جامعة بغداد
كلية العلوم
قسم التقنيات الاحيائية

Principles of Biotechnology laboratory syllabus

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Course Description:

Introduce students to:

1. Apparatus and instruments used in biotechnology lab.
2. Microbiology: Pure culture techniques, isolation, cultivation, maintenance and preservation of pure cultures, sterilization techniques, microorganism nutrition and growth requirements.
3. Industrial Microbiology (fermentation). It includes the principles and practices in the main applications of micro-organisms to the industrial production of foods, pure chemicals, proteins and other useful products.

Course objectives:

1. Students will learn aspects of experimental design.
2. Students will learn the purpose of the experimental methods they use.
3. Students will learn to interpret the results.

Course policy and Grading:

1. **Attendance** is mandatory. Three or more unexcused absence will result in grade reduction.
2. **No make-up mid and final exam.**
3. Students will be **evaluated** based on:
    - Attendance (10 %)
    - Reports (10%)
    - Class participation (10%)
    - Quizzes (10%)
    - Mid exam (60 %)

Lab reports

Each student should write his or her reports individually in an organized way. It is expected that students will write about 2 to 3 pages for each lab. Reports will be collected on a weekly basis. Lab reports should be written in the following format:

1. **Title and date:** provide a descriptive title of the week's lab and date of the work.
2. **Purpose:** Briefly describe the purpose of the lab work
3. **Materials and methods**: information about solutions and tools that had been used. **note**, in some cases there will be some modification or changes in the procedure which should be mentioned in your lab reports, such as information on volume, concentration and time.
4. **Results:** your result should be labeled and have enough information so that an outside reader can understand your result. The results should also be referenced appropriately (figure 1 or tables 1) so that you can discuss the results in the discussion section.

5. **Discussion:** summarize your results. Explain the outcome of the procedure. Was there anything you could done to better your results.

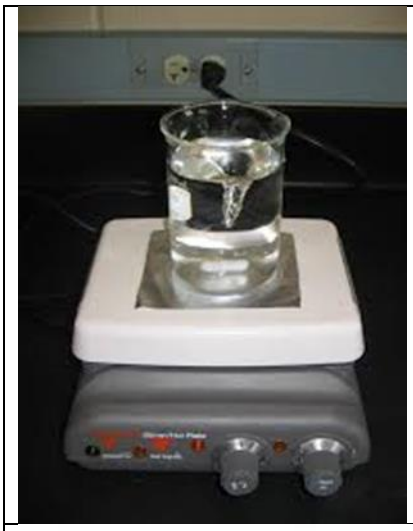
Principles of Biotechnology

Lab1: Laboratory Equipments

a- Instruments or apparatus

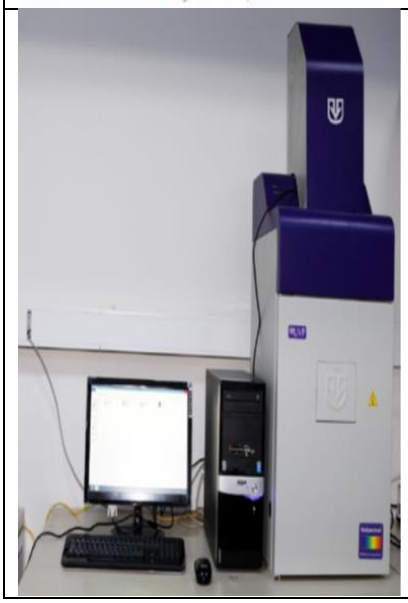
Image	Scientific name	purpose
	Centrifuge	Used to separate heavy material from light material by the help of the centrifugal force
	Balance	Determining weight

	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.

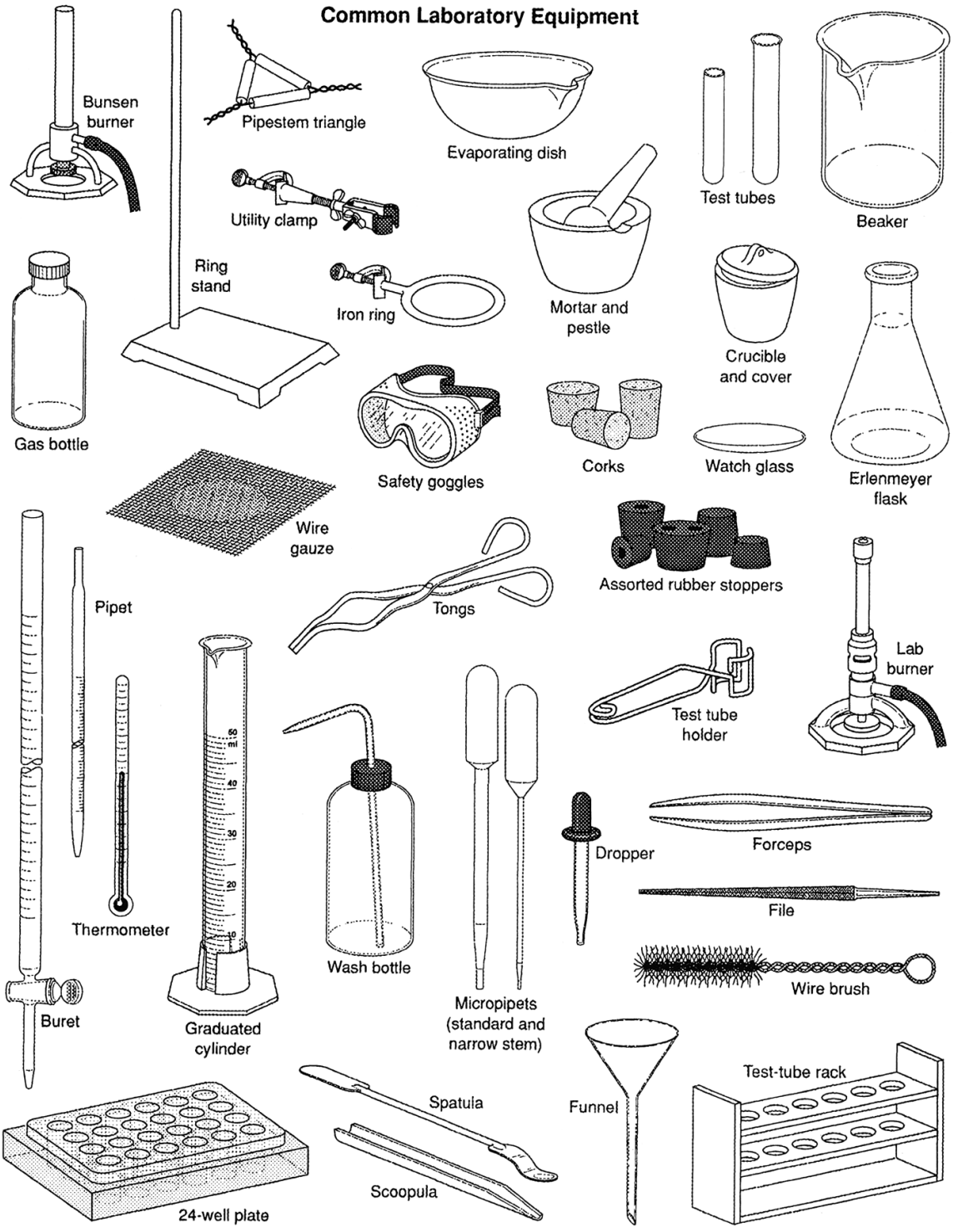
	Shaking incubator	In order to provide optimal conditions for cell growth, some type of agitation or shaking is necessary to incorporate oxygen and evenly distribute nutrients throughout the culture media. The incubator shaker can be used for growth of just about any kind of cell including bacterial cultures, 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 no.: 3

