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College of Science
Department of Biology

Practical Biotechnology
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المرحلة الرابعة – الدراستين الصباحية والمسائية

الفصل الدراسي الثاني

تدريسي المادة:

أ.م.د. ولاء شوكت علي

أ.م.د.محمد لفتة عطا الله

أ.م.د.علياء رزوقي حسين

أ.م. ديمه نزار فرج

أ.م.د. نجلاء نبهان ياسين

م.د. شيماء فاضل

م.د. حسام صباح اوهيم

م.د. شمم ناصر راضي

م. حسام محمود

Isolation& screening of Microorganisms

Biotechnology: is a technology that involves the use of living organisms, cells or cellular components for the production of biomaterials or could be defined as a precise genetic improvement of living organisms for the benefit of human beings.

Obtaining of living organisms for biotechnology

All living organisms (such as animals, plants and microorganisms that represent the important parts of biological system) can be used in biotechnology but the most common ones are the microorganisms. In specific, the important microorganisms utilised in biotechnology are bacteria, actinomycetes, fungi and algae. These organisms exist virtually everywhere, e.g., in air, water, soil, surfaces of plants and animals, and tissues of plant 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 particular organizations, which maintain culture collections, e.g., American Type Culture Collection (ATCC).

- **The first step** in obtaining a producer strain is the **isolation** of the microorganisms of interest from their **natural habitats**.
- **The second step** is **screening** the isolated M.Os.

M.Os. Screening: involves highly selective procedures used to allow further isolation & detection of **high-yielding M.Os** with industrial importance among large microbial population.

- During screening process many valueless M.Os. will be discarded.
- To provide a cell concentration with small amount of inoculation (except **crowded plate technique**), e.g. soil is diluted then their **aliquots are applied by spreading** on a nutrient agar surface.
- **Aliquots spreading: is a method in which the inoculum is sprayed or applied in some manner on the surface of the agar plates will yield well isolated colonies (30-300).**

Selection of the suitable M.Os. to produce the desired product. Such M.Os. must be:

1- High-yielding strain.
2- Have stable biochemical/genetical characteristics.
3- Should not produce undesirable substances.
4- Easily cultivated on large-scale.

Lab. screening techniques:

1- Primary Screening for Organic Acid/Amine Producers.

Soil sample is serially diluted then inoculated on plates of **Neutral Red Agar, Bromocresol Green Agar or $CaCO_3$ Agar**. After that, the plates are incubated at suitable temperature for a specific period of time.

a- Growth of Acid/Amine producers will change the colour of the indicating dye near the producing colonies.

b- Positive results:

Neutral Red Agar – (Nutrient Agar + Neutral Red)

The positive results on Neutral Red Agar show that Acid Producing colonies change the colour of the medium to **Red,** while Amine Producing colonies change the colour of the medium to **Yellow**.

Bromocresol Green Agar – (Nutrient Agar + Bromocresol Green)

The positive results on Bromocresol Green Agar show that Acid Producing colonies change the colour of the medium to **Yellow**, while Amine Producing colonies will not change the colour of the medium so it remains **Green**.

$CaCO_3$ Agar medium – (Nutrient Agar + 1-2% Calcium Carbonate)

Production of Organic Acid is detected by forming a clear zone due to dissolved $CaCO_3$ around the producing colony.

c- Disadvantages of this method:

e.g. If the Nitrogen source of the medium is **Ammonium Sulphate**, the M.O. may utilize the Ammonium ion leaving sulphate as Sulphuric acid**, ,** which gives **false result**, so further testing for the +ve results must be done to ensure the production of the desired product.

2- Primary Screening for Antibiotics Producers (Crowded plate techniques).

a- Serial dilution of the microbial source (e.g. soil **10^{-1} -10^{-8}**) is prepared then **0.1-0.2ml** is applied to the **nutrient agar** plates to be incubated in a suitable conditions **30˚C** for **2-5 days**.

b- Colonies showing antimicrobial activity are indicated by forming an inhibition zone surrounding the productive colony, which must be sub-cultured to be purified & tested against a sensitive M.O. (Test M.O.).

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

d- After that, a suspension of the test M.O. is prepared then sprayed on the surface of agar. Agar blocks, which are obtained by Pasteur pipette or a sterile cork borer and contained the antibiotic producing M.O., 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 Wells method will take place to detect the inhibition zones).

e- Disadvantages of this method are that of inhibition zones might be formed by a **change in pH values** resulting from the metabolism or rapid utilization of critical nutrients. This method also has limited applications because the isolated colonies have antimicrobial activity against specific M.O. but not others.

3- Primary Screening of Growth Factors (Amino Acids, Vitamins) Producers (Auxanography).

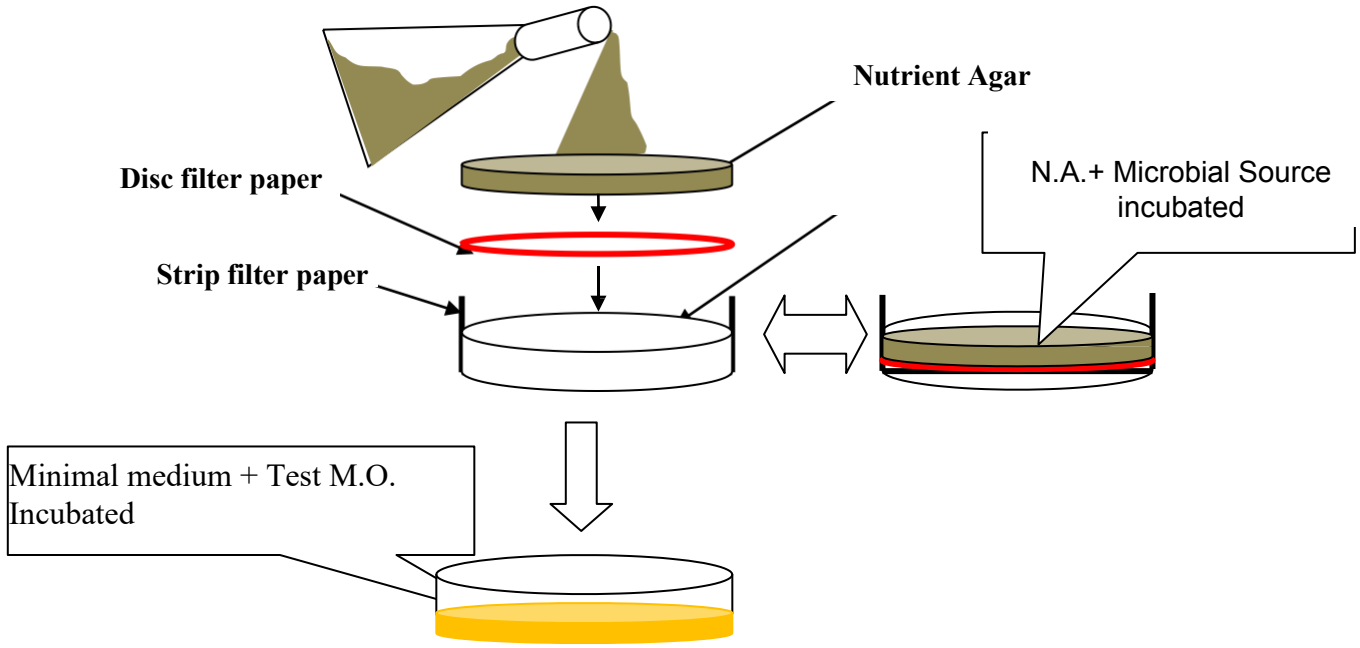
a- In this technique, 2 types of medium plates are prepared: In order to prepare the 1st plate, nutrient agar medium is poured on a **disc filter paper** placed over a **strip filter paper**.

b- Microbial source material such as soil is diluted to be inoculated on the prepared nutrient agar.

c- To prepare the 2nd plate, a minimal medium lacking a growth factor is inoculated with a test M.O.

d- Nutrient Agar prepared in step (a) is lifted aseptically by tweezers & spatula to be placed over the inoculated Minimal medium prepared in step (c). The growth factors produced in nutrient agar can diffuse into the lower layer of agar containing the test M.O. to stimulate its growth.

e- Productive colonies will be sub-cultured for further testing.



