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U.S. DEPARTMENT OF ENERGY

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

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March 8, 2017

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

	Total Costs FY 12 –FY 14	FY 15 Costs	FY 16 Costs	Total Planned Funding (FY 17-Project End Date)
DOE Funded	N/A	$150K	$540K	$540K ($35K funded)
Project Cost Share (Comp.)*	N/A	N/A	N/A	N/A

Barriers

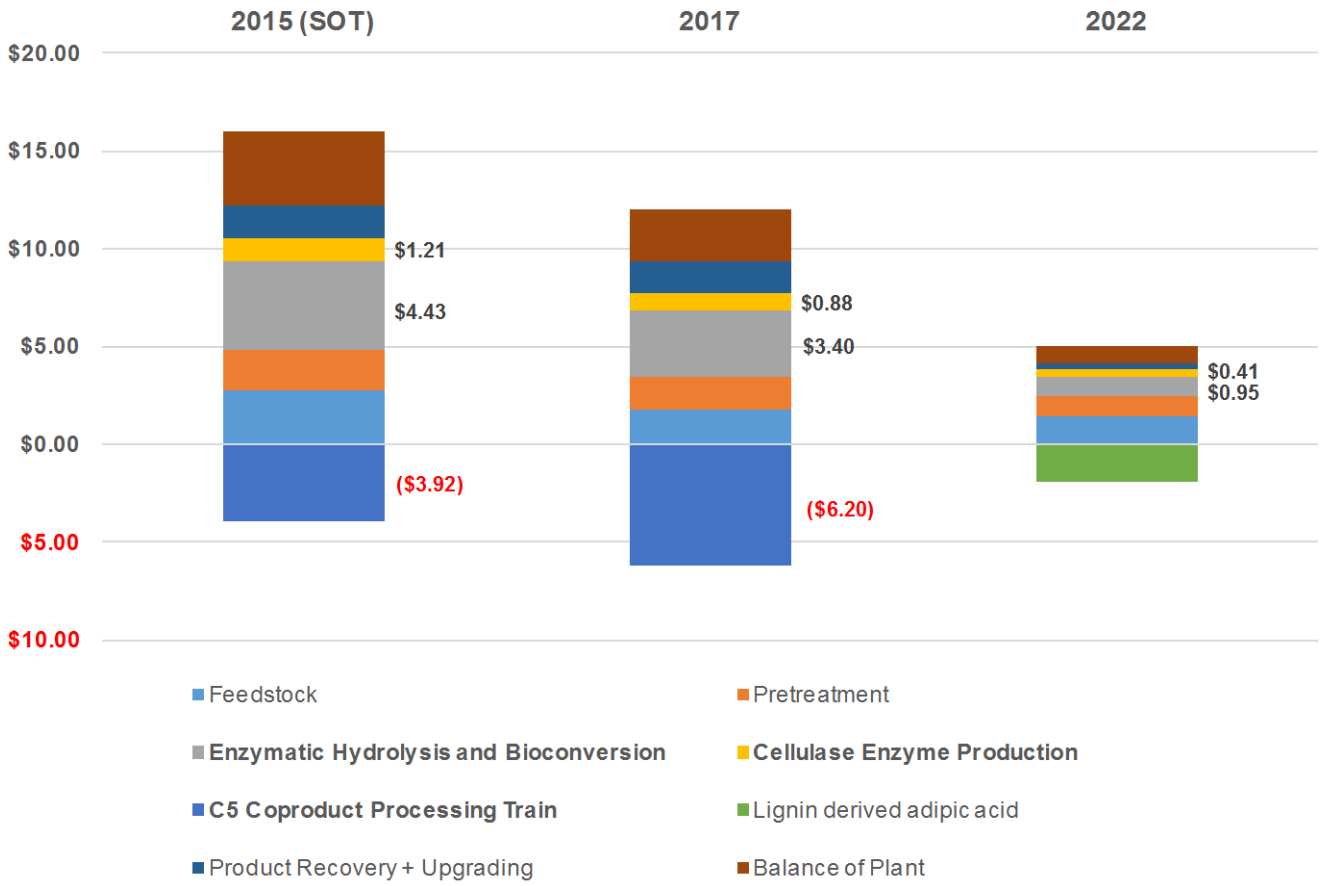
- Enzymatic Hydrolysis and Bioconversion (2022 target)
- Cellulase Enzyme Production (2022 target)
- C5 Coproduct Train (2017 target)

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)



