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Practical Biotechnology Fourth stage

University of Baghdad

College of Sciences

Department of Biology

Contents

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

Obtaining living organisms for biotechnology

Living organisms (biological systems) used in biotechnology are animals, plants, microorganisms, and their products, such as enzymes, toxins, etc. But the most common are microorganisms. The essential microorganisms used in biotechnology are bacteria, actinomycetes, fungi, and algae. These organisms occur virtually everywhere, e.g., in air, water, soil, surfaces of plants and animals, and tissues of plants and animals. But the most common sources of these microorganisms are soil, lakes, and river mud. Alternatively, microorganisms can be obtained as pure cultures from organizations that maintain culture collections, e.g., **A**merican **T**ype **C**ulture **C**ollection (**ATCC**).

In modern biotechnology, the gene encoding an interesting product (enzyme, protein, and toxin), could be identified and transferred into an expression vector by genetic engineering technology, then transformed into an expression system (microbial cell, animal cells, or plant cells) to produce the interested product in higher amount.

- The first step in obtaining a producer strain is the isolation of concerned M.Os. from their natural habitats.
- The second step after the isolation of concerned M.Os. is their screening. Screening for the highly desirable isolate.

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

Highly desirable isolate possessing the following four characteristics:

1- High-yielding strain.

2- Have stable biochemical and genetic characteristics.

3- Should not produce undesirable substances.

4- Easily cultivated on a large scale.

Examples of isolation and screening of some interesting M.Os

- **Primary Screening for Organic Acid/Amine Producers.**
  a- The soil sample dilutes serially and then inoculated on Neutral red agar, bromothymol blue agar, and $CaCO_3$ Agar. Plates are incubated at a suitable temperature for a period of time.
  b- The growth of acid/amine producers will change the colour of the indicating dye near the producing colonies.

  Positive results will be:

  Neutral Red Agar – (Nutrient Agar + Neutral Red)
  Acid producer colonies – change the colour of the medium to Red.
  Amine producer colonies – change the colour of the medium to Yellow.
  Bromothymol Blue Agar – (Nutrient Agar + bromothymol l Blue)
  Acid producer colonies – change the colour of the medium to Yellow
  Amine producer colonies – the colour of the medium remains Blue.
  $CaCO_3$ Agar medium – (Nutrient Agar + 1-2% Calcium Carbonate)
  Production of organic acid detected by clear zone from the dissolved $CaCO_3$ around the colony.
- **Primary Screening for Antibiotic Producers (crowded plate techniques).**
  a- Prepare the bacterial sample by adding one gram of soil into 9 ml of D.W, mixing well, and leaving for settle. Then apply 1, 0.5, and 0.2 ml

to the nutrient agar plates, then incubate in suitable conditions (30˚C for 2-5 days). **Why we use 30 ˚C**.

b- Colonies showing antimicrobial activity indicated by the colony's inhibition zone must be sub-cultured to be purified & tested against sensitive M.Os. (Test M.Os.).

c- Test M.Os. is the M.Os. that is used as an indicator for the presence of specific antibiotic activity.

d- A suspension of the test M.Os. is made and then sprayed on the surface of the agar. The same process needs to be done for the antibiotic-producing M.O, blocks from the antibiotic-producing M.O plate (made by Pasteur pipette or a sterile cork borer) are transferred to the inoculated medium to measure the diameter of the inhibition zones in mm after incubation. (If the Antibiotic producing M.O. was grown in a liquid medium, then the wells method will be used to detect the inhibition zones.

The disadvantage of this method is that inhibition zones might be caused by the change of pH values resulting from the metabolism or rapid utilization of critical nutrients, so further sensitive tests need to be done.

- **Isolation & Screening for Protease Producing M.Os.**

a- M.Os. that produce proteases include ***Bacillus subtilus***, ***Bacillus megaterium***, ***Aspergillus niger*** & ***Aspergillus oryzae***.

b- Milk agar medium used to detect protease-producing M.Os., which can be prepared by adding 10 ml of skim milk into 90 ml of nutrient agar. The medium should be autoclaved & cooled to add the skim milk to avoid casein denaturation (casein is a protein content of milk & the substrate for protease production).

c- Diluted microbial source (soil) or other sources (meat, cheese, legumes) inoculated on milk agar medium.

d- Positive results appear as **clear zones** around the colonies referring to casein utilisation.

e- Colonies with wide diameters can be isolated for further testing.

Protease enzyme has industrial applications:

1. Leather industry (dehairing).
2. Food applications (meat tenderization & improvement).
3. Medical application (digestive aid).

- **Isolation & Screening for Amylase Producing M.Os.**

a- Amylases can be produced by bacteria & fungi; it utilizes starch to dextrin, maltose, or glucose.

b- Diluted microbial source inoculated with 0.1-0.2ml on starch agar medium and incubated at 30-40˚C for 24-72hrs.

c- Starch agar medium (**nutrient agar +1% Starch**) can be used to isolate amylase-producing M.Os.; they can hydrolyze starch in the medium to dextrin, maltose, or glucose.

d- Iodine is used to detect starch utilization in the medium. Adding a few drops over the Petri dish, amylase non-producible colonies appear blue. In contrast, amylase-producing M.Os. will give **brown** colour for **dextrin**, and **yellow** colour for **maltose** or **glucose**.

Design of growth media

Types of growth media:

1- Microbial Growth Media:

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

Many bacteria can use glucose as an energy and carbon source. Some bacteria can use light as an energy source, and others can oxidize sulfur as an energy source. Most bacteria require protein, peptides, or amino acids as a nitrogen source, but many can use ammonia, nitrates, or nitrogen molecules. Bacteria capable of fermentation can oxidize one molecule and use another as an electron acceptor; thus, they are not able to obtain as much energy as would be available were the energy oxidized completely to carbon dioxide and water.

A. Synthetic media: are chemically defined media upon which microorganisms are grown and whose exact compositions are known.

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

One of these media is the glucose salt medium which consists of glucose as a carbon source and many inorganic salts.

Preparation of glucose salt medium:

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

0.5 gm NaCl + 0.02 gm $MgSO_4$ + 0.1 gm $NH_4H_2PO_4$ then sterilize by autoclave.

2- Add 10 ml of glucose (10 %) to the media after sterilizing it with a 0.2 Mm filter.

B. Natural media (Liquid and Solid media)

Natural or crude media are a 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 a liquid such as whey and molasses or solid such as soybean and grain bran.

Preparation of Media:

1- Liquid medium (whey):

Whey is a byproduct of the dairy process and one of the principal media in fermentation in the alcohol industry and lactic acid production. It consists of organic acids, proteins, peptides, amino acids, and vitamins.

Whey Preparation:

1- Warm a quantity of milk to about 60-70 °C.
2- Add rennin enzyme (milk clotting enzyme used in cheese making) at a ratio of 1:5 (v/v, 1ml enzyme solution to 5 ml milk solution); incubate at 37 - 40°C until curd is formed.
3- Separate the curd from the liquid (whey) by filtration with cheese clothing.
4- Collect the whey in a flask, adjust the pH to an appropriate value, and sterilize by autoclave. After this step, whey can be used as a nutrient medium to grow different microorganisms and produce many industrial products.

2. Solid medium (solid-state fermentation media):

Wheat bran medium:

1- Wet the wheat bran by adding water or suitable buffer (1:3 – 1:10 wt/vol) and mix well. The water or buffer should not be free (there is no liquid out of the medium)
2- Sterilize by autoclave for 30 min. This medium can be used to cultivate many kinds of fungi and bacteria and produce industrial products such as enzymes and fermented foods.

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 nitrogen source in microbial media. Some are made by cooking milk or meat products in acid. Still, 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, but most 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, and 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 temperatures with inorganic acids such as HCl and H_2SO_4, then neutralized.

Peptone preparation by acid hydrolysis:

1-Add 10 ml of 5N HCl to 100 gm of meat and heat until boiling for 15 min.

2-Adjust the pH with 3N NaOH until pH=7.

3-Filter and concentrate the extract under a vacuum to get Syrup (liquid medium).

