Document No.: SOP-002
Revision: 1.0

STANDARD OPERATING PROCEDURE
DEPARTMENT OF MICROBIOLOGY PILOT PLANT

TITLE: Ethanol Primary Flask Culture

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

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1. Scope

This procedure describes how to use dilute phosphoric acid pretreated biomass hydrolysate for propagation of the Ethanol Production Strain.

2. 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.

3. Related Documents and SOPs

A. Primary seed flask culture SOP
B. 10 L seed culture SOP
C. Culture transfer 10 L to 140 L SOP
D. Sampling procedure SOP
E. Strain storage SOP
F. Biomass pretreatment SOP
G. Transfer SOP
H. Sugar and inhibitor determination by HPLC SOP
I. UF Biosafety manual
J. MSDS sheets for chemicals listed in section D

4. Preparation/Materials/Equipment

The equipment used in this SOP is listed below:

A. Sterilized 500 ml flask
B. New Brunswick Scientific innova 40 shaker incubator
C. Sterile laminar flow hood
D. Nalgene filter sterilizing units (250 ml, 500 ml, 1000 ml)

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

E. Biomass Hydrolysate (SOP-xxx)
F. Ammonium hydroxide (5 N; Labchem Inc. 500 ml LC11110-1 or 4 liter LC11110-4)
G. Magnesium sulfate heptahydrate (1 M, filter-sterilized, Fisher 500 g M63-500)
H. AM1 trace elements (filter-sterilized)
I. AM1 media salts (50X, filter-sterilized; $(NH_4)_2PO_4$ Fisher 500 g A686-500; $NH_4H_2PO_4$ Fisher 500 g A684-500)
J. Sodium metabisulfite (0.5 M, Sigma-Aldrich ReagentPlus $\geq$ 99%, S9000-500G)
K. Tryptone (Bacto Tryptone 500 g, 211705)
L. Yeast extract (Bacto Yeast Extract 500 g, 212750)
M. Sodium chloride (Fisher 500 g, S271-500)
N. Xylose (50%, w/v, Spectrum 5 kg CAS 58-86-6)

5. Detailed Procedure

A. Preparation of Stock Solutions

1. Add 5 N ammonium hydroxide to increase the pH of the biomass hydrolysate to 6.3 (typically 38 ml 5 N NH_4OH per liter of 180°C hydrolysate).
2. Filter-sterilize the pH-adjusted hydrolysate.
3. Make 1 X Luria-Bertani broth by adding 10 g of Tryptone, 5 g of yeast extract, and 5 g of sodium chloride per liter of solution.
4. Make 50% (w/v) xylose by adding 500 g of xylose per liter of solution and filter-sterilize.
5. Make 1 M $MgSO_4$ by adding 246.47 g of magnesium sulfate heptahydrate per liter of solution and filter-sterilize.
6. 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.
7. Make 0.5 M sodium metabisulfite by adding 66.03 g of sodium metabisulfite per liter of solution.
8. Make 667 X AM1 trace elements.

B. Preparation of Seed Flask

1. Add 45 ml of pH-adjusted filter-sterilized hydrolysate to the sterilized 500 ml flask.
2. Add 50% xylose to a final total sugar concentration of 50 g/L taking into account the total sugar number obtained from hydrolysate HPLC analysis (usually 8 ml; 150 ml total fermentation volume).
3. Add 15 ml of Luria-Bertani broth.
4. Add 70.8 ml of sterilized deionized water (150 ml total fermentation volume).
5. Add 0.225 ml of 1 M Mg SO_4.
6. Add 0.225 ml of AM1 Trace elements.
7. Add 2.1 ml of 50X AM1 Salts.
8. Add 0.15 ml of 0.5 M sodium metabisulfite.
9. Add 1.5 ml of thawed glycerol stock.

C. Incubation

1. Place a sterile paper towel cap on top (refer to Autoclave Procedure for Seed Flask SOP) and place flask in a shaker incubator set to 37°C and 150 rpm.
2. Incubate for 6 to __ hours.
3. Sample flask according to SOP-xxxx to monitor growth and ethanol production.
4. Culture is ready for transfer when:
    a. OD =
    b. Ethanol =

6. 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.

7. 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		Experiment	

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

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

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