acid from Biomass	8.9%	12.2%

Technical targets guide research direction

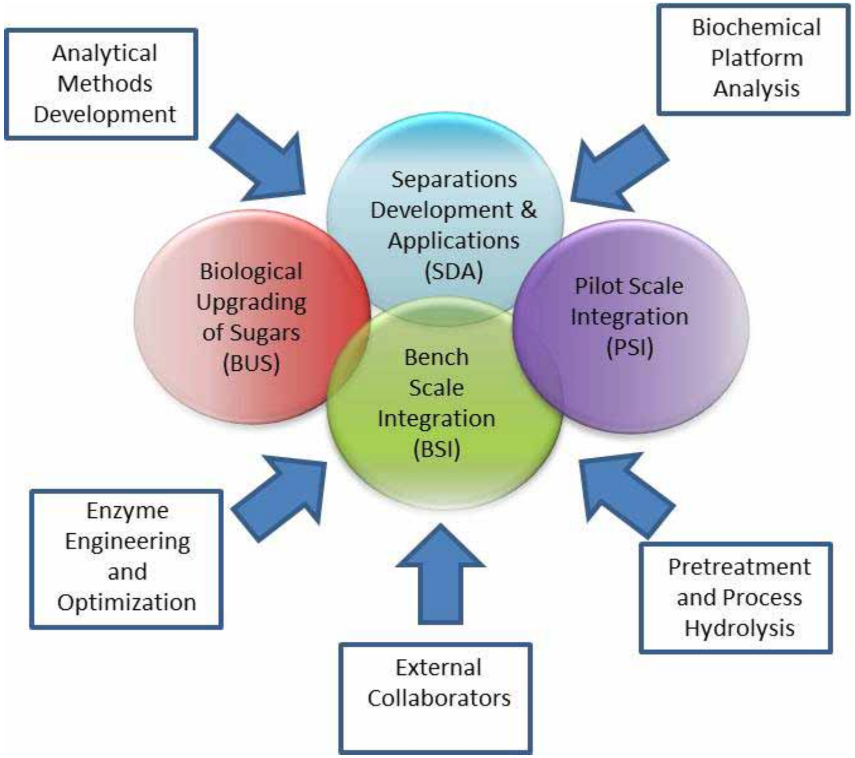
- Lipid fermentation targets:
  - 60% lipid content
  - 0.27 g lipid/g sugars
  - 100% sugar utilization
  - 0.4 g/L-hr Qp
- SA fermentation targets:
  - 0.74 g SA/g sugars
  - 2.0 g/L-hr Qp
- EH targets:
  - 10 mg enz/g cellulose
  - 90% glucose yield
  - 30% xylose yield
  - 17.5 wt.% solids
  - 3.5 days

Project Structure

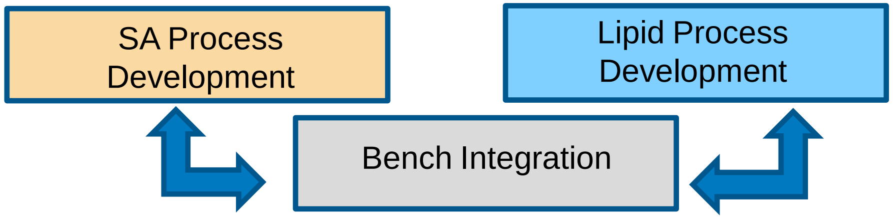


Management Approach

Integrated Project Approach Across Platform



BSI Task Structure



- Task structure aligned with process steps
- Researchers are shared between BSI and other related tasks
- Shared milestones between tasks ensure collaboration and hand-off of technology
- Engage outside collaborators for strains and enzymes
- Research is driven by technical targets needed to meet the 2017 cost target

Key Challenges

- Process complexity and deliverable schedule
- Dependency on other core projects to deliver technology ready for integration.

