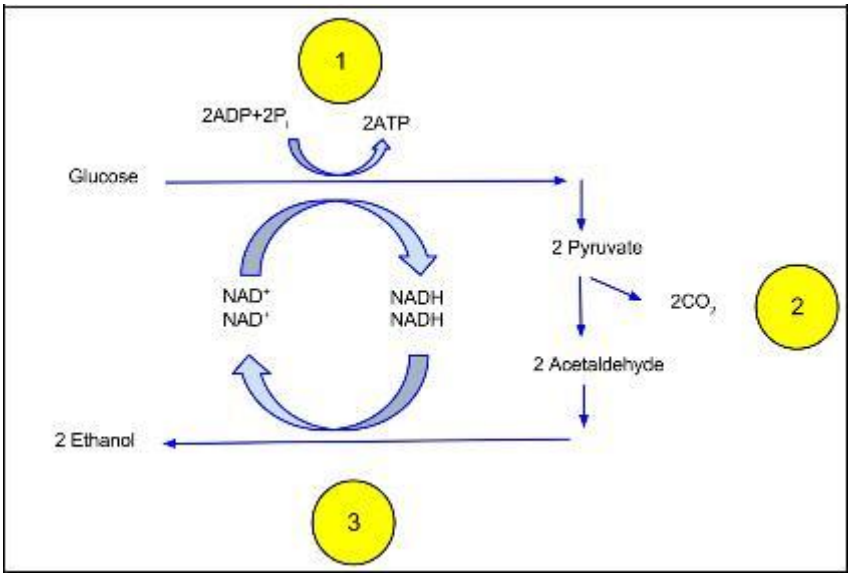
Fermentation:

The conventional definition of fermentation is the breakdown (metabolism) of the larger molecules, for example carbohydrates, into simpler ones under the influence of microorganisms for their enzymes. In a microbiological way, fermentation is defined as, any process for the production of useful products through culture of microorganisms, whereas in a biochemical sense, this would mean the numerous oxidation-reduction reactions in which organic compounds used as source of carbon and energy, act as acceptors or donors of hydrogen ions.



Although, fermentation was implied for example for brewing and wine production from ancient times and during the end of fifteenth century, brewing became partially industrialized in Britain. In the 19th century Cagniard-Latour and Schwan reported that the fermentation of wine and beer is accomplished by yeast cells. Finally it was Pasteur who observed that microorganism are associated with fermentation and are causing many diseases in human beings.

At present, there are more than one million microorganisms known to be present in nature out of which about a few hundred species synthesize the useful products some of these important microorganisms are enumerated as;

Algae: *Chlorella sorokiniara*, *Spiruline maxima.*

Fungi: *Aspergillus,Fusarium,Moniliforme,Gibberella,pencilliumchrysogenium, P.n otatum,Rhizopus,T.reesei* etc.

Actinomycetes: *Micromonospora purpurea,Nocardia mediterrnei* etc.

Bacteria: *Clostridium acticum, Pseudomonas denitrificans, Bacillus substilis, Bacillus polymyxa, Acetobacter lacti, Acetobacter woodi* etc.

Factors that influence fermentation:

It is essential to maintain optimal growth environment in the reaction vessel for maximum product formation. Maximal efficiency of the formation can be achieved by continuously monitoring the variables such as the pH, temp, dissolved oxygen, adequate mixing nutrient concentration, and foam formation. Improved sensors are now available for continuous and automated monitoring of these variables (i.e., on line measurement of pH)

1. pH: Most of the micro organisms employed in fermentation grow optimally between pH 5.5 and 8.5 in the bioreactor, as the microorganisms grow, they release metabolites into the medium which change pH. Therefore, the pH of the medium should be continuously monitored and maintained at the optimal level. This can be done by the addition of acid or alkaline base (as needed) and a thorough mixing of fermentation contents (sometimes an acid or alkaline medium components can be used to correct pH, besides providing nutrients to the growing microorganism.)

2.Temperature: Temperature control is absolutely essential for a good fermentation process. Lower temperature causes reduced product formation while higher temperature adversely affects the growth of microorganisms. The bioreactors are normally equipped with heating and cooling systems that can be used as per the requirement, to maintain the reaction vessel at optimum temp.

3. Dissolved O_2: O_2 is sparingly soluble in H_2O(0.0084 g/l at 25C). Continuous supply of O_2 in the form of sterilized air is carried out by introducing air into the bioreactor in the form of bubbles. Continuous monitoring of dissolved O_2 conc. is done in the bioreactor for optimal product formation.

4. **Adequate mixing:** Continuous and adequate mixing of the microbial cultures ensure optimal supply of nutrients and O_2, besides preventing the accumulation of toxic metabolic byproducts. Good mixing (by agitation) also

create favorable environment for optimal and homogenous growth environment and good product formation. However excessive agitation may damage microbial cells and increase the temperature of the medium, besides increased foam formation.

