

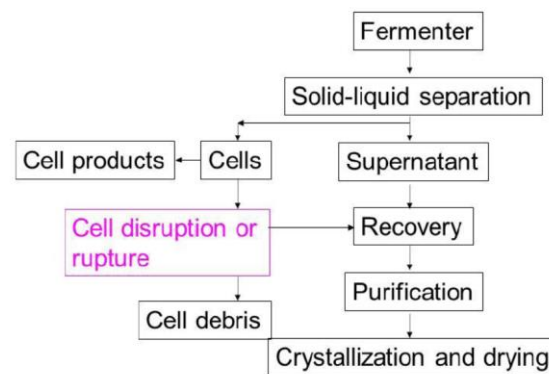
- 2- Extraction of high molecular weight: This technique is applied to compounds like proteins, which get inactivated in the presence of organic solvents like ethanol, methanol, chloroform, hexane etc. The solubility of the desired product is dependent on various properties of the protein e.g. ionic character, hydrophobicity, size etc.
3. - Membrane filtration: This technique involves the use of semi-permeable membrane. Many kinds of membranes are available based on the size of the pores and their chemical nature (polyether sulfone or polyvinyl difluoride). Unfortunately, these filters cannot be sterilized. Now membranes made up of ceramics and steel are being introduced which can be easily autoclaved.

V.RECOVERY AND PURIFICATION OF PRODUCTS

The main objective of the first stage for the recovery of an extracellular product is the removal of large solid particles and microbial cells usually by centrifugation or filtration. In the next stage, the broth is fractionated or extracted into major fractions further more precise chromatographic techniques and crystallization to obtain a product which is highly concentrated and essentially free from impurities

Steps involved

- Strategies to recovery and purify bio-products



The choice of recovery process is based on the following criteria:

1. The intracellular or extracellular location of the product.
2. The concentration of the product in the fermentation broth.
3. The physical and chemical properties of the desired product (as an aid to select separation procedures).
4. The intended use of the product.
5. The minimal acceptable standard of purity.
6. The magnitude of biohazard of the product or broth.
7. The impurities in the fermenter broth.
8. The marketable price for the product.

Supernatants are recovered organelle factions of the cell produced after crystallization, precipitation, centrifugation, or other processes that separate cellular extracts. It is also called the supernate.

The supernatant is typically free of precipitate, translucent, and of lower density than the precipitate, which typically settles in its layer or pellet within the solution. The process by which the supernatant separates from the precipitate is called decantation.

The supernatant is formed through several sequential steps in any given separation reaction; it typically becomes less complex as the procedure for separation progresses.

Cell Disruption

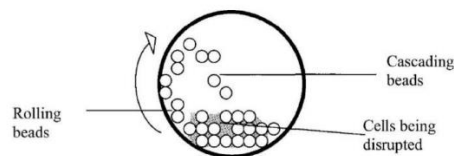
Disruption: the cell envelope is physically broken, releasing all intracellular components into the surrounding medium. The disruption of cells is necessary for the extraction and retrieval of the desired products, as cell disruption significantly enhances the recovery of biological products. Cell disruption cannot be considered an isolated process, as it affects the physical properties of the cell slurry.

Methods: mechanical and non mechanical

Mechanical physical methods

The main principle of the mechanical disruption methods, is that the cells are being subjected to high stress via pressure, abrasion with rapid agitation with beads, or ultrasound.

The main principle requires a jacketed grinding chamber with a rotating shaft, running in its center. Agitators are fitted with the shaft, and provide kinetic energy to the small beads that are present in the chamber. That makes the beads collide with each other. The choice of bead size and weight is greatly dependent on the type of cells. The diameter can affect the efficiency of cell disruption in relation of the location of the desired enzyme in the cell. The increased number of beads increases the degree of disruption, due to the increased bead-to-bead interaction. The increased number of beads, however, also affects the heating and power consumption. Main issues related to bead mills, are the high temperature rises with increase of bead volume, poor scale-up, and most importantly, there is a high chance of contamination.

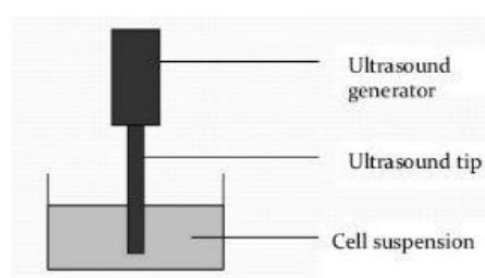


$$\ln \left(\frac{R_m}{R_m - R} \right) = kt$$

K is a function of the rate of agitation (1500-2250 rpm), cell concentration (30-60% wet solids), beads diameter (0.2-1.0 mm), and temperature. Where R - protein released (kg protein/kg biomass), R_m - max protein available, k -rate constant and is a function of temperature.

Ultrasound

Ultrasonic disruption is caused by ultrasonic vibrators that produce a high frequency sound with a wave density of about 20 kHz/s. A transducer then converts the waves into mechanical oscillations through a titanium probe, which is immersed into the cell suspension. Such a method is used for both bacterial and fungal cell disruption. Bacterial cell can be disrupted in 30 to 60 sec, and yeast between 2 and 10min. This method is usually used in combination with a chemical method.



