NREL
NATIONAL RENEWABLE ENERGY LABORATORY





DOE Bioenergy Technologies Office (BETO) 2017 Project Peer Review

Enzyme Engineering and Optimization

WBS 2.5.4.100

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National Renewable Energy Laboratory

Biochemical Platform Review
March 7, 2017
Denver, CO

This presentation does not contain any proprietary, confidential, or otherwise restricted information

Goal Statement

- **Goal 1 -** Technology to meet or exceed the target of 10 mg/g cellulose on pretreated feedstocks and feedstock blends. Attainment of this overall enzyme cost is consistent with BETO's technoeconomic evaluations for the 2022 biomass conversion target.
- **Goal 2 -** Enable novel/economic advanced biofuels through metabolic enzyme improvement and development of plug-and-play pathway engineering techniques.
- **Outcome 1 -** Public sector, cellulase design and formulation solutions enabling second generation biofuels from pretreated feedstocks and feedstock blends.
- **Outcome 2 -** Improve existing and enable new metabolic pathways by leveraging BETO's investment in cellulase technology toward metabolic pathway enzymes.
- **Relevance**
  - All four 2022 Biochemical Platform pathways require cellulase hydrolysis.
  - Cellulase related costs are between 20 and 25% for product MSRP.
  - 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: 2018
- Percent Complete: 66%

Budget

	Total Costs FY 15 – FY 16	FY 16 Costs	FY 17 Costs	Total Planned Funding (FY 16-Project End Date
DOE Funded	$1.6M*	$1.6M*	$1.6M*	$4.8M ($1.6M FY18)
Project Cost Share (Comp.)	0	0	0	0

*$1.1M Task 1
$0.5 M Task 2

Barriers

- Bt.C Biomass Recalcitrance—Better cellulases and flexibility for new (mixed) feedstocks.
- Bt.D Pretreatment Chemistry—Reduce severity/ cost of Pt via enhanced cellulase performance.
- Bt.G Cellulase Enzyme Loadings—Reduce cellulase cost through reduced loadings.

Partners

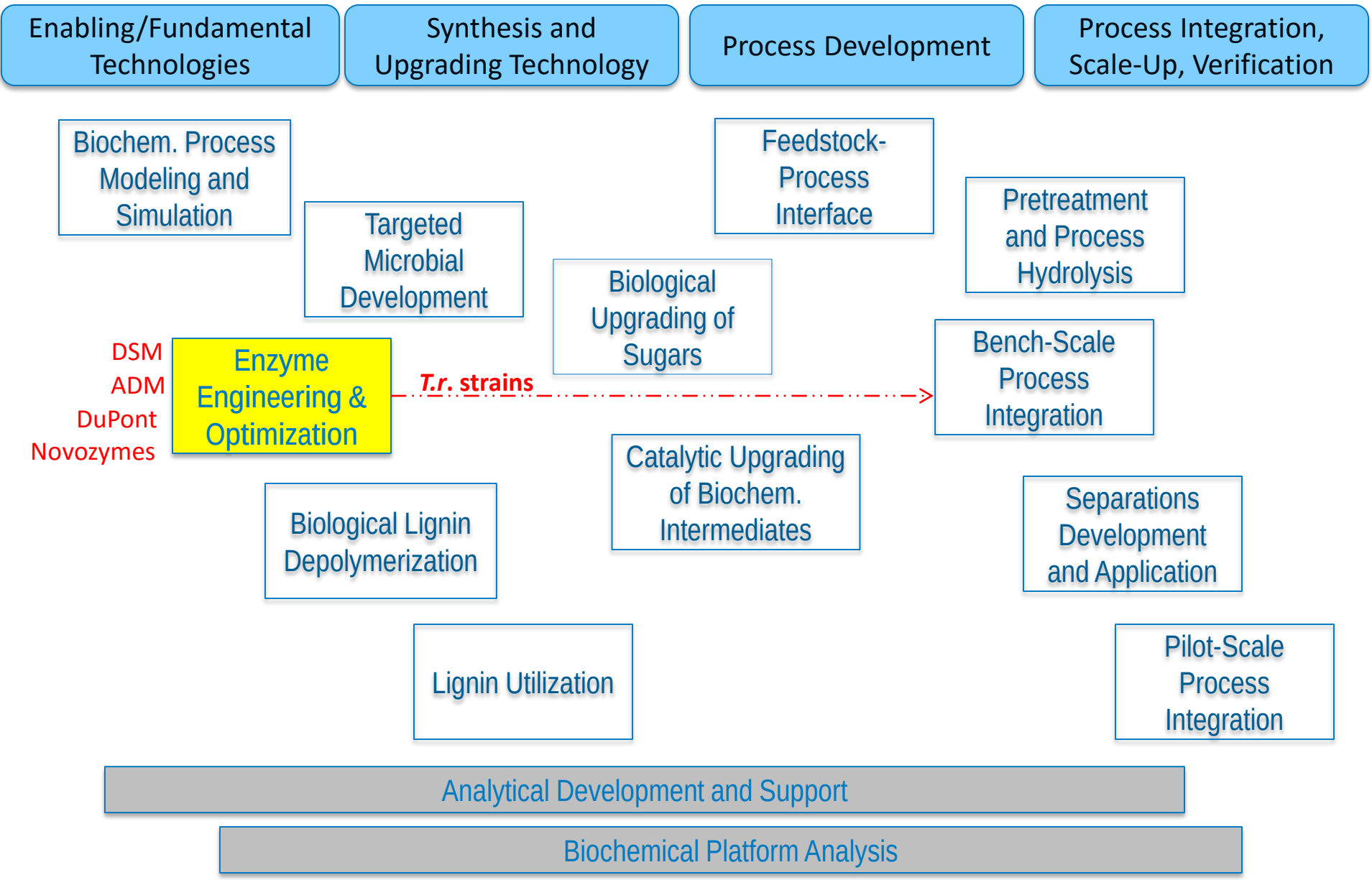
- Internal: BC Process Modeling and Simulation Project; Targeted Microbial Development Proj.
- Subcontractors: K. Reardon (Colorado State University), Z. Tan (University of Colorado—Boulder)
- Academic Collaborators: Zhongping Tan (CU), Jhih-Wei Chu (Berkeley), Lee Makowski (ANL), Paul Langan (LANL), Parastoo Azadi (UGA CCRC), Scott Baker (PNNL), Simon Cragg (University of York, UK), Jerry Ståhlberg, (Swedish University Ag Sci), John McGeehan (University of Portsmouth, UK), Joel Kaar (University of Colorado—Boulder), Michelle O'Malley (UCSB), Alan Darvill (CCRC/UGA)
- National Lab/Industry Collaborators: Galya Orr, EMSL (PNNL); Genencor DuPont, Novozymes, DSM, ADM
- DOE FOA to Genencor, subcontracted to NREL under CRADA ($150,000/3 years)

1—Project Overview

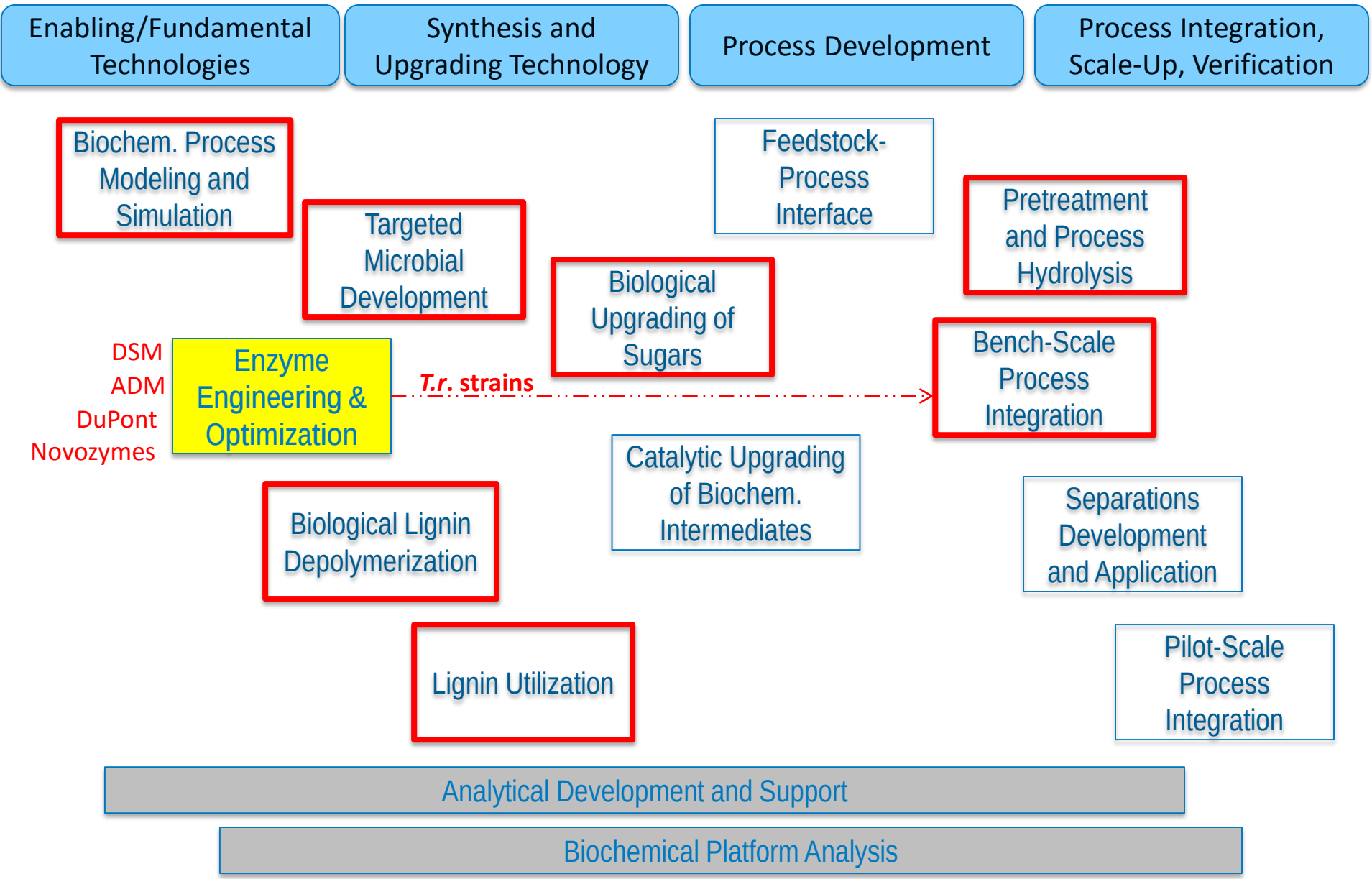
- For the past decade, we have used enzyme engineering approaches for improving enzymes and formulations critical to BETO's cost goals and a viable biorefinery industry.
  - Our strategy is to employ a knowledge based approach which permits linking protein structure to performance.
  - To this end, we have pioneered the close coordination of computational analysis and experimental validation for cellulases.
  - Our work is targeted to be specifically relevant to the process design and commercialization goals of biomass biorefineries.
  - We inform industry and the public via top tier publications (>50 since 2013).

- Currently, ~one fourth the biofuel cost is due to cellulase production and use.
  - We have confirmed that Nature harbors cellulases far superior to those currently used in many industrial formulations and that this performance can be explained on a structure/function basis.
  - *The remaining challenge is achieving viable expression levels of these enzymes in commercially relevant hosts for real-world testing.*
  - To meet these aggressive goals, we have established working relationships with the enzyme companies (DuPont, DSM, and Novozymes)
  - In FY2018, we will provide improved *T. reesei* hosts expressing improved enzymes to BETO researchers.

Biochemical Conversion Projects—NREL



Biochemical Conversion Projects—NREL



2—Approach (Management)

2015/2016—Enzyme Engineering and Optimization

2015 Task 1. Enzyme Improvement: We will 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, though promising enzymes also will have classical biochemical characterization to build structure-function models, including crystallography and computational analysis (QMMM).

2015 Task 2. Formulation and Application: Testing of the performance of enzymes and formulations on process feedstocks and schemes identified by the BETO 2017 base case. Enzyme synergy, feedstock-enzyme matching, high-temp hold, high-solids inhibition relief, and product-inhibition relief studies.

2016 Task 1. Enzyme Improvement ($1.1M): Standardize a functional selective screen in yeast for Cel7A activity and assay random mutations for activity. Also investigate novel, chimeric, random and site-directed Cel7A mutants; as well as multi-domain proteins (i.e., CelA or cellulosomes) synergistic with free fungal enzymes. Also establish a heterologous Cel7A-expressing *T. reesei* strain for consistent activity production as the "standard" benchmark cellulase for BETO. Random evolution of enzymes in yeast for the development of optimized cellulases with FY16 Go/No-Go.

2016 Task 2. Engineering Metabolic Pathway Enzymes ($0.5M): In collaboration with the Lignin Utilization, Biological Upgrading of Sugars, and Targeted Microbial Development projects, we leverage our experience in protein purification and enzyme characterization to critically evaluate metabolic enzymes (both wild type and engineered) needed for biofuels and bioproducts production. TMD has provided the highest priority problem to solve for FY16, the low activity of the heterologous Bhd and Aldc enzymes in the 2,3 butanediol pathway in *Zymomonas*.

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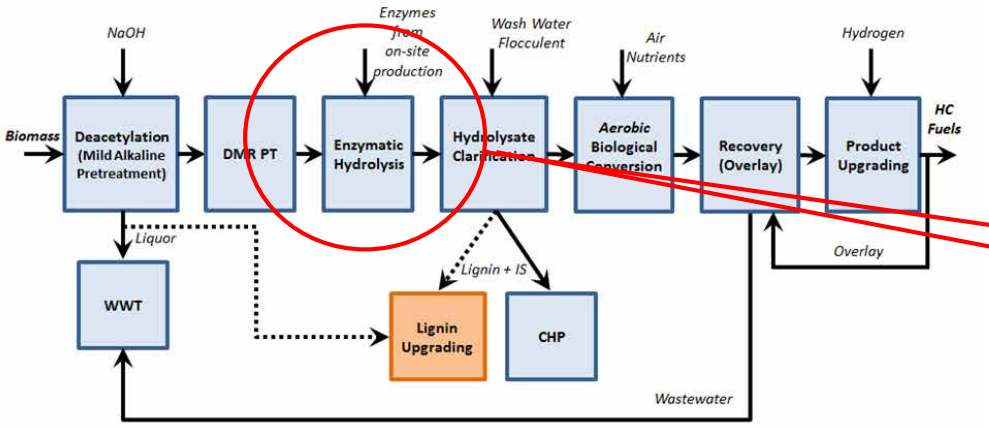
2—Approach (Technical)

- *Emphasize the structure of your approach. . . .*
  - Use RACI internal management which assigns researchers to milestone structure.
  - Academic and National Lab partners (CU Boulder, and PNNL-EMSL).
  - Milestones are Regular, Quarterly, and Annual, with several SMART milestones identified. One Go/No-Go decision is planned for FY2016.
  - Two Tasks (Task 1—Cellulase; Task 2—Metabolic Enzymes).
- *Explain the top 2 to 3 potential challenges. . . .*
  - To show that current, commercial enzyme cocktails can be reformulated for improved performance and reduced cost.
  - For the 2022 cost goals, good communication with the enzyme industry is important and thus we maintain relationships with ADM, DSM, DuPont, and Novozymes.
  - The current TEA analysis of the impact of cellulase cost is based on onsite production of a leading commercial formulation—a publicly available research strain and enzyme formulation would be a more robust solution for the overall biorefinery community! To this end, we are working to build such a strain.
- *Describe critical success factors. . . .*
  - The translation of (our) new and improved enzymes into the commercial arena.
    - It takes more than showing that new enzymes exist, they must be formulated commercially.
    - Continually reassess enzyme formulations with respect to ongoing process changes (pretreatment, lignin quality, new microbes).
  - Timely dissemination of technical achievements (publications and presentations).

