U.S. Department of Energy (DOE)
Bioenergy Technologies Office (BETO)
2019 Project Peer Review
Fermentative Production of Tricarboxylic Acid Cycle-Derived Chemicals Using Cellulosic Glucose

March 6th, 2019
Biochemical Conversion

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Principal Investigator: Jeffrey Dietrich

LYGOS

Goal Statement

Project Goal

- **Develop an integrated process from cellulosic glucose through fermentative production of aspartic acid, a high-value chemical derived from the tricarboxylic acid cycle (TCA)**

Anticipated Outcomes

- **Achieve key technical milestones (fermentation yield, titer, and productivity); progressing bio-based aspartic acid process toward techno-economics supporting cost-competitive production**

Relevance to Department of Energy and BETO goals

- **Reduce dependence on foreign oil and manufacturing**
  - **New technology to an industrial chemical that can be produced domestically using cellulosic biomass as the feedstock**
- **Develop biochemical processes that support and improve integrated biorefinery economics**
  - **Target products where biochemistry can outcompete incumbent petrochemical producers on price and quality**
  - **The TCA cycle enables access to chemicals where CO_2 is sequestered, valorizing a waste stream from many industrial processes.**

Quad Chart

Timeline

- **Start Date:** October 1st, 2016
- **End Date:** March 30th, 2019
- **% Completion:** 90%

Barriers

- Demonstration of novel, high-yielding metabolic pathway (Ct-H)
- Improving fermentation performance (e.g., yield, titer, productivity; Ct-H)
- Integrated process development, sugar through purified chemical (Ct-J)

Budget

	Budget Period 1 Funding	Budget Period 2 Funding	Budget Period 3 Funding	Total Funding
DOE Funded	$753,152	$956,312	--	$1,709,464
Project Cost Share	$188,288	$239,078	--	$427,366

Partners

- Commercial cellulosic sugar provider (confidential; no cost-share)

