DOE Bioenergy Technologies Office (BETO)
2019 Project Peer Review
Enzyme Engineering and Optimization
WBS 2.5.4.100

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Biochemical Platform Review

March 4-8, 2019

Denver, CO

Goal Statement

Goal

- Enzyme technology enabling the SOT 2022 goal of advanced biofuels for $3.00/gge ($2.50/gge for 2030).
- **Meet or exceed 90% conversion of glucan and 90% xylan to glucose and xylose, respectively, using 10 mg cellulase / gram cellulose loadings with DMR (deacetylated mechanically refined) treated corn stover at 20% solids.**
- Employ a knowledge based approach which permits linking protein structure to performance and targeting the new DMR solids previously considered recalcitrant.

Outcome

- **An enhanced "DMR grade" commercial enzyme formulation developed by our commercial partner, Novozymes.**
- Public sector TRL 2 level biochemical findings shared in publication and presentations which enhance general knowledge regarding cellulase structure/function relationships.

Relevance

- All 2022 Biochemical Platform pathways require cellulase hydrolysis.
- **Cellulase related costs are ~10% of current biofuel Base Case SOT (i.e., biofuels for $9.23 and $10.73/gal depending on pathway).**
- Understanding enzyme structure/function/activity technology enables industrial enzyme improvement while reducing company R&D cost.

Quad Chart Overview

Timeline

- Project start date 2016
- Project end date 2021
- Percent complete 60%

Barriers addressed

Ct-B. Efficient Preprocessing and Pretreatments – Trade off analysis to optimize prepossessing unit operations which increase the overall process energy intensity.

Ct-C. Process Development for Conversion of Lignin – approaches to increase lignin yield.

	Total Costs Pre FY17**	FY 17 Costs	FY 18 Costs Note 1	Total Planned Funding (FY 19-Project End Date)
DOE Funded	$1.6M	$0.9M	$0.9M ($0.7M CFIT)	$3.4 + $2.9M = $6.3M total
Project Cost Share*				Note 2

•Partners: If multiple DOE recipients are involved in the project, please list level of involvement, expressed as percentages of project funding from FY 17-18. [(i.e. NREL (70%); INL (30%)]

Note 1: For FY18 only Cell Free & Immobilization Technologies was funded under EEO.

Note 2: Novozymes is an unfunded partner to EEO but also a participating partner under the related TCF project "Improving Cel6A"

Objective

We propose combined research thrusts developing better cellobiohydrolases, xylanases, and lignin modifying enzymes to meet the glucan/xylan conversion goals projected in the FY2017 SOT. This novel approach is targeted specifically to dilute alkali deacetylation/mechanical refined corn stover (DMR).

End of Project Goal

In partnership with Novozymes, produce a DMR solids acting enzyme formulation that achieves a 90% conversion of cellulose to glucose and 90% conversion of xylan to xylose under standard conditions*. With Novozymes as partner, demonstrate and enable a route towards industrial feasibility for advanced fuels from biomass.

*percent glucose (and xylose) released in 5 days from DMR solids using an enzyme loading of 10 mg enzyme/g cellulose in DMR solids at 20% solids and 25°C.

1—Project Overview

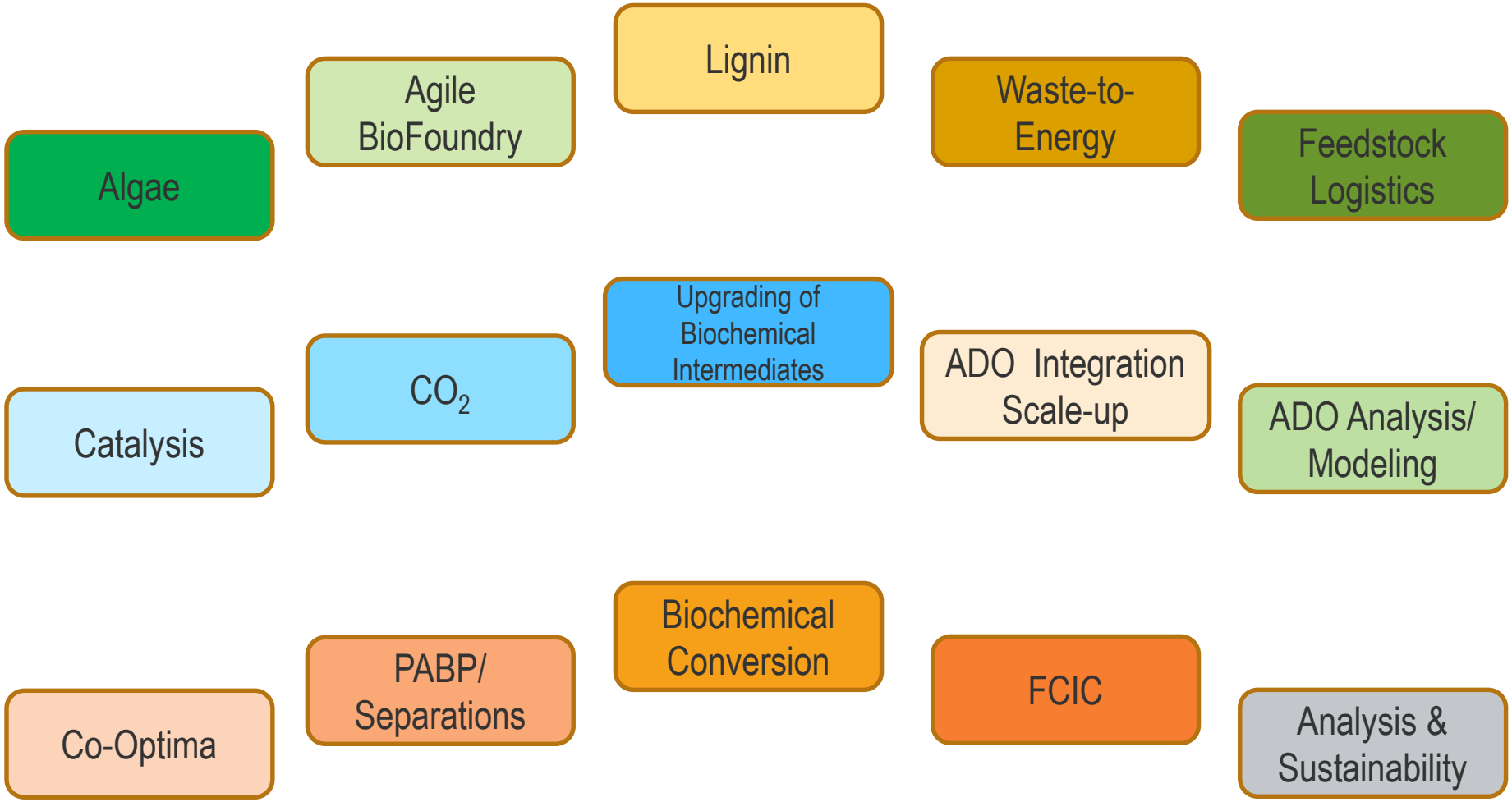
The Biochemical platform's new path forward requires enzyme formulations meeting specific performance goals with DMR solids.

- **Traditional cellulase preparations are less effective on DMR solids than on DDA (deacetylated dilute acid) solids!** DDA solids are reduced in xylan content relative to DMR solids. Also, *the ferulic acid esters (linked to lignins) are hydrolyzed during DMR which leaves the unsubstituted, insoluble xylan (previously deacetylated) to block enzymes from reaching cellulose.*
- Thus, effective hydrolysis of DMR solids requires **aggressive cellulases and higher titers of selected xylanase/lignin modifying enzymes.**
- **The increased soluble oligosaccharides in DMR liquors also require new enzyme activities to produce fermentable sugars.**
- New enzyme formulations are needed and thus we have established a **partnership with Novozymes (see the 2018 TCF award - R. Brunecky).**

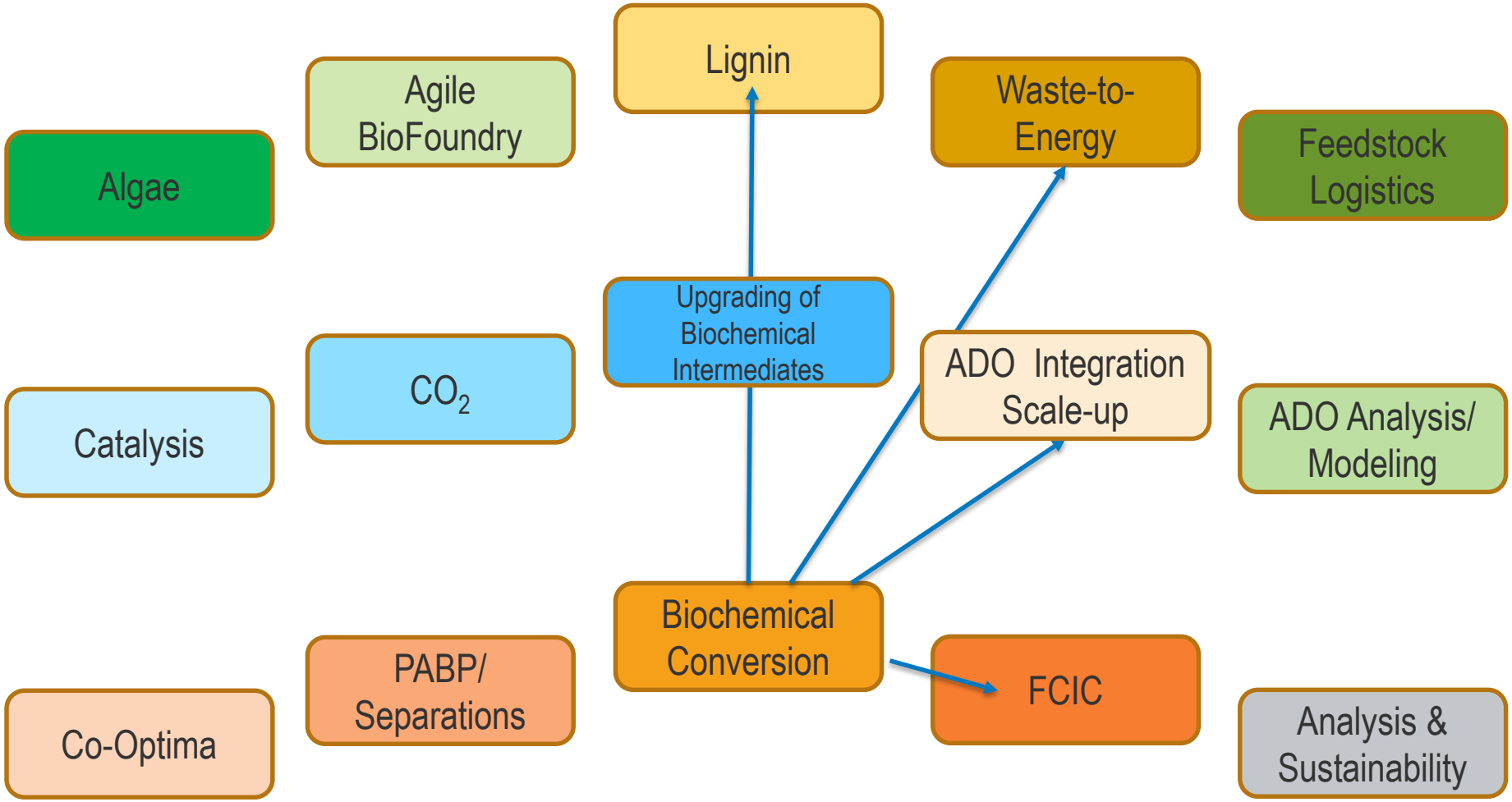
We demonstrated that enzyme engineering approaches are effective for improving commercial biofuels enzyme formulations.

- We have confirmed that **Nature harbors cellulases superior to those currently used in industrial formulations** and that this performance can be explained on a structure/function basis.
- **We will use engineering and genomics approaches to provide improved enzymes (cellulases, hemicellulases, and lignin modifying enzymes).**
- We inform industry and the public via top tier publications (>70 since 2013).

