STANDARD OPERATING PROCEDURE
STAN MAYFIELD BIOREFINERY PILOT PLANT

TITLE: Strain Performance Verification

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A. Scope

This procedure describes how to verify that the working stock of the ethanol production strain, *Escherichia coli* SL100, meets the performance targets under controlled conditions. This procedure should be run every time the results are suspect, or every six months, whichever comes first.

B. Safety and Training Requirements

Refer to UF lab safety policies and review the Material Safety Data Sheets (MSDS) for each material listed in section D below before starting any process work.

Refer to UF Biosafety guidelines and the NIH Guidelines whenever handling biological cultures/genetically modified organisms.

Review the location of fire extinguishers, fire blankets, safety showers, spill cleanup equipment and protective gear before beginning any process work.

During operations in the lab, the following safety gear will be utilized at all times:

- Lab Coat
- Safety Glasses
- Protective Gloves (nitrile, neoprene)

C. Related Documents and SOPs

1. Strain Storage and Handling SOP-0508
2. Sugars, Organic Acids, and Inhibitors Determination SOP-0505
3. UF Biosafety manual
4. MSDS sheets for chemicals listed in section D
5. Ethanol Concentration by Gas Chromatography SOP-0500
6. Optical Density measurement SOP-0513

D. Preparation/Materials/Equipment

1. 3 Sterile POM Fleakers equipped with pH control towers and 37°C water bath
2. Sterile 13 mm test tubes filled with water
3. Sterile laminar flow clean bench
4. D-xylose
5. Monobasic ammonium phosphate ($NH_4H_2PO_4$)
6. Dibasic ammonium phosphate ($(NH_4)_2HPO_4$)
7. Magnesium sulfate ($MgSO_4$)
8. Iron chloride hexahydrate ($FeCl_3 * 6H_2O$)
9. Cobalt chloride hexahydrate ($CoCl_2 * 6H_2O$)
10. Copper chloride dihydrate ($CuCl_2 * 2H_2O$)
11. Zinc chloride ($ZnCl_2$)
12. Sodium molybdate dihydrate ($Na_2MoO_4 * 2H_2O$)
13. Boric acid (H_3BO_3)
14. Manganese chloride tetrahydrate ($MnCl_2 * 4H_2O$)
15. Concentrated hydrochloric acid (HCl)
16. Sterile DI water
17. Nalgene rapid flow bottle top filter units 0.2 µM PES membrane
18. 3 sterile 1 L media bottles.
19. Aluminum foil
20. Laboratory balance
21. Analytical balance

E. Detailed Procedure

1. Add 131.5 mL of sterile DI water by weight (131.5 g) to all three POM Fleakers (final volume will be 200 mL).
2. Dispense 40 mL of 50% w/v aqueous xylose into each POM Fleaker (100 g/L of xylose final concentration).
    a. To make the xylose stock, weigh 250 g of D-xylose into a beaker and bring to 500 mL total volume using DI water. Mix until completely dissolved.
    b. Filter sterilize using Nalgene rapid flow bottle top filter units with 0.2 µM PES membrane. Store in the refrigerator for up to 6 months.
3. Dilute 100 mL of concentrated hydrochloric acid (~12.1 M) into 800 mL of DI water and complete to 1 L. The final concentration will be ~121 mM.
4. Add 0.20 mL of 1000X trace metal stock to each POM Fleaker.
    a. To prepare the 1000X stock, the following concentrations of elements are dissolved 121 mM HCl. Make sure to use the analytical balance for measuring these amounts.
        i. 2400 mg/L $FeCl_3 * 6H_2O$

ii. 300 mg/L $CoCl_2 * 6H_2O$
iii. 150 mg/L $CuCl_2 * 2H_2O$
iv. 300 mg/L $ZnCl_2$
v. 300 mg/L $Na_2MoO_4 * 2H_2O$
vi. 75 mg/L H_3BO_3
vii. 495 mg/L $MnCl_2 * 4H_2O$

b. Filter sterilize the solution using the Nalgene sterile filter top units into a 1 L sterile media bottle and store in the refrigerator wrapped in foil.

5. Dissolve 120.4 g of magnesium sulfate ($MgSO_4$) into 1 L of DI water and filter sterilize using the Nalgene sterile filter top units into a 1 L sterile media bottle.
6. Add 0.30 mL of 1 M magnesium sulfate to each POM Fleaker for a final concentration of 1.5 mM.
7. Add 20 mL of 10X ammonium phosphate stock to each POM Fleaker.
    a. To prepare the ammonium phosphate stock, add to a 1 L beaker containing 250 mL of DI water 8.6 g of monobasic ammonium phosphate and 26.3 g of dibasic ammonium phosphate.
    b. Bring to 1 L with DI water, ensuring everything is dissolved.
    c. Filter sterilize using Nalgene rapid flow bottle top filter units 0.2 µM PES membrane into a sterile 1 L media bottle.
    d. Store solution in the refrigerator for up to 6 months.
8. Remove one 8 mL cryovial from the strain storage freezer and defrost according to Strain Storage and Handling SOP-0508.
9. Inoculate each POM Fleaker using 2.0 mL of defrosted glycerol culture.
10. Right after inoculation, aseptically take a sample of the broth for analysis in order to measure sugars, inhibitors and OD, according to Sugars, Organic Acids, and Inhibitors Determination SOP-0505 and Optical Density Measurement SOP-0513.
11. Incubate the POM Fleakers at 150 rpm for 72 h in the 37 °C water bath.
    a. Place POM Fleakers in the water bath and turn the magnetic stir plate to on.
    b. Ensure the setting is at 150 rpm.
    c. Rinse pH probes (stored in fresh formaldehyde/KCl) 4 times each, using a new sterile-water filled test tube and carefully insert into the POM Fleaker top.
    d. Prime the base line using a 10 mL sterile syringe, and insert the line into the closed solenoid located on the top edge of the POM Fleaker tower.
    e. Carefully connect the Luer-Lock end of the base line to one of the two outer Luer-Lock needles on the POM Fleaker lid.
    f. Screw a Luer-Lock sterile syringe onto the other Luer-Lock needle on the POM Fleaker lid.
12. Every 24 h, aseptically sample the broth for sugars, OD, and ethanol according to the Sugars, Organic Acids, and Inhibitors Determination SOP-0505, the Optical Density

Measurement SOP-0513, and the Ethanol Concentration by Gas Chromatography SOP-0500.

13. Compare the results obtained to the performance criteria below (see attached table).
14. If the strain performance parameters fall outside the targeted criteria, contact the supervisor.

F. Data Archival and Analysis

Record all fermentation parameters, OD, sugars, and ethanol measurements and compare to established criterion.

Time (h)	pH		45% KOH used (mL)		Ethanol (g/L)		$OD_{550\ nm}$ (If applicable)	
	Actual	Target	Actual	Target	Actual	Target	Actual	Target
0		6.3		0		0		0.1
24		6.3		5-10		10-15		3-5
48		≥6.3		5-10		25-35		5-7
72		≥6.3		5-10		45-48		6-7