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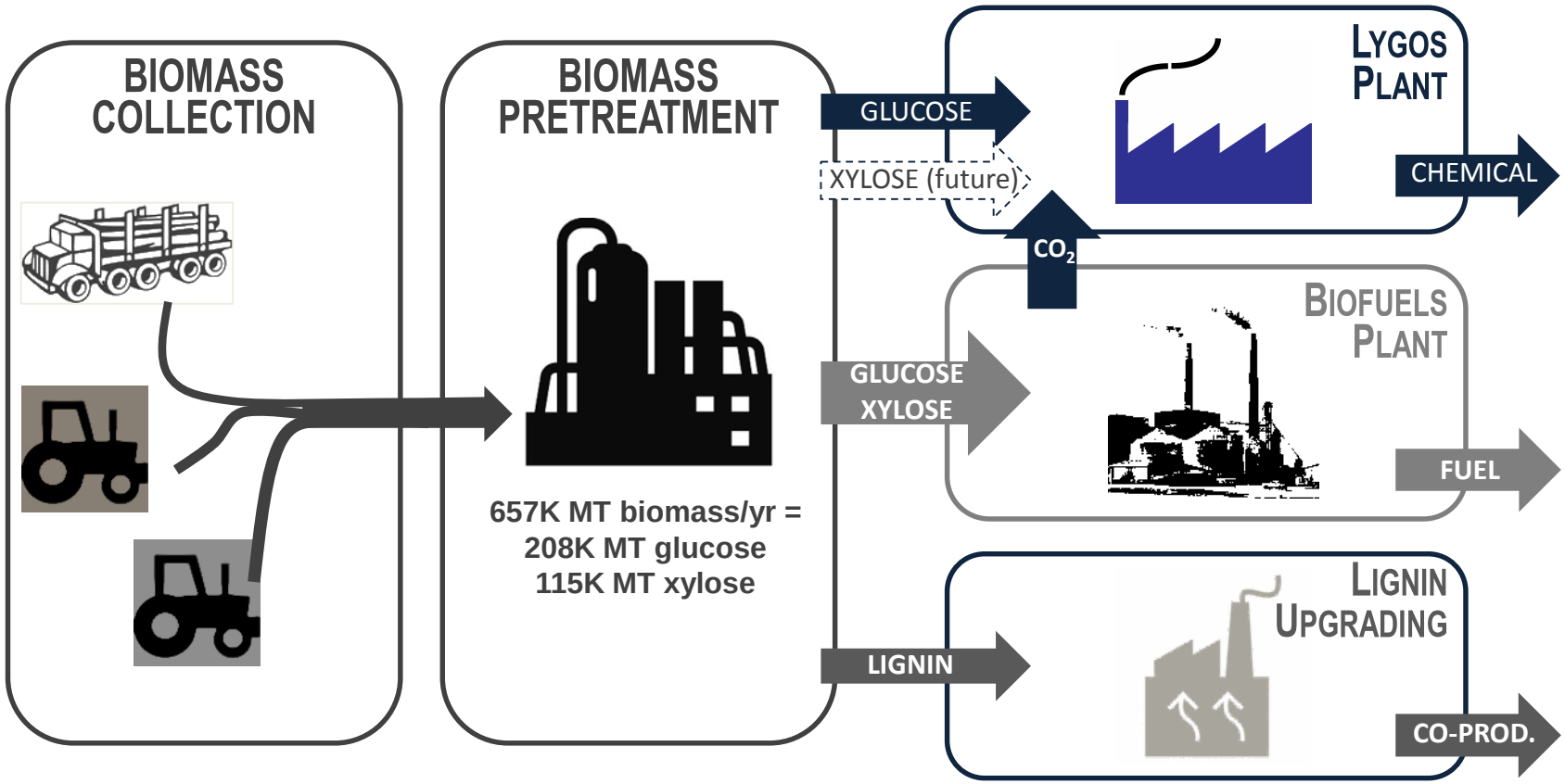
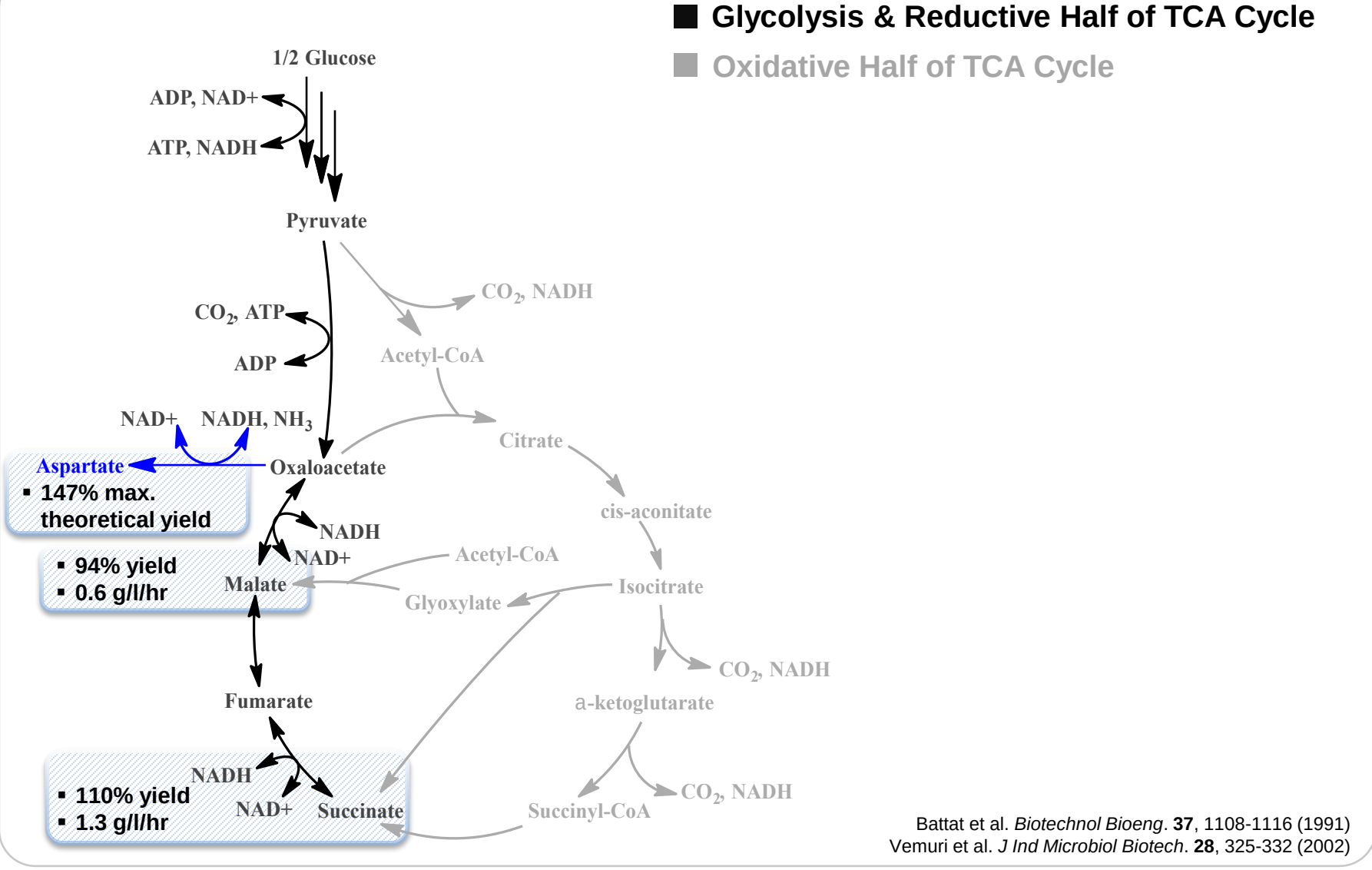
Project Overview: Integrated Biorefinery Vision



