NREL
NATIONAL RENEWABLE ENERGY LABORATORY





DOE Bioenergy Technologies Office (BETO) 2017 Project Peer Review

Targeted Microbial Development

WBS 2.4.3.102

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Targeted Microbial Development
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Goal Statement

- **Goal:** To investigate and recommend promising pathways for advanced biological upgrading of biomass sugars and lignin to hydrocarbons (HC) and co-products to support the DOE BETO 2022 goal of enabling advanced HC fuels at $3/GGE.
- **Outcome:**
  - Investigate leading technologies for production of reduced cost fuels and high carbon efficiency intermediates amenable to catalytic upgrading to HC fuels.
    - Engineer *Zymomonas* for 2,3 butanediol production
    - Lignin upgrading to muconic acid by *Pseudomonas*
    - Improve production of fatty alcohols in oleaginous yeast
  - Identify future sugar upgrading technologies; as well as a critical knowledgebase, for BETO and bioenergy industry to further R&D working towards production of third-generation HC biofuels from biomass.
- **Relevance:**
  - This project develops and improves metabolic pathways and strains for two of the four pathways for sugar upgrading and all four pathways for lignin upgrading .
  - NREL TEA shows that significant cost savings for advanced fuels are: product yield, titer and rate; secreted products; anaerobic culture.

Quad Chart Overview

Timeline

	Task 1	Task 2	Task3
• Project start date:	2015	2015	2015
• Project end date:	2018	2018	2018
• Percent complete:	66%	66%	66%

Budget

	FY 15 Costs	FY 16 Costs	FY 17 Costs	Total Planned Funding (FY16- 18) Project End Date
DOE Funded Task 1	$500K	$500K	$500K	$1.5M ($500K FY18)
Task 2	$500K	$500K	$500K	$1.5M ($500K FY18)
Task 3	$900K	$900K	$900K	$2.7M ($900K FY18)
Project Cost Share (Comp.)*	0	0	0	0

Barriers

- Ct-H. Efficient Catalytic Upgrading of Sugars/Aromatics, Gaseous and Bio-Oil Intermediates to Fuels & Chemicals
  - Efficient, highly active, selective, durable biological catalysts
  - Pathways that tolerate feedstock variability and inhibitors
  - Catalysts for lignin to central metabolism or upgrading
- Ct-E. Efficient Low-Temperature Deconstruction

Partners

- BETO Internal: BUS (Biological Upgrading of Sugars); EEO (Enzyme Engineering & Optimization); PPH (Pretreatment & Process Hydrolysis; BSI (Bench-Scale Integration); BPMS (Biochemical Process Modeling & Simulation)
- Collaborations
  - Hal Alper (Univ of Texas at Austin)
  - Lydia Contreras (Univ of Texas at Austin)
  - Neol BioSolutions (Spain) via BUS

1 - Project Overview

This project addresses three upgrading strategies

✓ ***Task 1* Anaerobic HC Intermediates from *Zymomonas mobilis***

- Anaerobic fermentation producing HC intermediates from both C6 and C5 sugars
  - Efficient and rapid carbohydrate utilization.
- High carbon efficiency
  - Amenable to separations and catalytic upgrading to HC fuels.

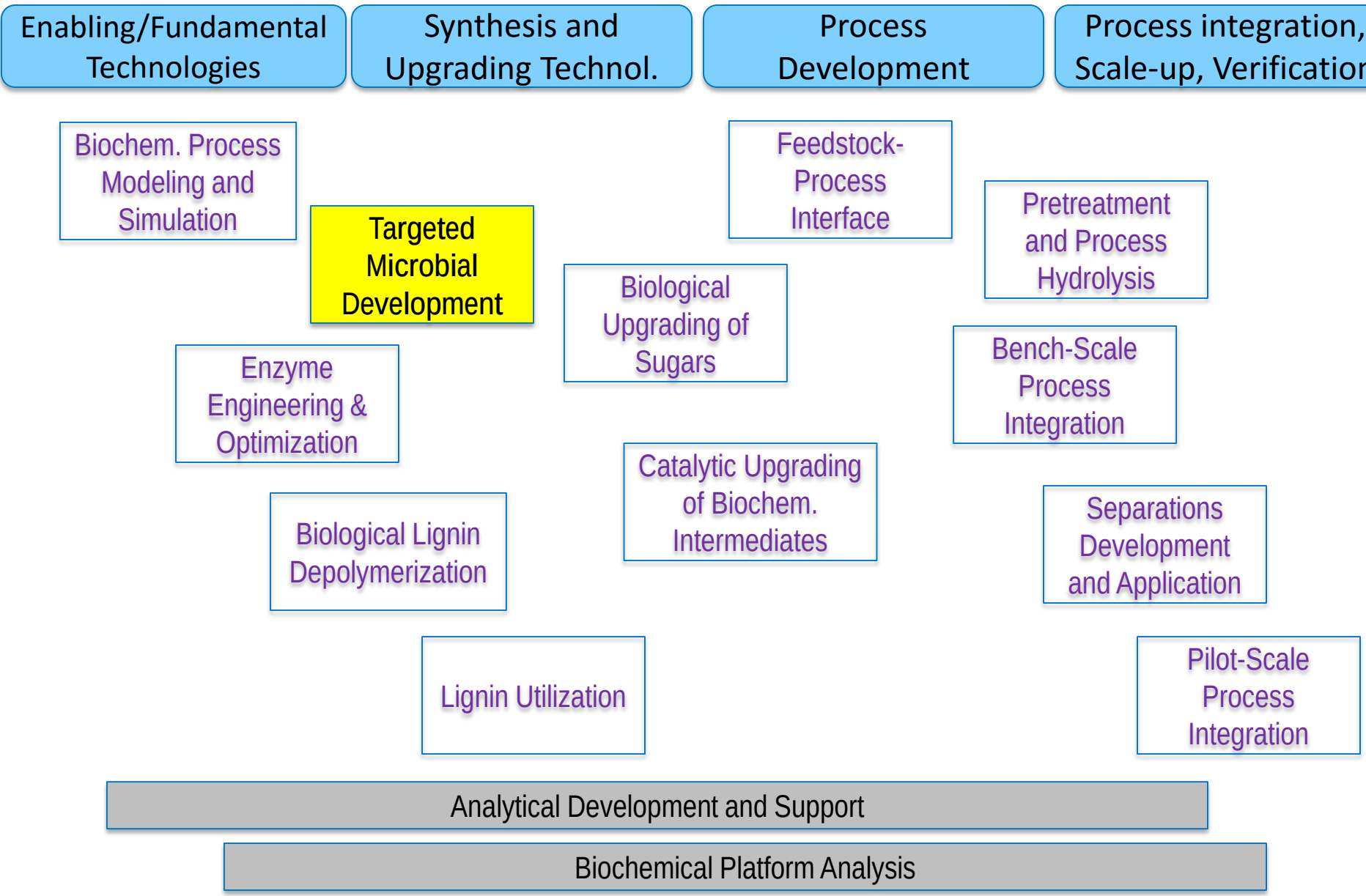
✓ **Task 2 Aerobic Aromatic Catabolism**

- Lignin upgrading to muconic acid by *Pseudomonas.*
- Provide value added co-product.

✓ ***Task 3 (Lower TRL)* Advanced Concepts for Producing HCs**

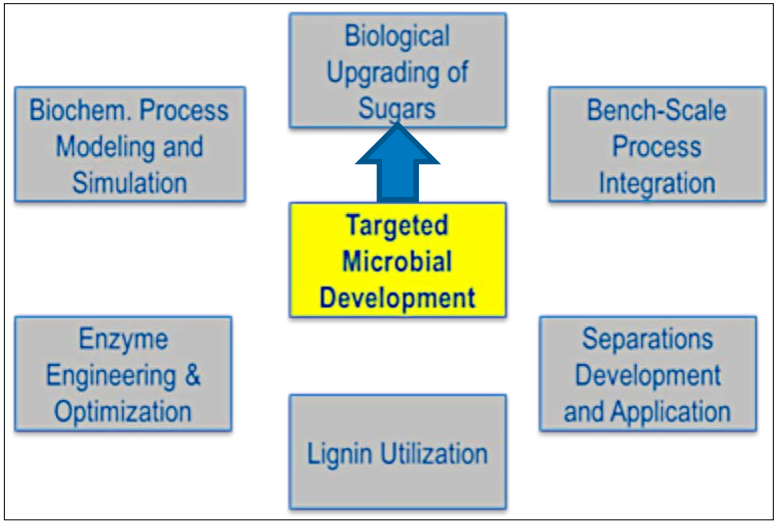
- Aerobic yeast fermentation for secreted fatty alcohol-based long chain hydrocarbon precursors readily for fuel product finishing.
- Aerobic yeast fermentation for consolidated bioprocessing (CBP) of fatty alcohols for process cost reduction.

Biochemical Conversion Projects - NREL



2 – Approach (Management)

- ✓ TEA analysis and collaboration
- ✓ Following DOE approved Regular (Smart) milestones and Go NoGo decisions
- ✓ Collaborations with multiple projects in the biochemical platform:



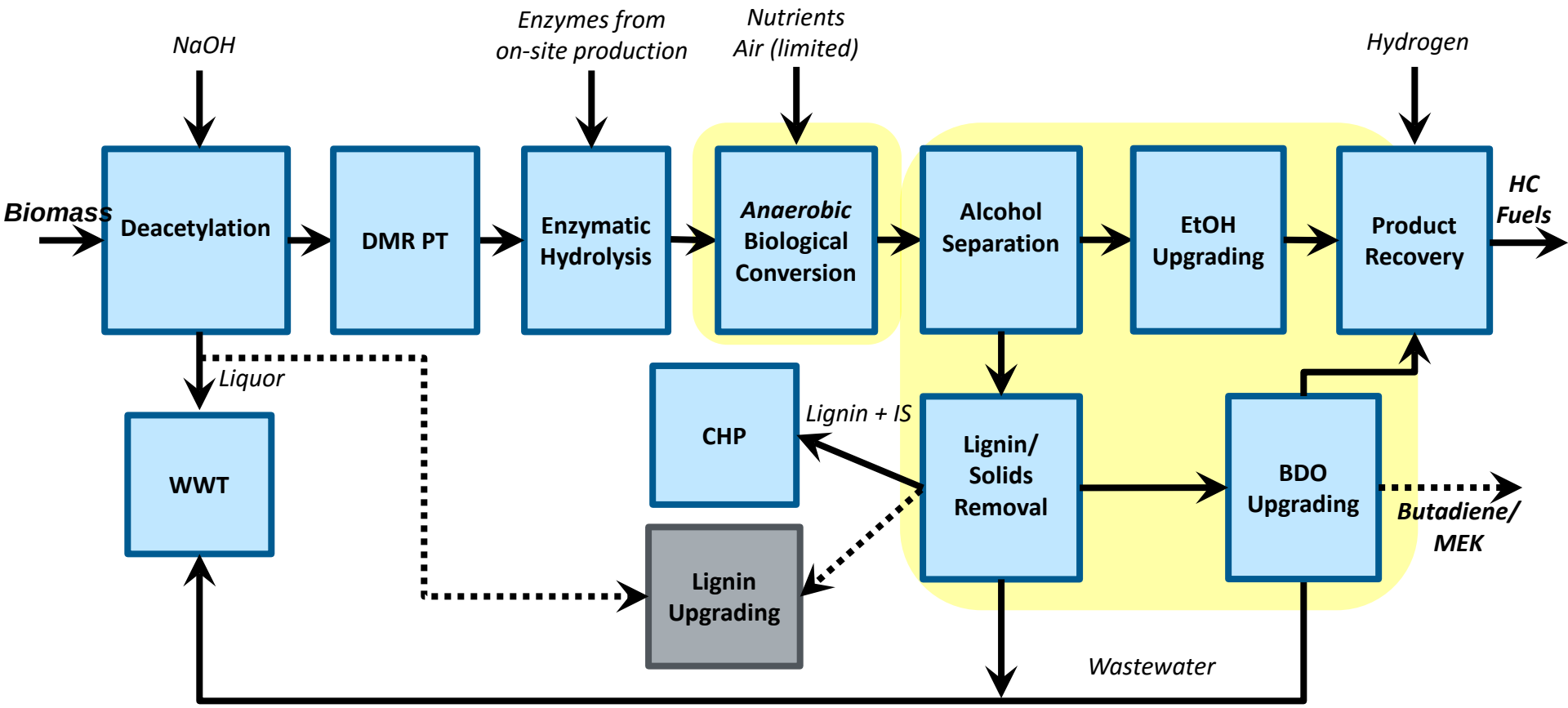
- ✓ Collaborations with workers outside of platform:
  - Hal Alper (University of Texas at Austin) – oleaginous yeast
- ✓ Publish all findings in peer reviewed journals

2 – Approach (Technical)

- *Explain the technical approach to achieving your goal(s)*
  - Apply metabolic engineering and synthetic biology tools to engineer microorganisms for efficient utilizing sugars to HC intermediates and valorizing lignin for chemicals production.
- *Explain the top 2-3 potential challenges (technical and non-technical) to be overcome for achieving successful project results*
  - Ability to change the pathway direction to direct carbon flow to desired products.
  - Demonstration of high yield, titer and productivity for achieving cost effective process.
- *Describe critical success factors (technical, market, business) that will define technical and commercial viability*
  - Demonstrate effective production of HC intermediates and chemicals in relevant yields, rates and titers (for example: 70 g/L BDO; 2 g/L/h; and 0.47 g/g).
  - Considerable of impact of bioproducts/fuels on existing markets
  - Timely dissemination of technical achievements (publications & presentations)

3 – Technical Accomplishments/ Progress/Results

Task 1: Engineer Z. mobilis producing 2,3 BDO

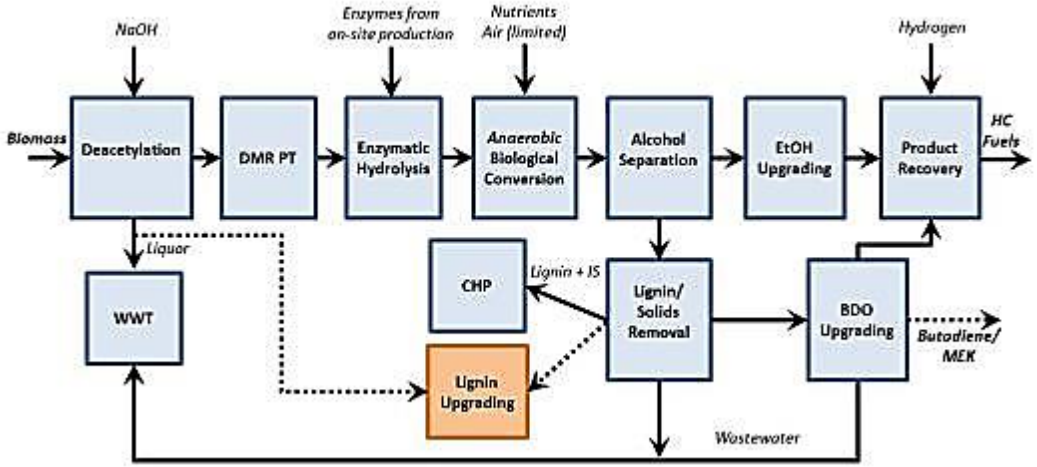


- Production of a mixed ethanol and 2,3-BDO product under anaerobic/microaerobic fermentation conditions
- High 67% theoretical carbon efficiency for either ethanol or 2,3-BDO from sugars (among the highest metabolic yield potential for any fermentative pathway)
- Product flexibility for upgrading the BDO intermediate to hydrocarbon fuels or chemicals (butadiene)

3 – Technical Accomplishments/ Progress/Results

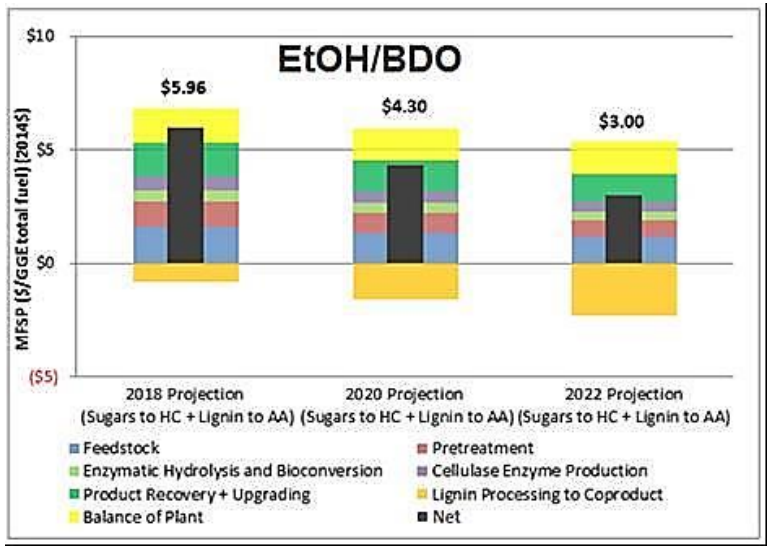
Task 1: TEA Analysis Results of the Mixed Alcohols/Diols Pathway

>>Requested by 2015 Review Panel<<



2022 targets: 20% total solids loading:
Mixed alcohol/diol titer: 70 g/L
Productivity: 2 g/L/hr
Yield (G+X): ~0.47 g/g

