**Fermentation technology**

Fermentation is basically an ancient food processing technology. The term fermentation was derived from the Latin verb ‘*fervere’*, which means ‘to boil’ but fermentation process not always required high temperature or boiling. It is diverse process and it use to indicate the development or propagation of microorganisms in various conditions or environments like- anaerobic aerobic etc. The fermentation process is initiated by the inoculation of substrate by desired microorganisms and held it under proper environmental conditions. Inoculated microorganisms will produce product of interest and this product may be used directly or it may be processed to isolate desired molecules. When the process of fermentation is used in a large field like- chemical, pharmaceutical, food industries for the production of large-scale products by utilizing different microorganism -it is called fermentation technology.

The basic principle of fermentation process includes three steps- a) upstream processing, b) fermentation of molecules by microorganisms and c) downstream processing. Upstream processing includes preparation and purification of medium and selection of microorganism to get desired products. Second phase includes biotransformation process by which selected medium will give rise to desired product and third step include collection and purification of products. All those three processes need a proper equipment, called fermenter in which biotransformation occur under optimal physical and chemical parameters or factors like- pH, temperature, oxygen etc.

1. **Upstream processing:**
2. Selection and isolation of suitable microorganisms:

 Selection of microorganism is the basic step of upstream fermentation process. For this purpose, screening or identifying of perfect strain of microorganism is an important part from the pool of microorganisms. The perfect strain will give proper desired product from the media. There are different industrially important bacteria, fungi, actinomycetes, algae which will yield high quantities of crude product. There are two strategies: shotgun and objective approaches by which suitable industrial microorganism are isolated. In the first approach sample of free-living microorganisms or biofilms or other microorganism communities are collected from soil, sewage, water sources, anthropogenic or natural habitat or from floral and faunal sources. After collection those microorganisms are isolated by different screening processes. Alternative is to take or more objective approach by sampling from specific sites where desired microorganisms are considered to be likely components of the natural microflora. There are some criteria for selecting proper strain of microorganisms. It should exhibit following criteria:

1. Efficient production of desired or target products
2. Genetic stability and liability,
3. Limited or no need for vitamins and additional growth factors
4. Utilization of wide range of carbon sources,
5. Non-pathogenic- should not produce any toxic products or metabolites,
6. Ready harvesting from fermentation.

Collected microorganism are then grown in suitable culture media.

1. Strain improvement:

Strain improvement is a vital part of the upstream processing of fermentation technology. Genetic recombination is basic method which is applied in this case. By the recombination technique, two different genomes will bring together to form single unit of new genotypes. Specific restriction endonucleases are applied in this case.

Some examples of recombinant DNA technology mediated changes in the production of different metabolites are given below:

|  |  |  |
| --- | --- | --- |
| Approach | Features | Yields |
| 1. Product modification
 | New enzymes modify the product of existing biosynthetic pathway. | Conversion of encephalosprin C into &-aminocephalosproranic acid by D amino biosynthetic pathway. |
| 1. New substrate utilization
 | Inaccessible substrate converted into accessible substrate | Beer fermentation by yeast: Cyclodextrins converted into glucose which is utilized by yeast |
| 1. Completely new metabolites
 | All the genes of a new pathway transferred | *E. coli*; transfer of two genes for polyhydroxybutyrate synthesis from *Alcaligenes eutrophus* |
| 1. Stimulated metabolic production
 | Amplification of genes or enzyme whose activity is rate limiting | Gene cefG of *C.acremonium* catalyzing the conversion of Penicillin N: increased cyclosporin yields. |
| 1. Enhanced growth
 | Enhanced substrate utilization | *E. coli* glutamate dehydrogenase into *M. methylotrophus*; carbon conversion increased from 4% to 7%. |

Mutagenesis is also a conventional tool for strain development. In general, spontaneous mutation rate is very low in bacterial cells (about one per 107 to 1011 cells) but by the use of mutagens, the mutation rate is accelerated many folds. For these purposes different physical mutagens (like- UV radiation, X radiation, gamma radiation) and chemical mutagens (Ethen methane sulphonate, nitroso methene guanidine, nitrous acid, acridine mustards) are use which induce modifications of base sequences in DNA.

Protoplast fusion is another technique for the strain improvement. The process is subjected to the application of PEG (polyethylene glycol) treatment by which combination of favourable traits occurs from two parental cultures. Protoplast fusion between non-producing strains of *Streptomyces griseus* and *Streptomyces tenjimariensis*, has formed a strain that produces

indolizomycin, a new indolizine antibiotic.

3. Strain stability:

stability is the prime factor for the newly developed strain. Stability is maintained by either storage in liquid nitrogen or lyophilization. Strains transformed by the plasmids must be maintained by under continual selection to confirm that plasmid stability is retained. Structural instability and segregational stability are two causes for the instability recombinant plasmids which are occurred by deletion or recombination and by the loss of total plasmid respectively.

