DOE Bioenergy Technologies Office (BETO)
2015 Project Peer Review

Advanced Enzyme Deconstruction/ Enzyme Engineering & Optimization

Date: March 23-27
Technology Area Review: Biochemical Platform

Principal Investigator: Michael E. Himmel
Task Leader: Steve Decker
Organization: NREL

On many slides, the slide notes section has important additional information

Goal Statement

- *Present the goal of this project and describe how the project fits with the goals/objectives of the Technology Area and ultimately the goals/objectives of the DOE Bioenergy Technologies Office, and the overall bioenergy industry.*
  - The goal of this project is to ensure that cellulase enzyme loadings can be reduced to the target of 10 mg/g cellulose on DA corn stover using modern enzyme engineering and formulation optimization strategies. The attainment of this overall enzyme cost is consistent with BETO's technoeconomic evaluations for meeting the 2017 biomass conversion target. Given that cost effective biomass conversion to sugars is a common theme for many conversion processes, the overall bioenergy industry also benefits from this work.
- *Explicitly state relevance and tangible outcomes for the United States.*
  - It is important that the U.S. shows leadership in the alternative fuels arena. This work will permit new science and technology to be developed in the U.S. which will benefit U.S. industry, as well as academic researchers seeking longer term solutions.
  - Ensure U.S. jobs and energy infrastructure

Quad Chart Overview

Timeline

- Project start date - 2001
- Project end date - 2017
- Percent complete - 82%

Barriers

- Barriers addressed
  - Bt.C Biomass Recalcitrance
  - Bt.D Pretreatment Chemistry
  - Bt.G Cellulase Enzyme Loadings

Budget

	Total Costs FY 10 – FY 12	FY 13 Costs	FY 14 Costs	Total Planned Funding (FY 15-Project End Date
DOE Funded*	$3.5M $3.4M $3.0M	$3.3M	$1.9M	$4.8M ($1.6M fy15)
Project Cost Share (Comp.)*	0	0	0	0

*for FY 10, 11, and 12

Partners

- Internal: BC Process Modeling and Simulation Project; Targeted Microbial Development Proj.
- Subcontractors: Colorado State University, NIST, Cornell University, Vanderbilt University, Weizmann Institute of Science, UC Berkeley
- Collaborators: Zhongping Tan (CU), Jhih-Wei Chu (Berkeley), Charles Brooks III (Univ Michigan), Lee Makowski (ANL), Paul Langan (LANL), Sunney Xie (Harvard), Parastoo Azadi (UGA CCRC), Scott Baker (PNNL), Simon Craig (Univ York, UK), Jerry Stahlberg (Swedish Univ Ag Sci), John McGeehan (Univ Portsmouth, UK), Joel Kaar (CU-Boulder), Vincent Eijsink (NMBU), Kiyo Igarashi (U Tokyo), Michelle O'Malley (UCSB)
- DOE FOA to Genencor, subcontracted to NREL under CRADA ($150,000/3 years)

1 - Project Overview

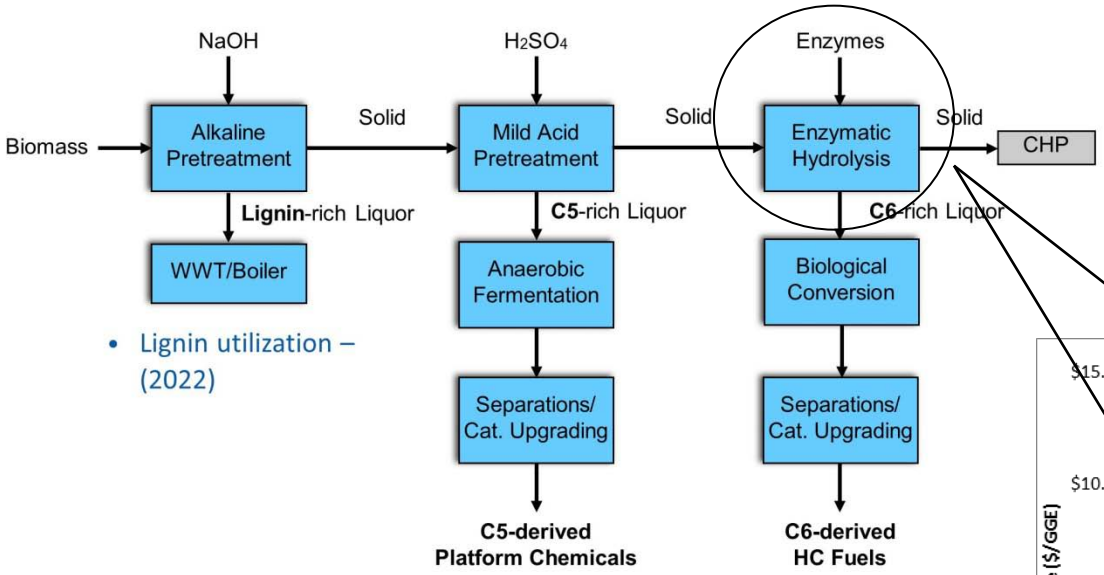
- For the past decade, we have used enzyme engineering approaches for improving enzymes and formulations critical to BETO's cost goals.
  - Our strategy is to employ a knowledge based approach which permits linking protein structure to performance.
  - To this end, we have pioneered the close coordination of computational analysis and experimental validation for cellulases.
- We will supply improved enzymes for the near term advanced biofuels platform and provide real-time coupling to feedstock and pretreatment efforts.
  - In contrast to other DOE supported work on cell wall degrading enzymes, our work will be targeted specifically to the needs of the BETO processes where ~one third the fuel cost is due to cellulases.
  - Our work will reduce the enzyme loading from the 2014 SOT of 14 mg/g cellulose to the target of 10 mg/g cellulose for 2017.
- To meet these aggressive goals, we have established working relationships with the enzyme companies (Genencor-DuPont, DSM, and Novozymes)
- To support BETO's longer term goals and mission, we will also work to ensure that the cost and protein loading targets for 2022 are met.
  - We will target reformulation of enzyme cocktails to respond to changes in feedstocks (new genetically engineered crops from BER, BES, ARPA-E), pretreatment chemistries and severities, and targeted products/co-products.

2 – Approach for FY15 (Technical)

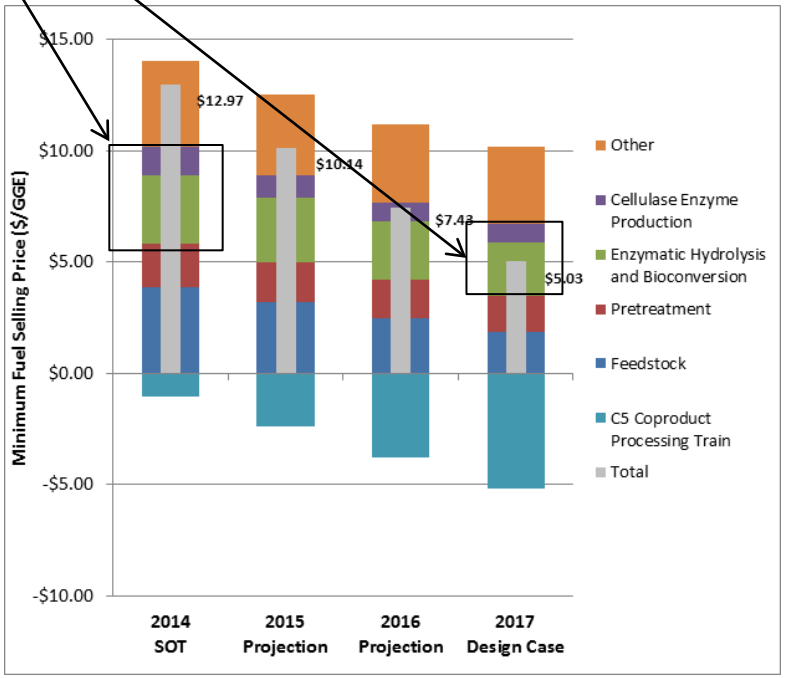
- *Describe critical success factors ….*
  - Meeting BETO's 2017 technology targets for cellulose conversion to glucose, cellulase cost, and solids concentrations for pretreatment.
  - Meet near term and future technical targets for BETO's 2022 goals for advanced biofuels. To some extent, these targets are still not fully defined, but reduced conversion cost is likely to be critical.
- *Explain the top 2-3 potential challenges ….*
  - To show that current commercial enzyme cocktails can be reformulated for improved performance and reduced cost.
  - The introduction of new processing steps in 2014 (DDR, deacetylation and disk refining and lignin centric pretreatments) may require new base line studies on optimizing biomass saccharification and fermentation.
  - To show that the sciences of biomass pretreatment, enzyme digestion, and microbial transformation to fuels can be understood sufficiently to enable industry to be successful in the near term.
  - To integrate properly the new fast pace of information coming from the DOE centers charged with conducting biomass conversion research (DOE EFRCs and BRCs).

2 – Approach (Technical)

2017 Biochemical Pathway – Biological Conversion



Enzyme Loading	Onsite Cellulase Production Cost	Total MFSP
10 mg/ gram cellulose	$.82/GGE	$5.03/GGE
20 mg/gram cellulose	$1.54/GGE	$5.78/GGE



	Design Report Basis	2014 SOT	2015 Projection	2016 Projection	New 2017 Target
Minimum Fuel Selling Price ($/GGE, 2011$)	***$5.10***	**$12.97**	**$10.14**	**$7.43**	**$5.03**
Feedstock Contribution ($/GGE, 2011$)	*$1.76*	$3.88 [1]	$3.20 [1]	$2.47 [1]	$1.87 [1]
Conversion Contribution ($/GGE, 2011$)	***$3.33***	**$9.09** [1]	**$6.93** [1]	**$4.97** [1]	**$3.16** [1]
RDB Fuel Yield (GGE/dry ton)	*45*	18	20	20	22
Succinic Acid Yield (lb/dry ton)	*NA*	197	206	232	270
Pretreatment/Separation					
Solids Loading (wt%)	*30%*	30%	30%	30%	30%
Xylan to Xylose (including conversion in C5 train)	*>73%*	73%	75%	78%	78%
Hydrolysate solid-liquid separation	*No*	Yes	Yes	Yes	Yes
Xylose Sugar Loss (into C6 stream after acid PT separation)	*NA*	5%	4%	2.5%	1%
Enzymes					
Enzyme Loading (mg/g cellulose)	*10*	14	12	10	10
Enzymatic Hydrolysis & Bioconversion – C6 Train					
Total Solids Loading to Hydrolysis (wt%)	*20%*	15%	15%	17.5%	17.5%
Enzymatic Hydrolysis Time (d)	*3.5*	3.5	3.5	3.5	3.5
Hydrolysis Glucan to Glucose	*90%*	77%	85%	85%	90%

Overview: Technical Objectives for 2015

- Multi-fold improvement in Cel7 activity for formulation in highly active commercial cellulases is needed for biomass conversion (targeting reduction in cellulase loading to 10 mg/gram cellulose by 2017).
- Understanding the role glycosylation plays in cellulase functionality and stability and the ability to manipulate/control these glycosylation profiles to improve biomass conversion.
- Identify and evaluate new enzymes for an ability to enhance the performance of *T. reesei* cocktails (non commercial).
- Evaluate new process scheme scenarios for enhanced saccharification of biomass cellulose
- Development of a yeast high throughput screening system for identification of superior Cel7s.

Cel7A is being used industrially now

~5,000 tons of enzyme/plant/year with a 2,000 ton/day plant

BETA RENEWABLES

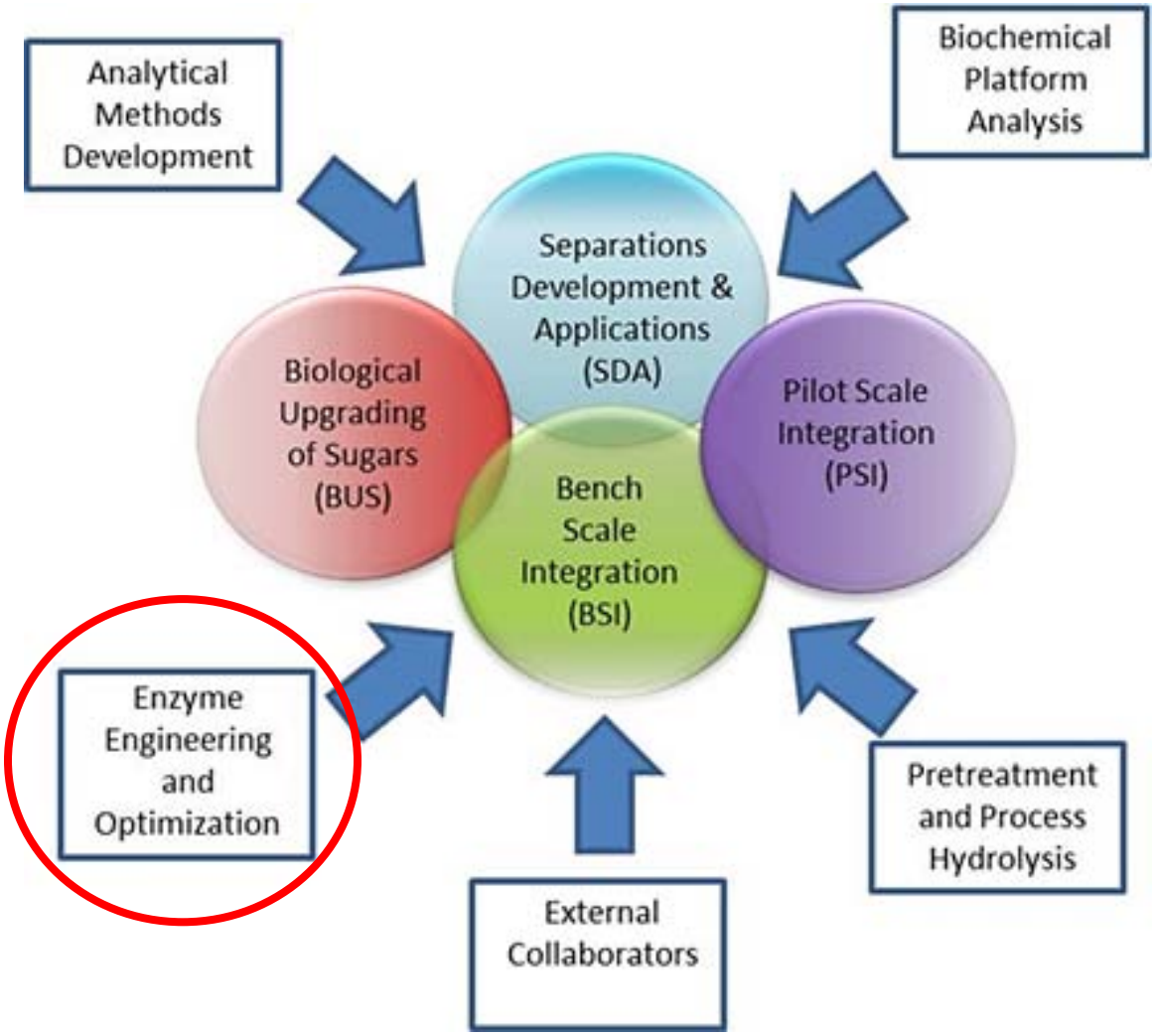
novozymes

DUPONT®

2 – Approach (Management)

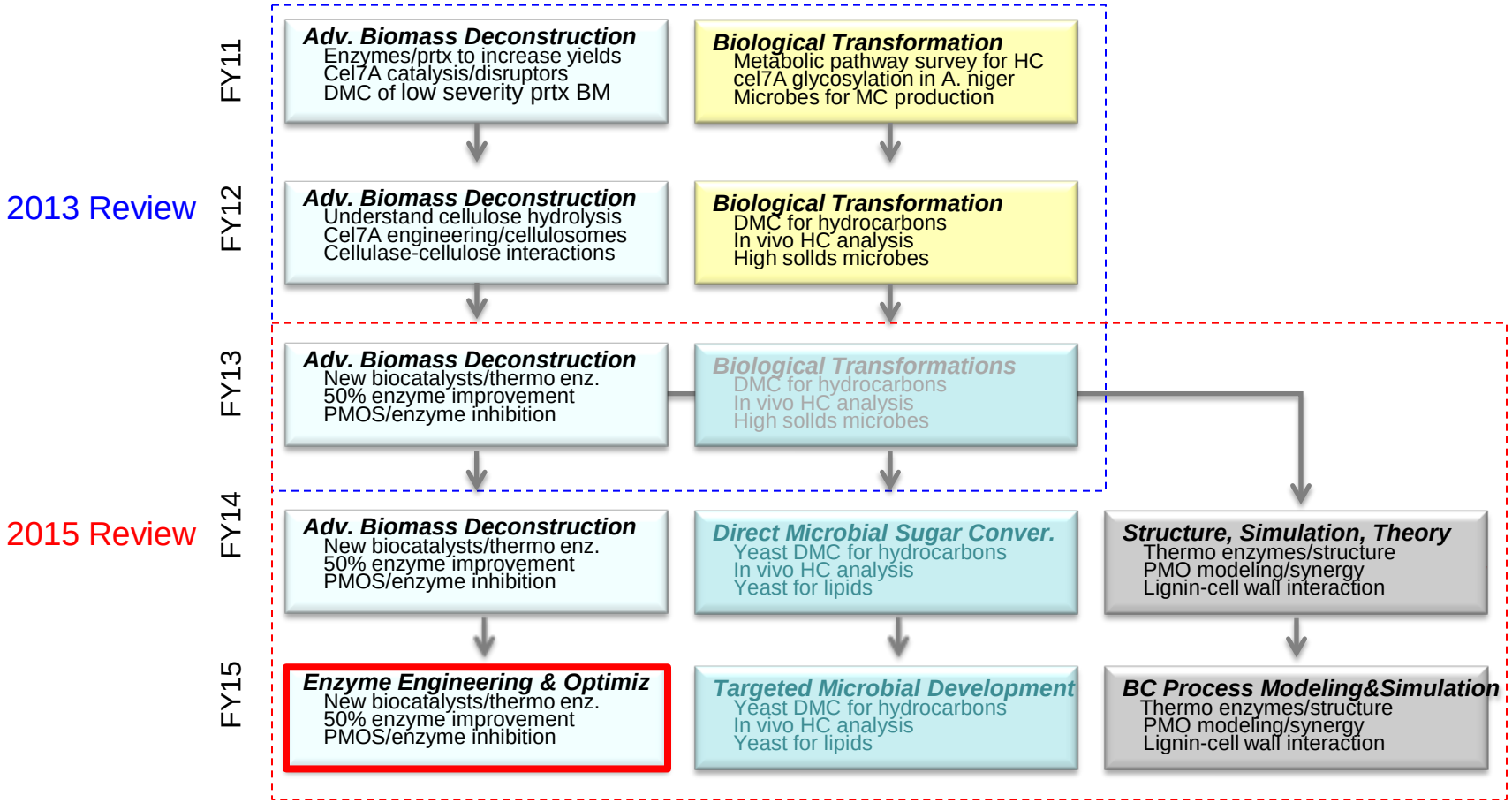
- *Describe critical success factors ….*
  - Meeting BETO's 2017 technology targets for cellulose conversion to glucose, cellulase cost, and solids concentrations for pretreatment.
  - Disseminating technical achievements in a timely manner (publications/presentations)
  - Meeting near term and future technical targets for BETO's 2022 goals for advanced biofuels. To some extent, these targets are still not fully defined, but reduced conversion cost is likely to be critical.
- *Explain the top 2-3 potential challenges ….*
  - For the 2017 cost goals, good communication with the enzyme industry is important and thus we maintain relationships with 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 strain and enzyme formulation would be more robust !
- *Emphasize the structure of your approach….*
  - Our approach is to use a basic RACI internal management plan which assigns work to researchers based on the milestone structure.
  - Milestones are Regular, Quarterly, and Annual, with several SMART milestones identified. One Go/No-Go decision is planned for FY2016.

