DOE Bioenergy Technologies Office (BETO)
2015 Project Peer Review

Targeted Microbial Development

Date: March 23-27
Technology Area Review: Biochemical Platform

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Task Leads: Min Zhang & Gregg Beckham
Organization: NREL

Goal Statement

- *Present the goal of this project and describe how the project fits with the goals/objectives of the Technology Area and ultimately the goals/objectives of the DOE Bioenergy Technologies Office, and the overall bioenergy industry*
  - The goal of this project is to develop novel pathways for advanced biological upgrading of sugars to hydrocarbons (HC) by investigating efficient and rapid carbohydrate utilization, high carbon efficiency, cost effective processes to support the DOE BETO 2022 goal of producing advanced HC fuels at $3/GGE.
- *Explicitly state relevance and tangible outcomes for the United States.*
  - Provide leading technology for fuels with reduced cost and high carbon efficiency intermediates amenable to separations and catalytic upgrading to HC fuels.
  - Identify cost effective sugar upgrading technologies including consolidated bioprocess and provide a critical knowledgebase for BETO and bioenergy industry to further R&D working towards the production of third-generation HC biofuels from biomass-derived sugars.

Quad Chart Overview

Timeline

	Task 1	Task 2	Task3
• Project start date:	2013	2015	2013
• Project end date:	2022	2017	2022
• Percent complete:	12%	33%	12%

Barriers

- Bt-F. Enzyme Production
- Bt-I. Cleanup/Separation
- Bt-J. Catalyst Development
- Bt-K. Biological Process Integration

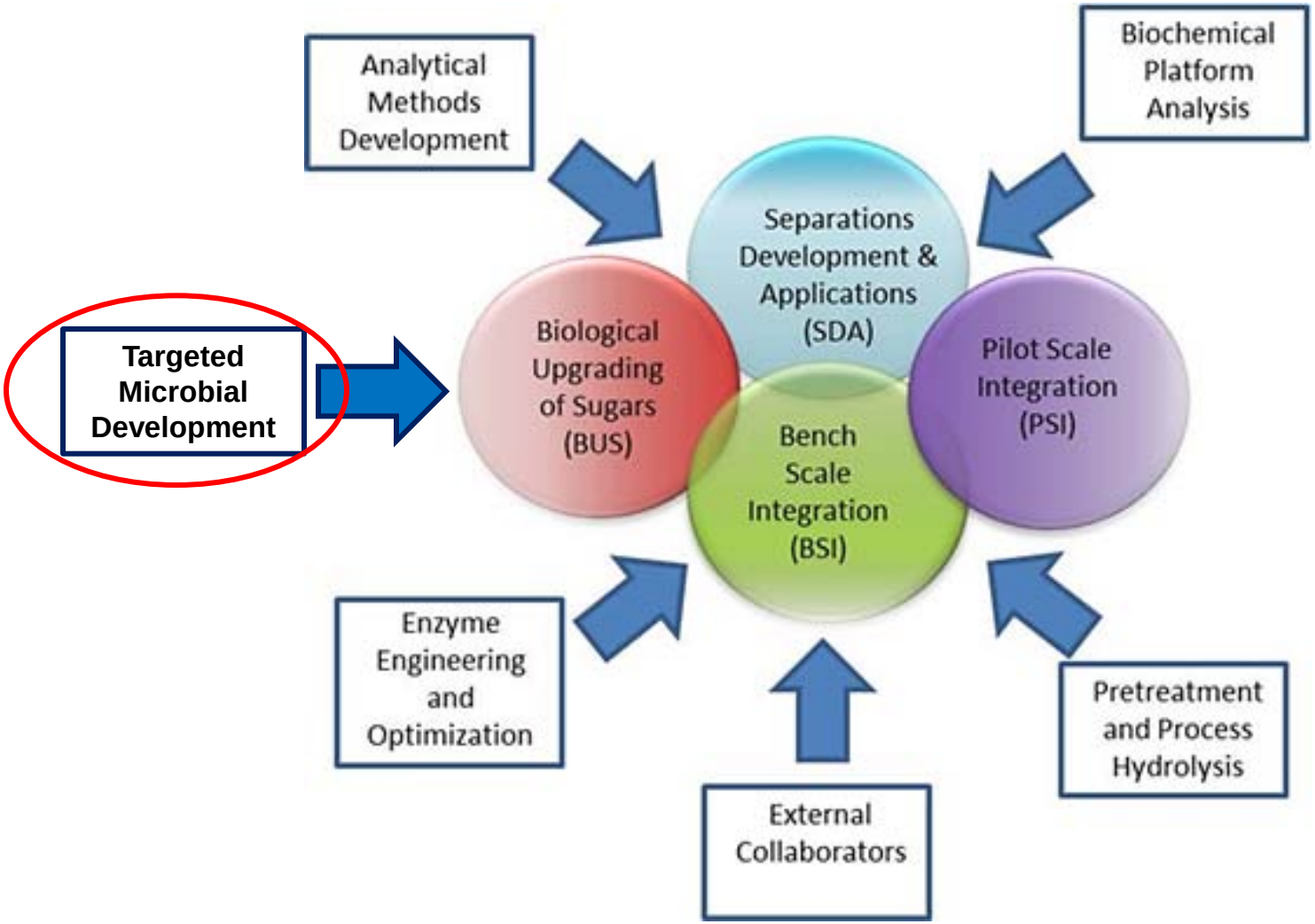
Budget

	Total Costs FY 10 –FY 12	FY 13 Costs	FY 14 Costs	Total Planned Funding (FY 15-Project End Date
DOE Funded				
Task 1	-	$500K	$500K	$4.0M ($500K FY15)
Task 2	-	-	-	$1.5M ($500K FY15)
Task 3	-	$1.5M	$1.7M	$7.2M ($900K FY15)
Project Cost Share (Comp.)*	0	0	0	0

Partners

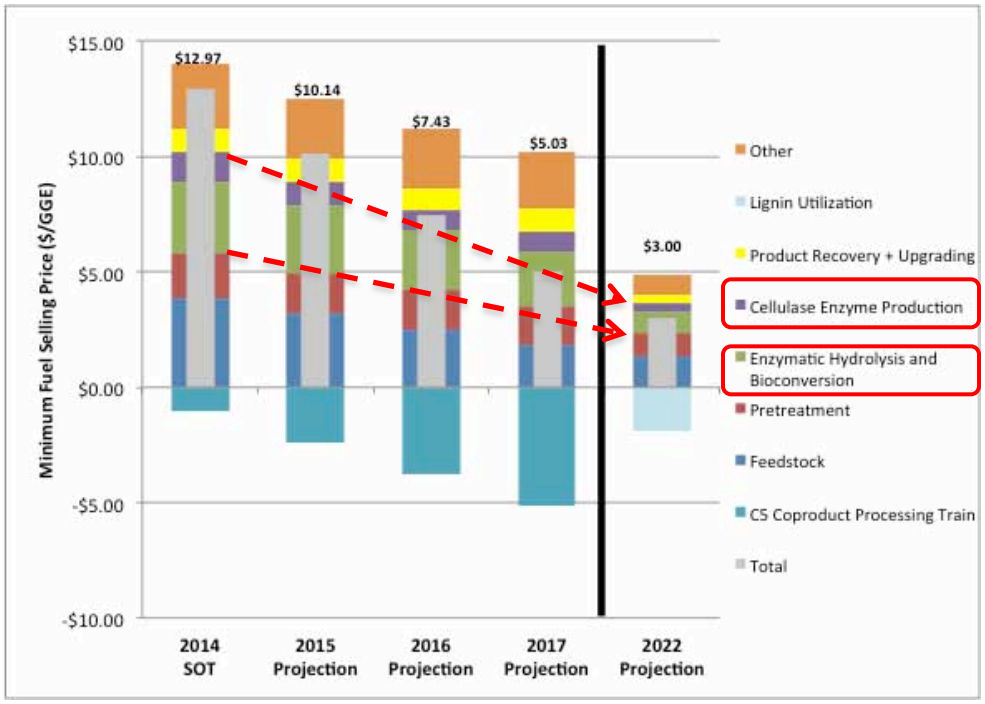
- BETO Internal: BUS (Biological Upgrading of Sugars); EEO (Enzyme Engineering & Optimization); PPH (Pretreatment & Process Hydrolysis; PI (Process Integration)
- Collaborations
  - Hal Alper (Univ of Texas at Austin)
  - Lydia Contreras (Univ of Texas at Austin)
  - MIT and Iowa State University for anaerobic bacterial fermentations

Biochemical Platform



1 - Project Overview

- NREL TEA shows that significant cost savings for advanced fuels lie in:
  - Product yield, titer and rate
  - Secreted products
  - Anaerobic culture
- This project consists of following Tasks:
  - Task 1 Anaerobic HC Intermediates from *Zymomonas mobilis*
  - Task 2 Anaerobic HC Intermediates from Other Bacteria
  - Task 3 Advanced Concepts for Producing HCs



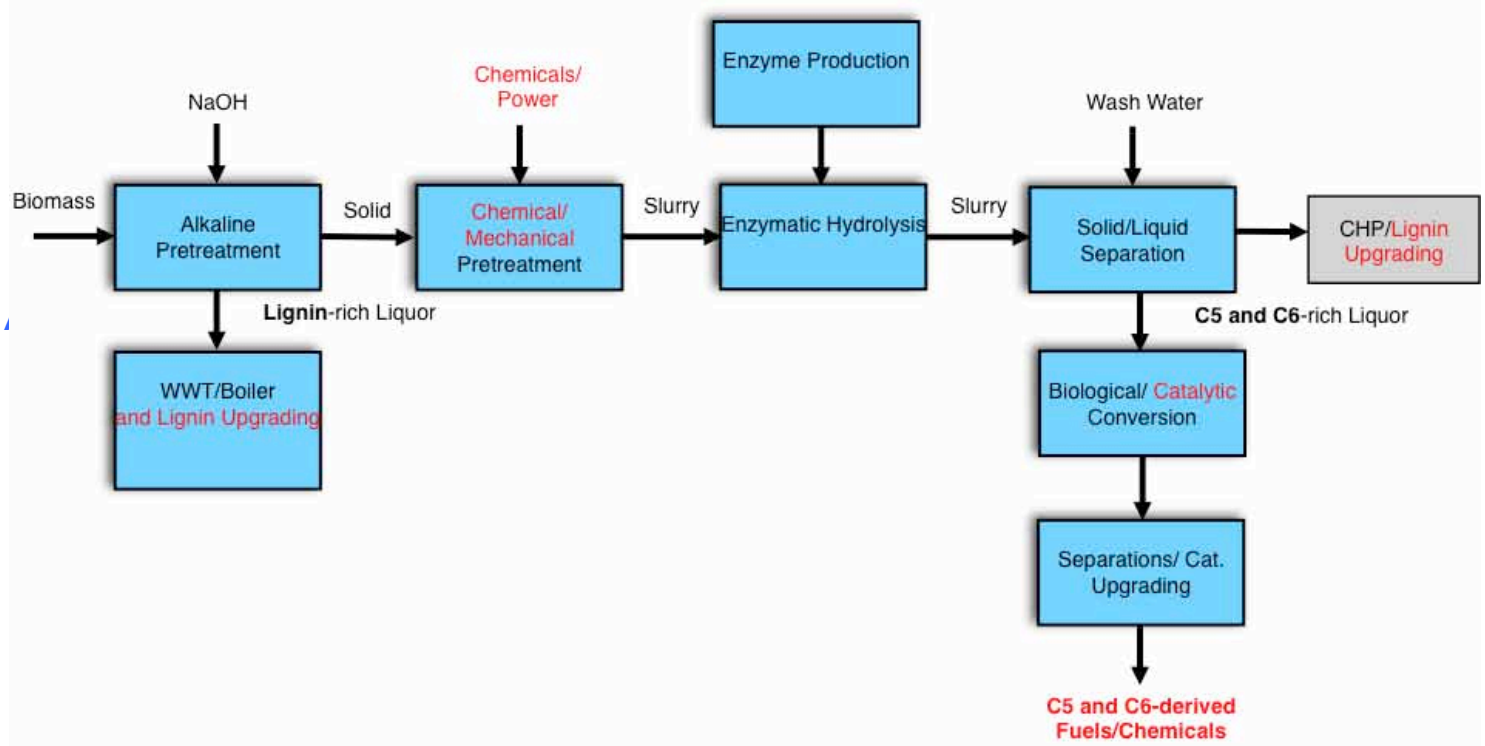
Primary targeting to reduce combined cost from cellulase enzyme production, enzymatic hydrolysis to bioconversion from $4.35/GGE (2014 SOT) to < $2/GGE (2022 Projection)

Supporting efforts in improving lipid production to meet the 2017 $5/GGE target.

1 - Project Overview

Tasks 1 and 2 focus on:

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

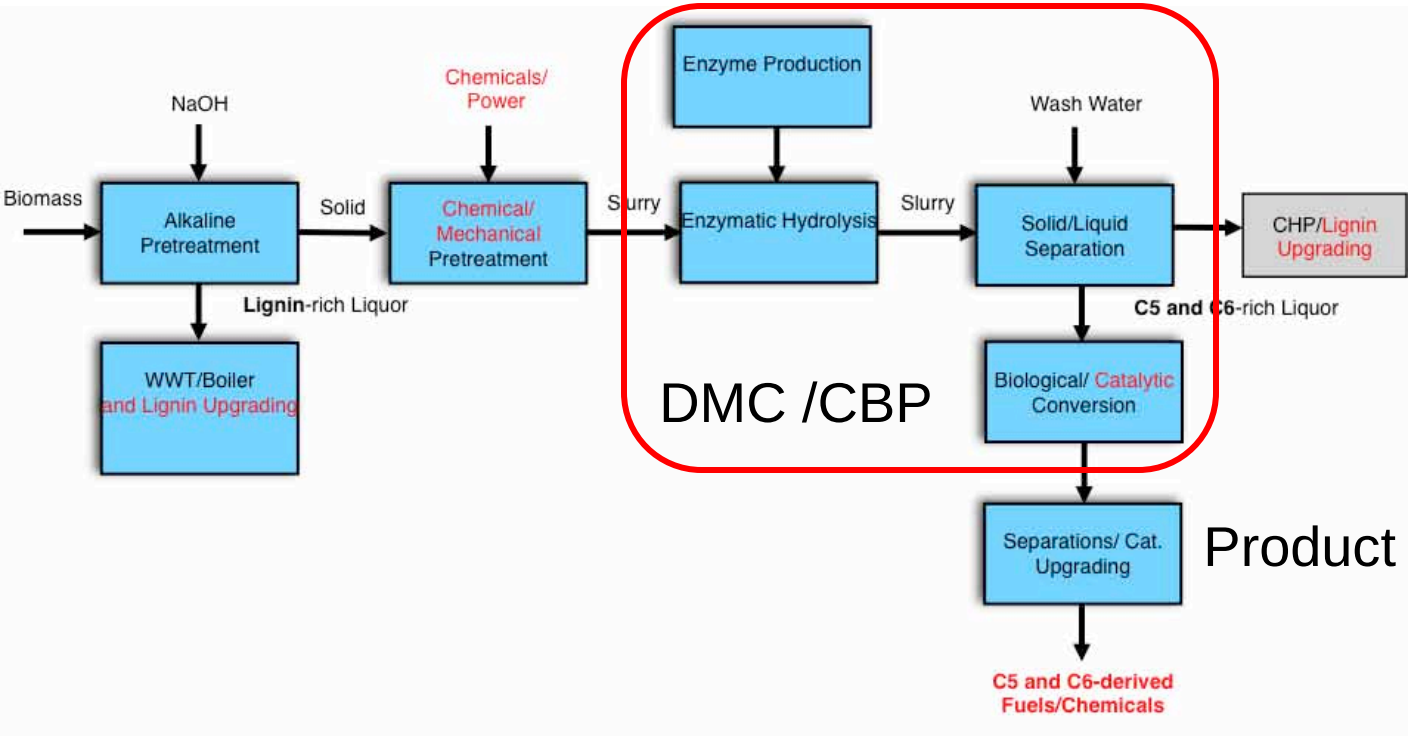


Process schematic for hydrocarbon production via anaerobic bioconversion

1 - Project Overview

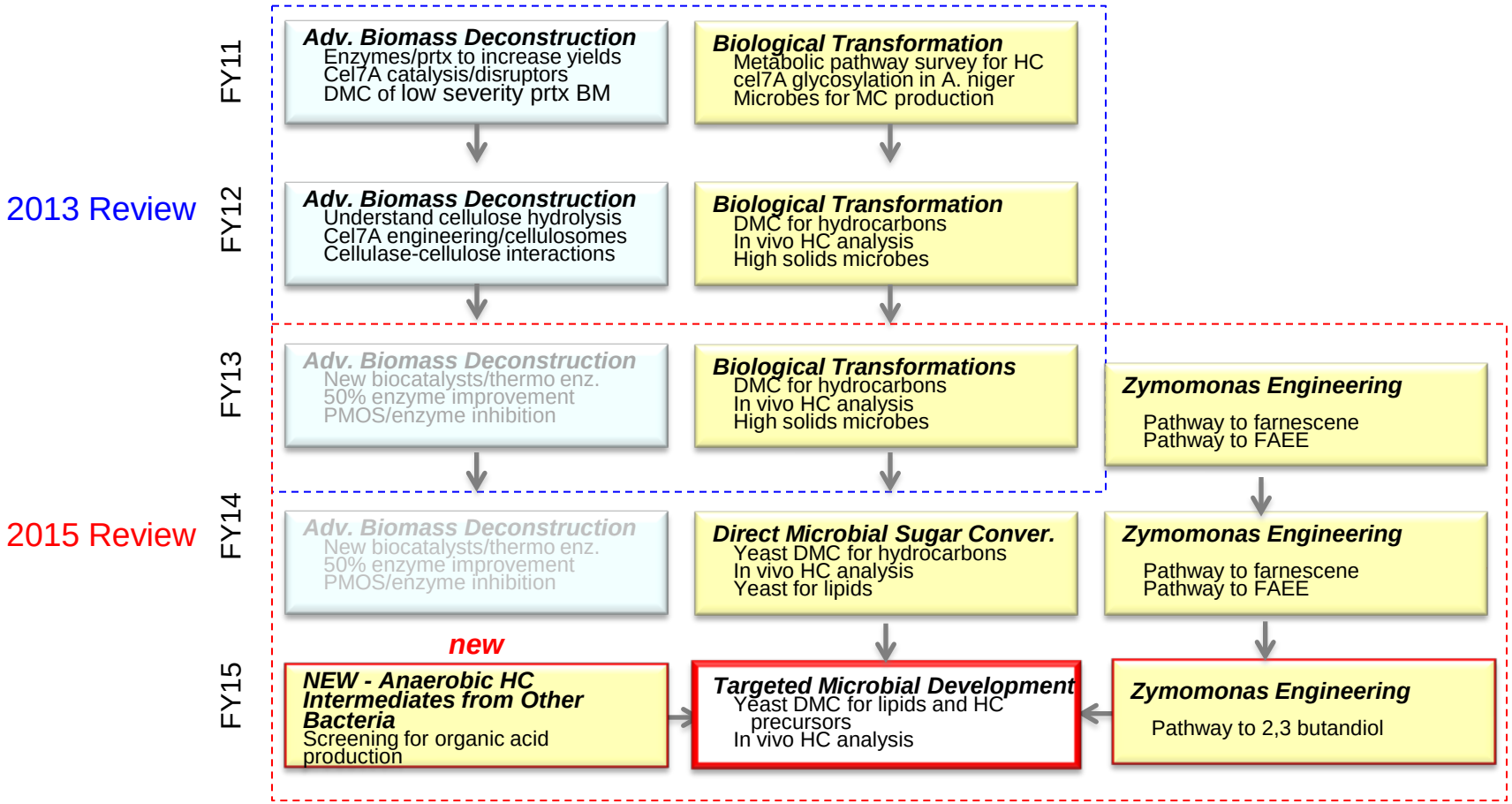
Task 3 focuses on:

- Direct Microbial Conversion (DMC) or Consolidated bioprocessing (CBP) for process cost reduction
- Efficient carbohydrate utilization
- Secreted fatty acid-based long chain hydrocarbon precursors readily for fuel product finishing.