1. Media selection:

Fermentation media is also very important part of the whole process. Both liquid or broth and solid media are used for these large-scale fermentation purposes. The main criteria for any medium are to maintain and satisfy the nutritional needs of microorganisms and all the added requirements should be increase the rate of product synthesis. Large scale fermentation process needs to develop proper media and proper inoculation step. The initial step of media preparation needs proper carbon and nitrogen sources, minerals, oxygen and their conversion to cell biomass, metabolites, carbon dioxide, water and heat. Generally, the chemical formula of microbial cells is approximately C4H7O2N (on the basis of dry weight is 48% C, 7%H, 32%O and 14% N).

In come industrial processes suitable nutrient sources can be added into the media to fulfil these demands. In some cases, certain nutritional and environmental conditions may affects the physiological, biochemical and also morphological changes in microorganisms. All single requirements are needed to fulfill the yields of the fermentation products. For example, carbon sources are essential for reproduction, product synthesis and overall maintenance of the cells. Most carbon sources also require as energy sources (ATP generation). Carbohydrates are considered as traditional sources of carbon and energy in industrial fermentation processes. Others sources of carbon includes: alkanes, alcohols, various organic acids etc. In most cases, many microorganisms utilize inorganic as well as organic sources of nitrogen. Ammonium salts such as ammonium sulfate, diammonium hydrogen phosphate or ammonia are good examples of inorganic sources of nitrogen, whereas amino acids, proteins and urea serve as organic sources of nitrogen in various industrial fermentation processes.

In broth or suspended fermentation process water is also major components of media which also provide the trace mineral elements, calcium carbonate, iron and chloride into the media. Water treatment is required before use from direct sources. Water treatment includes removal of suspended solid materials, unnecessary salts, and most importantly others microorganisms.

Other requirements for media formulation:

For the proper growth and production of desired quantity and quality of metabolites or fermented products some other factors or ingredients are also required in proper proportions for each selected fermentation process. Other requirements include: minerals, vitamins, growth factors, precursor molecules, inducers, inhibitors, oxygen, cell permeability modifiers, antifoams etc. For example – proper amount of copper, cobalt, iron, manganese, zinc, molybdenum are the basic requirements among minerals. Corn steep liquor is used in commercial product formation because it contains a wide range of minerals. On the other hand, phenylacetic acid or phenylacetamide is widely used as side chain precursor molecules in Penicillin production and D-threonine is used as a precursor in L-isoleucine production. In plant cell culture different secondary metabolites like- terpenoids, flavonoids productions are triggered by presence of elicitors which are isolated from plant pathogens. In case of genetically modified microorganisms, growth is accelerated by the addition of inducers which can switched on the cloned genes. Inducers are important in many cases where intermediate molecule formation need to redirect or to halt a pathway at a particular point to stop further metabolism of the target product. For example, in the production of glycerol by yeast, inhibitors are used to redirect metabolism of sodium bisulfite. Depending upon the aerobic and anaerobic fermentation process, oxygen is also necessary as per requirements and if required, it may be supplied in the for of air containing 21%(v/v) oxygen. Oxygen requirement is also depending upon the carbon sources. Cell permeability modifiers are the compounds which help to increase cell permeability by modifying cell membrane and/or cell wall. For example, modifiers such as penicillin and surfactants are also added in fermentation media for amino acid fermentations like L-glutamate fermentation from *Corynebacterium* and *Brevibacterium*. Foam formation is a major problem in many fermentation processes mainly due to presence of proteins in media. Foam formation actually lowered the rate of product synthesis. Certain surface-active agents called antifoams are used to reduce the foam formation. They actually reduce the surface tension that binds the foam together. They should be nontoxic, thermostable and work at low concentration with maximum activity. Soya oil, sunflower oil, rapeseed oil, deionized fish oil, mineral oils, tallow all are examples of natural antifoaming agents whereas synthetic antifoams include – silicon oils, poly alcohols and alkylated glycols.

**Types of fermentation processes:**

There are several types of fermentation processes: Submerged fermentation and Solid-state fermentation.

1. Solid state fermentation:

In this process of fermentation, solid materials are used s substrate and microorganisms are allow to grow on it. Substrate includes: saw dust, wheat bean, cereal grains, wood shaving and different animals and plant substrates. Different species of fungi and actinomycetes are used in this process. Fungi are generally grown in low water concentration and its hyphae easily penetrate the substrate to produce exoenzymes which decompose polysaccharides.