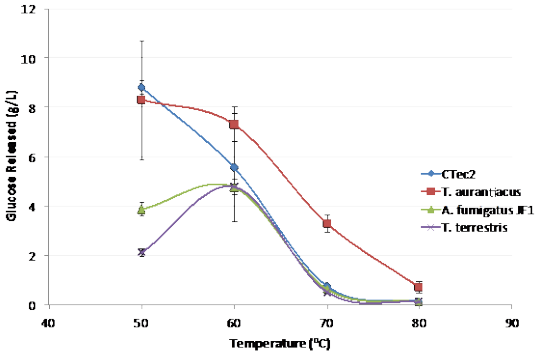
1-Overview-Cellulase enzyme production

- *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?**

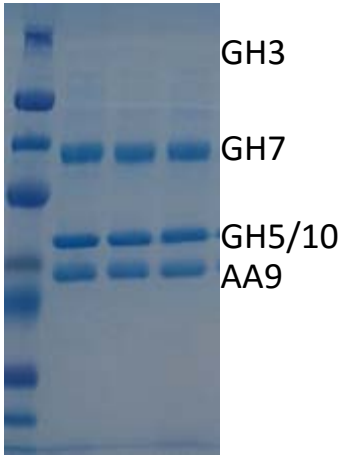
1-Overview-*Thermoascus aurantiacus*

Thermoascus aurantiacus

Saccharification of
Ionic liquid-pretreated switchgrass



T. aurantiacus supernatants (AFEX-pretreated corn stover)



Biotechnology for Biofuels, **2012**, *5*, 54

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

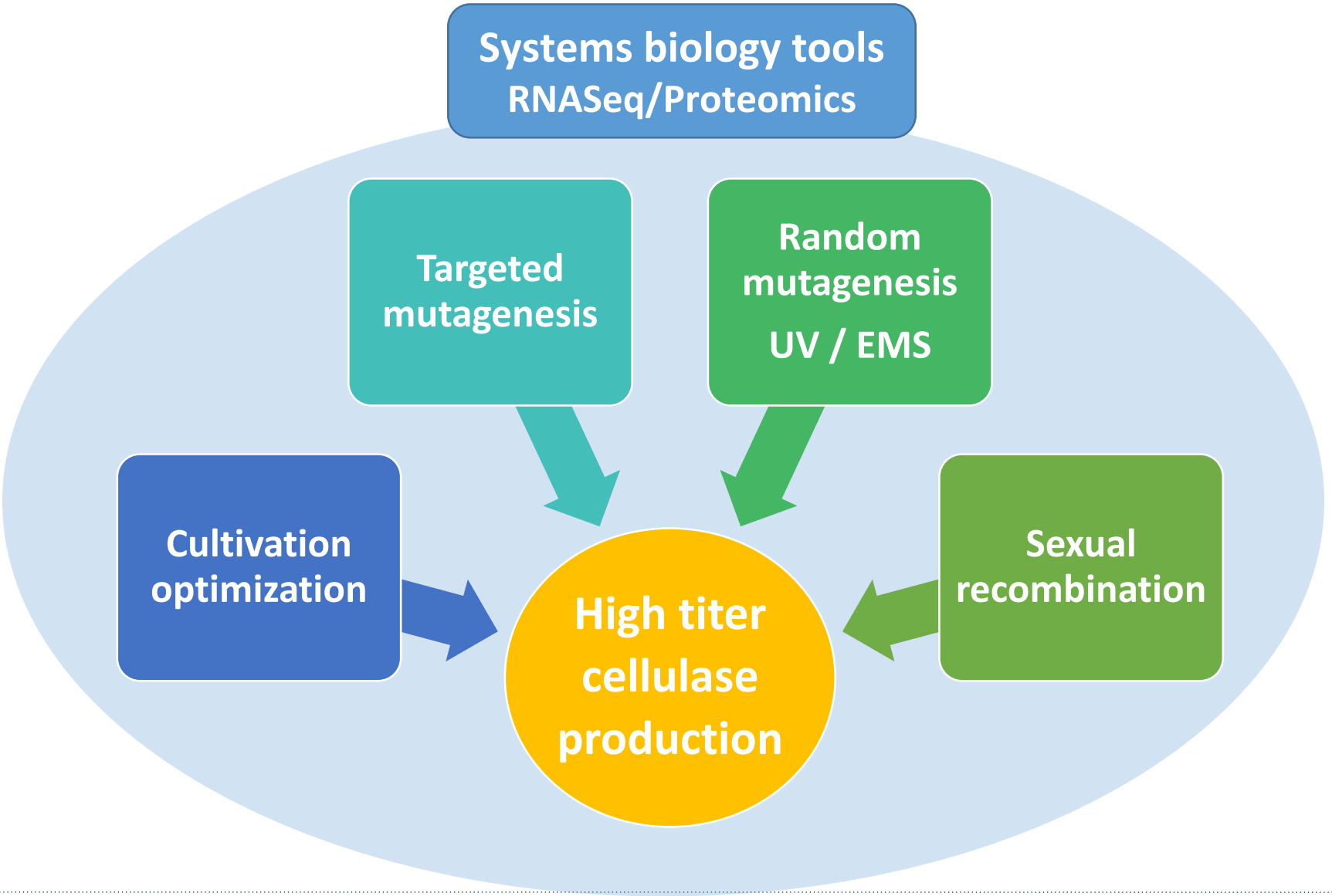
2 – Approach (Management)

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



2-Approach-Technical Goals and Challenges

Technical Goals

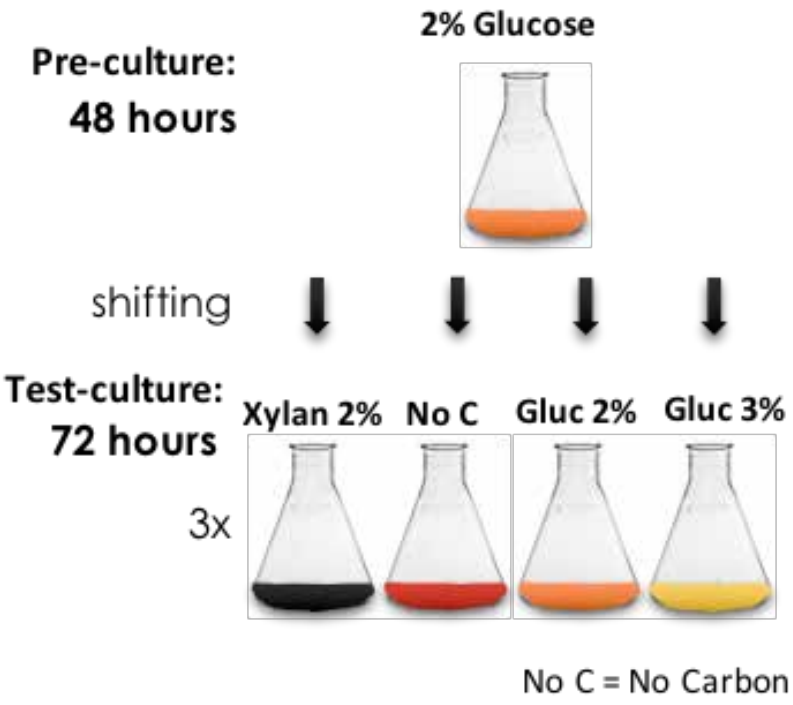
- Produce cellulases at >10 g/L (Go/no go milestone)
- Scale-up of *Thermoascus aurantiacus* cultivation to 300 L (w/ABPDU)
- Demonstrate >80% glucose release at 65◦C from pretreated corn stover (NREL-produced material)
- Generate cellulase hyperproduction mutants of *T. aurantiacus* (w/PNNL)