Critical Success Factors: Delivery of strains, enzymes, and biomass sugars that, when integrated, achieve the technical targets needed to meet BETO's 2017 HC fuel cost target

Technical Approach

Three research areas within BSI

Area 1: Develop an integrated SA fermentation process on biomass sugars

- Target: 2.0 g SA/L-hr rate, 0.795 g SA/g yield on C5-enriched sugars
- Approach
  - Screen natural strains on C5-enriched sugar stream
  - Develop fermentation process for high productivity
  - Evaluate new developed strains from BUS project using process relevant conditions

Area 2: Develop an integrated lipid fermentation process on biomass sugars

- Target: 0.4 g lipid/L-hr rate, 60% lipid content, 0.27 g lipid/g yield on C6-enriched sugars
- Approach
  - Screen natural oleaginous yeast on C6-enriched sugar stream
  - Develop fed-batch fermentation for higher productivity
  - Manipulate nutrients for higher lipid content
  - Evaluate new developed strains using process relevant conditions

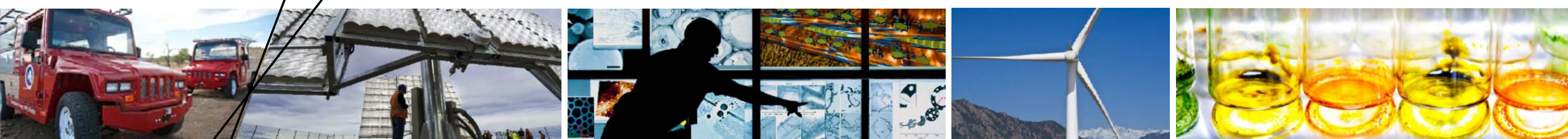
Area 3: Produce biomass sugars for the fermentation processes

- Target: 90% glucose yield,17.5% solids,10 mg enzyme/g cellulose
- Evaluate new pretreated feedstocks
- Work with enzyme companies to develop and test new enzymes on new feedstocks
- Determine sugar stream properties for fermentation processes

Critical Success Factors: Demonstrating fermentation productivities and yields on biomass sugars that meet technical targets

Key Challenges

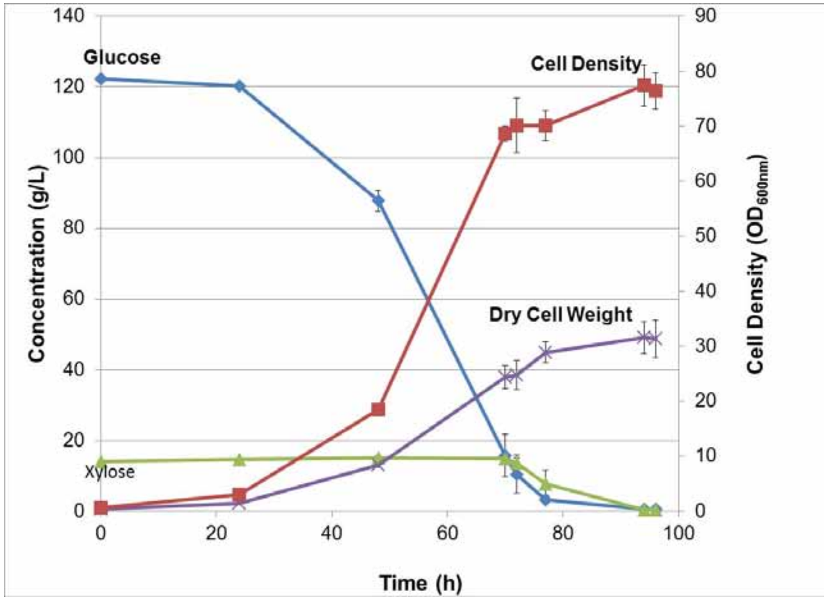
- Developed strains and enzymes will meet research targets
- Fed-batch or continuous operation will increase productivities



Technical Accomplishments

FY14 State of Technology for Lipid Production

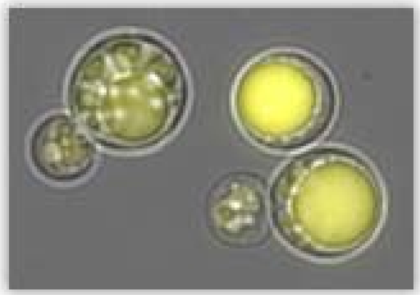
Lipid Production by *L. starkeyi* from Pretreated Corn Stover Hydrolysate