3—Project Integration

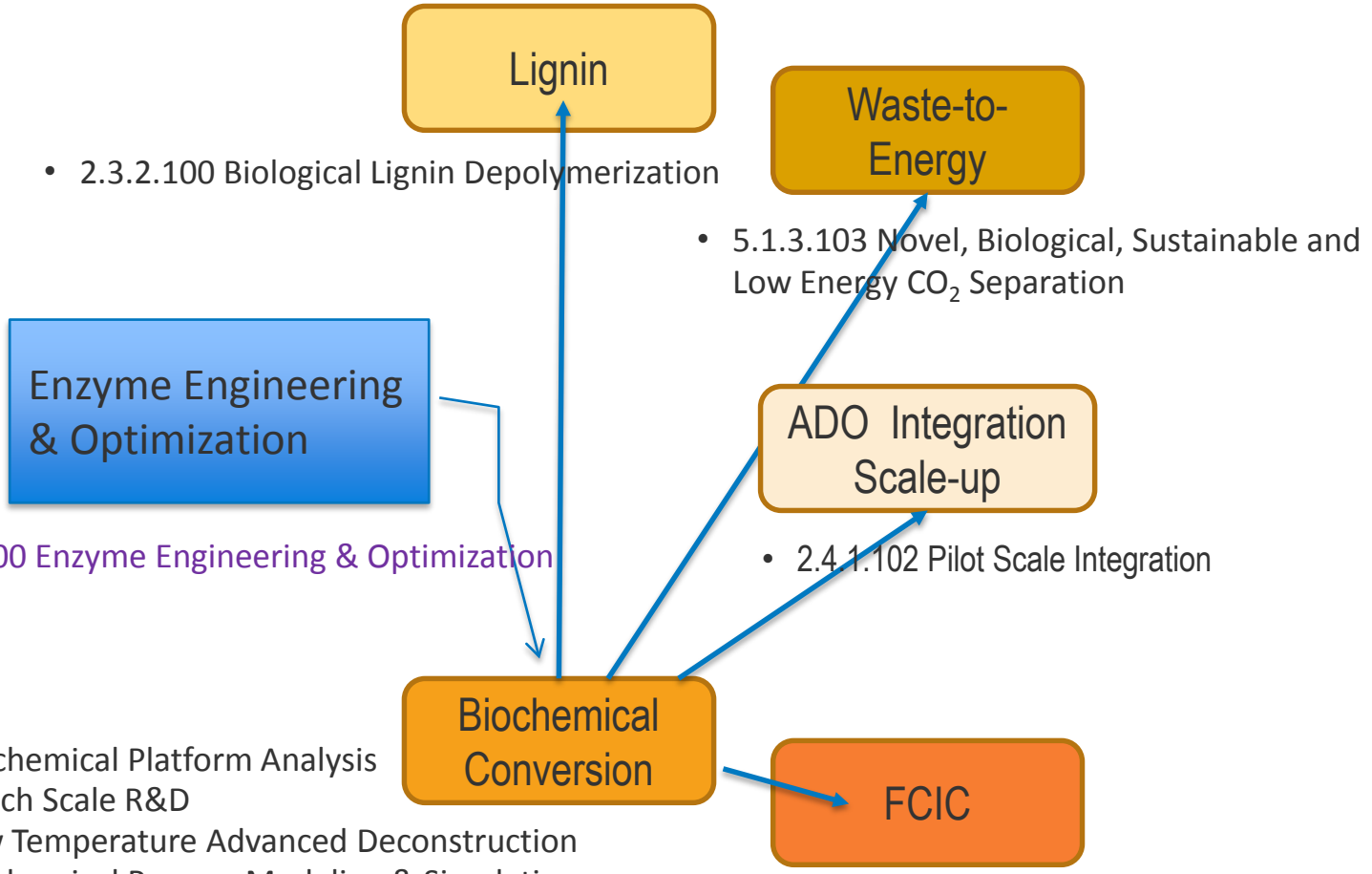


3—Project Integration



3—Project Integration

Our work impacts these other Projects



- 2.1.0.100 Biochemical Platform Analysis
- 2.4.1.100 Bench Scale R&D
- 2.2.3.100 Low Temperature Advanced Deconstruction
- 2.5.1.100 Biochemical Process Modeling & Simulation
- 2.5.4.101 Cell Free & Immobilization Technologies
- 2.4.3.102 Targeted Microbial Development
- 2.3.2.103 Fungal Genomics — Genetics

2—Approach (Management)

Emphasize the structure of your approach. . . .

- Work closely with BPMS (modeling/theory), LTAD, (pretreatment), and TEA.
- Use academic and national lab collaborators (CCRC-UGA) and PNNL-EMSL).
- **Milestones are Regular, Quarterly, and Annual, with several SMART milestones identified. One Go/No-Go decision is planned for FY2018 and FY2019.**
- Use industrial partners (Novozymes and Metgen Oy)

Describe critical success factors. . . .

- Technical: Demonstrate that superior CBHs exist in Nature and that protein design factors can be harnessed to create optimal industrial formulations.
- Business: **Contribute to an awareness that enhanced enzyme formulations are possible and cost effective when acting on DMR solids (or tuned to any new substrate).**
- Market: Promote timely dissemination of progress to key audience (publications and presentations) which will enable new biomass to fuels industry.

Explain the top 2 to 3 potential challenges. . . .

- To show that better CBHs exist in Nature requires a rigorous yet tactical screening approach followed by deep understanding of cellulase structure/function.
- **To identify new GHases able to break the recalcitrant linkages in DMR oligosaccharides.**
- To show that current, **commercial enzyme cocktails can be reformulated cost effectively** for improved performance of DMR solids (Novozymes partnership is critical).

2—Approach (Management) – FY19 MS

Task 1. Cellulase Improvement ($550K) – S. Decker

a. Megatron I (determine best CBHI enzymes acting on DMR solids). We intend to interrogate nearly new 100 enzymes to meet this goal.

b. Determine best formulations consisting of advanced cellobiohydrolases (CBHI and CBHII*), xylanases, and LMEs for effectiveness on DMR solids.

Annual SMART	**Tasks 1 and 2. Demonstrate that the new "enzyme triad" approach to reducing DMR solids recalcitrance can be effective by achieving an 80% conversion of glucose from glucan using improved cellobiohydrolases, xylanases, and lignin modifying enzymes under standard conditions*. We will be working closely with the LTAD and BPMS projects, as well as Novozymes to achieve this goal.**	**9/30/2019**

Task 2. Lignin Modifying Enzymes ($350K) – S. Decker

a. Interrogate industrial preparations and purified LMEs for effectiveness on DMR solids.

b. Determine best xylanases acting on DMR solids. It is anticipated that most of the new xylanases for this work will come from industry.

QPM1	**Task 2. LME and Xylanases. Determine if either pre or post DMR treatment of biomass with commercial LME enzymes improves the saccharification of DMR solids. Success is maintaining conversion levels with a reduction in loading of current Ctec2 or 3 (mg enzyme/g DMR solids), or higher extents of 5-day conversion of DMR solids with the same loading (10 mg/g DMR solids). Collaboration w LTAD.**	**12/31/2018**

*CBHI acts on reducing cellulose chain ends and CBHII acts on non reducing ends. Both are needed.

2—Approach (Technical)

2018/2019—Enzyme Engineering and Optimization

2018 Task 1. Enzyme Improvement: Use a combination of rational design, random evolution, and gene-shuffling techniques with selective screening of candidate enzymes. Data generated will be primarily under process-relevant conditions, although promising enzymes will be subjected to classical biochemical characterization to build structure-function models.

2018 Demonstration Project – Cell Free & Immobilization Technology CFIT): We used cell free concepts to design and demonstrate successfully that 1,3PDO can be produced directly from glycerol. New process designs using CFIT aggressively target the production of BDO from pyruvate, and ultimately from glucose **(see 2019 CFIT Project – Y. Bomble**).

2019 Task 1. Enzyme Improvement: Identify the best **Cel7A enzymes from natural diversity for enhanced digestion of DMR solids (Megatron I).** In FY2018, we noted the discovery of new CBH I enzymes from preliminary diversity studies which have improved kinetics compared to the *T. reesei* enzyme. **[The co-development of Megatron II, which delivers improved CBH II enzymes, is based on a separate TCF proposal w/Novozymes partnership.]**

2019 Task 2. Lignin Modifying Enzymes (LMEs) and Xylanases: Deliver **LME enzymes found to enhance the digestion of DMR feedstocks** in the presence of the improved cellobiohydrolases. Interrogate enzymes from commercial formulations (e.g., MetGen Oy). Direct characterization of **recalcitrant lignin and hemicellulose linkages (remaining after mechanical refining), which contribute to the approximately 10% glucose and xylose equivalents tied up in oligosaccharides.** Knowledge of protected linkages will allow us to choose more precisely the accessory enzymes able to release xylose and other sugars.

2—Approach (Technical)

2018/2019—Enzyme Engineering and Optimization

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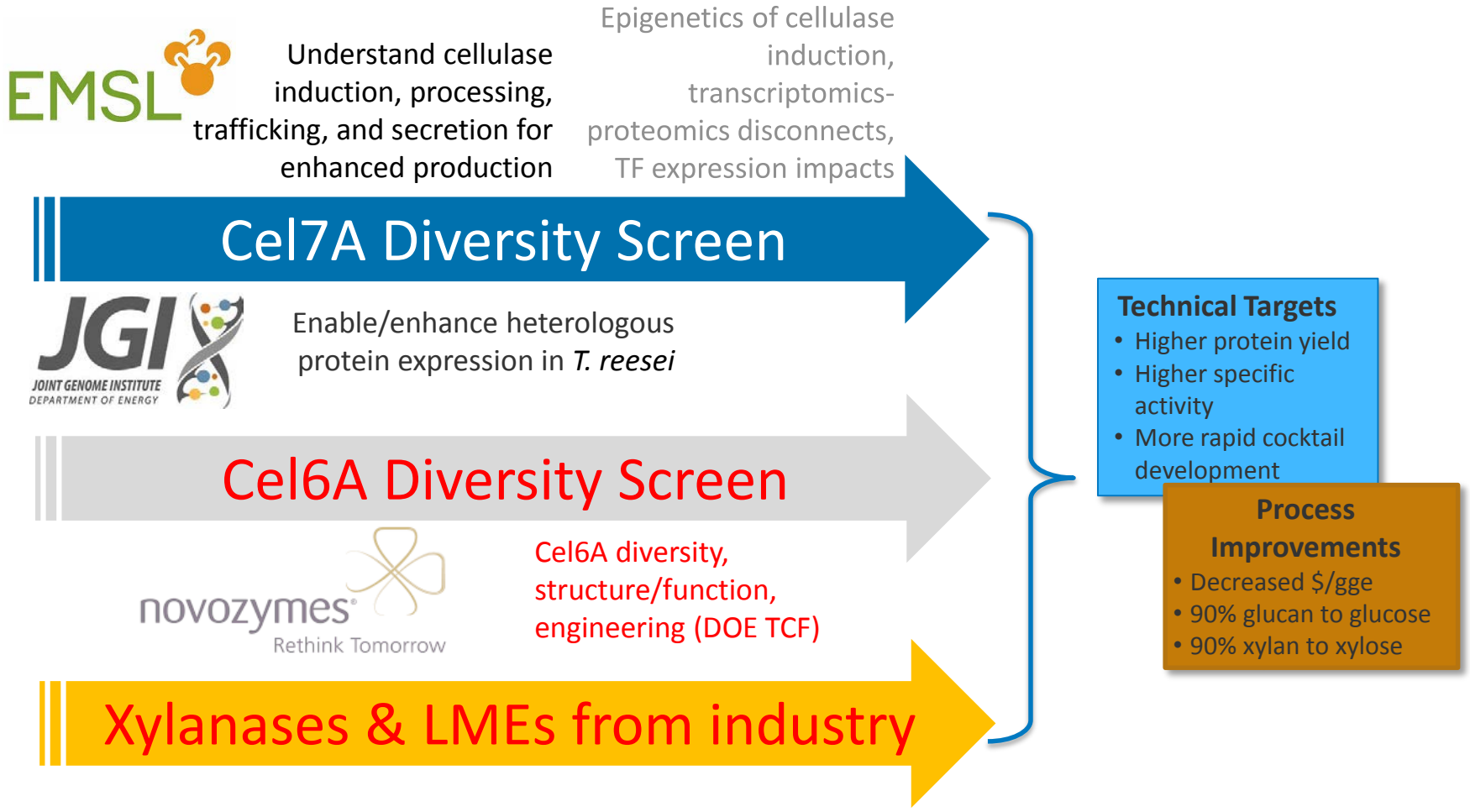
2—Approach (Technical)

Mapping our approach



3—Technical Accomplishments / Progress/Results

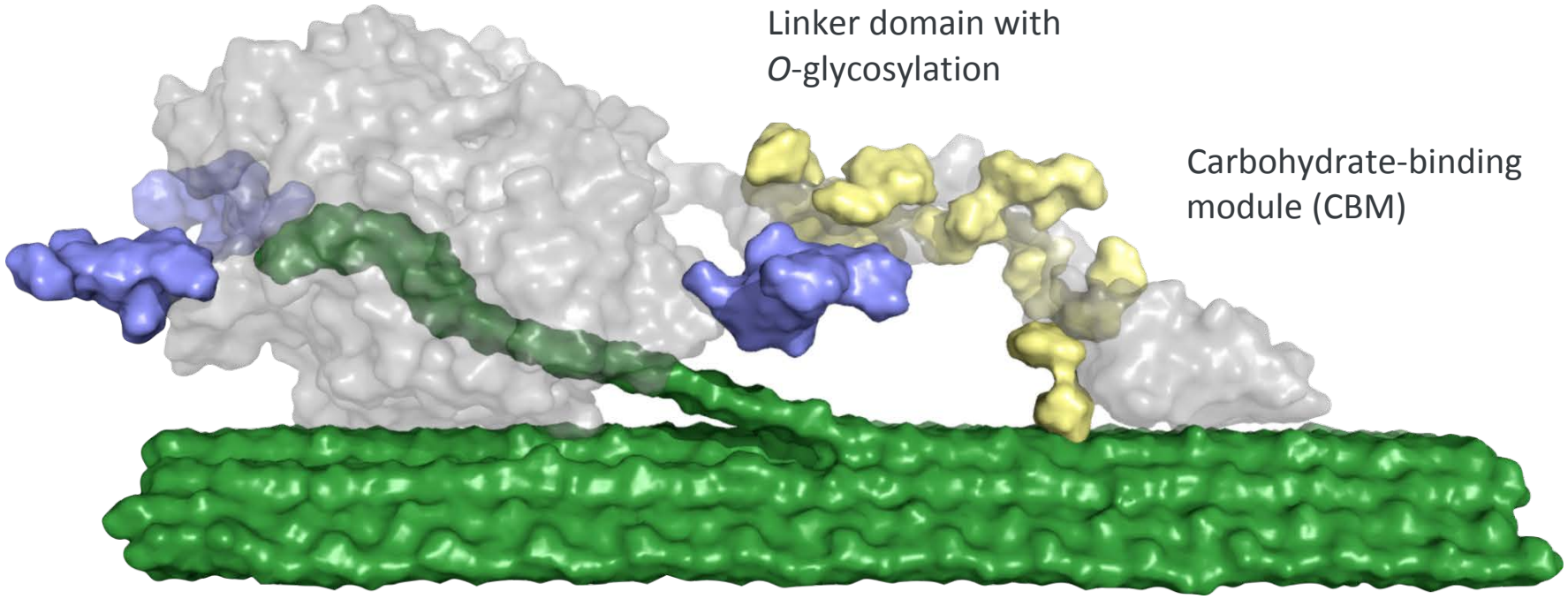
- **Understand the structure/function basis for cellulase performance.**
- **Employ the *T. reesei* chassis strain developed at NREL to prepare Cel7A enzymes.**
- **Leverage the EMSL collaboration regarding *T. reesei* expression mechanisms.**
- **Design and test novel chimeric and multi-domain Cel7A mutants.**
- **Test ~100 Cel7A enzymes from Nature (Megatron).**
- **Initiate work with lignin modifying enzymes (LMEs).**
- **Work closely with industrial collaborator (Novozymes).**

3—Technical Accomplishments / Progress/Results

Background: Understand the structure/function basis for cellulase performance.