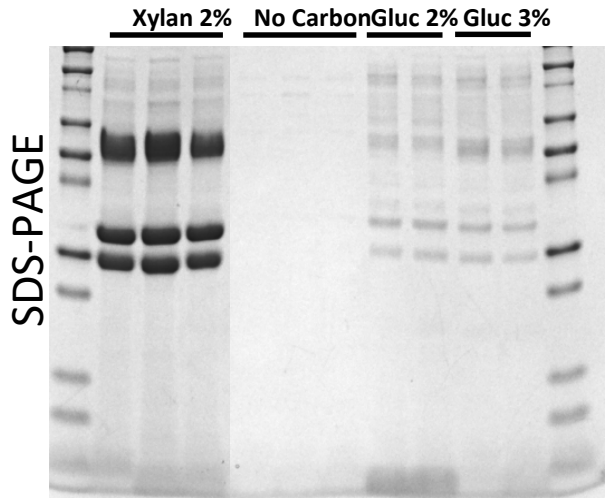
Technical Challenges

- 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*

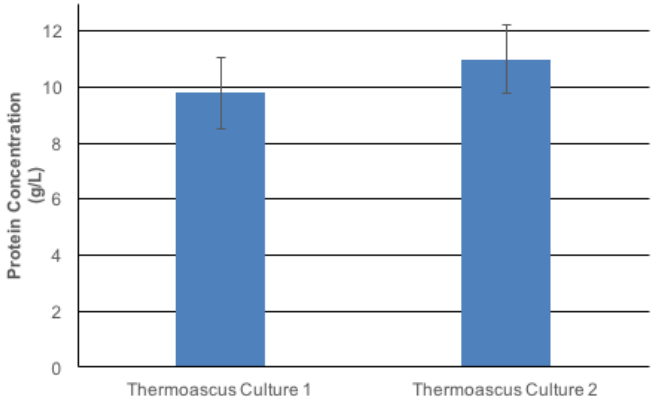
3 – Technical Accomplishments/ Progress/Results

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





Protein concentration (2% xylan)



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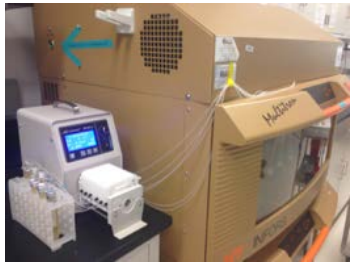
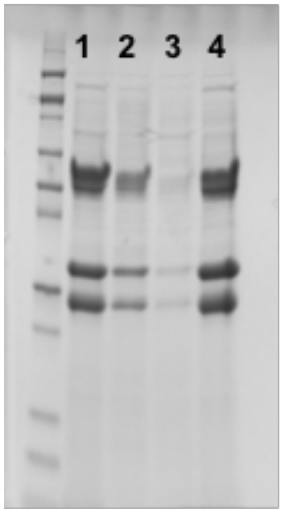
3 – Technical Accomplishments/ Progress/Results

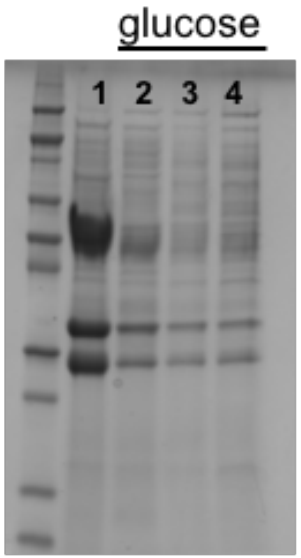
Fed-batch conditions that simulated bioreactors demonstrated that xylose induced production of cellulases





1: Xylan 2%
2: Xylose 2 %
3: Xylose 0.5 %
4: Low xylose feed

Low xylose feed:
$\dot{V}_{feed}$ = 3.2 uL min^{-1}
C_{feed} = 20 g L^{-1} xylose
→ 78 mg xylose h^{-1} L^{-1}



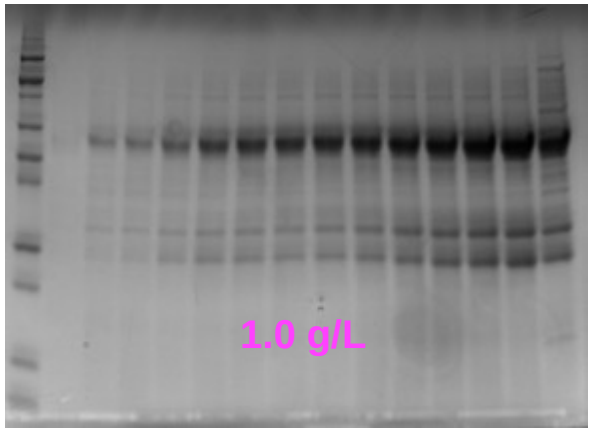
1: Xylan 2 %
2: Glucose 2 %
3: Glucose 0.5 %
4: Low glucose feed

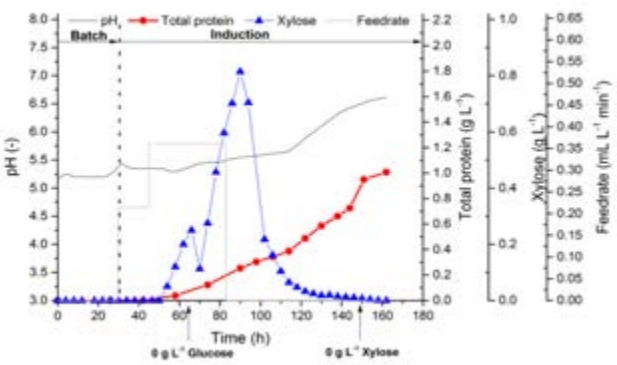
Low glucose feed:
$\dot{V}_{feed}$ = 3.2 uL min^{-1}
C_{feed} = 20 g L^{-1} glucose
→ 78 mg glucose h^{-1} L^{-1}