$/GGE MFSP Contribution (2014$)	2018 Projection (Sugars to HC + Lignin to AA)	2020 Projection (Sugars to HC + Lignin to AA)	2022 Projection (Sugars to HC + Lignin to AA)
Feedstock	$1.58	$1.32	$1.14
Pretreatment	$1.11	$0.88	$0.76
Enzymatic Hydrolysis and Bioconversion	$0.52	$0.44	$0.38
Cellulase Enzyme Production	$0.59	$0.53	$0.42
Product Recovery + Upgrading	$1.48	$1.38	$1.25
Lignin Processing to Coproduct	($0.83)	($1.62)	($2.34)
Balance of Plant	$1.50	$1.37	$1.39
MFSP	$5.96	$4.30	$3.00



3 – Technical Accomplishments/ Progress/Results

Task 1: Engineer Z. mobilis producing 2,3 BDO

- Anaerobic process
  need purge of air for the excess NADH for BDO
- Potential high TYR
- Upgrade to fuels and chemicals

DMR pretreatment and enzymatic hydrolysis containing mixed C5/ C6 sugars

Glucose

Xylose

Arabinose

Glucose

Xylose

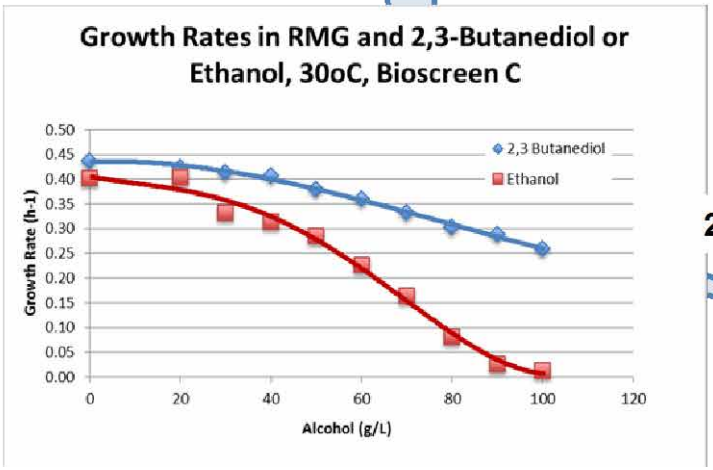
Arabinose

PPP pathway

G3P

Entner-Doudoroff (ED) pathway
"Catabolic Highway"

BDO is less toxic



2 Pyruvate

Als acetolactate synthase

Acetolactate

Aldc acetolactate decarboxylase

2 Acetaldehyde

R-acetoin

Bdh butanediol dehydrogenase

2,3 Butanediol

2 Ethanol

3 – Technical Accomplishments/ Progress/Results

Task 1: Impact of Als for increase BDO titer and BDO to ethanol ratio

<<Data from late 2015>>

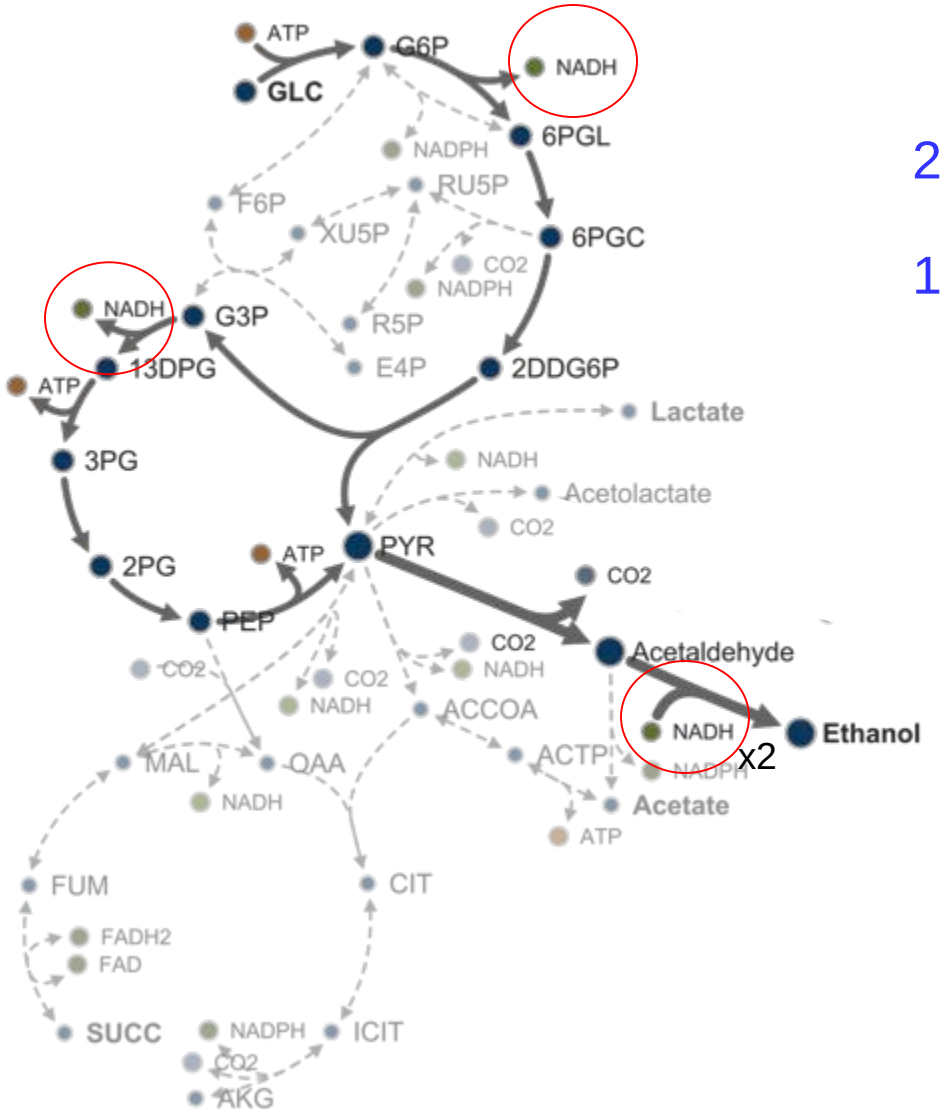
Strains	ALS Promoter	ALS Source	Acetoin (g/L)	2,3-BDO (g/L)	Ethanol (g/L)	BDO vs EtOH ratio (g/g)	Improved fold on g/g basis	BDO+Acetoin vs EtOH carbon flux (c mole/c mole)
BC5	Peno	*E. cloacea*	1.11	4.54	31.95	0.14	baseline	0.18
BC9	Ptet	*B.lichemiformis*	1.60	10.80	26.20	0.41	2.9	0.49
BC10	Ptet	*B. subtilis*	1.10	10.20	27.40	0.37	2.6	0.42
BC11	Ptet	*B. subtilis*	0.70	13.30	24.90	0.53	3.8	0.58

Flask study, 8% glucose, microaerophilic conditions

- ✓ Improved Als and Bdh enzymes selected by EEO are currently under testing!

3 – Technical Accomplishments/ Progress/Results

Task 1: Ethanol fermentation (anaerobic) has balanced redox



2 mol ethanol / mol glc

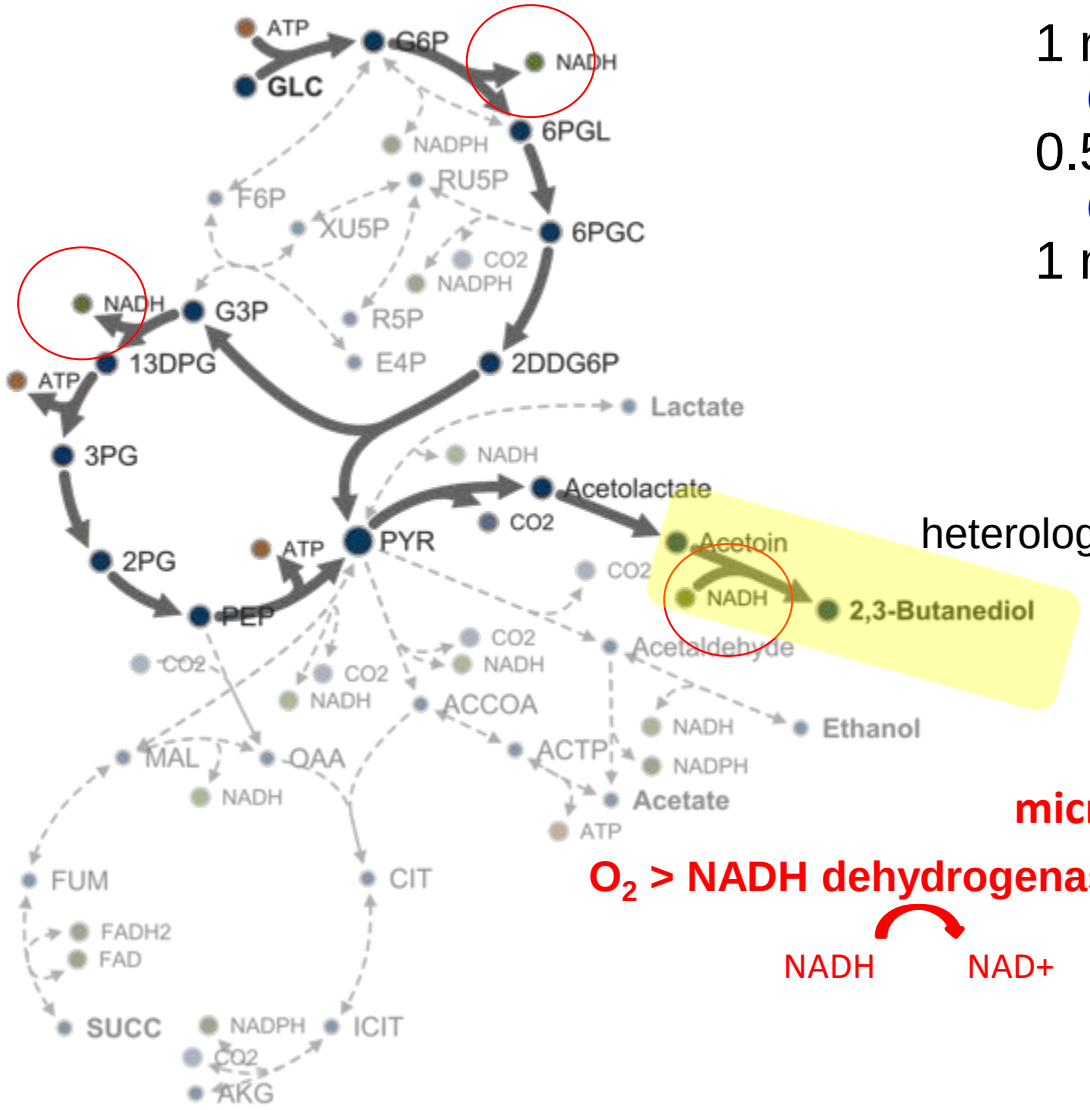
67% C-mol yield

1 mol ATP / mol glc

Bomble & St. John
BPMS

3 – Technical Accomplishments/ Progress/Results

Task 1: BDO fermentation leads to redox imbalance (predict O_2 rescue)



1 mol BDO / mol glc
(67% C-mol yield)

0.5 mol O_2 / mol glc
(1 missing NADH / mol glc)

1 mol ATP / mol glc

heterologous pathway

microaerophilic growth

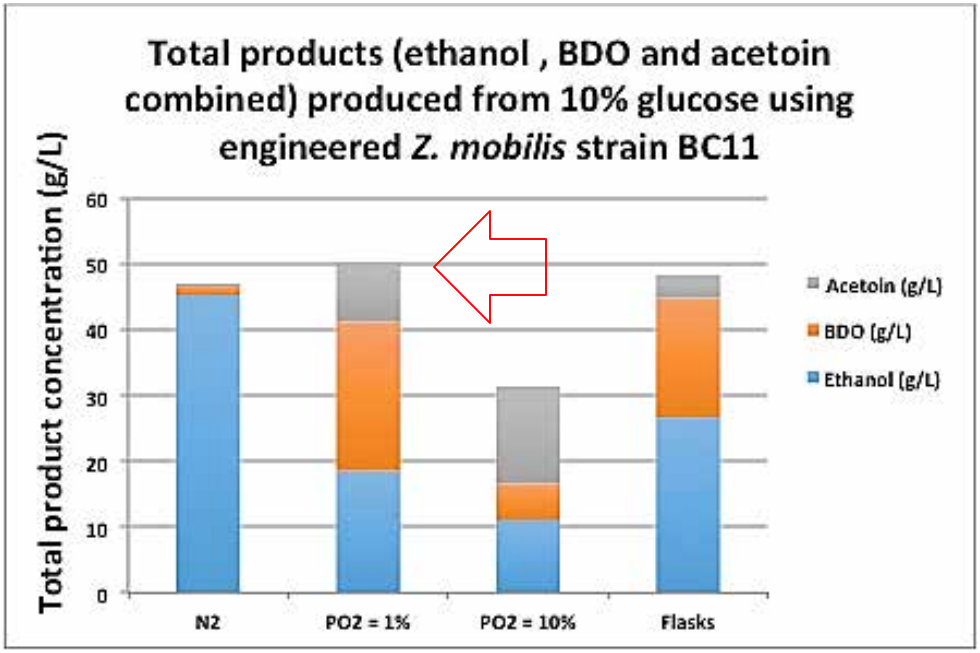
O_2 > NADH dehydrogenase > H_2O

NADH NAD+

Bomble & St. John
BPMS

3 – Technical Accomplishments/ Progress/Results

Task 1: Low concentrations of oxygen significantly increase BDO production

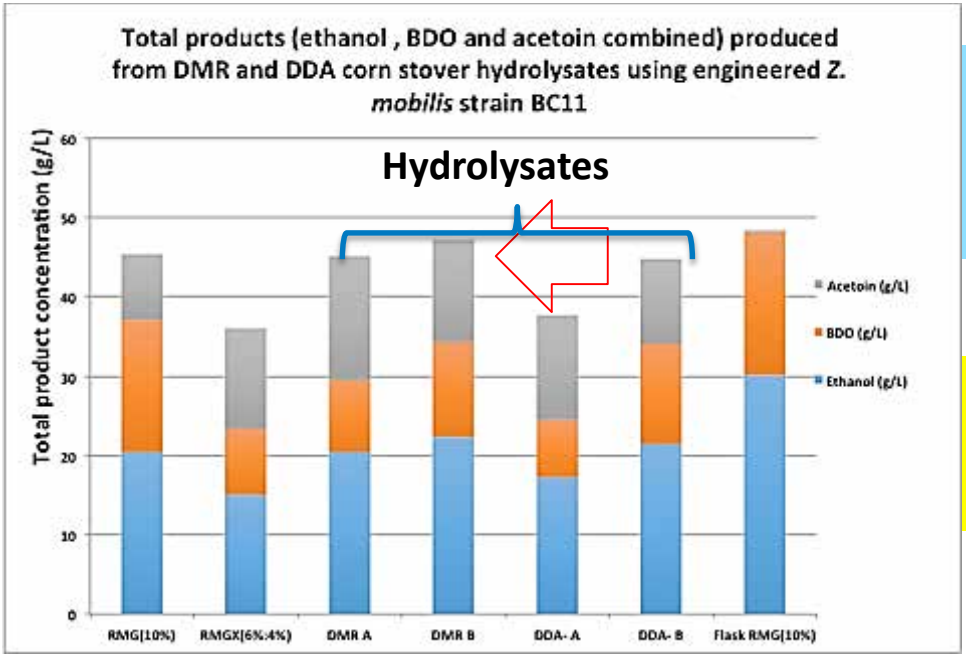


- Achieved the FY16 milestone target of producing BDO titer of 20 g/L.
- Also ~50 g/L of total products including ethanol, BDO and acetoin with 1.44 g/L/h productivity and 0.48 g/g yield.

