DOE Bioenergy Technologies Office (BETO) 2015 Project Peer Review

Maximizing multi-enzyme synergy in biomass degradation in yeast

March 26
Technology Area Review

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Goal Statement

- To demonstrate synthetic biology technologies for rapidly developing organisms suitable for industrial lignocellulose processing
- To develop strains for reducing saccharification costs
- To develop American technologies to be commercialized for making biomass-derived fuels and chemicals competitive with those derived from fossil fuels toward energy independence and sustainable development

Quad Chart Overview

Timeline

- 25-Apr-2013
- 25-Apr-2015
- 50% complete

Barriers

- Barriers addressed

Bt-F (Cellulase Enzyme Production Cost)
Bt-G (Cellulase Enzyme Loading)
Bt-J (Catalyst Development)
from Multi-Year Program Plan

Budget

	Total Costs FY 10 - FY 12	FY 13 Costs	FY 14 Costs	Total Planned Funding (FY 15 - Project End Date)
DOE Funded	$0	$60,539	$430,127	$775,558
Project Cost Share (Comp.)*	$0	$0	$132,647	$8,045

Partners

- Partners
  - NREL (Pin-Ching Maness)
- Other interactions/collaborations
  - Synthetic Genomics, Inc.
  - NREL (Dan Schell, Ling Tao)
  - Novozymes

1 - Project Overview

- Synergism among carbohydrate-active enzymes not sufficiently explored for biofuel production
- Synthetic biology technologies to rapidly construct yeast strains expressing many enzymes to discover novel synergy
- Reducing costs associated with saccharification

2 – Approach (Technical)

1. *Gene synthesis to enable the incorporation of any known cellulase sequence, as well as codon optimization for improved expression in yeast*
2. *Introduction of the constructs into yeast to confirm activity*
3. *'Green Monster' process to construct multi-enzyme strains*
4. *Yeast sexual cycling to shuffle introduced enzymes*
5. *Viability assay to select strains that contain optimal combinations of saccharification enzymes*
6. *Demonstration of improvements over the baseline strain in terms of ethanol produced in laboratory-scale SSF and HHF experiments*

- *Critical success factors: >33% reduction in required enzyme load to achieve the same saccharification level in lab-scale cultures*
- *Go/no-go criterion: functional expression of 20 cellulases (already met)*

2 – Approach (Management)

Maxim Kostylev, Ph.D.
(David Wilson's lab)

Timothy Hanly, Ph.D.
(Mike Henson's lab)

Small group of motivated researchers with correct skills

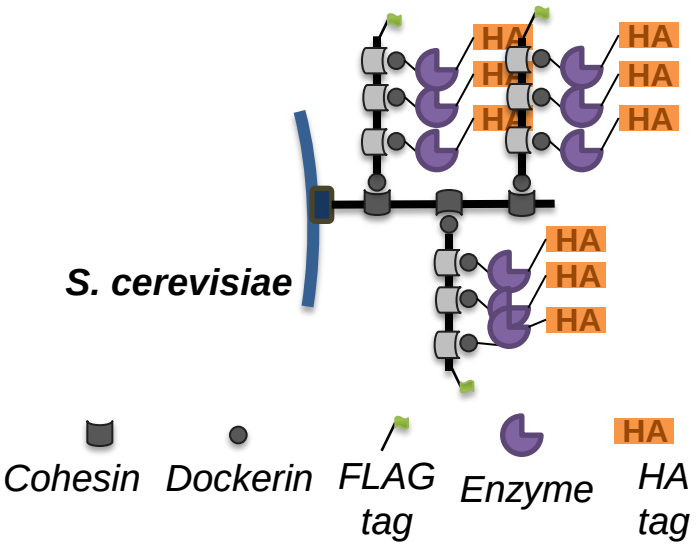
Weekly lab meeting, monthly note checking, monthly TC with DOE

- *Critical success factors:*
  - *Successful completion of all proposed tasks*
  - *High-impact publications*
  - *Patent application*
  - *Identification of funders or corporate partners for subsequent work*

- *Challenges:*
  - *Initial delays*

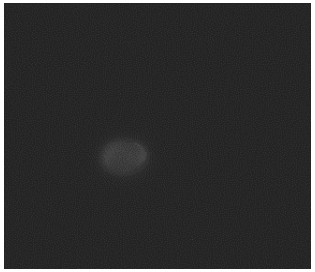
3 – Technical Accomplishments/ Progress/Results

- *Expression of mini-scaffoldins for accommodating cellulases on the yeast cell surface*

