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

2.4.3.100 Development of a thermophilic SSF system for butanol production

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Biochemical Conversion

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Goal Statement

- Decrease capital and operating costs in the biological conversion of lignocellulosic biomass to butanol through development of a thermophilic simultaneous saccharification and fermentation system
- Targets cost reductions in four of BETO's research focus areas from MYPP
  - Deconstruction Processes – *reduced severity pretreatment, potential combined pretreatment and saccharification, no thermal decomposition*
  - Biochemical Upgrading – *robust organism to produce chemical intermediate from all sugars present*
  - Integration and Intensification – *combined saccharification, fermentation and separation*
  - Conversion Enabling Technologies – *synthetic biology and metabolic flux analysis*
- Helps to meet U.S. goals to reduce dependence on fossil fuels and to reduce greenhouse gas emissions
  - Reducing costs for biochemical conversion
  - Developing an integrated conversion process

Quad Chart Overview

Timeline

- Project start date – 11/2013
- Project end date – 9/2017
- Percent complete – 30%

Budget

	Total Costs FY 07 –FY 12	FY 13 Costs	FY 14 Costs	Total Planned Funding (FY 15-Project End Date
2.4.3.100 INL	0	0	152K	693K
2.4.3.101 SNL	0	0	55K	165K
2.4.3.? NREL	0	0	0	100K
Xtreme Biochemicals	16 M			

Barriers

- Barriers addressed
  - Bt-D Pretreatment processing and selectivity
  - Bt-G Enzyme efficiency
  - Bt-J Biochemical conversion process integration
- Additional barriers Addressed
  - Bt-E, Bt-F, Bt-H, Bt-I

Partners

- Partners
  - INL (80%) and SNL (20%)
- Other collaborators
  - NREL
- Non-Technical partners
  - Xtreme Biochemicals, Inc
  - Green Biologics, Ltd.

1 - Project Overview

- During an internal project, INL researchers devised a strategy to lower biochemical conversion costs through development of a thermophilic saccharification and fermentation process
  - Identified and characterized an endoglucanase/endoxylanase with optimum activity at 70 C and pH 2-3.
- Attracted venture capital funding to expand the enzyme work
  - Formed company Xtreme Biochemicals, Inc.
  - $16 M funding starting in 2007 to present
  - Identified, expressed and characterized an almost complete suite of thermophilic lignocellulose hydrolyzing enzymes
- Partnered with SNL and obtained BETO Seed funding to develop optimized enzyme blends to deconstruct lignocellulose in FY14

1 - Project Overview cont'd

- Seed project was selected for Merit Review in FY14 and successfully passed the review
- Proposal written to incorporate thermophilic enzyme work with a proof of principle thermophilic fermentation organism and bioreactor system
  - SNL and NREL to continue enzyme blend development
  - INL focus on genetic engineering of fermentation organism and bioreactor development
  - Xtreme Biochemicals focus on business development of enzymes
  - Green Biologics focus on business development of fermentation process

2 – Approach (Technical)

- FY14 milestone was to develop an optimized thermophilic enzyme blend, test the blend on a pretreated feedstock and determine whether the hydrolysate was fermentable
  - SNL screened enzymes from thermophilic organisms
  - Optimized INL and SNL enzyme blends for maximum glucose and xylose yields from pretreated biomass
  - Prepared of hydrolysate for fermentibility assessment by Green Biologics
  - Hydrolysate fermentation and assessment of solvent production
- FY15 Q1 and Q2 milestones were to select a butanol tolerant thermophilic strain and obtain sequence information
  - Conduct literature search and assess GBL's thermophilic library
  - Obtain contract sequencing for selected organism if needed

2 – Approach (Management)

- INL is the lead for this project and coordinates efforts with partners
  - Teleconferences as needed
  - Quarterly and annual reports
  - Yearly meeting with partners to discuss progress
- Partner roles
  - INL will lead genetic engineering and reactor design proof of principle
  - SNL and NREL will each develop optimized blends for INL/SNL and INL/NREL enzymes using various pretreated feedstocks
  - Xtreme Biochemicals will work to commercialize enzymes and enzyme blends
  - Green Biologics will provide their expertise as a commercial manufacturer of biologically-produced butanol and their library of thermophilic butanol tolerant organisms
  - Green Biologics will also provide a scale-up and commercialization pathway for the processes developed in this project