3—Technical Accomplishments/ Progress/Results

Ce7A is the workhorse of the fungal cellulase world!



Single most important enzyme in industrial lignocellulosic biomass conversion

Approach: Separate the problem into components to study sub-domains and elementary steps

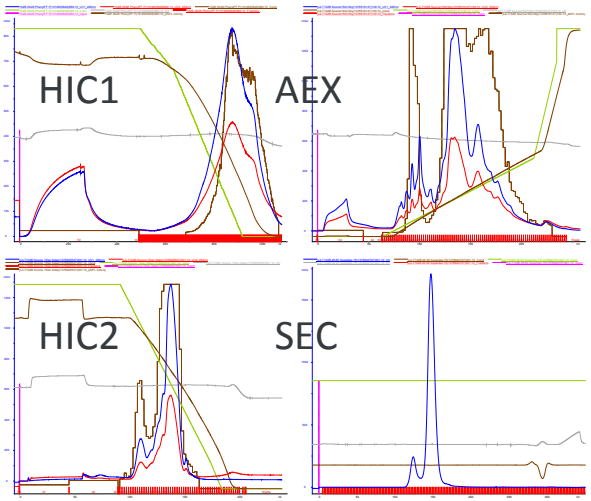
Overall aim: Understand function to aid protein-engineering efforts

3—Technical Accomplishments/ Progress/Results

Penicillium funiculosom Cel7A—Why so active?

We discovered *Pf*Cel7A has higher specific activity than *Tr*Cel7A.

- *Pf*Cel7A is amenable to industrial production.
- Probed domain functionality through "chimeras"
  - ✓ Swap Pf and Tr CDs, linkers, CBMs
  - ✓ Designed efficient purification schemes



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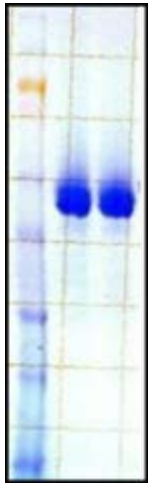
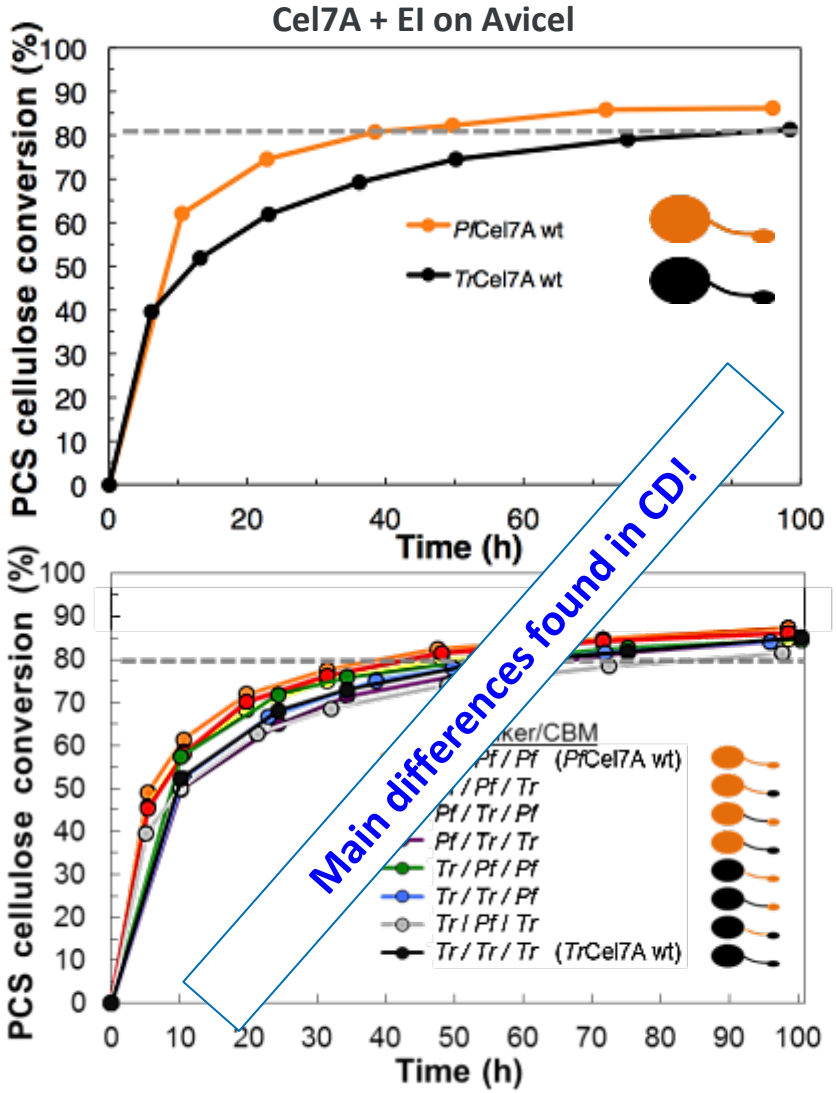
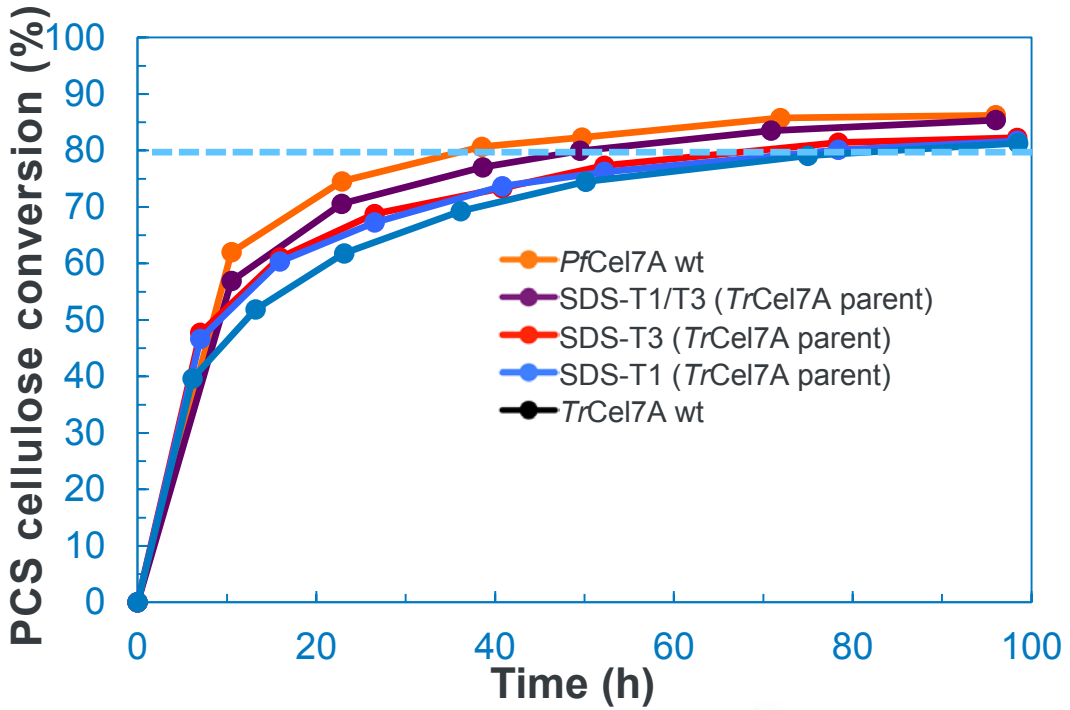


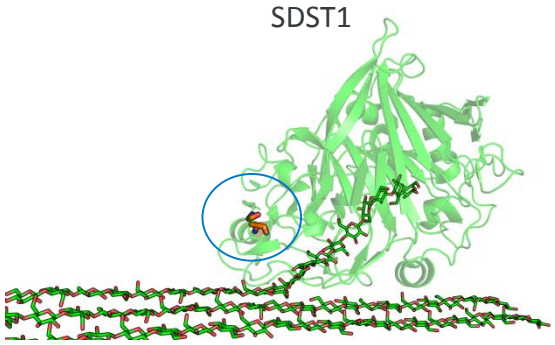


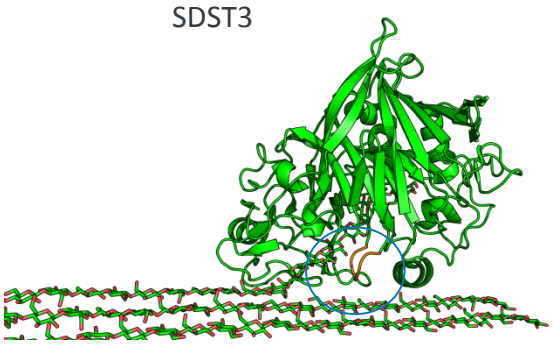
3—Technical Accomplishments/ Progress/Results

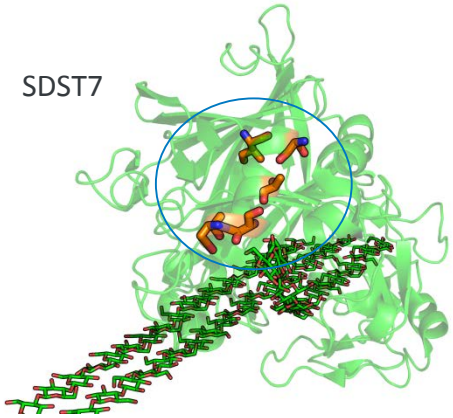
We can capture the design features of the P. funiculosom enzymes!

- Twelve subdomain swaps (*P*f→T*r* and *Tr*→*Pf*) were engineered and expressed in *T. reesei*
- Two *Pf*→*Tr* SDSs improved activity and were additive, nearly matching that of *Pf* parent.
- Conclusion: we can import subdomains from a superior enzyme to an inferior enzyme making it equivalent to the former.









3—Technical Accomplishments / Progress/Results

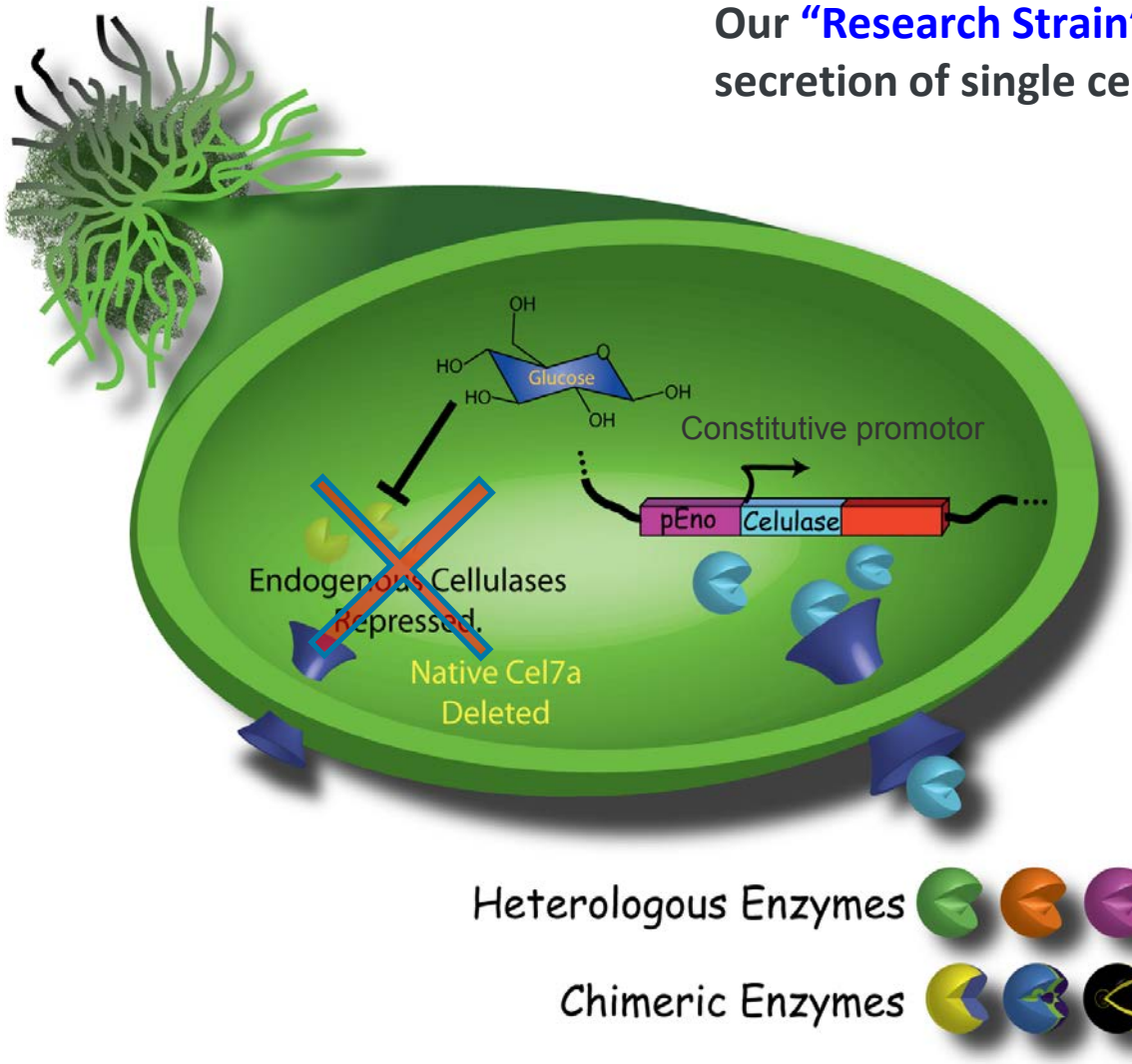
Understand the structure/function basis for cellulase performance.