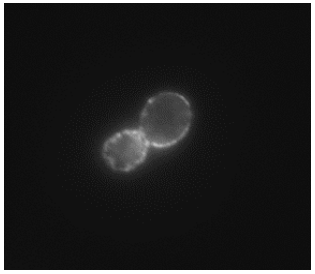


Anti-FLAG staining

Microscopy



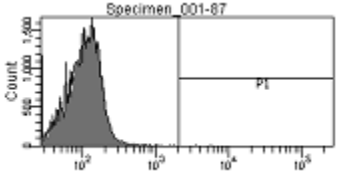
Parent strain



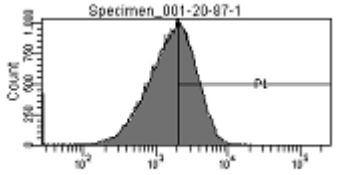
R. flavefaciens scaffoldins (codon-optimized by DNA2.0)

Flow cytometry

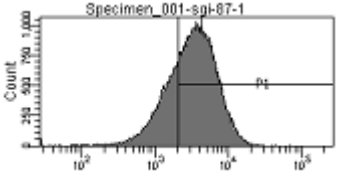
Parent strain



Codon-optimized by DNA2.0



Codon-optimized by SGI-DNA



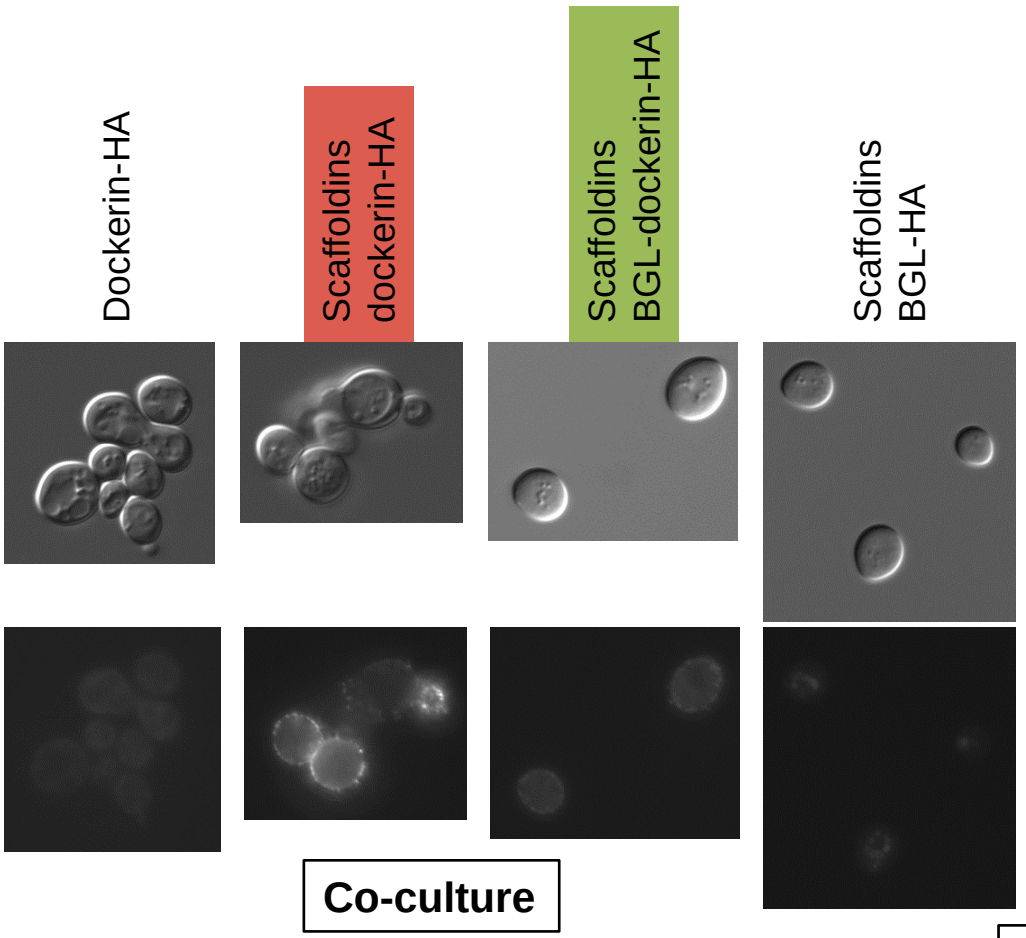
Fluorescence

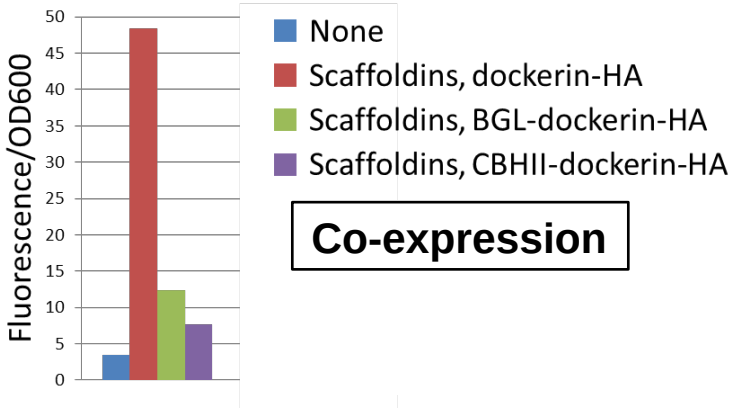
Inspired by nature and also:

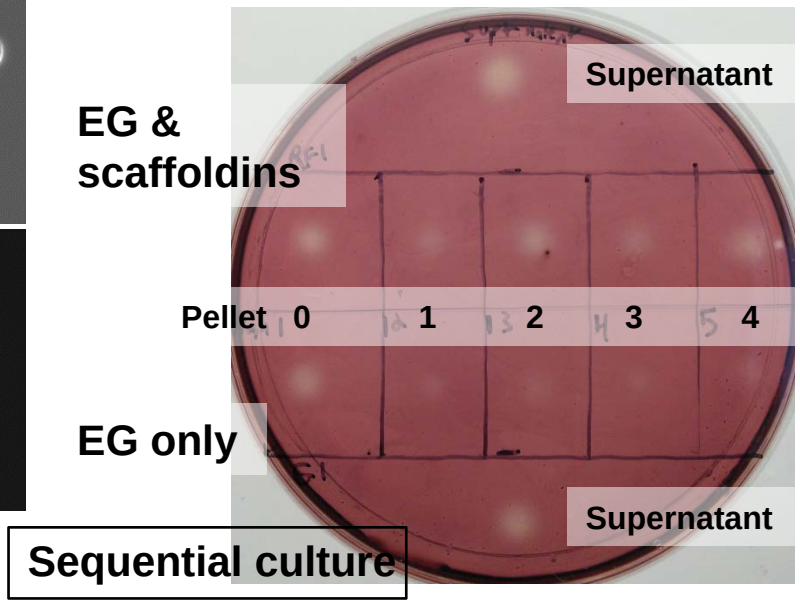
