2013 DOE Bioenergy Technologies Office (BETO) Project Peer Review

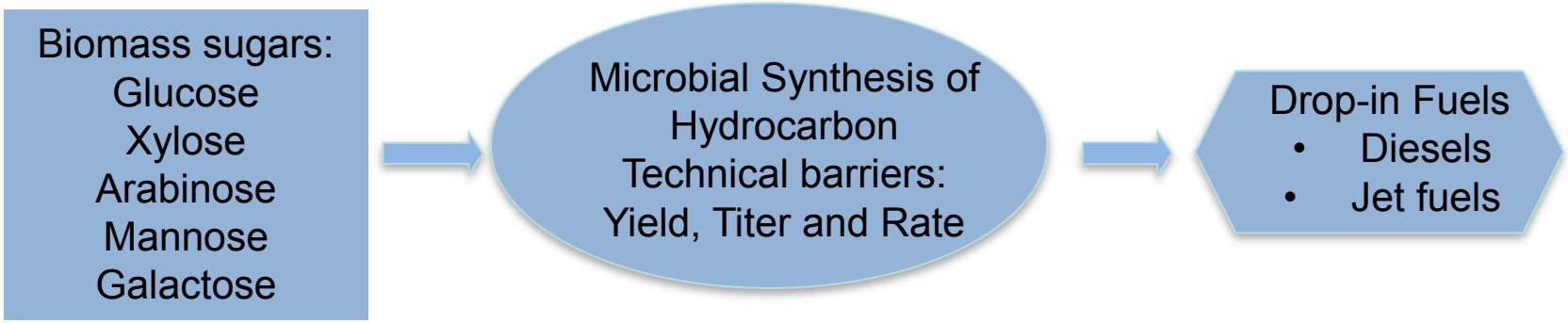
Engineering *Zymomonas mobilis* for Hydrocarbon Fuels Production

Date: May 22, 2013
Technology Area Review: Biochemical Conversion

Principal Investigator: Min Zhang
Organization: National Renewable Energy Laboratory

Goal Statement

- **Project objectives**
  - Develop biological catalysts for production of fuel molecules and/or intermediates for renewable gasoline, jet fuel or diesel from sugars derived from lignocellulosic biomass feedstocks.
  - Provide key technology fundamentals regarding microbial synthesis of hydrocarbon fuel.
  - Identify, understand and overcome the critical barriers for conversion of lignocellulosic feedstocks to hydrocarbons.



Quad Chart Overview

Timeline

- Project start date: 2013
- Project end date: 2017
- Percent complete: 10%

Barriers

- Barriers addressed
  - Bt-J. Catalyst Development
  - Bt-K. Biological Process Integration

Budget

Funding for FY11: N/A
Funding for FY12: N/A
Funding for FY13: $ 500 K / 0
(DOE / Cost share)

Years the project has been funded / average annual funding: N/A

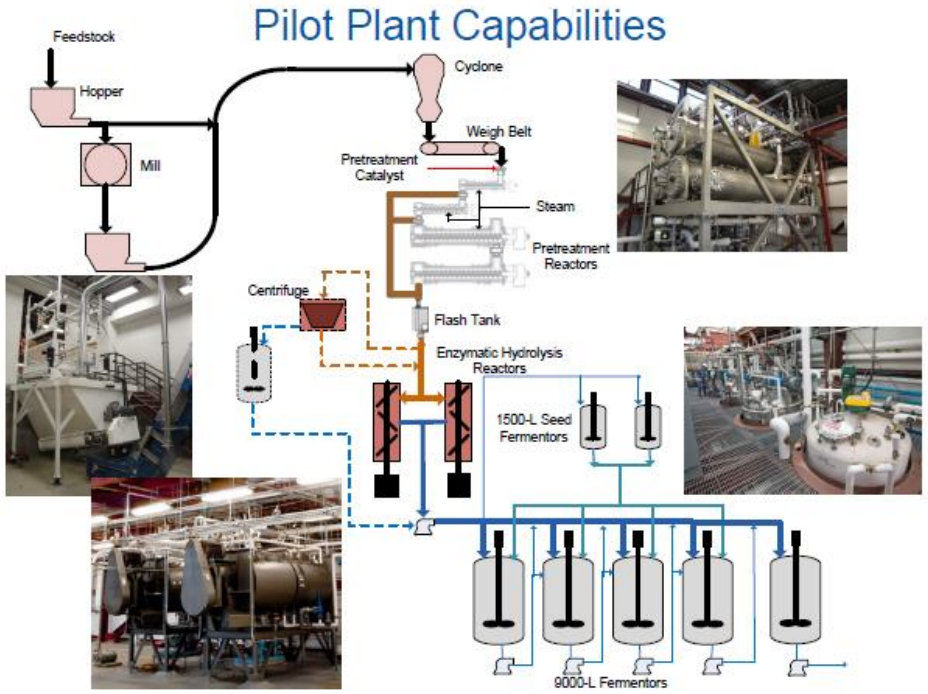
Partners

Interactions/collaborations: Analysis team, Pretreatment & Enzymatic Hydrolysis team, Biomass Process Integration team.

Project management: The project is managed under the Biochemical Platform at NREL

Project Overview

- Engineered the facultative anaerobe *Z. mobilis* to efficiently use the second and third most abundant plant derived sugars, xylose and arabinose and convert them to ethanol at high yield.
  - High specific glucose uptake rate
  - Rapid catabolism
- 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 2012 Demonstration for competitive ethanol production from cellulosic biomass at NREL pilot facility.



- Therefore, Zymomonas can serve as a good candidate host for making hydrocarbon molecules from biomass sugars.

1 - Approach

- 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 renewable fuels.
  - Isoprenoid pathway
  - Fatty acid pathway
  - Pyruvate derived pathway
- 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.

- FY13 D-milestone:
  - Demonstrate production of 0.001% to 1% at least one top candidate high energy fuel and/or intermediate using *Zymomonas mobilis* from glucose and xylose.