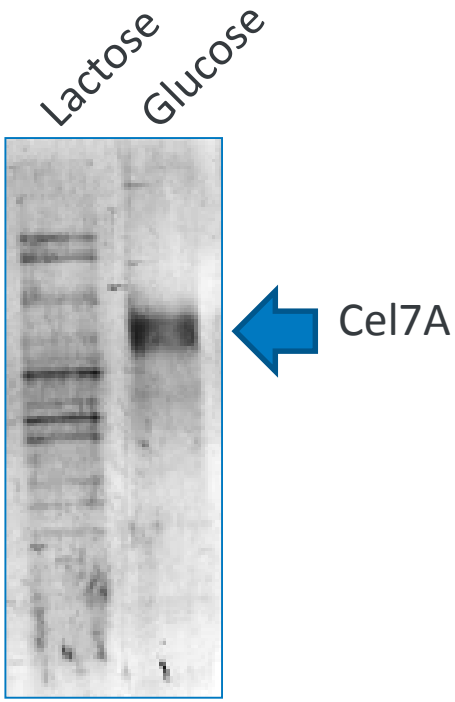
Employ the *T. reesei* chassis strain developed at NREL to prepare Cel7A enzymes.

3—Technical Accomplishments/ Progress/Results

T. reesei as a chassis strain for fungal cellulases

Our "Research Strain" enables the expression and secretion of single cellulases at modest titers.

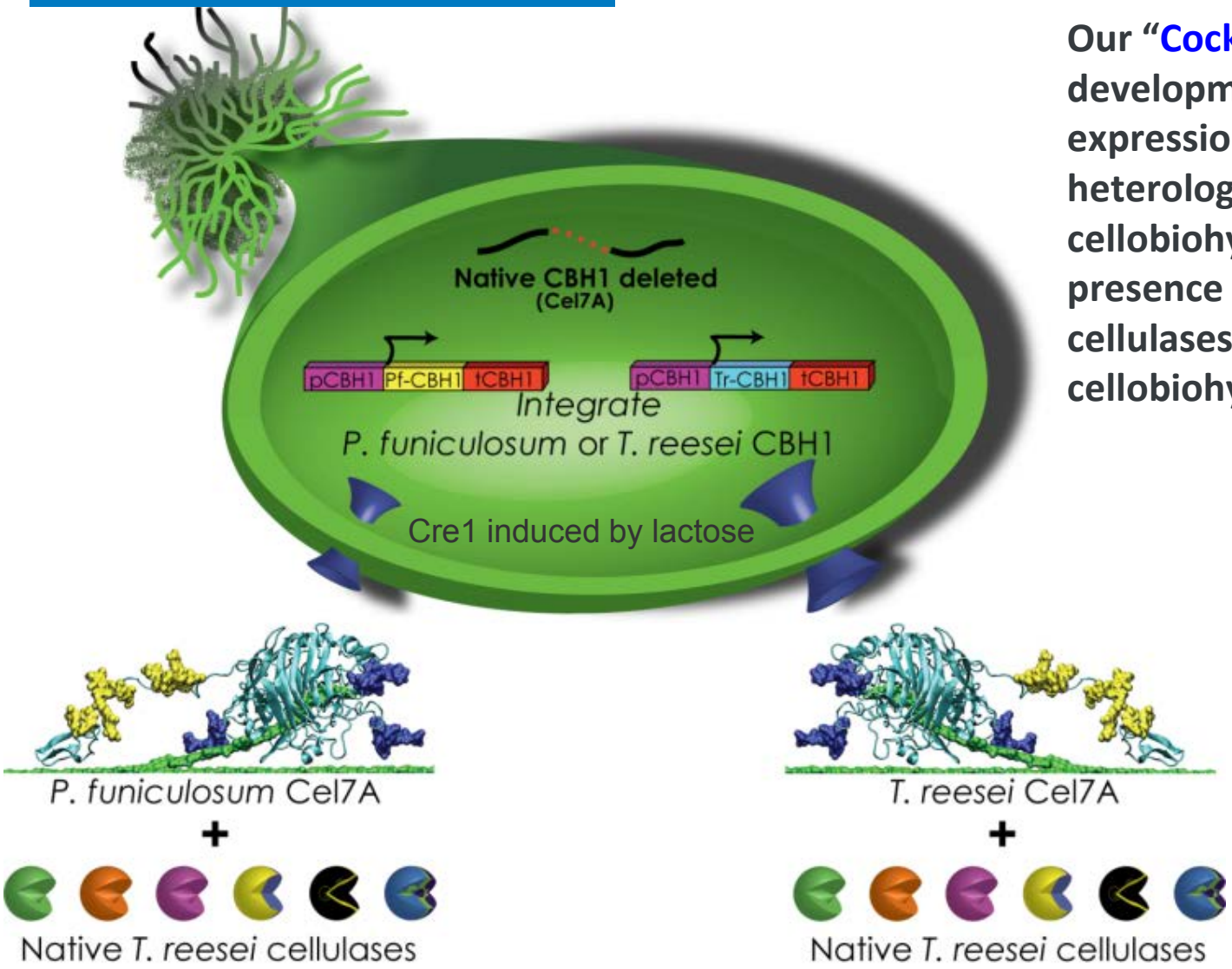




Linger et al. 2015
Singh et al. 2015

3—Technical Accomplishments/ Progress/Results

T. reesei as a chassis strain for fungal cellulases – longer term



Our "Cocktail Strain" (under development) enables the expression and secretion of heterologous cellobiohydrolases in the presence of a full suite of cellulases except the native cellobiohydrolase

3—Technical Accomplishments / Progress/Results

Understand the structure/function basis for cellulase performance.

Employ the *T. reesei* chassis strain developed at NREL to prepare Cel7A enzymes.

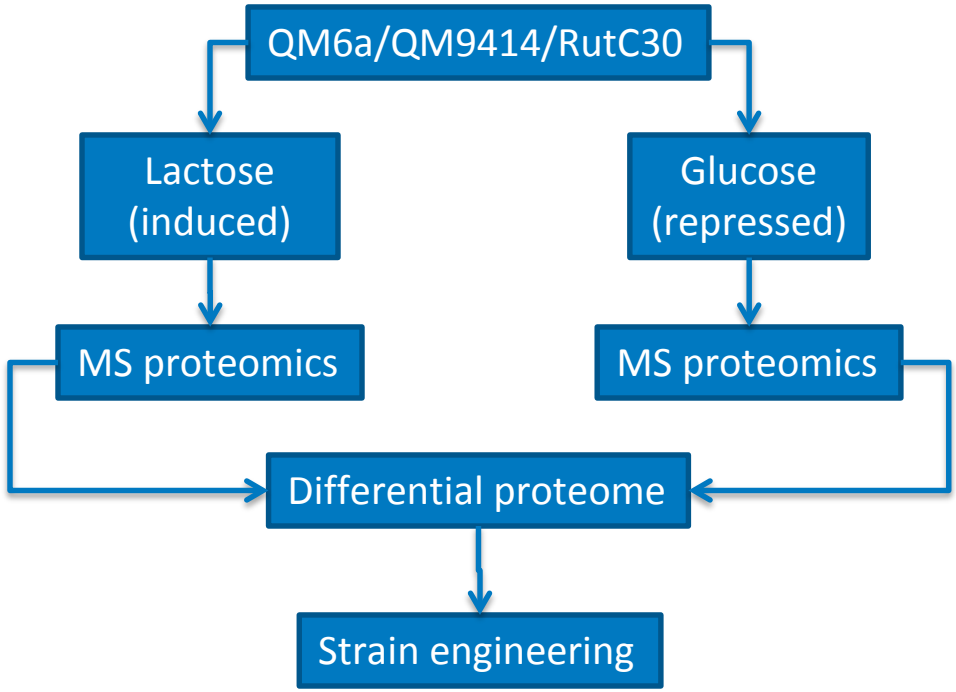
Leverage the EMSL collaboration regarding *T. reesei* expression mechanisms.

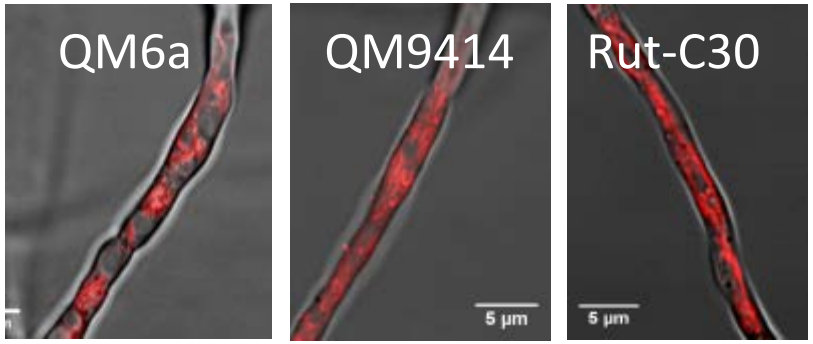
3—Technical Accomplishments/ Progress/Results

Understanding cellulase processing-EMSL collaboration

Cel7A requires considerable post-translational modification

- Co-induced processing enzymes
  - Disulfide isomerases, chaperonins, glycosylation, trimming, signal processing, secretion targeting
  - Growth stage proteomic analysis under inducing/repressing conditions
- Unfolded protein response by heterologous expression?
  - Follow Cel7A through ER/Golgi w/ HR imaging





3—Technical Accomplishments/ Progress/Results

Example: Extracellular Enzymes

Proteins that get induced under lactose alone

- transmembrane amino acid transporter [Trichoderma reesei QM6a]
- Non-Catalytic module family expansin [Trichoderma reesei QM6a]
- ribosomal protein P1 [Trichoderma reesei QM6a]
- adenylate cyclase associated protein [Trichoderma reesei QM6a]
- glycoside hydrolase family 35 [Trichoderma reesei QM6a]
- glycoside hydrolase family 54 [Trichoderma reesei QM6a]
- glycoside hydrolase family 5 [Trichoderma reesei QM6a]
- glycoside hydrolase family 11 [Trichoderma reesei QM6a]
- glycoside hydrolase family 67 [Trichoderma reesei QM6a]
- carbohydrate-binding module family 1 [Trichoderma reesei QM6a]
- glycoside hydrolase family GH30 [Trichoderma reesei QM6a]
- glycoside hydrolase family 7 [Trichoderma reesei QM6a]
- glycoside hydrolase family 5 [Trichoderma reesei QM6a]
- glycoside hydrolase family 27 [Trichoderma reesei QM6a]
- glycoside hydrolase family 55 [Trichoderma reesei QM6a]
- glycoside hydrolase family 65 [Trichoderma reesei QM6a]

