محاضرات اسس تقنيات احيائيه عملي

المرحلة الاولى

قسم التقنيات الاحيائيه

ا.د. غازي منعم عزيز

أ.م.د. سحر ارحيم حسين

أ.م.د. علي جبار رشك

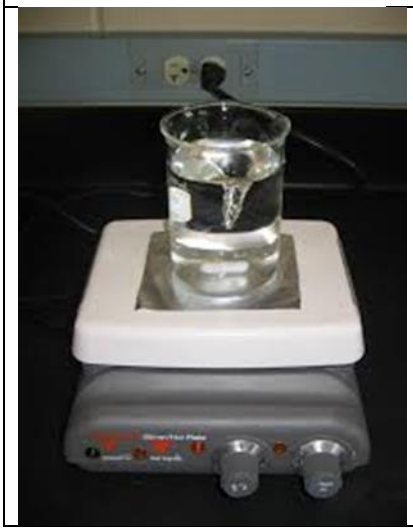
م.م. اسيل فخري خلف

University of Baghdad
College of Science
Department of Biotechnology

Laboratory Equipment's

Instruments or apparatus

Image	Scientific name	purpose
	**Centrifuge**	**Used to separate heavy material from light material by the help of the centrifugal force**
	**Balance**	**Determining weight**
	**Sensitive balance**	**Determining weight (small mass)**

	**Autoclave**	**An autoclave is a pressure chamber used to sterilize equipment and media by subjecting them to high pressure saturated steam at 121 °C for around 15–20 minutes.**
	**pH meter**	**is an electronic device used for measuring the pH (acidity or alkalinity) of a liquid**
	**Magnetic stirrer and magnetic bar**	**To uniformly mix the reaction material and balance temperature distribution, in order to speed up reaction, then shorten reaction time by the help of a magnetic bar.**

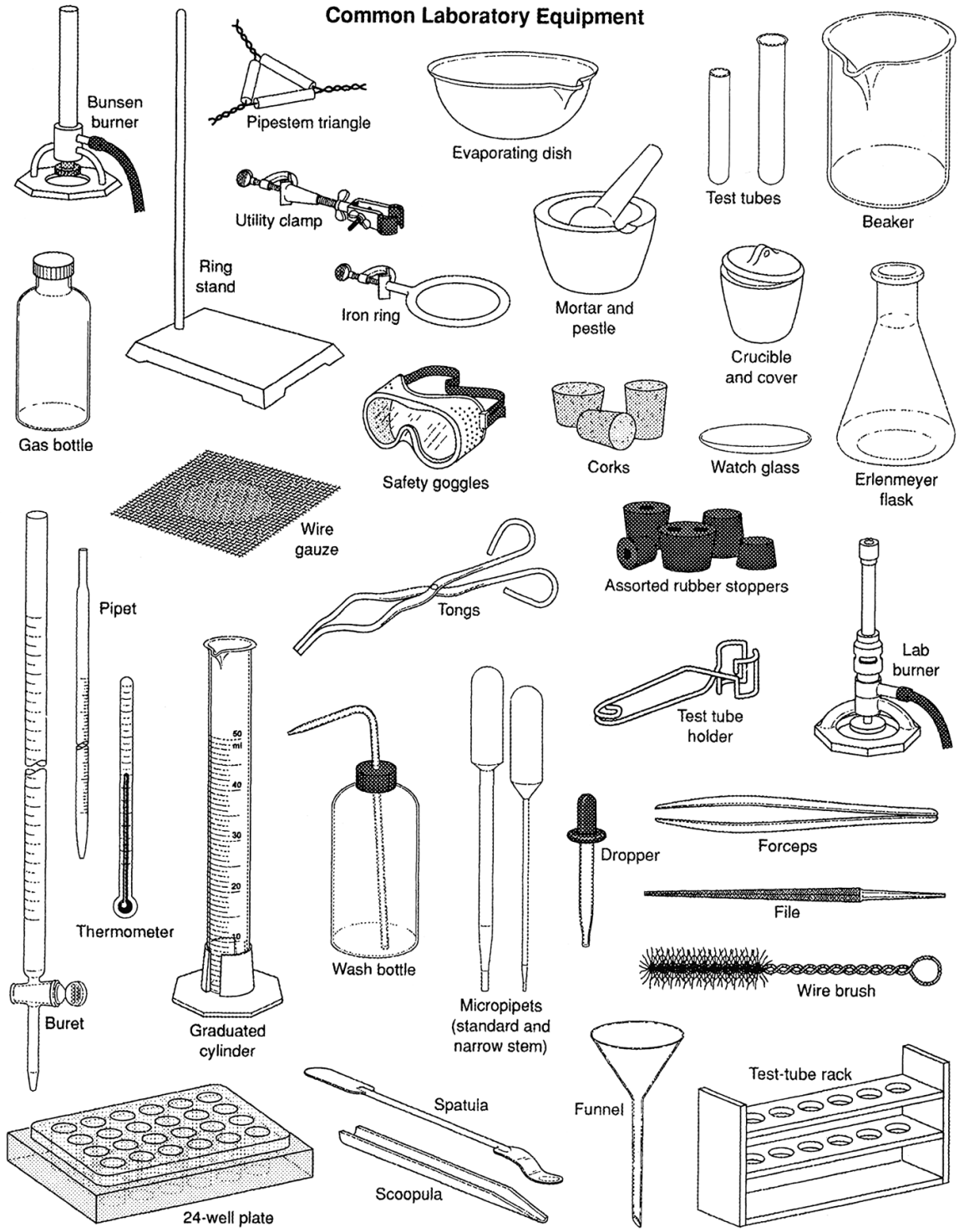
	**Vortex mixer**	**Used for mixing the solutions**
	**Incubator**	**It's a device used to grow and maintain microbiological cultures or cell cultures. The incubator maintains optimal temperature, humidity and other conditions such as the carbon dioxide (CO2) and oxygen content of the atmosphere inside.**
	**Shaking incubator**	**In order to provide optimal conditions for cell growth, some type of agitation or shaking is necessary to incorporate oxygen and evenly distribute nutrients throughout the culture media. The incubator shaker can be used for growth of just about any kind of cell including bacterial cultures, tissue cultures, and yeast.**

University of Baghdad
College of Science
Department of Biotechnology

	**Hood**	**Used to provide sterile conditions for culturing the microbes**
	**Spectrophotometer**	**is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength**
	**Refrigerator**	**Used for storing samples, solutions, media and bacterial strains.**

	**Water bath**	**Used to incubate samples in water at a constant temperature over a long period of time.**
	**Microscope**	**Used to see objects that are too small for the naked eye.**
	**Thermal cycler (PCR)**	**This device is used for the amplification of a specific region of any DNA sample with polymerase chain reaction in a test tube. It is also used for detection and constitution of genetically modified organism, as well as other genetic analysis.**

a- Laboratory tools:



Microorganism's growth requirements and culture media

A microorganism is defined as a living thing that is so small that must be viewed with a microscope or with electron microscope.

There are five different categories of microorganisms: bacteria, algae, protozoa, fungi, and viruses. Microorganisms exist throughout the world, from Antarctica to your kitchen, from inside animals, like humans, to an expansive wilderness. An initial aim of all microbiologists is the reproducible growth of their microbial cultures. Microbial growth requires suitable environmental conditions, a source of energy, and nourishment.

These requirements can be divided into two categories include: physical and chemical.

1. Chemical requirement's:

Chemical factors	Form usually found in nature	Chemical form commonly added media
Carbon	CO_2, organic compounds	Organic; simple sugars e.g. glucose, acetate or pyruvate; extracts such as peptone, tryptone, yeast extract etc.
Oxygen	Water (H_2O), organic compounds	
Hydrogen	Water (H2O) organic compounds	
Nitrogen	NH_3, amino acids	Organic; amino acids, nitrogenous bases. Inorganic; NH_4CI
Phosphorus	PO_4	KH_2PO_4, Na_2HPO_4
Potassium	K+	KCI, K_2HPO_4
Magnesium	Mg^{2+}	$MgCI_2$, $MgSO_4$
Calcium	Ca^{2+}	$CaCI_2$
Sodium	Na^+	NaCl
Iron	Fe^{3+} organic iron complexes	$FeCI_3$
Trace elements	Usually present at very low concentrations	$ZnCI_2$, $CuCI_2$
Organic growth factors	Usually present at very low concentrations	Vitamins, amino acids, purines, pyrimidines

2. Physical / Environmental requirements

2.1 Temperature

Most microorganisms grow well at the normal temperatures favored by man, higher plants and animals. However, certain bacteria grow at temperatures (extreme heat or cold) at which few higher organisms can survive. Depending on their preferred temperature range, bacteria are divided into three groups:

- Psychrophiles (cold-loving microorganisms): have an optimum growth temperature between 0°C and 15°C.
- Mesophiles (moderate-temperature-loving bacteria): have an optimum growth temperature between 25°C and 45°C.
- Thermophiles (heat-loving microbes): have an optimum growth temperature between 50°C and 65°C.

2.2 pH

- **Neutrophils:** pH range near neutrality between pH 6.5 and 7.5.
- **Acidophils (acid-loving)**: Grow at pH values below 4 with some bacteria still active at a pH of 1.
- **Alkalinophils (base-loving)**: Prefer pH values of 9-10 and most cannot grow in solutions with a pH at or below neutral.

2.3 Osmotic Pressure

Microbes contain approximately 80-90% water and if placed in a solution with a higher solute concentration will lose water which causes shrinkage of the cell (plasmolysis). However, some bacteria have adapted so well to high salt concentrations that they actually require them for growth. These bacteria are called halophiles (salt-loving) and are found in salt or in areas such as the Dead Sea.

Culture media

Culture media contains nutrients and physical growth parameters necessary for microbial growth. All microorganisms cannot grow in a single culture medium. Culture media can be distinguished on the basis of **composition, and consistency.**

1. Classification of culture media based on consistency

A. Solid medium

Solid medium contains agar at a concentration of 1.5-2.0%, solid medium has physical structure and allows bacteria to grow in physically informative or useful ways (e.g. as colonies or in streaks). Solid medium is useful for **isolating bacteria** or for determining the colony characteristics of the isolate.

B. Semi-solid media

It was prepare with agar at concentrations of 0.5% or less, it has soft custard like consistency and are useful for the determination of bacterial motility.

C. Liquid (Broth) medium

This media contains specific amounts of nutrients but don't have trace of gelling agents such as gelatin or agar and it serves various purposes such as propagation of large number of organisms, fermentation studies, and various other tests. e.g. sugar fermentation tests.

2. Classification of culture media based on composition

A. Synthetic or chemically defined medium: A chemically defined medium is one prepared from purified ingredients and therefore whose exact composition is known, like salt and sugar such as mineral salt medium.

B. Non synthetic (Natural) or chemically undefined medium, like: Molasses, and Whey.

C. Semisynthetic media the media of which chemical composition is partially known is as semisynthetic media, like: PDA and nutrient agar.

- **Preparation of nutrient broth and agar media**.

