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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Project Goals, Outcomes, Relevance

GOAL: Develop recombinant thermophilic microbial strains capable of metabolizing syngas (CO, CO_2, H_2) 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 carboxydotrophic 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 R&D

	Total costs	FY16	FY17 to End
DOE Funding		815,138	784,184
Project cost share		208,310	191,522
Kiverdi NREL		69% 31%	

Barriers Addressed

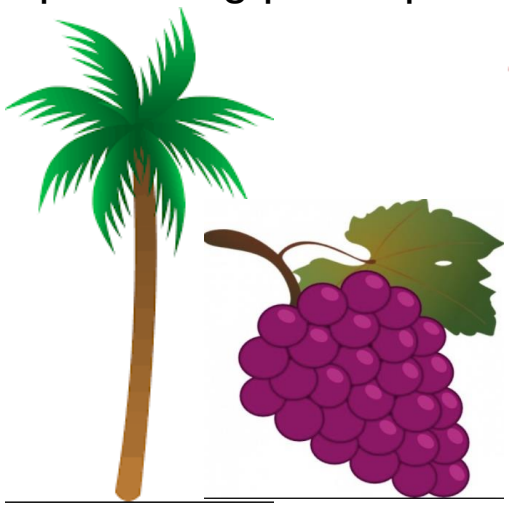
- Heterologous genetic transformation of a chemoautotrophic thermophile
- Thermostability of monoterpene synthases
- Syngas fermentation by engineered thermophilic strains

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

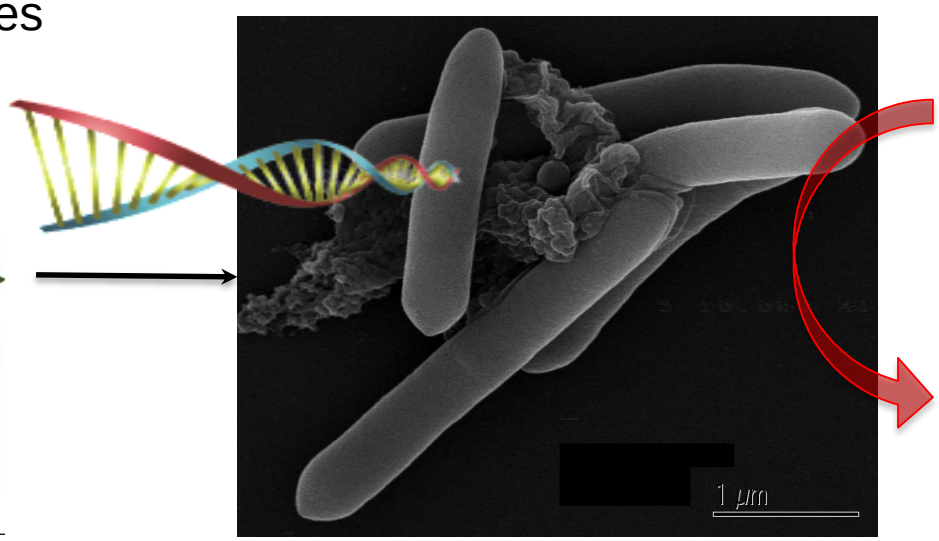
End of project goal: A thermophilic chemoautotroph using syngas engineered to produce a monoterpene

Project Overview

Genes from monoterpene producing plant species



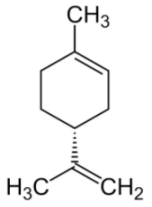
Genetically engineered chemoautotrophic thermophiles



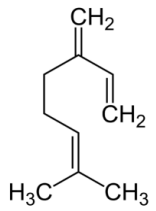
Monoterpene production

Syngas (H_2, CO, CO_2)

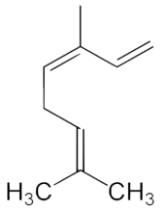
- Municipal waste
- Agricultural waste
- Industrial waste