Blenders

- The use of blenders which employ high speed can be used to disrupt cell walls.
- It is the same process used by centrifugation, which separates or concentrates materials suspended in a liquid medium.

Homogenization

- Liquid-based homogenization is the most widely used cell disruption technique for small volumes and cultured cells.
- Cells are lysed by forcing the cell or tissue suspension through a narrow space
- Homogenizers use shearing forces on the cell similar to the bead method.
- Homogenization can be performed by squeezing cells through a tube that is slightly smaller than beads beating.

Non Mechanical Cell Disruption

a)physical methods

1. Freeze-Thaw

- It is suitable when working with soft plant material and algae.
- Disruption is achieved via a series of freezing and thawing cycles.
- Freezing forms ice crystals, which expand upon thawing, and this ultimately causes the cell wall to rupture.

2. Microwave/ Thermolysis

- Microwave (along with autoclave and other high temperature methods) are used to disrupt the bonds within cell walls, and also to denature proteins.
- However, uncontrolled amount of heat can easily denature or damage target proteins and substances.

3. Osmotic Shock

- Through the process of osmosis, water can be moved into the cell causing its volume to increase to the point that it bursts.
- The method however, can only work with animal cells and protozoa, since they do not have cell walls.

4. Electric Discharges

- It is also possible to achieve cell disruption via electrical discharges in mammalian and other cells that are bounded by plasma membranes only.

b)chemical methods

- They are often used with plant cells (and sometimes in combination with shearing).
- Organic solvents such as toluene, ether, benzene, methanol, surfactants, and phenyl ethyl alcohol DMSO can be used to permeate cell walls.
- EDTA can be used specifically to disrupt the cell walls of gram negative bacteria, whose cell walls contain lipopolysaccharides that are stabilized by cations like Mg^{2+} and Ca^{2+} .

- EDTA will chelate the cations leaving holes in the cell walls.

c)Enzymatic methods

- Another strategy to achieve cell lysis is to use digestive enzymes which will decompose the microbial cell wall.
- Different cell types and strains have different kind of cell walls and membranes, and thus the used enzyme depends on microbe. For example, lysozyme is commonly used enzyme to digest cell wall of gram positive bacteria. Lysozyme hydrolyzes β -1-4-glucosidic bonds in the peptidoglycan.
- The cell wall of yeast and fungi differs significantly from the cell wall bacteria. One commonly used enzyme mixture for degradation of cell wall of yeast and fungi is Zymolyase.

Osmotic shock

Osmosis is the transport of water molecules from high- to a low-concentration region when these two phases are separated by a selective membrane.

Separation

- The separation of a component from a liquid mixture by treatment with solvent in which the desired component is preferentially soluble is known as liquid-liquid extraction.
- Polar liquids mix with each other and dissolve salts and other polar solids.
- The solvents for nonpolar compounds are liquids of low or nil polarity.
- The dielectric constant is a measure of the degree of molar polarization of a compound.

Liquid-Liquid Extraction (LLE),

It is also known as solvent extraction, is a process where a solute (or solutes) is transferred from one liquid phase to another. This transfer is driven by the difference in solute solubility between the two liquid phases. The two liquid phases are generally immiscible or partially miscible. One phase is typically water-based (the aqueous phase), while the other is an organic phase.

The partition coefficient is a measure of how a solute distributes itself between the two phases. It is defined as the ratio of the concentration of the solute in the organic phase to its concentration in the aqueous phase at equilibrium. The higher the partition coefficient, the greater the preference of the solute for the organic phase.

$$K_D = Y_L / X_H$$

Y_L and X_H are concentrations of the solute in light and heavy phases, respectively.

Precipitation

Precipitation may be conducted at various stages of the product recovery process. It is a particularly useful process in that it allows enrichment and concentration in one step, thereby reducing the volume of material for further processing.

Reduce the product solubility in the fermentation broth by adding

chemicals. Applicable: separate proteins or antibiotics from fermentation broth.

Methods:

- salting-out by adding inorganic salts such as ammonium sulfate, or sodium sulfate to increase high ionic strength (factors: pH, temperature). The solubility of hemoglobin is reduced with increased amount of ammonium sulfate.
- added salts interact more strongly with water so that the proteins precipitate.
- inexpensive
- Isoelectric (IE) precipitation: Precipitate a protein at its isoelectric point.

Adsorption

The biological products of fermentation can be concentrated by using solid adsorbent particles. In the early days, activated charcoal was used as the adsorbent material. In recent years, cellulose-based adsorbents are employed for protein concentration. And for concentration of low molecular weight compounds (vitamins, antibiotics, peptides) polystyrene, methacrylate and acrylate based matrices are used. The process of adsorption can be carried out by making a bed of adsorbent column and passing the culture broth through it. The desired product, held by the adsorbent, can be eluted.