4-Sterilize the product by autoclave.

A spray drier can be used to obtain peptone as powder.

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, soybean protein etc..) and incubated at 30-40°C for 30-60 min, and then the extract was taken after filtration.

2- Meat extract

The waste products from meat canning factories are used to prepare meat extract. Meat extract can be considered a source of organic carbon, nitrogen, vitamins, and inorganic salts. It is an integrated nutritional material with

peptone that contains minerals, phosphate, energy sources, and some essential factors that may not be found in peptone.

Meat Extract Preparation

1- Add 100 ml of tap water to 100gm of meat and boil the mixture for 5-10 min.

2- Filter under a vacuum to get protein lysate.

3- Centrifuge the mixture at 3000 rpm. discard the precipitate and take the supernatant

4- Sterilize the product by autoclave. It can be dried and used as powder.

3- Yeast extract: - Yeast extract is used in most media for microbes. Some 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.

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

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

Preparation of yeast extract by autolysis:

1- Suspend 5 gm of dry bakery yeast or cells (*Saccharomyces cerevisiea*) in 5 ml of D.W.

2- Incubate at 50°C for 24 hours with occasional mechanical stirring to autolysis the cells.

3- Then leave to cool, neglects the precipitate, and use the supernatant fluid.

4- Use 2 to 10 ml per liter of finished medium to supply growth factors. Use larger volumes to provide nitrogen and carbon sources

Preparation of yeast extract by plasmolysis:

1- Mix 10 gm of bakery yeast powder with 10 ml of normal saline.

2- Incubate for 1 h at 37 °C.

3- Add 20% NaCl to obtain 50 ml volume, then leave it for 10 min.

4- Filter the mixture to get the liquid extract, and sterilize by autoclave.

5- Dry the product under a vacuum.

Cell disruption technology

Cellular disruption is a biotechnological method to release biological molecules from inside a cell, including organelles, pigments, proteins, DNA, RNA, and lipids. Microorganisms produce many molecules or compounds that have biotechnological importance. Therefore, genetic engineering was applied to such microorganisms to produce certain proteins, plasmids, polysaccharides, and other biotechnological molecules which are either extracellular or intracellular.

Factors Affecting the Yield of Product

A. Location of a product within the cell.

B. Degree of disintegration.

C. The extent of denaturation of the product during a disruption.

To achieve a good yield.

1. Minimize the number of steps.
2. Choose appropriate disruption methods.

Cell disruption methods can be classified into two categories:

1. Mechanical Methods (physical methods).
2. Non-mechanical methods (chemical methods).

Mechanical Methods (physical methods)

1. **Mixers and blenders**, grind cells coarsely.

2. **Coarse grinding**: using a pestle and mortar is useful for disrupting a tissue sample, such as plant and animal tissue.

3. **Fine grinding**: the bead mills are useful for disrupting microorganisms by using glass beads with agitation.

4. **Homogenizations** by using a French press.

High-shear mechanical methods for cell disruption include fluid processing systems used extensively for homogenization and disaggregation of a wide range of biological materials. (Such as chloroplast materials, unicellular organisms, homogenates of animal tissue, and other biological particles)

5- **Ultrasonic vibrations by using a sonicator**. (Having a frequency greater than 18 kHz can be used to disrupt cells) useful for the disruption of microorganisms.

Mechanical disruption methods do not need chemicals that interfere with subsequent purification steps.

Non-mechanical methods (Chemical and Physicochemical Methods)

1- **Detergents**: disrupt the structure of cell membranes by solubilizing their phospholipids and are mainly used to rupture mammalian cells.
2- **Enzymes:** lysozyme is used for microorganisms; it lyses bacterial cell walls, mainly gram-positive. The combination of lysozyme and detergent is used for gram-negative types. Pectinase and cellulase treatment for plants, lipase and protease for the animal.
3- **Organic solvents**: like acetone, chloroform, toluene, and ether mainly act on the cell membrane by solubilizing its phospholipids and denaturing its proteins.

4- **Osmotic shock**: is mainly used to lyse mammalian cells.

Marker Substance for Cell Disruption

To determine the degree of cell disruption, marker techniques are used

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

General Considerations in Selecting Cell Disruption Methods

Certain disruption methods may not be suitable for all types of cells. For example, ultrasound methods generate heat that can destroy organelles and alter the configuration of biological molecules. At the same time, shearing systems like blenders and ball mills can shatter the cell contents and cell walls. Also, the amount of energy required to break the cell depends on the type of organism. Some cells can be easily broken by gentle treatment, such as osmotic shock (e.g., animal cells), while other types of cells require more force (e.g., yeast cells and plant cells).

Methodology:

A - Bacterial cell opening: (Non-mechanical method)

G+ve *Bacillus spp* lysis by using enzymatic disruption:

1. suspend the cells by 50mM Tris-HCL(PH=7) 1mMEDTA+10%Sucrose+ TES
2. Add a freshly prepared lysozyme solution at a final 100 µg / ml concentration and incubate the lysis mixture in ice for 30 min.
3. Centrifuge for 15min at 5000rpm
4. Detect bioproduct (protein) in supernatant
5. Detect the efficiency of destruction microscopically

B. Yeast cell opening:

Saccharomyces cerevisiae disruption by agitation (mechanical method)

1. Mix 0.4 ml of a cell sample in 1.6 mL of lysis buffer and vortex vigorously.
2. Add tiny glass beads and then 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 the efficiency of destruction microscopically for obtaining the bioproduct.

Lysis buffer Contents: 0.05 M Tris-HCL (PH=8)

12.5% glycerol (to prevent ice crystals formation).

1mM EDTA (Endonucleases inhibitor).

1.25mM Besamidine (proteases inhibitor).

C. Mammalian cells (R.B.C.s) disruption by osmotic shock (Non-mechanical method)

1. Resuspend the Pelleted RBCs in 3.5 ml of cold D.W. (or in hypoosmotic buffer (7.5 mM NaH_2PO_4, 1.0 mM $MgCl_2$,1.0 mM NaATP))
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 the efficiency of cell lysis microscopically.

D. Plant cell disruption by Cationic detergent

1. Grind 200 mg of plant tissue to a fine paste in approximately 500 µl 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 55o 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.
5. Detect the efficiency of destruction microscopically for obtaining the bioproduct.

Protein (enzyme) concentrate

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. (NaCl, $(NH_4)_2SO_4$, and $CaCl_2$).
2- Organic solvents precipitation (the most frequently used ethanol, methanol, and acetone).
3- Dialysis
4- Centrifugal filter unit, e.g. Amicon or Vivaspin (usually used for purified proteins).

Salt precipitation

Salt precipitation has been used for protein concentration because the salt ions interact so strongly with water, are low cost, have high solubility, and don't affect most proteins. The salt (ions) will attract H_2O molecules leaving protein particles to be aggregate and precipitate in a process called (salting out).

The most effective salt and widely used for the precipitation of proteins is $(NH_4)_2SO_4$. It has many advantages, such as high efficiency in protein precipitation, low cost, high solubility, and no effect on most enzymes.

The amounts of ammonium sulfate used are often expressed as percentage saturation, i.e., 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 the desired percentage saturation.

Procedure:

1-Take 10 ml of enzyme solution (crude extract) and add certain amounts (gms) of ammonium sulphate $(NH_4)_2SO_4$ to obtain the desired % saturation of the salt (20, 40, and 60 % etc.) obtained from standard tables.

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

3- Dissolve the precipitant in a small quantity of buffer solution, then measure the enzyme activity to the supernatant and add another quantity of $(NH_4)_2SO_4$ to get the 2nd, 3rd, and final saturation % according to the table.

Note: it is favourable 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 purification steps such as ion exchange chromatography.

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

Dialysis is one of the common operations in biochemistry to separate dissolved molecules by passing through a dialysis tube (semi-permeable membrane with specific molecular cutoff) according to their molecular dimensions. A semi-permeable membrane is used to remove small molecular components from protein solutions or concentrate the extract.

