Biotechnology

Biotechnology: the use of living organisms, cell or cellular components for the production of compounds or precise genetic improvement of living things for the benefit of human.

Obtaining of living organisms for biotechnology

Living organism (**biological system**) use in biotechnology are animal, plant and microorganisms, but the most common living organism are microorganisms.

The important microorganisms used in biotechnology are, generally, bacteria, actinomycetes, fungi and algae. These organisms occur virtually everywhere, e.g., in air, water, soil, surfaces of plants and animals, and plant and animals tissues. But most common sources of these microorganisms are soil, lake and river mud.

The first step in developing a producer strain is the **isolation of concerned microorganisms** from their **natural habitats.** Alternatively, microorganisms can be obtained as pure cultures from organizations, which maintain culture collections, e.g. American Type Culture Collection (ATCC).

- **It is highly desirable to use a strain possessing the following main characteristics:**

1- It should be high-yielding strain
2- It should have stable biochemical / genetical characteristics
3- It should not produce undesirable substance.
4- It should be easily cultivated on large-scale
5- Ability to grow on different natural media.

Biotechnological process design should take into account specific biotechnological constraints and minimize the cost of product during production and downstream operations

Design of growth media

Types of growth media

1- Microbial Growth Media:

Any medium and environment must provide everything the species under cultivation requires. These are oxygen (or other electron acceptor), water, nitrogen source, carbon source, energy source, minerals, vitamins and trace biochemicals.

Many bacteria can use glucose as energy and carbon sources. Some bacteria can use light as energy source and others can oxidize sulfur as energy source. As nitrogen source, most bacteria require protein, peptides, or amino acids, but many can use ammonia, nitrates or nitrogen molecules.

A. Synthetic media:

Are **chemically defined media** upon which microorganisms are grown and therefore whose exact compositions are clearly known.

These media contain pure sugar as a sole carbon source, ammonium salts, nitrate or amino acid as a sole source of nitrogen, in addition to inorganic salts. These media are uncommonly used in industry' due to the high coast compared of the metabolic products and are used for development and in laboratory experiments.

One of these media is **glucose salt medium** which consists of glucose as a carbon source in addition to many inorganic salts.

Preparation of glucose salt medium:

1- Dissolve the following salts in 90 ml of distilled water:

0.5 gm NaCI + 0.02 gm $MgS0_4$ + 0.1 gm NH4H2PO4

Then sterilize by autoclave.

2- Add **10** ml of **glucose** solution **(10%)** to the medium after sterilizing it by 0.2 mM

filter.

B. Natural media (Liquid and Solid media)

Natural or crude media are **complex mixture of nutrient materials** containing carbon and nitrogen source, mineral salts and growth factors (vitamins and amino acids). They are available in nature as primary or secondary byproducts of industrial processes of food manufacturing or as plant wastes, either as liquid such as whey and molasses or solid such as soy bean and grain bran.

Preparation of Media

1- Liquid medium (whey):

Whey is a **byproduct of dairy process,** and the one of principal media in fermentation in **alcohol industry** and **lactic acid production** .it is considered as a carbon source and one of crude sources of simple surges it consists of organic acids, proteins, peptides, amino acids and vitamins.

2. Solid medium (solid state fermentation media): Wheat bran medium

C. Supporting nutrients: The media may be supported by animal or plant nutrients and can be added to enrich the media with vitamins, amino acids and other growth supports.

These nutrients include (Peptones, meat extract and yeast extract)

1 - Peptones: Peptones are the most widely used **source of nitrogen** in microbial media. Some are made by **cooking milk or meat products in acid,** but most are made by **incubating milk or meat with trypsin, pepsin,** or other proteolytic enzymes to digest the protein to a mixture of amino acids, peptides, and polypeptides. Many microbes, called proteolytic, can digest proteins, and few of microbes can't.

Tryptone is a tryptic digest of casein. Casein is a complex of proteins found **in milk.** Trypsin is an important digestive enzyme produced by the pancreas. It cleaves proteins into shorter pieces called peptones. Tryptic digests of dried milk are called tryptones.

Tryptones are the best choice for bacteria media because they are used by most bacteria from animals and supply nitrogen, energy, and carbon.

Methods of protein hydrolysis:

There are two methods to prepare the peptones:

a- Acid hydrolysis: This process is achieved at high temperature with inorganic acids such as HCI and H_2S0_4, then neutralize,

b- Enzymatic hydrolysis:

This method is achieved by adding proteolytic enzymes (proteases produced by microorganisms, animals or plants). The protease is added to the protein (such as casein, meat, soy bean protein etc...) and incubate at 30-40°C for 30-60 min then filter and take the extract.

2- Meat extract

The waste products resulted from meat canning factories are used for preparation of meat extract .The extract must be free from fermented carbohydrate especially when it's used in testing of fermented carbohydrate, meat extract can be considered as a source of organic carbon , nitrogen, vitamins and inorganic salts, thus it is an integrated nutritional material with peptone which contains minerals ,phosphate, energy sources and some essential factors which may not found in peptone.

C- Yeast extract: -

Yeast extract is used in most media for microbes. Some of these microbes require vitamins and other growth factors from their plant or animal hosts and yeast extract is rich in vitamins,

minerals, and digested nucleic acids.

Yeast extract is very hydroscopic and is difficult to keep dry in the classroom and is sometimes difficult for students to obtain. It is easy to make. If necessary you can grow your own yeast, but you can usually buy compressed yeast at any bakery.

When a yeast cell is inactivated, a natural digestion process called **autolysis** starts. During this process the yeast's own enzymes break down proteins and other parts of the cell. This causes the release of peptides, amino acids, vitamins and other yeast cell components which, once the insoluble components have been removed, is called **"Yeast Extract"**

Yeast extract is often added to conventional media to supply growth factors for fastidious microbes.

Methods for preparation of yeast extract by autolysis and plasmolysis:

Screening of microorganisms

The use of highly selective procedures to allow the detection and isolation of only those microorganisms which are of interest from among a large microbial population.

- Screening allows the discarding of many valueless microorganisms, at the same time it allows the easy detection of the useful microorganisms that are present in the population in very less number

Primary screening

Primary screening allows the detection & isolation of microorganisms that possess potentially interesting industrial application.

- Primary screening separate out only a few microorganisms having real commercial value.
- Primary screening determines which microorganisms are able to produce a compound without providing much idea of the production or yield potential of the organisms.

Soil sampling:

1- The sample (about 500 g) is taken deep in soil (about 5 to 10 cm) by clean spatula.
2- The sample is put in sterile sac and all necessary information recorded on it.
3- The sacs are transferred into the lab and maintain in the refrigerator at 4°C until used.

There are three methods used for isolation and selection of the bacterial isolates that have the ability to produce certain substances:

1- Crowded plate technique
2- Auxotrophic technique
3- Enrichment culture technique

A- Crowded plate technique

The crowded plate technique is the simplest screening technique employed in detecting and isolating **antibiotic producers** or **growth factor**.