Fermentation conditions	Acetoin	BDO	BDO+ Acetion	Ethanol	Productivity			Yield			Time used
					BDO	BDO+ Acetoin	BDO+ Acetoin + Ethanol	BDO	BDO+ Acetoin	BDO+ Acetoin+ Ethanol	
	(g/L)	(g/L)	(g/L)	(g/L)	(g/L/hr)	(g/L/hr)	(g/L/hr)	(g/g)	(g/g)	(g/g)	(hrs)
N2	0.39	1.27	1.66	45.31	0.04	0.05	1.34	0.01	0.02	0.47	35.00
PO2 = 1%	9.09	22.64	31.74	18.50	0.65	0.91	1.44	0.22	0.30	0.48	35.00
PO2 = 10%	14.73	5.53	20.27	11.09	0.13	0.48	0.75	0.07	0.26	0.40	42.00
Flasks	3.48	18.13	21.61	26.67	0.52	0.62	1.38	0.18	0.21	0.48	35.00

3 – Technical Accomplishments/ Progress/Results

Fermentation using corn stover hydrolysates - product distribution, yield, productivity



Q4 2016 "SOT data" on CS hydrolysate (10% sugar, ~13% of the total solids loading) :

- Mixed alcohol/diol (with acetoin) titer: ~48 g/L
- Productivity: ~1 g/L/hr
- Yield (G+X): ~0.43 g/g

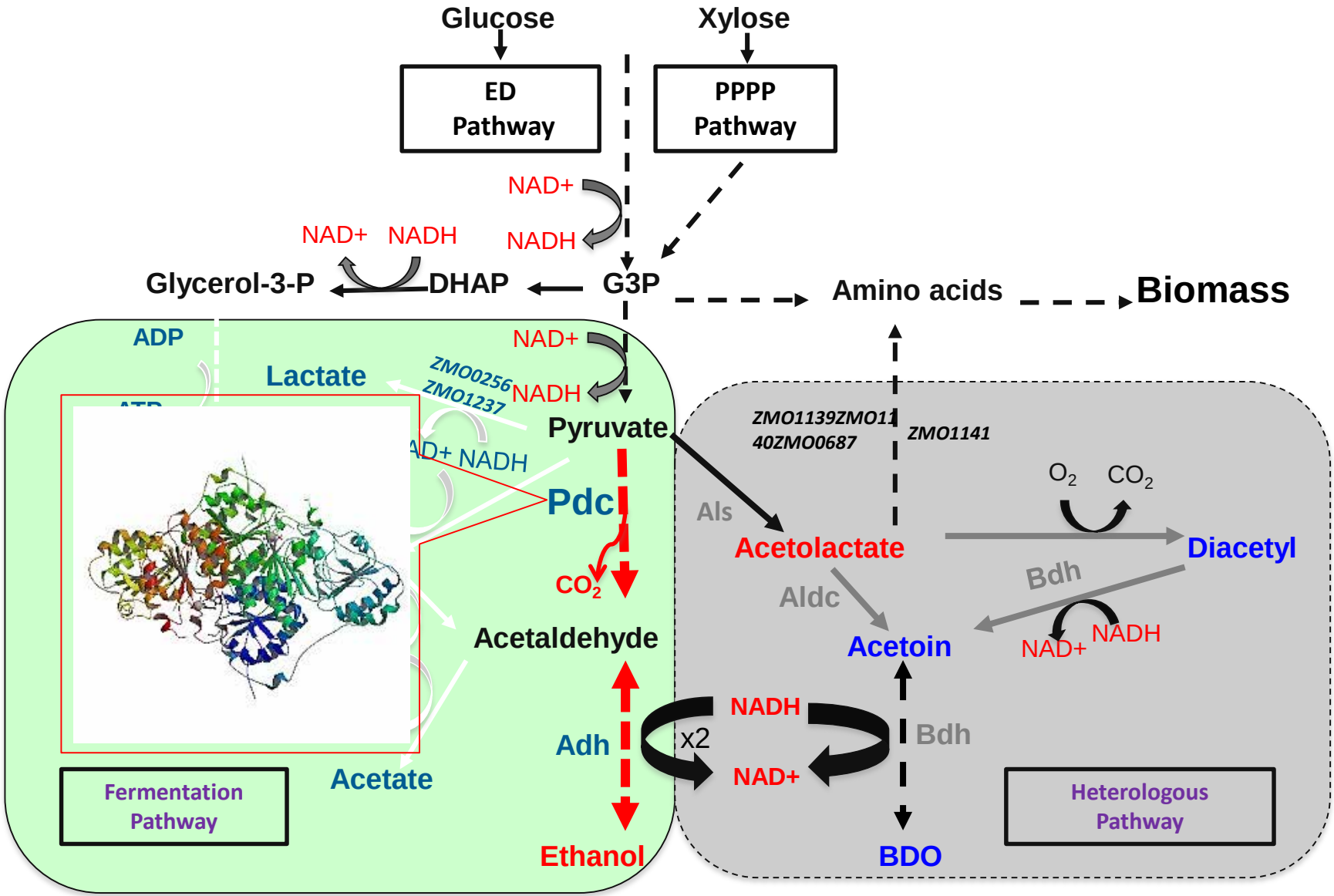
2022 targets: 20% total solids loading :

- Mixed alcohol/diol titer: 70 g/L
- Productivity: 2 g/L/hr
- Yield (G+X): ~0.47 g/g

					Productivity			Yield			
Fermentation medium	Acetoin	BDO	BDO+ Acetion	Ethanol	BDO	BDO+ Acetoin	BDO+ Acetoin+ Ethanol	BDO	BDO+ Acetoin	BDO+ Acetoin+ Ethanol	Time used
	(g/L)	(g/L)	(g/L)	(g/L)	(g/L/hr)	(g/L/hr)	(g/L/hr)	(g/g)	(g/g)	(g/g)	(hrs)
RMG(10%)	8.26	16.61	24.87	20.49	0.76	1.13	2.06	0.17	0.25	0.45	22
RMGX(6%:4%)	12.68	8.31	20.98	15.06	0.19	0.49	0.84	0.09	0.23	0.40	43
DMR (10% G+X) A	15.63	9.01	24.64	20.48	0.21	0.57	1.05	0.09	0.24	0.44	43
DMR (10% G+X) B	12.90	11.95	24.85	22.33	0.28	0.59	1.12	0.11	0.23	0.43	42
DDA (10% G+X) A	13.19	7.25	20.43	17.28	0.17	0.48	0.88	0.07	0.21	0.38	43
DDA (10% G+X) B	10.75	12.61	23.35	21.46	0.30	0.56	1.07	0.12	0.23	0.43	42
Flask RMG(10%)	0.28	17.95	18.23	30.16	0.75	0.76	2.02	0.18	0.19	0.49	24

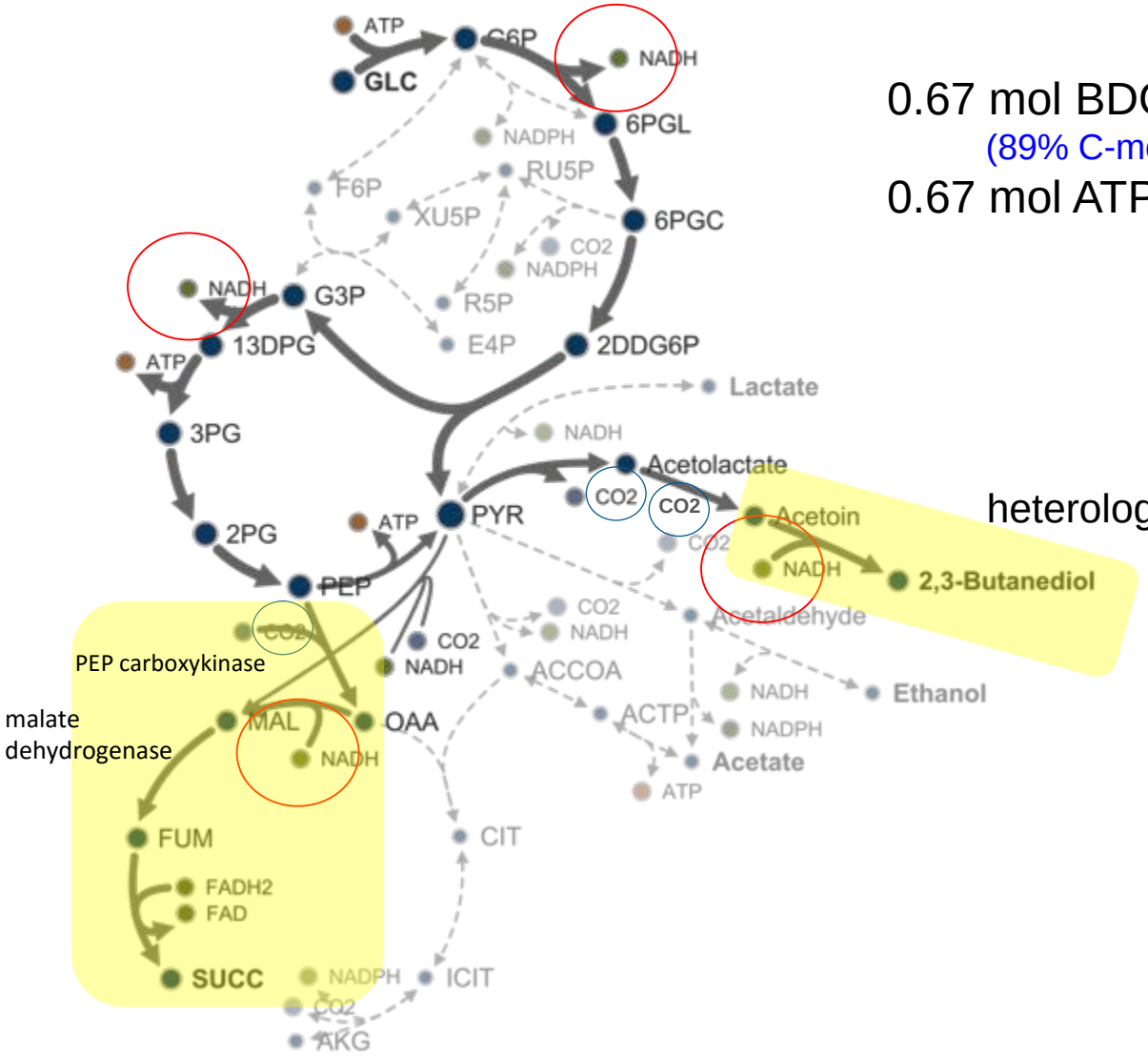
3 – Technical Accomplishments/ Progress/Results

Strategy 1. *How to further increase BDO titer - pdC knockout or reduction?*



3 – Technical Accomplishments/ Progress/Results

Strategy 2. *BDO + succinate fermentation (anaerobic)*



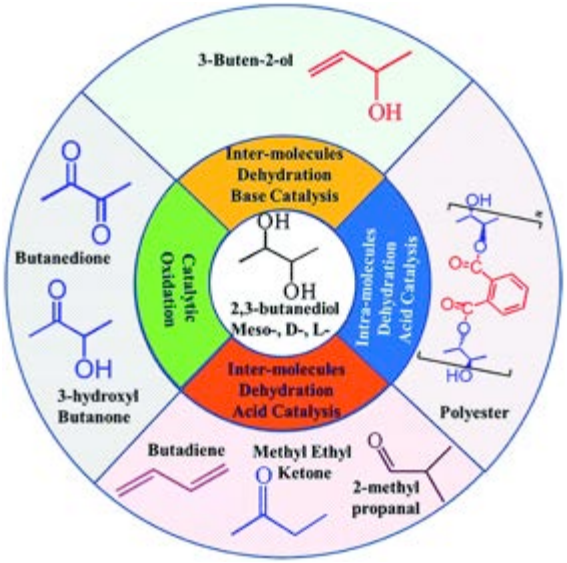
0.67 mol BDO + 0.67 mol succin/mol glc
(89% C-mol yield)
0.67 mol ATP / mol glc

heterologous pathway

Bomble & St. John
BPMS

3 – Technical Accomplishments/ Progress/Results

Task 1: Summary



Zhao et al. RSC Adv., 2016, 6, 16988-16995

Summary of accomplishments

- Demonstrated production of mixed alcohol/diol (with acetoin) using engineered *Zymomonas* strain titers of ~48 g/L with productivity of ~1 g/L/hr and yield at ~0.43 g/g from corn stover hydrolysate at 13% total solid loading.
- Illustrated the benefits of controlled microaerophilic fermentation to BDO production.

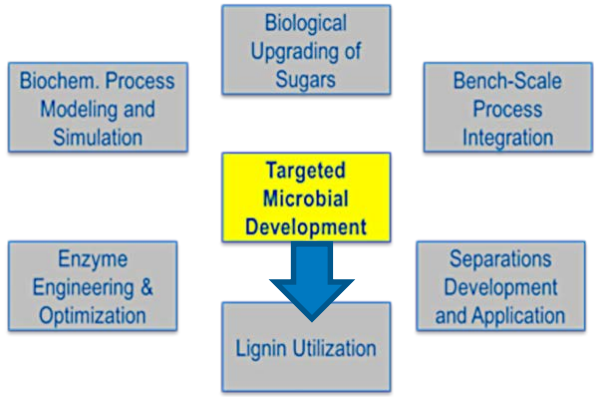
Future directions

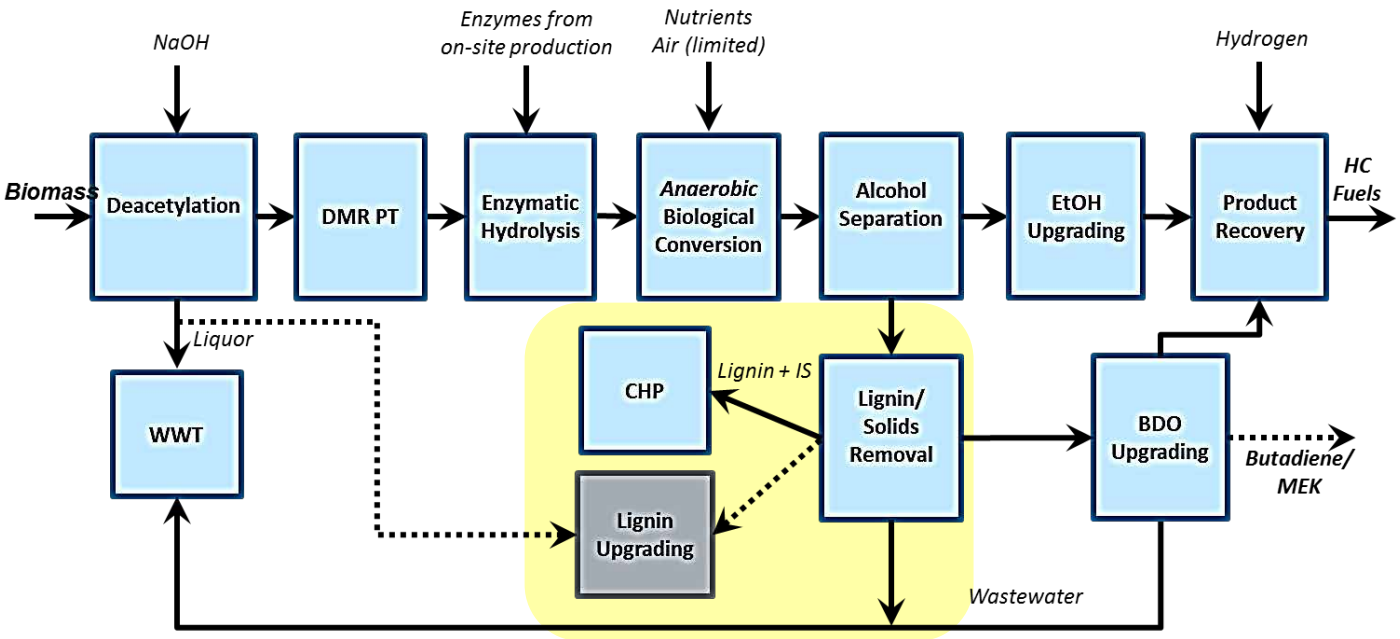
- FY18 Q4 milestone: Develop a strain of *Zymomonas mobilis* and a corresponding fermentation processes (with BSI) able to achieve 50 g/L BDO at a productivity of >1 g/L/hr from biomass.
- Pursue succinate pathway concept in coordination with TEA and BSI.
- Discover or engineer "tunable" PDC enzyme.