2 – Approach (Management)



2 – Approach (Management)

Evolution of Enzyme Engineering & Optimization



2 – Approach (Management)

2014 – Targeted Conversion Research – Rational Design – Advanced Biomass Deconstruction

There were three major focus areas for the ABD Task in FY14

T. reesei Cel7 Subdomain Engineering (informed by natural variants)

Improving and understanding Cel7 function remains critical. We are examining multiple aspects of Cel7 structure/function relationships including glycosylation, diverse evolutionary sources, point mutations, and subdomain swapping between diverse Cel7s. This rational approach will give us insight into understanding how this critical enzyme functions.

Evolutionary Engineering of Cellulases

Random evolution is a powerful force in enzyme improvement, however it requires an effective screen in order to harness its potential. Until now, there has not been an effective means of rapidly evaluating improvements in the tens of thousands of variants generated by these methods. We are developing such a screen in order to capture the potential of random evolution as a tool for the improvement of Cel7.

Multi-domain/Accessory Cellulases Expressed in Fungi

Bacterial and fungal biomass hydrolyzing machinery are very different; however, they are being shown to have highly synergistic behavior in degrading biomass. We are exploring the potential to functionally express these complex bacterial enzymes in fungi in order to develop a single enzyme production system with higher activity than either parent system.

3 – Technical Accomplishments/ Progress/Results

2014 ABD Engineering the Key Cellulase - Cel7A

2014 key milestones

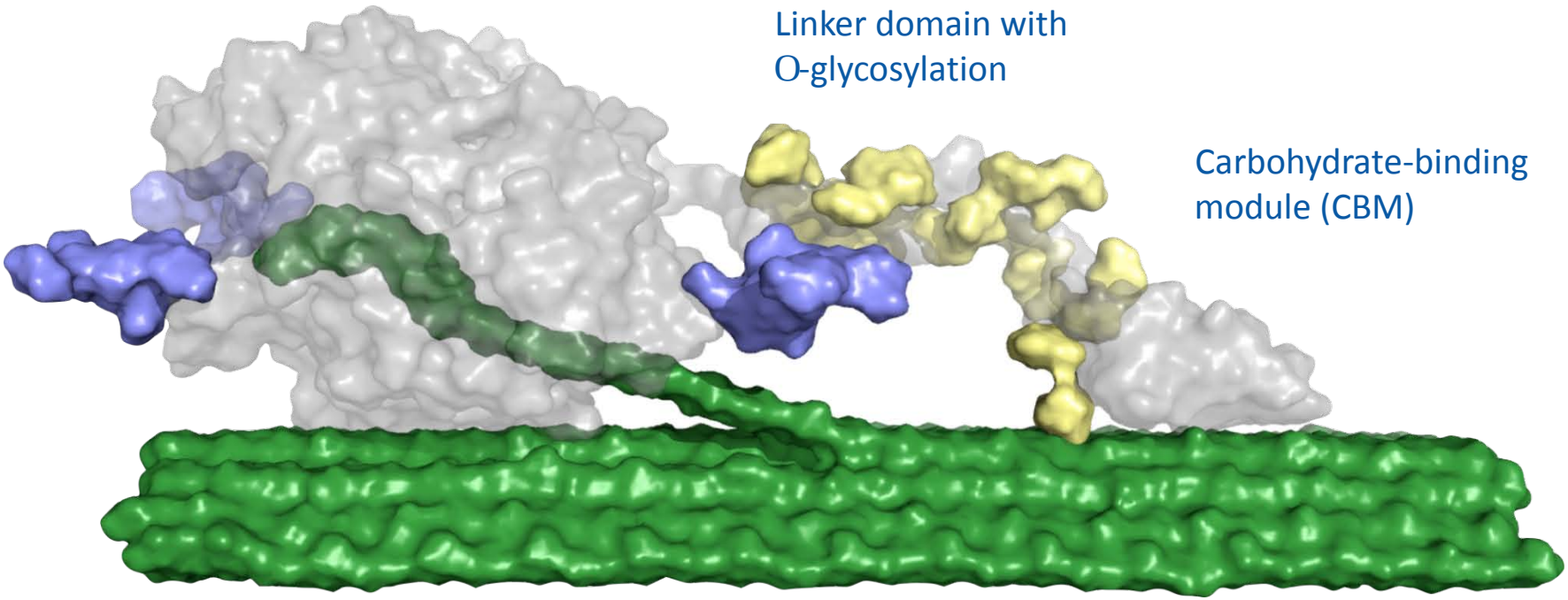
Q1	12/31/2013	Regular	**Structural Design Mutations for Improving Cel7 Cellulases (Joint with Structure, Simulation & Theory Project)**
Q4	9/30/2014	Regular	**Improved Cellulases for Biomass Conversion**

- Understand structure/function of catalytic domain
- Understand structure/function of CBM and linker
- Natural variants of GH7 cellobiohydrolases exhibit different properties
  - pH tolerance
  - thermal stability
  - binding affinities
  - activity on biomass
  - glycosylation patterns

✓ Requires a rigorous purification and testing scheme

3 – Technical Accomplishments/ Progress/Results

Cellulases are typically multi-modular glycoproteins (Cel7A)



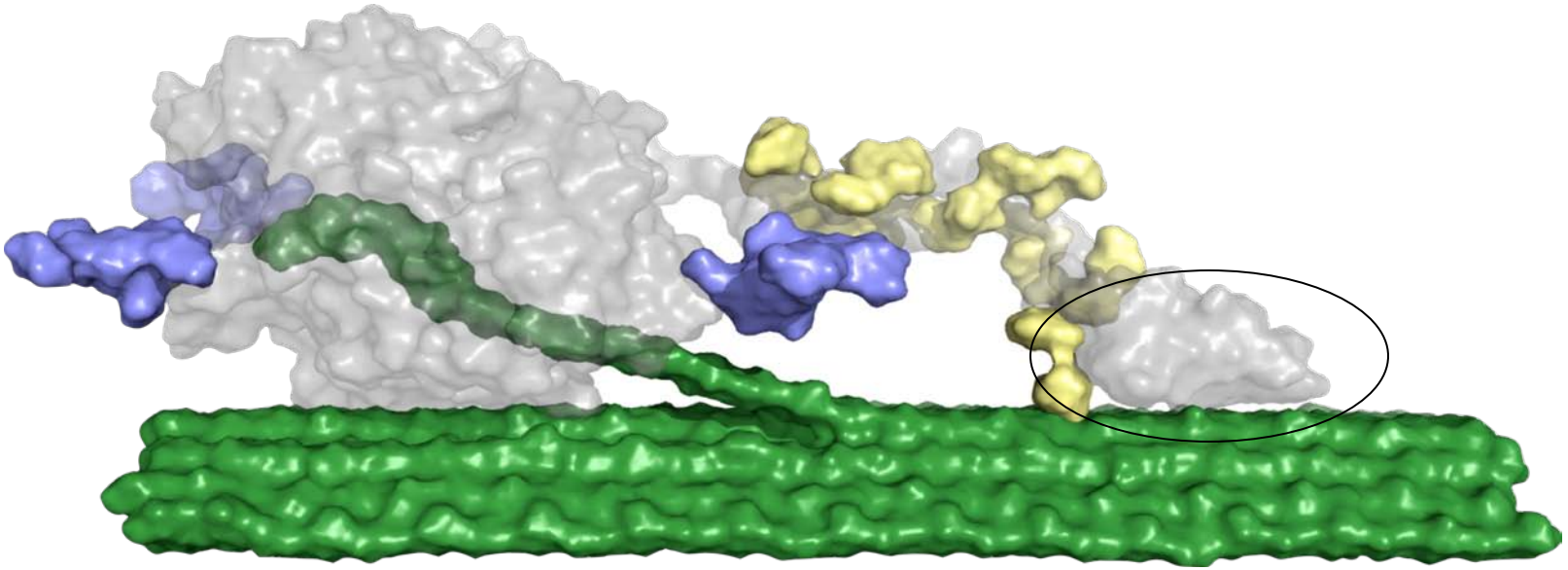
Single most important enzyme in industrial lignocellulosic biomass conversion

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

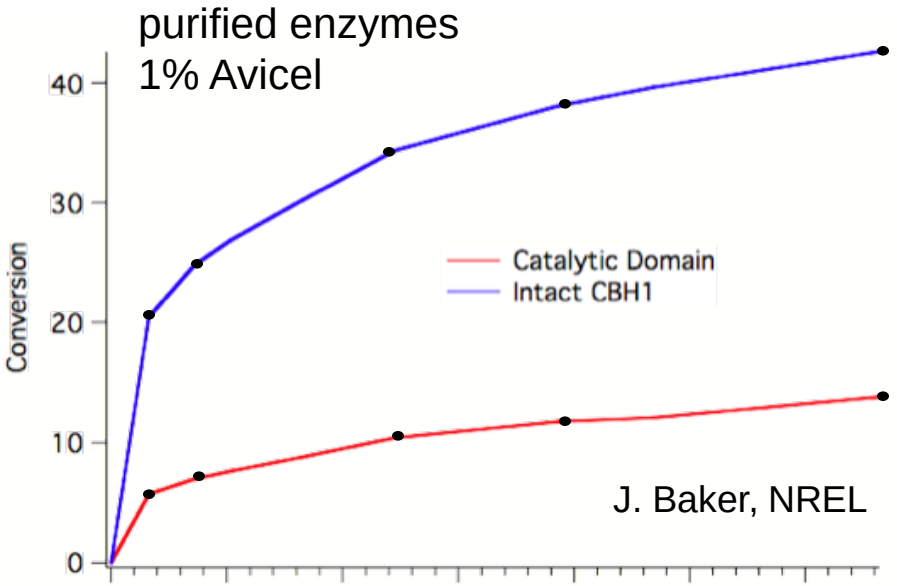
Function(s) of the carbohydrate-binding module

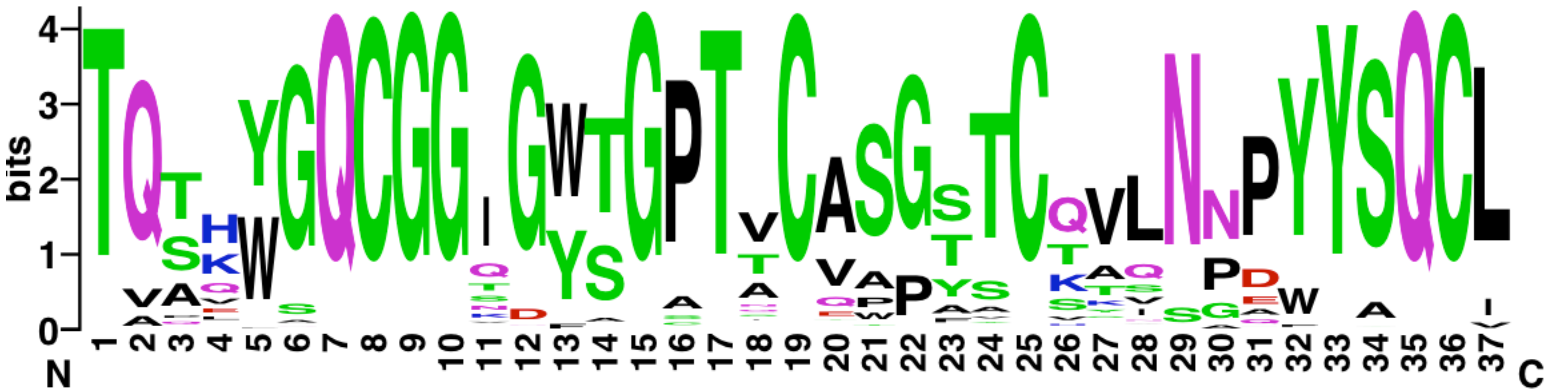


3 – Technical Accomplishments/ Progress/Results

What are the functions of CBMs and how can we engineer them?

- Increases proximity of enzyme to substrate
- Targets specific cellulose faces
- ✓ Higher CBM binding affinity leads to higher activity
- Many of the residues are highly conserved

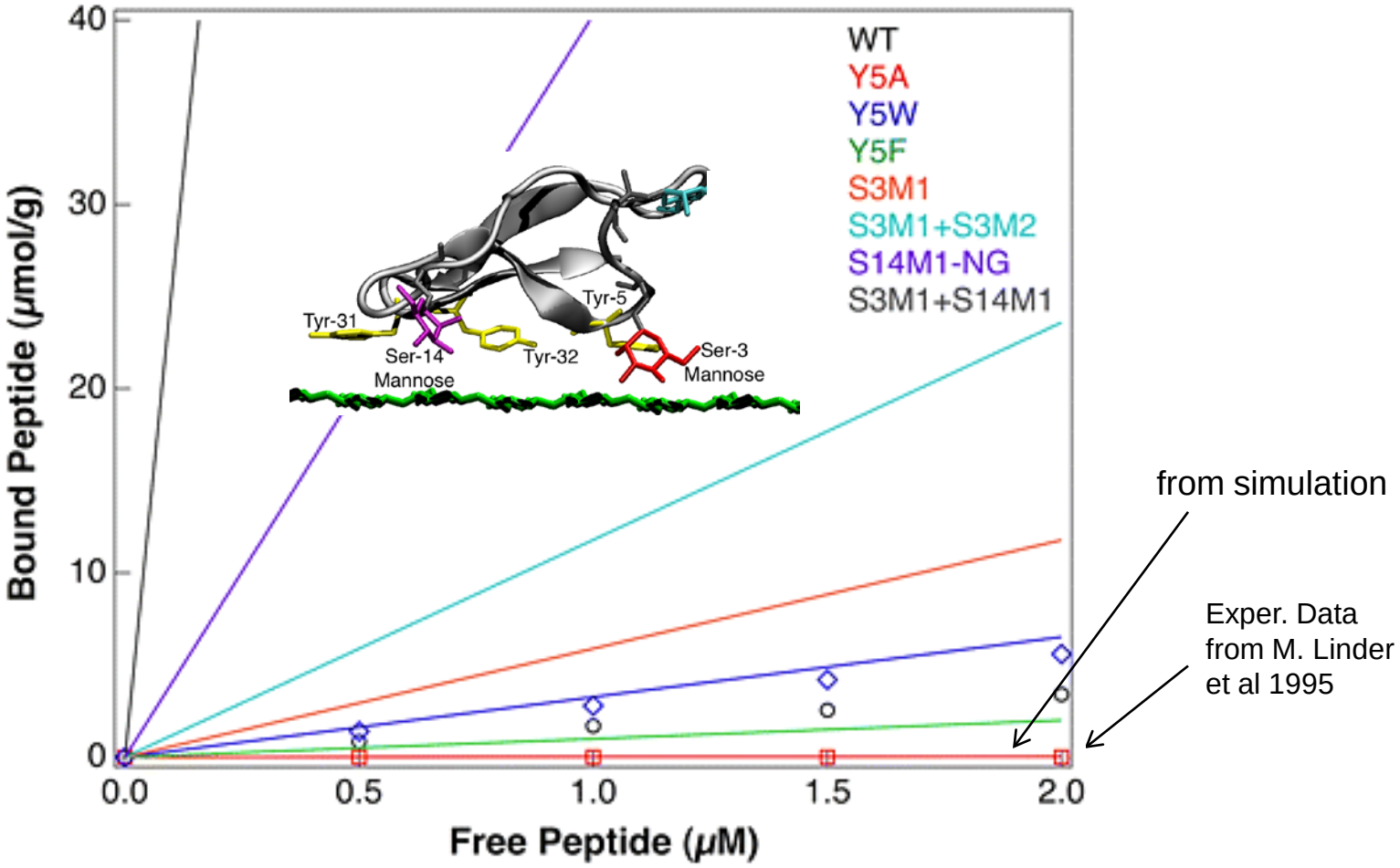




3 – Technical Accomplishments/ Progress/Results

CBM simulations predict improved binding by 2 to 140 fold over non glycosylated form

One *O*-glycan can alter the CBM binding affinity quite dramatically



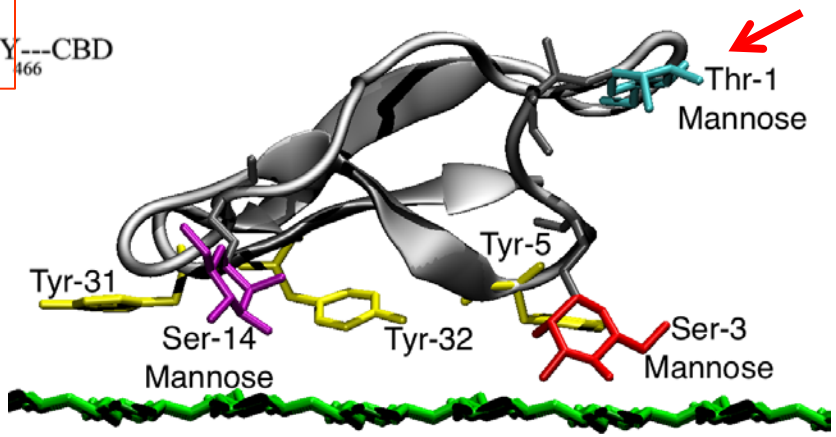
C.B. Taylor, M.F. Talib, C. McCabe, L. Bu, W.S. Adney, M.E. Himmel, M.F. Crowley, G.T. Beckham, J. Biol. Chem. 2012

3 – Technical Accomplishments/ Progress/Results

2014 - CBM engineering improves binding affinity by a factor of 8

Eur. J. Biochem. *256*, 119−127 (1998)