Procedure

1- Suspend 1.0 gram of soil sample in about 9 ml of sterile normal saline.
2- Mix well and allow the soil particle to settle down.
3- Prepare serial ten-fold dilutions (10^{-1} – 10^{-6}) of the supernatant using sterile normal saline.
4- Spread 0.1 ml from each of the last three dilutions on sterile nutrient agar plates with glass spreader.
5- Incubate the plates at room temperature or 30°C for 24 to 48 hours.
6- Select plate with 100-300 colonies
7- Collect 5 colonies from the bacteria want to be tested and suspend in 5 ml of normal saline (sterilize with autoclave)
8- Spread isolated bacteria (like *E.coli*) on agar plates after make 5 wells
9- Add 0.1 ml from each strain
10- Incubation plate at 37°C for 24 hour
11- Observe inhibition zone around the well or growth in case growth factor production

B- Auxotrophic technique

This technique is largely employed for detecting microorganisms able to produce **growth factors** (eg. Amino acid and Vitamins) extracellularly.

Auxotrophy is the inability of an organism to synthesize a particular organic compound required for its growth. An auxotroph is an organism that displays this characteristic.

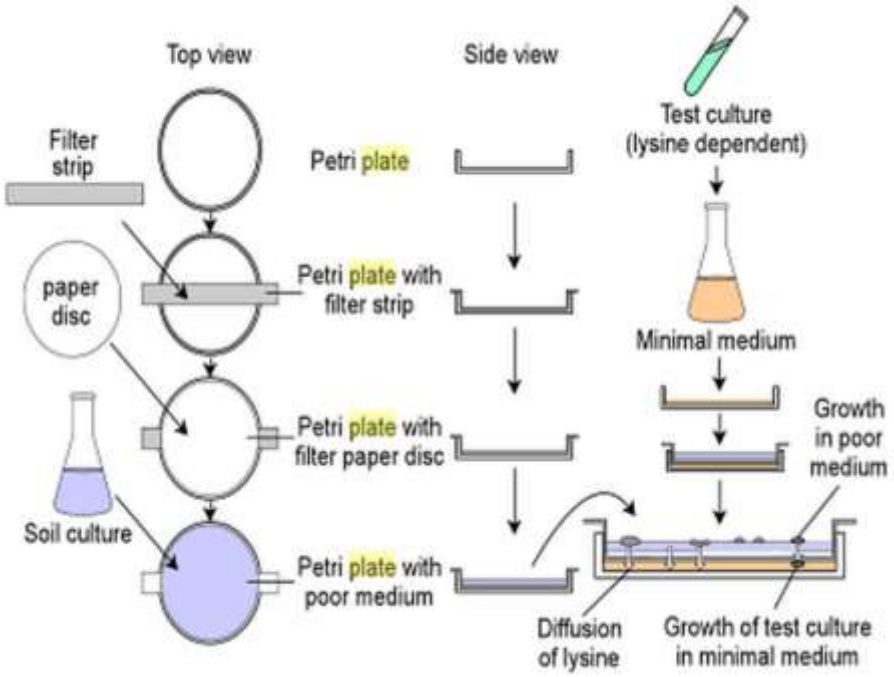
1- A filter paper strip is kept across the bottom of a petri dish in such a way that the two ends pass over the edge of the dish. A filter paper disc of petri dish size is placed over paper strip on the bottom of the plate. The nutrient agar is poured on the paper disc in the dish and allowed to solidify.

2- Microbial source material such as soil, is subjected to dilution such that aliquots on plating will produce well isolated colonies. Plating of aliquots of properly diluted soil sample is done.

3- A minimal medium lacking the growth factor under consideration is seeded with the test organism. The seeded medium is poured on the surface of a fresh petri dish and allowed to solidify.

4- The agar in the first plate as prepared in step- 1 is carefully and aseptically lifted out with the help of tweezers and a spatula and placed without inverting on the surface of the second plate as prepared in the second step.

5- The growth factor(s) produced by colonies present on the surface of the first layer of agar can diffuse into the lower layer of agar containing the test organism. The zone of stimulated growth of the test organism around the colonies is an indication that they produce growth factor(s) extracellularly. Productive colonies are sub cultured and are further tested.



C- Enrichment culture technique:

This technique was designed by a soul microbiologist, Beijerinck, to isolate the desired microorganisms form a heterogeneous microbial population present in soil. Either medium or incubation conditions are adjusted so as to favour the growth of the desired microorganism. On the other hand, unwanted microbes are eliminated or develop poorly since they do not find suitable growth conditions in the newly created environment. Today this technique has become a valuable tool in many screening program for isolating industrially important strains.

1- Process soli sample with heat in 80°C for 10 minute to kill all vegetative cell and left spore only.
2- Spread diluted sample (soil) on agar agar media contain casein 0.5%
3- Incubation for 24-48 hour at 37c
4- Observed growth of bacteria colonies as a result to consumption casein as the sole source of energy from the bacteria produce protease enzyme
5- Productive colonies are sub cultured and are further tested

Secondary screening

Secondary screening allows further sorting out of microorganisms obtained from Primary screening having real value for industrial processes and discarding of those lacking this potential

Some test are done: For micro organism

- Classification and identification
- Culture requirements
- Pathogenicity
- Genetic stability
- Scope for improvement e.g, by mutation, genetic manipulation

Tissue culture media

Tissue culture is often a generic term that refers to both **organ culture** and **cell culture** and the terms are often used interchangeably. Cell cultures are derived from either primary tissue explants or cell suspensions.

A. Animal tissue culture:

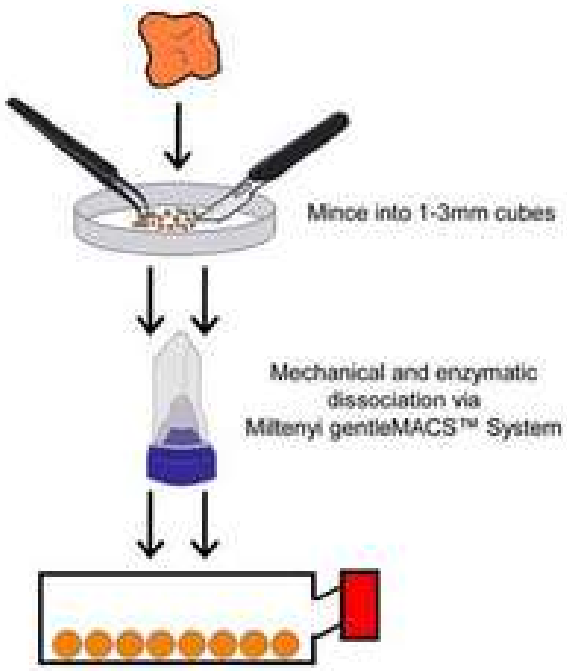
Animal tissue culture has important application in biotechnology for production of **vaccines, pharmaceuticals, and antibodies** which used in diagnostics.