4- 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 dissolving 10g of skim milk + 2g of Agar in 100ml of D.W., Medium should be autoclaved & cooled in order to add the skim milk, to avoid **Casein Denaturation** (Protein content of Milk & the substrate for Protease production).

c- Diluted microbial source (soil) or another source (meat, cheese or legumes) is inoculated on Milk agar medium.

d- **Positive results** appear as **clear zones** around the colonies referring to the utilization of Casein.

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

f- Protease enzyme has industrial applications

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

5- Isolation & Screening of Amylase Producing M.Os.

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

b- Diluted microbial source is inoculated with 0.1-0.2ml on Starch Agar Medium incubate at 30-40˚C for 24-72hrs.

c- **Starch Agar Medium** (Nutrient Agar +1% Starch) can be used to isolate amylase producing M.Os. that can hydrolyze starch in the medium to dextrin, maltose or glucose.

d- Iodine is used to detect the Starch utilization in the medium, adding few drops over the Petri dish, Amylase **non producible colonies** appear **blue**, while **Amylase producing M.Os.** will give **brown colour** for **dextrin**, and **yellow colour** for **maltose** or **glucose.**

Cell Disruption Technology

Cellular disruption is a biotechnological method to release the biological molecules including **organelles, proteins, DNA, RNA** and **lipids** from inside a cell. 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**.

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; while shearing systems like blenders and ball mills can shatter the cell contents as well as the 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).

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**: by using pestle and mortar, useful for disruption for a tissue sample, such as plant and animal tissue.
3. **Fine grinding**: the bead mills useful for disruption of 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** that are 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 frequency greater than 18 kHz can be used to disrupt cells) useful for disruption of microorganisms.

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

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 those of the gram-positive type. The combination of lysozyme and detergent used for gram negative type. **Pectinase** and **cellulase** treatment for plant, **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:** visible cell counting.
2. **Physical:** measuring the Vol. of intact cells, O.D., a viscosity of the sample.
3. **Chemical:** measuring protein concentration.

LAB Work:

A. Bacterial cell disruption (Non-mechanical method):

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

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

B. Yeast cell disruption (Mechanical method):

***Saccharomyces cerevisiae* disruption by fine grinding with agitation.**

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

Lysis buffer Contents: 0.05 M Tris-HCL (PH=8), 1mM EDTA (endonucleases inhibitor) 1.25 mM benzamidine (proteases inhibitor).

C. Mammalian cells (RBCs disruption) by osmotic shock.

1. Re-suspend the Pelleted RBCs in 3.5 ml of cold D.W.
   (or in hypoosmotic buffer (7.5mM NaH_2PO_4,1.0 mM $MgCl_2$ and 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 microscopically the efficiency of cell lysis.

D. Plant cell disruption by detergent.

1. Grind 200 mg of plant tissue to a fine paste in approximately 0.5 ml 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^{o} 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 microscopically the efficiency of destruction for obtaining the bioproduct.

Extraction & Purification of Enzymes

Enzymes: are proteins specialized to catalyze 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.

Extraction of Amylase enzyme

Amylases

Catalyze the cleavage of glycosidic bonds in starch and related carbohydrates, α-amylase is one of the water-soluble extracellular enzymes.

Extraction of α -amylase

- A strain of *Bacillus subtilis* (producing amylase) is cultured in starch-containing medium to induce enzyme production and incubate at 37- 40C° for 24-48 hrs.
- Extract the enzyme from the culture media by adding buffer (e.g. phosphate buffer with ratio 1:3 (solid media to water) (you don't need that in liquid media).
- Centrifuge at 5000 rpm for 30 min. The supernatant called (crude enzyme)
- Measure the enzyme activity in the extract (supernatant):

(2ml of crude enzyme solution) + (4ml of 1 % starch) and notice the disappearance of blue colour after adding some drops of iodine then record the results. Compare with control (solution of 1 % starch and iodine without enzyme)

Concentration of enzyme

1. Salt precipitation e.g. (NaCl, $(NH_4)_2SO_4$ and $CaCl_2$).
2. Organic solvents precipitation (alcohols and ketones)

Salt precipitation

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

Procedure

- Take 50 ml of supernatant (crude enzymes) and add certain amounts (grams) of ammonium sulphate $(NH_4)_2SO_4$ to obtain the desired % saturation of the salt (20,40,60 %, etc) may be obtained from standard table or calculated by equation.
- Centrifuge at 5000 rpm for 10-15 min or filter.
- Dissolve the precipitate in a little amount of buffer solution.
- Measure the enzyme activity.
- Take the supernatant and add another quantity of $(NH_4)_2SO_4$ according to the table or from the saturation law to get the 2nd, 3rd and the final saturation % as below:

Calculation of the saturation % for $(NH_4)_2SO_4$ salt:

$$X\ (g/100ml)\ ((NH_4)_2SO_4) = \frac{\mathbf{53.3\ (S2-S1)}}{\mathbf{100 - 0.3\ (S2)}}$$

S1= first saturation %
S2= second saturation%
X= grams of ammonium sulphate to be added to 100ml of protein solution.

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. **Semi-permeable membrane** used to remove small molecular components from protein solutions or to concentrate the extract.

Procedure:

- Wash the dialysis tubes by D.W and boil it for 10 min. or treat them by alcohol to remove the contaminated substance.
- Close one end of the dialysis tube by a thread.
- Pour the enzyme solution in dialysis tubes, leave a space in the tube (do not full it to protect it from rupturing and close the second end of the tube.
- Put the tube in a container (beaker) containing D.W or buffer solution (which is used to dissolve the precipitate after diluted 10times) and surround the container with an ice bath.
- Change the outside buffer (or water) from time to time (30-120) min, the dialysis process may take several hours or a day.
- When dialysis has finished, release the enzyme solution from dialysis tube. Measure enzyme activity and protein concentration.
- Concentrate the enzyme by dialysis with polyethene glycol, sucrose or by using Amicon tube.

Purification

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 column of the gel has 1 diameter to 20 lengths (1:20).

