U.S. Department of Energy (DOE)
Bioenergy Technologies Office (BETO)
2017 Project Peer Review

Maximizing multi-enzyme synergy in biomass degradation in yeast

March 9, 2017
Biochemical Conversion Session

Yo Suzuki
J. Craig Venter Institute

Goal Statement

- *Enzymatic hydrolysis and enzyme production comprise a substantial part of biofuel cost*
- *More efficient saccharification reactions or reduced quantitative needs for enzymes in cocktails help achieve cost-competitive production of biofuels*
- *Demonstrate synthetic biology technologies for rapidly discovering synergy among biomass-active enzymes and incorporating it into the saccharification process*
- *If successful, enzymes that synergize among themselves or with a current enzyme cocktail can be identified quickly and examined for inclusion in the future generation of enzyme cocktails*
- *Digital biological converter (Craig Venter)*
- *Improved cocktails will help reduce saccharification costs*

Quad Chart Overview

Timeline

- Apr 25, 2013
- Oct 31, 2016
- 100% complete

Barriers

- Bt-F (Cellulase Enzyme Production Cost)
- Bt-G (Cellulase Enzyme Loading)
- Bt-J (Catalyst Development)

from Multi-Year Program Plan

Budget

	FY 12–FY 14	FY 15	FY 16	FY 17 (Project ended)
DOE Funded	$513,934	$432,021	$318,802	$1,467
Cost Share	$132,647	$1,434	$6,611	$0

Partners

<u>Interactions/collaborations</u>

- NREL
  - Dan Schell
  - Ling Tao
  - Pin-Ching Maness
- Synthetic Genomics, Inc.
- Novozymes

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1 - Project Overview

Pioneering work:

- *Synergy among carbohydrate-active enzymes is prevalent*
- *Heterogenous enzymes can be expressed in yeast*
- *Designer cellulosomes can be produced in yeast*

Our work:

- *Use gene synthesis to access enzymes from various environments*
- *Combine multiple enzymes in Saccharomyces cerevisiae (rapid generation of expression constructs, CRISPR engineering)*
- *Shuffle enzyme constructs using mating and meiosis in yeast*
- *Express the enzymes and assemble them into yeast surface-displayed cellulosomes for activity testing*
- *Identify combinations of enzymes that are effective*

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*
- *Daily interaction, weekly lab meeting, monthly note checking, monthly TC with DOE*
- *Challenges: various delays (recruitment, design of validation conditions, institute move, vendor delivery, extended testing of pipeline, problem with an DNA assembly technique and replacement with a new technique for generating enzyme constructs)*
- *Critical success factors: completion of all proposed tasks*

2 – Approach (Technical)

Establish a synthetic biology pipeline and convert enzyme sequences to yeast strains with activity, via gene synthesis, generation of expression constructs, integration of the constructs into yeast chromosomes, and enzyme activity testing (achieved)

ML: Demonstrated activity in yeast for 45 enzymes ***(>20: go-no go criterion)***

Generate multi-enzyme strains and shuffle the enzyme constructs via mating and meiosis of yeast (unique goal, achieved)

ML: Demonstrated near random assortment of enzyme constructs in the first 2 successive crosses, but diversity loss was apparent by cross 5 due to strain fitness; decided to cross 2 strains just once to generate a pool of shuffled strains to screen

2 – Approach (Technical)

Establish an assay for screening strains for degradation of pretreated corn stover (challenging goal, achieved)

ML: Growth-based screen in a 96-well format was the only method that worked

Identify strains carrying optimal combinations of enzymes (achieved)