Proteins that get induced under both glucose and lactose

- Copper/Zinc superoxide dismutase [Trichoderma reesei QM6a]
- amidase [Trichoderma reesei QM6a]
- fructose bisphosphate aldolase [Trichoderma reesei QM6a]
- proteasome, subunit alpha type 4-like protein [Trichoderma reesei QM6a]
- mannose-6-phosphate isomerase [Trichoderma reesei QM6a]
- glycoside hydrolase family 76 [Trichoderma reesei QM6a]
- glycoside hydrolase family 5 [Trichoderma reesei QM6a]
- glycosyltransferase family 8, partial [Trichoderma reesei QM6a]
- glycoside hydrolase family 37 [Trichoderma reesei QM6a]
- glycoside hydrolase family 31 [Trichoderma reesei QM6a]
- glycoside hydrolase family 72 [Trichoderma reesei QM6a]

more under lactose

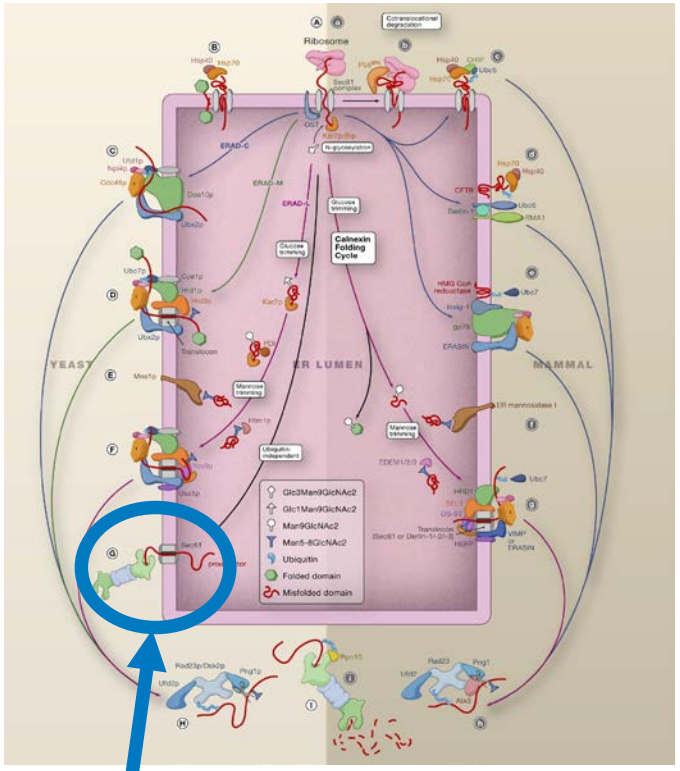
less under lactose

Proteins that get induced under glucose alone

- Mn superoxide dismutase [Trichoderma reesei QM6a]
- succinate dehydrogenase [Trichoderma reesei QM6a]
- 3-ketoacyl-CoA thiolase-like protein [Trichoderma reesei QM6a]
- isocitrate dehydrogenase, partial [Trichoderma reesei QM6a]
- peptidyl-prolyl isomerase [Trichoderma reesei QM6a]
- multicopper oxidase [Trichoderma reesei QM6a]
- phosphoenolpyruvate carboxykinase [Trichoderma reesei QM6a]
- X-Prolyl aminopeptidase [Trichoderma reesei QM6a]
- aldehyde dehydrogenase [Trichoderma reesei QM6a]
- 2-oxoglutarate dehydrogenase-like protein [Trichoderma reesei QM6a]
- serine protease-like protein [Trichoderma reesei QM6a]
- glucose-6-phosphate isomerase [Trichoderma reesei QM6a]
- phosphoenolpyruvate carboxykinase [Trichoderma reesei QM6a]
- peptidyl-prolyl isomerase [Trichoderma reesei QM6a]
- glycoside hydrolase family 18, chitinase [Trichoderma reesei QM6a]
- glycoside hydrolase family 13 [Trichoderma reesei QM6a]
- glycoside hydrolase family 72 [Trichoderma reesei QM6a]

3—Technical Accomplishments/ Progress/Results

Probing the *T. reesei* Secretory System to Enhance Heterologous Protein Production

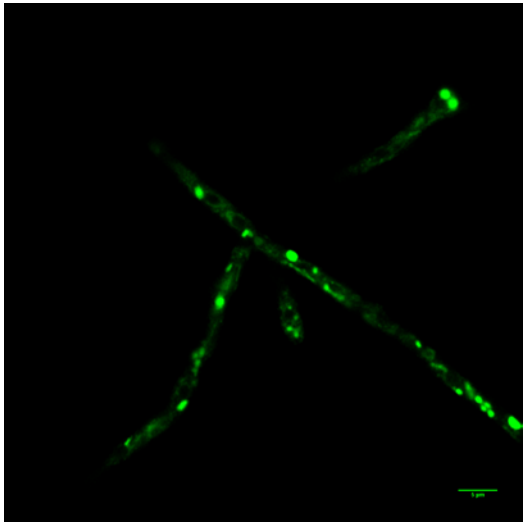


Sec61 forms a channel in the ER membrane

Fluorescence of mycelia

G	L	S	
18776	8764	8765	*T. reesei* secretion signal
2405	1422	2537	Rat FSH secretion signal
22962	8761	10672	Pfun secretion signal w/o A
8592	9019	10781	Pfun secretion signal with A

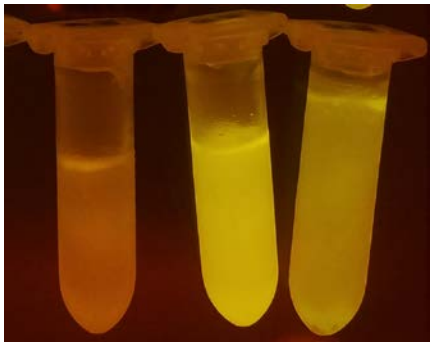
- Decreased GFP in mycelia when secretion is initiated by growth on Lactose (L)or Starch (S)
- Inverse GFP levels in extracellular medium
- Rat Follicle Stimulating Hormone is poor overall
- *P. funiculosum* Cel7A signal is sensitive to mutation



F-seGFP9

Fluorescence of culture broth

Glucose Lactose Starch



More on relationships between signal sequence and N-terminal amino acids…..

3—Technical Accomplishments / Progress/Results

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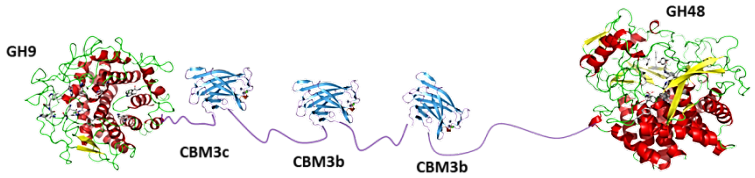
Employ the *T. reesei* chassis strain developed at NREL to prepare Cel7A enzymes.

Leverage the EMSL collaboration regarding *T. reesei* expression mechanisms.

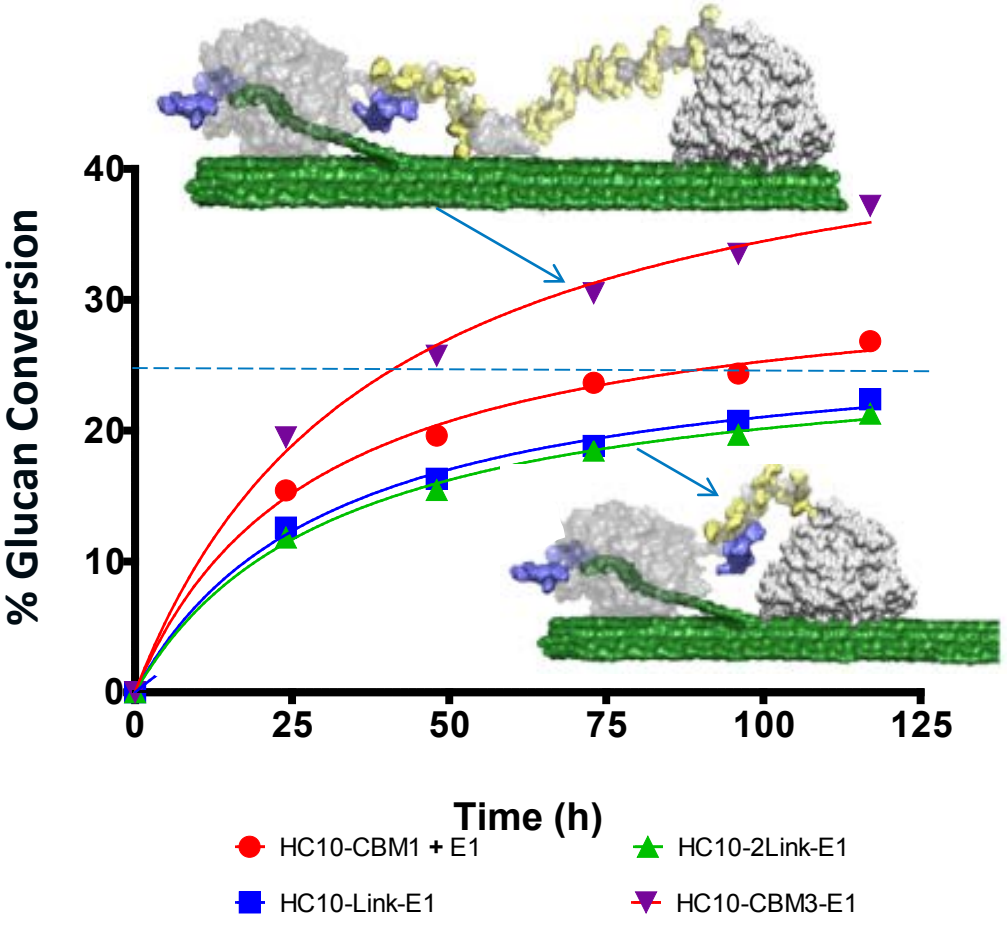
Design and test novel chimeric and multi-domain Cel7A mutants.

3—Technical Accomplishments/ Progress/Results

Taking another page from Nature: multi-domain mimics: aka Raptor



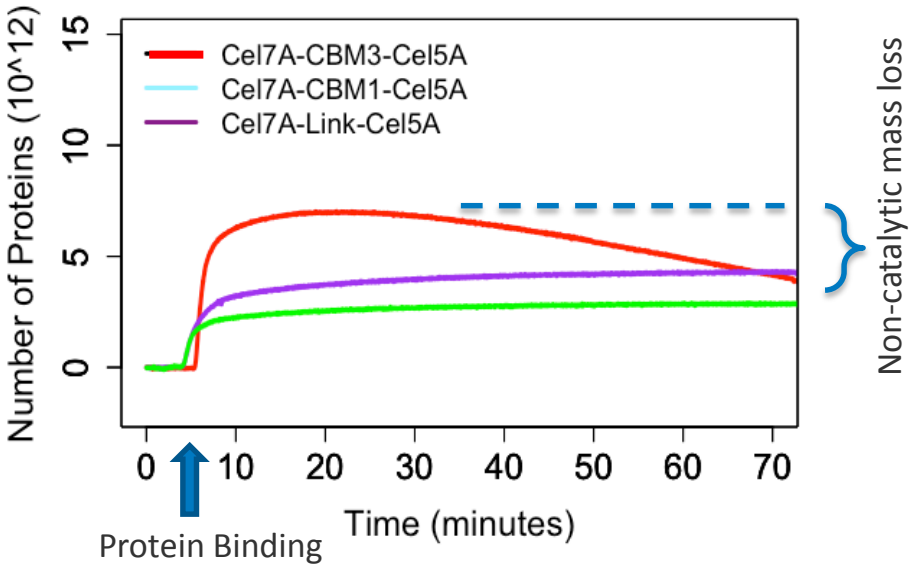
- Cel A cannot be expressed with full activity *in T. reesei*
- Developing strategies for *T. reesei* in FY2015-16
- HC10-CBM1 is outperformed by all multifunctional enzymes
- The combination of HC10-CBM1+E1 is more effective than linked HC10-E1 constructs
- HC10-CBM3-E1 also outperforms HC10-CBM1 + E1 **(37% increase in activity)**

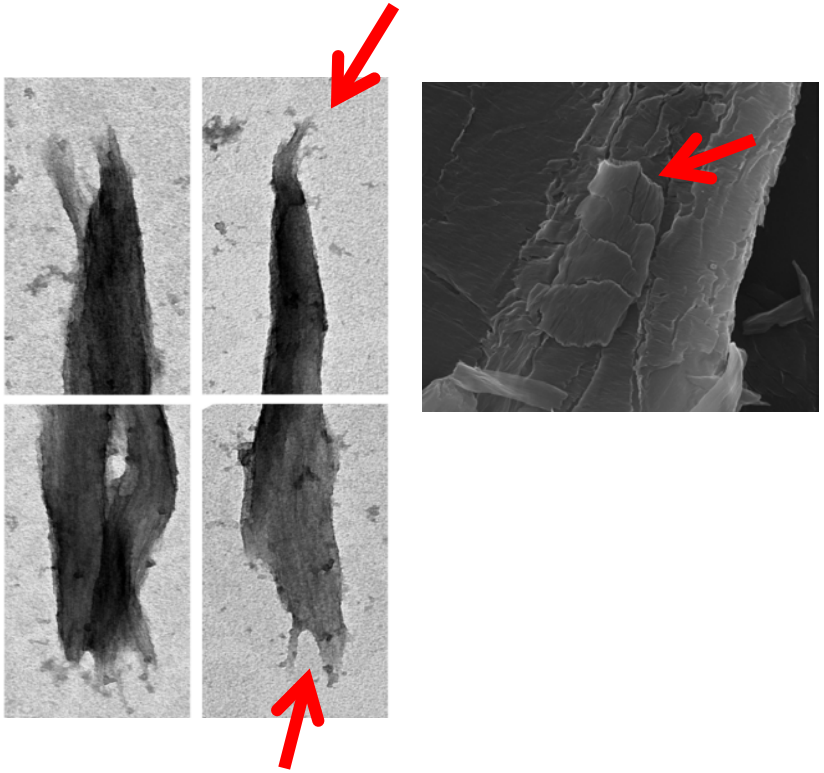


All digestions were run at a "equal"100 nmol active site loading (~10 mg/g) on Avicel 50°C

3—Technical Accomplishments / Progress/Results

The CBM3 multifunctional enzyme shows novel non-catalytic mass loss mechanism and novel deconstruction





- The CBM3 multifunctional enzyme highest linear binding rate observed among enzymes tested at NREL.
- Non catalytic mass loss may indicate cellulose disruptive role for CBM3!