Bayer EA *et al*. *Trends Biotechnol.* 1994
Wen F *et al*. *Appl. Environ. Microbiol.* 2010
Goyal G *et al*. *Microb. Cell Fact.* 2011
Tsai SL *et al*. *ACS Synth. Biol.* 2012
Fan LH *et al*. *PNAS* 2012

3 – Technical Accomplishments/ Progress/Results

- *Functional expression of mini-scaffoldins for accommodating cellulases on the yeast cell surface*



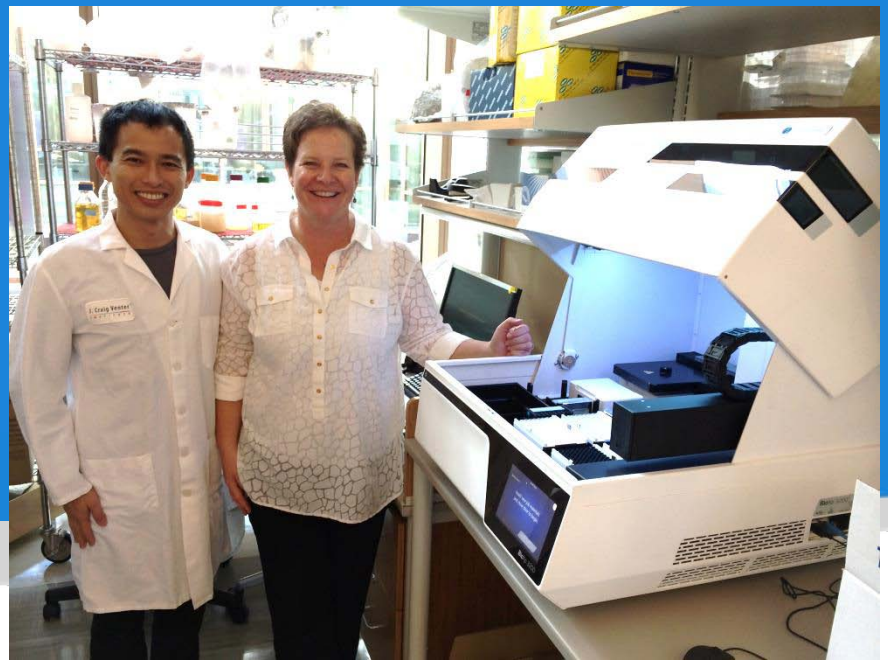




3 – Technical Accomplishments/ Progress/Results

- *Pipeline for generating and characterizing cellulase-expressing yeast strains*

