NREL
NATIONAL RENEWABLE ENERGY LABORATORY

DOE Bioenergy Technologies Office (BETO) 2015 Project Peer Review

Advanced Biofuels from Cellulose via Genetic Engineering of *Clostridium thermocellum*

March 26th, 2015

Technical Area Review: Biochemical Conversion

Principal Investigator: Pin-Ching Maness

National Renewable Energy Laboratory

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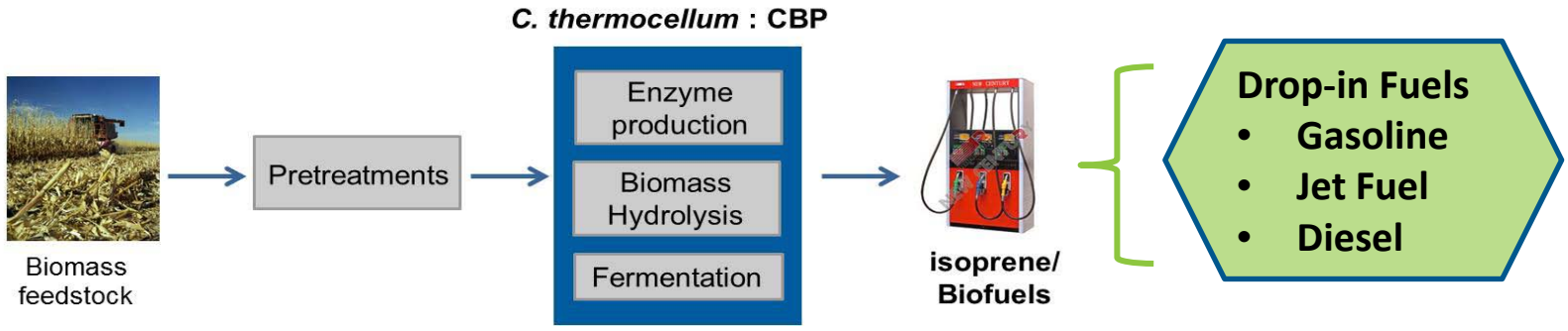
Project Goal

Goals

- This Seed project aims to produce isoprene (C_5H_8; 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

	Total Costs FY 10 – FY 12	FY 13 Costs	FY 14 Costs	Total Planned Funding (FY 15-Project End Date
DOE Funded	0	0	$163,500	$246,470
Project Cost Share (Comp.)*	0	0	0	0

NREL Partners

- Computational and Modeling Team (5%)
- Analysis Team (10%)

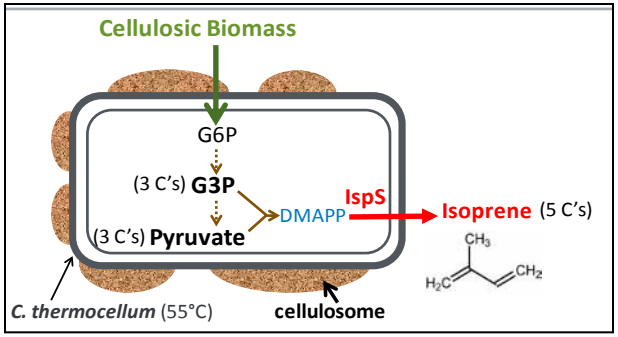
Project Overview

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

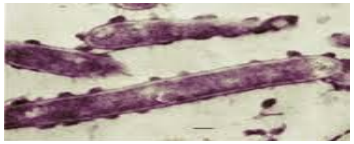
Context:

- 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.

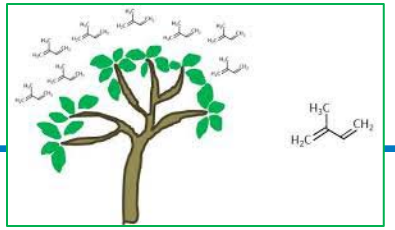
Objective: Develop *C. thermocellum* as the model catalyst for cellulosic isoprene, a precursor for both biofuels and biochemicals.



