Ministry of Higher Education
and Scientific Research
University of Baghdad
College of Sciences
Department of Biotechnology

Principles of Biotechnology/Practical/ First course/Year 1

مدرسو المادة

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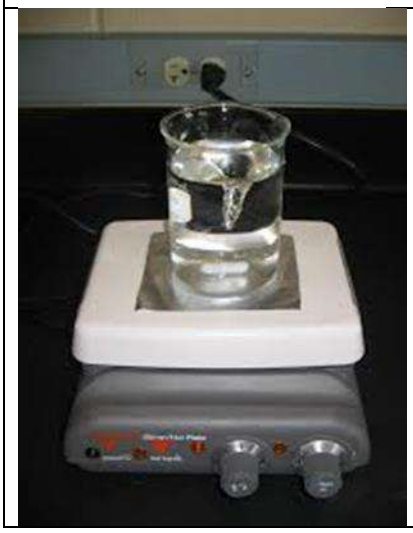
Lab.1 Laboratory Equipment

a- 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	It is a device which provides mixing and keeping the chemical solutions and mixtures at a certain time and temperature by the help of a magnetic bar.

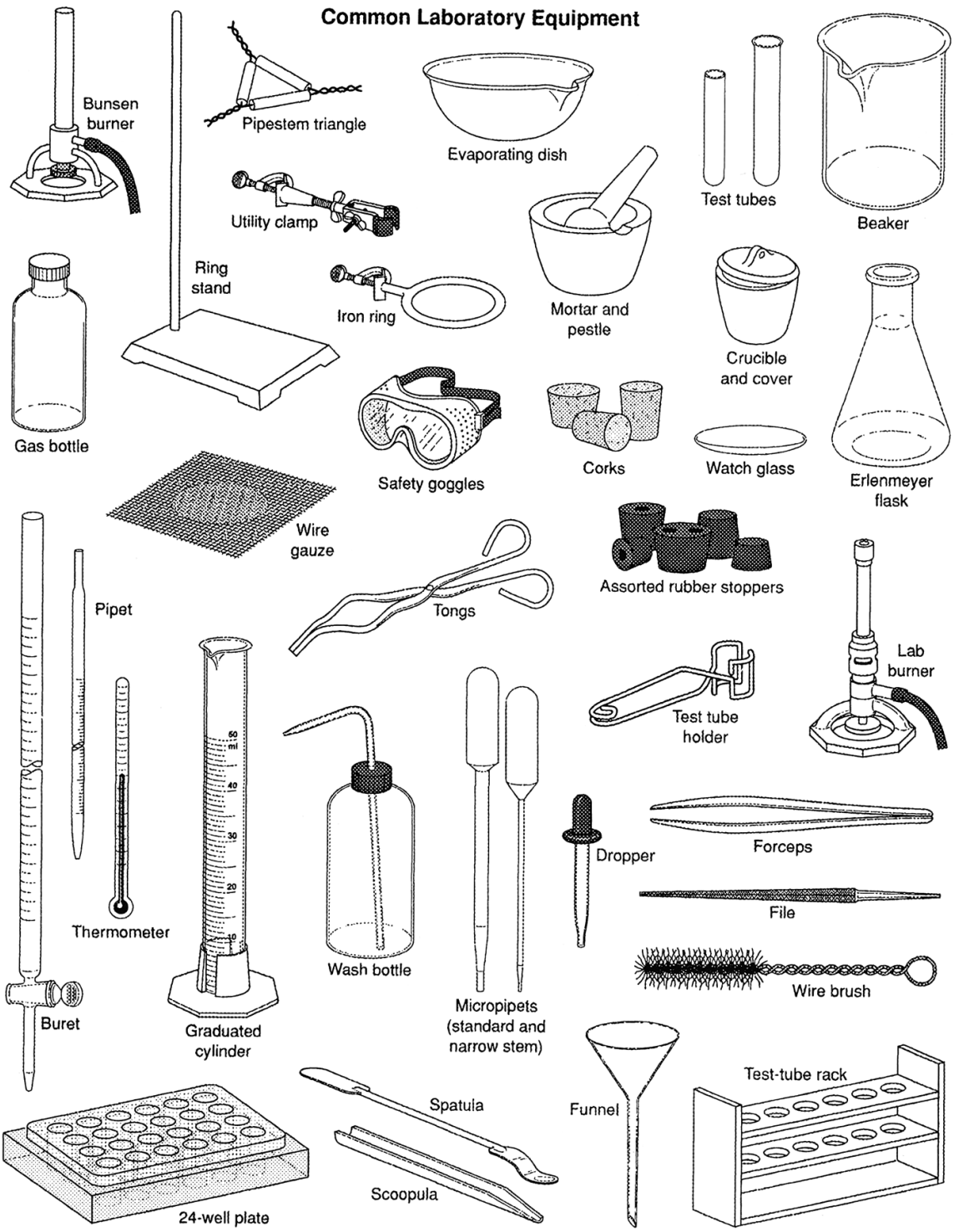
	Vortex mixer	Used for mixing the solutions
	Incubator	It's a device used to grow and maintain microbiological cultures or cell cultures. The incubator maintains optimal temperature, humidity and other conditions such as the carbon dioxide (CO2) and oxygen content of the atmosphere inside.
	Shaking incubator	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.

	Hood	Used to provide sterile conditions
	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.

	Electrophoresis system	This device is used for profiling DNA fragments according to their sizes after polymerase chain reaction (PCR).
	Gel documentation system (transilluminator)	This device is used to display DNA fragments after electrophoresis run.
	NanoDrop spectrophotometer	The NanoDrop is designed for measuring nucleic acid concentrations in sample volumes of one microliter.

b- Laboratory tools:



Lab2. Microorganisms 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, physical and chemical.**

1. **Chemical requirments.**

Chemical factors	Form usually found in nature	Chemical form commoly 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 (H2O), 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^+	NaCI
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 favoured 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 salterns 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**
    1. **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.
    2. **Semisolid media**
       They are prepared with agar at concentrations of 0.5% or less. They have soft custard like consistency and are useful for the determination of bacterial motility.
    3. **Liquid (Broth) medium**
       These media contains specific amounts of nutrients but don't have trace of gelling agents such as gelatin or agar. Broth medium 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**
    1. Synthetic or chemically defined medium
       A chemically defined medium is one prepared from purified ingredients and therefore whose exact composition is known.
    2. Non synthetic (Natural) or chemically undefined medium, like: Molasses, and Whey.
    3. 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

1. Wipe down lab bench carefully with Disinfectant to help prevent contamination of your media.
2. 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.
3. Weigh out 1.5 g beef extract and 2.5 g peptone and add into the flask.
4. Stir over gentle heat from a bunsen burner to dissolve completely.
5. Check the pH of the medium and adjust to pH 7.0, if necessary, using the HCl and/or NaOH.
6. Pour the mixture into the 1 L graduated cylinder and add warm water to the 500 ml mark. Pour back into the flask.
7. 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.
8. Add 6.0 g of agar to the flask and label it NA.
9. 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 the tubes using a 10 ml pipette. Make ten 10 ml tubes.
10. Close the flask with a Styrofoam plug covered.
11. Autoclave the flask and the tubes for 15 minutes at 121 °C and 15 lb/in2 pressure.
12. 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.
13. 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.
14. 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.
15. Flame the neck of the flask between each plate.
16. 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.
17. Clean all glassware and leave on paper towels beside sink.