Laboratory supplies

Flask (1 L), Graduated cylinder (1 L), Spatula, Weigh boats, Beef extract, Peptone, Agar powder, Test tube rack, Test tubes, Labeling tape, roll, Autoclave, Petri plates

Procedure:

Wipe down lab bench carefully with disinfectant to help prevent contamination of your media.

1. Measure approximately 250 ml of distilled water (located in 60°C water bath) in a 1 L graduated cylinder and pour into a 1 L flask.
2. Weigh out 1.5 g beef extract and 2.5 g peptone and add into the flask.
3. Stir over gentle heat from a bunsen burner to dissolve completely.
4. Check the pH of the medium and adjust to pH 7.0, if necessary, using the HCl and/or NaOH.
5. Add the mixture into the 1 L graduated cylinder and add warm water to the 500 ml mark, re add into the flask.
6. Using a 10 ml pipette, dispense 10 ml of the mixture into each test tube. Make 10 tubes and place in a test tube rack.
7. Add 6.0 g of agar to the flask containing some of medium above and label it NA.
8. Heat to just boiling for 1-2 minutes while stirring constantly. The agar will not dissolve unless it is boiled; the solution will become completely clear when it has dissolved. Allow agar to cool until there is no danger of you being burned and then dispense into another tubes using a 10 ml pipette. Make ten 10 ml tubes.
9. Close the flask with a Styrofoam plug covered.
10. Autoclave the flask and the tubes for 15 minutes at 121 °C and 15 lb/in2 pressure.
11. After removing the media from the autoclave, allow the broth tubes to cool, and store for later use. Place the flask in the 48°C water bath. Quickly lay the tubes of NA on the slant racks on the center table so that the medium forms a long slant and a short butt, and allow them to cool and solidify. Do not allow the agar to reach the top of the tube. Allow them to cool completely before returning to the rack. Store for later use.
12. Lay your petri dishes on the bench. The cover should be on top. Light your bunsen burner, then remove the NA flask from the water bath. Carefully wipe the bottom dry to prevent the dripping water from contaminating the plates.
13. Remove the tapes and cotton plug from the flask. Carefully flame the neck of the flask,

open the plate cover about half way and fill the plate about 1/2 full.

14. Flame the neck of the flask between each plate.
15. Allow plates to solidify completely, which will take 15 minutes. Then invert, label and incubate at 37 °C overnight to dry off excess moisture and check for contamination.
16. Clean all glassware and leave on paper towels beside sink.

The Isolation of Microorganisms from a different environments by a different techniques

The survival, growth & the ecological distribution of microorganisms is greatly affected by the chemical and physical nature of their environment. Therefore M.O. can be isolated from many different environments such as soil, water, air, food, plants & animals, M.O. grow and divide as rapidly as the environment permits.

When working with microorganisms, it is desirable to start with single, isolated colonies to ensure you are working with a pure culture, also in order to adequately study and characterize an individual M.O. species, one needs a **pure culture.**

The colony theoretically forms from a single cell, a colony should then represent a pure culture. A **colony** is a visible mass of microorganisms growing on an agar surface and usually originating from a single organism or arrangement of organisms.

Three different techniques can be used to obtain pure colonies or pure isolates:

1- The spread plate technique:

It's an easy, direct way to obtain pure colonies, in this technique, a small volume of dilute bacterial mixture containing 100 to 200 cells or less is transferred to the center of an agar plate and is spread evenly over the surface with a sterile L-shaped glass rod. After incubation, some of the dispersed cells develop into isolated colonies. In this procedure, one assumes that a colony is derived from one cell and therefore represents a copy of a pure culture.

After incubation, the general form of the colony and the shape of the edge or margin can be determined by looking down at the top of the colony. The nature of the colony elevation is apparent when viewed from the side as the plate is held at eye level. These variations are illustrated in figure1). After a well-isolated colony has been identified, it can then be picked up and streaked onto a fresh medium to obtain a pure culture.

2- The **streak-plate technique:**

In this technique, the bacterial mixture is transferred to the edge of an agar plate with an inoculating loop and then streaked out over the surface in one of several patterns. At some point on the streaks, individual cells will be removed from the loop on the agar surface and will give rise to separate colonies figure (2.), again, one assumes that one colony comes from one cell. The key principle of this method is that by streaking, a dilution gradient is established on the surface of the plate as cells are removed on the agar surface. Because of this gradient, the growth will occurs on a part of the plate where the cells are not sufficiently separated, and individual, well isolated colonies develop in other regions of the plate where few enough cells are removed to form separate colonies that can be seen with the naked eye. Cells from the new colony can then be picked up with an inoculating loop or needle and transferred to an agar slant or other suitable medium for maintenance of the pure culture.

3- The pour-plate technique:

Also will yield isolated colonies and has been extensively used with bacteria and fungi. The original sample is diluted several times to reduce the microbial population sufficiently to obtain separate colonies upon plating (figure. 3).

The small volumes of several diluted samples are added to sterile petri plates and mixed with liquid tryptic soy agar that has been cooled to about 48° to 50°C. Most bacteria and fungi will not be killed by the brief exposure to the warm agar. After the agar has hardened, each cell is fixed in place and will form an individual colony if the sample is dilute enough. Assuming no chaining or cell clusters, the total number of colonies are equivalent to the number of viable microorganisms in the diluted sample. To prepare **pure cultures,** colonies growing on the surface or subsurface can be inoculated into fresh medium.

Materials & Procedure

Brain heart infusion agar, 95% ethyl alcohol, L-shaped glass rod, petri plates, inoculating loop, bunsen burner, sterile pipettes, soil sample, water sample**.**

The spreading Procedure

1. Pipette 0.1 ml of the water sample or serial dilutes of soil sample onto the center of BHI agar plate.
2. Put the L-shaped glass rod into a beaker of ethanol and then briefly pass the ethanol-soaked spreader through the flame to burn off the alcohol and allow it to cool inside the lid of a sterile petri plate.
3. Spread the sample evenly over the agar surface with the sterilized spreader, making sure the entire surface of the plate has been covered.
4. Invert the plates and incubate for 24 to 48 hours at room temperature or 30°C.
5. After incubation, measure some representative colonies and carefully observe their morphology, then record your results in the report for lab 3.

The streaking Procedure

1. Aseptically take the sample from water or serial dilute of soil sample by a loopful.
2. Streak out the loopful containing the sample on the surface of BHI agar plate that you have prepared as follows:

a. Carefully lift the cover of petri plate just enough to insert your inoculating loop easily, in order to avoid contamination. Insert the inoculating loopful of sample and spread it over a small area (area 1) at one edge of the plate as shown in (figure 2).

b. Remove the inoculating loop and kill any remaining bacteria by flaming them.

c. Rotate the plate while carefully keeping in mind where the initial streaks ended (use the marked quadrants as a guide) and cross over the streaks in area 1.

d. Remove the loop, flame it, cool in the agar as before, and repeat the streaking process.

3. Incubate the plates at 30° to 37°C for 24 to 48 hours in an inverted position. Afterwards, examine each of the agar plates to determine the distribution and amount of growth in the three or four streaked areas and record your results in the report for lab 3.

The Pour-Plate Procedure

The original sample is diluted several times to decrease or dilute the population sufficiently. 1 ml of each dilution is then dispensed into the bottom of a petri plate. Agar pours are then added to each plate. Isolated cells grow into colonies and can be used to establish pure cultures. The surface colonies are circular and large, subsurface colonies are lenticular or lens shaped and much smaller.

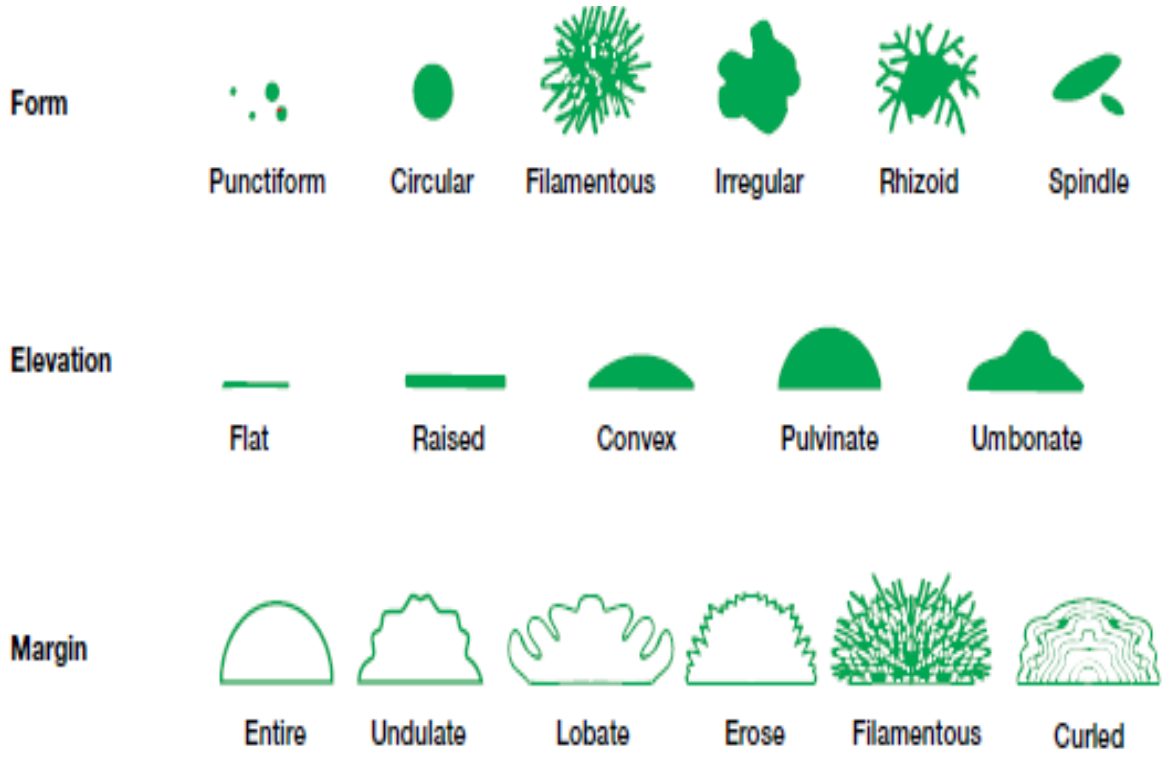


Figure 1: Bacterial colony characteristics on agar media as seen with the naked eye.

