

FOOD AND INDUSTRIAL MICROBIOLOGY

Down Stream Processing of Biologicals

Mani Shankar Bhattacharyya, Ashwini Kamble and Uttam Chand Banerjee
Department of Pharmaceutical Technology (Biotechnology)
National Institute of Pharmaceutical Education and Research
Sector 67, SAS Nagar-160 062, Punjab (INDIA)

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Filtration; Centrifugation; Chromatography; Spray drying; Cell disintegration; Solvent extraction.

Introduction

In biological processes after the production of the product, extraction and purification remain the next most important steps. The steps involved are most difficult and costly. Therefore, in biological processes the downstreaming processes should be efficient and cost effective to make the process economically viable. But, unfortunately, in general the recovery cost in microbial processes ranges from 15% to 75% of the total manufacturing cost. The recovery cost depends on the nature and purity of the product and the methods chosen for the recovery purpose. On the other hand, production parameters such as temperature, pH, process handling and aseptic conditions also affect the downstreaming process. It is known that production cost is less in the case of extracellular and simple products; on the other hand intracellular products cost more for the recovery. Therefore, the following criteria are taken into consideration while designing a recovery process:

1. Localization of the product (i.e. whether intracellular or extracellular)
2. Product concentration in the fermented broth
3. Physical and chemical nature of the product which may serve as an aid for the selection of separation process
4. Acceptable purity of the product by the competent authority (like FDA)
5. Marketable price of the product

Along with these, bio-safety consideration of the production process has also to be taken care of. The first step for the recovery of an extracellular product is to remove the cellular parts and media components. The preferred method that is used for the separation of cellular parts is centrifugation. The next steps are fractionation of the broth for the extraction of the product. Ultrafiltration, reverse osmosis, adsorption, ion exchange, gel filtration or affinity chromatography, liquid-liquid extraction, two phase aqueous extraction or precipitation are the methods used for isolation or purification of the desired product. The preference for the method depends on the nature of the product. Afterwards the product is crystallized to attain the required purity. By products may be isolated by the modification of this system. Product recovery may be made easier by taking care of the following steps in the upstream or bioconversion process.

1. By selecting microorganisms that do not produce any pigment during the fermentation or do not produce any undesirable metabolites.
2. By modifying the production conditions so that least amount of undesirable secondary metabolites are formed.

The following process parameters should be checked and maintained precisely:

1. harvesting time
2. pH control during fermentation and harvesting
3. temperature control and monitoring
4. addition of appropriate reagents for flocculation and separation

Purification of many of the compounds can be made by using a number of alternative routes. Ultimate choice of a particular process can be made by considering various criteria such as capital cost, processing cost, throughput requirement, yield potential, product quality and technical expertise available.

Removal of Microbial Cells and other Solid Matters

Microbial cells and other solid particles are separated from the broth by centrifugation or by filtration. In some cases, to increase the sedimentation rate, heat treatment is done or flocculating agents are added. In recent times, some modern techniques have also been investigated for the separation of the solid materials from the fermented broth, viz, electrophoresis and dielectrophoresis, to exploit the charge properties of the microbial cells. The other methods are ultrasonic treatment, used for the flocculating characteristics and the use of magnetic field. But these techniques suffer from high cost and difficulties of scale up. Two phase liquid extraction technique, in the more recent time, has been of much interest because of its easy scalability and cost effectiveness and can be used for the products that needs gentle conditions.

Foam Separation

Foam separation of solid materials like microbial cells or proteins and colloidal materials is mainly based on the surface properties of the materials. Cells or molecules are absorbed on surfaces of the gas bubbles arising with the bowling air and finally separated by skimming. Surfactants used generally are called collector and the material that is made surface active and collected are termed as colligends. For a process to be developed on the basis of this technique, the following parameters should be taken care of: pH, temperature, and air flow rate and surfactant and colligend collection ratio. Schematic representation of the foam separation process has been shown in the Fig. 1.

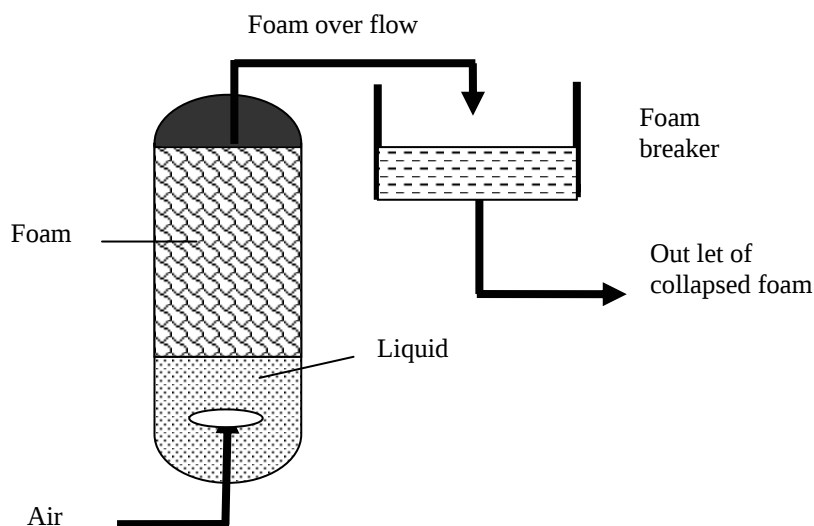


Fig. 1: Schematic representation of the foam separation process

Precipitation

Precipitation is mainly a product recovery process and can be applied at any stage of downstream processing. It is helpful to separate some product at one step or remove some impurities in the other. Sometimes the process allows enrichment and concentration of the

product and reduces the volume to be handled. The following techniques are used for the precipitation of various materials:

1. Change in the pH of the solution to reach the isoelectric point of the compound. The molecules precipitate due to a decrease in the solubility.
2. Ammonium and sodium salts (sulphates) are mainly used to precipitate the proteins. Salts remove the water molecules from the surface of protein molecules to facilitate the exposure of the non-polar surfaces of the molecules. Due to the non-polar interaction of the molecules that aggregate and precipitate.
3. Solvents (chilled ethanol, acetone) are used for the precipitation of proteins from the broth by changing the dielectric properties of the molecules. Non ionic polymers such as polyethylene glycol also precipitate proteins in the same way.
4. Poly-electrolytes are also used for the aggregation and precipitation of proteins.

Certain newer methods, such as, affinity precipitation or the selective precipitation of compounds and for the protein precipitation dye binding and aggregation are of particular interest.

Filtration

Filtration is one of the most widely used and efficient processes for solid-liquid separation with different types of filtration systems (Table 1). The process uses a porous medium that allows the flow of gas or liquids but not the solid material. It is amenable to scale up. But the following factors influence the choice of the most suitable equipment to meet the specific requirement with obvious cost effectiveness.

1. Viscosity and density of the filtrate
2. Nature of the solid particles: shape, size, distribution and packing characteristics
3. Solid: liquid ratio
4. Scale of operation
5. Material required to be recovered i.e. solid or liquid or both
6. Mode of operation: continuous or batch
7. Need for additional attachment for vacuum suction or need for low temperature

Theories of Filtration

A simple filtration apparatus (Fig. 2) consists of a filtration cloth supported by a porous material. When the broth passes through the filter cloth, the solid material is deposited on it and a cake is formed. Due to the gradual deposition of solid on the cloth, the cake increases in thickness and a resistance in the flow gradually builds up. To make the rate of flow constant, an increasing pressure has to be applied on the cloth with the increase in thickness of the cake and resistance of flow. Sometimes the pores of the filtering cloth may be closed due to the clogging or the flow may be stopped due to the compression of the particles. In that case pressure can not be applied for filtration especially when the particles are compressible.

