Title: Process Controlled Fed-Batch Fermentation of Recombinant HSA Secreting *Pichia pastoris* SOP

Approvals:
Preparer:_____Kari Britt_____ Date ____01Apr09_____
Reviewer:_____Sonia Wallman_____ Date____ 01Apr09_____

1. **Purpose:**
   1.1. To produce a fed batch culture of yeast cells.
2. **Scope:**
   2.1. Applies to producing a process controlled fed batch culture of *Pichia pastoris* recombinant for human serum albumin.
3. **Responsibilities:**
   3.1. It is the responsibility of the course instructor/lab assistant to ensure that this SOP is performed as described and to update the procedure when necessary.
   3.2. It is the responsibility of the students/technicians to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.
4. **References:**
   4.1. pH Meter SOP
   4.2. autoclave SOP
   4.3. shaking incubator SOP
   4.4. spectrophotometer SOP
   4.5. microscope SOP
   4.6. Gram stain SOP
   4.7. Biolyzer SOP
   4.8. BioFlo 3000 SOP
   4.9. HSA ELISA SOP
   4.10. centrifuge SOP
   4.11. Cino, Julia, *High Yield Protein Production from Pichia pastoris Yeast: A Protocol for Benchtop Fermentation.* May 1999 American Biotechnology Laboratory.
5. **Definitions:** N/A
6. **Precautions:**
   6.1. Use BL2 safety measures and discard culture waste in biohazard containers.
   6.2. Ammonium hydroxide is extremely corrosive. Wear safety glasses and transfer into containers in a fume hood. It is extremely damaging to eyes and mucous membranes. It causes burns. Avoid contact with skin. Harmful if swallowed or inhaled.
7. **Materials:**
   7.1. *Pichia pastoris* expressing Human Serum Albumin (Yeast strain: GS 1 15/HIS+/MUT-/SEC HSA by Invitrogen is recommended.)
   7.2. BioFlo 3000 bench-top fermenter (New Brunswick Scientific Co., Inc.), 5 liter working volume
   7.3. visible microscope with 100x magnification
   7.4. shaking incubator (37^{o}C and 30^{o}C)
   7.5. autoclave

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7.6. water bath (30°C)
7.7. pH meter
7.8. spectrophotometer
7.9. centrifuge
7.10. Biolyzer or glucose test strips (Such as: Urine Reagent Strips from LW Scientific, Item Number: URS-01PR-GL77)
7.11. Antifoam A (optional)
7.12. potassium phosphate dibasic (K_2HPO_4)
7.13. potassium phosphate monobasic (KH_2PO_4)
7.14. glucose
7.15. yeast nitrogen base (YNB) without amino acids
7.16. yeast extract
7.17. peptone
7.18. five 500mL shake flasks
7.19. 100mL glass bottle
7.20. 1L flask
7.21. 100% methanol feed solution (1 Liter)
7.22. 30% ammonium hydroxide solution (500mL)
7.23. compressed air
7.24. Gram stain kit

8. Procedure:

8.1. **Media Preparation for Seed Flask Cultures**

8.1.1. Prepare 0.1M Potassium Phosphate Media, pH 6, 1X YNB with 1% Yeast Extract and 2% Peptone.

8.1.1.1. Dissolve 1.3 ± 0.05g potassium phosphate dibasic (K_2HPO_4) and 5.8 ± 0.05g potassium phosphate monobasic (KH_2PO_4) in 500mL±5mL deionized water in a 1L vessel to make 0.1M potassium phosphate buffer.

8.1.1.2. Adjust 0.1M potassium phosphate buffer to pH 6 ±0.1.

8.1.1.3. Add 5±0.5g yeast extract, 10±0.5g peptone, and 10g±0.5g glucose to the 0.1M potassium phosphate buffer and stir to dissolve.

8.1.1.4. Transfer 90mL of the 0.1M Potassium Phosphate Media with 1% Yeast Extract and 2% Peptone into five 500mL shake flasks so that each flask contains 90mL media.

