Agile BioFoundry

U.S. DEPARTMENT OF ENERGY | Energy Efficiency & Renewable Energy

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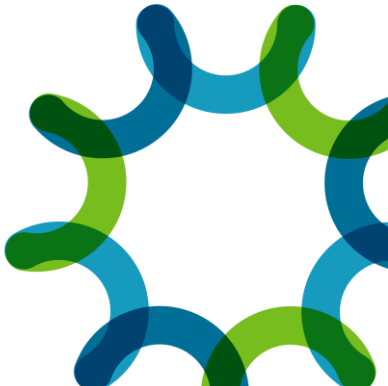
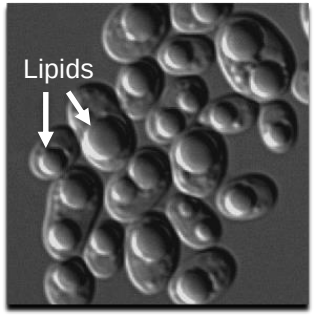
Host: *Rhodosporidium toruloides*

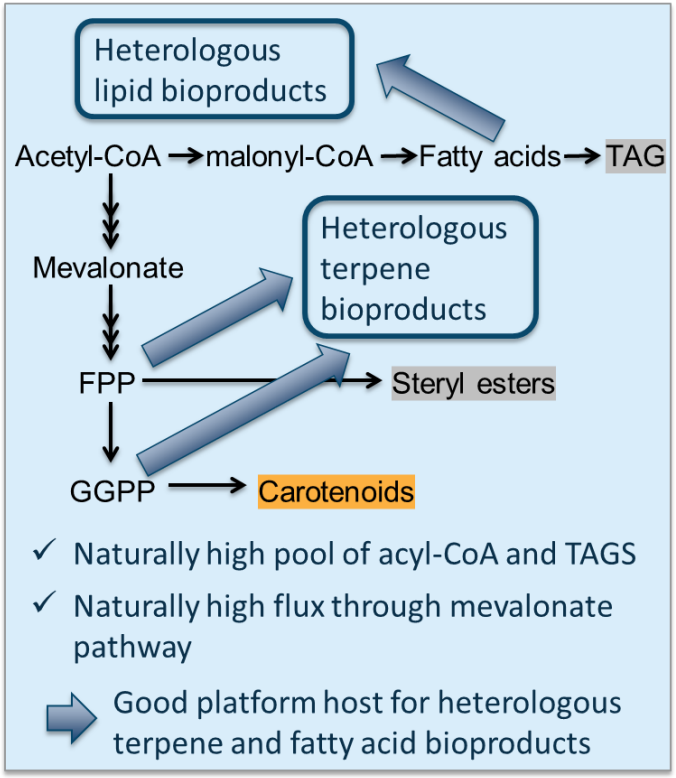
John Gladden

ABF IAB
October 4th, 2019



Rhodosporidium toruloides?



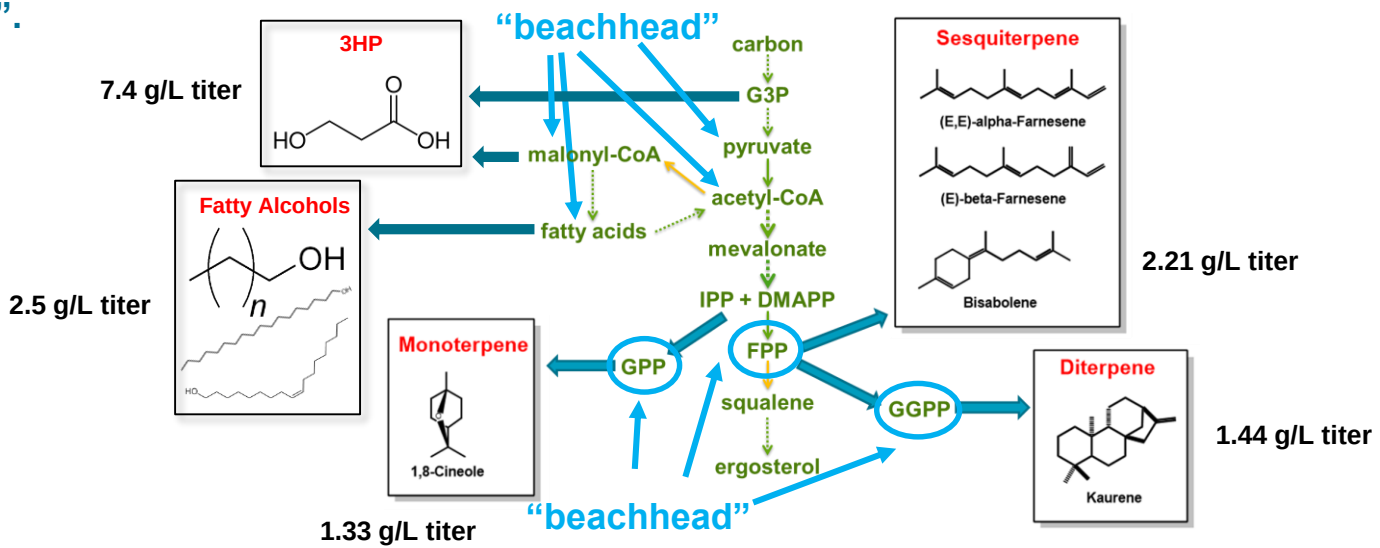


Rhodosporidium toruloides

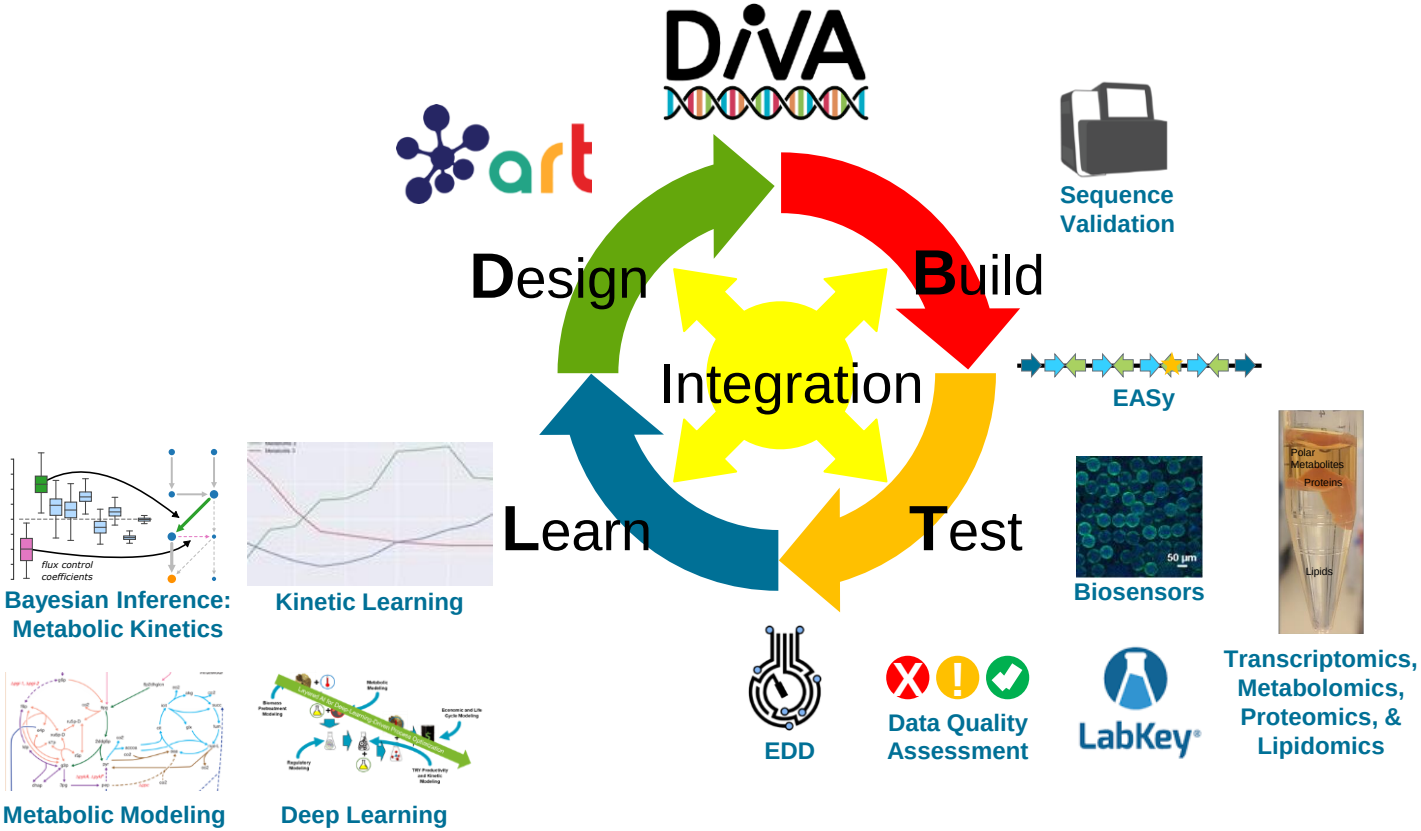
- Utilizes lignocellulose
- Fast growing
- Oleaginous, carotenogenic
- Metabolically versatile
- Genetically tractable

Current Targets and Potential Beachheads

- **Target 1: Terpenes**- biofuels and bioproducts (adhesives, insect repellents, polymers, fragrances, food additives)
- **Target 2: Fatty Alcohols**- Detergents, lubricants, plastics and cosmetics. $5.2 billion in 2011 globally. Grow at 4% CAGR in next decade.
- **Transfer Target: 3HP-** acrylate polymers, biodegradable polymers
- **Beachheads:** These targets pull from many versatile metabolites that provide opportunities to produce literally thousands of bioproducts. Beachhead stains could be an asset ABF offers to **enable industry to focus on the "last mile".**