Process schematic for hydrocarbon production via consolidated bioprocessing.

Evolution of Targeted Microbial Development



2 – Approach (Technical)

Task 1 - Anaerobic HC Intermediates from *Z. mobilis*

- *Describe critical success factors ...*
  - Demonstrate effective production of HC or HC intermediates in relevant rates and titers
  - Identify compatible co-product(s) suitable for 2022 goals
  - Product recovery strategies suitable for larger scale fermentation
  - Considerable of impact of bioproducts/fuels on existing markets
- *Explain the top 2-3 potential challenges…*
  - Ability to change the pathway direction to direct carbon flow to desired products
  - Ability to provide sufficient energy (e-) to support new or redirected pathways to synthesize needed energy intensive products.
  - Demonstration of industrial scale fermentations (DuPont bioethanol process)
  - Understand most cost effective conversion routes for BDO and related products to fuels

2 – Approach (Technical) cont.

Task 1 - Anaerobic HC Intermediates from *Z. mobilis*

- *Z. mobilis* is well known for both its high specific glucose uptake rate and rapid catabolism and is engineered to metabolize all the major biomass sugars.
  - Systematically studied for improving tolerance to inhibitors in biomass hydrolysates by applying the systems biology and genomic tools.
  - Improved xylose- and arabinose-utilizing *Z. mobilis* strain used in FY2012 demonstration for competitive ethanol production from cellulosic biomass at NREL pilot facility.
- Apply metabolic engineering and synthetic biology tools to engineer *Zymomonas* for synthesis of high-energy fuel molecules and/or intermediates that can be converted into hydrocarbon fuels.
  - Isoprenoid pathway (i.e. farnesene) FY13/14
  - Fatty acid pathway (i.e. FAEE) FY13/14
  - Pyruvate derived pathway (i.e. 2,3 butanediol) FY15
- Identify potential metabolic and energetic barriers, and further devise strategies to improve product yield and efficiency for the most promising fuel molecules and/or intermediates from both hexose and pentose sugars derived from plant biomass.

2 – Approach (Technical) cont

2015 – Targeted Microbial Development (TMD)

Task 1. Anaerobic HC Intermediates from *Z. mobilis*

- We will recruit three genes to channel pyruvate to acetolactate, acetoin and then 2,3 butanediol and further maximize its flux by considering various gene sources, optimizing the gene expression, and protein engineering if necessary. A strategy to knockout PDC to ethanol pathways will be pursued to eliminate the ethanol formation.
- BDO is a starting material for bulk chemicals and more importantly, it can be further chemically upgraded into jet fuel. Initial TEA is conducted to examine the feasibility of sugars (mixed C6/C5) to BDO and further catalytic upgrade to jet fuel and establish targets.
- Further optimization of xylose utilization to improve the rate and complete utilization will be also investigated. We will also further explore high value co-products to achieve the DOE BETO 2022 goals.

2 – Approach (Management)

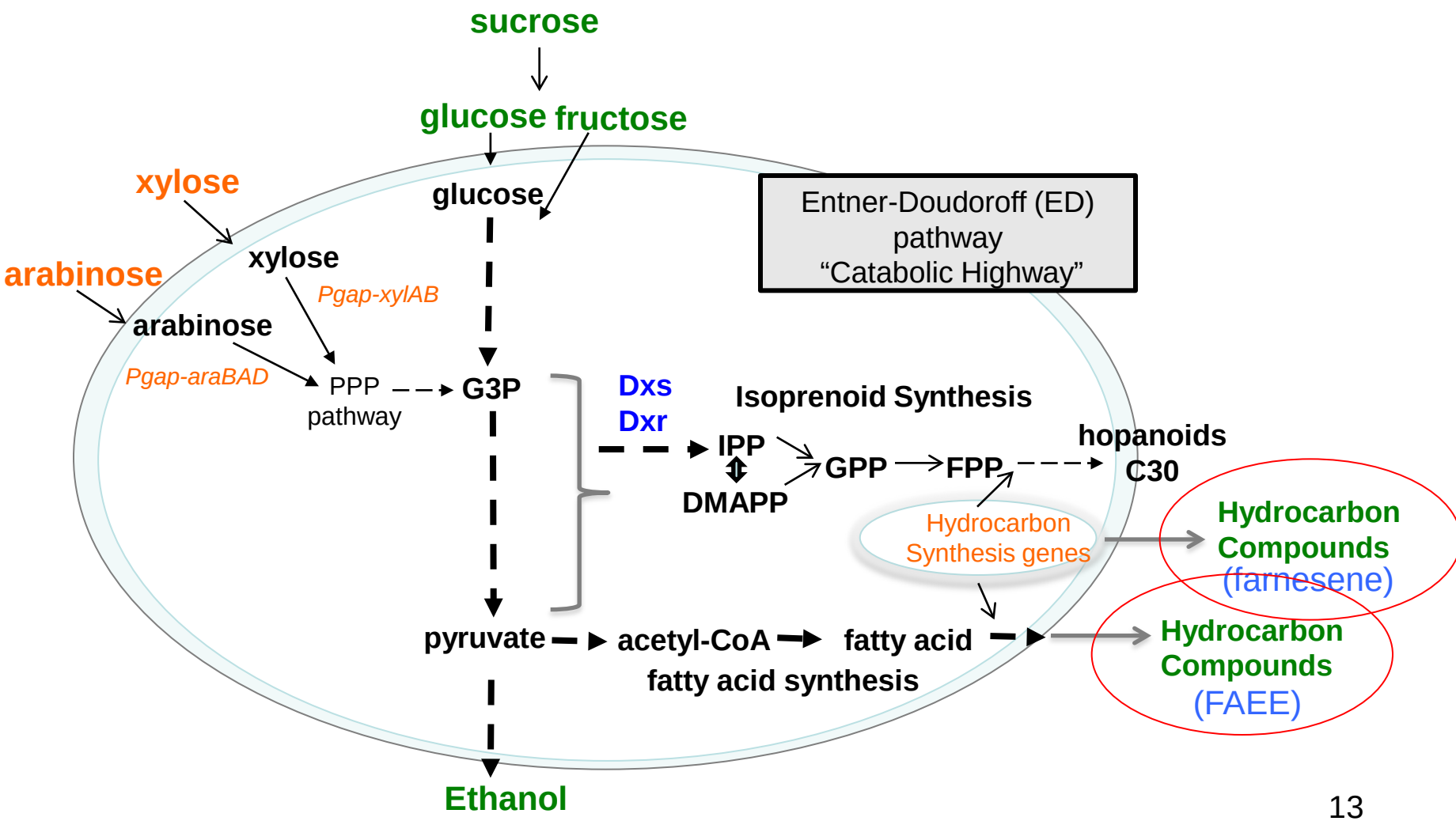
Task 1 - Anaerobic HC Intermediates from *Z. mobilis*

- *Describe critical success factors ...*
  - Set up key criteria for strain selection
    - Microorganism related capability (inherent properties)
    - Engineering potential (genetic tools, genome, systems biology knowledge and tools etc…
    - Hydrocarbon-producing potential – intracellular vs extracellular
    - Key performance metrics: **yield, titer, and rate**
    - Process advantages (fermentation and process options: aerobic vs anaerobic, liquid vs solid, batch, fed-batch, continuous, etc)
    - Easy of product recovery and upgrading
    - Biomass feedstock suitability (biomass sugars, particularly cellulose and xylan, xylose utilization capabilities, hydrolysate toxicity, etc..)
    - Compatibility with pretreatment methods
    - Potential to meet near term and long cost targets
    - Co-product potentials
    - Regulatory status for commercialization (GRAS)
    - Media requirements
- *Explain the top 2-3 potential challenges…*
  - Initial TEA analysis for evaluating technology gaps and its cost impact
- *Emphasize the structure of your approach ….*
  - Set up Regular, Quarterly, and Annual milestones with SMART performance metrics.
  - Go/No-Go decision point was made based on product titer as it is critical achieve process economics.

3 – Technical Accomplishments/Progress/Results

Task 1 Anaerobic HC Intermediates from *Z. mobilis*

Metabolic Engineering of *Zymomonas* for Hydrocarbon Production

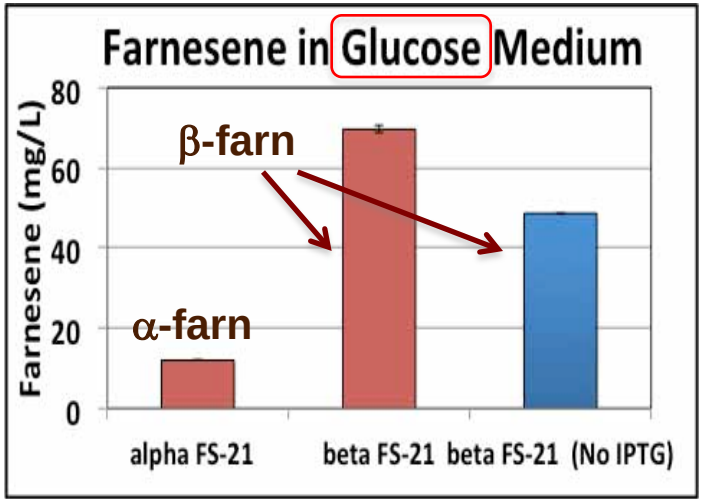


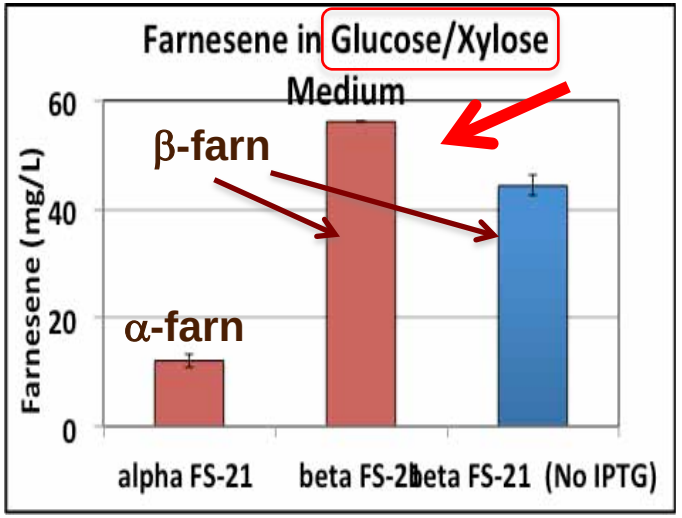
3 – Technical Accomplishments/Progress/Results

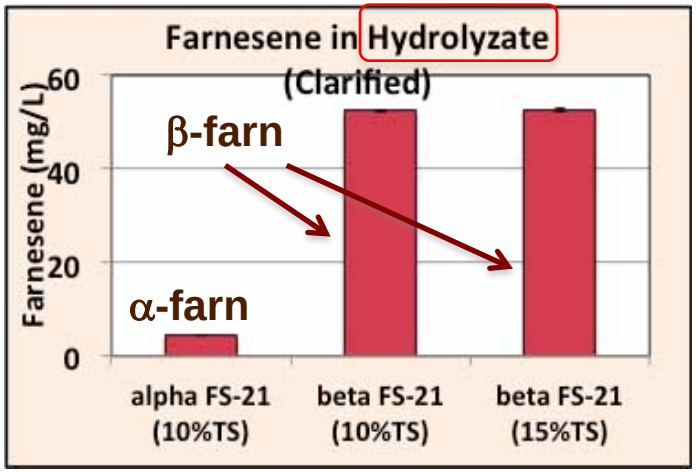
Task 1 Anaerobic HC Intermediates from *Z. mobilis*

D	Demonstrate production of 0.001% to 1% at least one top candidate high energy fuels and/or intermediates using *Zymomonas mobilis* from glucose and xylose	September 30, 2013

Farnesene Production in Fermenters (anaerobic)



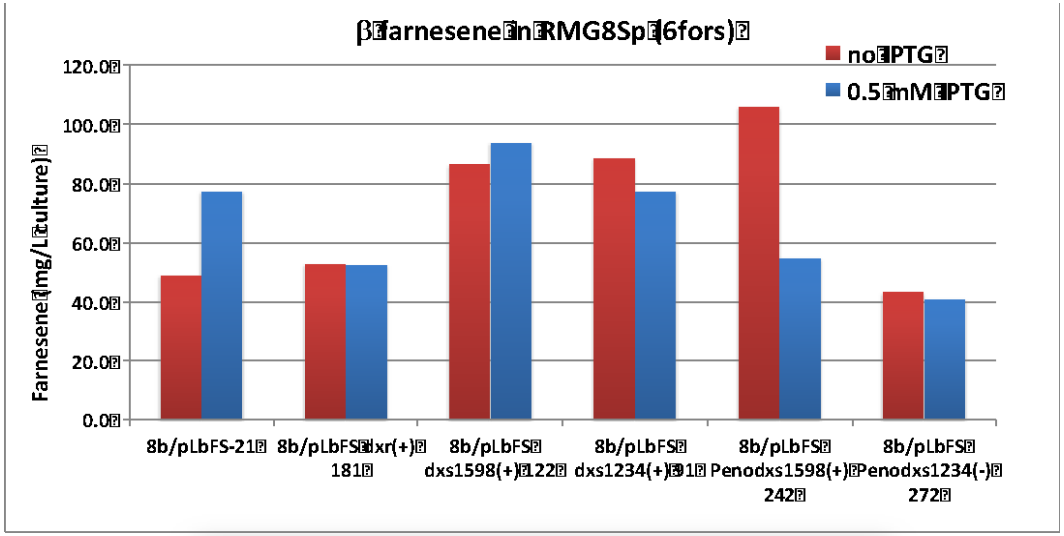




3 – Technical Accomplishments/Progress/Results

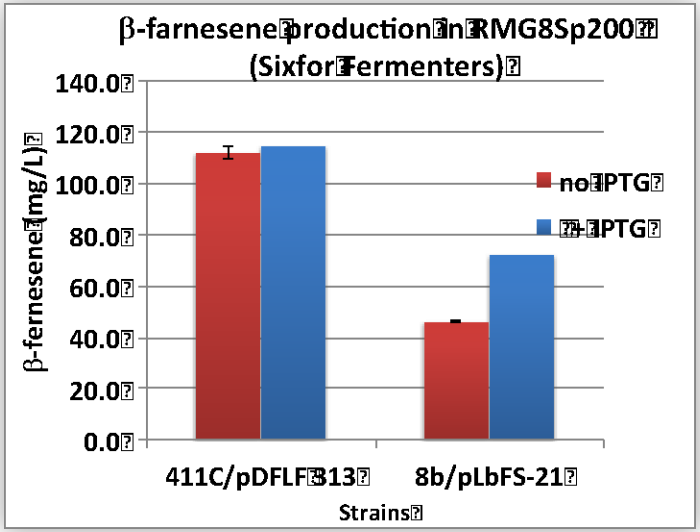
Task 1 Anaerobic HC Intermediates from *Z. mobilis*

Q4	9/30/2014	Stretch	Enhance production of hydrocarbon synthesis in Zymomonas mobilis by 3-fold of FY13 level by overexpressing and down-regulating key genes



- β-farnesene was increased via *dxs* or *dxr* overexpression in 8b (1.5 x).
- IPTG induction did not increase the farnesene level in strain 242.

Dxs – deoxy xylulose 5-phosphaste synthase
Dxr – deoxy xylulose 5-phosphaste reductoisomerase



We successfully improved the production of farnesene in *Z. mobilis* by overexpressing several key pathway genes, including *dxs*, *dxr*, *idi* and *fpp* in the isoprenoid pathway. A 1.6 to 2-fold improvement in titer and a better health of the organism was achieved when compared with the basal construct *Z. mobilis*/pLβFS that was constructed in FY13.

3 – Technical Accomplishments/Progress/Results

Task 1 Anaerobic HC Intermediates from *Z. mobilis*

Close Out! - Demonstrate *Zymomonas* production of farnesene

- **Significance and Impact**
  - Demonstrated the feasibility of advanced hydrocarbon production using *Zymomonas* under anaerobic condition, and the versatility of *Zymomonas* platform for heterologous high-value fuel/chemical production.
  - The detailed understanding of microbial responses to heterologous pathway engineering at molecular level will guide future metabolic engineering.
  - The tools and vectors established in this project will not only can be used for future work, but also help establish collaborative relationships with academic and industrial partners such as the one established with UT-Austin recently.
  - No-go decision was made not pursuing this direction due to low titer of these pathways.
  - A new strategy to redirect carbon from pyruvate to 2,3 butanediol (BDO) synthesis is proposed in FY15.
    - Product of the primary metabolism
    - Less toxic
    - Platform chemical
    - Can be catalytic upgraded to long chain hydrocarbons containing branch chain which is excellent for jet fuel application.