The characteristics of bacterial colonies are described using the following terms.
Appearance: Shiny or dull**, Optical property:** Opaque, translucent, transparent**, Pigmentation:** Pigmented (purple, red, yellow) Non-pigmented (cream, white), **Texture:** Rough or smooth

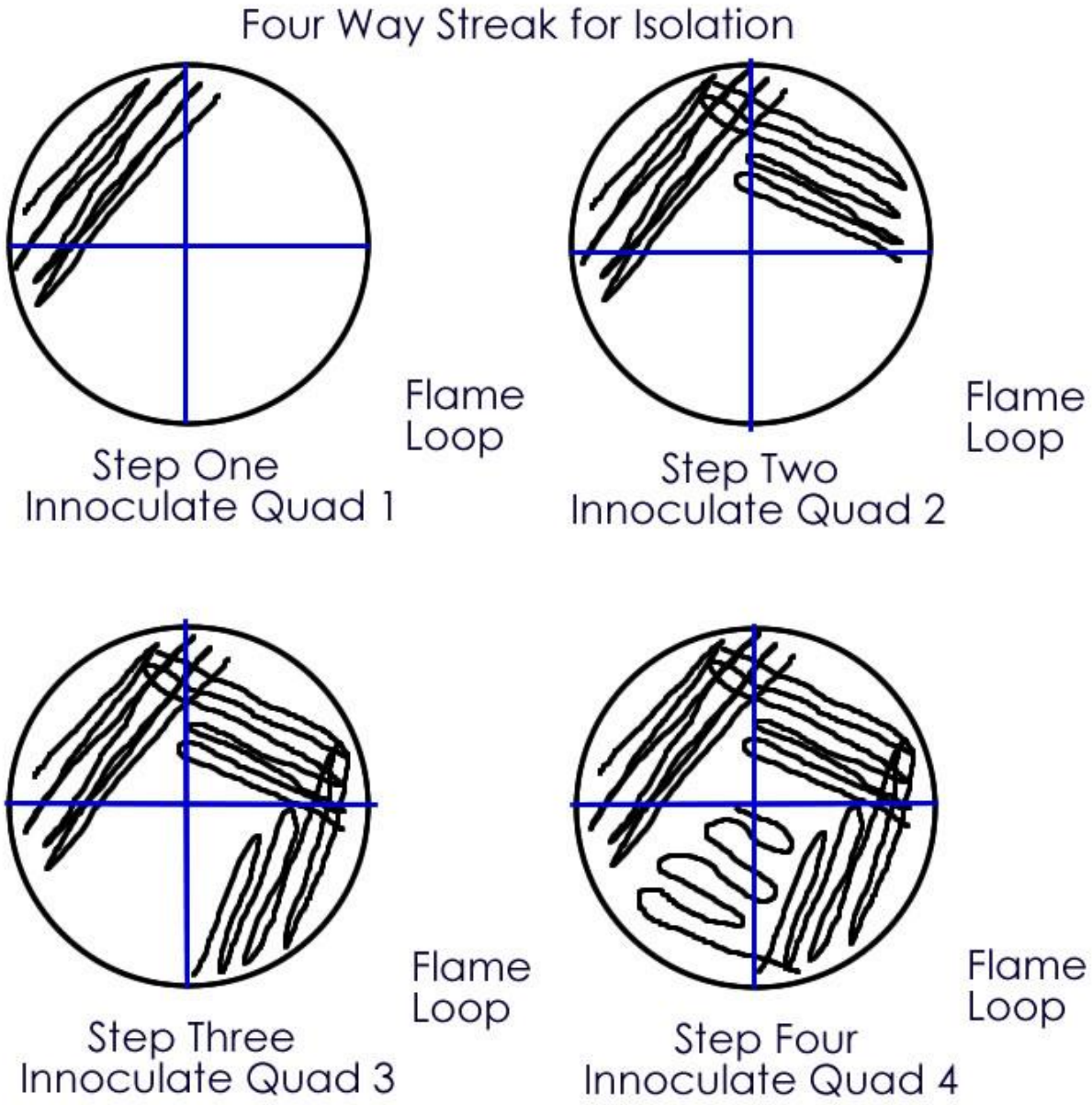


Figure 2: The streak-plate technique

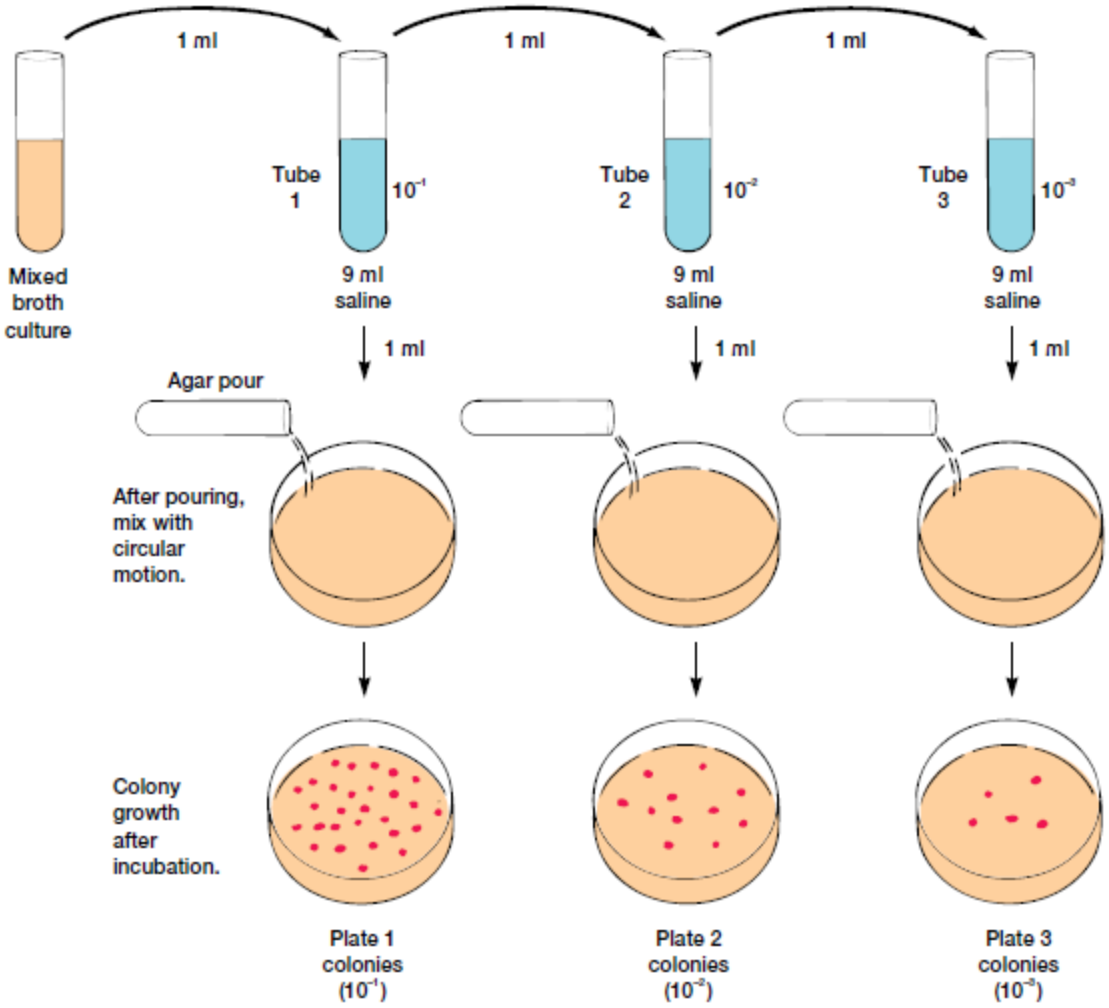


Figure 3: The Pour-Plate Technique

Maintaining and preserving pure cultures

Once microorganism has been isolated and grown in pure culture, it is necessary to maintain the viable culture; free from contamination, for some period of time. There are several methods available for maintaining and preserving pure cultures include:

1- Sub-culturing

The organisms may simply be sub-cultured periodically into a fresh medium to permit continued growth and to ensure the viability of a stock culture. Aerobes are maintained on agar slants. Anaerobes are maintained by growing the bacteria deep in the agar where air is excluded; this is achieved by stabbing an agar tube with an inoculating needle coated with a bacterial inoculum to produce a stab culture. For more stringent anaerobic conditions, cultures can be covered with 2-3cm of sterile mineral oil, and incubated in anaerobic chambers.

Unfortunately, frequent sub-culturing introduces high risk, since some genetic and physiological changes will occurred within the strain. Additionally; it is time consuming method, especially if large numbers of cultures are involved.

2-Maintenance at low temperature by refrigeration

Longer storage times can be achieved by lowering the temperature conditions for storage. Under these conditions, bacterial metabolism is sharply reduced and cultures can be maintained for 3-5 months at refrigerator temperatures 4-5 C˚.

3- Freezing

Much longer storage times are possible when cultures are stored at -196 to -20 c˚. These temperature requirements can be achieved by commercial ultra-cold freezers or by storing

cultures in containers of liquid nitrogen. The rapid freezing of cells is obligatory, as freezing induces ice crystal formation in cells that can lead to mechanical lyses and cell death, (often, protecting material such as glycerol is added to the culture). Glycerol is often employed as an antifreeze agent to prevent damage due to ice crystal and to ensure the ability to recover viable microorganisms when frozen cultures are thawed.

4-Drying

Removal of water also reduces rates of microbial metabolism, producing non metabolizing cultures that are not subjected to genetic or physiological changes. This method is particularly used to endospores forming bacteria. Endospores do not carry out active metabolism and are relatively dry. After drying process, the cultures must be covered to prevent air entrance.

In these types of cultures soil, sand, silica gel are used as a carrier. They have been known to remain viable for centuries. The procedure involve mixing of 20% soil ,78% sand and 2% calcium carbonate then sterilized in oven temperature 130 C° for 8-10 hours ,after cooling inoculate the tube with dried spore suspension and then store at room temperature.

5- Lyophilization

Simple desiccation of non-endospore forming bacteria is rarely used because of the loss of viability of most active bacterial cells during the drying process. Desiccation for long - term preservation of most cultures can be achieved by freeze-drying or lyophilization. Cell suspensions, usually in a medium containing a protecting substance, are quick frozen in a dry ice acetone bath. They are then desiccated in the frozen state using a high vacuum to sublime the water directly from the solid to the gaseous form. Cultures can be stored for many years without any appreciable loss of viability.

Maintaining stock cultures by sub-culturing

Materials

Petri plate with well isolated colonies, Nutrient agar slant, Inoculating loop, Bunsen burner.

Procedures

1-Under aseptic conditions, pick a well isolated colony and using the inoculating loop that you have sterilized in a bunsen burner flame, Transfer some of the cells to each of two labeled agar slants.

2- Place inoculated tubes into a 37 ˚C incubator for 24-48 hours.

3- At the next laboratory transfer one of the tubes to a refrigerator and the other to a room temperature or 28 ˚C incubator.

4- Store the cultures for six weeks.

5- After storage, compare the appearances of the culture stored at 25-28 ˚C and the culture stored at 5 C˚. Aseptically transfer material from each culture tube to fresh nutrient agar slants.

6-Incubate the inoculated tubes at 37 C˚ for 24-48 hours.

7- At the next laboratory, observe the new slants and describe their appearances.

The enumeration methods of Microorganisms

1- Direct Microscopic Method (Total Cell Count).

2- Spectrophotometric (turbidimetric) method.