The process of solid-state fermentation is mainly used for the production of various traditional foods (e.g.: cheese, natto, koji, sake, soy sauce, ragi etc. etc.), alcoholic products (e.g.: ethanol), enzymes (e.g.: alpha amylase, cellulase, rennin, protease, pectinase, beta galactosidase etc. etc.) and antibiotics (e.g.: penicillin, tetracycline, Cephalosporins, Iturin, surfactin etc etc). Air supply and temperature control are two basic requirements for the solid-state fermentation which is controlled by forced aeration. Forced aeration promotes oxygen and carbon dioxide transfer as well temperature transfer from the fermentation bed. In some cases, bioreactors those are used in the solid-state fermentation is placed in closed condition to control temperature and others factors. Maintenance of sterile condition is an integral part of the solid-state fermentation. Before inoculation, fermenter tray should be sterilized properly and after fermentation process is completed the trays are removed to collected total fermented mash to the downstream processing for product recovery. Tray fermenter is generally used to perform solid state fermentation and plastic, wooded or metallic trays with perforated bottom is used in this process to facilitated proper aeration.

Some examples of Solid-state fermentation products with required microorganisms and substrates:

|  |  |  |
| --- | --- | --- |
| Products | Microorganisms | Substrate |
| **Enzymes:**1. α- amylase

  | *Aspergillus oryzae, Rhizopus* sp*, Bacillus licheniformis*. | Wheat bran, cassava |
| 1. Cellulase
 | *Aspergillus niger, Trichoderma reesei* | Wheat bran, wheat straw |
| 1. Glucoamylase
 | *Aspergillus niger, Rhizopus* sp | Corn, cassava, wheat bran |
| 1. β -galactosidase
 | *Kluyveromyces laccis* | Whey, corn or wheat bran  |
| 1. Pectinase
 | *Talaromyces flavus, Aspergillus niger, Aspergillus, carbanerius* | Wheat bran, fruit pomace, wheat bran, coffee pulp |
| 1. Rennin
 | *Mucor pusillus, Mucor miehei* | Wheat bran |
| 1. Protease
 | *Penicillium caseicolum, martierella renispora* | Dried skim milk, wheat bran |
| **Metabolites** 1. Citric acid
 | *Aspergillus niger* | Sugarcane, fruit pomace, wheat bran. |
| 1. Ethanol
 | *Saccharomyces cerevisiae* | Fruit pomace, beet, carob pods, sweet sorghum. |
| 1. Gibberellic acid
 | *Gibberella fujikuroi* | Wheat bran |
| 1. Lactic acid
 | *Lactobacillus* sp*, Rhizopus oryzae* | Sugarcane, sweet sorghum |
| **Food products:** 1. Cheese
 | *Penicillium roqueforti* | Milk curd |
| 1. Bread dough
 | *Saccharomyces cereliisiae, Lactobacillus sanfrancisco* | Wheat powder |
| 1. Soy sauce
 | *Aspergillus sojae* | Soy bean. Wheat  |
| 1. Miso
 | *Spergillus oryzae* | Soy bean, rice |
| 1. Natto
 | *Bacillus natto*  | Soy bean |
| 1. Tempeh
 | *Rhizopus oligosparus*  | Soy bean  |
| 1. Tape
 | *Amylomyces rouxii*, *Rhizobium chinensis* | Rice, Cassava, maize |

There are some advantages in the process of solid-state fermentation:

1. The process is much easier,
2. Low-cost rate to carry down the whole process,
3. It is performed in low moisture condition which help for the lower contamination rate of bacterium and fungus,
4. Different waste (e.g., agricultural) utilization as substrate,
5. Product recovery much easier,
6. Product yields good.

There are also some disadvantages of solid-state fermentation:

1. Slower microbial growth
2. Bacterial contamination can hamper the process
3. Difficulties often encountered on scale-up
4. Substrate moisture level difficult to control.
5. Submerged fermentation:

It is a closed system of fermentation where microorganisms grow and differentiate in presence of free water and various nutrients in suspended or dissolved conditions. This type of culture media is called broth media. This type of fermentation is used for the production of different enzymes, antibiotics or others products.

Submerged fermentation process is mainly divided into three types- Batch fermentation, Fed batch fermentation and Continuous fermentation.

1. Batch fermentation:

It is a closed system fermentation process which is carried out in close fermenter vessel or bioreactor. Different medium, nutrients with selected microorganisms are inoculated under an aseptic condition of the closed bioreactor. The quantity of added media or broth should be constant. Minute change in volume of broth can change in pH level. In batch culture, microorganisms grow in classical growth rate with fresh sterilized medium. The growth rate commonly divided into lag phase, log phase, exponential phase and death phase respectively. In batch culture, it is very crucial to minimize the nonreproductive steps, achieve a high rate of product synthesis, optimize productivity, and maximize the yield of the end products. Extension of growth is very much advantageous for the efficient production of biomass (baker’s yeasts, feed biomass) or primary, metabolites (ethanol, acetic, citric or lactic acids), where as in case of secondary metabolite production, the exponential phase is shortened (by limitation of one nutrient, usually the nitrogen source) and the stationary phase is prolonged to achieve the maximum yield of the product.