Procedure:

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

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

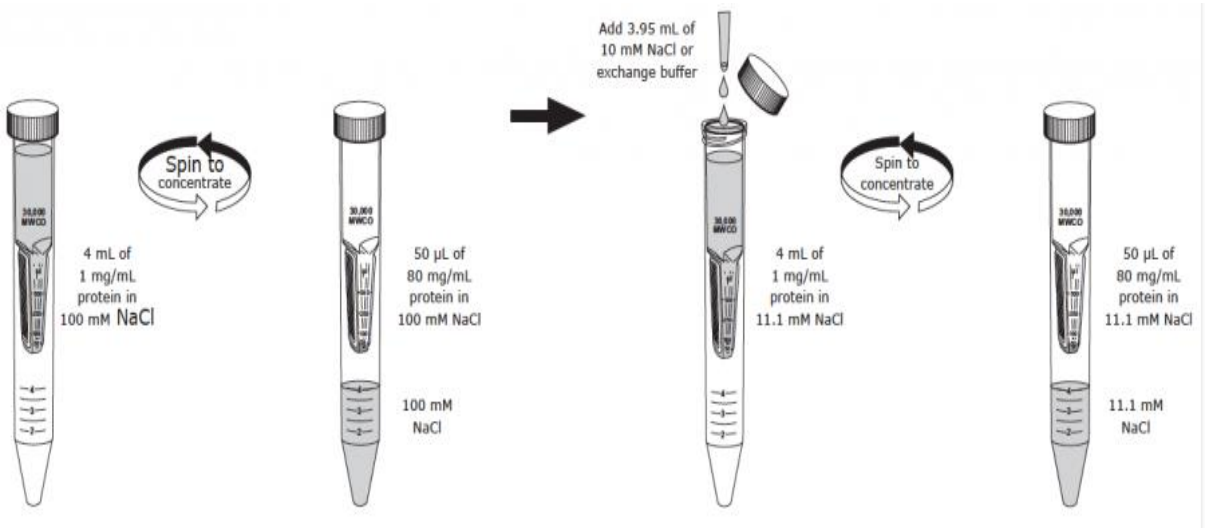
3-Pour the enzyme solution into dialysis tubes, leave a space in the tube (do not fill it to prevent it from rupturing and close the tube's second end.

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

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

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

- **Centrifugal filter unit, e.g. Amicon or Vivaspin**



Amounts of solid ammonium sulfate required to change the % saturation of 100 mL of solution: The table below is used to determine how much ammonium sulfate (in grams) must be added to 100 mL of solution to change from any particular % saturation to any other % saturation. Find the entry in the first column that gives the initial % saturation of your solution then find the entry in the top row that shows the desired final % saturation of ammonium sulfate for the solution. The value at the intersection of that row and column is the amount of solid ammonium sulfate that must be added to 100 mL of solution.

Final Concentration (% saturation at 0°C) of ammonium sulfate solution

Init %	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	10.6	13.4	16.4	19.4	22.6	25.8	29.1	32.6	36.1	39.8	43.6	47.6	51.6	55.9	60.3	65	69.7
5	7.9	10.8	13.7	16.6	19.7	22.9	26.2	29.6	33.1	36.8	40.5	44.4	48.4	52.6	57	61.5	66.2
10	5.3	8.1	10.9	13.9	16.9	20	23.3	26.6	30.1	33.7	37.4	41.2	45.2	49.3	53.6	58.1	62.7
15	2.6	5.4	8.2	11.2	14.1	17.2	20.4	23.7	27.1	30.6	34.3	38.1	42	46	50.3	54.7	59.2
20	0	2.7	5.5	8.3	11.3	14.3	17.5	20.7	24.1	27.6	31.2	34.9	38.7	42.7	46.9	51.2	55.7
25		0	2.7	5.6	8.4	11.5	14.6	17.9	21.1	24.5	28	31.7	35.5	39.5	43.6	47.8	52.2
30			0	2.8	5.6	8.6	11.7	14.8	18.1	21.4	24.9	28.5	32.3	36.2	40.2	44.5	48.8
35				0	2.9	5.7	8.7	11.8	15.1	18.4	21.8	25.8	29.6	32.9	36.9	41	45.3
40					0	2.9	5.8	8.9	12	15.3	18.7	22.2	26.3	29.6	33.5	37.6	41.8
45						0	3	5.9	9	12.3	15.6	19	22.6	26.3	30.2	34.2	38.3
50							0	3	6	9.2	12.5	15.9	19.4	23.5	26.8	30.8	34.8
55								0	3.1	6.1	9.3	12.7	16.1	20.1	23.5	27.3	31.2
60									0	3.1	6.2	9.5	12.9	16.8	20.1	23.9	27.9
65										0	3.2	6.3	9.7	13.2	16.8	20.5	24.4
70											0	3.2	6.5	9.9	13.4	17.1	20.9
75												0	3.3	6.6	10.1	13.7	17.4
80													0	3.4	6.7	10.3	13.9
85														0	3.4	6.8	10.5
90															0	3.4	7
95																0	3.5
100																	0

Purification of protein (enzymes)

Enzymes: are proteins specialized in catalyzing biological reactions.

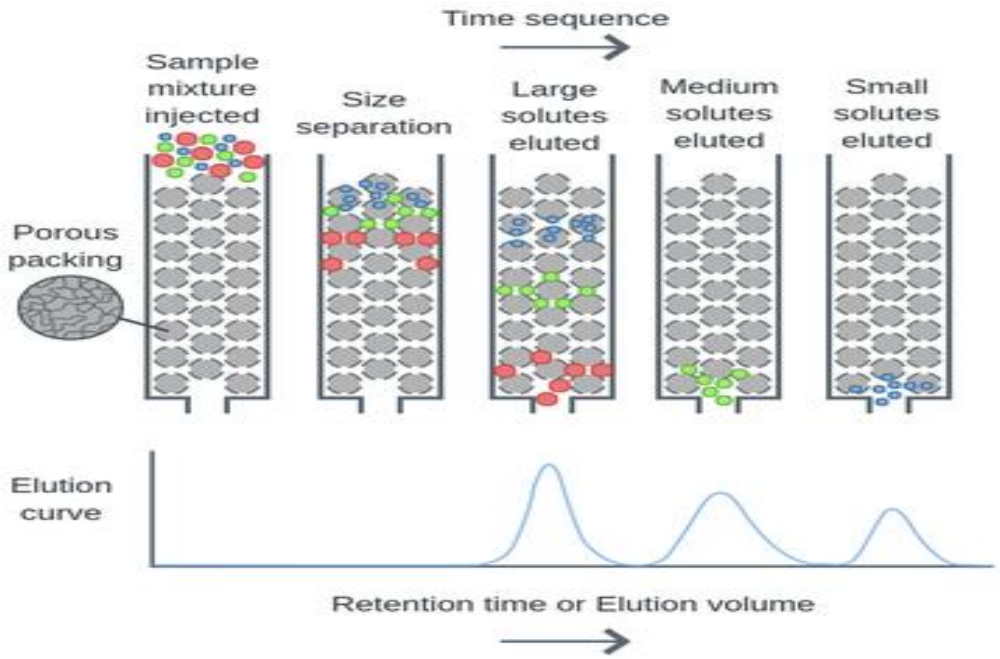
- Enzymes are found in all kinds of cells and may locate inside the cell (**Intracellular**) or secrete outside (**Extracellular**).
- Extraction methods differ according to the following:

1. Type of organisms (plant, animal, fungi, bacteria, etc.).
2. Location of an enzyme.

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

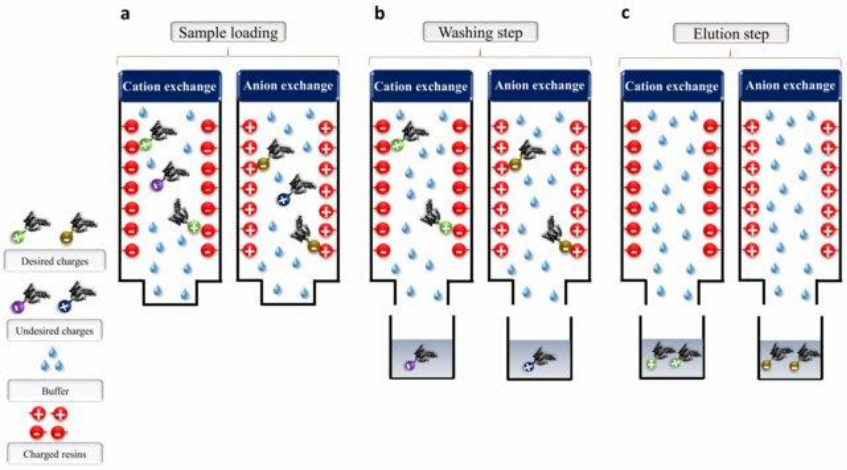
A- Gel Filtration Chromatography

It is known as gel permeation or size exclusion chromatography (SEC). The liquid phase passes through a porous gel which separates the molecules according to their sizes. The pores are generally tiny, excluding the larger solute molecules but allowing smaller molecules to enter the gel. This causes the larger molecules to pass through the column at a faster rate than the smaller ones. The column of the gel has 1 diameter to 20 lengths (1:20).