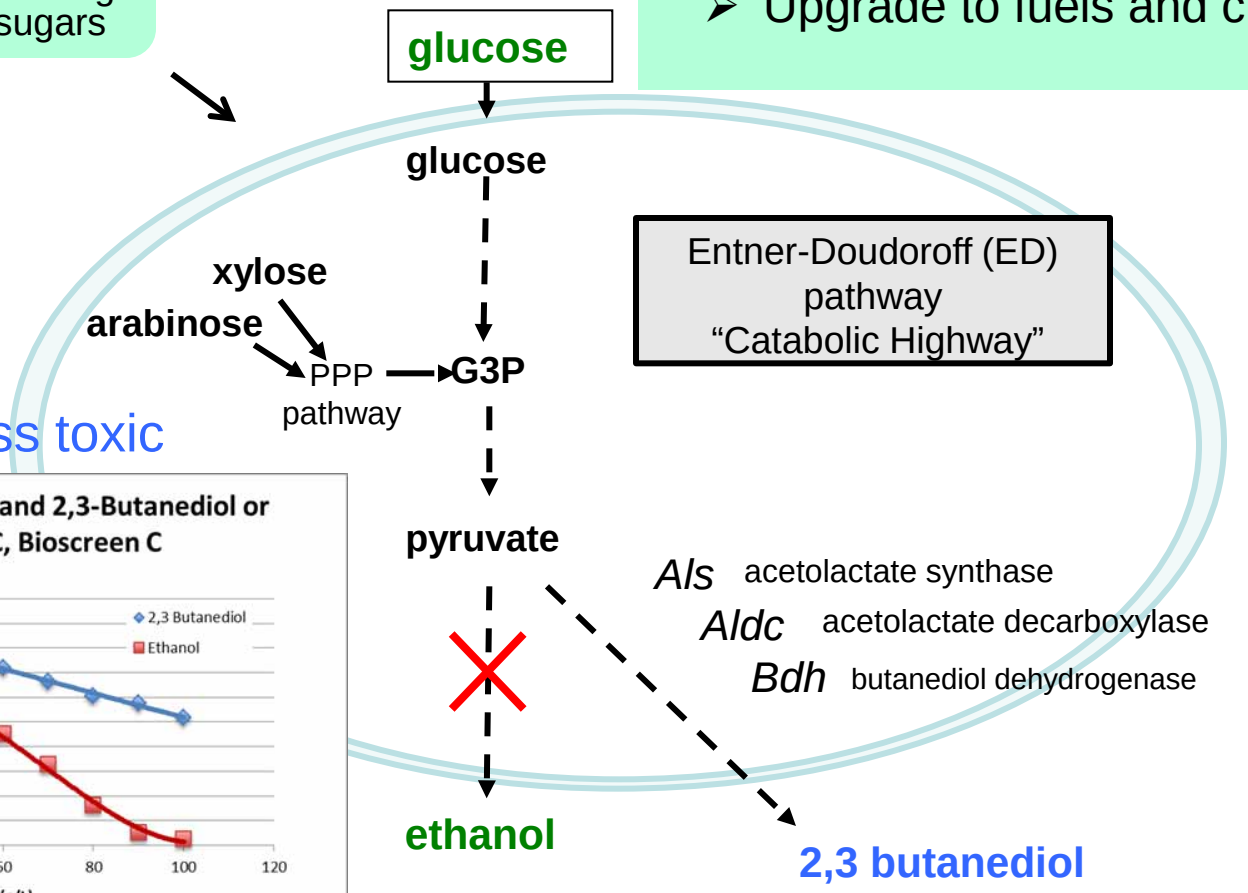
3 – Technical Accomplishments/Progress/Results

Task 1 Anaerobic HC Intermediates from *Z. mobilis*

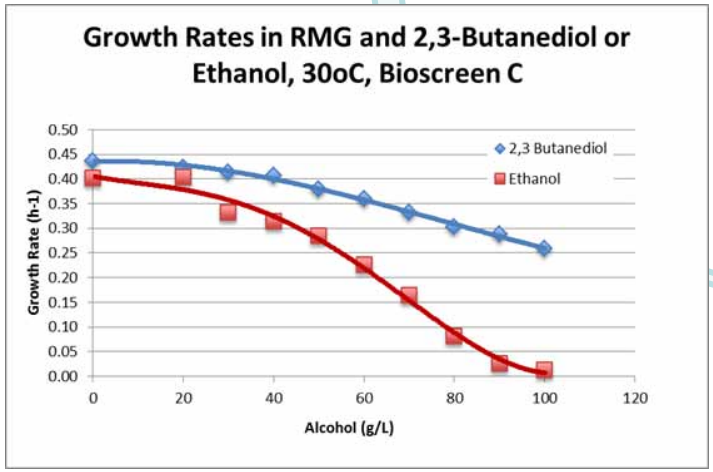
Metabolic Engineering of *Zymomonas* for 2,3 Butanediol production

DMR pretreatment and enzymatic hydrolysis containing mixed C5/C6 sugars

- Anaerobic process
- Potential high TYR
- Upgrade to fuels and chemicals



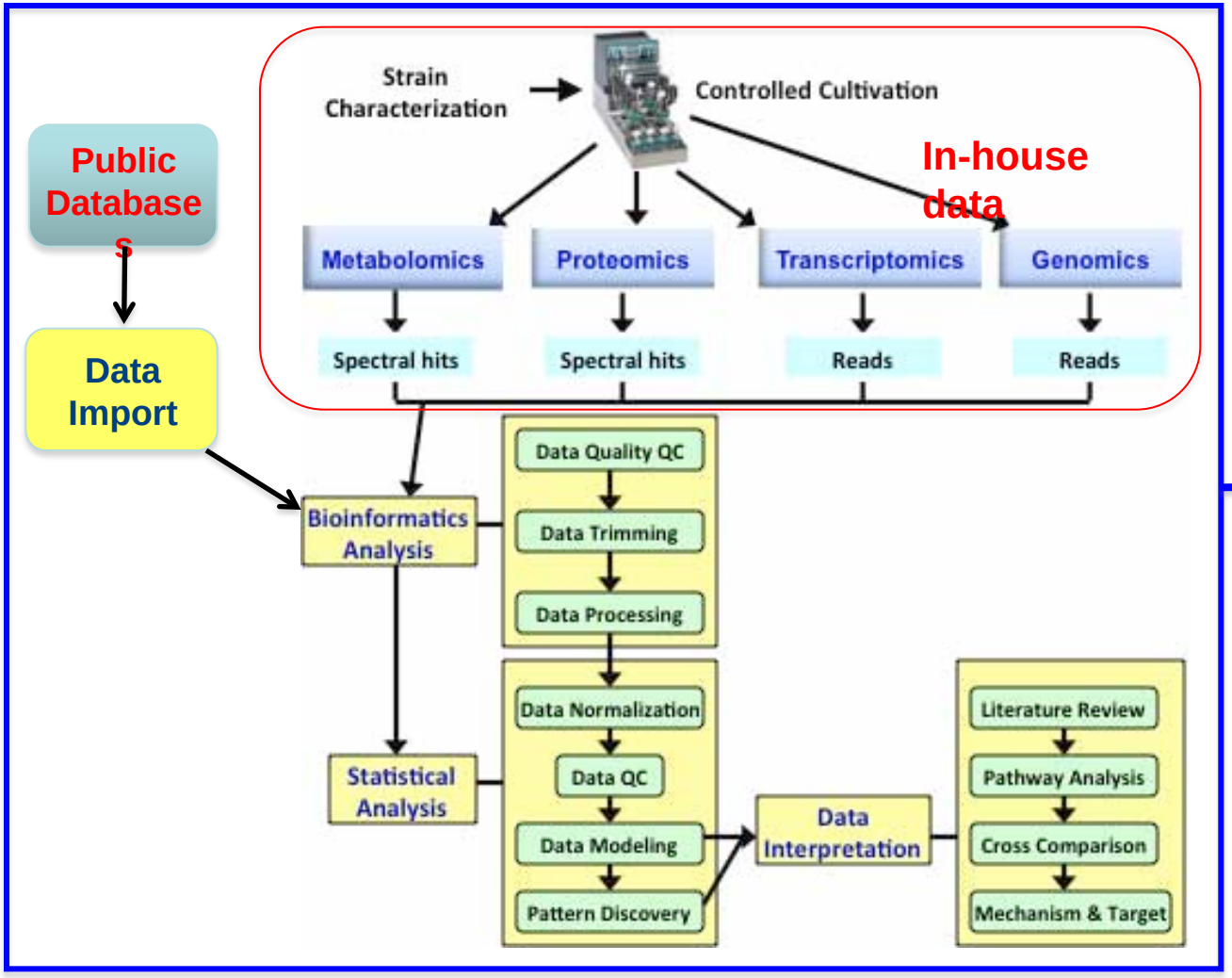
BDO is less toxic



3 – Technical Accomplishments/ Progress/Results

Task 1 Anaerobic HC Intermediates from *Z.mobilis*

In-house Bioinformatics/Systems Biology Platform
Guided Metabolic Engineering

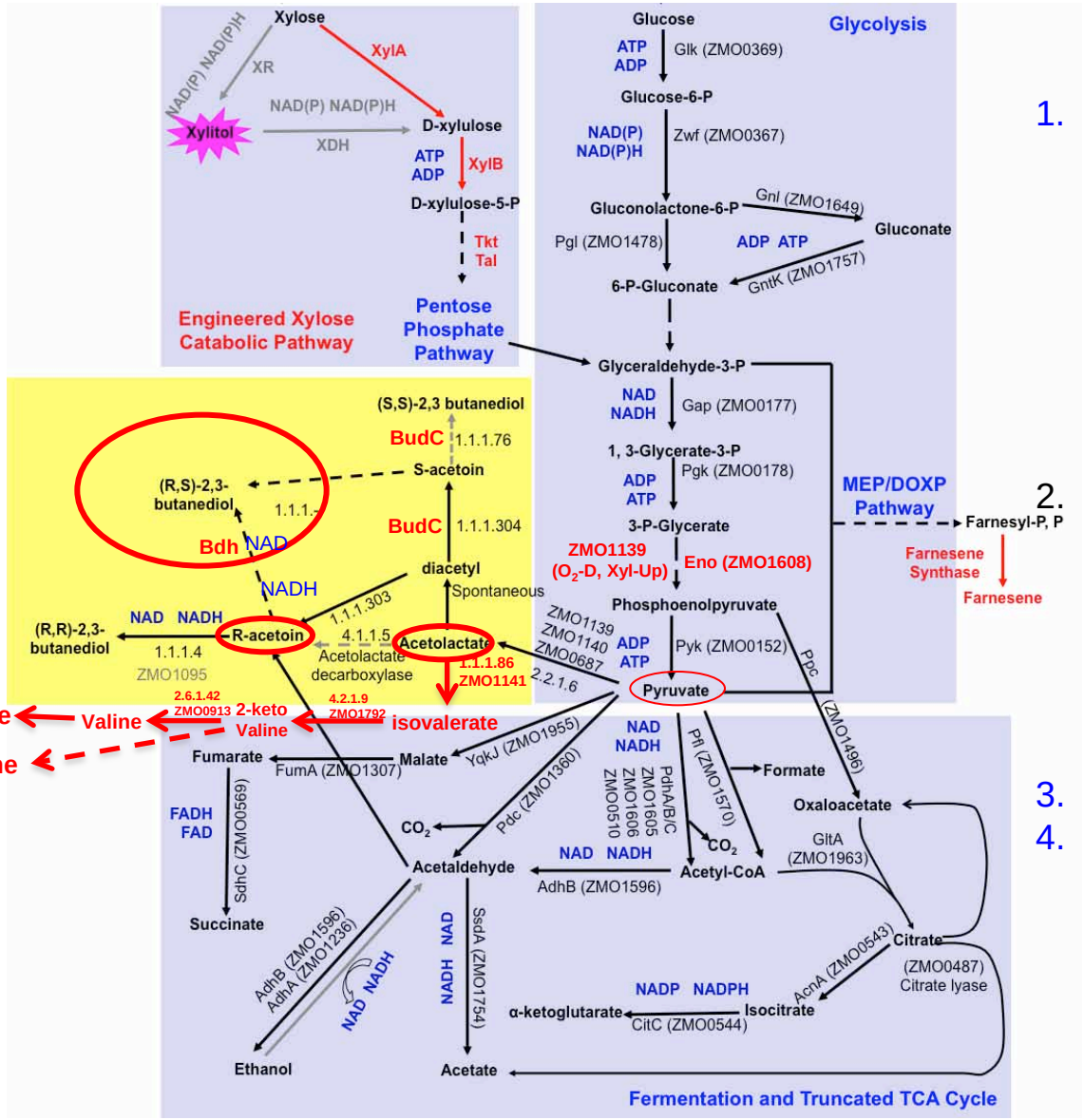


1. Characterize mutants.
2. Associate the genotype with phenotype.
3. Identify key metabolic targets (e.g. genes, promoters, signal peptides) for strain improvement.

3 – Technical Accomplishments/Progress/Results

Task 1 Anaerobic HC Intermediates from *Z. mobilis*

BDO Production in *Z. mobilis*: Pathway Construction and Analysis



1. Three *als* homologous genes exist, which are ZMO0687 (*budB*), ZMO1139 (*ilvB*), and ZMO1140 (*ilvH*). The high similarity among *budB*, *ilvB* and *pdc* indicates the potential competition among them, and the strategies to shift the carbon flux from pyruvate to acetolactate are needed.
2. ZMO1141 driving the acetolactate to branched amino acids biosynthesis has abundant transcript level indicating the need of a acetolactate decarboxylase (Aldc) with strong affinity to acetolactate.
3. No Aldc homologue in Z.
4. No butanediol dehydrogenase (Bdh) homologue identified in *Z. mobilis* indicating that it is essential to clone *bdh* for BDO production.

3 – Technical Accomplishments/Progress/Results

Task 1 Anaerobic HC Intermediates from *Z. mobilis*

Cutting-edge Toolbox Development to Expedite Metabolic Engineering Practice

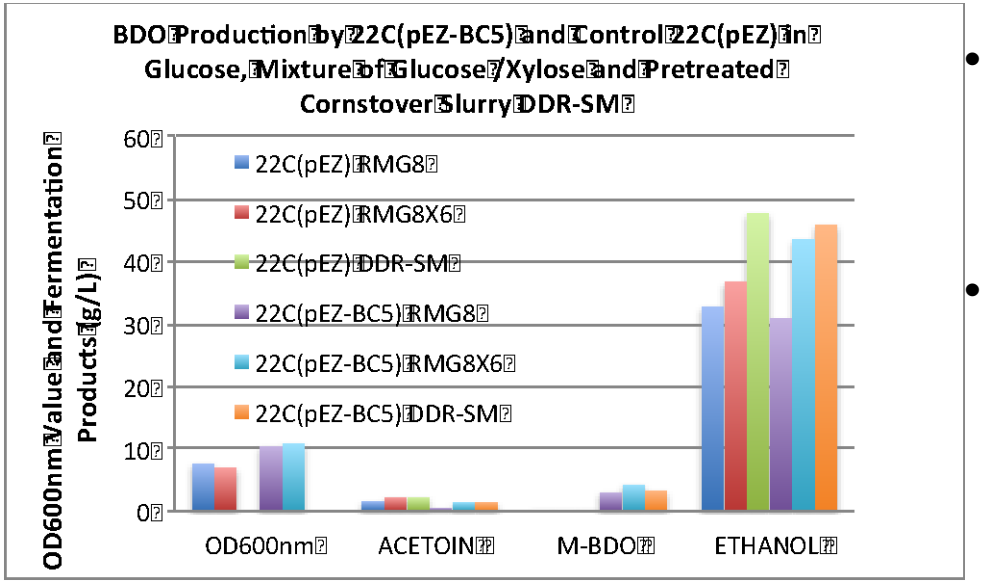
1. Customized rRNA depletion kit developed and further verified in various *Z. mobilis* wild-type 8b and mutant strains (e.g. 22C), reducing current RNA-Seq cost to **1/12th**.
2. A shuttle vector constructed and confirmed for *Z. mobilis*, which minimizes the vector size from 7.2-kb to 3-kb maximizing the pathway gene cloning capability.
3. Gibson and BioBricks assembly approaches were adapted for efficient pathway engineering.
4. Two reporter-gene systems controlled by tightly regulated promoters developed and inducible promoters, sRNAs were identified, which will be tested together with riboswitches identified for gene expression fine-tuning.
5. Genome editing tools are being explored including the emerging CRISPR/Cas9 system.

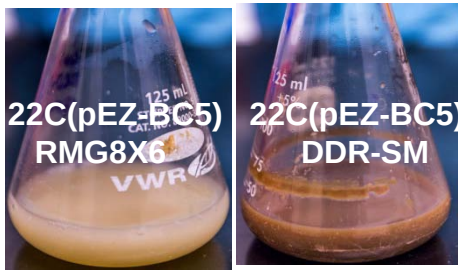
3 – Technical Accomplishments/Progress/Results

Task 1 Anaerobic HC Intermediates from *Z. mobilis*

Successful demonstration of BDO production in *Z. mobilis* in short time

- ✓ Butanediol biosynthesis pathway gene identification.
- ✓ Butanediol biosynthesis pathway engineering.
- ✓ Analytical method development and BDO production conditions.





- Three-gene construct had higher BOD titer than the two-gene construct in both 8b and 22C backgrounds
- BDO up to ~4 g/L can be produced in different conditions using pure sugar glucose, mixed sugar of glucose and xylose as well as DMR pretreated biomass sugars.
- Ethanol pathway knockout is ongoing

2 – Approach (Technical)

Task 2 - Anaerobic HC Intermediates from Other Bacteria

- *Describe critical success factors (technical, market, business) that will define technical and commercial viability*
  - Must successfully evaluate multiple anaerobic bacteria that naturally produce single or mixed products.
  - These bacteria must be amenable to separations and catalytic upgrading.
- *Explain the top 2-3 potential challenges (technical and non-technical) to be overcome for achieving successful project results*
  - To achieve high yields in the biological step
  - The co-design of organism and target product
  - Cost-effective separations
  - The needed strain improvements and adaptations may not be readily achievable
  - The cost economics for the deacetylation/disk refining (DDR) pretreatment process may not prove to be viable at large scale.