3 – Technical Accomplishments/ Progress/Results

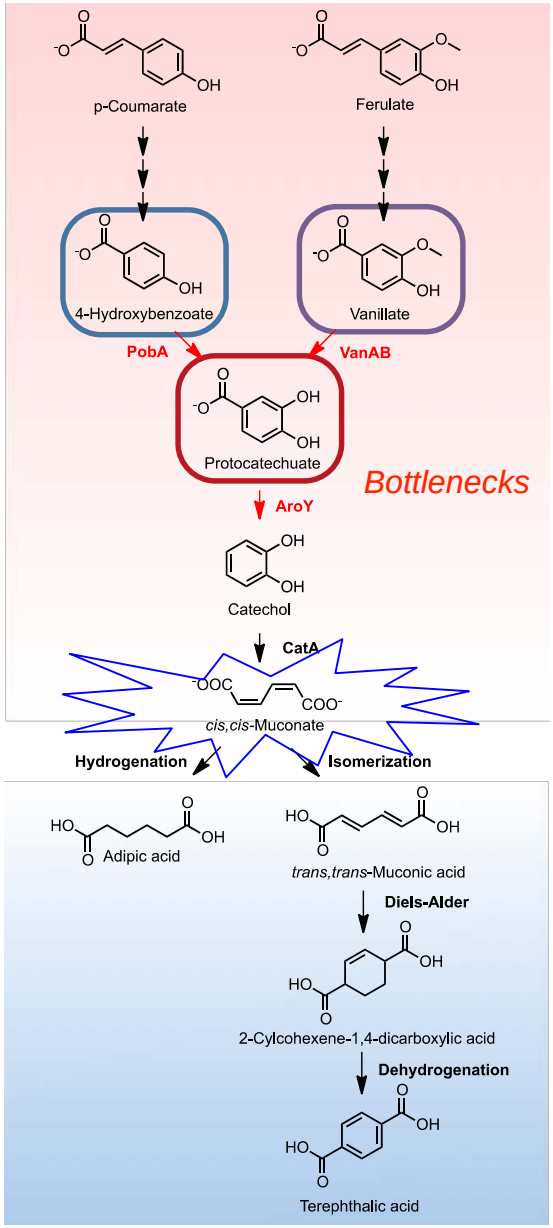
Task 2: Strain development for microbial lignin valorization

- TEA analysis and collaboration
- Following DOE approved Regular (Smart) milestones and Go NoGo decisions





3 – Technical Accomplishments/ Progress/Results

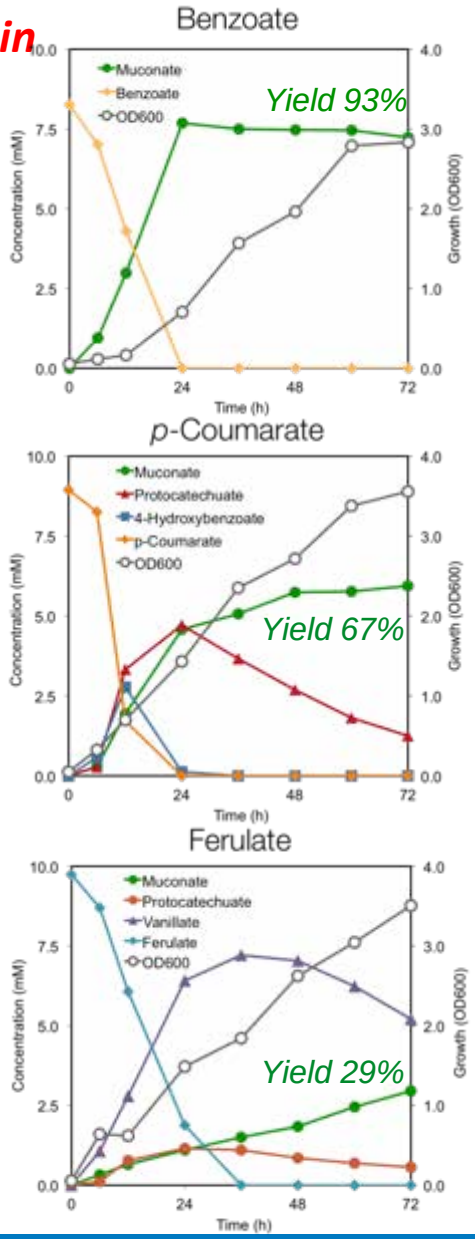


Task 2: Muconic acid production from lignin

Metabolic engineering of *P. putida* KT2440 for production of muconate

2022 targets: ~20% total solids loading:
- Muconate titer: ~50g/L
- Productivity: 1 g/L/hr
- Yield (aromatics): ~90-95% of target

- Initial strain demonstrated production of muconate from relevant lignin monomers, *p*-coumarate and ferulate
- Yields were limited by bottlenecks caused by the enzymes that metabolize protocatechuate, 4-hydroxybenzoate, and vanillate
- More recent metabolic engineering has focused on overcoming these metabolic bottlenecks to improve yields and productivity



Vardon, Franden, Johnson, Karp, *et al.*, *EES* 2015

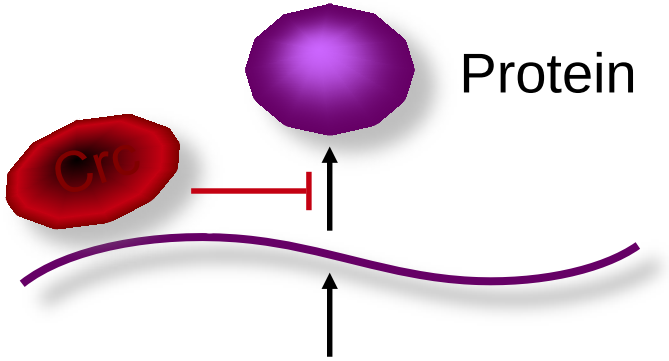
3 – Technical Accomplishments/ Progress/Results

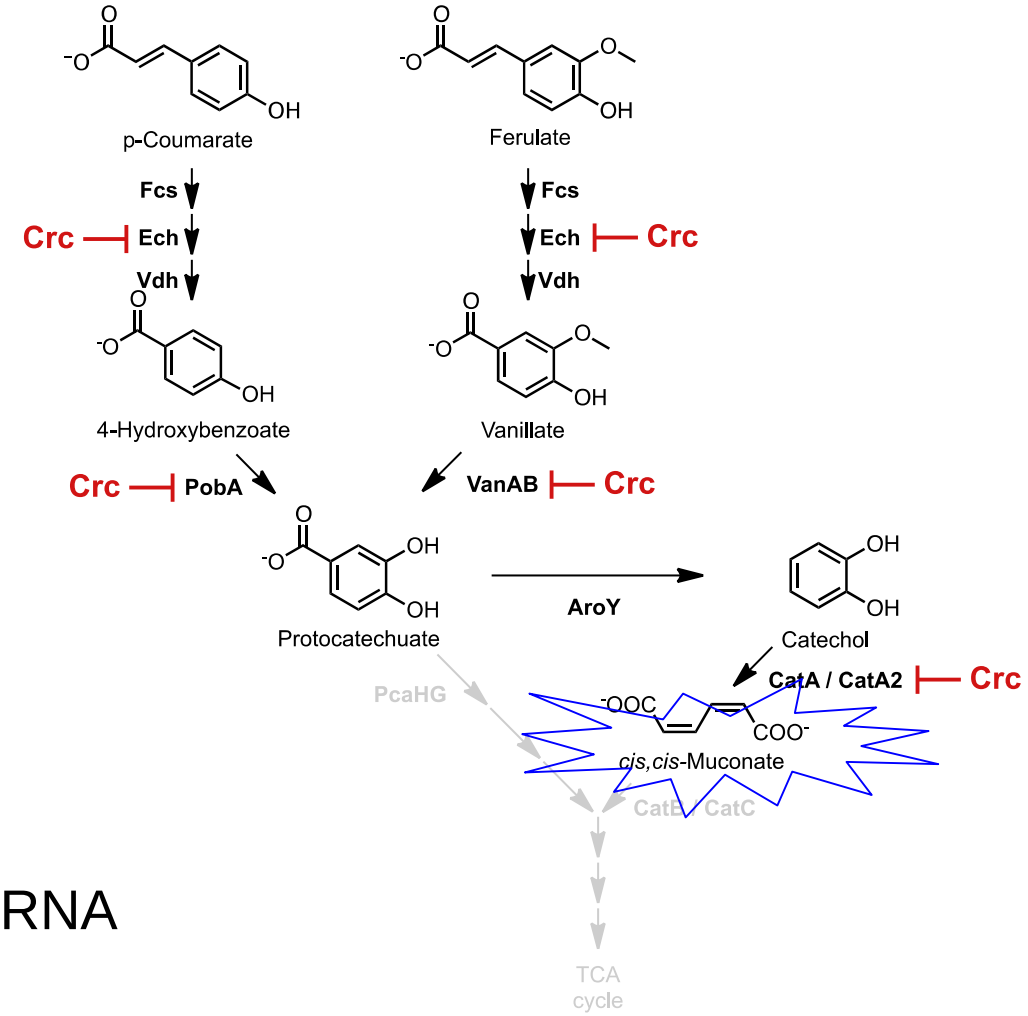
Task 2: Crc is a global regulator of catabolism in Pseudomonads

Carbon Repression Control (Crc) controls substrate preference in Pseudomonads

- Substrate preference enables microbial fitness in natural, competitive environments
- In Pseudomonads, this is largely regulated by a global regulator called the Carbon Repression Control (Crc) protein
- Crc is a translational regulator that binds to mRNAs at AAnAAnAA motifs near ATG, blocking their translation to protein
- Global regulator of catabolism:

Amino and organic acids > glucose > aromatics

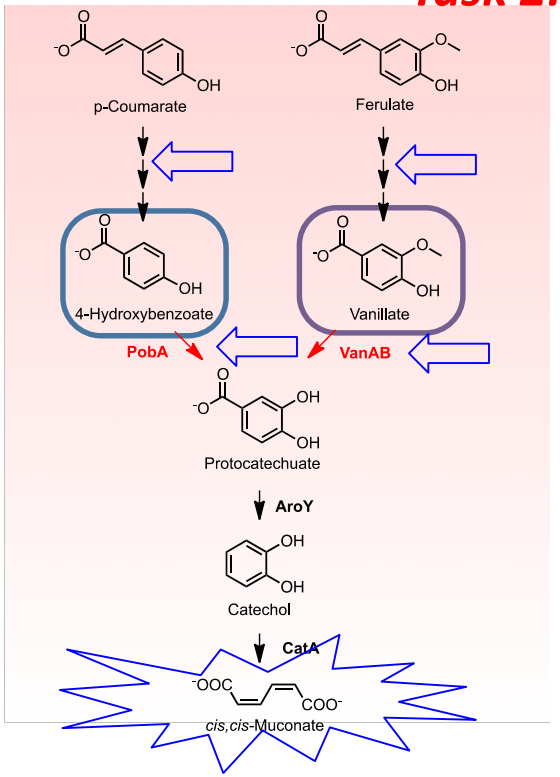




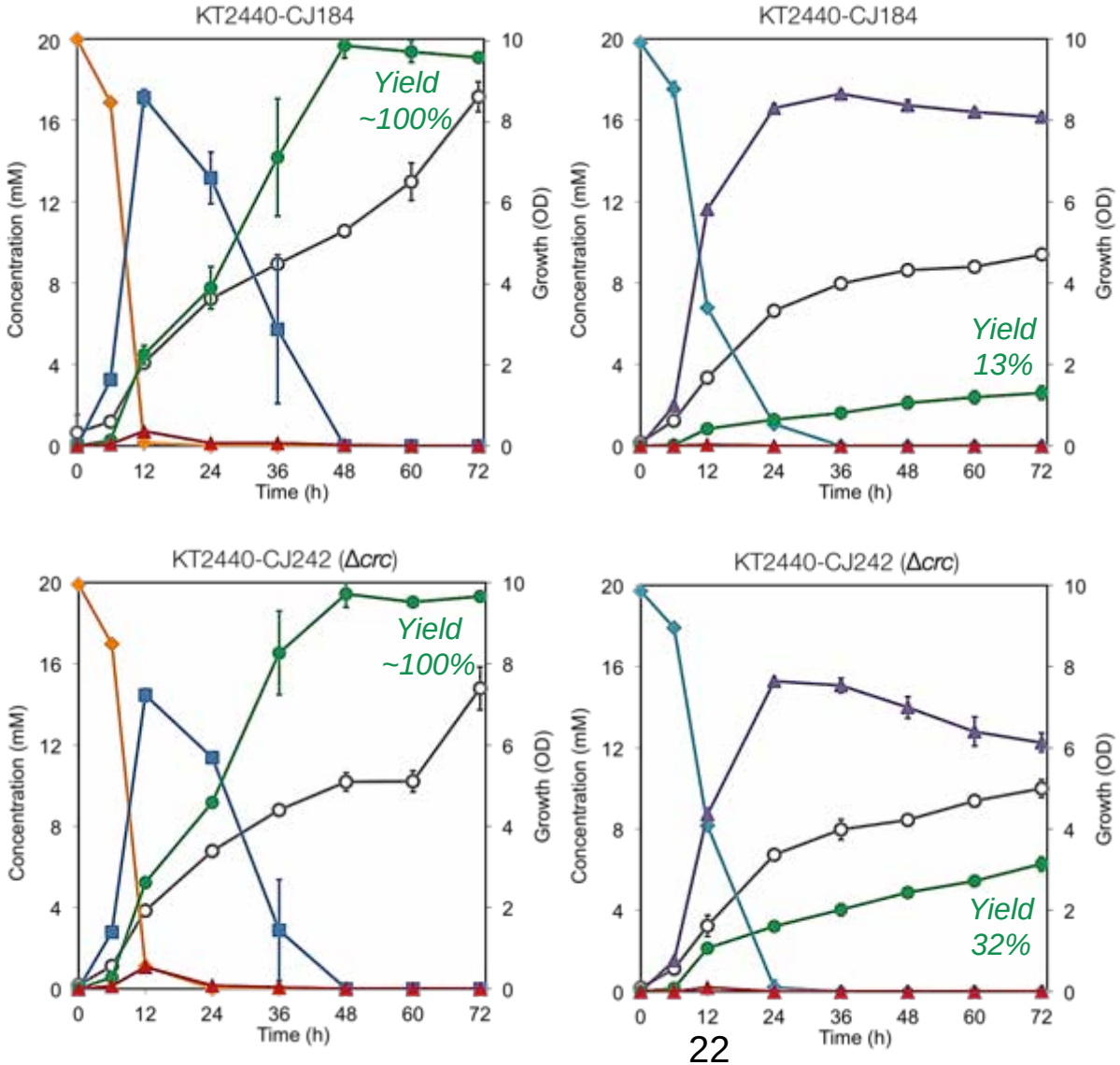
Johnson, *et al*., in revision: *Metabolic Engineering Communications*

3 – Technical Accomplishments/ Progress/Results

Task 2: Deletion of crc enhances aromatic metabolism

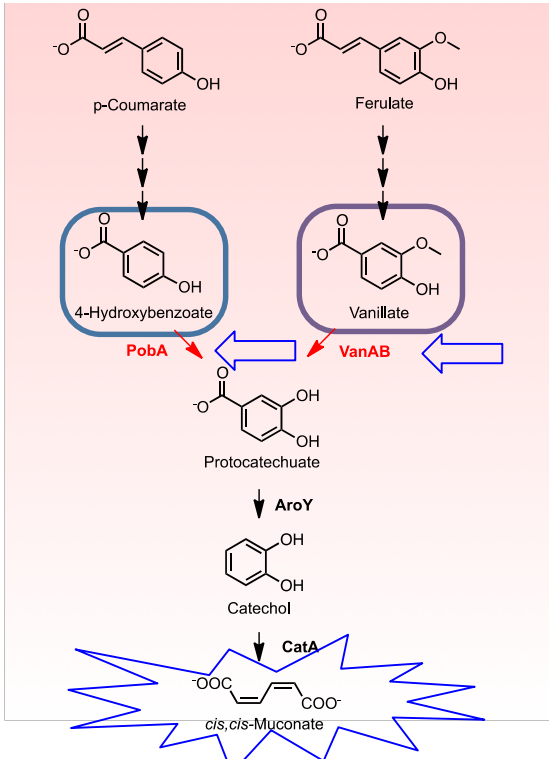


- Deletion of the gene encoding Crc led to reduced accumulation of 4-hydroxybenzoate and vanillate when strains were fed glucose for energy and growth.

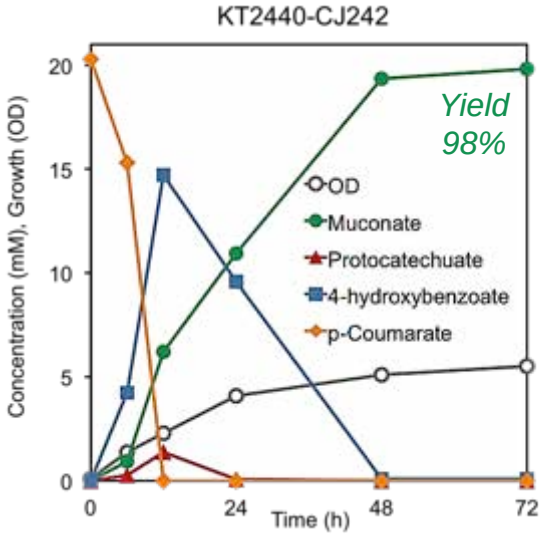


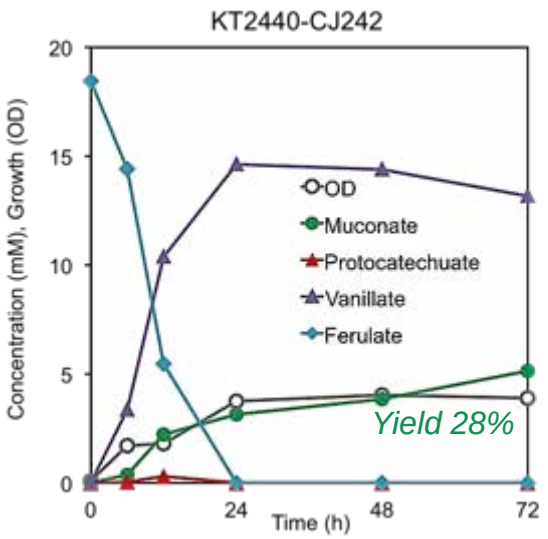
3 – Technical Accomplishments/ Progress/Results

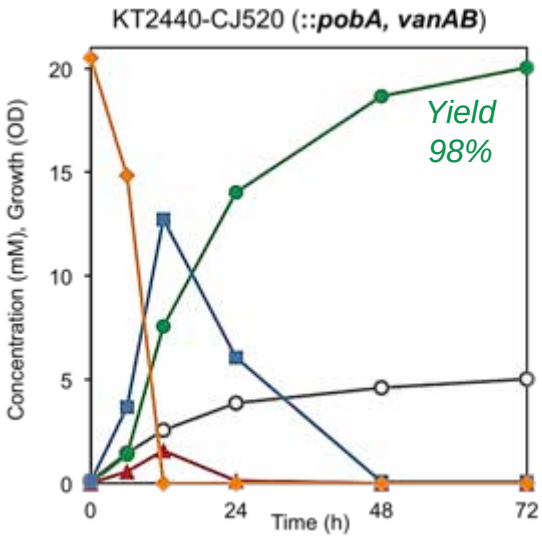
Task 2: Enzyme overexpression enhances aromatic metabolism