5. Nutrient concentration: The nutrient conc. in a bioreactor is limited so that its wastage is prevented. In addition limiting conc. of nutrients may be advantageous for optimal product formation, since high nutrient conc. are often associated with inhibitory effect on microbial growth. It is now possible to do on line monitoring of the nutrient conc. and suitably modify as per the requirement.

6. Foam formation: The media used in industrial fermentation is generally rich in proteins. When agitated during aeration, it invariably result in froth or foam formation that builds in head space of the bioreactor. Antifoam chemicals are used to lower surface tension of the medium, besides causing foam bubbles to collapse. Mineral oils based on silicone or vegetable oil are commonly used as antifoam agents.

Fermentation media composition:

The media composition is critical to product yields as high producing strains of microorganisms. The medium not only provide the nutrient needed for microbial growth but also for the metabolite production. The organisms vary greatly in their nutrient requirements from autotrophs which produce all the biochemicals required from simple inorganic nutrients deriving their energy from oxidation of some inorganic components of the medium to the difficult organisms like lactic acid bacteria which require many organic compounds for their growth. The various media may be grouped into two broad categories. A Synthetic or chemically defined medium is desirable for various studies, but product yield from such media are generally low. Foaming is not a problem with such media. The complex media contain undefined constituents like soybean meal, molasses, corn steep liquor etc and give much higher yields of metabolites. Carbon source can be simple e.g., sugar, alcohol etc. In many processes, precursors need to be provided e.g., phenylacetic acid for penicillin G, inorganic cobalt for vit. B_{12}. Buffers are also added to prevent drastic

changes in pH and an antifoam would often be needed when complex media is used. For many fermentations e.g., antibiotic production, medium suited for rapid cell growth is unsuitable for product formation .In such cases specialized media for production have to be devised. Some of the important microbial cultures are described below:

1. Batch culture: In this culture, microorganisms are grown in a vessel which is termed fermenter or bioreactor and is regarded to be the simplest culture. In the fermenter, biological reactions are carried out in a controlled manner. For large scale production fermenters, certain measures are necessary for maximizing the culture. Some of these consideration are as such:

a) Proper aeration and agitation.
b) pH control system.
c) Sampling facility.
d) Temperature control system.
e) Minimum evaporation losses from fermenter.

Batch culture shows different phases of growing stages. A microbe grows in a medium until the nutrients are exhausted or toxic metabolites secreted by it reach to an inhibitory level. After inoculation, the microbe takes some time to adjust in the new environment according to size of fermenter and hence does not grow in the medium. Thus, the time taken for adaption before it comes to its active growth is known as Lag phase. The microorganisms grow luxuriantly till nutrients are present. Therefore, nutrients dependent logarithmic or exponential active growth and thereby increase in biomass is represented as Log or exponential phase. As soon as the levels of nutrients decrease growth of culture gradually slow down. This stage of retardation the growth to reach to a stationary phase is known as deceleration phase.

However, during stationary phase microorganisms do not grow and thus fail to increase the biomass and ultimately the no. of microbial cells decline due to accumulation of toxic metabolites. This stage is known as death phase. At the end the amount of biomass depends upon the nutritional components and ability of microorganisms to utilize the substrate and convert into biomass. This type of growth is known as Sigmoidal growth curve.

2. Fed-batch culture: In this culture, it is principally a batch culture but the

difference is that, the culture is fed continuously with fresh medium without removal of the original culture medium from the fermenter. Therefore, it results in continuous increase in volume of medium in the fermenter.

3. Continuous culture: A continuous culture is that where a steady exponential phase for growth of culture retard due to depletion of nutrients rather than by accumulation of toxic products. Accumulation of toxicity is prevented by addition of fresh medium to the fermenter and removal of spent medium and microbial biomass from it as a result of which the exponential phase of culture is prolonged.

Sterilization:

Sterilization is the process of inactivating or removing all living organisms from a substance or surface. In concept, it is regarded as absolute in that all living cells must be inactivated/removed, usually in a single step at the given time. But in practice, the success of sterilization procedures is only a probability. Therefore, the probability of a cell escaping inactivation/filtration does exist although it is very small. When a closed system is sterilized once, it remains so indefinitely since it has no openings for the entry of microorganisms. But most fermentation vessels are open systems, such systems are initially sterilized and must be kept sterilized by ensuring the removal of living cells at their entry points, e.g., the cotton plug of a culture flask. Sterilization may be achieved by
(i) heating
(ii) irradiation,
(iii) chemicals or
(iv) filtration.

Heating: It is the most commonly used and the least expensive sterilizing agents. Dry heat is used in ovens and is suitable for sterilization of solids which can withstand the high temperatures needed for sterilization e.g., lab glass wares etc. Steam i.e., moist or wet heat, is used for sterilization of media and fermenter vessels. An autoclave uses steam for sterilization (at 121^{o}c and 15 p.s.i), the period of time at this temp-pressure depending on medium volume, e.g., 12-15 min for 200ml, 17-22 min for 500ml, 20-25 min for 1 lit. But sterilization of oil require a few hours, and concentrated Media (10-20% solid)

must be agitated for effective sterilization. Autoclaves can also be used to sterilize laboratory vessels, small volumes of media and even small fermenters. Large fermenters are sterilized by either a direct injection of steam or by indirect heating by passing steam through heat exchange coils or a jacket. The steam should always be saturated. Media sterilization may be achieved in a continuous flow sterilization system either by direct steam injection or by indirect steam heating and then filled in a sterile fermenter. Alternatively, the medium may be filled in the fermenter and steam-sterilized with the latter, in such a case ,the medium volume would increase by 10-20% for which provision must be made.