Biochemicals can improve integrated biorefinery economics

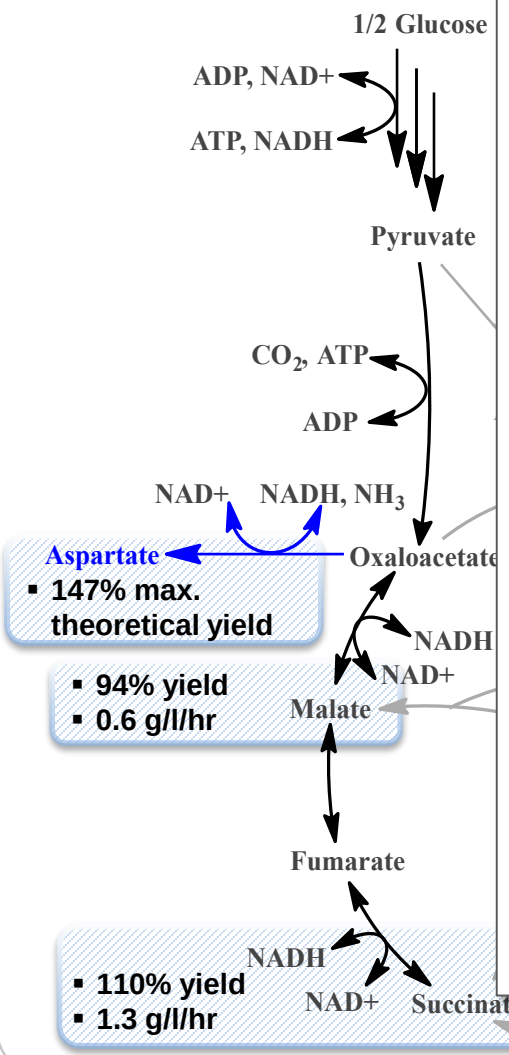
- Short-term : Separate C6/C5 sugar streams to reduce technical/commercial risk
- Long-term: Can incorporate C5 sugar stream in subsequent plant design
- Use of CO_2 waste stream as additional carbon source

Project Overview: Targeting TCA Cycle-Derived Chemicals



Battat et al. *Biotechnol Bioeng*. **37**, 1108-1116 (1991)
Vemuri et al. *J Ind Microbiol Biotech*. **28**, 325-332 (2002)

Project Overview: Targeting TCA Cycle-Derived Chemicals



- **Industrially relevant TCA cycle (reductive half)**
  - Commercial metrics reported on intermediates
  - 2:1 molar yields (stoichiometric from glucose)
  - CO_2 fixed during oxaloacetate biosynthesis
  - Pathways are redox balanced or near balanced
- **Technical approach is to express non-native enzymes to convert TCA cycle intermediates to desired product**
  - Design/select for new enzyme activities
  - Incorporate ATP-saving enzymatic activities
  - Utilize adaptive evolution and high-throughput screening for longer-term strain improvement
- **Organic acids have specific technical considerations**
  - Transport out of the cell
  - Host/product compatibility (*i.e.*, acid tolerance)
  - Purification (*i.e.*, salt reduction)

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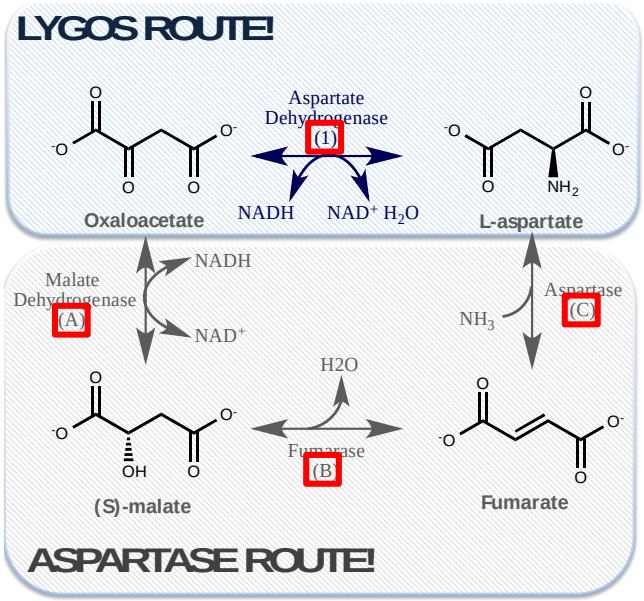
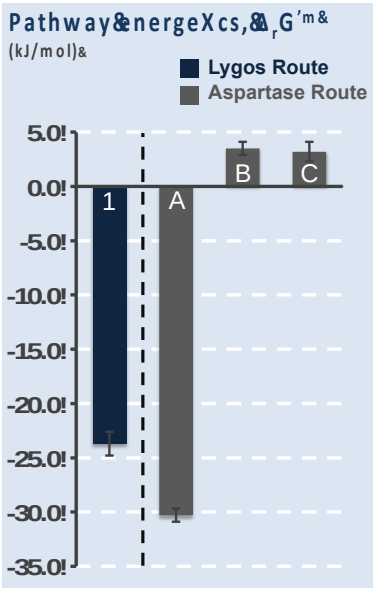
Lygos' Route Enables Efficient Production from Sugar