3 – Technical Accomplishments/ Progress/Results

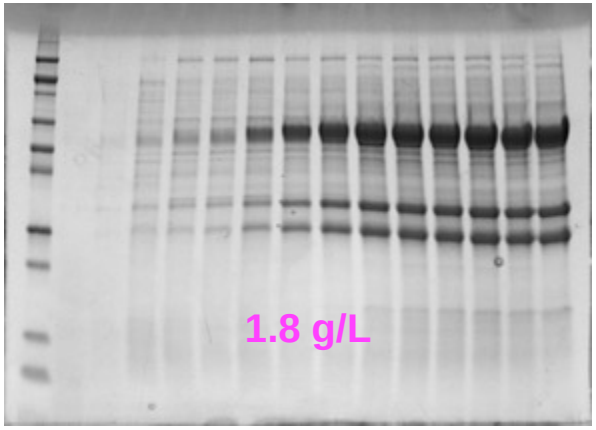
Multiple 2 L bioreactor campaigns have benn conducted at the ABPDU

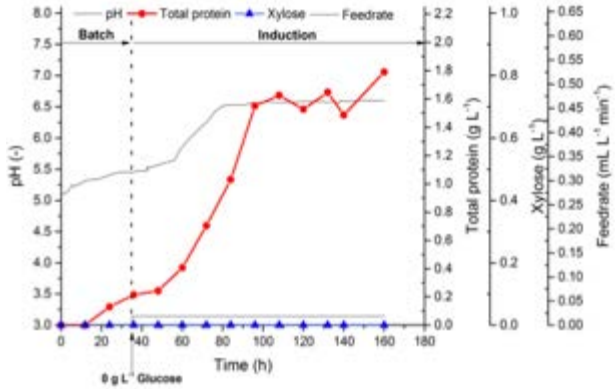
2 % xylan





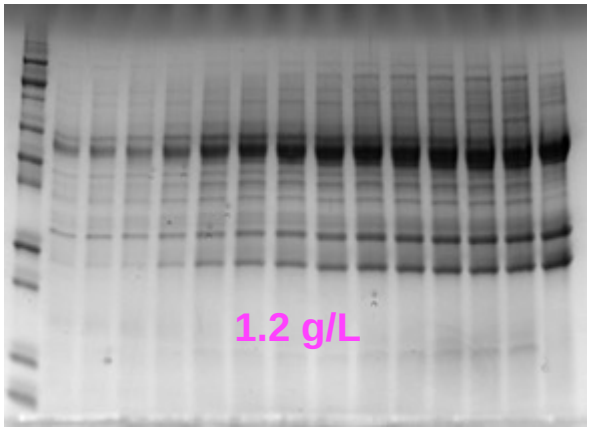
0.01% xylose

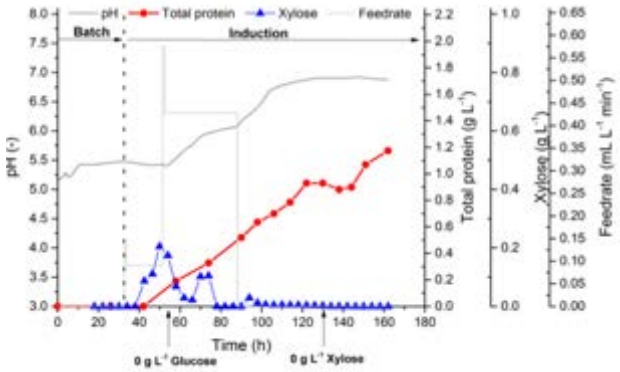




Xylose induction scaled to 20 L

0.01% C5 acid hydrolysate



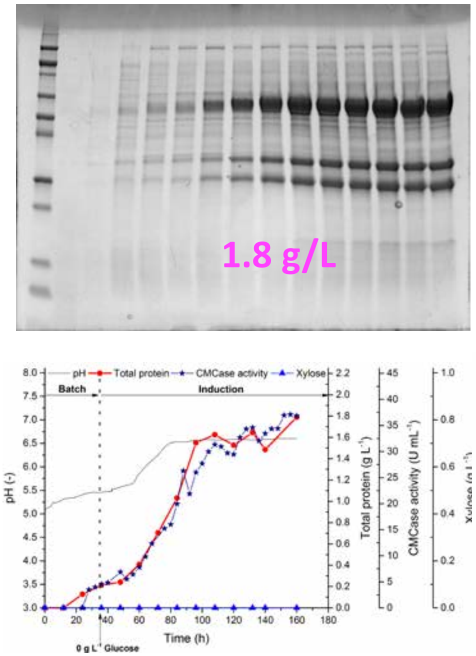


Hydrolysate prepared from corn stover (ABPDU)

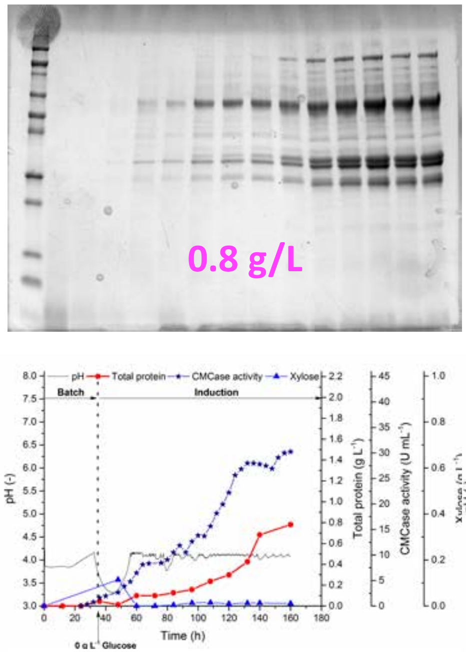
3 – Technical Accomplishments/ Progress/Results

pH dependence of protein production diverges from most filamentous fungi

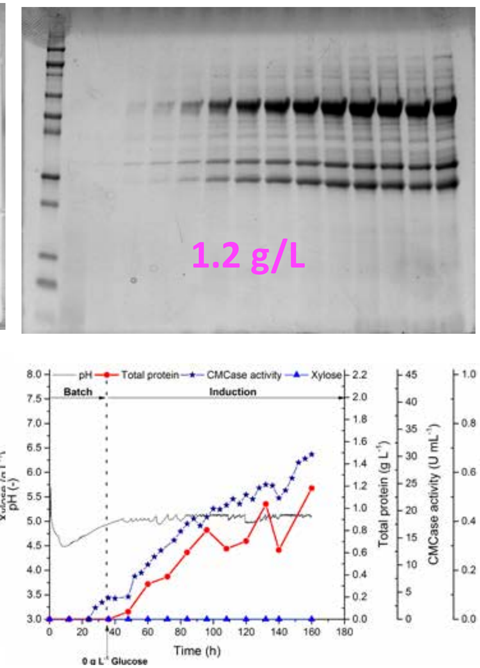
pH 5 (uncontrolled)