Lab.3 Isolation of Microorganisms from a different environments by a different techniques

Objective: A students should be able to understand the different Isolation techniques & the purpose of the Pure Cultures Isolation.

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** is an easy, direct way of achieving this result. 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 clone 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 as it glides along 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 deposited on the agar surface. Because of this gradient, confluent growth occurs on 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 deposited 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. Dip 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. Record your results in the report for lab 3.

The streaking Procedure

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

a. Carefully lift the top of the 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. Then Insert the loop under the lid and cool it at the edge of the agar near area 1.
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.

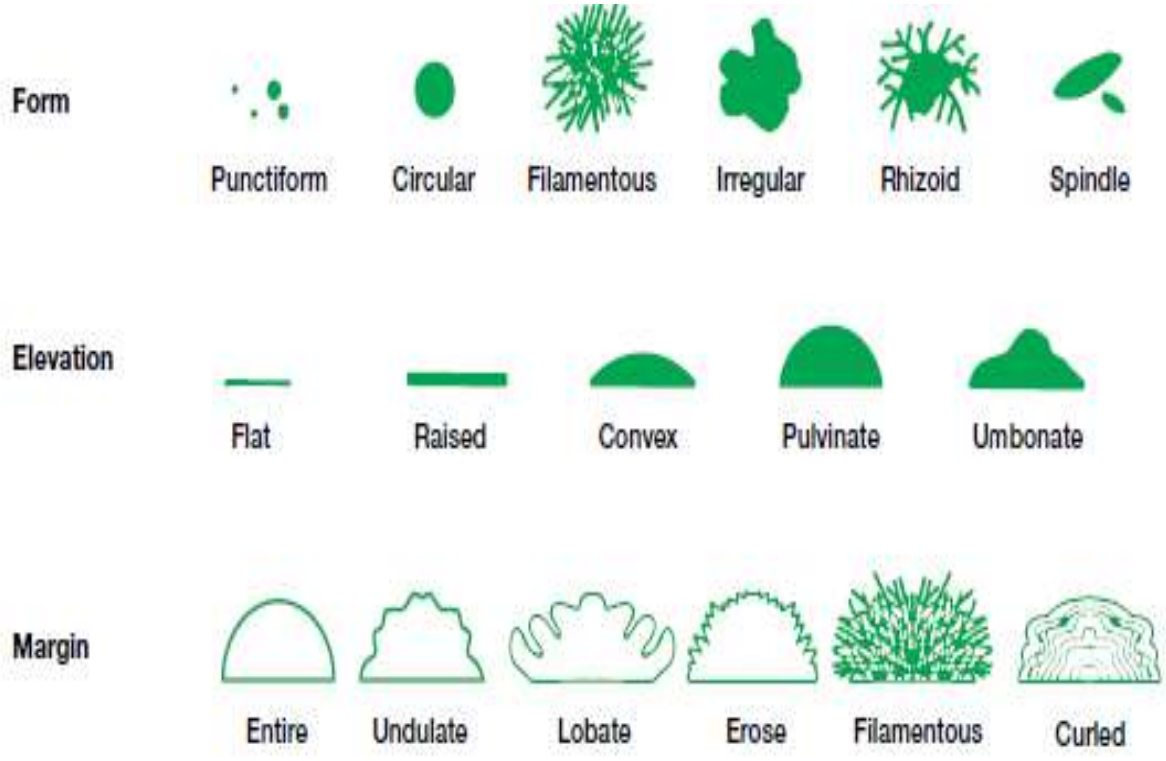


Figure 1: **Bacterial Colony Characteristics on Agar Media as Seen with the Naked Eye.** The characteristics of bacterial colonies are described using the following terms.
Appearance: Shiny or dull Optical property: Opaque, translucent, transparent
Pigmentation: Pigmented (purple, red, yellow) Non-pigmented (cream, tan, white)
Texture: Rough or smooth