3 – Technical Accomplishments/ Progress/Results

INL enzyme activities (U/mg) at optimum temperature and pH

Activities	T(°C)	pH	Substrate	Specific Activity (U/mg)
Endoglucanase	80	6	CMC	275
Endoxylanase	70	6	WAX	807
β-glucosidase	70	6	cellobiose	461
β-xylosidase	70	5	xylobiose	148
α-glucuronidase	70	6	Aldouronic acids	34
α-L-arabinofuranosidase	70	6	WAX	0.62
Acetylxylan esterase	70	6	Acetylated xylan	acetate
Ferulic acid esterase	70	6	Ferulic acid	1.1
Coumaric acid esterase	70	6	Coumaric acid	0.49

- Glycosylases
- Glycosidases
- Esterases
- Activity on natural substrates
- Worked with MSU to benchmark INL enzymes against commercial enzymes

3 – Technical Accomplishments/ Progress/Results (cont'd)

SNL and NREL Enzymes (U/mg)

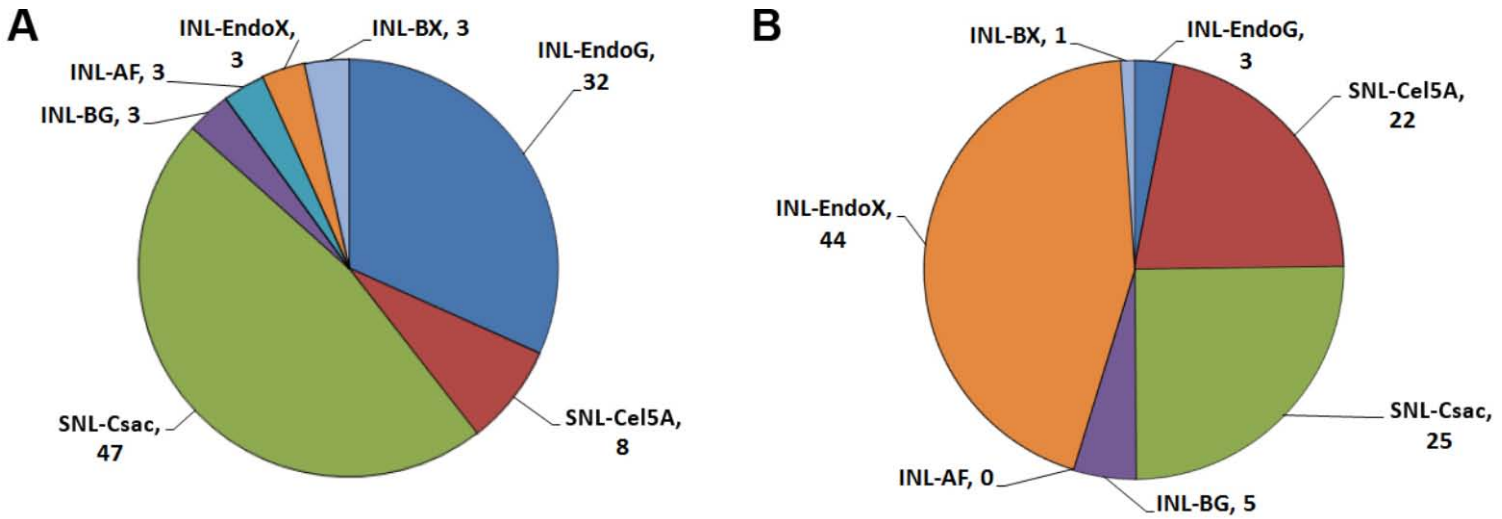
Activities	T(°C)	pH	Substrate	Specific Activity (U/mg)
SNL				
Cel5a	70	6	IL-pretreated switchgrass	0.06
Csac	70	6	IL-pretreated switchgrass	0.07
NREL				
CelA	72	6	Avicel	0.29

- INL enzymes lack CBH
- SNL enzymes have EG and CBH activities
- NREL CelA has a CBH activity

3 – Technical Accomplishments/ Progress/Results (cont'd)

- We identified 7 enzymes with $T_{opt} \geq 70$ C
- Enzyme mixtures were optimized using an augmented simplex lattice design of experiment