Establishing steps of tissue culture:

1. **Primary cell culture:**

Cells are dispersed mechanically then enzymatically (Proteolytic enzymes such as trypsin or collagenase) into a cell suspension, then cultured as a monolayer on suitable substrate. Primary cell passage (which has been obtained from the monolayer) can pass to secondary cultures by detaching the cells with **trypsin** and **EDTA.** Then reseeding them in fresh media.

This can be done multiple times to form a **Cell line.**



2. Secondary cell culture and cell line

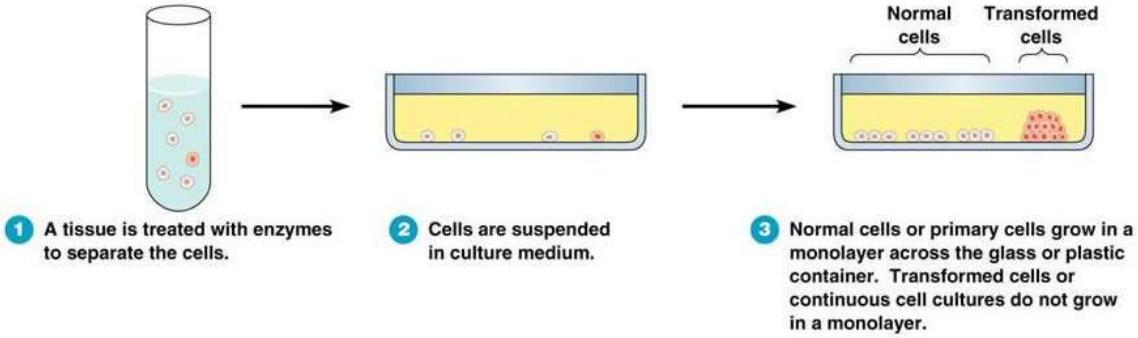
When a primary culture is sub-cultured, it is known as secondary culture or cell line or sub-clone. The process involves removing the growth media and disassociating the adhered cells (usually enzymatically).

❖ **On the basis of the life span of culture, the cell lines are categorized into two types:**

a) Finite cell lines
b) Continuous Cell lines

- Most primary cell lines have a **finite life,** and will eventually fail to divide and die, but not all cells of primary cultures die. Sometimes a few cells in the culture are going to change in **morphology and growth characteristics,** these cells are transformed.
- Transformed cells are usually neoplastic - form cancer.
- These cells are less adherent, fast growing, less fastidious in their nutritional requirements, able to grow up to higher cell density and different in phenotypes from the original tissue. Such cells grow more in suspension. They also have a

tendency to grow on top of each other in multilayers on culture-vessel surfaces.



- **Transformed cell lines can also be obtained by:**

a. Infecting cells with **oncogenic** viruses.
b. Treating cells with carcinogenic chemicals.
c. Propagating cells from tumors.

Applications of Animal Cell Culture

1- In vitro toxicity testing.
2- Production of viral vaccines.
3- Production of pharmaceuticals.
4- Cloning to produce rare proteins for research purposes or for pharmaceuticals and vaccines.
5- Production of monoclonal antibodies from hybridomas.

Preparation of tissue culture for mouse embryo:

1- Add small parts of mice fetus to blood plasma to yield a clot to stimulate adhesion of tissue cells.
2- Mix the fetus extract with serum.
3- Centrifuge the mixture repeatedly with BSS (Buffer Saline Solution) to wash a tissue cells, then supernatant is neglected and the precipitate is taken.

4- Transfer the tissue parts to the falcon flask and add 5 ml of **RPM1** (Roswell Park Memorial Institute medium) or **EMEM** (Eagle's Minimum Essential Medium)
5- Close the flask and incubate it for 24hr at 37°C for the purpose of cells adhesion.
6- Change the media weekly till the growth is spread and cover 50% of growth surface in the falcon, at this point we can obtain the growth of cells in steps called **(passages).**

B- Plant Tissue Culture (P.T.C)

This technique involves the growth of new plants from small parts of its tissue in tissue culture media under sterilized conditions.

When the conditions are convenient we can stimulate the plants quickly to produce a new shoots and this can be subdivided to produce many plants.
Adding of plant hormones such as IAA (Indol Acetic Acid) will stimulate the growth of roots. After that the plants are being ready to be cultured in the soil.

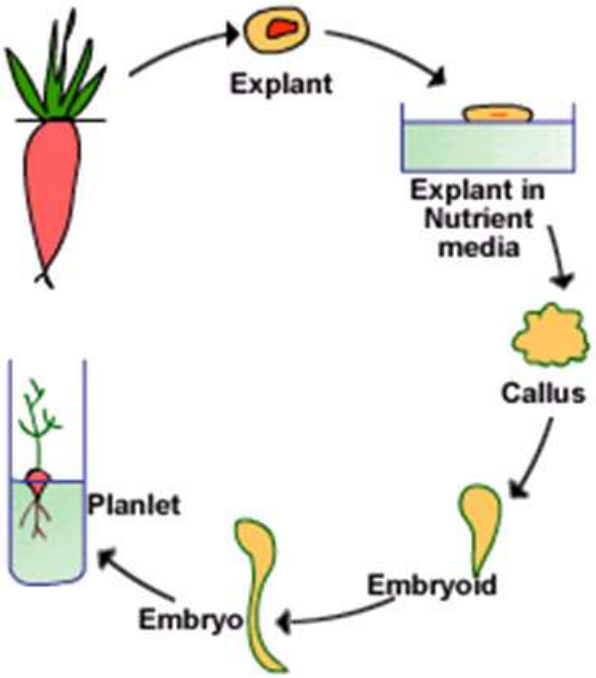
Preparation of plant tissue culture:

1. Preparation of Murashige minimal organic medium (MMOM)

- Dissolve a mixture of MMOM (mixture of inorganic salts, vitamins and growth factors) in 80 ml of D.W.
- Add some antibiotics (1 mg/ml) to the media to prevent microbial contamination.
- Add 0.6 gm. of agar to the MMOM solution.
- Distribute the media in slant tubes.
- Sterilize by filtration.

2. Preparation of plant material parts

- Cut 1cm area of the suitable (desired) plant parts (leaf, stem, etc.) especially the merestimic ends.
- Wash the plant parts with Anti septic disinfectant materials (hypo chlorate sol.) to remove fungal spores and bacteria.
- Transfer the plant parts to MMOM media and place it in contact with the lower surface of the leaf then press it slightly after that cover the slants.
- Incubate the tubes at the room temperature (avoid direct sun light) to yield **(callus),** the new shoot will grow in about two weeks.