Chemicals: The chemicals used for sterilization cause inactivation by oxidation or alkylation; these are formaldehyde,H_2O_2, ethylene oxide, propylene oxide, etc. H_2O_2 (10-20%w/v) is being increasingly used in the sterilization of milk and of containers for food products. It is a powerful oxidizing agents, kills both vegetative cells and spores, and is very safe. Ethylene oxide is used for sterilizing equipments, which are likely to be damaged by heat and is very effective, but it is highly toxic and violently explosive if mixed with air.

Radiation: High energy X-rays are used for sterilization of a variety of labware and of food. In general, vegetative cells are much more susceptible than bacterial spores (Clostridium spores can resist nearly 0.5M rad). But Deinococcus radiodurans vegetative cells can survive 6Mrad. Viruses are usually similar to bacterial spores, but some viruses, e.g., encephalitis virus, require upto 4.5 M rad for inactivation. In practice, 2.5M rad is used for sterilizing pharmaceutical and medical products. X-rays cause inactivation by inducing single or double strand DNA breaks, and by producing free radicals and peroxides to which –SH enzymes are particularly susceptible.

Filtration: Aerobic fermentation requires a very high rate of air supply often equaling 1 volume of air (equal to medium volume)every minute. It contains both fungal and bacteria, which are ordinarily removed by filtration using either a depth or a screen filter. Depth filters are made from fibrous or powdered materials pressed or bonded together in a relatively thick layer, the material used are fiberglass, cotton, mineral wool, cellulose fibers, etc in form

of mats, wads or cylinder. Modern depth filters are cylinders of bonded borosilicate microfibers. Depth filters allow higher filtration rates and efficiencies than do screen filters, they are not suitable for filtration of moist air.

Screen filters are membranes of cellulose esters or other polymers with pores of 0.45µm or smaller (bacterial contaminants are 0.5 µm or larger). Usually, a microfiber is used along with such filters in order to remove gross contamination. All filters themselves must be sterilized before they can be used to sterilize the air. Filters are also used to sterilize the effluent gases from fermenters, especially in case of pathogenic microorganisms.

Bioreactors:

The heart of fermentation (or bioprocessing) technology is the fermenter (or bioreactor). A bioreactor is basically a device in which the organisms cells are cultivated and motivated to form the desired products.

It is a system designated to give right environment for optimal growth and metabolic activity of the organism.

Traditional fermenters are made up of wood or slate. In recent years , stainless steel bioreactors are in use. A high quality stainless steel does not corrode or leak toxic metals into the growth medium. The size of the bioreactor is highly variable ranging from 20 liters to 250 million liters or even more.

Basic function of bioreactors:

The main function of fermenter is top provide a controlled environment for growth of a micro organism or a defined mixture of micro organisms, to obtain a desired product while bio reactor referred to production unit of mammalian cell cultures.

The vessel should be capable of being operated aseptically for a number of days and should be reliable for long term operation. The adequate aeration and agitation should be provided to meet the metabolic requirements of the microbes. However the mixing should not damage the micro organisms. The power consumption should be low and temperature and ph control system

should be provided. The evaporation losses from the fermenter should not be excessive. The vessel should be designed to require the minimal use of labor in operation, harvesting, cleansing and maintenance. It should have proper sampling facility. The vessel should be constructed to infuse instead of flange joints. The cheapest and the best material should be used and there should be adequate service provision for individual plants.

Types of Bioreactors.

Based on design of the bioreactors they can be grouped into the following types:

Continuous stirred tank bioreactor: A continuous stirred tank bioreactor consists of a cylindrical vessel with motor driver central shaft that supports one or more agitator(impeller).The shaft is fitted at the bottom of the bioreactor. The no. of impeller is variable and depends on the size of the bioreactor. Different types of impellers (concave bladed, marine propeller etc.,) are in use.

In stirred tank bioreactor ,the air is added to the culture medium under pressure through a device called sparger. The sparger may be the ring with many holes or a tube with single orifice. The sparger along with impeller(agitators) enable better gas distribution system throughout the vessel. The bubbles generated by sparger are broken down to small ones by impellers and dispersed throughout the medium. This enables the creation of a uniform and homogenous environment throughout the bioreactor.