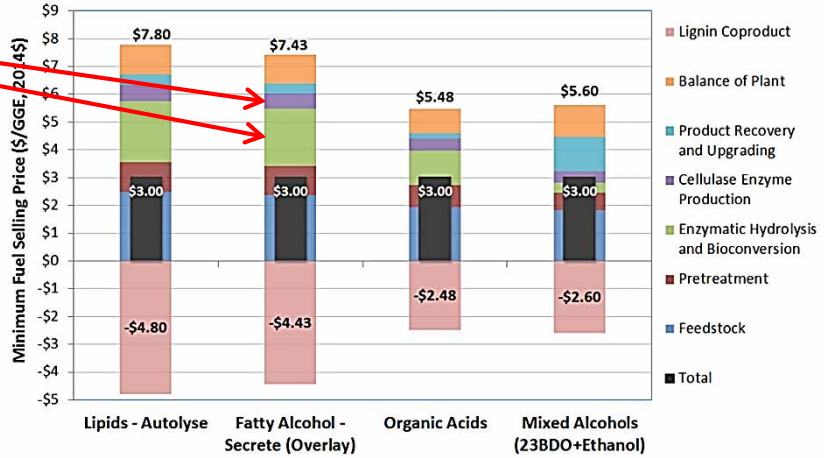
2—Approach (Technical)

2022 Biochemical Pathways

Cellulase cost remains significant



Fatty Alcohols Cost Projections (Example)



$/GGE MFSP Contribution (2014$)	2018 Projection (Sugars to HC + Lignin to AA)	2020 Projection (Sugars to HC + Lignin to AA)	2022 Projection (Sugars to HC + Lignin to AA)
Feedstock	$1.58	$1.32	$1.14
Pretreatment	$1.11	$0.88	$0.76
Enzymatic Hydrolysis and Bioconversion	$0.52	$0.44	$0.38
Cellulase Enzyme Production	$0.59	$0.53	$0.42
Product Recovery + Upgrading	$1.48	$1.38	$1.25
Lignin Processing to Coproduct	($0.83)	($1.62)	($2.34)
Balance of Plant	$1.50	$1.37	$1.39
MFSP	$5.96	$4.30	$3.00

~20-25% of overall process cost

3—Technical Accomplishments/ Progress/Results

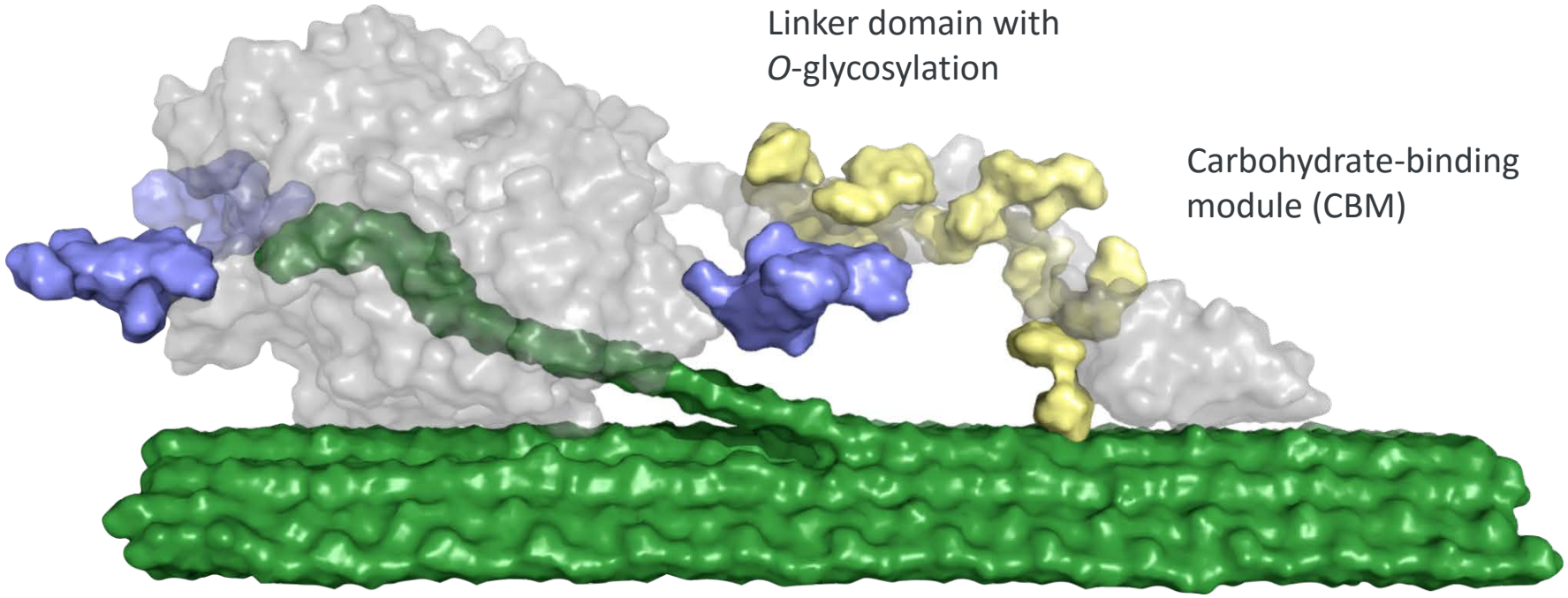
2016–17 **Task 1.** *Enzyme Improvement*

Evaluate variants and combine properties

- Understand the structure/function basis for cellulase (Cel7A) performance on pretreated corn stover.
- Design and test novel chimeric, multi-domain, and random/site-directed Cel7A mutants for effectiveness of pretreated biomass.
- Establish a heterologous Cel7A-expressing *T. reesei* strain for such testing. (Note that this strain can also be used as the "standard" benchmark or public sector strain for BETO.)
- Standardize a functional selective screen in yeast for Cel7A activity and assay random mutations for activity. Random evolution of enzymes in yeast for the development of optimized cellulases with FY16 Go/No-Go.
- Test ~100 Cel7A enzymes from nature following discovery of two examples superior to *T. reesei* enzyme (Megatron).

3—Technical Accomplishments/ Progress/Results

Cellulases Typically Are Multi-Modular Glycoproteins (Cel7A)



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

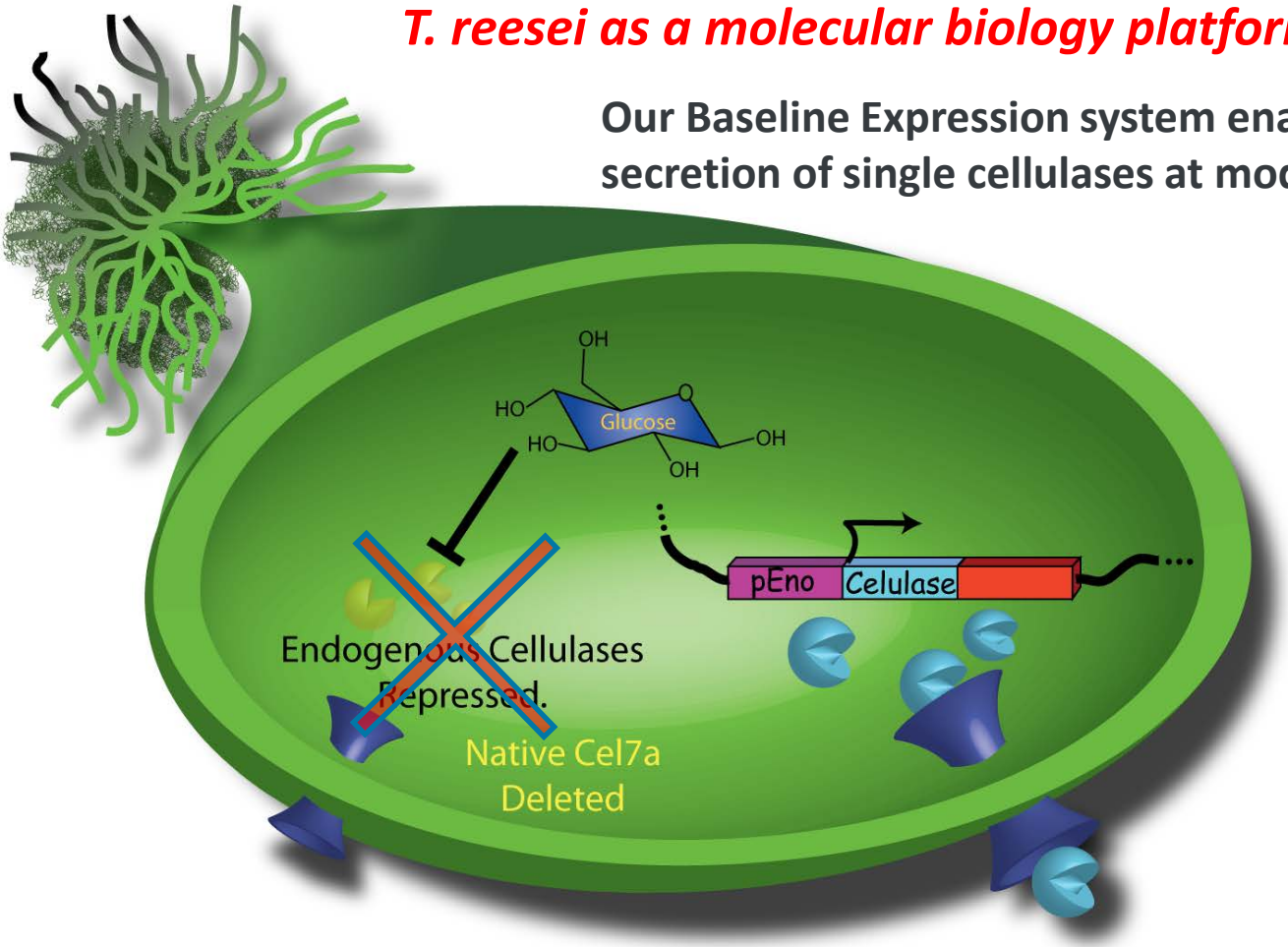
Critical Tool Development—Building a T. reesei expression system

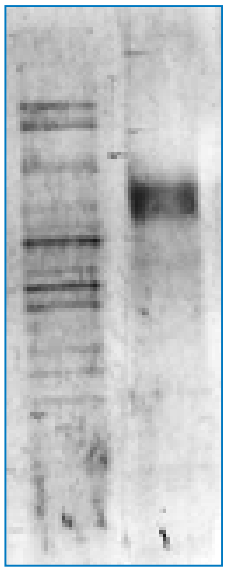
- Began working in *Aspergillus awamori*
  - "Industrial analog" strain
  - Did not produce native protein conformations
    - Wrong glycosylation/*N*-terminal processing
    - Unstable/susceptible to proteases
- Moved to *Trichoderma reesei (wild type QM6a)*
  - Several iterations required to get good productivity and correct folding; started with QM6a (base-strain, totally native)
  - Current system is good productivity, constitutive production, correct PTM; expression levels appear to be source-dependent
- Next step—increased production host strain (QM9414/RutC30)

3—Technical Accomplishments/Progress/Results

T. reesei as a molecular biology platform microbe

Our Baseline Expression system enables the expression and secretion of single cellulases at modest titers ("Research Strain")





Heterologous Enzymes

Chimeric Enzymes

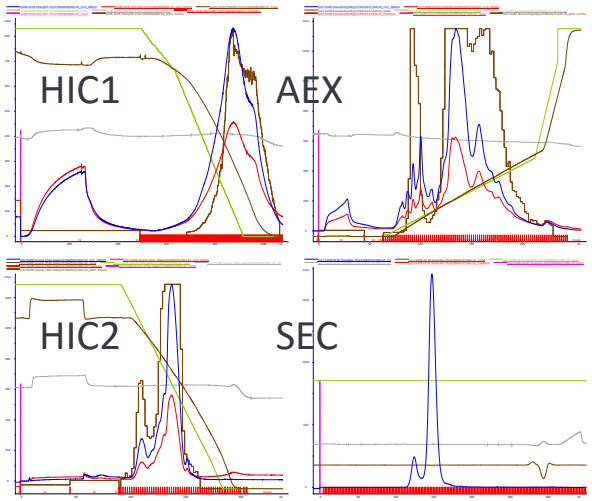
Linger et al. 2015
Singh et al. 2015

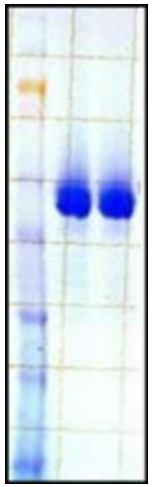
3—Technical Accomplishments/Progress/Results

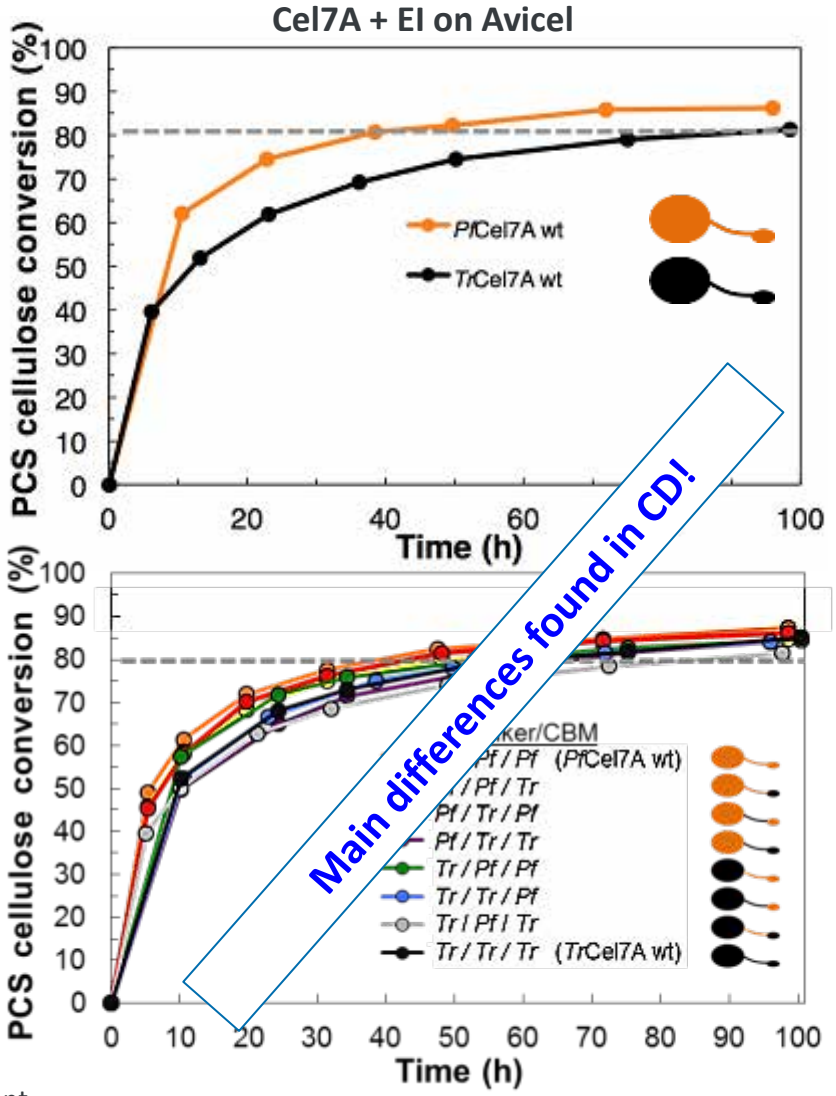
Penicillium funiculosom Cel7A—Why so active?

In 2013, we discovered *Pf*Cel7A has higher specific activity than *Tr*Cel7A.

