

# Enzyme Engineering and Optimization (EEO)

March 8, 2021

Biochemical Conversion and CO<sub>2</sub>  
Utilization

Michael Himmel\_NREL

# Project Overview

- ***Project History***

- We propose to enhance the performance of key process hydrolytic enzymes to **enable the higher sugar yields at lower enzyme loadings** set out in the FY2019 NREL Biochemical Platform State of Technology (SOT).
- Achieve a critical **understanding of the science underpinnings** regarding the structure and function of cellobiohydrolase (Cel7A and Cel6A) enzymes acting on pretreated biomass.

- ***Describe your project goals***

- **Meet or exceed 90% conversion of glucan and xylan** to monomers using 10 mg enzyme / gram loadings of DMR (deacetylated mechanically refined) pretreated corn stover which is required to meet economic targets.
- **Share superior enzymes with Novozymes** for testing and possible incorporation in improved Gen2 and Gen1.5 formulations.

# Project Overview

## ***Briefly frame the project with the Heilmeier Catechism:***

### ***1. What are you trying to do?***

Employ a combined discovery/protein engineering approach to improve the critical cellulases and generally share progress through publications

### ***2. How is it done today and what are the limits?***

Today's formulations employ well studied, naturally occurring enzymes; Nature harbors better examples and these templates can be used to engineer superior enzymes.

### ***3. Why is it important?***












To reduce overall process uncertainty and cost: enzymes are ~10% of MFSP.

### ***4. What are the risks?***

High Level – That superior enzymes don't exist in Nature.

Mid Level – That the identified enzymes don't express from *T. reesei* or are unstable.

# Market Trends

Product	 Anticipated decrease in gasoline/ethanol demand; diesel demand steady
	 Increasing demand for aviation and marine fuel
	 Demand for higher-performance products
Feedstock	 Increasing demand for renewable/recyclable materials
	 Sustained low oil prices
	 Decreasing cost of renewable electricity
	 Sustainable waste management
	 Expanding availability of green H <sub>2</sub>
	 Closing the carbon cycle
Capital	 Risk of greenfield investments
	 Challenges and costs of biorefinery start-up
	 Availability of depreciated and underutilized capital equipment
Social Responsibility	 Carbon intensity reduction
	 Access to clean air and water
	 Environmental equity

## NREL's Bioenergy Program Is Enabling a Sustainable Energy Future by Responding to Key Market Needs

### Value Proposition

- The new bioeconomy needs biomass deconstruction processes that **work cost effectively** to provide sugars for upgrading.
- Supports industry in formulating advanced cellulase preparations capable of **reaching 90+% conversion of glucan/xylan at minimal enzyme loadings.**

### Key Differentiators

- Superior cellobiohydrolase enzymes.
- We propose to find **enzymes from natural diversity that are better than currently used enzymes** and we have demonstrated that we can clone, express, and build chimeric cellulases in *Trichoderma reesei*.

## 2. Approach

### *Technical Approach*

- To improve Cel7A, the most important fungal cellulase, following a two-step strategy.
  - **Megatron.** Genes chosen from Nature are cloned and expressed in *T. reesei* using a constitutive promotor and the recombinant enzymes are tested for efficacy on various substrates. The top performers are subjected to structure studies and the subsites thought to confer enhanced performance are determined.
  - **King Chimera.** The subsites are then genetically combined to produce new mutants of the native “best in class” Cel7A we have found in Nature (*Penicillium funiculosom*) and then expressed in *T. reesei* and tested.
- **Substrate Preparation.** Improve Digestibility of Acidified DMR Solids. In pursuit of a reproducible biomass sample for enzyme testing we found that the DMR solids acidified by LTAD for disk refining at Andritz held a lignin barrier that is effectively removed by a final dilute alkali wash.
- ***T. reesei* Tools.** Evaluate 2A peptide technology for expressing multi-cistronic cellulase genes in *T. reesei* for research; as well as industrial application.

# 1. Management

## Project Structure

**Milestones** used to keep us on track and our goals clear. Includes: Regular, Quarterly, and Annual, with SMART milestones identified. One **Go/No-Go** decision is planned for March of each year. **Industrial partner** (Novozymes).

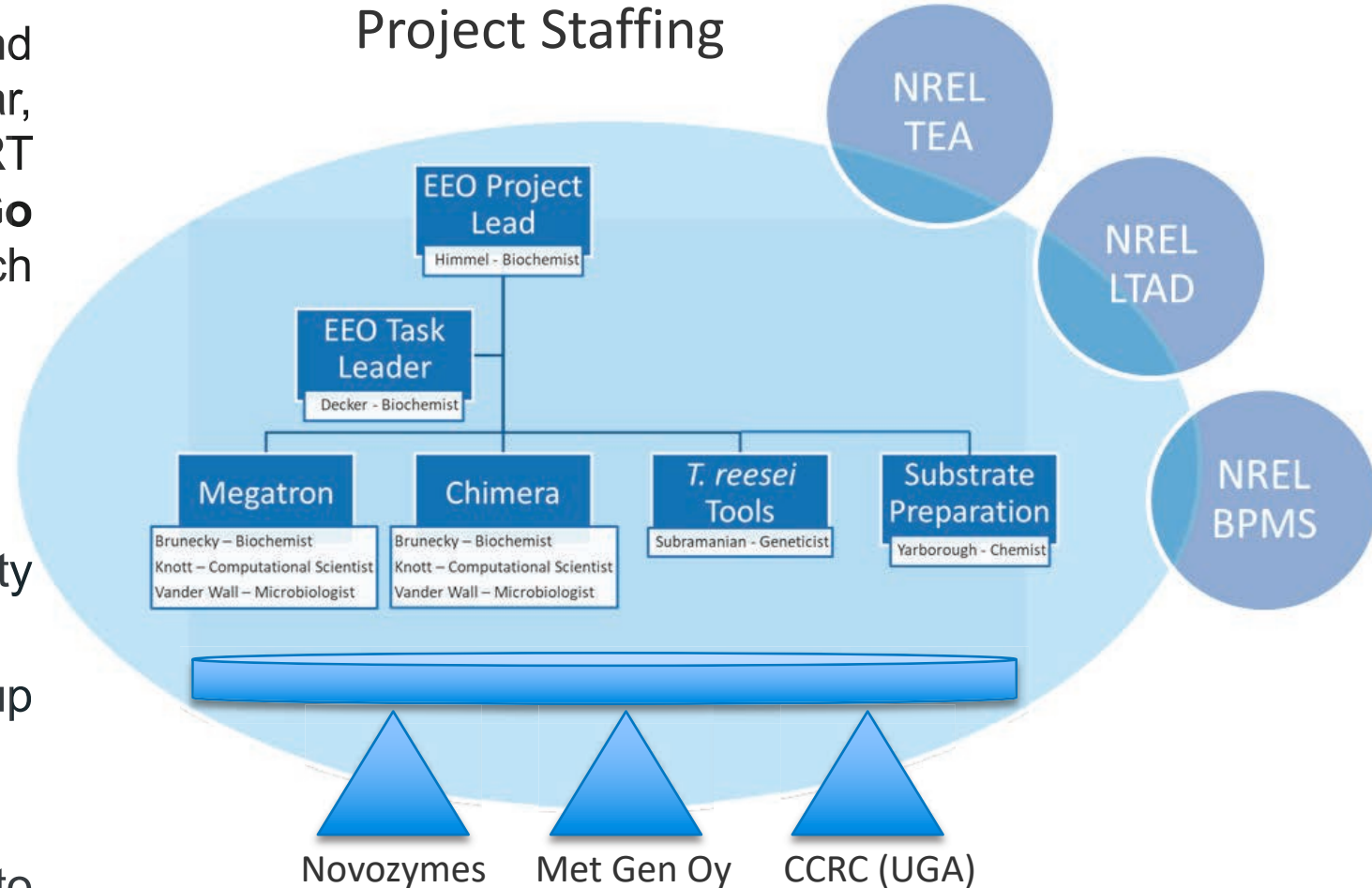
## Risks and Mitigation

**R:** Top Cel7A performers from diversity can be expressed in *T. reesei*.

**M:** Ensure at least one functioning backup host is in place (*A. niger*).

**R:** That all chimeras made are inactive.

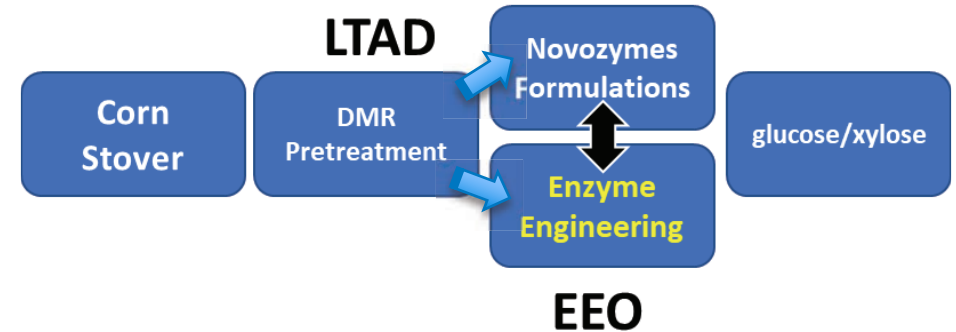
**M:** Reduce the size of the subsites to probe limit of structural disturbance.



## 2. Approach

- **Top 2-3 potential challenges (basis of GoNoGo)**

1. To show that better CBHs exist in Nature
2. To show that these “hot spots” or beneficial subsites can be combined into **uniquely superior enzymes**.



- **Go/No-Go decisions and why critical.**

1. Go confirmed by the initial random discovery of two superior enzymes in data bases and if the answer was NoGo, then an entirely new project strategy would have been needed.
2. Go confirmed by the testing of several chimeras that are active and performed much better than the parental enzyme (backbone structure). If NoGo, the subsite concept may have been flawed.

- **Economic and/or technical metrics used to measure progress.** Estimates are based on making further “isolated” enzyme loading/EH yield adjustments. From the NREL FY20 SOT (DMR, BDO production, and burning lignin).

- The FY19 case MFSP (enzyme contribution), \$/GGE = \$7.79 (\$0.59)
- The FY20 case MFSP (enzyme contribution), \$/GGE = \$6.80 (\$0.45)

## 2. Approach

**From TEA (R. Davis) All cases based on BDO production and burning lignin**

*New targets?*

Parameter	FY19 SOT	FY 20 SOT	FY 22 EEO Goals A	FY22 EEO Goals B	FY 30 EEO Goals
Enzyme loading (mg/g cellulose)	12	10	10	10	5
Glucan to glucose	84%	88%	95%	97%	97%
Xylan to xylose	82%	93%	95%	97%	97%
MFSP (enzyme contribution), \$/GGE	\$7.79 (\$0.59)	\$6.80 (\$0.45)	\$6.54 <sup>a</sup> (\$0.44)	\$6.51 <sup>a</sup> (\$0.43)	\$6.31 <sup>a</sup> (\$0.23)
MFSP reduction vs FY19 attributed to enzyme performance (enzyme use/EH yields alone)	NA	\$0.69	\$0.95	\$0.98	\$1.18

<sup>a</sup> Future EEO projections for FY22-FY30 are based on extrapolating from FY20 SOT basis, only modifying enzyme usage and EH yields in isolation vs FY20 SOT basis (further MFSP reductions are possible through other process improvements + lignin conversion to coproducts)



# 3. Impact

## **Project Impact** – Enable the cost-effective production of sugar monomers from recalcitrant lignocellulosic biomass

- Former commercial enzyme products were formulated for dilute acid hydrolyzed biomass. Solids from the DMR process differ considerably.
- The enzyme production and use contributions to BDO MFSP are about 1/10<sup>th</sup> the cost but contribute substantially to the de-risking needed to commercial this process.
- The ability to recover sugars, about 11-16% of soluble sugars released by Cellic enzymes, now locked in oligosaccharides poses new challenge.
- New enzyme formulations designed for Gen2 biomass conversion processes will also enhance Gen1.5 processes.

## **Discuss how you are disseminating your results**

- We publish, patent, and present BETO funded work whenever possible. See **19 published papers** and **two patents** in the Additional Slides.
- During COVID, public presentations were essentially nonexistent.
- We have an R&D CRADA with Novozymes to develop improved cellulases (**see Testimonials**)



# Evidence of Industrial Impact

## CRADA Novozymes - Advanced Cellobiohydrolases Supports

- EEO FOA Project (Himmel)
- Technology Commercialization Fund (TCF) - Cel6A Improvement project (Brunecky)

“NREL was and is a key partner in the development of enzymes for the biomass-to-biofuels industry. In the context of the current CRADA we see strong performance of NREL molecules in our current 1.5G product discovery work.” Sarah Teter, Global Manager, Novozymes. January 2021

SOLARBIOTECH

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[www.solarbiotech.com](http://www.solarbiotech.com)

January 22<sup>nd</sup>, 2021

Norton, VA, USA

Att. DOE Bioenergy Technologies Office (BETO)

Ref. Enzyme Engineering and Optimization Program, Michael Himmel, NREL

Dear Manager:

Over the past year and earlier, I have been closely following the progress made by Dr. Michael Himmel of NREL in the context of the Enzyme Engineering and Optimization program his team has been leading. The progress made by Dr. Himmel since the initiation of this program has had a great impact on the plant biomass conversion industry with the development of novel and outstanding enzyme candidates for the conversion of plant polysaccharides into valuable products, including chemicals and biofuels. The newly developed enzymes and the tools to improve them have shown great performance and present tremendous potential for further advancing biomass biorefinery towards techno-economic viability of a wide range of renewable fuels and chemicals technologies.

We look forward to witnessing continuous success of the Enzyme Engineering and Optimization Program and encourage further funding of this important project.