Bubble column bioreactor: In the bubble column bioreactor the air or gas is introduced at the base of the column through perforated pipes or plates, or metal microporous spargers. The flow rate of the air/gas influences the performance factors-O_2 transfer ,mixing .The bubble column bioreactor may be fitted with perforated plates to improve performance. The vessel used for bubble column bioreactors is usually cylindrical with an aspect ratio of 4-6 (i.e., high to diameter ratio)

Airlift bioreactors: Also known as tower reactor, an airlift bioreactor can be described as a bubble column containing a drought tube .Many types of airlift bioreactors are currently in use today. Air is typically fed through a sparger ring into the bottom of a central drought tube that controls the circulation of air and medium. Air flows up the tube forming bubbles and exhaust gas leaves at

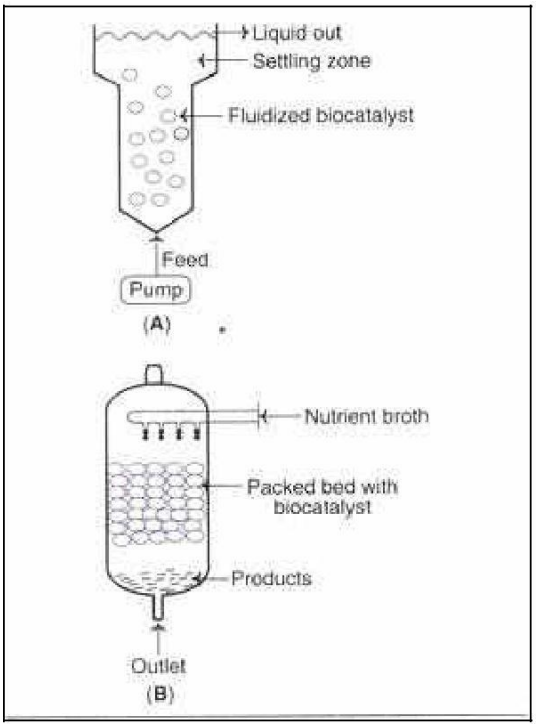
the top of the column. The degassed liquid then flows downward and the product is drained from the tank. There are two types

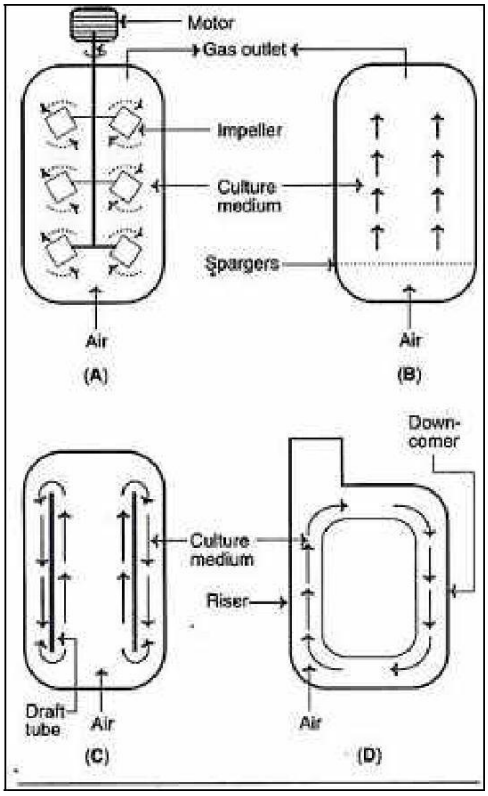
a.) Internal loop airlift bioreactor: Has a single container with a central draft tube that creates interior liquid circulation channels.

b.) External-loop airlift bioreactor: This system consists of a riser and an external down-comer, which are connected at the bottom and the top respectively. As the injected air at the bottom of the riser creates gas bubbles that begin to rise through the main tank, exhausted gas leaves at the top and the resulting heavier solution descends through the down comer.

4. Packed bed bioreactors: A bed of solid particles with biocatalysts on or within the matrix of solids, packed in a column constitute a packed bed bioreactor. The solid may be porous or non porous gels, and may be compressible or rigid in nature. A nutrient broth flows continuously over the immobilized biocatalyst. The product obtained in the packet bed bioreactor are released into the fluid and removed .While the flow of the fluid can be upward or downwards, downflow under gravity is preferred.

5. Photobioreactors: These are the reactor specialized for fermentation and can be carried out either by exposing to sunlight or artificial illumination. Since artificial illumination is expensive, only the outdoor photo-bioreactor are preferred. Certain important compounds are produced by employing photo-bioreactors e.g., B carotene, asthaxanthin. The photoreactors are made up of glass or more commonly transparent plastic. The array of tubes or flat panels constitute light receiving system (solar receiver).The culture can be circulated through the solar receiver by methods such as using centrifugal pumps or airlift pump. It is essential that the cells are continuous circulation without forming sediments. Further adequate penetration of sunlight should be maintained. The tubes should also be cooled to prevent rise in temp. Photobioreactors are usually operated in a continuous mode at a temp in the range 25-40^{o}C. Microalgae and cynobacteria are normally used. The organisms grow during day light while the products are produced during night.





Construction of fermenters:

The criteria considered before selecting materials for construction of a fermenter are (a) the material that have no effect on sterilisation, and (b) its smooth internal finish-discouraging lodging of contamination. The internal surface should be corrosion resistant.