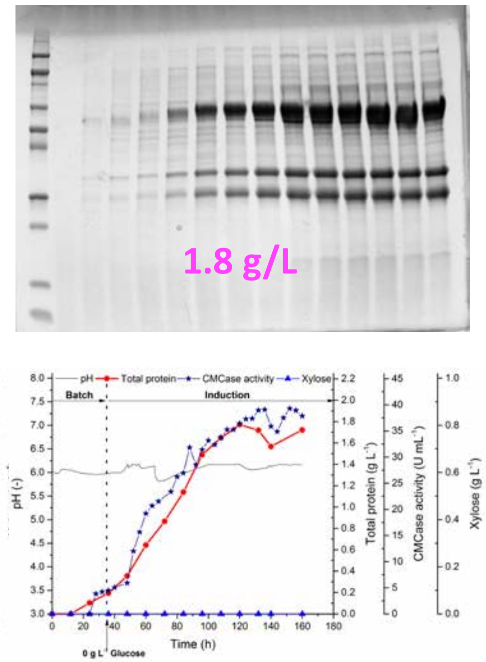
pH 4



pH 5



pH 6

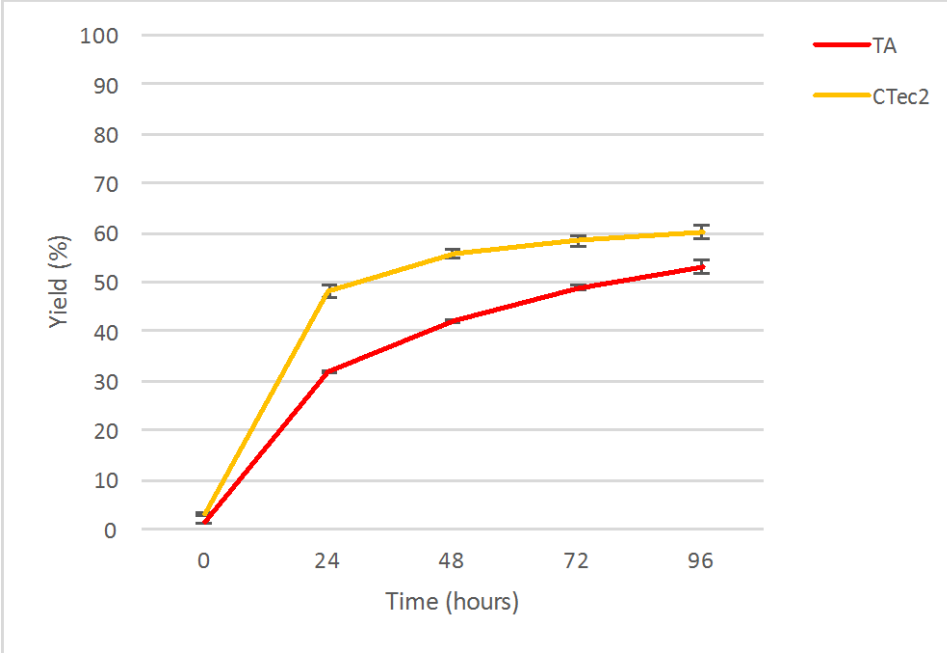


3 – Technical Accomplishments/ Progress/Results

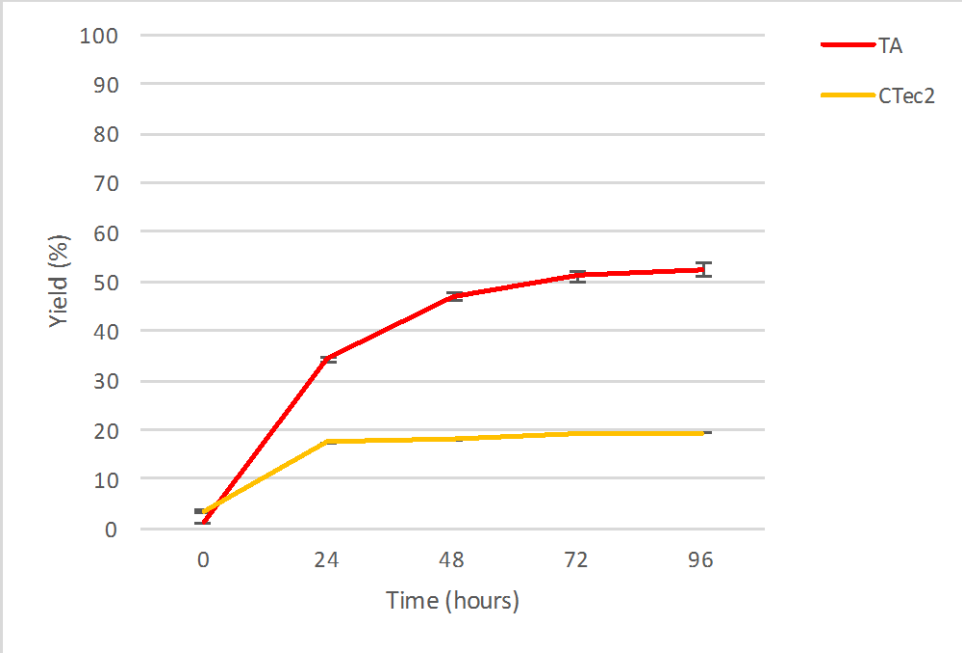
Saccharification of deacetylated, mechanically refined (DMR) corn stover