8.1.1.5. Transfer 36mL of the media into a 100mL autoclavable bottle.

8.1.1.6. Autoclave the 500mL shake flasks and 100mL bottle containing media per autoclave SOP.

8.1.1.7. Prepare 100mL 10X Yeast Nitrogen Base (YNB) Solution without amino acids.

8.1.1.7.1. Weigh out 6.7±0.02g YNB without amino acids and combine with 100±1mL deionized water in a 500mL vessel.

8.1.1.7.2. Filter sterilize the 10X YNB and label as: Sterile Filtered 10X YNB, [date], [initials], Store: 2-8°C, Dispose: drain.

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8.1.1.8. Aseptically add 10mL 10X YNB to each of the five autoclaved and COOLED shake flasks of media containing 90mL of 0.1M Potassium Phosphate Media with 1% Yeast Extract and 2% Peptone.

8.1.1.9. Aseptically add 4mL YNB to the 100mL bottle containing 36mL 0.1M Potassium Phosphate Media with 1% Yeast Extract and 2% Peptone and store at 4°C until needed to blank the spectrophotometer.

8.1.1.10. Label the five shake flasks as: 0.1M Potassium Phosphate Media, pH 6, 1X YNB, with 1% Yeast Extract and 2% Peptone, [date], [group], [initials], Store: 2-8°C, Dispose: drain.

8.1.1.11. Label the 100mL bottle as: 0.1M Potassium Phosphate Media, pH 6, 1X YNB, with 1% Yeast Extract and 2% Peptone, [date], [initials], Blanking Media for Spectrophotometer, Store: 2-8°C, Dispose: drain.

8.1.1.12. Proof the media in the shake flasks at 37°C ± 0.5C and shaking at approximately 200rpm for a minimum of 24 hours.

8.1.1.13. Visually check the media in the shake flasks for contamination. If no contamination is present, four of them can be used for inoculation and one of them should be stored at 2-8°C until the media is needed for cryopreservation. Add to the label: For Cryopreservation of *Pichia pastoris*.

8.1.1.14. If the media in any of the shake flasks becomes contaminated, add bleach and dispose down the drain.

8.2. **Seed Flask Culture**

8.2.1. Thaw the contents of four 1mL cryovials (one vial per shake flask) of *Pichia pastoris* cells in 30°C water bath. Record the Vial ID including the passage number of the cells. Passage number is indicated as P[#].

8.2.2. Prepare the biological safety cabinet (BSC) per the BSC SOP.

8.2.3. Spray the outside of the cryovials and the autoclaved 500mL shake flasks containing 100mL 0.1M Potassium Phosphate Media, pH 6, 1X YNB with 1% Yeast Extract and 2% Peptone with 70% isopropanol, allow to dry for at least 30 seconds, and place them in the BSC.

8.2.4. Spray all items that will be needed for step 8.2.5 with 70% isopropanol and allow to dry for at least 30 seconds before placing in the BSC.

8.2.5. Sterilely transfer the contents of each vial to an autoclaved shake flask containing media in the BSC.

8.2.6. Remove the shake flasks from the BSC and label as: Pichia Inoculum [group], [date], [initials], Dispose: Autoclave then drain.

8.2.7. Incubate shake flasks for 24-48 hours at 30°C and shaking at approximately 200 rpm. Note: Shake flask caps should be loose while shaking to promote aeration of the culture.

8.3. **Sampling the Seed Flask Culture**

Reminder: Record all sampling results in the batch record and in the data table at the end of the batch record as needed.

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8.3.1. Aseptically remove a 2mL sample from each seed flask and place into a corresponding labeled cuvette.
Note: Do not label cuvettes in an area that would interfere with OD reading.

8.3.2. Take an OD reading of cultures at 600nm per the spectrophotometer SOP using the Blanking Media as the blank. OD absorbance should be between 2 and 6.
Reminder: If the initial sample OD reading is greater than 1.0, the sample should be diluted until it reads below 1.0 and then multiply by the dilution factor to obtain the absorbance value.

8.3.3. Prepare a Gram stain of each culture per the Gram Stain SOP and examine the cultures for contamination using a microscope per microscope SOP.

