Developing *Thermoascus aurantiacus* as a Thermophilic Fungal Platform for Industrial Production of Cellulases (WBS 2.2.3.102-3)

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Biochemical Conversion
Goal Statement

• Develop a thermophilic fungal platform for cellulase production for application in the biofuel industry
• Develop a high titer cellulase producer whose enzymes can saccharify biomass at higher temperatures and efficiencies than current commercial systems
• Fungal strains and bioprocess conditions developed in this project will lower the cost of enzyme production and improve on-site generation of enzyme for biomass to biofuel conversion. 2016 revision of BETO MYPP specifically calls for “new, more efficient enzymes” and “improving enzyme temperature tolerance”. (Section 2, page 67-68)
Quad Chart Overview

Timeline

• Project start date- 10/1/14
• Project end date- 9/30/17
• 70% complete (paused 12/16)

Budget

<table>
<thead>
<tr>
<th></th>
<th>Total Costs FY 12-14</th>
<th>FY 15 Costs</th>
<th>FY 16 Costs</th>
<th>Total Planned Funding (FY 17-Project End Date)</th>
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<tr>
<td>DOE Funded</td>
<td>N/A</td>
<td>$150K</td>
<td>$540K</td>
<td>$540K ($35K funded)</td>
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<tr>
<td>Project Cost Share (Comp.)*</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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</tbody>
</table>

Barriers

• Enzymatic Hydrolysis and Bioconversion (2022 target)
• Cellulase Enzyme Production (2022 target)
• C5 Coproduct Train (2017 target)

Partners

• LBNL- FY15 (100%), FY16 (72%), FY17 (72%)
• PNNL- FY15 (0%), FY16 (28%), FY17 (28%)
• Collaborations:
  • NREL- biomass
  • TU Braunschweig- systems biology
  • Norwegian University of Life Sciences- LPMOs
  • National Corn Ethanol Research Center- Gen 1.5 ethanol
1 – Overview

Low temperature deconstruction and fermentation design case (2016 MYPP)

Legend:
- Feedstock
- Pretreatment
- Enzymatic Hydrolysis and Bioconversion
- Cellulase Enzyme Production
- C5 Coproduct Processing Train
- Lignin derived adipic acid
- Product Recovery + Upgrading
- Balance of Plant
1-Overview-Cellulase enzyme production

• *T. reesei* is most commonly used platform strain for cellulase production

• Limits of *T. reesei* mixtures:
  • Cellulase temperature optimum at ~50°C; requires heterologous expression of LPMO and beta-glucosidase for high performance
  • Cellulase production requires C6 disaccharide inducer (sophorose, lactose), which complicates on-site enzyme production
  • Production strains cannot be crossed to isolate hyperproduction genotypes

• Can a thermophilic fungal platform be developed that overcomes these limitations?
Properties of *T. aurantiacus*:

- Produces thermostable enzymes (1-2 g/L in shake flasks), enables biomass saccharifications at ≥60°C
- Remarkably simple glycoside hydrolase complement that dominates supernatant
- Fungus is self-fertile (homothallic); though sexual crossing has not been demonstrated
- Cellulase inducers are unknown
2 – Approach (Management)

Project management, laboratory fungal cultivation, enzymatic analysis, sexual crossing, random mutagenesis (Steven Singer, Timo Schuerg, Raphael Gabriel, Lauren Tom)

Bioreactor cultivation, scale-up, hemicellulosic hydrolysate, TEA model (Deepti Tanjore, ABPDU staff)

Targeted mutagenesis, culture optimization (Jon Magnuson, Ziyu Dai, Beth Hofstad)
2-Approach-Platform Development

- Systems biology tools
  - RNASeq/Proteomics

- Targeted mutagenesis

- Random mutagenesis
  - UV / EMS

- Cultivation optimization

- Sexual recombination

High titer cellulase production
2-Approach-Technical Goals and Challenges

Technical Goals

• Produce cellulases at >10 g/L (Go/no go milestone)
• Scale-up of Thermoascus aurantiacus cultivation to 300 L (w/ABPDU)
• Demonstrate >80% glucose release at 65°C from pretreated corn stover (NREL-produced material)
• Generate cellulase hyperproduction mutants of T. aurantiacus (w/PNNL)

Technical Challenges

• Production at 1-2 g/L in shake flasks with complex biomass as substrate (State of Technology in 9/14)
• No bioreactor or scale-up conditions for T. aurantiacus protein production
• No genetic system for T. aurantiacus
3 – Technical Accomplishments/ Progress/Results

By pre-growing biomass on glucose and inducing with xylan at low culture volumes, >10 g/L of protein was produced in shake flasks.

SDS-PAGE

Protein concentration (2% xylan)
Fed-batch conditions that simulated bioreactors demonstrated that xylose induced production of cellulases.
Multiple 2 L bioreactor campaigns have been conducted at the ABPDU

- 2% xylan
- 0.01% xylose
- 0.01% C5 acid hydrolysate

Xylose induction scaled to 20 L
Hydrolysate prepared from corn stover (ABPDU)
pH dependence of protein production diverges from most filamentous fungi.
3 – Technical Accomplishments/ Progress/Results

Saccharification of deacetylated, mechanically refined (DMR) corn stover

Conditions: 2% biomass loading, 20 mg protein/g glucan, 100 mM Na citrate pH 5, 4 days, 50 mL scale

50°C (Glucose) 60°C (Glucose)
Saccharification of deacetylated, dilute acid (DAAD) pretreated corn stover

Conditions: 2% biomass loading, 20 mg protein/g glucan, 100 mM Na citrate pH 5, 4 days, 50 mL scale

50°C (Glucose)  60°C (Glucose)
3 – Technical Accomplishments/ Progress/Results

Linking pretreatment and on-site protein production in a biorefinery.