ABF DBTL Infrastructure



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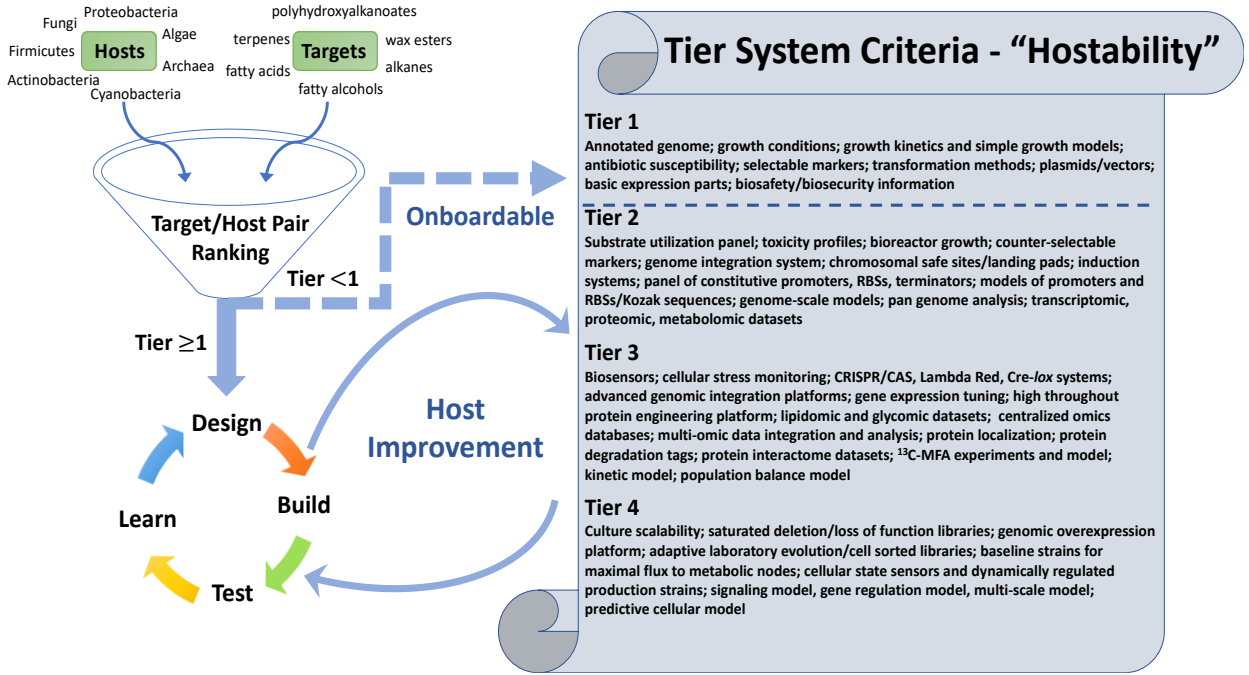
Host Onboarding -Tier system



- Tier 1 represents the basic tools needed for DBTL
- Hosts that do not meet Tier 1 require further development prior to usage
- Tools increase in sophistication as an organism moves up Tiers
- Not all tools in all Tiers are required for all organisms

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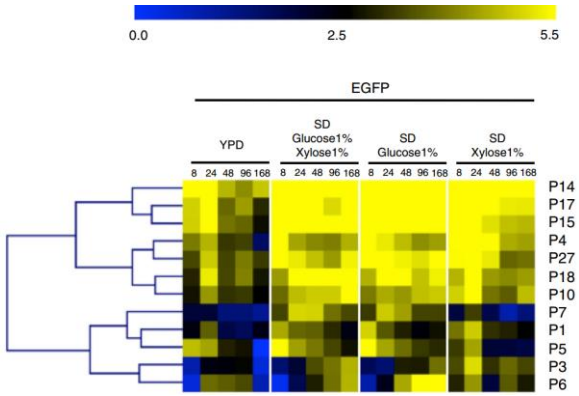
Host Development- Tier Elevation

- **Many tier elevation efforts currently under way for FY21Q1_HOD_R1**
  - FY21Q1_HOD_R1 Continue host improvement to elevate 2-3 onboarded hosts at least one Tier.
  - *FY21Q4_DBTL_AS1* (SOP coverage of core DBTL unit operations)

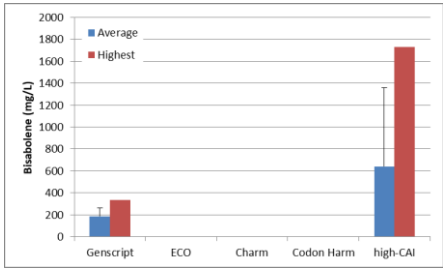
Expanding Parts: Promoters



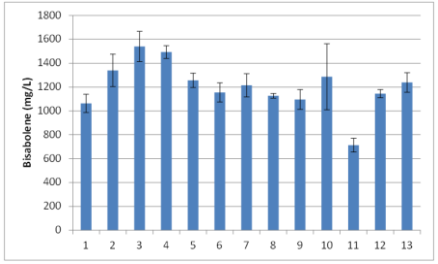
A toolset of constitutive promoters for metabolic engineering of *Rhodosporidium toruloides*

Luísa Czamanski Nora[1,2,3], Maren Wehrs[2,3,4], Joonhoon Kim[2,5], Jan-Fang Cheng[6,8], Angela Tarver[6,8], Blake A. Simmons[2,3], Jon Magnuson[2,5], Miranda Harmon-Smith[6], Rafael Silva-Rocha[1], John M. Gladden[2,3,7], Aindrila Mukhopadhyay[2,3,8], Jeffrey M. Skerker[8,9*] and James Kirby[2,3,7*]

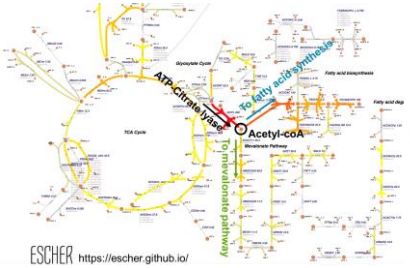
Codon Optimization



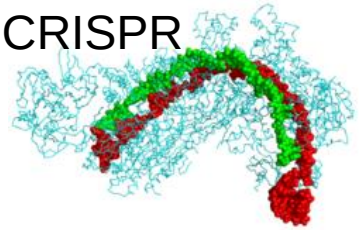
Media Optimization



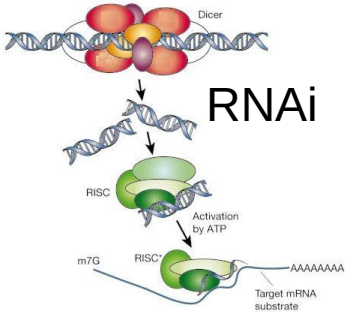
Metabolic Model



CRISPR



RNAi



Functional Genomics: TnSeq

ATMT
Agrobacterium tumefaciens mediated transformation

Map (Tnseq)
Associate barcodes with insertion locations

Count (Barseq)
Quantify strain abundance in experimental conditions

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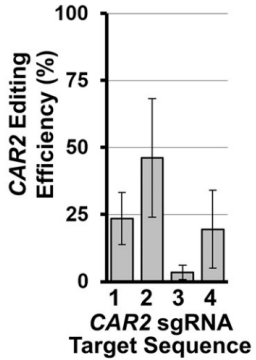
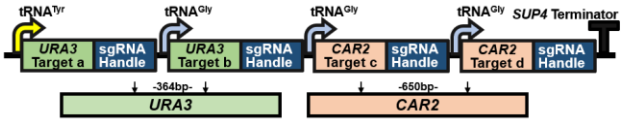
CRISPR Toolset in *R. toruloides*

- **Developed 1st approach for simultaneously deleting 2+ genes in *Rhodo***

Optimized CRISPR-Cas9 expression to achieve ~50% editing efficiency:

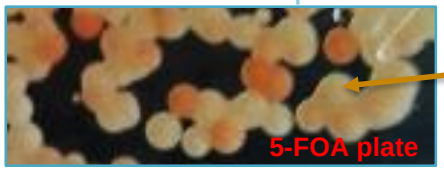


Designed a tRNA-driven sgRNA array for multiplexed edits:



Achieved efficient multiplexed editing:

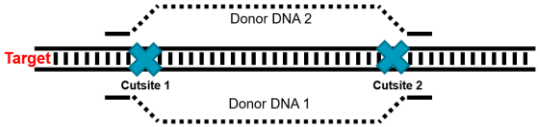
Colonies Forming = *URA3* Deleted

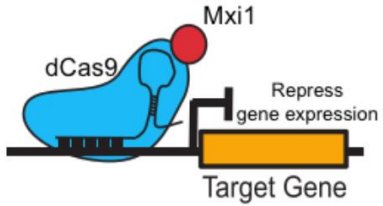


White = *CAR2* Deleted

- **ABF actively using this tool to expedite targeted gene deletion in *Rhodo***
- **Exploring advanced CRISPR techniques for enhancing editing of DNA/RNA**

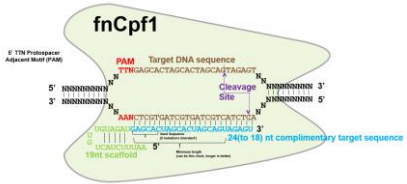
Utilizing co-transformed Donor DNA for targeted insertion





dCas9 constructs built to repress eGFP in *Rhodo*

Next-gen DNA-editing Cpf1 built, being tested in *Rhodo*



Next-gen RNA-editing Cas13 built, being tested in *Rhodo*

Otoupal et al. Multiplexed CRISRP-Cas9-Based Genome Editing of Rhodosporidium. mSphere. 2019.

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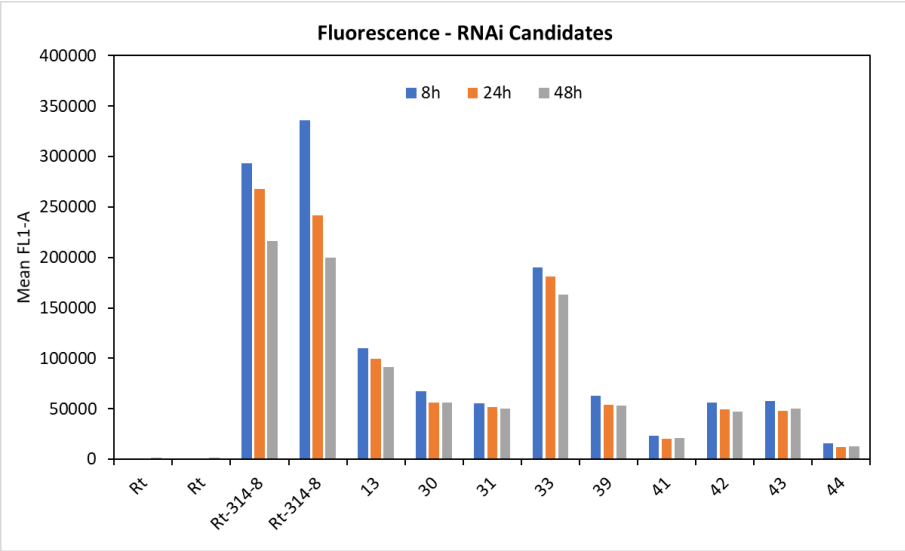
Establishing RNAi in *R. toruloides*

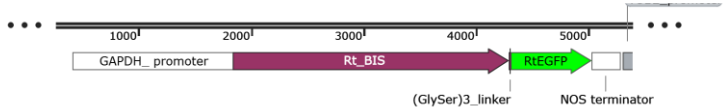
- RNAi can be a valuable screening tool, especially for partial knockdown of essential genes, like ACC1, the major switch for lipid metabolism.
- Can we develop this tool for *R. toruloides*?
  - FY21Q1_HOD_R1 Continue host improvement to elevate 2-3 onboarded hosts at least one Tier.