Can we redirect carbon to make hydrocarbon fuel molecules?

Zymomonas possess the active pathways to supply substrate for hydrocarbon synthesis.

- The organism intrinsically possesses advantageous properties, i.e. highest total hopanoid (C30) content at 30 mg/g DWC, and up to 70% of vaccenic acid (18:1n7), which could be well suited to serve as an anaerobic microbial platform for hydrocarbon production.

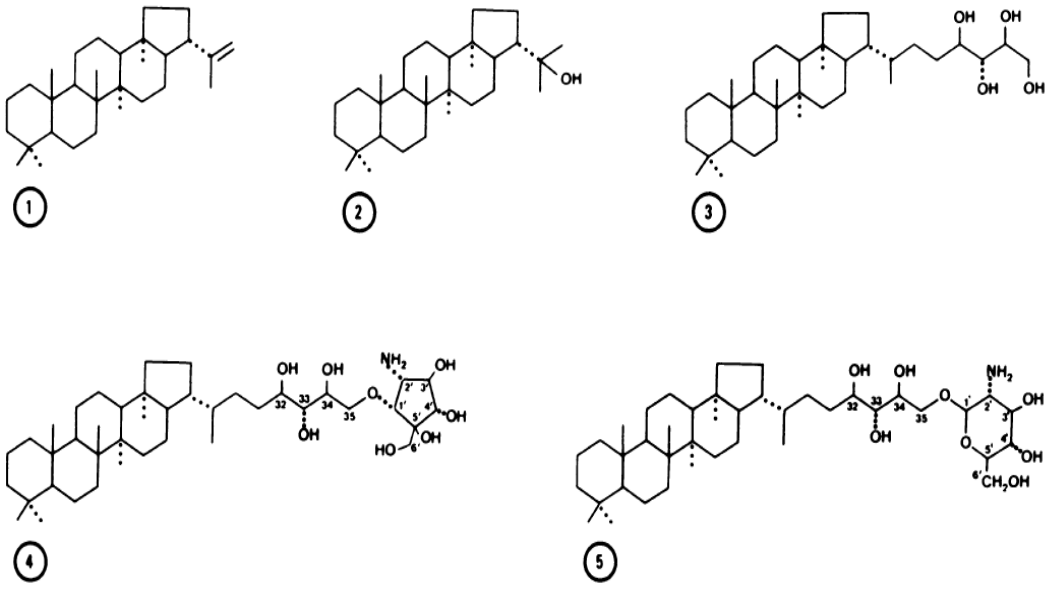
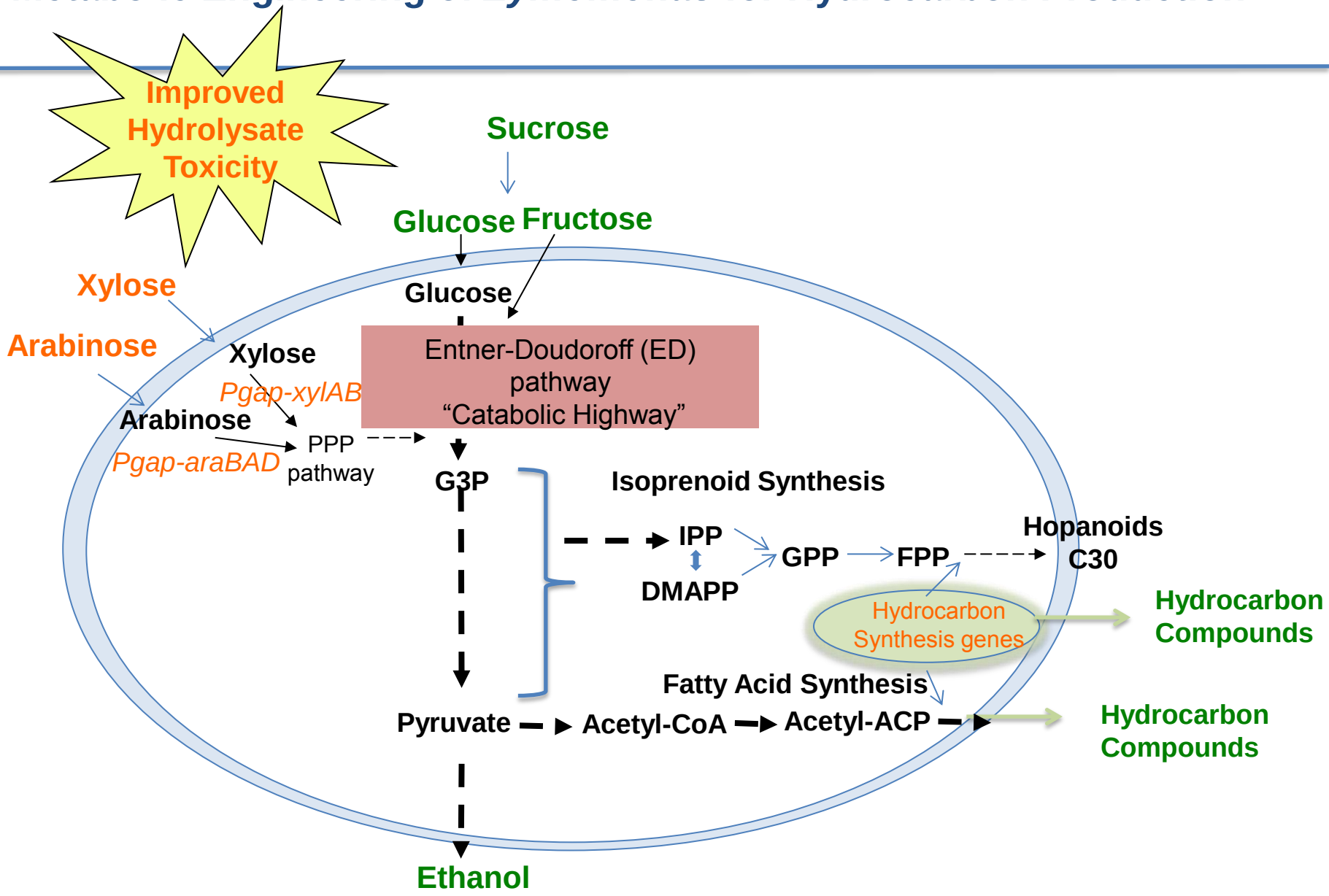


FIG. 1. Hopanoids from *Z. mobilis*. 1, hopene; 2, diplopterol; 3, bacteriohopanetetrol; 4, bacteriohopanetetrol ether; 5, glucosaminyl bacteriohopanetetrol.