Best Regards,



**Alex Berlin, Ph.D.**  
Founder, CEO & CTO  
Solar Bioprocessing, LLC  
[alex@solarbiotech.com](mailto:alex@solarbiotech.com)  
Phone# 530-304-2144  
[www.solarbiotech.com](http://www.solarbiotech.com)

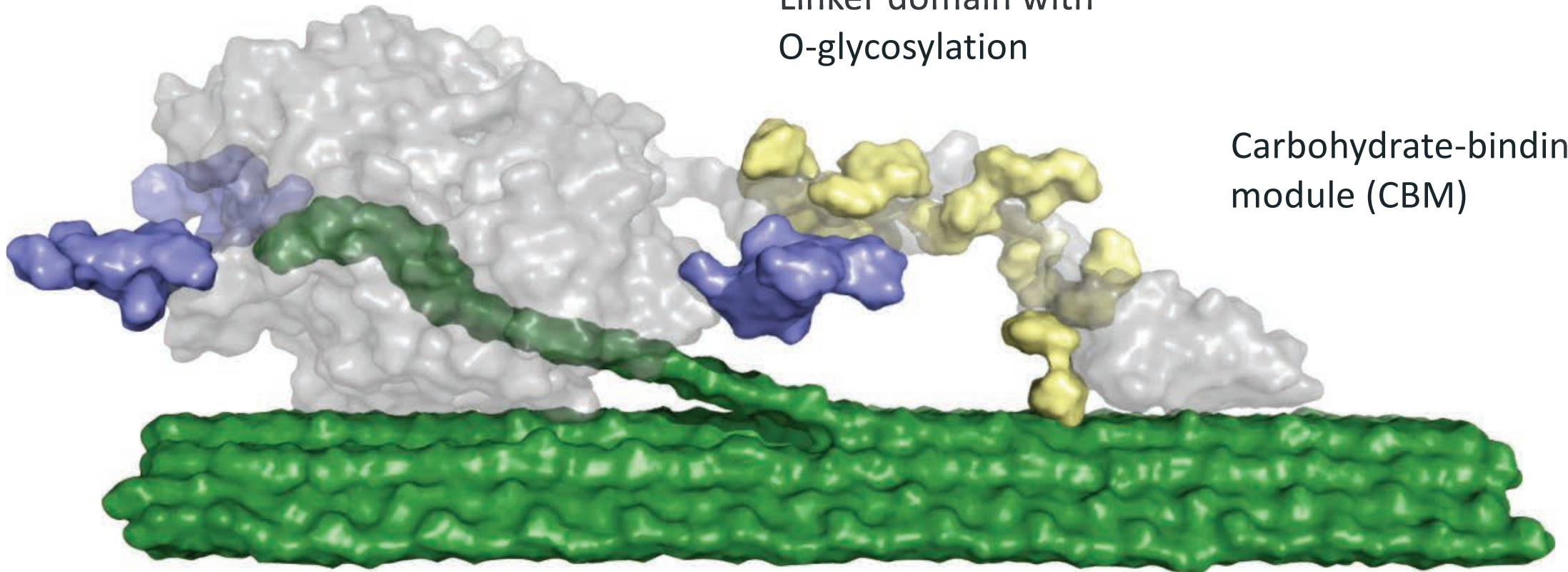
## 4. Progress and Outcomes

***Cellobiohydrolase I (Cel7A) is the most important enzyme in industrial lignocellulosic biomass conversion***

Catalytic domain with *N*-glycosylation

Linker domain with  
O-glycosylation

Carbohydrate-binding  
module (CBM)



## 4. Progress and Outcomes

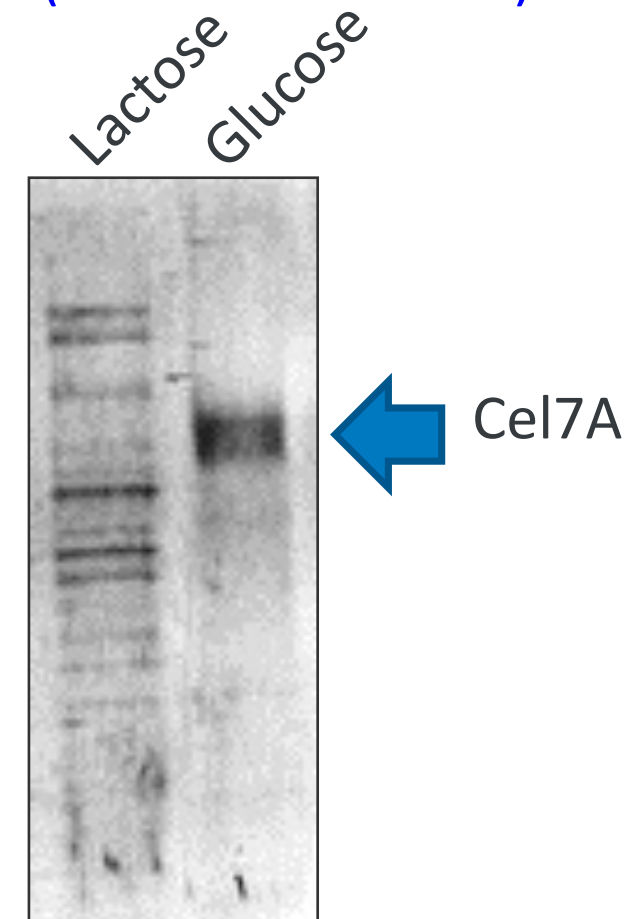
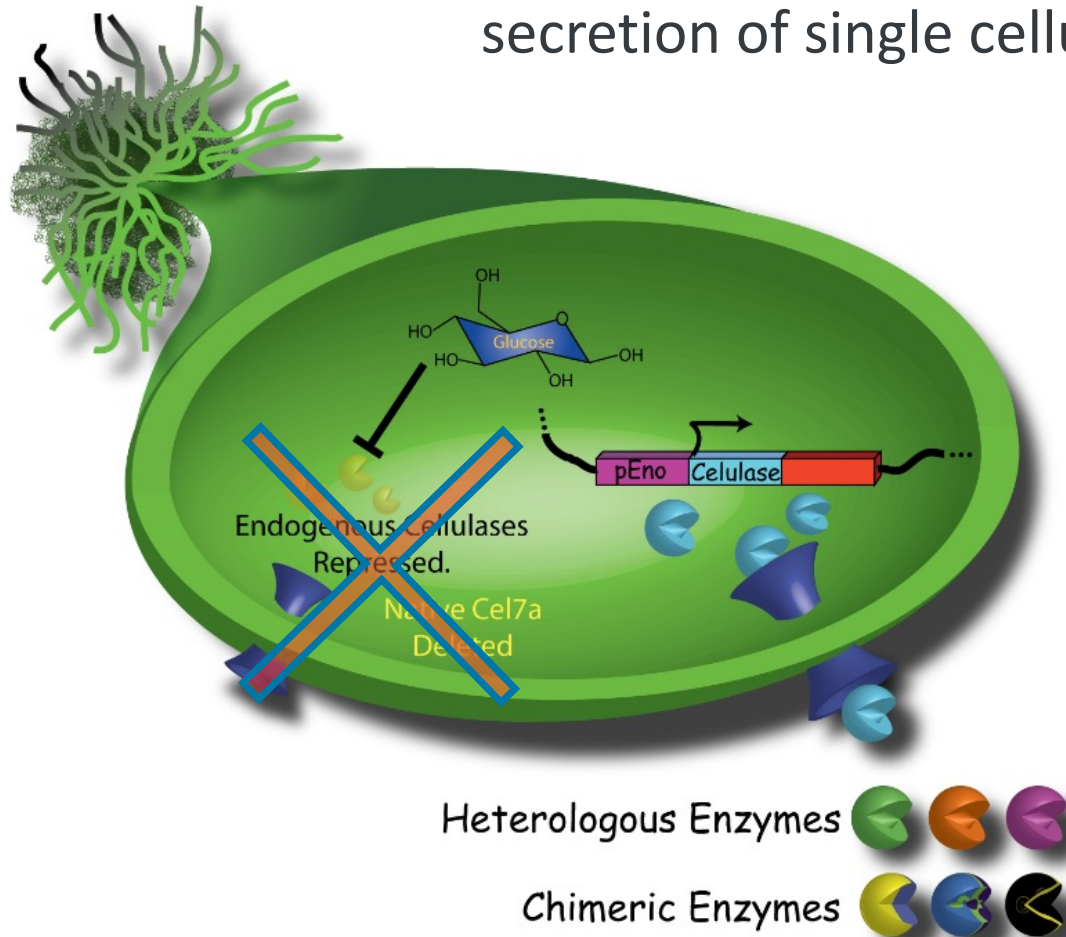
### *Critical Tool Development—Building a *T. reesei* expression system*

- Began working in *Aspergillus awamori*
  - “Industrial analog” strain
  - *A. awamori* 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)

# 4. Progress and Outcomes

## *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”)



# 4. Progress and Outcomes

## *Preparing DMR substrates in FY2020*

Substrate provided to EEO in FY2019.

- Dilute acid pretreated corn stover (DAPCS)
- Acidified DMR solids from LTAD (deacetylated pH ~10, acidified and shipped to Andritz, Sezgo milled at NREL w final pH ~2.0)

Substrate used by EEO in FY2020 was modified by EEO.

- Acidified DMR solids washed with dilute NaOH to remove residual/replated lignin just prior to final neutralization and enzyme addition.

*Conclusion: the DMR process is ideally an “always alkaline” pretreatment and ANY excursion to lower pH during handling causes reprecipitation of the residual lignins.*

- *This outcome can be completely restored by a simple wash with 200 mM NaOH before neutralization and enzyme addition.*

Substrate made available to EEO fall of FY2020 (Xiaowen Chen, LTAD).

- DMR solids from two stage (NaOH & Na<sub>2</sub>CO<sub>3</sub>) deacetylation followed by ozonolysis/disk refining

Substrates not available to EEO.

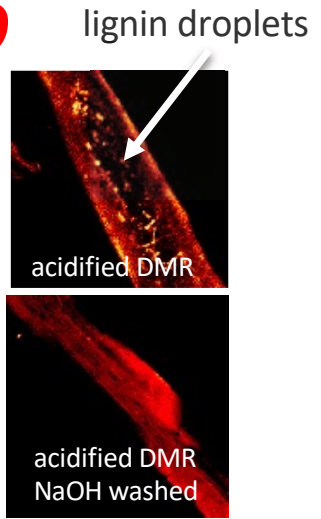
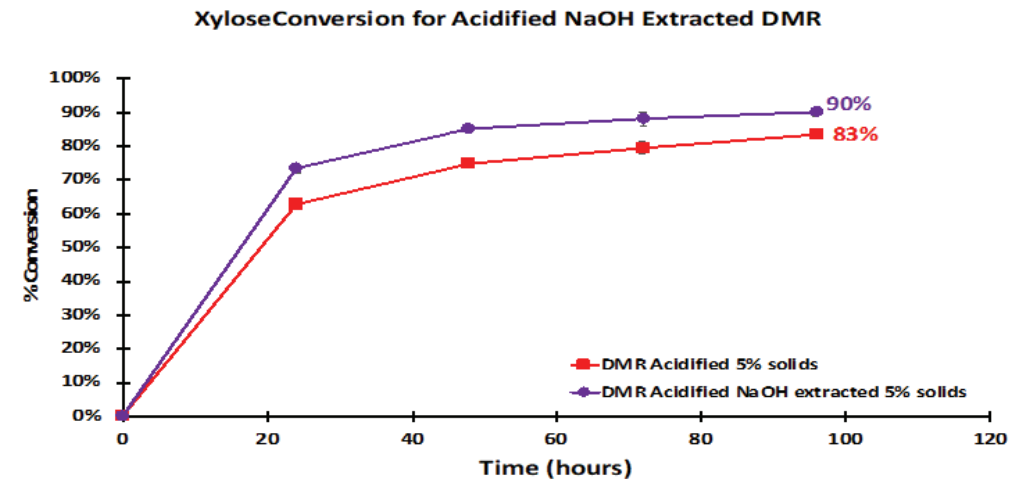
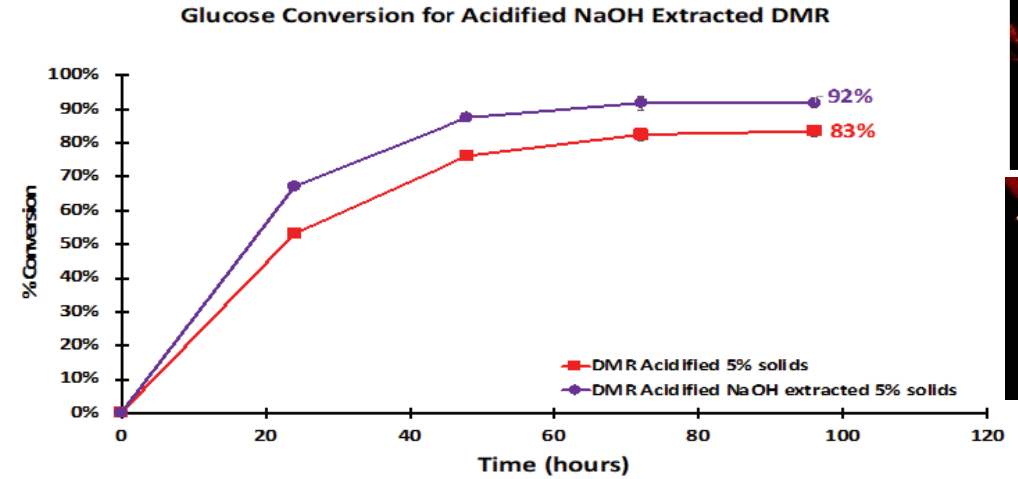
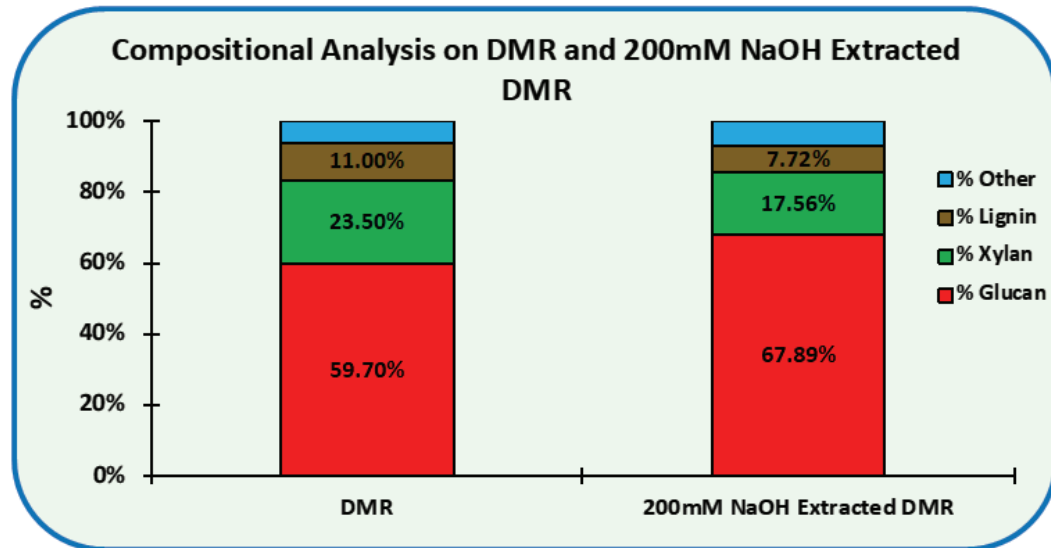
- DMR solids developed following the NREL SOT (always alkaline process from pilot plant).

