

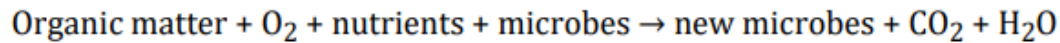
The sludge collection system moves the settled sludge to a common point where it can be pumped back to the aeration tank for further treatment. The volumetric flow of the returned sludge must be accurately measured in order to control the microbial population in the reactor.

Sludge Wasting System (Waste Activated Sludge (WAS))

While the microbes are in the aeration tank they reproduce very quickly, becoming overpopulated (increasing MLVSS concentration), removing the excess microbial population from the system. It is important to maintain a desired microbial population for process control, which is done through sludge wasting. This is linked with the Mean Cell Residence Time (MCRT), which is the amount of time microbes are in the process. The sludge can simply be dewatered if the sludge has already been stabilized through an extended aeration process.

Biochemical Reactions

In the activated sludge process, the BOD and suspended solids are reduced through a variety of biochemical reactions. In the first step, carbonaceous BOD (CBOD) is reduced through a chemoheterotrophic aerobic reaction:

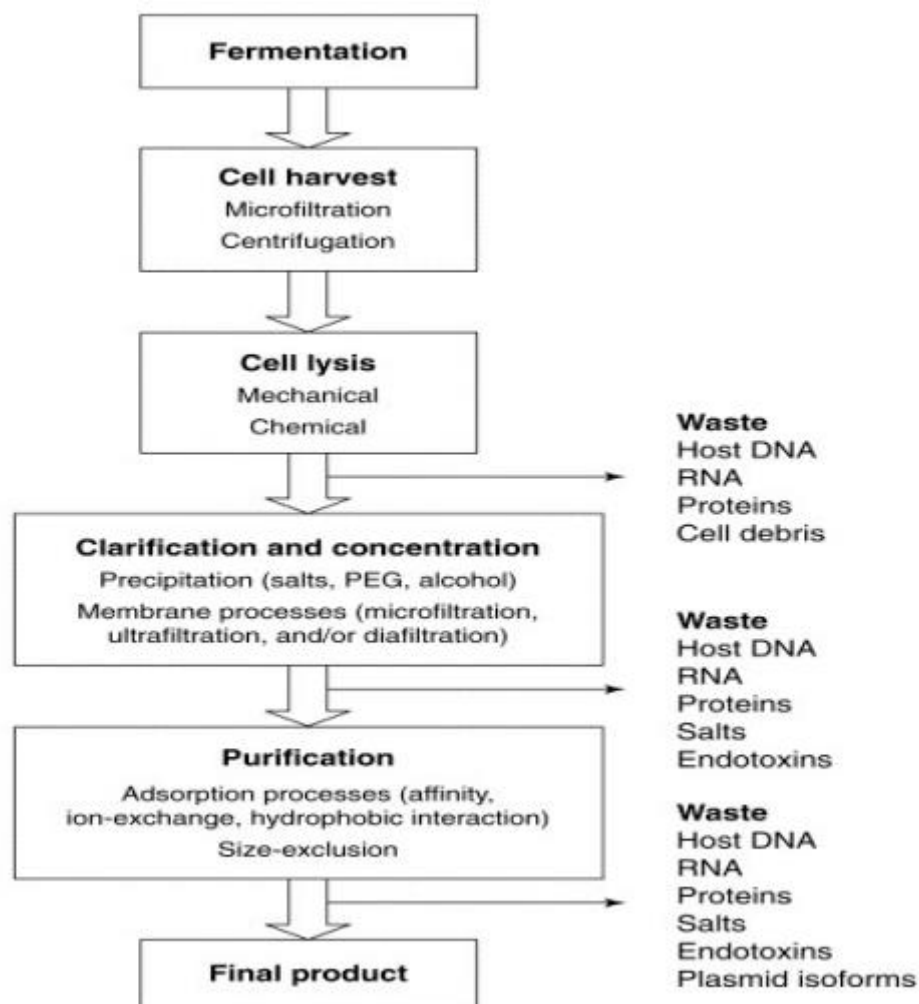


When the organic matter, nutrients and microbes in the aeration basin are given oxygen, it produces new microbes, water and carbon dioxide as a byproduct. These new microbes are either wasted (WAS) or returned (RAS) to do more work in the aeration basin. The second reaction that occurs helps to reduce the nitrogenous BOD (NBOD) in the waste stream. This occurs through a chemoautotrophic aerobic reaction known as nitrification. Nitrification is an aerobic process in which ammonia-oxidizing bacteria, known as Nitrosomonas, change the ammonia (NH_4) and organic nitrogen in the wastewater into oxidized nitrogen, usually nitrate (NO_2).