- Successful knockdown of GFP fluorescence in RNAi strains

- Double inverted promoter RNAi targets GFP in bisabolene synthase-GFP fusion gene

Target: Bisabolene synthase fused to GFP



RNAi: GFP double promoter

- Good correlation between GFP fluorescence and bisabolene titers indicates RNAi leads to full transcript degradation.

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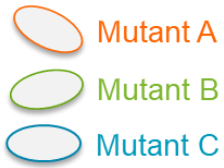
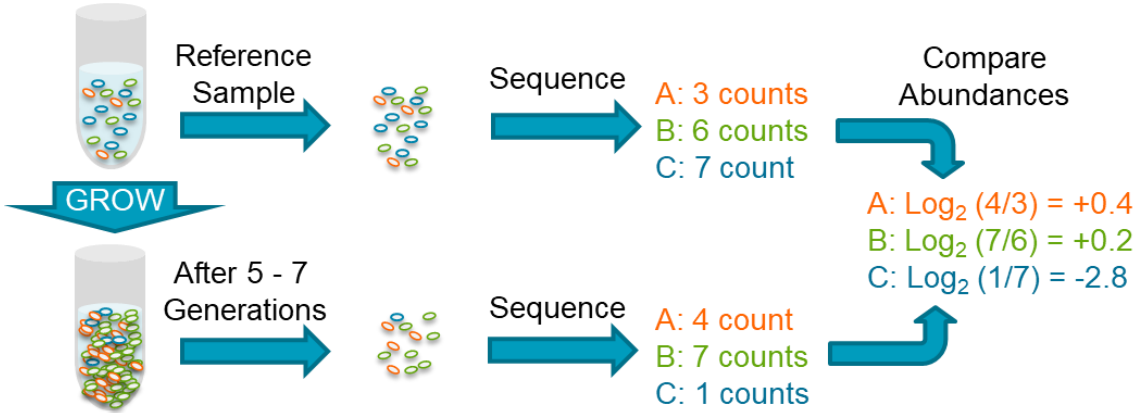
TnSeq: A Simplified View

- **Combines transposon-based mutagenesis with high throughput sequencing to identify genes involved in a biological function**

Starting population:
A mixed pool of mutant strains
Each strain has an insertion in a different location
Insertion locations have been mapped to different genes



Experiment:
Mutant pool is cultivated in condition of interest and re-sequenced
Changes in relative abundance for each mutant is calculated as a 'Fitness Score'



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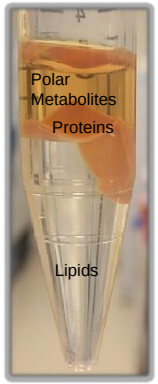
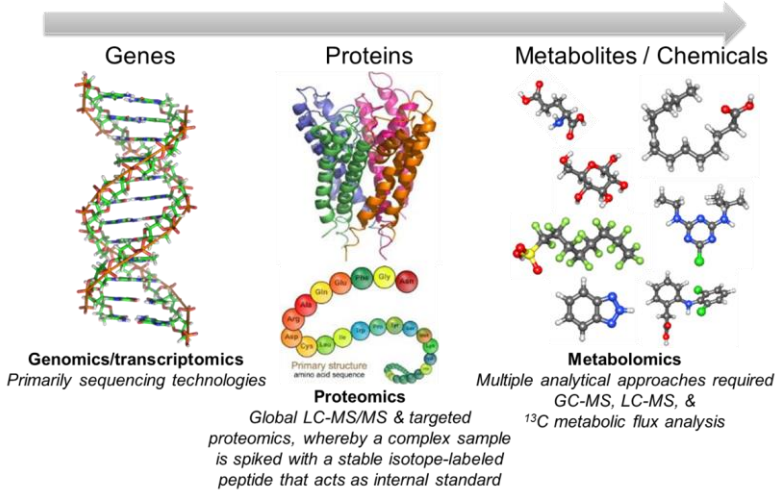
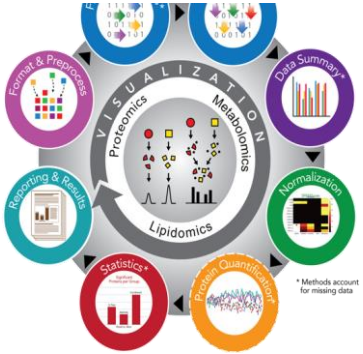
ABF– Multi-omics Analysis

- **Single-sample Metabolite, Protein and Lipid Extraction**
  - Method developed previously: Burnum-Johnson *et al.* Analyst, 2017, 142, 442-448
  - Now used at the ABF







Host	Proteomics (global + targeted)	Metabolomics/Lipidomics (Intra/Extracellular)
P. putida	>300 datasets	>500 datasets
A. pseudoterreus	>250 datasets	>450 datasets
R. toruloides	>250 datasets	>300 datasets

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Metabolic network modeling of *R. toruloides*

- The genome-scale metabolic network reconstruction of *R. toruloides* was generated and validated against high-throughput experimental data
- The metabolic model was used to model lipid accumulation and elucidate lignocellulosic carbon utilization pathways using omics-data
  - FY21Q1_HOD_R1 Continue host improvement to elevate 2-3 onboarded hosts at least one Tier.

Growth phenotype prediction

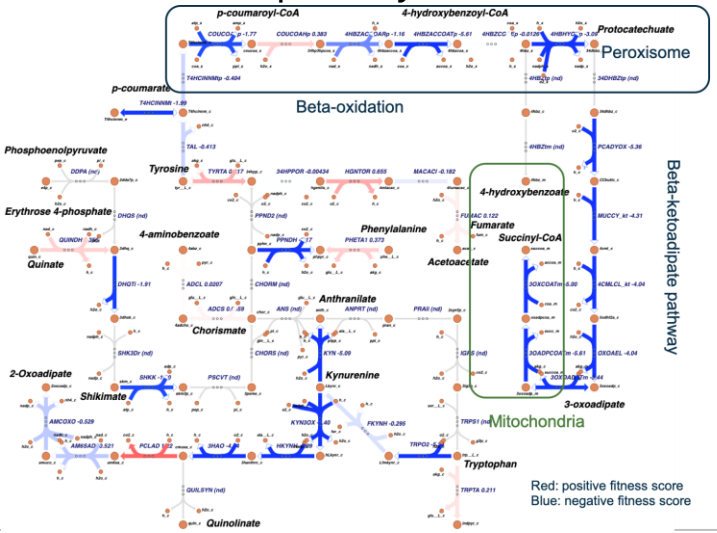
Biolog Data

PlateType	Row	Column 1	2	3	4	5	6	7	8	9	10	11	12
PM1	A	0	0.23	-0.02	-0.02	0.02	0.06	0.18	0.19	0.04	0.13	0.13	0.05
	B	0.06	0.04	0.14	0.01	-0.01	0.06	-0.01	0.23	0.04	0.09	0.03	0.21
	C	0	-0.03	0.03	0.23	0.31	0.01	0.17	0.16	0.13	0.02	-0.01	0
	D	0.19	0.01	-0	-0	0.26	0.02	0.07	-0.01	-0.01	-0	0.04	0
	E	0.23	-0.02	-0.01	0.01	0.32	-0.02	0.01	0.13	0.14	0.04	-0.02	-0.01
	F	-0	0	0.01	0.03	0.01	0.09	0.1	0	-0	-0.01	0.11	0.01
	G	0.07	-0.02	0.1	0.07	0.15	0.1	0.08	-0	0.11	0.08	0.04	0.02
	H	0.14	0.05	-0	-0.02	0.04	0.31	0.03	0.13	0.17	0.16	-0	-0.01
PM2	A	0	-0.02	-0.02	-0	0.02	0.05	0.05	-0.01	0.08	0.12	-0.02	0.08
	B	-0	-0.01	0.02	0.02	0.19	0.16	0.17	0.22	0.13	-0.01	0.03	0.03
	C	0.15	0.03	0	0	0.01	0	0.02	0.01	-0	0.01	-0	0.01
	D	-0.01	0.25	0.01	0.01	0	0.08	0.04	0.18	-0	0.16	0.02	0.18
	E	-0.08	0.1	-0.01	-0.01	0.09	0.02	0.1	0.11	0.13	0.07	-0.01	0.14
	F	0.03	-0.02	0.04	-0.01	0.06	0.07	-0.02	0.15	0.1	0.05	-0.01	-0.01
	G	0.01	0.17	-0.01	0.11	0.17	0.03	0.08	0.01	0.16	0.09	0.01	-0.02
	H	0.16	-0.02	0.19	0.1	-0.02	-0.04	0.01	-0.02	0.26	-0.01	0	-0.02
PM3B	A	0	0.12	0.06	0.11	0.13	0.01	0.12	0.13	0.11	0.14	0.06	0.24
	B	0.17	0.1	0.04	0.08	0.08	0.06	0.12	0.07	0.13	0.07	0.06	0.16
	C	0.1	0.1	0.05	0.01	0	-0.05	0.01	0.01	0.02	0.09	0.15	0.12
	D	0.01	0.12	0.1	0.05	-0.01	0.06	0.05	0.06	0.08	-0.02	0.02	0
	E	-0.03	0.02	0.05	0.05	0.06	0.1	0.11	0.03	0.08	0.03	0	-0.01
	F	-0	0.14	0.14	0.1	0.08	0.03	0.18	0.05	0.02	0.09	0.09	0.22
	G	0.22	0.1	0.14	0.21	0.09	0.11	0.06	0.16	0.04	0.13	0.1	0.21
	H	0.1	0.19	0.09	0.17	0.16	0.06	0.11	0.21	0.16	0.18	0.18	0.27
PM4A	A	0	0.15	0.23	0.24	0.32	-0	-0.01	0.23	0.17	0.15	0.14	0.21
	B	0.06	0.26	0.15	0.14	0.27	0.23	0.22	0.13	0.16	0.15	0.17	0.15
	C	0.24	0.17	0.15	0.18	0.01	0.15	0.24	0.23	0.01	0.25	0.13	0.13
	D	0.19	0.23	0.3	0.17	0.18	0.17	0.2	0.16	0.14	0.16	0.16	0.15
	E	0.17	0.12	0.15	0.15	0.14	-0.01	-0.01	0.11	0.13	0.12	0.22	0.06
	F	0.01	0.17	0.19	0.13	0.15	0.13	0.11	0.05	0.27	0.07	0.12	0.13
	G	0.05	0.09	0.12	0.11	0.07	0.04	0.2	0.14	0.2	0.11	0.19	0.11
	H	0.26	0.1	0.08	0.2	0.06	0.12	0.15	0.02	0.12	0.11	0.14	0.01