CelA-like scalloping and Cel7A-like tip sharpening morphologies observed in our ‘raptor’ multifunctional cellulase

3—Technical Accomplishments / Progress/Results

Understand the structure/function basis for cellulase performance.

Employ the *T. reesei* chassis strain developed at NREL to prepare Cel7A enzymes.

Leverage the EMSL collaboration regarding *T. reesei* expression mechanisms.

Design and test novel chimeric and multi-domain Cel7A mutants.

Test ~100 Cel7A enzymes from Nature (Megatron).

3—Technical Accomplishments/ Progress/Results

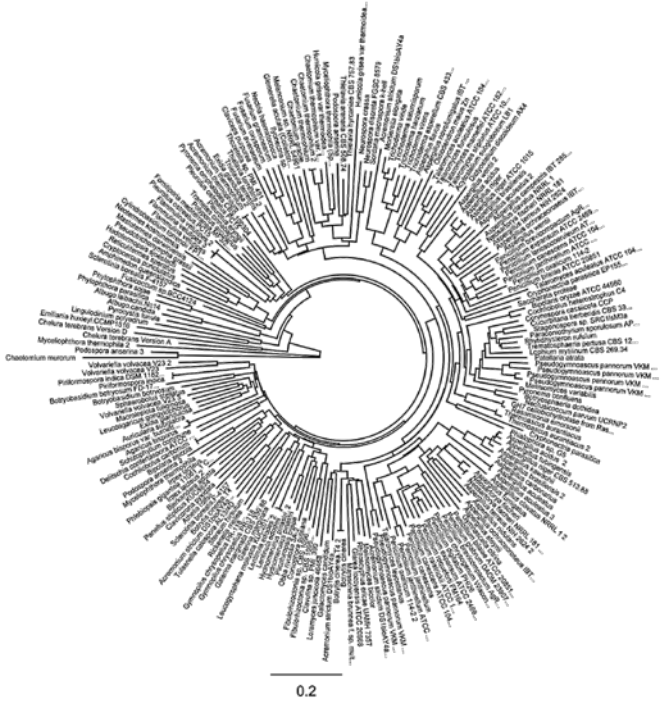
Cel7A "wheel of life"

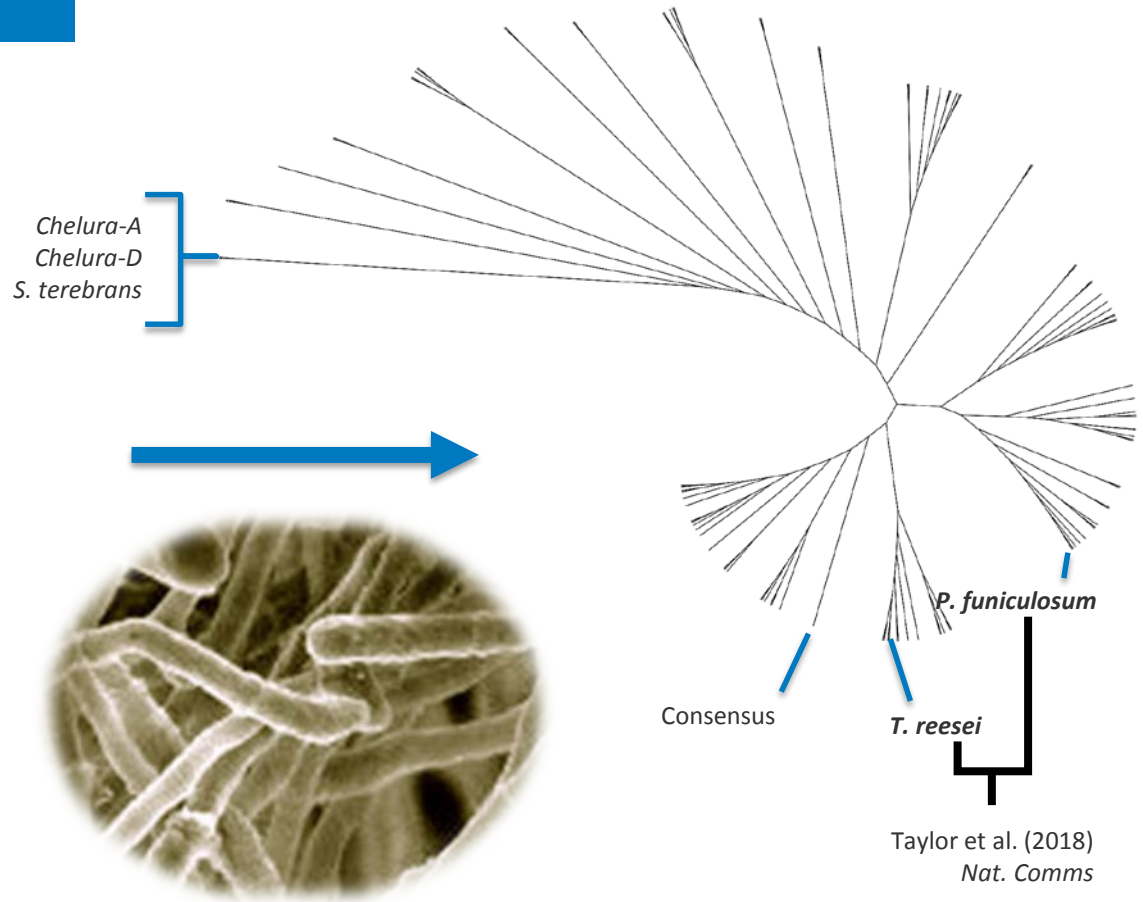
SLU

University of Portsmouth

3—Technical Accomplishments/ Progress/Results

Second Cel7A found in FY17 that out performs the *P. funiculosum* enzyme!





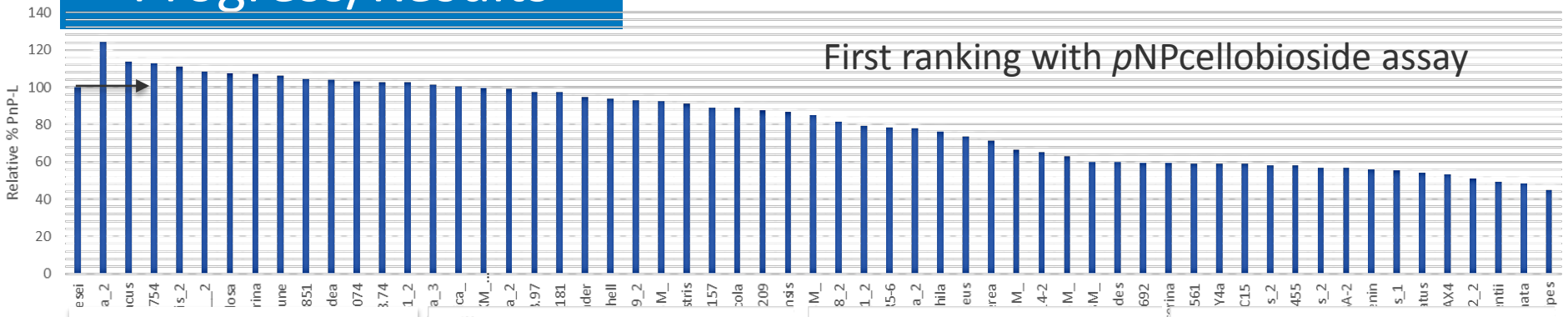
Cellulase Construction- FY18Q2 QPM

Produce and test 5 multi-functional variants of linker type and length, CBM number and type, and/or catalytic activities to demonstrate expression and activity. Demonstrate transformation and activity of >50 Cel7 enzymes from Cel7 diversity study.

3—Technical Accomplishments/ Progress/Results

Enzyme Characterization is underway

First ranking with *p*NPcellobioside assay

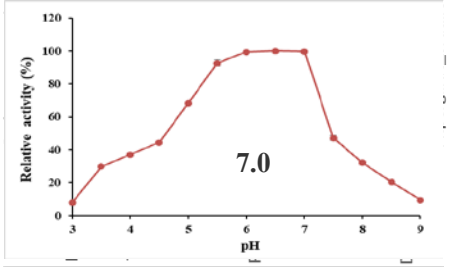


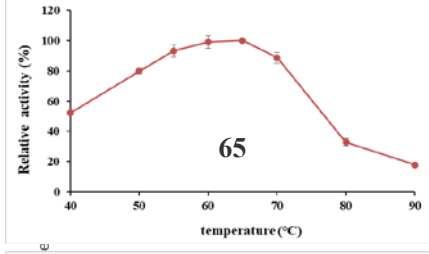
Examples

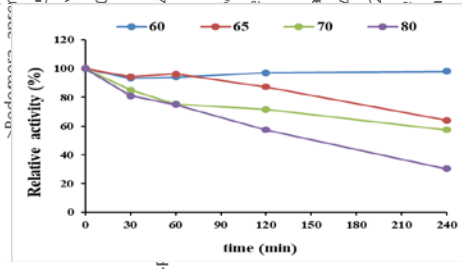
B3-Mycel7

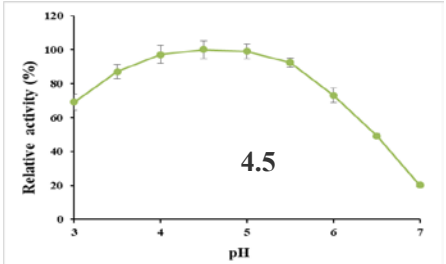
E12-Blender

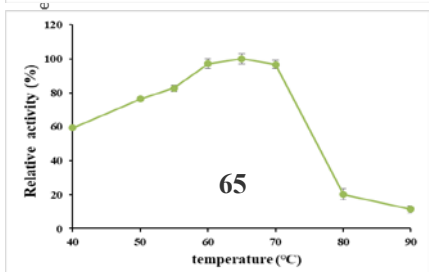
C5-Podospora

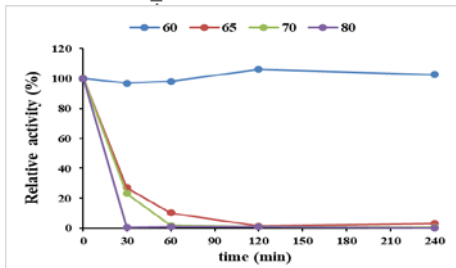


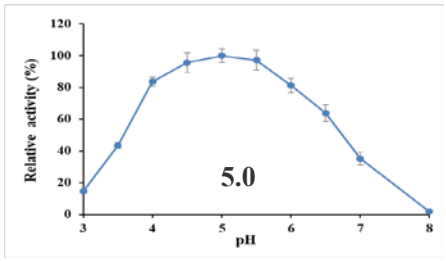


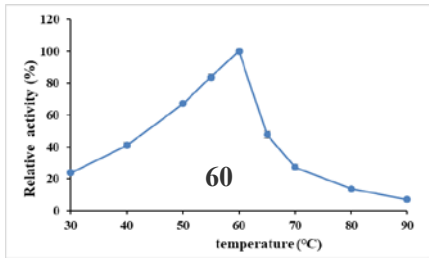


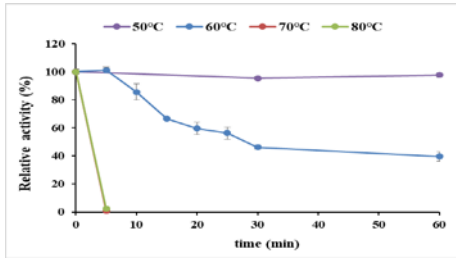












3—Technical Accomplishments / Progress/Results

- Understand the structure/function basis for cellulase performance.
- Employ the *T. reesei* chassis strain developed at NREL to prepare Cel7A enzymes.
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- Design and test novel chimeric and multi-domain Cel7A mutants.
- Test ~100 Cel7A enzymes from Nature (Megatron).
- **Initiated work with lignin modifying enzymes (LMEs).**
-

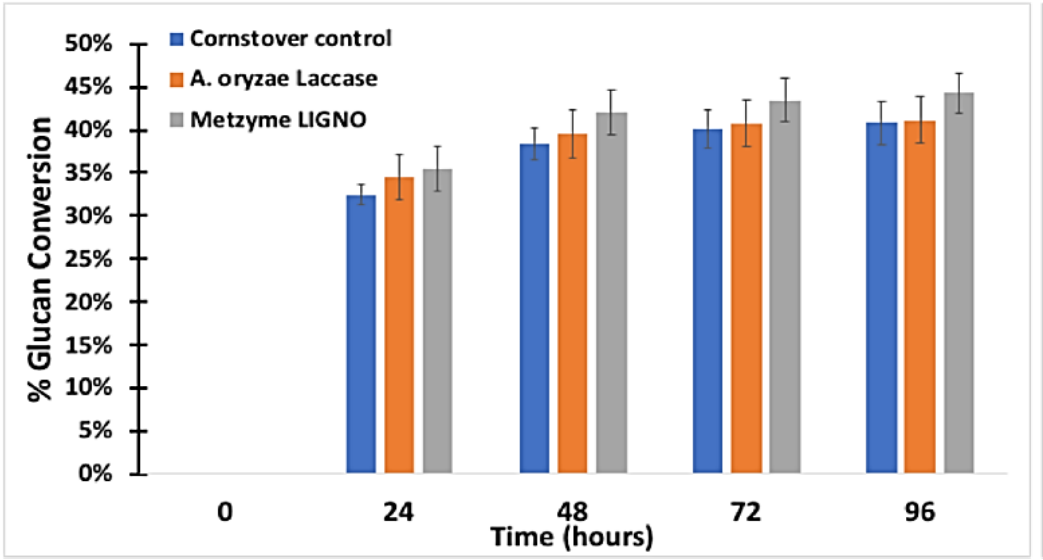
3—Technical Accomplishments/ Progress/Results