Not recognized that Thr1 is part of CBM before 2014



CBMs made by chemical synthesis
(Tan, CU Boulder)

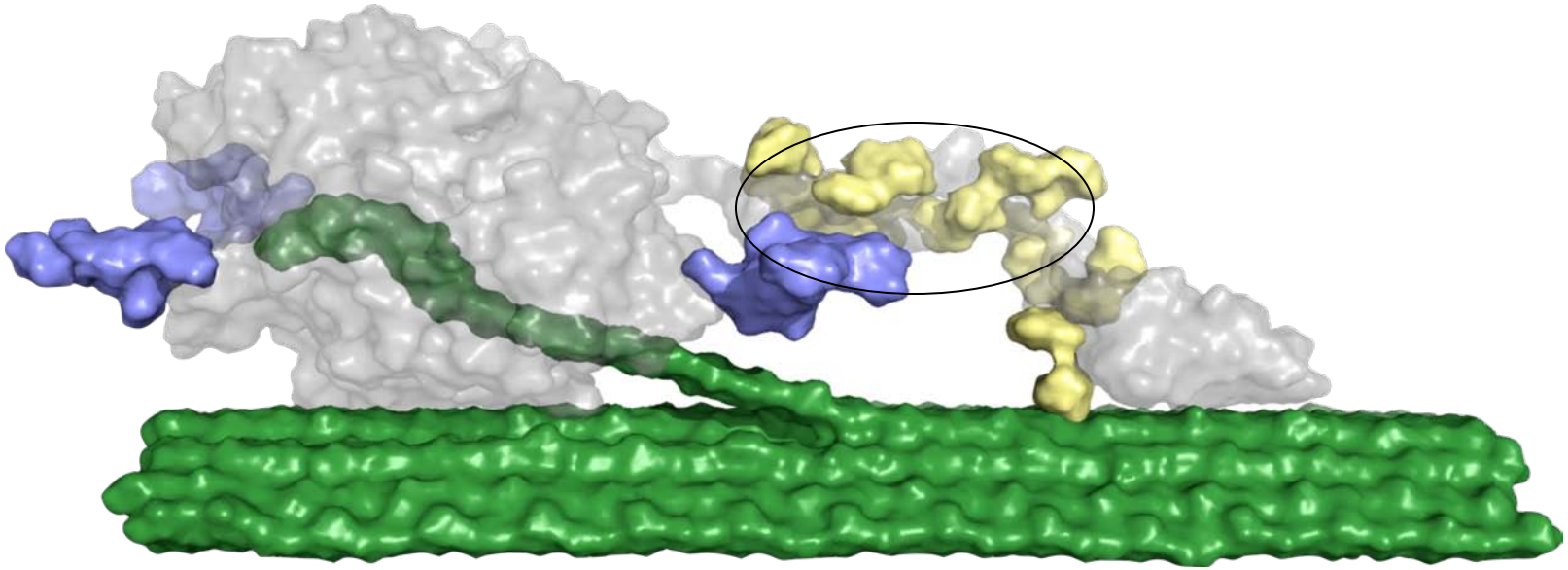
Thr-1	Ser-3	Ser-14	T_m (°C)	K_A (μM^{-1})	Half Life (hr)
H	H	H	62	0.095	0.18
Man	Man	H	72	0.21	3.31
Man	Man	Man	74	0.75	3.12

- T_m increase of 10-12 °C
- Binding affinity increase of 2 to 8-fold
- Proteolytic half life increase of 17-fold

L. Chen *et al.*, *PNAS* 2014

3 – Technical Accomplishments/ Progress/Results

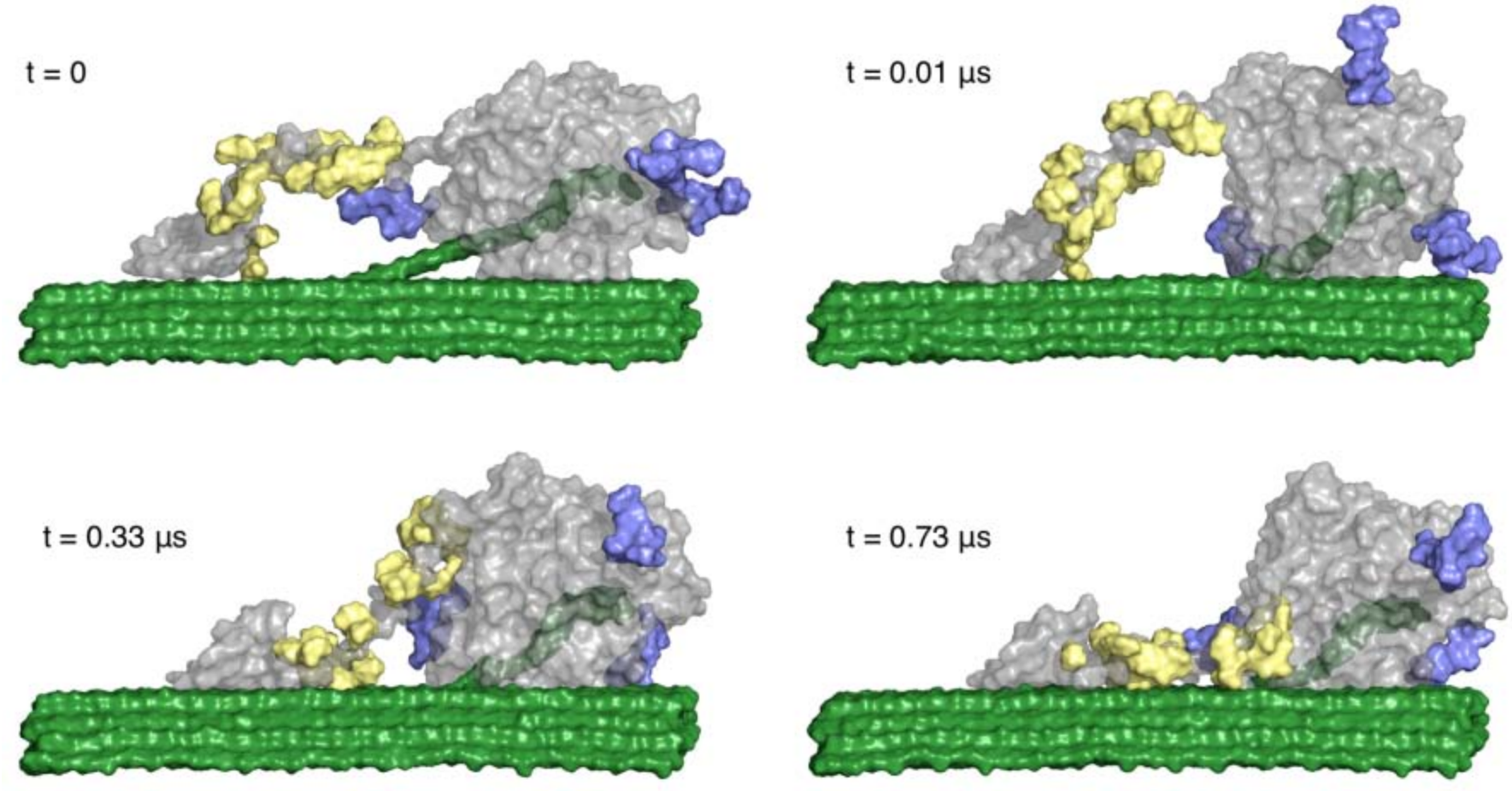
What does the linker do?



3 – Technical Accomplishments/ Progress/Results

What does the linker do when the enzyme is complexed on cellulose?

A: It deforms and binds strongly to the surface!



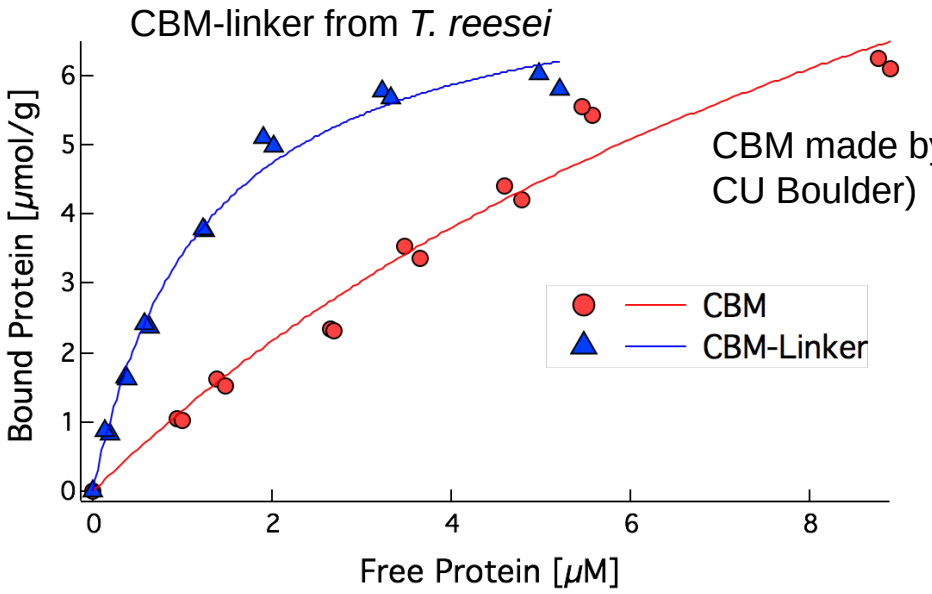
MD simulations using Kracken

C.M. Payne et al., PNAS, 2013

3 – Technical Accomplishments/ Progress/Results

Experimental binding affinity measurements

Linker imparts significant binding affinity improvements over CBM alone



CBM made by chemical synthesis (Tan, CU Boulder)

$$[B] = \frac{B_{max}[F]}{K_{ads}^{-1} + [F]}$$

System	K_{ads} [μM]$^{-1}$	B_{max} [μmol/g cellulose]
CBM	0.08 ± 0.02	15.4 ± 2.9
CBM-Linker	0.80 ± 0.07	7.7 ± 0.3

C.M. Payne et al., PNAS, 2013

2 – Approach (Management)

2015 – 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.
- Move toward 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; however, expansion of this new enzyme paradigm discovery has been curtailed due to reduced resources.
- 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

Task 1 2015 EEO Engineering the Key Cellulase - Cel7A

Task 1. Generate >12 *T. reesei* strains expressing phylogenetically diverse or mutant cellulases. (Joint with the BC Process Modeling & Simulation Project)	6/30/2015	Quarterly Progress Measure (Regular)
Task 2. Report the predicted partial attainment of a one-quarter 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)

- Evaluate variants and combine properties
  - Engineer subdomain swaps
  - Engineer mutants with reduced glycosylation
  - Examine natural sources - Other fungi, *Daphnia* spp. and social amoebae
  - Note: Challenges of microheterogeneity - differences in protein glycosylation produced in a single strain
- Correlate structural differences with observed properties
- Requires a robust and native-like expression system
  - Began developing *T. reesei* expression systems in 2013
  - NREL constitutive promotor system demonstrated in 2014
    - This system is now published and shared within the R&D community

3 – Technical Accomplishments/ Progress/Results

Penicillium funiculosom Cel7A – why so active?

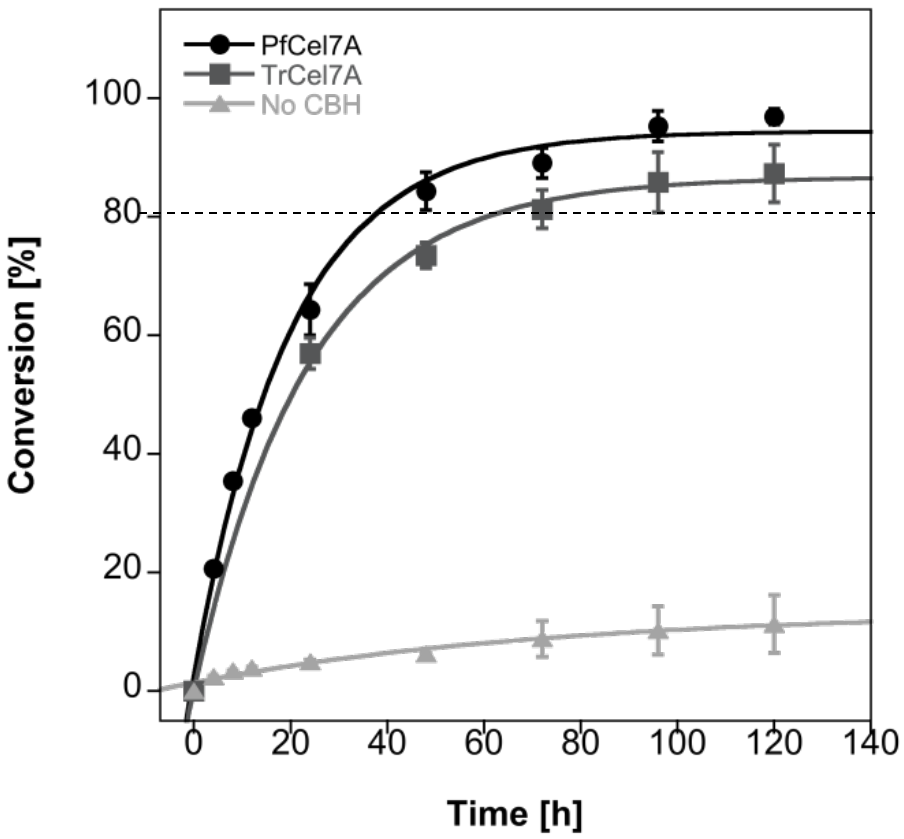
We discovered Pfun Cel7A has higher T^{o}_{opt} /higher specific activity on PCS than *T. reesei*

- Structure determined at NREL
- Differences are found in several regions of the protein (active site and subdomains)
- Amenable to industrial productivity

➢ Swapping 12 *P. funiculosom* subdomains into *T. reesei* and vice-versa

➢ Testing *P. funiculosom* Cel7A in Cel7A delete strains of *T. reesei*

DOE NREL US Patent

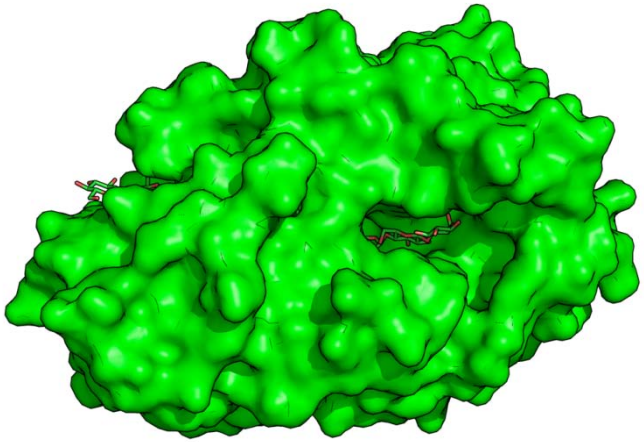


Cel7A + EI on Avicel

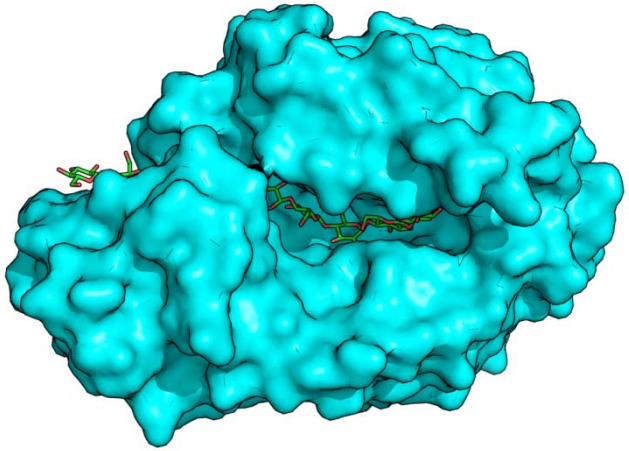
3 – Technical Accomplishments/ Progress/Results

Overall structure comparison with other key GH7s

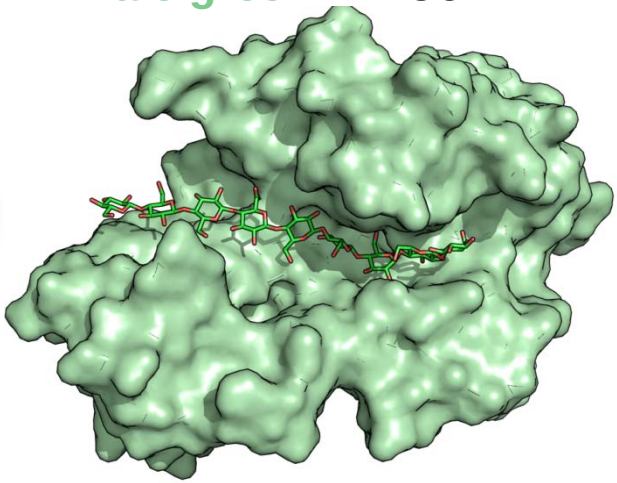
Green = *Tr*Cel7A (4C4C)



Cyan = *Pc*Cel7D (1GPI)

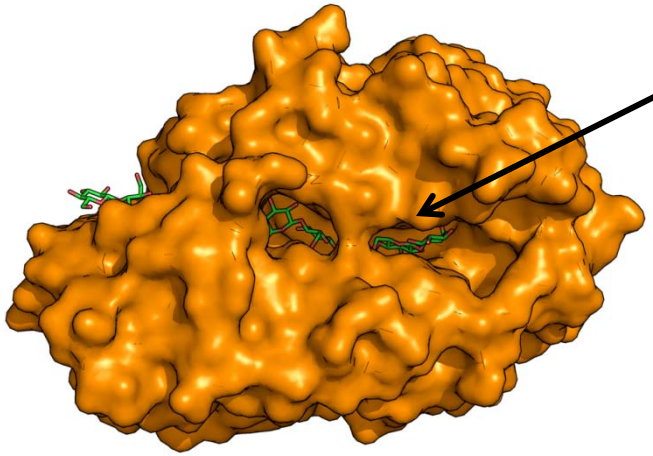


Pale green = *Tr*Cel7B



Active site cleft increasingly open

Orange = *Pf*Cel7A

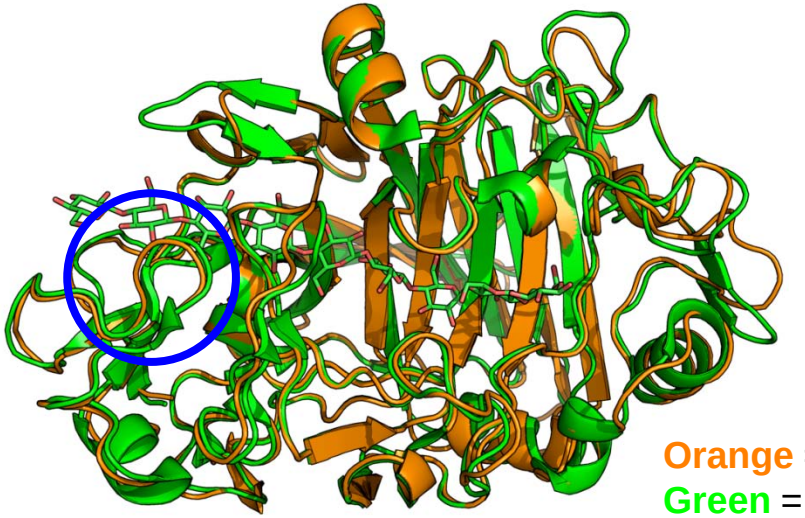


Distinctly different loop configuration over tunnel

4C4C ligand shown (cellononaose)