The 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.

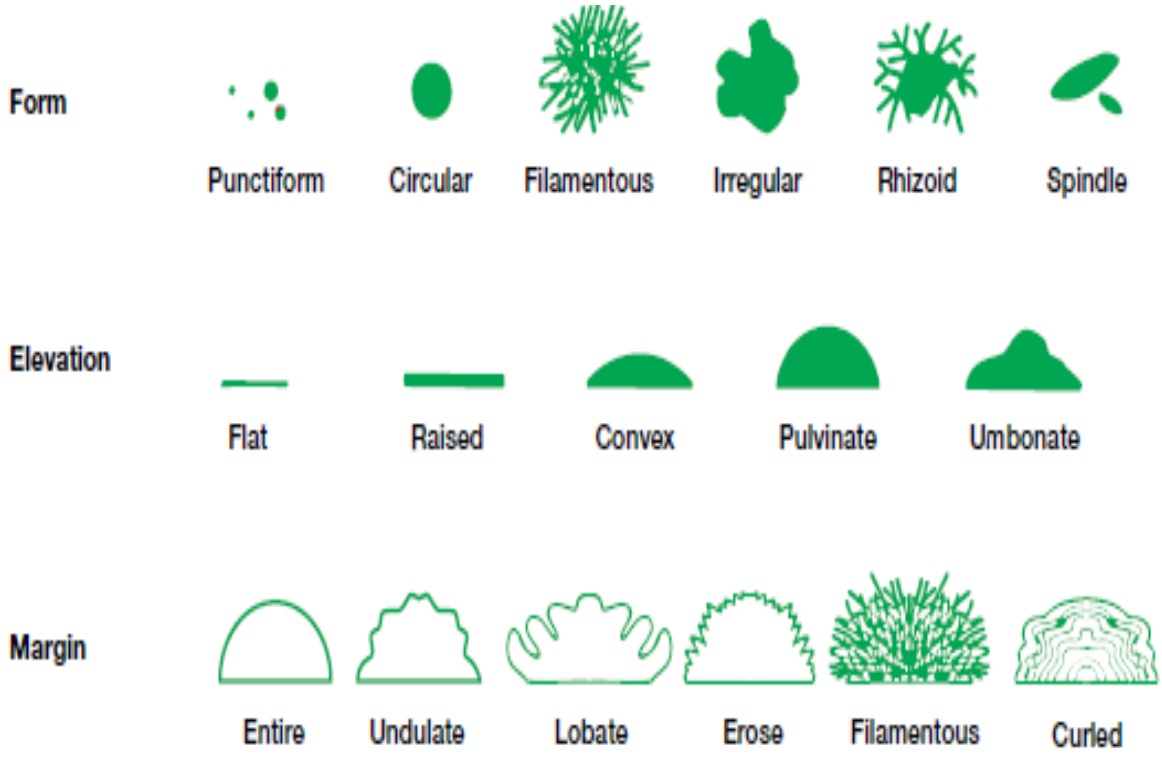


Figure 1: **Bacterial Colony Characteristics on Agar Media as Seen with the Naked Eye.** The characteristics of bacterial colonies are described using the following terms.
Appearance: Shiny or dull Optical property: Opaque, translucent, transparent
Pigmentation: Pigmented (purple, red, yellow) Non-pigmented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