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)

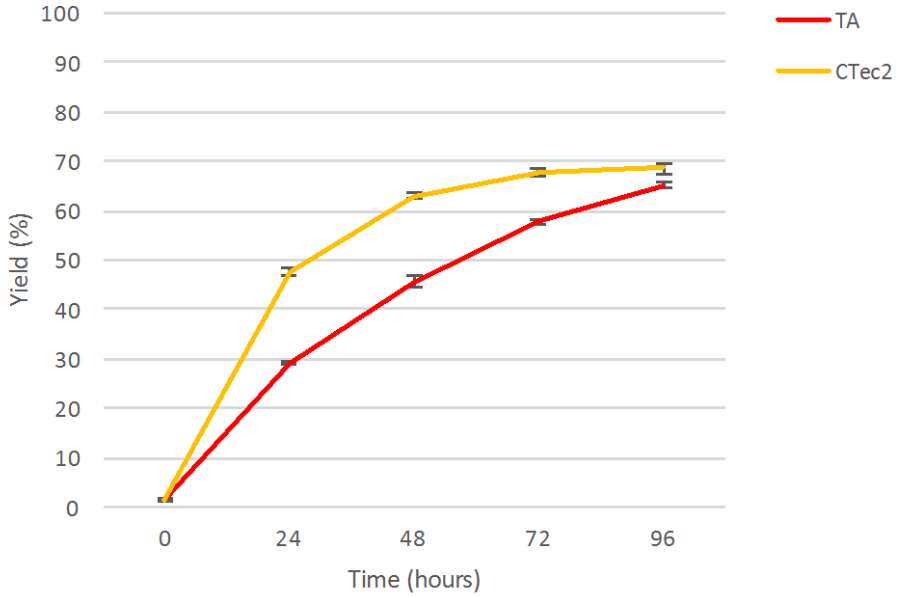


3 – Technical Accomplishments/ Progress/Results

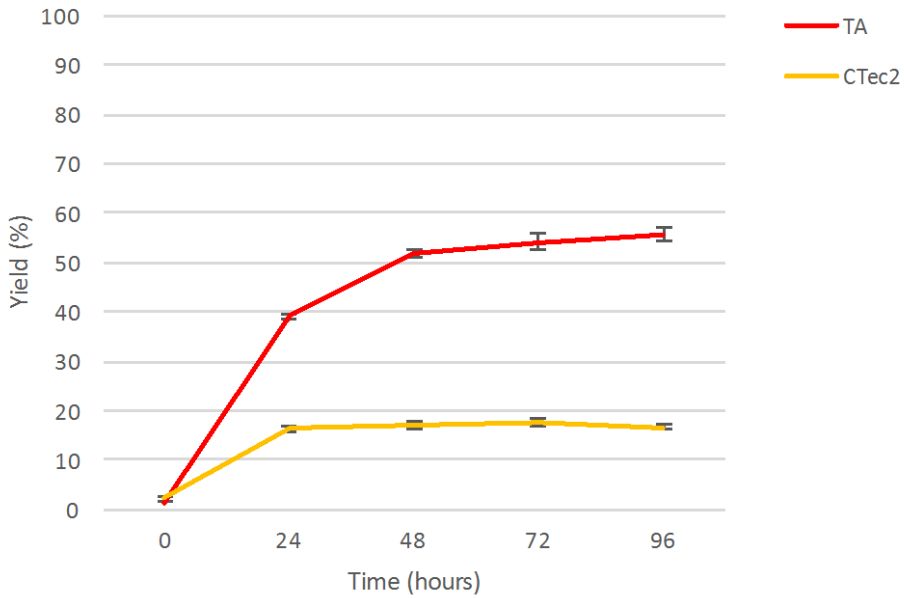
Saccharification of deacetylated, dilute acid (DAAD) pretreated corn stover

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

50∘C (Glucose)