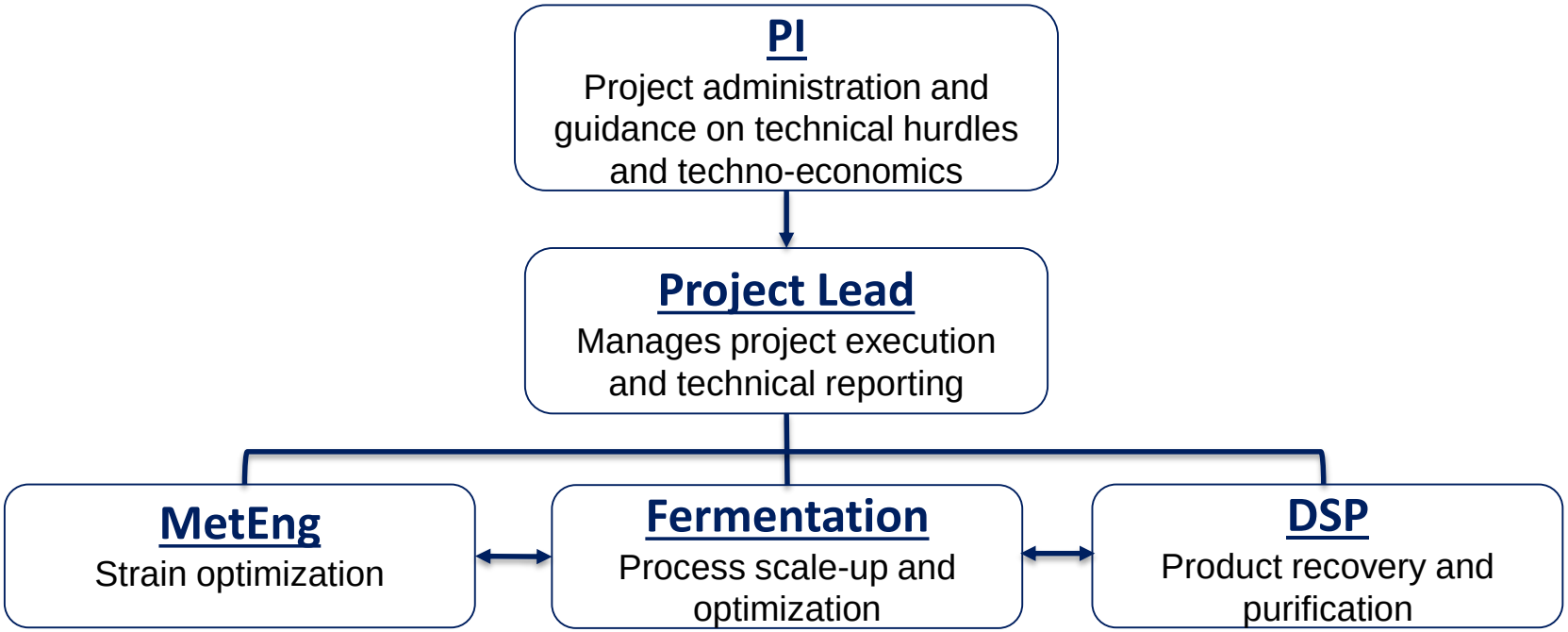




OUR ADVANTAGE OVER THE CURRENT ASPARTASE TECHNOLOGY

- Thermodynamics of Lygos' process strongly favors product formation
- Low-cost, anaerobic fermentation
- Bio-similars (*e.g.*, succinate) support project feasibility (i.e., good yields, productivities)