Advantages of plant tissue culture

1- Mass production of various plant cultivars

- 6 million plants per year from one explant.
- Much higher production rate than other asexual propagation methods.

2- Especially beneficial for:

- Plants in high demand or valuable plants.
- Plants that are slow or difficult to propagate.

- Endangered species.

3- Production of pathogen-free plants

- Maintaining disease-free plants by micropropagation.

4- Germplasm preservation

- Germplasm: the DNA of a species
- In the past: seeds
  - limited shelf-life
  - don't preserve uniform characteristic (variability)

5- Continuous year round production

- Unaffected by climate

6- Propagated in controlled lab conditions

- The ability to change specific conditions to meet the needs of a particular plant species.
- Mainly, nutrient, light and temperature requirements.

Cell disintegration and extraction techniques

To get the intracellular product → the disintegration (rupturing or breaking) of cells is need

The methods used to break cells depend largely on the fragility of the cells

Animal cells: burst by osmotic shock, freeze/thaw, enzyme digestion (lipase, protease), toluene

Plant tissue: → pectinase and cellulase treatment

Microbial cells → lysozyme treatment

How to achieve good yield

1- **Minimize the number of steps**

2- **Choose appropriate disruption method**

a) **Can a given disruptor be used for a particular cell type?**

b) **Which is the best method of extracting a product?**

Stability of the released protein

The disruption methods can impose great physical and chemical stress ex. heat generated by mechanical disruption may result in enzyme denaturation, activation of proteolytic enzyme can degrade target proteins &enzymes

The yield and kinetics of the process

Yield the quantity of enzyme released/unit starting materials Specific activity-the amount of enzyme (unit)/ released protein (g)

Factors affecting the yield of enzyme:

(a) location of product with in the cell→extracellular(high yield) or intracellular(low)

(b) degree of disintegration

(c) extent of denaturation of the product during disruption → consider the "optimum disruption time"

The need to consider subsequent steps

After disruption → clarification step is need for subsequent purification step usually, through centrifugation, clarifying cell lysate is difficult Mechanical disruption methods are not needed chemicals that interfere with subsequent purification step.

Assessing the extent of disruption

- Use a marker substance (estimate total intracellular proteins)

Marker substance for cell disruption→ to determine the degree of cell disruption, marker techniques are used

1- Biological**:** visible cell counting
2- Physical: measuring the Vol. of intact cells, O.D., viscosity of the sample
3- Chemical: measuring the protein concentration

Methods of disruption

1- Methods needing specialist equipment

(a) mixer and blender
(b) coarse grinding with pestle and mortar
(c) fine grinding in a bead mill
(d) homogenization
(e) ultrasonication

2- Methods using non-specialist equipment

(a) freezing and thawing
(b) osmotic shock
(c) detergents
(d) solvents
(e) enzyme lysis

Mechanical disruption methods

a) **Mixers and blenders** →Grind cells coarsely→Use the buffer containing inhibitors and reducing agents

b) **Coarse grinding: pestle and mortar:**

- Useful for disruption of tissue samples
- Samples were grinded to fine powder under liquid nitrogen
- Necessary to maintain the frozen state

c) **Fine grinding:** the bead mill Useful for disruption of micro-organisms

- Bead mills should have cooling jackets because of heat generation during disruption

- Optional parameters: bead size, bead volume, agitator speed,milling time

d) **Homogenization** → High-shear mechanical methods for cell disruption include **Fluid processing systems** that used extensively for homogenization and disaggregation of a wide range of Biological materials. → Animal cells are easily disrupted, but very inefficient

High -pressure homogenizer →suitable for large scale operation **principles:** sample → narrow orifice→ cell crushing →cell breaking

e) **Ultrasonication: Very** vigorous process, results in complete solubilization

- Principles → the vibrating titanium probe create cavities → collapse of the cavities →pressure changes and shear forces cause cell disruption

- Problem: heat-generation(cause thermal denaturation, alteration of Nz activity

Very useful for fragmentation of cellular DNA related with increasing of viscosity

Non-mechanical disruption methods

1- **Heat shock**

Consider the thermal denaturation → leading to loss of enzyme activity. Useful for purification of heat-stable proteins

2- **Freezing and thawing-** Very simple, but suitable for cells without a cell wall Repeating of freezing and thawing may cause denaturation of protein

3- **Osmotic shock-** Osmotic pressure results from a difference in solute concentration across a semi permeable membrane → suddenly transferring a cell from an isotonic medium to distilled water (which is hypotonic) → result is a rapid influx of water into the cell result in the rapid expansion in cell volume → followed by its rupture,

E.g. if red blood cells are suddenly introduced into D.W. they will be lyse, i.e. disrupt thereby releasing hemoglobin. Osmotic shock is mainly used to lyse mammalian cells.

4- **Lytic enzymes** - bacterial disruption with lysozyme→ useful for Gram positive cells

5- **Detergents:** Detergents disrupt the structure of **cell membranes** by solubilizing their phospholipids, increase protein solubility, mainly used to rupture **mammalian**

cells.

For disrupting **bacterial cells**→ detergents have to be used in conjunction with lysozyme.

6- **Solvents** → toluene, ether, isoamylalcohol, chloroform

Organic solvents like acetone 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

Methodology

A - **Bacterial cell opening: G+ve** *Bacillus spp* **lysis by using enzymatic disruption:**

1- Suspend the cells in 50mM Tris-HCl (pH=7) 10mM EDTA+ 10%Sucrose + TES + 300µg\ml of lysozyme.
2- Centrifuge for 15 min at 5000 rpm → Detect bio product (protein) in supernatant
3- Defect microscopically the efficiency of destruction.

B- G-ve *E.coli* **disruption using detergents:**

1. Suspend the cell pellet in 0.5 mL of 50mM TES **(Tris+ EDTA + Sucrose)**
2. Add 0.8% NaOH + 3% SDS.
3. Centrifuge the solution at 5,000rpm for 10 minutes → discard the precipitate
4. Transfer the supernatant to a new test tube.
5. Detect microscopically the efficiency of destruction for obtaining the bio product.

C- Yeast cells : *Saccharomyces cerevisiae* **disruption** *by* **Agitation**

1. Mix 0.4 mL of cell sample in 1.6 mL of lysis buffer and vortex vigorously.
2. Add small glass beads → vortex vigorously.

3. Centrifuge the lysate at 10,000 rpm for 10 minutes.
4. Transfer the supernatant to a new test tube leaving the cell debris.
5. Detect microscopically the efficiency of destruction for obtaining the bio product.

Lysis buffer contents: 0.05 M Tris-HCl (pH=8), 12.5% glycerol (to prevent ice crystals formation). 1mM EDTA (Endonucleases inhibitor). 1.25mM Bisamidine (proteases inhibitor).

D- RBCs disruption *by* osmotic shock.

