Isopentenol Production Lab using EZ Media (and not so EZ Media)

NSF ATE Project 1601636
Chemical and BioEnergy for Sustainability

I. Background:

Isopentenol (3-methyl-3-buten-1-ol, FW = 86.13 g/mol, d = 0.853 g/mL, BP = 130-132 ^{o}C) is an isoprenyl alcohol derived from isopentenyl diphosphate (IPP), a key intermediate in the mevalonate biosynthesis pathway. Due to its higher carbon to oxygen ratio and lower hygroscopicity, as compared to bioethanol, isopentenol has superior combustion properties and is considered a "drop-in" biofuel for spark ignition engines.[1] *E. coli* strain DH1 was engineered as a two-plasmid system for the heterologous expression of five genes (plasmid 1) encoding enzymes for the synthesis of mevalonate diphosphate, and two genes (plasmid 2) encoding enzymes for the conversion of mevalonate diphosphate to isopentenol.[2] The maximum observed titer for isopentenol was 1.5 g/L (46% theoretical yield) using EZ-Rich production media containing 1 g/L of glucose as the carbon source. The initial production cultures were performed on a 5mL scale using a rotary shaker. In the following experiment, you will optimize the isopentenol titer using a 2L microbial bioreactor by varying dissolved oxygen content and pH.

II. Objectives:

- Calculate the theoretical yield[3] of isopentenol from glucose
- Prepare a starter culture from a cryogenic stock using LB + antibiotic plasmid selection media.
- Assemble and autoclave the bioreactor containing production media (EZ Rich + 1% glucose)
- Innoculate and induce production of isopentenol using β-D-1-thiogalactopyranoside (IPTG)
- Operate the bioreactor and collect data on dissolve oxygen, temperature, pH and stir rate
- Analyze and quantify metabolite production (including isopentenol) via GC-MS at 12, 24, 36 and 48 hr time points.

III. Procedure:

A) Starter Cultures and LB + Antibiotic Media Preparation

Prepare 10 x 10 mL tubes containing Lenox Broth (LB) media (2.2 g/100 mL of DI water). ***Due to high-pressure expansion, be sure to autoclave media in vessels at least twice the volume of the media you are autoclaving – and do NOT screw caps on tightly.*** For example, use a 250 mL vessel to autoclave 100 mL of media and attach cap lightly. If available, use autoclave tape. Set autoclave to 121°C for 15 min. After autoclave cycle is complete, place LB media in a laminar flow hood *and allow to cool to room temperature*. Add 100 μL the following antibiotic solutions to achieve a final concentration of 25 mg/L and 100 mg/L, respectively.

- Chloramphenicol (25 mg/mL, prepared in 95% ethanol)
- Carbenicillin disodium salt (100 mg/mL, prepared in sterile DI water)

Note that the above solutions must be passed through 0.2 μm syringe filter to sterilize because they will decompose upon autoclaving. The antibiotic solutions can be stored in the freezer for future use.

Using a 10 mL sterile pipette, aliquot 10 mL of LB + antibiotic media from into 10 sterile test tubes and cap.

B) Inoculate Starter Cultures from cryogenic E. coli stock, grow overnight, and prepare cryogenic E. coli stock for future use.

Remove vial of cryogenic *E. coli* stock (JBEI strain 1A)[2] from -80°C freezer and place into an ice bath to slowly thaw.

Working in the laminar flow hood, inoculate 3 x 10 mL tubes of LB + Antibiotic media (prepared in part A) each with 250 μL of thawed *E. coli* stock

Set in incubator at 37°C and allow to shake at 200 RPM for 16 hr.

After 16 hr of incubation:

1) Draw one of the 10 mL tubes into a sterile syringe, this will be used to inoculate the bioreactor.

2) Using a sterile pipette, add 1 mL of bacteria culture and 1 mL of 50% sterile glycerol to 10 separate, sterile cryovials and place in 80°C freezer for future use.

3) Use the third tube to measure optical density (600 nm) of the overnight bacterial culture and record.