3- The plate count method (standard, or viable counting**)**

1- Direct Microscopic Method (Total Cell Count)

In the direct microscopic count, a counting chamber consisting of a ruled slide and a coverslip is employed. A small number of bacteria in a volume is directly counted microscopically while the larger number of bacteria in the original sample is determined by preparing serial dilutions.

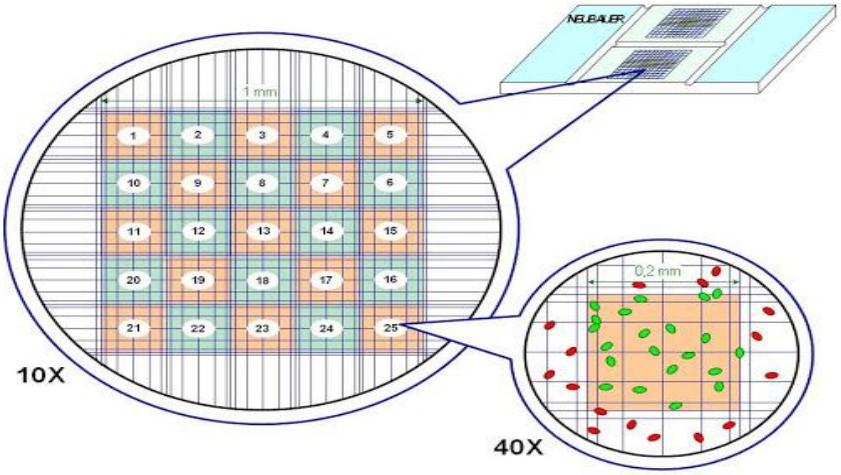
Materials Microscope, Hemocytometer & coverslip, Suspension of yeast

Procedure

1. Make a serial dilution series of the yeast suspension, from 1/10 to 1/10000.
2. Starting with the 1/10 dilution, use a Pasteur pipette to transfer a small aliquot of the dilution to the hemocytometer. Place the tip of the pipette into the H-shaped groove of the hemocytometer and allow the cell suspension to flow into the chamber of the hemocytometer by capillary action until the chamber is filled. Do not overfill the chamber.
3. Add a similar sample of diluted yeast to the opposite side of the chamber and allow the cells to settle for about 1 minute before counting.
4. Refer to the diagram of the hemocytometer grid in (Figure. 1) and note the following.
5. The 4 outer squares, marked 1-4, each cover a volume of 10^{-4} mL.
6. The inner square, marked as 5, also covers a volume of 10^{-4} mL, but is further subdivided into 25 smaller squares. The volume over each of the 25 smaller squares is 4.0×10^{-6} mL.
7. Each of the 25 smaller squares is further divided into 16 squares, which are the smallest gradations on the hemocytometer. The volume over these smallest squares is 25×10^{-6} mL.

8. The number of cells in a sample can be determined by counting the number of cells in one or more of the squares by using the following equation:

$$\textbf{Cells in 1 ml} = \frac{\textit{Number of the cells in the counted small square}}{\textit{Number of the}\ \textbf{considered small square}} \times 5 \times 10000$$



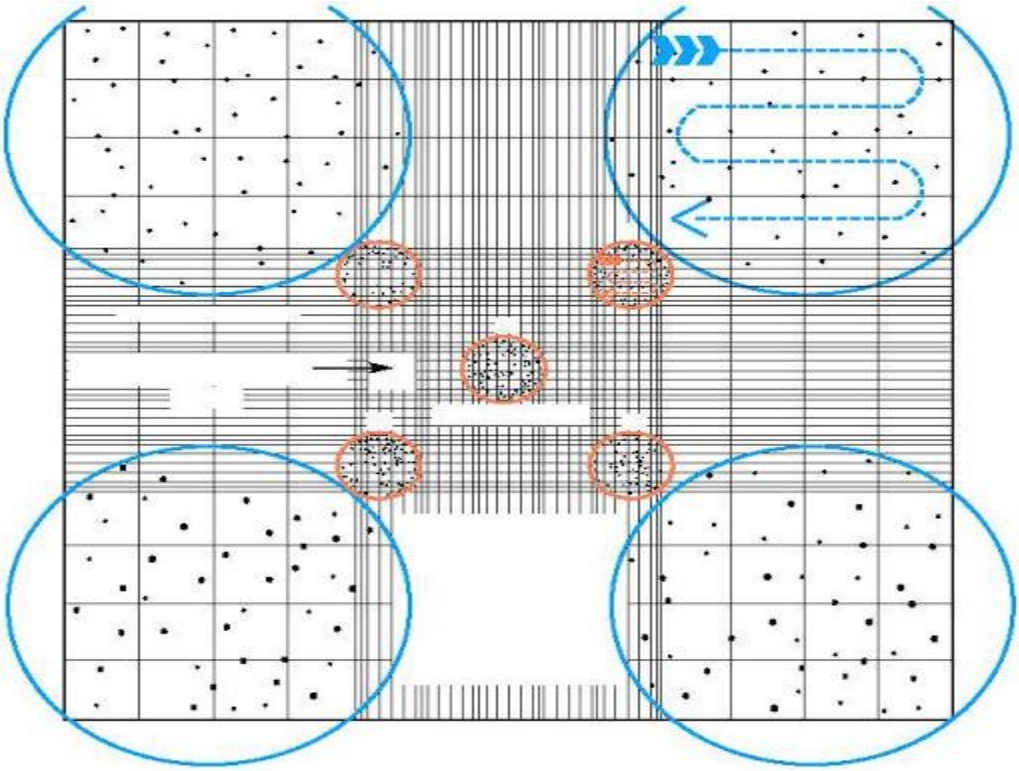


Figure (1)

2- The plate count method (standard, or viable counting)

It reveals information related only to live bacteria. It is an indirect measurement of cell density due to the number of bacteria in a given sample is usually too great to be counted directly, when the sample is serially diluted and then plated out on an agar surface, single isolated bacteria can form visible isolated colonies. The final plates in the series should have between 30 and 300 colonies. Each one distinguished as distinct **colony-forming units (CFUs).** The number of colonies can be used as a measure of the number of viable (living) cells in that known dilution.

Procedure

1-The original sample is diluted several times (10^{-1}-10^{-10}) to dilute the population sufficiently.

2- 1 ml of each dilution is then dispensed into the bottom of a petri plate.

3- Agar pours are then added to each plate. Isolated cells grow into colonies and can be used to establish pure cultures.

4- After the pour plates have cooled and the agar has hardened, they are inverted and incubated at 30°C for 24 hours or 25°C for 48 hours.

5. At the end of the incubation period, select all of the petri plates containing between 30 and 300 colonies (Figure.2).

6- By using a colony counter calculate the number of bacteria (CFU) per milliliter of original sample as follows:

$$\text{Number of CFUs / mL of sample} = \text{Number of colonies (30–300 plate)} \times \text{Inverse dilution } (10^{n})$$

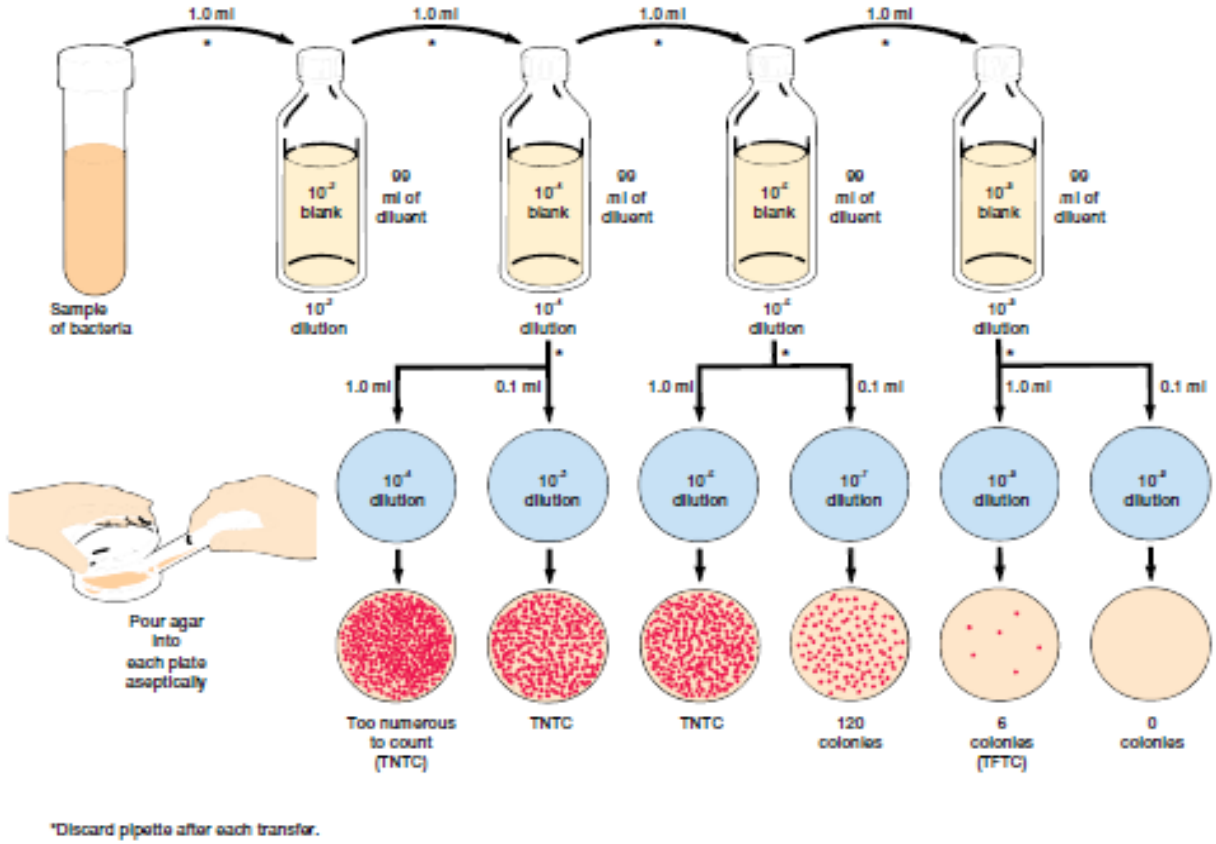


Figure (2): Quantitative Plating Procedure.

3- **Spectrophotometric (turbidimetric measurement) method.**

The spectrophotometric analysis is based on turbidity and indirectly measures all bacteria (cell biomass), dead or alive. The bacteria growing in a liquid culture appears turbid**.** This is <u>because</u> a bacterial culture acts as a colloidal suspension that blocks and reflects light passing through the culture. The instrument used to measure turbidity is a spectrophotometer. Within limits, the light absorbed by the bacterial suspension will be directly proportional to the concentration of cells in the culture.

Procedure

1. Twofold or tenfold serial dilutions made of a bacterial or yeast stock.
2. A counting chamber can then be used to perform a direct microscopic count on each dilution.
3. Then, a spectrophotometer that the correct wavelength in nanometers (550 - 600 nm) used to measure the absorbance of each dilution tube.

