Heterologous Expression of Terpene Synthase Enzymes in *Methanosarcina*

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Abstract

Methanogens are obligate anaerobes that utilize inexpensive, non-food substrates and make methane as a by-product. Previously in this lab, methanogens have been engineered to direct carbon to isoprene production. Methanogens then could be engineered to make terpenes which are made of several modified isoprene units. Terpenes are molecules of interest to engineer because they are currently synthesized from non-renewable petroleum or are harvested from their endogenous species at low expression levels. Terpenes are part of multi-billion dollar industries like flavoring, fragrance, pharmaceuticals, and have potential in the energy industry.

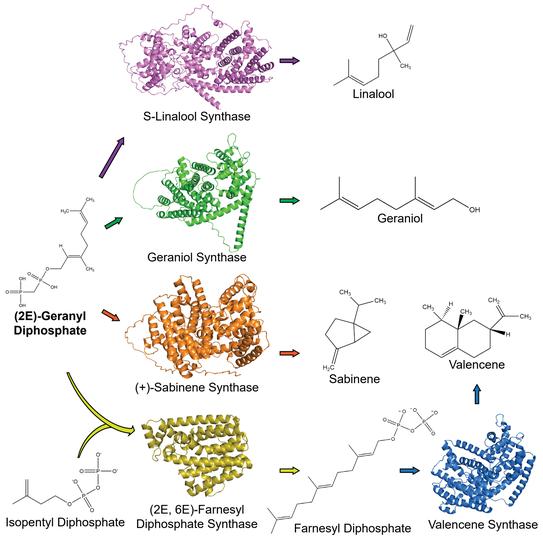
Methanogens are being investigated for engineering terpenes because they don't exihibit feedback inhibition in their mevalonate (MVA) pathway where terpene precursors come from. A higher percentage of their carbon could be directed to the product of interest than in other model species used for engineering. This project aims to reduce fossil fuel use and increase renewable energy by engineering methanogens to sustainably synthesize terpene compounds.

Research Questions

- Can *Methanosarcina acetivorans* synthesize farnesyl diphosphate, geraniol, linalool, sabinene, and valencene from inexpensive non-food feedstocks such as CO_2, methanol, and/or acetate?
- Can these terpenes be synthesized in appreciable amounts?
- Are these terpenes toxic to *M. acetivorans*?

Terpene Synthases

Figure 1: Biochemical pathway of terpenes and their enzymes. Enzyme structures are predicted by AlphaFold and visualized on Pymol. UniProt: Q96376, Q6USK1, O81193, A0A7J9PTF8, Q6Q3H2.



Why methanogens, and why terpenes?

- Methanogens have a naturally high-flux MVA pathway for isoprenoid lipids compared to model organisms.
- Terpenes are part of billion dollar industries of fragrance and flavoring.
- Terpenes are currently derived from native organisms or petroleum.
- Terepenes have potential in the pharmaceutical, materials, and energy industries:
  - Fuel blending.
  - Sustainable jet fuel with thermal stability and low pressure tolerance.

Cloning Terpene Expression Plasmids

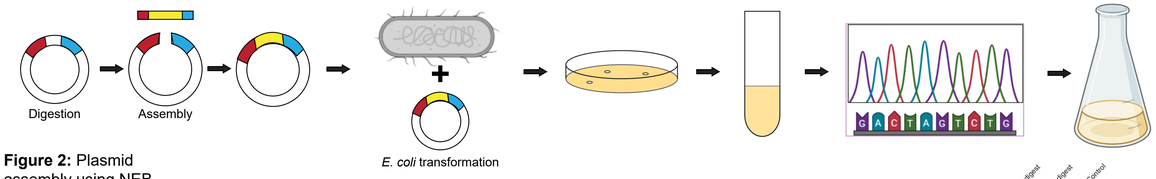


Figure 2: Plasmid assembly using NEB HiFi on digested backbone and terpene gene. Transform plasmid into *E. coli* via heatshock and plate. Select colonies into LB media with ampicillin. Harvest plasmids, restriction digest screen, and sequence. Correct plasmids are re-transformed and harvested in bulk for methanogen transformations.

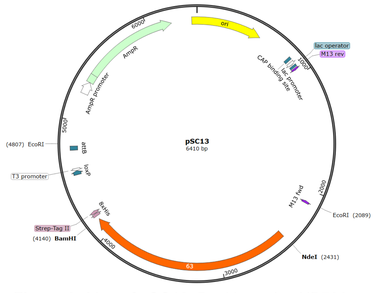


Figure 3: Map of pSC13. Backbone is pNB730 digested with NdeI and BamHI, and the insert is a terpene gene optimized for *M. acetivorans*.

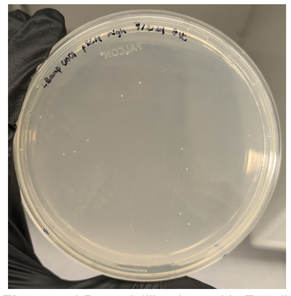


Figure 4: LB ampicillin plate with *E. coli* colonies containing pSC14.

