Enhancing Production of Isoprene from Engineered Methanogens

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Abstract

Methanogens are obligately anaerobic archaea noteworthy for producing methane from C1 compounds and acetate. The energetic limitations of these low-energy substrates require methanogens to utilize a highly efficient central metabolism which greatly favors respiratory byproducts over biomass. This metabolic strategy creates high substrate:product conversion ratios which is industrially relevant for the production of biomethane, but may also allow for the production of value-added commodities. Particularly of interest are terpene compounds, as methanogen membranes are composed of isoprenoid lipids resulting in a higher flux through isoprenoid biosynthetic pathways compared to Eukarya and Bacteria. To assess the metabolic plasticity of methanogens, our laboratory has engineered Methanosarcina acetivorans to produce the hemiterpene isoprene. We that isoprene producing strains would result in a decreased growth phenotype corresponding to a depletion of metabolic precursors needed for isoprenoid membrane production. We found that the engineered methanogens responded well to the modification, directing up to 4% of total towards isoprene production and increasing overall biomass despite the additional metabolic burden. Using flux balance analysis, RNA sequencing, and scale bioreactor growth we investigated how the engineered strains respond to isoprene production and how production can be enhanced.

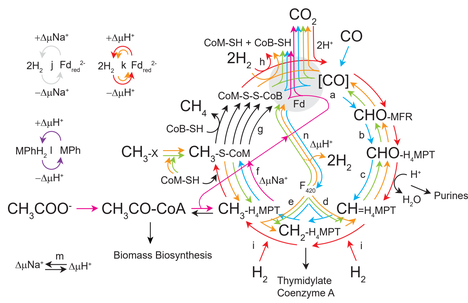


Figure 1. The Wolfe Cycle of Methanogenesis. (Buan 2018)

Primary pathways:

- **All methanogenesis**
- **Hydrogenotrophic pathway**
- **Methyl respiration**
- **Methylotrophic pathway**
- **Acetoclastic**
- **Carboxydotrophic pathway**

All pathways produce <1 ATP per mol of substrate

Why Use Methanogens to Produce Isoprene?

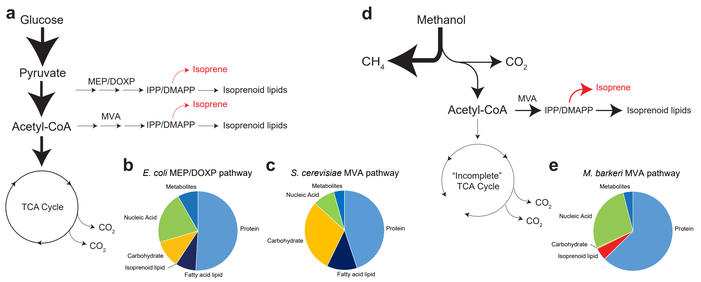
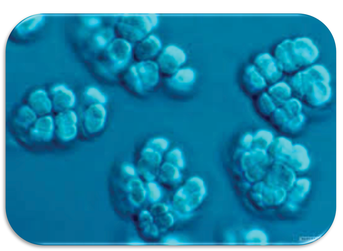


Figure 2. Isoprenoid biosynthesis pathways and macromolecular compositions of representative Bacteria, Eukarya, and Archaea. **a)** Isoprene is synthesized from isopentenyl pyrophosphate/dimethylallyl pyrophosphate (IPP/DMAPP) derived from glucose via the methylerythritol phosphate/deoxy xylulose phosphate (MEP/DOXP) pathway in bacteria or mevalonate (MVA) pathway in eukarya. **b & c)** relative amounts of macromolecules in *E. coli* bacterium (Egan & Vollmer, 2013) and S. cerevisiae yeast (Yamada & Sgarbieri, 2005), respectively. d) isoprenoid lipids are synthesized from IPP/DMAPP by the archaeal MVA pathway in methanogens. **e)** isoprenoid lipids in methanogens comprises 5% biomass dry weight. (Feist et al., 2006) Arrow sizes and line widths depict published carbon fluxes through each pathway. One or more genes is required for most organisms to produce isoprene monomer (red arrows).