DMAPP: dimethylallyl pyrophosphate

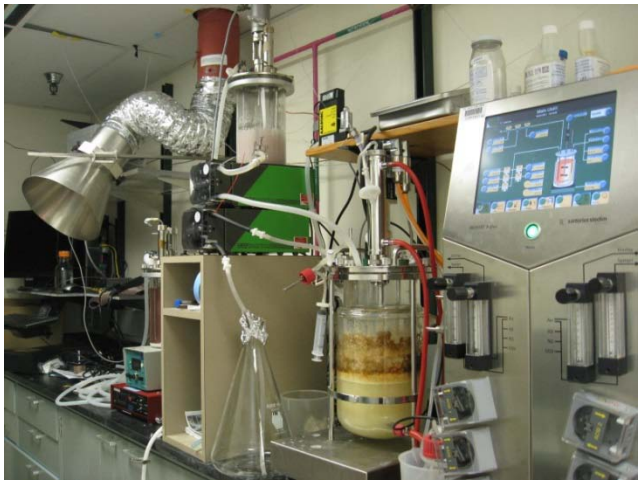


Technical Approach



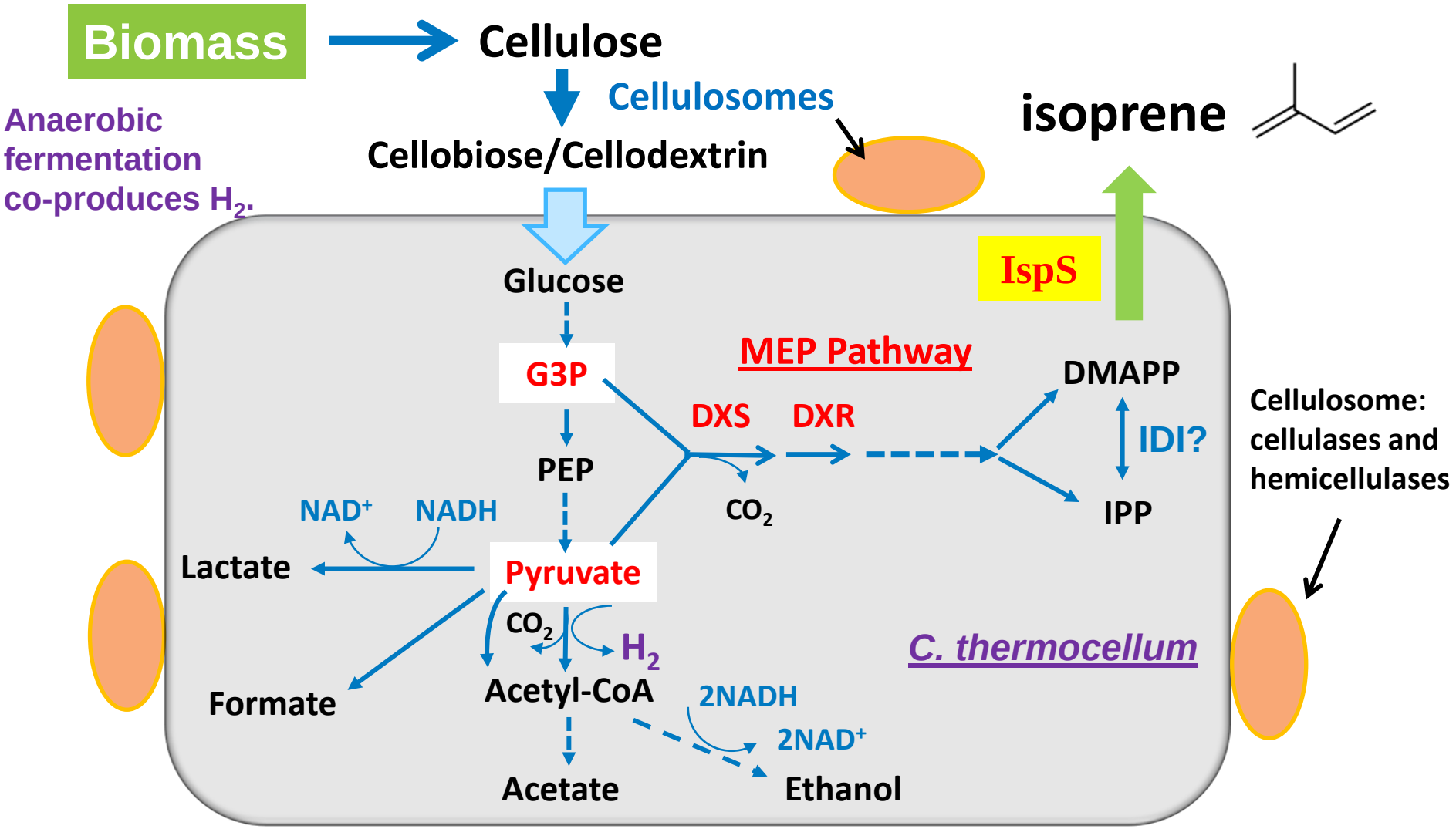
- **Approach:**
  - Express isoprene synthase from kudzu vine (*Pueraria montana*; good candidate based on literature) and hybrid aspen (*Populus canescens;* with crystal structure) in the thermophilic *C. thermocellum* (55-60 °C).
  - Increase productivity via metabolic pathway engineering.
  - Use 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.