8.3.4. Transfer the sample to a test tube to measure the pH per the pH meter SOP.

8.3.5. Transfer 1.5mL of sample to a microfuge tube and centrifuge at high speed for 5 minutes.

8.3.6. Remove the supernatant and transfer it to a clean microfuge tube.

8.3.7. Label the tube as: Seed Flask Sample, HSA, [lot number], [date], [group], [initials] and store at 2-8°C until needed for SDS-PAGE and ELISA.

8.4. **Media Preparation for Bioreactor**

8.4.1. Prepare 2.7 liters of 0.1M Potassium Phosphate Media, pH 6 and 300mL 10X YNB for use in the bioreactor.
Note: Yeast extract and peptone are purposefully left out of the bioreactor media to help reduce foaming. However, if you experience poor cell growth, 1% yeast extract and 2% peptone can be added to the bioreactor media in future runs. If you choose to use yeast extract and peptone, then be prepared to use an antifoaming agent such as Antifoam A in case of excess foaming during the run.

8.4.1.1. Dissolve 2.3±0.05g potassium phosphate dibasic and 10.4±0.05g potassium phosphate monobasic in 900±10mL deionized water in a 2L flask to make 0.1M potassium phosphate buffer, pH 6.

8.4.1.2. Adjust 0.1M potassium phosphate buffer to pH 6 ±0.1.

8.4.1.3. Add 20±0.5g glucose to the 0.1M potassium phosphate buffer and stir to dissolve.

8.4.1.4. Label flask as: 0.1M Potassium Phosphate Media, pH 6, [date], [initials], Store: 2-8°C, Dispose: drain.

8.4.1.5. Repeat steps 8.4.1.1 though 8.4.1.4 two times to make 2.7L of 0.1M Potassium Phosphate Media, pH 6.

8.4.1.6. Prepare 300mL 10x Yeast Nitrogen Base (YNB) Solution without amino acids.

8.4.1.6.1. Weigh out 20.1±0.05g YNB without amino acids and combine with 300±5mL deionized water in a 500mL vessel.

8.4.1.6.2. Filter sterilize the 10X YNB and label as: Sterile Filtered 10X YNB, [date], [initials], Store: 2-8°C, Dispose: drain.

8.5. **Assemble BioFlo 3000 per BioFlo 3000 SOP.**

8.5.1. Clean all bioreactor parts per BioFlo 3000 SOP.

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8.5.2. Assemble the vessel per BioFlo 3000 SOP.
8.5.3. Assemble the headplate (underside) per BioFlo 3000 SOP.
8.5.4. Aseptically add 2.7L of 0.1M Potassium Phosphate Media, pH 6 to the vessel per BioFlo 3000 SOP.
8.5.5. Attach the headplate to the vessel per BioFlo 3000 SOP.
8.5.6. Assemble the headplate (top side) per BioFlo 3000 SOP.
8.5.7. Connect the bioreactor to the cabinet per BioFlo 3000 SOP.
8.5.8. Calibrate the pH probe per the BioFlo 3000 SOP.
8.5.9. Install Dissolved Oxygen probe per BioFlo 3000 SOP.
8.5.10. Attach tubing per BioFlo 3000 SOP.
8.5.11. Autoclave the entire assembly for at a minimum of 121°C and at least 30 minutes per BioFlo 3000 SOP and autoclave SOP.
8.5.12. Once the bioreactor vessel has cooled, aseptically add 300mL of filtered 10X YNB through the inoculation port of the headplate (See section 9 of the BioFlo 3000 SOP for the position of the inoculation port in the headplate.).

8.6. **Prepare Feed Solutions for BioFlo 3000**
8.6.1. Assemble two 1L flasks (each with a sidearm) for feed solutions.
8.6.1.1. Insert a 2mL glass pipet into a rubber stopper. Apply a small amount of deionized water to the outside of the pipet before inserting, if needed. Repeat this step with a second pipet and rubber stopper.
8.6.1.2. Insert the rubber stopper with glass pipet into the top of a 1L flask with a sidearm. Repeat this step with the second rubber stopper and 1L flask.
8.6.1.3. Adjust the height of the glass pipets so that the tips are just above the bottom of the flasks.
8.6.1.4. Attach tubing with an air filter to the side arm of each flask.
8.6.2. Autoclave the two assembled feed solution flasks per autoclave SOP.
8.6.3. Allow the feed solution flasks to cool to room temperature before adding feed solutions.
8.6.4. Aseptically pour approximately 500mL of 30% ammonium hydroxide (NH_4OH) into an assembled feed solution flask. CAUTION: Wear safety glasses and pour in a fume hood.
8.6.5. Aseptically pour approximately 1L of 100% methanol into an assembled feed solution flask.