2 – Approach (Management)

Task 2 Anaerobic HC Intermediates from Other Bacteria

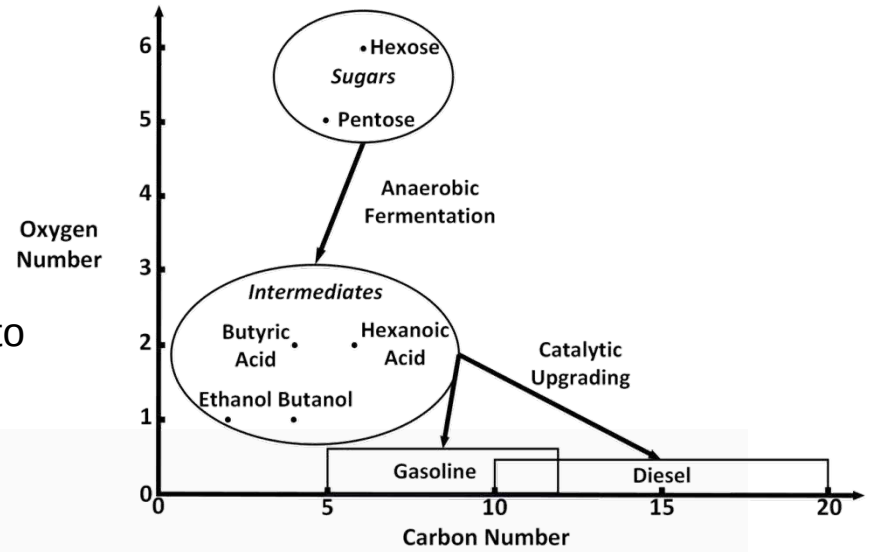
- *Describe critical success factors ...*
  - Evaluation of multiple anaerobic bacteria that produce single or mixed products that are amenable to separations and catalytic upgrading. Specific emphasis will be placed on short chain organic acids, aldehydes, ketones, and alcohols.
  - Evaluation and down selection will first be based on literature data, and subsequent evaluations will be based on bench scale batch fermentations using both mock and biomass derived mixed C5/C6 sugar streams (e.g., from the deacetylation/disk (DDR) refining pretreatment and enzymatic hydrolysis deconstruction approach being developed in the Pretreatment and Process Hydrolysis project).
- *Explain the top 2-3 potential challenges…*
  - Data from this task must be evaluated in TEA models to examine the initial viability of hybrid biological-catalytic processes for the production of HC fuels towards the DOE BETO 2022 goals.
- *Emphasize the structure of your approach ….*
  - Necessary strain improvements and adaptation will be pursued through the application of standard laboratory mutagenesis and evolution methods.
  - The goals will be met using a milestone assessment system (Regular, Quarterly, and Annual) as well as a Go/No-Go decision point.

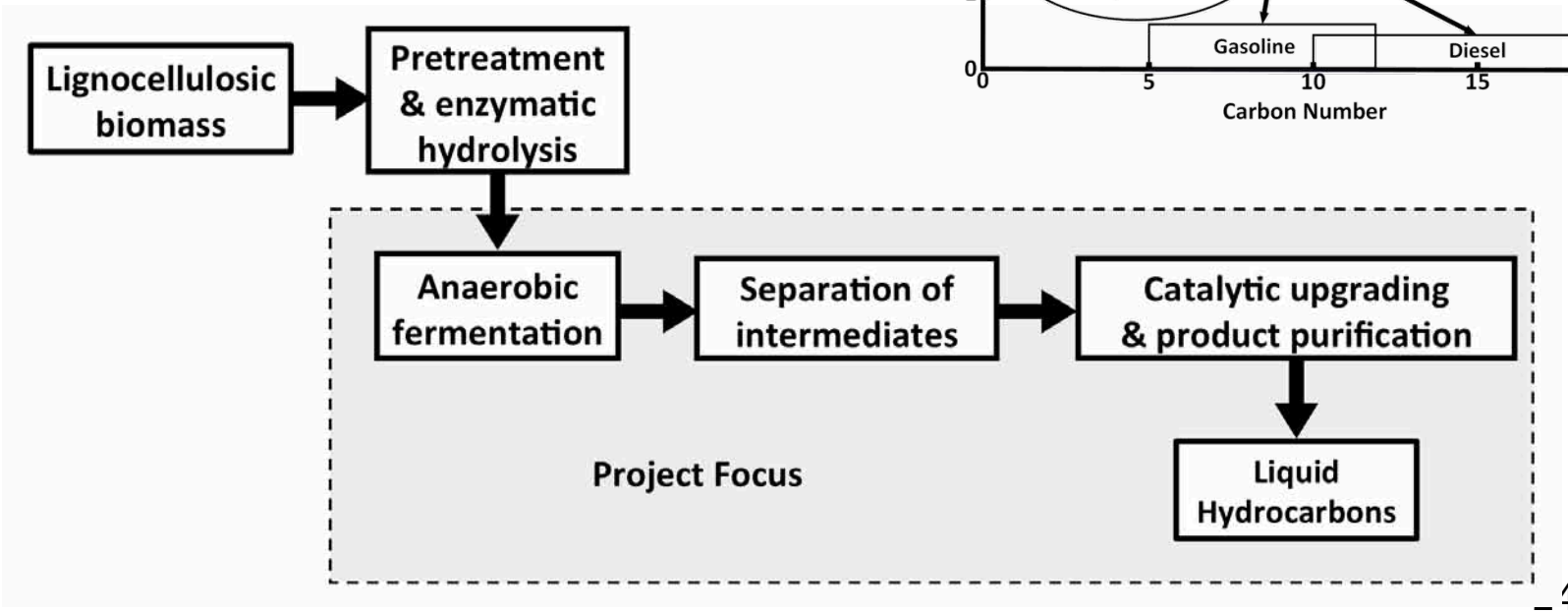
3 – Technical Accomplishments/ Progress/Results

Task 2 Anaerobic HC Intermediates from Other Bacteria

Ongoing work:

- Down-selected to hexanoic acid and butyric acid for initial strain evaluations
- Setting up continuous-loop fermentation for immobilized cell fermentors with *ex situ* separations
- Initiating molecular biology efforts to put HA production into a low-pH fermenting yeast strain to ease separations



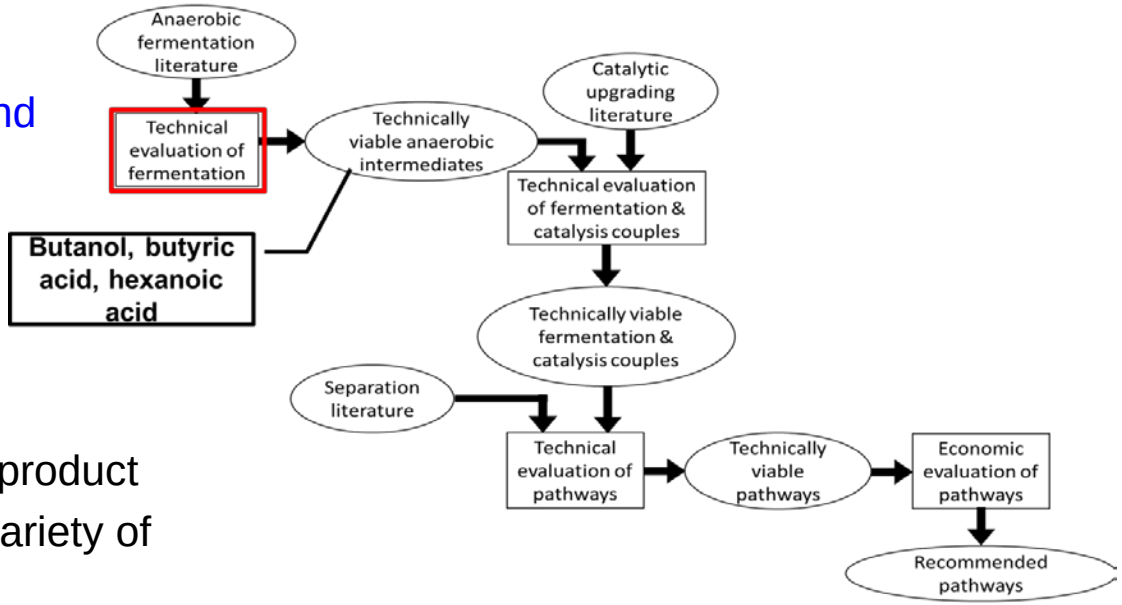


3 – Technical Accomplishments/ Progress/Results

Task 2 Anaerobic HC Intermediates from Other Bacteria

Pathway criteria

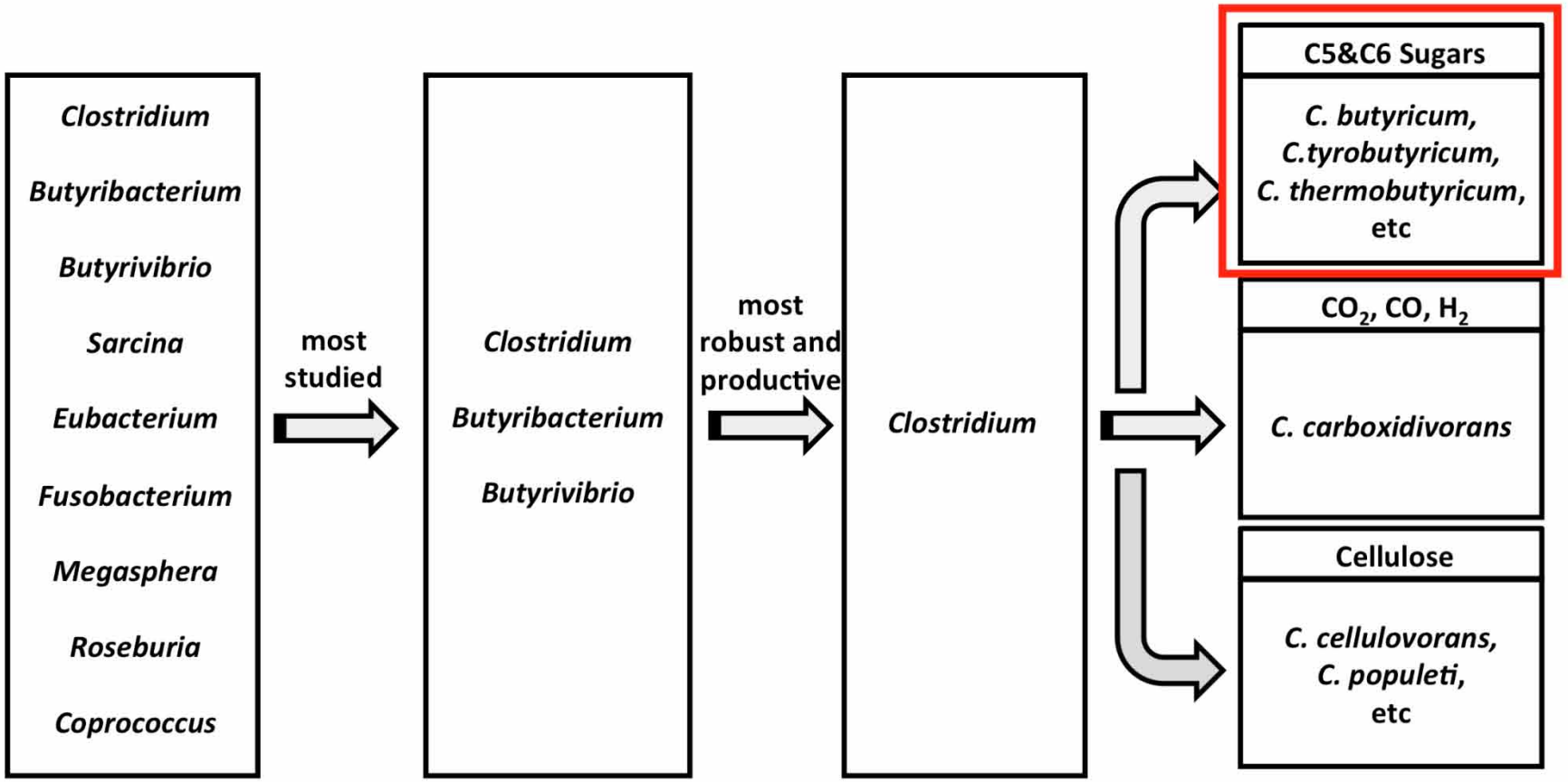
- Molecule quality of product
  - Chain length distribution
  - Energy content (volume and mass basis)
  - Oxygen content
- Overall carbon efficiency
- Overall hydrogen consumption
- Gallons of product/tons of feed
- Total production cost/gallon of product
- Substrate - ability to accept a variety of biomass feeds
- Toxicity & scale-ability
- Commercial readiness - SOT (lab/pilot/production scale), extent of testing (days, months, etc.)



3 – Technical Accomplishments/ Progress/Results

Task 2 Anaerobic HC Intermediates from Other Bacteria

Approach: Example of host down-selection approach – butyric acid



3 – Technical Accomplishments/ Progress/Results

Task 2 Anaerobic HC Intermediates from Other Bacteria

Hexanoic acid

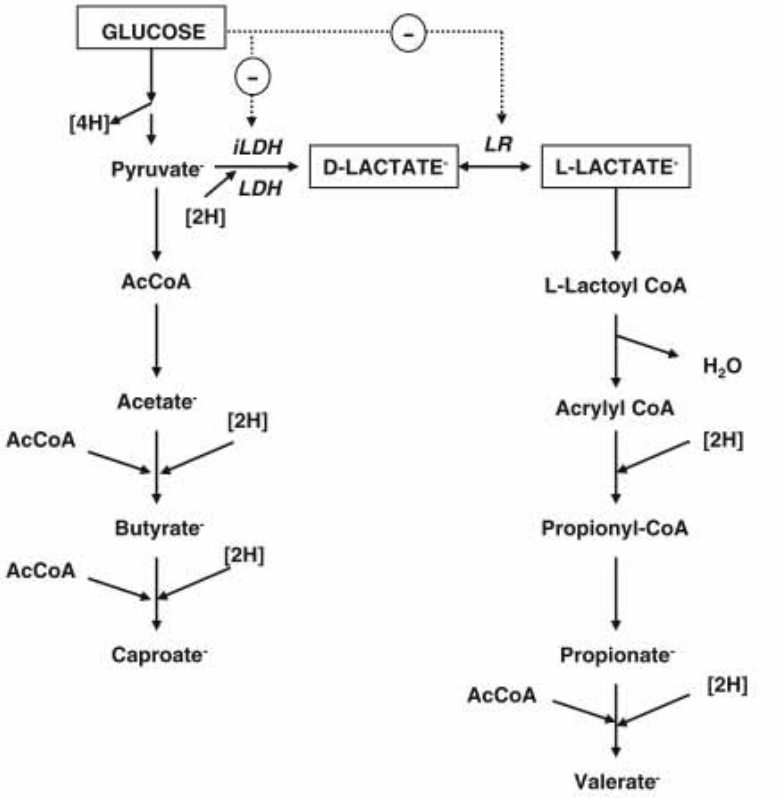


Fig. 1 Fermentation pathway of lactate and glucose by *M. elsdenii*. Glucose is known to repress synthesis of both lactate racemase (*LR*) and an NAD-independent lactate dehydrogenase (*iLDH*), but not the NAD-dependent lactate dehydrogenase (*LDH*). Adapted from Hino and Kuroda (1993)

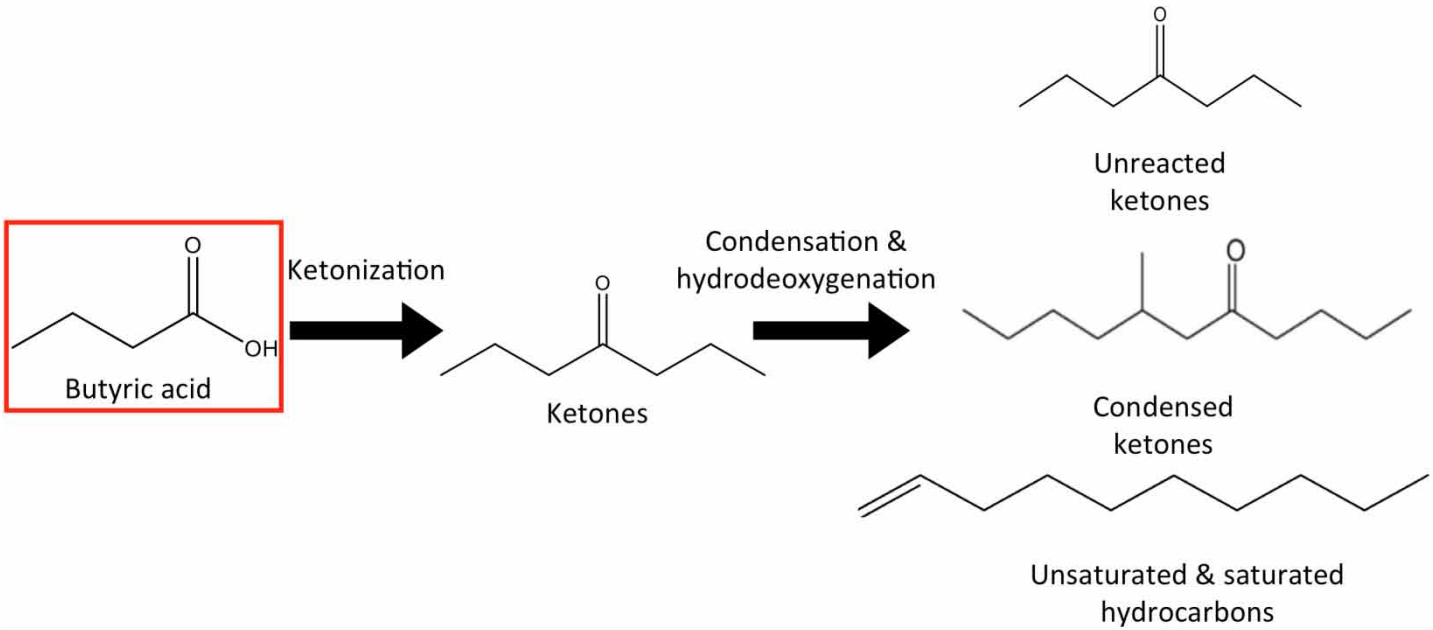
Ongoing work:

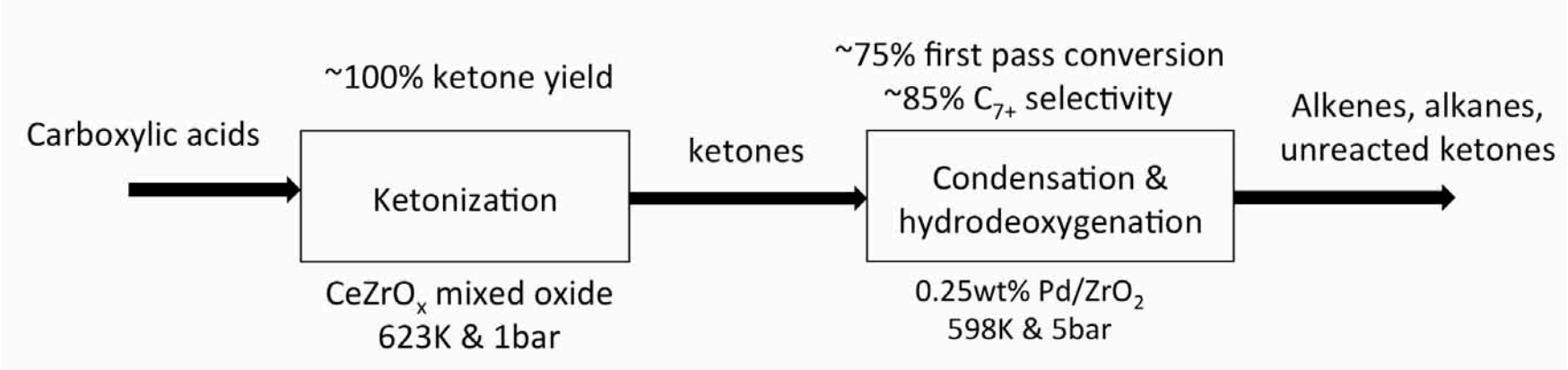
- Genes have been identified by collaborators for HA production
- Implementing these pathways into low pH fermenting yeast strains now (pH ~3)
- Solubility of HA is 10 g/L, enables *in situ* phase separation

3 – Technical Accomplishments/ Progress/Results

Task 2 Anaerobic HC Intermediates from Other Bacteria

Example of catalytic approach for acid upgrading to HC fuels





3 – Technical Accomplishments/ Progress/Results

Task 2 Anaerobic HC Intermediates from Other Bacteria

NREL fermentation and catalysis Infrastructure

Capabilities now online with immobilized bio-reactors

Single-bed reactors for evaluating chemistry in flow to obtain process relevant data

2 – Approach (Technical)

Task 3 Advanced Concepts for Producing HCs

- *Describe critical success factors ….*
  - Identify compatible co-product(s) suitable for 2022 goals.
  - Production of suitable cellulase titers in culture broth for DMC strains (note that for advanced biofuels these parameters are not known but are likely lower than needed for enzyme production goals).
  - Demonstration of growth on biomass with promising fuels production (many parameters can be optimized in out years, including particle size, product titers and production rates).
- *Explain the top 2-3 potential challenges ….*
  - Ability to change the pathway direction to direct carbon flow and sufficient energy to desired products.
  - The IP landscape for large scale DMC processes is somewhat unclear.
  - The optimal product recovery modality and range of product inhibition effects on DMC strains is not known.