High Solid Fermentation



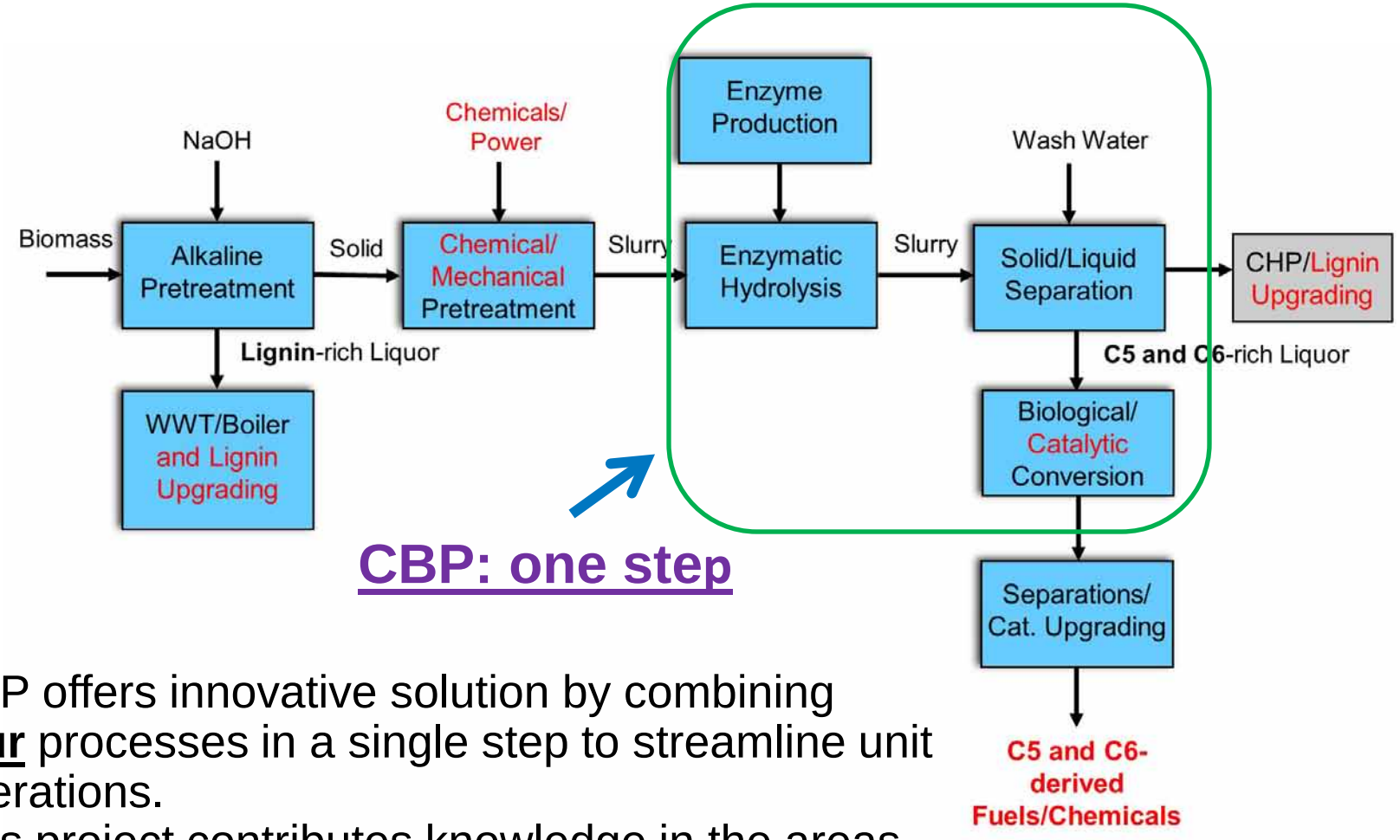
- **Critical Success Factors**
  - Improvements in isoprene carbon conversion efficiency, rate, and productivity.
  - Bioreactor scale-up fermenting high cellulose solid loadings (30%).

Cellulose to Isoprene in *C. thermocellum*



MEP: methylerythritol 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.