Hermans et al., 1991 J. Bacteriol.

Metabolic Engineering of *Zymomonas* for Hydrocarbon Production



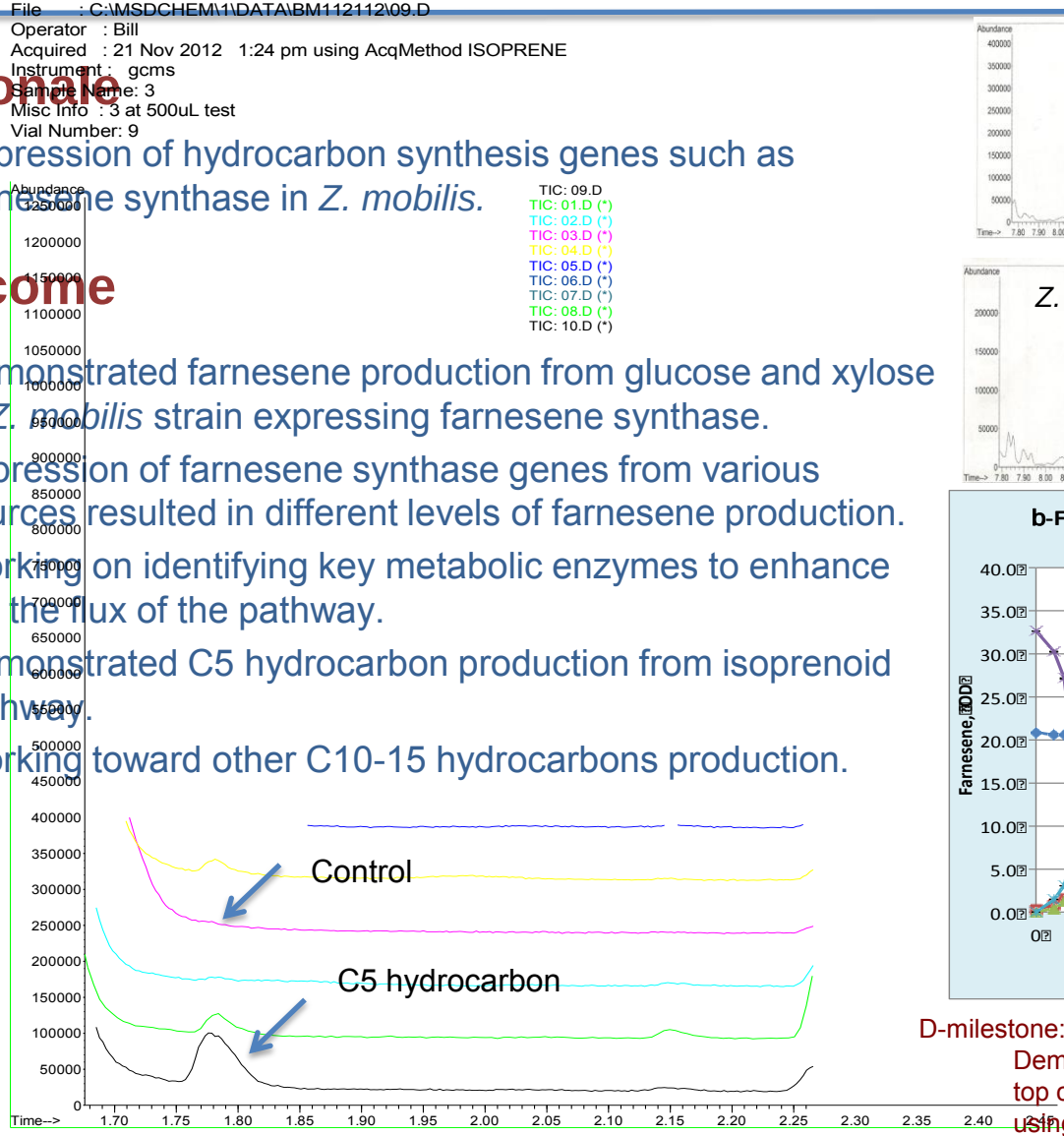
Hydrocarbons Derived from Isoprenoid Pathway

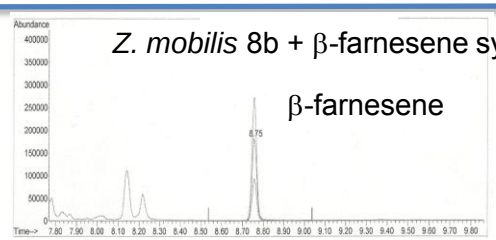
Rationale

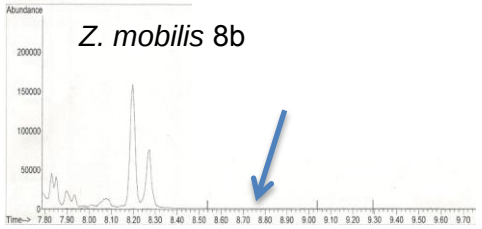
- Expression of hydrocarbon synthesis genes such as farnesene synthase in *Z. mobilis.*