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







DOE NREL US Patent

3—Technical Accomplishments/Progress/Results

Can we capture the design features of the P. funiculosom enzymes?

- Twelve subdomain swaps (*Pf*→*Tr* 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.
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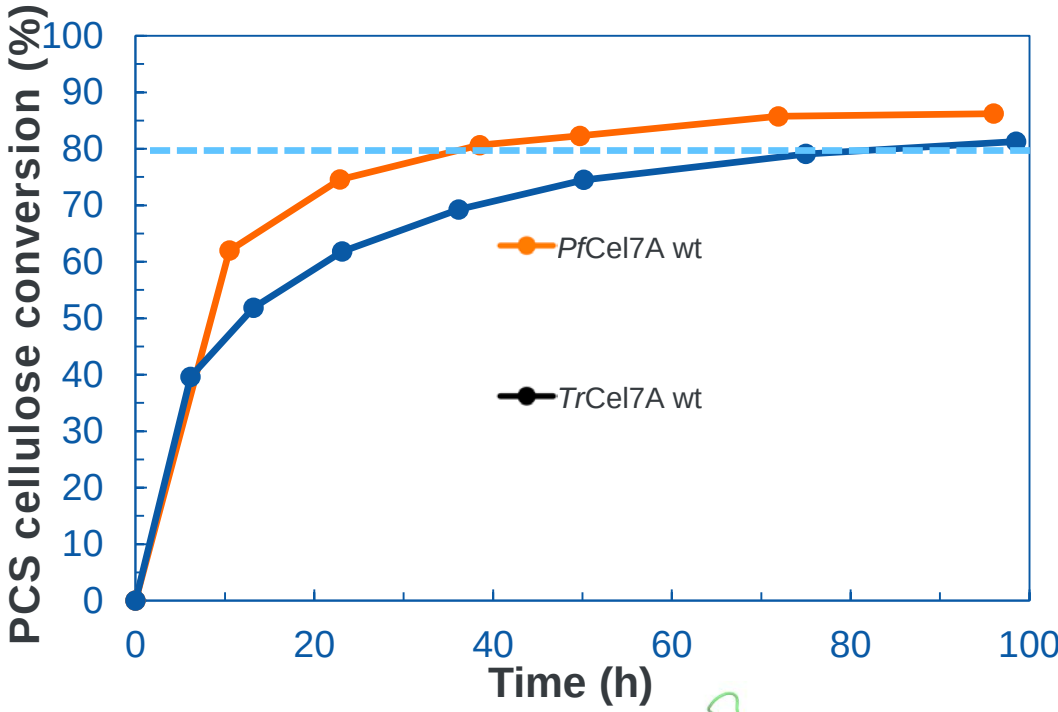

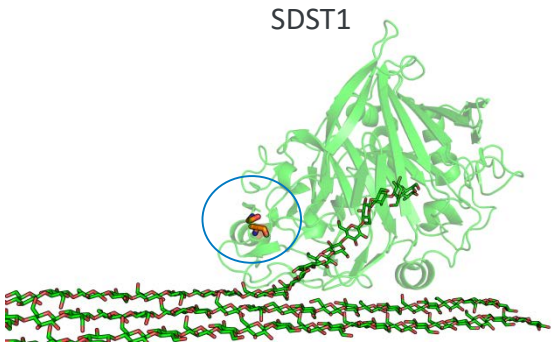

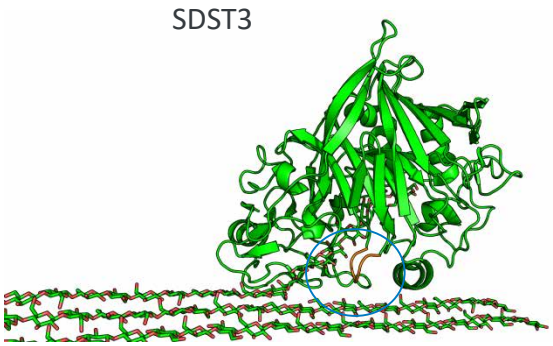

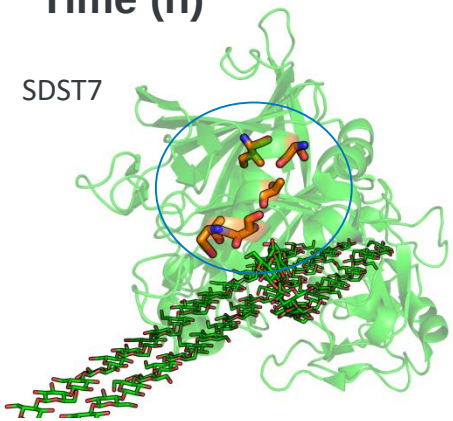

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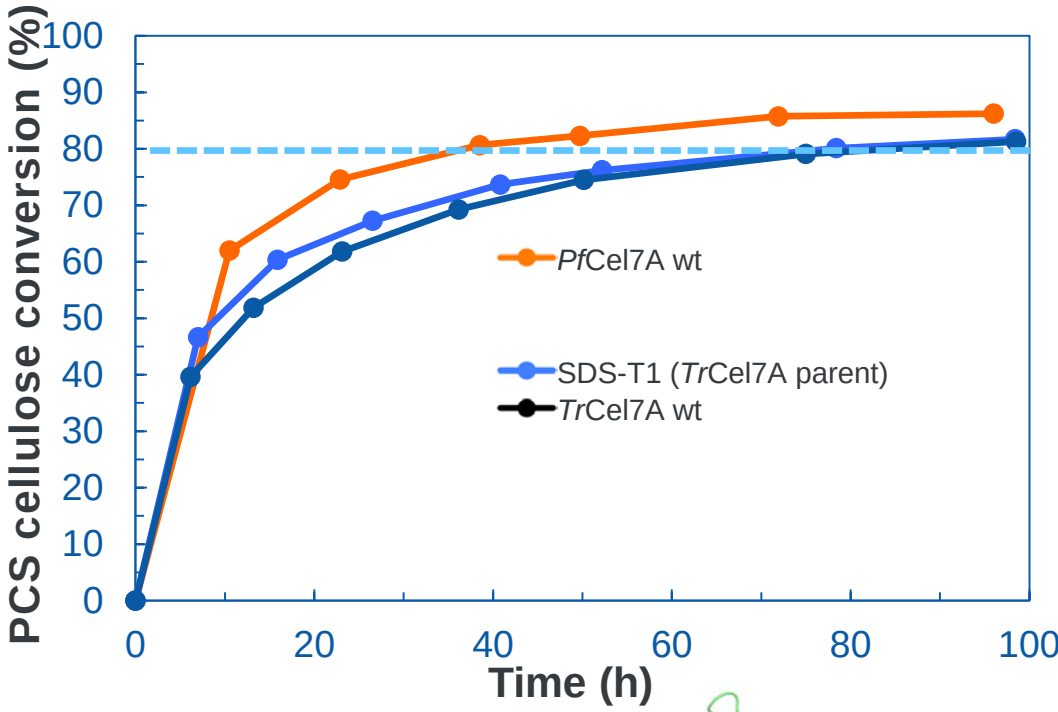

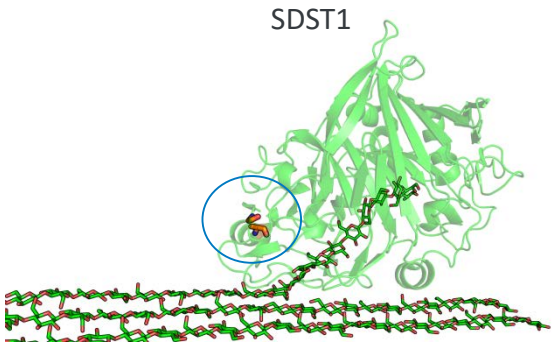

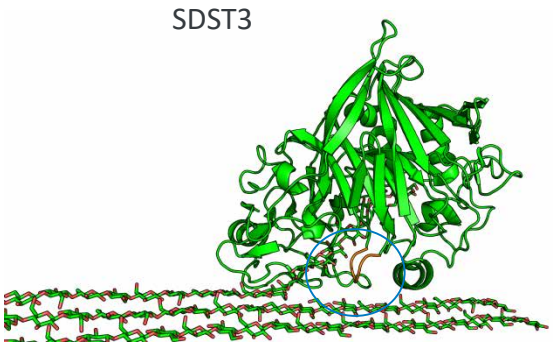

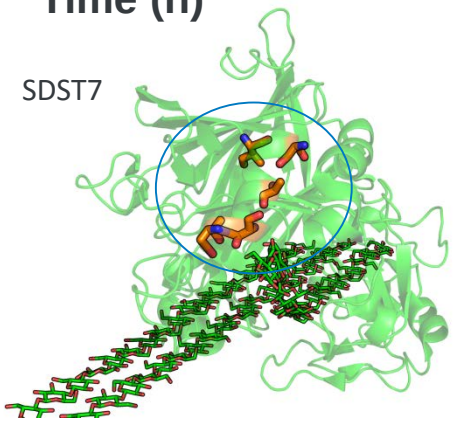

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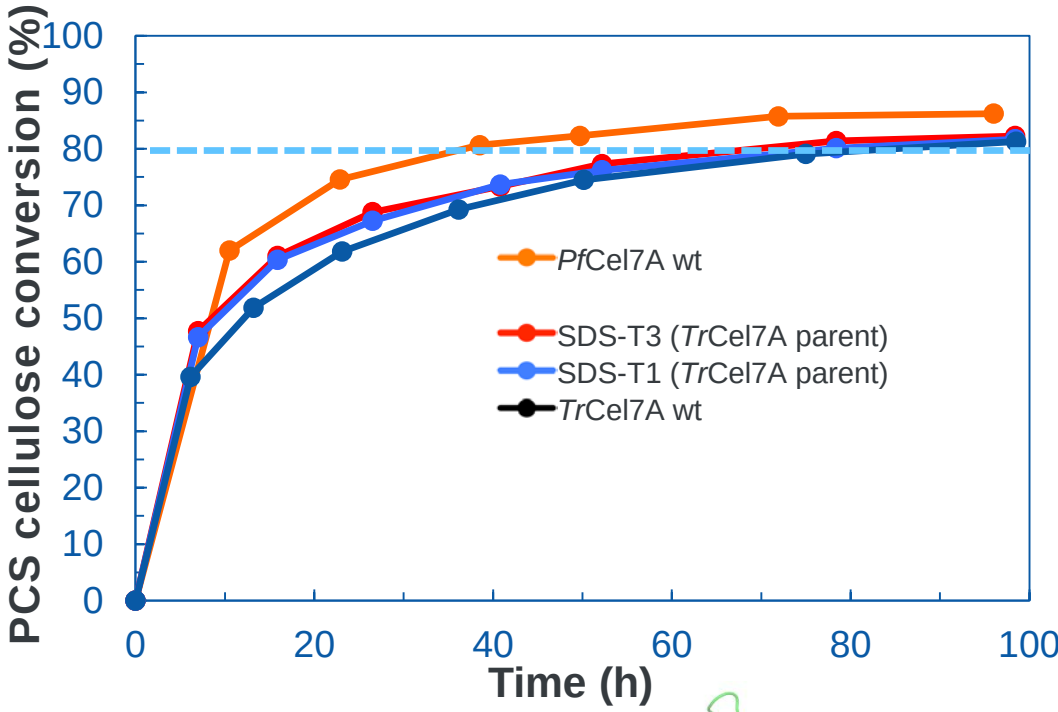

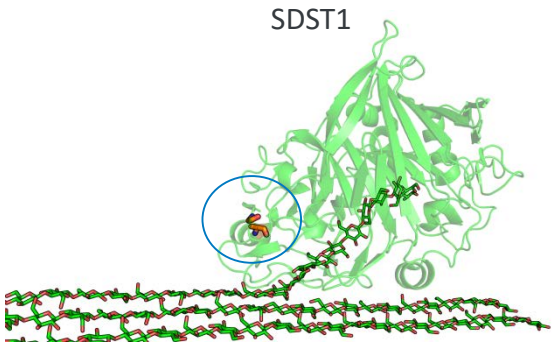

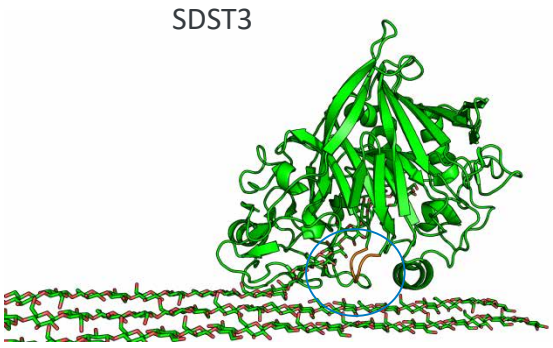

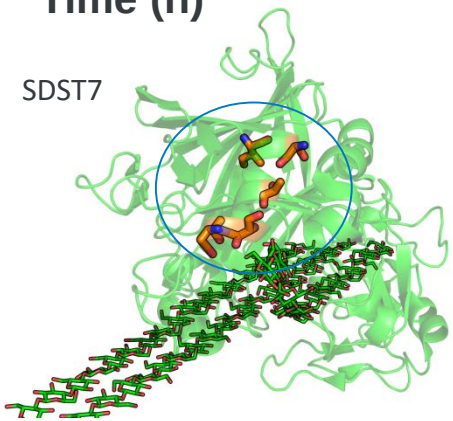

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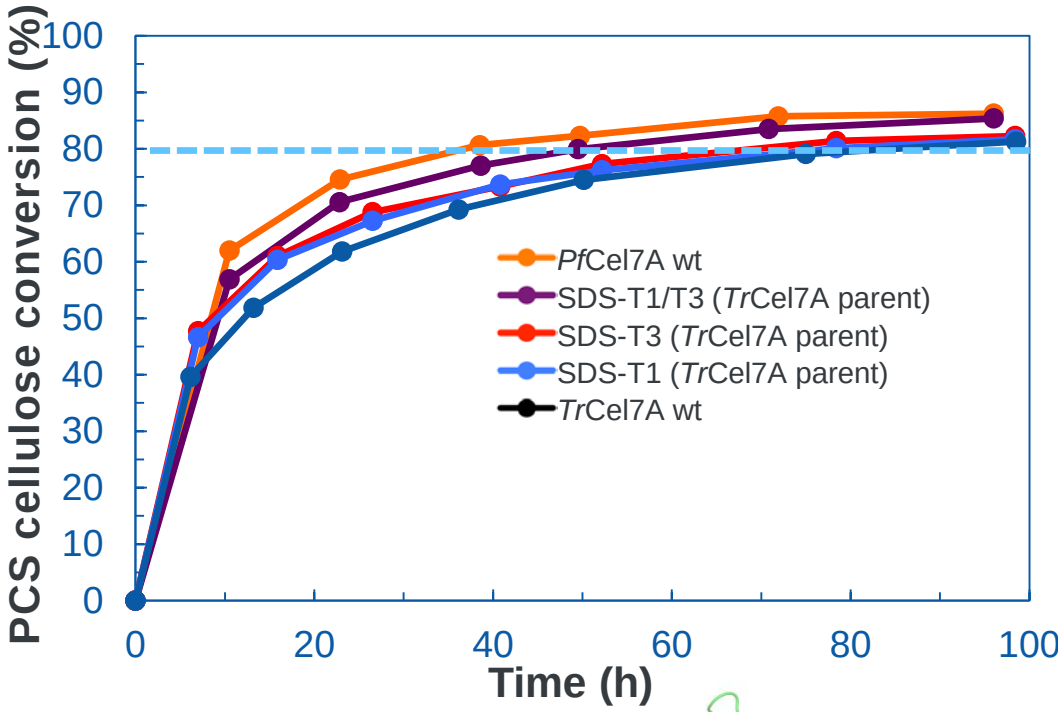
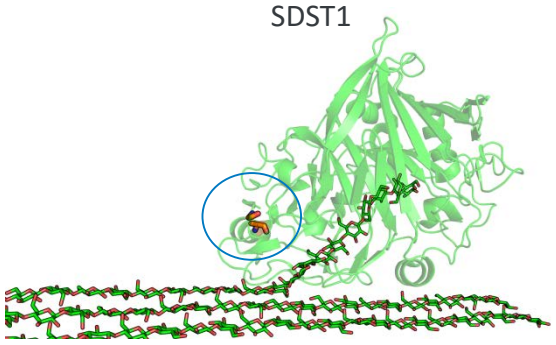
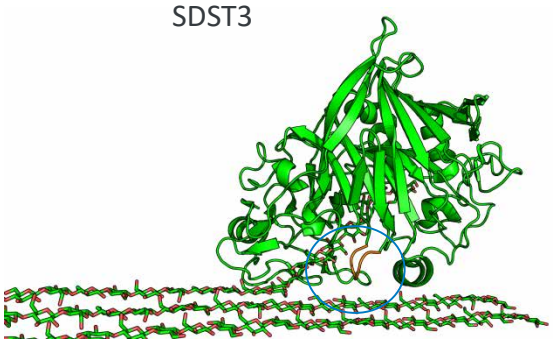
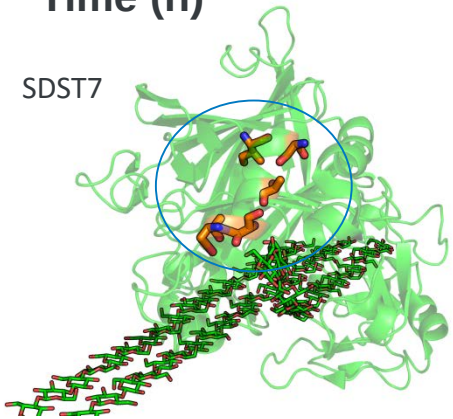