Model Prediction

PlateType	Row	Column 1	2	3	4	5	6	7	8	9	10	11	12
PM1	A	nan	0.07	0	nan	0.05	0.07	0.06	0	0	0.07	0.07	0.06
	B	0.06	0.06	0.06	nan	0.06	0.07	0.09	0.04	0	nan	0.07	
	C	0.14	nan	nan	0.09	nan	0.09	0.02	0.09	0.11	nan	nan	0
	D	0.06	nan	nan	0	nan	0.07	0.05	nan	nan	0.11		0
	E	0.07	nan	0.14	0.14	nan	nan	nan	nan	0.13	nan	nan	0.06
	F	0.06	0.08	0	nan	0.04	nan	0.04	nan	0	0.11		0
	G	0.07	nan	0.06	0.06	0	0	nan	nan	nan	nan	nan	0.07
	H	0	nan	nan	0	nan	nan	nan	0.06	nan	0	0	0
PM2	A	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan
	B	nan	nan	nan	nan	0	0.06	0.06	nan	0	nan	nan	nan
	C	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan
	D	nan	nan	nan	0	nan	nan	nan	0.06	nan	0.05	nan	nan
	E	0	0	nan	nan	0	0.03	nan	nan	nan	nan	nan	0.09
	F	nan	nan	nan	0	0.04	nan	nan	nan	nan	nan	nan	nan
	G	nan	nan	nan	0.05	0	0	0.06	nan	0.05	0.04	0	0
	H	0.05	0	nan	0.05	nan	nan	nan	0.05	0.07	nan	nan	nan
PM3B	A	nan	0.06	0.06	0.06	0.06	nan	0.02	0.06	0.06	0.06	0	0.07
	B	0.07	0.02	0	0.06	0.06	0	0	0	0	0.06	0.06	0.06
	C	0.06	0.06	0.02	nan	nan	nan	nan	0.06	nan	0.06	0.06	0.06
	D	nan	nan	nan	nan	nan	nan	nan	nan	0	nan	0.06	nan
	E	nan	0	0	0.06	0.06	nan	nan	0	nan	nan	0	nan
	F	nan	0.06	0.07	0.01	0.01	0.06	0.14	0	0	0	0	0
	G	0	0	0	nan	0.06	nan	nan	0.06	nan	nan	nan	nan
	H	0.06	0.07	nan	0.02	0.02	0.06	0.06	0.06	0.07	0.07	0.02	0.02
PM4A	A	nan	0.06	0.06	nan	0.06	nan	nan	nan	nan	0.11	nan	nan
	B	nan	nan	0.07	0.06	0.06	0.1	0.1	nan	nan	0.14	nan	0
	C	0.1	nan	0.14	0.14	nan	0	0.11	nan	nan	0.06	nan	nan
	D	0.14	0.14	nan	nan	0	nan	nan	nan	nan	0.06	nan	nan
	E	nan	nan	nan	0	0.06	nan	nan	nan	nan	0.06	nan	nan
	F	nan	0.06	0	nan	nan	nan	0.06	nan	0.06	0.06	nan	0.06
	G	nan	nan	0.06	nan	0.07	nan	0.06	nan	nan	nan	nan	nan
	H	nan	nan	nan	nan	0	0.06	0	nan	nan	nan	nan	nan

Metabolic pathway elucidation



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Roadmap to Automation in FY20 and Beyond

- Use demonstration project workflows as a baseline for developing automation platforms
- In FY20, define workflows and develop SOPs
- In FY20, start with Build workflows, and determine what can be automated

Automation
- *FY20Q3_DBTL_R5* (Identify DBTL tasks suitable for automation)
- *FY21Q4_DBTL_R5* (Workflow automation 2x efficiency improvements)
- *FY22Q4_DBTL_AS1* (5x efficiency improvements)

Standardization
- FY20Q1_DBTL_R1 (Standardization of 4 DBTL unit operations)
- *FY21Q4_DBTL_AS1* (SOP coverage of core DBTL unit operations)

Qpix
Used to spread cultures onto agar and pick colonies

Transformation workflow for *R. toruloides*

Biomek
Can be used for transformation, and soon for plasmid isolation

Biolector
Microfermentation
48 well plates measuring pH, Biomass, and %DO

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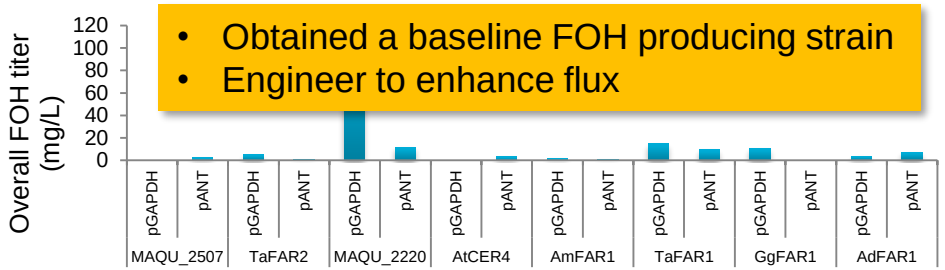
Fatty Alcohols in *R. toruloides*

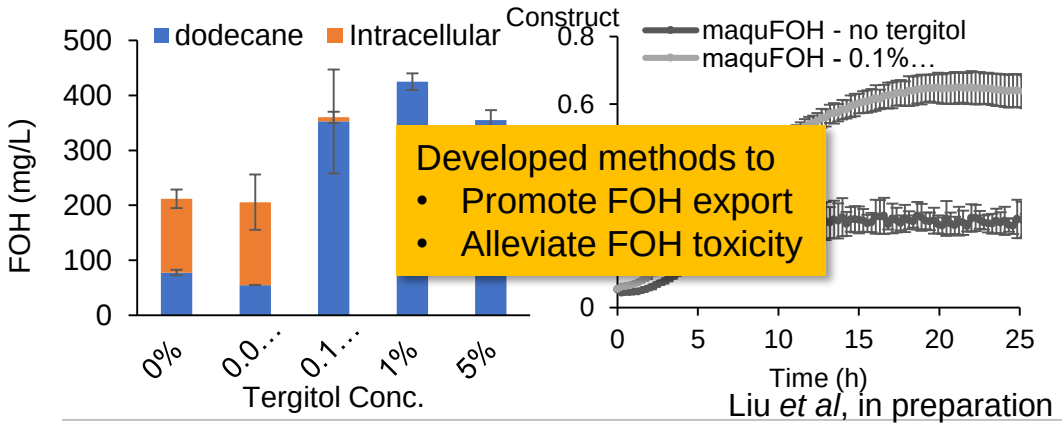
- Versatile family of chemicals
  - Detergents, surfactants, cosmetics and food applications
  - $5 billion global market with ~4% projected annual growth
- Current production methods require petrochemicals
  - Multiple processes from ethylene and alkenes
  - Hydrogenation of fatty acid methyl esters from natural oils
- Short enzymatic path from *R. toruloides'* efficient fatty acid biosynthesis pathway
- Potential platform for other fatty acid pathway derived products

FOH Strain Engineering and Media Optimization



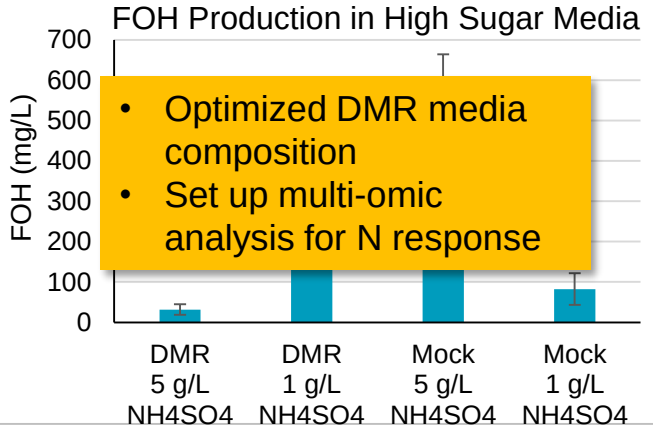
- Obtained a baseline FOH producing strain
- Engineer to enhance flux

Gene	Source organism
Rt_Maqu_2507	bacteria *M. aquaeolei*
Rt_TaFAR2	barn owl
Rt_Maqu_2220	bacteria *M. aquaeolei*
Rt_AtCER4	Arabidopsis
Rt_AmFAR1	honey bee
Rt_TaFAR1	barn owl
Rt_GgFAR1	domestic chicken
Rt_AdFAR1	domestic goose