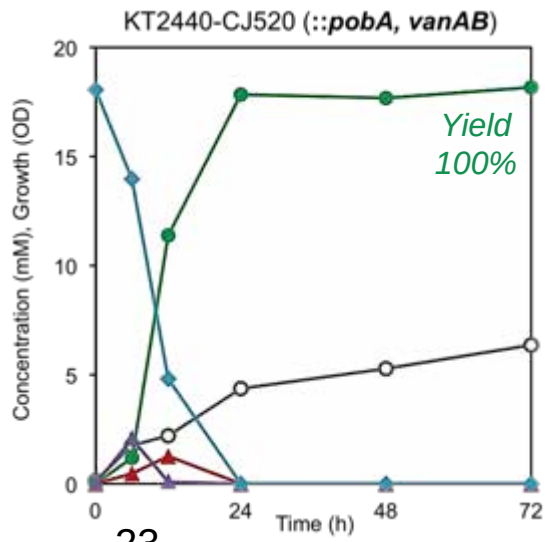


- Genes encoding PobA and VanAB (hydroxylases) were overexpressed from the genome.
- Overexpression of PobA reduced 4-HB accumulation and enhanced muconate production
- Overexpression of VanAB greatly reduced vanillate accumulation and enhanced muconate production



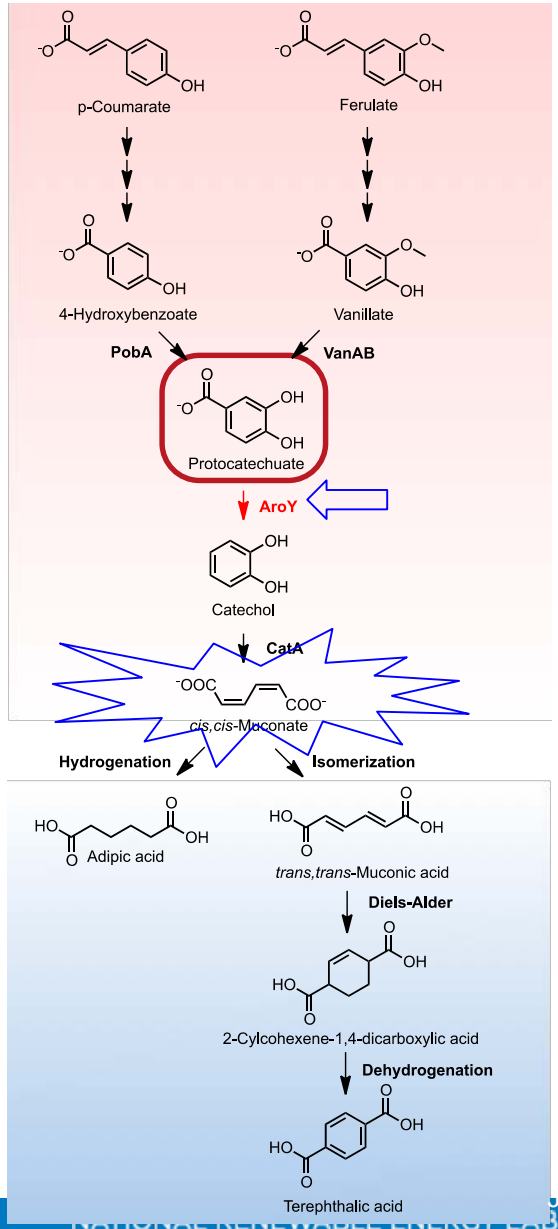






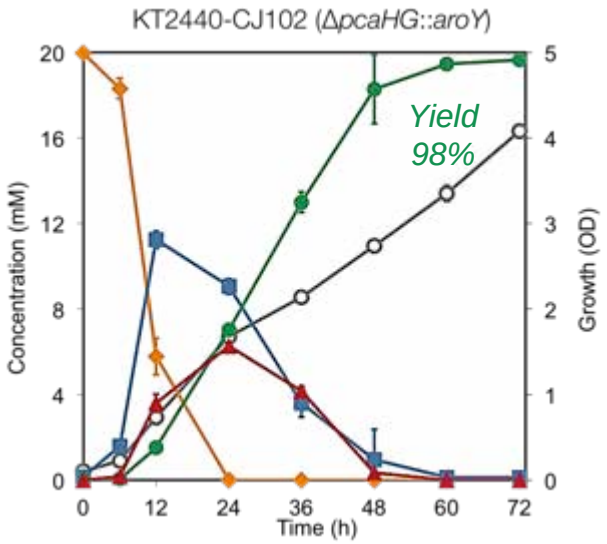
3 – Technical Accomplishments/ Progress/Results

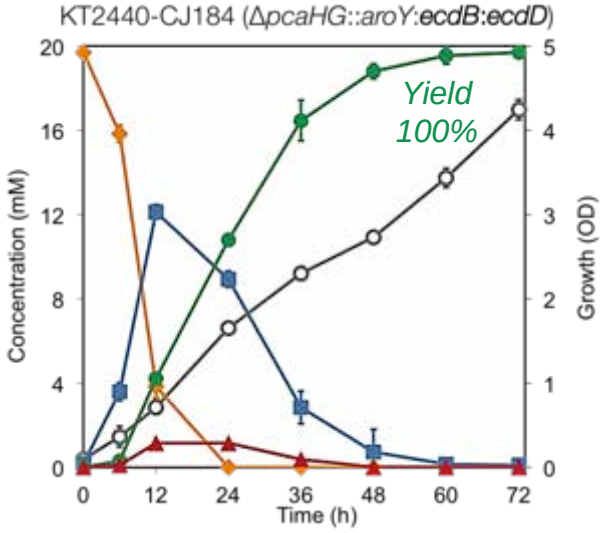
Task 2: Protocatechuate decarboxylase debottlenecking



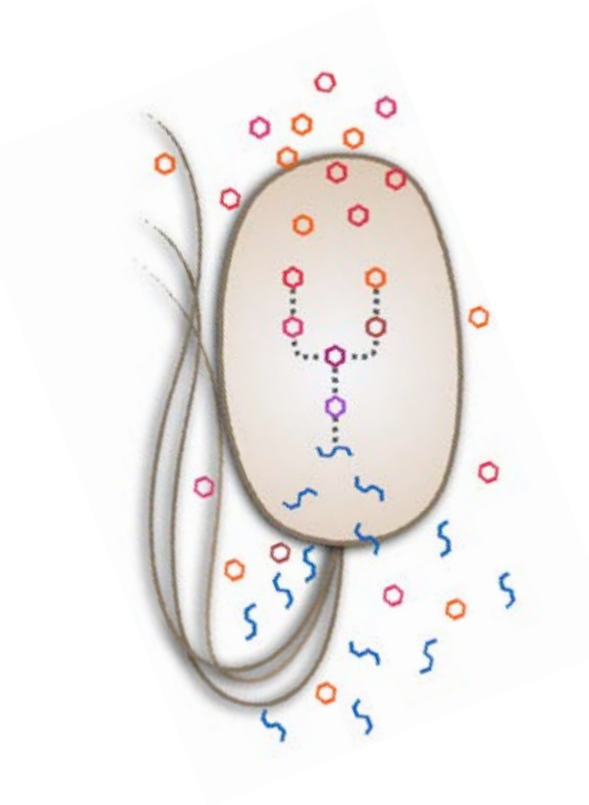
Metabolic engineering of *P. putida* KT2440 to enhance metabolism of protocatechuate

- EcdB and EcdD are genetically associated with the family of decarboxylases that includes the protocatechuate decarboxylase, AroY
- EcdB produces a specialized prenylated flavin co-factor required for activity of AroY
- The role of EcdD is unknown
- Co-expression of EcdB and EcdD increased activity of the AroY, reducing the accumulation of protocatechuate and increasing the productivity of muconate production.





Task 2: Summary



Summary accomplishments

- Co-expression of genetically associated proteins EcdB and EcdD reduced accumulation of protocatechuate.
- Deletion of Crc, a global regulator of carbon catabolite repression, reduced accumulation of 4-hydroxybenzoate and vanillate.
- The accumulation of 4-hydroxybenzoate and vanillate was further reduced by overexpression of the enzymes responsible for their conversion.
- Combined, the strain engineering above reduced major metabolic bottlenecks, enhancing muconate production dramatically.
- Achieved muconate production of 30-35 g/L and 0.6 g/L/hr (see BUS)

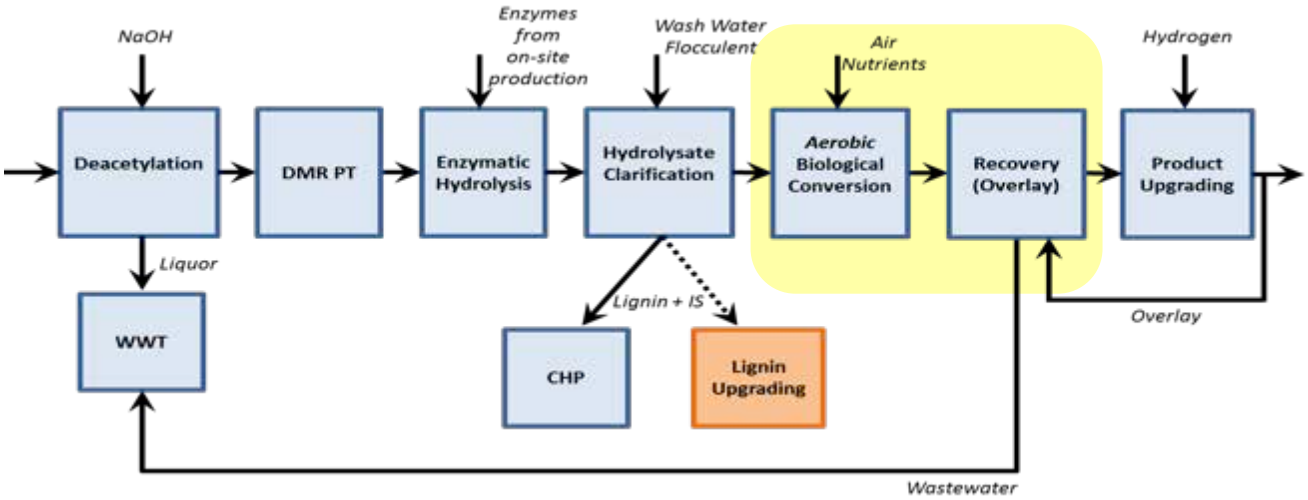
Future directions

- Understand genome expression of PobA to further reduce 4-hydroxybenzoate accumulation (already demonstrated using plasmid).
- Expand upper pathways to include other lignin relevant aromatic molecules such as guaiacol, phenol, syringol, etc.
- Examination of co-conversion of aromatic substrates to determine if further engineering is required to enable efficient conversion of complex mixtures of lignin-derived aromatic molecules.

Task 3 (TRL 3-4) Fatty Alcohols from Yeast

Block diagram schematic for the fatty alcohol pathway base case (considering combustion of lignin/residuals; then adding lignin-to-coproducts for achieving $3/GGE)

2022 targets: 20% total solids loading:
Fatty alcohols titer: ~72 g/L
Productivity: 1 g/L/hr
Yield (G+X): ~0.25 g/g

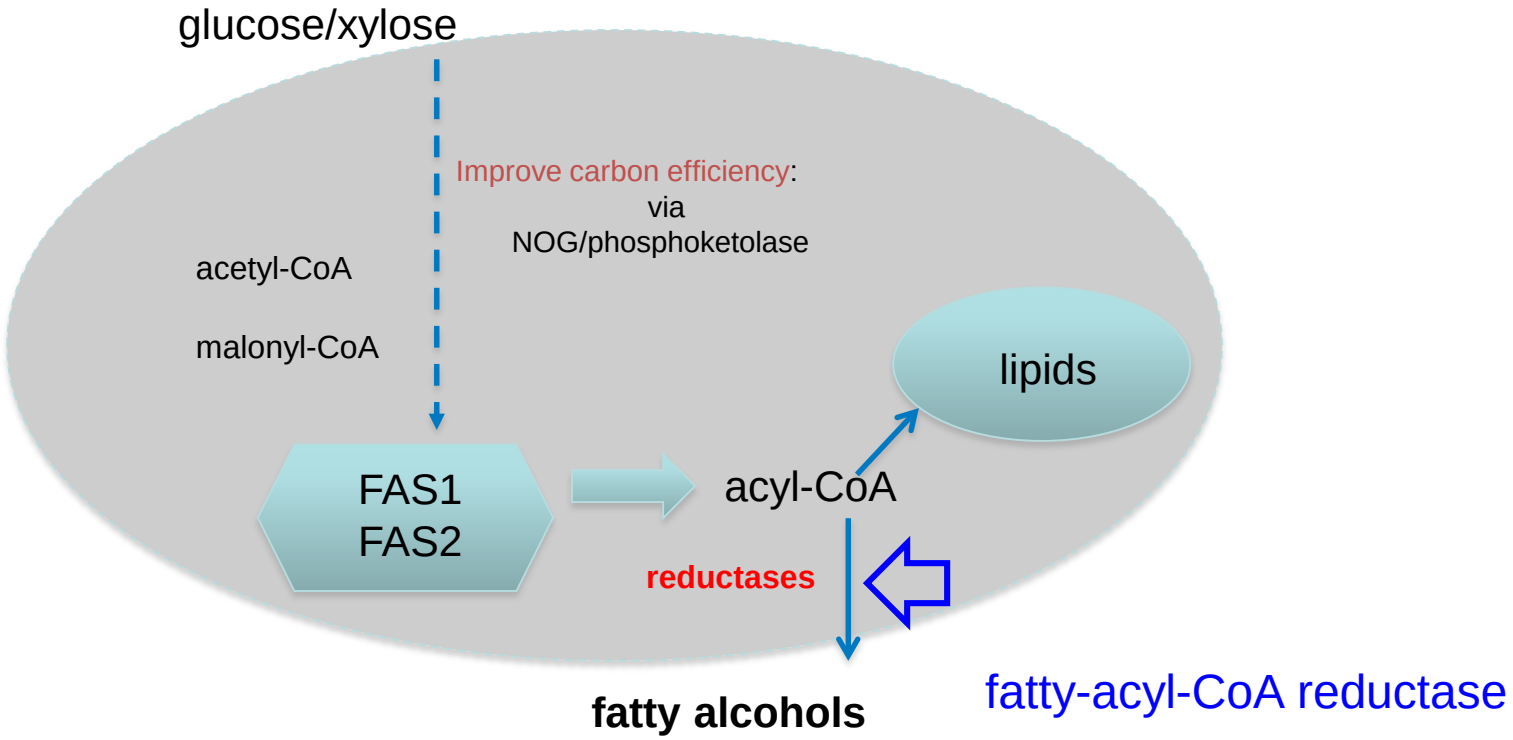


- Long chain fatty alcohols secreted by oleaginous yeast. Long chain fatty alcohols represent a more refined biofuel precursor molecule
- Naturally secreted (and/or leaked across the membrane, or easily extracted) molecule compared to larger intracellular TAG lipids.

