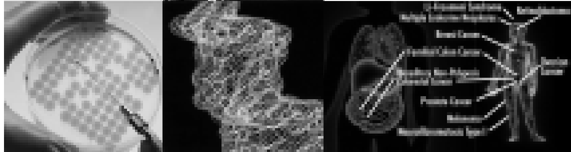
Bioseparation

Bio
Process
Engineering
Lab



Department of Chemical and Biological Engineering
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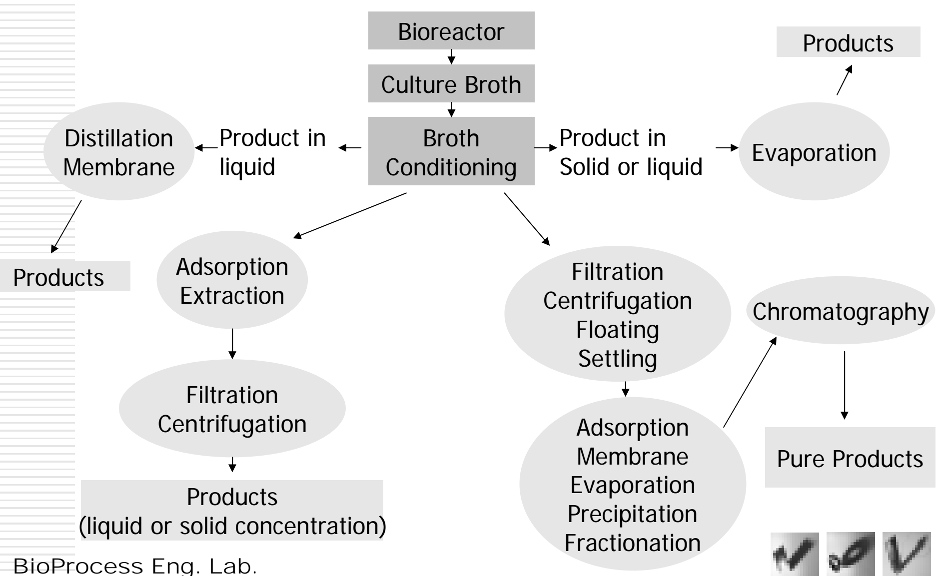
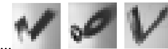
Schematic Diagram of Bioseperation Process

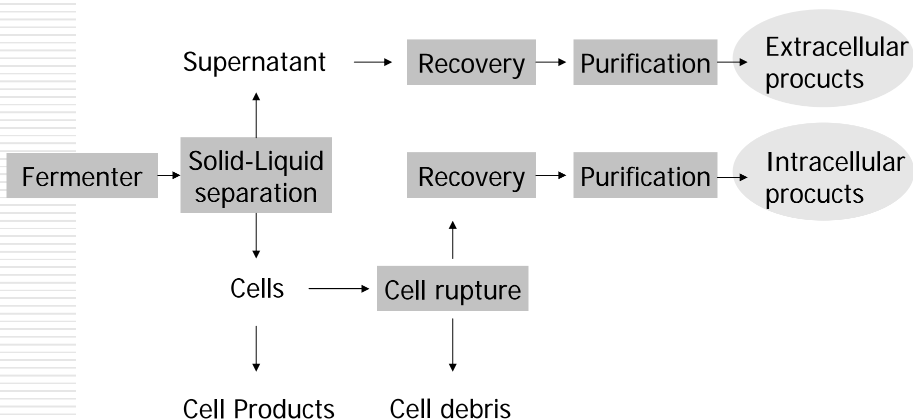


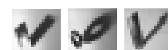
Examples of Bioprocessing Products And Their Concentrations

Types	Products	Concentration g/l
Cell Itself	bker's yeast single cell protein	30
Extracellular	alcohols, organic acids, amino acids, enzymes, antibiotics	100 20
Intracellular	Recombinant DNA proteins	10



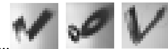
Major Process Steps In Downstream processing





Purification

- The preferential concentration & Isolation of a specific product from a mixture or biological molecules
- Extent of purification may depend on:
  1) Source of product
  2) Applications of product
  3) Cost of product



Product separation & recovery

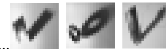
1. Product types
   Amino acids, Antibiotics, Enzymes, Polysaccharides
   Vaccines, Vitamins, Nucleotides, SCP
2. Sources
   Fermentation - Microbial / Animal / Plant
   Crops
   Wastes
3. Requirements - Separation, Fractionation,
   Purification, Concentration

The main criteria for efficient primary downstream processing

1) Maximum recovery of the desired product

2) The processing equipment must be reliable.
   The number of processing step should be kept to an absolute minimum (since capital and operating costs)

3) The process give large reductions in volume between the fermentation broth and the final fluid stages of recovery process(1000 : 1)



4) The processing should aim to provide finally a spent broth that will be easily utilised for recycling
   (unused nutrients for large scale operations)

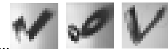
5) For all processes, downstream processing must be established as soon as the broth leaves the bioreactor.
   Delay or hold-ups will necessiate expensive holding systems with the definite possibility of deterioration of the product in the broth.

6) Since the product concentration in most biotechnological processes is low, the cost per unit volume of broth handled in primary recovery stages will represent a significant proportion of the final product cost.