8.7. **Prepare the bioreactor for operation per the BioFlo 3000 SOP.**
8.7.1. When prompted by the BioFlo 3000 SOP, input the working temperature into the control panel of the bioreactor.
8.7.1.1. Desired Working Temperature: 30°C
8.7.2. Set up the feed solution flask containing 30% ammonium hydroxide (NH_4OH) solution on Feed 1 per BioFlo 3000 SOP. Ammonium hydroxide is a basic solution.
8.7.3. Set up the feed solution flask containing 100% methanol solution on Feed 2 per BioFlo 3000 SOP.

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8.7.4. When prompted by the BioFlo 3000 SOP, input the desired pH into the control panel of the bioreactor.
8.7.4.1. Desired pH: 6.0
8.7.5. Calibrate the dissolved oxygen probe per BioFlo 3000 SOP.
8.7.6. Set DO mode to Controlling by Agitation Only per BioFlo 3000 SOP.
8.7.6.1. Set minimum agitation rpm to 200.
8.7.6.2. Set maximum agitation rpm to 1000.
8.7.6.3. Set agitation to DO control mode.
8.7.6.4. Set the dissolved oxygen level (DO) to 30%.
8.7.7. Disregard the use of the foam sensor.

8.8. **Fermentation Procedure**

8.8.1. Set up and start the BioCommand Lite program according to the instructions in the Fermentation Procedure section of the BioFlo 3000 SOP.

8.9. **Bioreactor inoculation**

Note: If excess foaming occurs during the run, an antifoaming agent can be added aseptically through the addition port. Dilute the antifoaming agent per the manufacturer's instructions. Alternatively, 1mL of soybean oil can be used as an antifoaming agent. There may be a small risk of contamination if you choose to use soybean oil. Therefore, aseptically remove the oil from a brand new container.

8.9.1. Allow all of the bioreactor time to reach all of its setpoints.
8.9.2. Choose the seed flask culture that has the highest OD and has NO contamination to inoculate the BioFlo 3000. Aseptically add the contents of the chosen flask through the inoculation port. The contents of more than one seed flask (with NO contamination) can be added if the OD readings are below 4.
Note: Unused seed flask cultures can be used for cryopreservation as directed in step 8.13.
8.9.3. Immediately take a sample of the culture following the instructions below.

8.10. **Sampling the Bioreactor Culture**

Reminder: Record all sampling results in the batch record and in the data table at the end of the batch record as needed.

8.10.1. Sample the culture a minimum of once per day.
8.10.1.1. Attach bulb to the sample port of the BioFlo 3000 (Be sure there is glass wool in tube before attaching.) and remove 2-8mL of culture.
8.10.1.2. Take an OD reading at 600nm per the spectrophotometer SOP using water as a blank for the spectrophotometer. Record the OD reading on the data table. **Reminder:** If the initial sample OD reading is greater than 1.5, the sample should be diluted until it reads below 1.5 and then multiply by the dilution factor to obtain the absorbance value.
8.10.1.3. Measure the glucose level per the Biolyzer SOP. Record the glucose reading on the data table.
8.10.1.4. Transfer 1.5mL of sample to a microfuge tube and centrifuge at high speed for 5 minutes.

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8.10.1.5. Remove the supernatant and transfer to a clean microfuge tube.
8.10.1.6. Label the tube as: Bioreactor Sample, HSA, [lot number], [date], [group], [initials] and store at 2-8°C until needed.
8.10.1.7. After 24-72 hours and when glucose levels reach an undetectable level move to Stage 2 of growth (described below). Ideally, the OD absorbance value should be approaching or greater than 20, but the culture can be can be moved into Stage 2 even if the OD is lower than 20.
8.10.1.8. From this point on, glucose levels do not need to be measured.