Ion-exchange Chromatography

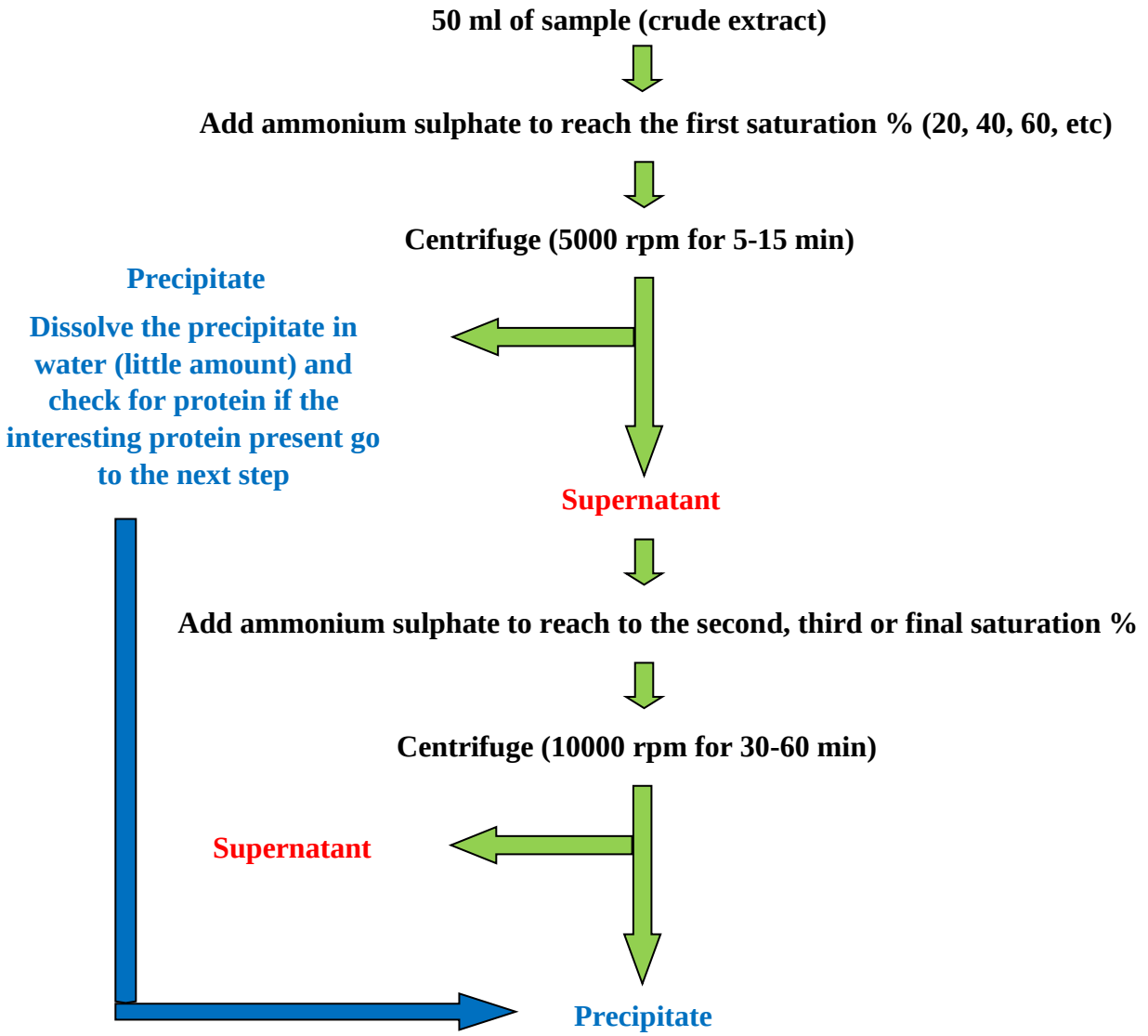
Separates proteins depending on the net charge of proteins in the gel carry either positive charges or negative charges.

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 phase.

Sample Application

1. Remove the storage buffer from the column by washing the column with 2 column volumes of filtered D.W.
2. Prior to apply the sample, equilibrate the column with 2 column volumes of filtered elution buffer.
3. Apply the sample (protein solution) carefully by 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 long storage period or 20% ethanol for short storage period). Keep the column with storage buffer, close the column outlet and store it at 4 C°.


50 ml of sample (crude extract)
Add ammonium sulphate to reach the first saturation % (20, 40, 60, etc)
Centrifuge (5000 rpm for 5-15 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 30-60 min)
Supernatant
Precipitate
(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

Is a technique of binding a biological system (**cells** or **its 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 biological systems in their natural state are immobilized. Many biotechnological processes need to be carried out using the immobilization of biocatalysts.

Why we use immobilization?

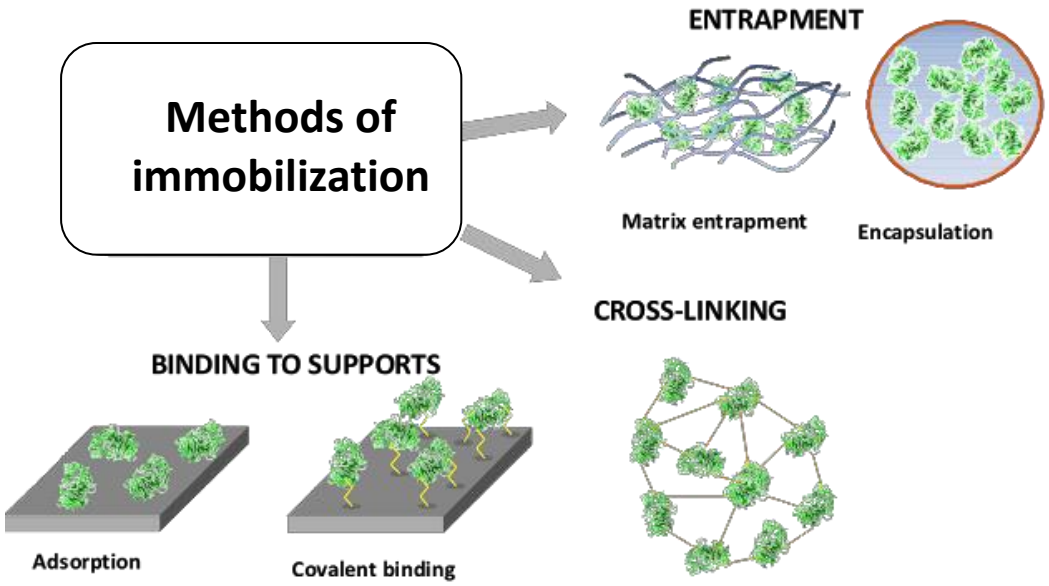
1. Easy separation of a biological system from the product.
2. Reuse of biological system or continued use.
3. Increasing the activity and stability of the biological system.
4. To retain high concentrations of the biological system within a certain defined region of space.

Materials used in Immobilization:

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

Methods of Immobilization

1. **Physical Methods:**
   - Encapsulation: inside 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 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 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 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 in a sterilized petri dish, leave for some time to solidify the agar.
4. Cut the agar to small squares or discs by a sterile tool.
5. Collect the agar pieces (containing the fungal spores) in a clean flask and wash with D.W. or suitable buffer for many times to remove the unentrapped spores.

B. Immobilization of yeast cell, spores or enzyme 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 suitable buffer for many times to remove the unentrapped cell.