Separations In Biotechnology

1. Large scale
   Organic acids, Amino acids, Antibiotics,
   Industrial Enzymes, SCP

2. Small scale
   Diagnostics, Therapeutics,
   Research probes and tools

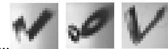


Large Scale Processes Exploit Chemical Engineering Unit Operations

- Flocculation / Flotation
- Continuous filtration / Centrifugation
- Disruption / Organic extracts
- Precipitation(Salt / Organic)
- Crude adsorption
- Evaporation / Ultrafiltration
- Spray drying / Drum drying

Small Scale Processes Utilise Scaled-Up Lab. Operation

- Batch / continuous centrifugation
- Mechanical disruption
- Fractional precipitation
- Fixed bed chromatography
- Dialysis / Diafiltration
- Ultrafiltration
- Freeze drying

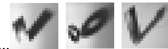


Scale Up 1kg Process To 1000kg(5000L) And Problems Multiply

- Increased process times
- Physical limitation of equipment
- Decreased efficiency of purification
- Decreased yields
- Increased material costs
- Repetitive decline in performance

Choice Of Recovery Methods

1. Product location / physical state
2. Product concentration
3. Physical/chemical properties of product
4. Product application
5. Minimum purity requirements
6. Physical/chemical characteristics of impurities
7. Product market price



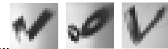
Cells + Aggregates

- Characters are important to
- ◆ fractionation / clarification,
- ◆ Size/ size distribution,
- ◆ Chemical composition,
- ◆ Specific gravity,
- ◆ Shape,
- ◆ Strength,
- ◆ Hydrophobicity,
- ◆ Toxicity,
- ◆ Reactivity,
- ◆ Surface properties,

Cell Separation & Broth Conditioning

- Cell separation
  - Settling
  - Flotation
  - Filtration
  - Centrifugation
  - Electokinetics
  - Two-phase liquid separations

- Broth conditioning
  - Conditioning or pretreatment of the contents of a bioreactor is normally designed to improve subsequent broth handling properties



Flocculation & Coagulation

Flocculation

The formation of cell agglomerates usually by means of bridging chemical molecules

Flocculation agent - a chemical or material, which when added to a particular suspension causes agglomeration to form

Coagulation

The destabilisation of cells by neutralisation of their charge

Mechanisms

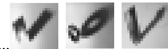
Surface charge neutralisation - ionic saltIonic bridging - Ca++ linkages

Polyelectrolyte bridging - synthetic charged polymers

Polymer entrapment - long chain polymers

Physical interaction - pellets

Chemico-physical method - surface chemistry interactions,agglomeration after lysis.

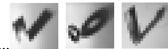


Factors Affecting Agglomeration

Genetic characteristics
Surface charge
Complex surface chemistry
Broth components(e.g. glucose, metal ions)
Complex surface physical characteristics
Time dependent physiological states of cells
Environmental dependent physiological states

Disadvantages Of Flocculations

Poorly understood mechanisms, 'Poor control'
Unpredictable batch fermentations
Lack of operational data
Low dewatering
Costs
No recycling
Less applicable to cell debris
Physical instabilities / Shear degradation



Cell Disruption

The extraction or liberation of products from cells by mechanical, chemical, microbial breakage of the cell wall or membrane(cell lysis)

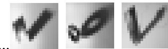
The choice of method for large scale cell disruption will depend on:

1) Susceptability of cells to breakage
2) Product stability characteristics
3) Ease of extraction from cell debris
4) Speed of the method
5) Cost of the process

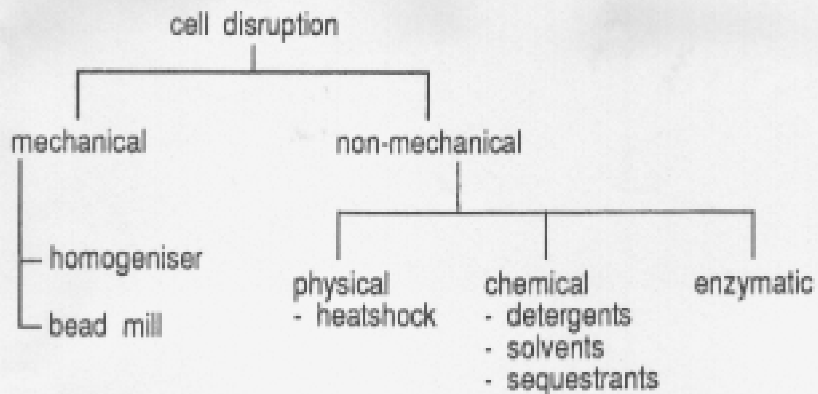
Mechanical & Physical
Liquid shear - high pressure homogenisation(homogenizer)
Solid shear - grinding / milling(agitation + abrasives)
Freezer / Thaw
Ultrasonics
Osmotic shock

Chemical
Cationnic and anionic detergents, Alkali, Organic solvents

Enzymatic
Lysozyme and related enzyme (The enzyme that catalyses the hydrolysis of polysaccharide layer of bacterial cell walls)



Methods For Cell Disruption

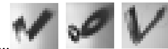


Separation Of Solid And Liquid Phases

Separation of solid from the liquid phase involves unit operations: Settling, Filtration, Cetrifugation, Flotation, Electrokinetics

* Settling - Traditional method in wastewater treatment and alcoholic beverage
  - Particles or cells settle to the bottom by simple gravitational processes

* Flotation - Traditional methods used in the alcoholic beverage industry and in wastewater treatment.
  - depends on the ability of the cell to rise to the surface and be collected (buoyancy of the cells).
  - Application of gas bubbles to concentrate particles at surfaces
  - Currently applied to cells / flocculated cells or debris



Centrifugation & Filtration

Centrifugation

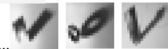
- utilised at a laboratory level for separating biological solid but are still difficult and costly to operate on a industrial scale by large density differences between particles and fluid, and low liquid viscosity.

