

Module III Manufacturing

Exercise 1. Good Manufacturing Practices

Estimated Time: 1 class period

- I. Data-recording rules that support good manufacturing practice.
 - A. Research and Development
 - B. Supplier Qualification Documentation
 - C. Batch Production Records
 - D. Quality Systems Records
 - E. Records Retention and Inspection
- II. Standard Operating Procedures

Exercise 2. Cell Growth

Estimated time: 2 class periods

- I. Prokaryotic expression systems
 - A. *Escherichia coli*—a typical prokaryotic expression system
 - B. Alternatives to the *E. coli* expression system
 - 1) *Bacillus subtilis*—secreted proteins
- II. Eukaryotic expression systems
 - A. Yeast—the expression system with the highest commercial value
 - B. Filamentous fungus—an excellent expression system for commercial production
 - C. Insect/baculovirus
 - D. Mammalian cells
 - 1) Relatively mature eukaryotic expression system
 - 2) Newly emerging expression system
 - F. Transgenic
 - 1) Animal expression systems
 - 2) Plant expression systems

MEW: Rupa, I intend this as a lecture series introducing different expression systems. Initially, they will all do *e. coli* expression—but ultimately we may be able at this point to let them pick (i.e. once you have different tracks, fully equipped labs, etc)

Exercise 3. Bacterial Fermentation: Upstream Processing

Estimated time: 1 – 2 class periods

Production of OPH by batch fermentation
Standard Operating Procedure
5L Scale Manufacture

Production Timeline

Day	Time	Operation	Comments
2-4 weeks	NA	Order supplies	Ensure that all reagents and supplies are ready for use
1-30 days	NA	Make trace element solutions	Make sufficient trace element solutions for all fermentations and seeds
2-5 days	NA	Plasmid transformation into host strain	The transformation should be completed with sufficient time for the agar plate to develop discrete colonies before it is needed to inoculate seed cultures.
2-14 days	NA	Make shake flasks for seed cultures	At least 2x50ml and 2x1L flasks are required
2-14 days	NA	Make antibiotic for seed cultures	At least 2.5ml of 10% antibiotic solution is required

Reagents, Supplies and Equipment

Reagents

Chemical	Supplier	Amount
Yeast extract	Difco	x g
Tryptone	Difco	x g
NaCl	Sigma	x g
TB Powder	VWR	x g
Ampicillin	Sigma	x g
<i>E. coli</i> DH5 α Competent cells	Invitrogen	1 vial
CoCl ₂ .6H ₂ O	Sigma	x g
NH ₄ OH	Sigma	x L
Glycerol	Sigma	x L

NOTE: The amount of each will depend on the class and fermenter size

Supplies

Item	Supplier	Amount
Sterile loops	Fisher/VWR	1 pack
Pipetter and tips	Fisher/VWR	
Fernbach Flasks (2.8 L)	Fisher/VWR	
Beaker (3 L)	Fisher/VWR	
1.5 ml epp tubes (sterilized)	Fisher/VWR	
Erlenmeyer flasks	Fisher/VWR	4 x 250 ml
Silicone tubing	Fisher/VWR	1 reel

NOTE: The supply list will depend on the class size and fermenter

Equipment

Item	Supplier	Amount
Shaking Incubator	Fisher/VWR	1-2
Low Temp Freezer (-80°C)	Fisher/VWR	1
Laminar Flow Hood	Fisher/VWR	1
Refrigerator/Freezer Combo	Fisher/VWR (Sears, etc)	1
Water Bath	Fisher/VWR	1
Light Microscope	Fisher/VWR	1
Bunsen Burners/Alcohol Lamps	Fisher/VWR	1
Plate Incubator	Fisher/VWR	1
Roller Tube/Tube Rack	Fisher/VWR	1

Plasmid Transformation into Host strain

I. Day 1: Prepare each of the following:

- A. Ampicillin (50 mg/mL stock; 100 μ g/mL working concentration)
 - 1) Add 2.5 g ampicillin to tube