C. <u>Immobilization of enzyme (amylase) by adsorption on a solid support.</u>

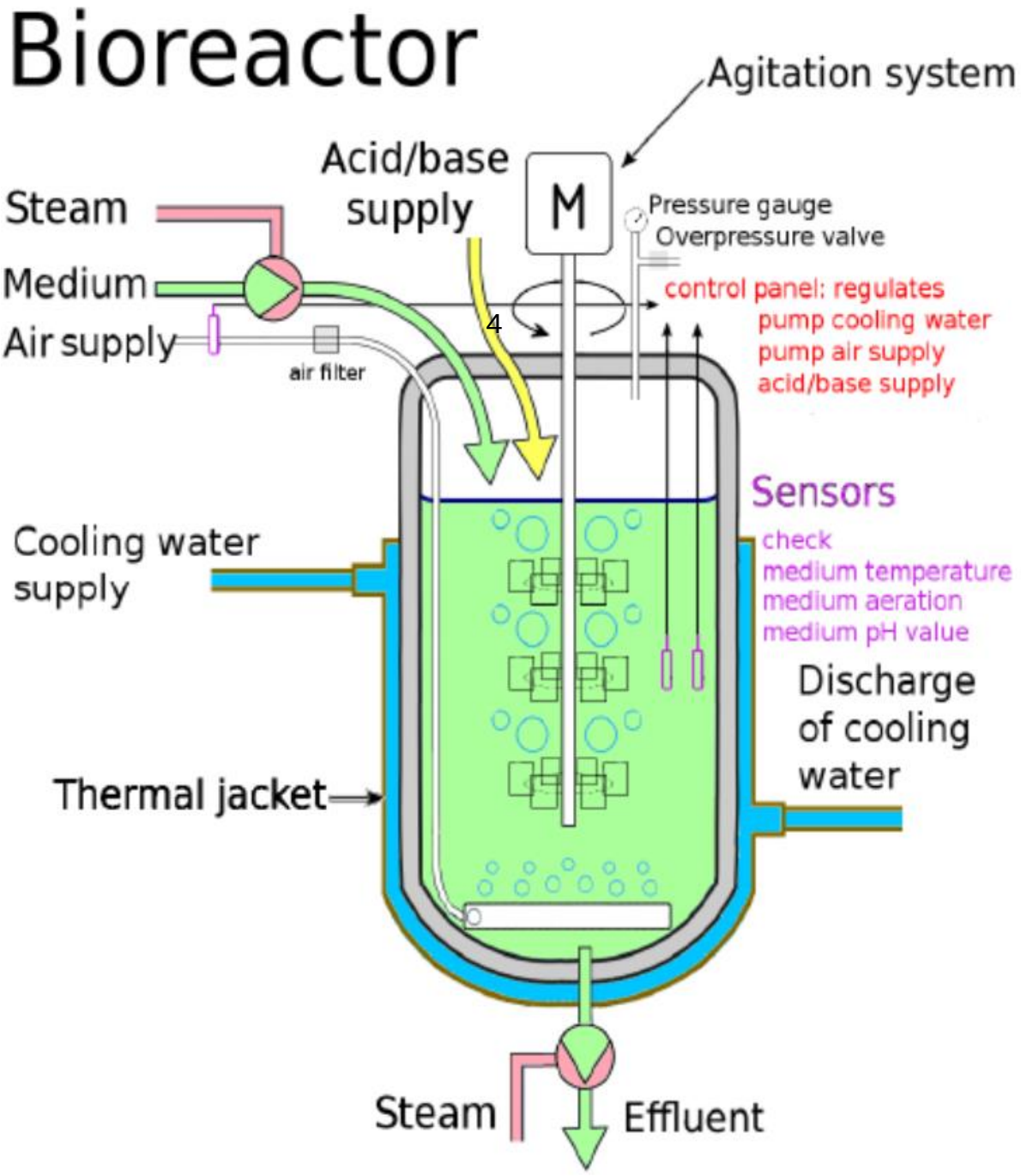
1. Extract the amylase from fungal (e.g. *A.niger*) or bacterial (e.g. *Bacillus subtilis*) culture.
2. Mix 3ml of enzyme solution with 5g of the solid support (e.g. silica gel, glass beads, charcoal, plastic particles, etc) with stirring for 1-2hrs. (or more)
3. Discard the liquid solution by filtration, 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.

Preparation of bioreactor containing the immobilized enzyme or cells:

A bioreactor is any container or vessel in which the biological reaction is carried out. The immobilized cells or enzyme can be used in bioreactors to achieve the industrial and biotechnological processes. There are many kinds of immobilized bioreactors such as **gas lift bioreactor, packed bed reactor and stirred tank reactor** 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 →collect the liquid (product) eluted from the column (bioreactor).
4. **Detect or measure the enzyme activity** (by iodine solution for amylase or hydrolysis of protein for protease).


Bioreactor
Agitation system
Acid/base supply
M
Steam
Medium
Air supply
air filter
Pressure gauge
Overpressure valve
control panel: regulates
pump cooling water
pump air supply
acid/base supply
4
Sensors
check
medium temperature
medium aeration
medium pH value
Cooling water supply
Discharge of cooling water
Thermal jacket
Steam
Effluent

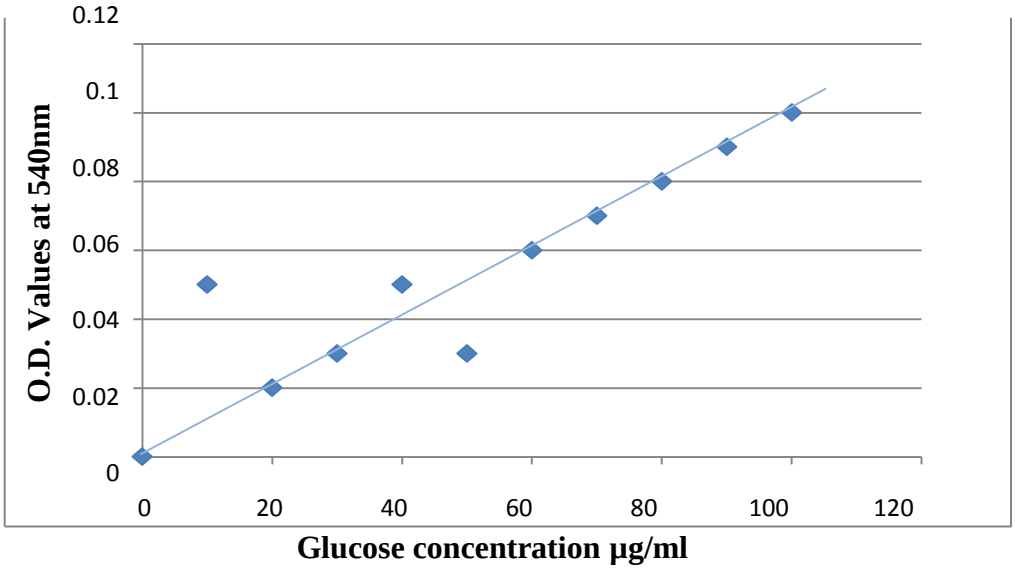
Production of Ethanol from Microorganisms

- **Ethanol** is one of the most important chemical & industrial from the economical 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 of M.Os. in the production.
- **M.Os.** used in Ethanol Production are:
  - **Yeast** was the first M.O. to be used, such as ***Saccharomyces cerevisiae*** **&** ***Saccharomyces uvarum***.
  - **Bacteria** such as ***Clostridium thermocelum*** which is anaerobic and thermophilic that can produce ethanol by converting waste cellulose directly to alcohol.
- **Natural Medium** for Ethanol Production include:
  - **Molasses** (waste of sugar industry).
  - **Dates** & **Debbis**.
  - Agricultural carbohydrates such as **Maize**, **Potatoes** & **Barley**.

