DOE Bioenergy Technologies Office (BETO) 2019 Project Peer Review

Engineering Thermophiles to Produce Drop-in Fuels from Syngas

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Bioconversion Technologies

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GOAL: Develop recombinant thermophilic microbial strains capable of metabolizing syngas (CO, CO₂, H₂) into monoterpenes to enable industrial production of fuels and solvents

OUTCOMES:
• Identified and characterized multiple terpene synthase enzymes
• Developed analytical methods for monoterpene products
• Identified new thermophile chassis strain and verified CO consumption
• Developed vectors and achieved transformation of thermophilic carboxydrotrophic bacteria with heterologous genes
• Demonstrated monoterpene synthesis from syngas in a mesophilic host

RELEVANCE:
• Syngas feedstocks are relatively inexpensive and diverse, thermophiles have several unique advantages, and end products have value as fuel and solvent applications among others.
Quad Chart Overview

Start: October 1, 2015  
End: September 30, 2017  
Completion: 86%  
Technical Target: Conversion 

<table>
<thead>
<tr>
<th>DOE Funding</th>
<th>Total costs</th>
<th>FY16</th>
<th>FY17 to End</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>815,138</td>
<td>784,184</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Project cost share</th>
<th>Total costs</th>
<th>FY16</th>
<th>FY17 to End</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>208,310</td>
<td>191,522</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Kiverdi NREL</th>
<th>Total costs</th>
<th>FY16</th>
<th>FY17 to End</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>69%</td>
<td>31%</td>
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</table>

Barriers Addressed
- Heterologous genetic transformation of a chemoautotrophic thermophile
- Thermostability of monoterpen synthases
- Syngas fermentation by engineered thermophilic strains

Objective: Develop microbial biocatalysts for industrial syngas fermentation to fuels and chemicals

End of project goal: A thermophilic chemoautotroph using syngas engineered to produce a monoterpenone
Project Overview

Genes from monoterpene producing plant species

Genetically engineered chemoautotrophic thermophiles

Monoterpene production

Syngas ($H_2$, $CO$, $CO_2$)

Municipal waste
Agricultural waste
Industrial waste

Limonene
Myrcene
Ocimene
### Project Relevance

**Inexpensive and flexible feedstocks**

- Flared Nat Gas
- Carbon Off-Gases
- Agricultural residue
- Wood waste

**Applications for Monoterpenes**

<table>
<thead>
<tr>
<th>Coupled Syngas and Bioprocess</th>
<th>Industrial Solvents</th>
<th>Fuels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limonene</td>
<td>Diluent</td>
<td>JP-10 jet fuel substitute (dimer)</td>
</tr>
<tr>
<td>Myrcene</td>
<td>Oil sand solvent</td>
<td>Bio-gasoline</td>
</tr>
<tr>
<td>Ocimene</td>
<td>Refining agent for crude petroleum</td>
<td>Gasoline or diesel additive</td>
</tr>
<tr>
<td></td>
<td>Asphalt grading</td>
<td>P-menthane (component of JP-4 / Jet B)</td>
</tr>
<tr>
<td></td>
<td>Industrial cleaner (e.g., marine vessels, concrete cleaners, parts washer)</td>
<td></td>
</tr>
</tbody>
</table>

#### Additional Advantages for Bio-derived Monoterpenes:

- Non-carcinogenic and non-toxic hydrocarbon
- High energy density (37.8 MJ/L, limonene)
- Bio-degradable
- Multiple products with the same process and equipment
- Consumer and home product applications (cleaners and insecticides)
Highly cooperative team science approach. Frequent meetings and offline communication.
Technical Approach

Integration of five objectives:
1. Identification and validation of chemoautotrophic host strains
2. Development of genetic tools and molecular biology approaches
3. Identification and recombinant expression of thermostable monoterpenic synthesis enzymes
4. Development of reliable analytic techniques to quantify monoterpenic molecules
5. Fermentation methods and solvent overlays optimized for monoterpenic production
Technical Tools

Analytical methods
• Detection and quantitation of monoterpene
• Detection of substrate molecules and side products

Microbiology
• Identification of new chassis strain (CO consuming thermophile)
• Routine manipulation of chassis strain(s)
• Liquid culture, cryogenics, plating, antibiotic selection

Biochemistry
• Biochemical assays to test enzyme activity, solubility, and performance
• Biochemical methods to detect enzyme substrates and precursor enzymes