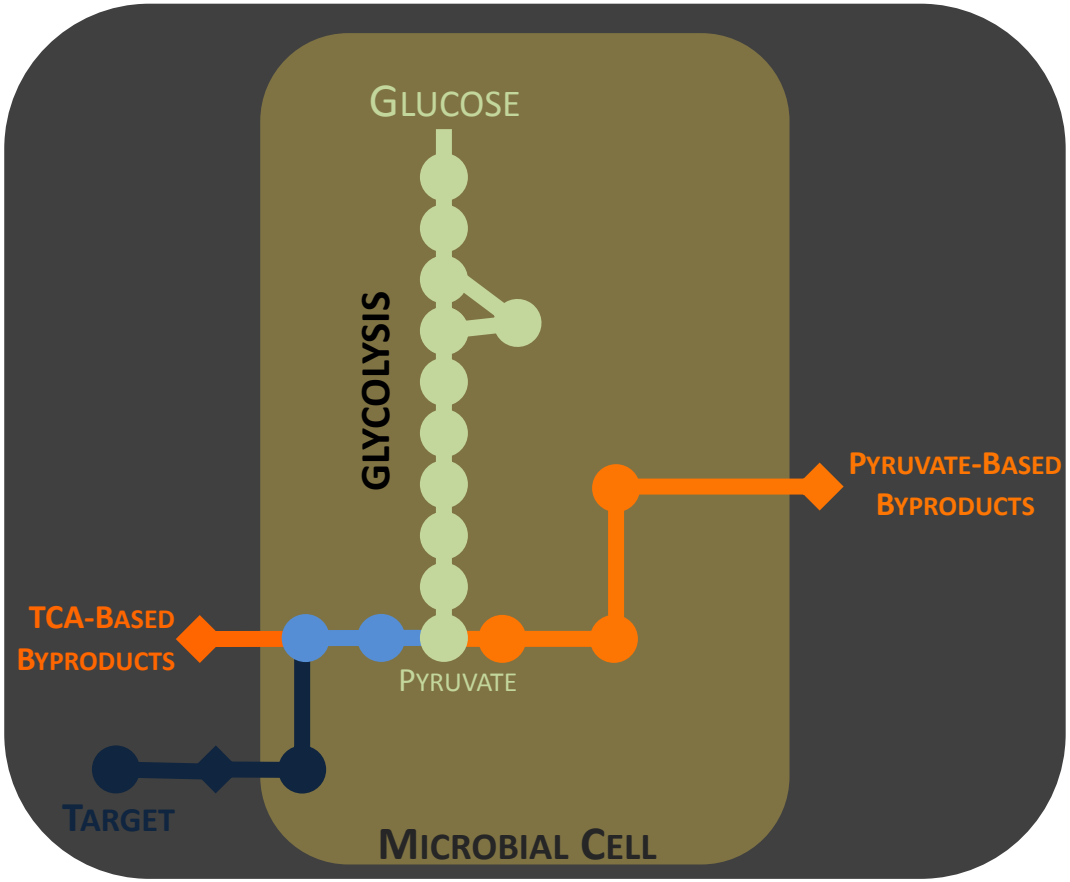
Management Approach



- Directors manage Departmental specific aims and appoint scientific leads to manage day-to-day completion of technical work in the laboratory
- All parties participate in contingency planning as unanticipated technical challenges are encountered

- **Quantitative milestones driven by techno-economic analysis and also address prioritized technical risks**
- **All strain and fermentation performance data is managed in a LIMS database**

Technical Approach: Strain Optimization



- Native pathways (decrease expression)
- Biosynthetic pathway (overexpress)
- Non-native enzyme screening

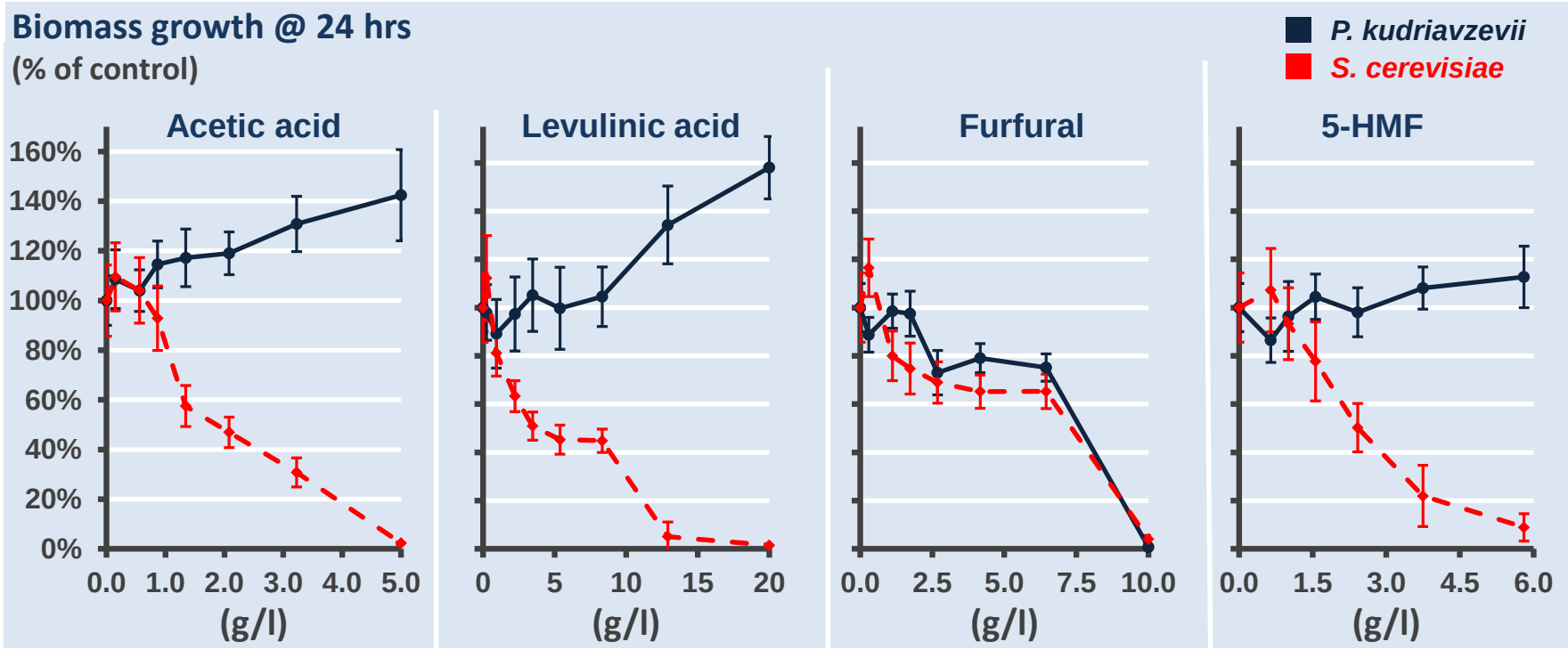
1. Decrease native byproduct formation (based on fermentation conditions)
2. Demonstrate non-native enzyme activities
3. Proof-of-concept pathway demonstration