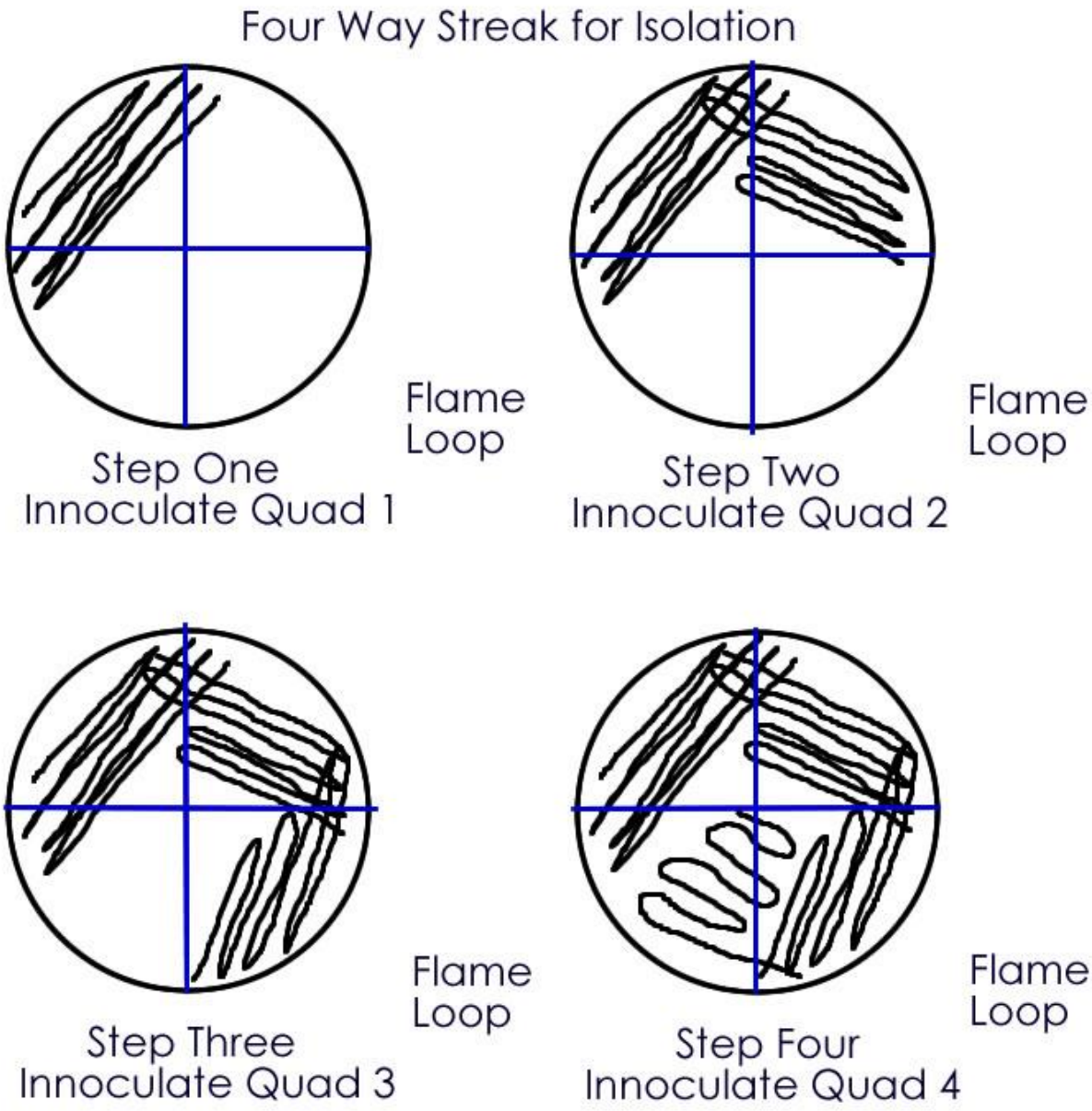


Figure 2: The **streak-plate technique**

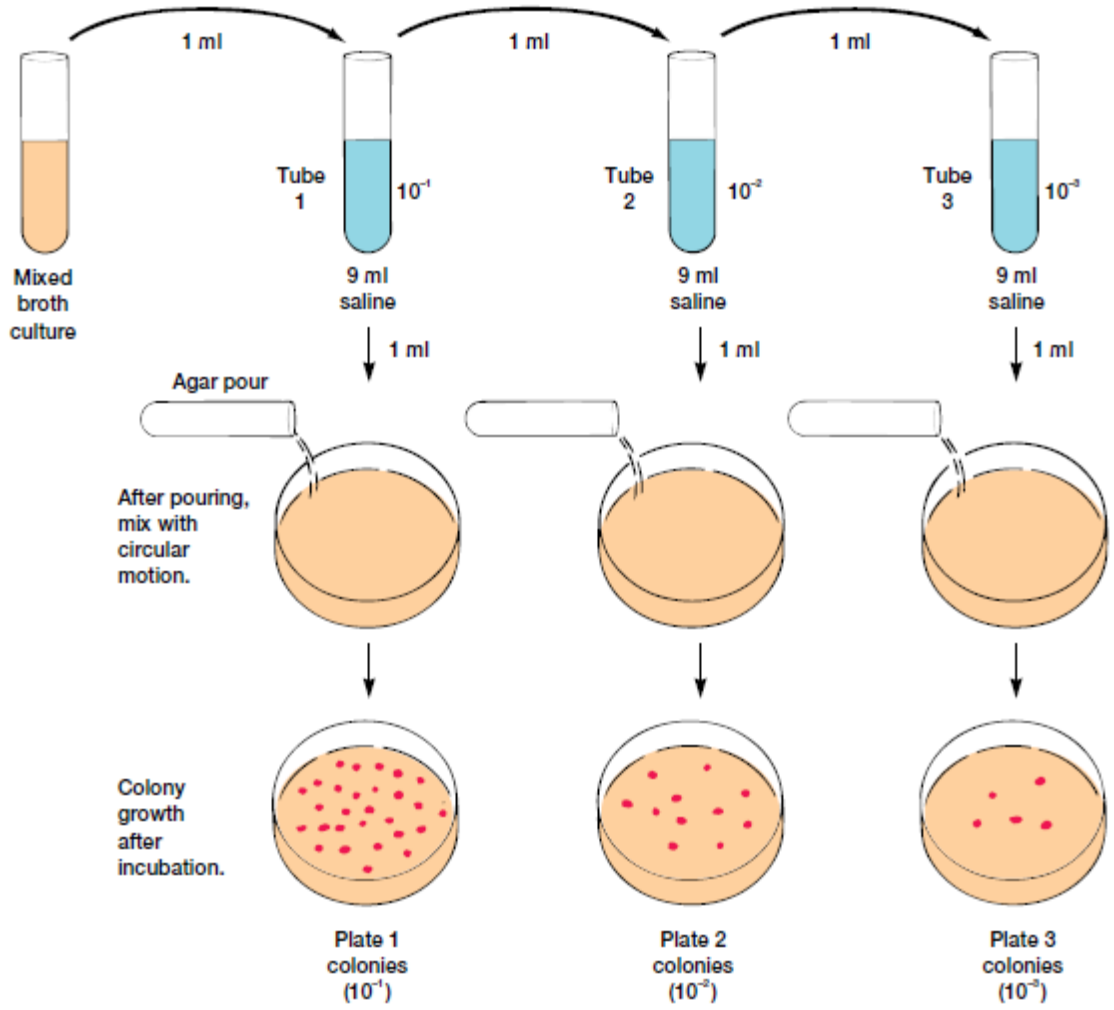


Figure 3: The Pour-Plate Technique. The original sample is diluted several times to decrease or dilute the population sufficiently. 1 ml of each dilution is then dispensed into the bottom of a petri plate. Agar pours are then added to each plate. Isolated cells grow into colonies and can be used to establish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

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.

Lab no.: 5

The enumeration methods of Microorganisms

Objectives: Each student should be able to describe several different ways to quantify the number of bacteria in a given sample.

The laboratory microbiologist often has to determine the number of bacteria in a given sample, as well as having to compare the amount of bacterial growth under various conditions. Which is important in dairy, food, & water microbiology.

The three most widely used methods for determining bacterial numbers are:
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. Given these volumes, the number of cells in a sample can be determined by counting the number of cells in one or more of the squares. Which square to use depends on the size of the object to be counted. Whole cells would use the larger squares, counted with 10X magnification. Isolated mitochondria would be counted in the smallest squares with at least 40X magnification.