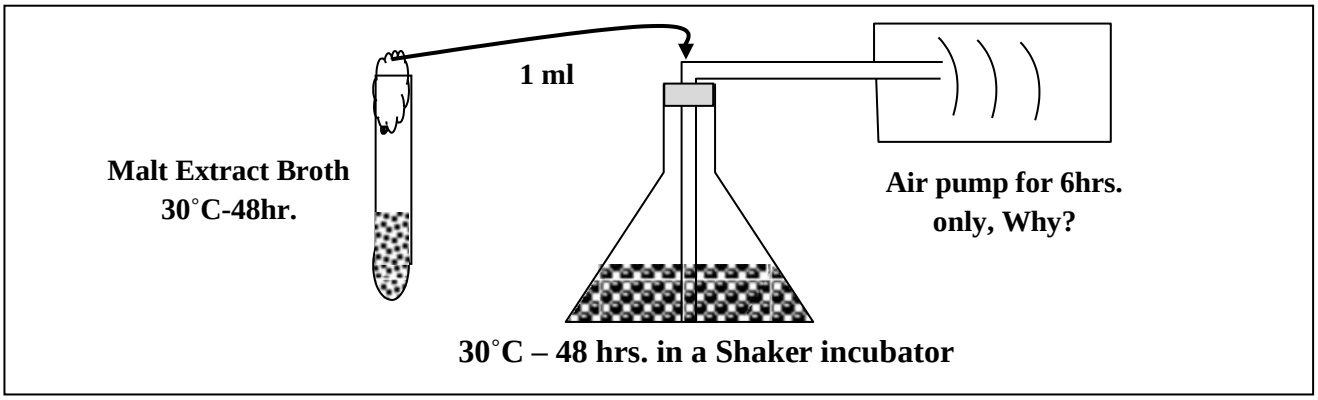
Ethanol Production

a. Prepare **Date Extract Medium** (Ethanol Production Medium) by mixing 100gm from the date with 100 ml tap water, **heat at 80°C** with constant stirring for **30min**. Filter by using **cheesecloth**, mix the residue with water by blender & repeat the filtration again.

b. Estimate the reducing sugars concentration in the filtrate using the **DNS solution (3, 5 Dinitro Salicylic acid)** then sterilize the filtrate and use it as ethanol production medium.



c. Inoculate the medium with **1%** *Saccharomyces cerevisiae* culture grown in **Malt extract broth** and incubate **at 30°C for 48hrs.**, during the first **6hrs.** the medium is **aerated** by using an air pump or a shaker incubator, then **stop aeration** to convert the condition to anaerobic which is suitable for ethanol production.



Ethanol Detection Methods

1. Ammonium Ceric Nitrate (ACN) Method

a- Take 0.5ml of ACN then add 3ml of D.W to it (dilution)

b- Add 3-5 drops from the sample (yeast fermented medium) to diluted ACN the appearance of **red colour** indicates the presence of ethanol.

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

2. Potassium Dichromate ($K_2Cr_2O_7$) Method

1.Add 5ml 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 colour with Acetaldehyde odor**.

(5ml) $K_2Cr_2O_7$ + (1ml) concentrated H_2SO_4 (1ml) from sample → Green colour

→ Acetaldehyde odor

Estimation of Ethanol Concentration

1- Physical Methods

a- Determination of Boiling point.

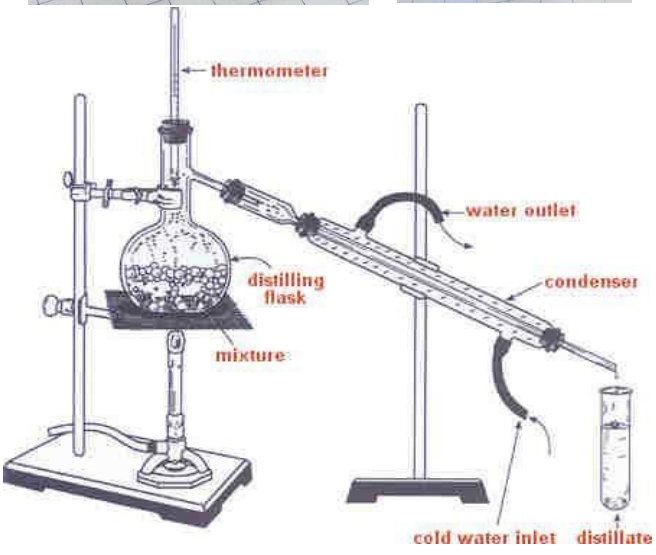
b- Estimation of Ethanol by Pycnometer.

Estimation of Ethanol by Pycnometer

1. Prepare different concentrations of ethanol ranging from **0.5-10%** by adding the required volume of **absolute ethanol** & **water** to 100 ml flask (apply the equation $C_1V_1=C_2V_2$.
2. Withdraw **50ml** from each concentration and mix with **25 ml of D.W**. in **500ml distillation flask** fitted with **40cm condenser** & collects the distillate in **50ml graduated cylinder**.
3. Apply the same process on the sample (fermented medium).
4. Determine the specific gravity of ethanol for each concentration as well as the sample with pycnometer & calculate the specific gravity by the following formula:

$$\text{Specific Gravity} = \frac{\text{Weight of Pycnometer with the sample - Weight of Empty Pycnometer}}{\text{Weight of Pycnometer with water - Weight of Empty Pycnometer}}$$

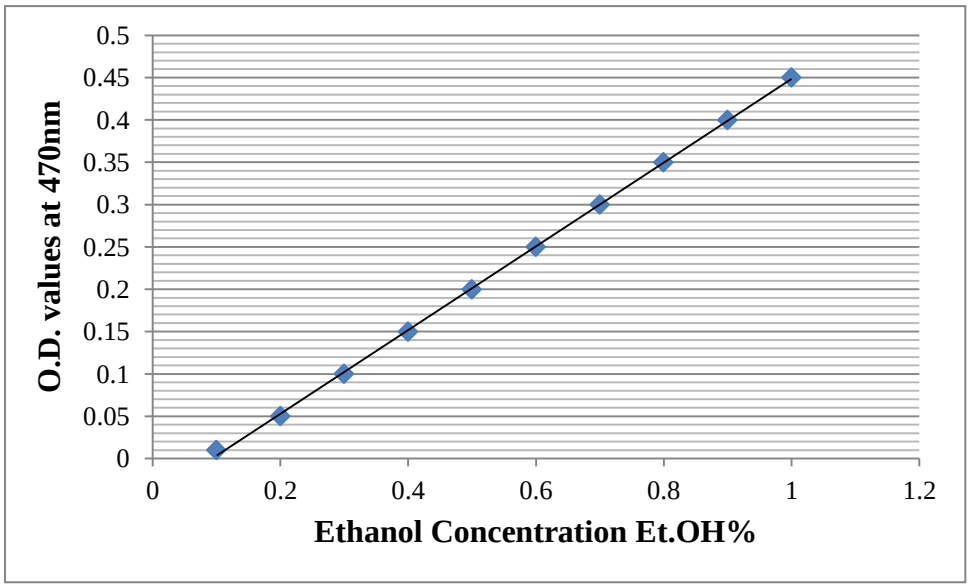
5. Compare the result with other concentrations & the specific gravity for the sample similar to one of them and thus know the concentration of the sample.