- 2) Bring to 50 mLs with ddH₂O
 - 3) Filter sterilize 1 mL aliquots into sterile 1.5 microcentrifuge tubes
 - 4) Store at 4 °C
- B. LB plates
- 1) Add 10 g Bacto-Tryptone , 5 g Bacto-yeast extract, 10 g NaCl and 15 g Bacto-agar to clean 2 L erlenmeyer flask(s)
 - 2) Bring to 1 L with ddH₂O
 - 3) Cap with aluminum foil; attach a 1 inch piece of autoclave tape to the aluminum foil cap
 - 4) Autoclave at 121 °C for 30 minutes – Liquid Cycle
 - 5) Cool to approximately 55 degree
 - 6) Add 2 mls of the sterile, 10 mg/mL ampicillin; Mix well
 - 7) Pour into sterile petri dishes (makes approximately 2 sleeves, or 40 plates)
 - 8) Let dry overnight
- C. TB liquid media
- 1) Add 104.72 g TB powder to a 3 L beaker
 - 2) Add 17.6 mLs 50% glycerol
 - 3) Bring to 2200 L with dH₂O
 - 4) Dispense 1000 ml to each of two fernbachs, and 50 mls to each of four 250 mL flasks
 - 5) Cap each flask with aluminum foil; attach a 1 inch piece of autoclave tape to the aluminum foil cap
 - 6) Autoclave at 121 °C for 30 minutes – Liquid Cycle
- D. 50% Glycerol
- 1) Add 50 mLs glycerol
 - 2) Add 50 mLs dH₂O
 - 3) Autoclave at 121 °C for 15 minutes – liquid cycle

II. Day 2: Transformation

- A. Purified OPH plasmid is stored at -20 °C
- B. Remove the relevant vial(s) and thaw.
- C. Transform into *E. coli* DH5 α (Invitrogen).
 - 1) Add 2 μ l of plasmids to 200 μ l Invitrogen DH5 α competent cells.
 - 2) Incubate cells on ice for 25 minutes.
 - 3) Heat shock the cells in a water bath @ 42 °C for 30 s, then return to the ice for 2 minutes.
 - 4) Aseptically add 500 μ l sterile media
 - 5) Incubate for 60 minutes at 37 °C.
 - 6) Plate 650 μ l and 50 μ l of the cells in onto LB agar with ampicillin (100 μ g/ml)
 - 7) Spread for single colonies and incubate @ 37°C overnight.

Day 3: Transformation

- A. Remove the plates from the incubator. Store upside down at 4 °C.

III. Day 3 (0 hr): Seed Production

- A. Pre-Seed culture growth

- 1) Approximately 30 min prior to use, turn on the laminar flow hood, swab with ethanol, and switch on the UV light for 10 minutes.
- 2) Select 2 x 250 ml LB flasks each containing 50 ml of TB medium
- 3) Aseptically add 100 μ l of 50 mg/mL ampicillin stock solution to each flask
- 5) In the laminar flow hood, aseptically pick several colonies from the transformation plate and resuspend in sterile medium
- 7) Incubate the flasks at 30°C and 250 rpm in an incubator/shaker for 9 h.

II. Day 3 (0930 hr): Seed Production

- A. Turn on the laminar flow hood, swab with ethanol, and switch on the UV light for 10 minutes.
- B. Remove 10 μ l of culture and check microscopically to confirm that there is no contamination.
 - 1) If the cultures pass the microscopic examination proceed to the next seed stage
- C. Select two 2L TB flasks each containing 1L of TB medium.
- D. Aseptically add 1 ml of 10% ampicillin stock solution to each flask

Day 3 (1800 hr): Seed Production

- A. Aseptically transfer 10-20 ml of the 50 ml pre-seed culture to each of the 2L flasks
- B. Incubate the flasks at 30°C and 240 rpm in a incubator/shaker overnight.

Exercise 4. Bacterial Fermentation: Bacterial Growth

Reagents

Chemical	Supplier	Amount
Yeast extract	Difco	x g
Tryptone	Difco	x g
NaCl	Baker	x g
Ampicillin	Sigma	x g
Zinc Sulfate, Heptahydrate	Baker	x kg
Manganese Chloride, Tetrahydrate	Baker	
Ferric Chloride, Hexahydrate	Baker	
Cupric Sulfate, Pentahydrate	Baker	
Boric Acid	Baker	
Sodium Molybdate, Dihydrate	Baker	
Sulfuric Acid (6 N)		
Magnesium Sulfate, Heptahydrate		
Breox Foam Control Agent FMT 30		x ml
CoCl ₂ .6H ₂ O	Fisher/sigma	x g
Potassium phosphate, monobasic		x g
Potassium phosphate, dibasic		x g
Ammonium Sulfate		x g
NH ₄ OH	Fisher	x L
Glycerol	Fisher	x L