1. Suspend the Pelleted RBCs in 3.5 ml of cold D.W. or in hypotonic buffer (7.5 mM **NaH_2P0_4+1.0 mM $MgCl_2$+1.0 mM Na-ATP)**
2. Transfer the sample immediately to a glass test tube immersed in a circulating 37°C water bath.
3. Centrifuge the lysate at 5,000 rpm for 10 minutes.
4. Remove the supernatant from the cell membranes.
5. Detect microscopically the efficiency of cell lysis

E- Plant cell disruption by Cationic detergent

1. **Grind** 200 mg of plant tissue to a fine paste in approximately 500 pi of CTAB buffer.
2. **Transfer** CTAB/plant extract mixture to a microfuge tube.
3. **Incubate** the CTAB/plant extract mixture for about 15 min at 55° C in a recirculating water bath. After incubation, spin the CTAB/plant extract mixture at 10000 rpm for 5 min to spin down cell debris.
4. **Transfer** the supernatant to clean microfuge tubes.

Detect microscopically the efficiency of destruction for obtaining the bioproduct

Extraction & Purification of enzymes

Enzymes: are proteins specialized to catalyze biological reactions. They are among the most remarkable biomolecules known because of their extraordinary specificity and catalytic power.

- Enzymes are found in all kinds of cells, and may locate inside the cell (Intracellular) or secrete outside (extracellular).
- Extraction methods differ according to the type of organisms (plant, animal, fungi, bacteria, etc...) and location of enzyme.

In this laboratory we will carry out the main steps of extraction and purification of a kind of an industrially important enzyme which can also applied for other enzymes or proteins.

Extraction of Amylase

Amylases: (α, β, and Glucoamylase) can be found in microorganisms, plants and higher organisms where they play a major role in carbohydrate metabolism. They catalyze the cleavage of glycosidic bonds in starch and related carbohydrates, these enzymes are among the most important enzymes with a wide variety of industrial applications.

- a-amylase is one of extra cellular enzymes, these enzymes are secreted in the culture medium outside the microbial cells (such as *Bacillus subtilis, A.niger,A.oryzae)* . They are often soluble in water, so they can readily be extracted from the culture medium and purified.

Method

- A strain of *Bacillus subtilis (producing* a- amylase) is cultured in starch containing medium to induce enzyme production and incubated at 37-40°C for 24-48 hrs.
- Extract the enzyme from the solid culture media by adding buffer (e.g phosphate

buffer with ratio 1:3 (solid medium: water). When the medium is liquid there is no need to addition the buffer but centrifugation.

- Centrifuge at 5000 rpm for 30 min the supernatant containing the enzyme is called (crud enzyme)
- **Measure the enzyme activity in the extract (supernatant) :**
  a) Add 1 ml of crude enzyme solution to 4ml of 1% starch→ incubate at 35˚C for 15 min
  b) Add few drops of iodine and notice the disappearance of blue color
  c) Compare with control (solution of 1% starch and iodine without enzyme)

Concentration of enzyme

Increasing the enzyme concentration and removing more water molecules is the most performed operation in enzyme purification, the most usually used methods to achieve this are:

1- **Salt precipitation** e.g. (NaCI, $(NH_4)_2So_4$ and $CaCI_2$).

2- **Organic solvents**: precipitation (the most frequently used are: ethanol methanol and acetone).

Salt precipitation

The most effective salt and widely used for precipitation of proteins is $(NH_4)_2So_4$, since it has many advantages such as: **1) high efficiency in protein precipitation, 2) low cost, 3) high solubility, 4) has no effect on most enzymes**. The salt (ions) will attract H_2o molecules leaving protein particles which will aggregate and precipitate, this process is called **salting out.**

The amounts of ammonium sulfate used are often expressed as percentage saturation, i.e. 30%, as a percentage of the amount required to saturate the solution. Convenient tables are

available that permit one to weigh out the correct amount of solid ammonium sulfate to give a desired percentage saturation.

Procedure

1- Take 10 ml of enzyme solution (crude extract), add certain amounts (gm.) of ammonium sulphate $(NH_4)_2So_4$ to obtain the desired % saturation of the salt (20, 40, 60 % etc) obtained from standard tables

2- Centrifuge at 5000 rpm for 10-15 min or filter to separate the supernatant

3- Dissolve the precipitant in small quantity of buffer solution then measure the enzyme activity to the supernatant

4- Add another quantity of $(NH_4)_2So_4$ to get the 2nd, 3rd, and the final saturation % according to the table

Note: it is favorable to achieve all steps of extraction and purification in a cold condition to avoid protein denaturation

Dialysis

After the ammonium sulfate precipitation step, the protein of interest may be in a high salt buffer. This may be undesirable for further steps of purification such as ion exchange chromatography.

One of the most common methods to get rid of salt in our sample is dialysis.

Dialysis is used to remove small molecules from protein solutions, or to exchange the medium. Dialysis is based on the fact that due to their size, protein molecules are unable to pass through the pores of a **semi permeable membrane** (prepared from cellulose acetate or nitro cellulose.) while lower-molecular substances distribute themselves evenly between the inner and outer spaces over time. After repeated exchanging of the external solution,

the conditions inside the *dialysis tube* (salt concentration, pH, etc.) will be the same as in the surrounding solution.

Procedure:

1- Wash the dialysis tubes by D.W and boil it for 10 min. or treat them by alcohol to remove contaminated substances.

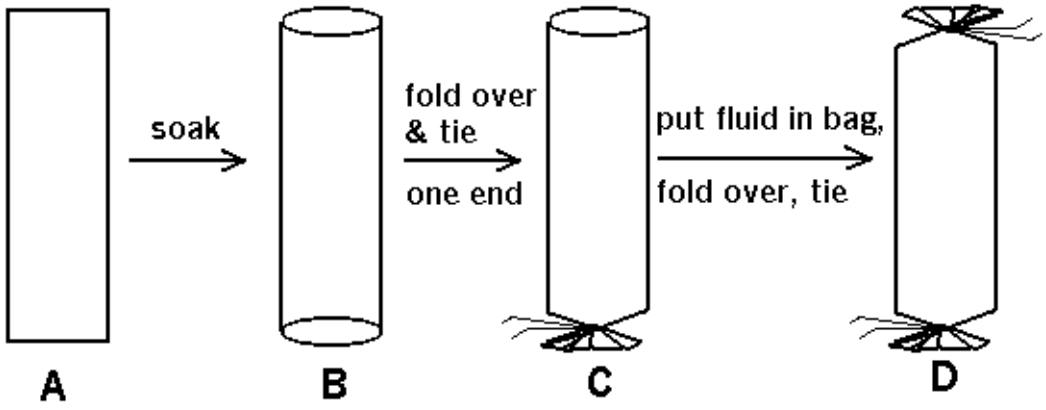
2- Close one end of the dialysis tube by a thread.

3- Pour the enzyme solution in dialysis tubes, leave a space in the tube (do not fill it to protect it from rupturing and close the second end of the tube.