4. A standard curve comparing absorbance to the number of bacteria can be made by plotting absorbance versus the number of bacteria per ml (figure 3).
5. Once the standard curve is completed, any dilution tube of that organism can be placed in a spectrophotometer and its absorbance read. Once the absorbance is determined, the standard curve can be used to determine the corresponding number of bacteria per ml (figure 4).

Note; A tube that contains just sterile broth. This tube is called the **blank** because it has a sample concentration equal to zero. It should therefore have an absorbance of zero.

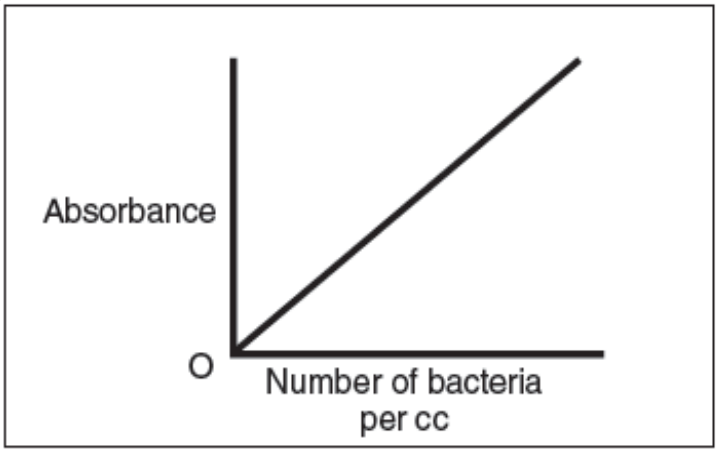


Figure (3): A standard curve plotting the number of bacteria per ml versus absorbance.

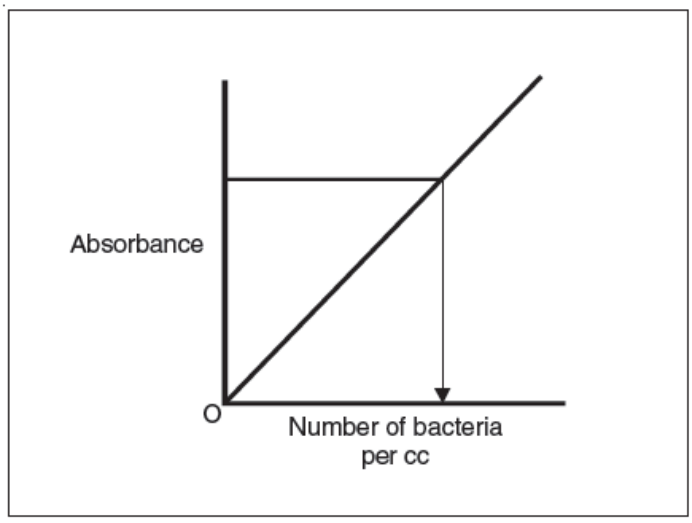


Figure (4): Using a standard curve to determine the number of bacteria per ml in a sample by measuring the sample's absorbance.

Solid state fermentation (SSF)

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms such as bacteria and fungi, to produce a wide variety of substances that are highly beneficial to individuals and industry like antibiotics, peptides and enzymes, in addition to the usual products of fermentation, such as carbon dioxide and alcohol.

There are two broad fermentation techniques have gained importance due to their economic and environmental advantages: Solid State Fermentation (SSF) and Submerged Fermentation (SmF).

Solid-State Fermentation (SSF)

This process involves the fermentation of solid substrate medium with microorganism in the absence of free flowing water.

SSF utilizes solid substrates, like wheat bran, rice and rice straw, hay, fruit and vegetable waste, paper pulp and bran. The main advantage of using these substrates is that nutrient-rich waste materials can be easily recycled as substrates. SSF is suited for fermentation techniques involving fungi and a number of bacteria requiring less moisture content. However, it cannot be used in fermentation processes involving organisms that require high water activity (a_w), such as most of bacteria. Thus, it is crucial to provide optimized water content, and control the (a_w) of the fermenting substrate for; the availability of water in lower or higher concentrations affects microbial activity adversely.

In SSF, microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture contents and the microbe is in contact with atmospheric oxygen unlike in Submerged Fermentation.

SSF are normally multistep processes involving the following steps

1- Pre-treatment of substrate raw materials either by mechanical, chemical or biochemical processing to enhance the availability of the bound nutrients and also to reduce the size of the components.

2- Hydrolysis of primarily polymeric substrates, e.g., polysaccharides and proteins.

3- Utilization (fermentation) of hydrolysis products.

4- Separation and purification of end products.

Advantages of SSF

1- Low cost & simple culture media, some substrates can be used directly as a solid media or enriched with nutrients

2-The product of interest is concentrated, that which facilitates its purification

3-The used inoculum is the natural flora of the substrates, spores or cells

4-The low humidity content and the great inoculum used in a SSF reduce vastly the possibility of a microbial contamination

5-The quantity of waste generated is smaller than the SmF (Low waste water output).

6-The enzymes are low sensitive to catabolic repression or induction

7- More energy economical.

8- No problems with foaming.

Disadvantages of SSF

1-The used microorganisms are limited those that grow in reduced levels of humidity

2- Reactor parameters such as humidity, pH, temp., free oxygen and carbon dioxide, need precise control.

3- Media are heterogenous, hence the mash is not properly mixture also substrate moisture level is difficult to control .

3-The scale up of SSF processes has been little studied and it presents several problems.

4- Continuous mixing or agitation of the medium required to overcome control parameters. Also is often damages the mycelia, retarding their growth and resulting in poor growth of the organisms.

Applications of SSF

1- Bioremediation & biodegradation of hazardous compounds.
2- Biological detoxification of agro-industrial residues.
3- Biotransformation of crops & crop-residues for nutritional enrichment.
4- Production of biologically active secondary metabolites, including antibiotics, alkaloids, plant growth factors, enzymes, organic acids & Biopharmaceuticals.
5- Production of bio-pesticides, including myco-pesticides & bio-herbicides.

Practical procedure

1- Prepare flask (250ml).
2- Weight 10gm of bran per flask
3- Add distill water to each flask in ratio of 1:1
4- Close the flask by cotton plugs then sterilize in autoclave
5- Prepare sterile vials some of them contain distilled water.
6- Prepare spores of *Aspergillus niger.*
8- Calculate the number of spore per ml by hemocytometer.
9- Place 1.5 ml from spore inoculate to the sterilized flask.
10 -incubate the flask in incubator at 28^{o}C for 3 -5 days.

Fermenter and Bioreactor

Bioreactor: Is a device, usually a vessel, used to direct the activity of a biological catalyst to achieve a desired chemical transformation.

Fermenter: type of bioreactor in which the biocatalyst is a living cell**.** Its refer to any devise that provides and maintains an optimum environment for the operation of fermentation. The fermenter is used to grow bacteria or fungi whereas bioreactor is used for eukaryotic cells such as plant cells. They also differ in the types of agitator and mixers used in them.

Proper design of ferment is essential for

1. Maintaining suitable environment required for controlled growth of the cells.
2. Maintaining septic condition and prevent contamination.
3. Regulation certain aspects like agitation, pH and aeration etc.
4. Easy isolation and purification for the desired product.
5. Easy cleaning of equipment.

Classification of fermenters

A- Based on size:

1- Laboratory (1-50 L).
2- Pilot plant (50-1000 L).
3- Production (> 1000 L).

B- Based on the type of growth system:

1- Submerged fermentation.
2- Solid state fermentation.

Components of fermenter

1- **Culture vessel**: commonly cylinder, range in size from one liter to many cubic meters and are often made of stainless steel or glass. The vessel is sterilized in an autoclave.
2- **Suppliers and feeders**: the vessel designed to contain 6 to 8 side necks to connect different sensors, air outlet, sample withdrawal etc.
3- **Measurement and control system**: they are monitoring devices to record different parameter (pH, O_2 and temperature etc.).

Temperature control

All fermentations generate heat from microbial activity and mechanical agitation of the media. Therefore fermenter must be cooled to prevent rise of temperature which damage the culture. Colling of the fermenters are slow process because the temperature of cooling water is only little degree lower than the fermenter temperature.

pH measurement and control

The pH is measured by a combined, serializable pH electrode with incorporated temperature sensor. The addition of acid or base is controlled by a micro-processor.

Oxygen measurement and regulation

A sterilize electrode measured the concentration of dissolved oxygen. Bases on that, the flow rate of air input can be set.

Air output

Used air is filtered. If necessary an optional condenser can be used to remove water vapor from the stream.

Sterilization

The fermentation vessel is sterilization as usual in an autoclave after disconnection of the cables from the electrodes and electromagnetic.