Developed methods to
- Promote FOH export
- Alleviate FOH toxicity

Liu *et al*, in preparation



- Optimized DMR media composition
- Set up multi-omic analysis for N response

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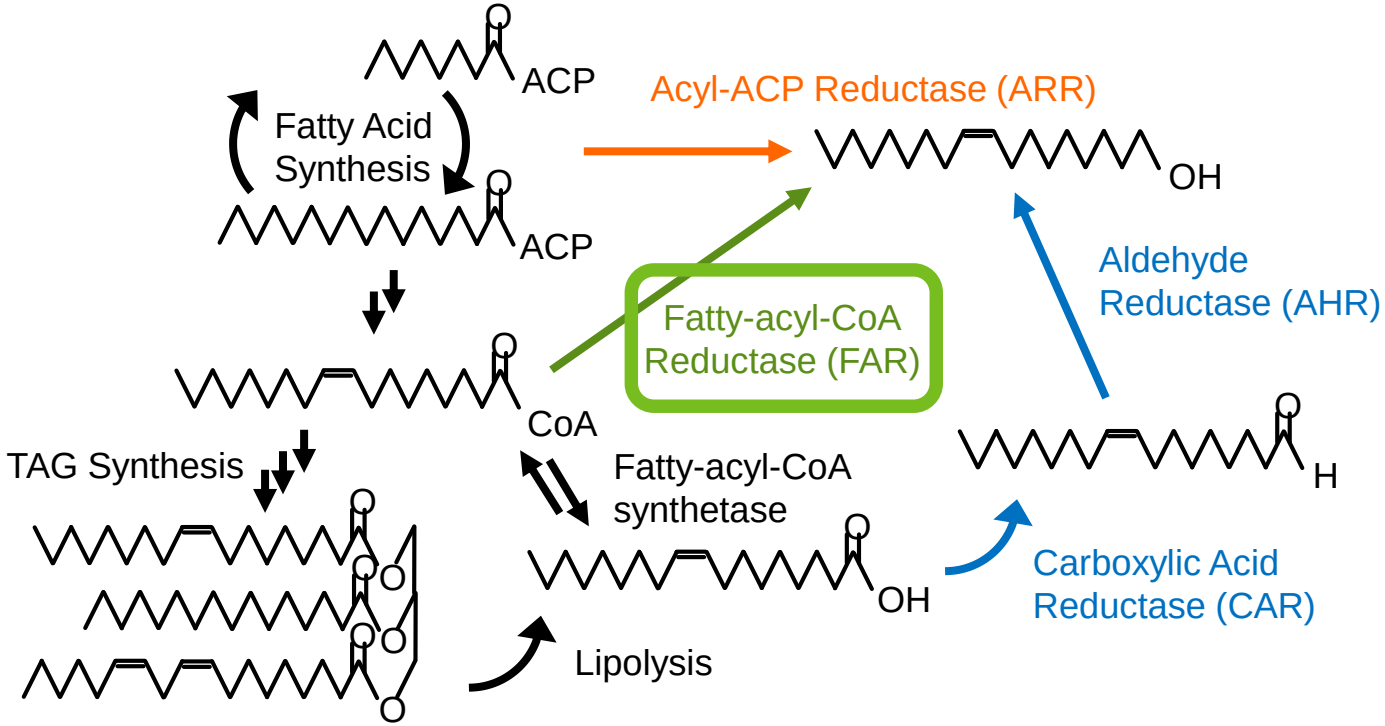
Microbial Fatty Alcohol Production

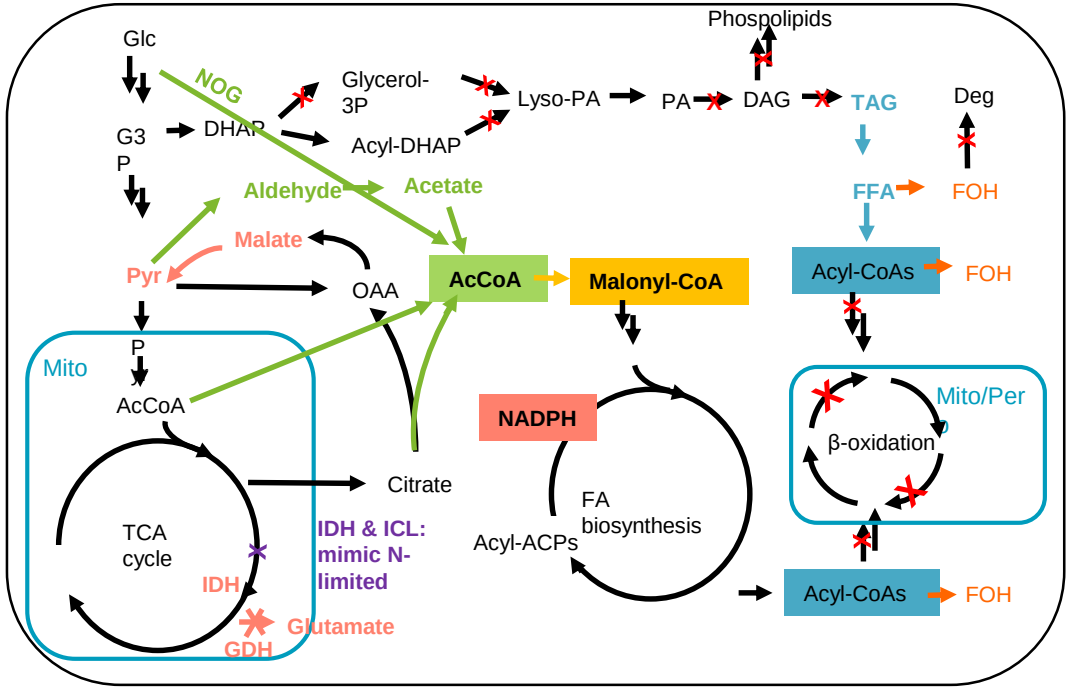


FOH Host Pathway Engineering: Rational Targets

Host engineering to

a. Enhance the pool of:
- acetyl-CoA
- malonyl-CoA
- acyl-CoA
- NADPH

b. Knock down competing pathways:
- TAG biosynthesis
- β-oxidation



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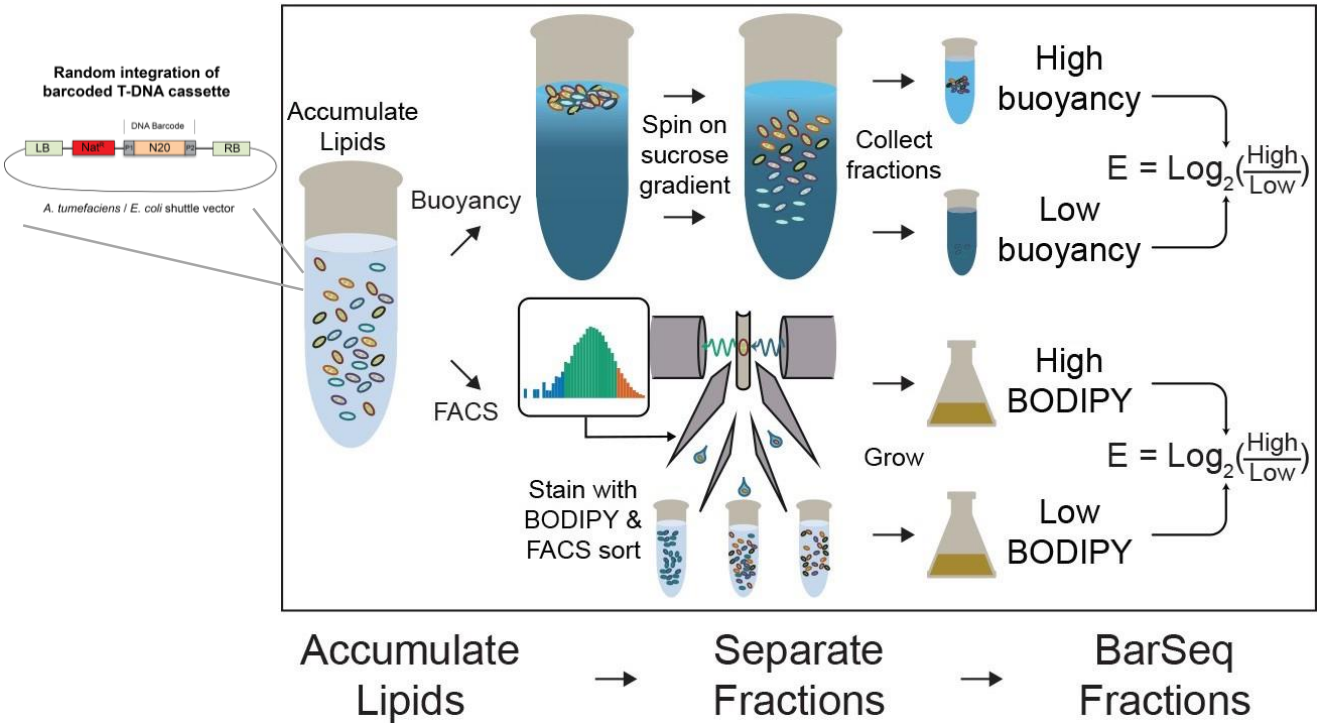
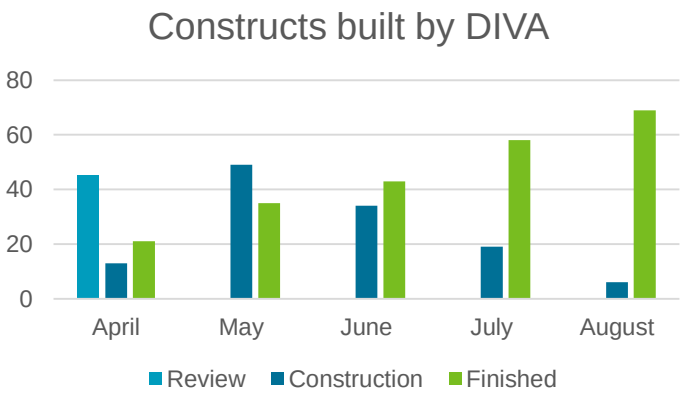
FOH Host Pathway Engineering: Non-intuitive Targets

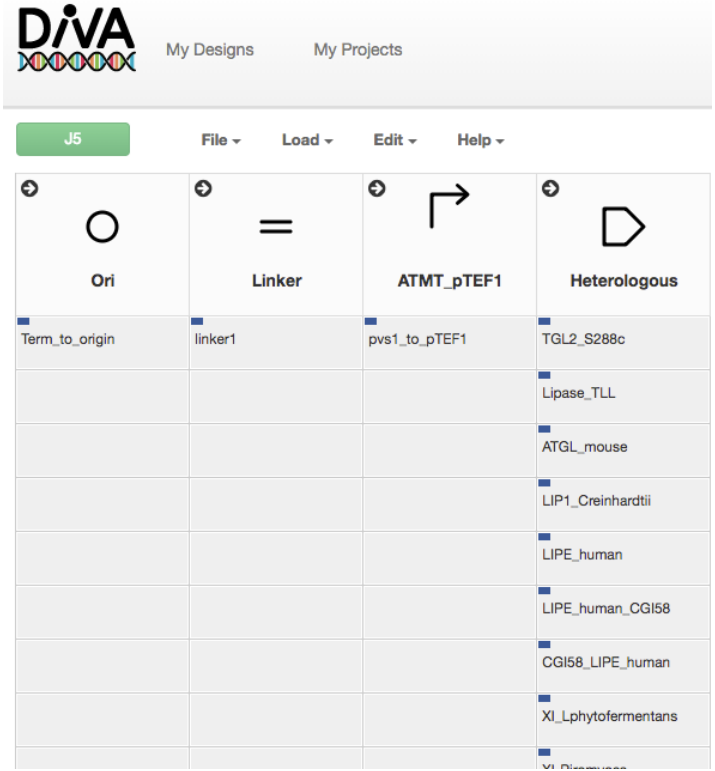


Coradetti *et al.*, 2018

ABF Design and Build Infrastructure

- Use ABF DIVA for Design and Build the majority of FOH constructs
- 106 constructs built





