

**STANDARD OPERATING PROCEDURE
STAN MAYFIELD BIOREFINERY PLANT**

TITLE: Applikon 140 L Fermentor Operating Procedure

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A. Scope

This procedure describes how to use dilute phosphoric acid pretreated bagasse that has been enzymatically saccharified (refer to Enzyme Hydrolysis SOP) for ethanol fermentation by *Escherichia coli*. The enzymatically saccharified slurry is weighed and added to an Applikon 140 L fermentor. The slurry is allowed to cool in the fermentor to 37°C. AM1 Salts, 1 M MgSO₄, trace metals, and ammonium hydroxide (5 N) are then added to pH adjust the slurry to 6.3. The slurry is then inoculated and the fermentation is followed to completion.

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.

C. Related Documents and SOPs

1. Primary seed flask culture SOP
2. 10 L seed culture SOP
3. Culture transfer 10 L to 140 L SOP
4. Sampling procedure SOP
5. Enzymatic saccharification of pretreated sugarcane bagasse SOP
6. Transfer of enzyme saccharified slurry to 140 L fermentor SOP
7. 140 L fermentor heat kill SOP
8. Deionized water sterilization SOP
9. UF Biosafety manual
10. MSDS sheets for chemicals listed in section D

D. Preparation/Materials/Equipment

The equipment used in this SOP is listed below:

1. Applikon In-situ sterilizable 140 L fermentor
2. Cole-Parmer Masterflex B/T peristaltic pump Model 77111-60 (12-321 RPM, 50/60 Hz, 6.5 amps, 0.5 HP)
3. Masterflex Tygon LFL Perfect Position autoclavable tubing (06429-91, 1.5" O.D., 0.75" I.D.)
4. Masterflex pump controller (Cole-Parmer Instrument Co., Model 7021-26)
5. Masterflex Norprene tubing (manufactured by Saint-Gobain, D6402-15)

6. Sterilized 2 L glass Pyrex bottle with screw cap and two spouts for exhaust and base tubing hook-up.

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

1. Enzyme saccharified cake slurry
2. Ammonium hydroxide (5 N)
3. Magnesium sulfate (1 M, filter-sterilized)
4. Trace elements (filter-sterilized)
5. AM1 media salts (50X, filter-sterilized)

E. Detailed Procedure

The working volume for this fermentor is 20 to 140 liters. It is composed of a Bio controller (ADI010), fermentation vessel, jacket, tubing, frame, and Pilot System (ADI1075).

STERILIZATION:

1. Sterilize the tubing listed in section D above.
2. Cover spouts on the left top side of the fermentor with aluminum foil.
3. Make sure all steam valves to fermentors are closed.
4. Start up small boiler. It should take 20-25 min. to heat up and get up to pressure.
5. Make sure water lines and air valves are OPEN. They are located on the wall behind the fermentors.
6. Turn power switch ON by turning clockwise all the way; there should be two clicks (like turning a car on). An alarm will go off for ~10 seconds (this is normal). If power on does not work then release/turn OFF the emergency stop button by turning clockwise.
7. Make sure stirrer is OFF. The stirrer will automatically be off if it is set on Remote and the loop is OFF.
8. Make sure the fermentor light is OFF.
9. Make sure every clamp and valve that needs to be closed is closed tight before starting the sterilization procedure.
10. Close the exhaust valve V11.02.
11. Make sure valves V10.07 and V10.08 are closed.
12. Make sure the three spouts in the front of the fermentor are closed. They are closed when they are pushed out. Push them out by pressing the pin on the side of the spout and pull the spout out. Pushing in opens the spouts.
13. Remove water return tubing (red tube). Close water valve to condenser (this is the red tubing) and move the tube from the water supply and hook up to the drain.

14. Close valves **V14.01**, V14.02, V11.02 (exhaust line), V14.03. During fermentation when you are sampling, open V14.02.
15. Close valve V10.08 (sparger) and V10.07 (overlay). These two valves provide steam to the fermentation chamber.
16. Slowly Open steam valve (in back of the fermentor) all the way.
17. Activate Temp. loop.
18. Menu → Sterilization Settings Value (use knob) → choose values by pressing menu after entering the desired temperature (e.g. 121°C) and time (e.g. 20 min.). The steam to filter is preset at 90°C. Menu → Sterilization → Start. Menu → Sterilize Routine → Sterilization Activation.
19. When you see steam coming out of the lines going into the drain behind the fermentor, OPEN the cooling water valve (by the wall in back of the fermentor) to help with the steam sterilization.
20. During sterilization, pressure should be between 17 and 20 psi. Use exhaust valve V11.02 to maintain pressure below 20 psi. Open and close it accordingly.
 - Valve V10.19 shuts OFF during sterilization which then shuts OFF air (blue tube) also.
21. Turn valve V11.03 ON. Turn it ON during growth also when steam is ON to superheat HEO2 to prevent filter F03 from getting wet. If the filter gets wet then F03 will not be able to close properly.
22. At 100°C-102°C crack valve V10.07 (overlay) open to sterilize the line. Pay attention to the pressure. It must be kept below 25 psi by opening exhaust valve V11.02. The exhaust valve may need to slightly cracked open during steps 20-21. Open valves V11.01 (exhaust filter condensate) and V14.04. Close valve V10.07 (overlay) at 112°C-115°C.
23. Open valve V10.08 (sparger). Close the valve at 121°C.
24. Close the exhaust valve V11.02 (exhaust valve) if it was open.
25. Open spouts on the top left side of the fermentor one at a time to allow some steam to exit for about 10 seconds (use autoclave gloves). Close each after the 10 seconds and cap with aluminum foil.
26. Once the sterilization cycle is done, close valves V14.04, V11.01, V14.03, and V11.03.
27. Activate Temp. loop.
28. Hook the red tubing back the way it was. Put the tube into “water return” and into “water supply.” Open the water supply valve.
29. Close the main steam valve (optional). You may need steam to sterilize line when sampling.
30. Open overlay valve V10.07 to make sure you have 15 psi air to prevent a vacuum from building up during cool down. It will take about 45 minutes for the fermentor to cool to 37°C.
 - Look into sterilizing in place using pump, citric acid, and water.