ML: Identified top performers and determined the genotypes for enzymes

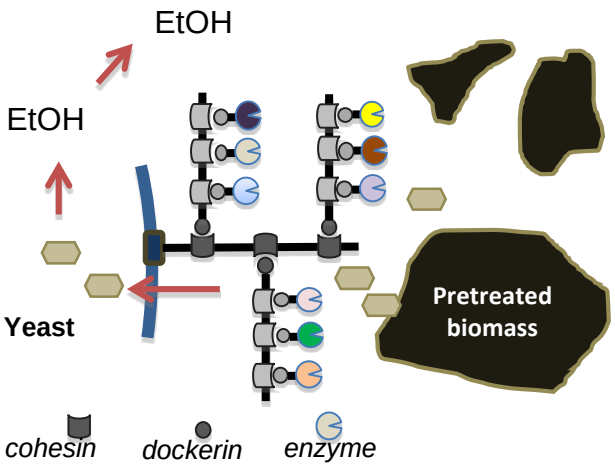
ML: Demonstrated 20-30% more ethanol production from pretreated corn stover with the engineered strains compared with a negative control strain in a selected condition

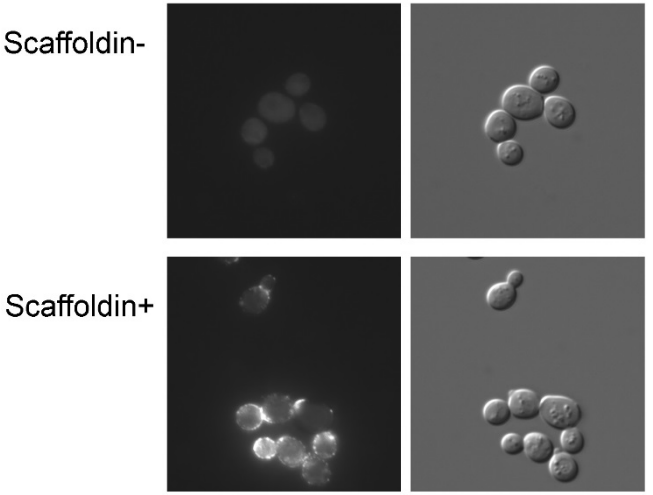
Target: 33% reduction in required enzyme load to achieve the same saccharification level in lab-scale cultures (not achieved)

The ability of yeast to produce enzymes is limited; the identified enzymes need to be recombinantly produced and mixed with enzyme cocktails to be evaluated

3 – Technical Accomplishments/ Progress/Results

- *Two-layered cellulosome with 9 sites for enzyme loading*





Inspired by

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

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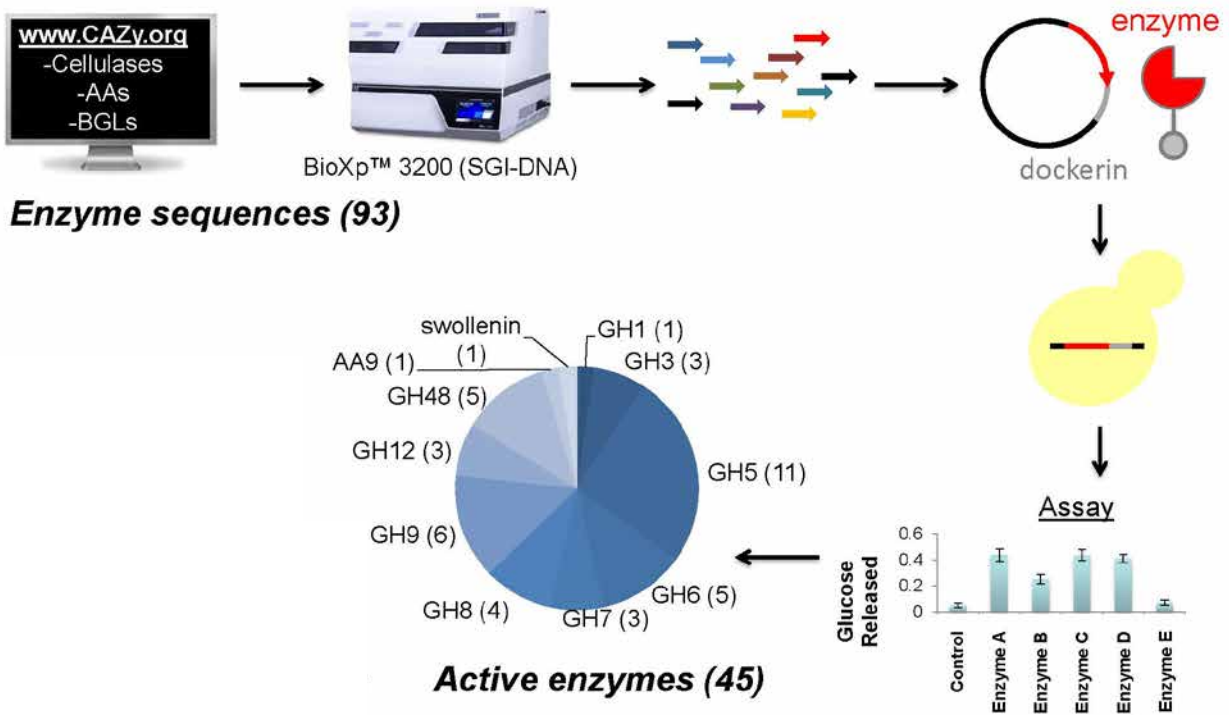
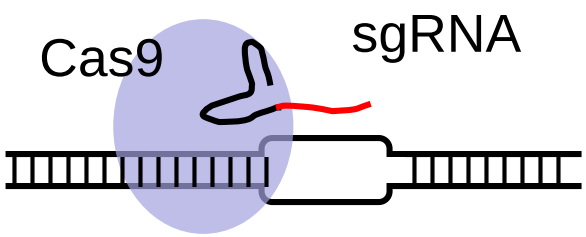
3 – Technical Accomplishments/ Progress/Results