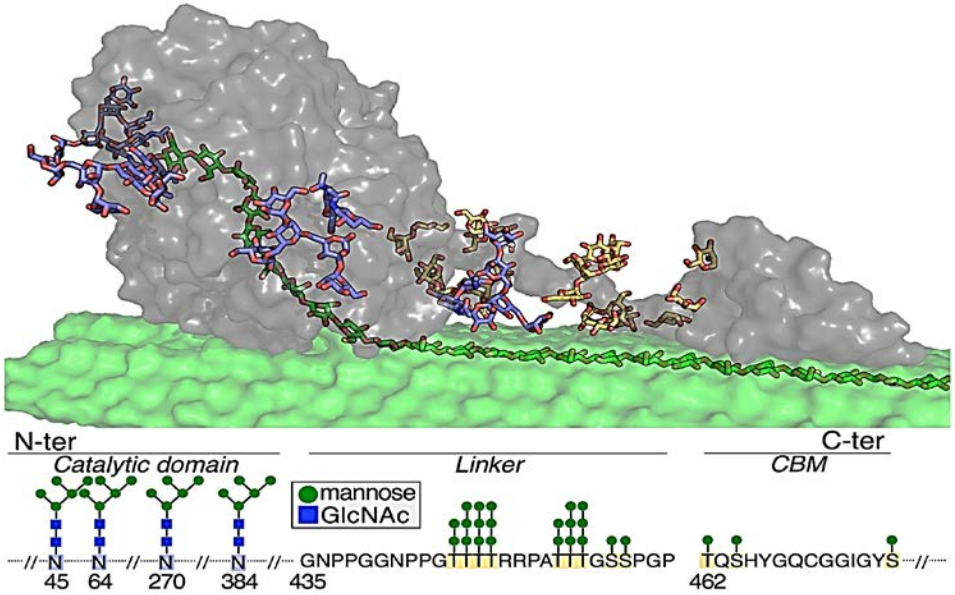




3—Technical Accomplishments/Progress/Results

Does glycosylation affect cellulase performance?

- Post-translational processing leads to differences in protein glycosylation
  - Computational modeling shows that increased glycosylation enhances linker and CBM binding to cellulose.
  - Glycosylation impacts substrate interaction, activity, pH and Topt parameters, protease susceptibility, etc.
- Structural analysis in partnership with UGA-CCRC
- Test with a SDM program targeting *N*- and *O*-linked glycans
  - Launched extensive glycosylation engineering program for Cel7A



A library of 25 mutants lacking glycosylation sites have been created to elucidate the role of *N*- and *O*-glycosylation in *Tr*Cel7A.

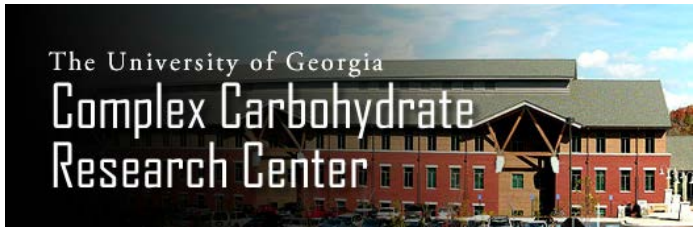
3—Technical Accomplishments/Progress/Results

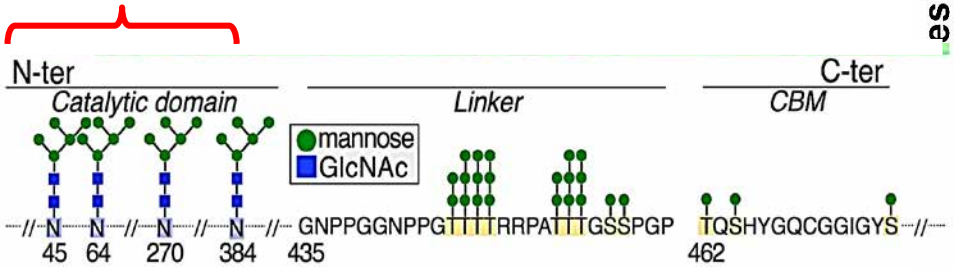
What about N-linked glycosylation?

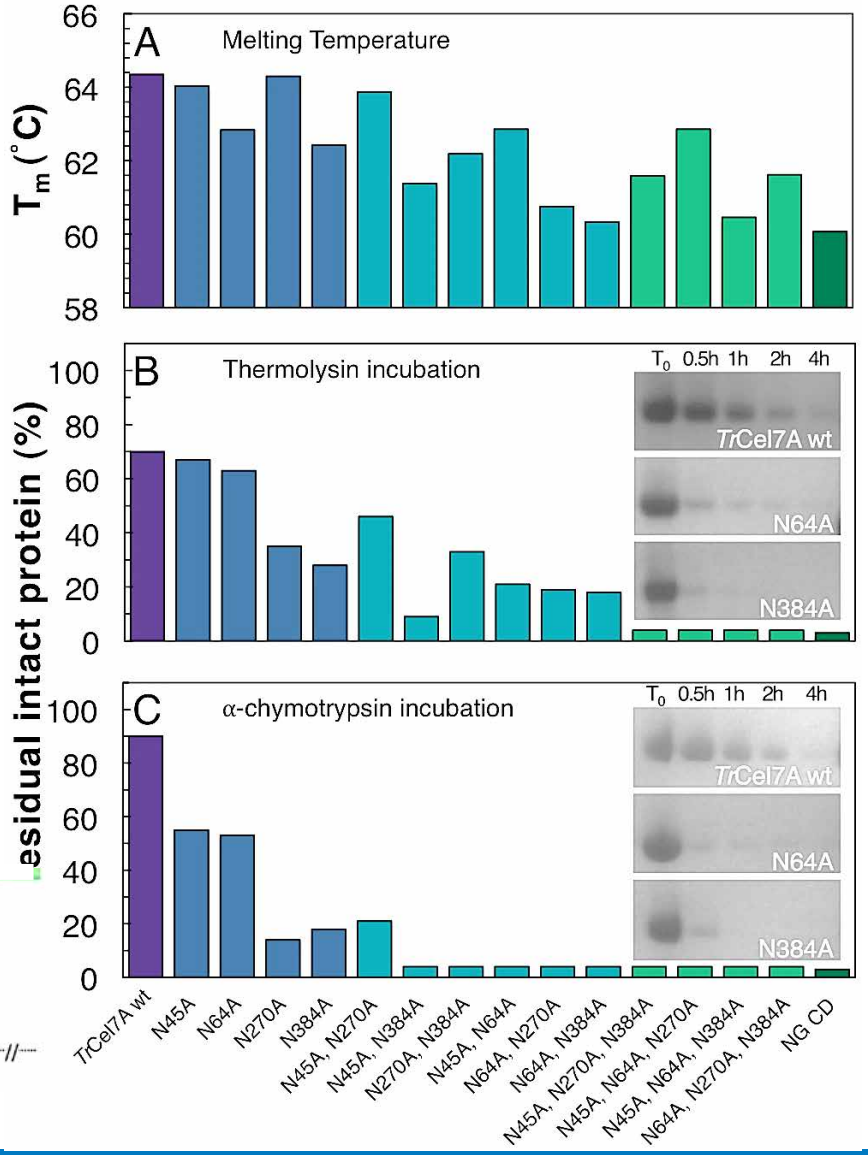
1) *Tr*Cel7A CD N-glycosylation

- Removal of *N*-glycosylation at the level of the catalytic domain affects thermal and proteolytic stability of *Tr*Cel7A.
- No impact on activity is found for CD glycosylation.

Collaboration with CCRC/UGA







3—Technical Accomplishments/Progress/Results

What about O-linked glycosylation?

2) *Tr*Cel7A Linker *O*-glycosylation

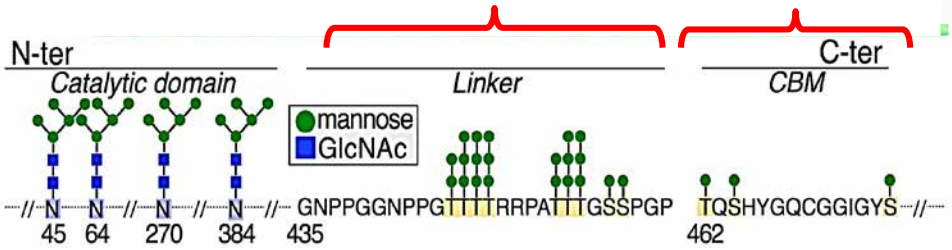
Removal of *O*-glycosylation at level of the linker peptide dramatically affects *Tr*Cel7A hydrolysis performances and binding to insoluble substrates.

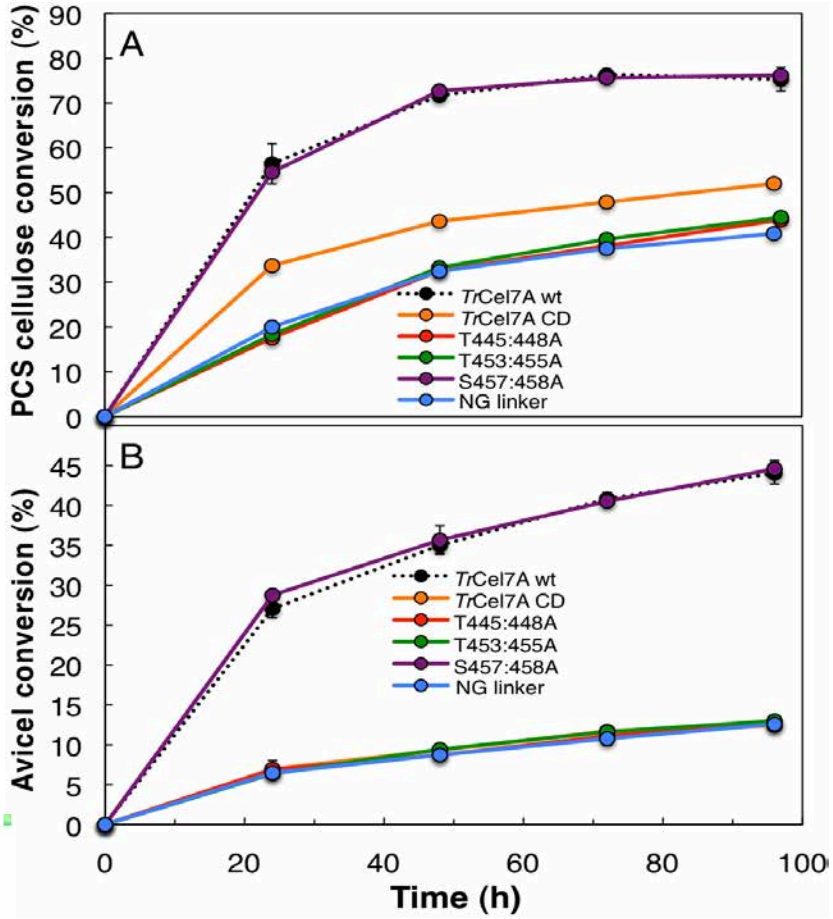
3) TrCel7A CBM O-glycosylation

No major effects on *Tr*Cel7A properties were revealed when *O*-glycosylation sites were deleted.

4) Linkage/Compositional Variation

Isoforms can have radically different activity. Studying linkage and composition of glycans at CCRC to understand impact





3—Technical Accomplishments/Progress/Results

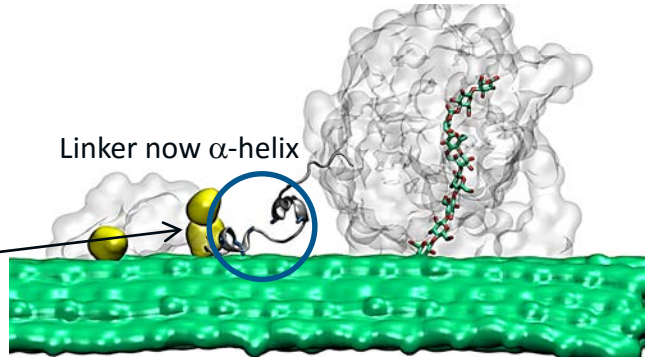
Can performance be improved using glycosylation?

Modulation of linker *O*-glycosylation to improve *Tr*Cel7A binding properties and increase its conversion rate.

These new *Tr*Cel7A linker mutants have been designed and production and characterization is in progress.

1) ---GNPPGGNPPGTTTTTTTTRRPATTTGSSPGP---
2) ---GNPPGGNPPGTTTTRRPATTTTTTGSSPGP---
3) ---GNPPGGNPPGTTTTRRPATTTTRRPATTTGSSPGP---
4) ---GNPPGGNPPGTTTTRRPATTTRRPATTTGSSPGP---
5) ---GNPPGGNPPGTTTTTTTTTTTGSSPGP---

---GNPPGGNPPGAAAAAAAAAAAGSSPGP---



Glycomics studies: revealing the glycan moieties of *Tr*Cel7A

- Identification of the *N*- and *O*-linked glycans decorating *Tr*Cel7A wild type is in progress, in collaboration with CCRC (University of Georgia, Athens).
- Elucidation of *Tr*Cel7A glycan patterning will enable further understanding of the role of glycosylation in *Tr*Cel7A and facilitate the design of improved *Tr*Cel7A mutants.