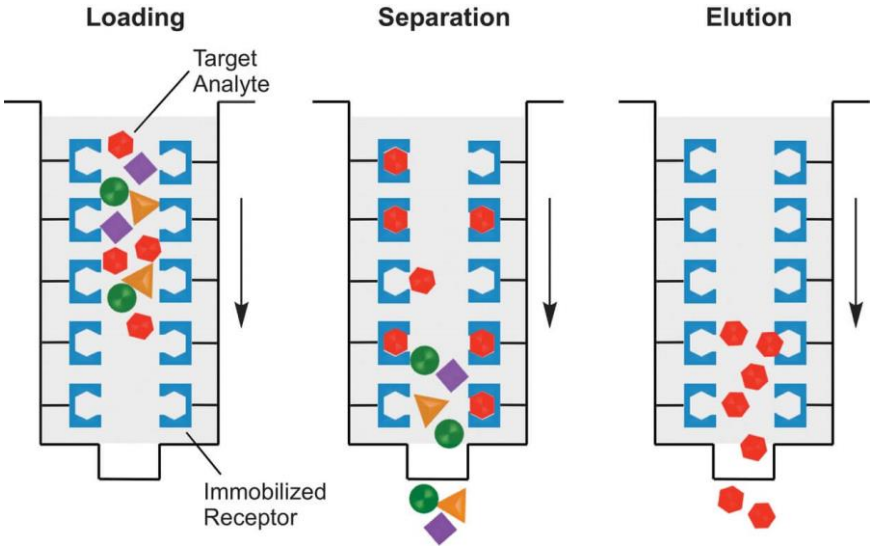
B- Ion-exchange chromatography

Separates proteins depending on the net charge of proteins in a gel carry either positive or negative charges. Proteins with the same net charge as the gel beads are repelled and flow out, whereas proteins with opposite charges will bind to the beads. Bounded proteins, negatively charged, are eluted by passing a gradient concentration of a salt solution (usually 1 -0.1M NaCl or KCl).



C- Affinity Chromatography

This is the most selective type of chromatography, and it utilizes the specific interaction between one kind of solute molecule and a second molecule immobilized on a stationary phase. The immobilized molecules may be antibodies to a specific antigen(s), enzyme, and substrate or an engineering protein with a specific purification tag.



The sample application in gel filtration chromatography

1. Remove the storage buffer from the column by washing the column with 2 column volumes of filtered D.W.

2. Prior to applying the sample, equilibrate the column with 2 column volumes of filtered elution buffer.

3. Apply the sample (protein solution) carefully with a Pasteur pipette on the top of the gel solution and allow entering the gel.

4. Add the filtered elution buffer above the gel. Connect the upper end of the column with the continuous supply of the elution buffer.

5. Run the filtered elution buffer through the column at the appropriate flow rate (1-2 ml per min)

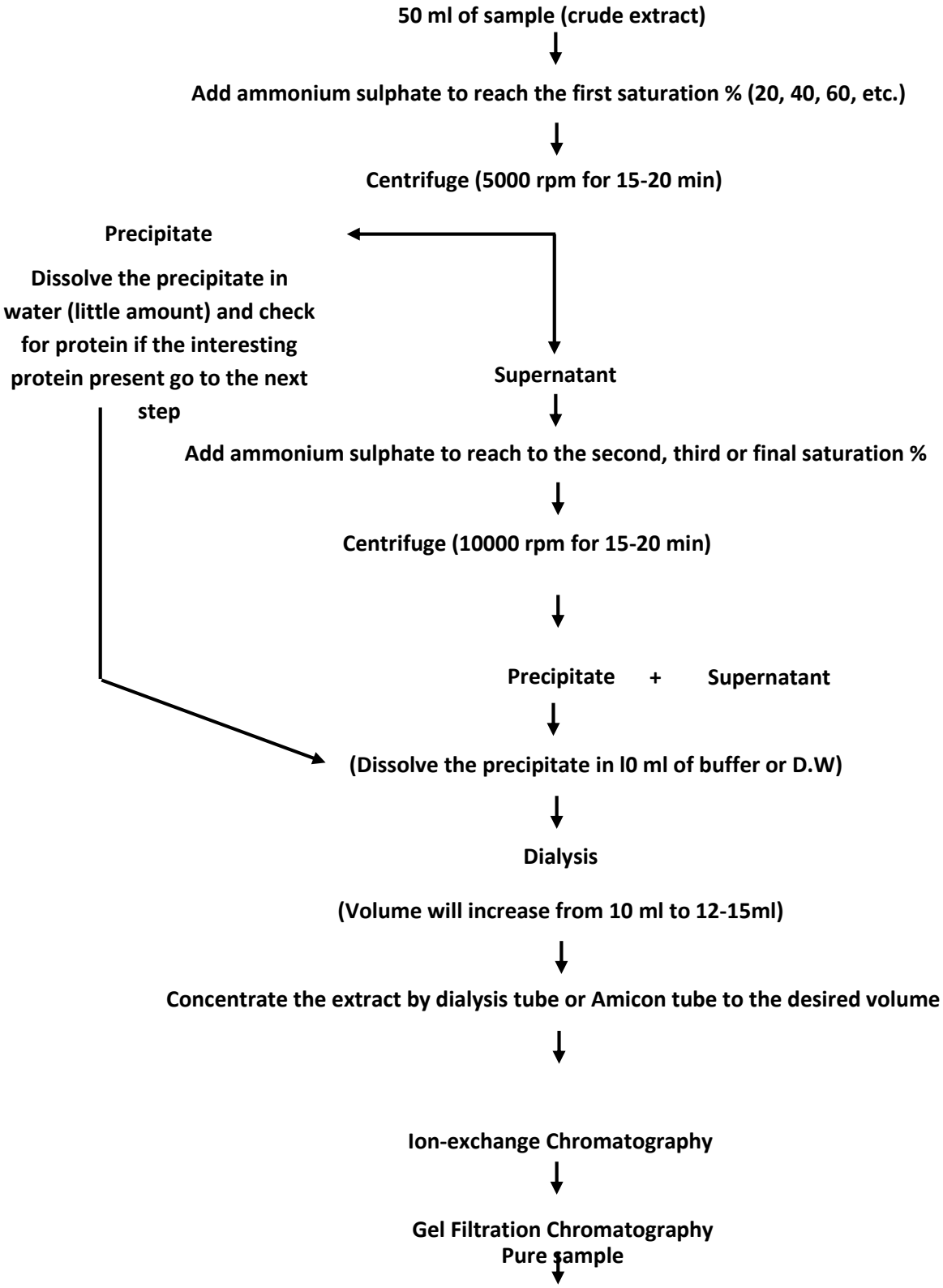
6. Collect the sample fractions eluted from the lower end of the column in tubes with the same volume of (2-5) ml. Close the lower outlet between the individual steps.

7. Measure the absorbance of diluted fractions at 280nm by UV spectrophotometer and draw the relationship between the number of fractions and absorbance.

