DOWNSTREAM PROCESSING

The exchaction and purification of fermentation products may be difficult and costly. The choice of recovery process is based on following criteria:

1. Location of Product → extra or intracellular.
2. Concentration of product in fermentation broth.
3. Physical and chemical Properties of the product.
4. Intended use of product.
5. Acceptable purity of product.
6. Magnitude of biohazard of product or broth
7. Impurities in fermentation broth
8. Marketable price for the product.

There are basically three stages distinct in recovery;

a] Removal of large solid particles and cells by centrifugation and filtration.

b] Broth is fractionated or extracted into major fractions using ultrafiltration, reverse osmosis, adsorption / ion-exchange / gel filtration or affinity chromatography, liquid-liquid extraction, two phase aqueous extraction or precipitation.

c] Purification is done by fractional precipitation, chromatographic techniques and crystallization to obtain highly concentrated and pure product.

It must be remembered that fermentation and product recovery are integral parts of complete process. Hence both the stages should be developed in a well planned manner to avoid unnecessary problems and expenses for this following techniques can be done;

1. Selection of micro organisms which does not produce pigments or undesirable metabolites.
2. Modification of fermentation conditions to reduce the production of undesirable metabolites.
3. Precise timing of harvesting.
4. pH and temperature control.
5. Addition of flocculating agents
6. Use of enzymes to attack cell wall.

The recovery and purification of compounds may be acheived by number of alternative routes. which involve comparing the following factors.

a) Capital Costs
b) Processing costs
c) Throughput requirements
d) Yield Potential
e) Product Quality
f) Technical expertise
g) Regulatory requirements
h) Waste treatment needs
i) Automation and Personal health and safety.

Foam Seperation :- Here the surface activity of materials is exploited. The material may be whole cells or molecules such a protein or colloide which is selectively adsorbed or attached to surface of gas bubbles which rise through liquid. Some materials can be made suface active by application of surfactants like long chain fatty acids, amines and qualeinary ammonium compounds.
Materials made suface active and collected → Colligends and the applied surfactants are termed → Collectors

Precipitation Method :- Usefull process that allows enrichment and concentration in one step, reducing the volume of material for further processing.

Typical agents used in precipitation make the compound insoluble and they include;

1. Acids and Bases — change pH of solution untill isoelectric point of compound reached and its solubility is decreased.
2. Salts :- Ammonium and Sodium Sulphate are used for recovery of proteins. Removal of water from surface of proteins makes them to come together causing precipitation.
3. Organic Solvents :- Chilled ethanol and acetone can be used for precipitation of proteins; they change the dielectric properties of the solution.

4. Non-Ionic Polymers :- Polyethylene Glycol which is similar in behaviour to organic solvents. Used in precipitation of proteins.

5. Polyelectrolytes :- Precipitation of many compound also used in cell aggregation.

6. Protein Binding Dyes :- Triazine dyes bind to a precipitate certain classes of proteins.

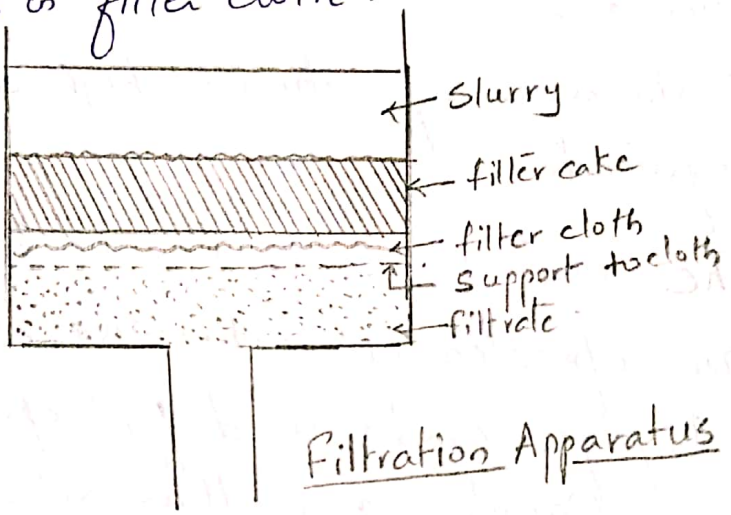
7. Affinity Precipitants :- Area of much current in they are able to bind and precipitate compounds selectively.

