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%

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

Budget

<table>
<thead>
<tr>
<th></th>
<th>Total Costs FY 07–FY 12</th>
<th>FY 13 Costs</th>
<th>FY 14 Costs</th>
<th>Total Planned Funding (FY 15-Project End Date)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4.3.100 INL</td>
<td>0</td>
<td>0</td>
<td>152K</td>
<td>693K</td>
</tr>
<tr>
<td>2.4.3.101 SNL</td>
<td>0</td>
<td>0</td>
<td>55K</td>
<td>165K</td>
</tr>
<tr>
<td>2.4.3.? NREL</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100K</td>
</tr>
<tr>
<td>Xtreme Biochemicals</td>
<td>16 M</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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
• 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**

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

- Glycosylases
- Glycosidases
- Esterases
- Activity on natural substrates
- Worked with MSU to benchmark INL enzymes against commercial enzymes
### SNL and NREL Enzymes (U/mg)

<table>
<thead>
<tr>
<th>Activities</th>
<th>T(°C)</th>
<th>pH</th>
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<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SNL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cel5a</td>
<td>70</td>
<td>6</td>
<td>IL-pretreated switchgrass</td>
<td>0.06</td>
</tr>
<tr>
<td>Csac</td>
<td>70</td>
<td>6</td>
<td>IL-pretreated switchgrass</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>NREL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CelA</td>
<td>72</td>
<td>6</td>
<td>Avicel</td>
<td>0.29</td>
</tr>
</tbody>
</table>

- INL enzymes lack CBH
- SNL enzymes have EG and CBH activities
- NREL CelA has a CBH activity
• We identified 7 enzymes with $T_{opt} \geq 70$ °C
• Enzyme mixtures were optimized using an augmented simplex lattice design of experiment

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Enzyme type</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNL-Cel5A</td>
<td>endoglucanase (Cel5A from <em>Thermotoga maritima</em>)</td>
</tr>
<tr>
<td>SNL-Csac</td>
<td>cellbiohydrolase (CSac from <em>Caldicellulosiruptor saccharolyticus</em>)</td>
</tr>
<tr>
<td>INL-EndoG</td>
<td>endo-1,4-β-glucanase</td>
</tr>
<tr>
<td>INL-BG</td>
<td>1,4-β-glucosidase</td>
</tr>
<tr>
<td>INL-AF</td>
<td>α-L-arabinofuranasidase</td>
</tr>
<tr>
<td>INL-EndoX</td>
<td>endo-1,4-β-xylanase</td>
</tr>
<tr>
<td>INL-BX</td>
<td>1,4-β-xylosidase</td>
</tr>
</tbody>
</table>

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 (C₅ and C₆ 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 C₅ and other C₆ 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
• 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
• 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 CO₂ 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 C₅ and C₆ 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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