98 carbohydrate-active enzymes
Gene synthesis
Gibson assembly or E. coli assembly
96-well-format yeast transformation



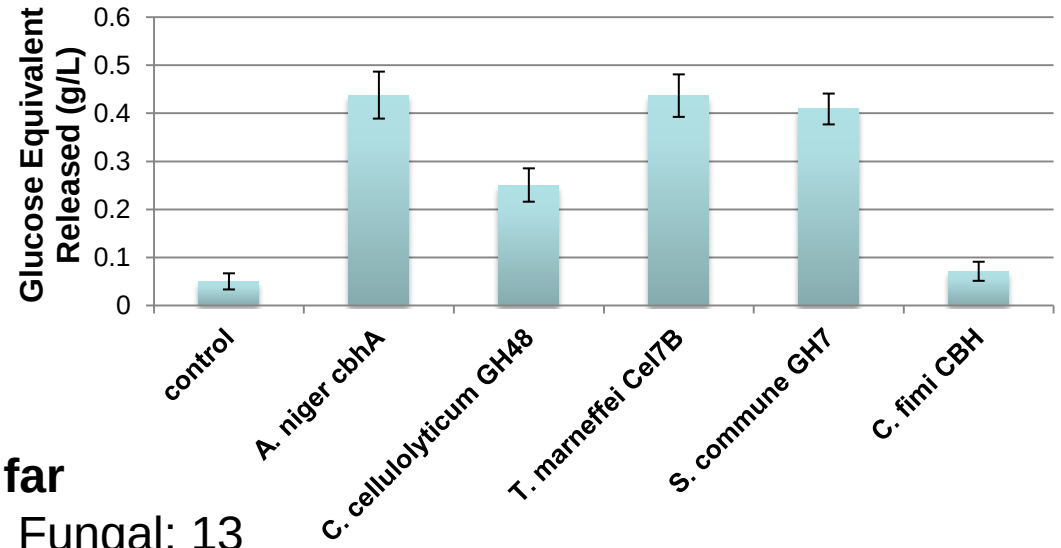
The first lab in the world to receive Synthetic Genomics BioXp™ 3200 (for beta-testing)

56 kb per day

3 – Technical Accomplishments/ Progress/Results

- *Pipeline for generating and characterizing cellulase-expressing yeast strains*