8. Wash the column with 2 column volumes of filtered D.W.

9. Wash the column with 2 column volumes of the storage buffer containing an antimicrobial agent (Na-azide for a long storage period or 20% ethanol for a short storage period). Keep the column with a storage buffer, close the column outlet and store it at 4 C°.

Note: after each step of purification, the activity of the protein must be measured.


50 ml of sample (crude extract)
Add ammonium sulphate to reach the first saturation % (20, 40, 60, etc.)
Centrifuge (5000 rpm for 15-20 min)
Precipitate
Dissolve the precipitate in water (little amount) and check for protein if the interesting protein present go to the next step
Supernatant
Add ammonium sulphate to reach to the second, third or final saturation %
Centrifuge (10000 rpm for 15-20 min)
Precipitate + Supernatant
(Dissolve the precipitate in l0 ml of buffer or D.W)
Dialysis
(Volume will increase from 10 ml to 12-15ml)
Concentrate the extract by dialysis tube or Amicon tube to the desired volume
Ion-exchange Chromatography
Gel Filtration Chromatography
Pure sample

Immobilization of a biological system

It is a technique of binding a biological system (cells or their derivatives, spores, enzymes, etc.) to an inert, insoluble carrier material. It allows the biological system to be held in place throughout the reaction. Many microorganisms own the capability to adhere to different kinds of surfaces in nature to get close to nutrients and easily supply food. Therefore, we can say that these natural systems are immobilized in their natural state. Many biotechnological processes need to be carried out using the immobilization of biocatalysts.

Why do we use immobilization?

1. Easy separation of a biological system from the product.
2. Reuse of biological system or continued use.
3. It increases the activity and stability of the biological system.
4. To retain high concentrations of the biological system within a certain defined region of space.

Materials used in Immobilization:

1. Organic materials: Ca-alginate, agar, polyacrylamide, etc.
2. Inorganic materials: glass, silicates, polystyrene, etc.

Methods of Immobilization

1- Physical Methods:

- Encapsulation: inside a semi-permeable membrane.
- Entrapment: within a porous matrix.
- Adsorption: in solid supports.

2- Chemical Methods:

- Cross-linking: with suitable agents to give insoluble particles.
- Covalent binding: to the surface of water-insoluble materials.

Encapsulation in insoluble Ca-alginate gel

It is recognized as a rapid, non-toxic, inexpensive, and versatile, the most often used method for the immobilization of cells (more than 80% of cell immobilization processes are still carried out using alginate).

Alginate is a kind of polysaccharide produced by some kinds of algae and bacteria. In the presence of monovalent cations, alginates form water-soluble salts, but with polyvalent cations, such as Ca^{2+} and Ba^{2+} they form an inert polymer network by binding the polyvalent cation (usually Ca^{2+}). This property of alginates is used for entrapping living cells in the alginate matrix.

LAB Work

A. Immobilization of amylase-producing fungal cells or spores by entrapment: (using organic material agar)

1. Prepare a thick spore suspension of *Aspergillus niger* by adding a few milliliters of D.W. to a culture of this fungus (on a slant), shake well, then transfer the suspension into a clean tube or flask.

2. Prepare 2-3% agar, sterilize by autoclaving, then cool to 40-45C°.

3. Mix 2-4ml of the suspension with 10-20 ml of melted agar and pour into a sterilized petri dish, leaving for some time to solidify the agar.

4. Cut the agar into small squares or discs with a sterile tool.

5. Collect the agar pieces (containing the fungal spores) in a clean flask and wash with D.W. or a suitable buffer many times to remove the unentrapped spores.

B. Immobilization of yeast cells, spores or enzymes by encapsulation with Ca-alginate.

1. Suspend the cells by D.W.
2. Prepare alginate solution 2% by dissolving 2g of sodium alginate in 100 ml D.W.
3. Prepare calcium chloride solution (0.4M) by dissolving 2.22g of $CaCl_2$ in 50ml of D.W.
4. Mix cell suspension with sodium alginate solution to form sodium alginate-cell solution.
5. Drop sodium alginate-cell solution by 1ml syringe into $CaCl_2$ solution with continuous stirring to form Ca-alginate beads with cells.
6. Collect Ca-alginate beads (containing the cells) in a clean flask and wash with D.W. or a suitable buffer many times to remove the unentrapped cell.

C. Immobilizes the enzyme (amylase) by adsorption on a solid support.

1. Extract the amylase from fungal (e.g. *A.niger*) or bacterial (e.g. *Bacillus subtilis*) culture.

2. Mix 3ml of enzyme solution with 5g of the solid support (e.g. silica gel, glass beads, charcoal, plastic particles, etc.) with stirring for 1-2hrs. (or more)

3. Discard the liquid solution by filtration, take the solid particles (containing the immobilized enzyme), and wash with D.W.to remove the unbounded enzyme molecules.

4. Perform the enzyme reaction: Add 5ml of starch solution to the immobilized enzyme, incubate for 15-30min, then add iodine solution to observe the colour.

Production of Amylase

What are amylases?

Amylases are enzymes that break down starch or glycogen. Amylases are produced by various living organisms, ranging from bacteria to plants and humans. Bacteria and fungi secrete amylases outside of their cells to carry out extracellular digestion. When they have broken down the insoluble starch, the soluble end products, such as (glucose or maltose) are absorbed into their cells.

Amylases are classified based on how they break down starch molecules

i. **α-amylase** (alpha-amylase) - Reduces the viscosity of starch by breaking down the bonds at random, therefore producing varied-sized chains of glucose (**dextrins and maltose**)
ii. **ß-amylase** (Beta-amylase) - Breaks the glucose-glucose bonds down by removing two glucose units at a time, thereby producing **maltose**
iii. **γ-amylase** (gamma amylase) - Breaks successive bonds from the non-reducing end of the straight chain, producing **glucose**

Many microbial amylases usually contain a mixture of these amylases.

Microbial amylases are used for the following purposes:

1. High Fructose Corn syrup preparation
2. Additives to detergents for removing stains
3. Saccharification of starch for alcohol production
4. Brewing

What organisms are responsible for amylase production?

Although many microorganisms produce this enzyme, the ones most commonly used for their industrial production are *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquifaciens* and *Aspergillus niger*

Lab work

❖ **Production of amylase using classical method**

- A strain of *Bacillus subtilis* (producing α- amylase) is cultured in a starch-containing medium to induce enzyme production and incubated at 37-40C˚ for 24-48 hrs.
- Extract the enzyme from the solid culture media by adding buffer (e.g phosphate buffer with a ratio 1:3 (solid medium: water). When the medium is liquid, there is no need to add 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).

Measure the enzyme activity:

Add 1 ml of crude enzyme solution to 4ml of 1% starch, incubate at 35°C for 15 min, then add a few drops of iodine and notice the disappearance of blue color, compared with control (solution of 1% starch and iodine without enzyme).

❖ Concentrate and partially purification using Salt precipitation $(NH_4)_2SO_4$

(see lab 3 and 4)

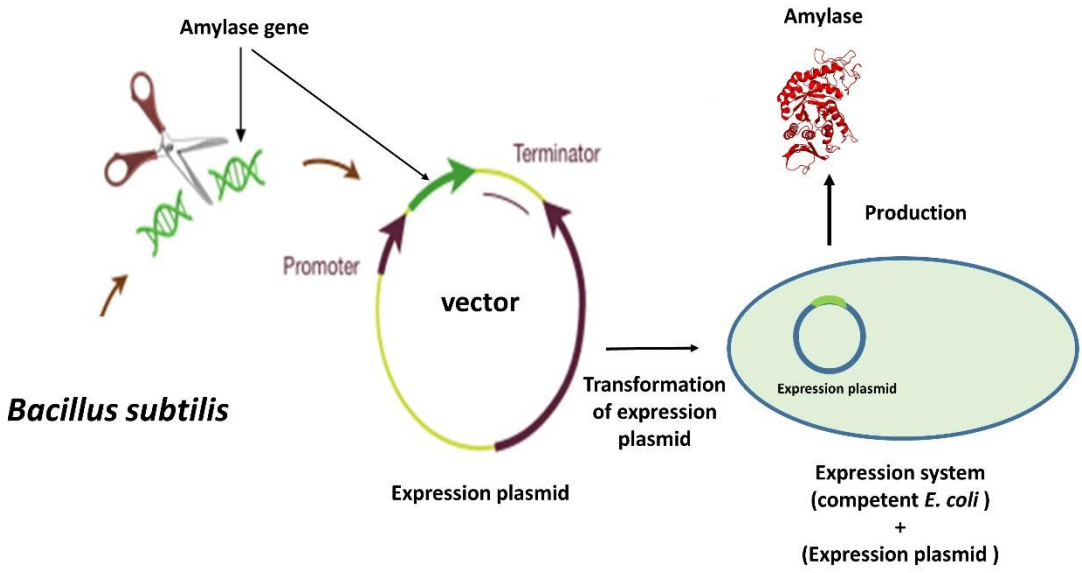
❖ Immobilize the amylases using alginate or agar.