2 – Approach (Technical) cont

2015 – Targeted Microbial Development (TMD)

Task 3. Advanced Concepts for Producing HCs

- Investigate novel concepts that can drastically reduce the cost of producing HCs. Critical technical challenges include: high carbon yield from glucose; as well as xylose, product secretion/recovery, and the high cost of cellulase/hemicellulose enzymes. Increased high carbon yield by incorporating phosphoketolase and enhanced lipid content by optimizing metabolic pathway enzymes and pathway regulation will be pursued in oleaginous yeast; including *Lipomyces* as we recently demonstrated transformation of this wild type strain, in addition to the engineered *Yarrowia* strain.
- Understanding of metabolic bottlenecks of the pathways for maximum sugar to hydrocarbon yield will be also pursued in collaboration with PNNL (on-going).
- Pathways and factors promoting extracellular production of HCs or facilitating recovery of products will be investigated and their impact on production cost will be evaluated in oleaginous yeast strains.
- Direct microbial sugar conversion (DMC) by aerobic (*Yarrowia* sp. and *Lipomyces*, *Saccharomyces*) will be evaluated for potential reduction of cost for HC intermediates.
- We will also identify suitable HC intermediates with high energy, easy separations, and/or high carbon production yields. TEA analysis of the potential impact of these technical barriers will be conducted to provide guidance for future work.

2 – Approach (Management)

Task 3 Advanced Concepts for Producing HCs

- *Describe critical success factors (technical, market, business) that will define technical and commercial viability*
  - Demonstrate technical and market pull for HCs and other drop in fuels from biomass.
  - Product recovery and cleanup are factors to be clarified in near term.
- *Explain the top 2-3 potential challenges (technical and non-technical) to be overcome for achieving successful project results*
  - Mixed perception of commercial readiness for DMC processes - Mascoma successes and the goals of BESC* should be considered.
  - Understand the cost sensitivities of yeast and possibly fungal (aerobic) fermentations in large fermenters (i.e., >500,000 gal)
- *Emphasize the structure of your approach ….*
  - Our approach is to use a basic RACI management plan which assigns work to researchers based on the milestone structure. Milestones are Regular, Quarterly, and Annual, with several SMART milestones identified.
  - Aspects of impacting DOE Barriers for both FY2017 and FY2022 technology are planned.

**The $25M/yr DOE BER BioEnergy Research Center (BESC) is based on DMC to ethanol*

3 – Technical Accomplishments/ Progress/Results

Task 3 – Advanced Concepts for Producing HCs

FY13

Type	Title/Performance Measure	Due Date
D	Demonstrate production of 0.1-1% hydrocarbon production of *T. reesei* strain growing on Avicel.	9/30/2013
E	Demonstrate that the heterologous enzymes in the hydrocarbon production pathway in *T. reesei* under examination are active.	5/30/2013
D	Demonstrate expression of hemicellulase and accessory enzymes in lipogenic yeast	8/30/2013
E	Incorporate new and improved glycoside hydrolases provided by the ABD subtask into lipogenic yeast.	7/30/2013

FY14

Qtr	Due Date	Type	Milestones, Deliverables, or Go/No-Go Decision	Decision Criteria
Q1	12/31/2013	Regular	Develop a transformation system using reusable selection marker URA3 in Yarrowia	
Q2	3/31/2014	Regular	Identify three target genes/enzymes that affect hydrocarbon production in Trichoderma	
Q3	6/30/2014	Regular	Co-express microbial cellulase genes in Yarrowia in various combinations to enable utilization of cellulolytic substrates (Avicel), such as: CBHI + (EGII or E1), CBHI+EGII+CBHII, CBHI+E1+CBHII. Exploit new genes as they become available during the year.	
Q4	9/30/2014	Stretch	Improve farnesene production in T. reesei by 2-fold relative to FY13	

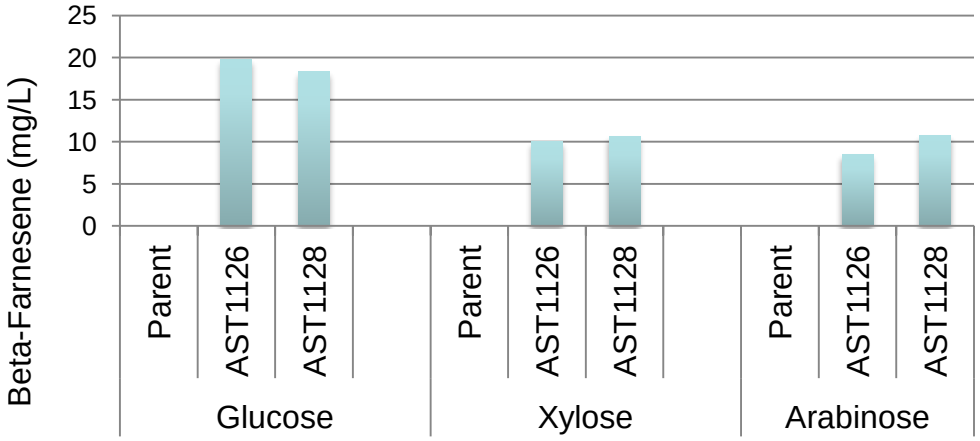
Focus on *Yarrowia/Saccharomyces* initially and move into *Lipomyces* in future

3 – Technical Accomplishments/ Progress/Results

Task 3 – Advanced Concepts for Producing HCs

Close Out! - Demonstrate direct microbial production of farnesene

- ✓ Production from 5% glucose, xylose and arabinose by *T. reesei* QM6a transformants
- ✓ Farnesene production from 2% Avicel by engineered *T. reesei* strains

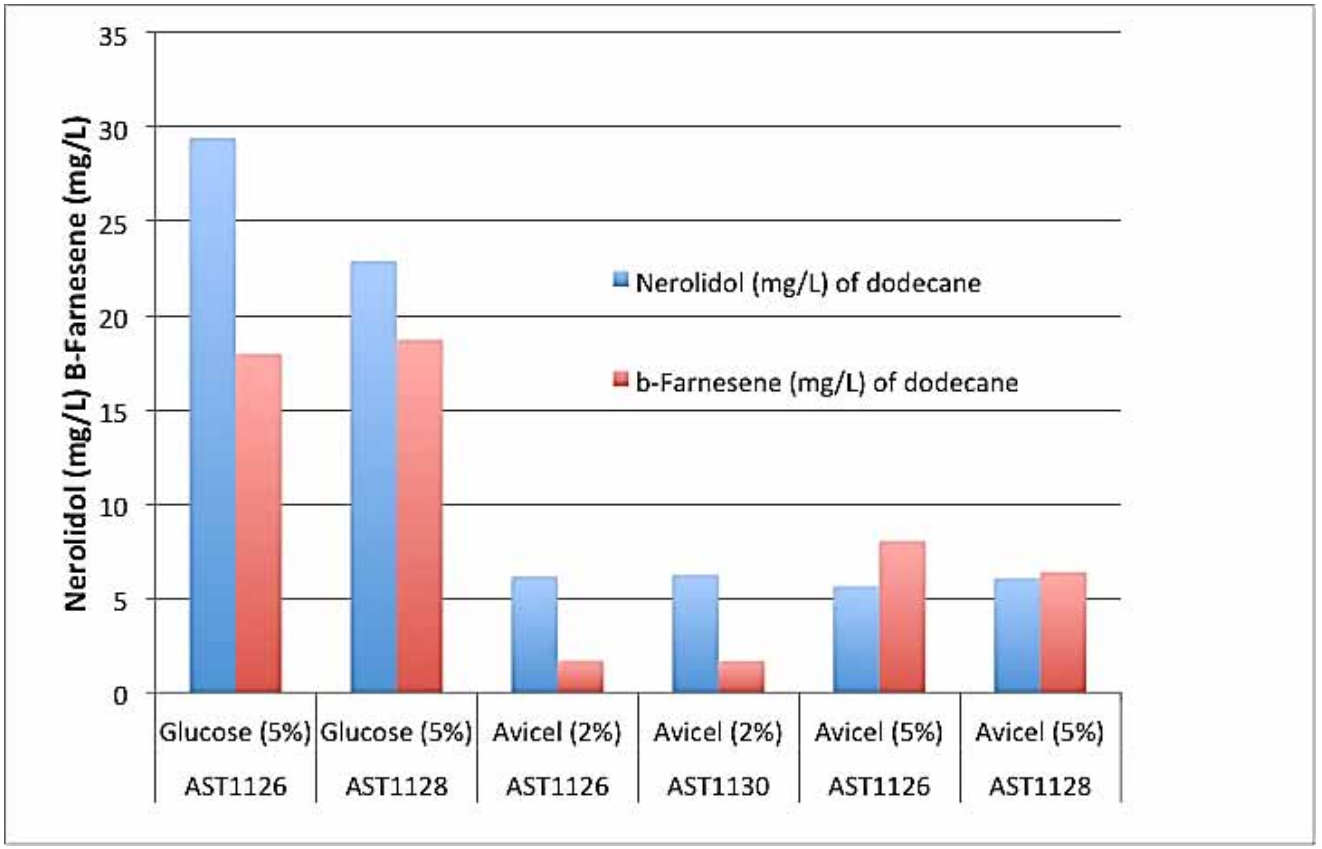


Strain	Parent strain	β-Farnesene in dodecane layer from 2% Avecel
		mg/L
QM6a		0
Rut-C30		0
AST1126	QM6a	1.67
AST1130	Rut-C30	1.64

3 – Technical Accomplishments/ Progress/Results

Task 3 – Advanced Concepts for Producing HCs

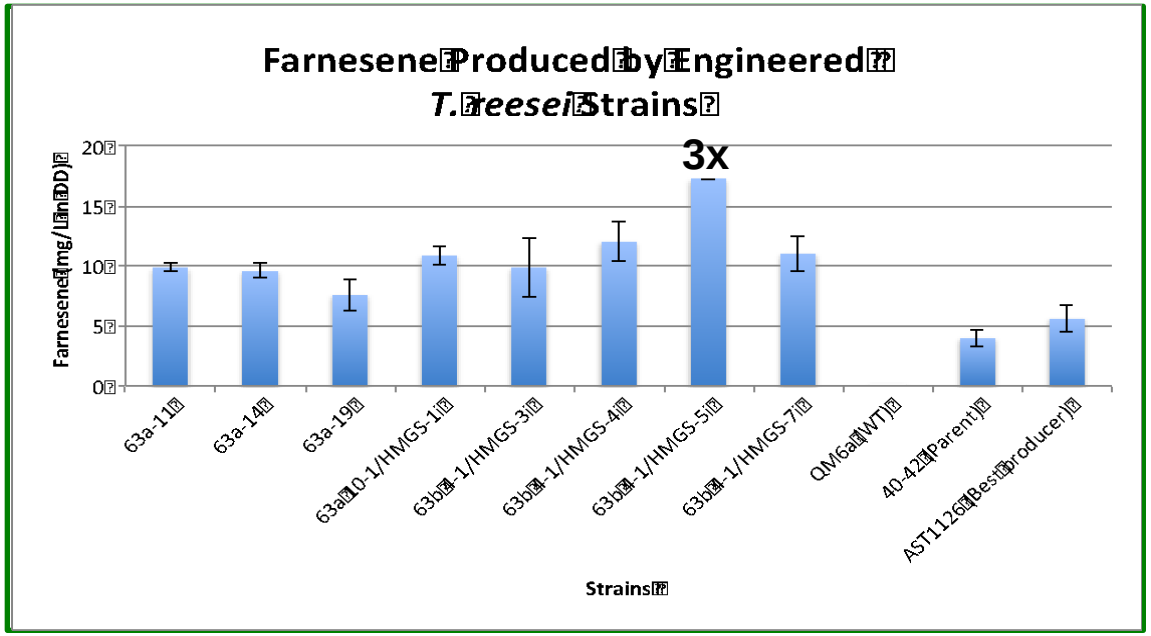
Nerolidol and farnesene produced by *T. reesei* transformants



3 – Technical Accomplishments/ Progress/Results

Task 3 – Advanced Concepts for Producing HCs

Through integrating multiple copies of the β-farnesene synthase gene as well as overexpression of the key enzymes, HMGS and HMGR, in the MVA pathway, we constructed *T. reesei* strains with β-farnesene production as high as 3-fold improvement when compared to our previous (FY13) "best producer" thus we exceeded our stretch milestone target (2-fold improvement) in Q4 FY14.

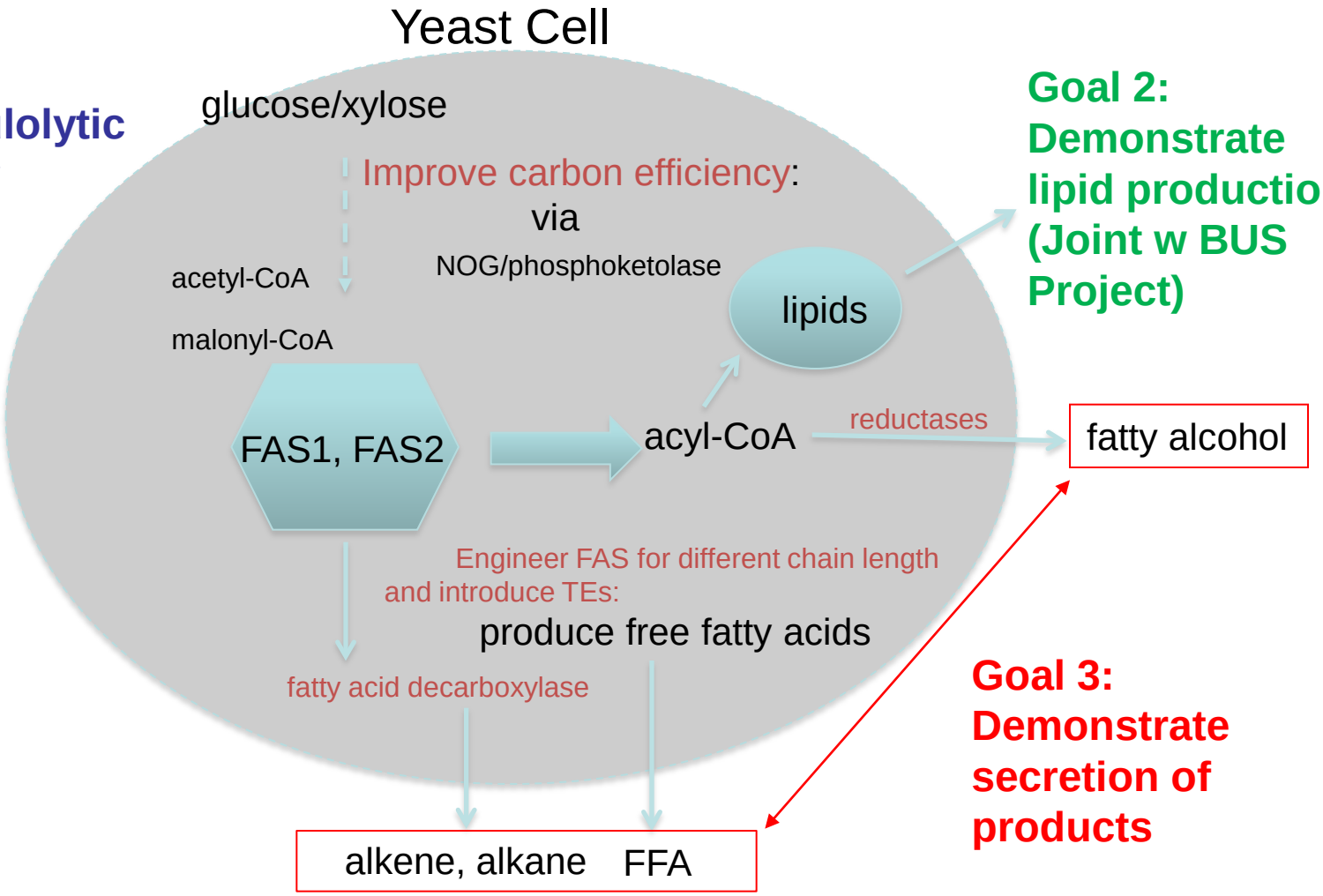


3 – Technical Accomplishments/ Progress/Results

Task 3 – Advanced Concepts for Producing HCs

Task 3. MS. Identify the most promising pathway for producing intracellular and extracellular HCs in yeast 9/30/2015

Goal 1: Improve lignocellulolytic capability



Goal 2: Demonstrate lipid production (Joint w BUS Project)