9. For the squares marked 1–4, the area of each is 1 mm2, and the volume is .1 mm3. Since .1 mm3 equals 10–4 mL, **the number of cells/mL = average number of cells per 1 mm2 × 104 × any sample dilution.**
10. For the 25 smaller squares in the center of the grid marked 5, each small square is 0.2 × 0.2 mm2, and the volume is thus 0.004 mm3. **For small cells, or organelles, the particles/mL equals the average number of particles per small square × 25 × 104 × any sampledilution.**

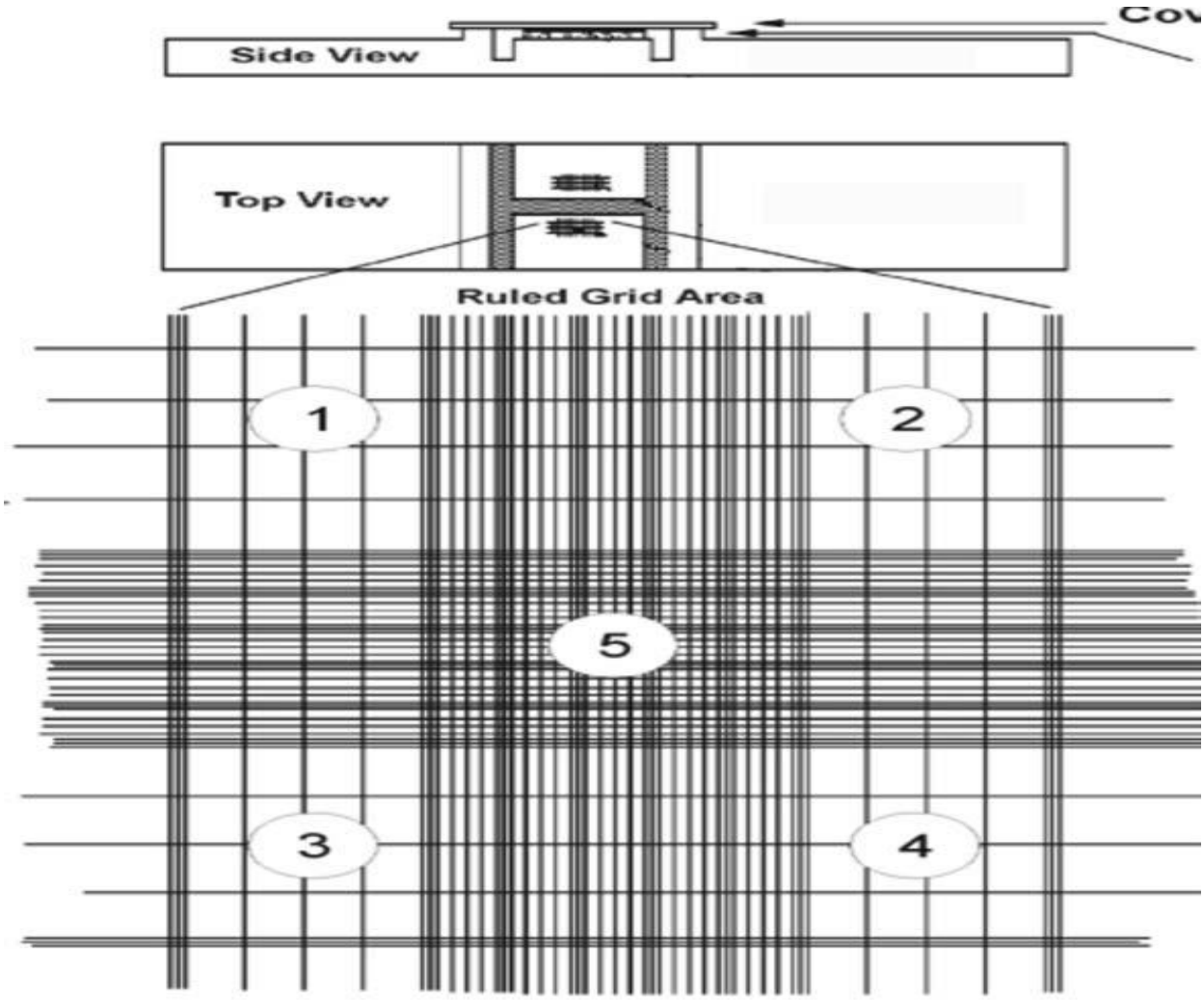


Figure 1

2- **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 because 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 2).
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 3).
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 .

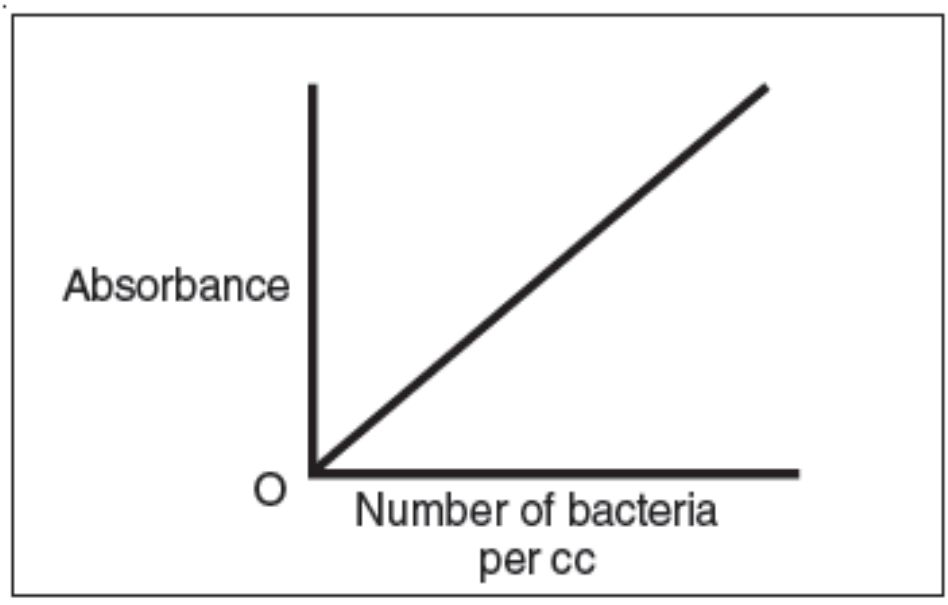


FIGURE 2: A standard curve plotting the number of bacteria per ml versus absorbance.