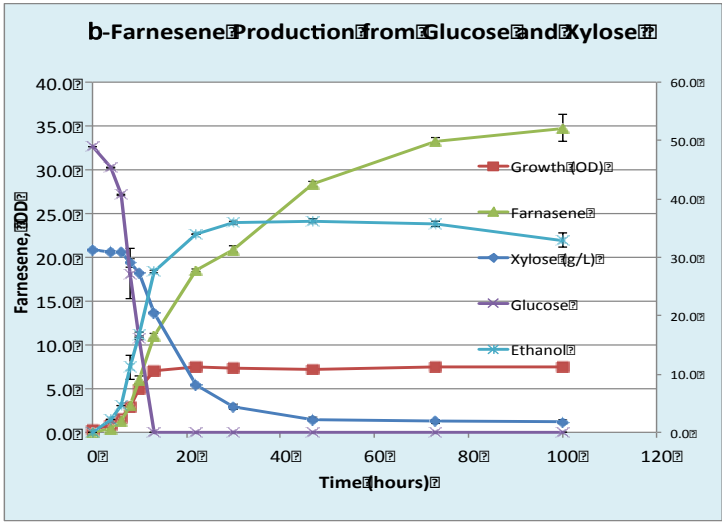
Outcome

- Demonstrated farnesene production from glucose and xylose in *Z. mobilis* strain expressing farnesene synthase.
- Expression of farnesene synthase genes from various sources resulted in different levels of farnesene production.
- Working on identifying key metabolic enzymes to enhance the the flux of the pathway.
- Demonstrated C5 hydrocarbon production from isoprenoid pathway.
- Working toward other C10-15 hydrocarbons production.









D-milestone:

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

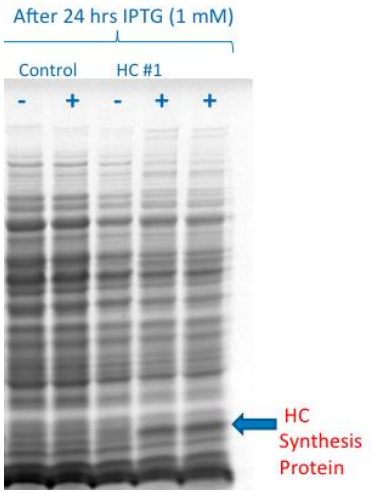
Hydrocarbons Derived from Fatty Acid Pathway

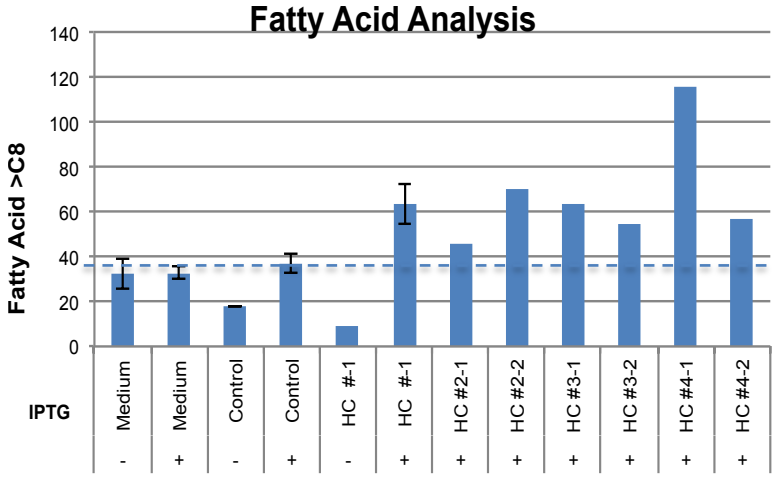
Rationale

- Expression of genes for enzymes required for synthesis of hydrocarbon intermediates from fatty acid pathway in *Z. mobilis.*

Outcome

- Demonstrated fatty acid production in *Z. mobilis* expressing genes for fatty acid synthesis.
- Production of various levels of fatty acids using different sources of fatty acid synthesis genes was observed.
- Different chain lengths of fatty acids (C14 and C18) were obtained which are suitable for jet and diesel applications.
- Working toward expressing synthesis genes for other other fatty acid derivative production.
- Working on identifying key metabolic enzymes to enhance the carbon flux of the pathway.