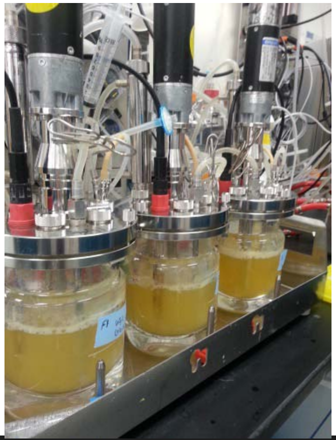


- Pretreatment: 160°C, 0.8% w/w acid, 10 minutes residence time, washed solids
- Enzymatic Hydrolysis: 25% TS, 60 mg protein/g cellulose CTec 3, 48°C, pH 5
- Fermentation: Biostat Q+, 300mL working volume, 0.6 initial OD, yeast extract/peptone medium, pH 5.1, 30°C, pO_2 10%, 134 g/L biomass sugar

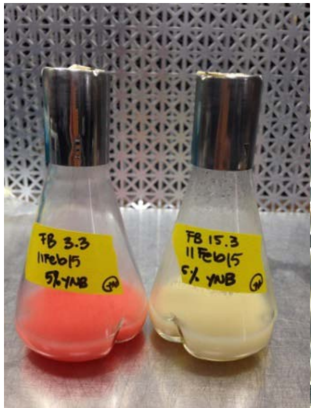
FY14 experimentally demonstrated values vs. 2017 targets for "C6 train" organism

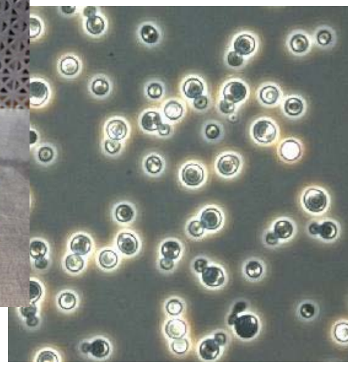
Metric	Pure Sugar - Glucose	C6 Biomass Sugars	2017 Target
Glucose utilization (total)	100%	100%	95%
Lipid content	56%	57%	60%
Volumetric productivity (g/L-hr)	0.28 (batch culture)	0.29 (batch culture)	0.40 (fed-batch)
Lipid process yield (total sugar-to-product , g/g)	0.17	0.20	0.23