C) Calibrate Probes, Assemble and Autoclave the Bioreactor

Assemble the bioreactor for autoclaving as shown below:

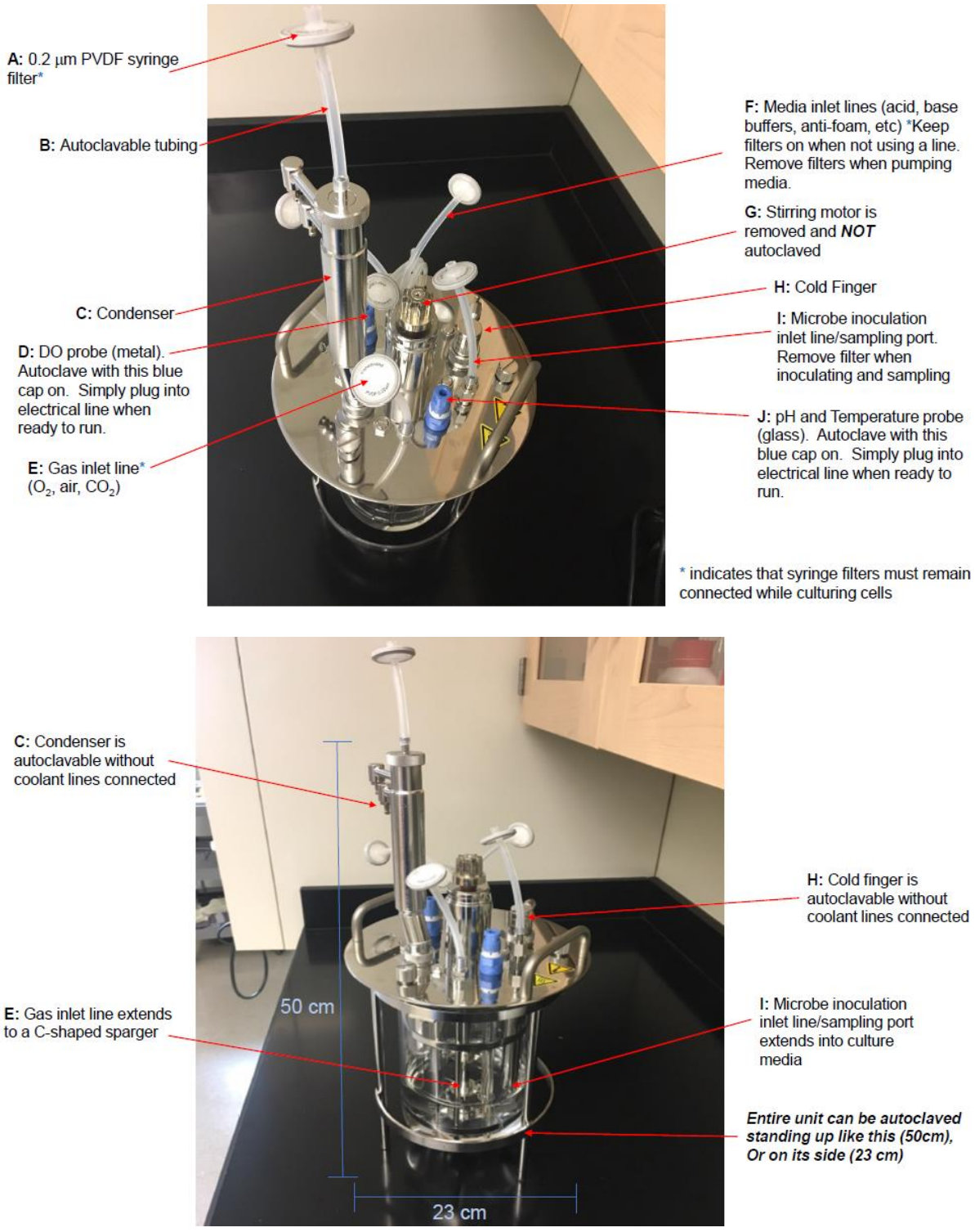

Figure 1: Notes on bioreactor assembly for autoclaving.

All holes in vessel head must be plugged. All tubing must be capped with 0.20 μm filters. Foam, pH and dissolved oxygen (DO) probes can be autoclaved inside of vessel. Screw all plugs, screws and probes "finger tight" only. You can tighten them all after autoclaving.