CMC- and barley β-glucan-overlay assays
PASC DNS assay
SDS-PAGE and Western



28 with demonstrated activity so far

Kingdom	Bacterial: 15	Fungal: 13	
Enzyme	Endo: 14	Exo: 11	BGL: 3

Glycoside Hydrolase Family

GH1: 1	GH3: 2	GH5: 5	GH6: 4	GH7: 3
GH8: 2	GH9: 5	GH12: 1	GH48: 5	

3 – Technical Accomplishments/ Progress/Results

Non–mutant

Marker 1

Single mutant

Marker 1
Marker 2

Double mutant

Marker 1
Marker 2
Marker 3

Triple mutant

Marker 1
Marker 2
Marker 3
Marker 4

Quadruple mutant

- *Multi-enzyme strains being constructed*

The Green Monster process

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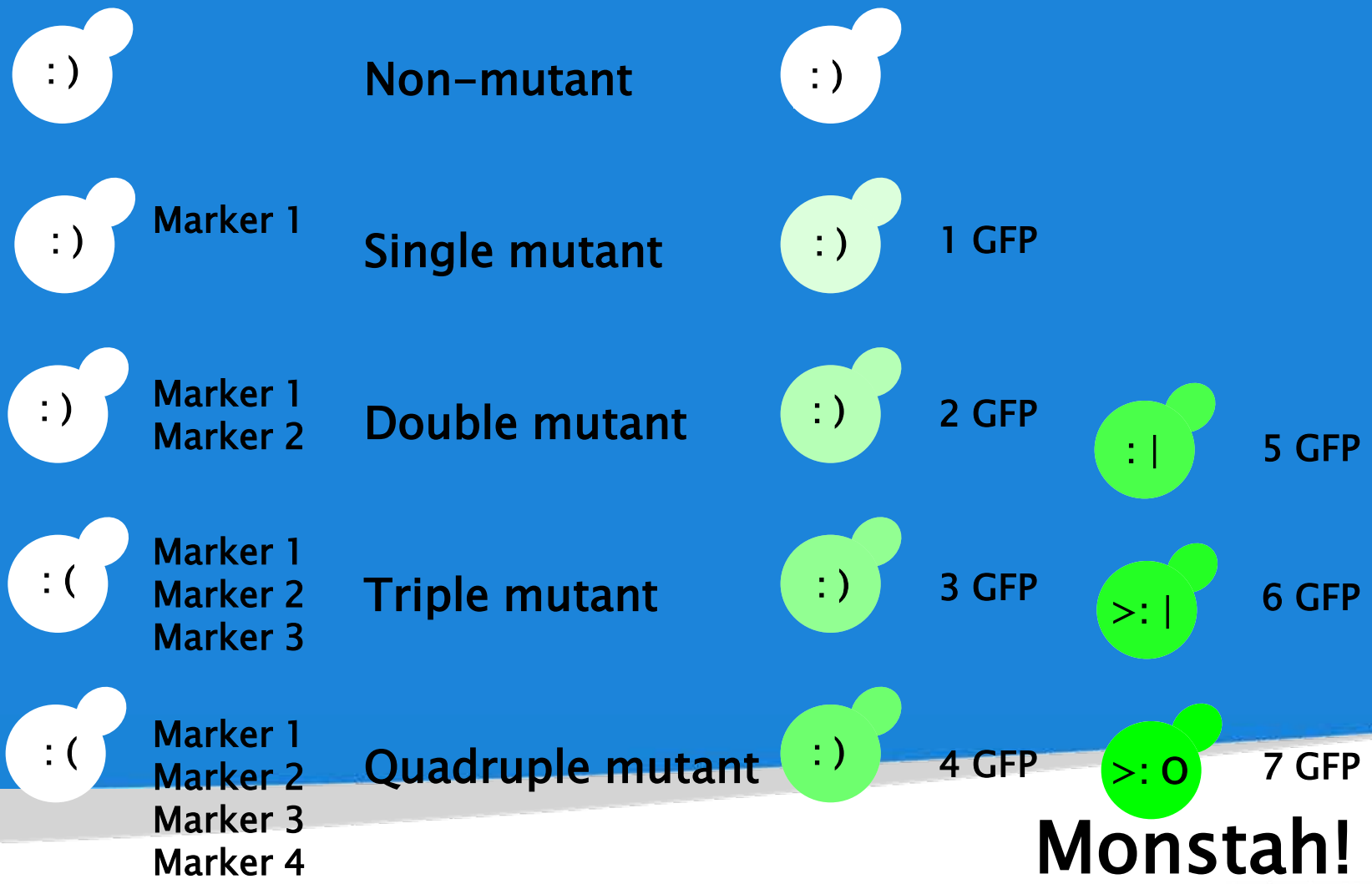
3 – Technical Accomplishments/ Progress/Results



Monstah!