Abbreviation	Enzyme type
SNL-Cel5A	endoglucanase (Cel5A from *Thermotoga maritima*)
SNL-Csac	cellobiohydrolase (CSac from *Caldicellulosiruptor saccharolyticus*)
INL-EndoG	endo-1,4-β-glucanase
INL-BG	1,4-β-glucosidase
INL-AF	α-L-arabinofuranasidase
INL-EndoX	endo-1,4-β-xylanase
INL-BX	1,4-β-xylosidase



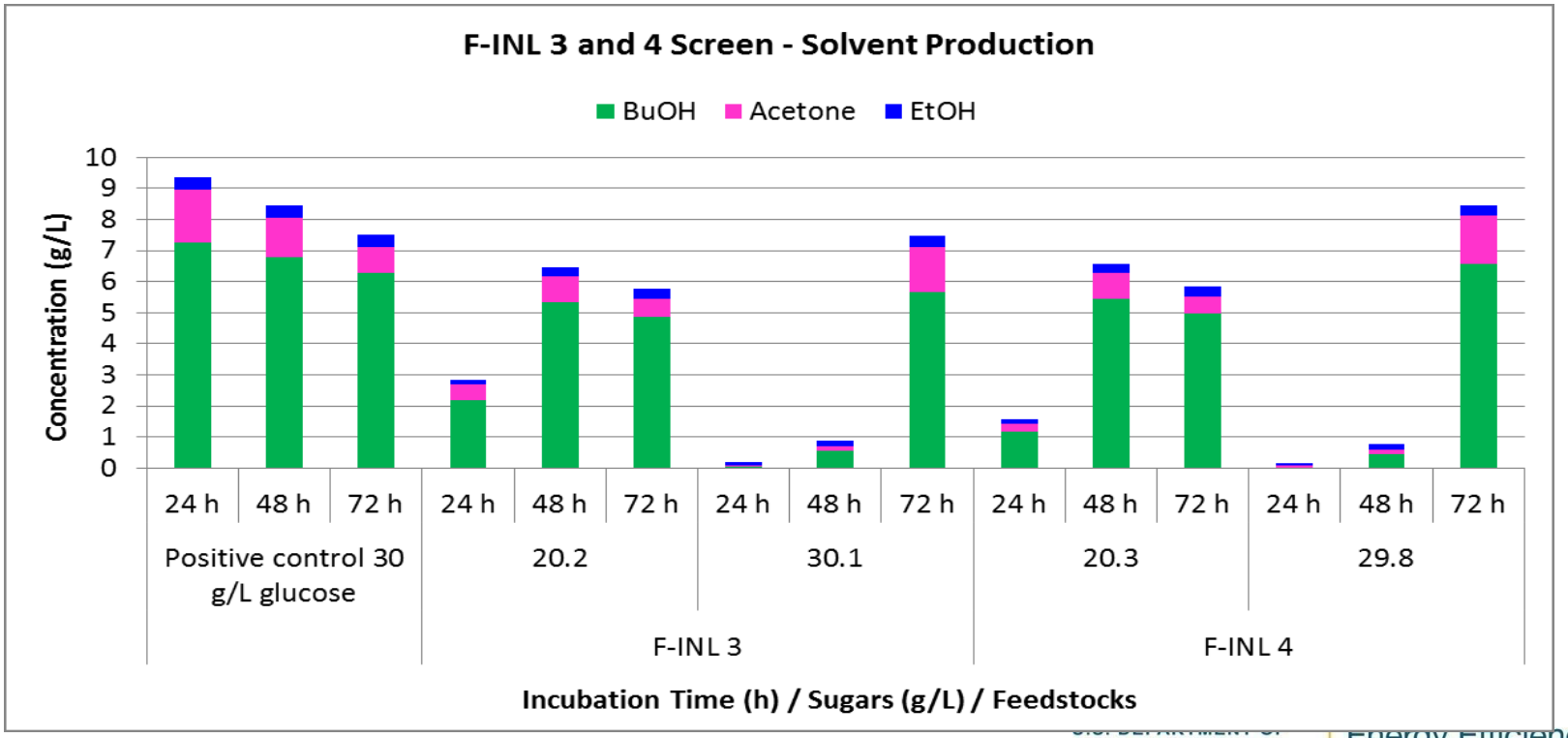
Optimal enzyme ratios for the degradation of pretreated corn stover (A) and switchgrass (B). Numbers following enzyme names indicate percentages.