Supplies

Item	Supplier	Amount
Capped Bottles (150 ml)		
Beaker (200 ml)		

Equipment

Item	Supplier	Amount
BioFlo310	New Brunswick	
Vessel (5.7 L)	New Brunswick	
Autoclave		

I. Day 1: Upstream -- Prepare each of the following:

- A. K12 trace metals solution
 1. Add the following to a clean 200 beaker:
 - 5 g/L NaCl
 - 4 g/L ZnSO₄-7H₂O
 - 4 g/L MnCl₂-4H₂O

- 4.75 g/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$
- 0.4 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
- 0.575 g/L H_3BO_3
- 0.5 g/L $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$
- 2. Bring to 0.1 L with ddH₂O
- 3. Add ~12.5 ml/L of 6 N H_2SO_4
- 4. Filter sterilize and dispense in 5 ml aliquots.
- B. 25% Magnesium Sulfate, Heptahydrate Stock Solution
 - 1. Add 25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ to clean 200 ml beaker
 - 2. Bring to 100 ml with ddH₂O, while stirring
 - 3. Filter sterilize and dispense in 20 ml aliquots
- C. 50% Glucose Solution
 - 1. Measure volumetrically 75 mls ddH₂O into a 200 ml beaker
 - 2. While stirring, add 50 g glucose
 - 3. Once dissolved, bring solution to 100 mls
 - 4. Transfer to a 150 ml autoclavable bottle and cap
 - 5. Autoclave at 121 °C for 30 minutes – Liquid Cycle
- D. 1 mg/ml Thiamine Stock Solution
 - 1. Add 11 mg thiamine to a 20 ml tube
 - 2. Add 11 ml of ddH₂O to the tube
 - 3. Cap and vortex until thiamine is dissolved
 - 4. Filter sterilize into a sterile 15 ml tube.
- E. 0.15 g/ml Calcium Chloride Solution
 - 1. Add 1.5 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to a 20 ml tube
 - 2. Add 10 ml of ddH₂O to the tube
 - 3. Cap and vortex until dissolved
 - 4. Filter sterilize into a sterile 15 ml tube.

II. Day 2: Upstream (Fermentor Set-up)

Production is done at 5.7 L scale. The approximate volumes break down as follows:

MEW: We will need to modify this for scale based on your fermentor size.

- A. Prepare the following in the 5.7 L fermentor vessel:
 - 1) Add the following:
 - 3.5 g/L KH_2PO_4
 - 5.0 g/L K_2HPO_4
 - 5.0 g/L $(\text{NH}_4)_2\text{SO}_4$
 - 5.0 g/L Yeast Extract
 - 1.00 ml/L Breox Foam Control Agent FMT 30
 - 2) Bring to approximately 3.5 L, to allow space in the 5.7 L working volume vessel for components to be added after sterilization (working volume: 5 L)
- B. After autoclaving and cooling the vessel, add:
 - 1) 4 ml/L of the 25% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
 - 2) 20 ml/L of the prepared 50% Glucose Solution
 - 3) 1 ml/L of the prepared K12 Trace Metals Solution
 - 4) 2.2 mg/L of the prepared Thiamine Solution

5) 0.15 g/L of the prepared Calcium Chloride, Dihydrate

C. Control Setpoints

- 1) Key setpoints into the controller prior to inoculation:
 - Temperature -- 30°C
 - pH -- 7.0
 - Dissolved Oxygen -- 30%
 - Agitation -- 200-1000 rpm (responding automatically to oxygen demand)

D. Dissolved Oxygen (DO) Control

- 1) Calibrate the DO probe at 0% (obtained by briefly disconnecting the cable) and at 100% (obtained using 1000 rpm agitation and 5 L/m airflow rate).
- 2) After calibration, DO should remain stable at approximately 100% until vessel is inoculated.
- 3) Program the DO cascade as follows:
 - Press **Casc** tab from the bottom of the screen.
 - Select DO from dropdown menu next to “Cascade From”
 - Select Agit, Gasflo and O2 as listed in the table below.
 - Select Yes, to activate the cascades