The Green Monster process

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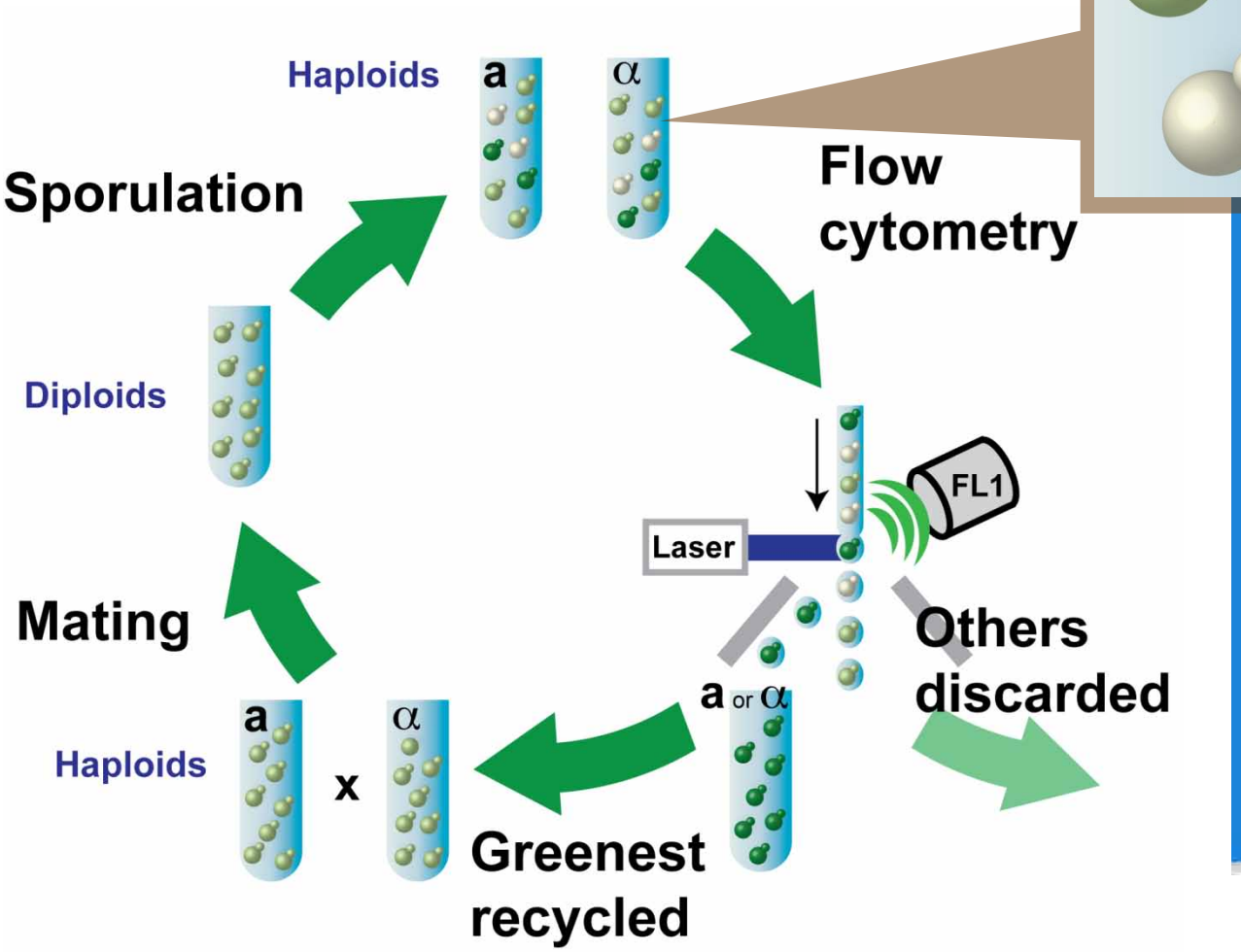
3 – Technical Accomplishments/ Progress/Results



Suzuki Y *et al*.
Nat. Methods 2011

The Green Monster process

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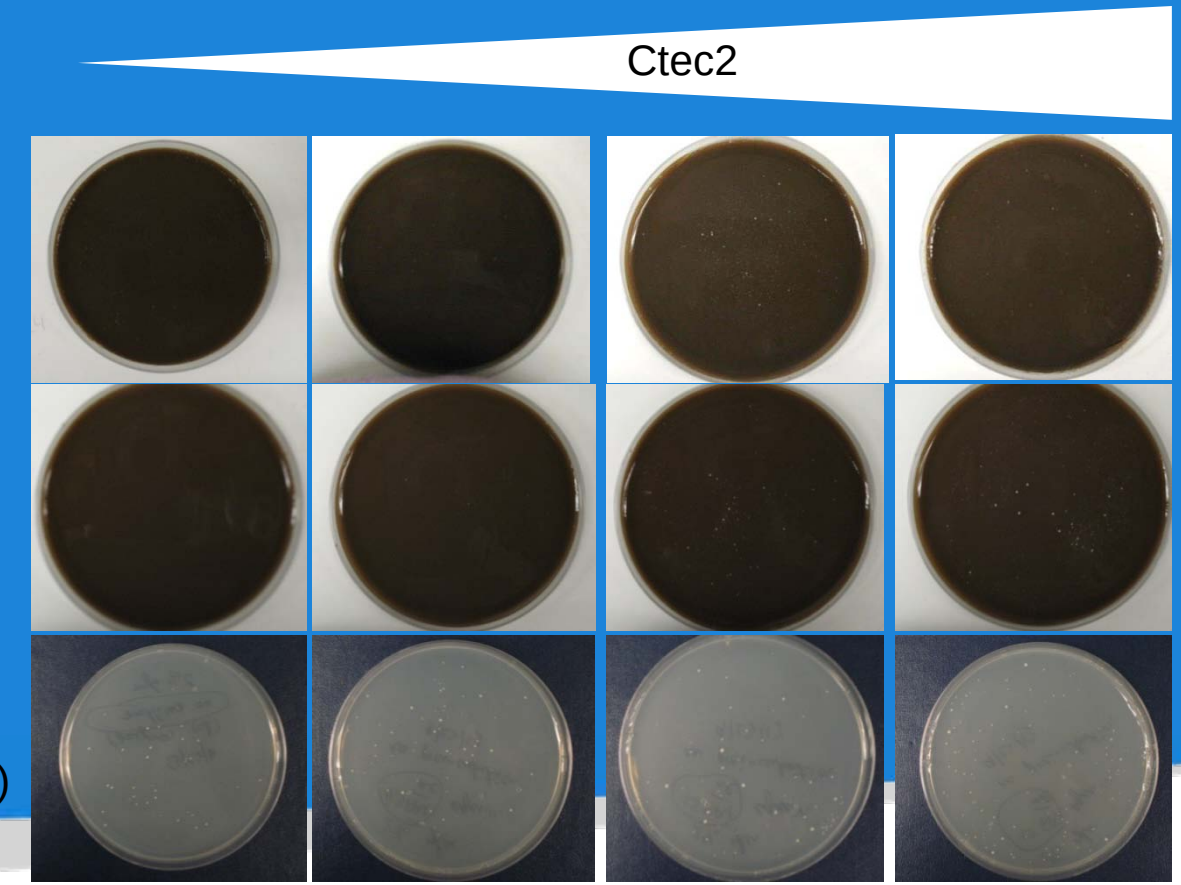
3 – Technical Accomplishments/ Progress/Results