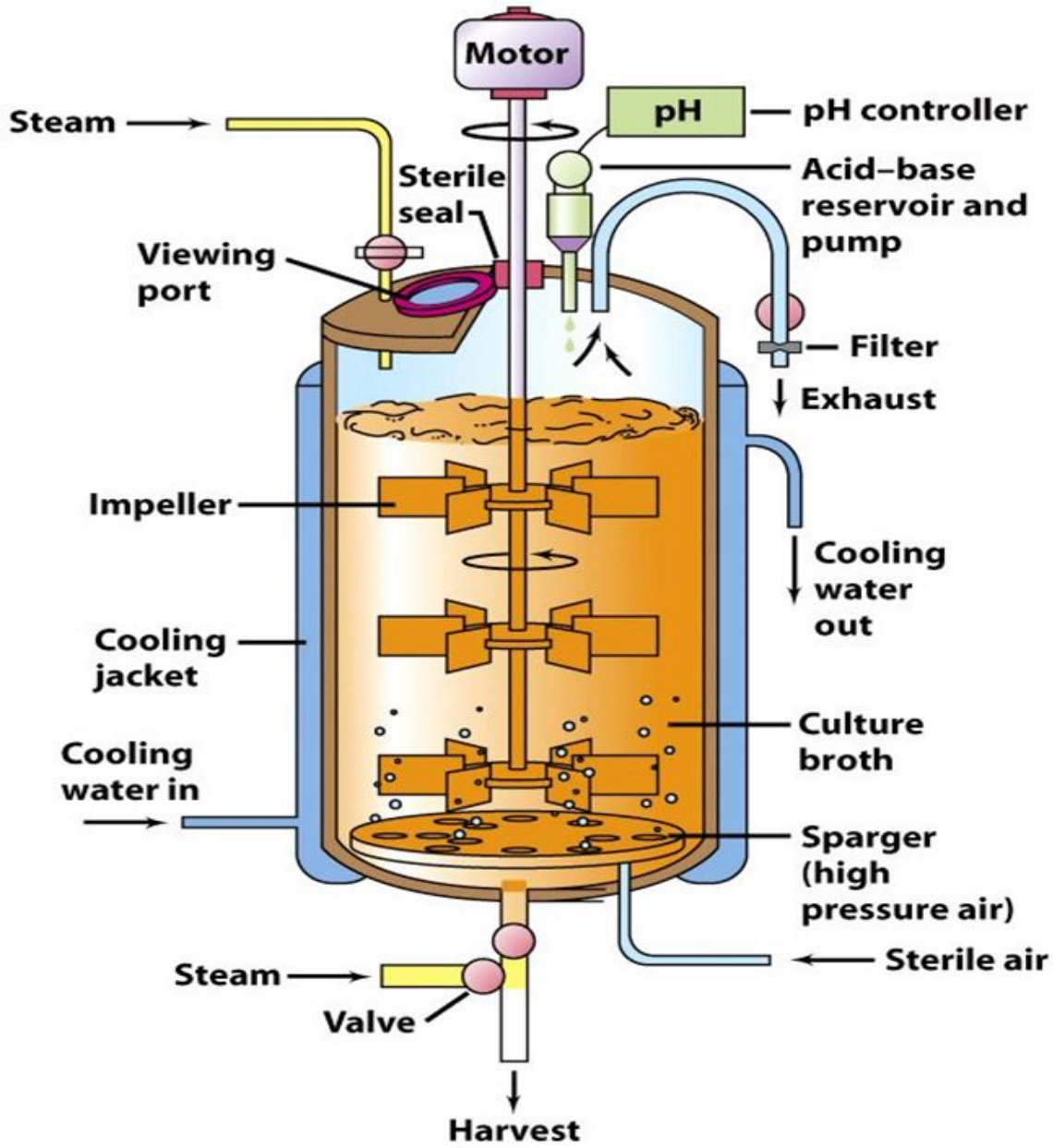


Figure: Show the fermenter and its sensors

The Roles of Enzymes in Biotechnology

What are Enzymes?

Enzymes form a special class of proteins produced by all living organisms. They act as catalysts (substances which in very small amounts are able to accelerate the rate of specific chemical reactions a million times or more) to increase the rate of chemical reactions. Consequently, enzymes are able to speed up the building up or breaking down of organic matter such as carbohydrates, fats and proteins.

Enzyme activity can be affected by **inhibitors** that decrease enzyme activity while **activators** are molecules that increase activity. Also affected by Temperature, **pH** & the **substrate concentration**.

The basic mechanisms of enzyme synthesis, including transcription, translation, and post translational processing, **Enzymes** are usually very specific as to which reactions they catalyze and the substrates that are involved in these reactions {each enzyme acts only on a restricted number of compounds (substances)}. Complementary shape, charge and hydrophilic/hydrophobic characteristics of enzymes and substrates are responsible for this specificity. For example, proteolytic enzymes present in the human digestive system, break down proteins into smaller molecules which can then be absorbed into the blood stream.

Peroxidases enzyme

The oxidoreductase class of enzyme, occurring in animal and plant tissues that catalyze the dehydrogenation (oxidation) of various substances in the presence of hydrogen peroxide. For many of these enzymes the optimal substrate is hydrogen peroxide, peroxidases can contain a hem cofactor in their active sites.

Peroxidase found in plant cells in to form

1- Soluble peroxidase: found in cytoplasm extracted by using neutral ionic strength buffer & neutral pH.

2- Bound peroxidase: found by covalent connection to cell wall or membrane extracted by using a high ionic strength buffer such as buffer contain 1M of NaCl.

Mechanism of peroxidase

Based on that of Bergmeyer method in which the rate of decomposition of hydrogen peroxide by peroxidase, with guaiacol as hydrogen donor, is determined by measuring the rate of color development spectrophotometrically at 436 nm and at 25˚C.

$$Guaiacol + 4H2O2 \xrightarrow{\text{Peroxidase}} Tetraguaiacol + 8H2o$$

Extraction of peroxidase from plants

Preparation of crude extract

1. Peel, wash, and cut plant tissues (radish, horseradish root) into small cubes.
2. Homogenize about 40 g in 200 ml (1:5) of distilled H_2O in a blender at high speed For 3-4 round/min. for 15 sec.
3. Clarify the extract by centrifugation (10-15,000 rpm/ 10 min.) and/or suction filtration through Whatman filter paper.
4. The extract may be stored for at least a week at 4°C.

Measurement of enzymatic activity

Preparation of substrate composed from

H_2O_2	(0.01 M)
Guaicol	(0.05 M)
Sodium acetate buffer pH 6	(0.1 M)
Distilled water	

Mix this components as shown:

H_2O_2	**Guaiacol**	**buffer**	**D.W**
1 ml	1 ml	1 ml	7 ml

Procedure

1- Add 3ml of substrate solution (prepared above) in the cell of spectrophotometer (Cuvate) (Consider as a blank).
2- Prepare a stop watch, and fix the spectrophotometer at wave length 436 nm.
3- Add 0.1 ml of crude extract to substrate solution in the cell (cuvate), mix well & then press on stop watch to start the measure.
4- Take the reading at 30 seconds (30, 60, 90,120,150,180 second).
5- Draw curve between the absorbance and the time(min.) to find:

Slope = $\Delta y/\Delta x$ =ΔAbs at 436nm/Δtime

Activity (unit/ml) = (3.1 x slope / 0.1 x 6.4) x DF

Enzyme Activity: Is the amount of enzyme which catalysis the conversion of one Micromole of hydrogen peroxide per minute at 25˚C.

Enzyme purification by ammonium sulfate precipitation

The raw materials for the isolation of enzymes are animal organs, plant material and M.O.s. The degree of purity of commercial enzymes ranges from raw enzymes to highly purified forms and depends on the application.

Downstream processing is a very important step in biotechnology because costs for collection, concentration and purification of the final product are substantial. High product concentrations in the supernatant or inside the cells and efficient purification are therefore important aspects in the overall economy of enzyme manufacture.

The choice of procedures for enzyme purification depends on their location. Isolation of intracellular enzymes often involves the separation of complex biological mixtures. While extracellular enzymes are generally released into the medium with only a few other components.

Cell disruption occur by **mechanical** methods such as high-pressure homogenization & the wet grinding of cells in a high-speed bead mill, and by **non-mechanical** methods ex. cells may frequently be disrupted by chemical, thermal, or enzymatic lysis.

After cell disruption, the next step is separation of extracellular or intracellular enzymes from cells or cellular fragments, respectively.

Purification is to be achieved, the volume of starting material must be decreased by one of the following concentration methods;

A- Thermal methods, B- Ultrafiltration, C- Precipitation

Precipitation with Salts

Ammonium sulfate precipitation

Is a method used to concentrate and purify proteins by altering their solubility. The solubility of proteins varies according to the ionic strength of the solution, and hence according to the salt concentration. At low salt concentrations, the solubility of the protein increases with decreasing salt concentration, an effect termed salting in.

As the salt concentration (ionic strength) is increased further, the solubility of the protein begins to decrease. At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution (salting out).

The ammonium sulfate amount to add can be determined from special tables. Each protein precipitate is dissolved individually in fresh buffer and assayed for total protein content and amount of desired protein. Salting-out is a very useful procedure to assist in the purification of a given protein. Ammonium sulfate is commonly used salt as it is very water soluble and has no adverse effects upon enzyme activity. It is generally used as a saturated aqueous solution which is diluted to the required concentration, expressed as a percentage concentration of the saturated solution (a 100% solution).

Practical part

1- Extraction of peroxidase from horseradish by using buffer (pH 6) in ratio 1:10.
2- To precipitation the desired protein (peroxidase) using ammonium sulfate at concentration 50% through addition of salt gradually to your sample to get the desired concentration, then stir for 1 hour to fully equilibrate.
3- Centrifuge at 10000 rpm for 30 minutes to pellet out protein.
4- Dissolve pellets in buffer to analyze proteins.

Purification

For many industrial applications, partially purified enzyme preparations will suffice; however, enzymes for analytical purposes and for medical use must be highly purified. Chromatography, is of fundamental importance to enzyme purification, molecules are separated according to their physical properties (size, shape, charge, hydrophobic interactions), chemical properties (covalent binding), or biological properties (bio specific affinity).

Immobilization of Enzymes

Enzyme immobilization is a process by which an enzyme is chemically or physically attached to a carrier to impart better physical and chemical properties than free enzymes would exhibit outside of its natural environment and give a longer life span. In addition, enzyme immobilization leads to increased stability, & ease of separation from product when applied to organic synthesis or industrial processes.

Enzyme immobilization is confinement of enzyme to a phase (matrix/support) different from the one for the substrates and products. The materials used for immobilization of enzymes, called carrier matrices which are grouped into three major categories:

1- Natural polymers: cellulose, gelatin, chitosan, collagen, pectin & starch.
2- Synthetic polymers: DEAE cellulose, PVC, PEG.
3- İnorganic polymers: Ceramic, Silica, Glass, Charcoal.

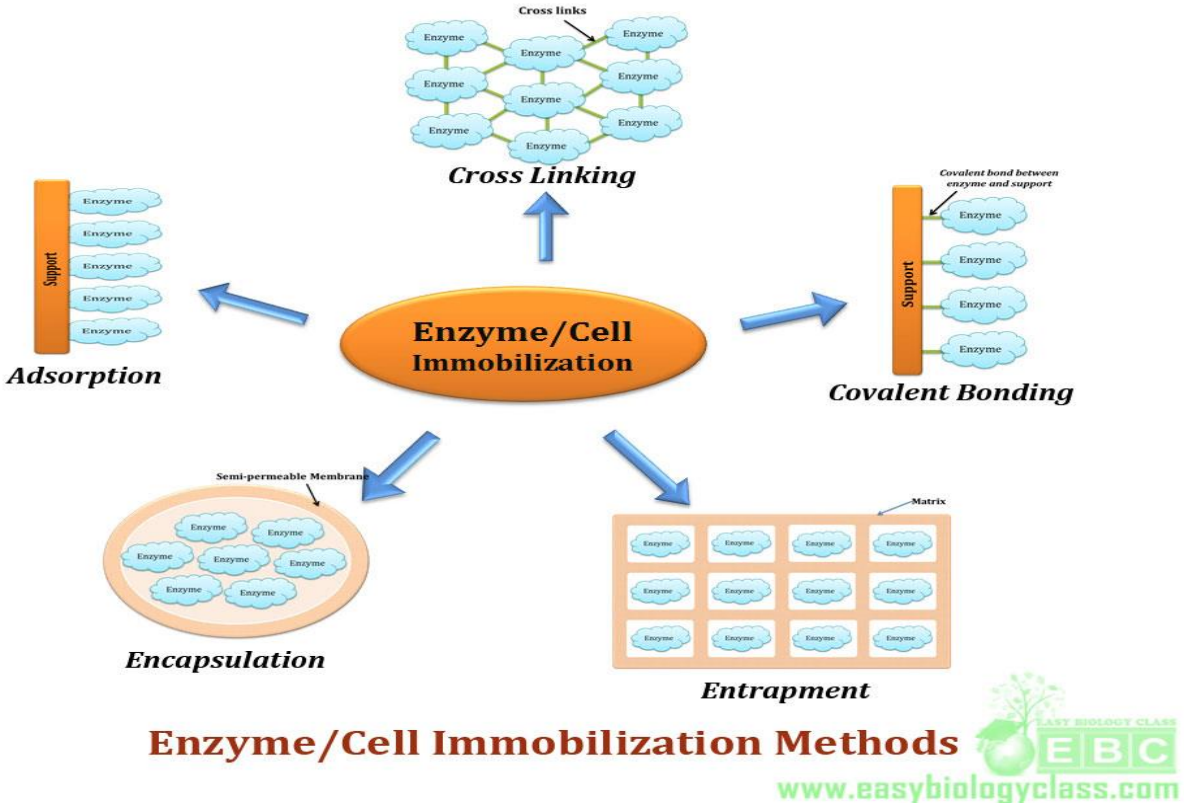
Benefits of immobilizing enzyme

1- Repetitive use of Enzymes.
2- Product is not contaminated with the enzyme.
3- Easy separation of enzyme from the product (food & pharmaceutical industries).
4- Continuous production systems can be used.
5- Thermal stability of Enzymes are usually increased by binding.
6- The ability to stop the reaction rapidly by removing the enzyme from the reaction solution, this led to improved process control.
7- Allows development of a multi-enzyme reaction system.