(see lab 6)

❖ Production of amylase using recombinant DNA technology

A recombinant protein is a protein that is encoded by recombinant DNA, which has been cloned into an expression system that allows the gene to be expressed and messenger RNA to be translated. Modifying the gene by recombinant DNA technology can lead to the expression of a mutant protein with desirable properties. A recombinant protein is an altered version of a native protein produced in a different environment to **enhance protein production**, **change gene sequence**, and **create commercially viable products**.

Amylase can be cloned into an expression vector and transformed into a competent *E. coli* strain by genetic engineering technology. This allow the production of amylase in large amount during a short time production.



❖ Preparation of bioreactor containing the immobilized enzyme or cells:

A bioreactor is any container or vessel in which a biological reaction is carried out.

The immobilized cells or enzymes can be used in bioreactors to achieve industrial and biotechnological processes. There are many kinds of immobilized bioreactors, such as gas lift bioreactors, packed bed reactors and stirred tank reactors, and others

To prepare a small and simple bioreactor for the laboratory experiments, apply the following steps:

1- A clean glass column or cylinder (with open sides) is used; the lower side is closed by a rubber stopper or plastic adapter connected with a rubber tube containing a screw to adjust the flow rate.

2- Pour or fill the column with the immobilized cells or enzymes carefully while the lower tube is closed.

3- Add the substrate solution (e.g. starch for amylase, protein solution for protease and so.) from the upper side, and collect the liquid (product) eluted from the column (bioreactor).

4- Measure the enzyme activity (by iodine solution for amylase or hydrolysis of protein for protease).

Production of Ethanol from Microorganisms

Ethanol is one of the most important chemicals in the industry from an economic perspective, as it comes immediately after water in terms of using it as a solvent in laboratories and chemical industries.

Alcohol was produced previously from the distillation of fermented plant materials, but it was costly in economic terms, so industrialists turned toward using microorganisms in the production. Yeast is the first microorganisms used, such as *Saccharomyces cerevisiae* and *Saccharomyces uvarum* also *Clostridium thermocelum,* which is an anaerobic and thermophilic bacterium that has the ability to produce ethanol by converting waste cellulose directly to alcohol.

Molasses (waste of the sugar industry), dates and debbis are the most important commercial production media for ethanol production as well as agricultural carbohydrates such as maize, potatoes and barley.

Experimental method

1. Preparation of ethanol production medium (Date extract medium): mix 100gm from date with 100ml tap water, heat at 80°C with constant stirring for 30min.
2. Filter through many layers of cheese cloth, mix the residue with water by blender and repeat the filtration again.
3. Estimate the reducing sugars concentration in the filtrate using the DNSA solution (3, 5 Dinitro Salicylic acid), then sterilize the filtrate and use it as an ethanol production medium.
4. Inoculate the medium (Date extract medium) with 1% *Saccharomyces cerevisiae* culture grown in Malt extract broth and incubate at 30°C for 48hrs, during the first 6 hours the medium is aerated by using an air pump or shaker incubator then stop aeration to convert the condition to anaerobic which is suitable for ethanol production.

Ethanol detection methods

1. Ammonium Cerric Nitrate (ACN) method

1. Take 0.5ml of ACN, add 3ml of D.W to it (dilution)

2. Add 3-5 drops from the sample (yeast fermented medium) to diluted ACN; the appearance of red color indicates the presence of ethanol.

0.5 ml ACN+ 3 ml D. W+ (3-5) drops from sample ⟶ Red color

2.Potassium dichromate ($K_2Cr_2O_7$) method

Ethanol is detected as follows:

1. Add 5 ml of $K_2Cr_2O_7$ solution to 1ml of concentrated sulfuric acid and heat the mixture slowly.

2.Add 1ml of the sample (yeast fermented medium) to the mixture. The positive result is the appearance of a green color with Acetaldehyde odor.

Heating

5ml $K_2Cr_2O_7$ + 1ml concentrated H_2SO_4 ⟶ 1ml from sample

Green color +Acetaldehyde odor ⟵

Estimation of ethanol concentration

- **Physical methods**

A) Determination of Boiling point

B) Estimation of ethanol by Pycnometer

1. Prepare different concentrations of ethanol ranging from 0.5 to 10% by adding the required volume of absolute ethanol and water to a 100 ml flask (apply the equation C1V1=C2V2.

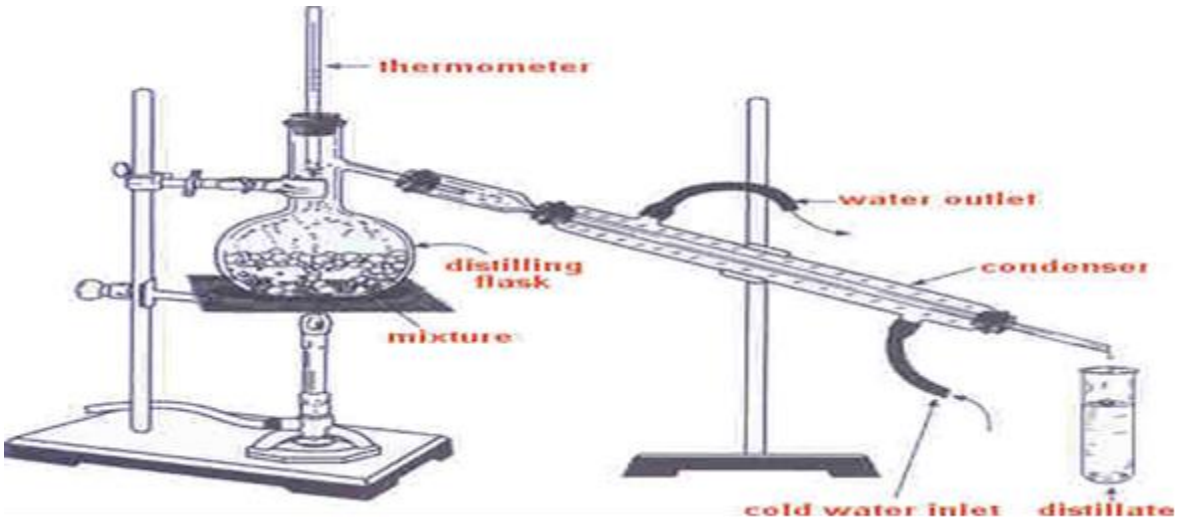
2. Withdraw 50 ml from each concentration and mix with 25 ml of D.W in a 500 ml distillation flask fitted with a 40cm condenser and collects the distillate in 50 ml graduated cylinder.

3. Apply the same process to the sample (fermented medium).

4. Determine the specific gravity of ethanol for each concentration as well as the sample with a pycnometer and calculate the specific gravity by the following formula:

$$\textbf{Specific Gravity} = \frac{\text{Weight of pycnometer with the sample – its weight without sample (empty)}}{\text{Weight of pycnometer with water - its weight without water (empty)}}$$

5. Compare the result with other concentrations and the specific gravity for the sample similar to one of them and thus know the concentration of the sample.