Purification by Chromatography

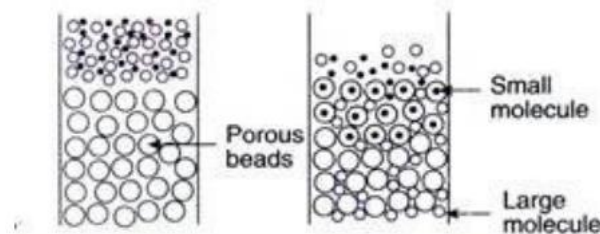
The biological products of fermentation (proteins, pharmaceuticals, diagnostic compounds and research materials) are very effectively purified by chromatography.

- Chromatography is basically an analytical technique dealing with the separation of closely related compounds from a mixture. Chromatography usually consists of a stationary phase and mobile phase.
- The stationary phase is the porous solid matrix packed in a column (equilibrated with a suitable solvent) on to which the mixture of compounds to be separated is loaded. The compounds are eluted by a mobile phase.
- A single mobile phase may be used continuously or it may be changed appropriately to facilitate the release of desired compounds. The eluate from the column can be monitored continuously (e.g. protein elution can be monitored by ultraviolet absorption at 280 nm), and collected in fractions of definite volumes

Gel-filtration chromatography:

This is also referred to as size-exclusion chromatography. In this technique, the separation of molecules is based on the size, shape and molecular weight. The sponge-like gel beads with pores serve as molecular sieves for separation of smaller and bigger molecules. A solution mixture containing molecules of different sizes (e.g. different proteins) is applied to the column and eluted. The smaller molecules enter the gel beads through their pores and get trapped. On the other hand, the larger molecules cannot pass through the pores and therefore come out first with the mobile

liquid as shown below. At the industrial scale, gel-filtration is particularly useful to remove salts and low molecular weight compounds from high molecular weight products.



Ion-exchange chromatography:

It involves the separation of molecules based on their surface charges. Ion-exchangers are of two types (cation- exchangers which have negatively charged groups like carboxymethyl and sulfonate, and anion- exchangers with positively charged groups like diethylaminoethyl (DEAE). The most commonly used cation-exchangers are Dowex HCR and Amberlite IR, the anionexchangers are Dowex SAR and Amberlite IRA. In ion-exchange chromatography, the pH of the medium is very crucial, since the net charge varies with pH. In other words, the pH determines the effective charge on both the target molecule and the ion-exchanger. The ionic bound molecules can be eluted from the matrix by changing the pH of the eluant or by increasing the concentration of salt solution. Ion-exchange chromatography is useful for the purification of antibiotics, besides the purification of proteins.

Affinity Chromatography

Affinity chromatography is a 'separation technique with many applications since it is possible to use it for separation and purification of most biological molecules on the basis of their function or chemical structure. This technique depends on the highly specific interactions between pairs of biological materials such as enzyme-substrate, enzyme-inhibitor, antigen-anti-body. The molecule to be purified is specifically adsorbed from, for example, a cell lysate applied to the affinity column by a binding substance (ligand) which is immobilized on an insoluble support (matrix). Eluent is then passed through the column to release the highly purified and concentrated molecule. The ligand is attached to the matrix by physical absorption or chemically by a covalent bond. The pore size and ligand location must be carefully.

Membrane Processes

Membrane processes include two major processes:

A. Ultrafiltration

B. Reverse Osmosis

Both processes utilize semi-permeable membranes to separate molecules of different sizes and therefore act in a similar manner to conventional filters.

Ultrafiltration

Ultrafiltration can be described as a process in which solutes of high molecular weight are retained when the solvent and low molecular weight solutes are forced under hydraulic pressure (around 7 a atmosphere) through a membrane of a very fine pore size. It is therefore used for product concentration and purification.

Reverse Osmosis

Reverse osmosis is a separation process where the solvent molecules are forced by an applied pressure to flow through a semi-permeable membrane in the opposite direction to that dictated by osmotic forces, and hence is termed reverse osmosis.

It is used for the concentration of smaller molecules than is possible by ultrafiltration. Concentration polarization is again a problem and must be controlled by increased turbulence at the membrane surface.

Drying

Drying: Drying is an essential component of product formulation. It basically involves the transfer of heat to a wet product for removal of moisture. Most of the biological products of fermentation are sensitive to heat, and therefore require gentle drying methods. Based on the method of heat transfer, drying devices may be categorized as contact, convection, radiation dryers. These three types of dryers are commercially available.

. Spray drying: Spray drying is used for drying large volumes of liquids. In spray drying, small droplets of liquid containing the product are passed through a nozzle directing it over a stream of hot gas. The water evaporates and the solid particles are left behind.

3. Freeze-drying/ Lyophilization Freeze-drying or lyophilization is the most preferred method for drying and formulation of a wide-range of products—pharmaceuticals, foodstuffs, diagnostics, bacteria, viruses. This is mainly because freeze-drying usually does not cause loss of biological activity of the desired product

Whole broth processing

- It is the recovering concept of the metabolite that is directly from the "**unfiltered fermentation broth**" and it is of in the considerable interest because of the simplicity.
- The **reduction in the "process stages"** and the savings in the potential cost.
- The downstream procession is very necessary for the **recovery of product and the product concentration.**
- The Downstream processing is really important for the **final product's purification** and it is used as a **useful product.**