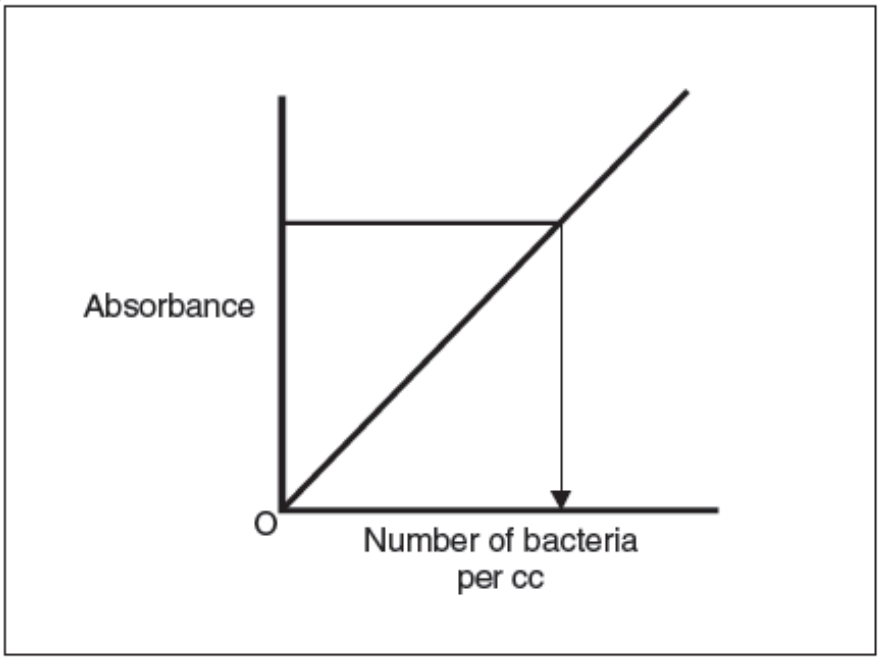


FIGURE 3 using a standard curve to determine the number of bacteria per ml in a sample by measuring the sample's absorbance.

3- 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 25 and 250 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 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 35°C for 24 hours or 20°C for 48 hours.
5. At the end of the incubation period, select all of the petri plates containing between 25 and 250 colonies.

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

Number of CFUs per mL of sample = Number of colonies (30–300 plate) × the dilution factor of the plate counted.

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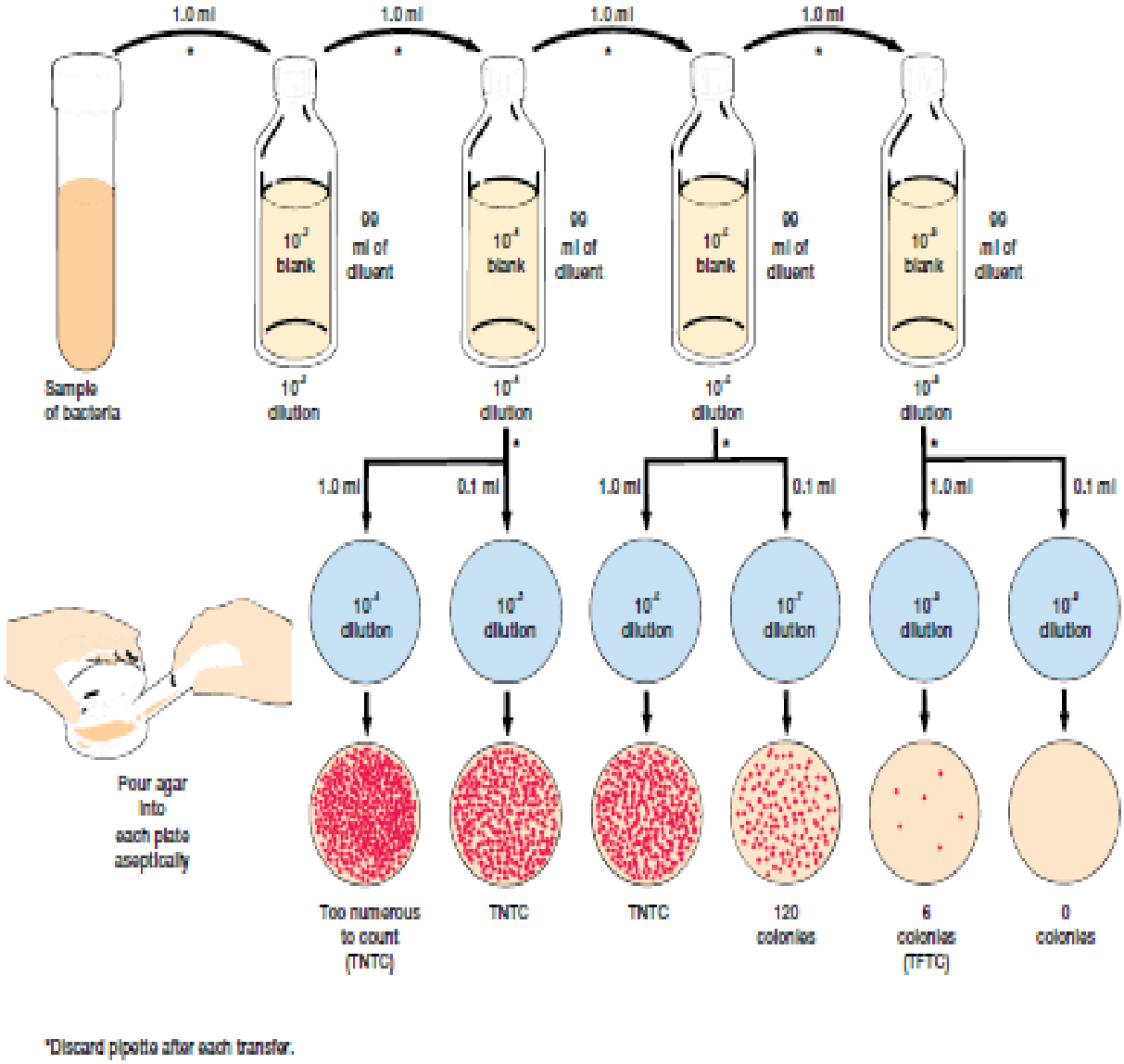


Figure 4 Quantitative Plating Procedure.

Second semester

Lab no.: 1
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 **Temp**., **pH** & the **substrate** concentration.

The basic mechanisms of enzyme synthesis, including transcription, translation, and posttranslational 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.

HOW DO ENZYMES WORK?
The remarkable property of enzymes is that they complete reactions without being destroyed, allowing a single enzyme molecule to catalyze many individual reactions. The action of enzyme that breaks down substrate is shown in Figure 1.
The first step in an enzymatic reaction is substrate binding to the enzyme at its active site. In this example, a protein binds to the reactive site of a

protease. This portion of the enzyme is shaped specifically to allow entrance of only certain substrates with the corresponding shape, much like a key fits into a lock. If the substrate does not fit the shape of the active site, it cannot attach itself to the enzyme and no reaction occurs. In the figure to the right, the enzyme breaks down the substrate bound to the active site into smaller parts while the enzyme itself is unchanged. Once the enzyme breaks the substrate, the resulting products are released from the enzyme. Thus the enzyme reaction process can take place over and over as the enzyme repeatedly binds to more substrate molecules. Some other types of enzymes can bind two substrates at a time and catalyze a reaction to link the substrates together.