4- Put the tube in a container (beaker) containing D.W or buffer solution (which used to dissolve the precipitant after diluted 10 times) and surround the container with ice bath to prevent protein hydrolysis and deactivation of enzymes.

5- Change the outside buffer (or water) from time to time (0.5-2 hrs) the dialysis process may take several hours or a day.

6- When dialysis has finished, release the enzyme solution from dialysis tube→ Measure the enzyme activity and protein concentration.



Purification:

There are many techniques used to purify enzymes and proteins, the most applicable techniques (chromatography) are:

a- Gel filtration Chromatography

Also known as gel permeation or size exclusion. The liquid phase passes through a porous gel which separates the molecules according to their sizes. The pores are normally small and exclude the larger solute molecules, but allow smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.

b- lon-exchange chromatography:

Separates proteins depending on the net charge of proteins in a gels carry either positive charges or a negative charges. Proteins having the same net charge as the gel beads are repelled and flow through out, whereas proteins having opposite charge will bind to the beads. Bounded proteins, negatively charged, are eluted by passing a gradient concentrations of salt solution (usually 0.1-0.5M NaCl or KCl).

c- Affinity Chromatography:

This is the most selective type of chromatography. It utilizes the specific interaction between one kind of solute molecule and a second molecule immobilized on a stationary phased the immobilized molecules may be an antibody to specific for certain antigen(s) or enzyme and its substrate.

Gel filtration technique:

There are different matrices (stationery phase/gel material) which may be used in
This technique → **Sephadex, Sepharose andSephacryl** are considered the commonest substances in gel filtration.

The gel using in this technique must have the desired characteristics such as:

1. Resistance to high pressure, temperature and pH.

2. Simplicity in treatment.
3. High flow rate (flow rate = 30 ml $cm^{-2}min^{-1}$).
4. High protein resolution.

Preparation of gel filtration column:

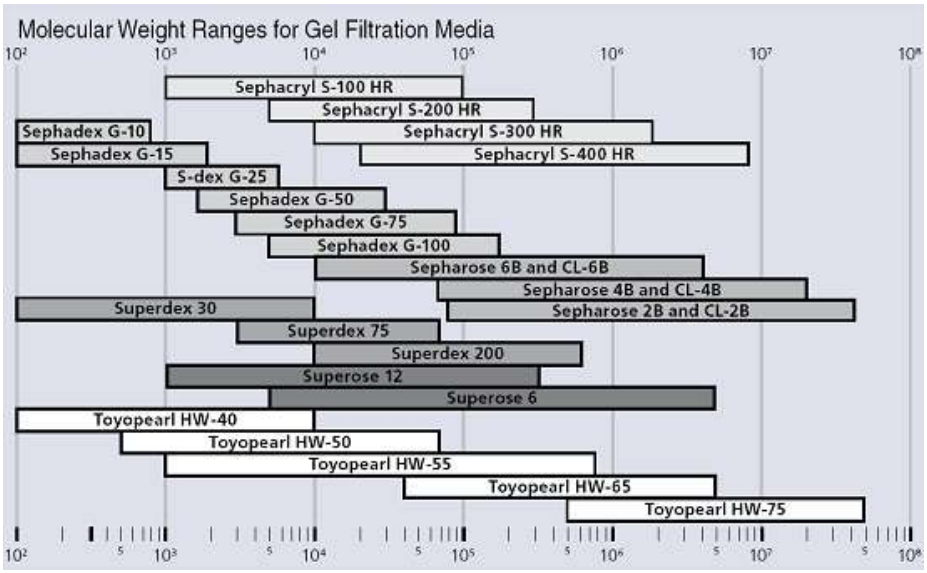
Packing a column is a very critical stage in any gel filtration experiment. A poorly packed column will give rise to uneven flow, peak broadening, and loss of resolution and can also affect flow rates.

a. Preparation of Gel

Gel may supplied by special companies as powder (e.g Sephadex, cellulose) or as liquid suspension (e.g Sephacryl, Sepharose).

To prepare a gel of sephadex: (there are many types of Sephadex, named as Sephadex G (e.g Sephadex G25, G50, G75 etc))

1. Weight an amount of dry powder and add appropriate amount of buffer (1:10 w/v) shake gently and allow to settle down for 1-2 hr (the gel bead will swell).
2. Decant the supernatant.
3. Repeat the steps 1-2 and measure the pH
4. Degass the gel by vacuum (a Buchner flask containing the gel is connected with vacuum for 15-30 min).



B- Packing of the gel in the column

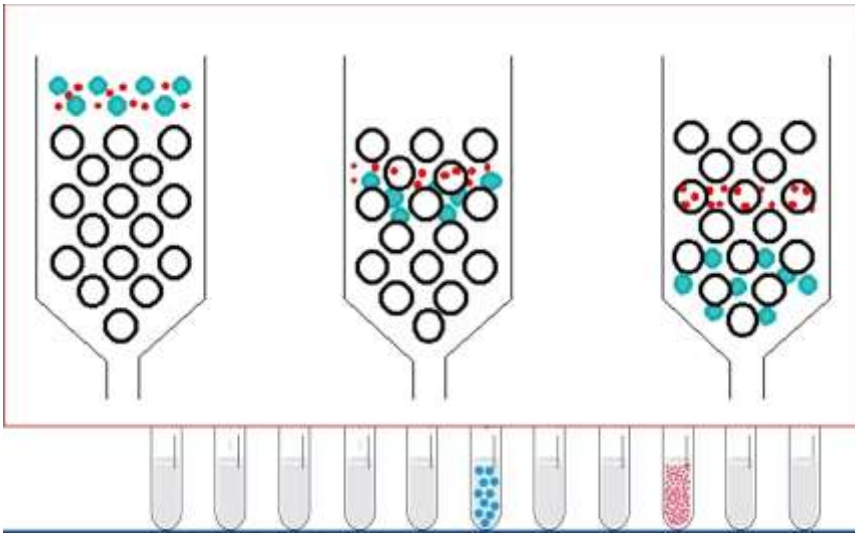
1. Mount the column (glass cylindrical apparatus) vertically and close it at its lower end (which is connected with glass tube containing a kind of filter (e.g glass wool).
2. Fill the tube (column) to one- third of its height with buffer.
3. Pour the well-mixed and well-degassed suspension gel inside the column using a glass rod. Avoid introducing air bubbles.
4. Open gradually the column outlet valve (screw) and start the flow of buffer.
5. Observe the sedimentation of gel and maintain the packing flow rate after a constant bed height is obtained.
6. Continue packing the column for approximately 10 minutes. Close the column outlet (screw).
7. Equilibrate the gel by passing the buffer for a time until pH becomes stable.

C- Sample application

1. Remove the eluent (buffer) above the gel.
2. Apply the (protein solution) carefully by Pasteur pipette on the top of the gel solution and allow to enter the gel.
3. Add the eluent (buffer) above the gel.