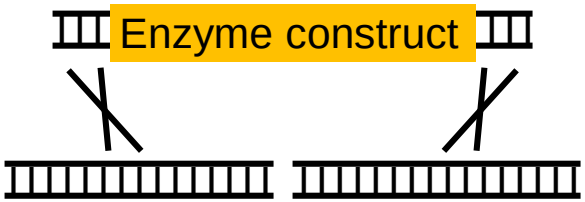


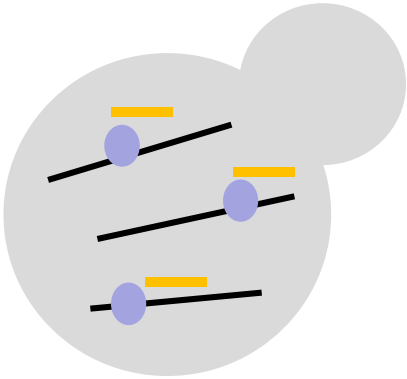
- *Synthetic biology pipeline for gene synthesis, construct assembly, strain construction, and strain testing*

3 – Technical Accomplishments/ Progress/Results

- *Multiplexed CRISPR-mediated genome engineering*







- *Multi-enzyme strains*

Locus / Strain	A	B	C	D	E	F	G	H	I
1	T cell 6A	Pbarc EndA	Tstip 5A	B.Lichen GH12	FpalGH12	Cter GH9d	Af swol	Chtherm cbhI	Piro E2 48
2	Rem cbhII	TW4	Tr EGII	B.Lichen GH12	Tkoningii EG	Cterm 9C	Treesei sw	Mtherm cbhII	Ccell 48
3	Tkoningii cbhII	Acell GH5	Aoryzae EndB	Sdegrade ns GH5J	Gtrabeum GH12	CT CelD	Tfusca AA10b	Tmarn Cel7B	Npat GH48
4	Tr CBHII	MH2	Cphy Cel5A	Cter GH5a	A. kawachii GH12	T. fusca 9A	Pchry AA9	T. emersonii cbhI	Cphy 48
5	Aniger cbhB	MH3	Cter GH5b	CT CelB	Tkoningii EG	T. fusca 9A	T. aurantiac us GH61a	Aniger cbhA	Acell 48