To	Start Setpoint	DO Startout %	End Setpoint	DO Endout %
Agit	200	0	1000	50
GasFlo	5	50	10	90
O2	0	90	100	100

E. pH Control

- 1) Liquid base is used to maintain pH at setpoint, relying on the acid-producing culture to lower pH if needed. The pH control parameters are:
 - Base: ammonium hydroxide, 30% solution
 - Pump 1
 - Transfer tubing: Narrow bore silicone tubing
 - Vessel inlet: Triport adapter in the vessel headplate.
- 2) Pump Calibration: To assure the most accurate flow rate, calibrate the pump each time you change tubing:
 - Use a sample tubing (about 2 feet long) of each line attached to a pump head.
 - Insert the tubing into the appropriate pump head.
 - Set the pump assignment to “None”.
 - Set the setpoint to “10%”.
 - Record the quantity of water flowing into a graduated cylinder for a definite time period.
 - Repeat for setpoints of 20%, 30%, etc., to 100% in order to establish the flow rates.
- 3) Controller Setup:
 - Pump 1: Select "Base" in “Assignment”
 - Pump 2: Select “Acid” in “Assignment”
- 4) To set up the cascade:
 - Press **Casc** tab from the bottom of the screen.

- Select **pH** from dropdown menu next to “Cascade From”
- Select **Pump 1 and Pump 2** as listed below:

To	Start Setpoint	DO Startout %	End Setpoint	DO Endout %
Pump 1	0	0	100	100
Pump 2	0	0	50	-100

NOTE: A “cascade” is used to regulate a variable automatically through adjustment of available inputs. For example, an agitation, gas flow, and oxygen “cascade” regulates the DO setpoint through automatic adjustment of agitation speed and gas flow rates. The term “cascade” means that if agitation alone could not maintain the DO at setpoint, GasFlo control (air flow or a combination of air and oxygen flow) will next be added. Finally, if agitation plus gas flow are not sufficient to maintain the DO setpoint, additional oxygen will enter the mix.

In the DO cascade, dissolved oxygen is expressed as a percentage – a DO output of 100% means that the DO setpoint has been fully attained. Until 100% DO is attained, the loop being cascaded will increase sequentially based on the minimum and maximum setpoints. So, when DO output reaches 50%, GasFlo is ramped up from 5 to 10; and when DO output then reaches 90%, pure O₂ is added until the DO output reaches 100%. Note the following:

- Regardless of DO Start Output %, the setpoint of any cascade loop should never go below its minimum setpoint value, (ie. Agitation will never drop below the starting setpoint of 200 rpm)
- Likewise, regardless of DO Output %, the setpoint of any cascade loop should never rise above its maximum setpoint, (ie. Agitation will not rise above 1000 rpm)
- Start Output % corresponds to the minimum value that will produce the minimum setpoint; lower outputs will not affect setpoint.

Exercise 5. Bacterial Fermentation: Downstream

Exercise 6. OPH Purification

Equipment:

Chromatography System (AKTA; located in cold room)

Fraction Collector

Fraction collection tubes (16 x 100 mM)

Sepharose Column [Amersham Biotech XK-26 Column (Long)]

Amersham cat # 19-0146-01

Packed with ~220 mLs of SP Sepharose Fast Flow

Amersham cat # 17-0729-01

DEAE Column [Amersham XK-26 Column (Short)]

Amersham cat # 19-0315-01

Packed with ~35 mLs of DEAE Sepharose Fast Flow

Amersham cat # 17-0709-01

Small (50 mL) and large (150 mL) sample loading columns

Dialysis Tubing (10,000 – 12,000 molecular weight cut off) & dialysis clips

Spectra/Por molecular porous membrane tubing (25 mm x 16 mM; 2 mL/cm;

MWCO 12-14,000)

VWR cat # 25225-226

2 and 4 L graduated cylinders & stir bars

Biorad MiniProtean II Electrophoresis Apparatus

Super speed centrifuge

SLA1500 rotor, bottles AND ADAPTERS

SS-34 rotor and bottles

GS-3 rotor and 500 mL bottles

Amicon stir concentrators

Amicon 8050; 50 mL capacity

Amicon 8200; 180 mL capacity

Ultracel Amicon YM10 Ultrafiltration Discs, regenerated cellulose – store in 95% EtOH at 4 °C