Limonene



Myrcene

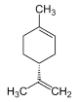


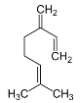
Ocimene

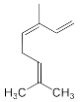
Project Relevance

Inexpensive and flexible feedstocks

Coupled Syngas and Bioprocess

Limonene 

Myrcene 

Ocimene 

Applications for Monoterpenes

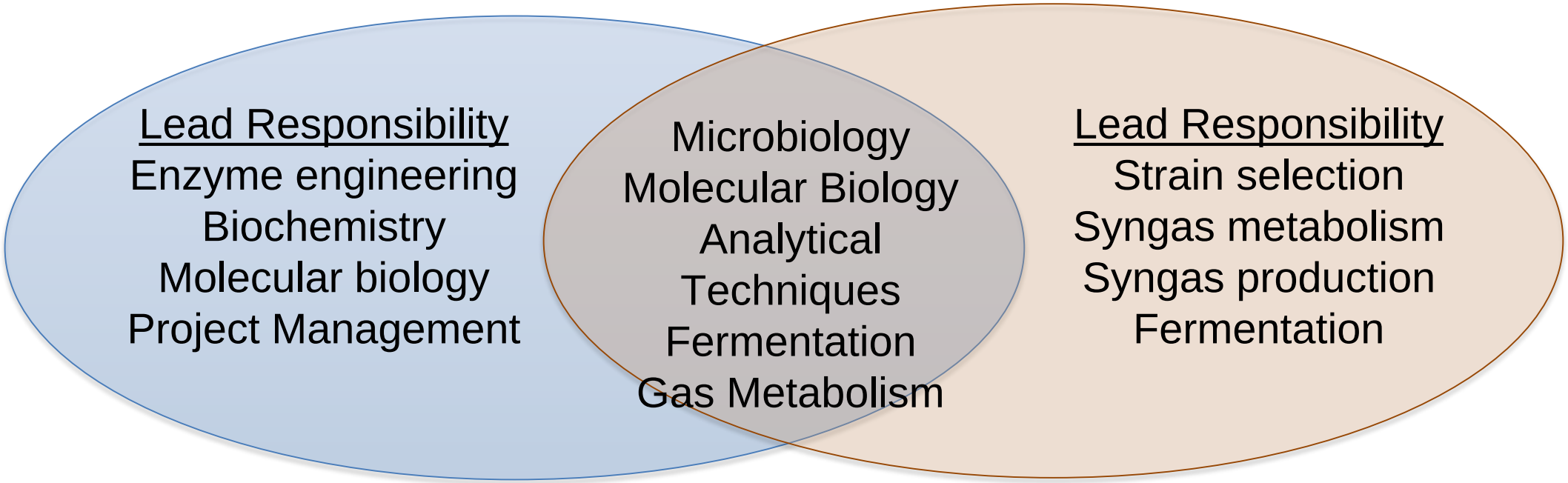
Industrial Solvents	Fuels
• Diluent • Oil sand solvent • Refining agent for crude petroleum • Asphalt grading • Industrial cleaner (e.g., marine vessels, concrete cleaners, parts washer)	• **JP-10 jet fuel substitute (dimer)** • **Bio-gasoline** • **Gasoline or diesel additive** • **P-menthane (component 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)

Project Management

Kiverdi NREL NATIONAL RENEWABLE ENERGY LABORATORY



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 monoterpene synthesis enzymes
4. Development of reliable analytic techniques to quantify monenterpene molecules
5. Fermentation methods and solvent overlays optimized for monoterpene 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

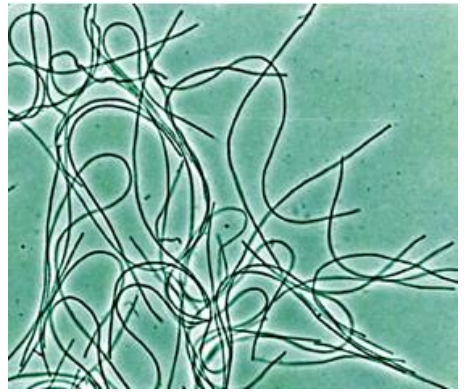
- Development of vectors to manipulate thermophilic chassis strain(s)
- Identified promoter to drive expression and thermo-tolerant selection method

Technical Achievements

- Research and management teams demonstrated the feasibility of the syngas bioprocess
- 90% of the work developing the recombinant chemoautotrophic thermophile was completed; monoterpene synthesis from CO 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