Goal 3: Demonstrate secretion of products

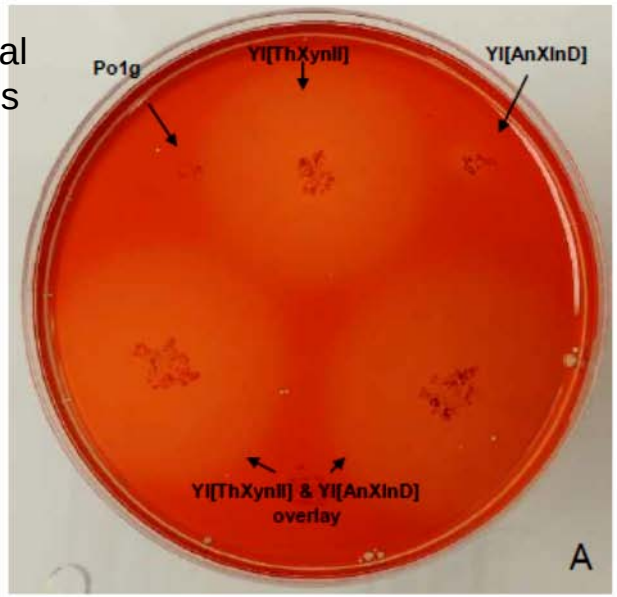
3 – Technical Accomplishments/ Progress/Results

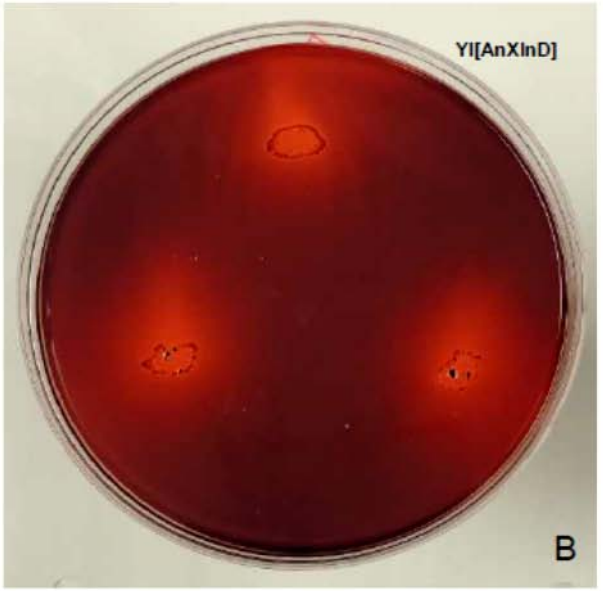
Task 3 – Advanced Concepts for Producing HCs

Goal 1 - Xylanase genes expressed in *Y. lipolytica*

Proteins; source	Amino acid number (MW)	Transformant
XynII; *T. harzianum*	**190 aa (21 kDa)**	**T131, Yl[ThXynII]**
XlnD; *A. niger*	**778 aa (85k Da)**	**T133, Yl[AnXlnD]**
AbfA; *A. niger*	**603 aa (65 kDa)**	**T128, Yl[AnAbfA]**

xylan mineral media plates



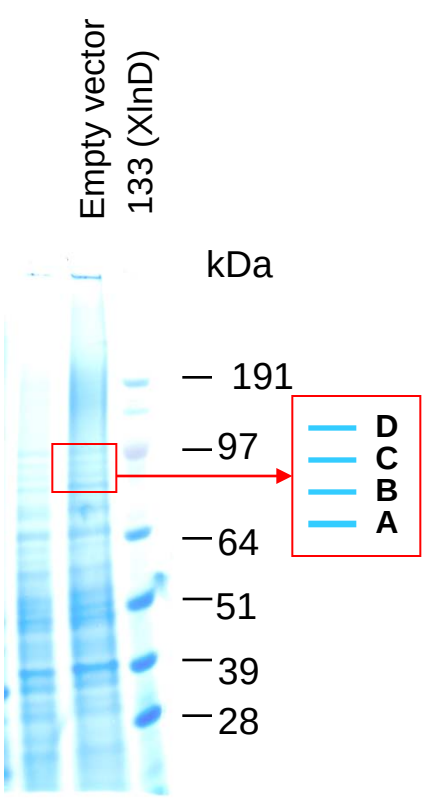


3 – Technical Accomplishments/ Progress/Results

Task 3 – Advanced Concepts for Producing HCs

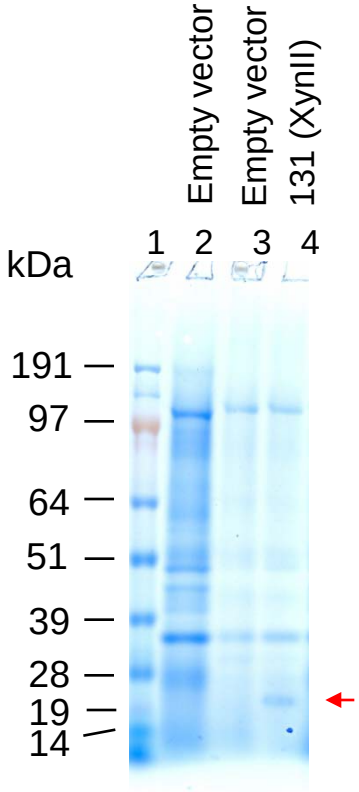
Goal 1 - Xylanase genes expressed in *Y. lipolytica*

exo-xylosidase



85 kDa

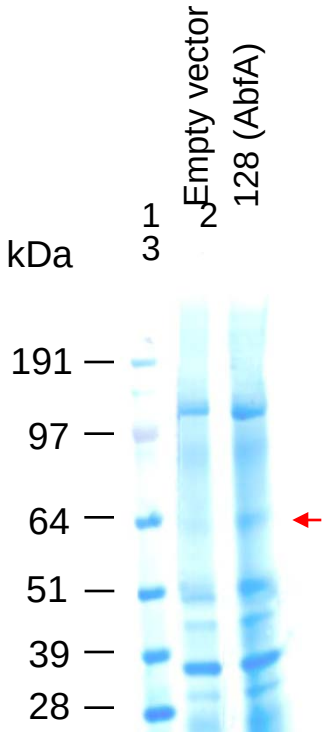
endo-xylanase



21 kDa

B, C, D: 94, 96, 99 kDa

arabinofuranosidase



65 kDa

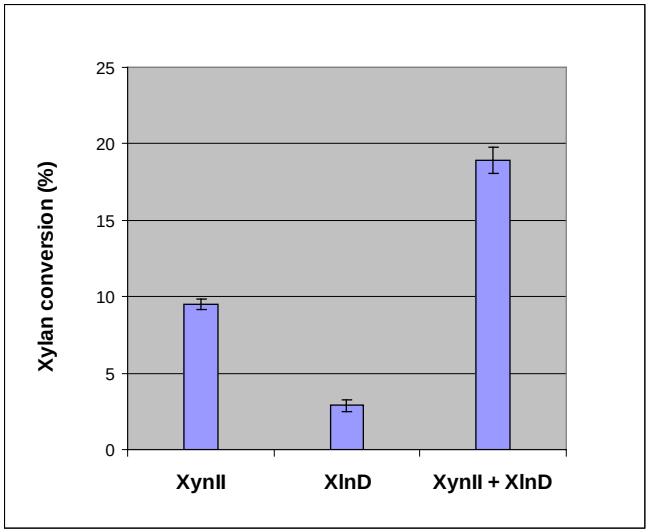
- All three xylanases were successfully expressed in *Y. lipolytica;*
- Xylanases expressed in *Y. lipolytica* were likely less glycosylated

3 – Technical Accomplishments/ Progress/Results

Task 3 – Advanced Concepts for Producing HCs

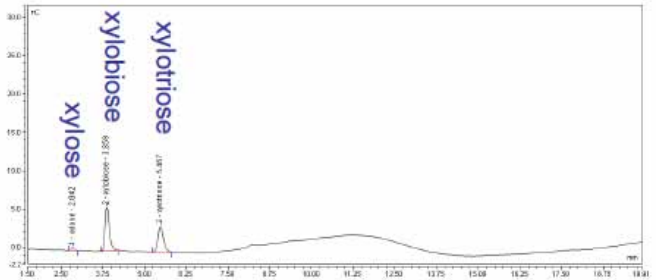
Goal 1 - Xylanase genes expressed in *Y. lipolytica*

Synergistic digestion of xylan with XynII and XlnD

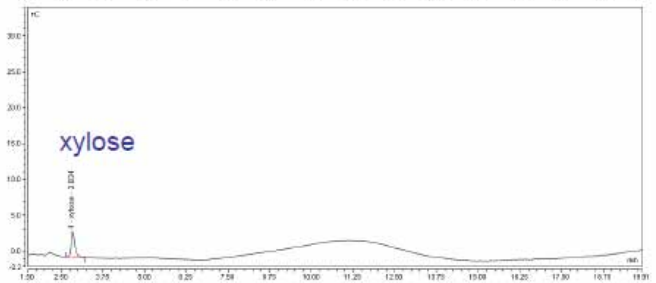


Xylan conversion by individual XynII, XlnD and combination of both at 5 min

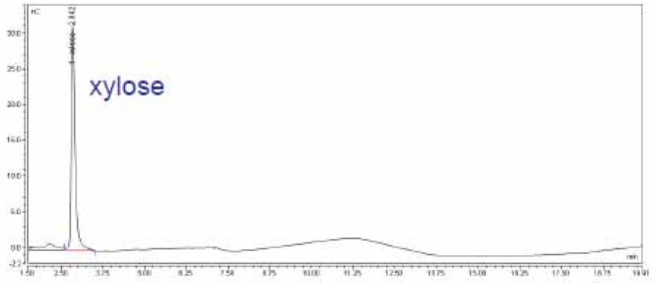
XynII



XlnD



XynII+XlnD



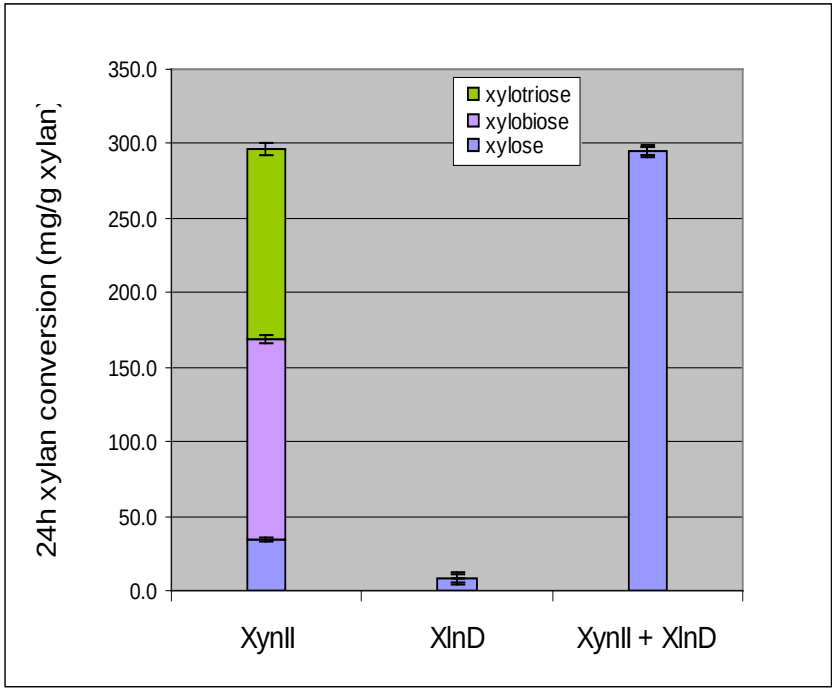
HPLC chromatograms of xylan digestion

3 – Technical Accomplishments/ Progress/Results

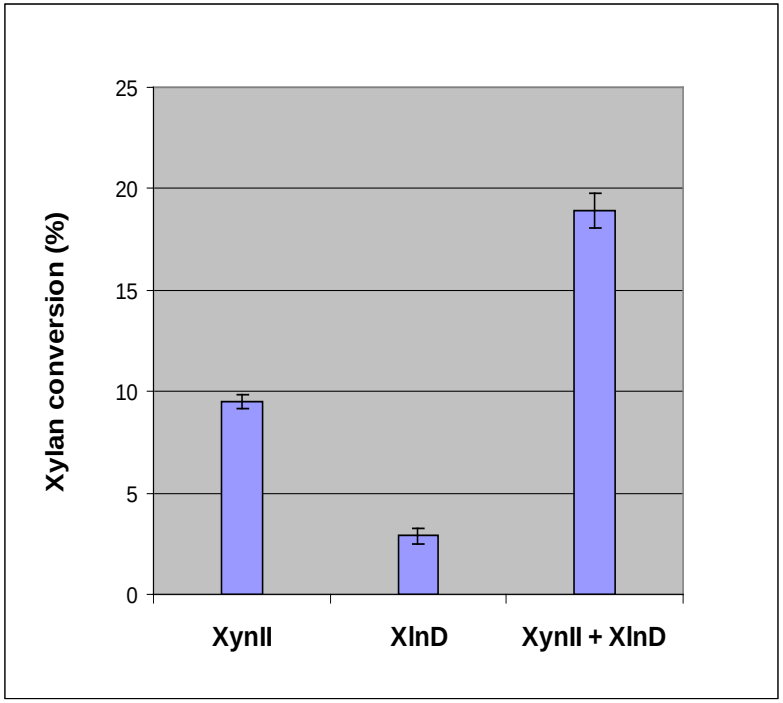
Task 3 – Advanced Concepts for Producing HCs

Goal 1 - Xylanase genes expressed in *Y. lipolytica*

Digestion of xylan in pretreated corn stover



Synergistic digestion of xylan with XynII and XlnD

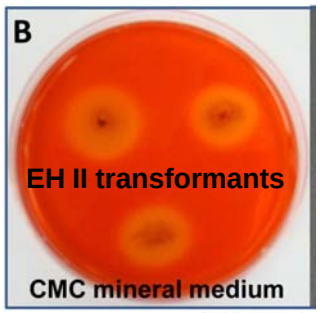


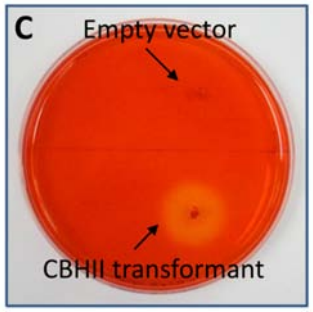
24h-digestion of PCS with individual XynII, XlnD and combination of both enzymes

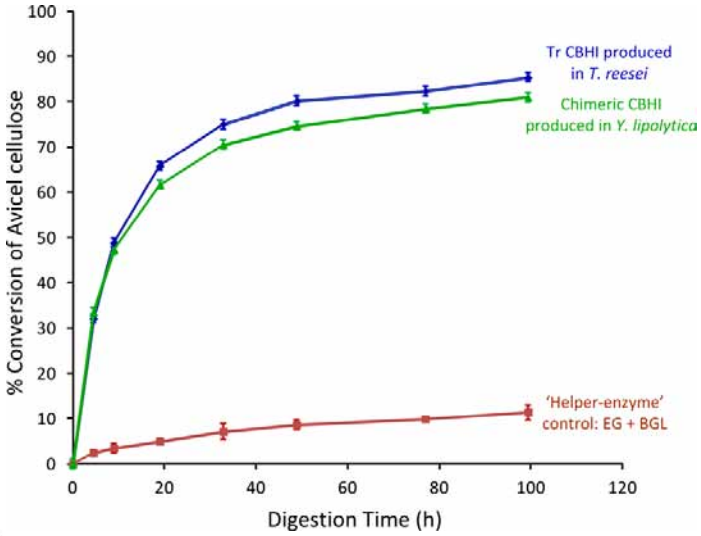
3 – Technical Accomplishments/ Progress/Results

Task 3 – Advanced Concepts for Producing HCs

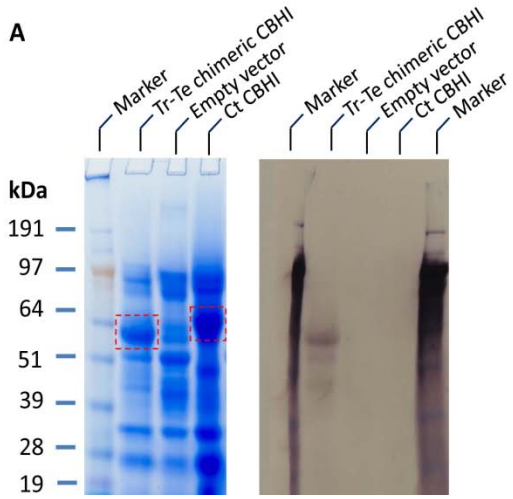
Goal 1- Expression of *T. reesei* EGII, CBH II and a chimeric *T. reesei-Talaromyces emersonii* CBH I in *Yarrowia*

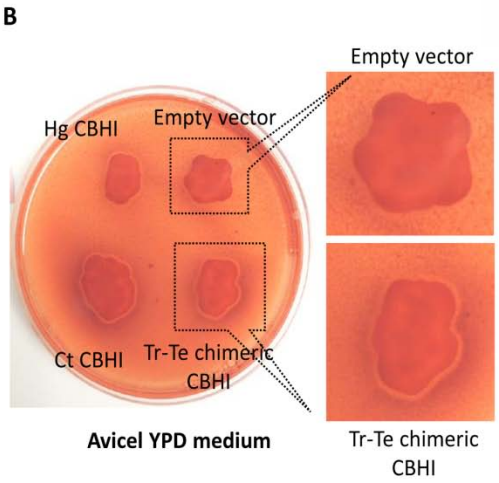






CBH I expression confirmed by Western blot



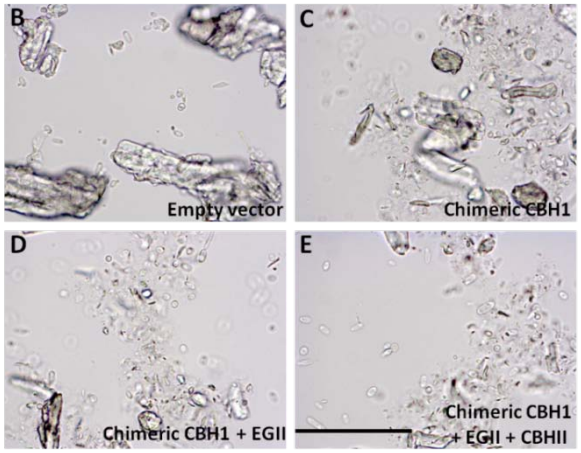


Specific Avicelase activity of purified Tr-Te chimeric CBHI from *Yarrrowia* is nearly as active as the native Tr CBH I