# 4. Progress and Outcomes

## Cleanup of acidified DMR solids for enzyme digestion R&D

Final NaOH extraction (wash) of DMR solids

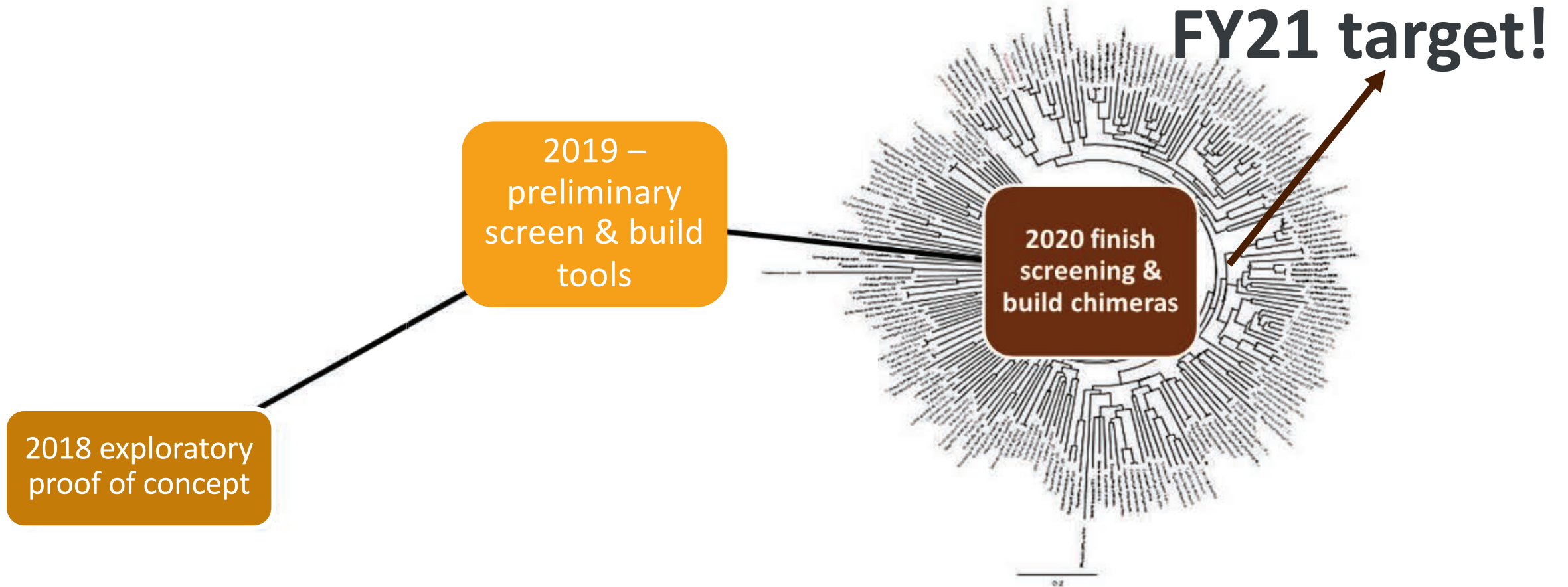
- 200mM NaOH
  - 2 hours at 80°C
  - Removal of liquor
  - Washed and titrated to pH 7
- Ctec3/Htec3
  - 96 hours at 50°C at pH 5
  - (8 mg Ctec3 + 2 mg Htec3)/g cellulose



SRS Imaging  
Y. Zeng

# 4. Progress and Outcomes

## *Megatron: Testing Nature's Cel7A Diversity*



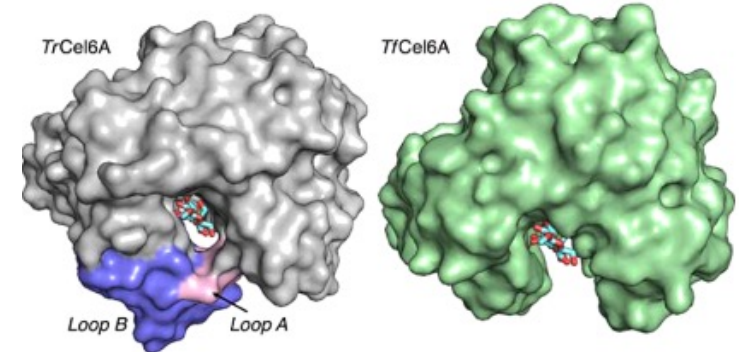


# 4. Progress and Outcomes

## *Megatron II: Cel6A diversity – Novozymes Partnership - Roman Brunecky TCF*



We constructed a library of ~1500 phylogenetically diverse GH6 enzymes and down selected a sample set of 100 processive exo-acting GH6 enzymes to test based on representative diversity of the genes and additional input from Novozymes.

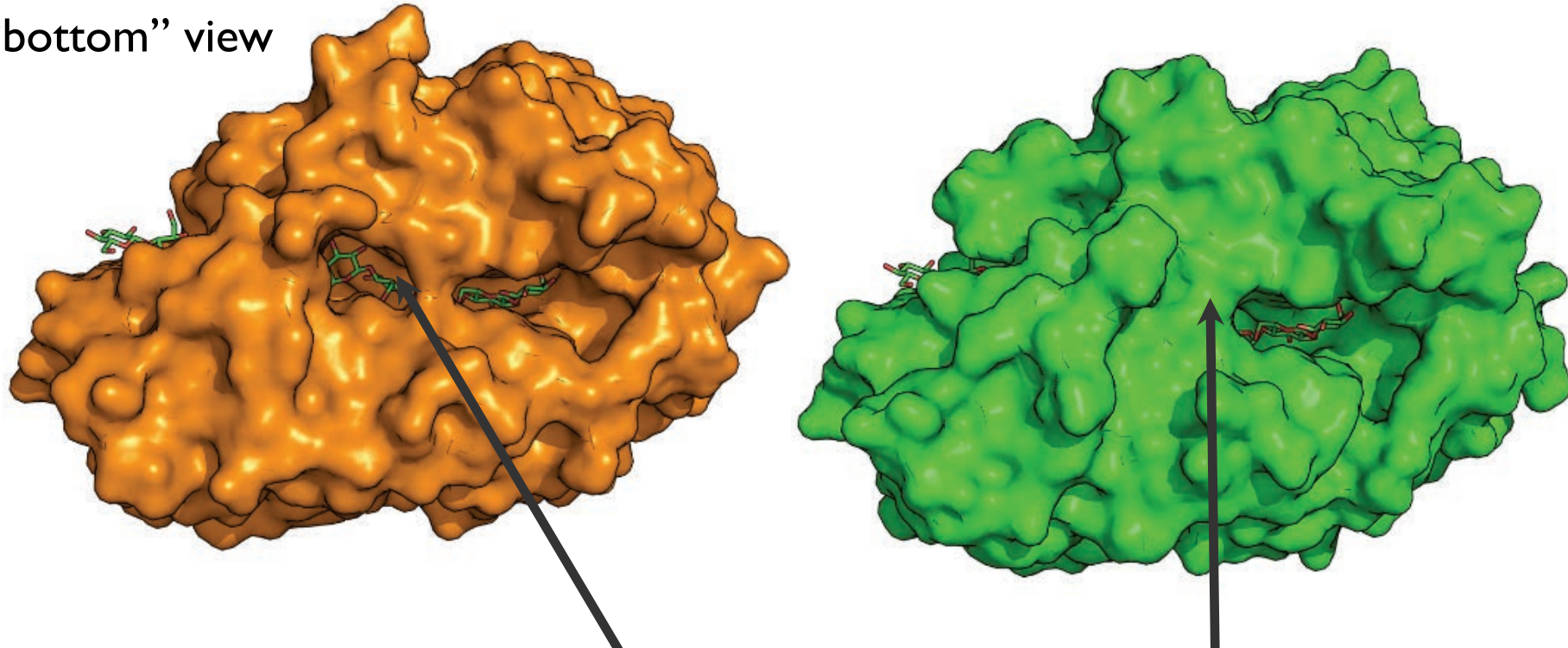


A and B loops were identified for each candidate and loop length was used to identify Cel6A exo vs endo activity

# 4. Progress and Outcomes

## *Focusing on the Pfun enzyme: comparison with TrCel7A*

“bottom” view



4C4C ligand shown (cellononaose)

**Orange** = PfCel7A

**Green** = TrCel7A (4C4C)

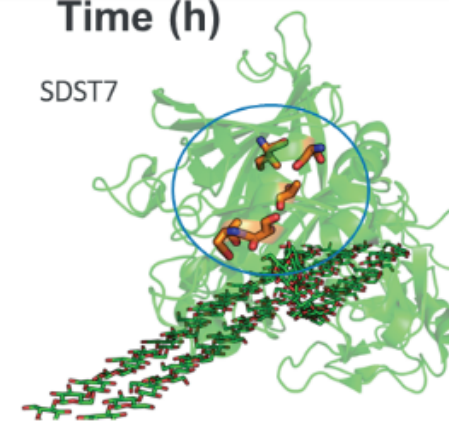
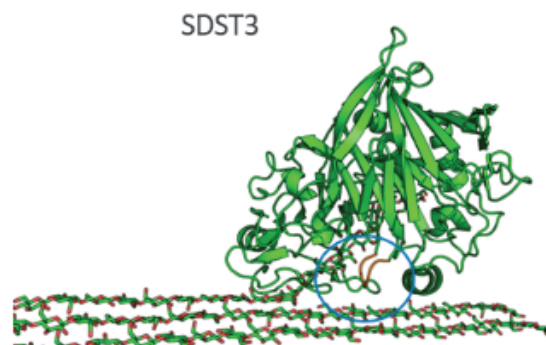
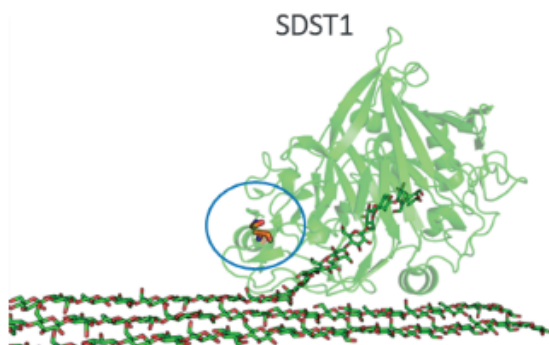
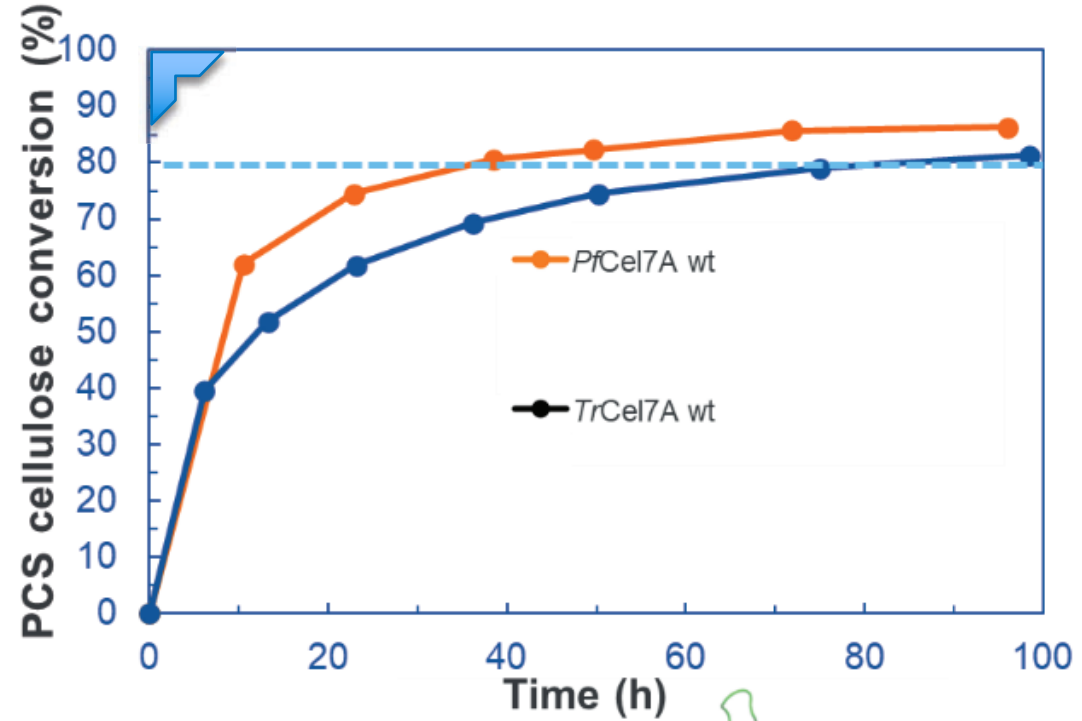
PfCel7A: alanine