Note: Metabolic pathways are outlined to be illustrative only

Technical Approach: Strain choice

Original host : *Pichia kudriavzevii,* a crabtree-negative yeast

- Wild type host has good acid, temperature, salt, and solvent tolerance
- Wild type host is resistant to common cellulosic hydrolysate inhibitors
- Extensive strain engineering and fermentation tools/methods @ Lygos
- Lygos strain evolved for a higher glucose consumption rate



Technical Approach: Strain choice

- **Engineered *P. kudriavzevii* produced low levels of aspartic acid**
  - Pathway enzyme activity was verified *in vitro*
  - Inherent regulation and export of amino acid are potential limiting factors
  - Transporter screening did not improve export to levels supporting strain optimization in pursuit of commercial targets
- **Decision made, with DOE input, to transition project to *Corynebacterium glutamicum* as the production host**
- **Switch to *C. glutamicum* addressed *P. kudriavzevii* technical challenges**
  - Used industrially for amino acid production at very large scale (*e.g.*, lysine)
  - Native amino acid exporters are present
  - Utilizes a diverse range of carbon sources and demonstrated ability to simultaneously co-utilize multiple carbon sources
  - Genetic toolbox available for host engineering
  - Decoupling of growth and production phase by transitioning from aerobic to microaerobic conditions (dual-phase fermentation)

Technical Approach: Process Optimization, Integration and Scaleup

Year 2: Process optimization, integration, and scaleup

- Cellulosic glucose (one supplier) used throughout the 2-Year workplan
- Progressive fermentation milestones on yield, titer, productivity; goals were driven by techno-economic model
- Purification milestones (yield) using integrated process materials (i.e., cellulosic glucose through to purified chemical)
- Final validation based on integrated process scaleup (10X scaleup)

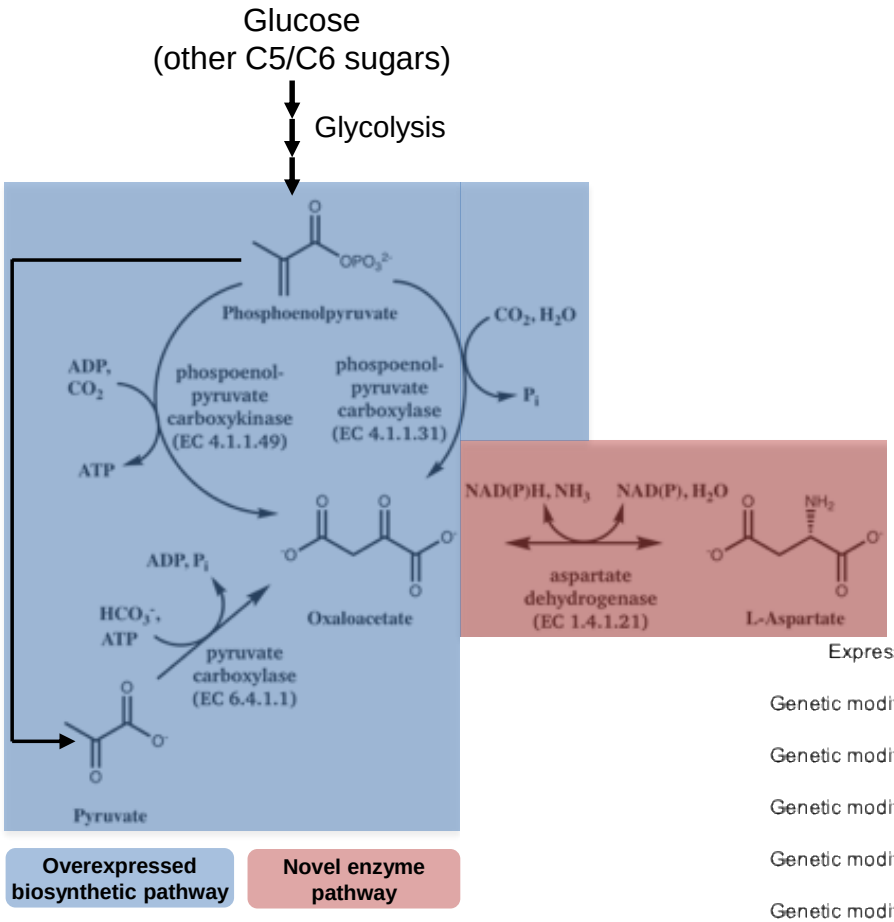
1000

10x

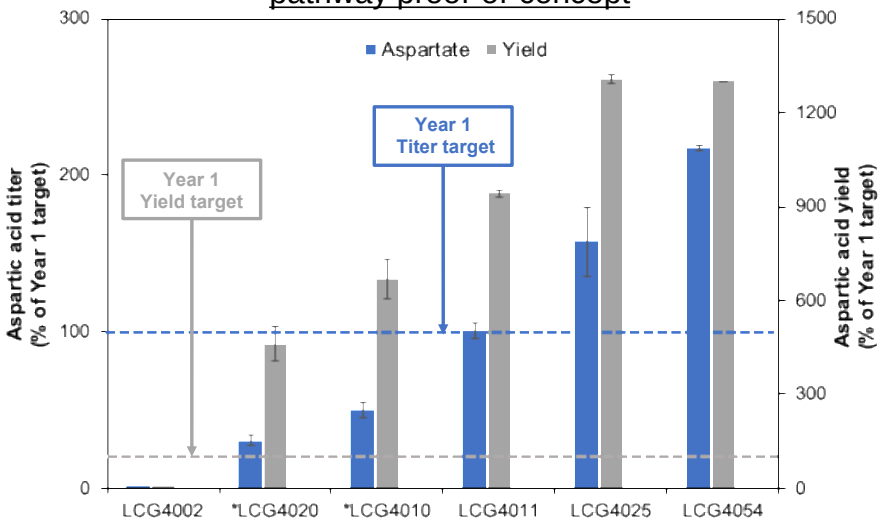
0.5 mL **0.5 Liter Reactors** **2-15 Liter Reactors**

