Project Goal

Goals

- This Seed project aims to produce isoprene (C₅H₈; 2-methyl-1,3-butadiene) from renewable biomass using cellulose-degrading microbes, a consolidated bioprocessing (CBP) approach.
- This project provides the proof-of-concept of isoprene production in genetically engineered Clostridium thermocellum, a cellulose degrader.

Relevance and Outcomes

- Isoprene is an important precursor to commodity chemicals and can be upgraded to branched alkane biofuels for blending into existing fuel infrastructure for gasoline, jet, and diesel fuels.
- This project provides an innovative solution to address BETO MYPP and performance goals ($3/gge, 2022 cost goal) toward developing commercially viable bioenergy and bioproduct technologies.
Quad Chart Overview

Timeline
• Project start date: November 2013
• Project end date: September 2015
• Percent complete: 50%

Barriers
• Barriers addressed
  – Bt-F: Hydrolytic Enzyme Production
  – Bt-G: Enzyme Efficiency
  – Bt-I: Catalyst Efficiency

Budget

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<th>Total Costs FY 10 – FY 12</th>
<th>FY 13 Costs</th>
<th>FY 14 Costs</th>
<th>Total Planned Funding (FY 15-Project End Date)</th>
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NREL Partners
• Computational and Modeling Team (5%)
• Analysis Team (10%)
Project Overview

**History:** A Seed project started in FY14, using a CBP approach aimed to streamline the biomass conversion process - a new paradigm.

**Context:**
- Isoprene can be produced in *C. thermocellum* via expressing a single foreign gene “isoprene synthase” (*ispS*) from plants.
- Proprietary genetic tools for *C. thermocellum* has been developed with funding support from EERE Fuel Cell Technologies Office, hence leveraging funding for BETO to accelerate progress.
- Isoprene is nontoxic and a gas at 34 °C - helping its harvesting.
- Theoretical carbon conversion efficiency is 83%, using glyceraldehyde-3-phosphate (G3P; 3C) and pyruvate (3C) as precursors.

**Objective:** Develop *C. thermocellum* as the model catalyst for cellulosic isoprene, a precursor for both biofuels and biochemicals.

DMAPP: dimethylallyl pyrophosphate
Technical Approach

• Approach:
  – Express isoprene synthase from kudzu vine (Pueraria montana; good candidate based on literature) and hybrid aspen (Populus canescens; with crystal structure) in the thermophilic C. thermocellum (55-60 °C).
  – Increase productivity via metabolic pathway engineering.
  – Use techno-economic analysis (TEA) to guide research directions.

• Potential Challenges:
  – IspS is of plant origin, its thermal tolerance is unknown.
  – Isoprene rate, yield, and titer may warrant improvements via metabolic pathway engineering.
  – Further improvement of genetic tools.

• Critical Success Factors
  – Improvements in isoprene carbon conversion efficiency, rate, and productivity.
  – Bioreactor scale-up fermenting high cellulose solid loadings (30%).
Cellulose to Isoprene in *C. thermocellum*

**Biomass** → **Cellulose** → **Cellulosomes** → **Cellobiose/Celldextrin** → **isoprene**

Anaerobic fermentation co-produces H₂.

**Glucose** → **G3P** → **PEP** → **Pyruvate**

NAD⁺ → **NADH**

**Lactate** ↔ **Formate**

**Pyruvate** → **Acetyl-CoA** → **Acetate** → **Ethanol**

**CO₂** → **H₂** → **2NADH** → **2NAD⁺**

**CO₂** → **IPP** → **IPPP** → **DMAPP** → **IspS** → **IspS** → **MEP Pathway**

**IspS** → **IDI?** → **Cellulosome: cellulases and hemicellulases**

**MEP**: methylerthritol phosphate; **DXS**: 1-deoxy-D-xylulose-5-phosphate synthase; **DXR**: 1-deoxy-D-xylulose-5-phosphate reductoisomerase; **DMAPP**: dimethylallyl pyrophosphate; **IPP**: isopentenyl pyrophosphate; **IDI**: isomerase.
Areas of Integration – 2022 Process

CBP: one step

- CBP offers innovative solution by combining **four** processes in a single step to streamline unit operations.
- This project contributes knowledge in the areas highlighted, interacting with research teams in biomass pretreatment, analysis, and bench- and pilot-scale integration.
Management Approach

- Multi-disciplinary team approach recruiting molecular and microbiologist, chemical engineer, and computational modeler.
- TEA guides research directions.
- Employ Quarterly Milestones and Go/No-Go Decision to manage progress and performance.
- Synergistic interactions with teams across the Biochemical Conversion Platform.
  - Integrate CBP approach with other enabling technologies.
Technical Accomplishments/Progress/Results
Dual Approaches to Address IspS Thermal Tolerance

(1) Bioinformatic Approach

- Use “Consurf” tool to predict conserved areas of 18 IspS sequences.
- Use “Pymol” tool to overlay Consurf results to IspS crystal structure.
- Identify four amino acid residues for mutation to improve thermal tolerance *(completed Q1 Milestone)*.
  - Alanine (102th; NEK’A’) to valine
  - Phenylalanine (163th; QHG’F’) to tyrosine
  - Leucine (241th; NHALA’L’) to isoleucine
  - Arginine (245th, LH’R’) to tryptophan

- **Next step:** build an IspS mutant library in *E. coli* and test IspS temperature profiles, *in vitro*.