Classification of immobilization methods for enzymes

The various methods used for immobilization of enzymes may be grouped into two main types:

1- Entrapment types like gel or fiber entrapment.
2- Microencapsulation.
3- Binding types like Crosslinking.
4- Covalent & metal binding.
5- Physical adsorption.



Enzyme immobilization by gel entrapment

The major components of an immobilized enzyme system are the enzyme, the matrix, and the mode of attachment of the enzyme to the matrix. The entrapment method is based on the occlusion of an enzyme within a polymeric network that allows the substrate and products to pass through but retains the enzyme.

An excellent matrix that has been extensively used in this method is agarose. In addition to its high porosity, which leads to a high capacity for proteins, some other advantages of using agarose as a matrix are hydrophilic character, absence of charged groups (which prevents nonspecific adsorption of substrate and products), and commercial availability. However, an important limitation in the use of agarose is the high cost.

Procedure of peroxidase entrapment by agarose

1- Extraction of peroxidase enzyme from radish (1:10) by 0.1M phosphate buffer pH 7).
2- Prepare 100ml of 1% agarose.
3- Mix 2 ml of enzyme with 10 ml of 1% agarose (chilled the agar to 45 C°).
4- Pour the mixture of enzyme with immobilization material (agarose) on the petri dish then cut the hardened gel into small cubes limits of 3 mm.
5- Wash the small cubes of gel with phosphate buffer.
6- Put the small cubes of immobilized enzyme in container with substrate of peroxidase, changing the color of substrate to brown indicates that the enzyme entrapped with agarose.

Production of Single cell protein from yeast

Single-cell protein refers to the crude, a refined or edible protein extracted from pure microbial cultures, dead, or dried cell biomass. They can be used as a protein supplement for both humans and animals. Microorganisms like algae, fungi, yeast, and bacteria have very high protein content in their biomass. These microbes can be grown using inexpensive substrates like agricultural waste viz. wood shavings, sawdust, corn cobs etc. and even human and animal waste. The microorganisms utilize the carbon and nitrogen present in these materials and convert them into high-quality proteins which can be used as a supplement in both human and animal feed. The single-cell proteins can be readily used as fodder for achieving fattening of calves, pigs, in breeding fish and even in animal husbandry poultry and Cattle Farming. Single Cell Protein (SCP) offers an unconventional but plausible solution to this problem of protein deficiency being faced by the entire humanity.

Advantages of using Microorganisms

1- M.Os grow at very fast rate under optimal conditions
2- Quality and quantity is better than higher plants and animals
3- Wide range of raw materials can be used (it causes less pollution)
4- Culture and fermentation conditions are simple
5- M.Os can be genetically manipulated
6- Independent on land & climate as plants & animals.

Properties of M.O.s that uses for produce of SCP

The selection of certain microbial strain is very important, some of the criteria are:

1- Non-pathogenic
2- Microorganisms have a high rate of multiplication, which means a large quantity of biomass can be produced in a comparatively shorter duration.

3- The microbes can be easily genetically modified to vary the amino acid composition.

4- A broad variety of raw materials, including waste materials, can be used as a substrate. This also helps in decreasing the number of pollutants.

5- Production is independent of climatic conditions.

The basic step for process of SCP production

1. Selected microorganism is inoculated in a pure state.
2. Fermentation.
3. Harvesting (Filtration the yield).
4. Post-harvest treatment (Drying the new microbial biomass).
5. Calculate the new weigh of biomass.

Procedure in the lab

A - Preparation of the inoculum

Prepare the yeast *Saccharomyces cerevisiae* by mixing 1gm yeast /25 ml of sterilized water in a flask.

B- Preparation of the growth medium

Dates extract is used in this experiment which prepare as follow:

1- Prepare 100 ml of the date extract with sugar concentrations 10% (by using the refractometer).

2- Add the following compounds to enhance the growth of the yeast:

0.1% KH_2PO_4 0.02% $MgSO_4.7H_2O$ 0.03% Urea

3- Adjust the pH to 4.5-5.5 (to prevent bacterial growth).

4- Plugged the flasks with cotton and covered it with aluminum foil. Then sterilize by autoclaving for 15 min at 121°C.

C- Cultivation conditions

Inoculate each flask with 2% of yeast cells suspension. Incubate the inoculated flasks in a shaker incubator at 30°C and 150rpm for 24-48 hours.

D- Analytical methods

1- Take 5 ml from each flask.

2- Centrifuge the samples at 6000 rpm for 10 min.

3-Take sample from the supernatant in each test tube and measure the concentration of sugar by refractometer to determine how much sugar was consumed.

4- Weight 3 filter papers and use them to collect the sediment from each test tube.

5- Place the filter papers in an oven for drying at 40°C for 24 hour. Next, weight the filter papers; the differences in weights represent the amounts of SCP produced in each flask.

6- Calculate the SCP produce using the following relation:

$$SCP\ (gram) = Weight\ of\ filter\ paper\ with\ dried\ sediment - weight\ of\ the\ paper$$

Calculate the productivity of SCP using the following relation:

$$Yield\ \% = Weight\ of\ SCP \times 100 \ / Consumed\ sugar$$

Antibacterial Activity of Ginger (*Zingiber Officinale*) Extract

The Nature is the greatest pharmacy on Earth, plants are the most formidable chemists. They are constantly producing an arsenal of chemical compounds, in order to respond to different challenges and threats in their environment.

Plants have provided humans with many of their essential needs, including life-saving pharmaceutical agents. Recently the WHO estimated that 80% people worldwide rely on herbal medicines for some aspect.

It is to be noted that most herbs can be used fresh; drying only to ensure that can make an herbal infusion of a given plant any time of the year. Herbs are used as raw materials in therapeutics, and this plant material is called **drug**.

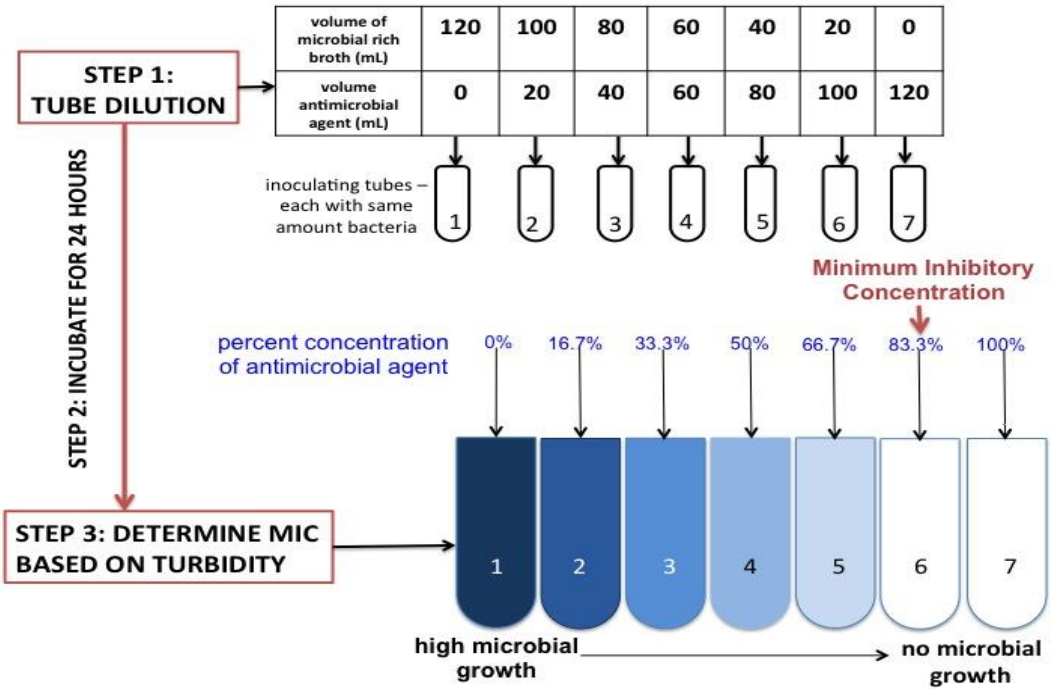
Medicinal plants used until now for the treatment of microbial infections as alternatives to chemically synthetic drugs to which many infectious M.O.s have become resistant. Antibiotic resistance is recognized by the World Health Organization (WHO) as the greatest threat in the treatment of infectious diseases.

An important potential strategy to help combat the resistance problem involves the discovery and development of new active agents capable of partly or completely suppressing bacterial resistance mechanisms.

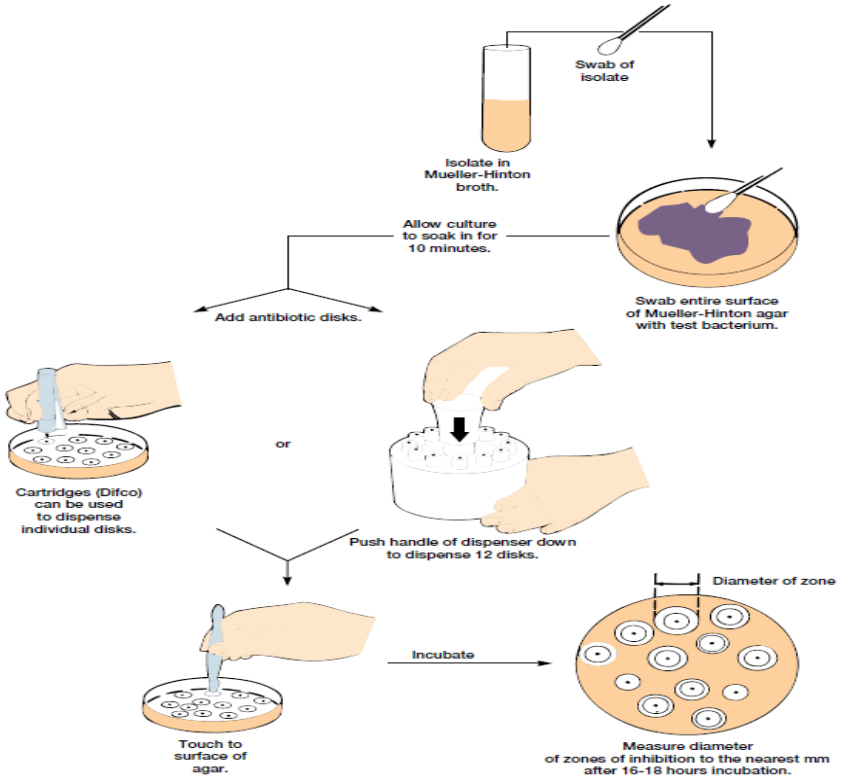
The lowest concentration at which the isolate is completely inhibited (as evidenced by the absence of visible bacterial growth) is recorded as the minimal inhibitory concentration or MIC.