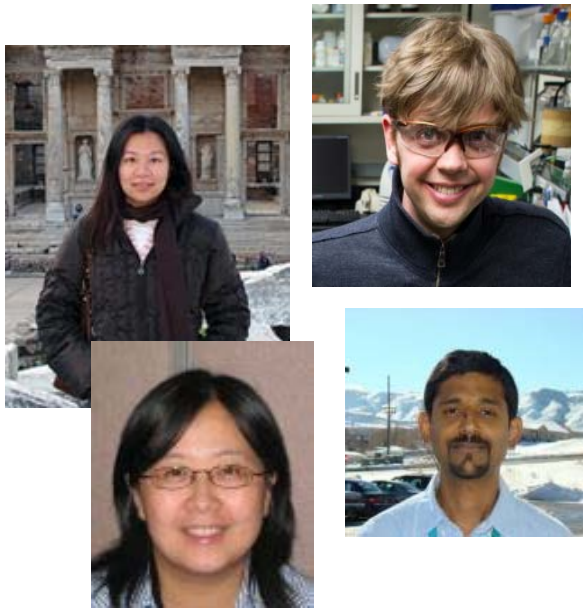
Areas of Integration – 2022 Process

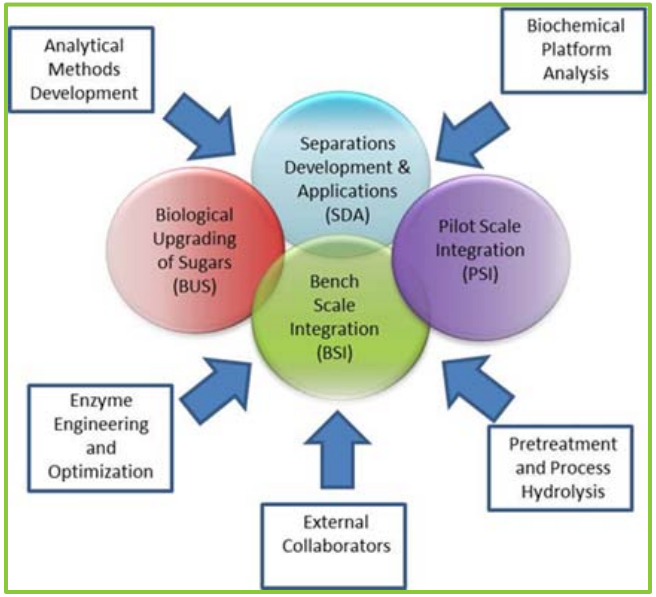


- CBP offers innovative solution by combining **four** processes in a single step to streamline unit operations.
- This project contributes knowledge in the areas highlighted, interacting with research teams in biomass pretreatment, analysis, and bench- and pilot-scale integration.

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.

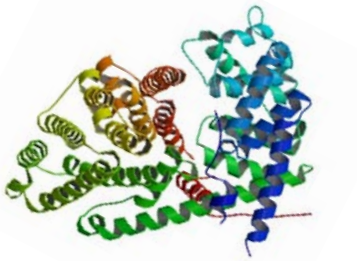




Technical Accomplishments/Progress/Results

Dual Approaches to Address IspS Thermal Tolerance

(1) Bioinformatic Approach

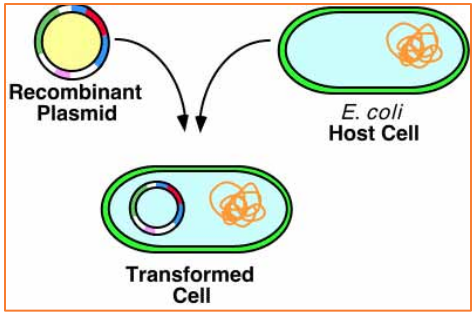


Crystal structure of gray poplar

Gregg Beckham

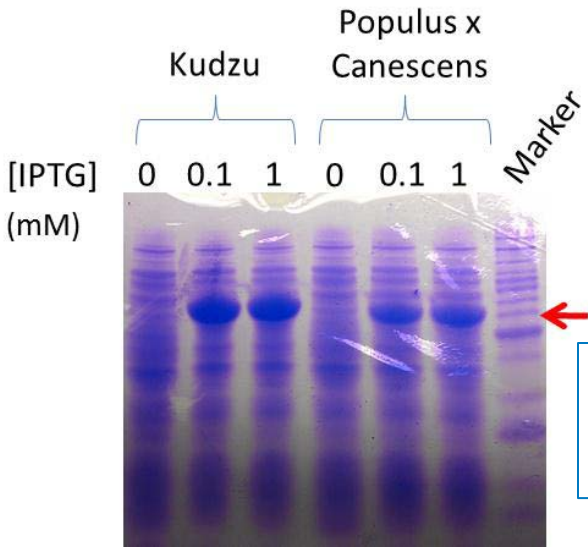
- Use “Consurf” tool to predict conserved areas of **18** IspS sequences.
- Use “Pymol” tool to overlay Consurf results to IspS crystal structure.
- Identify four amino acid residues for mutation to improve thermal tolerance **(completed Q1 Milestone)**.
  - Alanine (102th; NEK’A’) to valine
  - Phenylalanine (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*



Katherine Chou

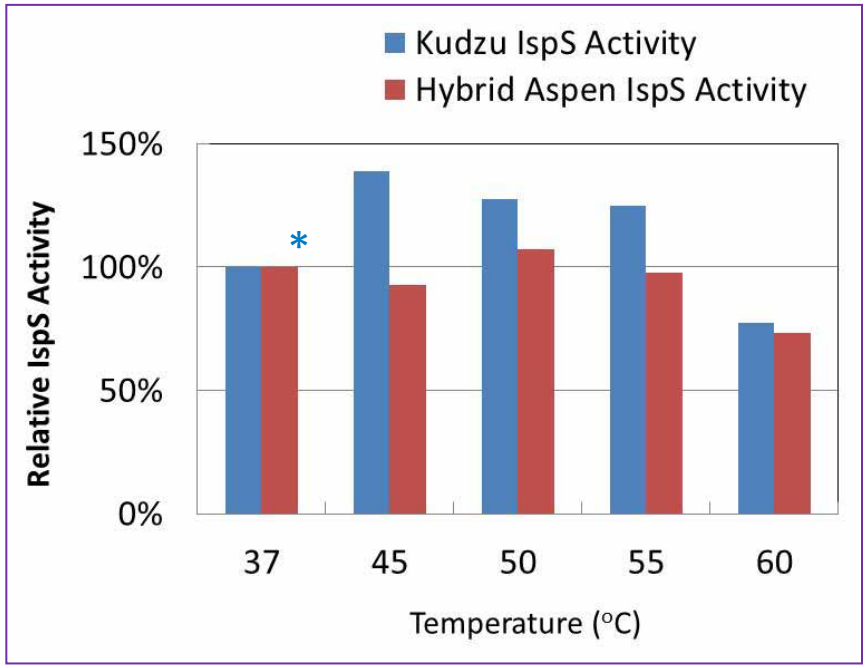
IPTG-inducible *ispS* gene overexpression in *E. coli* BL21



