STANDARD OPERATING PROCEDURE
STAN MAYFIELD BIOREFINERY PLANT

TITLE: 10 L Fermentation Operating Procedure

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APPROVALS: **Process Change Committee** **DATE:**
UF EH&S **DATE:**

CONTENTS

A. Scope

This procedure describes how to use dilute phosphoric acid pretreated bagasse hydrolysate (refer to Biomass Pretreatment and Screw Press Dewatering SOP) for seed propagation of *Escherichia coli*. The hydrolysate is pH adjusted to 6.3 using ammonium hydroxide (5 N) and added to a 10 L fermentor (refer to Autoclave Procedure for 10 L Fermentor SOP). The culture is maintained at 37°C for a predetermined amount of time (usually 24 h). Air (0.01 vvm), Luria-Bertani broth, sodium metabisulfite (0.5 M), AM1 Salts (50 X), 1 M $MgSO_4$, and AM1 trace elements are then added. The hydrolysate is then inoculated and incubated for a predetermined amount of time.

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.

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 plant, the following safety gear will be utilized at all times:

- Lab Coat
- Safety Goggles or Face Shield
- Protective Gloves (nitrile, neoprene)
- Autoclave Gloves

Avoid inhalation of vapors and wear nitrile or neoprene rubber gloves. Contain spills by using spill kits next to fermentors. The ammonium hydroxide must be tightly sealed at all times to avoid escape of vapors.

C. Related Documents and SOPs

1. New Brunswick Scientific Bioflo 3000 Bench-top Fermentor guide to operations manual No: M1227-0050.
2. Primary seed flask culture SOP
3. 10 L seed culture SOP
4. Culture transfer 10 L to 140 L SOP
5. Sampling procedure SOP
6. Strain storage SOP
7. Biomass pretreatment SOP
8. Transfer SOP
9. Sugar and inhibitor determination by HPLC SOP
10. UF Biosafety manual
11. MSDS sheets for chemicals listed in section D

D. Preparation/Materials/Equipment

The equipment used in this SOP is listed below:

1. New Brunswick Scientific Bioflo 3000 Batch Continuous Bioreactor
2. Brinkmann Instruments Lauda K-4/R Electronic chiller
3. Masterflex Norprene tubing (manufactured by Saint-Gobain, D6402-15)

4. 250 ml graduated cylinder (plastic)
5. Bradley James Corporation FermProbe (K23857)
6. Kuir Tec clearbraid K3150 RF tubing by Kuriyama (1/4" I.D., max. 250 psi, non-toxic PVC, NSF 51)
7. Cole Parmer air flow meter (0-0.5 LPM)
8. Fisherbrand nylon filter (0.45 µm)
9. SCHOTT screw-cap with two spouts
10. Pyrex 500 ml bottle
11. Sterile laminar flow hood
12. Nalgene filter sterilizing units (250 ml, 500 ml, 1000 ml)

The chemicals/materials used in this SOP are listed below:

1. Hydrolysate
2. Ammonium hydroxide (5 N; Labchem Inc. 500 ml LC11110-1 or 4 liter LC11110-4)
3. Magnesium sulfate heptahydrate (1 M, filter-sterilized, Fisher 500 g M63-500)
4. AM1 trace elements (filter-sterilized)
5. AM1 media salts (50X, filter-sterilized; $(NH_4)_2PO_4$ Fisher 500 g A686-500; $NH_4H_2PO_4$ Fisher 500 g A684-500)
6. Sodium metabisulfite (0.5 M, Sigma-Aldrich ReagentPlus $\geq$ 99%, S9000-500G)
7. Xylose (Spectrum 5 kg CAS 58-86-6)

E. Detailed Procedure

1. Autoclave a prepped 10 L fermentor with 346 g of xylose (will vary depending on the total sugar present in the hydrolysate used; 50 g/L total sugar required in fermentor) and 7,423.11 ml of deionized water for 60 minutes at 121°C.
2. Add 5 N NH_4OH to pH-adjust the hydrolysate to 6.3 (typically 38 ml 5 N NH_4OH per liter of 180°C hydrolysate).
3. Filter-sterilize the pH-adjusted hydrolysate.
4. Make 1 M $MgSO_4$ by adding 246.47 g of magnesium sulfate heptahydrate per liter of solution and filter-sterilize.
5. Make 50 X AM1 Salts by adding 131.5 g of $(NH_4)_2PO_4$ and 43.5 g of $NH_4H_2PO_4$ per liter of solution.
6. Make 0.5 M sodium metabisulfite by adding 66.03 g of sodium metabisulfite per liter of solution.
7. Make 667 X AM1 trace elements.
8. Add 2000 ml of pH-adjusted filter-sterilized hydrolysate to the autoclaved 10 L fermentor.
9. Add 15 ml of AM1 Trace elements.
10. Add 15 ml of 1 M Mg SO_4.
11. Add 160 ml of 50X AM1 Salts.
12. Connect the jacket water out and jacket water in (in this order; refer to Bioflo 3000 fermentor manual).
13. Activate temperature control set to 37°C (refer to Bioflo 3000 fermentor manual).
14. Turn chiller switch on and connect tubing to condenser water out and condenser water in connections on the fermentor (in this order; refer to Bioflo 3000 fermentor manual).
15. Set up base addition apparatus by hooking up Norprene tubing to "Feed 1" pump and placing one end in a 250 ml graduated cylinder filled with 5 N NH_4OH (refer to Bioflo 3000 fermentor manual).
16. Hook up the other end of the Norprene tubing to a spout on top of the fermentor.

17. Make sure base tubing is hooked up correctly so that base is pumped into the fermentor. The pumps move clockwise.
18. Place a calibrated pH probe in the fermentor and connect to controller.
19. Activate pH control set to 6.3 (refer to Bioflo 3000 fermentor manual).
20. Connect clearbraid tubing to house air supply and to the air flow meter.
21. Connect clearbraid to air flow meter and to a spout on a 500 ml pyrex bottle. This spout should have a plastic tube going into deionized water in the bottle (400 ml).
22. Conncet another plastic tube to the other spout a filter and to a tube connected to a spout on top of the fermentor.
23. Turn the air supply on and adjust the flow of air to 0.02 L/min. using the knob on the flow meter.
24. Once temperature is up to 37°C add 10 ml of 0.5 M sodium metabisulfite.
25. Add 150 ml of a 500 ml seed flask grown for 6 h at 37°C in a shaker incubator (150 rpm; refer to Primary seed flask culture SOP).

F. Data Archival and Analysis

Record all fermentation parameters, OD_{550nm}, 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.

G. **Fermenation Log Sheet**

Start date and time: _____

Hz concentration of seed: _____

EtOH concentration at time of inoculation: _____

Total hours the seed was grown: _____

Total volume of the seed: _____

Total volume of the inoculum: _____

Vessel used for growing the seed: _____

RPM of the seed: _____

RPM of the fermentation: _____

Comments:

Experiment				
Time	5 N NH_4OH	pH	OD_{550}	EtOH g/L
0				
24				
48				
72				
96				
120				
144				

Experiment				
Time	5 N NH_4OH	pH	OD_{550}	EtOH g/L
0				
24				
48				
72				
96				
120				
144				

Experiment				
Time	5 N NH_4OH	pH	OD_{550}	EtOH g/L
0				
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