Table 1: Different types of filtration systems

Mode of filtration	Types of filters	Characteristic features and applications
Batch filtration	Plate and frame filter: It is a type of pressure filter consisting of plates and frames arranged alternately; frames are fitted with filtering elements (filter pads or filter cloth). The assembly of filters and frames are held together by a horizontal frame and forms a series of liquid tight components. Liquid passes through the assembly through a hole in the corner, flows through the grooves and discharged through an outlet tap.	In industrial scale this is the cheapest filtration process and the area required is less. The main draw back of this process is the frequent wear and tear of the filtering cloth in batch operations due to its frequent dismantling. This system finds wide application for clarification in the breweries. It is also used for collecting high value solids for which the application of a continuous filtration apparatus is not suitable.
	Pressure leaf filter: These filters consist of a number of metal plates called leaves with metal framework of grooved plates. These are covered with fine wire mesh or sometimes a filter cloth, occasionally are pre-coated with a cellulose fiber coat. The slurry for the filtration is fed in to the filter by pressure or by suction. The system can be steam sterilized. Types: 1) Vertical metal-leaf filter 2) Horizontal metal-leaf filter 3) Stacked-leaf filter	Generally used for clarification of high volume liquid containing low solid load or in small batch filtration of valuable solids
Continuous Filtration	Rotary vacuum filter: The filter consists of a hollow rotating drum. The drum is segmented internally. The drum remains covered with a piece of fabric or metal filter and remains immersed in a trough containing the liquid to be filtered. Vacuum is applied inside the drum and the slurry is fed in to the container from out side. Due to the vacuum filtrate is sucked into the drum and through the compartments collected in a collecting vessel. To help the discharge of the filter cake air pressure is applied inside the drum and remaining broth is carefully washed out from the cake by water jet without allowing much dilution of the filtering liquid (broth). Depending on the cake discharge pattern a	This is applied in the industries for the filtration of large volume of liquids in a continuous process. Though the process can be applied for the separation of cells from the broth but disrupted cell slurry can also be effectively removed from the processing liquids.

	number of rotary vacuum filters are available. These are: 1. String discharge 2. Scraper discharge 3. Scraper discharge with precoating of the drum	
Cross flow Filtration (Tangential flow filtration)	Cross flow filtration system contains a media storage tank, a pump and a system of packs of membrane. To attain a much convoluted surface and an increased surface area the membrane is usually packed in a cassette of hollow fibers or flat sheet in a plate and frame type stack or a spiral cartridge. The broth is forced across the membrane surface, most of the bulk material sweeps the membrane and returns to the main tank and less than 10% pass through the membrane as permeate. As the process continues, the volume of the original feed reduces to 5-10% of their initial volume. [Two types of membranes can be used depending on the need of the process; microporous membrane with a specific pore size (0.45 μm or 0.22 μm) or an ultrafiltration membrane with a specific molecular weight cut-off]	The process is highly efficient and > 99.9% cell retention can be achieved. The system is a closed system and no aerosol is formed, the separation process is independent of media and cell densities and no addition of filter aid is needed. The system can be sterilized with minute modification. It is used for the filtration of fermentation broths, cell lysates etc. <i>Note: Factors that influence cross-flow filtration</i> Increased pressure drop increases the rate of filtration up to a certain limit. But as the system is based on the swept clean principle pressure drop should not be too high else it will lead to the blockage of the membrane. Therefore, to achieve the best results higher flow rate is recommended. Higher temperature increases the flow rate by lowering the viscosity of the system. Membrane may get clogged by the fowl material present in the medium. This can be controlled by modification of the constituents of membrane or modifying the media, like, minimizing the addition of antifoam reagent.

The flow through a bed having a constant and uniform depth can be represented by Darcy's equation:

$$\text{Rate of flow} = \frac{dV}{dt} = \frac{KA\Delta P}{\mu L} \quad (1)$$

Where, μ = liquid viscosity
 L = depth of the filtration bed
 ΔP = difference in the pressure across the filtration bed
 A = area of the filtration bed exposed to the liquid to be filtered
 K = constant for the filtration bed (known as Kozeny's constant)

K depends on the specific surface area s (specific surface area is defined as the surface area / unit volume) of the particles that make up the filtration bed and the voidage (Σ) when the particles are packed together. The voidage is defined as the fractional volume of the filter bed that is available for the liquid to pass through and it is generally 0.3 to 0.6. Therefore, K can be expressed as:

$$K = \frac{\Sigma^2}{5(1-\Sigma)^2 s^2}$$

Σ and s can not be determined easily and in most practical cases L also can not be measured directly. L can be expressed as:

$$L = \frac{vV}{A}$$

V = the broth volume passed in time t

v = volume of the cake deposited per unit volume of the broth

After substitution in equation (1)

$$\frac{dV}{dt} = \frac{KA^2\Delta P}{\mu vV} \quad (2)$$

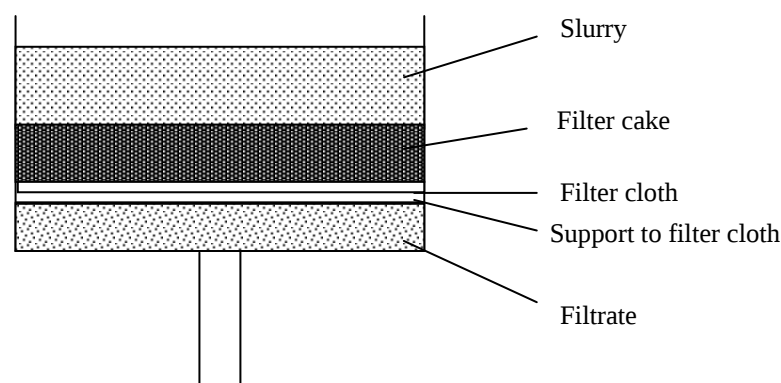


Fig. 2: Schematic presentation of a simple filtration apparatus

Equation (2) is a generalized equation relating the rate of filtration to the pressure drop, cross-sectional area of the filter and the filtrate retained. The integrated equation can represent filtration at constant pressure.

$$VdV = \frac{KA^2\Delta P dt}{\mu v} \quad (3)$$

Integrating equation (3)

$$V^2 = \frac{2KA^2\Delta Pt}{\mu v} \quad (4)$$

In equation (4) ΔP is a constant and μ is generally equal to 1 and v can be determined in the laboratory, where as, A^2 remains approximately constant. Therefore, it can be inferred that there is a linear relationship between V^2 and t . It is generally possible to calculate the pressure necessary to maintain a constant filtration rate, but is of little practical use.

Use of Filter Aids

Filter aid is generally used when the filtering material blocks the filter medium (filter cloth) due to bacterial broth or presence of some gelatinous material. Diatomaceous earth material (kieselguhr) is the most widely used filtration aid. It has a very high voidage (approximately 0.85) and the porosity of the material increases when it is mixed with the cell suspension. On the other hand, the filter aid should be added as less as possible to minimize the absorption of the filter material. It may be used as an initial bridging material in the wider pores of a filter medium to prevent or reduce the blinding of the pores. In order to attain an efficient filtering process using the filter aids i) a thin layer of the filter aid is applied on the filter medium to form the precoat before the filtration of the broth, ii) before starting the filtration, to build up a satisfactory filter bed an appropriate quantity of filter bed is mixed with the harvested broth and after building up of the bed, the raffinate is returned back to the remaining broth before starting the actual filtration (In case of vacuum drum filter, fitted with an advancing knife, a thick precoat filter is built-up on the drum). Generally, filter aids are used in case of intracellular products which facilitates further purification stages. Filter aids can not be used in case of microbial biomass production and for those cells where pretreatment procedures like flocculation or heating are considered.

Application

Cross-flow or tangential-flow filtration is commonly employed where the solution to be filtered is passed across the surface of the membrane without any appreciable phase change (Fig. 3). Filtration is almost exclusively operated within industry in a cross-flow mode due to its inherent resistance to fouling. The shearing effect of the fluid as it passes over the surface of the membrane acts to remove any particles which may have collected at the surface of the membrane. This helps to maintain a relatively steady flux across the membrane. Cross-flow membrane filtration technology is quickly gaining global acceptance as an important manufacturing step in many of the process lines in the food and fermentation, food ingredients, dairy, brewery, beverage, nutraceutical, and starch and sweetener industries. This membrane filtration process has large number of applications in food industry for processing of different beverages, dealcoholisation of beer and wine, processing of water reclamation, clarification of corn syrup and fructose. It has application in egg white or whole egg concentration. The ability to produce very specific separations at low or ambient temperature with no phase change can make membrane filtration a much more cost-effective process than more conventional methods such as rotary vacuum filtration or filter presses.

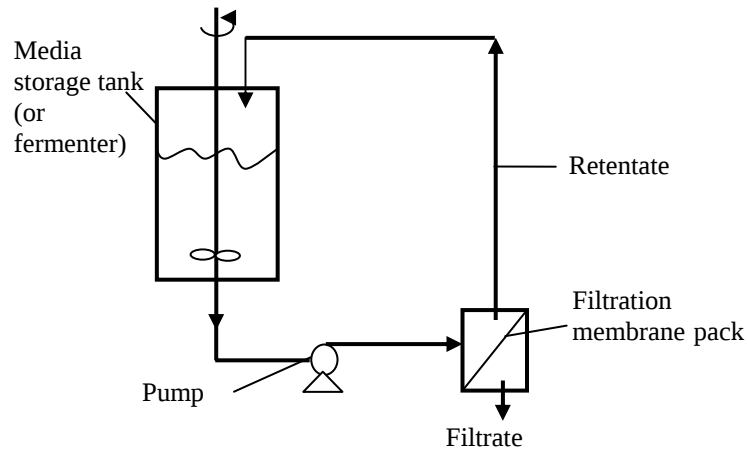


Fig. 3: Schematic presentation of a typical cross flow filtration system

Centrifugation

When solid separation is not satisfactory by filtration or a very high degree of separation is required, centrifugation is the method of separation. The main principle of centrifugation process is the sedimentation under centrifugation force.