3 – Technical Accomplishments/ Progress/Results

Task 3: (TRL 3-4) Oleaginous Yeast Cell (Yarrowia or Lipomyces)

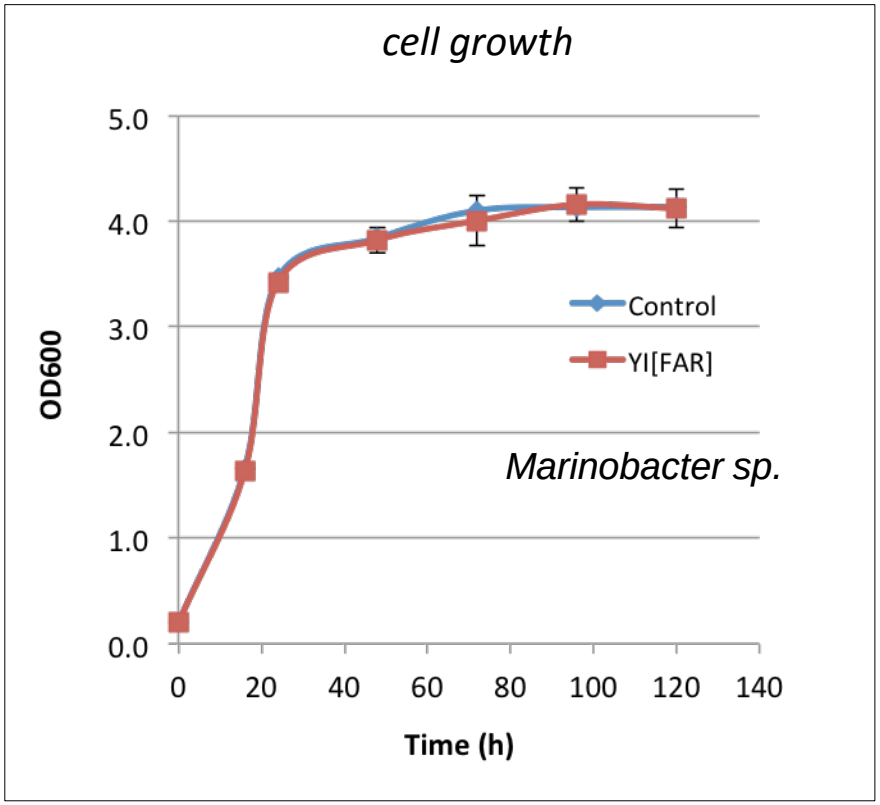
- **Long chain fatty alcohols represent a more refined biofuel precursor molecule**
- **Naturally secreted (and/or leaked across the membrane, or easily extracted) molecule compared to larger intracellular TAG lipids.**

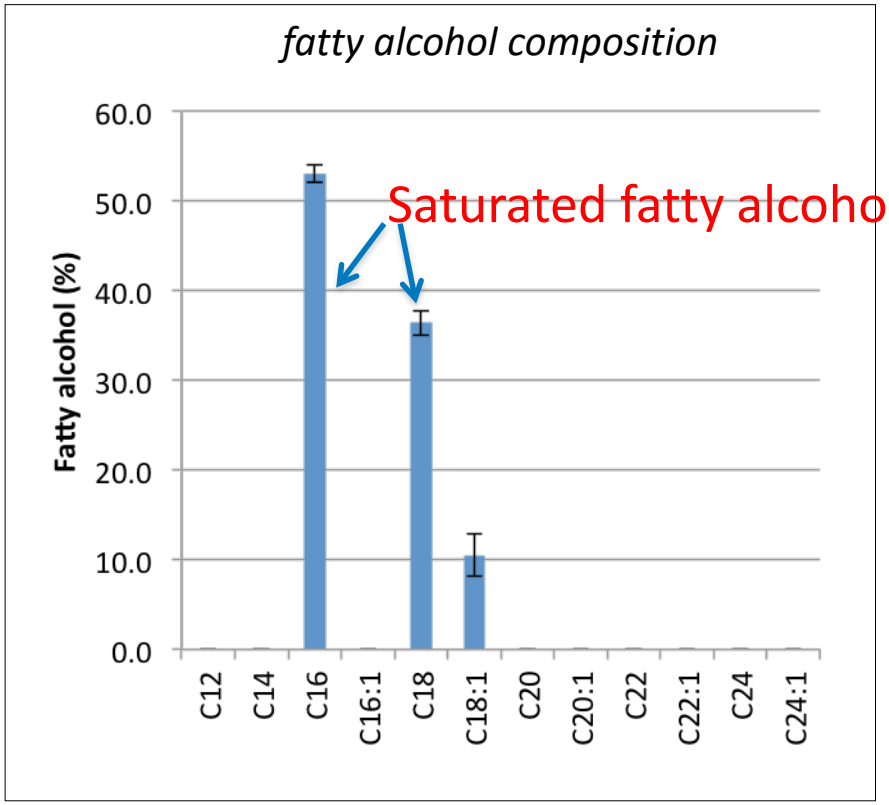


3 – Technical Accomplishments/ Progress/Results

Task 3: Expression of FAR gene in Y. lipolytica - *167 mg fatty alcohols/L*

- This titer was approximately 3-fold higher than observed with the mouse *mfar1* gene expression in *S. cerevisiae* (56.5 mg/L) as reported by Keasling's group (2013).
- Saturated fatty alcohols with a long chain length, i.e., hexadecanol (C16), octadecanols (C18) were predominant in all produced fatty alcohols, which could be beneficial for downstream upgrading to hydrocarbons.

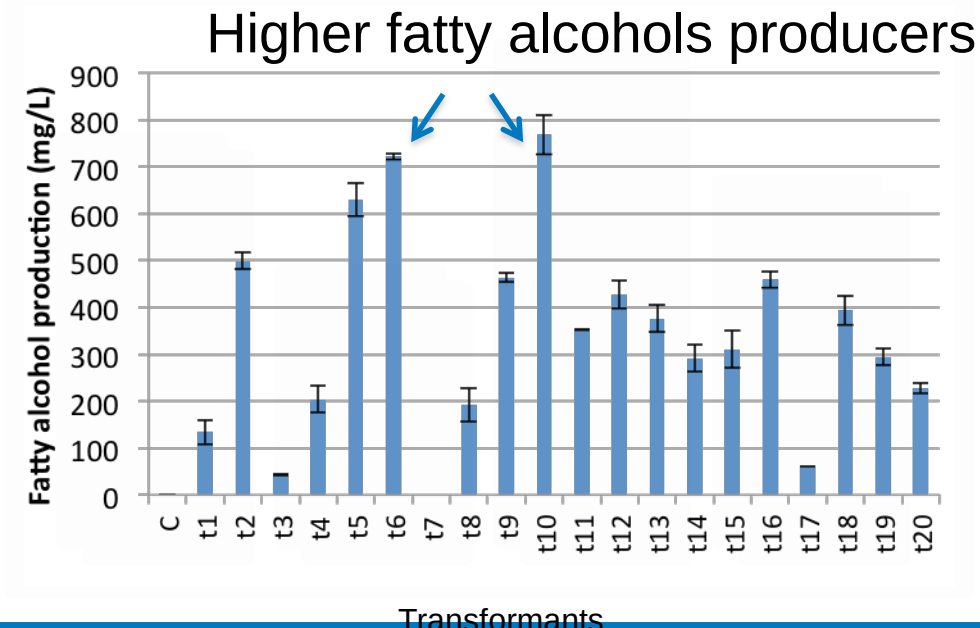


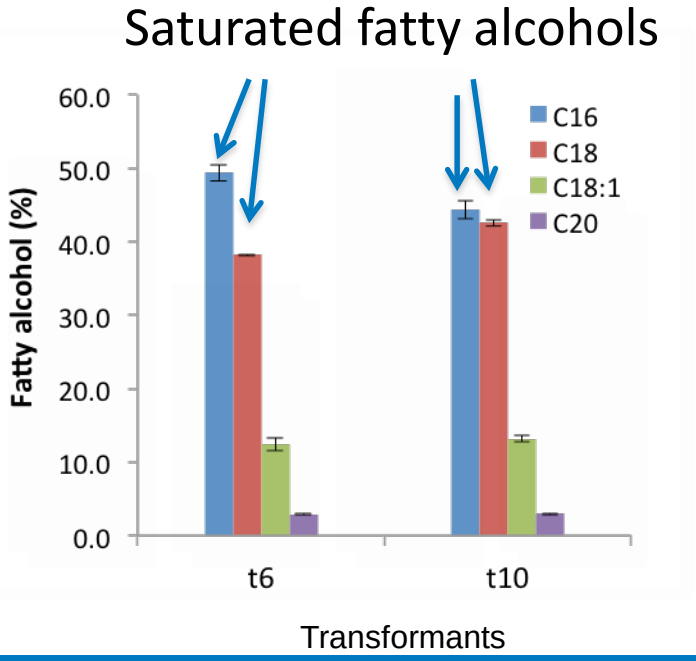


3 – Technical Accomplishments/ Progress/Results

Task 3: Expression of FAR gene in *L. starkeyi* - *770 mg fatty alcohols/L*

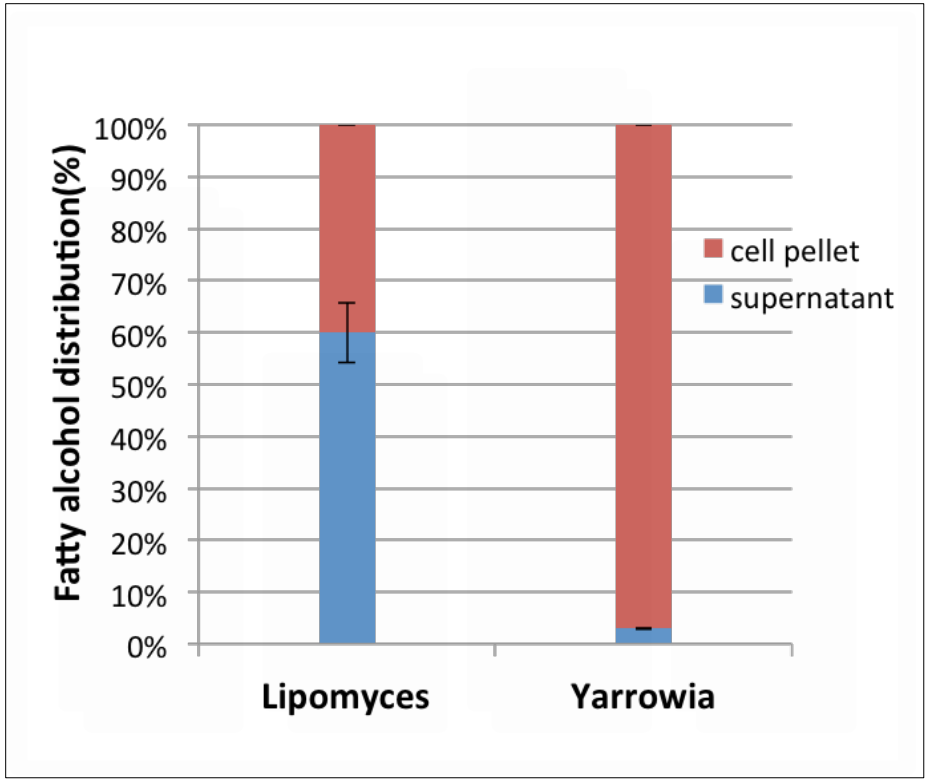
- As *L. starkeyi* accumulates high levels of TAG, we assume higher levels of fatty acyl-CoA accumulates in *L. starkeyi* than *Y. lipolytica*; therefore, more fatty alcohols should be produced when directing the carbon flux toward fatty alcohol synthesis by overexpressing the FAR gene.
- Transformants 6 and 10 produced more fatty alcohols, 720 mg/L and 770 mg/L at 5d, respectively which is about 5-fold more than in *Y. lipolytica*
- The patterns of fatty alcohols composition in *L. starkeyi* FAR transformants were similar to that of *Y. lipolytica*, saturated hexadecanol (C16) and octadecanol (C18) were the major constituents accounting for 85~88% of total fatty alcohols





3 – Technical Accomplishments/ Progress/Results

Task 3: Secretion of Fatty Alcohols Enabling Cost Effective Recovery

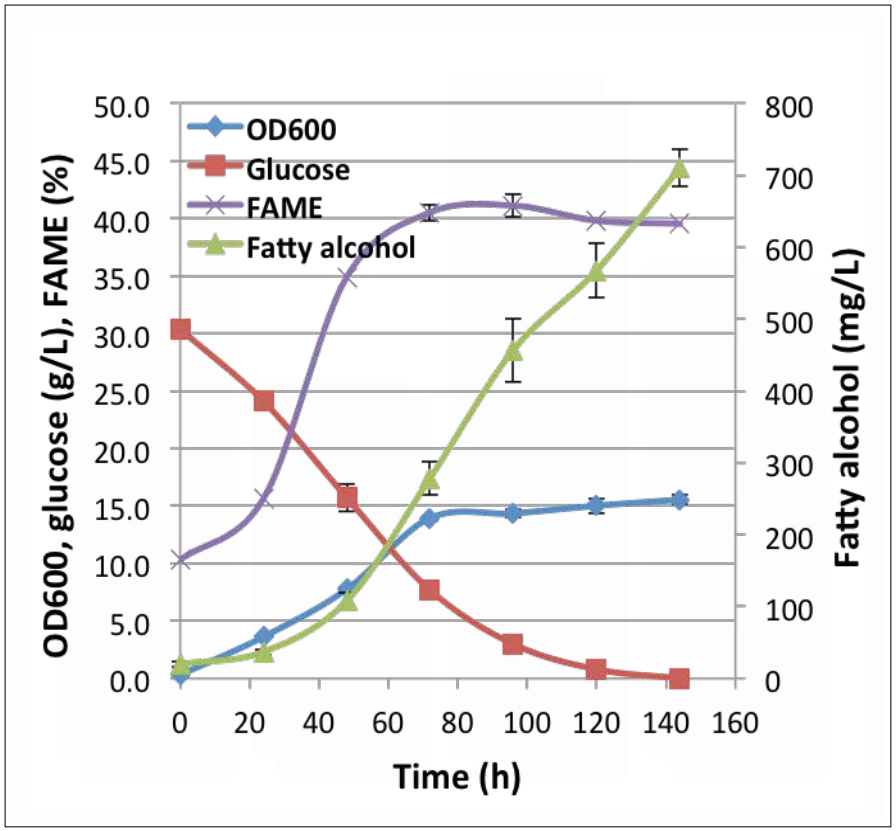


- When using a dodecane overlay during fermentation, 99% of total fatty alcohols (770 mg/L) produced by *L. starkeyi* were extracted into dodecane phase compared to 92% extraction total fatty alcohols (167 mg/L) produced in *Y. lipolytica.*
- In *L. starkeyi*, 60% of the total fatty alcohols were naturally secreted out of the cells into the medium. In *Y. lipolytica*, only 3% of total were secreted.
- Secretion of fatty alcohols should be beneficial for downstream products separation, as well as for the cells in terms of tolerance to fatty alcohol.

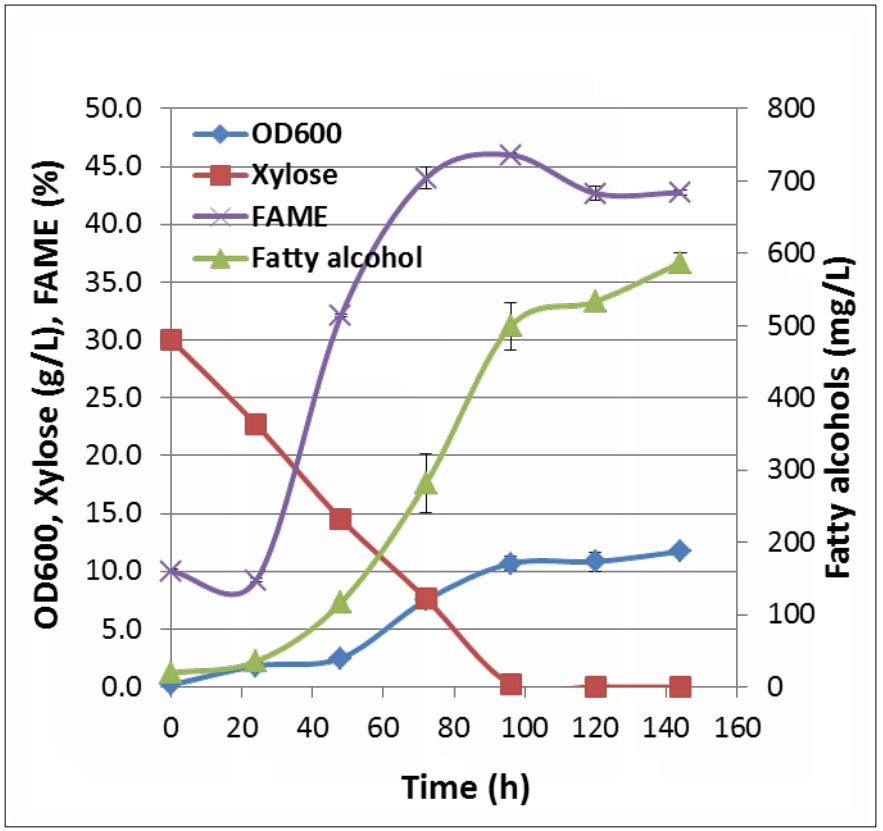
3 – Technical Accomplishments/ Progress/Results