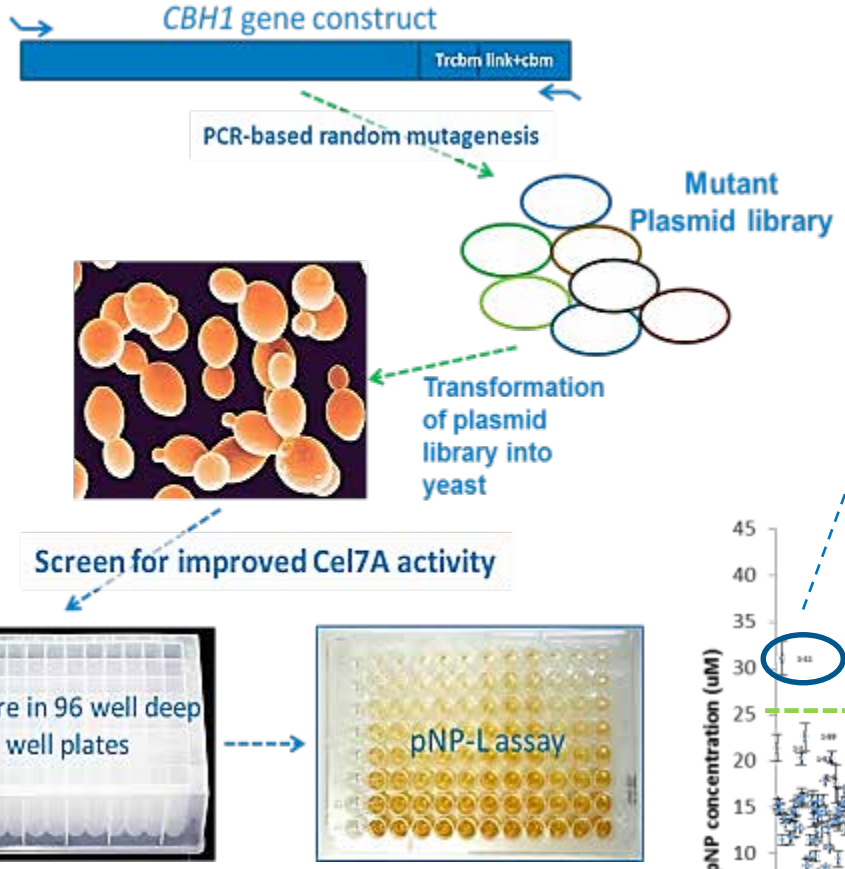
3—Technical Accomplishments/Progress/Results

Task 1: 2015 EEO yeast expression of GH7 cellobiohydrolase

- **Advantages of yeast expression of GH7s**
  - Powerful expression tools
  - HTP screening capable, allow for random evolution and screening of Cel7A.
- **Disadvantages**
  - Poor processing of TrCel7A, low yields
  - No growth on cellobiose *Saccharomyces.*
- **Overcoming barriers**
  - *Talaromyces* and *Humicola* GH7s express better than *Trichoderma*.
  - +β-G grow on cellobiose, +EG to grow on cellulose.
  - Add in glutaminyl cyclase to allow correct processing.
- **Progress**
  - B-G is been expressed →growth on cellobiose
    - Limited growth on cellulose but better than native
  - Glutaminyl cyclase gene identified and cloning is underway.

3—Technical Accomplishments/Progress/Results

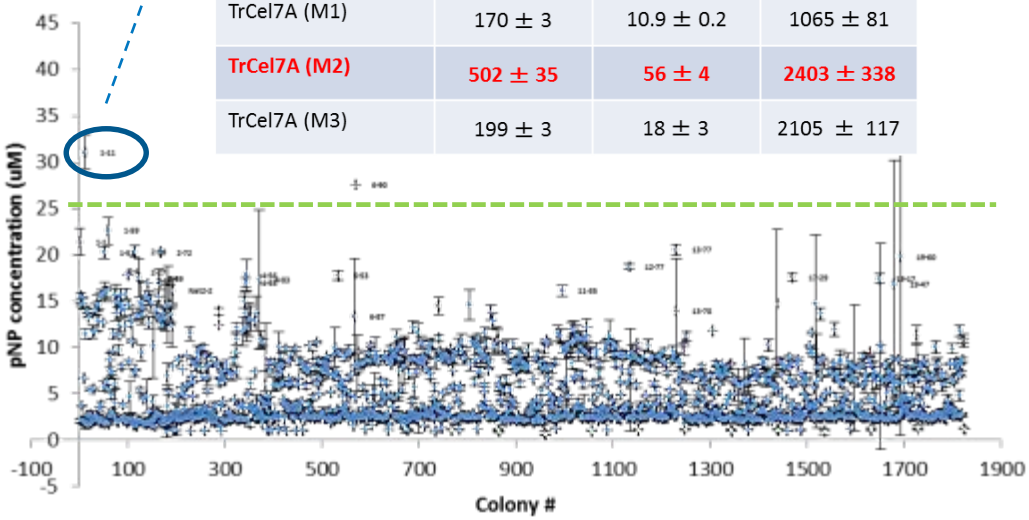
Identification of yeast mutants expressing higher Cel7A enzyme activities



Mutation in clone M2 leads to possible improved active site catalysis

pNPL Activity

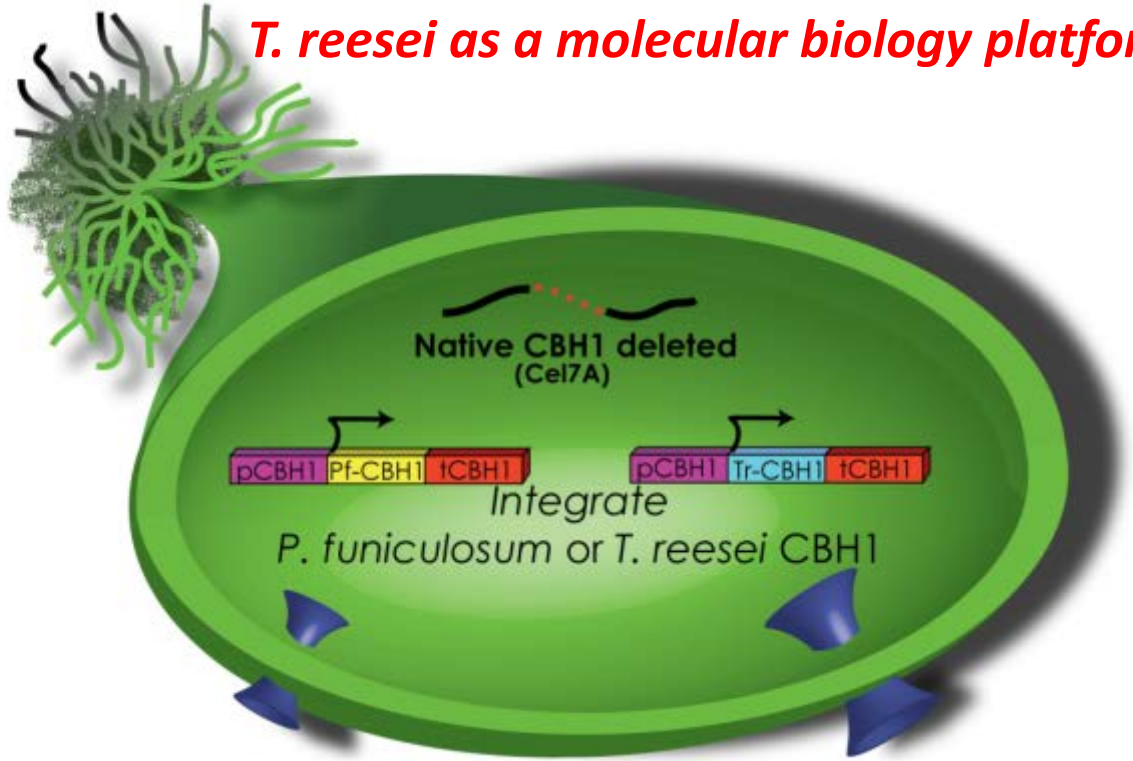
Protein	Specific Activity (U/mg)	K_{cat} (min^{-1})	kM (mM)
TrCel7A wt	204 ± 10	20 ± 1	1930 ± 112
TrCel7A (M1)	170 ± 3	10.9 ± 0.2	1065 ± 81
TrCel7A (M2)	502 ± 35	56 ± 4	2403 ± 338
TrCel7A (M3)	199 ± 3	18 ± 3	2105 ± 117



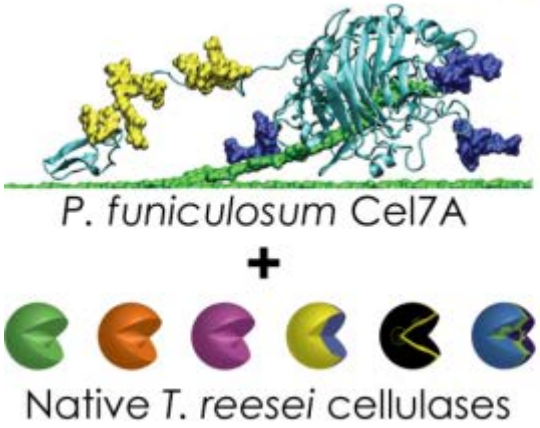
NREL Record of Invention (ROI) No. 16-76
U.S. provisional patent application No. 62/326,382

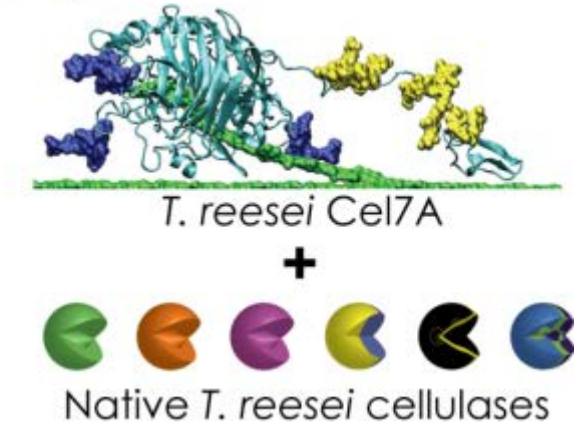
3—Technical Accomplishments/Progress/Results

T. reesei as a molecular biology platform microbe



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





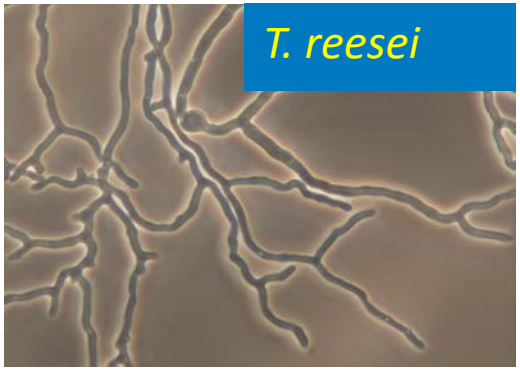
3—Technical Accomplishments/Progress/Results

Progress Towards Ideal Expression Strains

1) Single Cellulase Expression Strain "**Research Strain**"
2) Full Cellulase Suite Strain: "**Cocktail Strain**"

Desired Traits:

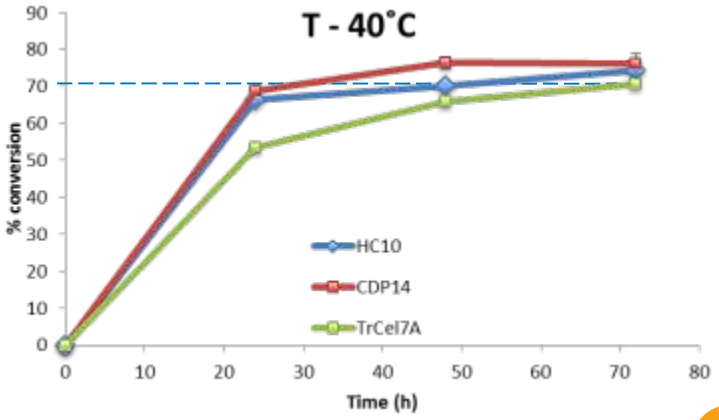
- Native CBHI Deleted ✓ ✓
- Endless Targeted Genetic Engineering
  - Recyclable selection markers Efficient gene-targeting for Knockouts/ins ✓ ✓
  - Deletion of NHEJ Pathway ✓
- High Enzyme Productivity
  - Multiple Protease knockout (Initiated)
  - Fundamental understanding of factors affecting Gene Expression (EMSL)
  - Elimination of "contaminating" proteins. (Have Been Identified)
  - Advanced Codon Optimization/Harmonization methods ✓ ✓
  - Increasing Secretory Capacity – enhanced ER?



3—Technical Accomplishments/Progress/Results

Megatron: Testing Nature's Cel7A Diversity

A pilot screen identified an improved Cel7A homolog (CPD14) from *Aspergillus oryzae*



2015 - Preliminary diversity screening

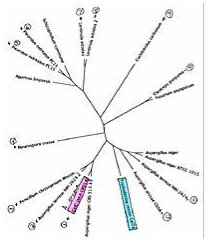
FY18 target!

2018 MS - Large-scale diversity screening

2016/17- Platform Development

- -NHEJ, 2A
- -codon usage
- -*lac* induction
- -Δprotease, ΔIAAO

2017 MS – Partial screen (12)



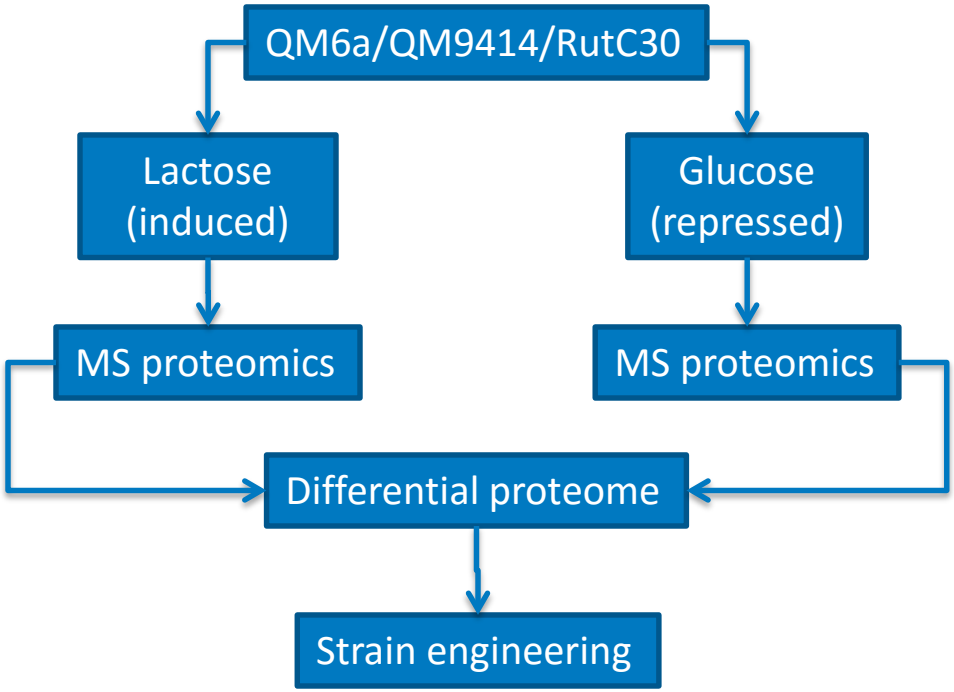
Synthesizing and Testing Expression of:

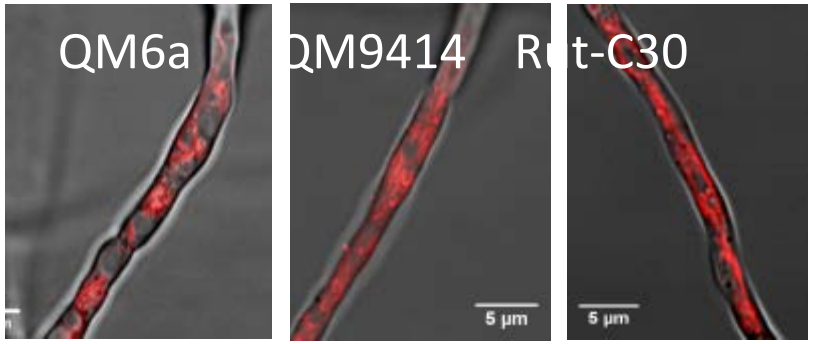
1) Twelve native sequences
2) Twelve "optimized" sequences
3) Twelve GoGREEN'ed sequences