Methanosarcina acetivorans

- Methanogenic archaea
- Produces methane as byproduct of respiration
- Obligate anaerobe
- 5.75 mbp genome
- Mesophilic
- Wides metabolic capacity of a methanogen
- Capable of autotrophic and heterotrophic growth on CO_2, methanol, methylamines, methyl sulfides and acetate
- NOT capable of growth on H_2:CO_2
- Genetically tractable

Engineering Methanogens to Produce Non-Native Metabolites

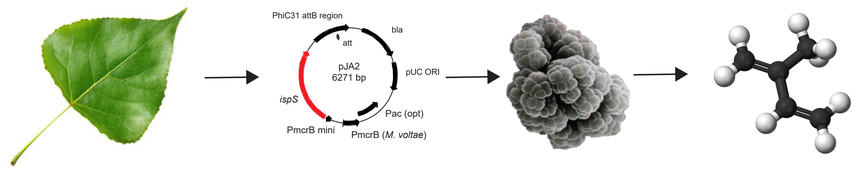
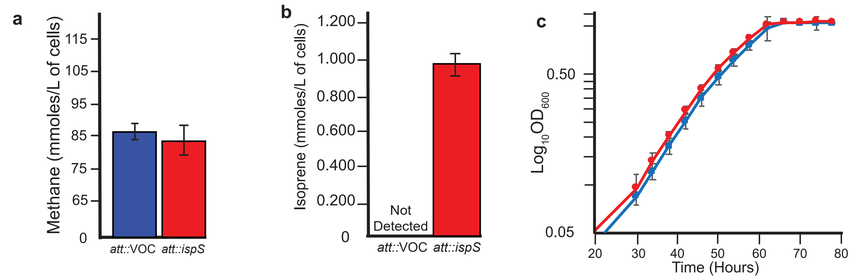


Figure 3. General overview of engineering a isoprene producing methanogen.
The gene for isoprene synthase was selected from *Populus alba*. The gene was codon optimized for expression in *Methanosarcina* species and is cloned into a plasmid containing an archaeal antibiotic resistance marker. The plasmid is transfected into *M. acetivorans* and selected for under antibiotic stress. Confirmation of gene insertion of attained by PCR and expression is confirmed via reverse transcription. The methanogens are cultivated and the volitile isoprene was collected in an oil overlay.



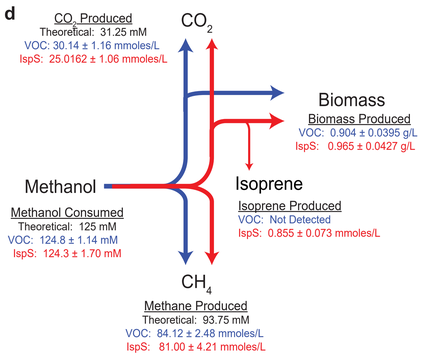
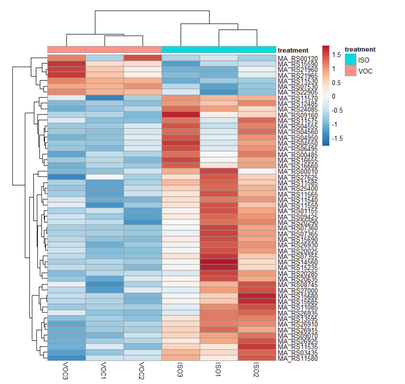


Figure 4. Effects of engineering methanogens to produce bioisoprene
a) Change in methane production in *M. acetivorans* engineered to produce bioisoprene. Methane quantification was achieved via GC-FID. The blue bars indicate methane production by a vector only control whereas the red bars indicate ispS+ strains.
b) Isoprene production from modified *M. acetivorans* strains. As expected the vector only control strain produced no detectable isoprene. The ispS+ strains produced nearly 1mM of isoprene per liter of cells.
c) Growth rates of engineered *M. acetivorans strains*. Growth rates of ispS+ and VOC strains were measured in HS+MeOH medium via absorbance at 600nm. There was no significant variance in growth rate between the two strains.
d) Mass balance of ispS+ and VOC strains of *M. acetivorans.* Methanol consumption as well as methane, CO_2, and isoprene production was measured by GC-FID. Cultures used for this experiment were desiccated and measured for dry weight. It was found that the carbon utilized for isoprene production was not diverted from biomass but rather CO_2 production.



RNAseq of Engineered Methanogens

RNAseq Overview	
# of Reads	396,282,199
Mean Quality Score	38.3
% of Bases >= 30	91.99
% Coverage of Genome	97.32
Protein-Coding Genes	4914
Significant DEGs	55
significant DE ncRNA	73

Figure 5. RNA was isolated from isoprene producing strains *of M. acetivorans* as well as a vector only control in triplicate (n=9). RNA samples were ribosomally depleated and treated with DNase before sequencing with an Illumina HiSeq sequencer. Reads were assembled and annotated onto the genome of *M. acetivorans* C2A and differential expression analysis was performed using DEseq2.