TrCel7A: tyrosine

# 4. Progress and Outcomes

**SUBSITES CONCEPT: Can we capture the design features of the *P. funiculosus* enzymes?**

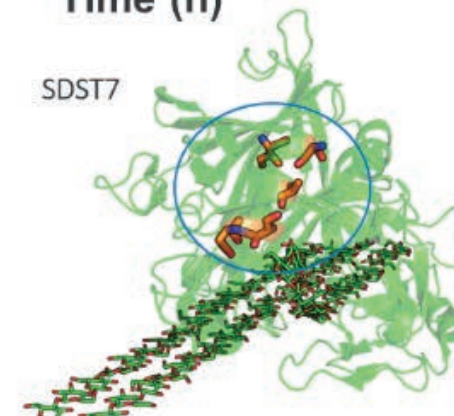
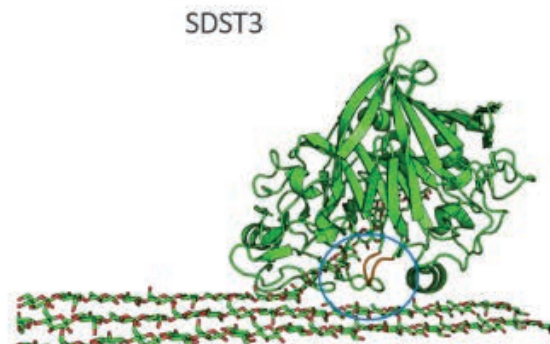
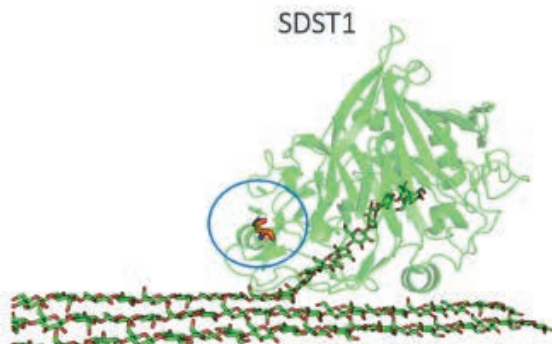
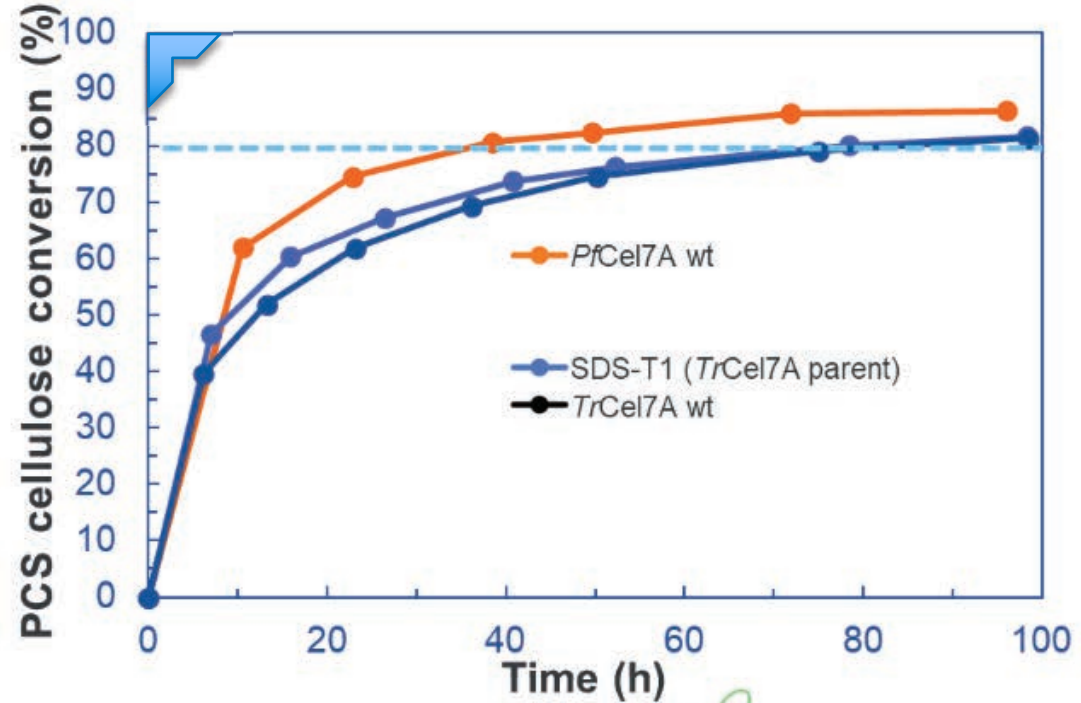
- Twelve subdomain swaps (*Pf*→*Tr* and *Tr*→*Pf*) were engineered and expressed in *T. reesei*
- Two *Pf*→*Tr* SDSs improved activity and were additive, nearly matching that of *Pf* parent.
- Conclusion: we can import subdomains from a superior enzyme to an inferior enzyme making it equivalent to the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



# 4. Progress and Outcomes

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

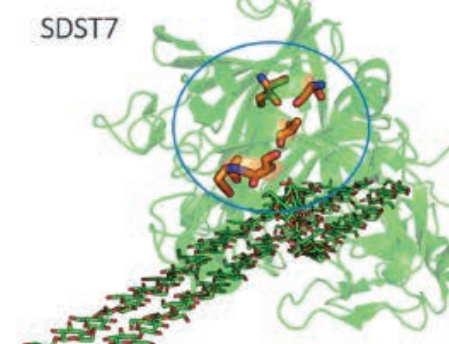
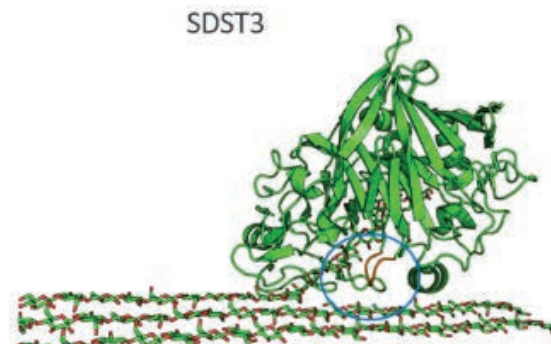
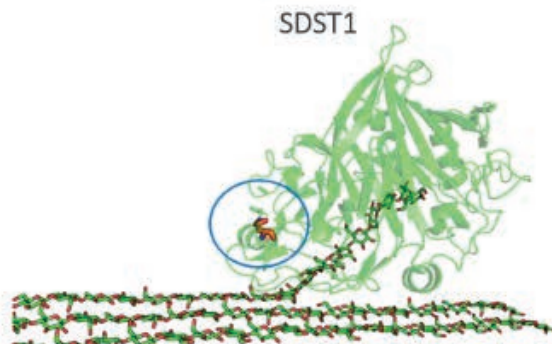
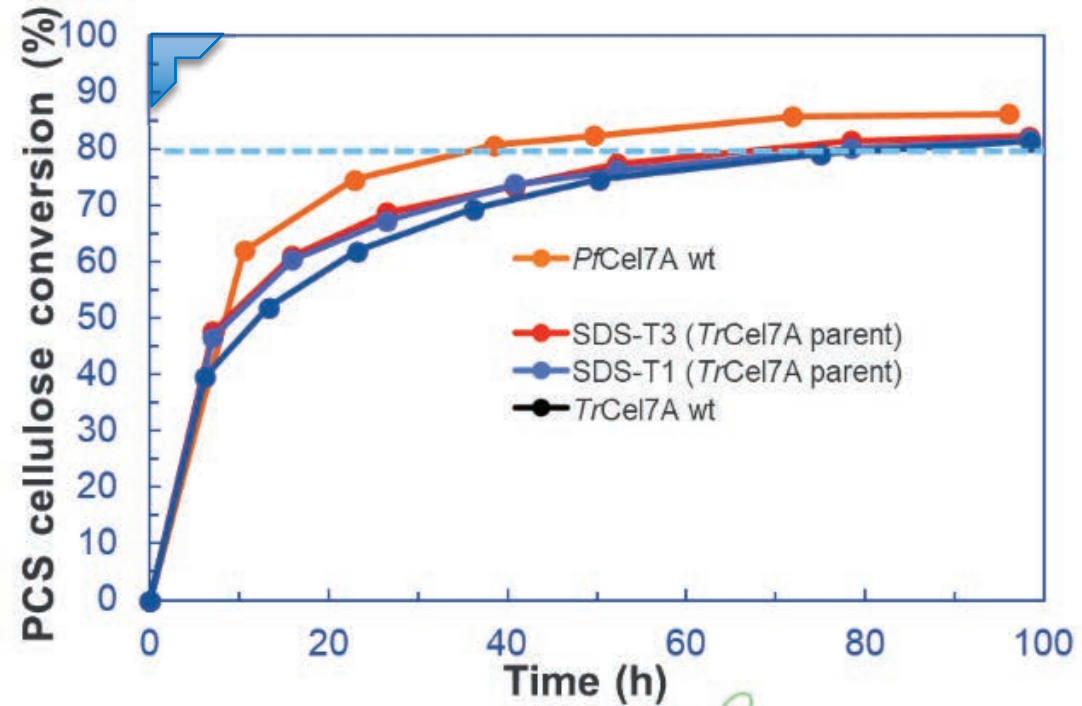
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# 4. Progress and Outcomes

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

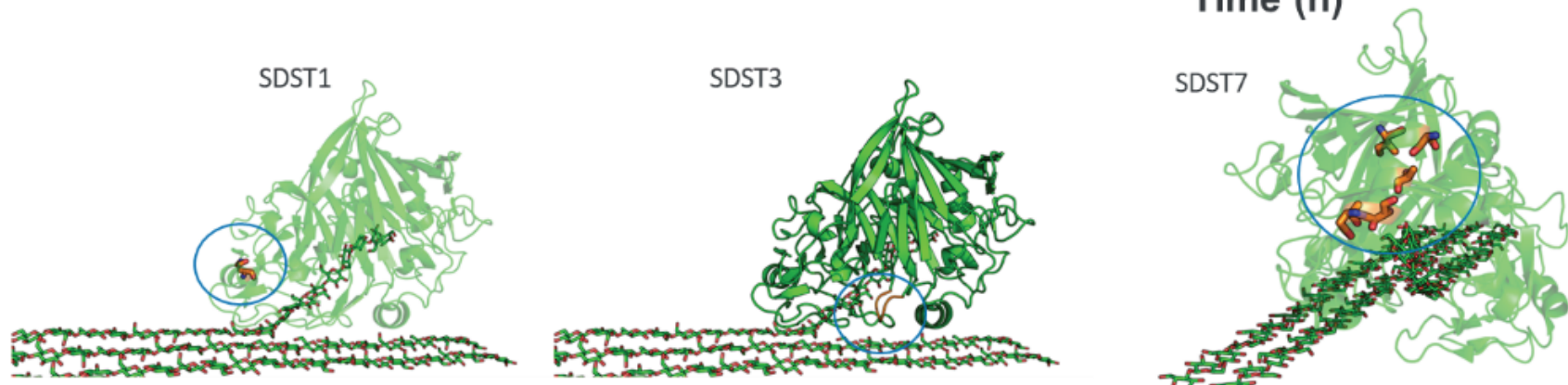
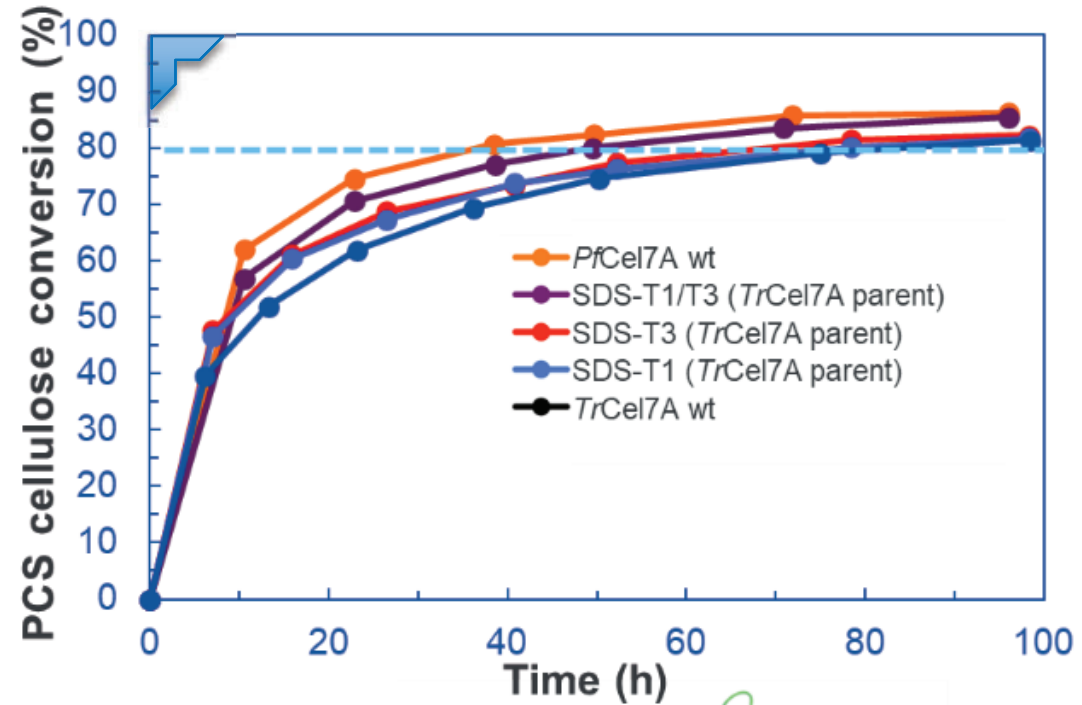
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# 4. Progress and Outcomes