44.5 mM diameter – for the 50 mL concentrator

NMWL 10,000

Millipore cat # 13622

63.5 mM diameter – for the 180 mL concentrator

NMWL 10,000

Millipore cat # 13632

Nitrogen tank and regulator

Sonicator with large flat tip

Reagents:

TB (Difco™ Terrific Broth)

50% Glycerol \geq 99.5%

50% glycerol 50% dH₂O

Sigma Cat # G7893

CoCl₂·6H₂O (Cobalt Chloride)

FW 207.3

Sigma cat # C-3169

Chemical Shelf 7

KH₂PO₄ (Potassium Phosphate Monobasic, Crystal)

FW 136.09

JT Baker cat # 3246-01

Chemical Shelf 4

K₂HPO₄ (Potassium Phosphate Dibasic, Powder)

FW 174.18

JT Baker cat # 3252-01

Chemical Shelf 4

KCl (Potassium Chloride)

FW 74.55

JT Baker cat # 3040-05

Chemical Shelf 4

KHCO₃ (Potassium Bicarbonate)

FW 100.12

Fisher cat # P-184

Chemical Shelf 4

Ampicillan (Sodium Salt)

FW 371.4

Sigma cat # A-9518

Freezer A

Streptomycin (Sulfate Salt)

FW 1,457

Sigma cat # S6501

Freezer A

Ammonium Sulfate, Molecular Biology Grade

FW 132.1

Calbiochem cat # 168356

Chemical Shelf 1 (extra is stored on top of the chemical shelves)

DIFCO Bacto™ Yeast Extract

DIFCO cat # 212720

Shelf above balance

NaCl

FW 58.44

EMD cat # 7760

Chemical Shelf #5

Bacto™ Tryptone

Fischer cat # 211699
Shelf above balance

I. Day 1: Preparation

- A. 1 M CoCl₂, (sterile)
 - 1) Add 23.79 g CoCl₂ to 100 ml ddH₂O
 - 2) Can be filter sterilized or autoclaved
- B. Buffer 6.7 (10 mM KPO₄, 50 μM CoCl₂, pH 6.7)
 - 1) Add 0.42 g Potassium Phosphate Dibasic
 - 2) Add 1.03 g Potassium Phosphate Monobasic
 - 3) Add 50 μL of 1 M CoCl₂
 - 4) Adjust pH to 6.7 with KOH, bring to 1 L with ddH₂O
- C. KHCO₃ Buffer (10 mM KPO₄, 50 μM CoCl₂, 10 mM KHCO₃, pH 6.7)
 - 1) Add 0.42 g Potassium Phosphate Dibasic
 - 2) Add 1.03 g Potassium Phosphate Monobasic
 - 3) Add 1.00 g Potassium Bicarbonate
 - 4) Add 50 μL of 1 M CoCl₂
 - 5) Adjust pH to 6.7 with KOH, bring to 1 L with ddH₂O
- D. Buffer 8.3 (10 mM KPO₄, 20 mM KCl, 50 μM CoCl₂, pH 8.3)
 - 1) Add 0.1 g Potassium Phosphate Monobasic
 - 2) Add 1.61 g Potassium Phosphate Dibasic
 - 3) Add 1.5 g of Potassium Chloride
 - 4) Add 50 μL of 1 M CoCl₂
 - 5) Adjust pH to 8.3 with KOH, bring to 1 L with ddH₂O
- E. 0.5 M Potassium Chloride pH 6.7 or 8.3
 - 1) Add 18.64 g KCl to 500 mL of either Buffer 6.7 or Buffer 8.3 (depending on step of protocol)
- F. Ampicillian stock (50 mg/mL stock; 50 μg/mL working concentration)
 - 1) Add 2.5 g ampicillian
 - 2) Bring to 50 mLs with ddH₂O
 - 3) Filter sterilize 1 mL aliquots into sterile 1.5 microcentrifuge tubes; store at 4 °C
- G. 10 % Streptomycin sulfate (w/v)
 - 1) Add 5.0 g streptomycin sulfate salt
 - 2) Bring to 50 mLs with Buffer 6.7
 - 3) Store unused portion at 4 °C
- H. Dialysis Tube Preparation
 - 1) Determine the appropriate length of dialysis tubing that you will need.
 - 2) Boil dialysis tubes for 10 minutes in 2% Sodium Bicarbonate, 1 mM EDTA, pH 8.0
 - 3) Rinse well with ddH₂O.
 - 4) Boil for 10 minutes in 1 mM EDTA, pH 8.0.
 - 5) Rinse well with ddH₂O.
 - 6) Store in 70% ethanol at 4°C.

