

Production of Protein Pharmaceuticals (Part 2)

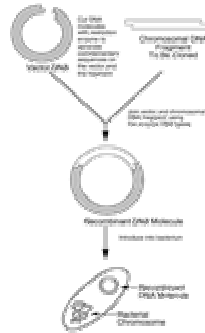
Dr. David Wishart
Athabasca Hall 3-41
david.wishart@ualberta.ca

Today's lecture notes are available at:

<http://redpoll.pharmacy.ualberta.ca>

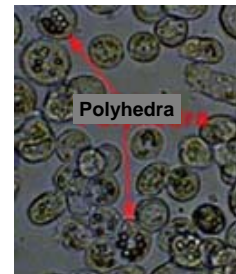
Review

- Gene of interest is cut out with restriction enzymes (RE)
- Host plasmid (circular chromosome) is cut with same REs
- Gene is inserted into plasmid and ligated with ligase
- New (engineered) plasmid inserted into a host cell



Review

- **Escherichia coli**
- **Other bacteria**
- **Pichia pastoris**
- **Other yeast**
- **Baculovirus**
- **Animal cell culture**
- **Plants**
- **Sheep/cows/humans**
- **Cell free**



Six Step Process

- Isolation of gene of interest
- Introduction of gene to expression vector
- Transformation into host cells
- Growth of cells through fermentation
- Isolation & purification of protein
- Formulation of protein product

Cell Growth Needs

- A sterile carbon, nitrogen, hydrogen and oxygen source (air and H₂O) + trace metals (Zn, Fe, Cu, Ca, Mg, Mn)
- A sterile energy source (light, sugar, acetate, methanol, ethanol)
- A constant (or near constant) temperature above 20 °C
- A growth regulating chemical (antibiotic)

Prototrophs vs. Auxotrophs

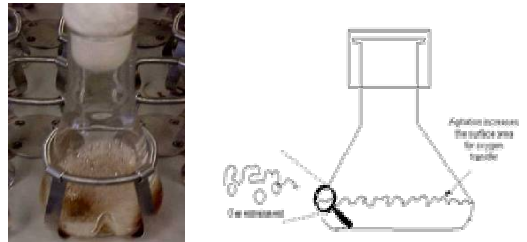
- Prototrophic cells (bacteria, plants) can produce all essential amino acids, nucleic acids, carbohydrates and lipids from simple nutrients (water, oxygen, nitrogen or ammonia, CO₂)
- Auxotrophic cells (yeast, insect cells, mammalian) need vitamins, essential amino acids (His, Cys), sugars, lipids, etc. to grow because they have lost this ability through evolution (bacterial symbiosis)

Features	Microbes	Animal Cells
Cell wall	Generally present	Generally absent
Cell membrane	Present	Present
Growth Rate	10-50% per hour	1-5% per hour
O ₂ Requirement	High	Low
Nutritional Rqmt	Usually simple	Complex
CO ₂ Requirement	Sometimes	Key for buffering
Environmental FX	Less affected	Very susceptible
Size	100-2000 nm	10000-100000 nm
Seeding density	1 cell	10 ⁵ cells/mL
Growth density	10 ⁹ -10 ¹⁰ cells/mL	10 ⁶ cells/mL

All Cells Can be Grown in Incubators or Fermentors



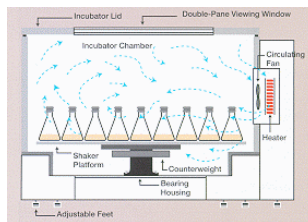
Shake Flask Incubator



Shake Flask Incubator



G25 New Brunswick Floor Model Incubator



Cutaway Model Incubator

Shake Flask Incubators

- Sometimes called environmental chambers
- Heavily insulated, heated with thermoregulation to keep temperature within 0.5 °C of set-pt.
- Rotatable platform to spin up to 500 rpm to facilitate aeration (dissolves N₂ and O₂ needed for growth)
- Designed for small-scale growth

Fermentors & Bioreactors

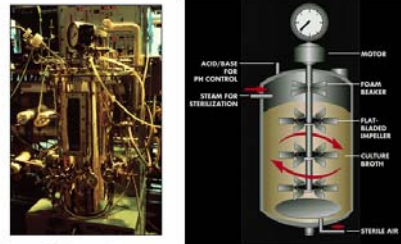
- Larger scale, sustained growth requires bioreactors & fermentors
- Fermentors have been used for centuries – primarily for brewing alcohol and making vinegar
- Modern technology and chemical engineering principles continue to improve fermentor design