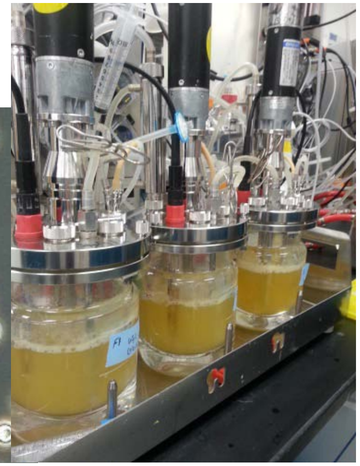


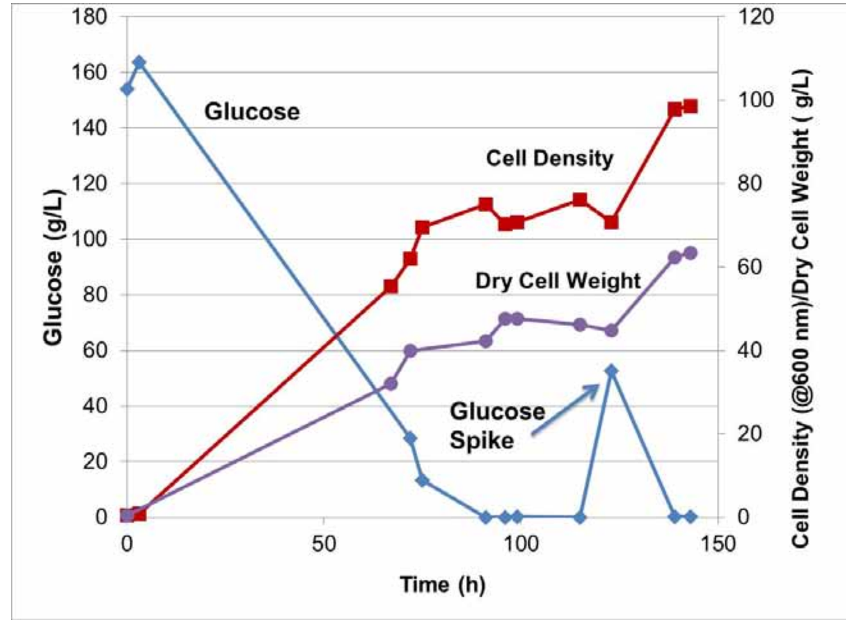


Lipid Fermentation Process Development - FY15









L. starkeyi batch fermentation spiked with extra glucose showed potential for increased productivity

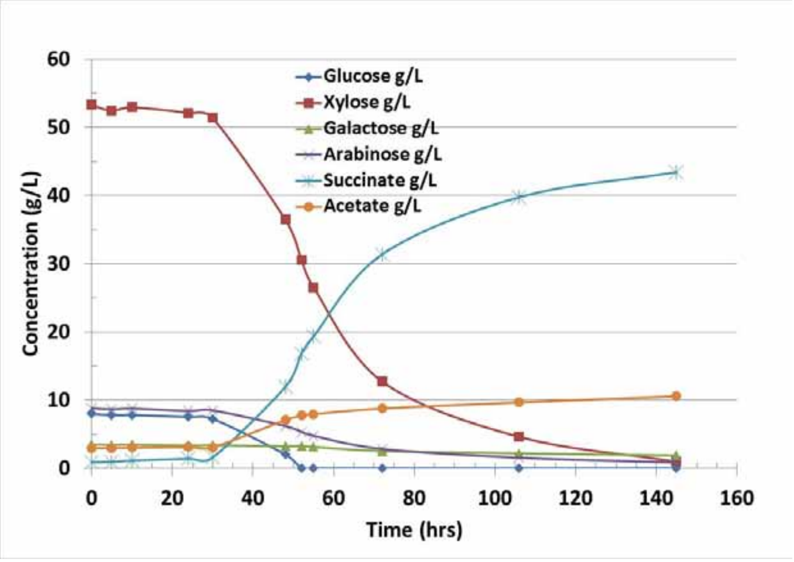
L. starkeyi screened in rich and minimal media yield different lipid content. Focus on media development during FY15/FY16 to increase lipid production.

- Rich Medium – 50% Lipid
- Minimal Medium – 74% Lipid

Metric	Spiked Batch	2017 Target
Glucose utilization (total)	100%	95%
Lipid content	56%	60%
Volumetric productivity (g/L-hr)	0.32 (if spiked at t=91h)	0.40 (fed-batch)
Lipid process yield (total sugar-to-product , g/g)	0.16	0.27

FY14 State of Technology for Succinic Acid Production

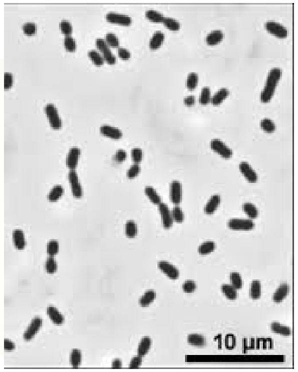
Succinic Acid Production by *Actinobacillus succinogenes* from Deacetylated Dilute Acid Pretreated Corn Stover Liquor



- Pretreatment: 160^{o}C, 0.8% w/w acid, 10 minutes residence time
- Fermentation: Biostat Q+, 300mL working volume, yeast extract/peptone/salts medium, pH 6.8 controlled with Na_2CO_3, 37^{o}C, CaO_2 supplementation, 300 rpm, batch fermentation

FY14 experimentally demonstrated values vs 2017 targets for "C5 train" organism, *A. succinogenes*