- *Screening method being developed - colony growth on corn stover agar plates*



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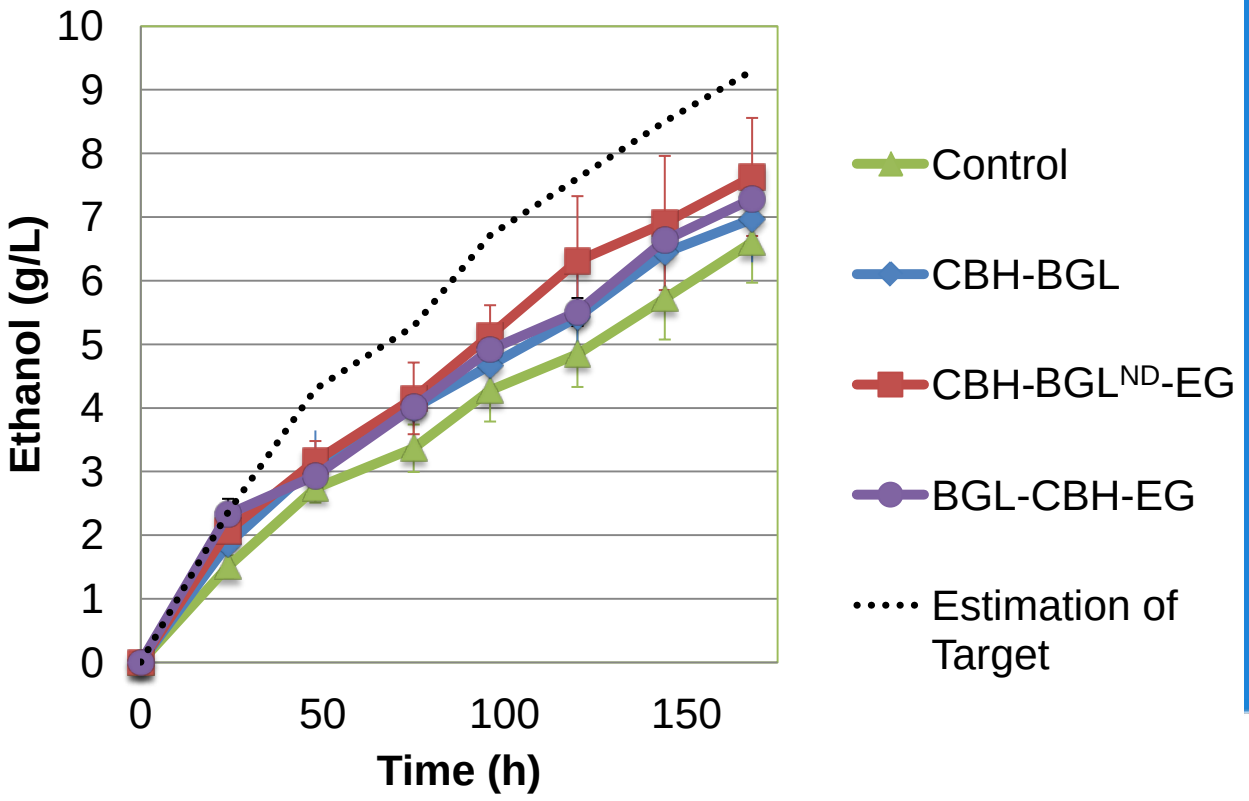
3 – Technical Accomplishments/ Progress/Results