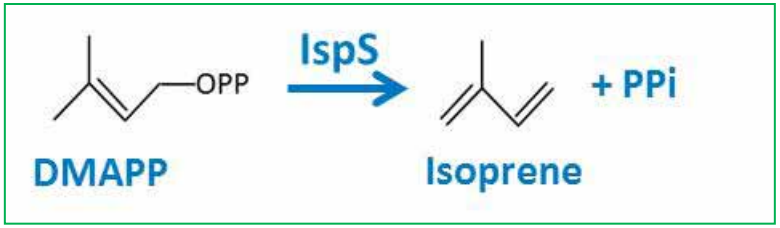
IspS Protein Detected

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*.



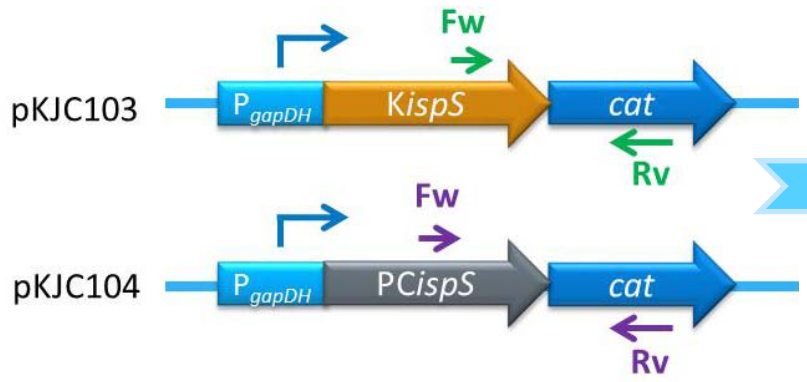
*Activity of PC-IspS is 22-30% higher than K-IspS

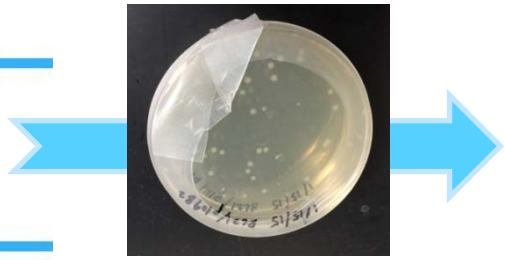


- **Completed FY14 Q2 Milestone.**
- The findings preclude the need of IspS protein engineering for thermal tolerance – accelerating progress.

Over-express Isoprene Synthase in *C. thermocellum*

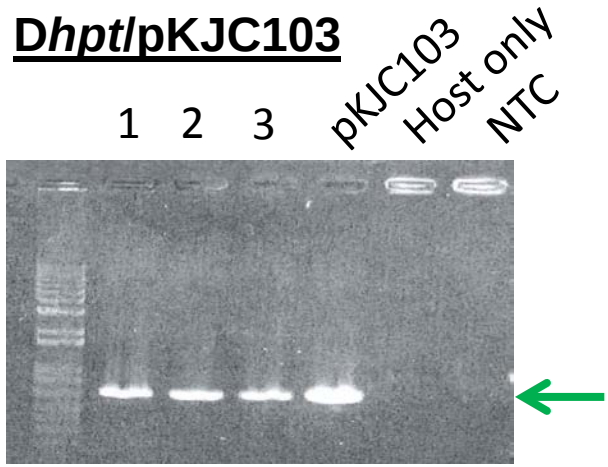
- Two *ispS* shuttle vectors constructed - **completed FY14 Q3 Milestone**
- Generated two *C. thermocellum* transformants harboring *ispS* gene.