Application of enzymes:
Enzymes are applied in various areas of application

1- Enzymes in technical applications such as detergents industry, Enzymes are applied to remove difficult stains and soil at low washing temperatures. Like proteases, lipases, amylases, & cellulases. In the leather industry, enzymes (proteases) are used in several steps in the processing of skins and hides for the production of leather.
Enzymes are also used in the production of fine chemicals and pharmaceuticals. For instance, Immobilized enzymes are used in the manufacturing of semi-synthetic penicillins and cephalosporins.

2- Enzymes in food industry such as Dairy Products: For the making of cheese the enzyme chymosin is used. Baking industry: Presently, enzymes are used to make up for deficiencies in some flours.

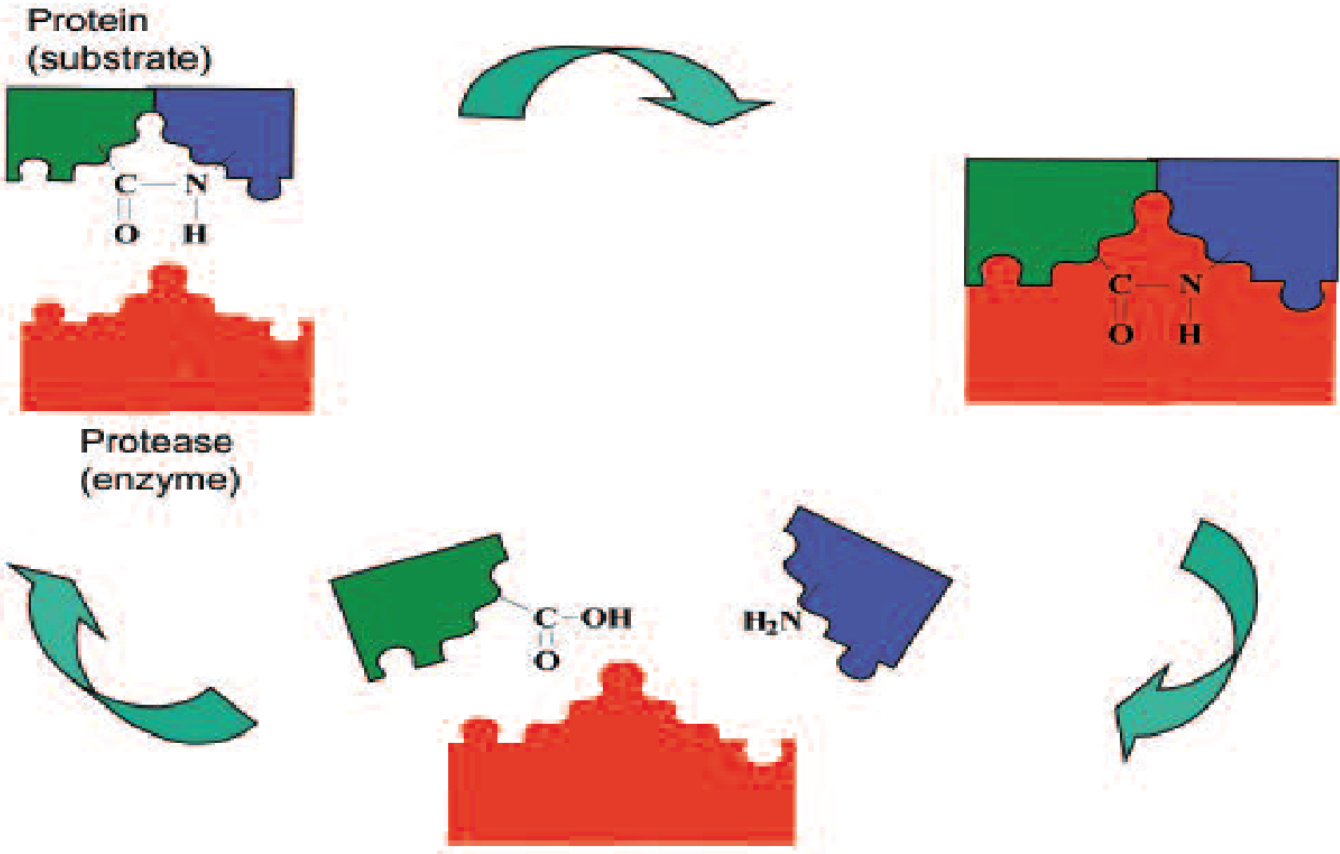
3- Enzymes in feed industry such as Feed enzymes are degrading specific feed components which are otherwise harmful or of little or no value. Feed enzymes in combination with endogenous enzymes degrade compounds So that can be utilized by animal.

4- Enzymes in cosmetics to clean and smoothen the skin, Enzymes with the ability to capture free radicals and thereby preventing damage to the skin

caused by environmental pollution, bacteria, smoke, sunlight or other harmful factors.

5- Enzymes in medicinal products as digestive aids is a wide spread application. Proteases are recommended for removal of fibrin layers from wound to improve healing.

6- Enzymes for scientific and analytical use enzymes are routinely used in determination of glucose, urea, & triglycerides in clinical diagnosis. In food analysis, Research and development in life science



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, but others are more active with organic hydro peroxides such as lipid peroxides. 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.

Peroxidase Guaiacol + 4H2O2 → Tetraguaiacol + 8H2o

Extraction of peroxidase from plants

Preparation of crude extract:

1. Peel, wash, and cut plant tissues (rutabaga, 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 of:

H2O2 0.01 M
Guaiacol 0.05 M
Sodium acetate buffer pH 6 (0.1 M)
Distilled water

Mix this components as shown:

H2O2	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 = Δy/Δx =ΔAbº436nm/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.

Lab no.: 2
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.
Note: Enzymes are very complex proteins and their high degree of specificity as catalysts is manifest only in their native state.
Enzymes will be purified as little as possible, only other enzymes and material likely to interfere with the process which the enzyme is to catalyze, will be removed.
Unnecessary purification will be avoided as each additional stage is costly in terms of equipment, manpower and loss of enzyme activity.

Downstream processing involves isolation and purification steps and ends up in the formulation of the enzyme preparation. (Figure 1)
Cell disruption by mechanical methods such as High-pressure homogenization & the wet grinding of cells in a high-speed bead mill
Cell disruption 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.