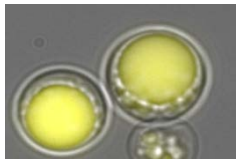
3 – Technical Accomplishments/ Progress/Results

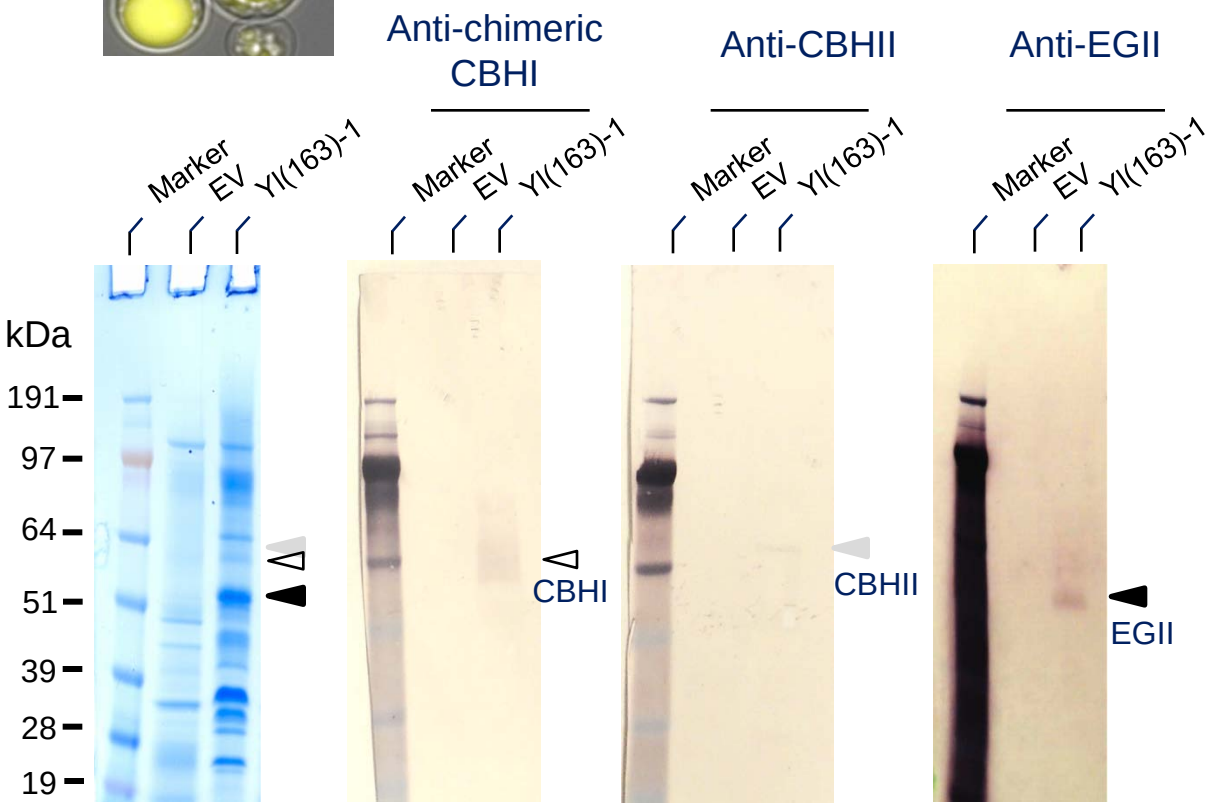
Task 3 – Advanced Concepts for Producing HCs

Goal 1 - Successfully co-expressed three microbial cellulase genes, chimeric CBHI, CBHII and EGII in *Y. lipolytica*



Morphology of co-culturing *Yarrowia* transformants expressing heterologous cellulases on mineral medium containing Avicel (2.7% w/v) as sole carbohydrates





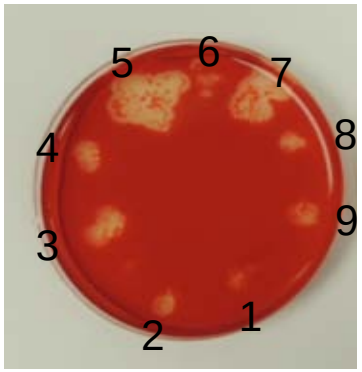
3 – Technical Accomplishments/ Progress/Results

Task 3 – Advanced Concepts for Producing HCs

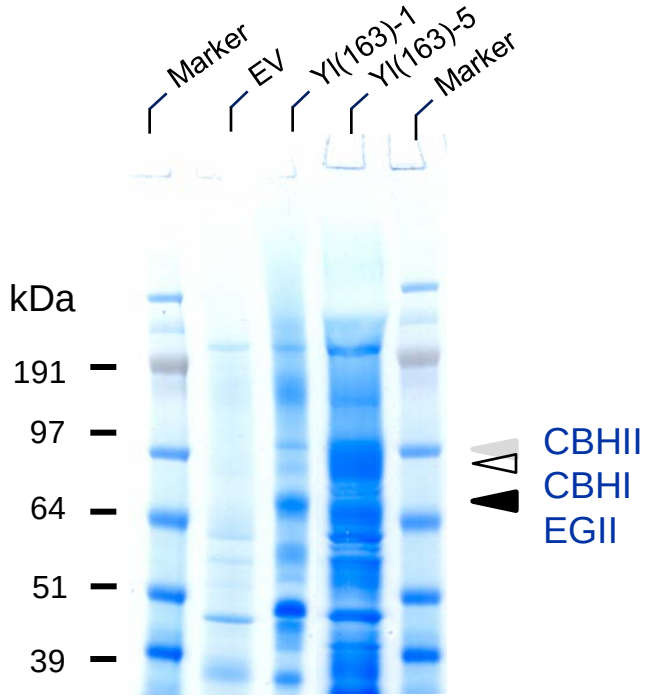
Goal 1 - Co-expression of CBHI, CBHII and EGII in oleaginous yeast *Yarrowia lipolytica* ACL/DGA1 mutant

- **Disruption of SNF1 in 163-1 (compared to previous CBHI single expression) lowered the co-secretion of CBHI and CBHII**

- **Random insertion (without disruption of SNF1) in 163-5 partially alleviated the above co-secretion "clog" in secretion pathway**



Transformants on YPDA/PASC plate:
Congo Red staining

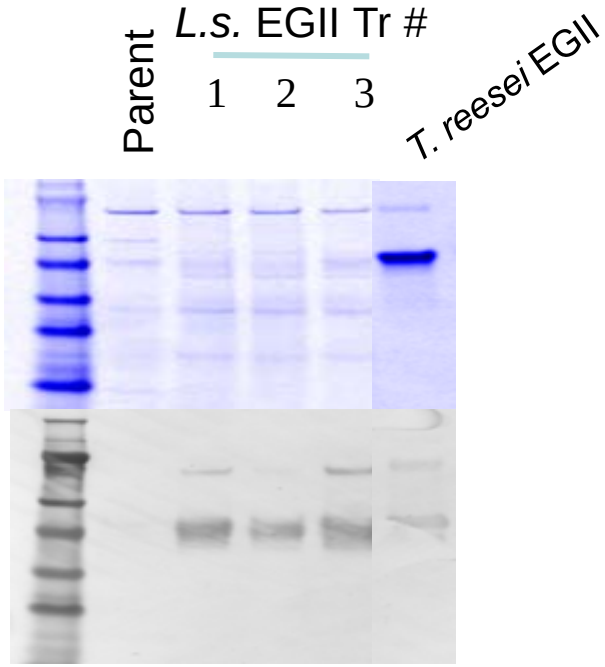


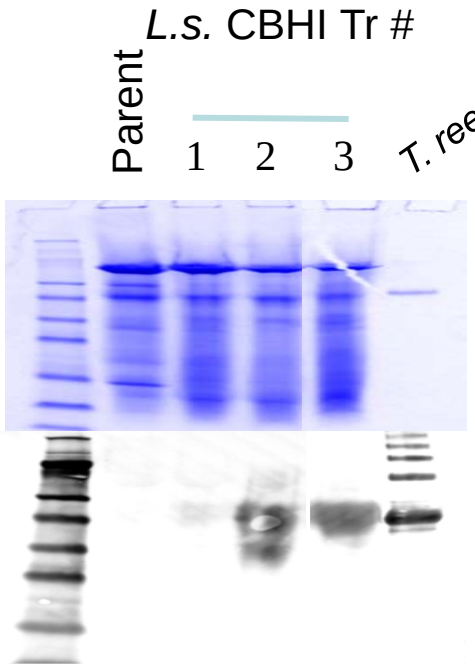
3 – Technical Accomplishments/ Progress/Results

Task 3 – Advanced Concepts for Producing HCs

Goal 1 - Example of Demonstrating Cellulase Expression in *Lipomyces starkeyi*

- ✓ Identify native signal peptides (SP) of *L. starkeyi* (amylase, dextranases, and protease)
- ✓ Selection of candidate gene: *T. reesei* EGII (*Tr* EGII), Tr-Te chimeric CBHI
- ✓ Design and synthesize the gene expression cassette using PYK1 promoter (P) and GAL1 terminator (T) of *L. starkeyi*





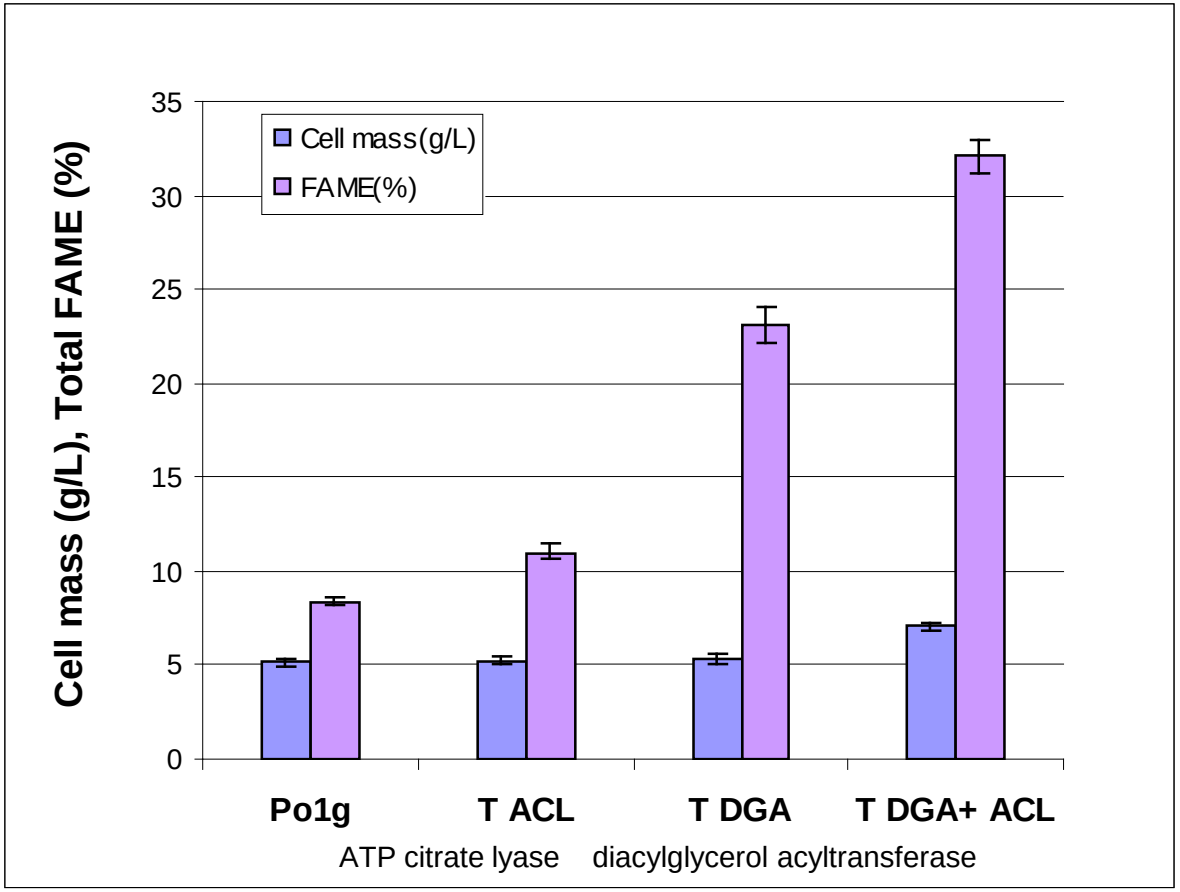
SDS-PAGE

Western blot probed by *T. reesei* EGII (right) and CBHI (left) antibodies

3 – Technical Accomplishments/ Progress/Results

Task 3 – Advanced Concepts for Producing HCs

Goal 2 - Lipid production in *Yarrowia* Po1g* transformants w overexpression of lipid synthesis pathway genes



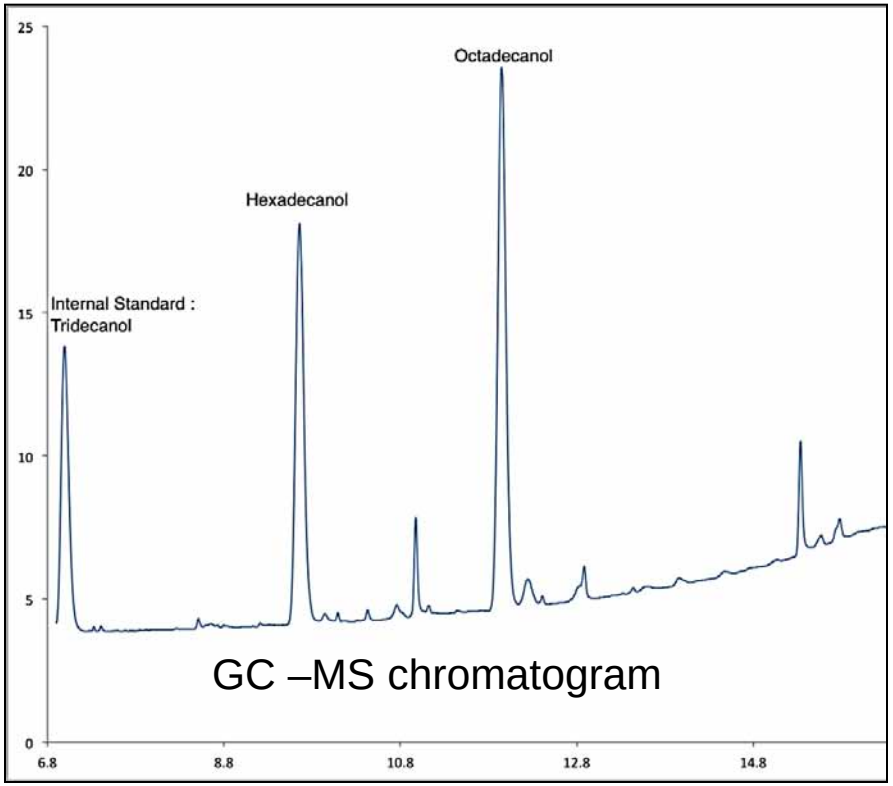
*baseline *Yarrowia* lipid production strain

3 – Technical Accomplishments/ Progress/Results

Task 3 – Advanced Concepts for Producing HCs

Goal 3 - Engineer *Yarrowia* for fatty alcohol production

- Fatty alcohols are important industrial chemicals
- Good biofuel additives because of their perfect fuel properties
- Fuel precursor for upgrading
- Naturally secreted to outside of the cells



GC –MS chromatogram

✓ Fatty alcohol was detected in the culture of FAR transformants

Overexpressed fatty acyl-CoA reductase (FAR) from *Marinobacter aquaeolei* in *Yarrowia*

Hexadecanol (palmityl alcohol,) and octadecanol (stearyl alcohol) detected in the culture supernatant

4 – Relevance

Task 1 Anaerobic HC Intermediates from *Z. mobilis*

- *Describe how project accomplishments contribute to meeting…*
  - Provide leading technology with reduced the cost and high carbon efficiency intermediates amenable to separations and catalytic upgrading to HC fuels.
- *Demonstrate how the project considers applications of the expected outputs in the emerging bioenergy industry*
  - Provides key technology to near term processes as well as longer term science for out year goals.
- *Your objectives should be clear regarding the relevance of your project …*
  - To develop novel pathways for advanced biological upgrading of sugars to hydrocarbons (HC) by investigating efficient and rapid carbohydrate utilization, high carbon efficiency, cost effective processes to support the DOE BETO 2022 goal of producing advanced HC fuels at $3/GGE.
  - MYPP goals addressed are: Bt-J. Catalyst Development and Bt-K. Biological Process Integration
- *Demonstrate that the successful project will advance the state of technology..*
  - Existing biomass conversion to biofuels processes demonstrate that *Zymomonas* technology is commercially viable today.
- *Tech transfer/marketability...*
  - DOE and NREL have an existing customer-base for *Zymomonas* centric technologies which can be considered for new achievements.