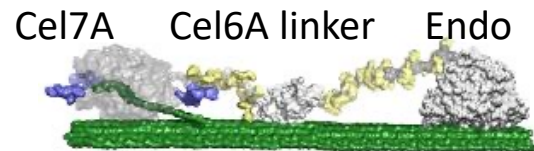
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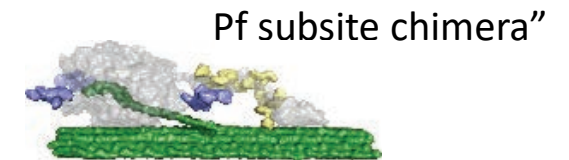
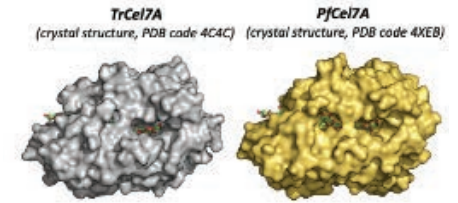


# 4. Progress and Outcomes

**Work in early 2020 on non-SOT substrates (DAPCS and acidified DMR solids)**



Campaign	ID	Activity Relative to Tr Cel7A
Control	<b>JLT102A (<i>T. reesei</i>)</b>	<b>1.0 (1)*</b>
1 <sup>st</sup> diversity Cel7A	HC10 ( <i>P. funiculosum</i> )	1.63 (1)
Tr/Pf Domain Chimeras (shows that linker is the least important)	TTP (all in <i>T. reesei</i> ) TPP PPT PTP PPP – fully restored	1.05 (1) 1.15 (1) 1.27 (1) 1.51 (1) 1.63 (1)
Pf Subsite Chimeras (on <i>T. reesei</i> backbone)	JLT102A ( <i>T. reesei</i> ) P1/P3 T1 T3 T1/T3 – not quite	1.00 (1) 0.78 (1) 1.10 (1) 1.17 (1) 1.35 (1)
Raptors (multifunctional)	PfC1Ac PfC3Ac	1.5* (2) 1.5 (2)
Thermostability	Species C Species D	1.10 (2) 1.29 (3)



\* (1) DAPCS - dilute acid pretreated corn stover, (2) - acidified DMR, (3) - Avicel

# 4. Progress and Outcomes

## Megatron: Robotic Screening and Down Selection

- ~100 putative Cel7 CBH genes were synthesized, cloned, and expressed in *T. reesei* *cel7a*-delete strain under a constitutive promoter.
- Clonal isolates screened for expression and then grown in 24-well plates (uniform conditions).

### Screen in the context of cellulase background

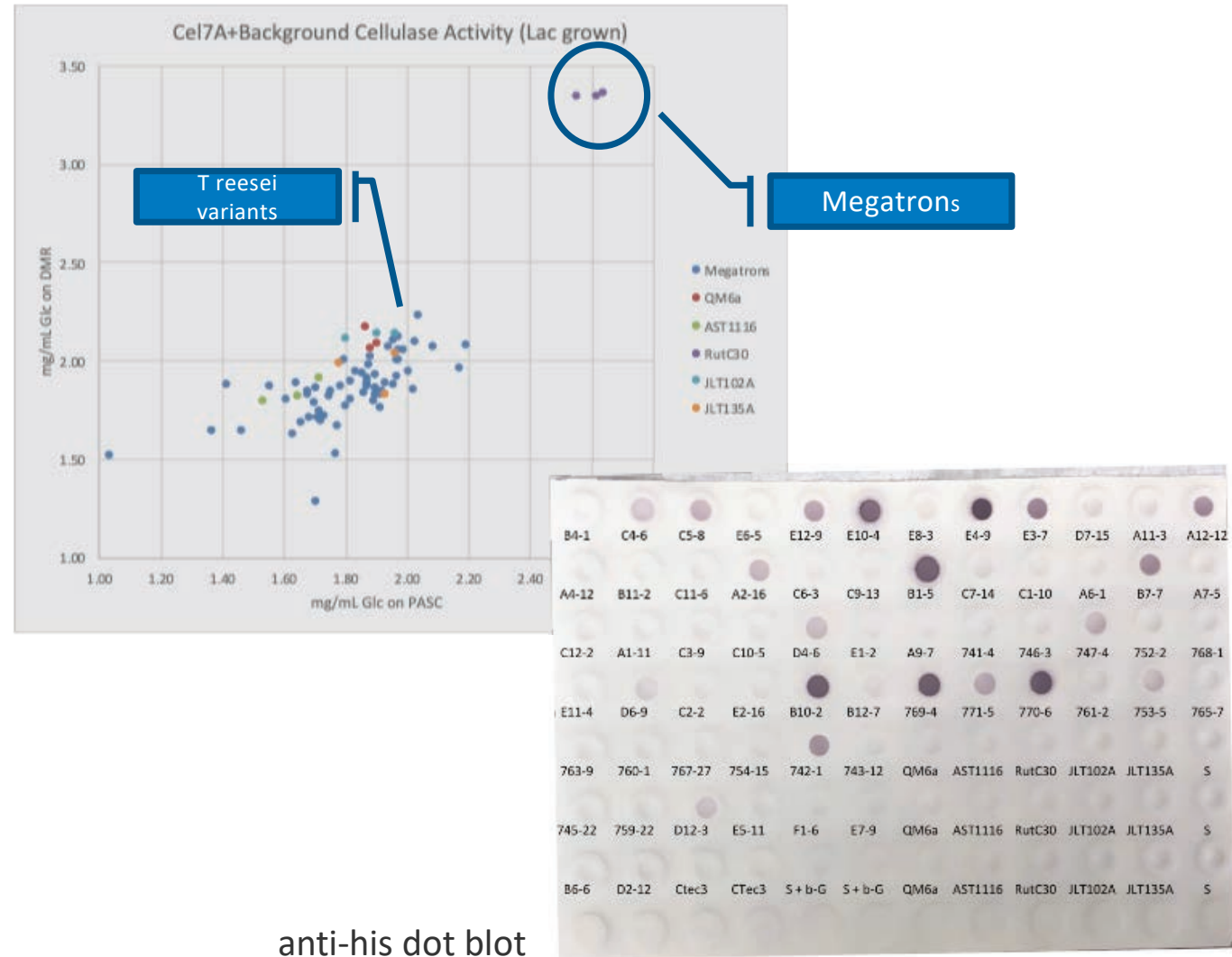
- Lactose: full cellulase system, including rCel7.

### Screening of Cel7 activity alone

- Glycerol: express only rCel7A

- Clarified broth was re-arrayed to deep well 96-well plates and screened

- PASC, Avicel, DMR matrices to assess activity
- Anti-his dot blots to estimate expression

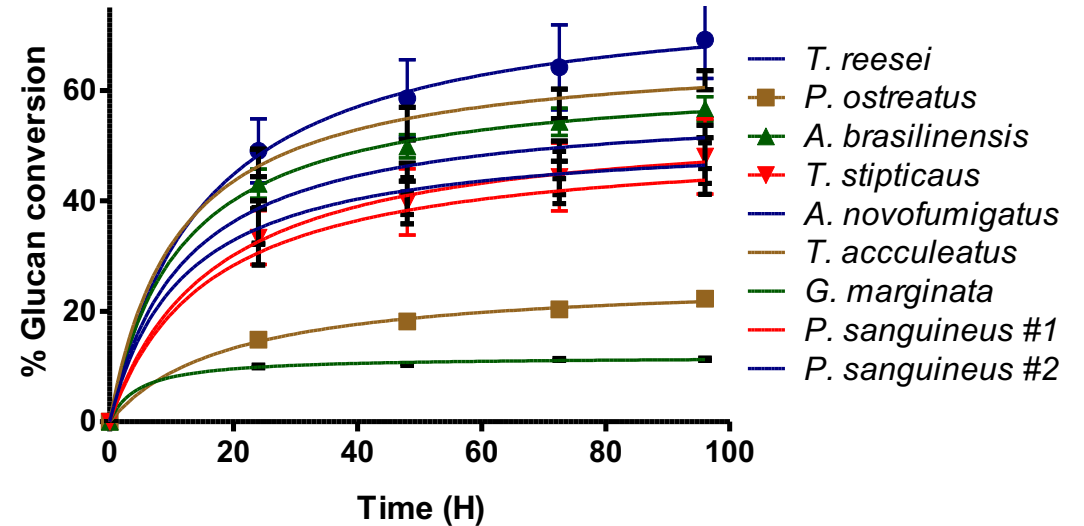




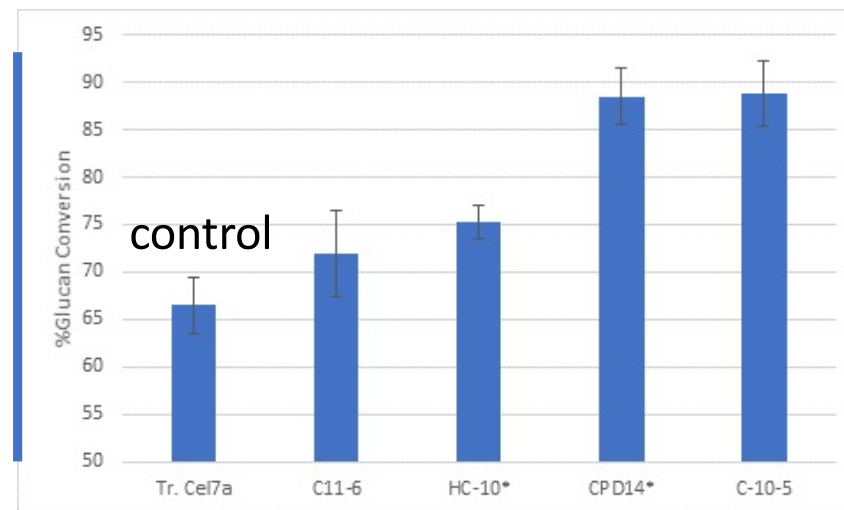
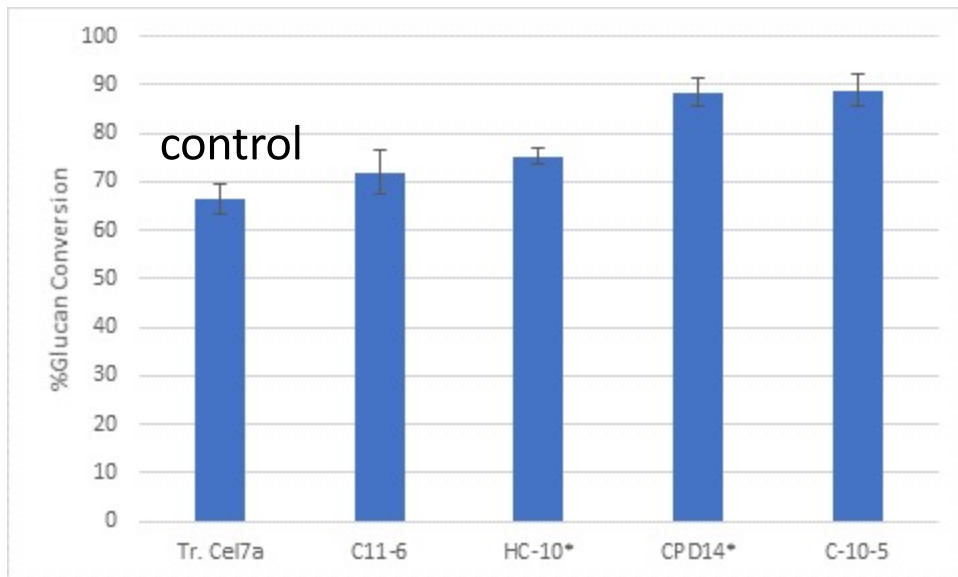
# 4. Progress and Outcomes