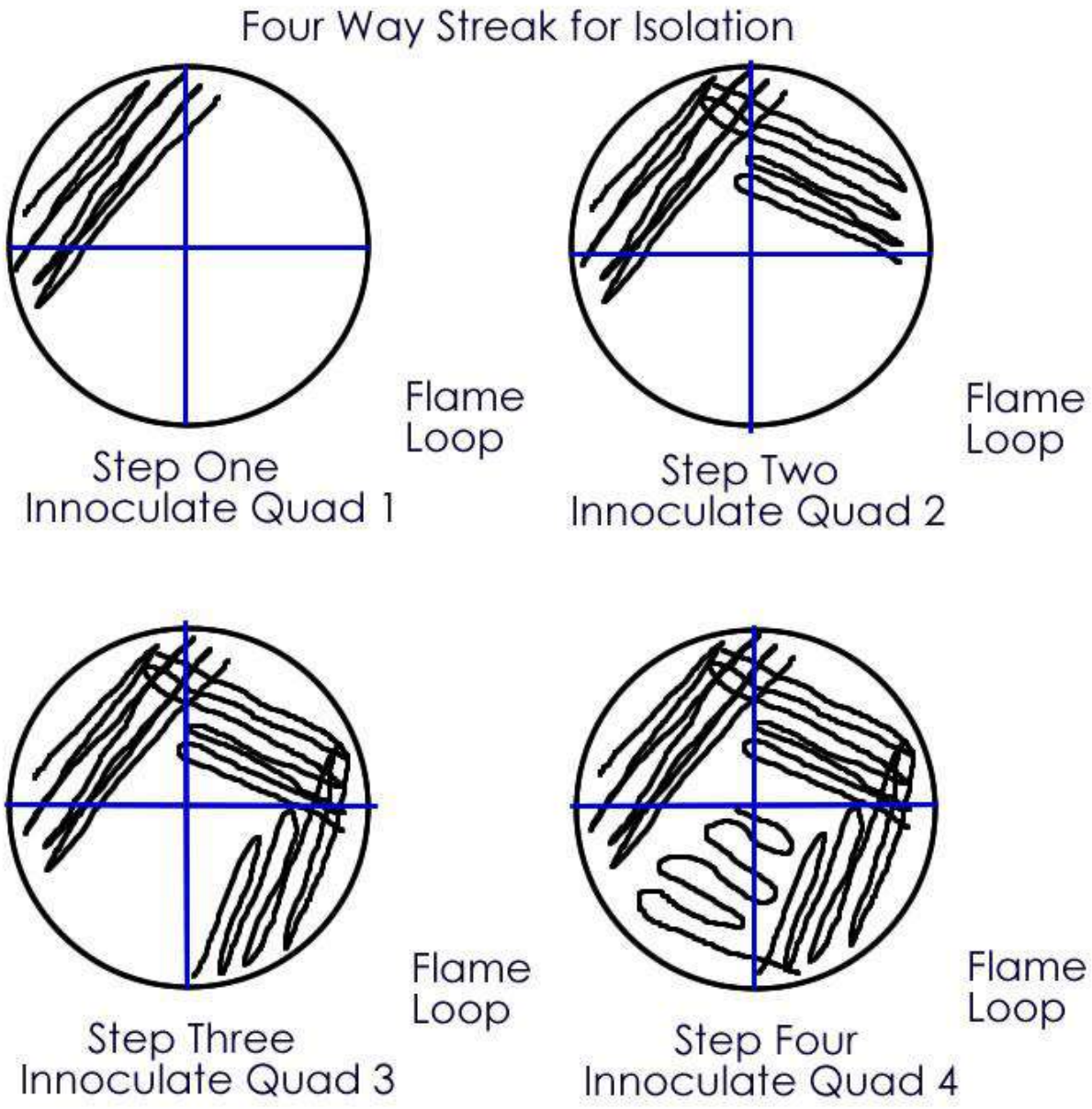


Figure 2: The **streak-plate technique**

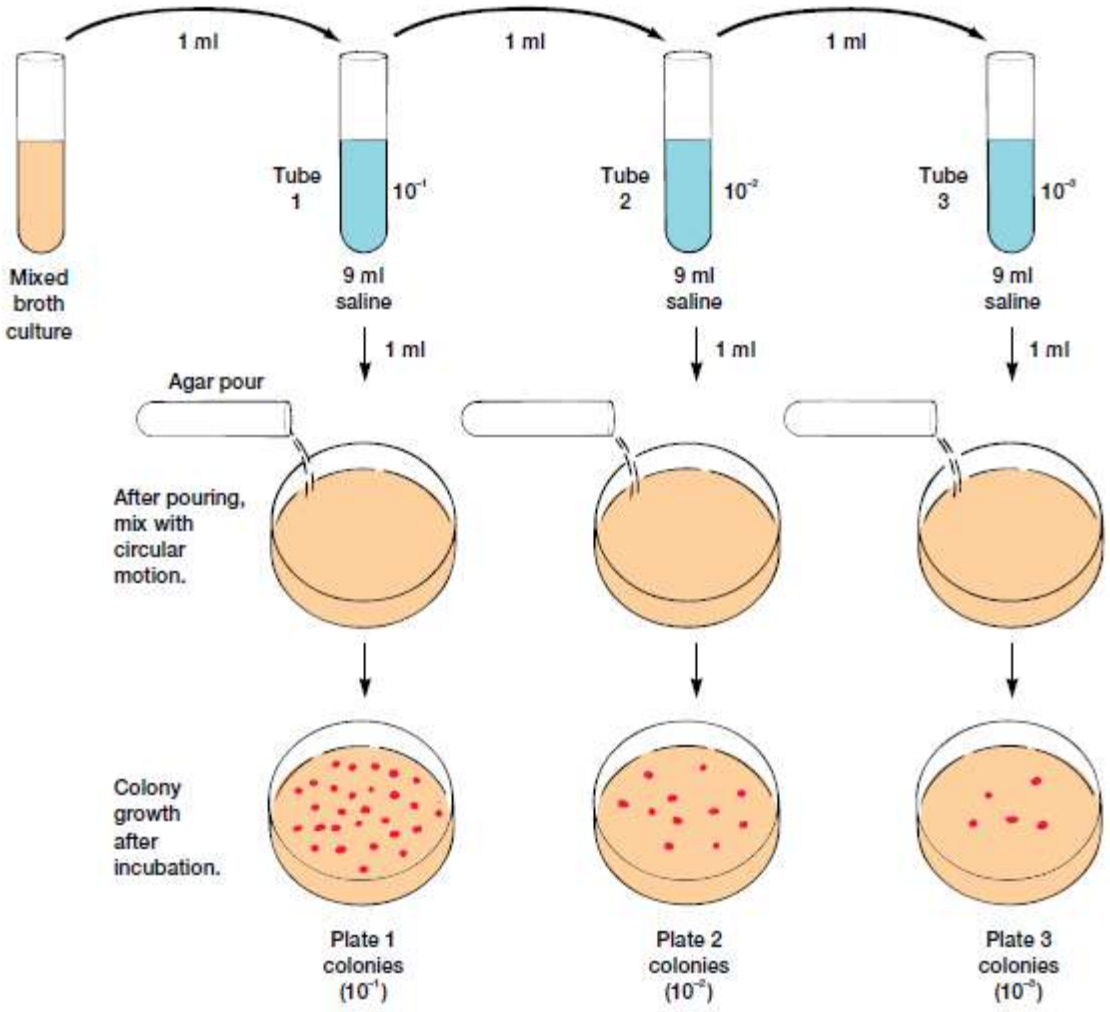


Figure 3: The Pour-Plate Technique. The original sample is diluted several times to decrease or dilute the population sufficiently. 1 ml of each dilution is then dispensed into the bottom of a petri plate. Agar pours are then added to each plate. Isolated cells grow into colonies and can be used to establish pure cultures. The surface colonies are circular and large, subsurface colonies are lenticular or lens shaped and much smaller.

Lab.4 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- Subculturing:

The organisms may simply be subcultured 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 subculturing 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 we use soil, sand, silica gel as 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 subculturing

Materials:

Petri plate with well isolated colonies

Nutrient agar slant

Inoculating loop

Bunsen burner

Procedures:

1-Using aseptic technique 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 session observe the new slants and describe their appearances.

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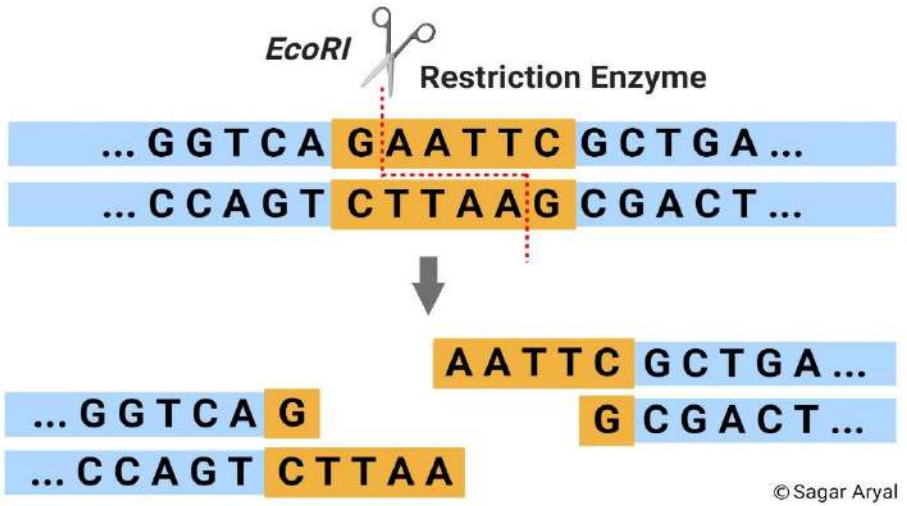
Lab. 2 Restriction Enzymes

Tuesday 18-05-2021

What is the restriction enzyme?