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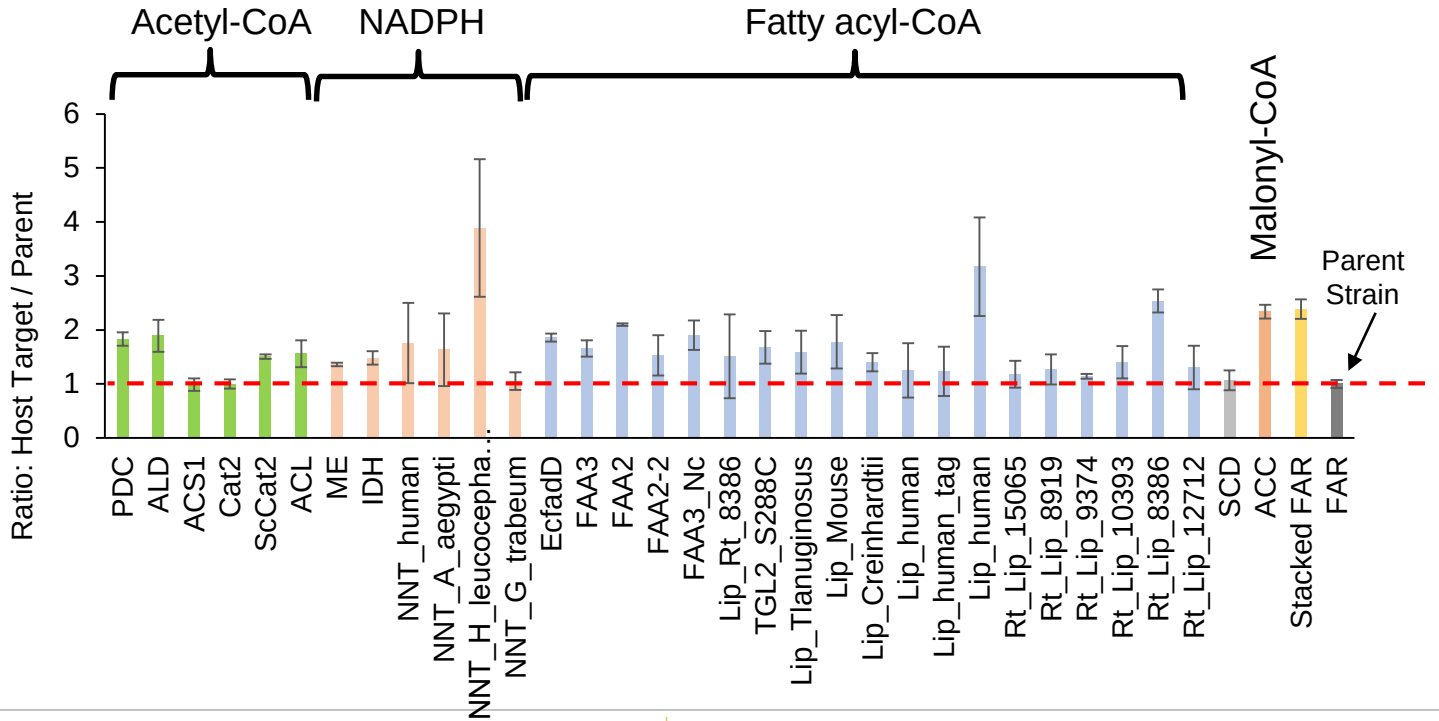
FOH Host Pathway Engineering

- **Overexpression of several host targets increases FOH titers**



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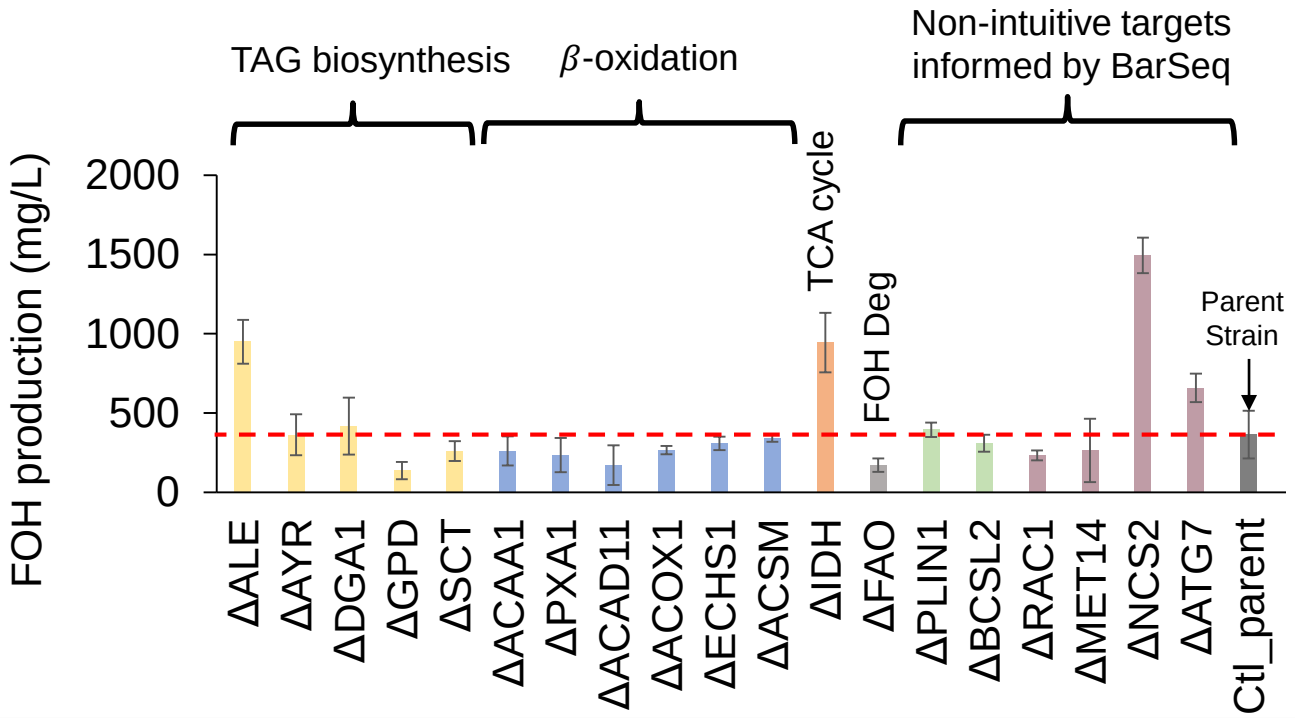
FOH Host Pathway Engineering

➢ **Knock-out of several host targets identified KO targets that improve FOH titers**



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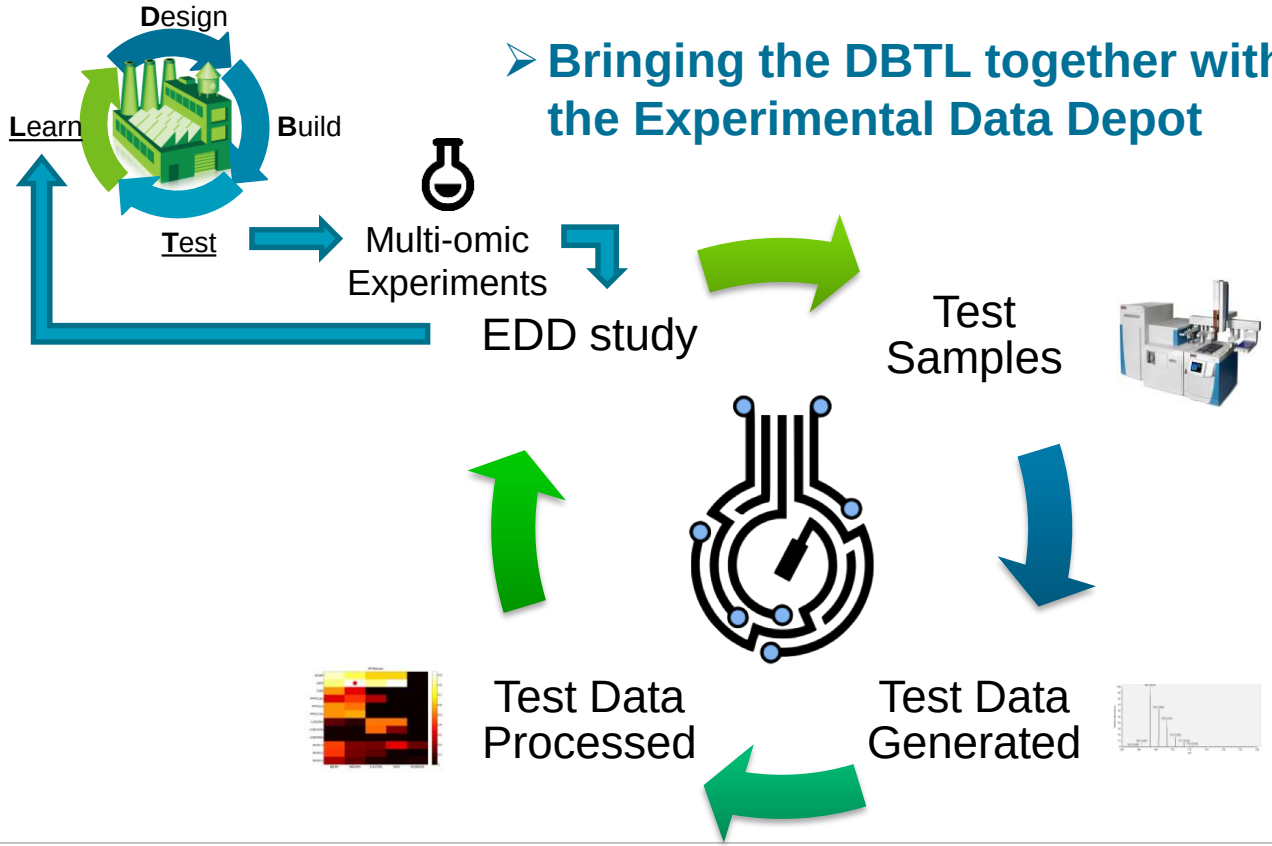
Test & Learn Workflow