Date	Products	Vessels	Process Control	Culture Method	Quality control
3000 BC to 1900	Alcohol Vinegar	Wooden Copper Trays	Thermometer heat exchanger	Batch Methods	Nil
1900 to 1940	Glycerol Citric acid Acetone	Steel with mechanical stirring	pH control Temp control	Batch and Fed-Batch	Almost Nil
1940 to present	Antibiotics Nucleotides Amino acids	Sterile mechanical aerated	pH & O ₂ electrodes Temp control	Fed-Batch and continuous	Very important
1960 to present	Native proteins & enzymes	Pressure jet vessels	Computer linked control loops	Continuous culture with recycler	Very important
1979 to present	Recombinant proteins & enzymes	Fermentors (all 4 types)	Computer linked control pH, O ₂ , Temp	Batch, Fed Batch & Continuous	Very important

Fermentors & Bioreactors

- Four basic bioreactor designs
 - Stirred tank reactors (mechanical agitation for aeration)
 - Bubble column reactors (bubbling air into media for aeration)
 - Internal loop airlift reactors (air and media circulate together)
 - External loop airlift reactors

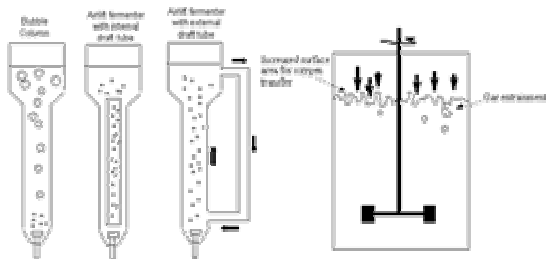
Stirred Tank Fermentor/Bioreactor



by Genentech, Corporate Communication

by Genentech, Graphics Department

Four Bioreactor Designs

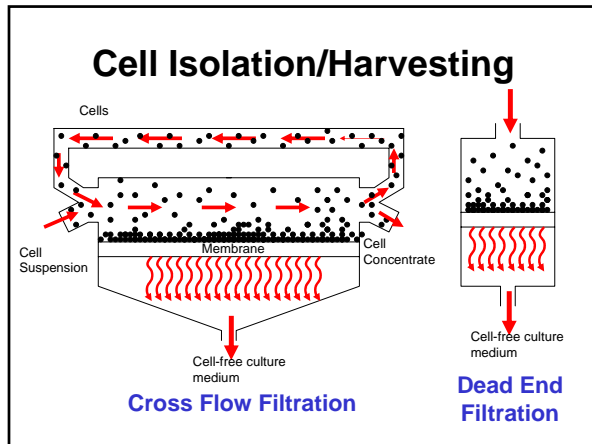


Airlift Reactors

Stirred Tank Reactor

Fermentor Scale Up

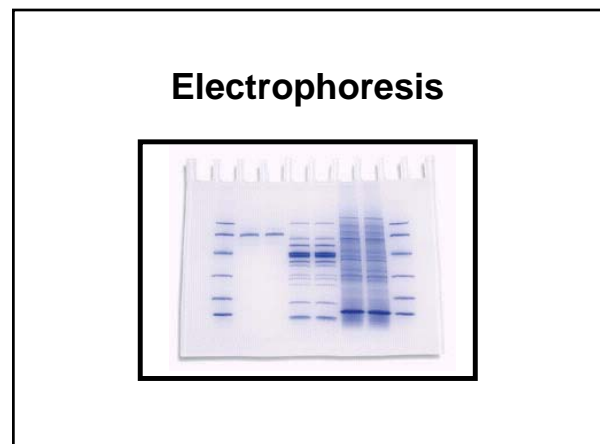
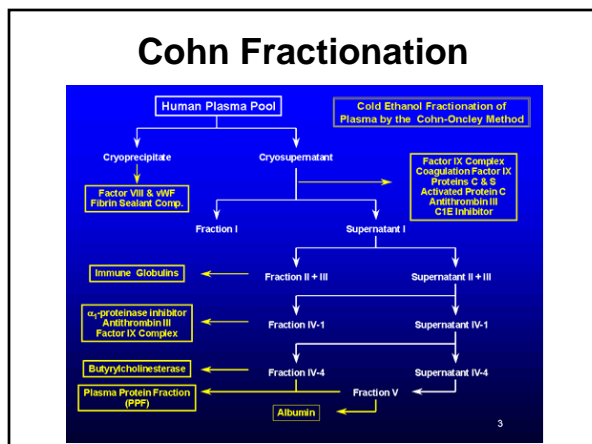
- Can't start cell culture in 100000 L, must do repeated, scaled inoculations
- Start with stock culture (5-10 mL)
- Then shaker flask (200-500 mL)
- Then seed fermentor (10L to 100 L)
- Then production fermentor (1000L to 100,000 L)