Steps of separation
1- Filtration
2- Centrifugation
3- Flocculation and flotation
4- Concentration
The enzyme concentration in starting material is often very low. The volume of material to be
Processed is generally very large, and substantial amounts of waste material must be removed.
Thus, if economic 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

Enzymes are very complex protein molecules possessing both ionisable and hydrophobic
Groups which interact with the solvent. Indeed, proteins can be made to agglomerate and, finally, precipitate by changing their environment. Precipitation is actually a simple procedure for concentrating enzymes.
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 concentrating enzymes.
Enzymes can also be fractionated, to a limited extent, by using different concentrations of
Ammonium sulfate. Sodium sulfate is another precipitating agent used.
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.
Precipitation with Polymers: The polymers generally used are polyethylenimines and polyethylene glycols of different molecular masses.

Purification
For many industrial applications, partially purified enzyme preparations will suffice; however,
Enzymes 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).

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 analyze proteins.

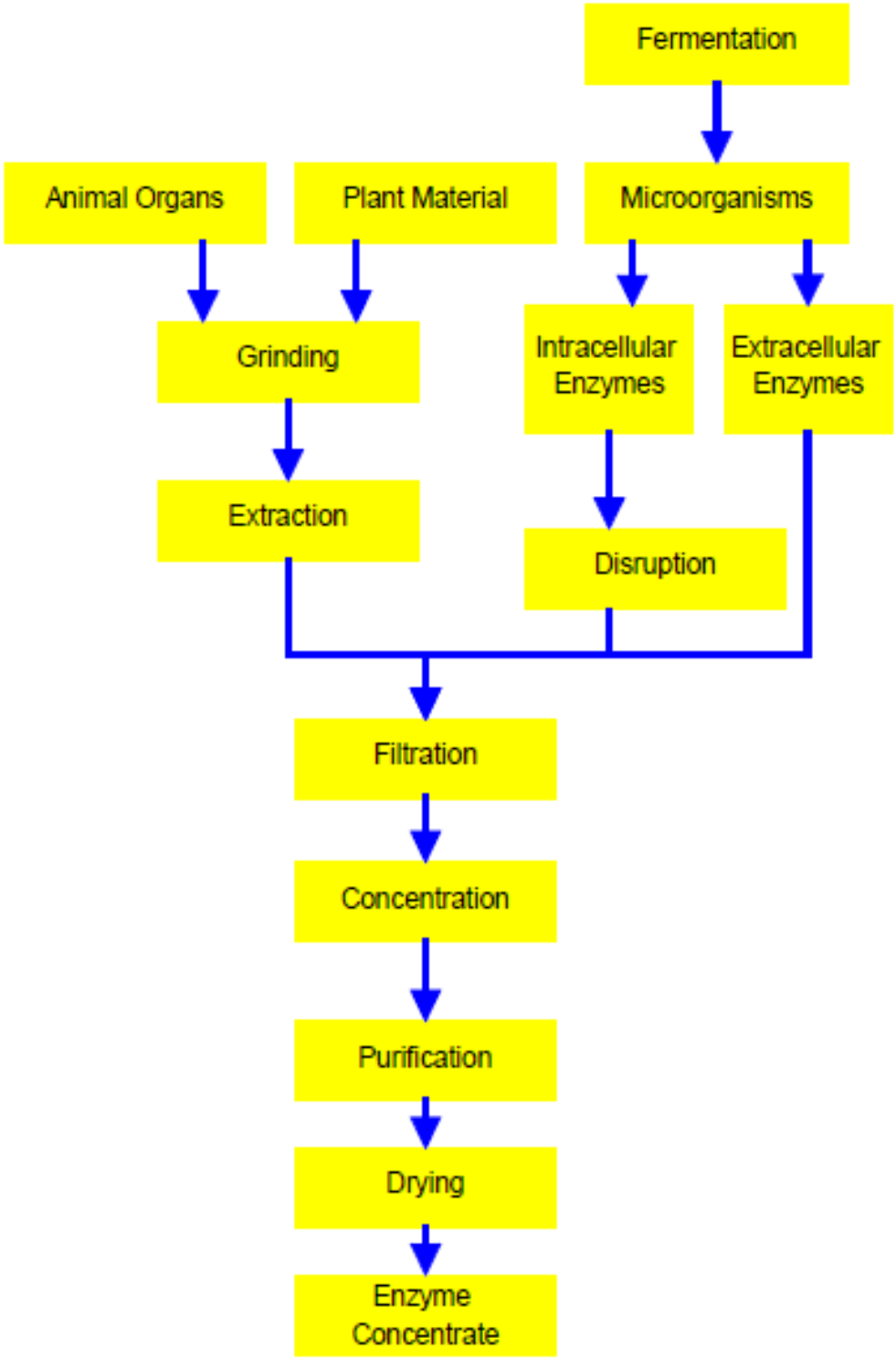

Fermentation
Animal Organs
Plant Material
Microorganisms
Grinding
Intracellular Enzymes
Extracellular Enzymes
Extraction
Disruption
Filtration
Concentration
Purification
Drying
Enzyme Concentrate

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

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.: 3

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.