II. Day 2 (0800): Purification Protocol

Cell free extractions

1. Chill the GS-3 rotor by placing it in the centrifuge and close the door. Make sure the temperature is set to 4 °C.
2. Place 500 mls of 6.7 Buffer and KHCO₃ Buffer in the refrigerator (4 °C) or on ice to cool.
3. Remove 50 g of cells from the freeze, add 500 mls of chilled KHCO₃ Buffer to the cells in a beaker and thaw on ice.
4. Transfer the resuspended cells to a plastic beaker on ice.
5. The sonicator should be set to 50-60% duty, 7 output control, HOLD time, and continuous. Place your cell solution (on ice) and lower the sonicator tip into the cell solution. The tip should be approximately 1 inch from the bottom of the plastic beaker. The tip must be submerged at least ½ of its diameter.
6. Turn on the sonicator for one minute. Turn the sonicator off for one minute. Repeat 5 more times for a total of 6 times.
7. Transfer 0.5 mLs to a sterile 1.5 mL microcentrifuge tube and label "Sample 1". Store tube at -20 °C.
8. Transfer the lysate (sonicated cell suspension) to centrifuge tubes. Don't fill the tubes more than ¾ full and balance the tubes with the scale balance.
9. Centrifuge the lysate for 45 minutes at 12,000 rpm at 4 °C.
10. Carefully transfer the supernatant to a clean graduated plastic beaker on ice, being careful not to disturb the pellet. Record the volume of the supernatant.
 - a) The pellet will need to be autoclaved. Add water to the bottle containing the pellet and using a glass or plastic rod, break up the pellet. Transfer the contents of the bottle to a Fernbach scheduled to be autoclaved. Used media and cell debris should be autoclaved at 121 °C for 60 minutes.

Streptomycin sulfate ppt

11. Using a stir plate and stir bar, slowly stir the supernatant on ice. SLOWLY add, in a drop wise fashion, 10% streptomycin sulfate to the supernatant so that the final concentration of streptomycin sulfate is approximately 1%. This should take approximately 5 to 10 minutes.
 - a) For example, if you have 300 mLs of supernatant, add 30 mLs of 10% S.S.
 - b) Once all of the streptomycin sulfate has been added, your solution should be white and milky in appearance. This indicates precipitation of nucleic acids.
 - c) Let the 1% S.S. solution stir gently, on ice, for at least 15 minutes.
12. Transfer the solution to clean centrifuge bottles, make sure the tubes are balanced. Centrifuge for 30 minutes at 12,000 RPM at 4 °C.
13. After centrifugation, transfer 0.5 mLs to a sterile 1.5 mL microcentrifuge tube and label "Sample 2". Store tube at -20 °C.

Ammonium sulfate ppt

14. Carefully transfer the supernatant to a clean graduated beaker on ice, being careful not to disturb the pellet. Make a note of the volume of the supernatant.

15. Using a stir bar and a stir plate, the supernatant solution should be stirring. Weigh out enough Ammonium Sulfate to bring final concentration of ammonium sulfate in the solution to 45%.
 - a) For every 100 mls of solution, add 25.8 grams of ammonium sulfate. For example, if you have 250 mLs of solution, add 64.5 grams of ammonium sulfate.
16. Add the ammonium sulfate VERY SLOWLY. Add a few grams and then let the solution reach equilibrium. Then add a few more grams. This process should take you at least 30 minutes.
 - a) Failure to add ammonium sulfate solution slowly will result in a failed purification.
17. Once all of the ammonium sulfate has been added, allow the solution to stir in the cold room overnight.

Day 2 (0800): Ion-exchange chromatography

1. Prepare 4 L of Buffer 6.7 and chill in the cold room (4 °C).
2. Prepare 6 L of Buffer 8.3 and chill in the cold room (4 °C).