Chassis Strain Selection

Originally Proposed Strain: *Chlororflexus aurantiacus*



- Thermophilic green non-sulfur bacteria (40-50^{o}C)
- Photosynthetic (anaerobically), but capable of aerobic growth in the dark
- Grows autotrophically on H_2/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)

Microbe	CO uptake	Aerobic/ Anaerobic	Autotrophic growth on syngas components (with O_2 for aerobic strains)				Genetic tractability	Genome Sequence	High flux to terpenes	Growth Temp (^oC): Optimum (Min)
			CO	H_2/ CO_2	H_2/CO_2 /CO	H_2/CO				
Chloroflexus aurantiacus	-	Both	-	+	ND	ND	?	+	+	48 (40)
Rubrivivax gelatinosus CBS	+	Both	++ +	+++	+++	+++	+	+	+	35
Cupriavidus necator	-	Both	-	+++	ND	ND	+	+	-	30
Cupriavidus metallidurans	-	Both	-	+++	ND	ND	(+)	+	-	30
Thermomicrobium roseum	+	Aerobic	-	-	-	ND	?	+	+	70 (55)
Moorella thermoacetica	+	Anaerobic	ND	ND	+	ND	(+)	+	+*	55 (45)
Hydrogenobacter thermophilus	ND	Aerobic	ND	+	ND	ND	?	+	?	70
Carboxydothermus hydrogenoformans	(+)	Anaerobic	ND	ND	ND	ND	?	+	+*	65 (40)
Carboxydocella thermautotrophica	(+)	Anaerobic	(+)	ND	ND	ND	?	-	ND	58 (40)
Caldanaerobacter subterraneus sp. yonseiensis	+	Anaerobic	ND	ND	ND	+	?	+	+*	75 (50)
Hydrogenibacillus schlegelii	+	Aerobic	ND	ND	+	ND	?	+	-*	65 (42)

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, H_2, CO_2)
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

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

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

(+) reported in literature; * based on annotated pathways in genomes

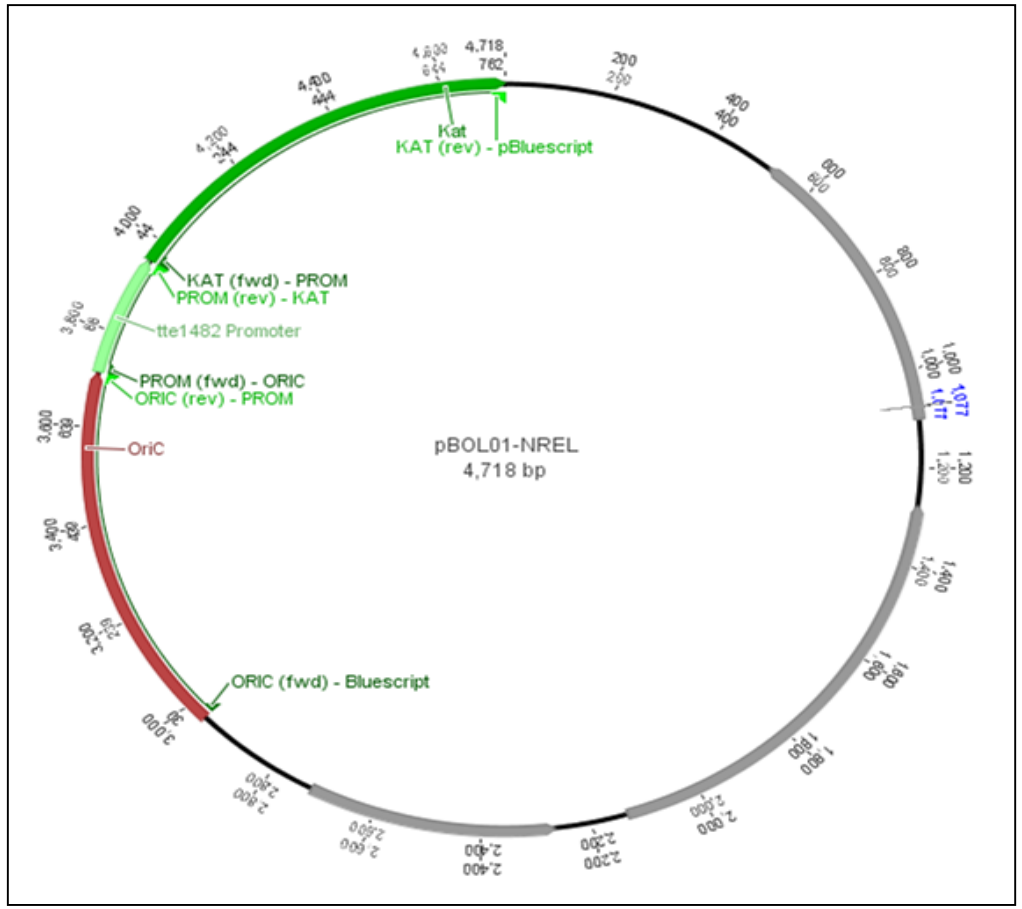
Thermophile Genetic Tools: *C.s. tengcongensis*