Filtration

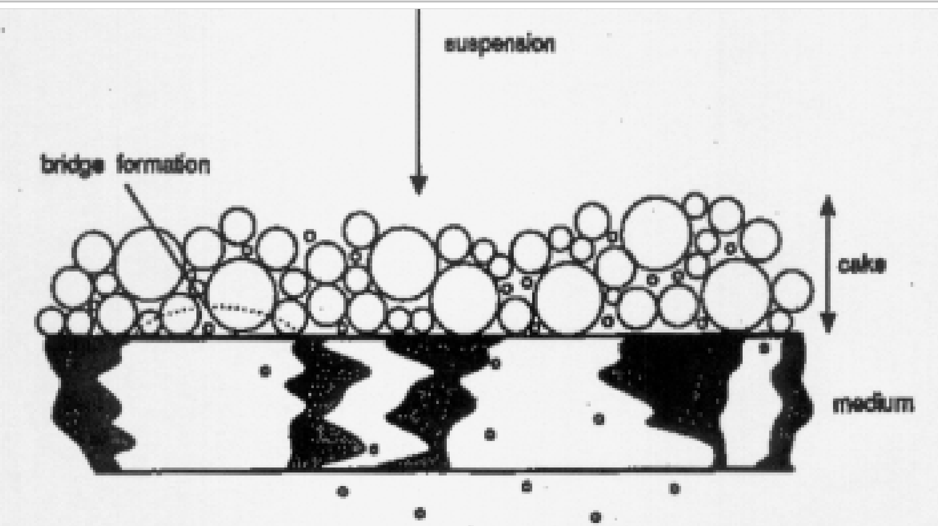
Some factors will influence the choice of the most suitable type of equipment which will meet the specified requirements at minimum overall cost.

1) The properties of the filtrate, particularly density and viscosity.
2) The nature of solid particles(size and shape, size distribution)
3) Scale of operation
4) The need of aseptic conditions

Centrifuge



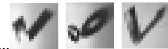
Basic Principle Of Cake Filtration



Filtration Methods

1) Coarse screen
2) Filter presses
3) Rotary drum filters
4) Membrane filtration

Performance affected radically by minor changes in feedstocks depends on :
broth characteristics, cell morphology, cell physiology, interactions of additives



Filter Aids

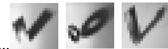
- Gelatinuous suspensions do not filter well.
- Filter aids such as Kisselguhr (Diatomaceus earth) may be added.
- Problems & Consequences
  - High voidage
  - Dosing is important
  - Improved porosity
  - Adsorption of some filtrate
  - Decrease of compressibility
  - Non-recoverable

Simple Filtration

1) Dead end filters :

The rate of passage of a liquid through a filter of unit area is dependent on :

- Pressure difference applied
- The resistance of the filter material
- The viscosities of the liquid
- The resistance produced by the cake already present



2) Plate and frame filters

It's a pressure filter, consisting of plates and frame arranged alternatively. The plates are covered with cloths or filter pads.

1. It's cheap per unit filtration space
2. Intermitant operation - requires frequent dismantling.
3. It's most suitable for fermentation broths with low solids content and low resistant to filtration.
4. It's widely used as a "polishing" device in brewers to filter out low residual numbers of yeast cells from beer inafterinitialclarification on a rotary vacuum filter.

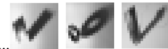
3) Pressure leaf filters

Intermitant batch filters, which incorporate a number of leaves : metal frame, covered in fine wire mesh or cloth. Maybe precoated with a layer of cellulose fibers. They're operated under pressure or vacuum.

- Vertical metal-leaf filters
- Horizontal metal-leaf filters
- Candle type filters

4) Rotary vacuum filtration

Performance - Rotation speed, Immersion, Pressure drop across filter, Solid content of feed stock, Temp./Viscosity of feed, Cake resistance/Compressbility, Precoating/Cloth resistance, Methods of discharge Cylindrical drum with the lower portion rotating through a trough filled with the biological suspension to be filtered.



Centrifugation

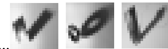
When filtration is not a satisfactory separation method, a centrifuge can be used to remove microorganisms and other similar size particles, from a broth.

It's more expensive, comparing with a filter, but may be essential when filtration is slow and difficult.

The cells or other suspended matters must be obtained free of filter aids.Continuous separation to a higher standard of hygiene is required.

Centrifugation Efficiency

- Cetrifugation efficiency is favoured by:
  - large particle diameter of cells
  - large density difference between cell and liquid
  - the liquid should have a low viscosity



In practice, particles of biological material are often small and of low density, which fermentation broths are often viscous, and high density.