IV. Separation of bioproducts (Downstream processing)

The extraction and purification of a biological product from the fermentation broth is called as downstream processing (DSP) or product recovery. DSP is quite complex and variable depending upon the type of the product.

Broadly it can be divided into five stages: 1) Cell harvesting 2) Lyses/breakage of cells 3) Concentration 4) Purification 5) Formulation



Definition of Downstream

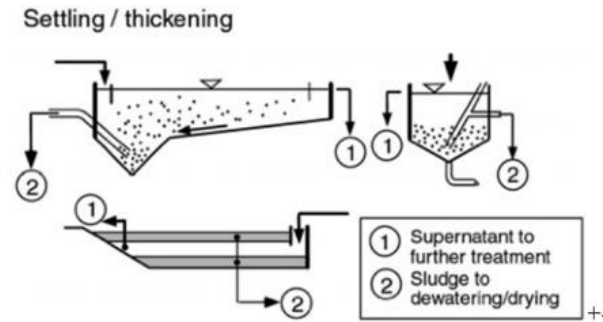
The downstream stage in the production process involves processing the materials collected during the upstream stage into a finished product. The downstream stage further includes the actual sale of that product to other businesses, governments or private individuals. The type of end user will vary depending on the finished product. Regardless of the industry involved, the downstream process has direct contact with customers through the finished product.

Steps of Downstream Processing

Downstream processing is generally considered to be a specialized field in biochemical/chemical engineering. It encompasses harvesting, purification and packaging of a specific industrial product synthesized in marketable quantities. Analytical bio separation refers to the separation or purification of biological products at different scales of operation, done at analytical scale, the purpose of which is to maintain the desired quality of the product. Downstream processing involves four important steps that result in the following progressive improvements in purity and concentration of the desired product:-

1.Cell harvesting or Solid-Liquid Separation:

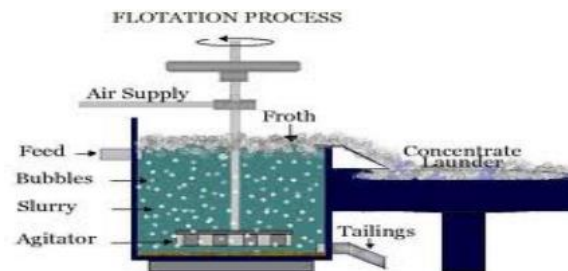
Once the fermentation is complete, the solid phase (i.e. cell bio-mass) is separated from the liquid phase by any of the following methods: Settling, Flotation, Flocculation, Filtration and Centrifugation. Settling: it depends on size and weight; it descends cells down by gravity and uses in alcohol industry and waste treatment.



Different types of settling tanks

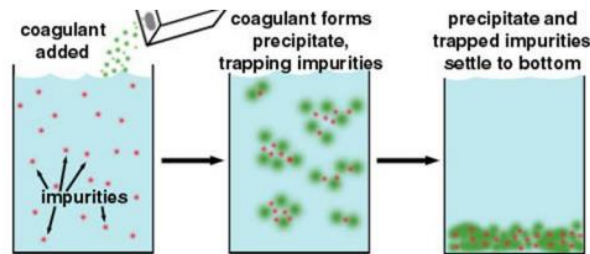
Flotation:

A gas is passed through the fermentation broth. This gas forms tiny bubbles to which cells get adsorbed. These bubbles rise to the surface of the broth and form foam. Sometimes along with the gas, collector substances (fatty acids) are added which facilitates foam fermentation. The foam containing the cells is removed. When a gas is introduced into the liquid broth, it forms bubbles. The cells and other solid particles get adsorbed on gas bubbles. These bubbles rise to the foam layer which can be collected and removed. The presence of certain substances, referred to as collector substances, facilitates stable foam formation e.g., long chain fatty acids, amines.