Applications:

1. The technique helps microbiologists to observe the metabolic activity of the bacteria. Also, it helps in studying the cell physiology of microbes.
2. It is efficient for the production of secondary metabolites such as antibiotics.

There are some advantages of batch fermentation process:

1. Simple and easy to operate; only one reactor is used.
2. Contamination rate low because of short growth periods and closed system.
3. Capital investment low as compared with others fermentation process.
4. High raw materials conversion level resulting from a controlled growth period.

Disadvantages of batch fermentation process:

1. Time consuming as compared with others processes.
2. Batch viability.
3. Organisms are not found in their specific growth phase.
4. Any kind of technical error changes the media composition which may affect the microbial exponential growth pattern
5. After each cycle of product recovery, system has to be cleaned and filled with fresh feed.
6. Fed batch fermentation:

It is a semi-open system in which one or more than one nutrient is added aseptically to the bioreactor and product retained inside the bioreactor with time. The feedback process in mainly applied in mammalian and insect cell cultures. More recently, advances have been made in high density fed-batch cultures by using sophisticated media designs and feeding schemes beyond the addition of carbon and energy substrates. Generally, in fed-batch culture, the culture is subsequently fed with fresh nutrient medium without removing the growing microbial culture and allows one to supplement the medium with such nutrients that are depleted or that may be needed for the terminal stages of the culture. There is basic four types of feed batch fermentation process: Fixed volume fed batch culture, variable volume feed batch culture, Repeated or cyclic fed-batch culture and single feed batch process.

* In fixed volume fed batch process, the limited substrate is taken in reactor in very concentrated form so that there is no marked increase in volume. Generally, growth limiting concentrated gas or liquid substrates are added and these substrates should be sterilized by dialysis or by radiation sterilization. A constant-volume fed-batch system has been used for hyper thermophilic Archaebacteria cultivated under aerobic conditions.
* In variable fed batch fermentation process in which addition of substrate feed changes the working volume with fermentation time. The volume change can be influenced by the requirements of the process, fermentation time and main objective of fermentation process. The variable feeding is based on some criteria which includes: similar medium composition could be used as per need and concentrated form should be added at a slow rate to prevent dilution of the fermentation broth.
* Cyclic or repeated fed batch culture is a process in which a certain amount of spent broth is removed followed by addition of fresh nutrient medium in the stationary phase. This removal and newly addition of the medium results in an increase specific growth rate. Repeated fed-batch cultivation was used to optimize conditions for penicillin G biosynthesis by the use of a phosphorous feeding strategy,
* Single feed batch culture process spent broth is not removed and, in this case, large quantity of fermentation broth is not utilized until the end of the total process. Thus, reactor volume can be a serious limiting factor for the duration of the fermentation.

Applications:

1. Fed-batch culture is used to produce antibiotics such as penicillin, which requires controlled feeding of nutrients.
2. Underfeeding or overfeeding of substrates may results in nutrient starvation and dilution of the product, respectively. Thus, the fed-batch provides a controlled flow of nutrients to achieve maximum production of desired products or to obtain high cell density.
3. It is also used in the production of baker’s yeast in order to avoid the Crabtree effect (repress respiration by the fermentation pathway above the limit of substrate concentration).

Advantages of fed batch fermentation includes:

1. Product recovery rate high because of higher cell density utilization which is proportional to the concentration of biomass.
2. Fed batch process limit the production of inhibitory product or desired inoculum.
3. Use for those which are toxic for inoculum.

Disadvantages of the fed batch include:

1. Lower productivity levels due to time for filling, heating, sterilization, cooling, emptying and cleaning the reactor.
2. Not easy to handle as like batch fermentation.
3. High investment for multiple tasks.
4. Productivity level low as compared with batch fed fermentation.
5. Continuous fermentation:

Continuous fermentation is completely open system where nutrients along with microorganisms are aseptically and continuously added to the specific bioreactors. Here culture volume remain constant and nutrients concentrations remain at constant steady state values. The process is totally controlled by either microbial growth activity or by-product formation. There is basic two types of continuous fermentation processes: Turbidostat and chemostat methods.

* In case of turbidostat methos a constant biomass concentration is controlled by the dilution rate but in this case flow rate of fresh media varies. Cell density is mainly controlled by controlling turbidity value (i.e., cells per unit volume) which is created by cell population.
* In chemostat methods a constant growth rate of cells is maintained which is equal to the dilution rate and is controlled by the availability of the limiting nutrient. In this case, growth rate is determined by adjusting the concentration of substrates like carbon, nitrogen, and phosphorus etc.

Applications:

It has some industrial applications. Primary metabolites are the main products of this process. Single-cell proteins (SCPs), for example.

Advantages of continuous fermentation includes:

1. Overcome the batch viability
2. Can produce primary metabolites in their large quantity
3. Can maintain the culture in fresh and healthy state by addition of nutrients.