Task 3: L. starkeyi utilizes both glucose & xylose for fatty alcohols production

Glucose (5%) as substrate

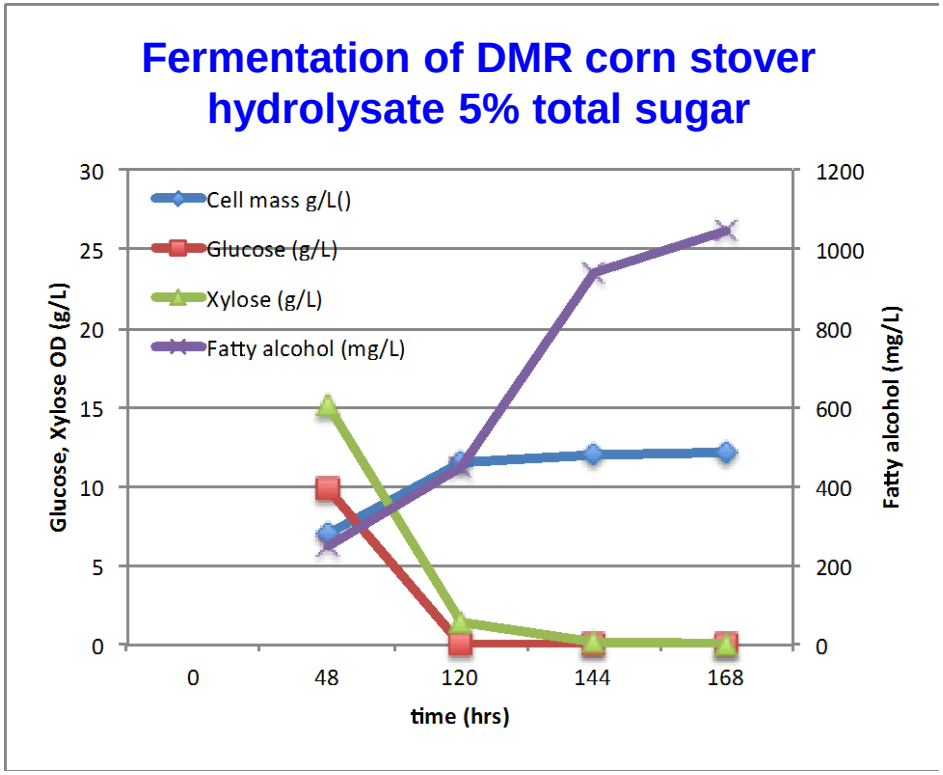


Xylose (5%) as substrate



3 – Technical Accomplishments/ Progress/Results

Task 3: DMR feedstock produces ~1.02 g fatty alcohols/L

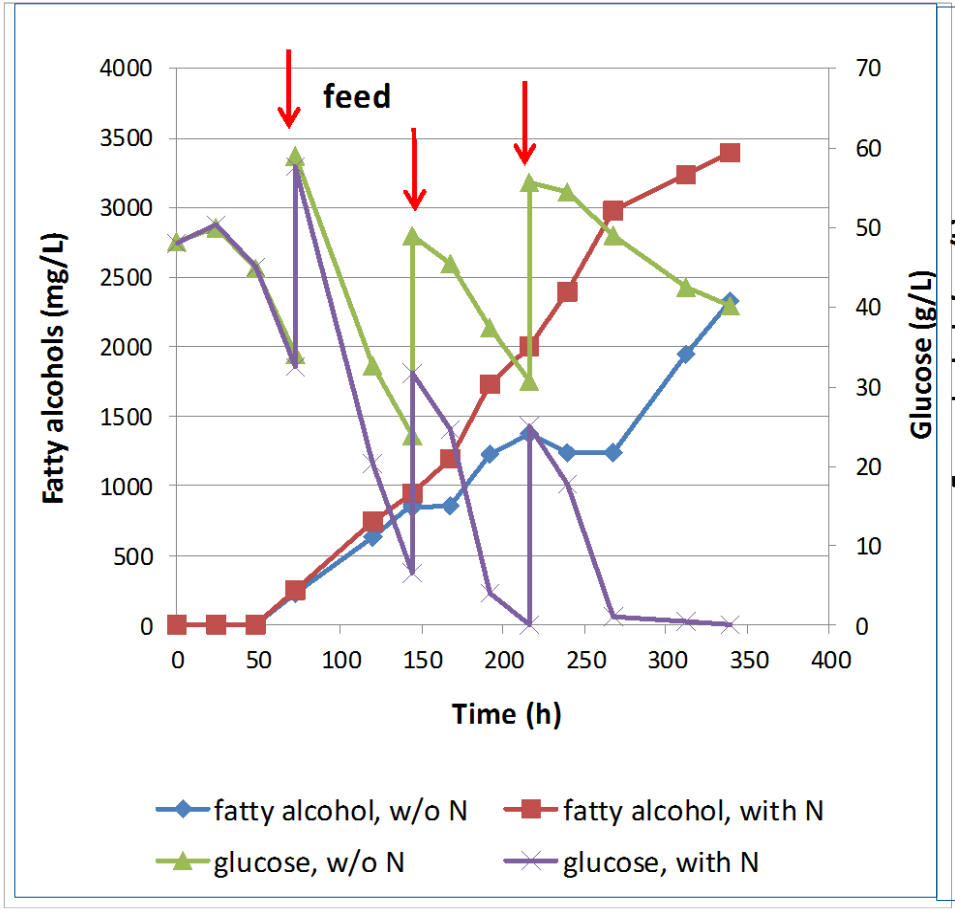


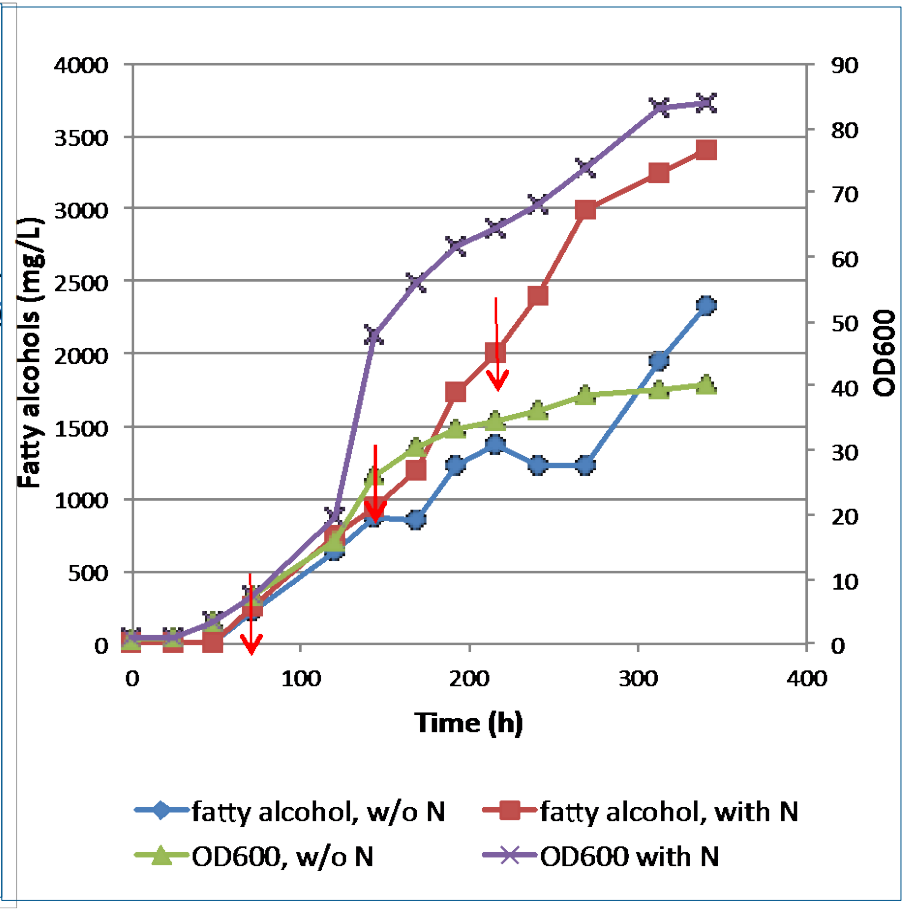
- Fatty alcohol Productivity on DMR hydrolysate is higher than on pure sugars
- *Lipomyces* FAR strain produced >1g/L of fatty alcohol from DMR containing 5% total sugars

3 – Technical Accomplishments/ Progress/Results

Task 3: Fed batch w nitrogen feeding produced ~ 3.5 g fatty alcohols/L

- *Lipomyces* FAR strain produced ~ 3.5 g/L of fatty alcohol from fed-batch fermentation
- Offers potential benefit of on-line extraction of the products



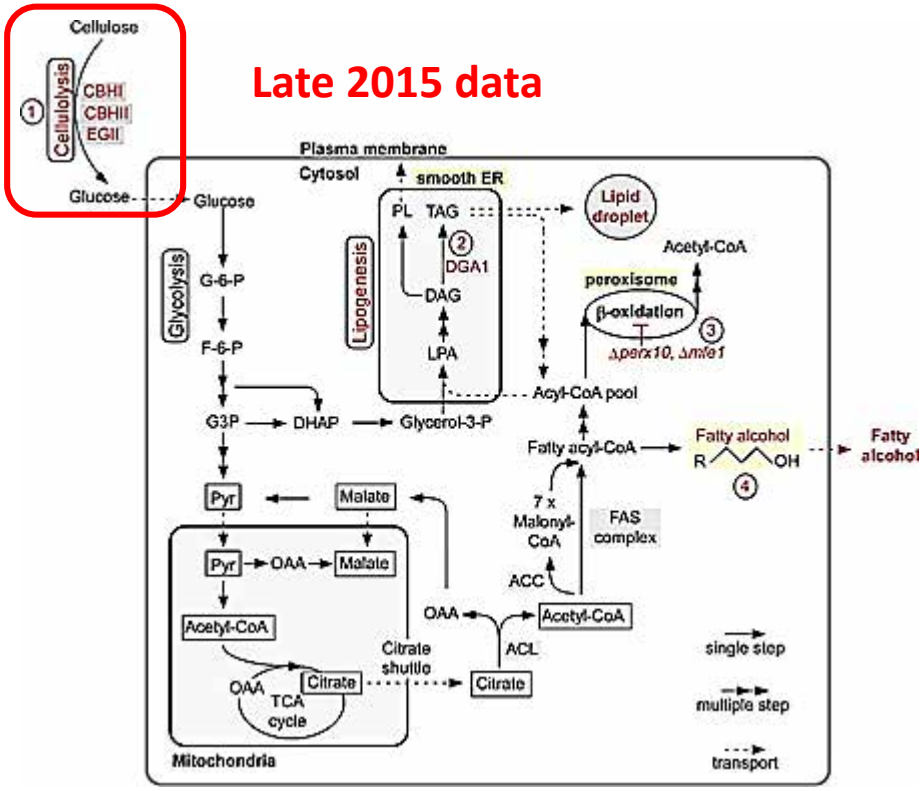


3 – Technical Accomplishments/ Progress/Results

Task 3 (TRL 2) Consolidated Bioprocessing

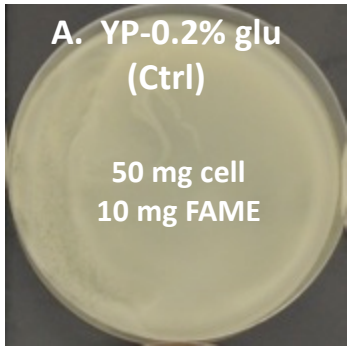
Goal: CBP of *Yarrowia* mutants utilizing cellulose (solid medium)

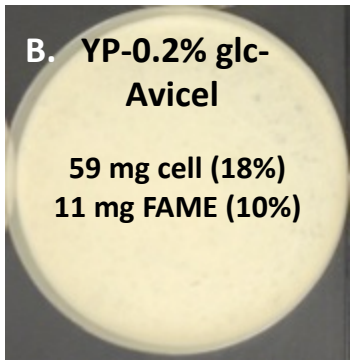
- A direct cell mat growth approach
- Cloned in *T. reesei* CBHI-CBHII-EGI

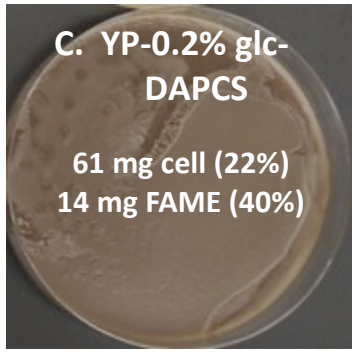


Late 2015 data

Transformant HA1(165-1) culture at Day 6







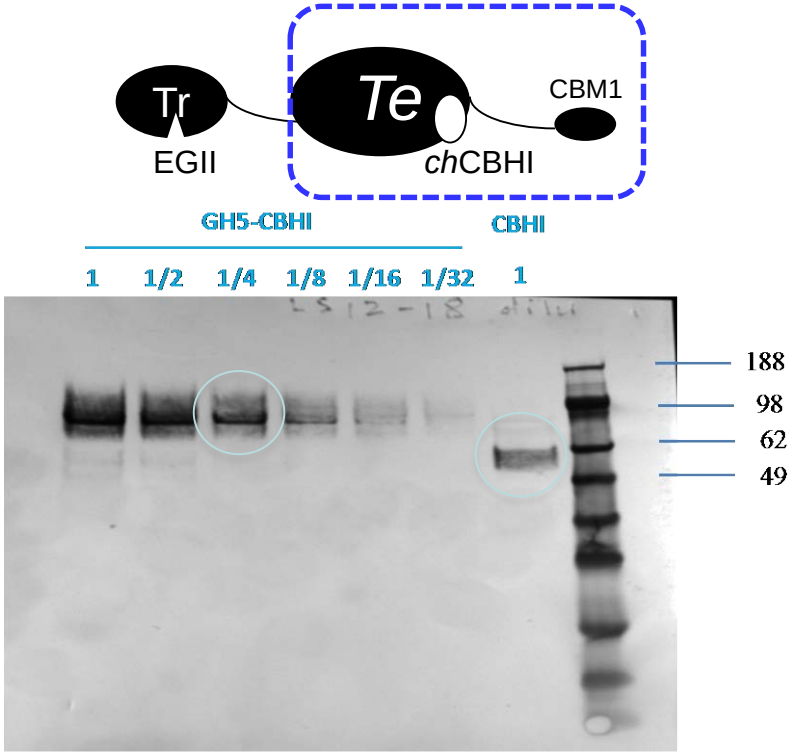
In DAPCS plate: *22% more cell growth and 40% more FAME than the control (per plate basis).*

Done with FAMES so move on with better enzymes!

Clone cellulase genes in *Lipomyces*!

3 – Technical Accomplishments/ Progress/Results

Task 3: Can we improve the secretion and activity of the key cellulases?



- *In Yarrowia,* production of the fusion protein GH5-*ch*CBHI was at least 8 times that of chimeric CBHI alone.
- In *Yarrowia*, activity of the fusion protein is comparable to the single protein mixture of *ch*CBHI and an endo.
- Next step, clone this chimera in yeast producing higher titers of fatty alcohols (*Rhodosporidium*).

Hydrolysis of 0.5% PCS		**chimeric CBHI + endo** (chimeric CBHI + endo)	**EGII-chimeric CBHI** (EGII chimeric CBHI)	**EGII-chimeric CBHI + endo** (EGII chimeric CBHI + endo)
Enzyme titer (percent of *T. reesei CBHI* activity)	*Yarrowia lipolytica*	32 mg/L (**93%**)	**250** mg/L (tbd)	planned to do next
	Lipomyces starkeyi	6 mg/L (80%)	22 mg/L (**116%**)	22 mg/L (80%)

Task 3: Summary

Summary of accomplishments

- Engineered *Lipomyces* FAR strain produced ~ 3.5 g/L of fatty alcohols from fed-batch fermentation.
- Showed that glucose and xylose are fermented by *Lipomyces.*
- Offers potential benefit of on-line extraction of the products.
- Showed that active endo-exo chimeras of fungal enzymes can be expressed in lipogenic yeast and are more active than individual enzyme counterparts.

Future directions

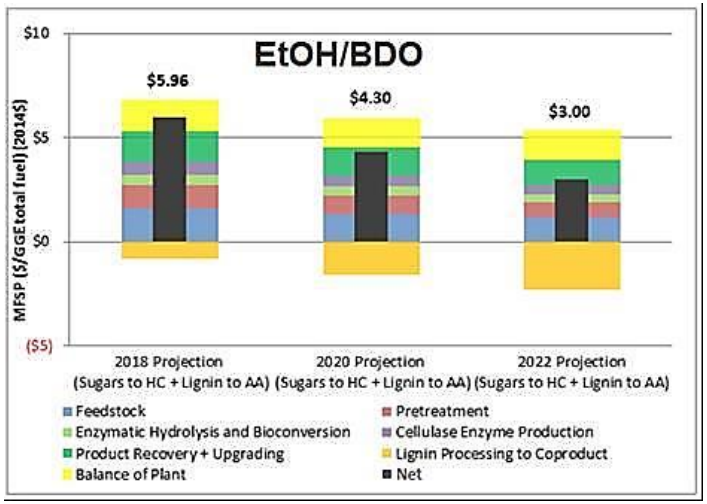
- FY17 Q3 milestone: Identify metabolic bottleneck(s) for enhanced fatty alcohols in oleaginous yeasts.
- FY17 Q2 Go/No-Go: "Evaluate cellulase expression in yeast for CBP." Can we get to sufficient cellulase titers?
- Pursue codon harmonization strategies to enhance cellulase production in yeast.
- Clone FAR genes in *R. toruloides* (Neol BioSol Via BUS)

4 – Relevance

Decreasing Biomass conversion costs through biocatalyst development

Relevance to Industry and Tech transfer

- NREL TEA shows that significant cost savings for advanced fuels are: product yield, titer and rate; secreted products; anaerobic culture
  - ROIs planned for FY2017-18
  - Numerous papers published in peer reviewed journals



- Enables *Zymomona*s as an effective and familiar microbial platform for fuels and chemicals in industry applications.

- Directly support BETO's mission: "Develop and transform our renewable biomass resources into commercially viable high performance biofuels."

- Project fulfills MYPP plan: "Efficient, highly active, selective, durable biological catalysts; Pathways that tolerate feedstock variability and inhibitors; Catalysts for lignin to central metabolism or upgrading."