Technical Accomplishments/Progress/Results

- **Demonstrated proof-of-concept aspartic acid pathway in *C. glutamicum***



Year 1 titer and yield metric targets for pathway proof-of-concept



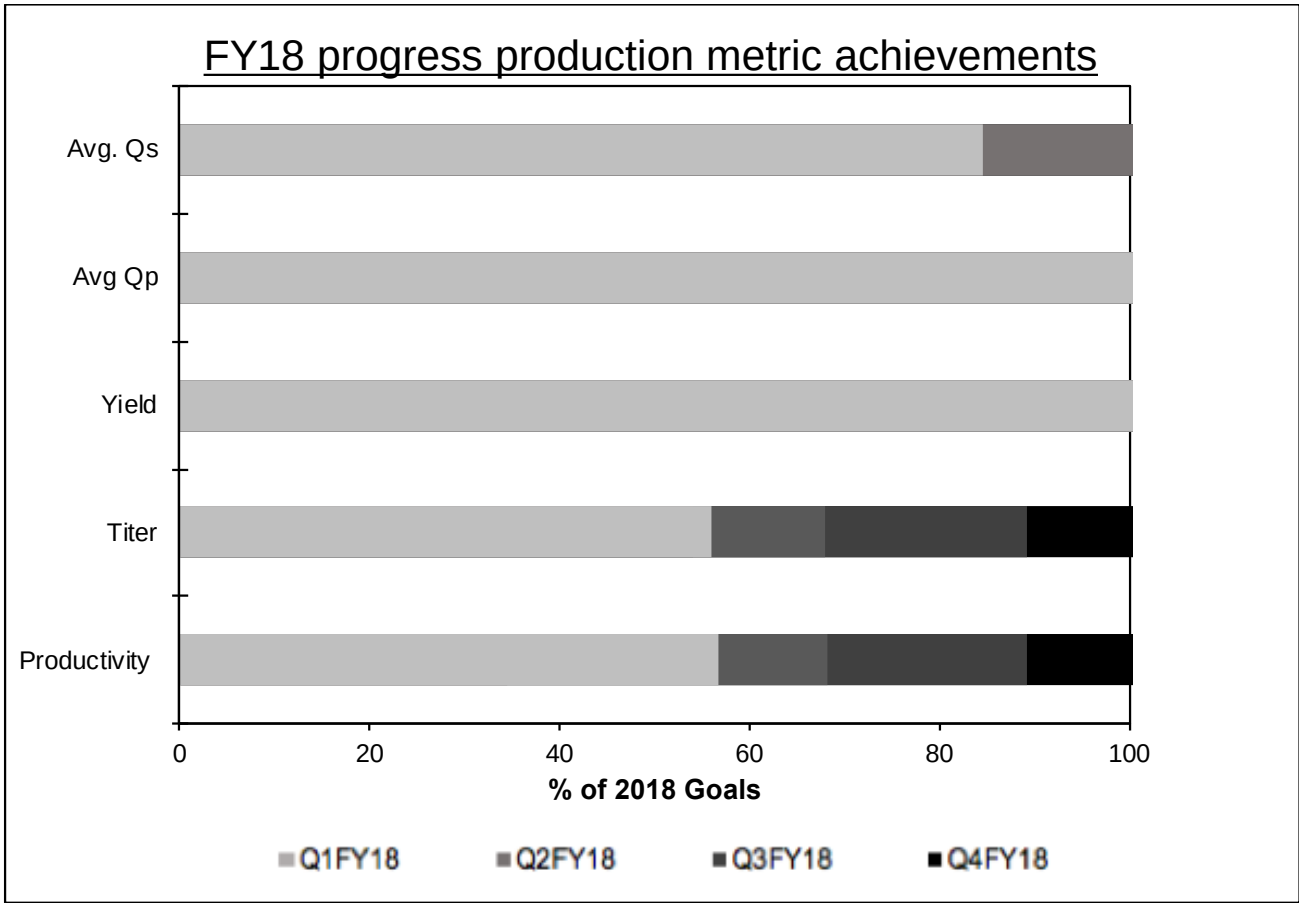
	LCG4002	*LCG4020	*LCG4010	LCG4011	LCG4025	LCG4054
Expression vector	✓	✓	✓	✓	✓	✓
Genetic modifcations #1		✓	✓	✓	✓	✓
Genetic modifcations #1		✓	✓	✓		
Genetic modifcations #2					✓	✓
Genetic modifcations #3			✓	✓	✓	
Genetic modifcations #4						✓
Genetic modifcations #5				✓	✓	✓