According to the Stokes law, for a spherical solid suspended in a liquid of Newtonian viscosity, the rate of sedimentation is proportional to the square of the diameter of the particles. Thus the rate of sedimentation of a particle under gravitational force can be expressed as:

$$V_g = \frac{d^2 g (\rho_p - \rho_L)}{18\mu} \quad (5)$$

where, V_g = rate of sedimentation

d = particle diameter

g = gravitational constant

ρ_p = density of the particle

ρ_L = density of the liquid

μ = viscosity

The equation can be modified for sedimentation under the centrifugal force as

$$V_c = \frac{d^2 \omega^2 r (\rho_p - \rho_L)}{18\mu} \quad (6)$$

V_c = rate of sedimentation under centrifugation force

ω = angular velocity of the rotor

r = radial position of the particle

Dividing equation (6) by equation (5) we get

$$\frac{V_c}{V_g} = \frac{\omega^2 r}{g} \quad (7)$$

This equation is a measure of sedimentation power of centrifugal force compared to the gravitational force and is referred as the relative centrifugal force (RCF) and denoted by the symbol Z.

From the above expression it is evident that other factors like viscosity difference between the cellmass and the liquid has little role to play in the rate of sedimentation. It is true that with increasing temperature the viscosity of liquid changes, but since the viscosity difference of the particles and the liquid is very less, the effect can be neglected. Therefore the diameter of rotor and the angular velocity of the rotor are the main factors to be considered while maximizing sedimentation by centrifugation. Generally, in the laboratory batch centrifugation with small volumes can be used, but they are of little practical use in the industry. In industries centrifuges are used in continuous or semi-continuous mode.

Different Types of Centrifuges

For the industrial application different types of centrifuges are available with different dimensions and modes of application. They differ mainly in their mode of operation, capacities, speed, mode of loading and discharging. The ultimate choice of the type of centrifuge depends on the type of application and effectiveness. Table 2 contains the description of different types of centrifuges used in the industries (excluding ultracentrifuge and other advanced mode of centrifugation used for the special type of application).

Table 2: Different types of centrifuges

Types of centrifuge	Description	Application
Basket centrifuge (perforated-bowl basket centrifuge)	Contains perforated bowls with a filter bag of nylon or cotton. Liquid is fed in continuous mode and it is operated at not more than 4000 rpm. Solid biological mass can be washed before removing the bowls. Rate of feeding is 50-300 dm ³ /min and have a solid holding capacity of maximum 500 dm ³	Used for the separation of mycelial mass, moulds and crystalline compounds.
Tubular-bowl centrifuge	The main component of this system is a cylindrical bowl which may be of variable design depending on their application. The system remains suspended by a shaft which is flexible and rotates by a motor fitted overhead. The inlet is at the bottom fitted with a nozzle which enters through the bottom bearing. The inlet consists of solid and a liquid phase; the liquid generally contains a light and a heavy phase. The	The system is applied for the separation of particles having dimension of 0.1µm to 200 µm in diameter and for a liquid having solid load of only 10% or less. Can be applied for 1) light phase/heavy phase liquid separation 2) for the separation of solid/light phase liquid/ heavy liquid phase and only for 3) solid/liquid separation. This

	solid particles sediment on the wall of the rotor and the two liquid phases get separated into two distinct zones. At the exit they are kept separated by the adjustable lips of the rings which may be of various sizes.	system can be operated at a speed of 50,000 rpm. Limitations are limited solid holding capacity, loss of efficiency etc.
Solid-bowl scroll centrifuge (Decanter centrifuge)	The main part of this system is a horizontal rotating solid bowl. Slurry is fed to the bowl through the spindle of an archimedean screw within the bowl. Solid is separated on the wall of the bowl and is scraped up to the conical end of the bowl. The slope of the bowl is so set that the excessive liquid is drained out from the solid. Liquid is discharged at the other end of the bowl.	The system is used in continuous mode for the separation of solids from the fermentation broth. The bigger version can be applied at a speed of maximum 5,000 rpm, smallest versions can be applied sometimes at a speed of 10,000 rpm. The system is available for various applications 1) with the facility of cake washing, 2) with vertical bowl decanting facility 3) with the facility of in-situ cleaning, and 4) the facilities for the containment of biohazards.
Multichamber centrifuge	This system consists of multiple chambers mounted within the chamber of the rotor. The slurry is fed in to the chambers through a system of spindles and travels through the system of chambers through a circuitous route. At the outer face of each chamber the solid is collected.	The system is applied for the separation of particles having dimension of 0.1 μ m to 200 μ m in diameter. Though the system is having greater solid handling capacity and there is virtually no loss of efficiency but its mechanical stability limits its larger application and speed maxima (6,500 rpm)
Disc-bowl centrifuge	This system consists of a central inlet pipe and a system of conical disc, made-up of stainless steel arranged in stacks with a spacer. The broth to be separated is injected in to the system through a central pipe and then it flows outward toward the discs. There it flows upward and inward in between the disc at an angle 45° with the axis of rotation. Within the closed system the solid sediments rapidly, accumulating on the inner wall of the bowl. The sediment formed is not slid in this case, forms slurry which flows and can be discharged continuously.	The system is highly efficient, has capacity of high volume liquid handling, easy removal of solid and <i>in-situ</i> cleaning facility. Solid can be removed by automatic opening of the solid collection bowl. Feed rate is as high as 1800 dm ³ /min and rotational speed ranges from 5000 rpm to 10000 rpm.

Application

Centrifugation is one of the preferred methods of clarification of wine, removal of solids from the fermented broths etc. In this, high speed rotation impels suspended material out of the wine. Speed of clarification is the primary advantages of centrifugation. It is especially useful when early bottling is desired. Centrifugation is also particularly valuable with very turbid wines. They frequently produce off-odours during spontaneous clarification and typically clog filter units. Centrifugation also increases the efficient use of polishing filtration by removing the most remaining suspended material.

Cell Disruption

Microbes are protected from the outside environment by rigid cell wall. The cell wall may be extremely hard and the recovery of the intracellular products requires the breakage of the cell wall. A number of cell disintegration methods are available (Fig. 4) but the choice of the method depends on its suitability for the particular substance. Though in some cases by the application of a particular method, a specific product can be recovered from inside the cells with greater yield and purity, most of the times these types of applications are not feasible. On the other hand, gross disruption of the cell releases a huge number of products and its down stream processing becomes difficult. Sometimes application of a particular process is only feasible in the laboratory scale and therefore, choice of suitable methods for the industrial application is a matter of investigation. In recent times, enzymes are the most important intercellular products of interest. But the disruption methods applied for the release of enzymes must keep them active and properly folded, at the same time release yield must be high. Followings are the some cell disruption methods:

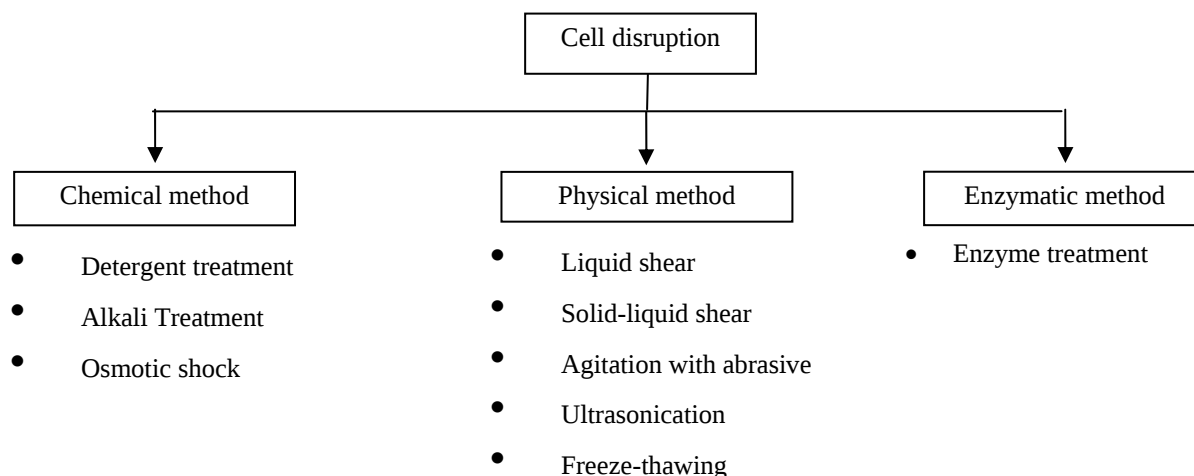


Fig. 4 : Different methods for the cell disruption

Chemical Methods

1. Detergent treatment

Detergents damage the cell wall by interacting with the lipoproteins of the microbial cell membrane and release the intracellular enzyme. The detergents used may be anionic or cationic or nonionic. The widely used detergents are quaternary ammonium salts, sodium

dodecyl sulphate, Triton X-100 etc. While applying these substances for the release of enzyme, it has to be kept in mind that the substances can cause protein denaturation and have to be removed from the cell free extract during further purification as soon as possible. The use of Triton X-100 is widely known to release membrane bound enzymes. The application of Triton X-100 along with guanidine-HCl is very effective for the release of a number of proteins.