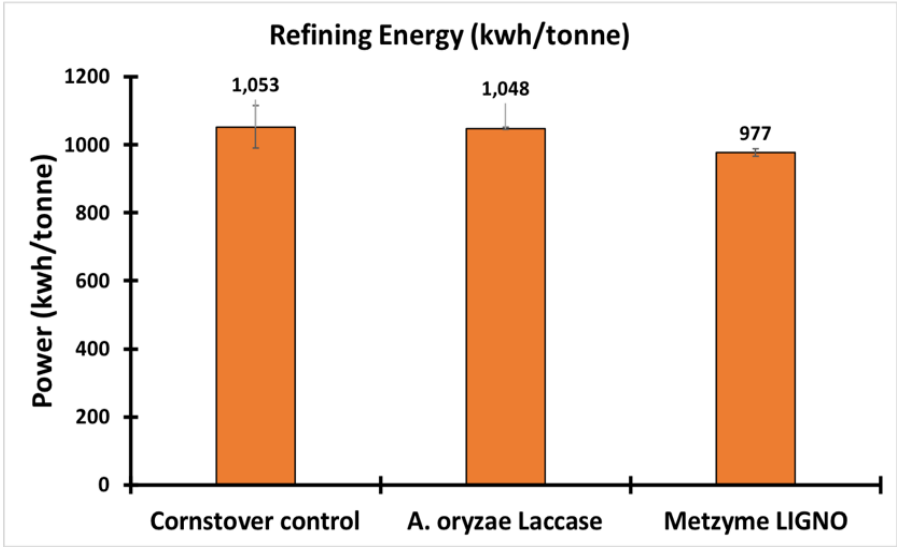
First look: LME enhanced saccharification

MetZyme® LIGNO™

An Enzyme product family for chemical pulp bleaching and extracts control

- Enzyme loading
  - 10 mg CTec2
  - 2 mg HTec2
  - 10 mg LME
- At 5% DMR solids and 96 h
  - ~9% increase in glucose digestion
  - ~7% decrease in milling energy
- Next Steps
  - Test multiple laccases and commercially available LME preparations
  - Examine various stages of the DMR pretreatment





3—Technical Accomplishments / Progress/Results

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- Design and test novel chimeric and multi-domain Cel7A mutants.
- Test ~100 Cel7A enzymes from Nature (Megatron).
- Initiate work with lignin modifying enzymes (LMEs).
- **Work closely with industrial collaborator (Novozymes).**

3—Technical Accomplishments/ Progress/Results

SCIENTIFIC REPORTS

OPEN

Received: 15 February 2017
Accepted: 11 May 2017
Published online: 29 August 2017

The Multi Domain *Caldicellulosiruptor bescii* CelA Cellulase Excels at the Hydrolysis of Crystalline Cellulose

Roman Brunecky[1], Bryon S. Donohoe[1], John M. Yarbrough[1], Ashutosh Mittal[1], Brian R. Scott[2], Hanshu Ding[2], Larry E. Taylor II[1], Jordan F. Russell[3], Daehwan Chung[1], Janet Westpheling[3], Sarah A. Teter[2], Michael E. Himmel[1] & Yannick J. Bomble[1]

The crystalline nature of cellulose microfibrils is one of the key factors influencing biomass recalcitrance which is a key technical and economic barrier to overcome to make cellulosic biofuels a commercial reality. To date, all known fungal enzymes tested have great difficulty degrading highly crystalline cellulosic substrates. We have demonstrated that the CelA cellulase from *Caldicellulosiruptor bescii* degrades highly crystalline cellulose as well as low crystallinity substrates making it the only known cellulase to function well on highly crystalline cellulose. Unlike the secretomes of cellulolytic fungi, which typically comprise multiple, single catalytic domain enzymes for biomass degradation, some bacterial systems employ an alternative strategy that utilizes multi-catalytic domain cellulases. Additionally, CelA is extremely thermostable and highly active at elevated temperatures, unlike commercial fungal cellulases. Furthermore we have determined that the factors negatively affecting digestion of lignocellulosic materials by *C. bescii* enzyme cocktails containing CelA appear to be significantly different from the performance barriers affecting fungal cellulases. Here, we explore the activity and degradation mechanism of CelA on a variety of pretreated substrates to better understand how the different bulk components of biomass, such as xylan and lignin, impact its performance.

Department of Energy Announces Technology Commercialization Fund Projects

AUGUST 22, 2018

Home » Department of Energy Announces Technology Commercialization Fund Projects

WASHINGTON, D.C. – Today, the U.S. Department of Energy (DOE) announced over $20 million in funding for 64 projects supported by the Office of Technology Transitions (OTT) Technology Commercialization Fund (TCF). With additional matching funds from the private sector, these projects will advance promising commercial energy technologies and strengthen partnerships between DOE's national labs and private sector companies to deploy energy technologies to the marketplace.

NATIONAL RENEWABLE ENERGY LABORATORY

- **Advanced Cellobiohydrolases, $750,000**
- **Novozymes Inc., Davis, Calif.**

4—Relevance)

Decreasing biomass conversion cost – improved enzyme formulations

Directly supports the needs of the biofuels industry

- Develop and transform our renewable biomass resources into commercially viable, high performance biofuels.
- **Current commercial enzyme formulations are not effective on DMR solids.**
- Novozymes partnership is key for applying research outcomes.

Addresses BETO's 2022 (and 2030) Performance goals:

- …verify integrated systems research at engineering scale for hydrocarbon biofuel technologies that achieve a mature modeled MFSP of $3/gge [and $2.50/gge respectively] with a minimum 50% reduction in emissions ……

Project metrics and technical targets are given by TEA.

- Produce a DMR solids acting enzyme formulation (CellicDMR?) that at a loading of 10 mg / gram cellulose achieves a 90% conversion of glucan to glucose and 90% conversion of xylan to xylose under standard conditions.

Reduction in conversion costs through improvements in:

- costs to **make and use enzyme** formulation,
- enhanced process **yield (glucose and xylose) from glucan and xylan**,
- enhanced process yields from **hydrolysis of recalcitrant oligosaccharides**,
- improvements in **quality of lignin** stream.

4—Relevance

Relevance to Industry

- Enzymatic conversion for sugar production is **required by most microbial product platforms.**
- New feedstocks, pretreatments, process schemes, and products are likely **to require reformulation of enzyme cocktails used in the process.**
- Availability of industrial enzyme formulation developed for DMR solids.

Advancing SOT

- The two leading commercial enzyme producers have noted our published technology in developing their commercial biomass enzymes.
- We intend that our **publications and presentations will continue** to be used by industry to design bench- and pilot-scale improvements to biomass handling, pretreatment, and enzyme saccharification.

Tech Transfer

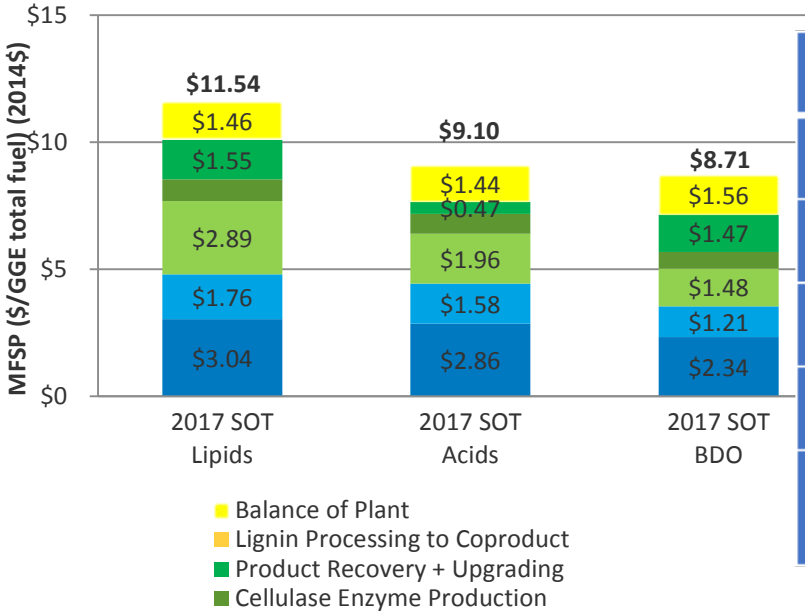
- NREL ROI: **Subdomain swaps enable** enhanced cellobiohydrolase performance.
- NREL ROI: Engineering **higher activity in the *Trichoderma reesei* family 7** cellobiohydrolase.
- NREL ROI: Enzymatic pre-conditioning for improved sugar release from biomass.
- NREL ROI: **Recombinant fungal multifunctional enzymes** or genes based on these enzymes outperform the native fungal enzymes for biomass conversion.
- Information will be available regarding biochemistry of improved cellulases.

4—Relevance

MFSP cost reduction strategies FY2017 SOT – Ryan Davis

Milestone Completion Report Agreement #: 8202 FY17 Level: Regular Milestone WBS #: 2.1.0.100	Completion Date: 9/30/2017 Scheduled Completion: 9/30/2017 Platform Area: Biochemical Con Milestone Completion Report
Project Title:	Biochemical Platform Analysis
Principal Investigator:	Ryan Davis
Milestone Title:	FY17 State of Technology
Authors:	Ryan Davis, Ling Tao, Mary Biddy, Eric Tan

- ✓ DMR solids are recalcitrant to enzymes developed for DDA solids!
- ✓ "Hotter" cellulases are required
- ✓ The ability to recover sugars locked in oligosaccharides poses new challenge



Parameter	FY17 SOT	FY 20 EH Targets	FY 22 EH Goals
Enzyme loading (mg/g cellulose)	12	10	10
Glucan to glucose	78%	85%	90%
Xylan to xylose	85%	85%	90%
MFSP (Enzyme contribution), $/GGE	$8.84 ($0.67)	$8.25 ($0.53)	$7.85 ($0.50)[1]
Total reduction in MFSP (yield plus enzyme cost to produce)		$0.74	$1.17

5—Future Work

What you plan to do through the end of the project (9-30-2019)

- Identify at least **5 superior performing cellobiohydrolases** from natural diversity.
- Use **Megatron studies to identify key "subsites" critical for building novel cellobiohydrolases** shown to have strong performance on DMR.
- We have shown a **decrease in power needed if we predigest w/ LMEs** before DMR. Next steps are looking at LMEs during deacetylation
- From the EMSL and collaboration, **understand protein secretion pathway in *T. reesei* sufficiently to demonstrate enhanced productivity of cellulases.**
- Develop (co-develop with Novozymes) cellulase, hemicellulose, and LME containing **formulation able to reach BETO's conversion goals for DMR solids.**

Highlight upcoming key milestones

- QPM2. (3/30/2019) "Report **hyperactive CBH I candidates from first 30 phylogenetically** novel sources. Because we have already found two non-*T. reesei* CBH I enzymes with about 10% better performance, success is finding more enzymes with comparable activity".
- Annual SMART. (9/30/2019). "**Demonstrate that the new "enzyme triad**" approach to reducing DMR solids recalcitrance can be effective by achieving an 80% conversion of glucose from glucan using improved cellobiohydrolases, xylanases, and lignin modifying enzymes under standard conditions".
- No-NoGo. (3/30/2020). "**Achieve intermediate conversion levels (83%) of glucose from cellulose/xylose from xylan in DMR solids** using improved cellobiohydrolases, xylanases, and LMEs. This can be done with CBH I delete *T. reesei* strains if the yield goals are met".

5—Future Work

Developing a *T. reesei* chassis strain for research – on going

Advantages:

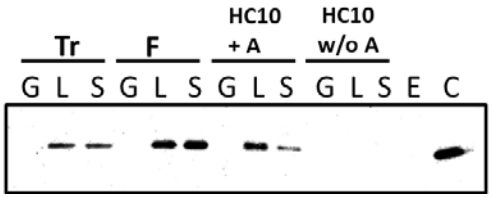
- Very high protein expression (up to 100 g/L)
- Commercially used today
- Potential for heterologous protein expression
- Can be used to engineer biochemical pathway for bioproduct synthesis

Disadvantages:

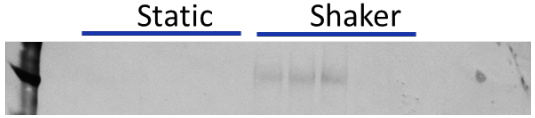
- Historically low heterologous protein expression levels
- Gene Knock-out and knock-in not available to research labs
- Regulation of enzyme expression not fully understood
- High expression of non target proteins
- Secretion pathways not exploited for heterologous expression

Understanding protein expression

Effect of C source on protein expression and secretion



Effect of culture parameters: static vs shaker

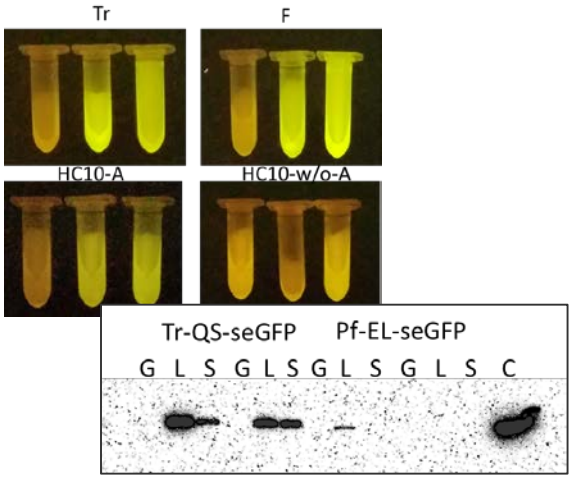


Strain engineering capabilities

- **Obtained a JGI Synthetic Biology award.**
- **Generating non-homologous end joining pathway mutants.**
- **Generation of cellulase mutants.**
- **Generation of protease (-) mutants.**
- **CRISPR/Cas9 in progress along with numerous knock-in/knock out genes to improve heterologous expression.**

Improving protein secretion

Effect of different secretion signals on heterologous protein secretion



5—Future Work

Address how you will deal with any decision points during that time (Go/No-Go Points)

(3/30/2020): Achieve intermediate conversion levels (83%) of glucose from cellulose/xylose from xylan in DMR solids…

- ✓ If candidates we feel are promising are not readily expressed from or folded by *T. reesei*, we will investigate alternative single use hosts, **such as insect or human cell lines (for research).**
- ✓ **Broaden range of xylanase and LME** candidates to natural diversity.
- ✓ Work with **LTAD (pretreatment team) to optimize DMR** conditions (fine tune).