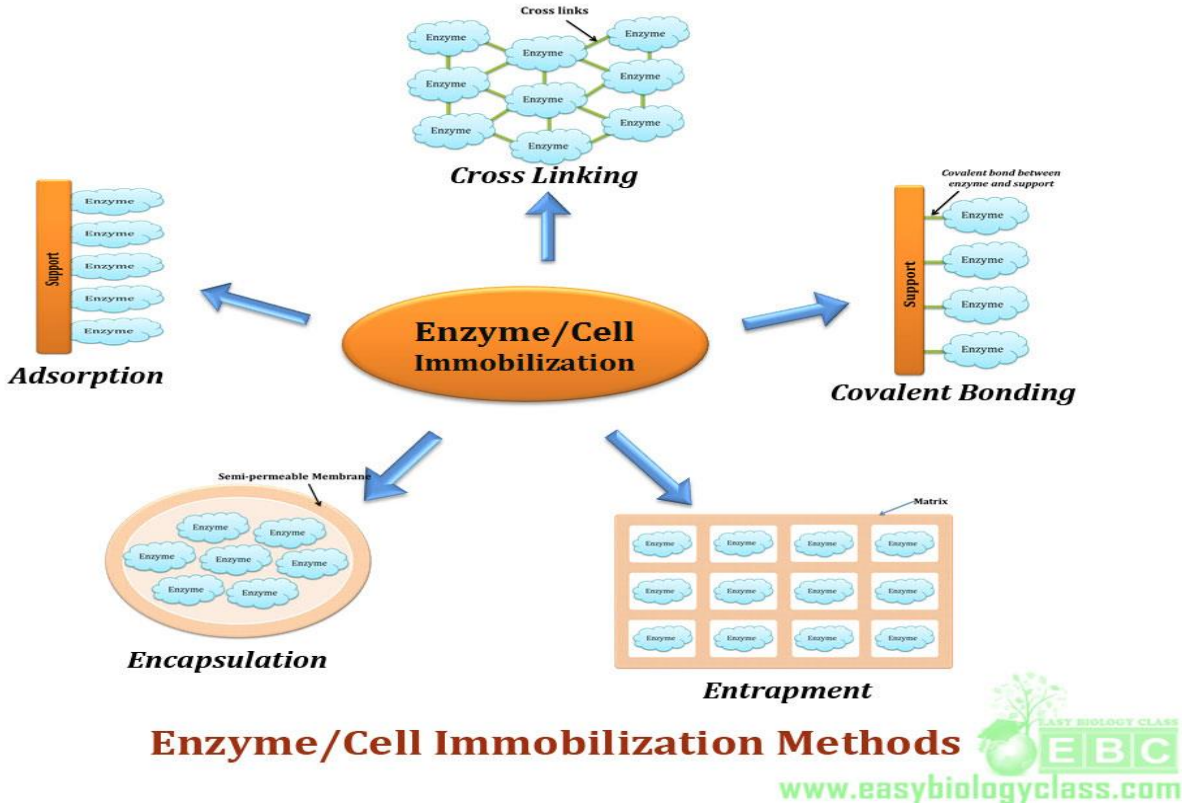
Enzyme immobilization is confinement of enzyme to a phase (matrix/support) different from the one for the substrates and products. The materials used for immobilization of enzymes, called carrier matrices which are grouped into three major categories**:**

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

The carrier being used, the enzyme and the immobilization method are the parameters for Standard Immobilization Method of Enzymes.

Benefits of Immobilizing an 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- Enzymes stability are usually increased by binding. Thermal
6- The ability to stop the reaction rapidly by removing the enzyme from the reaction solution. 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 & Microencapsulation.
2- Binding types like Crosslinking & Carrier binding which can be Ionic, Covalent & Metal binding or 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 (1:1) 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.

LAB: 4
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.

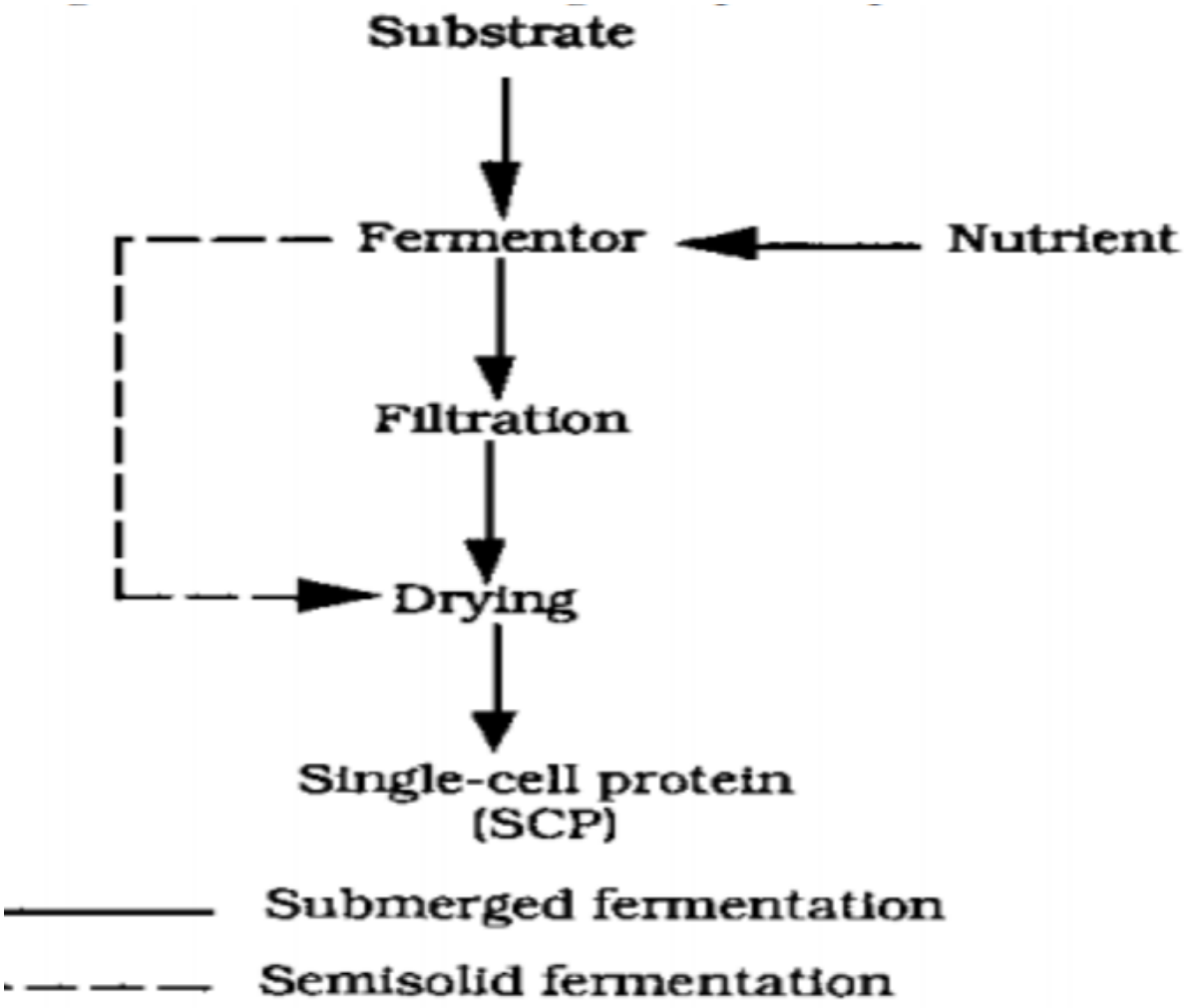
5- Place the filter papers in an oven for drying at 40°C for 24 hour. Next, weight the filter papers; the differences in weights represent the amounts of SCP produced in each flask.

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



Lab no.: 5 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. 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 1.5ml from spore inoculate to the sterilized flask.
10 -incubate the flask in incubator at 28c for 3 -5 days.