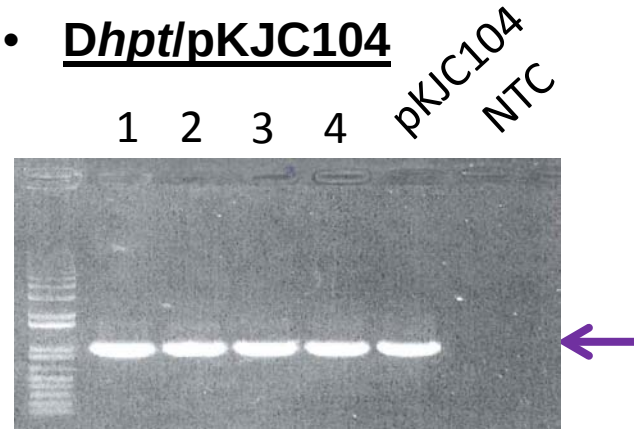


Colony PCR

- **D*hpt*/pKJC103**



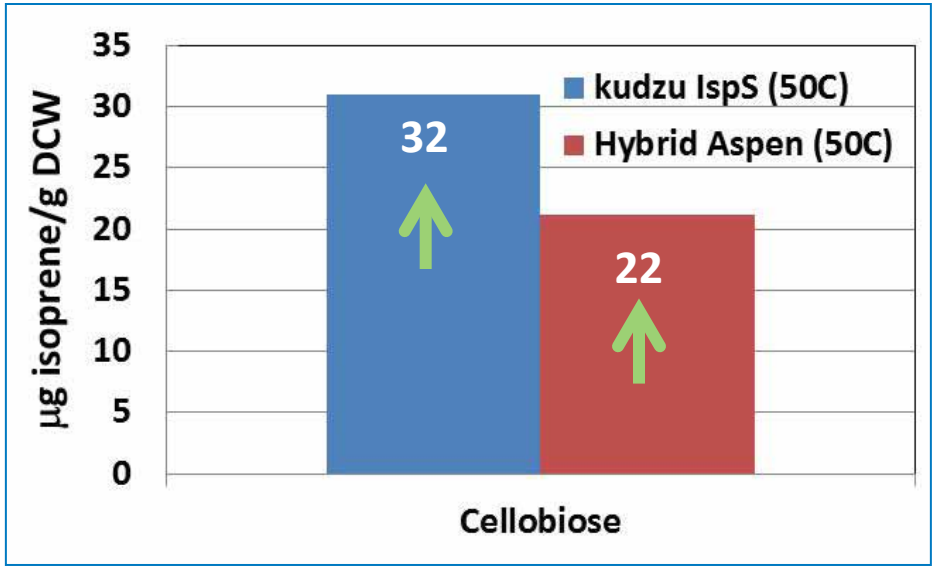
- **D*hpt*/pKJC104**

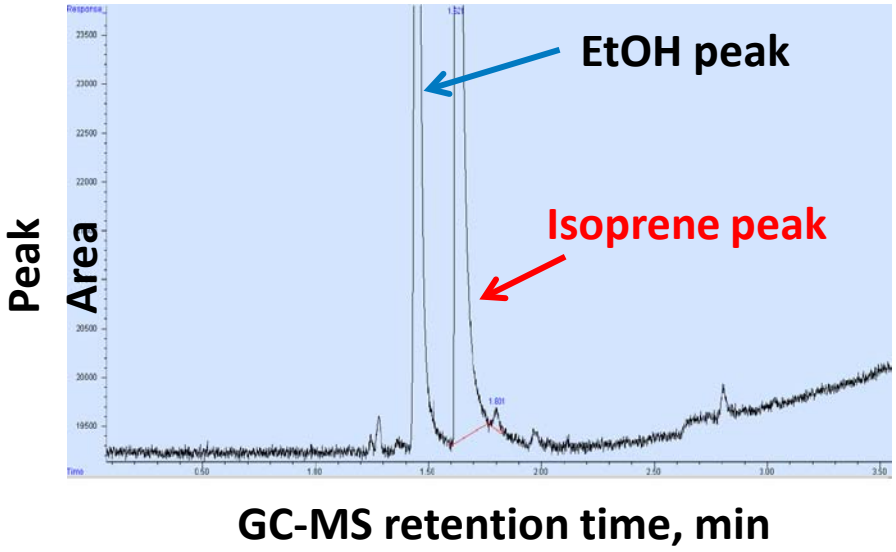


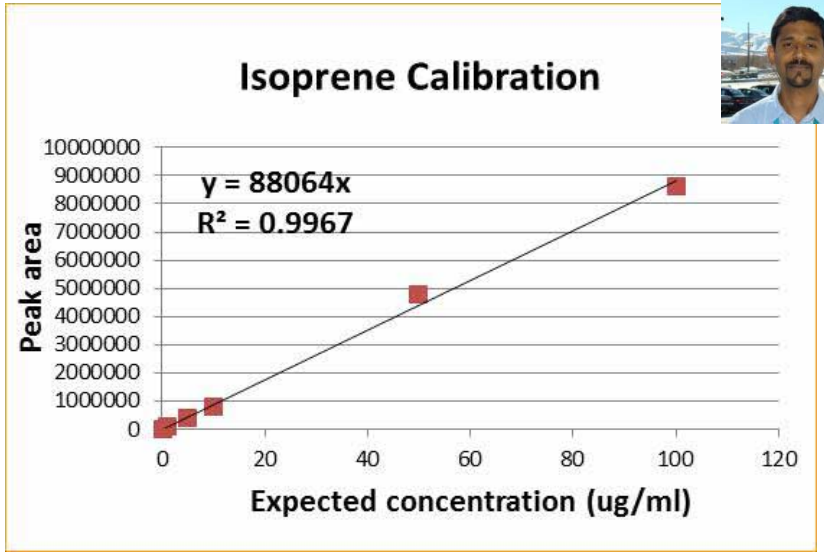
These *C. thermocellum* transformants will be assayed for isoprene production, both *in vivo* and *in vitro*.

cat: thiamphenicol antibiotic marker; NTC: no template control.