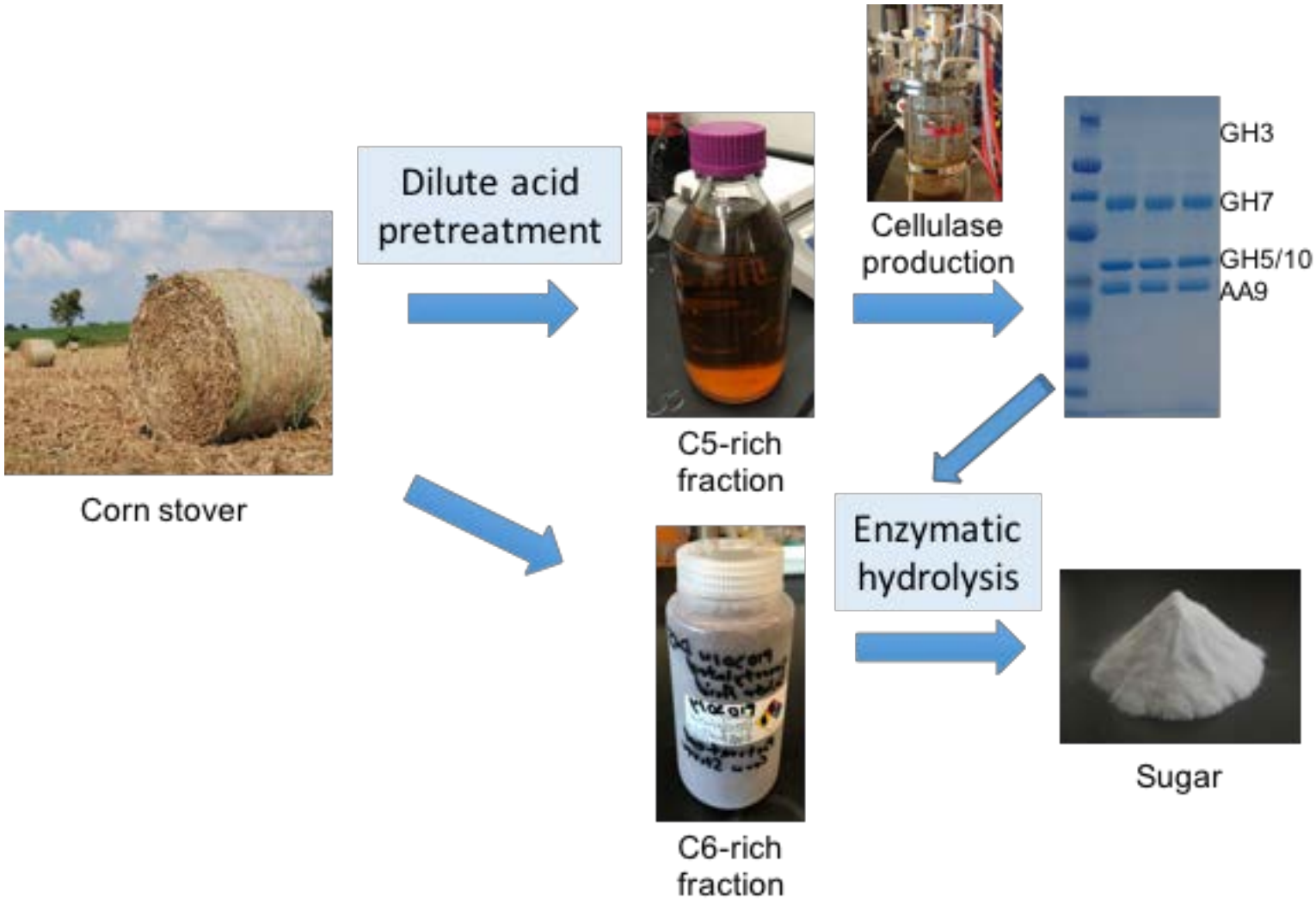


60∘C (Glucose)



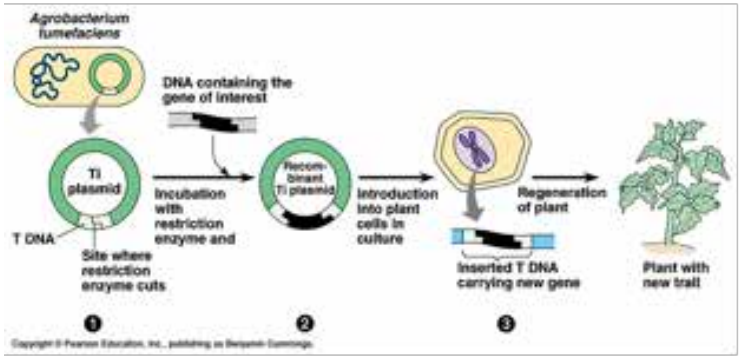
3 – Technical Accomplishments/ Progress/Results

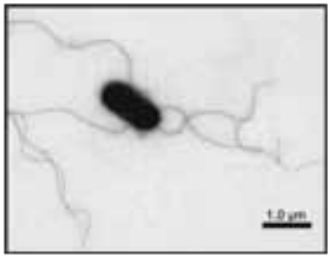
Linking pretreatment and on-site protein production in a biorefinery



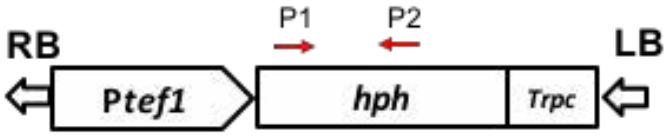
3 – Technical Accomplishments/ Progress/Results

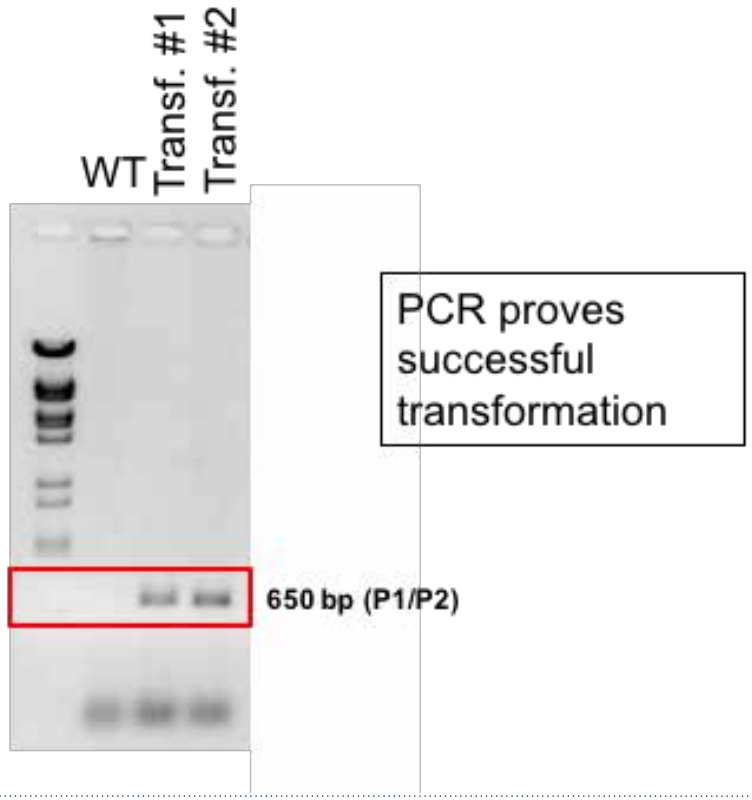
An *Agrobacterium tumefaciens* transformation system has been established for *T. aurantiacus*