Disadvantages of continuous fermentation include:

1. Risk of contamination is high due to in and out of medium.
2. In long term process, genetic mutation of strains may occur.
3. The process is expensive as compared with batch and fed batch process and need expert staffs to maintain.

Distinguish between batch fand fed batch fermentation process:

|  |  |
| --- | --- |
| Batch fermentation | Fed batch fermentation |
| Closed type process | Semi open type process |
| It is fill and forgot type | Not fill and forgot type |
| Easy to operate | Not easy as batch process |
| Fermenter is filled up to 3/4th portion with medium | Fermenter is partially filled |
| Organisms maintain in their log phase for short period of time | Organisms maintain in their log phase for more time period. |
| Contamination rate very low | Contamination rate higher than batch feed. |
|  |  |

|  |  |
| --- | --- |
| Feb batch fermentation | Continuous fermentation |
| Discontinuously addition of nutrients | Continuous addition of nutrients |
| No simultaneous product recovery | Product recovery simultaneous |
| Product toxicity cannot be avoided | Product toxicity can be avoided |
| Batch variability is found | Batch variability not found |
| It may produce lower quantity of primary metabolites | Produce large quantities of primary metabolites. |
| Content of vessel may vary with time | Content of vessel is fixed |
|  |  |

Distinguish between fed batch and continuous fermentation process:

1. **Downstream processing:**

The efficient working of bioreactor depends on proper operations in the upstream processing and product recovery of useful products after fermentation is performed by downstream processing. The product is either present in the cell, in the medium or in both. For the proper product recovery and product purification we need to go through the steps of the downstream processes. The whole downstream processing can be divided into some unit processes, linked together to achieved a final and purified product. So downstream is a multistage product recovery and purification process.



Figure: Examples of unit downstream processing (Ref: Industrial microbiology: An introduction by Michael J. Waites, Neil L. Morgan, John S. Rockey, Gary Higton).

**Cell harvesting:**

First step of downstream processing is cell harvesting where solid-liquid separation is carried out to remove or isolate cells from medium. The method of solid-liquid separation is chosen on the basis of different major factors, like: size of microorganisms, specific gravity, viscosity and rheology of the spent fermentation medium.

**Broth conditioning:**

Cell separation methods from the liquid media is associated with sedimentation and centrifugation which is called broth conditioning. Broth conditioning also used to promote floatation which occurs naturally in traditional ale and baker’s yeast fermentations. Certain floc precipitation methods are also used at the end of many traditional beer and wine fermentation processes, where the addition of finings (egg albumen, isinglass, etc.) may be employed to precipitate yeast cells. Major advantages of these techniques are their low cost and ability to separate microbial cells from large volumes of medium.

**Sedimentation:**

It is a low-cost technology and relatively slow process for large flocs (greater than 100µm diameter)- for example primary yeast separation in the production of alcoholic beverages and in waste water treatments. The rate of sedimentation depends on particle size and density of medium. Larger the particle and the greater the density, the faster the rate of sedimentation. The sedimentation rate obeys the Stokes’ Law:



where *V*g = rate of particle sedimentation (m/s); *d*p = diameter of the particle (m); *p*s – *p*l = difference in density between the particle and surrounding medium (kg/m3); ***g*** = gravitational acceleration (m/s2); and h=viscosity (Pascal seconds (Pa s)).

Therefore, for rapid sedimentation the difference in density between the particle and the medium needs to be large, and the medium viscosity must be low.

**Centrifugation:**

Centrifugation is well known and vastly applied technique in downstream processing. The technique is mainly based on the principle of density differences between the particles to be separated and the medium instead of using only gravitational force. A centrifugal field is applied here to separate efficiently solid particles from the liquid phase (fluid/ particle separation). The technique is well applied in both laboratory as well as in large scale industrial processes.



where *V*c = centrifugal sedimentation rate or particle velocity (m/s); w = angular velocity of the centrifuge (rad/s); and *r* = distance of the particle from the centre of rotation (m) (for h, *p*s – *p*l and*d*p). Hence, the faster the operating speed (w) and the greater the distance from the centre of rotation, the faster the sedimentation rate (*V*c). Centrifuges can be compared using the relative centrifugal force (RCF) or ***g*** number (the ratio of the velocity in a centrifuge to the velocity under gravity =w2 *r*/***g***).

In recent days, different types of centrifugation techniques are used to separate solid particles from liquid phase. Four main types of the centrifuges are used in industrial processes: tubular centrifuge, multi-chamber bowl centrifuge, Disc stack centrifuge and Screw-decanter centrifuge.

* 1. Tubular centrifuge: It is simple and small type of centrifuge which produce highest centrifugal force of 13000 to 17000*g*. It is commonly used in pilot plants. It consists of a hollow tubular rotor bowls providing a long flow path for the suspension, which is pumped in at the bottom and flows up through the rotor. Particulate material is thrown to the side of the bowl, and clarified liquid passes out at the top for continuous collection. There must be periodic removal of solids and solids are removed manually.