- We might want to take the dip tube out and use V17.01 and a pump to pump media into the fermentor.

FERMENTATION START-UP:

1. Make sure water lines and air valves are OPEN. They are located on the wall behind the fermentor (there are four valves).
2. Turn Power switch ON by turning clockwise all the way; there should be two clicks. An alarm will go off for ~15 seconds (this is normal). If power on does not work, release/turn OFF emergency stop button by turning clockwise.
3. Add enzyme saccharified cake slurry (6 h of enzyme digestion) to Applikon 140 L fermentor (refer to Enzymatic saccharification of pretreated sugarcane bagasse SOP).
4. If stirrer is not ON turn it ON. The stirrer should be off during sterilization and if there is no media/liquid in the fermentor.
5. Equilibrate the slurry to 37°C.
6. Calibrate probe by selecting the pH loop, then pressing the calib button and following the instructions. The pH loop does not have to be on to calibrate the probe. The slope should be 1. If the slope is reading 0.8 then you may need a new probe. The offset should be ~0.7. If the offset starts going to ~0.2 then you may need a new probe. Black cable with blue sticker connecting to the front of the fermentor is the pH cable. The one with the black sticker is the dO₂ cable. Disconnect the blue cable and take out your probe to calibrate it in buffer. You can also connect it to a pH stimulator such as the pH stimulator 112 from Mettler Toledo to calibrate. Match the buffer temp. to the temp. that is being measured by the fermentor thermocouple at the bottom of the screen. After calibrating in pH 7 and 4 check the pH of the buffers again to make sure the probe is working correctly. It is a good idea to always take a sample of the starting media, go to another pH probe and measure the pH then use the “sample correction” function. Sample correction → calibrate → use knob to change pH based on the sample you know the pH for. If you mess up while calibrating the probe just choose the default option. Default → calibrate → will now go back to slope: 1, offset: 0, sample correction: 0 default values. If you have taken the probe out make sure to squirt some de-ionized water on the O-ring to lube it up to make it easy to put the probe back in.
 - The pH probe can be autoclaved in place. Use a slow vacuum.
 - DON'T CALIBRATE YOUR SPEED.
 - The “Setp” button is like an escape or back key on a computer keyboard.
 - Press Start/Stop after you change settings. Use knob to change settings.
 - Green light means the loop is active and within limits.
 - Constant red light means active loop is out of limits.
 - Flashing red and green light means the loop is inactive and out of limits.
 - “View” button will show you a graph. You can change the x (time) axis with the knob but you can't change the y-axis.
 - Menu → Access control (Applikon default password is 5345; right now it is 0000).
 - Menu → Manual control → allows you to turn ON all components with * manually; use the knob to choose a component and press Start/Stop button. You can control pum time/delay/read as well as dosage. Menu → Control output → Set up base pump → low: 0% high: 50% for our purposes

- Before each fermentation and if you are using a new kind of tube you could use a cylinder to calibrate for that particular type of tubing. Menu → Dose monitor → Calibrate → Start → Stop → enter volume used. **Hit “reset” after each fermentation run.** Pumps have to be on REMOTE to be controlled by computer.
 - Config. tells you how controls are set up.
7. Record the pH of the slurry.
 8. Add sterilized deionized water (refer to deionized water sterilization SOP), trace elements, magnesium sulfate, AM1 salts, and sodium metabisulfite in this order.
 9. Turn on the pH control to control pH at 6.3 with 5 N NH_4OH .
 10. Take $t = 0$ sample and store at -20°C . Samples will be taken every 24 h and stored for sugar and acids HPLC analyses.
 11. Add inoculum after pH (6.3) and temperature (37°C) are at desired values.
 12. Open exhaust valve V11.02 during the fermentation.
 13. Open overlay valve V10.07 to maintain a positive pressure in the fermentation vessel.

F. Data Archival and Analysis

Record all fermentation parameters, $\text{OD}_{550\text{nm}}$, 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.