3—Technical Accomplishments/Progress/Results

Understanding cellulase processing-EMSL collaboration

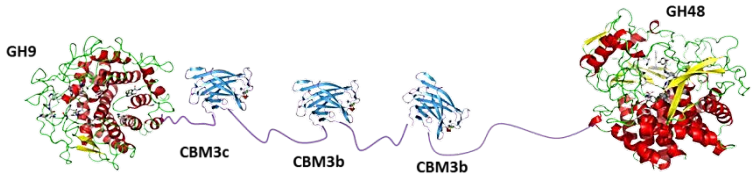
- Cel7A requires considerable post-translational modification
  - Co-induced processing enzymes (?)
    - Disulfide isomerases, chaperonins, glycosylation, trimming, signal processing, secretion targeting
    - Growth stage proteomic analysis under inducing/repressing conditions
  - Unfolded protein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



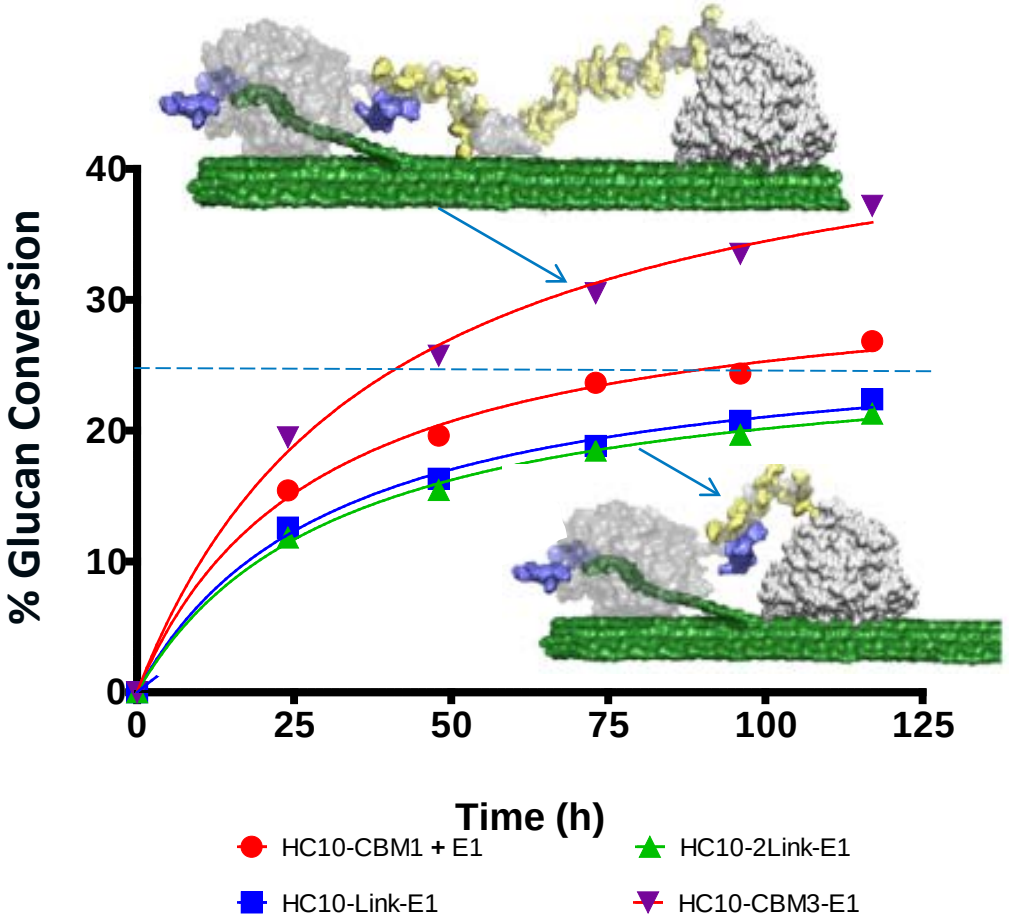


3—Technical Accomplishments/Progress/Results

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

2016 – Enzyme Engineering and Optimization (EEO)

Task 1. Enzyme Improvement

- Use enzyme engineering approaches for improving cellulases based on our significant record of published studies of fungal cellulase (Cel6A, Cel7A, Cel7B, GH61) kinetics and action at the molecular scale.
- Use a combination of rational design, random evolution, and gene-shuffling techniques with selective screening of candidate enzymes.

Task 2. Formulation and Application

- Screen and develop enzyme cocktails targeted to specific feedstock/pretreatment combinations by combinatorial mixing of single-activity expressing *T. reesei* strains, with successful formulations being introduced into a multi-enzyme *T. reesei* strain for pilot testing.
- New and improved enzymes will be mixed to identify synergistic properties in order to minimize protein loading while maximizing activity on selected feedstocks for 2017 targets. Some of this will work will include cellulosomes and multi-domain protein (i.e., CelA) synergy with free fungal enzyme systems.
- Enzyme synergy, feedstock-enzyme matching, high-temp hold, high-solids inhibition relief, and product inhibition relief studies will be conducted at NREL (1 to 500 mL scale), with scale-up by industry or NREL pilot-plant (est. 1 to 100L).

3—Technical Accomplishments/Progress/Results

2016–17 Task 2. Engineering Metabolic Pathway Enzymes

Evaluate variants and combine properties

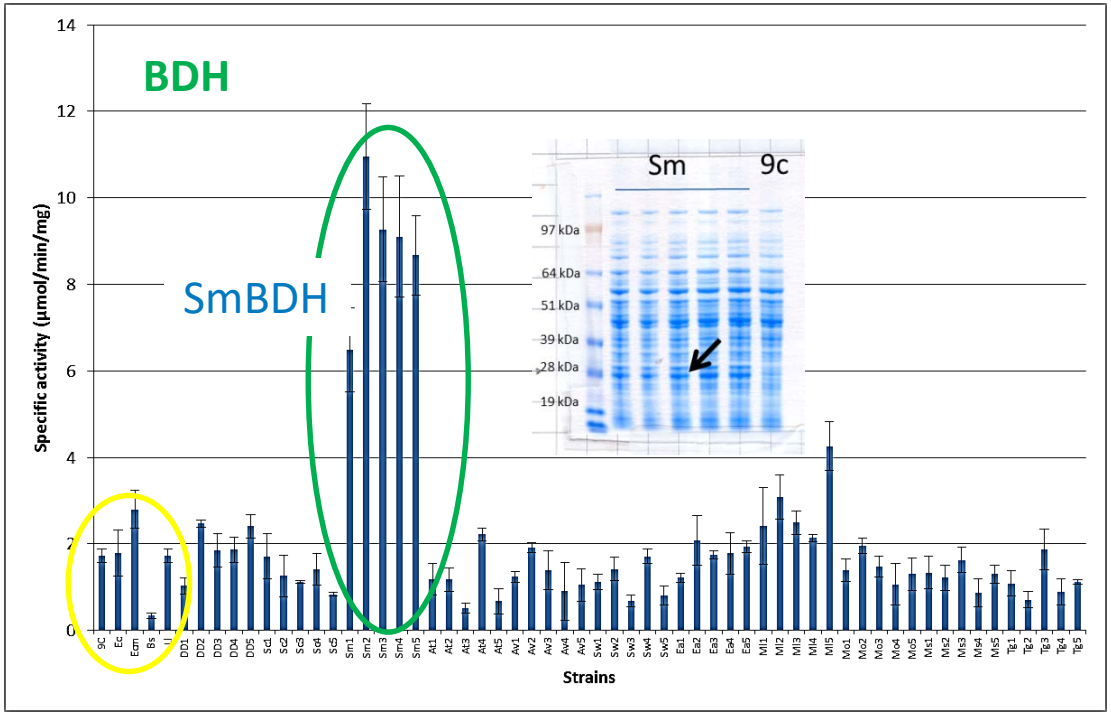
- In collaboration with the Lignin Utilization, Biological Upgrading of Sugars, and Targeted Microbial Development projects, leverage our experience in protein purification and enzyme characterization to critically evaluate metabolic enzymes (both wild type and engineered) needed for biofuels and bioproducts production.
- TMD has provided to us the highest priority problem to solve for FY16, the low activity of the heterologous Bhd and Aldc enzymes in the 2,3 butanediol pathway in *Zymomonas*.
- Determine the structure of critical pathway enzymes as needed (Bdh).
- Model the BDO production pathway (with BPMS).

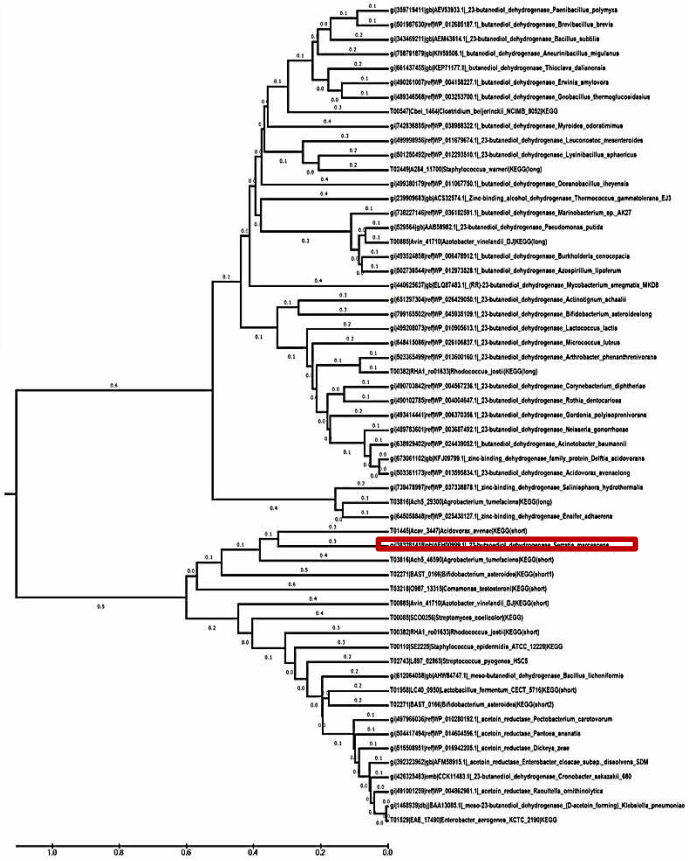
1. α-acetolactate synthase (Als)
2. α-acetolactate decarboxylase (Aldc)
3. acetoin reductase (2,3-butanediol dehydrogenase) - Bdh

3—Technical Accomplishments/Progress/Results

Bdh enzymes from natural diversity

- *S. marscens* was highest performer (kcat/Km)
- Forwarded to TMD for testing in *Z. moblis.*
- *Performance must be greater than E. cloacea enzyme.*
- Testing new round of clones currently.

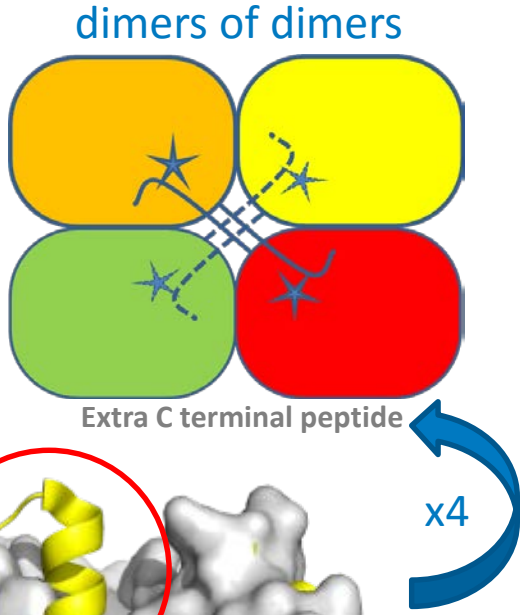


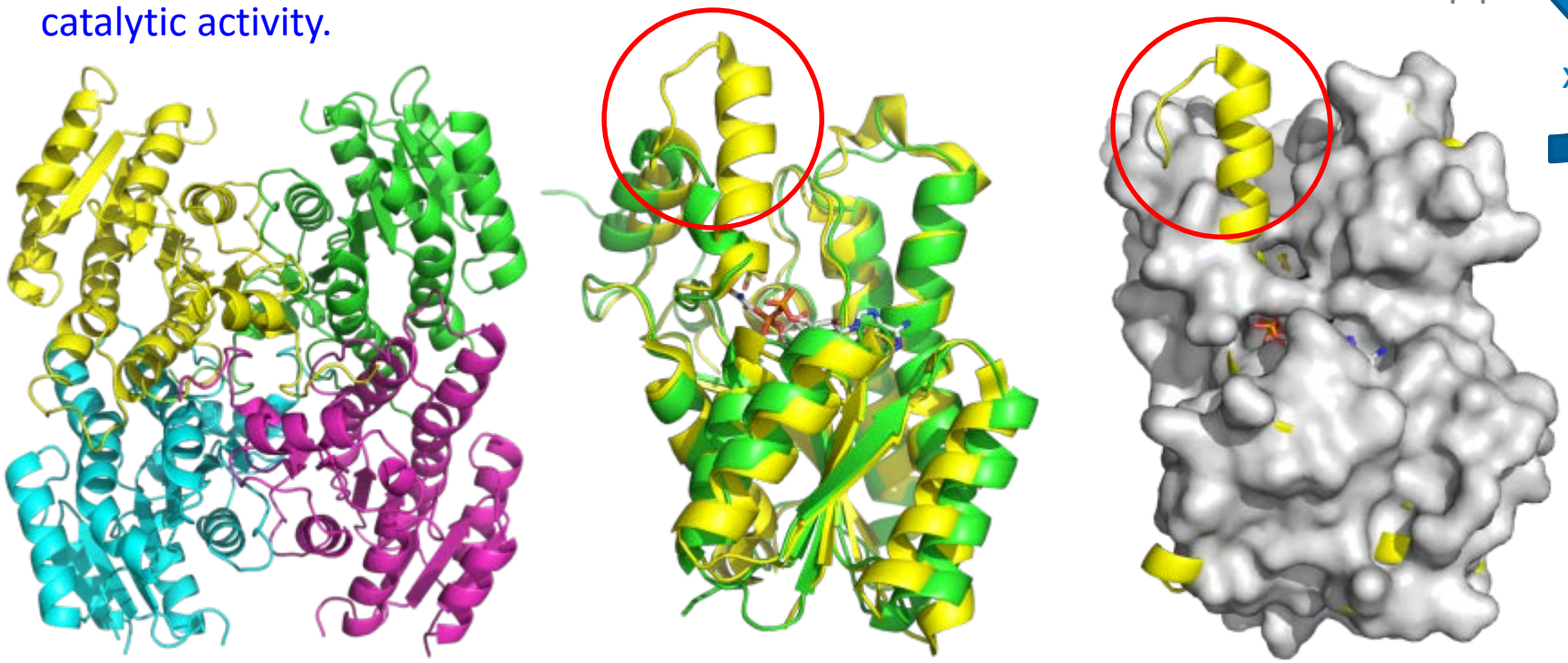


3—Technical Accomplishments/Progress/Results

Solved the Structure for Bdh at NREL

- Solved crystal structure of *Serratia marcescens* Bdh in apo-form at 2.3Å resolution.
- It is a homotetramer like other short-chain oxidoreductases.
- When compared to other know structures of S-acting Bdhs (*K. pneumoniae, B. saccharolyticum*) it shows more open access to the active site - likely explanation for the superior catalytic activity.