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*

Novel Vector Constructed (pBOL01-NREL)

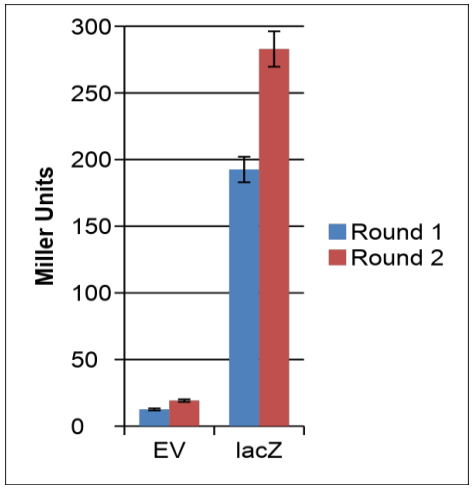


Thermophile Genetic Tools: *C.s. tengcongensis*

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

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

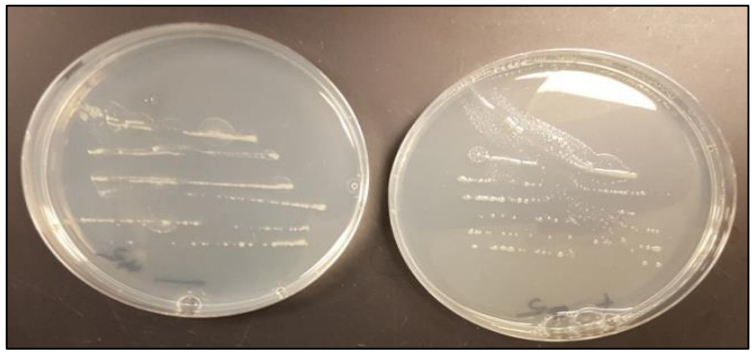
LacZ reporter assay



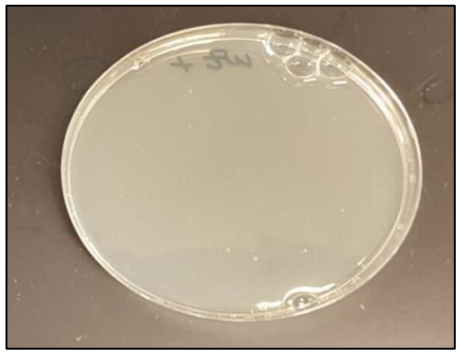
- Three constitutive promoters of varying expression strength were identified
- The range of strengths will be used to balance recombinant gene expression

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)