2. Alkali Treatment

This method is applied for the cell disruption in very limited cases only when the enzyme is highly alkali stable and can tolerate pH up to at least 11.5. Only very few applications of this method are found for the release of the enzyme and one of the classical examples is for the release of L-asparaginase.

Physical Methods

1. Liquid Shear

Liquid shear force for cell disruption is used mostly for the large scale release of enzymes or intracellular products from the microbial cells. The method is based on the shear force generated by cavitation in the cell slurry due to a large pressure drop. The machine consists of a hollow cylinder made up of stainless steel and a piston with an appropriate system of adjustable valves. Cell slurry is loaded inside the cylinder and the pressure inside the cylinder is increased to thousands of psi through a system of hydraulics. When the cell slurry is allowed to pass through a small orifice, the cells experience a sudden pressure drop. That pressure drop causes cavitation and the shock waves so produced disrupt the cells. The amount of pressure drop experienced by the cells has a direct influence on the disruptive release of the enzymes from the cells. Therefore, it can be concluded that higher pressure drop is more effective for cell disruption. Mechanical strength of the cell wall, shape and size of the microorganisms also play an important role in achieving effective disruption. The process of cell disruption is exothermic and to prevent heat denaturation of the enzyme, an effective cooling system is always needed. The whole process is operated within 0-4°C. To make the process less abrasive, it should be operated in multi-pass mode for a longer time. In an industrial process, proper balance has to be made between the maximum release due to effective breakage of the cells and the percentage of released enzyme to achieve cost effectiveness.

2. Solid-liquid shear

This is a suitable method for laboratory and can also be operated in semi-continuous mode in small scale in the industry. Frozen samples of microorganisms are passed at very low temperature (-25°C) through a small orifice at very high pressure. The breakage occurs due to the liquid-shear and the presence of ice crystals. The process is especially applicable for temperature labile enzymes.

3. Agitation with abrasives

In this method cells are disrupted with the help of mechanically resistant beads made up of glass, alumina or titanium compounds. Inside a hollow chamber, cells are agitated with beads

by a system of agitator shaft. The shear forces so generated cause cell disintegration. The process is exothermic and an efficient cooling system must be associated to prevent thermal denaturation of the enzyme. The process can be applied at a large scale after suitable investigation.

4. Ultrasonication

Ultrasonic waves are used to break the cells at small scale. The process relies on the cavitation generated due to the sonic waves and the shear force generated thereby. A high electrical power is converted in to mechanical energy in terms of sonic wave and propagates through a horn in the liquid generating cavitation. The system suffers from several drawbacks: high power consumption, heat generation, small operable volume, etc.

5. Freeze-thawing

This is a very mild process. Due to repeated freezing and thawing of the microbial cells, ice crystals generate, create pores in the cell wall and facilitate the release of enzyme. The process is applicable in combination with other methods.

6. Osmotic Shock

Osmotic shock is applied for the mild release for the enzymes from the cells. A sudden change in the salt concentration changes the osmotic balance within the cells and the cell is disrupted. But the method is not very efficient for the microbial cells having tough cell wall. To apply this method for the disruption of microbial cells, generally the cell wall is first made weak by some other method. The method has proved to very efficient and unique for the release of luciferase enzyme from *Photobacterium fischeri*.

Enzymatic method

Various enzymes can break different bonds present in the cell wall and facilitate the release of enzymes. The process is the mildest one used for the release of intracellular enzymes. The enzymes used are lysozyme, enzyme extracts from *Streptomyces* sp., *Penicillium* sp., *Trichoderma* sp., snail etc. The method is very costly but highly effective for the release of enzymes with lesser impurities. During the downstream processing, the used enzyme must be removed. Sometimes this process is also used in combination with other cell disintegration methods.

Liquid Chromatography

Chromatography is the science which studies the separation of molecules based on the differences in their shape, size structure and/or composition. In general, it is a technique by which the components in a sample, carried by the liquid or gaseous phase, are resolved by sorption-desorption steps on the stationary phase. The components which display tighter interactions with the support will tend to move more slowly through the support than those components with weaker interaction. In this way, different types of components can be separated from each other as they move over the support material. Chromatographic separations can be carried out using a variety of stationary and mobile phases including

immobilized silica on glass plates (thin layer chromatography), volatile gases (gas chromatography), paper (paper chromatography), and liquids which may incorporate hydrophilic, insoluble molecules (liquid chromatography). The most commonly used of these techniques is liquid chromatography, it is very versatile and is used to separate a wide variety of compounds ranging from small organic molecules to important biopharmaceuticals including enzymes, nucleic acids, carbohydrates, fats, vitamins, and more. Different modes of liquid chromatography are discussed below:

Ion-Exchange Chromatography

Principle

The ion-exchange chromatography relies on the attraction between the oppositely charged molecules. Ion-exchange separations are mainly carried out in columns packed with an ion-exchanger. There are two types of ion exchanger: cation exchanger in which the stationary phase carries a negative charge and the mobile phase carries the positive counter ion. On the other hand, anion exchanger, in which the stationary phase carries a positive charge and the mobile phase carries negative counter ion (Fig. 5). Molecules of acidic, basic and neutral nature can be easily separated by this technique. In this separation, matrix-bound charged groups reversibly adsorb oppositely charged sample molecules. Diffusion of the molecule through the matrix of the exchanger is dependent upon the degree of cross-linkages of the exchanger and the ionic strength of the buffer. This diffusion process can control the rate of the whole ion-exchange process. However, desorption of the bound ion is achieved by change in pH or changing salt concentration, or by adding competitive counter ions, since it tends to disrupt the electrostatic interaction of the matrix with the bound molecule. Ion-exchange chromatography is perhaps the most frequently used chromatographic technique for separation of proteins according to their net charges.

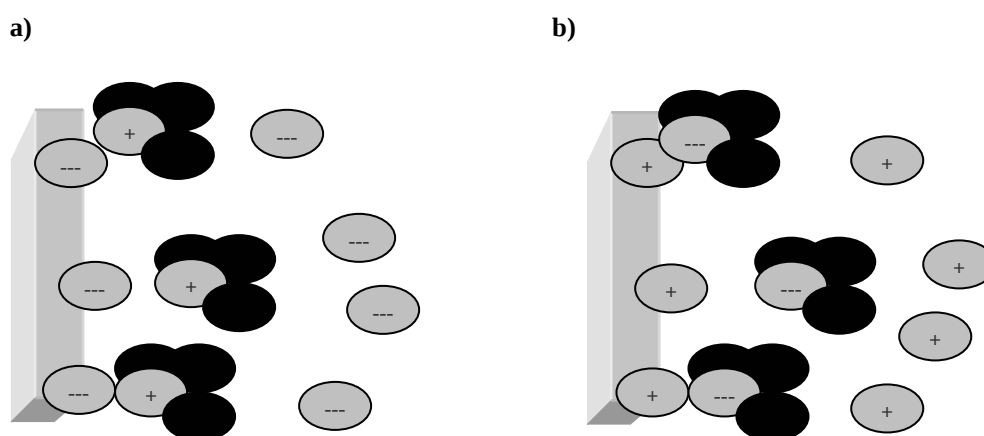


Fig. 5: a) Cation exchanger: stationary phase displays a negatively charged functional group that retains positively charged cations b) Anion exchanger: it retains negatively charged anions by displaying positively charged functional group

Components of Ion Exchange Chromatography

Ion exchange chromatography mainly consists of a column packed with ion-exchange resins bonded to inert polymeric particles of small diameter. Ion exchange chromatography can be carried out using a variety of matrices and ionic groups. Mostly, matrices used are polystyrene, cellulose and agarose. Functional ionic groups include sulphonic acid, carboxylic acid and quaternary ammonium group. As shown in Fig. 6a and 6b, anion exchanger may consist of diethylaminoethyl cellulose (DEAE-C) as the support material which has been derivatized with a positively charged amino group, while cation exchanger may consist of carboxymethyl cellulose (CMC) based support material which has been derivatized with a negatively charged carboxylic acid. Some examples of ion-exchangers are given in Table 3.