There are two of such materials (i) glass which are used in fermenter. According to American iron and steel institute(AISI), if a steel contains 4% chromium , it is called stainless. The long and continuous use of stainless steel sometimes shows pitting. It is also important to consider the materials used for aseptic seal. Sometimes it is made between glass and glass, glass and metal and metal joints between a vessel and detachable top or base plate. On pilot scale, any material to be used will have to be assessed on their ability to with stand pressure, sterilization, corrosion and their potential toxicity and cost.

(I) **Control of temperature**: Since heat is produced by microbial activity and mechanical agitation, then it is sometimes necessary to remove it. On the other hand, in certain processes extra heat is produced by using thermostatically controlled water bath or by using internal heating coil or

jacket meant for water circulation.

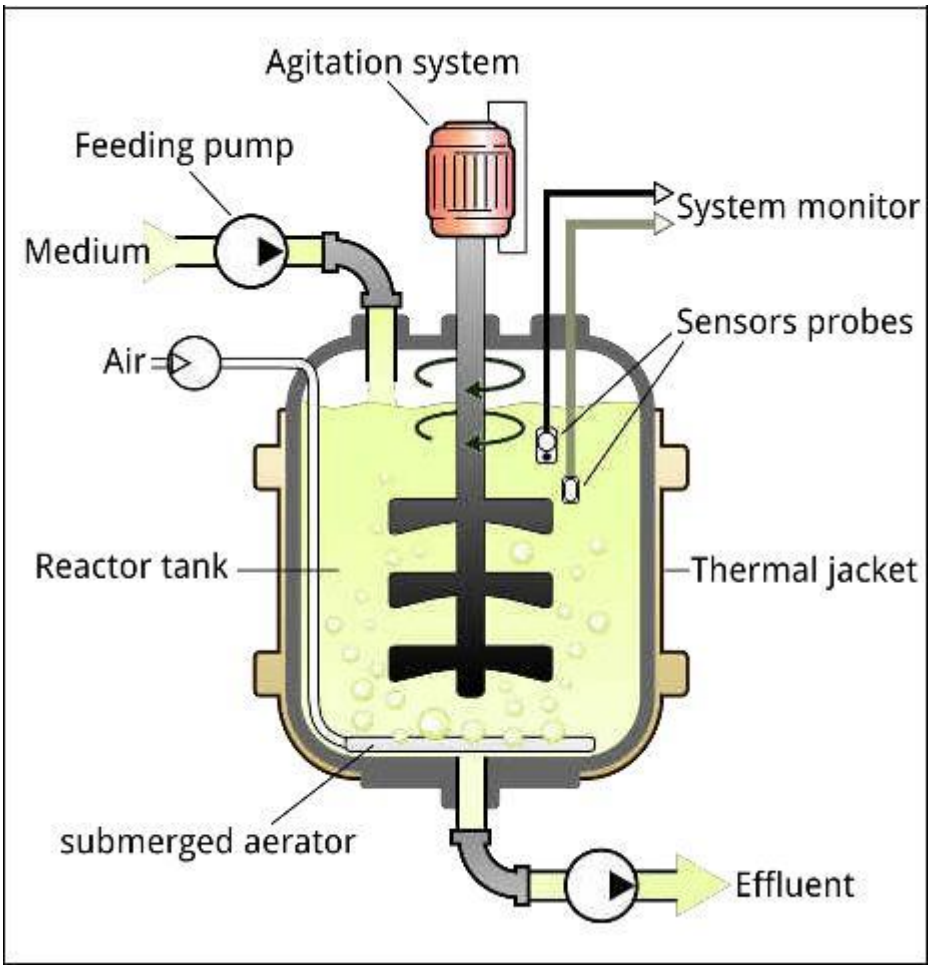
(ii) **Aeration and agitation.** The main purpose of aeration and agitation is to provide oxygen required to the metabolism of microorganisms. The agitation should ensure a uniform suspension of microbial cell suspended in nutrient medium. There are following necessary requirements for this purpose (a) the agitator (impeller)for mixing (b) stirrer glands bearing meant for aseptic sealing, (c) baffles for checking the vortex resulting into foaming (d) the sparger (aeration) meant for introducing air into the liquid.

a) **The agitator (Impeller):**The size and position of the impeller in the vessel depends upon the size of the fermenter. In tall vessels, more than one impeller is needed if adequate aeration agitation is to be obtained. Ideally, the impeller should be 1/3 or ½ of the vessel diameter above the base of the vessel. The number of impeller may vary from size to size to the vessel.

b) **Stirrer glands and bearing**: Four basic types of seals assembly have been used. The packed gland seal, the simple bush seal, the mechanical seal and the magnetic drive.

c)**The baffles** are normally incorporated into agitated vessel of all sizes to prevent a vortex and to improve aeration efficiency. They are metal strips roughly one-tenth of the vessels diameter and attached radially to the walls.

d) Sparger: A sparger may be defined as a device for introducing air into the liquid in a fermenter. It is important to know whether sparger is to be used on its own or with agitation as it can influence equipment design to determine initial bubble size. Three basic types of sparger have been used and may be described as the porous sparger, the orifice sparger and the nozzle sparger.