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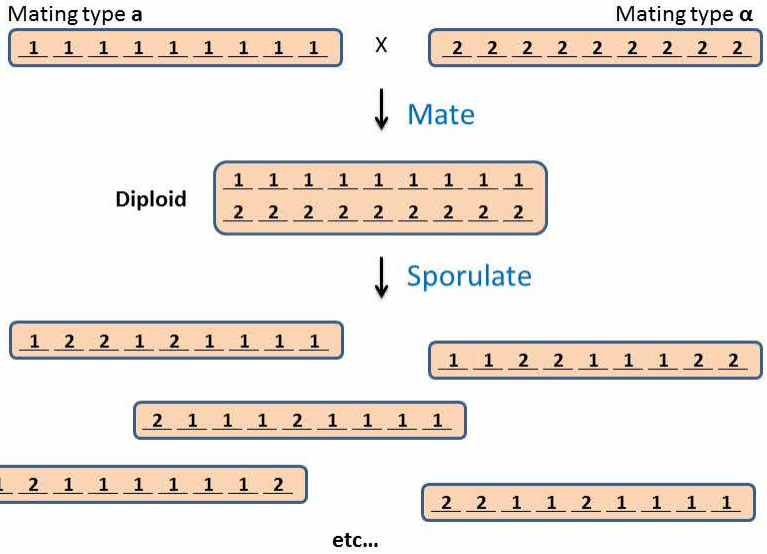
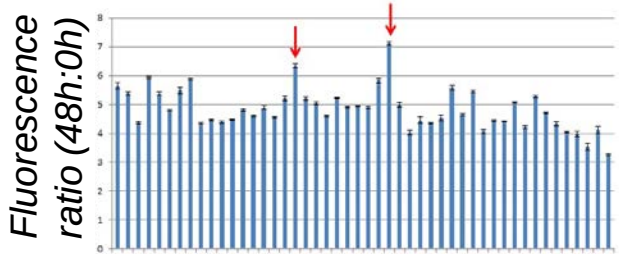
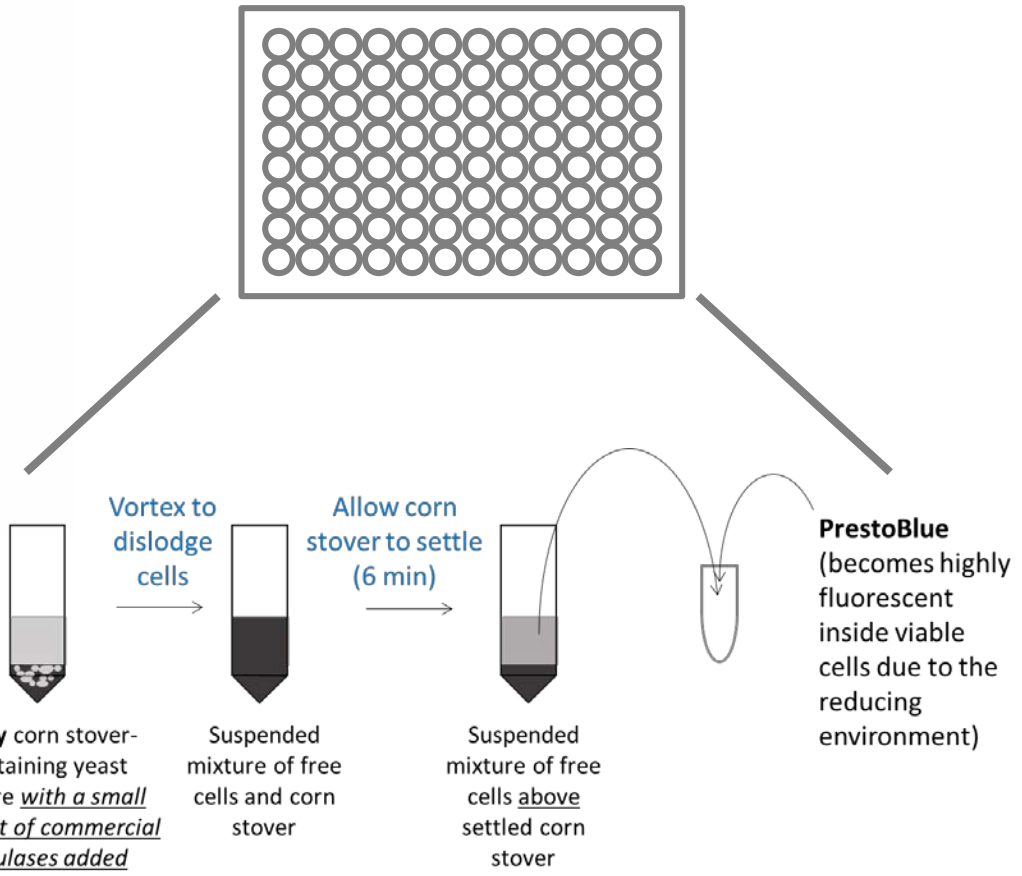
3 – Technical Accomplishments/ Progress/Results