(2) Express WT IspS in *E. coli*

IPTG-inducible *ispS* gene overexpression in *E. coli* BL21

Crystal structure of gray poplar

Gregg Beckham

Katherine Chou

![Image of IspS protein detection](image_url)

IspS Protein Detected

**IPTG**

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<th>0</th>
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<td></td>
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<td><strong>Populus x Canescens</strong></td>
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62.4 kDa
Isoprene Synthase is Thermal Stable at 55 °C

- IspS from kudzu (K-IspS) and hybrid aspen (PC-IspS) were successfully expressed in *E. coli* and assayed for isoprene production *in vitro*, by adding its substrate DMAPP (dimethylallyl pyrophosphate) at various temperatures.

- Both IspSs are thermal stable at up to 55 °C, the latter an optimal temperature for *C. thermocellum*.

- Activity of PC-IspS is 22-30% higher than K-IspS.

- Completed FY14 Q2 Milestone.

- The findings preclude the need of IspS protein engineering for thermal tolerance – accelerating progress.
Over-express Isoprene Synthase in *C. thermocellum*

- Two *ispS* shuttle vectors constructed - completed FY14 Q3 Milestone
- Generated two *C. thermocellum* transformants harboring *ispS* gene.

These *C. thermocellum* transformants will be assayed for isoprene production, both *in vivo* and *in vitro*.

*cat*: thiamphenicol antibiotic marker; NTC: no template control.
**In Vivo Isoprene Titer**

- **Average isoprene titer:** 27 µg/g dry cell wt. (arrows) – **meeting FY14 Q4 Go/No-Go Milestone.**
- **FY15 goal:** focus on metabolic engineering to increase titer and productivity.
Ongoing: Over-expression of dxs and/or dxr

- Literature reveals over-expression of dxs/dxr led to 2.3-fold increase in isoprene production and 8.8-fold increase in limonene production.
- Constructed dxr synthetic operon for its over-expression in C. thermocellum.

dxr operon constructed in E. coli

Construction of dxs synthetic operon is underway.
Isoprene Techno-Economic Analysis

**Objective:** Perform TEA to assess economic potential of biomass to Isoprene

**Findings from Preliminary TEA:**

- Isoprene averaged 153,000 metric ton/yr from 2000-2013 and projected at 200,000 metric ton/year in 2018.
- Isoprene price is averaged at $2.69/kg from 2010-2013.
- By eliminating the aeration step, anaerobic pathway has less CapEx comparing to aerobic pathway (compressor, small reactor, mass transfer).
- Isoprene gas separation will be simplified without air.
- H₂ co-production favors economic values for isoprene upgrade.
Relevance

- Relevant to BETO MYPP objective to produce renewable biofuels and chemicals at $3/gge by 2022.
- Specifically target three MYPP barriers: hydrolytic enzyme production (Bt-F), enzyme efficiency (Bt-G), and catalyst efficiency (Bt-I).
- CBP microbes could potentially simplify biomass pretreatment and fermentation, hence stimulate the emerging bioenergy industry.
- Advance the state-of-technology by applying an anaerobic microbe which eliminates aeration cost required in an aerobic process.
- High temperature fermentation (55 °C) helps isoprene harvesting (a gas) and minimizes contaminations.
- CBP concept is transformative and could boost commercial viability of biomass-to-biofuels conversion.
Future Work in FY15

- **2015 Q4 Go/No-Go Decision:** Demonstrate an isoprene titer of 10 mg/g dcw, a **370-fold** improvement over the 2014 baseline of 20 µg/g cdw via metabolic pathway engineering.
- Determine rate-limiting steps: by comparing isoprene production rates *in vivo* (substrate is limiting) vs. *in vitro* (enzyme is limiting).
- Already generated *C. thermocellum* mutants lacking both formate and lactate competing pathways, hence more pyruvate flux.
- Over-express *dxs/dxr/ispS* in the above host to increase flux from pyruvate and G3P to DMAPP.
- Future work will knock down the production of geranyl diphosphate and over-express the isomerase (*idi*), the latter was not found in the *C. thermocellum* genome.
Summary

• CBP microbe combines cellulase enzymes production, cellulose hydrolysis, and fermentation in one consolidated step.
• Address BETO MYPP technical barriers with metabolic engineering efforts toward improving microbial catalyst. NREL’s proprietary genetic tool is an enabling technology.
• Isoprene is non-toxic, easy to harvest, and a precursor for both chemicals and biofuels. Isoprene production requires the heterologous expression of isoprene synthase from plant.
• Both K-IspS and PC-IspS are thermal tolerant and expression in \textit{C. thermocellum} yielded a titer of 27 µg/g dcw – proof of concept.
• Over-expression of rate-limiting steps is underway in \textit{C. thermocellum} host lacking both lactate and formate competing pathways to boost isoprene yield, productivity, and titer.
• TEA revealed less CapEx for anaerobic pathway, with H₂ co-production supports isoprene upgrade and adds economic value.
The following slides are to be included in your submission for Peer Evaluation purposes, but will not be part of your oral presentation –

You may refer to them during the Q&A period if they are helpful to you in explaining certain points.
NREL: Katherine Chou, Ling Tao, Jennifer Markham, Venkat Subramanian, Gregg Beckham, Rick Elander, and Adam Bratis

Funding – DOE Bioenergy Technologies Office (BETO)

Thank you ....
Responses to Previous Reviewers’ Comments

• None, a new Seed Project started in FY14.

Note: This slide is for the use of the Peer Reviewers only – it is not to be presented as part of your oral presentation. These Additional Slides will be included in the copy of your presentation that will be made available to the Reviewers.
None.

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