Bioreactor

Design and operation.

These are designed to provide support to the best possible growth and biosynthesis for industrially important culture, and to allow ease of manipulation for all operations associated with the use of the fermenter. These vessels must be strong enough to resist the pressure of large volume of agitating medium. The product should not corrode the material nor contribute toxicity to the growth medium. This involves a meticulous design of every aspect of the vessel part and other opening accessories in contact, etc.

In fermentations, provisions should be made for the control of the contaminating organisms, for rapid incorporation of sterile air into the medium in such a way that the oxygen of air is dissolved in the medium and therefore, readily available to the microorganisms and CO_2 produced from microbial metabolism is flushed from the medium. Some stirring devices should be

available for mixing the organisms through the medium so as to avail the nutrients and oxygen. The fermenter has a possibility for the intermitted addition of antifoam agent. Some form of temperature control efficient heat transfer system is also there for maintaining a constant predetermined temperature in the fermenter during the growth of organism. The pH should be detected. Other accessories in the fermenter consist of additional inoculums tank or seed tank in which inoculums is produced and then added directly to the fermenter without employing extensive piping which can magnify contamination problems.

Downstream processing:

As the fermentation is complete. It is necessary to recover the desired end product .The end products include antibiotics , amino acids, vitamins, vaccine organic acids, industrial enzymes. The extraction and purification of a biotechnological product from fermentation is referred as downstream processing (DSP) or product recovery. Downstream processing is a complex and important as fermentation process. It often requires the expertise and technical skills of chemists, process engineer, beside bio scientists.

In the present date biotechnology the fermentation and downstream processing are considered as an integral system. The methodology adopted for down steam processing depends on the nature of the end product and on the following criteria

1. The intracellular or extracellular location of the product.
2. The conc. Of the product in the fermentation broth.
3. The physical and chemical properties of the desired product.
4. The magnitude of biohazard of the product or broth
5. The marketable price of the product.
6. The impurities in the fermenter broth.

The desired products for isolation by DSP are most frequently metabolites which may be present as follows:

1. Intracellular metabolites . These products are located within the cells e.g., vitamins, enzymes.

2. Extracellular metabolites: They are present outside the cells (culture fluid)e.g., most antibiotics (penicillin, streptomycin, amino acids, alcohol, citric acid, some enzymes(amylase, protease).

3. Both intracellular and extracellular e.g., vitamin B_{12}, flavomycin:
Sometimes the microorganism may itself be the desired end product for isolation e.g., cell protein.

If the fermentation broth is analyzed at the time of harvesting it will be discovered that the specific product may be present at the low conc. In an aqueous solution that contains intact microorganisms, cell fragments, soluble and insoluble medium components and other metabolic products. The product may also be intracellular heat labile and easily broken down by contaminating of microorganisms. All these factors tend to increase the difficulties of product recovery. To ensure good recovery of purification, speed of operation may be the overriding factor because of the labile nature of the product.

The main objective of the first stage for the recovery of an extracellular product is the removal of large solid particles and microbial cell usually by centrifugation or filtration. In the next stage, the broth is fractionated or extracted into major fractions using ion exchange, gel filtration, reverse osmosis, liquid-liquid extraction, two phase aqueous extract or precipitation. Afterwards the product containing fraction is purified by fractional precipitation further more precise chromatography technique and crystallization to obtain a product which is highly conc. and essentially free from impurities. Other products are isolated using modification of this flow stream.

Filtration:

Filtration is one of the most common processes used at all scales of operation to separate suspended particles from liquid or gas using a porous medium which retains the particles but allows the liquid or gas to pass through. 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, rotator drum filters, and membrane filters are in use.

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

2. Rotatory drum vacuum filters: These filters are frequently used for separation of broth containing 10-40% solids (by volume) and particles in size of 0.5-10um. Rotatory 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 rotator 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. The filter cake can be easily removed.

Centifugation:

Microorganisms and other similar sized particles can be removed from a broth by using a centrifuge when filtration is not a satisfactory separation method. Although a centrifuge may be expensive when compared with a filter, it may be essential when:-

1.Filtration is slow and difficult.

2.The cells or other suspended matter must be obtained free of filter aids.

3.Continuous separation to a high standard of hygiene is required.

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 the liquid phase(fluid/particle separation).Unlike the centrifugation that is conveniently carried out in the laboratory scale, there are certain limitations for large scale industrial centrifugation. However in recent years, continuous flow industrial centrifugation have been developed. There ia a continuous feeding of the slurry and the collection of clarified fluid while the solid deposits can be removed intermittently. The different types of centrifuges are briefly described here.