Transformant - kanamycin	Transformant + kanamycin



No-DNA control (WT) + kanamycin

Transformants + kanamycin	WT - kan	WT + kan

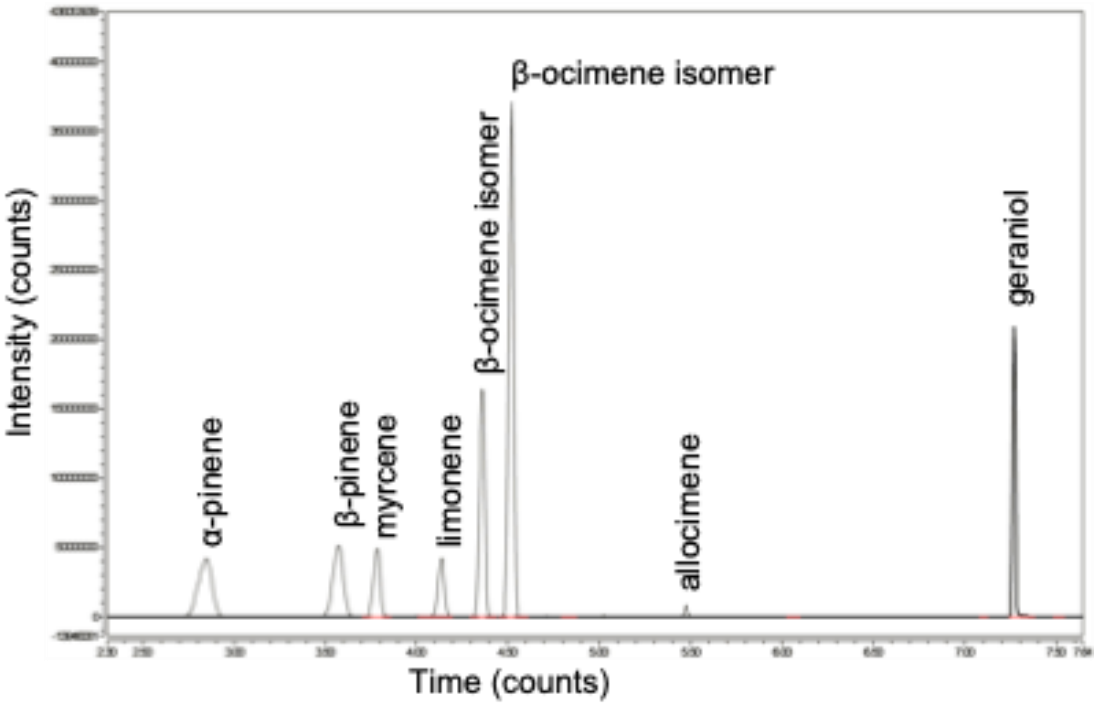
Milestones reached

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

Kanamycin resistance Transformant

Analytical Methods Development

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



- Re established standards for mercene, limonene, geraniol, pinene etc.)
- Tested overlays, dodecane, isopropypyl 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

Monoterpene Synthase Candidate Screening

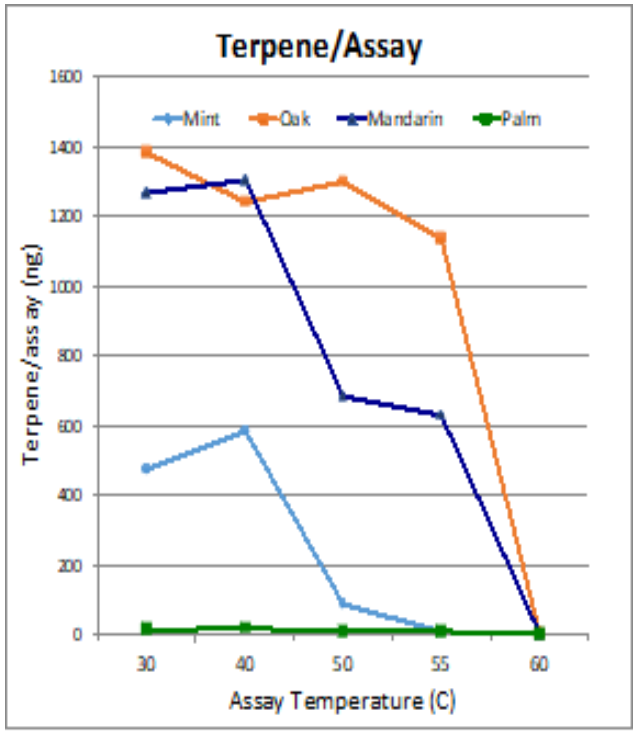
Goal: Identify at least one monoterpene synthase that would function at high temperature