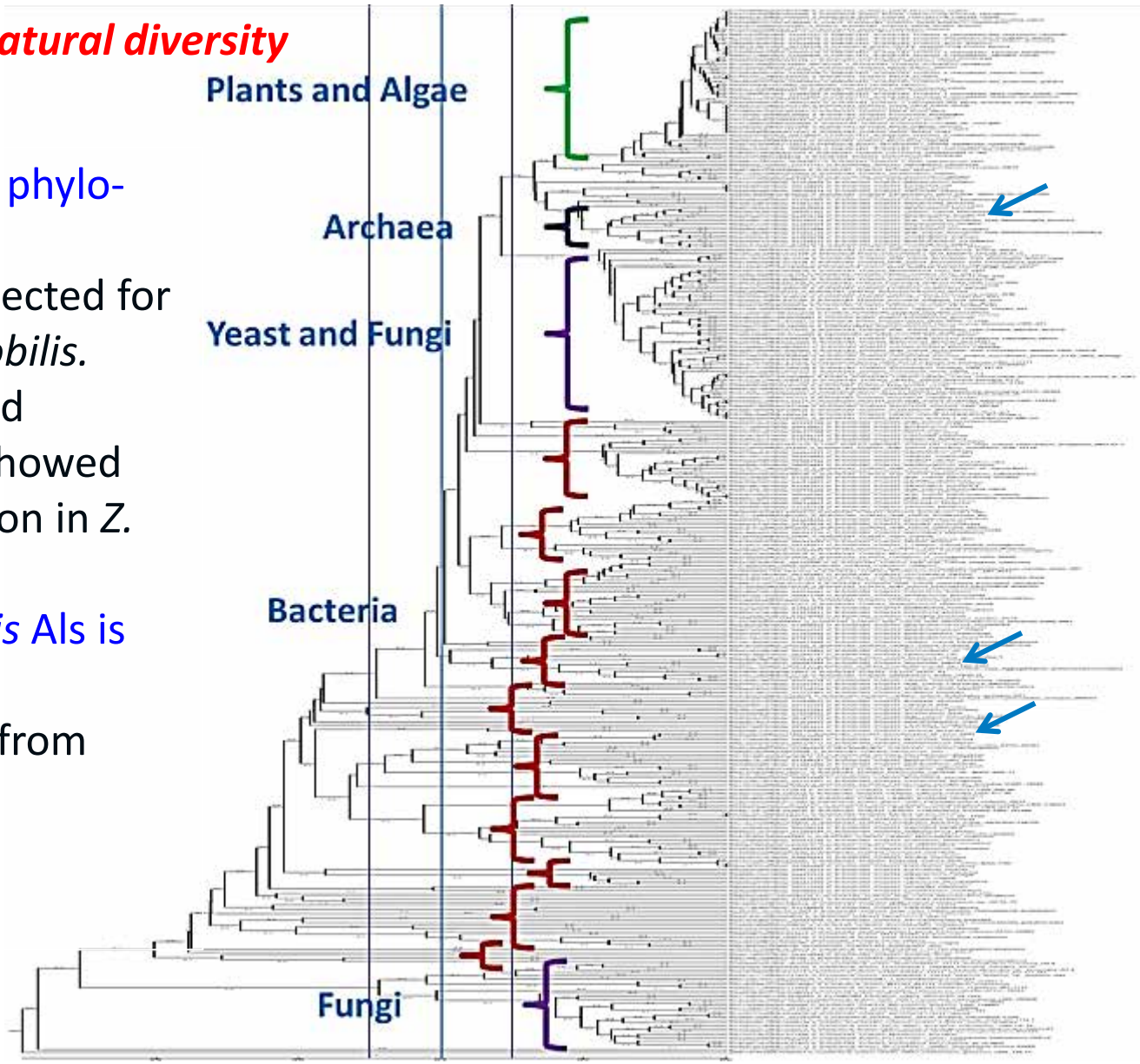


Yellow: *K. pneumoniae*, green/grey: *S. marcescens*

3—Technical Accomplishments/Progress/Results

Als enzymes from natural diversity

- Sought genes from phylo-diverse sources.
- Three Als genes selected for expression in *Z. mobilis.*
- *Methanoculleus* and *Synechocystis* Als showed successful expression in *Z. mobilis.*
- So far, the *B. subtilis* Als is best tested.
- Testing new genes from diversity currently.



4—Relevance

Overarching Goals

- Ensure that the 2022 TEA targets of 10 mg cellulase / g cellulose loading and BDO pathway enzyme improvements are met.

Importance

- All four 2022 BETO bioprocess scenarios require on-site hydrolysis enzyme production at lower than 2017 SOT cost and cellulase cost remains a barrier to commercialization.
- All four 2022 BETO bioprocess scenarios delivering new/improved advanced biofuels from pretreated biomass slurries will require significant metabolic pathway engineering. Lignin processing poses new challenges.
- The "knowledge based" approach we use—to first learn from nature and then engineer better enzymes—has become the science and technology standard in this field and will de-risk all processes going forward.

Relevance to BETO and the Biorefinery industry

- Public domain enzymes will enable the deployment of commercial biorefineries insulated from directional uncertainties in the enzyme business.
- Improved Cel7As decrease time-to-target, increase yield, reduce inhibitor formation, and reduce enzyme cost.
- We leveraged existing biochemical skills to metabolic pathway enzymes.

4—Relevance

- **Relevance to Industry**
  - BETO provides research that industry would not do (but needs).
  - New feedstocks, pretreatments, process schemes, and products are likely to require reformulation of enzyme cocktails used in the process.
  - Enzymatic conversion generates sugars for any microbial product platform and enabling/improving advanced metabolic pathways provides for new and/or more economical biofuels.
- **Advancing SOT**
  - The two leading commercial enzyme producers have noted our published technology in developing their commercial biomass enzymes.
  - Partnered with multiple industrial collaborators (DuPont, Novozymes, DSM, White Dog) to advance commercial enzyme technology.
  - We consider that our publications and presentations will 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: Conserved Codon mimicry for increased heterologous protein expression.
  - NREL ROI: Recombinant fungal multifunctional enzymes or genes based on these enzymes outperform the native fungal enzymes for biomass conversion.

4—Relevance

2016–17 NREL's Accomplishments Attracting Attention from Biofuels Companies

"Specifically, NREL's work related to improved, novel chimeric enzymes and/or enzymes with reduced of natural glycosylation may lead to the improved performance of current commercial cellulase formulations"

DUPONT

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biosciences.dupont.com

October 28, 2016

Michael Himmel, Ph.D.
National Renewable Energy Laboratory
15013 Denver West Parkway
Golden, CO 80401

Dear Mike,

DuPont Industrial Biosciences is one of the largest developers and manufacturers of industrial enzymes and has specific interest in improving performance and reducing the cost of enzyme formulations designed to convert lignocellulosic biomass to fermentable sugars. DuPont is a world leader in advanced biofuels and has been working to develop commercial enzymes for second generation biofuels; we introduced Accellerase™ enzymes for biomass-to-ethanol conversion and we are commissioning our commercial-scale cellulosic ethanol plant in Nevada, IA.

We have collaborated with NREL in developing second generation biofuels technology previously and we may have interest in further understanding NREL's progress in engineering improved fungal Cel7A cellobiohydrolases and other cellulose enzymes that may be useful for our plant and our customers. Specifically, NREL's work related to improved, novel chimeric enzymes and/or enzymes with reduced levels of natural glycosylation may lead to improving the performance of current commercial cellulase formulations. Such improvements may be of interest to us in the future. Moreover, we applaud NREL's structure-based approach to understanding mechanisms for improve cellulase enzyme performance – an undertaking well suited to the national labs.

We are watching the progress of this work with keen interest.

Kind regards,

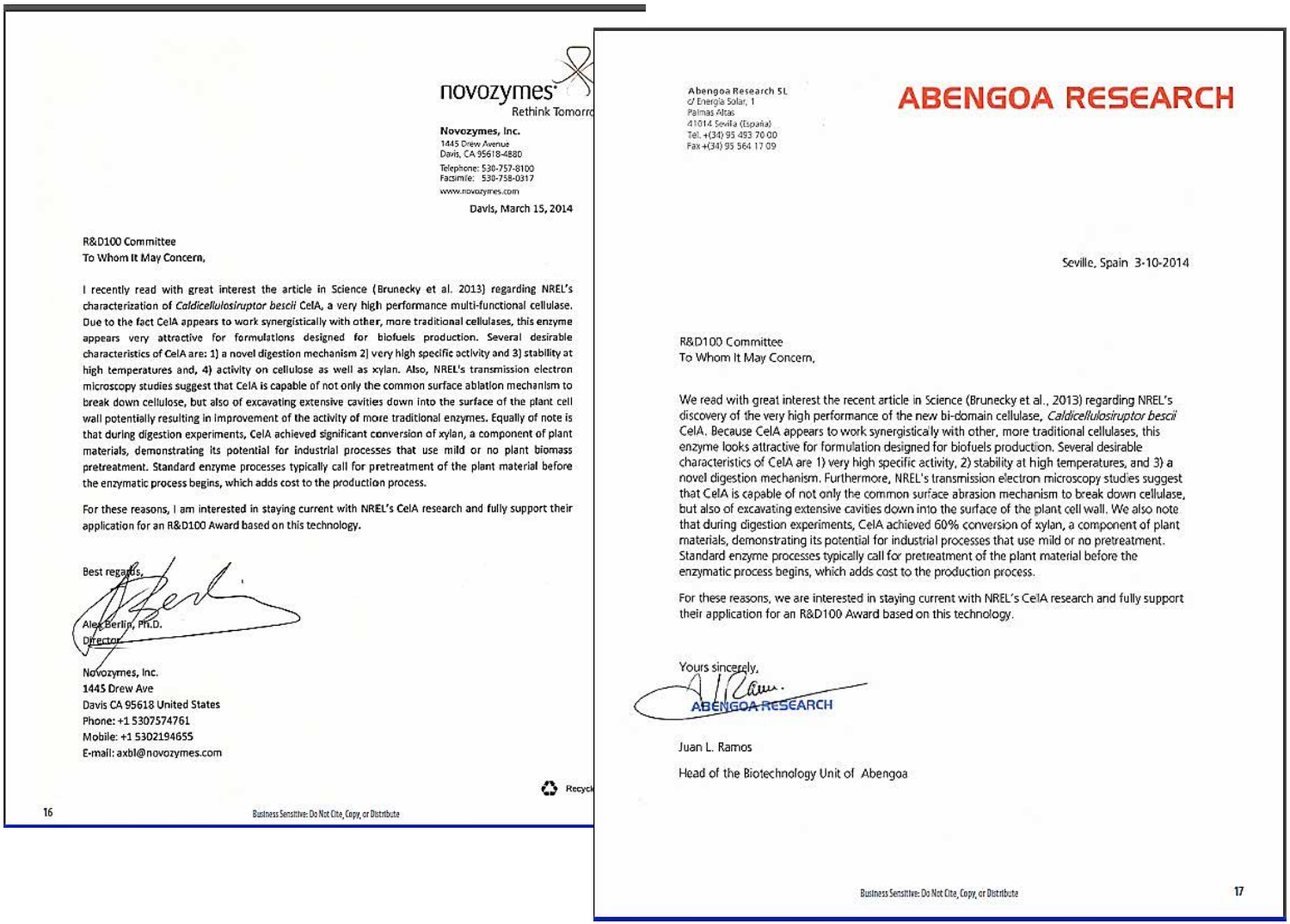
Michael Arbige

DuPont Industrial Biosciences

Vice President, R&D

4—Relevance

2014 – 15 NREL's Accomplishments Attracting Attention from Biofuels Companies



novozymes
Rethink Tomorro

Novozymes, Inc.
1445 Drew Avenue
Davis, CA 95618-4880
Telephone: 530-757-8100
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www.novozymes.com

Davis, March 15, 2014

R&D100 Committee
To Whom It May Concern,

I recently read with great interest the article in Science (Brunecky et al. 2013) regarding NREL's characterization of *Caldicellulosiruptor bescii* CelA, a very high performance multi-functional cellulase. Due to the fact CelA appears to work synergistically with other, more traditional cellulases, this enzyme appears very attractive for formulations designed for biofuels production. Several desirable characteristics of CelA are: 1) a novel digestion mechanism 2) very high specific activity and 3) stability at high temperatures and, 4) activity on cellulose as well as xylan. Also, NREL's transmission electron microscopy studies suggest that CelA is capable of not only the common surface ablation mechanism to break down cellulose, but also of excavating extensive cavities down into the surface of the plant cell wall potentially resulting in improvement of the activity of more traditional enzymes. Equally of note is that during digestion experiments, CelA achieved significant conversion of xylan, a component of plant materials, demonstrating its potential for industrial processes that use mild or no plant biomass pretreatment. Standard enzyme processes typically call for pretreatment of the plant material before the enzymatic process begins, which adds cost to the production process.

For these reasons, I am interested in staying current with NREL's CelA research and fully support their application for an R&D100 Award based on this technology.

Best regards,

Alex Berlin, Ph.D.
Director

Novozymes, Inc.
1445 Drew Ave
Davis CA 95618 United States
Phone: +1 5307574761
Mobile: +1 5302194655
E-mail: axbl@novozymes.com

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16

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Fax +(34) 95 564 17 09

ABENGOA RESEARCH

Seville, Spain 3-10-2014

R&D100 Committee
To Whom It May Concern,

We read with great interest the recent article in Science (Brunecky et al., 2013) regarding NREL's discovery of the very high performance of the new bi-domain cellulase, *Caldicellulosiruptor bescii* CelA. Because CelA appears to work synergistically with other, more traditional cellulases, this enzyme looks attractive for formulation designed for biofuels production. Several desirable characteristics of CelA are 1) very high specific activity, 2) stability at high temperatures, and 3) a novel digestion mechanism. Furthermore, NREL's transmission electron microscopy studies suggest that CelA is capable of not only the common surface abrasion mechanism to break down cellulase, but also of excavating extensive cavities down into the surface of the plant cell wall. We also note that during digestion experiments, CelA achieved 60% conversion of xylan, a component of plant materials, demonstrating its potential for industrial processes that use mild or no pretreatment. Standard enzyme processes typically call for pretreatment of the plant material before the enzymatic process begins, which adds cost to the production process.

For these reasons, we are interested in staying current with NREL's CelA research and fully support their application for an R&D100 Award based on this technology.

Yours sincerely,

ABENGOA RESEARCH

Juan L. Ramos

Head of the Biotechnology Unit of Abengoa

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17

5—Future Work

- *Explain what you plan to do through the end of the project (9-30-2018)*
  - Understand codon usage in GH7 cellobiohydrolase expression in *T. reesei.*
  - Apply codon understanding to expression and screening of >150 natural diversity Cel7s.
  - Identify co-induced helper proteins that enhance Cel7 expression in *T. reesei.*
  - Use proteomics and glycomics to determine optimal glycosylation/other PTM of Cel7 for enhanced activity.
  - Fine-tune best natural diversity Cel7s to reduce required enzyme loading for 2022 cost target.
  - Provide "tweaked" metabolic pathway enzymes to SDT for increased advanced biofuel production.
- *Highlight upcoming key milestones*
  - Fatty Alcohol Reductase Screening (3/31/2017) - provide genes, activity, and biochemical properties for 10 or more FAR genes to SDT for use in yeast-production of HCs
  - GH7 Natural Diversity Library Development (Go/No Go, 6/30/2017) - use diverse set of 5 Cel7 genes to optimize expression of Cel7 in *T. reesei.*
  - Diversity Screening of Cellobiohydrolases for Improved Sugar Yield from Biomass (9/30/2018) - clone, express, and screen >150 Cel7 genes in *T. reesei* and select top candidates for process testing.

5—Future Work

- *Address how you will deal with any decision points during that time (Go/No-Go Points)*
  - ✓ Finalizing development of the *T. reesei* cocktail strain is required in order to efficiently screen the natural diversity library.
  - ✓ If codon usage is key and if we do not solve it, we will fall back on classic expression enhancement and screening, which will be slower and less efficient.
  - ✓ Pursue enzyme diversity solutions first, and if insufficient, follow up with protein enzyme engineering approaches.
- *What is the remaining budget and is it sufficient to complete the remaining work? If not, what are the plans to accomplish the work?*
  - – FY17 budget is $1.6M, FY18 is expected to be slightly higher and we have planned accordingly.
  - – For reduced budget, we would need to drop further metabolic pathway enzyme development work and/or reduce the breadth of cellulase screening.