- *Very preliminary corn stover result*

SSF
Enzyme loading:
1 FPU/g cellulose
10% w/w solids

% conversion		
Control	20	2
CBH-BGL	21	2
CBH-BGLND-EG	23	3
CBH-BGL-EG	22	1



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4 – Relevance

- *This project addresses barriers Bt-F (Cellulase Enzyme Production Cost), Bt-G (Cellulase Enzyme Loading), and Bt-J (Catalyst Development) identified in the BETO Multi-Year Program Plan*
- *Strains with superior saccharification properties will be licensed to or further developed with industrial partners*
- *Yeast strains that require less enzyme loading will result in reduced cost for enzyme cocktail to positively impact the commercial viability of biomass and/or biofuels*
- *Preparation is ongoing for IP protection*

5 – Future Work

- *Generation and identification of strains carrying optimal combinations of enzymes using corn stover as a feedstock*
- *Identification of characteristic features of combinations that result in efficient saccharification*
- *Demonstration of increase in saccharification efficiency and decrease in necessary enzyme load*
- *Preliminary assessment of the effect of scale-up using a bench-top bioreactor*
- *Final validation*

- *No more go/no-go point*

Summary

1. Overview: Powerful synthetic biology methods enable the reconstitution of natural synergism among enzymes in laboratory/industrial processes
2. Approach: Mating and meiosis in yeast are used to construct numerous multi-enzyme strains
3. Technical Accomplishments/Progress/Results: We have designed and built a mini-cellulosome system, generated synthetic constructs for 98 enzyme genes, and so far confirmed enzyme activity for 28 enzymes
4. Relevance: Our research targets reducing costs associated with enzymes by reducing enzyme loading
5. Future work: We will generate and screen multi-enzyme strains to identify those containing optimal sets of enzymes

Additional Slides

Publications, Patents, Presentations, Awards, and Commercialization

Publications fully or partly supported by DE-EE0006109

(1) Karas, B.J., Jablanovic, J., Irvine, E., Sun, L., Ma, L., Weyman, P.D., Gibson, D.G., Glass, J.I., Venter, J.C., Hutchison, C.A. 3rd, Smith, H.O. & **Suzuki, Y.** Transferring whole genomes from bacteria to yeast spheroplasts using entire bacterial cells to reduce DNA shearing. ***Nature Protocols.* 2014** Apr; 9(4):743-750.

(2) Labunskyy, V.M., **Suzuki, Y.** (co-first author), **Hanly, T.J.**, Murao, A., Roth, F.P. & Gladyshev, V.N. The insertion Green Monster (iGM) method for expression of multiple exogenous genes in yeast. ***G3: Genes, Genomes, Genetics.* 2014** Apr 28; 4(7):1183-1191.

(3) Karas, B.J., Wise, K.S., Sun, L., Venter, J.C., Glass, J.I., Hutchison, C.A. 3rd, Smith, H.O. & **Suzuki, Y.** Rescue of mutant fitness defects using *in vitro* reconstituted designer transposons in *Mycoplasma mycoides*. ***Frontiers in Microbiology*. 2014** Jul 23; 5:369.

(4) Karas, B.J., **Suzuki, Y.** & Weyman, P.D. Strategies for cloning and manipulating natural and synthetic chromosomes. ***Chromosome Research*.** Published online.

(5) Suzuki, Y., Assad-Garcia, N., **Kostylev, M.**, Noskov, V.N., Wise, K.S., Karas, B.J., Stam, J., Montague, M.G., **Hanly, T.J.**, Enriquez, N.J., Ramon, A., Goldgof, G.M., Richter, R.A., Vashee, S., Chuang, R.Y., Winzeler, E.A., Hutchison, C.A. 3rd, Gibson, D.G., Smith, H.O., Glass, J.I. & Venter, J.C. Bacterial genome reduction using the progressive clustering of deletions via yeast sexual cycling. ***Genome Research*. 2015** Mar;25(3):435-444.

Three more papers in preparation

Meeting presentations

Kostylev, M., **Hanly, T.J.** & **Suzuki, Y.** Synthetic biology for biofuels: the engineering of a multivalent cellulosome on the cell surface of *Saccharomyces cerevisiae*. American Society for Microbiology 114th General Meeting (Boston, MA). 2014 May17-20.

Two meeting presentations in preparation for 37th Symposium on Biotechnology for Fuels and Chemicals

Technology transfer and commercialization efforts

Preparing for IP protection