## Four Megatron enzymes performed better than the Tr enzyme

Many candidates failed to perform better than Tr acting on biomass

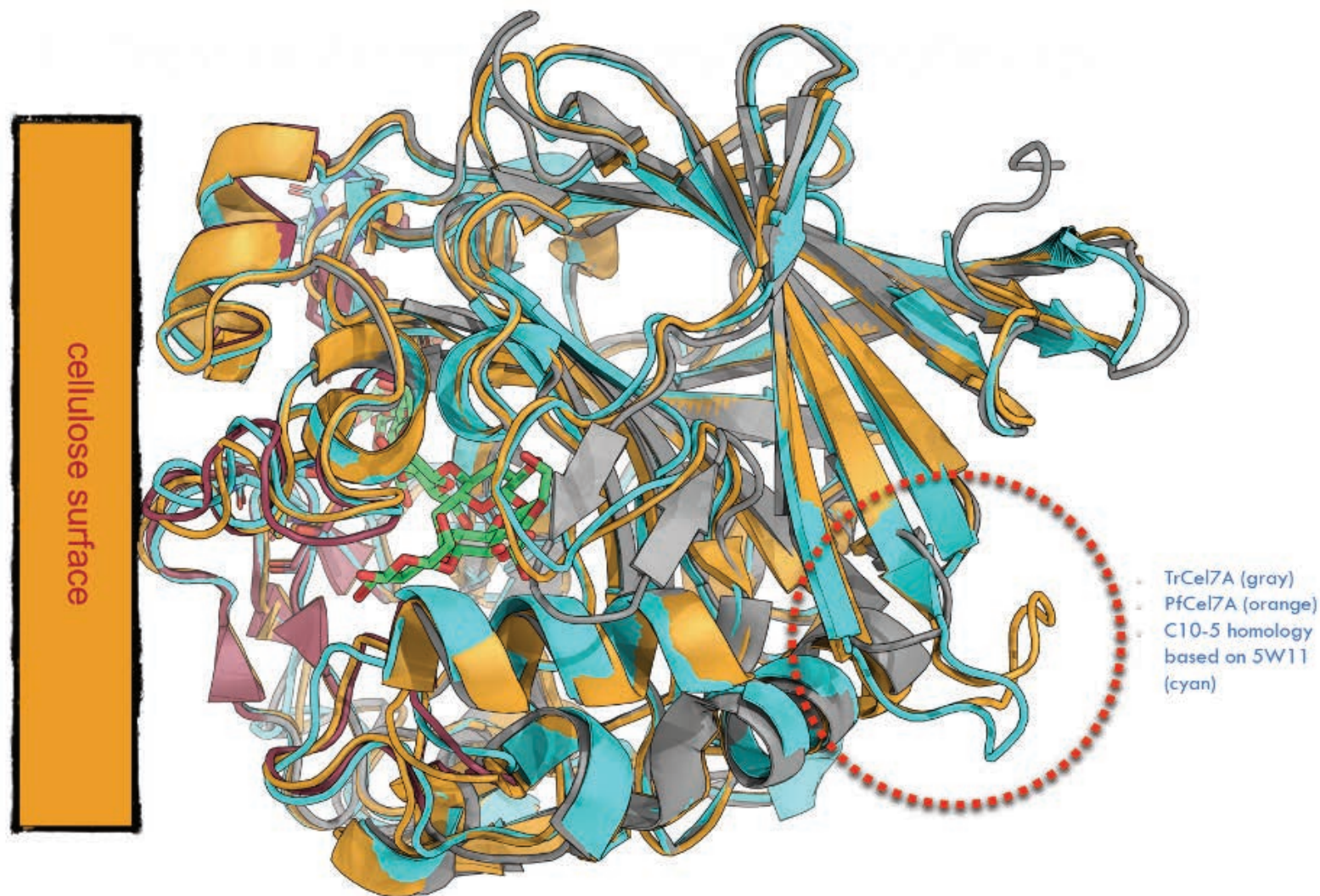


### Superior Megatron candidates



## 4. Progress and Outcomes

### *Early lessons from the first Megatron, PfCel7A*

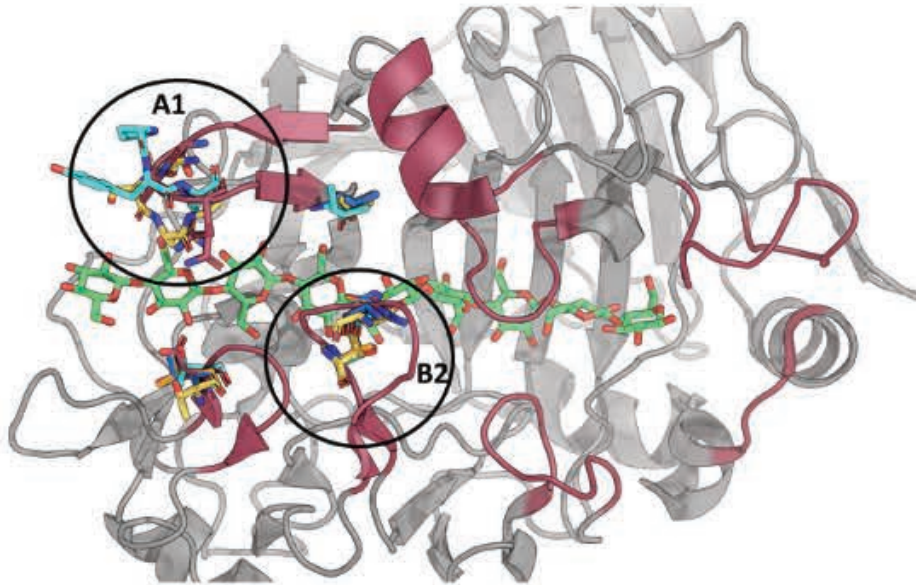


# 4. Progress and Outcomes

## *Important amino acid residues conferring improved activities to Cel7A*

### Two major mutational approaches

#### 1. Mutations in the A1 and B2 loops of PfCel7A



#### 2. Removal of additional disulfide near entrance to mimic PfCel7A

### Progress in mutational analysis

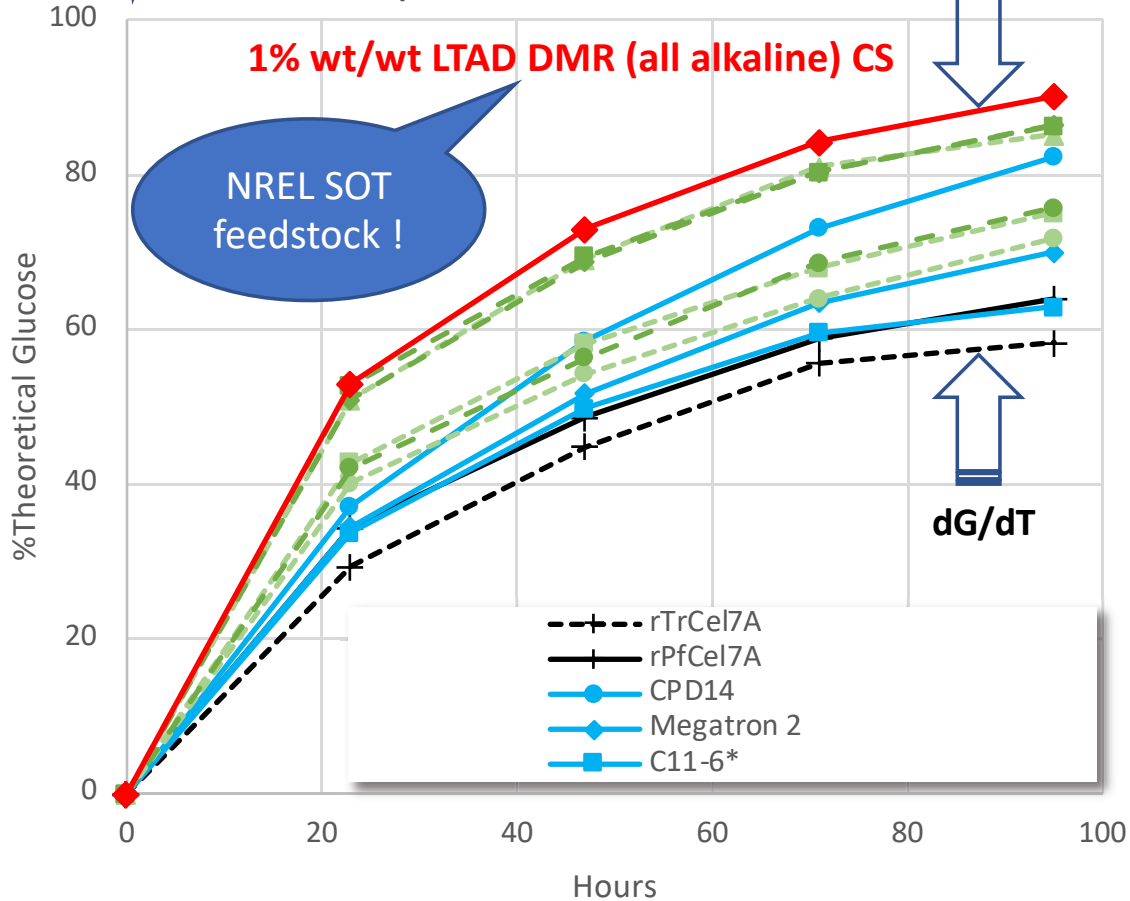
Construct #	Mutation	Base Cel7A protein	Constructs generated	Clonal isolates obtained	Protein purified	Protein amounts (mgs)
1	Disulfide mutation to remove additional in 10-5 Cel7A	10-5 Cel7A	Yes	No	No	-
2	B2 loop-1	PfCel7A	Yes	Yes	Yes	16
3	B2 loop-2	PfCel7A	Yes	Yes	Yes	35
4	Outside A1 loop	PfCel7A	Yes	Yes	Yes	24
5	B2 loop - 1+2	PfCel7A	Yes	Yes	Yes	12
6	B2 loop -1 + Outside A1 loop	PfCel7A	Yes	Yes	Yes	14
7	B2 loop - 2 + Outside A1 loop	PfCel7A	Yes	Yes	Yes	5.4
8	B2 loops 1, 2 + Outside A1 loop	PfCel7A	Yes	Yes	Yes	44
9	Add back disulfide mutations + best of 2-8	PfCel7A	No	No	No	-
10	B1 loop + best of 2-8	TrCel7A	No	No	No	-

# 4. Progress and Outcomes

Key  
Slide

## King Chimeras – single, double, and triple mutations

Improved Cel7 Variants



NREL mix: 28 mg/g Cel7A: 1.89 mg/g E1 endo: 0.5 mg/g BG

King Chimera Triple

King Chimera Doubles

Chimera Singles

Megatrons

*Penicillium funiculosum*

*Trichoderma reesei*

Cel7A	Mutations @ 95h-Tr = 1.0	X-fold Increase
V101I T198A N195D	1+2+3	1.55
N195D T198A	2+3	1.48
V101I T198A	1+2	1.30
N195D V101I	1+3	1.48
V101I	1	1.23
T198A	2	1.29
N195D	3	1.46

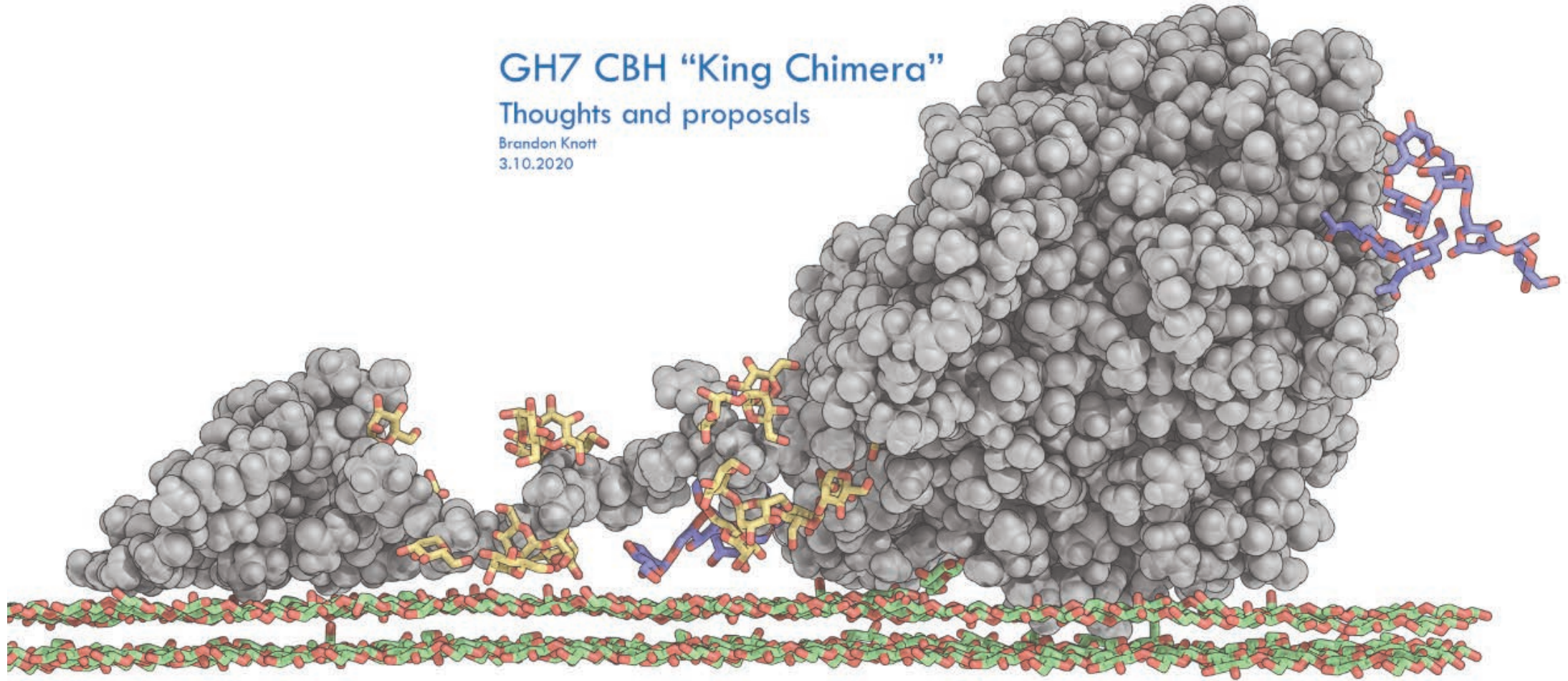
- Initially, we discovered that *Penicillium funiculosum* Cel7A was better than *Trichoderma reesei*
- We identified >1500 natural variants, built ~100, screened ~65, and produced/purified/assayed ~15 and found 3 better than *T. reesei* (Megatron)
- We identified 3 probable sites of improvement in best Megatron and moved them into PfCel7A (Chimera Singles)
- We combined singles into doubles (King Chimera Doubles)
- We combined all three singles into a King Chimera Triple