6—Summary

Overview

The goal is to provide technology that meets or exceeds cellulase enzyme loadings **of 10 mg cellulase/g cellulose** using enzyme engineering and formulation optimization strategies for 2022.

Approach

Employ a knowledge based approach which permits linking protein structure to performance which coordinates computational analysis and experimental validation.

Technical Accomplishments/Progress/Results

Reported the first activity improvement of Cel7A through enzyme/discovery engineering

- **~2.1 fold increase of ternary systems for non *T. reesei* enzymes**
- **~2.5 fold increase of Raptor chimeras comp. unlinked enzymes**

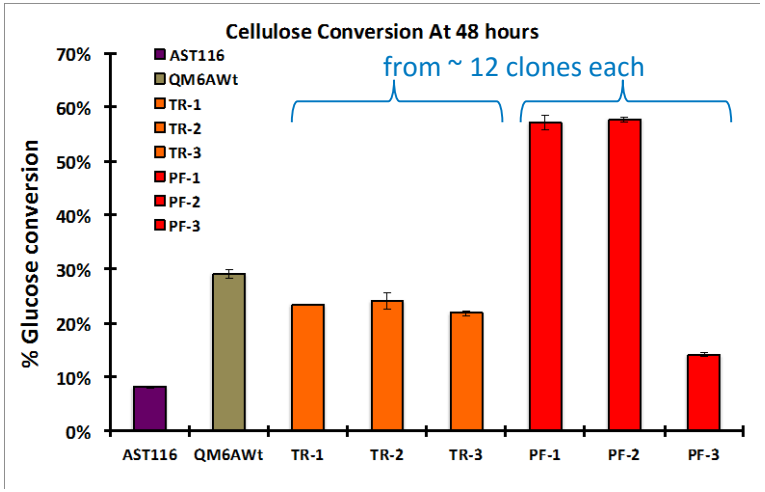
Importance of cellulase glycosylation for binding and activity

Significant process toward building a *T. reesei* "cocktail strain"

- **Constitutive (ENO) expression, 2A peptide, targeted gene insertion.**

Identified "most active from Nature" Bdh and Als enzymes

- **Transferred these genes to TMD for expression in *Z. mobilis***



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

The early stages of selection of glycosylation and Pf subdomain mutations suggested for future work in FY2015 are being accomplished in FY2017. Active mutations suggested by HTP yeast screening will be tested in FY2017. Express newly discovered, highly active Cel7 in *T. reesei* will be tested in FY2018.

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  - 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
Bryon Donohoe
Brandon Knott
Jeff Linger
Vladimir Lunin
Larry Taylor II
Todd Vanderwall
Todd Vinzant
Qi Xu
John Yarbrough

Additional Slides

Responses to Previous Reviewers' Comments

2015 Peer Review Report – Reviewers comments

- "This is as reasonable an approach as possible when trying to stay current with industry and be relevant. The challenge is keeping relevant with a commercial industry. However, NREL, with all of its expertise, experience and closeness to the materials has made a big contribution in this field over the years. Keep up the good work."
- "Lower enzyme loading and cost is an important goal. Huge effect on profitability in biochemical conversion facility."
- "This project could benefit greatly from a discussion of the work completed over the last 15 years and how it relates to progress as measured by a TEA."
- "This project continues to develop strategic insights and developments to improve the performance and cost of cellulases. The project is well connected with other BETO projects and applying innovative strategies. While the project clearly uses techno-economic analysis, the focus is very heavy on improving the biochemical activity of the enzymes. Recommend that the project put more focus on evaluating and improving the production economics of the new enzymes."
- "This is an extremely successful program with a lot of promise to improve cellulase enzyme performance and thereby decrease enzyme cost to the overall process. At some point, there should be some emphasis on reactor and process design to reduce enzyme loading and improve system performance. "

PI Response to Reviewer Comments

- Thank you for your comments. We have intentionally stayed away from commercial sector endeavors, such as large-scale enzyme production technologies and economics. Rather, we enable industry by tackling the difficult task of improving the key enzyme components in cellulase cocktails using rational design.

Also provide highlights from any Go/No-Go Reviews. None

Publications, Patents, Presentations, Awards, and Commercialization

- Publications
  - 25 publications from 2014 with impact factors above 6.0
- Patents
  1. 5 ROIs filed with DOE/NREL
  2. Software Copyright: *GoGREEN (Generate Optimized Genetic Rhythm for Enzyme Expression in Non-native systems).*
- Technology transfer or commercialization efforts
  - CRADA with Genencor-DuPont: Completed in 2013
  - Technical Service Agreement with DSM: Completed 2016
  - Collaboration with Novozymes: Co-authored manuscript – Under Review
  - Proposal submitted to 2017 EERE Small Business Venture by Avitar, Inc; NREL subcontractor if funded.
- Awards
  - 2014 Colorado State University, Department of Biochemistry & Molecular Biology, Distinguished Alumnus – Himmel

EEO: 2014-2016 Publications

- "*Acidothermus cellulolyticus* Glycoside Hydrolase Family 74 Structure at 1.82 Å," Markus Alahuhta, William, S. Adney, Michael, E. Himmel and Vladimir, V. Lunin, **Acta Crystallographica** Section F, 69(12), 1335-8 (2013).
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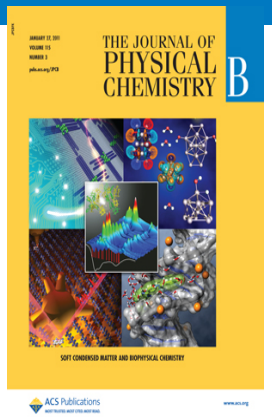

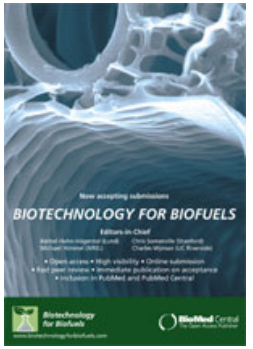

EEO: 2014-2016 Publications

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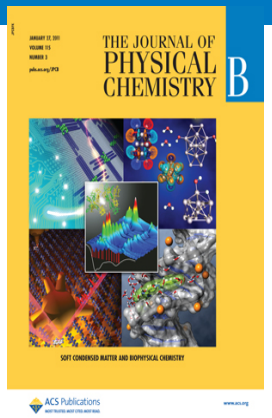

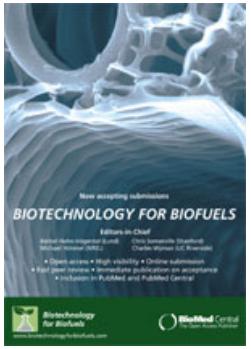

EEO: 2014-2016 Publications

- "Heterologous Protein Expression in *Hypocrea jecorina*: A Review and New Developments" Stephen R. Decker, Larry E. Taylor, Sarah E. Hobdey, Michael E. Himmel, Kara Podkaminer, Arjun Singh, and Jeff Linger, **Biotechnology Advances**, (2014). In Press.
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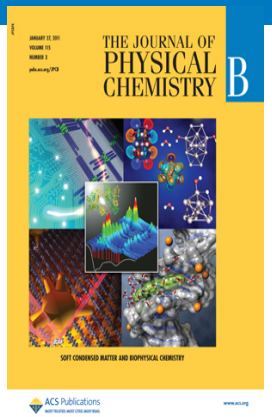

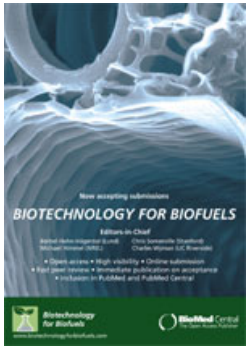

EEO: 2014-2016 Publications

- "Investigation of the Mechanisms of Biphasic Xylan Hydrolysis During Dilute Acid Pretreatment of Corn Stover", Ashutosh Mittal, Todd B. Vinzant, Roman Brunecky, Stuart K. Black, Michael E. Himmel, David K. Johnson; **RSC Green Chemistry** 17, 1546-1558 (2015).
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- "Distinct roles of N- and O-glycans in cellulase activity and stability," Antonella Amore, Brandon C. Knott, Sarah E. Hobdey, John M. Yarbrough, Michael F. Crowley, Michael E. Himmel, Stephen R. Decker, Gregg T. Beckham, Larry E. Taylor II, **EES** (2017) Submitted.
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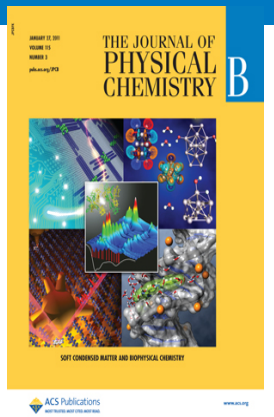

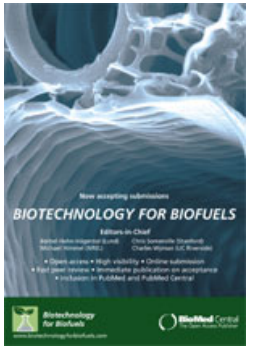

2016 EEO Milestones

Milestone Name/Description	End Date	Type
Task 1. Develop a strain of *S. cerevisiae* or *Yarrowia* spp. for HTP cellulase expression and optimization	3/30/2015	Quarterly Progress Measure (Regular)
Task 1. Generate >12 *T. reesei* strains expressing phylogenetically diverse or mutant cellulases.	6/30/2015	Quarterly Progress Measure (Regular)
Task 1. Test 5000 rCel7A expressing yeast colonies using the new HTP yeast screening method for enhanced activity or biochemical properties. Select top 10-20 strains for further analysis.	9/30/2015	Annual Milestone (Regular)
Task 1. Reduction in enzyme cost using improved or new enzymes or synergistic combinations (~$0.10 /g cellulose loading). SMART milestone.	10/1/2017	Annual Milestone (Regular)
Task 2. Assemble library of pretreated substrates. Evaluate with base fungal cocktail to identify needed activities and publish the results.	12/30/2014	Quarterly Progress Measure (Regular)
Task 2. Report analysis of novel enzyme addition schemes to reduce protein loading.	6/30/2015	Quarterly Progress Measure (Regular)
Task 2. Report the predicted partial attainment of a one-third reduction in enzyme cost (~$0.13 g/g cellulose loading) based on the combined approaches taken by the project. Share results with enzyme companies and publish. SMART milestone.	9/30/2015	Annual Milestone (Regular)
Task 2. Report improved high-solids digestion activity of a) 25% increase in rate of hydrolysis with 20% solids, b) 10% increase in final yield of sugar at 20% solids, or c) maintain rate and yield of 1% solids with 10% or higher solids loadings. SMART milestone.	9/30/2016	Annual Milestone (Regular)
Task 1. Publish new HTP yeast screening method and share results with industrial collaborators.	12/30/2015	Quarterly Progress Measure (Regular)

2017 EEO Milestones

Milestone Name/Description	End Date	Type
Impact of Cel7A Glycosylation on Binding to Biomass- Generate glycosylation-deficient and/or additive mutants of *T. reesei* Cel7A and evaluate them for changes in activity, stability, and binding to biomass, lignin, and/or cellulose. Mutations will be made in existing *T. reesei* expression host(s). Mutations will be made in catalytic, linker, and binding modules in both *N*- and *O*-glycosylation sites. Mutations will include single-site as well as combinatorial multiple-site mutants for each type. Deliverable: *Submission of journal article detailing methods, results, and interpretation of how these impact Cel7A engineering and industrial usage.*	12/31/16	Regular Quarterly
Fatty Alcohol Reductase Screening- Screen fatty alcohol reductase enzyme diversity for increased fatty alcohol production in *Lipomyces starkyii*. Genome mining of known microbial genomes for homology to FAR from nature will be used to generate a cladogram for relatedness classes. These genes will be divided into 10 or more clades. One gene from each clade will be selected for synthesis and expression in *Lipomyces starkyii*. Deliverable: *Provide activity numbers and gene construct(s) to TMD to test for improvement in FA production*.	3/31/2017	Regular Quarterly (Joint w/ TMD)
GH7 Natural Diversity Library Development- Design, have synthesized, and express in *T. reesei* a natural diversity set of 5 or more Cel7 genes in their native, optimized, and harmonized codon variants. Measure protein production rate and titer for each expression construct and determine specific activity and biochemical properties for each variant. Deliverable: ***Go*** *decision moves forward synthesis of 50-150 additional Cel7 natural diversity variants and subsequent screening in T. reesei.* ***No Go*** *triggers retool/redesign of expression system and gene design or abandons natural diversity screening efforts.*	6/30/2017	Regular Quarterly
Enhanced *Trichoderma reesei* Heterologous Cellulase Expression System- Develop enhanced *T. reesei* heterologous expression host and vector system. Specific strain traits likely to be included are: non-homologous end joining deletion (*mus53*), protease deletions/knock outs (3 or more), Isoamyl Alcohol Oxidase or other interfering protein deletion(s), and auxotrophic, counter-selectable phenotypes (*pyr4*, *amdS*). Vector properties should include strong inducible (*cbh1*) or constitutive (*eno*, other) promoters, *cbh1* locus integration flanking sequences, and recyclable markers (*pyr4*, *amdS*). The exact combination of above traits will depend on phenotypic characteristics of the combined mutations. Examples given of specific genes modifications given above are the most likely to be successful, but may change. Enhanced strain should have improved expression of heterologous proteins (2-fold or higher than parent strain) and simplified gene product purification (fewer chromatography steps). Deliverable: *T. reesei expression host and transformation vector system available for either public dissemination or licensing (pending NREL Legal)*	9/30/2017	Annual SMART

2016 EEO Go/NoGo Milestone

Name	Description	Criteria	Date
Determine value of single-enzyme production strains of *T. reesei* and potential need for additional single enzyme strains	(3/30/2016) Standard *Trichoderma* cellulase production is induced by a variety of compounds, each of which induces a different set of proteins, some required, some extraneous. By decoupling induction, we can produce single enzyme activities that can be mixed and matched to a given pretreated feedstock. By eliminating unneeded proteins, the total protein loading can be reduced and protein production is more efficient. If these criteria for continuing have not been met, we will multi-enzyme systems and/or new enzyme discovery options.	Three goals must be met in order to continue work on single-enzyme *T. reesei* strains. 1) a single enzyme production system with high production (>5 mg/L of heterologous protein) and minimal background protein (<20% of total secreted protein) needs to be established. 2) At least 12 heterologous proteins must have been expressed and assayed on at least 3 biomass feedstocks, and 3) a combinatorial screen capable of screening complex enzyme mixes (up to 5 activity components) must have been established and demonstrated to quantify biomass conversion on a % yield basis for glucan and xylan. Successful establishment of these three goals along with at least one mix demonstrating activity greater than Ctec2 (on a sugar yield/g protein basis) on pretreated biomass.	3/31/2016

2017 EEO Go/NoGo Milestone

Go/No-Go Decision (80 character limit)	Description	Criteria	Date	Actions
Proceed with Cel7 natural diversity expression screening in FY18	Design, synthesize, and test 5 new Cel7A genes under 3 codon strategies for enzyme rate, titer, and activity in *T. reesei* in order to screen a much larger natural diversity library for better activity and/or improved biophysical properties	For Go Decision: 1. Development of codon harmonization algorithm 2. Selection of screening vector 3. Contracted Gene Synthesis and cloning from external supplier 4. Determine best codon strategy	06/30/2017	Go: proceed with natural diversity enzyme testing in FY18 with existing codon strategy No Go: Use results to redesign and retool expression parameters or abandon natural diversity and focus on engineering TrCel7A improvements