2- **Chemical Methods**
a- Ammonium Ceric Nitrate (ACN) Method. b-
Potassium Dichromate ($K_2Cr_2O_7$) Method.

A) Ammonium Ceric Nitrate (ACN) Method.

1. Prepare different concentrations of ethanol ranged between **0.1-1%** from absolute ethanol.
2. Add 2ml of **(ACN)** to 5ml of each concentration; leave it for 5min to complete the reaction.
3. Read the absorbency at **470nm** by spectrophotometer, the blank consists of distilled water and **ACN** (without ethanol).
4. Draw the Standard Curve for the Estimation of Ethanol Concentration, O.D. results plotted on the Y-axis while the Ethanol Concentrations on X-axis & calculate the slope.
5. Apply the same steps on the sample to estimate & compare the result of the standard curve to determine the concentration of alcohol in the sample as shown in the figure.

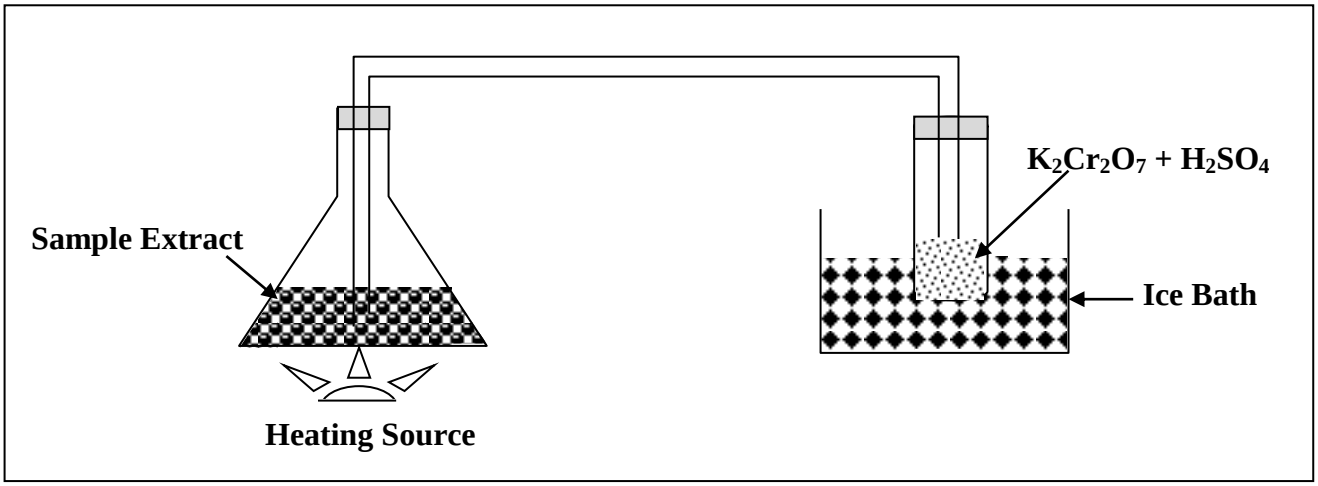


B) Potassium Dichromate ($K_2Cr_2O_7$) Method.

1. Transfer 1ml of the sample (yeast fermented medium) to a flask fitted with connection glass tube reaches to the bottom of another tube containing 5ml of ($K_2Cr_2O_7$ + H_2SO_4) solution and place in an ice bath.
2. Heat the flask containing the sample on a flame and boil until dryness.
3. Transfer the content of the tube containing ($K_2Cr_2O_7$ + H_2SO_4) to another beaker & titrate with **Mohr** solution (**sulfuric acid and ammonium ferric sulfate)** after addition of **Diphenylamine** reagent.
4. Stop the titration when the colour is turned to **bluish green** & record titration volume, calculate the percentage of ethanol as follows:

$$\textbf{Et.OH\%} = \frac{5(\textbf{Vol.of Mohr Sol. after Titration with Blank} - \textbf{Vol.of Mhor Sol. after Titration with Sample})}{\textbf{Vol.of Yeast fermented medium} \times \textbf{Vol.of Mohr Sol. after Titration with Blank}} \times 100\%$$

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



Manufacture of Antibiotics- Penicillin production

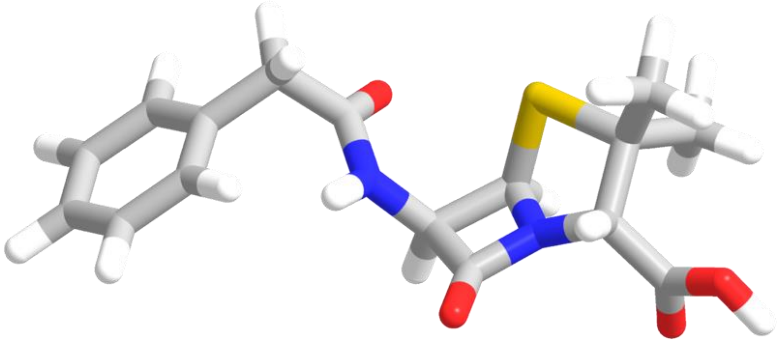
❖ Antibiotics are manufactured by using **Fermentation** Technique in which M.Os. are inoculated into large vessels (fermentor) containing the necessary media.

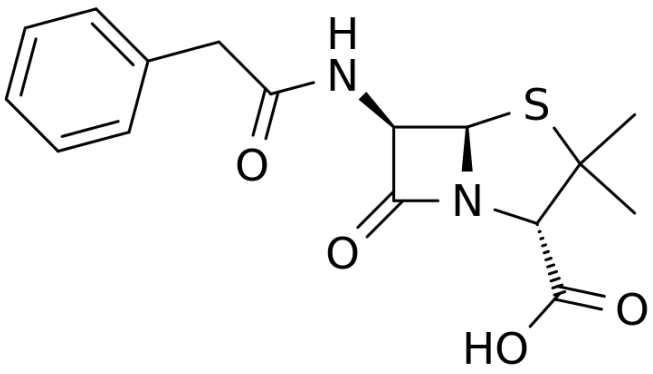
- There are two types of fermentation
  1- **Batch Fermentation.**
  2- **Continuous Fermentation**

	Batch Fermentation		Continuous Fermentation
1	Sterile medium is placed in a vessel, when the nutrients are exhausted the vessel must be emptied & then re-batched with the medium.	1	Sterile medium is added to the fermentor without interruption to the Fermentation System balancing it with the withdrawal of broth for product extraction.
2	Broth is fermented for a definite period for hours or a day.	2	Weeks to months.
3	e.g. Antibiotic production	3	e.g. Brewing & Single Cell Protein Production.