3 – Technical Accomplishments/ Progress/Results (cont'd)

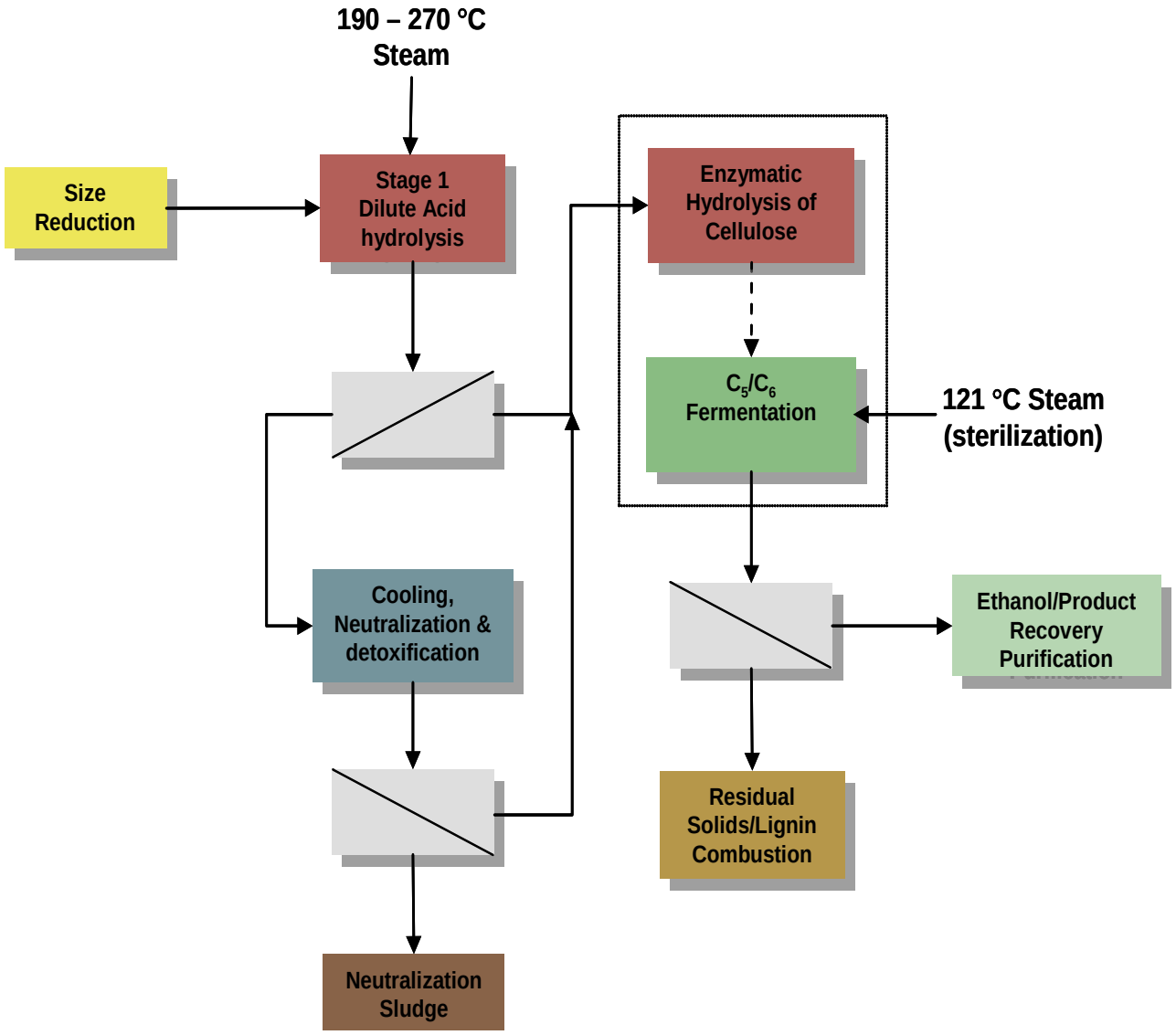
- Ionic liquid pretreated switchgrass was obtained from SNL
- Switchgrass at 8% solids was enzymatically hydrolyzed using blend from previous slide for 72 hours at 70 C with 30 mg/g loading
- Glucose yield 53 % and xylose yield 95%
- Hemicellulase system is working extremely well with high xylose yields
- Glucose yields were lower than expected
  - Cellulase system chosen needs other activities (i.e. CBH)
  - SNL is screening their thermophile library for these activities
  - Will work with NREL and utilize enzymes developed through BESC to develop a better cellulase system