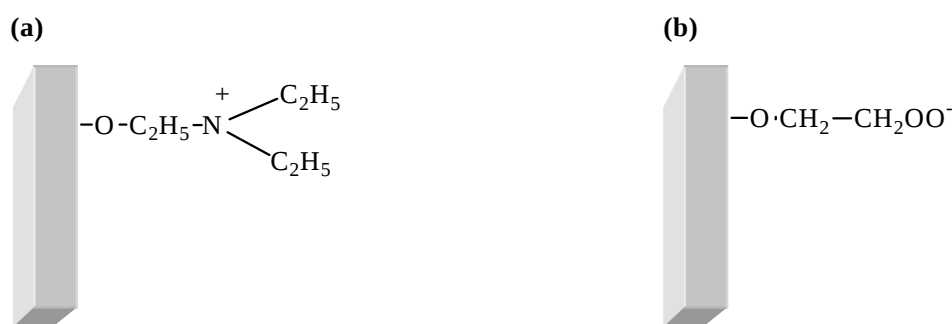


Fig. 6: a) Diethylaminoethyl cellulose based support material; b) CM-cellulose based support material

Table 3: Examples of commonly used ion-exchangers

Anion exchanger	Functional groups
Quaternary aminoethyl (QAE)	$\text{-O-CH}_2\text{CH}_2\text{N}^+(\text{C}_2\text{H}_5)_2\text{-CH}_2\text{-CHOH-CH}_3$
Quaternary ammonium (QA)	$\text{-O-CH}_2\text{-CHOH-CH}_2\text{-O-CH}_2\text{-CHOH-CH}_2\text{-N}^+(\text{CH}_3)_3$
Cation exchanger	Functional groups
Sulfopropyl (SP)	$\text{-O-CH}_2\text{-CHOH-CH}_2\text{-O-CH}_2\text{-CH}_2\text{-CH}_2\text{-SO}_3^-$
Methyl sulfonate (S)	$\text{-O-CH}_2\text{-CHOH-CH}_2\text{-CH}_2\text{-CHOH-CH}_2\text{-SO}_3^-$

Application

This is perhaps the most frequently used chromatographic technique used for separation of protein molecules, peptides and nucleotides according to their net charges. Proteins are composed of amino acids and have different overall charges at different pH values. At acidic pH, the amino groups bear a full positive charge, while at basic pH, carboxylic group bear negative charge. The pH at which the positive and negative charges on the protein are equal is known as the isoelectric point. At a pH value below the isoelectric point, a protein is retained by a cation exchanger while a protein will be retained by an anion exchanger at a pH above the isoelectric point. Mostly, protein purification is done on anion exchange columns because most proteins are negatively charged at physiological pH values (pH 6–8). Separation of amino acids is brought about either reducing the net charge by changing pH or

by increasing the ionic strength to break up the ionic interaction. Ion exchange chromatography has also application in the large scale separation of sugar for enriching fructose and separating cane molasses.

Affinity Chromatography

It is a technique enabling purification of a biomolecule with respect to biological function or individual chemical structure. This separation technique exploits the unique property of specific biological interaction to achieve separation and purification.

Principle

Separation is based on the binding affinity of biomolecules with their respective biologically active ligands (this ligand is bonded covalently to the solid matrix) which interacts with its analyte as a reversible complex that can be eluted by changing buffer or by addition of a competitive ligand. Affinity chromatography media are commonly used for applications such as purification of fusion proteins, mono- and polyclonal antibodies, and glycoproteins.

Components of Affinity Chromatography

The major components required for an affinity chromatography procedure are bead matrix, ligand, and a solution containing the substrate to be isolated, a wash to elute the non-bound impurities in the solution, and a final wash to elute the bound substrate from its ligand. Agarose, dextran, derivatives of cellulose, or other polymers can be used as the matrix, Sepharose (agarose-based) is the most widely used matrix, because the hydroxyl groups on the sugar residues can be easily manipulated to accept a ligand. Selection of ligand is in accordance with target molecule. To isolate a specific enzyme, a substrate analogue, an inhibitor, or even a cofactor can be used as ligand. For isolation of antibodies specific for antigen A from an antiserum, antigen A can be used as a ligand. This technique requires that the material to be isolated is capable of binding reversibly to a specific ligand that is attached to an insoluble matrix. Binding must not be too strong or weak. Ideal K_D (dissociation constant) value should be between 10^{-4} and 10^{-8} .

Once ligand-matrix gel is loaded into a column, sample mixture containing the desired isolate to be purified is added to the immobilized ligand (Fig. 7a). Proteins sieve through the matrix of affinity beads and only proteins with specific binding affinity towards ligand are bound (Fig. 7b). However, some of the impurities remain unbound or loosely bound in the gel column. In order to remove these impurities, a wash of different pH, salt concentration, or temperature is run through the gel (Fig. 7c). It is important to use a strong wash so that all the impurities are removed, but it is also just as crucial that the wash be not so strong that it removes the bound isolates.

Once the impurities are washed out, the remaining part is protein mixture which is still bound to the ligand-matrix in the gel, which is recovered by either specific or non-specific elution (Fig. 7d). Non-specific elution may be achieved by a change in either pH or ionic strength. This second wash relies on the reversible binding properties of the ligand, which allows the bound protein to dissociate from its ligand. The purified protein is eventually recovered in a buffered solution that may be contaminated with specific eluting agent or high concentration

of salts and these can be removed by techniques such as size exclusion chromatography before the isolation is complete.

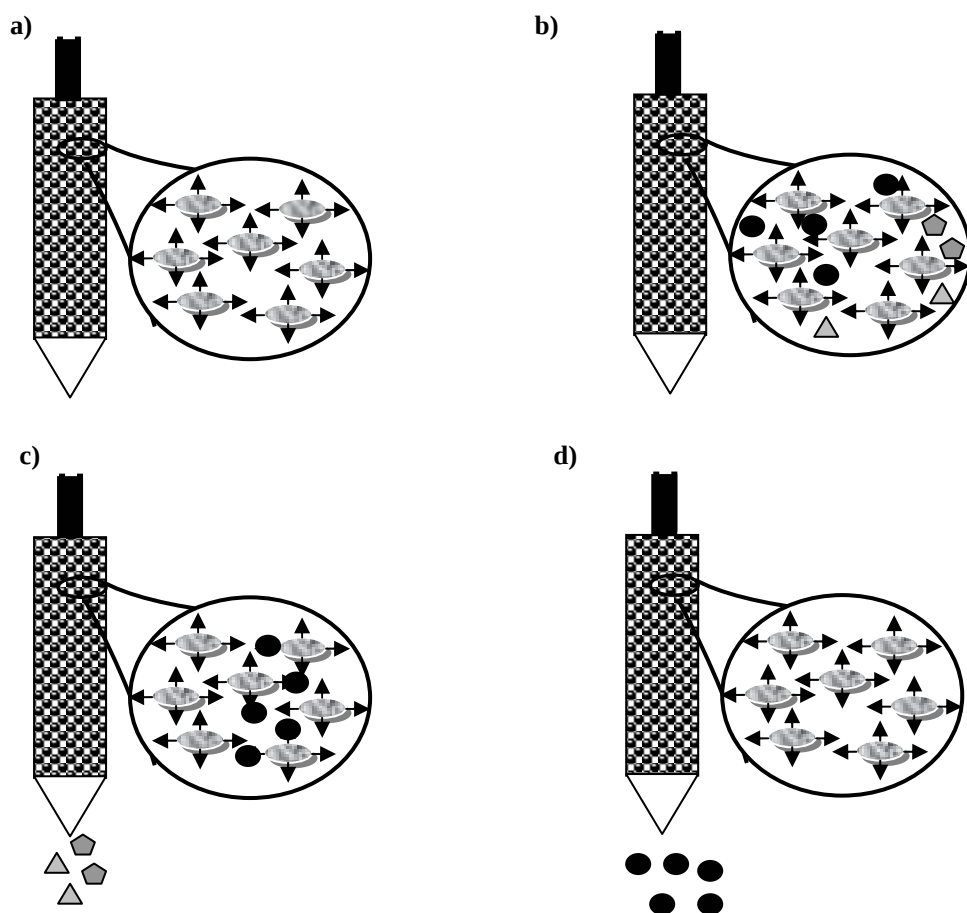


Fig. 7: a) Loading of ligand-matrix gel into elution column b) Proteins with specific affinity bind to ligand c) Unbound impurities get washed off d) Purified protein of interest

Application

Affinity chromatography can be used for purification of enzymes and other proteins, including receptor proteins and immunoglobulins. Affinity chromatography has application in separation and quantitation of glycosylated hemoglobins, fusion proteins, antibodies, etc. Immobilized single-stranded DNA can be used to isolate complementary RNA and DNA. Affinity chromatography has the application in separation of amino-acyl-ribonucleic acid synthetase I on tRNA conjugate of sepharose matrix.

Size Exclusion Chromatography (Gel Filtration)

Size exclusion chromatography is also called gel filtration; it is a method that separates molecules according to their size and shape. The separation of the components in the sample mixture is based on their molecular weight, shape and size. Gel filtration can be used as an analytical method to determine the molecular weight of an uncharacterized molecule. Gel filtration is also an important preparative technique in the purification of proteins, polysaccharides and nucleic acids.

