Engineering Thermophiles to Produce Drop-in Fuels from Syngas

U.S. Department of Energy (DOE), Bioenergy Technologies Office (BETO)
2017 Project Peer Review

Thursday, March 9, 2017
Biochemical Conversion

Steven M. Yannone
Kiverdi, Inc.
Goal Statement:

- **Goal:** To develop microbial biocatalysts to convert syngas into monoterpenes.

- **Outcome:** The project will engineer novel metabolic capabilities into thermophilic bacteria that use hydrogen for metabolic energy and CO$_2$ and CO as carbon sources to produce monoterpenes useful as solvents and fuels.

- **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.

The diversity of feedstocks available to syngas processes (including waste) together with the continuous fermentation potential of thermophiles makes the economics of producing monoterpenes in this bioprocess highly competitive and flexible in dynamic fuel and solvent markets.
Quad Chart Overview

Project Timeline
Start: October 1, 2015
End: September 30, 2017 (67% Complete)

Technical Barriers
1) Transform carboxydrotrophic thermophile
2) Identify thermostable monoterpene synthase
3) Circumvent monoterpene toxicity
Technical Target: Conversion R&D

Project Budget

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<th>FY 15 Costs</th>
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Partner Spending
Kiverdi (69%)
NREL (31%)
Project Overview

Genes from monoterpene producing plant species

Genetically engineered thermophiles

Monoterpene production

Syngas
\((H_2, CO, CO_2)\)

Municipal waste
Agricultural waste
Industrial waste

Limonene
Myrcene
Ocimene

- Identified and characterized multiple terpene synthase enzymes
- Developed analytical methods for monoterpene products
- Identified new thermophile chassis strain and verified CO consumption
- Developed vectors for thermophilic carboxydotrophic bacteria (Go/NoGo)
**Project Relevance**

**Inexpensive and flexible feedstocks**

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

**Applications for Monoterpenes**

### Industrial Solvents
- Diluent
- Oil sand solvent
- Refining agent for crude petroleum
- Asphalt grading
- Industrial cleaner (e.g., marine vessels, concrete cleaners, parts washer)

### Fuels
- JP-10 jet fuel substitute (dimer)
- Bio-gasoline
- Gasoline or diesel additive
- P-menthane (component of JP-4 / Jet B)

**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

**Concurrent Development of Required Technological Components**

<table>
<thead>
<tr>
<th>Field</th>
<th>Description</th>
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</table>
| **Analytical methods** | – Detection and quantitation of monoterpenes  
                           – Detection of substrate molecules and side products |}
| **Microbiology**       | – Identification of new chassis strain (CO consuming thermophile)  
                           – Development of routine manipulation of chassis strain(s)  
                           - liquid culture, cryogenics, plating, antibiotic selection |
| **Molecular biology**  | – Identification of candidate thermotolerant Terpen synthases  
                           – Development of vectors to manipulate thermophilic chassis strain(s)  
                           – Identified promoter to drive expression and thermo-tolerant selection method |
| **Biochemistry**       | – Biochemical assays to test enzyme activity, solubility, and performance  
                           – Biochemical methods to detect enzyme substrates and precursor enzymes |

Converge all technical groundwork in year 2 to accomplish goals.
Results: Quantitative Analytical Methods

Quantitative analytical assays developed for:

- limonene
- a-pine
- B-pine
- Myrcene

Geraniol and ocimene isomers can be identified but not yet precisely quantified

Limit of detection for monoterpenes pushed down to 1ng, limit of quantitation is at 10 ng
Identification of Candidate Genes

Grape and holm oak terpene Synthase alignment

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</table>

67% Identity, 81% Similarity, 0% Gaps

Literature and database searches identified set of candidate genes

Candidate genes were computationally compared, evaluated, and selected

Genes selected for cloning and evaluation

1) Mint limonene synthase
2) Mandarin orange limonene synthase*
3) Holly Oak pinene synthase*
4) Date Palm terpene synthase (ocimene)
5) Grape terpene synthase (new addition)

* Database annotations incorrect
Biochemical Evaluation of Enzymes

- Candidate genes were cloned into pET28 vectors (*E. coli* expression vectors).
- Epitope tags were added for detection of recombinant enzymes.
- Enzymes were expressed in *E. coli* and evaluated for a number of characteristics.