 

* 1. Multi-chamber bowl centrifuge: It is modified tubular bowl type centrifuge and consist of several vertically mounted chambers connected in such a way that feed flows in a zigzag manner and are capable of operating at 5000 to 10000*g.* the force is higher in periphery chambers as result liquid feed passes from the centre through each chamber in turn and comparatively smaller particles settle down in the outermost chamber.

 

* 1. Disc stack centrifuge: In this type of centrifuge, several discs separate the bowl into settling zone and it works at a centrifugal force of about 5000 to 13000*g.* As liquid enters the centrifuge, particulate material is thrown outwards to the bowl wall where they accumulate and clarified fluid moves upwards. These centrifuges usually have the facility to discharge the collected material periodically during operation.

 

* 1. Screw-decanter centrifuge: It is a type of centrifuge where the process is continuously operated at a centrifugal force of 1500 to 5000*g*. It is mainly used in sewage system for the separation of sludge and for collecting yeast and fungal mycelium.

 

**Filtration:**

Filtration is also most commonly used downstream process for separating the biomass and culture filtrate. The process and its efficiency is depend on different factors: organisms cell size, others organisms’ presence, medium viscosity and most importantly temperature. There is different form of filters such as: depth filters, membrane filters, rotary drum vacuum filters and absolute filters.

Depth filters:

Depth filters are widely used in downstream processing in which a porous filtration medium (glass wool, asbestos or filter papers) is applied to retain particles throughout the medium. The particles are trapped within the matrix and the fluid passes out. Filamentous fungi can be removed by using depth filters. The advantages of this process include the ability to produce large flocculent, which can then be filtered and the filtration method is the flexibility in the choice of filter arrangement, which allows high solid storage capacities to be obtained, while keeping the energy consumption rate within an acceptable range.

Membrane filters:

It is advanced type of filtration process in which supported membranes have specific pore sizes. Clogging of filters is a major limitation of this membrane filters. According to the pore size membrane filters are divided into: microfiltration, ultrafiltration and reverse osmosis membranes.

* 1. Microfiltration:

It is very effective method of clarification of liquid. Generally, particles or microbial cells of 10-2 to 10µm are separated from liquid by the process. Membrane which is used to microfilter is highly cost effective which make the process expensive but the process include quite operation, lower energy requirements. The products can easily wash by the process and containment is readily achieved and no bioaerosols are produced.

* 1. Ultrafiltration:

It is same as microfiltration but here smaller pore sizes of the filtrating membrane can filter or separate the molecules of 2000 to 50000 Da. The membrane has anisotropic structure and composed of a thin membrane with pores of specific diameter for selective filter. Sometimes, several of these ultrafiltration units can be linked together to produce a sophisticated purification system. Generally, it is applied for the removal of particulates or macromolecules in water treatment process, cheese processing, enzyme recovery, radiocarbon dating of bone collagen and many more. Another variation on the ultrafiltration system is diafiltration, where water or other liquid is filtered to remove unwanted low molecular weight contaminants. This can be used as an alternative to gel filtration or dialysis for removing ammonium sulphate from a protein preparation precipitated by this salt for changing a buffer or in water purification.

* 1. Reverse osmosis:

It is a process of dewatering applied mainly in desalinate sea water for drinking. In this process of osmosis water will cross a semipermeable membrane if the concentration of osmotically active solutes is higher on the opposite side of the filtrating semipermeable membrane from the salt side. The process needs high pressure. For example, a pressure of 30–40 bar is needed to dewater a 0.6mmol/L salt solution (note:1 bar=100kPa=0.987atm). a strong metal casting is also needed to house this equipment.

**Cell disruption:**

There are some intracellular target products (like enzymes, recombinant proteins) and they form inclusion bodies. So, we need to disrupt the microorganisms and release the products. Some cells have strong cell walls so problem arises to disrupt the cell wall or envelops. For example, to disrupt the yeast cell wall, a pressure of about 650 bar is needed. Major problem is associated with cell disruption include the liberation of DNA which can increase the viscosity of the suspension. DNase can solve the problem. Cell disruption can be performed by mechanical and non-mechanical methods. Disruption process is controlled by some other factors like: particle size, total protein concentration or the activity of a specific intracellular enzymes released into the disrupting suspension.