# 4. Progress and Outcomes

## GH7 CBH “King Chimera”

Thoughts and proposals

Brandon Knott  
3.10.2020



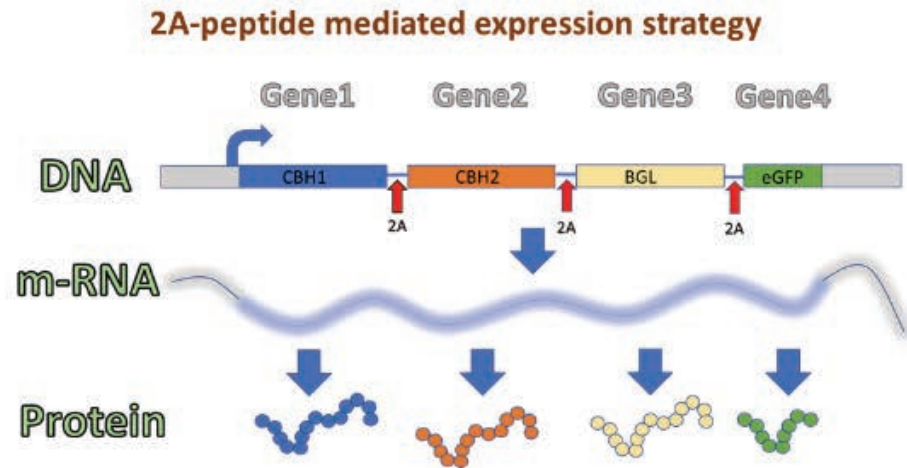
# 4. Progress and Outcomes

## Recommendations for future subsites based on emergent insight from current results

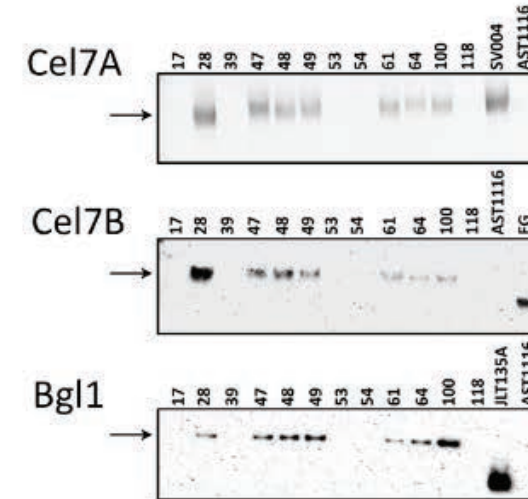
	site	# mutations	source	comments
	C4G, C72A	2	10-5	removing disulfide near entrance to make C10-5 like <i>PfCel7A</i> ; similar to what was done with <i>TrCel7A</i> in <b>2018 Nature Comm</b>
	N198D	1	<i>PfCel7A</i>	N198 in <i>TrCel7A</i> is N195 in <i>PfCel7A</i> ; so this is <b>N195D</b> . D is what C10-5 has here. On B2 loop.
	T201A	1	<i>PfCel7A</i>	T201 in <i>TrCel7A</i> is T198 in <i>PfCel7A</i> ; so this is <b>T198A</b> . A is what C10-5 has here. On B2 loop.
	V104I	1	<i>PfCel7A</i>	V104 in <i>TrCel7A</i> is V101 in <i>PfCel7A</i> ; so this is <b>V101I</b> . I is what C10-5 has here. Located at the base of A1 loop.
	N198D, T201A	2	<i>PfCel7A</i>	Combining 2 and 3 from above. <b>N195D/T198A</b>
	N198D, V104I	2	<i>PfCel7A</i>	Combining 2 and 4 from above. <b>N195D/V101I</b>
	T201A, V104I	2	<i>PfCel7A</i>	Combining 3 and 4 from above. <b>T198A/V101I</b>
	N198D, T201A, V104I	3	<i>PfCel7A</i>	Combining 2, 3, and 4 from above. <b>N195D/T198A/V101I</b>
	G4C, A72C, best of 2-8	multiple	<i>PfCel7A</i>	Putting <i>TrCel7A</i> 10th disulfide into <i>PfCel7A</i> + best of 2 through 8
	D52T, best of 2-8	2-5	<i>TrCel7A</i>	<i>TrCel7A</i> has D52, whereas the other four have T. B1 loop.

# 4. Progress and Outcomes

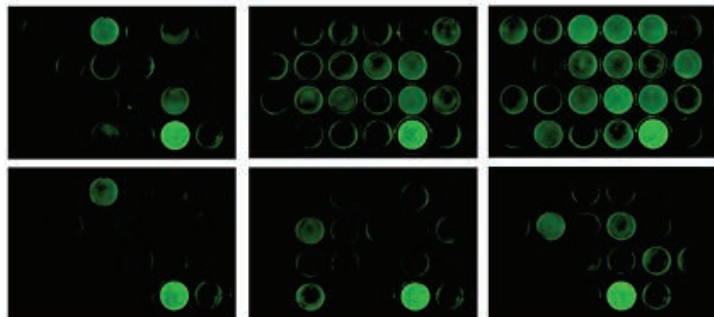
## Multi-cistronic protein expression platform for production of cellulase cocktails



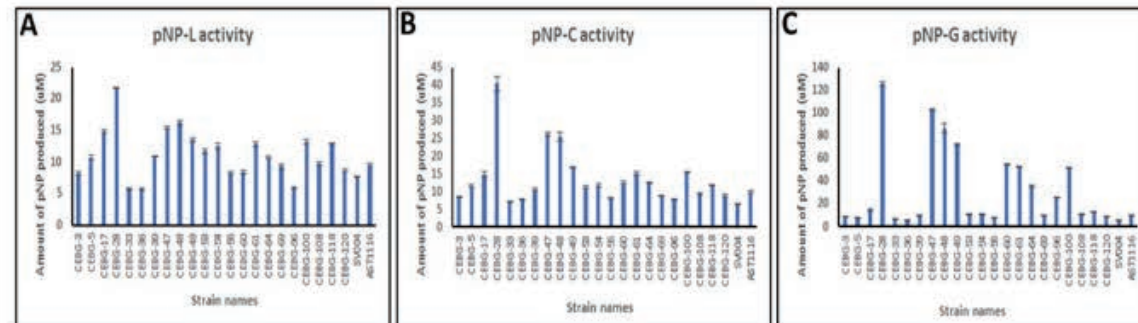
Confirmation of simultaneous expression of 3 independent proteins from a single promoter



GFP-based screening of expressing transformants



Confirmation of functional protein expression by cellulase activity analysis



Manuscript (in preparation)

## 4. Progress and Outcomes – Summary

Type	2019 EEO Milestones Description and Criteria	End Date
QPM1	LME and Xylanases. Determine if either pre or post DMR treatment of biomass with commercial LME enzymes improves the saccharification of DMR solids. <b>LME enzymes were found to increase digestibility of Ctec/Htec by only a few percent and these commercial enzymes are costly.</b>	12/31/2018
QPM2	Report hyperactive Cel7A candidates from first 30 phylogenetically novel sources. Choose 100, screen 68, tested top 10, and picked 4. <b>We reported several wild type Cel7A enzymes with activity greater than TrCel7A using the NREL enzyme mix and DAPCS.</b>	3/31/2019
QPM3	LME and Xylanases. Working with NREL's Cel7A delete <i>T. reesei</i> strain (augmented with new Megatron Cel7A candidates), screen commercial and purified xylanases for enhanced performance on DMR solids. <b>We abandoned this approach considering the modest performance enhancement (QPM1).</b>	6/30/2019
Annual SMART	Achieve <b>80% conversion</b> of glucose from cellulose in <b>DAPCS</b> using improved cellobiohydrolases, xylanases, and lignin modifying enzymes under standard conditions. <b>We reported the Megatron Cel7A (Pfun) reaching 85% conversion on DAPCS in 80 hrs (TrCel7A reached 80% conversion) both with NREL enzyme mix and standard loadings.</b>	9/30/2019
EOP MS	Demonstrate that Megatron enzymes and NREL SOT DMR solids can achieve <b>90% conversion</b> of glucose/xylose from glucan/xylan under standard conditions. Collaboration w LTAD and BPA and Novozymes.	9/30/2021



# 4. Progress and Outcomes – Summary

Type	2020 EEO Milestones Description and Criteria	End Date
QPM1	Prepare representative sample of DMR solids for EEO. Determine optimal conditions for washing/extraction with NaOH. <b>We determined that at least 200 mM NaOH will produce the maximally digestible DMR solids using Ctec/Htec enzymes.</b>	12/31/2019
QPM2	Determine which GAX glycosidic linkages remain intact following treatment of DMR slurries with Novo enzymes. <b>NMR chemical linkage analysis of these oligosaccharides (16% of soluble sugars) identified 9 discrete species (CCRC collaboration). Working to identify enzymes to hydrolyze these bonds.</b>	3/31/2020 – delayed till FY21
QPM3	Wrap up Megatron research (CBH I) to develop/engineering the most effective cellobiohydrolases to be used by Novozymes. <b>Demonstrated conversion of glucan using Megatron/King Chimera enzymes and the NREL enzyme mix that are as high as 1.55x of the conversion when Tr Cel7A is used in the mix. Enzymes sent to Novozymes for evaluation.</b>	6/30/2020 – proposed continuation
Annual SMART	Achieve a goal of <b>85% conversion</b> of glycan and xylan in <b>DMR solids</b> to free sugars using improved cellobiohydrolases, xylanases, and lignin modifying enzymes and dilute alkali washing set. <b>Demonstrated 92% glucan conversion of NaOH extracted acidified DMR solids at 10 mg Ctec/Htec loading.</b>	9/30/2020
EOP MS	Demonstrate that Megatron enzymes and NREL SOT DMR solids can achieve <b>90% conversion</b> of glucose/xylose from glucan/xylan under standard conditions. Collaboration w LTAD and BPA and Novozymes.	9/30/2021

# 4. Progress and Outcomes

## *Summary and Future Outlook*

### FY21 AOP

#### **Task 1) Bring to completion the Cel7A work with the planned End of 3-Year Project Smart Milestone**

- The full range of Cel7A mutations identified during FY20 were not anticipated and thus not tested.
- Focus on hand-offs to Novozymes to permit testing of new Cellic formulations *vis a vie* conversion and loading goals suggested in the NREL SOT.

#### **Task 2) Transition from cellulase to support of Cell Free Technology (CFiT)**

Cofactor cost is the main cost driver in cell free biocatalysis. Biomimetics can be more than 100-fold cheaper than natural cofactors (Joint with CFiT). Q2

- Enable the use of biomimetic cofactors, to reduce the cost of cell free biocatalysis, in at least two classes of redox enzymes using a combination of design principles derived from natural/engineered enzyme sequence/structure diversity and de novo enzyme engineering.

# Quad Chart Overview (for AOP Projects)

## Timeline

- Project start date - 2016
- Project end date - 2021

	FY20	Active Project
DOE Funding	(10/01/2019 – 9/30/2020) \$900,000	Three-year project = \$2.7M

## Project Partners (unfunded collaborators)

- S Teter – Novozymes (CRADA)
- S Rantanen - MetGen Oy

## Barriers addressed

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

## Project Goal

In partnership with Novozymes, produce a DMR solids acting enzyme formulation that achieves a 90% conversion of cellulose to glucose and 90% conversion of xylan to xylose under standard conditions. If these goals are met early, push on to higher conversions – i.e., 92% or 95%.

## End of Project Milestone

Demonstrate that Megatron approach with dilute alkali wash can achieve 90% conversion of glucose/xylose from glucan/xylan under standard conditions (10 mg enzyme/gram cellulose in DMR solids; 5 days; 10% solids; 50°C). Collaboration w LTAD and BPA.

## Funding Mechanism

AOP as WBS# - 2.5.4.100

# Market Trends

## Product

- Anticipated decrease in gasoline/ethanol demand; diesel demand steady
- Increasing demand for aviation and marine fuel
- Demand for higher-performance products
- Increasing demand for renewable/recyclable materials

## Feedstock

- Sustained low oil prices
- Decreasing cost of renewable electricity
- Sustainable waste management
- Expanding availability of green H<sub>2</sub>
- Closing the carbon cycle

## Capital

- Risk of greenfield investments
- Challenges and costs of biorefinery start-up
- Availability of depreciated and underutilized capital equipment

## Social Responsibility

- Carbon intensity reduction
- Access to clean air and water
- Environmental equity

# EEO Project Summary

## Management

- PI (single POC): Task leader/staff assigned roles
- Milestone and TEA/LCA driven
- Feedback from industrial partner

## Approach

- Improve the most critical cellulase, Cel7A, to achieve specific conversion goals.
- Screen from natural diversity to identify critical subsites and build superior Cel7A chimeras.
- Share improved enzymes with Novozymes.

## Impact

- De-risk Gen2 and Gen1.5 processes by providing more effective/less costly cellulases to industry.

## Progress and Outcomes

- Demonstrated that Nature harbors superior Cel7As.
- New Cel7A 3x mutant is 1.55x *T. reesei* performance.
- Achieved 90% glucan goal by several approaches

# Acknowledgments

## Funding

U.S. DOE EERE Bioenergy Technologies Office

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

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

Mike Himmel (PI)

Stephen Decker (Task Lead)

Lilly Amore

(HerbalifeNutrition\*, Milano)

Markus Alahuhta

Gregg Beckham

Roman Brunecky

Brandon Knott

Venkat Subramanian

Todd Vanderwall

John Yarbrough

### **Unfunded collaborators**

Christy Payne, Kentucky

Clare McCabe, Vanderbilt

Mats Sandgren

Jerry Ståhlberg



Al Darvill

Parastoo Azadi

Maria Pena

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[www.nrel.gov](http://www.nrel.gov)

This work was authored by the National Renewable Energy Laboratory, operated by Alliance for Sustainable Energy, LLC, for the U.S. Department of Energy (DOE) under Contract No. DE-AC36-08GO28308. Funding provided by U.S. Department of Energy Office of Energy Efficiency and Renewable Energy, Bioenergy Technologies Office. The views expressed in the article do not necessarily represent the views of the DOE or the U.S. Government. The U.S. Government retains and the publisher, by accepting the article for publication, acknowledges that the U.S. Government retains a nonexclusive, paid-up, irrevocable, worldwide license to publish or reproduce the published form of this work, or allow others to do so, for U.S. Government purposes.