Principle

The stationary phase consists of porous glass granules or gel particle which is in equilibrium with mobile phase. Molecules of large size are completely excluded from the pores. They have access only to the mobile phase between the beads and, therefore, elute first, while, molecules of intermediate size are partially included. They can fit inside some but not all of the pores in the beads. These molecules elute between the large (excluded) and small (totally included) molecules. However, molecules of small size are completely included. They get distributed between the mobile phase inside and outside the molecular sieve and elute last in a gel filtration separation (Fig. 8).

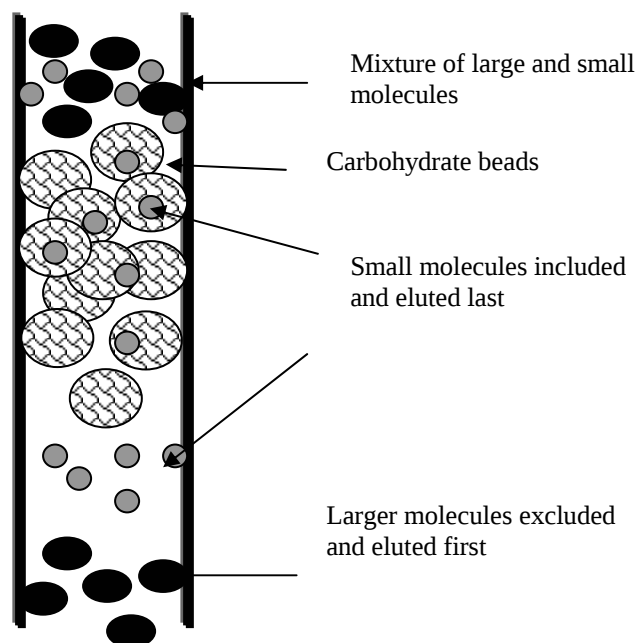


Fig. 8: Size exclusion chromatography

In gel filtration, the separations are described by the following equation:

$$V_r = V_0 + K_d V_i$$

where V_r is the retention volume of the protein, V_0 (void volume) is the volume of mobile phase between the beads of the stationary phase inside the column, V_i (included volume) is the volume of mobile phase inside the porous beads and K is the partition coefficient; the extent to which the analyte can penetrate the pores in the stationary phase, with values ranging between 0 and 1. If the analyte is larger and completely excluded from the mobile phase within the gel, $K_d = 0$, whereas, if the analyte is sufficiently small to gain complete access to the inner mobile phase, $K_d = 1$. The variation of K_d between these two limits makes possible the separation of analytes within a narrow molecular size range on a given gel.

For two substances of different relative molecular masses and K_d values, K'_d and K''_d and the difference in their elution volumes, V_s , can be given as

$$V_s = (K'_d - K''_d) V_i$$

Where V_i is the inner volume within the gel available to a compound whose $K_d = 1$.

Components of Size Exclusion Chromatography

The basic components of the size exclusion experiment are the matrix, chromatography column and the elution buffer. The matrix is the porous bead which is actually the separation medium, for example, agarose porous bead as shown in Fig. 9. It is the stationary phase of the gel chromatography. There are many different types of gel filtration matrices available in different fractionation ranges (Table 4). Matrices that are commonly used include cross-linked dextrans (e.g. Sephadex), agarose (Sepharose, Bio-Gel) and polyacrylamide (Sephacryl, Bio-Gel P).

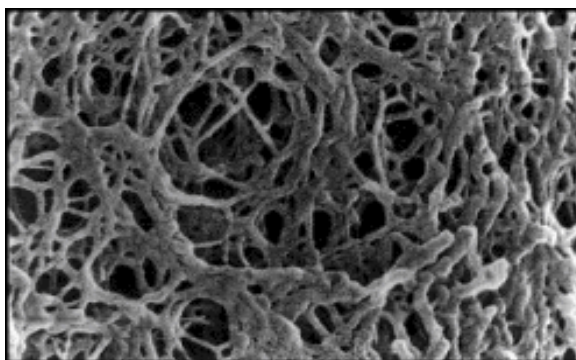


Fig. 9: Porous bead structure of size exclusion chromatography. (Scanning electron micrograph taken from the Department of Biochemistry, Uppsala University, Uppsala, Sweden)

Table 4: Matrices commonly used for size exclusion chromatography

Matrix	Trade name	Fractionation range for globular protein (Da)
Dextran	Sephadex	$7 \times 10^2 - 3 \times 10^4$
Agarose	Sepharose	$1 \times 10^4 - 4 \times 10^4$
	Bio-gel A	$1 \times 10^4 - 15 \times 10^6$
Polyacrylamide	Bio-Gel P	$1 \times 10^2 - 1 \times 10^5$

The spectrum of molecular weights a matrix is capable of separating is called its fractionation range. For example, consider a matrix that has a fractionation range (in molecular weight) of 1000 to 100,000 daltons. Molecules in the range of 1000 to 100,000 daltons will enter the beads with varying efficiencies and be partially or completely separated from one another. Molecules greater than 100,000 daltons will not enter the beads and will be eluted in the void volume. Molecules with an average molecular weight of 1000 or less will not be separated from each other since they all penetrate the beads completely and with equal efficiency. These molecules require the maximum volume of buffer for elution.

Exclusion chromatography columns tend to be longer than those for other forms of chromatography in order to increase the amount of stationary phase and hence pore volume. The elution buffer is the mobile phase of the chromatography and flows through the matrix and out of the column. The column, with the matrix and applied sample, is developed by the elution buffer. This means that the molecules in the sample are carried by the flow of buffer into the matrix where they are gradually separated. Exclusion chromatography requires a single mobile phase and isocratic elution. The separated zones of molecules then flow out of

the column where they are collected for analysis which is most commonly done with ultraviolet absorption spectrophotometric detectors.

Application

Size exclusion chromatography is mainly used for purification of biomolecules such as proteins, peptides, and hormones, antibodies, polysaccharides on the basis of size. Other than purification, this technique can also be used for determination of relative molecular mass (M_r). Solutions of high molecular mass can be desalted by using column of Sephadex G-25. Exclusion chromatography has also been used to study the protein-ligand binding.

Ultrafiltration

Ultrafiltration is a form of filtration which is based on membrane separation process driven by pressure gradient, in which the membrane fractionates the dissolved components of a liquid as a function of their solvated size and structure. Ultrafiltration is most commonly used to process a solution that has a mixture of components differing in molecular weight. Ultrafiltration membranes allow passage of solutes depending on the nominal molecular weight cut off (NMWCO) of the membrane being used.

Principle of ultrafiltration

Ultrafiltration is based on porous semi-permeable membrane. A pressure (driving force) is applied to force the solvent and small molecules through the membrane to the permeate and thereby macromolecules larger than the pore size are retained by membrane and concentrated in the retentate reservoir. The membrane mass transport mechanism is called permeation and can be described in basic terms by Fick's Law:

$$J = P \frac{\Delta DF}{l}$$

Where: J = flux

P = permeability coefficient

l = membrane thickness

ΔDF = driving force across the membrane

Flow mode in filtration

Membrane filtration can be operated in both cross flow and dead-end flow mode. In dead-end filtration (conventional filtration), the feed flow is perpendicular to the membrane surface (Fig. 10a) which causes build up of debris at the membrane surface and leads to blinding of the membrane and therefore reducing the fluid permeation. On the contrary, cross flow filtration (tangential flow) provides a continuous turbulent flow (Fig. 10b) and hence reduces the membrane fouling layer formed due to feed stream debris and macromolecule. Particles deposited on the membrane surface are swept away by the feed flow, thus minimizing choking of the membrane.

As compared to the dead-end filtration mode cross flow mode promotes consistent, long-term productivity. Crossflow filtration is influenced by a great number of parameters such as crossflow velocity, transmembrane pressure, membrane resistance, cake layer resistance, size distribution of the suspended particles and particle form.