Flocculation:

At high cell density some cells (yeast cells) aggregate and thus settle down at the bottom of the fermentors. This process can be accelerated by the addition of flocculating agent like salts, organic polyelectrolyte and mineral hydrocolloid. In flocculation, the cells or cell debris form large aggregates to settle down for easy removal. The process of flocculation depends on the nature of cells and the ionic constituents of the medium. Addition of flocculating agents is often necessary to achieve appropriate flocculation.



Conceptual illustration of the flocculation process

Filtration:

Filtration is the most commonly used technique for separating the biomass and culture filtrate. The efficiency of filtration depends on many factors— the size of the organism, presence of other organisms, viscosity of the medium, and temperature. Several filters such as depth filters, absolute filters, rotary drum vacuum filters and membrane filters are in use.

Depth Filters:

They are composed of a filamentous matrix such as glass wool, asbestos or filter paper. The particles are trapped within the matrix and the fluid passes out. Filamentous fungi can be removed by using depth filters.

Absolute Filters:

These filters are with specific pore sizes that are smaller than the particles to be removed. Bacteria from culture medium can be removed by absolute filters.

Rotary Drum Vacuum Filters:

These filters are frequently used for separation of broth containing 10-40% solids (by volume) and particles in the size of 0.5-10 μ m. Rotary drum vacuum filters have been successfully used for filtration of yeast cells and filamentous fungi. The equipment is simple with low power consumption and is easy to operate. The filtration unit consists of a rotating drum partially immersed in a tank of broth. As the drum rotates, it picks up the biomass which gets deposited as a cake on the drum surface. . This filter cake can be easily removed.

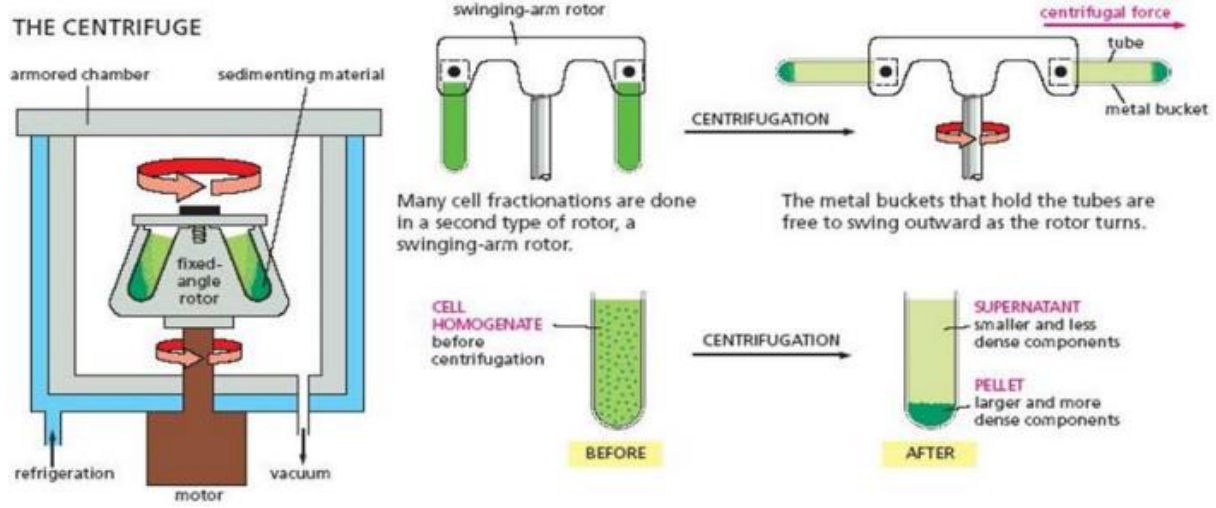
Membrane Filters:

In this type of filtration, membranes with specific pore sizes can be used. However, clogging of filters is a major limitation. There are two types of membrane filtrations—static filtration and cross-flow filtration. In cross-flow filtration, the culture broth is pumped in a crosswise fashion across the membrane. This reduces the clogging process and hence better than the static filtration.

There are 3 major types of filtrations based on the particle sizes and other characters. These are microfiltration, ultrafiltration and reverse osmosis.