How Does *Methanosarcina acetivorans* Respond to the Production of Isoprene?

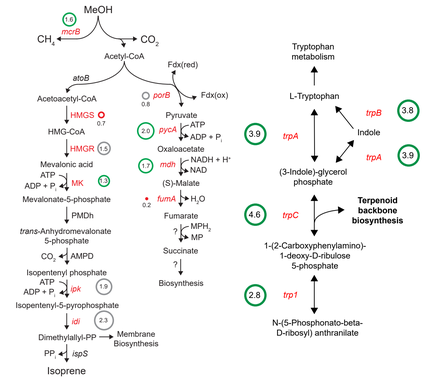
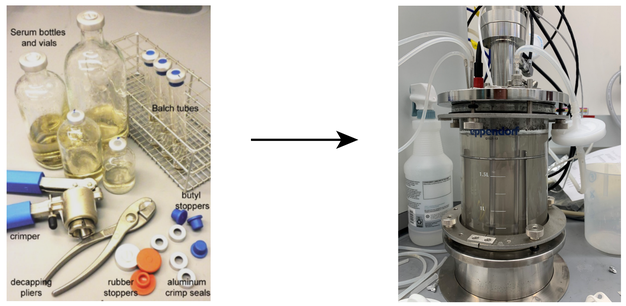


Figure 6. Differnetially expressed genes in ispS+ *M. acetivorans*. Folde changes of differentially expressed genes are represented in colored circles. Green circles indicate significant upregulated genes, red circles indicated significantly downregulated genes, whereas not significantly significantly variable genes (p>0.01) are indicated in grey. Increases in expression of genes associated with the Mevalonate Pathway was expected given the increased demand for mebrane precursors in ispS+ strains. The increase in biomass observed and decrease in CO_2 production despite producing the same amount of methane leads us to propose that these engineered strains of *M. acetivorans* are utilizing the production of isoprene as a means of removing electrons for the regeneration of methanogenic cofactors.

Increasing Isoprene Production and Scale-up



Reduction time	Inoculation Volume (ml)	pH control (6.8)	Agitation (RPM)	N_2 Overlay (SLPM)	CO_2 Sparge (SLPM)	Volume (Liter)	MeOH (mmol/min)	Cysteine (mmol/min)	NH_4CL (mmol/min)	Final OD
Before	10	Y	150	0.1	0.1	1	1.2	0	0	**0.165**
Before	10	N	50	0.1	0.1	1.5	1.2	0	0	**0.033**
Before	40	N	50	0.1	0.1	1.5	0	0	0	**0.042**
After	50	N	50	0.05	0.1	1.5	0	0	0	**0.062**
After	50	Y	50	0	0.05	1.5	1.2	0	0	**0.404**
After	100	Y	50	0	0.05	1.5	1.2	2.1	0	**0.456**
After	100	Y	150	0	0.1	1.75	1.2	2.1	0	**0.532**
After	100	Y	150	0	0.1	1.75	1.2	2.1	2.1	**0.520**
After	**200**	**Y**	**200**	**0**	**0.15**	**1.75**	**1.2**	**2.1**	**2.1**	**0.705**

Figure 7. Scale-up conditions for *M. acetivorans* and increases in biomass yield. Laboratory strains of *M. acetivorans* were grown in an Eppendorf BioFlo® 320 in high salt (HS) medium supplemented with MeOH. Cultures grown in the bioreactor showed final optical density (OD600) significantly lower than those observed in cultures grown in sealed Balch tubes. The cultivation of *M. acetivorans* in the bioreactor was optimized by adjusting both the preparation of the media as well as growth conditions and feed supplementation. We observed that the optimal conditions for *M. acetivorans* growth require the media to be made anaerobic in the vessel before reducing the media and an inoculation size of ~10%. The culture should be agitated at 200rpm while maintaining a sparge of CO2 at 0.15SLPM. Once cultures reached exponential growth (OD600 >0.4) a feed mixture containing a carbon, nitrogen, and sulfur source was drip fed until maximum growth was observed. When isoprene producing M. acetivorans were cultivated at 1.75L scale with an incorporated oil trap isoprene was detecctable via GC-FID analysis. A maximum yield of 4.1mM/L of isoprene has been achieved so far, though over time the volatility of isoprene results in a loss of captured isoprene as the culture progressess and more air is flowed through the oil trap. This loss of isoprene can be corrected with more sophisticated isoprene capture methods including an chilling setup to keep the isoprene below its volatility temperature.

References

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