➢ **Bringing the DBTL together with the Experimental Data Depot**

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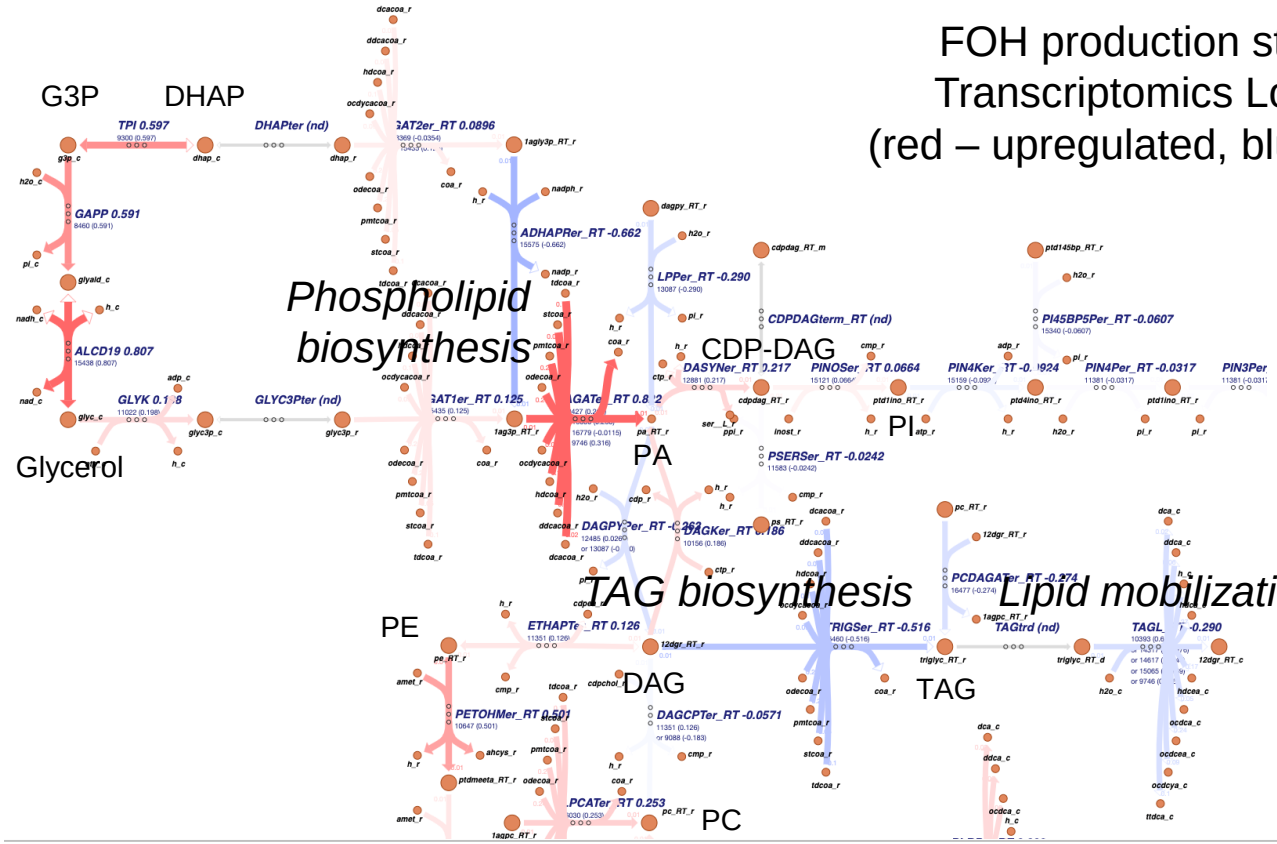
Multi-omics data on metabolic maps



FOH production strain vs wild-type
Transcriptomics Log2 Fold Change
(red – upregulated, blue – downregulated)

Lipid metabolism

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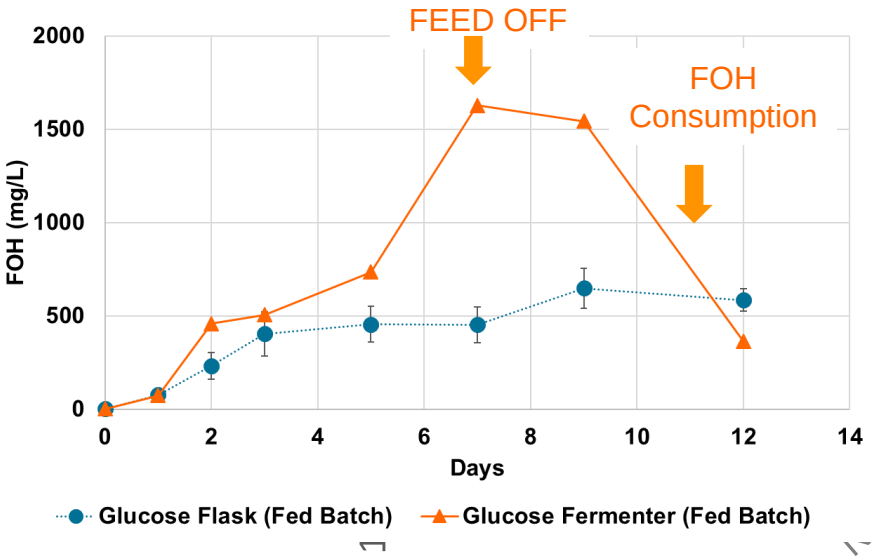
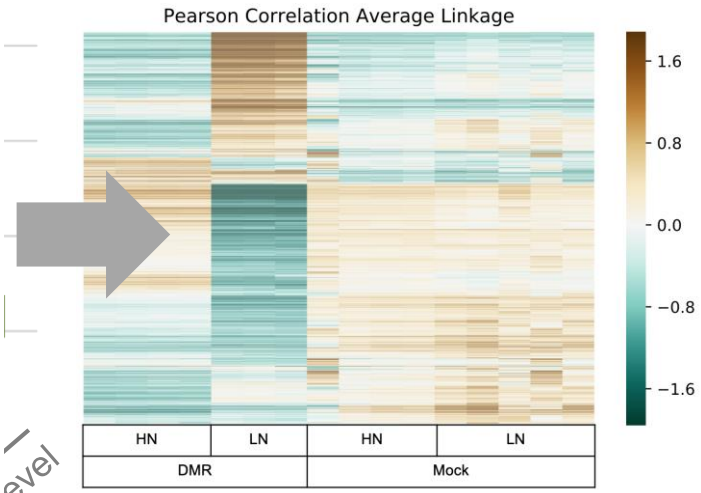
Optimizing FOH Production in DMR/Mock Media



DMR/Mock

Massively Different N response on DMR



- Fatty alcohol dehydrogenases induced on DMR

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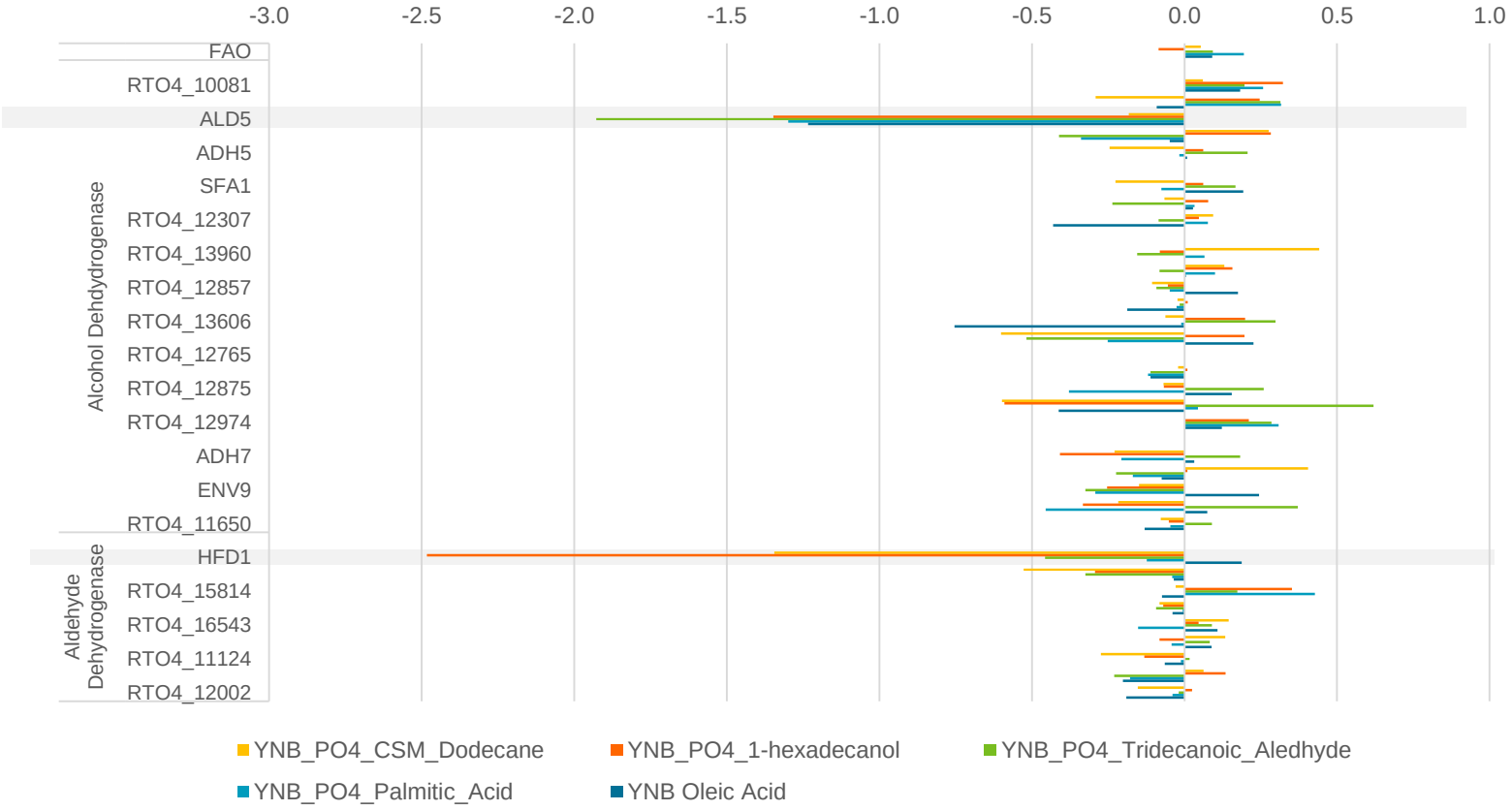
TnSeq Alcohol Dehydrogenase Fitness Scores