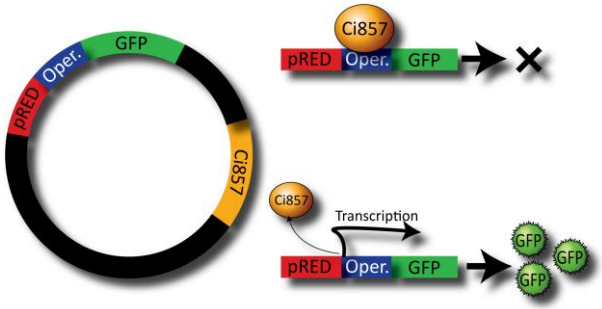
Developing Synthetic Biology Tools

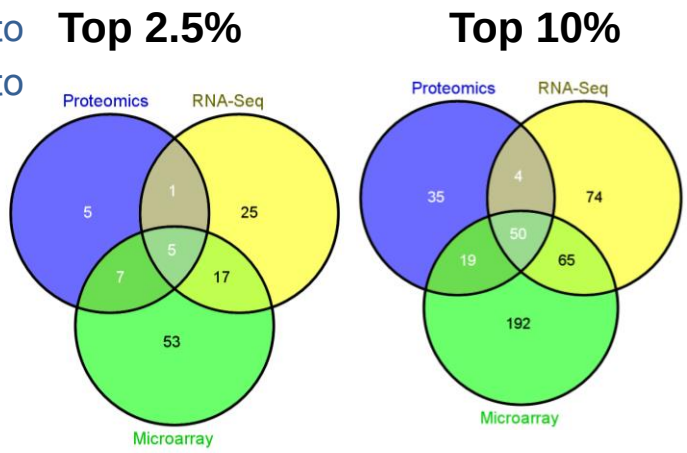
Rationale

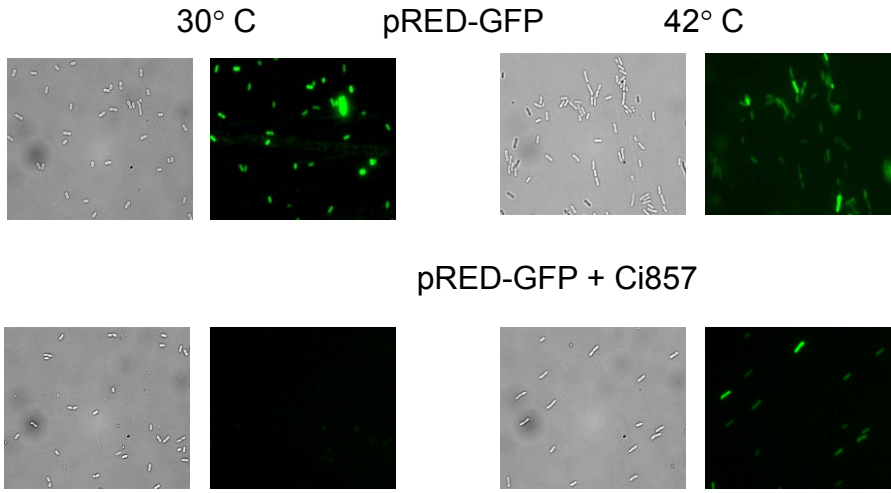
- In order to effectively engineer *Z. mobilis*' metabolic pathway to produce novel biofuel molecules, tools that enable us to redirect the carbon flows are essential.

Outcome

- Promoter Engineering in *Z. mobilis.*
  - Several classes of promoters with various strength were identified based on both transcriptomics and proteomics analysis and will be evaluated.
- Inducible promoter systems
  - Repressor-gene fusion achieved using Gibson Assembly technology.
  - Ptac with IPTG induction is functioning but the expression is leaky.
  - The Lambda red system is functional for GFP induction in *Z. mobilis*.



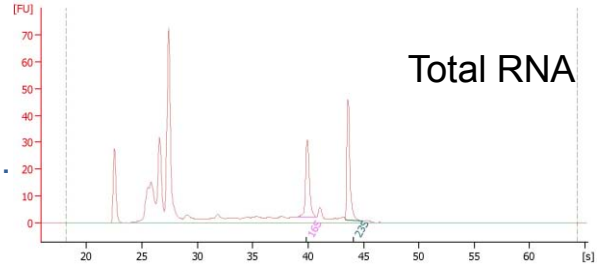




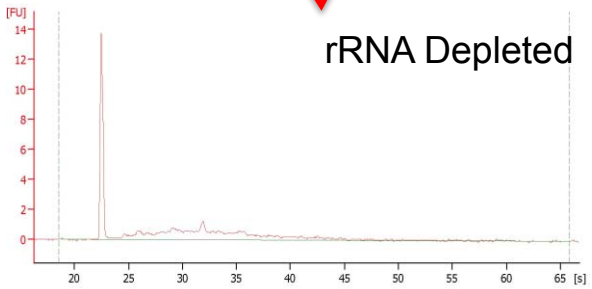
Improving Omics Analysis Tools

Rationale

- RNA-seq provides depth of knowledge on gene expression and small RNA regulations but more than 90% of total RNA is ribosomal RNAs which makes RNA-seq less cost effective.



Total RNA



rRNA Depleted

Outcome

- Worked with scientists in Life Technologies to design customized probes for effective ribosomal RNA removal.
  - Expected total yield after removal (2.5-7.5%)
  - Absence of 16s and 23s rRNA bands by Bioanalyzer analysis
  - qPCR results indicate dramatic rRNA reduction (70 to 3000-fold) after treatment.
- *Z. mobilis* 8b genome sequence finalized and annotated.
  - Corrected genome sequence by adding missing a 2.4-kb fragment.
  - Completed 4 plasmid (41, 39, 33, 33-kb) sequences and annotation.

qPCR Result:

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3 - Relevance

Meeting the goals and objectives of the Biomass Program Multi-Year Program Plan

- Provides direct support for achieving goals to produce renewable gasoline, renewable diesel, and renewable jet from cellulosic biomass at $3/gge by 2022 by developing biocatalysts capable of producing hydrocarbons from biomass sugars.
- Enables dramatically new technologies providing 36 billion gallons of renewable fuels by 2022 (EISA)

- Relevance to the Bioenergy Technologies Office, alignment with MYPP goals, and relevance for the overall bioenergy industry
  - Bt-J. Catalyst Development: There is a need for biological or chemical catalysts for production of advanced biofuels (such as ethanol, renewable gasoline, or diesel) and other bioproducts from the sugar mixture and inhibitors in the hydrolysate broth produced during biomass pretreatment and hydrolysis.

Applications of the expected outputs

- Improved biocatalysts capable of producing hydrocarbons from biomass sugars will be validated with the process integration team. Once superior performance is demonstrated, the biocatalysts can be transferred to be used by industry.
- We consider that our planned publications and presentations will be used by industry to design improved microbial conversion process of sugars to advanced biofuels.

4 - Critical Success Factors

Critical success factors (technical, market, business) which will define technical and commercial viability.

Meet near term and future technical targets for DOE's 2017 goals for advanced biofuels. To some extent, these targets in near term are yet to be defined.

Demonstrate that the successful project will advance the state of technology and positively impact the commercial viability of biomass and /or biofuels.

This will lead to the development of robust strains that are capable of producing hydrocarbons at high yield, rate and titer from biomass sugars. This work leverages the recent successful 2012 demonstration of cellulosic ethanol conversion process using this organism.

Top 2-3 potential challenges (technical and non-technical) to be overcome for achieving successful project results.

- To have effective synthetic biology tools enabling regulation of genes to direct carbon flow toward hydrocarbon production in near term.
- To understand bioenergetics and redox status of the hydrocarbon-producing cells and devise strategy for improving hydrocarbon flux.
- To seek collaboration with academic and industrial partners for enhanced tool development and learning.