4. Connect the upper end of column with the continuous supply of Buffer.
5. Notice the flow rate of the column.
6. Receive and collect the samples (fractions) eluted from the gel flow from the lower end of the column in tubes with same volume (2-5) ml.
7. Measure the absorbance of diluted fraction at 280 nm by UV-Vis spectrophotometer and draw the relationship between No. of fractions and absorbance (O.D).
8. Close the lower outlet between the individual steps.

    ❖ maintain the gel by addition buffer containing Na-azide to the column and keep it wet (**Na- azide is an inhibiter of microbial growth**)



Gel filtration Chromatography

Immobilization

Is a technique of binding biocatalysts (enzymes , cells , or its derivatives) to an inert, insoluble carrier material , It allows biocatalysts to be held in place throughout the reaction, following which they are easily separated from the products and may be used again.

Benefits of Immobilization

1. Increasing their activity and stability.
2. Improving the biotechnological applications (e. g. bioseparation of the products from the biocatalysts).
3. Increasing the number of times of a reaction which can be performed.

The Principle of Immobilization

The technique used for the physical or chemical fixation of cells, organelles, enzymes, or other proteins onto a solid support, into a solid matrix in order to increase their stability and make possible their repeated or continued use.

Methods of Immobilization

1. Physical methods e.g. Encapsulation , Adsorption or Entrapment
2. Chemical methods Cross Linking or Covalent binding

Immobilization by the gel:- depend on the large size of biocatalysts, so it couldn't out from the pores of the gel beads. This method is suitable to the cell Entrapment.

The principles of cell Entrapment by use the gel

The biocatalysts (i.e. Cells) are adding to the gel then mixing well. Then will be polymerization to gel. From this method we will be obtained on the cell or enzymes coated beads are ready for use.

The main advantage of Immobilization by the gel

Technique used for the immobilization of whole cells is the generation of a hard matrix with

several forms. Also, it is easy the control of some physical characteristics such as porosity.

Types of gel using in immobilization

1- Alginate: - The basic materials to Alginate are Glucouronic acid or manouronic acid. The polymerization induced by use Ca^{+2}. **Alginate uses in cell immobilization**.

2- Silica gel: The structure of the silica layer enables the immobilized enzyme to react with substrates in a solution. The immobilized enzyme can be quantitatively recovered without loss of the activity **because** the enzyme molecules are immobilized inside the silica -gel layer.

3- Polyacrylamide gel: - The gel forms from two Materials; bis acryl amide and acryl amide. The second one is important to forming across-linkage with the first material by used Ammonium per sulfate because ammonium per sulfate uses as inducer for polymerization.

4- Agar- Agar: - immobilized cells using agar-agar as. entrapment materials(gel concentration 3%, initial biomass concentration in the gel 0.8% (W/V) is useful for extracellular enzymes production in batch fermentations, beads (cells / Agar) are obtaining from injection the mixture in cooled toluene.

Prepare immobilized cells

1. Collect spores of *Aspergillus niger* by filtration from Stock culture maintained on potato dextrose agar (PDA) plates at 4 °C.
2. filter and wash the pellet suspension with phosphate buffer to eliminate the remaining culture medium
3. Resuspend in 5ml phosphate buffer to use for the immobilization.
4. Dissolve 2 gm of Sodium alginate in 100 ml of 0.9 % NaCl, the solution is then autoclaved.
5. Add 1ml of spore suspension to 10ml Sodium alginate solution.
6. Add the Cell / Na - alginate solution to 90ml CaC1 solution with stirring, then gel beads will form.

Enzyme Immobilization on Silica Gel advantages:

1. Enzymes are usually stabilized by binding.
2. Product is not contaminated with the enzyme (especially useful in the food and pharmaceutical industries).
3. Easy separation of enzyme from the product

Prepare immobilized enzyme

1. Add 2 g of silica gel and 4 ml of polyethyleneimine to 32 ml of phosphate buffer, for amylase coupling to silica gel, so Amylase is covalently bound to the surface of silica gel.
2. Mix with 2 ml of 0.1M acetate buffer solution (pH 5.5) containing 2 ml of amylase
3. The immobilization is carrying out at 5°C for 30 min.

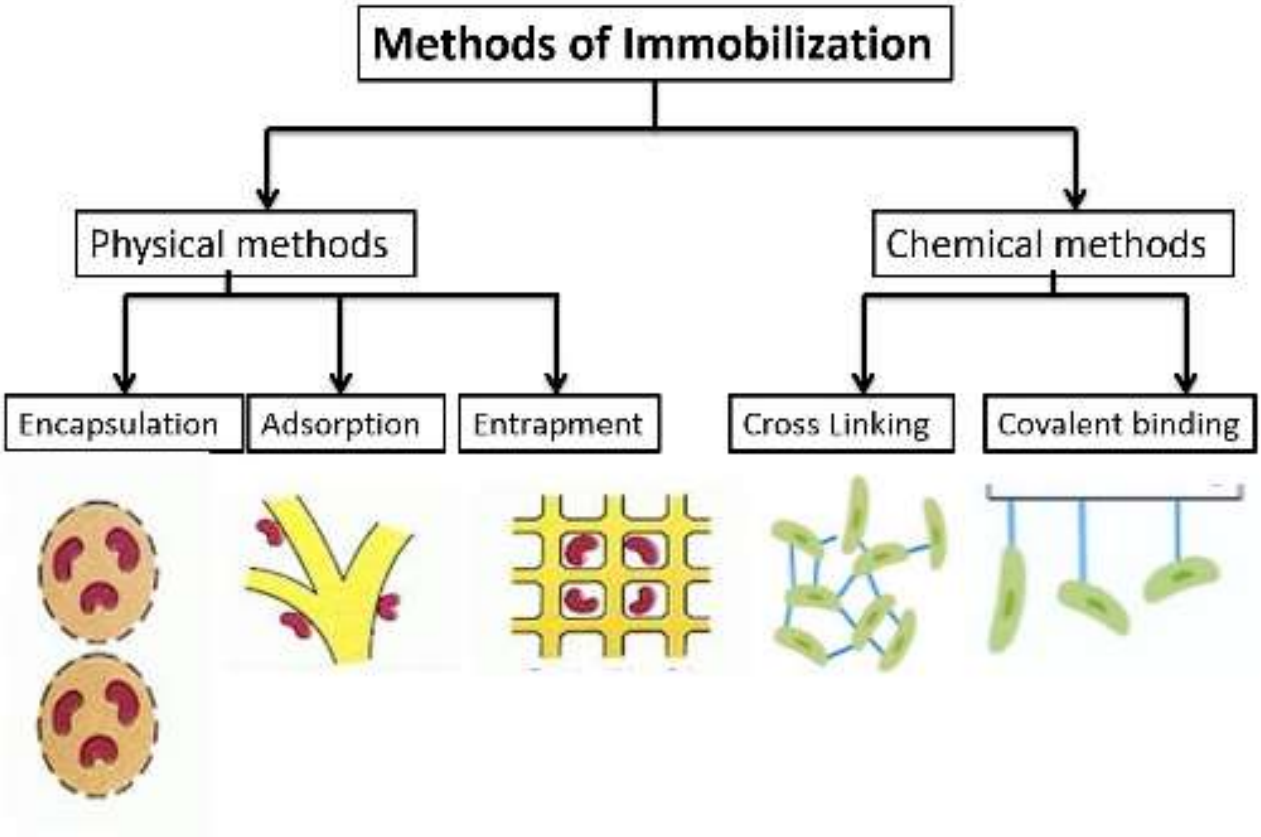
Bioreactor design

1- After make the beads of gel (as mentioned above) which contain the biocatalysts the beads put in column to make packed Bioreactors.