- **Chemical methods**

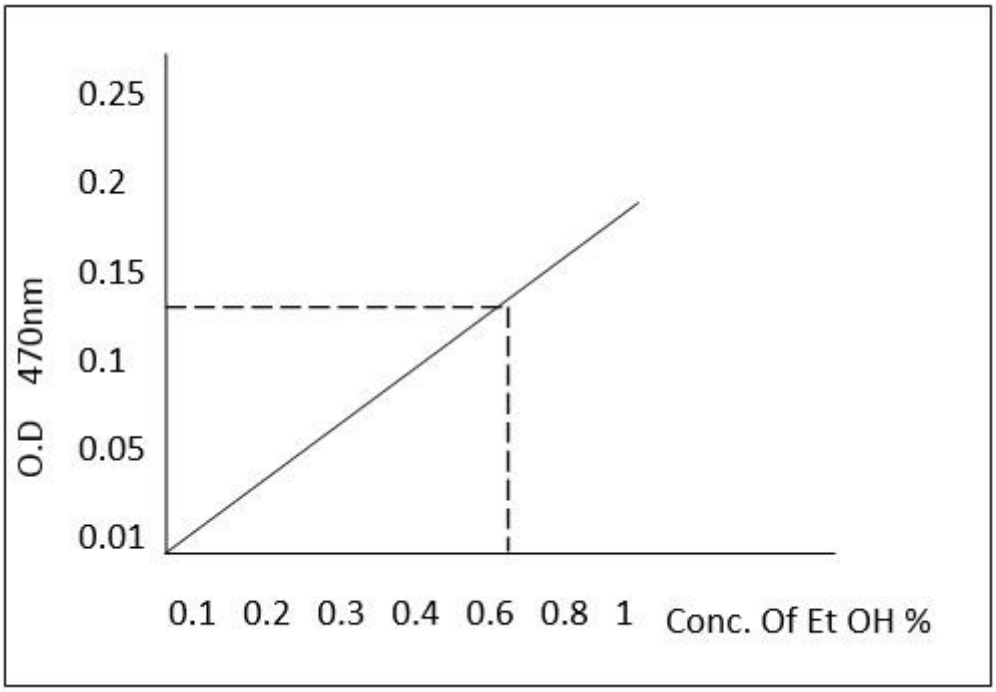
A) Preparation of Ethanol standard curve

1. Prepare different concentrations of ethanol ranging between 0.1-1% from absolute ethanol.

2. Add 2 ml of Ammonium Ceric Nitrate (ACN) to 5 ml of each concentration and leave for 5 min to complete the reaction.

3. Read the absorbance at 470 nm by spectrophotometer; the blank consists of distilled water and ACN (without ethanol).

4. Apply the same steps on the sample to be estimated and compare the result with the standard curve to determine the concentration of alcohol in the sample as shown in the figure.



B) Ethanol estimation by Potassium dichromate $K_2Cr_2O_7$

1. Transfer 1ml of the sample (yeast fermented medium) to a flask fitted with a connected glass tube reached to the bottom of another tube containing 5ml of ($K_2Cr_2O_7$ + H_2SO_4) solution and place in ice bath.

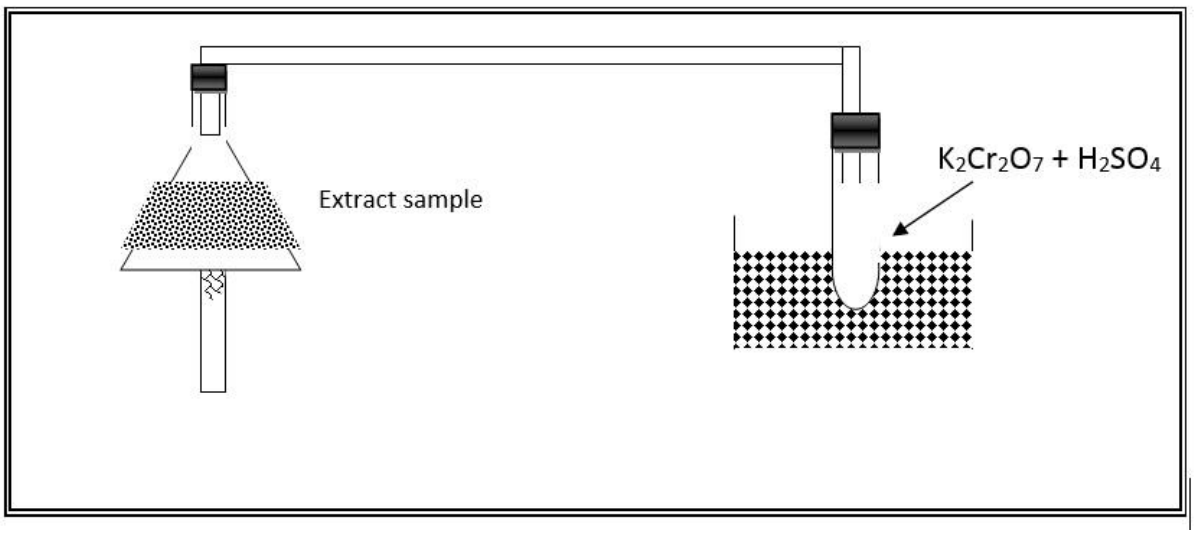
2. Heat the flask containing the sample on a flame and boil until dry.

3. Transfer the content of the tube containing (potassium dichromate and H_2SO_4) to another beaker and titrate with Mohr solution (sulfuric acid and ammonium ferric sulfate) after the addition of Diphenylamine reagent.

4. Stop the titration when the color turns to bluish-green and record titration volume; calculate the percentage of ethanol as follows:

$$\text{Et OH\%} = \frac{\text{(Read Mohr solution after titration with blank - read Mohr solution after titration with sample)}}{\text{The volume of yeast fermented medium x read Mohr solution after titration with blank}} \times 100$$

Note: Blank is consisting of (Diphenylamine + Mohr + $K_2Cr_2O_7$).



Manufacture of Antibiotics (Penicillin production)

Antibiotics are manufactured using the fermentation technique, where microorganisms are inoculated in large vessels (fermentor) containing the necessary media.

There are two types of fermentation

1- Batch fermentation

2- Continuous fermentation

In batch fermentation, a volume of sterile medium is placed in a vessel, inoculated with microorganisms, and the broth is fermented for a definite period. The tank is then emptied, and the antibiotic is extracted. The vessel is then rebatched with medium to repeat the process. Duration can be hours or days. It is used widely in the manufacture of antibiotics.

In continuous fermentation, a sterile medium is added to the fermentor without interruption to the fermentation system with balancing withdrawal (harvesting) of broth for product extraction. The duration is weeks to months. It is widely used in brewing and single-cell-protein production.

Production of Benzyl Penicillin

The organism

The original organism for the production of Penicillin was *Penicillium notatum*, which was isolated by Fleming in 1926. Today, penicillin is produced by *P. chrysogenum*. Mutagenic variants produce larger amounts of penicillin than the original wild strain that has been selected and maintained. Production strains are stored in dormant form using culture preservation techniques.

All laboratory manipulations are carried out in laminar flow cabinets; operators wear sterilized clothing and work aseptically.

Oxygen supply

Supplied as filter-sterilized air from a compressor. Air is introduced at the bottom of the fermenter via a ring sparger that breaks the flow into a myriad of bubbles and increases the transfer area. These bubbles lose oxygen as they rise up the tank. At the same time, CO_2 diffuses into them.

Impellers also help the transfer of oxygen. Baffles are also used to promote intimate contact with cells and nutrients.

Temperature control

Since penicillin is very sensitive to heat generated by metabolism, the temperature has to be reduced by controlled cooling. This is achieved by circulating chilled water through banks of pipes inside the vessel.

Defoaming agents and instrumentation

During vigorous mechanical stirring and aeration, the microbial cultures foam. This causes loss of culture by entrapment in exhaust gases. Defoaming agents are added, and temporary backpressure is applied to contain the culture within the vessel.