FILTRATION :- One of the most common process to seperate suspended particles from a liquid or gas using porous medium which retains the particles and allows the liquid or gas to pass through.

filtration can be carried out under variety of conditions but faces certain factors which influence the choice of equipment like;

i) Properties of filtrate – viscosity and density
ii) Nature of solid particles – size, shape and packing characteristics.
iii) Solid : Liquid Ratio
iv) Need for recovery of solid or liquid fraction or both.
v) Scale of Operation
vi) Need for Batch or Continuous Operation
vii) Need of aseptic conditions.
viii) Need for vacuum or pressure suction.

A simple filtration apparatus has a support covered with a porous filter cloth. As filtrate passes through the cloth filter cake builds which reduces the flow due to its thickness. To keep the flow rate constant "pressure" has to applied. Also flow rate may be reduced due to blocking of holes in filter cloth.



Filtration Apparatus

To avoid blocking of filters it is common to use filter aid Kieselguhr is the most widely used material. It is mixed with the cell suspension to improve porosity of filter cake. It is not cheap and also absorbs filtrate which will be lost when cake is disposed. Hence the following method is used

→ A thin layer of kieselguhr is applied to filter to form a precoat prior to filtration

→ Appropriate quantity of filter aid is mixed with the harvested broth. filtration is started and a satisfactory filter bed is build. The raffinate is again mixed with broth and than true filtration is started.

→ In vaccum drum filters a thick precoat filter is initially build up on drum.

→ If product is intracellular filter aids are not normally used and they may require purification.

CENTRIFUGATION :- The method is employed wh
filtration is not a suitable method for seperation.
It is expensive but is important under conditions when,

1. filteration is slow and difficult.
2. Cells or other suspended particles must be free of filter aids.
3. Continuous seperation to high standard of hygiene is required.

The centrifuges used in harvesting fermentation broths are operated on continuous or semi continuous basis.
Centrifuges can be used for seperating heavy and light-phase liquids, as well as solid fractions and also for breaking emulsions.

The rate of sedimentation is influenced by difference in density between cells & liquid, diameter of cells, viscosity of liquid.
Ideally cells should have large diameter, large density difference and low viscosity. In practicality the rate of sedimentation is increased by taking into consideration two major factors; angular velocity and diameter of centrifuge.

In industrial fermentations it is common to add flocculating agents to aid de-watering. The aggregate of cells have same density as a single cell but have increased diameter.

During flocculation one or more mechanism can induce cell flocculation;

a) Neutralization of anionic charges which allow the cells to aggregate. This includes changes in pH or presence of compounds which alter anionic environment.

b) Reduction in hydrophilicity.

c) The use of high molecular weight polymer bridges. Anionic, non-ionic and cationic polymers can be used.

Flocculating agents used for bacteria, yeast and algae are alum, calcium salts and ferric salts, tannic acid, titanium tetrachloride etc. Flocculation also depends on choice of additive, dosage and conditions of floc formation. Majority of flocculating agents currently used are polyelectrolytes which act by charge neutralization and hydrophobic interactions.

Another method to coagulate microbial proteins is by heating them for a short period of time.
Also alterations in cell surface properties is used.

CELL DISRUPTION :- Micro organisms are protected extremely tough cell wall. To release their cellular contents number of methods of cell disintegration have been developed. Although many techniques are availabl only few have been proved to be suitable for large-scal applications, particularly for intracellular enzyme extractio These methods which are available fall into two categorie

I - Physico - Mechanical Methods:	II Chemical Methods.
a) Liquid shear	a) Detergents
b) Solid shear	b) Osmotic shock
c) Agitation with abrasives	c) Alkali treatment
d) Freeze - thawing	d) Enzyme treatment.
e) Ultrasonication.	

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a) **Liquid Shear** (Breakoff) → Makes use of high-pressure Homogenizers. To carry out cell disruption. During use the microbial slurry passes through a non-return valve and impinges against operative valve. The cells than pass through a narrow channel followed by a sudden pressure drop at exit of narrow orifice. This causes cavitation in slurry and shock waves so produced disrupt the cells. There is a need to cool the slurry between 0-4°c to minimize loss in enzyme activity because of heat-generation during the process.

b) **Solid Shear** → Pressure extrusion of frozen micro-organisms at -25°c through a small orifice is used to obtain small samples of enzymes or microbial cell walls. Disruption is due to liquid shear through narrow orifice and presence of ice-crystals.

c] Agitation and Abrasives :- cell disruption by means of rotating discs and charge of small beads.
Beads are made of glass, alumina, ceramics and some titanium compounds. Dissipation of heat generated in the mill is one of the major problems in scale up though it can be controlled by using cooling jackets.

d] Freezing And Thawing :- freezing and thawing of microbial cells paste will cause ice crystals to form and their expansion followed by thawing will lead to disruption of cells. The technique is slow and causes limited release of cellular materials and cannot be used on its own but requires a combination with other techniques.

e] Ultrasonication :- Application of high frequency vibrations (~20KHz) leads to cavitation and shock waves are produced which cause cell disruption.
Method is highly effective on small scale, but not for large scale operations. Power requirements are high. Large heating effect so cooling needed.