Centrifugation:

The technique of centrifugation is based on the principle of density differences between the particles to be separated and the medium. Thus, centrifugation is mostly used for separating solid particles from liquid phase. Unlike the centrifugation that is conveniently carried out in the laboratory scale, there are certain limitations for large scale industrial centrifugation. Centrifuge used to separate bacteria, and can be used efficiently in the case of significant differences in density between the solid particles and liquid portion.



Centrifugation process

Tubular bowl centrifuge:

This is a simple and a small centrifuge, commonly used in pilot plants. Tubular bowl centrifuge can be operated at a high centrifugal speed, and can be run in both batch and continuous mode. The solids are removed manually.

Disc centrifuge:

It consists of several discs that separate the bowl into settling zones. The feed/slurry is fed through a central tube. The clarified fluid moves upwards while the solids settle at the lower surface.

Multi-chamber centrifuge:

This is basically a modification of tubular bowl type of centrifuge. It consists of several chambers connected in such a way that the feed flows in a zigzag fashion. There is a variation in the centrifugal force in different chambers. The force is much higher in the periphery chambers, as a result smallest particles settle down in the outermost chamber.

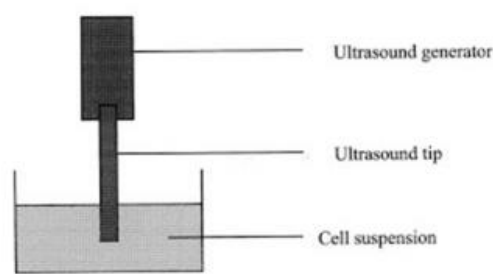
2-Lyses/breakage of cells

If the desired product is located inside the cell, the cells are first recovered from the fermentor by any of the cell harvesting methods.

Methods that used to break the cells are:

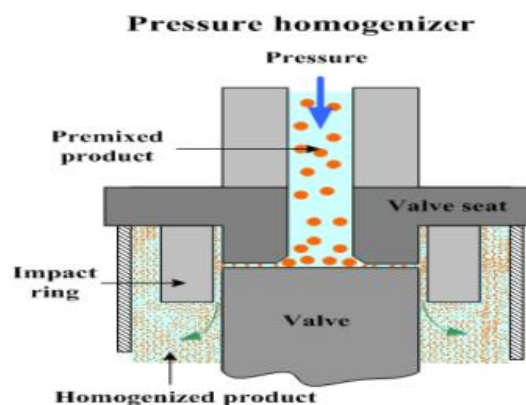
- Physical methods:

Ultrasonication: The cells are disrupted by passing ultra-waves through samples. This technique is ideal in laboratory where sample size is small.

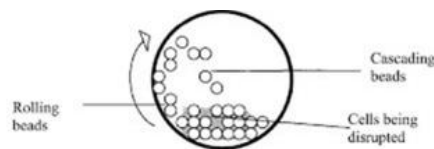


Cell disruption by sonication

2. Osmotic shock: The cells are suspended in a viscous solution like 20% (w/v) sucrose or glucose. The cell suspension is then transferred to the cold water (4°C) which results in cell lyses.
3. Heat shock (Thermolysis): The cells are exposed to heat which results in disintegration of the cells. It is an economical method but the product has to be heat stable.
4. Freeze-Thaw method: It is commonly used to lyse bacterial and mammalian cells. The technique involves freezing a cell suspension in a dry ice/ethanol bath or freezer and then thawing the material at room temperature or 37°C. This method of lysis causes cells to swell and ultimately break as ice crystals form during the freezing process and then contract during thawing.
5. High pressure homogenization: The cell suspension is forced to pass through a narrow pore at a high pressure which results in breakage of cells



6. Grinding with glass beads: The cell suspension containing glass beads is subjected to a very high speed in a vessel. The cells break as they are forced against the walls of the vessel by the beads.



Cell disruption by Bead Milling

Chemical methods:

1. Detergents: disrupt the structure of cell membranes by solubilizing their phospholipids disrupting lipid:lipid, lipid:protein and protein:protein interactions. These chemicals are mainly used to rupture mammalian cells. For disrupting bacterial cells, detergents have to be used in conjunction with lysozyme. With fungal cells (i.e. yeast and mould) the cell walls have to be similarly weakened before detergents can act.