4 – Relevance

Task 2 Anaerobic HC Intermediates from Other Bacteria

- *Describe how project accomplishments contribute to meeting the platform goals and objectives of the BETO Multi-Year Program Plan*
  - Anaerobic microbes that produce C3-C8 species suitable for upgrading to fuels support the FY2017 and MYPP goals identified by BETO for advanced biofuels
- *Demonstrate how the project considers applications of the expected outputs in the emerging bioenergy industry*
  - Provides key technology for near and mid term product and fuels intermediate formation processes; as well as longer term science for out-year goals
- *Your objectives should be clear regarding the relevance of your project to the Bioenergy Technologies Office, MYPP goals*
  - The MYPP goals impacted by this work are : Bt-I. Cleanup/Separation, Bt-J. Catalyst Development, and Bt-K. Biological Process Integration.
- *Demonstrate that the successful project will advance the state of technology and positively impact the commercial viability of biomass and/or biofuels*
  - Data from this task must be evaluated in TEA models to examine the initial viability of hybrid biological-catalytic processes for the production of HC fuels towards the DOE BETO 2017 and 2022 goals
- *Tech transfer/marketability should be discussed here (not separately)*
  - ROIs and US patents will be filed as the technology is demonstrated

4 – Relevance

Task 3 Advanced Concepts for Producing HCs

- *Describe how project accomplishments contribute to meeting …*
  - Provide leading technology with reduced cost and high carbon efficiency intermediates amenable to catalytic upgrading to HC fuels for FY 2022.
- *Demonstrate how the project considers applications of the expected outputs in the emerging bioenergy industry .*
  - Provides key technology for mid term processes as well as longer term science for out-year goals
- *Your objectives should be clear regarding the relevance of your project..*
  - MYPP goals addressed are: Bt-F. Enzyme Production, Bt-J. Catalyst Development and Bt-K. Biological Process Integration
  - To develop novel pathways for advanced biological upgrading of sugars to hydrocarbons by investigating efficient sugars utilization, high carbon efficiency, cost effective processes supporting DOE BETO 2022 goal of producing advanced HC fuels at $3/GGE.
- *Demonstrate that the successful project will advance the state of technology and positively impact the commercial viability of biomass…*
  - Existing DMC biomass conversion to biofuels processes demonstrate that this general technology is commercially viable.
  - HC production from DMC strains is a stretch goal compared to bioethanol
- *Tech transfer/marketability...*The IP space is largely unknown but this provides opportunities for forward viewing industry

5 – Future Work

Task 1 Anaerobic HC Intermediates from *Z. mobilis*

- *Explain what it is you plan to do through the end of the project with emphasis on the next 18 months (through September 30, 2016)*
  - Demonstrate production of 2,3 butanediol at 20 g/L (18 months)
  - Demonstration of production of BDO at 50 g/L from mixed C5/C6 sugar streams from DDR pretreated corn stover. SMART milestone (2017)
- *Highlight upcoming key milestones*

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)

- *Address how you will deal with any decision points during that time (Go/No-Go Points) and any remaining issues with proposed abatement actions*
  - None identified in 2015 AOP
  - **Newly defined: Choose diversity or engineering approach for Bdh & Aldc – 3/30/2016**

5 – Future Work

Task 2 Anaerobic HC Intermediates from Other Bacteria

- *Explain what it is you plan to do through the end of the project with emphasis on the next 18 months (through September 30, 2016)*
  - Employ separations and chemical catalysis to obtain final target molecule.
  - Initiating molecular biology to put HA production into a low-pH fermenting yeast to ease separations
- *Highlight upcoming key milestones*

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)

- *Address how you will deal with any decision points during that time …*

Name	Description	Criteria
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.

5 – Future Work

Task 3 – Advanced Concepts for Producing HCs

- *Explain what it is you plan to do through the end of the project with emphasis on the next 18 months (through September 30, 2016)*
  - Improve the lipid titer to 75% (g lipid/g cell) by pathway optimization in oleaginous yeast. SMART (Joint w BUS milestone -18 months)
  - Identify pathways and factors promoting extracellular production of HCs or facilitating recovery of products.
- *Highlight upcoming key milestones*

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)

- *Address how you will deal with any decision points during that time (Go/No-Go Points) and any remaining issues with proposed abatement actions*
  - None identified in 2015 AOP
  - **Newly defined: Choose diversity or engineering approach for GHs – 3/30/2016**

Summary

Task 1 Anaerobic HC Intermediates from *Z. mobilis*

Overview

Using *Z. mobilis*, we will investigate novel concepts that can drastically reduce the cost of producing fuels intermediates using anaerobic pathways. Critical technical challenges are high carbon yield from glucose and xylose, as well as production titers and rates.

Approach

- Recruit three genes to channel pyruvate to acetolactate, acetoin and then 2,3 butanediol and further maximize its flux by considering various gene sources, optimizing the gene expression, and protein engineering if necessary.
- Knockout PDC to eliminate the ethanol formation.

Technical Accomplishments/Progress/Results

- Successful demonstration of BDO to 4.5 g/L produced by engineered *Z. mobilis* in using glucose, mixed sugar of glucose and xylose as well as DMR pretreated biomass sugars.

Relevance

BDO is a starting material for bulk chemicals and more importantly, it can be further chemically upgraded into jet fuel. Initial TEA will be conducted to examine the feasibility of sugars (mixed C6/C5) to BDO and further catalytic upgrade to jet fuel and establish targets

Future work

- BDO pathway optimization (enzymes and e-) and redirect carbon from ethanol to BDO production.
- Conduct TEA analysis to identify technical targets to reduce the cost of production.

Summary

Task 2 Anaerobic HC Intermediates from Other Bacteria

Overview

Evaluate anaerobic microbes and processes that produce C3-C8 species amenable for separation and upgrading

Approach

- Conduct systematic survey of scientific and patent literature for fermentation, separations, and catalysis readiness.
- Employ separations and chemical catalysis (if needed) to obtain final target molecule.

Technical Accomplishments/Progress/Results

- Down-selected to hexanoic acid (HA) and butyric acid for initial strain evaluations
- Setting up continuous-loop fermentation for immobilized cells with *ex situ* separations

Relevance

Anaerobic microbes that produce C3-C8 species suitable for upgrading to fuels support the AOP and MYPP goals identified by BETO for advanced biofuels.

Future work

•Molecular biology to achieve HA production in low-pH fermenting yeast to ease separations

•Down select to 2-3 organisms based on performance in batch anaerobic fermentations for further adaptation, evolution, and evaluation.

Summary

Overview — Task 3 – Advanced Concepts for Producing HCs

Investigate novel direct microbial conversion (DMC) based concepts that can drastically reduce the cost of producing hydrocarbon fuels to meet FY2022 goals. Critical technical challenges are high carbon yield from glucose and efficient product secretion/recovery; active and adequate cellulase expression.

Approach

- Direct microbial sugar conversion (DMC) by aerobic (*Yarrowia* sp. and *Lipomyces*) will be evaluated for potential reduction of cost for HC intermediates.
- Pathways and factors promoting extracellular production of HCs or facilitating recovery of products will be investigated and their impact on production cost will be evaluated in oleaginous yeast strains.

Technical Accomplishments/Progress/Results

- Successfully co-expressed three xylanases and three microbial cellulase genes, chimeric CBHI, CBHII and EGII in *Yarrowia lipolytica* to enable the utilization of cellulolytic substrate after demonstrating heterologous expression of individual chimeric CBHI
- Successfully transformed the highly oleaginous yeast *Lipomyces* with the EG II and CBH I cellulase genes from *T. reesei. This is the first reported heterologous cellulase gene expression of Lipomyces.*

Relevance

DMC yeast species that produce lipids may be suitable for upgrading to fuels support the 2022 AOP and MYPP goals identified by BETO for advanced biofuels.

Future work

- Improve GH expression to support DMC; stack all GH genes in target strain
- Identify HC intimidates with high energy, easy separations, and/or high carbon production yields.
- TEA analysis of the potential impact of these technical barriers will be conducted to provide guidance for future work.

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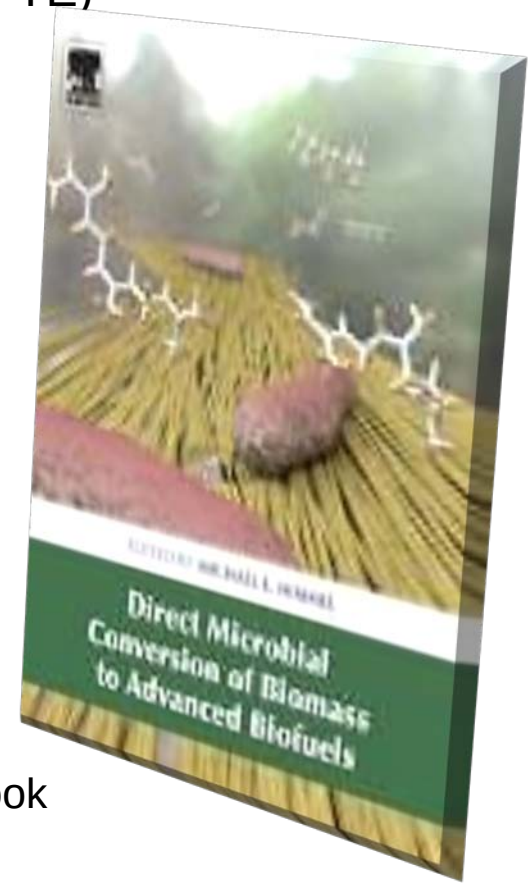


New 600 pg book

Additional Slides

Responses to Previous Reviewers' Comments

- If your project is an on-going project that was reviewed previously, address 1-3 significant questions/criticisms from the previous reviewers' comments.
  - "I question using *Z. mobilis* as a production host in this case. It seems that it was chosen because there has already been a lot of work done with Z. mobilis at NREL. It was originally isolated in the production of tequila, so it is very appropriate for cellulosic ethanol, but not necessarily for other hydrocarbons. The physiology of Zm may not be able to support high level production of a non-ethanol product."
  - Answer: *Z. mobilis* intrinsically possesses several unique properties (the membrane lipids contain very abundant triterpenic isoprenoids and unique fatty acid composition) that potentially make it well suited to serve as an anaerobic microbial platform for hydrocarbon (HC) production from lignocellulosic biomass. *Z. mobilis* is well known for both its high specific glucose uptake rate and rapid catabolism and is engineered to metabolize all the major biomass sugars. We seek to take the advantages of the organism's metabolic capabilities and are attempting to redirect the carbon flow to other energy-dense fuel products.
  - "NREL has done a tremendous amount of research on *Zymomonas*. Building on this knowledge makes some sense, but looking ahead at the go / no-go decision points and objectively making a decision to stay the course or look at alternative organisms will be important."

Responses to Previous Reviewers' Comments

- If your project is an on-going project that was reviewed previously, address 1-3 significant questions/criticisms from the previous reviewers' comments.
  - This project, the work to date, and the proposed future work are appropriate for this early-stage scoping project. Effort should be placed on completing the TEA as soon as possible so that it may inform future research targets. In light of the significant economic challenges of producing a hydrocarbon fuel from biomass sugar feedstock, it is recommended that the performer consider higher value products. Comments to DOE: DOE needs to fish or cut bait on these approaches. If DOE decides that there is value in establishing an "in-house" strain/platform to evaluate and validate the various proposed hydrocarbon approaches, then significantly more resources should be deployed to develop the necessary metabolic engineering tools to support this effort. The RDD&D path from POC to commercial relevance is incredibly long and the amount of funding necessary to advance this work will be significant.
  - Answer: Concerns about using *Zymomonas* for HC production are well taken. There are a number of go/no go decision points in Year 2 and Year 3 to help us measure whether we should continue the research activities on *Zymomonas* or redirect resources to investigation of alternative organisms. TEA analysis will be conducted to provide guidance on economically-relevant research targets. Researchers showed very promising results using both *E. coli* and *S. cerevisiae* through metabolic engineering approaches to enhance the production of HC from a few milligrams per liter to hundreds of milligrams per liter. Our initial attempt of introducing the farnesene synthase gene has so far resulted in achieving a titer of ~35 mg/L. Our goal is to improve the product titer and yield using the available genetic tools developed in the ethanologen project and apply new metabolic tools as they become available.

DMSC and TMD: 2012-2015 Publications

- “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. E. Himmel, Ed.,) Chapter 10, Springer Publishers, London/New York, NY. 2015. In press.

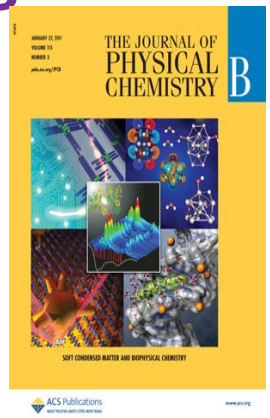
- "Heterologous Expression of Xylanase Enzymes in Lipogenic Yeast *Yarrowia lipolytica*,” Wei Wang; Hui Wei; Markus Alahuhta; Xiaowen Chen; Deborah Hyman; Min Zhang; Michael E Himmel, PloS One, (2014), In Press.

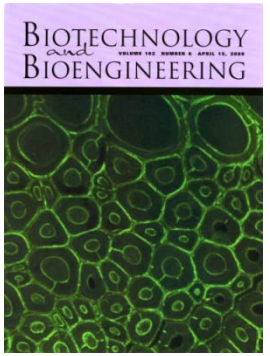
- “Engineering Towards a Complete Heterologous Cellulase Secretome In *Yarrowia lipolytica* Reveals Its Potential For Consolidated Bioprocessing,” Hui Wei, Wei Wang, Markus Alahuhta, Todd Vander Wall, John O. Baker, Larry E. Taylor II, Stephen R. Decker, Michael E. Himmel, Min Zhang, **Biotechnology for Biofuels**, (2014) In Press.

- “Improved Ethanol Yield and Reduced Minimum Ethanol Selling Price (MESP) by Modifying Low Severity Dilute Acid Pretreatment With Deacetylation and Mechanical Refining: 2) Techno-Economic Analysis,” Ling Tao, Xiaowen Chen, Andy Aden, Eric Kuhn, Michael E Himmel, Melvin Tucker, Mary Ann A Franden, Min Zhang, David K Johnson, Nancy Dowe and Richard T Elander, **Biotechnology for Biofuels** 5, 69 (2012) DOI:10.1186/1754-6834-5-69.

- Biomass Conversion,” S.R. Decker, J. Sheehan, D.C. Dayton, J.J. Bozell, W.S. Adney, B. Hames, S.R. Thomas, R.L. Bain, S. Czernik, M. Zhang, and M.E. Himmel, in Reigel’s Handbook of Industrial Chemistry 2nd Ed,” (J.A. Kent, Ed.), Kluwer Academic/Plenum Publishers, New York, pp. 1249-1323, (2012).

- *Direct Microbial Conversion of Biomass to Advanced Biofuels*, (M. E. Himmel, Ed.,) Springer Publishers, London/New York, NY. 2015, 600 pages.





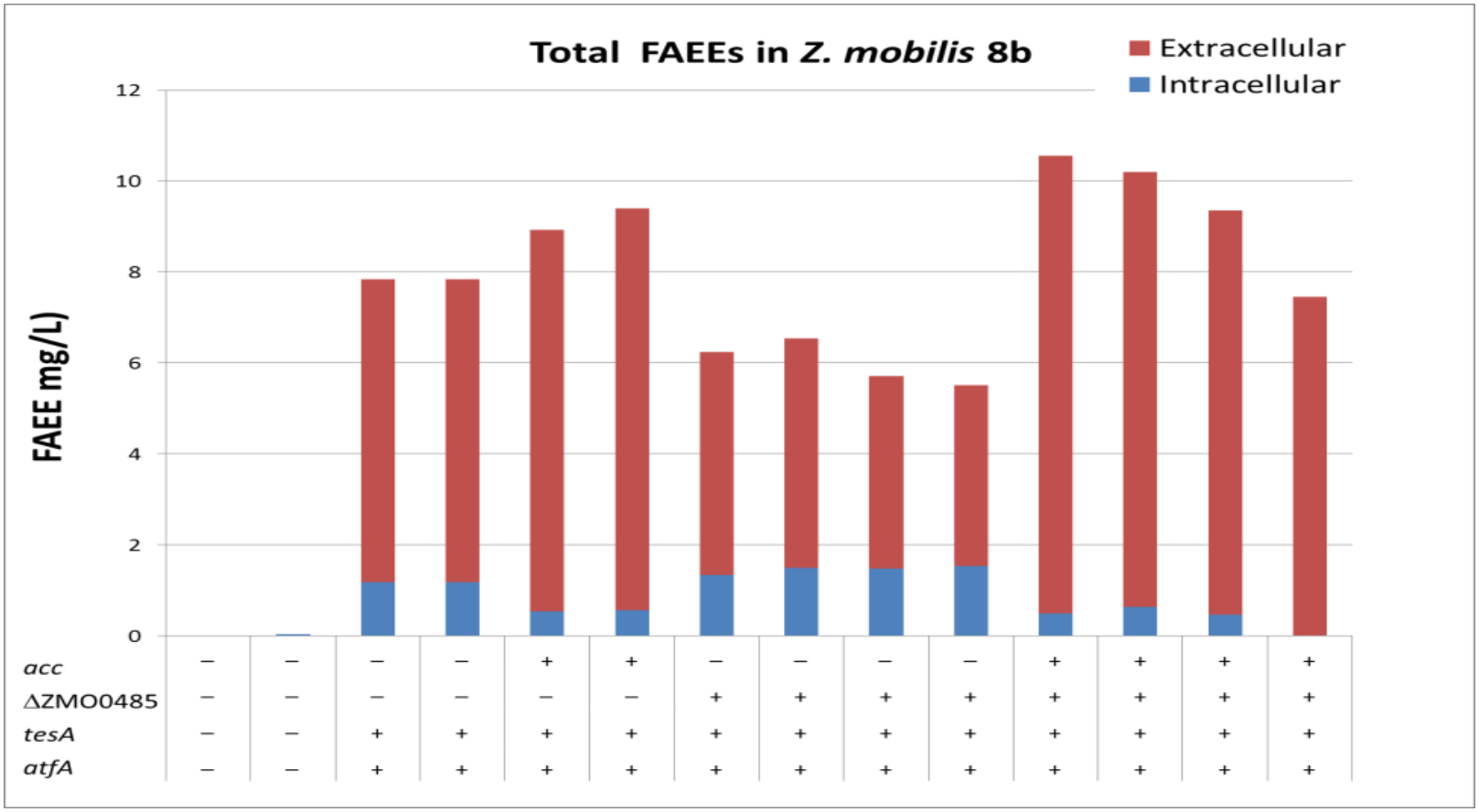
Detailed Milestone chart for 2015

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)

3 – Technical Accomplishments/Progress/Results

Task 1 Anaerobic HC Intermediates from *Z. mobilis*

Z. mobilis 8b was metabolically engineered to produce 10 mg/L Fatty Acid Ethyl Ethers (FAEEs), primarily extracellular, by modifications to several key enzymes in the fatty acid pathway as well as introducing two heterologous genes encoding wax ester synthase and thioesterase.



TMD Abstract

The goal of this project is to develop novel pathways for advanced biological upgrading of sugars to lipids/lipid alcohols (FY2017) and to hydrocarbons (FY2022) by developing efficient and rapid carbohydrate utilization, high carbon efficiency, cost effective processes to support the DOE BETO 2022 goal of producing advanced hydrocarbon fuels at $3/GGE. To achieve this goal, we have designed three experimental tasks: Working in *Z. mobilis*, Task 1 will recruit genes to channel pyruvate to acetolactate, acetoin and then to 2,3 butanediol and further maximize its flux by considering various gene sources, optimizing the gene expression, and protein engineering if necessary. Task 2 will evaluate anaerobic microbes that produce C3-C8 species amenable for separation and upgrading; as well as conduct systematic survey of scientific and patent literature for fermentation, separations, and catalysis readiness. Task 3 will investigate novel DMC concepts impacting FY2022 goals that can drastically reduce the cost of producing hydrocarbons. Critical technical challenges include: high carbon yield from glucose; as well as xylose, product secretion/recovery, and the high cost of cellulase/hemicellulose enzymes. We recently demonstrated transformation of the wild type and engineered *Yarrowia* strains to produce a fully active trio of cellulase and duo of xylanase enzymes from *T. reesei*.