3 – Technical Accomplishments/ Progress/Results (cont'd)

- Hydrolysate (C_5 and C_6 sugars) yielded as much or more butanol than the glucose control at 72 hours
- Fermentation organism showed a lag in solvent production compared to glucose control
  - Likely due to presence of C_5 and other C_6 sugars

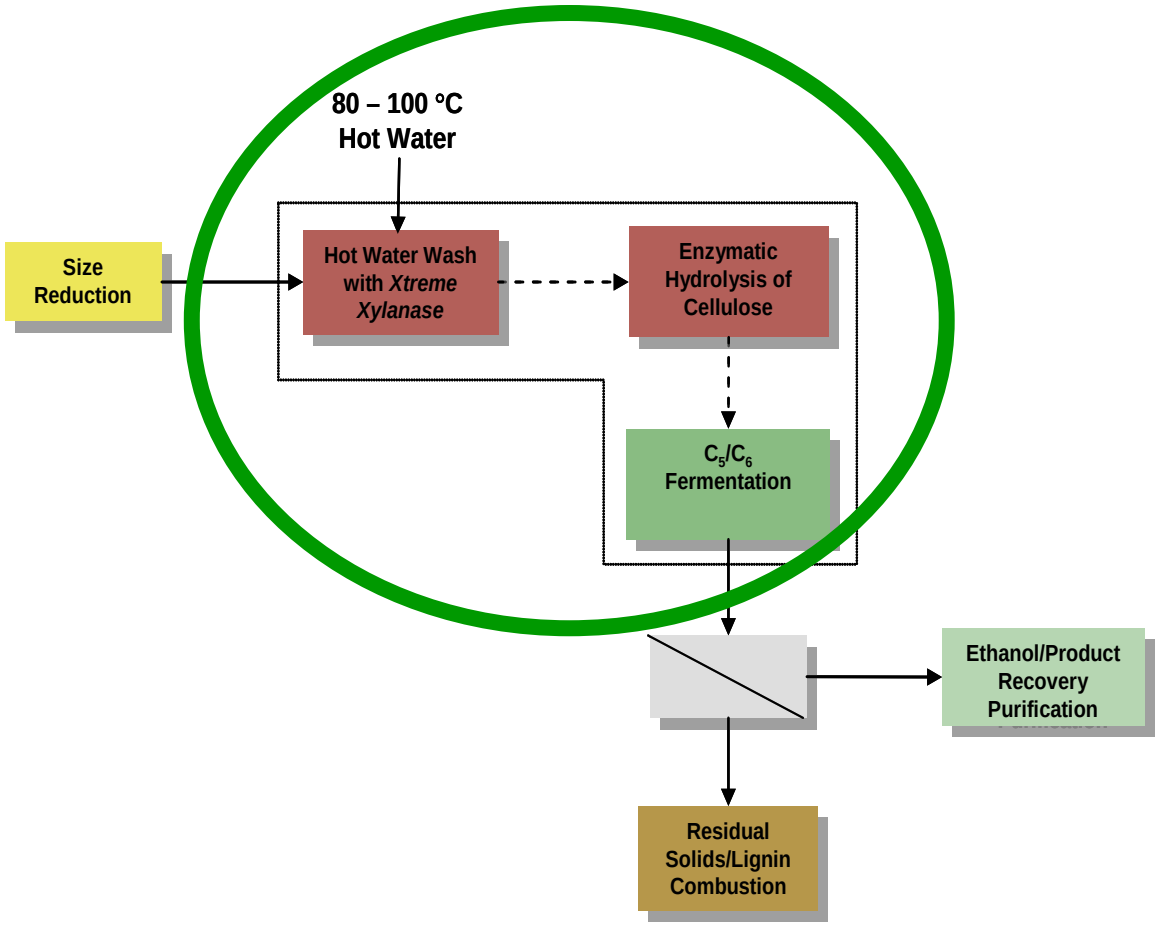


3 – Technical Accomplishments/ Progress/Results (cont'd)



- NREL dilute acid hydrolysis model
- Expensive materials
- Thermal decomposition
- High pressure
- Cooling and neutralization needed
  - Mismatched enzyme and fermentation conditions
- No integration of unit operations