Restriction enzyme is a protein that recognizes a specific, short nucleotide sequence and cuts the DNA only at that **specific site**, which is known as **restriction site** or **target sequence**.



More than 400 restriction enzymes have been isolated from the bacteria that manufacture them.

In live bacteria, restriction enzymes function to defend the cell against invading viral bacteriophages. Restrictions sites in the viral genome (a "happy accident" of nature, as far as the bacteria are concerned, since they don't appear to have any specific function in the virus) are cleaved by the bacterium's restriction enzymes, fragmenting and destroying the DNA of invading bacteriophages before it can incorporate into the host's genome and take over the cell.

A bacterium is immune to its own restriction enzymes, even if it has the target sequences ordinarily targeted by them. This is because the bacterial restriction sites are highly methylated, making them unrecognizable to the restriction enzyme.

Lab. 2 Restriction Enzymes Tuesday 18-05-2021

Types of Restriction Enzymes

Based on enzyme subunit composition, cofactor requirements, nature of their target sequence, and position of restriction site relative to the target sequence, restriction enzymes are of four types (Types I, II, III, and IV):

1. Type I restriction enzyme – It cuts at sites far from a recognition site (asymmetrical in nature) and requires both ATP and S-adenosylL-methionine (AdoMet) as cofactor to function. It has a feature of both restriction digestion and methylase.

2. Type II restriction enzymes – It cleaves within or at short-specific distances from recognition site (palindromic in nature) and mostly requires magnesium to function. It has only feature of restriction digestion independent of methylase.

3. Type III restriction enzymes – It recognizes two separate non-palindromic and inversely oriented sequences and cleaves at 20–30 base pairs away from a recognition site. It contains more than one subunit and requires ATP for restriction digestion and S-adenosyl-Lmethionine for DNA methylation.

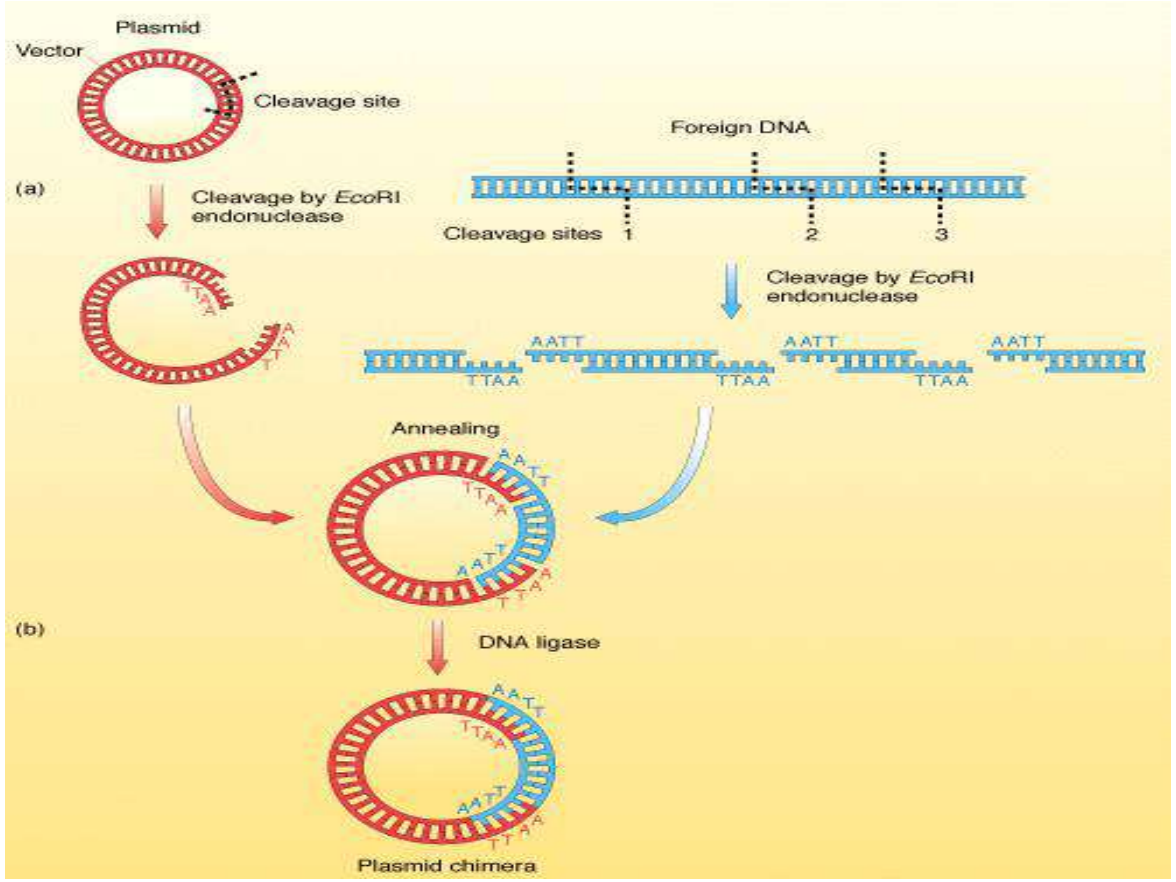
4. Type IV restriction enzymes – It recognizes modified DNA (methylated, hydroxymethylated, and glucosyl-hydroxymethylated). Examples are McrBC and Mrr systems of ***E. coli.***

Lab. 2 Restriction Enzymes

Tuesday 18-05-2021

When a restriction enzyme cleaves a restriction site, the reaction creates highly reactive "sticky ends" on the broken DNA. This is useful to the biotechnologist!

By cutting open vector DNA with the same with restriction enzymes used to cleave the target DNA, complementary "sticky ends" are created. This fosters the insertion of the target DNA into the vector:



The fragment is "glued in" with DNA ligase, which creates the phosphordiester bonds necessary to complete the sugar-phosphate backbone of the newly transgenic DNA.

Lab. 2 Restriction Enzymes

Tuesday 18-05-2021

Identity of Restriction Enzymes:

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

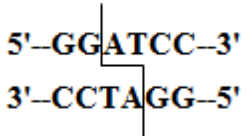
- **EcoRI** is isolated from E. coli strain RY13
  - **Eco** refers to the genus and species (1st letter of genus; 1st two letters of specific epithet)
  - **R** is the strain of E. coli
  - **I** (Roman numeral) indicate it was the first enzyme of that type isolated from E. coli RY13.
- **BamHI** is isolated from Bacillus amyloliquefaciens strain H

- **Sau3A** is isolated from Staphylococcus aureus strain 3A.