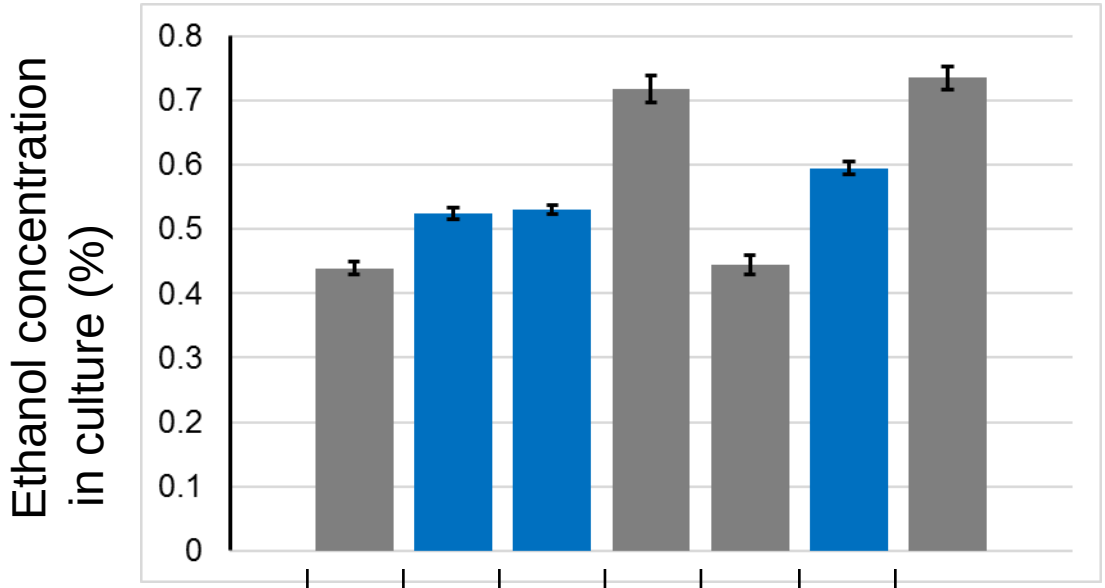






- *Shuffling of enzyme constructs and selection of top performers*

3 – Technical Accomplishments/ Progress/Results



Strain	B	E1	E2	B	B	E1	B
Ctec2 (mg/g glucan)	1.4	1.4	1.4	2.1	1.4	1.4	2.1
Supernatant	-	-	-	-	+	+	+

- *Simultaneous saccharification and fermentation, 10% solids, 96 h, 100 mL*
- *If the same enzymes are provided as recombinant proteins, the titer may be higher*

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4 – Relevance

- *Goal: demonstrate synthetic biology technologies for rapidly discovering synergy among biomass-active enzymes toward incorporating it into the saccharification process*
- *Novel synergy would improve enzyme cocktails, and the improved cocktails would be more efficient or needed in a smaller amount, resulting in the reduction of the cost of saccharification*

- *This project addresses BETO's goal to reduce costs for enzymatic hydrolysis and enzyme production*

- *Our synthetic biology approach can be expanded to additional enzymes (e.g., lignin-modifying enzymes) and feedstocks. Mechanisms of enzyme synergy can be dissected in our system, and the validated enzymes can be examined for suitability for inclusion in future enzymes cocktails*
- *We wish to collaborate with industrial partners*