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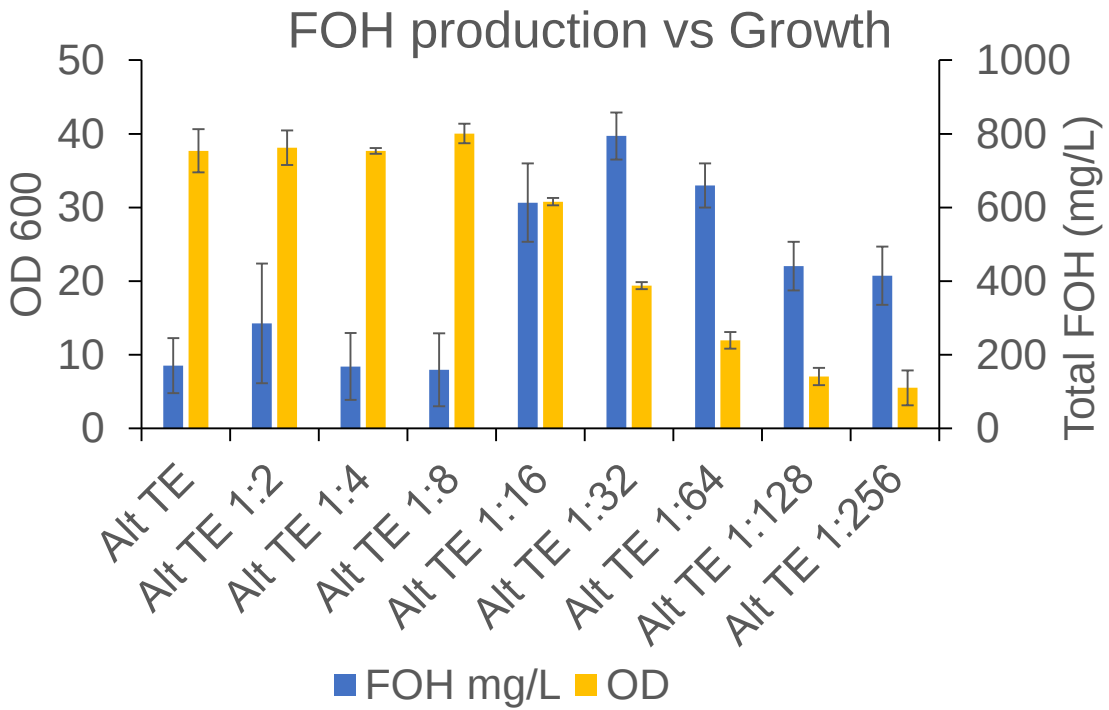
Media Optimization to ID Factors that Affect FOH



- Higher trace elements lead to decreased FOH production
- Individual metal titrations revealed the FOH/Biomass effect is dominated by **Zn**

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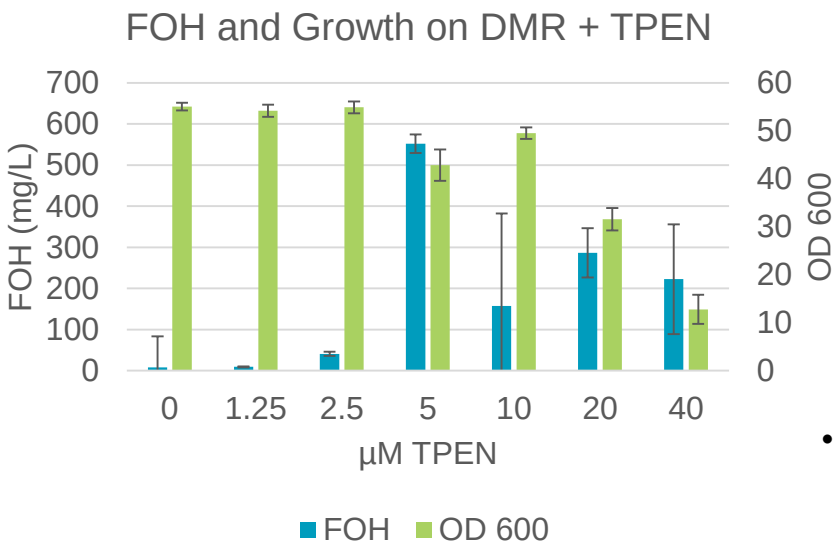
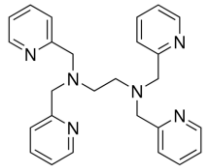
DMR Hydrolysate Has Too Much Zinc



TPEN:
N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediaminex



- Chelating agent with high affinity for zinc

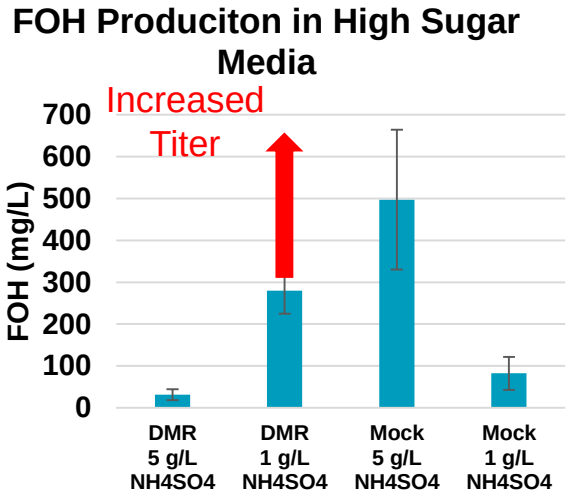
- Follow-on studies will focus on exploring
  - Zn-dependent fatty alcohol dehydrogenases
  - global response to Zn

ART for Media Optimization

- We collaborate with LBNL to use Automated Recommendation Tool (ART) to help optimize FOH titers in DMR

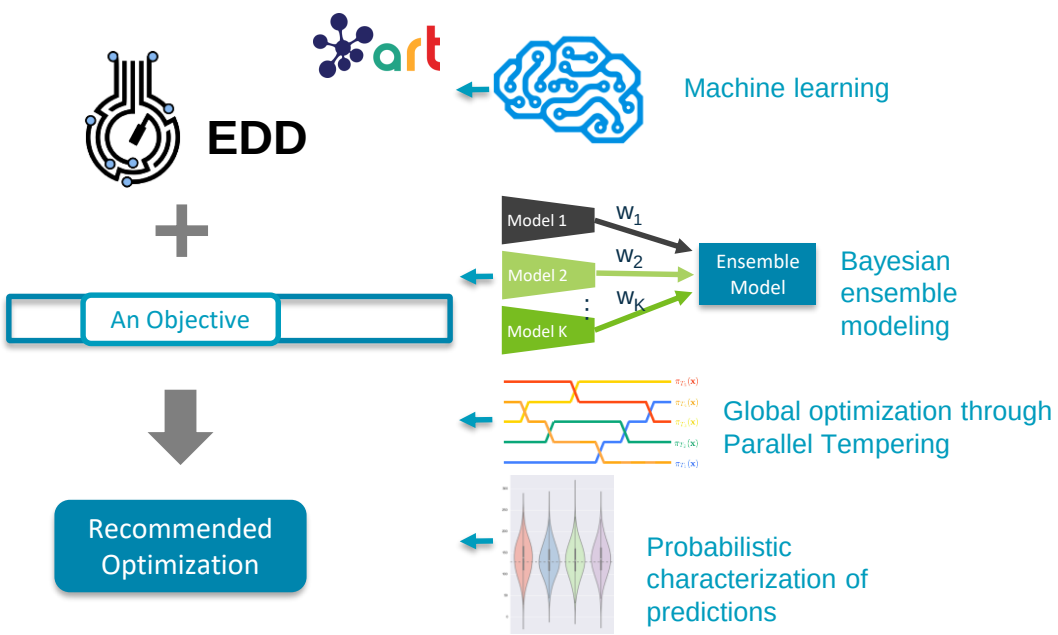
Use biolector or Ambr 250 to test many media additions to DMR

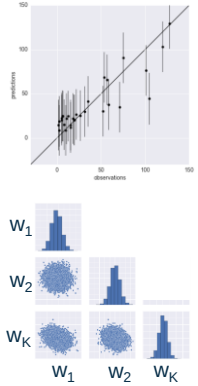
Use ART to predict optimal medium for FOH production in DMR



Learn: Automated Recommendation Tool (ART)

- ART uses machine learning to analyze variant data sets and make predictions for optimal configurations
- Flexible, can be used to make pathway recommendations, optimize media, etc.





Acknowledgements

Sandia National Laboratory
James Kirby
Gina Geiselman
Derek Clay
HeeJin Hwang
Sam Coradetti
Di Liu
Peter Otoupal
Xun Zhuang
Mary Tran-Gymafi
Anne Ruffing
Anthe George

NREL
Gregg Beckham
Davinia Salvachua

PNNL
Joonhoon Kim
Jon Magnuson
Nathalie Munoz Munoz
Young-Mo Kim
Kristin Burnum-Johnson
Yuqian Gao
Swarnendu Tripathi
Jeremy Zucker
Mark Butcher

ANL
Phil Liable
Peter Larson

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Edward Baidoo
Veronica Benites
Anna Fuhrmann
Jeffrey M. Skerker
Nathan Hillson
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Masakazu Ito
Hector Garcia Martin
Tijana Radivojevic
Chris Petzold
Zach Costello
Adam P. Arkin
Jay D. Keasling
Blake A. Simmons

ABPDU
Eric R. Sundstrom
Jan-Philip Prahl
Mona Mirsiaghi
Carolina Barcelos
Isaac Wolf
Ning Sun
Gabriella Papa
Deepti Tanjore
Todd Pray