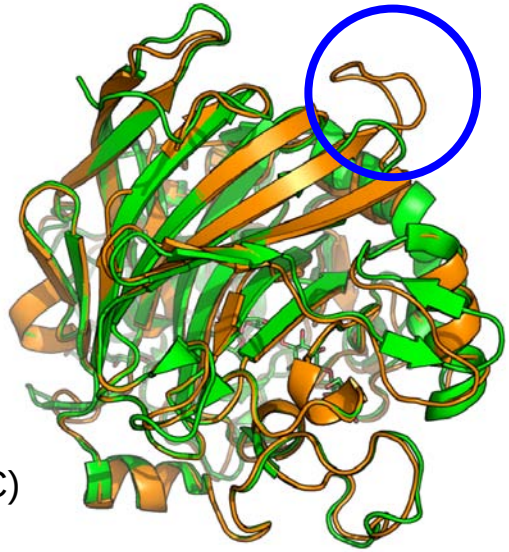
3 – Technical Accomplishments/ Progress/Results

A1 loop - compared to *Tr*Cel7A

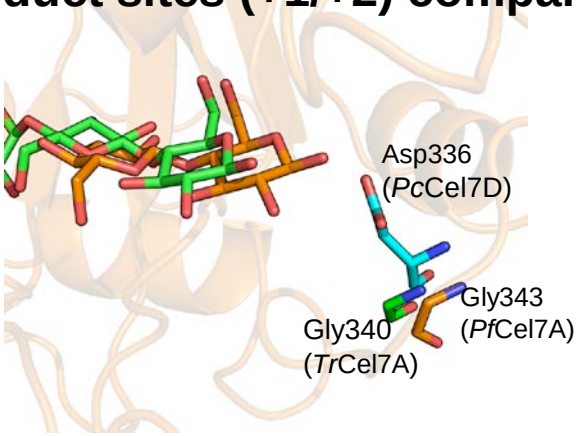


Orange = *Pf*Cel7A
Green = *Tr*Cel7A (4C4C)

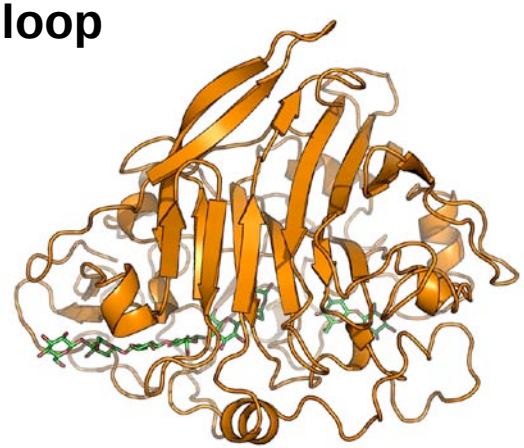
'Top' loop - compared to *Tr*Cel7A



Product sites (+1/+2) compared



*Pf*Cel7A and *Pc*Cel7D have an 'exo' loop

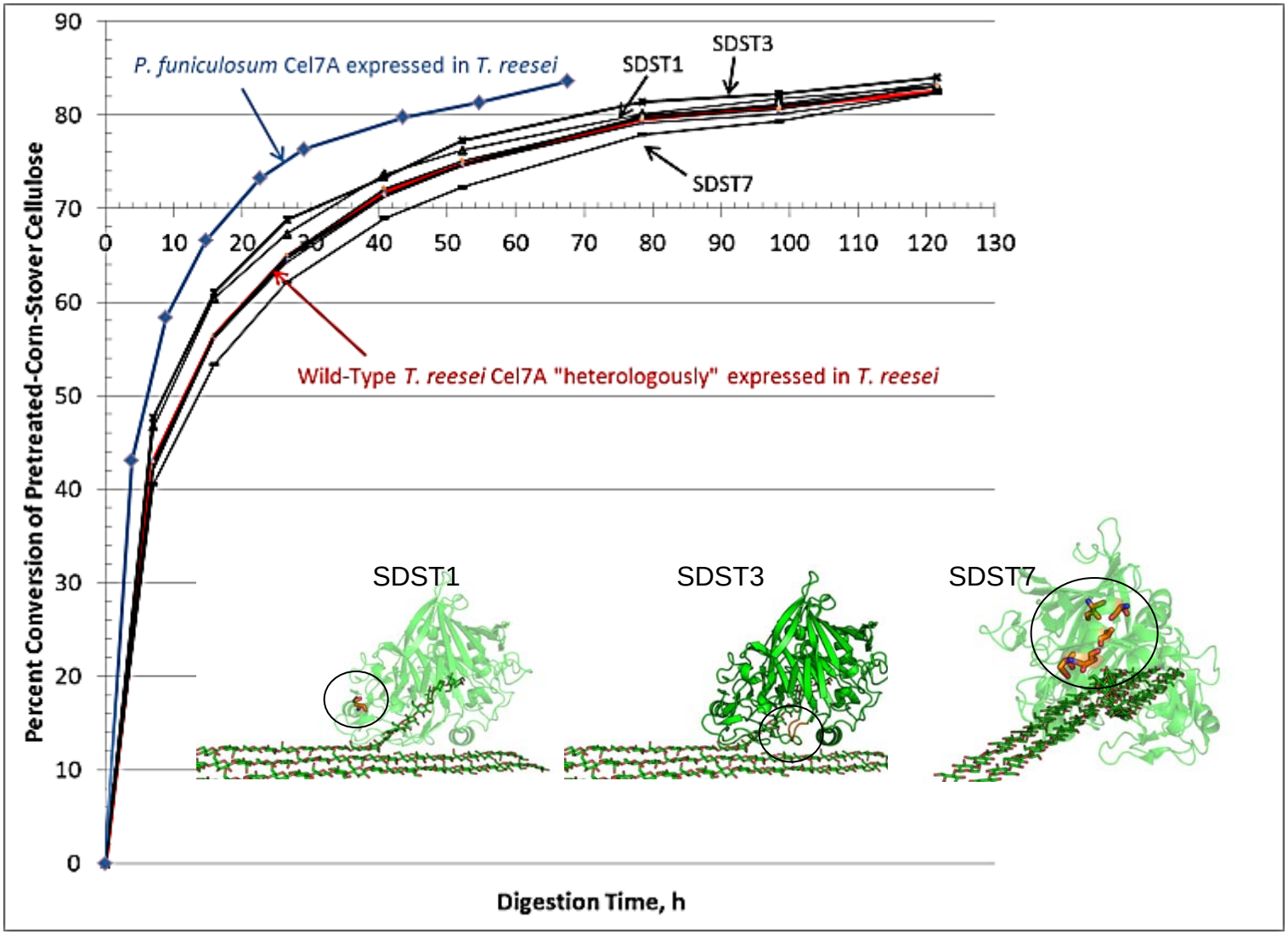


Orange = *Pf*Cel7A
Green = *Tr*Cel7A (4C4C)
Cyan = *Pc*Cel7D (1GPI)

4C4C ligand shown (cellononaose)

3 – Technical Accomplishments/ Progress/Results

Can we capture the design features of the P. fun enzymes?
(*P. fun* subdomains into *T. reesei* Cel7A)

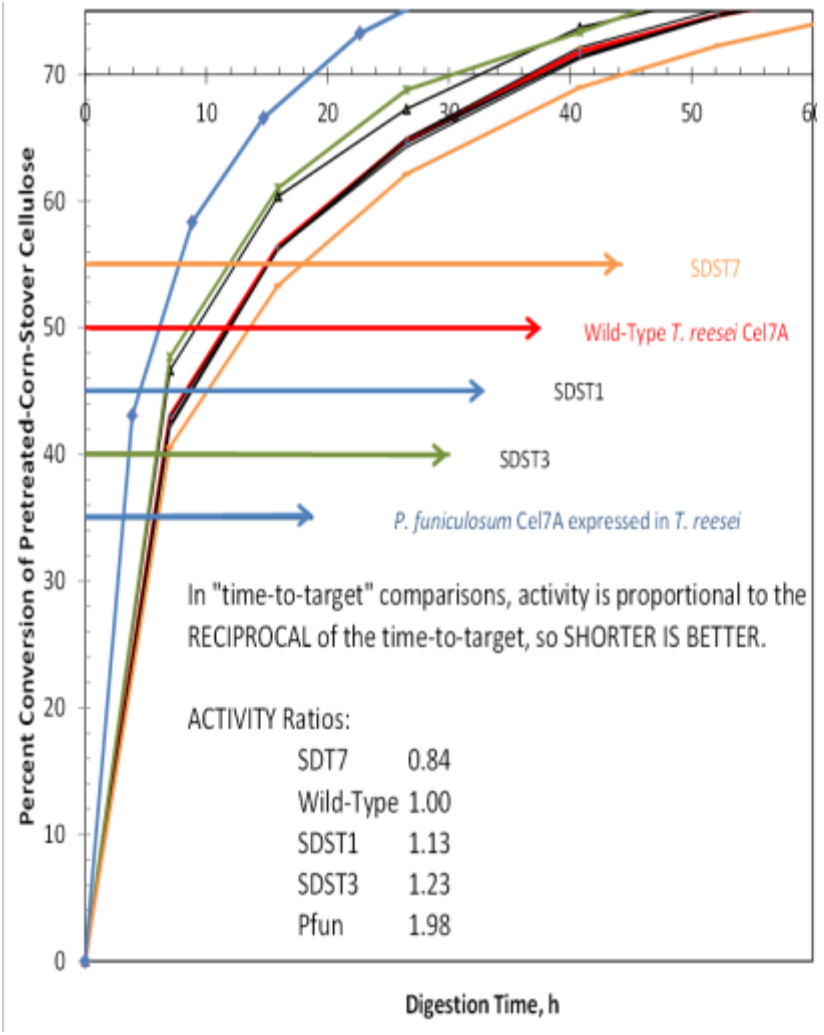


3 – Technical Accomplishments/ Progress/Results

Yes, we can capture the design features of the P. fun enzymes

- Preliminary single-subdomain swaps (*P. funiculosum* into *T. reesei*) have given us two swaps that significantly enhance activity of *T. reesei* Cel7A.

- Follow-up efforts (currently underway)
  - We have to date examined 3 of 12 swaps identified from structure
  - Determine whether combining the two productive swaps results in effects that are additive (or even synergistic).
  - Express and evaluate the analogous "*T. reesei* to *P. funiculosum*" swaps.



3 – Technical Accomplishments/ Progress/Results

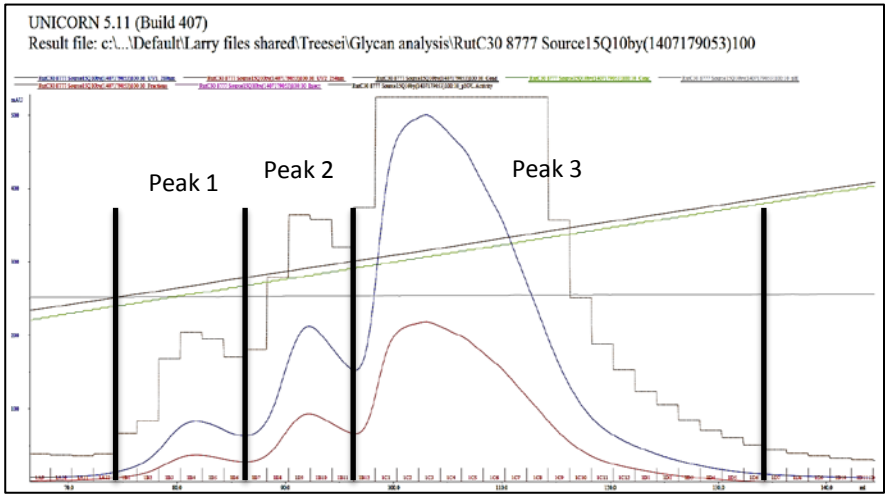
Does glycosylation affect cellulase performance?

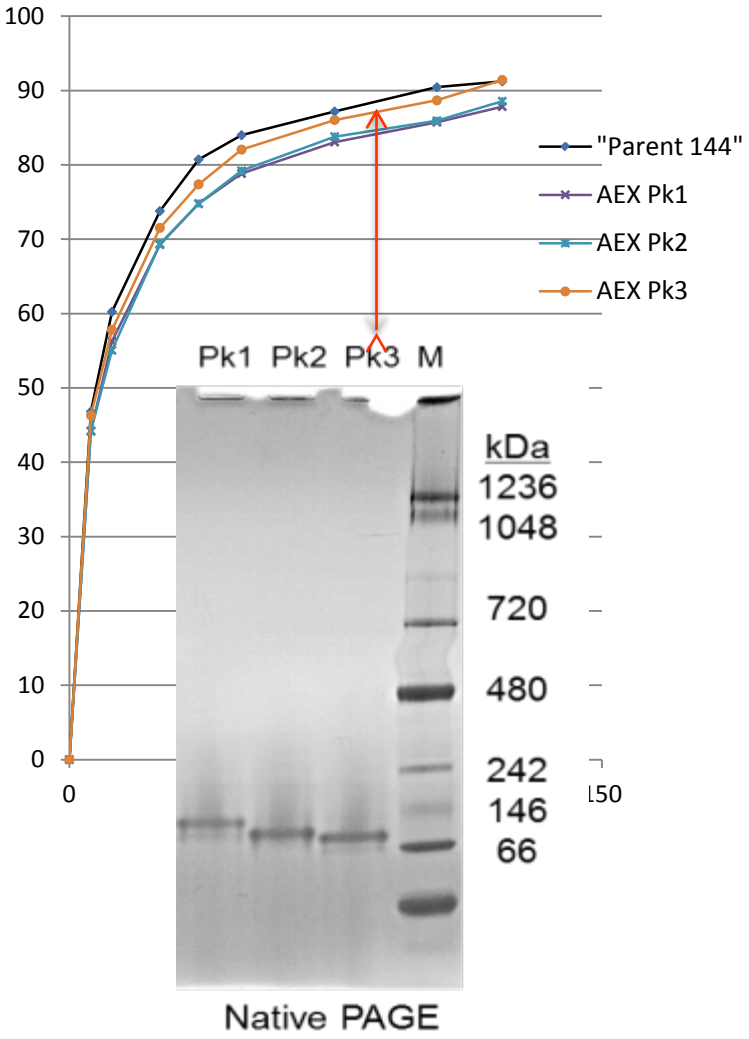
- Post-translational processing leads to differences in protein glycosylation
  - Affects substrate interaction, activity, pH and Topt parameters, protease susceptibility, etc.
  - Can be separated based on charge or hydrophobicity
  - Long HIC or AEX gradients reveal distinct activity subpopulations
- Structural analysis in partnership with UGA-CCRC
- Test with a SDM program targeting *N* and *O*-linked glycans

3 – Technical Accomplishments/ Progress/Results

Activity of AEX fractions also suggest less glycosylated species are more active

- AEX is ion exchange but also mixed partition chromatography
  - Three distinct fractions generated
  - Late eluting fraction is most active
  - Late eluting fraction is lowest MW (SDS gels not shown)
  - Dilemma: no fraction equals the loading sample