- 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, BamHI recognizes the double stranded sequence to form "sticky ends".



Lab. 2 Restriction Enzymes

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Notes:

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

2- Restriction sites are recognized no matter where the DNA came from

3- 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.

4- A fragment of DNA produced by a pair of adjacent cuts is called a restriction fragment.

5- A particular restriction enzyme will typically cut an organism's DNA in to many pieces, from several thousand to more than a million!

- Raw materials of enzymes isolation are animal organs, plant materials or microorganisms

- The purity of commercial enzymes ranging from raw to highly purified forms and it is depend on the application

- Downstream processing is a very important step in biotechnology because the costs of isolation, 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

- **Enzymes are very complex proteins and their high degree of specificity as catalysts is manifest only in their native state**

Enzymes should be purified as little as possible, only other enzymes and material likely to interfere with the process which the enzyme is to catalyse, should be removed.

Unnecessary purification should be avoided as each additional stage is costly in terms of equipment, manpower and result in loss of enzyme activity.

Enzymes purifications steps

1- Isolation

The choice of procedure for enzymes purification are depends on their location

- **Intracellular enzymes** from cells or cellular fragments includes can be isolated via **cell disruption, which can be done as following:**

A- Mechanical methods such as High-pressure homogenization & the wet grinding of cells in a high-speed bead mill.

B- Non-mechanical methods such as chemical, thermal, or enzymatic lysis.

- **Extracellular enzymes** are generally released into the medium with only a few other components can be processed directly to the next step

2- Separation for this purpose the following methods can be used: filtration or centrifugation

3- Concentration can be achieved by using evaporation, ultrafiltration, precipitation or adsorption

Precipitation is actually a simple procedure for enzymes concentrating. Enzymes are complex protein molecules possessing both ionisable and hydrophobic groups which interact with the solvent. Proteins can be made to agglomerate and, finally, precipitate by changing their environment.

Precipitation methods

A- Precipitation with Salts: High salt concentrations act on the water molecules surrounding the protein and change the electrostatic forces responsible for solubility. Ammonium sulfate is commonly used for precipitation; hence, it is an effective agent for the enzymes concentration. Enzymes can also be fractionated, to a limited extent, by using different concentrations of ammonium sulfate. Sodium sulfate is another precipitating agent used.

B- Precipitation with Organic Solvents: Organic solvents influence the solubility of enzymes by reducing the dielectric constant of the medium. The solvation effect of water molecules surrounding the enzyme is changed; the interaction of protein molecules is increased; and therefore, agglomeration and precipitation occur. Commonly used solvents are ethanol and Acetone.

C- Precipitation with Polymers: The polymers generally used are polyethylenimines and polyethylene glycols of different molecular masses.

4- Purification

For many industrial applications, partially purified enzyme preparations will suffice; however, nzymes for analytical purposes and for medical use must be highly purified.

Special procedures employed for enzyme purification are crystallization, electrophoresis, and chromatography. However crystallization and electrophoresis are not relevant for large scale purifications. Chromatography, in contrast, 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).

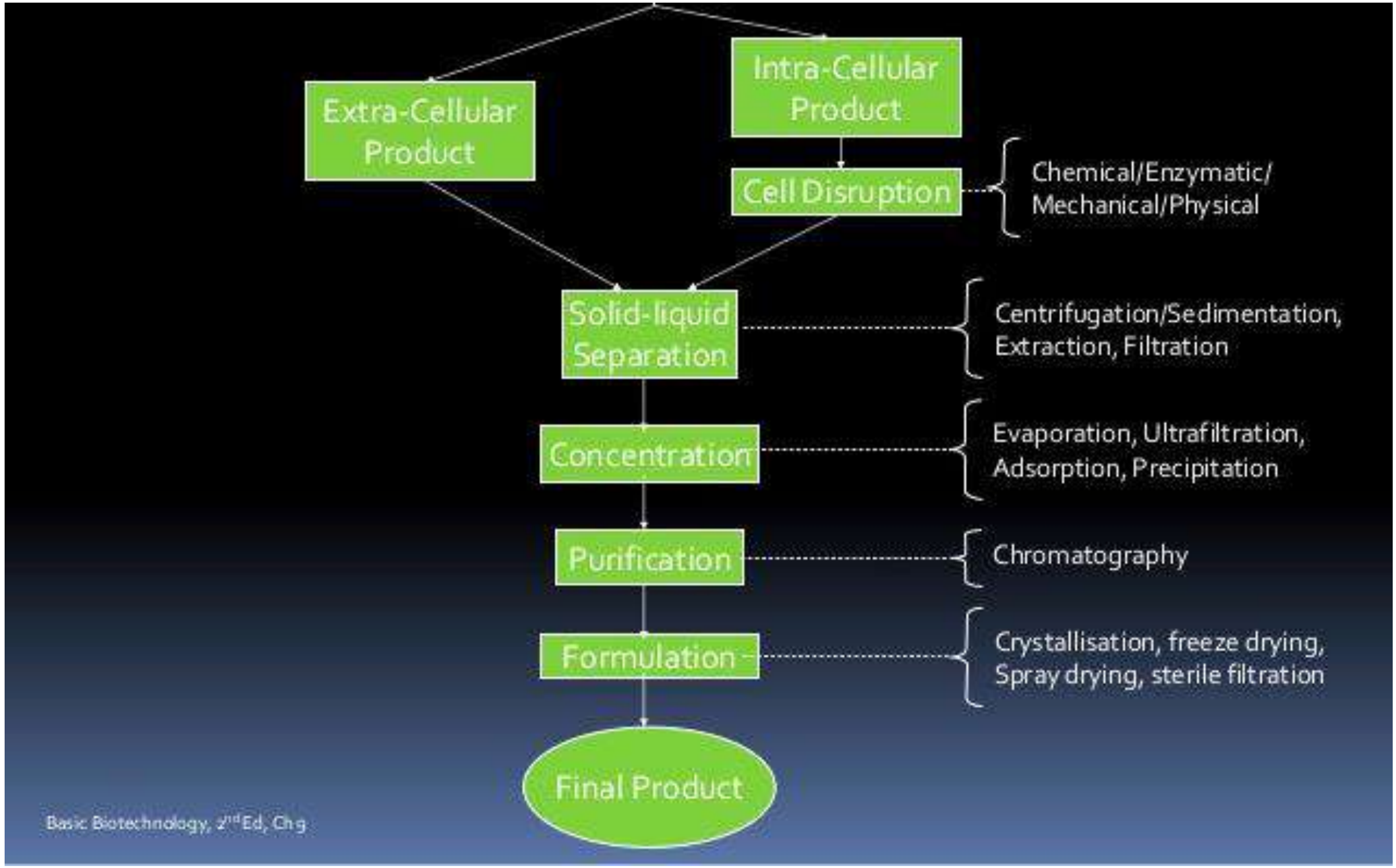


Figure 1: Flowchart of the downstream processing of enzymes (shows the sequence of steps involved in the recovery of enzymes.