Day 2 (1300): Sepharose Chromatography

1. Retrieve your ammonium sulfate ppt
 - a) The solution should be cloudy in appearance. This indicates precipitation (“salting out”) of proteins.
 - b) Transfer 0.5 mls to a sterile 1.5 mL microcentrifuge tube and label “Sample 3”. Store tube at -20 °C.
2. Transfer the ammonium sulfate solution to clean centrifuge tubes. Make sure that they are balanced. Centrifuge for at least 60 minutes at 12,000 RPM at 4 °C.
 - a) After centrifugation, transfer 0.5 mLs to a sterile 1.5 mL microcentrifuge tube and label “Sample 4”. Store tube at -20 °C.
3. Discard the supernatant.
4. Gently resuspend the pellets in 40 mls of chilled Buffer 6.7. Do not vortex. (Note: if you do a 5 L prep, resuspend the pellet in 50 mLs of Buffer 6.7. Use 60 mLs for a 6 liter prep and so on).
5. Carefully transfer the resuspension to prepared dialysis tubes (rinse with dH₂O prior to use) and make sure that both ends of the tubing are sealed using dialysis clips.
 - a) Dialyze against chilled 2 L of Buffer 6.7 for 3 hours in the cold room.
 - b) Transfer dialysis bag to fresh Buffer 6.7 (2 Liters) and dialyze overnight.
 - c) The buffer change is very important because OPH will NOT bind to the sepharose column if ammonium sulfate is still present.
6. Equilibrate your Sepharose Column overnight with Buffer 6.7 at 1 mLs/minute. Pass the buffer through both A and B tubes (i.e. set “B” to 50%). Make sure you have enough buffer!
 - a) Allowing 16 hours at 1 mL/min, you will need at least 960 mL of buffer (60 minutes x 16 hours x 1 mL/min).
 - b) Failure to use enough buffer will result in a dry (and ruined) column that will need to be repacked.

Day 3 (0800): Sepharose Chromatography

- Carefully transfer the dialysate to centrifuge tubes. Centrifugation at 10,000 rpm for 20 min.
 - After centrifugation, transfer 0.5 mLs to a sterile 1.5 mL microcentrifuge tube and label "Sample 5". Store tube at -20 °C.
- Verify that the column is equilibrated by checking the conductivity and pH of the column wash buffer. Both should be the same coming out of the column as going in.
- The supernatant should be yellow-orange and free of particulates. Load the supernatant into the large, glass super-loop (making sure that no air bubbles are present) and attach to the AKTA.
- Prepare the fraction collector (collect 6 ml fraction) and chart recorder
 - Generally, you want the chart recorder to record the protein elution as a definable peak between the 0 – 100 % marks (on the paper). If the recorder is set to low, you won't see a protein peak. If it is too high, you won't be able to determine where the fractions eluted from the column. This may require close monitoring during the initial run to determine optimal settings.
- Set the AKTA to run at 3 mL/min, 0% B (i.e. 100% A) with Buffer 6.7.
 - Pump A line should be in Buffer 6.7, pump B line should be in 0.5 M Potassium Chloride
- As the protein elutes from the column, peak(s) will be displayed on the chart recorder. Once the signal has returned to the starting point, your column has been washed of excess protein. Since this is a cation exchange column, OPH should be retained in the column. Stop the AKTA.
- Set the AKTA to run at 3 mL/min, with a gradient of 0-400 mls of 0 – 100% B (0.5 M Potassium Chloride).
- When the cycle is finished, wash the column with 1 M Potassium Phosphate pH 6.7 and then re-equilibrate in Buffer 6.7.
 - Check the conductivity of the buffer eluting off the column to make sure that it is the same as that going into the column. The column may be stored in Buffer 6.7 for 2 weeks at 4 °C. If stored longer, equilibrate the column in 40% ethanol and Buffer 6.7.
- OPH elutes from the column at 125-175 mM KCl (approximately between fraction # 30 – 35, under the described conditions)
- Using a paraoxonase spot assay and SDS-PAGE gel, screen fractions for the presence of the enzyme.

Paraoxonase Spot Assay

- Using one well per fraction, add 50 µl of 1 mM paraoxon, 20 mM CHES (pH 9.0) to each well of a 96 well plate. Add 5 µl of sample from a fraction tube and mix well.
- Determine OD readings immediately at 460 nm in a plate reader. As a general rule of thumb, if the solution turns yellow within 20 seconds, you have sufficient OPH to proceed.