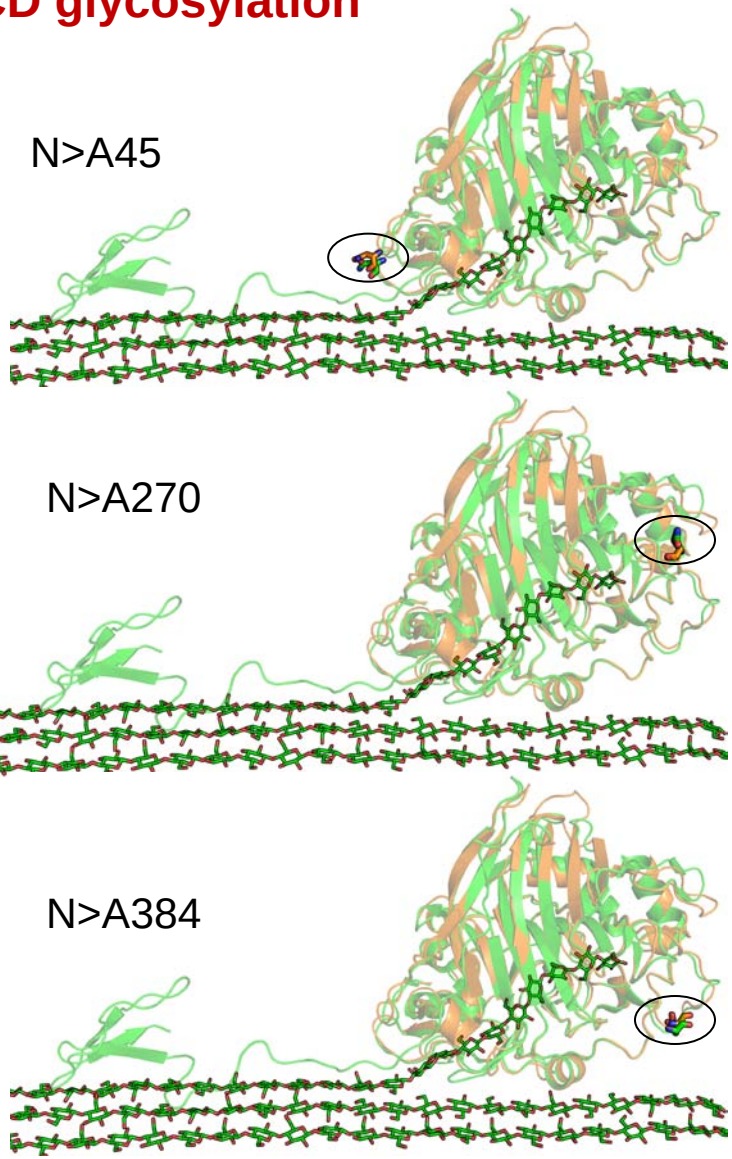


3 – Technical Accomplishments/ Progress/Results

Engineering Cel7A CD glycosylation

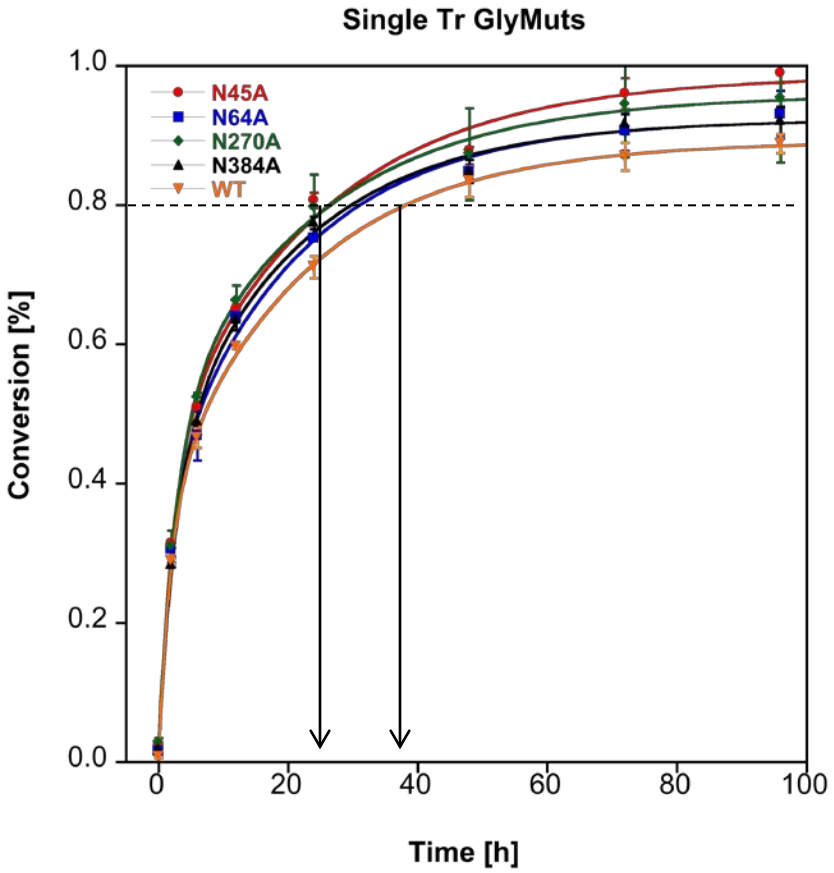
Build a Cel7A glycan mutation data base

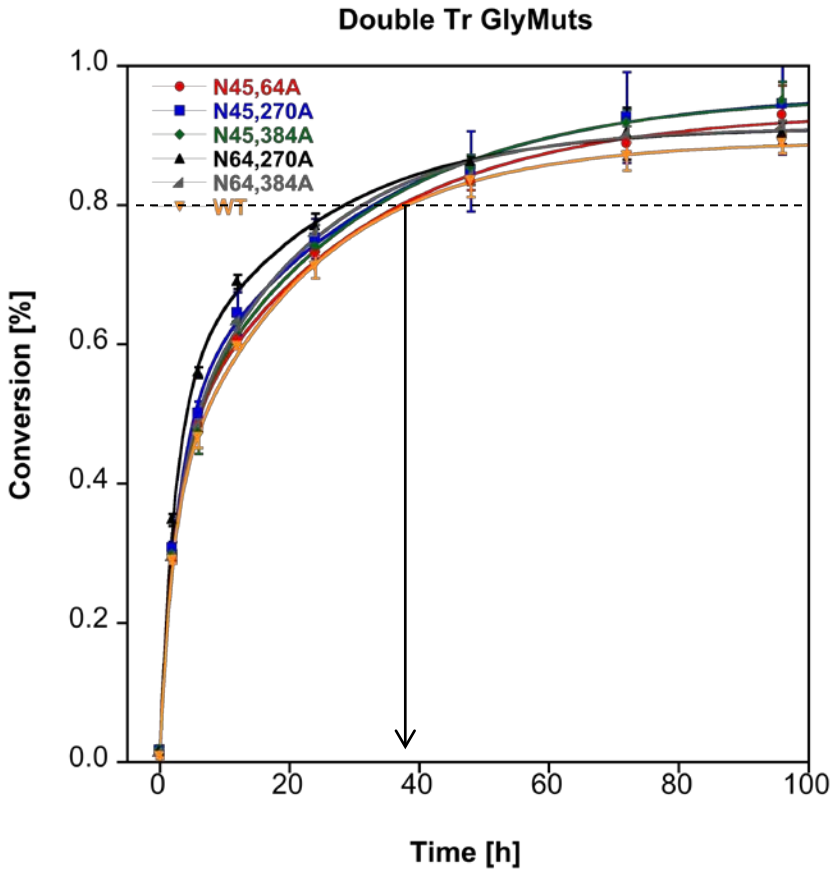
- N (asparagine) on catalytic domain
  - Point N→A mutations on cat
  - 9 mutants tested to date
- O (serine) on linker and CBM
  - Block O→A on linker
  - Experiments still underway



3 – Technical Accomplishments/ Progress/Results

Engineering Cel7A CD glycosylation





- Single mutations result in 1.5 fold improvement on Avicel
- N>A mutant don’t appear to be additive

3 – Technical Accomplishments/ Progress/Results

"Other" Cel7A Enzymes

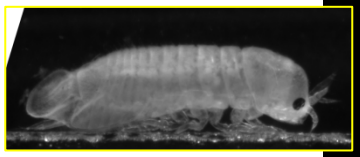
- *Daphnia pulex*- the water flea
  - Collaboration between NREL and U. Portsmouth
  - Genomic GH7 Cellobiohydrolase
    - higher K_m, lower K_{cat}, lower T^o_{opt}, higher pH_{opt} compared to *T. reesei*
    - No native linker or CBM

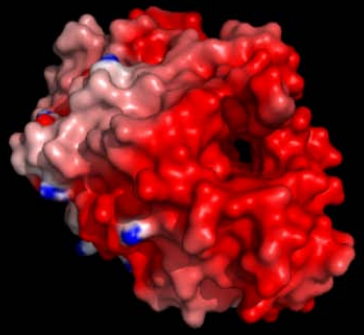
- Social amoebae cellobiohydrolases
  - Slime molds- *Dictyostelium discoideum* and *D. purpureum*
  - Equivalent activity to *T. reesei* on PCS
  - Reduced sensitivity to end product inhibition
    - Potentially better on high solids

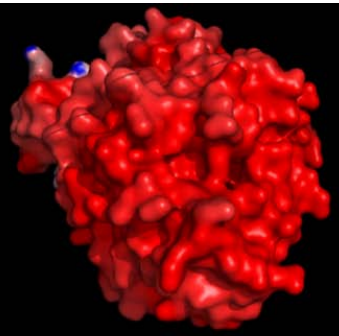
3 – Technical Accomplishments/ Progress/Results

L. quadripunctata Cel7B surface is highly negatively charged

Surface features may impart benefits to biomass digestion



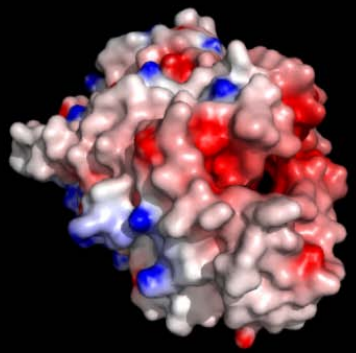


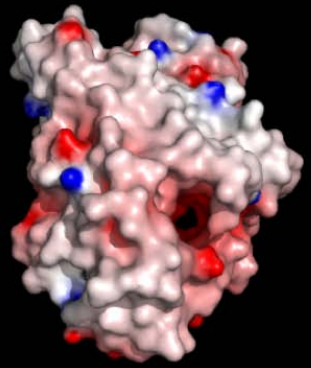


Limnoria quadripunctata
Marine crustacean

Chelura terebrans
Marine crustacean

Images courtesy of S. Cragg, J. McGeehan.





Daphnia magna
Fresh-water crustacean

Trichoderma reesei
Fungus

Seidl 2009

M. Kern, J. McGeehan et al. PNAS 2013

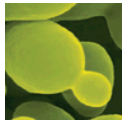
3 – Technical Accomplishments/ Progress/Results

Challenges with Potential Host Organisms

Source

- *E. coli*
  - Instability, unglycosylated, difficulty with disulfide bonds, proteolysis

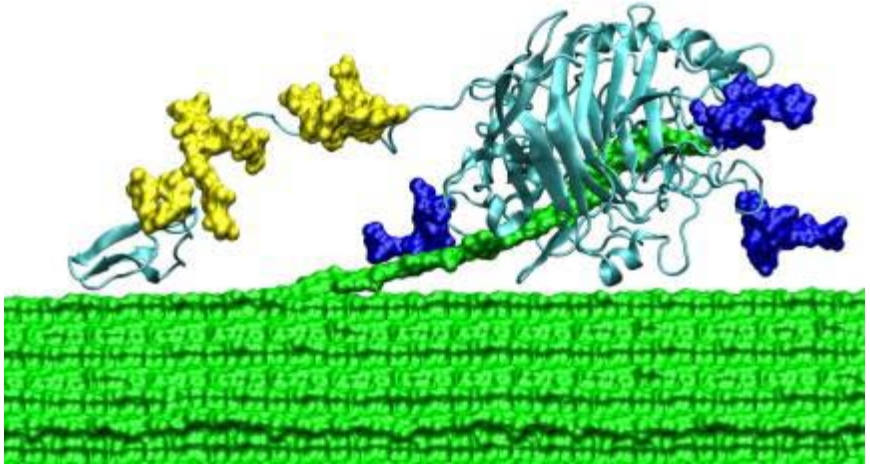
- *S. cerevisiae:*
  - Productivity and efficient secretion challenging
  - Hyperglycosylation
  - Lower enzyme stability and activity, folding issues



- *Pichia pastoris*
  - Productivity
  - Hyperglycosylation

Source

- *Aspergillus*
  - Different glycosylation
  - proteolysis



3 – Technical Accomplishments/ Progress/Results

Critical Tool Development - Building a *T. reesei* expression system

- Began 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*
  - 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 is increased production host strain (QM9414)

3 – Technical Accomplishments/ Progress/Results

T. reesei as an Expression Host

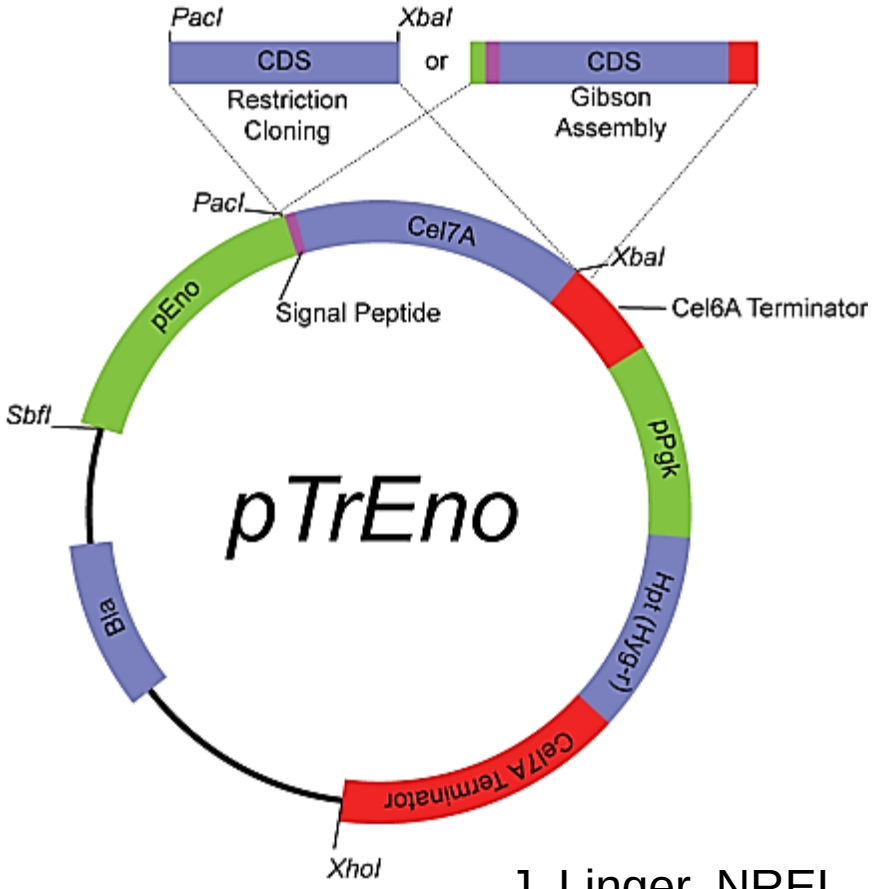
Pros

- Native producer of a powerful cellulolytic suite
- A proven industrial organism
- High levels of production and secretion
- Native glycosylation patterns

Cons

- Lower throughput strain engineering than many microbes
- Cellulase suite is co-induced making individual enzyme purification challenging

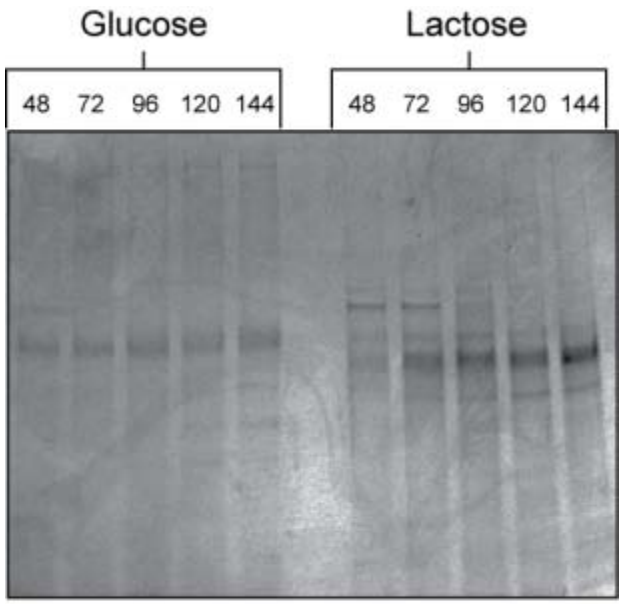
T. reesei Expression NREL System V 2.0

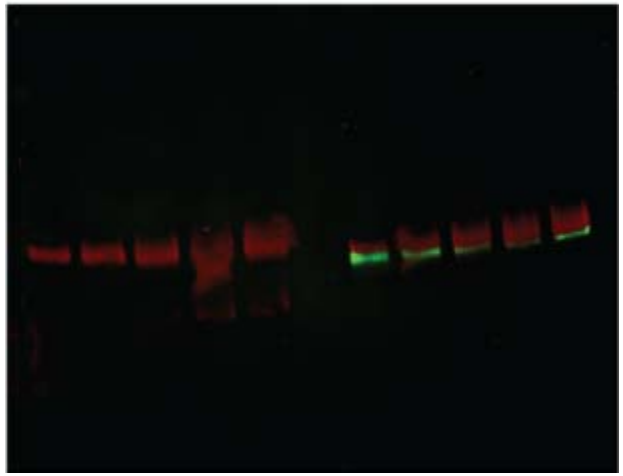


J. Linger, NREL

3 – Technical Accomplishments/ Progress/Results

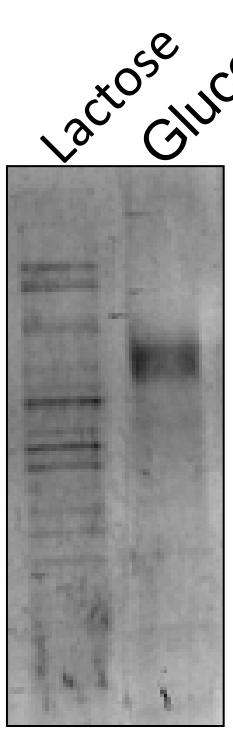
Constitutive Expression in the Absence of "Contaminating" Cellulases





Cel7A

Cel6A

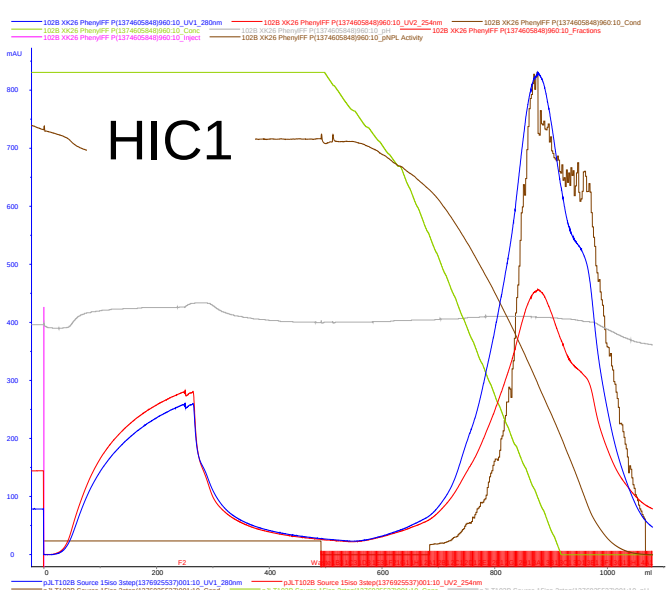


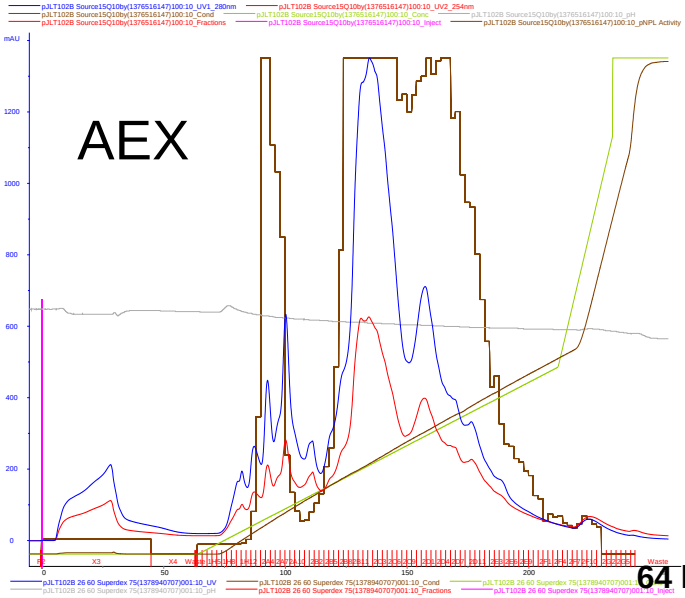
Cel7A

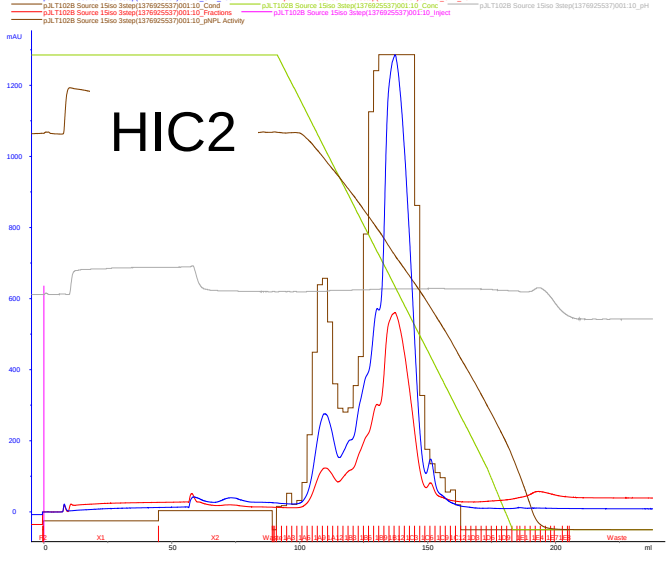
Catabolite repression serves as a surrogate for a comprehensive cellulase deletion strain