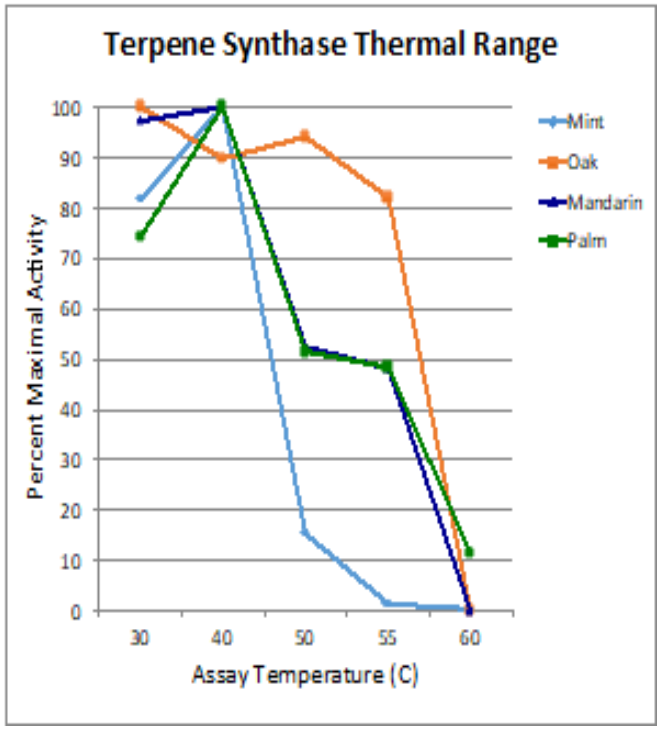
- Candidate genes were screened for expression and solubility in *E. coli*.
- Genes from poplar, mint, date palm, mandarin, grape and holm oak.
- Mint, mandarin, holm oak and date palm expressed sufficiently to be detected by blotting.
- Candidates were further analyzed by *in vitro* assay for conversion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

TS Construct	CBB	West.	Soluble	Activity (Insol.)	Activity (solub.)	Other Products	Product (ng)
Mint LS*	No	Yes	No	Yes	Yes	-	**413**
Mint LS	No	Yes	No	Yes	Yes	-	**1093**
Date palm LS	No	Yes	No	No	Yes	ocimene	**30**
Mand. LS	No	Yes	Yes	Yes	Yes	myrcene	**11,173**
Oak TS	No	Yes	Yes	Yes	Yes	myrcene pinene	**1564**

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^{o}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

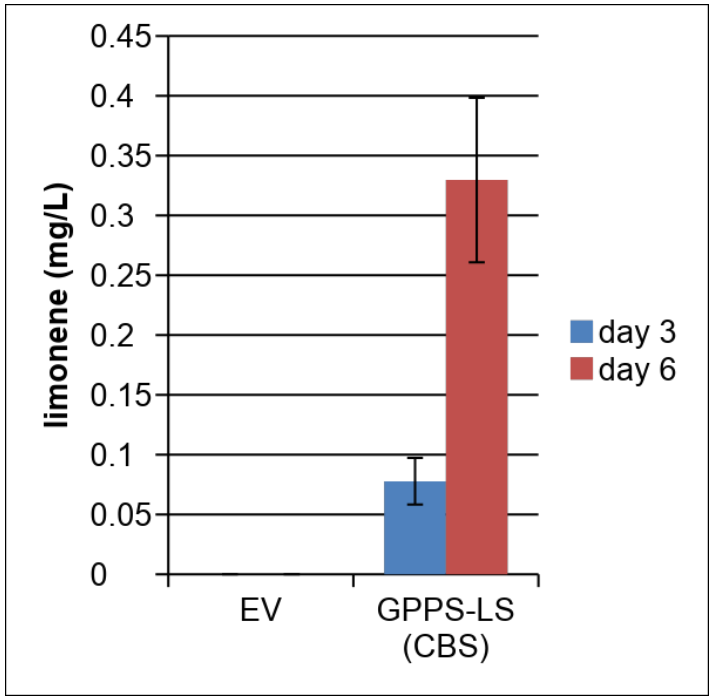
Goal: Demonstrate recombinant organism growth and monoterpene production using syngas

- 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 monoterpene into isopropyl myristate