SDS-PAGE electrophoresis

1. Based on the spot assay results, select the 10 – 20 fractions with paraoxonase activity for SDS-PAGE analysis.

Protein Concentration

1. Load a 180 mL capacity Amicon stir concentrator with a YM10 membrane. Transfer the fractions containing OPH to the concentrator
2. Concentrate the enzyme in the cold room.
 - a) Test both the flow-through and the retained samples for activity against 1 mM Paraoxon.
 - b) Reduce the volume of OPH fractions to ≤ 50 mLs.
 - c) Transfer 0.5 mLs to a sterile 1.5 mL microcentrifuge tube and label “Sample 6”. Store tube at -20°C .
3. Carefully transfer the concentrated enzyme solution to a clean dialysis tube and make sure that both ends of the tubing are sealed using dialysis clips.
4. Dialyze against chilled 2-4 L of Buffer 8.3 for 3 hours in the cold room.
5. Transfer dialysis bag to fresh Buffer 8.3 (2-4 Liters) and dialyze overnight.
6. After dialysis, transfer 0.5 mLs to a sterile 1.5 mL microcentrifuge tube and label “Sample 7”. Store tube at -20°C .

Column Preparation

1. Equilibrate the DEAE Column overnight with Buffer 8.3 at 1 mL/min.
 - a) Pass the buffer through both A and B tubes (i.e. set “B” to 50%).
 - b) Make sure you have enough buffer to last the night. Allowing 16 hours at 1 mL/min, you will need at least 960 mL of buffer (60 minutes x 16 hours x 1 mL/min).
 - c) Failure to use enough buffer will result in a dry (and ruined) column that will need to be repacked.
 - d) Check the conductivity of the buffer eluting off the column to make sure that it is the same as that going into the column.

Day 4 (0800): DEAE chromatography

1. Transfer the dialysate to the 50 mL glass super-loop. The solution should be clear. Prepare the super-loop and the fraction collector.
2. Both the A and B line should be in Buffer 8.3.
 - a) Set the AKTA to run at 3 mL/min, collect fractions equal to 6 mLs, and the AKTA to INJECT. This will ‘inject’ the sample from the super-loop into the column.
 - b) Since this is an anion-exchange column and the charge of OPH is neutral at this pH, OPH should flow through the column, while contaminating proteins remain bound to the column.
3. Monitor the progress of the elution with the chart recorder. OPH should begin eluting from the column by the second or third fraction collection tube.
4. Using a paraoxonase spot assay and SDS-PAGE gel, screen fractions for the presence of the enzyme.

- a) When the cycle is finished, wash the column with 1 M Potassium Phosphate, pH 8.3, and then re-equilibrate in Buffer 8.3.

Paraoxonase Spot Assay

1. Using one well per fraction, add 50 μ l of 1 mM paraoxon, 20 mM CHES (pH 9.0) to each well of a 96 well plate. Add 5 μ l of sample from a fraction tube and mix well.
2. Determine OD readings immediately at 460 nm in a plate reader. As a general rule of thumb, if the solution turns yellow within 20 seconds, you have sufficient OPH to proceed.

SDS-PAGE electrophoresis

1. Based on the spot assay results, select the 10 – 20 fractions with paraoxonase activity for SDS-PAGE analysis.

Protein Concentration

1. Transfer the fractions containing OPH to a small 50 ml capacity Amicon stir concentrator with a YM10 membrane.
 - a) Concentrate the enzyme in the cold room and test both the flow-through and the retained samples for activity.
 - b) Concentrate until the volume of OPH is \leq 10 mLs.
 - c) Transfer the OPH to a 15 ml falcon tube and store at 4 °C; OPH must be stored at a concentration \geq 1.0 mg/ml.
 - d) Transfer 0.5 mLs to a sterile 1.5 mL microcentrifuge tube and label "Sample 8". Store tube at -20 °C.
52. You may concentrate your samples to 2-5 mg/ml and store at -20°C in 40% glycerol for extended periods of time. You may also store you enzyme at 4°C if used within two weeks of time. It is a good idea to perform your kinetic and structural studies within 24 hours of purification.