1. Mechanical cell disruption methods:

There are different cell disrupting methods for recovery of products, like: solid shear method, liquid shear method etc. In solid shear method frozen cell preparations are extruded through a narrow orifice at high pressure. Solid shear methos are used in laboratory conditions only. Liquid shear method is more effective than solid shear method which is used in both laboratory as well as in large scale conditions. Manton and Gaulin homogenizer (APV-type mill) is a kind of device which is applied in liquid shear cell disruption method. In small scale, manual cell grinding with abrasives (alumina, glass beads, kieselguhr, silica) is effective cell disruption method but result is not up to mark. In different industries, high-speed bead mills are applied where cell suspensions are agitated with small beads (of diameter 0.5 to 0.9µm diameter) of zirconium oxide, glass or titanium carbide. After the collision with the beads, cells become disrupted. The disruption is controlled by agitation speed, beads size, bead density, diameter, broth density, flow rate and temperature. Another process of mechanical disruption is ultrasonic disruption which involves cavitation, microscopic bubbles and cavities generated by pressure waves. Ultrasonic vibrators are used for cell disruption which produce a high frequency sound with a wave density of approximately 20 kilohertz/s. This technique also generates heat which denature thermolabile proteins. It is reported that rod shaped bacteria are easier to break than cocci and Gram-negative organisms are easily disrupted than Gram-positive cells.

1. Non-mechanical cell disruption methods:

A wide range of methods are applied in nonmechanical cell disruption methods like: autolysis, osmotic shock, rupture with ice crystals (freezing/thawing) or heat shock. For example, autolysis is used in extraction of yeast products. It has the advantages of lower cost and uses the microbes’ own enzymes, so that no foreign substances are introduced into the product. Osmotic shock is often useful for releasing products from the periplasmic space. This may be achieved by equilibrating the cells in 20% (w/v) buffered sucrose, then rapidly harvesting and resuspending in water at 4°C. for small scale microbial disruption, various chemicals and enzymes are applied for cell disruption. Some organic solvents like: acetone, butanol, methanol, chloroform have been used to separate enzymes and other substances from the microorganisms. Those organic chemicals create pores on cell membrane and help to get out the enzymes from the cells. Alkali and detergent like: sodium lauryl sulphate or Triton X-100 can also be effective for this cell disruption. Cell wall degrading enzymes, like: lysozymes can easily degrade the cell membranes and release the desired products. Snail gut enzymes contain a mixture of b-glucanases which is applied in the disruption of yeast cell wall. Sometimes in case of actively growing microorganisms, antibiotics (e.g.: Penicillin, Cycloserine) are used to disrupt the cell membrane in combination of osmotic shock.

**Product recovery processes:**

1. **Chromatography:**

Several Chromatography techniques are applied to recover high value products. Choosing of particular type of chromatographic technique is very important and this selection process is depended on several factors like: molecular weight, biological affinities, hydrophobicity, isoelectric point and chromatographic technique depends on some parameters like: capacity, recovery, resolving power(selectivity).

Some types of chromatography techniques: Basic principle and applications in down steam processing:

1. Absorption chromatography:

It is the oldest type of chromatography process in which mobile phase consist of liquid and gaseous form and the mobile phase is absorbed onto the stationary phase solid surface. Basic principle includes analytical separation of the chemical mixture based on the interaction of absorbate with the absorbent. Silica gel H, silica gel G, silica gel N, silica gel S, hydrated gel silica, cellulose microcrystalline, alumina, modified silica gel is generally used as absorbent. This technique involves hydrogen bonding and/or van der Waals forces.

Different types of protein molecules are eluted by this process.

1. Affinity chromatography:

It is also an oldest and highly selective chromatography technique. It is highly selective technique. In this process the difference in absorption depends on the specific affinity between a substance fixed in the separation materials, termed absorbent and the desired components in the mixture, called ligand. Affinity chromatography has been applied in largescale industrial bioprocessing. Different types of affinity ligands are used to different extents; antibodies and their fragments, receptors and their binding substances, avidin/biotin systems, textile and biomimetic dyes, (oligo)peptides, antisense peptides, chelated metal cations, lectins and phenylboronates, protein A and G, calmodulin, DNA, sequence-specific DNA, (oligo)nucleotides and heparin. Likewise, there are several support types developed and used; natural, synthetic, inorganic and composite materials.

1. Gel filtration chromatography:

This chromatography technique essentially involves separation of the molecules on the basis of molecular size and shape. The stationary phase consists of porous beads composed of acrylic

polymers, agarose, cellulose, cross-linked dextran, etc., which have specific pore size. These materials contain some ionic groups. The initial choice of stationary phase material is also a key factor, as some may interact with the target product, e.g., carbohydrate-based matrices interact with glycoproteins. Mainly protein materials are eluted by this process.

1. Ion exchange chromatography:

This type of chromatography is most promising technique in bioprocessing on which ionic interactions are basic principle of the technique. The matrix materials are mainly based on cellulose substituted and consists of positively and negatively charged ions. A commonly used example is the anion-exchange resin diethylaminoethyl (DEAE) cellulose. Generally, molecules undergo an electrostatic interaction with opposite charges on the stationary phase. Maintenance of pH is primary factor of the process. Ion exchange chromatography is mainly used in antibiotics and protein separation.