EX; 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 increasing 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.

The aim is to find the ammonium sulfate concentration which will precipitate the maximum proportion of undesired protein, whilst leaving most of the desired protein still in solution, the precipitated protein is then removed by centrifugation and then the ammonium sulfate concentration is increased to a value that will precipitate most of the protein of interest whilst leaving the maximum amount of protein contaminants still in solution. The precipitated protein of interest is recovered by centrifugation and dissolved in fresh buffer for the next stage of purification.
This technique is useful to quickly remove large amounts of contaminant proteins, as a first step in many purification schemes.

Practical part:

1- Extraction of peroxidase from horseradish by using buffer (pH 6) in ratio 1:1.2- Precipitation of desired protein (peroxidase) by using ammonium sulfate in concentration of 50%:

3- Add solid ammonium sulfate to your sample to get the desired concentration, stir for 1 hour to fully equilibrate.

4- Centrifuge at 10000 rpm for 30 minutes to pellet out protein.

5- Dissolve pellets in buffer to analyse proteins.

Table 20.1 Final concentration of ammonium sulfate: Percentage saturation at 0 °C[a]

Initial concentration of ammonium sulfate (percentage saturation at 0 °C)	Percentage saturation at 0 °C																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (g) to be added to 1 l of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

Lab no.: 5

Antibacterial Activity of Ginger (*Zingiber Officinale)* (Roscoe) 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 we 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**. In order to avoid misunderstanding, it is better to use the expression “phytogenic drug”, or **crude drug**, the word drug comes from the Dutch word **droog, droge** which means dry, or to dry.

Medicinal plants includes all plants any or all parts of which are used for therapeutically purposes due to the active ingredients contained in them. They can be wild plants or cultivated ones.
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.
Higher plant-derived products represent approximately 25% of drugs in current clinical use. The more than 350 000 species of higher plants currently recognized, only 5–10% have been investigated and considering that each plant species may contain 500–800 different secondary metabolites, the Potential for the discovery of new therapeutic products in this largely untapped resource is considerable.

Phytochemical products that produce minimum inhibitory concentrations (MIC) in the range 100–1000 mg mL in in vitro susceptibility tests can be classified as antimicrobials. many of these products are produced as a mechanism of plant defense in response to tissue disruption or pathogen attack.

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.

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

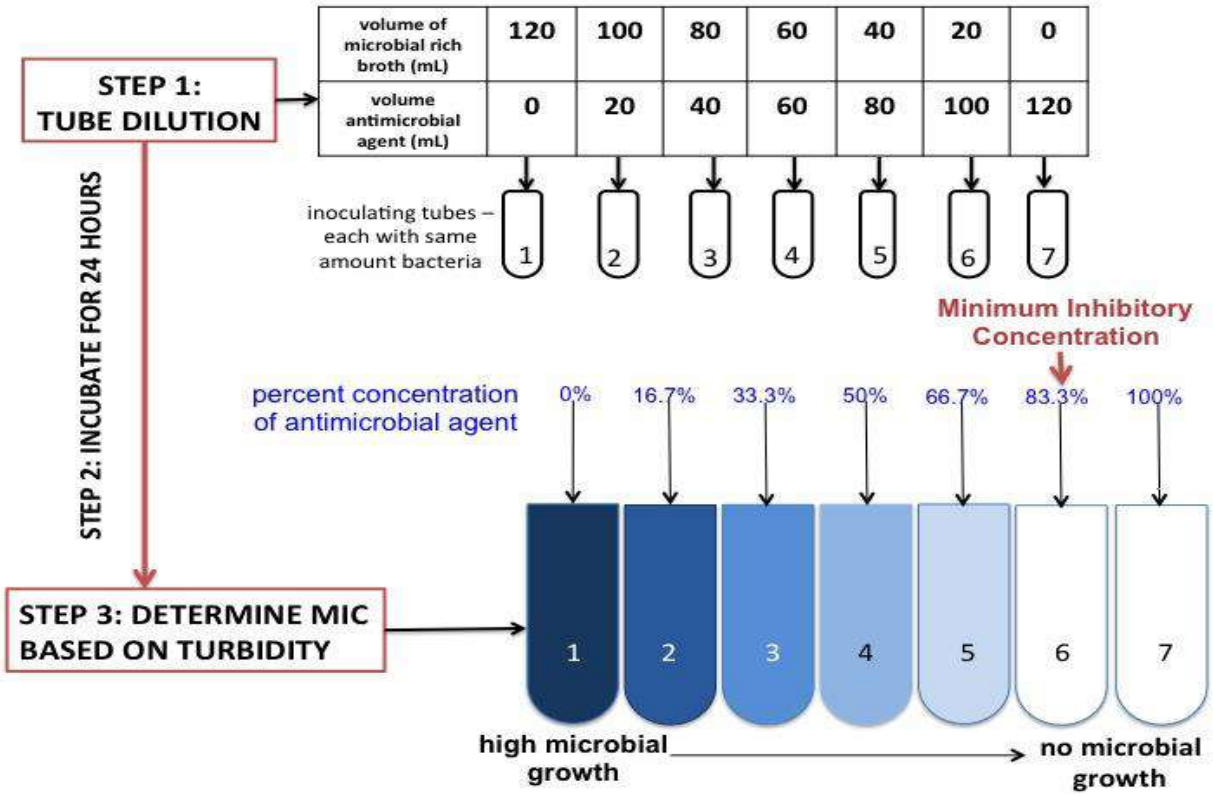
3. incubation period
4. depth of the agar
5. diffusion rate of the antibiotic
6. concentration of antibiotic in the disk
7. Growth rate of the bacterium