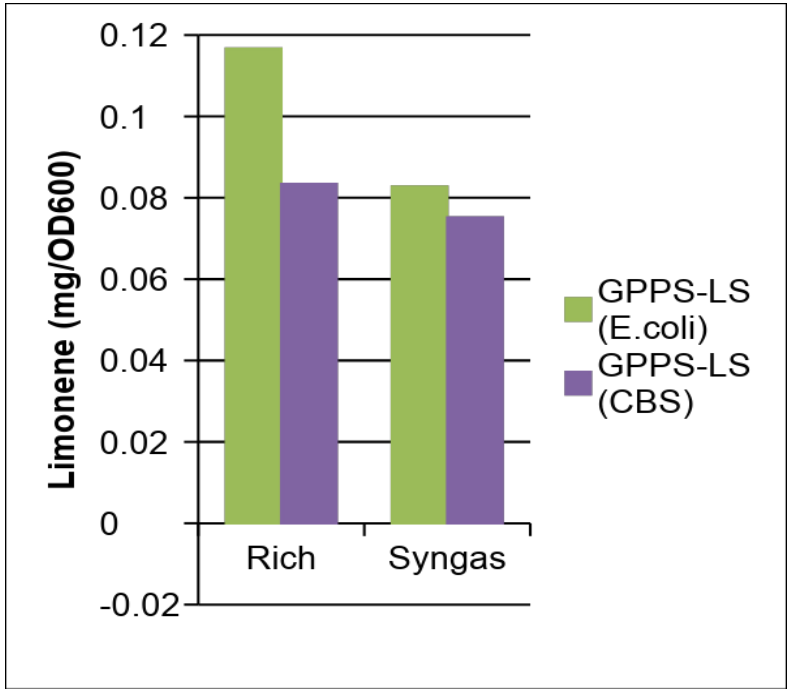
Terpene production *in vivo*: *R gelatinosus* CBS

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

B. Start-Up		Notes
B.1 Ordering of equipment/strains/consumables		Completed
B.2 Project syncup: Visit to NREL		Completed
1.3 Materials and equipment received		Completed
1. Host strain characterization and screening		
1.1 Establish base case fermentation process		Completed
1.2 Syngas impurity tolerance tests		Completed
1.3 Test strain variant options		Completely new host strains identified
1.4 Bioinformatics		Completed
1.5 Metabolic modeling		Completed
1.6 Chloroflexus aurantiacus growth on CO (C. *tencongensis)*		Tested a dozen strains in searching for new host/platform microbes
2. Development of a genetic system in host strain		
2.1 Analysis of selection/counterselection methods		Completed
2.2 Test transformation methods		Completed (Multiple strains)
2.3 Apply selection protocols for clone isolation		Completed -Go/No-Go
2.4 Genetic Tool Development		Completed
3. Terpene Synthase Development		
3.1 Identify and test thermostable terpene synthases		Completed - Multiple enzymes identified
3.2 Engineer terpene synthase(s)		Determined to be unnecessary due to native thermostability
3.3 Demonstrate enzyme specificity		Completed
3.4 Express engineered enzyme in host strain		Completed - success in *E. coli, CBS and C. necator* Not in Thermophile
4. Biocatalyst process integration, development and optimization		
4.1 Screen primary production strains in bottles		Nearly completed - developed fermentation and overlay methods
4.2 Test recombinant Task 1 strains under optimized conditions		Fermentation and overlay optimized, final strain not available
4.3 Demonstrate capability to produce product from syngas		Completed for CBS,
5. Techno-economic Analysis (TEA)		
5.1 Verify base case TEM assumptions for commercial success		Began work
5.2 Complete integrated process TEA and process flow diagrams		Incomplete