Summary

1. Overview: digital biological converter, multi-enzyme strains, enzyme shuffling, identification of synergy
2. Approach: successful management despite some delays; milestone-driven approach to prepare enzyme constructs, shuffle these constructs, establish an assay for screening, and identify strains carrying optimal combinations of enzymes
3. Technical Accomplishments/Progress/Results: two-layered scaffold with nine enzyme sites, functional expression for 45 out of 93 enzymes using a synthetic biology pipeline, ten nine-enzyme strains, viability-based screen with pretreated corn stover, enhanced ethanol production with the selected set of enzymes
4. Relevance: technologies aimed at improving enzyme cocktails to reduce saccharification costs

Additional Slides

Responses to Previous Reviewers' Comments

Yeast is incapable of expressing high enough amounts of enzymes for consolidated bioprocessing

- We have emphasized our method as a discovery tool for enzyme synergy including positive interactions with current enzyme cocktails

The relationship with the previous work is not clear

- Our research builds on pioneering work of others

Publications, Patents, Presentations, Awards, and Commercialization

Publications Fully or Partly Supported by DE-EE0006109

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.
http://www.nature.com/nprot/journal/v9/n4/full/nprot.2014.045.html

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.
http://www.g3journal.org/content/4/7/1183.long

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.
http://journal.frontiersin.org/Journal/10.3389/fmicb.2014.00369/abstract

Karas, B.J., **Suzuki, Y.** & Weyman, P.D. Strategies for cloning and manipulating natural and synthetic chromosomes. *Chromosome Research*. 2015 Feb; 23(1): 57-68.
http://rd.springer.com/article/10.1007%2Fs10577-014-9455-3

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.
http://genome.cshlp.org/content/25/3/435.long

Publications, Patents, Presentations, Awards, and Commercialization

Zheng, H., Lefebvre, S.C., Smith, S.R., **Hanly, T.J.**, **Suzuki, Y.** & Allen, A.E. Successful diatom transcription factor synthesis and downstream cloning using the BioXp™ 3200 system. *BioTechniques*. 2015 July; 59(1):46-47. http://www.biotechniques.com/multimedia/archive/00250/BTN_A_000114311_O_250253a.pdf

Kostylev, M., Otwell, A.E., Richardson, R.E., & **Suzuki, Y.** Cloning should be simple: *Escherichia coli* DH5α-mediated assembly of multiple DNA fragments with short end homologies. *PLOS ONE*. 2015;10(9):e0137466. http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0137466

Manuscript in preparation:
Kostylev, M., **Hanly, T.J.**, & **Suzuki, Y.** Maximizing multi-enzyme synergy in biomass degradation in yeast (tentative title).

Publications, Patents, Presentations, Awards, and Commercialization

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.

Suzuki, Y. Maximizing multi-enzyme synergy in biomass degradation in yeast. 2015 Project Peer Review (Alexandria, VA). 2015 Mar 23-27.

Kostylev, M., **Hanly, T.J.** & **Suzuki, Y.** The engineering of a multivalent cellulosome on the cell surface of *Saccharomyces cerevisiae*. 37th Symposium on Biotechnology for Fuels and Chemicals (San Diego, CA). 2015 Apr 27-30. Oral presentation.

Hanly, T.J., **Kostylev, M.** & **Suzuki, Y.** Toward conversion of corn stover to ethanol by *Saccharomyces cerevisiae* displaying a cellulosomes. 37th Symposium on Biotechnology for Fuels and Chemicals (San Diego, CA). 2015 Apr 27-30.

Lectures

Video conferencing. Instructor Michael Gallagher. Saratoga Springs High School (Saratoga Springs, NY). 2015 Oct 23.

Lecture. Synergy among cellulases. Instructor Matt Leader. High Tech High of North County (San Marcos, CA). 2015 Dec 10.

Lecture. Genetic interaction. BIOL 549 Microbial Genetics. Instructor Marina Kalyuzhnaya. San Diego State University (San Diego, CA). 2016 Oct 27.