❖ **Penicillins** are a class of antibiotics

- Produced by *Penicillium notatum*, *Penicillium chrysogenum*.
- Its produced as **α-aminopencillanic acid** linked via an **amide bond** to **various side chains**.
  a- Strong Acid can be extracted from an aqueous solution at pH=2-3.
  b- It is soluble in H_2O, methanol & ethanol.
  c- Its major activity on Gram positive bacteria e.g. *Staphylococcus aureus*.





Production of Benzyl Penicillin

a- The organism

The production of Penicillin was from ***Penicillium notatum***, isolated by Fleming in1926. Today penicillin is produced by ***P. chrysogenum.*** **Mutagenic variants** produce large amounts of penicillin than the original wild strain. Production strains are **stored in dormant form** using culture preservation techniques. All laboratory manipulations are carried out in **laminar flow cabinets** (Why?), operators wear sterilized clothing and work aseptically (Why?).

b- Oxygen supply

Supplied as filter sterilized air from a compressor. **Air** is pumped at the bottom of the fermentor via a ring sparger that breaks the flow into myriad (عشرات الآلاف) of bubbles & to increase transfer area. These **bubbles** loose oxygen as they rise up the tank, at the same time CO_2 diffuses into them. **Impellers** (الدوافع) also help the transfer of oxygen. **Baffles** (الشوارد) are also used to promote intimate contact of cells & nutrients

c- Temperature control

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

d- De-foaming Agents & Instrumentation

During vigorous (قوي) **mechanical stirring** & **aeration** the microbial cultures form foam. This cause **loss of culture** by entrapment in exhaust gases. **Defoaming agents** are added & **Temporary backpressure** is applied to contain the culture within the vessel.

e- Media additions

Nutrients required may be added during the process.

f- Transfer & sampling systems

Aseptic systems are provided to transfer the inoculum to the vessel, to allow the taking of routine samples during fermentation & to transfer the final contents to the extraction plant (مصنع). **Sampling monitors:** growth, levels of nutrients, the concentration of penicillin, microbial contaminants.

g- Control of fermentation

- **Aeration** must be controlled **because** Fall in oxygen cause reduced production of penicillin G while cultures continue to grow.
- **Sugar** is the key nutrient that controls growth.

❖ **Natural Medium**

- **Corn steep liquor**, a by-product of the maize starch industry is used to provide nutrients such as nitrogen, carbon compounds such as acids, sugars, ions (Ca^{+2}, Mg^{+2}, SO_3^{-2}, PO_3^{-2}, K^+), trace metals & growth factors. The medium is sterilized with steam at 120°C either in the fermenter itself or in ancillary (ملحق) plant.
- **Fed nutrients**
  - The sterile medium is **stirred**, **aerated** & its **pH**, **temperature** are set, then inoculated.
  - All **feeds** must be sterilized **(Why?)** to avoid contaminants (β-lactamase producing bacteria) that can destroy penicillin or utilize nutrients intended for the fungus.
  - **Stimulation** by **Phenylacetic acid** or **Amino acids that build penicillin** (α-aminoadipidic acid, cysteine & valine). Supply side of benzyl penicillin because:
    - It stimulates the synthesis of benzyl penicillin.
    - It prevents other unwanted forms of penicillin.
- **Termination**

  Are done when raw materials **(sugar)** are thought to be exhausted & no more conversion to penicillin.
- **Extraction**
  - Cells are removed by filtration
  - Isolation of Penicillin G by **solvent extraction** using amyl acetate, butyl acetate & an aqueous buffer.
  - Then solvent recovered by distillation.
  - Treatment of crude extract.
  - Formation of appropriate salt.
  - Removal of pyrogens (Any substance that can cause a rise in body temperature).
  - Packaging.

In another study, Two local strains of *Penicillium chrysogenum* were selected for enhancement of Penicillin G production under **Solid-State Fermentation** using **Sugar beet pulp & Agro-industrial residue**.

Detection & Estimation for Penicillin Production

a- Vacuum-Filter 100ml of the production medium, take the filtrate which contains penicillin & dry the mycelia at 50-70°C to determine its dry weight.

b- Penicillin activity & concentration were measured by **Plate Diffusion Test**, as follows:

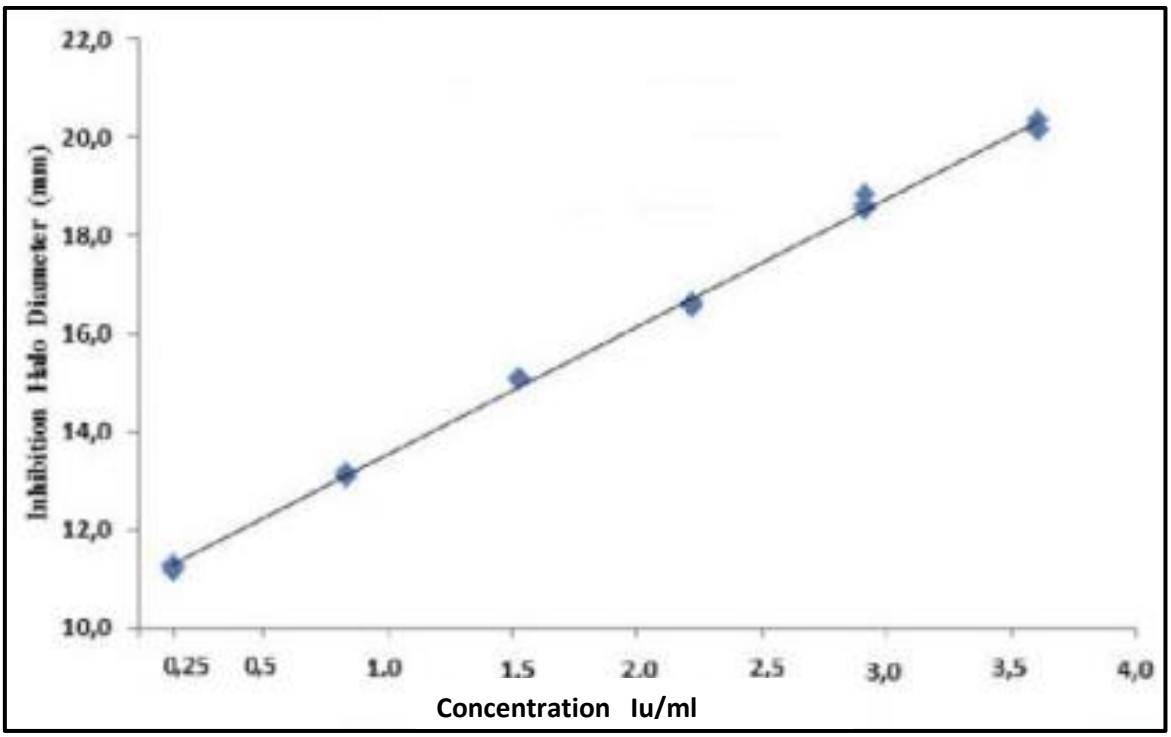
1- Prepare **Nutrient Broth** (50ml) to inoculate it with the **Test Strain** ***Staphylococcus aureus*** incubated in shaker incubator for 3hrs. at 28°C.

2- Prepare **Nutrient Agar** Petri dishes to streak the **Test Strain** with 0.1-0.2ml & leave for 5-10min.