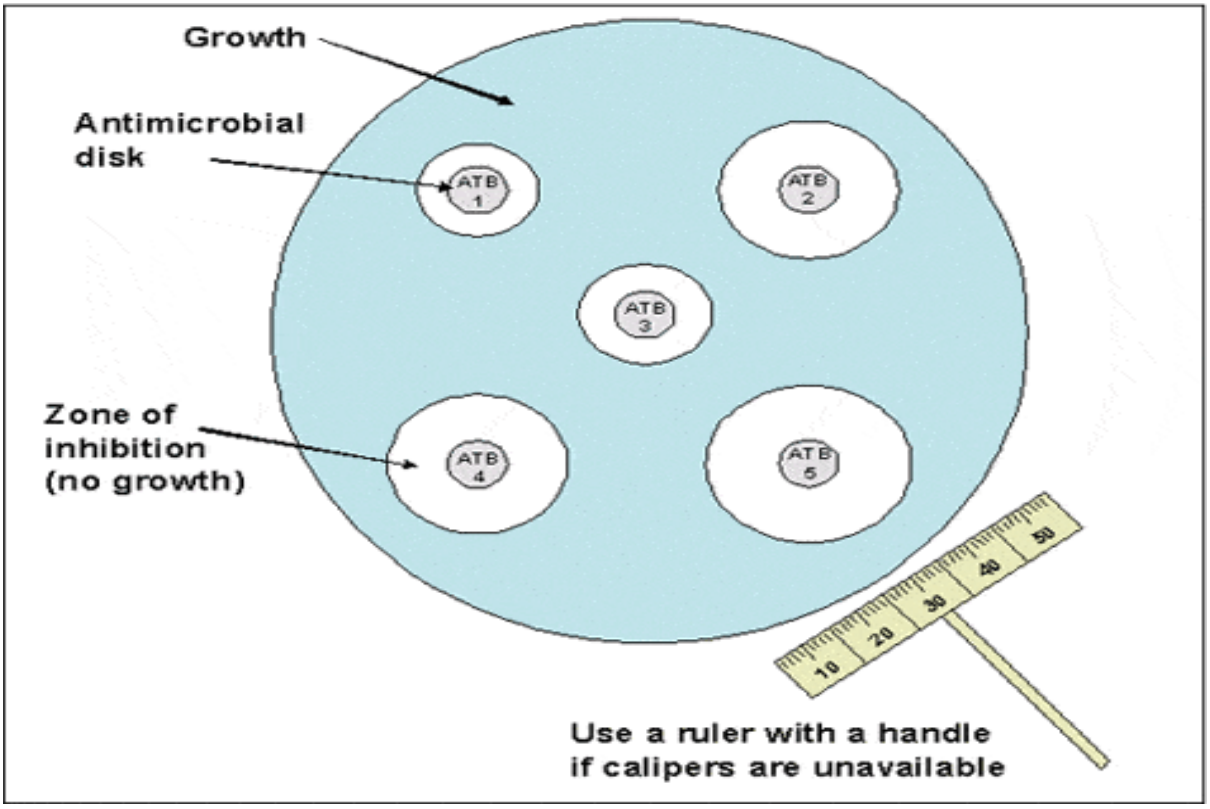
Procedure:

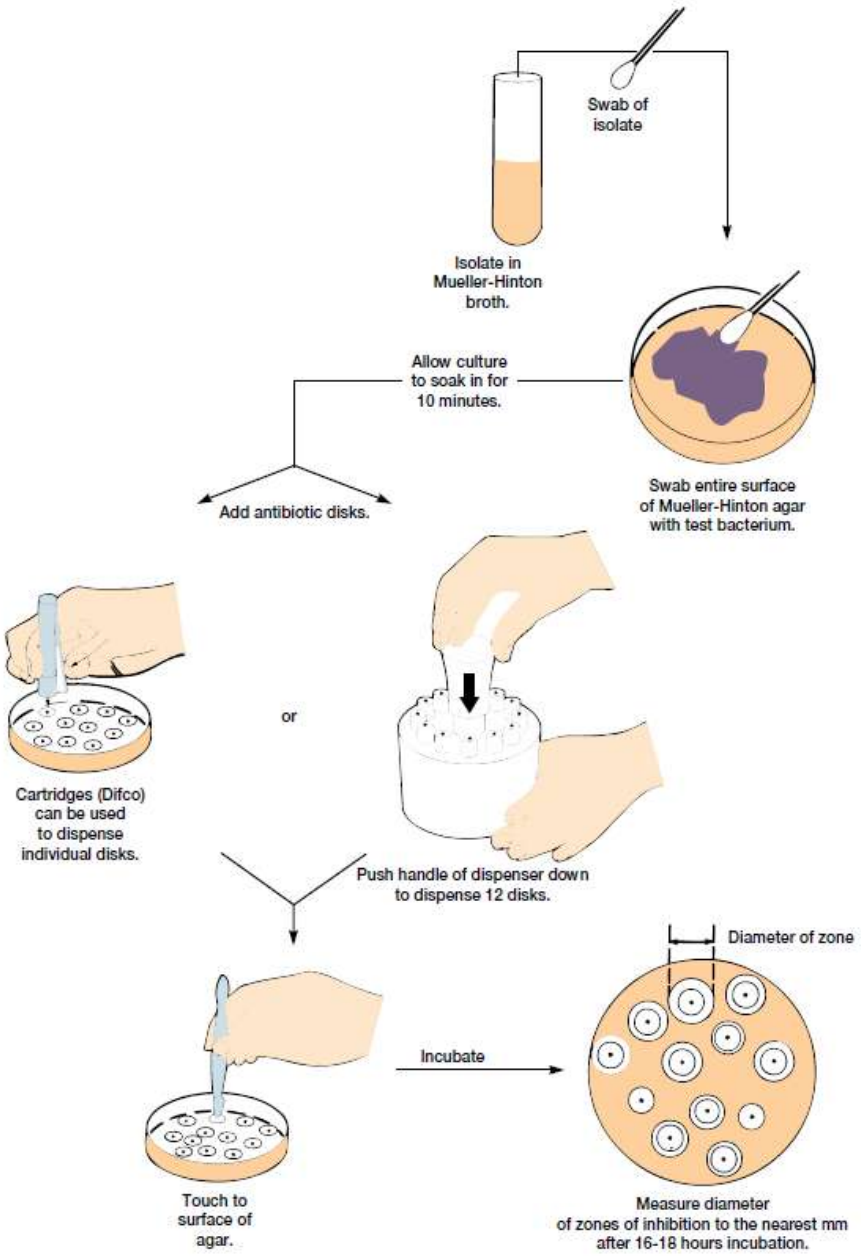
Preparation of plant crude extract:

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 and diluting 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 the 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.

Antimicrobial activity assay can be carried out in 2 ways:
A. broth dilution
B. Agar dilution


STEP 1:
TUBE DILUTION
volume of microbial rich broth (mL)
120
100
80
60
40
20
0
volume antimicrobial agent (mL)
0
20
40
60
80
100
120
inoculating tubes – each with same amount bacteria
1
2
3
4
5
6
7
STEP 2: INCUBATE FOR 24 HOURS
Minimum Inhibitory Concentration
percent concentration of antimicrobial agent
0%
16.7%
33.3%
50%
66.7%
83.3%
100%
STEP 3: DETERMINE MIC BASED ON TURBIDITY
1
2
3
4
5
6
7
high microbial growth
no microbial growth


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


Swab of isolate
Isolate in Mueller-Hinton broth.
Allow culture to soak in for 10 minutes.
Swab entire surface of Mueller-Hinton agar with test bacterium.
Add antibiotic disks.
Cartridges (Difco) can be used to dispense individual disks.
or
Push handle of dispenser down to dispense 12 disks.
Touch to surface of agar.
Incubate
Diameter of zone
Measure diameter of zones of inhibition to the nearest mm after 16-18 hours incubation.

Lab. 6
Production of Single cell protein from yeast

The term “single cell protein’’ also known as MICROBIAL PROTEIN refers to the total protein extracted from the pure culture of M.O.s (yeast, algae, fungi & bacteria) which can be used for humans & animals as protein - rich food supplements.