What is the remaining budget and is it sufficient to complete the remaining work? If not, what are the plans to accomplish the work?

- – FY19 budget is $0.9M, FY20/21 may be slightly higher and we have planned accordingly.
- – In the case of an opportunity for enhanced budgets, we would propose to bring **key subcontractors on board** to assist with processing of the large enzyme libraries produced in Megatron.

6—Summary

Overview

To provide enzyme formulation technology that meets or exceeds 90% conversion of glucan and xylan to sugars, respectively, using cellulase loadings of 10 mg cellulase/g cellulose in DMR solids.

Approach

Employ a knowledge based approach, which constitutes linking protein structure to performance; as well as the coordination of computational analysis and experimental validation.

Technical Accomplishments/Progress/Results

- Published high impact studies describing the importance of specific subsites in Cel7s which confer enhanced performance. This finding launched new interest from industry in cellulases.
- Discovered that some specific orientations of CDs and CBMs from fungi and bacteria (Raptor) can be constructed to produce hyper active enzymes that are expressible in *T. reesei*.
- Secured partnership with EMSL and JGI to use omics tools to enhance the secretion of cellulases from *T. reesei*.
- New partnership with Novozymes to develop new formulations by combining superior Cel7 and Cel6As with optimally active xylanases and lignin modifying enzymes.

Relevance

Attainment of this enzyme cost is consistent with **TEA evaluations for meeting the 2022 biomass conversion target.** Publications and patents are used to disseminate progress to biorefinery industry and industrial benefit is shown by letters of support from industry (see slides).

Future work

Show progressive success throughout the three year POP by achieving 85% and 90% glucan conversion; and maintaining this level of conversion with the additional goal of 90% conversion of xylan in DMR solids; show that combinations of key subsites can be used to build improved enzymes.

Acknowledgments

Funding

U.S. DOE EERE Bioenergy Technologies Office

- HQ: Jonathan Male, Kevin Craig, Ian Rowe
- NREL LPM and Platform Lead: Zia Abdullah, Rick Elander

NREL Project Members (all between 10 and 50% FTE)

Stephen Decker (Task Lead)
Lilly Amore
Markus Alahuhta
John Baker
Gregg Beckham
Roman Brunecky
Brandon Knott
Jeff Linger
Vladimir Lunin
Venkat Subramanian
Todd Vanderwall
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Clare McCabe, Vanderbilt
Mats Sandgren, Jerry Ståhlberg, Henrik Hansson, SLU
John McGeehan, Simon Cragg, Portsmouth
Simon McQueen-Mason, Neil Bruce, York
Steve Withers, UBC

Parastoo Azadi
Nitin Supekar
Lance Wells
Linda Zhao

Thank You

www.nrel.gov

Publication Number

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Responses to Previous Reviewers' Comments

2017 Peer Review Report – Representative reviewers comments

- "The project is built on the idea that detailed study of individual enzymes through basic research on structural biology will lead to improvements in industrial enzyme mixtures. The project has generated some scientifically interesting results (making the performance of the Tr Cel7A like that of the Pf Cel7A by swapping sub-domains, investigating the role of glycosylation sites in Cel7A activity and stability), but there is a weakness is in closing the loop and demonstrating the performance of single enzymes and improved mixtures in deconstruction of pretreated biomass and in measuring expression of native and mutated variants in a relevant host."
- "The PIs have developed a highly interesting program to improve the generation of reactive cellulases and development of new cellulase systems for the production of sugars in the biorefinery. The work has clear industrial potential, and the team would benefit from including additional descriptions of this work's links to commercial application. The dots are all there, and just a little more time spent connecting them will really prove the utility of this work….."
- "The primary focus of the EEO program is to develop better cellulase activity. Since this enzyme represents up to 25% of biorefinery cost, this is a very important task. One might think that after 10 years of active research, there is not room for significant improvement in cellulase activities. However, their results indicate that this is not the case and that there are several exciting avenues to more active and robust enzymes….."

PI Response to Reviewer Comments

- The Review Panel made very good points regarding cellulase metrics and industrial relevance, which we will correct in future AOP planning. The challenge for NREL is that only the enzyme companies know what it costs to produce commercial cellulase formulations. We thus necessarily take the approach that improving the key component enzyme performance will always result in a reduction in cellulase cost, regardless of the exact production and formulation path taken by a particular company. The enzyme companies appear to agree that this assumption is valid. In planning for the FY2019/2021 EEO project we ensured strong and interactive collaboration with two industrial enzyme companies to ensure aggressive plugin of science results to technology outcomes. Mike Himmel
- ***Also provide highlights from any Go/No-Go Reviews.*** None

Outreach & Communication

- *Publications*
  - 12 publications from 2016
- *Patents*
  - Six cellulase relevant ROIs filed with DOE/NREL from 2016
- *Technology transfer or commercialization efforts*
  - Collaboration with Novozymes: Co-authored manuscript in 2018
  - Posters given at meetings likely to attract industrial interaction (SIM, ACS, Gordon Conferences, AICHE, etc)
  - 2019 - DOE BETO Technology Commercialization Fund project funded (R. Brunecky, PI); Novozymes partner
- *Awards (which leverage BETO's investment in EEO)*
  - 2018 – EMSL award to S. Decker for project entitled "Continuation of EMSL award to Steve Decker for project entitled "Post-Translational Modification and Subcellular Trafficking Effects on *Trichoderma reesei* Cellulase Production, Secretion and Efficacy"
  - 2018 – JGI award to V. Subramanian for project entitled "Molecular Engineering of *Trichoderma reesei* for improved cellulase production"

EEO Publications 2015-present

- "Investigating The Role of Lignin on Biphasic Xylan Hydrolysis During Dilute Acid and Organosolv Pretreatment of Corn Stover," Ashutosh Mittal, Todd B. Vinzant, Roman Brunecky, Stuart K. Black, Michael E. Himmel and David K. Johnson, **Green Chemistry** 17: 1546-1558 (2015).

- "Fungal Cellulases," Christina M. Payne, Brandon C. Knott, Heather B. Mayes, Henrik Hansson, Michael E. Himmel, Mats Sandgren, Jerry Ståhlberg, and Gregg T. Beckham, **Chem. Rev.** 115(3): 1308–1448 (2015).

- "A Heterologous Cellulase Expression System for *Hypocrea jecorina*." Linger, J. L., L. E. Taylor II, T. A. VanderWall, S. E. Hobdey, J. O. Baker, K. K. Podkaminer, M. E. Himmel, S. R. Decker. **Biotechnol. Biofuels** 33(1): 142-54 (2015).

- "New Insights into Microbial Strategies for Biomass Conversion," In Direct Microbial Conversion of Biomass to Advanced Biofuels, Sarah E. Hobdey, Bryon S. Donohoe, Roman Brunecky, Michael E. Himmel, Yannick J. Bomble, (M. E. Himmel, Ed.) Chapter 7, Elsevier Publishers, London/New York, NY. 2015, pp 111-127.

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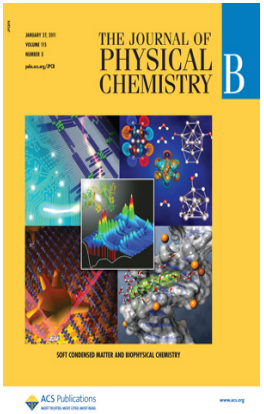

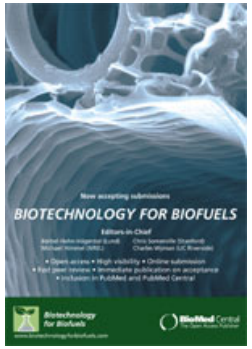

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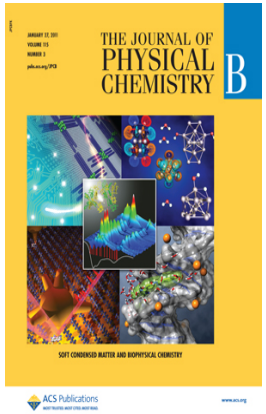

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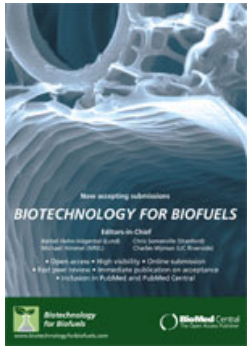

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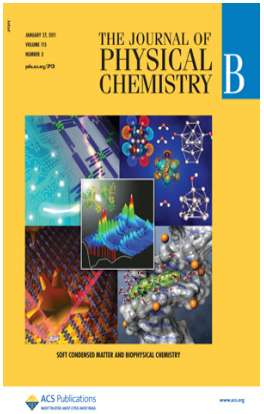

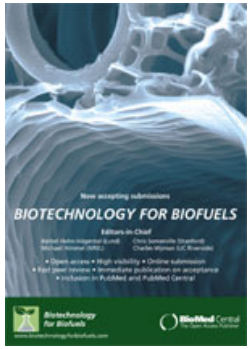

2019 EEO Milestones

Type	Description and Criteria	End Date
QPM1	Task 2. LME and Xylanases. Determine if either pre or post DMR treatment of biomass with commercial LME enzymes improves the saccharification of DMR solids. Success is maintaining conversion levels with a reduction in loading of current Ctec2 or 3 (mg enzyme/g DMR solids), or higher extents of 5-day conversion of DMR solids with the same loading (10 mg/g DMR solids). Collaboration w LTAD.	12/31/2018
QPM2	Task 1. CI. Report hyperactive CBH I candidates from first 30 phylogenetically novel sources. Because we have already found two non-T. reesei CBH I enzymes with about 10% better performance, success is finding more enzymes with this or better activity on cellulose.	3/31/2019
QPM3	Task 2. LME and Xylanases. Working with NREL's CBHI delete T. reesei strain (augmented with new Megatron CBHI candidates), screen commercial and purified xylanases for enhanced performance on DMR solids. Report formulations leading to enhanced glucose release under standard conditions*.	6/30/2019
Annual SMART	Tasks 1 and 2. Demonstrate that the new "enzyme triad" approach to reducing DMR solids recalcitrance can be effective by achieving an 80% conversion of glucose from glucan using improved cellobiohydrolases, xylanases, and lignin modifying enzymes under standard conditions*. We will be working closely with the LTAD and BPMS projects, as well as Novozymes to achieve this goal.	9/30/2019
Annual SMART	Tasks 1 and 2. Achieving a goal of 85% conversion of glucose from cellulose in DMR solids using improved cellobiohydrolases, xylanases, and lignin modifying enzymes under standard conditions*. For this goal to be successful, the partnership with Novozymes must be enabled. Our improved enzymes must be incorporated into the new Cellic formulations by the company for testing to be relevant. Collaboration w LTAD and BPA.	9/30/2020
EOP MS	Tasks 1 and 2. In partnership with Novozymes, produce a DMR solids acting enzyme formulation ($Cellic_{DMR}$) that achieves a 90% conversion of cellulose to glucose and 90% conversion of xylan to xylose under standard conditions*. With Novozymes as partner, demonstrate and enable a route towards industrial feasibility for advanced fuels from biomass. Collaboration w LTAD and BPA.	9/30/2021

2019 EEO Go/NoGo Milestone

Decision	Description	Criteria	Date
A GO decision indicates continuation on as planned. A NOGO decision can take different directions, pursuant to discussion with DOE. One direction could be investigation of modified DMR protocols to render pretreated biomass more digestible, another direction could be more deeply into why fungal cellulases for any source (Megatron) behave poorly on DMR, and a final option, abandon the project.	Achieve intermediate conversion levels of glucose from cellulose/xylose from xylan in DMR solids using improved cellobiohydrolases, xylanases, and lignin modifying enzymes. This can be done with CBH I delete (In-house) T. reesei strains if the yield goals are met. This caveat is given in case Novozymes needs more time to incorporate new enzymes into Cellic strains.	Achieve 83% conversion of glucose from cellulose and 83% of xylose from xylan in DMR solids using improved cellobiohydrolases, xylanases, and lignin modifying enzymes under standard conditions*.	3/30/2020

END