Fermentative Production of Tricarboxylic Acid Cycle-Derived Chemicals Using Cellulosic Glucose

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Biochemical Conversion

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This presentation does not contain any proprietary, confidential, or otherwise restricted information
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\textsubscript{2} is sequestered, valorizing a waste stream from many industrial processes.
Quad Chart

Timeline
- **Start Date:** October 1\(^{st}\), 2016
- **End Date:** March 30\(^{th}\), 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

<table>
<thead>
<tr>
<th></th>
<th>Period 1 Funding</th>
<th>Period 2 Funding</th>
<th>Period 3 Funding</th>
<th>Total Funding</th>
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<td><strong>DOE Funded</strong></td>
<td>$753,152</td>
<td>$956,312</td>
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<td>$1,709,464</td>
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<td><strong>Project Cost Share</strong></td>
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Partners
- Commercial cellulosic sugar provider (confidential; no cost-share)
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 CO₂ waste stream as additional carbon source
Project Overview: Targeting TCA Cycle-Derived Chemicals

- Glycolysis & Reductive Half of TCA Cycle
- Oxidative Half of TCA Cycle

1/2 Glucose

- ADP, NAD+
- ATP, NADH

Pyruvate

- CO₂, ATP
- NAD+
- NADH, NH₃

Oxaloacetate

- NADH
- NAD+

Malate

- NADH
- NAD+

Fumarate

- NADH
- NAD+

Succinate

- NADH
- NAD+

Aspartate

- 147% max. theoretical yield
- 94% yield
- 0.6 g/l/hr

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)
  - CO₂ 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)

Project Overview: Bio-advantage of Lygos’ process

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

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.

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

**Biomass growth @ 24 hrs** (% of control)

**Acetic acid**
- 0% - 160%
- 0.0 - 5.0 g/l

**Levulinic acid**
- 0% - 160%
- 0 - 20 g/l

**Furfural**
- 0% - 100%
- 0.0 - 10.0 g/l

**5-HMF**
- 0% - 100%
- 0.0 - 6.0 g/l

*P. kudriavzevii* and *S. cerevisiae*
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)
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)
- Demonstrated proof-of-concept aspartic acid pathway in *C. glutamicum*

Glucose
(other C5/C6 sugars)

Glycolysis

Overexpressed biosynthetic pathway

Novel enzyme pathway

- 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.

FY18 progress production metric achievements

- Avg. Qs
- Avg Qp
- Yield
- Titer
- Productivity

% of 2018 Goals

- Q1FY18
- Q2FY18
- Q3FY18
- Q4FY18
Demonstrated “Ver1” integrated process for purification of aspartic acid from fermentation broth

Strain engineering & optimization

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.
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
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:**

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