It requires :

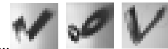
- high angular velocity
- large radius(of centrifuge)
- large volume
- thin sedimentation layer

Types Of Centrifuge

1) Tubular bowl

Hazards to the enzyme are aeration and the consequent foaming of the clarified solution, which aerosol formation may be a hazard to the user. This occurs because of turbulence in the bowl.

Very high angular velocity ➔ turbulence ➔ foaming & aerosol



2) Multichamber

They have large radius, low angular velocity, thus sedimentation occurs with high efficiency over large surface area.
They're widely used for Baker's yeast.
Some heating(because the bowl is located above the gearbox) represents a danger to the enzyme.

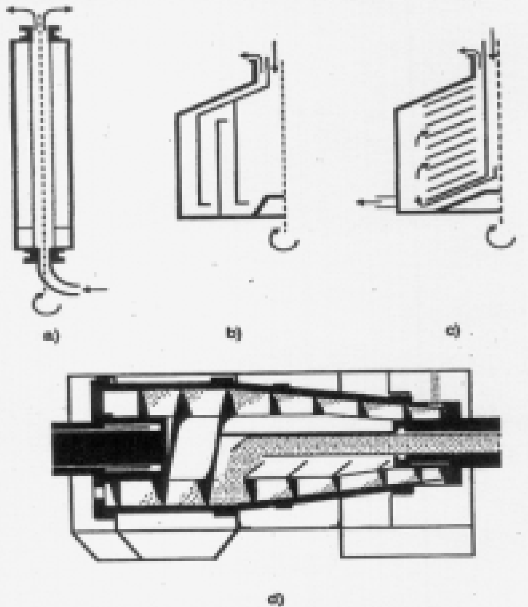
3) Scroll

They used for continuously handling coarse material(such as sewage sludge. They're few hazards to enzymes.)

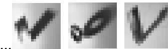
4) Basket

They used for food.

Types Of Centrifuges



a) Tubular centrifuge
b) Multichamber
c) Disk stack centrifuge
d) Decanter centrifuge



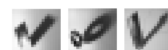
Membranes Filtration

Reverse osmosis, ultrafiltration, nanofiltration :
Concentration of enzymes

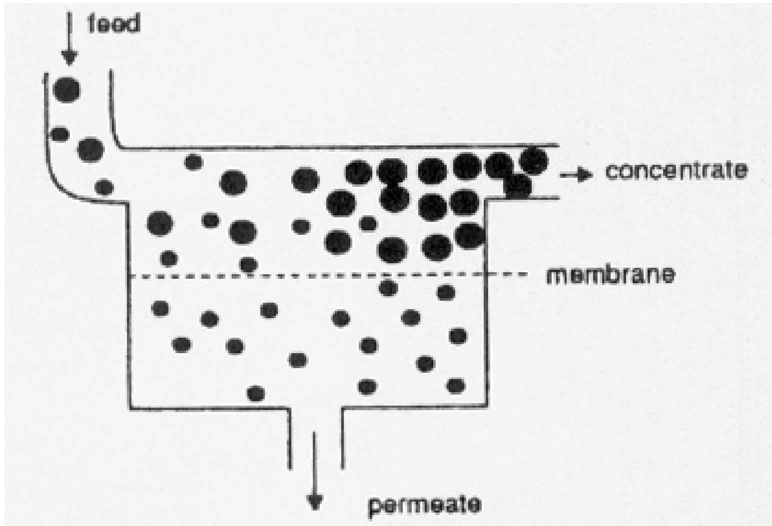
Ultrafiltration :
molecules are forced hydraulically through a membrane of very small pore size.

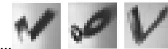
Reverse osmosis :
A ultrafiltration using a membrane with pores small enough to allow the passage of solvent molecules only, as in the desalination of sea water.

Ultrafiltration is a particularly useful method for enzyme work.