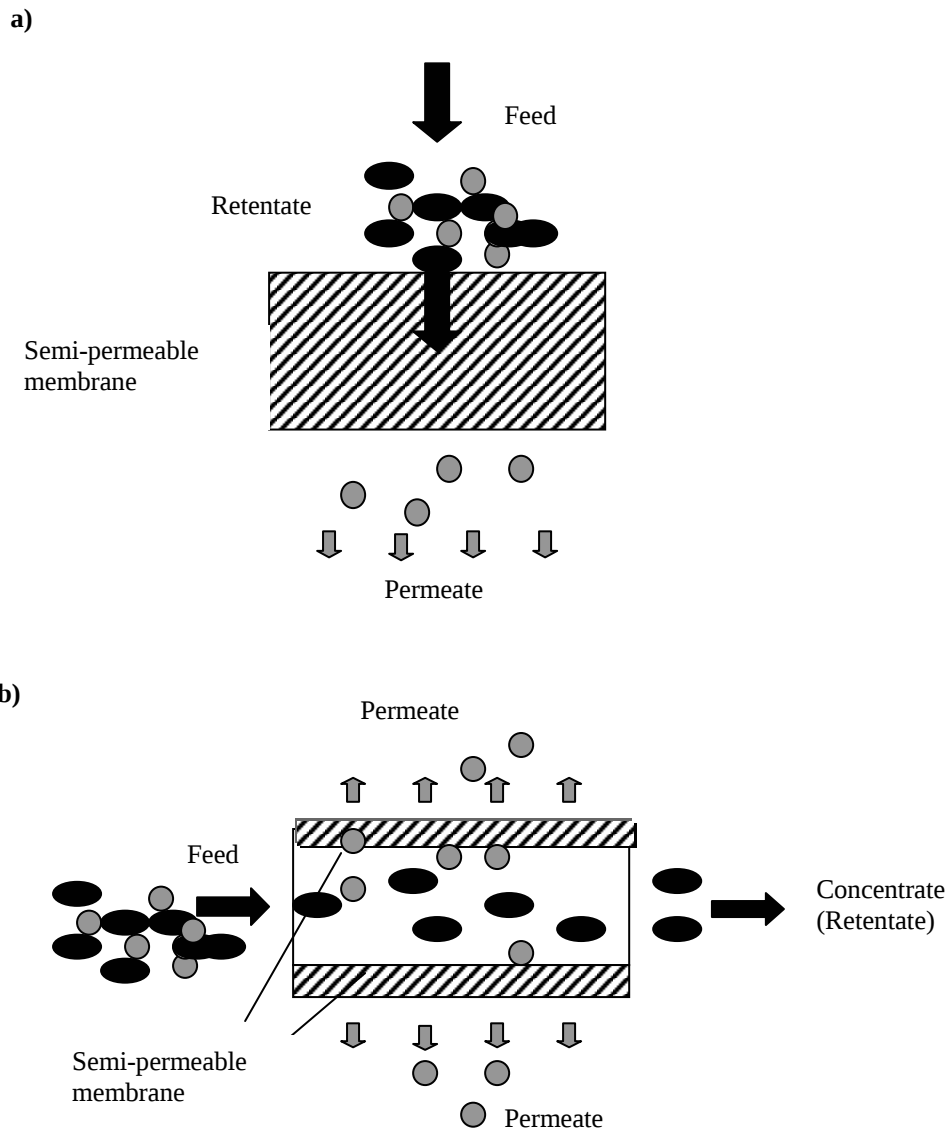


Fig. 10: Schematic diagram showing a) dead-end and b) crossflow mode in ultrafiltration

Application

Ultrafiltration is an important process for separation of colloids and particulate matter from liquid suspensions in many fields of engineering and applied science. This separation process is used for a broad variety of applications, ranging from the processing of biological macromolecules to removal of suspended particles and pathogenic microorganisms in water and wastewater treatment, food processing, separation processes in the beverage and cosmetic industries, harvesting of bacterial cells, separation of plasma from whole blood, and dewatering of suspended solids such as mineral slurries.

Food and biotechnological applications account for nearly 40% of the current total usage of ultrafiltration membranes. Protein bioseparation is an important component of this application segment. Ultrafiltration is used for protein concentration, desalting, clarification and fractionation (i.e. protein–protein separation). Concentration, desalting and clarification

are technologically less demanding and have been in use in the bioprocess industry for some time. In food industry, the ultrafiltration process is mainly used for the separation of critical flavor compounds from the elements that can not be heat pasteurized. In modern biotechnological process there are lots of applications of ultrafiltration in terms of separation of cell mass, proteins of different molecular weights etc.

Spray Drying

Spray drying is a three-step drying process involving spray of droplets, drying and separation of dried powder from the air stream. It is the most widely used industrial process involving particle formation and drying. There are many applications of spray drying in the pharmaceutical as well as in the food industry. Pharmaceutical spray dryer is a flexible, modern, and easy-to-handle dryer available with different levels of control systems. It dries solutions, suspensions, and emulsions into powders, granule or agglomerate. It can be used as a laboratory dryer for testing and development work or for batch production. Therefore, spray drying is an ideal process where the end-product must comply with precise quality standards regarding particle size distribution, residual moisture content, bulk density, and particle shape. Operating conditions and dryer design are selected according to the drying characteristics of the product and powder specifications.

Spray Drying System

As shown in Fig. 11, spray drying system consists of nozzle atomizer, feed pump, air heater, air disperser, drying chamber, and systems for exhaust air cleaning and powder recovery.

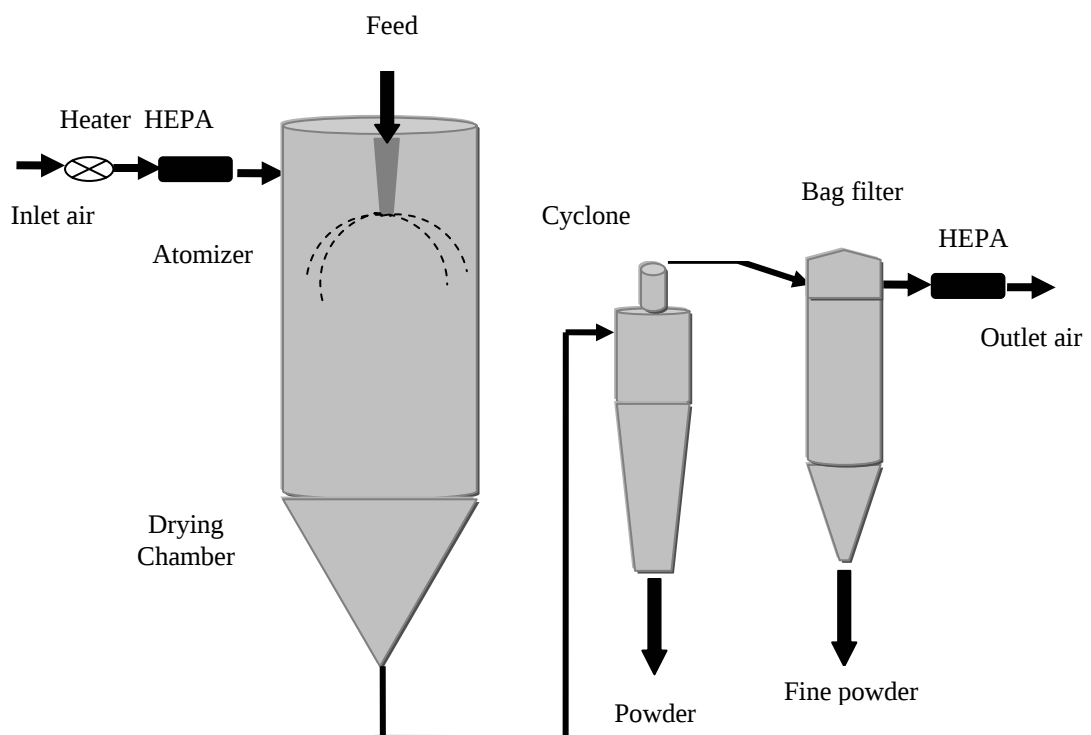


Fig. 11: Schematic diagram of typical spray drying system

Operating principle

Every spray drying process consists of the following unit operations:

- Pre-concentration of liquid
- Atomization
- Air flow
- Separation of powder from moist air
- Cooling

1. Pre-concentration of liquid

The liquid feed is generally pre-concentrated by evaporation to reduce the water content economically.

2. Atomization

It is the process of formation of droplets from a liquid feedstock. Spray drying involves pumping a concentrate of the liquid product into the atomizing device where it is broken into small droplets and these droplets come in contact with hot air in a drying chamber. The spray of droplets is produced by either rotary or nozzle atomizers.

Atomizing devices are the distinguishing characteristic of spray drying. They provide a large surface area for exposure to drying forces. This is an important parameter as it responds readily to changes in the process and reflects the quality of the product. Hence it is necessary to monitor the exit air temperature. It should be high enough to produce desired moisture without heat damage. There are two controls that may be used to adjust the exit air temperature:

- altering feed flow rate
- altering inlet temperature

If heat damage occurs before the product is dried, the particle size must be reduced; smaller particle dries faster, therefore, experiences less heat damage. This can be accomplished in three ways:

- smaller orifice
- increase atomizing pressure
- reduce viscosity - by increasing feed temperature or reducing solids

3. Airflow

The spray droplets produced in atomization process meet a stream of hot air and they lose their moisture very rapidly while still suspended in the drying air. The initial contact between spray droplets and drying air controls evaporation rates and product temperatures in the dryer. There are three modes of contact:

i) Co-current spray drying

The spray droplets to be dried are pumped to the top of the drying chamber by a variable speed progressing cavity pump which provides a very smooth flow and constant flow rate. Drying air and particles move through the drying chamber in the same direction (Fig. 12). Hot air is blown into the chamber, on to the droplets causing flash evaporation of the surface moisture. By the time the particles fall to the discharge point at the base of the cone, the

bound moisture in the particles is evaporated. In this configuration the particle temperature will never exceed the temperature of the exhaust air and hence this is an ideal mode for drying heat sensitive products. The added advantage of co-current spray drying is handling both low and high viscosity feeds. Powders produced with the co-current two-fluid nozzle have a mean particle size in the range of 10-40 μm .