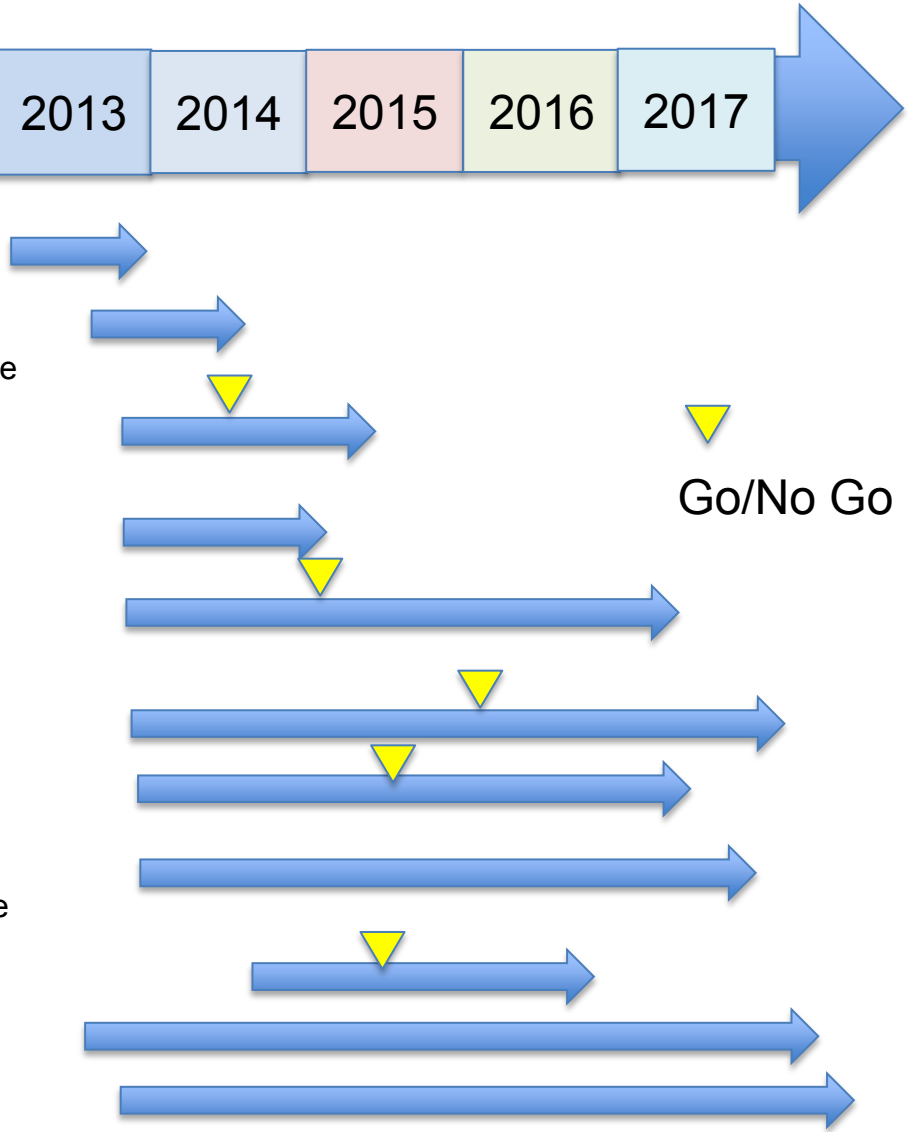
5. Future Work

- Explain what it is you plan to do through the end of the project.
  - We will continue to develop effective promoters and controlled gene expression system (i.e. by small RNA) to regulate the gene expression.
  - We plan to down regulate the carbon flux from pyruvate to ethanol formation and up-regulate hydrocarbon producing pathways.
  - We will overexpress key pathway genes to enhance the hydrocarbon production.
  - Improve xylose utilization rate.
- Provide a Gantt Chart to show estimated timeline.
  - FY13 D-milestone (9/30/2013): Demonstrate production of 0.001% to 1% at least one top candidate high energy fuels and/or intermediates using *Z. mobilis* from glucose and xylose.
- Highlight upcoming key milestones – go/no go decision points.
  - Need to identify what are the key targets that affect hydrocarbon production. If there is bioenergetics limitation, we would devise strategies to overcome the barrier.
- Address how you will deal with any decision points during that time and any remaining issues.
  - Develop genome engineering tools in *Zymomonas.*
  - Engineer efficient energy generation pathways to provide sufficient metabolic energy for hydrocarbon synthesis.

5. Future Work-Gantt Chart

Activities:

- Demonstrate production of 0.001% to 1% at least one top candidate high energy fuels and/or intermediates using *Z. mobilis* from glucose and xylose.
- Develop effective promoters and controlled gene expression system (i.e. by small RNA) to regulate the gene expression.
- Develop genome engineering tools in Zymomonas.
- Down regulate the carbon flux from pyruvate to ethanol formation and up-regulate hydrocarbon producing pathways.
- Identifying key metabolic genes that impact the hydrocarbon synthesis in both isoprenoid and fatty acid pathways (omics)
- Overexpress key pathway genes to enhance the hydrocarbon production.
- Improve xylose utilization rate.
- Need to identify what are the key targets that affect hydrocarbon production: experimental measurement and metabolic modeling. If there is bioenergetics limitation, we would devise strategies to overcome the barrier.
- Engineer efficient energy generation pathways to provide sufficient metabolic energy hydrocarbon synthesis.
- Fermentation evaluation
- TEA analysis



Summary

Approach

- Applying metabolic engineering and synthetic biology tools to engineer *Z. mobilis* for synthesis of high-energy fuel molecules and/or intermediates that can be converted into renewable fuels.
- Understand the technical barrier associated microbial hydrocarbon synthesis in *Z. mobilis*.