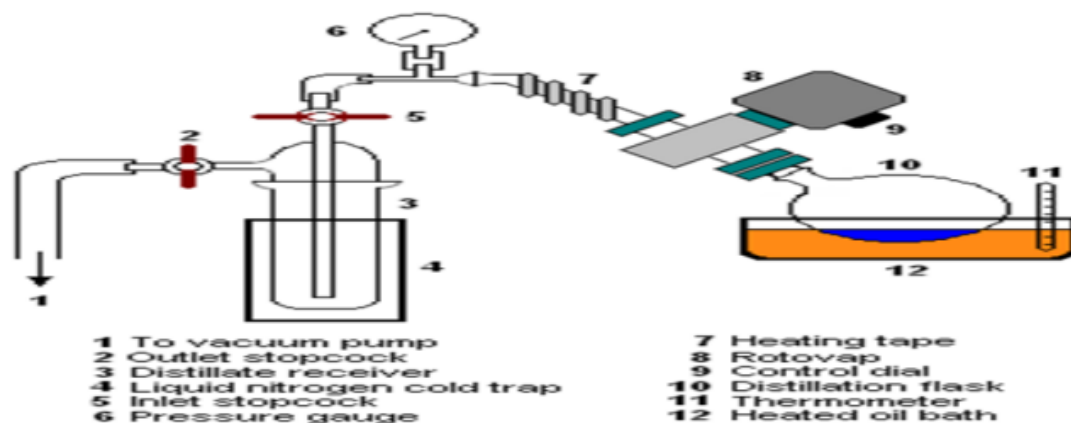
2. Organic solvents: mainly act on the cell membrane by solubilizing its phospholipids and by denaturing its proteins. Some solvents like toluene are known to disrupt fungal cell walls. Others are ether, phenylethly alcohol, DMSO, benzene, Methanol, chloroform etc.

3- Acid/Alkali treatment: It is the easiest and least expensive method available in general lab. The method is fast, reliable and relatively clean way to isolates DNA from cells. It can be used for both laboratory and industrial scale.

4- Enzymatic lysis: The cells are broken with the help of enzymes. Bacterial cells are lysed by the addition of lysozyme. This enzyme hydrolyses β -1,4 glycosidic linkage of peptidoglycan layer of bacterial cell wall. Fungal cells are lysed by the addition of chitnases, cellulases and mannases.

3- Concentration

More than 90% of the cell free supernatant is water and the amount of desired product is very less. Methods of concentration are: a- Evaporation process: concentrates the requisite product. Water is evaporated by applying heat to the supernatant with/without vacuum. The amount of heat applied is decided by the heat stability of the product.



Rotary evaporation laboratory setup

b- Liquid-liquid extraction: A desired product (solute) can be concentrated by the transfer of the solute from one liquid to another liquid. This process also results in partial purification of the product. Liquid-liquid extraction is basically of two types:

- 1- Extraction of low molecular weight products: This is primarily applied for extraction of lipid soluble compounds.

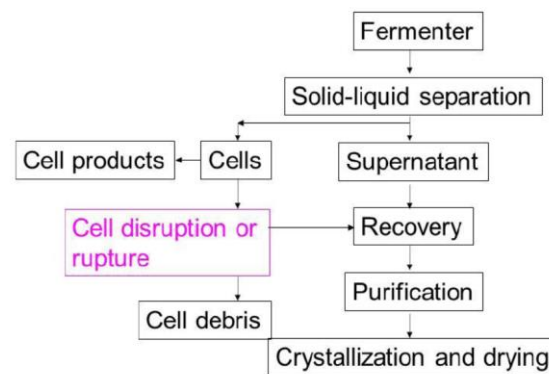
- 2- Extraction of high molecular weight: This technique is applied to compounds like proteins, which get inactivated in the presence of organic solvents like ethanol, methanol, chloroform, hexane etc. The solubility of the desired product is dependent on various properties of the protein e.g. ionic 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

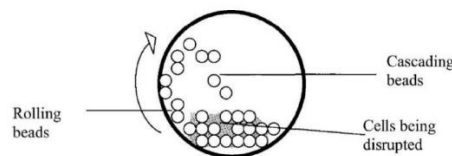
Disruption: the cell envelope is physically broken, releasing all intracellular components into the surrounding medium. The disruption of cells is necessary for the extraction and retrieval of the desired products, as cell disruption significantly enhances the recovery of biological products. Cell disruption 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.