2- The substrates are flow from down to up of the Bioreactor with suitable flow rate.

3- In this test the substrates are starch & serum albumin & the bio products are amylase & protease Respectively

Note: The biological activity of protease depends on the dissolved of the serum albumin clots.


Methods of Immobilization
Physical methods
Chemical methods
Encapsulation
Adsorption
Entrapment
Cross Linking
Covalent binding

Penicillin production

Is a group of antibiotics derived from *Penicillium* fungi (*Penicillium notatum ,Penicillium chrysogenum)* . Penicillin effective against many previously serious diseases, such as syphilis and infections caused by staphylococci and streptococci. Penicillins are still widely used today, though many types of bacteria have now become resistant. All penicillins are β-lactam antibiotics and are used in the treatment of bacterial infections caused by susceptible, usually Gram-positive, organisms.

Chemical structure of the Penicillin core

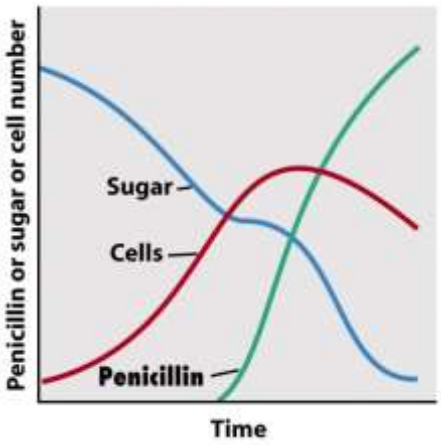
- This core has the molecular formula R-$C_9H_{11}N_2O_4S$, where R is the variable side chain that differentiates the penicillins from one another
- Penicillin was the first important commercial product produced by an aerobic, submerged fermentation
- First antibiotic to have been manufacture in bulk.

The yield of penicillin can be increased by:

- Improvement in composition of the medium.
- Isolation of better penicillin producing mold sp. *Penicillium chrysogenum* which grow better in huge deep fermentation tank.
- Development of submerged culture technique for cultivation of mold in large volume of liquid medium through which sterile air is forced.

Commercial Production of Penicillin

- Like all antibiotics, penicillin is a secondary metabolite, so is only produced in the stationary phase.



Industrial Production of Antibiotic- Penicillin

The industrial production of penicillin was broadly classified in to two processes namely,

1. Upstream processing
2. Downstream processing

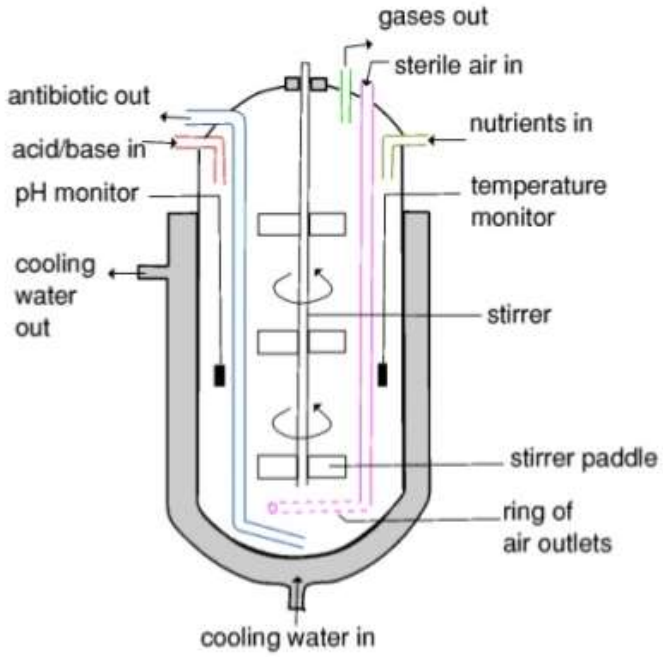
- **Upstream processing** encompasses any technology that leads to the synthesis of a product. Upstream includes the exploration, development and production.
- The extraction and purification of a biotechnological product from fermentation is referred to as **downstream processing**.

Inoculum Preparation

- The medium is designed to provide the organism with all the nutrients that it requires.
- Inoculation method- submerged technique
- Spores -major source of inoculum
- Carbon Sources: Lactose acts as a very satisfactory carbon compound, provided that is used in a concentration of 6%.
- Nitrogen Sources: Corn steep liquor (CSL)
- PAA- precursor

FERMENTATION PROCESS

- The medium is inoculated with a suspension of conidia of *Penicillium chrysogenum.*
- The medium is constantly aerated and agitated, and the mould grows throughout as pellets.
- After about seven days, growth is complete, the pH rises to 8.0 or above, and penicillin production ceases



Stages in downstream Processing

- Downstream processing is relatively easy since penicillin is secreted into the medium (to kill other cells), so there is no need to break open the fungal cells.
- However, the product needs to be very pure, since it being used as a therapeutic medical drug, so it is dissolved and then precipitated as a potassium salt to separate it from other substances in the medium.

Removal of cells

The first step in product recovery is the separation of whole cells and other insoluble ingredients from the culture broth by technique such as filtration and centrifugation.

Isolation of Benzyl Penicillin

1. The pH is adjusted to 2-2.5 with the help of phosphoric or sulphuric acids.
2. In aqueous solution at low pH values there is a partition coefficient in favor of certain organic solvents such as butyl acetate.
3. This step has to be carried out quickly for penicillin is very unstable at low pH values.
4. Antibiotic is then extracted back into an aqueous buffer at a pH of 7.5, the partition coefficient now being strongly in favor of the aqueous phase. The resulting aqueous solution is again acidified & re-extracted with an organic solvent.
5. These shifts between the water and solvent help in the purification of penicillin.

6. The treatment of the crude penicillin extract varies according to the objective, but involves the formation of an appropriate penicillin salt.
7. Pure metal salts of penicillin can be safely sterilized by dry heat, if desired. Thereafter, the aqueous solution of penicillin is subjected to crystallization