	Pure Sugar C5 Liquor	C5 Liquor – Deacetylated PCS	2017 Target
Succinic Acid volumetric productivity (g/L-hr)	0.66 (batch culture)	0.30 (batch culture)	2.0 (continuous culture)
Process yield (total sugar-to-product, g/g)	0.58	0.59	0.73
Succinic Acid Concentration (g/L)	47.3	43.4	
Sugar utilization	Xylose - 98% Glucose - 100% Arabinose - 93% Galactose - 59%	Xylose - 98% Glucose - 100% Arabinose - 90% Galactose - 46%	Xylose - 92% Glucose - 100% Arabinose - 92% Galactose - 100%

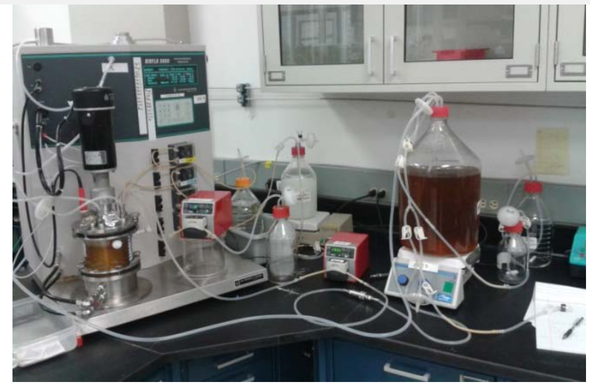


SA Fermentation Process Development – FY15

Small scale reactor modifications to promote biofilm formation by *A. succinogenes*. Biofilm can be used as a form of cell retention for increasing volumetric productivity

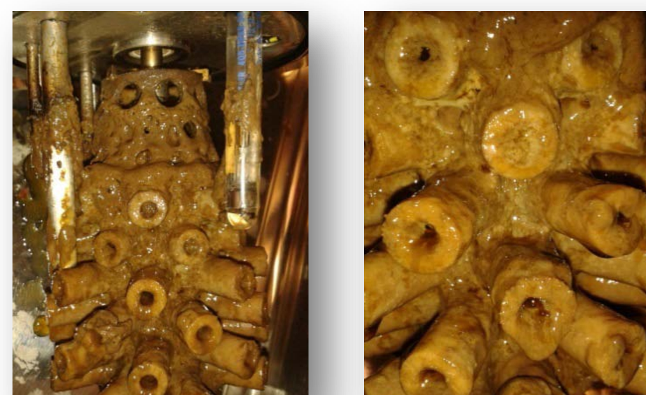
Modifications to fermentor agitator shaft

Bench scale continuous fermentor set up



Time course of biofilm formation

Fermentation post mortem



SA Fermentation Process Development

Continuous DCS hydrolysate fermentation – First Run

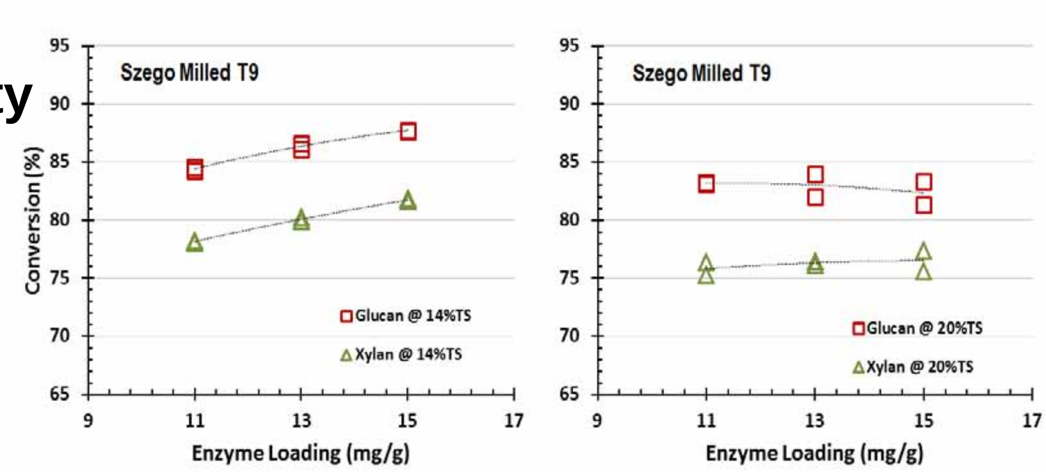
- **Representative data, pseudo steady state**
- **After apparent adaptation of culture**
- **Linear increase in productivity due to constant titer**