- ### Protein Isolation & Purification
- After cells (or media) are harvested proteins may be purified/isolated
 - Intracellular (inside cell) proteins are harder to purify
 - Require cell disruption, separation, removal of cell debris, DNA, RNA, lipid
 - Extracellular (outside cell) proteins are easier to purify
 - No cell disruption needed, just isolate

- ### Cell Disruption Methods
-
- Vigorous Methods**
 - Sonication
 - French press
 - Glass bead disruption
 - Gentle Methods**
 - Enzymatic lysis
 - Detergent lysis
 - Freeze-thaw
 - Osmotic lysis

- ### Protein Isolation Methods
- Differential salt precipitation
 - Differential solvent precipitation
 - Differential temperature precipitation
 - Differential pH precipitation
 - Two-phase solvent extraction (PEG)
 - Preparative electrophoresis
 - Column chromatography
- Most purifications require combinations of 2-3 steps*



Electrophoresis

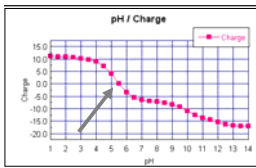
- Principle is to separate proteins (in tact) on the basis of their charge and their ability to migrate within a gel (jello-like) matrix
- A strong electric field is applied to the protein mixture for an extended period of time (hours) until the proteins move apart or migrate

Isoelectric Focusing (IEF)



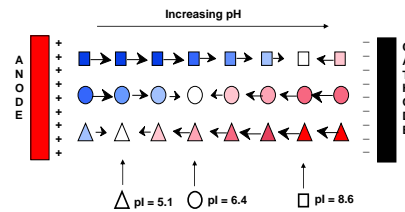
Isoelectric Point (pI)

- The pH at which a protein has a net charge=0
- $$Q = \sum Ni / (1 + 10^{pH-pKi})$$
 Transcendental equation



pKa Values for Ionizable Amino Acids			
Residue	pKa	Residue	pKa
C	10.28	H	6
D	3.65	K	10.53
E	4.25	R	12.43

IEF Principles



Isoelectric Focusing

- Separation of basis of pI, not Mw
- Requires very high voltages (5000V)
- Requires a long period of time (10h)
- Presence of a pH gradient is critical
- Degree of resolution determined by slope of pH gradient and electric field strength
- Keeps protein structure intact
- Can be scaled up to isolate mg to gms of protein in a single "tube" gel run

Column Chromatography



Column Chromatography

- Most common (and best) approach to purifying larger amounts of proteins
- Able to achieve the highest level of purity and largest amount of protein with least amount of effort and the lowest likelihood of damage to the protein product
- Standard method for pharma industry

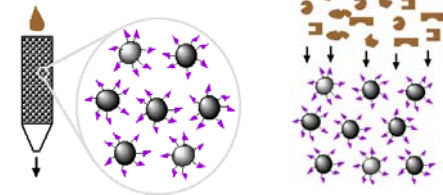
Column Chromatography

- Can be done either at atmospheric pressure (gravity feed) or at high pressure (HPLC, 500-2000 psi)
- Four types of chromatography:
 - Affinity chromatography
 - Gel filtration (size exclusion) chrom.
 - Ion exchange chromatography
 - Hydrophobic (reverse phase) chrom.

Affinity Chromatography

- Adsorptive separation in which the molecule to be purified specifically and reversibly binds (adsorbs) to a complementary binding substance (a ligand) immobilized on an insoluble support (a matrix or resin)
- Purification is 1000X or better from a single step (highest of all methods)
- Preferred method if possible

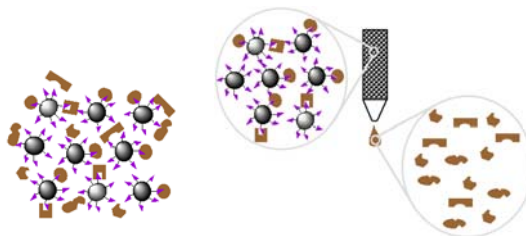
Affinity Chromatography



Step 1: Attach ligand to column matrix

Step 2: Load protein mixture onto column

Affinity Chromatography



Step 3: Proteins bind to ligands

Step 4: Wash column to remove unwanted material, elute later

Affinity Chromatography

- Used in many applications
- Purification of substances from complex biological mixtures
- Separation of native from denatured forms of proteins
- Removal of small amounts of biomaterial from large amounts of contaminants