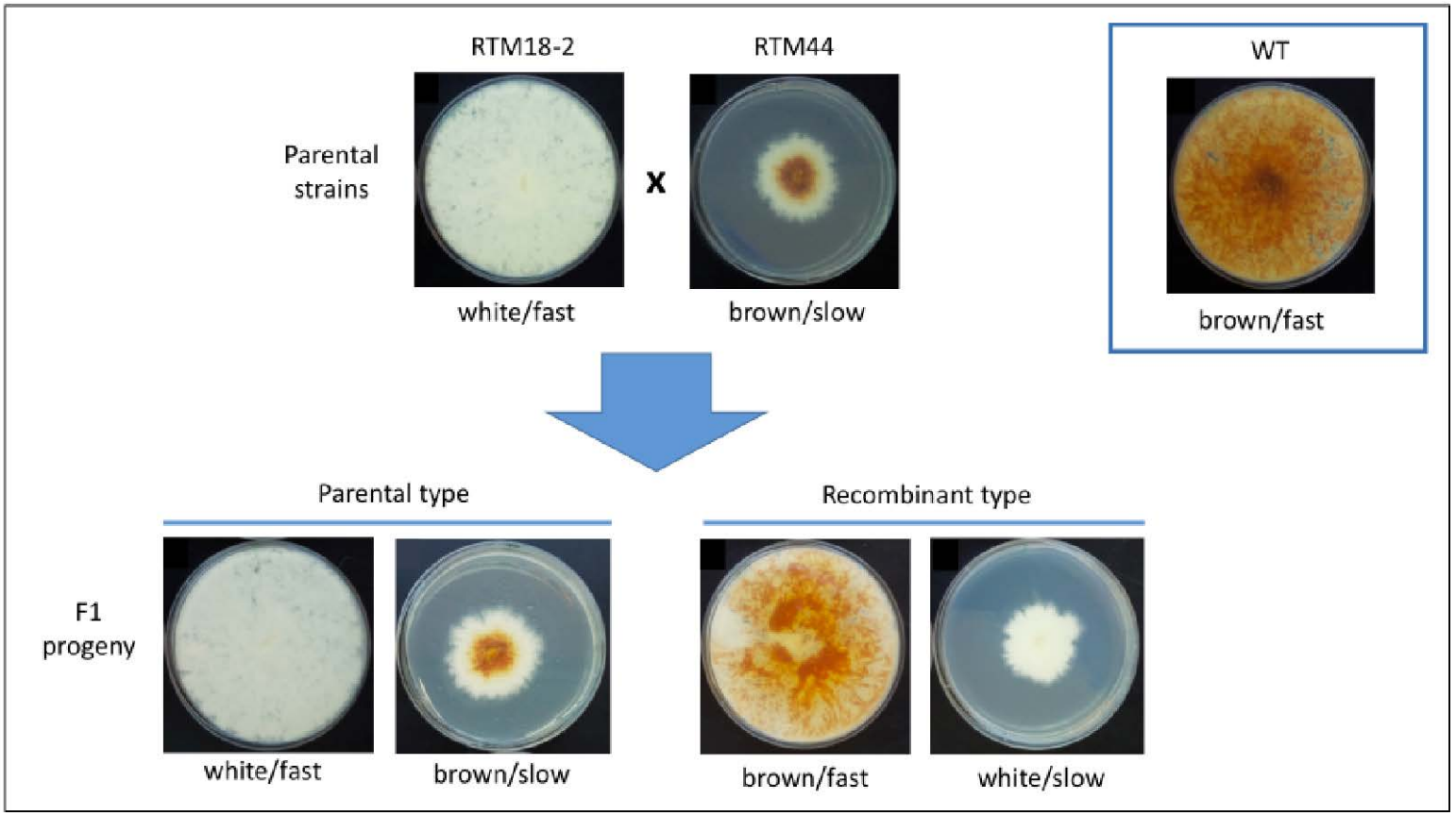
A. tumefaciens
Jeon et al. 2008





3 – Technical Accomplishments/ Progress/Results

Sexual crossing is demonstrated using UV mutagenized strains of *T. aurantiacus*



3 – Technical Accomplishments/ Progress/Results

Preliminary economic model predicts lower enzyme cost for enzymes from *T. aurantiacus*

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

3 – Technical Accomplishments/ Progress/Results

Publications and Patents

1) "*Thermoascus aurantiacus*: an Intriguing Thermophilic Fungus for Cellulase Production" Schuerg, T.; Gabriel, R.; Baecker, N.; Baker, S.E.; Singer, S.W. 2016, *Current Biotechnology*, *5* doi: 10.2174/2211550105666160520123504
2) "Xylose induces cellulase production in *Thermoascus aurantiacus*" Schuerg, T.' Gabriel, R.; Prahl, J.P.; Masson, F.; Tachea, F.; Miller, M.; Hubbard, S.; Pray, T.; Tanjore, D. Singer S.W. *Biotechnology for Biofuels*, submitted.
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) “Upscaling of cellulase enzyme production using Thermoascus aurantiacus” 29th Fungal Genetics Conference, Asilomar, CA, 3/15/17.

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 enyzme 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

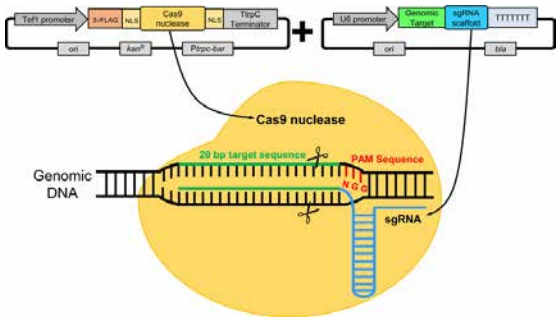
- **Industrial Relevance**: Provides a new platform for cellulase production that has distinct advantages over current technologies, including the production of cellulases from a soluble biomass fraction that perform at high temperature and enable on-site enzyme production using biomass components. Simplicity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

5 – Future Work

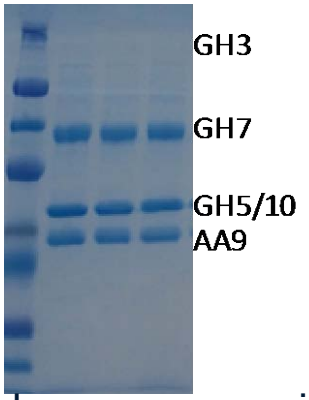
FY17 (project currently paused):

- Test *Agrobacterium*-mediated knockouts of genes responsible for carbon catabolite repression (*cre*1)
- 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 temprature; 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.