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STANDARD OPERATING PROCEDURE
STAN MAYFIELD BIOREFINERY PILOT PLANT

TITLE: Primary Flask Culture

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APPROVALS: Process Change Committee **DATE:**

A. Scope

This procedure describes how to use hydrolysate obtained from dilute phosphoric acid pretreatment for inoculation of the primary propagators.

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 for Research Involving Recombinant DNA Molecules 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 Goggles
- Protective Gloves (nitrile, neoprene)

C. Related Documents and SOPs

1. Ethanol measurement SOP-0500
2. Strain storage SOP-0508
3. Optical Density Measurement SOP-0513Sugar and inhibitor determination by HPLC SOP-0505
4. UF Biosafety manual
5. MSDS sheets for chemicals listed in section D
6. Experimental Plan

D. Preparation/Materials/Equipment

1. 5 sterilized 2 L flasks

2. Sterile 500 mL graduated cylinders
3. 2 2 L side arm flasks
4. 2 New Brunswick Scientific Innova 40 shaker incubator
5. Sterile laminar flow hood
6. Laboratory Balance
7. Biomass Hydrolysate
8. Ammonium hydroxide (5 N)
9. 1 M magnesium sulfate
10. 1 L volumetric flask
11. 120 mM hydrochloric acid
12. 0.5 M sodium metabisulfite
13. Stir plate
14. Automatic Pipette (200-1000 µL)
15. Sterile Pipette tips (200-1000 µL)
16. Sterile beaker/flask to hold 5 liters
17. Nalgene disposable filter sterilization unit (Catalog # 567-0020)
18. Buchner funnel
19. GF/D filter

E. Detailed Procedure

1. Prepare the Trace Metals Stock solution.
    a. Add 500 mL of 120 mM HCl to a 1 L volumetric flask.
    b. Dissolve in the 120 mM solution 2.4 g of $FeCl_3*6H_2O$, 300 mg of $CoCl_2*6H_2O$, 150 mg of $CuCl_2*2H_2O$, 300 mg of $ZnCl_2$, 300 mg of $Na_2MoO_4*2H_2O$, 75 mg of H_3BO_3, and 495 mg of $MnCl_2*4H_2O$.
    c. Complete the volume to 1 L using 120 mM HCl.
    d. Filter-sterilize the solution using a 1 L Nalgene disposable sterilization unit.
2. Pre-filter 1.5 L of hydrolysate through a Buchner funnel using a GF/D filter and 2 L side arm flasks.
3. Adjust the pH of 1.5 L of hydrolysate to pH 9.0 using 5 N ammonium hydroxide and filter-sterilize it.
4. Let the hydrolysate sit overnight at room temperature.
5. In the sterile laminar flow hood, dispense aseptically 300 mL of hydrolysate into each sterile 2 L flask.
6. Aseptically, add 1.0 mL of trace metal stock to each flask using automatic pipette.
7. Aseptically, add 1.50 mL of 1 M magnesium sulfate to each flask using automatic pipette.

8. Still working inside the laminar flow hood, weigh 687 g of sterile DI water into the flask. Alternately, a sterile 500 mL graduated cylinder can be used.
9. Add 2.0 mL of 0.5 M sodium metabisulfite to each flask for a final concentration of 1 mM.
10. Inoculate each flask using 8 mL of frozen glycerol culture that has been quickly thawed in cool water. Right after inoculation, aseptically take a 5 mL sample of the broth for analysis (sugars, inhibitors, OD).
11. Incubate in the shake incubator at 37 °C and 150 rpm for 24 h.
12. After 24 h, aseptically sample the broth for analysis (ethanol, sugars, inhibitors, OD). After ensuring that the ethanol and OD requirements are met (according to the Experimental Plan) the broth is ready to be used for inoculation of the primary propagator.

F. Data Archival and Analysis

Record the information of the hydrolysate used and all fermentation parameters (OD, sugars, inhibitors, and ethanol measurements) in batch record and fermentation log sheet. Store all log sheets, batch records, HPLC chromatograms, and fermentation log sheets in a folder labeled with Run Number.