Antimicrobial activity assay can be carried out in 2 ways:

A. broth dilution



B. Agar dilution



Many factors are involved in sensitivity disk testing and must be carefully controlled these include:

1. size of the inoculum
2. distribution of the inoculum
3. incubation period
4. depth of the agar
5. diffusion rate of the antibiotic
6. concentration of antibiotic in the disk
7. Growth rate of the bacterium

Procedure

Preparation of plant crude extract:

1- Peel, wash, and cut plant tissues (**Ginger:** *Zingiber officinale* Roscoe) into small cubes.

2- Homogenize about 10 g in 50 ml (1:5) of D.W in a mortar by grinding.

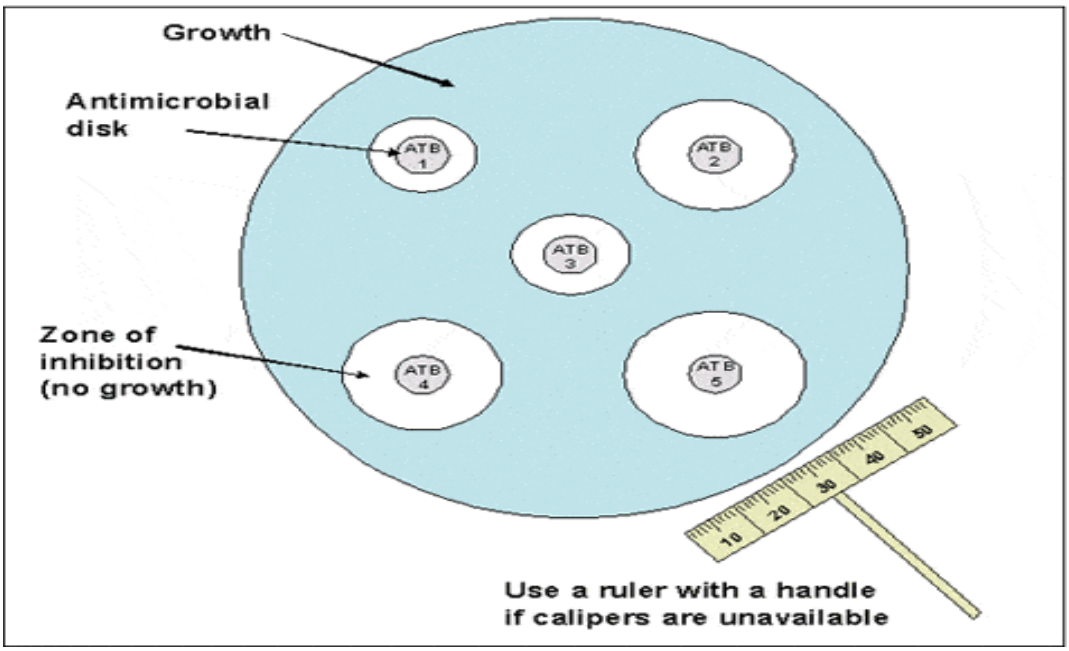
3- Clarify the extract by filtration through (Whatman No. 1) filter paper the extract may be stored at 4°C. & later used to detect it is activity against *Escherichia coli*.

4- Nutrient broth was used for growing the microorganism (*Escherichia coli*) suspension, with cells density of the inoculum was approximately 10^5 cells/ml.

5- The cells suspension was inoculated on nutrient agar.

6- The antibacterial activity of the extract was determined by using agar disc diffusion method.

7- The activity was determined by the measurement of the inhibitory zone diameter in mm after incubation at 37°C of bacteria, for 48 hours. The disc without plant extract was used as negative control.


Growth
Antimicrobial disk
ATB 1
ATB 2
ATB 3
ATB 4
ATB 5
Zone of inhibition (no growth)
10
20
30
40
50
Use a ruler with a handle if calipers are unavailable

Solid state fermentation (SSF)

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms such as bacteria and fungi, to produce a wide variety of substances that are highly beneficial to individuals and industry like antibiotics, peptides and enzymes, in addition to the usual products of fermentation, such as carbon dioxide and alcohol.

There are two broad fermentation techniques have gained importance due to their economic and environmental advantages: Submerged Fermentation (SmF) and Solid State Fermentation (SSF).

Solid-State Fermentation (SSF)

This process involves the fermentation of solid substrate medium with microorganism in the absence of free flowing water. SSF utilizes solid substrates, like wheat bran, rice and rice straw, hay, fruit and vegetable waste, paper pulp, bagasse and bran involving fungi and a number of bacteria. The main advantage of using these substrates is that nutrient-rich waste materials can be easily recycled as substrates. In SSF, microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture contents and the microbe is in contact with atmospheric oxygen unlike in submerged fermentation.

SSF are normally multistep processes involving the following steps:

1- Pre-treatment of substrate raw materials either by mechanical, chemical or biochemical processing to enhance the availability of the bound nutrients and also to reduce the size of the components.
2- Hydrolysis of primarily polymeric substrates, e.g., polysaccharides and proteins.
3- Utilization (fermentation) of hydrolysis products.
4- Separation and purification of end products.

Advantages of SSF

1- Low cost & simple culture media, some substrates can be used directly as a solid media or enriched with nutrients

2-The product of interest is concentrated, that which facilitates its purification.

3-The used inoculum is the natural flora of the substrates, spores or cells.

4-The low humidity content and the great inoculum used in a SSF reduce vastly the possibility of a microbial contamination

5-The quantity of waste generated is smaller than the SmF (Low waste water output).

6-The enzymes are low sensitive.

7- More energy economical.

8- No problems with foaming.

Disadvantages of SSF

1-The used microorganisms are limited those that grow in reduced levels of humidity.

2- Reactor parameters such as humidity, pH, temp., free oxygen and carbon dioxide, need precise control.

3- Media are heterogenous, hence the mash is not properly mixture also substrate moisture level is difficult to control.

4-The scale up of SSF processes has been little studied and it presents several problems.

5- Continuous mixing or agitation of the medium required to overcome control parameters. Also is often damages the mycelia, retarding their growth and resulting in poor growth of the organisms.

Applications of SSF

1- Bioremediation & biodegradation of hazardous compounds.

2- Biological detoxification of agro-industrial residues.

3- Biotransformation of crops & crop-residues for nutritional enrichment.

4- Production of biologically active secondary metabolites, including antibiotics, alkaloids, plant growth factors, enzymes, organic acids & Biopharmaceuticals.

5- Production of bio-pesticides, including myco-pesticides & bio-herbicides.

Practical procedure:

1- Prepare flask (250ml).

2- Weight 10gm of bran per flask

3- Add distill water to each flask in ratio of 1:1

4- Close the flask by cotton plugs then sterilize in autoclave

5- Prepare sterile vials some of them contain distilled water.

6- Prepare spores of *Aspergillus niger*.

8- Calculate the number of spore per ml by hemocytometer.

9- Place 1.5ml from spore inoculate to the sterilized flask.

10 -Incubate the flask in incubator at 28c for 3 -5 days.

What is a restriction enzyme?

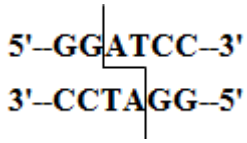
Restriction enzyme is a protein produced by bacteria that cleaves DNA at specific sites along the molecule. In the bacterial cell, restriction enzymes cleave foreign DNA, thus eliminating infecting organisms. Restriction enzymes can be isolated from bacterial cells and used in the laboratory to manipulate fragments of DNA, such as those that contain genes; for this reason they are indispensable tools of recombinant DNA technology (genetic engineering). A bacterium uses a restriction enzyme to defend against bacterial viruses called bacteriophages, or phages. When a phage infects a bacterium, it inserts its DNA into the bacterial cell so that it might be replicated. The restriction enzyme prevents replication of the phage DNA by cutting it into many pieces. Restriction enzymes were named for their ability to restrict, or limit, the number of strains of bacteriophage that can infect a bacterium. Each restriction enzyme recognizes a short, specific sequence of nucleotide bases (the four basic chemical subunits of the linear double-stranded DNA molecule—adenine, cytosine, thymine, and guanine). These regions are called recognition sequences, or recognition sites, and are randomly distributed throughout the DNA. Different bacterial species make restriction enzymes that recognize different nucleotide sequences.

Identity of Restriction Enzymes

Restriction enzymes are named for the organism from which they were first isolated. For example

- *Eco*RI is isolated from *E. coli* strain RY13.
- *Eco* refers to the genus and species (1st letter of genus; 1st two letters of species).
- R is the strain of *E. coli*
- I (Roman numeral) indicate it was the first enzyme of that type isolated from *E. coli* RY13.
- *Bam*HI is isolated from *Bacillus amyloliquefaciens* strain H
- Sau3A is isolated from *Staphylococcus aureus* strain 3A.
- And so on.

- ✓ Some restriction enzymes also cut DNA to form "blunt" ends (without single-stranded tails), which also can be inserted into target DNA via the action of DNA ligase.
- ✓ DNA ligase isn't picky: it can't tell the difference between foreign and host DNA (who'd figure it would ever have to?), and this enables the creation of chimeric DNA--DNA from two separate sources.
- ✓ Each enzyme recognizes and cuts specific DNA sequences. For example, *Bam*HI recognizes the double stranded sequence to form "sticky ends".



Notes:

- Most restriction enzymes are specific to a single restriction site.

- Restriction sites are recognized no matter where the DNA came from.
- The number of cuts in an organism's DNA made by a particular restriction enzyme is determined by the number of restriction sites specific to that enzyme in that organism's DNA.
- A fragment of DNA produced by a pair of adjacent cuts is called a **restriction fragment**.
- A particular restriction enzyme will typically cut an organism's DNA in to many pieces, from several thousand to more than a million!
- There is a great deal of variation in restriction sites even within a species.
- Although these variations do not have phenotypic expression beyond the base sequences themselves, the variants can be considered molecular "alleles," and they can be detected with sequencing techniques.
- As such, they can be used in mapping studies similar to the way true genes with known phenotypic effects can be used, but skipping the breeding steps and going straight to the molecules.