5 – Future Work

- **Enabling *Zymomonas* as a new microbial platform for chemical and fuels production**:
  - FY18 Q4 milestone: Develop a strain of *Zymomonas mobilis* and a corresponding fermentation processes (with BSI) able to achieve > 50 g/L of BDO at a productivity of >1 g/L/hr from biomass
  - Pursue succinate pathway concept in coordination with TEA and BSI.
  - Discover or engineer "tunable" PDC enzyme
- **Chemicals from biological lignin upgrading:**
  - *FY17 Q2* milestone*: Demonstrate a reduction of protocatechuate buildup of ≥10% in the engineered strain at a productivity of at least 0.3 g/L/hr*
  - Study intermediate inhibition in *P. putida*; study conversion of real substrates (lignins)
  - Improve the tolerance of *P. putida* to muconic acid via evolution
  - Increase productivity and titers from *p*-coumaric through strain and fermentation development. We are still finding bottlenecks (Q2)
- **Long chain hydrocarbon production:**
  - *FY17 Q3* milestone: *Identify metabolic bottleneck(s) for enhanced fatty alcohols in oleaginous yeasts. Get TEA on FA pathway.*
- **Effective cellulase expression in CBP host:**
  - Right Goal? FY17 Q2 Go/No-Go: "Evaluate cellulase expression in yeast for CBP (50 mg Cel7A/L)."
  - Pursue codon harmonization strategies to enhance cellulase production
- *What is the remaining budget and is it sufficient to complete the remaining work? If not, what are the plans to accomplish the work? On track.*

TMD Summary

Overview

We are investigating promising pathways for advanced biological upgrading of biomass sugars and lignin to hydrocarbons (HC) and co-products.

- ***Approach***
  - Apply metabolic engineering and synthetic biology tools to engineer microorganisms for efficient utilizing sugars to HC intermediates and valorizing lignin for chemicals production.
- ***Technical Accomplishments/Progress/Results***
  - Demonstrated production of mixed alcohol/diol (with acetoin) using engineered *Zymomonas* titers ~48 g/L; productivity ~1 g/L/hr ; and yield ~0.43 g/g from corn stover hydrolysate at 13% total solid loading.
  - Co-expression of genetically associated proteins EcdB and EcdD and deletion of Crc, a global regulator of carbon catabolite repression. The engineered *Pseudomonas* strain reduced major metabolic bottlenecks, enhancing muconate production dramatically.
  - TEA analysis showed a combined mixed alcohol/diol from sugars and muconate pathway can reach $3/GGE with improved conversion process.
  - Improve the secretion and activity of the key cellulases by fusion protein engineering.
- ***Relevance***
  - This project supports the DOE BETO 2022 goal of enabling advanced HC fuels at $3/GGE.
- ***Future work***
  - Continue to use engineering strategies to improve the mixed alcohol/diol and muconate production with a titer, rate and yield to reduce cost (ca 50-70 g/L for 2018).
  - Further improve the secretion and activity of the key cellulases to enable CBP (ca~50 mg Cel7A/L).
  - Improve fatty alcohol production using oleaginous yeast.

Acknowledgements

- *Funding*
  - U.S. DOE EERE Office of the Biomass Program
    - HQ: Jonathan Male, Kevin Craig, Ian Rowe
  - NREL LPM and Platform Lead
    - Zia Abdullah and Rick Elander
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John Baker
Yat-Chen Chou
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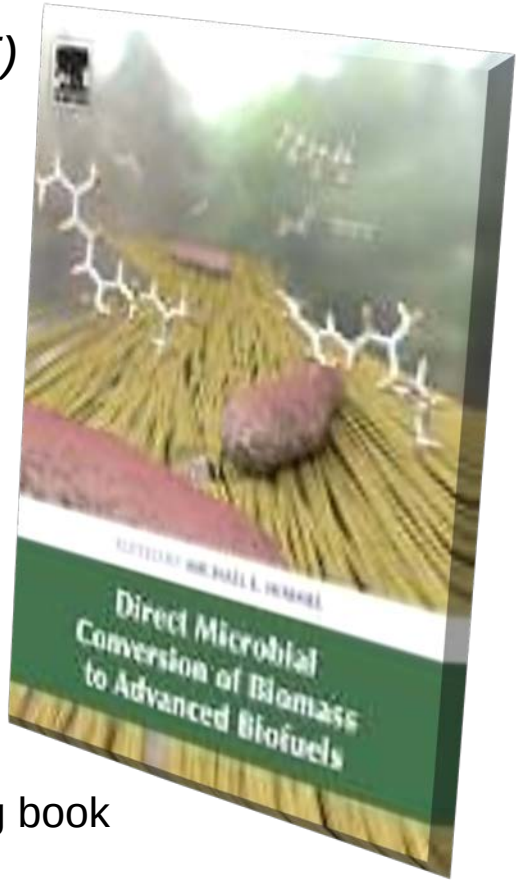
Lieve Laurens
Eric Knoshog
Todd Vanderwall
Stefanie Van Wychen
Qi Xu
Shihui Yang
Wei Wang
Hui Wei
Gregg Beckham (Task 2 Lead)
Davinia Salvachua
Chris Johnson
Holly Smith
Darren Peterson



New 600 pg book

Additional Slides

Responses to Reviewers' Comments 2015

Overall Impressions

- This is a good project and it is focused on what the overall 2017-2022 project plan needs. I would question the 2017-2022 direction regarding aerobic lipid fermentation to make fuels directly from the fermentation. I would suggest fermentation to a platform chemical followed by potentially much easier and more well-known chemical processing. An example would be fermentation to isobutanol followed by chemical transformation (dehydration, oligomerization, hydrogenation) to jet fuel. There are many examples of chemical transformations like this to useful products from platform chemicals. This happens to be the Gevo route, but other chemicals are similar platforms, succinic acid for example, but it is also somewhat difficult to recover.
- Future work seems like a logical continuation of the current path, based on lessons learned from the initial research.
- It is never too early to perform a techno-economic analysis on a project; indeed, it is often a requirement prior to funding any industrial R&D project. There is promise in several aspects of this research, but it has a feel that there is research going on because there has always been this research going on. It is strongly encouraged, given the extended timeline on two of the three tasks, that time be spent on an objective economic analysis, potentially with direct industry input, to validate the effort that has taken place. There have been the same concerns expressed over the last two reviews, project management needs to pause and consider them seriously.
- This project has a wide scope of activities and is making good progress towards well-defined goals. NREL continues to champion *Zymomonas* as a host for biofuels/biochemicals, but it is not clear if this platform organisms is industrially relevant. Engineering oleagenous yeast for direct microbial conversion to lipids is an innovative, high impact approach.

PI Response to Reviewer Comments

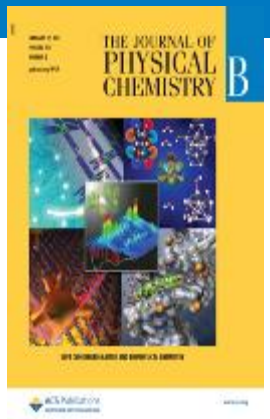
- We thank the reviewers for their comments. We agree with the suggestion to conduct TEA analysis for Tasks 1 and 3 (preliminary TEA has been conducted and will be revisited going forward).
- It is our strategy to work toward the direction recommended by the reviewer; however, the aerobic oleaginous yeast project was the BETO platform for 2017. Pursuant to more recent guidance from DOE, we are now focusing on 2,3-butanediol (BDO) from *Zymomonas* (or isobutanol as suggested by the reviewer) and fatty alcohols (a secreted product that can be recovered readily from the fermentation broth) from oleaginous yeast. This latter process can be further extended to direct microbial conversion (DMC) approaches.
- We do consider *Zymomonas mobilis* to be an industrially relevant organism today, as shown by the U.S. Patent record where 18 companies have filed patent applications directed to *Z. mobilis* as an industrial microbe. Moreover, a 30 million gallons / year commercial demonstration plant using *Zymomonas* technology is being built by DuPont.

Publications, Patents, Presentations, Awards, and Commercialization

- Publications
  - 11 publications from 2014 to today
- Patents
  1. One ROI filed with DOE/NREL
  2. NREL ROI under evaluation
- Technology transfer or commercialization efforts
  - MTA with Hal Alper (University of Texas at Austin)
  - MTA with Neol BioSolutions (Spain) for *R. toruloides* (FA producer)
- Awards
  - 2014 Colorado State University, Department of Biochemistry & Molecular Biology, Distinguished Alumnus – Himmel
  - 2015 Battelle Inventor of the Year (for *Zymomonas*) – Min Zhang
  - 2015 NREL Special Recognition Award for *Zymomonas* IP – Min Zhang

TMD: 2014-2016 Publications

- "Heterologous Expression of Xylanase Enzymes in Lipogenic Yeast *Yarrowia lipolytica*," Wei Wang, Hui Wei, Markus Alahuhta, Xiaowen Chen, Deborah Hyman, Min Zhang, and Michael E Himmel, **PloS One** (2014) **9**(12) e111443.
- "Engineering Towards a Complete Heterologous Cellulase Secretome In *Yarrowia lipolytica* Reveals Its Potential For Consolidated Bioprocessing," Hui Wei, Wei Wang, Markus Alahuhta, Todd Vander Wall, John O. Baker, Larry E. Taylor II, Stephen R. Decker, Michael E. Himmel, Min Zhang, **Biotechnol. Biofuels**, (2014) **7**:148 doi:10.1186/s13068-014-0148-0
- "Identification of Genetic Targets to Improve Lignocellulosic Hydrocarbon Production in *Trichoderma reesei* Using Public Genomic and Transcriptomic Datasets," In Direct Microbial Conversion of Biomass to Advanced Biofuels, Shihui Yang, Wei Wang, Hui Wei, Michael E. Himmel, Min Zhang, (M. Himmel, Ed.,) Chapter 10, Springer, London/New York 2015. In press.
- Direct Microbial Conversion of Biomass to Advanced Biofuels, (M.Himmel, Ed.,) Springer Publishers, London/New York, NY. 2015, 600 pages.
- "Impact of Nitrogen Deficiency Strategies on Lipid Production for Yeast and Fungal Consolidated Bioprocessing Candidates," Yang, S., Wang, W., Wei, H., Wychen, S. V., Pienkos, P. T., Zhang, M., and Himmel, M., **Energie**s **9**, 685 (2016) doi:10.3390/en9090685.
- "Metabolic Engineering of *Zymomonas mobilis* for Production of 2,3-Butanediol from Lignocellulosic Biomass Sugars," Yang, S., Mohagheghi, A., Chou, Y-C., Franden, M. A., Dowe, N., Himmel, M., and Zhang, M., **Biotechnol. Biofuels 9**:189 (2016) doi: 10.1186/s13068-016-0606-y.



TMD: 2014-2016 Publications

•"*Zymomonas mobilis* as a Model System for Production of Biofuels," Shihui Yang, Qiang Fei, Yaoping Zhang, Lydia M. Contreras, Sagar Utturkar, Steven D. Brown, Michael E. Himmel, and Min Zhang, **Microbial Biotechnol**. (2016) doi:10.1111/1751-7915.12408.

•"Fatty Alcohol Production in *Lipomyces starkeyi* and *Yarrowia lipolytica*," Wei Wang, Hui Wei, Eric Knoshaug, Stefanie Van Wychen, Qi Xu, Michael E. Himmel, and Min Zhang, **Biotechnol. Biofuels** (2016) **9**:227 doi:10.1186/s13068-016-0647-2

•"Biomass Conversion," Stephen R. Decker, John Sheehan, David C. Dayton, Joseph J. Bozell, William S. Adney, Andy Aden, Bonnie Hames, Steven R. Thomas, Richard L. Bain, Roman Brunecky, Chien-Yuan Lin, Antonella Amore, Hui Wei, Xiaowen Chen, Melvin P. Tucker, Stefan Czernik, Amie Sluiter, Min Zhang, Kim Magrini, and Michael E. Himmel, In Handbook of Industrial Chemistry and Biotechnology 13th Edition," (J.A. Kent and S.D. Barnicki, Eds.), Springer Publishers, New York, Chapter 33, pp. xxx-xxx, (2016) In Press.

•. Qi Xu, Eric P. Knoshaug, Wei Wang, Markus Alahuhta, John O. Baker, Shihui Yang,, Todd Vander Wall, Stephen R. Decker, Michael E. Himmel, Min Zhang, Hui Wei. Expression and secretion of fungal endoglucanase II and chimeric cellobiohydrolase I in the oleaginous yeast *Lipomyces starkeyi*. **Microbial Cell Factories** (submitted in Dec 2017; under review).

•. Hui Wei, Wei Wang, Andrew B. Hill, Qi Xu, Stefanie Van Wychen, Eric Knoshaug, Chien-Yuan Lin, Yonghua Luo, Xiaowen Chen, Melvin P. Tucker, Stephen R. Decker, Michael E. Himmel, Hal S. Alper, Min Zhang. Co-expression of cellobiohydrolases I, II and endoglucanase II in oleaginous yeast *Yarrowia lipolytica:* complex art of balance for cellulase production and lipid accumulation. **Biotechnol. Biofuels** (submitted in Feb, 2017; under review).

FY2016 TMD Milestones

Milestone Name/Description	End Date	Type
Task 1. Identify the BDO pathway genes for expression in *Z. mobilis*.	12/30/2014	Quarterly Progress Measure (Regular)
Task 1. Down select best gene combination to demonstrate BDO production at 10 g/L in *Z. mobilis* from glucose and xylose. SMART milestone.	9/30/2015	Annual Milestone (Regular)
Task 1. Demonstrate the redirect of carbon flux from ethanol to BDO production.	12/30/2015	Quarterly Progress Measure (Regular)
Task 1. Demonstration of production of BDO at 50 g/L from mixed C5/C6 sugar streams from DDR pretreated corn stover. SMART milestone.	9/30/2017	Annual Milestone (Regular)
Task 2. Identify and procure multiple anaerobic organisms that can convert mixed sugars to high carbon efficiency intermediates	3/30/2015	Quarterly Progress Measure (Regular)
Task 2. Down select to 2-3 organisms based on performance in batch anaerobic fermentations on biomass derived substrates for further FY16/17 adaptation, evolution, and evaluation.	3/30/2016	Quarterly Progress Measure (Regular)
Task 3. Evaluate *C. bescii* for utilization selected pretreated biomass feedstocks and identify suitable HC intermediate to be produced in *C. bescii*.	6/30/2015	Quarterly Progress Measure (Regular)
Task 3. Identify the most promising pathway for producing intracellular and extracellular HCs in yeast.	9/30/2015	Annual Milestone (Regular)
Task 3. Improve the lipid titer to 75% (g lipid/g cell) through pathway optimization in oleaginous yeast. SMART milestone.	9/30/2016	Annual Milestone (Regular)
Task 3. Identify pathways and factors promoting extracellular production of HCs or facilitating recovery of products.	9/30/2017	Annual Milestone (Regular)

FY2017 TMD Milestones

Milestone Name/Description	End Date	Type
Task 2. Conduct Bioscreen C and/or flask measurements to determine the relative utilization rates of p-coumarate and ferulate in native *Pseudomonas putida* KT2440. Q1	12/31/2016	Regular Quarterly
Task 1. Provide improved Zymomonas strain to BSI for fermentation optimization and testing Q2	3/31/2016	Regular Quarterly
Task 2. Conduct a crc knockout along with the EcdB, EcdD, or EcdBD genes (to overcome the protocatechuate decarboxylase bottleneck). Demonstrate a reduction of protocatechuate buildup of ¾ 10% in the engineered strain at a productivity of at least 0.3 g/L/hr. Q2	3/31/2016	Regular Quarterly
Task 2. Fatty Alcohol Reductase Screening- Screen fatty alcohol reductase enzyme diversity for increased fatty alcohol production in *Lipomyces starkyii*. Genome mining of known microbial genomes for homology to FAR from nature will be used to generate a cladogram for relatedness classes. These genes will be divided into 10 or more clades. One gene from each clade will be selected for synthesis and expression in *Lipomyces starkyii. D*eliverable: Provide activity numbers and gene construct(s) to TMD to test for improvement in FA production.	3/31/2017	Regular Quarterly (Joint w/ EEO)
Task 3. Identify metabolic bottleneck(s) for enhanced fatty alcohols in oleaginous yeasts Q3	6/30/2017	Regular Quarterly
Task 1. Demonstrate production of 2,3-butanediol from glucose & xylose from DMR-EH hydrolysate at 35 g/L using engineered Zymomonas mobilis using batch and/or fed-batch fermentations Annual SMART Milestone Q4	9/30/2017	Annual SMART (Joint w/ BSI)

TMD: 2016 Go NoGo Milestone

Name	Description	Criteria	Date
Down select to 2-3 organisms based on performance in batch anaerobic fermentations on biomass derived substrates	(3/30/16) We will evaluate a large number of strains to produce intermediates with high carbon efficiency, including with preliminary techno-economic and feasibility analysis regarding downstream separations and catalytic upgrading. The "No-Go" will discard intermediates and strains that do not enable direct upgrading to hydrocarbon fuels.	Identify 2-3 strains from feasibility studies that are able to produce high carbon efficiency anaerobically derived intermediates on biomass-derived sugars and that demonstrate ability to separate and upgrade. High carbon efficiency tentatively means (from C5 and C6 sugars) with titers of >50 g/L, rates > 0.75 g/L/hr, and yields of at least 0.5 g/g.	3/31/2016

TMD: 2017 Go NoGo Milestone

Go/No-Go Decision (80 characterlimit)	Description	Criteria	Date	Actions
Evaluate cellulase expression in Yeasts for CBP	FY17 Go/no go decision: Evaluate cellulase (CBH I) expression in *Lipomyces, yarrowia* and *Saccharomyces* for activities and secretion level	For Go Decision: 1. rCBH I activity same as *T. reesei* CBH I 2. Secretion level 100 mg /L	3/31/2017	No Go: Consider abandon CBP direction