D (/h)	Productivity (g/L.h)	Yield ($g/g_{total\text{-}sugars}$)	Titer (g/L)	Sugar conversion (%)
0.020	0.79	0.82	39.9	82
0.025	1.05	0.77	41.6	90
0.031	1.36	0.80	43.2	90
0.044	1.83	0.79	41.6	83
FY17	2.00	0.795	-	-

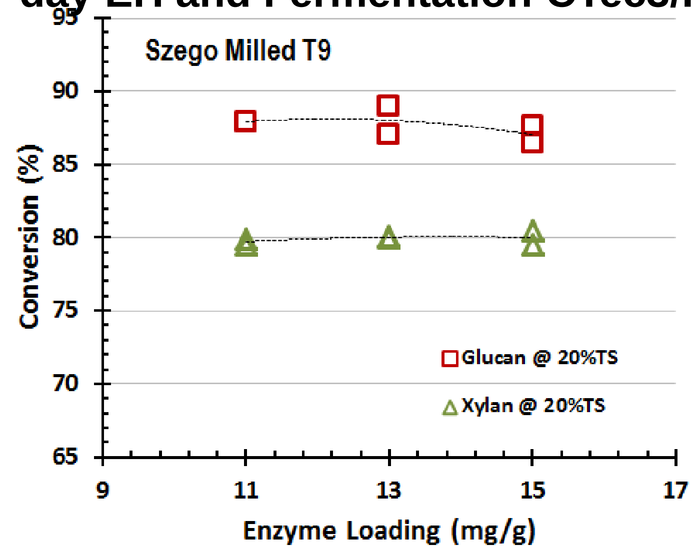
Bench Scale Integration – Enzymatic Hydrolysis Work

- **Evaluated different pretreated CS for improved cellulose digestibility and fermentability**
  - DMR PCS (deacetylated/mechanically milled)
  - Alkaline/Dilute Acid PCS
  - Alkaline PCS
- **Alkaline/Dilute Acid PCS achieved >90% cellulose conversion, but significant pretreatment xylose loss**
- **Glucose yield from DMR PCS reached 85% at 11 mg enzyme loading from EH alone (14% TS) and 87% at 11 mg with fermentation (20% TS)**

5 day EH only CTec3/HTec3 (80:20)



7 day EH and Fermentation CTec3/HTec3 (80:20)



2015 Current Progress Towards Targets

	2015 Current	2017 Target
Enzyme Loading (mg/g cellulose)	11	10
Total Solids Loading to Hydrolysis (wt%)	14%	17.5%
Enzymatic Hydrolysis Time (d)	5	3.5
Hydrolysis Glucan to Glucose	85%	90%
Expt'l bioconversion scale/method	Bench scale/ Batch spiked	Pilot scale/ Fed-batch
Bioconversion Volumetric Productivity (g/L-hr)	0.32	0.4
Lipid Content (wt%)	56%	60%
Glucose to Product [total glucose utilization]	52% [100%]	78% [100%]
C6 Train Bioconversion Metabolic Yield (Process Yield) (g/g sugars)	0.16 (0.16)	0.27 (0.27)
Bioconversion Volumetric Productivity (g/L-hr)	1.8	2.0
C5 Train Bioconversion Metabolic Yield (Process Yield) (g/g sugars)	0.66(0.79)	0.795 (0.74)

- Enzyme loading and glucose yield from DMR material using CTec3/HTec3
- Lipid yield and productivity based on *L. starkeyi* pure glucose batch fermentation with rich medium and spiked with additional glucose
- Succinic acid productivity and yields from *A. succinogenes* continuous biofilm fermentation on C5 biomass sugar