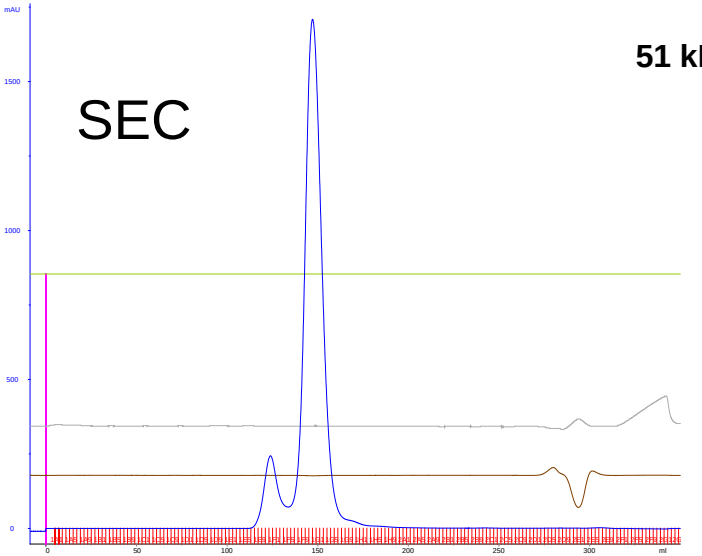
3 – Technical Accomplishments/ Progress/Results

Purification of pTrEno-Driven Cel7A





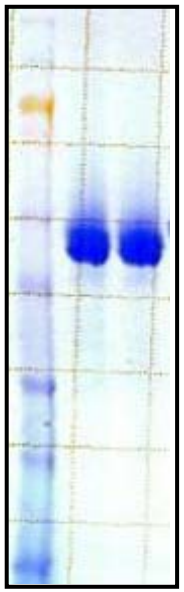




wildtype Cel7A
pTrEno-Cel7A

64 kDa-

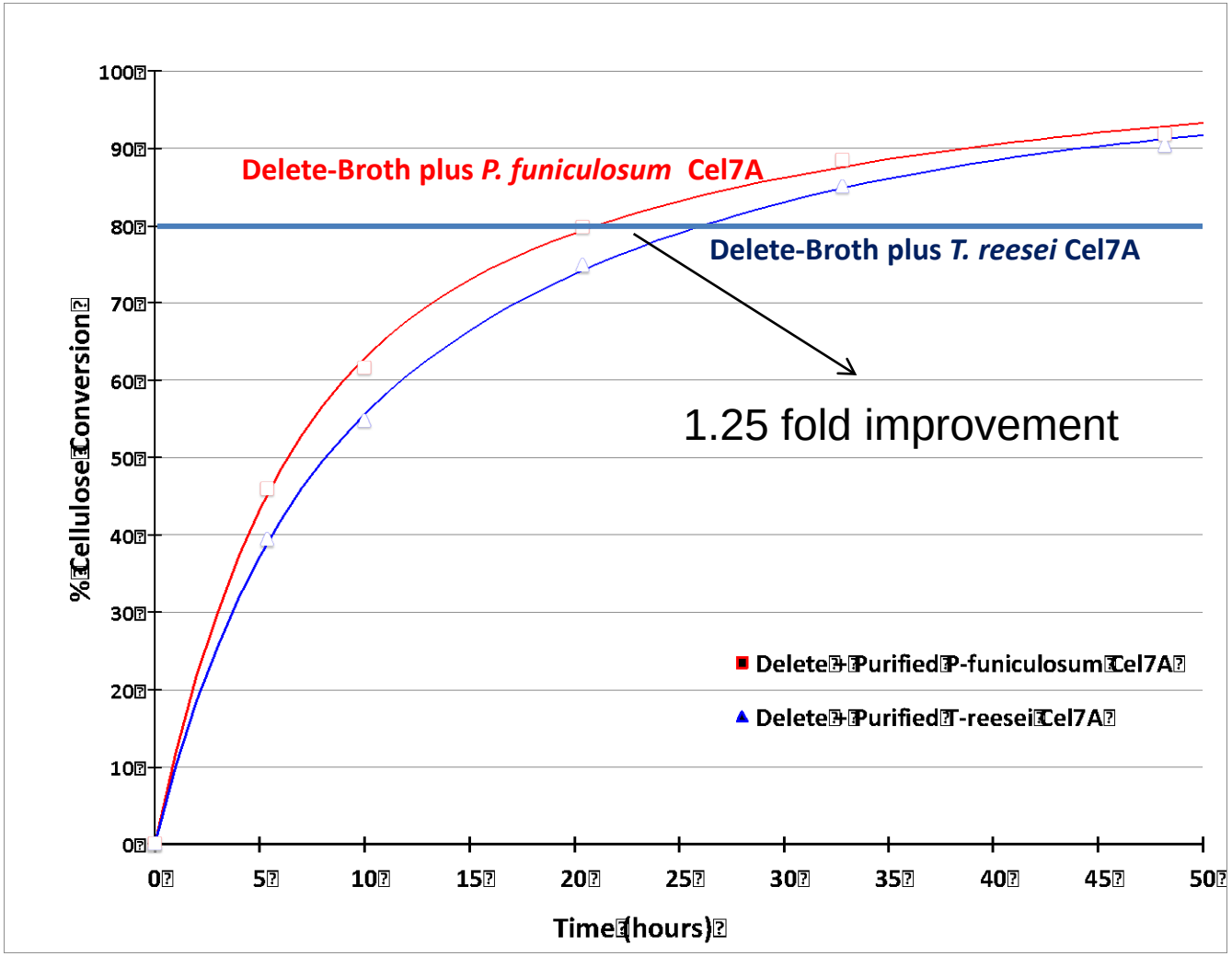
51 kDa-



Typical purification outcome

3 – Technical Accomplishments/ Progress/Results

Digestion of Pretreated Corn Stover by Purified *T. reesei* and *P. funiculosum* Cel7As Added Back to Cel7A-Delete *T. reesei* Broth



2 – Approach (Management)

2015 – 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

Task 2. Improving the *T. reesei* secretome

Q3	6/30/2014	Stretch	Demonstrate Functional Expression of Multi-component Cellulases in Fungal Host

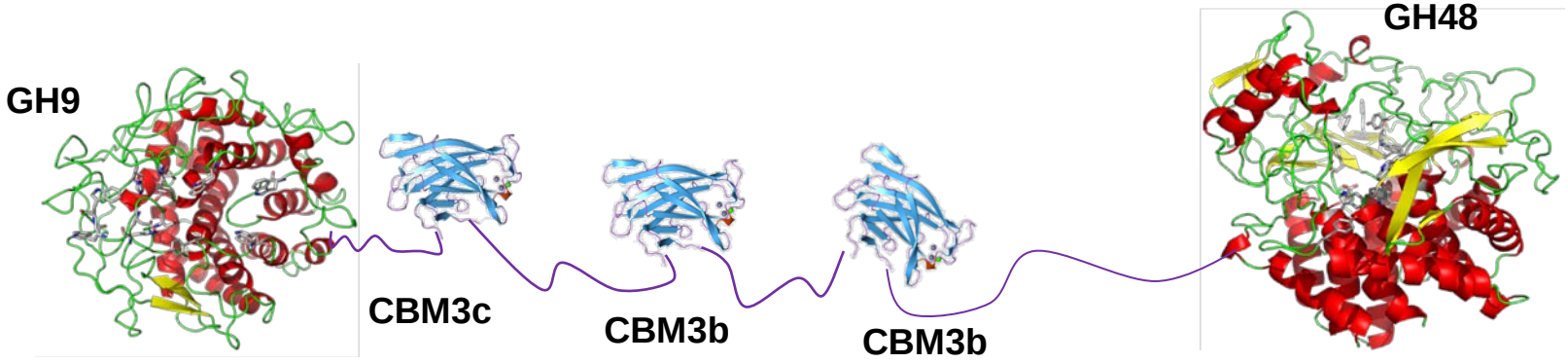
Task 2. Report analysis of novel enzyme addition schemes to reduce protein loading.	6/30/2015	Quarterly Progress Measure (Regular)

- Use yeast or filamentous fungi to express multi-domain cellulases (MDCs) that are functional in hydrolyzing cellulose and/or biomass.
- MDCs are bacterial in origin and comprise either multiple catalytic and/or binding domains joined together by linker peptides (single gene product) or through a separate "connector" protein (scaffoldin) in cellulosomes.
- Current attempts in this area have had very limited success; however, the advantages of these enzymes, including high thermal tolerance, high specific activity, and multi-substrate hydrolysis, make pursuing this work worthwhile.
- In FY2015, focus is on *C. bescii* CelA – the most active single cellulase we have encountered.
- Optimize cellulase addition strategies – SHF in high temp hold mode

3 – Technical Accomplishments/ Progress/Results

Commercially relevant expression host for *C. bescii* CelA

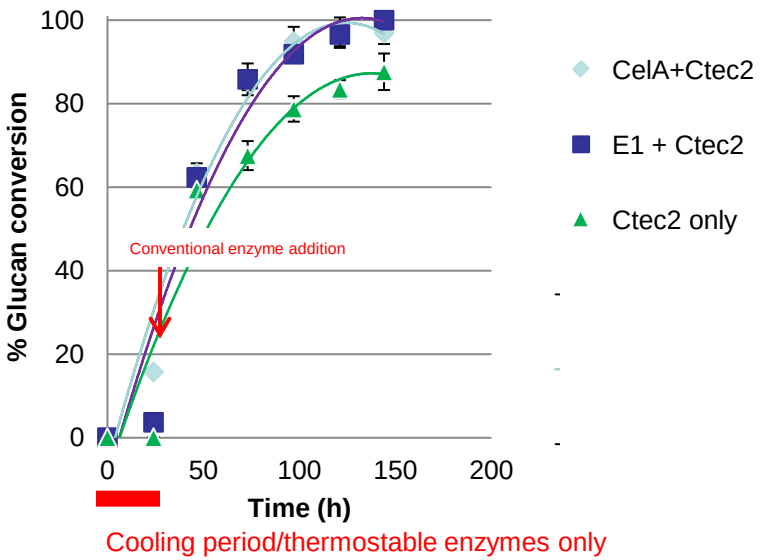
- Design commercially relevant expression system for multi-domain glycoside hydrolases
- Focus on *C. bescii* CelA initially
  - Cannot be expressed with full activity in *E. coli*
  - Cannot be expressed with full activity in *T. reesei*
  - Developing strategies for *T. reesei* in FY2015-16



3 – Technical Accomplishments/ Progress/Results

High temperature pre-digestion of corn stover biomass for improved product yields

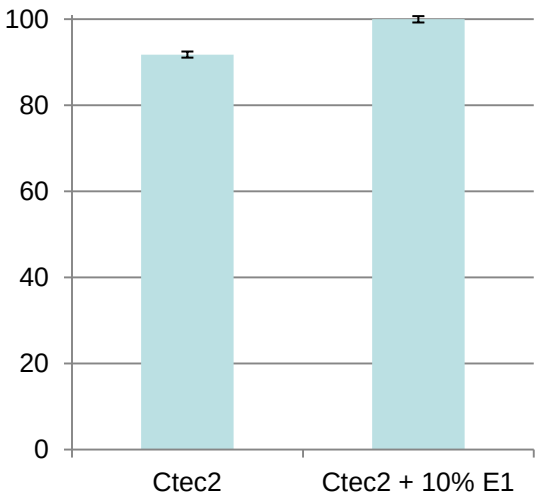
Low solids digestion curves



Cooling period/thermostable enzymes only

High solids endpoint conversions (5d)

20% DA CS



Biomass coming from pretreatment reactors take time or energy to cool.

By utilizing a small loading of thermostable enzymes, 15% of total loading, earlier in the process, we can pre-digest the biomass to enable higher extents of conversion which impacts process economics.

Brunecky et al *BfB*, 2014

TEA analysis (L. Tao)

MESP ($/gal)			
	3day	4day	5day
CelA-mix	$2.50	$2.22	$2.12
E1-mix	$2.52	$2.21	$2.15
Ctec2	nd	$2.44	$2.30

3 – Technical Accomplishments/ Progress/Results

ADB-EEO: Key Accomplishments

- Reported the first activity improvement of Cel7A through enzyme engineering
  - 1.6 fold improvement using ternary systems on biomass (HIC fractions - low glycan forms)
  - 1.5 fold improvement using ternary systems on biomass (*N*-linked glycan mutants)
  - 1.25 fold improvement using fungal broth on biomass (P fun add back to delete QM6a broth)
- Discovery of the critical nature of CBM glycosylation and binding
  - 2-140 fold improvement in binding for single and double *O*-linked mutations (computational)
  - 8 fold improved binding for Thr1 oligosaccharide *O*-linked mutations (experimentally verified)
  - 10 fold improved binding of CBM + linker mutants (experimentally verified)
- Developed a constitutive promotor driven *Trichoderma reesei* expression system to enable enzyme engineering in a native/industrially relevant host.
- Methods for hydrophobic surface area developed and predictions made for lignin binding – not shown.
- Demonstrated utility of a high temperature hold process step using *C. bescii* CelA and *A. cellulolyticus* E1.

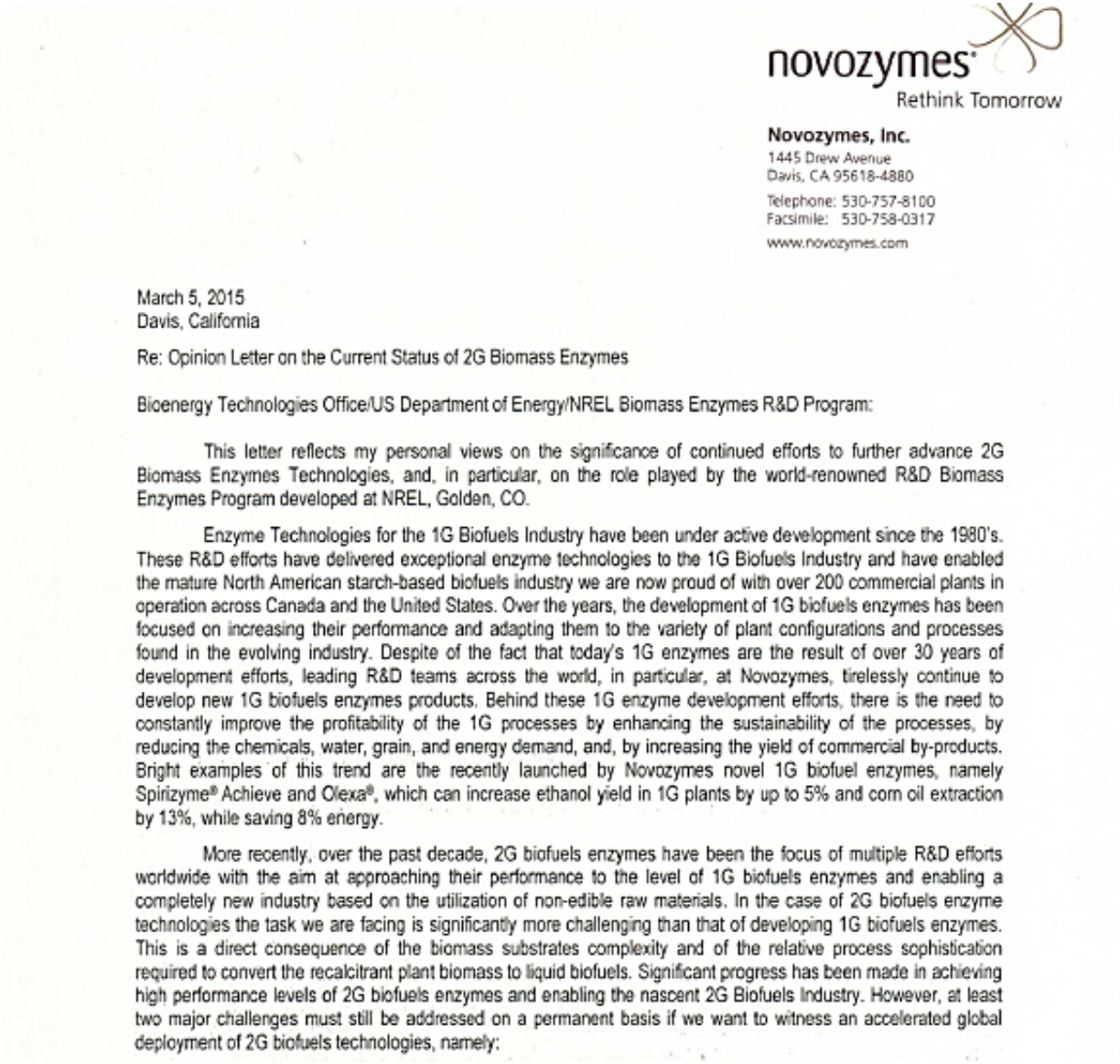
4 – Relevance

- *Describe how project accomplishments contribute to….*
  - This project provides fundamental and applied science strategies to enable the process engineering targets listed by the Multi-Year Program Plan to achieve advanced biofuels.
  - This overarching goal:“ *Enable the production of biofuels nationwide and reduce dependence on oil through the creation of a new domestic bioenergy industry supporting the EISA goal of 36 bgy of renewable transportation fuels by 2022”.*
- *Demonstrate how the project considers applications ….*
  - Increase confidence (reduce risk) regarding process implementation by demonstrating that the major process unit operations are underpinned by considerable technical “know how”; in some cases even to extending the molecular level. **>>Knowledge is Forever<<**
  - 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.
- *Your objectives should be clear regarding the relevance of your project …*
  - Bt-C. Biomass Recalcitrance: We are working to understand the biomass recalcitrance problem at the range of length scales and chemistries necessary to reduce processing costs.
  - Bt-D. Pretreatment Chemistry: Pretreatment is necessary to render biomass more susceptible to hydrolysis by cellulase enzymes and we are working to better define the critical process parameters most likely to reduce costs and increase yield (see slide 24).
  - Bt-G. Cellulase Enzyme Loading: Reducing the cost of enzymatic hydrolysis depends on identifying more efficient enzymes and we are working to improve the specific performance of cellulases using rational design strategies, based on informatics and mechanistic models.