3- Make **5 wells** in the inoculated Nutrient Agar using a sterile cork borer or Pasteur pipette & fill it with 0.2ml of standard solutions containing 0.25,0.5, 1, 1.5, 2 iu/ml Penicillin G then incubate at 28˚C for **16-18 hrs**. record the results by measuring the diameters of the inhibition zones to draw a standard curve based on 10-20 standard plates to determine the iu/ml for the culture filtrates.

4- The **sample** is prepared in the same manner & inoculated with dilutions then incubated at 28°C for **16-18 hrs**., which permits the diffusion of Penicillin from the wells to the agar leading to the inhibition of the Test Strain causing clear zones of inhibition around the wells.

5- Measure the diameter of **Inhibition Zones**, plot the measurements on the standard curve to estimate the concentration of the produced Penicillin.



Production of Citric Acid by *Aspergillus niger*

❖ Citric Acid

- $C_6H_8O_7$ nH_2O (n=1 or 0)
- Mwt. monohydrate =210.14 Anhydrous = 192.13
- Weak organic acid
- Natural preservative/conservative
- Used to add an acidic, or sour, taste to foods, soft drinks & cakes.
- It can also be used as an environmentally benign cleaning agent.
- It is very corrosive (مادة أكلة) when contacts the skin surface & can burn severely.
- In biochemistry, the conjugate base of citric acid, **Citrate**, is important as an intermediate in the citric acid cycle, & therefore occurs in the metabolism of virtually all living things.
- Citric acid exists in greater amounts in a variety of **fruits & vegetables,** most notably citrus fruits. Lemons & limes have particularly high concentrations of 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.

Production Technique

- Cultures of ***A. niger*** are fed on **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 M.Os. in a low-water-activity environment on non-soluble materials acting as both nutrient source & physical support.

- The **major advantages** of solid-state fermentation over submerged fermentation include:
  - **Higher yields**.
  - **Low water requirement.**
  - L**ower operating costs**.

❖ Many M.Os. 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 & Methods:

1- **Preparation of inoculum (*A. niger* spore suspension)**

A. niger stock culture is reactivated & 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 addition of 10ml D.W. to the *A. niger* culture then shake well & use this suspension to inoculate the production media.

2- **Production Medium (Molasses**)

Molasses (beet or cane molasses) is **diluted** with water to a **sugar content of 12-20%** some nutrient salts may be added to support acid production then **adjust pH** using 1N of HCl and/or NaOH, the initial pH of the fermentation culture must be 3, then sterilize the medium.

3- **Inoculation**

Inoculate the medium with spore suspension by adding **5% (5ml)** of spore suspension to **100ml medium** then incubate at 25-30°C for 5-7 days.

4- **Extraction & Recovery of citric acid**

Extraction - Filter the culture to remove the mycelia & solid particles, take the filtrate (containing citric acid).

Recovery of CA

- Take 25-50ml of the filtered broth.
- Add **$Ca(OH)_2$ solution** with heating in a water bath until **pH** becomes neutral & a **precipitate** will form as **Ca Citrate**.

- **Filter off** by filter paper, collect the precipitate & **treat** it with 2M of **H_2SO_4** with a ratio of 1:8 (gm. of precipitate : ml H_2SO_4) to precipitate **Ca as insoluble sulfate.**
- **Filter** to remove the precipitate **(citrate)**, the filtrate solution will contain the dissolved citric acid.
- **Evaporate** to obtain CA crystals.
- **Calculate** the weight of CA crystals by measuring the dry wt. of the container before & 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**

The citric acid (CA) is determined titrimetrically by using (0.1N) NaOH & phenolphthalin as
indicator:

10 ml sample + 2-3drops of phenolphthalein $\xrightarrow{\text{titrate with 0.1N NaOH}}$ until pink color appears

& record the volume of titritable NaOH.

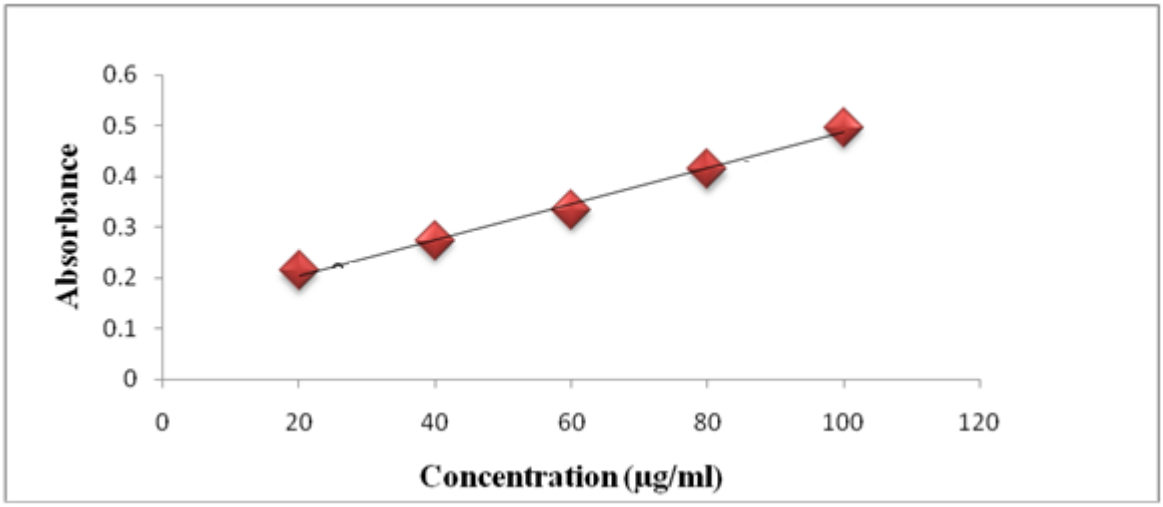
- **Assay of CA**: Calculate on the anhydrous basis.
  1 ml of 0.1N sodium hydroxide = 6.404 mg of **Citric Acid** ($C_6H_8O_7$)

e.g.: If the volume of the sample (containing CA) is 10 ml & the volume of titritable NaOH= 3ml, therefore, mg of **Citric Acid in the sample (10ml) = 3×6.404**
Calculate in 1ml or 100(%)?

6- **Determination of Sugar Concentration**

- **Estimate the sugars** (or total carbohydrates) in the medium before & after the fermentation process (medium before & after culturing) as follows:
  - **Prepare a standard curve of sugar**: prepare a certain concentration of glucose (0-100µgm/ml).
  - Take 1ml of each concentration + 1ml of 5% phenol solution + 5ml conc. **H_2SO_4** (added carefully on the wall of glass tube) then cool in an ice bath & read the O.D at 490nm, the blank is 1ml of D.W.+ reagent.
  - Plot the relationship between the O.D & glucose concentrations, obtain the slope of (= $y_2 – y_1/x_2-x_1$).

- Take 1ml of the samples(sample for the medium before culturing & sample for medium after culturing), treat it as above then read O.D at 490nm to calculate the concentration of the sugars depending on the standard curve (Note: the unit is µgm, 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)**

$$\textbf{Yield of CA\%} = \frac{Conc.of\ CA}{Conc.of\ Consumed\ Sugar\ (Conc.of\ Sugar\ in\ the\ medium - Residual\ Sugar\ after\ Fermentation} \times 100\%$$