Calibration of pH probe should be performed with 2 standards (pH 7.00 and pH 4.00). pH probe does not require electrolyte prior to assembly in the vessel.

Calibration of dissolved oxygen probe should be performed with water or media saturated in O_2. This can be achieved by sparging O_2 for 5 mins with stirring at 250 RPM. Probe requires electrolyte prior to assembly in the vessel. Unscrew cap from bottom of probe shaft and add electrolyte to membrane basin holding the sensor. The membrane basin is located within the middle part shown in Figure 2. The cap on the right can be filled with electrolyte and used to store the DO probe when not in use. The pH probe has an identical cap for storage with electrolyte.

Figure 2: DO Probe disassembly for electrolyte addition.

Bioreactor should be autoclaved with 10-15 mL of DI water inside on 30 min liquid cycle at 121°C.

D) Prepare EZ Rich Media

A 5L supply of EZ Rich Media can be purchased from Teknova (www.teknova.com).

It arrives sterile and therefore does not require autoclaving. Some components of the media must be remain frozen, while other components can be stored at RT.

Prepare 1L of EZ Rich Media as follows:

Thaw individual bottles of "10x MOPS" (100 mL), "10x ACGU" (100 mL), and "5x Supplemental EZ" (200 ml).

In laminar flow hood, add the components above to 580 mL of sterile, DI water at room temperature. Sterile DI water is not included in EZ Rich Media from Teknova. Add 10 mL of sterile "0.132 M K_2HPO_4" solution supplied by Teknova.

Add 50 mL of sterile "20% glucose solution" to achieve a final concentration of 1% glucose in 1 L of EZ Rich Media.

Measure pH of media in the bioreactor and record: _____

E) Inoculation of Bioreactor

Remove a syringe filter at an open port and add the 10 mL culture in a syringe from part B1 to the bioreactor.

Set Temperature to 30^{o}C

Set stirring to 200 RPM

Allow the cells to grow at 50% O_2 saturation for 48 hours.

Sample 1-2 mL of culture broth by 0.2 μm syringe filtration followed by 1:1 dilution with acetonitrile into GC vial. Inject 2.0 μL onto GC.

Using the following GC-MS method, prepare a calibration curve for isopentenol.

Time (min)	Temperature (^{o}C)	Ramp rate (^{o}C/min)	Hold time
0	50		2.5
2.5	50	50	
5	175		2.0

- Run time ~ 7min. Inlet temperature = 275 ^{o}C. Transfer temperature = 250 ^{o}C
- Column = Thermo Scientific TG SQC 30 m x 25 mm x 0.25 μm
- 1 min solvent delay time.
- Isopentenol retention time is approximately 2.85-2.90 min.
- Major fragments are seen at m/z = 67 and 68
- Other possible mevalonic acid lactone derivatives detected at 5.53 and 6.00 min.
- See Appendix Figure A1 for chromatogram.

IV. Conclusions

Complete the following table:

Reaction time (hr:min)	O_2 saturation (%)	pH of media	Isopentenol concentration (mg/L)	Identity of other metabolites (m/z)
12:00				
24:00				
36:00				
48:00				

Assuming the hat is the theoretical yield of isopentenol from 1% glucose?

Based on your data, calculate the yield, rate and titer of isopentenol.

Time (hr:min)	Yield (mg/L)	Rate (mg/hr)	Titer (mg/L/hr)
0:00-12:00			
12:01-24:00			
24:01-36:00			
36:01-48:00			

How do these results compare to the theoretical yield?

V. Possible Variations on this experiment:

1) Allow culture to go anoxic (0% O_2 saturation) and determine metabolite profile. *E. coli* is a facultative anaerobe. Is O_2 required for isopentenol production?

2) Maintain pH at different levels (e.g. 6, 7, 8) over the 48 hr incubation period and determine the metabolite profile. Is there an optimum pH for isopentenol production, or production of other metabolites?

3) Instead of 1% glucose from Teknova, use different amount of glucose (e.g. 1%, 2%, 3%) and/or glucose generated from soda pulping/enzyme hydrolysis lab. Is there a difference in the yield, rate and titer of isopentenol production? Glucose must be sterile filtered as a solution, NOT autoclaved.