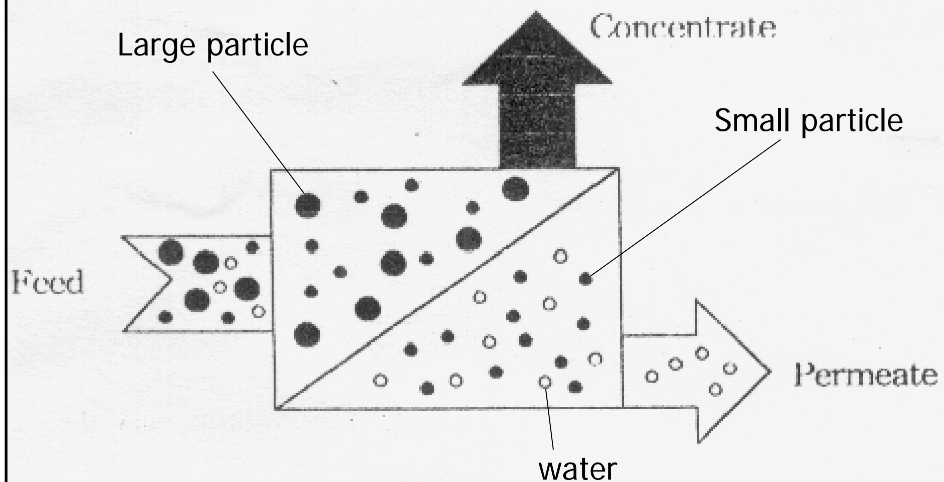


Basic Principle Of A Membrane Separator

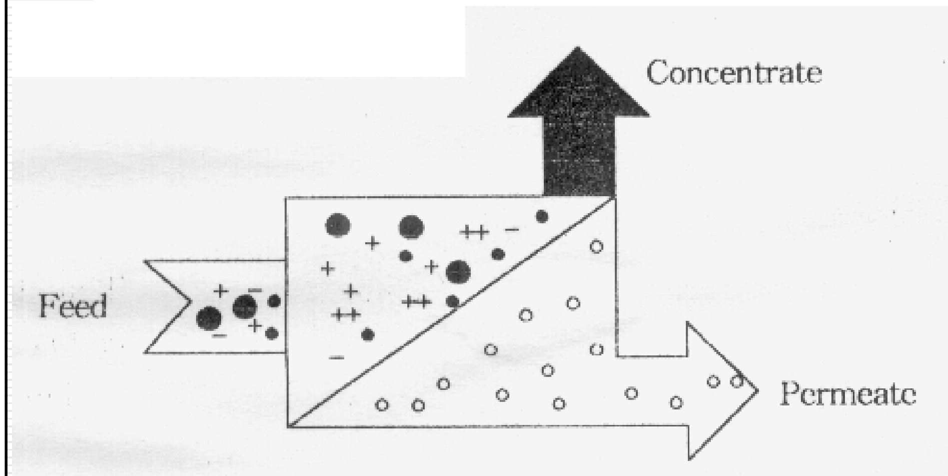


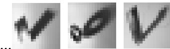


Ultrafiltration

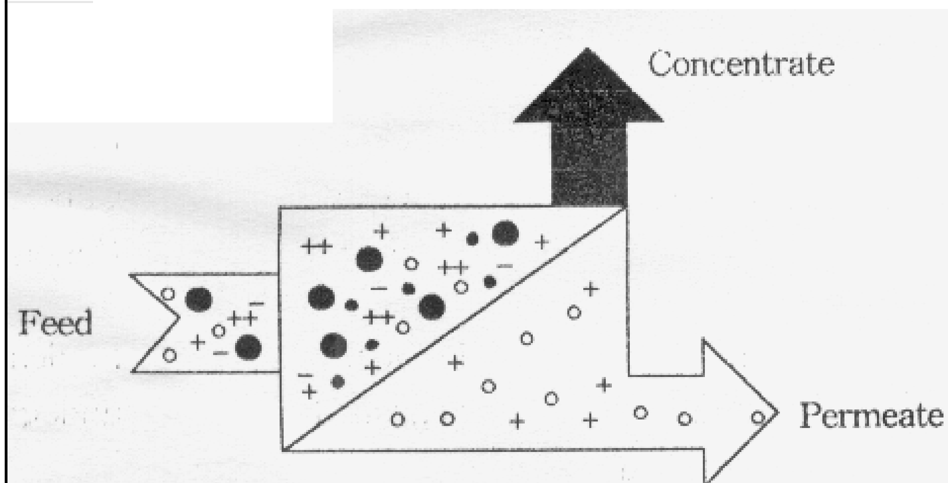


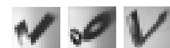
Reverse Osmosis





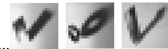
Nanofiltration





Advantages Of Ultrafiltration

1) It is gentle and non-destructive(though shaer damage of large molecules is possible)
2) No chemical reagents are used.
3) No phase change is required.
4) It can be operated at low hydrostatic pressures.
5) Low temperature operation is possible(though cooling is necessary in equipment where high flow rates are maintained over membranes)
6) Constant pH and ionic strength may be maintained if required.
7) Simultaneous concentration and purification can be achieved.
8) The process is economical in use.



For enzymes,

1) Little effect on the bioactivity of the protein molecules
2) High recovery rates(up to 99.9%)
3) Rapid processing time
4) Little ancillary equipment

Disadvantages of ultrafiltration

1) High concentration is a problem.
2) Shear damage can occur.
3) Quantitative recoverys are rare.

For enzymes,

1) Rapid clogging of the membrane
2) Viscous solution leads to decrease of flow rates and prolong of processing time

Modules : Shell and Tube(Tubular), Flat sheets(Flat plate)
Spiral wound, Hollow fiber

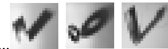
Products In Solid Phase

Separation of the solids from the broth

A cream consistency(about 15%(w/v) solids)
A dewatered sludge(20 - 25%(w/v) solids)
A damp solid(40% or more(w/v) solids)
➔ suitable for immediate scale

Need of further drying for product stability

Actually the moisture content must be reduced to approximately 5% to avoid deterioration



Drying

Drying (Microorganisms or other products)
- last stage of a manufacturing process
- long drying time with low temperature (heat-sensitive material)
- short drying times with high temperature (better, generally)

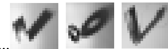
Purpose of drying
1) The cost of transport can be reduced.
2) The material is easier to handle and package.
3) The material can be more conveniently stored in the dry state.

BioProcess Eng. Lab.

Spray dryer

- Complete drying in a few seconds
  (rapid rate of evaporation)
- Evaporative cooling effect prevents the material from becoming overheated and damaged.
- Hot gas(150oC - 250oC), Cooling air exhaust temp. 77-100oC

Drum dryer - The material is more heat stable.