*Experiments performed with cellulosic glucose

- **Decoupling growth from production resulted in high aspartic acid yield**

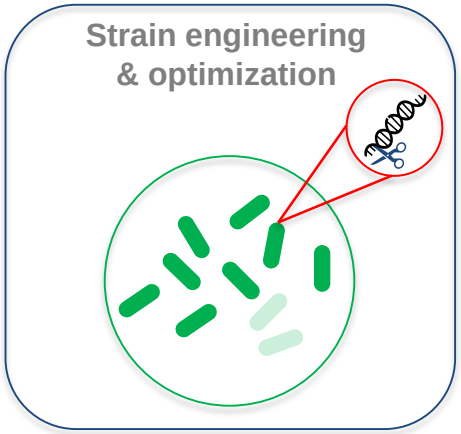
Technical Accomplishments/Progress/Results

Continued strain engineering and fermentation process optimization resulted in rapid improvements in small-scale production metrics. All fermentation yield, titer, and productivity metrics for the project were met using cellulosic glucose.



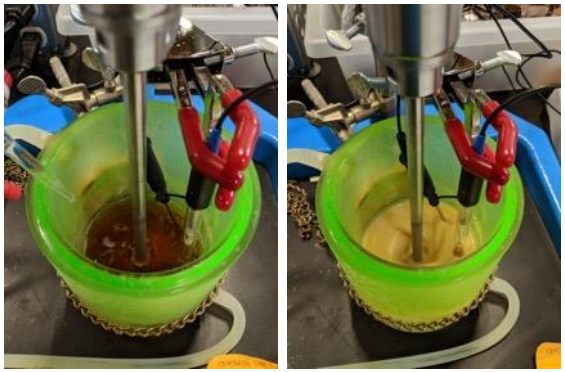
Technical Accomplishments/Progress/Results

Demonstrated "Ver1" integrated process for purification of aspartic acid from fermentation broth



Fermentation & Production

Fermentation broth containing product



Purification Process

Final purified product

Technical Accomplishments/Progress/Results

Year 1:

- **Switch of production host from *P. kudriavzevii* to *C. glutamicum***
- **Demonstrated proof-of-concept aspartic acid production using cellulosic glucose**
  - Demonstrated efficient *in vivo* activity of L-aspartate dehydrogenase
  - Prototyped pathway and achieved Year 1 Go/No-Go yield milestone

Year 2:

- **Further strain optimization resulted in performance metrics that met or exceed all project yield, titer, and productivity targets**
- **Demonstrated an integrated process for fermentative production of aspartic acid**
  - Product was recovered from fermentation broth at high yields and purity
  - Produced gram quantities of on-spec, purified product
- **Demonstrated fermentation process at 2-L bioreactor scale**
  - Currently working on bioreactor optimization for better replication of small-scale, anaerobic performance
- **Current process yields, titers, and productivities warrant pilot scaleup for further integrated process optimization and sample preparation.**

4 – Relevance

By the completion of this BETO project, demonstration of process commercial viability is anticipated

Relevance to BETO

- **Demonstration of process from cellulosic glucose to higher-value chemicals, improving integrated biorefinery economics**
- **TCA cycle-derived chemicals sequester CO_2, providing a route to valorize waste CO_2 streams**
- **Integrated process development**
  - **Target products where biochemistry can outcompete incumbent petrochemical producers on price and quality**
  - **TCA cycle derived molecules enable access to chemicals where CO_2 is sequestered during production**

Future Work: End of Project Goals

- **Metabolic pathway is de-risked**
  - No pathway “discoveries” remain
  - Incremental strain and process optimization/improvements
- **Improvement of 2- and 15-liter fermentation processes to demonstrate better process scaling from 5-ml assay**
- **Further optimization of purification process using larger volumes of fermentation broth**

0.5 mL

1000

0.5 Liter Reactors

10x

2-15 Liter Reactors

Summary

- **Overview:** Engineered microbe to consume cellulosic glucose and produce aspartic acid, a TCA cycle-derived organic acid. Developing an integrated process from feedstock to purified chemical.
- **Approach:** Prototype strain demonstration in Year 1, transitioning to process optimization and integration in Year 2. Final validation based on process scale-up.
- **Technical Accomplishments:**
  - Demonstrated production of aspartic acid at yields, titers and productivities that merit pilot scaleup of the integrated process
  - Purified product from fermentation broth at grams quantity
- **Relevance**
  - Ct-H: efficient catalytic upgrading of sugars to chemicals
  - Ct-J: process integration
- **Future Work**
  - Optimization of fermentation process at the 2-L and 15-L scale
  - Process integration and de-risking of all units operations for the purification process

Additional Slides

Not applicable

Responses to Previous Reviewers' Comments

Not applicable

Publications, Patents, Presentations, Awards, and Commercialization

Patents:

- U.S. Patent Application publication US2018/0258437

Awards:

- U.S. Department of Energy (DOE), Bioenergy Technologies Office (BETO), Award number: DE-EE0007565