Corn stover → Dilute acid pretreatment → C5-rich fraction → Cellulase production

C6-rich fraction → Enzymatic hydrolysis → Sugar
An *Agrobacterium tumefaciens* transformation system has been established for *T. aurantiacus*
Sexual crossing is demonstrated using UV mutagenized strains of *T. aurantiacus*.
Preliminary economic model predicts lower enzyme cost for enzymes from *T. aurantiacus*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NREL 2011 Design Model</th>
<th>Current Model</th>
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</thead>
<tbody>
<tr>
<td>Fermentation Temperature</td>
<td>28°C</td>
<td>50°C</td>
</tr>
<tr>
<td>Water supply</td>
<td>Chilled water</td>
<td>Cooling water</td>
</tr>
<tr>
<td>Fermentation time</td>
<td>120 h</td>
<td>96 h</td>
</tr>
<tr>
<td>Fermentation Mode</td>
<td>Fed-batch</td>
<td>Fed-batch</td>
</tr>
<tr>
<td>Conversion of sugar to enzyme (g/g)</td>
<td>0.24</td>
<td>0.24</td>
</tr>
<tr>
<td>Glucan conversion to glucose</td>
<td>90% of theoretical</td>
<td>90% of theoretical</td>
</tr>
<tr>
<td>Enzyme cost ($/kg)</td>
<td>4.24</td>
<td>3.99</td>
</tr>
<tr>
<td>Protein loading (mg/g glucan)</td>
<td>20 mg</td>
<td>8 mg</td>
</tr>
<tr>
<td>Expected enzyme (mg) required to produce 1 gallon ethanol</td>
<td>117.18</td>
<td>46.87</td>
</tr>
<tr>
<td>Expected volume of enzyme producing fermenter</td>
<td>80,000 gal</td>
<td>32,000 gal</td>
</tr>
</tbody>
</table>
3 – Technical Accomplishments/ Progress/Results

Publications and Patents


3) “Production of cellulase by a thermophilic fungal cell induced by a soluble xylan, or analog thereof” Patent Application Ser. No: 62/457,685
3 – Technical Accomplishments/ Progress/Results

Presentations
1) “Engineering a thermophilic filamentous fungus into a high performance host for cellulase production” 38th Symposium on Biotechnology for Fuels and Chemicals, Baltimore, MD 4/28/16
2) “High temperature saccharification of pretreated corn stover with enzymes from *Thermoascus aurantiacus*” 39th Symposium on Biotechnology for Fuels and Chemicals, San Francisco, CA 5/1/17
3) “Unraveling cellulase and xylanase induction in *Thermoascus aurantiacus* for improved enzyme production” 39th Symposium on Biotechnology for Fuels and Chemicals, San Francisco, CA 5/1/17
4 – Relevance

- **Project Goal**: Develop a thermophilic fungal platform for cellulase production for application in the biofuel industry

- **Importance**: Fungal strains and enzymes developed in this project will allow biomass saccharifications to be performed at higher temperatures and lower enzyme loadings than current technologies

- **Accomplishments**: Demonstrated scalable cellulase production using xylose as inducer, enabling utilization of a C5 pretreatment stream for enzyme production; demonstrated that cellulases showed comparable performance to commercial mixtures at higher temperatures; developed genetic system for thermophilic cellulolytic fungus (only one other reported)

- **BETO MYPP relevance**: contributes to 2022 targets for lowering biochemical conversion to $0.95/GGE and enzyme price to $0.41/GGE; unexpectedly contributes to 2017 goal of valorizing C5 hydrolysate (-$6.20/GGE)
4 – Relevance

- **Industrial Relevance**: Provides a new platform for cellulase production that has distinct advantages over current technologies, including the production of cellulases from a soluble biomass fraction that perform at high temperature and enable on-site enzyme production using biomass components. Simplicity of enzymatic mixture and ability to engineer *T. aurantiacus* permits feedstock-specific design of high temperature cellulase mixtures.
5 – Future Work

FY17 (project currently paused):

• Test *Agrobacterium*-mediated knockouts of genes responsible for carbon catabolite repression (*cre1*)
• Perform high solids saccharification (20% biomass loading) with DMR and DAAD corn stover
• Identify limiting reactions in saccharification by enzyme supplementation
• Perform updated TEA using C5 hydrolysate as substrate and align with design case

Beyond FY17

Advanced engineering with CRISPR/Cas9

Heterologous expression for designer enzymatic mixtures

Enzyme production scale-up (kgs) for testing
Summary

- **Overview** - *T. aurantiacus* provides a promising thermophilic fungal platform for cellulase production

- **Approach** - a combination of cultivation improvements and development of a genetic system were proposed to develop *T. aurantiacus* as a cellulase production platform

- **Technical Accomplishments/Progress/Results** - high titer cellulase production using xylose as inducer; saccharification of NREL corn stover substrates comparable to commercial enzymes at high temperature; development of *Agrobacterium*-mediated transformation

- **Relevance** - this work enables cellulase production using C5 stream from dilute acid pretreatment; provides an alternative to current commercial enzymatic mixtures that performs at higher temperatures, which is directly related to BETO MYPP goals.