Molecular biology
• Development of vectors to manipulate thermophilic chassis strain(s)
• Identified promoter to drive expression and thermo-tolerant selection method
Technical Achievements

- Research and management teams demonstrated the feasibility of the syngas bioprocess
- 90% of the work developing the recombinant chemoautotrophic thermophile was completed; monoterpenes synthesis from CO was not achieved
- Monoterpenes production from syngas was demonstrated in a mesophilic chemoautotroph; titers reached 25% of original target
- Genetic tools and analytical methods were developed that support ongoing biocatalyst R&D
- Data were recorded for future technoeconomic process analysis
Chassis Strain Selection

Originally Proposed Strain: *Chlororflexus aurantiacus*

- Thermophilic green non-sulfur bacteria (40-50°C)
- Photosynthetic (anaerobically), but capable of aerobic growth in the dark
- Grows autotrophically on \( \text{H}_2/\text{CO}_2 \), and heterotrophically (organic acids, sugars)
- Putative CO dehydrogenase identified
- High natural terpenoid pathway flux (photosynthetic pigments)

Early evaluation revealed *Chlororflexus aurantiacus* to be unsuitable host for project goals.
- No/poor growth under dark aerobic conditions regardless of carbon source
- No evidence of any CO/Syngas uptake under any growth condition

- Effort was targeted to identification of a new host strain candidate
- This was not part of the original scope but clearly essential for project goals
Candidate Strain Screening

12+ potential strains identified, 9 strains underwent extensive testing on criteria.

Strain evaluation criteria:

1) CO/Syngas consumption

2) Thermal range of growth

3) Available genome sequence

4) Genetic tractability

5) Metabolic properties (e.g. terpene flux)
Identification of New Chassis Candidates

Variables tested during growth optimization experiments:

1) Autotrophic vs. mixotrophic growth
2) Media composition
3) Uptake of Syngas components (CO, H₂, CO₂)
4) Culture: headspace ratio (optimized gas mixing)
5) Temperature (minimal/maximal, optimal)

35+ growth experiments for top 6 candidate strains, each lasting 3-5 days

Three candidate thermophilic strains with sequenced genomes were identified as project chassis strains

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Aerobic/Anaerobic</th>
<th>CO uptake</th>
<th>Growth and utilization of synthetic syngas</th>
<th>Genetic tractability</th>
<th>Genome Sequenced</th>
<th>High flux to terpenes</th>
<th>Optimum growth temp °C (Minimum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moorella thermoacetica</td>
<td>Anaerobic</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>55 (45)</td>
</tr>
<tr>
<td>Thermomicrobium roseum</td>
<td>Aerobic</td>
<td>+</td>
<td>?</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>70 (55)</td>
</tr>
<tr>
<td>Caldanaerobacter subterraneus sp. tengcongensis</td>
<td>Anaerobic</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>75 (50)</td>
</tr>
</tbody>
</table>

Note: Two mesophilic backup strains also identified as contingency strains for this project.

(+): reported in literature; *: based on annotated pathways in genomes
Expression Vector Development

- *tengcongensis* expression vectors built
- Amylase promoters driving:
  - myrcene synthase (thermostable)
  - GPPS
  - Both MS and GPPS as polycistronic
  - Both MS and GPPS independently
- Phosphate acetyltransferase promoter driving:
  - myrcene synthase
  - limonene synthase
  - GPPS and LS as polycistronic

Constructs were built and used for transformation of *C.s. tengcongensis*
Thermophile Genetic Tools: *C.s. tengcongensis*

**Goal:** Identify independently controlled promoters for expression strength and inducibility; Demonstrate expression of at least 2 genes in *C.s. tengcongensis*

<table>
<thead>
<tr>
<th>Promoter for Gene</th>
<th>Constitutive/Inducible</th>
<th>Construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate acetyltransferase</td>
<td>strong constitutive</td>
<td>✓</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>strong constitutive</td>
<td>✓</td>
</tr>
<tr>
<td>Glucoamylase</td>
<td>Potentially inducible (starch)</td>
<td>✓</td>
</tr>
<tr>
<td>Alpha-amylase</td>
<td>Potentially inducible (starch)</td>
<td>✓</td>
</tr>
<tr>
<td>Fructose-specific phosphotransferase</td>
<td>Potentially inducible (fructose)</td>
<td>✓</td>
</tr>
<tr>
<td>Maltose-binding protein</td>
<td>Potentially inducible (maltose)</td>
<td>✓</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxykinase</td>
<td>Potentially repressible (mannoheptulose)</td>
<td></td>
</tr>
<tr>
<td>bgaR-PbgαL</td>
<td>lactose-inducible promoter</td>
<td>✓</td>
</tr>
<tr>
<td>pRpls1</td>
<td>strong constitutive</td>
<td></td>
</tr>
<tr>
<td>pRpls10</td>
<td>mid constitutive</td>
<td></td>
</tr>
<tr>
<td>pRpls20</td>
<td>weak constitutive</td>
<td></td>
</tr>
</tbody>
</table>