Media additions

Nutrients required may be added during the process

Transfer and sampling systems

Aseptic systems are provided to transfer the inoculum to the vessel, to allow the taking of routine samples during fermentation and to transfer the final contents to the extraction plant. Sampling monitors growth and levels of nutrients and concentration of penicillin. Also, microbial contaminants must be avoided.

Control of fermentation

Aeration must be controlled. A fall in oxygen causes reduced production of penicillin G while cultures continue to grow. Growth should be limited by controlling key nutrients (sugar)

Medium

Corn steep liquor, a by-product of the maize starch industry, is used to provide nutrients such as nitrogen and carbon compounds such as acids, sugar ions and growth factors.

The medium is sterilized with steam at 120 °C either in the fermenter itself or in an ancillary plant.

The medium contains calcium, magnesium, sulfate, phosphate, potassium and trace metals.

Fed nutrients

The sterile medium is stirred and aerated, and its pH and temperature are set. Then inoculated.

All feeds must be sterilized to avoid contaminants (b-lactamase-producing bacteria) that can destroy penicillin or utilize nutrients intended for the fungus.

Stimulation by phenylacetic acid

Supply-side of benzyl penicillin

Simulates the synthesis of benzyl penicillin and prevents other unwanted forms of penicillin. Amino acids that build penicillin are a-aminoadipidic acid, cysteine, valine and phenylacetic acid.

Termination

Are done when raw materials (sugar) are thought to be exhausted and no more conversion to penicillin

Extraction

Removal of cells ⟶ Cells are removed by filtration ⟶ Isolation of Penicillin G by solvent extraction using amyl acetate, butyl acetate etc. and an aqueous buffer ⟶ Then solvent is recovered by distillation ⟶ Treatment of crude extract ⟶ Formation of appropriate salt ⟶ removal of pyrogens ⟶ and packaging.

Production of citric acid by *Aspergillus niger*

Citric Acid $C_6H_8O_7$ nH_2O (n=1 or 0) Mol. Wt. monohydrate =210.14

Anhydrous = 192.13

Citric acid is a weak organic acid. It is a natural preservative/conservative and is also used to add an acidic or sour, taste to foods and soft drinks and cakes. It can also be used as an environmentally benign cleaning agent. It is very corrosive to the touch of the skin and can burn severely.

In biochemistry, the conjugate base of citric acid, citrate, is important as an intermediate in the citric acid cycle, and therefore occurs in the metabolism of virtually all living things.

Citric acid exists in greater amounts in a variety of fruits and vegetables, most notably citrus fruits. Lemons and limes have particularly high concentrations of the acid. At room temperature, citric acid is a white crystalline powder. It can exist either in an anhydrous (water-free) form or as a monohydrate. Certain strains of the mold *Aspergillus niger* could be efficient citric acid producers. In this production technique, which is still the major industrial route to citric acid used today, cultures of *A. niger* are fed on a sucrose or glucose-containing medium to produce citric acid. The source of sugar is corn steep liquor, molasses, hydrolyzed corn starch or other inexpensive sugary solutions. After the mold is filtered out of the resulting solution, citric acid is isolated by precipitating it with lime (calcium hydroxide) to yield calcium citrate salt, from which citric acid is regenerated by treatment with sulfuric acid.

Commercial production of citric acid is generally by submerged fermentation of sucrose or molasses using the filamentous fungus *A. niger* or synthetically from acetone or glycerol.

In recent times, solid state fermentation (SSF) as an alternative to submerged fermentation in the production of microbial metabolites. Solid-state fermentations refer to the cultivation of microorganisms in a low-water-activity

environment on non-soluble materials acting as both a nutrient source and physical support. The major advantages of solid-state fermentation over submerged fermentation include higher yields, low water requirement and lower operating costs.

Many microorganisms have been evaluated for the production of citric acid, including:

- Bacteria such as *Bacillus licheniformis*, *B. subtilis*, *Corynebacterium spp.*
- Fungi such as *A. niger*, *A. awamori*, *Penicillium restrictum*
- Yeast such as *Candida lipolytica*, *C. intermedia* and *Saccharomyces cerevisiae*

However, *A. niger,* a filamentous fungus, remained the organism of choice for citric acid production due to

1- Ease of handling
2- Its ability to ferment a variety of cheap raw materials
3- High yields.

Materials and methods:

1- Preparation of inoculum (*A. niger* spore suspension) *A. niger* stock culture is reactivated and cultivated by streaking a loopfull of the culture on Petri dishes or slants containing potato dextrose agar (PDA) incubated at 25° C for 5 days.

- Spores suspension is prepared by the addition of 10 ml distilled sterilized water to the *A. niger* culture shake well and use this suspension to inoculate the production media.

2- Production medium (molasses): the molasses (beet or cane molasses) is diluted with water to a sugar content of 12-20%...some nutrient salts may added to support acid production; adjust pH: The initial pH of the fermentation culture is adjusted to 3 using 1 N of HCl and/or NaOH sterilize the medium.

3- Inoculation: Inoculate the medium with spore suspension, adding 5% 5ml of spore suspension to 100 ml medium and incubate at 25-30°C for 5-7 days.

4- Extraction and recovery of citric acid : filter the culture to remove the mycelia and solid particles and take the filtrate(containing citric acid). Recovery of CA: the usual procedure is to precipitate CA from hot neutral aqueous solution followed by the addition of sulfuric acid to remove the calcium as Ca-sulfate.

Procedure : the filtered broth ml is treated with Ca(oH)2 Ca citrate will be precipitated filter off and treated with H_2SO_4 to precipitate Ca as insoluble sulfate and release the citric acid.

Take 25-50 ml of culture filtrate add Ca(oH)2 solution with heating in a water bath until pH becomes neutral and a precipitate forms filter collect the precipitate (Ca- citrate) on the filter paper in a beaker add 2M H_2SO_4 at a ratio 1:8 (g of ppt: ml H_2SO_4) filter to remove the precipitate (Ca- citrate) the filtrate solution will contain the dissolved citric acid evaporate to obtain CA crystals calculate the weight of CA crystals (measure the dry wt of the container before and after this step, wt of CA= wt of the container with crystals (after evaporation) – wt of the container. Calculate the concentration of CA as gm/ml , gm/L or as % (gm/100ml of medium or sample)

5- Citric Acid Determination

Citric acid (CA) is determined titrimetrically by using 0.1 N NaOH and phenolphthalin as indicator :

10 ml sample + 2-3drops of phenolphthalein titrate with 0.1N NaOH until a pink color is appeared; record the volume of titritable NaOH.

Assay of CA: Calculate on the anhydrous basis.

1 ml of (0.1N) sodium hydroxide = 6.404 mg of Citric Acid ($C_6H_8O_7$)

Example: if the volume of the sample (containing CA) is 10 ml and the volume of titritable NaOH= 3ml therefore mg of Citric Acid in the sample (10ml) = 3x 6.404 Calculate in 1ml or in 100(%)?

6- Determination of sugar concentration

Estimate the sugars (or total carbohydrates) in the medium before and after the fermentation process (medium before and after culturing) by phenol -- H_2SO_4 method as follows:

1) Prepare a standard curve of sugar: prepare a certain concentration of glucose (0- 100 Micro gm/ml)

2) Take 1ml of each concentration + 1ml of 5% phenol solution + 5 ml conc. H_2SO_4 (added carefully on the glass tube wall) cool in an ice bath read the O.D at 490 nm, the blank is 1ml of DW+ reagents plot the relationship between the O.D and glucose concentrations standard curve obtain the slope of the st. curve:(= y2 – y1/x2-x1)

3) Take 1ml of the samples (sample for the medium before culturing and sample for medium after culturing) and treat it as above read O.D at 490 and calculate the concentration of the sugars depending on the standard curve (Note: the unit is micro gm , you can convert it to mg or gm as needed)

7- Estimation of CA yield: the yield is calculated as % of the product (CA)/ consumed substrate (sugar)

$$\textbf{Yield of CA\%} = \frac{\text{conc. of CA} \quad X \quad 100}{\text{conc. of consumed sugar(medium sugar conc.-residual sugar after fermentation}}$$