[Cavitation is phenomenon in which rapid changes of pressure in liquid lead to formation of small vapor-filled cavities in places where the pressure is low. When subjected to high pressure these cavities collapse to generate intense shock waves]

CHEMICAL METHODS :-

a] **Detergents** :- They damage the lipoproteins of the microbial cell membrane and lead to release of intracellular components.
Compounds used as detergents includes quaternary ammonium compounds, sodium lauryl sulphate, (SDS), and Triton-X-100. These agents can cause protein denaturation and have to be removed before purification.
Use of **Triton X-100** in combination with **guanidine-HCl** is effectively used for release of cellular proteins.

b] **Osmotic Shock** :- Osmotic shock caused by sudden change in concentration of salt can cause disruption of cells. However effect on microbial cells is minimal.

c] **Alkali Treatment** :- It can be used for hydrolysis of microbial cell wall material provided the desired enzyme can tolerate a pH of 11.5 to 11.5 for 20-30 minutes.

d] **Enzyme Treatment** :- A number of enzymes hydrolyse specific bonds in cell wall of limited microorganisms. Though it is a gentle method it is very expensive and also presence of enzyme can complicate the further purification processes. However they have potential for selective release of products. They may also be used for pretreatment; to partially hydrolyse cell walls before cell disruption by mechanical methods.

LIQUID - LIQUID EXTRACTION →

Seperation of product from liquid mixture by treatment with a solvent in which the desired component is soluble is known as liquid-liquid Extraction

Before starting large scale extraction, the solubility characteristics of component in various solvents should be known. for this reason the polarity of molecules should be known. Polar liquids mix with each other and dissolve salts and other polar solids.

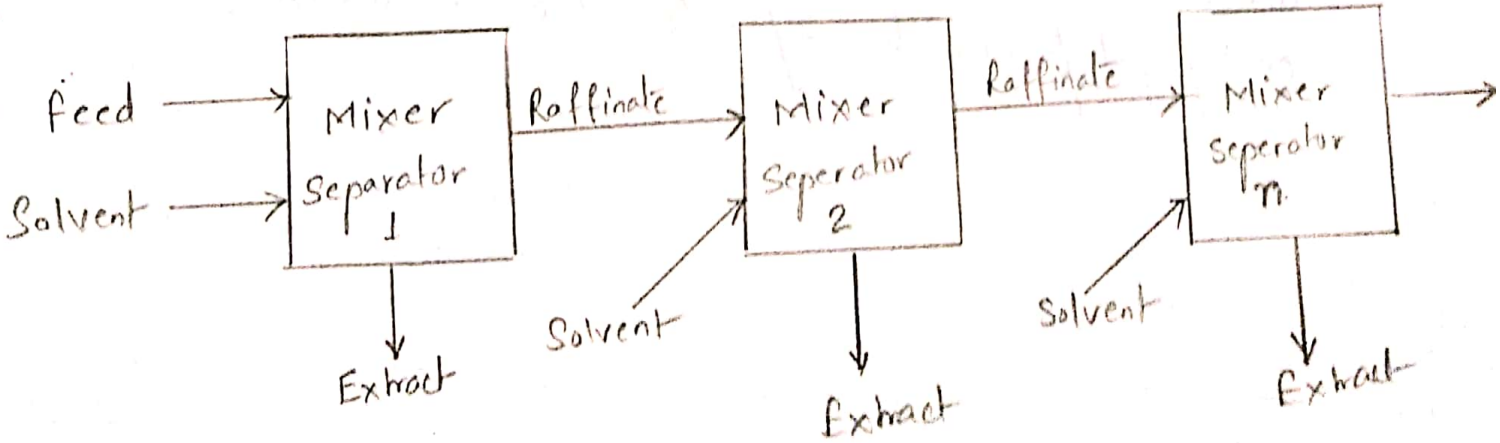
Non-polar compounds requie solvents with low or nil polarity.