Technical accomplishments

- Demonstrated farnesene (C15) production from glucose and xylose in *Z. mobilis* as *well* as fatty acids (C14-C18) synthesis strain expressing respective hydrocarbon synthesis genes.

Relevance

- Provides direct support for achieving goals to produce renewable gasoline, renewable diesel, and renewable jet from cellulosic biomass at $3/gge by 2022 in the MYPP.

Critical success factors and challenges

- Meet near term and future technical targets for DOE's 2017 goals for advanced biofuels.
- Establish effective synthetic biology tools enabling regulating the genes directing carbon flow to hydrocarbon production in near term.
- Understand bioenergetics and redox status of the hydrocarbon producing cells and devise strategy for improving hydrocarbon flux.

Future Work

- Continue to develop effective synthetic biology tools to down-regulate the carbon flux from pyruvate to ethanol formation and up-regulate hydrocarbon producing pathways and overexpress key pathway genes to enhance the hydrocarbon production
- Understand the impact of hydrocarbon synthesis on bioenergetics and redox through experimental measurement and metabolic modeling.

Technology transfer

- Protect IPs, publish papers and present at conferences and open to collaborate with partners.

Acknowledgments

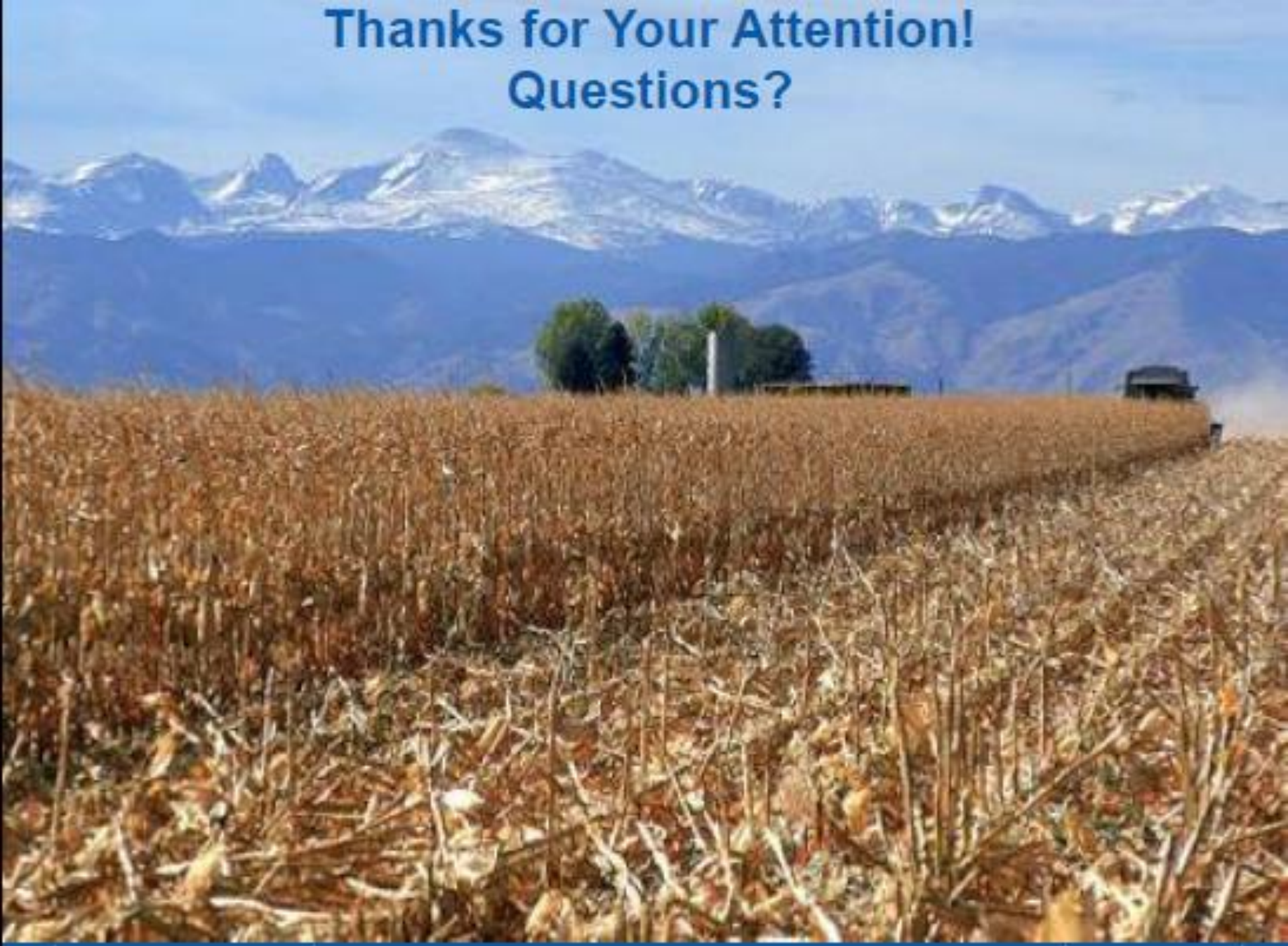
Funding – DOE Bioenergy Technologies Office (BETO)

Thank you ….

BETO Technical Managers: Joyce Yang, Leslie Pezzullo

NREL: Yat-Chen Chou, Mary Ann Franden, Shihui Yang, William Michener, Michael Guarneri, Jeff Linger, Lieve Laurens, Stefanie Van Wychen and Adam Bratis,

Thanks for Your Attention!
Questions?



Additional Slides

Responses to Previous Reviewers' Comments

- If yours is an on-going project that was reviewed previously, address 1-3 significant questions/criticisms from the previous reviewers' comments (refer to the 2011 reviewer comments if needed).