1. High-performance liquid chromatography (HPLC):

This type of chromatography technique is mainly applied for the separation of organic molecules from non-aqueous solvents and proteins from aqueous solution. In this process, densely packed columns with small rigid particles of 5–50μm diameter of silica or a cross-linked polymer are used in high pressure. This method is fast and gives high resolution of solute molecules. This is mainly use in large scale industrial bioprocessing.

Dialysis and electrodialysis:

Dialysis is a kind of separation process by which low molecular weight solutes and inorganic ions are separated from the solutions. The membrane used here are size selective with specific molecular weight cut-offs. By the process of osmosis, low molecular weight solutes move across the membrane from high to low concentration. In electrodialysis, charged molecules get separated from the solutions by application of a direct electrical current carried by mobile counter-ions. They are mainly used for the desalination of water.

1. **Distillation:**

It is a recovery process by which fuel alcohol, acetone and other solvents get separated from the fermentation media. Batch distillation in pot stills continues to be used for the production of some whiskies (see Chapter 12), but for most other purposes continuous distillation is the method of choice. With ethanol, for example, the continuous system produces a product with a maximum ethanol concentration of 96.5% (v/v). This azeotropic mixture is the highest concentration that can be achieved from aqueous ethanol, unless a dehydration step is introduced using a water entrainer such as benzene or cyclohexane.

**Finishing steps of downstream processing:**

1. **Crystallization**

Product crystallization may be achieved by evaporation, low-temperature treatment or the addition of a chemical reactive with the solute. The product’s solubility can be reduced by adding solvents, salts, polymers (e.g., nonionic PEG) and polyelectrolytes, or by altering the pH.

1. Lyophilization:

Lyophilization or freeze drying is a water removal process which is mainly used to preserve perishable materials to increase self-life and to make the product more convenient for transport. This technique consists of three basic stages: freezing phase, primary drying or sublimation and secondary drying or absorption.

In the first phase, products are allowed to get freeze by placing on a shelf in freeze dryer or in a freezer or chilled bath (shell freezer). Lyophilization is easiest to accomplish using large ice crystals, which can be produced by slow freezing or annealing. However, with biological materials, when crystals are too large, they may break the cell walls, and that leads to less-than-ideal freeze-drying results. To prevent this, the freezing is done rapidly. For materials that tend to precipitate, annealing can be used. This process involves fast freezing, then raising the product temperature to allow the crystals to grow.

Second phase or sublimation is a drying process by allowed in low pressure heat. About 95% of water molecules in the materials is removed in the steps. It can be a slow process.

Excessive application of heat can alter the structure of products.

Third phase or absorption phase is the final step of lyophilization in which inonically bound water molecules are removed by raising temperature higher than in the primary drying phase. Freeze dried materials retain a porous structure. After the lyophilization process is complete, the vacuum can be broken with an inert gas before the material is sealed. Most materials can be dried to 1-5% residual moisture.

Problems To Avoid During Lyophilization

1. Heating the product too high in temperature can cause melt-back or product collapse,
2. Condenser overload caused by too much vapor hitting the condenser.
3. Too much vapor creation
4. Too much surface area
5. Too small a condenser area
6. Insufficient refrigeration
7. Vapor choking – the vapor is produced at a rate faster than it can get through the vapor port, the port between the product chamber and the condenser, creating an increase in chamber pressure.

Lyophilization is highly applied process in different bioprocessing industries. For examples: Food processing, diary industries, pet food processing industries etc.

**References:**

1. K.G. Clarke, Downstream Processing, Science Direct, 2013.

2. T.R. Bansod, L.D.Hingane, Fermentation Technology, World Journal of Pharmacological Research, 2021.

3. U. Satyanarayana, Biotechnology, books & allied (P) Ltd, 2005.

4. Vyas, S.P and Dixit V.K. Pharmaceutical biotechnology, CBS publishers and distributors New Delhi, 1998.

5. W.Sun, M.H. Shahrajabian and M. Lin, Research Progress of Fermented Functional Foods and Protein Factory-Microbial Fermentation Technology, MDPI, 2021.

6. N.A.Castillo, A.L.Valdez, J. I. Fariña, Microbial production of scleroglucan and downstream processing, Frontiers, 2015.

7. V. K. Singh, S. Jha, P. Rana, R.P.Sinha, Application of Synthetic Biology Approaches to High-Yield Production of Mycosporine-like Amino Acids, Research Gate, 2023.

8. W.Y. Chai, K. T. K. Teo, M. K. Tan, H. J. Tham, Fermentation Process Control and Optimization, Wiley Online Library, 2022.

9. M. J. Waites, N. L. Morgan, J, S. Rockey, G. Higton, Industrial Microbiology: An Introduction, School of Applied Science, South Bank University, 2001.

10. P. B.K. Kishor, A. M. Rao, I.S. Kumar, Biotechnology, Himalaya Publishing House, 2011.