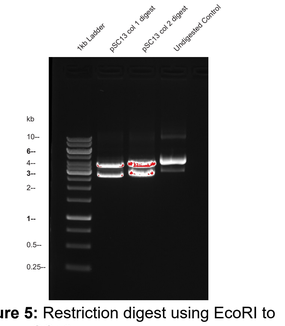


Figure 5: Restriction digest using EcoRI to screen pSC13 colonies visualized on a gel.
-With insert bands at 3.5kb and 2.7kb.
-No insert bands at 3.5kb and 1kb.

Methanogen Transformation

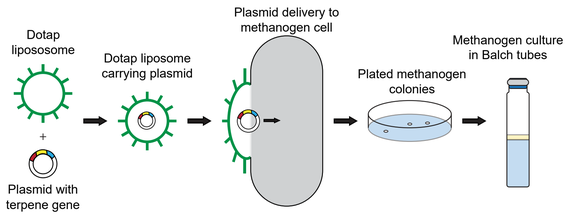


Figure 6: Method of transforming methanogens with terepene plasmids. Successful colonies are streaked for isolation then grown in Balch tubes containing high-salt media with a layer paraffin oil layer.

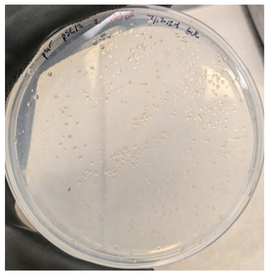


Figure 7: Puromycin selection plate with pSC12 *M. acetivorans* transformation colonies.

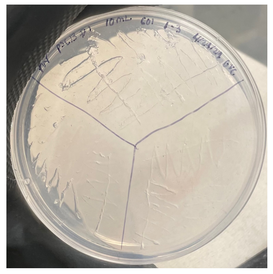


Figure 8: pSC13 transformants streaked for isolation on puromycin selection plates.

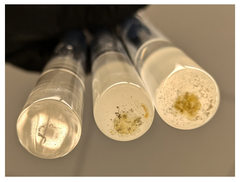


Figure 9: Left tube contains uninoculated media. Right tubes contain potential *M. acetivorans* pSC35 transformants. Not yet PCR screened.

Toxicity Tests

Figure 10: Toxicity determined by adding pure terpene to a normal *M. acetivorans* culture and measuring optical density over time.

Results:
- 20mM geraniol has no significant effect on growth rate (p=0.64) or carrying capacity (p=0.64).
- 20mM valencene has no significant effect on growth rate (p=0.30) or carrying capacity (p=0.83).

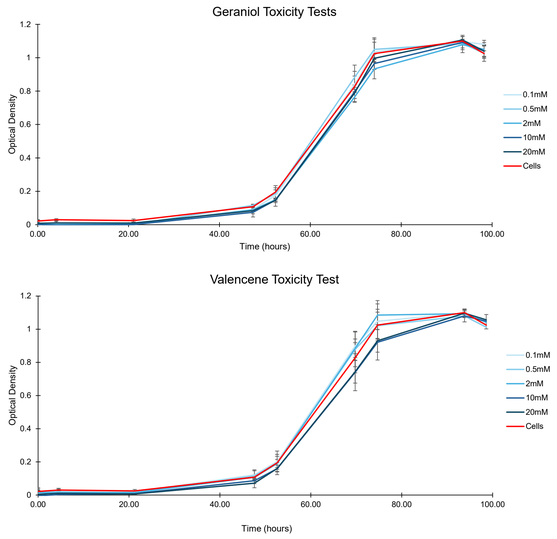


Figure 11: Growth curves of valencene and geraniol over a time period of 100 hours. Methanol media tubes are inoculated with untransformed *M. acetivorans* and paraffin oil containing the terpene. Incubated at 35°C and measured on Spectronic 20 at λ=600nm. N=3

Next Steps

- Continue methanogen transformations.
- Toxicity tests using farnesyl diphosphate, linalool, and sabinene.
- Diagnostic PCR of methanogen transformants.

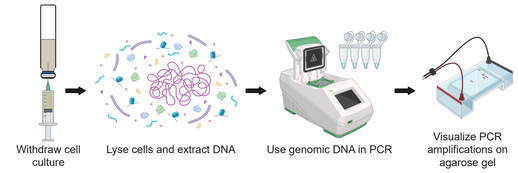


Figure 12: Method of diagnostic PCR to screen transformants for integration of terpene gene into the methanogen genome.

- RT-qPCR Assays

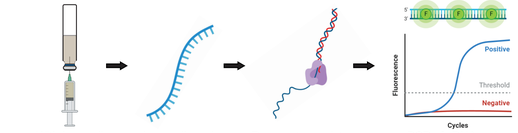


Figure 13: RT-qPCR procedure to check transcription of the terpene genes.

- Enzyme Assays
- Gas Chromatography

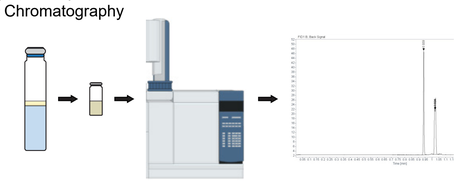


Figure 14: Gas chromatography is performed on the paraffin oil from stationary phase culture. Compare gas chromatogram to the standard terpene in paraffin oil to identify terpene quantities made by methanogens.

Acknowledgements

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