**Amount of Active Enzyme**

**Thermal Stability of Enzymes**

Holly Oak is leading candidate enzyme.
# Biochemical Evaluation of Enzymes

Characterization pipeline established for recombinant enzymes

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<th>Soluble</th>
<th>Activity (Insol.)</th>
<th>Activity (solub.)</th>
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<th>Product (ng)</th>
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<td>Date palm LS</td>
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<td>e pinene</td>
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* Ocimene quantitation is approximate

** One-off experiment has not been repeated yet
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 H\textsubscript{2}/CO\textsubscript{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

Early strain vetting revealed a need to identify suitable “chassis” strain for project goals
Chassis 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)

<table>
<thead>
<tr>
<th>Microbe</th>
<th>CO uptake</th>
<th>Aerobic/Anaerobic</th>
<th>Autotrophic growth on syngas components (with O_2 for aerobic strains)</th>
<th>Genetic tractability</th>
<th>Genome Sequence</th>
<th>High flux to terpenes</th>
<th>Growth Temp (°C): Optimum (Min)</th>
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<tbody>
<tr>
<td>Chloroflexus aurantiacus</td>
<td>-</td>
<td>Both</td>
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<td>?</td>
<td>+</td>
<td>-</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>35</td>
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<tr>
<td>Cupriavidus necator</td>
<td>-</td>
<td>Both</td>
<td>+ H_2/CO, ND/ND</td>
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<td>+</td>
<td>-</td>
<td>30</td>
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<tr>
<td>Cupriavidus metallidurans</td>
<td>-</td>
<td>Both</td>
<td>+ H_2/CO, ND/ND</td>
<td>(+)</td>
<td>+</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>Thermomicrobiurn roseum</td>
<td>+</td>
<td>Aerobic</td>
<td>- H_2/CO, ND/ND</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>70 (55)</td>
</tr>
<tr>
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<td>Anaerobic</td>
<td>ND/ND</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>55 (45)</td>
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<td>?</td>
<td>70</td>
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<tr>
<td>Carboxydothema thermosoler</td>
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<td>Anaerobic</td>
<td>ND/ND</td>
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<td>65 (40)</td>
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<td>Carboxydolota thermطورifica</td>
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<td>ND/ND</td>
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<td>75 (50)</td>
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<tr>
<td>Hydrogenibacillus schlegelli</td>
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<td>ND/ND</td>
<td>?</td>
<td>+</td>
<td>-</td>
<td>65 (42)</td>
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</table>
Identification of New Chassis Strains

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 suitable candidate thermophilic strains identified as project chassis strains

<table>
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<tr>
<th>Microbe</th>
<th>Aerobic/Anaerobic</th>
<th>CO uptake</th>
<th>Growth and utilization of synthetic syngas</th>
<th>Genetic tractability</th>
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(*) reported in literature;  * based on annotated pathways in genomes

Note: Two mesophilic backup strains also identified as contingency strains for this project.
Genetic Tool Development: *Moorella thermoacetica*

Transformation protocol reported in literature was not reproducible in ATCC and DSM strains. Despite a number of attempts and strategies to optimize conditions, no Moorella transformants were verified.

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<td>+</td>
<td>0</td>
<td>NREL</td>
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</tbody>
</table>

Outgrowth observed in selective medium after transformation. No exogenous DNA detected in cells.

Despite a number of attempts and strategies to optimize conditions, no Moorella transformants were verified.
Genetic Tool Development: *Thermicrobium roseum*

Development of electroporation protocols for transformation unsuccessful to date.