8.11. **Bioreactor Growth Stages**

8.11.1. **Stage 1: Batch Growth**

8.11.1.1. Maintain starting conditions for approximately 24-72 hours.
8.11.1.2. When the OD reaches approximately 20 and glucose levels are undetectable, move to Stage 2.

8.11.2. **Stage 2: Fed-Batch Production of Human Serum Albumin**

8.11.2.1. Change the setpoint for Feed 2 (100% methanol) to 1 (for 1%) by following the directions to "Activate additional feed loops at the appropriate time as indicated by the process SOP" section in the BioFlo 3000 SOP.
8.11.2.2. Feed for 12-48 hours and then harvest the culture.

8.12. **Data Collection and Cell Harvest**

8.12.1. Retrieve data generated by Biocommand Lite per BioFlo 3000 SOP.
8.12.2. Using the sampling assembly, collect 1L of culture into sterile bottles through the harvest port.
8.12.3. Transfer approximately 50mL of the culture into individual centrifuge tubes.
8.12.4. Centrifuge at approximately 3000xg for 5-8 minutes.
8.12.5. Remove the supernatant by pouring into sterile bottles.
8.12.6. Store supernatant at 2-8°C for use in downstream processing SOPs.
8.12.7. Harvest remaining culture through the harvest port into bottles for autoclaving, then disposal.
8.12.8. Shut down and clean the BioFlo 3000 per BioFlo 3000 SOP.

8.13. **Cryopreservation**

Note: It is recommended to cryopreserve cells from the unused seed flask cultures rather than the bioreactor, since treatment with methanol can be toxic to the cells.

8.13.1. Autoclave 50mL of 100% glycerol in a 100mL bottle per autoclave SOP.
8.13.2. Prepare the Biological Safety Cabinet (BSC) per the BSC SOP.
8.13.3. Spray the outside of all items that will be needed for steps 8.13.4 through 8.13.10 with 70% isopropanol, allow to dry for at least 30 seconds, and then place in the BSC.
8.13.4. In the BSC, sterilely transfer about 50mL of the culture into individual centrifuge tubes.
8.13.5. Remove the centrifuge tubes from the BSC to centrifuge at approximately 3000xg for 5 minutes.

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8.13.6. Spray the outside of the tubes with 70% isopropanol and allow to dry for at least 30 seconds before returning them to the BSC.
8.13.7. In the BSC pour off the supernatant into a waste container.
8.13.8. Sterilely add 11mL of autoclaved glycerol to the 100mL of 0.1M Potassium Phosphate Media, pH 6, 1X YNB with 1% Yeast Extract and 2% Peptone set aside for cryopreservation in step 8.1.1.13. to make the storage media.
8.13.9. Aseptically add 5mL of the storage media to each centrifuge tube and resuspend the pelleted *Pichia* cells.
8.13.10. Aseptically dispense 1mL aliquots to sterile 1.5mL cryovials. Label the cryovials: P. pastoris, HSA, [date], [initials], P[#]. Increase the passage number by one from the recorded Vial ID used in the seed flask culture.
8.13.11. Place cryovials in a Styrofoam tube rack. Label container: P. pastoris, HSA, Working Cell Bank, [date], [initials], P[#]. Store at -86°C.

9. **Attachments:**
9.1. Data table

10. History:

Name	Date	Amendment
Deb Audino Laura Hyson	31Aug07	Initial Release
Deb Audino	04Apr08	College name change and rearranged steps.
Kari Britt	01Apr09	Added in descriptions of growth stages and cryopreservation directions. Changed media components for the seed flask culture. Added additional steps to the sampling sections. Added information regarding antifoam. Rearranged steps for consistency to Batch Record and other upstream processing documents. Also made general grammar and formatting edits as needed throughout the document.

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Elapsed Time (Hours)	pH	Temp (^{o}C)	%DO2	Agitation (rpm)	Methanol Feed	OD (600nm)	Glucose (mg/dL)	Operator/Verifier