Tabular bowl centrifuge: This is a centrifuge using particle size range of 0.1 to200µm and up to 10% solids in the in going slurry. This is a simple and a small

centrifuge. Tabular bowl centrifuge can be operated at a high centrifugal speed, and can be run in both batch and continuous mode. The main components of the centrifuge is a cylindrical bowl(or rotor) which may be a variable design depending on applications, suspended by a flexible shafts , driven by a overhead motor or air turbine. The inlet to the bowl is via a nozzle attached to the bottom. The feed which may consists of solids and light and heavy phases is introduced by the nozzle. During operation solids sediment on the bowl wall while the liquid separate into the heavy phase in zone (a) and the light phase in the central zone. The two liquid phases are kept separate and exit from the bowl by an adjustable ring.

Disc centrifuge: This centrifuge relies for its efficiency on the presence of disc in the rotor or bowl. A central inlet pipe is surrounded by a stack of stainless steel conical disc. Each disc has spacers, the broth to be separated flows outward from the centre feed pipe, then upwards and inwards between the discs at an angle of 45 degree Celsius to the axis of rotation. The close packing of the disc assists rapid sedimentation and the solids then slide to the edge of bowl provided that there is no gums or fats in the slurry, and eventually accumulate on the inner wall of the bowl.

Multi-chamber centrifuge: This is basically a modification of tabular bowl type of centrifuge. It consists of several chambers connected in such a way that the feed flows in the zigzag fashion. There is a variation in the centrifugal force in t6he chambers. The force is much higher in the periphery chambers, as a vesicle smallest particle settled down in the outmost chamber.

Scroll centrifuge or decanter: It is composed of a rotating horizontal bowl tapered at one end. That decanter is generally used to concentrated fluids with high solid concentration (biomass content 5-80%) The solids are deposited on the walls of the bowl which can be scrapped and removed from the narrow end.

Chromatography:

The biological products of fermentation (proteins, pharmaceuticals, diagnostic compounds and research materials) are very effectively purified by chromatography. It 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 to be loaded. The compounds are eluted by a mobile phase. Thus in a commonly practiced elusion type chromatography, a column is packet with adsorbed gel. A fluid mixture is injected followed by an eluent. Different solvents interact differently with the adsorbent material. Solutes interacting weakly with the matrix pass out rapidly while other interacting strongly exit slowly. The differential migration rates are used to separate different components. Some of the important chromatography methods are:

1. Adsorption chromatography: Is based on the different adsorption of solute particles on to solid particles like aluminium and silica gel by Vander wall and steric interaction.

2. High pressure liquid chromatography: Is based on general chromatography except that it is done under high pressure which provides fast and high resolution.

3. Ion exchange chromatography: It involves the separation of molecules based on their surface charges. Ion exchangers are of two (cation exchangers which have negatively charged group like carboxy-methyl and sulphonate, and anion exchanger with positively charged groups like di ethyl amino ethyl.

Ion exchange can be defined as the reversible exchange of ions between a liquid phase and a solid phase which is not accompanied by any radical change in the solid structure. The strength of attachment depending on the net charge of the solute at the pH of the column field. After deposition solutes are subsequently washed off by the passage of buffers of increasing ionic strength or pH.

4. Gel filtration: This is also referred to as size exclusive chromatography. In this technique the separation of molecules is based on the size, shape and molecular weight. The sponge like gel beads with pores serves as molecular sieves for separation of smaller and bigger molecules. A solution mixture containing molecules of different sizes 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 comes out first with the mobile phase. At the industrial scale gel filtration is particularly useful to remove salts and low molecular weight compounds from higher molecular weight products.

Separation of cells:

Disruption of microbial cells is usually difficult due to their small size, strong cell wall and high osmotic pressure inside cells. Generally, cell disruption is achieved by mechanical means, lysis or drying. The method of cell disruption must not damage the product of interest, the suitability of various methods is usually assessed in terms of recovery of a cellular enzyme activity following cell disruption.

Mechanical cell disruption: This approach uses shear, e.g., grinding in a ball mill, colloid mill, etc., pressure and pressure release, e.g., homogenizer, and ultrasound. A widely used methods is as follow: the cell suspension is forced through a fine nozzle; the cell disintegrate due to hydrodyanamic shear and cavitations.

Drying: The cells may be dried, e.g., by adding the cells into a large excess of cold acetone and subsequently extracted using buffer or salt solution. Drying induces changes in cell wall structure, which facilitate extraction. This method is widely used.

Lysis: Microbial cells may be lysed by chemical means, e.g., salt or surfactants, osmotic shock, freezing or by lytic enzymes, e.g., lysozyme ,etc.

In general, recovery of enzyme activity is the best following cell disruption using enzymes or ultrasound, followed by thermal and osmotic methods, while mechanical methods are the least desirable. However, ultrasound method is confined to laboratory mainly due to difficulties in heat removal on large scale.

Solvent Extraction:

The process of recovering a compound or a group of compounds from a mixture or from cells into a solvent phase is called extraction. Extraction usually achieves both separation as well as concentration of the product. It is especially useful for the recovery of lipophilic substances, and in antibiotic recovery, it is often an early step after cell separation.