<table>
<thead>
<tr>
<th>OD600</th>
<th>Plasmid (1 ug)</th>
<th>Wash</th>
<th>Electroporation</th>
<th>Recovery time</th>
<th>Thiamphenicol (liquid medium)</th>
<th>Growth in liquid?</th>
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<td>1000 V, exp</td>
<td>20 hrs</td>
<td>15</td>
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<td>pBBR1MCS</td>
<td>Sucrose</td>
<td>none</td>
<td>20 hrs</td>
<td>15</td>
<td>—</td>
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<tr>
<td>0.435</td>
<td>none</td>
<td>Sucrose</td>
<td>none</td>
<td>20 hrs</td>
<td>15</td>
<td>—</td>
</tr>
</tbody>
</table>

Attempts using various transformation strategies failed to result in transformants to date.
Genetic Tool Development: *C.s. tengcongensis*

- **Anaerobic plating protocol developed**
- **Antibiotic selection on plates established**

- *C.s. tengcongensis*: anaerobic thermophile that consumes CO
- Growth and transformation at 60°C
- Incubation of plates in gasket-sealed anaerobic container
- Sensitivity to 400 µg/ml kanamycin (plate and liquid)
Shuttle Vector Development

- *tengcongensis* origin of replication
- *tengcongensis* promoter: phosphate acetyltransferase
- Thermostable resistance to kanamycin (kat):
  - from pMK18 (de Grado et al., 1998)
- Backbone: pBluescript II KS +
- Gibson assembly:
  - Designed PCR primers with Geneious software
  - PCR amplified origin and promoter from *tengcongensis* genomic DNA (Q5 polymerase)
    - PCR amplified Kat gene from pMK18
    - Assembled fragments (NEB Gibson Assembly kit)
- *E. coli* strain JM109 for propagation:
  - recA- and endA- to improve stability
  - Amp selection, blue-white screening

Novel Vector Constructed (pBOL01-NREL)
Transformation method: Natural competence

Grow liquid culture to OD (600 nm) 0.8-1.2 (in TTE + starch)
Dilute and incubate culture +/- plasmid, 8 hours at 60oC (unshaken)
(1) Apply to plates +/- kanamycin and incubate at 60oC for 66 hours
(2) Inoculate liquid +/- kanamycin, incubate at 60oC

Transformants: growth on selective plates and liquid (Kan)

Four identified colonies on selective plates transferred to liquid selection and propagated

Vector: - + +
Transformed kanamycin resistance is stable

4 transformants retain antibiotic resistance when re-streaked or grown in selective liquid (400 µg/ml Kan)

No-DNA controls remain sensitive to Kan and fail to grow (mock transformations)
Validation of Transformation: *tengcongensis*

Direct probe for exogenous DNA in host strain

PCR from transformed cells to probe for thermostable kanamycin resistance gene (KAT)

PCR product of KAT gene (761 bp)

1. Size standards-2-log ladder
2. Transformant #2
3. Transformant #3
4. Transformant #4
5. Transformant #5
6. Cells with no plasmid
7. pMK18 DNA (+ control)

Unambiguous validation of exogenous DNA present in host cells
Current and Future Work

**Year one accomplishments:**

- New chassis strain identified
- Thermostable enzymes characterized
- Vector and transformation established
- Gas fermentation established

**Year two: Technology integration for monoterpene production from syngas.**

**Identified Thermostable Genes**
- Myrcene synthase
- Limonene synthase
- Ocimene synthase
- Limonene synthase

**Validated microbial and molecular tools**
- Identified chassis strain
- Shuttle vector, transformation, selection
- Functional promoter

**H₂, CO, CO₂**  Syngas Fermentation

Monoterpenes Production
Current and Future Work

Metabolic Pathway Manipulation strategy

- Clone terpene synthases into *C.s. tencongensis*
- Probe for monoterpene production
- Probe for GPP pools
- Probe for GPP synthase activity
- Identify thermostable GPP synthase genes
Acknowledgements

Pin-Ching Maness
Carrie Eckert
Sharon Smolinski
Emily Freed
John Reed
Ken Zahn
Sonali Hande
Laura Huskins