Clarification Requirements

1) Coarse debris removal - Centrifugation and Filtration
2) Fine particulates - depends on concentrating procedures
   - Biomass, Extracellular or Intracellular products, Membrane products, Cell wall products

Biomass and cell wall product require broth clarification

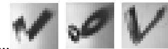
- Intracellular(soluble product) and cell wall product require **homogenate clarification.**

- Unit operation for membrane product and cell wall product for extracellular and intracellular soluble product may be considered together - **Concentration & Fractionation**

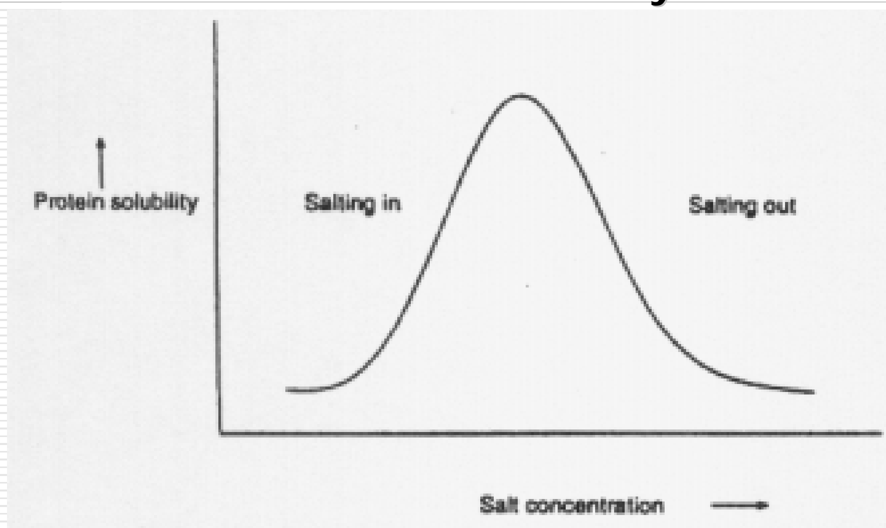
Salting Out

Enzymes may be pricipitated and fractionated by "salting out", usually by ammonium sulphate, very soluble, self-cooling on dissolving in water, and harmless to most enzymes.

1) High concentration of $(NH_4)_2SO_4$
2) Purity specification for salt
3) pH/temperature control is important
4) Contact mode is critical/ also time scale
5) Requires clarification - high capacity centrifuge / membrane process
6) De-salting problem



Effect Of Salt Concentration On Protein Solubility



Affinity Separation

Another kind of precipitation

Exploit specific interactions associated with biological activity/ Biospeificity

Ex) Enzyme may be interact with; substarte, Inhibitor, Cofactor, Antibiotic

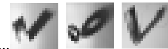
Such interactions may be highly specific - Triazines Dyes

Precipitation with:

Organic solvents - water miscible

(1) Lower dielectric constant
- hydrophilic interaction at protein surface

(2) Hydrophobic residues may refold
- denatured product concentration increases with increasing chain length/ Require low temperature(0℃)



Methanol, Ethanol, Isopropanol, are common.

➔ increases hydrophobicity

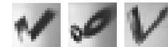
➔ increases solvent effects

Organic-Two-Phase

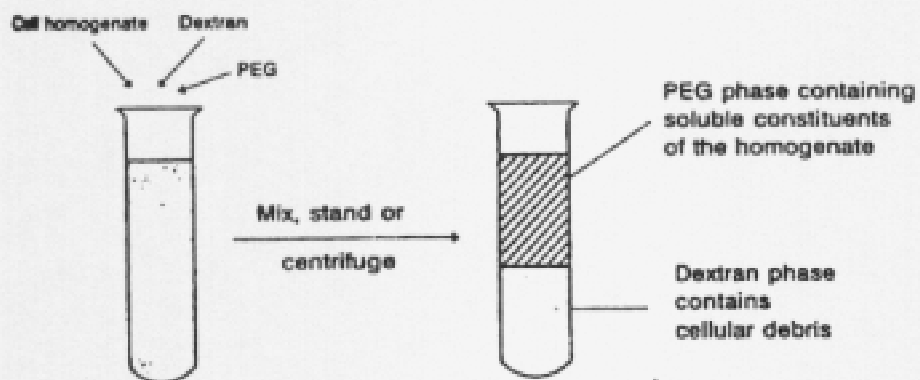
- not generally applicable to biochemical systems.
  Increase interest in aqueous 2-phase partition

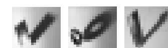
  e.g. Mix 2 species of polymer(PEG/Dextrans) or polymer-salt
  (PEG-phosphate)
  (1) homogeneous mix at low concentration
  (2) immiscible at high concentration

  Biochemicals and/or particles may preferentially partition into top or bottom phases dependent upon various physical and biochemical characters



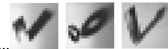
Principle Of Aqueous Two-Phase Partitioning System





Advantages;

(1) High water content for both phases
(2) Low interfacial tension between phases
(3) Equilibrium occurs rapidly in dispersed phases
(4) Liquid-liquid extraction long stabilized/ unit operation



Disadvantages ; polymers are expensive

Partition characteristics of biopolymers depends on :
Surface charge, Surface hydrophobicity/hydrophilicity,
Surface area, Conformation
Charges in ionic component and pH influence both carrier and biopolymer partition.
interdependence of parameters makes quantitative correlation of partition coefficients with molecular properties for a defined molecule presently impossible.
Influences : Polymer types, Polymer molecular weights,
Polymer conc., Ionic strength/nature, pH, Temp.,
Molecular properties.