**Additional Slides**

# Responses to Previous Reviewers' Comments

## **Address significant questions/criticisms from the previous reviewers' since addressed**

- RE: Project Critical Path. “For a project with this importance and level of funding it would be warranted to have an earlier thorough review.”
  - Such ‘thorough reviews’ have occurred in the context of CRADA meetings with our partner.
- “Probably need a better "Plan B" than insect/human cells, if *T. reesei* work is not productive.”
  - *T. reesei* was found to be effective for essentially for all fungal Cel7A mutants attempted.
- “Do learnings from this program help in developing formulations for other feedstock sources? Would be worth broadening the scope of enzyme engineering to include pathway enzymes?..”
  - Yes, these learnings based on lignocellulose apply to corn fiber and other agricultural residues. We are working in FY21 on pathway enzymes.
- “There is a need to expand enzyme developed beyond DMR Corn Stover to other feedstocks that are available from paper and pulp industry.”
  - Our charter is to support the DOE BETO Biofuels Design reports, which specify feedstocks, pretreatment, products. However, more recently we have initiated limited work on corn fiber.
- **Go/No-Go (3/31/2020)**
  - Determine if final NaOH washing of DMR solids achieves the 85% conversion using Novozymes enzymes. If Go, prepare a new series of conversion targets based on DMR extraction and replan EEO for FY21 (e.g., increase current FY21 glucan target from 90% to 92% conversion). This was done. May be a stretch goal!



# Peer Reviewed Publications

- “Translational and Secretory Mechanisms: Two Independent Events Required for Successful Protein Expression in *T. reesei*,” Subramanian, V., Heiland, K.L., Farmer, S.F., Moore, K.T., Ho, A., Himmel, M.E., and S.R. Decker, **2021** (In preparation).
- “A Multi-plex Protein Expression System for Production of Complex Enzyme Formulations in *Trichoderma reesei*,” V. Subramanian, S.F. Farmer, K.L. Heiland, K.T. Moore, T.A. Vander Wall, W. Sun, Y.B. Chaudhari, M.E. Himmel, and S.R. Decker, *Biotechnol. Biofuels*, **2021** (In Review)
- “Towards Sustainable Production and Utilization of Plant Biomass Based Nanomaterials,” J.Y. Zhu, U.P. Agarwal, P.N. Ciesielski, M.E. Himmel, R. Gao, Y. Deng, M. Morits, M. Österberg, *Biotechnol. Biofuels*, **2021**, (In Review).
- “Chimeric Cellobiohydrolase I Expression, Activity, and Biochemical Properties in Three Oleaginous Yeast,” Markus Alahuhta; Qi Xu; Eric Knoshaug; Wei Wang; Hui Wei; Antonella Amore; John O. Baker; Todd Vander Wall; Michael E. Himmel; Min Zhang. *Biotechnol. Biofuels*. BBIO-D-20-00335. **2021**.
- “Carbohydrate-Binding Module O-mannosylation Alters Binding Selectivity to Cellulose and Lignin,” Y. Li; X. Guan; P.K. Chaffey; Y. Ruan; B. Ma; S. Shang; M.E. Himmel; G.T. Beckham; H. Long; Z. Tan, *Chemical Science* (IF 9.346): **2020**. doi: 10.1039/d0sc01812k.
- “Reduced Type-A Carbohydrate-Binding Module Interactions to Cellulose I Leads to Improved Endocellulase Activity,” B. Nemmaru, N. Ramirez, C.J. Farino, J.M. Yarbrough, N. Kravchenko, S.P.S. Chundawat, *Biotechnol. Bioeng.*, **2020**. doi.org/10.1002/bit.27637
- “Phylogenetics-based Identification and Characterization of a Superior 2,3-butanediol Dehydrogenase for *Zymomonas mobilis* Expression,” V. Subramanian, V.V., Lunin, S. Farmer, M. Alahuhta, K.T. Moore, A. Ho, Y.B. Chaudhari, M. Zhang, M.E. Himmel, and S.R. Decker, *Biotechnol. Biofuels*, 13:186, **2020**.
- “The Hydrolysis Mechanism of a GH45 Cellulase and its Potential Relation to Lytic Transglycosylase and Expansin Function,” V.S. Bharadwaj, B.C. Knott, J. Ståhlberg, G.T. Beckham, M.F. Crowley,” *Journal of Biological Chemistry* 295 (14), 4477-4487, **2020**.
- “Structure of the Xylan O-Acetyltransferase AtXOAT1 Reveals Molecular Insight into Polysaccharide Acetylation in Plants,” V. Lunin, H.-T. Wang, V.S. Bharadwaj, P.M. Alahuhta, M.J. Peña, J.Y. Yang, M.E. Himmel, K. Moremen, W.S. York, Y.J. Bomble, B. Urbanowicz, *The Plant Cell*, **2020**. doi.org/10.1105/tpc.20.00028.
- Bomble Y.J., Himmel M.E., (**2020**) “Overview and Future Directions in Metabolic Pathway Engineering,” In: Himmel M. and Bomble Y. (eds) Metabolic Pathway Engineering. Methods in Molecular Biology, vol 2096. Humana, New York, NY. [https://doi.org/10.1007/978-1-0716-0195-2\\_1](https://doi.org/10.1007/978-1-0716-0195-2_1)

# Peer Reviewed Publications

- Alahuhta M., Himmel M.E., Bomble Y.J., Lunin V.V., (2020) “Crystallography of Metabolic Enzymes.” In: Himmel M., Bomble Y. (Eds) Metabolic Pathway Engineering. Methods in Molecular Biology, vol 2096. Humana, New York, NY. [https://doi.org/10.1007/978-1-0716-0195-2\\_10](https://doi.org/10.1007/978-1-0716-0195-2_10).
- Chou Y.C., Singh A., Xu Q., Himmel M.E., Zhang M. (2020) “Methods for Metabolic Engineering of a Filamentous *Trichoderma reesei*,” In: Himmel M., Bomble Y. (Eds) Metabolic Pathway Engineering. Methods in Molecular Biology, vol 2096. Humana, New York, NY. [https://doi.org/10.1007/978-1-0716-0195-2\\_4](https://doi.org/10.1007/978-1-0716-0195-2_4).
- “The Dissociation Mechanism of Processive Cellulases,” J.V. Vermaas, R. Kont, G.T. Beckham, M.F. Crowley, M. Gudmundsson, M. Sandgren, J. Ståhlberg, P. Våljamäe, B.C. Knott,” *PNAS*,116(46), 23061-23067, **2019**.
- “Synthetic Fungal Multifunctional Enzymes Exhibit Unique Cellulose Deconstruction Properties,” Roman Brunecky, Venkat Subramanian, John Yarbrough, Todd Vinzant, Bryon Donohoe, Todd Vanderwall, Michael E. Himmel, Stephen R. Decker, *Green Chemistry*, **2019**.
- “Nanomechanics of Cellulose Deformation Reveal Molecular Defects that Facilitate Natural Deconstruction” by Peter N. Ciesielski, Ryan Wagner, Vivek S. Bharadwaj, Jason Killgore, Ashutosh Mittal, Gregg T. Beckham, Stephen R. Decker, Michael E. Himmel, and Michael F. Crowley, *PNAS*, 116 (20), 9825-9830, **2019**.
- “Glycosylation Is Vital for Industrial Performance of Hyperactive Cellulases,” Daehwan Chung, Nicholas S. Sarai, Brandon C. Knott, Neal Hengge, Jordan F. Russell, John M. Yarbrough, Roman Brunecky, Jenna Young, Nitin Supekar, Todd VanderWall, Deanne W. Sammond, Michael F. Crowley, Christine Szymanski, Lance Wells, Parastoo Azadi, Janet Westpheling, Michael E. Himmel, and Yannick J. Bomble, *ACS Sustainable Chemistry & Engineering*. (2019) 7 (5), 4792-4800 DOI: 10.1021/acssuschemeng.8b05049.
- “Ameliorating the Metabolic Burden of the Co-Expression of Secreted Fungal Cellulases in a High Lipid-Accumulating *Yarrowia lipolytica* Strain by Medium C/N Ratio and a Chemical Chaperone,” Hui Wei, Wei Wang, Hal S Alper, Qi Xu, Eric Knoshaug, Stefanie Van Wychen, Chien-Yuan Lin, Yonghua Luo, Stephen R Decker, Michael E Himmel, Min Zhang, *Frontiers Microbiol.* (2019) 9:3276. doi: 10.3389/fmicb.2018.03276.
- “Chemical and Structural Effects on the Rate of Xylan Hydrolysis during Dilute Acid Pretreatment of Poplar Wood,” Mittal, Ashutosh, Pilath, Heidi M., Parent, Yves, Chatterjee, Siddharth G., Donohoe, Bryon S., Yarbrough, John M., Black, Stuart K., Himmel, Michael E., Nimlos, Mark R., and Johnson, David K, *ACS Sustainable Chemistry & Engineering*, 7(5), (2019). Web. doi:10.1021/acssuschemeng.8b05248
- “Expression of an Endoglucanase-Cellobiohydrolase Fusion Protein in *S. cerevisiae*, *Y. lipolytica*, and *L. starkeyi*,” Q. Xu, M. Alahuhta, H. Wei, E.P. Knoshaug, W. Wang, J.O. Baker, T. Vander Wall, M.E. Himmel, M. Zhang, *Biotechnol Biofuels*, 2018 Dec 3; 11:322. (2018).

# Patents and Presentations

## Patents

- United States Patent No.10,538,753 “Multifunctional Microbial Cellulases” Roman Brunecky, Venkat Subramanian, Steve Decker, and Michael E. Himmel 2020.
- United States Patent No.10,329,548 “Fungal Cellobiohydrolases,” Venkat Subramanian, Michael E. Himmel, Stephen E. Decker, Gregg T. Beckham, and Jeff G. Linger, June 2019.

## Presentations

- April 2019, 41st SFBC Seattle “Synthetic multifunctional cellulases in fungi,” Roman Brunecky, Venkat Subramanian, John Yarbrough, Bryon Donohoe, Todd Vinzant, Brandon Knott, Mike Himmel, and Steve Decker.
- July 2019, SIMB Annual Meeting, “The role of lignin modifying enzymes in deacetylation and mechanical refining of corn stover,” John Yarbrough et al.
- July 2019, Gordon Research Conference: Carbohydrate-Active Enzymes for Glycan Conversions, “Molecular Interfaces and Assemblies that Drive CAZyme Action,” Mike Crowley et al.

# Quad Chart for EEO FY2019

## Timeline

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

## Barriers addressed

Ct-B. Efficient Preprocessing and Pretreatments – Trade off analysis to optimize preprocessing unit operations which increase the overall process energy intensity.  
Ct-C. Process Development for Conversion of Lignin – approaches to increase lignin yield.

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

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

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

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

## Objective

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

## End of Project Goal

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

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

Mike Himmel / National Renewable Energy Laboratory

WBS# - 2.5.4.100

## Project Objectives

Achieve the targeted extents of conversion of glucan/xylan in DMR solids and the glucuronoarabinoxylans (GAXs) in DMR liquors.

- Develop effective DMR delignification/extraction strategies.
- Improve cellulases/hemicellulases targeting DMR solids.
- Identify and demonstrate debranching enzymes for DMR liquors

## Technical Approach

Determine optimal NaOH titer for washing/extraction of DMR solids.

- Work with TEA to determine process impact/tradeoffs.

Identify best cellobiohydrolases from diversity (Megatron).

- Cel7A (this proposal) and Cel6A (TCF award w/Novozymes).

Characterize recalcitrant linkages in the GAXs currently not hydrolyzed

- These GAXs represent about 12% of the original carbon
- Identify and test new hemicellulases needed and communicate to Novozymes for formulation.

## Project Attributes

<b>Project Start/End</b>	10/1/2018 to 9/31/2021	
<b>FY20 Budget at House Mark</b>	Total: \$900,000	
<b>Collaboration</b>	S Teter - Novozymes S Rantanen MetGen Oy	650-641-9264 +358 40 8374354
<b>DOE TM Lead</b>	Ian Rowe	202-586-7720

## Project Milestones and Outcomes

### FY20 milestones

- QPM1. Prepare representative sample of DMR solids for EEO. Determine optimal conditions for washing/extraction with NaOH. 12/31/2019
- QPM2. Determine which GAX glycosidic linkages remain intact following treatment of DMR slurries with Novo CTec enzymes. Identify enzymes that excel at breaking these bonds and share with Novozymes, 3/31/2020
- QPM3. Wrap up Megatron research (CBH I) and TCF project (CBH II) to develop/engineering the most effective cellobiohydrolases to be use by Novozymes. 6/30/2020
- Annual Smart: Achieve 85% conversion of glucan in DMR solids using improved cellobiohydrolases, xylanases, and extraction methods under standard conditions. 9/30/2020

### End of project goal/milestones (must be SMART)

- Demonstrate new extraction/Megatron approach can achieve 90% conversion of glucose/xylose from glucan/xylan under standard conditions. 9/30/2021.

### Go/No-Go w/date (3/31/2020)

- Determine if NaOH washing/extraction of EEO-grade of DMR solids achieves the 85% conversion target under standard assay conditions using current Novozymes formulation. If yes, prepare a new series of conversion targets based on DMR extraction and replan EEO for FY21 (e.g., increase current FY21 glucan target from 90% to 95% conversion). May be a stretch goal!

### Risk Abatement

- Replace ligninase treatment with NaOH extraction to “normalize” prep to prep variations recently identified as problematic.
- Novozymes partnership will ensure new formulations become commercial.

### Significant Interest/Concerns

- Discovery that NaOH extraction is needed to overcome LCC shield on DMR solids can be overcome experimentally but has process implications.