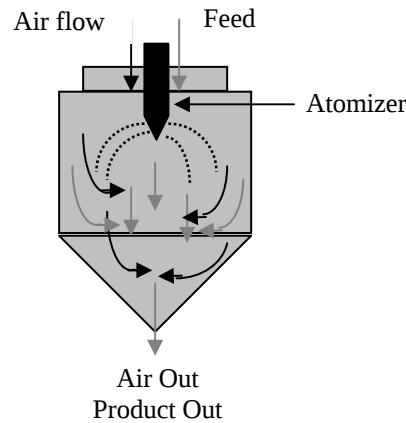


Fig. 12: Co-current spray flow dryer

ii) Counter-current spray drying

Drying air and particles move through the drying chamber in opposite directions as shown in Fig. 13. In counter-current configuration the nozzle is positioned at the bottom of the chamber and sprayed upwards into the flow of hot air. This mode is suitable for products which require a degree of heat treatment during drying. The temperature of the powder leaving the dryer is usually higher than the exhaust air temperature.

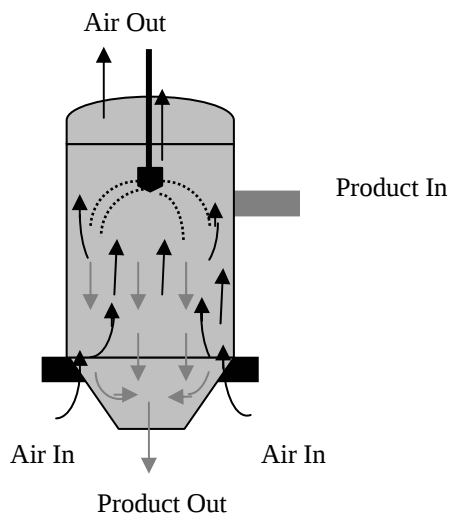


Fig. 13: Counter current flow dryer

iii) Mixed flow (fountain mode)

Particle movement through the drying chamber experiences both co-current and counter-current phases (Fig. 14). This mode is suitable for heat stable products where coarse powder requirements necessitate the use of nozzle atomizers, spraying upwards into an incoming airflow, or for heat sensitive products where the atomizer sprays droplets downwards towards an integrated fluid bed and the air inlet and outlet are located at the top of the drying chamber. The fountain mode has the advantage of spray drying high solids feeds, and producing free-flowing powders of a mean particle size in the range of 60-100 μm .

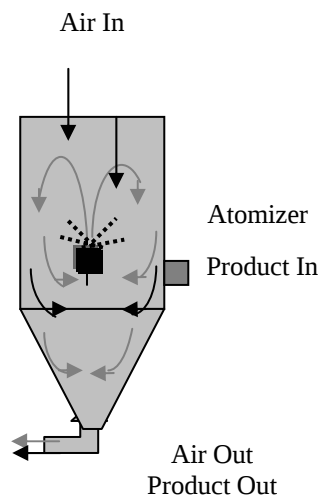


Fig. 14: Mixed flow (fountain mode) dryer

4. Powder Recovery

It is essential for both economic and environmental reasons that as much powder as possible be recovered from the air stream. The dry powder is separated from the moist air by collecting system. Bag filters are very efficient but not as popular due to labor costs, sanitation, and possible heat damage because of the long residence times. They are not recommended in the case of handling high moisture loads or hygroscopic particles. Cyclones are not as efficient as bag filters but several can be placed in series. Cyclone dryers are capable of drying ton-lots of powder per hour. The dry powder is separated from the moist air in cyclones by centrifugal action. The centrifugal action is caused by the great increase in air speed when the mixture of particles and air enters the cyclone system. The dense powder particles are forced toward the cyclone walls while the lighter moist air is directed away through the exhaust pipes. The powder settles to the bottom of the cyclone where it is removed through a discharging device. Wet scrubbers usually act as a secondary collection system following a cyclone. Wet scrubbers are the most economical outlet air cleaner. It dissolves any dust powder left in the air stream into either water or the feed stream by spraying the wash stream through the air. This also recovers heat from the exiting air and evaporates some of the water in the feed stream (if used as the wash water).

5. Cooling system

In some spray drying systems, the air-conveying ducts for the dry powder are connected with cooling systems which admit cold air for transport of the product through conveying pipes.

There are two most common types of spray dryers, namely, open cycle and close cycle spray dryers, as shown in Fig. 15a and 15b. The open cycle heats the incoming air and cleans it by using cyclones and scrubbers. After drying the food product, the hot air goes back to the environment and its heat is wasted. The close loop system is more efficient since the hot air is recycled.

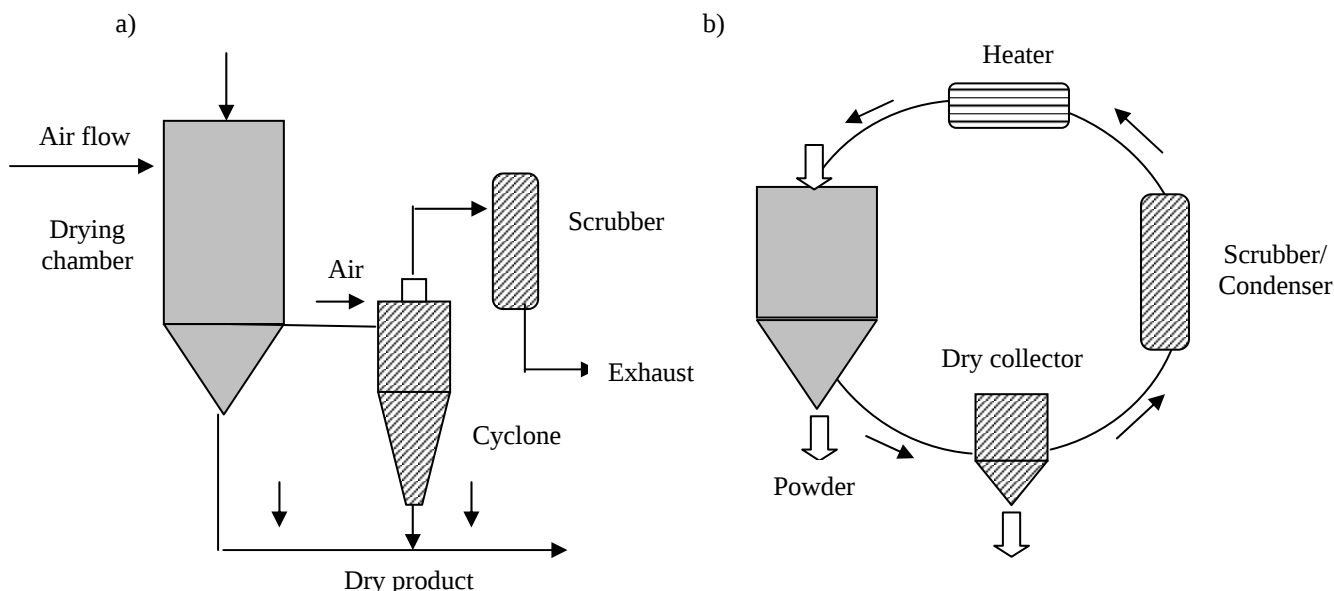


Fig. 15: a) Open cycle spray drying layout b) Closed cycle spray drying layout

Application

Spray drying system has a broad spectrum of application in pharmaceutical and food industries. In the pharmaceutical sector, spray drying is the most attractive method for the preparation of micro-encapsulated formulations of active pharmaceutical ingredients including peptides, proteins and DNA. Biodegradable microparticles such as dl-lactic/glycolic acid microparticles for sustained release of a water-soluble drug (Thyrotropin releasing hormone: TRH) can be prepared by spray drying method. In food industries, it is an important method to dry heat sensitive products such as fruits and vegetables. For instance, in obtaining high quality tomato powder at a low cost, spray drying seems to be the most efficient process. Another interesting application of spray drying is in the production of skim milk powder. It is required to apply low pasteurization temperature and gentle treatment during the whole process. For this purpose two-stage drying is preferred. It provides a gentler drying because the particle temperature is much lower, especially during the critical drying phase from 20 to 10% moisture, which results in a powder with no protein denaturation.

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