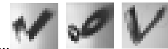
Column Chromatography

The initial step in purification process ;
a) to liberate and concentrate the protein of interest
b) to remove particularly undesirable contaminant

Ex) lipids etc.: results in fouling or clogging of chromatographic system

Further purification by column chromatography
- Characteristics of protein are important;

Ex) Size and shape
Overall charge
The presence of surface hydrophobic groups
The ability to bind various ligands



Types Of Chromatography

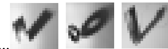
Ion-exchange chromatography – based on **charge**
Gel-filtration chromatography – based on **size and shape**
Hydrophobic interaction chromatography
– based on **degree of hydrophobicity**
Affinity chromatography – based on ability to bind **specific ligands**

Chromatographic techniques
In general, 75 – 95% recovery
Minimization of chromatographic step
(Optimization: 3 – 5 steps, in general)

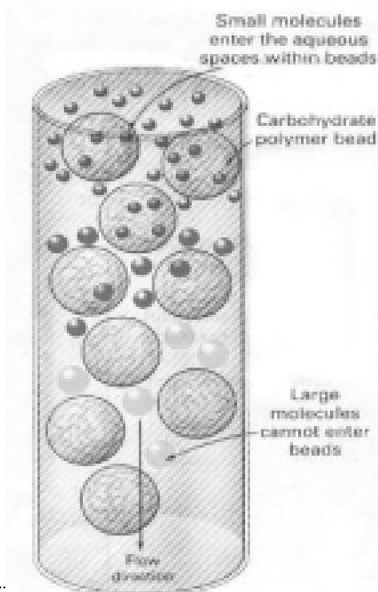
Gel Filtration Chromatography

(Size exclusion chromatography or molecular sieving)

- packed with a porous gel matrix in bead form
- large proteins cannot enter the gel beads and hence are quickly eluted
- the smaller the protein, the more potential internal routes (like maze), and the longer it is retained within the bead structure
- Gel matrices; chemically cross-linking polymeric molecules
  (Dextran, Agarose, Acrylamide, Vinyl polymers)
  Ex) Sephadex G-25 or Bio-Gel P2(Dextran particles)

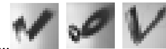


Gel Filtration Chromatography



Ion Exchange Chromatography

- Amino acids of proteins exhibit charged side chains due to N-terminal amino group and C-terminal carboxyl group
- At pH 7.0, aspartic acid and glutamic acid; overall negatively-charged acidic side groups
  lysine, arginine, histidine ; positively-charged basic side groups
- Ion-exchange chromatography is based on the principle of reversible electrostatic attraction of a charged molecule to a solid-matrix which contains covalently attached side groups of opposite charge.
- Anion exchangers contain covalently attached positive groups; Adsorption of anionic proteins(negative charge)
- Cation exchangers contain covalently attached negative groups; Adsorption of cationic proteins(positive charge)



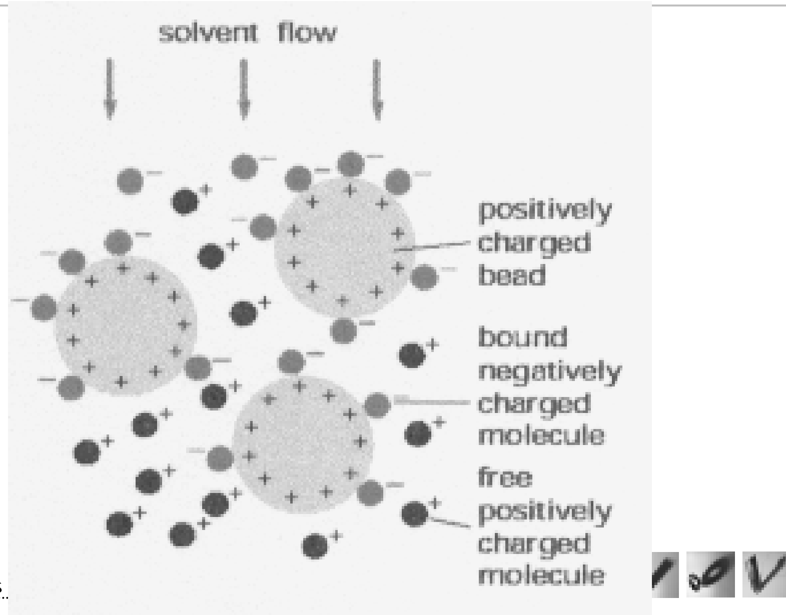
-Elution may be achieved using a salt-containing irrigation buffer
(Salt cation, Na+ displaces the protein from the ion-exchange matrix)

Advantages

High level of resolution
Straight-forward scale-up
Ease of use
Ease of column regeneration
Concentration of protein
Inexpensive