The final choice of solvent depends on partition coefficient K which is; ~~the ratio~~

$$K = \frac{\text{Concentration of solute in extract}}{\text{Concentration of solute in raffinate}}$$

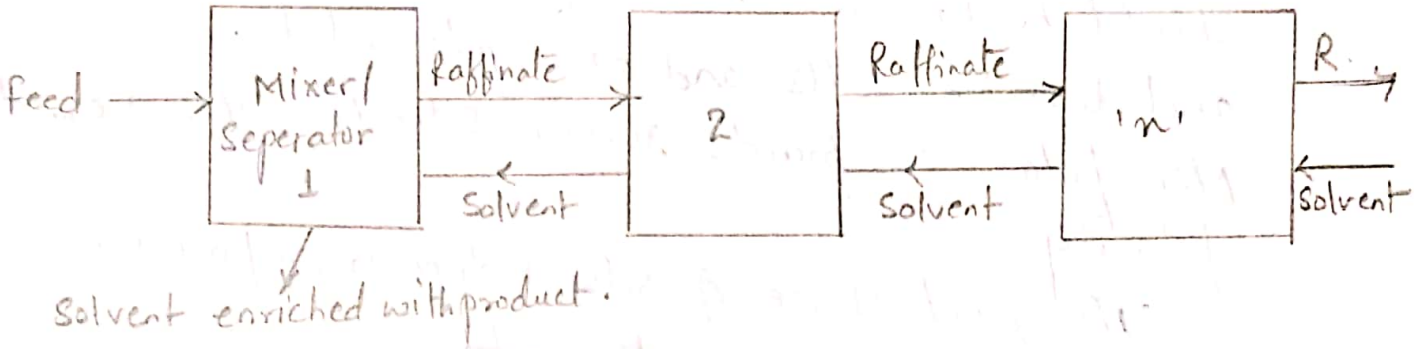
More the value of K means good stability of product and good seperation in a single stage extraction system. low the value of K means difficult in the extraction and requires a multistage process which may be co-current or counter-current system.

Co-Current Extraction System



In a co-current system 'n' mixers / seperator vessels are in line and the raffinate goes from vessel 1-to-n fresh solvent is added at each stage and feed and solvent pass in the same direction. Extract is collected at each stage. It ~~used~~ uses a high amount of solvent to acheive high degree of extraction.

Counter-Current Extraction System.



In a counter-current system 'n' number of mixers or seperators are connected in series. The extracted raffinate passes from vessel 1 to vessel 'n' while the product rich solvent passes from vessel 'n' to 1. The feed and the extracting solvent pass in opposite direction. It is the most efficient system. In practice series of ~~etc~~ counter current extractions are conducted in single continuous extracted using the centrifugal forces to seperate the two liquid phases.

SOLVENT RECOVERY → The solvent recovery plant is usually a distillation Unit. This distillation is achieved in three stages viz;

1. Evaporation :- Removal of solvent from a solution by application of heat to the solution. Most of the industrial evaporators use tubular heating surfaces. Circulation of liquid over the heating surfaces may be induced by boiling or agitation. In batch distillation the vapour from the boiler passes up the column and than it is condensed. Some part of condensate is returned back to the column as reflux of counter-current contact with rising vapour.
   In case of continuous distillation no condensate is collected but it is refluxed back to column until ideal operating conditions are established. Than feed is added into column at specific levels, the more volatile components ~~are~~ move upwards followed by reflux of condensate and less volatile fractions move down.

2. Vapour - Liquid seperation is a column, to seperate the lower boiling more volatile compounds from other less volatile component.

3. Condensation of vapour to recover more volatile solvent fraction.

TWO PHASE AQUEOUS EXTRACTION →

The use of organic solvents has limited applications in the processing of sensitive biologicals. Hence two phase aqueous system which have a high water content and low interfacial surface tension are regarded as being biocompatible. Here a large number & variety of natural and synthetic hydrophilic polymers are added to aqueous solution. When concentrations exceed a certain value two immiscible phases are formed. Also phase seperation can be improved by centrifugal seperations. The various aqueous systems are;

1. Non-ionic polymers / non-ionic polymer / water i.e. polyethylene glycol dextran.
2. Polyelectrolyte / non-ionic polymer / water i.e. Sodium carboxymethyl cellulose / polyethylene glycol.
3. Polyelectrolyte / Polyelectrolyte / water i.e. Sodium dextran sulphate / sodium carboxy methyl Cellulose.
4. Polymer / low molecular weight-component / water dextran / propyl alcohol

The distribution of solute between two phases is characterized by partition coefficient & number of factors like temperature, polymer, salt concn. pH and properties of solute.