Relevance

- **Project Impacts**
  - BSI plays a key initial role in **scaling up** new integrated bio-refining processes and **identifying operating conditions** more closely **aligned with** envisioned **commercial-scale** processes
  - Provides **performance data** to support **TEA** activities
  - **Data from integrated process** development experiments are more relevant for **tracking performance improvements** aimed at achieving annual SOT cost reductions to achieve cost targets
  - Project **works closely with industrial entities** like the commercial enzyme companies by providing process-relevant data to aid the industry in developing technologies.
  - We **focus on BETO's programmatic goals**, and the information provided is made publically available through publications and presentations
- **Envisioned Outcomes**
  - The **demonstration**, at bench scale, of **BETO's 2017 fuel cost target of $5/GGE** and the 2022 fuel cost target of $3/GGE in an integrated process using biomass sugars, commercial ready enzymes, and strains that can be commercially scaled
- **Key Stakeholders and Beneficiaries**
  - Industrial entities developing technologies for hydrocarbon biofuels and co-products are both stakeholders and beneficiaries
  - This project can **provide a means to test strains, enzymes, and equipment** from a variety of organizations **in an integrated fashion using biomass sugars**. Information will be publically available through BETO and peer-reviewed journals
- **BSI project underwent BETO's AOP Merit Review in 2014**
  - A three-year project plan was submitted and accepted
  - Go/No-Go decisions and major milestones developed for FY15, 16 and 17

Future Work

Lipid Process Development

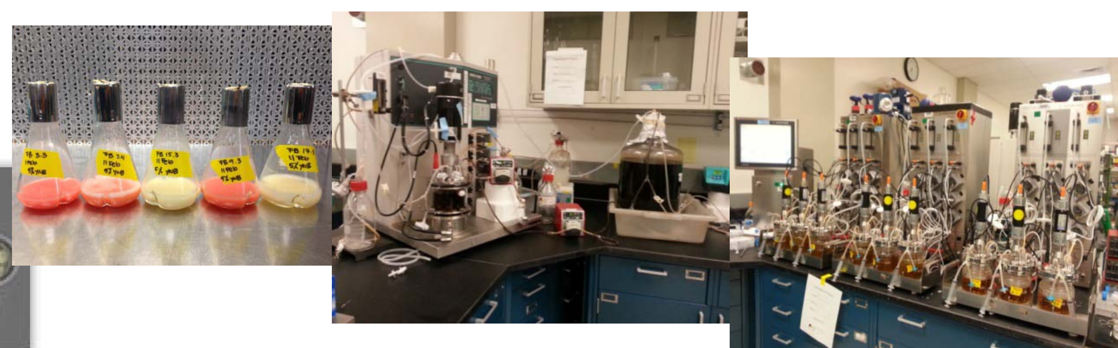
- Choose 2-3 strains by end of FY15
- Develop fermentation process
- Define sugar stream
- Quantify aeration requirements
- Demonstrate 0.4 g/L/hr rate, 60% lipid content, and a 0.27 g/g yield on C6-enriched sugars

Succinic Acid Process Development

- Down select from current strains
- Develop fermentation process
- Continue to evaluate improved strains
- Demonstrate 2 g/L/hr, 0.795 g/g yield on C5-enriched sugars

Bench Scale Integration

- Fermentation performance on biomass sugars
- Evaluate new enzyme preparations
- Test new feedstocks for 2017 and 2022 processes
- Demonstrate 90% glucan to glucose yield at 10 mg/g cellulose enzyme loading at 17.5% solids in 3.5 days