- At physiological pH, most proteins have negative charges
  Anion exchange chromatography is most commonly used
- Ideal matrix; inert, rigid, highly porous
  Ex) Cellulose-based ion-exchangers(DEAE Sephacel)

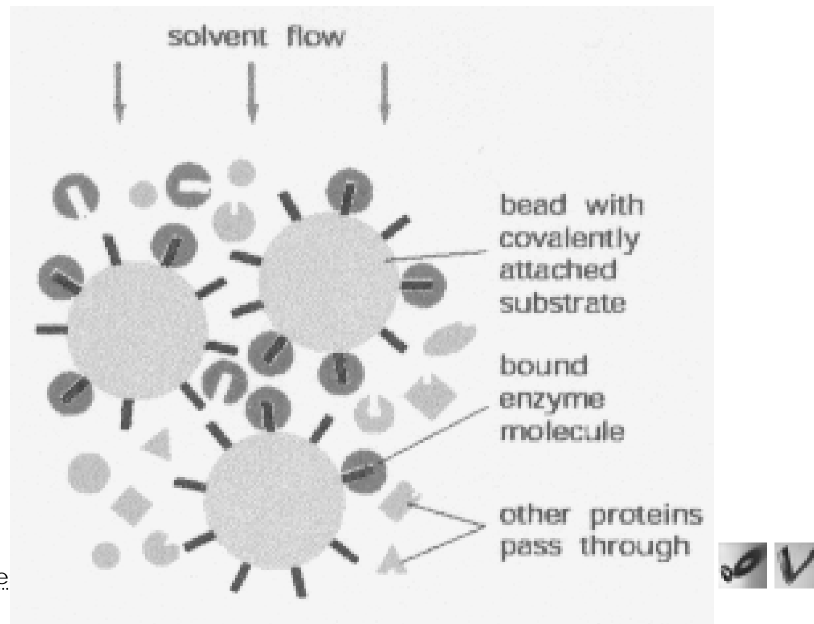
Principle Of Ion Exchange



Affinity Chromatography

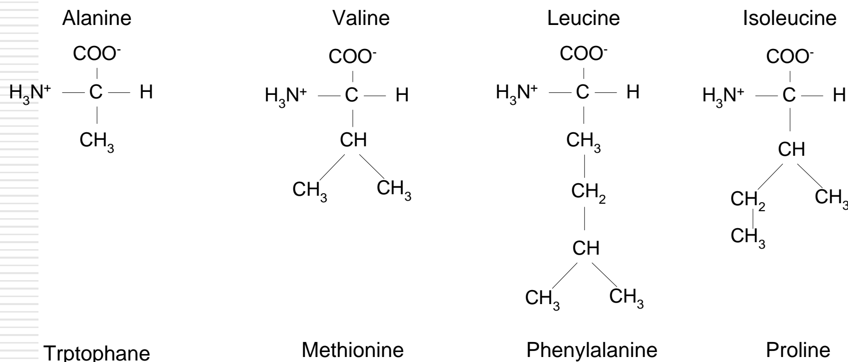
- The most powerful and highly selective method
- This technique relies on the ability of most proteins to bind specifically and reversibly to other compounds(ligands)
- A wide variety of ligands may be covalently attached to an enert support matrix and packed into a chromatographic column
- Only the protein molecules that selectively bind to the immobilized ligand will be retained on the column
- Washing the column with a suitable buffer will flush out all unbound molecules
- An appropriate change in buffer composition result in desorption of the retained proteins

Principle Of Affinity Chromatography



Hydrophobic Interaction Chromatography (HIC)

Eight amino acids are classified as hydrophobic, due to non-polar nature of their side chains



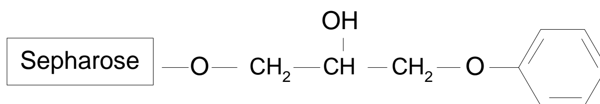
Trptophane Methionine Phenylalanine Proline

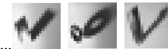
- Majority of hydrophobic amino acid residues are buried internally in the molecule.

- Minority of hydrophobic (Protein hydrophobicity) amino acid ➔ Protein surface

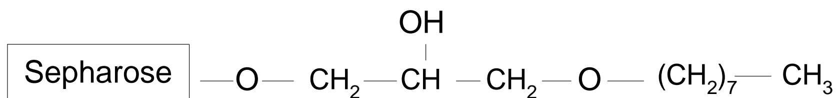
- HIC fractionates protein molecules by exploiting differing degrees of surface hydrophobicity. This hydrophobic groups covalently attached to a suitable matrix

Ex) Phenyl Sepharose



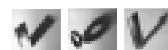


Octyl Sepharose



Application area

- Water treatment
- Pharmaceutical manufacturing
  (Separation of various antibiotics)
- Waste treatment



Characteristics Of Chromatography

Types	Objective	Capacity	Productivity	Step	Efficiency
Gel Filtration	Fractionatin Desalting	Low Moderate	Low High	Final Choice	Final purification Removal of low MW salt
Ion Exchange	Concentration Fractionation	Very high High	Very high High	First Choice	Volume reduction or removal of impurities First purification or a high purity
HIC	Fractionation	High	High	First or middle	Removal of impurities or high purity
Affinity Purification	fractionation	Very high	Very high	choice	Removal of impurities, Final purification or high purity

Analysis Of The Purification Methods Used At Successive Steps