In Vivo Isoprene Titer



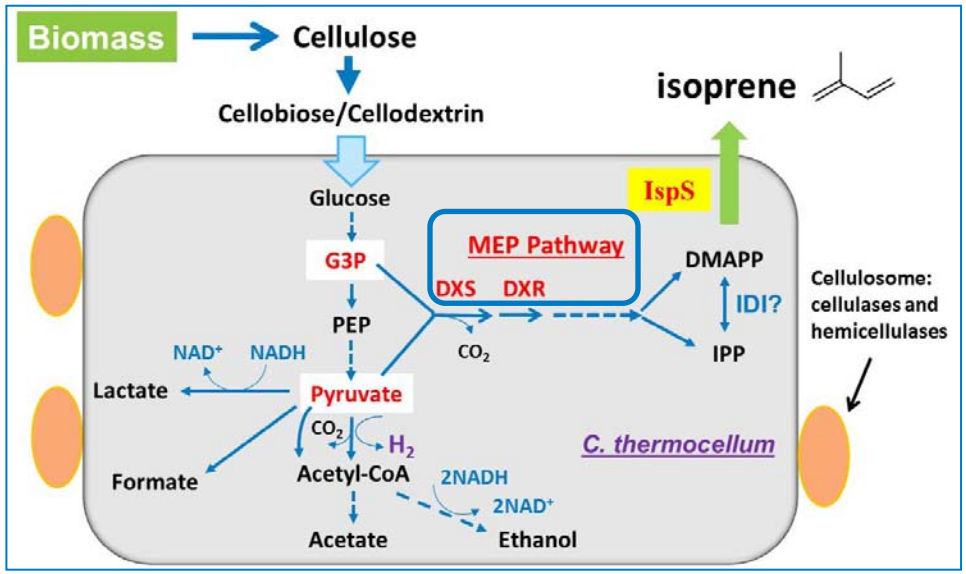




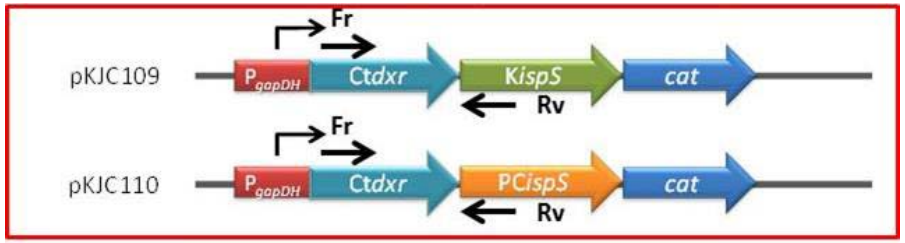
- Average isoprene titer: 27 µg/g dry cell wt. (arrows) – **meeting FY14 Q4 Go/No-Go Milestone**.
- FY15 goal: focus on metabolic engineering to increase titer and productivity.

Ongoing: Over-expression of *dxs* and/or *dxr*

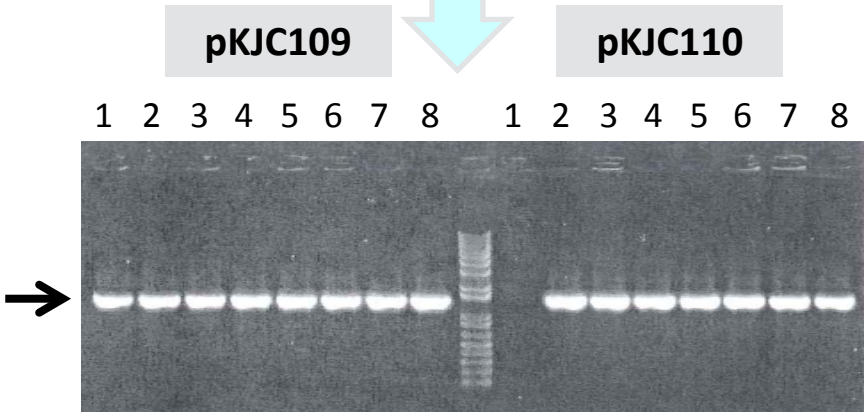
- Literature reveals over-expression of *dxs/dxr* led to 2.3-fold increase in isoprene production and 8.8-fold increase in limonene production.
- Constructed *dxr* synthetic operon for its over-expression in *C. thermocellum.*