3 – Technical Accomplishments/ Progress/Results (cont'd)



- Combined thermophilic saccharification and fermentation (possibly pretreatment)
- Less expensive materials
- Less high pressure steam
- Lower cooling/neutralization requirement
- Fermentation/enzyme conditions are matched
- Reduced sterilization/contamination
- Integration of unit operations
- 27% reduction in cost of gallon of ethanol over NREL model

3 – Technical Accomplishments/ Progress/Results (cont'd)

- Cannot directly compare to butanol case due to differences in vapor pressure and volatility
- An examination of gas stripping for butanol case
  - Comparing 30 C fermentation with gas stripping to 70 C fermentation with gas stripping results in 77-fold decrease in CO_2 requirements
  - Current commercial 30 C fermentations do not incorporate gas stripping
  - A 70 C fermentation with gas stripping would result in a 210% increase in butanol yield over commercial fermentations due to decreased toxicity to fermentation organism

4 – Relevance

- Bt-D and Bt-E Pretreatment Processing and Selectivity and Pretreatment Reactor Design and Optimization
  - Thermophilic lignocellulose enzymes allow pretreatment severity to be reduced
  - Lower temperatures and pressures mean less expensive construction materials
  - Reduces or eliminates thermal decomposition products
  - Reduces or eliminates cooling requirements
- Bt-F and Bt-G Hydrolytic Enzyme Production and Enzyme Efficiency
  - Enzymes have high specific activity on natural lignocellulose substrates
  - Unique enzyme activities discovered by INL, SNL and NREL
  - Complete suite of lignocellulose hydrolyzing enzymes
  - High throughput screening allows testing of many blends
  - Enzyme production has been tested in commercial production systems
- Bt-I Catalyst Efficiency
  - Synthetic biology and metabolic modeling used to more efficiently target genetic engineering

4 – Relevance

- Flux analyses to improve carbon flow toward butanol production
- Organism able to utilize both C_5 and C_6 sugars

- Bt-J Biochemical conversion process integration
  - Saccharification enzymes and fermentation organism are matched for temperature and pH optimum allowing higher production rates
  - Higher temperature operation reduces contamination and sterilization issues
  - Thermophilic organisms are generally more robust and solvent tolerant
  - Higher vapor pressure of butanol at elevated temperature allows use of cost effective gas stripping
    - Product purification/reduces separation costs
    - Removes butanol toxicity and facilitates continuous operation instead of batch
  - Potential to combine saccharification, fermentation and product recovery in one reactor

5 – Future Work

- Enzyme blends
  - Identify improved endoglucanases and cellobiohydrolases to increase glucose and xylose yields from blends (6/30/2015)
  - Demonstrate 50% increase in glucose yield over current blends (6/30/2016)
- Genetic engineering of thermophilic organism
  - Identify at least one gene from the metabolic flux analyses that can increase butanol production (9/30/2015)
  - Introduce thermotolerant selectable markers into organism (12/20/2015)
  - Go/No-Go Demonstrate butanol production increase of 25% with gas stripping (3/31/2016)
  - Demonstrate optimized butanol pathway (9/30/2016)

Summary

- A proof of principle integrated thermophilic simultaneous saccharification and fermentation system will be developed
- This leverages previous work at INL, SNL and NREL
- System will demonstrate the capability to reduce capital and operating costs
  - Pretreatment
  - Enzyme saccharification
  - Fermentation
  - Product separation

Responses to Previous Reviewers' Comments

- This project has not been previously reviewed
- There have not yet been any Go/No-Go decisions

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Publications, Patents, Presentations, Awards, and Commercialization

- No publications, presentations, patents or awards yet on current work
  - Thermoacidophilic xylanase from previous work won an R&D 100 award and a Federal Laboratory Consortium award in 2006
  - Previous work with Xtreme Biochemicals has yielded 23 U.S. and foreign patents with more pending on thermophilic enzymes and the organism from which they originated
  - FY14 work has been accepted for presentation at the 37th Symposium on Biotechnology for Fuels and Chemicals
- Describe the status of any technology transfer or commercialization efforts
  - Patents generated previously have been licensed to Xtreme Biochemicals
  - It is expected that intellectual property generated for this project would also be licensed by either Xtreme Biochemicals or Green Biologics

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