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- Genetic tools and analytical methods were developed that support ongoing biocatalyst R&D
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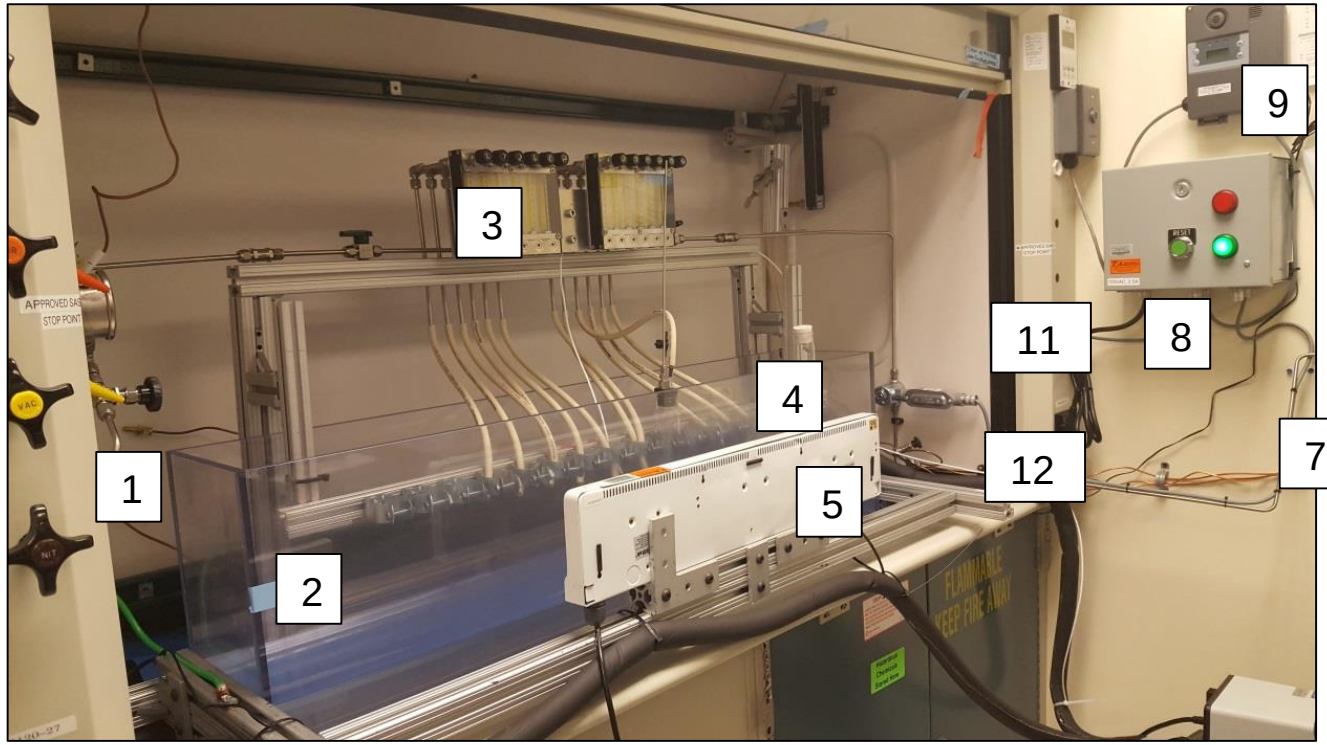
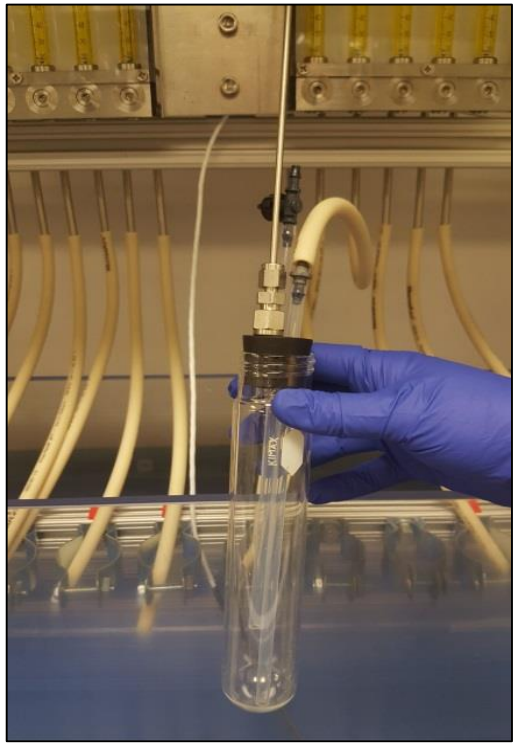
Slides Not Shown

Air-lift Syngas Bioreactor





1) 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 H_2 monitor (outside hood, audible alarm), 10) relay to solenoid valve (connected to CO/H_2 and ventilation monitors), 11) solenoid valve (to shut off syngas if CO/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

Publications, Patents