VI. References (to read before starting this experiment):

[1] A. Khan, T.S. Lee, Converting Sugars to Biofuels: Ethanol and Beyond. *Bioeng*., 2 (2015) 184-203.

[2] K.W. George, A. Chen, A. Jain, T.S. Batth, E.E.K. Baidoo, G. Wang, P.D. Adams, C.J. Petzold, J.D. Keasling, T.S. Lee, Correlation Analysis of Targeted Proteins and Metabolites to Assess and Engineer Microbial Isopentenol Production, *Biotechnol. Bioeng.*, 111 (2014) 1648-1658.

[3] D. Dugar and G. Stephanopoulos Relative potential of biosynthetic pathways for biofuels and bio-based products. *Nat. Biotech*. (2011) 29, 12, 1074-1078.

VII. Appendix:

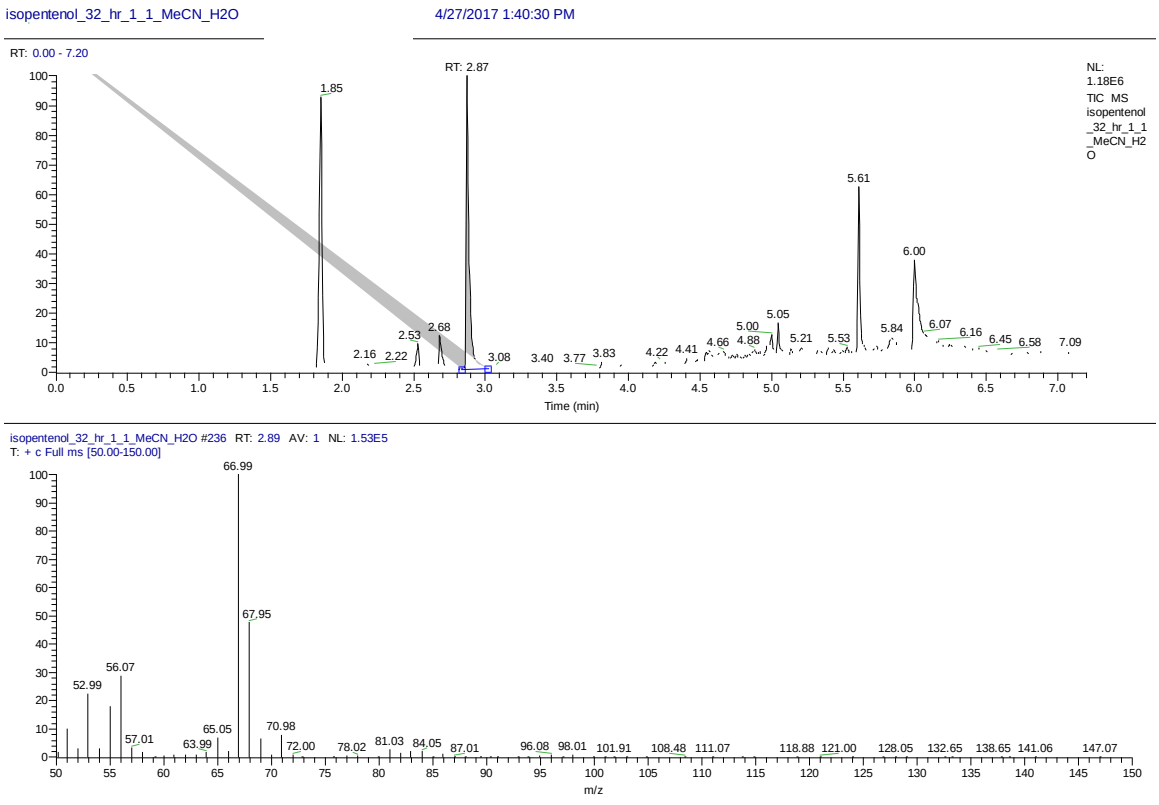

Figure A1: Chromatogram (top) and Mass Spectrum (bottom) of 2 μL injection of 1 mL of 32 hour E. coli JBEI Strain 1A culture broth filtered through 0.2 μm syringe filter into 1 mL of acetonitrile in a GC vial.