4 – Relevance

- *Demonstrate that the successful project will advance the…..*
  - Both the near term (2017) and longer term (2022) BETO goals for advanced biofuels require continued reduction in conversion (pretreatment, cellulase production, saccharification)
  - New feedstocks, pretreatments, process schemes, and products are likely to require reformulation of enzyme cocktails used in the process
  - The knowledge based approach we are taking to improving enzyme cellulase/hemicellulase performance will provide the portfolio of enzymes needed for these goals.
- *Tech transfer/marketability*
  - NREL ref. no. 12-28 U.S. patent application 13/941,754 "Enzymes for Improved Biomass Conversion."
  - Material Transfer Agreements (MTA) executed with:
    - Novozymes – see following letter (2015)
    - DuPont
    - Dyadic
    - DSM
  - Technical Services Agreement (TSA) executed with California Safe Soil
  - Negotiating CRADAs with enzyme companies

4 – Relevance



novozymes
Rethink Tomorrow

Novozymes, Inc.
1445 Drew Avenue
Davis, CA 95618-4880
Telephone: 530-757-8100
Facsimile: 530-758-0317
www.novozymes.com

March 5, 2015
Davis, California

Re: Opinion Letter on the Current Status of 2G Biomass Enzymes

Bioenergy Technologies Office/US Department of Energy/NREL Biomass Enzymes R&D Program:

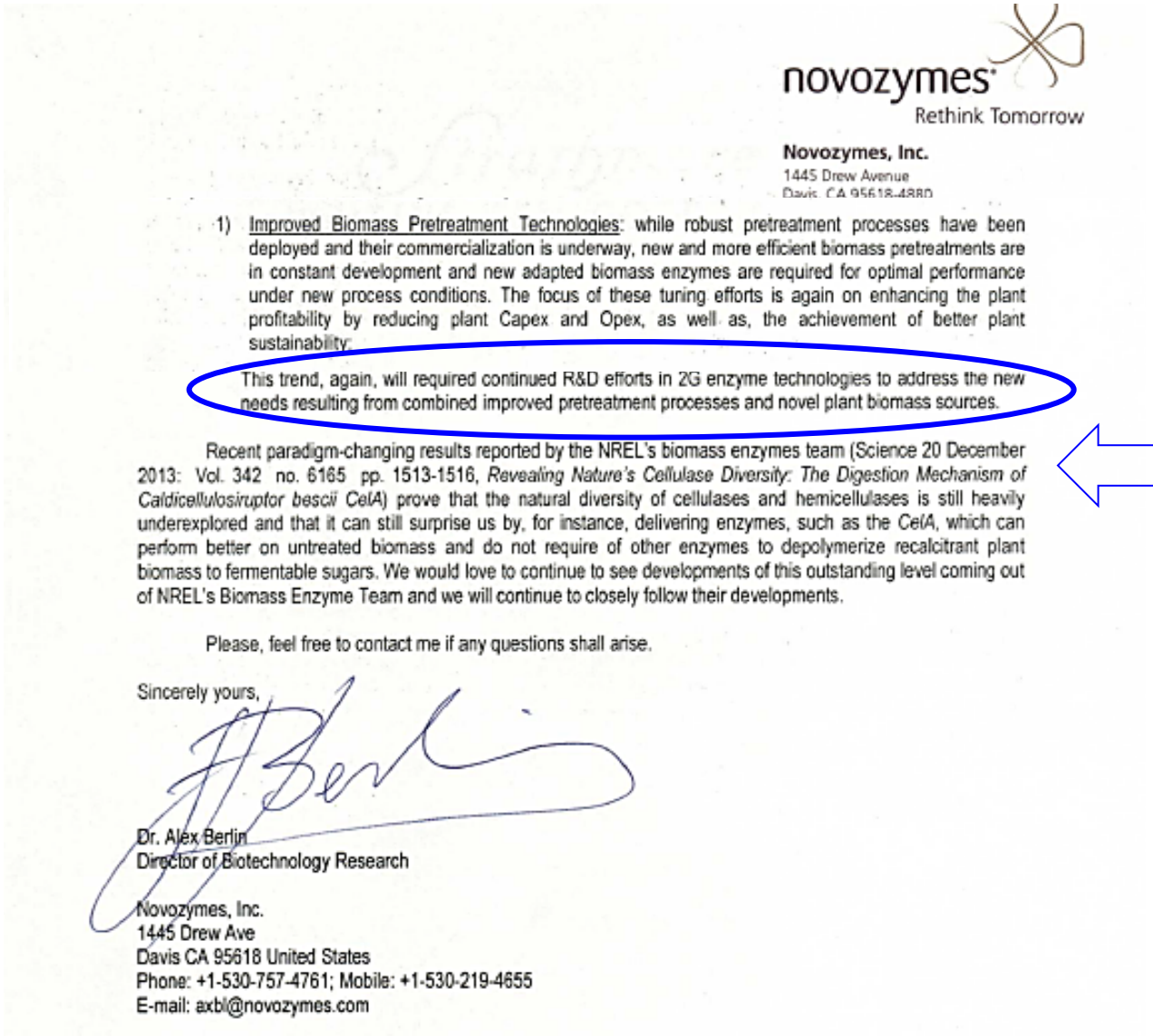
This letter reflects my personal views on the significance of continued efforts to further advance 2G Biomass Enzymes Technologies, and, in particular, on the role played by the world-renowned R&D Biomass Enzymes Program developed at NREL, Golden, CO.

Enzyme Technologies for the 1G Biofuels Industry have been under active development since the 1980's. These R&D efforts have delivered exceptional enzyme technologies to the 1G Biofuels Industry and have enabled the mature North American starch-based biofuels industry we are now proud of with over 200 commercial plants in operation across Canada and the United States. Over the years, the development of 1G biofuels enzymes has been focused on increasing their performance and adapting them to the variety of plant configurations and processes found in the evolving industry. Despite of the fact that today's 1G enzymes are the result of over 30 years of development efforts, leading R&D teams across the world, in particular, at Novozymes, tirelessly continue to develop new 1G biofuels enzymes products. Behind these 1G enzyme development efforts, there is the need to constantly improve the profitability of the 1G processes by enhancing the sustainability of the processes, by reducing the chemicals, water, grain, and energy demand, and, by increasing the yield of commercial by-products. Bright examples of this trend are the recently launched by Novozymes novel 1G biofuel enzymes, namely Spirizyme® Achieve and Olexa®, which can increase ethanol yield in 1G plants by up to 5% and corn oil extraction by 13%, while saving 8% energy.

More recently, over the past decade, 2G biofuels enzymes have been the focus of multiple R&D efforts worldwide with the aim at approaching their performance to the level of 1G biofuels enzymes and enabling a completely new industry based on the utilization of non-edible raw materials. In the case of 2G biofuels enzyme technologies the task we are facing is significantly more challenging than that of developing 1G biofuels enzymes. This is a direct consequence of the biomass substrates complexity and of the relative process sophistication required to convert the recalcitrant plant biomass to liquid biofuels. Significant progress has been made in achieving high performance levels of 2G biofuels enzymes and enabling the nascent 2G Biofuels Industry. However, at least two major challenges must still be addressed on a permanent basis if we want to witness an accelerated global deployment of 2G biofuels technologies, namely:

"It is common to distinguish between first generation (1G) biofuels made from feedstock also suitable for human food production and second generation (2G) biofuels made from cellulosic material not useable as a food source." *Energies* **2014**, *7*, 4430-4445; doi:10.3390/en7074430

4 – Relevance



novozymes
Rethink Tomorrow

Novozymes, Inc.
1445 Drew Avenue
Davis, CA 95618-4880

1) Improved Biomass Pretreatment Technologies: while robust pretreatment processes have been deployed and their commercialization is underway, new and more efficient biomass pretreatments are in constant development and new adapted biomass enzymes are required for optimal performance under new process conditions. The focus of these tuning efforts is again on enhancing the plant profitability by reducing plant Capex and Opex, as well as, the achievement of better plant sustainability.

This trend, again, will required continued R&D efforts in 2G enzyme technologies to address the new needs resulting from combined improved pretreatment processes and novel plant biomass sources.

Recent paradigm-changing results reported by the NREL's biomass enzymes team (Science 20 December 2013: Vol. 342 no. 6165 pp. 1513-1516, *Revealing Nature's Cellulase Diversity: The Digestion Mechanism of Caldicellulosiruptor bescii CelA*) prove that the natural diversity of cellulases and hemicellulases is still heavily underexplored and that it can still surprise us by, for instance, delivering enzymes, such as the *CelA*, which can perform better on untreated biomass and do not require of other enzymes to depolymerize recalcitrant plant biomass to fermentable sugars. We would love to continue to see developments of this outstanding level coming out of NREL's Biomass Enzyme Team and we will continue to closely follow their developments.

Please, feel free to contact me if any questions shall arise.

Sincerely yours,

Dr. Alex Berlin
Director of Biotechnology Research

Novozymes, Inc.
1445 Drew Ave
Davis CA 95618 United States
Phone: +1-530-757-4761; Mobile: +1-530-219-4655
E-mail: axbl@novozymes.com

"It is common to distinguish between first generation (1G) biofuels made from feedstock also suitable for human food production and second generation (2G) biofuels made from cellulosic material not useable as a food source." *Energies* **2014**, *7*, 4430-4445; doi:10.3390/en7074430

5 – Future Work

- *Future work to the end of the project*
  - We will stack the successful N>A mutations in Cel7A with the subdomain swaps
  - We will also stack the above mutations with those for *O*-glycan deletions (available this spring)
  - We will design and generate a targeted gene insertion *T. reesei* strain for *in vivo* testing.
  - We will test all successful Cel7A mutations for performance *in vivo* using both add back and transformed *T. reesei* strains.
  - Demonstrate high throughput Cel7A screening system using *S. cerevisiae* host
- *Highlight upcoming key milestones*

Task 2. Report the predicted partial attainment of a one-quarter 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 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)

- *Go/No-Go Points in next three years*

Go/No Go 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

Summary

Overview

The goal of this project is to ensure that cellulase enzyme loadings can be reduced to the DOE 2017 target of 10 mg/g cellulose using modern enzyme engineering and formulation optimization strategies. Publications, presentations, and patents are stressed to disseminate progress to industry

Approach

To employ a knowledge based approach which permits linking protein structure to performance. We have pioneered the close coordination of computational analysis and experimental validation for cellulase improvement and achieved positive results.

Technical Accomplishments/Progress/Results

We have successfully demonstrated the ability to model cellulase action, design structural features likely to impact performance, build targeted mutations, and show improved performance on pretreated biomass. We have also demonstrated the utility of the high temperature hold biomass conversion scheme using caldiphilic bacterial cellulases.

Relevance

The attainment of this overall enzyme cost is consistent with BETO's technoeconomic evaluations for meeting the 2017 biomass conversion target. Given that cost effective biomass conversion to sugars is a common theme for many conversion processes, the overall bioenergy industry also benefits from this work.

Future work

The early stages of selection of glycosylation and P fun subdomain mutations suggested for future work in FY2013 are being accomplished in FY2015. Active site mutations and mutations suggested by newly initiated HTP yeast screening will be accomplished in FY2015-16. Work to express newly discovered, highly active bacterial cellulases in *T. reesei* will be accomplished in FY2015-17. If Out-Years are funded, the newly discovered LPMOs should be subjected to enzyme engineering studies to improve their native performance under process conditions.

Acknowledgements

- Funding
  - U.S. DOE EERE Office of the Biomass Program
  - HQ: Jonathan Male, Valerie Sarisky-Reed, Leslie Pezzullo, Bryna Guriel
  - NREL LPM and Platform Lead: Adam Bratis and Rick Elander
- NREL Project Members (all between 10 and 50% FTE)

Markus Alahuhta
John Baker
Gregg Beckham
Yannick Bomble
Roman Brunecky
Mike Crowley
Stephen Decker (Task Lead)
Bryon Donohoe
Sarah Hobdey
Brandon Knott (PD)
Jeff Linger
Vladimir Lunin
Kara Podkaminer (PD)
Mike Resch
Logan Schuster (student)
Larry Taylor II
Todd Vanderwall
Todd Vinzant
Qi Xu
John Yarbrough

	Impact Factor	2013	2014	2015
Chem Rev	45.6			1
Science	34		2	
EES	15.5	1		
JACS	11.4	1	2	
Biotechnol Advances	11.2			1
PNAS	9.8	2	2	
Curr Opin Biotechnol	8.5	1		
Green Chem	6.85		1	
BfB	6.3		5	1
JBC	4.7	2	1	
J Phys Chem B	3.4	2		

Additional Slides

Responses to Previous Reviewers' Comments

- *2013 Peer Review Report – Reviewers comments*
  - "Extensive amount of research going on in this project. It seems like there are numerous tasks and findings all going on simultaneously. The cycle back an forth between enzyme development and pretreatment is important. Several publications generated from this research to date are significant."
  - "The inclusion of basic, fundamental research in the portfolio is a good investment in the long term viability of BETO's mission. The output of this work may not be immediately viable for commercialization, but the knowledge that is created may prove to be invaluable down the road. The government is uniquely positioned to fund this type of work and it should be maintained."
  - "This is very fundamental research to get a better understanding of what's happening at the molecular level, which will result in improved enzyme and decreased costs."
  - This is a very impressive body of work demonstrating how fundamental knowledge may benefit applied project goals. This reviewer just wished there had been more highlights demonstrating technology impact." Me too (MEH)
  - This is not a single unified project, but rather an evolving group of projects. It has a strong track record and will certainly continue to be productive. The advanced biomass deconstruction aspect is the strongest. The structure component (both x ray and bioinformatics) of the structure, simulation and theory aspect is strong, but this reviewer has reservations about the practical knowledge arising from application of simulation and theory to these complicated systems. The direct microbial sugar conversion aspect will be very challenging, but it is important to gain this fundamental knowledge despite the extreme technical difficulty." Several important points introduced by this reviewer require comment. We also agreed that this Project was becoming too large, so the Simulation and Theory and Biochemical Transformations tasks became new Projects in FY2014 (MEH).
  - M. Himmel: As one can see from the diverse comments above, the applied fundamental (and especially Theory) aspects of this work were both heralded and questioned in terms of near term value to BETO. However, we believe that a well planned near term technology program should have some element of longer term R&D in its portfolio - all reviewers seem to agree to this point.
- *Also provide highlights from any Go/No-Go Reviews.* None

Publications, Patents, Presentations, Awards, and Commercialization

- Publications:
  - 20 publications from 2013 with impact factors above 6.0
- Patents
  - 2014 NREL ROI: “In planta enzymes for reduced recalcitrance traits.”
  - 2014 NREL ROI: “Enzymatic pre-conditioning for improved sugar release from biomass”
  - U.S. Patent: “Enzymes for Improved Biomass conversion” US2014/0017735A1 Jan 16, 2014
- Technology transfer or commercialization efforts
  - CRADA with Genencor-DuPont: completed in 2013
  - CRADA with DSM: ongoing
  - CRADA with Novozymes: under negotiation
- Awards
  - 2014 Colorado State University, Department of Biochemistry & Molecular Biology, Distinguished Alumnus
  - 2013 Battelle Memorial Institute Inventor of the Year

ABD and EEO: 2013-2015 Publications

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- "Fungal Cellulases and Complexed Cellulosomal Enzymes Exhibit Synergistic Mechanisms In Cellulose Deconstruction," Michael G. Resch, Bryon S. Donohoe, John O. Baker, Stephen R. Decker, Edward A. Bayer, Gregg T. Beckham, Michael E. Himmel, **Energy Environ. Sci**. 6, 1858-1867 (2013).
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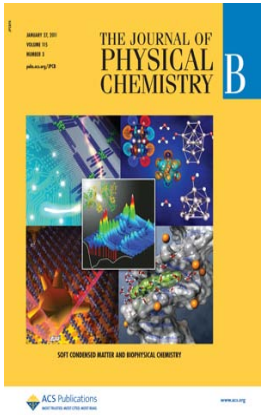

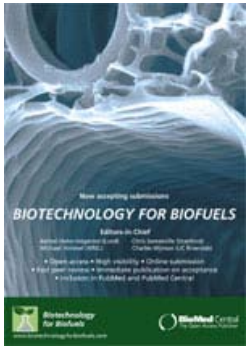

ABD and EEO*: 2013-2015 Publications

- "Revealing Nature's Cellulase Diversity: The hyperactive CelA from *Caldicellulosiruptor bescii*," Roman Brunecky, Markus Alahuhta, Qi Xu, Bryon S. Donohoe, Michael F. Crowley, Irina A. Kataeva, Sung-Jae Yang, Michael W. W. Adams, Vladimir V. Lunin, Michael E. Himmel, and Yannick J. Bomble, **Science**, 342(6165), 1513-6 (2013). *Attributed to BESC funding but key to on going work for BETO.
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- "Glycosylated Linkers in Multi-Modular Lignocellulose-Degrading Enzymes Dynamically Bind to Cellulose," Christina M. Payne, Michael G. Resch, Liqun Chen, Michael F. Crowley, Michael E. Himmel, Larry E. Taylor II, Mats Sandgren, Jerry Ståhlberg, Ingeborg Stals, Zhongping Tan, and Gregg T. Beckham, **PNAS**, 110(36), 14646–14651 (2013).
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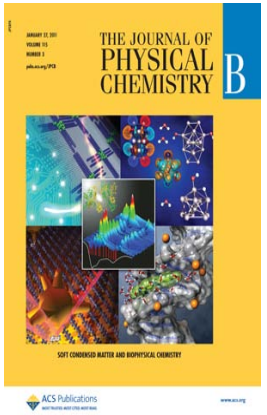