Summary

1. Approach
   - Develop an integrated fermentation process using oleaginous yeast for lipid production from C6-rich biomass streams for hydrocarbon fuel production
     - SMART goal of 0.4 g/L-hr Qp, 60% lipid content, 0.27 g lipid/g sugars process yield
   - Develop an integrated fermentation process to produce a model chemical co-product (succinic acid) from C5-rich biomass hydrolysate
     - SMART goal of 2.0 g/L-hr Qp, 0.74 g succinic acid/g sugars process yield
2. Technical accomplishments
   - Produced baseline data on real substrates for FY14 SOT which set out-year technical targets
   - Demonstrated reduction in enzyme loading from 20 mg/g to 11 mg/g cellulose
   - Demonstrated improved lipid productivities with feeding glucose to produce higher cell density (0.32 g/L-hr Qp)
   - Demonstrated high succinic acid yield and productivity on biomass sugars using a continuous biofilm fermentation (1.8 g/L-hr Qp and 0.79 g SA/g sugars)
3. Relevance
   - Demonstrating BETO's fuel cost target for 2017 at bench scale in an integrated process using biomass sugars
   - Providing the data for TEA and Life Cycle Analysis
4. Critical success factors and challenges
   - Development of the strains, enzymes, and pretreatment, that when integrated, will meet the technical targets needed to be economical in a short period of time (3 years)
5. Future work:
   - Down-select strains and develop the fermentation process with biomass sugars to meet the 2017 cost target
6. Technology transfer:
   - Collaborate with enzyme companies to develop improved enzymes for new pretreated feedstocks
   - Work with outside developers to test strains in an integrated process
   - Disseminate process information to DOE and commercial entities

Acknowledgements

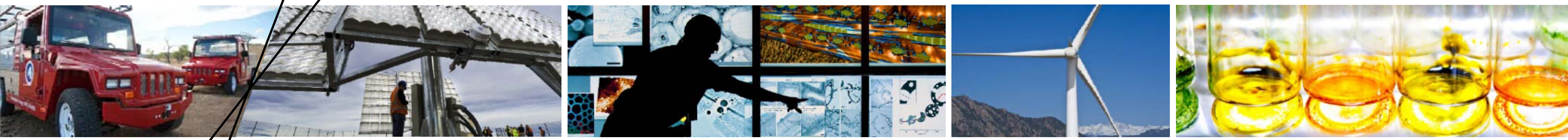
The FY14 BSI Team

U.S. DEPARTMENT OF ENERGY | Energy Efficiency & Renewable Energy | BIOMASS PROGRAM

- Holly Smith
- Darren Peterson
- Andrew Lowell
- Ed Jennings
- Rob Nelson
- Ali Mohagheghi

Not pictured

- Davinia Salvachua (*honorary member*)
- Ryan Spiller (*student intern*)



Additional Slides

Acronyms

- BSI – Bench Scale Integration
- DCS – Deacetylated Pretreated Corn Stover
- EH – Enzymatic Hydrolysis
- GGE – Gallons Gasoline Equivalents
- HC – Hydrocarbon
- PCS – Pretreated Corn Stover
- Qp – Volumetric Productivity
- SA – Succinic Acid
- SOT – State of Technology
- TEA – Techno-economic Analysis
- WWT – Waste Water Treatment

Previous Reviewers' Comments

- **Project was peer reviewed under the BPI task in FY13**
- **FY15 merit review comments**
  - Goals of the project are clearly outlined and tied to BETO programmatic goals
  - Milestones and deliverables have been tied back to advancing state of the art
  - Role of data in SOT and benchmarking is of substantial impact
  - Project is ambitious in scope given the timeline
  - Challenge is the number of parties who will need to perform well and for the benefit of the integrated goal
  - It is necessary to engage outside stakeholders to ensure knowledge transfer outside of NREL

Publications, Patents, Presentations, Awards, and Commercialization

- **Ali Mohagheghi, Jeff Linger, Holly Smith, Shihui Yang, Nancy Dowe, and Philip T Pienkos. 2014, "Improving xylose utilization by recombinant *Zymomonas mobilis* strain 8b through adaptation using 2-deoxyglucose". Biotechnology for Biofuels. 7:19.**
- **Ali Mohagheghi, Jeffrey G. Linger, Shihui Yang, Holly Smith, Nancy Dowe, Min Zhang and Philip T. Pienkos. 2015 "Improving a recombinant Zymomonas mobilis strain 8b through continuous adaptation on dilute acid pretreated corn stover hydrolysate." Biotechnology for Biofuels.**