1. **Liquid-liquid extraction.** It employs two immiscible liquids into which the product is differentially soluble. Usually, successively smaller volumes of the solvent are used for repeated extraction of a given sample, back extraction also tend to increase the selectivity of extraction. The extraction may be performed in a single step, by multi stage parallel-flow extraction, or by counter current extraction(most complex but most effective)
2. **Whole broth (medium +cells) extraction.** It should be used wherever possible since it reduces the number of steps as well as product loss. The effectiveness of extraction may , however, be reduced due to the presence of cells.
3. **Aqueous multiphase extraction.** It is used for separation of enzymes from cells/cell debris. The enzymes are extracted in an aqueous polyethylene glycol-dextran mixture, which form separate phases. Recovery of enzymes from these phases is rather easy and free from some of the difficulties encountered in centrifugation.

Immobilization of enzymes:

Enzyme immobilization may be defined as confining the enzyme molecules to a distinct phase from the one in which the substrates and products are present, this may be achieved by fixing the enzyme molecules to or within some suitable material. It is critical the substrates and the products move freely in and out of the phase within which the enzyme molecules are confined. Immobilization of enzymes molecules does not necessarily render them immobile, in some methods of immobilizations eg entrapment and membrane confinement, the enzyme molecules move freely within their phase while in case of adsorption and covalently bonding they are in fact immobile.

The materials used for Immobilization of enzymes, called carrier matrices are usually inert polymers or inorganic materials. The ideal carrier matrices have the following properties

a.) Inertness
b.) Low cost
c.) Physical strength

d.) Stability
e.) Regenerability after the useful lifetime of the immobilized enzyme
f.) Enhancement of enzyme specificity
g.) Reduction in period inhibition
h.) Shift in the pH optimum for enzyme action to the desired value for the process
i.) Reduction in microbial contamination and non specific adsorption

Clearly, most matrices possess only some of the above features. Therefore, carrier matrix for the immobilization of an enzyme must b chosen with care keeping in view the properties and limitations of various matrices.

Methods of Immobilization: The various methods used for immobilization of enzymes may be grouped into the following four types

1. **Adsorption:** In case of adsorption the enzyme molecules adhere to the surface of carrier matrix due to a combination of hydrophobic effects and the formation of several salt links per enzyme molecule. The binding of enzyme molecules to the carrier matrix is usually very strong but it may be weaken during use of many factors eg addition of substrate, ph or ionic strength etc. Therefore the matrix should be carefully chosen keeping these factors in mind. Some of the commonly used matrix are Ion exchange matrix, porous carbon, clays, hydrous metal oxides etc.
   Adsorption of enzymes to the matrices is very easy and widely used. The enzyme is mixed with a suitable adsorbent under appropriate conditions of ph and ionic strength. After incubation for a sufficient period of time, the carrier is washed to remove un adsorbed enzyme molecules and the immobilized enzyme is ready for use. This method usually produce a high loading of the enzyme.

2. **Covalently Binding**: In this system, the enzyme molecules are attached to the carrier matrix by formation of covalent bonds. As a result, the strength of binding is very strong and there is no enzyme loss during use. T he covalent bond formation occurs with the side chains of amino acids of the enzymes, their degree of reactivity being dependent on their charged status.

Lysine residues are the most usefully in covalent binding of enzymes since they are usually exposed on the surface, are highly reactive and only very occur at active sites of enzymes. Enzyme loading is quite low, only in exceptional cases it may be high.

3. **Entrapment**: In this approach, enzyme molecules are held or entrapped within suitable gels or fibers and there may or may not be covalent bond formation between the enzyme molecules and the matrix. A non covalent entrapped may be viewed as putting the enzyme molecule in a molecular cage just as a caged bird or animal. When covalent binding is also to be generated, the enzyme molecules are usually treated with a suitable reagent eg Acryloyl chloride is used to prepare lysine residues for binding by forming acryloyl amides. These are then co polymerized and cross linked with acryl amide and bisacryl amide to form a gel containing the entrapment enzyme which may be used to form small beads or a film on a solid support. Enzyme loading in the entrapment procedure is very high. However, diffusion of the substrate to the enzyme and of the product away from the enzyme creates difficulties.
4. **Membrane confinement:** Enzyme molecules ,usually in an aqueous solution, may be confined within a semi permeable membrane, which, ideally, allows a free movement in either direction to the substrates and products but, does not permit the enzyme molecules to escape.

Industrial advantage of Immobilization:

1. Enzymes are costly items, and can be used repeatedly only if they can be recovered from the reaction mixture. Immobilization permits their repeated use since such enzyme preparations can be easily separated from the reaction system.
2. The product is readily freed from the enzyme. This saves on the cost of downstream processing of the product.
3. Immobilized enzymes can be used in non-aqueous system as well which

may be highly desirable in some cases as well.

4. Continuous production system can be used, which is not possible with free enzymes.
5. Thermo stability of some enzymes may be increased eg Glucose Isomerase denatures at 45 degree Celsius in solution, but is stable for about 1 year even at 65 degree Celsius when it is suitably immobilized.
6. Recovery of enzymes may also reduce effluent handling problems.
7. Enzymes can be used at much higher concentration than free enzymes.