- Three constitutive promoters of varying expression strength were identified
- The range of strengths will be used to balance recombinant gene expression

**LacZ reporter assay**
**Transformation Go/NoGo**

- Moorella thermoacetica and Thermomicrobium roseum were untransformable
- Transformed kanamycin resistance is stable in *C. tengcongensis*
- No-DNA controls remain sensitive to Kan and fail to grow (mock transformations)

Milestones reached
- ✓ “Isolation of 1 or more transformants of the host strain”
- ✓ “functional expression of the antibiotic selection marker”
- ✓ “expression of heterologous beta-galactosidase enzyme”
Analytical Methods Development

Goal: Develop a protocol to extract, simultaneously detect and quantify monoterpenes

- Reestablished standards for mercene, limonene, geraniol, pinene etc.
- Tested overlays, dodecane, isopropyl myristate, heptane on live cultures
- Developed “universal” analytical method for monoterpenes, precursor, and overlays
- Determined IPM is leading overlay for methods and performance
- Detected bio-derived limonene from E. coli cultures over time with overlay
- Established reliable LOD and LOQ for all molecules of interest
Goal: Identify at least one monoterpene synthase that would function at high temperature

- Candidate genes were screened for expression and solubility in *E. coli*.
- Genes from poplar, mint, date palm, mandarin, grape and holm oak.
- Mint, mandarin, holm oak and date palm expressed sufficiently to be detected by blotting.
- Candidates were further analyzed by *in vitro* assay for conversion of geranyl pyrophosphate (Gpp) to a monoterpene.

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### Monoterpene Synthase Candidate Screening

<table>
<thead>
<tr>
<th>TS Construct</th>
<th>CBB</th>
<th>West.</th>
<th>Soluble</th>
<th>Activity (Insol.)</th>
<th>Activity (solub.)</th>
<th>Other Products</th>
<th>Product (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mint LS*</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>413</td>
</tr>
<tr>
<td>Mint LS</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>1093</td>
</tr>
<tr>
<td>Date palm LS</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>ocimene</td>
<td>30</td>
</tr>
<tr>
<td>Mand. LS</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>myrcene</td>
<td>11,173</td>
</tr>
<tr>
<td>Oak TS</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>myrcene pinene</td>
<td>1564</td>
</tr>
</tbody>
</table>
Monoterpene Synthase Thermostability

- Four enzymes showing activity in E coli extracts were tested by challenging at successively higher temperature: 30, 40, 50, 55 and 60°C
- Gpp conversion to terpene products was measured in the *in vitro* assay

- Primary candidate enzyme: Holly Oak myrcene synthase
- Highest activity and thermostability
- Second candidate: Mandarin myrcene synthase
Fermentation

Goal: Demonstrate recombinant organism growth and monoterpenes production using syngas

- Developed an air-lift bioreactor system (Appendix)
- Continuous delivery of syngas at controlled temperatures
- Demonstrated growth of *C. c tengcongesis* w/w/o solvent overlay
- Developed an organic solvent culture overlay to continuously extract monoterpenes into isopropyl myristate
Terpene production in vivo: *R. gelatinosus* CBS

Goal: Demonstrate monoterpene production in a recombinant chemoautotroph

*Rubrivivax gelatinosus* CBS strain expressing mint LS and grand fir GPP-synthase (codon optimized for CBS or *E. coli*); grown with dodecane overlay

**Day 3 vs. day 6**

- **Rich vs. minimal media (day 6)**
  - CBS produced limonene
    - 1.2mg/L on rich medium + syngas
    - 0.2mg/L on min media + syngas
### Summary