dxr* operon constructed in *E. coli



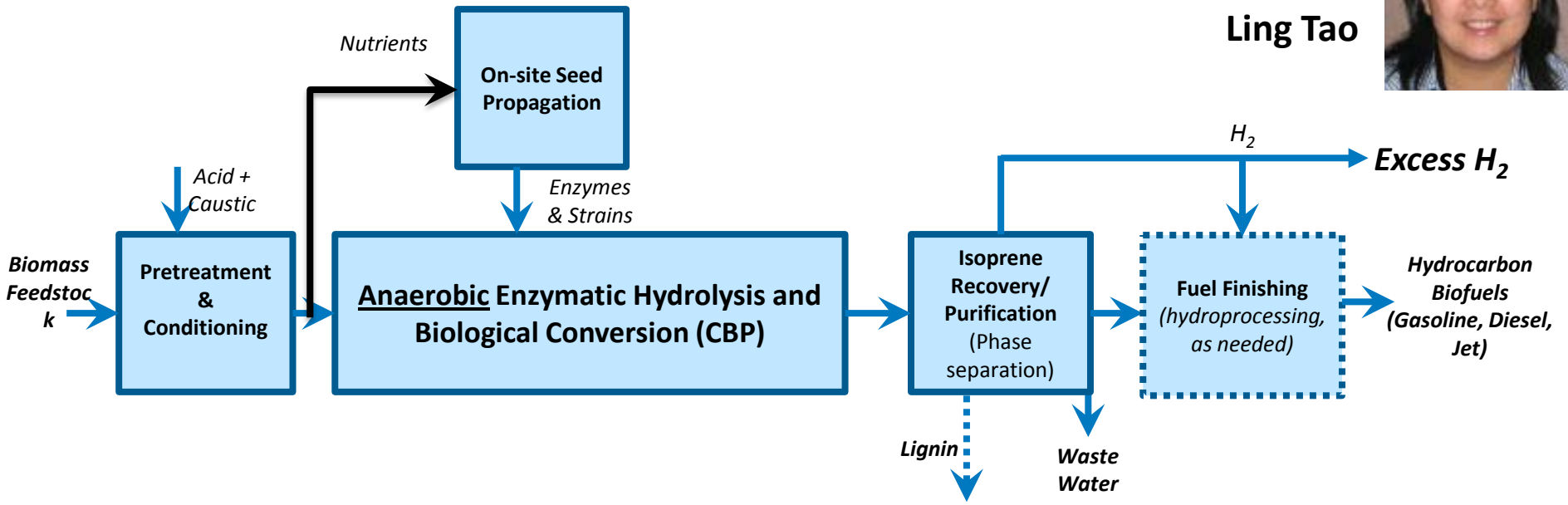
PCR



- Construction of *dxs* synthetic operon is underway.

Isoprene Techno-Economic Analysis

Ling Tao



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

Findings from Preliminary TEA:

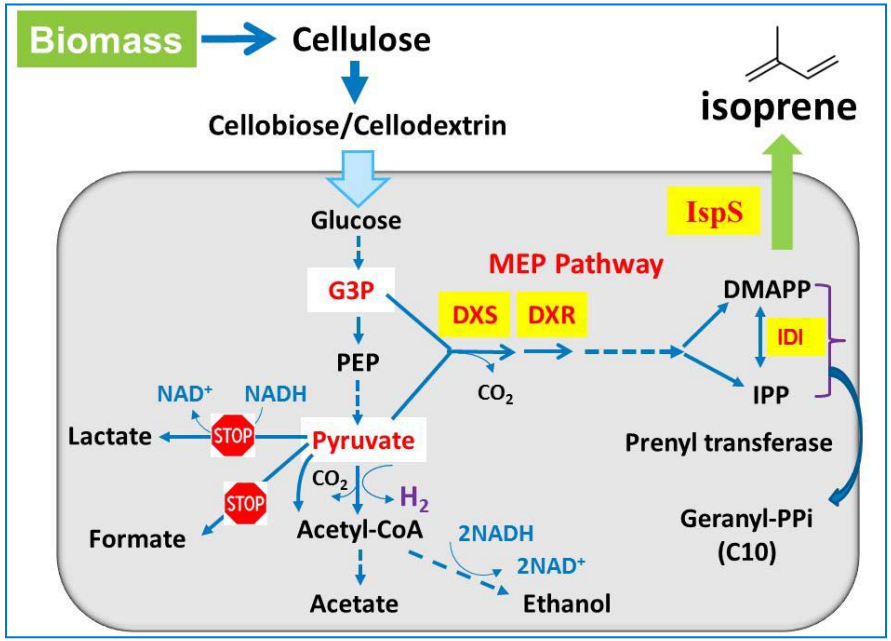
- Isoprene averaged 153,000 metric ton/yr from 2000-2013 and projected at 200,000 metric ton/year in 2018.
- Isoprene price is averaged at $2.69/kg from 2010-2013.
- By eliminating the aeration step, anaerobic pathway has less CapEx comparing to aerobic pathway (compressor, small reactor, mass transfer).
- Isoprene gas separation will be simplified without air.
- H_2 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

- **<u>2015 Q4 Go/No-Go Decision:</u>** 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 *C. thermocellum* yielded a titer of 27 µg/g dcw – proof of concept.
- Over-expression of rate-limiting steps is underway in *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 H_2 co-production supports isoprene upgrade and adds economic value.

(Not a template slide – for information purposes only)

- ***The following slides are to be included in your submission for Peer Evaluation purposes, but <u>will not be part of your oral presentation</u> –***
- ***You may refer to them during the Q&A period if they are helpful to you in explaining certain points.***

Acknowledgements

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.**

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Publications, Patents, Presentations, Awards, and Commercialization

- **None.**

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