Affinity Chromatography

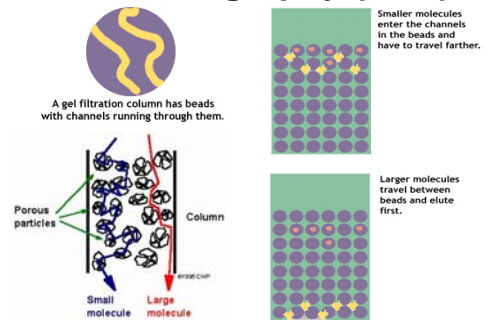
- The ligand must be readily (and cheaply) available
- Ligand must be attachable (covalently) to the matrix (typically sepharose) such that it still retains affinity for protein
- Binding must not be too strong or weak
- Ideal K_D should be between 10^{-4} & 10^{-8} M
- Elution involves passage of high salt or low pH buffer after binding

Ligand	Specificity
AMP	Enzymes with NAD cofactors and ATP dependent kinases
Arginine	Proteases such as prothrombin, kallikrein, clostripain
Cibacron Blue Dye	Serum Albumin, Prealbumin
Heparin	Growth factors, cytokines, coagulation factors
Protein A	Fc region of immunoglobulins
Calmodulin	Calmodulin regulated kinases, cyclases and phosphatases
EGTA-copper	Proteins with poly-Histidine tails

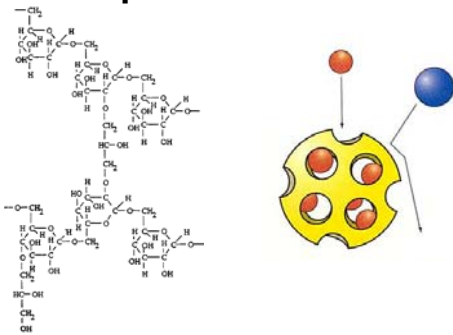
Size Exclusion Chrom.

- Molecules are separated according to differences in their size as they pass through a hydrophilic polymer
- Polymer beads composed of cross-linked dextran (dextrose) which is highly porous (like Swiss cheese)
- Large proteins come out first (can't fit in pores), small proteins come out last (get stuck in the pores)

Size Exclusion Chromatography (SEC)



Sephadex Structure



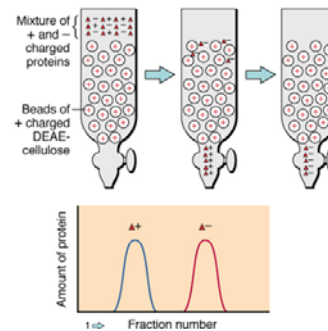
Ion Exchange Chromatography (IEC)

- Principle is to separate on basis of charge "adsorption"
- Positively charged proteins are reversibly adsorbed to immobilized negatively charged beads/polymers
- Negatively charged proteins are reversibly adsorbed to immobilized positively charged beads/polymers

Ion Exchange Chromatography

- Has highest resolving power
- Has highest loading capacity
- Widespread applicability (almost universal)
- Most frequent chromatographic technique for protein purification
- Used in ~75% of all purifications

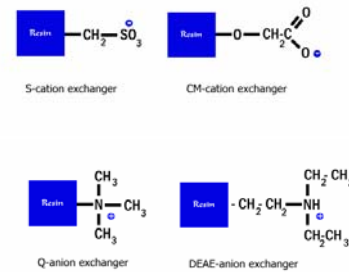
IEC Principles



IEC Nomenclature

- Matrix is made of porous polymers derivatized with charged chemicals
- Diethylaminoethyl (DEAE) or Quaternary aminoethyl (QAE) resins are called anion exchangers because they attract negatively charged proteins
- Carboxymethyl (CM) or Sulphopropyl (SP) resins are called cation exchangers because they attract positively charged proteins

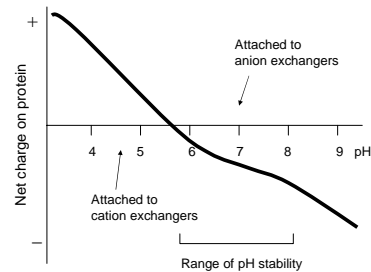
IEC Groups



IEC Techniques

- Strong ion exchangers (like SP and QAE) are ionized over a wide pH range
- Weak ion exchangers (like DEAE or CM) are useful over a limited pH range
- Choice of resin/matrix depends on:
 - Scale of separation
 - Molecular size of components
 - Isoelectric point of desired protein
 - pH stability of the protein of interest

Protein pH Stability Curve



IEC Rules of Thumb

- If a protein is most stable below its pI, a cation exchanger should be used
- If a protein is most stable above its pI, an anion exchanger should be used
- If stability of the protein is known to be good over a wider pH range then either type of ion exchanger can be used

Conclusion

- Isolation of gene of interest
- Introduction of gene to expression vector
- Transformation into host cells
- Growth of cells through fermentation
- Isolation & purification of protein
- Formulation of protein product