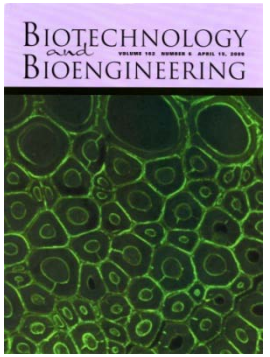

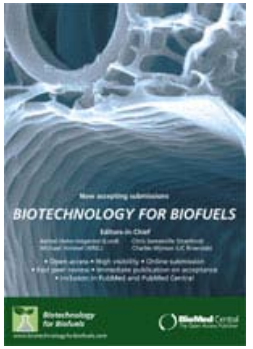

ABD and EEO: 2013-2015 Publications

- “Response to Comment on “Revealing Nature’s Cellulase Diversity: The Digestion Mechanism of *Caldicellulosiruptor bescii* CelA,” Roman Brunecky, Markus Alahuhta, Qi Xu, Bryon S. Donohoe, Michael F. Crowley, Irina A. Kataeva, Sung-Jae Yang, Michael G. Resch, Michael W. W. Adams, Vladimir V. Lunin, Michael E. Himmel, Yannick J. Bomble, **Science** 344, 578 (2014) DOI: 10.1126/science.1251701.
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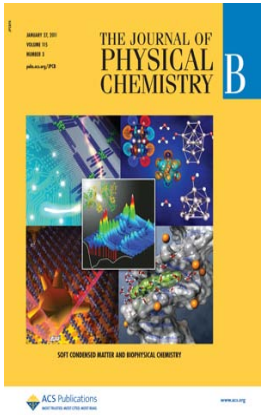
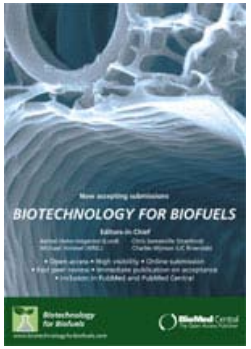
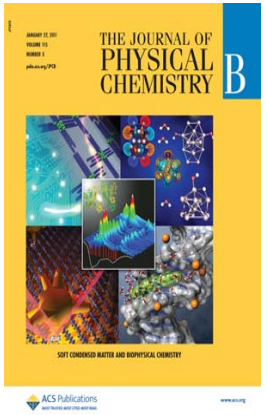
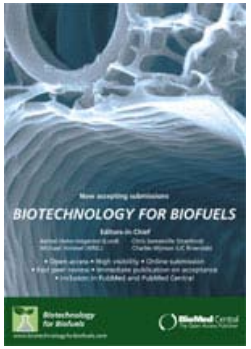




ABD and EEO: 2013-2015 Publications





- "Carbohydrate Protein Interactions That Drive Processive Polysaccharide Translocation in Enzymes Revealed From a Computational Study of Cellobiohydrolase Processivity," Brandon C. Knott, Michael F. Crowley, Michael E. Himmel, Jerry Stahlberg, and Gregg Beckham, **JACS** (2014) In press.
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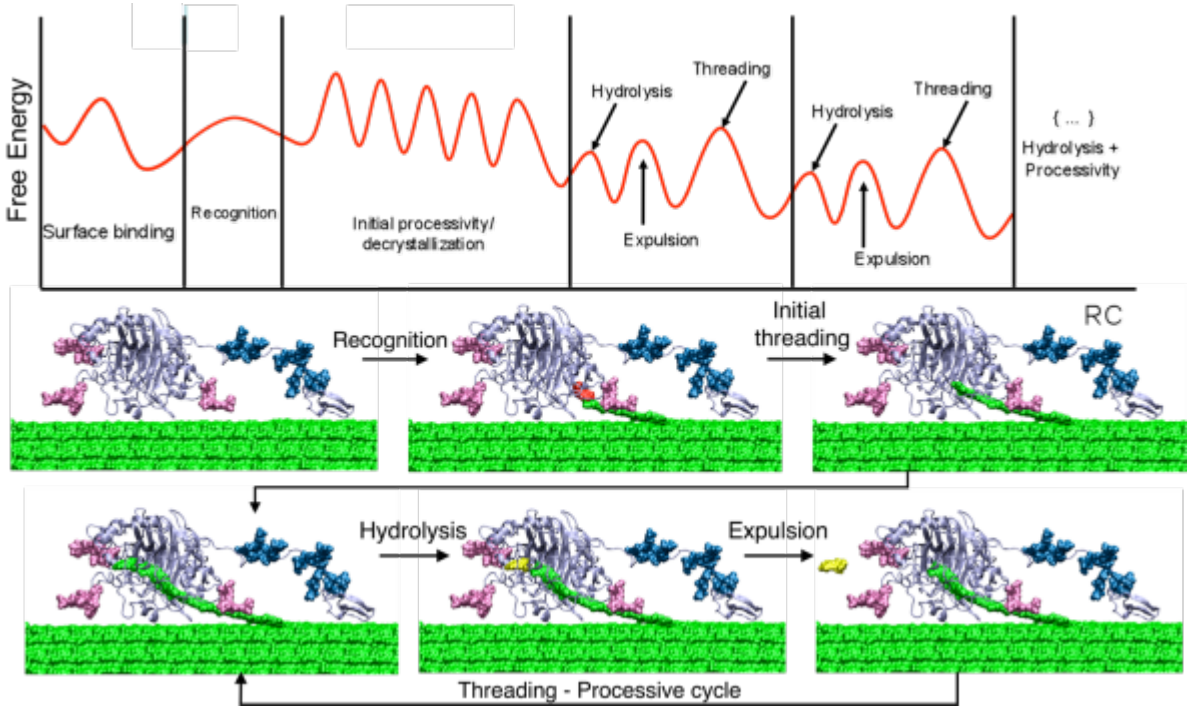
Additional accomplishments

3 – Technical Accomplishments/ Progress/Results

Our hypothesized action of a processive enzyme on cellulose

See BC Process Modeling and Simulation (Crowley)



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3 – Technical Accomplishments/ Progress/Results

Task 1 2015 EEO Yeast Expression of GH7 Cellobiohydrolase

Task 1. Develop a strain of *S. cerevisiae* or *Yarrowia* spp. for HTP cellulase expression and optimization	3/30/2015	Quarterly Progress Measure
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)

- 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 expressing →growth on cellobiose
    - Limited growth on cellulose but better than native
  - We have expressed several GH7s in *Saccharomyces* and *Yarrowia*; however, titers are very low.
  - Glutaminyl cyclase gene identified and cloned.

Task 1. milestone 3/30/2015

test#	1	2	3	4	5	6	7	8	9
carbon source	D	-	CB	GA	CB	CB GA	GA	D GA	-
enzyme					bG	bG	bG		bG
result	+	-	-	-	+	-	-	+	-

D- Dextrose, CB = cellobiose, GA = gluconic acid, bG = beta-glucosidase expressed in yeast

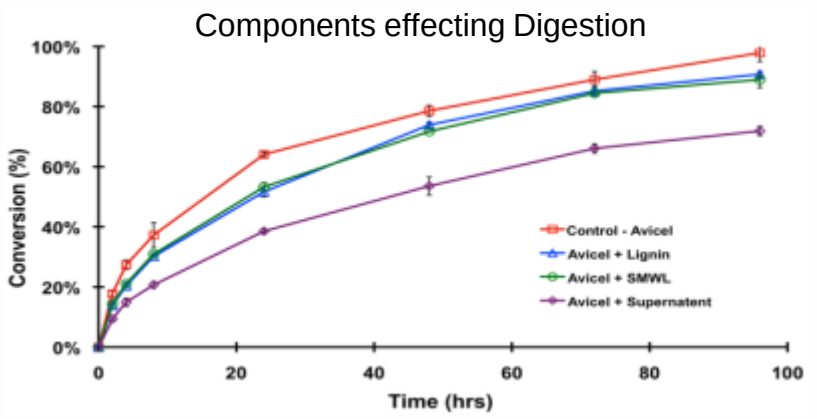
Conclusions:

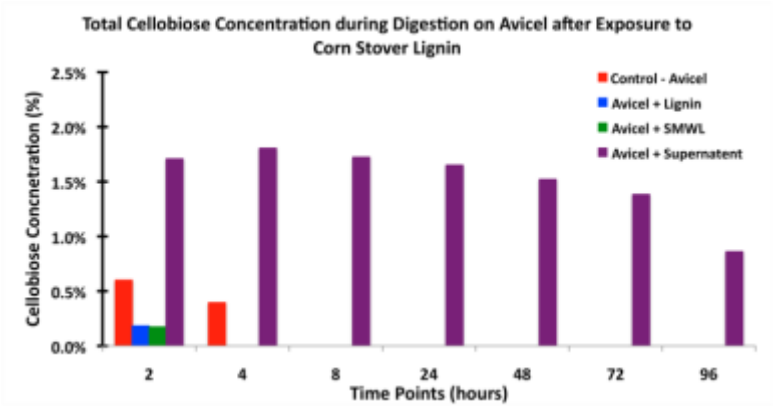
- *S. cerevisiae* cannot grow on cellobiose (col. 3)
- bG expression allows growth on cellobiose (col. 5)
- GA inhibits bG activity on cellobiose (col. 6)
- GA is not a suitable carbon source for growth (col. 7)

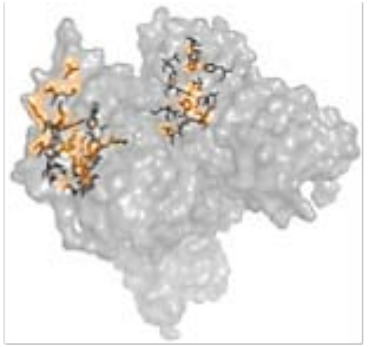
3 – Technical Accomplishments/ Progress/Results

Differential binding to biomass GHs to lignin

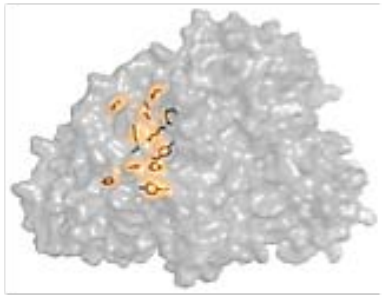
Task 2 2015 EEO Formulations can be improved by engineering bG



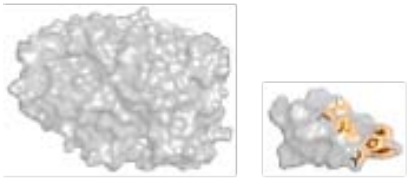




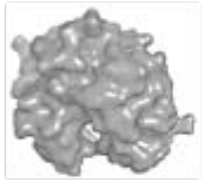
BSA – showing 2 of the 4 hydrophobic patches



GH3 – *Aspergillis niger*



Cel7A, including CBM1 - *Trichoderma reesei*



Xylanase - *T. lanuginosus*

Conclusions

- Glycosyl hydrolases are adsorbing to lignin under process conditions
- Temperature, pH and ionic conditions influence this phenomena
- The predominate enzymes affected are β-glycosidase and xylanases
- The bulk of β-glycosidase activity remains functional when bound to lignin
- Mechanisms of enzyme adsorption to lignin appear to be hydrophobic and electrostatic in nature

Detailed Milestone chart for 2014

Qtr	Due Date	Type	Milestones, Deliverables, or Go/No-Go Decision	Decision Criteria
Q1	12/31/2013	Regular	**Structural Design Mutations for Improving Cel7 Cellulases** Design phase of mutations to improve cel7 cellulase. Use homology and molecular mechanics modeling to generate in silico mutations that are predicted to alter one or more biophysical properties of Cel7A from T. reesei, P. funiculosom, or other GH7 exocellulase. Twelve or more variants will be designed to improve processivity, kon/koff parameters, lignin binding, and/or temperature stability.	
Q2	3/31/2014	Regular	**High Throughput Mutation and Functional Expression Screening for Cellulase Improvement** Success is to develop new transformation/expression methodology in yeast to functionally screen tens of thousands of random mutations in cel7A from Talaromyces emersonii, T. reesei, or other fungal source. The critical development is the screening methodology whereby non-active or low-activity variants are not propagated and variants with higher-than-baseline activity can be differentiated and ranked by activity. The screen must be stringent enough to reject the lowest 90%+ of the variants based on functional expression. It is expected that the host yeast will need to be engineered to functionally express Cel7 enzymes. The addition or deletion of post-translational modification genes such as glutaminyl cyclase, glycosyl transferases, chaperonins, or disulfide bond facilitators may be required for host yeast development.	
Q3	6/30/2014	Stretch	**Demonstrate Functional Expression of Multi-component Cellulases in Fungal Host** Use yeast or filamentous fungi (T. reesei or A. awamori) to express multi-domain cellulases (MDCs) that are functional in hydrolyzing cellulose and/or biomass. At high level, functionality is defined by performance similar to the enzyme from its native host. MDCs are bacterial in origin and comprise either multiple catalytic and/or binding domains joined together by linker peptides (single gene product) or through a separate "connector" protein (scaffoldin) in cellulosomes. We will examine at least two examples. In this case, functionality of the expressed cellulase is determined by hydrolysis of cellulose and/or cellulosic biomass (depends on which catalytic domains are present) at levels that are meaningful, i.e. >60% conversion at 120 h with protein loadings below 50 mg protein/g substrate. Current attempts in this area have had very limited success; however the advantages of these enzymes, including high thermotolerance, high specific activity, and multi-substrate hydrolysis, make pursuing this work worthwhile.	
Q4	9/30/2014	Regular	**Improved Cellulases for Biomass Conversion** Cellulase improvement is incremental, as we continue to explore and engineer diverse enzyme systems. Random and rational-design Cel7A engineering, HTP functional screening in yeast, and multi-component cellulase expression all hold promise for improved activity, defined here as a meaningful increase in specific activity (>10%), thermotolerance (>50 C), decreased protein loading requirements (10% or more less protein/g substrate for same conversion level), increased initial rate (>10% reduction in time-to-target of 80% converision), increased extent of conversion (>2% increase in conversion yield of glucose or xylose), or increased activity by engineering decreased non-specific lignin binding.	

Detailed Milestone chart for 2015

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-quarter 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)

4 – Relevance

2014 NREL's Accomplishments Attracting Attention from Biofuels Companies

novozymes
Rethink Tomorro

Novozymes, Inc.
1445 Drew Avenue
Davis, CA 95618-4880
Telephone: 530-757-8100
Facsimile: 530-758-0317
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

Recyck

Abengoa Research SL
c/ Energía Solar, 1
Palmas Altas
41014 Sevilla (España)
Tel. +(34) 95 493 70 00
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

Testimony from Novozymes in 2013

Examples illustrating how TCR task's fundamental discoveries impact commercial enzyme development

novozymes
Rethink Tomorrow

Key TCR Discoveries

"Accessory components" might be limiting in *T. reesei* secretome:

- Extensive work published 2008-2009 indicated that overall catalytic performance of *T. reesei* cocktails could be improved by addition of non-cellulolytic components
- These findings inspired further work among enzyme development companies, including Novozymes

2-D gel fingerprinting - powerful enzyme discovery tool:

- Very early work on 2-D fingerprinting of *T. reesei* proteins was influential in the development of Novozymes' secretome analysis capabilities
- Use of the tool ultimately led to a number of important discoveries including the abundance and diversity of GH61 proteins in *Thielavia terrestris.*

Glycosylation is important factor impacting the specific activity of heterologous cellulases

- Increased awareness of role of glycosylation, and impacted selection of expression hosts in discovery pipeline

Relevant TCR Publications

Accessory components:

Selig, M. J., T. B. Vinzant, M. E. Himmel, and S. R. Decker. 2009. "The Effect of Lignin Removal by Alkaline Peroxide Pretreatment on the Susceptibility of Corn Stover to Purified Cellulolytic and Xylanolytic Enzymes *Appl. Biochem. Biotechnol.* 155:397-406.

Decker, S. R., M. Siika-aho, and L. Viikari. 2008. Enzymatic Depolymerization of Plant Cell Wall Hemicelluloses. In: M. E. Himmel (ed*). Biomass Recalcitrance.* Blackwell Publishing, Oxford, UK.

Selig, M. J., E. P. Knoshaug, W. S. Adney, M. E. Himmel, and S. R. Decker. 2008. "Synergistic Enhancement of Cellobiohydrolase Performance on Pretreated Corn Stover by Addition of Xylanase and Esterase "*Bioresource Technol*. 99:4997-5005.

Selig, M. J., S. R. Decker, E. P. Knoshaug, J. O. Baker, M.E. Himmel and W. S. Adney 2008. "Heterologous Expression of *Aspergillus niger* β-d-Xylosidase (XlnD): Characterization on Lignocellulosic Substrates *Appl. Biochem. Biotechnol.* 146:57-68.

Knoshaug, E. P., M. J. Selig, J. O. Baker, S. R. Decker, M. E. Himmel, and W. S. Adney. 2008. "Heterologous Expression of Two Ferulic Acid Esterases from *Penicillium funiculosum Appl. Biochem. Biotechnol*. 146:79-87.

2-D gel fingerprinting:

Vinzant, T.B., W. S. Adney, S. R. Decker, J. O. Baker, M. T. Kinter, N. E. Sherman, J. W. Fox, and M. E. Himmel. 2001. "Fingerprinting *Trichoderma reesei* Hydrolases in a Commercial Cellulase Preparation" *Appl. Biochem. Biotechnol.* 91-93:99-107.

Glycosylation:

Jeoh, T., Michener, W., Himmel, M. E., Decker, S. R., & Adney, W. S. 2008. Implications of cellobiohydrolase glycosylation for use in biomass conversion. *Biotechnol Biofuels*, 1:10.

EEO Abstract

The goal of this project is to ensure that cellulase enzyme loadings can be reduced to the DOE 2017 target of 10 mg/g cellulose using modern enzyme engineering and cocktail formulation optimization strategies. Given that cost effective biomass conversion to sugars is a common theme for many conversion processes, the overall bioenergy industry also benefits from this work. We are using a knowledge based approach, which links protein structure to performance. We have pioneered the close coordination of computational analysis and experimental validation for cellulase improvement and have achieved positive results (1.25 to 1.6 fold improvement in Cel7A performance). We have successfully demonstrated the ability to model cellulase action, design structural features likely to impact performance, build targeted mutations, and show improved performance on pretreated biomass. We have also demonstrated the utility of the high temperature hold biomass conversion scheme using cocktails of caldiphilic bacterial cellulases. The early stages of selection of glycosylation and *P. funiculosum* subdomain mutations in *T. reesei* Cel7A suggested for future work in FY2013 are being accomplished in FY2015, with successful outcomes. Active site mutations and mutations suggested by newly initiated HTP yeast screening will be accomplished in FY2015-16. Work to express newly discovered, highly active bacterial cellulases in *T. reesei* will be accomplished in FY2015-17.