It is possible to use different ligands in two phases leading to increase in selectivity or the simultaneous recovery & seperation of several components

Two phase system have found application in purification of many solutes viz proteins, enzymes, cells and subcellular particles. Large scale protein extraction using this system has emerged which employs PEG as upper phase and lower phase having dextran or salt-solution.

CHROMATOGRAPHY → Used to isolate and purify low concentrations of metabolic products. The scale up of chromatographic processes is difficult and much current work is being done on mathematical models & computer programmes to convert the small scale data into operating conditions for large scale operations.

The various chromatographic techniques are;

1. Adsorption chromatography
2. Ion-Exchange chromatography.
3. Gel Permeation Chromatography.
4. Affinity chromatography.
5. Reverse Phase Chromatography.
6. High Performance Liquid Chromatography (HPLC).

I. Adsorption Chromatography :- Binding of solute to solid phase by weak Van der Waals forces. The columns are packed with inorganic adsorbants like active carbon, aluminium oxide, aluminium hydroxide, silica gel etc and organic macroporous resins.

Active carbon may be used to clarify broths from pigments and other impurities.

The first organic macroporous adsorbants was Amberlite XAD Resin. They have surface polarities which vary from non-polar to highly polar but they do not have ionic functional groups. It was shown that these resins can be used for recovery of Cephalosporin C (acidic amino acid) cefotiam (basic amino acid) desferrioxamine B (basic hydroxamic acid) and paramethasone (neutral steroid).

II Ion-Exchange Chromatography → Reversible exchange of ions between a liquid phase and a solid phase which is not accompanied by any change in solid structure.

Cationic ion exchange resins normally contain sulphonic acid, carboxylic acid or phosphonic acid active groups. The most common cation exchange resin is Carboxymethyl cellulose.

Positively charged solutes (proteins) will bind to resin & strength of attachment depends on the net charge of solute, at pH of the column feed.

The solutes are sequentially washed off by passage of buffers of increasing ionic strength or pH.

Anionic ion exchange resins contain a secondary amine, quaternary amine or quaternary-ammonium active group.

Common anion ion exchange resin is DEAE (diethylaminoethyl) cellulose. It is used in the same manner to elute negatively charged solutes. (examples on page 302 of whitaker)

III. Gel Permeation Chromatography → Also known as Gel exclusion or gel filtration. It seperates molecules on basis of their size. Smaller molecules diffuse faster as compared to larges ones and penetrate into the pores of gel at greater degree.

When elution starts larger molecules elute first.

Wide range of gels are available — cross linked dextrans like sephadex and cross-linked agarose like sepharose

This technique yields a fairly pure fraction which can than be concentrated by pressure dialysis or any other method.

IV. Affinity Chromatography → Use for seperation and purification of most biological molecules on the basis of their function or chemical structure.

The technique depends on highly specific interactions between pairs of biological materials such as enzyme-substrate, enzyme-inhibitor, antigen-antibody etc.

The molecule to be purified from ~~cell lysate~~, eg:- cell lysate applied to affinity column by a binding substance (ligand) which is immobilised on insoluble support (matrix). Eluent is then passed through column to release highly purified and concentrated molecule.

The ligand is attached to matrix by physical adsorption or chemically by a covalent bond.

Material for matrix → agarose, cellulose, dextrose & polyacrylamide.

The method is costly and time consuming so alternative affinity methods such as affinity cross-flow filtration, affinity precipitation and affinity partitioning are used.

V. Reverse Phase Chromatography (RPC) → It utilizes a solid phase which has hydrophobic alkyl chains. This allows seperation of proteins according to their hydrophobicity More hydrophobic proteins bind most strongly to stationary phase and are eluted later than less-hydrophobic protein. Alkyl groups are normally 8-18 Carbons in length. RPC can also be combined with affinity techniques.

VI HPLC → It is high resolution column chromatography. There is improvement in nature of packing materials of column which yield smaller, more rigid and uniform beads. This allows packing in columns with minimum spaces betⁿ beads It is used to seperate and purify wide range of solutes including biomolecules.

HPLC is distinguished from liquid chromatography by use of
a) improved media (selectivity & physical properties),
b) for solid phase (stationary) through which mobile (fluid) phase passes.