- **Research and management teams demonstrated the feasibility of the syngas bioprocess**
- **90% of the work developing the recombinant chemoautotrophic thermophile was completed; monoterpene synthesis was not achieved**
- **Monoterpene production from syngas was demonstrated in a mesophilic chemoautotroph; titers reached 25% of original target**
- **Genetic tools and analytical methods were developed that support ongoing biocatalyst R&D**
- **Data were recorded for future technoeconomic process analysis**

#### 8. Start-Up

<table>
<thead>
<tr>
<th>Task</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1 Ordering of equipment/strains/consumables</td>
<td>Completed</td>
</tr>
<tr>
<td>8.2 Project syncup: Visit to NREL</td>
<td>Completed</td>
</tr>
<tr>
<td>1.3 Materials and equipment received</td>
<td>Completed</td>
</tr>
</tbody>
</table>

#### 1. Host strain characterization and screening

<table>
<thead>
<tr>
<th>Task</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Establish base case fermentation process</td>
<td>Completed</td>
</tr>
<tr>
<td>1.2 Syngas impurity tolerance tests</td>
<td>Completed</td>
</tr>
<tr>
<td>1.3 Test strain variant options</td>
<td>Completely new host strains identified</td>
</tr>
<tr>
<td>1.4 Bioinformatics</td>
<td>Completed</td>
</tr>
<tr>
<td>1.5 Metabolic modeling</td>
<td>Completed</td>
</tr>
<tr>
<td>1.6 Chloroflexus aurantius growth on CO (C. fennuglossis)</td>
<td>Tested a dozen strains in searching for new host/platform microbes</td>
</tr>
</tbody>
</table>

#### 2. Development of a genetic system in host strain

<table>
<thead>
<tr>
<th>Task</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Analysis of selection/counterselection methods</td>
<td>Completed</td>
</tr>
<tr>
<td>2.2 Test transformation methods</td>
<td>Completed (Multiple strains)</td>
</tr>
<tr>
<td>2.3 Apply selection protocols for clone isolation</td>
<td>Completed - Go/No-Go</td>
</tr>
<tr>
<td>2.4 Genetic Tool Development</td>
<td>Completed</td>
</tr>
</tbody>
</table>

#### 3. Terpene Synthase Development

<table>
<thead>
<tr>
<th>Task</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Identify and test thermostable terpene synthases</td>
<td>Completed - Multiple enzymes Identified</td>
</tr>
<tr>
<td>3.2 Engineer terpene synthase(s)</td>
<td>Determined to be unnecessary due to native thermostability</td>
</tr>
<tr>
<td>3.3 Demonstrate enzyme specificity</td>
<td>Completed</td>
</tr>
<tr>
<td>3.4 Express engineered enzyme in host strain</td>
<td>Completed - success in E. coli, CBS and C. necator Not in Thermophile</td>
</tr>
</tbody>
</table>

#### 4. Biocatalyst process integration, development and optimization

<table>
<thead>
<tr>
<th>Task</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Screen primary production strains in bottles</td>
<td>Nearly completed - developed fermentation and overlay methods</td>
</tr>
<tr>
<td>4.2 Test recombinant Task 1 strains under optimized conditions</td>
<td>Fermentation and overlay optimized, final strain not available</td>
</tr>
<tr>
<td>4.3 Demonstrate capability to produce product from syngas</td>
<td>Completed for CBS</td>
</tr>
</tbody>
</table>

#### 5. Techno-economic Analysis (TEA)

<table>
<thead>
<tr>
<th>Task</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Verify base case TEM assumptions for commercial success</td>
<td>Began work</td>
</tr>
<tr>
<td>5.2 Complete integrated process TEA and process flow diagrams</td>
<td>Incomplete</td>
</tr>
</tbody>
</table>
Slides Not Shown
Air-lift Syngas Bioreactor

1) \( \text{N}_2 \) line (for purging samples), 2) water bath, 3) rotameters, 4) tubing and tube bioreactor, 5) halogen lights (outside hood, sash will be closed), 6) water recirculator for temperature control of water bath, 7) syngas line (stainless steel), 8) hood ventilation monitor (internal), 9) CO and \( \text{H}_2 \) monitor (outside hood, audible alarm), 10) relay to solenoid valve (connected to CO/\( \text{H}_2 \) and ventilation monitors), 11) solenoid valve (to shut off syngas if CO/\( \text{H}_2 \) detected outside hood or ventilation failure), 12) anti-static mat in hood (and equipment grounded/bonded, second anti-static mat will be in front of hood).

Materials chosen for compatibility with flammable gas.
Responses to Reviewers