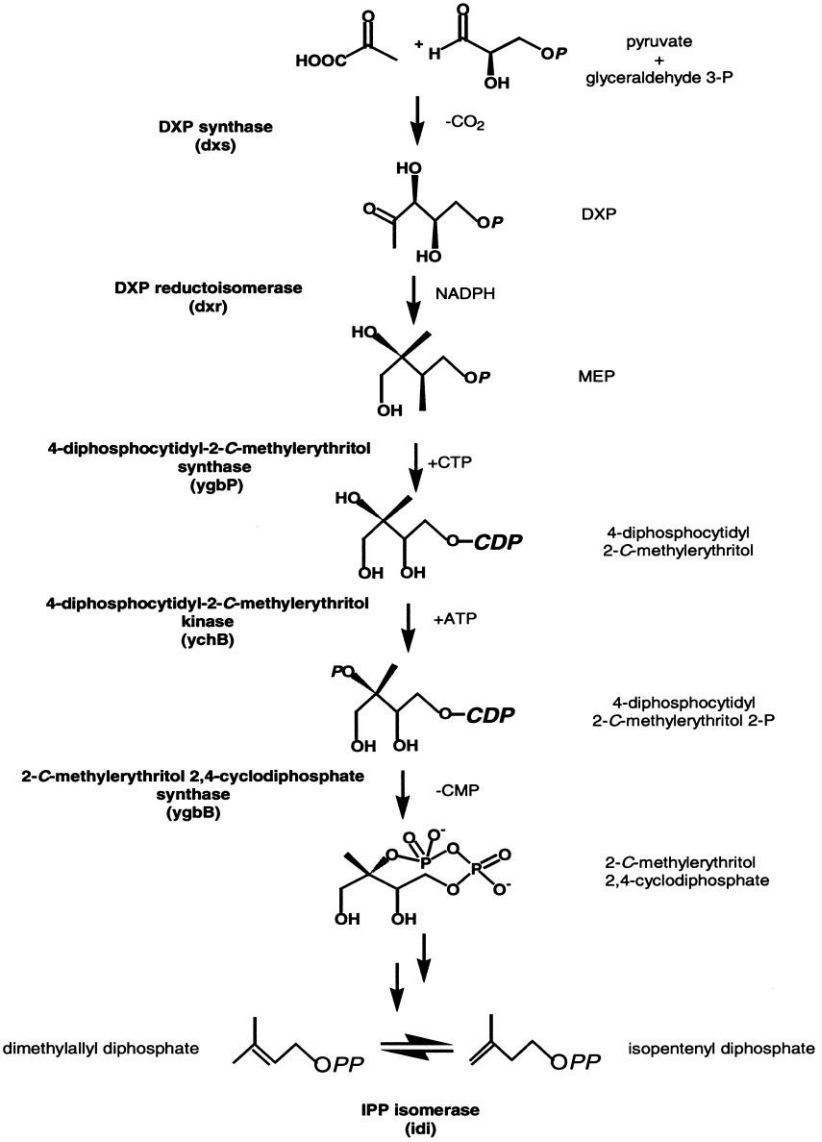
- N/A

Note: This slide is for the use of the Peer Review evaluation only – it is not to be presented as part of your oral presentation, but can be referenced during the Q&A session if appropriate. These additional slides will be included in the copy of your presentation that will be made available to the Reviewers and to the public.

Publications, Presentations, and Commercialization

- "Essential Synthetic Biology Tools for Engineering Zymomnas mobilis for hydrocarbon production" poster presentation by by Min Zhang, Jeffrey Linger, Shihui Yang, Yat-Chen Chou and Michael Guarnieri at the Keystone Symposia: Precision Genome Engineering and Synthetic Biology: Designing Genomes and Pathways (C5), March 17-22, 2013, Breckenridge, CO
- Submitted an abstract on "Engineering Zymomonas for Hydrocarbon Production" by Yat-Chen Chou and Min Zhang for 2013 SIMB Annual meeting, August 11-15, 2013.
- ROI on hydrocarbon production by Zymomonas submitted.

Isoprenoid Synthesis Pathway in Z. mobilis



- Possess methylerythritol phosphate pathway (MEP) or deoxyxylulose 5-phosphate pathway(DXP) (no evidence of mevalonate pathway (MVP))

- Identification of *dxr* gene and characterization
- of the 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXP) (Grolle *et al.* FEMS Microbiology Letters (2000)

- Synthesis of the C5-precursor isopentenyl diphosphate (IPP) and isomerization to dimethylallyl diphosphate (DMPP)
- Elongation of DMPP by head to tail condensation of two IPP to farnesyl diphosphate (C15, FPP)

Isoprenoid end products

General Presentation Guidelines

(not to be included in your presentation)

*Draft presentations should be uploaded to the Peer Review Portal website by **April 22, 2013**. More information on using the Peer Review Portal will be forthcoming.*

*BETO will review your draft presentations and provide feedback to you on any requested changes to your powerpoint slides between **April 29th-May 3rd***

*Final presentations should be submitted in Adobe PDF or Microsoft PowerPoint (PPT preferred) format by **May 3rd.** The file you submit will be made available for download by the general public through the meeting agenda webpage.*

Please remember to double check the meeting agenda webpage for your presentation time. As a general guideline, please plan on 1 min per slide to leave enough time for Q&A.

Suggested fonts are Times New Roman, Arial, and Tahoma.

To ensure ease of reading, we STRONGLY recommend that you use a minimum font size of 20 pt.

Highlight your main point or heading with a dominant color.

Format with landscape orientation.

Contrasting colors work best. Use dark colored fonts on a white or light colored background

Avoid intensely bright or saturated colors that compete with the text.

Keep color scheme consistent throughout your presentation. Changing colors and type styles can be confusing and distract from your message.

Do not include external files (such as video clips) in your presentation. To share additional files and/or information with the review panel only, use the "other" file upload option.

Consider adding a slide that gives definitions for any acronyms you use during your presentation, for those of our attendees who aren't as fluent in certain areas.

Though your presentation will be in color, it is best to choose colors and data symbols so that they can be easily distinguished in black and white for those reviewers with hardcopies.