Single cell protein (SCP) a protein produced or derived from the culture of a single-celled organism, by Submerged & Semisolid state fermentation based on using a different Raw materials for SCP production such as:

A. High energy sources e.g. Alkanes, methane, methanol, ethanol, gas oil.
B. Waste product e.g. molasses, whey, sewage, animal manures, straw.
C. Agriculture and forest sources e.g. cellulose, lignin.
D. Carbon dioxide

Advantages of using Microorganisms:
1- MO 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- MO can be genetically manipulated
6- Independent on land & climate as plants & animals.

Organism	Mass Doubling
Bacteria and yeast	10-120 min
Mold and algae	2-6 h
Grass and some plants	1-2 wk
Chickens	2-4 wk
Pigs	4-6 wk
Cattle	1-2 mo
People	0.2-0.5 yr

The doubling time of cells (td) or Mass doubling time (S) of different organisms

Organism (1,000 kg)	Amount of protein
Beef cattle	1.0 kg
Soybeans	10.0 kg
Yeast	100.0 tn
Bacteria	100x10,000,000 tn

Efficiency of protein production of several protein sources in 24 hours

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- Have high growth rate, productivity and yield, on the specific low-cost substrates to be used

3- Tolerance changes of temperature, pH, and heat generation during fermentation and oxygen requirements.

4- Growth morphology and genetic stability in the fermentation

5- Ease of recovery, and requirements for further downstream processing.

Nutritional Value of SCP

Single Cell Protein is basically composed of proteins, fats, carbohydrates, ash ingredients, water and other elements such as potassium and phosphorus.

It contains more lysine less amount of methionine and cysteine. Microbiological proteins are deficient in the sulphur amino acids cysteine and methionine and require supplementation, while they exhibit better levels of lysine

	Fungi	Algae	Yeasts	Bacteria
Protein	30-45	40-60	45-55	50-65
Fat	2-8	7-20	2-6	1.5-3.0
Ash	9-14	8-10	5-9.5	3-7
Nucleic acids	7-10	3-8	6-12	8-12

Table 1- Average composition of the main groups of M.O.s (% dry weight)

The basic step for process of SCP production:

1. Selected microorganism is inoculated in a pure state.
2. Addition raw materials as carbon source, sources of nitrogen, phosphorus and other nutrients needed to support optimal growth of the selected microorganism.
3. Prevention of contamination by maintaining sterile, the medium components may be heated or sterilized by filtration and fermentation equipment may be sterilized.
4. Filtration the yield.
5. Drying the new microbial biomass.
6. Calculate the new weigh of biomass.

Procedure in the lab:

1- Preparation of the inoculum: prepare the yeast *Saccharomyces cerevisiae* by mixing 1gm yeast /25 ml of sterilized water in a flask.

2- 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.

3- 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.

4- 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.

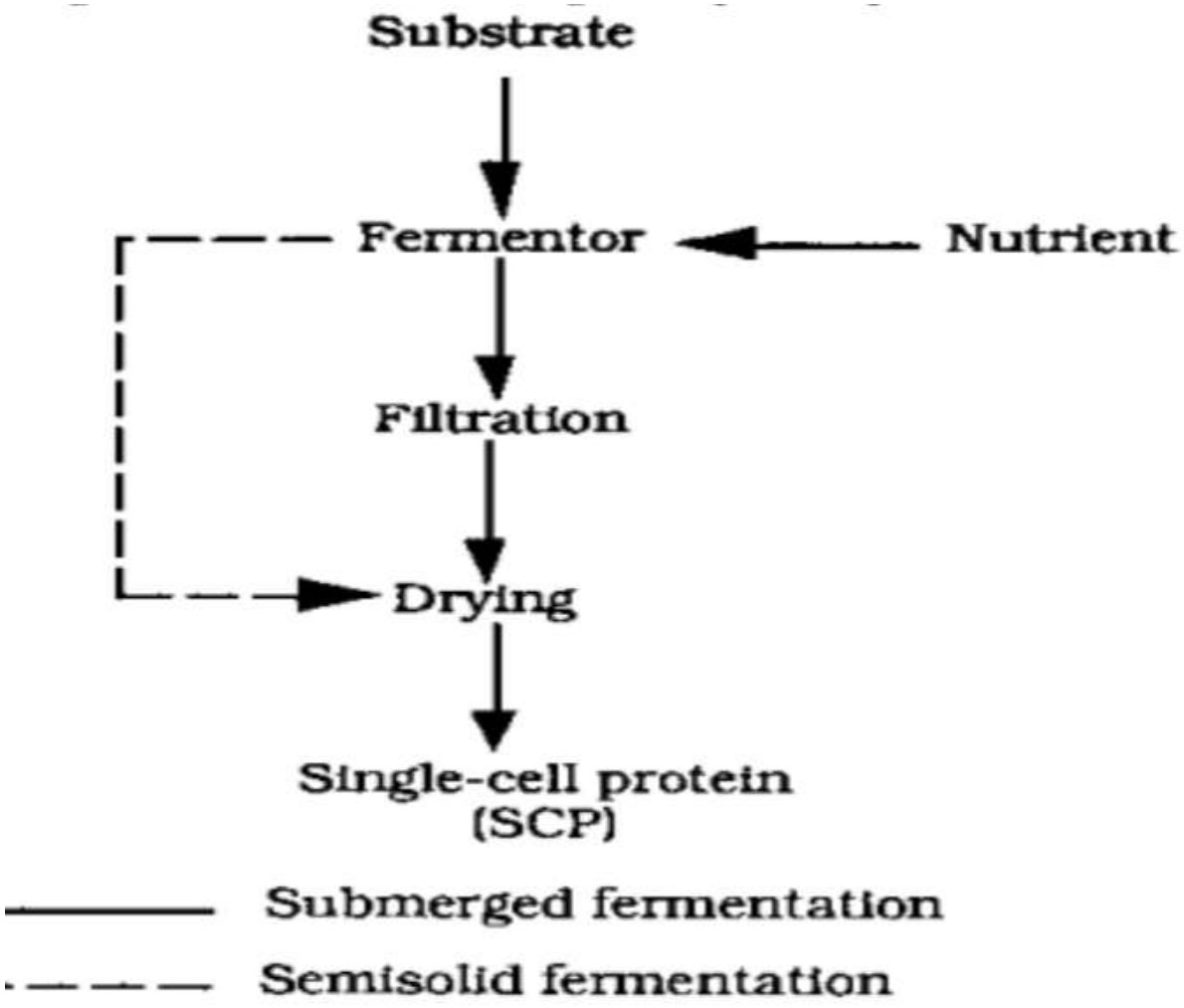
5- Place the filter papers in an oven for drying at 40°C for 24 hour. Next, weight the filter papers; the differences in weights represent the amounts of SCP produced in each flask.

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 × 100 / Consumed sugar


Substrate
Fermentor
Nutrient
Filtration
Drying
Single-cell protein
(SCP)
Submerged fermentation
Semisolid fermentation